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**Full title: Digestibility and oxidation stability of plant lipid assemblies:
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21	List of abbreviations
22	1-lysoPC 1-lysophosphatidylcholine
23	ALA α -linolenic acid
24	ANSES French National Agency for Food, Environmental and Work Health Safety
25	AO Antioxidant
26	ARA Arachidonic acid
27	BSSL Bile-salt simulated lipase
28	CCK Cholecystokinin
29	CEH Carboxyl ester hydrolase (pancreatic)
30	CLSM Confocal laser scanning microscopy
31	CMC Critical Micelle Concentration
32	COB Crude OB
33	CRF Chloroplast rich fraction
34	DBE Dissociating Bond Energy
35	DGDG Digalactosyldiacylglycerol
36	DGMG Digalactosylmonoacylglycerol
37	DHA Docosahexaenoic acid
38	EPA Eicosapentaenoic acid
39	FA Fatty acid
40	FFA Free Fatty acid
41	GI Gastrointestinal
42	GL Galactolipid
43	GLP-1 Glucagon-like peptide 1
44	GPLRP2 Guinea pig pancreatic lipase related protein 2
45	HGL Human gastric lipase
46	HII Inverse hexagonal phase
47	HPL Human pancreatic lipase
48	HPLRP2 Human pancreatic lipase related protein 2
49	LA Linoleic acid
50	Lα Lamellar phase
51	LC-PUFA Long chain polyunsaturated fatty acid

52	MGDG Monogalactosyldiacylglycerol
53	MGMG Monogalactosylmonoacylglycerol
54	OB Oil body
55	PA Phosphatidic acid
56	PC Phosphatidylcholine
57	PE Phosphatidylethanolamine
58	PG Phosphatidylglycerol
59	PI Phosphatidylinositol
60	PL Phospholipid
61	PLA2 Phospholipase A2 (pancreatic)
62	PLRP2 Pancreatic lipase related protein 2
63	PO Prooxidant
64	PS Phosphatidylserine
65	PUFA Polyunsaturated fatty acid
66	PYY Peptide YY
67	ROS Reactive oxygen specie
68	SDA Stearidonic acid
69	SM Sphingomyelin
70	SQDG Sulfoquinovosyldiacylglycerol
71	TAG Triacylglyceride
72	TLC Thin layer chromatography
73	UV Ultraviolet
74	WOB Washed OB
75	WPI Whey protein isolate

ABSTRACT

Most lipids in our diet come under the form of triacylglycerols, that are often redispersed and stabilized by surfactants in processed foods. In plant however, lipid assemblies constitute interesting sources of natural bioactive and functional ingredients. In most photosynthetic sources, polar lipids rich in ω 3 fatty acids are concentrated. The objective of this review is to summarize all the knowledge about the physico-chemical composition, digestive behaviour and oxidative stability of plant polar lipid assemblies to emphasize their potential as functional ingredients in human diet and their potentialities to substitute artificial surfactants/antioxidants. The specific composition of plant membrane assemblies is detailed, including plasma membranes, oil bodies, and chloroplast; emphasizing its concentration in phospholipids, galactolipids, peculiar proteins and phenolic compounds. These molecular species are hydrolysed by specific digestive enzymes in the human gastrointestinal tract and reduced the hydrolysis of triacylglycerols and their subsequent absorption. Galactolipids specifically can activate ileal break and intrinsically present an antioxidant (AO) activity and metal chelating activity. In addition, their natural association with phenolic compounds and their physical state ($L\alpha$ state of digalactosyldiacylglycerols) in membrane assemblies can enhance their stability to oxidation. All these elements make plant membrane molecules and assemblies very promising components with a wide range of potential applications to vectorise ω 3 polyunsaturated fatty acids, and equilibrate human diet.

Keywords: plant membrane, polar lipids, digestive lipolysis, antioxidants, surfactants

97 1 INTRODUCTION

98 **Lipids are very important nutrients for human health but also for living 's growth**, playing
99 several key roles in the homeostatic regulation of metabolism in both plants and animals, in
100 particular through three main functions: i) Lipids can be used for energy storage in their neutral
101 form (triacylglycerides, TAG); ii) lipids also play a crucial role in membrane structure. Indeed,
102 polar lipids possess amphipathic structure and self-association properties, which allow them to
103 define the cells and to segregate cellular compounds into organelles; iii) finally, many lipids are
104 signalling molecules or at the basis of primary and secondary messengers such as eicosanoids
105 or resolvins, which play a key role in signal transduction and molecular recognition processes.

106 **The digestion of lipids from the diet must provide two essential polyunsaturated fatty**
107 **acids (PUFA):** linoleic (LA, C18:2, ω 6) and α -linolenic acid (ALA, C18:3, ω 3). These two
108 essential fatty acids (FA) are at the basis of an elongation and desaturation pathway, resulting
109 in highly bioactive long chain (LC) PUFA such as arachidonic acid (ARA, C20:4, ω 6),
110 eicosapentaenoic acid (EPA, C20:5, ω 3) and docosahexaenoic acid (DHA, C22:6, ω 3).
111 Nevertheless, the synthesis pathway of EPA and DHA from ALA exhibit a low conversion
112 efficiency, thus it is also recommended to consume foods rich in its two essential FA. These
113 LC-PUFA play indeed a crucial role in the homeostatic regulation of the body by producing
114 signalling oxygenated lipids called eicosanoids and pro-resolvines [1]. ω 3 and ω 6 FA exhibit
115 opposite metabolic effects and compete for the synthesis of lipid mediators through their
116 biosynthetic enzymes. Thus, a balanced consumption of ω 3 and ω 6, with an optimal ω 6/ ω 3
117 ratio of 4 and an adequate intake of each type of FA is recommended [2].

118 **Nevertheless, over the last three decades, the ω 6/ ω 3 ratio has considerably increased in**
119 **Western diets**, going from 1:1 up to 20:1 in very rich diets, due to a significant increase in the
120 intake of ω 6 and a concomitant decrease in the intake of ω 3 [3]. Meanwhile, an increase in

overweight and obesity incidence has been observed. This increase is certainly multifactorial, but very likely linked to metabolic imbalances, including changes in the FA composition of diets. It is therefore essential to maintain our efforts and set up nutritional strategies to decrease the $\omega 6/\omega 3$ ratio in our diets and recover normal homeostasis, in particular by increasing our intake of LC-PUFA $\omega 3$, such as EPA and DHA, while limiting the intake of total lipids [4]. Such strategy can be achieved by favouring fish rather than meat in the diet, but also by increasing our $\omega 3$ rich vegetable oils intake. More recently, it has also been proposed to enrich our diet with green vegetal-derived lipids, as an alternative source of essential PUFA [5].

Indeed, some plant membrane assemblies or polar lipids blends are able to improve the assimilation of specific $\omega 3$ PUFA, that are better absorbed under the form of phospholipids (PL) than TAG [6]. However, this better absorption has been proved for long chain $\omega 3$ PUFA but disproved for ALA [7]. In addition to PL, plant and algae membranes also include galactolipids (GL), associated to micronutrients, whose consumption leads to the production of essential $\omega 3$ [8]. The great abundance of plants and algae on earth explains that these GL represent the major class of acylglycerol lipids in nature, and thus the principal source of FA (80% versus 20% in PL and vegetal TAG), including essential PUFA [9]. Furthermore, green vegetal and seeds such as walnuts or flax contain significant quantities of ALA (60-70% wt. of total fatty acids) [1] and are rich in phenolic compounds and other phytochemical derivatives.

As a consequence, the consumption of PUFA-rich vegetal drinks has grown steadily in recent years. This trend is boosted by new nutritional recommendations and an awareness of the beneficial environmental and health effect of plant derived fats and proteins. Vegetal drinks are generally under the form of emulsions, containing proteins, fibres but also oil bodies, which are lipoprotein assemblies composed of a TAG core encapsulated in a phospholipid-protein monolayer. The interest of these vegetal beverages is that they naturally contain surfactant

145 molecules that can stabilize these emulsions while presenting a good PUFA and global
146 nutritional profile. If seed-based beverages have been developed, they are not very diversified
147 in terms of botanical sources and the leafy parts of plants remain, to date, under-exploited in
148 the field of human nutrition. In addition to their fibers and bioactive pigments contents, these
149 leafy parts do contain lipids although it is most of the time ignored – many leafy juices are for
150 instance wrongly labelled “lipid-free”. The digestibility of these specific plant lipids is also
151 poorly known, in particular concerning GL, and despite their human consumption can reach
152 200 mg by day [10] and could be boost up by using plant assemblies as functional ingredients.

153 However, in order to use properly plant polar lipids and lipoproteic assemblies, it is crucial to
154 understand their digestive behaviour and their oxidation stability. It is thus the objective of the
155 present review to summarize most of the recent data about plant lipid assemblies (membrane
156 and OB), their physico-chemical composition and digestive behaviour. These elements will be
157 detailed in three complementary paragraphs to unveil the potentials of these lipids as vectors of
158 omega 3 PUFA and to modulate the digestion in given nutritional targets.

159

2 PLANT MEMBRANE ASSEMBLIES' DESCRIPTION

2.1 Plasma membranes of plant cells

The plasma membrane is the most complex plant cell membrane, containing lipids and proteins in proportions that vary according to the type of cell and plant species. The plasma membrane defines an interface between cells and their environment. Although many cellular functions are performed by protein compounds (e.g. signal recognition or transduction), lipids also play a crucial role in the membrane or in the signal transduction. Three main classes of lipids are present in the plasma membrane of plant cells, namely PL (28-50% wt.), sphingolipids (6-30% wt.), and sterols (23-52% wt.). The proportions and acyl chain lengths of these lipids influence the physical state and the properties of the formed lipid bilayer [11]. Compared to other cell membranes in the animal kingdom, plant plasma membranes are predominantly rich in phytosterols, with a sterol/PL ratio ranging from 0.6 to 1.5 wt.

The structural PL composing the plant plasma membrane are mainly phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (68-80% wt.), as well as phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA); regarding FA composition, the membrane is mainly composed of palmitic acid (C16:0, 18-70% wt.) and LA (C18:2, ω 6, 20-60% wt.), as well as ALA (C18:3, ω 3, 7-26% wt.) [11]. A greater diversity of sphingolipids and sterols is observed in plants than in animals. These two compounds are essential structural compounds of membranes since they regulate the lipid packing and the phase transitions of the membrane bilayers [12].

2.2 Chloroplast membranes

Chloroplasts are flattened disc-shaped organelles of 2 to 10 μm in diameter, present in the cell's cytoplasm of photosynthetic plants (Figure 1). They contain grana, which are characteristic cylindrical stacks composed of 400 nm diameter membrane discs, comprising between 5 and 20 layers of thylakoid membranes [13]. The inner chloroplast envelope and the thylakoid membrane exhibit a similar lipid composition, in contrast to the outer chloroplast envelope, which exhibits a typical "eukaryotic cells" composition. Thylakoids are the photosynthetic membrane of chloroplasts and exhibit a fluid architecture, based on more than 100 different globular proteins (60-70% wt.) dispersed in a lipid matrix. This lipid matrix self-organizes as a stack of bilayers, composed mainly of PL and pigments (30-40% wt.). The main pigments are chlorophyll, the key element of photosynthesis, and carotenoids, which display an AO action, protecting the chlorophyll and the chloroplast membrane against oxidation [14]. Diverse phenolic compounds are also present in chloroplast and plant membranes, and contribute to the plant defence against external stresses. Thylakoids specificity is their unique lipid composition, that is very well preserved in higher plants and cyanobacteria. Thylakoids are made up of neutral GL in the form of monogalactosyldiacylglycerols (MGDG) (53% wt.) and digalactosyldiacylglycerols (DGDG) (27% wt.), which differentiates them from most other biological membranes. These GL corresponds to 80% of the total non-pigmented lipids of these membranes in higher plants and algae [15]. In addition to these two glycolipids, thylakoids contain the less abundant anionic glycerolipids sulfoquinovosyldiacylglycerol (SQDG) and PG, whose proportions vary according to the species.

This quartet of lipids shown in Figure 2 exhibits different phase behaviours, which govern the overall membrane behaviour and thus the thylakoids architecture. Indeed, DGDG, PG and SQDG form lamellar phases ($L\alpha$), regardless of the membrane hydration rate and allow the formation of a bilayer; whereas the most abundant MGDG tends to form an inverse hexagonal

structure (HII) in aqueous solution. The MGDG/DGDG ratio, hydration and lipid profile therefore modulate the bilayers self-organization, including the coexistence and transition between L α and HII phases. In particular, the critical role of DGDG has been highlighted as one of the main factors in the membranes stacking by establishing hydrogen bonds between the polar heads of adjacent bilayers, leading to the dense packing of GL bilayers in thylakoids membrane. Additionally, the regularity between the stack induced by DGDG and the repulsive electrostatic forces triggered by the charged lipids PG and SQDG [16] allow a greater repulsion of water molecules. The lipids (SQDG being anionic) but also the proteins entangled in the membrane can strongly modulate the isoelectric point of thylakoids.

A distinction regarding the FA composition of GL exists between the so-called "eukaryotic" or "prokaryotic" biosynthetic pathway. Indeed, the distribution of C18 and C16 FA on the glycerol backbone depends on the site of synthesis (chloroplast or endoplasmic reticulum) of the diacyl precursors of GL. MGDG and DGDG resulting from the prokaryotic pathway (chloroplast) are mainly C18:3/C16:3 and C18:3/C16:0 respectively (as abundantly found in spinach for instance); while MGDG and DGDG from the "eukaryotic" pathway (endoplasmic reticulum) are mainly found with a composition C18:3/C18:3 [17, 18]. The pathways of synthesis of GL, especially DGDG and MGDG, are described in detail in the recent review of Sahaka et al. [8].

In comparison, the outer membrane of chloroplast is depleted in MGDG and contains other glycerophospholipids (PC, PE, PS, PI...), in addition to PG. In this sense, it is much more similar in functional and compositional terms to plasma membranes or non-photosynthetic organelles.

2.3 Oil body assemblies and oil body membranes

In seeds, nuts, and some other plant tissues, lipids can be found in the form of lipoprotein assemblies - called oleosomes or oil bodies (OB) -, ranging in size from 500 nm to 2.5 μ m [19], depending on growing conditions and environmental factors (Figure 3). These OB are very

promising functional food ingredients as recently underlined by Abdullah et al. [20]. The main physiological function of these entities is the storage of energy in the form of TAG, but they also play a role in many cellular processes, including the lipid transport, the membrane synthesis, and the protein storage and degradation [21, 22].

Representing up to 75% of the seed volumes, particularly in oilseeds and protein crops, OB are composed by a TAG core (94-98% wt.), covered by a monolayer of proteins (0.6-3% wt.) embedded in a PL monolayer (0.6-2% wt.) [23] (Figure 1 C). This membrane protects the OB from chemical or mechanical stress. The membrane of OB is composed by strong amphiphilic proteins (mainly oleosins), that confers natural emulsifying properties to OB and allows them to be located at the water/oil interface [24].

Oleosins are alkaline proteins ranging in size from 15 to 26 kDa depending on the seeds and isoforms [25]. Oleosins exhibit N- and C-terminal hydrophilic domains, presumably oriented horizontally or extended at the surface of the OB, that frame a highly hydrophobic core domain. It is assumed that this domain of 72 uncharged and uninterrupted residues fold into an hydrophobic hairpin with two branches of about 30 residues penetrating into the TAG core of OB [24]. The secondary and tertiary structures of this central domain remain however unknown, probably due to the fact that purified oleosins or oleosins present in artificial OB may not adopt their native conformation [26-28]. The presumed hairpin structure would extend over a length of 5 to 6 nm. Analyses performed on 1000 oleosins from various plants and green algae revealed no exceptions to this corresponding sequence $X_{30}PX_5SPX_3PX_{30}$ (with X representing a large non-polar residue in X_5 and X_3 and a hydrophobic residue in X_{30}) [29]. A transcribed gene coding for an oleosin-like protein has been observed in two species of primitive modern green algae, *Chlamydomonas* and *Volvox*. This protein could be the precursor of oleosin, which is believed to have evolved in green algae to give rise to universal oleosins, whose genes are

present in all evolved plant species. Despite these evolutionary diversifications, these specialized oleosins conserve the central hydrophobic hairpin domain.

The isoelectric point of OB oleosins determined on seven species by isoelectric focusing indicated a range of 5.7-6.6 (rapeseed 6.5, mustard 5.7, cotton 6.3, flax 6.0, maize 6.3, peanuts 6.6, sesame 6.4) [30]. This acid pI contributes to the stability of OB by bringing negative charges at physiological pH to the membrane, which lead to electrostatic repulsion and steric hindrance between objects, thus avoiding aggregation and coalescence phenomena. Depending on the content of protein and PL, which vary according to the type of seed, there is an increase in the overall size of the OB when these percentages decrease. The size and mass composition of OB isolated from different seeds are listed in Table 2.

Many studies have analysed the lipid composition of different oilseeds but there is less chemical characterization data on OB from these seeds, as their composition are not exactly similar. The FA profile of polar lipids in OB is generally enriched in unsaturated FA compared to the TAG profile but keeps the same pattern in terms of predominant PUFA. The PL classes present in OB include five types: PC, PE, PI, PG and PS. PC is consistently in the largest proportion, representing between 40 and 65% wt. of PL. PE and PI are then present at 5-15% wt. of PL. PE is functionally very important due to its small polar head, that favours membrane curvatures [31]. PL distribution in different oilseed species is shown in Table 3.

OB extraction from plant seed by aqueous extraction results in a naturally emulsified product with a higher stability than emulsions prepared using surfactants such as Tween 20, or proteins such as caseinates or whey protein isolate (WPI) [32]. The natural stability of OB [33] has raised interest for their application in many areas, from cosmetic and pharmaceutical to food industry [34, 35], which explains why their use is quite widespread in the preparation of food emulsions such as creams, salad dressings, mayonnaise or liquors [36].

278 Plant polar lipids take part into the human diet, either under concentrated form (lecithin) or as
279 naturally-assemblies (nut consumption, green vegetable consumption...), but are differently
280 digested than TAG and can modulate TAG digestion.

281

3 DIGESTIBILITY AND DIGESTION MODULATION PROPERTIES OF PLANT MEMBRANE LIPIDS

The digestion of lipids depends on their chemical nature, but also on their dispersion state and on the stability of this dispersion during digestion. Indeed, the hydrolases (lipases, phospholipases and galactolipases) that are involved in the digestion, act at lipid-water interfaces.

The molecular characteristics of these lipid molecules have an impact on their hydrolysis and adsorption kinetics but also on their metabolic fate. Lipids represent an extremely wide range of molecules with diverse structures and functions, whose behaviour, due to their different physico-chemical properties, is not the same in the digestive system [37]. The gastrointestinal behaviour of molecules, membrane fragments and assemblies, as well as their effect on digestion are presented in the schematic overview Figure 4.

3.1 Digestibility of plant polar lipids: enzymes at work, influence of chemical and physical properties

3.1.1 Enzymes involved in plant polar lipid hydrolysis in Humans

In humans, no lipolytic enzyme acting on polar lipids is produced in the upper part of the gastrointestinal tract (oral cavity, oesophagus and stomach). Hence, the lipolysis of these polar lipids starts in the small intestine. Several lipolytic enzymes are secreted by the exocrine pancreas into the duodenum, and work together to digest dietary lipids: colipase-dependent pancreatic lipase (HPL), pancreatic phospholipase A2 (PLA2), pancreatic carboxyl ester hydrolase (CEH) also called bile-salt simulated lipase (BSSL), and pancreatic lipase related protein 2 (PLRP2) [38]. Lipolytic enzymes exhibit a more or less extensive specificity for their substrate. Human PLA2 (14000 Da, 125 AA) is responsible for the intestinal hydrolysis of glycerophospholipids, including the conversion of PC into 1-lysophosphatidylcholine (1-

lysoPC). In adults, PLA2 represents only a small portion of the lipolytic enzymes, with HPL and CEH accounting for the largest part [39]. HPL is the main enzyme responsible for the efficient intestinal digestion of dietary TAG in humans. Its enzymatic activity is dependent on its specific cofactor colipase. It has been estimated that HPL is responsible for the release of 56% of TAG acyl chains in the lumen, whereas human gastric lipase (HGL) only achieves 10 to 25% in the stomach, depending on the type of ingested meal.

Galactolipase activity has also been found in human pancreatic juice and in duodenal content [40-42]. In contrast to HPL, PLRP2 (50 kDa) has been shown to efficiently hydrolyse GL in the human pancreatic juice, to a similar level to PLRP2 of rats *in vivo* where little or no tritium-labelled GL were found intact in the chyle [43]. De Caro et al. [41] characterized the specificity of human PLRP2 (HPLRP2), and demonstrated that HPLRP2 activity towards TAG and PL was very low, but was high on MGDG monomolecular films. PLRP2 with similar structure has also been found in the intestines of other species, with higher galactolipase activities in particular in monogastric herbivores, such as guinea pig (GPLRP2), whose diets contain a significant amount of GL due to their herbivore dietary habits. The structural features of enzymes with galactolipase activity in humans, monogastric herbivorous species but also in some fish, foliovorous insects and microorganisms have been reviewed in detail by Sahaka et al. [8].

CEH (72 kDa, 753 AA) has also been shown to possess galactolipase activity [44], but to a lesser extent than PLRP2. Like HPLRP2, this non-specific enzyme is able to hydrolyse various lipid substrates including TAG, PL, cholesterol and vitamin esters, in addition to GL. CEH is present in higher concentration than HPLRP2 in human pancreatic juice and duodenal content, which suggests a significant contribution to the digestion of GL [38, 45]. Like PLRP2, CEH is more active on its substrates in the presence of bile salts. CEH is also able to hydrolyse the three TAG positions without distinction, but has a strong preference for MAG, thus contributing to

the total hydrolysis of TAG in synergy with other lipases. This specificity distinguishes CEH from other digestive esterases and allows it to hydrolyse partial acylglycerols into final reaction products (free fatty acids (FFA) and glycerol) in new-borns [46].

3.1.2 Digestibility of plant polar lipids: the influence of i) the structural organization of the substrate and ii) the enzyme specificity within the same substrate group

Lipolysis is a complex interfacial process, where the bioaccessibility of lipids depends on several parameters, including the structure and the composition of the food matrix, the lipid physical states and the enzyme specificities. The lipase action in the duodenum depends on the structure of its substrate but also on the structural changes that the substrate undergoes during digestion.

In the diet, lipids are mostly found in the emulsified form, which is constitutively closest to their native structure. Recent studies in the field of nutrition have shown that the organization of lipids in an emulsified form would modulate the digestion and bioavailability of fatty acids [47]; these studies have highlighted the importance of the interfacial activation of enzymes involved in the digestion of emulsified lipid droplets [48, 49]. Indeed, some lipases are inactive in the bulk phase, the lid covering the active site being in a close conformation. When the lipolytic enzyme encounters an interface, the lid undergoes conformational changes, allowing the substrate access to the hydrophobic cavity surrounding the active site of the enzyme. One has to be cautious however with the assignment of interfacial activation to the opening of a lid controlling the access to the enzyme active site. PLA2 for instance shows interfacial activation on PL micelles although it has no lid. A more general definition of the interfacial activation of lipolytic enzymes is a much higher activity on their lipid substrates when forming a supramolecular assembly, *i.e.* emulsion for lipases and micelles or liposomes for phospholipases. PLA2 for instance is weakly active on monomeric medium-chain PL but its

activity is drastically increased when the substrate concentration exceeds the Critical Micelle Concentration (CMC), above which micelles are formed [38]. In addition, the hydrolytic activity of pancreatic PLA2 requires the presence of a calcium ion that interacts with the substrate and will activate the catalytic effect of the active site of the enzyme. This interfacial activation process is different for human pancreatic lipase (HPL), which does not require calcium and is active at the oil-water interface. The presence of a lid in lipases and its conformational changes are certainly associated with this drastic environmental change for a water-soluble protein. Changes in the lid conformation result in the exposure of a large hydrophobic surface that allows lipase adsorption at the oil-water interface. While HPL can bind to the lipid droplets and hydrolyse TAG on its own *in vitro*, it requires the presence of a colipase to access its substrate in the presence of bile salts. Indeed, it has been established that the action of HPL on TAG emulsions *in vitro* is inhibited by bile salts, which hinders the adsorption of lipase at the lipid-water interface. The presence of a colipase, a 10 kDa protein produced by the pancreas, allows the specific anchoring of the lipase through the formation of a 1:1 complex in the presence of bile. However, some gastrointestinal lipases do not need interfacial activation, such as HPLRP2, which, on the contrary to HPL, acts preferentially on substrates forming monomers or small aggregates dispersed in solution, such as MAG, PL and GL [50].

The physical state of the emulsified lipids is also of importance for the hydrolytic activity of the enzymes. It seems indeed quite intuitive that solid state lipids will not get hydrolysed due to the reduced molecular motions and diffusion rate in solid substrates. More importantly, a study on the action of pancreatic lipase on various water-soluble and water-insoluble substrates revealed that the hydrolysis activity only occurred when lipid saturation was exceeded, the rate of reaction depending on the surface concentration of the substrate per unit volume [38]. Additionally, it is now well-established that the specific surface area of the emulsion is often

381 correlated with lipolytic activity. Nevertheless, the presence of surfactants - bile salts, PL,
382 proteins - can modulate this correlation by promoting the reversibility of interfacial adsorption
383 of lipase by forming a barrier or simply by competitive effects.

384 The enzymes specificity towards their substrates is also important and may not be equivalent
385 within the same lipid group. Overall, several PLA2 have been shown to have a PL head group
386 specificity and a preference for anionic substrates such as PG and PE, more than for zwitterionic
387 PL such as PC [51]. The substrate specificity is slightly different in the case of pancreatic PLA2,
388 whose physiological substrates in the small intestine are the dispersions of long-chain PC in
389 bile salts. A study by [52] has investigated the aqueous long-chain PC/sodium cholate system
390 as a substrate for PLA2. The results showed that PC was a good substrate for PLA2 in the form
391 of large mixed micelles of PC and bile salts, with a PC/cholate molar ratio of approximately
392 0.8. Similar results were obtained by [53], who showed an optimum activity of pancreatic PLA2
393 on mixed micelles of bile salts and PC at a PC/bile salt ratio of 0.5.

394 [54] studied the *in vitro* HPLRP2 hydrolysis of natural long-chain MGDG and DGDG extracted
395 from spinach leaves, compared to the hydrolysis of synthetic medium acyl chain MGDG and
396 DGDG. A continuous pH-stat assay was established, based on the titration of FFA released
397 from the hydrolysis of mixed micelles of GL and bile salts. The galactolipase activity of PLRP2
398 was found to be weakly affected by the acyl chain length, in contrast to the bile salt/substrate
399 ratio, which governs the micellar solubilization of DGDG and MGDG. This ratio is therefore
400 an important parameter in determining the optimum conditions for measuring the activity of
401 PLRP2. Overall, both the acyl chain length and size of the polar head have no major impact on
402 the active site of PLRP2 compared to classical HPL [50]. The analysis of lipolysis product of
403 MGDG and DGDG including the FA profile analysis also confirmed the specificity of HPLRP2
404 for the hydrolysis of the ester bond at the *sn*-1 position of acylglycerolipids [54]. Since ALA is

the main FA (>60%) found at this position in MGDG and DGDG isolated from chloroplast, it is the main FA released upon the hydrolysis of GL by PLRP2.

3.2 Digestibility of natural assemblies

3.2.1 Chloroplasts

The enzymatic hydrolysis of galactolipids from thylakoid membranes and their effects on the digestive system have been the subject of several studies. As previously mentioned, GL, including chloroplast rich fraction (CRF) and thus thylakoid membranes, are digested by enzymes in the pancreatic juice, including PLRP2 and CEH to a lesser extent [38]. Indeed, [42] have studied the *in vitro* digestion of CRF from pea vine field residue and spinach leaves by the human pancreatic juice, where MGDG and DGDG were rapidly hydrolysed in monogalactosylmonoacylglycerol (MGMG) and digalactosylmonoacylglycerol (DGMG). The impact of thylakoid supplementation upon digestion have been summarized in a recent review by [55]. Overall, the reviewed studies indicate that the thylakoid membranes have been found to reduce the digestion and absorption of TAG. Indeed, the thylakoid supplementation in humans induces an increase in the secretion of the satiety hormones cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1) as well as the suppression of the hunger hormone ghrelin, allowing the blood glucose regulation through the increase of insulin secretion and the decrease of appetite. The reduced appetite due to the secretion of satiety hormones suggests a delayed absorption of the nutrients in the intestine, which causes the prolongation of lipid digestion, as demonstrated by *in vivo* studies on animal [56] but also on human cohorts [57]. The observed effects on the hepatic metabolism lead to a decrease in circulating LDL and total cholesterol, as well as in the plasma lipid and glucose levels. Also, a decrease in the secretion of leptin was observed, which regulates the storage of fat and glucose in the body, raising the interest of the probable anti-diabetes effect of thylakoids. A decrease in serum TAG levels as a consequence of thylakoid supplementation in mice was observed by [58]. The effects of spinach thylakoids

on the distribution of fat content in the body, the faeces and the liver were studied. The results showed in mice an increase in faecal fat content (23 mg/day compared with 10 mg/day in the control), a decrease in body fat deposition and a reduction of liver fat accumulation; this led to an overall weight loss in mice supplemented with thylakoids compared to the control. Reduced distribution of fat cells in these mice was also observed, ranging from 30-100 μ m compared to 50-150 μ m in the control mice, suggesting that a thylakoid supplementation would stimulate lipid storage in smaller cells through precursor recruitment, thus avoiding inflammation of over-extended cells and alteration of metabolic homeostasis. A similar phenomenon was also reported by [59], who observed a decrease in the cholesterol absorption in the human subjects supplemented with milk PL and sphingomyelin (SM), but also a decrease in the degree of reabsorption of endogenous cholesterol from the intestinal mucosa. Indeed, 20 to 25% of the ingested SM had been found intact in the ileum, suggesting that their interactions by the formation of a complex with cholesterol could explain the significant cholesterol reflux in the ileum after meals (1510-1750 mg).

The hypothesis of the absence of polar lipid digestion must be excluded to explain the overall decrease in intestinal absorption of TAG induced by thylakoid membrane supplementation. Indeed, thylakoid membranes have been shown to have an inhibitory effect on the HPL complex *in vitro*, even in the presence of bile salts, which reduced the fat digestion by 80% [60]. The analyses performed on thylakoids treated with trypsin showed that the inhibitory effects on HPL were provided by intrinsic transmembrane protein complexes, and especially by the presence of several hydrophobic groups. One of the possible mechanisms put forward by [60] to explain the regulation of appetite was based on a competitive phenomenon at the lipid-water interface. The hypothesis was that thylakoid membranes can adsorb directly to the surface of the lipid substrate because of their tendency to be at a liquid-liquid interface, preventing access of the lipase-colipase complex by steric hindrance. Nevertheless, the hypothesis of the inhibition of

intestinal lipolysis by GL was based on *in vitro* digestion studies performed with classical pancreatic lipase only by and the possible hydrolysis of GL of the thylakoid membrane by PLRP2 and CEH was not considered. The observed satiety effects observed by [60] could thus have been generated by other thylakoids components (proteins and liposoluble compounds).

The intestinal absorption and the metabolic fate of GL in rats were studied by [43]. In these experiments, rats were fed with [³H]FA-labelled DGDG and [¹⁴C]-labelled DGDG mixed with soybean oil enriched or not in PC to determine whether the GL were completely hydrolysed and re-esterified to TAG and PL in the chylomicrons, or whether some could be absorbed as such. After the recovery of total lipid extracts and aqueous phases from blood and gastrointestinal organs, the individual lipid classes were separated by thin layer chromatography (TLC) and the radioactivity was measured in the different extracts. In most tissues the presence of [³H] radioactivity was associated with TAG and PL, indicating that DGDG given orally to rats were hydrolysed and their FA further re-esterified in TAG and PL synthesized in the enterocytes and subsequently found in the chyle and the plasma. These studies excluded a direct absorption of lyso-GL in the intestine nor their use for the resynthesis of GL in enterocytes.

Most of the answers concerning the digestion of GL were obtained on MGDG, DGDG and SQDG purified from leaf lipid extracts such as spinach. However, further studies on the mechanisms of GL digestion by direct action of pancreatic enzymes are still required on more varied and complex sources, such as not purified whole plant material, in order to elucidate complex mechanisms regarding the inhibition and synergistic effects. Studies performed with CRF represent one step forward in this direction.

3.2.2 Oil bodies

OB are not highly concentrated in plant polar lipids, but they form quasi-natural or at least minimally processed emulsions when OB have been extracted from their natural matrix and

redispersed in a water phase. For this reason, their digestion has attracted a lot of attention over the last decade.

Several data have been collected on the stability and the behaviour of OB during digestion [36]. [61, 62] studied the behaviour of almond OB during their *in vitro* gastric and intestinal digestion. Acid aggregation was the most important phenomena observed under gastric conditions, reflecting that gastric pH brought OB close to their pI. A partial proteolysis of at least a part of the oleosins composing the membrane was also postulated. During the transit through the intestinal compartment, the transition to a neutral pH and the addition of bile salts led to the rupture of the aggregates formed in the gastric phase of digestion. The results indicated a lag phase before the onset of lipolysis, due to a delayed adsorption of HPL, suggesting that the preserved membrane structure of the OB could hinder the pancreatic lipase access to the oil/water (o/w) interface. Similar results had been obtained by [63], who studied almond OB as substrates for several purified lipases, including HPL and GPRLP2. These two lipases were found to be active on OB, but with a lower activity (18 to 38%) than that observed on artificial almond oil emulsions. The protein/PL layer covering the OB was found to slow down the hydrolysis by decreasing the specific activity of the enzyme, and a more or less long lag phase before the onset of hydrolysis was observed. This lag phase was quite characteristic of high packing lipid systems, such as chylomicrons, which have a relatively close structure to OB, as well as milk fat globules. GPLRP2 and the HPL/colipase complex had also been found to have phospholipase activity on PL of the PL/oleosin layer, despite the presence of the proteins. The phospholipase activity, even low, could be one of the mechanisms that could explain the action of lipases on OB TAG. Indeed, the introduction of defects in the PL layer would allow the lipase to penetrate the membrane and hydrolyse the TAG core. This hypothesis had been put forward by [64], who showed that the action of a phospholipase was required before the action of the plant lipase to access the core of TAG during the growth of cucumber

seeds. Another possible mechanism would be related to the TAG partitioning at the OB o/w interface, which would be hydrolysed by lipases bound to the interface without penetrating the membrane.

[65] studied the impact of exogenous proteins on the fate of OB from sunflower seeds in the gastrointestinal (GI) tract during their *in vitro* digestion. SDS-PAGE analyses of crude OB (COB) and washed OB (WOB) emulsions indicated a more or less extensive degradation of oleosins after the gastric digestion step in the presence of pepsin, followed by the intestinal digestion in the presence of bile salts and porcine pancreatic lipase. A qualitative analysis of the SDS-PAGE results concluded to a quasi-complete degradation of WOB proteins after two hours of digestion under gastric conditions, suggesting hydrolysis and/or removal of all oleosins by pepsin cleavage. In the case of WOB nevertheless, the SDS-PAGE results indicated the presence of a 6.7 kDa protein fragment after the gastric digestion phase, which may represent the central hydrophobic domain (as well as residual fragments of terminal hydrophilic domain) of oleosins, which remained anchored to the oil phase despite the pepsin action on the groups exposed to the aqueous phase. Additionally, data indicate the presence of oleosins that remained "intact" despite gastric digestion. The exogenous proteins present in sunflower seed could explain this only partial hydrolysis of oleosins on the surface of COB. Since sunflower OB are able to associate with exogenous proteins, the latter could have formed a protective layer covering the OB, thus affecting their surface potential as confirmed by ζ -potential measurements, and partially shielding the oleosin domains exposed to digestion. This "apparent protection" from enzymatic digestion was not found at such level in the case of WOB enriched in WPI or caseinates, which raises the interest of using OB in their native non-isolated form. The different behaviour of OB during the gastric phase induced significant changes in the droplet morphology at the beginning of the duodenal phase. Although bile salts were dominant at the interface of all droplets, COB exhibited significantly smaller droplet sizes than WOB

emulsions, whether or not enriched with WPI or caseinates; this may have impacted the rate of digestion of OB. Finally, a net reduction of the protein band was observed in all types of emulsions (COB, WOB, WOB-WPI, WOB-caseinates) after the intestinal digestion phase *in vitro*, indicating the removal of the oleosins remaining on the surface of OB by the bile salts.

The formation of an exogenous protein layer on the surface of unwashed OB droplets may explain the similar protection by oleosins observed in the case of *in vitro* GI digestion of almond OB [61]. Indeed, SDS-PAGE analyses indicated the presence of amandine peptides, resistant to pepsin hydrolysis on OB surface even after 15 minutes under gastric conditions. Confocal laser scanning microscopy (CLSM) images supported this observation, showing that the surface of the almond OB was completely covered with proteins, with the almond proteins and their digestion peptides remaining attached to the surface of OB under gastric conditions. During the duodenal phase, despite the hydrolysis of some surface proteins into smaller fragments, a 2-minutes lag phase was observed before the release of the FFA, suggesting a slower penetration of pancreatic lipase at the droplet interface, probably hindered by the membrane assembly of the almond OB and the protective layer formed by exogenous proteins.

3.3 Effect of the addition of isolated constituents from plant assembly on TAG digestion

3.3.1 Effect of GL addition on TAG emulsion digestion

The digestion behaviour of lecithin-stabilized olive oil emulsion droplets coated with various amounts of spinach GL was studied by [66], the objective of this work being to quantify the effects of MGDG and DGDG on the *in vitro* activity of porcine pancreatic lipase under duodenal conditions. The results indicated that DGDG adsorbed at the droplet interface interfere with the adsorption and penetration of the lipase-colipase complex, preventing close contact with the substrate, and thus may have an inhibitory effect on the intestinal lipolysis. Interface measurements indicated significant molecular interactions between the heads of the DGDG, resulting in a tighter molecular arrangement, slowing down the interfacial penetration of

pancreatic lipase. The inhibitory effect persists under physiological conditions, since DGDG are highly surfactant molecules, they are not entirely displaced by bile salts. As a result, it slows down the enzymatic hydrolysis of TAG by pancreatic lipase and may be used to trigger the secretion of satiety hormones by the delivery of undigested fat in the distal ileum, subsequently leading to a loss of appetite. Because of their ability to delay and slow down the lipolysis of lipid droplets, DGDG appear to be effective tools in the development of novel food emulsions to regulate the fat intake and in the treatment of metabolic disorders such as obesity. Such inhibition effects of GL on the pancreatic lipase activity are interesting but they should be tested under conditions involving all pancreatic lipolytic enzymes, including PLRP2 and CEH, to validate this approach. Indeed, [66] only tested these effects of GL on the HPL-colipase complex without taking into account the possible lipolysis of GL under normal physiological conditions.

3.3.2 Effect of the addition of glycerol-PL as plant lecithin on TAG emulsion digestion

Plant lecithins are also believed to modulate the lipid digestion, thus influencing the lipid metabolism, as oil emulsification with lecithin has been shown to increase the bioavailability of FA during intestinal absorption [6]. Indeed, as previously mentioned, the amphiphilic nature of PL brings them natural emulsifiers properties, which results in the spatial arrangement of PL at the surface of lipid droplets, either in the course of the emulsion preparation or in the GI tract upon mixing of dietary lipids with that contains endogenous PC (95% wt.). By promoting the emulsification of dietary fat, PL allow increasing the specific area available for the lipase adsorption thereby increasing the rate of lipolysis and the subsequent intestinal adsorption. The direct dose-dependent impact of rapeseed lecithin just blended in bulk oil to increase the lymphatic lipids and the ALA bioavailability was also very recently reported in rat by [67].

Regarding the impact of various interfaces on lipid digestion, [68] showed that the *in vitro* gastric lipolysis of a linseed oil emulsion was increased by 30% when the lipid droplets were coated with soy lecithins instead of caseinate or Tween 80. These results were confirmed *in vivo*, with the faster intestinal absorption and bioavailability of ALA observed with soy lecithin, compared to other emulsifiers. Plant lecithins have also been shown to exhibit a potential beneficial impact on lipid absorption and a subsequent enhancement of lipid profile in plasma, liver and adipose tissues [6]. [69] have shown that the long-term supplementation of soy-derived PC in hyperlipidaemic mice prevented lipid accumulation by lowering the plasma TAG, cholesterol and leptin levels, thereby lowering the LDL/HDL ratio and alleviating obesity-related complications. These results suggest the potential of soy lecithins in the delivery of certain specific FA to certain tissues and in the prevention of obesity-related cardiometabolic disorders. However, further research is needed to determine whether these effects are found under normal human lipidemic conditions and to investigate the effects of lecithins from other sources with different lipid composition. To balance these potential beneficial effects, it is however worth noting that the use of lecithin as emulsifier may bypass the satiety mechanisms triggered in the distal part of the small intestine due to a faster gastrointestinal lipolysis and a subsequent fat absorption [68]. Liquid test meals containing a lecithin stabilized emulsion are digested at a much higher rate than normal solid-liquid meals with non-pre-emulsified fat [38]. Overall the lipolysis measured at the Angle of Treitz (end of duodenum) can thus reach around 60% with this type of liquid meal while it is only around 30% with a normal meal. Other studies in healthy volunteers and obese patients [70, 71] have shown a faster absorption of emulsified dairy fat compared to the same amount of non-emulsified fat. Altogether, these findings may lead to lower levels of undigested fat and FFA in the lower part of the small intestine and thus impair the release of satiety hormones like GLP-1 and peptide YY (PYY). Thus, a risk/benefit assessment should be performed before developing food product with additional plant lecithins.

Improving the fat digestion and absorption could be interesting to avoid malnutrition in premature infants and elderly for instance, while it could be an aggravating factor of obesity in other subjects.

3.3.3 Effect of oleosin addition on TAG emulsions digestion

To our knowledge, the effect of the addition of purified oleosins on the digestion of lipid emulsion droplets has not been documented to date. Nevertheless, [63] have investigated the susceptibility of almond OB to the *in vitro* lipolysis by various purified lipases. From the known behaviour of these lipases on PL-stabilized emulsions like Intralipid (soybean oil emulsified with egg lecithins), it is possible to deduce some effects of oleosins on lipolysis. Depending on the lipase, the specific activity measured on OB was found to range from 18 to 38% of the specific activity measured on almond oil emulsified by Arabic gum, indicating that oleosins combined with PL have an overall negative effect on lipolysis. For instance, human gastric lipase (HGL) shows a specific activity of 71 ± 3 U/mg on almond OB versus 202 ± 21 U/mg on Arabic gum-emulsified almond oil, and around 600 U/mg on Intralipid [72]. While PL favour the activity of HGL on soybean oil (Intralipid), their combination with oleosins in OB results in an 8-fold lower activity. These effects on HGL can be compared to those of casein that also reduces the activity of gastric lipase on rapeseed [73] and flaxseed [68] oils compared to lecithin alone. With human pancreatic lipase (HPL), colipase is required to measure the activity on almond OB in the absence of bile salts, while HPL alone is active on almond oil emulsified by Arabic gum. As seen with HGL, PL activity on OB (1079 ± 98 U/mg) is reduced compared to its activity on Arabic gum-emulsified almond oil (2865 ± 269 U/mg), but only 23-fold. This activity is characterized by a lag-time before steady state kinetics are observed. It is worth nothing that, on the contrary to HGL, HPL is not readily active on Intralipid and other emulsions presenting PL at their surface like milk fat globule [72]. HPL activity on these substrates usually needs to be triggered by the addition of FFA or the combined action of other

629 enzymes like HGL or PLA2. FFA favour the adsorption of HPL at the oil-water interface.
630 Similarly, the combination of PL and oleosins in OB allows the binding and action of HPL
631 associated with colipase. Therefore, oleosins may also have positive effects on lipolysis.

632 Oleosins are known to have a stabilizing role in OB, and this interfacial part very likely
633 modulate their digestion. Indeed, as natural surfactant components of OB, they provide an
634 electrostatic and steric stabilization of OB assembly at a physiological pH. Their degradation
635 has been observed to decrease the OB emulsions stability by [74], which was illustrated by an
636 increase in the polydispersity of the OB size, a decrease in the interfacial layers elasticity and
637 alterations of interactions between droplets.

638 The behaviour at the air-water interface of purified oleosins extracted from maize germ OB was
639 studied by [75]. It appeared that the behaviour of maize oleosins at the air-water interface was
640 comparable to that of milk proteins and egg yolk alipoproteins, which are used to stabilise many
641 food preparations. The high surface activity of these proteins is of interest to their use as
642 emulsifiers to stabilize foods. Nevertheless, a review by [76] reported the allergenicity of
643 peanut, hazelnut and sesame oleosins, identified and characterized as major lipophilic allergens
644 in susceptible individuals. This explains why the use of oleosins as emulsifiers or encapsulants
645 for protein or lipidic drug substances raises certain health issues, particularly for cosmetic,
646 pharmaceutical or food applications. For example, [77] proposed that increasing the oleosin
647 content in cocoa beans would promote a mechanism to enhance cocoa flavour by generating
648 more hydrophilic peptides and hydrophobic amino acids, the precursors of key components of
649 cocoa flavour. Depending on the application, the patents submitted raise the importance of
650 removing contaminants such as other proteins from the OB during purification, with the aim of
651 reducing allergenicity, colour and odour and specially to increase the stability.

3.4. Potential of plant membranes to vectorize compounds of interest in food applications

3.4.1. GL composition and ability to vectorize essential ω 3 PUFA

Due to their abundant presence in nature, thylakoid lipid membranes represent an important source of essential fatty acids, especially thanks to the unique composition of GL, which are rich in PUFA and in particular ALA. Among the numerous botanical sources offered by vegetal biodiversity, spinach thylakoids have been the subject of many scientific studies [78-80]. The thylakoid lipids of spinach are composed of the quartet MGDG, DGDG, SQDG, and PG, in proportions presented in table 1, according to a review by [81]. In comparison, non-photosynthetic sources, such as seeds, possess a different lipid profile, with still GL, but in a lower proportion and different distribution and FA composition. For example, in wheat flour, DGDG and MGDG account for 77% wt. of total GL, but DGDG are present in higher amounts than MGDG, contrary to what is found in the leaves. GL are mainly found in amyloplasts, whose number and size thus influence the concentration of GL found in wheat flour . As a comparison, the GL composition of wheat flour in spring is provided in table 1, according to [82].

3.4.2. Ability of plant lecithins to encapsulate molecules of interest

In a recent study, Vegara et al. [83] have evidenced the ability of liposomes made from rapeseed PL and stigmasterol (ST) at low concentration to protect lactoferrin during gastrointestinal digestion *in vitro*. The stability, size and organization of the liposomal vesicles were studied. The good balance in the membrane composition of the formulated liposomes made it possible to delay their hydrolysis during the gastric and intestinal digestion phases, thus making it possible to obtain a significant quantity of intact lactoferrin in the intestine after 120 minutes of digestion. Overall, liposomes made with rapeseed PL, properly combined with other lipids, have shown their potential to be used as an oral delivery system for the molecules sensitive to

677 the digestive enzymes, such as lactoferrin, thanks to their protective capacity throughout the
678 digestive process.

679

4 OXIDATION OF MEMBRANE COMPOUNDS AND MODULATING EFFECTS ON THE DIFFERENT COMPARTMENTS OF THE DIGESTIVE SYSTEM

4.1 Susceptibility to oxidation of plant polar lipids

4.1.1 Lipid oxidation

To increase the PUFA intake and especially dietary $\omega 3$, it is of the upmost importance that the plant membranes lipids are stable to oxidation. Reactive oxygen species (ROS) are generated in cell membranes and are responsible for the degradation of many cellular compounds, including lipids, proteins and prooxidant (PO) or antioxidant species such as chlorophyll, carotenoids and phenolic compounds. Treatments such as heating or exposure to light, or the presence of trace metals in raw materials also play a catalytic role in triggering the oxidation of lipids in food matrix during their storage [84].

Lipid oxidation is a phenomenon whose main mechanisms, kinetics, and factors of variation are well known today. This radical chain reaction affects the unsaturated fatty acids present in oil, fat or structural lipids and is generally schematized according to three main steps. i) The first step of initiation leads to the formation of a first alkyl radical by the abstraction of a hydrogen atom on methylene group either between two double bonds (at a bis-allylic position) or adjacent to a double bond (allylic hydrogen atom) by the ROS. This removal thus constitutes the limiting step of the reaction since it requires the availability of a hydrogen, which is only the case in lipids having one or more unsaturated chains. This explains the susceptibility of PUFA to oxidation, whose stability is inversely proportional to the number of unsaturation they contain. ii) During the second step of propagation, the alkyl radicals formed during the initiation step react with molecular oxygen, leading to the formation of a peroxy radical; which in turn react with other unsaturated FA to form hydroperoxides. The latter primary oxidation products

are highly unstable and can be converted into secondary oxidation products. iii) The final termination step then closes the reaction chain when two radical products react with each other to form a non-radical product.

The oxidation of lipids is a phenomenon that one seeks to avoid since it is responsible for 1) irreversible loss of essential PUFA, 2) early reactions that favour the faster propagation of oxidation, and 3) the organoleptic degradation of food products, through the formation of secondary oxidation products, sometimes toxic, that can enter the blood circulation through the diet and be further absorbed in body tissues, inducing the development of cancer, atherosclerosis and inflammatory diseases [85].

Delaying the onset of lipid oxidation is thus crucial, since it can cause the deterioration of food products and reduce their shelf life [86, 87].

4.1.2 The structural factors within the plant polar lipids that conditions their sensitivity to oxidation

The sensitivity of lipids to oxidation increases with their level of unsaturation whatever their form: homogeneous lipid medium (oil), biphasic system (o/w emulsion), dispersion (liposomes in aqueous phase). Nevertheless, many exceptions to this simple rule can be observed depending on the chemical structure of the lipid containing the unsaturation (FA, TAG or PL esters), its physical state and its environment (presence of PO metal ions, initial oxidation state, initial degree of hydrolysis, presence of other AO, partition coefficient in oil/water emulsion and proximity with interface...) [86, 87]. At a given unsaturation level and in a given environment, the susceptibility of the lipid molecule to oxidation will mainly depend on the Dissociating Bond Energy (DBE) necessary to break the carbon-hydrogen (C-H) bond of allylic or bi-allylic hydrogen atom. The lower this DBE, the faster the oxidation of the molecule. However, the access to this data for GL in comparison with phospholipids or TAG is not straightforward but can be inferred from bibliographic data obtained on molecules having

similar acyl chains. Within PL, PE, which is often the most unsaturated and plasmalogen-rich, is the most rapidly degraded. Conversely, PC is more resistant. The oxidative stability of GL has been less documented although their PUFA content makes them potentially very sensitive to oxidation [88, 89]. The comparisons of GL, PL and TAG reactivity have been conducted using sources with different unsaturation levels and they tend to indicate a higher stability of GL over PL, which are also more stable than TAG. In these experiments, the impact of natural PO or AO is limited by the fact that the extracts have been purified, but differences in the physical state of molecule can also modulate the oxidative reactivity. The physical state (solid/liquid) of TAG, PL or GL (either in hexagonal packing in micelles or in bilayers under condensed liquid, expanded liquid or gel) will determine the phase distributions and the local concentrations of substrates. PO or AO agents can also influence the oxidation kinetics, as well as the quality of the local environment around the molecule. For instance, hydrolysis reactions generating FFA promote oxidation via interaction phenomena with metal ions that boosts the initiation and propagation of the oxidation reaction. The control of all these factors is difficult and may explain some opposition in studies seeking to link the molecular form and the sensitivity to oxidation.

4.2 Reactivity of polar lipids in natural plant membrane assembly

In the plant kingdom, membrane defence systems are set up to control the ROS concentration and thus protect the cells. The plants possess several types of AO but also innate enzymes that are able of neutralizing ROS or regenerating the active forms of AO species. This explains why a relative chemical stability to oxidation is observed in the different native forms of plant membrane assemblies.

4.2.1 Chloroplasts

Thylakoid GL are continuously exposed to oxidative stress due to their very high levels of PUFA as well as their light exposure during photosynthesis [90]. GL may have a different response to oxidative stress than other classes of lipids such as PL or TAG.

The composition of various AO molecules in different spinach extracts was recently investigated by [91], who studied the AO properties of thylakoids and their possible application in the cells protection against ultraviolet (UV) radiation. The examined thylakoid extracts contained phenolic compounds (*i.e.* flavonoids, tannins) as well as pigments (*i.e.* chlorophyll and carotenoids). All thylakoid extracts tested by the ORAC assay in this study demonstrated AO capacities that were maintained during storage at 4°C for 1 to 79 months, although these decreased over time. In addition to various AO molecules, the presence of AO enzymes was reported in spinach thylakoids, including superoxide dismutase, which regulates the level of damaging radicals by scavenging superoxide anions, and whose activity does not appear to have been affected by storage. The enzymatic activity and structural constituents of thylakoids are believed to be responsible for the potential antioxidant activity of chloroplast in their native form *in situ*.

4.2.2 Oil bodies

In addition to their significant physical stability, OB exhibit relatively high chemical stability to oxidation during storage, despite the presence of a significant amount of unsaturated fatty acids in the majority of oilseeds [92-95]. The presence of endogenous proteins in the mixed PL/protein membrane surrounding the TAG core, but also of exogenous storage proteins in the continuous phase in nut-derived juice for instance; partly explains the oxidative stability of unsaturated FA [23]. In addition to proteins and lipids, OB also constitute a rich source of AO bioactive compounds, including tocopherols and phytosterols, which have been shown to be associated with the surface of objects. In particular, [96] reported the presence of a high amount

of tocopherol in sunflower OB (0.57 mg/g lipid) extracted by a single wash cycle with sodium phosphate buffer. Further extraction of the organelles with a 9 M urea solution removed the OB exogenous proteins as well as some phenolic compounds less hydrophobic than tocopherols, resulting in a significant increase in the OB tocopherols concentration, supporting the hypothesis that the tocopherols are intrinsically bound to the structure of oilseed OB.

The presence of a high amounts of tocopherols and phenolic AO compounds has been found in several types of oilseeds, allowing the protection of OB PUFA from oxidation and thus contributing to their oxidative stability *in vivo* in seed grain.

4.2.3 Lecithins

In addition to their multiple above-mentioned biological and biochemical properties, plant lecithins have shown AO properties regardless of the source, which is partly attributable to their high concentration of PL. Indeed, PL possess various AO properties, the mechanisms of which were clearly detailed in a recent review by [97] and also elucidated in terms of chemical mechanisms by [98]. In particular, PL exhibit an AO role by chelating the PO metal species and are able to displacing or regenerating the primary AO species. In addition, scientific studies have shown that the presence of phenols in plant lecithins, in synergy with PL, increased the AO capacity of lecithins [99]. For instance, the reactive polar head of PL (amine for PE and PC, reducing sugar for PI) have been shown to develop synergy with tocopherols, enhancing the capacity of free radical termination of this latter [100]. To explain such synergy, it was hypothesized that PL could facilitate the hydrogen or electron transfer to tocopherols and its regeneration. However, as for OB, these AO capacities are highly dependent on the quality and the structure of food matrix, and can thus be altered by processing.

4.3 Uses of plant polar lipids protection against oxidation reported in literature

In emulsions, the molecules are distributed according to their polarity and surface activity within the different phases, which are the oil phase, the water phase and the interfacial region.

In these systems, lipid oxidation is a phenomenon that occurs at the droplet interface, where the presence of a large surface area facilitates interactions between lipids and water-soluble PO species. Many factors influence the rate of lipid oxidation in these emulsified systems, including the physico-chemical properties of the interface (thickness, charge, composition, rheology, permeability) and those of the continuous phase (oxygen concentration, AO and PO composition, pH, etc.). The current trend towards PUFA-rich, but also minimally-processed products limits the use of traditional oxidation control methods such as synthetic AO; therefore, the natural stable systems where lipid oxidation can be controlled, such as plant membrane, have raised interest over the last few years.

4.3.1 Chloroplasts membrane

The emulsions stabilized by chloroplast thylakoid membrane have been proposed as functional food to promote satiety and were characterized in terms of stability, interfacial tension and droplet size but not in terms of stability to oxidation [101]. The stability to oxidation of chloroplast membrane polar lipids with different levels of unsaturation has been studied. [90] compared the oxidative stability of GL (MGDG, DGDG and SQDG) from spinach and algae to PC from salmon oil and to TAG from soybean and sardine oil. Data were obtained after purification of all lipids to avoid the influence of potentials AO and PO which are naturally present in these products, such as chlorophyll, carotenoids or tocopherols. The results of the analyses indicated that MGDG and DGDG of spinach and algae had an overall higher oxidation stability than that of TAG or PC from marine animal sources. MGDG and DGDG from spinach and algae showed the same oxidative stability as TAG from soybean oil, whereas the latter had a lower number of unsaturation numbers. SQDG from spinach and algae showed even a greater oxidative stability than MGDG and DGDG but presented on average a lower number of unsaturation than GL fraction of the same sources. Altogether, these results suggested that the galactosyl and sulfoquinovosyl residues of GL could protect the PUFA unsaturation by

enhancing the DBE of allylic hydrogen and/or through different intermolecular interactions which could modify the molecules packing and reactivity.

More recently, [88] have evaluated the oxidative stability of three types of lipids: MGDG and DGDG of spinach and algae, as well as TAG from linseed oil, previously purified from AO and PO impurities. The greater oxidative stability of DGDG and MGDG compared to TAG from linseed oil was evidenced, regardless of the source and despite higher unsaturation levels than in the TAG fraction. Within GL classes, a greater oxidative stability of DGDG from spinach and algae compared to MGDG from the same sources was evidenced. In addition to the modulation of allylic hydrogen BDE, differences in self-assembly structure and physical state between MGDG and DGDG cannot be excluded. Indeed, [102] established that MGDG form at the interface a less compact structure than DGDG, with galactosyl headgroup extended at the interface. On the other hand, DGDG could pack their galactosyl moieties at the interface, adopting a lamellar phase $L\alpha$ with parallel orientation of the headgroups with respect to the plane of the bilayer. This organization of DGDG enhanced their compacity and steric hindrance at the interface in comparison with MGDG, possibly limiting the interaction with ROS.

4.3.2 Oil bodies

Scientific studies have raised the interest of natural OB emulsions for the preparation of stable emulsions, since they have a remarkable chemical stability compared to synthetic OB emulsions prepared with other surfactants. In particular, the stability of OB extracted from *Echium Plantagineum* has been studied *ex vivo* by [95]. The interest of this plant is that its consumption has a potentially beneficial effect on health since *E. Plantagineum* contains a high concentration of PUFA, half of which is part of $\omega 3$ and includes a significant amount of stearidonic acid (SDA, C18:4, $\omega 3$) (14% wt.), which is converted to up to 30% in EPA in the human body. Despite the natural oxidative stability of oilseeds *in vivo* during storage, a high concentration of PUFA results in a susceptibility to oxidation after oil extraction from the seed. The oxidative

stability of emulsions of *E. Plantagineum* washed OB dispersed in an aqueous phase was compared to that of synthetic emulsions of *E. Plantagineum* oil stabilized by emulsifiers: SDS and Tween 20; as well as to that of bulk oil. After 7 days of incubation at 40°C, a significantly less marked oxidation was evidenced in the case of the natural *E. Plantagineum* OB emulsion. Different hypotheses have been proposed to explain this conservation of the oxidative stability of natural OB emulsions *ex vivo*: (i) the potential AO role of oleosins on the surface of OB by metal chelation or through sacrificial oxidation; (ii) the formation of a thin and compact layer by protein (oleosin) arrangement on the surface of OB, which stabilizes the droplet interface and provides a barrier to oxygen and reactive species ; (iii) the differences in surface area/volume ratio between the natural OB emulsion and the stabilized synthetic emulsions, which would reduce the effective surface concentration of tocopherols in the processed emulsions and explain the observed oxidation differences.

Similar results had been previously obtained by [103], who compared the oxidative stability of natural suspensions of washed sunflower OB with that of formulated sunflower oil emulsions stabilized with SDS and Tween 20. The results indicated higher concentrations of primary and secondary oxidation products in the formulated emulsions. The presence of a stable lipid-water interface was suggested as an argument for the oxidative stability of the natural sunflower OB emulsion, in addition to the presence of seed protein residues in the continuous phase, which are preferentially oxidized or chelated by free metal ions. However, it should be pointed out that the method of OB extraction may affect their physical integrity, and therefore their recovery method is crucial in maintaining the emulsion chemical stability [104].

OB therefore appear as a natural form of oil encapsulation, providing significant protection against oxidative stress. As developed in paragraph 4.2.2, the presence of tocopherols and phenolic compounds closely associated with OB in significant concentrations also raises the interest in the application of OB as a deliver agent for AO such as vitamin E.

4.3.3 Lecithins

Lecithins have been shown to be suitable for use as emulsifiers but also because of their AO activity, especially in bulk vegetal oils. Indeed, some PL present in plant lecithins showed the ability to act in synergy with tocopherols, thus improving AO activity [105]. This property would be related to the ability of PL to give a hydrogen atom of their amine function, allowing the regeneration of the oxidized tocopherol quinone in its initial form. The amine functions of PC, PS or PE, and the sugar residue of PI have also been shown to exhibit metal chelation properties.

[100] studied the AO effects of soybean lecithins on the oxidative stability of several vegetal bulk oils, as well as their synergistic effect with different forms of tocopherols. The best protective effects against oxidation were observed after incorporation of 5% wt. of lecithin containing high proportions of PC and PE, especially in oils rich in LA, such as rapeseed, walnut or soybean. Also, the combination of these lecithins with the γ - and δ -tocopherol forms was shown to be more effective than with the α -tocopherol or the compounds in their individual forms. This study showed in particular the potential use of lecithins in the protection of refined vegetal oils against oxidation. Nevertheless, PC was also shown to have no synergistic effect with tocopherols, and even in some cases to exhibit PO properties. Indeed, the study by [106] on the AO activity of PL on perilla oil had shown the AO efficacy of PE and PS in synergy with a mixture of tocopherols, whereas PC had shown little effect. Also, PE had been shown to increase the activity of α -tocopherol, but not PC [107]. Due to its intermediate hydrophilic-lipophilic balance (HLB), PC exhibits the ability to form reverse micelles when its concentration is above the CMC, which themselves have been reported to have PO activity [97].

In emulsified fish oil systems, lecithins appear to promote the dispersion of other active AO, thereby limiting the free-radical propagation in the medium [108]. Nevertheless, studies are

900 needed on the synergistic effects of lecithins with phenolic compounds in emulsified vegetal
901 oil systems, as data are, to our knowledge, still lacking.

902 **4.4. Plant polar lipids oxidation through digestive system**

903 Oxidation of plant lipids can occur prior the ingestion, but can also takes place during the
904 digestion [109]. During the oral phase, oxidation phenomenon is quite limited in time and the
905 enzyme activity is supposed to be low. Conversely, the gastric digestion phase takes place at
906 low pH in the presence of oxygen and catalysts found in food, and the oxidation phenomenon
907 can thus be quite high and leads to vegetal oil peroxidation and oxidation of vitamin E, C and
908 beta-carotene. Some AO of food such as polyphenols and glutathione peroxidases can help to
909 reduce the hydroperoxides production in gastric phase. Gastrointestinal glutathione peroxidases
910 are also important actors limiting gastric hydroperoxides. TAG monohydroxyperoxydes can be
911 hydrolysed by HPL almost similarly to non-oxidized TAG but non-absorbable oxidized lipids
912 can also reach the distal part of the intestine and may favour oxidation and inflammation. The
913 peculiar oxidative behaviour and AO effect of PL from plant membrane in the gastrointestinal
914 part is still unknown and should be further investigated. Main data and hypothesis of structural
915 level that determine plant polar lipids susceptibility to oxidation are summarize in Table 4.

5 CONCLUSION

The plant membrane gathers a diversity of GL and phospholipids, selectively acylated with PUFA and are already organized as lamellar structure, embedded with proteins and phenolic compounds.

Among these polar lipids, GL concentrating ALA and phospholipids can be used as specific vector of PUFA. They are hydrolysed by specific intestinal enzymes (PLRP2, PLA2 and CEH) and allow the reduction of the TAG digestion and absorption. GL specifically can activate the ileal break. Concerning the potential inhibition activity on pancreatic lipase, *in vitro* studies involving all pancreatic enzymes are needed to determine the exact involved mechanisms.

These plant polar lipids also intrinsically present AO activity (higher stability to oxidation than TAG) and metal chelating activity. In addition, their natural association with the phenolic compounds in membrane assemblies and their physical state ($L\alpha$ state of DGDG) can enhance their stability to oxidation.

Minimally-processed plant assemblies such as OB or chloroplasts which have acidic isoelectric point and are not hydrolysed by gastric enzyme can thus be preserved during this phase and be conveyed with minimal transformation to duodenum. Their stability to oxidation in gastric phase remains to be elucidated.

Altogether, all these elements make these plant membrane molecules and assemblies very promising components with a wide range of application to vectorise $\omega 3$ PUFA and equilibrate human diet.

936 **Author Contributions** – C. Bourlieu, V. Vié and J. Kergomard determined the outline and
937 content of the review, designed the tables and figures, J. Kergomard wrote the review, all
938 authors corrected and implemented the review.

939

940 **Conflicts of Interest** – none to declare.

941

943 **TABLES**

944 **Table 1** Lipid composition (in percent by weight) of spinach leaves chloroplasts membrane and wheat
945 flour

		MGDG	DGDG	SQDG	PG	Others
Chloroplast	Thylakoids ^a	53.0	27.0	7.0	7.0	6.0
	Inner	49.0	30.0	5.0	8.0	8.0
	membrane ^b					
	Outer	17.0	29.0	6.0	10.0	38.0
membrane ^b						
Amyloplasts	Wheat flour ^c	0.4	1.0	-	-	0.4
^a Isolated from spinach leaves [% wt.], [110]						
^b Isolated from spinach leaves [% wt.], [111]						
^c Spring wheat flour [% w.],[82]						
Source: [81]						

946

947

Seed	Rapeseed	Flaxseed	Mustard	Cotton	Corn	Peanut	Sesame	Soybean	Sunflower
	d	d	d	n		t		n	
Diameter (μm)	0.6 ^a	1.3 ^a	0.7 ^a	1.0 ^a	1.4 ^a	2.0 ^a	2.0 ^a	0.4 ^b	1.0-2.0 ^d
Neutral Lipids [% wt.]	94.2 ^a	97.6 ^a	94.6 ^a	97.0 ^a	97.6 ^a	98.2 ^a	97.4 ^a	85.9 ^c	96.0 ^d
Proteins [% wt.]	3.5 ^a	1.3 ^a	3.2 ^a	1.7 ^a	1.4 ^a	0.9 ^a	0.6 ^a	8.2 ^c	(/)
PL [% wt.]	2.0 ^a	0.9 ^a	1.6 ^a	1.2 ^a	0.9 ^a	0.8 ^a	0.6 ^a	5.8 ^c	2.5 ^d
FFA [% wt.]	0.4 ^a	0.1 ^a	0.2 ^a	0.1 ^a	0.1 ^a	0.1 ^a	0.1 ^a	(/)	(/)

^a [30]

^b [112]

^c [113]

^d [31]

950 **Table 3** Mass percentage composition of PL of OB isolated from various seeds

P	Rapesee	Flaxsee	Mustar	Cotto	Corn	Peanut	Sesame	Sunflow	Soybea
L	d	d	d	n				er	n
P	59.9 ^a	57.2 ^a	53.1 ^a	58.9 ^a	64.1 ^a	61.6 ^a	41.2 ^a	81.0 ^c	58.0 ^d
C						60.8 ^b			
P	5.9 ^a	2.8 ^a	15.5 ^a	4.6 ^a	8.1 ^a	5.0 ^a	15.8 ^a	13.0 ^c	15.0 ^d
E						5.1 ^b			
PI	14.0 ^a	6.9 ^a	13.1 ^a	18.1 ^a	7.6 ^a	8.4 ^a	20.9 ^a	5.0 ^c	14.0 ^d
						8.6 ^b			
P	(/)	(/)	(/)	(/)	(/)	(/)	(/)	(/)	(/)
G									
P	20.2 ^a	33.1 ^a	18.3 ^a	18.7 ^a	20.2 ^a	25.0 ^a	22.1 ^a	(/)	(/)
S						25.5 ^b			

^a [30]

^b [114]

^c [31]

^d [115]

951

952

953 **Table 4** Main data and hypothesis of structural level that determine plant PL susceptibility to oxidation

Compounds assembly	/ Molecular oxidation	stability to	Partition in O/W system	Chelating effect	Physical assembly	ref
Galactolipids	Galactolipids > PC > TAG; SQGD > galactolipids					[90]
Galactolipids	DGDG > MGDG				Potential effect of L α packing of DGDG	[88]
Phospholipids	PL > TAG			Chelating pro-oxidant metals	Segregate PC in membrane	[97]
Phospholipids			Synergy with tocopherol: facilitate its regeneration through H transfer from amine functions of the PC, PS or PE, and the sugar residue of the PI			[100, 116]
OB	OB > reconstituted artificial emulsion with same TAG core		Potential AO effect of oleosin at interface			[95, 103]

954

955

956

FIGURES

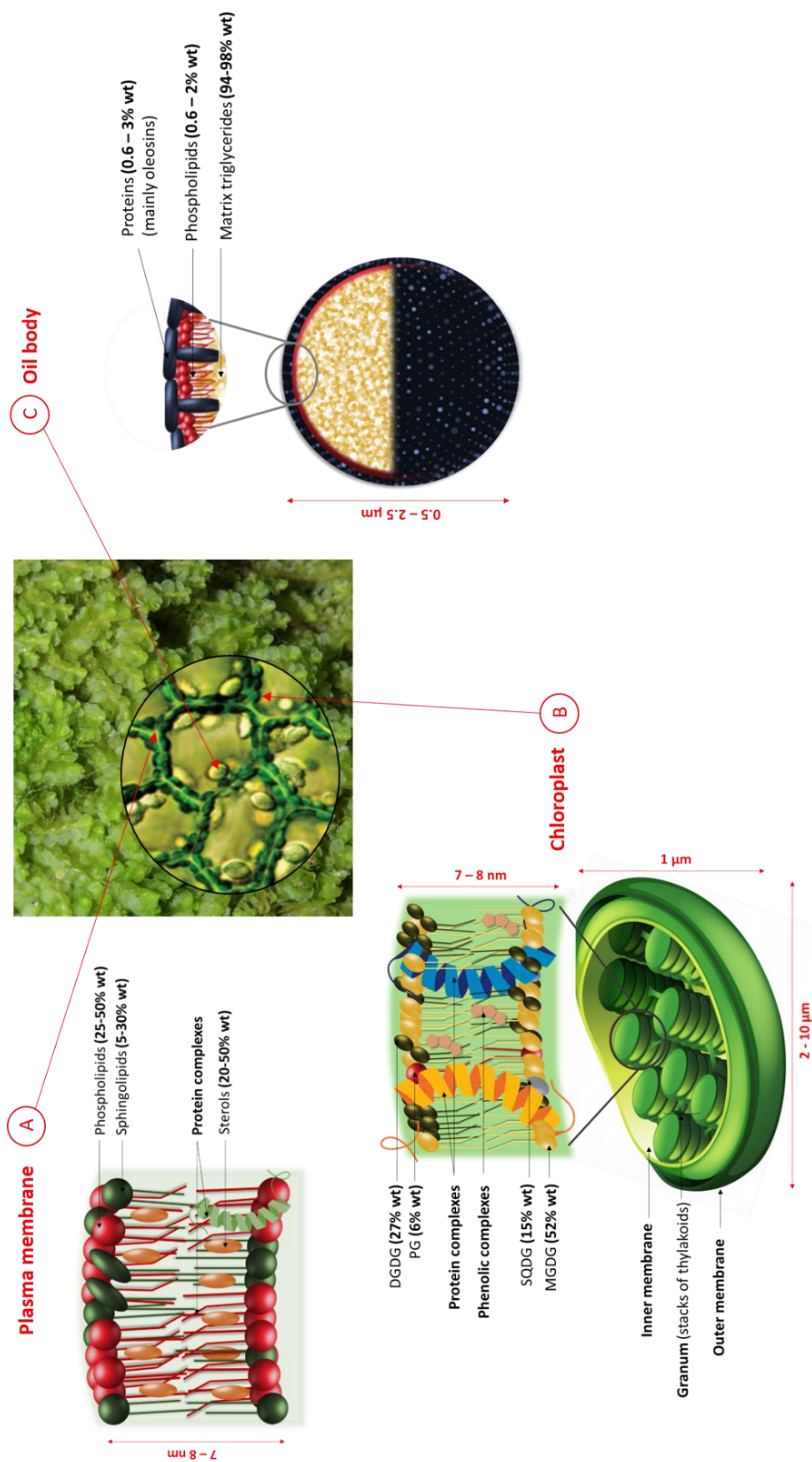
Figure captions.

Figure 1 Vegetal assemblies' description: (A) Chloroplast, (B) Oil Body, (C) Plasma membrane - Micrograph adapted from [117].

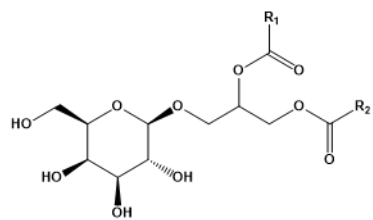
Figure 2 Structure of the main plant membrane lipids: A) Monogalactosyldiacylglycerol (MGDG), B) digalactosyldiacylglycerols (DGDG), C) sulfoquinovosyldiacylglycerol (SQDG), D) Phosphatidylglycerol (PG), E) Phosphatidylcholine (PC), F) Phosphatidylethanolamine (PE), G) Phosphatidylinositol (PI) and H) Phosphatidylserine (PS).

Figure 3 Micrographs of vegetal tissues with embedded OB: A) Walnut OB (CLSM, red: PL, blue: proteins and green: TAG), unpublished results; B) Cashew Nut OB (CLSM, red: PL, blue: proteins and green: TAG), unpublished results; C) Almond OB (TEM), reproduced with authorization from [118]; D) Rice OB (TEM) · reproduced with authorization from [119]. Abbreviations. CLSM, confocal laser scanning microscopy; TEM, transmission electron microscopy.

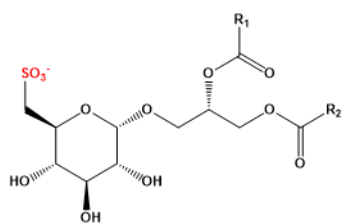
Figure 4 Schematic overview of the gastrointestinal behaviour of plant membrane molecules, fragments and assemblies, and their modulating digestion effects.



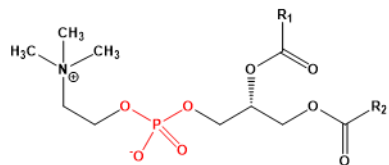
(A)



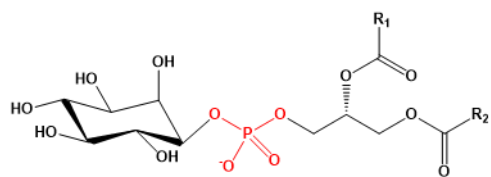
(C)



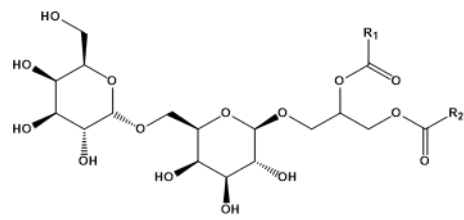
(E)



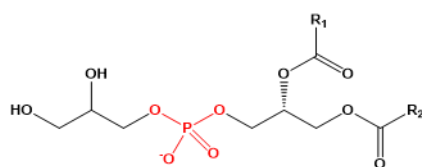
(G)



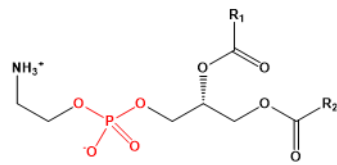
(B)



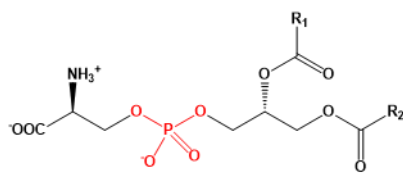
(D)



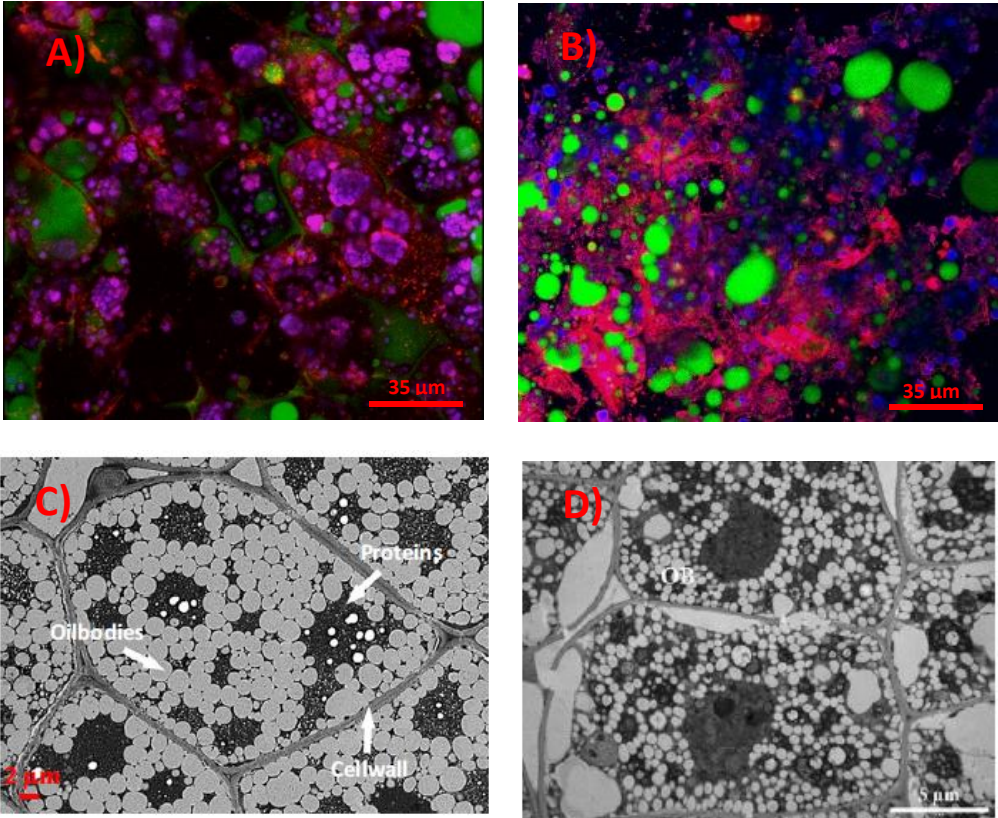
(F)



(H)



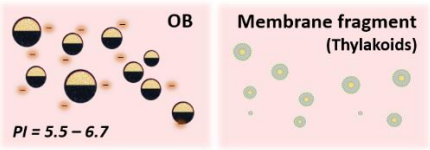
981 **Figure 3**



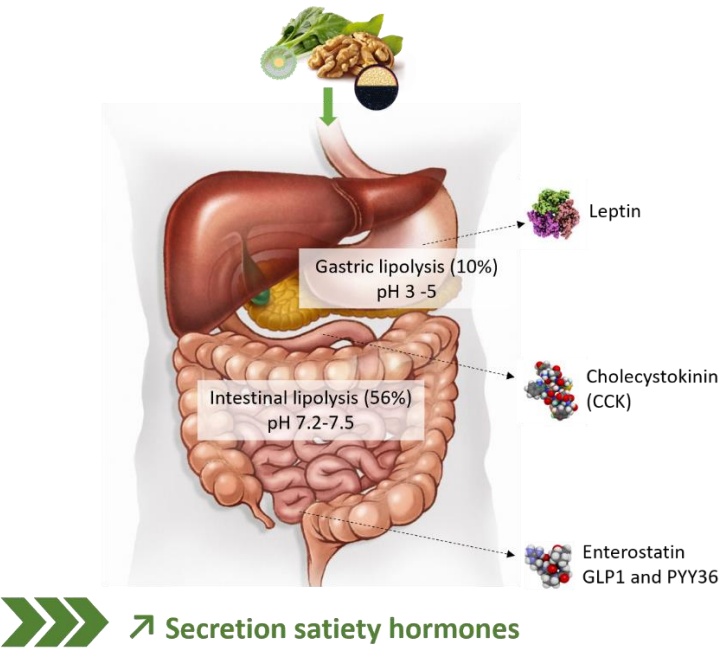
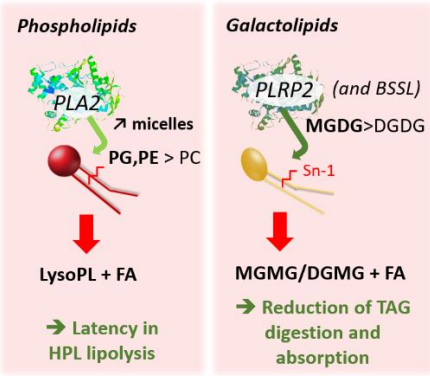
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Gastric behavior: aggregation but stability



Intestinal behavior: aggregation but stability



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