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Review

# Lipidomic approaches to the study of phospholipase $A_2$-regulated phospholipid fatty acid incorporation and remodeling☆

David Balgoma [a,b], Olimpio Montero [a,b], María A. Balboa [a,b], Jesús Balsinde [a,b,*]

[a] Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas (CSIC), 47003 Valladolid, Spain
[b] Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), 08036 Barcelona, Spain

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ABSTRACT

The distribution of fatty acids among cellular glycerophospholipids is finely regulated by the CoA-dependent acylation of lysophospholipids followed by transacylation reactions. Arachidonic acid is the fatty acid precursor of a wide family of bioactive compounds called the eicosanoids, with key roles in innate immunity and inflammation. Because availability of free AA constitutes a rate-limiting step in the generation of eicosanoids by mammalian cells, many studies have been devoted to characterize the processes of arachidonate liberation from phospholipids by phospholipase $A_2$s and its re-incorporation and further remodeling back into phospholipids by acyltransferases and transacylases. These studies have traditionally been conducted by using radioactive precursors which do not allow the identification of the phospholipid molecular species involved in these processes. Nowadays, lipidomic approaches utilizing mass spectrometry provide a new frame for the analysis of unique phospholipid species involved in fatty acid release and phospholipid incorporation and remodeling. This review focuses on the mass spectrometry techniques applied to the study of phospholipid fatty acid trafficking and the recent advances that have been achieved by the use of this technique.



## 1. Introduction

Once regarded as mere building blocks or as energy-storage depots, lipids are now universally recognized to play key roles in cell communication and a number of physiological and pathophysiological states [1–8]. Among the various lipid families are the fatty acids, which are frequently found esterified in the *sn-1* and *sn-2* positions of the glycerol backbone of glycerophospholipids (GPs). Interestingly, saturated fatty acids are primarily found in the *sn-1* position, whereas unsaturated fatty acids are primarily esterified in the *sn*-2 position [9]. This particular distribution of the apolar fatty acid chains together with the polar headgroup bonded to the phosphate provides these compounds with an amphiphilic structure that is essential to bilayer organization of cell membranes. Moreover, the distribution of fatty acids in cells is achieved by a controlled movement of incorporation, remodeling and liberation of fatty acids into, among and from GPs, respectively [10]. In these three processes, $PLA_2$ enzymes play important roles, since they release free fatty acids by cleaving the ester bonds at the *sn*-2 position of GPs [11,12], and help maintain the 2-lysoglycerophospholipid pool that acts as free fatty acid acceptor for further remodeling reactions [13,14].

Mammalian $PLA_2$ enzymes involved in long-chain fatty acid metabolism have frequently been classified into three groups according to biochemical commonalities, namely, $Ca^{2+}$-independent cytosolic $PLA_2$ ($iPLA_2$), $Ca^{2+}$-dependent cytosolic $PLA_2$ ($cPLA_2$), and $Ca^{2+}$-dependent secreted $PLA_2$ ($sPLA_2$) [11,12]. The role of $iPLA_2$ in phospholipid fatty acid homeostasis has been thoroughly studied, and appears to be strikingly dependent on cell type [15–23]. When the cells are stimulated via surface receptors, $cPLA_2$ becomes a major regulator of phospholipid fatty acid turnover. $cPLA_2$ is regulated at a cellular level by mitogen-activated protein kinase-mediated phosphorylation cascades and by $Ca^{2+}$ transients,

*Abbreviations:* AA, arachidonic acid; GP, glycerophospholipid; $PLA_2$, phospholipase $A_2$; $cPLA_2$, cytosolic group IVA $PLA_2$; $iPLA_2$, $Ca^{2+}$-independent $PLA_2$; $sPLA_2$, secreted $PLA_2$; CoA-IT, CoA-independent transacylase; ACS, acyl-CoA synthetase; LPCAT, lysophosphatidylcholine:acyl-CoA acyltransferase; ESI, electrospray ionization; HPLC, high performance liquid chromatography; MS, mass spectrometry; TLC, thin layer chromatography; PC, choline glycerophospholipids; PE, ethanolamine glycerophospholipids; PI, phosphatidylinositol.

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* Corresponding author at: Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas (CSIC), 47003 Valladolid, Spain. Tel.: +34 983 423 062; fax: +34 983 184 800.
*E-mail address:* jbalsinde@ibgm.uva.es (J. Balsinde).

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which induces translocation of the enzyme to various organelles, to initiate phospholipid hydrolysis and free fatty acid release [23–30]. In activated cells, particularly those of immunoinflammatory origin, a $sPLA_2$ may also participate in stimulated phospholipid hydrolysis by acting in concert with $cPLA_2$ [31–33].

Due to the importance of AA in inflammation, the trafficking of this fatty acid in cells involved in immunoinflammatory reactions (e.g. phagocytes, mast cells) has been thoroughly studied [34]. In these cells, free AA usually incorporates in phospholipids, mainly PC, via the Lands cycle of phospholipid deacylation/reacylation by the action of an acyl-CoA lysoglycerophosphocholine acyltransferase (LPCAT) [34]. Next, the AA is transferred in a remodeling process [34] from PC to PE by the action of CoA-independent transacylase (CoA-IT). In these two steps, $PLA_2$ enzymes, particularly $iPLA_2$, play regulatory roles by maintaining the levels of 2-lyso acceptors [13,14,35,36]; lysoPC as a transient acceptor, and lysoPE as a final acceptor (Fig. 1). The process of CoA-IT AA remodeling is a key regulatory point in the AA trafficking mechanism because blockade of this enzyme has been associated to induction of apoptosis [37,38].

Traditional studies on the remodeling of GP fatty acids have been carried out by incubating the cells with a radiolabeled fatty acid, extraction of the sample, separation of GP classes by TLC and quantification by scintillation counting. Using this methodology, determination of the particular species within every GP class is achieved through a laborious protocol that may involve derivatization and multiple separation steps before final quantification by scintillation counting [39,40]. Current advances in the coupling of mass spectrometry to liquid chromatography through electrospray ionization (ESI), as well as the development of MS tandem triple quadrupole analyzers have made the high-throughput analysis of GPs possible. This breakthrough is not only quantitative, but also qualitative, leading to the emergence of a new field called lipidomics [41–44]. Lipidomics is not solely focused towards analysis and chemometrics, but aims at the knowledge of lipid changes and their interacting partners in a biological context [45–47]. In addition, the term metabolipidomics has been coined to emphasize the dynamical aspects of lipids in cells, organs, tissues and whole organisms, as lipidomics is focused on profiling the "static" composition of molecular species in biological samples [48]. As the main tool for metabolipidomics is stable isotope labeling, this strategy takes advantage of the previous radiolabeling studies in addition to the MS analysis capability, thus making possible the identification of minor species with rapid turnover rates that may be involved in phospholipid fatty acid remodeling [49].

## 2. Mass spectrometry approaches to the study of phospholipid fatty acid remodeling

Preparation of the samples for MS analysis will generally require liquid–liquid extraction, evaporation of the organic extract under vacuum or a nitrogen stream, and resuspension in a proper solvent mix. Historically, the most widely used extraction methods for GPs are the chloroform-based method of Bligh and Dyer for cell samples [50] and the Folch method for tissue samples [51].

Taking into account the technical developments of MS and the structure of GPs, two main strategies for the study of fatty acid remodeling can be used. The first strategy combines the direct infusion to the ESI chamber of the resuspended lipid extract with the use of precursor ion scanning of characteristic ions in fragmentation experiments (ESI-MS/MS) [52,53]. The mass spectrometry analyzer used in precursor ion scanning is the triple quadrupole. The first quadrupole scans the range of m/z for GPs; in the second chamber the ions selected in the previous one are fragmented by collision-induced dissociation (CID), and the third quadrupole transmits a fragment with a specific m/z, so that the ions isolated in the first quadrupole that yield a specific fragment are the only ones to be detected [54,55]. Therefore, direct infusion and precursor ion scanning procedures are suitable for analyzing families of compounds whose m/z values corresponding to the precursor ion are different but share a common structural part and, therefore, render a common identifying fragment (Table 1). For instance, when the ionization is carried out in positive mode, all PC species, whose m/z in MS depends on the acyl substituents (number of carbon atoms and double bonds), have the phosphocholine unit that yields m/z 184 in CID; hence, if m/z 184 is fixed in the third quadrupole and different m/z are filtered in the first one, the species with a phosphocholine unit but different acyl chains can be detected [56,57]. Similarly, families of compounds can be characterized in CID by a specific neutral loss (NL). In such analysis, the first quadrupole and the third quadrupole must be in scan mode but coupled with the NL difference of m/z.

The strategy described above is quite straightforward and not too time- or solvent-consuming. However, a major problem is that, inasmuch as the mass information obtained corresponds to the sum of carbons and double bonds of the two fatty acids initially

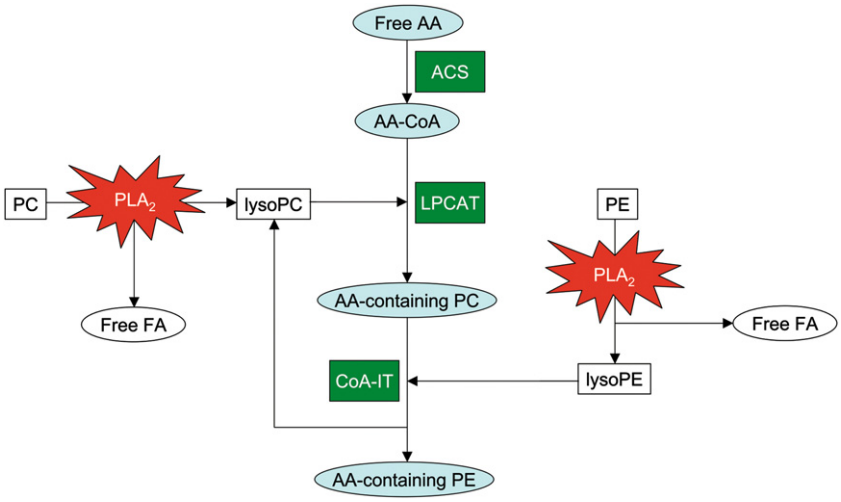


**Fig. 1.** Dual role of $PLA_2$ enzymes in glycerophospholipid AA incorporation and remodeling. AA, arachidonic acid; PC, choline glycerophospholipids; PE, ethanolamine glycerophospholipids; FA, fatty acid; ACS, acyl-CoA synthetase; LPCAT, lysophosphatidylcholine:acyl-CoA acyltransferase; CoA-IT, CoA-independent transacylase; $PLA_2$, phospholipase $A_2$.

**Table 1**
Specific fragments produced by glycerophospholipid classes depending on the polar headgroup in MS/MS experiments.

| GP class | Precursor ion | Identifying fragment |
|---|---|---|
| PA | $[M-H]^-$ | m/z 153 |
| PI | $[M-H]^-$ | m/z 241 |
| PIP | $[M-H]^-$ | m/z 321 |
| $PIP_2$ | $[M-H]^-$ | m/z 401 |
| PS | $[M-H]^-$ | NL 87 |
| PE | $[M-H]^-$ | m/z 196 |
| PC | $[M+H]^+$ | m/z 184 |

present, it is not possible to unequivocally identify the fatty acids present in each GP. An additional drawback of this procedure is that complex matrix effects can produce ion suppression and the signal of species present at low concentrations in the sample can be lost.

By using the scheme of serial product ion scanning (ESI-MS/MS) [54], the length and double bonds of both acyl chains present in a GP can be determined by quadrupole-time of flight (q-ToF) mass spectrometry [58,59]. However, in these spectrometers, ion suppression in direct infusion analysis can still eliminate minor signals of GPs. On the other hand, triple quadrupole instruments achieve a better performance than q-TOF instruments for quantification purposes.

In order to obtain more accurate structural information and to minimize matrix effects, a second strategy has been developed which consists in introducing a step of separation before MS detection, by coupling an HPLC instrument to the MS instrument. Separation of GPs by HPLC can be carried out by using two stationary phases, namely normal phase (NP) and reversed phase (RP) [60]. In normal phase, where molecules interact with a polar stationary phase – typically silica gel [61] or diol-modified columns [62]–, GPs are primarily separated by their polar headgroup, the less polar compounds eluting first and the most polar eluting later. Reversed phase liquid chromatography consists of silica coated with hydrocarbon chains – typically ocadecyl and octyl [63–65]– and, since the stationary phase is apolar, GPs are primarily retained and separated by acyl chains, eluting first those with less number of carbons and more double bonds [66]. This implies that GPs belonging to the same class (the same polar headgroup) and producing the same m/z but containing different acyl chains, can be separated by this procedure (e.g., PE(18:0/18:0) and PE(P-18:2/20:4)).

Coupling of HPLC with mass spectrometry has been mostly done with an ESI interface and, with regard to ion separation, several analyzer types have been used [60]. Ion-traps have been used in full scan mode (HPLC-ESI-MS) [63,67]. In this type of MS analysis, analyzer saturation problems are avoided by the previous chromatographic separation step. Quadrupole analyzers have been used both in multiple reaction monitoring (HPLC-ESI-MS/MS) with triple quadrupole spectrometers [68,69], and in full scan with single quadrupole spectrometers (HPLC-ESI-MS) [70]. Although ion-traps are not suitable for MS/MS quantification analysis, they offer the possibility of performing more than one cycle of fragmentation, which permits in-depth structural characterization [71,72].

Both NP and RP-HPLC offer a good separation and avoid putative analytical interferences. However, since GPs co-elute by polar headgroup in NP-HPLC, this mode offers the same analytical information as direct infusion with precursor ion scanning. When minor compounds and/or specific species analysis is required, RP-HPLC is the preferred separation technique. Notwithstanding, while good separation of PE, PC and PI is possible in RP-HPLC, PA and PS show high-grade peak tailings that frequently prevent quantification. Important improvements in the separation and analysis of PA and PS have recently been reported [73].

To conclude this section, the analytical strategy to be utilized, namely ESI-MS/MS or HPLC-ESI-MS/MS depends on the aim pursued. A summary of choices is depicted as a flowchart in Fig. 2.

## 3. Research applications: $PLA_2$-regulated phospholipid fatty acid trafficking

Two different approaches can be undertaken to study the remodeling of GP fatty acids by MS. The first one focuses on the polar headgroup and addresses the variation of apolar chains. The second approach deals with variations of the apolar chains among defined GP classes. Both approaches may take advantage of the use of stable isotope-labeled compounds (metabolipidomics) to study the pathways of incorporation/remodeling of a specific fatty acid or headgroup.

### 3.1. Headgroup-based studies

In these studies the aim is to analyze the recycling of fatty acids within a specific GP class (same headgroup). Thus, precursor ion scanning of headgroup-containing specific fragments appears as the most straightforward approach

By using tandem MS, Rouzer et al. studied GP fatty acid remodeling in murine resident peritoneal macrophages and RAW264.7 cells stimulated with yeast-derived zymosan [74]. A deficiency of AA in cultured cells as compared to their primary counterparts was evidenced, and AA-supplemented RAW264.7 cells showed a distribution of AA among GPs that was different from that found in resident murine macrophages [74]. Moreover, when the macrophages were exposed to zymosan, which activated $cPLA_2$, AA-containing PC species were found to act as net donors of the $cPLA_2$–mediated free AA mobilization, and AA-containing PE species did not show significant changes [75].

To specifically study those GPs that have been recently synthesized, a metabolipidomic approach can be followed by incubating the cells with a stable isotope-containing compound that labels the polar headgroup of certain phospholipids. In these samples, precursor ion scanning of headgroup-derived fragments in tandem MS would be the technique of choice. For example, addition of $[^2H]_9$-choline to the cells would generate newly-synthesized $[^2H]$-labeled PC which could be identified by ESI-MS/MS scan of m/z 193 in positive mode. Since the fragment of 184 corresponds to endogenous PC, it would be possible to compare the composition of newly-synthesized PC with that of pre-existing PC. Such an approach has recently been carried out by Postle et al. [76] to compare the composition and dynamic remodeling of endonuclear GP species in cultured cells. Experiments with alternative MS/MS precursor scans of 184 and 193 in positive mode when IMR32 neuroblastoma cells are exposed to $[^2H]_9$-choline, revealed a higher grade of saturation of PC in the endonuclear space, which remodels with time into an even more saturated profile [77]. In addition, the profile of GPs in whole cells in the equilibrium was found to be more sensitive to culture conditions than endonuclear GPs. These latter showed a highly regulated synthesis and remodeling of acyl chains in the endonuclear compartment [78].

Using a similar metabolipidomic technique, two newly-synthesized different pools of PC have been described in yeast [79]. A pool comes from PE methylation and can be characterized by labeling with (methyl-$[^2H]_3$)-methionine, whereas the other one comes from the *de novo* route. None of the two newly-synthesized PC sources matches the PC profile in the equilibrium, suggesting that a remodeling process via PLA is necessary [79].

Another approximation to the study of fatty acid remodeling is the one described recently by Kainu et al. [80], where a whole GP, labeled in the headgroup, is introduced in the cell by

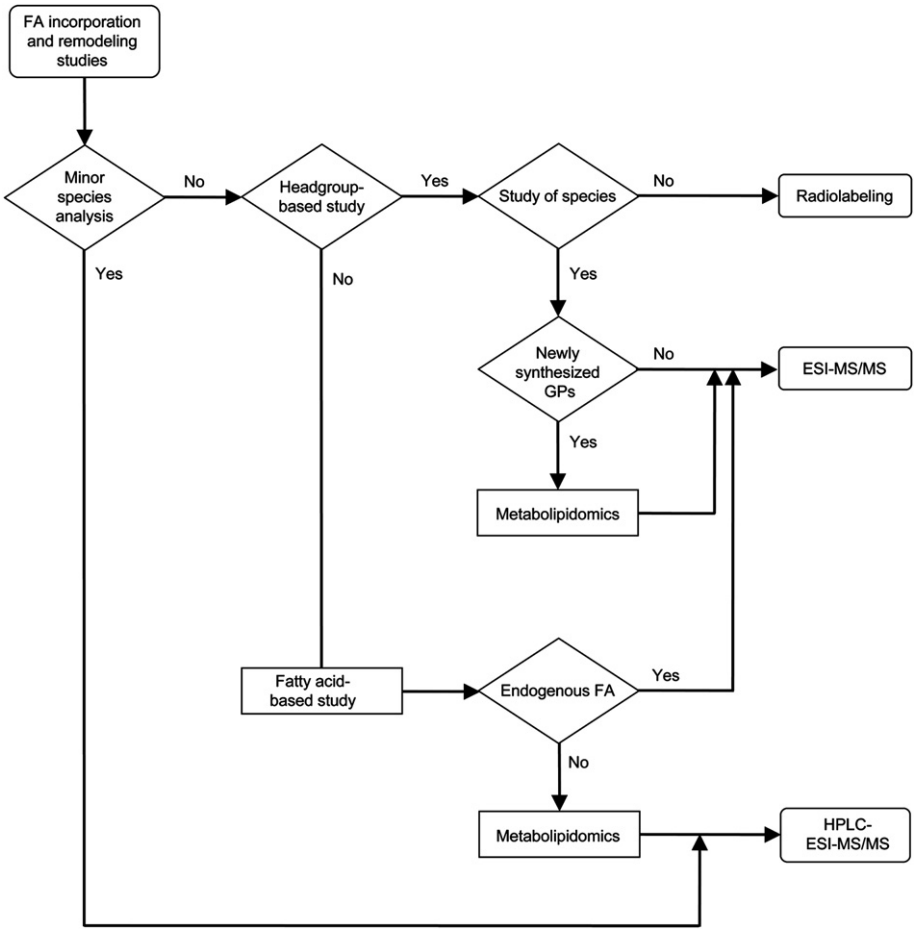


**Fig. 2.** Flowchart to choose the appropriate analytical strategy to study fatty acid remodeling by $PLA_2$. Metabolipidomics refers to MS experiments with stable isotope-labeled compounds.

a cyclodextrin-based shuttling procedure. Using this approach, the remodeling of acyl chains at both *sn-1* and *sn-2* positions was characterized in PE and PS in BHK21 and HeLa cell lines. When the exogenous GP had a fatty acid composition not originally present in the cell, the remodeling process was carried out very quickly, to ultimately reach a fatty acid composition similar to that existing in the endogenous headgroup counterparts [80]. Conversely, when the exogenous GP had a fatty acid composition already present in the cell, remodeling was rather slow [80]. By double-labeling both headgroup and *sn-2* fatty acid of the exogenously-added GP, it was found that the extent of remodeling was directly related to the extent of $PLA_2$-mediated hydrolysis on this particular GP. Based on the finding that PE and PS species with identical acyl chain composition remodeled at different rates, the possibility was suggested that the $PLA_2$ enzymes involved were able to discriminate not only among fatty acid residues, but also among headgroups [80].

### 3.2. Fatty acid-based studies

To study the recycling of a particular fatty acid, MS/MS precursor ion scanning experiments would constitute the most straightforward approach. For example, remodeling of AA can be studied by scanning the precursor ions of m/z 303 in negative mode. It is important to note that when labeled fatty acids are used, interferences of endogenous fatty acids with the same m/z may occur. Commercial deuterium-labeled AA contains eight deuterium atoms ($[^2H]_8$AA). Thus the m/z to carry out the precursor ion scan in negative mode is 311. Unfortunately, arachidic acid (C20:0) produces the same m/z in tandem MS detection. This could be a major problem when studying GP species enriched in both arachidic and AA, e.g PE. Since these two fatty acids notably differ in the number of double bonds (0 vs 4, respectively), a manner to circumvent this problem would be to conduct an RP-HPLC separation step before MS analysis.

By using RP-HPLC-ESI-MS and deuterium-labeled AA, we have recently identified the PI species involved in the initial incorporation of AA in human monocytes and U937 cells [49]. Four major species were identified, namely PI(16:0/$[^2H]$AA), PI(18:1/$[^2H]$AA), PI(18:0/$[^2H]$AA) and PI($[^2H]$AA/$[^2H]$AA). Pulse-labeling with $[^2H]$AA for short periods of time and monitoring of these species in monocyte-like U937 cells, showed that the levels of PI($[^2H]$AA/$[^2H]$AA) experience an exponential decrease, disappearing within 3 h. In contrast, the levels of the other $[^2H]$AA-containing PI molecular species decrease only slightly with time [49].

Stimulation of the monocytes with the phagocytosable stimulus zymosan, activates $cPLA_2$, which results in extensive recycling of AA among GPs. We have used RP-HPLC-ESI-MS to track these changes and, consistent with previous studies [75], we have found that all major AA-containing PC and PI species show a decrease that is $cPLA_2$-dependent, but AA-containing PE major species do not show significant variation (Balgoma et al., manuscript in preparation). In addition, these studies have allowed us to identify three minor species (i.e. those that comprise less than 5% of a given GP class in

unstimulated cells) that, unlike all other species, increase rather than decrease after cellular activation. Two of the three species are PC(20:4/20:4) and PI(20:4/20:4), are apparently produced in a deacylation/reacylation pathway via cPLA$_2$ and acyl-CoA synthetase-dependent reactions. The third minor species, PE (16:1/20:4), is undetectable in unstimulated cells and may be produced via coenzyme A-independent transacylation reactions (Balgoma et al., manuscript in preparation).

## 4. Concluding remarks

Mass spectrometry offers selectivity and sensitivity to study fatty acid remodeling within individual GP species and the role of PLA$_2$ in this process. The analytical method to utilize, *i.e.* ESI-MS/MS or HPLC-ESI-MS/MS, will depend on the information that is desired to obtain.

Since AA is found primarily in the *sn-2* position of GPs, PLA$_2$ enzymes are key regulators of phospholipid AA incorporation and remodeling. PLA$_2$ enzymes may act on various points in the remodeling process of AA; by acting upon GPs and liberating free AA, and indirectly by producing lysoPC and lysoPE that act as transient and final acceptors of AA. The use of RP-HPLC-ESI-MS in the study of AA incorporation and remodeling has allowed the detection of minor species in human monocytes, namely PC (20:4/20:4), PE(16:1/20:4) and PI(20:4/20:4) which are generated as specific products of fatty acid remodeling reactions. Thus, mass spectrometry lipidomic approaches allow the possibility of studying the process of fatty acid remodeling in depth, with emphasis on the differential remodeling of the *sn-1* and *sn-2* positions of the glycerol backbone, even when both positions are esterified by the same fatty acid.

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