Scanning densitometry and mass spectrometry for HPTLC analysis of lipids: The last 10 years

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[AQ7] Vicente L. Cebolla[a] Carmen Jarne[a] Jesús Vela[b] Rosa Garriga[c] Luis Membrado[a] Javier Galbán[d] [AQ1]

[a]. Instituto de Carboquímica, ICB-CSIC, Zaragoza, Spain; [AQ2]

[b]. Departamento de Química Analítica, EINA, Universidad de Zaragoza, Zaragoza, Spain;

[c]. Departamento de Química Física, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, Spain;

[d]. Departamento de Química Analítica, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, Spain

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CONTACT *Vicente L. Cebolla vcebolla@icb.csic.es Instituto de Carboquímica, ICB-CSIC, C/Miguel Luesma, 4, 50018 Zaragoza, Spain.* [AQ3]

ABSTRACT

This work is a review on HPTLC contribution to lipid analysis in complex matrices, in the period from 2010 to now, lapse of time where hyphenation with other techniques, especially Mass Spectrometry, has experienced an important growth.Scanning densitometry (SD)-UV/FL of separated lipids, as the core of detection and centerpiece for hyphenation, and its coupling with MS using soft ionization techniques (ESI, APCI, MALDI, DESI and others), are the central axis of this work. The occasional intercalation in this coupling of an on-plate biological assay for effect-direct analysis (EDA) of lipids, as well as the combination of SD with radiodensitometry (RD) using isotopically labeled-lipids are also covered topics.The described techniques make possible to develop strategies for obtaining qualitative and different levels of quantitative information, including untargeted lipids species.HPTLC of lipids has been used for: comparative purposes; fingerprinting; semi-quantitative determination, identification of species; or quantitative determination of a given individual lipid. Goals in molecular biology and biochemistry-related samples have been: preparative isolations; control of purity; verification of metabolic products; on-plate biological assays; profiles in cells; analysis of products from the cellular metabolism; measurement of enzymatic activities; monitoring lipid transport across membranes or at biological interfaces; or monitoring lipid dynamics.

Keywords: Densitometry ; electrospray mass spectrometry ; HPTLC-MS ; lipidomics ; lipids MALDI ; DESI

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Introduction

Thin-Layer Chromatography (TLC) has been very popular in lipid analysis. According to Han, the term lipidome appeared for the first time in the literature in 2001, in an article devoted to TLC-densitometry.[1,2] The reason for this popularity lies in its simplicity, speed and high sample throughput due to the development of several samples and standards in parallel, being a technique particularly well adapted to lipid class separations. Its instrumental evolution, usually termed High-Performance Thin-Layer Chromatography (HPTLC) or Planar Chromatography, is a modular, step-automated and computerized analytical technique, using special equipment for sample application, chromatographic separation and detection.

The possibility of performing multiple detection in a planar format is a strong differential feature of HPTLC versus other techniques. Scanning Densitometry (SD) is usually the central detection system that takes advantage of the properties of the plate as storage medium. Thanks to the planar storage format, SD offers different detection possibilities for separated native or fluorophore-labeled lipids under different UV/FL conditions, with optional derivatization or staining using a wide range of reagents. Likewise, the coupling of HPTLC with MS through soft ionization methods (e.g., ESI, MALDI, DESI) have recently opened new possibilities for analyzing lipid-related samples.[3–5] It should be remembered that in 2016 only few references on HPTLC-MS were considered (see, for example, Ref.[6]), and all were based on MALDI.

Although MALDI has been the most used ionization technique coupled to HPTLC for lipid analysis, the use of ESI-MS has progressed in the last years mostly thanks to the introduction of extraction/elution-based HPTLC-MS interfaces. Likewise, other ambient ionization methods have been applied to HPTLC of lipids, in particular DESI, a spray-based method.

SD is an intermediate and necessary step in hyphenation. This coupling can additionally intersperse an on-plate biological assay for effect-directed analysis (EDA). This may include an on-line characterization of selected biologically active bands because, if required, chosen zones of the plate can be selectively scanned by SD and/or transferred to MS.

On the other hand, SD has also been combined with radio-densitometry (RD) using isotopically labeled-lipids for HPTLC of complex samples. This was mostly related to lipid trafficking in biological systems, where lipids in low concentrations are involved.

All these aspects of HPTLC related to lipid analysis will be discussed in this review.

In the fields of molecular biology and biochemistry, the contribution of HPTLC to analytical results adds to that of a cohort of techniques that have greater visibility.[7–9] In spite of its current technical possibilities, HPTLC has often been undervalued. This may be due in part to the fact that HPTLC successfully works even under very modest experimental conditions. Not a few analytical problems concerning lipids have been solved using a modest HPTLC configuration, e.g., manual sample application, separation using a conventional, home-made vertical chamber, UV lamp detection, and band isolation after scraping off the layer. Nonetheless, at present time, various instrumental options from different levels of sophistication and performance can be selected for each step (sample application, chromatographic development and detection) depending on the issue. HPTLC has become a reliable and GMP-compliant analytical technique.

Despite the technical progress in detection, lipid identification is still based in many cases on migration distance of lipid standards. In the same way, it is not infrequent that the isolation of the separated lipids is based on the scraping off the band, followed by extraction and subsequent filtration prior to MS analysis, a time-consuming procedure with a poor recovery of the lipids which is somewhat tedious and unrealistic when a large number of samples is to be processed.

The purpose of this review is to show how HPTLC has contributed to lipid analysis, mostly focused on the last 10 years which is the period where the coupling has experienced a more important growth. We discuss here advances in detection, identification and determination of main classes of lipids and their corresponding subclasses, separated by HPTLC: SL, NL, FA, and PL, in different matrices. Qualitative and quantitative determinations, including untargeted lipids are also discussed.

Scanning densitometry (SD)

Lipids have been separated in bands, mostly on silica gel plates, according to their polar head. SD converts the bands on a plate either into a chromatogram similar to those obtained from other chromatographic techniques or into an image. Scanning of the plate is very fast, and re-detection (under other conditions) or scanning of a partial zone of the plate is also possible. All sample components are detected on the plate even those which are irreversibly adsorbed that would not elute when using column-based techniques.[6] This is important with regard to quantitative analysis.

Techniques for SD of lipids

Direct detection and on-plate staining

After HPTLC separation, cholecalciferol (Vitamin D3) was detected at 280 nm.[10] Unsaturated lipids can be detected by UV densitometry at 190 nm, for example, from biodiesel and human plasma samples.[11–14] However, saturated lipids cannot be detected by UV. As lipids are not usually naturally colored or fluorescent, chromogenic or fluorogenic procedures can be envisaged, pre- or post-chromatographically. Detection of bands through scanning of the plates using UV-Vis or Fluorescence mode allows a qualitative and quantitative evaluation of chromatograms to be carried out. Therefore, revealing agents have been used for general lipid detection. In general, they are quite unspecific although some of them have certain specificity toward some lipid-classes. Readers interested in TLC staining, and practical guides for general staining in HPTLC and for lipid staining can be found in a pioneering work[15] and URL addresses.[16,17]

Inorganic revealing agents

Mo-based Inorganic agents have been used for the detection of phosphorylated molecules in PL at different UV wavelengths which depend on the absorption spectra of formed compounds. They are not completely selective because other classes of lipids have responses in these systems. Mo-blue[18–23] and Dittmer and Lester's reagent provide similar results and are based on solutions of MoO_3 in H_2SO_4 and metallic Mo. A most modern modification includes the addition of tin (II) chloride to Dittmer and Lester's reactive.[24]

Phosphomolybdic acid in EtOH (drying at 120 °C and scanning at 190 nm),[25–29] and cupric sulfate or acetate in phosphoric acid (e.g., 140 °C/30 min, and UV at 620 or 450 nm)[30–40] are popular reagents for PL.

In general, all these revealing agents are destructive with regard to the sample, and they are usually applied as a recipe, under fixed conditions. As the stoichiometry of these Mo-based reactions are usually complex and not well understood, it is difficult to modify reaction conditions to obtain an optimized response for the lipid detection. In most cases, heating is usually necessary to complete the corresponding chemical reaction, e.g., complexation, oxidation, thermal aromatization.

H_2SO_4 with subsequent charring was used for general lipid detection, in MeOH,[41] in water,[21,42] or in EtOH.[43] Likewise, general detection using iodine has frequently been used.[43–45] Other employed oxidant, in this case for neutral lipids in biodiesel, was $KMnO_4$/NaOH.[46]

Neu's reagent was used to detect endocrine-disruptor lipids in food supplements. This borate salt forms complexes with certain groups of phenolic compounds giving them specific fluorescence emission wavelengths depending on molecular structure.[47]

In general, when using inorganic reagents separation is to be duplicated on another parallel plate before band scraping or MS spectrum acquisition.

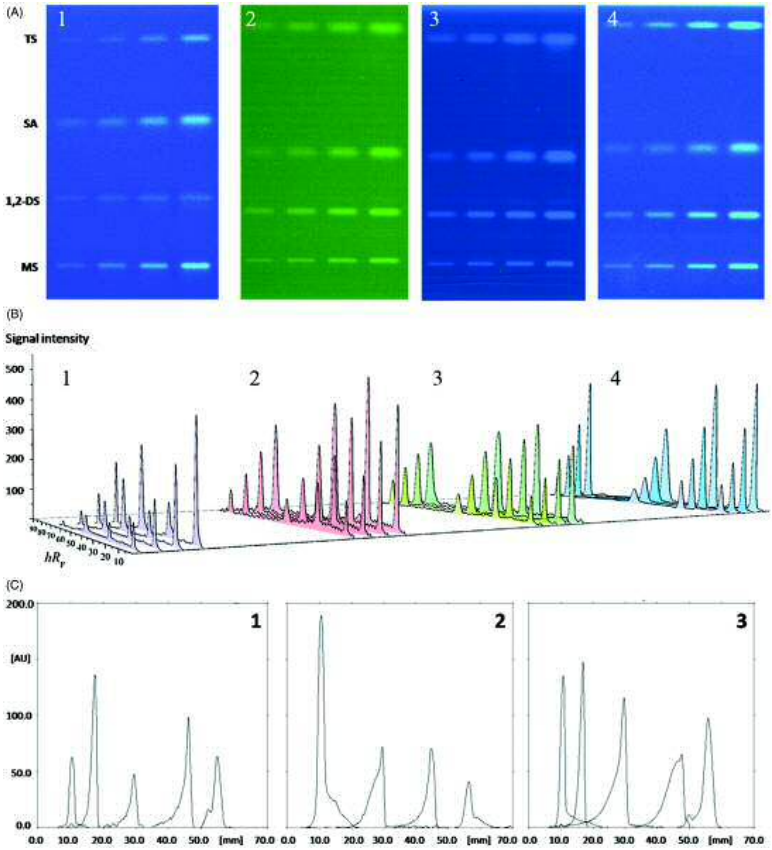
Organic revealing agents

Among organic revealing agents (Figure 1), orcinol (5-methylbenzene-1,3-diol) allows selective detection of sugar groups in GSL, PL, GL to be performed.[48,49] Lipids without sugar units give no response when using orcinol at 550 nm.

Many other organic revealing agents were used for lipid detection. Among them, Azure A, (a cationic methylated thiazine) with H_2SO_4 was useful to qualitatively detect sulfatides and sulfo-GL.[21] GL were detected by the same authors using α-naphthol in EtOH/H_2SO_4 [22], and dimethyl p-phenylenediamine monohydrochloride[20] have shown certain specificity for PL- hydroperoxides.

Figure 1. (A) Images of HPTLC separation of a standard-mix containing monostearin (MS), 1,2-distearin (1,2-DS), stearic acid (SA) and tristearin (TS) (125–1000 ng/zone, from left to right) on LiChrospher silica gel plates under UV 366 nm illumination; after post-chromatographic derivatization with primuline (1: 250 mg/L in methanol), and pre-chromatographic impregnation with berberine (2: 60 mg/L in methanol including 0.1% formic acid), coralyne (3: 12 mg/L in methanol including 0.1% formic acid), and primuline (4: 250 mg/L in methanol including 0.1% formic acid). (B) Corresponding 3D densitograms of the fluorescence scan at FL 366/>400 nm for both primuline and berberine, and at FL 435/>460 nm for coralyne. (A) and (B) reprinted from reference [879] with permission. (C) Chromatograms of SL standards illustrates selective detection of glycosphingolipids using orcinol

(SM is not detected). Densitometry by: (1) UV 190 nm; (2) post-impregnation using primuline in metanol (200 mg/L; UV 366/ >400 nm); and (3) post-impregnation using orcinol (0.2 g orcinol/100 mL of 10% H2SO4, 15 min, 100°C, Vis-550 nm). Migration distances (m.d. in mm): lyso-Gb3 (10.0 mm); SM (17.4 mm); Gb3 (29.6 mm); LacCer (47.6 mm); GlcCer (55.9 mm). A seven-step AMD sequence over 90 mm total migration distance was used using a MeOH-DCM gradient according to reference [12].



Likewise, different GL were detected using periodic acid Schiff (PAS) reaction, a common procedure in histochemistry. Thus, Gal-DG, normal and hydroxyl fatty acid ceramide monohexosides which contain 1,2, glycols (vicinal OH), were specifically hydrolyzed under mild condition by aqueous periodic acid with the production of an aldehyde group detectable with Schiff reagent.[50]

Ninhydrin was used for PL or, in general, lipids having a free amino group.[18,21,22]

The presence of plasmalogen PL, i.e., plasmenyl-phospholipids which have an ether bond in position *sn*-1 to an alkenyl group, can be detected by spraying 2,4-dinitrophenylhydrazine in HCl although they are not separated from the di-acyl or alkyl forms by HPTLC.[17]

Alkenyl lipids can also be detected using Schiff reaction. After chromatography, and previous to a second dimension development, the plate was submitted to hydrolysis step (1% HCl). This hydrolysis by HCl cleaves the alkenyl ether bond and the resulting aldehydes migrate with the second dimension mobile phase providing facile identification by their color when stained with leucofuscin. PE plasmalogens were detected in this manner in chicken and rat brains during myelination.[50]

AmidoBlack 10B/NaCl is an amino acid staining azo dye used in biochemical research for multiple purposes, included blue stain of blood proteins. In HPTLC, it was used for SL, PL but also for lipids in general.[51] Lupeol, *β*-sitosterol[52] and phytosterols[53] were detected using anisaldehide-H_2SO_4. Other unspecific, used revealing agents for lipids was dichlorofluorescein/EtOH.[54]

There is a group of strong dipolar heterocyclic-related fluorophores (e.g., primuline, berberine, coralyne) that experience important increases in their fluorescence intensity in the presence of long hydrocarbon chains, for example, lipids and alkanes.[11,55] It was demonstrated that this fluorescence is generated by weak, nonspecific electrostatic interactions between the fluorophore and the hydrocarbon chain, i.e., ion dipole-induced ones in the case of berberine or coralyne cation, and dipole-induced dipole interactions in the case of primuline.[55] This is not strictly a derivatization as only non-covalent interactions are involved. Operationally, no heating is required but simply a pre or post-chromatographic impregnation of the plate, by dipping.

Among these fluorophores, primuline (0.02–0.05% in MeOH, in acetone) is the most sensitive for lipids and is used for general lipid detection, including PL.[12,47,56–59] Many examples are shown throughout this work. Not only saturated but unsaturated lipids are also detected (λ_{exc} = 365 nm; λ_{em} > 400)[9] (Figure 1). The increase in fluorescence is in proportion to the mass of alkane applied and to the length of the aliphatic chain. Knowledge of fluorescence mechanism allows the approximate prediction of the signal generated by these fluorophores in an analytical system. The magnitude of the emission can be modulated by the choice of the fluorophore, its concentration and the chromatographic parameters.[11]

Lipids do not form adducts with primuline[60] which is compatible with MS for characterization, either in MALDI,[61,62] DESI,[56,57] or ESI and APCI through an elution-based HPTLC-MS interface.[12] Reversibility of staining was also mentioned.[60,63]

The effect of on-plate stabilization and increasing of analyte fluorescence by dipping the plate in PEG 400–4000,[47] paraffin or Triton X100, was explained by the same mechanism of fluorescence generation through the non-covalent interaction of the corresponding fluorophore (the analyte in this case) with the long-hydrocarbon chains of PEG, paraffin or surfactants.[55]

Immunostaining of glycosphingolipids (GSL)

HPTLC overlay or immunoassay consists of two essential steps: separation of GSLs on an HPTLC plate and detection of the GSLs in situ with specific ligands (e.g., antibodies). The use of specific anti-GSL antibodies makes it possible to detect the lipid-bound oligosaccharides with structural specificity. Immunostaining procedures for GSL in HPTLC have been described in detail.[23,32,64–66]

After HPTLC of GSLs, the silica gel was fixed with poly(isobutyl methacrylate) to prevent detachment of the silica gel layer. Primary antibodies and secondary antibodies were mostly used in TLC overlay assays for single detection of respective GSL. An antibody mixture composed of identical aliquots of the four antibodies was recently introduced. This antibody cocktail was used in the same way than the individual single antibodies.[65] Blotting of thus stained sphingolipids from the plate to a plastic membrane can be done, and bound antibodies are visualized by the color developed using 0.05% (w/v) 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt in glycine buffer.

Labeled lipids

Another way to detect lipids is to derivatize them to give synthesized fluorescent analogues before HPTLC. NBD, the fluorescent group N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino], was used in an enzymatic activity assay for recombinant acyl-CoA:diacylglycerol acyltransferases which are essential for TG biosynthesis. The fluorescent substrate was incubated with various fatty acyl-CoAs, and the lipids were extracted and separated using HPTLC. Enzymatic activity was expressed as picomoles of TG formed per minute and mg of protein, using a calibration curve for the NBD-DG substrate.[67]

Sphingosine was modified to obtain a bifunctional or trifunctional probe to investigate lipid metabolism as well as lipid localization in intact cells.[68,69] The probe has a coumarin cage group, i.e., a photo-cleavable protection group, that can be cleaved using UV light with wavelengths >400 nm. It also contains a diazirine crosslinking group which requires shorter wavelengths, around 355 nm. This enables sequential photoreactions (uncaging and crosslinking) to be performed on the same molecule. An additional alkyne click moiety is used for post-crosslinking functionalization with fluorophores. This probe design is also useful for following lipid metabolism dynamics since the modified lipid (clicked to a fluorophore) can be visualized by HPTLC. These fluorescent modifications do not change lipid proper-

ties, and allowed to reveal sphingosine subcellular localization via crosslinking, fixation, and specific staining by click reaction with a fluorophore.

HPTLC was used as a tool for analyzing products from the synthesis of fluorescent membrane-spanning lipids resistant to spontaneous as well as protein-mediated intermembrane transfer.[33] They consist of a fluorescent tag either directly or via a phosphoethanolamine spacer to the lipid core, a tetraether lipid caldarchaeol, prepared from cultures of archaea *Thermoplasma acidophilum*.

Analysis of lipid classes and subclasses by SD

Although greener solvents as ethanol and ethyl acetate were proposed as potentially suitable for the substitution of methanol and chloroform methods, lipid extracts from samples of different origins are mostly obtained from Bligh-Dyer or Folch, which are the gold standard extraction methods.[70]

Tables 1–4 show densitometry-based HPTLC methods for analyzing lipid sub-classes mostly from PL (Table 1); SL (Table 2); NL + FA (Table 3). Table 4 (Total lipid classes) refers to lipid subclasses from all the main classes (PL, SL, NL) when they are involved in the same sample/analysis.

Table 1. HPTLC-SD of PL from different samples.

Involved sub-classes	Original samples	Type of development/Staining systems	Type of analysis	Biological or analytical significance	[Reference] author
PE, PC-hydroperoxides	Enzymatically and thermally oxidized egg yolk	Isocratic/VIS: $CuSO_4$-H_3PO_4; Mo-Blue	Qualitative	Comparison of profiles with LC-MS	[20] Parchem
PL, gangliosides	Dermal fibroblasts	Isocratic/VIS: H_2SO_4; UV: Mo-Blue; FL: primuline	SQ. No details on standards were provided. Scraping off (MALDI)	Lipid alterations associated with the lack of parkin protein	[19] Lobasso
PC, PE	Sunflower and soybean lecithins in chocolate	Isocratic/FL: primuline	SQ, external standardCoupled to ESI-MS (interface) and MALDI	Influencing effects of PC, PE on rheological parameters in chocolate production	[71] Krüger
PG, CL, LPG, PC, PA, hydro- and dihydroperoxy CL	*S. pastorianus syn.* Carlbergensis strains	Isocratic/FL: primuline	Scraping off (preparative, MS)	Membrane PL of bacterial cells	[72] Alves
Polar lipids (PL, SL)	Mammalian cell lines (SP2/O, CHO, HEK)	2D; Isocratic/UV: 2,7-dichloro-fluorescein	Qualitative	Profiling in mammalian cells	[54] Zhang
PL, TG (PC interferences)	Adipose tissue from mice	Isocratic/FL: primuline	CompaprativeScraping off (MALDI)	HPTLC to remove PC interferences in nutrition changes of adipose tissue	[73] Popkova
CL, monolyso-CL	Acinetobacter baumanii	Isocratic/VIS: H_2SO_4; Mo-Blue; UV: ninhydrin	QualitativeScraping off (preparative)	Identification of unique CL and mono-lyso-CL	[18] Lopalco

Involved sub-classes	Original samples	Type of development/Staining systems	Type of analysis	Biological or analytical significance	[Reference] author
PL, NL, FA	Control and Conjugated Linoleic Acid (CLA)-rich eggs	Isocratic/FL: primuline	Scraping off (separate plate by MALDI)	CLA-rich yolks contained more LPC than did control eggs	[74] Shinn
PC, PE, CL, PG	Membrane proteins and extracts from photosynthetic purple bacteria	Sequential (7 steps)[a]/UV 190 nm	Coupling (interface) with ESI-MS	PL associated to membrane proteins	[14] Lapieza
PL sub-classes	Airborne strain *Pseudomonas fluorescens* in air dust clouds	Isocratic/FL: primuline	Coupling with MALDI imaging	Presence of PC in airborne *P. fluorescens*	[75] Kondakova
PL sub-classes	LS174T colorectal adnocarcinome xenografts treated with a vascular disrupting agent	Sequential/UV: Amido Black 10B	Coupling with MADI imaging	Increase of LPC in solid tumors in the central tumor area	[51] Batubara
PL composition	Bee queen sperm	Isocratic/FL: primuline	SQ with external standardCoupling MALDI	Conservation of honey bee sperm PL during storage in bee queen	[76] Wegener
PL, Chol	Synovia from Ultra High MW polyethylene knee joint implants	Sequential/FL: primuline	Scraping off (MALDI)	Changes in joint implants with lipid adsorption	[77] Fröhlich
Trehalose 6-monomycolate (TMM), trehalose 6,9-dimycolate(TDM), CL	Bacteria and protease-treated soluble *Rhodococcus equi* antigen	Sequential/VIS: iodine	Scraping off (by weight, purification)	Identification of *R. equi* lipids	[43] Harris
Diphytanylglycerols, PI, PG, archaeol lipids, cardalchaeol lipids ether lipid-CL	*Pyrococcus furiosus*	Isocratic/VIS: H_2SO_4; FL: primuline	Scraping off (MALDI)	Descriptive analysis	[21] Lobasso
PL	Egg yolk extract	Isocratic/primuline	Scraping off (31P- NMR)	HPTLC lipid isolation may be used without any risk of sample alteration	[78] Teuber
PG, PE, PC, PI	Mdm 12 protein purified from bacteria and yeast	Sequential/VIS: CuSO4-H_3PO_4	Descriptive (biological structure)	Identification of PL binding ERMES domain (see text). HPTLC-based PL exchange *in-vitro* assay using liposomes	[31] AhYoung
PL profile	Mammary tumor cells, hepatocites mitochondria	Isocratic/VIS: CuSO4-H_3PO_4	SQ of PL with external standards	Separation and semi-quantification of eight subclasses of PL	[79] Pinault
PL, glucosaminyl-PG	*Pseudomonas aeruginosa* bacterium	Isocratic/VIS: iodine	Scraping off (ESI-MS)	Off-line identification of glucosaminyl-PG	[80] Abbes

In Tables 1–4, we refer as to “isocratic” when development was done using a single solvent (or a mixture of solvents). Isocratic developments were done in one, two, or three different plates (using the same or other mobile phase). We distinguish it from “sequential” development when successive solvents are developed, either in order of increasing or decreasing polarity.

[a]Gradient AMD.

Table 2. HPTLC-SD of sphingolipids from different samples.

Involved subclasses	Original samples	Type of development/Staining systems	Type of analysis	Biological or analytical significance	[Reference] author
SL, sphingosine	Intact cells in well plates	Isocratic/previous lipid derivatization	Qualitative	Monitoring SL dynamics	[68] Höglinger
SM, Cer, Glu-Cer	Skin homogenates	Isocratic; sequential/VIS: $CuSO_4$-H_3PO_4	SQ with External calibration	Lipids in epidermis or Stratum Corneum	[34] Nomoto
Dimeric Cer (dCer)	Stratum Corneum and other skin layers	Sequential (18 steps)[a]/VIS: $CuSO_4$-H_3PO_4	Quantitative using dCer as standard	Determination of novel dimeric Cer in Stratum Corneum	[35] Neubert
Cer profiles, NL	Skin Stratum Corneum	Sequential (8 steps)[a]/VIS: $CuSO_4$-H_3PO_4	SQ with External CalibrationMS-coupling (interface)	Deep characterization of Ceramides	[39] Kabrodt
Glycosyl-Cer	Plant seeds from different species	Sequential (18 steps)[a]/VIS: $CuSO_4$-H_3PO_4	SQ with External calibrationScraping off (preparative)	Plants as alternative sources for competitive Cer production	[81] Reisberg
Gangliosides and glycosylinositol phosphor-Cer	Lipid extracts	Isocratic/VIS: orcinol; FL: primuline	Scraping off (preparative)	Removal of background interferences	[48] Kamimiya
Gb_4	Rat polycystic kidney disease	Isocratic/FL: primuline	Qualitative/ComparativeScraping off (MALDI)	Structural characterization of Gb_4 and other neutral SL	[82] Ruh
Glu-Cer, Glc-Cer	*A. fumigatus, A. nidulans, S. apiospermum*	Isocratic/VIS: orcinol; FL: primuline	Scraping off	Characterization	[63] Sicard
Sphinganine, sphingosine, ceramides	Cultured cells	Isocratic/VIS: orcinol; $CuSO_4$-H_3PO_4; radiodensitometry	Quantitative	Indirect lipid transfer protein activity using quantification of glyco-SL	[32] Backman
PL removal before SL analysis	*In vitro* propagated human monocytic THP-1 cells	Isocratic/VIS: orcinol; inmunostaning	Coupling to MALDI	On-plate enzymatic PL disintegration before HPTLC	[65] Kouzel
SM, Gb_3	Human plasma from healthy individual and Fabry’s patient	Sequential (7steps)[a]/UV 190nm; FL: primuline	QualitativeCoupling to ESI-MS/MS (interface)	MS profiling of SM and Gb_3 molecular species	[13] Jarne

Involved subclasses	Original samples	Type of development/Staining systems	Type of analysis	Biological or analytical significance	[Reference] author
SM, Gb_3	Human plasma from healthy individual and Fabry's patient	Sequential (2 steps)[a]/FL: primuline	SQ with Standard AdditionCoupling to APCI-MS (interface)	SQ of SM and MS profiling	[12] Domínguez
Acidic GSL	Bovine brain extracts	Isocratic/No densitometry	Coupling comparison (interface/LESA)	No loss of sialic acids that occurs in MALDI	[83] Park
(G)SL profiles	Skeletal muscle, brain tissues and serum	Isocratic/UV: Amido Black 10B	QualitativeCoupling to MALDI	Strategy for biomarker discovery	[58] Torretta
GSL	C2C12 mouse myoblasts	Isocratic/No densitometry	ComparativeCoupling to MALDI	High-throughput characterization method	[59] Torretta
SL	Mouse kidney, spleen and small intestine	Isocratic/VIS: orcinol; FL: primuline; inmunostaining	Coupling to MALDI (parallel plate)	Profiles of SL	[84] Suzuki
Galactosyldiglycerides, normal and hydroxyl fatty acid ceramide monohexosides, SM, and also PC, PE_1 and PE_2	Chicken and rat brains	isocratic; 2D/VIS: thionine; PAS reactions	Comparative	Variation in lipid composition during myelinization	[50] Helmy
SL	Human lens	Isocratic/FL: primuline	Detailed identification by DESI-MS	30 SL species from 11 different SL subclasses, including novel ether-linked PA species were identified by DESI-MS. Only LacCer with a sphinganine backbone were observed.	[56] Seng
Ganglioside species (GQ1, GT1, GD1, GM1) and sulfoglyco-SL	thaw-mounted, thin tissue slices of rat brain	Isocratic/VIS: orcinol	Detailed identification by DESI-MS	Identification of species directly from tissue slices by DESI-MS, including GD1a and GD!b isomers	[49] Wiseman
SL profiles	Sera from normolipidemic normal weight (NW), vitamin D deficient dyslipidemic normal weight (vitDNW), and vitamin D deficient dyslipidemic obese (vitDO) men and women	Isocratic/FL: primuline	HPTLC-primuline profiling was used for SQ purposes	Changes in ceramides and SM levels among NW, vitDNW, vitDO	[85] Al-Dahgry[86] Torretta

[a]Gradient AMD.

Table 3. HPTLC-SD of NL and FA from different samples.

Involved subclasses	Original samples	Type of development/Staining systems	Type of analysis	Biological or analytical significance	[Reference] author
MG, DG, TG, FFA	E471 emulsifiers	Sequential/FL: primuline	SQ with External calibration	1,2-distearin as standard. Response factor system. Accurate and sensitive method	[87] Oellig
MG, DG, TG, FFA, fruit acid esters, free fruit acids	E472 emulsifiers	Sequential/FL: primuline	Fingerprint videodensitometry. Coupling to ESI-MS (interface)	Fingerprint approach to composition of different samples, batches and E472 categories with identification of constituents	[88] Oellig
Lupeol, β-sitosterol	Stem bark of Bombax ceiba	Isocratic/VIS: anisaldehide-H_2SO_4	Quantitative, External calibration with target standard	Osteogenic activity study	[52] Chauhan
MG, DG, TG, FFA	Biodiesel samples of rapeseed oil	Sequential/VIS: $KMnO_4$-NaOH	Comparative	Visual inspection	[46] Fedosov
MG, DG, TG, FFA	Transesterified refined soybean oil	Isocratic/FL: primuline	Comparative	Estimation of esterification reaction	[89] Chattopaday
Chol, CholE, FFA	*In vivo* skin surface	Sequential (17 steps)[a]/VIS: $CuSO_4$-H_2SO_4	SQ with External Standard	Profiling of Stratum Corneum lipids	[37] Opitz
α-Linolenic (18:3), Eicopentaenoic (20:5), docosahexaenoic (22:6) acids (ω-3 FA)	Market dietary supplements and cooking products	Isocratic/VIS: iodine	Quantitative. External calibration using target standard	Validation according ICH guidelines	[45] Dabrowska
Lupeol, β-sitosterol, oleanolic acid	*Leptademia pyrotechnica* plant	Isocratic/VIS: *p*-anisaldehyde-H_2SO_4	Quantitative using target External calibration	Botanical source of ayurvedic drug Jivanti	[90] Preet
Free sterols, FFA, TG, Methyl Esters, Steryl Esters	Pitcherplant mosquitoes (*W. smithii*)	Isocratic/VIS: PMA	SQ with External Standard calibration	Effect of diapause on NL	[27] Counihan
Vitamin D3	Purified fish oil	Isocratic/UV280 nm	Quantitative. External calibration using target standard	Validated method for Vitamin D3 quantification	[10] Demchenko

Involved sub-classes	Original samples	Type of development/Staining systems	Type of analysis	Biological or analytical significance	[Reference] author
NL, FA profiles	Microalgae, higher plant cells	Isocratic; 2D/UV: 2% 8-anilino-naphtalenesulfonic acid in MeOH	Scraping off (for GC)	Proposal of a lipid fraction, previously analyzed by HPTLC-GC, as external standard	[91] Jouhet
MG	FAME-biodiesel	Sequential (3 steps)[a]/FL: primuline	Coupling to ESI-MS (interface)	Profiling and characterization	[92] Jarne
MG, DG, FFA	FAME-biodiesel	Sequential (4 steps)[a]/FL: primuline	Coupling to ESI-MS/MS, HR-MS (interface)	Profiling and structural identification of molecular species	[13] Jarne
Estrogen-effective E1, E2, E3, EE2	Food samples	Isocratic/UV; FL: Neu's reagent; EDA: HPTLC-pYES	QualitativeCoupling to ESI-MS (interface)	Information of estrogens	[47] Morlock
MGDG, DGDG (glycol-glycerolipids)	*Erigeron canadensis* plant	Isocratic/VIS: H_2SO_4-MeOH	SQ with external calibration	MGDG and DGDG semiquantification at E. Canadensis collected at different stages of growth	[41] Ellnain
FFA	Lipids extracted from liver of mice	Isocratic/UV254 photographic image	QualitativeCoupling to EASI-MS	Distinct free fatty acid profile in thelivers of genetic hypertriglyceridemic mice with regard to control normotriglyceridemicmice	[93] Alberici

[a]Gradient AMD.

Table 4. HPTLC-SD of total lipid classes from different samples.

Involved sub-classes	Original samples	Type of development/ Staining systems	Type of analysis	Biological or analytical significance	[Reference] author
NL, FA, SL, PL	Urinary extracellular vesicles	Sequential/iodine vapor	ComparativeScraping off (MALDI)	Discrimination of urinary exosomes from microvesicles. SL profiles only detectable in exosomes	[94] Singtho
TG and others sub-classes	Human skin	Sequential/VIS: CuAcO-H_3PO_4	Comparative	Level of TG with severity of ichthyosis	[30] Ujihara
Free sterols, FFA, TG, SterylE, PC, PE, PI	*Sacaromyces cerevisiae* yeast	Isocratic/VIS: PMA; $CuSO_4$-H_3PO_4	SQ with External calibration	Changes in lipid content between strains possessing or lacking [RNQ^+] prion	[25] Bui
Polar and neutral sub-classes	*Rhodosporilium toruloides* yeast oil	Sequential/FL: primuline	SQ with External calibration	Manothermosonication is useful for lipid extraction	[95] Meullimiestre

Involved sub-classes	Original samples	Type of development/ Staining systems	Type of analysis	Biological or analytical significance	[Reference] author
Cer, Chol, PA, CholS	Model membrane from lipid mixtures	Sequential (16 steps)[a]/ VIS: $CuSO_4$-H_3PO_4	SQ with External calibration	Model lipid membranes for studying impact of Cer on drug diffusion and penetration	[36] Ochalek
Total lipids	Cells from *Haloferax volcanii* strain WR-340	Isocratic/VIS: H_2SO_4	ComparativeScraping off (MALDI)	Lipids in this Archaeon as a cell host system for producing mammalian olfactory receptors	[42] Lobasso
Total lipids	Pig olfactory epithelia and cilia extracts	Isocratic; 2D/VIS: H_2SO_4; Mo-Blue	QualitativeScraping off	Descriptive analysis	[22] Lobasso
SM, PC, PS, PE, DG, Chol, Cer, PI, FA	Neutrophils from blood	Sequential/VIS: $CuSO_4$-H_3PO_4	Coupling with ESI-MS (interface)	Lipid alterations in neutrophils lead to formation of Neutrophil Extracellular Traps (NETs)	[38] Neumann
Cer, Chol, FFA	Stratum corneum	Sequential (11 steps)[a]/ VIS:$CuSO_4$-H_3PO_4	SQ with External standardCoupling with ESI-MS (interface)	semiquantification of ceramides, cholesterol, and free fatty acid in Stratum corneum	[40] Jamin
Neutral (free sterols, FFA, TG) and polar (PC, PE) lipids	Biomphalaria glabrata snail[b]	Isocratic/VIS: PMA; $CuSO_4$-H_3PO_4	SQ with External standard	Schistosoma mansoni infection and temperature had deleterious effects in lipid metabolism	[28] Hunsberger
Polar and neutral subclasses	Mice liver, spleen and intestine	Isocratic/VIS: PMA	SQ with External calibration	Effects of *Schistoma mansoni* infection	[29] O'Sullivan
Phytosterols, phenolic lipids	Marine algae and seagrass	Isocratic/EDA: DPPH radical; α-amylase; acetylcholine esterase (AChE) VIS: *p*-anisaldehyde-H_2SO_4; Fast Blue B;	Identification of bioactive lipids	Presence of lipids. EDA for antioxidant, α-amylase and AChE inhibitory activities	[53] Agatonovic-Kustrin
TG, squalene, PL	Lipids from Yeast strain expressing Diacylglycerol acyltransferase (DGAT2)	Sequential/VIS: PMA (5%)	SQ with External calibration	DGAT proteins are associated with these lipids. The FA composition was affected by the nature of the acyltransferase expressed	[96,97] Aymé
Chol, FA, sulfatides, PE, PI, PS, PC, SM, ganglioside	Brain porcine extract	2D-separation/FL: primuline	Direct identification with DESI-MS	Reactive-DESI using betaine aldehyde for Chol identification. DESI-based molecular imaging of lipid subclasses	[57] Paglia

Involved sub-classes	Original samples	Type of development/ Staining systems	Type of analysis	Biological or analytical significance	[Reference] author
CholE, Chol, wax esters, TG, PE, PG, PI, PS, PC, SM	Zebrafish eleutheroembryos (ZE) exposed to different bisphenol A concentrations from day 2 to day 6 postfertilization	Sequential/VIS: 3% $Cu(AcO)_2$-8% H_3PO_4	Relative lipid intensity (normalization of each subclass	Together with other techniques, HPTLC contributed to demonstrate that differences in some subclasses with time and BPA-related patterns depended on the unsaturation degree (mostly DG and PC) and fatty acid chain length (mostly DG and PC)	[98] Martínez
Sterols, PL, SL	Extracts from wild-type and spf1 cells (*Saccharomyces cerevisiae)*	Isocratic for sterols; Isocratic for PL; 2D for PL/FL: primuline/RD [^{32}P] [^{3}H] labels (see text)	Absolute quantification of ergosterol and lanosterol using external standard calibration	Contribution to the elucidation of the physiological role of Spf1p, a P5A ATPase, an enzyme which are involved in regulation of homeostasis in the endoplasmic reticulum	[99] Mollerup

[a]Gradient AMD.

The type of information obtained and the biological and/or analytical significance of the results derived from HPTLC-SD are given in Tables 1–4. Selected examples are detailed in the text. Details on development conditions for each example are provided in the Supplementary Information (Tables 1S–4S).

SD has sometimes been the only detection technique used for analysis[20,29,30,46,54,68,89] by UV and/or FL. Alternatively, preparatively isolated bands have also been scraped off the stationary phase and usually characterized by other techniques (see subsection "Scraping off").

Comparative, qualitative analysis and fingerprinting

Densitometry has often been used for qualitative characterization of lipids in descriptive works, or for obtaining comparative information on the effect of sample-related variables when experiments were compared under given HPTLC conditions. In these cases, identification of lipids has usually been done on the basis of m.d. (in mm) or retardation factor (hRf = 100 × band m.d./solvent front m.d.), using standards.

An illustrative example of descriptive use of SD is the profiling of PC and PE hydroperoxides in enzymatically and thermally oxidized egg yolk.[20] They were obtained using HPTLC with N,N-dimethyl-p-phenylenediamine staining. Profiles were in reasonable agreement with results from a database created from a two-step LC-MS based method which was designed for qualitative assessment of oxidized phospholipids (oxPL) in foods.

Another example is the work on the involvement of an endoplasmic reticulum (ER)-mitochondrial encounter structure (ERMES), a complex that bridges ER and mitochondria, in phospholipid transport in yeast.[31,100] Authors showed that a conserved ERMES domain preferentially binds PC. Phospholipids bound to MdmM12, one of the ERMES proteins, were separated by HPTLC using a classic two-step sequential development and detected by UV densitometry after charring using a 10% (w/v) solution of $CuSO_4$ in an 8% aqueous solution of phosphoric acid (145 °C, 4.5 min). PE and PG were identified, on the basis of m.d. of standards, as the primary phospholipids co-purifying with Mdm12. No direct HPTLC-MS coupling was used. Results were validated using parallel, independent ESI-MS measurements from extracts of isolated proteins.

An HPTLC-based exchange in-vitro assay using liposomes to mimic biologic membranes was also developed in the same work in order to monitor the lipid exchange activity of Mdm12, after incubation with liposomes. PL were separated, detected and identified under the previously described HPTLC-densitometry conditions, and the result was that Mdm12 exchanges nearly all its endogenously PG and PE with PC and PI extracted from the liposomes.

Videodensitometry has been used for developing a sensitive and selective method for fingerprinting comparison of samples, batches and categories of E472 emulsifiers, food additives.[88] Categories involves MG and DG of fatty acid

esters from acetic (ACETEM), lactic (LACTEM), citric (CITREM), tartaric (TATEM), mono- and di-ethyl tartaric (DATEM), and mixed acetic and tartaric (MATEM) acids. After separation, post-impregnation of the plate with primuline and illumination under 366 nm on a visualizer allowed an evaluation of the emulsifiers composition of several samples on the same plate in a single run.

Semi-quantitative and quantitative analyses

Absolute quantification of a lipid by densitometry was achieved in certain samples when it was possible to separate the target and its related pure standard was available, as in the cases of lupeol and *β*-sitosterol in stem bark of Bombax ceiba plant in petroleum ether and MeOH extracts.[52] These analytes, together with oleanolic acid, were also quantitatively determined in *Leptademia pyrotechnica* bacterium.[90] In this case, Linear range was 2–10 µg, with LOD = 0.41 µg (β-sitosterol); 0.55 µg (lupeol); and 0.30 µg (oleanolic acid).

Likewise, eicosapentaenoic (20:5, EPA) and docosahexaenoic (22:6, DHA) ω-3 fatty acids were accurately identified and quantitatively determined in a collection of Polish market dietary supplements and cooking products.[45] Vitamin D3 was also quantified in purified fish oil, and the on-silica UV spectrum from the sample was superimposed to this standard.[10] In all cases, appropriate standards were used for both characterization of migration distance (Rf) and for external calibration, using the Area of peaks.

However, as a substantial part of compounds in lipidomics have not yet been identified, it is not possible to have standards for all possible unknown compounds. Thus, a semi-quantitative (SQ) approach make sense for untargeted lipids, which is based on the idea that any unknown analyte can be quantified using a different known analyte as calibrant, assuming a certain degree of uncertainty. For a given detection system, the Response Factor (RF) of a compound depends on chemical structure. Based on this approach, the error will be directly proportional to the difference in RF between the standard and the analyte.[101,102]

When separations of lipid-samples are performed on silica gel HPTLC plates, lipids are grouped in sub-classes according to their polar head, each sub-class including species that only differ in their fatty acid chain lengths. This makes the SQ approach suitable for lipids in HPTLC-UV/FL densitometry. Therefore, a suitable standard (or standard mixture) may be selected per sub-class which minimizes the difference in RF between it and the untargeted lipids for a given detection system. Analysis reliability strongly depends on the choice of an appropriate standard. It is in that sense that we have used in Tables 1–4 the term SQ. Semi-quantitative and quantitative analysis have often been frequently misused in the literature when analyzing profiles that include untargeted lipids.

SQ-related error may be assumable taking into account that uncertainties around 10% were considered acceptable in lipidomic ESI-MS quantification,[103] or between 5% and 15% (or even greater) for most lipid species analyzed in exosomes.[104,105] Errors come from sampling, sample preparation, use of normalizers, internal standards, and analysis itself, affecting quantitation in determining a particular category of lipids.

External calibration for SQ analysis using densitometry has usually been performed using representative standards of the corresponding lipid sub-class.[10,19,27,28,34–37,40,46,52,73,79,85,86,95–98]

A response factor system for quantitation was employed for a rapid and sensitive determination of MG, DG, TG and FFA in E471 emulsifiers by HPTLC with fluorescence densitometry using plates pre-chromatographically impregnated with primuline.[87] Calibration was done with 1,2-distearin and the amounts of lipid classes were calculated using response factors. Taking into account the response factors for the respective 18:0 MG, DG, TG and FFA, the lipid class quantities of the simulated emulsifiers were calculated as 18:0 fatty acid (ng/zone) and expressed as monostearin, distearin, tristearin and stearic acid, applying the molar conversion factors. Limits of detection and quantitation were 1 and 4 ng/zone, respectively, for 1,2-distearin.

According to Bui et al., HPTLC-densitometry is a reproducible and accurate method for quantifying lipids in yeast cells.[25,26,107] Thus, the influence of the prion [RNQ^{+}] and its determinant prion protein Rnq1 on yeast lipid profiles (*Saccharomyces cerevisiae*) was studied. Using different development schemes, free sterols, FFA, TG, SterylE, methyl esters, Sq, PC, PE, PI were separated. Quantifications of relative levels of free sterols, FFA, and TG were done using 5% PMA in ethanol and UV at 610 nm. In a similar way, SterylE, methyl esters and Sq; and PC, PE, PI were

detected using 10% cupric sulfate in 8% phosphoric acid at 370 nm. Calibration was performed using standards from the different classes involved, and polynomial regression calibration curves were used.

Calibration by standard addition has scarcely been used in HPTLC-densitometry, although it is usually a recommended method for complex samples that contain a low concentration of target analyte, such as biological samples. Thus, an accurate semiquantitative determination of sphingomyelins (SM) in human plasma using SM (d18:1/16:0) as standard was carried out.[12] This was done after a 2-step HPTLC-AMD separation of plasma samples and detection of separated SL by primuline-fluorescence densitometry. The selected SM standard was also used to plate-to-plate correction of fluorescence signal. Operationally, six plasma calibration solutions with different SM standard concentrations on the plasma sample were submitted to the corresponding preparation procedure, and, then, were employed for calibration. Standards and samples should undergo the same treatment and analysis conditions in order to minimize errors in the determination of lipids in biological samples. This is particularly important in the case of SLs since their analysis usually includes a previous step of PL removal.

Scraping off

It is usual to scrap off the band on the plate to preparatively isolate a lipid class in order to characterize their molecular species, or to control its purity. This involves filtering, solvent removal and drying. The procedure is tedious, time consuming and yields a poor yield, mainly due to experimental losses.

ESI-MS was used for structural identification of isolated fractions.[72,80,81] HPTLC profiling, scraping off and off-line, direct infusion MS revealed that there were major differences in the lipid content across the three mammalian cell lines (mouse myeloma SP2/0, Chinese Hamster Ovary CHO, and human embryonic kidney HEK-293).[54]

Differential lipidome profiles of exosomes vs urinary microvesicles, potential biomarkers in various kidney diseases, were obtained by scrapping off in combination with MALDI.[94]

Profiling of PL, and identification of unique cardiolipin and monolyso-cardiolipin species in *Acinetobacter baumannii*, an opportunistic human pathogen which is responsible for an increasing number of nosocomial infections and exhibits broad antibiotic resistances, was obtained using HPTLC and off-line MALDI-MS.[18] PL classes were separated with chloroform/methanol/acetic acid/water (85:15:10:3.5, by volume), and the following staining systems were used: (1) spraying the plate with sulfuric acid in water; (2) molybdenum blue spray reagent for phospholipids; and (3) ninhydrin solution, for phosphatides or lipids having a free amino group; (4) iodine vapor was used in a parallel plate for visualizing lipids before recovering them from the scraped silica. Using this combined technique, similar lysocardiolipin levels were found in the two clinical strains *A. baumannii* ATCC19606T and AYE whereas in the nonpathogenic strain *Acinetobacter baylyi* ADP1 lysocardiolipin levels were highly reduced.

Other off-line MALDI examples can also be found.[19,21,22,42,43,48,73,74,76,77,82] Moreover, scraping off was used with other characterization techniques, such as gravimetry,[44] or GC.[91] In this case, glycerolipids from three different types of cells, two microalgae (*Phaeodactylum tricornutum*, *Nannochloropsis gaditana*) and one higher plant (*Arabidopsis thaliana*) were separated using a 2 D-scheme, and detected by UV densitometry after spraying the plate with 2% 8-anilino-naphtalenesulfonic acid in methanol.[91] The lipids were scraped off the plate in a parallel plate. Lipids were quantified by methanolysis and GC-FID directly from the scraped silica. Authors proposed to use a lipid extract from a qualified control (QC) of each type of cells, previously analyzed by TLC-GC, and used it as an external standard to quantify the MS results.

SD and Effect-Direct Analysis (EDA)

A strong point of the planar format of HPTLC analysis is the possibility of combining SD with on-plate biological (cell-based)/biochemical (enzymatic) and chemical staining assays, usually for target-directed identification of biologically active molecules in complex samples. This is referred as to Effect-Direct Analysis (EDA) which can be performed on a chromatographic plate because the mobile phase can be easily removed after plate development and before bioassay application.

EDA has been developed for a variety of assays and samples, although their application has been scarce for lipids. In a search of bioactive compounds in 19 marine algae and 1 seagrass, a battery of microchemical (DPPH•, i.e., 2,2-Diphenyl-1-picrylhydrazyl radical, *p*-anisaldehyde-H_2SO_4, and Fast Blue B) and biochemical (α-amylase and acetylcholine esterase (AChE) enzymatic) assays was developed, by dipping the plates in the corresponding solution after

separation of bands.[53] This allowed to evaluate antioxidant activity (DPPH•), presence of phytosterols (p-anisaldehyde-sulfuric acid), phenolic lipids, i.e., alkyl resorcinol derivatives (Fast Blue), α-amylase and AChE inhibitory activities of separated bands.

Antioxidant activities in the samples were found to be correlated to phytosterol content. This was expressed in *β*-sitosterol equivalents, from a calibration curve (*β*-sitosterol with *p*-anisaldehyde-sulfuric acid; linearity: 0.5–5.0 µg/band). LOD and LOQ were 0.5 and 1.6 µg, respectively.

α-Amylase inhibitory activities were correlated to AChE inhibition and also attributed to the phytosterol content. Some samples were found to contain phenolic lipids with significant antioxidant activities.

Detection of lipid-related endocrine disrupting compounds (EDC), such as E1, E2, E3, EE2 in food as sharp-bounded zones by the combination of reversed-phase HPTLC with detection by specific microorganisms was reported.[47] For performing the bioassay (HPTLC-pYES), the plate was immersed into a suspension of recombinant Saccharomyces cerevisiae cells which carry the DNA sequence of the human estrogen receptor (hERα) together with the reporter gene lac-Z. Endocrine disrupting compounds induced lac-Z gene expression, which encoded ß-galactosidase, reacting at pH >7 with 4-methylumbelliferyl-ß-D-galactopyranoside (MUG) to produce 4-methylumbelliferone (MU). This was detected by densitometry at UV 366 nm as blue fluorescence. Consequently, EDC are detected as a blue fluorescent MU zone, and their identity can also be elucidated by direct coupling to MS from the zone of interest on the plate.

SD and radio-densitometry (RD)

Radioactive isotopes led to the development of a nuclear-based technology, usually referred to as Thin-Layer Radiochromatography.[108] RD has been used for HPTLC-lipid detection in bioresearch, often as unique detection technique, without SD.[109–117] Due to considerations of safety, need of skills in nuclear methodology, and equipment cost, its implementation has been restricted to the laboratories working in nuclear techniques.

Combination of SD and RD has proved to be useful in the fields of biochemistry and molecular biology. As early as 1998, Kobayashi et al.[118] published a landmark paper that demonstrated that lysobisphosphatidic acid (LBPA) is one of the physiological antigens that is recognized by the sera of patients with antiphospholipid syndrome, that it is found in high concentration in the inner membranes of endosomes, and regulates their structure and function. HPTLC was key in obtaining some of these conclusions. A versatile use of HPTLC included: LBPA 2 D-separation using ^{32}P-labeled PL; preparative isolation of LBPA; control of its purity by phosphate staining; verification of products from on-plate mild alkaline methanolysis of ^{32}P-labeled LBPA (glycerophosphorylglycerol, bis(glycerophosphoryl)glycerol, glycerophosphate); and on-plate biological assays (resistance to phospholipase-A and negative derivatization against ninhydrin).

More recently, the physiological role of Spf1p, a P5A ATPase, an enzyme which are involved in the regulation of homeostasis in the ER, was recently studied.[99] In the absence of one of P5 ATPase gen of yeast *Saccharomyces cerevisiae*, cells accumulate sterols at the plasma membrane and have disturbances in ergosterol homeostasis, and these changes affect protein function in the ER and plasma membrane. There is also an increase in the total sphingolipid content. HPTLC experiments allowed to obtain PL and SL profiles and concluded that lanosterol content decreased, whereas ergosterol content increased in spf1 cells.

Backman et al.[32] showed that GSL production in cells can be followed using metabolic labeling with radiolabeled lipid precursors and that this is an indirect measurement of lipid transfer protein activity. Sphingolipid labeling in cells with previously synthesized ^{3}H-sphinganine, ^{3}H-sphingosine, and ^{3}H-palmitic acid was used. Different mobile phase systems were used for separating GSL (Table 2-SI). Therefore, the products from the cellular lipid metabolism can be quantitatively analyzed with HPTLC-RD and SD, and this work is an excellent example of combination of detection techniques: orcinol for glycosphingolipids; iodine or copper acetate in 8% phosphoric acid for PL; and primuline in acetone/water (4:1, v/v) for general lipid detection. The quantitative estimation was done from the scraping off the silica spots into a scintillation fluid and the radioactivity was measured using a liquid scintillation counter. Likewise, bands were scrapped off and submit to immunoblotting for glycosphingolipid identification.

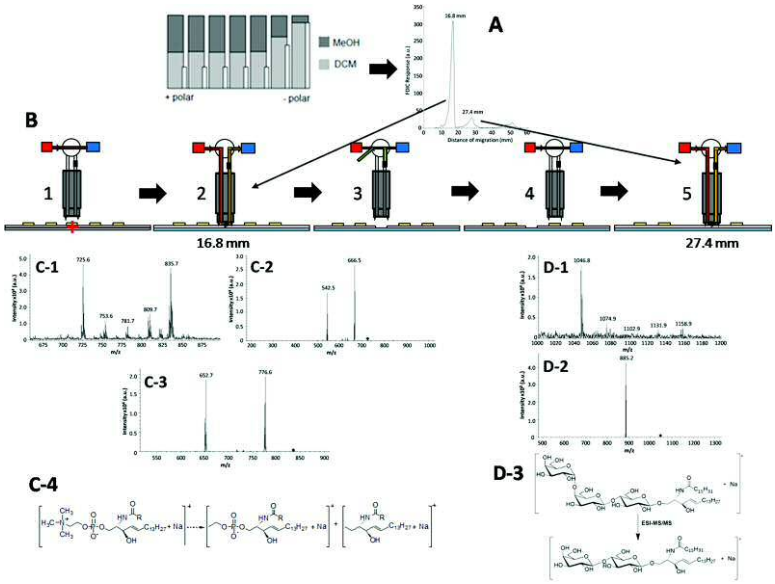
Coupling HPTLC with MS

Extraction-based interfaces

Once the lipid sample is separated on the plate, the mobile phase removed, and the separated bands detected, they can be located on the dry plate, extracted using an appropriate solvent, and directly transferred from the plate to MS equipment through a modular TLC-MS interface. A review on its general application has recently been published. [119] They are commercialized by CAMAG (TLC-MS Interface) and Advion (plate Express).

TLC-MS interface operates as follows: based on SD, an oval or circular elution head is positioned and lowered on the peak. Solvent is pumped through the elution head. The band is subsequently dissolved and extracted via inlet capillary. Silica gel is filtered and the eluate is then directed via the outlet capillary into any mass spectrometer. The operation is completed in less than 1 minute. Using this interface, a hands-free transfer of the zone of interest in the HPTLC chromatogram into the MS was enabled, making it possible the direct identification of lipid species (Figure 2).

Figure 2. (A) AMD separation sequence and densitogram (UV 190 nm) of a plasma extract of neutral sphingolipids. (B) Automatic extraction of HPTLC bands using TLC-MS interface. Blue: HPLC pump for MeOH delivery (0.2 ml/min); red: MS equipment; black: frit for silicagel filtering; +: laser crosshair. Way of operating: (1) bypass; (2) band extraction (16.8 mm); (3) air cleaning; (4) bypass; (5) band extraction (27.4 mm). (C) Sphingomyelin molecular species. HPTLC-ESI(+)-MS spectrum (C-1) of band at 16.7 mm, which most abundant ions were fragmented to obtain the respective HPTLC-ESI+-MS/ MS spectra of the precursor ions at (C-2) m/z 725 and (C-3) m/z 835, confirming SM(d18:1;C16:0) and SM (d18:1;C24:1) structures, respectively. (C-4) General scheme of SM fragmentation in trimethylamine and phosphocholine groups. (D) Gb3 isoforms. HPTLC-ESI(+)-MS spectrum (D-1) of band at 27.4 mm shows sodiated adducts d18:1;C24:0 (m/z 1158), d18:1;C22:0 (m/z 1131), d18:1;C20:1 (m/z 1102), d18:1;C18:0 (m/z 1074). The most abundant ion at m/z 1046 was fragmented (D-2) to obtain the HPTLC-ESI+-MS/MS spectrum of this precursor ion, showing the loss of a hexose. This confirms Gb3 (d18:1;C16:0) structure. (D-3) Scheme of the latter Gb3 fragmentation.



An advantage of this interface is its independence from the mass spectrometer. Any MS equipment can be connected to it. Other detection systems may be connected, either directly or through off-line extraction into vials. Unlike desorption-based techniques, the interface does not allow continuous scanning along the plate track. However, it allows a direct and on-demand access to hot zones on the plate.

Liquid Extraction Surface Analysis (LESA) is another approach which is based on the formation of a stable wall-less liquid microjunction between the analyte extracted on the HPTLC surface and a solvent pipette tip within a small distance. Subsequent to the analyte extraction, the solvent-containing pipette tip is moved to a nanoelectrospray infusion chip and the sample solution is sprayed into the MS equipment.[120] In the case of lipids, the use of LESA has

been scarce. When used in combination with silica gel plates, a coating of Carfa Magic Silicone oil must be sprayed before MS.[121] It has been limited to hydrophobic RP-C8 and C18 HPTLC plates.[122] Some TG were not detected because solubility of very apolar lipids is limited in the LESA extraction solvent. The necessity of a liquid junction formation and a certain amount of water in the extraction system results in a limitation for lipid analysis. This technique has potential for medium-polar lipids.

Ionization of lipids: APCI, ESI-MS

The interface has been mainly used in combination with APCI or ESI for lipid analysis. Molecular species of sodium adducts of SM species were identified from the plate by comparing ESI-MS and APCI-MS spectra zones with those of a selected SM standard. Although this method was useful, it did not provide an unequivocal identification of the species.[12]

It is usually accepted that sodium adducts give poor fragmentation using tandem mass spectrometry techniques and consequently little information on their fragmentation pathway can be obtained by MS/MS. For this reason, obtaining MS/MS spectra by tandem techniques directly from the chromatographic plate has been a very little used resource so far. Recording of MS/MS spectra of lipids had been hampered by the ubiquitous presence of sodium ions during the chromatographic process, and their coexistence with protonated ions. This may cause a poor fragmentation of the isolated precursor ions, which complicates or prevents the structural interpretation of the product ions. However, it was possible to obtain working conditions so that the sodium adducts from a wide variety of lipids are stable and can be fragmented in positive ESI mode directly from the plate.[10] This was useful for unequivocal structural identification of lipid species from complex samples as sodium adducts by ESI^+-MS/MS, such as MG and DG in a FAME-based biodiesel, as low-concentration impurities. Fatty acids were identified using ESI in negative mode, ESI (−).

Likewise, fruit acid esters of MG and DG present in E472 emulsifiers were identified by ESI (+)-MS using the interface. Obtained adducts were $[M + Na]^+$, $[M-H + 2Na]^+$, and $[M + NH_4]^+$, the latter due to the presence of formic acid in the development solvent.[88]

Molecular species of SM and Gb_3 were unequivocally identified in human plasma samples by ESI(+)-MS/MS.[13] Analytical conditions were also compatible with ionization in negative mode (ESI^-) for lipid classes that did not show an adequate ionization in ESI(+). For example, ceramides from stratum corneum were separated and ionized in negative mode. MS/MS complex fragmentation patterns of standards and the HPTLC-separated ceramides were explained through charge-driven fragmentation, i.e., generation of fatty acid-related fragment ions fragment ions via stepwise pathways involving deprotonated CER isomerization into an ion-dipole complex prior to dissociation.[40]

Simultaneous detection of neutral, acidic and sulfo-GSL using positive and negative ESI modes was performed in sperm from different freshwater fishes.[60]

The use of the interface does not produce loss of sialic residues that frequently occurs in the structural analysis of GSL species by MALDI.[83]

Concerning PL, HPTLC-ESI (+)-MS spectra of PC and CL also mostly provided sodium adducts, $[M + Na]^+$ and $[M-2H + 3Na]^+$ respectively, and the corresponding molecular species were identified by MS/MS.[14] However, positive ionization for PE was not achieved. Likewise, ESI (−)-MS and MS/MS spectra of CL were also obtained as $[M-2H]^{2-}$, as well as those of PE and PG species as $[M-H]^-$ and $[M]^-$, respectively. The same ions were obtained for the corresponding PL separated from bacterial extracts. In other work[71] Krüger et al. reported that PC and PE ionized as $[M + Na]^+$ and $[M+2Na-H]^+$.

In general, MeOH was used as eluant for interface operation at a flow rate of 0.1 ml/min. Adding 5 mM ammonium acetate to MeOH, extracted lipid bands from different classes were analyzed by ESI-MS/MS using a hybrid triple quadrupole LIT (linear ion trap) mass spectrometer.[38] Thus, fatty acyl species in subclasses were determined in the positive (SM, PC, PS, PE, DG, Chol, Cer) or the negative (PI, FA) ion mode by specific precursor ion or neutral loss scans. Precursor ions were monitored for the determination of SM and PC (*m/z* 184), for cholesterol and CE (*m/z*

369), and for PI subspecies (*m/z* 241). A neutral loss of 185 was selected for the determination of PS species, a loss of 141 for PE species, and a loss of 35 for the analysis of DG species. Fatty acids were analyzed in the full scan mode.

SD and MS coupling

Several separation methods were developed to directly transfer the bands separated on HPTLC to MS, via the interface.[12–14,38,39,47,60,71,92]

Sphingolipids

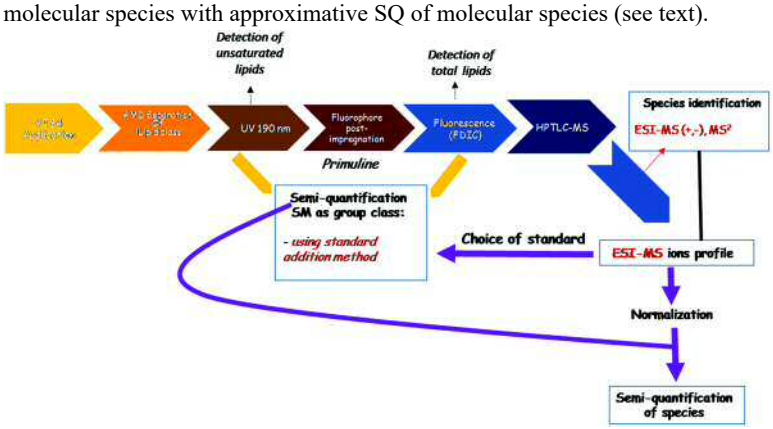
Some developed methods were based on AMD. Two of them[39,40] were detected at 450 nm after charring with copper sulfate-phosphoric acid reagent. Another[12] used primuline-induced fluorescence or UV at 190 nm. In all cases, a twin plate was used under the same chromatographic conditions for HPTLC-MS coupling with the interface.

Ten CER subclasses Chol and FFA were separated on LiChrospher HPTLC silica gel plates by AMD using an 11-step gradient elution based on mixtures of chloroform, acetone, and methanol-water, with previous AcH plate preconditioning.[40] Ceramides (CER) subclasses are integral parts of the intercellular lipid lamellae of the stratum corneum and play an important role in the barrier function of mammalian skin. They are based on the sphingoid base and fatty acyl chains. Base may include Sphingosine (S), phytosphingosine (P), 6-hydroxysphingosine (H), or dihydrosphingosine (dS). Fatty acyl chains may be non-hydroxylated (N), α-hydroxylated (A), ω-hydroxylated (O). The latter may be esterified to a fatty acid (E). Ceramide analyzed include: AH, AP, NH, EOH/AS, AdS, NP, OS, EOP, NS/NdS, EOS. AS was chosen as external standard for CER semiquantitative analysis.

The combination of semi-quantitative SD analysis of a given lipid class using a representative standard and its ESI-MS profiling allows semi-quantitative analysis of molecular species. A SQ analysis of SM subclass was performed on silica gel HPTLC plates by densitometry (UV190), using SM (d18:1/16:0) as standard.[12] From ESI-MS profiles of SM, a normalization of ion species in ESI-MS profiling can be done because interface solvent was removed before MS analysis.[123] Thus, the obtained ESI-MS spectrum is a representative profile of individual molecular species for a given lipid-class because, in ESI ionization, response factors in MS are similar for molecular species belonging to a given lipid class. It is the corresponding class-polar head that defines ESI response in lipids, and the aliphatic chain length does not modify it substantially.[124]

In HPTLC-MS experiments, unlike LC-MS, the mobile phase (either a gradient or a mixture of solvents) is evaporated before detection, and peaks are extracted and sent to MS equipment through the interface. This eluting solvent is a pure solvent or has a constant composition and, in this way, similar ionization efficiencies are obtained for the individual lipids of a given separated class on silica gel plates. This allows to perform a semi-quantification of each SM species. In addition, the information provided by ESI-MS profiles can be of help to improve the previous selection of representative standard needed for SQ. This feedback may be useful to improve SQ analysis[123] (Figure 3). Other works state that differences in the relative signal intensity for the diverse constituents (with different FA) represented their relative quantity.[57,88]

Figure 3. Scheme of a lipid-class analysis which involves SQ analysis by densitometry using an external standard; identification of molecular species by HPTLC-ESI-MS, MS/MS; using ESI-MS profile for: selecting an adequate standard; and for normalizing molecular species with approximative SQ of molecular species (see text).



Neutral lipids and fatty acids

In a similar way, MG impurities were determined in FAME-biodiesel.[13,92,123] FAME can be used alone or mixed with petroleum diesel, and contains lipid impurities that affect motor performance, such as MG, FFA, DG. For example, MG can produce obstruction in fuel filters. SQ of MG subclass in FAME was performed by fluorescence densitometry using primuline-impregnated plates, and 1-oleoyl glycerol as a representative standard. MG and FA species were identified by ESI-MS (+ and −, respectively) and high resolution MS. MG species were semi-quantitatively determined by combination of SD (fluorescence in primuline) and the ESI-MS profile.

An approach based on the HPTLC-SD-Interface-ESI-MS coupling and HPTLC-bioassay was reported to obtain an effect-directed profiling of endocrine disruptors in food.[47]

Neumann et al. demonstrated that cholesterol alteration in human blood-derived neutrophils lead to formation of NET (Neutrophil Extracellular Traps), a host innate immune defense mechanism.[38] They obtained comparative profiles of cholesterol and different classes of PL, SLs and other neutral lipids by coupling HPTLC-SD to ESI-MS, MS/MS using the interface. SD was carried out using $CuSO_4/H_3PO_4$ (170 °C, 10 min), using a parallel plate.

Separated zones corresponding to fruit acid esters of MG and DG present in E472 emulsifiers were analyzed by ESI-MS using the interface. The combination of fingerprint by SD and ESI-MS characterization is a rapid and useful tool to relate composition and technical functionality of emulsifiers.[88]

In a work that studies the mechanism by which cholestasis impairs the storage of liver lipids in transgenic mice with surface protein of the hepatitis B virus, HPTLC-FL (primuline)-APCI-MS was used to monitor the levels of TG and FFA in liver lipid extracts.[8]

Phospholipids

The interface-ESI-MS was used for characterizing lecithins (PC and PE) from soybean and sunflower, raw materials for chocolate production.[71] SD-interface-ESI-MS allowed to obtain comparison of lecithin fingerprints. Primuline was used for an accurate semi-quantification of PC and PE by fluorescence SD, using as external standards PC34:1 and PE34:1, respectively. An Independent calibration was also performed using ESI (+)-MS. Limits of detection (LODs) and limits of quantification (LOQs) of seven PL were studied for the three employed detection techniques. Mean LODs ranged from 8 to 40 mg/kg for HPTLC-FLD and, using a single-quadrupole MS, from 10 to 280 mg/kg for HPTLC-ESI+-MS as well as from 15 to 310 mg/kg for HPTLC-FLD-ESI(+)-MS, after derivatization with the primuline reagent.

Molecular species of PC, PE, PG bound to membrane proteins in photosynthetic purple bacteria were identified even though the separation was not completely resolved.[14] PL bound to MP have influence on protein activity, and in protein crystallization for isolation of MP. Thus, photosynthetic membranes of *Rhodobaca (Rbc.) bogoriensis, Rhodobacter (Rb.) blasticus*, and *Rhodospirillum (R.) rubrum* were extracted using a special detergent, dodecyl β-maltoside (DDM). Extracts were further purified to obtain the Protein-Detergent-Lipid complex corresponding to *Rbc. bogoriensis*. A 7-step AMD gradient was used based on MeOH:water:Ethyl acetate (with plate conditioning using AcH) for separation. Bands were detected at 190 nm by SD and video-SD (UV366). Based on standard m.d., the respective zones corresponding to each PL subclass were selected and directly transferred to tandem ESI-MS via the interface.

On-plate matrix assisted laser desorption and ionization (MALDI)

In MALDI-MS, the plate is coated with a matrix, which absorbs the energy of laser irradiation and is necessary for the generation of ions. For lipid analysis, MALDI is frequently combined with time-of-flight (TOF) mass analyzer. For getting a clear idea of the evolution of MALDI in lipid analysis, please refer to the reviews published by Fuchs et al. in the 2009–2012 period, which were devoted to: the state of lipid analysis by HPTLC;[125] the use of MALDI-TOF in general lipid research;[126] the combination of MALDI-TOF with HPTLC;[127] and the analysis of PL and glycolipids by HPTLC-MALDI.[128]

HPTLC separation of lipid in classes allowed to conveniently analyze them by MALDI, resolving the difficulties in the analysis of acidic PL species due to ion suppression by PC species.[124]

Ionization and matrices

An advantage of MALDI is that it allows exhaustive structural identification of untargeted lipids which are ionized from the near-surface zone of a lipid-sample, previously separated into classes, by a continuous scanning along the plate track. A TLC-plate adapter and software were introduced in 2007 by Bruker Daltonics[61] for directly accomplishing qualitative structural characterization and screening of lipids on the plate.[21,23,51,58,59,62,65,71,75,78,84,117,126–132] In a similar way that MS imaging allows the recording of spatially resolved mass spectra from slices of biological tissues, MALDI has also been used to monitor the distribution of selected lipids on the HPTLC plate. For this, peak intensities, in dependence of the position, were converted into gray or a color scale.[75]

Although most HPTLC-MALDI studies were performed with UV lasers using a matrix, other alternatives were studied. Thus, GSL were separated on an HPTLC plate and transferred to a plastic membrane which was fixed on a MALDI adapter to be directly analyzed, in a process referred as to blotting.[133–137] Likewise, direct HPTLC-MALDI, without any matrix, were employed using an IR laser,[65,138–140] which offers the advantage of ablating more material per laser pulse on the order of a few micrometers in depth with regard to UV lasers, thus improving detection sensitivity.

In general, the selection of an adequate matrix for lipids is crucial and has been considered as an empirical, "trial and error" process. Criteria that MALDI matrices should fulfill and their pros and cons with regard to different lipid classes are summarized and discussed in the review by Leopold et al..[138] Some lipid classes are not detectable if the "wrong" matrix is used. A careful selection of matrix is necessary to overcome ion suppression effects.

The plates were usually sprayed or dipped with a relatively large amount of organic matrix which is usually overlaid through multiple deposition steps. The most popular matrix was DHB,[58,59,62,71,74–77,94,117] which allows detection of virtually all lipids from apolar to polar species, although it is not very sensitive and is a poor matrix for negative ionization.[138] Other employed matrices in HPTLC-MALDI were: 9-aminoacridine[18,19,21,22,42,127] which is the matrix of choice for detecting PE in the negative ionization mode; α-cyano-4-hydroxycinnamic acid CHCA;[51,77] 1,4,6-trihydroxyacetophenone monohydrate (THAP);[77] or glycerol.[65] Recently, an ionic liquid-stabilized nanomatrix was used for direct oligosaccharide profiling by HPTLC-MALDI.[129] This has interest for analyzing complex glycolipids, such as gangliosides. Spin-coating is one of the most common techniques for generating uniform thin films on substrates, the film thicknesses ranging from a few nanometers to a few microns. In this application, an ionic liquid (di-isopropyl ethylamine) provided good dispersion and stabilization for the spin coating of DHB-functionalized magnetic nanoparticles (DHB@MNP) on the plate. DHB@MNP is 2,5-dihydroxybenzoic acid conjugated Fe_3O_4 nanoparticle.

Because the sure presence of matrix and, potentially, of other staining, immunostaining or additive agents, the interpretation of MALDI spectra may not be easy as fragmentation is complex.[23,65]

Quantitative issues

Different lipid classes are detected in MALDI with different sensitivities.[59,65] An attempt was made to compensate for this by using calibration curves based on the intensities of the MALDI ion peaks.[59] Thus, calibration curves for Gb_3 (using the signal from C16:0 $[M + Na]^+$ ion peak), and for GM3 (using that of C16:0 $[M-H]^-$ ion peak) were done from their respective HPTLC bands. However, according to Schiller et al.,[139,140] the difficulty in obtaining quantitative information from MALDI is related to the current configuration of MALDI equipment, because the distribution of analytes is not homogeneous across the band. Therefore, the mass spectra produced are dependent on the position of laser irradiation zone which is significantly smaller that the TLC band. This is needed to offer increased resolution in MS imaging.

In almost all works reviewed, the relative intensity of each lipid species identified was normalized with regard to total intensity of all lipid species found in each sample. These results should be interpreted as comparative rather than as quantitative.[94]

Other aspects related to the quality of MALDI spectra were studied.[78,130–132]

Identification of sphingolipids

Usually, complex glyco-SL are separated on silica gel plates according to their polar groups and structural identification is done through MS identification of SL-fatty acid chains. Addressing glycan identification by HPTLC-MALDI may be another way of analyzing lipids such as gangliosides or sulfatides. However, this is not well established due to the difficulties in their separation caused by their high structural similarity and their poor detection due to their low ionization efficiency. Likewise, loss of sialic residues frequently occurs in structural analysis of GSL by MALDI, due to acidic matrix.[59,83] A method was reported that involves reversed-phase HPTLC and using *n*-propanol:acetonitrile:water (1:5:1, v/v/v) as mobile phase. The above mentioned DHB-functionalized magnetic nanoparticles (DHB@MNP) matrix was used for MALDI. Although isomeric glycans were not separated, the proposed method generated glycosidic and cross-ring cleavage ions, enabling on-spot structural elucidation of composition, sequence, branching and linkage of glycans in each separated spot, and allowed the identification of 25 oligosaccharides from human milk, as well as a rapid screening of other oligosaccharide-rich samples.[129]

Facing the problem of previous removal of PL for GSL analysis in total lipid extracts which were prepared from in vitro propagated human monocytic THP-1 cells, Kouzel et al. developed a method that combines on-plate enzymatically disintegration of PL by treatment of crude lipid extracts with phospholipase C, with a subsequent chromatographic development and overlay immunostaining detection of individual GSLs with a mixture of various anti-GSL antibodies.[65] This sample pretreatment provided enhanced detection sensitivity of GSLs and allowed for their structural assignment directly from the plate by infrared (IR)-MALDI MS and MS/MS.

GSL as GlcCer, LacCer, Gb_3, Gb_4 and other more complex structures were identified by HPTLC-MALDI in mouse kidney ($GalGb_4$ and other globo-SL), spleen (Gg_4Cer) and small intestine (Gg_4Cer with ceramides containing 4-hydroxysphinganine and α-hydroxyl fatty acids), using parallel plates with orcinol, primuline, and immunostaining.[84] Profilings of SL and GSL from skeletal mice muscle, brain mice tissue, human serum, and murine myoblasts were also performed by HPTLC-MALDI MS/MS to create a database of molecules to be searched in preclinical steps.[58] Likewise, glycosphingolipid profiles were studied in the search for specific markers of the differentiation of human bone marrow mesenchymal stem cells toward osteoblasts,[117] by combining HPTLC-radiodensitometry-MALDI using metabolically ^{3}H-gangliosides labeled in the sphingosine moiety.

Identification of neutral lipids and phospholipids

HPTLC-MALDI-MS was used to analyze TG and PL in chicken conjugated-linoleic acid rich egg yolk with regard to control eggs.[74] Two LPC, 1 SM, 17 PC, 19 TAG, 9 PE molecular species were identified.

Short-time screening on main PL classes and mapping of individual PL species by MALDI in biological materials were reported.[62] Likewise, PG, PC PE PI, LPE and SM were analyzed in an airborne strain of *Pseudomonas fluorescens* from air in dust clouds.[75] The resulting profiling of bacterial lipidome showed the unexpected presence of PC.

Other works also use MALDI to obtain comparative profiles. Concentration of LPC was higher from a 24 h-treatment with a drug in a colorectal adenocarcinoma xenograft grown in mice.[51]

A detailed PL composition of honey bee sperm during storage in the bee queen was reported.[76] Comparative profiles of PE, PS and cholesterol in synovial fluid were obtained, and demonstrated that these species vary from different stages of inflammation produced by polyethylene-based knee joint implants.[77]

The lipidome of the marine archaeon *Pyrococcus furiosus* was studied from lipid extracts by means of MALDI from lipid solutions and directly from the plate.[21] Most of the major polar compounds were diether-archaeol lipids

and the remaining part by tetraether-caldarchaeol lipids, some of which were different from those previously described. Moreover, results suggest that cardiolipins are ubiquitous in archaea.

Desorption electrospray ionization (DESI)

Other different ambient ionization MS methods were applied to HPTLC of lipids, such as DESI, a spray-based method, in which ambient ionization is concomitant with extraction.[141] A pneumatically assisted ESI is directed at the plate surface. The stream hitting the surface is a solvent mixture. Charged droplets impact the HPTLC plate where they facilitate analyte dissolution and generate secondary droplets which are scattered off the surface of the plate under of a nebulizing gas by an ESI-mechanism which allows soft ionization with minimal sample preparation.

HPTLC-SD-DESI-MS provided a simple but powerful approach for the detailed structural elucidation of lipidome of human lens. It revealed minor components, such as glycolipids (1% of total lens lipids approximately), which were not identified by direct-infusion MS of the lipid extract due to ion suppression effects.[56] HPTLC-SD (primuline)-DESI-MS, MS/MS allowed detection of 30 species from 11 classes of SL in human lens, including sulfatides, dihydrosulfatides, lactosyl- and dihydrolactosyl ceramide sulfates, including novel ether-linked phosphatidic acid species. As an example of the performance of this technique, LacCer with a sphinganine backbone were exclusively observed by HPTLC-SD-DESI whilst in the case of other GSL-classes only sphingosine analogues were detected. All this was accomplished despite the limited resolution of DESI experiments, as lipids could only be separated over a short distance because the DESI source has a maximum mobile distance of 35.8 mm. Likewise, HPTLC separation reduced mass spectral complexity and then provided detailed identification of lipid species by DESI-MS, reducing the ion suppression effects when compared to direct-infusion MS.

The same research group found that developed plates exposed to ambient ozone prior to DESI-MS analysis produces ozonolysis products rather than oxidation ones, allowing for the unambiguous identification of lipid-double bond positions even in the case of low abundant, unsaturated lipids. Difference in double bond positions yields distinctly ozonolysis products.[142]

Ozonolysis of the unsaturated lipids on silicagel plates involves the formation of the corresponding aldehyde, and the subsequent hemiacetal formed via the addition of methanol to the aldehyde moiety during the desorption/ionization process. Thus, CID spectra shows a characteristic loss of MeOH ($\Delta m = -32$ Da). This was observed by DESI-MS, MS/MS analysis of a variety of unsaturated lipids including PC, SM, PS, PG, in both positive and negative modes.

Lipids were directly identified from tissue slices of rat brain by HPTLC-DESI-MS, MS/MS even if separation was partially resolved.[49] Optimal planar separations are not required given the high sensitivity, specificity, and spatial resolution of the DESI-MS. Thus, complex gangliosides (GQ1, GT1, GD1, GM1) were identified in negative mode. DESI-MS/MS revealed the presence of both GD1a and GD1b isomers. Staining with orcinol was the intermediate densitometric step.

DESI-MS was also successfully applied to the direct analysis of PL from porcine brain lipids which were separated in two dimensions on silica gel HPTLC plates.[57] Molecular imaging of separate but still incompletely resolved spots on HPTLC plates was used for the direct analysis of sample lipids by DESI-MS, MS/MS. Eight classes containing more than fifty lipids were imaged in the negative ion mode. Likewise, mapping of the distribution of nonpolar lipids (e.g., cholesterol) was done by reactive DESI using betaine aldehyde in acetonitrile. As in the previous cases, although the spots corresponding to different lipid classes are not completely resolved, the specificity and selectivity achieved by the MS and MS/MS detection allow the resolution of overlapping spots. The derivatization of the lipids on the HPTLC plate with primuline allows visualization of the spot by fluorescence. In this way, the DESI experiment can be performed in the spot sampling mode, by directly positioning the sprayer on a spot which one wishes to investigate.

DESI-Ion mobility separation (IMS)

Ion Mobility is a post-ionization technique used to separate ionized molecules in a gas-filled mobility drift cell in which ions drift at a velocity obtained from an electric field based on their shapes or dipoles.[143] Roughly, the different time of each ion drift provides the separation of different shaped molecules. IM-MS, usually coupled to LC-MS,

has been useful for the analysis of individual molecular species of a lipid class based on their molecular size (including chain length and unsaturation), and of isobaric/isomeric species possessing different conformational structures.

Recently, IMS has been orthogonally added to HPTLC-DESI-High Resolution TOF to study ecdysteroids in insect molting hormones. This equipment was useful for detection, identification, and imaging these polar polyhydroxylated steroids, as well as to enable co-migrating and isobaric compounds to be resolved thanks to Drift time parameter.[144]

Other techniques

An MS technique based on Induced Desorption/Ionization by neutral SO_2 clusters (DINeC) recently proved to produce extremely soft desorption, giving clear and fragmentation-free spectra of PL extracted from egg yolk. No sample preparation and no additional matrix were required. A LOD in the nanomol range was obtained although the SO_2 beam of DINeC probes only the surface of the uppermost layer particles on the HPTLC plate.[145]

Desorption atmospheric pressure photoionization (DAPPI) is an ambient ionization technique that involves a thermal mechanism of desorption. Ionization is initiated by photons emitted from a vacuum UV lamp. It has been an excellent tool for identifying polar as well as completely nonpolar lipids from vernix caseosa in combination with HPTLC, using normal or reversed phase.[146] Vernix caseosa is a white, greasy biofilm which covers large skin areas of the fetus during the last trimester of the pregnancy and often remains on the baby skin at birth. Detected lipids using an orbitrap MS-equiment were Chol, TG, 1,2 diol-diesters, and wax esters. Reported LOD values were in the ng or pmol range. According to the authors, previous HPTLC is needed as, in the absence of a previous chromatographic separation, detection may be prone to matrix effects that may lead to significant background disturbances, decreased sensitivity, as well as undetection of existing compounds in the sample (e.g., squalene and its oxidation product). Desorption temperature must be carefully optimized as there was a strong effect of the lipid MW on the desorption efficiency. Likewise, some of lipids fragmented during the ionization, which may complicate their identification.

On spot-EASI (Easy ambient sonic-spray ionization)-MS was combined with HPTLC for obtaining representative FFA profiles of livers from genetic hypertriglyceridemic mice compared with control normolipidemic mice.[93] It is performed by spraying methanol on the plate along each HPTLC track to promote desorption/ionization of lipid peaks.

Ellis et al. addressed in 2013 that DART (Direct Analysis in Real-Time) was limited to direct analysis of non-polar lipids. DART had not been applied in combination with HPTLC.[141] Since then, the situation has not changed substantially. DART is based on the interaction of helium with a glow or corona discharge that leads to the production of electrons, ions and metastable species.

Conclusions and outlook

Lipids have usually been analyzed in complex matrices mostly from lipidomics, energy processes, and food analysis. After HPTLC separation into lipid classes/subclasses, quantitative analyses of separated individual lipids are possible by SD when appropriate standards are available, as in other chromatographic techniques. Likewise, strategies for accurate analysis of lipid classes and their-derived untargeted lipid species are possible using SD combined with MS. Hyphenations, such as HPTLC-SD-MS, HPTLC-SD-EDA-MS, HPTLC-SD-RD-MS, have not been fully exploited, especially in biological fields.

A separation of sample into lipid-classes, followed by SD and MS coupling provides a simple but powerful approach for the detailed structural elucidation of lipids present in complex biological extracts, allowing exact recognition of lipids by their *m/z*, and confirmation by their collision-induced dissociation MS/MS data. Several examples presented in this work show that, regardless of the MS equipment used, HPTLC separation reduces mass spectral complexity, which, in turn, reduces in part undesirable effects such as ion suppression or matrix effects. In addition, reduction in spectral complexity allows for a comprehensive examination of samples, even whether not completely resolved separations are obtained.

Rapid recording of MS spectra is of special interest in the analysis of complex lipid samples, either by selective access to hot zones using devices for the extraction of selected areas of the plate, or by scanning using ambient desorption/ionization techniques along the corresponding plate track.

Given the works published in the last 10 years, HPTLC has proved to be a powerful tool in constant development and has a special and a complementary role to contribute to lipid analysis together with the other chromatographic techniques coupled to MS.

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Abbreviations

AcH	acetic acid
AMD	Automated Multiple Development
APCI	Atmospheric Pressure Chemical Ionization
Cer	Ceramides
Chol	Cholesterol
CholE	Cholesteryl esters
CholS	Cholesteryl sulfate
DCM	Dichloromethane
DESI	Desorption Electrospray Ionization
DG	Diacylglycerides
E1	estrone
E2	17-β estradiol
E3	estriol
EE2	17-α ethynylestradiol
EDA	Effect-Direct Analysis
ESI	Electrospray Ionization

EtOH	ethanol
FA	Fatty Acids
FAME	Fatty acid-methyl esters
FFA	Free Fatty Acids
GalCer	Galactosyl-Ceramides
Gb_3	Globotriaosylceramides
Gb_4	Globotetraosylceramides
GC	Gas Chromatography
GL	Glyco-lipids
GlcCer	Glucosyl-Ceramides
GSL	Glycosphingolipids
HPTLC	High-Performance Thin-Layer Chromatography
IMS	Ion-Mobility Separation
LacCer	Lactosyl-Ceramides
LC	Liquid Chromatography
LESA	Liquid Extraction Surface Analysis
LPC	Lyso-Phosphatidylcholines
LPG	Lyso-Phosphatidylglycerols
MALDI	Matrix Assisted Laser Desorption Ionization
m.d.	Migration distance
MeOH	Methanol
MG	Monoacylglycerides
NL	Neutral Lipids
PA	Phosphatidic Acids
PC	Phosphatidylcholines
PE	Phosphatidylethanolamines
PG	Phosphatidylglycerols
PI	Phosphatidylinositols
PL	Glycerophospholipids
PMA	Phosphomolybdic acid
PS	Phosphatidylserines
SD	Scanning Densitometry
SL	Sphingolipids
SM	Sphingomyelins
SQ	Semi-quantitative
Sq	squalene
SterylE	Steryl-esters
TG	Triacylglycerides

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