Journal of Chromatography A, 1280 (2013) 92–97

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# Top–down lipidomic analysis of human lipoproteins by chip-type asymmetrical flow field-flow fractionation–electrospray ionization-tandem mass spectrometry

Ki Hun Kim, Ju Yong Lee, Sangsoo Lim, Myeong Hee Moon*

*Department of Chemistry, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-749, Republic of Korea*

ARTICLE INFO

*Article history:*
Received 12 September 2012
Received in revised form
24 December 2012
Accepted 10 January 2013
Available online 15 January 2013

*Keywords:*
Asymmetrical flow field-flow fractionation (AF4)
Chip-type AF4
cAF4–ESI-MS-MS
Top–down lipidomics
Lipoproteins
LDL

ABSTRACT

This study demonstrates the potential utility of on-line chip-type asymmetrical flow field-flow fractionation (cAF4) and electrospray ionization tandem mass spectrometry (ESI-MS-MS) for the top–down lipidomic analysis of human lipoproteins. Utilizing a cAF4, which is a miniaturized AF4 channel operated with a micro flow rate regime, enabled high density lipoprotein (HDL) and low density lipoprotein (LDL) to be separated by hydrodynamic diameter in an aqueous solution with the simultaneous desalting of lipoproteins. On-line desalting was found to enhance the ionization of lipoproteinic lipid molecules during the feeding of cAF4 effluent to ESI-MS when compared to the direct infusion of lipoproteins to MS. An evaluation of top–down lipidomic analysis was performed to test the efficiency of in-source fragmentation during cAF4–ESI-MS in the dissociation of lipoprotein particles into individual lipid molecules. This study demonstrates the structural identification of the following lipid classes: phosphatidylcholines (PCs), cholesteryl esters (CEs), and regioisomers of triacylglycerols (TAGs) having an identical mass but different acyl chains and dimeric forms of TAGs in the positive ion mode, and phosphatidylglycerols (PGs), phosphatidic acids (PAs), phosphatidylinositols (PIs), and their lyso species in the negative ion mode. The developed method was applied to plasma samples from patients with coronary artery disease (CAD) for the separation of HDL and LDL and for the simultaneous analysis of lipoproteinic lipids, resulting in the identification of 11 PCs, 9 PGs, 4 PAs, 2 PIs, 2 PEs, 18 TAGs, and 6 CEs.



## 1. Introduction

Lipoproteins, vehicles to carry fats and cholesterol in the human blood system, are globular complexes composed of triacylglycerol (TAG) and cholesteryl esters (ChE) in the core with phospholipids (PLs), free cholesterol, and proteins in the outer layer [1]. It is known that smaller and denser low density lipoprotein (LDL), as well as low levels of high density lipoprotein (HDL), are risk factors for atherosclerosis and other cardiac diseases [2–5]. Among the lipoprotein components other than cholesterol, phospholipids (PLs) are a major lipid class composed of various molecular categories and differ according to the polar head groups and lengths and degree of unsaturation of the acyl chain [6]. Since clinical analysis of LDL and HDL is important for diagnosis or risk assessment, the composition and concentration of lipid species in HDL/LDL are important to understanding the function of lipoproteins and their metabolism in relation to diseases. Moreover, individual PL species could serve as potential biomarkers of adult diseases such as breast cancer [7], ovarian cancer [8,9] and prostate cancer [10].

The analysis of lipid molecules contained in lipoproteins first requires the proper isolation or fractionation of HDL and LDL particles from the blood sample. Density gradient ultracentrifugation (DGU) [11–13] and polyacrylamide gel electrophoresis (PAGE) [14,15] yield accurate fractionation based on densities or sizes, respectively. However, in addition to the time-consuming process, DGU requires a considerable amount of sample and PAGE requires the retrieval of LDL particles from the gels. Size exclusion chromatography (SEC) such as fast performance liquid chromatography (FPLC) offers rapid and reproducible separation of lipoproteins for cholesterol determination [16], but the possible interaction of lipoprotein particles with packing materials cannot be prevented completely. Molecular characterization of PLs has been greatly accelerated with the development of sophisticated mass spectrometry (MS) techniques with which chromatographic separation of lipid molecules is commonly interfaced [17,18]. Capillary liquid chromatography (LC) with tandem MS analysis has been shown to be efficient with regard to both the speed and accuracy of molecular identification of PL species [19–21].

Recently, flow field-flow fractionation (FlFFF) has been successfully utilized for the size fractionation of HDL and LDL

* Corresponding author. Tel.: +82 2 2123 5634; fax: +82 2 364 7050.
*E-mail addresses:* mhmoon@yonsei.ac.kr, mhmoon@hotmail.com (M.H. Moon).

0021-9673/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2013.01.025

particles from human plasma samples, resulting in the selective detection of decreased HDL levels and reduced LDL sizes in patients with coronary artery disease (CAD) [22–24]. FlFFF is an elution-based method capable of separating nanoparticles and biomacromolecules such as proteins and cells by hydrodynamic diameter [25–27]. Hollow fiber FlFFF (HF5) was first hyphenated with electrospray ionization-mass spectrometry (ESI-MS) for the analysis of intact proteins [28] and was utilized for the separation of serum lipoproteins in conjunction with multiangle light scattering (MALS) [29]. With the implementation of a multiplexed hollow fiber FlFFF (or MxHF5), it was demonstrated that HDL and LDL fractions can be isolated and collected in their intact forms on a semi-preparative scale, and the number of PL species in each fraction can be analyzed with structural identification using nanoflow liquid chromatography–electrospray ionization-tandem mass spectrometry (nLC–ESI-MS-MS)[30]. The latter work was a bottom–up analysis of PL mixtures requiring the extraction of PLs from each lipoprotein fraction using organic solvents.

This study introduces for the first time the top–down analysis of various lipid molecules of LDLs from plasma samples using a direct interface of the chip-type asymmetrical FlFFF (cAF4), a miniaturized AF4 channel developed to use the microflow regime, with ESI-MS-MS in an aqueous phase. HDL and LDL particles from a human blood sample were separated by cAF4 according to differences in hydrodynamic diameter in an aqueous solution, and the lipoprotein particles eluted with on-line desalting during cAF4 were directly analyzed by ESI-MS-MS. The effects of in-source fragmentation during ESI and desalting using cAF4 were examined first with LDL standards. The developed method was applied to the separation of LDL particles from blood plasma samples from patients with CAD and for the simultaneous characterization of various PLs, TAGs, and CEs.

## 2. Materials and methods

### 2.1. Materials and reagents

The LDL standard material was obtained from Sigma (St. Louis, MO, USA). Human blood plasma samples from healthy volunteers and CAD patients were obtained with written consent from the Yonsei University School of Medicine. HPLC grade water and $CH_3CN$ were purchased from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals ($HCO_2H$, $NH_4OH$, $HCO_2NH_4$, and $NH_4HCO_3$) were from Sigma. The LDL standard contained in the buffer solution (150 mM NaCl, 0.01% EDTA, pH 7.4) was directly injected for cAF4–ESI-MS-MS analysis. However for comparison with direct infusion of LDL to MS, the background salts in the LDL standard solution were removed as follows. The LDL standard was first diluted ten-fold with a 10 mM $NH_4HCO_3$ solution (carrier solution of cAF4) and centrifuged twice using a centrifugal filter unit with a 100 kDa pore size (Millipore, Danvers, MA, USA) at 12,000 g for 15 min twice. The retentate was suspended with $NH_4HCO_3$ solution to the original volume. Capillary tubes (50-, 75-, and 100-μm I.D. having a 360-μm O.D.) for the connection of flow to and from the cAF4 system and for the MS emitter were purchased from Polymicro Technology (Phoenix, AZ, USA). Plasma samples were treated using the depletion spin column from Sigma to remove albumin and IgG prior to analysis. A 100 μL aliquot of plasma sample diluted in equilibration buffer was loaded on the spin column that had been equilibrated prior to use and incubated for 10 min. Then, proteins depleted with albumin and IgG were collected by centrifugation (5,000 g, 1 min.). The eluate was re-applied to the spin column for 10 min and washed with 200 μL of Tris buffer (pH 7.4) for optimal depletion.

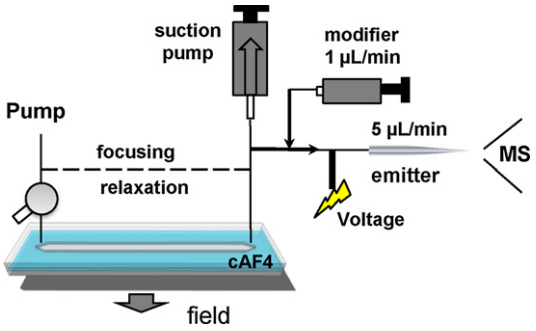


**Fig. 1.** Schematic of cAF4–ESI-MS-MS. The cAF4 effluent was split into two parts (4:1) with the aid of a syringe pump in suction mode, and the resulting flow directed toward the ESI emitter was premixed with ionization modifier liquid from another syringe pump.

### 2.2. cAF4 channel

The chip-type AF4 channel utilized in this study was the same as previously reported [31]. Briefly, cAF4 was constructed by stacking four 1.5-mm-thick stainless steel (SS) plates (13 cm × 4.5 cm) together with a 254-μm-thick PVC spacer in which a rectangular ribbon-like space was cut out (0.5 cm wide and 7 cm long) with triangular ends. The top and bottom layers were the binding blocks used to clamp a series of spacers including an SS plate inserted with a sintered SS frit (10 cm × 1.5 cm × 0.15 cm, 5-μm pore size) and another SS layer in which an inner space was cut out to provide a flow reservoir between the frit containing the plate and the bottom block. A regenerated cellulose membrane with a molecular mass cut-off of 20 kDa (Millipore Corp., Danvers, MA, USA) was inserted between the PVC spacer and the frit wall to keep the sample from penetrating through the frit. The total thickness of the cAF4 channel assembly was about 6 mm. For the inlet and outlet of flow, a Nanoport assembly from Upchurch Scientific (Oak Harbor, WA, USA) was thermally fixed onto the hole drilled at the SS surface of the top block in order to connect the silica capillary tubes. For the crossflow exit, a narrow conduit was cut at one end of the reservoir plate through which a PEEK tube was inserted and sealed with epoxy.

### 2.3. cAF4–ESI-MS-MS of lipoproteins

Separation of LDL particles by cAF4 was achieved in two different carrier solutions (10 mM ammonium bicarbonate ($NH_4HCO_3$) for positive ion mode or 5 mM ammonium formate ($NH_4HCO_2$) for negative ion mode) in HPLC grade water. A model 510 HPLC pump from Waters (Milford, MA, USA) was utilized to deliver the carrier solution to the cAF4 channel through a model VI-12 loop injector with a 20 μL loop from Flom (Tokyo, Japan). The sample injection was made during focusing/relaxation mode in which two counter-directing flow streams were introduced from both the channel inlet and outlet and exited through the channel wall only. The ratio of sample flow rate and focusing flow rate from the channel outlet was set to be 1:4 so that the sample materials could be focused at a position 1/5 from the channel inlet. The period of focusing/relaxation including sample delivery from the injector to the channel was 2 min for the LDL separation at a total flow rate of 0.4 mL/min. The outflow/crossflow rate ($\dot{V}_{out}/\dot{V}_c = 0.02/0.38$ mL/ min) was controlled with length control of a narrow bore capillary tube at the channel outlet. The cAF4 outflow stream (20 μL/min) was split to reduce into 4 μL/min toward the MS emitter through a micro-Tee from Upchurch Scientific (see Fig. 1). The rest of the flow (16 μL/min) was unpumped to a model Legato 110 syringe pump from KD Scientific (Holliston, MA, USA). The feed flow was then mixed with ionization modifier liquid (1.0%

formic acid in $CH_3CN$ for positive or 0.5% $NH_4OH$ for negative ion mode of MS analysis) and delivered by a syringe pump equipped in the MS system at a flow rate of 1 μL/min, resulting in a total feed rate to the MS source of 5 μL/min. Modifier liquids were used to enhance the ionization of lipids during ESI since the cAF4 effluent was an aqueous solution. The modifier liquid and feed flow were mixed with the use of a NanoTight Y-connector with dual lumen sleeves from Upchurch Scientific.

Lipoprotein particles eluted from the cAF4 were directly introduced to an LTQ Velos ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA) via a pulled tip capillary tube (1 cm × 100 μm i.d., 360 μm o.d.) as an emitter for ESI. During ESI, an in-source fragmentation method was applied with 40 V to enhance dissociation of intact LDL particles. ESI was achieved by applying an electrical voltage through a Pt wire (shown in Fig. 1) at 3.0 kV with the capillary temperature set at 200 °C, and CID for MS-MS of lipid molecules was accomplished with 40% of the normalized collision energy. A precursor MS scan followed by three data-dependent CID runs were both monitored at m/z 400–2000 and 200–1200, respectively. MS data were collected using Xcalibur software from Thermo Fisher Scientific. The mass tolerance between the measured mono-isotopic mass and the calculated mass for the identification of lipids was 1.0 Da. Identification of lipids was made manually.

## 3. Results and discussion

The ability of cAF4–ESI-MS-MS to perform top–down lipidomic analysis of lipoproteins was evaluated using an LDL standard. Fig. 2 shows the comparison of MS and MS-MS spectra of the LDL standard solution obtained with (a) direct ESI-MS-MS and (b) cAF4 separation followed by ESI-MS-MS in the positive ion mode. The precursor scan in Fig. 2a shows prominent ion peaks at m/z 600–1000, which are expected to be sodium adducts (marked in the figure) of PL molecules. However, the individual CID spectra of these sodiated precursor ions were too complicated to distinguish the characteristic fragment ions for the identification of PL species as shown on the right side of Fig. 2a due to the presence of salts in the LDL standard solution. Injecting 2.0 μg of LDL into the cAF4 resulted in somewhat broad elution of LDL in 18–35 min as indicated by the base peak fractogram (BPF, fractogram recorded by the base peak signal at each time slice) of cAF4–ESI-MS-MS on the left in Fig. 2b. The MS spectra of eluted LDL particles at the peak maximum time ($t_r$ = 24.7 min) was relatively simple with low background noise compared to that in Fig. 2a due to the on-line removal of salts during cAF4 separation. Moreover, the ion peaks marked in the figure were protonated PL ions ($[M+H]^+$) at m/z 758.7, 786.6, and 810.6, which were found to be the sodium adduct ions (m/z 780.8, 808.8, and 832.9, respectively) in Fig. 2a. These ions were identified as 16:0/18:2-PC, 18:0/18:2-PC, and 18:0/20:4-PC, respectively, from the MS-MS experiments. The CID spectra of m/z 758.7 is shown on the right side of Fig. 2b with the characteristic fragment ions identifying the molecular structure as 16:0/18:2-PC; m/z 699.2 for $[M+H-N(CH_3)_3]^+$, m/z 575.3 for the loss of choline ($HPO_4(CH_2)_2N(CH_3)_3$, 183 amu), m/z 496.1 for the loss of an acyl chain in the form of a ketene ($[M+H-R_2'CH{=}C{=}O]^+$), and m/z 502.2 and 478.2 for the loss of fatty acids (sn-1 and sn-2, respectively) in carboxylic acid. Since on-line desalting of the sample can be achieved during cAF4 separation in which the sample solution is washed off by the crossflow movement, a separate desalting procedure is not required.

The numerous ion peaks above m/z 1000 in Fig. 2 are thought to be from the formation of ions as multimeric forms of PL species, and the generation of such ions can be minimized by applying 40 V of in-source fragmentation during ESI. These ions were expected to be produced from incomplete dissociation of LDL particles into individual lipid molecules during ESI, resulting in the formation of dimeric or multimeric ions. The presence of these multimeric ions was reduced, as indicated in Fig. 2b. When the in-source fragmentation was not applied, the formation of multimeric ions increased. The MS spectra of LDL particles applied by direct infusion that were obtained at different energies of in-source fragmentation are shown in Fig. S1 of Supplementary Data. When in-source fragmentation was not applied (0 V in Fig. S1), the relative intensities of multimeric ions above m/z 1000 were much higher than those of individual ions (presumably smaller than m/z 1000). At 80 V of in-source fragmentation, it was found that the intensities of the multimeric ions were greatly reduced, but precursor ions were destroyed by the high energy. After varying the in-source fragmentation energy, 40 V was selected as the optimum condition to exhibit relatively large intensities of individual ion peaks.

During the cAF4–ESI-MS-MS analysis of LDL standard particles in the positive ion mode, six PCs and three LPCs were identified with the molecular structure in the same way as shown in the CID spectra of Fig. 2b. The structures of the identified PC and LPC molecules are listed in Table 1a. These belong to the identified list of plasma PLs from the bottom–up analysis of a human plasma sample, the PL mixture for which was extracted using organic solvent followed by nanoflow LC–ESI-MS-MS analysis [32]. Table 1a also lists the 6 identified cholesteryl esters (CEs) and 16 triacylglycerols (TAGs). Since 10 mM ammonium bicarbonate solution was utilized as the carrier solution for cAF4 in the positive ion mode along with the modifier liquid (0.1% formic acid in $CH_3CN$), the precursor ions of CE and TAG molecules were detected as ammonium adduct ions, $[M+NH_4]^+$. Fig. 3a shows the CID spectra of an ammonium adduct ion having m/z 666.5 ($[M+NH_4]^+$), representing the loss of ammonia in the neutral form ($[M+NH_4-NH_3]^+$) at m/z 648.5, along with m/z 369.0, the characteristic cholestane ion which results from the loss of water and an acyl chain ($[M+NH_4-RCOONH_4-H_2O]^+$) from CE. It was also found that the heterodimeric form of CE could be

**Table 1**
List of phospholipids, cholesteryl estercholesteryl esters (CEs), and triacylglycerols (TAGs) identified from the LDL standard by top–down lipidomic analysis using AF4–ESI-MS-MS in (a) positive and (b) negative ion mode.

| Class | Acyl chain type (observed m/z) |
|---|---|
| (a) Positive ion mode (type of precursor ion: [1] $[M+H]^+$, [2] $[M+NH_4]^+$) | |
| PC[1] | 16:0/18:2 (758.7), 16:0/18:1 (760.7), 16:0/20:4 (782.6), 18:0/18:2 (786.6), 16:0/22:6 (806.7), 18:0/20:4 (810.6) |
| LPC[1] | 16:0 (496.4), 18:1 (522.4), 18:0 (524.0) |
| CE[2] | 16:0 (642.7), 18:2 (666.7), 18:1 (668.8), 18:0 (690.7), 16:0/18:2 (1291.4), 20:4/18:2 (1339.4) |
| TAG[2] | 18:2/16:0/14:0 (846.8), 16:0/18:1/16:1 (848.8), 18:1/14:0/18:1 (848.8), 16:0/16:0/18:1 (850.8) 18:2/18:2/16:1 (870.9), 18:2/18:2/16:0 (872.9), 16:0/18:2/18:1 (874.9), 16:0/18:1/18:2 (874.9) 18:1/16:0/18:1 (876.8), 18:1/16:0/20:4 (898.9), 18:2/20:3/16:0 (898.9), 18:3/20:2/16:0 (898.9) 18:1/18:1/18:2 (900.9), 18:1/18:1/18:1 (902.9), 18:1/18:1/18:0 (904.9), 18:1/16:0/20:0 (906.9) |
| (b) Negative ion mode (type of precursor ion: [1] $[M+HCOO]^-$, [2] $[M-H]^-$) | |
| PC[1] | 16:0/18:2 (804.8), 16:0/20:4 (826.7), 18:0/18:2 (830.7), 18:0/20:4 (854.7) |
| LPC[1] | 14:0 (540.5), 18:0 (568.2) |
| SM[1] | d18:1/16:0 (747.0) |
| PE[2] | 16:0/18:2 (715.8) |
| LPE[2] | 14:0 (424.5) |
| PA[2] | 18:0/18:2 (699.6), 18:2/18:1 (697.8), 18:0/18:1 (701.6) |
| LPA[2] | 18:0 (437.5) |
| PG[2] | 16:0/18:2 (742.6), 16:0/18:1 (744.7), 16:0/20:4 (766.7), 18:0/18:1 (770.7) 16:0/22:6 (790.7), 20:4/18:0 (794.6) |
| LPG[2] | 14:0 (455.4), 16:1 (481.1), 18:1 (511.1) |
| PI[2] | 18:0/18:2 (861.1), 18:0/20:4 (885.7) |

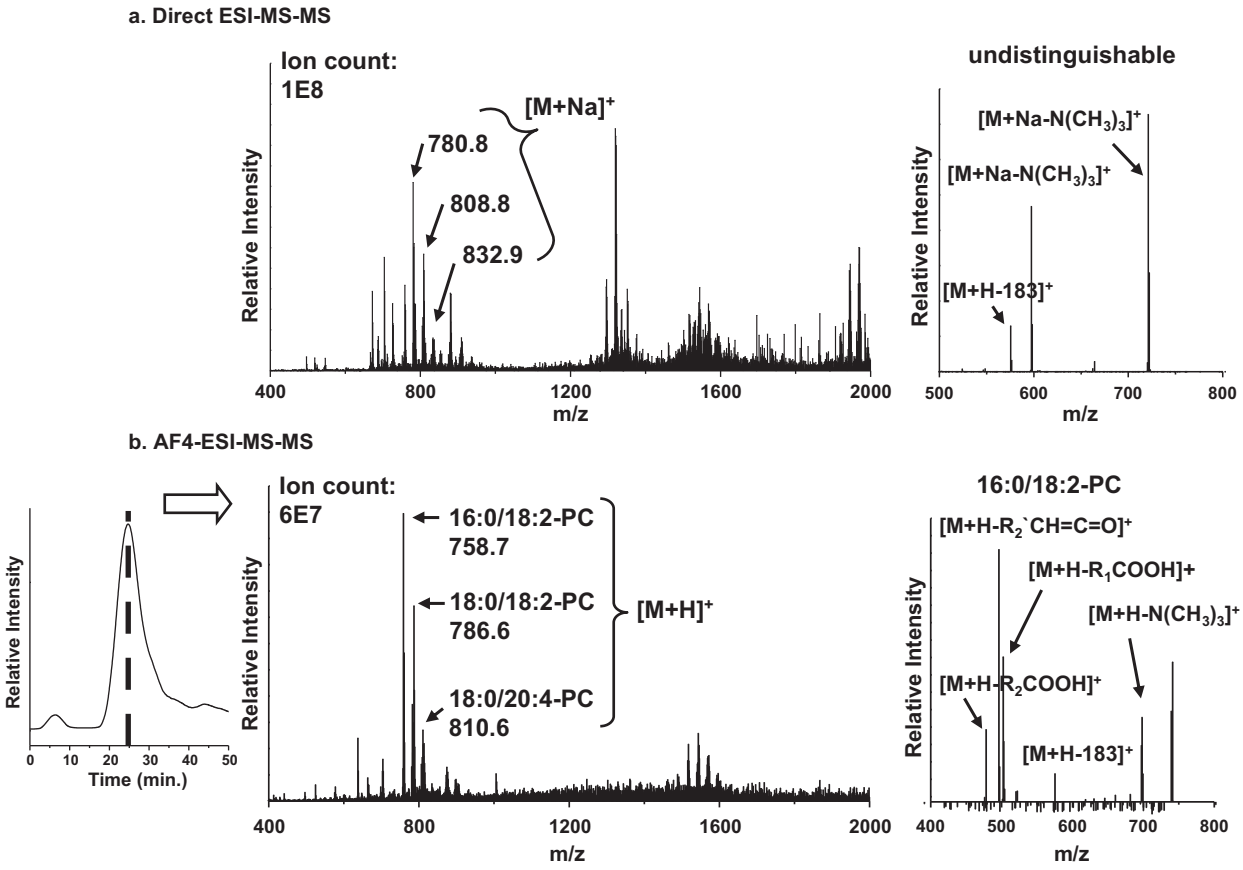


**Fig. 2.** Comparison of MS and MS-MS spectra of LDL standard particles from (a) direct infusion to ESI-MS and (b) cAF4–ESI-MS-MS (base peak fractogram (BPF) of LDL sample shown at the bottom left).

identified by the larger m/z regime. Fig. 3b shows the CID spectra of heterodimeric CE species (m/z 1291.4, $[M+NH_4]^+$). The parent ion is presumed to be the aggregate of two CE molecules with different acyl chains in which fragment ions represent the dissociation of each acyl chain (marked as $R_1$ and $R_2$); ions of m/z 993.1 and 1018.1 for the dissociation of the acyl chain in the form of ammonium carboxylate ($[M+NH_4-RCOONH_4]^+$), m/z 666.6 and 640.6 for the ammonium adduct of each CE molecule ($[R_1Chl+NH_4]^+$ and $[R_2Chl+NH_4]^+$, respectively), and the characteristic cholestane ions at m/z 369.4. The molecular structure of the heterodimers was identified as 18:2-CE/16:0-CE ($R_1Chl/R_2Chl$).

The top–down analysis of LDL particles by cAF4–ESI-MS-MS enabled identification of isomeric TAG molecules. Fig. 4 shows the CID spectra of precursor ions of m/z 898.9, $[M+NH_4]^+$, obtained from Fig. 2b. It represents the combination of characteristic fragment ions from different TAG isomers with a total carbon number of acyl chains as 54:5. Fragment ions clustered in m/z 573.6–625.5 represent the similar type of fragments resulted from the loss of an acyl chain from TAG isomers in the form of ammonium carboxylate as $[M+NH_4-RCOONH_4]^+$. For instance, the fragment ion of m/z 601.6 represents the loss of acyl chain 18:2 from the parent ammoniated ion. Likewise, a possible set of dissociated acyl chains contained in the TAG isomers (m/z 898.5) can be expected as 16:0. 18:1, 18:2, 18:3, 20:2, 20:3, and 20:4 from fragment ions m/z 625.6, 599.6, 601.6, 603.6, 573.6, 575.6, and 577.6, respectively. Due to the relatively low intensities of fragment ions below m/z 550, MS-MS spectra in m/z 250–550 were illustrated with 10× magnification. In this m/z region, acylium ions, $[RCO]^+$, and ions from the

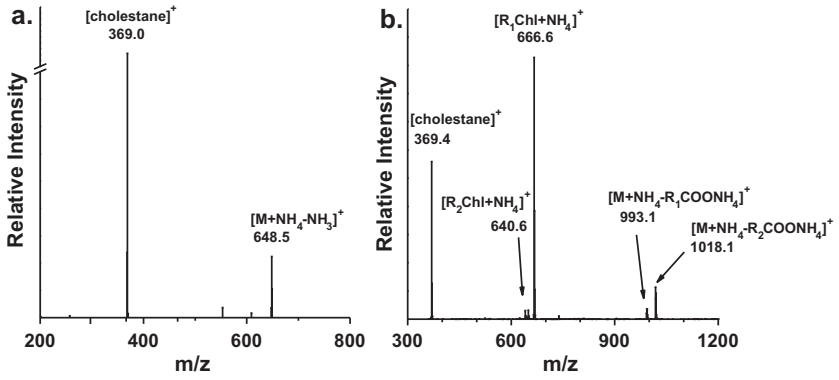


**Fig. 3.** CID spectra of (a) 18:2-CE (m/z 666.5, $[M+NH_4]^+$) and (b) CE heterodimer, 18:2($R_1$)–CE/16:0($R_2$)–CE (m/z 1291.4, $[M+NH_4]^+$).

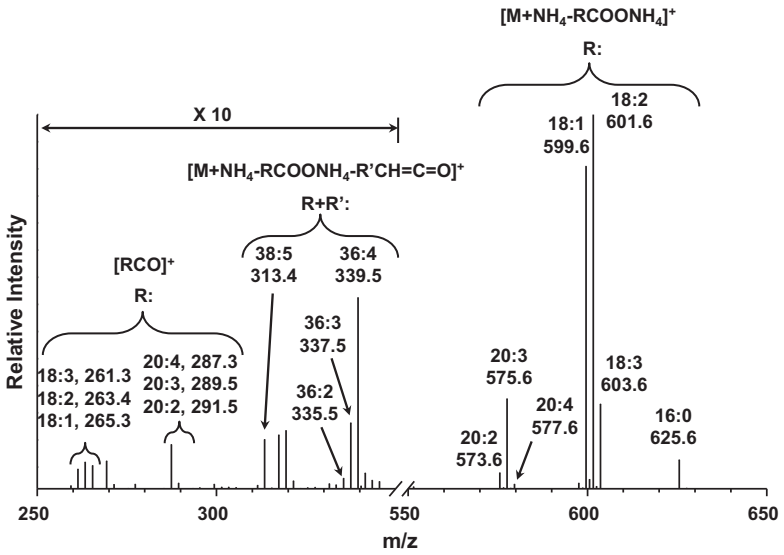


**Fig. 4.** CID spectra of TAG isomers (m/z 898.9, $[M+NH_4]^+$) having different acyl chains obtained by cAF4–ESI-MS-MS.

loss of two adjacent acyl chains in the form of carboxylic acid and ketene, $[M+NH_4-RCOONH_4-R'CH{=}C{=}O]^+$, were observed where R and R' denoted acyl chains without a positional order For instance, ions of m/z 335.5, 337.5, and 339.5 were generated from the loss of two acyl chains as 36:2, 36:3, and 36:4, respectively. From these spectra, a possible combination can be expected with following isomeric TAG molecules without a positional order expressed as 16:0/18:1/20:4, 16:0/18:2/20:3, 16:0/18:3/20:2, 18:1/18:2/18:2, and 18:1/18:1/18:3. A direct infusion of the LDL standard in ESI-MS-MS results to be failed in their successful identification. This may be due to the influence of salts contained in the LDL standard solution on ionization, leading to noisy background signals. However, the detection of TAG isomers in Fig. 4 demonstrates the potential utility of cAF4–ESI-MS-MS in identifying isobaric TAG species simultaneously.

Top–down analysis of the LDL standard by cAF4–ESI-MS-MS in the negative ion mode enabled the identification of various molecular classes of PLs. The precursor ion spectra are shown in Fig. S2 of the Supplementary Data. With the use of a mixed modifier solution, 5 mM ammonium formate and 0.5% ammonium hydroxide in acetonitrile, four PCs, two LPCs, and one sphingomyelin (SM) (Table 1b) were detected as formate adduct ions ($[M+HCOO]^-$) while the rest of the PLs including one phosphatidyl ethanolamine (PE), one LPE, three PAs, one LPA, six PGs, three LPGs, and two PIs were detected as $[M-H]^-$. All lysophospholipids were identified in the form of an acyl chain at the sn-1 position since regioisomers (acyl/lyso and lyso/acyl) cannot be differentiated from the mixed MS-MS spectra unless they are separated as reported in the literature [33].

The developed method was applied to human plasma samples from CAD patients after depletion of albumin. Plasma aliquots of 2 μL were injected into cAF4–ESI-MS-MS. Fig. 5a shows the BPF of a healthy human plasma sample. Due to the use of a very low outflow rate (20 μL/min at the channel outlet) that is essential for ESI, lipoprotein separation in the cAF4 channel took somewhat longer than that can be observed with a higher flow rate condition in a conventional channel, but it resulted in complete separation of HDL and LDL particles. Fig. 5b-d show extracted ion fractograms (EIFs) of 54:2-TAG (m/z 876.8, $[M+NH_4]^+$), cholestane (m/z 369.5) for cholesteryl esters, and apolipoprotein A-I (ApoA-1, m/z 1081.2, $[M+26H]^{+26}$). The latter ion was selected due to the highest peak intensity among the other multi-protonated ions. While the apparent peak area of 54:2-TAG in HDL is larger than in LDL (Fig. 5b), the peak for cholestane appears to be much larger in LDL as shown in Fig. 5c. ApoA-1 (28 kDa) was detected only in HDL peak. This is because ApoA-1 is one of the major proteins in HDL while ApoB-100 (513 kDa) is found exclusively in LDL [34]. However, the multi-charged ion peak of ApoB-100was not observed under the present ESI conditions.

Once the ionization of lipoprotein particles can be consistently maintained, the EIF of target molecules can be utilized for the quantitative comparison between normal and diseased states. For example, the peak area of the EIF of 32:0-PC as well as 34:3-PC (Fig. 6) indicated the standard LDL, the ten healthy control plasma samples, and the ten CAD patient samples had similar concentrations. The relative peak area of individual PC species was calculated from the total peak area of the target species from LDL divided by that of a highly abundant PC species (34:2-PC, the third species from the left in Fig. 6) commonly found in each sample. The selection of 34:2-PC as an internal reference was due to the relatively large intensity compared to that of other PC species. In Fig. 6, the relative intensity of 34:2-PC for each sample was set to be 100. We chose to express acyl chains as the sum of the total carbon number along with the number of double bonds (i.e., 34:3-PC) because of the limitation in resolving PC isomers having different acyl chain structures but the same m/z value from the ESI-MS spectra. While

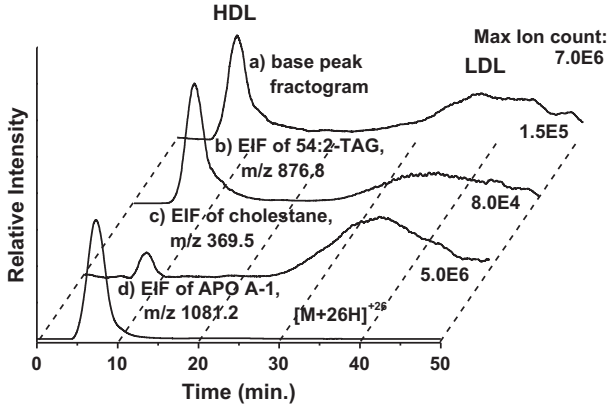


**Fig. 5.** (a) The BPF of a human plasma sample obtained by cAF4–ESI-MS-MS superimposed with extracted ion fractograms (EIFs) of (b) 54:2-TAG (m/z 876.8, $[M+NH_4]^+$), (c) cholestane (m/z 369.5) for CE, and (d) apolipoprotein A-1 (m/z 1081.2, $[M+26H^+]^{+26}$).

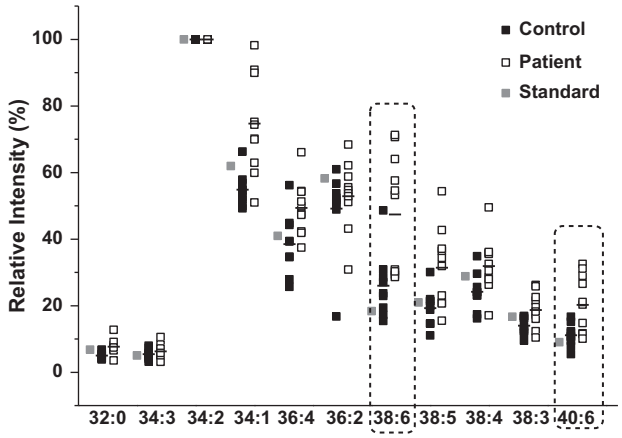


**Fig. 6.** Relative abundance of ten PC species (compared to the peak area of 34:2-PC of each individual sample as an internal reference) from healthy controls ($n = 10$) and CAD patients ($n = 10$) calculated from the peak area of an extracted ion fractogram by cAF4–ESI-MS.

the PC species having 32:0 and 34:3 showed small variations, the PCs of 38:6 and 40:6 showed significant differences between CAD patients and controls: $1.94 \pm 1.01$ fold ($p = 0.002$ by student's $t$-test) for 38:6-PC and $1.89 \pm 0.96$ ($p = 0.006$) for 40:6-PC ($n = 10$ for each group) as listed in Table S1 of Supplementary Data. The major acyl chain structures of the two PC species found in the MS-MS spectra were 16:0/22:6 and 18:0/22:6, respectively. Notably, the relative peak intensities of the control samples (black squares) and the standard LDL sample (gray square) were close to each other for all 10 PC species examined in Fig. 6. However, the patient samples showed some statistical differences in the relative amounts of PC species. Though the current work dealt with ten patients and ten control samples, the method used shows a potential to be utilized for high speed screening of target lipid species in LDL depending on the disease status once biomarkers of CAD or other cardiovascular diseases are established.

## 4. Conclusions

This study demonstrates the possibility of top–down lipidomic analysis for lipoprotein particles from human plasma samples. In the developed method, HDL and LDL are separated by cAF4 according to differences in hydrodynamic diameter, and the eluting lipoprotein particles are analyzed on-line by ESI-MS-MS for the characterization of PLs including lyso-PLs, TAGs, and CEs. cAF4 separation of lipoprotein particles followed by shotgun analysis provides an advantage of on-line desalting of samples that enhances CID experiments for the structural identification of lipid molecules. The extracted ion fractogram of a specific precursor ion can be utilized to quantify the relative regulation of targeted lipid molecules among samples depending on the disease status. While an off-line combination of MxHF5 and nLC–ESI-MS-MS for the PL analysis of lipoprotein in the previous studies [30,32] provides a semi-preparative separation of lipoprotein particles followed by a high throughput and non-targeted analysis PLs with high sensitivity in different lipoproteins, cAF4–ESI-MS-MS offers a high speed screening of targeted lipid molecules without a separate lipid extraction and a possibility of comparing the relative abundance of specific lipid molecules present between HDL and LDL simultaneously. However the latter approach bears a limitation in detecting the relatively low abundance lipids due to the spectral congestion during ESI.

The current study shows the potential utility of cAF4–ESI-MS in high speed screening of specific lipids contained in blood plasma samples from patients with CAD, further optimizations are needed to improve the separation speed of the cAF4 system without sacrificing the resolution. Moreover, the ionization efficiency of a single lipid molecule from globular particles needs to be enhanced by controlling in-source fragmentation and the composition of the ionization modifier so that the formation of multimeric lipid ions can be minimized.

## Acknowledgment

This study was supported by a grant (No. 2011-0016438) from the National Research Foundation (NRF) of Korea funded by the Korean government (MEST).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.01.025.

## References

[1] C.A. Converse, E.R. Skinner, Lipoprotein Analysis: A Practical Approach, Oxford University Press, New York, 1992.
[2] P.W.F. Wilson, R.B. D'Agostino, D. Levy, A.M. Belanger, H. Silbershatz, W.B. Kannel, Circulation 97 (1998) 1837–1847.
[3] W.E. Boden, Am. J. Cardiol. 86 (2000) 19L–22L.
[4] M.A. Austin, B.L. Rodriguez, B. McKnight, M.J. McNeely, K.L. Edwards, J.D. Curb, D.S. Sharp, Am. J. Cardiol. 86 (2000) 412–416.
[5] M.C. Bakogianni, C.A. Kalofoutis, K.I. Skenderi, A.T. Kalofoutis, J. Diabet. Complications 15 (2001) 265–269.
[6] J.F.H.M. Brouwers, E.A.A.M. Vernooij, A.G.M. Tielens, L.M.G. Van Golde, J. Lipid Res. 40 (1999) 164–169.
[7] M.O. Leach, M. Verrill, J. Glaholm, T.A.D. Smith, D.J. Collins, G.S. Payne, J.C. Sharp, S.M. Ronen, V.R. McCready, T.J. Powles, I.E. Smith, NMR Biomed. 11 (1998) 314–340.
[8] M. Okita, D.C. Gaudette, G.B. Mills, B.J. Holub, Int. J. Cancer 71 (1997) 31–34.
[9] R. Sutphen, Y. Xu, G.D. Wilbanks, J. Fiorica, E.C. Grendys Jr., J.P. LaPolla, H. Arango, M.S. Hoffman, M. Martino, K. Wakeley, D. Griffin, R.W. Blanco, A.B. Cantor, Y.J. Xiao, J.P. Krischer, Cancer Epidemiol. Biomarkers Prev. 13 (2004) 1185–1191.
[10] M.G. Swanson, D.B. Vigneron, Z.L. Tabatabai, R.G. Males, L. Schmitt, P.R. Carroll, J.K. James, R.E. Hurd, J. Kurhanewicz, Magn. Reson. Med. 50 (2003) 944–954.
[11] B.A. Griffin, M.J. Caslake, B. Yip, G.W. Tait, C.J. Packard, J. Shepherd, Atherosclerosis 83 (1990) 59–67.
[12] J. Schiller, O. Zschörnig, M. Petković, M. Müller, J. Arnhold, K. Arnold, J. Lipid Res. 42 (2001) 1501–1508.
[13] H.Y. Sun, S.F. Chen, M.D. Lai, T.T. Chang, T.L. Chen, P.Y. Li, D.B. Shieh, K.C. Young, Clin. Chim. Acta 411 (2010) 336–344.
[14] R.M. Krauss, D.J. Burke, J. Lipid Res. 23 (1982) 97–104.
[15] S.B. Alabakovska, B.B. Todorova, D.D. Labudovic, K.N. Tosheska, Clin. Chim. Acta 317 (2002) 119–123.
[16] P. Wiesner, K. Leidl, A. Boettcher, G. Schmitz, G. Liebisch, J. Lipid Res. 50 (2009) 574–585.
[17] R. Taguchi, J. Hayakawa, Y. Takeuchi, M. Ishida, J. Mass Spectrom. 35 (2000) 953–966.
[18] G. Isaac, D. Bylund, J.E. Månsson, K.E. Markides, J. Bergquist, J. Neurosci. Methods 128 (2003) 111–119.
[19] R. Taguchi, T. Houjou, H. Nakanishi, T. Yamazaki, M. Ishida, M. Imagawa, T. Shimizu, J. Chromatogr. B Biomed. Sci. Appl. 823 (2005) 26–36.
[20] D.Y. Bang, D. Kang, M.H. Moon, J. Chromatogr. A 1104 (2006) 222–229.
[21] H. Kim, H.K. Min, G. Kong, M.H. Moon, Anal. Bioanal. Chem. 393 (2009) 1649–1656.
[22] I. Park, K.J. Paeng, Y. Yoon, J.H. Song, M.H. Moon, J. Chromatogr. B Biomed. Sci. Appl. 780 (2002) 415–422.
[23] D.C. Rambaldi, P. Reschiglian, A. Zattoni, C. Johann, Anal. Chim. Acta 654 (2009) 64–70.
[24] J.Y. Lee, D. Choi, C. Johan, M.H. Moon, J. Chromatogr. A 1218 (2011) 4144–4148.
[25] J.C. Giddings, Science 260 (1993) 1456–1465.
[26] K.G. Wahlund, J.C. Giddings, Anal. Chem. 59 (1987) 1332–1339.
[27] P. Reschiglian, M.H. Moon, J. Proteomics 71 (2008) 265–276.
[28] P. Reschiglian, A. Zattoni, B. Roda, L. Cinque, Anal. Chem. 77 (2005) 47–56.
[29] D.C. Rambaldi, A. Zattoni, S. Casolari, P. Reschiglian, D. Roessner, C. Johann, Clin. Chem. 53 (2007) 2026–2029.
[30] J.Y. Lee, K.H. Kim, M.H. Moon, J. Chromatogr. A 1216 (2009) 6539–6542.
[31] K.H. Kim, M.H. Moon, Anal. Chem. 83 (2011) 8652–8658.
[32] S.K. Byeon, J.Y. Lee, M.H. Moon, Analyst 137 (2012) 451–458.
[33] J.Y. Lee, H.K. Min, M.H. Moon, Anal. Bioanal. Chem. 400 (2011) 2953–2961.
[34] M. Heller, D. Stalder, E. Schlappritzi, G. Hayn, U. Matter, A. Haeberli, Proteomics 5 (2005) 2619–2630.