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REVIEW

# Flow Field-Flow Fractionation with Mass Spectrometry for Top-Down and Bottom-Up Lipidomics

**Seul Kee Byeon**[1] · **Myeong Hee Moon**[1]

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**Abstract** As lipidomics has attracted increased attention in life science, advanced mass spectrometry (MS) technologies have been combined with other separation techniques to improve and expand the branch of study. This review intends to provide general knowledge of offline and online coupling of flow field-flow fractionation (FlFFF)—a technique that encompasses the separation of nano- to micro-scale biomolecules—with MS for analysis of blood plasma lipoproteins, processes that are considered bottom-up and top-down approaches, respectively. The first part of this review focuses on the bottom-up method using multiplexed hollow fiber FlFFF (MxHF5) and nanoflow liquid chromatography electrospray-ionization tandem mass spectrometry (nLC-ESI–MS/MS) for non-targeted identification of lipids. In this protocol, plasma lipoproteins of different types are collected using MxHF5, and the lipids within the lipoproteins are then extracted and analyzed via nLC-ESI–MS/MS. The second part of the review describes the top-down approach, which uses online coupling of miniaturized FlFFF to ESI–MS for a fast screening of targeted lipids. Here, the separation of lipoproteins and detection of their component lipids are achieved simultaneously. While both methods aim to quantify the lipids within lipoproteins, the bottom-up approach provides an extensive lipidome, whereas the top-down method is suitable for high-speed targeted lipidomic analysis. This review discusses variants of FlFFF-ESI–MS/MS that offer effective analytical technologies for lipidomics.

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**Keywords** Lipids · Lipoproteins · Flow field-flow fractionation · Nanoflow liquid chromatography electrospray-ionization tandem mass spectrometry

## 1 Introduction

Because lipids are engaged in various cellular processes such as cell signaling, energy storage, and cell structure composition, they can be regarded as potential disease markers, as their abnormal metabolism is related with the onset of many illnesses [1–3]. Among the different classes of lipids, the most abundant are the phospholipids (PLs), which are amphipathic structures consisting of non-polar fatty acyl chains connected to a polar phosphate group by a glycerol backbone. Another branch consists of the sphingolipids (SL), the backbone of which—sphingosine—contains one alkyl chain bonded to an amino alcohol. PLs and SLs can be classified into further subtypes depending on their head groups, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PS), and phosphatidylglycerol (PG) for PLs; and ceramide (Cer), sphingomyelin (SM), monohexosylceramide (MHC), and dihexosylceramide (DHC) for SLs.

Lipids are combined with proteins to form spherical macromolecules called lipoproteins. Since the polar heads of their lipids face outwards, lipoproteins travel freely in the bloodstream while carrying insoluble fats and cholesterol; consequently, their levels are reportedly correlated with states of health [4]. Abnormal levels of lipoproteins trigger various health problems such as atherosclerosis and cardiovascular diseases. Lipoproteins are classified according to their densities, with each class playing a different role. High-density lipoproteins (HDLs) carry

cholesterol and fats from various tissues into the liver for degradation and in contrast, low-density lipoproteins (LDLs) carry lipids and cholesterol from the liver to other tissues. As these lipoproteins play critical roles in human health, maintaining the proper ratio of HDL to LDL (or even very low-density lipoproteins, VLDL) by increasing the level of HDL and lowering that of LDL is crucial in prevention of general disorders [5].

Lipoproteins can be separated using various techniques, such as density gradient ultracentrifugation (DGU), polyacrylamide gel electrophoresis (PAGE), size exclusion chromatography (SEC), and ion exchange chromatography (IEC); however, each technique has limitations that cannot be overlooked. DGU requires relatively large amounts of samples and is time-consuming [6–8]. Samples subjected to PAGE require intricate procedures to be retrieved from gel for further analysis [9, 10]. Separation using SEC or IEC can be performed rapidly, but requires the use of packing materials that may undergo problematic interactions with samples [11–13]. An alternative analytical technique that can overcome the drawbacks of these techniques is flow field-flow fractionation (FlFFF), of which separation takes place inside an empty channel as shown in Fig. 1; the lack of any packing material dramatically lessens potential sample loss.

FlFFF is an elution-based technique that utilizes two flow streams that are perpendicular to each other to achieve the size-based separation of the sample components [11, 14–16]. The migration flow carries the sample through the FlFFF channel as the perpendicular crossflow moving across the channel drives sample components towards their equilibrium heights. When a sample is injected into the FlFFF channel, a focusing/relaxation procedure is applied to the sample components via two opposing flow streams emerging from the inlet and outlet of the channel. This causes sample components smaller than the size of the membrane pores to exit through the crossflow, while components larger than the pores are retained inside the channel and become equilibrated. Sufficient time (usually 3–5 min) needs to be allocated for the focusing/relaxation period to ensure that the sample components reach their respective equilibrium positions, as smaller components diffuse faster and reach a higher equilibrium position than do larger ones. During the separation step—in which the migration flow enters the system from the inlet channel and drives the retained sample components toward the outlet—the smaller components elute earlier than do the larger ones. This is because the velocity of the flow stream farther from the channel wall is greater than that of the parts closer to the wall, creating a parabolic flow. Using this principle, HDLs can be separated from the larger LDLs not through their difference in density, but rather their significant difference in size.

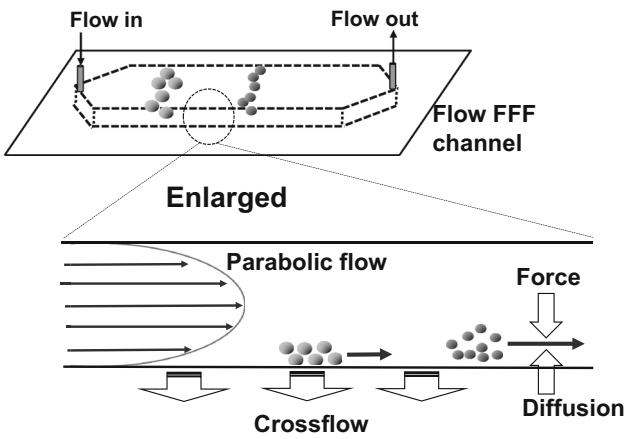


**Fig. 1** Schematics of the flow FFF channel

Lipids within lipoproteins can be analyzed by mass spectrometry (MS) with high accuracy, resolution, and sensitivity. Electrospray ionization–MS (ESI–MS) is the most frequently used soft ionization technique and allows for the sample to be injected into MS by direct infusion [17]. ESI–MS readily detects lipids within complex samples such as plasma but only lipids that are easily ionized tend to be detected. In other words, lipids that do not yield strong MS response are not likely to be detected due to suppression of ionization caused by spectral congestion of other lipids. To overcome this, chromatographic techniques such as thin layer chromatography (TLC), gas chromatography (GC), and liquid chromatography (LC) are often coupled to MS. TLC is simple, fast and relatively cheap, but its low resolution and sensitivity limits its value to lipidomics [18]. GC offers high resolution and sensitivity when applied to non-polar lipids such as triacylglycerol (TG); however, relatively polar lipids must be derivatized before analysis is possible. On the other hand, a wide range of lipids—regardless of their polar or non-polar characteristics—can be analyzed by LC in intact conditions. Fittingly, LC–ESI–MS has been shown to be a highly efficient analytical platform for lipidomics studies. In case of normal-phase LC (NPLC), lipids are separated according to their head groups, though individual lipids with identical head groups cannot be differentiated. However, reversed-phase LC (RPLC) distinguishes lipids based on the hydrophobic interaction between their fatty acyl chains and a stationary phase, which eventually leads to a comprehensive profile of all the lipids in a sample [19]. The studies described in this review employed RPLC-ESI–MS/MS for the identification and quantification of lipids.

Lipids play critical roles in disease progression and lipoprotein levels, particularly those of HDL and LDL [4]. Plasma lipoproteins must first be fractionated, then subjected to LC–ESI–MS/MS to study the composition and concentration of the lipids within them and thus better

understand the relation between lipids and disease progression. In recent years, FlFFF has been coupled online and offline to MS for top-down and bottom-up lipidomic analysis. In a bottom-up approach, lipoproteins are fractionated and collected from plasma using FlFFF; the lipids are then extracted and analyzed using nanoflow LC–ESI–MS/MS to determine their structures and quantities. The top-down approach employs online FlFFF-ESI–MS/MS for simultaneous separation of lipoproteins and detection of the constituent lipids. This review aims to provide an understanding of the bottom-up and top-down FlFFF-MS techniques that can be used to investigate the lipidome and its connection to disease states at the molecular level.

## 2 Bottom-Up Lipidomic Analysis of Lipoproteins

### 2.1 Separation of Lipoproteins Using FlFFF

Among the different types of FlFFF channels that can be utilized to separate lipoproteins, multiplexed hollow fiber FlFFF (MxHF-FlFFF, MxHF5) channels are suitable for semi-preparative purposes [20]. Hollow fiber FlFFF (HF5) is a variant of FlFFF that utilizes a hollow fiber (HF) membrane as the separation channel. HF module can be prepared by inserting a HF membrane in a glass tubing using a low pressure union and connection tee. One end of the channel is connected to an injector, while the other end is connected to a detector. For separation of lipoproteins, 0.1 M of phosphate buffered saline (PBS) or Trizma buffered saline (TBS) is used as a carrier solution to maintain pH and keep metabolites in their native states. As Fig. 2 demonstrates, three-way and four-way valves are used to switch the direction of the flow between focusing and separation [21]. In MxHF5, instead of using a single HF channel, six to eight channels are simultaneously connected to the inlet and outlet to increase the throughput [21]. Moreover, as hollow fibers are significantly cheaper than other types of membranes utilized in FlFFF, they are highly cost-effective and can be easily replaced while working with a large sample set. Separation of lipoproteins can be detected by UV/Vis as shown in Fig. 3 [21]. As the size of HDL is substantially smaller than that of LDL and very low-density lipoproteins (VLDL), it elutes at earlier retention times, separating itself from the latter two. VLDL requires a step elution in which the flow rate is reduced by decreasing the crossflow rate to zero after the elution of LDL, as applying the flow rate used for separation of HDL/LDL to VLDL would take an excessive amount of time. As Fig. 3 illustrates, VLDL can be fractionated using FlFFF and collected for further analysis. The fractionated lipoproteins are collected and concentrated in a volume of approximately 500 μL for lipidomic analysis [21].

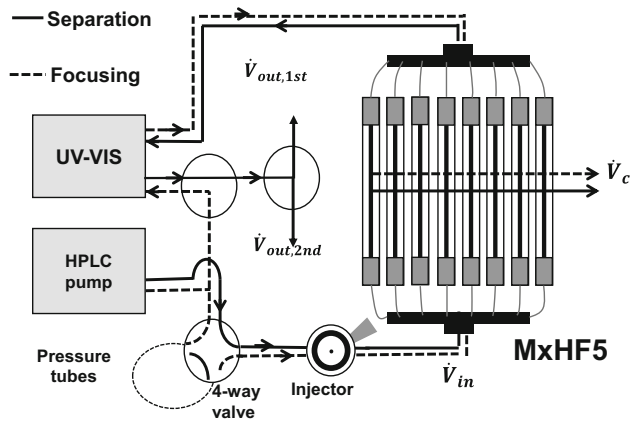


**Fig. 2** System configuration of MxHF5-UV–Vis for collection of lipoproteins from plasma. Reprinted with permission from [21]. Copyright 2014 American Chemical Society

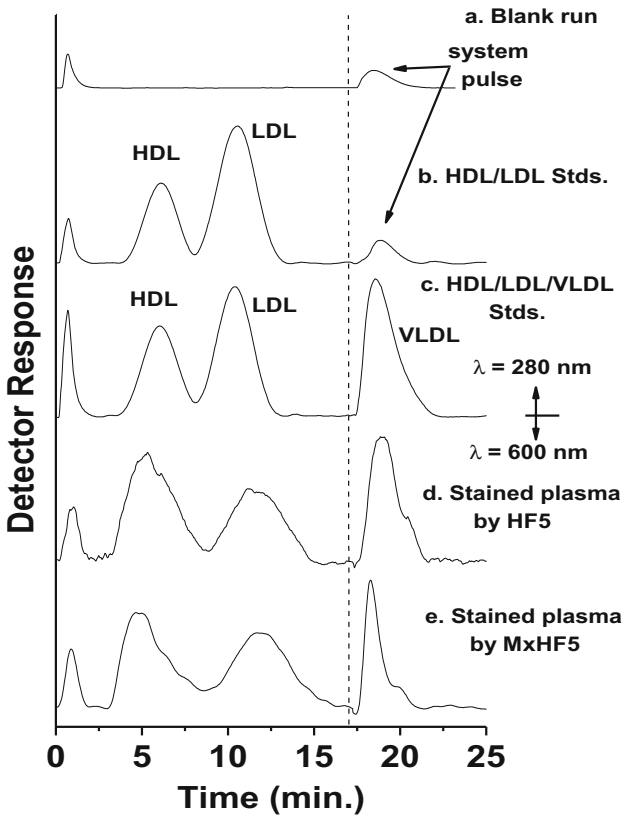


**Fig. 3** Fractograms of lipoproteins using a single HF-FlFFF (*a–d*) and MxHF5 (*e*). *a* Blank run with flow condition changed at 17 min. *b* Separation of HDL and LDL standards. *c* Separation of HDL, LDL, and VLDL standards. *d* Separation of SBB-stained HDL, LDL, and VLDL from plasma. *e* Separation of SBB-stained HDL, LDL, and VLDL from plasma using MxHF5. Reprinted with permission from [21]. Copyright 2014 American Chemical Society

### 2.2 Lipid Extraction

Following fractionation of lipoproteins using FlFFF, the lipids within each fraction must be isolated from accompanying salts, proteins, and metabolites, as they can suppress ionization and consequently interfere with MS detection of lipids. Among the different types of commonly used lipid extraction techniques that are conventionally utilized, a modification of the Folch method incorporating MTBE/$CH_3OH$ has been shown to yield the highest recoveries of various PLs and SLs [22, 23]; therefore, Folch modified with MTBE/$CH_3OH$ is used to extract the lipids from lipoproteins for studies introduced in this review.

### 2.3 Nanoflow LC–ESI–MS/MS for Analysis of Lipids

As mentioned, the use of LC prior to ESI–MS/MS decreases sample complexity, reduces ionization suppression, and thus provides a comprehensive profile of lipids including those found in trace amounts. Nanoflow LC (nLC) consumes far less sample while increasing the sensitivity of ESI–MS: the smaller droplets formed at the junction between the nLC column and the ESI–MS apparatus leads to a greater number of ions entering the MS. For nanoflow analysis, a pulled-tip capillary tubing (75 μm ID and 360 μm OD) packed with C18 particles is used for the analytical column and is directly attached to the MS without an emitter. A polar mobile phase composed of water and organic solvents, such as $H_2O$:$CH_3CN$ (9:1, v/v), is used to load the sample into the analytical column, while an organic mobile phase such as isopropanol:$CH_3OH$:$CH_3CN$ (6:2:2, v/v/v) is used to gradually elute the sample off the column. Over various tests, a flow rate of 300 nL/min was found to be optimal for the separation and subsequent ESI–MS analysis of lipids. With nLC-ESI–MS/MS, a vast library of lipids can be derived from a few μg or μL of sample; even lipids that exist in ultra-trace amounts such as femtomoles can be analyzed.

Lipid structures can be determined via collision-induced dissociation (CID) spectra obtained through the data-dependent MS/MS analysis and analyzed by the algorithm-based software LiPilot [24]. Based on a library of *m/z* values and $MS^n$ spectra corresponding to typical fragmented ions, lipids can be rapidly identified through an automated search that compares differences between the experimental and theoretical *m/z* values of precursor and their corresponding fragment ions. A confidence score is calculated for each result to enhance the identification accuracy and discard any false-positive matches. Because of the impact of lipid head groups and fatty acyl chains on MS signals—and the limited number of lipid standards available on the market—absolute quantification of all lipids in complex biological samples such as plasma is impossible; but relative quantification can be performed using internal standards (IS). Lipids with odd numbers of carbon atoms in fatty acyl chains are used as ISs because they are not found in human; thus, the corrected peak area of lipids can be calculated with respect to the peak area of the IS. Multiple standards representing each lipid class should be used when analyzing samples for accurate relative quantification of lipids.

### 2.4 Investigation of Phospholipids from Lipoproteins of Patients with Coronary Artery Disease

Coronary artery disease (CAD) develops from deposition of plaque that are caused by oxidized lipoproteins onto the arterial walls; as the walls thicken over time, an insufficient amount of blood is delivered to the heart, which can cause a heart attack if the arteries become completely blocked. Low levels of HDL and high levels of LDL are purported to increase the risk of CAD, and FlFFF-based studies of lipoproteins have shown that, compared to control sets of healthy individuals, the size of LDLs decreases among patients with CAD; concurrently, there is an increase in LDL concentration [25]. Using MxHF5, the separated lipoproteins from healthy controls and patients with CAD were collected, after which the lipids within them were extracted and characterized using nLC-ESI–MS/MS [26]. The levels of lipoproteins and the composition of lipids differed between the control and patients with CAD in both the HDL and LDL fractions. A total of 19 and 10 lipids from HDL and LDL, respectively, showed significant variations in patients with CAD compared to healthy individuals; some lipids were found exclusively in the HDL or LDL fractions of one patient group or the other (as shown in Fig. 4), suggesting their potential use as biomarkers of CAD [26]. 16:0/16:1-PC, 20:1/20:4-PE, and 16:1-lysophosphatidic acid (LPA) were exclusively detected in the HDL fraction of patients with CAD, while 16:0/22:3-PG was exclusively found in their LDL fractions. 16:1/18:2-PC was found in both the HDL and LDL samples of the control group, but was not detected in patients with CAD. On the other hand, 18:0-lysophosphatidylcholine (LPC) and 18:0/20:4-PA were found in the HDL fractions of both healthy individuals and patients with CAD, though they were increased more than fivefold in the former; however, 22:6/16:0-PG and 20:1/20:4-PA were elevated significantly in the HDL of patients with CAD. These results generally agree with those of another lipidomic study that analyzed intact and oxidized lipids from patients with CAD [21]; however, as that study was based on ten healthy individuals and ten patients with CAD, additional

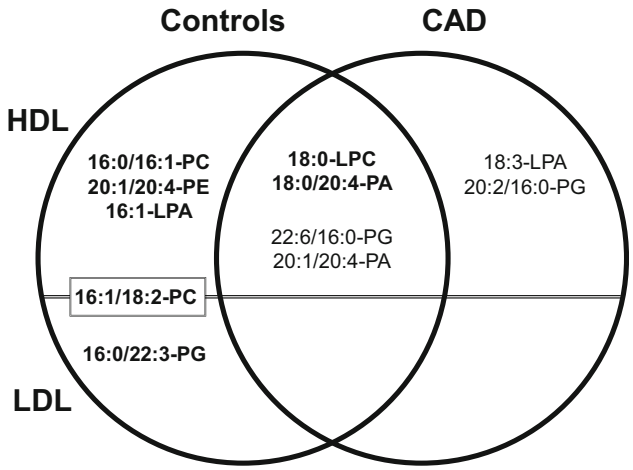


**Fig. 4** Venn diagram showing distribution of lipids across samples and lipoprotein fractions. The levels of lipids in *bold* were significantly increased in control samples; levels of lipids in *plain text* were elevated significantly in patient samples. Reprinted with permission from [26]. Copyright 2012 Elsevier B.V

analysis from a larger pool is needed to validate the efficacy of lipids as biomarkers. Still, using FlFFF and nLC-ESI–MS/MS as part of a bottom-up lipidomic study produced a comprehensive profiling of lipids from HDL and LDL fractions. Lipidomic analysis of VLDL was not performed in this study [26].

### 2.5 Characterization of Oxidized Phospholipids from Patients with Coronary Artery Disease

Many studies have discovered that oxidation of LDL plays a critical role in the onset of CAD, as oxidized LDLs (Ox-LDLs) are more easily and rapidly taken up by macrophages [27, 28]. Oxidation of LDLs occurs through the oxidation of lipid molecules and Ox-LDLs are associated with inflammatory diseases and alterations in biological membranes [28]. Using MxHF5, HDL, LDL, and VLDL were size fractionated as shown in Fig. 3 [21], which demonstrated that VLDL eluted after the field (radial flow) was turned off at 17 min. While lipoprotein standards were clearly detected at 280 nm (Fig. 3b, c), lipoproteins from plasma sample were detected at 600 nm after staining with 1% Sudan Black B in dimethyl sulfoxide. Figure 3d and e represent the separation within a single HF5 channel and MxHF5 module, respectively [21]. Profiling of Ox-PLs in each lipoprotein fraction was performed using nLC-ESI–MS/MS after extraction of lipids. From CID spectra, Ox-PLs of all classes of PLs in patients with CAD were structurally identified and their relative quantities were determined. As a total of 283 PLs including 123 Ox-PLs from controls and 315 PLs including 169 Ox-PLs from patients were identified, supporting that oxidation of PLs progressed more in CAD. The numbers of Ox-PLs in the LDL and VLDL fractions were higher in patients with CAD because of some Ox-PLs being present only in those samples. Among Ox-PLs, singly hydroxylated PLs were more abundant than other forms of Ox-PLs (such as hydroperoxylated PLs or short-chain products). Figure 5 illustrates PLs with 16- and 18-carbon saturated acyl chains hydroxylated at the sn-1 position from all fractions of HDL (H), LDL (L), and VLDL (V) the relative abundance ratios of singly oxidized PLs to intact PLs (lipids that had not gone oxidized) were greater in all fractions of lipoproteins isolated from patients with CAD than controls [21]. In patients with CAD, the relative portion of singly hydroxylated species of 22:5/18:0-PC, 18:0/18:2-PI, and 18:0/20:4-PI from HDL, 18:0/18:2-PA from LDL, and 18:0/20:4-PI from VLDL were elevated by twofold, while singly hydroxylated species of 16:0/20:5-PC and 18:0/20:4-PA from VLDL were newly found from patients with CAD. With proper separation of lipoproteins using FlFFF and use of nLC-ESI–MS/MS, analysis of low-abundance Ox-PLs was successfully achieved.

## 3 Top-Down Lipidomic Analysis

### 3.1 Online Coupling of Miniaturized FlFFF to MS

Lipids in lipoproteins can be analyzed without extraction by directly injecting lipoproteins eluted from an FlFFF channel into an ESI–MS as shown in Fig. 6. To carry out

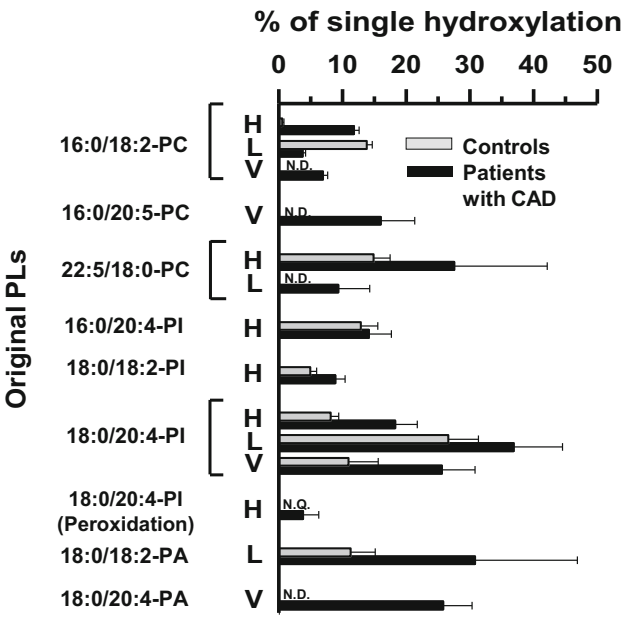


**Fig. 5** Percentage of singly hydroxylated PLs from HDL, LDL, and VLDL fractions of patients with CAD compared to intact PLs. *N.D.* not detectable; *N.Q.* not quantifiable. Reprinted with permission from [21]. Copyright 2014 American Chemical Society

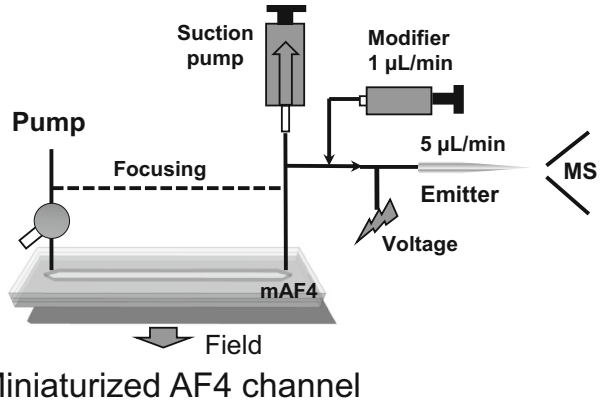


**Fig. 6** Schematics of miniaturized AF4-ESI–MS/MS for top-down lipidomic analysis of lipoproteins. Reprinted with permission from [31]. Copyright 2013 Elsevier B.V

top-down lipid analysis, two requirements must be met. The first is the depletion of high-abundance plasma proteins such as albumin and immunoglobulin G (Ig), which can suppress the ionization of targeted lipids from lipoproteins. In FlFFF, the HDL peak usually overlaps with the albumin peak, as they are similar in size. Altering the flow rate conditions can separate the aforementioned peaks but will result in LDL being eluted much later, extending the overall analysis time. As one of the merits of top-down lipidomic analysis is high speed, such actions eliminate one of the core advantages of the approach. In addition, as albumin constitutes 50–60% of plasma proteins, its removal serves to enrich the lipids in the collected fractions. These issues can be solved by depleting albumin and IgG using a depletion kit before injecting a plasma sample into the FlFFF channel.

Second, the flow rate of the FlFFF effluent should be reduced to a few µL/min for a direct coupling with ESI–MS/MS. This can be achieved by implementing a miniaturized asymmetrical FlFFF (mAF4) channel to ESI–MS (a standard FlFFF channel typically outputs a few tenths of a mL/min and is, therefore, insufficient for the task). The mAF4 channel is assembled by stacking frit-inserted, 1.5-mm-thick stainless steel plates to form a 13-cm-long and 6-mm-wide channel [29–32]. Unlike the MxHF5, in which the channel is cylindrical, the rectangular channel design of mAF4 forces the field to travel in one direction (from the top of the channel to its bottom) through a permeable wall (a regenerated cellulose membrane sheet layered above the frit). With the use of FlFFF prior to ESI–MS, online desalting effect is achieved as salts and other types of metabolites with small molecular weights permeate through the membrane and frit during the focusing/relaxation step along with the crossflow, enhancing the ESI of lipids [31]. Figure 6 shows a schematic of mAF-ESI–MS/MS with suction pump, which is employed to adjust the rate of the outflow. Without the pump, the outflow rate would be too high, impeding ionization; however, decreasing the outflow would lengthen analysis time, necessitating the use of a pump [31]. As the carrier liquid of mAF4 was aqueous $NH_4HCO_3$ solution, ionization modifier (1% formic acid in $CH_3CN$ for positive ion mode and 0.5% $NH_4OH$ for negative ion mode) was mixed at a constant rate through the micro-tee prior to ESI–MS analysis.

Top-down lipid profiling via online coupling of mAF to ESI–MS/MS is used for targeted analysis because ionization suppression resulting from other species is expected to be higher than that found in bottom-up analysis with nLC-ESI–MS/MS. As all lipids present within lipoproteins are eluted together at their corresponding retention times, those with low-abundance or low MS response that are detected in nLC-ESI–MS/MS are not likely to be detected in mAF4-ESI–MS/MS. Therefore, top-down analysis is highly recommended to be performed in selective reaction monitoring (SRM) for targeted lipidomic analysis as only selective precursor ions with specific product ions are scanned.

To compensate for the fluctuation in ESI–MS intensity, carbonic anhydrase (CA) is utilized as an IS for top-down analysis. Unlike bottom-up analysis—in which the internal standards consist of lipids that are not found in samples of interest—top-down mAF4-ESI–MS/MS uses protein standards for two reasons. First, addition of lipid standards to plasma sample would not assure of uniform adsorption of lipids at the surface of lipoproteins. Second, they would permeate the channel membrane during focusing/relaxation. CA can be utilized as an IS for the quantitation of lipids since the amount of natural CA found in plasma is negligible: the peak area corresponding to 500 ng of standard CA added to plasma was 193.91 ± 2.98 ($n = 3$), while CA in intact plasma was undetectable in triplicate [30]. Figure 7a shows the elution of HDL and CA, represented with the SRM fractograms of 34:2-PC from HDL of a plasma sample based on SRM transition of $m/z$ 758.7 → 575.7 overlapped with the SRM fractogram of CA, an IS (0.5 µg), based on $m/z$ 1613.1 ($[M+18H]^{+18}$) → 1521.4 ($y_{67}^{+5}$), respectively. Though the CA peak overlaps to some degree with the HDL peak as illustrated in Fig. 7a, the effect of peak overlap on ionization efficiency of lipids has proven to be insignificant. By varying flow rate conditions, the degree of CA and HDL peak overlap varied, but the variation in the peak areas of lipids from HDL were acceptable, with an average relative difference of 3.77 ± 1.71% [30]. CA can be detected in both positive (Fig. 7b, c) and negative ion modes (Fig. 7d, e) by selecting the most intense fragments as precursor and product ions in SRM mode [30].

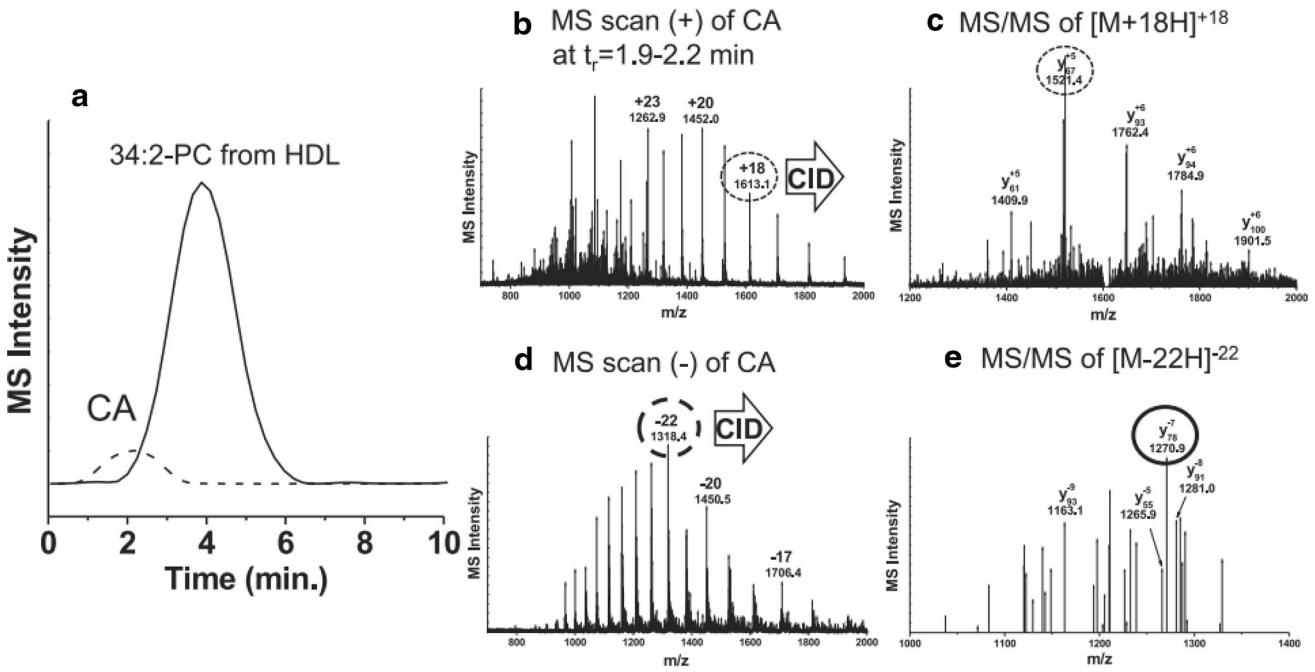


**Fig. 7** **a** SRM fractograms of 34:2-PC from HDL of a plasma sample (2.5 μL) based on the SRM transition of *m/z* 758.7 → 575.7 overlapped with the SRM fractogram of CA, an internal standard (0.5 μg), based on *m/z* 1613.1 ($[M+18H]^{+18}$) → 1521.4 ($y_{67}^{+5}$), **b** MS spectra of CA during $t_r$ = 1.9–2.2 min in positive ion mode, **c** MS/MS spectra of $[M+18H]^{+18}$, d) MS spectra of CA in negative ion mode, and e) MS/MS spectra of $[M-22H]^{-22}$ (*m/z* 1318.4). Reprinted with permission from [30]. Copyright 2013 Elsevier B.V

### 3.2 Rapid Screening of Lipids from Lipoproteins of Patients with Coronary Artery Disease

Alterations in the lipid compositions of HDL and LDL from patients with CAD were assessed using mAF4-ESI–MS/MS by selectively quantifying 39 lipids [30]. By observing the decrease of retention times for LDLs from patients with CAD, a decrease in the sizes of LDLs can be expected along with significant changes in the concentrations of 13 lipids from patients with CAD. While only a handful of lipids that were detected with high MS response were analyzed, many of these showed dramatic changes in patients with CAD, showing the value of the top-down method for lipidomic analysis of lipoproteins. In addition, the targeted analysis of apolipoprotein-A1 (ApoA1)—a major protein in HDL that is frequently used as a biomarker for diagnosis of cardiovascular diseases owing to its critical role in cholesterol homeostasis [33]—was performed in SRM mode. ApoA1 was exclusively detected in HDL fractions, and its level was decreased by 2.5-fold in patients with CAD, demonstrating that mAF4-ESI–MS/MS has the potential to be utilized as an integrated analytical platform for monitoring not only lipids but specific protein as well. This can be an alternative feature of mAF4-ESI–MS/MS for the selective detection and quantitation of ApoA1 in the drug intervention study without isolating/purifying ApoA1 from plasma proteins, which is quite complicated.

### 3.3 Lipidomic Analysis of Lipoproteins of Rabbits Grown Under Metabolic Stress

Both bottom-up and top-down methods were used to investigate variations in the lipidomes of HDLs and LDLs from rabbits grown under conditions that would stimulate metabolic stress [32]: inflammation (I), dehydration (D), high cholesterol diet (HC), and HC with inflammation (HCI). Figure 8a compares the lipoprotein profiles of rabbit sera grown under metabolic stress obtained by HF5-UV detection, showing that relative levels of lipoproteins between the groups. The amount of HDL decreased dramatically in HC and HCI subjects, while that of LDL rose [32]. The increase in the retention times of LDL from the HC and HCI groups indicates that LDL sizes become larger, while the broader LDL peaks show that an HC diet induces a greater range of LDL particle sizes. Lipids from the pooled lipoprotein fractions were analyzed quantitatively using nLC-ESI–MS/MS; those that changed significantly were discovered through statistical evaluation. Subsequently, these lipids were selectively quantified using mAF4-ESI–MS/MS as shown in Fig. 8b and c [32]. Given that significant differences in peak areas were observed for

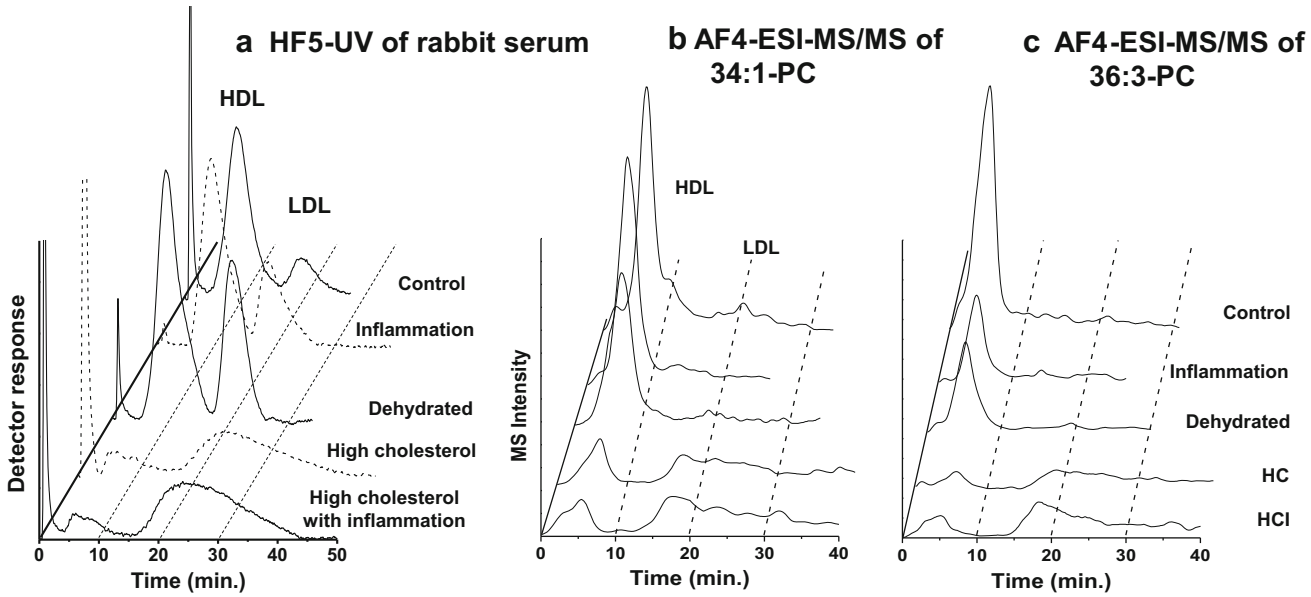


**Fig. 8** **a** HF5-UV fractograms of lipoproteins from rabbits grown under stress conditions. SRM fractograms of lipoproteins based on the detection of **b** 34:1-PC and **c** 36:3-PC from lipoprotein using mAF4-ESI–MS/MS. Reprinted with permission from [32]. Copyright 2015 Elsevier B.V

the targeted HDL and LDL lipids under HC and HCI treatments, it can be concluded that an HC diet induces alteration of lipids in both lipoprotein fractions to a greater extent than do inflammation or dehydration. Additionally, while the same types of lipids may exist in both HDL and LDL, they can be affected differently by metabolic factors, a phenomenon that emphasizes the need for the separate profiling of lipids from the various lipoprotein classes. When the quantification results from the bottom-up and top-down methods were directly compared, their results did not exhibit significant statistical differences, indicating that the top-down method using mAF4-ESI–MS/MS is a potential alternative to the bottom-up method of nLC-ESI–MS/MS for the rapid screening of targeted lipids.

## 4 Conclusion

The assessment of lipids within lipoproteins can be achieved using online and offline coupling of FlFFF to MS for top-down and bottom-up applications, respectively. A non-targeted, bottom-up lipidomic analysis involves: off-line separation and collection of lipoproteins using MxHF5; a 24-h extraction process including purification of lipids from salts and other metabolites; and nLC-ESI–MS/MS for identification and quantification of the recovered lipids, generating a comprehensive profile of the lipidome under study. Using CID spectra obtained from the MS data-dependent mode, lipids are structurally identified and quantified with respect to the peak areas of IS lipids, which are added to compensate for fluctuations in MS intensity. Profiles of lipids from HDL, LDL, and VLDL fractions can be created using this method. On the other hand, top-down analysis using mAF4-ESI–MS/MS allows for rapid screening of targeted lipids from lipoproteins. Lipoprotein fractionation and lipid detection are achieved simultaneously, as FlFFF is coupled to MS. To reduce ionization suppression, albumin and IgG must be removed from plasma samples. With the addition of CA to the plasma or serum sample as an IS, lipoprotein-derived lipids can be quantified without being subjected to extraction or nLC-ESI–MS/MS. However, as all lipids within lipoproteins are eluted at the same time, only those with a relatively high abundance or high MS response are likely to be well quantified under SRM mode in the top-down approach. As this limits the ability to analyze extremely low-abundance lipids, top-down analysis using mAF4-ESI–MS/MS is best suited for the rapid screening of targeted lipids.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflicts of interest.

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