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New Lipidomic Approaches in Cystic Fibrosis

Mario Ollero, Ida Chiara Guerrera, Giuseppe Astarita, Daniele Piomelli, and Aleksander Edelman

Abstract

Lipid analysis has been a crucial source of information in cystic fibrosis (CF). New methodologies for qualitative and quantitative lipidomics allow evaluation of a large number of samples, of special interest in patient screening for diagnostic and prognostic biological markers, as well as in cell physiology. In this chapter, two new complementary approaches are described: matrix-assisted laser desorption coupled to time of flight (MALDI-TOF-ClinProTools^{TM}) and liquid chromatography coupled to ion trap mass spectrometry (LC-MS^n). MALDI-TOF-ClinProTools^{TM} offers a large unbiased screening for the discovery of potential lipid alterations in diseased patients. LC-MS^n represents a state-of-the-art lipidomic tool for the identification and quantification of such alterations. The combination of both may open new perspectives in the quest for lipids participating in CF pathogenesis, therapy targets, and biomarkers.

Key words: Lipidomics, biomarkers, mass spectrometry, MALDI, electrospray.

1. Introduction

Lipid alterations in cystic fibrosis (CF) patients have been extensively reported since the advent of analytical techniques in the 1960s. These observations have arisen from partial lipidomic approaches, which mainly consisted of either fatty acid profiling or targeted analysis of individual lipid species (lipid methods in the context of CF reviewed in (1, 2)).

The recent development of mass spectrometry (MS) techniques has given rise in parallel to a series of lipidomic applications of outstanding potential in the search for novel diagnostic or prognostic biomarkers in CF. In this case the goal is not necessarily the finding of a specific molecule or to identify a differentially

displayed compound, but to determine complex molecular signa-tures that correlate with a particular pathologic status. The draw-backs of current lipidomic approaches are the slowness of analy-sis, the complexity of data interpretation, the lack of a database for an easy identification, and the problems associated with quantification.

In this chapter we describe a novel lipidomic strategy for biomarker discovery in CF, which uses two complementary techniques: matrix-assisted laser desorption, coupled to time of flight/time of flight (MALDI-TOF/TOF-ClinProTools^{TM}) together with liquid chromatography coupled to ion trap mass spectrometry (LC-MS^n) (**Fig. 16.1**).

MALDI-TOF/TOF-ClinProTools^{TM} ([3]) provides a finger-print of the molecular species present in biological samples with high level of accuracy and resolution. Therefore, this tech-nique is particularly suitable for molecular profiling, and it has been proven useful in the search for protein signatures associ-ated with a number of disorders ([4–10]). Roughly, lipid extracts are separated by solid phase extraction and then analyzed by MALDI-TOF/TOF. The data obtained by MALDI-TOF/TOF analysis are subjected to a thorough statistical analysis with the ClinProTools^{TM} software, which determines those lipid species able to significantly segregate two or more populations (i.e., healthy individuals from patients or patients at different severity status of the disease).
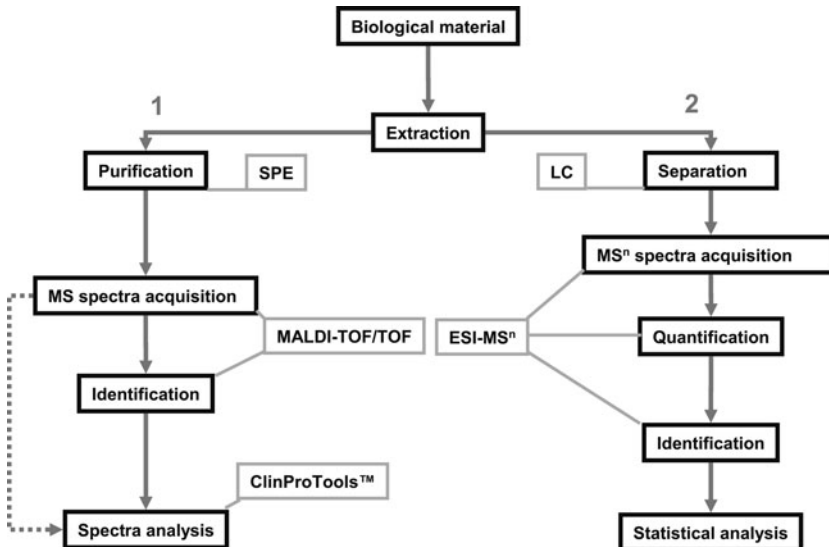

Fig. 16.1. Experimental flowchart. Numbers "1" and "2" denote the ClinProTools^{TM}-MALDI-TOF/TOF and LC-MS^n methods, respectively. The *discontinuous arrow* indicates the possibility of bypassing identification when the primary goal of screening is to obtain significant signatures. In this case a MALDI-TOF/TOF instrument may be used and identification, if desired, can be performed on an equivalent sample by ESI-MS^n. SPE, solid phase extraction; LC, liquid chromatography; ESI, electrospray ionization.

LC-MS^n is used to obtain structural information and quantify lipid species in complex biological matrices ([11], [12]). Briefly, lipid species are chromatographically separated by LC and ionized by either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). The molecular ions generated in the ion source are then fragmented by MS^n into a set of diagnostic fragments, which are used for the chemical characterization and quantification of the lipid species.

2. Materials

2.1. Biological Material

These techniques can be applied to the study of cell lines, animal tissues, human tissues, and body fluids. CF cell models, such as those stably transfected with CFTR/F508del-CFTR (e.g., HeLa, HEK, FRT), immortalized cells derived from CF patients (e.g., HBEo-, CFBEo-), cells derived from adenocarcinoma and expressing large amounts of CFTR (e.g., Calu-3, T84, HT29, Caco), are suitable for lipidomic analysis. Adherent or non-adherent cells must be devoid of incubation medium and washed several times in either water or saline solution (PBS), by sequential centrifugations at $800 \times g$. The final pellet must be resuspended in water (ideally 0.5 ml) and maintained at 4°C. Tissues must be homogenized in PBS or water. Blood plasma and serum are ideally diluted in water to a final volume of 0.5 ml. The suggested initial volume is 0.1 ml. Other fluids may be used, but their lipid content is likely to be very limited.

2.2. Organic Extraction and SPE

– Solvents must be at least of HPLC quality (CHROMASOLV, Sigma-Aldrich, Lyon, France). The following solvents are used: chloroform, methanol (some butylated hydroxytoluene is added to the methanol stock, *see* **Note 1**), isopropanol, diethyl ether, acetic acid, sodium acetate, and hexane. Mixtures of solvents are prepared freshly before use to avoid changes in volume proportions due to accidental evaporation, which may occur during storage. The following mixtures are used:

1. Chloroform–methanol (2:1, v/v)

2. Chloroform–isopropanol (2:1, v/v)

3. Diethyl ether–acetic acid (98:2, v/v)

4. Chloroform–methanol–0.8 N sodium acetate in water (60:30:4.5, v/v/v).

– Glassware and glass tubes must be used in all cases. Ten milliliter conical tubes with phenolic screw caps are recommended (Kimble, Vineland, NJ, USA).

– For SPE extraction, a vacuum device is recommended (Varian). Alternatively, compressed air can be applied to accelerate elution. SPE is performed on direct phase aminopropyl columns (Supelclean LC-NH2-SPE, Supelco, Bellefonte, PA, USA).

– For sample evaporation, prior to SPE and to mass spectrometry analysis, a vacuum apparatus can be used (Savant SC210A SpeedVac concentrator). Alternatively, a stream of nitrogen gas can be applied.

2.3. Matrix and Calibrants for MALDI-TOF

Matrix must be prepared freshly before use. Dihydroxybenzoic acid (DHB) (Sigma-Aldrich) is suggested as a universal matrix for lipid ionization. Nevertheless, it favors the ionization of PC vs. other phospholipid moieties (13):

1. DHB matrix is prepared as a 0.5 M solution in methanol. Trifluoroacetic acid (0.1%) (TFA, Sigma-Aldrich) is added as a counter ion.

2. Calibrant mixture: peptide calibration Mix5 (LaserBio Labs, Sophia Antipolis, France). The mixture covers the mass range of 500–2000 Da. It contains bradykinin (aminoacids 1–5), bradykinin (aminoacids 1–7), bradykinin, angiotensin I human, and neurotensin. Thaw a stock aliquot (10×) before use and dilute 1/10 in matrix solution.

2.4. Instruments for MALDI-TOF-ClinProTools^{TM}

All materials and instruments are from Bruker Daltonics (Bremen, Germany):

1. AutoFlex III or UltraFlex MALDI-TOF/TOF mass spectrometers. These instruments allow the study of ion fragments by MS/MS and subsequently the determination of molecular structure and ulterior identification of some molecules.

2. ClinProTools^{TM} and FlexControl software packages.

3. MALDI target.

2.5. Equipment for LC/MS^{n} Analysis

1. Agilent 1200-LC system (with autosampler) coupled to Ion-Trap XCT detector interfaced with ESI or APCI (Agilent Technologies).

2. Gas: ultra-high purity compressed helium (for MS fragmentation) and high-purity N_2 (for drying samples and for atmospheric pressure ionization functioning).

2.6. Reagents

A representative list of internal standards used to quantify endogenous lipid species may include the following lipids:

1. Fatty acyls:
   – Fatty acids and eicosanoids: heptadecanoic acid from Nu-Chek Prep (Elysian, MN, USA); d8-arachidonic

acid from Cayman Chemicals (Ann Arbor, MI, USA); prostaglandin: d4-prostaglandin E2 from Cayman Chemicals.

– Fatty acid ethanolamide: heptadecenoylethanolamide (synthesized as previously reported (11)).

2. Glycerolipids:
   – Triacylglycerol: trinonadecenoin from Nu-Chek Prep.
   – Diacylglycerol: dinonadienoyl-sn-glycerol from Nu-Chek Prep.
   – Monoacylglycerol: monoheptadecanoyl-sn-glycerol from Nu-Chek Prep; d8-2-arachidonoyl-sn-glycerol from Cayman Chemicals.

3. Glycerophospholipids (all from Avanti Polar Lipids, Alabaster, AL, USA):
   – Phosphatidylethanolamine: 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine.
   – Phosphatidylglycerol: 1,2-diheptadecanoyl-sn-glycero-3-phosphoglycerol.
   – Phosphatidylcholine: 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine.
   – Phosphatidylserine: 1,2-diheptadecanoyl-sn-glycero-3-phosphoserine.
   – Phosphatidylinositol: 1,2-dipalmitoyl-sn-glycero-3-phosphoinositol.

4. Sphingolipids (from Avanti Polar Lipids):
   – Ceramide: N-lauroyl-ceramide.
   – Sphingomyelin: N-lauroyl-sphingomyelin.

5. Sterol lipids (from Avanti Polar Lipids):
   – Cholesterol: d7-cholesterol.

6. Solvents and chemicals for HPLC mobile phases: water, methanol, chloroform (HPLC grade) are purchased from Thermo Fisher Scientific (Somerset, NJ, USA). Acetic acid and ammonium acetate are from Sigma (St Louis, MO, USA).

2.7. Supplies (All from Agilent Technologies)

1. LC columns:
   – Zorbax XDB Eclipse C-18 (50 × 4.6 mm i.d., 1.8 μm particle size, 80 Å of porous diameter).
   – Poroshell 300SB C-18 (2.1 × 75 mm i.d., coating layer of 0.25 μm on total particle diameter of 5 μm, 300 Å of porous diameter).

2. Glass vials (1.5 ml for autosampler and LC analysis).

3. Caps with teflon liner.

4. Conical insert for reducing the volume of autosampler vials.

3. Methods

3.1. MALDI-TOF/TOF-ClinProTools™

3.1.1. Lipid Extraction

1. Based on Folch's method (14), extraction is performed under the principle of addition of six volumes of chloroform–methanol (2:1, v/v) to a liquid or semi-liquid sample. The latter can be a body fluid, a cell suspension, or a tissue homogenate (*see* **Section** 2.1). The optimal volume of sample is 0.5 ml, but smaller volumes down to 0.2 ml can be easily handled.

2. The mixture is vortexed for at least 10 s and centrifuged at $800 \times g$ for 5 min at 4°C. After centrifugation, two phases can be distinguished: an aqueous phase (upper) containing polar molecules, an organic phase (lower) containing hydrophobic molecules, and an interphase where proteins remain as a solid precipitate.

3. The lower phase (organic) is transferred to a disposable glass tube.

4. Alternatively, to increase extraction efficiency, 1.5 ml of chloroform–methanol (2:1, v/v) is added to the remaining upper phase and step 2 is repeated. Both lower phases are combined (*see* **Note 3**).

5. Samples can be stored at –20°C until further processing (*see* **Note 2**). If so, fill tubes with either CO_2 or nitrogen (*see* **Note 1**).

3.1.2. SPE Fractionation

This methodology was described by Kaluzny et al. (15). More than a purification method it represents a rapid way of reducing the complexity level of lipid samples:

1. Total extracts are evaporated either under a stream of nitrogen gas or by means of a vacuum apparatus (*see* **Note 4**).

2. The aminopropyl column (1 ml) is conditioned with two volumes (2 ml) of hexane. The elution speed is adjusted to 2–4 ml/min.

3. Two hundred microliters of chloroform is added to each dry sample residue. The solution is vortexed for 10 s.

4. Vacuum is broken and the dissolved extract is loaded onto the column. The extract penetrates the column by gravity. The solvent level is allowed to reach the top of the column.

5. Two column volumes of chloroform–isopropanol (2:1, v/v) are loaded. Vacuum is applied and this allows the elution of non-polar lipids (cholesterol; cholesteryl esters;

mono-, di-, and tri-glycerides). This fraction is collected and named as fraction I.

6. Two column volumes of 2% acetic acid in diethylether are loaded under vacuum. This allows elution and collection of free fatty acids (fraction II).

7. Two column volumes of methanol are loaded under vacuum. This allows elution of neutral phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM) (fraction III).

8. Two column volumes of chloroform–methanol–0.8 N sodium acetate (60:30:4.5, v/v/v) are loaded under vacuum. This allows elution of acidic phospholipids, such as phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), and phosphatidylinositol (PI) (fraction IV).

9. Sodium acetate of fraction IV can interfere with analysis. To eliminate this salt, fraction IV must be evaporated either under a nitrogen stream or by a concentrator. Three milliliters of chloroform–methanol (2:1, v/v) is added to the extract, followed by 0.5 ml of water. The mixture is vortexed for 10 s and centrifuged at $800 \times g$ for 3 min. The lower phase is aspirated and transferred to a new glass tube.

10. Fractions can be stored at –20°C until further processing (*see* **Note 2**). If so, fill tubes with CO_2 (*see* **Note 1**).

3.1.3. MALDI-TOF/TOF

1. On the day of the analysis, fraction extracts are dried and resuspended in 10 µl of chloroform–methanol (1:1, v/v) by vortexing and sonicated with 3×10 s pulses in a sonication bath (*see* **Note 5**). One microliter is thoroughly mixed with 1 µl of matrix solution in a separate Eppendorf, and 0.5 µl of the mixture is spotted on a classical MALDI target (Bruker). Ideally the same sample should be spotted three times to obtain technical triplicates. The spotting has to be fast because of the hydrophobic nature of the target surface and the fast rate of evaporation of this small volume. When the spot is dry the sample is ready to be analyzed by MALDI-TOF/TOF MS. It is necessary to add blank spots containing a 1:1 (v/v) mixture of chloroform–methanol (1:1, v/v) and the matrix solution (mixed and spotted as described for samples).

2. The MALDI profiling is done in reflectron mode over a detection range of 0–2000 Da, or less, according to the class of lipids analyzed. The spectra can be acquired in both positive and negative modes. The laser power is chosen to guarantee the best signal to noise ratio and it must be kept at

the same intensity throughout the experiment. For the best reproducibility, the spectra are acquired in automatic mode, using FlexControl 3.0 (Bruker), accumulating only spectra with the resolution or the most intense peak higher than 4000.

3. Calibrants mixed with the matrix (1:1, v/v) are also spotted along the sample and their spectra acquired in automated mode using the same parameters. The spectra obtained allow an external calibration which can assure a mass precision within 200 ppm. In order to refine the mass precision to 50 ppm, an additional internal calibration is performed using the signal from the matrix present in the sample spectrum. Both calibrations are performed using FlexAnalysis 3.0 (Bruker).

3.1.4. ClinProTools^{TM} Analysis

Randomly acquired spectra are grouped into classes of patients before being subjected to statistical analysis by ClinProt Tools 2.0 (Bruker). Higher versions of this software can be used when available. Spectra are automatically processed when opened. They undergo baseline subtraction, normalization to the corresponding TIC (total ion current), and finally recalibration. The spectra that do not contain most of the reference masses used for recalibration are automatically excluded.

Parameters of peak picking have to be reset to adjust to the detection range and resolution. Further manual elimination of isotopic isoforms for the same compound is necessary because the software is developed for proteins/peptides acquired in linear mode, therefore with a much lower non-isotopic resolution.

Different classes of patients can simultaneously be compared with the controls. The statistical analysis chosen is a univariate sorting algorithm (QC). The results can be displayed by a 2D plot using the two most discriminant peak intensities in all classes (**Fig. 16.2**). However, a complete Excel list, containing the mass value, the intensity, and the ANOVA p-value for each peak, can also be exported. Raw relative abundance values can also be exported in an Excel file. Once the list of relevant peaks is obtained, it is important to eliminate those that are present in the blank spectrum, which correspond to either contaminants or matrix ions (*see* **Notes 5** and **6**). Identification of relevant peaks can be performed either by ion fragmentation using the MALDI-TOF/TOF apparatus (*see* **Note** 7) or by LC/MS^n analysis (*see* **Section 3.2**).

3.2. LC/MS^n Analysis

3.2.1. Sample Preparation

Lipid samples are prepared as described in **Section 3.1.1**, with the addition of a mixture of internal standards into the chloroform–methanol solution before the extraction process.
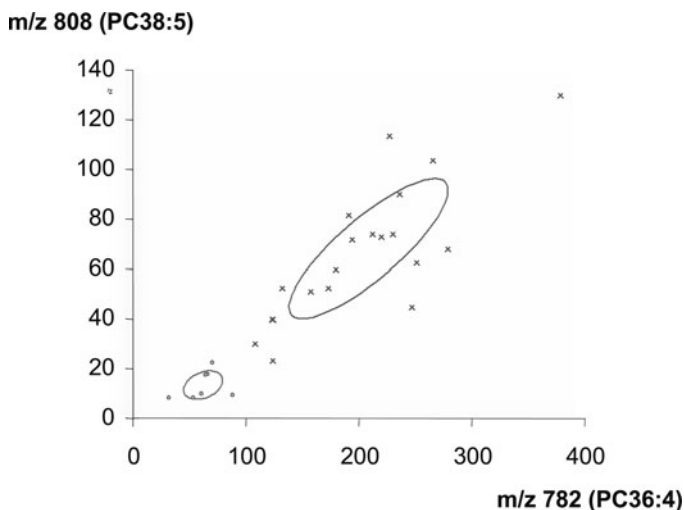
m/z 808 (PC38:5)

m/z 782 (PC36:4)

Fig. 16.2. ClinProTools^{TM} 2D peak distribution diagrams corresponding to a couple of ions (*m/z* 808 and *m/z* 782) able to segregate two populations of samples (*circles* and *crosses*). *Ellipses* correspond to 95% confidence intervals. The values represented correspond to arbitrary units of relative intensity.

Internal standards are constituted by non-endogenous lipid species representative of each lipid class (*see* **Section 2.6**). Lipid molecular species are quantified by normalizing the individual molecular ion peak intensity with an internal standard for each lipid class. These internal standards allow the lipid levels to be normalized for both extraction efficiency and instrument response.

3.2.2. Chromatographic and Intrasource Ionization Separation of Lipid Molecules

In order to simplify the analysis in biological tissues, lipids are schematically divided into three main classes: (a) small lipids, defined here as molecules containing one aliphatic group such as fatty acids and their derivatives (amides, esters, oxygenated compounds); (b) large lipids, molecules containing two or more aliphatic groups, such as phospholipids, diacylglycerols, triacylglycerols, sphingolipids; and (c) sterol lipids, molecules containing a rigid four-ring backbone such as cholesterol and its derivatives. To identify and quantify the different classes of lipids by LC/MS, two separate chromatographic approaches are applied, using different reversed-phase C-18 stationary phases. A chromatographic separation of lipid species helps reduce the isotopic effects, which affect the actual mass abundance allowing a more accurate quantification. Furthermore, ESI set in either positive or negative mode and APCI set in positive mode are used to separate the lipid classes on the basis of different ionization efficiencies of their functional groups. The combination of chromatographic resolving power in conjunction with ionization source selection and mass detection allows the identification and quantification of lipid species present at very low concentration.

3.2.2.1. Small Lipids

To separate lipids containing one fatty acyl group, a reversed-phase C-18 column packed with conventional porous silica particles of small spherical diameter (sub-2 μm) is used. Fatty acyl species are separated both by chain length and by degree of unsaturation of their fatty acid chains. Generally, in positive ESI mode small lipids are detected as protonated molecular ions or sodium and ammonium adducts. In contrast, in negative mode small lipids are detected as deprotonated molecular ions. Mobile phase A consists of methanol containing 0.25% acetic acid and 5 mM ammonium acetate; mobile phase B corresponds to water containing 0.25% acetic acid and 5 mM ammonium acetate. Small lipids are identified based on their retention times and MS^n properties.

1. Fatty acyls: Fatty acyls are separated using a reversed-phase Zorbax XDB Eclipse C-18 column. Lipids are eluted using a linear gradient from 90% A to 100% B in 2.5 min at a flow rate of 1.5 ml/min with column temperature at 40°C. ESI is in the negative mode, capillary voltage is set at –4 kV, and fragmentor voltage is 100 V. N_2 is used as drying gas at a flow rate of 13 l/min and a temperature of 350°C. Nebulizer pressure is set at 60 psi. We use commercially available fatty acyls as reference standards. Fatty acids are analyzed monitoring the mass-to-charge ratio (m/z) of the deprotonated molecular ions $[M–H]^-$ in selected-ion monitoring mode. Detection and analysis are controlled by Agilent Chemstation and Bruker Daltonics softwares.

3.2.2.2. Large Lipids and Sterol Lipids

To separate large (glycerolipids, glycerophospholipids, and sphingolipids) lipids and sterol lipids, a reversed-phase C-18 column packed with superficially porous particles is used. This allows for fast flow rates and good peak shape for large lipid molecules. Although lipids are separated when differing in a single fatty acyl chain, their combinatorial nature makes only a partial separation of the isomeric species possible. Therefore, to obtain more information on the lipid structure, LC separation is coupled with MS^n fragmentation data. Generally, large lipids are detected in the positive ESI mode as sodium or ammonium adducts or as deprotonated molecular ions in the negative mode. For sterol lipids, which are highly hydrophobic and hard to ionize, APCI is used in positive mode and the protonated molecular ions are detected after loss of water.

1. Glycerolipids, glycerophospholipids, and sphingolipids: Large lipid molecules are separated using a reversed-phase Poroshell 300SB C-18 column. A linear gradient is applied from 85% A to 100% B in 5 min at a flow rate of 1.0 ml/min with column temperature set at 50°C. MS detection is performed both in the positive and in the negative ionization modes. The capillary voltage is set at 4.0 kV and skimmer

voltage at 40 V. N_2 is used as drying gas at a flow rate of 10 l/min, temperature at 350°C, and nebulizer pressure at 60 psi. Helium is used as collision gas, and fragmentation amplitude is set at 1.2 V. Ion charge control is on, smart target set at 50,000, maximum accumulation time at 50 ms, scan range of 100–1500 amu, 26,000 m/z per second. Lipids are identified based on their retention times and MS^n properties (**Fig. 16.3**). Detection and quantitative analysis are controlled by Agilent Chemstation and Bruker Daltonics software.

2. Sterol lipids: Sterol lipid molecules are separated using a reversed-phase Poroshell 300SB C-18 column. Lipids are separated using a linear gradient from 75% A to 100% B in 4-min period at a flow rate of 1.0 ml/min with column temperature at 50°C. APCI is set in positive mode. Drying gas is set at 350°C and a flow rate of 8 l/min. Nebulizer gas pressure is set at 30 psi and vaporizer temperature at 475°C. Capillary voltage is set at 300 V with the corona current set at 5 µA. Lipids are identified based on their retention times and MS^n properties (**Fig. 16.3**). Detection and quantitative analysis are controlled by Agilent Chemstation and Bruker Daltonics software.
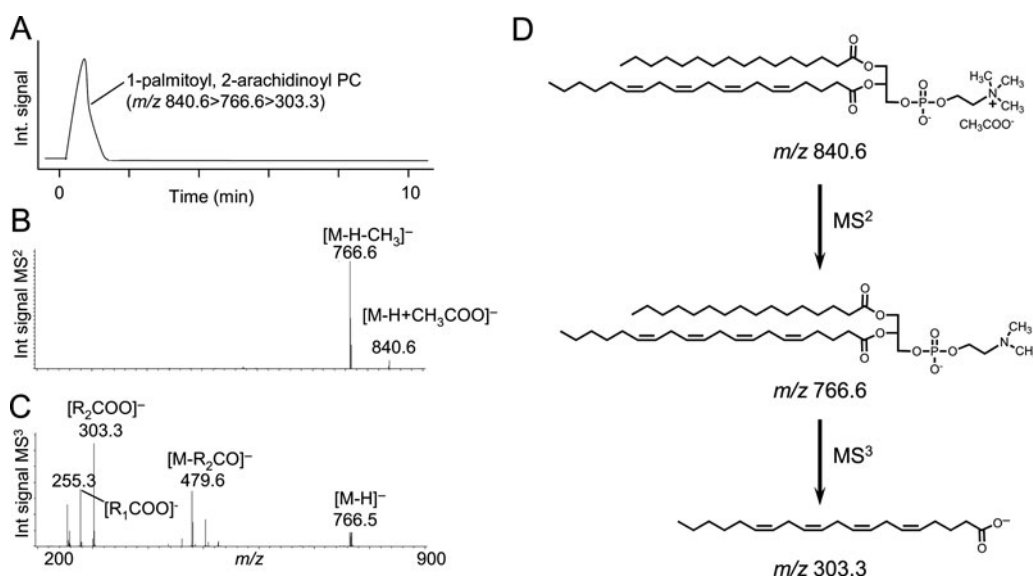

Fig. 16.3. Identification of 1-palmitoyl,2-arachidonoyl phosphatidylcholine (PC) (m/z 840.6) in biological samples. Representative extracted LC/MS^3 chromatogram (*panel A*) and fragmentation pattern in MS^2 (*panel B*) and MS^3 (*panel C*) using an ion trap instrument. PC species are detected as acetate adducts of the molecular ions using ESI set in the negative mode. The MS^2 fragmentation pattern is characterized by neutral loss of the acetate adduct of the *N*-methyl group (*panel D*). MS^3 of the ion with m/z 766.5 yields the lysophospholipid with neutral loss of ketene in combination with the *sn*–1 and *sn*–2 carboxylate anions (*panels C and D*). Abbreviations: R_1 = *sn*–1 aliphatic chain; R_2 = *sn*–2 aliphatic chain.

4. Notes

1. Peroxidation is the major cause of degradation of lipid extracts, particularly those containing high amounts of unsaturated chains. It is recommended to use butylated hydroxytoluene (BHT) (Sigma) as antioxidant. This is added to the methanol stock. Other actions conducted to minimize peroxidation are to perform all work on ice, fill tubes with either CO_2 or nitrogen to eliminate molecular oxygen, and store extracts at low temperature (*see* **Note 2**). Although nitrogen is a more inert gas, CO_2 presents the advantage of being heavier than air and remains in the interior of the tube.

2. Storage of extracts: Lipid extracts and SPE fractions can be stored before evaporation at –80°C for several months. Higher temperatures –20°C are acceptable for shorter storage times (up to 2 weeks). In general, long storage is not recommended. Shipping of extracts or samples must be assured in dry ice.

3. For MALDI-TOF/TOF-ClinProTools^{TM} analysis, normalization is automatically performed to total ion current by the software and there is no need for internal standards, which may also interfere with the MS detection of isomeric endogenous lipids, as no previous separation is performed.

4. Sample evaporation must be performed in the absence of molecular oxygen to minimize lipid oxidation. Nitrogen or any other inert gas is an acceptable option. Nevertheless, vacuum concentrators provide a cleaner preparation, as the lipid extract is better confined to the bottom of the tube.

5. Specificity of MALDI analysis: Some matrices favor the detection of particular lipid classes. DHB is especially indicated in the analysis of neutral phospholipids (PC, SM) in the positive mode. Other matrices can be used in the negative mode. A drawback of MALDI is the interference of matrix peaks with lipid signals. DHB is particularly noisy in the negative mode and it is not recommended for the analysis of fatty acids (see (13) for review).

6. Plastic contaminants: Long-term storage of body fluids in plastic vials may result in the presence of polymer contaminants in the sample. These contaminants are extracted by organic solvents and may be found as ghost peaks in spectra. They can be recognized as a regular sequence of peaks differing in 44 m/z. The use of plastic vials and the length of storage should be minimized.

7. Identification by MALDI-TOF/TOF: Relevant ions resulting from ClinProTools^{TM} analysis can be identified by

tandem MS using the LIFT application integrated in the FlexControl^{TM} (Bruker) software associated with the MALDI-TOF/TOF apparatus. For most lipid classes, analysis of fragmentation patterns, as described in **Fig. 16.3** for ESI-MS^{n}, allows molecular structure determination. At present, the most comprehensive general database of lipid mass spectra is that of the Lipid Maps initiative (http://lipidmaps.org/data/databases.html). Search engines compatible with MS/MS data are also available (http://lipidmaps.org/tools/index.html; Lipid MS Predictor software), but a manual analysis of the fragmentation spectra is needed to assure identification.

Acknowledgments

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