Review

Lipid analysis by thin-layer chromatography—A review of the current state

Beate Fuchs[a], Rosmarie Süß[a], Kristin Teuber[a,b], Mandy Eibisch[a], Jürgen Schiller[a,b,*]

[a] University of Leipzig, Medical Department, Institute of Medical Physics and Biophysics, Härtelstr. 16/18, D-04107 Leipzig, Germany
[b] LIFE – Leipzig Research Center for Civilization Diseases, Universität Leipzig, Germany

ARTICLE INFO

ABSTRACT

High-performance thin-layer chromatography (HPTLC) is a widely used, fast and relatively inexpensive method of separating complex mixtures. It is particularly useful for smaller, apolar compounds and offers some advantages over HPLC. This review gives an overview about the special features as well as the problems that have to be considered upon the HPTLC analysis of lipids. The term "lipids" is used here in a broad sense and comprises fatty acids and their derivatives as well as substances related biosynthetically or functionally to these compounds. After a short introduction regarding the stationary phases and the methods how lipids can be visualized on an HPTLC plate, the individual lipid classes will be discussed and the most suitable solvent systems for their separation indicated. The focus will be on lipids that are most abundant in biological systems, i.e. cholesterol and its derivates, glycerides, sphingo- and glycolipids as well as phospholipids. Finally, a nowadays very important topic, the combination between HPTLC and mass spectrometric (MS) detection methods will be discussed. It will be shown that this is a very powerful method to investigate the identities of the HPTLC spots in more detail than by the use of common staining methods. Future aspects of HPTLC in the lipid field will be also discussed.

© 2010 Elsevier B.V. All rights reserved.

Contents

Abbreviations: AA, arachidonic acid; AMD, automated multiple development; APCI, atmospheric pressure chemical ionization; CDP-DAG, cytidine diphosphate diacyl-glycerol; CDH, ceramide dihexoside; CER, ceramide; CHO, cholesterol; CHO-ET, cholesteryl ester; CL, cardioplipin; CMH, ceramide monohexoside; DAG, diacylglycerols; DCC, monodansyl cadaverine chloride; DEAE, delayed extraction; DA, diethylaminoethyl-cellulose; DESI, desorption electrospray; DGDG, digalactosyl-diacylglycerol; DHB, 2,5-dihydroxybenzoic acid; EDTA, ethylene-diamine-tetraacetic acid; EI, electron ionization; ESI, electrospray ionization; FA, FFA, fatty acid; FAB, fast atom bombardment; FAME, fatty acid methyl ester; FT, Fourier transform; GC, gas chromatography; GPL, glycerophospholipid; GSL, glycosphingolipid; GSu, glycolipid sulfate; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; IR, infrared; LD, laser desorption; LPA, lyso-phosphatidic acid; LPase, lipoprotein lipase; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; LPL, lyso-phospholipid; LPS, lyso-phosphatidylserine; MAG, monoacylglycerols; MALDI, matrix-assisted laser desorption and ionization; MGDG, monogalactosyl-diacylglycerol; MLCL, monolysocardiolipin; MS, mass spectrometry; m/z, mass over charge; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidylinositol-monophosphate; PIP$_2$, phosphatidylinositol-bisphosphate; PIP$_3$, phosphatidylinositol-trisphosphate; PL, phospholipid; PLA$_2$, phospholipase a$_2$; PLC, phospholipase C; PPI, (poly-)phosphoinositides; PS, phosphatidylserine; RF, retardation factor; ROS, reactive oxygen species; RP, reversed phase; SM, Sph, sphingomyelin; S/N, signal to noise; sn, stereospecific numbering; SQDG, sulfoquinovosyl-diacylglycerol; TAG, triacylglycerol; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; TOF, time-of-flight; UTLC, ultra-thin-layer chromatography; UV, ultraviolet.

* Corresponding author at: Universität Leipzig, Medizinische Fakultät, Institut für Medizinische Physik und Biophysik, Härtelstr. 16/18, D-04107 Leipzig, Germany. Tel.: +49 341 9715733; fax: +49 341 9715709.
E-mail address: juergen.schiller@medizin.uni-leipzig.de (J. Schiller).

1. Introduction

About 20 years ago, William Christie, one of the most respected scientists in the field of lipids and particularly lipid analysis asked the provocative question "Has thin-layer chromatography had its day?" [1]. Now, after more than 2 decades, it is evident that the most appropriate answer is "No". The reasons for this answer can be found in detail in the excellent "Lipid Analysis" book by Christie and Han [2], the book chapter by Shantha and Napolitano [3] or the report by Nikolova-Damyanova [4] and only two very important reasons why planar chromatography is still alive shall be given here: The increasing commercial availability of pre-coated TLC plates has significantly improved the achievable reproducibility of separation that was quite limited in the past when home-made TLC plates were primarily used. Additionally, the availability of many different absorbent materials including high-performing silica, bonded phases and impregnated layers have increased the versatility of HPTLC for numerous and quick separations particularly in the lipid field.

Thus, methods of thin-layer chromatography (TLC) and its refined version high-performance thin-layer chromatography (HPTLC) are even nowadays indispensable tools of modern analytical chemistry [5]. The most important difference between TLC and HPTLC is (a) the different particle sizes of the stationary phases and (b) the care that is used to apply the samples and to process the obtained data [6]. However, in this review both terms will be used as synonyms because many older references will be provided that used only "TLC" because "HPTLC" has not yet been available.

The aim of this review is to provide a survey of applications of TLC in the lipid field. This review is in these authors' opinion strongly needed because the majority of reviews dedicated to lipids were published some time ago [7,8]. Nevertheless, there were of course reviews on TLC in general [9], reviews focused on topics such as two-dimensional TLC in the analysis of secondary plant metabolites [10,11] or TLC in food and agricultural analysis [12] as well as the analysis of biological samples in general [13] that covered of course aspects of lipids, too.

Although there were (and still are) several potential concerns against the wider application of TLC (e.g. the lower chromato-graphic resolution in comparison to HPLC and the potential oxidation of the analyte caused by exposition to atmospheric oxygen), there are many advantages that make TLC clearly competitive to liquid chromatography [14]:

(1) TLC is convenient and simple. If commercially available ready-made TLC plates are used, even the less experienced user is able to perform high quality separations.
(2) The equipment that is needed for TLC is rather inexpensive and can be, thus, easily established in each laboratory.
(3) TLC is already certified in many different industrial and especially pharmaceutical processes. A particular important advantage in that field is that TLC can be easily used to determine different analytes quantitatively (at least if reliable standards are available).
(4) TLC does not provide any "memory" effects as a completely new stationary phase is used in all cases. This is a significant advantage in comparison to LC, where remaining contributions of a previous run can be never completely excluded.
(5) TLC consumes by far smaller amounts of solvents than HPLC. Therefore, TLC is less expensive regarding the required consumables and particularly more environment-friendly.
(6) As many different samples may be simultaneously applied onto a single TLC plate, TLC is in practice often faster than LC (although there are recent "multiplexing" LC solutions available that enable the analysis of several samples in parallel).
(7) After TLC separation, lipids can be easily visualized by staining, for instance, with dyes that bind specifically to characteristic functional groups such as amino or carbohydrate residues. This is a significant advantage in comparison to HPLC where "post column derivatization" is normally more difficult.
(8) TLC may also be used for the analysis of "suspicious" samples that might easily damage an HPLC system. This is particularly important in food chemistry where often less defined mixtures have to be characterized.

In the field of lipids, TLC is classically used for routine separations, identification of the individual lipids and their quantitative determinations. With the advent of the "automated multiple devel-
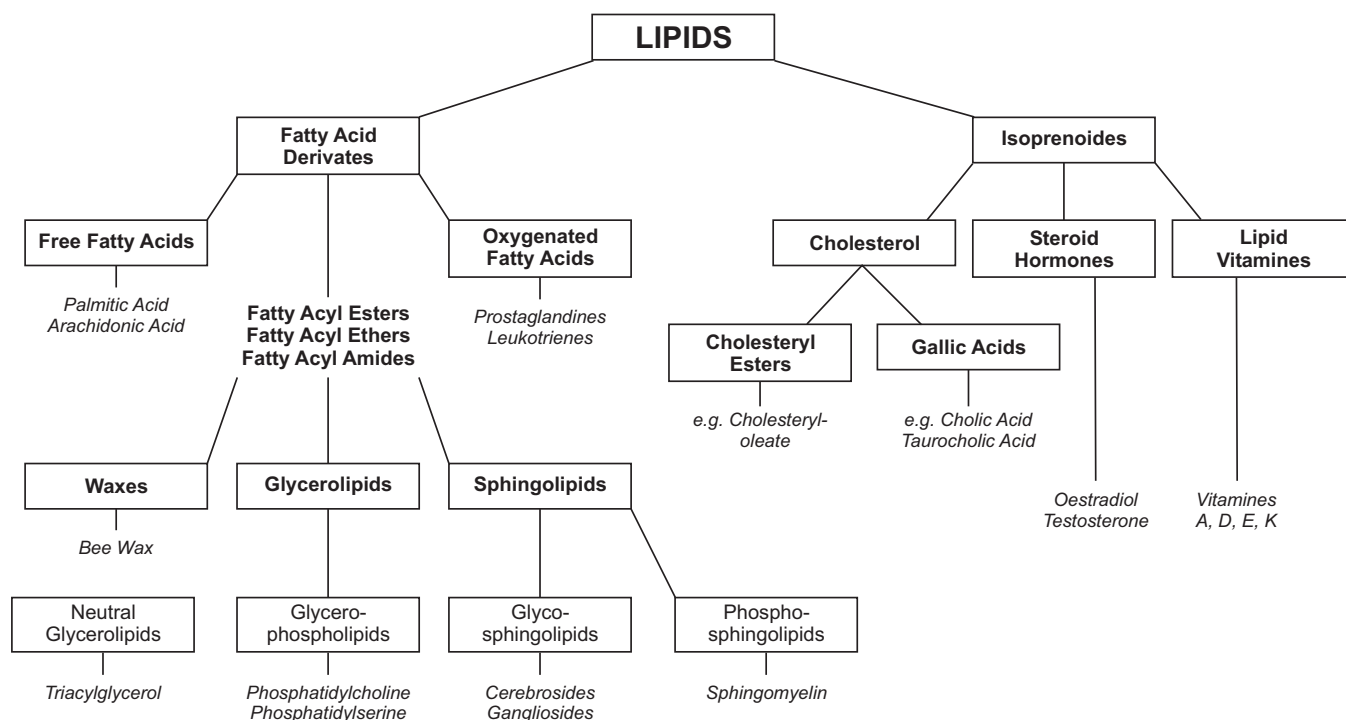
LIPIDS

Fatty Acid Derivates

Isoprenoides

Free Fatty Acids

Palmitic Acid
Arachidonic Acid

Fatty Acyl Esters
Fatty Acyl Ethers
Fatty Acyl Amides

Oxygenated Fatty Acids

Prostaglandines
Leukotrienes

Cholesterol

Steroid Hormones

Lipid Vitamines

Cholesteryl Esters

e.g. Cholesteryl-oleate

Gallic Acids

e.g. Cholic Acid
Taurocholic Acid

Oestradiol
Testosterone

Vitamines
A, D, E, K

Waxes

Bee Wax

Glycerolipids

Sphingolipids

Neutral Glycerolipids

Triacylglycerol

Glycero-phospholipids

Phosphatidylcholine
Phosphatidylserine

Glyco-sphingolipids

Cerebrosides
Gangliosides

Phospho-sphingolipids

Sphingomyelin

Fig. 1. Short survey of the different classes of "lipids" that are relevant to this review. Some selected examples of the most important subclasses such as triacylglycerols and phosphatidylcholines are also provided. This schema summarizes the data given in the well-known textbooks by Stryer [18] and Lottspeich [19].

opment" (AMD) technique, all steps from application to mixing solvents, development and drying could be automated. Particularly, a reproducible gradient elution became available. The use of gradients (i.e. mixtures of different solvents and/or different salt concentrations that change time-dependent) is very common in HPLC but was not very common in TLC over decades. One selected example of this technique is available in [15]. Although such automation is obviously expensive, it is a prerequisite for the wider use of HPTLC in the pharmaceutical, cosmetic and – most important – the oils and fats industries. These aspects will be discussed below in more detail and a survey of the equipment that is nowadays available in the TLC field is available from the CAMAG (a leading supplier of TLC equipment) homepage (http://www.camag.com) and is described in more detail in the book by Hahn-Deinstrop that gives an excellent survey of practical aspects of TLC [16].

1.1. Structures of lipids relevant to this review

Traditionally, "lipids" can be defined as apolar compounds that are insoluble in water and may be enriched by the treatment/extraction with organic solvents such as chloroform or hexane [2]. Another definition has been introduced by Christie [17]: According to this definition, lipids are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds.

A survey of the methods of lipid extraction that are commonly used in daily practice will be given below, but first, a short survey of the lipids relevant to the topic of this review will be provided. We will focus primarily on compounds that occur in biological systems and have, thus, physiological relevance. According to our experience, we will focus on animal-derived lipids, while plant lipids will only be loosely treated. The structures of the relevant glycolipids typically occurring in plants will be introduced at the appropriate places. A coarse overview of the lipids relevant to this paper is given in Fig. 1. Please note that in addition to the compounds illustrated

in Fig. 1 there are many detergents that may be also considered as lipids. However, these compounds do not play a major role in our review.

Lipids are not only of relevance regarding the storage of energy, but are also massively involved in signal transduction processes [20]. Thus, the determination of selected lipid species such as lysophospholipids (lacking one fatty acyl residue in comparison to phospholipids) [21], diacylglycerols, phosphatidic acids or phosphoinositides is of considerable interest because this allows conclusions on metabolic processes and has, thus, significant diagnostic relevance. Some glycolipids are nowadays also considered as disease markers and markers of the differentiation of e.g. stem cells into cancer cells.

There are nowadays also a lot of known membrane proteins that are located in the cellular membrane. Since many of these proteins represent enzymes, their activity is assumed to be regulated by the lipid composition of the membrane and slight changes of the lipid composition may strongly affect the enzymatic activity. This holds, for instance, for the well-known enzyme phospholipase A_2 (PLA_2) [22]. Thus, the knowledge of the lipid composition is of interest from different points of view.

1.2. Methods of lipid analysis

Basically, this paragraph could have been completely omitted because there is a recent book entirely dedicated to this topic [2] as well as different excellent journal reviews [5,23]. Therefore, Table 1 will provide only a very crude survey of instrumental methods so far applied in lipid analysis.

Mass spectrometric aspects will be discussed below in more detail because they are nowadays one of the key methods in successful lipid analysis. Despite their relatively low sensitivity, however, NMR spectroscopic methods are unequivocally very powerful tools in lipid research and have the particular advantage that they may be even used under *in vivo* conditions, i.e. without the

Table 1
Overview of important techniques of lipid analysis. The advantages and drawbacks of the various methods are listed.

	Principle	Advantages	Drawbacks	Remarks
Thin-layer chromatography (TLC)	Separation is achieved on a "stationary phase" (normally silica gel) due to polarity differences of the analytes	TLC is quite inexpensive and fast. Variations of the mobile phase enable separation of even complex mixtures. Different stainings can be easily performed.	Oxidation (of unsaturated lipids) may occur if the TLC plate is stored for a while since a large (lipid) surface is exposed to atmospheric oxygen. Preparative applications are limited.	Often used as initial method if a complex lipid mixture has to be analyzed. For more details see below.
High-performance liquid chromatography (HPLC)	Separation on a "stationary phase" under high pressure by elution with different solvents	High quality separations are achievable. Also applicable on a preparative scale. Coupling with MS is well established.	More time-consuming and expensive than TLC. Detection of saturated lipids (lack of UV absorptions) is difficult. Post-column derivatization is challenging.	Routine method of lipid isolation in many laboratories. However, "fine-tuning" of the composition of the mobile phase to the lipid mixture of interest is challenging.
Gas chromatography (GC)/GC/MS	Separation of volatile compounds on a carrier gas. Detection often performed by means of mass spectrometry	Highly established in fatty acid analysis. Automated devices are commercially available.	Only volatile compounds can be analyzed. Thus, derivatization of the analyte is required.	Most widely applied technique for examination and quantifying the fatty acyl compositions of lipids. However, increasingly replaced by soft ionization MS techniques.
Soft ionization mass spectrometry	MALDI and ESI MS enable the characterization of lipids without major analyte fragmentation	Both techniques are highly sensitive and enable direct analyte detection. Handling is normally quite simple.	Ion suppression may occur, i.e. different lipid classes are detectable with strongly different sensitivities. Impurities affect spectral quality significantly.	This field is currently strongly developing. ESI is so far most frequently used in lipid analysis, but applications of MALDI are increasing. Screening of biological tissues is possible.
^{1}H/^{13}C NMR	Differences in electron densities lead to different chemical shifts of the observed nucleus within a given compound	Basically all lipids are detectable. Correlation (2D) experiments can be performed to obtain further information, for instance to differentiate isomers. Interaction studies (e.g. with proteins) can be easily performed.	Complex spectra are obtained if mixtures are analyzed. Very limited sensitivity (^{13}C) and need of deuterated solvents. Expensive equipment.	NMR can be basically also used under *in vivo* conditions (NMR imaging).
^{31}P NMR	Differences in electron densities lead to different chemical shifts of the observed nucleus within a given compound	Direct absolute quantitation is possible. Isomeric lipids can be differentiated.	Only lipids containing phosphorous are detectable. Limited sensitivity. Expensive equipment.	Detergents or solvent mixtures have to be used in order to suppress the aggregation of phospholipids.

need of previous extraction. Another important advantage is the capability to locate the position of double bonds within a given lipid, to differentiate cis/trans isomers and positional isomers of e.g. conjugated linoleic acids – often within a single spectrum. An excellent survey can be found under http://lipidlibrary.aocs.org and there are of course many reviews of these analytical aspects available [24].

One particular powerful method is in our opinion ^{31}P NMR. Although this method can be exclusively used in the case of phosphorous containing compounds (or at least only after suitable derivatization [25]), it allows the differentiation and quantitation of all PL classes within a single spectrum according to differences in their headgroups. However, differences in fatty acyl compositions can exclusively be monitored if the lengths or the degree of unsaturation of the fatty acyl residues differ significantly [26]. ^{31}P NMR spectra are normally recorded in the presence of a suitable detergent (e.g. sodium cholate) that suppresses the aggregation of PL that would result in severe line-broadening and loss of resolution. It is a particular advantage of ^{31}P NMR that extraction of the sample of interest with organic solvents is not absolutely necessary but the sample may be directly solubilized in the detergent. This helps to avoid PL losses that may easily occur by the extraction process [27].

The instrumental methods summarized above are nowadays well-established. However, one should keep in mind that the structures of quite complex lipids were elucidated long time ago without these techniques. Thus, classical methods of lipid analysis such as determination of the iodine number or the determination of the

phosphorous content should be even nowadays considered as useful methods and they are surely not only of historical interest.

1.3. How to extract lipids from biological samples

Independent of the subsequent analysis, the first step of lipid analysis is normally the extraction of lipids. This sounds rather trivial but is actually a very important point because many lipids cannot be extracted so easily since they are associated in the original biological material with other compounds. For instance, the hydrophobic aliphatic moieties of lipids interact with the non-polar regions of proteins, especially with the amino acids valine, leucine or isoleucine. Additionally, the acidic phosphate groups of lipids strongly interact with metal ions that are normally bound to proteins. These both examples provide already evidence that lipid extraction is really a sophisticated but widely neglected task [28].

Here, we will provide only a crude survey of established extraction methods of tissues and body fluids although it is highly recommended that each user adopts these basic methods to the analytical problem of interest. Some established methods are summarized in Table 2:

We do not want to go further into details since this topic would be worth a review of its own. However, it should be kept in mind that slight modifications of lipid extractions may lead to severe losses of at least some lipid classes. A more comprehensive survey of this topic is available in [36] and from http://lipidlibrary.aocs.org/topics/extract/index.htm.

Table 2
Established methods to extract lipids from biological samples. Please note that these methods may only be used as very coarse guidelines. Any particular tissue or body fluid may require specific solvent mixtures.

Solvent system	Particularly suitable for	Comments/reference
CHCl$_3$/CH$_3$OH (2:1, v/v) "Folch Method"	Lipids from animal, plant and bacterial tissues	The tissue water is the ternary component and its amount is very important in order to avoid loss of lipids [29].
CHCl$_3$/CH$_3$OH (1:1, v/v) "Bligh and Dyer Method"	Useful for water-rich systems, particularly body fluids	Partial loss of apolar lipids such as TAG may occur [30]
Butanol saturated with water	Plant lipids, i.e. lipids entrapped in starch and rather polar lipids	Provides very good recovery of lysolipids [31]
Hexane/2-propanol (3:2, v/v)	Low content of non-lipids (proteins, pigments, small molecules) in the extract because the used solvent mixture is highly apolar	In comparison to CHCl$_3$, hexane and isopropanol are solvents of low toxicity only [32]. Plastic material can be used.
Chloroform/isopropanol (7:11, v/v).	Particularly suitable for erythrocytes with a high lipid content	Indicated to provide high lipid yields [33]
Chloroform/methanol/12 N HCl (2:4:0.1, v/v/v)	Acidic phospholipids such as PS and particularly phosphoinositides	Addition of HCl leads to charge screening and improves extraction yields of acidic lipids [34]. This method, however, leads to complete hydrolysis of plasmalogens [35].

2. Thin-layer chromatographic lipid analysis

Since technical aspects of TLC are comprehensively discussed elsewhere [11,16,37] and will be also discussed in other papers appearing in this special issue, only a very coarse survey of technical aspects of TLC separation of lipids will be given here.

2.1. The stationary phase

The most popular stationary phases for lipid separations are silica gel, alumina and kieselguhr, whereby silica gel is unequivocally the absolutely dominant phase. Silica can be additionally modified by impregnation with other substances to provide optimum results regarding the separation of a certain lipid class. Based on their surface characteristics these phases can be classified as "normal" or "reversed" phases.

In normal phase TLC, the stationary phase (normally silica gel) is polar and the mobile phase is quite apolar (i.e. the used solvent system contains significant amounts of solvents such as hexane or chloroform). 10–50 μm particles are regularly used for purposes of TLC, while about 5 μm particles with narrow size distributions are used in HPTLC. These smaller particles result in higher separation quality. Additionally, smaller sample amounts are sufficient in the case of HPTLC and, thus, smaller detection limits can be achieved. Normal phase chromatography is the standard method of lipid separation according to polarity differences caused by differences of the headgroups of the PL of interest. In addition to the separation of the individual lipid classes, separation within lipid classes, i.e. according to differences in fatty acyl compositions is also possible by normal phase chromatography [38] although this is normally the domain of reversed phases.

Among the different modifications of the stationary phase, silver nitrate and boric acid impregnations are most popular. AgNO$_3$ is primarily used to separate lipids with different fatty acyl compositions based on the degree of unsaturation [39] because the Ag$^+$ forms a complex with the π electrons of the double bonds of unsaturated fatty acids leading to a decreased mobility of these fatty acids. In selected cases, even the determination of the positions of the double bonds is possible by this approach. This topic will be discussed below in more detail although there are already some comprehensive reviews of this topic that are highly recommended if a comprehensive survey of this important field is required [40–43].

In contrast, boric acid (H$_3$BO$_3$) is primarily useful for the detection of the different isomers of DAG as well as the separation of isomeric PL. H$_3$BO$_3$ forms complexes with compounds containing vicinal hydroxyl groups and leads to a slower migration of these compounds. Apart from this, boric acid does also bind to acidic compounds and modifies their migration properties as well [44]. Another much more rarely used modification of the stationary phase is the addition of EDTA that has been indicated to improve the separation of acidic PL [45] such as phosphatidylserine (PS) that often results in a quite diffuse spot (a review exclusively dedicated to PS analysis is available in [46]). In the same manner ammonium sulfate has been indicated to improve the separation between phosphatidylinositol (PI) and PS. Using silica gel plates impregnated with 0.4% ammonium sulfate and chloroform–methanol–acetic acid–acetone–water (40:25:7:4:2, v/v/v/v/v) as the mobile phase it could be shown that 5 different PL (PS, PE, PI, PC and SM) and three lysophospholipids (LPS, LPE and LPC) can be easily separated [47]. Finally, please note that in some (particularly older) references, the authors are talking often about "silica gel H". The "H" has nothing to do with the silica gel as such but simply indicates that the binder is composed of fine particles of silicon dioxide or alumina and that the plates do not contain calcium sulfate binder. The type of the binder is sometimes very important.

Nowadays, there are many different stationary phases commercially available that are potentially useful in the lipid field. Although this list is surely incomplete, these phases comprise Celite^{TM} (Supelco Inc., PA), cellulose powder, ion exchange cellulose, starch, polyamides and Sephadex^{TM} (Supelco Inc., PA). However, all these potentially useful phases do not play a major role in the lipid field and only a handful of papers have so far reported about such applications. Thus, we will focus here primarily on unmodified silica gel.

2.2. Detection systems

It is a major advantage of TLC that the separated lipid fractions can be easily visualized by binding to a dye. A lot of different reagents are nowadays available – often even as ready-made spray agents – at moderate prices. A comprehensive recent review of commonly used dyes is also available in [48]. These agents can be sorted according to their specificity and if they are destructive or non-destructive.

2.2.1. Non-destructive, non-specific

One of the most frequently used methods is the exposure of the developed TLC plate to iodine vapors that forms a non-covalent, brown complex with lipids. Unfortunately, however, completely saturated lipids can hardly be stained, while the iodine cannot be completely removed from highly unsaturated lipids (containing e.g. arachidonoyl residues) because the iodine is chemically bound to the double bonds [49]. Staining with 2,7-dichlorofluorescein or rhodamine 6G [50] provides yellow or pink spots, respectively, if the TLC plate is illuminated with UV light. The rhodamine is partic-

Table 3
Commonly used methods of lipid staining. Please note that this list is not aimed to be complete but just to provide a survey of the most common staining methods.

Lipid class	Reagent	Results/remarks
Cholesterol and cholesteryl esters	Acidic ferric chloride	Red to violet spots are generated. The reaction is faster with free cholesterol than with esters [57]. Does also react with free fatty acids.
Free fatty acids	2′,7′-Dichlorofluorescein/AlCl$_3$/FeCl$_3$	Rose color after a few minutes [57]
Phospholipids	Molybdic oxide/molybdenum	Phospholipids form blue spots on a white background [58]. A modified version has been described in [59].
Phospholipids containing choline	Dragendorff reagent (bismuth nitrate + KI)	PC, LPC and SM become detectable as orange-red spots [60]
Phospholipids containing free amino groups	Ninhydrin in butanol	PE, PS and the corresponding lysolipids are detected as red-violet spots [61]
Glycolipids	α-Naphthol/sulfuric acid	All glycoplipids are characterized by a yellow spot. Although cholesterol is also reactive, it provides a red spot [62].
Plasmalogens (alkenyl-acyl lipids)	2,4-Dinitrophenylhydrazine in 3 M HCl	The alkenyl ether is highly sensitive to acids [35] and the initially generated aldehyde reacts with the reagent. Yellow-orange spots on a white background are generated due to hydrazone formation [63].
Glycolipids	Orcinol/sulfuric acid	Blue-purple spots on a white background [64]
Glycolipids	5-Hydroxy-1-tetralone in 80% sulfuric acid	Glycolipids give yellow spots easily distinguishable from the light blue spots of phospholipids [65]
Gangliosides	Resorcinol–HCl reagent	Only gangliosides appear as violet-blue spots, while other glycolipids appear as yellow spots [66]
Sphingolipids	Sodium hypochlorite/benzidine reagent	Blue spots with a white background are generated with all lipids containing a secondary amine group [67]
Cardiolipin	10-N-nonyl-3,6-bis(dimethylamino)acridine (10-N-nonyl acridine orange)	This dye was indicated to be specific of cardiolipin but interaction with other acidic lipids may also easily occur [68]

ularly useful when alkaline solvent systems have been used and 2,7-dichlorofluorescein is to be preferred with acidic solvents due to the stabilities of the dyes. Both dyes can be easily removed if the polarity of the solvent is changed or the lipid (with the bound dye) is passed over a short column. This is also true for the dye primuline [51,52] that can be used in a similar way and gives sensitivities in the low nanomole range [52] that is comparable to rhodamine [50].

It was also shown that polyunsaturated lipids show intense darkening when the separation was performed on AgNO$_3$-impregnated TLC plates [53] that must be regarded as a consequence of the reduction of Ag$^+$ to colloidal silver. Remarkably, this darkening is dependent on the composition of the solvent system and seems to require the presence of aromatic hydrocarbons such as toluene.

2.2.2. Destructive, non-specific

Spraying the complete TLC plate with a corrosive reagent and charring the plate to render the lipids visible is a very common method [54]. 50% sulfuric acid either in methanol or water is a typically used solvent system and the plate is heated afterwards to about 120 °C for approximately 1 h. Although mechanistic details are so far widely unknown, it should be noted that saturated and unsaturated lipids require different times to be completely reduced to carbon. The intensities of these black spots can be also quantitatively analyzed (detection limits about 25–50 ng per lipid class) using videodensitometric equipment [55].

A lot of different reagents such as potassium dichromate (5%) in 40% sulfuric acid or a 3–6% solution of cupric acetate in 8–10% phosphoric acid were also indicated to be potentially useful.

2.2.3. Destructive, specific

Different reagents are known that react selectively with a specific lipid moiety under generation of colored products. A survey of the most frequently used reagents is summarized in Table 3. A detailed survey of staining agents is also available from the excellent internet side www.cyberlipid.org and from the quite old but nevertheless excellent review by Sherma and Bennett [56].

Many different dyes were recently compared regarding the achievable sensitivity [69]. The most sensitive stain could be achieved with 0.2% amido black 10B in 1 M NaCl: After pre-soaking in water, the TLC plate was immersed in the staining solution for a

few minutes. The sensitivity is about 15 ng regarding DAG, TAG, and PS, while only about 100 ng of free fatty acids and 500 ng of phorbol esters can be detected. There is also an ongoing debate whether dipping and spraying methods give really comparable results [70].

3. Applications for lipid separations by TLC

This chapter gives an overview about the most important lipid classes and how they can be separated by means of TLC. Lipids are sorted according to increasing complexity. Please note that the majority of the provided data was obtained with complex lipid mixtures. Therefore, information regarding a certain lipid class may also appear at a different position than expected by the reader. Please also note that the focus of this review will be the analysis of physiologically relevant lipids according to the lipid definition suggested above. Hydrocarbons and waxes, for instance, will not be discussed here.

3.1. Fatty acids

Fatty acids can be found in huge amounts in fat tissues of the organism although the majority of them are esterified with alcohols, particularly glycerol. It must be explicitly stated that the salts of free fatty acids (that are the prevalent forms at pH 7.4 and in the presence of physiological saline) represent (as well as lysolipids) detergents that are deleterious for the ordered cellular membrane structure. Therefore, the concentration of free fatty acids must be kept by the organism as small as possible and the concentration of free fatty acids (together with lysolipids) is often considered to be indicative of pathological conditions [21].

3.1.1. Determination of differences in length and number of double bonds

We will focus here particularly on normal phase chromatography because this technique is most commonly used and we are no real experts in reversed phase (RP) chromatography. Readers particularly interested in RP-phase HPTLC are referred to the excellent reviews by Nikolova-Damyanova [71,72]. Free fatty acids can be easily separated from the residual lipids by means of TLC and subsequently determined by GC eventually in combination with MS. Impregnation of TLC plates with AgNO$_3$ is very often used for more

detailed analysis. For instance, argentation TLC was used by Wilson and Sargent [73] to separate polyunsaturated fatty acids that are of enormous physiological interest. Silica gel 60 TLC plates were coated with $AgNO_3$ by a simple spraying technique. Surprisingly, it was indicated by these authors that spraying results in coating as uniform as dipping [73] although this is often denied [70].

The plates were air-dried in subdued light, heated at $110\,°C$ for 30 min to achieve activation and used within 1 h. After isolation by column chromatography, fatty acid methyl ester (FAME) mixtures were applied to the impregnated TLC plates. The plates were developed with toluene–acetonitrile (97:3, v/v). The plates were dried, lightly sprayed with 3% copper acetate—8% orthophosphoric acid in water and charred at $180\,°C$ for 20 min to visualize the esters. Using this procedure dienes could be well separated from trienes and in turn from tetraenes.

Additionally, even saturated fatty acids can be separated by TLC [74]. This method requires, however, derivatization of the fatty acids into monodansyl cadavarides that can be conveniently done by treating RP18 plates with monodansyl cadaverine chloride (DCC) solution, while methanol–acetonitrile–THF (18:2:1, v/v/v) served as mobile phase. Typical RF values of saturated fatty acids achievable under these conditions are: 20:0 (0.28), 17:0 (0.45), and 15:0 (0.58).

The best practical method is often to separate fatty acids with 0–2 double bonds on one silver ion TLC plate and those with 3 and more double bonds on another plate. Hexane–diethylether (9:1, v/v) is a suitable eluent for the first plate and hexane–diethylether (2:3, v/v) for the second plate. In general, however, this separation is quite difficult and does not give well reproducible results.

Schwertner and Mosser [75] have shown that the quantitative determination of different fatty acids is possible using this method: Lipids were first extracted with chloroform–methanol and butylated hydroxytoluene (BHT) as antioxidant. The total lipid extract was separated on silica gel with n-hexane–diethyl ether–acetic acid–BHT (95:5:1:0.1, v/v/v/v). Plates were sprayed with rhodamine 6G and the individual lipid classes monitored under UV light. The lipids were saponified with KOH, then esterified with BF_3 and subsequent GC was used for quantitation.

Nowadays, TLC is only seldom used alone but normally in combination with additional methods. The recently performed evaluation of the fatty acid composition of some *Lycoperdaceae mushrooms* is a typical example [76]: Polar lipids (primarily PL), free fatty acids, sterols, TAG, and fatty acid esters were separated by TLC on silica gel with n-hexane–acetone (25:4, v/v). Afterwards GC was used to determine the fatty acyl compositions of the individual lipid classes. It must be stressed that Ag-TLC is even nowadays one of the most powerful methods to separate cis- and trans-isomeric fatty acids and to isolate specific fractions in quantities suitable for further structural analysis. Further information on these aspects is available in [72]. Finally, it should be noted that even in recent papers on fatty acyl compositions of lipids by GC or GC/MS, preliminary separation by Ag-TLC (or Ag-column chromatography) is performed [42].

3.1.2. Oxidation products of fatty acids

It should be noted that not all fatty acids are of equal interest but highly unsaturated fatty acids are much more interesting than the more saturated ones because particularly arachidonic acid (AA) that is released under catalysis of the enzyme phospholipase A_2 (PLA_2) from PL is the starting material for many further products such as leukotrienes and thromboxanes. These compounds have considerable regulatory physiological activity particularly under inflammatory conditions. However, many methods for separating AA and its metabolites involve derivatization. For instance, Rao et al. [77] described a method for separating monohydroxy acid metabolites of AA by extraction with ethyl acetate after acidifica-

tion with 0.5 M citric acid. Aliquots were applied to a common silica gel G plate. The mobile phase for separation of thromboxane B 2 was diethyl ether–methanol–acetic acid (135:3:3, v/v/v). Hydroxy acids could be separated using petrol ether–diethyl ether–acetic acid (60:39:1, v/v/v). For more detailed characterization of the identified compounds, TLC separation was combined with GC. However, prostaglandins and thromboxanes formed from AA seem to require 2D TLC [78] and cannot be easily separated in a single dimension.

Threo- and erythro isomers of vicinal dihydroxy esters can be separated quite easily on silica gel impregnated with boric acid as complexing agent with hexane–diethyl ether (60:40, v/v) as the solvent system, whereby the threo isomer migrates more rapidly [79]. The hydrogenation of fat plays a significant industrial role but is accompanied by isomerization of fatty acids into the trans forms that are assumed to have harmful effects. Thus, the differentiation of cis and trans isomers of isolated fatty acids by Ag TLC plays also a significant role [80].

3.2. Cholesterol and esters

Although cholesterol is nowadays often regarded as a "bad" molecule, it represents a very important constituent of biological membranes. Additionally, cholesteryl esters are important for the transport of fatty acids through the blood flow in the form of lipoproteins. As lipoproteins are of high interest in the context of atherosclosis research, there are many papers dedicated to the analysis of cholesterol and its derivatives. There are also many very early papers dealing with cholesteryl ester analysis by TLC [81] and these are summarized in the excellent review by Touchstone [8].

A very sensitive, fluorescence-based method allowing the determination of cholesterol in amounts of about 5 ng was described in 1996 [82]: TLC separation of extracts from human lipoproteins was performed on silica gel with hexane–ethyl ether–acetic acid (80/15/1, v/v/v). Dried plates were rehydrated for 30 sec in phosphate-buffered saline and then incubated in a filipin (a strong fluorophore) suspension. Spots could be visualized at 365 nm and fluorescence intensity was linear between 5 and 3000 ng cholesterol. It was indicated that there are major intensity differences between native and oxidatively modified cholesterol and, thus, the method might be also useful to estimate the extent of lipid peroxidation.

Kawai et al. determined cholesteryl ester hydroperoxide isomers by a combination between TLC and GC/MS [83]. Amounts of less than 1 nmol could be detected on silica gel TLC plates developed with n-hexane–diethyl ether–acetic acid (70/30/1, v/v/v). Similarly, cholesterol oxidation products in meat during cooking and frozen storage could be determined by a combination of TLC and GC [84]. TLC of the unsaponifiable meat extract was initially performed on silica with hexane–ether to separate oxysterols from sterols. After elution of oxysterol, development on silica with hexane–ether–ethyl acetate (1:1:1, v/v/v) was performed. Subsequently the sample was extracted from the silica, derivatized in order to enhance its volatility and its detailed composition monitored by GC.

Autoxidation products of cholesterol can be identified by TLC [85]: A two step-TLC on silica was performed first with toluene–methanol (1:1, v/v) for 5 cm and subsequently after drying with pure toluene to 16 cm. The detection of tri-, di- and monoacylglycerols as well as free fatty acids was performed by exposing the TLC plate to iodine vapor while cholesteryl esters, cholesterol, cholesterol oxides and PL were monitored by spraying with 50% H_2SO_4.

Finally, TLC is also an important tool regarding the analysis of bile acids. Bile acids are major metabolites of cholesterol and facilitate its elimination in the feces by the formation of micelles that solubilize the cholesterol in the bile [86].

Table 4
Survey of some selected TLC separations of different sterols. Please note that this is just an arbitrary selection and there are many additional useful methods available from the literature.

Analyte	Stationary phase	Mobile phase	Remarks	Reference
Cholestrol, allylestrenol, pregnanediol etc.	RP-HPTLC plates	Acetronitrile–methanol–acetonitrile–water and methanol–water in different binary mixtures	Investigation of the retention behavior of 12 different steroids. $CuSO_4$ in H_3PO_4 used for visualization.	[90]
Androgens and gestagens	Silica	Cyclohexane–ethylacetate–ethanol (24:16:1) and chloroform–benzene–ethanol (36:4:1) in one direction; chloroform–acetone (9:1) and hexane–dichloromethane–acetonitrile (4:3:2) in the second dimension	HPTLC separation of anabolic androgens. Fluorescence detection.	[91]
Oxo-steroids	Silica gel F254	Chloroform–methanol (97:3)	Measurement of 17-oxo steroids in biological fluids with TLC and fluorometric scanning detection. Dansylhydrazine was used for derivatization. Linearity of fluorescence detection between 30 and 1000 ng.	[92]
Progesterone, trenbolone acetate, melengestrol acetate, 17-β-estradiol, 19-nortestosterone, fluoxymesterone, norethandrolone, 4-chloro-δ-1-metestosterone, cholesterol acetate, 6-β-hydroxymethandienone and oxymetholone.	Silica gel 60 F254	Chloroform–acetone	Simultaneous separation of eleven steroid hormones and synthetic anabolics. The investigated steroids were successfully visualized under UV light.	[93]
Steroids	Silica impregnated with AgNO₃	1. Hexane–ethylacetate (3:1.2:1) 2. Hexane–ether (10:1.5:1) 3. Hexane 4. Hexane–toluene (10:1)	Silver nitrate impregnated silica layers were used for the separation of a variety of steroids	[94]
Estradiol, hydrocortisone, testosterone and cholesterol	Diol F254s	Chloroform	The densitometric detection of these compounds with and without the use of sulfuric acid as visualizing reagents was compared.	[95]

3.3. Steroids (non cholesterol)

TLC is an established method of sterol analysis in urine and plasma. Unfortunately, due of the largely different chemical structures of sterols, there is no generally applicable TLC method and optimum conditions depend on the sample of interest. There were already some reviews dealing with sterol analysis by TLC. A book chapter including detailed information on sample preparation, stationary-phase and mobile-phase systems useful for the separation of steroidal pharmaceuticals was contributed by Szepesi and Gazdag [87]. Somewhat later Dreassi et al. [88] have also reviewed the application of TLC to steroids and there is also one very recent review available [89]. Although surely incomplete, Table 4 gives a survey about thin-layer chromatographic systems that have been successfully used for the analysis of steroids.

A comparative study has also been performed regarding corticosteroids [96]. In this study twelve different mixtures of organic solvents were compared to assess their efficiency as mobile phases for the separation of eighteen glucocorticosteroids along with four different spray reagents. It was found that chloroform–methanol (92:8, v/v) or chloroform–acetone (90:10, v/v) were the mobile phases of choice, while a mixture of 2,4-dihydroxybenzaldehyde, sulfuric acid and acetic acid represents the most useful spray reagent to visualize all compounds of interest [96].

Of course, antibody techniques are also a suitable method of alkaloid glycoside detection [97]. This has been explicitly shown in a chromatographic study of 36 different estradiols and estrones on silica and RP-18 silica with non-aqueous and aqueous-organic mobile phases. More recently [98], desmosterol, campesterol, brassicasterol, β-sitosterol, ergosterol, cholesterol and stigmasterol could be identified in the bodies of different snails. Among a lot of different solvent systems that were investigated for their suitability, C18 layers with acetonitrile–chloroform (40:35, v/v) or petrol ether–acetonitrile–methanol (2:4:4, v/v/v) gave optimal sterol separations. A quite similar method was also suggested to evaluate the content and composition of free sterols and free fatty alcohols in Jojoba oil [99].

Very recently, TLC has been used to investigate potential interactions between the vitamins A and D (that will be discussed below in more detail) with frequently used therapeutics (estrogens, corticosteroids, non-steroidal anti-inflammatory drugs, etc.) [100]. A comprehensive and timely survey of TLC separations of sterols, steroids and related triterpenoids is available in [101].

Due to the structural similarities of steroids, there is significant interest in combining the power of TLC separation with MS detection methods (particularly MS/MS) and this important field has been reviewed [102].

3.4. Vitamins

There are basically water and "fat" soluble vitamins. Although TLC separation of water-soluble vitamins is of course also possible, we will focus here exclusively on the properties of some apolar vitamins. A more detailed review of this topic is available from the basic reviews in [103–105] and a rather recent review [106].

3.4.1. Vitamin A (retinol)

This is a natural antioxidant that occurs in huge amounts in the human eye lens where it prevents oxidation processes induced by sunlight. The oxidation susceptibility of vitamin A and its derivates

confers many analytical problems and is most probably one reason why TLC is even nowadays frequently used because it can be faster performed than HPLC. Both, reversed phase (C8 and C18) and adsorption (silica and alumina) TLC plates can be used to separate vitamin A from its characteristic oxidation products and other vitamins. In a pioneering study, Kouimtzis and Papadoyannis [107] described the separation and quantitation of vitamin A on silica plates developed with acetone–methanol–benzene. Under these conditions RF values of 0.96, 0.85 and 0.63 were obtained for vitamins A, D3 (cholecalciferol) and B2 (riboflavin), respectively.

Separation of vitamin A from other vitamins was also described by Thielemann [108]. Silica plates were developed with a mixture of benzene–petrol ether–acetic acid (35:65:1, v/v/v). Vitamin A (RF 0.71) could be easily separated from other lipophilic vitamins such as D2 (ergocalciferol, RF 0.18). Under the applied conditions, all water-soluble vitamins remained at the origin and did not interfere with the more lipophilic vitamins.

TLC is also a very useful method if derivates of vitamin A (all-trans retinal, all-trans retinic acid, vitamin A acetate, vitamin A palmitate, etc.) have to be differentiated [109]. TLC either on silica RP-2 or Kieselguhr impregnated with 10% paraffin in cyclohexane with methanol–water (19:1, v/v) gave a good separation. It is a significant advantage that all vitamin A derivates exhibit strong UV absorptions and can be, thus, directly detected at 254 nm without the need of staining procedures.

A TLC procedure for carotenoids in fruits and vegetables was reported by Premachandra [110], who claimed applicability of this approach for quantitative measurement of vitamin A in matrices of complex compositions. Vitamin A esters could be separated from vitamin A alcohol by developing on alumina layers with 5% diethyl ether in petrol ether. As already indicated above, TLC binding studies between vitamins and other compounds were also provided. A more detailed survey of this important topic is available in [105].

3.4.2. Vitamin D (cholecalciferol)

This vitamin is considered as one of the most important vitamins and it has been even claimed that insufficient nutritional supply with this vitamin would lead to increased mortality rates. TLC is widely used to separate picogram amounts of vitamin D and its analogues on silica gel. TLC is particularly used for the pre-purification of saponified samples in order to separate vitamin D from cholesterol by means of GC [111]. However, in a similar manner as discussed above, care must be taken to minimize oxidation of vitamins on the TLC plate. It has been shown that such oxidation reactions can be minimized by developing the plate at 0 °C under a N_2 atmosphere [112].

Some rather unconventional stationary phases (such as corn starch, rice starch, talc and impregnated corn starch layers) were recently also applied to the separation of fat soluble vitamins [113]. It should finally be noted that TLC is an indispensable routine analytical method in the chemical laboratory for the fast screening of synthesis products related to vitamins [114].

3.4.3. Vitamin E (tocopherol)

Vitamin E is an antioxidant that seems to play important roles in many physiological processes and is also related to many diseases [115]. Tocopherol is particularly able to act as a lipid soluble antioxidant that protects vulnerable polyunsaturated fatty acids (or fatty acyl residues of lipids) in cell membranes and lipoproteins from harmful peroxidation processes [116].

1D TLC systems are already capable of separating tocopherol and tocotrienol homologues [117]. However, only few silica-based systems are capable of distinguishing between positional isomers. Although 1D separations have been described, these are very accident-sensitive because complex mobile phases containing four or five different solvent components have to be used. A more simple, but 2D method that can be also applied to more complex matrices, i.e. extracts from human plasma or red blood cells has been described by Lovelady [118]: Silica gel G plates were developed in the first dimension with chloroform to achieve a separation of the various homologues. Differentiation of α- and β-isomers was subsequently accomplished in the second dimension using a mixture of diisopropyl ether–petrol ether.

α-Tocopherol in food and oils can be directly (i.e. without clean-up) determined by HPTLC [119]. Simple silica does also allow the separation of α-tocopherol from its oxidized decomposition products, including α-tocopherylquinone, α-tocopherylhydroquinone, and various other compounds that occur in different tissues such as rat liver in significant concentrations [120]. For a more detailed survey please consult the reviews indicated above.

3.4.4. Vitamin K

Vitamin K is an essential cofactor in mammals and has important Ca^{2+}-binding properties. Silica gel is undoubtedly still the most common sorbent for TLC of vitamin K. One of the major advantages of silica gel is that it has (in contrast to alumina) little or no tendency to catalyze its unwanted degradation. Most interest has been paid to Vitamin K1 (2-methyl-3-phythyl-1,4-naphthochinone) that is also known as Phyllochinone. We will focus here exclusively on this compound and a more comprehensive review on vitamin K in general is available in [121].

Silica gel H plates developed with chloroform were successfully used by Baczyk et al. [122] in their study of the decomposition of vitamins D2 and K1 by exposition to UV light. Because of the lability of the vitamin K1 molecule, degradation on the TLC plate is a serious problem, even working under subdued light [123] and in an inert gas atmosphere. Carbon tetrachloride and benzene or ethyl acetate in combination with methylethylketone are suitable solvents.

Vitamin K1 can be easily separated from α-tocopherol, β-carotene, and the vitamins A and D2 in food. Plates were developed with mixtures of petrol ether–benzene (6:1, v/v), or hexane–diethyl ether (70:30, v/v). In the same manner as with fatty acids, argentation as well as RP-TLC are complementary techniques for the determination of the length and the degree of unsaturation of the side chains in these naphthochinone derivates [124]. For instance, vitamin K1 (one double bond) can be easily separated from vitamin K4 (four double bonds) on AgNO₃-impregnated silica gel plates. As silver ions are not destructive for vitamin Kl-related compounds, samples can be collected from the plates for further analysis.

3.5. Glycerides

Triacylglycerols (TAGs) are extremely important for the storage of energy in the organism. Additionally, diacylglycerols (DAGs) represent important second messenger molecules. TAGs can be formally regarded to be generated from glycerol and three free fatty acids via ester condensation. TAGs (primarily from vegetable oils such as palm or olive oil) are also extremely important chemicals in food, cosmetic as well as pharmaceutical industries. Therefore, methods allowing their fast analyses are of immense relevance. TLC is normally assumed to be less expensive than HPLC-based methods that can be of course also used and provide similar results.

3.5.1. Separation of the different acylglycerols

There is significant *in vivo* relevance of TAG: Since different lipases play important roles under pathological conditions, the simultaneous presence of mono-, di-, and triacylglycerols is evident and their analysis is unequivocally of considerable medical interest.

For instance, the enzyme lipoprotein lipase (LPase) hydrolyzes circulating lipoprotein TAG [125]. The action of LPase is assumed to play a key role in the modulation of plasma lipid levels and
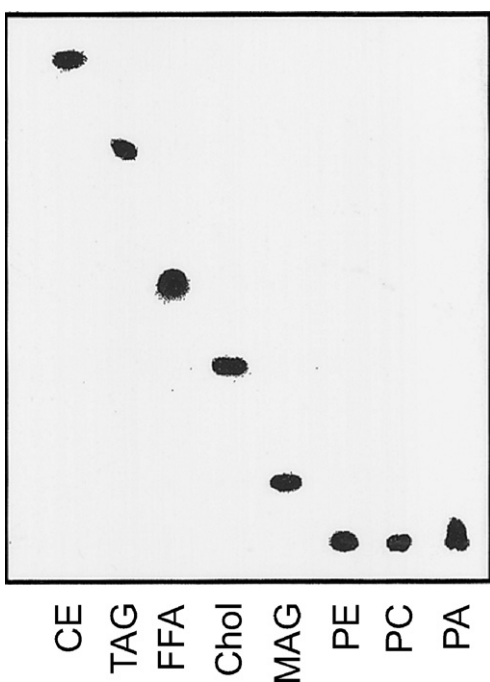
Fig. 2. Chromatogram showing the separation of different lipid classes by TLC. First the plates were run to 5 cm from the bottom in chloroform–methanol–acetic acid (90:10:1, v/v/v). After drying the plates were run in hexane–diethyl ether–acetone (60:40:5, v/v/v) to 16 cm. Again, the plates were dried and then run in hexane–diethyl ether (97:3, v/v) to 19 cm.
Reprinted with modification and permission from [52].

atherosclerotic risk. Unfortunately, this enzyme does not cleave the fatty acyl residues selectively, but generates a mixture of different isomers: LPLase action *in vitro* first produces 1,2- and 2,3-DAG; further subsequent hydrolysis leads to the formation of 2-MAG. Finally, 2-MAG undergoes isomerization into 3-MAG leading finally to complete hydrolysis [126].

During LPase *in vitro* action, partial hydrolysis products of TAG may accumulate. These products may represent almost 10% of the total acylglycerols in human plasma and as much as 30% in rat plasma [127]. Therefore, the determinations of their moieties by chromatographic or other analytical methods are highly needed because some commonly applied enzymatic assays are not capable of differentiating the individual species.

The products of lipase-catalyzed TAG hydrolysis can be easily separated on common silica plates. A mobile phase of hexane–diethyl ether–acetic acid (70:30:1, v/v/v) gives a good separation of TAG, free fatty acids, 1,2- and 1,3-DAG as well as MAG. The approximate RF values of these compounds are 0.7, 0.45, 0.26, 0.23, and 0.05, respectively. The plate may be first developed twice with diethyl ether to 3 cm. This enables the MAG to migrate from the origin, and separates them from PL and other more polar lipids which remain near to the origin [128]. It could also be shown that Na_2CO_3 impregnated silica plates can be advantageously used for such types of separations: Using a solvent consisting of diethyl ether–hexane–methanol (65:35:3, v/v/v), free fatty acids, mono-, di-, and triacylglycerols can be well resolved and the achieved RF values are about 0.0, 0.18, 0.79–0.85 and 0.98, respectively [129]. A selected chromatogram is shown in Fig. 2. Please note that multiple development is necessary in order to obtain highly resolved chromatograms.

Fielding et al. [130] developed a useful method for the separation of different glycerides that were initially extracted from human plasma with chloroform–methanol (2:1, v/v) according to the method by Folch [29]. After the addition of 1 M NaCl, the extrac-

tion tubes were centrifuged in order to speed up phase separation and the lower solvent layer (primarily $CHCl_3$) containing the acylglycerols was concentrated. The mobile phase for TLC consisted of toluene–diethyl ether–ethyl acetate–glacial acetic acid (8:1:1:20, v/v/v/v). After development, the layers were dried and the acylglycerol spots were visualized with iodine. The areas of interest were cut out and eluted with chloroform–methanol (2:1, v/v). A subsequently performed quantitative assay relied on the measurement of the glycerol released by enzymatic hydrolysis of acylglycerols.

Bilyk et al. [131] reported the successful separation of acylglycerols, fatty acids and amides as well as cholesterol using a complex solvent mixture. The separation between DAG and ceramides containing phytosphingosine and sphingosine bases has been also described [132] but the necessary solvent mixture was very complex. Elliott et al. [133] have shown that hexane–diethyl ether–acetic acid (65:35:1, v/v/v) is a suitable solvent mixture for HPTLC (on silica gel) to separate cardiolipin, cholesterol, oleate, triolein, and cholesteryl oleate. This quite remarkable lipid mixture occurs particularly in human aortic lipids.

It must be explicitly noted that migration of the acyl groups from the *sn*-2 position to the *sn*-1 and -3 positions may occur and falsify the obtained results. This migration can be easily suppressed using boric acid (H_3BO_3) impregnated silica gel TLC plates. H_3BO_3 interacts weakly with the free hydroxyl groups and prevents in this way the acyl migration nearly completely. The TLC plates are developed with chloroform–acetone (96:4, v/v). This resolves both, the 1,2- and 1,3-DAG and the 1(3)- and 2-MAG.

3.5.2. Separation in dependence on the degree of saturation

Although silver ion TLC or silver column chromatography is an extremely powerful tool regarding the acyl compositions of TAG, this topic will be only loosely treated here because there are some excellent reviews dealing with these aspects [42] available. $AgNO_3$ TLC enables separation of TAG in dependence on the content of unsaturated fatty acyl residues. Since oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) predominate in common TAGs, up to nine double bonds are present within one molecule. Denoting S = saturated, M = monoenoic, D = dienoic and T = trienoic acids, the following order of chromatographic separation can be obtained:

SSS > SSM > SMM > SSD > MMM > SMD > MMD > SDD > SST > MDD > SMT > MMT > DDD > SDT > MDT > DDT > STT > MTT > DTT > TTT

This is schematically illustrated in Fig. 3:

Normally, it is impossible to separate all the fractions listed above within a single TLC run. Therefore, it is common practice to separate the least polar fractions first with hexane–diethyl ether (80:20, v/v) or chloroform–methanol (197:3, v/v) and to separate the remaining fractions with more polar solvents such as diethyl ether alone or chloroform–methanol (96:4, v/v) [2]. The isolated bands can be easily detected by spraying with 2′,7′-dichlorofluorescein. In addition, separation of isomeric compounds is also possible to some extent. For instance, TAG of the type SSM where the monoenoic component is in the 2-position can be separated from compounds where this residue is located in the 1- or 3-position.

Please note that the nomenclature used above does not take into consideration the positions of the double bonds (although this may throughout affect the achievable RF values) and this will be outlined below in more detail. Higher unsaturated fatty acyl residues as present in fish oil cannot be easily resolved, but silver ion chromatography is also helpful with this respect [134].

Finally, the reader should note that enzymatic or chemical degradation of TAG is often required in order to determine the fatty acyl compositions and particularly the positions of the different fatty acyl residues unequivocally. One major prob-
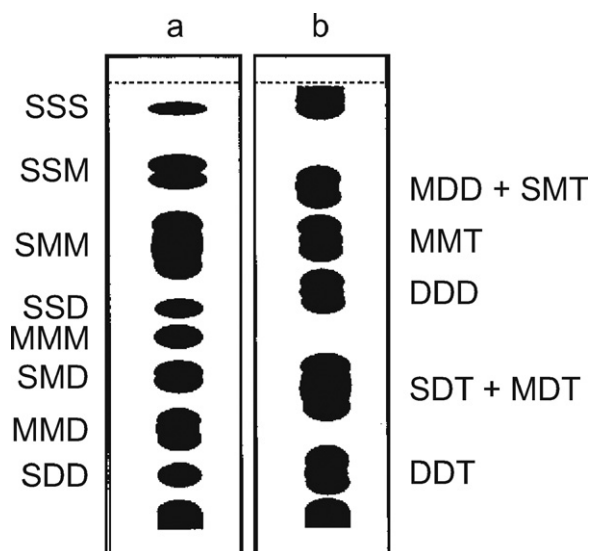
Fig. 3. Schematic separation of soybean TAGs on silica gel G impregnated with 10% AgNO$_3$. Plate (a) was developed with chloroform–methanol (99:1, v/v) while plate (b) was developed with chloroform–methanol (96:4, v/v). Abbreviations denote: S = saturated, M = monoenoic, D = dienoic and T = trienoic acids esterified to glycerol. Reprinted with modification and permission from [2].

lem in comparison to phospholipids where PLA$_2$ is specific of the *sn*-2 position [135] is that most lipases do not cleave a fatty acyl residue absolutely specifically [136]. Pancreatic lipase though not absolutely specific is the enzyme of choice due to its simple and inexpensive availability. In contrast, lipase from *rhizopus arrhizus* is nearly specific for the primary bonds of glycolipids.

Chemically, TAG may be cleaved using a Grignard reagent but this aspect is already described elsewhere in much more detail [137].

3.5.3. Vegetable oil analysis

Possible adulteration of edible vegetable oils (such as virgin olive oil) is nowadays a considerable problem in the European Union and, thus, evaluation of authenticity of oils is an important issue: Quantitative Ag-TLC of eight samples of sunflower oil [138] with different linoleic acid contents was performed on silica gel (impregnated by dipping into a 0.5% methanolic solution of AgNO$_3$) with petrol ether–acetone (25:1, v/v), petrol ether–acetone–ethyl acetate (100:5:2, v/v/v and 50:3:2 (v/v/v)). Detection was achieved by consecutive treatment with bromine and sulfurylchloride vapors (30 min each) followed by heating at 180–200 °C. Quantitative evaluation was performed by absorbance measurement at 450 nm. In a similar way, compositional changes of some oils upon a roasting process could be evaluated [139]: Ag-TLC provided the quantitative data for the TAG classes differing in unsaturation while RP-TLC was used for the differentiation of TAG species differing in chain-lengths. Afterwards, detailed fatty acyl compositions were determined by GC. The combination of TLC and GC/MS enabled Myher et al. [140] to identify and quantify about 100 different TAG species from butter.

In a very remarkable work it could be shown [141] that the lipid composition of Brazilian coffee beans can be accurately analyzed by TLC. Briefly, TAG classes differing in unsaturation were separated on 19 cm × 4 cm glass plates coated with 0.2 mm silica gel G layer and impregnated by dipping into a 5 g/l methanolic solution of AgNO$_3$ and afterwards developed using petrol ether–acetone (25:1, v/v) or hexane–acetone–ethanol (50:2:1, v/v/v). Afterwards, the plate was dried and treated consecutively with bromine and sulfuryl chloride vapors to ensure the quantitative charring of the

separated TAG classes. Alternatively, reversed-phase TLC (RP-TLC) using kieselguhr G and acetone–acetonitrile–water with a constant ratio between acetone and acetonitrile of 7:3 (v/v) and variable contributions of water gave also acceptable results on fatty acyl compositions [141]. This work is a clear proof that even rather complex lipid samples can be analyzed by means of HPTLC.

Very recently, the TAG contents in biodiesel were also determined by TLC [142]. Hot acidic p-anisaldehyde was used to specifically stain lipid contaminants such as TAG, DAG, and MAG in biodiesel and good agreement with simultaneously obtained GC data was achieved. However, detection limits achievable by this approach were rather poor.

TLC solvent mixtures are often somewhat difficult because the individual constituents possess limited miscibility. A completely stable solvent mixture (dichloromethane–ethyl acetate–methanol–acetic acid (27:22:38:13, v/v/v/v)) [143] has been shown to be excellent for monitoring oils from plant sources (including blended oils used in frying) together with the separation of mono-, di-, and triacylglycerides, and fatty acids on RP18 HPTLC silica gel layers. The detection limits for the individual components were about 0.4 µg.

Frying processes are commonly used in food preparations and it has been shown that even completely saturated TAG undergo oxidation processes when exposed to high temperatures [144] leading to potentially harmful products. An enrichment of oxidized lipids could be detected by TLC subsequent to feeding of different animals with peroxidized vegetable oils [145]. A very comprehensive review dealing with the enrichment of oxyacylglycerols as well as oxycholesteryl esters for further MS characterization has been recently published [146].

3.6. Sphingolipids and glycolipids

Due to the increasing interest that spingolipids are currently experiencing, there are many review papers dedicated to this field [147,148]. The most common compound – sphingomyelin – is normally detected within the phospholipid fraction using the same eluents and will be discussed below. Chloroform–methanol–water mixtures are commonly used to separate sphingolipids. Ratios between 70:30:4 and 50:40:10 are typically recommended for neutral sphingolipids. It should be noted that solutions of higher ionic strength must be used instead of pure water if gangliosides have to be simultaneously separated. However, isopropanol–6 M aqueous ammonia–methyl acetate (15:5:1, v/v/v) is also a very common mobile phase and the achievable separation quality is illustrated in Fig. 4. Obviously, GSL with one to four sugar residues can be easily separated. Additionally, isomeric glycopyranose residues such as glucose and galactose can be also differentiated [149]. It is also obvious that some fractions (for instance the galactosylceramides) are split into two different bands. Although this aspect has not yet been completely clarified, it seems likely that this is due to the presence of normal fatty acyl and 2-hydroxy fatty acyl residues.

In addition to the solvent systems mentioned above, CHCl$_3$–acetone–methanol–acetic acid–water (46:17:15:14:8, v/v/v/v/v) and CHCl$_3$–CH$_3$OH–acetic acid (65:25:10, v/v/v) represent also appropriate mobile phases. These systems are useful for the separation of sulfatides that are also present in significant amounts in many tissues such as brain [150]. Excellent HPTLC separation enabling the separation of 9 different sulfatides was recently obtained [151]: Silica gel 60 HPTLC plates and the solvent system chloroform–methanol–0.2% CaCl$_2$ (55:45:10, v/v/v) was used in the first step and then chloroform–methanol–acetone–acetic acid–water (5:2:4:2:1, v/v/v/v/v) in the second step. The glycolipids were visualized either using iodine vapors or by spraying the plate with orcinol/H$_2$SO$_4$ reagent and heating for 5 min at 120 °C.
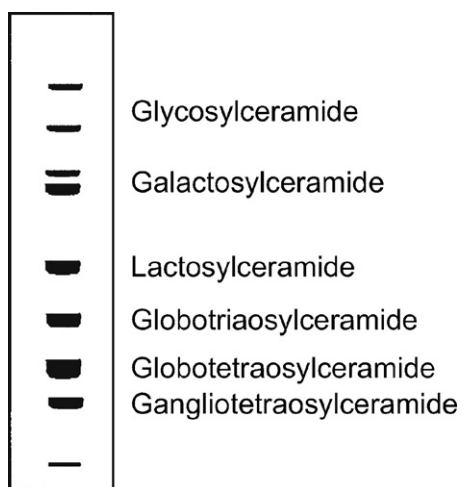
Fig. 4. Schematic separation of neutral sphingolipids by HPTLC on silica gel using isopropanol–6 M aqueous ammonia–methyl acetate (15:5:1, v/v/v) as mobile phase. Reprinted with modification and permission from [2].

Finally, a comprehensive review on skin (stratum corneum) lipids has also been published [152]. Skin lipids are rich in glycolipids as well as sphingolipids.

There is currently significant interest regarding the role and the diagnostic relevance of glycolipids in different diseases [153]. Additionally, the glycolipid composition undergoes remarkable changes during e.g. cellular growth and cell differentiation, particularly from normal to tumor cells [154].

An important class of glycolipids that does particularly occur in the brain is represented by gangliosides. Gangliosides are complex glycolipids containing ceramide polyhexosides and one or more sialyl acyl residues [155]. Due to this highly polar moiety, the extraction step is very important and great care is needed to avoid losses of the glycolipids of interest because they may easily remain (at least partially) in the aqueous phase.

Although acetonitrile–isopropanol–50 mM KCl (10:67:33, v/v/v) is also a useful mobile phase [156], the most widely used mobile phases for glycosphingolipids (GSL) are mixtures of chloroform, methanol and water, because they form a single phase at the range of hydrophobicities well suited for GSL resolution on silica gel TLC plates. Chloroform–methanol–water mixtures ranging from 70:30:4 (low analyte polarity) to 50:40:10 (high analyte polarity) are normally chosen depending on the particular GSL under study. Smaller, less polar GSL are better resolved in lower polarity solvents, whereas larger, more polar species require more polar solvents. The use of aqueous salt solutions rather than pure water is recommended for resolving gangliosides (see above) and other anionic GSL. Salts alter the ganglioside mobility and improve their resolution. The addition of ammonia (1–5 M) to the aqueous phase results in changes in the relative mobilities of different gangliosides compared to a neutral aqueous phase and is particularly useful when multiple solvent mixtures are used. It should be noted that chloroform–methanol–water mixtures are rather difficult to handle because they form complex vapor-liquid equilibria in the development tank. Therefore, tank geometry and conditions during development may alter chromatographic migration and achievable resolution significantly and must be, thus, carefully controlled.

A common procedure is the extraction of the glycolipids from the tissue of interest with chloroform–methanol (2:1, v/v), and chloroform–methanol–water (30:60:8, v/v/v), successively, and the glycolipid fraction is afterwards enriched by column chromatography. Månsson et al. [157] used chloroform–methanol–0.25% KCl (50:40:10, v/v/v) on sil-

ica gel for acidic lipids, while for neutral glycolipids, chloroform–methanol–water (65:35:8, v/v/v) is more appropriate.

Due to the complex composition of glycolipids, 1D TLC is normally not sufficient but 2D TLC has to be used and this has been explicitly shown for HL-60 cells [158]: First, the cells were extracted with chloroform–methanol (2:1, v/v). Development in the first dimension used chloroform–methanol–water (65:25:4, v/v/v). Separation in the second dimension was achieved using tetrahydrofuran–dimethoxymethane–methanol–water (10:6:4:1, v/v/v/v). Plates were air dried and subsequently analyzed by specific stainings for the presence of carbohydrates. We have used TLC to analyze complex lipid mixtures from algae that are a rich source of different glycolipids [159]. After lipid extraction according to a slightly modified Folch method, chlorophyll, carotenoids and triacylglycerols were first removed by column chromatography. Afterwards, HPTLC silica gel 60 plates (20 cm × 20 cm in size) were developed using the system introduced by Olsen and Henderson [160] with slight modifications. The first eluent (methyl acetate–isopropanol–chloroform–methanol–0.25% KCl in a ratio of 25:25:25:10:4 (v/v/v/v)) ran to a height of 13 cm from the origin. After drying, the plates were developed with the second eluent (hexane–diethylether–acetic acid in a ratio of 70:30:2 (v/v/v)) to a height of 18 cm from the origin. This resulted in convincing separation quality of all major algal lipid classes and a typical TLC plate is shown in Fig. 5:

The reader should finally note that generalizations regarding glycolipids are rather difficult since some glycolipids may contain very long oligosaccharide chains that change the chromatographic properties significantly.

In contrast, it is an advantage that monoclonal antibodies to GSL are available in some cases [161]. This makes the selective detection of compounds on TLC plates very simple and can be directly combined with MS detection [162]. This will be outlined below in more detail. In particular the high sensitivity of this approach – pmol amounts – will be emphasized [163].

3.7. Phospholipids

Although there is another paper in this special issue that is dedicated to phospholipids, some comments are here also surely necessary since otherwise a paper dealing with TLC analysis of lipids would be incomplete. Phospholipids (PL) constitute a highly important and widespread class of biomolecules [164], of which glycerophospholipids (GPLs) are of particular significance. All GPLs consist of a glycerol backbone, esterified with two varying fatty acids and one molecule of phosphoric acid. Under physiological conditions (about pH 7.4), the mono- and diprotonated forms of phosphoric acid are in rapid equilibrium. The resulting phosphatidic acid (PA) is again able, via ester condensation with different alcohols, to form a large variety of phospholipids, namely phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as neutral representatives (Fig. 6), as well as phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI) and derived higher phosphorylated compounds (e.g. PIP_2) as negatively charged phospholipids.

Lipid nomenclature is surely science of its own and the interested reader is referred to the excellent and timely survey recently provided by Fahy et al. [165]. Here, it should be only mentioned that the term "phosphatidylcholine", for instance, implies the presence of two acyl residues. As there are also huge amounts of alkyl and alkenyl lipids (termed "plasmalogens") [35], the more general term "glycerophosphocholine" should be used because these species possess only a single ester linkage.

Phospholipids are (beside cholesterol and minor amounts of some defined membrane proteins) primary components of membranes and very essential to cell function. There is nowadays also
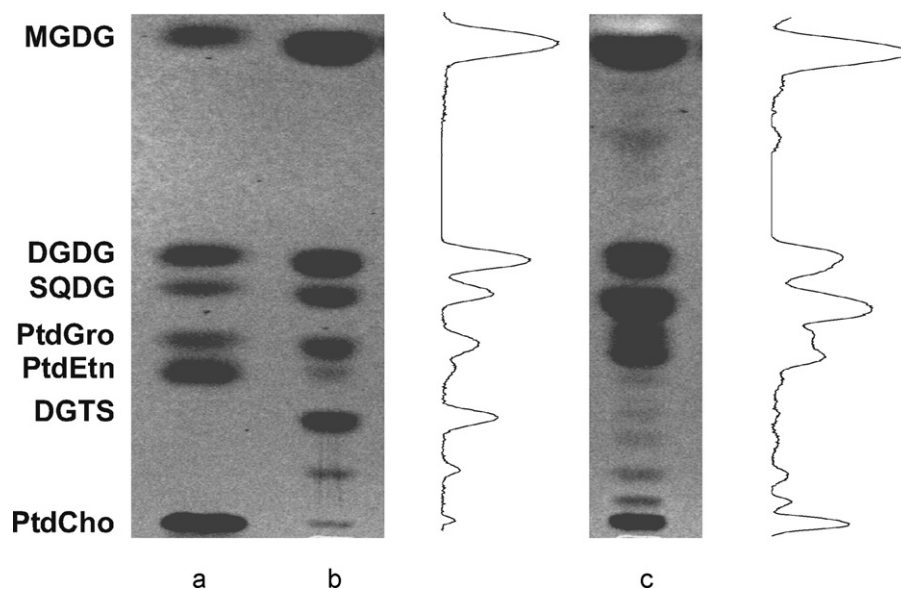
Fig. 5. Videoimage and densitometric evaluation of a typical TLC plate of algal lipid extracts subsequent to primuline staining. Lane (a) represents a mixture of some relevant lipids and is used to compare the RF values of the individual lipids. Lane (b) corresponds to *C. reinhardtii* and lane (c) to the extract from *C. meneghiniana*. DGTS which is not commercially available is absent in the reference mixture. Abbreviations: DGDG: digalactosyldiacylglycerol; DGTS: l,2-diacylglyceryl-3-O-4β-(N,N,N-trimethyl)-homoserine; MGDG: monogalactosyl-diacylglycerol; PtdCho: phosphatidylcholine; PtdEtn: phosphatidylethanolamine; PtdGro: phosphatidylglycerol; SQDG: sulfoquinovosyldiacylglycerol.
Reprinted with permission from [159].

increasing interest in using PL or compounds derived thereof as disease markers [21]. Thus, it is likely that lipid analysis by TLC will further increase because TLC is even nowadays considered to represent the method of choice if large numbers of samples have to be routinely screened. Silica gel is used in the majority of cases and many excellent solvent systems have been described [7,8,166]. Detection limits of about 20 ng per phospholipid are realistic [167].

Basically, there are one- and two-dimensional approaches to separate complex PL mixtures and both will be discussed below in more detail.

3.7.1. One-dimensional TLC

This type of separation is primarily used if non-acidic PL mixtures are of prime interest. Under these conditions, 1D TLC is often sufficient to obtain reasonable separation quality—as already reported in some very early reports [168]. Silica gel containing 7.5% magnesium acetate was used with a mobile phase of chloroform–methanol–ammonia (65:25:4, v/v/v). Chloroform–methanol–water (25:10:1, v/v/v) is still nowadays a widely used solvent mixture [169] if there are only small contributions of acidic lipids. If there is a major contribution of acidic lipids, however, PS and PE fractions can easily interfere as well as PI and PC. The order in which PL appear under these conditions is: LPC, SM, PC, PE and cardiolipin (CL). A more suitable solvent system for complex lipid mixtures is methyl acetate–isopropanol–chloroform–methanol and 0.25% aqueous KCl (25:25:25:10:9, v/v/v/v/v) [170]. This system is suitable for many complex lipid mixtures although PA and PE are not well resolved. It has been reported that this can be improved by drying the plate after the first run and re-developing using the same solvent system. A more detailed comparison of eight different mobile phases for separation of PL by 1D TLC has also been performed [171] and it turned out that chloroform–methanol–water (65:25:4, v/v/v) provided the best overall separation quality of PL standards. However, if acidic PLs are of particular interest, a slight modification of the solvent system helps to overcome problems with the quality of separation [172].

The power of the different solvent systems is schematically illustrated in Fig. 7:

However, it should be noted that such high separation quality is only achievable if great care is taken regarding the composition of the solvent mixtures as well as the activity of the used TLC plates. This important topic has been recently reviewed [173]. If glycolipids are of higher interest, then it is often advisable to perform TLC in two steps. First, common PLs are separated from the residual lipids and afterwards, glycolipids are separated. This is particularly important regarding the analysis of plant lipids that normally contain much higher amounts of glycolipids than PLs [174]. Reasonable resolution could be obtained using diisobutyl ketone–acetic acid–water (40:25:3.7, v/v/v). The addition of acetone was also indicated to be extraordinarily helpful in order to improve the quality of separation between glycolipids and PLs.

In addition to these solvent mixtures, chloroform–ethanol–water–triethylamine (30:35:6:35, v/v/v/v) [175] gives also high separation quality. Although this method was originally described for H_3BO_3 impregnated TLC plates, common TLC plates may also be used without a significant loss of quality. Independent on the method of separation, PL in nanogram amounts can be easily identified: HPTLC of CL, PA, PC, PE, PG, PI, PS, and SM was performed on silica gel by fourfold automated multiple development with chloroform–methanol–2-propanol–triethylamine–0.25% aqueous KCl (60:18:50:36:9, v/v/v/v/v) while the chamber was preconditioned with 0.1 N ammonia. Visualization was performed by spraying with (1) 1,6-diphenyl-1,3,5-hexatriene, and after intermediate drying with (2) molybdenum blue reagent according to Dittmer and Lester. Visual detection limits down to 10 ng could be reproducibly achieved [176]. Even lower detection levels could be obtained using an altered staining method [177]: TLC of PL and neutral lipids was performed on EDTA-impregnated silica gel and after pre-concentration with chloroform–methanol–water (60:40:10, v/v/v) with five step-wise developments: (i) chloroform–methanol–water 65:40:5 to 2 cm, (ii) ethyl acetate–2-propanol–ethanol–chloroform–methanol–0.25% KCl 35:5:20:22:15:9 to 5 cm, (iii) toluene–diethyl ether–ethanol 60:40:3 to 7.5 cm, (iv) n-heptane–diethyl ether 94:8 to 10.5 cm,
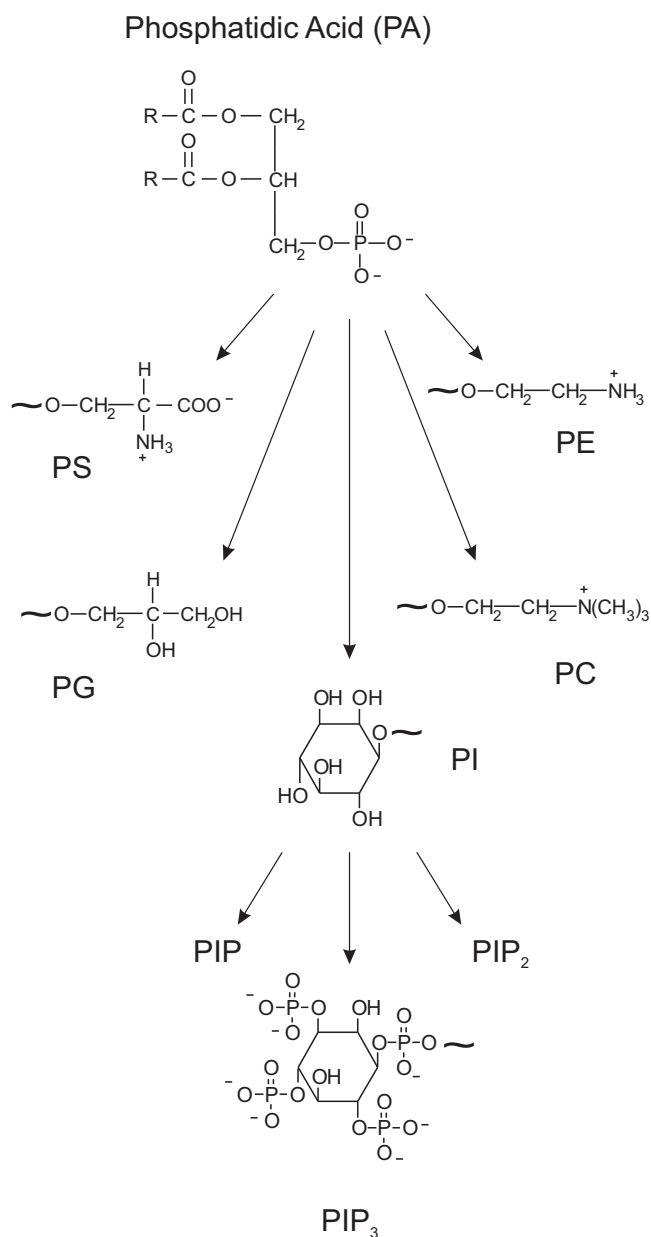
Phosphatidic Acid (PA)

Fig. 6. The chemical structures of the glycerophospholipids relevant to this review. They are basically formed from glycerol esterified with two organic fatty acids ("R" represents a varying fatty acyl residue) and with phosphoric acid. The resulting phosphatidic acid (PA) can react again with a variety of small organic molecules. Accordingly, the following compounds are formed: Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylserine (PS) and phosphatidylinositol (PI). PI can be further phosphorylated on the inositol ring.

Fig. 7. Schematic HPTLC separations of complex lipid mixtures from animal tissues. A: chloroform–methanol–water (25:10:1, v/v/v); B: methyl acetate–isopropanol–chloroform–methanol–0.025% KCl (25:25:25:10:9, v/v/v/v/v); C: First development with pyridine–hexane (3:1, v/v) and second development in the same direction with chloroform–methanol–pyridine–2 M ammonia (35:12:65:1, v/v/v/v). Abbreviations: CL, cardiolipin; CDH, ceramide dihexoside; GSu, glycolipid sulfate; CMH, ceramide monohexoside; SQDG, sulfoquinovosyl-diacylglycerol.
Reprinted with modification and permission from [2].

(v) pure n-heptane to 12.5 cm. Charring of the separated spots was performed by dipping in a solution of 10% cupric sulfate in 8% phosphoric acid for 10 s and heating at 200 °C for 2 min.

Finally, it is remarkable that all major lipid classes of human plasma can be resolved in a single 1D TLC step and even without previous extraction of the plasma. A development with chloroform–methanol–water (65:30:5, v/v/v) was first performed over a short distance in order to remove protein-bound lipids. Afterwards, the TLC plate was developed over the full length with hexane–diethyl ether–formic acid (80:20:1.5, v/v/v) to separate the individual lipid classes [178]. Similar data could be also obtained for SM, PC, PE and PS using TLC on silica with chloroform–methanol–acetic acid–water (100:55:16:6, v/v/v/v)
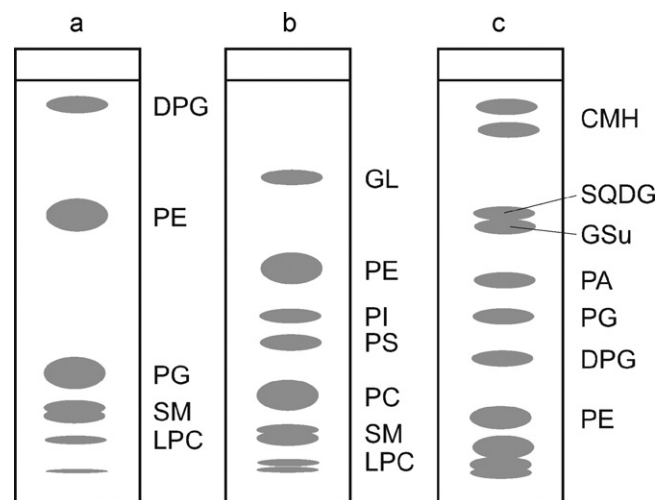
and subsequent staining with Naphtho Blue Black [179]. Acceptable data could be also obtained from urine samples although urine is much more diluted than blood or plasma [180] and contains by far lower amounts of lipids (particularly PE, PC and LPC as well as cholesterol): HPTLC was accomplished on silica gel plates with a concentration zone with chloroform–methanol–water (65:25:4, v/v/v). The detection was performed by spraying with aqueous copper sulfate reagent followed by heating. Additionally, Ninhydrin spray reagent was used to confirm the presence of PE by its characteristic amino group. The limit of quantification was about 250 ng/spot.

A quite simple method to separate complex lipid mixtures has been suggested by Vaden et al. [181]. These authors were particularly interested in PLs from yeast and acidic lipids. They could show that the used boric acid concentration has a significant impact on the separation between PG and PE as well as MLCL and PA. 1.8% boric acid seems the optimum concentration. A typical example is illustrated in Fig. 8.

3.7.1.1. Determination of enzymatic activities. There are the physiologically highly relevant phospholipases A₂, C and D that generate lysophospholipids, diacylglycerols and phosphatidic acids, respectively. One important field is the determination of the related enzymatic activities by measuring the corresponding educts and products. This can be done by means of TLC and one of the first investigations related to PLC was performed by Goldfine [182]. Chloroform extracts were separated using chloroform–methanol–acetic acid (65:25:8, v/v/v) and gave a clear separation between PL and the related DAG. A more detailed investigation of PLC was reported in [183]: TLC of membrane PL (SM, PC, PI, PS, PE, and CL) was performed on silica with chloroform–methanol–acetic acid–water (30:15:4:2, v/v/v/v) and subsequent detection by spraying with 0.05% rhodamine 6G in acetone. Subsequently, TLC of acetylated DAG was performed on silica impregnated with 10% aqueous AgNO₃ solution and chloroform–methanol (99:1, v/v) or chloroform–acetic acid (19:1, v/v). Alternatively TLC separation could be also performed on RP18 plates with acetone–acetonitrile–chloroform (5:4:2, v/v/v).
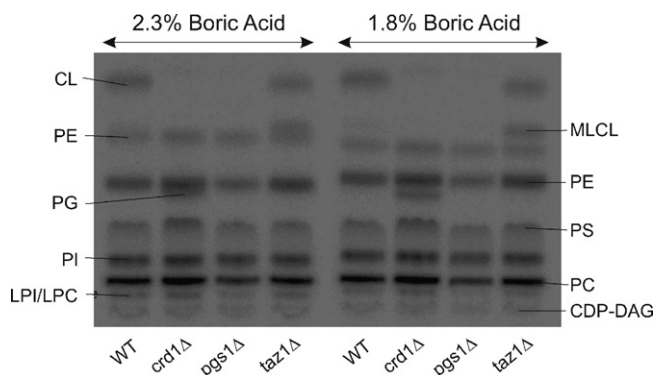
Fig. 8. Effect of boric acid concentration on separation of yeast PLs. PLs from CL pathway mutants were separated on a 2.3% (left) or 1.8% boric-acid-washed TLC plate (right). Chloroform–ethanol–water–triethylamine (30:35:7:35, v/v/v/v) served as mobile phase. Abbreviations: CL, cardiolipin; MLCL, monolysocardiolipin; CDP-DAG, cytidine diphosphate diacylglycerol. The labels along the x-axis indicate the wild-type (WT) and different mutant yeast strains.
Reprinted with modification and permission from [181].

Fig. 9. Typical two-dimensional high-performance thin-layer chromatography of total lipids extracted from the cortical P2 fraction from brain mitochondria. The (silica) plate was first developed with a solvent system consisting of chloroform–methanol–28% ammonia (65:25:5, v/v/v). After drying the plate was developed in the second dimension with a solvent system consisting of chloroform–acetone–methanol–glacial acetic acid–water (50:20:10:10:5, v/v/v/v/v). PLs were visualized by exposure to iodine vapors. "NL" means neutral lipids such as triacylglycerols. All further abbreviations are defined in the list of abbreviations.
Reprinted with modification and permission from [195].

An investigation of PLA$_2$ has also been performed by Wang and Gustafson [47]. A large variety of PL and three LPL could be separated on silica containing 0.4% ammonium sulfate with chloroform–methanol–acetic acid–acetone–water (40:25:7:4:4:2, v/v/v/v) and subsequently detected by exposing to iodine vapor. The important topic of chromatographic enzyme activity determinations has been recently reviewed [184].

3.7.1.2. Phospholipid oxidation. This is a very important aspect and has been recently comprehensively reviewed [185]. However, the majority of studies do actually not deal with the detection of intact lipids but only of oxidatively modified fatty acids [186]. Nevertheless, PC hydroperoxides could be isolated by means of TLC with chloroform–methanol–water (10/5/1, v/v/v) [187]. The aldehyde group present in PC subsequent to oxidation was visualized by spraying with Schiff's reagent and the hydroperoxide group was detected by spraying with potassium iodide and starch [188]. The detection of lipid peroxidation products in meat samples was also performed by means of TLC [189]: HPTLC of PL hydroperoxides and their parent PL (e.g. SM, PC, PE) was initially performed on silica with hexane–ether (3:2, v/v) for the removal of all neutral lipids. Subsequently, HPTLC of PL and their corresponding hydroperoxides was accomplished with chloroform–ethanol–methanol–triethylamine–water (30:25:10:35:8, v/v/v/v/v). Detection was performed by dipping in a freshly prepared solution of N,N-dimethyl-p-phenylenediamine and densitometry at 654 nm.

TLC is also a useful method to separate headgroup-modified (for instance chlorinated) PLs such as PE: PE was eluted with chloroform–methanol–acetic acid (80:12:8, v/v/v) and free fatty acids with diethyl ether–petrol ether–acetic acid (70:30:1, v/v/v) [190]. Air-dried plates were sprayed with Ninhydrin and heated at 100 °C to visualize the amine groups of PE, or were charred at 180 °C to visualize all lipids.

A detailed review dealing with analytical methods dedicated to oxidized lipids is available since 2000 [191].

3.7.1.3. Bacterial phospholipids. Bacteria such as *Escherichia coli* possess a very different lipid composition in comparison to human and mammalian cells. For instance, PC, a highly abundant PL in human cells is present in bacteria in small amounts only while PG, PE and CL are highly abundant. A method suitable of separating these lipid mixtures has been recently suggested [192]: One- and two-dimensional TLC of PE, PG, CL, and N-acyl PE
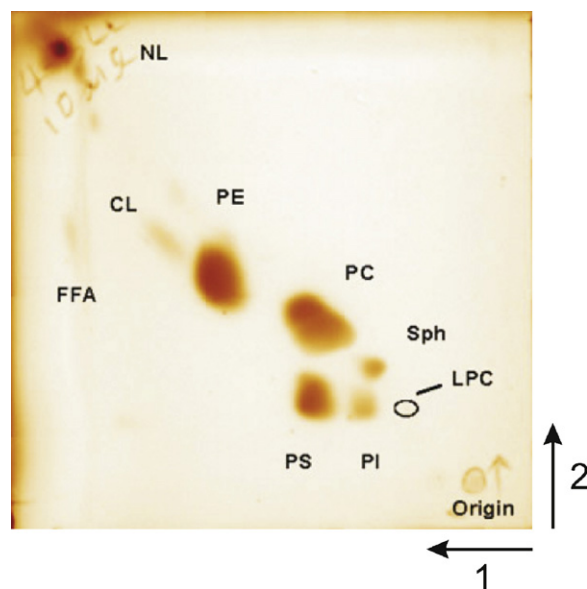
was performed on silica gel with 1-propanol–chloroform–ethyl acetate–methanol–water (50:50:50:21:18, v/v/v/v/v) for 1D separation. Spots were visualized by treatment for 20 min with aqueous thionine and differential staining with 0.05 M sulfurous acid [193]. Additional PL profile information was obtained by separation on aluminium oxide with chloroform–methanol–2–propanol–water (100:25:2:2, v/v/v/v) and staining with the Biebrich Scarlet reagent as explained in more detail in [194].

In addition, 2D-TLC was performed with 1-propanol–chloroform–ethyl acetate–methanol–water (50:50:50:21:18, v/v/v/v/v) in the first direction, treatment with 1% HCl directly on the TLC plate in order to hydrolyze the plasmalogens and development with hexane–diethyl ether (5:1, v/v) in the second direction. The aldehydes generated upon plasmalogen hydrolysis were visualized with Schiff's leukofuchsin reagent.

3.7.2. Two-dimensional TLC

2D TLC is a powerful tool to separate even very complex lipid mixtures and a typical example from lipids from brain mitochondria is shown in Fig. 9 [195].

Although the quality of separation is highly improved by 2D TLC, this method has also serious disadvantages limiting their applications significantly. First, only a single sample can be investigated and, thus, 2D TLC is much more time-consuming than 1D TLC. Second, as only a single sample can be applied, the simultaneous application of standards is impossible. This makes spot assignments as well as quantitative data analysis highly difficult.

Therefore, multiple development in a single dimension is often used as an alternative to 2D HPTLC. Normally, a solvent mixture with high elution power is used first, followed by eluents with lower elution power. This confers the advantage that the analyte is concentrated in each step and the gradient development often helps to overcome problems related to limited resolution.

Nevertheless, there is impressive analytical power in 2D TLC: For instance, more than 100 different lipid species could be identified in brown algae [196] by 2D-TLC on silica with (1) chloroform–methanol–water (65:25:4, v/v/v) and (2) chloroform–methanol–isopropylamine–concentrated ammonia (130:70:1–10, v/v/v/v). Detection was performed by spraying with dichlorofluorescein while betaine lipids were additionally monitored with the Dragendorff's reagent. 2D TLC is frequently used in the field of plant lipids that have a more complex lipid composition than animal cells.

2D TLC is also the method of choice if polyphosphoinositides (PPI) have to be identified in a complex extract from cell membranes [197]: After cell extraction with chloroform–methanol–HCl, aliquots were applied to HPTLC layers of silica gel. In order to facilitate resolution of the PPI, the plates were dipped in a solution of 1% potassium oxalate. The first development used chloroform–methanol–4.3 M ammonia (90:65:20, v/v/v) to resolve the PPI. Development was carried out to 0.5 cm below the top edge of the plate. After drying, a second development in the first dimension was performed with chloroform–methanol–concentrated ammonia (130:50:10, v/v/v) in order to separate lysophosphatidylethanol from PC. Next, the plates were rotated and developed in the second dimension with chloroform–methanol–acetic acid–water (100:30:35:3, v/v/v/v) to resolve the remaining PL classes. After being developed in each solvent system, the individual spots were visualized by charring.

2D TLC is also a powerful method for the separation of lipid oxidation products in complex lipid mixtures [198]. 2D TLC of PC, PE, PI, PS, SM, CL, LPC and LPE was performed on silica impregnated with 7.5% magnesium acetate with chloroform–methanol–ammonia (5:25:5, v/v/v) in the first dimension and chloroform–acetone–methanol–acetic acid–water (6:8:2:2:1, v/v/v/v/v) in the second dimension. Quantitative data were obtained by exposure to iodine vapor and comparison with a standard mixture.

Extracts of *Clostridium butyricum* protoplasts [182] could be also successfully separated by 2D TLC. The plates were exposed to HCl fumes for 20 s between the first and second dimension to hydrolyze the plasmalogens, permitting separation of the resulting LPL in the second dimension. This is, thus, a nice example where sample derivatization was performed *in situ* on a TLC plate.

3.8. Phosphoinositides

Phosphoinositides (PPI) play extremely important roles in the cellular metabolism and are, thus, of significant physiological interest. However, these polar lipids have two important disadvantages that massively complicate their analysis [199]. First, they occur only in very small amounts. Second, they are much more difficult to extract than the majority of other PL, in particular the zwitterionic lipids such as PC or PE. The addition of acids to the extraction solvents is mandatory in order to compensate the charges of the phosphate groups and, thus, to avoid potential losses into the aqueous layer. Radioactive labeling by ^{32}P (on the phosphate residues) or ^{3}H (on the inositol ring) is often used in order to detect low abundant PPI with sufficient sensitivity.

Due to their significant polarities, separation of PPI from all other PL is normally not a major problem and can be performed in one or two dimensions [200]: Two-dimensional TLC, for instance, resolved many different classes of major and minor PL species on silica with chloroform–methanol–acetic acid (55:25:5, v/v/v). This solvent system has been also applied successfully to human erythrocytes, human platelets and BL/VL3 murine lymphoma cells.

TLC methods have been described that enable the separation of different phosphatidylinositol isomers phosphorylated in 3-, 4- or 5 positions [201]. The most common method uses
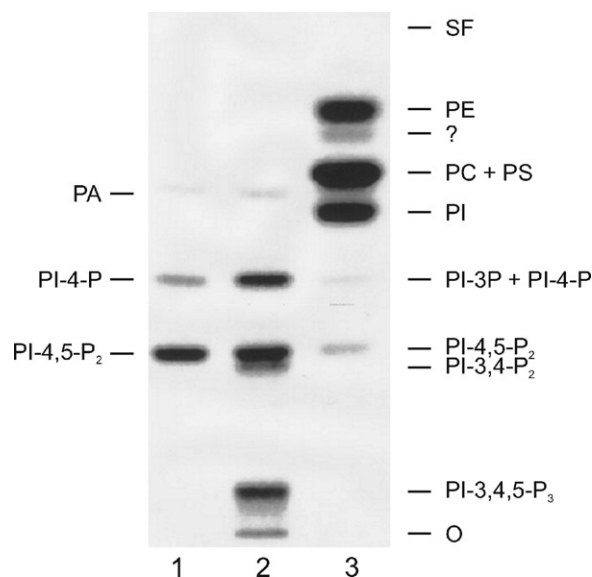
Fig. 10. Phosphor screen autoradiography of lipid compounds separated on boric acid-impregnated HPTLC plates silica gel 60. Development was performed in 1-propyl acetate–2-propanol–absolute ethanol–6% aqueous ammonia (3:9:3:9, v/v/v/v). ^{32}P-labeled erythrocyte (lane 1), ^{32}P-labeled erythrocyte PL incubated additionally with PI 3-kinase γ and Mg–[γ-^{32}P] ATP (lane 2), and ^{32}P-labeled A431 cell PL (lane 3). Abbreviations: O, origin; PI 3,4,5-P₃, phosphatidylinositol 3,4,5-trisphosphate; LPI 3,4-P₂, lysophosphatidylinositol 3,4-bisphosphate; LPI 4,5-P₂, lysophosphatidylinositol 4,5-bisphosphate; PI 3,4-P₂, phosphatidylinositol 3,4-bisphosphate; PI 4,5-P₂, phosphatidylinositol 4,5-bisphosphate; PI 3-P, phosphatidylinositol 3-phosphate; PI 4-P, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; SF, solvent front. Reprinted from [201] with permission.

HPTLC plates impregnated with boric acid and development in 1-propyl acetate–isopropanol–absolute ethanol–6% aqueous ammonia (3:9:3:9, v/v/v/v). The achievable separation power is exemplarily illustrated in Fig. 10.

4. Coupling TLC with mass spectrometric detection

LC/MS is nowadays an established method that enables the recording of mass spectra directly from the fractions of an HPLC run and this topic has been comprehensively reviewed [202]. Consequently, there are many papers dealing with "lipidomics" studies and these will not be discussed here [203].

There is a clear difference between TLC and LC: In LC, the sample is dissolved in a suitable solvent, while in TLC, the sample is dispersed in a "matrix" of the stationary phase. There were considerable attempts in the past to combine TLC with MS [summarized in 204], but this method couple became first really successful with the invention of soft ionization and desorption MS techniques that are only available since about 20 years.

Of course, TLC may be combined with MS in the following way: The spot of interest is eluted from the silica gel with suitable solvents and the obtained fractions are afterwards characterized independently by MS. This works quite well but is obviously tedious and time-consuming as soon as many different samples have to be analyzed. In order to overcome this problem, a method enabling a more direct coupling between TLC and ESI (electrospray ionization) MS was recently successfully established [205]. A plunger based extraction interface (now commercially available as the "ChromX-tract" from the CAMAG company) combined with an HPLC pump was shown to provide good results for quantitative TLC/ESI MS from HPTLC silica gel plates regarding repeatability of the MS spectra and the achievable sensitivities. This device works well for ana-

lytes from glass backed as well as aluminum backed TLC and HPTLC plates, layers with thicknesses up to 100 μm and different stationary phases [206]. However, we will not focus on this application in more detail, but will deal exclusively with real surface desorption MS techniques with the focus on matrix-assisted laser desorption and ionization mass spectrometry (MALDI MS). Desorption electrospray ionization (DESI) [207] and APCI ion sources [208] have been recently also combined with TLC and such methods are discussed in [204]. Independent of the applied MS method, such MS/TLC couplings offer several important advantages:

(a) There is no need to extract the sample from the TLC plate prior to analysis. This is on the one hand very convenient and avoids the risk of loosing some material upon the extraction process on the other hand.
(b) Since this type of analysis can be performed very fast, risks of sample alteration, for instance by oxidation, can be minimized.
(c) There is much higher resolution in comparison to visual inspection of the TLC plate because the achievable MS resolution is determined primarily by the laser spot size that is normally of the order of only about 50 μm. This means that 20 individual MALDI mass spectra can be recorded from a TLC spot of a diameter of 1 mm. Thus, this approach provides the possibility to resolve different components that could never be resolved by common staining protocols.

A short survey of the methods so far used in TLC/MS is available in [209]. Corresponding to our matter of expertise, we will focus on MALDI MS. Another reason why MALDI MS is emphasized here is the vast abundance of these devices because a lot of them were purchased in the context of recent "Proteomics" initiatives. Finally, TLC MALDI can also be performed as an "imaging approach" and this is a hot topic of current research [210].

4.1. TLC combined with MALDI MS

The characteristics of MALDI MS were recently reviewed and, thus, there is no need to explain them here in detail. The interested reader is referred to our recent review that was dedicated to MALDI MS of lipids [211] or the excellent book by Hillenkamp and Peter-Katalinić [212]. It shall be only emphasized that MALDI analysis is fast and convenient and provides spectra that can be relatively simply analyzed because nearly exclusively singly charged ions are generated. Additionally, MALDI tolerates relatively high sample contaminations such as salts. In contrast, MALDI provides also significant disadvantages. Although there are nowadays also matrix-free approaches available, classical "MALDI" requires a matrix. This is normally a small organic molecule such as 2,5-dihydroxybenzoic acid (DHB) that absorbs the laser energy [213]. However, this matrix undergoes photochemical reactions and, thus, MALDI spectra normally exhibit a background of matrix-derived signals that complicates data analysis—particularly in the low mass range. Although there were considerable attempts to overcome this problem for instance using microparticles or graphite as matrix [214], it must be explicitly noted that MALDI is less suitable for the low mass range. This is the reason why only moderate efforts were made to analyze smaller lipids such as free fatty acids so far. This is also the reason why we will focus here on glyco-, sphingo- and phospholipids.

4.1.1. Glyco- and sphingolipids

The majority of applications of TLC/MALDI have so far been dedicated to the analysis of glycolipids, while phospholipids have been investigated to a much lesser extent [14]. Although there were earlier investigations, most intriguing data were obtained in the last few years.

For instance, it could be shown in 2004 that the analysis of gangliosides is possible by direct TLC/MALDI without major fragmentations of the analyte [215]. Although rather sophisticated instrumentation was used, the achieved high spectral quality is surprising since the high contribution of carbohydrates makes these molecules very refractive to MS. Recently is was also shown that glycolipids from brain can be analyzed using a commercially available MALDI-TOF MS device equipped with a standard N_2 laser [150]. One very important aspect is the application of the matrix. This must be applied as homogeneous as possible and without compromising the achieved chromatographic resolution. Special spray devices to meet these requirements are now available from the majority of MS device manufacturers, for instance, the "ImagePrep" device from the Bruker Daltonics Company.

The majority of TLC/MALDI studies of glycolipids have used UV lasers but there are also some studies where infrared lasers were applied although these are primarily available on homebuilt MALDI devices. IR lasers have two advantages. First, glycerol may be used as matrix. As glycerol is a liquid, there are no problems regarding inhomogeneous matrix/analyte co-crystallization. Second, IR radiation penetrates deeper into the sample than UV radiation. Therefore, bringing the complete analyte from the inner of the plate to the TLC surface is less important. Using an IR laser, Dreisewerd et al. [216] were able to show that even minor gangliosides can be identified in a complex lipid mixture.

Nevertheless, UV lasers may also be used. In a recent study, DHB in acetonitrile/water (1:1, v/v) was used as matrix for the analysis of glycosphingolipids. Sensitivities of the order of 50 pmol could be obtained [217]. As already mentioned above, the use of antibodies, i.e. oligosaccharide-specific proteins, is a straightforward approach to identify GSL. Using this approach in combination with TLC/MALDI detection limits of less than 1 ng could be obtained [162]. It is a particular advantage of this approach that crude lipid extracts of biological origin can be directly used for TLC-IR-MALDI-MS and no laborious, previous GSL purification is needed. The important field of glycolipid analysis particularly by combined TLC/MALDI has been recently comprehensively reviewed [218] and matrix-free approaches that overcome the problem of interfering matrix background are also discussed in this review.

4.1.2. Glycerophospholipids

Much less interest has been paid so far to PL analysis by combined TLC/MALDI. However, two different methods were recently reported: One approach was based on the use of an IR laser and glycerol as matrix [219]. This approach has the significant advantage that quantitative data of some selected PL could be obtained but as well confers the disadvantage that abundant glycerol adducts (and to a minor extent even NaCl adducts) of the PLs of interest are detected and complicate the interpretation of the recorded spectra. Therefore, another approach used a readily available N_2 laser and standard DHB as matrix [220].

One selected lane of a TLC-separated hen egg yolk extract and some selected positive ion MALDI mass spectra (directly recorded from the TLC plate) are shown in Fig. 11.

In the context of these data, two aspects have to be emphasized: First, even low abundant lipids (e.g. phosphatidylinositol (PI) that makes out less than 1% of the PL from the egg yolk) can be easily detected. Thus, the detection limit is about 400 pmol [220]. Second, in dependence on the position where the laser beam hits the spot corresponding to a certain lipid fraction, different mass spectra are obtained. This is particularly evident for the PE fraction where shorter and longer fatty acyl residues can be differentiated. This clearly indicates that changes of the fatty acyl compositions slightly affect the migration properties of the
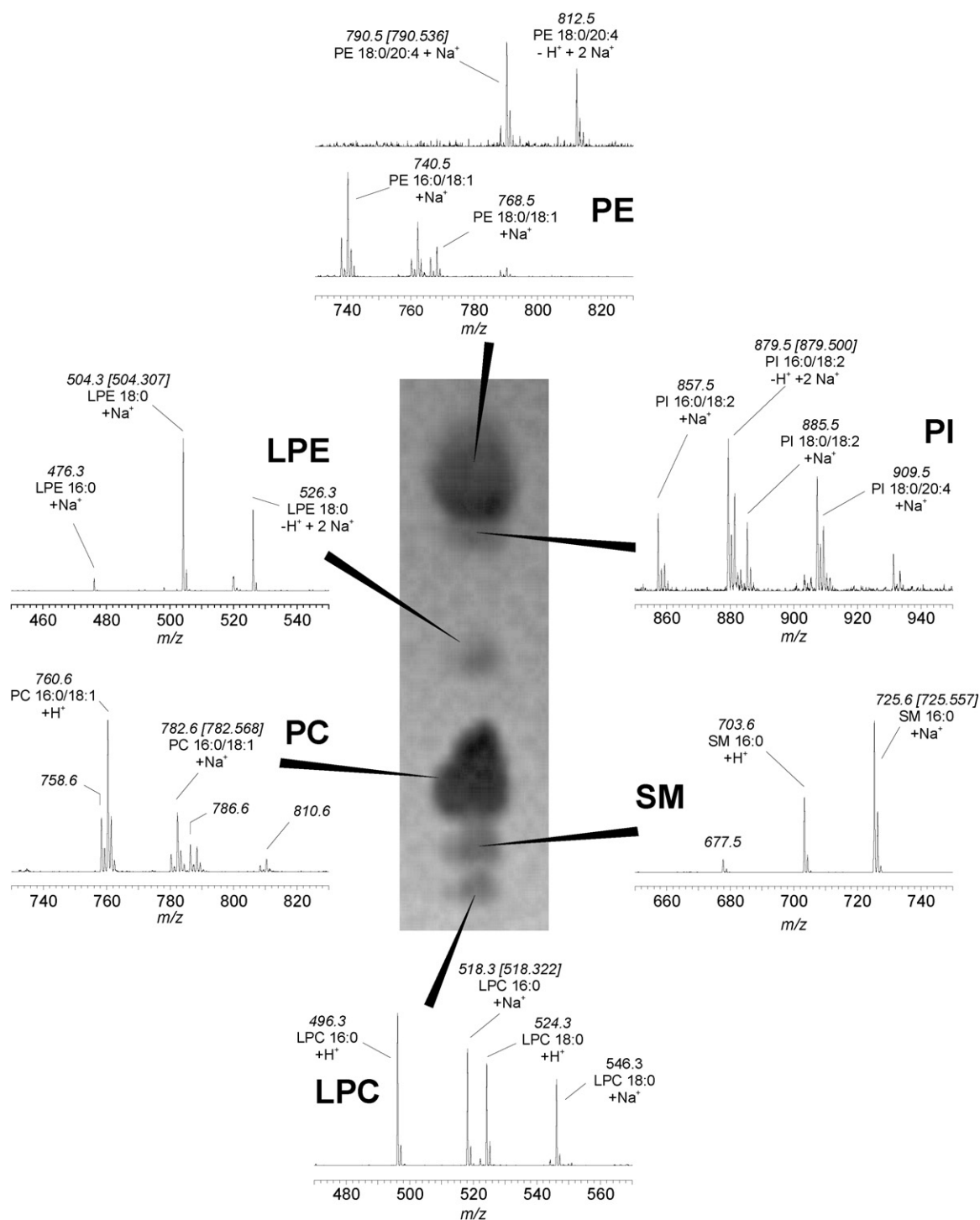
Fig. 11. Expanded region of a TLC-separated egg yolk extract and the corresponding positive ion MALDI-TOF mass spectra recorded directly from the indicated positions on the plate. Only the relevant mass regions of each PL class are shown and assignments are provided directly in the individual traces. Data given in parentheses correspond to theoretical masses and were introduced to enable comparisons with the experimental data in selected cases. Please also note that the PE fraction provides different spectra, depending on the position where the laser hits the PE spot. The only marked fragmentation is the loss of the headgroup of SM (leading to $m/z = 677.5$). Reprinted with permission from JPC: J. Planar Chromatogr. 22 (2009) 35–42 [221].

PL. This difference would have never been resolved by manual inspection of the TLC plate. TLC/MALDI is also rather fast and allows the "rasterizing" of such a TLC lane within a few minutes. This method has also been already successfully applied to more complex lipid mixtures such as extracts from stem cells [222].

5. Summary and outlook

There is unequivocally considerable interest in lipid analysis. It may even be expected that lipids will experience additional interest in the future because an increasing number of diseases is recognized to be accompanied by alterations of the lipid composi-

tions of the affected tissues and/or body fluids such as blood. This particularly concerns widespread diseases of significant socioeconomic interest such as atherosclerosis or rheumatic diseases where changes of the lipid patterns are of considerable diagnostic relevance [223].

Many different methods of lipid analysis based on chromatographic and spectroscopic methods are nowadays established and among these methods HPTLC is often considered to be of traditional interest only because more sophisticated and more modern methods are available. Nevertheless, HPTLC is in these authors' opinion one of the most versatile and reliable technique of lipid analysis.

Hopefully, we were able to provide sufficient evidence that HPTLC is an extremely powerful tool and can be applied to all relevant lipid classes of physiological and diagnostic interest ranging from apolar cholesteryl esters or triacylglycerols to highly polar poly-phosphoinositides. HPTLC is accepted as a time-saving and economical method that may be used with minimum trouble shootings. It speeds up analysis work which is usually not possible with other parallel chromatographic techniques. Finally, HPTLC may be also applied to "suspicious" samples (for instance from food) that may easily plug or even damage an HPLC column. Due to the immense number of staining methods, even minor compounds of complex mixtures may be identified although there are still strong efforts to establish even more powerful staining methods that would improve quantitative data analysis. Another important issue is the replacement of environment-hostile solvents such as chlorinated hydrocarbons by "green" solvents of reduced toxicity. Nevertheless, HPTLC is more beneficial to the environment because much smaller solvent volumes in comparison to HPLC are required.

In our opinion, there were some significant milestones in the history of planar chromatography. TLC is known since about 1938. One major progress was achieved in 1975 when HPTLC was introduced that enabled the performance of separations with much higher quality and higher sensitivity. Further improvements could be achieved by the introduction of Ultra-Thin-Layer Chromatography (UTLC) in 2001 that enabled even higher precision separations. Nowadays, a large variety of pre-coated TLC plates are commercially available that enable much higher reproducible, time-saving separations in comparison to handmade plates. The first decade of the 21st century, however, was dedicated to the establishment of improved detection techniques.

The scope of hyphenation of HPTLC with other analytical techniques such as spectroscopic methods and particularly mass spectrometry appears to hold considerable promise for the analysts who previously had reservation towards the use of planar chromatography. Although this was not discussed in this review, optical methods such as infrared (IR), Raman and laser spectroscopy are very useful to evaluate complex analyte structures. An even more powerful approach is the use of mass spectrometry and this important topic has been recently extensively reviewed [204,209]. Although a lot of different methods are nowadays already commercially available, further significant progress can be expected in this field. So far, there are basically methods based (a) on extraction of the analytes of interest prior to MS and (b) different desorption methods that allow the characterization of the analytes directly on the TLC plate. Of course, the selection of the most appropriate method depends on the analytical problem and the achievable equipment. To date, methods based on extraction seem to provide the more reliable quantitative data, while MS desorption methods normally provide higher resolution. For instance, different lipids with different acyl compositions can be identified within a single spot on the TLC plate. This obviously opens a new dimension which makes HPTLC highly competitive with so far most common LC/MS methods.

It was one aim of this review to illustrate potential applications of HPTLC regarding lipid analysis. Hopefully, it turned out that basi-

cally all naturally occurring lipid classes can be analyzed by TLC. Thus, each potential user must decide whether he would like to use TLC or if HPLC is his/her method of choice. It is the result that counts and according to these authors' knowledge both methods will provide similar results. Thus, we are confident that TLC will have an important future development that has been claimed already by Morlock and Schwack [224].

Acknowledgments

References

[1] W.W. Christie, Lipid Technol. 2 (1990) 22.
[2] W.W. Christie, X. Han, Lipid Analysis, 4th ed., PJ Barnes & Associates, Bridgwater, 2010.
[3] N.C. Shantha, G.E. Napolitano, in: R.J. Hamilton (Ed.), Lipid Analysis in Oils and Fats, Chapman and Hall, London, 1998, p. 1.
[4] B. Nikolova-Damyanova, J. Liq. Chromatogr. Relat. Technol. 22 (1999) 1513.
[5] B.L. Peterson, B.S. Cummings, Biomed. Chromatogr. 20 (2006) 227.
[6] L.S. Ettre, Chapters in the Evolution of Chromatography, 1st ed., Imperial College Press Co., London, 2008.
[7] R.J. Henderson, D.R. Tocher, in: R.J. Hamilton, S. Hamilton (Eds.), Lipid Analysis. A Practical Approach, IRL Press, Oxford, 1992, p. 65.
[8] J.C. Touchstone, J. Chromatogr. B. Biomed. Sci. Appl. 671 (1995) 169.
[9] C.F. Poole, J. Chromatogr. A 856 (1999) 399.
[10] L. Cieśla, M. Waksmundzka-Hajnos, J. Chromatogr. A 1216 (2009) 1035.
[11] S. Momchilova, B. Nikolova-Damyanova, in: M. Waksmundzka-Hajnos, J. Sherma, M. Kowalska (Eds.), Thin-layer Chromatography in Phytochemistry, CRC Press, Boca Raton, 2008, p. 277.
[12] J. Sherma, J. Chromatogr. A 880 (2000) 129.
[13] J. Sherma, B. Fried, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 2297.
[14] B. Fuchs, R. Süß, A. Nimptsch, J. Schiller, Chromatographia 69 (2009) 95.
[15] S. Zellmer, J. Lasch, J. Chromatogr. B Biomed. Sci. Appl. 691 (1997) 321.
[16] E. Hahn-Deinstrop, Applied Thin-layer Chromatography, 2nd ed., Wiley-VCH, Weinheim, 2007.
[17] W.W. Christie, High-performance Liquid Chromatography and Lipids: A Practical Guide, 1st ed., Pergamon Press, Oxford, 1987.
[18] L. Stryer, Biochemie, 4th ed., Vieweg, Braunschweig, 1988.
[19] F. Lottspeich, J.W. Engels, A. Simeon, Bioanalytik, 2nd ed., Spektrum Akademischer Verlag, Heidelberg, 2006.
[20] T. Yeung, S. Grinstein, Immunol. Rev. 219 (2007) 17.
[21] B. Fuchs, J. Schiller, Mini-Rev. Med. Chem. 9 (2009) 368.
[22] R.B. Cornell, R.S. Arnold, Chem. Phys. Lipids 81 (1996) 215.
[23] A. Carrasco-Pancorbo, N. Navas-Iglesias, L. Cuadros-Rodriguez, Trends Anal. Chem. 28 (2009) 263.
[24] F.D. Gunstone, in: W.W. Christie (Ed.), Advances in Lipid Methodology—Two, Oily Press, Bridgwater, 1993, p. 1.
[25] A. Spyros, P. Dais, J. Agric. Food Chem. 48 (2000) 802.
[26] J. Schiller, M. Müller, B. Fuchs, K. Arnold, D. Huster, Curr. Anal. Chem. 3 (2007) 283.
[27] M.H. Nouri-Sorkhabi, L.C. Wright, D.R. Sullivan, P.W. Kuchel, Lipids 31 (1996) 765.
[28] A.I. Carrapiso, C. García, Lipids 35 (2000) 1167.
[29] J. Folch, M. Lees, G.H. Sloane-Stanley, J. Biol. Chem. 226 (1957) 497.
[30] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 3 (1959) 911.
[31] A.J. Colborne, D.L. Laidman, Phytochemistry 14 (1975) 2639.
[32] A. Hara, N.S. Radin, Anal. Biochem. 90 (1978) 420.
[33] H.G. Rose, M. Oklander, J. Lipid Res. 6 (1965) 428.
[34] T.W. Honeyman, W. Strohsnitter, C.R. Scheid, R.J. Schimmel, Biochem. J. 212 (1983) 489.
[35] J. Leßig, B. Fuchs, Curr. Med. Chem. 16 (2009) 2021.
[36] W.W. Christie, in: W.W. Christie (Ed.), Advances in Lipid Methodology—Two, Oily Press, Dundee, 1993, p. 195.

[37] J. Sherma, G. Morlock, JPC: J. Planar Chromatogr. 21 (2008) 471.
[38] W. Steele, W. Banks, Milchwissenschaft: Milk Sci. Int. 49 (1994) 372.
[39] G. Dobson, W.W. Christie, B. Nikolova-Damyanova, J. Chromatogr. B Biomed. Appl. 671 (1995) 197.
[40] B. Nikolova-Damyanova, J. Chromatogr. A 1216 (2009) 1815.
[41] S. Momchilova, B. Nikolova-Damyanova, J. Sep. Sci. 26 (2003) 261.
[42] B. Nikolova-Damyanova, in: R.O. Adlof (Ed.), Advances of Lipid Methodology—Five, Oily Press, Bridgwater, 2003, p. 43.
[43] B. Nikolova-Damyanova, in: W.W. Christie (Ed.), Advances in Lipid Methodology—One, Oily Press, Bridgwater, 1992, p. 81.
[44] Y. Ando, M. Satake, Y. Takahashi, Lipids 35 (2000) 579.
[45] D. Allan, S. Cockcroft, J. Lipid Res. 23 (1982) 1373.
[46] S.L. Abidi, J. Chromatogr. B Biomed. Sci. Appl. 717 (1998) 279.
[47] W.Q. Wang, A. Gustafson, J. Chromatogr. 581 (1992) 139.
[48] Y. Wang, I.S. Krull, C. Liu, J.D. Orr, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 793 (2003) 3.
[49] E. Vioque, R.T. Holman, Arch. Biochem. Biophys. 99 (1962) 522.
[50] K. Kishimoto, R. Urade, T. Ogawa, T. Moriyama, Biochem. Biophys. Res. Commun. 281 (2001) 657.
[51] V.P. Skipski, Methods Enzymol. 35 (1975) 396.
[52] T. White, S. Bursten, D. Frederighi, R.A. Lewis, E. Nudelman, Anal. Biochem. 10 (1998) 109.
[53] M.J. Martínez-Lorenzo, I. Marzo, J. Naval, A. Piñeiro, Anal. Biochem. 220 (1994) 210.
[54] D. Kritchevsky, L.M. Davidson, H.K. Kim, Clin. Chim. Acta 46 (1973) 63.
[55] T.R. Klein, D. Kirsch, R. Kaufmann, D. Riesner, Biol. Chem. 379 (1998) 655.
[56] J. Sherma, S. Bennett, J. Liq. Chromatogr. 6 (1983) 1193.
[57] A.E. Dudziński, J. Chromatogr. 31 (1967) 560.
[58] J.C. Dittmer, R.L. Lester, J. Lipid Res. 15 (1964) 126.
[59] E.K. Ryu, M. MacCoss, J. Lipid Res. 20 (1979) 561.
[60] H. Wagner, L. Hoerhammer, P. Wolff, Biochem. Z. 334 (1961) 175.
[61] J.E. Valls, R.A. Bello, M.S. Kodaira, J. Food Qual. 25 (2002) 165.
[62] J. Kawanami, J. Biochem. 62 (1967) 105.
[63] K.S. Rhee, R.R. Del Rosario, L.R. Dugan, Lipids 2 (1967) 334.
[64] N. Neskovic, L. Sarlieve, J.-L. Nussbaum, D. Kostic, P. Mandel, Clin. Chim. Acta 38 (1972) 147.
[65] K. Watanabe, M. Mizuta, J. Lipid Res. 36 (1995) 1848.
[66] F. Šmíd, J. Reinišová, J. Chromatogr. 86 (1973) 200.
[67] V.P. Skipski, A.F. Smolowe, M. Barclay, J. Lipid. Res. 8 (1967) 295.
[68] P.F. Gallet, A. Maftah, J.M. Petit, M. Denis-Gay, R. Julien, Eur. J. Biochem. 228 (1995) 113.
[69] A.Y. Plekhanov, Anal. Biochem. 271 (1999) 186.
[70] J.D. Vasta, J. Sherma, Acta Chromatogr. 20 (2008) 15.
[71] B. Nikolova-Damyanova, in: W.W. Christie (Ed.), Advances in Lipid Methodology—Four, Oily Press, Dundee, 1997, p. 193.
[72] B. Nikolova-Damyanova, S. Momchilova, J. Liq. Chromatogr. Relat. Technol. 24 (2001) 1447.
[73] R. Wilson, J.R. Sargent, J. Chromatogr. 623 (1992) 403.
[74] H.G. Frank, R. Graf, JPC: J. Planar Chromatogr. 4 (1991) 134.
[75] H.A. Schwertner, E.L. Mosser, Clin. Chem. 39 (1993) 659.
[76] D. Nedelcheva, D. Antonova, S. Tsvetkova, I. Marekov, S. Momchilova, B. Nikolova-Damyanova, M. Gyosheva, J. Liq. Chromatogr. Relat. Technol. 30 (2007) 2717.
[77] G.H. Rao, K.R. Reddy, J.G. White, J. Chromatogr. 232 (1982) 176.
[78] K. Tsunamoto, S. Todo, S. Imashuku, J. Chromatogr. 417 (1987) 414.
[79] L.J. Morris, J. Chromatogr. 12 (1963) 321.
[80] F. Destaillats, P.A. Golay, F. Joffre, M. de Wispelaere, B. Hug, F. Giuffrida, L. Fauconnot, F. Dionisi, J. Chromatogr. A 1145 (2007) 222.
[81] L.J. Morris, J. Lipid Res. 4 (1963) 357.
[82] G.B. Smejkal, G. Hoppe, H.F. Hoff, Anal. Biochem. 239 (1996) 115.
[83] Y. Kawai, M. Miyoshi, J.H. Moon, J. Terao, Anal. Biochem. 360 (2007) 130.
[84] J.E. Pie, K. Spahis, C. Seillan, J. Agric. Food Chem. 39 (1991) 250.
[85] J. Nourooz-Zadeh, J. Agric. Food Chem. 38 (1990) 1667.
[86] D. Panveliwalla, B. Lewis, I.D.P. Wootton, S. Tabaqchali, J. Clin. Pathol. 23 (1970) 309.
[87] G. Szepesi, M. Gazdag, in: J. Sherma, B. Fried (Eds.), Handbook of Thin Layer Chromatography, Marcel Dekker, New York, 1991, p. 907.
[88] E. Dreassi, G. Ceramelli, P. Corti, in: B. Fried, J. Sherma (Eds.), Practical Thin-layer Chromatography—A Multidisciplinary Approach, CRC Press, Boca Raton, 1996, p. 231.
[89] S.A. Bhawani, O. Sulaiman, R. Hashim, M.N. Mohamad Ibrahim, Trop. J. Pharm. Res. 9 (2010) 301.
[90] H. Lamparczyk, R.J. Ochocka, P. Zarzycki, J.P. Zielinski, JPC: J. Planar Chromatogr. 3 (1990) 34.
[91] H.F. De Brabander, J. Van Hoof, JPC: J. Planar Chromatogr. 3 (1990) 236.
[92] Z. Tomsová, J. Chromatogr. Biomed. Appl. 570 (1991) 396.
[93] B. Bagocsi, Z. Vegh, K. Ferenczi-Fodor, JPC: J. Planar Chromatogr. 21 (2008) 107.
[94] T. Momose, M. Mure, T. Iida, J. Goto, T. Nambara, J. Chromatogr. A 811 (1998) 171.
[95] C. Cimpoiu, A. Hosu, S. Hodisan, J. Pharm. Biomed. Anal. 41 (2006) 633.
[96] S.M. Petrovic, M. Acanski, V.M. Pejanovic, J.A. Petrovic, JPC: J. Planar Chromatogr. 6 (1993) 29.
[97] T. Hiroyuki, P. Waraporn, T. Chiyumi, S.A. Yukihiro, FEBS Lett. 404 (1997) 279.
[98] J. Jarusiewicz, J. Sherma, B. Fried, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 2607.
[99] M. Van Boven, P. Daenens, K. Maes, M. Cokelaere, J. Agric. Food Chem. 45 (1997) 1180.
[100] A. Ramic, M. Medic-Saric, S. Turina, I. Jasprica, JPC: J. Planar Chromatogr. 19 (2006) 27.
[101] L. Dinan, J. Harmatha, R. Lafont, in: M. Waksmundzka-Hajnos, J. Sherma, M. Kowalska (Eds.), Thin-layer Chromatography in Phytochemistry, CRC Press, Boca Raton, 2008, p. 575.
[102] I.D. Wilson, W. Morden, JPC: J. Planar Chromatogr. 9 (1996) 84.
[103] T.R. Watkins, Chromatogr. Sci. 36 (1987) 195.
[104] A.P. De Leenheer, W.E. Lambert, H.J. Helis, Chromatogr. Sci. 55 (1991) 993.
[105] A. Rizzolo, S. Polesello, J. Chromatogr. 624 (1992) 103.
[106] C. Cimpoiou, A. Hosu, J. Liq. Chromatogr. Relat. Technol. 30 (2007) 701.
[107] T.A. Kouimtzis, I.N. Papadoyannis, Microchim Acta 1 (1979) 145.
[108] H. Thielemann, Pharmazie 36 (1981) 574.
[109] J. Sliwiok, A. Podgorny, A. Siwek, B. Witkowska, JPC: J. Planar Chromatogr. 3 (1990) 429.
[110] B.R. Premachandra, Int. J. Vit. Nutr. Res. 55 (1985) 139.
[111] D. Sklan, P. Budowski, M. Katz, Anal. Biochem. 56 (1973) 606.
[112] V. Justova, L. Starka, J. Chromatogr. 209 (1981) 337.
[113] N. Perisic-Janjic, B. Vujicic, JPC: J. Planar Chromatogr. 10 (1997) 447.
[114] T.G. Lobastova, S.M. Khomutov, L.L. Vasiljeva, M.A. Lapitskaya, K.K. Pivnitsky, M.V. Donova, Steroids 74 (2009) 233.
[115] M. Wojcik, I. Burzynska-Pedziwiatr, L.A. Wozniak, Curr. Med. Chem. 17 (2010) 3262.
[116] U. Singh, S. Devaraj, I. Jialal, Annu. Rev. Nutr. 25 (2005) 151.
[117] A.P. De Leenheer, V.O. De Bevere, A.A. Cruyl, A.E. Claeys, Clin. Chem. 24 (1978) 585.
[118] H.G. Lovelady, J. Chromatogr. 85 (1973) 81.
[119] J.L. Hess, M.A. Pallansch, K. Harich, G.E. Bunce, Anal. Biochem. 83 (1977) 401.
[120] J.G. Bieri, T.J. Tolliver, Lipids 16 (1981) 777.
[121] M.F.L. Fefevere, A.E. Claeys, A.P. De Leenheer, Chromatogr. Sci. 30 (1985) 201.
[122] S. Baczyk, L. Duczmal, I. Sobisz, K. Swidzinska, Mikrochim. Acta 2 (1981) 151.
[123] R.M. Wilson, T.F. Walsh, S.K. Gee, Tetrahedron Lett. 21 (1980) 3459.
[124] H.K. Lichtenthaler, K. Borner, C. Liljenberg, J. Chromatogr. 242 (1982) 196.
[125] G. Olivecrona, T. Olivecrona, Curr. Opin. Lipidol. 21 (2010) 409.
[126] P. Nilsson-Ehle, T. Egelrud, P. Belfrage, T. Olivecrona, B. Borgström, J. Biol. Chem. 248 (1973) 6734.
[127] P. Singer, U. Gerike, W. Gödicke, V. Moritz, R. Baumann, Biomed. Biochim. Acta 43 (1984) 233.
[128] H.K. Mangold, D.C. Malins, J. Am. Oil Chem. Soc. 37 (1960) 383.
[129] R. Schuch, K.D. Mukherjee, J. Chromatogr. 450 (1988) 448.
[130] B.A. Fielding, S.M. Humphreys, R.F. Allman, K.N. Frayn, Clin. Chim. Acta 216 (1993) 167.
[131] K.A. Bilyk, G.J. Piazza, R.G. Bistline, M.J. Haas, Lipids 26 (1991) 405.
[132] J. Bodennec, G. Brichon, J. Porookalian, G. Zwingelstein, J. Liq. Chromatogr. Relat. Technol. 22 (1999) 1493.
[133] R.J. Elliott, G.W. Moore, J.D. Biggart, Biochem. Soc. Trans. 13 (1985) 471.
[134] N.R. Bottino, J. Lipid Res. 12 (1971) 24.
[135] B. Fuchs, J. Schiller, Eur. J. Lipid Sci. Technol. 111 (2009) 83.
[136] W.C. Byrdwell, in: W.C. Byrdwell (Ed.), Modern Methods for Lipid Analysis by Liquid Chromatography/Mass Spectrometry and Related Techniques, AOCS Press, Champaign, 2005, p. 1.
[137] P.R. Redden, X. Lin, D.F. Horrobin, Chem. Phys. Lipids 79 (1996) 9.
[138] I. Marekov, R. Tarandjiiska, S. Momchilova, B. Nikolova-Damyanova, J. Liq. Chromatogr. Relat. Technol. 31 (2008) 1959.
[139] S. Momchilova, B. Nikolova-Damyanova, J. Liq. Chromatogr. Relat. Technol. 30 (2007) 2267.
[140] J.J. Myher, A. Kuksis, L. Marai, P. Sandra, J. Chromatogr. 452 (1988) 93.
[141] B. Nikolova-Damyanovaa, R. Velikova, G.N. Jham, Food Res. Int. 31 (1998) 479.
[142] J.D. Fontana, G. Zagonel, W.W. Vechiatto, B.J. Costa, J.C. Laurindo, R. Fontana, L. Pelisson, B.H. Jorge, F.M. Lanças, J. Chromatogr. Sci. 47 (2009) 844.
[143] J. McSavage, P.E. Wall, JPC: J. Planar Chromatogr. 11 (1998) 214.
[144] J. Schiller, R. Süß, M. Petković, G. Hanke, A. Vogel, K. Arnold, Eur. J. Lipid Sci. Technol. 104 (2002) 496.
[145] J.P. Suomela, H. Ahotupa, H. Kallio, Lipids 40 (2005) 349.
[146] A. Kuksis, Front. Biosci. 12 (2007) 3203.
[147] R.L. Schnaar, L.K. Needham, Methods Enzymol. 230 (1994) 230.
[148] G. van Echten-Deckert, Methods Enzymol. 312 (2000) 64.
[149] K. Ogawa, Y. Fujiwara, K. Sugamata, T. Abe, J. Chromatogr. 426 (1988) 188.
[150] B. Fuchs, A. Nimptsch, R. Süß, J. Schiller, J. AOAC Int. 91 (2008) 1227.
[151] Y. Niimura, I. Ishizuka, Glycobiology 16 (2006) 729.
[152] K. Raith, H. Farwanah, S. Wartewig, R.H.H. Neubert, Eur. J. Lipid Sci. Technol. 106 (2004) 561.
[153] Y.H. Xu, S. Barnes, Y. Sun, G.A. Grabowski, J. Lipid Res. 51 (2010) 1643.
[154] C.A. Reis, H. Osorio, L. Silva, C. Gomes, L. David, J. Clin. Pathol. 63 (2010) 322.
[155] J. Müthing, J. Chromatogr. A 720 (1996) 3.
[156] R.K. Yu, T. Ariga, Methods Enzymol. 312 (2000) 115.
[157] J.E. Månsson, P. Fredman, O. Nilsson, L. Lindholm, J. Holmgren, L. Svennerholm, Biochim. Biophys. Acta 834 (1985) 110.
[158] F.M. Platt, G.R. Neises, R.A. Dwek, T.D. Butters, J. Biol. Chem. 269 (1994) 27108.
[159] A. Vieler, C. Wilhelm, R. Goss, R. Süß, J. Schiller, Chem. Phys. Lipids 150 (2007) 143.
[160] R.E. Olsen, R.J. Henderson, J. Exp. Mar. Biol. Ecol. 129 (1989) 189.
[161] C. Dubois, J.L. Magnani, G.B. Grunwald, S.L. Spitalnik, G.D. Trisler, M. Nirenberg, V. Ginsburg, J. Biol. Chem. 261 (1986) 3826.

[162] J. Souady, J. Soltwisch, K. Dreisewerd, J. Haier, J. Peter-Katalinić, J. Müthing, Anal. Chem. 81 (2009) 9481.
[163] M. Sugita, Y. Sanai, S. Itonori, T. Hori, Biochim. Biophys. Acta 962 (1988) 159.
[164] L.D. Roberts, G. McCombie, C.M. Titman, J.L. Griffin, J. Chromatogr. B Biomed. Sci. Appl. 871 (2008) 174.
[165] E. Fahy, S. Subramaniam, H.A. Brown, C.K. Glass, A.H. Merrill Jr., R.C. Murphy, C.R. Raetz, D.W. Russell, Y. Seyama, W. Shaw, T. Shimizu, F. Spener, G. van Meer, M.S. VanNieuwenhze, S.H. White, J.L. Witztum, E.A. Dennis, J. Lipid Res. 46 (2005) 839.
[166] B. Fried, in: J. Sherma, B. Fried (Eds.), Handbook of Thin Layer Chromatography, Marcel Dekker, New York, 1991, p. 183.
[167] N.T.K. Thanh, G. Stevenson, D. Obatomi, P. Bach, JPC: J. Planar Chromatogr. 13 (2000) 375.
[168] J.D. Turner, G. Rouser, Anal. Biochem. 38 (1970) 423.
[169] G. Wang, T. Wang, J. Agric. Food Chem. 58 (2010) 2554.
[170] F. Vitiello, J.P. Zanetta, J. Chromatogr. 166 (1978) 637.
[171] J. Aloisi, B. Fried, J. Sherma, J. Liq. Chromatogr. 14 (1991) 3269.
[172] V.P. Skipski, M. Barclay, E.S. Reichman, J.J. Good, Biochim. Biophys. Acta 137 (1967) 80.
[173] D. Hanloser, V. Widmer, E. Reich, J. Liq. Chromatogr. Relat. Technol. 31 (2008) 1857.
[174] B.W. Nichols, Biochim. Biophys. Acta 70 (1963) 417.
[175] C. Leray, X. Pelletier, S. Hemmendinger, J.-P. Cazenave, J. Chromatogr. Biomed. Appl. 420 (1987) 411.
[176] J. Muething, M. Radloff, Anal. Biochem. 257 (1998) 67.
[177] J.I. Ruiz, B. Ochoa, J. Lipid Res. 38 (1997) 1482.
[178] I.R. Kupke, S. Zeugner, J. Chromatogr. 146 (1978) 261.
[179] J. Vaysse, P. Pilardeau, M. Garnier, Clin. Chim. Acta 147 (1985) 183.
[180] D.R. Massa, J.D. Vasta, B. Fried, J. Sherma, JPC: J. Planar Chromatogr. 21 (2008) 337.
[181] D.L. Vaden, V.M. Gohil, Z. Gu, M.L. Greenberg, Anal. Biochem. 338 (2005) 162.
[182] H. Goldfine, N.C. Johnston, C. Knob, J. Bacteriol. 175 (1993) 4298.
[183] U. Stoll, Fat Sci. Technol. 97 (1995) 229.
[184] H.S. Hendrickson, Methods Mol. Biol. 109 (1999) 1.
[185] A. Kuksis, J.P. Suomela, M. Tarvainen, H. Kallio, Mol. Biotechnol. 42 (2009) 224.
[186] K. Oette, J. Lipid Res. 6 (1965) 449.
[187] H. Itabe, H. Yamamoto, M. Suzuki, Y. Kawai, Y. Nakagawa, A. Suzuki, T. Imanaka, T. Takano, J. Biol. Chem. 271 (1996) 33208.
[188] J. Sherma, Handbook of Chromatography—Analysis of Lipids, CRC Press, Cleveland, 1993.
[189] L. Bruun-Jensen, L. Colarow, L.H. Skibsted, JPC: J. Planar Chromatogr. 8 (1995) 475.
[190] A.C. Carr, J.J. van den Berg, C.C. Winterbourn, Biochim. Biophys. Acta 1392 (1998) 254.
[191] G. Subbanagounder, A.D. Watson, J.A. Berliner, Free Radic. Biol. Med. 28 (2000) 1751.
[192] F.M. Helmy, T.E. Mueller, A. Juracka, JPC: J. Planar Chromatogr. 15 (2002) 23.
[193] M.H. Hack, F.M. Helmy, J. Chromatogr. Biomed. Appl. 525 (1990) 339.
[194] F.M. Helmy, L. Nosavanh, H. Haynes, A. Juracka, Cell. Biochem. Funct. 26 (2008) 434.
[195] H. Bayir, V.A. Tyurin, Y.Y. Tyurina, R. Viner, V. Ritov, A.A. Amoscato, Q. Zhao, X.J. Zhang, K.L. Janesko-Feldman, H. Alexander, L.V. Basova, R.S. Clark, P.M. Kochanek, V.E. Kagan, Ann. Neurol. 62 (2007) 154.
[196] W. Eichenberger, S. Araki, D.G. Müller, Phytochemistry 34 (1993) 1323.
[197] A.G. Mallinger, J.K. Yao, A.S. Brown, C.S. Dippold, J. Chromatogr. Biomed. Appl. 614 (1993) 67.
[198] J. Piluki, F.A. Kummerow, J. Agric. Food Chem. 39 (1991) 451.
[199] A.K. Singh, Y. Jiang, J. Chromatogr. B Biomed. Appl. 671 (1995) 255.
[200] K.T. Mitchell, J.E. Ferrell Jr., W.H. Huestis, Anal. Biochem. 156 (1986) 447.
[201] H. Hegewald, Anal. Biochem. 242 (1996) 152.
[202] M. Jemal, Z. Ouyang, Y.Q. Xia, Biomed. Chromatogr. 24 (2010) 2.
[203] X. Han, Biochim. Biophys. Acta 1801 (2010) 774.
[204] G. Morlock, W. Schwack, Trends Anal. Chem. 29 (2010) 1157.
[205] H. Luftmann, M. Aranda, G.E. Morlock, Rapid Commun. Mass Spectrom. 21 (2007) 3772.
[206] A. Alpmann, G. Morlock, Anal. Bioanal. Chem. 386 (2006) 1543.
[207] G.J. Van Berkel, M.J. Ford, M.A. Deibel, Anal. Chem. 77 (2005) 1207.
[208] S. Peng, M. Edler, N. Ahlmann, T. Hoffmann, J. Franzke, Rapid Commun. Mass Spectrom. 19 (2005) 2789.
[209] G. Morlock, W. Schwack, J. Chromatogr. A 1217 (2010) 6600.
[210] K. Schwamborn, R.M. Caprioli, Nat. Rev. Cancer 10 (2010) 639.
[211] B. Fuchs, R. Süß, J. Schiller, Prog. Lipid Res. 49 (2010) 450.
[212] F. Hillenkamp, J. Peter-Katalinić, MALDI MS—A Practical Guide to Instrumentation, Methods and Application, 1st ed., Wiley-VCH, Weinheim, 2007.
[213] B. Fuchs, J. Schiller, Curr. Org. Chem. 13 (2009) 1664.
[214] M. Rainer, M.N. Qureshi, G.K. Bonn, Anal. Bioanal. Chem. (2010), doi:10.1007/s00216-010-4138-1, in press.
[215] V.B. Ivleva, Y.N. Elkin, B.A. Budnik, S.C. Moyer, P.B. O'Connor, C.E. Costello, Anal. Chem. 76 (2004) 6484.
[216] K. Dreisewerd, J. Müthing, A. Rohlfing, I. Meisen, Z. Vukelić, J. Peter-Katalinić, F. Hillenkamp, S. Berkenkamp, Anal. Chem. 77 (2005) 4098.
[217] K. Nakamura, Y. Suzuki, N. Goto-Inoue, C. Yoshida-Noro, A. Suzuki, Anal. Chem. 78 (2006) 5736.
[218] J. Müthing, U. Distler, Mass Spectrom. Rev. 29 (2010) 425.
[219] A. Rohlfing, J. Müthing, G. Pohlentz, U. Distler, J. Peter-Katalinić, S. Berkenkamp, K. Dreisewerd, Anal. Chem. 79 (2007) 5793.
[220] B. Fuchs, J. Schiller, R. Süß, M. Schürenberg, D. Suckau, Anal. Bioanal. Chem. 389 (2007) 827.
[221] B. Fuchs, J. Schiller, R. Süß, A. Nimptsch, M. Schürenberg, D. Suckau, JPC: J. Planar Chromatogr. 22 (2009) 35.
[222] B. Fuchs, J. Schiller, R. Süß, M. Zscharnack, A. Bader, P. Müller, M. Schürenberg, M. Becker, D. Suckau, Anal. Bioanal. Chem. 392 (2008) 849.
[223] B. Fuchs, J. Schiller, U. Wagner, H. Häntzschel, K. Arnold, Clin. Biochem. 38 (2005) 925.
[224] G. Morlock, W. Schwack, LC–GC Eur. 21 (2008) 366.