



Masterarbeit

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“Characterization of membrane lipids from virus-based vaccines using thin layer chromatography in combination with matrix-assisted laser desorption/ionization mass spectrometry”

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1 Summary

Vaccines have been one of the major breakthroughs of medical history. A wide variety of viral vaccines exists today, combating most of viral infections ailing humanity, making some, like the pox, become extinct. From antigen synthesis over extraction of natural antigens from different sources to high tech methods like biotechnological production of engineered viral vaccines, type of vaccines and methods of vaccine production are very diverse. This diversity, along with the necessity of comparison and control methods requires a proven and reproducible, easy to use and accurate analytical method to evaluate vaccine properties.

Most prominent biomolecules of our time can be analyzed with mass spectrometry. A lot of research concerning virus action, reproduction and function has been done (1-5). Nevertheless viral membranes and therefore the first part of the virus or viral vaccine that contacts the innate and adaptive immune system, has only sparsely been analyzed. Especially since the lipid membrane is among the most complex system within the world of organisms and bio-objects, this is somewhat disappointing. Much, especially information concerning replication, invasion and immune defense is only sparsely understood and the extremely complex lipid composition of the membrane has only recently begun to gain attention and research activity so far focuses mainly on a few viruses such as HIV (2).

The emerging field of lipidomics, the newest addition to the family of “omics” has led to a renewed interest in virus features, this time focusing on the viral envelop, that a large percentage of viruses possess.

Due to the low concentration of lipids in virus extracts (mass-wise), and desorption/ionization suppression through prominent classes of lipids due to the ever present phosphatidylcholines with their quaternary positively charged ammonia group, the need for a pre-separation was clear early on. This pre-separation technique should have qualities such as easy handling, high reproducibility, low cost and MALDI-MS compatibility.

This method of pre-separation is easily found in one of the standard methods of lipid analysis, 1D and 2D high performance thin layer chromatography (HPTLC).

This thesis evaluates the pros and cons, possibilities and strengths of a lipid analysis method based on HPTLC with „matrix assisted laser desorption ionization – time-of-flight mass spectrometry”, in short MALDI-TOF mass spectrometry. Aim of the work was to develop a strategy for analysis of viral vaccines.

In the course of this work optimal extraction conditions for maximum of extracted complete lipidome is found to be the extraction method of Bligh&Dyer. Comparison of widely used staining methods the staining with primuline dye was chosen, as well as separation conditions for 1D and 2D chromatography. Application of 2D HPTLC plates directly on a MS target with conductive tape was successfully tested and imaging of the HPTLC spots revealed that the spots have an additional separation within the spot according to their acyl side chain lengths.

2 Introduction

2.1 History of Lipidomics

*“the full characterization of lipid molecular species and their biological roles
with respect to expression of proteins involved in lipid metabolism and function,
including gene regulation (6, 7)”*

Among the prominent classes of bio-molecules such as nucleotides, proteins and carbohydrates, lipids stand out because they are not easily defined by a shared chemical structure, like peptides are defined by the peptide bond. They are defined by their physico-chemical behavior, as every biological substance that is not soluble in water and soluble in organic solvents is defined as a lipid. Therefore the name “lipids” stands for a wide array of structures that have very different chemical features, for example simple hydrophobic lipids such as fatty acids or waxes over complex and diverse structures like sphingolipids, up to compounds like vitamins and sterols (8). Since lipids are such a diverse group of substances, general approaches for their complete analysis have been lacking. Among the more prominent methods used with some success are thin layer chromatography (TLC) (9), high performance liquid chromatography (HPLC) (10), gas chromatography GC (11), GC coupled with mass spectrometry (12) or nuclear magnetic resonance spectroscopy (NMR) (13). Chromatographic methods lack in resolution, since lipids are categorized due to their solubility and therefore at least have similar behavior in that regard. Therefore it is often difficult, or in some cases impossible to reliably separate lipids using chromatography, especially if they are very similar to each other. In addition chromatographic methods lack in easy ways to identify lipids with high reliability which in a field of substances as versatile as lipids often only allows an identification of lipid classes. NMR, being able to identify lipid structures quite well suffers from the high cost of the device as well as the structural diversity of the sample. In addition, NMR spectra of complex mixtures of structurally similar species are very challenging to interpret and often the method suffer of too low sensitivity. With the emergence of novel techniques in the fields of analytics, especially with modern soft ionization mass spectrometry, this changed dramatically. Mass Spectrometry, especially with some kind of preliminary separation process, has made analysis of the lipidome relatively easy and led to the new field of lipidomics(14).

The scientific analysis of lipids has long endured a shadowy existence outside the world of food (fat) chemistry. Among the large classes of bio-molecules carbohydrates, proteins, nucleic acids and lipids, the latter where long deemed the scientifically least interesting. Proteins where seen as the prominent and scientifically most interesting agents of metabolic pathways, resulting in the growing fields of proteomics. Nucleic acids as the carrier of our genetic information in the same way led to the establishment of the field of genomics

Lipids on the other hand where seen as simple molecules whose sole function has been seen as a barrier, building block and energy storage. For a complete understanding of the metabolism of

biological systems however, it became more and more clear that lipids play very diverse roles. Later studies revealed that especially the glycerophospholipids have important roles in the organism such as signal transduction (15), compartmentalization (16) and membrane trafficking (17). Newly available interest concerning lipids in major diseases like diabetes, obesity or stroke (18, 19) led to the establishment of a growing new research field, carrying the new label of “lipidomics” (14) and focusing on the role of lipids in the whole metabolism and its occupants, like bacteria and virus.

2.2 Lipids

Lipids are, due to their definition, among the most structurally diverse bio-molecules. They are defined as every molecule that is insoluble in water and soluble in organic solvents (8). This results in a heterogeneous group of molecules such as fatty acids, waxes, eicosanoide, monoglycerides, diglycerides, triglycerides, phospholipids, sphingolipids, sterols, terpenes, prenols, fat soluble vitamins (such as vitamin A, D, E and K) and more(20). In order to understand the mass spectrometric analysis of lipids it is necessary to take a closer look at the chemical and structural characteristics of the lipids examined in this work.

Lipids are divided into eight major classes of lipids: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids and prenol lipids. This categorization is due to their corresponding chemical properties and the definition after the LipidMaps project (8). For a better clarity these major groups were further refined into 81 general lipid classes and 276 sub categories by the Lipid Maps Consortium (21).

This work focuses mainly on glycerophospholipids, sphingolipids and sterol lipids since they are the most prominent lipids in viral membranes.

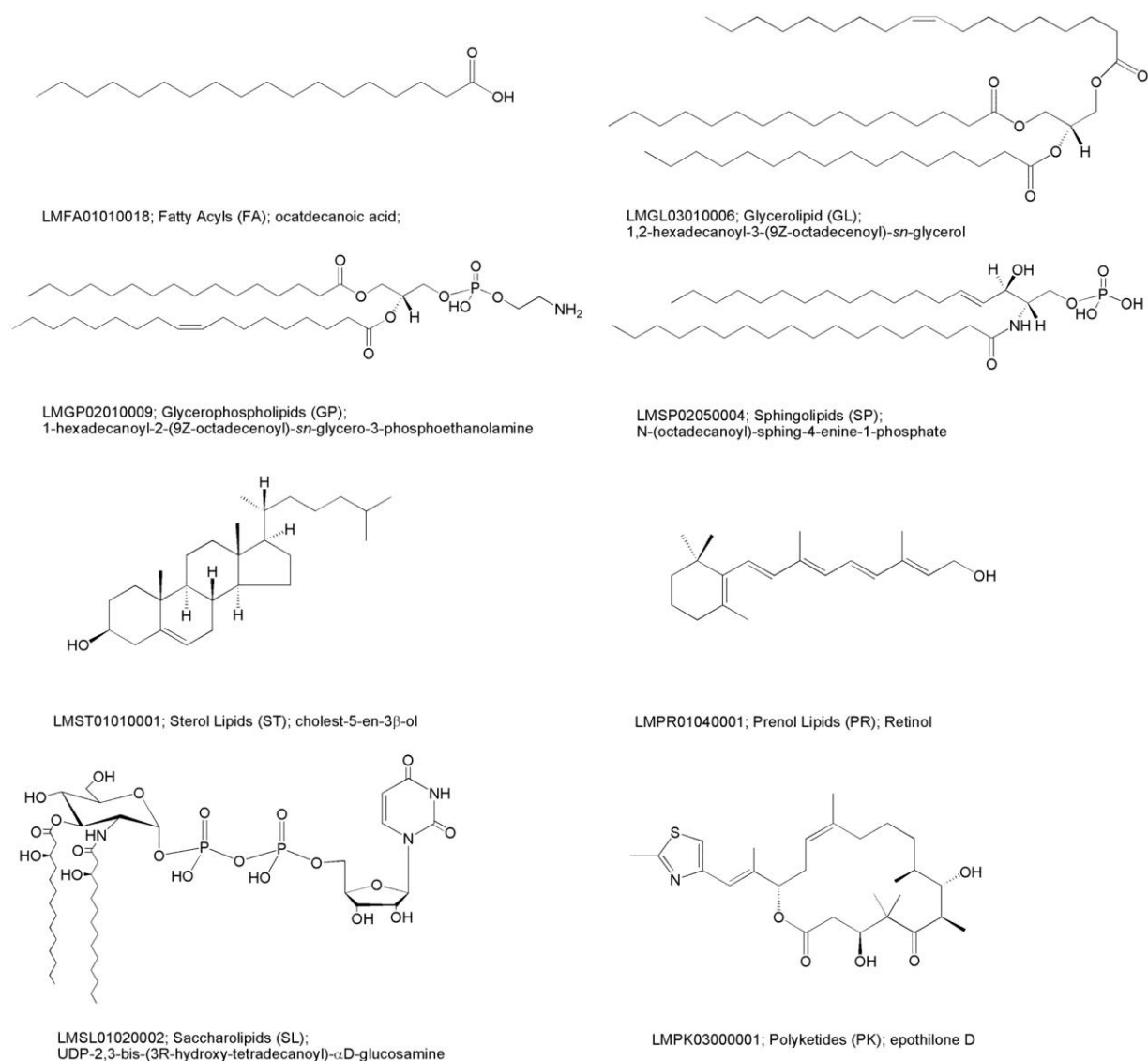


Figure 1 Main classes of lipids: Overview with one example of each class and corresponding LipidMaps ID. Reuse with permission from (21)

Glycerophospholipids and sphingolipids are the two groups of lipids that are, in addition to cholesterol, the building blocks of membranes in biological systems.

The first two are structurally rather similar in that they have a phosphate and only two fatty acid chains. Sphingolipids in addition to that, have always at least one nitrogen from the amine bond. As will be shown in chapter MALDI-TOF-MS of lipids those two atoms are important for their mass spectrometric detection since they are points where ion adducts are likely to form.

2.3 Virus Composition, Rafts and Budding

Enveloped viruses are a special kind of viruses that differ from their simpler variants in the way that the virus capsid is enveloped within a lipid bilayer membrane. The virus capsid is a very sophisticated protein construct similar in strength to Plexiglas® and deformable by 30% before breaking occurs (22). It is therefore very durable and a good protection for the virus DNA or RNA respectively. Normal viruses have only one general mode of replication. They enter the cell by receptor mediated endocytosis or other receptors mediated invasion methods, replicate in the cell and leave it by initiating apoptosis. During cell death the membrane breaks and the virus is able to leave the cell again. Enveloped viruses have the additional possibility to leave the cell by producing their own membrane and then leave the cell by budding of from the membrane.

Viruses do this for several reasons. Although it requires a lot of additional work on part of the virus having no lipid membrane leaves it only the capsid for protection. The viral capsid however has several disadvantages over a lipid membrane from the host cell. The virus capsid is easier to destroy for host organisms and easier to detect, since it differs greatly from protein surfaces normally found in the organism. By being very different from host organisms it is more susceptible to detection from the host immune system. In addition to that, most viruses replicate by killing the cell and releasing all the virus capsids into the host organism. This is a lacking strategy in most cases, since for reproduction the virus has to enter new cells constantly and will soon run out of viable host cells. The answer to this is to leave the cell without actually killing it. This however means the virus needs a method to bypass the protective layer of the cell without destroying it. The most prominent way to do so is enveloping the capsid in a part of this membrane and “bud” of from the cell with a small part of the membrane.

This membrane can originate from several parts of the cell; there are viruses that seem to bud from the inner plasma membrane, the nuclear core membrane, the ER or the Golgi apparatus(22).

Figure 2 shows an overview of the budding process from the host cell membrane as far it is understood today. All required parts are assembled at the site of viral budding through steering the host cell metabolism towards producing the required parts by viral DNA or RNA. Upon completion of all parts the viral budding is initiated where the viral particle becomes coated by a modified version of the host cell lipid bilayer. For some virus budding and assembly occur simultaneously, for other a preformed core pushes out through the membrane (22).

For the virus this approach leads to a maximized protection since it is enveloped in a layer of membrane that looks exactly like the host cell and therefore is not detectable to the immune system. It would however be completely inert, so the virus would be inactivated from then on since it has no mode to break the membrane or enter other cells for re-infection. To avoid this enveloped viruses modify the lipid membrane of the cell with their own membrane proteins and also by changing the lipid membrane composition to their needs. How they do so and in what way is only roughly understood, but it definitely leads to an increase in cholesterol for strengthening the membrane form.(22)

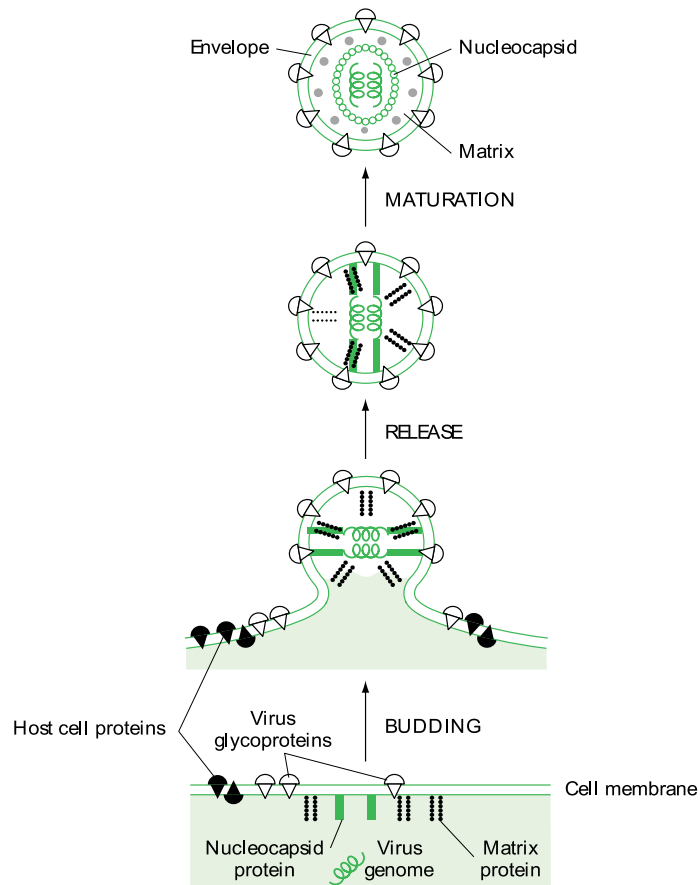


Figure 2 Schematic of the budding process from the cell membrane. Used with permission from (22)

Depending on type and origin of the newly formed virus these membranes differ greatly in lipid composition. 50 to 75 % of the total viral lipids are phospholipids (23). Virus on the lower end of this percentage are plasma membrane budding viruses which all contain excessive amounts of cholesterol, peaking at a cholesterol/phospholipid ratio of greater than 1 (23). Major components of the viral envelope are cholesterol, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphatidylserine. Phosphatidylinositol is frequently not present in RNA virus, whereas sphingomyelin plays a less important role in DNA viruses (23). Generally it can be said, that a higher percentage of phosphatidylcholine goes along a lower percentage of sphingomyelin and phosphatidylethanolamine and vice versa (23).

At the time being, it is only roughly known how enveloped viruses build their membrane, where they do so, and how they bud of. Several studies have been made that led to several theories concerning this question. The most followed theory as of today claims that assembly and budding occur in so called membrane rafts, located for example at the plasma membrane, a theory that emerged due to the compositional similarities between the plasma membrane and the virus membrane (22).

	Cell	PC	PE	SM	PS	PI
Newcastle Disease	CEF	25	28	24	17	-
	EG	12	35	18	12	7
Rous Sarcoma	CEF	20	33	29	12	-
	CEF	25	31	25	14	4

Table 1 Samples of different virus and their reported membrane lipid composition in mass percentages (23) CEF = chicken embryo fibroblasts, EG = embryonated egg.

Table 1 shows mass percentages of lipids for lipid membranes of two different RNA viruses regarding the most prominent lipids in the membranes. Newcastle disease virus has been harvested from two different sources. Large differences can be seen that seem to depend on the host cell, up to the fact that chicken embryo fibroblasts lack PI altogether. Unfortunately results for these percentages vary from laboratory to laboratory. Rous sarcoma virus was harvested in two different laboratories using the same cell line. Although the differences are not as large as with the different cell lines from Newcastle disease, they are there. This may result from the fact that no cell-line stays the same when cultivated in laboratories.

There is therefore a notable difference concerning lipid membrane composition between different virus, different cell lines, and even different mutants of the same cell line.

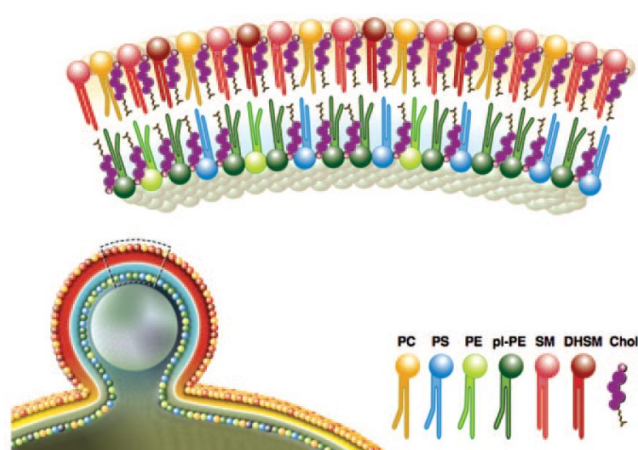


Figure 3 Visualization of the lipid membrane of HIV. Reused with permission from(2). PI-PE = plasma PE, DHSM = Dihydro SM.

Figure 3 shows a scheme of the composition of the lipid membrane of HIV. The virus most likely buds from the plasma membrane of the cell, using its own metabolism to change the existing plasma

membrane in a way that benefits the virus (22). This means, for example, a large increase in cholesterol. There is also a great difference between outer and inner membrane.

A lot of work has been performed towards the discovery of the properties of prominent virus like HIV. Less research has been done towards a general view of the properties of virus. Due to this comparatively little is known about the properties of lipid membranes of the tick-borne encephalitis virus (TBEV) virus used in this work.

2.3.1 Tick-borne encephalitis virus (TBEV)

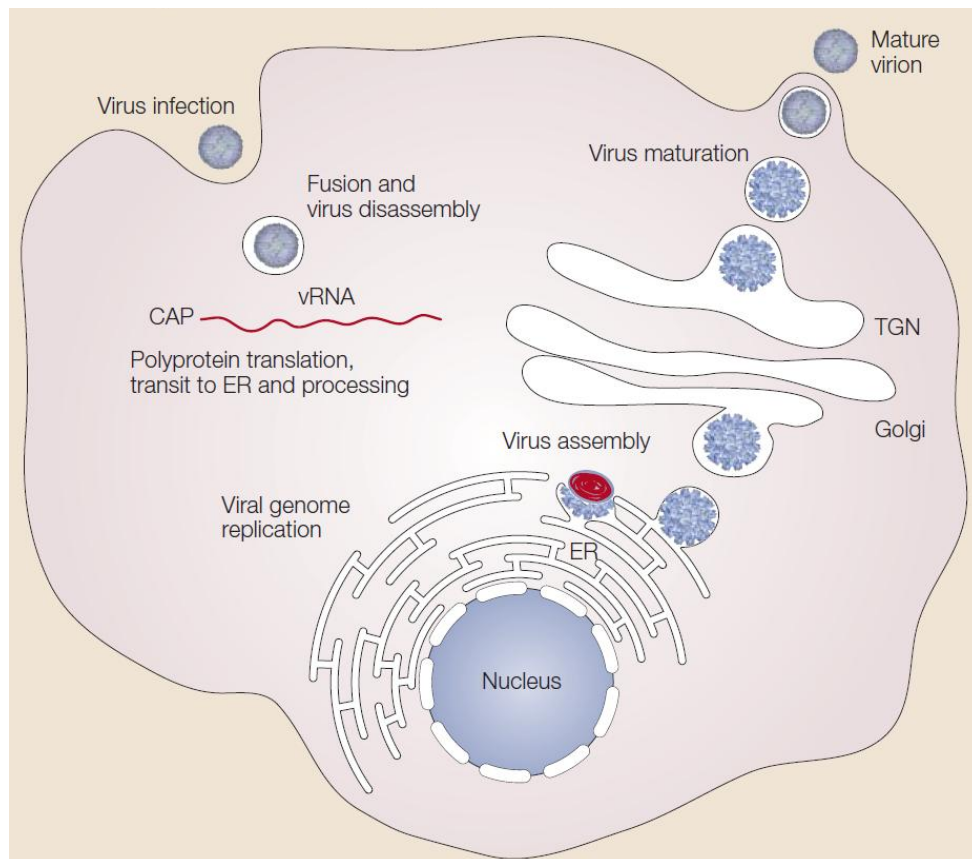


Figure 4 TBEV mode of replication. Figure used with permission from (24)

Tick-borne encephalitis virus is the virus associated with the tick borne encephalitis; a virus induced neurological disorder normally transported by the ordinary tick. TBEV is a virus of the genus flavivirus of the flavivirae family. Like all flavivirus it is a positive sense RNA virus that enters cells via receptor mediated endocytosis, where a pH change towards a more acidic environment initiates conformational changes in the virion, and finally leads to release of the viral RNA into the host. Here it is translated into a single polyprotein, transported to the ER and processed into new virus. These non infectious immature particles are transported through the Golgi apparatus and cleaved by host furin, a protein whose function in the cell is to transform proteins into their active state, into mature and infectious flavivirus that are released again by exocytosis.

2.3.2 Bovine Synovial Fluid

Early on in this work it became apparent, that the virus samples contained only very low levels of lipid, therefore making the detection of sparsely present lipids very difficult. Throughout the proof of experimental concepts synovial fluid was used in order to obtain data on the lipid composition of the samples. In addition this work also had the benefit to be able to work with a sample containing high amounts of lipids. Especially the MSI experiments have been performed only with bovine synovial fluid.

2.4 Thin Layer Chromatography (TLC) for lipid separation

TLC is a method developed in the early 1950 and first brought to a wider audience in 1956 by E. Stahl (25). Its widespread usability and advances in automation made it a standard method in a lot of scientific fields.

Distribution of overall TLC usage

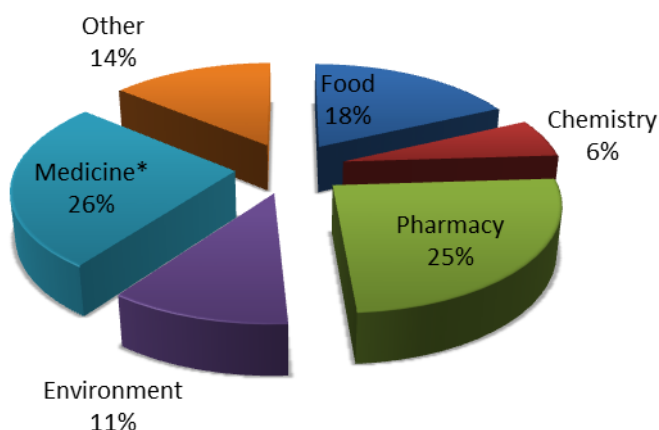


Figure 5 Fields of science where TLC plays a significant role today. 100% equals all sold TLC plates worldwide and the single percentages the amount of usage in the respective fields of science. Medicine includes toxicology, biochemistry and pharmacology. Figure after (26)

B. Spangenberg et al. (27) describe the principles and theoretical background of TLC measurements. TLC has a lot of advantages and unique qualities that set it apart from other widely used separation methods like high performance liquid chromatography. While the needed manual steps to make a TLC separation are easy to understand they are challenging to completely automate. The experimental setup makes it however very easy to analyze a lot of samples in one run and using sample application robots a close resemblance to complete automation can be achieved. Every separation experiment needs a new TLC plate on which new samples need to be applied. This however also means every separation works with a new stationary phase eliminating concerns like how to clean the stationary phase but on the other hand needs a kind of activation every time. It is easily possible to work with unfavorable samples that will contaminate the stationary phase and make further experiments on the same stationary phase impossible since the plate is disposed of after each separation, reducing risk of cross contamination due to contaminated stationary phase to zero. TLC will therefore be not optimal for consecutive experiments with the exceptions of 2D techniques and multidevelopment plates whereas the latter is very suitable for parallel experiments. While with other chromatographic methods detection often takes place after the sample passed the stationary phase, the whole stationary phase is scanned during TLC experiments. This has the advantage that substances that did not move along the stationary phase but remained immobile can be detected.

A standard TLC experiment consists of a stationary phase glued homogeneously on a durable backing like aluminum or glass positioned vertically in a sealed glass container. The mobile phase is present as a liquid reservoir on the bottom of the glass container. Just above the liquid level the sample is applied to the stationary phase on the durable backing and the mobile phase is soaked up through

the stationary phase by capillary forces. Several variations of these standard setup exist varying the material of the stationary phase, using electricity to maintain the flow of the mobile phase (electro-planar chromatography) or even setups with a “lid” on the stationary phase using pressure to propel the mobile phase through the stationary phase with a constant defined velocity (overpressure chromatography). These methods however are not discussed in this work since only the standard setup was used.

In the standard TLC the stationary phase can be seen as bundle of extremely fine capillaries. The mobile phase cohesion here is distinctively superior to the capillary wall adhesion. This results in reduced surface tension for the mobile phase accounting for a pressure difference that accelerates the liquid through the capillaries. In the setup used in this work, called “dipped” vertical chamber TLC, the flow is only stopped by a counter pressure of the rising fluid versus the surface tension forces. This results in a fast flow of mobile phase up the stationary phase that is getting slower and slower over the experiment. The uppermost point to which the mobile phase so far advanced is called front.

Samples are transported along the stationary phase until they adsorb to the material and stop advancing. Adsorption in TLC occurs at the surface of the particles of the stationary phase which are in contact with the mobile phase. Forces involved here are van der Waals’ forces, dipole type interactions and hydrogen bonding. It is important for chromatographic separation that this process is reversible and only involves these physical interactions, not chemical ones. Steric factors as well as temperature play an additional role in adsorption. The adsorption balance depends on temperature, solute concentration at the surface of the adsorbent, and its concentration in the mobile phase. The relationship between these factors results in a Gaussian shaped peak. Asymmetric zones are overloaded with sample and the mobile phase cannot bind more solute in spite of increasing concentration, resulting in a faster than expected migration through the stationary phase. This is called tailing since it produces a trail line like a small tail. If more substance is absorbed by the stationary phase than expected this is called a fronting. Here a small tail is running in front of the main peak.

Migration of the sample along the TLC can be summarized as follows. The Sample spot is applied on the TLC and the TLC immersed in the mobile phase as can be seen in Figure 6. At this moment the entire sample is still on the spot that is in the stationary phase. The moment the first solvent reaches the sample spot, equilibrium is achieved between the amount of sample still adsorbed in the stationary phase and the one in the mobile phase. The sample in the mobile phase then moves along and fresh mobile phase without sample reaches the spot. A new equilibrium is formed, while some sample moves along with the mobile phase. Along the plate several equilibriums are reached. Depending on viscosity of the solvents, the sample, the adsorbent interactions, the adsorbent, time and temperature this results in the movement of the sample spot along the plate.

Standard materials for TLC are silica gel, aluminum oxide, and Kieselguhr or magnesium silicate. These materials are polar and easily store water. This blocks the active sites of the stationary phase. Therefore it is mandatory to preheat the plates in order to reactivate them. Plates wetted with water cannot be used for TLC experiments.

A wide range of different products for different applications is available today from classical silica gel plates on glass or aluminum, aluminum oxide, Kieselguhr, reverse phase plates, modified plates with different polar groups such as CN- or NH-, or cellulose in addition to special features like

concentration zones with different layer composition in order to concentrate the samples prior to separation.

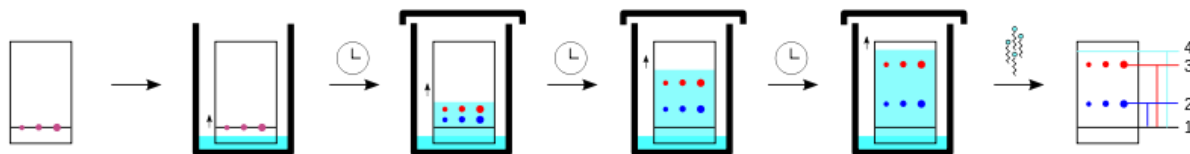


Figure 6 Schematic of a TLC procedure (28). A TLC plate with a mixture of two different samples is inserted in a glass chamber filled with the solvent. A lid ensures a saturation of the chamber atmosphere with solvent. Through solvent movement the mixture separates into the fast moving red and slow moving blue sample. At the end of the TLC procedure sample blue moved from the baseline 1 to its current position 2, whereas sample 3 moved up to point 3. Line 4 indicates the solvent front.

Components are characterized according to their R_f values. The R_f value is a value used to qualitatively evaluate TLC. It is calculated by dividing the distance from starting point to sample by the distance from starting point to solvent front. R_f values vary from 0 to 1, whereas zero means the component did not move at all, and 1 indicates a sample that moved as fast as the solvent.

In ideal conditions the R_f value is the same for a given substance in each experiment. Since this value however depends on so many factors, from solvent quality, to plate integrity over temperature to purity of samples, it is almost impossible to achieve that. It is therefore often prudent to use the R_f values in comparison to substances with known behavior.

One important theoretical parameter to describe the separation abilities of a given TLC plate is the number of theoretical plates. This number describes the number of equilibria occurring between stationary and mobile phase along the whole plate. The higher the number the more equilibria occur and the better the separation is. It is a value correlating retention factor and distribution of the sample.

$$N = \frac{1}{R_f} \left(\frac{z_s}{\sigma_s} \right)^2$$

Where N is the theoretical plate number at the solvent front migration distance and z_s is the substance migration distance and σ_s is the standard deviation of the substance peak. For substances staying at the starting point the migration distance is zero. For those substances that migrate with the solvent front there is no interaction with the stationary phase and the number of equilibrium steps is zero. In either case N is zero as well. N is larger than zero only for substances with R_f values with $0 < R_f < 1$. Plate numbers up to 5000 can be achieved with TLC. The standard deviation of a peak is mostly determined by the peak width at half height.

Introducing the effective retention factor one can calculate effective ranges of R_f values in order to achieve good separation of spots. Looking at two spots having retention factors of R_{f1} and R_{f2} using the equation of the effective retention factor NQ^2 one gets

$$NQ^2 = \left[\sqrt{NR_f(1 - R_{f2})} \right]^2$$

Whereas Q is defined as

$$Q^2 = R_f(1 - R_f)^2$$

Evaluating the effective plate number graphically one gets the following curve.

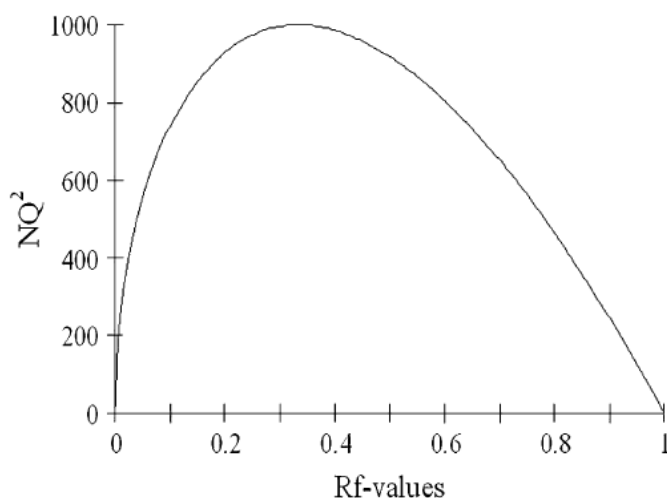


Figure 7 Plot of effective plate number as a function of Rf values with N = 6,751 according to (29)

As can be seen in Figure 7 solvent mixtures leading to R_f values of around 0.33 are ideal, and only ones with values between 0.05 and 0.9 are satisfactorily usable. It is very difficult to improve plate quality by more than a factor of 2-3. Changing the mobile phase can however improve the selectivity by more than a factor of 50. In practice it is therefore more productive to optimize the solvent mixture.

Guiochon and Siouffi (30-33) adapted theoretical ideas and formulas like the van Deemter equation to the TLC method. Using their work it was possible to develop so called high performance TLC plates (HPTLC). For optimal phase separation the van Deemter equation illustrates the relationship of molecular diffusion, mass transport and the local plate height. It allows a forecast of the optimum separation distance in TLC and indirectly optimal particle and pore diameters. With these works optimized TLC could be produced that improved TLC chromatography greatly. Throughout this work only HPTLC plates have been used. In order to illustrate their advantage a short explanation on the theoretical work using the modified van Deemter equation is necessary (30-33). First the expression of H is needed. H is the so called local plate high. It is described as the total separation distance divided by the theoretical number of plates N . It is in theory the imaginary fraction of the plate over which one equilibrium step in the separation is achieved.

$$H = A d_p \left(\frac{d_p}{D_m} u \right)^{1/3} + \frac{B D_m}{u} + C \frac{d_p^2}{D_m} u$$

Here

- D_m solute diffusion coefficient (mobile phase)
- d_p average particle size
- u mobile phase velocity
- A-C Knox equation coefficients

The Knox coefficients will not be explained in great detail and are explored in detail in Spangenberg's work (27). In general A characterizes the quality of the stationary Phase and describes the diffusion and mass transport in the mobile phase and is dependent on the flow velocities through the stationary phase. B characterizes the axial diffusion and describes the effect of the mobile phase on molecular diffusion. C characterizes the resistance to mass transport in the layer. It takes into consideration that some molecules are adsorbed and therefore fixed in position while others move forward with the mobile phase, resulting in zone dispersion in the flow direction. It is directly dependent on the particle diameter. Using the van Deemter equation it was possible to get optimum conditions for given TLC experiments. Separation distances could be optimized. For standard 10 μm pore diameter this would be 10 cm for substances with high molecular weight and 5 cm and 5 μm for those of low molecular weight. (27)

In order to maximize the use of available space on an existing layer Consden et al. experimented with a two dimensional technique (34) as early as the times of paper chromatography. The technique is most often used in order to analyze complex samples. The principle is to use two different solvent mixtures with different properties and turning the TLC plate by 90° after the first development.

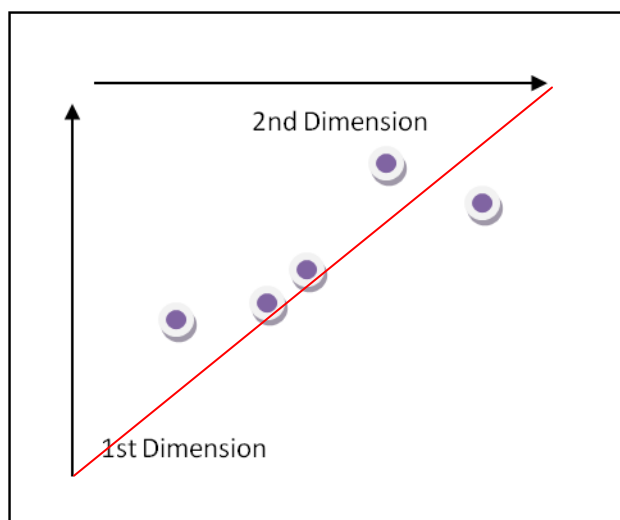


Figure 8 Schematic of a typical two dimensional thin-layer chromatography

If the same solvents would be used for both dimensions the spots of the TLC would be all aligned along the red line in Figure 8. Using 2D TLC made early identification of complex samples like pharmaceuticals possible, where one dimension would not be enough to separate the high number of different components.

2.5 Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry

The matrix assisted laser desorption/ionization (MALDI) method for mass spectrometry (MS) was introduced in 1988 by Karas and Hillenkamp as well as Tanaka et al. (35, 36). It has since gathered a huge interest because of its relative easy sample preparation, high tolerance for contaminants and its soft ionization. It is best suited for non-volatile, polar but thermo labile substances and prominently used for protein analysis (37). In the last years it grew more prominent as a method in lipid analysis (38).

In his work about MALDI-MS Hillenkamp et al. wrote that in a typical experimental setup like the one used in this work the sample is mixed with a near saturated matrix solution and applied onto the target surface. The solvent evaporates rather rapidly and a homogeneous crystal layer remains on the surface. One of the surprising qualities of the matrix is that it incorporates the analyte quantitatively and relatively homogenous. This has been verified for several matrixes so far (39).

The optical absorption of the matrix in the transfer of energy from the laser beam to the sample is governed by Beer's law (40).

$$H = H_0 * e^{-\alpha z}$$

H is the laser fluence, which is the energy in Joule per area of the laser applied at a depth of z into the sample. H_0 is the laser fluence at the sample surface and α is the absorption coefficient, a material characteristic of the absorbing sample. The absorption coefficient defines how far a given wavelength can penetrate the material before it is completely absorbed.

Regarding the amount of energy absorbed per unit of volume Hillenkamp gives the equation

$$\frac{E}{V} = \alpha * H$$

where E is energy and V is volume. Choosing a matrix with a sufficiently high absorption coefficient one can apply a relatively low fluence H_0 , leading to less direct impact on the sample and therefore to more soft desorption/ionization.

The first step in MALDI-MS encompasses the dissolution of the analyte and the matrix in a suitable solvent. Both solvents are then mixed and applied to a suitable surface to be taken in the MS device, and dried until the matrix crystallizes. This method is called the "dried droplet" method(39). Other methods of applying sample and matrix exist (39) but they are not used in this work and will not be discussed. The ratio of matrix to analyte is chosen in a way that the analyte molecule is surrounded mainly by matrix crystals, which is achieved by using molar ratios of analyte to matrix of 10^{-2} to 10^{-4} . The matrix must be a substance that has a high absorbance at the used laser wavelength. At the end of the first step a matrix crystal layer with widespread analyte embedded in the crystals is present. This supports the fact that the solution excess in respect to the analyte is maintained also in the crystal. Should the addition of analyte hamper matrix crystallization in any way it is most often also impossible to get any signal at all. Most of the time this effect is due to a concentration of analyte in

matrix that is too high.

During experiments one can however often experience differences in signal intensity on the spot. Some areas seem to show better signal intensity than others, so called “sweet spots”. This was attributed to an inhomogeneous analyte distribution in the matrix layer. Experiments with fluorescent analyte however showed that this is not the case (41) and that other explanations, like different ionization states of the analyte in different locations or heterogeneous orientation of the matrix crystal surfaces relative to the spectrometer axis and perpendicular to which the ions are ejected in conjunction with the limited angular acceptance of the mass spectrometer.

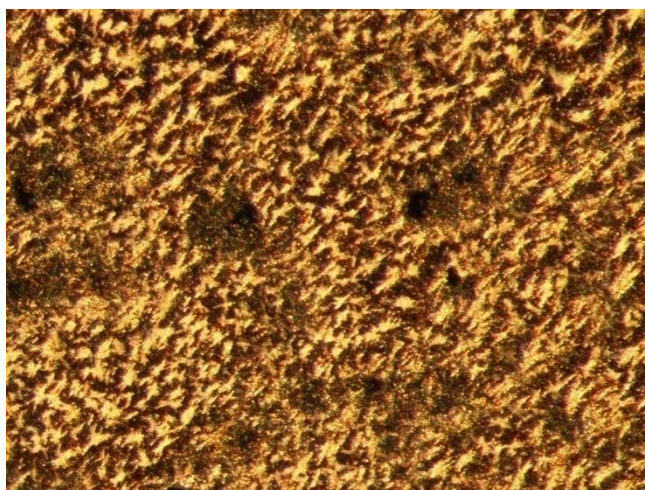


Figure 9 Crystals of the 2,4,6-trihydroxyacetophenone (THAP) matrix under the microscope (12x magnification)

The second step is the ablation of matrix-crystals by means of high frequency (e.g. 20 Hz) and energetic laser pulses directed towards the sample on the target. The mechanism of MALDI-MS is not fully understood, especially the ionization process itself remains under constant discussion(39).

Explained briefly, the laser pulses or beam or photons hits the matrix/analyte solid layer, transfers its energy on the wavelength susceptible matrix. This leads to an ablation of matrix and analyte over a whole after activation with the laser and a plum of solid fragments and activated ions in the gas phase results. First of all it is necessary to understand the difference between matrix and analyte ionization. Although no precise numbers have been determined Hillenkamp proposes ion yields of up to 10^{-3} for matrix molecules and on average 0.1-1% for analyte molecules, in very exceptional cases even up to 10%. Intensities of ion signals are not independent from each other and maybe already in the solid state but definitely in the gas phase charge transfer processes take place. The older model assumes that the analyte is present as a neutral molecule in the matrix by photo ionization of the matrix and charge transfer, the analyte molecules are ionized (42).

The current idea of ionization however is called the “lucky survivor” model (43). The lucky survivor model assumes that the analyte is incorporated in the matrix already charged. In order to maintain the charge, the analyte must be incorporated in a, at least partially, solvated form. In a second step the model assumes a breakup of the crystal lattice into small clusters upon desorption. Statistically some of these clusters carry a positive or negative charge by deficit or an excess of a single counter ion. In the expanding plume the clusters are assumed to lose neutral matrix and solvent molecules as well as counter ions after their proton-transfer neutralization with analyte (de)protonation sites. This results in a molecule that is neutral except for the only remaining excess charge. These singly charged

ions are the lucky survivors of the neutralization process. This model explains the phenomenon of mostly singly charged ions in MALDI (39).

2.5.1.1.1 Time of flight analyzer

Due to the pulsed nature of the MALDI technique, a time of flight (TOF) analyzer is most prominently used in combination with MALDI. The TOF analyzer has the additional advantage of having a wide range of analytes that can be detected, being able to detect masses of more than 2.000.000 Daltons (44). In addition the TOF detector detects the whole mass range so simultaneous detection of the whole mass range is possible.

The general principle of a TOF device is that after ionization the molecule is accelerated towards an evacuated field free drift by a difference of potential between an electrode and the extraction grid. Since all the ions acquire the same kinetic energy, they differ by their velocity according to their mass. Upon leaving the acceleration region they enter a field free region where they are separated according to their velocity before reaching the detector. An ion of mass m with a charge of $q=ze$, where e is the elementary charge and z is the charge number, is accelerated with a potential of V_s along a flight path of the length of L in a time of t resulting in the following formula for the m/z values in linear detection.

$$\frac{m}{z} = \left(\frac{2eV_s}{L^2} \right) t^2$$

Since the time of flight depends on mass and charge it delivers an accurate mass of the sample as long as a calibration with samples of known mass has occurred.

The resolution of the TOF analyzer is, among others, dependent on the mass, energy of the ions and flight time. The resolution is therefore proportional to flight path and time, so to get more resolution these values have to be increased.

The most prominent drawback of the TOF technique was its poor resolution which could be solved by introducing two new techniques to the TOF, delayed pulsed extraction and the reflectron.

2.5.1.1.2 Delayed / pulsed extraction

Further improvements of the MALDI-TOF mass spectrometer were achieved after reviving a quite old technique, first introduced by Wiley and McLaren in 1950 (45). Delayed pulsed extraction deals with the problem that after ionization there are ions with the same m/z ratio, but with a velocity distribution letting ions with the same mass arrive at slightly different times, thus reducing mass resolution. In order to address this, the ions are initially separate according to initial velocity in a field free region for a given time, normally within tens of nanoseconds. Ions with the same m/z ratio but different velocities then separate and the higher velocity ions move further towards the detector than the others. When the electric field is switched on again the ions that were lagging behind are now longer in the extraction field since they are further away from the detector and therefore are accelerated more so they catch up to their counterparts with more velocity. Using this method equalizes the velocity differences of ions after the ionization. Unfortunately, time focusing by delayed extraction is mass dependent so the improved mass resolution is only achieved for a limited mass range. The delayed time has to be set according to the m/z value. Since this optimizes the

process towards a single m/z value it gets more inaccurate the further the detected ion deviates from the set m/z .

2.5.1.1.3 Reflectron ion detection mode

A significant breakthrough in mass spectrometry was the so called “reflectron”, a device invented by Mamyrin et al. in St. Petersburg (46, 47). Like the pulsed delayed extraction the device was invented to compensate for the velocity distribution and focus the ions on the detector. The reflectron uses an electrostatic reflector, normally made of ring electrodes that slow down the ions and turn them around like in a mirror to “reflect” the ion stream towards a second detector, thus increasing resolution. Reflectron does two things mainly. First, it increases flight time without increasing device length and therefore giving a longer flight path without a lot of spatial requirement. A longer flight path, as in longer separation length in every separation, increases separation and therefore resolution. Second, and more important it equalizes velocity differences in the ions since higher energy ions penetrate the ion mirror more deeply and therefore have to cover a larger distance and reach the detector in the same time as the slower ions with the same m/z that do not penetrate the mirror as deep and therefore have a shorter travel distance. While delayed extraction compensates mostly for the initial velocity distribution, reflectron compensates more for energy dispersion that may result from uneven surfaces.

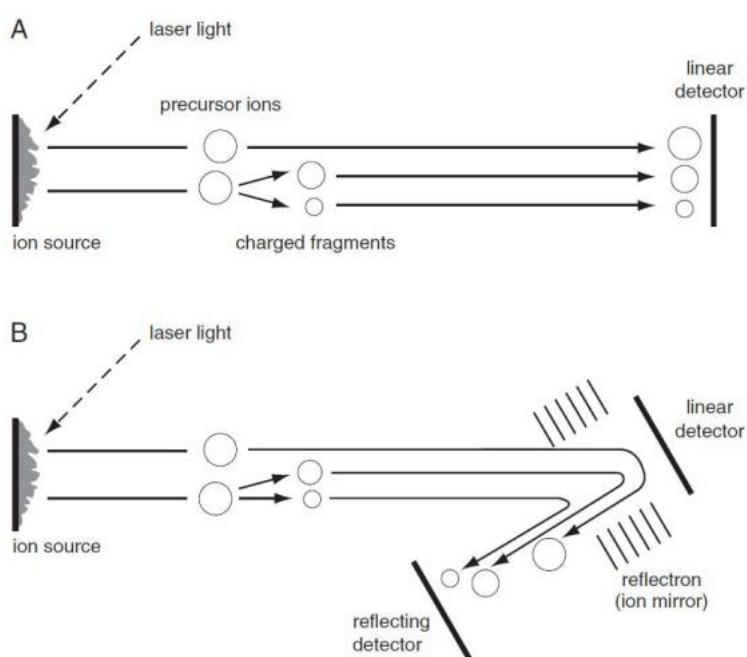


Figure 10 Schematic of a linear (A) and reflectron (B) device. Figure kindly provided from colleague Paul Rigger

Reflectron however introduces a loss of sensitivity and a mass range limit of maximum 50.000 Daltons in exchange for an increase of resolution by ~5 times. (44)

2.5.1.1.4 Post source decay, in source decay and collision induced dissociation

Ions on their way to the detector can be in varying states. The internal energy of the ion can be sufficient so that already fragments of the molecular ions are extracted into the plume. This form of fragmentation or decay is called in source decay (ISD). The internal energy might be so low that no fragmentation can occur during the time the ion needs to reach the detector, whereas no fragmentation takes place at all. It is possible that the molecular ion starts intact, but the internal energy is enough for the molecular ion to fragment during the time it flies towards the detector. In this case fragmentation or decay occurs, but after the ion is leaving the ion source. This is called post source decay (PSD) (39).

With ISD the original ion, called precursor ion, has the same kinetic energy as the fragment ions and therefore they reach the detector according to their m/z value (39).

With PSD the fragmentation happens in the mass spectrometer on the ions way to the detector, after the ion source. This has the disadvantage that the fragments and the precursor ion have the same velocity. In order to reliably distinguish between them the reflectron mode is used. Hereby the precursor ion with its higher mass, enters the ion mirror more deeply than the smaller fragment ions and therefore needs more time to reach the detector (39).

Measuring in linear mode only allows to see ISD and in reflectron mode enables the scientist to see whether PSD occurred. In the linear mode mass spectrum both the precursor ion and its fragments reach the detector simultaneously. This results in peak broadening, but not in several well-separated different fragment ion peaks (39).

By applying a so called ion deflection gate before the reflectron but after the ion source, a group of ions with well-defined m/z values can be selected as precursor ions. Since a time-of-flight device is used an easy way to select only certain masses for detection is to use a reflectron and only activate it for a given set of flight times. As long as the reflectron remains deactivated all masses will pass it and are lost to the detector of the reflectron since they are not reflected towards the reflectrons detector. Upon activation masses are reflected towards the reflectrons detector. If the reflectron is only activated for certain flight times this effectively results in a so called ion deflection gate. The result is a small time of flight range that works as a gate that only certain masses can pass. A sequence then would occur as follows: The reflectron field is deactivated and all ions pass the gate unstopped and hit the detector of the linear flight path. The moment masses of a given mass, or more precisely time of flight would pass the gate it is activated for a short duration and the ions now reaching the reflectron field are reflected in their flight path towards the reflectrons detector. Deactivating the field in the reflectron closes the gate and only the masses in the time of flight window where the reflectron field was active are seen at the reflectron detector. It has to be noted however that this might also eliminate fragment ions since this gate only lets certain masses pass. If the fragmentation of the molecule occurs before selection of the masses, the fragments might be lost. This effect can be partially avoided with a curved field reflectron. In a curved field reflectron the ion mirror of the reflectron field depends non-linear on the distance to the entry point of the ion mirror. By adjusting this field accordingly heavy ions do not penetrate as deep in the field as light ones and therefore trajectory deviation is reduced compared to a linear field reflectron. Utilizing this

effect one can stop ions from being lost an effect that normally would occur for ions that enter the field to deep and are absorbed at the devices wall by flying through the field completely.

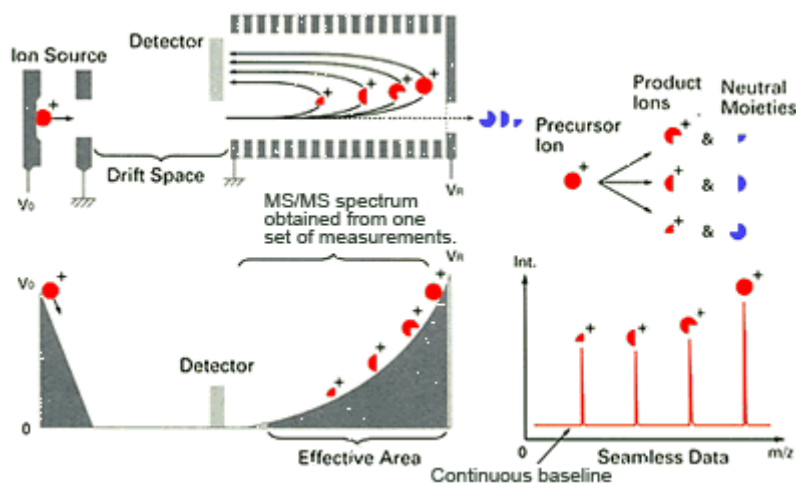


Figure 11 Schematic of the functions of a curved field reflectron kindly provided from Shimadzu©.

As can be seen in Figure 11, using a curved field reflectron results in spectra that are seamless despite a wide range of different masses due to the non linear reflectron field. This allows for seamless spectra of PSD or collision induced dissociation (CID) over a wide range of masses without the need to stitch several spectra together.

Using only metastable decay such as PSD does however result in less than satisfying fragmentation of some classes of biomolecules (e.g. certain classes of lipids) due to the fact that PSD is similar to low energy CID fragmentation in its results. In order to enhance the ability to look upon fragmentation of ions tandem mass spectrometry (MS/MS) with collision induced dissociation was used. Tandem mass spectrometry with CID describes a device with a collision cell. A collision cell describes a module where, after acceleration of the ions those ions are confronted with a collision gas. After separation in the mass spectrometric analyzer 1 (linear TOF) ions are accelerated and confronted with a collision gas stream. Accelerating the ions to high kinetic energies of up to 20 keV and colliding them with a neutral collision gas, in this work nitrogen, will lead to strong fragmentation of the precursor ions. Letting the ions collide with gas molecules increases fragmentation compared to PSD.

The idea of this technique is to select a certain precursor ion and let it collide with neutral gas molecules resulting in more fragmentation as in for example PSD. After that the fragments are separated by the mass spectrometric analyzer 2 (reflectron) and are detected with high resolution. Depending on the collision energy in the CID cell and the selected gas even C-C bonds can be broken(48).

2.5.1.1.5 MALDI-TOF-MS of lipids

Looking back to the chapter Lipids a closer look on sphingolipids and glycerophospholipids reveals interesting features important to the researcher interested at lipids. Structurally, especially at the phosphate and nitrogen atoms, covalent bonds are very likely to break. In addition the bond energy at these bonds is low enough that covalent bond dissociation can occur by applying only the energy available during the desorption/ionization process of MALDI without applying further fragmentation methods (ISD, PSD).

For a definite identification of molecules using MALDI mass spectrometry, CID was used. After fragmentation several different fragment species are present. Depending on the parent molecule and its complexity several different bonds break and depending on which one different fragments result. Depending on conditions (mainly high collision energies) even C-C single bonds can be broken. Analyzing fragmentation patterns it is possible to identify the fragmented molecule unambiguously. It is harder to identify molecules with unknown fragmentation schemes. Acceleration towards collision gas, laser energy, and especially the associated ion (H^+ , Na^+ etc) define fragmentation efficiency. The fragmentation of a given molecule differs greatly depending of the molecular ion being a $[M-H]^-$, $[M+H]^+$, $[M+Na]^+$ or even molecular ions with more than one sodium $[M+xNa-(x-1)H]^+$. For experiments with Lipids in MALDI-RTOF-MS using the setups of this work multiply charged ions of lipids are not considered since they do not or rarely occur at the used experimental settings.

Fragmentation data for proteins exists in abundance and proteomics researchers developed computer databases where one can fill in a single MS/MS spectrum online send it to the proteomics database and get hits what protein most likely was fragmented. A database for Lipidomics with this ability is however only now emerging.

A growing database is build by the Nature Lipid Maps project from researchers all around the world, and already for a growing number of lipids the same is possible as with proteins (49). Still for a significant number of lipids no complete MALDI fragmentation schemes are available yet. Fragmentation schemes for the most prominent Glycerophospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) have been described by Pittenauer and Allmaier (7). His work also proposed a universal nomenclature for the fragments which will be used throughout this work.

Pittenauer and Allmaier used Pittenauers previous work with Cheng (50), where a universal nomenclature for triacylglycerols and their fragments was proposed, and based on that they devised a universal product nomenclature for glycerophospholipids (7).

2.6 The Objective

During the last decade it has become more and more clear that lipids play a more significant role in the function of enveloped viruses as previously believed (2, 51, 52). Enveloped viruses not only possess the most diverse lipid envelope known to the biochemist (23) but several functions of lipid budding, infection pathways and envelope production in the host cell are linked to the virus lipids and its envelope (52). Still, effective and fast methods to analyze and quantify these lipids are still only emerging.

Several approaches such as ESI MS, MALDI MS, NMR, chemical analysis (e.g. modification and precipitation, chemical modification especially with radio nuclides) as well as gravimetric and chromatographic methods have been developed and evaluated. None of the methods alone so far has allowed scientists to reliably analyze and quantify lipid compositions of biological samples available in low concentrations as in viral and vaccine research. This thesis focuses now on the idea that a *off-line* and *semi-on-line* coupled method of high performance thin layer chromatography (HPTLC) with MALDI mass spectrometry could be used to easily identify lipids in viral samples and deliver fingerprint mass spectra of virus lipid envelopes that could be used to later identify virus (vaccine) classes or even the host organisms of the virus. In addition the pre-separation of the lipids should allow for a better detection of the individual lipid classes and in the future quantification or at least a semi-quantitative analysis.

3 Experimental

3.1 Materials and Methods

3.1.1 Instrumentation

Mass spectrometry measurements were done using a MALDI TOF/RTOF instrument, namely: AXIMA-ToF² from Shimadzu Biotech Kratos Analytical (Manchester, UK).



Figure 12 The Axima ToF² from Shimadzu

Software for all experiments with this device was the Shimadzu Biotech Launchpad version 2.8.5. For data processing we used the same software as well as the version 5.0.1 of mMass (53)

Photometric quantification of lipids was done by using a Nanophotometer from Implen (Munich, Germany) using 1.5 ml semimicro cuvettes from Plastibrand (Wertheim, Germany)

UV detection of lipids was done using a handheld UV lamp model UVGL-58 from UVP (Cambridge, UK)

Photometric quantification of lipids in 96 well plates was done using an ELx808 ELISA reader from BioTek (Winooski, Vermont, USA) using Maxisorp 96 well plates from Thermo Fisher (Waltham, MA, USA)

Concentrating of sample volumes was done using a Univapo 100H vacuum concentrator from Uniequip (Planegg, Germany)

Sample Storage was done using Chromacol FISV C049 storage vials with a glass insert from Chromacol (Herts, UK)

MALDI Matrix application for imaging mass spectrometric experiments was done using The “Profi airbrush set” from Conrad (Vienna, Austria)

3.1.2 Chemicals.

From Sigma Aldrich, St. Louis, MO, USA

- Cholesterol, >99%
- Sphingomyelin from bovine brain >97%, order nr. S7004
- 2-a-phosphatidylcholine C 16:0/16:0 >99% (no longer available from Sigma)
- 2-a-phosphatidylcholine C 22:0/22:0 >99% (no longer available from Sigma)
- Phosphatidylcholine from chicken egg >99%, order nr. P3556
- Phosphatidylethanolamine >99% (no longer available from Sigma)
- Phosphatidylinositol from soybean >99% P5954
- Coomassie Blue R-250
- Potassiumchloride >99%
- Acetic acid 100%
- 2,4,6-trihydroxyacetophenone monohydrate (THAP) >99,5% HPLC grade
- 6-aza-2-thiothymine (ATT) >99% HPLC grade
- 9-aminoacridine (9-AA) >99,5% HPLC grade
- Vanilin >99%
- Sodium sulfate >99%
- Potassium hydroxide >99%

From Merck, Darmstadt, Germany

- HPTLC Silica Gel plates 20x20cm, 200µm strength, order nr. 1.05547.0001
- 2-Propanol gradient grade for liquid chromatography
- Methanol gradient grade for liquid chromatography
- Chloroform for analysis
- 1,2-dichloropropane gradient grade for liquid chromatography
- Hexane gradient grade for liquid chromatography
- Isopropyl-alcohol gradient grade for liquid chromatography
- 1-propanol gradient grade for liquid chromatography
- Sulfuric acid 96% for analysis
- Phosphoric acid 85% for analysis
- Methyl acetate gradient grade for liquid chromatography
- Ammonia 2N titriPUR

From J.T. Baker, center valley, PA USA

- Ethyl acetate gradient grade for liquid chromatography

From Aponorm, Hilscheid, Germany

- Castor oil for pharmaceutical uses.

From 3M, St. Paul, MI, USA: double sided adhesive tape

From Whatman (Kent, UK):

- PVDF Westran[®] Membrane order nr. 732-4020

From Baxter (Wien, Austria)

- TBEV (tick-born encephalitis virus)Vaccine samples - inactivated viral particles in carbohydrate solution

Bovine synovial fluid was acquired from a local slaughterhouse and instantly frozen to -20°C after collection.

3.2 Extraction methods

3.2.1.1 *Bligh & Dyer*

Lipid extraction was done using a modified Bligh & Dyer (54) method.

- For complete lipid extraction, 240 μ l of the lipid sample were incubated for one hour with 300 μ l chloroform and 600 μ l methanol by putting them together and letting them rest in the closed cup.
- After incubation 300 μ l each of chloroform and water were added in order to initiate phase separation.
- It turned out to be more efficient to prolong incubation time up to two hours. This is reported to increase the amount of extracted PI from the sample, because PI are reported to take long times in order to be transferred to the organic solvent phase (55).
- For complete phase separation the solution was centrifuged at 15000 rpm for 15 min and the lower chloroform phase contained the lipids. The upper phase was removed with a pipette and the lower phase was used for further experiments.

Before usage the chloroform of the lower phase was evaporated using a vacuum concentrator and the dried lipids are resuspended in 10 μ l chloroform in order to increase the lipid concentration.

3.2.1.2 *Hara & Radin*

Lipid extraction was performed after the method of Hara & Radin(56).

- 100 μ l of the lipid sample were treated with 1.8 ml of a hexane / isopropyl alcohol mixture containing 3 parts hexane and 2 parts isopropyl alcohol (v/v).
- After washing the solution for at least 1 min with 12 ml of sodium sulfate (1 g in 15 ml water) and phase separation the upper hexane layer contained the lipids of interest.

3.2.1.3 *Merill*

Lipid extraction after the method described by Merrill et al. (57).

- 0.1 ml of the lipid sample was mixed with 0.5 ml methanol and 0.25 ml chloroform.
- The mixture is shaken vigorously for 30 seconds.
- Then the mixture was incubated over night at 48°C using an Eppendorf heating block in closed Eppendorf cups. After letting the cups cool to room temperature care has to be taken when opening the cups because of the still existing overpressure.
- After that the mixture was cooled down to room temperature, 75 μ l 1 M potassium hydroxide in methanol were added.
- The mixture was incubated for two hours at 37°C in the Eppendorf heater again and after that neutralized by adding 3 μ l 100% acetic acid.
- After adding 1 ml chloroform and 2 ml water the formed mixture was centrifuged at 15000 rpm for 15 min. The lower organic phase containing the lipids was used for further experiments.

3.2.2 Storage

Storage of lipids was first done, after drying lipid solutions in the desiccators, in the Chromacol vials and storing the liquid free lipids in the refrigerator at 4°C and under exclusion of light. Later on storage was done storing all lipids dissolved in a mixture of 1,2-dichloropropane/1-propanole 1:1 (v/v).

The final storage method we used was done using chloroform/methanol 1:2(v/v) in Chromacol vials.

3.2.3 Quantification of lipids

Lipids were quantified by a sulfo-phospho-vanillin method used to measure complete lipid content (58). It has to be mentioned that this method targets *double bonds* present in lipids or possible to be formed during the photometric assay. The reaction itself is a sulfuric acid induced radical reaction (Figure 13).

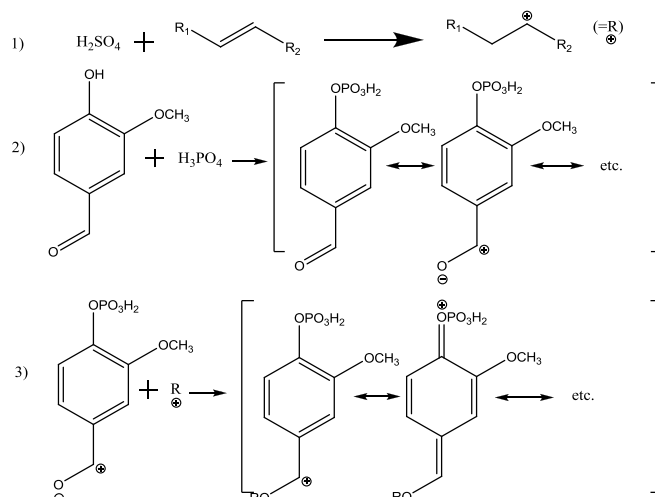


Figure 13 Schematic of the proposed reaction sequence of the sulfo-phospho-vanillin reaction. Reaction scheme adapted after Knight et al (59)

1) Unsaturated compounds react with sulfuric acid to form a carbonium ion

2) Vanillin reacts with phosphoric acid to an aromatic phosphate ester.

3) The carbonium ion from 1) reacts with the activated carbonyl group of phospho vanillin to produce the charged phosphor-vanillin complex seen in 3). This complex is responsible for the color change that can be used to quantify the sample.

Since this method needs double bonds in the lipid to work, it is, as nearly all known lipid quantification methods, a rough estimate when used for small quantities of lipid mixtures of unknown composition.

- 100 µl lipid sample dissolved in the storage solvent mixture (chloroform/methanol) were pipetted directly into the wells of a 96 well plate. For optimum assay conditions a lipid content of 5-120µg in each well is suggested (58).

- After the solvent was evaporated at 90°C, 100 µl concentrated sulfuric acid is added to each well.
- Then the complete microtiter plate is incubated at 90°C for 20 min using a tightly attached plate lid in an oven.
- Background absorbance is measured at 540 nm after the plate is cooled to room temperature (preferably fast cooling using ice water or refrigerator) using the complete reaction chemicals without adding actual sample.
- 50 µl of a vanillin-phosphoric acid reagent (0.2 mg vanillin per ml of 17% aqueous phosphoric acid) is added to each well for color development (10 min reaction time at room temperature) and then absorbance is again measured at 540 nm.
- Heating and cooling steps are crucial for good linearity of the calibration. Therefore it is crucial to cool the plate as fast as possible by using ice and take great care to heat the plate exactly for 20 min.

3.3 TLC

3.3.1 Evaluated mobile phases for 1D HPTLC

One dimensional HPTLC chromatography was done using variations of a widely used method in lipid separation.

A 10 cm diameter cylindrical TLC chamber was utilized and pre-saturation of the chamber with the solvent for 45 min.

As mobile phase 10 ml of a solvent mixture consisting of

1. methyl acetate, 1-propanol, chloroform, methanol, 0,25% aqueous potassium chloride (25/25/25/10/9 v/v/v/v/v)
2. ethyl acetate, 2-propanol, chloroform, methanol, 0,25% aqueous potassium chloride (25/25/25/10/9 v/v/v/v/v)
3. ethyl acetate, 1-propanol, chloroform, methanol, 0,25% aqueous potassium chloride (25/25/25/10/9 v/v/v/v/v)

was applied.

5 or 10 µl of the sample was applied (5 µl for 1mg/ml standards, 10 for sample) using a glass syringe.

3.3.2 Lipid Detection on HPTLC plates

Several staining methods are widely used to visualize lipids after separation.

Two detection systems which were widely used (60, 61)staining with Coomassie Brilliant Blue R 250 and primuline, were selected.

3.3.2.1 Coomassie Brilliant Blue staining:

Coomassie Brilliant Blue R 250 (see Figure 18) is a triphenylmethane dye used originally to dye cotton tissue but nowadays is mainly used to dye biomolecules in biochemistry (60, 62, 63).

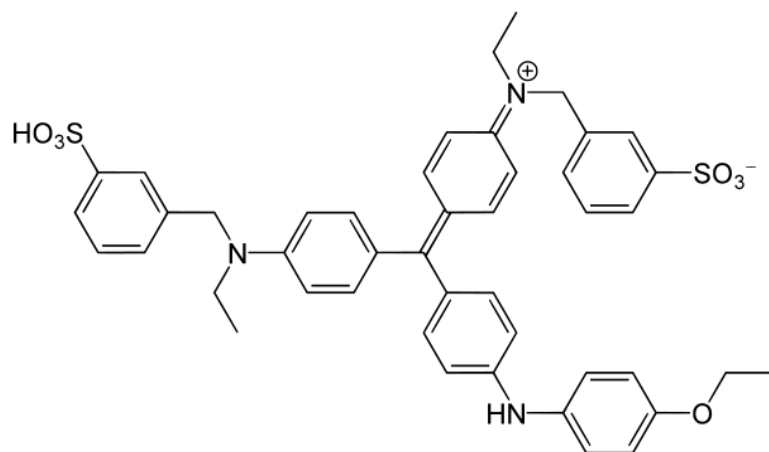


Figure14 Chemical structure of Coomassie Blue R-250

- A mixture of 10 ml methanol, 40 ml water and 15 mg Coomassie Brilliant Blue R-250 was prepared and mixed vigorously for several min.
- After the development and drying of the HPTLC plate the plate was immersed in a solution containing 0.03% Coomassie Brilliant Blue R-250 and 20% methanol (the above solution) for 20 min, using a low height glass chamber with a tight lid in order to reduce methanol evaporation

After the staining bath a destaining step followed: immerse the plate in 20% methanol for 5-10 min. After that only the sample spots are blue and the rest of the plate remains white. If the plate shows a strong blue colour all over it was not thoroughly cleaned before.

According to the reference (61) the detection limits (shown in Table 2) of Coomassie Brilliant Blue R-250 for lipids are:

Lipid	Detection limit
Cholesterol	0.5µg
Cholesterol stearate and glycerol tripalmitate	0.05µg
Saturated fatty acids	0.05µg
Phospholipids	0.05-0.1µg
Ceramides	0.1µg
Unsaturated fatty acids	Hardly detectable

Table 2 detection limits of Coomassie blue R250 when staining lipids

3.3.2.2 Primuline Staining

Primuline is a dye with a benzothiazole ring (See Figure 15) also known as Carnotine or Direct Yellow 7 that is widely used in lipid analysis especially for MS (60, 62).

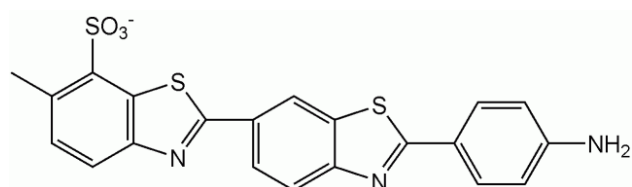


Figure 15 Chemical structure of primuline dye

- Prepare a 5% primuline in water solution (50 mg/ml)
- Dilute the primuline solution 100-fold with an acetone : water (8:2 v/v) mixture to give a final concentration of 0.05% primuline
- For a 10x10cm plate spray a minimum 4 ml of the solution on a HPTLC plate until it the plate is wet using an airbrush.
- Dry briefly with a hair dryer, until the solvent is evaporated.



Figure 16 Example of a 2D HPTLC stained with primuline dye seen under UV light. Lower right was the starting point. For detail on the corresponding spots see chapter 4.1.1. Plate size 10x10cm.

3.3.3 2D thin layer chromatography

In order to enhance resolution and separation a two dimensional thin layer chromatography technique using HPTLC plates was used (2D-HPTLC). This adds another separation dimension in 90° to the first and by using different mobile phases in the first and second dimension different separation efficiency was achieved. Two dimensional HPTLC was done using the following solvent mixtures for the first and second dimension:

1. Chloroform/methanol/ammonia(25%) with 20:10:2 v/v/v
2. Chloroform/methanol/acidic acid/water with 15:2:3:0,6 v/v/v/v

Sample application was done exactly as with the 1D chromatography (see chapter 3.3.1); detection was carried out using primuline. For an example see Figure 16

3.4.2 Lipid extracts for mass spectrometry

Samples, standards and calibration substances were all applied to a stainless steel target (Shimadzu Kratos Analytical, Manchester, UK).



Figure 18 MALDI metal target used for the MS experiments. Samples were applied into the small ringed areas.

- Lipid extract was dissolved in methanol/chloroform (2:1 v/v)
- MALDI matrix THAP was dissolved in methanol (20 mg/ml)
- 10 μ l of dissolved lipid extract solution and 10 μ l of MALDI matrix solution were mixed in a small Eppendorf tube
- 0.8 μ l of this mixture were applied to one of the provided target application circles. Care has to be taken not to apply larger volumes because the sample droplet then tends to flow over the engraved ring. Afterwards the solution was dried at room temperature
- The standard was applied using the method mentioned in 3.4.1.
- 10 μ l of the castor oil/methanol mix and 10 μ l of MALDI matrix mix were mixed in an Eppendorf tube using a pipette.
- Again 0.8 μ l of this mixture were applied to the plate. It was tried to put calibration substance into the first and last application circle of each column (Columns defined here as the vertical dot lines). In between the sample was applied in order to be able to compensate for calibrant deviations.

3.4.3 Lipid extracts from thin layer chromatography

First experiments coupling HPTLC with MALDI-TOF MS were done by extracting the lipids from the HPTLC plate material by scratching out the material around a spot from the plate. The so obtained material was then treated using the Bligh & Dyer extraction method and the resulting extract was measured by MALDI-MS using the same procedure as described in chapter 3.4.2.

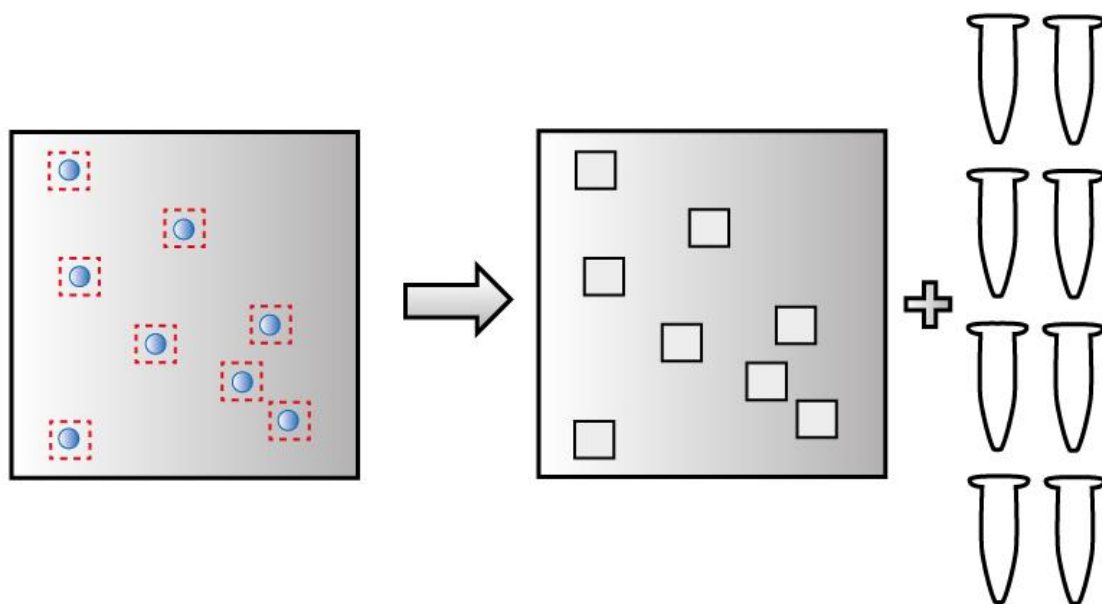


Figure 19 Schematic of the process of extraction from the 2D HPTLC plate.

The HPTLC plate was stained either with Coomassie Brilliant Blue R 250 or Primuline and each spot material was then carefully removed from the aluminum backing with a scalpel and transferred into an Eppendorf tube. The best practice here was to accept a bit of extra silica gel material without sample and scratch out a square area around the spot because this was easier to handle (see Figure 19). The lipids were then extracted from the silica gel using the Bligh & Dyer method used also to extract the lipids from the TBEV vaccine samples (see chapter 3.2).

3.4.4 Lipids detected directly from the HPTLC plate

Lipids were measured directly from the HPTLC plate taping the plate to a MALDI-MS metal target and applying a small amount of castor oil to a place (without any disturbing other compounds) on the plate itself.

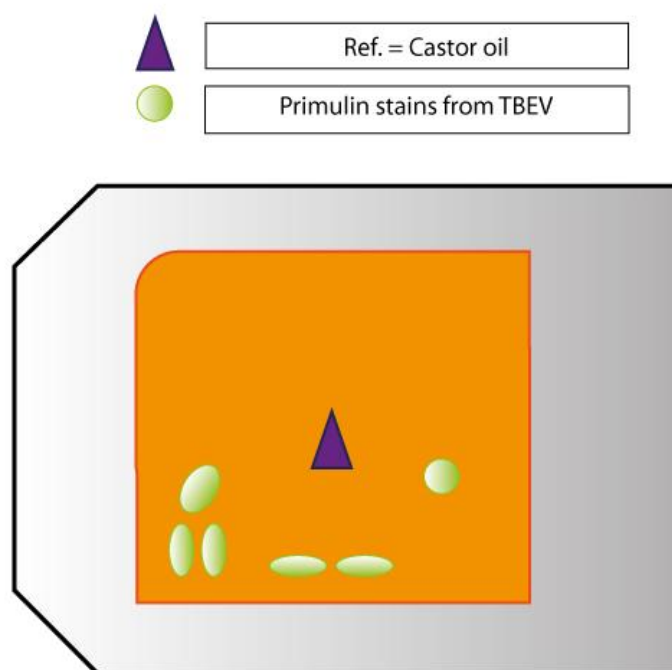


Figure 20 Schematic showing how the HPTLC plate (orange) and calibrations substance (purple) are located on the metal target (grey).

- The HPTLC plate is stained with primuline dye and all spots are marked with a pencil in order to find them again later during measurement.
- In the center of the plate a castor oil calibration spot was carefully applied. It was necessary to perform the deposition with great care so that the oil was only deposited on a very small area since it would otherwise disperse too much and signal would only be present at the outer borders of the spot where there is still a sufficient amount per area present. For later experiments several calibration spots were put on free spaces close to sample spots in order to increase accuracy.
10 μ l are administered using a Hamilton syringe to apply the sample in small doses till all sample is on one single spot. This is done in order to have a concentrated small spot. Since the MALDI matrix is sprayed on the target later on, the required sodium ions have to be applied with the oil, using the same technique for calibration substance preparation as in 3.4.1. The whole plate is coated with a 20 mg/ml MALDI matrix solution by spraying approximately 3 ml of MALDI matrix solution on a plate of the size of 5 x 5 cm using an airbrush. Care should be taken to keep a distance from the plate of approximately 20 cm and spray in an S-like pattern very homogeneously.
- The plate is then fixed on the metal target using double sided tape.

3.4.5 PVDF membrane with the so called far eastern blotting method

Far eastern blotting is a blot method first established 1998 by Ishikawa and Taki (64) using a PVDF membrane. In addition to a reported increase in signal quality it allows for easy application of techniques not possible on HPTLC plates such as antibody binding studies or enzyme activity reactions. Due to its origin this blotting method was named “far eastern blotting”. Far eastern blotting utilizes a heat induced blotting method, working as follows:

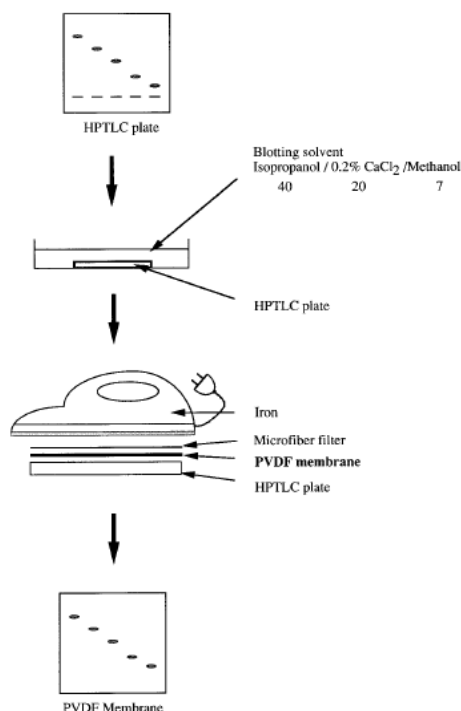


Figure 21 Schematic of the eastern blotting procedure. Schematic reused with permission from (65)

- Immerse the HPTLC plate in a blotting solution of isopropyl alcohol / 0.2% CaCl_2 / methanol 40/20/7 v/v/v for 20 seconds.
- The HPTLC plate is then covered with a PVDF membrane and a microfiber filter. The filter is responsible to hinder drying and stop direct contact between membrane and the hot iron surface, since the PVDF membrane would melt when it would be in direct contact with the iron too long.
- The spots are blotted to the membrane using a normal household iron at a temperature of 180°C. This is done by pressing the iron onto the fiber filter for 30 s.

The membrane is then prepared like the HPTLC plate for measurements directly from the HPTLC plate (see chapter 3.4.4).

3.5 Mass spectrometric measurement setups

The AXIMA-TOF² is equipped with a 337.1 nm nitrogen laser from LTB Lasertechnik (Berlin, Germany). Pulse setup is 3 ns pulse duration with a pulse rate of 20 Hz. Each shot has a maximum energy of approx. 160 μ J. The device controls the actual irradiation in arbitrary units from 0 to 180, where 180 (arbitrary units, au) is the maximum possible energy. Laser beam radius is 60 μ m, acceleration voltage is 20 kV and acceleration distance is 1.27 meter.

According to manufacturer data the maximum achievable resolution is 4000 (FWHM) for linear mode and 18000 for reflectron. Statistical experiments towards the maximum achievable resolution were not made in the course of this work.

For all experiments the delayed extraction function was set to m/z 955, except for identification of defined peaks with CID (MS/MS), where the function was set to the corresponding precursor ion m/z value.

For non HPTLC measurements the laser irradiation was set to 80 au and for HPTLC experiments it was set to 90 au.

Mass range was set from m/z 1 to 1200, except for experiments with different matrixes and PVDF membrane where it was set to m/z 400 to 1200 in order to treat the detector with care.

Average mass spectra are based on 250 to 500 single mass spectra and were accumulated depending on the signal quality. For mass spectra obtained from TBEV samples 1000 mass spectra were accumulated.

Average CID spectra are based on 250 to 1000 single mass spectra and were accumulated depending on signal quality.

All spectra were smoothed using the Savitzky-Golay smoothing filter with a very small smoothing degree of 5.

MALDI experiments were performed using automatic laser movement (s-pattern) over the whole sample spot

Imaging experiments were done by setting the device to accumulate mass spectra with a resolution of three mass spectra per pixel point and 250×250 points over a space of 2.5×2.5 cm resulting in a resolution of 60 μ m spot per 0.1 mm.

3.5.1.1 CID experiments

CID was done with collision energy of 20 keV by introducing helium into a collision cell, which is differentially pumped, at an approximate pressure of 5×10^{-6} mbar. Ion gate was set to a symmetric 4 Da mass window. By symmetric it is understood that the range below the parent mass is as large as above, for a mass of 500 the gate would for example be 498 to 502. Depending on spectral quality 250 to 1000 single spectra were accumulated for a single average spectrum. Laser irradiation was done as with non CID experiments.

4 Results and discussion

4.1 HPTLC

First HPTLC experiments were done using slight variations of a solvent mixture. A conclusive overview of the developments can be found in (66). The variations explained in chapter 3.3.1 for HPTLC were used trying to separate a mixture of known lipid standards. All plates were 5 cm x 10 cm,

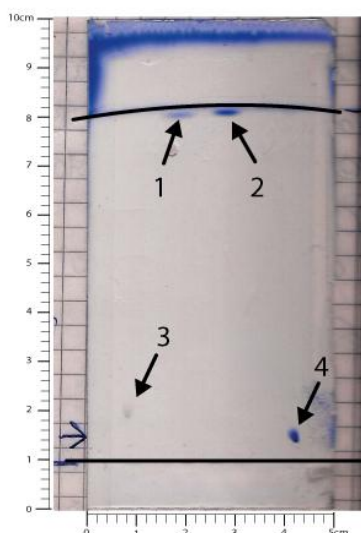


Figure 22 HPTLC using solvent mixture 2 described in chapter Evaluated mobile phases for 1D HPTLC. Spots are (1) PI, (2) cholesterol, (3) non hydroxyl ceramide (NHC) and (4) PC (Coomassie Brilliant Blue R-250 detection). Spots administered as dots.

As can be seen in Figure 22 NHC and cholesterol are running with the solvent front and are not separated at all, and also the more polar substances PI and PC are only marginally separated.

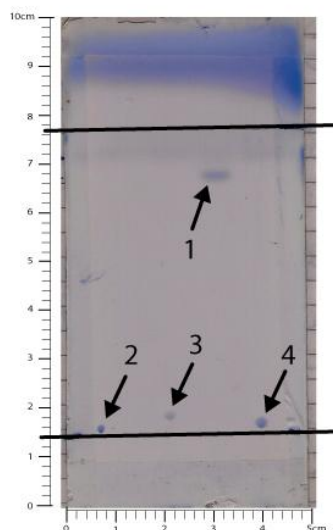


Figure 23 HPTLC using solvent mixture 3 described in chapter Evaluated mobile phases for 1D HPTLC. Spots are (1) SM (2) PI (3) cholesterol (4) PC (Coomassie Blue R-250 detection). Spots administered as dots.

In Figure 23 no usable separation was reached by changing the isomer of propanol to the one used in solvent mixture 1. Polar substances SM, PC and PI are hardly separated.

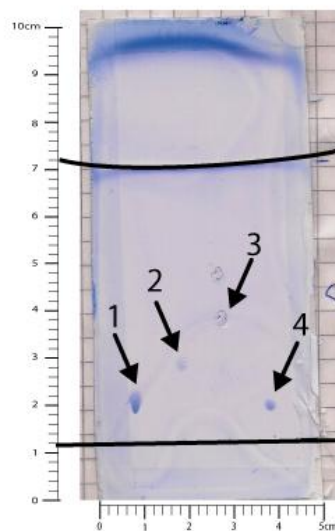


Figure 24 HPTLC using solvent 1 from chapter Evaluated mobile phases for 1D HPTLC. Spots are 1) PC 16, 2) PI, 3) PE, 4) PC 22 (Coomassie Blue R-250 detection). Spots administered as dots.

Using the solvent mixture 3 (Figure 24) it was possible to see good separation of the main polar components PI, PE and PC respectively. Since to the date of this work, fragmentation and characterization of cholesterol and structures similar has not been achieved with MALDI-MS the focus of the further HPTLC experiments was shifted completely towards the more polar components of the lipids.

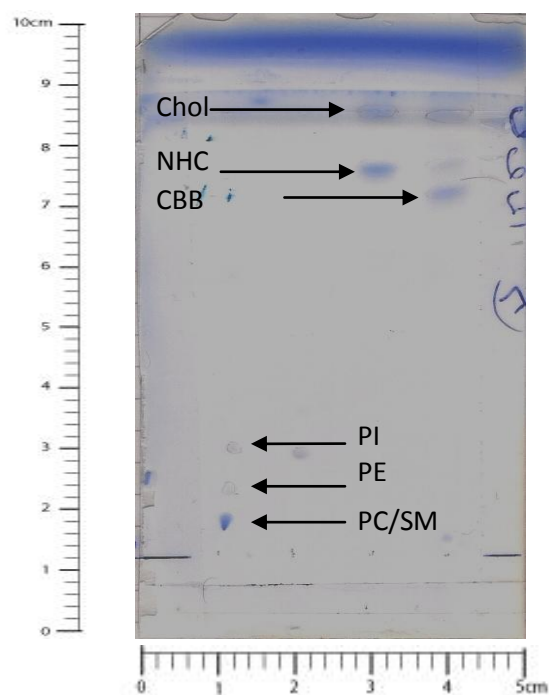


Figure 25 A one dimensional HPTLC separation of lipid samples.

Using the solvent mixture 1 (

Figure 25) it was possible to gather average R_f values of several classes of lipids as shown in Table 3.

	AV	STD	in %
PI	0,15	0,00	1,60
PE	0,25	0,02	6,74
PC22	0,07	0,01	8,14
Chol	0,95	0,01	0,76
NHC	0,82	0,06	7,06
CBB	0,76	0,05	6,63

Table 3 Overview of average Rf values of tested standard lipids AV = average value, STD = standard deviation, in % is standard deviation in percent of the average. Average with 10 experiments.

The other solvent mixtures were not applied anymore due to the fact that they lacked separation performance. Switching ethylacetate to methylacetate, the solvent mixtures polarity was increased and the separation power of the solvent increased dramatically. In general it can be said that both solvent mixtures 2 and 3 are not suited for the purposes of this work since they are too apolar.

4.1.1 Evaluation of staining methods

In order to decide whether to use primuline or Coomassie Brilliant Blue R-250 a sensitivity experiment was done. The compatibility of both staining methods with MS was not a criterion since both are reported to be working just fine with MALDI-MS (60)

Dilutions from a stock 1 µg/µl solution of PC 22:0/22:0 were prepared. The pure solution could easily be detected by both staining methods so dilutions of 1:100, 1:10.000 and 1:1.000.000 (all v/v) were created. Of each dilution except the 1:100, 1 µl, 5 µl and 10 µl were applied resulting in a row of dilutions with the following lipid content

Spot number	1	2	3	4	5	6	7
Amount of lipid	1 pg	5 pg	10 pg	0.1 ng	0.5 ng	1 ng	10 ng

Table 4 Comparison experiment Primulin to Coomassie Blue R-250 dilution steps. Amount of lipid on one spot on HPTLC plate.

Using this scheme, the following two plates resulted but without any prior separation step.

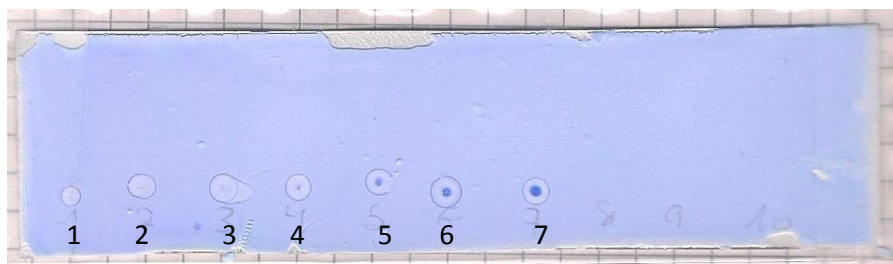


Figure 26 Dilution experiment with Coomassie Blue R-250.

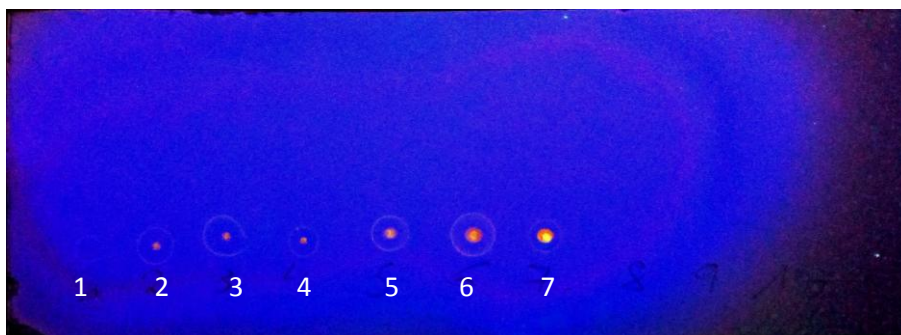


Figure 27 Dilution experiment with primuline dye seen under UV light.

As can be easily seen in Figure 26 and Figure 27, by means of the primuline dye already 5 pg of lipid was detected compared to the just 0.1 ng by means of the Coomassie Blue R-250 dye. The circles around the actual spots are not sample but from the solvent during application. Coomassie Blue R-250 therefore was discarded as a staining reagent in favor of primuline dye.

4.1.2 2D HPTLC of Lipids

With 2D-HPTLC it was possible to get close to 20 different classes of lipids out of the B&D extract of TBEV. Although the spots contained enough lipid to be detected by primuline staining most of them did not contain enough lipid to be detected using MALDI-MS. Those are marked as unknown in the schematic below (Figure 28). For the unknown spots sample signal could not be discerned from background noise. The schematic is a composition of several experiments. Experiments were done using the standard lipid amount found in the description of the Bligh & Dyer method (see chapter 3.2) and with a preparation using ten times the amounts of materials mentioned there (2.4 ml). All of these plates were analyzed using mass spectrometry and a map of the resulting lipids and their relative position to each other was produced. As explained in the chapter Imaging, one spot contains often several lipids of the same species, for example only PC's but with varying fatty acid side chains separated along one spot. While the definite lipids are different between synovial fluid and TBEV, the position and class of lipids along the 2D separation are the same.

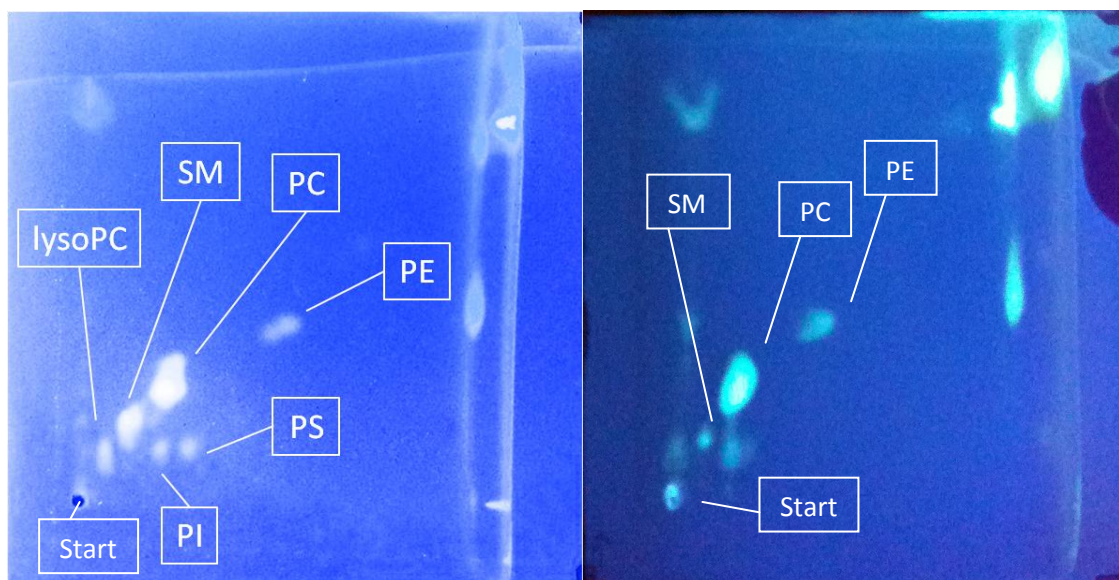
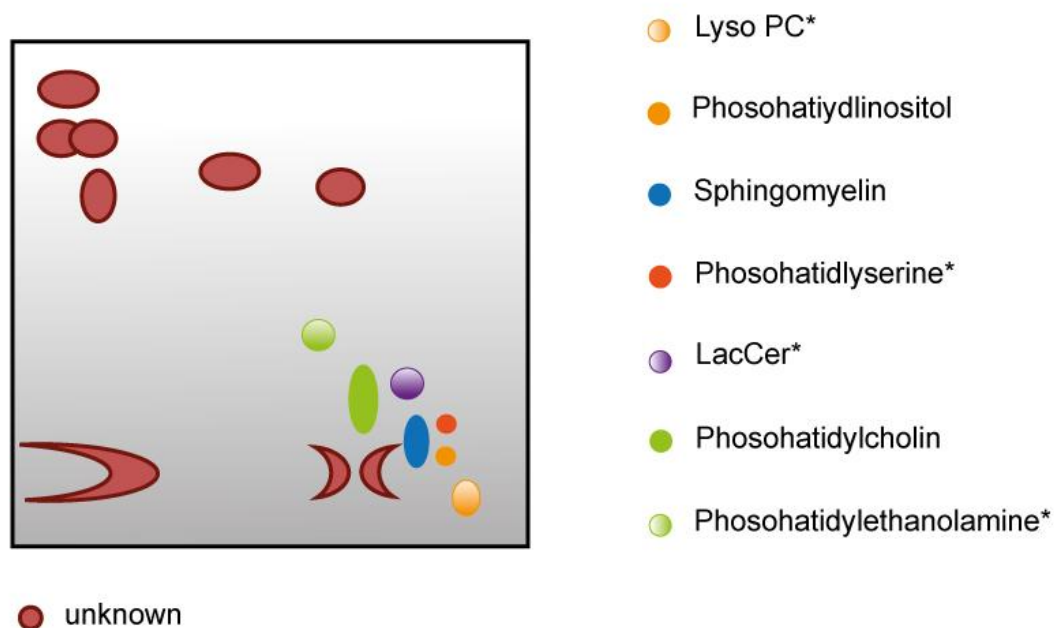


Figure 28 Sample 2D HPTLC plates stained with primuline dye (10x10cm) Synovia fluid on the left and TBEV on the right.

As the 2D HPTLC plates (Figure 28) show, synovial delivers more spots (left side). The general pattern of lipid species is of course the same for both samples for those classes of lipids that are present in both samples. A schematic that summarizes the results of all done experiments in one theoretical plate is shown below (Figure 29).



* Not seen with TBEV but synovial fluid

Figure 29 Schematic of the results from 2-D HPTLC and all the identified species areas. One area encompasses a single class of lipids and within several different lipids of the same class. See also Figure 55 LacCer = Lactosyl Ceramide. Lyso PC is at the starting point.

The main number of experiments was done using TBEV vaccines. Using lipid standards and synovial fluid as additional references a map of relative coordinates for the prominent lipid species could be created. Since the great differences of exact locations on the plate from experiment to experiment, only the relative positions of the spots to each other were used to estimate what spot is what. This was necessary to attribute known locations on the plate from standards to some species (see Figure 29) of TBEV extractions. The synovial fluid was used since an abundance of synovial fluid was available with relative high lipid content and a test with real samples was required. With the high lipid content synovial fluid it was easier to get first results on position of lipid classes compared to the low lipid content viral extracts and positive identification of otherwise not identifiable spots like PE.

4.2 Mass Spectrometry

4.2.1 Sample preparation

4.2.1.1 Influence of the solvent and composition of matrix mixtures

At the beginning of this work two matrixes were used for the MS experiments, 6-aza-2-thiothymine (ATT) and 2',4',6'-Trihydroxyacetophenone monohydrate (THAP). THAP is the standard matrix used in all prior lipid experiments of the group due to its good performance with lipids in general (7, 67). ATT was reported to be a suitable matrix for lipid analysis and was tested also as a comparison (68).

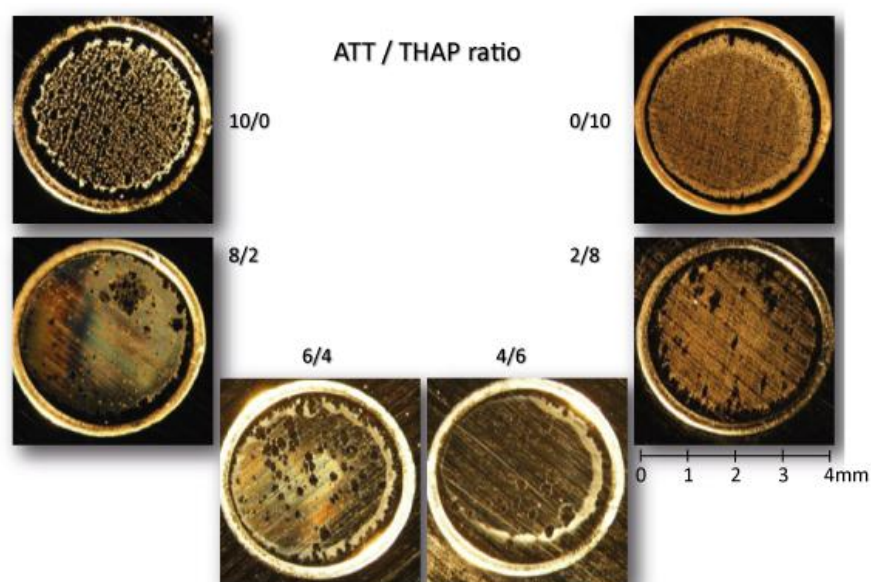


Figure 30 Microscope pictures of Kratos Analytical metal target spots with dried matrix. Shown are variant matrix mixtures of ATT and THAP matrix.

During the experiments it was difficult to obtain any signal using the ATT matrix analyzing PC standards. Closer examination with a microscope showed that using the sample preparation techniques described in chapter 3.4.2 ATT showed very weak signals due to its inhomogeneous crystallization. In order to cancel this effect out we tried to create a mixture of both matrices. All mixtures of THAP with ATT resulted in inhomogeneous layers of the matrix. Only pure THAP matrix crystallizes to a homogeneous matrix layer (see Figure 30)

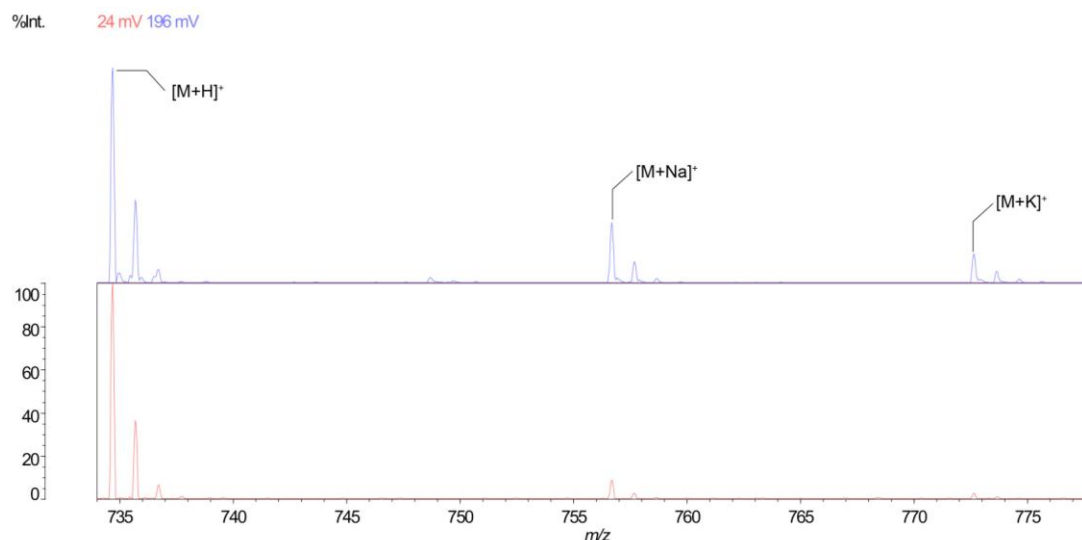


Figure 31 Comparison of the molecular ion region of the positive ion MALDI mass spectrum of PC (32:0) prepared with the MALDI matrix THAP (upper) or ATT (lower) matrices.

The inhomogeneous matrix crystallization of ATT (Figure 30) as well as the comparatively low performance regarding signal to noise ratio (Figure 31) where ATT delivered several magnitudes less signal than THAP were reasons to no longer use ATT as a matrix.

During the first experiments crystallization of pure THAP was often not satisfying in the form of very inhomogeneous crystallization. Further research on this topic showed that the solvent seemed to evaporate in several steps. According to their volatility, several evaporation steps seem to occur. This results in smaller droplets of less volatile solvent remain concentrated on a smaller area of the spot. The matrix mainly concentrates in these remaining solvent islands that then evaporate also resulting in the creation of something that can be described visually as “islands of matrix”.

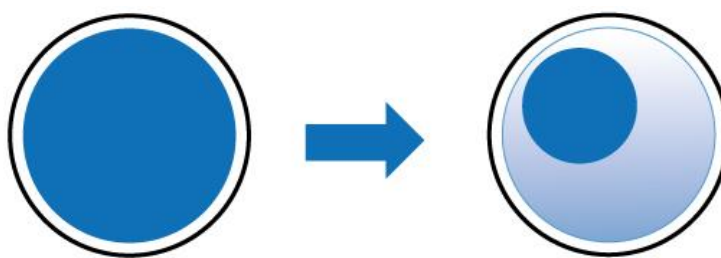


Figure 32 Schematic of the effect of different volatile solvents during matrix crystallization.

As can be seen in Figure 32, instead of evaporating homogeneously, the more volatile solvent evaporates, shifting the less volatile one around on the spot, where it, and its contents, concentrate, creating an “island of matrix”. Since THAP dissolves better in methanol than in chloroform less matrix is on the larger part of the spot (highlighted in lighter blue), where the more volatile chloroform evaporated, concentrating in the smaller methanol spot.

This was thought to be an effect of an unfavorable amount of chloroform in the sample mixture, since chloroform is more volatile than methanol and evaporates faster. Different mixtures of chloroform/methanol were therefore used with the same amount of THAP in order to see this effect under the microscope.

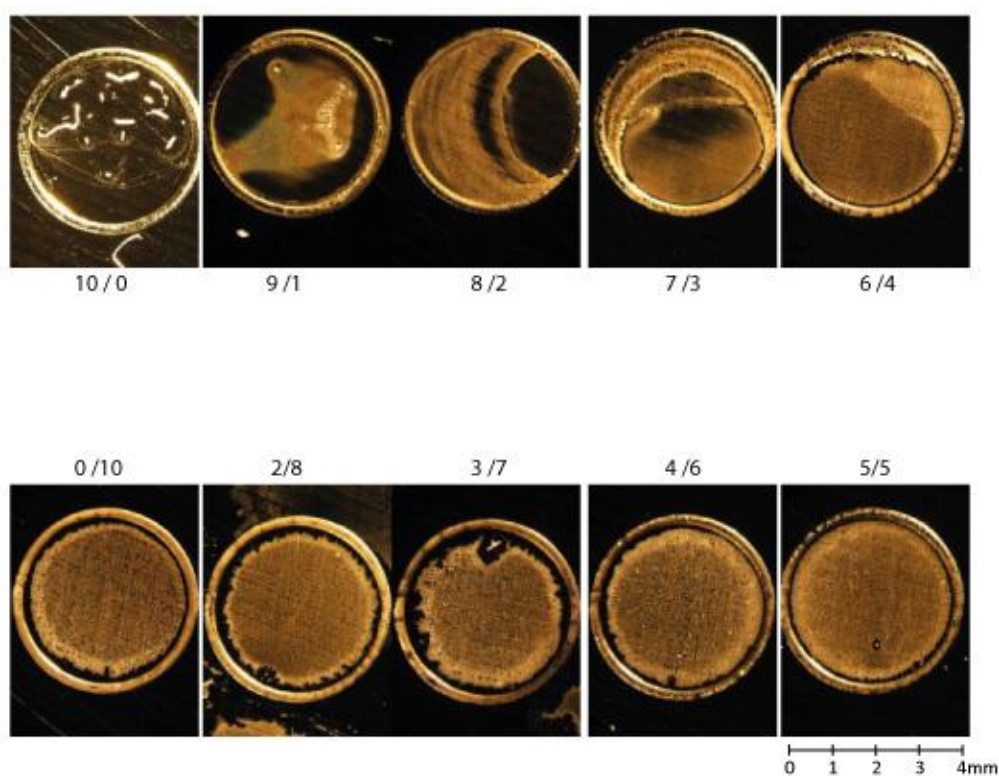


Figure 33 Microscope pictures of the influence of chloroform on matrix crystallization. Ratios given in parts chloroform/methanol. .

Percentages of more than 50% of chloroform in the sample mixture resulted in inhomogeneous crystallization behavior, whereas more than 70% chloroform resulted in the crystallization into hot spots of matrix. Unfortunately those areas are weak in sample since most lipids dissolve better in chloroform, whereas the THAP matrix due to its higher polarity dissolves better in methanol. This leads most probably to an unfavourable sample/matrix ratio for efficient analyte desorption/ionisation samples in areas with low matrix content, where the sample can no longer be detected due to a lack of matrix.

4.2.1.2 Further matrices tested for MALDI

In literature several matrixes can be found for lipid analysis with MALDI – MS (69). The three most prominent matrices were tested against THAP as the well known standard matrix for lipids of our group. Due to its unsatisfying crystallization behavior the ATT Matrix was discarded earlier on so two additional matrixes, 9-aminoacridine (9AA) (69) and para-nitroaniline (PNA) (69) remained.

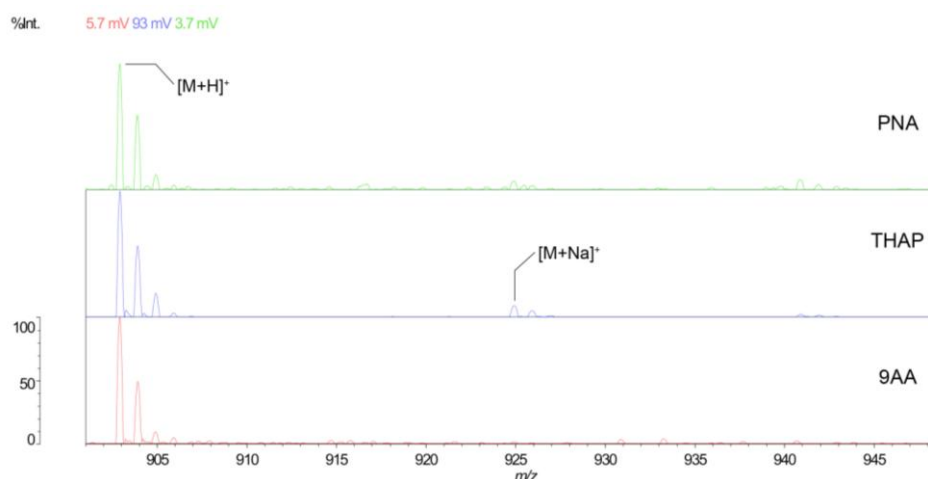


Figure 34 Comparison of molecular ion region of the positive ion MALDI mass spectrum of PC22:0/22:0 in order to show the performance of p-nitroaniline (PNA), (THAP) and 9-aminoacridine (9AA)

All matrixes were used as a 20 mg/ml solution dissolved in methanol. During measurement PNA showed a two times higher sensitivity to the matrix-assisted laser desorption/ionization so experiments with PNA were done using half the laser power used for the other matrices in order to treat our detector with care. Interestingly this increase in signal intensity by using PNA did not result in an increase in sample signal intensity but only in increase in matrix signal intensities. Therefore we were forced to use a higher laserpower (80) and use the ion gate to blank the MALDI matrix signals from reaching the detector to prevent overload. With identical standards THAP gave ~20 fold higher intensities without a loss in signal quality. With 9AA and PNA the sodium ion products could not be detected. This behavior is an advantage under normal conditions since less ion adducts result in more signal from the singly protonated molecule. For further experiments using positive ion mode all but THAP were discarded as standard matrix.

PNA has been known to decay over a very short time (69). Measuring a sample that was in the mass spectrometer for more than 30 min showed no signal any more despite visual existing substance still on the target. Kristin Teuber reported (69) that PNA is not stable under vacuum conditions and decays rapidly. This, in addition to PNA's high carcinogenic nature led to its drop out for further experiments. 9AA was also discarded as a MALDI matrix for positive ion mode measurements because its performance was inferior regarding signal intensity to the one gained by using THAP.

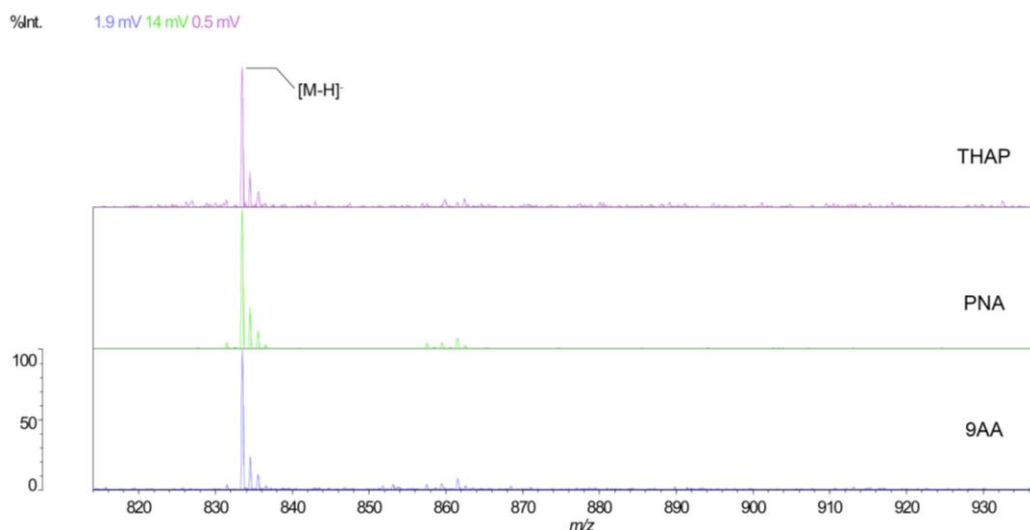


Figure 35 Comparison of the molecular ion region of the negative ion MALDI mass spectrum of a commercial PI sample from chicken egg to see the performance of PNA, 9AA and THAP

Doing the same experiment in the negative ion mode resulted in completely different performances. THAP showed less performance, whereas 9AA surpassed THAP ten-fold. As in the experiment before PNA was discarded due to its instability and high toxicity and 9AA was chosen for all further experiments using negative ion mode. Influence of solvents on the results was not investigated.

4.2.1.3 Influence of matrix concentration

Matrix to analyte ratios are crucial in MALDI mass spectrometry, since the analyte must be surrounded by only matrix molecules without being so low in concentration as to no longer be detectable. Starting from the concentration values used in prior experiments done in the group, three concentration steps were evaluated in order to find the most suited matrix to analyte ratio.

Prior experiments were done using concentrations of 20 mg/ml matrix and mix the matrix in a 1:1 ratio with the analyte solution. In order to see whether an effect could be seen when this ratio was halved or taken up by half we used solutions of 10 mg/ml, 20 mg/ml and 30 mg/ml of THAP in methanol.

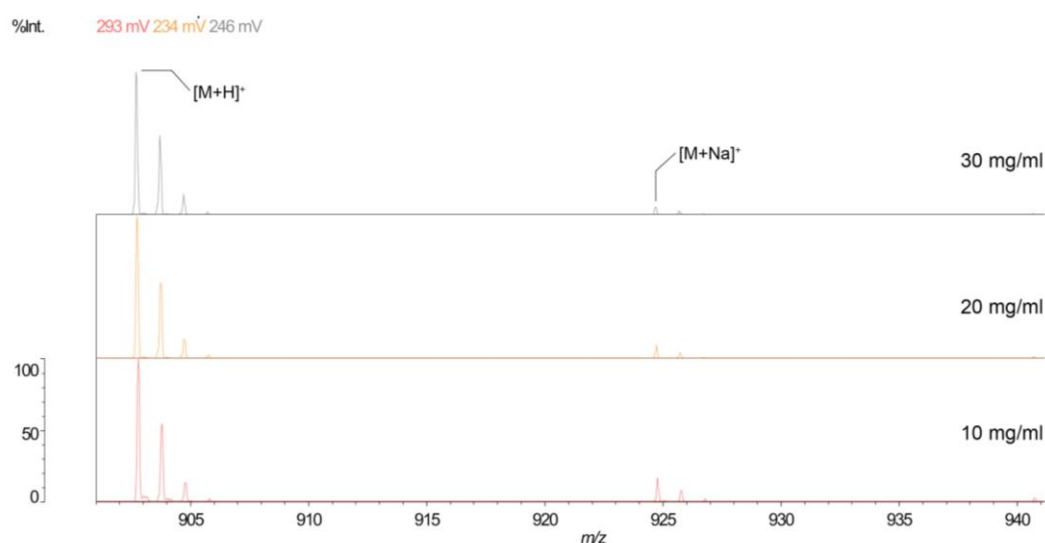


Figure 36 Comparison of the molecular ion region of the positive ion MALDI mass spectrum of 8 μ g of PC 22:0/22:0 mixed with different concentrations of THAP matrix dissolved in methanol.

As can be seen in Figure 36 indicated by the intensity values in the upper left corner the influence of matrix concentration within the range of matrix/solvent ratio we used is negligible. For the experiment the same volume of mixture was applied but with differing concentration.

Experiments showed that intensity values are all in the same magnitude. Therefore 20 mg/ml THAP in methanol was used for the rest of the experiments.

4.2.1.4 Influence of storage solvents

A mixture of 1,2-dichloropropane/1-propanol 1:1 (v/v) was evaluated in order to reduce the amount of volatile solvents in the storage mixture. It was reported that the above mixture reduced loss of solvent and therefore change of concentration of lipid samples over time could be avoided (70). During the optimization process differences in signal quality due to different solvent mixtures was taken in as an experiment and the improved storage method was compared to the standard method 2 parts chloroform/ 1 part methanol. HPTLC experiments did not suffer any changes because of storage solvents, but mass spectrometric experiments did.

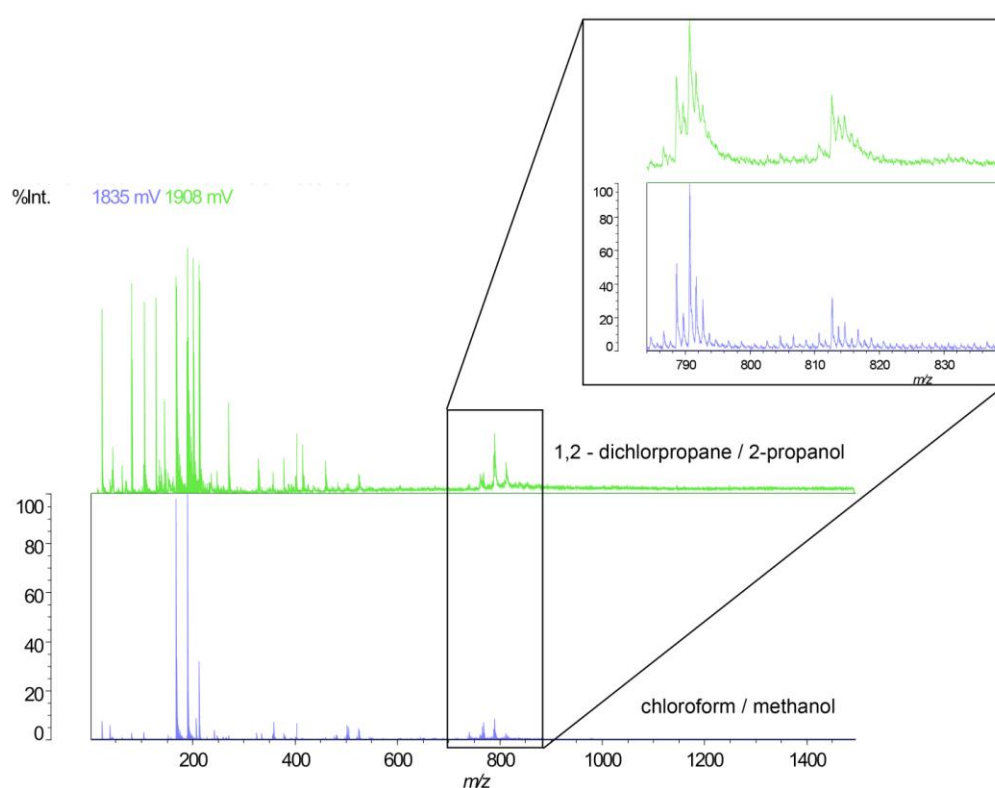


Figure 37 Comparison of the positive ion MALDI mass spectrum in order to see the influence of the storage solvent on the measurements. The green spectrum is the previously used 1,2-dichloropropane/2-propanol mixture, blue is the chloroform/methanol mixture. The close up shows the relevant lipid peaks cleaned from the blank spectrum (Lipid mixture from PC standard samples)

PC was dissolved in the both storage solvents and taken to the mass spectrometer using THAP matrix. As the above figure shows, drastic differences exist between the two storage solvents. Whereas the standard chloroform/methanol storage solvent exhibits little matrix signals in the lower mass range, the 1,2-dichloropropane/2-propanol mixture shows less resolved signals. Close up of the lipid signals shows a loss in signal resolution, a lot of the peaks can no longer be discerned.

4.2.1.5 Influence of storage time

Glycerophospholipids, as esters, are instable in water and oxygen containing environments over longer times. Therefore stability over time is a crucial factor during these experiments. One lipid sample, a PC22:0/22:0 was taken aside for these experiments and evaluated after certain times. The sample was stored in the chloroform/methanol mixture ratio described earlier, in order to hinder air and water to oxidize it, in light protected vials to hamper light induced breaking of bonds and at -20°C in order to stop heat induced fragmentation.

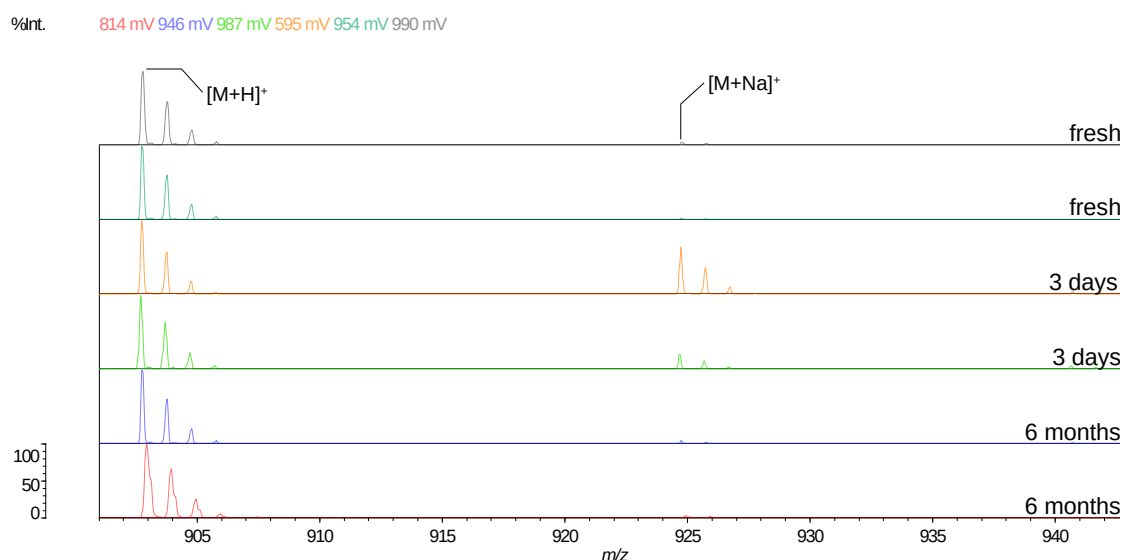


Figure 38 Comparison of the molecular ion region of the positive ion MALDI mass spectrum of PC22:0/22:0 to see the influence of storage on the sample measured under the exact same conditions directly after production of the aliquot, 3 days after and 6 months after.

As can be seen in the upper left corner, intensity values range mainly between 800-1000 mV. Counting in the reproducibility of MALDI in general no real change of the sample within 6 months could be detected. Also there is no sign of any degradation, which would have resulted in additional peaks.

Considering the drastic variations that can occur in MALDI experiments due to analyte distribution in the sample, intensity values started at maximum of 946mV for a fresh sample. After three days, in order to see whether there are any short term issues with the chosen storage method the maximum intensity was at 1058 mV, and after 6 months is peaked at 954mV. Differences in sodiated ions signal intensity are most likely due to impurities of solvents and freshly prepared MALDI matrix solutions.

4.2.1.6 Reproducibility of MALDI – TOF measurements

As was explained in chapter Matrix Assisted Laser Desorption Ionization Time of Flight Mass despite homogenous crystal distribution inhomogeneous signal intensity distribution with so called “sweet spots” can occur. As was also seen in chapter Sample preparation the crystallization is not always perfectly homogenous. The effect of these phenomenons on the spectra were examined.

The problem of having to search for “sweet spots” can be avoided by averaging measurements from shots taken from all over the whole spot. This however, because a lot of mass spectra without useful signal are taken into consideration, has the drawback to increase noise in the averaged mass spectrum, but has the advantage to be automatable. With very inhomogeneous intensity distribution over the spot this will however most likely result in loss of information since for the averaged mass spectrum the small number of intense sample signals will be lost in the noise of the averaged mass spectrum. A somewhat better method is to manually scan the sample spot for high signal intensities and scan intensively around these.

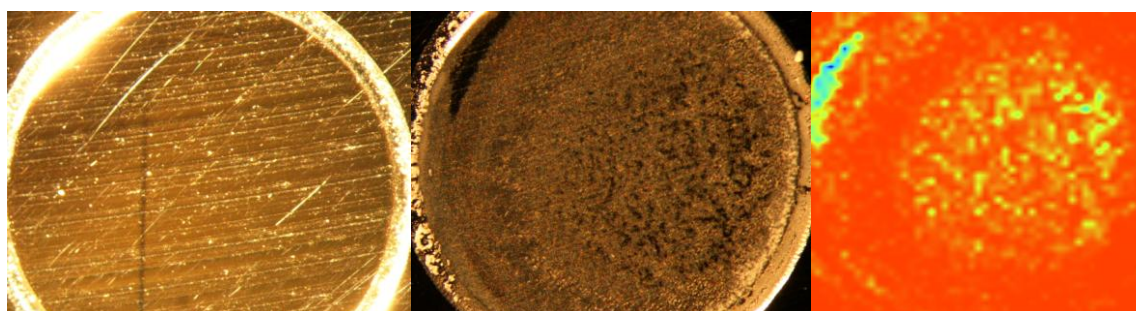


Figure 39 Picture of the blank spot (left), matrix covered spot (middle) and the heat map of the signal intensities of the applied sample (right) whereas orange is low intensity and yellow to white is very high intensity (10 times enlarged, whole spot 4mm diameter).

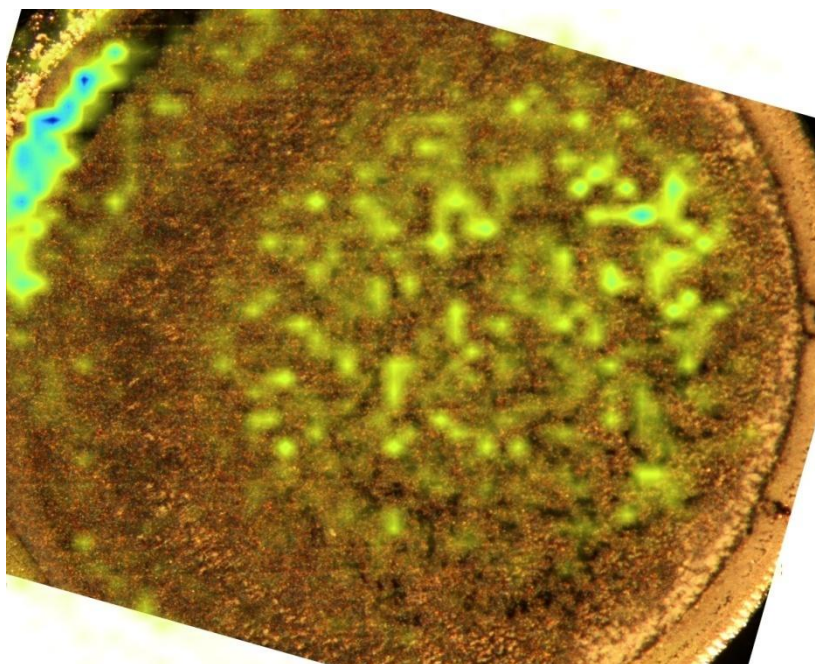


Figure 40 Sample spot of the previous MALDI measurement merged. The highlighted areas are where the sample is the most intense throughout the matrix covering the spot. (10x enlarged, whole spot 4mm diameter)

Sample intensities tend to be higher along edges in the matrix layer and less intense the more homogenous the layer is. In Figure 40 an overlay of a microscope picture with an intensity map for the sample is shown, highlighting the uneven sample distribution, with maxima of sample intensity in the upper left corner where there is a large rift in the matrix layer. During the measuring process samples deviating from this observation have been recorded, and examples of homogeneous matrix regions with high sample intensity could also be seen. This may however be the result of a jagged surface. Matrix here would pile high on itself and produce the same jagged structure but with an underlying matrix carpet. Nevertheless the reasons for this behavior remain little understood.

In order to evaluate the effect of this phenomenon for our matrix and experiment setup a row of samples was prepared exactly in the same manner and measured in the same way, using automated rastering of the whole sample spot and averaging the measured data.

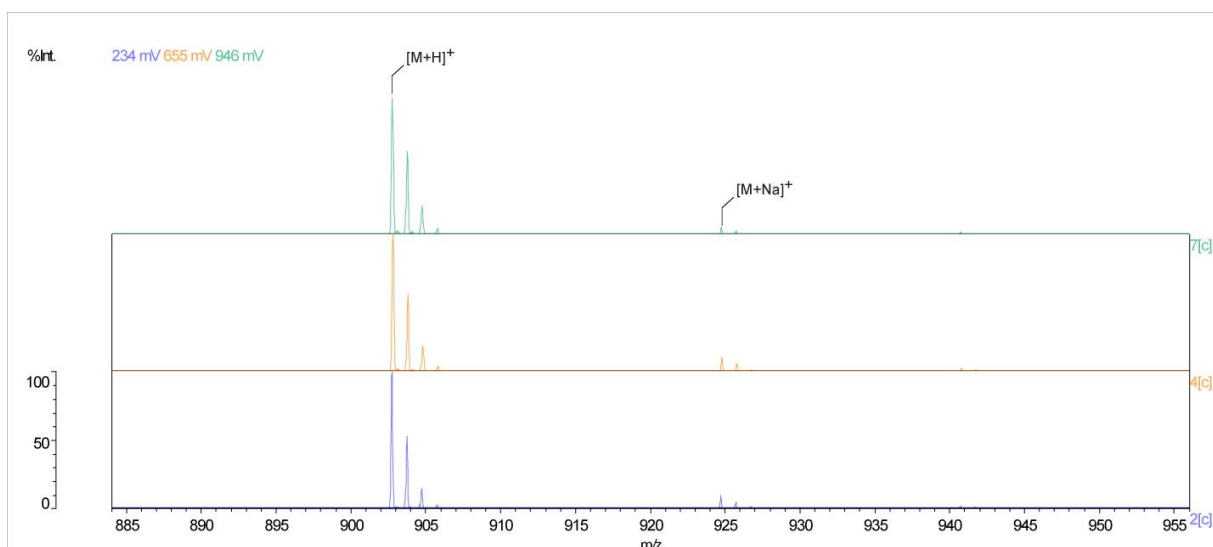


Figure 41 Comparison of the molecular ion region of the positive ion MALDI mass spectrum of 8 μ g of PC 22:0/22:0 with 20mg/ml THAP matrix, prepared exactly in the same way and measured consecutively.

Averaged intensity values differed greatly during the experiment series (averaged over 10 mass spectra), averaging on 590.4 mV with a standard deviation of 312.6 mV. These data was gathered using exactly the same setup, preparation, samples, concentrations and measurement variables. Multiple measurements are therefore preferable all the time in order to get the best possible results.

4.2.1.7 Influence of buffered matrix solutions

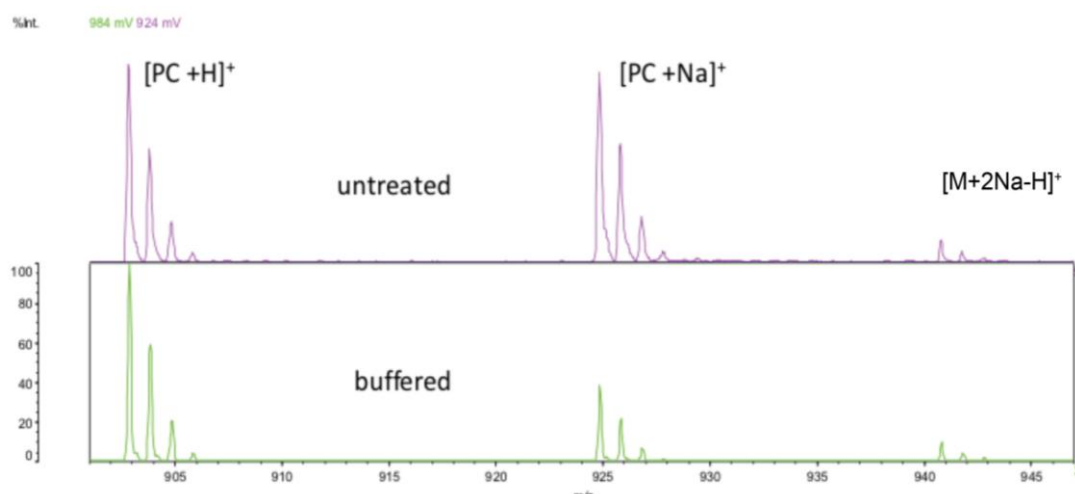


Figure 42 Comparison of the molecular ion region of the positive ion MALDI mass spectrum of a PC 22:0/22:0 lipid samples using buffered (ammonium phosphate) or non-buffered

During MALDI - MS measurements there is always a large amount of salts to be taken care of. A normal MALDI - MS measurement shows positive ions of $[\text{PC (M)}+\text{H}]^+$, $[\text{PC (M)}+\text{Na}]^+$ and sometimes $[\text{PC (M)}+\text{K}]^+$. This means that the given amount of sample is distributed among three different peaks and each signal is less intensive than it could be, in case of no sodium and potassium adducts present. Additionally, resolution of unbuffered mixtures is found to be (FHMW) in the range of 3750 for the $[\text{M}+\text{H}]^+$ peak and in buffered (ammonium phosphate buffer) mixtures it was found to be in the range of 8200, that is a more than twofold increase in resolution. Sometimes a high salt concentration is wanted in order to obtain signals of samples that need sodium in order to desorb/ionize, or to help destabilize the ion during CID. Most of the time however the additional ion peaks of the same sample with different salt adduct ions are unwanted. An easy method to avoid this is using buffered solvent mixtures which “push” out the majority of the detectable salt ions in favor of undetectable ones like ammonium salts. As the mass spectrum shows, the intensity of the sodium adduct ions is significantly reduced, whereas the protonated molecule is increased in resolution and intensity.

4.2.2 Influence of extraction method

Tests on the performance of the three extraction methods from Wenk (10), Merrill (57) and Bligh&Dyer (54) using the same TBEV sample and measuring the lipid extract without preliminary separation with MALDI-MS delivered very different results.

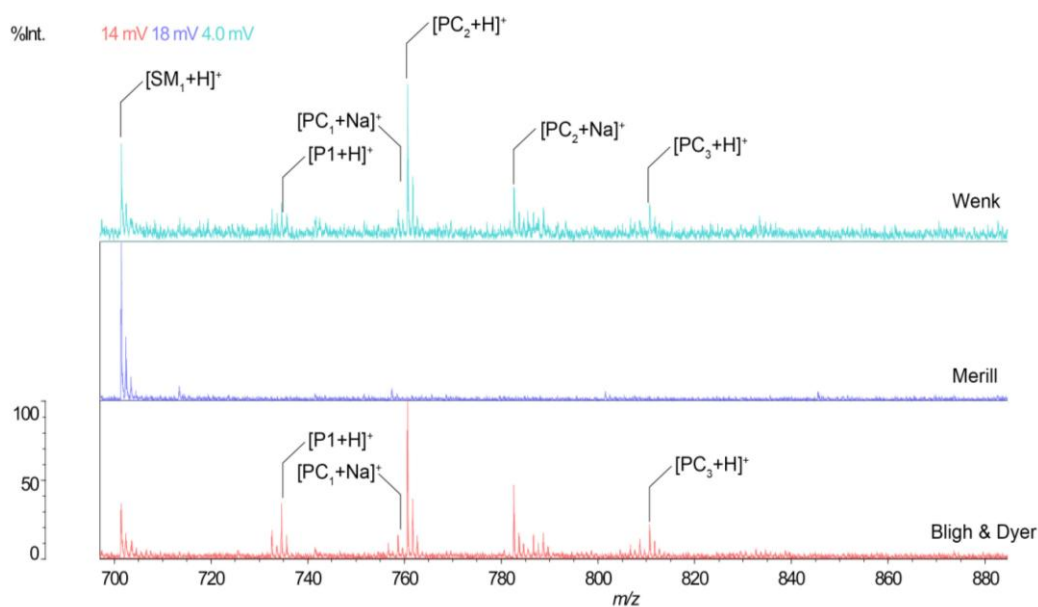


Figure 43 Comparison of the molecular ion region of the positive ion MALDI mass spectrum of TBEV to show the tested extraction methods from Bligh & Dyer, Merrill and Wenk.

Using the Bligh & Dyer method seems to extract the most lipids resulting in the most peaks in the mass spectrum which goes along well with the reported effectiveness of the method.

The Method described by Wenk, was reported to enhance extraction of phosphoinositides, while reducing the extraction efficiency of prominent lipids such as PC and SM. As can be seen in the spectra, Wenk's extraction method results in a decrease of signal intensity of the relevant lipid classes of PC by more than three times. Although no PI's could be detected with the other methods, no PI's could be detected in the Wenk extraction. This is surprising since Wenk claims that his extraction method increases PI extraction significantly. Whether this is due to a lack of PI in the sample in general, the method itself or the most probably low content of it in the sample could not be discerned in this work.

Merrill claims his method is more powerful for the extraction of sphingolipids. This could be verified since the intensity of the SM signal increased noticeably. Because the main focus of the work is, however on the phospholipids, the method was not further used. Another reason for this is the unwanted side effect that the treatment of the sample with an alkaline solution during the extraction accounts for the loss of all phospholipids through chemical destruction.

4.2.3 Calibration

The MALDI-TOF technique requires a very well done calibration with known substances. During this experiment castor oil was used to do so. Of particular interest was the consistency of the surface of the HPTLC plate we used to measure samples. Since correct correlation of an ion peak with its lipid molecular mass using a TOF analyzer requires a planar surface with a perfectly smooth and planar surface. Measurements were done in order to evaluate the height inconsistencies of the HPTLC plate. It was reasoned that a porous material like silica gel would exhibit a lot of small rifts or holes.

The whole plate was coated with calibration substance and the plate was measured using the same calibration for all acquired mass spectra. Deviations from the expected mass then have been converted into height.

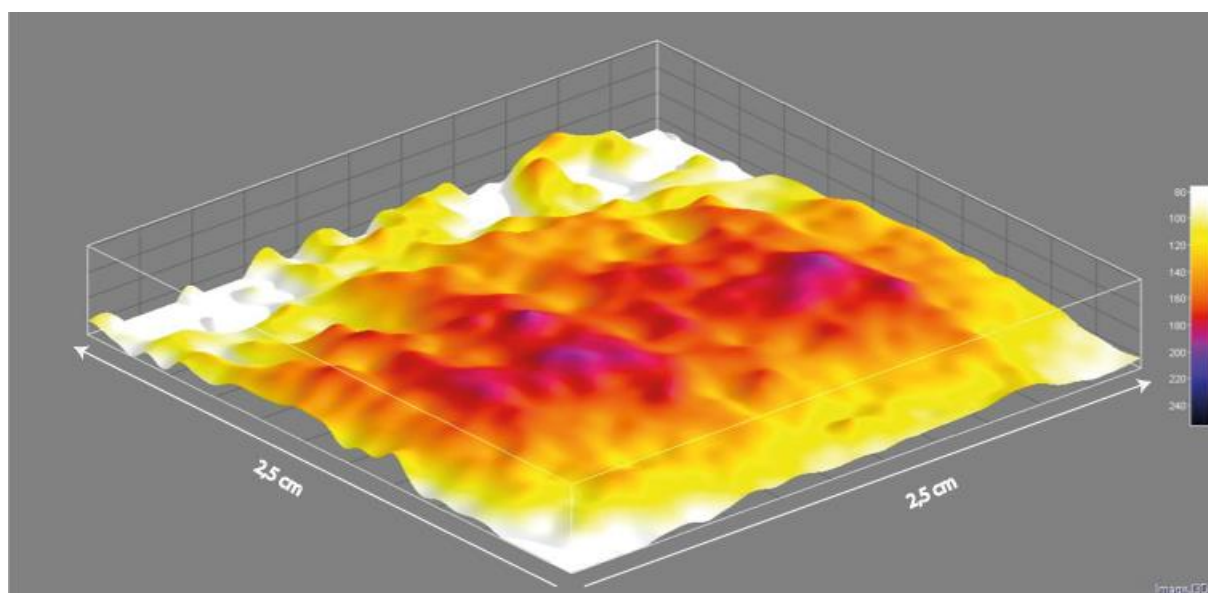


Figure 44 Visualization of mass deviations on the HPTLC plate. Used with friendly permission from Max Bonta. Mass deviations are from 0 (lowest) to 0.24 (highest) Daltons. Scale unit are mDa

Surprisingly the deviations on the HPTLC plate were rather low with a maximum deviation of 0.24 Da. Even more astonishing, the highest deviations, the “mountains” on the visualization above, only occur at spots where there is no tape under the HPTLC plate. In the above picture you can see the effect when underneath the HPTLC plate there are two adjacent pieces of double-sided tape not exactly together. As long as a complete layer of tape without any free space between the tapes is present, the deviations are even smaller and range only to 0.14 mDa.

4.2.4 Quantification of Lipids

Reliable quantification of the lipid content in each step of the experiments of this work would have been very important. Therefore it was an early focus of this work. Quantification of the diverse classes of lipids in the lower μg to ng range used in the experiments proved to be difficult to impossible in the timeframe of this work.

To this date the best method to at least evaluate roughly the complete lipid content of our samples at the beginning of the experiment was the sulfo-phospho-vanilin method. Prominent methods such as ashing of the samples or gravimetric methods were not tested due to the fact that they destroy the sample.

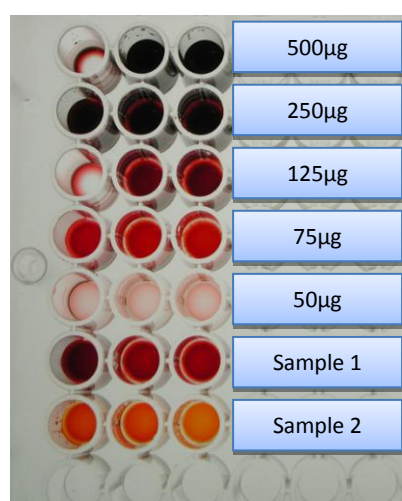


Figure 45 Example of a sulfo-phospho-vanilin quantification using decreasing amounts of cholesterol for calibration. Numbers are complete content per well.

While the method uses an elegant chemical method to detect the samples it brings with itself several technical problems and an unfavorable detection limit that resulted in the discontinuation of quantification experiments in this work. The chemicals of the method destroy the sample holder rather quick by dissolving part of the well material so optic measurements were impossible or at least hampered since the transparent well got cloudy through the acid and the solvents. Because of the clouding of the plate it cannot be said with certainty that the gathered data is reliable. The experiment seems to work better with large quantities of sample with low solvent content. However even very careful handling with a lot of material and little hazardous solvents led us quickly to the insight that detection limits are in the medium μg range per ml.

It was however possible to do some rough estimates using high volumes of TBEV vaccine samples. They contain an estimate of $\sim 60\mu\text{g}/\text{ml}$ of lipid.

Figure 45 also shows sample 1 and 2. Sample 1 is a lipid mix without treatment. Sample 2 is the same lipid sample applied to a HPTLC plate and extracted from the plate again using the Bligh & Dyer method. While quantification was not possible reliably, it could be shown that qualitatively an optically detectable amount of lipid was not retrievable from the HPTLC plate. This is in accordance with loss in signal intensity in MS experiments using extractions from the HPTLC plate.

4.2.5 MS/MS

In order to identify and then verify what each spot of the 2D HPTLC separation (see chapter 3.3.3) is, each significant lipid peak was identified as far as this was possible using CID. Due to the fact that all recorded spectra of viral samples have comparatively low intensities a reliable identification of lipids that included their exact acyl side chain composition was not possible. Clear CID spectra normally deliver signals for the broken carbon-carbon bonds. With this information a definite identification of acyl side chains is possible. For that however very intense parent peaks are needed. This was only possible with standard samples, not with virus samples. Therefore only the identification of the lipid class was possible by identifying the fragments related to the head groups of the various lipid classes. Identified lipid classes from viral samples were: PC, PE, PI.

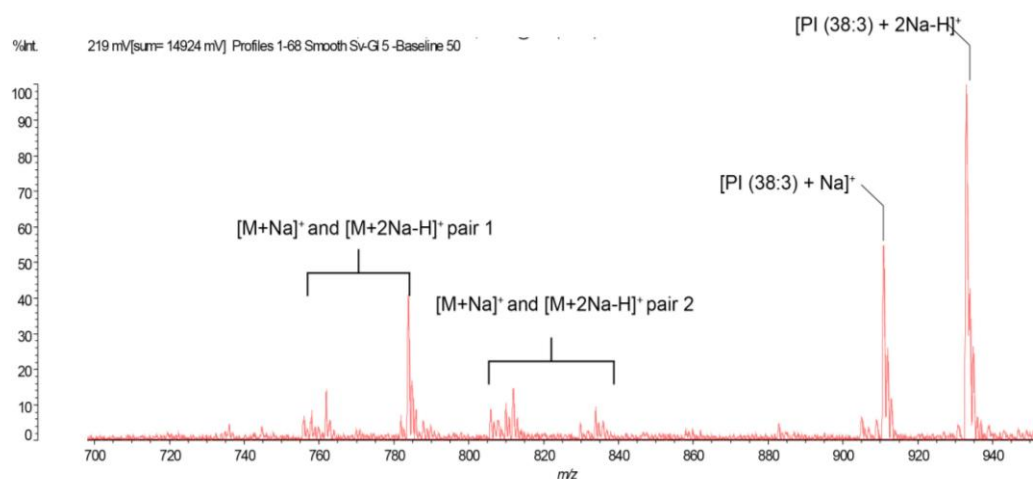


Figure 46 spectrum of the positive ion region of the PI spot with the peaks of the parent ion PI (38:3) as well as two unidentified lipid species. Only the PI (38:3) delivered signals that made a secure identification possible.

PI is found with very low signal intensities on the respective spot (see Figure 28, Figure 29) with the $[M+H]^+$ peak at 911.0 m/z and the $[M+2Na-H]^+$ at 933 m/z. Due to their lower stability, and therefore better fragmentation behavior, the $[M+2Na-H]^+$ ion was selected as precursor ion. From the wide spectrum of fragments normally found in high intensity CID spectra, such as loss of the inositol at parent mass -162 or loss of the complete polar head group with a neutral loss from the parent mass of -232.

This however is most likely due to the fact that the CID spectrum suffers from the low intensity of the parent peak.

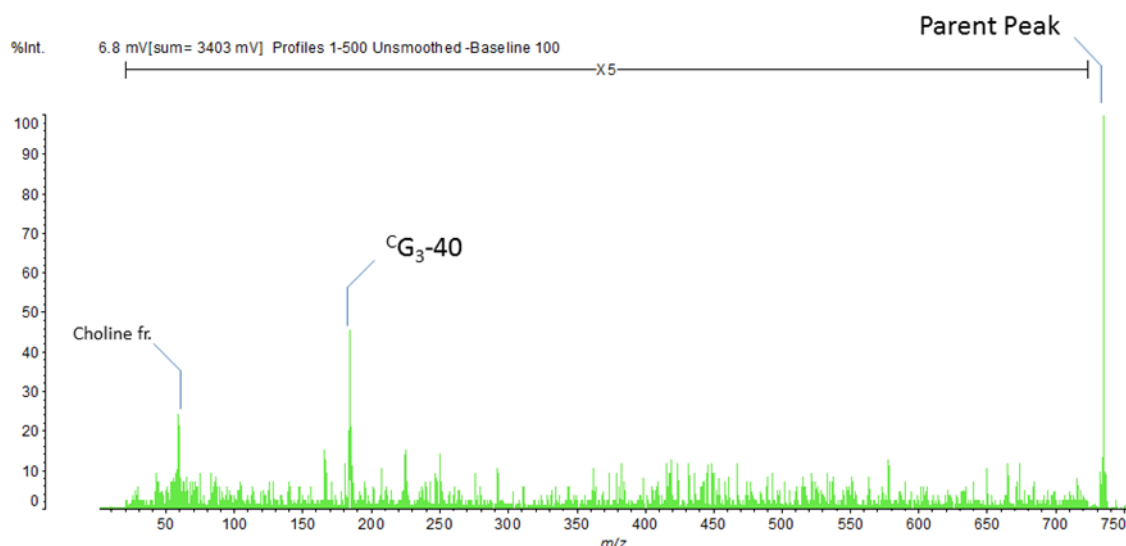


Figure 47 Positive mode MALDI CID spectrum of PI, parent peak $[M+2Na-H]^+$

Only the $^{21}G_3-40$ fragment at m/z 306.4 and the same fragment minus water at m/z 288.4 were found in the CID spectrum. This however suffices to safely identify the lipid as a phosphatidylinositol.

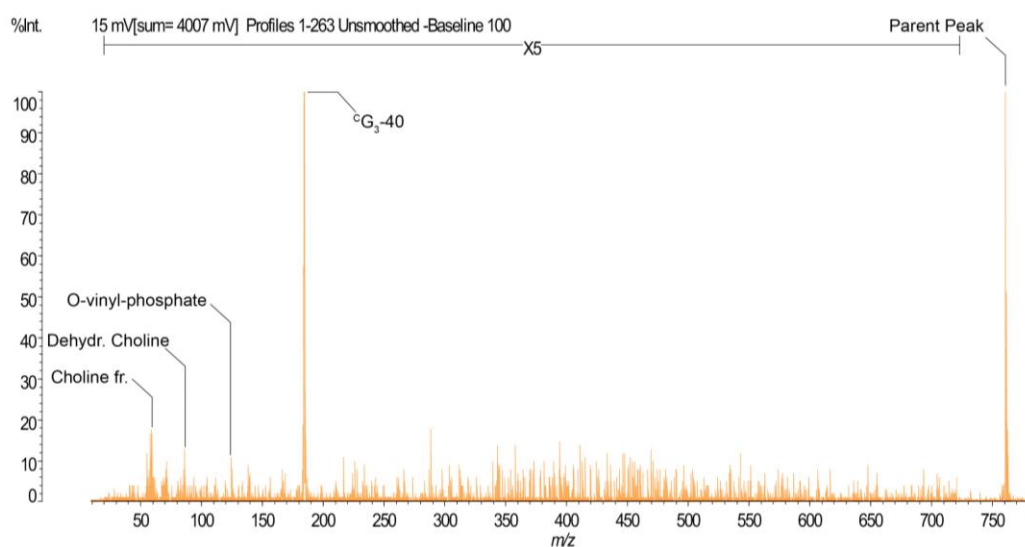


Figure 48 Positive mode MALDI CID spectrum of PC 34:1 $[M+H]^+$

Phosphatidylcholine, due to its quaternary ammonia group exhibits a fragmentation scheme that differs slightly from the other phospholipids. The same is true for sphingomyelin, a molecule that shares structural features with PC, such as the same head group. The differentiation between the two of them has, since CID spectra only allowed for the identification of the lipid class by head group fragment masses, not easily been possible. Only the presence of different spots indicated what SM was by looking at the fragmentation spectra and telling which one was definitely PC. The other one was then identified as SM. Due to the second nitrogen in the sphingomyelin however we have the phenomenon that SM ions always has an uneven positive mass in the spectrum, whereas PC always has an even mass. Differing from the standard fragmentation scheme of all other species PC shows

very low abundance of the ${}^cA_{12}$ of ${}^cA_{23}$ as well as product ions such as ${}^cC_{12}+18$ and ${}^cC_{23}+18$ or the loss of one neutral fatty acid ${}^cC_{12}$ and ${}^cC_{23}$. Fragments where both fatty acid substituent are at least partially fragmented, such as cD_3 (m/z 354.1) or cF_3 (m/z 282.1) are reported to be more prominent (7). Most abundant are characteristic fragments of cG_3 (m/z 224.1), ${}^cG_3-40$ (m/z 184.1) ${}^cG_3-58$ (m/z 166.1), protonated O-vinyl-phosphate or the cyclized form of it (m/z 125.0) and the protonated dehydrated choline (m/z 86.1). The most characteristic fragments all PC CID spectra, especially the ${}^cG_3-40$, protonated O-vinyl-phosphate and the protonated dehydrated choline were found. cG_3 as well as ${}^cG_3-58$ could not be found, however the existence of the mass peak at m/z of 58 is the loss of the 58 mass choline fragment, hinting at the appropriate fragment as well as the ${}^cG_3-40$ indicating that the ${}^cG_3-40$ fragment would most probably found with more concentrated samples. The fragments of cD_3 or cF however could not be found as well as the low abundance fragments of ${}^cA_{12}$, ${}^cA_{23}$, ${}^cC_{12}+18$, ${}^cC_{23}+18$, ${}^cC_{12}$ and ${}^cC_{23}$. This however was expected since the parent peaks exhibit low intensity values leading to statistically less signal resulting in to little signal of most less-abundant fragment peaks. As the label says however, the found fragments of PC are signature fragments securely identifying all PC clearly as such. Since no single fatty acid loss fragments could be found a closer identification however was not possible. All respective data was found with real TBEV samples.

4.2.6 Far Eastern Blotting

Far eastern blotting was primarily used to enhance sensitivity and concentrate samples in order to detect species not detectable prior to the blotting.

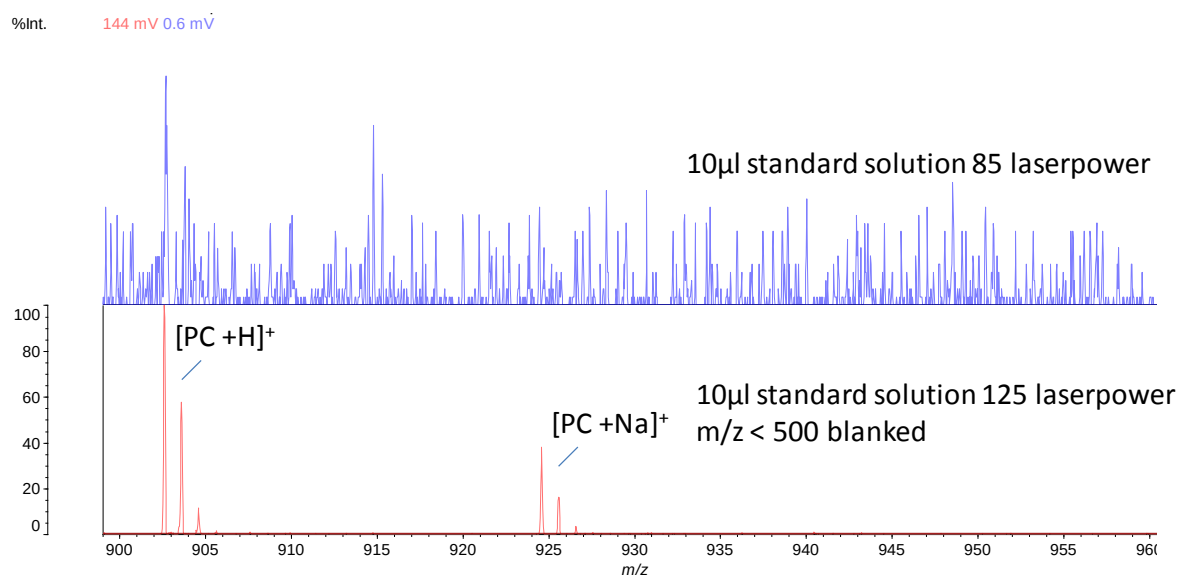


Figure 49 Comparison of the molecular ion region of the positive ion MALDI spectrum of PC 44:0. Spectra from PVDF membrane gained using “normal” experimental conditions and conditions with a 47% increase in laser power.

Figure 49 shows the effect of the blotting on MS measurements using standard lipid samples. A ~50% increase in laser energy is needed (from 80 to 120) in order to get a reasonable signal, which in turn leads to a signal overload at the detector in the mass range below 500. In addition a ~50% increase in laser power results in more in source decay, another unwanted side effect. An increase in sensitivity could not be detected. The main reason for this is most likely the fact that the used

samples already have a very low lipid content and loss during transfer results in lipid concentrations that are too low to gain useful spectra anymore.

4.2.7 Mass spectra directly from HPTLC compared to samples extracted from the HPTLC

The measurements directly from HPTLC was done by applying the HPTLC plate directly to the MALDI-MS target using double sided tape. It delivered mass spectra with up to three times higher intensities, and allowed the identification of phosphatidylinositol from the plate, including MS/MS measurements, which was not possible with the scratch method due to lack of signal intensity. A drawback however is the increase in noise most probably originating from the sometimes needed higher laserpower for these kind of experiments.

Early experiments have indicated that lipids cannot be quantitatively regained from silica using the Bligh & Dyer method as can be seen in chapter 3.2.3. Because these experiments are only qualitatively and not quantitatively the verification is only a rough statement. Therefore a loss of material is not only coming from an additional separation step but also from a loss of material due to the irreversible adsorption to the silica gel. On the other hand impurities from the silica gel, the binder of the silica gel and so on are most probably extracted and result in more noise and impurities signals. Since the TBEV already contains very little lipid from the start this drastically reduces signal information for experimental use.

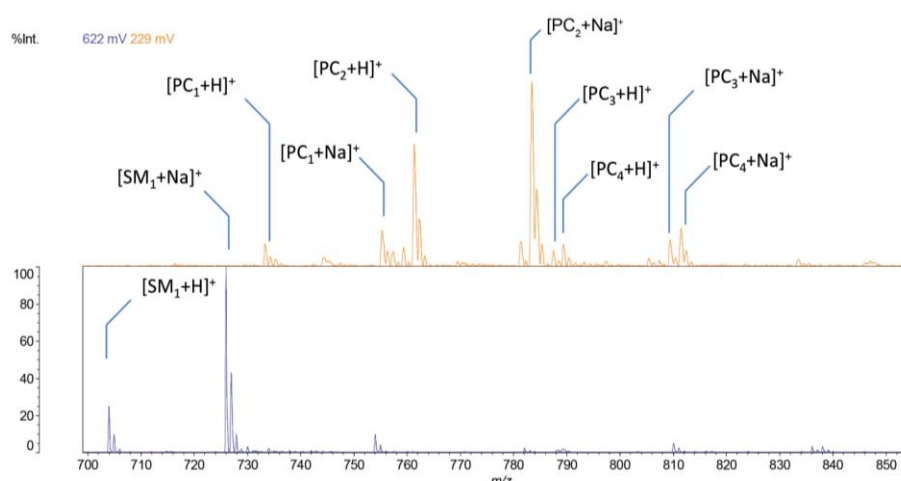


Figure 50 Positive ion MALDI mass spectrum from 2D HPTLC separation of TBEV vaccine. Spectra show sphingomyelins (lower spectrum) and phosphatidylcholine (upper spectrum)

The loss of resolution is possibly a result of the additional contaminants within the HPTLC plate, such as the silica, the glue with which it is attached to the aluminum backing, as well as a higher amount of salts that are trapped within the silica. Most probably the main reason for this is however the fact that the laserenergy that is needed to obtain reasonable spectra is slightly higher for HPTLC plate experiments which also results in loss of resolution.

Using TBEV vaccines the first measurement from HPTLC was done using a method that removed the whole solid phase from the plate using a scalpel (Figure 19). This method, having drawbacks such as a preparation in several steps, and therefore several steps where loss or contamination could occur, was easier to implement. The MALDI-MS measurement itself needs no change of the measurement procedure at the mass spectrometer.

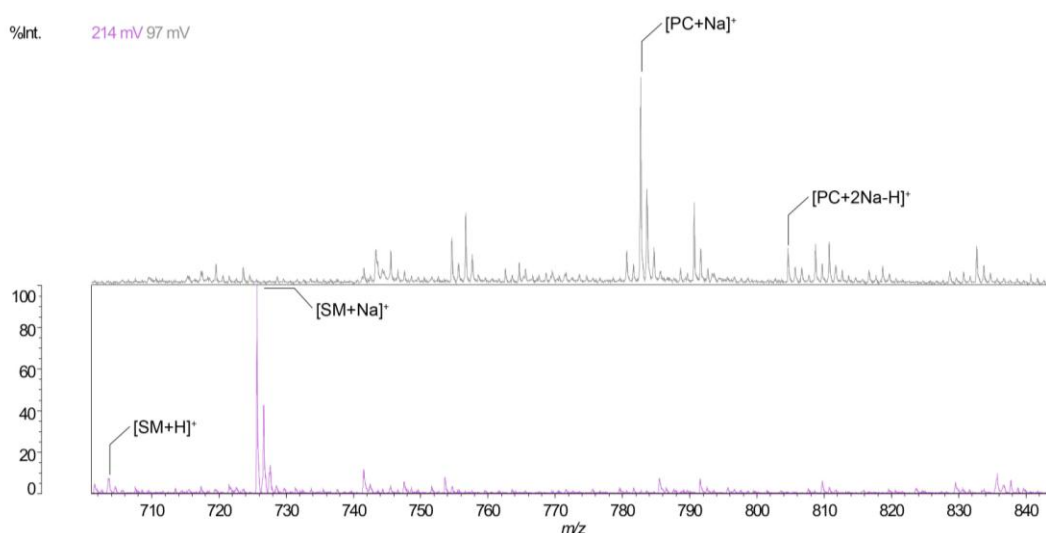


Figure 51 Molecular ion region of the positive ion MALDI mass spectrum of SM and PC. Spectra from 2D HPTLC of TBEV gained through lipid extraction from the 2D HPTLC spots containing sphingomyelin (lower spectrum) and phosphatidylcholine (upper spectrum).

Despite the several additional steps that have to be taken in order to be able to measure the samples, such as the scratching from the plate, extraction and reduction in volume after that, the spectra have satisfying intensity, at least for SM and PC. Most of the spots however delivered no identifiable signals. So far only the two species above could be clearly identified using this method as far as TBEV samples are concerned.

In comparison to the measurement directly from the HPTLC plate the extraction method delivered mass spectra with better resolved peaks and with good mass accuracy.

In comparison the direct method results in a loss of resolution but retains high signal intensity, whereas the extraction method results in loss of signal intensity while remaining resolution. Since a lot of the lipids were thought to be hardly detectable due to lack of intensity the direct method was chosen for further experiments.

The loss of mass accuracy however was not easily explained since the mass shift due to height changes on the HPTLC plate, as seen earlier in Figure 44 the height differences of the plate account for only small changes in mass. Spectra obtained directly from the HPTLC plate however deviate up to one 1.0 m/z from the expected m/z value.

Shooting the laser beam on the same spot repeatedly containing a known sample (PC22:0/22:0) showed a rather surprising change in mass. These experiments showed that the laser ablated the silica gel layer over the first 60 shots, therefore changing the distance from target to detector resulting in wrong m/z values masses if several measurements or a lot of shots for one spectrum are done. In short, the deviation occurs due to a change of dissociation/ionization height compared to the calibration, a phenomenon that has to be taken in to account.

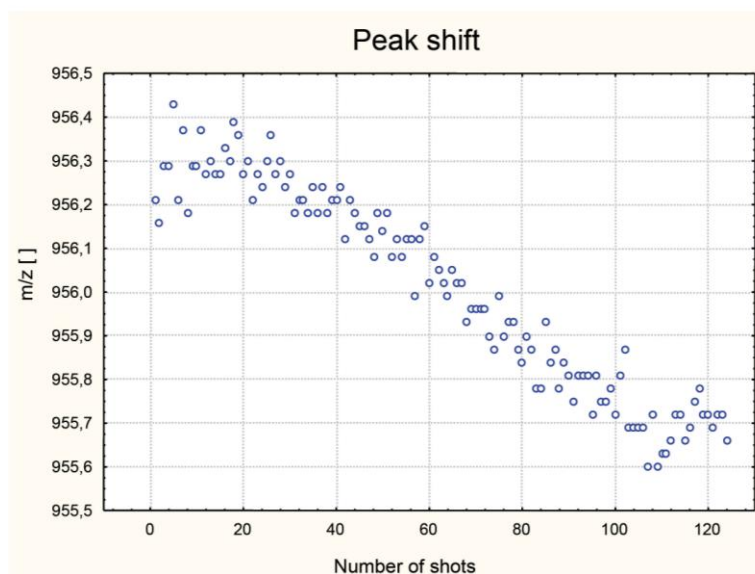


Figure 52 Mass changes during measurement from HPTLC plates depending on number of shots.

The laser ablates the silica on the plate resulting in a loss of signal after approximately 120-140 shots. Also after 120 shots the mass shift of the sample was close to one Dalton before the device stopped to get any signal at all at this spot (Figure 52).

This results from the embedment of the lipid in the silica gel material that gets ablated by the laser. Due to the lower height, the time of flight in the device is significantly longer resulting in the mass shift. With the mass shift of up to 0.24 Dalton from the plate itself this amounts to 1.24 Dalton mass shift in the worst case of a heavily ablated HPTLC plate.

Since for example the determination whether a peak is a sphingomyelin or phosphatidylcholine is mainly done with the exact (accuracy of at least ± 0.1 Da necessary) molecular mass, since both have very similar fragmentation schemes but differ always in whether they have an even or uneven mass number, special consideration have to be taken into this effects while measuring HPTLC plates directly. Special caution should therefore also be taken to not shoot too many times on the same space of the plate.

4.2.8 Complete lipid extract without HPTLC

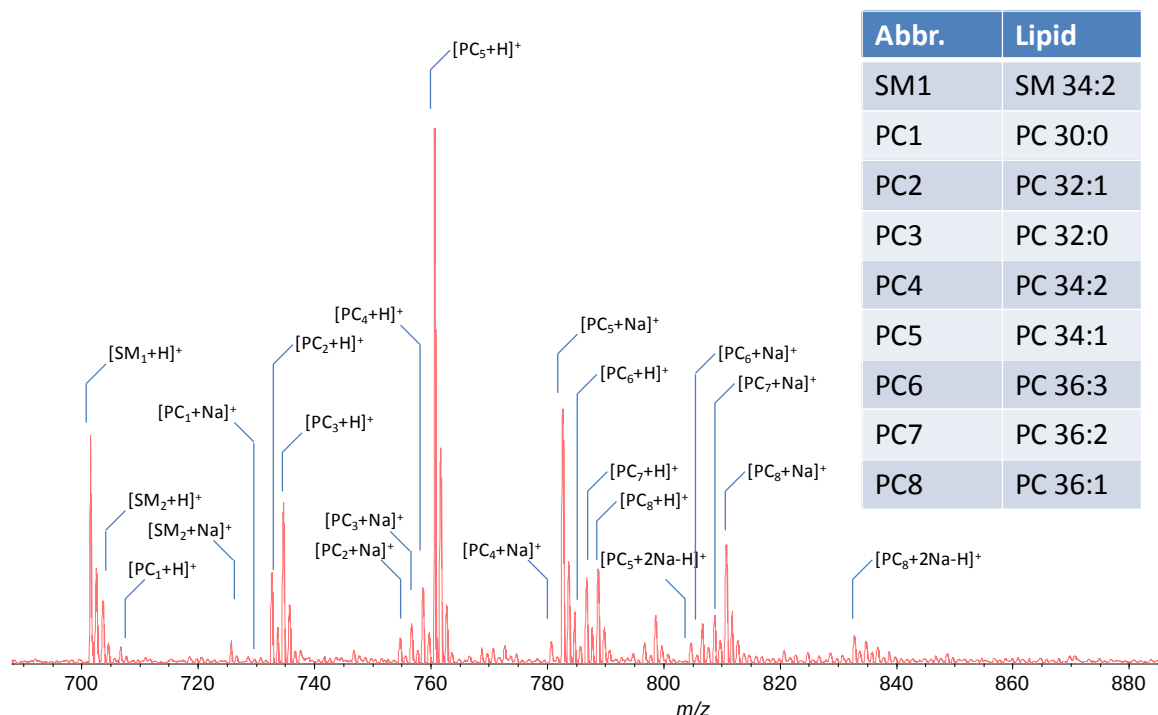


Figure 53 Positive ion MALDI mass spectrum of lipids from a complete scan of the whole TBEV virus.

Using all the information gained during preparation evaluation it is possible to get the following 9 species confirmed by CID in the complete lipid TBEV spectrum. Since the concentration and therefore the signal intensity is very low for all TBEV vaccine extracts, it was only possible to confirm the lipid class not the exact acyl side chains because the parent ion peaks are too weak in intensity for CID experiments. In addition to this PI 38:3 could be found and identified on the 2D HPTLC. Other species, such as PS or PE could not be verified by mass spectrometry but only estimated with HPTLC measurements and comparisons with synovial fluid extracts. Therefore more definite statements on side chain length are not possible so far.

4.2.9 Imaging Mass Spectrometry

Several imaging experiments were done aiming for a proof of principle on the capacities not only to gain MS spectra directly from a HPTLC plate but also to gain a spatial resolution on where exactly which signal was obtained on the plate. Mass spectrometric Imaging (MSI) was developed in order to be able to get the advantages of mass spectrometry during analysis of organic and biological samples with additional information upon spatial distribution of samples. Today, from imaging of whole organs to pharmaceutical experiments a whole world of MSI exists (71, 72). Since separation of lipids on HPTLC was done using 2D techniques it seemed natural to look for methods to scan the plate eliminating the need to stain it.

First experiment was done scanning areas of the 1D HPTLC plate after developing TBEV vaccine samples in one dimension with a PC (32:0) standard as comparison.

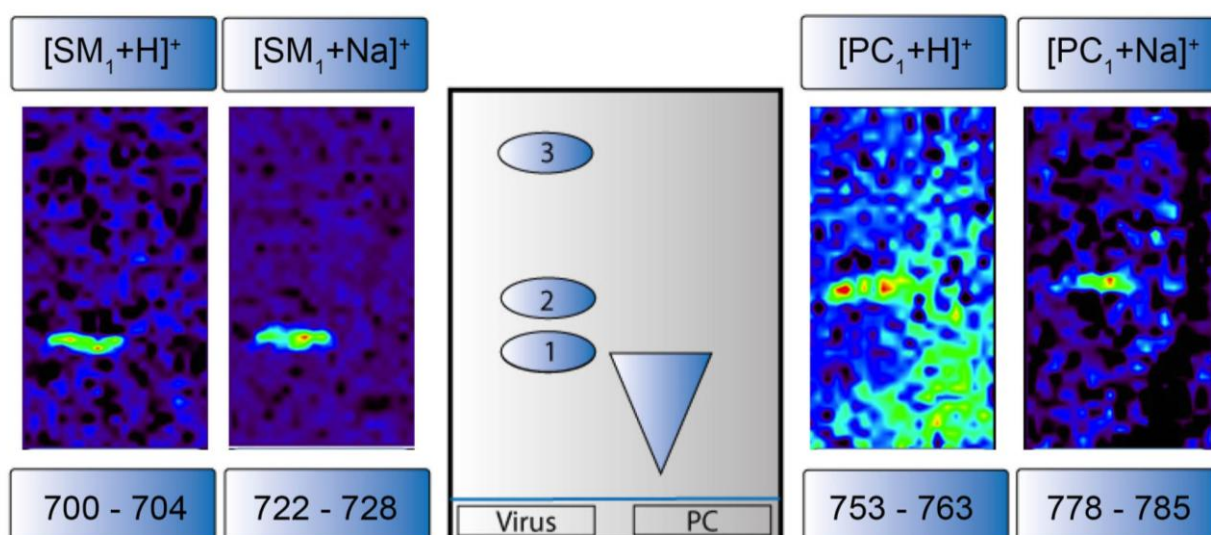


Figure 54 IMS data from the HPTLC plate with TBEV virus. Numbers below the images are m/z values. Colors in blue to red show high signal intensities (red) over medium (green) to low signal intensities (blue to black) of the mass range given below the image. Left side of the schematic are the $[M+H]^+$ and $[M+Na]^+$ peaks for SM_1 and right side are the same for PC_1 .

Figure 54 shows the result of a scanning of a one dimensional HPTLC plate separation of TBEV vaccine extract, where three different spots could be detected in the scanned area. Mapping the found masses resulted in two prominent species found on the plate exactly where the spots on the HPTLC plate had been detected through staining. The masses found corresponded to the sphingomyeline ($SM_{34:4}$) ions and the phosphatidylcholine ($PC_{34:1}$) ions respectively. Molecules were verified by means of HE-CID experiments.

With the possibility to detect the spots on the plate verified, a closer look to the single spots on the two dimensional HPTLC plates was taken. Due to technical limitations of the device a complete scan of the whole HPTLC plate was not possible, since the plate is too big.

Early experiments showed that during rastering the m/z values seemed to shift. This was first attributed to changes in surface, but surface analysis (see chapter 4.2.3) showed that these changes

were too big to come from the height changes in the HPTLC plate. Imaging data obtained from synovial fluid of the spot revealed that there is a clear separation of lipid species within every single spot on the HPTLC plate. Whereas one single spot contains lipids of the same species, that is for example only sphingomyelin, within the spot there are several different types of sphingomyelin, differing in the acyl side chains. These different sphingomyelins had been separated spatially on the spot.

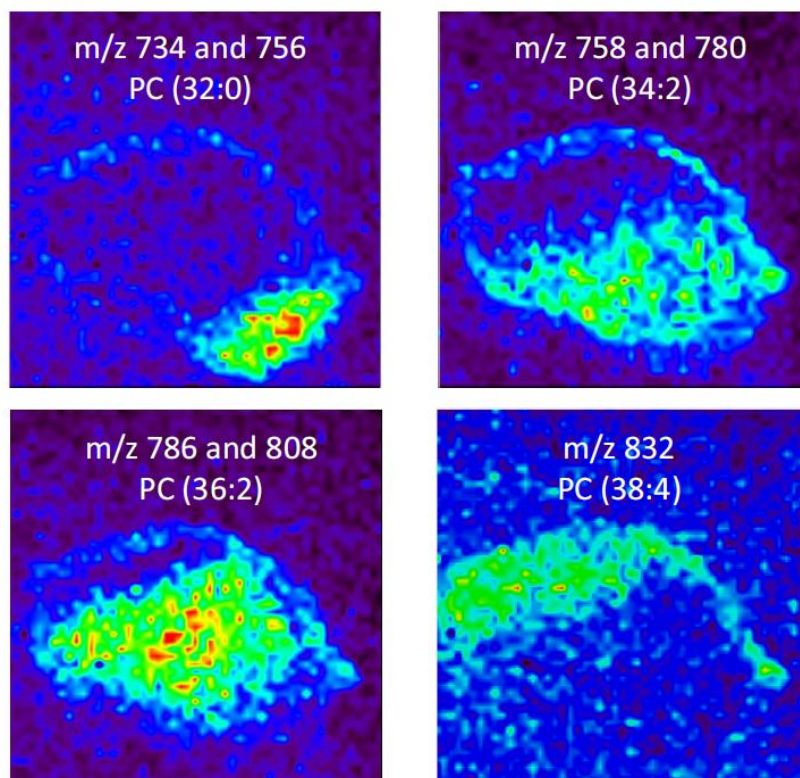


Figure 55 Intensity distribution of masses from a real bovine synovial fluid sample spot imaged directly from 2D HPTLC plate.

Figure 55 shows a especially good example from our experiments with bovine synovial fluid showing the distribution of four different phosphatidylcholine species within one spot on the two dimensional HPTLC plate. The species PC (32:0), PC (34:2), PC (36:2) and PC (38:4) can be clearly detected.

5 Outlook

A general proof of principle of the method coupling has been shown. The main challenges that remain are sensitivity. Most probably this can be tackled by purification of the lipid samples, enrichment and concentrating the samples significantly in front of the HPTLC separation.

Further experimentation will and is done using 1D HPTLC and a multi step development in one direction in order to enhance separation while maintaining the possibility of analyzing several samples on one HPTLC plate. Here the solvent mixture 1 from chapter 3.3 as first development step is successfully used.

Additional consideration should and is given to lateral resolution improvements in the MSI experiments and therefore better identification of species could be achievable.

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7 Abbreviations

Abbreviation	Name
9AA	9-aminoacridine
AFM	atomic force microscopy
ATT	6-aza-2-thiotymine
Cer	Ceramide
Chol	Cholesterol
Da	Dalton
DESI	Desorption electrospray ionization
HPTLC	High performance TLC
LacCer	Lactosylceramide
LysoPC	enzymatically hydrolyzed PC
MALDI	Matrix assisted laser desorption ionization
MS	Mass spectrometry
PC	Phosphatidylcholin
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PNA	para-nitroaniline
PS	Phosphatidylserine
SM	Sphingomyeline
THAP	2,4,6-trihydroxyacetophenone
TLC	Thin layer chromatography
TOF	Time of flight

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7.2 Abstract

Vaccines have been one of the major breakthroughs of medical history. A wide variety of viral vaccines exist today, combating most of viral infections ailing humanity, making some, like the pox become extinct. From antigen synthesis over extraction of natural antigens from different sources to high tech methods like biotechnological production of engineered viral vaccines, type of vaccines and methods of vaccine production are very diverse. This diversity, along with the necessity of comparison and control methods requires a proven and reproducible, easy to use and accurate analytical method to evaluate vaccine properties.

Most prominent biomolecules of our time can be analyzed with mass spectrometry. A lot of research concerning virus action, reproduction and function has been done (1-5). Nevertheless viral membranes and therefore the first part of the virus or viral vaccine that contacts the innate and adaptive immune system, has only sparsely been analyzed. Especially since the lipid membrane is among the most complex system within the world of organisms and bioobjects, this is somewhat disappointing. Much, especially information concerning replication invasion and immune defense is only sparsely understood and the extremely complex lipid composition of the membrane has only recently begun to gain attention and research activity so far focuses mainly on a few viruses such as HIV (2).

The emerging field of lipidomics, the newest addition to the family of “omics” has led to a renewed interest in virus features, this time focusing on the viral envelop, that a large percentage of viruses possess.

Due to the low concentration of lipids in virus extracts (mass-wise), and desorption/ionization suppression through prominent classes of lipids due to the ever present phosphatidylcholines with their quaternary positively charged ammonia group, the need for a pre-separation was clear early on. This pre-separation technique should have qualities such as easy handling, high reproducibility, low cost and MALDI-MS compatibility.

This method of pre-separation is easily found in one of the standard methods of lipid analysis, 1D and 2D high performance thin layer chromatography (HPTLC).

This thesis evaluates the pros and cons, possibilities and strengths of a lipid analysis method based on HPTLC with „matrix assisted laser desorption ionization – time-of-flight mass spectrometry”, in short MALDI-TOF mass spectrometry. Aim of the work was to develop a strategy for analysis of viral vaccines.

In the course of this work optimal extraction conditions for maximum of extracted complete lipidome is found to be the extraction method of Bligh&Dyer. Comparison of widely used staining methods the staining with primuline dye was chosen, as well as separation conditions for 1D and 2D chromatography. Application of 2D HPTLC plates directly on a MS target with conductive tape was successfully tested and imaging of the HPTLC spots revealed that the spots have an additional separation within the spot according to their acyl side chain lengths.

7.2.1 Zusammenfassung

Impfstoffe stellen einen der größten Durchbrüche der medizinischen Geschichte dar. Eine große Vielfalt an Impfstoffen gegen eine ganze Armee an Krankheiten existiert heute, und hat gefährliche Krankheiten wie die Pocken heute nahezu ausgerottet. Von der synthetischen Herstellung von Antigenen über die Extraktion natürlicher Antigene bis hin zur biotechnologischen Produktion designer Antigene gibt es eine ganze Reihe an modernen Impfstoffherstellungsverfahren. Diese Vielfalt, einhergehend mit der Notwendigkeit einer einfachen Vergleichs- und Kontrollmethode unter den Verfahren zur besseren Erforschung und auch Zulassung, erfordert neue, moderne Analysenmethoden.

Die Mehrheit der vorkommenden Biomoleküle kann heute mit Massenspektrometrie untersucht werden. Ein großer Forschungsaufwand wurde in die Untersuchung der Infektionswege und Methoden von Viren getätigt (1-5), ohne jedoch einen für viele Viren wichtigen Bestandteil genauer zu untersuchen; die virale Lipidmembran. Diese erste Anlaufstelle für das Angeborene und das Adaptive Immunsystem bei allen lipidumhüllten Viren schaffte es erst vor relativ kurzer Zeit größere Aufmerksamkeit auf sich zu ziehen, und das obwohl die Viruslipidmembran zu den komplexesten Systemen in der Welt der Organismen und Bioobjekten gehört. Vieles, vor allem hinsichtlich auf die genaue Rolle der Membran und ihrer Lipide auf Replikation, Invasion und Immunabwehr ist kaum bekannt und die meiste Forschung hier fand bisher mehrheitlich hinsichtlich des HIV Virus statt (2).

Das neugeborene Feld der „Lipidomik“, des jüngsten Zuwachses der „Omik“ Forschungsbereiche führte hier zu einem erneuerten Interesse in der Forschung rund um umhüllte Viren.

Aufgrund der recht geringen Probenmengen, des geringen Anteils den das Lipidom (massenmäßig) am gesamten Virus oder Impfstoffpartikel ausmacht und der Ionensuppression durch prominente Bestandteile der Lipidmembran während der massenspektrometrischen Messung, wurde früh die Notwendigkeit einer vorgeschalteten Trennmethode ersichtlich. Diese Trenntechnik sollte Eigenschaften wie leichte Bedienung, hohe Reproduzierbarkeit und geringe Kosten in sich vereinen und MALDI-MS-kompatibel sein.

Diese Methode zur Trennung fand sich schnell in der standardmäßig in der Lipidchemie verwendeten Dünnschichtchromatographie, und dort wiederum in der optimierten Form der „High Performance“ Dünnschichtchromatographie, im englischen abgekürzt als HPTLC.

Diese Arbeit beschäftigt sich mit den Vor- und Nachteilen, den Möglichkeiten und den Stärken einer Lipidanalytikmethode basierend auf HPTLC mit „matrix assisted laser desorption ionization – time-of-flight mass spectrometry“, kurz MALDI-TOF Massenspektrometrie genannt. Ziel war es diese Strategie für die Untersuchung von Virusvakzinen zu entwickeln.

Im Laufe dieser Arbeit wurden optimalen Konditionen gefunden um eine möglichst große Gesamtheit der Lipide einer Präparation mit der Methode von Bligh & Dyer zu extrahieren. Ein Vergleich weitverbreiteter Färbungsmethoden für Lipide wurde gemacht und mit Primulin ein universeller und sensitiver Farbstoff als geeignet für Vakzine und Synovialflüssigkeit gefunden. Trennmethoden basierend auf 1D und 2D HPTLC wurde evaluiert. Eine direkte Kopplung von HPTLC Platte mit MALDI-MS wurde erfolgreich getestet. Im Zuge von Imaging Massenspektrometrie direkt von 1D HPTLC

konnte auch festgestellt werden, dass über die Trennung in Spots nach Lipidklassen hinaus eine zusätzliche Trennung der Lipidspezies innerhalb des jeweiligen Spots stattfindet.

7.2.2 Curriculum Vitae



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Date of Birth/Location 27.11.1982 in Mannheim, Germany

Experience

01.02.12-31.12.12 SCIENTIFIC EMPLOYEE: DEPARTMENT OF PHYSICAL CHEMISTRY, TU WIEN. TOPIC: WHOLESAL ANALYSIS OF VARIOUS SAMPLES IN COOPERATION WITH INDUSTRY PARTNERS FINDING NOVEL METHODS FOR IMPROVEMENT IN MATERIAL SCIENCE.

01.01. 11-31.01. 12 MASTER THESIS, DEPARTMENT OF BIO- AND POLYMER ANALYSIS, TU WIEN. TOPIC: DEVELOPMENT OF A NOVEL MASS SPECTROMETRIC METHOD FOR LIPIDOMIC ANALYSIS OF VIRAL PARTICLES.

01.09.09-30.11.09 BACHELOR THESIS AT DEUTSCHES KREBSFORSCHUNGSZENTRUM, HEIDELBERG
TOPIC: DEVELOPMENT OF PNA-MONOMERS IN ORDER TO ENHANCE THE SPECTRA OF PEPTIDE NUCLEIC ACID FUNCTIONS IN IMAGING

01.10.08-15.03.09 INTERNSHIP AT ROCHE DIAGNOSTICS MANNHEIM,
TOPIC: CHEMICAL MODIFICATION OF BIOSENSORS

18.04.08-30.09.08 INTERNSHIP AT PHYSICIANS WORLD GMBH MANNHEIM

01.12.06-30.08.08 SELF EMPLOYED INSTRUCTOR FOR SCHÜLERHILFE GMBH,
HOCKENHEIM

Summer 05,-07,-10

INTERNSHIP IN QS AND ANALYTICS, JUNGBUNZLAUER AG;
PERNHOFEN

Studies

March 10 - today

UNIVERSITY OF VIENNA, AUSTRIA: MASTER OF SCIENCE IN
BIOLOGICAL CHEMISTRY

March 07- November 09

UNIVERSITY OF APPLIED SCIENCE MANNHEIM, GERMANY:
BACHELOR OF SCIENCE IN BIOLOGICAL CHEMISTRY

October 06 - February 07

UNIVERSITY OF APPLIED SCIENCE DARMSTADT, GERMANY:
BIOTECHNOLOGY (GUEST-SEMESTER)

March 04 - June 06

KARLSRUHE INSTITUTE OF TECHNOLOGY, GERMANY: CHEMISTRY
(FOUNDATION COURSE)

October 03 - February 04

KARLSRUHE INSTITUTE OF TECHNOLOGY, GERMANY: INDUSTRIAL
ENGINEERING SPECIALIZED IN BIOTECHNOLOGY.