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Top-down and bottom-up lipidomic analysis of rabbit lipoproteins under different metabolic conditions using flow field-flow fractionation, nanoflow liquid chromatography and mass spectrometry

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ABSTRACT

This study demonstrated the performances of top-down and bottom-up approaches in lipidomic analysis of lipoproteins from rabbits raised under different metabolic conditions: healthy controls, carrageenan-induced inflammation, dehydration, high cholesterol (HC) diet, and highest cholesterol diet with inflammation (HCI). In the bottom-up approach, the high density lipoproteins (HDL) and the low density lipoproteins (LDL) were size-sorted and collected on a semi-preparative scale using a multiplexed hollow fiber flow field-flow fractionation (MxHF5), followed by nanoflow liquid chromatography-ESI-MS/MS (nLC-ESI-MS/MS) analysis of the lipids extracted from each lipoprotein fraction. In the top-down method, size-fractionated lipoproteins were directly infused to MS for quantitative analysis of targeted lipids using chip-type asymmetrical flow field-flow fractionation-electrospray ionization-tandem mass spectrometry (cAF4-ESI-MS/MS) in selected reaction monitoring (SRM) mode. The comprehensive bottom-up analysis yielded 122 and 104 lipids from HDL and LDL, respectively. Rabbits within the HC and HCI groups had lipid patterns that contrasted most substantially from those of controls, suggesting that HC diet significantly alters the lipid composition of lipoproteins. Among the identified lipids, 20 lipid species that exhibited large differences (>10-fold) were selected as targets for the top-down quantitative analysis in order to compare the results with those from the bottom-up method. Statistical comparison of the results from the two methods revealed that the results were not significantly different for most of the selected species, except for those species with only small differences in concentration between groups. The current study demonstrated that top-down lipid analysis using cAF4-ESI-MS/MS is a powerful high-speed analytical platform for targeted lipidomic analysis that does not require the extraction of lipids from blood samples.

1. Introduction

Lipoproteins are globular complexes present in blood that are composed of lipids with few proteins and are classified into density-dependent subclasses, including high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) [1]. The major roles of lipoproteins are transportation of lipids and cholesterols in the bloodstream and their functions within the human body are related to cellular structure, intercellular signaling, cell proliferation, and apoptosis [2,3]. Since altered metabolism of lipids can often trigger the pathogenesis of metabolic disease [4,5], methods for lipid analysis have become of increasing interest in clinical fields for diagnostic and prognostic applications. Among the various risk factors for metabolic diseases, consumption of an unhealthy diet is a major factor [6]. The various types of unhealthy diet and their influences are discussed below. High cholesterol diets often cause metabolic diseases that lead to obesity and diabetes, and can even lead to the development of cancer [7,8]. Accumulation of carrageenan, a common additive utilized in the food industry to improve taste and keep ingredients from separating, can eventually cause inflammation in the human body, which is a known contributor to serious metabolic diseases

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such as heart diseases [9]. Dehydration, caused by insufficient intake of water, promotes low blood pressure and decreases the overall amount of blood in the human body. In extreme cases, it can affect the brain and cause minor or major headaches, along with diminished memory and impaired nerve function [10]. Since decreased levels of HDL and increased levels of LDL, along with a size reduction in LDL, are common signs of an increased risk for various types of metabolic disease, it is important to study the relative levels of lipoproteins. Therefore, a separate lipid profiling of HDL and LDL is necessary to discover the specific lipid species involved in the development of metabolic disorders.

Analysis of lipids from lipoproteins has been performed in using a bottom-up method, which requires the isolation of different lipoproteins, extraction of lipids from the HDL/LDL fractions, and analysis of the lipids with sophisticated mass spectrometry (MS) methods in conjunction with liquid chromatography (LC). While HDL and LDL can be separated by size exclusion chromatography (SEC), gel electrophoresis, and ultracentrifugation [11–14], flow field-flow fractionation (FlFFF) methods provide robust and high speed size separation of HDL/LDL in an empty channel without the concern of sample interaction with the packed bed or gels [15–18]. Recently, multiplexed hollow fiber FlFFF (MxHF5) [19] was successfully utilized for size-sorting of HDL and LDL particles from plasma samples on a semi-preparative scale (~50 μL plasma per injection) and quantitative analysis using nanoflow LC-ESI-MS/MS of the extracted phospholipids (PLs) in different lipoproteins was performed using samples from patients with coronary artery disease (CAD) [20]. Lipid analysis can also be made using a top-down approach in which plasma or serum lipoproteins can be injected directly into electrospray ionization-tandem MS (ESI-MS^n) during the separation of lipoproteins. Development of a chip-type asymmetrical FlFFF (or AF4) channel offered an on-line hyphenation with MS (cAF4-ESI-MS/MS) to carry out a direct analysis of the lipids in different lipoproteins [21,22]. Use of miniaturized FlFFF channel prior to ESI-MS offers great advantages such as the on-line desalting of plasma samples, which can enhance the ionization of lipid species, and a high-speed lipid screening capability (~200 min per sample) with the bypass of the time consuming analytical steps (~36 h): isolation of the HDL/LDL fraction and extraction of the lipids from each lipoprotein fraction prior to LC–MS/MS analysis. Moreover, lipid extraction from each lipoprotein fraction, an essential step for chromatographic separation, is not necessary in the top-down analysis method. While sophisticated LC–MS/MS analysis of lipids is a beneficial platform that provides a comprehensive analysis of lipids, including isomeric forms of lipids, with the minimization of ion suppression from highly abundant species, the pre-chromatographic steps limit the throughput, and this often becomes a problem when the sample set is large. However, top-down lipidomic analysis by cAF4-ESI-MS/MS offers great potential for high speed screening of targeted lipids with increased throughput.

In this study, comprehensive lipid profiling was carried out for the HDL and LDL fractions of serum samples from New Zealand white rabbits raised under different metabolic conditions: normal diet (control), inflammation induced by injecting carrageenan, dehydrated diet, high cholesterol (HC) diet, and high cholesterol diet with inflammation (HCI). Changes in the lipid distribution in rabbit sera under these different metabolic conditions were characterized by both the bottom-up and top-down analysis methods: an off-line combination of MxHF5 with nLC-ESI-MS/MS and a direct analysis using cAF4-ESI-MS/MS, respectively, as illustrated in Fig. 1. Initially, qualitative and quantitative lipid profiling was carried out using pooled serum samples from each of the different diet groups of rabbits using an off-line combination of MxHF and nLC-ESI-MS/MS. Then, lipid species showing greater than 10-fold differences among the groups were selected and these targeted

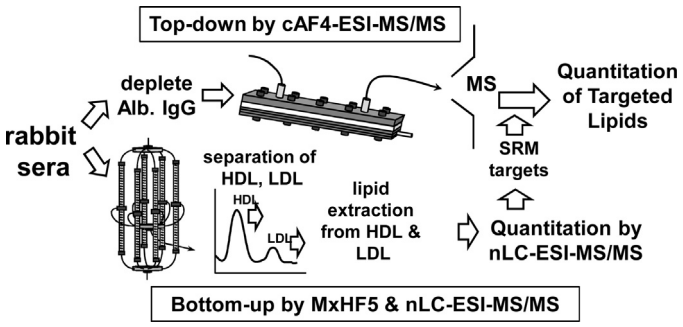

Fig. 1. Schematic of the top-down and bottom-up approaches for lipoprotein specific lipid analyses from rabbit serum samples using an on-line cAF4-ESI-MS/MS and an off-line combination of MxHF5 with nLC-ESI-MS/MS.

species were quantified by cAF4-ESI-MS/MS from individual samples using the selected reaction monitoring (SRM) method in order to confirm the differences and to validate the top-down approach as an alternative high speed screening platform for the analysis of lipids from lipoproteins.

2. Experimental

2.1. Materials and reagents

Bovine serum albumin (BSA), apoferritin, thyroglobulin, carbonic anhydrase (CA), NH_4HCO_2 and NH_4OH as ionization modifiers for MS, and methyl-*tert*-butyl ether (MTBE) for lipid extraction were purchased from Sigma–Aldrich (St. Louis, MO, USA). Serum samples from New Zealand white rabbits raised under five different metabolic conditions were obtained from Korea University Guro Hospital (Seoul, Korea). The first animal group consisted of four 12-week-old healthy rabbits, the second group consisted of five rabbits with inflammation induced by hypodermic injection with 3 mL of 1% carrageenan solution for 3 weeks, the third group of three rabbits was given 1/3 of the water supply given to the other groups to induce a mild dehydration effect, the fourth group of five rabbits was maintained on a high cholesterol diet, and the last group consisted of six rabbits with inflammation induced by carrageenan and maintained on a high cholesterol diet. Serum samples from a healthy human control and a patient with coronary artery disease (CAD) were obtained under informed consent from Severance hospital (Seoul, Korea) to allow for a comparison of the concentrations and sizes of lipoproteins with those of rabbits. The ProteoPrep® Immunoaffinity Albumin & IgG Depletion Kit from Sigma–Aldrich was used to deplete albumin and immunoglobulin G (IgG) from rabbit serum, as these can interfere with lipoproteins or lipids in UV detection and on-line MS analysis. HPLC grade H_2O, $CHCl_3$, CH_3CN, C_3H_7OH, and CH_3OH for LC were purchased from Avantor Performance Materials (Center Valley, PA, USA). Nineteen lipid standards (16:0-lysophosphatidylcholine (LPC), 18:0-lyso phosphatidylethanolamine (LPE), 13:0/13:0-phosphatidylcholine (PC) as an internal standard (IS), 18:0/18:0-PC, 18:0/18:0-phosphatidylethanolamine (PE), 18:0-lysophosphatidic acid (LPA), 18:0-lysophosphatidylglycerol (LPG), 18:0-lysophosphatidylserine (LPS), 16:0/16:0-phosphatidic acid (PA), 18:0/18:0-phosphatidylglycerol (PG), 15:0/15:0-PG, 16:0/18:1-phosphatidylinositol (PI), 16:0/16:0-phosphatidylserine (PS), $(18:1)_4$-cardiolipin (CL), d18:1/12:0-sphingomyelin (SM), d18:1/16:0-monohexosylceramide (MHC), d18:1/16:0-dihexosylceramide (DHC), d18:1/14:0-ceramide (Cer), d18:1/22:0-ceramide (Cer), and d18:1/24:0-trihexosylceramide (THC)) used to establish the run conditions of nLC-ESI-MS/MS were purchased from Avanti

Polar Lipids, Inc. (Alabaster, AL, USA) and Matreya, LLC. (Pleasant Gap, PA, USA).

2.2. MxHF5

Polyacrylonitrile (PAN) hollow fibers (0.85 mm × 1.55 mm, ID × OD) with a molecular weight cut-off of 30 kDa were purchased from Synopex Water, Inc. (Pohang, Korea). A 25 cm length of the fiber was inserted inside a 20 cm-long glass tube (4.0 mm × 5.0 mm, ID × OD) on which an ETFE tee from IDEX Corp. (Oak Harbor, WA, USA) was stabilized on one end using an 1/8″ ferrule and nut. The middle end of the ETFE tee was connected for radial flow. Six hollow fiber modules were connected in parallel for the assembly of MxHF5 and each of the ends of the modules were connected together using a PEEK 7-port Manifold in parallel. All tubing, ferrules, nuts, and manifolds used in MxHF5 were purchased from IDEX Corp. A Waters model 590 HPLC pump from Waters (Milford, MA, USA) and a UV 730D detector from Young-Lin Instrument (Seoul, Korea) were utilized. Operation of MxHF5 is described in Supplementary data.

2.3. Lipid extraction from HDL and LDL of pooled rabbit sera

The modified Folch method using MTBE/CH_3OH [23] was used to extract lipids from the collected lipoproteins. The collected HDL and LDL particles were concentrated to a final volume of approximately 1 mL using Amicon Centrifugal Filters with a molecular weight cut-off of 10 kDa from Merck Millipore (Darmstadt, Germany). The concentrated HDL and LDL were transferred to 2 mL centrifugal tubes with open lids and their tops were sealed with MilliWrap Membrane Seal, purchased from Merck Millipore. Samples were dried under vacuum centrifugation to a powdery form and were then mixed with 300 μL of CH_3OH, followed by the addition of 1000 μL of MTBE. The mixture was vortexed for 30 min and 250 μL of MS-grade H_2O was added and samples were vortexed for another 10 min. The aqueous and organic layers of the mixture were partitioned by centrifuging at 1000 × *g* for 10 min and the upper organic layer was pipetted and saved in a new centrifugal tube. To the aqueous layer, 300 μL of CH_3OH was added and the mixture was tip sonicated for 2 min. The sonicated mixture was centrifuged at 1000 × *g* for 5 min and the supernatant was pipetted and combined with the previously saved upper organic layer. The combined mixture, in a tube sealed with MilliWrap Membrane Seal, was dried under vacuum centrifugation to a powdery form, dissolved in $CHCl_3$:CH_3OH (1:1, v/v) and diluted with CH_3OH:CH_3CN (9:1, v/v) to a final concentration of 20 μg/μL for analysis by nLC-ESI-MS/MS.

2.4. Nanoflow LC-ESI-MS/MS

A LC binary pump, 1200 capillary pump from Agilent Technologies (Santa Clara, CA, USA), was equipped with a LTQ Velos ion trap mass spectrometer from Thermo Scientific (San Jose, CA, USA). Mobile phase solutions consisted of H_2O:CH_3CN (9:1, v/v) as mobile phase A and C_3H_7OH:CH_3OH:CH_3CN (6:2:2, v/v/v) as B with a mixed modifier of 5 mM NH_4HCO_2 and 0.5% NH_4OH. The mixed modifier enabled the detection of lipids in both the positive and negative modes using a single set of mobile phases, as published in the previous study [24]. Details of nLC-ESI-MS/MS operation are described in Supplementary data. Identification of lipids was aided by LiPilot, software developed in the laboratory, followed by confirmation by manual evaluation [25].

2.5. cAF4-ESI-MS/MS

The cAF4 channel was assembled in the same manner as with the previous model [22] and details of channel are in Supplementary data. The Waters model 590 pump was connected to the inlet of the cAF4 channel via the 7725i injector. Carrier solutions of 10 mM NH_4HCO_3 with 1.0% HCO_2H as modifier in the positive ion mode and 5 mM NH_4HCO_2 with 0.5% NH_4OH in the negative ion mode were used to detect lipids from HDL and LDL. During focusing/relaxation, the sample was introduced to the cAF4 channel through the injector at a flow rate of 400 μL/min and salts and particles less than 10 kDa were expected to exit through the crossflow. During elution, the outflow from the channel outlet was set to 20 μL/min using capillary tubing and a suction syringe pump set at 10 μL/min withdrew around half of the outflow as the feed was too fast for MS. The remaining 10 μL/min of the outflow was mixed with the modifier (2.5 μL/min) via a MicroTee, resulting in a final feed of 12.5 μL/min reaching the MS. Unlike the bottom-up method of MxHF5 and nLC-ESI-MS/MS where pooled serum samples were used, analysis by individual sample was conducted in the top-down approach to individually quantify the targeted lipids. Ten microliters of depleted sample (albumin and IgG depleted),

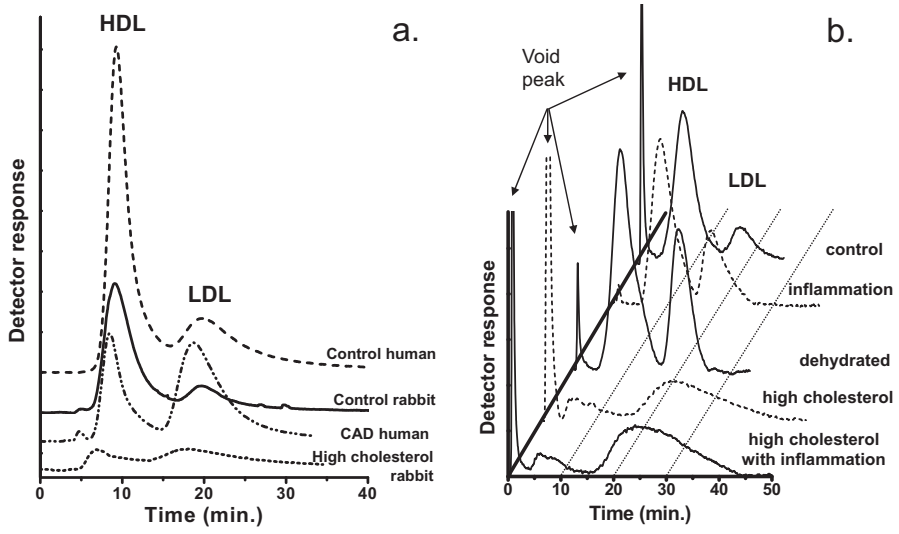

Fig. 2. (a) Fractograms of depleted human and rabbit serum samples depicting the separation of HDL and LDL particles by HF5 at $\dot{V}_{in}/\dot{V}_{out}$ = 0.4/0.20 mL/min and (b) fractograms of pooled samples of serum from five different rabbit groups at $\dot{V}_{in}/\dot{V}_{out}$ = 0.4/0.15 mL/min by HF5. Lipoproteins were monitored at 600 nm.

along with 500 ng CA as an IS, and 30 μL of depleted sample, along with 2 μg CA, were analyzed in the positive and negative ion modes, respectively. After each run, the channel was washed for 30 min to avoid the sample carry-over phenomenon. The lipid species that showed significant differences between the groups by nLC-ESI-MS/MS were selected for analysis by top-down FFF-MS in the SRM mode. The ESI voltage was set to 3.0 kV and in-source fragmentation of 40 V with a collision energy of 40% was applied.

3. Results and discussion

FlFFF separations of HDL and LDL from rabbit sera (control and high cholesterol group) and human plasma (control and CAD patient) samples are compared in Fig. 2a which was obtained at $\dot{V}_{in}/\dot{V}_{out} = 0.4/0.20$ mL/min using a single module of HF5. Each serum sample was stained with SBB for detection at 600 nm. In Fig. 2a, the level of HDL from CAD patient was decreased while that of LDL was increased along with a reduction in LDL sizes (decrease in retention time), compared to those of healthy human controls, as shown in an earlier report [15]. Compared to the human control, the concentration of lipoproteins from the rabbit control (same injection volume, 20 μL of depleted plasma) was a lot lower but similar profiles of the relative level of each lipoprotein and the retention times of HDL and LDL were observed. The latter supports the assumption that the lipoprotein sizes in rabbit serum are approximately the same as those in human serum. For the CAD patient and the rabbit with HC, the relative level of HDL was decreased while that of LDL was increased, compared to that of each control. As the peak of LDL from rabbits with HC was observed to be very broad, unlike that of other samples, LDLs under HC conditions can be assumed to exist in a wide range of particle sizes. Details of the elution patterns, obtained at $\dot{V}_{in}/\dot{V}_{out} = 0.40/0.15$ mL/min, of other rabbit sera from the five groups of rabbits that were pooled by group are shown in Fig. 2b. A slightly different flow condition was utilized in Fig. 2b in order to ensure the complete separation of HDL and LDL and avoid any overlap as much as possible. Fig. S1 in Supplementary data shows the fractograms of lipoproteins from 20 μL individual serum samples (depleted) and a pooled sample from rabbits with HC by HF5 (single channel), and demonstrates that they all share similar retention times for the lipoproteins whether the samples are pooled or not. This supports the conclusion that the fractograms presented in Fig. 2b (all pooled samples) are a good reflection of the individual samples. As the retention times of HDL from the control, inflammation, and dehydrated models are similar to each other (8.8 ± 0.4, 9.2 ± 0.4, and 9.0 ± 0.3 min ($n = 3$) in Fig. 2b, respectively), they share similar sizes of HDL particles. However, the HDL amount of dehydrated models was the highest among all groups. This can be partially explained by the increased concentration of all lipoproteins due to the reduced amount of water in the body. A similar result was observed in the LDL peaks; the retention times of LDL were detected at 19.5 ± 1.0, 19.0 ± 0.9, and 20.1 ± 0.7 min ($n = 3$) for control, inflammation, and dehydrated models, respectively. However, the levels of LDL, calculated based on the peak area, were different such that LDL from the inflammation model was increased

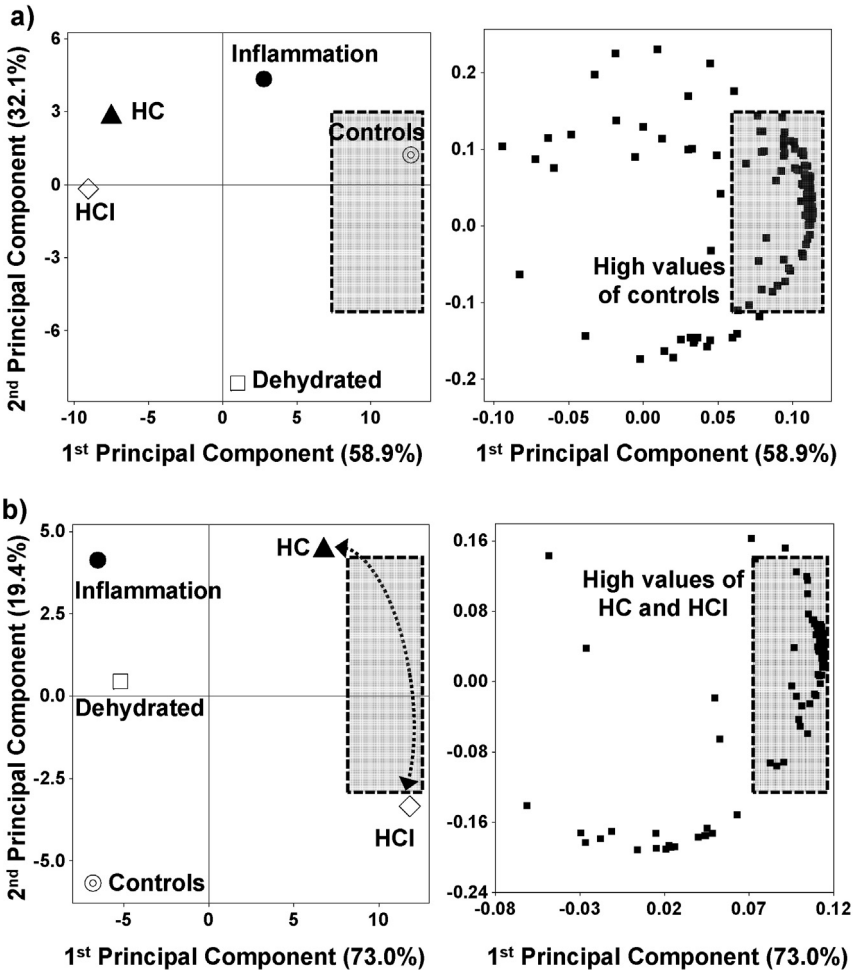

Fig. 3. Principle component analysis (PCA) plots of all lipids quantified from (a) HDL and (b) LDL from pooled serum samples of the different rabbit groups using nLC-ESI-MS/MS. The loading plot on the right depicts relatively high distribution of lipids according to the scores plot on the left.

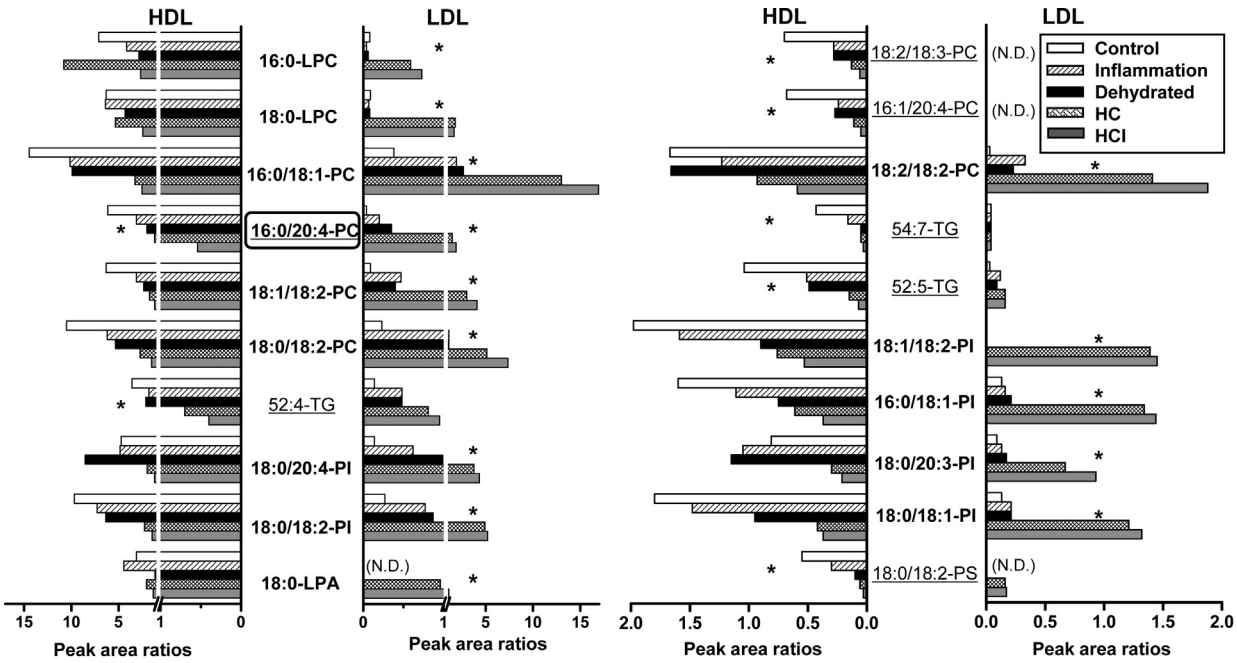

Fig. 4. Relative peak area (vs. IS) of lipid species from HDL (underlined and star marked) LDL (bold and star marked) exhibiting more than a 10-fold difference between rabbit groups, based on the analysis by nLC-ESI-MS/MS.

about twofold compared to that of control, while LDL in the dehydrated model was increased by more than threefold. While the sizes of the lipoprotein particles from both the inflammation and dehydration models remained unchanged based on the slight change in retention times, it would be expected that the increased level of LDL may play a role in the development of metabolic diseases. On the other hand, a HC (high cholesterol) diet affected both the size and the level of lipoproteins in rabbit groups with or without induced inflammation. While the levels of HDL for both the HC and HCI groups was significantly lower, those of LDL were increased by greater than three- to fourfold for the HC and HCI groups, respectively. It is apparent that the changes in HDL and LDL were more intense when the inflammation effect was combined with HC.

Based on the separation of the single HF5 in Fig. 2b, the lipoproteins from 100 μL of pooled serum samples were separated for a semi-preparative scale collection of lipoproteins by the MxHF5 modules ($\dot{V}_{in}/\dot{V}_{out} = 2.4/0.9$ mL/min). Lipids from these HDL and LDL fractions were analyzed by nLC-ESI-MS/MS, yielding the identification of a total of 122 and 104 lipid species from HDL and LDL, respectively, as listed in Table S1 in Supplementary data. The data from each lipid species listed in Table S1 represent the relative peak areas which are the ratios of the measured peak areas of the target species to that of an internal standard (IS, 1 pmol of 13:0/13:0-PC), after the peak areas from each species were multiplied by the volume of the dissolving solvent added to the dried lipids at the end of the extraction in order to adjust the lipid concentration. The possible combinations of the three acyl chains of triacylglycerol (TG) confirmed by CID spectra are listed in Table S2 in Supplementary data, without information on the exact chain locations. For a brief overview of the statistical difference in the distribution of lipids among groups, results of principal component analysis (PCA) were plotted as shown in Fig. 3, based on the relative peak area ratios (variables) from all five groups of pooled samples (subjects) in Table S1. The scores plots on the left of Fig. 3a and b show five subjects of groups scattered at different positions and the corresponding loading plots on the right demonstrate the distribution of all 122 and 104 lipids from HDL and LDL, respectively. As components on loading plots reflect the high abundance of variables on the equivalent location of scores plot, the clustered points (lipids) on farther right from the loading plot of Fig. 3a indicate relatively high amount of lipids among controls for HDL. In Fig. 3b, the clustered components on the loading plot denote HC and HCI groups to show relatively high abundance of lipids for LDL. During this analysis, several important findings were obtained. 18:0-LPA and 18:1/18:2-PI derived from LDL were not detected at all in the control, inflammation, and dehydrated groups, but appeared in two other groups (HC and HCI) at relatively high concentrations, as their peak area ratios were close to or over 1 (relative to 1 pmol of IS) in Table S1. Also, the results of nLC-ESI-MS/MS support the idea that the level of lipoproteins does not represent the overall level of lipids, and consequently suggests that an analysis of lipoprotein specific lipids is required. For example, the overall concentrations of LPC from HDL were generally highest in HC group, unlike other species. For PC from HDL, the controls had the highest concentrations, followed by the inflammation and dehydrated groups which shared similar levels, and the HC and HCI groups. The differences between the HC and HCI groups were considerable with respect to PC levels, but insignificant for LPE, PE, SM, PA, PG, Cer and MHC levels. For LDL, the HCI group had the highest overall concentrations of most lipid classes. While many species showed differences in concentration between the different rabbit groups, 7 species (3 PC, 3 TG, 1 PS) from HDL and 14 species (2 LPC, 5 PC, 1 LPA, 6 PI) from LDL exhibited significant differences of greater than 10-fold between the groups with the highest and lowest concentrations of the corresponding species with p-values from t test smaller than 0.01, and these species are marked in bold in Table S1. These species with significant differences were selected for targeted analysis using the top-down method and are listed in Table 1, which will be described later in detail. Also, peak area ratios of these species are plotted in Fig. 4 and it clearly illustrates that levels of most lipids from LDL were much higher in HC or HCI groups than the other three groups. This supports the hypothesis that a HC diet affects the concentrations of lipids in LDL more than in HDL, as the 16:0/20:4-PC species was increased in the HCI group by approximately 45-fold compared

Table 1
Comparison of quantification of lipids from lipoproteins between nLC-ESI-MS/MS and cAF4-ESI-MS/MS. The relative peak areas ratios from control, inflammation, dehydrated, and HC groups are written in respect to those of HCI for direct comparison.

Class	Molecular species	*m/z*	Lipo-proteins	Control/HCI		Inflammation/HCI		Dehydrated/HCI		HC/HCI	
				nLC–MS/MS	cAF4-MS/MS	nLC–MS/MS	cAF4-MS/MS	nLC–MS/MS	cAF4-MS/MS	nLC–MS/MS	cAF4-MS/MS
LPC	16:0	496.5	HDL	2.65 ± 0.68	2.30 ± 1.14	1.55 ± 0.23	1.78 ± 0.98	1.06 ± 0.21	1.34 ± 0.67	4.03 ± 1.00	2.67 ± 1.47
			LDL	0.11 ± 0.02	N.Q.	0.05 ± 0.01	N.Q.	0.08 ± 0.01	N.Q.	0.81 ± 0.18	N.Q.
	18:0	524.5	HDL	2.60 ± 0.77	2.43 ± 0.61	2.63 ± 0.38	2.33 ± 0.82	1.77 ± 0.29	2.02 ± 0.50	2.20 ± 0.35	1.61 ± 0.42
			LDL	0.05 ± 0.01	N.Q.	0.04 ± 0.01	N.Q.	0.05 ± 0.01	N.Q.	1.07 ± 0.21	1.04 ± 0.59
PC	36:5	780.5	HDL	6.46 ± 0.44	10.08 ± 3.20	2.88 ± 0.21	6.35 ± 1.86	4.08 ± 0.19	6.89 ± 2.45	1.50 ± 0.14	1.95 ± 0.74
			LDL	N.D.	N.Q.	0.17 ± 0.15	N.Q.	0.24 ± 0.08	0.29 ± 0.08	0.86 ± 0.34	0.98 ± 0.21
	36:4	782.5	HDL	6.92 ± 0.44	9.61 ± 2.47	3.85 ± 0.33	4.33 ± 0.58	3.26 ± 0.24	4.18 ± 0.73	1.88 ± 0.15	1.13 ± 0.31
			LDL	0.02 ± 0.00	N.Q.	0.14 ± 0.02	N.Q.	0.15 ± 0.03	0.31 ± 0.09	0.77 ± 0.07	0.92 ± 0.21
	34:1	758.5	HDL	5.74 ± 0.80	6.08 ± 1.73	4.03 ± 0.29	4.65 ± 1.31	3.94 ± 0.40	3.85 ± 1.13	1.30 ± 0.07	1.20 ± 0.29
			LDL	0.02 ± 0.00	N.Q.	0.11 ± 0.02	N.Q.	0.16 ± 0.05	0.15 ± 0.03	0.77 ± 0.11	0.82 ± 0.20
	36:3	784.5	HDL	5.36 ± 0.62	7.88 ± 2.78	2.64 ± 0.36	3.71 ± 0.94	1.98 ± 0.23	2.96 ± 0.67	1.47 ± 0.19	1.17 ± 0.32
			LDL	0.02 ± 0.00	N.Q.	0.12 ± 0.03	0.16 ± 0.07	0.10 ± 0.03	0.18 ± 0.08	0.73 ± 0.11	0.69 ± 0.21
	36:2	786.5	HDL	6.82 ± 0.91	7.11 ± 1.54	3.98 ± 0.43	4.58 ± 1.37	3.39 ± 0.34	3.58 ± 0.77	1.51 ± 0.16	1.16 ± 0.28
			LDL	0.03 ± 0.01	N.Q.	0.15 ± 0.03	N.Q.	0.15 ± 0.01	0.24 ± 0.06.	0.70 ± 0.06	0.59 ± 0.21
TG	54:7	894.8	HDL	14.33 ± 1.00	11.37 ± 3.09	4.67 ± 0.57	4.22 ± 1.46	3.33 ± 0.87	2.61 ± 0.95	1.33 ± 0.09	1.05 ± 0.33
			LDL	1.00 ± 0.00	N.Q.	1.00 ± 0.25	N.Q.	1.00 ± 0.00	N.Q.	1.00 ± 0.00	N.Q.
	52:4	872.9	HDL	10.53 ± 0.96	13.53 ± 5.17	5.26 ± 0.47	7.58 ± 2.07	6.24 ± 0.57	8.29 ± 2.55	2.06 ± 0.18	1.37 ± 0.35
			LDL	0.15 ± 0.01	0.66 ± 0.26	0.51 ± 0.11	0.62 ± 0.23	0.51 ± 0.09	0.68 ± 0.24	0.85 ± 0.03	0.81 ± 0.31
PI	18:1, 18:2	859.5	HDL	3.74 ± 0.66	4.43 ± 0.73	3.00 ± 0.64	4.28 ± 1.15	1.70 ± 0.29	3.31 ± 0.57	1.43 ± 0.25	1.46 ± 0.52
			LDL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.96 ± 0.07	0.76 ± 0.21
	16:0,18:1	835.6	HDL	4.32 ± 0.43	4.00 ± 1.46	3.00 ± 0.64	3.72 ± 1.34	2.03 ± 0.38	3.35 ± 1.33	1.65 ± 0.23	1.40 ± 0.54
			LDL	0.09 ± 0.01	N.Q.	0.11 ± 0.01	0.20 ± 0.07	0.15 ± 0.01	N.Q.	0.93 ± 0.09	0.95 ± 0.14
	18:0,20:4	885.6	HDL	3.97 ± 0.35	3.37 ± 1.03	4.06 ± 0.33	3.97 ± 1.29	7.17 ± 0.51	6.76 ± 3.22	1.66 ± 0.31	1.21 ± 0.44
			LDL	0.03 ± 0.00	N.Q.	0.14 ± 0.02	0.10 ± 0.03	0.23 ± 0.01	0.29 ± 0.11	0.87 ± 0.08	0.86 ± 0.32
	18:0, 18:2	861.6	HDL	6.68 ± 0.83	6.33 ± 1.97	5.01 ± 1.81	5.63 ± 1.68	4.40 ± 0.40	4.76 ± 2.52	1.57 ± 0.40	1.08 ± 0.30
			LDL	0.05 ± 0.00	N.Q.	0.15 ± 0.02	0.17 ± 0.05	0.17 ± 0.03	0.20 ± 0.05	0.95 ± 0.06	0.86 ± 0.38
	18:0,20:3	887.6	HDL	3.86 ± 0.88	N.Q.	5.00 ± 1.76	N.Q.	5.48 ± 1.66	N.Q.	1.43 ± 0.28	N.Q.
			LDL	0.10 ± 0.01	N.Q.	0.14 ± 0.02	N.Q.	0.18 ± 0.02	N.Q.	0.72 ± 0.07	0.83 ± 0.43
	18:0, 18:1	863.6	HDL	4.86 ± 0.85	5.89 ± 0.93	4.00 ± 0.50	5.32 ± 0.75	2.57 ± 0.50	2.88 ± 1.44	1.14 ± 0.11	1.59 ± 0.33
			LDL	0.10 ± 0.04	N.Q.	0.16 ± 0.03	N.Q.	0.16 ± 0.02	0.54 ± 0.26	0.92 ± 0.12	0.96 ± 0.22
PS	18:0, 18:2	786.5	HDL	18.33 ± 2.97	25.91 ± 9.27	10.00 ± 2.54	13.42 ± 5.12	6.33 ± 0.81	24.96 ± 10.6	2.00 ± 0.25	1.86 ± 1.11
			LDL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.94 ± 0.34	0.97 ± 0.24
LPA	18:0	437.5	HDL	2.30 ± 0.29	1.31 ± 0.97	3.29 ± 0.43	2.14 ± 0.62	0.86 ± 0.11	2.15 ± 0.98	1.51 ± 0.22	1.67 ± 0.78
			LDL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.87 ± 0.16	N.Q.

with controls. According to a study that investigated total TG levels from participants who were fed diets with either 1400 or 400 mg of cholesterol per day for a month, TG levels increased slightly but not as dramatically as 10-fold [26]. Similar result was observed from this study in agreement, in which levels of only 3 TG species were significantly altered (> 10-fold) by metabolic factors.

While the 20 lipid species (16:0/20:4-PC selected for both HDL and LDL) in Fig. 4 were selected as target lipid molecules from the bottom-up quantitative analysis by nLC-ESI-MS/MS, the same species were quantified again by the top-down lipid analysis of individual samples using cAF4-ESI-MS/MS. Fig. 5 shows the comparison of the extracted ion fractograms (EIFs) obtained by injecting individual sample into cAF4-ESI-MS/MS at a flow rate condition for cAF4, $\dot{V}_{in}/\dot{V}_{out} = 0.40/0.013$ in mL/min, using the SRM method. For instance, the fractograms in Fig. 5a were generated from the detection of 34:1-PC which was obtained by extracting the peak intensity of a specific product ion (*m/z* 575.6, $[M+H-183]^+$) from the SRM transition of the precursor ion *m/z* 758.6 ($[M+H]^+$). In cAF4-ESI-MS/MS, PC species were selectively monitored by the SRM transition of $[M+H]^+ \rightarrow [M+H-183]^+$, a typical fragment ion resulting from the loss of a phosphocholine head group ($HPO_4(CH_2)_2N(CH_3)_3$, 183 Da). The data in Fig. 5b is for 36:3-PC and was based on the SRM transition of (*m/z* 784.6 → 601.6). Due to the detection of PC as a loss of a head group, the chain structure of the PC species from cAF4-ESI-MS/MS analysis was written in the form of the total carbon number of acyl chains and double bonds as 36:3-PC instead of the R1/R2 forms. Therefore, the summed signals from the possible isomers, such as 18:1/18:2-PC, 18:0/20:3-PC, etc., which were not distinguishable in cAF4-ESI-MS/MS are listed in Table 1. However, other lipid species are expressed with the full chain structures as their quantifier ions enable one to distinguish chain structures. For the compensation of run-to-run variation in cAF4-ESI-MS/MS, carbonic anhydrase (CA) was added to each rabbit sample as an IS (500 ng and 2 μg for positive and negative ion modes, respectively, for each injection) so that the concentration of each lipid species could be expressed as a relative peak area to that of CA (based on a SRM transition of *m/z* 1001.5 ($[M+29H]^{+29}$) → 983.5 (y_{69}^{+8}) in positive ion mode and of 1318.3 ($[M-22H]^{-22}$) → 1301.3 (y_{141}^{-12}) in negative ion mode). A lipid standard could not be included to sample as an IS since the membrane from cAF4 would not be able to retain it (10 kDa MWCO), while a protein standard like CA (29 kDa) would be easily retained. Although CA exists in human blood plasma, the effect of natural CA in plasma on quantitation can be treated as negligible compared to the amount added and its addition as IS holds no effect on ionization efficiency of lipids, according to a previous report [22]. In Fig. 5, the targeted lipid species detected by cAF4-ESI-MS/MS exhibited distinct differences in concentration between lipoproteins (HDL and LDL) and between the different rabbit groups. The HC and HCI groups exhibited extremely small HDL peaks compared to those from the other three groups, while they showed much larger and broader peaks of LDL. In particular, the 34:1-PC molecules appeared to be similar in their abundances in HDLs from control and inflammation groups (Fig. 5a), however, 36:3-PC decreased significantly in the inflammation group compared to that of the control sample (Fig. 5b). This demonstrates the feasibility of top-down lipidomic analysis with cAF4-ESI-MS/MS using SRM to distinguish and quantify lipids that exist in different

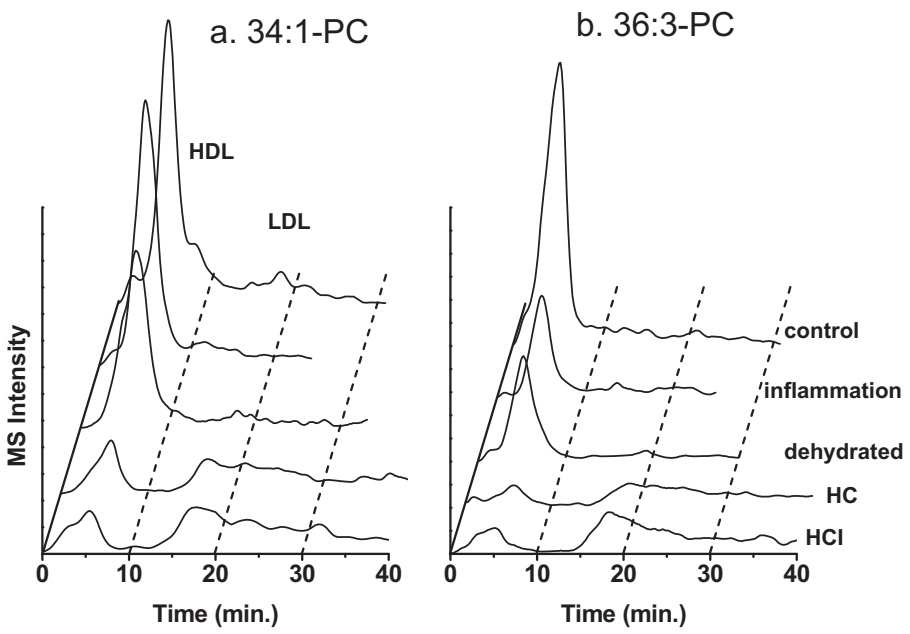

Fig. 5. Extracted ion fractograms (EIFs) from SRM transition of (a) 34:1-PC (of m/z 758.6 → 575.6) and (b) 36:3-PC (m/z 784.6 → 601.6) by cAF4-ESI-MS/MS for the five different rabbit groups.

concentrations. Results from the quantification of the 20 selected lipids by cAF4-ESI-MS/MS were compared with those obtained by nLC-ESI-MS/MS (listed in Table S1). In order to overcome differences in the measured values between the two analytical methods, results were normalized by calculating the ratio of the peak area of a species to that of the HCI group as a normalization reference, as listed in Table 1. Since the HCI group contained most of the lipid species that were selected for the quantitative comparison while other rabbit groups did not, data from the HCI group was utilized as a reference to compare the relative amounts obtained between nLC-ESI-MS/MS and cAF4-ESI-MS/MS. Quantified ions of other lipid classes were as follows: $[M+NH_4]^+ \rightarrow [M+NH_4\text{-}RCOONH_4]^+$ for TGs, $[M-H]^- \rightarrow [M-H\text{-}RCOOH]^-$ for PIs, of which the product ions were produced from the loss of an acyl chain in the form of ammonium carboxylate or carboxylic acid, respectively, and $[M-H]^- \rightarrow [M-H-87]^-$ for PSs, in which 87 amu was from serine ($C_3H_5NO_2$). The average peak area ratio of each species with respect to HCI group is plotted in Fig. 6, with filled symbols for the LC–MS results and open symbols for the cAF4-MS results. For instance, the average peak area ratios of 34:1-PC from HDL with respect to the HCI group were 5.74 ± 0.80 vs. 6.08 ± 1.73 (nLC-ESI-MS/MS vs. cAF4-ESI-MS/MS methods) for the control, 4.03 ± 0.29 vs. 4.65 ± 1.31 for the inflammation group, 3.94 ± 0.40 and 3.85 ± 1.13 for the dehydrated group, and 1.30 ± 0.07 (nLC-ESI-MS/MS) and 1.20 ± 0.29 for the HC group. The lipid species plotted in Fig. 6 are those found in Fig. 4 and show relatively large differences in comparison to the HCI group. Analyses of these species demonstrated that the nLC-ESI-MS/MS and cAF4-ESI-MS/MS results were similar. Paired t-test to compare individual differences between the two methods was carried out for the selected species and the resulting p-values are listed in Table S3 in Supplementary data. As the values are mostly higher than 0.05, the two methods are not significantly different at the 95% confidence level for most of the selected species, except for a few with less than a fivefold of difference with respect to the HCI group. These exceptions found with a few species may originate from spectral congestion when the different lipids were simultaneously introduced to MS in the form of lipoprotein particles by cAF4-ESI-MS/MS, and in part from relatively poor ionization due to the introduction of the aqueous carrier solution of cAF4 to MS. Even though a stream of CH_3CN with ionization modifiers was mixed

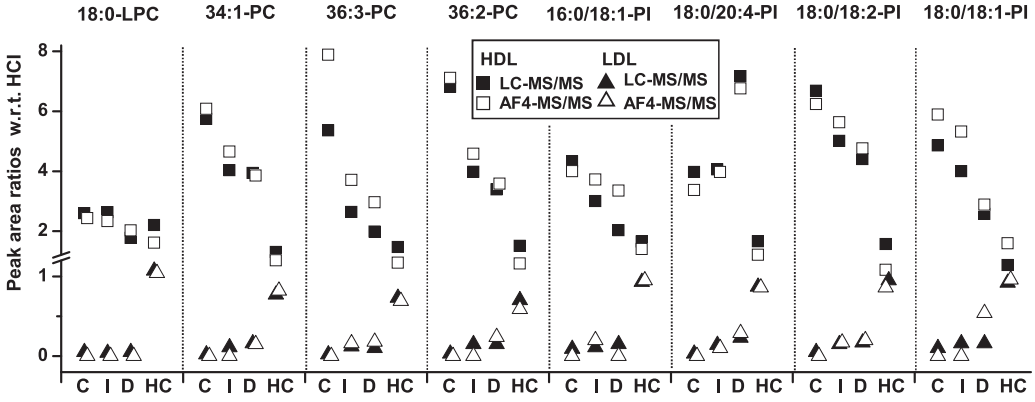

Fig. 6. Comparison of peak area ratios of lipids species from HDL and LDL, with respect to those of the corresponding species from the HCI group, determined by nLC-ESI-MS/MS and cAF4-ESI-MS/MS methods.

with the cAF4 effluent on-line prior to ESI, a considerable amount of aqueous solvent lowers the ionization efficiency compared to the injection with organic solvents to ESI when nLC was used. These two effects have a significant influence on the quantitation of low abundant lipid species by cAF4-ESI-MS/MS. Nonetheless, the lipid species shown in Fig. 6 support the conclusion that the top-down cAF4-ESI-MS/MS method can be utilized as an alternative platform for targeted lipidomic analysis once the target species are well established.

4. Conclusion

In this study, lipidomic analysis of lipoproteins from rabbit serum samples were carried out to elucidate the lipid species affected by different metabolic conditions using both bottom-up and top-down approaches. As expected, the PLs of rabbits varied with their metabolic conditions, and the HC and HCI groups (high cholesterol-treated groups) showed the most contrasting amount of PLs when compared to those of healthy controls. The overall concentrations of lipids from HDL were highest in controls and lowest in HC-treated groups, while those from LDL were highest in both of the HC-treated groups and lowest in controls. This study shows that a HC diet causes profile of most PLs and TGs to differ from those of healthy controls far more seriously than inflammation or dehydration. Also, multiple factors, such as consumption of a HC diet combined with inflammation, are found to be even more threatening than a HC diet alone. Of the 122 and 104 lipid species identified by nLC-ESI-MS/MS from HDL and LDL, respectively, from pooled serum samples of rabbits, 7 species from HDL and 14 species from LDL showed greater than a 10-fold difference between the groups with the lowest and highest concentration of these corresponding species (p-values less than 0.01). Among these species, 16:0/20:4-PC was the only species that exhibited greater than a 10-fold difference in both HDL and LDL between the groups, while the remaining 19 species showed significant differences in only HDL or LDL. This indicates that lipids in different classes of lipoproteins are differentially affected by metabolic conditions. When they were quantified again from individual samples using cAF4-ESI-MS/MS as a top-down direct analysis method, the quantitative results for the selected lipids from both methods were statistically equivalent for most of the selected species, with a few technical limitations. The latter may originate from a relatively poor ionization of lipids due to the use of an aqueous solution for cAF4 and possible spectral congestion during the direct injection of lipoprotein. Nonetheless, the top-down approach has the advantage of being a high-speed screening method for targeted lipids. For instance, lipid extraction of lipoprotein fractions from MxHF5 requires more than half a day of effort prior to nLC-ESI-MS/MS, while only 30 min were required for the depletion of highly abundant proteins from a blood sample for cAF4-ESI-MS/MS. When a large set of samples needs to be run, analysis by cAF4-ESI-MS/MS can be done in substantially shorter analysis time with simple preparation. As nLC-ESI-MS/MS provides a comprehensive and untargeted analysis of lipid species, it can serve as a primary platform to “scan” the lipidome of samples while the top-down platform of cAF4-ESI-MS/MS can be utilized to “spot” the targeted species during a high-speed screen from a large set of samples.

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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.2015.05.059

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