Universidade de Aveiro Departamento de Química
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Caracterização do perfil de lípidos polares da polpa da azeitona com recurso à lipidómica

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Doutora Eliana Sousa Cruz Ferreira Alves, Investigadora Júnior do Departamento de Química da Universidade de Aveiro e da Professora Doutora Maria do Rosário dos Reis Marques Domingues, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro

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o júri

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palavras-chave

Azeitona, óleo, lípidos polares, ácidos gordos, lipidómica, extração em fase sólida, cromatografia, espectrometria de massa, origem botânica, origem geográfica.

resumo

O consumo da azeitona, fruto da oliveira (*Olea europaea* L.), na forma de azeitona de mesa e de azeite aumentou significativamente nas últimas duas décadas a nível mundial. Contudo, o valor nutricional desta matriz complexa e rica em lípidos, cuja polpa representa ca. 75 % do seu peso seco, ainda não é totalmente conhecido. A sua fração lipídica compreende essencialmente triacilgliceróis mas também ácidos gordos livres, esteróis e lípidos polares. No entanto, o perfil destes lípidos polares, como fosfolípidos e glicolípidos, compostos com diversos benefícios para a saúde e propriedades bioativas, é praticamente desconhecido, havendo a necessidade da sua caracterização. Como tal, este estudo caracterizou o lipidoma polar da polpa da azeitona de três variedades e duas regiões geográficas através de técnicas emergentes de alta resolução e sensibilidade, baseadas em cromatografia e espetrometria de massa. O trabalho começou pela extração dos lípidos totais da polpa, seguida pelo fracionamento do extrato total e caracterização da fração enriquecida em lípidos polares. O processo de fracionamento por extração em fase sólida foi testado e confirmou-se a eficiência deste protocolo na recuperação de lípidos polares em termos qualitativos e quantitativos. A análise do extrato lipídico total e das frações enriquecidas em lípidos polares por cromatografia em camada fina permitiu verificar a complexidade do lipidoma da polpa. A análise por HILIC HPLC-ESI-MS/MS da fração de lípidos polares permitiu a identificação e caracterização estrutural de cento e oitenta e oito espécies lipídicas: cento e trinta e dois fosfolípidos, trinta e cinco glicolípidos, quinze esfingolípidos e seis betaínas. Para além da identificação de diversas novas espécies moleculares, várias classes de lípidos foram reportadas na polpa da azeitona pela primeira vez. Este trabalho também revelou a composição em ácidos gordos do lipidoma polar da polpa de azeitona, compreendendo ácidos gordos monoinsaturados, polinsaturados, saturados, monohidroxi e dihidroxi. Por fim, a análise estatística multivariada permitiu identificar com sucesso marcadores lipídicos que conseguem discriminar a origem botânica e/ou geográfica das azeitonas, demonstrando assim a unicidade do perfil do lipidoma polar de cada grupo de azeitonas estudadas. Com a identificação de potenciais lípidos bioativos, este trabalho também permitiu valorizar a azeitona como um alimento benéfico para a saúde e com diversas potenciais aplicações industriais como as indústrias alimentar, cosmética, farmacêutica, nutracêutica e de rações.

keywords

Olive, oil, polar lipids, fatty acids, lipidomics, solid-phase extraction, chromatography, mass spectrometry, botanical origin, geographical origin.

abstract

The consumption of olives, the fruits of the olive tree (*Olea europaea* L.), in the form of table olives and olive oil has increased significantly in the last two decades worldwide. However, the nutritional value of this complex matrix rich in lipids, whose pulp represents ca. 75% of its dry weight, is not yet fully known. Its lipid fraction comprises essentially triacylglycerols but also free fatty acids, sterols and polar lipids. However, the profile of these polar lipids, such as phospholipids and glycolipids, compounds with various health benefits and bioactive properties, is practically unknown, with the need for their characterisation. As such, this study characterised the polar lipidome of the olive pulp of three varieties and two geographic regions through emerging techniques of high resolution and sensitivity, based on chromatography and mass spectrometry. The work began by extracting total lipids from the pulp, followed by fractionation of the total extract and characterisation of the polar lipid-rich fractions. The fractionation process by solid-phase extraction was tested, and the efficiency of this protocol in the recovery of polar lipids in qualitative and quantitative terms was confirmed. The analysis of the total lipid extract and polar lipid-rich fractions by thin-layer chromatography allowed verification of the pulp's lipidome complexity. The analysis of the polar lipid-rich fractions by HILIC HPLC-ESI-MS/MS allowed the identification and structural characterization of one hundred and eighty-eight lipid species: one hundred and thirty-two phospholipids, thirty-five glycolipids, fifteen sphingolipids and six betaines. In addition to identifying several new molecular species, several lipid classes have also been reported in the olive pulp for the first time. This work has also revealed the fatty acid composition of the polar lipidome of the olive pulp, comprising monounsaturated, polyunsaturated, saturated, monohydroxy and dihydroxy fatty acyl chains. Finally, the polar lipid-based multivariate statistical analysis allowed the successful identification of lipid markers that can discriminate the botanical and or geographic origin of olives, thus demonstrating the uniqueness of the polar lipidome profile of each group of olives under study. With the identification of potentials bioactive lipids, this work has also allowed valuing the olive fruit as a beneficial food for health and several potential industrial applications such as the food, cosmetics, pharmaceuticals, nutraceutical and feed industries.

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List of abbreviations

AD - *Arbequina* from Dão
APCI - atmospheric pressure chemical ionisation
ASG - acylated sterol glycoside
AT - *Arbequina* from Trás-os-Montes
BL - betaine lipid
CE - cholesteryl ester
Cer - ceramide
Chol - cholesterol
CL - cardiolipin
CPA - cyclic phosphate anion
CT - *Cobrançosa* from Trás-os-Montes
DAG - diacylglycerol
DGDG - diglycosyldiacylglycerol
DGMG - diglycosylmonoacylglycerol
DGTS - diacylglyceryl-*N*,*N*,*N* trimethylhomoserine
DGTS - diacylglyceryl-*N*,*N*,*N* trimethylhomoserine
DHA - docosahexaenoic acid
dLGG - 1,2-di-*O*-α-linolenoyl-3-*O*-ß-galactopyranosyl-*sn*-glycerol
dMPA - 1,2-dimyristoyl-*sn*-glycero-3-phosphate
dMPC - 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
dMPE - 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine
dMPG - 1,2-dimyristoyl-*sn*-glycero-3-phospho-(10'-rac-glycerol)
dMPS - 1,2-dimyristoyl-*sn*-glycero-3-phospho-l-serine
DPA - docosapentaenoic acid
dPPI - 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylinositol
ELSD - evaporative light-scattering detection
EPA - eicosapentaenoic acid
ESI - electrospray ionisation
FA - fatty acid
FA(OH) - hydroxy fatty acid
FAME - fatty acid methyl ester
FDR - false discovery rate
FFA - free fatty acid
FID - flame ionisation detector
GC - gas chromatography
GD - *Galega Vulgar* from Dão
GL - glycolipid
GPL - glycerophospholipid
HC - hydrocarbon
HDL - high-density lipoprotein
HexCer - hexosylceramide
HILIC - hydrophilic interaction liquid chromatography
HPLC - high-performance liquid chromatography
LC - liquid chromatography

LDL - low-density lipoprotein
LPA - lysophosphatidic acid
LPC - lysophosphatidylcholine
LPE - lysophosphatidylethanolamine
LPG - lysophosphatidylglycerol
LPI - lysophosphatidylinositol
LSI - lipidomics standards initiative
MAG - monoacylglycerol
MALDI - matrix assisted laser desorption/ionisation
MGDG - monoglycosyldiacylglycerol
MGDG – monoglycosyldiacylglycerol
MGTS - monoacylglyceryl-*N,N,N* trimethylhomoserine
MS - mass spectrometry
MS/MS - tandem mass spectrometry
MUFA - monounsaturated fatty acid
NACE - non-aqueous capillary electrophoresis
NARP - non-aqueous reversed-phase
nhPC - 1-nonadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine
NL - neutral lipid
NMR - nuclear magnetic resonance
NPSM - *N*-palmitoyl-D-*erythro*-sphingosylphosphorylcholine
PA - phosphatidic acid
PAF - platelet-activating factor
PC - phosphatidylcholine
PC 1 - principal component 1
PC 2 - principal component 2
PCA - principal component analysis
PDO - protected designation of origin
PE - phosphatidylethanolamine
PE-N(FA) - *N*-acyl phosphatidylethanolamine
PG - phosphatidylglycerol
PI - phosphatidylinositol
PL - phospholipid
PUFA - polyunsaturated fatty acid
QqQ - triple quadrupole
R_f - retention factor
RP - reversed-phase
RR - recovery rate
RSD - relative standard deviation
SD - standard deviation
SFA - saturated fatty acid
SFC - supercritical fluid chromatography
SG - sterol glucoside
SHL - sulpholipid
SL - sphingolipid
SLE - solid/liquid extraction

SM - sphingomyelin
sn - stereospecific number
SPE - solid-phase extraction
SPL - sphingophospholipid
SQDG - sulphoquinovosyldiacylglycerol
SQMG - sulphoquinovosylmonoacylglycerol
TAG - triacylglycerol
TIC - total ion current
TLC - thin-layer chromatography
TLCL - 1,1,2,2-tetramyristoyl cardiolipin
TOF - time-of-flight
t_R - retention time
UV - ultraviolet
w/w - weight per weight
XIC - extracted ion chromatogram

1. Introduction

1.1. The olive tree and the olive fruit

1.1.1. Tree

The olive tree (*Olea europaea* L.) (Fig. 1) is a subtropical evergreen plant indigenous to the Mediterranean Basin (1). It is a species easily found in several countries around the Mediterranean region. It has continued spreading outside this region and, nowadays, exists in many places worldwide such as Southern Africa, China, Japan and Australia (2).

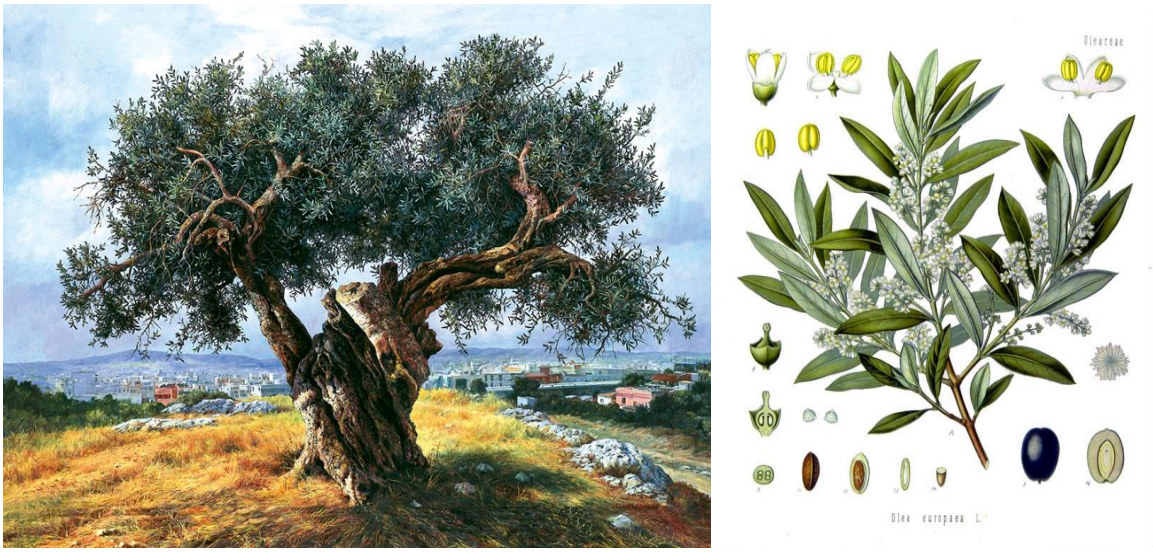


Figure 1 - The olive tree (Olea europaea L.) (on the left) and a representation of the olive tree's flower and fruit (on the right) (3).

It is estimated that about 6,000 years ago, the first fruit trees were cultivated in the Near East. However, the cultivation of fruit trees, like the olive tree, only began when innovative techniques were mastered, as vegetative propagation. The olive tree is one of the oldest cultivated trees worldwide, with indications of its cultivation dating, at least, from the Early Bronze Age (3,100-2,000 BC) (1). When olives spread to the central and Mediterranean coastal areas of the Iberian Peninsula, the olive tree was introduced in Portugal, 146 AD (2). The dissemination and cultivation of this tree in the Iberian territory were enhanced by the Arabic culture. This civilisation was also responsible for the origin of the Portuguese words for olive, "azeitona", and olive oil, "azeite" (2).

The olive tree is the only edible species belonging to *Oleaceae*, its botanical family containing 30 species such as jasmine, the ash tree and privet (4). Generally, this tree does not grow more than 10 meters high, and it grows slowly, reaching its exponential production in 25–30 years. Besides, it can live for several centuries with the ability to reach up to 1,000 years old. The rejuvenation pruning allows the olive tree to maintain the normal production levels, making it possible to obtain its fleshy and oil-containing fruit for an extended period (4).

1.1.2. Fruit

1.1.2.1. Morphology

The olive fruit is an ovoid drupe weighing between 2 and 12 g, but some varieties can reach up to 20 g (4). Typically, it has a 36-53% water content, and oil content usually ranges between 18% and 27% (5). It is commonly divided into three distinct anatomical parts (Fig. 2), that influence the quality of the end-product: epicarp or skin (2.0-2.5% dry weight), mesocarp or pulp (71.5-80.5% dry weight) and endocarp or stone (17.3-23.0% dry weight) that contains the seed (2.0-5.5% dry weight) (6,7).

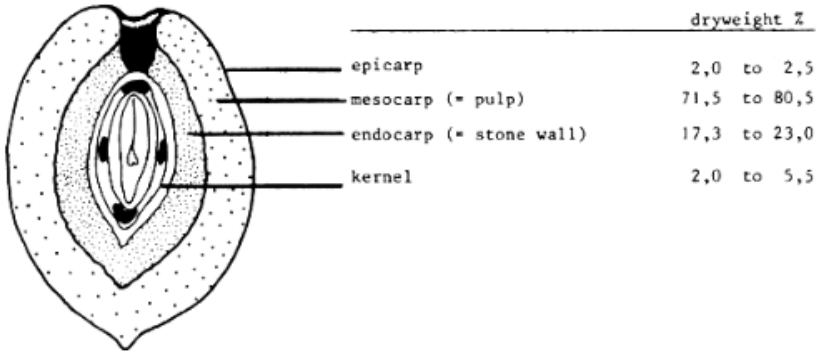


Figure 2 - Cross section of an olive fruit and its physical composition (7).

The epicarp turns from green to black as the fruit's maturation goes on, due to the concentrations of the primary pigments in olives: chlorophylls, carotenoids and anthocyanins (8).

The mesocarp is the central part of the olive fruit and represents, together with the epicarp, the edible portion of the olives, comprising 70-80% of the whole fruit (7,8). The pulp is the reserve supply of all the constituents and contains mainly water (70-75% of the mesocarp weight) and a high oil content (14-15% in green olives and up to 30% in black, ripe olives). This composition is variable depending on the olive's variety (4,8). The endocarp (stone wall) of the olive fruits is formed by resistant and fibrous lignin and has a characteristic ovoid shape. Some parameters as its weight, size, and shell conformation are essential to determine the end-product quality, and the kernel (olive seed) may contain 2-4% of oil (4,8).

1.1.2.2. Olive varieties

The intercrosses between wild and domesticated *O. europaea* resulted in many different cultivars generating almost 1,000 recorded varieties among the immensity of olive trees around the globe (4,9). Consequently, there are many varieties conserved and cultivated depending on the agro-climatic region, *i.e.*, this high variability in the grown olive trees depends on their origin and geographical distribution. It is well known that olive fruits are highly variable in their shape, size, microclimate requirements and chemical constituents (9).

Several countries have their autochthonous varieties, for example, Portugal, Spain, Italy, France, Greece and Tunisia. Outside the Mediterranean region, different olive varieties are also found in countries with a distinct climate and geographic traits such as the United States, Australia and Argentina (10). Table 1 enumerates some olive varieties and their countries of origin.

The purpose of a particular olive variety for the production of table olives or olive oil is closely related to its chemical composition, oil content and size (11). Some cultivars are more appropriate for table olives' production in the existing diversity of varieties, while others are used to extract the oil, and others can serve both purposes (11,12). Normally, olives with a lower pulp to kernel ratio (4:1-7:1) are used for olive oil production, while olives with pulp to kernel ratios of 7:1-10:1 are used to prepare table olives (13). Table 2 shows some olive varieties cultivated in Portugal with their respective main purpose (table olive, olive oil or both).

Table 1 - Examples of olive varieties and their country of origin (10,11)

Country	Olive varieties	Country	Olive varieties
Albania	Kalinjot	Italy	Ascolana Tenera, Biancolilla, Carolea, Coratina, Cucco, Dolce Agogia, Dritta, Frantoio, Leccino, Moraiolo, Ogliarola Barese, Oliva di Cerignola, Pendolino, Pisciottana, Rosciola, Sant'Agostino, Santa Caterina, Taggiasca
Argentina	Arauco	Morocco	Haouzia, Menara, Meslala, Picholine marocaine
Croatia	Lastovka, Levantinka, Oblica	Portugal	Bical, Carrasquenha, Cobrançosa, Cordovil de Castelo Branco, Cordovil de Serpa, Galega Vulgar, Maçanilha Algarvia, Madural, Negrinha, Redondal, Verdeal
Cyprus	Ladoelia	Spain	Aloreña, Arbequina, Bical, Blanqueta, Cornicabra, Changlot Real, Empeltre, Farga, Gordal de Granada, Hojiblanca, Lechín de Sevilla, Manzanilla Cacereña, Morisca, Morona, Picual, Royal de Cazorla, Verdial de Badajoz, Verdiell, Villalonga
Egypt	Aggezi Shami, Hamed, Toffahi	Syria	Abou-Satl, Doebli, Kaissy, Sorani, Zaity
France	Aglandau, Bouteillan, Grossane, Lucques, Picholine Languedoc, Salonenque, Tanche	Tunisia	Chemlali de Sfax, Chétoui, Gerboui, Meski, Oueslati
Greece	Adramitini, Amigdalolia, Chalkidiki, Kalamon, Koroneiki, Mastoidis, Megaritiki, Valanolia	Turkey	Ayvalik, Çekiste, Domat, Erkence, Izmir Sofralik, Memecik, Uslu
Israel	Barnea, Kadesh, Merhavia	United States	Mission

Table 2 - Major purposes of some olive varieties cultivated in Portugal (11,12,14–17)

Olive Variety	Purpose	
	Table Olives	Olive Oil
Arbequina	x	x
Bical de Castelo Branco	x	x
Blanqueta	x	x
Carrasquenha	x	x
Cobrançosa		x
Cordovil de Castelo Branco	x	x
Cordovil de Serpa	x	x
Galega Vulgar	x	x
Gordal	x	
Hojiblanca	x	
Maçanilha	x	x
Maçanilha Algarvia	x	x
Madural		x
Negrinha	x	
Picual		x
Redondil	x	x
Verdeal de Serpa	x	x
Verdeal Transmontana		x

Galega Vulgar (Fig. 3A) is an autochthonous olive variety in Portugal, representing almost 80% of the national olive groves area. It is diffused in different regions of Portugal, namely Beira Interior, Ribatejo, Alentejo and Algarve (18,19). This variety is characterised by a small fruit, smooth consistency of mesocarp and low adherence to the endocarp. It is prone to tuberculosis and susceptible to the attack of the olive fly, but the roots own great resistance to the disease caused by the fungus *Verticillium* (14). Even though the fruit is suitable for table olives' production and despite the low oil yield (less than 18%), *Galega Vulgar* is used to obtain a soft, sweet, slightly bitter and slightly spicy olive oil having high stability and quality (14,15,18). This cultivar is associated with several olive oils with a protected designation of origin (PDO) such as Azeites da Beira Interior, Azeites do Ribatejo, Azeites do Norte Alentejano, Azeites do Alentejo Interior, and Azeites de Moura (18).

Cobrançosa (Fig. 3B) is mostly cultivated in the north of Portugal (Trás-os-Montes region), but it is also accessible in Beira Alta, Ribatejo, and Alentejo (18,20). It has a slightly bitter and spicy taste when greener or sweet and soft when ripe. Being a fruit of medium size, the mesocarp has a tough consistency and high adherence to the endocarp

(14,15). *Cobrançosa* is a very common and productive cultivar, suitable for vibration harvesting, with low susceptibility of foliage diseases and weather accidents, high susceptibility to drought and salinity, tolerance to cold and limestone and alkaline soils (14,18,20). This cultivar is not commonly used for conserving, but widely used for oil production with a medium olive oil yield (18-22%) (14,18). The PDO olive oils associated with *Cobrançosa* are Azeites de Trás-os-Montes, Azeites da Beira Alta, Azeites do Norte Alentejano and Azeites do Alentejo Interior (18).

Arbequina (Fig. 3C) is originally from the Spanish region of Catalunya (Tarragona and Lérida) and Alto Aragón and is disseminated in the Portuguese regions Alentejo and Ribatejo. It is a cultivar easily adaptable to new environmental conditions, resistant to the cold and susceptible to ferric chlorosis in very limestone soils (16,17,21). It is very appreciated for its early entry into production, high oil yield, excellent oil quality and other agronomic characteristics. The fruit has a small size, and it is particularly suitable for new high-density olive plantations due to its reduced vigour (16,17).

Figure 3 - Some olive varieties cultivated in Portugal: Galega Vulgar (A), Cobrançosa (B) and Arbequina (C) (19–21).

1.1.3. Table olives and olive oil

As mentioned earlier, olives are consumed in two forms: table olives and olive oil. These fruits must be processed before human consumption due to the strong bitter flavour. It makes them unpalatable by the chemical component oleuropein (11,22), as will be described next.

1.1.3.1. Table olives

According to the Codex Alimentarius, table olives are understood as a product "prepared from the sound fruits of varieties of the cultivated olive tree *(Olea europaea* L.)". The olive fruits must reach the appropriate degree of development for table olive

processing. The fruits are chosen according to their volume, shape, flesh-to-stone ratio, fine flesh, taste, firmness, and ease of detachment from the stone. Furthermore, the olive fruits must be treated to remove their bitterness and preserved. Preservation can be done through natural fermentation, and or by heat treatment, and or by other means to prevent spoilage and to ensure the product's stability under appropriate storage conditions with or without the addition of preservatives (23).

There are several possible processing methods to prepare table olives. They include the alkaline treatment, partial or total fermentation, dehydration and darkening by oxidation, and these processes depend on the cultivar, ripeness stage, cultivation conditions, and the processing technology (22,23). Among the various trade preparations and or treatment types of table olives, the most economically important international trade are the Spanish-style green olives, the California-style black ripe olives, and Greek-style natural black olives (22).

1.1.3.2. Olive oil

Olive oil is "the oil obtained solely from the fruit of the olive tree (*Olea europaea* L.) or the exclusion of oils obtained using solvents or re-esterification processes and of any mixture with oils of other kinds", as referred to in the Codex Alimentarius (24). However, to obtain virgin olive oils, it is only allowed to use physical means and mechanical approaches under conditions that do not alter the oil. Only a few treatments are permitted, namely washing, decantation, centrifugation and filtration (24). In terms of productivity, generally speaking, to make one litre of olive oil, an average of 5 Kg of olives is required (4).

Olive oils are categorised according to chemical and organoleptic factors. Some examples include: extra virgin olive oils (owner of the highest quality), that have the requirement mentioned above for virgin olive oils, with no defects relatively to organoleptic characteristics (odour and taste) and free acidity (expressed as oleic acid) up to 0.8%; virgin olive oils, with the same requirement but with a median of the sensory defect up to 2.5 and free acidity of not more than 2.0%; ordinary virgin olive oils, with a similar requirement as the previous virgin olive oils, a median of sensory defect between 2.5 and 6.0 and a permitted maximum of acidity of 3.3%; refined olive oils, obtained from virgin olive oils by refining methods that do not alter the initial glyceridic structure and its free acidity, expressed as oleic acid, can reach up to 0.3%; and olive oils, a mixture of

virgin and refined olive oils that is suitable for human consumption, with a free acidity not higher than 1% (24). There are other categories of olive oil of lower quality, as lampante olive oil and refined olive pomace oil, whose differences are, as well, associated to the obtaining method, presence of defects and free acidity (25).

Olive oil, a food product known by its organoleptic characteristics, health benefits and high commercial price, ends up being an ideal target for fraudsters (25,26). Accidental contamination can occur somewhere during processing, *e.g.*, using the same processing machinery for other foods. However, traders may often intentionally mislabel less expensive olive oil as higher quality olive oil or add less expensive edible oils to virgin olive oils to increase profits (25,27). Alongside authenticity, food traceability plays an important role in the quality and safety of foods and has been increasingly required by consumers and governmental organisations (28). Olive oil's origin is expected to be known since certain regions or cultivars possess well-known organoleptic characteristics that influence consumers' sensory experience. However, factors such as the olive's cultivation spreading around the world, or the production of *coupage* olive oils obtained from several olive varieties to achieve a special flavour or aroma, have made difficult the traceability of olive oil which can lead consumers to hesitate about the quality of the commercialised end-product (28).

Olive oil is a complex matrix, highly influenced by numerous factors. The efforts made so far, as tighter trade regulations, have not proved very effective to assure the authenticity and the origin of olive oil. The same applies to the scientific research on olive oil's major and minor compounds or studies of its traceability of botanical and geographical origin (25,28,29). Recently, some relevant results have been evidenced by using mass spectrometry (MS)-based approaches. First, it has been shown that extra virgin and virgin olive oils have unique chemical fingerprints based on their polar lipid profile or phospholipid profile. Significant differences have been found between olive oil categories based on polar lipids as biomarkers (29–31). Likewise, the olive fruit's detailed neutral and polar lipid profiles (*Galega Vulgar*) have been recently decoded by lipidomic approaches. It has revealed to be a key tool for the chemical fingerprinting of these matrices, enhancing the study of olive products of known origin and controlled production (29,32). There is still much work to do in the study of olives and olive oils. New approaches are being developed with recent sensitive and robust analytical methods, which could lead to finding molecular markers able to reveal their specific chemical

identity. These advances would detect fraud and certify the geographical and varietal origin of olives and olive oils worldwide (25,26,33).

1.1.3.3. Worldwide production and consumption of table olives and olive oil

Between 1998/1999 and 2018/2019, the worldwide olive oil production increased from 2.40 million tonnes to 3.18 million tonnes (estimated), corresponding to an increase of 32.25% (34). In 2016/2017, the European Union (EU) was the major producer with 68.41% of the 2.56 million tonnes of olive oil produced worldwide, followed by Turkey (6.95%), Morocco and Australia (both 4.29%) and Tunisia (3.90%). Among the EU-members, 99% of the olive oil production is centred in a group of four countries: Spain (73.63%), Greece (11.13%) Italy (10.40%) and Portugal (3.96%). (34,35) Although the available data on the 2018/2019 campaign is estimated, it indicates that olive oil production in this period, compared to the average of the last five years, has increased about 11% in the EU and has decreased 1% in non-EU members (36). Worldwide olive oil consumption increased by approximately 22% in the last two decades. As for the olive oil production, the EU is the largest olive oil consumer with 51.43% of the 2.73 million tonnes of olive oil consumed worldwide in 2016/2017. Next comes the United States (11.56%), Turkey (5.50%), Morocco (4.40%) and Syria (3.60%). Within the main olive oil consumers inside the EU, there is Spain (31.59%), Italy (31.30%), France (7.77%), Greece (7.49%), Portugal (4.99%) and the United Kingdom (4.96%) (37,38). In 2018, 1.6 million tonnes of olive oil were exported by the EU members to other EU countries (63%) and outside the EU (37%). The group formed by Spain, Italy, Portugal and Greece (by descending order of contribution) was responsible for 99% of all extra-EU exports of olive oil. The EU imported 1.2 million tonnes of olive oil, with only 15% coming outside the EU. In 2018, the main importers of olive oil from the EU were the United States (35%), Brazil (11%), Japan (9%), China (6%), Canada (5%) and Australia (5%) (39).

The worldwide production of table olives increased from 1.20 million tonnes to 2.75 million tonnes (estimated) between 1998/1999 and 2018/2019, an increase of around 129%. In 2016/2017, the worldwide production was 2.9 million tonnes, and the main producers were the EU (29.03%), Egypt (19.00%), Turkey (13.81%), Algeria (10.11%), Syria (6.55%) and Morocco (4.14%). About the EU's 0.84 million tonnes, in 2016/2017, 70.80% originated from Spain and 21.30%, 4.74% and 2.58% from Greece, Italy and Portugal, respectively (40,41). Indeed, the production of table olives is lower than the

production of olive oil. In Portugal, there was a sharp difference between productions: the olive oil production was 55 times higher than the production of table olives, in 2018 (42). Compared with the average of the last five years, the 2018/2019 campaign estimations indicate a decrease of table olives' production of 7.33% in the EU and 6.90% in the non-EU members (36). Relatively to table olive consumption, it registered a growth of around 125% between 1998/1999 and 2018/2019 (estimated). In 2016/2017, the largest consumers of table olives (world total of 2.72 million tonnes) were the EU (21.00%), Egypt (14.70%), Turkey (12.19%), Algeria (10.90%), the United States (7.56%) and Syria (6.79%). Among the EU members, Spain (33.39%) was the major consumer, followed by Italy (14.81%), France (11.32%), Germany (7.44%) and the United Kingdom (6.39%). Portugal (1.05%) was the fourteenth major consumer of table olives within the EU. The main exporters of table olives are the EU, Egypt, Morocco and Argentina, totalling 82.82% of the global exports (0.54 million tonnes), in 2016/2017 (43). The most relevant importers are the United States, Brazil and the EU, representing, in 2016/2017, 56.40% of the global imports (0.36 million tonnes) (44).

The production, consumption and trade of olives and olive oil could change substantially in the future. That may happen mainly due to climate change, the modernisation of the machinery, the investment in irrigation systems, the new harvesting methods, and the new trade agreements as the EU-Mercosur Deal (45–47). Mercosur is a major market for EU exports of olive oil. Its founding members are Argentina, Brazil, Paraguay and Uruguay. So, exporters of these mostly high-value-added exports could benefit significantly from removing tariffs under the referred deal (48). According to a recent study, Portugal is foreseen to become the third-largest world olive oil producer by 2030. This achievement will be driven by investments in high-density groves and modern mills, especially at the Alqueva dam in the Alentejo region, which has allowed super-intensive groves to proliferate (49,50).

1.2. Chemical composition of the olive fruit and other oily fruits

1.2.1. Global composition

The olive fruit's chemical composition is strongly influenced by several factors such as the cultivar, the geographical area, and inorganic and organic microcomponents present in the soil (51). The olive fruit is mainly made up of water, lipids (oil) and

carbohydrates (mainly cellulose), and contains minor components such as proteins and minerals (Fig. 4) (13,52). The pulp of this fruit is mainly composed by water (up to 75% of the pulp weight) and lipids (up to 30% of ripe olives' weight).

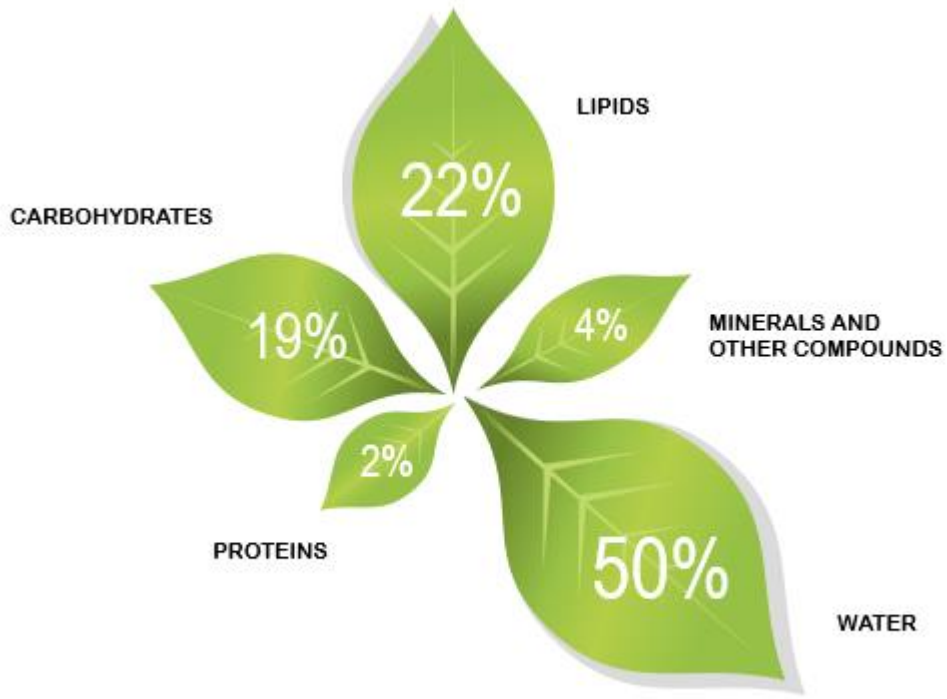


Figure 4 – Chemical composition of the whole olive fruit (%, weight by weight) (13,52).

1.2.2. Lipid composition

In the olive fruit, as in other oily fruits, like avocado, or oleaginous fruits, like almond, hazelnut, chestnut or peanut, lipids are abundant chemical components. These lipids are a complex mixture of compounds consisting mainly of triacylglycerols (TAG). Minor constituents include free fatty acids (FA), sterols, triterpene-derived compounds and polar lipids (6,32,53). In the olive pulp (mesocarp), the lipid content is high (23 g of lipids in 100 g of fruit), followed by the seed (0.60%), the endocarp (0.21%) and the epicarp (0.11%) (6). Vegetable oils may be obtained from different matrices. From seeds with low oil content as wheat germ and soybean (10-20% of oil, on a fresh basis), from oleaginous fruits that possess a high oil content, like peanuts, almonds, walnuts (45–65%

of oil, on a fresh basis), or from the pulp of oily fruits as olive (22% of oil, on a fresh basis), or avocado (20% of oil, on a fresh basis) (5,54,55).

1.2.2.1. Neutral lipids

Fatty acids (FA) are aliphatic monocarboxylic acids. There are hundreds of FA with distinct configurations, chain lengths, types of unsaturation and substituents along their chain. In plants and fruits, the FA chain lengths range between 4 and 22 carbons, being the most common the FA with 18 carbons. In oils (liquid) and fats (solid), the primary FA are oleic (C18:1), palmitic (C16:0), and linoleic (C18:2) acids (56). Olives and olive oil have a high content in C18:1, a monounsaturated FA (MUFA) that comprises around 74% of the total FA composition of the olive pulp (32,54,57,58). The chemical structures of some FA are shown in Fig. 5 (59).

The series of FA with 18 carbons is one of the most abundant and relevant in several matrices. C18:1*n*-9 is the most abundant FA in plant and animal kingdoms, being also found in microorganisms. C18:2*n*-6 is a polyunsaturated FA (PUFA) found in plants. In animals, the presence of this compound is mainly due to dietary plant or seed oils. C18:3*n*-3 is a PUFA found in higher plants and algae (60). C18:2*n*-6 and C18:3*n*-3 are essential FA, that is, FA that cannot be synthesised by the body so must be obtained through the diet. Other relevant FA found in food sources are docosahexaenoic acid, with six unsaturations (DHA, C22:6*n*-3), eicosapentaenoic acid (EPA, C20:5*n*-3) and docosapentaenoic acid (DPA, C22:5*n*-3), both with five unsaturations (61). These *n*-3 PUFA are not found in plants but in marine organisms (oily fish as mackerel, salmon or sardines, shellfish, and seaweed).

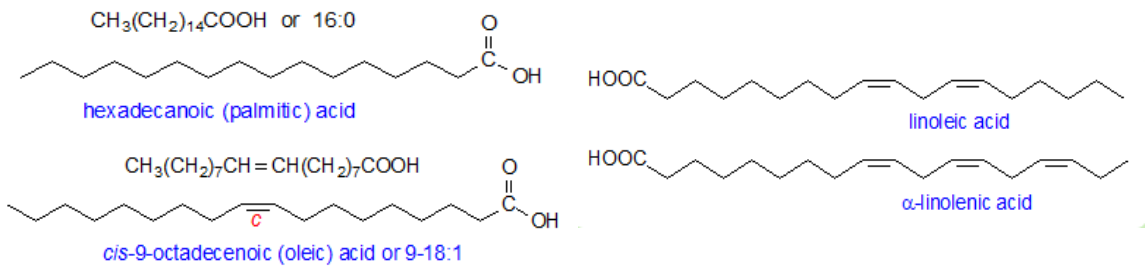


Figure 5 - Chemical structure of some fatty acids: palmitic (C16:0), oleic (C18:1), linoleic (C18:2) and alfa-linolenic (C18:3) acids (59).

The major portion of the FA in the olive fruit is esterified to other lipids, mainly in TAG, while free FA are found in low proportions (up to 5%, w/w, in the pulp) (6). In the olive fruit, TAG represent the main lipids of the neutral lipid fraction: 92% (w/w) of the pulp's lipids; 80% (w/w) of the seed's lipids; and 78% (w/w) of the whole stone (6). In other oily fruits, for instance, in the avocado pulp, around 90% of the neutral lipids are TAG (55,62). Almond oil has a TAG content of 98% (w/w) and peanuts, when fully ripe, have a TAG content of 95.8% (w/w) (63,64). TAG result from the fusion of three FA with a glycerol molecule by esterification (Fig. 6), wherein the FA may be all alike, two may be different or all may be different (54,56).

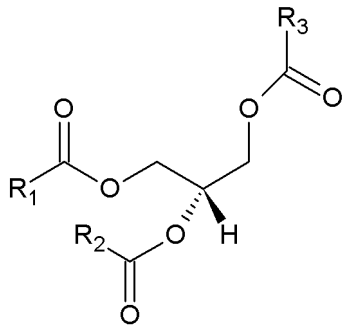


Figure 6 - Generic structure of a triacylglycerol.
R1, R2, and R3 represent fatty acyl chains.

TAG provide energy which is deposited in fat tissues and serve as thermal and mechanical protectors, being found in the organs' surrounding layer. TAG are also a source of essential FA. As the esterification of the FA is possible in three different sites in the glycerol molecule, the stereospecific number (*sn*) of the carbon atoms of the glycerol backbone is significant to the characterisation of acylglycerols (mono-, di- and tri-). The "*sn*" prefix indicates the FA's position in the glycerol backbone and is mentioned before the compound's name (65). With some exceptions, the distribution of the FA in the TAG molecule is characteristic for vegetable oils, with saturated FA occupying the *sn*-1 and or *sn*-3 positions, and unsaturated FA occupying the *sn*-2 position (66).

The olive fruit contains several molecular species of TAG that are mainly composed by C18:1, followed by C16:0 (a saturated FA – SFA), C18:2, stearic (C18:0) (a SFA) and C18:3 (32,54,57,58). Olives have the main content of C18:1 in the TAG, while compared with hazelnut (ca. 68%), almond (ca. 62%) and avocado (ca. 61%) (54).

With the advance of the MS-based analytical techniques, it has been possible to identify more TAG and FA molecular species in olives and other oily fruits. Alves *et al.* (2019) successfully identified 71 TAG species in the olive pulp (*O. europaea* L. cv. *Galega Vulgar*), from C40:0 to C60:2, with FA chains ranging from C11:0 to C26:0 (32). Lee *et al.* (2014) described 70 TAG species in avocado and canola oil, with TAG going from C46:0 to C60:2 (67). Lieb *et al.* (2019) identified 125 TAG in Costa Rican *Acrocomia aculeata* fruits with FA chain length ranging between 28 and 52. They verified that mesocarp oils mainly contained unsaturated TAG composed of long-chain FA (more than 14 carbons) (68).

Triolein (OOO), a TAG composed of three C18:1, is the most abundant TAG in olives, ranging between 21-55% of the TAG fraction (54,57,58). High contents of triolein are also found in hazelnut (32-60%), almond (33-44%) and avocado (11-29%), as shown in Table 3. On the other hand, walnut, peanut and palm oils have other major TAG, being the most common the LLL (ca. 16%), OLL (ca. 16%) and POP (up to ca. 25%) respectively (54). Lísa *et al.* (2008) verified that only six TAG were common to palm, peanut, cottonseed and olive oils and cocoa butter (PLP, OOO, OOP, POP, SOO and SOP, where P stands for palmitic acid, L for linoleic, O for oleic, and S for stearic acid) (54).

Table 3 - Relative abundance (% w/w) of some triacylglycerols in different oleaginous fruits

Source	**TAG**							**Reference**
	OOO	OOP	POP	SOO	OLO	LLL	OLL	
Olive	21-55	17-25	2.6-3.5	2.7-4.9	10-22	0.1-2.5	0.6-4.8	(54,57,58)
Avocado	11-29	20.2	4.2	1.0	10.3	0.4-0.9	2.3-5.0	(54,69)
Palm	3.8-5.3	20.3	2.1-24.5	1.1-3.0	2.5	0.3-0.7	1.0-1.3	(54,70)
Peanut	11.8	6.3	0.9	1.6	14.3	4.6	16.2	(54)
Hazelnut	32-60	18.5	1.8	5.4	17.1	2.0	7.6	(71,72)
Walnut	2.2	1.2	0.2	0.4	6.2	16.3	13.2	(71)
Almond	33-44	11.5	0.5	2.6	20.1	3.3	13.9	(71,73)

TAG, triacylglycerol; O, oleic acid; P, palmitic acid; S, stearic acid; L, linoleic acid

Besides TAG, the olive fruit and the other oily fruits have several minor lipid classes: monoacylglycerols, diacylglycerols, waxes, sterol esters, free sterols, and polar lipids.

The latter will be discussed in more detail since they have been little explored in these foodstuffs and will be studied in this work.

1.2.2.2. Polar lipids

As mentioned before, the olive fruit has other lipid components, as polar lipids. Polar glycerolipids include phospholipids (PL) and glycolipids (GL) (56). Polar lipids may be divided into several classes and subclasses, depending on their structure, being the polar head group the main differentiator.

PL, a major component of all cell membranes forming lipid bilayers, commonly found in foodstuffs as cereals and nuts, may be divided into two sub-groups: glycerophospholipids (GPL), containing glycerol, and sphingophospholipids (SPL), having a sphingosyl backbone (56,74). GPL structures are based on 3-*sn*-phosphatidic acid deriving mainly from *sn*-1,2-diacylglycerols (Fig. 7). The polar head group (a carbohydrate, an amino acid, an amino alcohol, or another functional moiety) is attached to the phosphate residue at the *sn*-3 position. The *sn*-1 and *sn*-2 substituents on the glycerol backbone are responsible for the division of GPL into more classes and subclasses. The same applies to SPL. They consist of PL containing sphingosine or a related amino alcohol instead of glycerol, like sphingomyelins (56).

Glycolipids (GL), such as glycosylglycerides and sulpholipids, consist of acylglycerols attached to a carbohydrate moiety by a glycosidic linkage at the *sn*-3 position (Fig. 7) (56). They are membrane components that influence several biological processes like cell-cell communication or signal transduction and appear in very distinct organisms, such as bacteria, plants, and animals, including humans (75). Among the GL classes, the most common are monoglycosyldiacylglycerols (MGDG), diglycosyldiacylglycerols (DGDG) and sulphoquinovosyldiacylglycerols (SQDG) (56).

Sphingolipids (SL), beside SPL, also include other compounds such as ceramides and glycosphingolipids. Ceramides (Cer) consist of sphingosine or other related amino alcohol with a FA attached to the amino group. Neutral glycosphingolipids are SL with sugar residues. These sugars, either glucose or galactose, are linked to the primary alcohol of the sphingosyl moiety by an *O*-ester linkage. These structures are called hexosylceramides (HexCer) or cerebrosides (56). SL are a significant class of eukaryotic cell membranes' constituents, considered building blocks of cell architecture and regulators of vital biological functions (76).

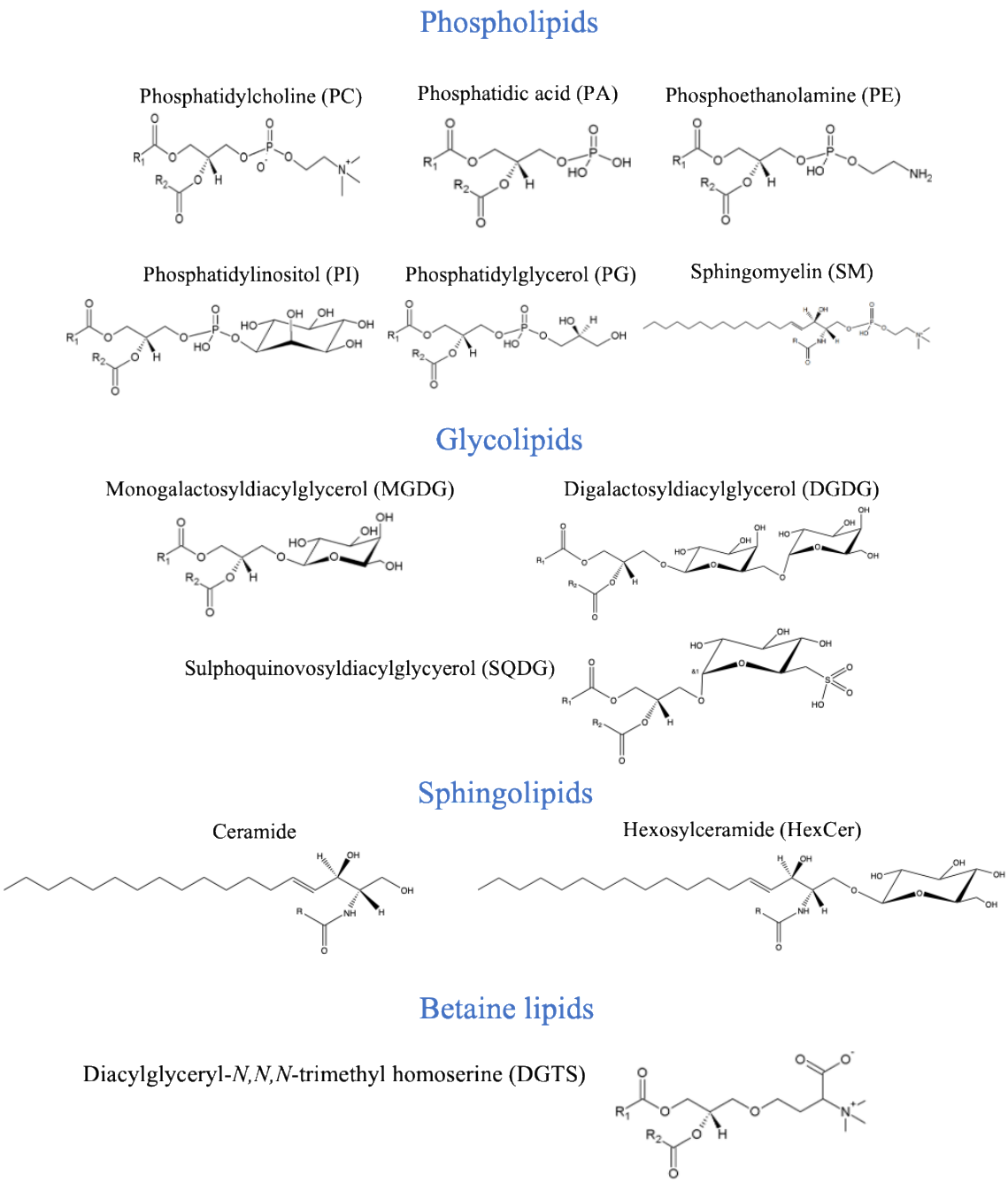


Figure 7 - Chemical structures of some polar lipids found in olives and olive oil. R, R1, and R2 represent fatty acyl chains.

Betaine lipids (BL) are glycerolipids containing a betaine moiety linked to the *sn*-3 position of the glycerol backbone and FA esterified in the *sn*-1 and *sn*-2 positions (Fig. 7) (56). The most common betaine lipids are diacylglyceryl-*N*,*N*,*N* trimethylhomoserine (DGTS). DGTS are commonly found in lower plants, algae, marine eukaryotic

phytoplankton, fungi, and bacteria (77–79). Recently, they have been reported in olives and olive oil (29,32).

The olive fruit's polar lipids have been poorly studied. Their identification is difficult because they are minor components, and they have a chemical diversity corresponding to a wide range of lipid classes of cationic, anionic and zwitterionic nature. Also, there are not still optimised analytical methods to recover these lipids from such a complex matrix as the olive fruit, which contains a high level of TAG, either for fractionation of the oil or to separate, identify and characterise the polar lipid molecules. The studies from Moussaoui *et al*. (2008) and Bianco *et al*. (1998) estimated that the content of polar lipids in the olive pulp accounted for 0.12% (w/w, on a dry basis) of PL and 0.028% (w/w, on a fresh basis) of GL respectively (51,80). The latter identified GL, such as DGDG(18:3-18:3) and DGDG(18:1-18:3) (51). Until the 2010s, most of these studies were based on low-tech approaches, such as thin-layer chromatography (TLC) (33). Montealegre *et al*. (2013) identified different PL classes in the olive fruit while using non-aqueous capillary electrophoresis coupled with electrospray ionisation-mass spectrometry (NACE-ESI-MS) (81). The olive pulp and seed showed distinct polar lipidomes. Some classes, such as phosphatidylethanolamine (PE) and lyso-phosphatidylethanolamine (LPE), were detected only in the olive pulp, not in the seeds (81). Cutting-edge analytical approaches based on by high-performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC-MS) enabled revealing a more in-depth identification and relative quantification of polar lipid classes and molecular species in the olive fruit (32). After fractionation of the olive pulp oil by solid-phase extraction (SPE), dozens of molecular species were found in the polar lipid-rich fraction by HPLC-MS analysis. Before separation by HPLC-MS, the fractionation procedure was necessary to get rid of TAG and sterols that would interfere with the analysis. The compounds identified belonged to four main groups: PL (71.96%), GL (25.44%), SL (2.33%), and BL (0.54%) (32). Table 4 summarises the polar lipid classes identified and quantified in the olive fruit or pulp, based on different studies.

Table 4 - Summary of the polar lipid classes identified and quantified in the olive fruit and pulp, based on various studies

Reference	Sample of interest	Polar Lipid Classes
(82), as cited by (81)	Olive fruit	PL, GSL: HexCer
(83), as cited by (84)	Olive fruit	GL, GSL: HexCer, SG and SHL
(51)	Olive fruit (*Carolea* and *Ottobratica*), both from Calabria region (Italy)	GL (0.028%): DGDG(18:3-18:3) and DGDG(18:1-18:3)
(81)	Olive pulp and fruit: *Arbequina* from Córdoba, Jaén, and Toledo, in Spain, and *Empeltre and Lechín de Sevilla* both from Córdoba, Spain	7 GPL: PC, PE, LPE, PI, PA, LPA and PG
(80)	Olive pulp (*Chemlal*) from Kabylia (Argelia)	PL (0.12% w/w)
(32)	Olive pulp (*Galega*) from Nelas region (Portugal)	5 PL (71.96%): PC (96.96%), LPC, PE, SM and PG; 3 GL (25.44%): MGDG (52.29%), DGDG (46.97%) and DGMG; GSL (2.33%): HexCer; 2 BL (0.54%): DGTS (91.56%) and MGTS

PL, phospholipid; GSL, glycosphingolipid; HexCer, hexosylceramide; GL, glycolipid; SG, sterol glucoside; SHL, sulpholipid; DGDG, diglycosyldiacylglycerol; GPL, glycerophospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPE, lyso-phosphatidylethanolamine; PI, phosphatidylinositol, PA, phosphatidic acid; LPA, lyso-phosphatidic acid; PG, phosphatidylglycerol, SM, sphingomyelin; MGDG, monoglycosyldiacylglycerol; DGMG, diglycosylmonoacylglycerols; DGTS, diacylglyceryl-*N,N,N*-trimethylhomoserine; MGTS, monoacylglyceryl-*N,N,N*-trimethylhomoserine. Percentages are expressed in relative abundance, as weight per weight (w/w).

Polar lipids have also been quantified and identified in other lipid-rich fruits. In avocado (*Persea americana* Mill.), PL represented 0.7–2.1% and GL 2.5–3.2% of the pulp's lipid fraction (55,62). PE and phosphatidylcholine (PC) are the major PL in the avocado pulp with an abundance of 40% and 36% (w/w), respectively. Phosphatidic acid (PA), phosphatidylglycerol (PG) and phosphatidylinositol (PI) were also reported (55,62). Among GL, MGDG is the main class with an abundance of 40% (w/w), while DGDG and cerebrosides were also reported (55,62). In *Florunner* peanut oil, the PL content was ca. 0.6% (w/w) (63). Unfortunately, the lipidome of this fruit was not studied extensively, with no mention of PL classes. The authors concluded that the FA C18:1 and C18:2 are the most common in the PL pool (63). The identification of GPL in almond and

rapeseed oils was also reported. PE and PC are the most abundant polar lipid classes in almond oil, 45% w/w each, and PI and PG the most abundant in *Hybridol* and *Pactol* rapeseed oils respectively, with 55% and 30% (w/w) (85).

1.3. Health effects of neutral lipids and polar lipids from olives and other food sources

The epidemiological, clinical and experimental knowledge derived from decades of research evidenced that dietary patterns, namely the Mediterranean diet, and consumption of specific foods deeply influence health outcomes and prevent chronic diseases, like cardiovascular diseases (86). Therefore, lipids have been a target of several studies wherein biological activity was investigated. TAG, free FA and polar lipids from diverse food sources have been explored as bioactive compounds. Nevertheless, the influence of these compounds in human health is not yet fully understood. High-level scientific proof is needed before formulating nutritional recommendations based on guidelines from evidence-based medicine (33,86–89).

The *n*-9 MUFA, *n*-6 and *n*-3 PUFA, PL and GL have been studied relative to their biological activity to discovering associations of these lipids to numerous biological functions (Table 5). Studies that explored the effects of the whole matrix (*e.g.*, olive oil) and did not refer to the effects of specific classes or molecular species from the neutral and or polar lipid fractions are not mentioned in Table 5.

Table 5 - Biological activity of fatty acids, triacylglycerols and polar lipids from various food sources

Reference	Source*	Lipids	Health effects
(90–93)	NA	MUFA (as *n*-9)	Improvement of several cardiovascular and diabetic risk factors (*e.g.*, HDL-cholesterol and glycosylated haemoglobin)
(61,93–96)	NA	PUFA (as *n*-6 and *n*-3)	Anti-inflammatory activity, maintenance of homeostasis and membrane fluidity, inhibition of LDL synthesis, reduction in blood pressure, essential for normal visual and brain function and beneficial involvement in several diseases as cardiovascular diseases, asthma, arthritis and multiple sclerosis.
(97)	Olive oil and seed oils (soybean, corn, sunflower, and sesame)	NL, polar lipids	Antithrombotic activity (PAF antagonists)
(98)	*Crassocephalum rubens* (Asteraceae)	dLGG (GL)	Cancer chemoprevention (suppression of inflammatory mediators)
(99–104)	*Spinacia oleracea* (spinach), *Chlorella vulgaris* (green alga) and *Rosa canina* (rose hip)	MGDG, DGDG	Antitumoral activity (human gastric and pancreatic cancers) and anti-inflammatory effects in arthritis and osteoarthritis
(105)	*Dicentrarchus labrax* (olive pomace-fed sea bass)	FA, GPL	Cardioprotective activity (influence in PAF activities)
(106) **as cited by** (107)**,** (87,107–109)	Salmon oil, seed oils (corn, cottonseed and soybean)	PL	Antioxidant activity (improving oxidative stability)
(110,111)	Rice bran, maize seeds, soybeans, and rye and wheat grains	SL	Connection to cell growth, differentiation and apoptosis in most type of cells (*e.g.*, colon and skin cells) from several origins (*e.g.*, mice and human), cancer inhibitory action, improvement of the skin's barrier function and reduction of plasma levels of cholesterol and TAG
(76,112–114)	Egg yolk, meat, fish, shellfish, grains, oily seeds	PL, SL	Positive effects in cognitive function, immunity, inflammation, hepatopathies and prevention of cardiovascular diseases and colon cancer

NA, not applicable; MUFA, monounsaturated fatty acid; HDL, high-density lipoprotein; GL, glycolipid; dLGG, 1,2-di-*O*-α-linolenoyl-3-*O*-ß-galactopyranosyl-*sn*-glycerol; MGDG, monoglycosyldiacylglycerol; DGDG, diglycosyldiacylglycerol; FA, fatty acid; GPL, glycerophospholipid; PAF, platelet-activating factor; NL, neutral lipid; PL, phospholipid, PUFA, polyunsaturated fatty acid; LDL, low-density lipoprotein; SL, sphingolipid; TAG, triacylglycerol; BL, betaine lipid. * Lipid source used in the study, if applicable.

Although it is not fully clear yet how lipid compounds act on some biological processes, it is clear that lipids are essential compounds involved in several distinct biological functions. Each lipid class can have more than one biological function/activity. MUFA and PUFA are involved in diverse mechanisms in the organism, and their biological activity is associated with the prevention of diseases like arthritis or cardiovascular diseases. In fact, their dietary consumption is essential (90). Based on the FA content of a food or dietary pattern, dietary lipid indices can be calculated, as the index of atherogenicity or the index of thrombogenicity, as a measure of the propensity of the diet to influence the risk of coronary heart disease. These indices consider MUFA, PUFA and SFA ratios in their formulas (93). Moreover, studies about dietary polar lipids have shown various beneficial health effects. PL have antioxidant, anti-inflammatory and cardioprotective activities and show influence in some many other factors (76,87,106–109,112–114). GL present anti-tumour and anti-inflammatory activity in arthritis, osteoarthritis and cancer prevention (98–104). SL are intrinsically involved in different organisms' parts as skin, plasma, colon and brain. The evidence suggests they also have cancer prevention activity (76,110–114). Studies indicate that betaines have antioxidant activity and beneficial effects on lipid metabolism, glucose homeostasis/insulin resistance and influence in different organs as the brain, liver, kidneys and heart (115–122). However, as far as is known, there are no studies reporting bioactive activities and health effects of BL.

Besides the referred lipid compounds, it is widely acknowledged that the health benefits provided by the consumption of olives (53,123), olive oil (53,86,88,91) and other plant-derived foodstuffs (124) are also due to other components, like phenolic compounds. Phenolic compounds have a high antioxidant activity and play an essential role in reducing cardiovascular risk factors. However, the study of these compounds is outside the scope of this work.

1.4. Potential biotechnological applications of olives and olive derived by-products

Alongside the health benefits of lipids that suggest the beneficial consumption of olives and olive-derived products, these foods and their by-products have been associated with other relevant uses.

There is a growing request for manufacturing non-dairy probiotic food products in the food market. Most foods containing probiotics are milk-based products, such as cheeses and yoghurts, and the individuals facing lactose intolerance and milk proteins allergy are increasing. Table olives, as a plant origin product, may well be an alternative probiotic owner. Several studies suggest the olive fruit as a potential probiotic food (123,125,126). The bacterial flora either autochthonous from the fruit or not, when inoculated onto the fruit, successfully adheres to and colonises the olive, which may be valuable to human health (123,125,126). With this new possible use for the olive fruit as a probiotics source, its nutritional features represent a relevant opportunity for olives in the food market.

Olive processing industrial by-products may be relevant to other industries in several ways. As a lipid source rich in FA and polar lipids, olive pomace and stoned olive cakes may be useful to produce animal feeds, such as functional fish feeds or as an ingredient to include in animal feedstocks, improving the nutritional and nutraceutical properties of various foods such as mammal's meat, milk and cheese (127–130). Also, olive pomace, leaves and stones have a potential role in skincare products and cosmetics, due to the positive effect of the FA at distinct levels such as the composition of the lipid film in the skin surface, the protective barrier and the maintenance of hydration (131,132).

Thus, more research is needed on the lipid composition from olives and olive-derived by-products and their beneficial effects to value these lipids. There are several potential biotechnological applications for these compounds, including the animal feed, pharmaceutical, nutraceutical and cosmetic industries.

1.5. Analytical methods to analyse lipids in olives

1.5.1. Lipidomics

Lipidomics, a rapidly growing field of research, describes and quantifies all lipid molecular species and tries to comprehend their biological functions (133). To ensure that lipid structural identification and quantitation are properly performed and to compare published papers, standardised approaches and guidelines are needed, as recognised by the lipidomics community (133). To encourage this standardisation, some guidelines provided by the Lipidomics Standards Initiative (LSI), for example, cover several analytical steps like extraction, analysis, processing, identification, quantification and reporting the data. This is to improve the overall knowledge about lipid biology and analytical chemistry like mass spectrometry analysis, being especially useful for new researchers in lipidomics (133,134).

1.5.2. Step-by-step analysis of lipids and their methodological approaches

The lipidomics workflow, either for targeted or untargeted approaches, is essentially built into four steps after preparation of the biological sample (Fig. 8). It begins with the extraction of the total lipids and is followed by fractionation, data analysis and data processing (134,135). The untargeted analysis is used to identify and quantify as many lipid species as possible and, consequently, find a specific lipid signature of the biological sample. Analysing the lipid extracts using LC-MS platforms, for example, can generate a high amount of data that needs to be interpreted and validated. On the other hand, with the information acquired from untargeted strategies, it is possible to design targeted lipidomic approaches where the goal is to detect and quantify a set of specific lipids (135). Although there is a certain consensus about the workflow steps, relatively to the techniques used in each step, a harmony between studies reveals itself hard to achieve due to the absence of standard procedures with the current use of distinct approaches to analyse neutral or polar lipids.

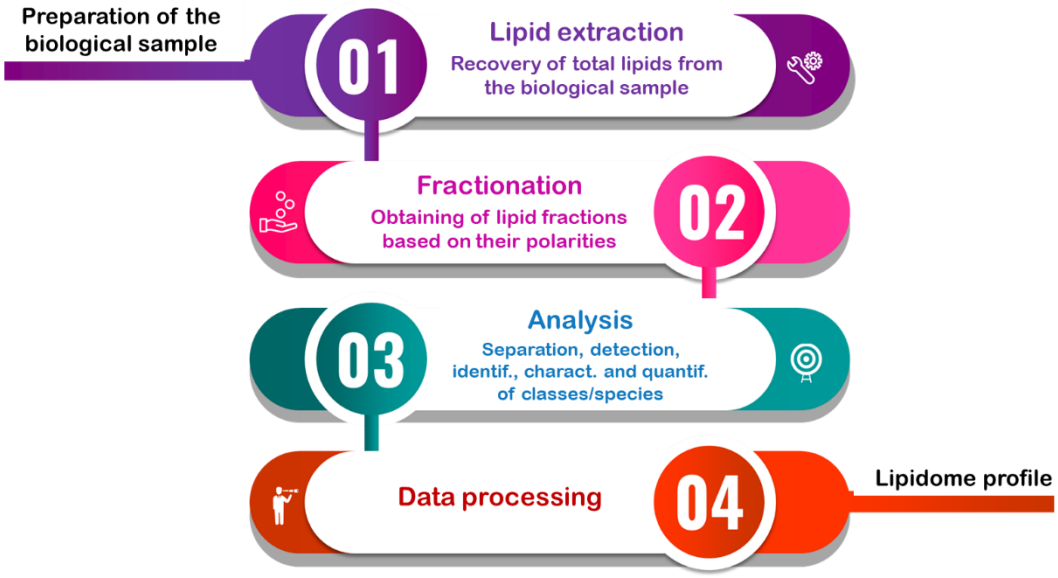


Figure 8 – An example of a lipidomics workflow.

Solid/liquid extraction (SLE) has been the elected approach to extract total lipids from olives. The most utilised methods use organic solvents with distinct polarities, namely the Folch method and the Bligh and Dyer method. The former uses chloroform, or a mixture of dichloromethane/methanol (2:1, by volume). The latter uses chloroform, or a mixture of dichloromethane/methanol (1:2, by volume), and an additional volume of chloroform or dichloromethane. In these methods, lipids are recovered from the lower phase, in the organic fraction, after adding water to the mixture and centrifuging. Other methods to avoid chlorinated solvents have emerged as the MTBE method that uses methyl-*tert*-butyl ether (MTBE)/methanol/water (10:3:2.5, by volume), or ultrasound, microwave or CO_2-assisted techniques. However, these approaches have been poorly reported to analyse lipids in olives or olive oil, with no data available regarding the study on polar lipids (33,135).

Fractionation is not a mandatory step but may be necessary after total lipid extraction when a high level of purity of interest compounds is required. If these components exist in low concentration, the sample must be concentrated. SPE allows fractionating lipids from the remaining compounds or recover different fractions from a total lipid extract. This technique is a useful lipid fractionation or enrichment approach. Silica cartridges are used to recover neutral lipids and polar lipids or fractionate the lipid extract into neutral lipid classes (free fatty acids, sterols, and TAG) and a PL-rich fraction. Aminopropyl-bonded (NH_2) silica is commonly used to separate total lipid extracts into TAG, sterols,

and polar lipids. SPE has been used for polar lipid recovery from olives (32) and olive oil (29–31). However, the authors' procedures were based on previous protocols and were not optimised for recovering the complex polar lipidome of these matrices. SPE is a crucial step to obtain the polar lipid-rich fraction from oils or oily matrices to be further analysed by sensitive techniques of identification and structural characterisation, so fine-tuned protocols are aimed.

Also used for fractionation, thin-layer chromatography (TLC) is a low-resolution technique that allows obtaining separation of lipids into their classes. PL classes can be fractionated by one-dimension TLC using a solvent system composed by chloroform/ethanol/water/triethylamine (35:30:7:35, by volume). Acylglycerols can be fractionated using *n*-hexane/diethyl ether/acetic acid (80:20:1, by volume). Spraying with a revealing agent as a solution of primuline in acetone and visualisation using a UV lamp allows detecting the lipid spots in the TLC plate. It is possible to identify the PL classes by comparing the retention factors (R_f) of each PL class with commercial PL standards applied to the same plate. The determination of the relative content of each PL and or GL class in the total lipid extract is also reachable with TLC by scrapping off the individual lipid spots and quantifying phosphorus or glucose in each spot. Additionally, the lipids from each TLC spot can be further analysed regarding lipid class identification and molecular characterisation or for FA analysis, after recovery by scraping the spots and using proper extraction methods (33,135). After SPE or TLC, the lipid fractions may be analysed through several techniques like gas chromatography (GC), high-performance liquid chromatography (HPLC), mass spectrometry (MS), tandem mass spectrometry (MS/MS), nuclear magnetic resonance (NMR) spectroscopy or non-aqueous capillary electrophoresis (NACE) (33,135).

GC has high selectivity and sensitivity, being the elected method for FA analysis for a long time (135,136). One of the challenges of GC relies on the need for volatilisation of the compounds on samples that contain non-volatile compounds, as FA. A commonly required step previously to GC analysis is sample derivatisation. Derivatisation is important because it changes the analytes' volatility, avoids degradation during the process and increases the thermal stability, sensibility and specificity by blocking certain functional groups of the analytes or introducing appropriate functional groups (135,137). The transesterification of FA with methanol into fatty acid methyl esters (FAME), *i.e.*, the addition of a methyl group to the carboxyl group of FA resulting in a methyl ester, is a process widely used that allows knowing the FA composition of simple or more

complex matrices (135,138). GC is a separation technique that may be coupled to flame ionisation detectors (GC-FID), MS analysers (GC-MS) or both for the identification and quantification of FAME (135,137).

HPLC is a separation technique that is often coupled to different detectors, like ultraviolet detectors (HPLC-UV) or analysers, like mass spectrometers (HPLC-MS). NMR and NACE can also be attached to mass spectrometers. These hyphenated techniques allow for the detection and molecular characterisation of TAG and polar lipids. Notwithstanding the diversity of approaches, LC coupled to electrospray ionisation mass spectrometry (LC-ESI-MS) represents nowadays the most popular way to analyse total lipid extracts or lipid fractions. More recently, high-resolution HPLC-MS and HPLC-MS/MS have provided detailed PL and TAG profiles of olives and olive oil (30,31,33,135). MS data gives information on the polar head of PL and the fatty acyl composition of PL and TAG. These techniques represent a powerful tool for lipid fingerprinting.

Different extraction and characterisation approaches on neutral and polar lipids were carried out in several studies using olives and other oleaginous fruits, as summarised in Tables 6 and 7. Among the distinct approaches to extract total lipids from oleaginous fruits, the most widely used is solvent extraction using the reference method in a Soxhlet apparatus with *n*-hexane, while the Bligh and Dyer method, the Folch method or SPE have been less used. GC-FID and HPLC-MS have been the main approaches to study the FA and TAG profile, respectively (Table 6). LC-MS approaches have been the elected analytical strategy for polar lipid profiling in oily fruits such as olive, avocado and almond, with still poor but promising results (Table 7).

Regarding the olive fruit, with the recent advances on the detailed lipidomic profiling carried out by high-resolution LC-MS analysis (32), a long road has been opened that will deepen the knowledge of this food at the molecular level. These analytical tools will help better understand the influence of several parameters, such as the geographical and botanical origin in the olive's composition in further studies.

Table 6 - Summary of lipidomic approaches used to analyse neutral lipids in olives and other oleaginous fruits in different studies

Reference	Subject	Relevant purpose	Approaches	
			Extraction	Analysis
(58)	Olive fruit (*Olea europaea* L.)	Acquisition of compositional isomer profiles and regiospecific analysis of	In a Soxhlet apparatus with petroleum ether	RP-HPLC and ^{13}C NMR spectroscopy
(57)	Olive fruit (*Olea europaea* L.)	Comparison of two different techniques for TAG analysis	In a Soxhlet apparatus with petroleum ether	RP-HPLC, MALDI-TOF-MS and GC-FID
(32)	Olive fruit (*Olea europaea L.*)	Characterisation of TAG profile	Bligh and Dyer method and SPE with aminopropyl cartridges	RP-HPLC-ESI-MS and RP-HPLC-ESI-MS/MS
(80)	Olive fruit (*Olea europaea L.*)	Fatty acid composition	Two methods (Soxhlet and open reactor) using hexane as solvent	GC-FID
(54)	Palm, peanut, cottonseed, olive oils and cocoa	Characterisation of TAG profile	Extraction with hexane, followed by filtration and dissolution in an acetonitrile–2-propanol–	NARP-HPLC, APCI-MS
(71)	Walnut, hazelnut and almond	Profiling of fatty acids and TAG	Extraction with hexane, followed by filtration and dissolution in an acetonitrile–2-propanol–hexane mixture	NARP-HPLC, APCI-MS ELSD and UV
(67)	Palm and canola oils	Profiling of regioisomeric TAG	Simple dissolution in chloroform, and triheptadecenoin	SFC, QqQ-MS
(68)	Costa Rican *Acrocomia aculeata* fruits	Profiling of fatty acids and TAG	In a Soxhlet apparatus with *n*-hexane	GC, HPLC-MS
(139)	Avocado fruit (*Persea americana* Mill.)	Comparison of fatty acid compositions	Extraction with *n*-hexane	GC-FID
(55)	Avocado fruit (*Persea americana* Mill.)	Fatty acid composition	Folch method. followed by fractionation with silicic acid column chromatography	TLC, GC-FID
(70)	Date palm (*Phoenix canariensis*)	Fatty acid and TAG composition	In a Soxhlet apparatus with *n*-hexane	GC-FID
(140)	Almond (*Prunus dulcis*)	Fatty acid composition	Supercritical CO_2 extraction	GC-MS

TAG, triacyclglycerol; RP-HPLC, Reversed-Phase High-Performance Liquid Chromatography; NMR, Nuclear Magnetic Resonance; MALDI-TOF-MS, Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry; GC-FID, Gas Chromatography coupled to a Flame Ionisation Detector; SPE, Solid-Phase Extraction; RP-HPLC-ESI-MS; Reversed-Phase High-Performance Liquid Chromatography coupled with Electrospray Ionisation-Mass Spectrometry; RP-HPLC-ESI-MS/MS; Reversed-Phase High-Performance Liquid Chromatography coupled with Electrospray Ionisation Tandem Mass Spectrometry; GC, Gas Chromatography; NARP-HPLC, Non-Aqueous Reversed-Phase High-Performance Liquid Chromatography; APCI-MS, Atmospheric Pressure Chemical Ionisation-Mass Spectrometry; ELSD, Evaporative Light-Scattering Detection; UV, Ultraviolet; SFC, Supercritical Fluid Chromatography; QqQ-MS, Triple Quadrupole Mass Spectrometry; MS, Mass Spectrometry; TLC, Thin-Layer Chromatography

Table 7 - Summary of lipidomic approaches used to analyse polar lipids in olives and other oleaginous fruits in different studies

Reference	Subject	Relevant purpose	Approaches	
			Extraction	Analysis
(81)	Olive fruit (*Olea europaea* L.)	Identification and quantification of glycerophospholipids	Modified Folch method	NACE-ESI-MS and MS/MS
(51)	Olive fruit (*Olea europaea* L.)	Identification of DGDG	Ethanol and "charcoal method"	HPLC-UV
(32)	Olive fruit (*Olea europaea* L.)	Characterisation of the polar lipid profile	Bligh and Dyer method and SPE with aminopropyl cartridges	HILIC-ESI-MS and HILIC-ESI-MS/MS
(85)	Rapeseed, olive, almond, and sunflower oils	Identification of glycerophospholipids	Bligh and Dyer method	LC-MS and LC-MS/MS
(55)	Avocado fruit (*Persea americana* Mill.)	Characterisation of phospholipids and glycolipids	Folch method followed by fractionation with silicic acid column chromatography	TLC, GC-FID
(62)	Avocado fruit (*Persea americana* Mill.)	Characterisation of phospholipid and glycolipid molecular species	Extraction with acetone and chloroform/methanol followed by silica gel column chromatography using LC-Si tubes	HPLC–ion trap–MS/MS

NACE-ESI-MS, Non-aqueous Capillary Electrophoresis coupled with Electrospray Ionisation Mass Spectrometry; MS/MS, Tandem Mass Spectrometry; DGDG, Diglycosyldiacylglycerol; HPLC-UV, High-Performance Liquid Chromatography coupled with an Ultraviolet Detector; HILIC-ESI-MS, Hydrophilic Interaction Liquid Chromatography coupled with Electrospray Ionisation Mass Spectrometry; HILIC-ESI-MS/MS, Hydrophilic Interaction Liquid Chromatography coupled with Electrospray Ionisation Tandem Mass Spectrometry; LC-MS, Liquid Chromatography coupled with Mass Spectrometry; LC-MS/MS, Liquid Chromatography coupled with Tandem Mass Spectrometry; TLC, Thin-Layer Chromatography; GC-FID, Gas Chromatography coupled with a Flame Ionisation Detector; HPLC–ion-trap–MS/MS, High-Performance Liquid Chromatography coupled with Ion Trap Tandem Mass Spectrometry

1.6. Aim of the study

This work aims to characterise the polar lipid fingerprint of the olive pulp using a lipidomic approach. This work is expected to provide a molecular signature for olives, based on polar lipid markers, useful for identity and authenticity purposes related to olives and olive oil. Also, to evaluate the olive pulp's nutritional and bioactive potential, based on its lipid content, for valuing this matrix as food and as an agro-industrial raw material for application in other industries. The proposed work will involve the lipid characterisation of samples from three varieties cultivated in Portugal (*Galega Vulgar*, *Cobrançosa* and *Arbequina*) from two geographical regions (Beira Interior and Trás-os-Montes). The total lipid composition of the extracted oil will be analysed and further fractionated using an in-house proof of concept SPE method to recover the polar lipid-rich fraction. The polar lipids will be identified and structurally characterised using HPLC-ESI-MS and HPLC-ESI-MS/MS in a high-resolution mass spectrometer. The chemical fingerprint data will undergo statistical discriminant analysis to verify which lipid markers can distinguish the botanical and or the geographical origin of the olives.

2. Material and Methods

2.1. Reagents and equipment

The organic solvents used in this work had HPLC purity and they were purchased from Fisher Scientific Ltd. (Loughborough, UK): dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), methanol (MeOH), *n*-hexane, acetonitrile, and diethyl ether. The remaining reagents were purchased from major commercial sources. Purified water (Synergy, Millipore Corporation, Billerica, MA, USA) was used whenever necessary. Phospholipid (PL) internal standards were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA): 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (dMPC); 1-nonadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (nhPC); *N*-palmitoyl-D-*erythro*-sphingosylphosphorylcholine (NPSM); 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (dMPE); 1,2-dimyristoyl-*sn*-glycero-3-phospho-(10'-rac-glycerol) (dMPG); 1,2-dimyristoyl-*sn*-glycero-3-phospho-l-serine (dMPS); 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylinositol (dPPI); 1,2-dimyristoyl-*sn*-glycero-3-

phosphate (dMPA); 1,1,2,2-tetramyristoyl cardiolipin (TLCL); and, *N*-heptadecanoyl-D-*erythro*-sphingosine (Cer d18:1/17:0).

The equipment used in this work was the following: bench top centrifuge (PrO-Analytical, Centurion Scientific Ltd., West Sussex, United Kingdom), water bath (Precisterm, JP Selecta, Barcelona, Spain), heating plate (SBH200D/3, Stuart, Bibby Scientific Ltd., Stone, UK), UV-visible spectrophotometer (Multiskan GO, Thermo Scientific, Hudson, NH, USA), microcentrifuge (Gusto High-Speed Mini Centrifuge, Heathrow Scientific, IL, USA), ultraviolet lamp (UV 254 nm, Camag, Berlin, Germany), vacuum concentrator centrifuge (Univapo-100H, Uniequip, Planegg, Germany), vacuum manifold (Visiprep SPE Vacuum Manifold, ref. Supelco-57030-U, Sigma-Aldrich), and HPLC system (UltiMate 3000™ UHPLC, Thermo Fisher Scientific, Germering, Germany) coupled online to a quadrupole-orbitrap hybrid mass spectrometer (Q Exactive™ HF , Thermo Fisher Scientific, Bremen, Germany).

2.2. Sampling and sample preparation

Olive samples from different botanical varieties and geographical origins were collected from traditional olive orchards in the 2018/2019 campaign: *Galega Vulgar* cv. (*n*=5) and *Arbequina* cv. (*n*=5) from the Dão region (Nelas, Portugal), and *Cobrançosa* cv. (*n*=5) and *Arbequina* cv. (*n*=5) from the Trás-os-Montes region (Mirandela, Portugal). The samples were kindly provided by Cooperativa de Olivicultores de Nelas, C.R.L. and Escola Superior Agrária, Instituto Politécnico de Bragança. The olives from Nelas and Mirandela were harvested at the end of November and early December respectively. *Galega Vulgar* and *Arbequina* samples from Dão were collected from five different trees each of random olive groves. *Cobrançosa* and *Arbequina* samples from Trás-os-Montes were randomly collected from one olive grove. The samples were transported at 4 ºC to the laboratory, where the olives were washed with tap water and distilled water, and then dried with a cloth. Olives showing signs of degradation or disease were discarded. Then, the samples were frozen at -80 ºC until lyophilisation. Before lyophilisation, the pulp was removed with a knife and immediately frozen in liquid N_2. Subsequently, it was transferred to screw-cap vials and lyophilised. The lyophilised olives were stored in the vials, away from light, heat and moisture, until lipid extraction.

2.3. Total lipid extraction of the olive pulp

The olive pulp's total lipids were extracted using the Folch method (141), with some modifications. About 150 mg of lyophilised pulp were transferred to an iced Pyrex extraction glass tube, and 1.5 mL of ultrapure water were added. The mixture was then crushed using a homogeniser (Heidolph DIAX 900, Sigma Aldrich, Darmstadt, Germany) until obtaining an homogenised paste (Fig. 8A). Next, 6 mL of extraction solvent (CH_2Cl_2/MeOH, 2:1, by volume) were added, homogenised by manual inversion and by a vortex mixer. After centrifuging for 5 min at 2000 rpm (Fig. 9B and 9C), the organic (lower) phase was collected to a new glass tube, and dried under a gentle N_2 stream. The lipid extract (oil) was redissolved in CH_2Cl_2 and transferred to a 4 mL amber glass vial (previously dried at 105 ºC in a drying over to remove any moisture, kept for at least 30 min in a desiccator, and weighed). After drying completely in the vial, the dried extract was weighed in the vial and stored at -20 ºC until the following procedures. Lipid extraction was performed in four days in a row (four sample groups, five biological replicates each group, one group per day, *n*=20).

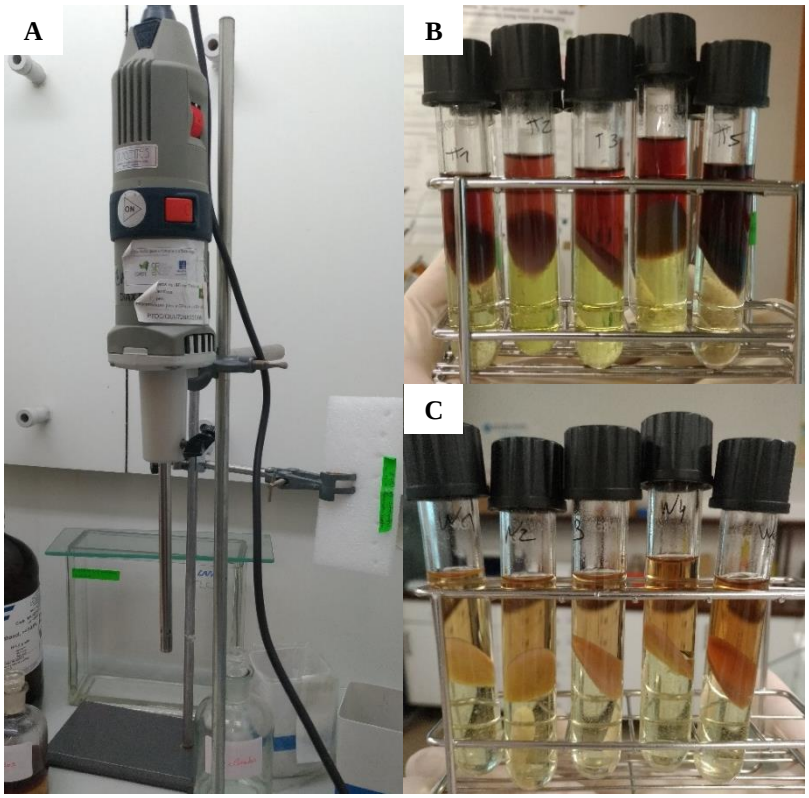


*Figure 9 – **A)** Ultraturrax, instrument used for olive pulp milling; **B and C)** Lipid extraction of the olive pulp by a modified Folch method, showing the phase separation after centrifugation. B) shows the separation of the organic (bottom) phase from the aqueous (upper) phase of* Galega Vulgar *olives from Dão, and C) shows the phase separation observed for* Arbequina *olives from Dão.*

2.4. Estimation of the phospholipid amount in the oil

The PL content in the oil was estimated through inorganic phosphorus measurement (142). The oil was dissolved in 3.0 mL of CH_2Cl_2 in the amber glass vial, and ten µL were transferred to a Pyrex glass tube. The sample was dried under a N_2 stream, and then 125 µL of 70% perchloric acid were added to the tube. After that, each tube was sealed with a crystal ball and heated at 180 ºC for 40-60 min for lipid hydrolysis. Meanwhile, a set of standards was prepared, duplicated, for the calibration curve (0.0-0.2 µg of phosphorus). The standard solution of monobasic sodium phosphate dihydrate (NaH_2PO_4, 10 µg/mL) was pipetted to glass tubes, followed by 125 µL of 70% perchloric acid and variable volumes of ultrapure water to make up 1.0 mL of solution. After lipid hydrolysis, the lipid samples were totally clear and left to cool down at room temperature. Then, 825 µL of ultrapure water were added to each sample tube. Afterwards, 125 µL of 2.5% aqueous ammonium molybdate solution and 125 µL of 10% aqueous ascorbic acid solution were added to the samples and standards, homogenising well before, between and after the addition of these two reagents. The samples and standards were then placed in a water bath with boiling water for exactly 10 min and put on cold water afterwards. Finally, 200 µL were transferred from the tubes to a 96-well microplate, in duplicate, and the absorbance of the samples and standards was measured at 797 nm (Fig. 10). A calibration curve was drawn with the absorbance values of the standards and the known phosphorus concentrations to determine the amount of phosphorus in the samples. The amount of phosphorus was calculated through the curve equation. The obtained value (in µg of inorganic phosphorus) was multiplied by 25 to estimate the amount of PL, a conversion factor representing the average mass of a PL when divided by the phosphorus mass (m=30.97).

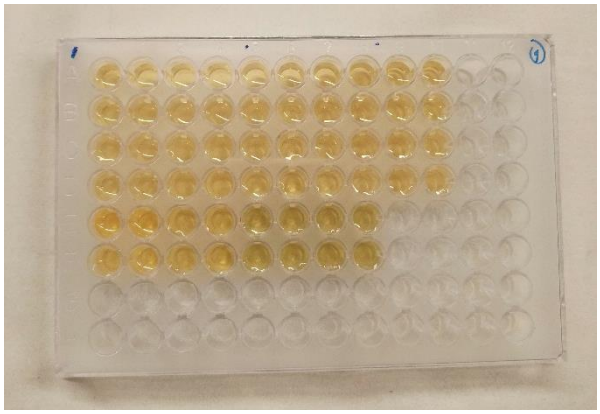


Figure 10 – The microplate of the phosphorus assay with samples and standards subjected to absorbance measurement at 797 nm.

2.5. Estimation of the glycolipid amount in the oil

The content of GL of the oil was estimated by using the orcinol (1,3-dihydroxy-5-methylbenzene) method (143–145). The oil was dissolved in 3.0 mL of CH_2Cl_2 in the amber glass vial, and ten µL of each sample were transferred to a Pyrex glass tube. The sample was dried under a N_2 stream. The standards were prepared in duplicate for the calibration curve (0.0-75 µg of glucose) by measuring different volumes of a standard solution of D-glucose in MeOH (2.0 mg/mL) into glass tubes. Then, 1 mL of orcinol solution (0.2% in 70% H_2SO_4, mass/volume) was added into each glass tube in the fume hood, top sealed with a crystal ball. After heating the samples and standards at 80 ºC for 20 min in a heating block, the tubes were cooled in a water bath. Then, 200 µL of each tube were transferred to a microplate (Fig. 11). The absorbance of the microplate was measured at 505 nm. A calibration curve was drawn with the values of the absorbance of the known standards and glucose concentrations to determine the amount of glucose. The amount of glucose in the samples was calculated through the curve equation. The estimated amount of GL in the samples was obtained by multiplying the mass of glucose obtained by the conversion factor 100/35 (c 2.8) (143).

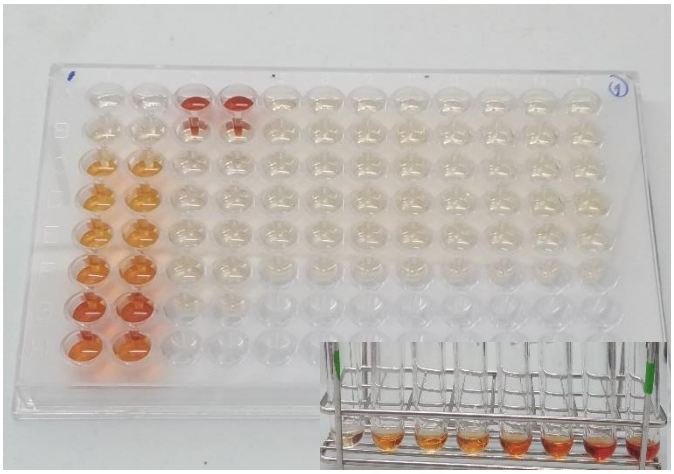


Figure 11 – The microplate and tubes of the orcinol assay for determining the total sugar residues in the oil, with samples and standards subjected to absorbance measurement at 505 nm.

2.6. Thin-layer chromatography (TLC) of the oil

A thin-layer chromatography (TLC) was performed to separate and visualise the main lipid classes present in the oil extracted from the olive pulp (146). The TLC silica plate (glass plates coated with silica gel 60, and with concentration zone, 20 x 20 cm, Merck, Darmstadt, Germany) was cleaned by a washing procedure to remove silica

interferents by elution in a chamber with a mixture of $CHCl_3$/MeOH (1:1, by volume). After the elution, the plate was allowed to completely dry upside down in the fume hood. The samples (1.5 mg of oil dissolved in CH_2Cl_2) and the lipid standards (10.0 µL, 1.0 mg/mL dissolved in $CHCl_3$) were applied in the concentration zone of the TLC plate and dried under N_2. Next, the plate was placed into a TLC chamber saturated with the solvent mixture (hexane/diethyl ether/acetic acid, 80:20:1, by volume). After the acylglycerols migrated to the top of the plate, it was removed from the chamber and dried in the fume hood. It was sprayed with a primuline solution (50.0 mg in 100.0 mL of acetone/water, 80:20, by volume). Then, the plate was left to dry for 15 min, and the lipid classes were revealed under UV light.

2.7. Solid-phase extraction of the oil

In order to fractionate the olive pulp oil to obtain different lipid classes (neutral lipids, intermediate polarity lipids, and polar lipids), the samples were subjected to solid-phase extraction (SPE) by using ionic exchange cartridges (Discovery DSC-NH_2, ref. 52637-U Supelco, Sigma-Aldrich) adapted to a vacuum manifold (Fig. 12). This SPE procedure has been improved in a previous master's thesis on the olive seeds' lipidomics to efficiently extract polar lipids from oils and oily matrices (147).

Figure 12 – Solid-phase extraction (SPE) fractionation system consisting of ionic exchange (NH_2) cartridges adapted to a vacuum manifold, volumetric glass beakers and glass tubes for sample collection.

The SPE cartridge was first conditioned with *n*-hexane. The sample was dissolved in CH_2Cl_2, and it was loaded onto the cartridge (a volume equivalent to 200 μg of polar lipids, as estimated by the results obtained in the PL and GL measurements, described in Sections 2.4 and 2.5, respectively). The neutral lipids were eluted with CH_2Cl_2 (fraction #1) with the solvent being delivered by decanting ampoules. The intermediate polarity lipids were eluted with diethyl ether/acetic acid (98:2, by volume, fraction #2). The polar lipids were recovered in three different fractions. First, they were eluted from the cartridge with CH_2Cl_2/MeOH (1:6, by volume, fraction #3), CH_2Cl_2/MeOH/ammonium acetate in 28% sodium hydroxide (40:10:1, by volume, fraction #4), and hexane/2-propanol/ethanol/ammonium acetate in milli-Q water/formic acid (844:704:202:101:1, by volume, fraction #5).

The fraction #1 was collected into a volumetric glass beaker, transferred to glass tubes, dried in a vacuum concentrator centrifuge, redissolved in CH_2Cl_2, transferred to 2 mL amber glass vials (previously dried at 105 ºC and weighted), completely dried under N_2 and weighted. Fraction #2 was discarded because intermediate polarity lipids were out of the scope of this work. The fractions #3, #4 and #5 were collected into glass tubes and dried under N_2. Subsequently, a volume of 3.0 mL of a mixture of CH_2Cl_2/MeOH (2:1, by volume) and 750 μL of Milli-Q water was added to fractions #4 and #5 to remove the salts. These two fractions were homogenised and centrifuged for 5 min at 2000 rpm, and then, the organic phase (the bottom layer) was collected into new tubes and dried under N_2. This extraction/wash process was carried out once again to remove most of the SPE eluents' salts. Finally, fractions #3, #4 and #5 were redissolved in CH_2Cl_2, transferred to 2 mL amber glass vials (previously dried at 105 ºC), and dried under N_2. All fractions were stored at -20 ºC until further analysis.

An initial set of experiments was carried out by submitting one random sample to fractionation to qualitatively evaluate the improved SPE procedure in olive pulp oil. The polar lipid-rich fractions #3, #4 and #5 were collected both mixed and separated in order to ascertain which lipid classes were recovered and by which eluent.

After the initial set of experiments, which confirmed the successful recovery of the polar lipid classes in qualitative terms, SPE fractionations were done for all the samples referred in the Section 2.2 (four groups of five replicates each, n=20). At this stage, polar fractions #3, #4 and #5 of each sample were collected into the same vial for subsequent analyses. To quantitatively evaluate the recovery of PL and GL, they were

estimated by following the procedures already described (Sections 2.4 and 2.5, respectively).

2.8. TLC of the polar lipid fractions

A TLC was performed with a polar eluent to verify the lipid classes present in the polar fractions resulting from SPE (148). The TLC silica plate was pre-washed in a chamber with $CHCl_3$/MeOH (1:1, by volume). After the plate had dried in the fume hood, it was sprayed with a solution of 2.3% boric acid in ethanol (w/v) to activate the silica's silanol groups. The plate was air-dried and, then, it was taken to the drying oven at 100 ºC for 15 min. When the plate reached room temperature, the samples, previously dissolved in 60 to 145 μL of CH_2Cl_2, and the polar lipid standards (10 μL each, 1.0 mg/mL in $CHCl_3$) were applied in the concentration zone, followed by drying in N_2. Next, the plate was placed into a TLC chamber saturated with the solvent mixture ($CHCl_3$/ethanol/water/triethylamine, 30:35:7:35, by volume). After the lipids' migration and the plate had been air-dried in the fume hood, it was sprayed with primuline solution as referred in Section 6. Then, the plate was allowed to dry, and the lipid classes were revealed under UV light. After visualisation, the bands were scraped off from the silica and placed in sealed glass tubes for further procedures (Section 2.9).

2.9. Estimation of the phospholipid and glycolipid amounts in the TLC bands

The estimation of PL and GL in the respective TLC bands was performed in the same way as described in Sections 2.4 and 2.5, respectively. An additional step was added to the referred procedure because of the silica spots. Before transferring the sample to the microplate, 1.0 mL of each sample was transferred to plastic microtubes and spun down for 5 min at 1000 rpm to concentrate the silica and avoid interferences in absorbance reading. Then, 200 μL of the supernatant were transferred to the microplate to measure the absorbances at 797 nm.

2.10. Analysis of the polar lipid fractions by high-performance liquid chromatography coupled to high-resolution mass spectrometry

The polar lipidome was identified while using high-performance liquid chromatography coupled with electrospray ionisation-high-resolution mass spectrometry (HPLC-ESI-MS). The polar lipid molecular species were analysed and structurally characterised while using tandem mass spectrometry experiments (MS/MS). The HPLC system (UltiMate 3000, Thermo Fisher Scientific, Germering, Germany), while using a hydrophilic interaction liquid chromatography (HILIC) column, was coupled online to a Q-Exactive Orbitrap mass spectrometer (Fig. 13).

Figure 13 – The High-Performance Liquid Chromatograph (UltiMate 3000™ UHPLC system, Thermo Fisher Scientific, Germering, Germany) (on the right) coupled online to the Q-Exactive Orbitrap mass spectrometer (on the left).

The solvent system consisted of two mobile phases (eluent A and eluent B). Eluent A consisted of acetonitrile/MeOH/water, 50:25:25, by volume, with 5 mM ammonium acetate. Eluent B consisted of acetonitrile/MeOH, 60:40, by volume, with 5 mM ammonium acetate. Initially, 5% of mobile phase A was held isocratically for 2 min, followed by a linear increase to 48% of A within 8 min. A new linear increase to 65% A within 5 min was followed by a maintenance period of 2 min, returning to the initial conditions in 3 min and held for more 10 min.

A volume of 20 µL of each sample was introduced into the HPLC system (Ascentis Si HPLC Pore column, 15 cm × 2.1 mm, 2.7 µm, Sigma-Aldrich). The sample contained: 20 µL of the lipid fraction (c 10 µg of PL) in CH_2Cl_2, 4 µL of a mixture of pure PL standards, and 76 µL of starting eluent (5% mobile phase A plus 95% of mobile phase B) to a final volume of 100 µL. The flow rate was 200 µL/min, at 35 ºC. The mixture of standards contained: dMPC, 0.02 µg; dMPE, 0.02 µg; NPSM, 0.02 µg; nhPC, 0.02 µg; dPPI, 0.08 µg; dMPG, 0.012 µg; dMPS, 0.04 µg; dMPA, 0.08 µg; and C17 Cer(d35:1), 0.02 µg. Five injections (n=5) from each of the four groups were performed for the pulp's polar lipid-rich fractions for the full scan MS run.

A mixture of the five samples from each group was used for the MS/MS experiments. The mass spectrometer was simultaneously operated in positive (electrospray voltage was 3.0 kV) and negative (electrospray voltage was −2.7 kV) ionisation modes, with a high resolution (70,000), automatic gain control (AGC) target of 1×10^6, and maximum injection time (IT) of 100 ms. The capillary temperature was 250 ºC, and the sheath gas flow was 20 arbitrary units. For the MS/MS experiments, a resolution of 17,500, AGC target of 1×10^5, and maximum IT of 50 ms were used. The cycles consisted of one full scan mass spectrum and ten data-dependent MS/MS scans that were continuously repeated throughout the experiments with the dynamic exclusion of 60 s and an intensity threshold of 2×10^4. Normalised collision energy ranged between 25, 30, and 35 eV (149). The Xcalibur data system (V3.3, Thermo Fisher Scientific, Waltham, MA, USA) was used for data acquisition and analysis.

2.11. Data Analysis

The polar lipid molecular species were identified based on the assignment of the precursor ions that were observed in the HPLC-MS spectra, considering the expected retention time, the mass accuracy (mass error of ≤ 5 ppm), and the identification of the well-known fragmentation pattern of each class that was observed in the MS/MS spectra of each ion, as described in the literature. MZmine software version 2.32 was used for raw data processing (150,151). Initially, the analysis protocol in this software comprised the mass list filtration, followed by peak detection and peak processing, wherein only peaks with raw intensity upper than 1×10^4 and having a mass tolerance of ≤ 5 ppm were considered. Then, the peak assignment and ion identification were performed based on the LIPIDMAPS database.

2.12. Statistical Analysis

An analysis of variance (one-way ANOVA) followed by Tukey's multiple comparisons (post hoc) test was performed to determine if there were differences between the group means (group effect) on the total oil content, total PL content and total GL content ($p < 0.05$). The Shapiro-Wilk test assessed the normality of the data. GraphPad Prism version 9.0.1 for macOS, GraphPad Software, San Diego, California USA, was used for the statistical evaluation of the results.

Different statistical tests analysed the HPLC-MS data to tentatively identify polar lipid molecular species that could discriminate the olive's botanical and geographical origin. One-way ANOVAs were performed to compare normalised peak areas of lipid classes in varieties from the same region. Assumptions of normality and homogeneity of variance were verified before analysis through Shapiro-Wilk and Levene's tests, respectively. Whenever these assumptions were not verified, the Kruskal-Wallis test was employed. These statistical analyses were performed using R 3.6.0 with a statistical significance level of $p < 0.05$ (152).

Furthermore, lipidomic data assessment was performed using Metaboanalyst (153). Missing values were replaced by 1/5 of minimum positive values of their corresponding variables. A data filtering process was performed to remove variables that show low repeatability, using the relative standard deviation (RSD = SD/mean). Normalised data were transformed and auto-scaled. Principal component analysis (PCA) was performed to visualise the general 2D clustering of samples from two different locations and three different varieties. A heatmap was performed using Euclidean distance measure and Ward clustering algorithm to identify the molecular species that contributed the most to the differences between groups. The top 25 variables were ranked using ANOVA. All experimental data are shown as mean ± standard deviation ($n = 5$).

3. Results and Discussion

3.1. General lipid characterisation of the olive pulp oil

In this work, the olive pulp lipidome was characterised by analysing samples from three cultivars grown in Portugal (*Galega Vulgar*, *Cobrançosa* and *Arbequina*) and two geographical regions (Dão and Trás-os-Montes). The extraction of the total lipids from

the olive pulp was carried out, followed by the fractionation of the same and subsequent analysis of the polar lipids. Liquid chromatography and MS techniques were used to study the lipid components to identify potential biomarkers of identity and botanical and geographical origin. Lipids identified were analysed based on literature data to pinpoint putative bioactive molecules.

3.1.1. Oil yield

The oil content of the olive pulp of the four groups was determined after total lipid extraction, and the results were expressed in g/100 g of dry matter (Table 8).

Table 8 - Oil content of olive pulp's samples, expressed in g/100 g of dry matter (% w/w)

Samples		% oil extracted from olive pulp (w/w)	Average oil content of groups ± standard deviation (g) *	Average of oil content of olives ± standard deviation (g)
***Galega Vulgar* from Dão**	1	27.27	36.69 ± 5.29a	41.67 ± 10.60
	2	34.59		
	3	39.31		
	4	41.03		
	5	41.26		
***Arbequina* from Dão**	1	48.75	49.22 ± 2.00b	
	2	51.40		
	3	50.10		
	4	45.58		
	5	50.29		
***Cobrançosa* from Trás-os-Montes**	1	24.30	27.81 ± 2.55c	
	2	26.23		
	3	31.57		
	4	29.61		
	5	27.35		
***Arbequina* from Trás-os-Montes**	1	47.83	52.94 ± 3.20b	
	2	53.18		
	3	57.87		
	4	52.36		
	5	53.45		

* Values in this column followed by different letters are significantly different ($p < 0.05$).

The four groups showed significantly different oil contents. *Galega Vulgar* from Dão and *Cobrançosa* from Trás-os-Montes showed lower oil yields, 36.69% (w/w) and

27.81% (w/w), respectively, compared to *Arbequina*. *Arbequina* from Trás-os-Montes had the highest oil content, approximately 52.94% (w/w), followed by *Arbequina* from Dão, with about 49.22% (w/w) of oil. The difference between both *Arbequina* samples was not significant, but it was significantly different from the other varieties ($p < 0.05$). On average, the gravimetric yield of the olive pulp's total lipid extraction (n=20) was 41.67 ± 10.60% (w/w), on a dry basis.

The literature reports a total lipid content ranging between 44.3% (w/w) and 54.21% (w/w), on a dry basis for the olive fruit (80,154). A previous study showed that *Arbequina* from Tarragona (Spain) has an average oil content of 49.5% (w/w), reinforcing together with these data from Portuguese cultivated olives the high oil content in *Arbequina* olives regardless of the geographic origin (154). The results obtained of the lower oil yields for *Galega Vulgar* and *Cobrançosa* agree with previous reports since both varieties have medium to low oil content on a fresh basis (14,15,18). No studies could reveal the dry-based oil contents for these varieties to make further relevant comparisons. The variation observed for the oil content between botanical varieties was expected because it is well known that the botanical origin contributes considerably to the olive fruit' characteristics. Among the variables that influence the oil content are the olive size, shape, mesocarp adhesion or resistance to biotic and abiotic stress (14,15,155).

Furthermore, although significant differences were not found for the oil content between the two regions considering the *Arbequina* variety, the olive fruit's chemical composition may also depends on agro-climatic traits and geographical distribution (9). In this work, two different regions were studied (Dão and Trás-os-Montes). These region's microclimate and edaphological conditions will naturally influence the olive fruit's chemical composition of these botanical varieties and may have a greater effect on some varieties than in others.

3.1.2. Separation into major lipid classes

A thin-layer chromatography (TLC) was carried out on the olive pulp oil, allowing to visualise the most abundant lipid classes. The TLC plate showing these lipid classes is represented in Fig. 14.

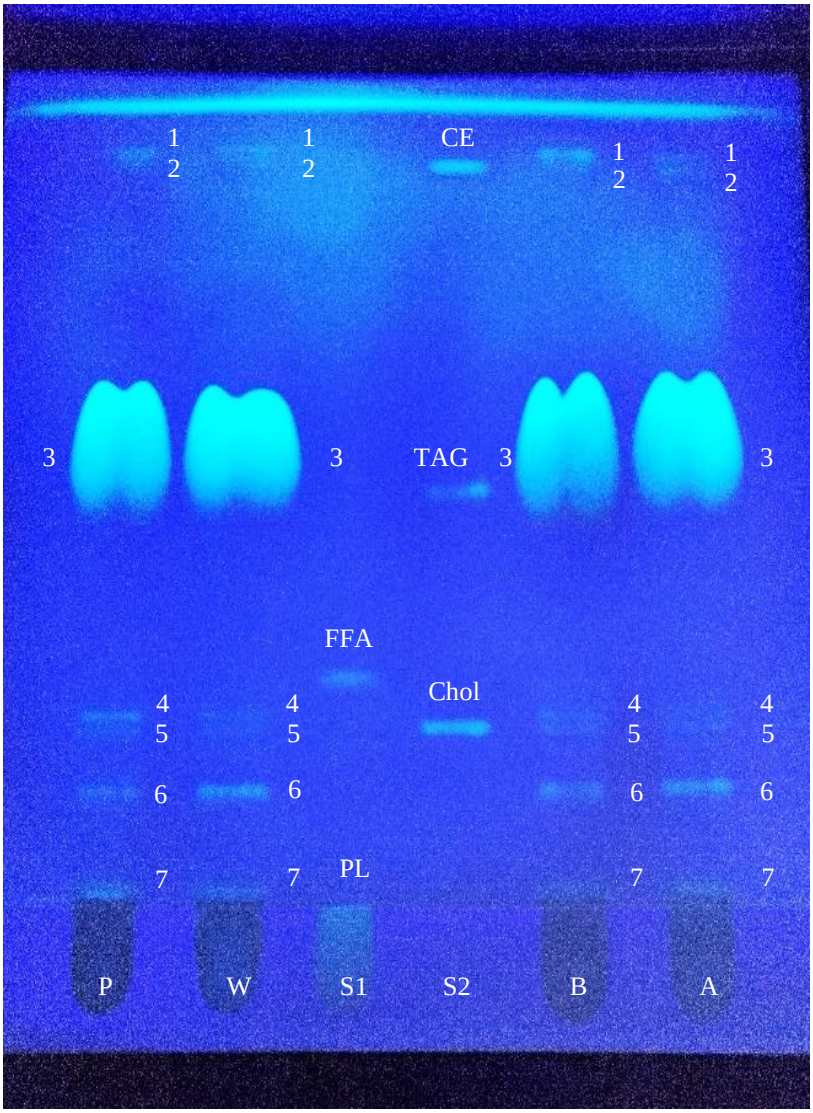


Figure 14 - The thin-layer chromatography plate under ultraviolet light allows to observe different lipid classes present in the olive pulp oil.
Samples: Galega Vulgar *(P) and* Arbequina *(W) from the Dão region,* Cobrançosa *(B) and* Arbequina *(A) from the Trás-os-Montes region. Lipid Standards: polar lipids (PL) and free fatty acids (FFA) were applied on spot S1, and cholesterol (Chol), triacylglycerols (TAG) and cholesteryl ester (CE) were applied on spot S2.*
Band identification: 1, 4, 6 – Unidentified; 2 – CE; 3 – TAG; 5 – Sterols; 7 – Polar lipids.

The TLC has shown that triacylglycerols (TAG, band #3) are the most abundant lipids in the olive pulp and several distinct lipid classes are also present in this oil. By comparing with the lipid standards, band #2 was assigned to cholesteryl esters (CE), band #5 to sterols and band #7 to polar lipids. The TLC bands #1, #4 and #6 did not have a corresponding standard. However, according to the literature for this chromatographic separation of acylglycerols (146), band #1 might represent hydrocarbons (HC), being

squalene one of the most abundant HC in the olive fruit (53). Band #4 might be 1,3-diacylglycerols (1,3-DAG) and band #6 might represent monoacylglycerols (MAG) (146). Although limited in terms of information, the TLC of the oil allowed verifying the main lipid classes of olive pulp in a fast and simple way, where the TAG stand out.

3.1.3. Total phospholipid and glycolipid contents

The contents of phospholipids (PL) and glycolipids (GL) were estimated in the olive pulp oil using colourimetric methods. The PL/GL ratio was calculated accordingly (Table 9). The olive pulp oil had about 0.40 ± 0.15% (w/w) of PL and 2.73 ± 0.69 % (w/w) of GL. The estimated average PL amount is more than three times higher than the amount of PL already reported for the olive pulp (*Chemlal* variety cultivated in Algeria), about 0.12% (w/w) (80).

Dão samples had the highest PL content. Both *Galega Vulgar* and *Arbequina* from Dão had higher PL levels, with 0.46 ± 0.08% (w/w) and 0.56 ± 0.16% (w/w), respectively. *Cobrançosa* and *Arbequina* varieties from Trás-os-Montes had 0.32 ± 0.05% (w/w) and 0.29 ± 0.07% (w/w) of PL, respectively. Interestingly, the PL content in the *Arbequina* variety was different between both regions, showing the highest and the lowest PL content among the four groups. Based on the statistical analysis performed for the total PL content, significant differences were only observed between *Galega Vulgar* from Dão and *Cobrançosa* from Trás-os-Montes groups ($p = 0.0129$).

In average terms, *Cobrançosa* had the highest GL content, 3.17 ± 0.46% (w/w), followed by *Galega Vulgar*, 3.13 ± 0.88% (w/w), *Arbequina* from Dão, 2.38 ± 0.23% (w/w), and *Arbequina* from Trás-os-Montes, 2.23 ± 0.40% (w/w). The post hoc tests for the total GL content showed significant differences between *Cobrançosa* from Trás-os-Montes and *Arbequina* from Trás-os-Montes groups ($p = 0.0407$), indicating a possible distinction between different varieties from the same region. No studies could be found on the GL content of the olive pulp on a dry basis. Only one study addressed the amount of GL in olives and reported 0.028% (w/w) of GL. In particular, two specific molecular species of GL were quantified in the whole olive fruit, on a fresh basis, neither being allusive to the olive pulp nor on a dry basis (51).

The average GL/PL ratio was 7.65 ± 3.11, revealing that GL are between three and ten times more abundant than PL, by mass, in the olive pulp. The two groups with the lowest PL contents, both from Trás-os-Montes region, were the two groups with the

highest GL/PL ratios, ca. 11.1 in *Cobrançosa* and ca. 8.4 in *Arbequina*. *Galega Vulgar* had a ratio of ca. 6.7 and *Arbequina* from Dão had a GL/PL ratio around 4.5. These ratios have been calculated for the olive fruit for the first time.

Table 9 - Total amount of phospholipids and glycolipids in the olive pulp oil, expressed in mg/100 mg oil (% w/w)

Samples		PL content in the oil	PL ± SD	Global PL ± SD*	GL content in the oil	GL ± SD	Global GL ± SD*	Ratio GL/PL ± SD	Global ratio GL/PL ± SD*
***Galega Vulgar* from Dão**	1	0.6040	0.4633 ± 0.0807	0.4022 ± 0.1541	4.7976	3.1287 ± 0.8824	2.7271 ± 0.6939	6.69 ± 0.96	7.65 ± 3.11
	2	0.4526			3.2154				
	3	0.3935			2.3422				
	4	0.4876			2.5651				
	5	0.3786			2.7232				
***Arbequina* from Dão**	1	0.8668	0.5645 ± 0.1594		2.2087	2.3769 ± 0.2339		4.50 ± 1.12	
	2	0.5450			2.4852				
	3	0.4266			2.0100				
	4	0.4382			2.6530				
	5	0.5459			2.5277				
***Cobrançosa* from Trás-os-Montes**	1	0.3308	0.3174 ± 0.0515		3.9647	3.1687 ± 0.4575		11.06 ± 2.23	
	2	0.3108			2.6911				
	3	0.2799			2.8102				
	4	0.3483			3.3553				
	5	0.2020			3.0223				
***Arbequina* from Trás-os-Montes**	1	0.1669	0.2869 ± 0.0732		2.3420	2.2340 ± 0.4019		8.36 ± 2.94	
	2	0.2736			2.1211				
	3	0.2670			1.5458				
	4	0.3557			2.7668				
	5	0.3711			2.3945				

PL, phospholipids; SD, standard deviation; GL, glycolipids. *Global data were calculated based on the values of all samples (*n*=20).

3.2. Fractionation of the olive pulp oil

The fractionation of the total lipid extract represents one of the most critical steps of the entire workflow of this lipidomic study. This stage allowed to obtain the polar lipid-rich fractions, which consequently allowed the in-depth characterisation of the polar lipid profile at the molecular level. Polar lipids exist in trace amounts in the oil and thus are difficult to analyse without previous fractionation. Therefore, this procedure was first evaluated in one sample and was afterwards performed for all samples (n=20).

3.2.1. Evaluation of the polar lipid efficiency recovery by solid-phase extraction (SPE)

At first, it was evaluated if the solid-phase extraction (SPE) of the oil could recover all polar lipid classes, including cationic, zwitterionic and anionic lipids. A qualitative analysis of the polar lipid classes recovered after SPE was carried out in the enriched fractions (fractions #3, #4 and #5, both mixed and separated) by using TLC analysis.

After revealing the TLC plate with the primuline solution and visualising with a UV lamp, the polar lipid classes were tentatively identified by comparing with the polar lipid standards applied in the same plate (Fig. 15).

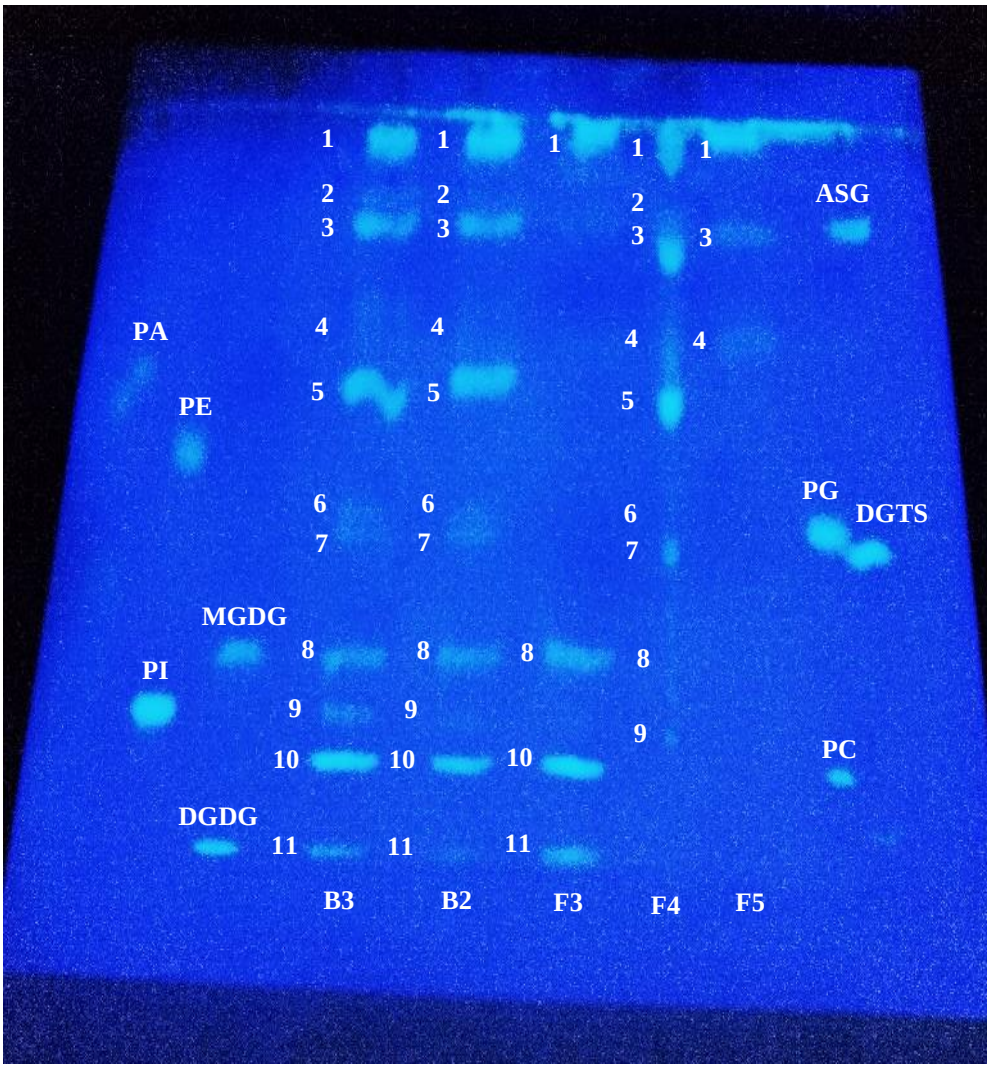


Figure 15 – The thin-layer chromatography (TLC) plate under UV light showing the migration of the polar lipid fractions of the olive pulp obtained after solid-phase extraction (SPE). Lipid standards: phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol (PI), diglycosyldiacylglycerol (DGDG), monoglycosyldiacylglycerol (MGDG), phosphatidylcholine (PC), phosphatidylglycerol (PG), diacylglyceryl-N,N,N trimethylhomoserine (DGTS) and acylated sterol glycoside (ASG). B2 and B3 represent the polar lipid-rich fractions 3, 4 and 5 mixed together from experiment 2 and 3, respectively. F3, F4 e F5 represent the polar lipid-rich fraction 3, 4 and 5 from experiment 2, respectively. Band identification: 1, 2, 3, 4, 5 – Unknown; 6 – PG; 7 – DGTS; 8 – MGDG; 9 – PI; 10 – PC; 11 – DGDG.

Three classes of PL were tentatively identified in the polar lipid-rich fraction, namely PG (band #6), PI (band #9) and PC (band #10). Moreover, two classes of GL, MGDG (band #8) and DGDG (band #11), and one class of betaine lipids, DGTS (band #7), could be presumably identified. Band #3 could be assigned to acylated sterol glycoside (ASG); however, this class has not been previously identified in the olive pulp but in the olive seeds (147). The bands #1, #2, #4 and #5 could not be identified. Band #5

could be assigned to PA, but the band was not well-defined, and the R_f was slightly different.

This SPE approach had a clear advantage compared to the previous work carried out on the olive pulp in our laboratory (32), because of the new eluent mixtures used. In contrast to the previous work, this improved approach allowed the recovery of anionic polar lipids such as PG and PI, as visualised in the TLC bands. Eluent 3, a mixture of CH_2Cl_2/MeOH (1:6, by volume), allowed mostly the recovery of neutral GL (MGDG and DGDG) and the cationic PL class PC. On the other hand, eluent 4, a mixture of CH_2Cl_2/MeOH/ammonium acetate in 28% sodium hydroxide (40:10:1, by volume), allowed the recovery of lipids such as DGTS and the anionic classes PI, PG, and possibly PA. Eluent 5, a mixture of hexane/2-propanol/ethanol/ammonium acetate in milli-Q water/formic acid (844:704:202:101:1, by volume) recovered the most anionic lipids due to its ionic strength, as expected.

Depending on the elution conditions, aminopropyl (NH_2)-functionalised chromatography as in the SPE columns applied in this work for the oil fractionation, works as normal-phase or ion-exchange chromatography with low strength and may retain weak anions. Elution with methanol, or with mixtures composed mainly of methanol, allows recovering cationic and neutral (or zwitterionic) PL, like PC, PE, SM and glycosphingolipids. However, acidic PL such as PI, PA and PG are retained in the column, making it necessary to use lower pH (acidic) solvents or greater ionic strength (by adding salts) to recover them. Plant matrices also have the anionic sulpholipids that co-elute with PG. Using a gradient of high ionic strength, pH, or both, allows eluting compounds with different charges or polarities. Molecules with acidic (anionic) functional groups are extracted from matrices in adsorbents with basic (cationic) surface groups. When the surface of the stationary phase is positively charged, the negatively charged analyte (anion), in this case, anionic PL, is attracted to the positive surface of the modified silica, being necessary to use an ionic strength or pH gradient to elute the analytes that bind more strongly to the solid-phase anion exchangers. The silica columns functionalised with NH_2 groups have been used in many biological matrices for fractionation and obtaining polar lipids for decades (32,156,157). The retention of polar lipids in these columns is conditioned by the analyte's interactions with the adsorbent (polarity, pH and elution strength). Since acid lipids have a negative electrical charge, they are more strongly retained in these columns, and an eluent of greater ionic strength is required to release these compounds.

After this procedure, the amount of PL and GL in each TLC bands was estimated using colourimetric assays to obtain the relative abundance of each lipid class (Table 10).

Table 10 - Estimation of phospholipids and glycolipids in the thin-layer chromatography bands (relative abundance in % w/w)

TLC band	Lipid Classes	% PL (± SD)	% GL (± SD)
1	-	13.86	31.10
2	-	5.48	11.92
3	ASG*/ others	10.56	12.21
4	-	17.4 ± 5.45	
5	-	28.29 ± 4.28	
6	PG	5.02 ± 1.72	
7	DGTS/ others	5.36	10.88
8	MGDG		29.73 ± 14.32
9	PI	9.81 ± 4.11	
10	PC	21.87 ± 2.06	
11	DGDG		37.23 ± 18.73

PL, phospholipid; GL, glycolipid; *ASG were not identified previously in the olive pulp but in the olive seeds (147).

This quantification suggested that the most abundant identified PL classes were PC and PI, representing 21.87 ± 2.06% and 9.81 ± 4.11% of the total PL. For GL, DGDG and MGDG were the most representative classes, representing 37.23 ± 18.73% and 29.73 ± 14.32% of the total GL, along with band #1 (31.10%). In fact, several unidentified bands also contained glucose, suggesting the presence of other GL that could not be identified. That was the case of bands #1 and #2 that quantified both phosphorus and glucose, suggesting the simultaneous presence of PL and GL, or more complex lipids such as phosphoglycolipids or glycophospholipids, that have both phosphate and glycosidic moieties in the same molecule. Another example is spot #7 that quantified phosphorus and glucose, but it was assigned to DGTS (betaine lipids), by comparing with the lipid standard with the same R_f. However, DGTS has neither phosphorus nor glucose in its structure, but these lipids have already been identified by LC-MS/MS in olives (32) and

olive oil (29) in trace amount. In this case co-elution with another lipid class cannot be excluded.

These results evidenced the limitation of this technique in characterising the olive pulp's polar lipid fraction. It only indicated the lipid classes by comparing them with the most common plant lipid standards. Another critical factor underlying one-dimensional TLC is that one band may represent more than one lipid class and lipids with the same R_f, co-elute. For instance, this happens with PG and SGDG, that migrate in the same band in the TLC run, as found in the olive seeds (158). Moreover, it was impossible to estimate PL and GL simultaneously in all spots due to the scarcity of experimental replicates.

Notwithstanding, this TLC allowed to evaluate semi-qualitatively and semi-quantitatively the olive fruit's polar lipidome quickly and straightforwardly, giving further clues for the LC-MS analysis of the polar lipid classes. This technique allows a subsequent separation of the fractions resulting from SPE and allows to observe in a simple way the complexity of the polar lipidome of the olive pulp, that could not be unravelled so far with such detail. Combining this technique with other higher sensitivity and resolution techniques, such as LC-MS, make lipidomics' potential undeniable for olive's polar lipid characterisation.

3.2.2. Fractionation of the samples by SPE

After the initial set of SPE experiments, an SPE procedure was performed for each sample of the four groups (4 x n=5) to recover the polar lipids, following the procedure described in Section 2.7.

3.2.2.1. Phospholipid and glycolipid contents in the polar lipid fractions and evaluation of polar lipids recovery rate

The PL and GL contents in the polar lipid-rich fractions were estimated and compared with predicted PL and GL estimates considering the amount of oil applied in the SPE column and the estimated GL/PL ratio in each sample. This comparison allowed to assess the recovery rate of PL and GL after oil fractionation.

The average recovery of PL and GL after fractionation was 56.49 ± 24.23 % and 13.78 ± 7.61%, respectively (Table 11). It was possible to obtain a better recovery rate in

the varieties of the Trás-os-Montes region, regarding PL. *Cobrançosa* had a recovery of 65.54 ± 14.62%, and *Arbequina* had a recovery of 77.50 ± 18.10%. For the Dão region, the varieties had a lower recovery rate, with *Galega Vulgar* achieving a recovery of PL of 47.62 ± 16.86% and *Arbequina* achieving 30.91 ± 20.42% (Table 11).

Table 11 - Recovery rates of phospholipids and glycolipids after fractionation of the olive pulp oil by solid-phase extraction

Samples		RR of PL (%)	RR of PL ± SD (%)	Global average of PL ± SD (%)	RR of GL (%)	RR of GL ± SD (%)	Global average of GL ± SD (%)	Ratio GL/PL ± SD	Global Average Ratio GL/PL ± SD
Galega Vulgar from Dão	1	20,21	47.62 ± 16.86	56.49 ± 24.23	5,27	13.00 ± 7.26	13.78 ± 7.61	1.77 ± 0.30	1.81 ± 1.06
	2	60,51			12,26				
	3	62,36			22,71				
	4	35,54			11,02				
	5	59,46			12,62				
Arbequina from Dão	1	19,19	30.91 ± 20.42		12,48	10.97 ± 6.34		1.18 ± 0.45	
	2	12,65			NA				
	3	65,23			17,88				
	4	26,55			2,56				
	5	NA			NA				
Cobrançosa from Trás-os-Montes	1	47,80	65.54 ± 14.62		21,16	16.21 ± 5.01		2.84 ± 1.27	
	2	66,13			18,99				
	3	88,14			20,61				
	4	60,10			10,35				
	5	62,00			9,93				
Arbequina from Trás-os-Montes	1	71,64	77.50 ± 18.10		3,80	13.51 ± 9.65		1.20 ± 0.75	
	2	96,39			26,81				
	3	99,71			13,65				
	4	51,74			1,95				
	5	68,00			21,34				

Percentages are expressed in w/w. RR, recovery rate; PL, phospholipids; GL, glycolipids, SD, standard deviation; NA, not available (these estimations were not possible to obtain due to experimental errors). Global averages were calculated based on the values of all samples (*n*=20).

The results suggest that most PL can be recovered after SPE fractionation. There was no uniformity in the results obtained both within each group and between groups. However, this SPE method showed an efficiency of up to 99.71 % in the PL recovery after fractionating the olive pulp oil (Table 11, sample #3 of *Arbequina* from Trás-os-Montes). On the other hand, the recovery of GL was much lower. *Cobrançosa* showed a recovery of 16.21 ± 5.01%, followed by *Arbequina* from Trás-os-Montes, 13.51 ± 9.65%, *Galega Vulgar*, 13.00 ± 7.26%, and *Arbequina* from Dão, 10.97 ± 6.34% (Table 11). As a result of these GL quantifications, the GL/PL ratio obtained after SPE was about 1.81, much lower than the GL/PL ratio obtained for the oil, about 7.65 (Section 3.1.3).

Comparing the GL content between the polar fraction and the oil may be biased, for example, due to the presence of lipid or lipid-soluble compounds with sugar residues that have intermediate polarity. These compounds having sugar residues were quantified in the oil samples, but they were eluted before the polar lipid fraction had been collected due to their polarity. For example, free sterol glucosides and acylated sterol glucosides, which are phytosterol conjugates wherein a glucose moiety (esterified to a fatty acid, in the second class) is linked to the sterol moiety, have been reported in olive oil (159,160). They may contribute to higher glucose levels in the pulp oil than the fraction obtained from the SPE. As such, it is not because we have a lower GL recovery rate that the GL has not been significantly recovered, and the comparison of the GL content between the oil and the polar lipid fraction may be less relevant. However, it must also be considered that the SPE system with the volumes used in this work, may not yet be the ideal for the recovery of these lipid classes in the olive pulp. As stated earlier, this new SPE procedure has been optimised in a previous master's thesis on lipidomics of the olive seeds, where recovery rates of PL and GL were above 90%. However, it had not been tested in the olive pulp, where the abundance of PL is relatively lower than the abundance found in the seed (147), and the GL content is much higher.

Another factor that can be considered is the fact that this fractionation process is not automated. The SPE was not all done strictly under the same conditions of flow or pressure because it was a semi-automated procedure, thus depends mostly on the operator and is time-consuming.

Despite all this, this improved fractionation process is very relevant because it allows obtaining the polar lipid-rich fractions with a broader lipidome that includes cationic,

zwitterionic and anionic classes, which has highly improved the method used for olive pulp polar lipid fingerprinting in our laboratory (32) and was one of the main goals of this work. Additionally, it effectively removes the largest lipid class from the olive pulp in the first elution, the TAG fraction. It represents one of the significant problems encountered for untargeted lipidomics analysis of polar lipids in olives and olive oil that implies a previous fractionation step, as SPE, to eliminate TAG and sterols, and other non-polar lipids. This problem has now been solved with the current SPE protocol, thus enabling an improved LC-MS analysis.

3.3. Characterisation of the polar lipidome of the olive pulp

After obtaining the polar lipid-rich fractions from SPE, the olive pulp's polar lipid classes and molecular species were identified and structurally characterised by LC-MS/MS.

3.3.1. Polar lipid profile

The polar lipid-rich fractions obtained from SPE were analysed by HILIC-ESI-MS/MS while using an Orbitrap mass spectrometer. The identification of the polar lipid molecular species was based on their retention time compared with internal standards, mass accuracy (mass error $\leq$ 5 ppm), and the MS/MS spectra analysis of each ion. The elution of the different polar lipid classes is shown in the total ion chromatogram (TIC) in the positive-ion mode (Fig. 16A) and the negative-ion mode (Fig. 16B). Twenty-one classes and sub-classes (lyso forms) of polar lipids were identified, including PL, GL, sphingolipids (SL), and betaine lipids (BL) (Fig. 16). In total, one hundred and eighty-eight lipid species could be identified: one hundred and thirty-two corresponding to PL, thirty-five to GL, fifteen to sphingolipids, and six to betaines (Table 12).

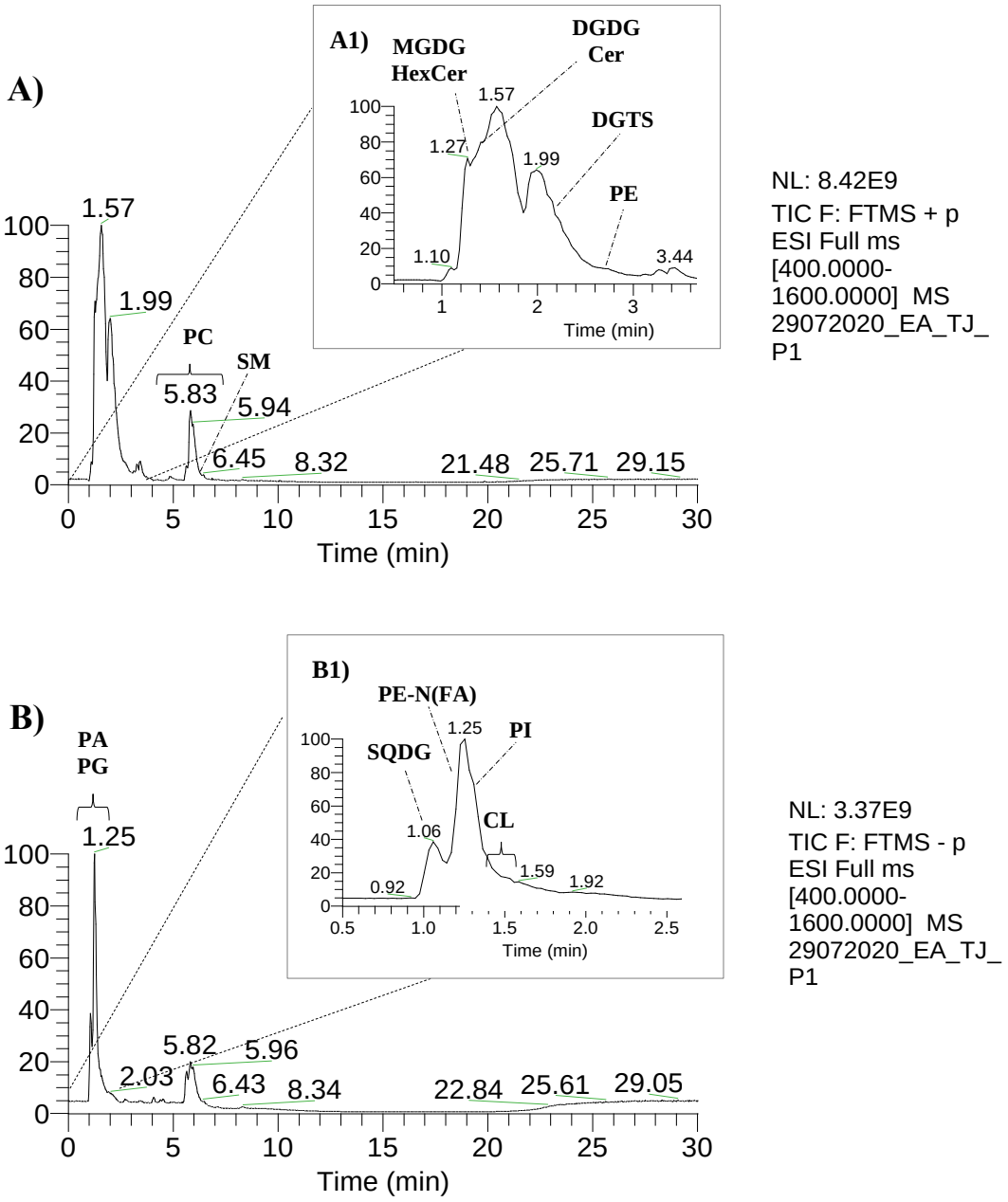


*Figure 16 - Total ion current (TIC) chromatograms of the polar lipid-rich fraction of the olive (*Olea europaea *L. cv.* Galega Vulgar*) pulp obtained by HILIC-ESI-MS in the positive-ion mode (A and A1) and negative-ion mode (B and B1). Polar lipid classes identified include phosphatidylcholines (PC), sphingomyelins (SM), monoglycosyldiacylglycerols (MGDG), hexosylceramides (HexCer), diglycosyldiacylglycerols (DGDG), ceramides (Cer), diacylglyceryl-N,N,N trimethylhomoserines (DGTS), phosphatidylethanolamines (PE), phosphatidic acids (PA), phosphatidylglycerols (PG), sulphoquinovosyldiacylglycerols (SQDG), N-acyl PE [PE-N(FA)], phosphatidylinositols (PI) and cardiolipins (CL).*

*Table 12 - Molecular species of polar lipids (phospholipids, glycolipids, sphingolipids and betaine lipids) identified in the olive (*Olea europaea *L.) pulp oil by HILIC HPLC-ESI-MS and HILIC HPLC-ESI-MS/MS*

Lipid Name	Formula	Observed Mass	Exact Mass	Mass error (ppm)	t_R (min)	Acyl Chain Composition
Class(C:N)		**$[M-H]^-$**				
PG(30:0)[a)]	C36H70O10P	693,4696	693,4707	-1,59	1,2	NA
PG(32:1)	C38H72O10P	719,4880	719,4863	2,36	1,23	14:0-18:1 and 16:0-16:1
PG(32:0)	C38H74O10P	721,5032	721,5020	1,66	1,23	16:0-16:0
PG(34:4)	C40H70O10P	741,4707	741,4707	0,00	1,2	16:0-18:4 and 16:1-18:3
PG(34:3)	C40H72O10P	743,4897	743,4863	4,57	1,20	16:1-18:2 and 16:0-18:3
PG(34:2)	C40H74O10P	745,5032	745,5020	1,61	1,20	16:0-18:2 and 16:1-18:1
PG(34:1)	C40H76O10P	747,5189	747,5176	1,74	1,20	16:0-18:1 and 16:1-18:0
PG(34:0)	C40H78O10P	749,5305	749,5333	-3,74	1,20	16:0-18:0
PG(36:6)*	C42H70O10P	765,4677	765,4707	-3,92	1,25	NA
PG(36:4)	C42H74O10P	769,5027	769,5020	0,91	1,20	18:2-18:2 and 18:1-18:3
PG(36:3)	C42H76O10P	771,5180	771,5176	0,52	1,20	18:1-18:2 and 18:3-18:0
PG(36:2)	C42H78O10P	773,5334	773,5333	0,13	1,20	18:0-18:2 and 18:1-18:1
PG(36:1)	C42H80O10P	775,5486	775,5489	-0,39	1,20	18:1-18:0
PG(36:0)	C42H82O10P	777,5608	777,5646	-4,89	1,2	18:0-18:0
		$[M-H]^-$				
LPG(14:0)	C20H40O9P	455,2416	455,2410	1,32	1,34	C14:0
LPG(16:0)	C22H44O9P	483,2739	483,2723	3,31	1,34	C16:0
LPG(18:1)	C24H46O9P	509,2873	509,2879	-1,27	1,34	C18:1
		$[M+H]^+$				
PE(30:3)*	C35H65NO8P	658,4419	658,4448	-4,40	2,77	NA
PE(32:2)	C37H71NO8P	688,4902	688,4917	-2,23	2,63	16:1-16:1
PE(32:0)	C37H75NO8P	692,5224	692,5230	-0,91	2,74	16:0-16:0
PE(34:2)	C39H75NO8P	716,5209	716,5230	-2,98	2,63	16:0-18:2, 16:1-18:1 and 17:1-17:1

PE(34:1)	C39H77NO8P	718,5374	718,5387	-1,78	2,63	16:0-18:1
PE(36:5)[a)]	C41H73NO8P	738,5051	738,5074	-3,09	2,55	NA
PE(36:4)	C41H75NO8P	740,5201	740,5230	-3,92	2,63	NA
PE(36:3)	C41H77NO8P	742,5379	742,5387	-1,05	2,55	18:2-18:1
PE(36:2)	C41H79NO8P	744,5528	744,5543	-2,01	2,57	18:1-18:1 and 18:0-18:2
		$[M+H]^+$				
LPE(16:1)	C21H43NO7P	452,2792	452,2777	3,28	4,02	C16:1
LPE(16:0)	C21H45NO7P	454,2929	454,2934	-1,03	4,05	C16:0
		$[M-H]^-$				
PE-N(FA) 52:4*	C57H103NO9P	976,7397	976,7370	2,72	1,14	NA
PE-N(FA) 52:3	C57H105NO9P	978,7536	978,7527	0,92	1,14	18:2-18:1-16:0 and 18:2-18:0-16:1 and 18:1-18:1-16:1 and 18:3-18:0-16:0
PE-N(FA) 52:2	C57H107NO9P	980,7686	980,7683	0,26	1,14	16:0-18:2-18:0 and 16:0-18:1-18:1
PE-N(FA) 54:6	C59H103NO9P	1000,7389	1000,7370	1,85	1,14	18:2-18:2-18:2 and 18:1-18:2-18:3
PE-N(FA) 54:5	C59H105NO9P	1002,7539	1002,7527	1,20	1,14	18:1-18:2-18:2 and 18:1-18:3-18:1 and 18:3-20:2-16:0 and 18:2-20:3-16:0
PE-N(FA) 54:4	C59H107NO9P	1004,7684	1004,7683	0,10	1,14	18:1-18:1-18:2N and 18:0-18:2-18:2N and 18:3-18:0-18:1N and 18:1-18:2-18:1N [b)]
PE-N(FA) 54:3	C59H109NO9P	1006,7844	1006,7840	0,40	1,14	18:1-18:1-18:1N and 18:2:18:0-18:1N [b)]
		$[M-H]^-$				
PI(32:1)	C41H76O13P	807,5049	807,5024	3,10	1,34	16:0-16:1
PI(34:3)	C43H76O13P	831,5024	831,5024	0,00	1,31	16:0-18:3
PI(34:2)	C43H78O13P	833,5173	833,5180	-0,84	1,34	16:0-18:2 and 16:1-18:1
PI(34:1)	C43H80O13P	835,5343	835,5337	0,72	1,31	16:0-18:1 and 16:1-18:0
PI(34:1(OH))*	C43H80O14P	851,5305	851,5286	2,26	1,34	NA
PI(36:4)	C45H78O13P	857,5167	857,5180	-1,52	1,31	18:2-18:2 and 18:3-18:1
PI(36:3)	C45H80O13P	859,5344	859,5337	0,81	1,31	18:1-18:2
PI(36:2)	C45H82O13P	861,5499	861,5493	0,70	1,31	18:1-18:1 and 18:0-18:2

PI(36:1)	C45H84O13P	863,5626	863,5650	-2,78	1,31	18:0-18:1
		[M-H]$^-$				
LPI(16:0)	C25H48O12P	571,2901	571,2883	3,08	1,48	C16:0
		[M-H]$^-$				
PA(34:2)	C37H68O8P	671,4661	671,4652	1,34	1,06	16:0:18:2 and 16:1-18:1
PA(34:1)	C37H70O8P	673,4816	673,4809	1,04	1,20	16:0-18:1
PA(36:4)	C39H68O8P	695,4672	695,4652	1,37	2,87	18:2-18:2 and 18:3-18:1
PA(36:3)	C39H70O8P	697,4815	697,4809	0,86	1,23	18:2-18:1
PA(36:2)	C39H72O8P	699,4976	699,4965	1,57	1,17	18:2-18:0 and 18:1-18:1
PA(38:2)*	C41H76O8P	727,5264	727,5278	-1,92	1,20	NA
PA(38:1)*	C41H78O8P	729,5464	729,5435	3,98	1,20	NA
		[M-H]$^-$				
LPA(16:0)	C19H38O7P	409,2374	409,2355	4,64	1,31	C16:0
		[M+H]$^+$				
PC(24:0(OH))[a)]	C32H65NO9P	638,4367	638,4397	-4,70	6,81	NA
PC(26:2)[a)]	C34H65NO8P	646,4434	646,4448	-2,17	6,84	NA
PC(16:0/C8CHO)[a)]	C33H65NO9P	650,4406	650,4397	1,38	6,78	NA
PC(26:2(OH))	C34H65NO9P	662,4422	662,4397	3,77	7,14	18:1-8:1(OH)
PC(26:1(OH))*	C34H67NO9P	664,4553	664,4553	0,00	6,23	NA
PC(27:2(OH))[a)]	C35H67NO9P	676,4560	676,4553	1,03	5,81	NA
PC(28:4(OH))[a)]	C36H65NO9P	686,4391	686,4396	-0,73	5,52	NA
PC(28:3(OH))[a)]	C36H67NO9P	688,4583	688,4553	4,36	5,67	NA
PC(30:3)	C38H71NO8P	700,4891	700,4917	-3,71	5,31	NA
PC(32:2)	C40H77NO8P	730,5375	730,5387	-1,64	5,75	16:1-16:1
PC(32:1)	C40H79NO8P	732,5525	732,5543	-2,46	5,72	16:0-16:1 and 14:0-18:1
PC(32:0)[a)]	C40H81NO8P	734,5677	734,5700	-3,11	6,25	NA
PC(31:0(OH))[a)]	C39H79NO9P	736,5525	736,5492	4,48	6,75	NA
PC(33:1)	C41H81NO8P	746,5718	746,5700	2,41	6,32	16:0-17:1
PC(32:1(OH))[a)]	C40H79NO9P	748,5465	748,5492	-3,61	5,77	NA

PC(34:5)[a)]	C42H75NO8P	752,5212	752,5230	-2,43	5,69	NA
PC(34:4)[a)]	C42H77NO8P	754,5361	754,5387	-3,45	5,47	NA
PC(34:3)	C42H79NO8P	756,5539	756,5543	-0,53	5,30	18:3-16:0 and 16:1-18:2
PC(34:2)	C42H81NO8P	758,5695	758,5700	-0,66	5,30	16:0-18:2 and 16:1-18:1
PC(34:1)	C42H83NO8P	760,5850	760,5856	-0,79	5,30	16:0-18:1 and 16:1-18:0
PC(35:4)[a)]	C43H79NO8P	768,5491	768,5486	0,65	6,06	NA
PC(34:4(OH))	C42H77NO9P	770,5313	770,5336	-2,98	6,22	NA
PC(35:3)	C43H81NO8P	770,5678	770,5700	-2,86	5,94	18:2-17:1 and 18:1-17:2
PC(34:3(OH))	C42H79NO9P	772,5482	772,5492	-1,29	6,17	16:0-18:3(OH) and 16:1-18:2(OH)
PC(35:2)	C43H83NO8P	772,5837	772,5856	-2,46	5,89	18:1-17:1
PC(34:2(OH))	C42H81NO9P	774,5640	774,5649	-1,16	6,08	16:0-18:2(OH)
PC(35:1)	C43H85NO8P	774,5990	774,6013	-2,97	5,94	18:1-17:0
PC(36:7)[a)]	C44H75NO8P	776,5200	776,5230	-3,86	6,31	NA
PC(34:1(OH))	C42H83NO9P	776,5773	776,5805	-4,12	6,28	16:0-18:1(OH)
PC(36:6)[a)]	C44H77NO8P	778,5353	778,5387	-4,37	6,03	NA
PC(36:5)	C44H79NO8P	780,5511	780,5543	-4,10	6,03	18:2-18:3
PC(35:5(OH))	C43H77NO9P	782,4914	782,4918	-0,51	6,72	NA
PC(36:4)	C44H81NO8P	782,5672	782,5670	0,26	5,97	18:2-18:2 and 18:3-18:1
PC(36:3)	C44H83NO8P	784,5846	784,5856	-1,27	5,86	18:1-18:2
PC(36:2)	C44H85NO8P	786,6003	786,6013	-1,27	5,83	18:1-18:1 and 18:2-18:0
PC(34:1$(OH)_2$)[a)]	C42H83NO10P	792,5735	792,5755	-2,52	6,83	NA
PC(37:5)[a)]	C45H81NO8P	794,5655	794,5670	-1,89	5,64	NA
PC(36:5(OH))	C44H79NO9P	796,5461	796,5492	-3,89	5,75	18:3-18:2(OH)
PC(36:4(OH))	C44H81NO9P	798,5648	798,5649	-0,13	5,30	18:1-18:3(OH) and 18:2-18:2(OH)
PC(36:3(OH))	C44H83NO9P	800,5795	800,5805	-1,25	5,27	18:1-18:2(OH) and 18:2-18:1(OH)
PC(37:2)	C45H87NO8P	800,6150	800,6169	-2,37	5,08	18:1-19:1
PC(36:2(OH))	C44H85NO9P	802,5946	802,5962	-1,99	5,30	18:1-18:1(OH)
PC(38:2)	C46H89NO8P	814,6304	814,6326	-2,70	5,41	18:1-20:1 and 18:2-20:0
PC(36:3$(OH)_2$)	C44H83NO10P	816,5747	816,5755	-0,98	6,17	18:1-18:2$(OH)_2$

PC(38:1)	C46H91NO8P	816,6500	816,6482	2,20	5,04	18:1-20:0
PC(36:2(OH)$_2$)	C44H85NO10P	818,5907	818,5911	-0,49	6,56	18:1-18:1(OH)$_2$ and 18:1(OH)-18:1(OH)
PC(37:0(OH))[a)]	C45H91NO9P	820,6426	820,6431	-0,61	5,97	NA
PC(40:5)[a)]	C48H87NO8P	836,6133	836,6169	-4,34	6,01	NA
PC(40:2)[a)]	C48H93NO8P	842,6674	842,6639	4,15	4,98	NA
PC(40:1)[a)]	C48H95NO8P	844,6829	844,6795	4,03	4,96	NA
PC(41:1)[a)]	C49H97NO8P	858,6948	858,6952	-0,47	5,22	NA
PC(42:1)[a)]	C50H99NO8P	872,7090	872,7108	-2,06	5,16	NA
PC(43:1)[a)]	C51H101NO8P	886,7245	886,7265	-2,26	5,08	NA
PC(44:1)[a)]	C52H103NO8P	900,7414	900,7421	-0,81	5,33	NA
		[M+H]$^+$				
LPC(16:1)	C24H49NO7P	494,3237	494,3247	-2,02	8,75	C16:1
LPC(16:0)	C24H51NO7P	496,3393	496,3403	-2,01	8,94	C16:0
LPC(18:3)	C26H49NO7P	518,3245	518,3247	-0,39	8,67	C18:3
LPC(18:2)	C26H51NO7P	520,3392	520,3403	-2,11	8,50	C18:2
LPC(18:1)	C26H53NO7P	522,3555	522,3560	-0,96	8,50	C18:1
LPC(18:0)[a)]	C26H55NO7P	524,3713	524,3716	-0,57	8,12	NA
LPC(18:2(OH))	C26H51NO8P	536,3353	536,3352	0,19	9,08	C18:2(OH)
LPC(18:1(OH))	C26H53NO8P	538,3518	538,3509	1,67	9,11	C18:1(OH)
LPC(20:1)*	C28H57NO7P	550,3871	550,3873	-0,36	8,44	NA
LPC(20:0)*	C28H59NO7P	552,4034	552,4029	0,91	7,46	NA
LPC(22:0)	C30H63NO7P	580,4360	580,4342	3,10	7,42	C22:0
LPC(24:0)*	C32H67NO7P	608,4658	608,4655	0,49	8,07	NA
		[M+H]$^+$				
SM(d36:1)*	C41H84N2O6P	731,6070	731,6067	0,41	7,31	NA
SM(t38:1)[a)]	C43H88N2O7P	775,6314	775,6329	-1,93	6,98	NA
SM(t40:0)[a)]	C45H94N2O7P	805,6796	805,6799	-0,37	6,28	NA
SM(t41:1)[a)]	C46H94N2O7P	817,6788	817,6799	-1,35	6,09	NA

		$[M-H]^-$				
CL(70:4)	C79H145O17P2	1427,9947	1427,9960	-0,91	1,42	18:0-(18:2)2-16:0 and (18:1)2-18:2-16:0 and 18:0-18:2-18:1-16:1 and (18:1)3-16:1
CL(70:3)	C79H147O17P2	1430,0075	1430,0110	-2,45	1,48	(18:1)3-16:0
CL(72:8)	C81H141O17P2	1447,9641	1447,9640	0,07	1,75	(18:2)4 and (18:3)2-(18:1)2 and (18:2)2-18:3-18:1
CL(72:7)	C81H143O17P2	1449,9807	1449,9800	0,48	1,42	(18:3)2-18:1-18:0 and 18:3-(18:1)2-18:0 and (18:2)2-18:3-18:0 and (18:2)3-18:1
CL(72:6)	C81H145O17P2	1451,9976	1451,9960	1,10	1,45	(18:2)2-(18:1)2
CL(72:5)	C81H147O17P2	1454,0115	1454,0110	0,34	1,42	18:2-(18:1)3 and (18:2)2-18:1-18:0
CL(74:10)[a)]	C83H141O17P2	1471,9608	1471,9640	-2,17	1,62	NA
CL(74:9)[a)]	C83H143O17P2	1473,9746	1473,9800	-3,66	1,53	NA
CL(74:8)[a)]	C83H145O17P2	1475,9901	1475,9960	-4,00	1,56	NA
		$[M+NH_4]^+$				
DGDG(34:3)	C49H90NO15	932,6290	932,6310	-2,14	1,38	16:0-18:3
DGDG(34:2)	C49H92NO15	934,6459	934,6467	-0,86	1,21	16:1-18:1 and 16:0-18:2
DGDG(34:1)	C49H94NO15	936,6657	936,6623	3,63	1,91	16:0-18:1
DGDG(36:6)	C51H88NO15	954,6134	954,6154	-2,10	1,35	18:3-18:3 and 18:2-18:4
DGDG(36:4)	C51H92NO15	958,6447	958,6467	-2,09	1,35	18:1-18:3 and 18:2-18:2
DGDG(36:3)	C51H94NO15	960,6580	960,6623	-4,48	1,35	18:1-18:2
DGDG(36:2)	C51H96NO15	962,6759	962,6780	-2,18	1,35	18:1-18:2
		$[M+NH_4]^+$				
MGDG(34:4)	C43H78NO10	768,5628	768,5626	0,26	1,24	16:1-18:3
MGDG(34:3)	C43H80NO10	770,5763	770,5782	-2,50	1,24	16:0-18:3
MGDG(34:1)*	C43H84NO10	774,6112	774,6090	2,84	1,27	NA
MGDG(36:6)	C45H78NO10	792,5618	792,5625	-0,88	1,27	18:3-18:3
MGDG(36:4)	C45H82NO10	796,5916	796,5933	-2,13	1,27	18:2-18:2
MGDG(36:3)	C45H84NO10	798,6060	798,6095	-4,41	1,24	18:1-18:2

MGDG(36:2)	C45H86NO10	800,6237	800,6252	-1,84	1,27	18:1-18:1 and 18:0-18:2
MGDG(38:1)*	C47H92NO10	830,6688	830,6721	-4,00	1,27	NA
MGDG(40:4)*	C49H90NO10	852,6565	852,6565	0,04	1,24	NA
		$[M-H]^-$				
SQDG(28:0)*	C37H69O12S	737,4537	737,4510	3,69	1,09	NA
SQDG(30:0)*	C39H73O12S	765,4830	765,4823	0,94	1,06	NA
SQDG(32:1)	C41H75O12S	791,4978	791,4979	-0,16	1,09	16:0-16:1
SQDG(32:0)	C41H77O12S	793,5149	793,5136	1,67	1,06	16:0-16:0
SQDG(34:3)	C43H75O12S	815,4984	815,4979	0,58	1,06	16:1-18:2 and 18:3-16:0
SQDG(34:2)	C43H77O12S	817,5169	817,5136	4,07	1,06	16:1-18:1 and 16:0-18:2
SQDG(34:1)	C43H79O12S	819,5301	819,5292	1,07	1,06	18:1-16:0
SQDG(36:6)	C45H73O12S	837,4827	837,4823	0,51	1,06	18:3-18:3
SQDG(36:5)	C45H75O12S	839,4979	839,4979	-0,03	1,09	18:3-18:2
SQDG(36:4)	C45H77O12S	841,5141	841,5136	0,62	1,06	18:1-18:3
SQDG(36:3)	C45H79O12S	843,5291	843,5292	-0,15	1,06	18:3-18:0 and 18:1-18:2
SQDG(36:2)	C45H81O12S	845,5453	845,5449	0,50	1,06	18:1-18:1
SQDG(36:1)	C45H83O12S	847,5602	847,5605	-0,39	1,06	18:1-18:0
SQDG(37:2)	C46H83O12S	859,5620	859,5605	1,75	1,76	18:1-19:1
		$[M-H]^-$				
SQMG(14:0)*	C23H43O11S	527,2507	527,2526	-3,63	1,12	NA
SQMG(16:0)	C25H47O11S	555,2842	555,2839	0,52	1,14	C16:0
SQMG(18:1)	C27H49O11S	581,2969	581,2996	-4,58	1,12	C18:1
SQMG(22:1)*	C31H57O11S	637,3594	637,3622	-4,33	1,06	NA
SQMG(22:0)[a)]	C31H59O11S	639,3751	639,3778	-4,24	1,09	NA
		$[M+H]^+$				
Cer(t40:1(OH))	C40H80NO5	654,6028	654,6036	-1,22	1,30	t18:1-22:0(OH)
Cer(t42:2)*	C42H82NO4	664,6215	664,6244	-4,36	1,24	NA
Cer(t41:1(OH))	C41H82NO5	668,6187	668,6193	-0,90	1,30	t18:0-23:1(OH) and t18:1-23:0(OH)
Cer(t42:1(OH))	C42H84NO5	682,6340	682,6349	-1,32	1,30	t18:1-24:0(OH)

Cer(t43:1(OH))	C43H86NO5	696,6500	696,6506	-0,86	1,27	t18:1-25:0(OH)
		$[M+H]^+$				
HexCer(d34:2)*	C40H76NO8	698,5540	698,5510	4,29	1,30	NA
HexCer(d34:2(OH))	C40H76NO9	714,5510	714,5520	-1,40	1,30	d18:2-16:0(OH)
HexCer(d34:1(OH))	C40H78NO9	716,5670	716,5676	-0,84	1,66	d18:1-16:0(OH)
HexCer(t34:1)	C40H76NO9	716,5670	716,5676	-0,84	1,66	t18:1-16:0 and t18:0-16:1
HexCer(t40:1(OH))	C46H90NO10	816,6550	816,6565	-1,84	1,66	t18:1-22:0(OH)
HexCer(t42:2)	C48H92NO9	826,6740	826,6772	-3,87	1,24	t18:1-24:1
HexCer(t41:1(OH))	C47H92NO10	830,6689	830,6721	-3,85	1,30	t18:1-23:0(OH)
HexCer(t42:1)	C48H94NO9	828,6902	828,6929	-3,26	1,27	t18:1-24:0
HexCer(t42:1(OH))	C48H94NO10	844,6860	844,6878	-2,13	1,30	t18:1-24:0(OH)
HexCer(t44:1(OH))	C50H97NO10	872,7169	872,7191	-2,52	1,30	t18:1-26:0(OH)
		$[M+H]^+$				
DGTS(34:2)	C44H82O7N	736,6073	736,6091	-2,44	2,35	NA
DGTS(34:1)	C44H84O7N	738,6223	738,6248	-3,38	2,32	16:0-18:1 and 14:0-20:1
DGTS(36:4)	C46H82O7N	760,6084	760,6091	-0,92	2,18	18:2-18:2
DGTS(36:3)	C46H84O7N	762,6223	762,6248	-3,28	2,27	18:1-18:2
DGTS(36:2)	C46H86O7N	764,6381	764,6404	-3,01	2,30	18:1-18:1
		$[M+H]^+$				
MGTS(16:0)	C26H52O6N	474,3781	474,3795	-2,95	3,33	C16:0

All molecular species were identified by MS/MS, exact mass (*m/z*) and retention time (t_R), except those with an asterisk (*) that were identified only by exact mass and by retention time. [a] The polar head of these species was confirmed by MS/MS, however the fatty acyl chains could not be confirmed by MS/MS since the informative MS/MS spectrum could not be obtained in the negative-ion mode. [b] The letter N joined to a fatty acid represents the binding of that fatty acid to the amine group of the polar head. Numbers in parentheses (C:N) indicate the number of carbon atoms (C) and double bonds (N) in the fatty acid side chain.

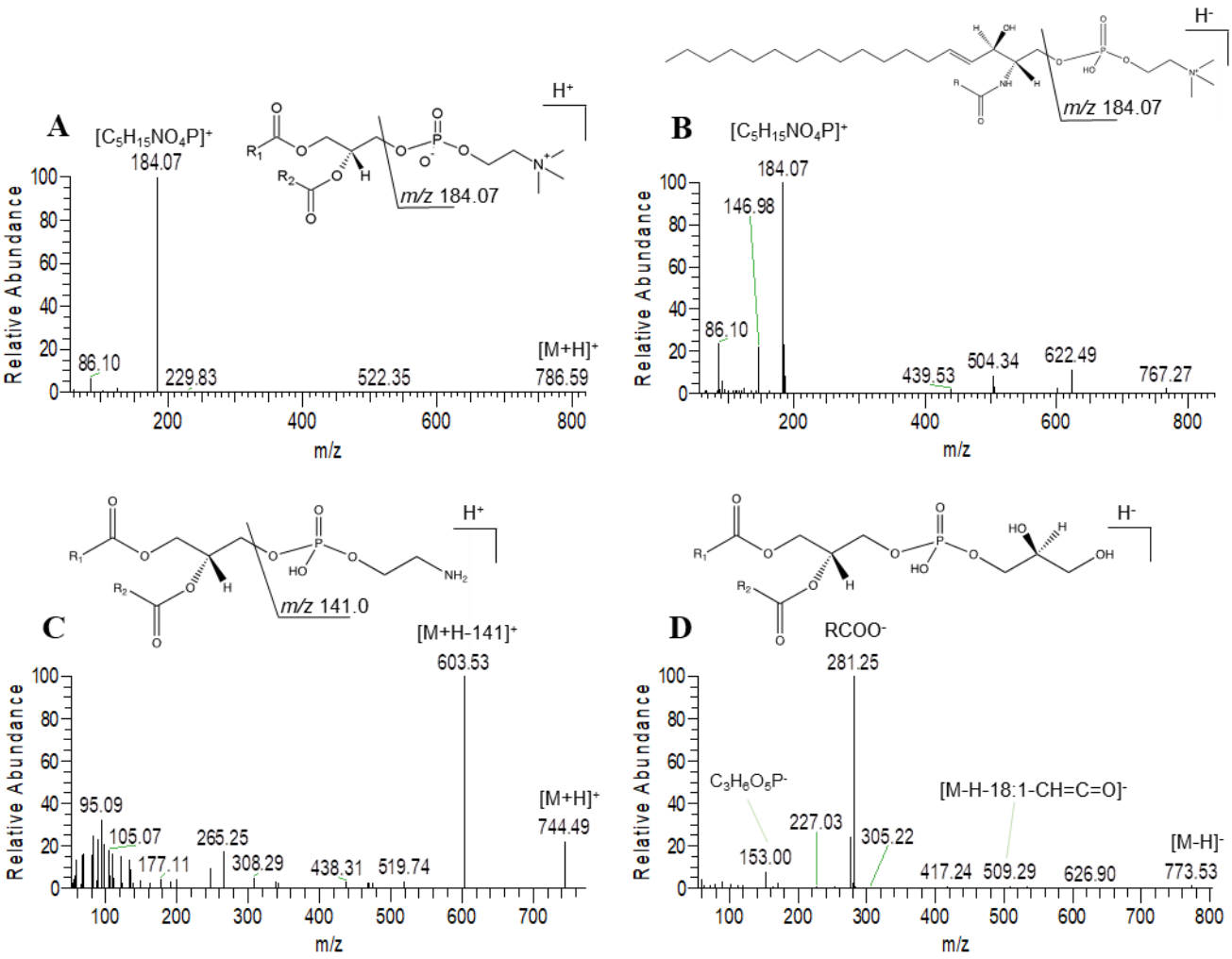
3.3.1.1. Phospholipids

In this study, the highest diversity of classes was represented by PL (thirteen classes and sub-classes), which comprised molecular species belonging to: phosphatidylglycerols (PG, fourteen species); lysoPG (LPG, three species); phosphatidylethanolamines (PE, nine species); lysoPE (LPE, two species); *N*-acyl phosphatidylethanolamines [PE-N(FA), seven species]; phosphatidylinositols (PI, nine species); lysoPI (LPI, one species); phosphatidic acids (PA, seven species), lysoPA (LPA, one species); phosphatidylcholines (PC, fifty-four species); lysoPC (LPC, twelve species); sphingomyelins (SM, four species); and cardiolipins (CL, nine species), as shown in Table 12.

PC, LPC, SM, PE and LPE were identified in the positive-ion mode as $[M + H]^+$ ions and the analysis of the corresponding ions in the MS/MS spectra allowed to confirm the identity of the polar head groups (158,161). PC, LPC, and SM were also identified in the negative-ion mode as $[M + CH_3COO]^-$ adducts. Molecular species of PG, LPG, PE-N(FA), PI, LPI, PA, LPA and CL were identified in the negative-ion mode, as $[M - H]^-$ molecular ions. Their MS/MS spectra showed the typical fragmentation, as characteristic diagnostic ions corresponding to the polar head group or typical neutral losses. The MS/MS spectra of the PL molecular species in the negative-ion mode allowed to confirm the fatty acyl composition by the carboxylate anions ($RCOO^-$) (161).

Representative MS/MS spectra of the PL classes found in the olive pulp are shown in Fig. 17. Their interpretation was made according to well-known fragmentation patterns, as described in the literature. Briefly, the MS/MS of PC (Fig. 17A), LPC and SM (Fig. 17B) as $[M + H]^+$ ions showed the characteristic product ion at *m/z* 184.1, corresponding to the phosphocholine cation (polar head group) (158,161). The MS/MS spectra of PE (Fig. 17C) and LPE as $[M + H]^+$ ions yielded an abundant ion that corresponds to a neutral loss of 141 Da, due to the loss of the phosphoethanolamine (polar head group). The MS/MS spectra of PG as $[M - H]^-$ ions (Fig. 17D) showed product ions at *m/z* 153.0, corresponding to the cyclic phosphate anion (CPA), that is common to all glycerophospholipid classes, and a product ion at *m/z* 171.1, corresponding to the glycerol phosphate anion. PE-N(FA) as $[M - H]^-$ ions (Fig. 17E) showed product ions originating from the loss of the fatty acyl chains, first from the amino-group linked to the PE polar head, and subsequent fragmentation typical of PE, with the neutral loss of the fatty acyl chains both in the form of acids and ketenes (162).

Also, it was possible to observe a product ion at *m/z* 153.0, corresponding to CPA. The MS/MS spectra of PI as $[M - H]^-$ ions (Fig. 17F) showed a characteristic precursor ion at *m/z* 241.0, corresponding to the inositol-1,2-cyclic phosphate anion (polar head group) and the product ion at *m/z* 153.0, corresponding to CPA. PA were identified as $[M - H]^-$ ions (Fig. 17G), in which the MS/MS spectra showed the product ion at *m/z* 153.0, corresponding to CPA. MS/MS of CL as $[M - H]^-$ ions (Fig. 17H) showed the presence of a product ion at *m/z* 153.0, corresponding to CPA, and the product ions derived from different combined losses of fatty acyl chains, phosphate groups and glycerols (163).



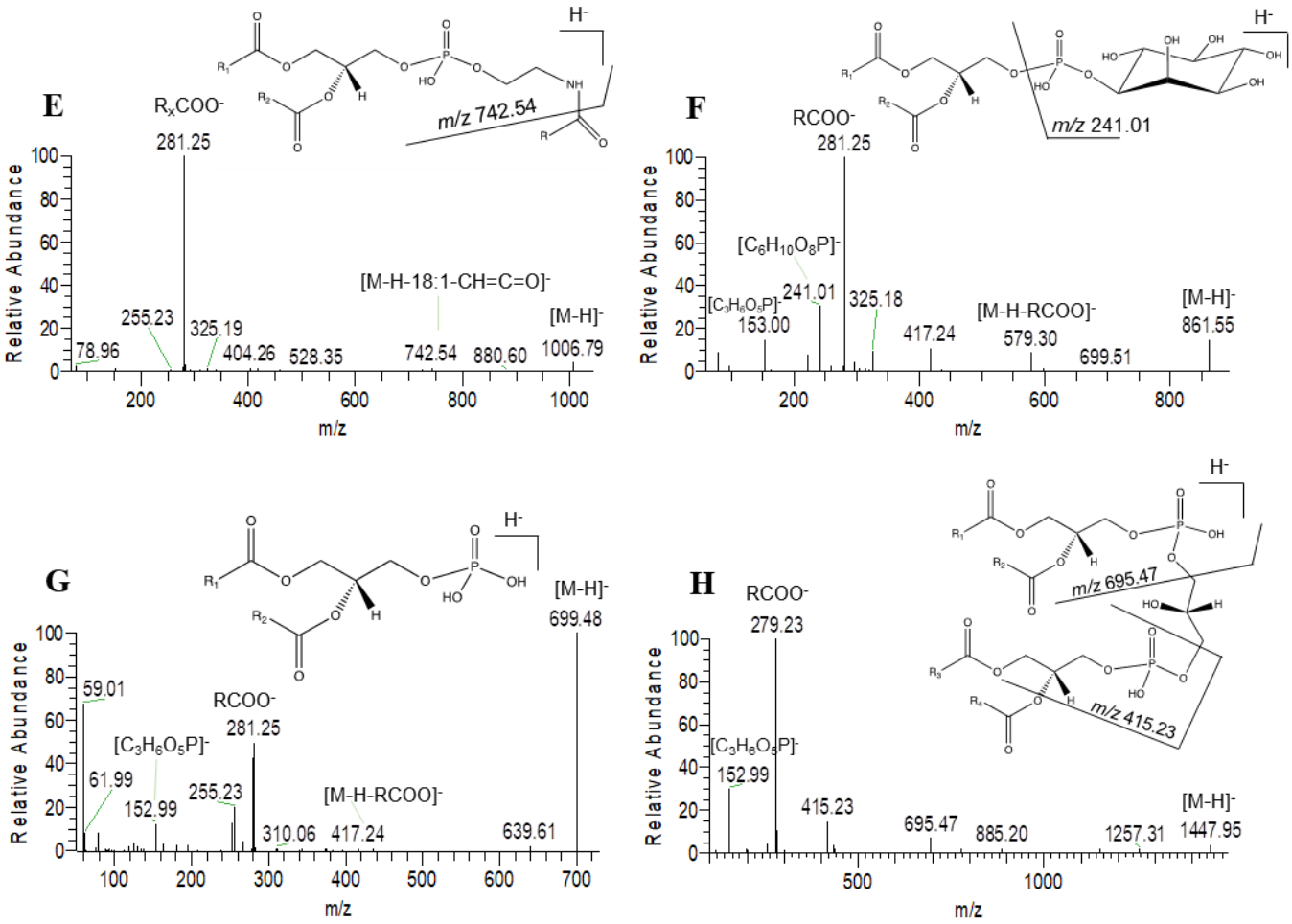


*Figure 17 – LC-MS/MS spectra of the phospholipid classes identified in the olive (*Olea europaea *L.) pulp. One MS/MS spectrum illustrative of each class is shown:* ***A)*** *PC(36:2) at m/z 786.59 as* $[M + H]^+$; ***B)*** *SM(t40:0) at m/z 767.27 as* $[M + H]^+$; ***C)*** *PE(36:2) at m/z 774.49 as* $[M + H]^+$; ***D)*** *PG(36:2) at m/z 773.53 as* $[M - H]^-$; ***E)*** *PE-N(FA)54:3 at m/z 1006.79 as* $[M - H]^-$; ***F)*** *PI(36:2) at m/z 861.55 as* $[M - H]^-$; ***G)*** *PA(36:2) at 699.48 as* $[M - H]^-$; *and* ***H)*** *CL(72:8) at m/z 1447.95 as* $[M - H]^-$. *A representative molecular structure of each lipid class is also shown.*

The most common fatty acyl chains esterified to the PL were the monounsaturated FA (MUFA) 18:1 and 16:1, the polyunsaturated FA (PUFA), 18:2 and 18:3, and the saturated FA (SFA), 18:0 and 16:0. PL also had a wide range of minor FA in their structures, with the carbon chains ranging between 8 to 24, and having up to four unsaturations, namely 14:0, 17:1, 17:2, 18:4, 19:1, 20:0, 20:1, 20:2, 20:3, and 24:0 (Table 12). In addition to these less common FA, hydroxy FA [FA(OH)] have also been identified, like 8:1(OH), 18:1(OH), $18:1(OH)_2$, 18:2(OH), $18:2(OH)_2$ and 18:3(OH) (Table 12). FA(OH) have been reported in higher plants in low amount. However, in this work, since the FA profile of the polar lipid

pool has not been analysed by GC-MS, other FA with the same exact mass as FA(OH) cannot be excluded, as they have been described in higher plants esterified to the polar lipids (164).

Fatty acyl chains between C14 and C18, with up to four unsaturations, were identified in the PG class. FA ranging from C16 to C18 were found in PI species, wherein the unsaturations were up to three. LPG molecular species esterified with FA ranging from C14 to C18 were observed. A single LPI was identified, containing the FA 16:0. PE had a variation of FA between C16 and C18, with up to two unsaturations. Only two species of LPE were reported, containing the FA 16:1 and 16:0. In its structure, PE-N(FA), a lipid class with three FA, bears FA from C16 to C20, which reached up to three unsaturations. PA comprised FA between C16 and C18, with up to two unsaturations. Only one species of LPA was reported, bearing the FA 16:0. PC was the polar lipid class with the highest number of molecular species identified, composed of fatty acyl chains ranging from C8 to C20, with up to three unsaturations, and several hydroxy FA. LPC molecular species bearing FA from C16 to C22 were observed, including hydroxylated LPC, as 18:1(OH) and 18:2(OH). SM with dihydroxy and trihydroxy long-chain bases were identified: one molecular species with a dihydroxy long-chain base ("d" species) and three with a trihydroxy (phytosphingosine) long-chain base ("t" species). CL, a lipid class with four FA in its structure, was composed by FA C16 and C18, with up to three unsaturations (Table 12).

Only two studies were published reporting the identification of PL molecular species in the olive fruit or the olive pulp by ESI-MS. In one study, the authors used non-aqueous capillary electrophoresis coupled to ESI-MS (NACE-ESI-MS) to analyse the PL in *Arbequina* olives' pulp (81). Seven PL classes and sub-classes were identified (PG, PI, PE, LPE, PA, LPA, and PC), bearing eight different FA (14:1, 16:0, 18:0, 18:1, 18:2, 18:3, 20:1 and 20:2) (81). All PL classes and sub-classes, and six lipid species reported in the study, namely PG(34:0), PG(34:1), PG(36:1, PG(36:2), PI(34:1), and PE(36:2), were common to the present study. In the second study, Alves *et al.* (2019) while using LC-MS approaches reported five PL classes and sub-classes in the pulp of *Galega* olives (PG, PE, PC, LPC, and SM) and seventy-seven lipid species, with FA chains ranging from C8 to C25, with up to three unsaturations, and with several PC and LPC bearing hydroxylated FA (32). All PL classes and sub-classes and most of the molecular species and fatty acyl chains reported by Alves *et al*. (2019) were also reported in this study.

Considering the scientific work that has been done so far on the detailed study of the polar lipidome of the olive pulp, the present work has provided a step forward in the knowledge of the PL pool. Significant findings can be highlighted as follows. Anionic PL classes PG, PI and PA and the anionic GL class SQDG could be accurately identified, with dozens of molecular species reported for the first time in the olive pulp. It was also the first time that complex PL classes, as PE-N(FA) and CL, and lysoPL (sub-classes), like LPG, LPI and LPE, were identified and structurally characterised in the olive pulp. It is also worth noticing that several molecular species of PE, PC and LPC classes have not been described previously.

Some of this information is corroborated by recent studies on olive oil while using LC-MS and NACE-MS. These studies reported PE, PC, LPC, PA, PG, PI, LPG, LPI, and LPE (29–31), which have been poorly reported or not reported in the olive fruit. As far as it is concerned, PE-N(FA) and CL have not been reported either in the olive fruit or in olive oil.

A recent study analysed the PE-N(FA) profile in several plants- and animal-food products by LC-MS, but could not found these lipids in extra virgin olive oil (165). PE-N(FA) is a nitrogen-containing lipid class composed of 1,2-diacyl-*sn*-glycero-3-phosphatidylethanolamine (PE) with a third fatty acyl chain linked to the amino group of the polar head through an amide bond. PE-N(FA) is a membrane PL, and it is found in animals, plants, bacteria and yeasts (165). In plants, PE-N(FA) are involved in supporting the membrane's structural integrity and function as precursors of lipid mediators, as *N*-acylethanolamines (166). These lipids are intrinsically involved in various physiological processes in plants, such as the elongation of the roots, regulation of seed germination, and defence from pathogenic attacks (162). It is also worth noticing that the studies suggest that PE-N(FA) are not abundant under physiological conditions, although their level increases under abiotic and biotic stresses, revealing a protective role in intracellular membranes of plants (162). Biomedical studies indicate that this PL class, together with its originating mediators, may have various biological functions such as regulating inflammation, appetite and pain, and regulating lipid metabolism, blood glucose and body weight (165).

On the other hand, CL is an anionic membrane PL composed of a dimeric structure with four acyl chains bound to two glycerol moieties linked via two phosphate groups to a third glycerol. CL can be found in the cytoplasmic membrane in prokaryotes and exclusively

located in eukaryotes' inner mitochondrial membrane (167,168). In animals and yeasts, the role of CL in maintaining the functional integrity and dynamics of mitochondria is well known. In these matrices, CL play a central role in mitochondrial metabolism, biogenesis and fusion-division dynamics, by maintaining the proper architecture and morphology of the mitochondrial membranes, by regulating the activity of several proteins and enzymes involved in mitochondrial function, and by influence events of apoptotic programmed cell death, among other actions (168,169). However, in plants, wherein mitochondria have a distinct morphology, CL's content, profile, and role are still poorly studied (163). Plant CL appear to be involved in several processes, such as the maintenance of the mitochondrial ultrastructure, the regulation of the respiratory function, the development of the plant embryo, and the protoplast response to UV light and heat shock (163,170,171). It has also been shown that plant CL mediate specific mitochondrial processes by stabilising protein complexes and having a protective role in the plant response to stresses that induce, for example, programmed cell death (163,168). Knowing the vital role of CL, it is not surprising that these lipids are relevant in maintaining good human health. Indeed, the literature reports correlations between alterations in CL profile, such as oxidations, and mitochondrial dysfunction in multiple tissues in many physio-pathological conditions, including heart failure, myocardial injury, diabetes and neurodegenerative diseases (169).

3.3.1.2. Glycolipids

Four GL classes and sub-classes were identified and structurally characterised in the olive pulp: diglycosyldiacylglycerols (DGDG, seven species), monoglycosyldiacylglycerols (MGDG, nine species), sulphoquinovosyldiacylglycerols (SQDG, fourteen species), and sulphoquinovosylmonoacylglycerols (SQMG, five species), as shown in Table 12. DGDG and MGDG were identified in the positive-ion mode, as $[M + NH_4]^+$ adducts (172,173). SQDG and SQMG were identified in the negative-ion mode as $[M - H]^-$ molecular ions (161). Illustrative MS/MS spectra of the GL classes are shown in Fig. 18.

As for PL, GL's structural characterisation was done by the interpretation of the MS/MS spectra according to their typical fragmentation under ESI-MS/MS conditions. MGDG as $[M + NH_4]^+$ ions (Fig. 18A) showed product ions resulting from the combined neutral loss of a hexose (180 Da) with ammonia (17 Da) from the molecular ion. For DGDG

as $[M + NH_4]^+$ ions (Fig. 18B), the MS/MS spectra showed product ions resulting from the combined neutral loss of two hexoses (162 Da + 180 Da) with ammonia (17 Da) from the molecular ion. In the MS/MS spectra of DGDG and MGDG, the identification of the acyl chains was possible due to the characteristic acylium ions plus 74 ($[RCO + 74]^+$), which were formed by the combined loss of one FA and the hexose moiety (161). SQDG and their lyso form, SQMG, as $[M - H]^-$, showed the characteristic product ion at *m/z* 225.0 (Fig. 18C), corresponding to the sulphoquinovosyl anion ($[C_6H_9O_7S]^-$, polar head group) (172,173). Fatty acyl chains were identified as product ions in the deprotonated carboxylic form ($RCOO^-$) (161).

In the GL pool, the more common FA were the MUFA 18:1 and 16:1, the PUFA 18:2 and 18:3, and the SFA 18:0 and 16:0. The FA 19:1 was also identified in SQDG (Table 12). DGDG comprised C16 and C18 FA, with up to four unsaturations. MGDG also had C16 and C18, with up to three unsaturations. FA ranging from C16 to C19, with up to three unsaturations, were found in SQDG. SQMG bear the FA 16:0 and 18:1.

In previous studies carried out on the identification of GL species in the olive fruit, Bianco *et al.* (1998) identified two molecular species of GL, namely DGDG(18:3/18:3) and DGDG(18:1/18:3), by HPLC-UV (51). Both lipid species were identified in the present work. Alves *et al*. (2019) reported three GL classes and sub-classes in the pulp of *Galega* olives (MGDG, DGDG, and DGMG) and nineteen lipid species bearing C16 and C18 FA, with up to four unsaturations (32). MGDG and DGDG and most of the molecular species were also reported in the present study. The current work allowed the identification and structural characterisation of species that were not previously identified within these classes for the olive pulp, e.g. MGDG(34:3). Likewise, the identification and structural characterisation of SQDG and SQMG species in olive pulp were reported herein for the first time. SQDG and two lipid species of SQMG were found in extra virgin olive oil by Alves *et al.* (2016) (29), corroborating the data obtained in this work.

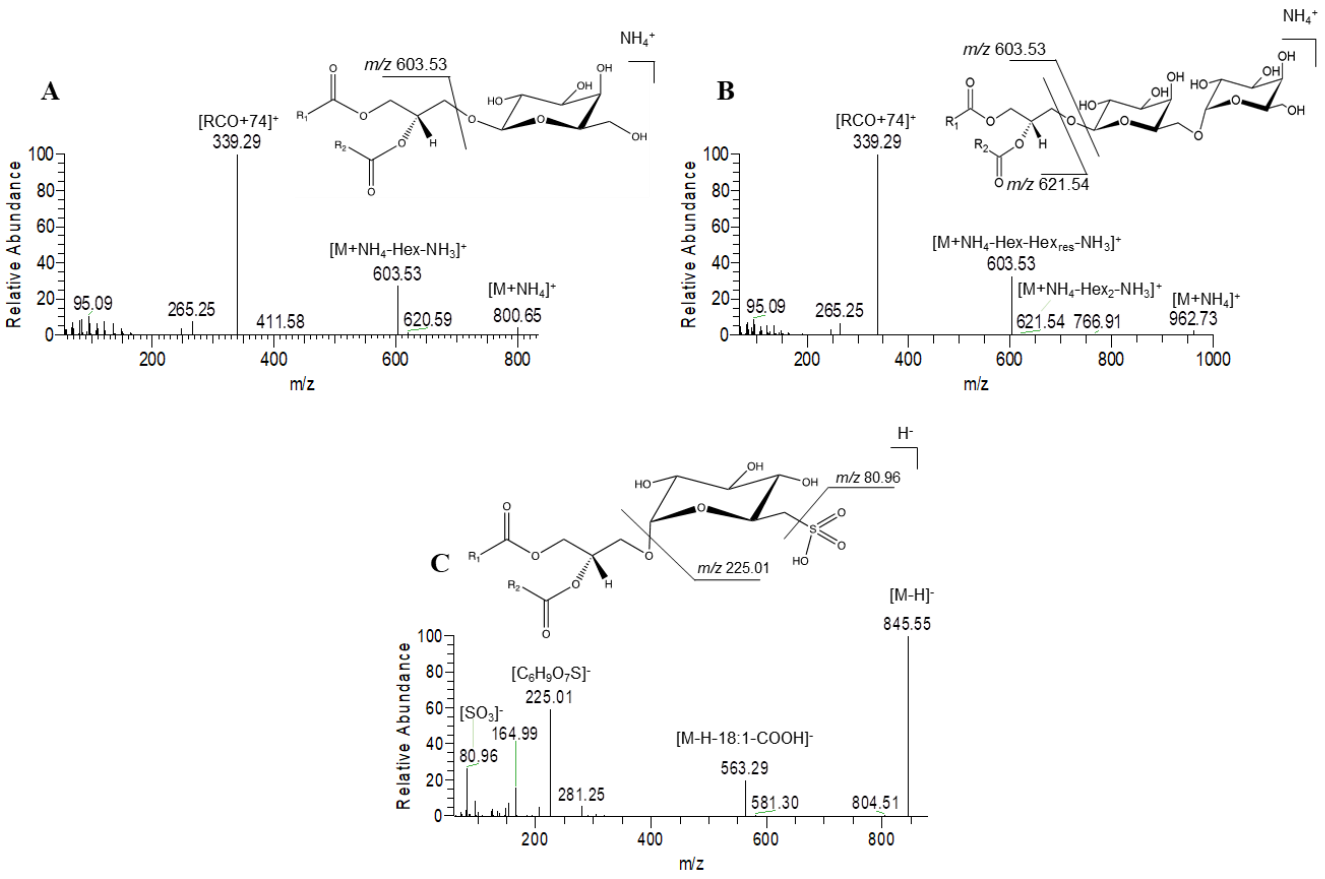


*Figure 18 – LC-MS/MS spectra of the different glycolipid classes identified in the olive (*Olea europaea *L.) pulp. One MS/MS spectrum illustrative of each class is shown:* ***A)*** *MGDG(36:2) at m/z 800.65 as* $[M + NH_4]^+$*;* ***B)*** *DGDG(36:2) at m/z 962.73 as* $[M + NH_4]^+$*; and* ***C)*** *SQDG(36:2) at m/z 845.55 as* $[M - H]^-$*. A representative molecular structure of each lipid class is also shown.*

3.3.1.3. Sphingolipids

Several lipid species from two SL classes could be identified and structurally characterised: ceramides (Cer, five species) and hexosylceramides (HexCer, ten species) (Table 12). Cer and HexCer were identified in the positive-ion mode, as $[M + H]^+$ molecular ions (174,175).

Illustrative MS/MS spectra of species from these two classes are shown in Fig. 19. The typical fragmentation of Cer molecules species as $[M + H]^+$, as observed in the MS/MS spectra (Fig. 19A), showed a neutral loss of one water molecule (-18 Da) from the precursor

ion, and product ions that were assigned to the sphingoid base at *m/z* 262.25 (loss of three water molecules from the long-chain base ($[t18:1+H-3H_2O]^+$), at *m/z* 280.26 (loss of two water molecules from the long-chain base ($[t18:1+H-2H_2O]^+$), and at *m/z* 298.27 (loss of one water molecule from the long-chain base ($[t18:1+H-H_2O]^+$). In the case of HexCer, the MS/MS spectra of protonated molecular ions (Fig. 19B) showed a neutral loss of a hexose molecule (-180 Da, -Hex) and the combined loss of hexose plus water (-198 Da, -Hex-H_2O) from the precursor ion, and the same product ions assigned to the sphingoid base as Cer. The acyl chains were verified by the neutral loss of the fatty acyl chain in the carboxylic form, from the product ion $[M-Hex-H_2O]^+$, in relation to the sphingoid base without the water molecules $[t18:1+H-3H_2O]^+$ (174,175).

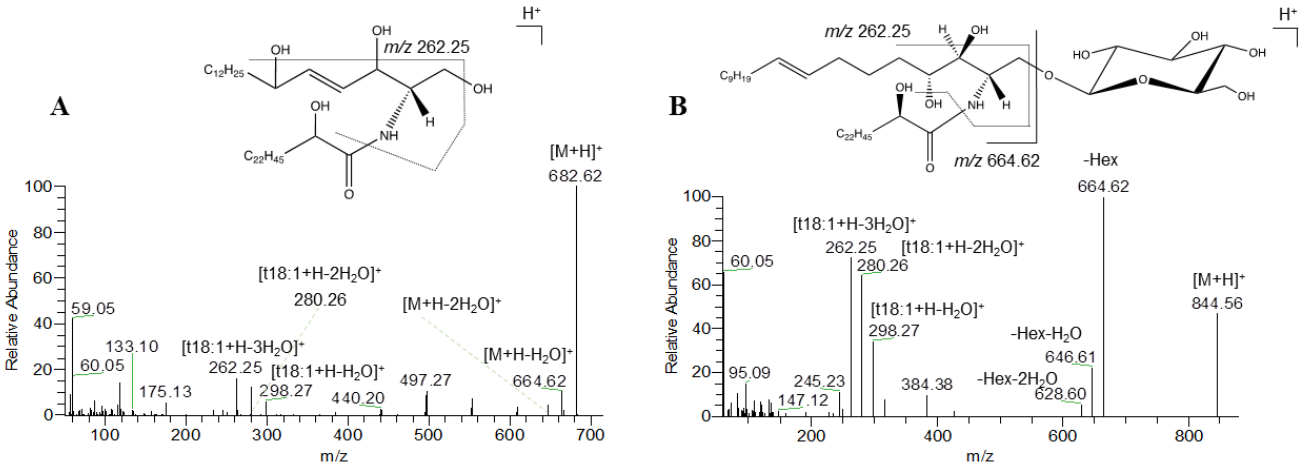


*Figure 19 - LC-MS/MS spectra of two sphingolipid classes identified in the olive (*Olea europaea *L.) pulp. One MS/MS spectrum illustrative of each class is shown:* ***A)*** *Cer(t42:1(OH)) at m/z 682.62 as* $[M + H]^+$*, and* ***B)*** *HexCer(t42:1(OH)) at m/z 844.56 as* $[M + H]^+$*. A representative molecular structure of each lipid class is also shown.*

The sphingoid bases in the SL pool mainly contained the C18 amino alcohol, while the fatty acyl chains ranged between C16 and C26 and were almost all hydroxylated. Only three species of HexCer did not have hydroxylated fatty acyl chains (Table 12). The sphingoid base of Cer contained t18:1 and t18:0, while the sphingoid base of HexCer contained d18:1, d18:2, t18:1 and t18:0.

As far as is known, there is only one study that identified and characterised SL species by LC-MS in the olive fruit. In that study, five lipid species of HexCer were reported, with the sphingoid backbone composed by d18:1, d18:2, and t18:1, and the FA chains

ranging between C16 and C26, and all were hydroxylated (32). For the first time, the present work allowed the identification and structural characterisation of several species of Cer in the olive fruit. In fact, ceramides and hydroxyceramides with long-chain FA, or phytoceramides, have been reported in plants (176,177). Furthermore, species of HexCer unreported in the olive fruit were also identified, e.g., HexCer(t41:1(OH)), HexCer(t34:1), HexCer(t42:2), and HexCer(42:1).

3.3.1.4. Betaine lipids

Finally, molecular species corresponding to two classes and sub-classes of betaine lipids (BL) were identified and structurally characterised: diacylglyceryl-*N*,*N*,*N* trimethylhomoserine (DGTS, five species) and monoacylglyceryl-*N*,*N*,*N* trimethylhomoserine (MGTS, one species), as shown in Table 14. Both were identified in the positive-ion mode, as $[M + H]^+$ molecular ions (178). Representative MS/MS spectra of both classes are shown in Fig. 20. The typical MS/MS spectra of these lipid classes showed a product ion at *m/z* 236.15, corresponding to the ion $C_{10}H_{22}O_5N^+$ generated from the ester bonds' cleavage and the loss of both acyl chains. The neutral loss of each acyl chain in the ketene form (-RCO) and in the carboxylic acid form (-RCOOH) from the precursor ion was also seen in DGTS (178).

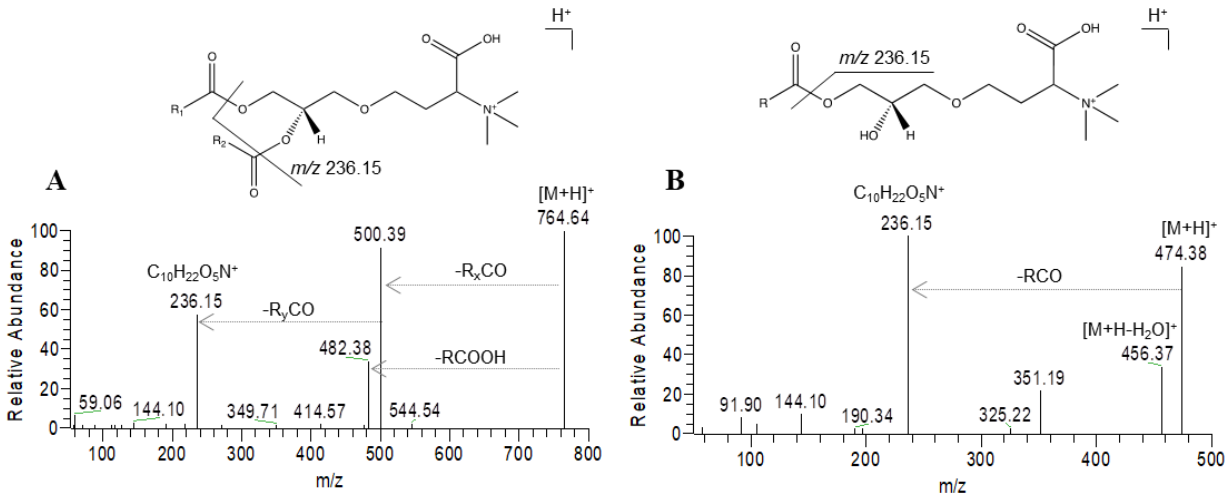


*Figure 20 - LC-MS/MS spectra of the two classes of betaine lipids identified in the olive (*Olea europaea *L.) pulp. One MS/MS spectrum illustrative of each class is shown:* ***A)*** *DGTS(36:2) at m/z 764.64 as* $[M + H]^+$*, and* ***B)*** *MGTS(16:0) at m/z 474.38 as* $[M + H]^+$*. A representative molecular structure of the lipid class and sub-class is also shown.*

The BL comprise the group of lipids with fewer species reported in this study, six lipid species. FA within the BL pool ranged from C14 to C20, with up to two unsaturations. Only one lysoDGTS molecular species was found, MGTS(16:0).

As for SL, only one study identified and structurally characterised BL molecular species in the olive pulp, reporting five species of DGTS and one molecular species of MGTS, the same six BL molecular species reported in the present study (32).

3.4. Polar lipid-based botanical and geographical discrimination of the olives

After the olive pulp's polar lipidome characterisation, the possibility of discriminating olives through their botanical and geographical origins was investigated. For this purpose, statistical analyses were performed involving the lipidomic data from the four studied groups.

The principal component analysis (PCA) of the samples by variety showed a two-dimensional scores plot with the eigenvalues describing 57.8 % of the total variance, including PC 1 (43.6 %) and PC 2 (14.2 %) (Fig. 21A). *Galega* and *Cobrançosa* were well discriminated each other alongside PC 2, with *Cobrançosa* located at positive values of PC 2, and *Galega Vulgar* located at negative values of PC 2. These two groups seem to have their own identity, with the biological replicates well grouped in the PCA scores plot. On the contrary, *Arbequina* samples did not group in the PCA, exhibiting a high dispersion of these samples. In this case, ten *Arbequina* samples were used for the multivariate analysis (five from Dão and five from Trás-os-Montes). The PCA shows five dots in the positive values of PC 2 and five dots, with an outlier, in the negative values of PC 2. These results suggest that other factors than the variety factor only are affecting the grouping of *Arbequina* olives.

A total of thirty-nine polar lipid species showed significant differences between botanical varieties (Annex I, Table S1). The top 25 lipid species that mostly contributed to the discrimination between varieties were used for explaining the observed differences. The clustering of the top 25 individual lipid species concerning their similarity in the abundance variation among varieties showed some aspects to be highlighted (Fig. 21B). *Galega Vulgar* had the highest abundance of most of these top 25 lipids. *Cobrançosa* showed lower abundance in a set of species which comprised almost all the anionic polar lipids in this top

(PG, SQDG and PI). Finally, *Arbequina* samples presented lower abundance in most species, mostly PC and HexCer, and LPC, LPE, PI and MGDG. This information based on the PCA, dendrograms and heatmap of the polar lipid profile suggests possible discrimination of the olives by their botanical variety.

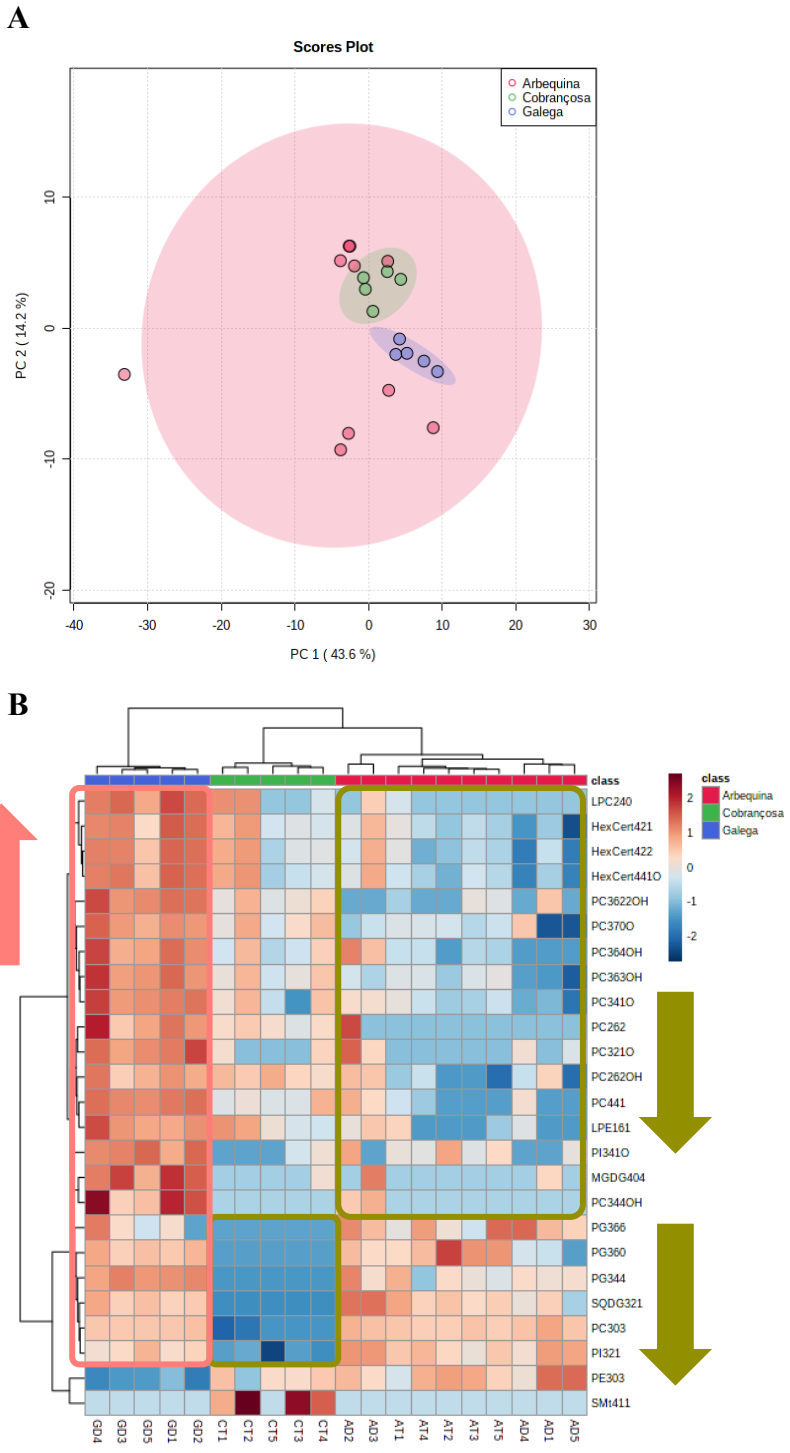


Figure 21 – Principal Component Analysis (PCA) scores plot of the first two principal components of the lipid classes data set obtained by LC-MS for the three botanical varieties ***(A)*** *and two-dimensional hierarchical cluster heatmap of the top 25 lipid species discriminating botanical varieties* ***(B)****.*

The dendrogram at the top of the heatmap represents the clustering of the sample groups, and the dendrogram on the left represents the clustering of the lipid species. Relative abundance levels are indicated on the colour scale, and the numbers indicate the fold difference from the mean. Lipid species are labelled as follows: AAAAxxig (AAAA = lipid class; xx = number of carbon atoms; i = number of double bonds; g = hydroxyl group).

Afterwards, a PCA was performed for the lipid species dataset considering the olives' botanical and geographical origin (Fig. 22A). The eigenvalues of the first two principal components represented 58.0 % of the total variance, summing PC 1 (43.5 %) and PC 2 (14.5 %). In this PCA scores plot, the two regions were clearly separated from each other alongside PC 2, with Trás-os-Montes (*Arbequina* and *Cobrançosa*) located at positive values of PC 2, and Dão (*Arbequina* and *Galega Vulgar*) located at negative values of PC 2. All groups were well clustered, except *Arbequina* from Dão that presented a high dispersion of the samples. In fact, in the PCA scores plot of the botanical varieties (Fig. 21A), the *Arbequina* samples were not very well grouped. However, in the PCA scores plot wherein both variety and region were considered (Fig. 22A), the two *Arbequina* groups could be discriminated. The samples from Trás-os-Montes were well grouped, revealing that the origin may be a discriminating factor.

A total of sixty-four polar lipid species showed significant differences between the samples from the four groups (Annex I, Table S2). The clustering of the top 25 individual lipid species that mostly explain the differences observed for the botanical and geographical origin of the olives showed apparent differences among the four groups with a close association in samples from the same region (Fig. 22B). Samples of *Arbequina* from Trás-os-Montes had a higher abundance in fourteen of to the top 25 lipid species, corresponding to the lipid classes CL, SQDG, DGTS, Cer, PE, PG, PC and PI, while showed lower abundance mainly in PC and LPC molecular species. *Cobrançosa* from the same region showed lower abundance in part of this set, comprising mostly PG, PI, SQDG and PC. On the other hand, *Arbequina* from Dão presented lower abundance in the other part of the set, composed by SM, CL, SQDG, DGTS, Cer and PE species. Once again, *Galega Vulgar* represented the group with the most remarkable diversity in the abundance of species, being the only detachable group with a greater abundance of PC and LPC species. Indeed, *Galega Vulgar* from Dão was the group with the most diversified polar lipidome concerning the other groups, with only five lipid species missing (Table 13). *Cobrançosa* and *Arbequina* from both regions showed similar differences in numerical terms of the total polar lipid species identified (Table 13). Additionally, from the nineteen polar lipid classes analysed statically, there are ten which lipid species are present in all samples (i.e., LPG, LPE, PI, LPI, PE-N(FA), CL, DGDG, SQMG, HexCer and MGTS).

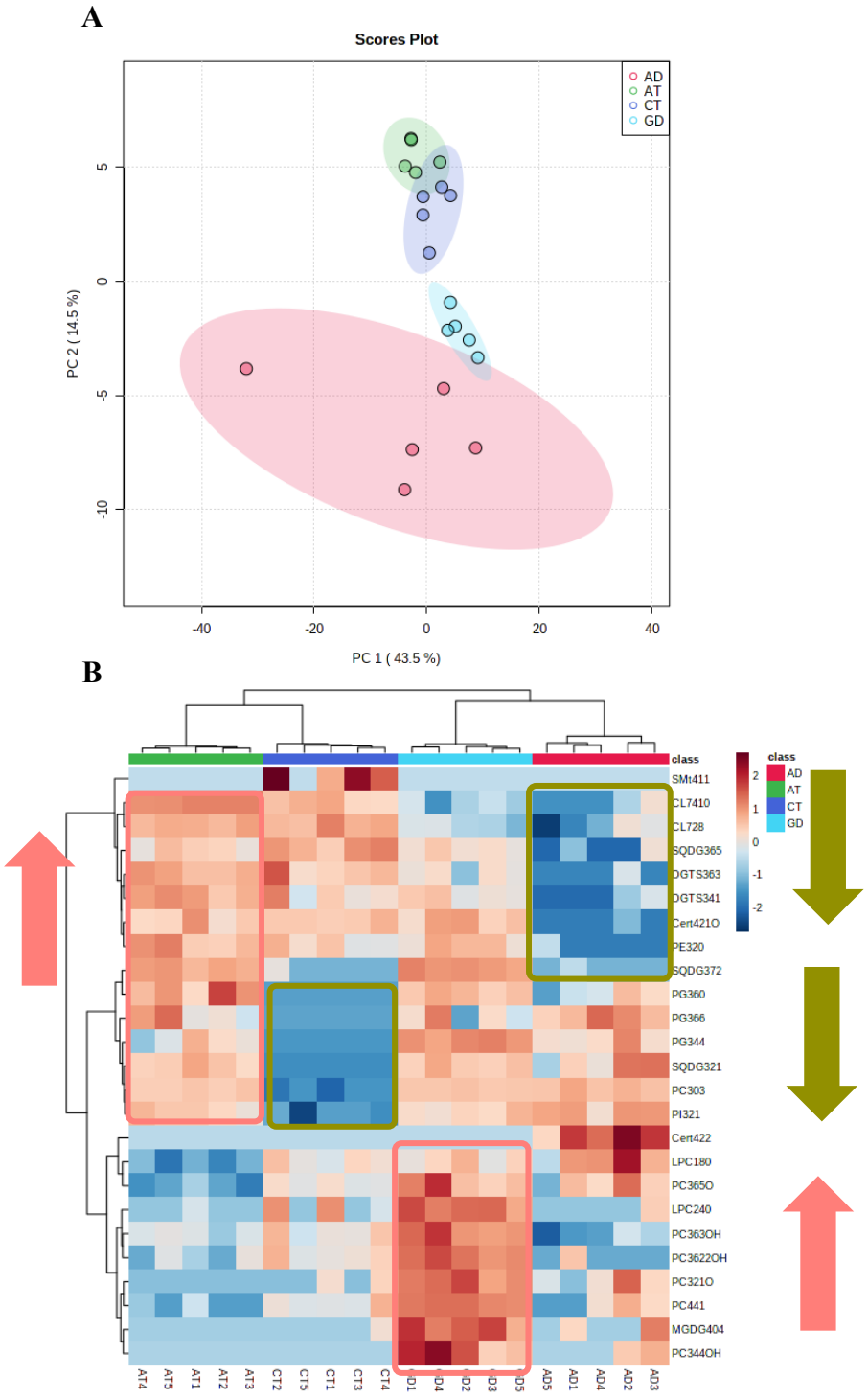


*Figure 22 - Principal Component Analysis (PCA) scores plot of the first two principal components of the lipid classes data set obtained by LC-MS for the three botanical varieties from the two regions **(A)** and two-dimensional hierarchical cluster heatmap of the top 25 lipid species discriminating the four groups **(B)**.*

The four groups are labelled as follows: AD = Arbequina *from Dão; AT =* Arbequina *from Trás-os-Montes; CT =* Cobrançosa *from Trás-os-Montes; GD =* Galega Vulgar *from Dão. The dendrogram at the top of the heatmap represents the clustering of the sample groups, and the dendrogram on the left represents the clustering of the lipid species. Relative abundance levels are indicated on the colour scale and the numbers indicate the fold difference from the mean. Lipid species are labelled as follows: AAAAxxig (AAAA = lipid class; xx = number of carbon atoms; i = number of double bonds; g = hydroxyl group).*

*Table 13 - Missing species of polar lipids identified in the olive (*Olea europaea *L.) pulp oil from two regions (Dão and Trás-os-Montes) with two different botanical varieties in each region (Dão:* Galega, Arbequina*; Trás-os-Montes:* Arbequina, Cobrançosa*)*

Variety / **Region**	***Galega*** **Dão**	***Cobrançosa*** **Trás-os-Montes**	***Arbequina*** **Dão**	***Arbequina*** **Trás-os-Montes**
PG(34:4)				
PG(36:0)				
PG(36:6)				
PE(32:0)				
PC(24:0(OH))				
PC(26:2)				
PC(32:1(OH))				
PC(34:4(OH))				
PC(36:2(2OH))				
PC(41:1)				
PC(42:1)				
PC(43:1)				
LPC(20:0)				
LPC(20:1)				
LPC(22:0)				
LPC(24:0)				
SM(d36:1)				
SM(t40:0)				
SM(t41:1)				
MGDG(40:4)				
SQDG(32:1)				
SQDG(37:2)				
Cer(t42:2)				
Cer(t42:1(OH))				
DGTS(36:3)				
Total missing species	**5**	**12**	**11**	**15**

The green colour means that the lipid species has been identified in the respective group, while the red colour means species that were absent in the respective group.

Lastly, a univariate statistical analysis was used to test for significant differences between the relative abundance of the polar lipid classes, comparing the groups from the same region (Fig. 23).

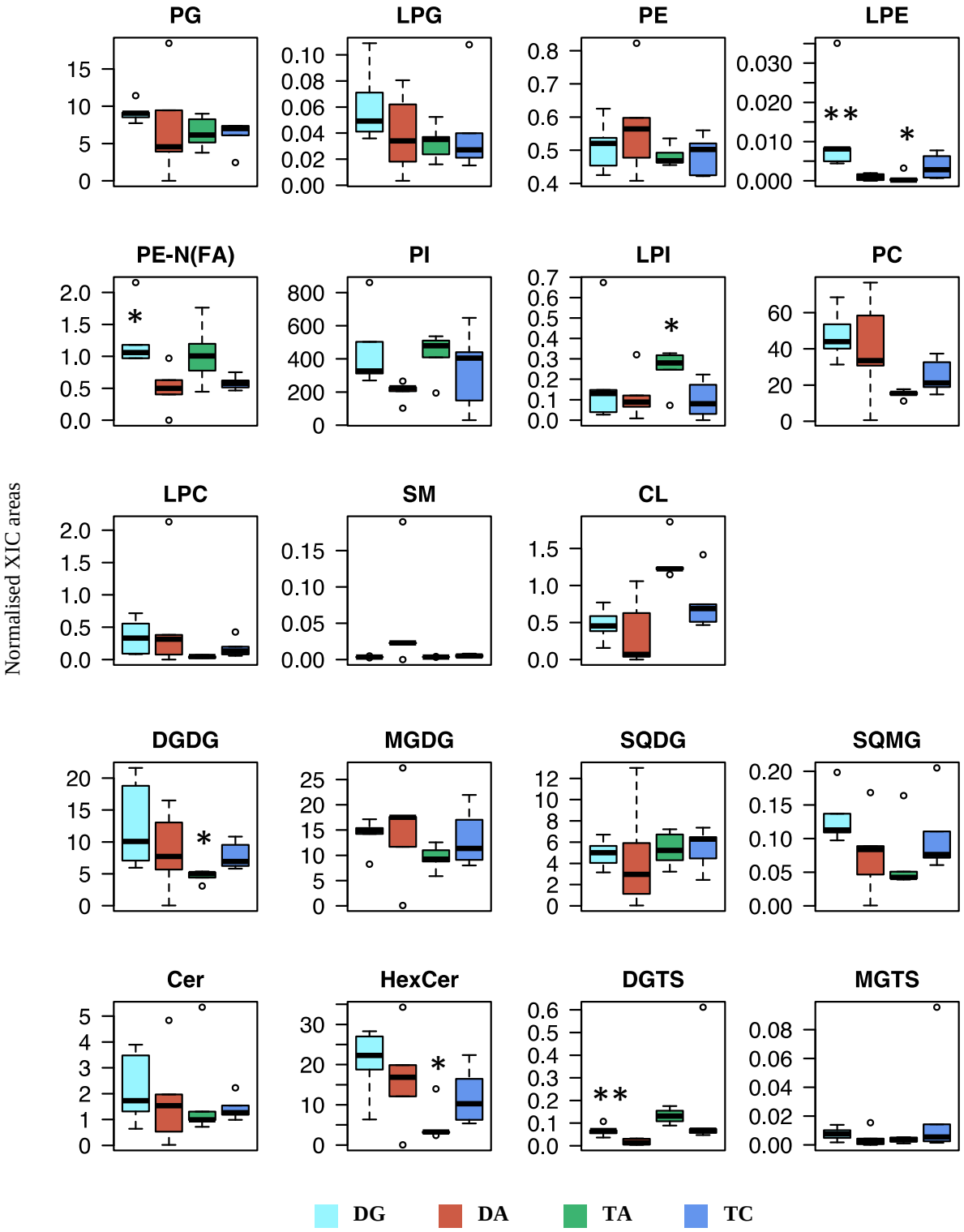


Figure 23 – Box plots of normalised peak areas of polar lipid classes of olive pulp samples from two different regions (Dão and Trás-os-Montes) with two different varieties in each region [Dão: Galega *(DG),* Arbequina *(DA); Trás-os-Montes:* Arbequina *(TA),* Cobrançosa *(TC)]. The boxes represent medians and 25th and 75th percentiles. Error bars represent range (from the minimum to the maximum value), and dots are outliers. Asterisks represent significant differences between groups from the same region as determined by one-way ANOVA, * $p < 0.05$, ** $p < 0.01$. XIC, extracted ion chromatograms.*

Six polar lipid classes showed significant differences between varieties from the same region (Fig. 23). LPE and DGTS presented significant differences between varieties from Dão ($p < 0.01$), wherein was a significant decrease in *Arbequina* comparing to *Galega Vulgar*. LPE also showed significant differences in Trás-os-Montes ($p < 0.05$), significantly higher in *Cobrançosa* than *Arbequina*. PE-N(FA) showed significant differences between varieties from Dão ($p < 0.05$), wherein was significantly higher in *Galega Vulgar* compared to *Arbequina*. The LPI, DGDG and HexCer classes presented significant differences between varieties from Trás-os-Montes ($p < 0.05$). LPI was significantly higher in *Arbequina* comparing to *Cobrançosa*, but DGDG and HexCer were significantly lower.

All these data retrieved from different statistical analyses revealed that olives from the same region or the same variety have significant differences in their polar lipidome fingerprint. Additionally, comparing lipid markers among groups, it has been shown that olives have their own identity, which depends on the botanical and geographical origin. Thus, the polar lipid-based botanical and geographical discrimination of olives revealed unprecedented and relevant information. It has shown the potential to guide new paths that will lead to accurate day-to-day discrimination of olives and combating fraud.

3.5. Concluding remarks and future perspective

The main goal of this work was to characterise the polar lipid fingerprint of the olive pulp to evaluate its nutritional potential while using emerging high-sensitivity techniques based on chromatography and mass spectrometry. This goal has been achieved. The polar lipid fingerprint of the olive pulp revealed one hundred and eighty-eight lipid species belonging to several lipid classes and sub-classes divided into four families of lipids: phospholipids (PL), glycolipids (GL), sphingolipids (SL) and betaine lipids (BL). The workflow started with the extraction of the total lipids from the pulp using an adaptation of the Folch method. It was followed by fractionation of the oil using solid-phase extraction (SPE). Then, lipid analysis, using high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) and data processing using the Xcalibur data system and MZmine software.

The gravimetric yield of the olive pulp's total lipid extraction was around 42% (w/w), on a dry basis. The oil extraction data showed substantial differences among *Galega Vulgar*,

Cobrançosa and *Arbequina*' oil content, confirming the variability from variety to variety. In this part of the workflow, a TLC of the oil revealed the olive fruit's complex lipidome, comprising several lipid classes of different polarities, including the polar lipids. PL and GL represented about 0.40% (w/w) and 2.73% (w/w) of total lipids respectively. Although the quantification methods are not standard methods, it was possible to observe that these two lipid families are trace compounds of the olive pulp, as expected.

The fractionation of the oil through SPE, one of the most critical steps of this lipidomic study, allowed to successfully obtain polar lipid-rich fractions. As shown through the TLC performed with the lipid-rich fractions, and later, through LC-MS, this improved process allowed recovering a vast set of polar lipid classes, including anionic classes, which had not been recovered in previous reports on the olive pulp. Although TLC is a less sensitive technique, it allowed evaluating semi-qualitatively and semi-quantitatively the olive pulp's complex polar lipidome. The improved SPE fractionation protocol allowed the recovery of up to 100% of PL (w/w), and up to 27% of GL (w/w). GL recovery was not high and can be derived from the fact that there may be lipid compounds with sugar residues in the oil that elute in the intermediate fraction or that were retained in the SPE cartridges, leading to a misleading estimative of the GL content in the polar lipid-rich fraction.

The improved SPE process had not been previously tested on the olive pulp, representing both an advantage and a disadvantage. Although this protocol was efficient in recovering polar lipids from the olive's seeds, the pulp's PL content is substantially lower than that found in the seed. Additionally, the SPE procedure might be fine-tuned for a better recovery of these lipids, mostly GL. Another limitation is the time-consuming of this protocol, allowing the preparation of around four samples a day for analysis. An automated SPE system would be a better option to achieve a high-throughput procedure for preparing samples for LC-MS analysis of polar lipids from olives, olive oil and other oily matrices.

Notwithstanding, this improved fractionation procedure is very relevant because it allowed obtaining polar lipid-rich fractions representing the most significant polar lipid classes with their chemical complexity and nature. It also effectively removed triacyclglycerols (TAG), one of the problems found in previous studies during MS data acquisition and analysis. Thus, this remarkable achievement allowed an analysis and data processing with less interference and more sensitivity.

Combining SPE with HPLC-MS allowed an unprecedented level of detail of the olive pulp's polar lipidome. It was possible to identify and structurally characterise twenty-one polar lipid classes and sub-classes with potential biological activity. PG, PI, LPE, PA and LPA have been little studied in the olive pulp and fruit. Seven classes and sub-classes had not been previously reported in these matrices, namely LPG, LPI, PE-N(FA), CL, SQDG, SQMG, and Cer. Moreover, worth noticing that several molecular species of PE, PC, LPC, MGDG and HexCer were also identified and structurally characterised for the first time in this foodstuff. In the polar lipid pool, the most common FA were 18:1, 16:1, 18:2, 18:3, 18:0 and 16:0. Nevertheless, FA comprised C8 and C26, with up to four unsaturations and hydroxylations in some classes.

Lastly, the second main goal of this work has been achieved. The statistical discriminant analysis on the chemical fingerprint data allowed successfully identifying lipid markers that can distinguish the botanical and or the olives' geographical origin. The polar lipid-based botanical and geographical discrimination of olives showed that each olive group has a unique polar lipid profile, revealing unprecedented information that is a starting point for establishing fingerprints for olive cultivars from different origins, with traceability purposes.

The present work has provided a big step forward in knowing the polar lipid pool and valorising the olive fruit's nutritional value. Lipidomics is a key tool for fingerprinting the minor lipid profiles and discovering new compounds with potential biological activity. Polar lipids contain MUFA and PUFA, having shown anti-inflammatory, antithrombotic, antitumoral, and antioxidant activities. The literature also suggests the ability of these lipids, when in food, to: influence lipid metabolism; improve several cardiovascular and diabetic risk factors; reduce blood pressure, cholesterol and TAG levels; regulate cell growth, differentiation and apoptosis in most type of cells (e.g., colon and skin cells); improve the barrier function of the skin; maintain the homeostasis and membrane fluidity (e.g., in myocardium and skin); enhance power performance; and have beneficial involvement in visual and brain functions and several diseases as cardiovascular diseases, asthma, arthritis, Alzheimer's, multiple sclerosis, and renal injury. As such, due to the wide range of biological action of PL, GL, SL and BL, olive and their derived products not only are relevant for consumption but also as an ingredient or raw material for several industries, such as food, nutraceutical, pharmaceutical, dermo-cosmetic, and animal feed industries.

For future work, several aspects could be considered. It would be interesting to gather more olive varieties from different regions and perform statistical analyses of their oil, PL and GL content. The same approach would also be relevant to know better the identity of olives from the varieties and regions studied in this work, and new ones.

It would also be interesting to retrieve more information from the TLC analysis of the polar lipid-rich fraction by scraping off the TLC bands and analysing each by direct ESI-MS/MS analysis, which could provide new information on the complex polar lipidome of the olive pulp. It would be worthy because, as stated earlier, certain bands of the TLC performed in these fractions had no reference standard and also in this low-tech approach more than one lipid class can migrate in the same band. This complementary approach would allow obtaining even more detailed information about the results already provided by LC-MS/MS and provide new insight into future tasks. The same applies to the TLC of the oil to better know its lipid composition at the molecular level and deeply understand its nutritional value.

Another relevant task would be to study the fatty acid profile of the oil and polar lipid fractions by GC-MS comparing both. The fatty acid profile of the olive fruit's polar lipids has never been reported, and it would be timely to know. The fatty acids reported in this study were identified through MS/MS data analysis of the polar lipid molecular species.

Additionally, another meaningful way forward would be to evaluate *in vitro* biological activities of the lipid extracts recovered from the olive pulp through biological tests with cell lines. These studies would contribute to a better knowledge of the impact of the olive's lipids on human health.

Finally, with the information obtained in this work, new studies can be delineated to study the polar lipid pool changes originated from table olives' production process. It is because the samples used herein did not represent olives for consumption, and it is necessary to take into account that the food product is subjected to fermentation processes, among others, which will necessarily modify the lipid composition.

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Annex

Table S1 - List of all lipid species that showed significant differences between different olive botanical varieties.

Lipid Species	f.value	p.value	-log10(p)	FDR	Tukey's HSD
PC(30:3)	303.46	5.01E-14	13.3	8.47E-12	C-A; G-C
PI(32:1)	60.551	1.85E-08	7.7329	1.56E-06	C-A; G-C
PG(34:4)	52.278	5.47E-08	7.2618	3.08E-06	C-A; G-A; G-C
SQDG(32:1)	40.555	3.38E-07	6.4707	1.43E-05	C-A; G-C
PC(36:2(OH)$_2$)	25.499	7.63E-06	5.1174	2.58E-04	C-A; G-A; G-C
MGDG(40:4)	21.168	2.43E-05	4.6144	6.84E-04	G-A; G-C
PC(34:4(OH))	20.465	2.98E-05	4.5259	7.19E-04	G-A; G-C
PC(36:3(OH))	18.96	4.69E-05	4.3288	9.76E-04	C-A; G-A; G-C
LPC(24:0)	18.629	5.20E-05	4.2841	9.76E-04	G-A; G-C
SM(t41:1)	16.788	9.45E-05	4.0246	1.60E-03	C-A; G-C
PG(36:6)	15.729	1.36E-04	3.8668	2.09E-03	C-A; G-C
PC(44:1)	15.25	1.61E-04	3.793	2.11E-03	G-A; G-C
PG(36:0)	15.232	1.62E-04	3.7903	2.11E-03	C-A; G-C
PC(34:1(OH))	13.284	3.36E-04	3.4742	4.05E-03	G-A; G-C
PC(26:2)	12.73	4.18E-04	3.3791	4.71E-03	G-A
LPE(16:1)	12.008	5.61E-04	3.2513	5.73E-03	C-A; G-A
HexCer(t42:2)	11.943	5.76E-04	3.2396	5.73E-03	G-A
HexCer(t44:1(OH))	11.519	6.88E-04	3.1622	6.46E-03	G-A
PI(34:1(OH))	10.995	8.62E-04	3.0643	7.67E-03	G-A; G-C
PC(32:1(OH))	10.786	9.45E-04	3.0245	7.82E-03	G-A; G-C
PC(37:0(OH))	10.672	9.94E-04	3.0026	7.82E-03	G-A
HexCer(t42:1)	10.617	1.02E-03	2.992	7.82E-03	G-A
PC(36:4(OH))	9.761	1.50E-03	2.8229	1.10E-02	G-A; G-C
PC(26:2(OH))	8.5983	2.63E-03	2.58	1.85E-02	C-A; G-A
SQDG(37:2)	7.8822	3.78E-03	2.4221	2.46E-02	G-A; G-C
PC(28:3(OH))	7.7417	4.07E-03	2.3903	2.46E-02	G-A
PG(36:1)	7.7413	4.07E-03	2.3902	2.46E-02	C-A
PC(34:1(OH)$_2$)	7.6721	4.22E-03	2.3744	2.46E-02	G-A
LPC(20:1)	7.667	4.23E-03	2.3733	2.46E-02	G-A; G-C
SQDG(36:6)	7.6093	4.36E-03	2.3601	2.46E-02	C-A; G-C
MGDG(34:3)	7.5421	4.52E-03	2.3447	2.47E-02	G-A; G-C

SQMG(22:1)	7.297	5.15E-03	2.2878	2.72E-02	C-A; G-A
PC(16:0/C8CHO)	7.0965	5.75E-03	2.2407	2.88E-02	G-A
LPE(16:0)	7.0801	5,80E-03	2.2368	2,88E-02	G-A
PC(24:0(OH))	6.9787	6,13E-03	2.2127	2,96E-02	G-A
PC(43:1)	6.7936	6,79E-03	2.1683	3,19E-02	G-A
PC(34:2(OH))	6.3401	8,77E-03	2.0571	4,00E-02	G-A
PE(32:2)	6.2727	9,11E-03	2.0403	4,05E-02	G-A
MGDG(34:1)	5.9572	1,09E-02	1.9607	4,74E-02	C-A; G-C

This table shows F-value, p value, p value adjusted and false discovery rate (FDR) correction. Tukey's HSD column shows significant differences between botanical varieties. C, *Cobrançosa*; A, *Arbequina*; G, *Galega Vulgar*.

Table S2 - List of all lipid species that showed significant differences between samples of the four groups studied, two different regions (Dão and Trás-os-Montes) with two different botanical varieties in each region [Dão: Galega *(DG),* Arbequina *(DA); Trás-os-Montes:* Arbequina *(TA),* Cobrançosa *(TC)].*

Lipid Species	**f.value**	**p.value**	**-log10(p)**	**FDR**	**Tukey's HSD**
PC(30:3)	223.97	2.83E-13	12.548	4.78E-11	CT-AD; CT-AT; GD-CT
SQDG(37:2)	66.243	3.07E-09	8.5135	2.40E-07	AT-AD; GD-AD; CT-AT; GD-CT
Cer(t42:1(OH))	63.348	4.26E-09	8.3707	2.40E-07	AT-AD; CT-AD; GD-AD
PI(32:1)	45.822	4.44E-08	7.3527	1.88E-06	CT-AD; CT-AT; GD-CT
PG(34:4)	38.359	1.55E-07	6.8086	5.25E-06	CT-AD; CT-AT; GD-AT; GD-CT
Cer(t42:2)	34.267	3.39E-07	6.4697	9.55E-06	AT-AD; CT-AD; GD-AD
PE(32:0)	26.502	1.91E-06	5.7187	4.61E-05	AT-AD; CT-AD; GD-AD
SQDG(32:1)	25.456	2.49E-06	5.604	5.26E-05	CT-AD; CT-AT; GD-CT
CL(74:10)	23.749	3.91E-06	5.4082	6.85E-05	AT-AD; CT-AD; GD-AT; GD-CT
PG(36:0)	23.615	4.05E-06	5.3923	6.85E-05	AT-AD; CT-AD; CT-AT; GD-CT
PC(36:3(OH))	20.599	9.66E-06	5.0151	1.48E-04	CT-AD; GD-AD; GD-AT; GD-CT
DGTS(36:3)	19.938	1.18E-05	4.9267	1.67E-04	AT-AD; CT-AD; GD-AD
MGDG(40:4)	17.001	3.13E-05	4.5041	4.07E-04	GD-AD; GD-AT; GD-CT

PC(36:2(OH)2)	16.572	3.65E-05	4.4378	4.15E-04	CT-AD; GD-AD; GD-AT; GD-CT
SQDG(36:5)	16.543	3.69E-05	4.4333	4.15E-04	AT-AD; CT-AD; GD-AD
DGTS(34:1)	15.65	5.11E-05	4.2912	5.40E-04	AT-AD; CT-AD; GD-AD
PC(34:4(OH))	15.153	6.17E-05	4.2094	6.14E-04	GD-AD; GD-AT; GD-CT
LPC(18:0)	14.142	9.18E-05	4.0369	8.62E-04	AT-AD; CT-AT; GD-AT
PC(32:1(OH))	13.773	1.07E-04	3.9718	9.49E-04	AT-AD; GD-AD; GD-AT; GD-CT
PC(44:1)	13.384	1.25E-04	3.9019	1.06E-03	GD-AD; CT-AT; GD-AT; GD-CT
PC(36:5(OH))	12.86	1.57E-04	3.8054	1.26E-03	AT-AD; CT-AT; GD-AT
CL(72:8)	12.466	1.86E-04	3.7311	1.43E-03	AT-AD; CT-AD; GD-AT; GD-CT
LPC(24:0)	11.816	2.48E-04	3.6047	1.83E-03	GD-AD; GD-AT; GD-CT
PG(36:6)	10.99	3.65E-04	3.4376	2.57E-03	CT-AD; CT-AT; GD-CT
SM(t41:1)	10.533	4.55E-04	3.3415	3.08E-03	CT-AD; CT-AT; GD-CT
PC(31:0(OH))	10.355	4.97E-04	3.3034	3.23E-03	AT-AD; CT-AD; GD-AD
PC(43:1)	10.119	5.60E-04	3.2521	3.50E-03	AT-AD; GD-AT
PI(34:1(OH))	9.7798	6.65E-04	3.1772	4.01E-03	GD-AD; GD-CT
PC(26:2(OH))	9.5251	7.59E-04	3.1198	4.42E-03	CT-AT; GD-AT
PC(26:2)	9.2882	8.60E-04	3.0657	4.84E-03	GD-AD; GD-AT
PC(35:4)	9.057	9.73E-04	3.012	5.30E-03	AT-AD; CT-AD; GD-AD
PC(37:0(OH))	8.9636	1.02E-03	2.99	5.40E-03	CT-AD; GD-AD; GD-AT
PC(34:1(OH))	8.809	1.11E-03	2.9535	5.70E-03	GD-AD; GD-AT; GD-CT
SQDG(36:6)	8.6714	1.20E-03	2.9206	5.97E-03	CT-AD; GD-CT
LPE(16:1)	8.41	1.39E-03	2.8572	6.69E-03	GD-AD; GD-AT
PG(36:1)	8.3181	1.46E-03	2.8347	6.69E-03	CT-AD; GD-AD
PE-N(FA) 54:6	8.3175	1.46E-03	2.8346	6.69E-03	AT-AD; CT-AD; GD-AT; GD-CT
PC(28:3(OH))	7.8865	1.88E-03	2.7269	8.34E-03	CT-AD; GD-AD
PC(36:4)	7.6521	2.15E-03	2.667	9.33E-03	AT-AD; CT-AD; GD-AD
HexCer(t42:2)	7.4998	2.36E-03	2.6275	9.96E-03	GD-AD; GD-AT
HexCer(t44:1(OH))	7.261	2.72E-03	2.5646	1.12E-02	GD-AD; GD-AT
$PC(34:1(OH)_2)$	7.0671	3.07E-03	2.5128	1.24E-02	GD-AD
HexCer(t42:1)	6.9982	3.20E-03	2.4942	1.26E-02	GD-AD; GD-AT
PC(24:0(OH))	6.9318	3.34E-03	2.4762	1.28E-02	GD-AT

PC(32:0)	6.803	3.62E-03	2.441	1.36E-02	AT-AD; CT-AD; GD-AD
DGTS(36:4)	6.5783	4.18E-03	2.3787	1.54E-02	AT-AD; CT-AD
PC(42:1)	6.3127	4.97E-03	2.3037	1.79E-02	AT-AD; GD-AT
DGTS(36:2)	6.2573	5.15E-03	2.2878	1.79E-02	AT-AD; CT-AD
PC(36:4(OH))	6.2509	5.18E-03	2.286	1.79E-02	GD-AD; GD-AT
CL(74:8)	6.1679	5.47E-03	2.2621	1.85E-02	AT-AD; GD-AD
PC(16:0/C8CHO)	5.8154	6.94E-03	2.1589	2.30E-02	GD-AT
CL(72:7)	5.6516	7.76E-03	2.1099	2.52E-02	AT-AD; CT-AD
PE(32:2)	5.5812	8.15E-03	2.0886	2.60E-02	GD-AT
DGTS(34:2)	4.9533	1.28E-02	1.8933	3.97E-02	AT-AD; CT-AD
PC(34:2(OH))	4.9397	1.29E-02	1.8889	3.97E-02	GD-AT
PC(33:1)	4.8123	1.42E-02	1.8479	4.14E-02	CT-AD; GD-AD
LPC(20:1)	4.8111	1.42E-02	1.8476	4.14E-02	GD-AD; GD-AT; GD-CT
PC(36:3(OH)$_2$)	4.8094	1.42E-02	1.847	4.14E-02	AT-AD; GD-AT
MGDG(34:3)	4.7598	1.48E-02	1.8309	4.23E-02	GD-CT
CL(70:3)	4.7116	1.53E-02	1.8152	4.31E-02	AT-AD
SQMG(22:1)	4.6271	1.63E-02	1.7875	4.52E-02	GD-AT
PC(34:3(OH))	4.525	1.76E-02	1.7538	4.80E-02	GD-AT
LPE(16:0)	4.4709	1.84E-02	1.7358	4.89E-02	GD-AT
PE-N(FA) 54:5	4.4618	1.85E-02	1.7328	4.89E-02	AT-AD

This table shows F-values, p value, p value adjusted and false discovery rate (FDR) correction. Tukey's HSD column shows significant differences between four groups.