LC-MS-based method for the qualitative and quantitative analysis of complex lipid mixtures[S]

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Abstract A simple and robust LC-MS-based methodology for the investigation of lipid mixtures is described, and its application to the analysis of human lipoprotein-associated lipids is demonstrated. After an optional initial fractionation on Silica 60, normal-phase HPLC-MS on a YMC PVA-Sil column is used first for class separation, followed by reversed-phase LC-MS or LC-tandem mass spectrometry using an Atlantis dC18 capillary column, and/or nanospray MS, to fully characterize the individual lipids. The methodology is applied here for the analysis of human apolipoprotein B-associated lipids. This approach allows for the determination of even low percentages of lipids of each molecular species and showed clear differences between lipids associated with apolipoprotein B-100-LDL isolated from a normal individual and those associated with a truncated version, apolipoprotein B-67-containing lipoproteins, isolated from a homozygote patient with familial hypobetalipoproteinemia. The methods described should be easily adaptable to most modern MS instrumentation.—Sommer, U., H. Herscovitz, F. K. Welty, and C. E. Costello. **LC-MS-based method for the qualitative and quantitative analysis of complex lipid mixtures. *J. Lipid Res.* 2006.** 47: **804–814.**

Supplementary key words low density lipoprotein • intermediate density lipoprotein • normal-phase high-performance liquid chromatography-mass spectrometry • reversed-phase liquid chromatography-tandem mass spectrometry • familial hypobetalipoproteinemia

A large variety of methods have been published for the separation of lipids, either by TLC or by LC; the methods have usually been described for the analysis of specific classes of compounds (www.cyberlipid.org). MS methods for the characterization of lipid mixtures have also been published in recent years, most of them centered on the use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and electrospray ionization (ESI) MS (1) (in addition to the references cited in the text, important Web-based sources of information were www.cyberlipid.org, www.lipidlibrary.co.uk, and www.lipidmaps.org). Sophisticated methods like the characterization of complex glycolipids directly from TLC plates by vibrationally cooled MALDI Fourier transform-ion cyclotron resonance MS (2) require instrumentation that is not yet widely available. A variety of elegant nanospray MS methods have been described (3–5) that are generally a good choice for the characterization of lipids, but they may not be fully capable of both qualitative and quantitative analysis of highly complex mixtures. LC-MS offers possibilities for a better determination of minor compounds whose signals might otherwise be suppressed. It also allows for an additional level of characterization of components based on their chromatographic behavior as well as the MS results.

Existing HPLC methods for the separation of lipids are limited, however, in that they either target only selected classes or are not compatible with subsequent MS. Pulfer and Murphy (6) suggested that, for a complete separation of lipids, normal- and reversed-phase chromatography should be combined. Because of its high sensitivity and the additional information it provides, MS is widely recognized as a superior detection method compared with the classic methods of ultraviolet or light scattering. We demonstrate here a simple, robust, and reproducible methodology for lipid analysis, which has been achieved by adapting to LC-MS several separation systems described in the literature for the thin-layer and liquid chromatography of lipids. After an optional initial cleanup and prefractionation on Silica 60, we use normal-phase HPLC-MS for class separation first, then an optional reversed-phase LC-MS or LC-tandem mass spectrometry (MS/MS) system for further analysis. Application of the methodology for the

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Abbreviations: B67, apolipoprotein B-67 (N-terminal 67% of apolipoprotein B); B100, apolipoprotein B-100 (full-length apolipoprotein B); ESI, electrospray ionization; IDL, intermediate density lipoprotein; IPA, *iso*-propyl alcohol, 2-propanol; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MeOH, methanol; MS/MS, tandem mass spectrometry; MTBE, methyl *t*-butyl ether; PC, glycerophosphocholine; PE, glycerophosphoethanolamine; PI, glycerophosphoinositol; QoTOF, quadrupole orthogonal time-of-flight; QQQ, triple quadrupole.

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[S] The online version of this article (available at http://www.jlr.org) contains an additional figure.

analysis of human LDL lipids is demonstrated here. We compared the lipids associated with normal apolipoprotein B-100 (B100)-containing LDL with those associated with mutant apolipoprotein B-67 (B67)-containing lipoproteins, which are found in certain cases of familial hypobetalipoproteinemia (7).

MATERIALS AND METHODS

Materials and instrumentation

Lipid standards were obtained from Avanti (Alabaster, AL), Matreya (Pleasant Gap, PA), or Sigma (St. Louis, MO) and were prepared for use in this study as 1 mg/ml total lipid stock solutions. Whereas the simple acylglycerols (monoacylglycerol, diacylglycerol, and triacylglycerol), cholesterol, cholesteryl esters, free fatty acids, and fatty acid methyl ester standards and the glycolipids (prepared as 1 mg/ml stock solutions) each consisted of only one species, typically the oleoyl (C18:1) derivatives, the phospholipid standards each contained multiple components. Lipid structures are provided in the Supplemental Figure. The solvents used were HPLC-grade. Most experiments were carried out using a Hewlett-Packard (now Agilent) 1090 HPLC system coupled to a Waters/Micromass (Beverly, MA) Quattro II triple quadrupole (QQQ) mass spectrometer. Both were controlled with Waters/Micromass MassLynx 3.4 software, which was also used for data interpretation, including quantitation. Also used were two different Applied Biosystems/Sciex (Toronto, Ontario, Canada) QStar Pulsar *i* quadrupole orthogonal time-of-flight (QoTOF) mass spectrometers, both controlled by Analyst QS SP8, and this software was also used for data interpretation. One of the QStar spectrometers was equipped with a nanospray source, and the other was connected to a Waters CapLC that was controlled by MassLynx 4.0.

B100-LDL derived from a normal individual was isolated by sequential ultracentrifugation. A narrow cut of d = 1.025–1.050 g/ml was isolated and washed at d = 1.063 g/ml. B67-containing lipoproteins, chylomicrons, intermediate density lipoprotein (IDL), and LDL derived from a patient homozygous to apoB were isolated by density gradient centrifugation. Lipids were extracted according to Bligh and Dyer (8). The human samples were gathered with institutional approval and the informed consent of the donor.

Separation of polar and nonpolar lipids on Silica 60

Lipid standards and the isolated and extracted LDL lipids were passed stepwise onto Silica 60 resin (200–400 mesh; EMD Chemicals, Inc., Gibbstown, NJ) and were eluted off with methyl *t*-butyl ether (MTBE), followed by methanol (MeOH) containing 10 mM ammonium acetate. Pasteur pipettes with glass wool plugs, or 6 ml glass solid-phase extraction columns (Supelco, Bellefonte, PA), were used. The samples were dried under a stream of nitrogen.

Normal-phase LC-MS

Either the two fractions from the previous step, or the full sample (or a set of standards), were separated on a YMC microbore PVA-Sil column (1 mm × 25 cm; Waters Corp., Milford, MA) on the HP1090 HPLC/Waters Quattro II LC/MS system, with ion extraction, separation, and detection in both positive and negative ion modes. Typically, ~50 ng per compound class was injected as lipid standards for these LC-MS runs. Two different gradient methods were used. For the determination of polar lipids, the solvents were 5 mM ammonium formate in MTBE/MeOH/*iso*-propyl alcohol, 2-propanol (IPA)/water (80:10:7:3) (A); 5 mM ammonium formate in MeOH/IPA/water (90:7:3) (B); or pure MeOH (C). At a flow rate of 0.1 ml/min, solvent A was held for 2 min, followed by a gradient over 15 min to solvent B, which was held for 5 min, and 3 min of solvent C before reequilibration. The postcolumn split diverted only ~10% of the flow to the mass spectrometer. In the ESI ion source, spray voltage in positive and negative ion modes was set to ±4 kV, and the cone (skimmer) voltage was ±40–60 V for polar lipids and ±25–30 V for nonpolar lipids. For the determination of the more nonpolar lipids, the solvents were 0.5% MTBE in heptane (D); 0.02% HOAc and 10% IPA in MTBE (E); or 10 mM ammonium acetate in MeOH/water (9:1) (F). The gradient method (0.1 ml/min) consisted of 5 min of solvent D, a gradient over 5 min to 80% solvent E, which was held for 5 min, 5 min of solvents D/E/F (20:70:10), 5 min of solvent E, and reequilibration. A postcolumn feed of 2.5 μl/min of 10 mM ammonium acetate in IPA/MeOH (60:40) was added with a syringe pump to the 10% split toward the mass spectrometer to achieve optimum ionization conditions. Collected fractions were dried under a stream of nitrogen before further use.

Reversed-phase LC-MS

Fractions obtained from the normal-phase column could be further characterized by reversed-phase LC-MS/MS using a 300 μm × 15 cm Atlantis dC18 capillary column (Waters Corp.) with either the Quattro II-based system described above or with a Waters CapLC system interfaced to an Applied Biosystems/Sciex QStar Pulsar *i* QoTOF mass spectrometer. Flow rates were either 100 μl/min with a preinjector split to 5 μl/min or 5 μl/min without a split; typical injections of standards were ~1 ng per compound class. For the separation of both the glycerophosphoethanolamines (PEs) and glycerophosphocholines (PCs), the following solvents and gradient were used: 10 mM ammonium acetate in MeOH/IPA/water (90:5:5) (G); and 10 mM ammonium acetate in MeOH/IPA/water (94:5:1) (H). The LC method consisted of holding solvent G for 10 min, a gradient over 15 min to solvent H, and holding H for 5 min. On the QQQ mass spectrometer, spray voltage was typically set to −4 kV and collision voltage to −80 V in negative ion mode and to 4 kV and 60–80 V in positive ion mode. LC-MS precursor ion scans for *m/z* 184 in positive ion mode were performed at a cone voltage of 80 V and a collision energy of −35 eV, with neutral loss scans (−141 mass units) in positive ion mode at 60 V (cone) and −20 eV (collision energy).

Nanospray MS

Nanospray MS was performed on either type of mass spectrometer. Nanospray tips were prepared from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) using a model P-97 tip puller (Sutter Instrument Co., Novato, CA). Samples were screened on the QQQ instrument using MS and MS/MS in the product ion, precursor ion, or neutral loss scan mode, essentially as described by Brügger et al. (3). In addition, precursor ion scans were performed for acyl fragments in the negative ion mode and for sugar fragments and cholesterol in the positive ion mode, as well as an additional neutral loss scan (−260 mass units) for glycerophosphoinositol (PI) in the positive ion mode. For selected samples, data were also obtained with the QStar QoTOF MS system using MS and MS/MS in the product and precursor ion modes.

Quantitation

Quantitation was based on the separations on the PVA-Sil column. For the experiments with biological samples, data ob-

tained for external standards run on the same day were used for estimation of the sample quantities. These standards contained a defined amount, typically 50 ng per compound class for each injection. To compensate for significant differences in sensitivity, amounts were adjusted for some classes (e.g., usually 50% less standard was used for monoacylglycerophosphocholines and two to four times the amount was used for cholesterol and gangliosides). The reported values were based on signal height or signal area of the single ion chromatogram at the appropriate retention time. The results were not corrected for variation in ionization efficiencies within a given compound class. Compounds evaluated for use as internal standards included dimyristoyl-*sn*-glycero-3-phosphocholine-d_{54} and a rare diacylglycerophosphoethanolamine (C16:0, 17:1).

RESULTS

Our goal in this study was to develop a robust methodology that can be used with simple as well as sophisticated instrumentation and that is especially suitable for the analysis of complex mixtures that are difficult to analyze by conventional nanospray MS. Additionally, for biological samples containing some known or potential nonlipid contamination, or in the case of very small amounts of polar lipids in the presence of large amounts of nonpolar lipids (or vice versa), we used an initial separation on conventional Silica 60. For the primary separation step, we chose the route of separation by compound classes by normal-phase LC-MS (which already delivers the information necessary for a more reliable quantitation of minor components, as well as a level of information about the molecular composition that might be sufficient to fulfill the analytical requirements in many cases). Fractions collected from this normal-phase column can then be further characterized by reversed-phase LC-MS and/or by nanospray MS.

Separation of polar and nonpolar lipids on Silica 60

Lipid standards containing diverse nonpolar phospholipids and glycolipids have been reproducibly separated on the basis of polarity by elution from Silica 60 resin with MTBE and MeOH. Compounds such as cholesteryl esters, triacylglycerol, and glycerophospholipids were found to be well separated, but compounds of medium polarity were detected in different amounts in both fractions (monoacylglycerols, short cerebrosides). Use of the 6 ml solid-phase extraction columns generated inconveniently large volumes of solvent that had to be evaporated, but self-plugged Pasteur pipettes did not always deliver reproducible results; therefore, these were used only for samples containing small amounts (<1 μg). Substitution of chloroform for MTBE, or addition of 10 mM ammonium acetate to the MeOH elution, did not significantly alter the profile. Even the most polar compound tested, the trisialoganglioside GT1b, could be eluted successfully with MeOH with or without ammonium acetate. For reasons discussed below, MTBE and ammonium acetate were selected for general use.

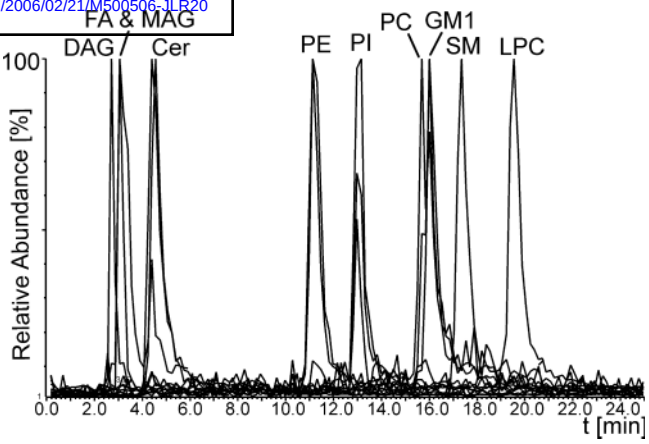

Fig. 1. Overlaid and normalized single ion currents from a separation of a standard mixture by compound classes on a PVA-Sil column in negative ion mode using the methyl *t*-butyl ether (MTBE)/methanol-based gradient for polar lipids (see Materials and Methods). DAG, diacylglycerol; FA, free fatty acid; MAG, monoacylglycerol; Cer, stearoyl dihydrogalactocerebroside; PE, glycerophosphoethanolamine; PI, glycerophosphoinositol; PC, glycerophosphocholine; GM1, ganglioside GM1; SM, sphingomyelin; LPC, monoacylglycerophosphocholine (PC, SM, and LPC as formate adducts; all others as $[M\text{-}H]^-$). Lipid solutions containing 12–75 ng per standard were injected onto the column, with one-tenth split toward the mass spectrometer.

Gradient for polar lipids on a normal-phase column

A mixture of phospholipid and glycolipid standards was applied to the PVA-Sil column. As shown in **Fig. 1**, the phospholipid groups could be well separated from one another. The larger amounts of accompanying nonpolar compounds elute first from the column, before the lipids of interest. The first class of polar lipids to elute are PEs (at ~10 min). We observed some separation within a single class, especially for sphingomyelins, as a result of the minor hydrophobic interaction properties of this column, but even the sphingomyelins still separated from diacylglycerophosphocholines and monoacylglycerophosphocholines that eluted before and after them, respectively. Cerebrosides and gangliosides appear at distinctive positions over the same gradient (Figs. 1, 2). Although the cerebroside shown here, *N*-stearoyl-DL-dihydrogalactocerebroside, clearly eluted earlier than the phospholipid standards, *N*-stearoyl-DL-dihydrolactocerebroside, which is one hexose larger and therefore more polar, runs slightly slower than the PE standards (data not shown). As expected, the palmitoyl species are not separated from their respective stearoyl homologs. **Figure 2** also demonstrates the usefulness of this gradient for the determination of another important lipid class, the gangliosides.

In earlier experiments, ammonium acetate was evaluated as the stabilizing salt. Although its separation and ionization efficiency were found to be similar to those of ammonium formate, some of the lipid species formed double peaks, likely as a result of incomplete protonation (data not shown). These double peaks were not observed in the presence of sufficient acetic acid or upon substitution with ammonium formate. Under the conditions

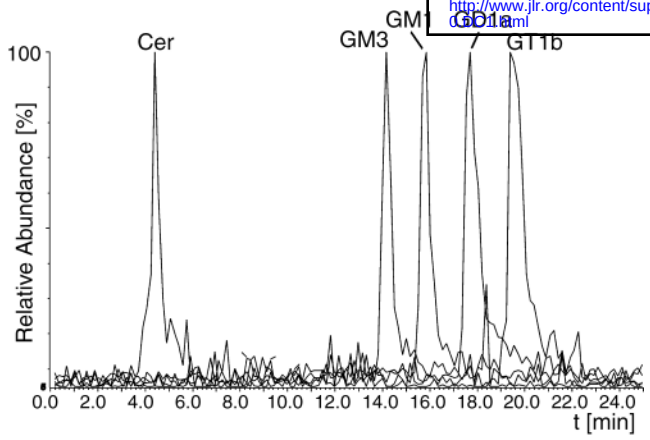

Fig. 2. Overlaid and normalized single ion currents in negative ion mode from a separation of gangliosides GM1 and GM3 (both as $[M\text{-}H]^-$), gangliosides GD1a and GT1b (both as $[M\text{-}2H]^{2-}$), and stearoyl dihydrogalactocerebroside (Cer; as $[M\text{-}H]^-$) on the PVA-Sil column (50 ng each, with one-tenth split toward the mass spectrometer). The gradient is identical to that used in Fig. 1.

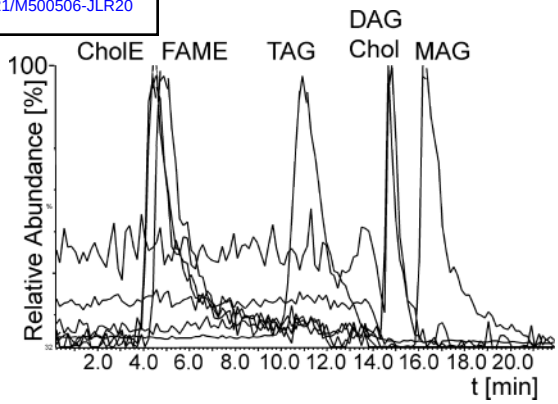

Fig. 3. Overlaid and normalized single ion currents in positive ion mode from a separation of nonpolar lipid standards on the PVA-Sil column (heptane/MTBE-based gradient for nonpolar lipids; see Materials and Methods). CholE, cholesteryl ester; FAME, fatty acid methyl ester; TAG, triacylglycerol; DAG, diacylglycerol; Chol, free cholesterol; MAG, monoacylglycerol (FAME as $[M+Na]^+$; all others as $[M+NH_4]^+$). All esters are oleoyl esters. The high background, especially for the cholesterol sample at *m/z* 404, is attributable to background signal likely derived from the solvents. Standards (50 ng each) were injected onto the column, with one-tenth split toward the mass spectrometer. An organic ammonium acetate solution was fed into this split to promote ionization (see text).

chosen, the $[M+H]^+$ species were observed for all of these classes in the positive ion mode spectra (the $[M+2H]^{2+}$ was observed for gangliosides GD1a and GT1b), and only PI showed a considerable amount of the $[M+NH_4]^+$ species. In the negative ion mode, the $[M\text{-}H]^-$ and $[M\text{-}2H]^{2-}$ ions dominated the spectra of the glycolipids, PE and PI, whereas the formate adducts ($[M+COOH]^-$) dominated the spectra of the glycerophosphocholine (PC) and sphingomyelin species.

Gradient for nonpolar lipids on a normal-phase column

The use of isooctane, heptane, hexane, or pentane as the nonpolar phase of the gradient gave similar chromatographic results; heptane was chosen for convenience. Additions of salts or buffers to the heptane reservoir in amounts sufficient to allow for proper ionization were found to interfere with the quality of the separation, so a 2.5 μl/min feed of 10 mM ammonium acetate in IPA/MeOH/water (60:40:1) delivered from a syringe pump was added, after the postcolumn split, to the flow (~10 μl/min, or 10%) directed toward the mass spectrometer. This addition could be expected to slightly degrade the appearance of the chromatogram but should not significantly affect the separation itself or the fraction collection. A mixture of nonpolar lipids and a PC was applied to the PVA-Sil column. As shown in **Fig. 3**, all species were retained on the column to a certain degree. Fatty acid methyl esters and cholesteryl esters were barely resolved from one another, and diacylglycerols eluted together with cholesterol and free fatty acids. The acids are only visible in negative ion mode and therefore do not appear in the positive ion spectrum shown in Fig. 3. The related groups (triacylglycerol, diacylglycerol, and monoacylglycerol; cholesterol and its esters) were each well separated. It must be noted that normal column washes were not sufficient for reequilibration; the system had to be purged for ~3 min at 1 ml/min without the column before reequilibration of the column between runs. This problem might be specific for this LC system or assembly.

Gradients for PE and PC lipids on a reversed-phase column

Separation of the molecular species of diacyl-PE and -PC standards could be achieved on a reversed-phase column. The characterization by LC-MS could be accomplished on the QQQ instrument using in-source fragmentation (skimmer dissociation), instead of one of the collision-induced decomposition MS/MS modes, to generate product ions. The cone voltage was selected such that PCs could be confirmed in the positive ion mode by aligning the profile of their $[M+H]^+$ ions with the single ion chromatogram corresponding to the head group fragment at *m/z* 184, and PEs could be determined by the neutral loss of the head group, leading to a $[M+H\text{-}141]^+$ product ion associated with the $[M+H]^+$ ion. This enabled us to detect ions in addition to those expected in these particular fractions. Alternatively, a precursor ion scan for the *m/z* 184 cation for PCs and a scan for a neutral loss of 141 mass units for PEs could be deployed online to specifically detect the signals from these species. For both classes of lipids, partial characterization of the molecular species could be achieved in the negative ion mode by aligning the molecular weight-related ions ($[M\text{-}CH_3]^-$ for PCs, $[M\text{-}H]^-$ for PEs) with the corresponding profiles of the acyl fragments observed during the run (**Fig. 4**). Depending on the chromatographic resolution of peaks and data points acquired, all of the more abundant components could be characterized in this way. Definition of the molecular composition of the lower abun-

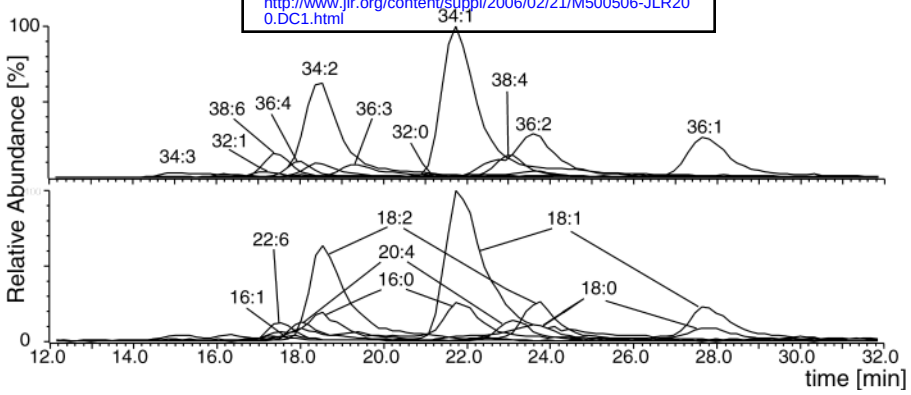

Fig. 4. Overlaid single ion currents from separations of a standard PC mixture on an Atlantis dC18 column (gradient from 5% to 1% water; see Materials and Methods) normalized to the largest signal in each overlay. These negative ion mass spectra were acquired at a cone voltage of −80 V. The top chromatogram shows the $[M\text{-}CH_3]^-$ ions, which dominate the spectrum at this voltage, with the total acyl composition indicated. The bottom chromatogram shows the abundant acyloxy fragments derived from these compounds (the main maxima for each fragment ion are indicated). Correlating ion intensities and retention times, therefore, allows for the determination of the fatty acid composition in the major compounds. All of the injected PC standard mix (100 ng total) was directed toward the mass spectrometer.

dance components requires LC-MS runs performed on one of the more sensitive, automated instruments, as described below.

Analysis of B100-LDL and B67-lipoproteins

The data obtained for a normal LDL lipid sample was compared with that acquired for three different samples (from the same individual) of lipids extracted from B67-containing lipoprotein fractions. Preliminary nanospray MS survey spectra already indicated distinct differences: most obviously, a lower amount of cholesterol and cholesteryl esters and a higher amount of triacylglycerols. **Figure 5** shows the mass spectra, summed over the elution window 2–24 min, of the gradient for polar lipids on the PVA-Sil column for the B100-LDL sample and the B67 lipid samples. Even though the appearance is different from the nanospray spectrum, its information content is the same. However, the possibility of signal suppression of the polar lipids by the more abundant cholesterols and triacylglycerol can now be excluded. More information was gained by summing up the ion chromatograms for both PVA-Sil gradients, fraction by fraction.

Distinct pattern differences could be seen between lipids derived from human B67-LDL and B67-IDL and those from normal B100-LDL, especially for cholesteryl esters (**Fig. 6**) and PCs (**Fig. 7**). The B67-chylomicron sample gave patterns similar to the B67-LDL and B67-IDL samples. Although the most dominant species were the same in the B67 and B100 samples, clear differences were apparent in the less abundant components. With respect to the cholesteryl esters, the B67 lipids clearly contained a lower amount of 16:1 (likely palmitoleic acid) and 20:4 (likely arachidonic acid) species. According to its MS/MS spectrum, the signal at *m/z* 714 is derived from a cholesteryl ester corresponding to the 22:6 species. Signals 5 Da higher than the dominant ammonium adducts $[M+NH_4]^+$ can be assigned to the sodium adduct ions $[M+Na]^+$. In Fig. 7A, C, the same kind of spectra for the LDL PCs are shown, whereas in Fig. 7B, D, the virtually identical nanospray MS spectra of the same isolated components are presented. Although the interpretation is not so straightforward, because there are two acyl residues per molecule, here also a pattern with a lower amount of palmitoleic acid and arachidonic acid can be assumed for the B67-LDL sample. Analysis of these fractions on the reversed-phase column, using the QQQ instrument operated at high cone voltage, allowed matching of the major fatty acyl components, as shown in **Fig. 8**. The results suggest a narrower distribution of fatty acids in the PCs from B67-LDL: they are more centered on the 18:2 species (likely linoleic acid) than are those from normal LDL. The 16:1 and 20:3 species do not yield peaks having signal-to-noise levels of >1:1. Not all $[M\text{-}CH_3]^-$ ions are matched by all of the expected acyl ions by this method, but most can be deduced from the ions found or by following the spectra point-by-point over the single ion chromatograms. (A less labor-intensive approach is obviously to couple the column to a mass spectrometer capable of automated MS/MS data acquisition.) Between the major series of characterized diacyl-PC peaks, groups of signals from another series with approximately one-twentieth the signal intensity can be detected; these components run only slightly faster on the PVA-Sil column and are likely to be plasmalogens (see Discussion).

According to our semiquantitative estimates, PEs account for maximally 1% of the lipids and are therefore more difficult to determine. They were virtually invisible during nanospray MS of the original samples, and even in the isolated fraction, their signal-to-noise values were low. Summing the signals over the range from 9.2 to 10.3 min from the polar lipid runs on the PVA-Sil column (**Fig. 9**) provided additional insight. The molecular weights of one group, giving presumed $[M+H]^+$ signals at *m/z* 716, 718, 742, 744, 746, 766, and 768, correspond to known diacyl-

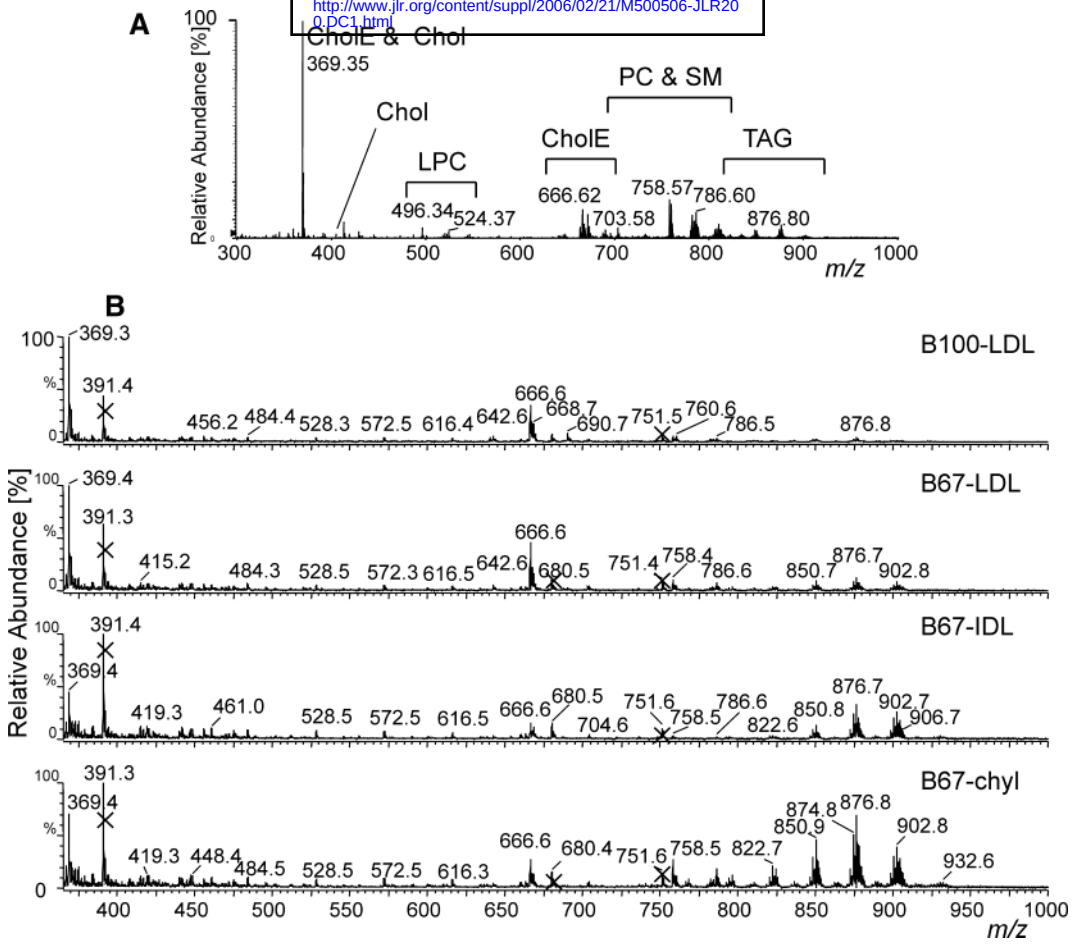

Fig. 5. A: Nanospray MS spectrum of normal apolipoprotein B-100 (B100)-LDL (QStar), with the approximate *m/z* range of the main components indicated. B: MS spectra of lipids associated with B100 (normal) and apolipoprotein B-67 (B67) (mutant), summed over the 2–24 min range of the ion chromatogram (for PVA-Sil column and the gradient for polar lipids, see Fig. 1). CholE, cholesteryl ester; Chol, free cholesterol; LPC, monoacylglycerophosphocholine; SM, sphingomyelin; TAG, triacylglycerol; chyl, chylomicron fraction; IDL, intermediate density lipoprotein. A variety of signals, especially those at *m/z* 391 and 751, originate from the solvent background. Lipid amounts injected were 750 ng for B100 and 320–600 ng for B67 lipids.

PE, and their retention times also match those of the standards. The retention times of species at *m/z* 724, 726, 728, 730, 750, and 752 are each ~0.3–0.4 min shorter, indicating that they are slightly less polar. In the positive ion mode, the nanospray MS/MS spectra used for confirmation show less abundant $[M+H-141]^+$ ions; these fragments result from the neutral loss of the head group. In the negative ion mode, MS/MS spectra show only one dominant type of fragments: the free acyloxy moieties. In the case of the most abundant species, these are *m/z* 279 (corresponding to linoleic acid) for $[M-H]^-$ *m/z* 726 and *m/z* 303 (arachidonic acid) for $[M-H]^-$ *m/z* 750. Both findings suggest that these are plasmalogens or other 1-*O*-alkyl-2-*O*-acylglycerophosphoethanolamines (9). No clear differences between the PE species in B67-LDL and B100-LDL were found.

Quantitation

Standard mixtures containing ~50 ng of lipid per class and injection were run under identical conditions on the PVA-Sil column before and after the biological samples, and a blank run was performed when the switch was made from standard to unknown samples. Calculations of the quantities reported here were based on either peak heights or peak areas for single ion current signals at the expected retention times. Based on the reproducibility of standards run on the same day, including tests of linearity, the error margin is currently ~20% for individual polar lipids and up to 50% (for cholesterol) for nonpolar lipids, whereas the error margins for relative amounts during the same run are approximately half these amounts.

When the molecular species for normal LDL lipids were added back together, total cholesterol (cholesterol and cholesteryl esters) accounted for 50–60%, acylglycerols for 10–15%, and total phospholipids for ~30% of total lipids. B67 lipids were variable and consisted of 15–45% total cholesterol, 15–55% total acylglycerol, and 25–35% total phospholipids (see Discussion).

Initially, we explored internal quantitation using a deuterated PC species, dimyristoyl -*sn*-glycero-3-phosphocholine-d_{54}, but this lipid exhibited incomplete deuteration. This feature made the interpretation unnecessarily

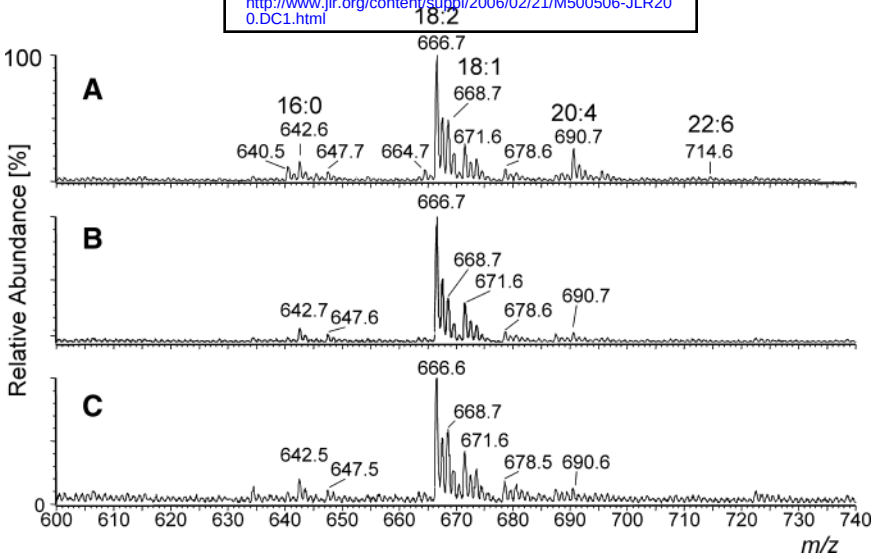

Fig. 6. MS spectra of cholesteryl esters in B100 (A), B67-LDL (B), and B67-IDL (C), derived by summing the total ion chromatograms from 4 to 6 min (see Fig. 3).

complex and increased the likelihood of interference with compounds of interest during the runs.

DISCUSSION

The characterization of many lipid samples can be accomplished with ESI-MS (5, 6). However, samples are often encountered that have insufficient material or are too complex to be reliably analyzed with standard nanospray methodology. As outlined below, LC and LC-MS methods have been developed for a variety of compound classes, but a robust LC-MS methodology to characterize a broad range of lipids has been lacking. Recent sophisticated advances in ESI-MS (5) address these issues, but the LC-MS methodology presented here has the advantage that it is based on relatively simple instrumentation that should be available in all MS laboratories. The primary goal of these

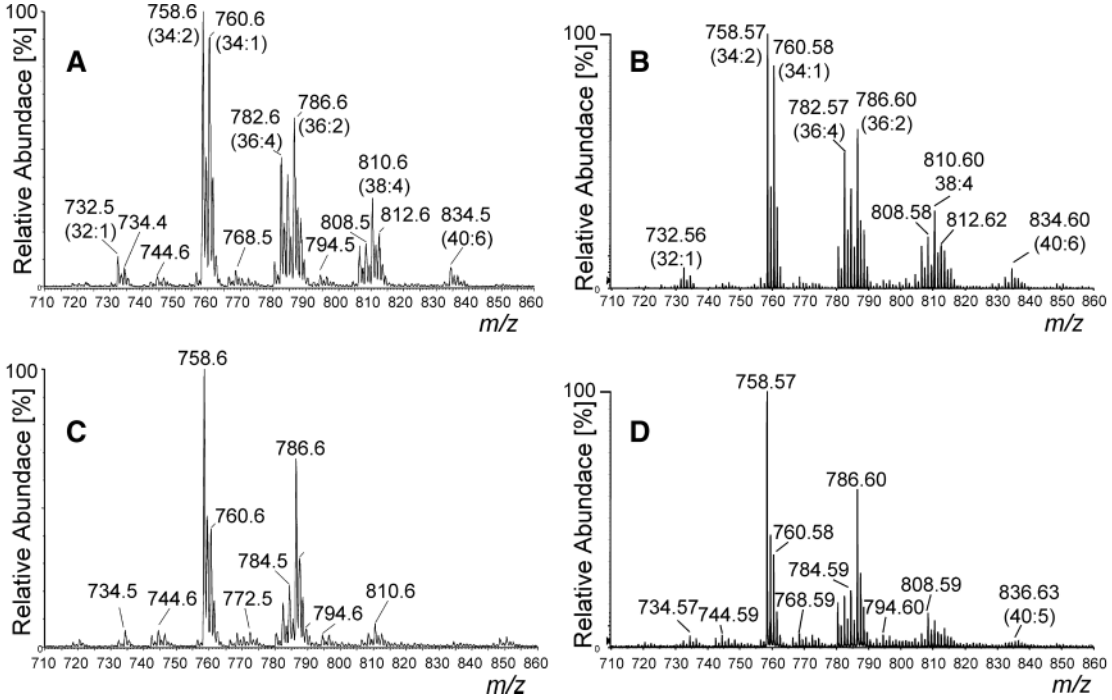

Fig. 7. MS spectra of PCs, derived by summing the total ion chromatograms over 12–15 min in the gradient for polar lipids on the PVA-Sil column (A, C) and from nanospray MS of the isolated fraction on the QStar mass spectrometer (B, D) from B100-LDL (A, B) and B67-LDL (C, D). The graphs show that summing the total ion chromatogram gives a good depiction of what is found in the fraction after isolation.

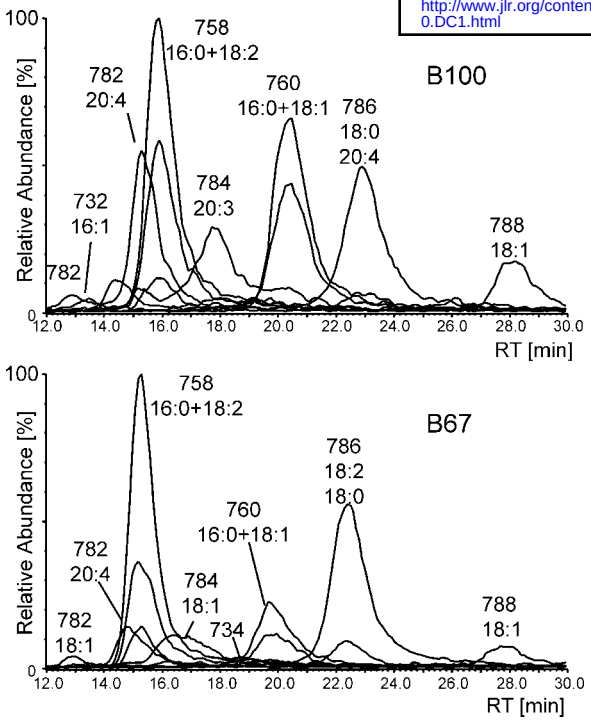

Fig. 8. Separation of the PC fractions from B100-LDL and B67-LDL on the dC18 reversed-phase column. Shown are the single ion chromatograms of the $[M+H]^+$ ions and the alkyl assignments obtained by matching the maxima to those in the single ion chromatograms of the acyloxy ions recorded in parallel negative ion mode analyses, as demonstrated in Fig. 4. Even though the chromatograms had different absolute retention times (RT), the fidelity of the appearances is obvious. For further interpretation, see the text.

studies was to devise an efficient and reliable methodology for routine use in our own laboratory; it should be easily reproducible elsewhere and should provide a basis for developments involving further automation and more advanced instrumentation.

The initial separation of lipids on Silica 60 is, in different variations, a well-established procedure. A laborious but useful separation of most classes is theoretically possible even by this step alone (10), and it has been used, for example, for the enrichment of one or more phospholipid classes before reversed-phase HPLC (11, 12). We use it for a simple two-step elution protocol such that nonpolar lipids are eluted with MTBE and polar lipids are eluted with MeOH. Addition of 0.2% acetic acid to MTBE can improve the elution of certain lipids (10) but might be problematic when very sensitive compounds are being analyzed. This procedure is especially useful for the analysis of contaminated samples, especially those that may contain particulate matter (to protect the following column and LC system), for samples containing very small amounts of polar lipids together with abundant nonpolar lipids or vice versa, or when, in the subsequent step, pure fractions need to be collected. As described for the following steps, this initial separation does not always have to precede methods for the quantitation and characterization of compounds.

The gradients used on both columns were adapted from established procedures, not using MS detection, published for the separation of lipids by thin-layer chromatography and on HPLC columns, especially as described by Hamilton and Comai (13) and Christie and Urwin (14). Also useful was information on the separation of different groups of compounds on normal-phase (15–17) or reversed-phase (18–21) resins, with subsequent MS detection. A review of the published methodology is available at www.cyberlipid.org. Our selection of solvent systems was based not only on their chromatographic properties but also on their compatibility with mass spectrometry (no nonvolatile salts, no ion pairing reagents, etc.) and, where possible, on their lesser toxicity.

We chose the PVA-Sil column, with polymeric vinyl alcohol bonded silica as the resin, because of the robustness of the material. It can be washed with water, as well as hexane, and functions stably over a wide pH range. The column assembly has been used intensively for more than a year on a broad variety of samples that differed widely in purity, and to date we have not seen any decrease in the quality of the analytical results.

Ammonium formate is added to the solvents for two reasons. Ammonium adduct ions, $[M+NH_4]^+$, are known to be more stable than $[M+H]^+$ ions in the positive ion mode and are easier to fragment than the metal-cationized species, for example, the sodium adduct ions $[M+Na]^+$. Formate or acetate ions serve a similar function in the negative ion mode. In the presence of ammonium acetate, however, abundant double peaks were observed in the chromatograms for some of the species, most likely as a result of incomplete protonation; this effect disappeared with the addition of buffering amounts of acetic acid or upon substitution with ammonium formate. The latter was chosen because of simplicity and ease of removal (together with the solvent) under vacuum.

The most frequently used solvent, chloroform, was replaced by MTBE, the solvent that had been reported to be chromatographically superior to chloroform by Hamilton and Comai (13). MTBE is slightly less polar but is more miscible with small amounts of water in a gradient, and it is also slightly less toxic (material safety data sheets).

The necessity of a postcolumn feed for highly nonpolar solvents, as well as the technical difficulties involved in serving all lipid classes well over a single gradient, suggested the use of different solvent gradients for nonpolar and polar lipids. In general, it was unnecessary to separate the compound classes in advance, because polar lipids can be simply washed from the column after the first gradient, and the flow-through containing the bulk of nonpolar lipids elutes several minutes before the first of the phospholipids tested, so long as the column is not overloaded. The flow-through fraction in the polar lipid gradient can also be used as the starting material for the nonpolar lipid gradient (12). Monoacylglycerols can be determined with either gradient, or likely with both, if polar and nonpolar lipids have been separated in advance. The chromatogram for the polar lipid gradient features well-resolved, sharp signals and is quite reproducible. In the chromatogram for

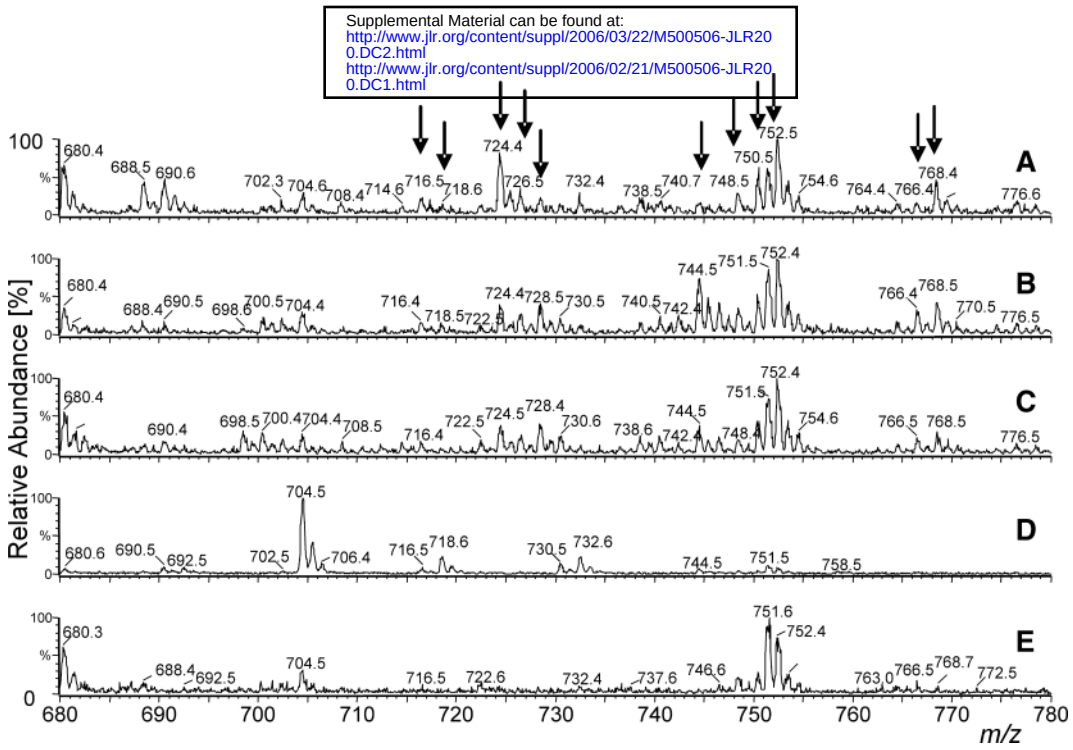

Fig. 9. MS spectra of the PE fraction of normal LDL lipids (A), B67-LDL lipids (B), B67-IDL lipids (C), a diacyl-PE standard mix (D), and a blank run (E), derived by summing the total ion chromatograms from 9–11 min (see Fig. 1). Candidates for the determination of retention times and further characterization by tandem mass spectrometry (MS/MS) could be directly determined from these spectra. Arrows indicate species confirmed by nanospray MS/MS.

the nonpolar lipids, however, the peaks for the acylglycerols and cholesteryl esters exhibited significant tailing, and the retention times sometimes shifted, especially for triacylglycerols. The use of another column (e.g., a diol column) could possibly solve the first problem; the second could be addressed by automating the washes between runs, if this is allowed by the chromatographic system being used. In any case, the gradient described above separated the species well enough to distinguish among them and, therefore, allowed for quantitation and further characterization.

Reversed-phase separation of lipids has been typically published for one or a few compound classes at once. We used it in a way that enabled us to further characterize the PVA-Sil fractions, developing the method by using the readily available QQQ instrument. The goal was to use the same LC method later with a more sensitive QoTOF instrument with automated MS/MS data acquisition. After consideration of the components likely to be encountered, the Atlantis dC18 column was chosen for its complete silanol end-capping, to avoid unwanted interactions, and for its diameter of 300 μm; its optimal flow rate of 5 μl/min can be directly sprayed toward either the Quattro II QQQ or the QStar QoTOF mass spectrometer. The solvents are compatible with those used for peptide analysis, so that no completely separate system is required for the analysis of lipids in a laboratory usually set up for peptide sequencing by LC-MS/MS. As shown here for PEs and PCs, the mass spectra obtained during the reversed-phase LC-MS experiments using high cone voltage in the ESI source (Figs. 4, 8) gave sufficient information about the major molecular species in a mix to enable, in positive ion mode, assignment to the PE or PC group, and to determine, in negative ion mode, which acyloxy fragments are derived from it. For PCs, a high cone voltage in the negative ion mode also helps to avoid generating heterogeneity in the signals as a result of variation in acetate addition and/or methyl group loss. The appropriate precursor or neutral loss scans could be applied for better selectivity when only the ions of interest were to be detected. The isolated PC fractions from the biological samples were also characterized by this method. Nevertheless, nanospray MS/MS, as described elsewhere (3–5), performed on the QStar QoTOF mass spectrometer is clearly a more reliable method than LC/MS on the Quattro II QQQ mass spectrometer, because of the higher sensitivity this mass spectrometer allows and the definite correlation of MS signals to their fragments. Still, it does not provide the additional characterization of the acyl backbone by retention time. The LC method described here should be directly applicable to automated LC-MS/MS on QoTOF, ion trap, and Fourier transform-ion cyclotron resonance (22) instruments, especially with an LC system designed for the lower flow rate.

Alternative LC methods have been published for certain compound classes, such as silver ion chromatography (23) or the addition of other metal ions for ionization and fragmentation (1, 6). These likely should only be tried on dedicated columns; regarding either choice, we were reluctant to risk contaminating our system, which is used for many other purposes; therefore, we did not have the opportunity to obtain information about the long-term influence of metal ions on mass spectrometer performance.

B67 is a truncated version of B100 that corresponds to its N-terminal 67%. It has been found in some cases of familial hypobetalipoproteinemia characterized by low levels of VLDL and LDL and high HDL cholesterol (7). The lipids analyzed in this study were obtained from a B67 homozygote. Although this variant does not seem to be harmful, detailed characterization of the lipids associated with this mutated species may nevertheless afford some insights on the interaction of apoB with certain lipid classes. In this study, one sample of B100-LDL and three B67-containing lipid fractions from the same patient were analyzed, and the results were compared with published data for B100-LDL. A larger number of samples will need to be processed before broad medical conclusions can be made. Although the absolute amounts were expectedly variable, especially for the nonpolar lipid groups in the B67 samples, the molecular pattern within each group was surprisingly consistent, and the distribution in the B67 lipids was clearly different from that of the corresponding B100 lipids.

For each analysis, the total ion chromatograms of the normal-phase LC-MS runs were summed up fraction-by-fraction; this process allows for a direct comparison by compound classes. For most lipid classes, in spite of different total amounts, differences in the molecular pattern between the B67 and B100 lipids were small, but they were more obvious in the case of cholesteryl esters and PCs. The most dominant species in the two cases were the same: they distinguished, for example, LDL lipids from HDL lipids. In a comparison not shown here, the oleic acid-containing compounds were found to dominate in HDL. Differences between B67 and B100 lipids existed in the distribution of less abundant components and were very similar among all three B67 samples. The relative abundances of minor species containing acyl fragments (e.g., 16:1, 20:4, or 22:6 fatty acids) were even lower in the B67 samples. The only fatty acid species clearly not reduced in abundance in the B67 samples was 18:2. The phosphatidylcholine signal at *m/z* 786 contained mostly the fatty acids 18:2 and 18:0.

In the spectra of the PCs, a group of compounds not matching common diacylglycerophosphocholines was detected and, overall, their abundances were not reduced relative to the base signal at *m/z* 758 (18:2, 16:0). The $[M+H]^+$ signals of low abundance components, especially those observed at *m/z* 744, 746, 768, 770, 794, and 796, appeared slightly earlier in the LC runs than the diacyl standards having similar *m/z* values. These are likely plasmalogens which are reported to comprise up to 4.5% of total LDL phospholipids (24).

PE species contribute ≤1% of the total LDL samples. They are virtually invisible by MALDI-TOF MS (25) or nanospray MS of the original samples as a result of suppression, especially by PC signals. Even in the isolated fractions, their signal-to-noise values are low. Besides signals derived from diacyl PEs, as with the PCs, peaks that could be attributed to another group of compounds were detected at somewhat shorter retention times, suggesting that these compounds were slightly less polar. In contrast to the PCs, this group seems to have higher abundances than the diacyl PEs. MS/MS experiments indicated these compounds to be 1-*O*-alk-1-enyl PEs (plasmalogens) or 1-*O*-alkyl PEs. Plasmalogens are known to be constituents of LDL PE as well as LDL PC (24) and are often present in tissue PE at considerable levels (1). One possible approach to confirm these structures would be mild acid hydrolysis of the vinyl ether linkage (9); this step will likely be part of a more detailed analysis of these LDL extracts.

Overall, it appears that the B67 mutant has a somewhat higher binding selectivity for certain acyl groups on lipids than does normal B100. Whether these differences are attributable to the apoB forms or are diet-related remains to be determined, because the present data set is too small to draw further conclusions.

Quantitation is generally difficult for complex lipid mixtures. Although the major factor that determines ionization efficiencies in positive or negative ion mode is the compound class (for polar lipids, the nature of the head group), the length and degree of saturation of the acyl chains also play a role. An example of the challenge has been provided by Brügger et al. (3), who used four different, well-characterized internal standards to correct for acyl group chain length variations in a precursor ion scan for PCs. Relative quantitation of aminophospholipids can be achieved by isotope tagging (26). Although the accuracy of relative abundance determinations is sufficient for the characterizations and comparisons described in this work, the estimations were performed only semiquantitatively, in that the data set used for the evaluation and the sample characterization is small, only external standards were used, and no correction for the bound fatty acids was performed. According to Han and Gross (5), in this context, the unequal response is a minor issue. Although internal standards were evaluated, they were not used in the final analysis because the deuterated compound was not sufficiently homogeneous, and the PE species we had considered as a candidate for the internal standard might have interfered with compounds to be identified. For general use, carrying out comparative runs with and without internal standards would likely be too time-consuming. If so, internal standards that may be used to achieve more quantitative results include monoacylglycerols, which can be used in both gradients on the normal-phase column, or other, nonnatural PEs, which are likewise efficiently ionized in both ion modes. The quantitation as outlined here needs to be optimized to compare its potential against existing methods, especially also by the use of more automated instrumentation.

As an additional note, we observed that the effect of varying the cone voltage depends on the specific sample cone supplied by the manufacturer of the mass spectrometer: different results were obtained for sample cones that could not be distinguished from one another by visual examination. In some cases, similar in-source fragmentation was observed with cone voltages 20 V lower (absolute value) than reported here.

The LC-MS methodology presented provides a fairly robust and technically simple method for the investigation

of complex lipid mixtures. The method described here for quantitation via external standards allows for a semiquantitative characterization of the total composition and can, when necessary, be improved by an appropriate choice of internal standards. The methodology should be easily adaptable for higher throughput and sensitivity using more sophisticated LC and MS instrumentation than we used for this initial approach.

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