# Proteomics and lipids of lipoproteins isolated at low salt concentrations in D$_2$O/sucrose or in KBr

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**Abstract** There is much interest in the significance of apolipoproteins and proteins that are noncovalently associated with lipoproteins. It is possible that the high ionic strength used for isolation of lipoproteins with KBr and NaI could alter the pattern of associated exchangeable proteins. Here we describe lipoprotein classes fractionation from up to 0.5 ml of serum or plasma with buffers of physiological ionic strength and pH prepared with deuterium oxide (D$_2$O) and sucrose. An advantage of the D$_2$O/sucrose procedure was that the lipoproteins could be directly analyzed by the techniques described without need for desalting. We compared the isolated lipoproteins with those obtained using ultracentrifugation in KBr from the same plasma pool. Electrophoretic homogeneity of the lipoproteins was very similar using the two methods, as well as their lipid composition evaluated by HPLC. Two-dimensional electrophoresis and surface-enhanced laser adsorption/ionization time-of-flight mass spectrometry indicated that the patterns of exchangeable proteins of VLDL isolated using with the two procedures were very similar. However, significant differences were found in the profiles of LDL and HDL, indicating that the D$_2$O/sucrose method allowed a more complete characterization of its exchangeable apolipoproteins and proteins.—Ståhlman, M., P. Davidsson, I. Kanmert, B. Rosengren, J. Borén, B. Fagerberg, and G. Camejo. **Proteomics and lipids of lipoproteins isolated at low salt concentrations in D$_2$O/sucrose or in KBr.** *J. Lipid Res.* 2008. **49:** 481–490.

Many of the established metabolic functions and interactions of the plasma lipoproteins are dependent on the complement of nonexchangeable and exchangeable apolipoproteins that reside mostly on the surface of the particles (1). Apolipoprotein B-100 (apoB-100) and apoB-48 can be considered structural and nonexchangeable, but the other major apolipoproteins (apoA-I, apoA-II, apoC-I, apoC-II, apoC-III, apoA-IV, apoD, and apoE) can be exchanged between lipoproteins and probably with cell membranes. The association of the exchangeable apolipoproteins with the surface of the micro-emulsion that constitutes the lipid moiety of the particles appears to be mediated by the secondary and tertiary organization of the apolipoproteins. The main motifs responsible are amphipathic α-helices present in all major apolipoproteins (2). In such associations, nonpolar and ionic interactions are involved, and the use of fractionation procedures with solutions of high ionic strength may cause, in the isolated particles, a distribution of exchangeable lipoproteins different from that existing in blood, plasma, or serum. In addition to the classic apolipoproteins, all lipoprotein classes also carry numerous other proteins that are enzymes and exchangers involved in their own metabolism as lipoprotein lipase, phospholipases, cholesterol ester transport protein, and phospholipid exchange proteins. Furthermore, proteins of the acute-phase response and the immune system are also partially associated with circulating lipoproteins (3, 4).

It is believed that several of the exchangeable proteins on the lipoprotein surface are associated with the atherogenic properties of LDL and with the anti-atherosclerotic effects of HDL (3–5). Immunological evaluations of apoC-III and apoE in individual lipoprotein classes showed that these measurements were more specific in terms of evaluation of cardiovascular risk than are conventional lipid and lipoprotein measurements (6). A high content of apoC-III in LDL is of special interest, because subjects with this phenotype have a remarkable increase in the risk for cardiovascular disease (7). However, evaluation of only selected exchangeable proteins associated with lipoprotein classes can miss important changes involving several other accompanying proteins and thus introduce a bias in our efforts to uncover the profiles associated with cardiovascular disease. Efforts to quantitatively and qualitatively evaluate most of the exchangeable proteins of given lipo-

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proteins are gathering momentum, thanks to developments in the field of proteomics. Recently, our laboratory found, using this approach, that the small, dense LDL of patients with the metabolic syndrome and peripheral atherosclerosis has a significant increase in all the apoC-III isoforms and a decrease in apoA-I, the apoC-I isoforms, and apoE when compared with small, dense LDL of matched controls (8). For the fractionation of LDL, we used buffers prepared with $D_2O$ and physiological NaCl that allowed direct surface-enhanced laser adsorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) procedures as well as electrophoresis without need for desalting (8, 9).

Proteomics techniques recently applied to HDL and to the $HDL_3$ subclass of a group of coronary artery disease patients and controls showed significant differences in the distribution of conventional apolipoproteins, acute-phase response proteins, proteinase inhibitors, and members of the complement activation (10). These promising initial efforts indicate that much can be gained by studying with unbiased techniques the role of lipoproteins as carriers of exchangeable proteins and its association with cardiovascular disease. Here we described how, with the aid of sucrose, the $D_2O$-based method using buffers with physiological ionic strength could be extended into the HDL density range, therefore providing a favorable alternative to the use of high concentrations of neutral salts. Details are given for lipoprotein fractionation of up to 0.5 ml plasma or serum using a tabletop ultracentrifuge. We compared the lipid composition, homogeneity, and complement of exchangeable proteins evaluated by two-dimensional electrophoresis (2-DE) and SELDI-TOF-MS of VLDL, LDL, and HDL obtained with the $D_2O$/sucrose procedure and those obtained with KBr-based fractionation.

## MATERIALS AND METHODS

### Plasma and serum samples

To compare the results from the $D_2O$/sucrose and KBr procedures, a single EDTA plasma pool from five normolipidemic healthy donors was used. In addition, individual sera from normolipidemic and dyslipidemic subjects were also used. Plasma and serum samples were stored as soon as obtained at $-80°C$ and thawed only once prior to fractionation.

### Chemicals

Deuterium oxide 99.9% D, $d_{20°C}$ 1.12 g/ml, sucrose 99.5% Sigma Ultra, KBr Sigma Ultra, $Na_2$-EDTA, and HEPES were from Aldrich-Sigma. Agarose Ultra Pure, Gel-Bond films, ready polyacrylamide gels, reagents, and chambers for electrophoresis were from Invitrogen. Solvents for lipid extraction and HPLC were from Rathburn (Walkenbur, Scotland).

### Ultracentrifugal procedures

The optima TLX centrifuge, TLA 120.2 rotor, polycarbonate tubes (11 × 34 mm), and the CentriTube slicer were from Beckman Coulter (Palo Alto, CA). Density measurements were made by gravimetry using volumes calibrated with $H_2O$ at $20.0 ± 0.2°C$. All the solutions were used within 2 days of preparation and maintained in glass containers with Teflon-lined caps.

Three stock solutions were prepared and their density checked for the fractionation with $D_2O$/sucrose. Buffer A contained 140 mM NaCl, 10 mM HEPES, 10 μM EDTA, and 10 μM butylated hydroxyl toluene in deionized ultrapure water, and it was buffered to pH 7.0, final $d_{20°C} = 1.006 ± 0.005$ g/ml. Buffer B contained the same composition as buffer A but was prepared in $D_2O$, $d_{20°C} = 1.126 ± 0.006$ g/ml. Buffer C contained 50.0% (w/w) sucrose in buffer B, $d_{20°C} = 1.325 ± 0.010$ g/ml. The exact density of these solutions may vary, and this appears to be caused by the hygroscopic nature of the sucrose. This is not a problem, because the measured density value is inserted into a spreadsheet and the volumes required to achieve the desired densities and final volumes are calculated automatically, once the simple equations are inserted into the respective columns. We use the equation Vh = Vi(Dr-Di/Dh-Dr), where Vh is the volume to add of the densest solution (buffer C), Vi is the volume of the initial less-dense solution, Dr is the density desired, Di is the density of the initial solution of the initial less dense solution, and Dh is the density of the densest solution (buffer C). **Table 1** presents an example using as the heaviest solution 50% (w/w) sucrose in buffer B ($d_{20°C} = 1.325$ g/ml) and starting with 0.5 ml plasma or serum. A similar scheme was used for the fractionation with KBr, using as the densest solution 35% (w/w) KBr prepared in buffer A, $d_{20°C} = 1.325$ g/ml. **Table 2** gives the volumes required to prepare small amounts of solutions of density 1.019, 1.063, and 1.210 g/ml required to complete the samples to 1.00 ml.

The fraction with density less than 1.019 g/ml (here called VLDL) was obtained after a 2.5 h ultracentrifugation at 118,000 rpm (495,000 g, $r_{avg}$) at 30°C. The tube slicer was calibrated in order to recover 0.5 ml of the floated fraction, with 0.5 ml of the bottom fraction remaining. Careful withdrawing of the fractions with a Pasteur pipette recovered routinely more than 0.47 ml. The volumes recovered were evaluated by gravimetry, taking into account their densities, to calculate exact volumes, and the bottom fraction was adjusted to 0.5 ml with solution d = 1.019 g/ml before raising the density to 1.063 g/ml and completing to 1.00 ml. The fraction 1.019–1.063 g/ml (LDL) was obtained after centrifugation at 118,000 rpm for 3 h at 30°C. After separation of the upper fraction containing LDL, 0.4 ml of the recovered fraction with d > 1.063 was adjusted to density 1.210 g/ml, completed to 1.0 ml, and centrifuged at 118,000 rpm for 15 h at 30°C. The fraction with density 1.063–1.210 g/ml (HDL) was recovered by slicing the upper 0.3 ml of the tubes. For fractionation of the lipoproteins using KBr, the conditions described above were also used.

### SELDI-TOF-MS of exchangeable apolipoproteins in VLDL, LDL, and HDL

Lipoprotein samples obtained with the $D_2O$/sucrose procedure were directly profiled in duplicates as described (8). Those

TABLE 1. Example of additions to adjust densities in a run with buffers prepared with $D_2O$ and sucrose starting with 0.500 ml of plasma or serum

| Vi (Vol Bottom Fraction) | Dr | Di (Bottom Fraction) | Dh (Buffer C) | Vh (Buffer C) | Vol to 1.00 ml (Equal Density) |
|---|---|---|---|---|---|
| *ml* | | | | *g/ml* | |
| 0.500 | 1.019 | 1.006 | 1.325 | 0.021 | 0.479 |
| 0.500 | 1.063 | 1.019 | 1.325 | 0.084 | 0.416 |
| 0.400 | 1.210 | 1.063 | 1.325 | 0.511 | 0.089 |

Dh, density of the densest solution; Dr, density desired; Vh, volume to add of the densest solution; Vi, volume of the initial less-dense solution.

TABLE 2. Example of additions required to prepare solutions of density 1.019, 1.063, and 1.210 g/ml using buffer A and the heavy $D_2O$/sucrose buffer C

| Vol (Buffer A, d = 1.006) | Dr | Di (Buffer A) | Dh (Buffer C) | Vh (Buffer C) |
|---|---|---|---|---|
| 10.0 | 1.019 | 1.006 | 1.325 | 0.425 |
| 10.0 | 1.063 | 1.006 | 1.325 | 2.176 |
| 10.0 | 1.210 | 1.006 | 1.325 | 17.739 |

obtained with KBr were previously desalted by dialysis. In brief, the samples were analyzed on the anionic protein chip array Q10, and protein chip arrays were equilibrated with 100 mM Tris-HCl, pH 9.0, using a Biomek Laboratory workstation (Beckman Coulter) modified to make use of a protein chip array bio processor (Ciphergen Biosystems). All the lipoprotein samples were diluted 1:1.5 (v/v) with a urea buffer (9 M urea, 2% CHAPS, 50 mM Tris-HCL, pH 9). A volume of 10 μl of each lipoprotein sample was mixed with 90 μl binding buffer, and the mixture was added to the protein chip surfaces and incubated for 30 min. A saturated solution of sinapinic acid (Aldrich Chemical; Milwaukee, WI) diluted 1:2 (v/v) with 50% acetonitrile containing 0.5% trifluoroacetic acid was used as matrix. The arrays were subsequently read in a protein chip reader system (PBS II; Ciphergen Biosystems). Data handling was performed using Ciphergen Express (version 3.0.6; Ciphergen Biosystems).

### Identification of proteins representing specific *m/z* peaks

Aliquots of LDL/HDL/VLDL fractions were pooled and concentrated by vacuum centrifugation, dissolved in 200 μl NuPAGE sample buffer (0.14 M Tris, 0.10 M Tris-HCL, 0.4 mM EDTA, pH 8.5, containing 10% glycerol, 2% LDL, and 3% DTT), boiled for 3 min, and then separated by the NuPAGE system (Novex precast gels; San Diego, CA) using 4–12% Bis-Tris gels (1/well). The NuPAGE MES buffer system (1 M MES, 1 M Tris, 69 mM SDS, 20 mM EDTA) was used as running buffer. The mini whole-gel eluter (Bio-Rad; Hercules, CA) was used for electro-elution following the manufacturer's instructions. An elution buffer (25 mM histidine, 30 mM MOPS, pH 6.5) was used, and the elution was performed at 100 mA for 30 min. Fourteen fractions of approximately 0.5 ml were harvested, and aliquots of 250 μl/fraction were concentrated and analyzed by the NUPAGE system, followed by SYPRO Ruby™ (Molecular Probes; Eugene, OR) staining for subsequent identification of protein bands with MS. The remaining part of the gel eluter fractions was mixed with ice-cold ethanol in 1:4 (v/v) ratios, precipitated

at −20°C for 2 h, centrifuged at 10,000 g for 10 min at 4°C, dissolved in 10 μl of 25 mM $NH_4HCO_3$, and then analyzed on NP20 protein chip arrays in order to follow the purification strategy by SELDI analysis.

To identify bands or spots from 1-D or 2-D gels, they were punched out and then digested by sequencing grade-modified trypsin (Promega; Madison, WI), and the digests were analyzed by MS. Analysis was performed on a 4700 Proteomics Analyser MALDI-TOF/TOF mass spectrometer (Applied Biosystems; Framingham, MA) in reflector mode. MS and tandem mass spectrometry (MS/MS) data analysis was performed using the GPS Explorer™ software (Applied Biosystems), which utilizes the Mascot peptide mass fingerprinting and MS/MS ion search software (Matrix Sciences; London, UK). Identification was considered positive at a confidence level of 95%. The search method used database information from in-house Protein Data Bank, Protein Information Resource, SwissProt and TREMBL databases searching mouse/rat/human sequences.

### HPLC of lipoprotein lipid classes and lipid/protein composition

Aliquots of the lipoprotein fractions were extracted using the procedure of Folch, Lees, and Sloan Stanley (11) and analyzed by HPLC in a Dionex ternary pump (P680) and a Chromeleon-Dionex chromatography data system equipped with a PL-ELS 1000 light-scattering detector (Polymer Laboratories; Shropshire, UK). The system was calibrated with external standards of each lipid class prepared by Larodan Fine Chemicals (Malmö, Sweden). The ternary gradient was essentially that described by Homan and Anderson (12) but with *n*-heptane-tetrahydrofuran (99:1; v/v) as solvent 1.

To evaluate the percentage composition of isolated lipoproteins, aliquots with similar amounts of protein measured colorimetrically (8) were lipid extracted using the method of Folch, Lees, and Sloan Stanley (11), and the chloroform phase was dried under $N_2$ and redissolved in 200 μl of hexane-isopropanol (1:2; v/v). Aliquots of 50 μl were placed in tared aluminum pans, and the solvent was evaporated under $N_2$ while heating them at 80°C. The weight of dried lipids was measured in a microbalance with a ±0.01 mg resolution.

### One-dimensional gel electrophoresis

Agarose electrophoresis of the isolated lipoproteins was carried out as described using a "submarine" procedure (9). One-dimensional PAGE in native conditions and in SDS buffers was performed in Novex™ (Invitrogen) 3–8%, 4–12%, and 4–20% Tris-glycine gradients according to the manufacturer's instruc-

TABLE 3. Percentage protein content and number of peaks (SELDI-TOF-MS) measured in lipoprotein fractions isolated from six samples of the same plasma pool using ultracentrifugation in KBr and $D_2O$/sucrose buffers

| Method | VLDL | | LDL | | HDL | |
|---|---|---|---|---|---|---|
| | Protein | Peaks | Protein | Peaks | Protein | Peaks |
| | % | No. | % | No. | % | No. |
| KBr | 10.60 ± 1.03 | 29 (14%) | 19.40 ± 0.79 | 33 (6%) | 47.58 ± 0.72 | 35 (13%) |
| $D_2O$/Sucrose | 10.08 ± 0.98 (ns) | 29 (11%) (ns) | 22.60 ± 1.00[a] | 42 (12%)[a] | 50.04 ± 0.77[a] | 49 (9%)[a] |

Protein percent content was calculated by adding the protein values measured spectrophotometrically to those of total lipids evaluated gravimetrically in lipid extracts: (prot/prot+lipids)100. The values are expressed as means ± SEM. The number of peaks was measured in the *m/z* range 2,000 to 10,000 of the SELDI-TOF-MS profiles. The figures in parentheses are the percent coefficient of variation for the number of peaks: (SD/mean)100. ns, not significant.

[a] $P < 0.05$, significance of differences between corresponding lipoproteins isolated using the two procedures.

tions. The lipoprotein fractions obtained with the $D_2O$/sucrose and KBr procedure were desalted and equilibrated in buffer A with the aid of Illustra™ micro-spin sephadex G-25 columns (GE Healthcare; Uppsala, Sweden) before electrophoresis.

## 2-DE of proteins in LDL and HDL

LDL and HDL fractions containing 400 μg of protein from KBr and $D_2O$/sucrose ultracentrifugation were precipitated using the Universal Protein Precipitation Agent (UPPA) kit (Gene Technology, Inc.; St Louis, MO) according to the manufacturer's instructions. The air-dried pellets were dissolved in 20 μl of a buffer containing 2.5% SDS and 2.3% DTT, denatured at 95°C for 3 min, and left to dissolve for 1 h prior to addition of Destreak solution (GE Healthcare). 2-DE was performed using IPGphor (Amersham Biosciences) and Protean Plus Dodeca cell (Bio-Rad). Samples were applied by in-gel rehydration for 12 h in pH 3–11 non-linear Immobiline (TM) DryStrips (24 cm; GE Healthcare). The proteins were then focused at 50,000 Vh at maximum voltage of 8,000 V. The second dimension was carried out using precast Optigel 10–20% gradient Tris-HCL (NextGen Sciences; Alconbury, UK) with Tris-glycine-SDS (24 mM Tris base, 0.2 M glycine, and 0.1% SDS) running at 60 mA for each gel at a maximum of 600 V and 300 W, at 15°C for about 6 h. The gels were stained with SYPRO Ruby™ according to the supplier's protocols. Image acquisition and analysis were performed using a Molecular Imager FX System (Bio-Rad) with a 532 nm laser.



**Fig. 2.** Native PAGE in a 4–12% acrylamide gradient gel of HDL isolated with the $D_2O$/sucrose method from serum samples from two hypercholesterolemic subjects (HC1, HC2), two subjects with the combined dyslipidemia of insulin resistance (IR1, IR2), and two apparently healthy subjects (C1, C2). The gels were loaded with 20 μg HDL protein per well and stained with Coomasie Brilliant Blue.

## RESULTS AND DISCUSSION

### Ultracentrifugation

Plasma lipoprotein fractionation in KBr solutions with the use of a tabletop ultracentrifuge and the rotor TL-100.2 (Beckman Coulter) at 405,000 $g_{av}$ has been described previously (13, 14). We introduced several modifications to the described procedure in order to adapt the fracti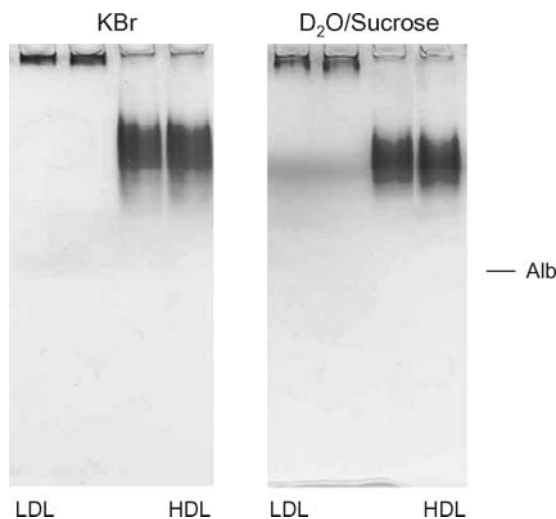onation to the use of $D_2O$/sucrose solutions. One drawback of using sucrose instead of KBr for lipoprotein fractionation is that the viscosity is higher than that of KBr solutions of equivalent densities. This obviously increases the time required to float or sediment macromolecules through a given distance. Thus, we used the newer rotor TL-120.2 at a higher relative centrifugal force ($g_{av}$), 496,000, and increased the temperature of the run to 30°C. This reduced the viscosity of 46% (w/w) $D_2O$/sucrose solutions



**Fig. 1.** Native PAGE in a 4–20% acrylamide gradient gel of LDL and HDL lipoproteins isolated with the KBr or the $D_2O$/sucrose ultracentrifugal procedures from the same plasma pool. The gels were loaded with 20 μg of LDL or HDL protein per well and stained with Coomasie Brilliant Blue. Alb, albumin.
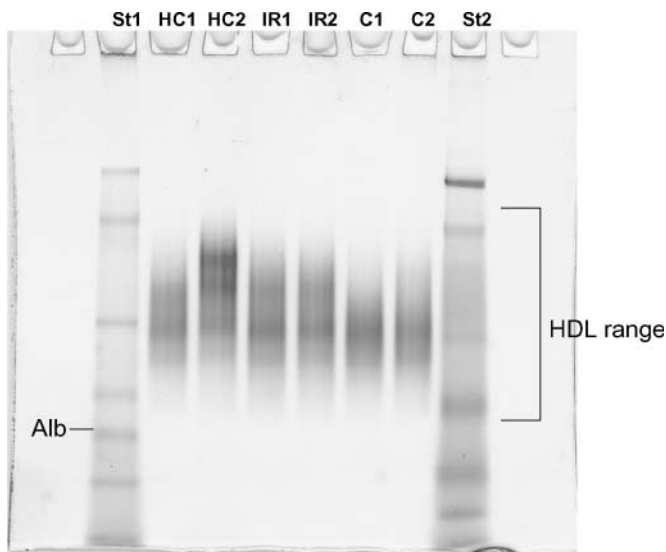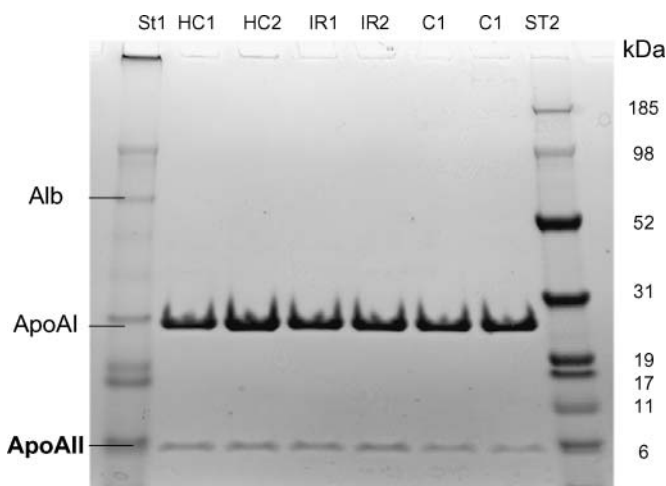


**Fig. 3.** SDS-PAGE in a 4–20% acrylamide gel of HDL isolated with the $D_2O$/sucrose method from serum samples from two hypercholesterolemic subjects (HC1, HC2), two subjects with combined dyslipidemia (IR1, IR2), and two apparently healthy subjects (C1, C2). The gels were loaded with 20 μg HDL protein per well and stained with Coomasie Brilliant Blue.

($d_{20°C} = 1.21$) from 11.3 mPA.s to 7.1. At this temperature, 12 h at 118,000 rpm were sufficient to float HDL though the short pathway of the tubes used. Lipoproteins are currently fractionated with several types of rotors. For density gradients and in 44 h-long runs at $g_{av}$ of 288,000, the SW41Ti (Beckman Coulter) is commonly recommended (14). At present, there is a trend toward the use of shorter runs with rotors such as the TLA-120.2 and the NVT90 (Beckman Coulter) that can use smaller samples and that are run at higher $g_{av}$, 465,000 and 645,000, respectively (15). High ultracentrifugal fields applied for long periods ($g_{av} \times$ time) can strip exchangeable proteins from lipoproteins. Thus, the selection of time and speed used represents a balance between the need to reach acceptable homogeneity of the fractions, within a reasonable time, without excessive depletion of exchangeable apolipoproteins, as discussed by Fless (16). Thus, each lipoprotein class represents an operational definition whose detailed composition depends on density ranges, but also on the conditions used for ultracentrifugal separation. In the present work, we are comparing fractions subjected to the same $g_{av} \times$ time but in solvents of different ionic strength and viscosity.

To compare the effects of the KBr and $D_2O$/sucrose procedures on the general composition of the isolated lipoproteins, we measured the total lipid and protein content from fractions obtained from the same plasma pool using the two procedures. **Table 3** presents results of this evaluation. There were no differences in the composition of VLDL obtained with the two methods. On the other hand, the LDL and HDL prepared in KBr have a slightly lower percentage protein content, indicating that in the $D_2O$ buffers, the lipoproteins retain a higher content of exchangeable lipoproteins. The above results are in line with the results of the SELDI-TOF-MS analyses discussed below.
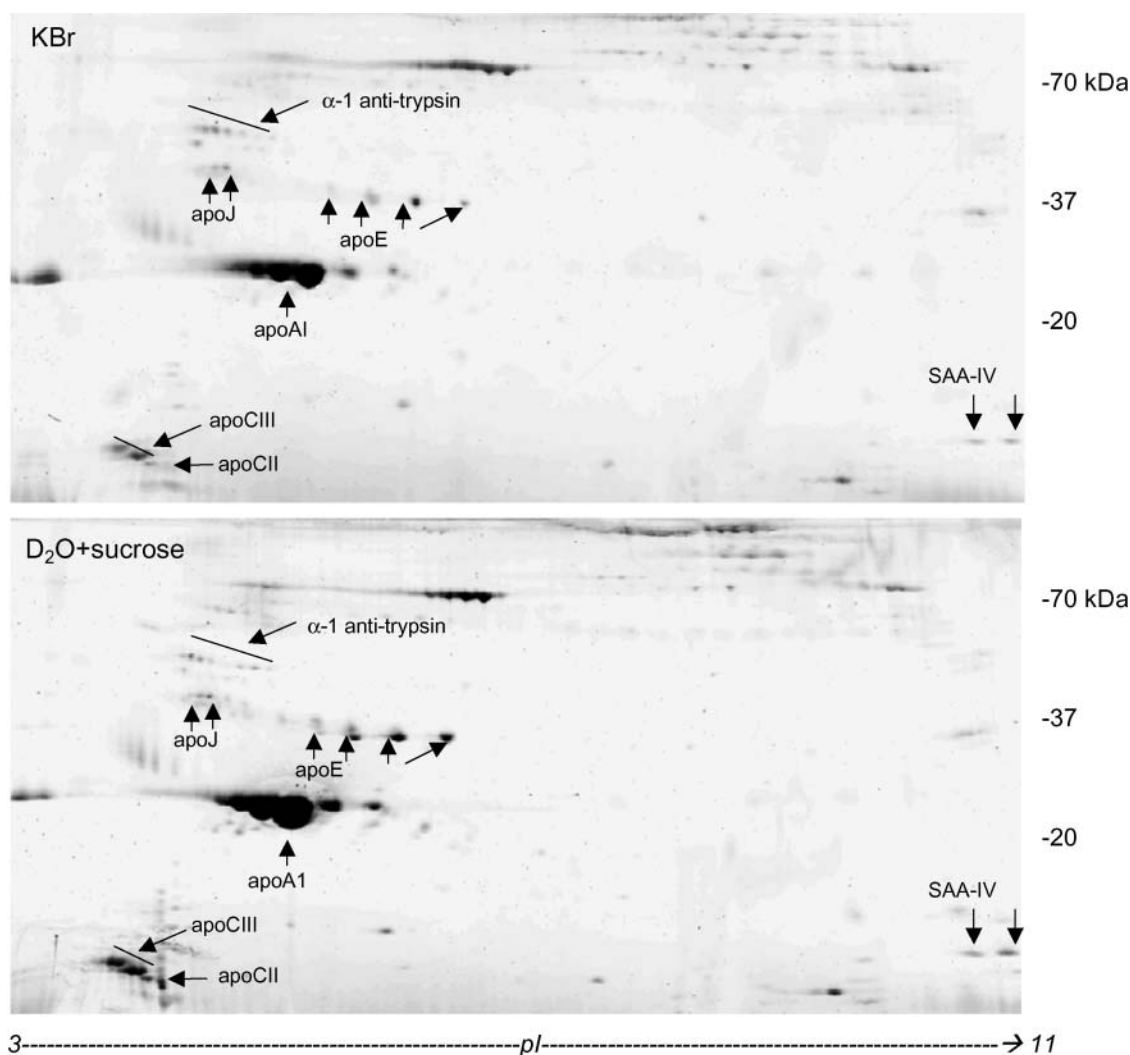


**Fig. 4.** Two-dimensional electrophoresis (2-DE) of associated proteins from LDL isolated from the same plasma pool using KBr and $D_2O$/sucrose ultracentrifugation. The same amounts of LDL protein (400 μg) were loaded in the gels. The most prominent identified spots are indicated. ApoB-100 does not enter this gel (details in the Materials and Methods section). Apo, apolipoprotein.

### Homogeneity of the isolated lipoproteins

There were no differences in the agarose electrophoretic patterns of VLDL, LDL, and HDL obtained with the two procedures (not shown), and native PAGE in 4–20% gradients illustrates the homogeneous bands for LDL and HDL isolated from the same plasma pool, with no visible evidence of albumin contamination when 20 μg of lipoprotein protein was loaded in the gels (**Fig. 1**). The $D_2O$/sucrose method was also applied to serum samples from two subjects with type 2a hypercholesterolemia, two normolipidemic subjects, and two subjects with combined dyslipidemia. **Figure 2**, showing a 4–12% native PAGE, indicates that the HDL from all six subjects was contaminated with neither LDL nor albumin. These results were confirmed by SDS-PAGE in 4–20% gradients of the HDL from the same subjects (**Fig. 3**). No band corresponding to albumin was detectable loading 20 μg of HDL protein in each gel well.

### 2-DE of proteins in VLDL, LDL, and HDL

The 2-DE patterns for VLDL-associated proteins obtained from the same plasma pool with KBr and $D_2O$/sucrose were almost identical (not shown). For LDL, the maps were qualitatively similar, but differences in the intensity of some of the identified spots were visible in gels loaded with the same amount of LDL protein (**Fig. 4**). The LDL isolated with $D_2O$/sucrose showed more-intense spots, corresponding to α-1 anti-trypsin isoforms, apoE isoforms, and apoC-II and apoC-III isoforms, but less-intense spots of an unidentified acidic protein of ~25 kDa. Surprisingly, the two-dimensional patterns of proteins associated with HDL that were isolated using the
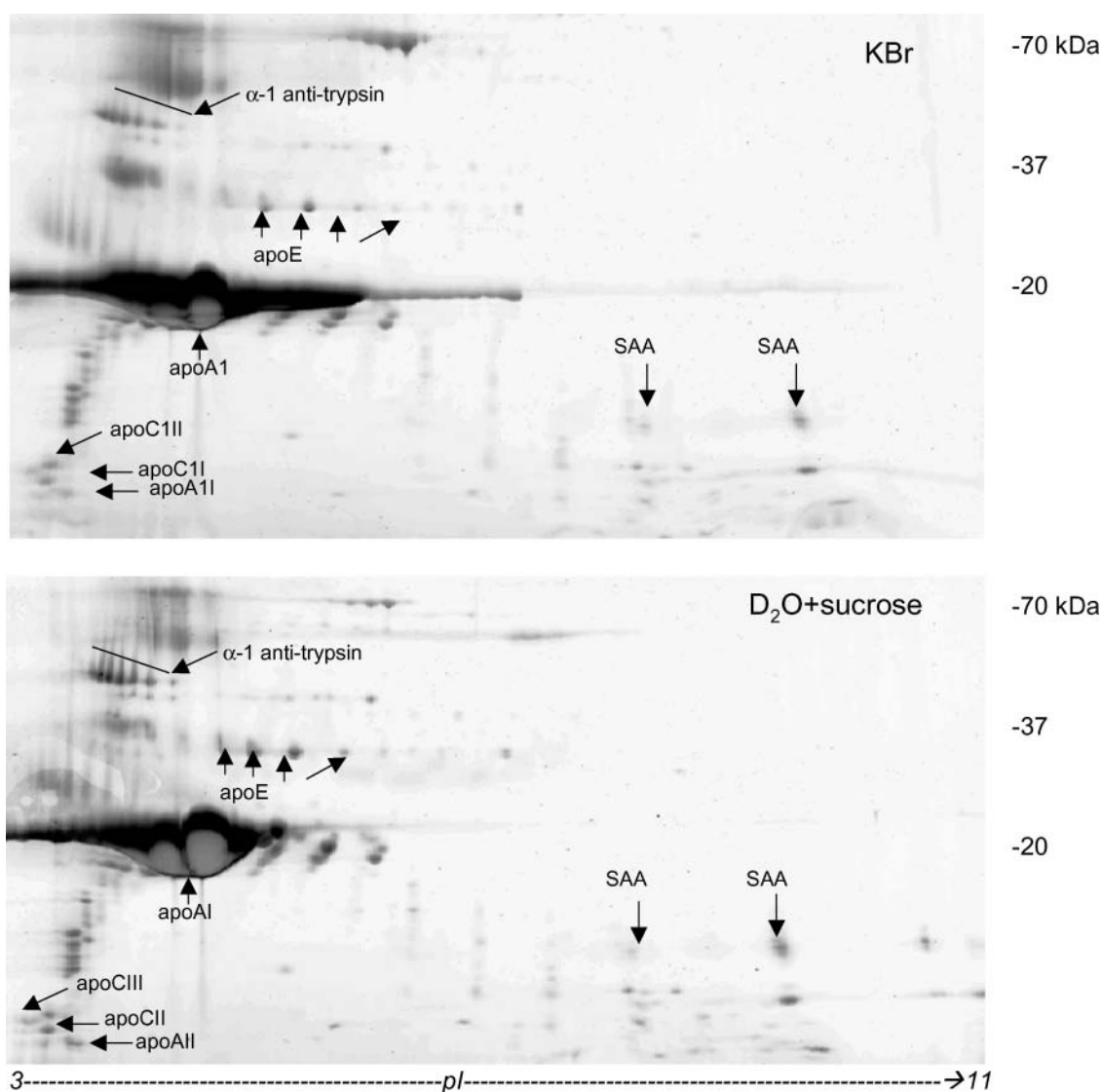


**Fig. 5.** DE2- of associated proteins from HDL isolated from the same plasma pool using KBr and $D_2O$/sucrose ultracentrifugation. The same amounts of HDL protein (400 μg) were loaded in the gels. The most prominent identified spots are indicated (details in the Materials and Methods section).

two methods showed fewer differences than those between LDL obtained from the same plasma pool with the two procedures (**Fig. 5**). The total number of stainable spots measured in duplicate gels was higher in the LDL isolated with $D_2O$ (81) than that in the images from LDL isolated with KBr (66). Also, in the HDL fractions, the number of spots was significantly higher in the $D_2O$ samples (108) than in the KBr-isolated samples (90).

### SELDI-TOF-MS of lipoprotein proteins

Several of the proteins associated with the lipoprotein particles were identified as isoforms of apoC-I (with a molecular mass of 6,430 and 6,630 Da), apoC-II+ apoC-III (with a molecular mass of = 8,920 Da), and isoforms of apoC-III (with a molecular mass of 8,920, 9,120, 9,420 and 9,720 Da). The two largest proteins, apoA-I (molecular mass = 28,100 Da) and SAA-IV (with $m/z$ = 12,890)

were identified using a combination of 1-D gel electrophoresis, gel elution, and MS/MS. The protein identities were also validated by immunoblotting. The profiles for the 2–20 kDa range of VLDL-associated proteins from individual samples from the same plasma pool obtained with KBr and the $D_2O$/sucrose procedures were indistinguishable and are not shown. This was expected, because the ionic strength of the background solution for isolation of VLDL is not very different in the two isolation procedures, ~0.08 M for the $D_2O$/sucrose buffer and 0.16 M for the NaCl-KBr buffer. For isolation of LDL, the differences in ionic strength become considerable between the two procedures, 0.08 M for the $D_2O$/sucrose buffer and 0.37 M for the KBr buffer. The SELDI-TOF-MS profiles of LDL samples obtained from the same plasma pool using the two procedures show significant differences in this lipoprotein class. Shown in **Fig. 6** are the profiles
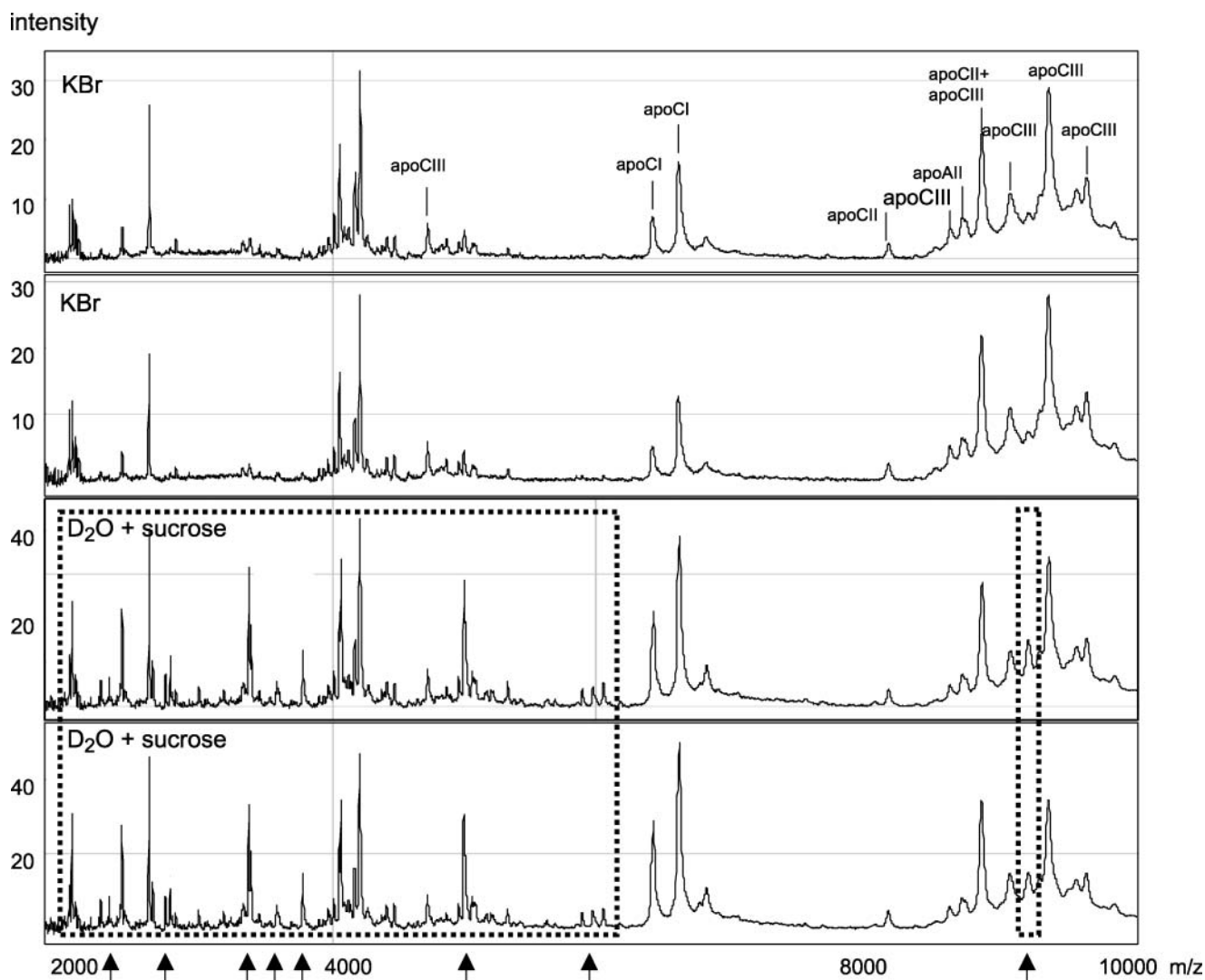


**Fig. 6.** Surface-enhanced laser adsorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) of proteins in the 2,000–10,000 $m/z$ range associated with LDL. The results are from two independent analyses of LDL fractionated from the same plasma pool by ultracentrifugation using KBr or $D_2O$/sucrose solutions to adjust densities. The dashed-line rectangles indicate molecular mass regions showing significant differences between LDL fractions isolated using the two methods.

for two individual samples obtained with KBr and two samples obtained with the $D_2O$/sucrose method using the same plasma pool as starting material. The profiles can be compared directly, because equal amounts of LDL protein were bound to the chips. The associated proteins from the LDL isolated with $D_2O$/sucrose show a more complex array below the 6,000 $m/z$ region than do the proteins from the LDL isolated with the KBr method. Also, in the region occupied by the apoC-II and apoC-III isoforms, the $D_2O$/sucrose fraction showed higher levels of these proteins (indicated by the dashed-line rectangles, Fig. 6). These results are in line with those obtained with the 2-DE (Fig. 5). The SELDI-TOF-MS results from the 2,000 to 10,000 $m/z$ region for two individual HDL lipoprotein samples obtained with the KBr method and two

samples of HDL obtained with the $D_2O$/sucrose scheme are shown in **Fig. 7**. In the HDL class, the isolation methods yield fractions with some quantitative differences in this mass range that surprisingly were less marked than those shown by LDL in the same region (Fig. 6). Here apoC-III$_2$ was more prominent in the HDL isolated with KBr (Fig. 7, dashed line rectangle), indicating that the ionic strength of the background solution used for flotation of the particles cannot be the only explanation for the observed dissimilarities in the associated proteins in the lipoprotein classes isolated by the two procedures. We have compared the number of peaks detected in the $m/z$ range 2,000–10,000 in the lipoproteins isolated using the two methods from six individual samples of the same plasma pool. Table 3 shows that in terms of this
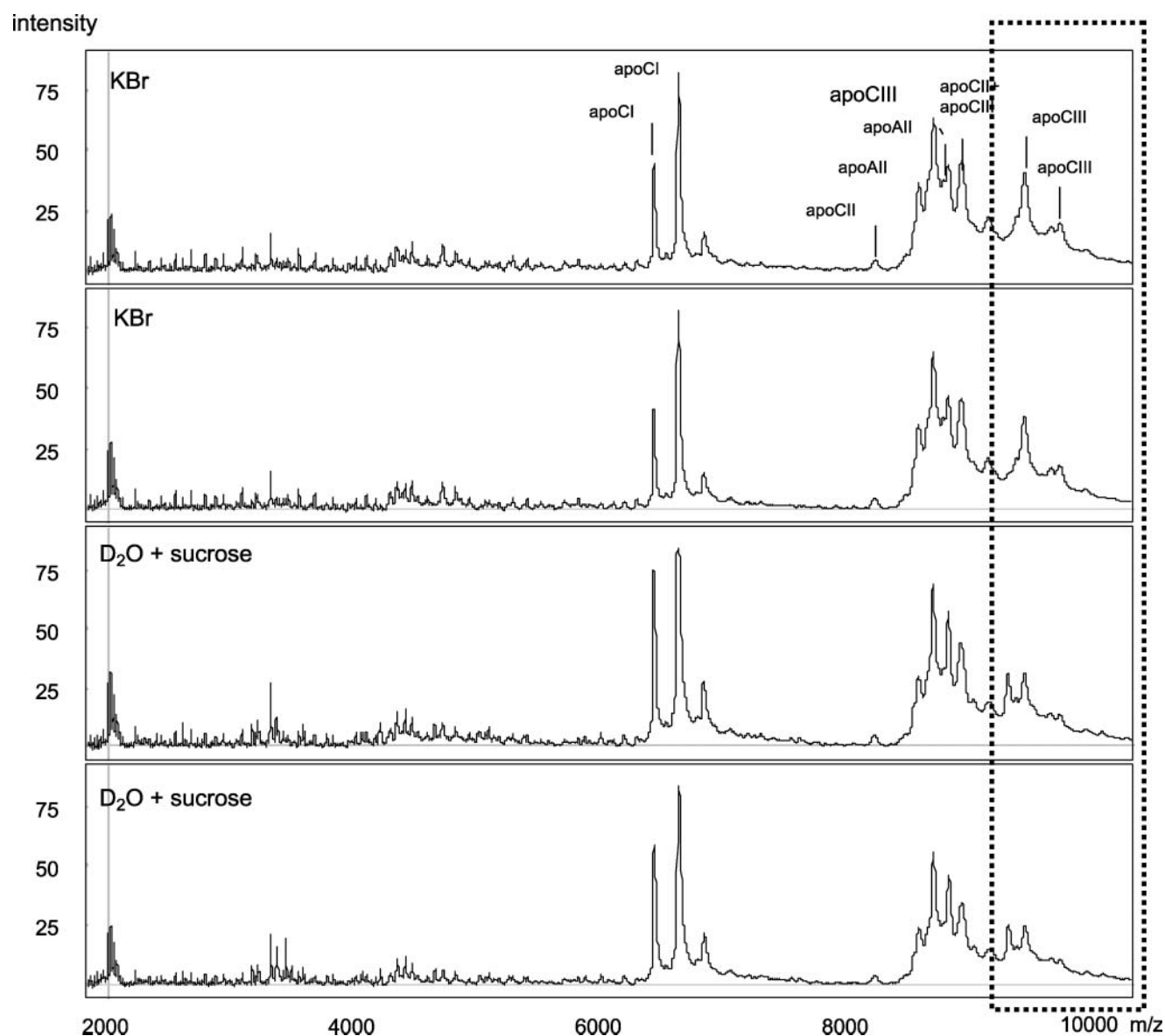


**Fig. 7.** SELDI-TOF-MS of proteins in the 2,000–10,000 $m/z$ range associated with HDL. The results are from two independent analyses of HDL fractionated from the same plasma pool using ultracentrifugation in KBr or $D_2O$/sucrose. The dashed-line rectangle indicates molecular mass regions showing significant differences between HDL fractions isolated with the two methods.

parameter, together with the percent coefficient of variation for the number of peaks, the largest difference resides in the LDL fraction, followed by HDL, whereas as in the 2-D gels, the number of components in VLDL was the same.

## Lipids of lipoproteins obtained with the KBr and $D_2O$/sucrose methods

There was no difference in the percentage lipid composition of each lipoprotein class isolated using the two methods from the same plasma pool (**Fig. 8**). This confirmed that the two methods separate very similar density ranges of the lipoprotein spectrum. The lipid analyses indicated that in terms of cholesterol, the recovery, adding the content of each lipoprotein class, was 78 ± 5% (mean ± SEM) and 74 ± 4%, respectively, for the KBr and the $D_2O$/sucrose methods. Lipid analysis indicated that less than 1% of the total plasma lipids remained in the lipoprotein-free fraction with d > 1.210 g/ml from the two fractionation procedures.

## Conclusions

Our results indicate that fractionation of plasma or serum lipoproteins using $D_2O$/sucrose buffers of physiological ionic strength for adjusting the background density is particularly suited for qualitative and quantitative evaluation of their exchangeable apolipoproteins and associated proteins. The procedure described here can be used for fractionation of up to 0.5 ml of plasma or serum. Although deuterium oxide is more expensive than potassium bromide, it cost less than 10 U.S. dollars to fractionate 10 samples of plasma or serum using the tubes and rotor described. The low salt levels in the LDL and HDL, or their subclasses, isolated with $D_2O$/sucrose, are compatible with direct analyses using most electrophoretic, chromatographic, and MS procedures. Another advantage of the presence of sucrose in the LDL and HDL fractions is that it allows their storage frozen until further use, because sucrose improves the cryopreservation of the biological and physico-chemical integrity of plasma lipoproteins (17, 18). ▄▄
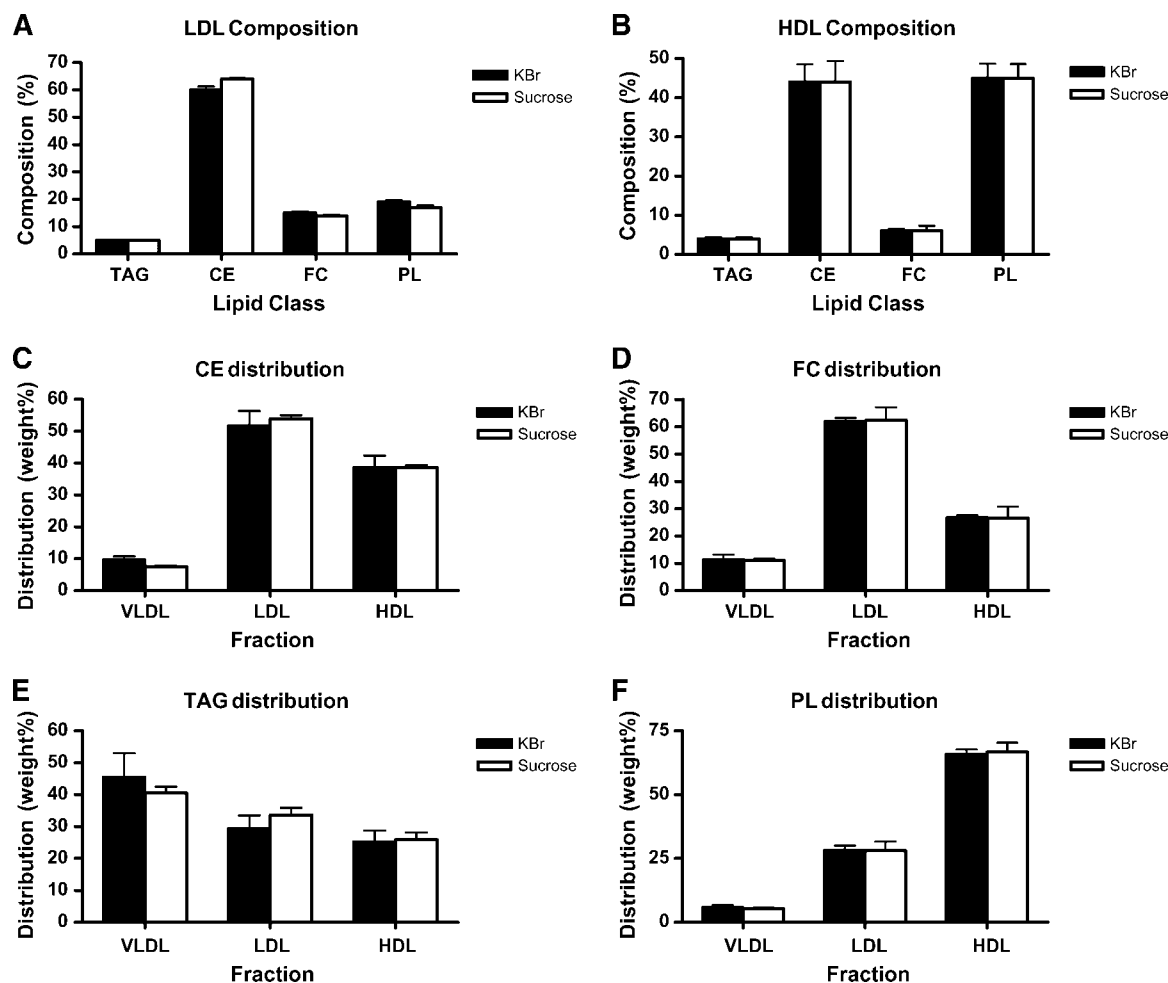


**Fig. 8.** Panels A and B show the percentage lipid composition of LDL and HDL isolated with the KBr (filled bars) and the $D_2O$/sucrose (empty bars) methods. Panels C–F show the percent weight distribution of cholesterol esters (CE), free cholesterol (FC), triacylglycerol (TAG), and phospholipids (PL) in each lipoprotein fraction. The error bars indicate the means ± SEM.

## REFERENCES

1. Jonas, A. 2002. Lipoprotein structure. *In* Biochemistry of Lipids, Lipoproteins and Membranes. D. E. Vance and J. E. Vance, editors. Elsevier, Amsterdam. 482–504.

2. Segrest, J., M. Jones, H. De Loof, C. Brouillete, Y. Venkatachalapathi, and G. Ananatharamaia. 1992. The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. *J. Lipid Res.* **33:** 141–166.

3. Rohrer, L., M. Hersberger, and A. von Eckardstein. 2004. High density lipoproteins at the intersection of diabetes mellitus, inflammation and cardiovascular disease. *Curr. Opin. Lipidol.* **15:** 269–278.

4. Kontush, A., and M. J. Chapman. 2006. Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. *Pharmacol. Rev.* **58:** 342–374.

5. Chait, A., C. Y. Han, J. F. Oram, and J. W. Heinecke. 2005. Thematic review series: the immune system and atherogenesis. Lipoprotein-associated inflammatory proteins: markers or mediators of cardiovascular disease? *J. Lipid Res.* **46:** 389–403.

6. Sacks, F. M., P. Alaupovic, L. A. Moye, T. G. Cole, B. Sussex, M. J. Stampfer, M. A. Pfeffer, and E. Braunwald. 2000. VLDL, apolipoproteins B, CIII, and E, and risk of recurrent coronary events in the Cholesterol and Recurrent Events (CARE) trial. *Circulation.* **102:** 1886–1892.

7. Lee, S-J., H. Campos, L. A. Moye, and F. M. Sacks. 2003. LDL containing apolipoprotein CIII is an independent risk factor for coronary events in diabetic patients. *Arterioscler. Thromb. Vasc. Biol.* **23:** 853–858.

8. Davidsson, P., J. Hulthe, B. Fagerberg, B-M. Olsson, C. Hallberg, B. Dahllof, and G. Camejo. 2005. A proteomic study of the apolipoproteins in LDL subclasses in patients with the metabolic syndrome and type 2 diabetes. *J. Lipid Res.* **46:** 1999–2006.

9. Hallberg, C., M. Håden, M. Bergstrom, G. Hansson, K. Pettersson, C. Westerlund, G. Bondkjers, A-M. Lindqvist-Östlund, and G. Camejo. 1994. Lipoprotein fractionation in deuterium oxide gradients: a procedure for evaluation of antioxidant binding and susceptibility to oxidation. *J. Lipid Res.* **35:** 1–9.

10. Vaisar, T., S. Pennathur, P. S. Green, S. A. Gharib, A. N. Hoofnagle, M. C. Cheung, J. Byun, S. Vuletic, S. Kassim, P. Singh, et al. 2007. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. *J. Clin. Invest.* **117:** 746–756.

11. Folch, J., M. Lees, and G. H. Sloan Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.

12. Homan, R., and M. K. Anderson. 1998. Rapid separation and quantitation of combined neutral and polar lipid classes by high-performance liquid chromatography and evaporative light-scattering mass detection. *J. Chromatogr. B Biomed. Appl.* **708:** 21–26.

13. Brousseau, T., V. Clavey, J. Bard, and J. Fruchart. 1993. Sequential ultracentrifugation micromethod for separation of serum lipoproteins and assays of lipids, apolipoproteins, and lipoprotein particles. *Clin. Chem.* **39:** 960–964.

14. Caslake, M., and C. Packard. 1997. The use of ultracentrifugation for the separation of lipoproteins. *In* Handbook of Lipoprotein Testing. N. Rifai, W. Russel, G. Russell Warnick, and M. Dominiczak, editors. American Association of Clinical Chemistry, Washington, D. C. 509–529.

15. Ordovas, J. 1998. Fast ultracentrifugation methods for the separation of plasma lipoproteins. *In* Methods in Molecular Biology-Lipoprotein Protocols. J. Ordovas, editor. Humana Press, Totowa. **110:** 93–111.

16. Fless, G. M. 1991. General preparative ultracentrifugation and considerations for lipoprotein isolation. *In* Analyses of Fats, Oils and Lipoproteins. E. G. Perkins, editor. American Oil Chemists' Society, Urbana. 512–523.

17. Rumsey, S. C., N. F. Galeano, Y. Arad, and R. J. Deckelbaum. 1992. Cryopreservation with sucrose maintains normal physical and biological properties of human plasma low density lipoproteins. *J. Lipid Res.* **33:** 1551–1561.

18. Rudling, M., S. Vitols, M. Masquelier, and C. Peterson. 1996. Cryopreserved LDL in the presence of sucrose to protect the biologic properties. *J. Lipid Res.* **37:** 2266–2267.