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Liquid Extraction Surface Analysis (LESA) of Hydrophobic TLC Plates Coupled to Chip-Based Nanoelectrospray High-resolution Mass Spectrometry

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Abstract: Direct identification and structural characterization of analyte spots on TLC plates have always been of great interest and the development of interfaces that allow TLC to be combined with MS is making steady progress. The recently introduced liquid extraction surface analysis (LESA) approach has the potential to hyphenate TLC with MS. A mixture of lipid standards was separated on HPTLC RP-18 glass plates using chloroform:methanol :acetonitrile 2:1:1 (v:v:v) as mobile phase. After visualization with primuline dye (0.02% in acetone:water 8:2 (v:v)), LESA was performed, followed by a chip-based nanoflow infusion in combination with FTICRMS. The optimized extraction solvent composition was methanol:chloroform:water:formic acid 52:24:24:0.2 (v:v:v:v). A nanoelectrospray voltage of 1.6 kV and a gas pressure of 0.2 psi were applied in all experiments. All phospholipids were extracted successfully and detected unambiguously using the optimized TLC-LESA-FTICRMS procedure. Sampling the tricaprylin spot gave the most intense signals and also tricaprin was detected. Three other triacylglycerols of higher molecular mass have logP values between 15.5 and 21.6, which are the highest among all investigated compounds and are not detected from their corresponding spots, due to the fact that the solubility of very apolar lipids is not high enough in the extraction solvent. It was demonstrated that TLC can be elegantly combined with mass spectrometry based on the LESA approach. In general, apart from the analysis of lipids, TLC-LESA-MS has a high potential for medium-polar compounds separated on reversed-phase TLC plates, but limitations are present when very apolar compounds have to be extracted.

Keywords: Chip-based infusion · LESA · Lipids · Liquid extraction surface analysis · Thin-layer chromatography

1. Introduction

Thin-layer chromatography (TLC) is a rather simple, fast and relatively inexpensive chromatographic technique that has been used in laboratories for decades to separate various classes of chemical compounds. Usually the spots on the TLC plate are detected by optical methods, which in most cases are sufficient for analyte assignment and even quantification in routine analysis, but structural characterization and unequivocal identification are not possible with these techniques. Mass spectrometry (MS) is able to provide significantly more information regarding the spots on a plate but combining TLC with MS is not a straightforward process because of the off-line character of the TLC-MS hyphenation. Nowadays the two most successful elution-based TLC-MS hyphenations are the surface sampling probe (SSP)[1] by Van Berkel, which is a variation of the model described by Wachs and Henion,[2] and an extraction system based on the work of Luftmann.[3] Both devices elute the TLC spot rapidly with a continuous flow of extraction solvent that is then directed to the MS ion source. The SSP is based on the formation of a stable liquid micro junction (LMJ) between the TLC surface and the probe within a small distance, whereas the design by Luftmann uses a cutting edge to establish a tight sealing. The latter instrumentation has been continuously improved[4] and also represented a more versatile approach.

The Liquid Extraction Surface Analysis (LESA)[5] mode on the commercially available TriVersa NanoMate chip-based infusion nanoelectrospray system[6] is yet another very recent addition to the field of surface sampling methods. An adaptation of a robotic pipette tip system was made which enabled the device to establish a LMJ between the conductive pipette tip and the sample. Subsequent to the extraction of the analyte the solvent-containing pipette tip is automatically moved to the nanoelectrospray infusion chip and the sample solution is sprayed into the mass spectrometer. Although LESA is a fairly new analysis tool there are already several publications that implemented this technique to sample mainly hydrophobic and plain surfaces. Examples are the determination of glucocorticoid receptor agonists in porcine ear sections,[7] lipid profiling of human atherosclerotic plaques,[8] the direct sampling of dried blood spots,[9] solid-phase extraction cards,[10] food surfaces,[11] tissue sections[11,12] and the detection of additives and degradation products from polymers.[13] Establishing the LMJ on wettable or absorbing surfaces is difficult[1] and in this present work we will evaluate the suitability of the LESA feature regarding its applicability for the analysis of TLC plates. A mixture of different lipid class standards was used to demonstrate

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the potential of the TLC-LESA-MS method because lipid samples are in most cases complex mixtures and therefore analysis greatly benefits from a chromatographic separation carried out prior to ESI-MS detection.[14] Furthermore the diverse chemical properties of different lipid classes allow the investigation of benefits as well as limitations of the procedure. The structures of the selected lipid standards are shown in Fig. 1.

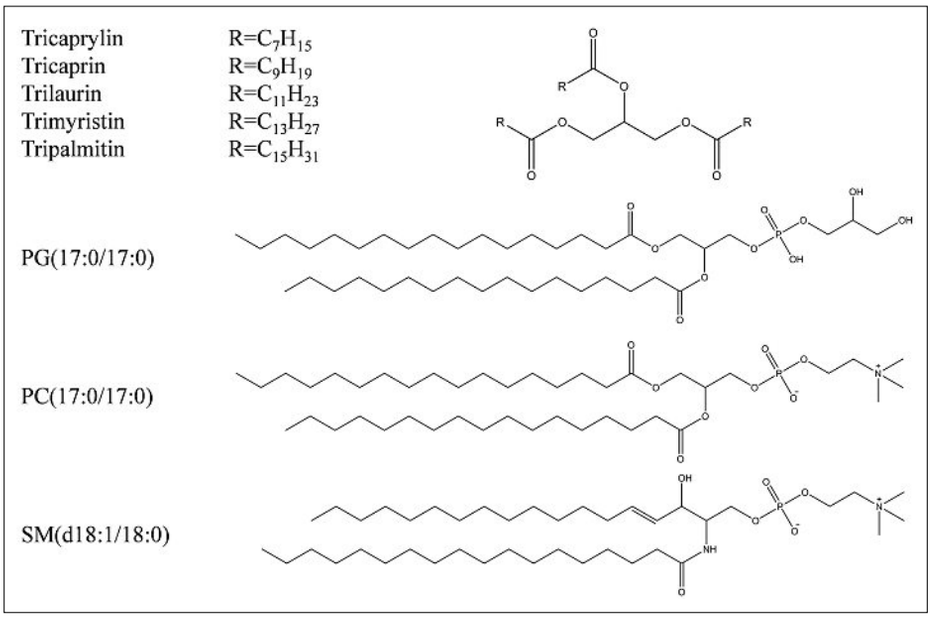

Fig. 1. Chemical structure of the selected lipids for the TLC-LESA-FTICRMS experiments.

2. Experimental

2.1 *Chemicals and Reagents*

HPLC-grade acetonitrile and methanol as well as analytical reagent grade chloroform and acetone were purchased from VWR International (Nyon, Switzerland). Formic acid was obtained from Merck (Darmstadt, Germany). Primuline dye, sphingomyelin SM(d18:1/18:0) and a lipid standard triglyceride mixture containing equal amounts of tricaprylin, tricaprin, trilaurin, trimyristin and tripalmitin was purchased from Sigma (Buchs, Switzerland). 1,2-diheptadecanoyl-*sn*-glycero-3-[phosphatidyl-*rac*-(1-glycerol)] PG(17:0/17:0) and 1,2-diheptadecanoyl-*sn*-glycero-3-phosphatidylcholine PC(17:0/17:0) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The solution for visualizing the lipid spots on the HPTLC plate consisted of 0.02 % primuline dye in 500 mL acetone:water 8:2 (v:v). Stock solutions of lipid standards were prepared at a concentration of 10 mg/mL in methanol/chloroform 1:1 (v:v) and were stored at –20 °C. Working standards were prepared daily prior to use. Ultrapure water was provided by a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA).

2.2 *Thin-Layer Chromatography*

HPTLC separation was performed on 10 × 10 cm HPTLC silica gel 60 RP-18 F_{254}s glass plates purchased from Merck (Darmstadt, Germany). All HPTLC plates were cut into half using a glass cutter in order to fit the LESA plate holder, washed one time with mobile phase and air-dried before their use. 1 µL of the respective lipid standard solution containing an absolute amount of 10 µg in case of TLC optimization experiments, and 1 µg absolute amount of lipid in case of TLC-LESA-MS experiments was applied to the HPTLC plates. The optimized mobile phase that baseline separated all selected analytes consisted of chloroform:methanol:acetonitrile 2:1:1 (v:v:v). The plates were air-dried and dipped into the primuline dye solution (0.02% in acetone:water 8:2 (v:v)), to allow visualization of the spots under a UV lamp at 366 nm. Sphingomyelin and phospholipids appeared as light blue spots whereas the triacylglycerols were visualized as dark spots. The spots on the plate were marked with a pencil and then subjected to LESA-MS analysis.

2.3 *LESA – Nanoelectrospray – MS Instrumentation*

Sample spots on the HPTLC plate were analyzed using a TriVersa NanoMate system (Advion BioSciences, Inc. Ithaca, NY, USA) that was equipped with the LESA feature. The operating mode of the TriVersa NanoMate system and the LESA technique has been described in detail previously[5,6] and is shown in Fig. 2. Briefly, the TLC plate is mounted onto a universal adaptor plate and a robotic arm picks up a conductive pipette tip, moves to a solvent reservoir and aspirates a defined volume. The tip is then moved to the TLC spot location of interest and an adjustable volume of the extraction solvent is dispensed. The microjunction created allows extraction of the analyte into a small volume of solvent before it is aspirated back into the pipette tip, which is then moved to the electrospray ionization chip to generate a nanoelectrospray directed towards the inlet of the mass spectrometer. The optimized extraction solvent composition was methanol:chloroform:water:formic acid 52:24:24:0.2 (v:v:v:v). Depending on the spot size to sample a volume between 5 and 9 µL was dispensed and consequentially a volume between 1 and 4 µL was aspirated. When 5 µL solvent was used a circular sampling area with a radius of 1.5 mm was extracted. This was sufficient to sample the center of a typical HPTLC band. By increasing the extraction solvent volume up to 9 µL the radius of the circular sampling area can be extended to 3.5 mm to cover larger spots. A nanoelectrospray voltage of 1.6 kV and gas pressure of 0.2 psi was applied in all experiments.

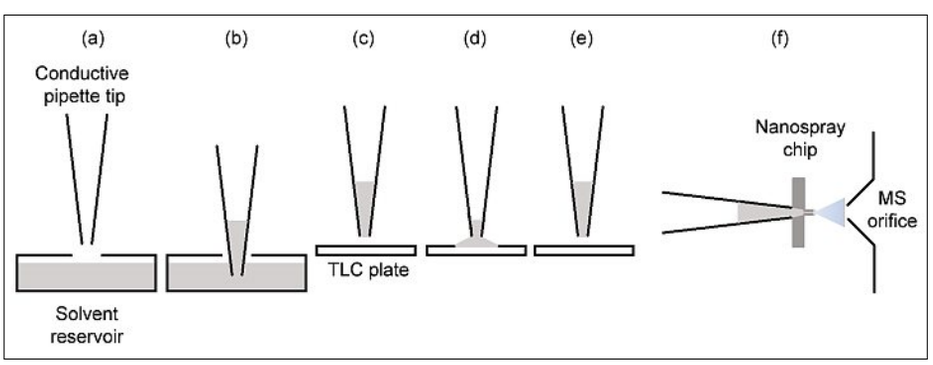

Fig. 2. Operating mode of the TriVersa NanoMate LESA technique. (a) a robotic arm picks up a conductive pipette tip and moves to a solvent reservoir; (b) it aspirates a defined extraction solvent volume; (c) the tip is moved to the TLC spot location of interest; (d) an adjustable volume of the extraction solvent is dispensed and the microjunction created allows extraction of the analyte into a small volume of solvent; (e) the solvent is aspirated back into the pipette tip; (f) the tip is then moved to the nanospray chip.

The TriVersa NanoMate system was coupled to an APEX III FTICRMS from Bruker Daltonics (Bremen, Germany) equipped with a 9.4-Tesla/160 mm bore actively screened superconducting magnet system from Magnex Scientific (Yarnton, Oxford, UK) and with a cylindrical ICR cell that has equipotential-line-segmented trapping plates ('infinity cell'). Hexapole ion accumulation was of 0.5 sec and the ions transfer time-of-flight was set to 2.5 msec. MS spectrum was resulting from 16 accumulated spectra acquired in broadband detection from *m/z* 187 to *m/z* 3000. The time domain (FID) size was of 2M data points with a transient length of 1.3631 sec. The FID signal was baseline corrected and Fourier transformed with the power calculation mode to produce a frequency spectrum finally converted into an *m/z* spectrum.

3. Results and Discussion

3.1 *Reversed-phase HPTLC of Lipids*

In the LESA approach a crucial point is the formation of a stable liquid junction with the extraction solvent systems so a C18 modified reversed-phase stationary phase was selected. In order to achieve complete separation of all eight selected lipid standards different mobile phase compositions based on the solvents chloroform, methanol, acetonitrile and acetone were investigated. As one would expect on a reversed-phase system, the separation of the triglyceride mixture was readily achieved according to their carbon number of the fatty acyl residue chains and corresponding logP values. Mobile phase compositions with high acetonitrile or acetone content resulted in a short migration distance, whereas high chloroform percentages caused the analytes to travel close to the solvent front. The phosphatidylglycerol also behaved as expected from its logP value and migrated between tricaprin and trilaurin, but the situation was different in case of phosphatidylcholine and sphingomyelin. Both compounds possess a nitrogen atom with a positive charge that seems to have a strong interaction with the silica backbone of the stationary phase material; therefore adsorption as well as hydrophobicity determines the retention behavior. This resulted in low retardation factors and spots with a noticeable tailing, whereas the spot shape in case of the triacylglycerols and the phosphatidylglycerol was symmetrical. Finally the optimized mobile phase that clearly separated all selected analytes consisted of chloroform: methanol:acetonitrile 2:1:1 (v:v:v). Table 1 lists the retardation factors of all analytes as well as their logP values.

Table 1. Retardation factors and logP values of the selected lipid standards

Compound	*Retardation factor*	*logP*[a]
Tricaprylin	0.82	9.39
Tricaprin	0.74	12.44
PG(17:0/17:0)	0.69	13.58
Trilaurin	0.62	15.50
Trimyristin	0.51	18.56
Tripalmitin	0.38	21.61
PC(17:0/17:0)	0.29	11.11
SM(d18:1/18:0)	0.18	9.92

[a]Calculated with ACD/Labs software (v. 12)

3.2 *Formation of the Liquid Junction*

Establishing a liquid junction is the most critical step throughout the whole TLC-LESA-MS experiment. The requirement for a successful analysis is the formation of a stable droplet that penetrates the stationary phase and extracts the analyte. At the same time spreading of the solvent across the TLC layer must be avoided because that would potentially transfer the analyte out of the sampling zone and additionally the aspiration of the extraction solution back into the conductive tip would become impossible. The key parameters are the hydrophobicity of the separation layer and the extraction solvent composition, in particular the water proportion.[1]

To define the range of solvent compositions that fulfills the required needs methanol–water mixture droplets were placed on the RP-18 TLC plate and the behavior of the liquid was evaluated. Fig. 3 shows a photographic image of these droplets for solvent composition ranges from 100% water to 100% methanol. The picture on top is taken from the side, to visualize the penetration of the stationary phase and the remaining droplet height which is needed to allow aspiration, whereas the bottom picture is taken from above to show the spreading of the liquid. As one would expect the droplet consisting of 100% water is completely repelled by the hydrophobic TLC plate. There is no penetration at all and the droplet can be moved around the plate like a ball on a flat surface. Even at a composition of 75:25% water:methanol the solvent is not wetting the plate and therefore these mixtures containing high percentages of water are not best suited to extract the analyte, even though dispensing and aspirating the liquid *via* the conductive pipette tip is achieved easily. As can be seen from Fig. 3, the droplets with a water content of 50% and 30% clearly penetrate the RP-18 stationary phase particles and therefore are able to successfully extract the analyte while still providing a defined shape and enough height for the aspiration process. From the bottom picture it can be seen that the spreading of the liquid on the TLC plate becomes noticeable at 30:70% water:methanol, which is generally an unwanted characteristic, therefore the mix-

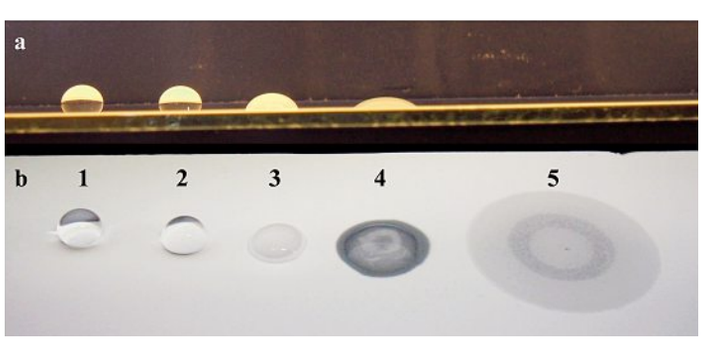

Fig. 3. Photographic images of solvent droplets with different compositions on a non-wettable RP-18 HPTLC plate. (a) side view; (b) top view; droplet solvent composition: (1) 100% water; (2) 75:25% water:methanol; (3) 50:50% water:methanol; (4) 30:70% water:methanol; (5) 100% methanol.

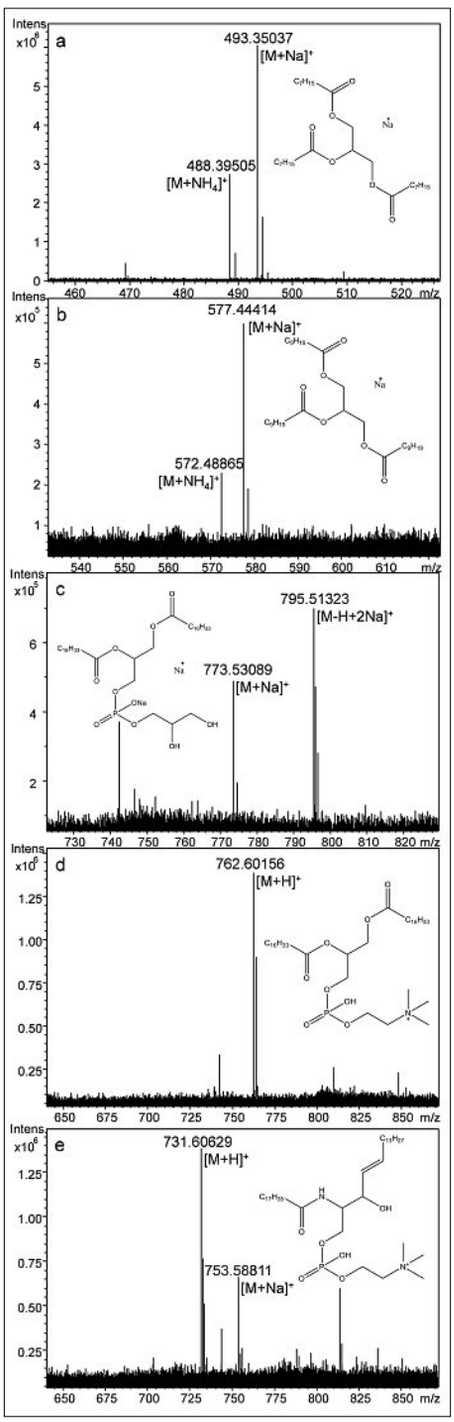

Fig. 4. FTICRMS spectra obtained by LESA surface sampling of analyte spots directly from a developed HPTLC plate. (a) spot R_f 0.82, tricaprylin, *m/z* 493.35037, mass error 0.75 ppm; (b) spot R_f 0.74, tricaprin, *m/z* 577.44414, mass error 0.42 ppm; (c) spot R_f 0.69, PG(17:0/17:0), *m/z* 795.51323, mass error 1.17 ppm; (d) spot R_f 0.29, PC(17:0/17:0), *m/z* 762.60156, mass error 1.13 ppm; (e) spot R_f 0.18, SM(d18:1/18:0), *m/z* 731.60629, mass error 0.12 ppm. The inserted chemical structures correspond in each case to the most intense *m/z* signal.

ture with 50% water would be best suited, when only the liquid junction formation is taken into account. When a droplet of 100% methanol was dispensed on the plate it immediately flowed radially from the spot out into the TLC stationary phase making a proper aspiration process impossible. These results are in good agreement with those described by Van Berkel for the SSP.[1]

3.3 *Optimized Extraction Solvent and LESA Parameters*

Establishing the liquid junction may be the basic requirement, but the solvent has to be suitable for the subsequent nanoelectrospray analysis and, even more important, it needs to be optimized regarding solubility of the target analytes. In the case of lipid analysis this also can be very challenging, because as described earlier, 50% water in the extraction solvent would form a perfect droplet for the LESA process, but unfortunately lipids are extracted very poorly with aqueous solutions and therefore the organic proportion should be kept as high as possible. Finally the optimized extraction solvent composition for the analysis of the selected lipid standards was methanol: chloroform:water:formic acid 52:24:24:0.2 (v:v:v:v). With this high organic content the liquid junction showed a considerable radial extension that depends on the amount of solvent dispensed. As a result the sampling area could be controlled by the volume released from the pipette tip. Depending on the TLC spot size, volumes between 5 and 9 µL were dispensed which corresponds to a diameter of the sampling area between 3 and 7 mm. The liquid junction was held in place for 3 sec before 1 to 4 µL of extraction solvent were aspirated and moved to the nanoelectrospray chip.

3.4 *LESA Sampling of Lipid Spots directly from the HPTLC Plate*

The optimized procedure was applied to separate all compounds listed in Fig. 1 followed by the LESA sampling of all spots directly from the HPTLC plate and high-resolution FTICRMS detection. MS spectra of all detected analytes are shown in Fig. 4. All phospholipids were extracted successfully and unambiguously detected with high mass accuracy. The average absolute mass error was 0.68 ppm and the exact value for every signal is shown in the figure caption. Sampling the tricaprylin spot gave the most intense signals but already the next glyceride spot of tricaprin showed rather poor signal intensities with a signal-to-noise ratio of only 13. The other three triacylglycerols such as trilaurin, trimyristin and tripalmitin are of higher molecular mass with the highest logP values among all investigated compounds and were not detected from their corresponding TLC spots. Since the procedure in general is suited to extract analytes directly from the plate, as shown for all phospholipids and the smaller triacylglycerols, and given the fact that signal intensities significantly de-

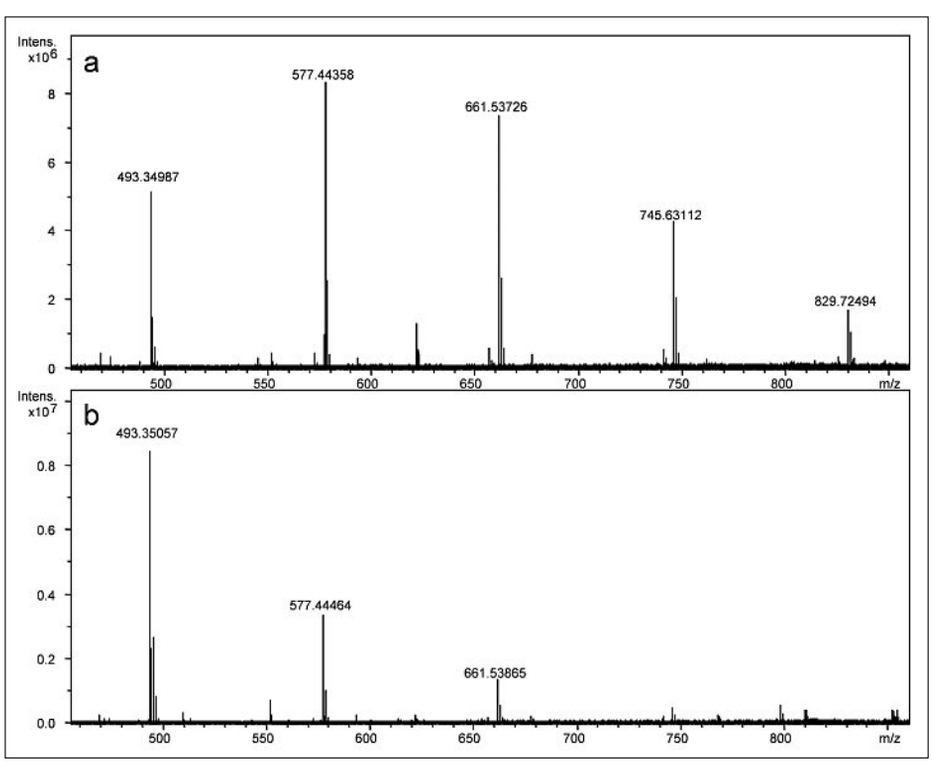

Fig. 5. Comparison of FTICRMS spectra obtained by infusing a 50 µg/mL glyceride mix standard solution in (a) methanol:chloroform:formic acid 50:50:0.2 (v:v:v) and (b) in the LESA extraction solvent methanol:chloroform:water:formic acid 52:24:24:0.2 (v:v:v:v). Signal assignment: *m/z* 493.34987 [tricaprylin+Na]+, mass error –0.26 ppm; *m/z* 577.44358 [tricaprin+Na]+, mass error –0.55 ppm; *m/z* 661.53726 [trilaurin+Na]+, mass error –0.82 ppm; *m/z* 745.63112 [trimyristin+Na]+, mass error –0.78 ppm; *m/z* 829.72494 [tripalmitin+Na]+, mass error –0.79 ppm.

creased already from tricaprylin to tricaprin, one would expect that the solubility of very apolar lipids is not high enough in the extraction solvent. To be able to judge to what extent the solubility decreases when water is added to the solvent, a standard solution containing the five triacylglycerols at a concentration level of 50 µg/mL was prepared in methanol:chloroform:formic acid 50:50:0.2 (v:v:v) as well as in the LESA extraction solvent methanol:chloroform:water:formic acid 52:24:24:0.2 (v:v:v:v). Both solutions were directly infused using the TriVersa NanoMate system and the resulting MS spectra are shown in Fig. 5. In the chloroform–methanol solution all compounds are detected whereas in the solution with 24% water the signal intensities decrease rapidly corresponding to the fatty acyl residue chain length and therefore hydrophobicity. This clearly reveals that the necessity of a liquid junction formation, and therefore a certain amount of water in the extraction solution, can result in limitations due to solubility problems when very apolar compounds are analyzed from TLC plates.

4. Conclusions

In this work lipid standards are separated on RP-18 HPTLC plates and LESA is used for the first time to analyze spots directly from a TLC plate. The selection of the extraction solvent turned out to be the crucial factor that affects the outcome of the analysis by far the most. When selecting the LESA solvent, three important aspects have to be considered: i) the system must be able to extract the analytes, ii) it has to provide stable and sensitive conditions for the subsequent nanospray-MS detection, and iii) it has to be suitable to establish a liquid junction during the LESA process. Small apolar lipids as well as lipids with a polar head group were detected directly from the plate. Very apolar lipids with logP values of 15.5 and above could not be extracted from the plate by the solvent composition needed for a stable liquid junction. As a general conclusion, apart from the analysis of lipids, TLC can be elegantly combined with high-resolution mass spectrometry based on the LESA approach and has high potential for medium-polar compounds separated on reversed-phase TLC plates.

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