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Lipidomic Studies Based on High-Performance Thin-Layer Chromatography

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Abstract

The last triennium has shown a significant contribution of High-Performance Thin-Layer Chromatography (HPTLC) to lipidomics. HPTLC separation in combination with radio- and /or Ultraviolet (UV)-Fluorescence (FL) densitometry was the technique of choice for tracking the transport of phospholipids among different cellular compartments in a number of biological systems, e.g., Gram-negative bacteria, yeast membranes, endoplasmic reticulum/mitochondrial membrane interface, and also to monitor the lipid transfer activity of a protein. Likewise, a significant number of HPTLC methods were developed to determine variations in the content of different lipid classes and subclasses after using genetic knockouts of cells. Radiolabeled control cells and cells whose genes were deleted using genomic editing allowed to study by HPTLC the effect of the disruption of the related proteins on the corresponding biosynthetic pathways.

As well, direct interface-based coupling of HPTLC to Mass Spectrometry (MS) using mostly Electrospray (ESI) ionization and a variety of mass analyzers, has gained new momentum in this period. Obtaining a large amount of information online in a very short time from complex biological samples, and the structural identification of target and non-target, unknown lipids, make this technique a useful tool for lipidomics. Quantitative issues related to HPTLC-MS are also discussed in this work.

Keywords: HPTLC, densitometry, HPTLC-MS, lipidomics, phospholipids, sphingolipids

1. Introduction

Lipids are involved in energy storage in cells, are structural constituents of cell membranes to which also provide shape and fluidity, and they are also signaling molecules in thousands of metabolic pathways. Therefore, lipids play important roles in many human diseases. The current scientific paradigm to understand their role is based on the hypothesis that some diseases have distinctive lipid profiles with respect to the respective healthy status. For example, unique sphingolipid profiles were shown to represent bio-signatures for various cancer types [1]. In a similar context, the identification of one lipid or a set of lipids that exemplify a

pathological state, generally referred to as biomarker screening, were associated to different lysosomal storage disorders [2], and to Alzheimer's disease [3], among others.

In general, lipidomic research involves the identification and quantification of the thousands of cellular lipid molecular species. Despite High-Performance Thin-Layer Chromatography (HPTLC) being a popular technique in lipid analysis, its contribution to this research field has often been limited in the past to the separation of lipid classes and subclasses, and their subsequent determination by densitometry. This stereotype of HPTLC as a limited technique began to change with the development of direct coupling of HPTLC to Mass Spectrometry (MS)-soft ionization techniques, which allowed the structural identification of target and non-target lipids in complex samples to be obtained, combined with the classical densitometric analysis of lipid classes. This has been reviewed and described in two works published in 2021 on different aspects of general lipid analysis by HPTLC-densitometry-MS [4,5].

HPTLC is currently present in numerous works dedicated to lipidomic analysis. Those of us who are dedicated to the development of analytical methods are not usually aware of their frequent use in other biological disciplines. However, HPTLC contribution, modest but necessary, is often devalued, undervalued and buried under the visibility of other techniques. One reason may be that, as Professor Rudolf Kaiser proudly commented [6], HPTLC perform chromatography at a high level with absolutely simple equipment and under very simple experimental conditions.

Despite the decrease in activity associated with the pandemic period, research published in the 2019-2021 triennium has shown a significant contribution of HPTLC to lipidomic analysis. Some of these papers are very recent and have not even been collected by the two 2021 reviews mentioned above [4,5]. They include special "solo" HPTLC-based techniques for phospholipids (PL) tracking (section *3.1*), and for studying the effect of protein deletion, by genetic knockout, which allowed to draw conclusions on mechanisms of biosynthetic pathways (section *3.2*). Likewise, HPTLC-MS was used for obtaining Electrospray (ESI)-MS lipid profiles, and for identifying target and untarget molecular species of lipids in complex biological samples (section *4*).

Separation conditions for PL, sphingolipids (SL), and neutral lipids (NL) are also discussed (section 2), as well as unaddressed quantitative aspects (section *4.3*). This article aims to make the reader aware of the advances, limitations and challenges faced by the application of HPTLC for lipidomic analysis, in order to be able to design research strategies that must be addressed so that this technique is more valued and accepted in this field.

2. Separation for HPTLC-based lipidomics

Most of papers refer to the separation of various subclasses of PL and SL. Table 1 details the separated sub-classes, samples, development conditions and references.

Although PL usually designates glycerophospholipids, it is common for sphingomyelin (SM), a SL-based on a ceramide backbone, to be considered for practical purposes also a PL due to its phosphocholine group. Although the separation of PL subclasses does not usually present complications, the particular separation of SM, phosphatidylcholines (PC) and lyso-SL, highly retained on silica gel, requires an acidic or basic medium. The most used developing systems are isocratic and consist of mixtures of chloroform ($CHCl_3$):methanol (MeOH):acetone (AcO):water:glacial acetic acid (AcH) (acid medium, typically 6:2:8:1:2, v/v) [7-12] or $CHCl_3$:ethanol (EtOH):trimethylamine:water (basic medium, typically 3:3.5:3.5:0.7, v/v, [8,13-16]. Figure 1 shows separation profiles for different PL classes.

Figure 1

Proportions of the solvents in both types of systems can vary slightly in works from different authors, as shown in Table 1. These systems were used for PL subclasses separation (SM, PC, phosphatidylserines PS, phosphatidylinositols PI, phosphatidylethanolamines PE, phosphatidylclycerols PG, phosphatidic acid PA, cardiolipins CL, mono-lyso cardiolipins MLCL, etc.). Likewise, it has been reported that the solvent system in basic medium allows for the separation into lyso-lipids, acyl-acyl, alkyl-acyl and alkenyl-acyl PL, as well as glycosphingolipids (GSL) [5].

Both the acidic and basic systems have also been combined for two-dimensional separations [10,17] that have been used to track PL traffic in biological systems [10] (see *section 3.1*).

As can be seen in Table 1, other isocratic systems were also used inspired by the previous ones, but with the absence of any of the solvents (AcO [11,18] or water [18]), or changing trimethylamine by ammonia solution [19]. Likewise, two-stage sequential developments were also used, covering a wider range of polarity [20-22].

The separation of SL, including neutral SL, as hexosyl-ceramides (HexCer), dihexosyl-ceramides (DiHexCer), globotriaosylceramides (Gb_3) [23,24], SL with sialic acids or sulfoglyco-SL in its structure and lyso-SL [11,14,15,23], was performed using basic polar systems, similar to those used for PL, often with the inclusion of $CaCl_2$ or KCl in water.

These development systems, applied to a GSL metabolic analysis of KO cells allowed to separate glucosyl-ceramide (GlcCer) from galactosyl-ceramide (GalCer), and lactosyl-ceramide (LacCer) from other DiHexCer [23]. According to the authors this was confirmed using the corresponding pure standards. It should be noted that these compounds are hardly separable by Liquid Chromatography (LC) and indistinguishable by MS.

It is quite frequent that publications do not specify neither the development chamber used, nor the distances of total migration nor of each of the chromatographic developments. It is necessary that this information be specified in the publications due to the influence of separation conditions on the migration distance of PL.

Table 1

The relatively high concentrations of acid or base necessary to separate PL precluded complete separation of these lipids in Automated Multiple Development (AMD), a technique for gradient development which allows fine-tuned separations to be obtained. However, it was possible to use a seven-step AMD gradient based on MeOH, ethyl acetate (AcOEt) and water for the separation of PC, PE, PG and cardiolipin (CL) as pure standards and as associated to membrane proteins in photosynthetic purple bacteria [25] (Figure 2A). This was done carrying out a previous preconditioning of the plate in AcH 1N.

Mobile phases compatible with AMD were used for separating globotriaosylceramide (Gb_3) isoforms in human plasma [26], and ceramide subclasses in skin Stratum Corneum [27] under the conditions specified in Figure 2B,C.

Figure 2

In the case of Gb_3 (a nine-step AMD gradient, [26]), five saturated Gb_3 isoforms migrated on LiChrospher plate in one of the separated peaks corresponding to the migration zone of Gb_3 standard. Likewise, other seven methylated and seven three-unsaturated Gb_3 species co-migrated with SM species.

In the case of Stratum Corneum [27], free fatty acids (FFA), cholesterol (Chol) and ten ceramide subclasses were separated using an eleven-step AMD gradient (Figure 1). Ceramide subclasses were combination of sphingoid bases (di-hydrosphingosine dS; sphingosine S; phytosphingosine P; and 6-hydroxysphingosine H) and fatty acyl chains (non-hydroxy-FA, N; α-hydroxy-FA, A; ω-hydroxy-FA, O; esterified ω-hydroxy-FA, E). Resulting ceramides, in increasing order of migration distance were: AH, AP, NH, EOH/AS, AdS, NP, OS, EOP, NS/NdS, EOS.

Table 2 details separation conditions for total lipid classes which cover a wide range of polarity. In addition to PL and SL (ceramides), different NL subclasses have been separated using the conditions described there (diacylglycerides, DG; triacylglicerides, TG; wax esters; sterols; cholesterol (Chol), chosteryl esters (CholE), FFA, hydroxy-FA, ecdysteroids) from different biological samples, using either isocratic [13,17,28-32] or sequential development [33].

Table 2

In general, normal-phase was mostly used for separations, in the form of silicagel HPTLC or Lichrospher plates. One work was published in which reverse phase is used [34]. This was superior to normal-phase for the separation of the primary products (epoxides and hydroperoxides) and secondary products of oxidation of 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine (POPC).

3. HPTLC- (radio or UV/FL) densitometry

HPTLC methods for separating lipid classes and subclasses, combined with semi-quantification by Ultraviolet (UV)/Fluorescence (FL) densitometry using revealing agents and appropriate standards, were mostly used for doing descriptive research about the effect of different variables on a lipidomic system. Recent papers studied the effect of: acne and non-acne in adolescent with dark skin on facial sebum lipidome profile [31]; dyslipidemia

and vitamin D on the sphingolipid serum profiling in normal weight and obese subjects [24]; the photoperiod on the fatty acid profile in hatchery-reared underyearlings and yearlings of Atlantic salmon [32]; the effect of bisphenol A on total lipid classes of zebrafish eleutheroembryos during the yolk sac absorption stage [33]; the influence of 21-benzylidene digoxin, a cardiotonic steroid, on Chol and PL content of the membrane of HeLa cells [12].

A special fluorescent labeled lipid was used in combination with HPTLC as a probe to investigate sphingolipid metabolism as well as lipid localization in cells [20]. This is a probe based on modified SM, and includes: i) a photo-cleavable protection group (coumarin cage, that can be cleaved at UV > 400 nm); ii) a diazirine crosslinking group (cleaved at 355 nm); and iii) an additional alkyne click moiety for post-crosslinking functionalization with fluorophores. This enables to reveal sphingosine subcellular localization via crosslinking, fixation, and specific staining by click reaction with a fluorophore, without changing lipid properties.

3.1. Phospholipid tracking

HPTLC has been the technique of choice for tracking PL in different biological systems, often by radio-densitometry using isotopically labeled lipids, and sometimes using fluorescent lipids.

PL traffic refers to the transfer of PL for the site of synthesis in cell to the respective cellular membrane destination, as this occurs in an organized way. How phospholipids travel between these organelles has not been studied extensively, probably due to the lack of a practically useful in vitro assay system to analyze these processes [16].

Yeast (*Sacharomices cerevisiae*) is frequently used in research because is an efficient model to study metabolic changes under different physiological conditions. PL traffic in yeast was tracked using a bidimensional (2D)-HPTLC method based on the previously described acidic (first dimension) and basic (second dimension) developments for PL [10]. Second dimension was performed by turning the plate 90°. In this case, detection was carried out charring with 10% cupric sulfate in 8% aqueous phosphoric acid solution for 10 min at 180 ºC. Likewise, HPTLC can be combined with enzymatic assays for tracking PL within a cell organelle. Microsomal preparations (from endoplasmic reticulum and ribosomes that are isolated together when homogenized cells are centrifuged), and isolated mitochondria are the source of enzymes. Radiolabeled or fluorescent tagged PL can

be used as substrates. For example, acyltransferase assay on lyso-phospholipids (LPL) results in the formation of its corresponding PL by acylation using radiolabeled or fluorescent oleoyl-coenzyme A [10]. Likewise, hydrolysis of CL, which has four fatty-acyl chains, by lipases results in the formation of their fluorescent tagged mono-lyso cardiolipin (MLCL) and di-lyso cardiolipin (DLCL) [10]. For both cases, products can be further quantified after being separated on the HPTLC plate.

Other in vitro HPTLC methods for PL tracking were recently published [11,16,19,21]. HPTLC development conditions can be found in the respective references in Tables 1 and 2.

A method for measuring LPL transport across the membrane was performed using *Escherichia coli* spheroplasts which are laboratory-prepared cells without an external cell wall [19]. In the inner membrane of Gram-negative bacteria there is a coupled enzyme tandem, formed by a LPL protein transporter (LpIT) and an acyltransferase (Aas) which facilitates rapid retrograde translocation of lyso forms of LPE, LPG and CL across the cytoplasmic membrane. In this system, Aas catalyzes acyl-transfer to LPL, generating the diacyl form of the corresponding PL.

In this proposed HPTLC-based translocation assay, radioactive LPL were mixed with cold counterparts. Assays were initiated by adding substrates into spheroplast solutions. Reactions were stopped at the indicated time. Total lipids were extracted and then separated by HPTLC. The previously mentioned Aas-dependent formation of diacyl form of lipids from corresponding lyso form can be quantified by radiodensitometry from the HPTLC plate, which reflects those LPL that have been translocated across the membrane.

On the other hand, two in-vitro assay HPTLC methods related to PL “come and go” traffic in this biological system were developed [16,21]. Most PL are synthesized between the endoplasmic reticulum (ER) and mitochondrial inner membrane thanks to a set of several enzymes which found in both structures. For this, the precursor PL have to shuttle between them. A complex structure called ERMES (ER-Mitochondria Encounter Structure), which directly tethers the ER and mitochondrial outer membranes, facilitates phospholipid transfer from the ER to mitochondria. A method [16] is able to measure PS and PE transport at ER/Mitochondria interface. This uses membrane fractions isolated from yeast cells. They were incubated with ^{14}C-serine for the indicated time periods. PL were extracted and analyzed by HPTLC and radio-imaging. Results showed PS transport from the ER to mitochondria as well as PE transport from mitochondria to the ER.

Other *in vitro* exchange, HPTLC assay [21] using liposomes to mimic biologic membranes was also developed in order to monitor the lipid exchange activity of Mdm12, one of the subunit proteins of ERMES complex. After incubation with liposomes, PL were separated 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 H_3PO_4 (145 ºC, 4.5 min). The result was that Mdm12 exchanges nearly all its endogenously PG and PE with PC and PI extracted from the liposomes.

According to [11], no assays are available that directly address the lipid transfer activity of a protein *in vivo*. These authors developed an indirect measurement by monitoring protein impact on glycosphingolipid homeostasis, based on HPTLC. For this purpose, GSL production was followed using metabolic labeling with radiolabeled lipid precursors. SL labeling in cells with previously synthesized ^{3}H-sphinganine, ^{3}H-sphingosine, and ^{3}H-palmitic acid was used. Two different mobile phase systems were used for separating GSL (Table 1, [11]). Therefore, the products from the cellular lipid metabolism were quantitatively analyzed with by radio-densitometry and densitometry using orcinol for GSL; iodine or copper acetate in 8% phosphoric acid for PL; and primulin in acetone/water (4:1, v/v) for general lipid detection.

3.2. HPTLC and genetic knockouts

This is an emerging field for HPTLC application in lipidomics.

For a given biosynthetic pathway, control cells and cells whose genes were deleted using CRISPR/Cas9 genomic editing can be radiolabeled using an isotope, and then total lipids can be extracted and analyzed by HPTLC. Thus, preparing genetic knockouts combined with HPTLC analysis allowed to obtain conclusions on mechanisms of biosynthetic pathways.

An example of HPTLC application in this field is the elucidation of Gb_3 biosynthesis mechanisms, in which the roles of two transmembrane proteins (LAPTM4A and TM9SF2) were unknown. Thus, knockout (KO) cell clones of each corresponding gene were generated using the CRISPR/Cas9 system.

To determine if Gb_3 biosynthesis was affected by the disruption of LAPTM4A and TM9SF2, lipids were metabolically labeled with [^{14}C]-galactose and separated by HPTLC [23]. Quantification was carried out by using radioactive imaging. Relative to parent cells, LAPTM4A- and TM9SF2-KO cells produced lower levels

of labeled Gb_3 and had higher levels of labeled LacCer, the direct precursor of Gb_3. Therefore, results suggested that both LAPTM4A and TM9SF2 were involved in Gb_3 synthase-dependent conversion of LacCer to Gb_3.

On the other hand, measurement of the enzymatic activity of endogenous Gb_3 synthase in vitro was done using HPTLC. Cell lysates were incubated with or without LacCer in addition to tritiated uridine-5'-diphosphogalactose ([^{3}H]UDP-galactose). Then, labeled lipids were separated on a HPTLC plate under the conditions detailed elsewhere [23]. Relative Gb_3 synthase activities are expressed as percentage of the value in control cells by radio-imaging. Results showed that Gb_3 synthase activity was markedly decreased in LAPTM4A-KO cell lysates relative to wild-type. In contrast, TM9SF2 disruption did not affect *in vitro* activity of Gb_3 synthase. Results indicated that LAPTM4A was involved in the regulation of Gb_3 synthase activity, whereas TM9SF2 regulation of Gb_3 synthesis was independent of these mechanisms.

Other examples different in the objective but similar in terms of methodology can be found in the recent literature. Thus, sphingolipid biosynthetic pathway was studied using CRISPR/Cas9 for gene deletion, and further using 3-L-[^{14}C]-serine labeling. Subsequent total lipid extraction and analysis were carried out by HPTLC [22].

Another example deals with the *GCR1* transcription factor, which is involved in yeast in the regulation of glycolysis [18]. A transcription factor is a protein that binds to specific sequences of DNA, thereby controlling the transcription of genetic information from DNA to messenger RNA. Results show that *GCR1* gen deletion exhibited, among other effects, reduced phosphatidylinositol (PI) levels compared to wild-type cells. Variation of PI levels from control and gen-deleted samples were quantified after separation of lipid extracts on silica gel HPTLC plates using $CHCl_3$:MeOH:AcH (65:25:8; v/v) as development system. The plate was exposed to iodine vapor to visualize the lipids, scraped from the HPTLC plate and quantified.

Similarly, the physiological role of Spf1p, a P5A ATPase, an enzyme which is involved in regulation of homeostasis in the ER, was studied by deleting one of P5 ATPase gen of yeast *Saccharomyces cerevisiae* [17]. HPTLC experiments allowed to obtain PL and SL profiles, and concluded that lanosterol content decreased, whereas ergosterol content increased in spf1 cells. There is also an increase in the total sphingolipid content. These changes affect protein function in the ER and plasma membrane.

Another work was published whose the aim was to investigate the effect of *Abcb4* gene knockout-induced cholestasis on lipid metabolism, in hepatitis B virus surface protein (HBs) transgenic mice [30]. The *Abcb4* gene

provides instructions for making a protein that helps move PL across the membranes of liver cells and release the PL into a digestive fluid called bile. HPTLC-based runs of hepatic lipid extracts revealed a significant decrease in the amount of TG, while the amount of FFA was increased with Abcb4 knockout in comparison to wild-type and HBs mice.

4. HPTLC-MS

Before the introduction of current HPTLC-MS technology, the separated HPTLC bands had to be scraped off from the plate, extracted with a solvent, the extract filtered and the solvent distilled off, and after subsequent drying, the sample was ready to be introduced into a mass spectrometer. This was time consuming and susceptible to experimental errors.

Thanks to the development of coupling devices, and the possibility of connecting them to soft ionization MS techniques and to any mass analyzer, HPTLC-MS is currently a technology that allows obtaining a large amount of information online in a very short time from complex lipidomic-related matrices. The HPTLC separation in lipid classes contributes to reduce ion suppression problems in MS detection, allowing for a comprehensive identification of individual molecular species for each class. This can be performed even if incompletely resolved separations are obtained.

4.1. Coupling through extraction-based interfaces

In the last three years, the most used coupling devices have been Advion Express™ TLC plate reader [5,13-15,28,34] and Camag TLC-MS Interface [9,25-27,30]. Both are inspired on the same principle. Separated bands are automatically extracted with solvent through a piston equipped with an elution head, and then the extract is sent to mass spectrometer via an outlet capillary [4,5]. These devices allow for selecting the desired target bands with precision. The complete operation takes less than one minute.

All the examples described in this subsection have been carried out with one of these two interfaces that are coupled with MS equipment bearing different ionization sources and mass analyzers.

ESI was the most popular among the MS ionization techniques employed in combination with the described interfaces [5,9,13-15,25-28,34]. ESI-MS ion profiles of lipid classes/sub-classes can be obtained. From these profiles, target and non-target individual lipids can be structurally identified by tandem mass spectrometry (MS/MS). Validation with analytical standards (either commercially available or from custom synthesis) was also essential.

In most of works reviewed, ESI ionization was combined with ion trap technology as mass analyzer. Using this technology, precursor ESI ions are first trapped, and then fragmented. Thus, MS/MS identification takes place over time so that only the identity confirmation of one selected ion can be done per extracted band. Using this technology, PL species (PC, PE, PG) associated to membrane proteins (MP) were identified in non-sulfur, purple bacteria having photosynthetic activity [25]. Likewise, five PC and three PC species were demonstrated to be bound to a purified membrane protein photosynthetic complex from *Rhodobaca bogoriensis*.

The new generation of ion-trap instruments allows the simultaneous positive and negative ESI ionization (ESI^+ and ESI^-, respectively) within the same chromatographic run. This is a big advantage for ion identification, via *m/z* of its respective positive and negative ions. This favors identification of isobars, and is also compatible with further MS/MS analysis. Several examples corresponding to recent research are described below.

Lipids from exosomes, i.e. spherical extracellular nanovesicles from endocytic origin with a diameter between 20 and 150 nm, are currently being considered as potential biomarkers of some specific types of cancer although no specific structures have been proposed. A study was carried out to evaluate the variation in content and composition of phospholipids from exosomes of progressively more metastatic cells (NIH-3T3, B16-F1, B16-F10 murine cell lines) [9]. Quantification of PL classes from exosomal lipid extracts was achieved by UV/FL densitometry, expressed as μg PL/100 μg exosome protein. Identification of individual SM, PC, PS, and PE species coming from lipid extracts of exosomes were performed by simultaneous ESI^+ and ESI^-, in combination with tandem ESI-MS/MS and using appropriate standards (Figure 3). A number of ions were identified based on their $[M+Na]^+$ and $[M+AcO]^-$ dual assignation. Acetate ions came from acetic acid that was part of the mobile phase used for HPTLC separation.

Figure 3

This technology was also useful to select the ionization conditions for a given PL class without the need to repeat the application of the sample or the chromatography. In an investigation of PL pattern in the maturation of 3T3-L1 adipocytes, identification of SM and PC species were performed by ESI^+ and that of PI and PE, by ESI^- [13].

A series of papers were published on sperm lipid composition of different fish species [13-15] which illustrate the power of HPTLC-MS to separate unknown lipid bands which contained untargeted lipids. Thus, GSL-related unknown bands were separated from sperm and untargeted lipids were identified through simultaneous ESI^+ and ESI^-. Thus, neutral Gal-Cer(d18:1/16:0) was identified in spermatozoa from sterlet, a fish with external fertilization. On the other hand, acidic sulpho-Gal-Cer(d18:1/16:0) were identified from stingray, a fish with internal fertilization [14].

In general, although the sperm of different freshwater fish species (carp, northern pike, rainbow trout and burbot) contain high amounts of neutral and acidic GSL and sulfo-GSL, there are major differences in their GSL composition, as was evidenced in unknown separated HPTLC bands [15]. Two mono-sialodihexosylgangliosides (GM3, d18:1/18:0 and d18:1/16:0) were identified in carp sperm. In northern pike sperm, GM3 species showed longer fatty acyl chains d18:1/22:1 and d18:24:1, as well as several sulfo-GSL. Likewise, burbot sperm contains acidic GSL with a sialylated oligosaccharide chain but different fatty acyl chains.

ESI was not only used with ion trap but has also been combined with triple quadrupole technology. When using this equipment, tandem MS takes place in space. Molecular species from ten ceramide subclasses were separated from skin stratum corneum on HPTLC silica gel plates. See section 2 for structures of separated ceramide classes [27]. They were identified using ESI^- ionization and tandem MS. Fragmentation pathways specific to ceramide types were proposed to explain the produced fragment ions and determine their structure.

ESI was also combined with a single quadrupole mass spectrometer to study the potential cytotoxic effect of royal jelly and its bioactive free fatty acids on a melanoma cell line (B16-F10) [28]. 8-Hydroxyoctanoic acid; 3,10-dihydroxydecanoic acid; 10-hydroxy-2-decenoic acid; decanedioic acid; 10-hydroxydecanoic acid were identified using ESI^-.

An ESI-APCI approach was used with ion trap to identify nineteen Gb_3-related biomarkers of Fabry disease, a lysosomal storage disorder [26]. Thus, ESI^+, ESI-MS/MS and Atmospheric-pressure chemical ionization in

positive mode ($APCI^+$) were used for identifying five isoforms of saturated Gb_3 (d18:1/C16:0, C18:0, C22:0, C24:0); seven isoforms of methylated-Gb_3; and seven species with two additional double bonds to that of sphingosine basis. $APCI^+$ was specifically used for confirming the presence of ions coming to originating from fragmentation of Gb_3 species to give the corresponding ceramides, in the *m/z* zone lower than 1000. Likewise, Ion intensity ESI-MS profiles were useful to demonstrate that saturated Gb_3 species in a Fabry's patient plasma were in higher concentration than in a control healthy individual sample.

In another work [30], APCI was used as a unique ionization source in combination with an orbitrap mass analyzer to obtain high-resolution MS spectra of TG and FFA in total hepatic lipid extracts.

4.2. On-plate HPTLC-MS

On-plate matrix assisted laser desorption ionization (MALDI) has been an established technique for lipid identification in complex mixtures [4,5]. A recently published methodological work is of interest for application of HPTLC-MALDI to lipidomics [35]. As background ions in MALDI-MS spectra often cover true lipid ions providing low MS intensities or low MS-imaging resolutions, this method consisted of prewashing of HPTLC plates with 1, 2-dichloroethane before development. This removed background signals when applied to the MALDI imaging analysis of TG, DG, MG and glucosyl-ceramide (Glc-Cer) standards [36].

Other desorption-ionization techniques that do not need the use of interfaces were recently used and have future potential for lipidomic analysis. DESI (Desorption Electrospray Ionisation), is a spray-based, ambient soft ionization technique that was combined with Time-of-Flight high-resolution MS and, orthogonally, with Ion Mobility Spectroscopy (IMS) for identifying ecdysteroids (poly-hydroxylated steroids) in a number of plant extracts [29]. The use of IMS allows co-migrating and isobaric compounds to be resolved thanks to drift time parameter.

A sensitive and extremely soft, induced Desorption/Ionization by neutral SO_2 clusters (DINeC) provided fragmentation-free spectra of PL extracted from egg yolk, without any sample preparation or matrix [36]. However, some issues related to inhomogeneity of probing should be solved.

4.3. Quantitative issues

Regardless of the technique used, a common problem in lipidomics is that relative quantification data is often presented as absolute quantitative levels, so instigating a disservice in obtaining high quality data, which are essential for the translation of biomarker research to clinical practice.

HPTLC could allow to carry out a double quantification, i.e. lipid classes by densitometry and individual species by MS. This could give HPTLC a high added value. However, no research was done in this direction.

Lipid classes are usually (semi)-quantified by HPTLC-densitometry. Semi-quantitativeness does not mean that the analysis is not reliable, but rather since it is carried out using a single standard per lipid class considered representative for all the lipids that make up that class, which differ in the length of the fatty acyl groups. Therefore, the analysis will be more or less accurate depending on the appropriateness of the choice of the standard(s).

MS quantitation of individual species is a hard task. It is only possible when a stable isotope, usually deuterated, of the individual lipid to be quantified (or a representative of a given class) is used as an internal standard in the sample to be analyzed. This should correct errors for extraction, adsorption losses and ion suppression, as the lipid to-be-analyzed and its isotopic counterpart have virtually identical composition for this purpose. Thus, the corresponding deuterated standard should be added to the sample in different concentrations within a suitable range, for example using the standard addition method. And both compounds (deuterated and non-deuterated) have a sufficient mass difference to be distinguished in MS spectra. The amount of the lipid could then be calculated from the calibration curve of its deuterated counterpart.

But to accomplish this, HPTLC-MS coupling technologies should be quantitative. In its current configuration, MALDI is not a quantitative technique as poor “shot-to-shot” reproducibility due to irregularities of the matrix/analyte co-crystals [5]. In the case of HPTLC-interface-ESI-MS systems, quantitativity depends on an adequate extraction of the band. It is not so much that the band extraction is complete, as that it is carried out in a precise manner on a representative surface of the band and that it is repeatable from sample to sample. This was evaluated and confirmed in a work on Gb_3 standard [26]. Positioning of interface was precise and its head drilled the same surface on two extracted bands (control and Fabry’s plasma), which represented in each case an important percentage of the sample peak. The aim of that work was not MS absolute quantification. However, a reliable relationship between ESI-MS ion intensities and concentration of lipids belonging to the same lipid

class can be established. This relationship is straightforward for several reasons, which have to do with the nature of sample and the mode of interface operation. Thus, for a given lipid class, ESI-MS responses per mass unit are similar for all the molecular species, as the influence of fatty acyl chain length is less significant than that of head lipid class. On the other hand, in HPTLC-MS there are no variations in ionization conditions for molecules of the same lipid class, as the automated band extraction and elution was performed using MeOH, and subsequent ESI ionization was carried out using the same solvent. No gradients were used, unlike LC-MS. It is known that LC-MS quantification is complicated as there are variations in ionization of molecules of the same lipid class at different retention times due to differences in the chemical environment ionization because of the gradient.

The correct quantification of lipid species by MS is a challenge for HPTLC-based lipidomics and it should be a research priority for HPTLC to become a completely accepted technique in this field.

Statements and Declarations

The authors declare no conflict of interest.

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CAPTIONS FOR FIGURES

Fig. 1 Representative separation of phospholipid standards by HPTLC using the basic mobile phase using $CHCl_3$:EtOH:trimethylamine:water (3/3.5/3.5/0.7, v/v) on HPTLC silica gel plates which were previously immersed in a solution of 2.3% boric acid in ethanol, prior to sample application. Detection was performed using 0.5% copper sulphate (w/v) in 1.16 m orthophosphoric acid and then, heating 15 min at 155 ºC. A) Separation profile of the individual PL standards (SM, PC, PI, PS, PE, CL, MLCL, PA, 6 µg per track; see text for abbreviations). Image obtained using CAMAG TLC visualizer. B) Separation of the corresponding PL-standard mixture (4 µg of each PL per track). Chromatogram was obtained by UV densitometry and WinCats software. *Reproduced with permission from Ref. [8]. Copyright John Wiley & Sons*

Fig. 2 Automated Multiple Development (AMD) conditions. A) a seven-step AMD gradient based on MeOH, AcOEt and water for separating PC, PE and PG associated to membrane proteins in photosynthetic purple bacteria, on an AcH 1N-preconditioned HPTLC silica gel plate. B) a nine-step AMD gradient based on MeOH and DCM for separating Gb_3-related biomarkers from a Fabry's plasma patient on a silica-based Lichrospher plate without any fluorescence indicator. C) an eleven-step AMD gradient based on MeOH:water:AcH (97:3:1,

v/v/v), AcO and $CHCl_3$ for separating ten ceramide subclasses from skin stratum corneum on a silica-based Lichrospher plate with a fluorescence indicator

Fig.3 (A) HPTLC chromatograms (UV 190 nm) of exosomes lipid extracts showing separation of phospholipid classes (SM, PC, PS, PE) using acidic separation (see text). B16-F1-EXOs: peak 1: SM (m.d. = 14.6 mm); peak 2: PC (m.d. = 20.3mm); peak 3: PS (m.d. = 23.8 mm) and peak 4: PE (m.d.=25.9 mm). B16-F10-EXOs: peak 1: SM (m.d. = 14.5 mm); peak 2: PC (m.d. = 18.0 mm); peak 3: PS (m.d. = 22.6 mm) and peak 4: PE (m.d.=25.6 mm) NIH-3T3-EXOs: peak 1: SM (m.d. = 14.0 mm); peak 2: PC (m.d. = 19.1 mm); peak 3: PS (m.d. = 21.4 mm) and peak 4: PE (m.d. = 24.8 mm). (**B**) Automatic extraction of HPTLC bands using the TLC-MS elution-based interface. Red: HPLC pump for MeOH delivery (0.2 mL/min); blue: ion trap MS equipment; black: frit for silicagel filtering; +: laser crosshair. Idealized operation of peaks 1 and 2 extraction: (**a**) bypass; (**b**) first band extraction; (**c**) air cleaning; (**d**) bypass; (**e**) second band extraction. *Reproduced with permission from Ref. [9]. Authors' Copyright.*

Table 1.- Separation of phospholipids (PL) and/or sphingolipids (SL) on silica gel HPTLC plates from different samples in lipidomic studies

Involved sub-classes	*Original samples*	*Development conditions*	*[Reference]*
		ACIDIC ISOCRATIC DEVELOPMENT	
PE, PC-hydroperoxides	Enzymatically and thermally oxidized egg yolk	$CHCl_3$:MeOH:glacial AcH:acetone:water (35:25:4:14:2)	[7]
SM, PC, PS, PI, PE, MLCL, PA, CL	Mammary tumor cells, hepatocites mitochondria	$CHCl_3$:MeOH:glacial AcH:acetone:water (6:2:2:8:1)	[8]
SM, PC, PS, PE	Exosomes from metastatic murine cells	$CHCl_3$:MeOH:glacial AcH:acetone:water (6:2:2:8:1)	[9]
PL tracking	Yeast	$CHCl_3$:MeOH:glacial AcH:acetone:water (50:10:15:20:5)	[10]
GSL	Cultured cells	$CHCl_3$:MeOH:glacial AcH:acetone:water (10:2:2:4:1)	[11]
PL	HeLa and Caco-2 cancer cells	$CHCl_3$:MeOH:glacial AcH:acetone:water (40:13:12:15:8)	[12]
		OTHER ACIDIC ISOCRATIC DEVELOPMENTS	
PC, PI, PE	Yeast (wild type and *grc1Δ* cells)	$CHCl_3$:MeOH:acetic acid (65:25:8)	[18]

PL	Cultured cells	$CHCl_3$:MeOH:acetic acid:water (50:30:8:3)	[11]
		BASIC ISOCRATIC DEVELOPMENT	
SM, PC, PS, PI, PE, MLCL, PA, CL	Mammary tumor cells, hepatocites mitochondria	$CHCl_3$/ethanol/triethylamine/water (3:3.5:3.5:0.7)	[8]
SM, PC, PE, PI	3T3-L1 adipocytes	$CHCl_3$/ethanol/triethylamine/water (30:35:35:7)	[13]
LPC, SM, PS, PI, PE, PG, Untarget GSL	Fish sperm	$CHCl_3$/ethanol/triethylamine/water (30:35:35:7)	[14,15]
PC, PS, PE, PDME	Mitochondrial membranes	$CHCl_3$/ethanol/triethylamine/water (30:30:35:7)	[16]
		ANOTHER BASIC ISOCRATIC DEVELOPMENT	
Lyso-PL	Bacterial membranes	$CHCl_3$:MeOH:water:ammonia (60:37:5:3.1)	[19]
		BI-DIMENSIONAL DEVELOPMENT	

1D) PA, PC, PE, PI, PS, CL, PG 2D) CL, PG	Yeast	2D: 1) $CHCl_3$:MeOH: AcH:water (85:15:10:3.5) 2) $CHCl_3$:MeOH: AcH (65:25:8)	[10]
PL separation	Yeast	2D: 1) $CHCl_3$:MeOH:25% ammonia (65:35:4) 2)) $CHCl_3$:MeOH: AcH:water (85:25:5:4)	[10]
PL separation	Extracts from wild-type and spf1 cells (*Saccharomyces cerevisiae)*	2D: 1) $CHCl_3$:MeOH:NH_4OH (65:25:4) 2) $CHCl_3$:MeOH:acetone:AcH:water (50:10:20:10:4)	[17]
		SEQUENTIAL DEVELOPMENTS	
SL, sphingosine	Intact cells in well plates	1) $CHCl_3$:MeOH:water:AcH (65:25:4:1) 2) Ethyl acetate/cyclohexane (1:1)	[20]
PG, PE, PC, PI	Mdm 12 protein purified from bacteria and yeast	1) DCM:ethyl acetate:acetone (80:16:4) 2) $CHCl_3$:acetone:isopropanol: ethyl-acetate:EtOH:MeOH:water:AcH (30:6:6:6:16:28:6:2)	[21]
SM, GSL	Cultured cells	1) Acetone 2) $CHCl_3$:MeOH:25% ammonia (50:25:6)	[22]

GlcCer, GalCer, LacCer, Gb2, Gb3, GM3	KO cells	methyl acetate:*n*-propanol:chloroform:methanol:0.25% KCl (50/50/50/20/18)	[23]
SL profiles	Sera	$CHCl_3$:MeOH:water (55:20:3)	[24]
Globosides	Cultured cells	$CHCl_3$:MeOH:CaCl2 (w/v) in water (45:55:10). $CHCl_3$:MeOH:0.25% CaCl2 (65:35:8)	[11,23]
AUTOMATED MULTIPLE DEVELOPMENT (see Figure 2)			
PC, PE, CL, PG	Membrane proteins and extracts from photosynthetic purple bacteria	7-step AMD gradient based on MeOH:water:ethyl acetate, with a previous plate preconditioning using HAc 1N	[25]
10 Cer subclasses, Chol, FFA	Stratum corneum	11-step AMD gradient based on $CHCl_3$/acetone/ methanol:water (acidic conditions)	[27]
19 Gb_3 species	Human plasma	9 step-AMD gradient (MeOH/DCM)	[26]

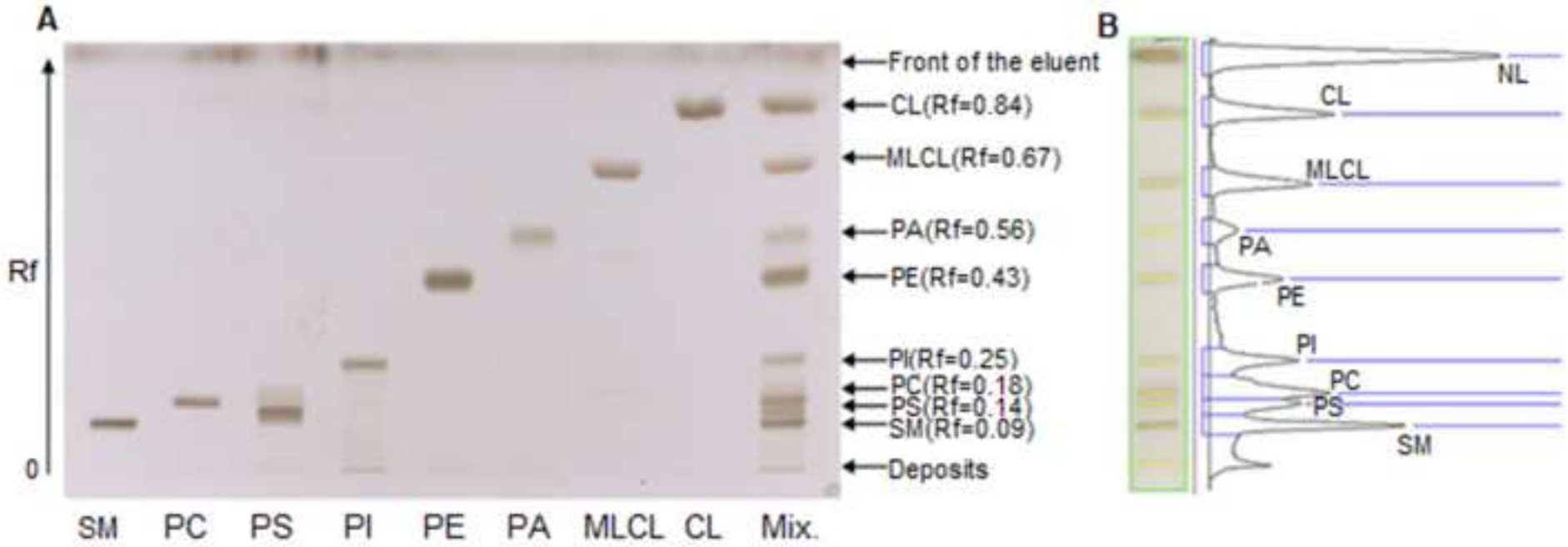
Table 2.- Separation of Total Lipid classes from different samples in lipidomic studies

Involved sub-classes	*Original samples*	*Development conditions*	*[Reference] Author*
		ISOCRATIC DEVELOPMENTS	
Sterols, PL, SL	Extracts from wild-type and spf1 cells (*Saccharomyces cerevisiae)*	For sterols: *n*-hexane:diethylether:AcH (80:15:1) For SL: $CHCl_3$:MeOH:4.2 M ammonia (9:7:2)	[17]
PL, TG	3T3-L1 adipocytes	For TG: *n*-hexane:diethylether:AcH (80:20:1)	[13]
Hydroxy-FA	Royal jelly	Toluene:ethyl acetate (7.5:2.5)	[28]
Ecdysteroids	Plant extracts	$CHCl_3$:EtOH (4:1)	[29]
TG, FA	Mice liver extracts	*n*-hexane:diethyl ether: AcH (8:2:0.4)	[30]
TG, wax esters, CholE	Facial sebum	Benzene: n-hexane:acetic acid (70:30: 0.1)	[31]
PL, DG, TG, Chol, CholE, FFA	Salmon flesh	n-hexane/diethyl ether/AcH (32:8:0.8)	[32]

SEQUENTIAL DEVELOPMENTS			
CholE, Chol, wax esters, TG, PE, PG, PI, PS, PC, SM	Zebrafish eleutheroembryos (ZE)	1) methyl acetate:isopropanol:$CHCl_3$:methanol/0.25% KCl (25:25:25:10:9) 2) n-hexane:diethyl ether:AcH (80:20:2)	[33]

Figure

Click here to access/download;Figure;Fig1.tif



Figure

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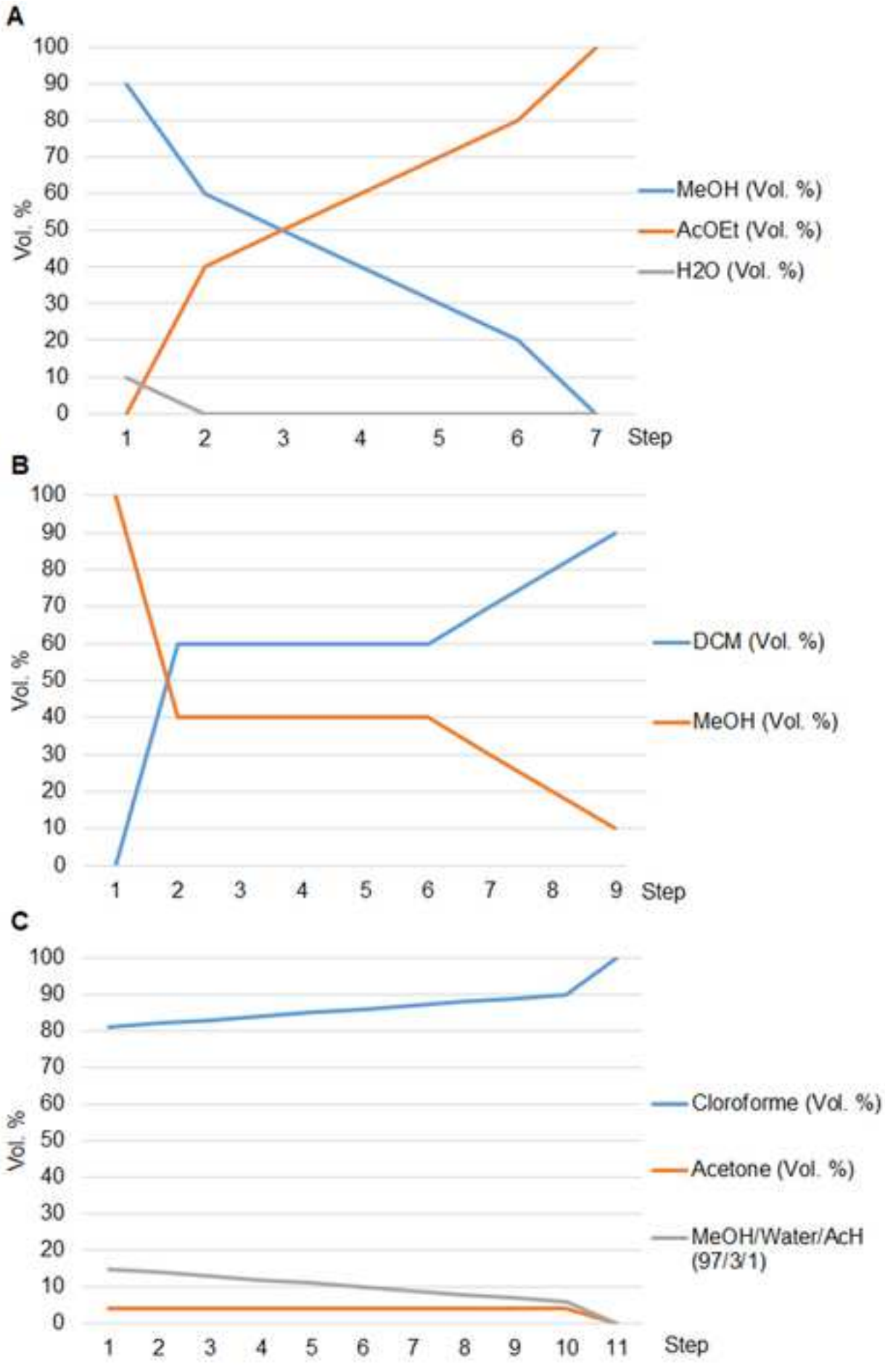

Figure

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