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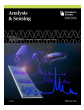

Thin-Layer Chromatography and Coomassie Staining of Phospholipids for Fast and Simple Lipidomics Sample Preparation

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Lipids play major roles in basic cellular functions. Their analysis, therefore, gained importance; however, it is complicated by the high complexity of natural lipidomes. To overcome this challenge, pre-separation of the lipids by thin-layer chromatography (TLC) and high-resolution mass spectrometry (MS) are often employed. Here, we explore Coomassie staining for TLC-separated phospholipids and provide an extraction protocol of stained lipids for subsequent MS analysis. We exemplify our approach by analyzing lipid mixtures varying in complexity and found that TLC pre-separation increases the number of identified lipid species and lipid classes. In addition, we identify and quantify lipids from polymer nanodiscs. In summary, Coomassie staining of TLC-separated lipids is well-suited for phospholipids, is compatible with MS, does not require specialized equipment and can be performed independently of subsequent MS experiments. We envision many future applications of our workflow.

By definition, lipids are apolar biomolecules that are insoluble in water. Accordingly, there is a variety of lipid classes including fatty acids, glycerolipids, glycerophospholipids (also termed 'phospholipids'), sphingolipids, saccharolipids, sterol lipids, prenol lipids and polyketides.[1] Many of these lipids are involved in basic cellular functions such as energy storage or signaling. Due to their amphiphilic structure, phospholipids form micelles, vesicles or lipid bilayers in an aqueous environment.[2] They consequently are the main constituents of cell and organelle membranes.[3] Sterols and glycolipids are additional components of lipid membranes and manipulate their physical properties such as thickness or curvature.[4]

The analysis of lipids is usually performed after their extraction from cells, tissue or fluid. Normal-phase thin-layer chromatography (TLC) is the classical technique to separate and analyze complex lipid extracts (for review see [5]). For this, a polar stationary phase (TLC plate) and an apolar mobile phase (solvents) are used. Based on their polarity, the different lipid classes are then separated on the TLC plate. Lipid spots are usually visualized by staining with various dyes or by illumination with UV light.[5a]

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Even though TLC identifies the lipid classes of complex mixtures, the exact composition including the identity of lipid species or modifications such as oxidation remain elusive.[6] This information is usually obtained from mass spectrometry (MS) experiments. Shotgun lipidomics, which is the direct-infusion of extracted lipids into the mass spectrometer followed by their subsequent analysis by tandem-MS, is most commonly employed to identify and quantify lipids in complex mixtures.[7] Nonetheless, due to overlapping isotope envelopes and different ionization efficiency of the different lipid classes their identification is often complicated.[7a] Pre-separation of the lipid classes is therefore advantageous.

Several studies showed that liquid chromatography-coupled MS analysis enhanced the lipid coverage, sensitivity and dynamic range.[8] Most of these studies used reversed phase, HILIC or anion exchange chromatography and mostly employed flow rates in the microliter range. A recent study used nanoflow liquid chromatography and further increased the dynamic range of phospholipid analysis.[9] Nonetheless, the application of liquid chromatography requires specialized equipment and experience and, therefore, complicates extensive lipid analysis. TLC, on the contrary, is easy to perform, does not require specialized equipment, can be performed in independent experiments and is versatile in terms of the analytes and their detection. Coupling traditional TLC separation with MS hence presents a valuable alternative to liquid chromatography-coupled MS analysis.

After several initial attempts coupling TLC with MS detection directly from TLC plates, a first study successfully coupled TLC separation with electrospray ionization MS for caffeine identification and quantification.[10] TLC-MS was then also developed for lipidomic applications and a commercial TLC extraction system is nowadays available.[11] However, the available dyes used for staining of lipid spots after TLC

separation, might be hazardous, require specialized equipment such as a sparying chamber or covalently modify the lipids. Dyes that non-covalently bind various lipid classes are therefore desirable. One example is Primuline yellow, which was successfully employed during TLC-MS.[12]

A comparably simple, cost-effective and non-hazardous dye for staining lipids after TLC separation is Coomassie.[13] Coomassie staining of lipids followed by MS analysis using matrix-assisted laser desorption/ionization was previously introduced.[14] However, due to interference of matrix ions and uncontrolled fragmentation, matrix-assisted laser desorption/ionization complicates lipid analysis of complex lipid mixtures.[15] Here, we further explore the application of Coomassie staining of TLC-separated membrane lipids and provide an optimized staining protocol for a multitude of lipid classes. We established an extraction protocol of various Coomassie-stained lipid classes from TLC plates and validated their identification by MS. We then explored the applicability of Coomassie staining and subsequent electrospray ionization MS analysis for lipid mixtures of varying complexity including a commercially available lipid extract as well as a eukaryotic cell extract prepared in-house. Finally, we explored additional applications of our workflow and verified lipid compositions from polymer-based nanodiscs.

Results and Discussion

Coomassie staining of lipids separated by TLC

Our aim was to establish a fast and simple workflow for sample pre-separation and preparation of phospholipids prior to direct-infusion MS experiments. To achieve this goal, we chose TLC separation and optimized a staining protocol compatible with MS analysis. There are numerous specific and unspecific staining protocols available, however, the utilized reagents often modify the lipid molecules and therefore complicate subsequent MS analyses. A simple alternative is Coomassie staining of lipids after TLC separation.[13] Similar to protein identification of Coomassie-stained proteins following gel electrophoresis,[16] we explored recovery of lipids after TLC separation and Coomassie staining for subsequent MS-based identification.

Coomassie staining was described for several lipid classes including phosphatidylethanolamine (PE) and phosphatidylcholine (PC).[13] In our assessment, we included additional phospholipids such as phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), cardiolipin (CL) and phosphatidic acid (PA). We also explored Coomassie staining of cholesterol (Chol), an important component of biological membranes. Specifically, PS 18:1/18:1, PG 18:1/18:1, PC 18:1/18:1, PE 18:1/18:1, CL 18:1/18:1/18:1/18:1 and Chol as well as natural lipid extracts of Soy PI and Egg PA were applied to silica plates, separated by TLC and subsequently stained with Coomassie (see **Methods** for details). Following this workflow, separation of all lipids was achieved and sufficient staining using Coomassie staining solution was observed for all lipid classes (Figure 1A). Of note, migration properties of PC and PA as well as PI and PS under the conditions applied here are similar and the lipids are, therefore, difficult to distinguish. We also explored the detection limit of the various lipid classes by titrating increasing amounts of the lipids (Figure 1B). We found that PE, PC, and Chol were sufficiently stained even at low amounts <200 ng. Negatively charged lipids such as PG, CL, PS, PA and PI were not detected at low concentration and required minimal amounts of approx. 1 μg for sufficient staining.

Noteworthy, overloading of some lipids was observed affecting their separation and causing elongated lipid spots. This effect was reduced when lower amounts of the respective lipids were loaded onto the TLC plate. In addition to overloading, lipid modifications such as oxidation due to sample storage and exposure to air as well as differences in the fatty acyl chain length (e.g., egg PA as observed in Figure 1B) might contribute to the extended peak shape of some lipids. To separate these lipid species, reversed-phase TLC might be employed.[17]

To explore whether differences in fatty acyl chain length affect Coomassie staining of the lipids, we examined a range of PG lipid species with increasing fatty acyl chain length (Figure 1C). While separation of the different species was only minimally affected, we found that only PG species with fatty acyl chains >14 carbon atoms were observed after Coomassie staining. PG species with shorter fatty acyl chains were not stained.

In summary, we found that Coomassie staining is a reliable and simple staining alternative for a variety of phospholipids as well as Chol. The detection limit of our set-up is comparable with the detection limit described before[13] and allows sufficient staining of all phospholipids tested here. While Coomassie staining of proteins mostly relies on positively charged amino acid residues,[18] staining of lipids is less understood. According to our experiments and in agreement with previous studies on Coomassie-binding to amino acids, peptides, proteins and other compounds such as detergents or flavonoids,[19] we assume that Coomassie preferentially binds positively charged moieties of the head groups of zwitterionic lipids and additionally interacts with aromatic and hydrophobic parts of the lipids. Accordingly, Chol contains neither positively nor negatively charged groups; however, pronounced staining of cholesterol is observed. In addition, the length of the fatty acyl chain has an impact on the staining efficiency confirming that positive charges alone do not account for the staining. We therefore assume that both positively charged moieties of the head groups as well as hydrophobic parts of the lipids contribute to Coomassie staining. Importantly, during TLC separation, lipids are separated according to their head groups. Short-chain species, which have been found to be less sufficiently stained, will be detected together with their long-chain counterparts therefore enabling the analysis of complex lipid extracts from biological samples.

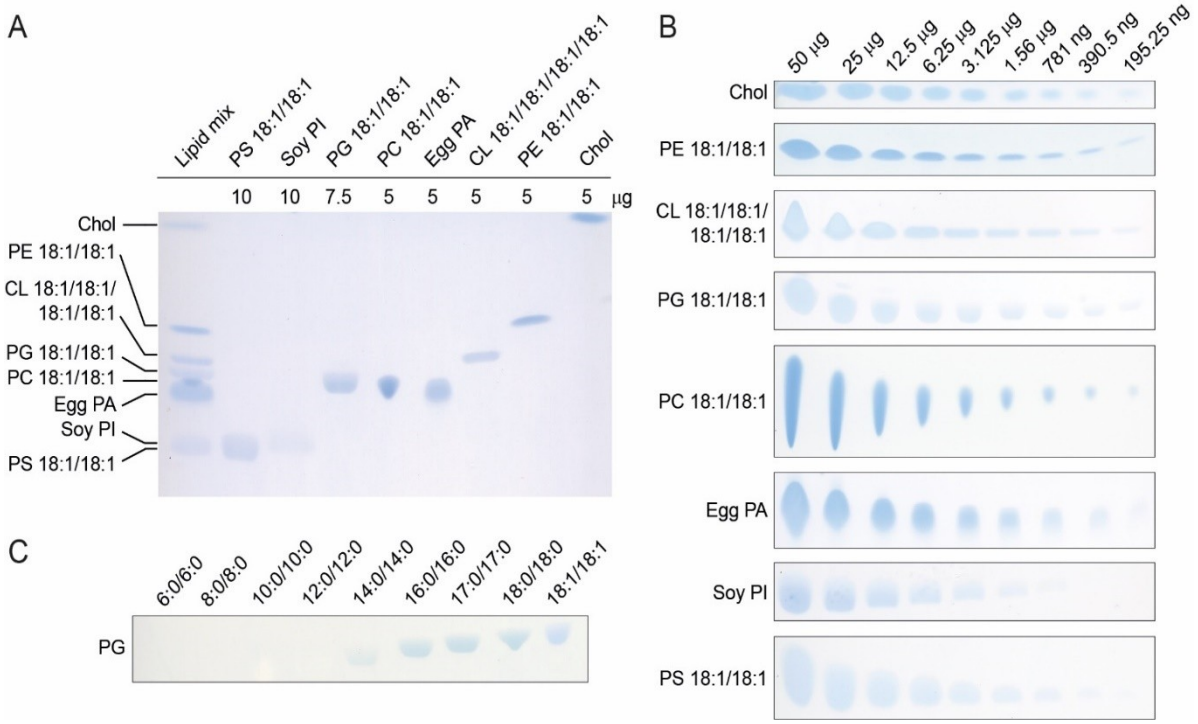

Figure 1. Coomassie staining of lipids separated by TLC. (A) PS 18:1/18:1, Soy PI, PG 18:1/18:1, PC 18:1/18:1, Egg PA, CL 18:1/18:1/18:1/18:1, PE 18:1/18:1 and Chol as well as a mixture of all lipids were separated by TLC and subsequently stained with Coomassie. Amounts applied to the TLC plates were adjusted to achieve visible staining. **(B)** Decreasing amounts of the lipids ranging from 50 µg to 195.25 ng were applied to the TLC plates and, after separation, stained with Coomassie. **(C)** 5 µg of phosphatidylglycerol species varying in length of the fatty acyl chains were loaded onto a silica plate, separated by TLC and explored for Coomassie staining.

Extraction of lipids from Coomassie-stained TLC spots

Having evaluated Coomassie staining for lipids separated by TLC, we next established an extraction protocol for subsequent MS-based lipid identification (Figure 2). For this, we applied four phospholipid species to silica TLC plates and, following TLC separation, stained the lipids with Coomassie as described (Figure 2 – step I). The stained lipid spots were then scraped off the TLC plate and the silica material was collected in a sample tube (Figure 2 – step II). Next, the lipids were extracted from the silica material by sequential addition of chloroform, methanol and water (see **Methods** for details). The addition of water induced formation of two phases: an upper, predominantly aqueous phase and a lower, organic phase. Extracted lipids reside in the organic phase while salts and most of the Coomassie migrate to the aqueous phase (Figure 2 – step III). The silica gel remains in the aqueous phase or at the interface of the two phases. The lipids extracted from the different spots are subsequently analysed by direct-infusion MS. According to their charge, PG and PS lipids were analysed in negative ion mode while PC and PE lipids were analysed in positive ion mode. The acquired mass spectra showed the expected mass-to-charge ratios (*m/z*'s) of the dioleoyl-species (Figure 2 – step IV).

TLC pre-separation of lipid extracts leads to higher identification rates

We next set out to compare direct-infusion lipid analysis with TLC pre-separation prior to MS analysis. For this, we used a commercially available *E. coli* extract containing PG, PE and CL phospholipids,[20] therefore, representing a natural lipid extract of intermediate complexity. We first analyzed the lipid extract by direct-infusion MS in negative and positive ion modes. For this, the sample was loaded into the emitter and, following electrospray ionization, lipid species were observed in the acquired mass spectra. Abundant precursor ions were then selected for fragmentation to identify their lipid head groups and fatty acyl chain composition. Importantly, dependent on the growth phase of *E. coli*, unsaturated fatty acids are modified by addition of methylene groups to *cis* double bonds yielding cyclopropane fatty acids.[21] Employing HCD or CID fragmentation, does not allow discrimination between odd-numbered fatty acyl chains with one double bond and cyclopropane fatty acids. Identification of lipid species with odd-numbered fatty acyl chains and one double bond is therefore ambiguous (Table S1).

In negative ion mode, multiple lipid signals were observed between *m/z* 700–800 and *m/z* 1350–1500 corresponding to PG and CL lipids, respectively (Figure 3). Following tandem MS, we assigned ten and 16 PG and CL lipid species, respectively (Table S1). Note that additional signals corresponding to PG

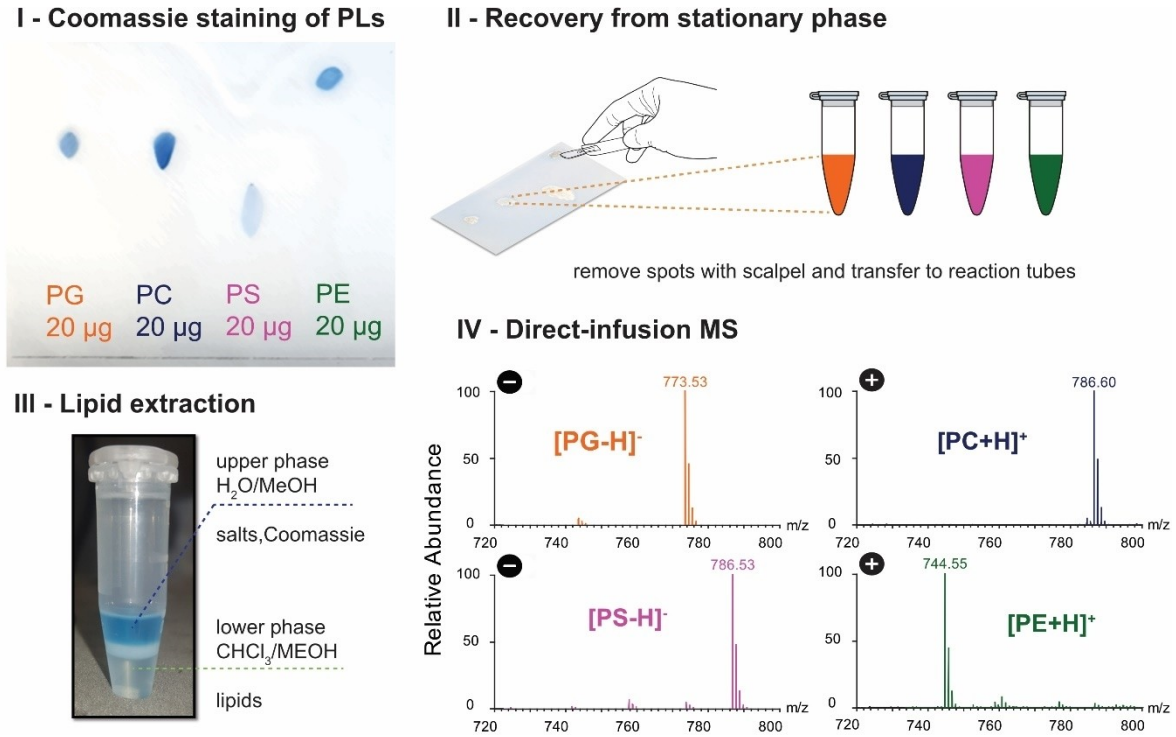

Figure 2. Workflow for MS-based identification of lipids from Coomassie-stained TLC spots. I: 20 µg of PG 18:1/18:1, PC 18:1/18:1, PS 18:1/18:1 and PE 18:1/18:1 were applied to a silica plate and subsequently separated by TLC and stained with Coomassie as described. **II**: Stained spots were removed from TLC plates using a scalpel. **III**: Lipids were extracted from TLC silica gel using chloroform, methanol and water. The lipids were obtained from the lower, organic phase. **IV**: MS of extracted lipids. PG 18:1/18:1 and PS 18:1/18:1 were analysed in negative ion mode. PC 18:1/18:1 and PE 18:1/18:1 were analysed in positive ion mode. All lipid species were identified by their correct m/z.

and PE gas phase dimers ($[PG+PG-H]^-$ and $[PE+PE-H]^-$) were also observed at *m/z* 1400–1500. These dimers most likely form during electrospray ionization and originate from high concentration of PG and PE lipids in the electrospray droplets; however, to identify low intense CL species, we refrained from diluting the lipid extract. In positive ion mode, 23 PE lipid species were identified at *m/z* 700–800 (Table S1).

We then used the same lipid extract, and separated the lipids according to their class by TLC. Using a lipid mixture of known composition for comparison, Coomassie staining confirmed the presence of PG, PE and CL (Figure 3). Stained lipid spots were then recovered from the TLC plate and lipids were extracted from the silica material. Subsequently, extracted lipids were analyzed as described above for direct-infusion experiments. Again, PG and CL species were identified in negative ion modes in the *m/z* range of 700–800 and 1350–1500 (Figure 3). However, pre-separation of the lipid extract increased the identification rate to eleven and 36 individual PG and CL lipid species. In positive ion mode, again, 23 PE lipids were identified at *m/z* 700–800 (Figure 3 and Table S1). In summary, TLC pre-separation of the lipid extract increased the identification from 49 to 70 individual lipid species.

These experiments showed, as expected, that pre-separation of a lipid mixture of intermediate complexity into lipid classes increases the number of individual lipid species identified from the same sample amount. This was particularly striking for CL species. Here, signals of CL species overlapped with gas-phase dimers of PG and PE lipids complicating correct identification by direct-infusion MS. Overlap of isotope distributions of lipid species from different lipid classes is, naturally, prevented when separating lipid classes. The observed mass spectra are therefore less complex and peak selection and annotation are unambiguous. In addition, when using automated peak selection for precursor fragmentation as performed here, less complex mass spectra allow selection of more precursor ions corresponding to the same lipid class. A similar effect can be observed when increasing instrument resolution. High-resolution mass spectra allow separation and differentiation of isobaric compounds and consequently yield higher identification rates.

We conclude that TLC pre-separation and extraction of lipids from Coomassie-stained lipid spots, proved advantageous for the analysis of lipid mixtures. In particular, complexity of mass spectra comprising overlapping lipid species was reduced when compared with shotgun experiments therefore allowing selection and identification of additional lipid species. Similar effects were previously observed when including liquid chromatography pre-separation into the analysis workflow.[8a,9] While liquid chromatography has many advantageous over other pre-separation techniques, its application requires experience and specialized equipment. Our workflow, on the contrary, is simple and fast, and can be performed independently of subsequent experiments. In addition, TLC pre-separation provides knowledge on the lipid classes present in the

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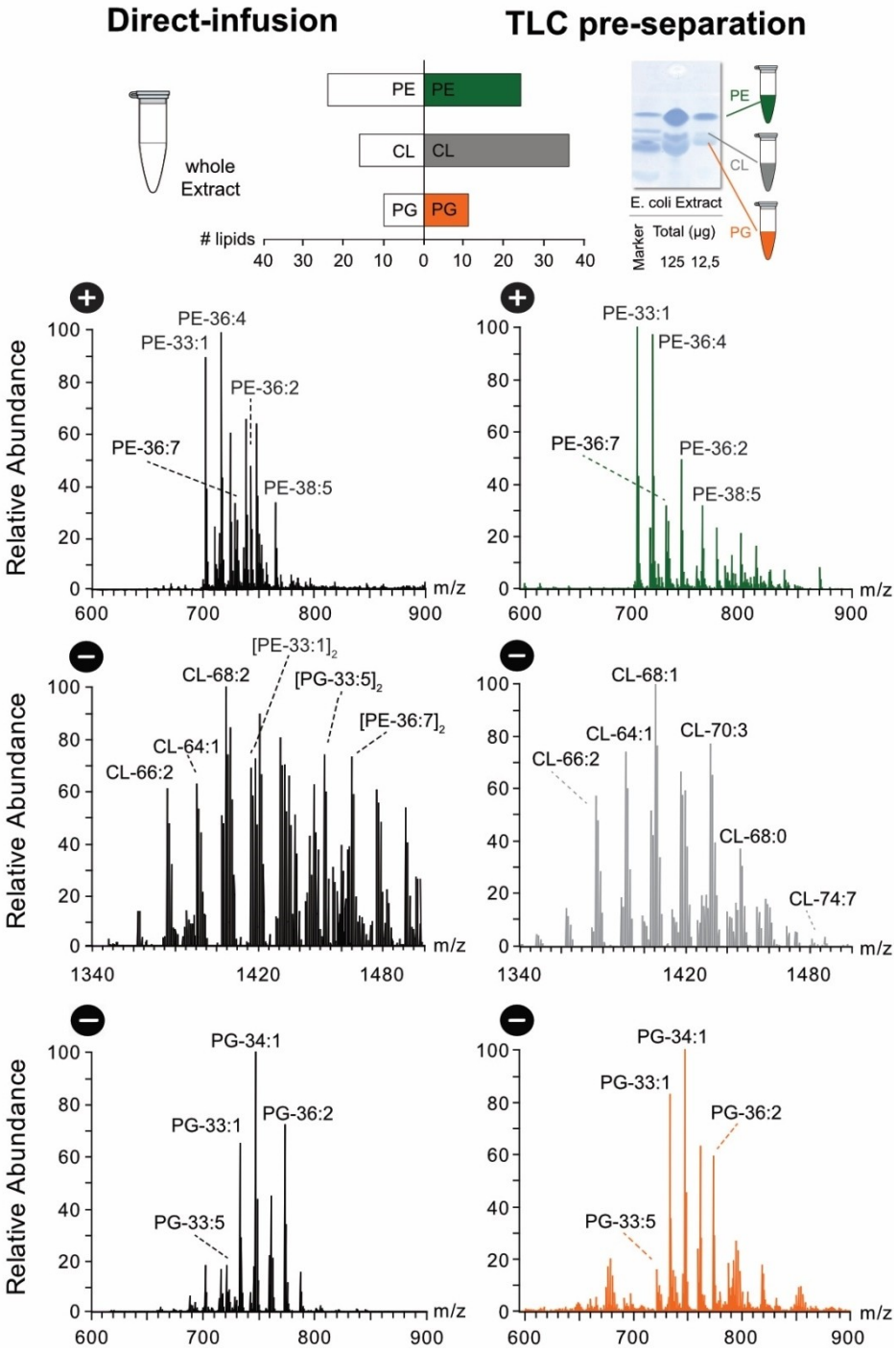

Figure 3. Lipid analysis of E. coli extract. E. coli extract was analysed in negative and positive ion modes after direct-infusion (left-hand side). 23 PE species (positive ion mode, top spectrum), 16 CL species (negative ion mode, middle spectrum) and 10 PG species (negative ion mode, bottom spectrum) were identified. For MS analysis after TLC pre-separation, lipids were extracted from Coomassie-stained spots as described. Lipid extracts were then analysed in negative and positive ion modes after direct-infusion (right-hand side). 23 PE species (positive ion mode, top spectrum), 36 CL species (negative ion mode, middle spectrum) and 11 PG species (negative ion mode, bottom spectrum) were identified. Note that identified lipid species are annotated at 'lipid species level' according to Liebisch et al.[22] (see **Methods** for details) because fragmentation of several m/z's identified different lipid isomers. Lipid species including identified isomers are listed in Table S1.

sample; selection of the ion mode, the m/z mass range or other experimental settings can be optimized before starting the actual measurements.

TLC pre-separation of complex lipid extracts uncovers additional lipid classes

We then moved forward and analysed a complex lipid mixture, namely a lipid extract from SH-SY5Y neuroblastoma cells. For

this, SH-SY5Y cells were grown under standard conditions and, following cell lysis, lipids were extracted as described (see **Methods** for details). Again, we compared direct-infusion MS analysis with TLC pre-separation of the lipid extract prior to MS analysis. Direct-infusion analysis revealed several PS, PC and PE lipid species. PS species were identified in negative ion mode while PE and PC lipids were identified in positive and, as Cl^--adducts, in negative ion modes. While in positive ion mode merely the lipid class could be confirmed through neutral loss of the choline (from PC) or ethanolamine (from PE) head groups, the analysis in negative ion mode allowed specification of the fatty acyl chains. In total, 42 lipid species corresponding to five PS, ten PC and 27 PE species were assigned (Table S2).

We then separated the lipid extract by TLC and stained the lipids with Coomassie as described. Several lipid spots were observed and, following extraction from the silica material, the lipids were analyzed by MS. Depending on the lipid class, negative or positive ion modes were employed. When compared with direct-infusion MS analysis, TLC pre-separation yielded additional lipid species resulting in 13 PS, 36 PC and 54 PE lipids (Figure 4). Importantly, TLC separation and Coomassie staining revealed two additional lipid spots allowing identification of additional lipid classes which were not identified by direct-infusion of the lipid extract; these include 5 PI and 15 SM species. In total, TLC pre-separation increased the number of identified lipid species from 42 to 123 (Table S2).

When analyzing complex lipid mixtures by direct-infusion MS, lipid species from different classes with similar masses overlap in the mass spectra and precursor selection and subsequent identification by tandem-MS might be hampered. This is particularly true for SM lipids which, due to their atomic composition, appear at odd *m/z*'s while abundant phospholipids such as PE and PC appear at even *m/z*'s.[7a] Therefore, the isotope envelopes of SM species likely overlap with those of other phospholipids in mass spectra obtained from direct-infusion analysis; consequently, low abundant SM species escape identification. Note that we employed automatic selection of precursor ions for tandem MS analysis. Lipids with overlapping isotope envelopes might have been missed during precursor selection. Similarly, PI lipids are often low abundant and abundant species might be preferred during MS/MS precursor selection. Pre-separation of the lipid classes, on the other hand, reduces the overall signal intensities and therefore allows selection of low-abundant lipids.

A recent study used LC-MS/MS and NMR spectroscopy to identify and quantify the lipidome of SH-SY5Y cells.[23] In this study, PC and PE lipids were most abundant (55% and 18%, respectively); low abundant lipids include PS (5%), CL (8%), PG (4%), PI (7%) and SM (3%) lipids. Even though our analysis was not quantitative, the number of lipid species identified for PC, PE, PI, PS and SM lipids after TLC separation agrees well with these findings. Note that we did not identify PG or CL species neither by direct-infusion analysis nor after TLC pre-separation. Reasons might be that Coomassie staining of negatively charged lipids requires higher sample amounts (see above) and, therefore, these lipids which were found to be under-represented in SH-SY5Y cells[23] were not visualized by Coomassie staining or, even though we observed low-intense signals in the *m/z* range of CL species by direct-infusion analysis, the ion intensities were not sufficient to acquire high-confident mass spectra. We conclude that pre-separation of complex lipid mixtures increases the number of identified lipid classes and species and enables identification of low-abundant lipids presupposing that lipid spots are sufficiently stained and visible to the naked eye.

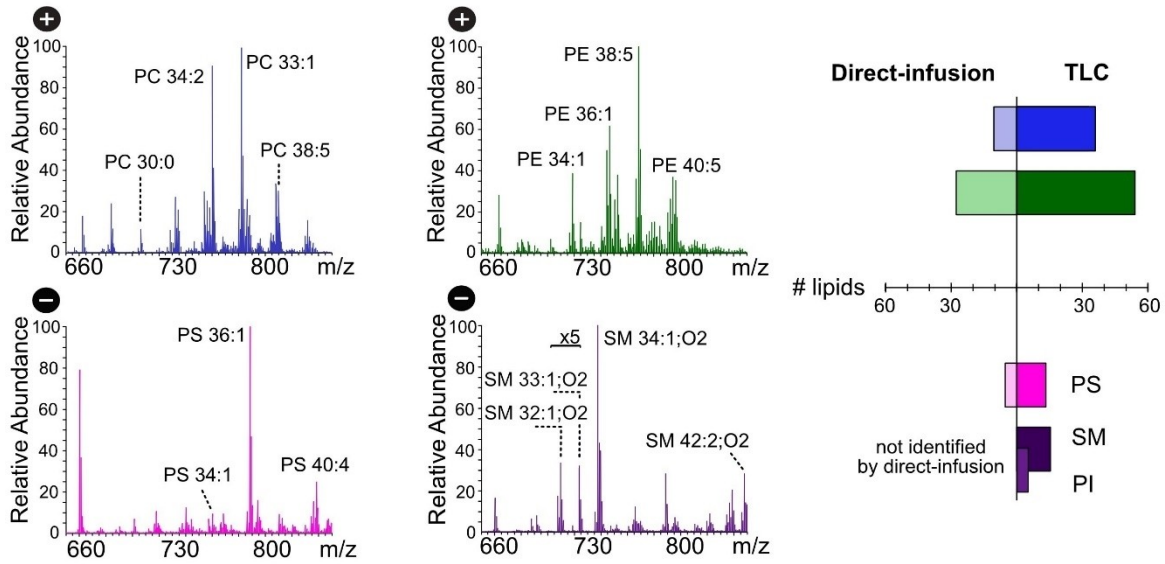

Figure 4. Lipid analysis of SH-SY5Y lipid extract. Example spectra of PC (blue), PE (green), PS (pink) and SM (purple) lipid species are shown. The number of identified lipid species after direct-infusion (left-hand side) or after TLC pre-separation (right-hand side) is compared (bar diagram). SM and PI lipids were only identified after TLC separation. Note that identified lipid species are annotated at 'lipid species level' according to Liebisch et al.[22] (see **Methods** for details) because fragmentation of several m/z's identified different lipid isomers. Lipid species including identified isomers are listed in Table S2.

Identification and quantification of the lipid composition of SMA(2:1) polymer nanodiscs

Finally, we applied our workflow for identification and quantification of lipids in polymer nanodiscs. To mimic a native-like membrane environment, nanodiscs are often prepared from natural lipid extracts. We therefore prepared nanodiscs from lipid vesicles composed of natural PC, PE, PI and PG extracts with a proportion of 17.65% PC, 25.2% PE, 3.15% PI and 54% PG using the SMA(2:1) polymer (see **Methods** for details). Nanodisc preparation was confirmed by dynamic light scattering and negative stain electron microscopy (Figure S1). The lipids of assembled nanodiscs were then extracted from the discs, separated by TLC and subsequently analyzed by MS as described above (Figure 5). Again PG and PI lipids were analyzed in negative ion mode, while PE and PC lipids were analyzed in both, negative and positive, ion modes. Following this workflow, we identified a total of 78 lipid species corresponding to the four lipid classes used for nanodisc preparation. Specifically, we identified 19 PC, 28 PE, 14 PI and 17 PG lipids (Table S3).

We then quantified the proportion of each lipid class after extraction from the nanodiscs. For this, the lipid extract of each TLC spot was spiked with a mixture of standard lipids containing 10 pg of deuterated or short-chain lipid analogues of each lipid class. Each lipid species was then quantified by

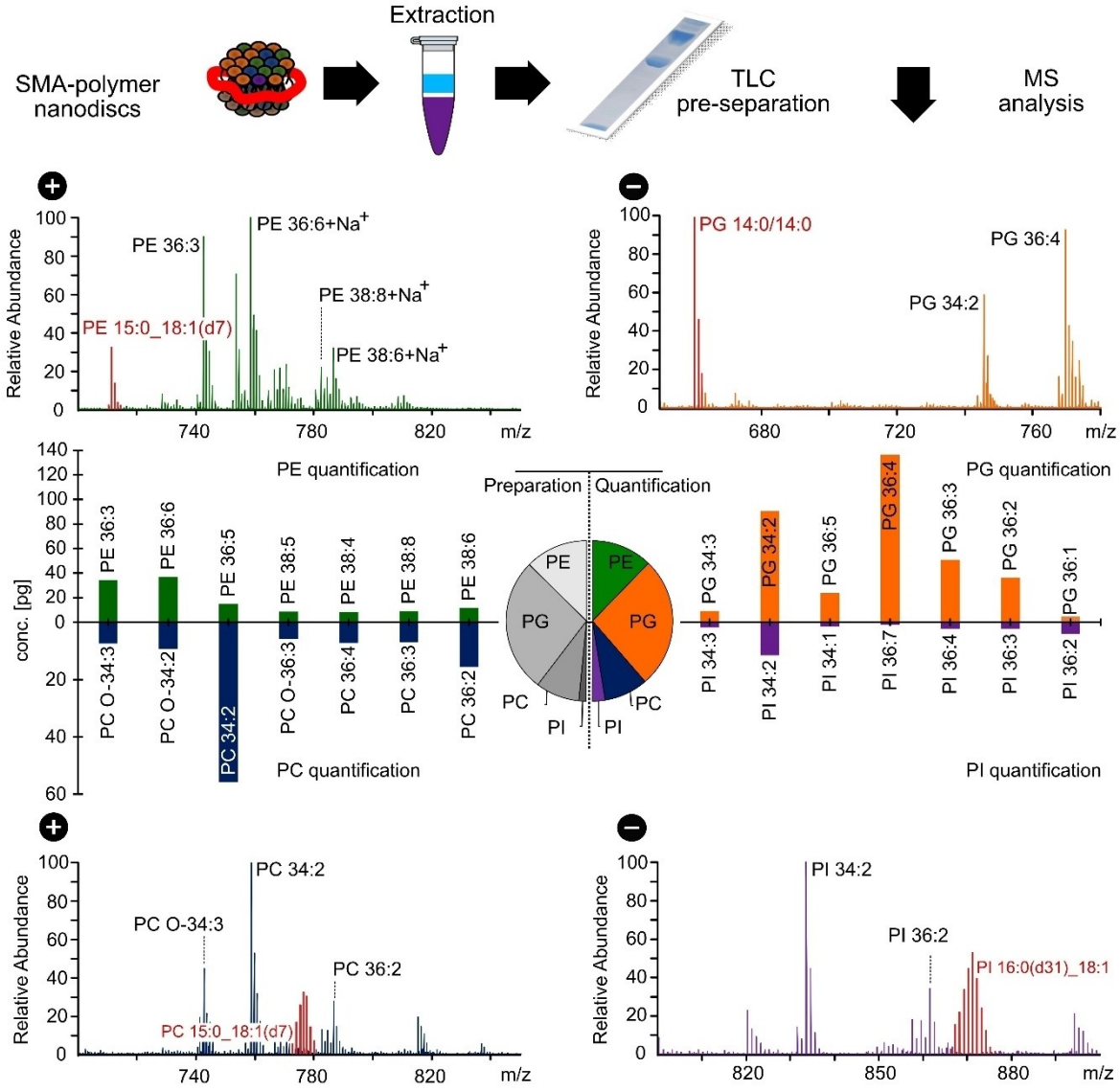

Figure 5. Lipid analysis of SMA(2:1) polymer nanodiscs. Lipids were extracted from the nanodiscs and pre-separated by TLC. Subsequent MS analysis was performed in positive or negative ion mode. To determine the proportion of each lipid class, extracted lipid spots were spiked with deuterated or short-chain standard lipids. The lipid species were quantified by comparing peak intensities of the corresponding standard lipid (red) with the respective lipid species. Mass spectra for PE (green), PG (orange), PC (blue) and PI (purple) lipids as well as quantitative amounts (bar diagrams) of the identified lipid species are shown. The pie chart visualises the relative lipid proportions of nanodisc preparation (grey shades) and the experimentally determined relative proportions (PE, green; PG, orange; PC, blue; PI, purple). Note that identified lipid species are annotated at 'lipid species level' according to Liebisch et al.[22] (see **Methods** for details) because fragmentation of several m/z's identified different lipid isomers. Lipid species including identified isomers are listed in Table S3.

comparing the intensity of the respective standard lipid with the intensity of the lipid species (see **Methods** for details, Table S3). For this, all isotope peaks were included during quantification and isotope impurities can therefore be excluded. We then calculated the relative proportion of each lipid class in the nanodisc by summing up the intensities of all lipids species of each lipid class. Following this procedure, we determined relative proportions of 17.7% PC, 25.5% PE, 5.08% PI and 52.5% PG corresponding well with the lipid proportions employed for nanodisc preparation (Figure 5).

These experiments showed that our workflow can, in principle, be applied to any biological or biochemical sample. The identification and quantification of lipids in nanodiscs, as shown here, is of particular interest for two reasons: First, the integration of different lipid classes and species into the nanodiscs during its assembly has to be carefully evaluated and verified to ensure that the desired membranes are used for subsequent experiments. Second, nanodisc polymers can be employed to solubilize natural lipid membranes; the identification and quantification of the lipid content in these membrane sections provides valuable insights into the composition of the natural membranes, including, for instance, the lipid environment of specific membrane proteins.[24] The analysis of the lipid content of nanodiscs was previously assessed by high-resolution native MS.[25] Identifying unknown lipid mixtures, our approach is a valuable addition to the available toolbox of MS for studying lipid nanodiscs. We envision that our workflow is also applicable to separate proteins and lipids co-purified from biological membranes.

Conclusions

We introduced a workflow combining TLC separation of lipid mixtures with MS analysis of extracted lipids. We demonstrate that Coomassie staining of the lipids is compatible with subsequent MS measurements, and that TLC separation increases the identification rate of lipid species as well as simplifies analysis of different lipid classes with overlapping *m/z* ratios. We successfully applied our workflow to complex lipid mixtures as well as to polymer nanodiscs composed of natural lipid extracts and showed that identification of low abundant lipids is improved.

Comparing Coomassie staining with commonly applied separation techniques such as liquid chromatography-based pre-separation or TLC separation with subsequent Primuline staining, it becomes apparent that all techniques have their own advantages and disadvantages as opposed in Table 1. Briefly, Primuline and Coomassie staining are rather similar and comparable (Figure S2). Primuline is very popular because it does not covalently modify the lipids and MS analysis can be performed without destaining of the TLC plates.[12] However, Primuline staining requires additional equipment such as a UV detector/lamp and preferably a spraying chamber; lipid spots are not visible by eye. Coomassie staining, on the other hand, is in principle also applicable without destaining of the TLC plates and stained lipids are immediately recognized (Figure S2). A disadvantage of both techniques is, however, sample loss due to strong binding of lipids to the silica material of TLC plates.[26] Nonetheless, both strategies are easy to perform, cost-efficient and compatible with MS analysis. Comparing the two staining protocols with liquid chromatography-based separation, the latter requires expensive equipment and an experienced operator. However, sample losses can be neglected, sample preparation is simplified and high-through analyses are possible when using liquid chromatography-separation (see Table 1 for details).

In summary, our workflow described here has the following advantages: (i) A simple set-up is employed for lipid separation; specialized experience and equipment such as a UV detector or a spraying chamber are not required. (ii) Coomassie is non-hazardous and easy to dispose. (iii) Coomassie does not covalently modify the lipids. (iv) Coomassie is cost-efficient and commonly available in most biochemical laboratories. (v) Lipid separation, extraction from TLC plates and subsequent MS analysis can be performed in independent experiments as required and feasible. (vi) Pre-separation of lipids provides previous knowledge on the sample allowing to optimize specific experimental parameters such as ion mode or *m/z* scan range prior to MS measurements. Moreover, the workflow can

Table 1. Comparison of TLC separation with Coomassie or Primuline staining as well as liquid chromatography (LC)-based pre-separation.[g]

	TLC + Coomassie-staining	TLC + Primuline-staining	LC pre-separation
Destaining of TLC plate	-[a]	+	n/a
Extraction from TLC plate	- -	- -[b]	n/a
Pre-separation independent of MS analysis	+ + +	+ + +	- - -
Non-covalent modification of the lipids	+ + +	+ + +	+ + +
Sample information prior to MS analysis	+ + +	+ + +	- - -
Sample loss	- -[c]	- -[c]	+ + +[d]
Specialized (expensive) equipment	+ + +	+ +[e]	- - -
Pricing of consumables	+ + +	+ +	- - -
Special experience required	+ + +	+ + +	- - -[f]
High-throughput	- - -	+[b]	+ + +
Total time consumption	- - -	- -[b]	+ + +

[a] Destaining not necessarily required (see Figure S2); [b] On-plate analysis by MALDI-MS is possible; [c] Relative composition of lipid mixtures remains after TLC[26]; [d] In theory, there is no sample loss during LC-separation; however, specialized experience is required for optimal experimental performance; [e] UV lamp required; [f] Maintenance and method development require an experienced operator; [g]Different aspects are listed and the methods are classified as advantageous (+ + +, + + and +) and disadvantageous (- - -, - - and -).

be easily adjusted to any additional sample requirements. We envision that this protocol will be adapted to many different applications in future.

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Conflict of Interest

The authors declare no conflict of interest.

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