

References

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- [2] B. Fuchs et al. (2008) Analysis of stem cell lipids by offline HPTLC-MALDI-TOF, ABC 392: 849-860
- [3] Bruker Daltonics Application Note MT-94 (2009) Direct Read-out of Thin Layer Chromatography (TLC) using MALDI-TOF
- [4] I.C. Guerrero et al. (2009) A Novel Lipidomic Strategy Reveals Plasma Phospholipid Signatures Associated with Respiratory Disease Severity in Cystic Fibrosis Patients, PLoS One 4, 11, e7735

*The use of larger TLC plate formats and of glass or plastic backing instead of aluminum is discouraged, as the quality of the read-out might be impaired.

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HPTLC-MALDI Adapter Target

Bruker Daltonics



Application Note # MT-101

High Resolution Lipid Profiling and Identification by Hyphenated HPTLC-MALDI-TOF/TOF

Combining HPTLC with MALDI-TOF MS provides a new method for high-resolution molecular analysis direct from a HPTLC plate. The highly automated, software-supported technique is suitable for analysis of lipids in clinical, nutritional, pharmaceutical and cosmetic studies. Two dimensional analysis by HPTLC and MALDI-TOF MS greatly simplifies the structural elucidation of lipids in mixtures and a subsequent targeted MS/MS acquisition further increases specificity.

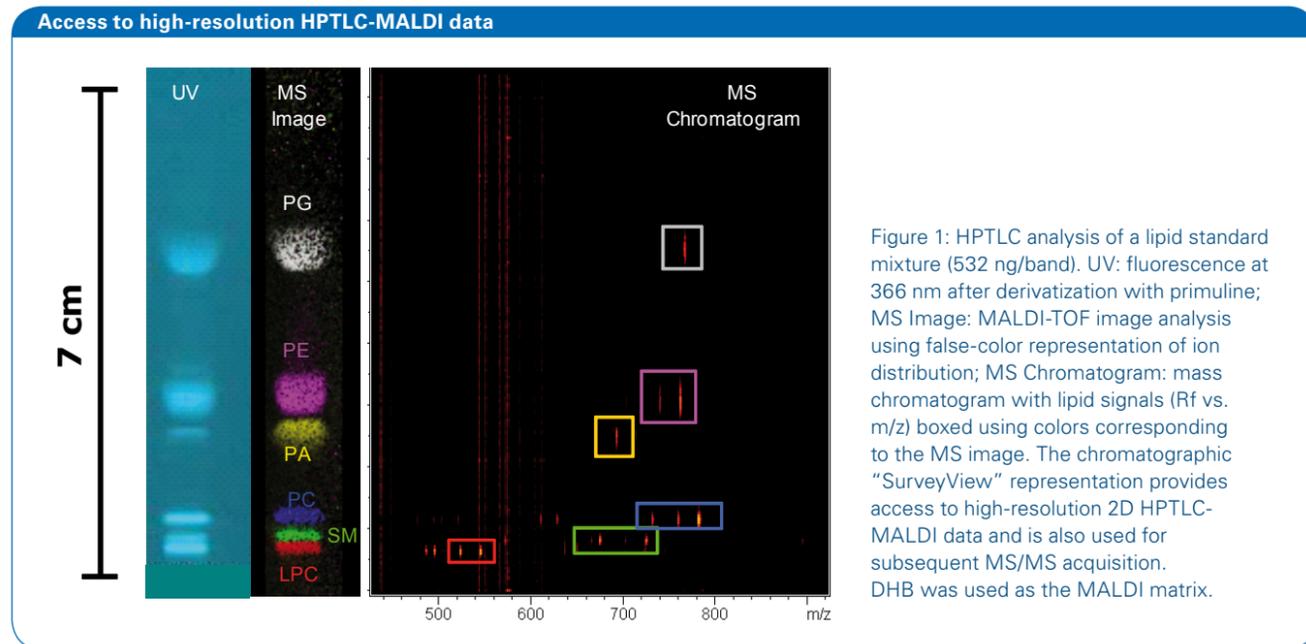
Introduction

High-performance thin layer chromatography (HPTLC) is an efficient method routinely used for analysis of biological lipids in complex mixtures. The method is mature and low-cost, robust automation is available. However, derivatization reactions used for visualization do not provide sufficient distinction between closely related groups of lipids – such as phosphatidylcholines (PC) and phosphoethanolamines (PE) – or lipids with small differences in acyl chain length. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) together with HPTLC provides high-resolution molecular analysis direct from HPTLC plates [1, 2]. Here we demonstrate HPTLC-MALDI-MS/MS analysis of lipid mixtures, a technique that provides additional structural information on headgroup and

acyl chain composition and enables detailed assignment of lipid structures – even if mixtures are only partially resolved by the chromatographic separation.

Experimental/Methods

HPTLC: HPTLC silica gel 60 F₂₅₄ aluminum backed sheets* (Merck, #1.05556.0001) were pre-run with water and methanol (1:3, v/v) and dried at 120 °C for 20 min. Lipid standards (10 mg/ml solutions in chloroform) were obtained from Avanti Polar Lipids and further diluted 1:7.5 with chloroform. Samples were applied as bands (band length 4 mm, track distance 8 mm, dosage speed 90 nl/s, application volume 1-4 µl) using the Automatic TLC Sampler (ATS 4, CAMAG). Chromatography of lipids and phospholipids was performed in a Twin Trough Chamber (10 x 10 cm, CAMAG) with 5 ml chloroform, ethanol, water and triethylamine (5:5:1:5, v/v/v/v) as the developing solvent, up to a migration distance of 70 mm from the bottom edge. Tracks were marked with a soft pencil after visualization under UV illumination (366 nm, TLC Visualizer [CAMAG]). For visualization with primuline (100 mg primuline dissolved in 200 ml water and acetone [1:4, v/v]), the plate was dipped (vertical speed 2.5 cm/s, immersion time 1 s) using the Chromatogram Immersion Device (CAMAG). The plate was dried in a stream of warm air for 1 min after application, development and derivatization.



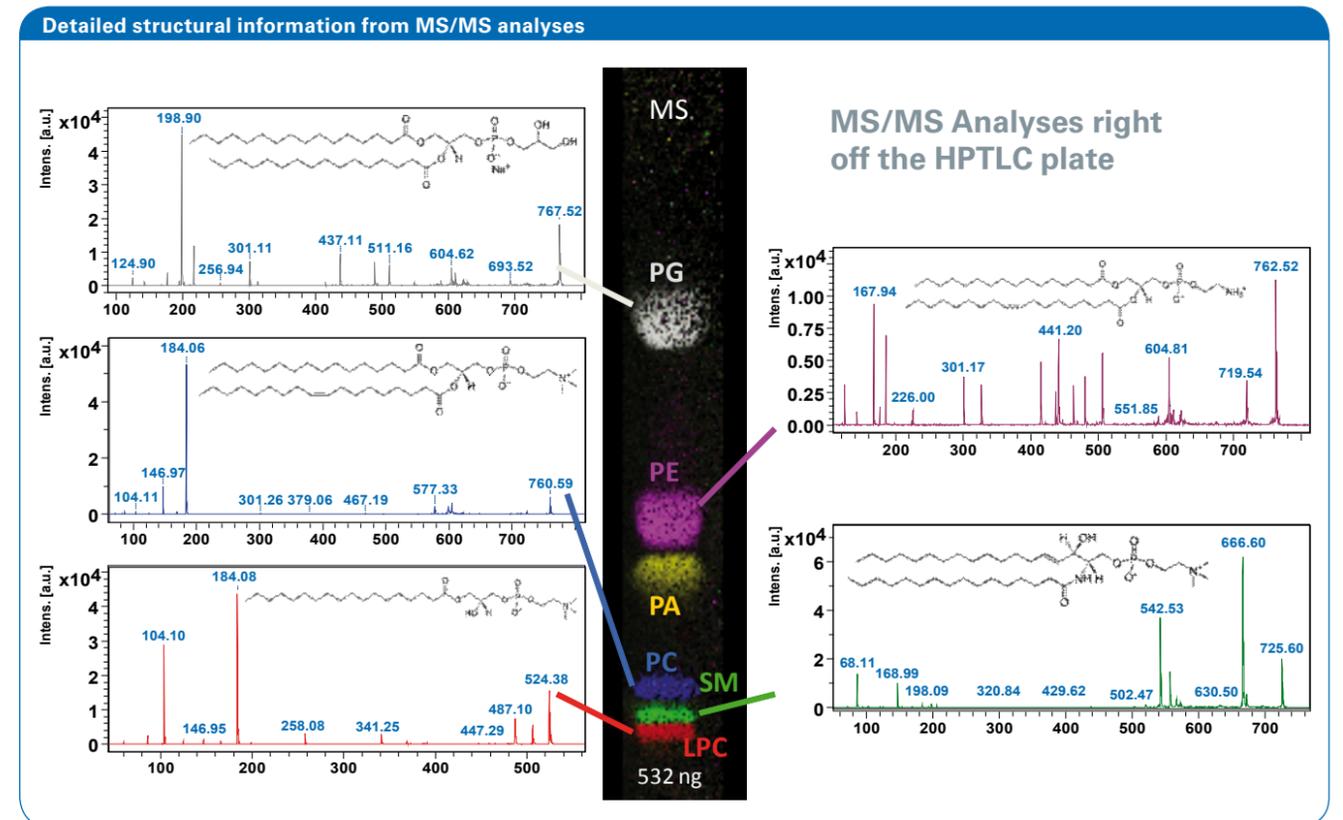
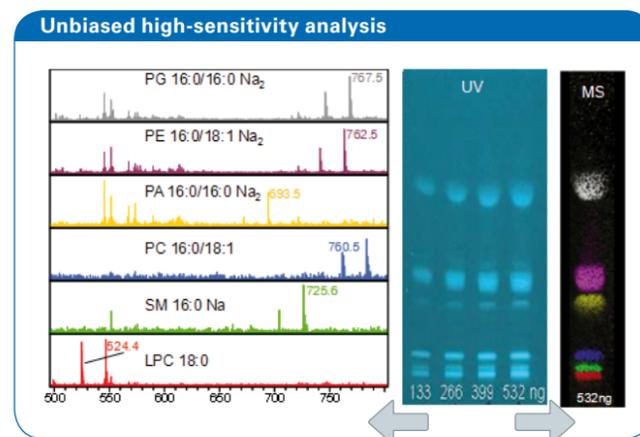
Coupling of HPTLC with MALDI-TOF MS:

HPTLC plates were homogeneously coated with DHB matrix (50 mg/ml in 50% acetonitrile) using the ImagePrep™ coating device (Bruker Daltonics) and the standard preparation method "TLC_DHB". The HPTLC plate was mounted into a TLC-MALDI adapter target (Bruker Daltonics, Part No. # 255595) and analyzed by MALDI-MS with raster widths of 100-400 μm. MALDI-MS imaging of HPTLC tracks was performed using an ultrafleXtreme™ MALDI-TOF/TOF (Bruker Daltonics) with 1 kHz smartbeam™ laser in reflector mode and an acquisition time of around 5 min per track. The MS Image was generated using flexImaging™ 2.0 with a pixel raster of 400 μm x 400 μm spots and 200 laser shots per pixel. The MS Chromatogram was created using a new dedicated software tool for TLC-MALDI (supplied with Compass 1.3) that derives the geometry of chromatographic tracks on the HPTLC plate from x- and y-scales on the adapter target. MS/MS spectra were obtained without the addition of a collision gas using Compass 1.3, which, guided by the MS information, provided direct access to any HPTLC fraction.

Figure 2: Sensitivity of HPTLC-MALDI: A dilution series of lipid standards containing 133-532 ng of each lipid/band was analyzed. Spectra were acquired from the 133 ng/band sample and MS imaging was performed on the 532 ng/band sample. PG: Phosphatidylglycerol; PE: Phosphatidylethanolamine; PA: Phosphatidic Acid; PC: Phosphatidylcholine; SM: Sphingomyelin; LPC: Lysophosphatidylcholine.

Results

Analysis methods: The HPTLC-separated lipids were analyzed using three different approaches (see Figure 1). Primuline, which fluoresces under UV light, was used for direct visualization of lipids on the TLC plate. Because primuline's interaction with lipids is non-covalent, it does not impair MALDI analysis and is compatible with generation of high-quality lipid mass spectra. However, the optical readout does not resolve overlapping lipids on the HPTLC plate. TLC-MALDI imaging of entire chromatographic tracks enables molecular analysis of separated lipids and distinction between overlapping molecular species based on their Extracted Ion Chromatograms. The close correlation between the UV and MS images indicates that the matrix application step does not delocalize the analytes.



Although the MS imaging readout compares favorably with classical stains, this method is too laborious and time-consuming for routine analyses. Recording along chromatographic tracks significantly reduces acquisition times and provides immediate access to the information contained in the HPTLC-MALDI dataset. Acquisition times are shortened from hours (MS images) to ~5 min/track (MS chromatogram) and the size of data files is dramatically reduced [3]. Interestingly – and to the surprise of many researchers interested in this technique – even after several years of TLC-MALDI experience no instrument problems caused by chromatographic phase debris in the MALDI ion source have been observed.

Sensitivity: An important advantage of hyphenated HPTLC-MALDI-TOF MS is the elimination of manual scraping off and elution of bands that are detected by staining. Therefore, the structural analysis can take full advantage of the chromatographic separation and does not focus just on analytes of interest. Figure 2 demonstrates that even bands containing just 133 ng lipid provided intense MALDI spectra that could be easily interpreted. The significant parent ions are marked and indicate [M+H]⁺ or sodiated signals.

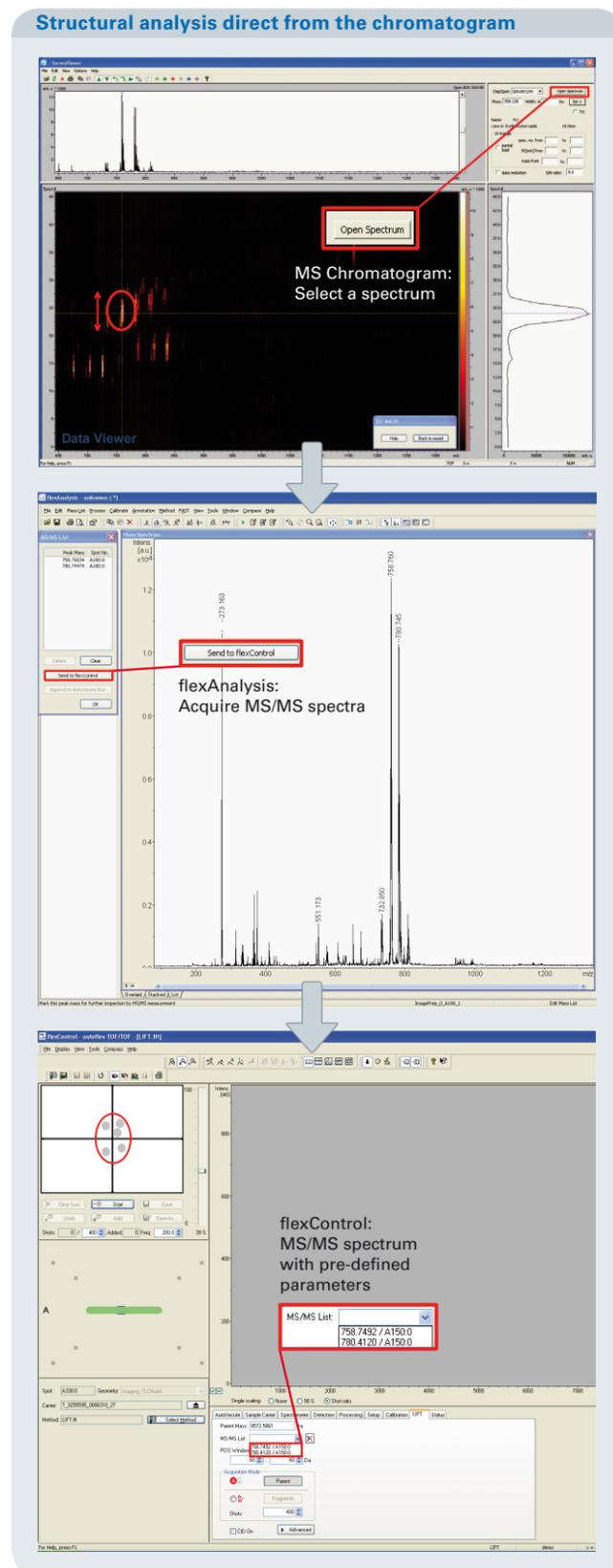


Figure 4: Workflow of MS/MS spectra acquisition based on the MS Chromatogram representation. Software-supported MALDI-TOF/TOF MS/MS spectra acquisition after MS acquisition from the HPTLC plate.

Structural analysis: After determination of parent ion molecular weights, further structural information can be obtained by MS/MS on a MALDI-TOF/TOF mass spectrometer as shown in Figure 3. The TLC-MALDI software supplied with Compass™ 1.3 enables selection of parent ions generated from TLC plates and directly obtaining MS/MS information, without any loss of chromatographic resolution. For the straightforward analysis of bands from an unstained HPTLC plate, the TLC-MALDI DataViewer™ (Figure 4) enables manual “peak picking” and acquisition of MS/MS spectra from the respective parent ions within a few seconds. The band’s spectrum is opened in flexAnalysis™ and a precursor ion selected for MS/MS. In flexControl™, position and parent mass are automatically pre-defined for MS/MS acquisition.

A detailed analysis of the MS/MS spectrum of lysophosphatidylcholine is shown in Figure 5. Several fragments could be assigned to lipid moieties (marked in the lipid structure representation), including the headgroup (m/z 184). The quick and simple recording of MS/MS spectra from developed HPTLC-MALDI datasets can increase the specificity of lipid identification, even when samples are chromatographically unresolved. Table 1 lists MS/MS fragment ions and structural elements of the reference lipids used in this study.

Conclusions

The chromatographic resolution of HPTLC is maintained throughout the MALDI analysis due to automated matrix application methods that do not delocalize analytes after separation. Reproducibility of HPTLC-MALDI band intensity is in the range of $\pm 10\%$ (data not shown) and sensitivity for lipids is in the 100 ng/band range. A major advantage of this technique is that MS/MS analysis can be triggered directly after completion of the HPTLC-MALDI-MS scan to provide additional, detailed structural information. The numerous benefits and easy implementation offered by HPTLC-MALDI make it extremely attractive for clinical [4], nutritional, pharmaceutical and cosmetic lipid analyses.

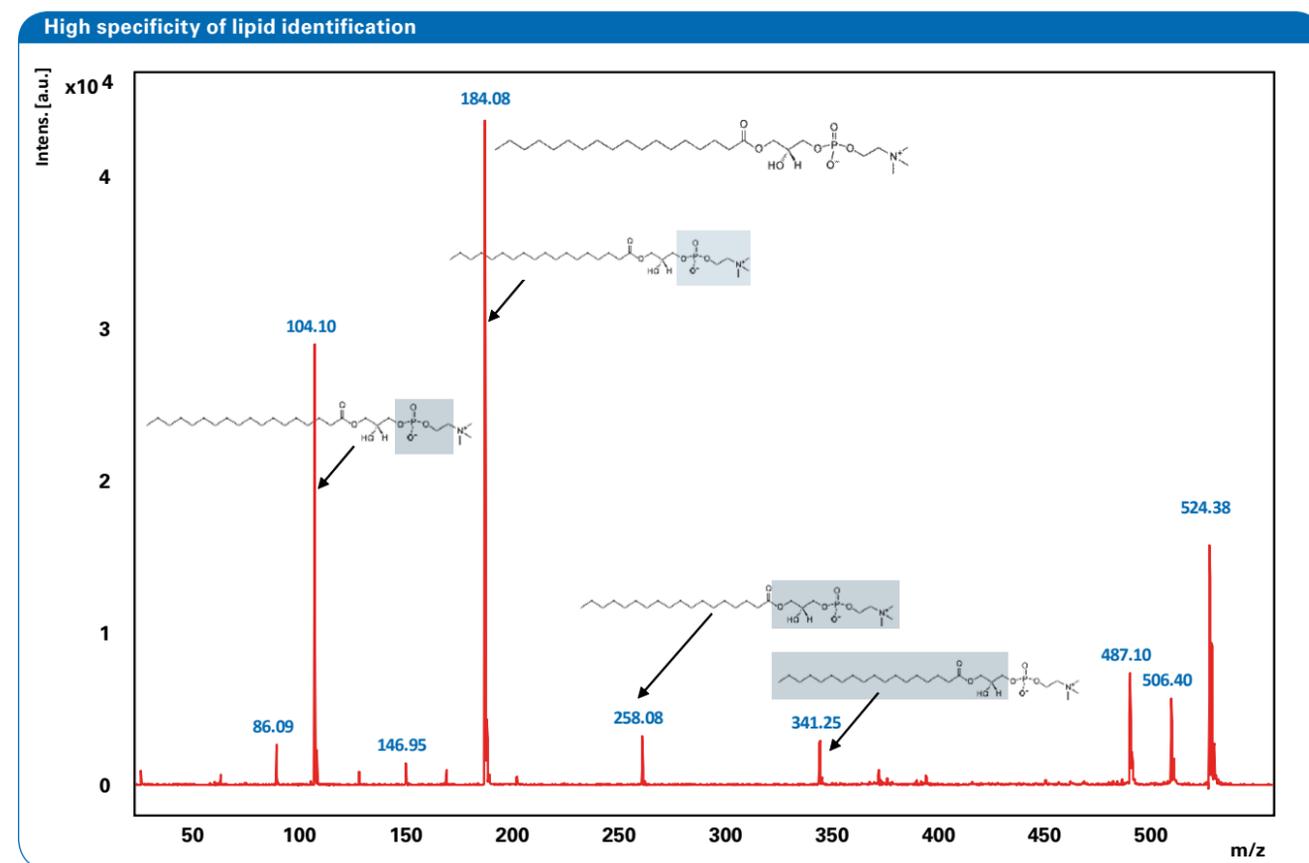


Figure 5: MS/MS spectrum of LPC with assignments of structural elements to the major fragment ions.

Parent Ion	Typical Fragments	
	Headgroup	Tail*
PG 16:0/16:0 [M+2Na-H] ⁺ 767.480	198.9, 216.9	437.1, 489.3, 511.2
PE 16:0/18:1 [M+2Na-H] ⁺ 762.502	167.9	301.2 (sn ₁), 327.2 (sn ₂), 415.2 (sn ₁), 441.2 (sn ₂)
PA 16:0/16:0 [M+2Na-H] ⁺ 693.444	124.9, 142.9	437.2
PC 16:0/18:1 [M+H] ⁺ 760.585	184.1	239.2 (sn ₁), 265.2 (sn ₂), 577.5
SM 16:0 [M+Na] ⁺ 725.556	86.1, 146.9	542.3, 666.4
LPC 18:0 [M+H] ⁺ 524.363	184.1	258.1, 341.2

* Fragment ions specific for the sn₁ and sn₂ substituents are indicated.

Table 1: Reference lipids used in this work and characteristic parent and fragment ion m/z values obtained from TLC-MALDI-TOF/TOF measurements.