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MALDI-TOF MS in lipidomics

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1. ABSTRACT

So far, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) seemed to be nearly a synonym for protein analysis. However, there is growing evidence that this technique is also an useful tool in lipid analysis and lipidomics because of its fast, simple and convenient performance allowing to record mass spectra of cells, crude tissue or body fluid extracts or even intact tissue slices in a few minutes. On the negative side, however, the reproducibility of MALDI-TOF mass spectra depends significantly on the homogeneity of the co-crystals between matrix and analyte and different lipid classes are detected with different sensitivities. This is especially important because lipids with quaternary ammonia groups (e.g., GPCho) may prevent the detection of other lipid classes (e.g., GPEtn). This review starts with a short overview on traditional methods of lipid analysis with the focus on mass spectrometric methods and compares MALDI-TOF MS with other important ionization techniques. Afterwards, some landmarks in the development of MALDI-TOF MS will be introduced and some important examples in the field of tissue and body fluid lipid analysis will be discussed. This review ends with a short outlook and summary focusing on the advantages and drawbacks of MALDI-TOF MS in lipidomics.

2. INTRODUCTION

Lipids are important cellular components. Without cells there is no life, and, consequently, lipids are essential constituents of all living organisms (1). The typical bilayer structure of the phospholipids (PL) in the membrane and the interaction with membrane proteins have been a broad focus of biophysicists for many years (2). Additionally, the interests in lipids to biochemists and life scientists are continuously increasing as lipids and especially PL have been recognized as important second messenger molecules (3). Additionally, it is nowadays also accepted that many diseases (e.g. arteriosclerosis) are accompanied by changes of the lipid composition of cells and tissues (4).

The cellular membrane structure is maintained by different lipids (Figure 1), particularly glycerophosphocholine (GPCho), glycerophosphoserine (GPSer), glycerophosphoethanolamine (GPEtn), sphingomyelin (SM), and cholesterol. Glycerophosphate (GPA), glycerophosphoglycerol (GPGro), diacylglycerol (DG), glycerophosphoinositol (GPIns) and its phosphorylated derivatives are also important, but less abundant constituents (1). In contrast, apolar lipids like cholesteryl esters and triacylglycerols are only minor

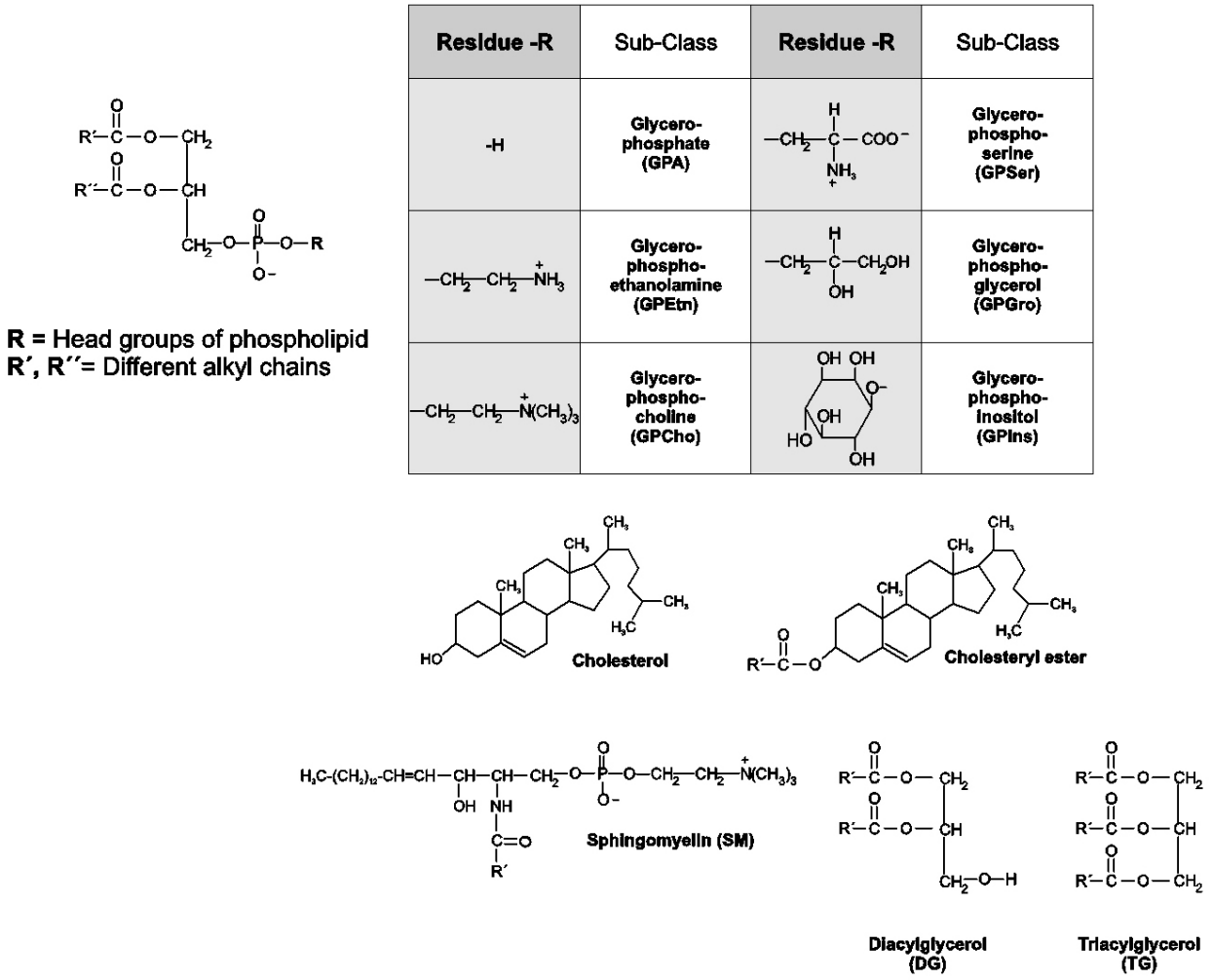

Figure 1. Chemical structures of selected lipids and phospholipids. The characteristic headgroups of the most relevant glycerophospholipids are shown in the table. Lipids are sorted into glycerophospholipids, cholesterol derivatives and other lipids. "R" and "R´" denote different alkyl chains. Please note that the majority of physiologically relevant lipids possess a saturated moiety at the *sn*-1 position and an unsaturated fatty acid residue at the *sn*-2 position. Reprinted with modification and permission from Elsevier (16).

constituents of the cell membrane, but occur in vast amounts in the lipoproteins of blood (5). In addition to the protein profile of body fluids and tissues from healthy donors and diseased persons, the corresponding lipid profile attracts increasing interest (6).

Nowadays, the term "lipidomics" (indicating the simultaneous determination of all lipid species in a given sample) is increasingly used and this emerging field has been recently reviewed (7). Of course, "lipidomics" requires sensitive and reliable analytical methods: The structural inhomogeneity of lipids stemming from differences in the fatty acid composition, the different linkage types between the fatty acid residues and the glycerol backbone (diacyl-, alkyl-acyl- and alkenyl-acyl-) and differences in the structure of the headgroup have hampered detailed lipid analysis over a long time. These are most probably still the reasons why such generally accepted protocols as in protein analysis do not exist for lipids. It is one major aim of this review to show that mass spectrometry (MS) and especially the invention of the soft-ionization methods have revolutionized lipid analysis (7).

3. TRADITIONAL METHODS OF LIPID ANALYSIS

Traditionally, lipid analysis is based on chromatographic methods, particularly liquid chromatography (LC) and thin-layer chromatography (TLC) (8). Both techniques are widely used but require long-standing experience (LC) or are limited by low resolution (TLC). A comprehensive discussion of chromatographic techniques in lipid research is out of the scope of this paper, but a timely review of these methods is available (9). Another modern technique of phospholipid analysis that is increasingly used is high resolution ^{31}P NMR spectroscopy (10). However, exclusively PL, but not lipids in general may be detected by ^{31}P NMR. Additionally, this technique has the disadvantage of being

less sensitive than established methods and requires expensive equipment (11).

4. APPLICATION OF MASS SPECTROMETRY TO LIPID ANALYSIS

Although MS for lipid analysis has been established for a long time, recording mass spectra of intact lipids was impossible for decades. This drawback was coming from limitations of the electron impact (EI) ionization process that was formerly exclusively available. The only available approach for "lipid analysis" using EI was to hydrolyze the lipids of interest and to analyze the released free fatty acids after derivatization (transformation into methyl or silyl esters in order to enhance their volatility). Thereby, all information on fatty acid composition of a given lipid is completely lost. However, this technique (i.e., GC-MS) is still widely used nowadays (7).

Applications of individual MS methods in lipid analysis have particularly been increasing in the last 15 years after the invention of the so-called "soft-ionization" techniques, allowing the analysis of intact lipid molecules with only a small extent of fragmentation. A short survey of these techniques is provided in Table 1. In contrast to electrospray ionization (ESI), atmospheric-pressure chemical-ionization (APCI) and matrix-assisted laser desorption and ionization (MALDI), fast-atom bombardment (FAB) ionization can hardly be termed as "soft-ionization" technique. However, historically and in comparison to EI, FAB must be actually considered as a relatively "soft" technique. One should note that even if the detection of the intact lipid molecules is possible by the soft-ionization methods, MS/MS techniques are employed in many studies (19). For example, a precursor-ion scan is used to selectively analyze a certain class or group of lipids.

4.1. Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry

Although ESI MS is currently the most frequently used technique in lipid research, this review is dedicated exclusively to MALDI-TOF MS. MALDI-TOF MS has the considerable advantage that this method can be performed very rapidly (less than one minute per sample) and in a very convenient, user-friendly way. These advantages are the prime reasons why MALDI-TOF MS became also popular for lipid analysis in the last decade (Figure 2), although the prime application is still the analysis of high molecular weight biopolymers, especially proteins. The focus on proteins is one reason why the majority of commercially available MALDI devices are equipped with a time-of-flight (TOF) analyzer. Large biopolymers can be only analyzed by a TOF instrument because the mass range of these detectors is nearly unlimited (20). An additional reason why MALDI is very often combined with TOF analyzers is the pulsed (but not continuous) ion generation.

A simplified scheme of the MALDI ion generation and time-of-flight detection is shown in Figure 3. MALDI-TOF MS is based on the utilization of an ultraviolet-absorbing matrix, whereby DHB (2,5-dihydroxybenzoic acid) was so far most frequently used for lipids (16, 21). UV lasers (often emitting at 337 nm) and, accordingly, matrices absorbing in the UV spectral range will be exclusively discussed here because IR lasers were so far primarily used for the analysis of glycolipids (22). The matrix (in a 100-1000 fold excess over the lipid) has two important tasks: (a) to absorb the laser energy (i.e., to avoid direct "hits" on the analyte) and (b) to prevent aggregation of the lipid molecules. Very homogeneous matrix/analyte co-crystals can be obtained when the sample preparation is carefully performed. In order to indicate this fact, the term "solid solution" is often used (23). Homogenous co-crystals are essential for obtaining reproducible MALDI mass spectra.

Unfortunately, despite of its profound importance, the process of ion generation in MALDI-TOF MS is so far only poorly understood (24). In contrast to ESI, singly-charged, but not multiply-charged ions are primarily generated under MALDI conditions (16). When the pulsed laser beam hits the sample, the matrix is vaporized and carries intact lipid molecules into the vapor phase. During the expanding process of this gas cloud, H^+ and other ions present in the system (e.g., Na^+) are exchanged between the lipid and the matrix molecules, leading to the formation of quasi-molecular ions (e.g. $[M+H]^+$ or $[M+Na]^+$). Besides cation generation, anions can also be generated by abstracting H^+ or Na^+ from the analyte. The ratio between cations and anions is determined by the (gas phase) acidities of the lipid and the matrix (16).

After being formed, ions are accelerated in a strong electric field with typical accelerating voltages of the order of 20 kV. After passing a charged grid, the molecules are drifting freely over a field-free space where mass separation occurs: Low mass ions arrive at the so-called "linear" detector in a shorter time than high mass ions (25). Positive and negative ions can be easily differentiated by inverting the direction of the applied electric field. Nevertheless, the detection of positive ions is much more popular in MALDI-TOF MS than the detection of negative ions. As indicated in Table 1, the achievable resolution on TOF mass spectrometers is relatively poor in the linear mode. However, it is possible to improve the resolution through increase in the length of the TOF path. Therefore, resolution can be enhanced by using either longer flight tubes or a so-called "electrostatic mirror", where ions are reflected at the end of the flight tube and reach the "reflector-detector" at the opposite direction in addition to being focused (25). This is schematically shown in Figure 3.

The comparably poor mass resolution obtained with common TOF devices is also caused by the fact that ions exhibit a broad kinetic energy distribution as a result of the initial velocity imparted to the ions during the desorption/ionization process. In order to minimize the influence of the initial velocity of the ions, modern MALDI-TOF spectrometers can be operated (in addition to the "continuous" extraction mode that is particularly used

Table 1. Overview of important MS ionization methods allowing the analysis of intact lipids

Method	Description	Application	Reference
ESI, Electrospray ionization	An ESI ion source consists of a fine capillary ("needle") at a high electric voltage. The sample solution is sprayed into the ion source chamber to form droplets. The droplets carry charge when they exit the capillary and as the solvent vaporizes the droplets disappear leaving highly charged analyte molecules. ESI mass spectra are characterized by the generation of multiply charged ions.	Today, ESI is most frequently used for lipid analysis but has the disadvantage that it is rather sensitive to impurities (e.g. salts and detergents). ESI is particularly suitable for analyzing highly polar and even ionic lipids. LC/MS coupling is possible.	12, 13
APCI, Atmospheric-pressure chemical-ionization	APCI is a relative of ESI and its ion source resembles the ESI one. In addition to the electrohydrodynamic spraying process, a plasma is created by a corona-discharge needle. In this plasma H^+ transfer reactions and (to a moderate small extent) fragmentations occur. Only quasi-molecular ions like $[M+H]^+$or $[M+Na]^+$ (depending on the solvent) and/or fragments are produced. Multiply charged molecules $[M+nH]^{n+}$, as in ESI, are not observed.	APCI is particularly used for analyzing less polar (smaller) lipids compared to ESI. However, increased analyte fragmentation occurs. APCI can be easily coupled to LC.	14, 15
MALDI, Matrix-assisted laser desorption ionization	MALDI uses a pulsed (in the majority of cases) UV laser (often N_2) for vaporizing and ionizing the sample. The analyte is dispersed in a solid matrix such as 2,5-dihydroxybenzoic acid (DHB). The laser pulse ablates the matrix which carries some of the large molecules into the gas phase in an ionized form so they can be extracted into the mass spectrometer. The MALDI technique can be coupled with a time-of-flight analyzer (resolution and accuracy of the spectra are low but easy to handle and hence, most commonly used) or a Fourier-transform mass spectrometer (expensive, difficult to handle, low dynamical range, but very accurate).	In contrast to the other methods, MALDI gives a pulsed ion generation. Suitable for analyzing polar and even ionic compounds, but also apolar lipids. Quantitative data interpretation is limited and depends particularly on the even co-crystallization between matrix and analyte. Particularly used in "proteomics".	16, 17
FAB, Fast-atom bombardment ionization	In FAB MS fast Xe (or Ar) atoms bombard the sample mixed with a matrix compound such as glycerol. The FAB mass spectrum contains often an intense signal for the pseudo molecular ions. However, the lower *m/z* region is crowded with signals resulting from the matrix (often glycerol) and fragments.	FAB was nearly exclusively used for the analysis of polar lipids but its importance is decreasing. In comparison to other "soft" methods, the extent of fragmentation is most pronounced in FAB spectra.	18

for the analysis of very large molecules) in a special "delayed extraction" (DE) mode, where the velocity distribution of the generated ions is minimized and, therefore, the mass resolution is enhanced. This technique is also termed as "time-lag energy focusing" (26) and applies a smaller accelerating voltage to faster ions. All spectra in this paper were recorded under DE conditions using a reflectron. The reflectron is also an indispensable requirement for recording post-source decay (PSD) spectra (27) that will be discussed below in more detail.

4.2. Characteristics of maldi-tof mass spectra of lipids

Some important milestones of the development of MALDI-TOF MS of lipids are listed in Table 2. These data are arbitrarily collected, but give a characteristic overview and acknowledge of the most important lipid classes (in these authors opinion). More complex structures as glycolipids will not be discussed in this paper. Because of the limited space of this review, further details cannot be given. More detailed survey is available in a recent review article (16).

In Figure 4 the MALDI-TOF mass spectra of some typical lipids are shown. Spectra on the left hand were recorded in the positive ion mode, whereas on the right hand the corresponding negative ion spectra are shown. All lipids possess exactly the same fatty acid composition, namely a palmitic acid residue (16:0) at the *sn*-1 position and an oleic acid residue (18:1) at the *sn*-2 position. TG (3×18:1 (9Z), triolein) was selected as a typical triacylglycerol (4d and 4h). The lipid class that has so far been most frequently studied by MALDI-TOF MS is GPCho. The interest in this lipid is not exclusively coming from its significant abundance in biological tissues and body fluids, but also from the fact that GPCho is commercially available for a variety of fatty acid compositions. GPCho (16:0/18:1 (9Z)) gives two peaks in the positive ion mode (4a) at *m/z* = 760.6 and 782.6 corresponding to the H^+ and the Na^+ adduct, respectively. The intensity ratio between these peaks depends on the buffer composition and can be altered by the salt content (21).

Although the adduct patterns seem less important, it is of interest to know that the yield of the fragment ions depends on the adduct from which they are derived, although details of this process are not yet understood: The H^+ adduct leads nearly exclusively to a cleavage of the choline headgroup (*m/z* = 184), while the Na^+ adduct gives a larger variety of fragmentation products (27). Accordingly, one might argue that the small fragment ion at *m/z* = 735 (loss of the quaternary ammonia group (21)) is stemming from the Na^+ adduct. The yield of fragment ions is also determined by the applied laser energy that has a considerable impact. The applied laser strength should be as low as possible but as high as necessary (25). Although GPCho gives an intense positive ion signal (4a), no negative ion spectrum can be obtained. The only signals detectable in the negative ion spectrum (4e) contribute to the DHB matrix (32) that undergoes oligomerization under conditions of laser irradiation.

The positive ion mass spectrum of GPEtn (16:0/18:1 (9Z)) (4b) exhibits a peak pattern similar to the GPCho, but is further characterized by a clear fragment ion signal at *m/z* = 577.5 corresponding to the loss of the phosphorylethanolamine headgroup (27). GPEtn (16:0/18:1 (9Z)) is also detectable in the negative ion mode (4f, *m/z* = 716.5), but with lower sensitivity in comparison to that in the positiveion mode. The negative ion mass spectrum is usually dominated by the DHB adduct of GPEtn (16:0/18:1 (9Z)) (*m/z* = 892.5).

Table 2. Applications of MALDI-TOF MS to isolated lipids and phospholipids as well as reaction products derived thereof

Lipid classes investigated	Comments	References
GPCho, LGPCho, GPEtn, GPSer, GPGro, GPIns, GPA, CL	First comparative study of different PL classes by MALDI-TOF MS. Special focus is on the suitability of different matrix compounds (e.g. sinapinic acid) and with what sensitivity the individual lipid classes can be detected.	28
GPCho, GPEtn, GPSer, GPIns	First study of PL by the MALDI- FT-ICR technique. Comparison of positive and negative ion spectra. Evaluation of characteristic fragmentation patterns.	29
Free fatty acids (particularly saturated)	Mixtures of fatty acids were obtained by alkaline saponification of triacylglycerols from different sources. By using meso-tetrakis-(pentafluorophenyl)-porphyrin as matrix the determination of saturated fatty acids (FA) was possible, whereas unsaturated FA were much more difficult to analyze. The applied matrix has the advantage that it does not give any signals at $m/z < 900$.	30
1-alkyl/alkenyl-2 -acyl-glycero-phosphocholine	First structural analysis of a plasmalogen (linkage of one fatty acid residue by an alkenyl ether) by MALDI-TOF MS and further methods. Plasmalogens can be detected in the same way as diacyl compounds.	31
GPCho, GPEtn, GPSer, GPA, GPIns and derived lysolipids	Determination of the relative detectabilities of different PL classes. It is shown that PL with quaternary ammonia groups (GPCho, LGPCho) are most sensitively detectable and may suppress the signals of less readily detectable lipids. Potential way outs are discussed.	32
$GPInsP_x$ and GPCho	The detection limits of differently charged phosphoinositides were investigated by using the signal-to-noise (S/N) ratio of the negative ion MALDI-TOF mass spectra. It is shown that $GPInsP_2$ is about two orders of magnitude less sensitively detected than GPIns. These differences are correlated with the charge of the corresponding lipid.	33
GPCho and diacylglycerols	The problem of the overlap between the individual adducts (H^+, Na^+ and K^+) and differences in the fatty acid composition of GPCho mixtures is discussed. It is suggested to convert the GPCho by PLC digestion into the corresponding DGs that give exclusively Na^+ adducts and can be, thus, quantitatively analyzed.	34
GPCho and LGPCho	The signal-to-noise ratio is introduced as a convenient and reliable measure of GPCho and LGPCho concentrations in mixtures. Some time later this technique was also used to determine phospholipase activities (36).	32,35
Chlorohydrins and hydroperoxides of GPCho and SM	Chlorohydrins and hydroperoxides can be chemically generated from unsaturated PL. It is shown that both species are sufficiently stable to become detectable by MALDI-TOF MS.	37, 38
Cluster ions between GPCho and the DHB matrix	It is reported that negatively-charged cluster ions between GPCho and the DHB matrix can be generated. Since these signals are shifted for 153 Da in comparison to the neutral GPCho, caution is needed to avoid misinterpretation of these signals. Such matrix clusters are also detectable in the positive ion mode (mass difference of 177 or 199 Da in comparison to the neutral analyte)	39
GPCho (PSD fragment ion spectra)	The "post source decay" spectra of GPCho species with varying fatty acid composition are compared. It is concluded that the majority of fragment ions is stemming from the Na^+ adducts, whereas the corresponding H^+ adducts provide only one fragment ($m/z = 184$) according to the cleavage of the choline headgroup.	27, 40
PSD fragment ion spectra of GPEtn, GPSer, GPIns, TG, CL, GPA, MGDG, DGDG	Comprehensive study of the fragmentation behavior (PSD spectra) of a variety of different lipid classes. The majority of lipids is characterized by the cleavage of the polar headgroup. The fragmentation behavior of GPCho differs significantly from all other PL.	40
GPCho and triacylglycerols	First study where the lipid composition of a complete animal (zooplankter) was investigated. Even without previous lipid extraction, GPCho and TG species could be easily monitored. This study emphasizes the considerable robustness of MALDI against sample impurities.	41
GPCho, SM and GPEtn	Introduction of *para*-nitro-aniline (PNA) as useful matrix for lipid analysis. This matrix possesses a higher basicity than DHB because of its amino group and allows, therefore, the detection of GPEtn as negative ion. Since GPCho is not detectable as negative ion, suppression effects of GPEtn by GPCho (32) do not play a role.	17
Gangliosides from brain	MALDI MS cannot be directly coupled to LC but very easily with TLC because a solid sample is used. When the TLC plate is directly used as the target, however, resolution and mass accuracy is reduced. This paper describes a method (vibrational cooling) how this loss of resolution can be avoided.	42
Phospholipid mixtures	The reproducibility of MALDI mass spectra is determined by the homogeneity of the matrix/lipid co-crystals and depends on the deposition onto the MALDI target. In this paper, the application of "ionic liquid matrices" is described for the first time for lipid analysis. Such matrices provide a higher reproducibility than standard lipid matrices as DHB.	43

The positive ion mass spectrum of GPSer (16:0/18:1 (9Z)) is shown in Figure (4c). This spectrum exhibits one additional peak because the GPSer is a negatively charged PL and undergoes H^+/Na^+ exchange. Accordingly, the peak at m/z = 828.5 corresponds to M-H^++2Na^+. One should also note that the same fragment ion (m/z = 577.5) as in the case of the GPEtn is detectable. This indicates that the cleavage of the polar headgroup is the most pronounced fragmentation pattern of PL (27). The negative ion mass spectrum of GPSer (16:0/18:1 (9Z)) (4g) is in agreement with the positive ion mass spectrum, but shows intense adduct ion peaks with the DHB matrix (m/z = 958.5 and 1134.5).

Finally, the mass spectra of triolein are shown in traces (4d) and (4h). This compound differs from PL because triolein does not possess a polar headgroup. This is the reason, why triolein is exclusively detectable as the corresponding Na^+ adduct (m/z = 907.8). In contrast to PL, the spectra of triacylglycerols do not show any H^+ adducts. Therefore, the tendencies of H^+ and Na^+ ions to bind to the PL headgroup or to the glycerol backbone of TG differ significantly (16,27). The peak at m/z = 603.5 corresponds to the loss of sodium oleate (27).

In addition to the m/z value of the analyte of interest, further information on the fragmentation patterns of the analyte is also available. This is achieved by selecting a precursor ion and detecting the corresponding fragment ions derived thereof. This method is known as "PSD" but is sometimes also called "FAST" (fragmentation analysis by structural TOF). In Figure 5 the negative (a) and positive ion (b) PSD spectra of GPEtn (16:0/18:1 (9Z)) are shown as representative examples. The precursor ions were m/z = 716 (5a) and 740 (5b). Since the mass resolution decreases under PSD conditions, no decimals are provided in these spectra. The assignment of the individual peaks is given in the corresponding spectra (27). It is

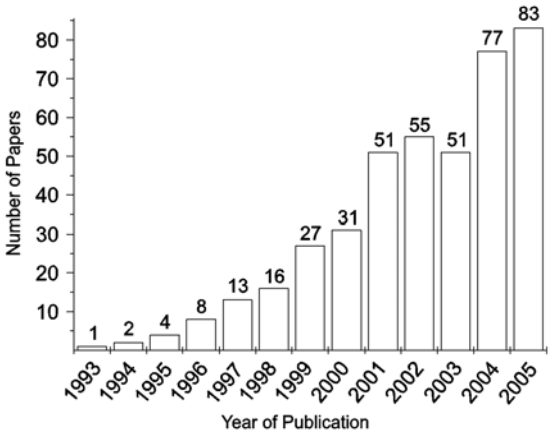

Figure 2. Development of the scientific interest in lipid analysis by MALDI-TOF MS. The number of available papers containing the term ("MALDI" or "matrix-assisted") AND ("lipid" or "phospholipid") in their abstracts is plotted against the year of publication. Data were retrieved from the "Web of Science"® database (http://isiknowledge.com).

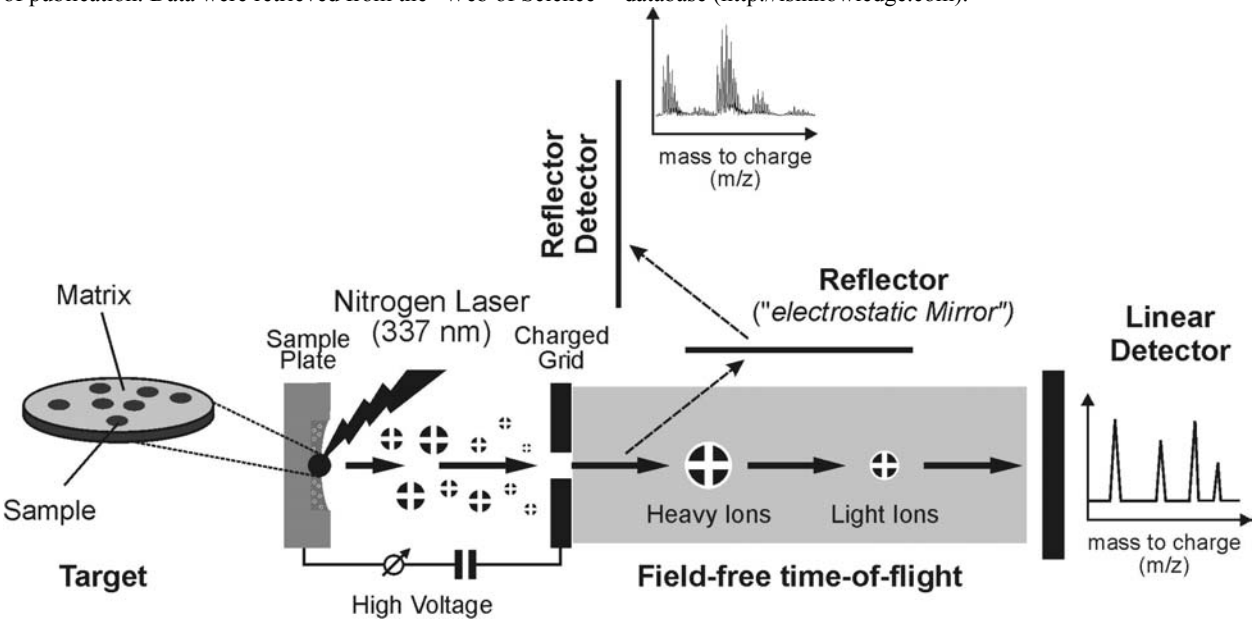

Figure 3. Scheme of the processes occurring during the MALDI-TOF ionization process in the mass spectrometer (for details see text). The influence of the detection using the "linear" and "reflector" mode are emphasized in the figure. Reprinted with modification and permission from Elsevier (16).

evident that the negative ion mass spectrum (5a) exhibits much less informative fragments which correspond to the deprotonated palmitic (*m/z* = 255) and oleic acid (*m/z* = 281). It is evident from the peak intensities that the tendency of cleaving the fatty acid residue at the *sn*-1 position is lower than that at the *sn*-2 position. In contrast, the positive ion PSD mass spectrum (5b) exhibits an increased number of fragments that are attributable to the loss of the phosphorylethanolamine headgroup. A more comprehensive discussion of fragment ion spectra under MALDI-TOF MS conditions has been reported (27).

5. SELECTED EXAMPLES OF COMPLEX MIXTURES

A considerable number of MALDI-TOF MS studies on physiologically relevant lipid mixtures has so far been performed that cannot be discussed in detail due to the limited space of this review. In order to give a representative overview, a selection of some papers is presented in Table 3. Although the survey given in Table 3 represents only a small selection, these data clearly indicate that MALDI-TOF MS is very useful in lipid research. However, one might wonder why so much work is focused on the analysis of GPCho. This is stemming from an important drawback of MALDI (i.e., the detectability of individual PL classes differs in dependence on the headgroup structure and PL containing quaternary ammonia groups are most sensitively detectable) (32). This confers problems especially in the context of GPEtn. When DHB is used as matrix, GPEtn is detected only with low sensitivity in the negative ion mode (54). In the positive ion detection mode, however, the signals of GPEtn are completely suppressed by GPCho even when both PL are

Table 3. Selected applications of MALDI-TOF MS to lipid mixtures obtained by organic extraction from tissues, cell cultures or body fluids. This list is not exhaustive but provides just an overview of some recent reference data

Tissue or body fluid	Comments	References
Liver, spleen, cerebrum and cerebellum	First application of "delayed extraction" MALDI-TOF MS to the analysis of complex tissue extracts. Although the focus was on sphingolipidosis and sphingolipids, some further lipid classes are also discussed.	45
Human neutrophilic granulocytes	The effect of stimulatory drugs on the PL composition of neutrophils could be monitored by MALDI-TOF MS.	21
Human spermatozoa	The effect of cryo-storage on the composition of human spermatozoa was assessed by measuring the GPCho/LGPCho content. Evidence is provided that the GPCho and ceramide content are reliable indicators of cryo damage. Some time later this method (47) was also used to assess the content of apoptotic spermatozoa in mixtures.	46
Human lipoproteins	Differences in the lipid as well as the fatty acid composition of different lipoprotein fractions could be monitored. Additionally, the effects of HOCl-treatment and PLA_2 digestion were investigated.	48
Bull spermatozoa	The high contribution of alkyl-acyl and particularly alkenyl-acyl PL in animal spermatozoa was verified by MALDI-TOF MS. The use of trifluoroacetic acid (TFA) should be avoided because of the sensitivity of plasmalogens to traces of acids. Some degradation is already observed subsequent to TLC separation due to the acidic groups on the TLC plate (50). Similar results were also obtained with boar spermatozoa (51).	49
Human and animal surfactant	The fatty acid composition of GPGro and GPCho from the bronchoalveolar lavage from different species (man, rat, etc.) were determined. The relative moieties of GPCho and GPGro were additionally determined by ^{31}P NMR. Later, even minor PL were assayed in the surfactant from minipig (53)	52
Porcine Lenses	A quantitative analysis of the major PL classes in the eye lenses was performed. GPGro, GPEtn and GPSer could be quantitatively determined from the negative ion spectra by using PNA as matrix.	54
Blood plasma and joint (synovial) fluid	The GPCho/LGPCho ratio was monitored in the plasma and the joint (synovial fluid) from patients suffering from rheumatoid arthritis. The higher the severity of the disease, the lower the GPCho/LGPCho ratio. Using this approach, the success of medical treatments could also be monitored.	55
PC species from brain	A comprehensive investigation of the GPCho composition of brain was performed. The authors noted that in the presence of Li^+ ions, the tendency of GPCho to fragmentations is elevated.	56

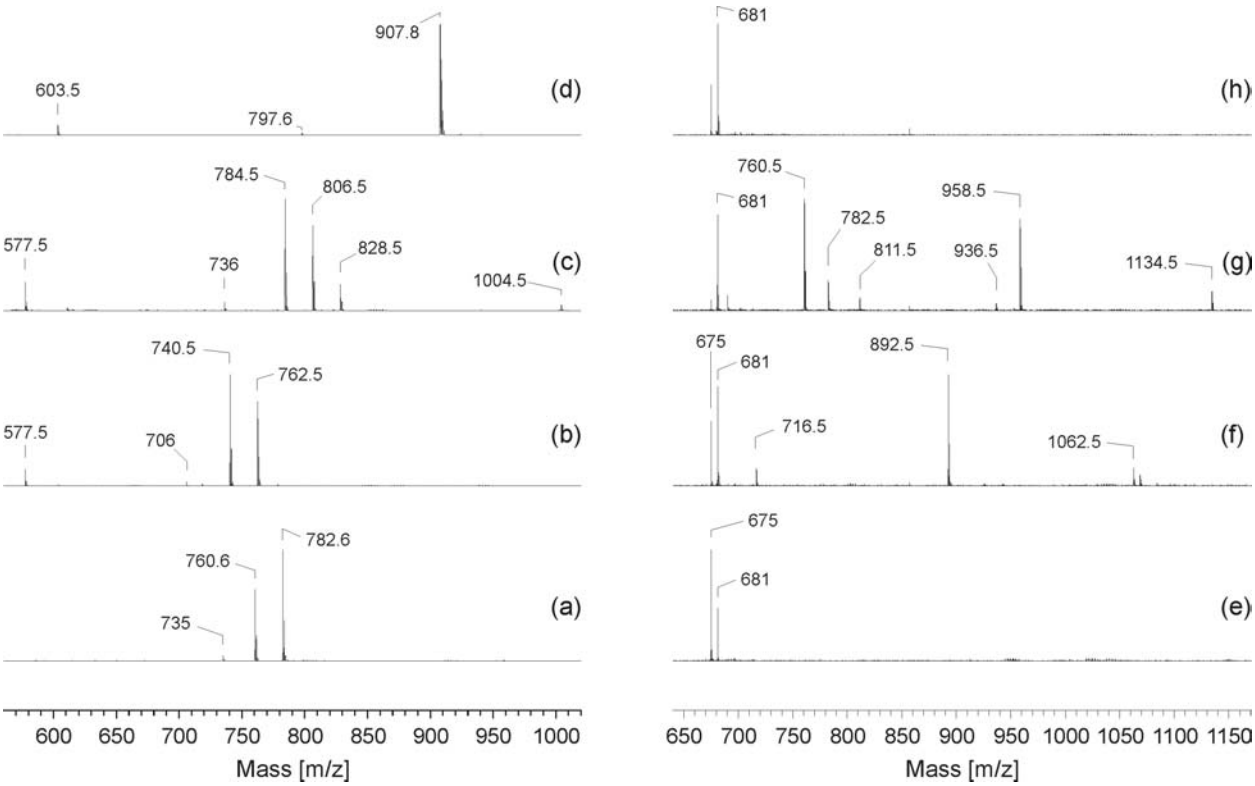

Figure 4. Positive (left) and negative (right) ion MALDI-TOF mass spectra of some selected lipids: 1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-3-phosphocholine (a,e), -phosphoethanolamine (b,f) and -serine (c,g). In (d) and (h) the spectra of triolein are shown. Spectra are labeled according to their *m/z* ratio. All spectra were recorded with DHB as matrix. The amount of lipid on the MALDI target was about 0.5 µg in all cases.

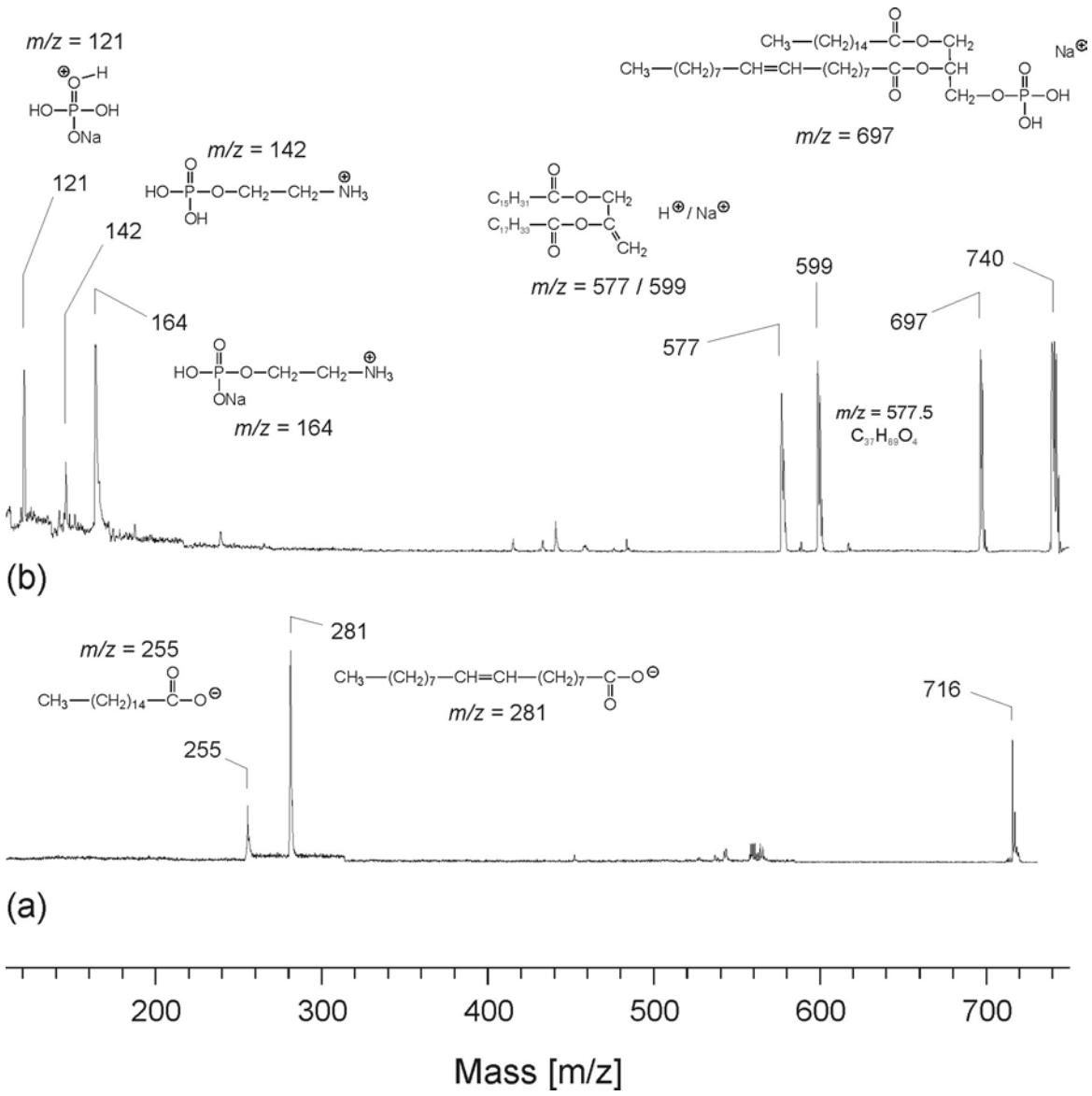

Figure 5. Positive (top, b) and negative (bottom, **a**) ion MALDI-TOF post-source-decay (PSD) mass spectra of 1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine. Because of the increase of the peak-widths under PSD conditions, no decimals are given. Besides the observed *m/z* values, the chemical structures of some selected fragment ions are also shown. Only the most intense signals are considered. Detailed peak assignments can be found in ref. (27).

present in comparable amounts. This problem shall be illustrated by using an organic extract from hen egg yolk as a typical lipid mixture. Four typical mass spectra of an organic extract of hen egg yolk are shown in Figure 6. Traces (6a-6c) represent positive ion spectra and were recorded with DHB as matrix, whereas trace (6d) is a negative ion mass spectrum and was recorded using PNA that possesses a lower acidity and is, therefore, more suitable for the detection of negative ions (54). Trace (6a) corresponds to the total extract whereas trace (6b) represents the GPCho ion peaks and trace (6c) shows the GPEtn species when both fractions were obtained by TLC separation from the total extract. A detailed assignment of all peaks can be found in ref. (32).

Although (6a) should represent the sum of spectra (6b) and (6c), it is obvious that the total extract exhibits nearly exclusively GPCho peaks, i.e. GPEtn peaks (GPEtn (18:0/20:4 (5Z, 8Z, 11Z, 14Z)) at *m/z* = 768.5 and 790.5 according to the H^+ and Na^+ adduct, respectively, is an particularly suitable marker) are suppressed in this mixture. In contrast, in the negative ion mass spectrum recorded in the presence of *para*-nitro-aniline (PNA) (6d) GPEtn is detected very easily because there is no interfering contribution of GPCho at all. GPCho is exclusively detectable in the positive ion mode, but not in the negative ion mode independent of what matrix is used.

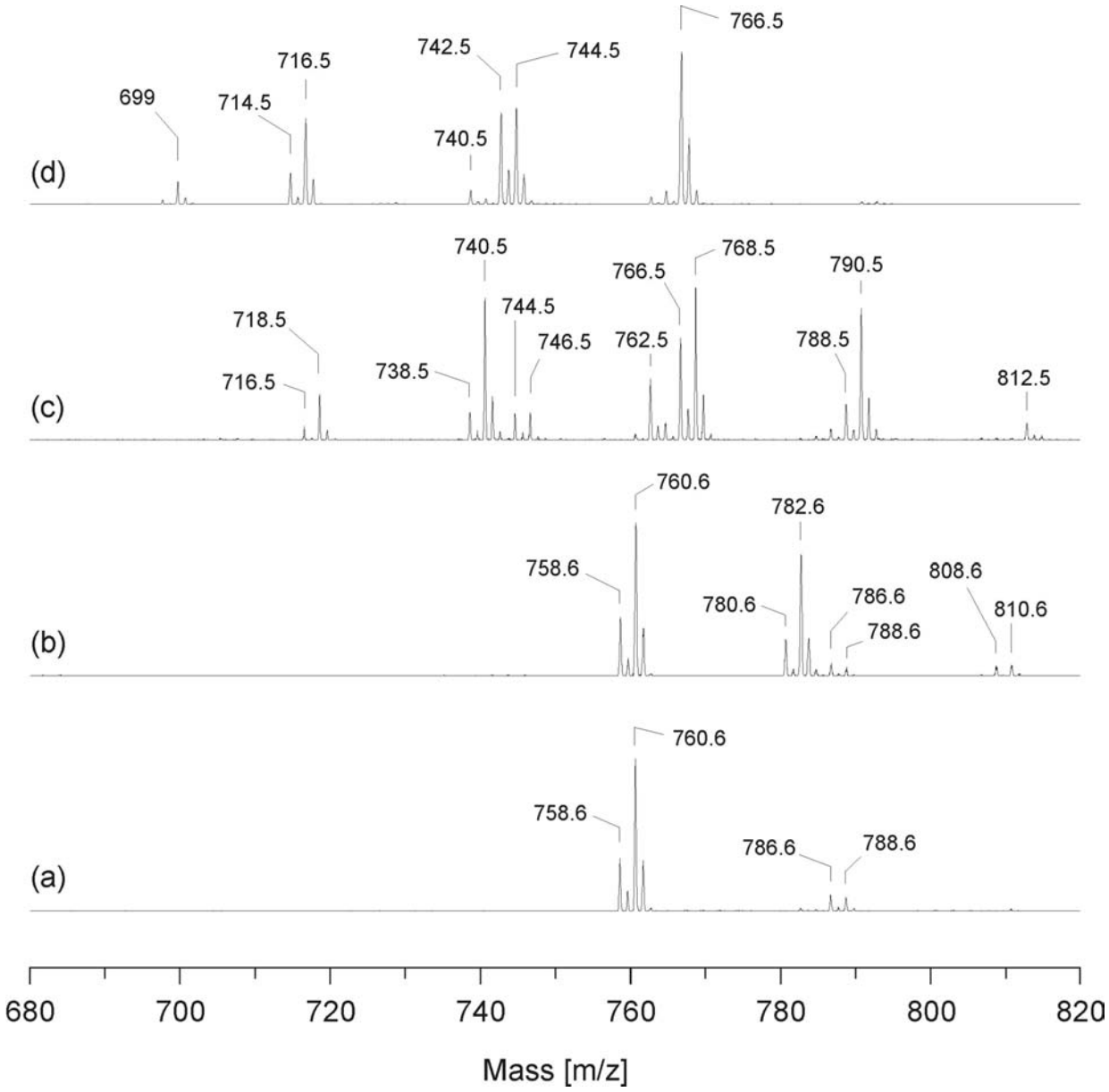

Figure 6. MALDI-TOF mass spectra of an organic extract (obtained according to Bligh & Dyer (62)) of hen egg yolk. Trace (a) represents the positive ion mass spectrum of the total extract, whereas trace (b) corresponds to the GPCho fraction and trace (c) corresponds to the GPEtn fraction, respectively. Separation of lipids was performed by TLC prior to MS analysis. Although some further TLC fractions could be obtained, only the most relevant lipid classes are shown here. Trace (d) represents the negative ion mass spectrum of the complete extract, but was recorded with *para*-nitro-aniline as matrix because of its lower acidity in comparison to DHB.

6. SUMMARY AND OUTLOOK

MALDI-TOF MS is an established analytical tool - especially in protein and peptide research (25). Its special advantages are the simple and fast performance, its high sensitivity (11) in the picomolar range, the low tendency to give fragmentation products and its robustness against sample impurities. The same advantages can be used in lipid analysis and there is nowadays growing evidence that MALDI-TOF MS is also useful in this field. However, even if there are already papers dealing with "lipidomics" by using MALDI-TOF MS (57), further work is necessary to evaluate the capabilities of the technique in this field: The majority of the so far available data is dedicated to the analysis of GPCho and this is - among other reasons - caused by its high detection sensitivity. PL with quaternary ammonia groups (GPCho, Monoacyl-GPCho, SM and ceramide) are generally more sensitively detectable than other lipids because of their permanent positive charge. Therefore, it is very difficult to assess the

concentration of further PL species because these species are at least partially suppressed by the GPCho. This particularly holds for GPEtn because this PL is hardly detectable as negative ion, whereas acidic PL as GPSer or GPGro can be advantageously detected as negative species. It should, however, be noted that both positive and negative ion detection modes are not independent of each other, i.e. the presence of huge amounts of GPCho decreases the sensitivity of the negative ion mode (33). Therefore, current research aims to establish new matrix compounds in order to overcome these problems. One promising matrix is PNA (17) that possesses a lower acidity than DHB leading to sensitive detection of GPEtn in the negative ion mode.

Although relative peak intensities are readily available from the mass spectra, internal standards are a must for the determination of absolute amounts of lipids. Surprisingly, reliable quantitative data on the detection limits of the most relevant PL classes are not yet available. However, the problem of different ionization efficacies of the individual PL classes is not only a problem of MALDI-TOF MS, but also holds for other ionization techniques when analysis is performed with a relatively higher concentration (58). Although the reproducibility of MALDI-TOF mass spectra depends on the homogeneity of the formed matrix/lipid co-crystals, reproducibility of spectra is sufficient and is expected to be further improved by the application of the recently introduced ionic liquid matrices (43).

Using a solid instead of a fluid sample provides the significant advantage over other MS techniques that spatially-resolved spectra may be recorded. This MALDI imaging is unequivocally a hot topic for the future (59). MS/MS or ion trap devices with ESI or APCI seem so far the methods of choice when the simultaneous determination of all lipids in a given lipid extract is necessary (15, 60, 61). However, it is often sufficient to monitor relative changes in the lipid profile between a patient suffering from a certain disease and the healthy control. Under these circumstances MALDI is a real alternative to the other MS methods.

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Abbreviations and acronyms: AP: atmospheric pressure; BAL: bronchoalveolar lavage; CI: chemical ionization; CL: cardiolipin; DE: delayed extraction; DG: diacylglycerols; DGDG: digalactosyl-diacylglycerol; DHB: 2,5-dihydroxybenzoic acid; EI: electron impact; ESI: electrospray ionization; FAB: fast atom bombardment; FAST: fragmentation analysis structural TOF; FT: Fourier transform; GC-MS: Gas chromatography - mass spectrometry; GPA: glycerophosphate; GPCho: glycerophosphocholine; GPEtn: glycerophosphoethanolamine; GPGro: glycerophosphoglycerol; GPIns: glycerophosphoinositol; GPInsP_x: glycerophosphoinositol-polyphosphate; GPInsP_2: glycerophosphoinositol-bisphosphate; ICR: ion cyclotron resonance; IR: Infrared; LC: liquid chromatography; LGPCho, lysoglycerophosphocholine (monoacyl-glycerophosphocholine); *m/z*: mass-to-charge ratio; MALDI: matrix-assisted laser desorption and ionization; MGDG: Monogalactosyl-diacylglycerol; MS: mass spectrometry; NMR: nuclear magnetic resonance; PL: phospholipid; PLA_2: Phospholipase A_2; PLC: Phospholipase C; PNA: *para*-nitro-aniline; PSD: post source decay; SM: sphingomyelin; *S/N*: signal-to-noise; *sn*: stereospecific numbering; TFA: trifluoroacetic acid; TG: triacylglycerol; TLC: thin-layer chromatography; TOF: time-of-flight; UV: ultraviolet

Key Words: MALDI-TOF MS; Phospholipid; Tissues; Body fluids; Mass spectrometry; Lipidomics; Review

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