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<Review>

## Chiral separation of glycerolipids by high-performance liquid chromatography

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### Summary

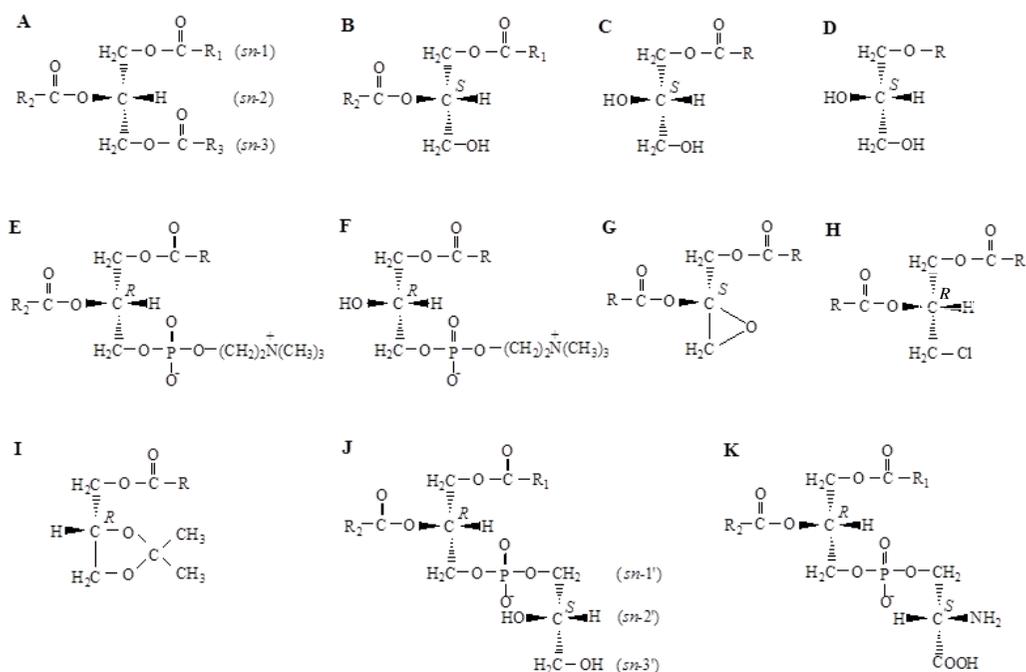
Since the enantiomer resolution of synthetic mono- and diacylglycerols as 3,5-dinitrophenylurethanes was achieved by chiral-phase high-performance liquid chromatography (HPLC) in the 1980s, the methodology has been extended to the resolution of various synthetic and naturally occurring chiral glycerolipids. The recent development of polysaccharide-based chiral stationary phases along with optimized chromatographic elution conditions has permitted direct enantiomer resolution of mono-, di-, and triacylglycerols without previous derivatization. However, there are still some compounds for which enantiomer resolution is difficult to achieve, such as phosphatidylcholine and phosphatidylethanolamine. With the development of new derivatization reagents and chromatographic techniques, resolution of these difficult compounds may be possible in the near future. Chiral-phase HPLC will become considerably important in the future as a valuable tool for further expansion of the field of lipidomics and a better understanding of lipid metabolism.

Key words: Chiral separation; Diastereomers; Enantiomers; Glycerolipids; High-performance liquid chromatography; Mass spectrometry.

### 1. Introduction

Glycerolipids in living organisms are complex mixtures of different types of molecules that contain stereoisomers. Although the technology of chromatography has advanced rapidly in recent years, there are still many lipid molecules for which separation is difficult to achieve and in which physiological significance and biological activities remain unknown. The development of accurate and concise methods for the analysis of lipids is therefore essential for many research studies. In the present “post-genomic era”, the focus of investigation is shifting from gene sequences and gradually returning to the gene products themselves (proteomics). It

is well recognized that an understanding of pathophysiological changes that occur in cells and tissues requires complementary profiling of lipid-derived metabolic products (lipidomics). This has led to the development of mass spectrometry (MS)-based lipidomics methodologies<sup>1)</sup>. However, MS is unable to differentiate among enantiomers, and in many instances, among diastereomers. This shortcoming is especially evident in the glycerolipids because of the prochiral nature of glycerol<sup>2)</sup>. At present, the resolution of a number of stereoisomeric lipid molecules has become possible through chiral-phase chromatography, especially high-performance liquid chromatography (HPLC). This chapter focuses on the applications of chiral-phase HPLC to support the MS of chiral glycerolipids. Readers are referred to earlier comprehensive reviews for background information relating to chiral analysis of



**Fig. 1** Structures of some chiral glycerolipids. (A) 1,2,3-triacyl-*sn*-glycerol; (B) 1,2-diacyl-*sn*-glycerol; (C) 1-acyl-*sn*-glycerol; (D) 1-alkyl-*sn*-glycerol (glyceryl ether); (E) 1,2-diacyl-*sn*-glycero-3-phosphocholine; (F) 1-acyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine); (G) (*S*)-1-acyl-2,3-epoxypropane (glycidol fatty acid ester); (H) (*R*)-1,2-diacyl-3-chloropropane; (I) 2,3-*O*-isopropylidene-*sn*-glycerol; (J) 1,2-diacyl-*sn*-glycero-3-phospho-1'-*sn*-glycerol (phosphatidylglycerol); (K) 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (phosphatidylserine).

glycerolipids<sup>2-8)</sup>.

## 2. Structures of chiral glycerolipids

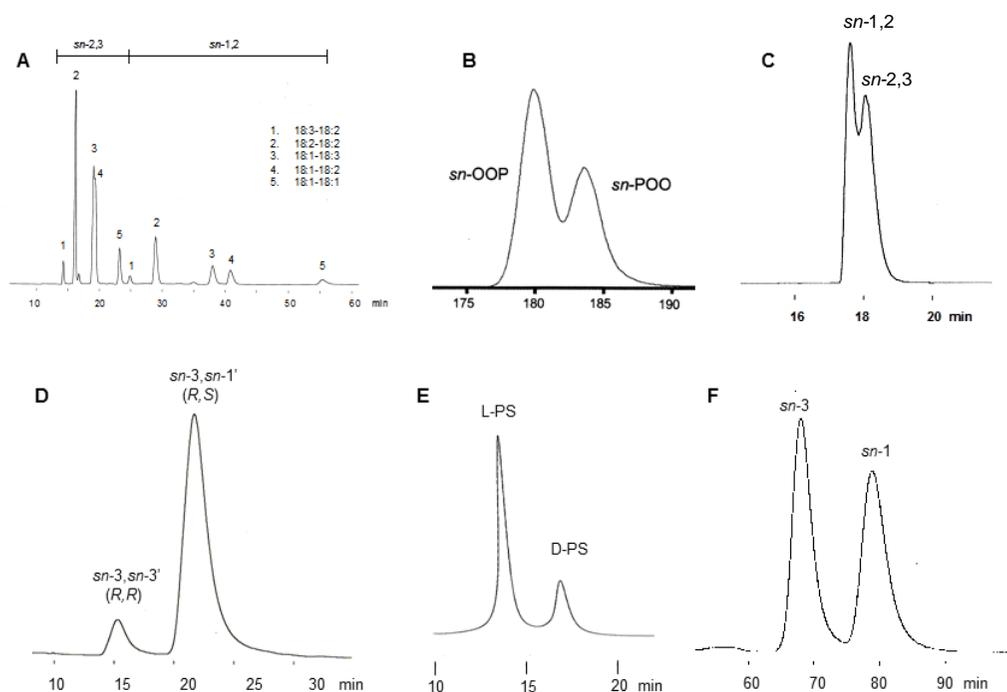
**Fig. 1** illustrates the structures of one enantiomer of the glycerolipids adopted in this review. Glycerol and common fatty acids are achiral compounds that have no chiral centers in their molecules, while glycerolipids become chiral molecules when the C-1 and C-3 positions of the glycerol backbone have different fatty acids, or when the C-2 position becomes a chiral center. Almost all naturally occurring glycerolipids are chiral and can exist as both enantiomers and diastereomers. Four stereoisomers (two sets of enantiomers) can exist for phosphatidylglycerol (PG, Fig. 1J) and phosphatidylserine (PS, Fig. 1K), whose molecules have two chiral centers.

## 3. Separation of enantiomeric mono-, di-, and triacylglycerols

1-Monoacylglycerol (MAG, Fig. 1C) was the first chiral glycerolipid to be resolved by HPLC<sup>9)</sup>. Enantiomeric 1-MAG was converted into the bis(3,5-dinitrophenylurethane) (DNPU) derivatives and then separated on a chiral stationary phase (CSP) column, (*S*)-2-(4-chlorophenyl)-isovaleroyl-D-phenylglycine (Sumipax OA-2100). Later, improved resolution was achieved using a Sumichiral OA-4100<sup>3,4)</sup> column, which contained chiral 1-(1-naphthyl)ethylamine as a chiral selector. This method has been extensively applied in the stereospecific analysis of triacylglycerols (TAG, Fig. 1A)<sup>5)</sup> and in the determination of the positional placement of an ether linkage in ether lipid molecules (Fig. 1D)<sup>4)</sup>. Recently, direct HPLC separation of enantiomeric MAG was achieved without previous derivatization on cellulose tris(4-chlorophenylcarbamate) (Chiralcel OF), amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak IA), and cellulose tris(3,5-dichlorophenylcarbamate) (Chiralpak IC), using hexane/2-propanol eluent mixtures<sup>10,11)</sup>. Separations of both enantiomers and their molecular species could be improved by chiral reversed-phase HPLC on Chiralpak IA and Chiralpak IC using CH<sub>3</sub>CN containing 10%–30% MeOH as the mobile phase<sup>8)</sup>.

Enantiomer resolution of 1,2-diacylglycerol (DAG, Fig. 1B) by chiral-phase HPLC is also based on the conversion of DAG into the DNPU derivatives, with optimal separation achieved using an (*R*)-1-(1-naphthyl)ethylamine polymeric phase (A-K03)<sup>4)</sup>. This method was applied to

determine the stereo configuration of the glycerol moieties in glycoylglycerolipids, in which 1,2-DAGs were released from glycosyldiacylglycerols (monogalactosyl-, digalactosyl-, and sulfoquinovosyldiacylglycerols) by periodate oxidation followed by hydrazinolysis<sup>12</sup>). The results clearly showed that the glycerol moieties in the glycosyldiacylglycerols isolated from higher plant leaves and seaweeds have an *S*-configuration (*sn*-1,2-DAG). As with MAG, enantiomeric 1,2-DAG can be separated directly without derivatization on a cellulose tris(3,5-dimethylphenylcarbamate) column (Chiralcel OD). This method was used for the evaluation of the stereospecificity of *Candida antarctica* lipase B and *Rhizomucor miehei* lipase, which possessed opposite stereospecificity<sup>13</sup>). Recently, improved resolution of the enantiomeric DAG in a free form has been obtained on a Chiralpak IA column using a



**Fig. 2** HPLC resolution of chiral glycerolipids. (A) a mixture of 1,2-diacyl-*rac*-glycerols<sup>8</sup>); (B) 1,2-dioleoyl-3-palmitoyl-*sn*-glycerol and its enantiomer from lard<sup>15</sup>); (C) 1,2-dipalmitoyl-*rac*-glycero-3-phosphocholine; (D) 1,2-diacyl-*sn*-glycero-3-phospho-1'-*sn*-glycerol (*R,S*) and its diastereomer (*R,R*) from *Escherichia coli*<sup>9</sup>); (E) 1,2-diacyl-*sn*-glycero-3-phospho-L-serine and -D-serine<sup>2</sup>); (F) 1-acyl-2-hydroxy-*rac*-glycero-3-phosphocholine<sup>8</sup>). (A) – (C) free form; (D) bis(3,5-dinitrophenylurethane) derivatives; (E) 3,5-dinitrophenylurea derivatives; (F) 3,5-dinitrophenylurethane derivatives. Column: (A) Chiralpak IA; (B) and (F) Chiralcel OD-RH; (C) Chiralpak ID; (D)–(F) A-K03. For other HPLC conditions, see text and refs. 2, 8, 9, and 15.

CH<sub>3</sub>CN/MeOH eluent mixture (**Fig. 2A**)<sup>8)</sup>.

Some asymmetric TAGs (Fig. 1A) with very different acyl groups have been successfully resolved by chiral-phase HPLC on polysaccharide-based CSP columns and excellent resolution was achieved for enantiomeric 1-docosahexaenoyl-2,3-dicapryroyl-*sn*-glycerol (22:6-8:0-8:0) and 1,2-dicapryroyl-3-docosahexaenoyl-*sn*-glycerol (8:0-8:0-22:6) on a Chiralcel OD column using hexane/2-propanol (200:1, by vol) as the mobile phase<sup>14)</sup>. Recently, a recycling HPLC technique was applied in the separation of TAG enantiomers with minor different acyl groups. Using this method, effective separations were obtained in 150 min for enantiomer pairs of 16:0-16:0-18:1/18:1-16:0-16:0, 18:1-18:1-16:0/16:0-18:1-18:1, and 16:0-16:0-18:2/18:2-16:0-16:0 on a Chiralcel OD-RH column using MeOH as the mobile phase (**Fig. 2B**), but neither 18:1-18:1-18:2/18:2-18:1-18:1 nor 16:0-16:0-18:1/18:1-16:0-16:0 were resolved<sup>15)</sup>. These techniques are simple and useful for the determination of the enantiomeric purity of synthetically structured lipids and some natural TAG.

#### 4. Separation of diastereomeric and enantiomeric glycerophospholipids

Phospholipids, such as phosphatidylcholine (PC, Fig. 1E) and phosphatidylethanolamine (PE), are compounds difficult to resolve enantiomers by chromatography, and no satisfactory resolution has been achieved to date on any CSP column, although a partial resolution of racemic PC has been obtained on an amylose tris(3-chlorophenylcarbamate) (Chiralpak ID) using CH<sub>3</sub>CN/MeOH/(C<sub>2</sub>H<sub>5</sub>)<sub>2</sub>NH (85:15:0.1, by vol) as the mobile phase (**Fig. 2C**). On the other hand, some phospholipids with hydroxyl or amino groups, such as lyso-PC (Fig. 1F), PG, and PS, react readily with 3,5-dinitrophenyl isocyanate to form the corresponding urethane or urea derivatives, respectively, are separable on chiral columns. This derivatization method was applied in determining the configuration of PG prepared by phospholipase D (PLD)-catalyzed transphosphatidylation from PC and glycerol<sup>16)</sup>. It was concluded that a bacterial PLD-catalyzed reaction proceeds to a large extent stereospecifically, showing a preference for the *sn*-3'-position of the glycerol molecule, while cabbage and peanut PLDs showed no positional preference. Based on these studies, a simple method for synthesizing pure PG stereoisomers from enantiomeric PC and isopropylidenglycerols (Fig. 1I) was developed using *Actinomadura* PLD<sup>17,18)</sup>. The chiral-phase HPLC also revealed that some bacteria have a significant amount of a PG diastereomer (the *R,R* form; **Fig. 2D**), whose proportion increased

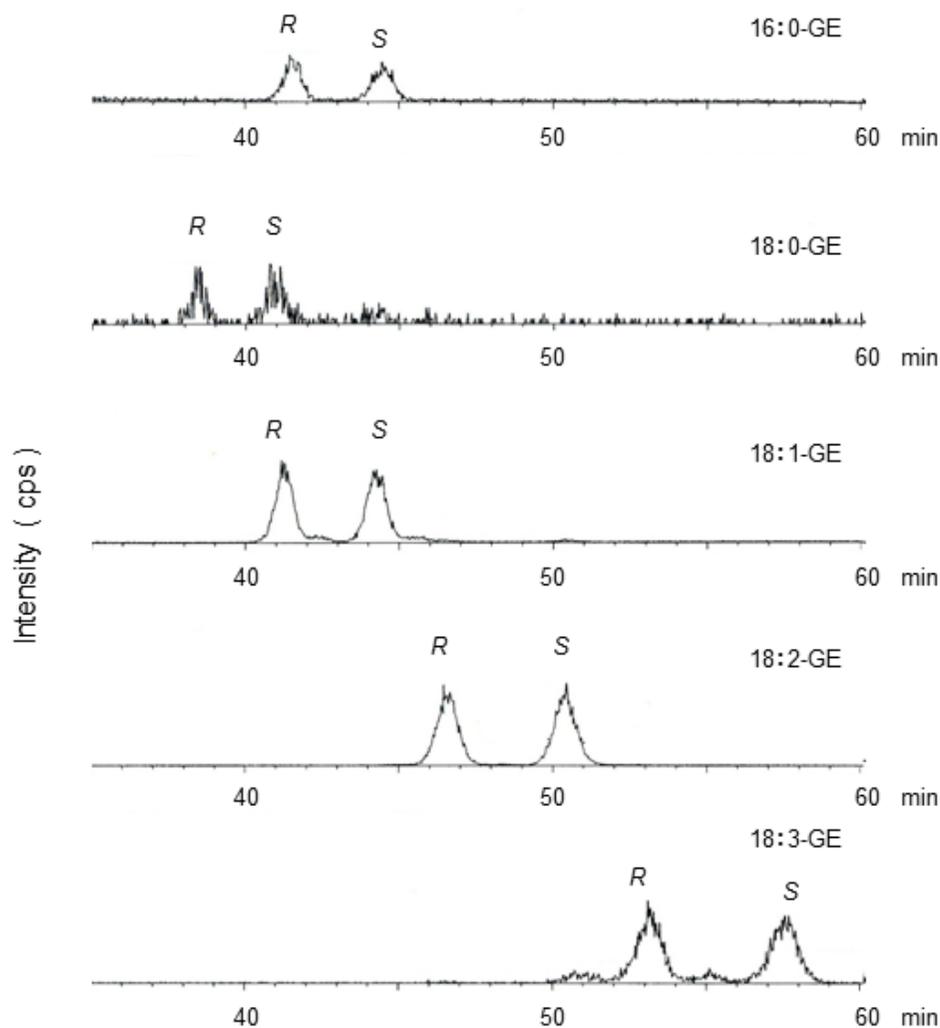
gradually with increasing growth temperature, suggesting adaptation toward environmental change<sup>19-21)</sup>.

Complete resolution of diastereomeric phosphatidyl-D-serine (D-PS) and L-serine (L-PS) as 3,5-dinitrophenylurea derivatives has also been achieved on A-K03 columns, using hexane/CH<sub>2</sub>Cl<sub>2</sub>/MeOH/TFA (70:20:10:0.2, by vol) as the mobile phase (**Fig. 2E**)<sup>7)</sup>. Using this method, Itabashi et al. have demonstrated that the serine moiety of the PS isolated from rat brain, rat liver, mackerel eye, and mackerel liver only had the L-configuration<sup>8)</sup>, although approximately 1% of the total PS was detected in rat cerebrum by reversed-phase HPLC of the fluorescent derivatives of serine liberated from PS by hydrolysis<sup>22)</sup>.

Lyso-PC is an abundant component of plasma and oxidized low-density lipoprotein (LDL) that displays several biological activities, some of which may occur through the platelet-activating factor (PAF) receptor<sup>23)</sup>. Near baseline resolution of enantiomeric 2-lyso-PC as DNPU has been achieved on Chiralcel OD-RH columns using a simple solvent system with MeOH/H<sub>2</sub>O (90:10, by vol) as the mobile phase (**Fig. 2F**)<sup>8)</sup>.

## 5. Separation of enantiomeric glycidol fatty acid esters

Glycidol fatty acid esters (GE, Fig. 1G), which are undesirable contaminants formed mainly during the refining processes of edible oils and have a high health risk potential<sup>24)</sup>. Although GE has enantiomers, no studies have been conducted on their resolution and composition in refined edible oils. A column-switching chiral-phase HPLC-MS method was recently developed for determining the composition of enantiomeric GE<sup>8)</sup>. An edible diacylglycerol-rich oil dissolved in hexane was injected into a silica column and GE was fractionated as a mixture of palmitate, stearate, oleate, linoleate, and linolenate. The GE mixture was introduced to an analytical column (Chiralpak IC), and individual GE molecules were separated and detected by on-line APCI-MS. The HPLC analysis was performed under isocratic elutions with hexane/2-propanol (99.5:0.5, by vol). Post-column addition of EtOH was used to assure APCI in the positive mode<sup>8)</sup>. Quantification was successfully performed in multiple reaction monitoring (MRM) mode using a precursor ion  $[M + H]^+$ . Using this method, Yoshioka et al.<sup>25)</sup> demonstrated that the GE molecules in some refined edible oils were composed of approximately equal amounts of *R* and *S* enantiomers (**Fig. 3**). The concentrations of palmitate, stearate, oleate, linoleate, and linolenate were 6.6, 2.6, 74, 118, and 49 μg/g,



**Fig. 3** Column-switching HPLC/APCI-MS profiles of glycidol fatty acid esters in a diacylglycerol-rich oil. 1st column: silica (250 x 4.6 mm ID); 2nd column: Chiralpak IC (250 x 4.6 mm ID); Mobile phase: isocratic elution with hexane/2-propanol (99.5:0.5, by vol) at 5°C (1st column) and 20°C (2nd column). MS: API 2000 Q Trap MS/MS. Scan mode: MRM (precursor ion,  $[M + H]^+$ ). Injection volume: 20  $\mu$ L (30 mg/mL hexane). From ref. 8.

respectively. The total amount of GE (250  $\mu$ g/g) was less than that reported previously (295  $\mu$ g/g), where a combination of double SPE and reversed-phase HPLC-MS was used<sup>26</sup>. As with GE, fatty acid esters of racemic 3-chloro-1,2-propanediol (Fig. 1H), a glycidol related substance, could be also clearly resolved into enantiomers using a Chiralpak IA column (Y. Itabashi et al., unpublished results).

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