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Ultrahigh-Performance Capillary Liquid Chromatography-Mass Spectrometry at 35 kpsi for Separation of Lipids

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Abstract

Improvements in sample preparation, separation, and mass spectrometry continue to expand the coverage in LC-MS based lipidomics. While longer columns packed with smaller particles in theory give higher separation performance compared to shorter columns, the implementation of this technology above commercial limits has been sparse due to difficulties in packing long columns and successfully operating instruments at ultrahigh pressures. In this work, a liquid chromatograph that operates up to 35 kpsi was investigated for the separation and identification of lipid species from human plasma. Capillary columns between 15-50 cm long were packed with 1.7 μ m BEH C18 particles and evaluated for their ability to separate lipid isomers and complex lipid extracts from human plasma. Putative lipid class identifications were assigned using accurate mass and relative retention time data of the eluting peaks. Our findings indicate that longer columns packed and operated at 35 kpsi outperform shorter columns packed and run at lower pressures in terms of peak capacity and numbers of features identified. Packing columns with relatively high concentration slurries (200 mg/mL) while sonicating the column resulted in 6 to 34 % increase in peak capacity for 50 cm columns compared to lower slurry concentrations and no sonication. For a given analysis time, 50 cm long columns operated at 35 kpsi provided a 20-95% increase in chromatographic peak capacity compared with 15 cm columns operated at 15 kpsi. Analysis times up to 4 h were evaluated, generating peak capacities up to 410 ± 5 ($n = 3$, measured at 4σ) and identifying 480 ± 85 lipids ($n = 2$). Importantly, the results also show a correlation between the peak capacity and the number of lipids identified from a human plasma extract. This correlation indicates that ionization suppression is a limiting factor in obtaining sufficient signal for identification by mass spectrometry. The result also shows that the higher resolution obtained by shallow gradients overcomes possible signal reduction due to broader, more dilute peaks in long gradients for improving detection of lipids in LC-MS. Lastly, longer columns operated at shallow

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Conflict of interest
None

gradients allowed for the best separation of both regional and geometrical isomers. These results demonstrate a system that enables the advantages of using longer columns packed and run at ultrahigh pressure for improving lipid separations and lipidome coverage.

Keywords

UHPLC; LC-MS; Capillary chromatography; Lipidomics; Lipid isomers

1. Introduction

Lipids are important biological molecules with functions that include energy storage, cell signaling, and membrane formation [1]. A number of diseases such as Alzheimer's, Parkinson's, diabetes, and chronic kidney disease have been associated with altered lipid profiles [2]. Improved lipidomics methods are therefore of interest for better understanding of normal and pathological states and developing potential therapeutic targets. In this work we describe preparations and use of capillary LC columns up to 50 cm long operated at 35 kpsi for improved separation and coverage of complex lipid samples.

Analyzing the lipidome is challenging due to the diversity and complexity of the lipidome. Lipids can be divided into eight classes and many sub-classes spanning a wide range of physicochemical properties [3]. Additionally, they can be present in a wide concentration range within a biological system. A number of techniques are employed to characterize lipidomes, including spectroscopy, separations, and mass spectrometry (MS) [4]. Direct infusion (e.g. shotgun lipidomics) MS-based methods are particularly powerful and popular due to their speed, sensitivity, ability to identify compounds, and mass resolving power [5]. Coupling separation techniques to MS can improve lipidome coverage by reducing ionization suppression [6] and separating isomeric and isobaric species [7]. Chromatographic data can also aid in lipid identification based on retention time [8].

Although a variety of separation methods have been used for lipids [9–11], high pressure and ultrahigh pressure liquid chromatography ((U)HPLC) is perhaps the dominant separation form used for lipid separations due to the wide range of lipid species amenable to this approach. A number of different HPLC modes have been used for lipid analysis, including reversed phase (RP) [12], normal phase (NP) [13], and hydrophilic interaction liquid chromatography (HILIC) [14,15]. NP-LC and HILIC separate lipids primarily by their head group, effectively separating lipid classes. RP-LC predominantly separates lipids by hydrophobicity, allowing for separation by chain length, number of double bonds, and occasionally by the head group.

Studies suggest that separation performance can be a bottleneck for further gains in lipidomic coverage. Multidimensional separations such as LC x LC that offer higher peak capacities than single dimension separations have been shown to improve lipidomic coverage, likely due to alleviation of ionization suppression due to less co-elution and improved spectral quality [16,17]. Smaller diameter columns and nanoelectrospray ionization (nESI) emitters with low flow rates can also alleviate ionization suppression [18],

corroborated with a recent report showing increased lipidomic coverage using capillary LC-MS compared to a larger-bore 2.1 mm column [19].

Continuing improvements in HPLC have allowed for higher efficiency separations than previously possible. It is well known from chromatographic theory that reducing the particle size of the column packing material and increasing the column length lead to higher efficiency separations, assuming that the column is well-packed [20]. The pressure needed to flow liquid through a packed column is directly proportional to the column length and inversely proportional to the square of the particle diameter. Pressure can therefore be a limiting factor in further improving separation efficiency in HPLC.

Instrument manufacturers have steadily increased system pressure limits, with state-of-the-art instruments capable of operation at 15-20 kpsi [21,22]. The higher pressure limits have allowed for fast and efficient separations using 5-15 cm long columns packed with sub-2 μ m particles [21]. Other researchers have developed instruments capable of operation at even higher pressures, from 25 to 100 kpsi [23–25]. Capillary columns ranging from 30 to 200 cm long have generated peak capacities of 300-1500 in 100-2000 min long gradient analyses [23,24]. Investigation of the column packing process has led to a better understanding of the conditions needed for high performance so that reduced plate heights below 2 have been achieved even for meter-long columns [26,27].

Despite these impressive results, ultrahigh pressure separations have only been performed on peptide and protein samples or with small molecules [28]. In this work, we investigated preparation of columns that could achieve the expected benefits of UHPLC operation at 35 kpsi on lipid separations and lipidome coverage. The results demonstrate substantial peak capacity improvements for lipid separations at 35 kpsi when compared to 15 kpsi for the same stationary phases and mobile phase gradients. Our results also show a strong correlation between the chromatographic peak capacity and the number of lipids identified from a human plasma extract so that longer columns, which require higher pressure, allowed more lipid identifications. Mass spectra of eluting peaks were cleaner due to less co-elution compared to lower resolution separations. Additionally, several different lipid isomers were investigated; longer columns operated at shallow gradients allowed for the best separation of both regional and geometrical isomers. These results demonstrate the benefits of using longer columns packed, using appropriate methods and operated at ultrahigh pressure for improving lipid separations and lipidome coverage.

Materials and methods

2.1. Chemicals and standards

HPLC grade water, acetone, methanol, and acetonitrile were purchased from VWR (Radnor, PA). Potassium chloride, acetic acid, HPLC grade 2-propanol, chloroform, formamide, formic acid and ammonium formate were purchased from Sigma Aldrich (St. Louis, MO). Potassium silicate (Kasil 2130) was purchased from PQ corporation (Valley Forge, IA). All lipids were purchased from Avanti Polar Lipids Inc (Alabaster, AL). A complete list of the lipids used in this work and their abbreviations are listed in Supplemental Material Table 1. Abbreviations for all lipids were according to those reported by Liebisch et al. [29]. A lipid

standard mixture was prepared in mobile phase B. The mixture contained PC 14:0/16:0, PC 14:0/18:0, PC 16:0/18:0, PC 18:0/18:1, PC 18:1(9Z)/18:1(9Z), PC 18:1(9E)/18:1(9E), and PC 16:0/2:0, all at a concentration of 5 μ M. The mixture also contained the Splash Lipidomix Mass Spec Standard (Avanti, Alabaster, AL), diluted 2:1 in the final mixture. This mixture contains 14 deuterium labeled lipids.

2.2. Human plasma extraction

Pooled human plasma was provided by the Michigan Regional Comprehensive Metabolomics Resource Core. Lipids were extracted using a modification of Bligh and Dyer extraction protocol [30]. To 50 μ L of plasma, 200 μ L of 0.15 M KCl in water, 400 μ L of methanol, 200 μ L of chloroform, and 1 μ L of acetic acid were added to an Eppendorf tube and mixed well. An additional 200 μ L of water and 200 μ L of chloroform were added, vortexed briefly, and centrifuged at 12,100 $\times g$ for 5 minutes at room temperature. The organic layer was carefully collected and transferred to an HPLC vial, dried under nitrogen gas, and reconstituted in 400 μ L of mobile phase B.

2.3 Column packing

Fused-silica capillaries with inner diameters of 100 μ m and outer diameter of 360 μ m were purchased from Polymicro Technologies, Inc. (Phoenix, AZ). Columns were prepared as previously described, with slight modifications [31]. Column outlet frits were prepared using the Kasil method [32]. An equal amount of potassium silicate and formamide were applied to a glass microfiber filter (Reeve Angel; Clifton, NJ) and the capillary tip was dabbed on the wetted paper to form the frit. The particles for all columns were 1.7 μ m BEH (130 Angstrom) from Waters Corporation (Milford, MA). For the 15 cm columns operated at 15 kpsi, columns were packed at 15 kpsi using a 75 mg/mL slurry in acetone. After packing, columns were flushed at 20 kpsi for 1 h, depressurized, and an inlet frit was applied using the Kasil method [32]. For the ultra-high performance columns, two packing methods were used to compare the effect that sonication has on column performance of gradient separations. For the non-sonicated columns, 75 mg/mL slurries containing 1.7 μ m C18 BEH particles from Waters Corporation (Milford, MA) were prepared in acetone as this concentration has previously been reported as near optimal when not sonicating to balance between particle size segregation and void formation [33]. Columns of 25 and 50 cm in length were packed by steadily increasing the packing pressure to 30 kpsi in order to maintain packing flow rate and limit packing voids [33]. After, columns were flushed with 50/50 (v/v) acetonitrile/water at 40 kpsi for 1 h, and slowly depressurized for another hour before applying an inlet frit using the Kasil method [32]. For the 50 cm sonicated columns, a 200 mg/mL slurry containing the same 1.7 μ m C18 particles was prepared in acetone. The empty column was placed in a sonicator bath as previously described [27]. After a couple centimeters of bed was formed, the pressure was immediately increased to 30 kpsi. The columns were flushed with 50/50 (v/v) acetonitrile/water at 50 kpsi for 1 h, depressurized for 1 h, and an inlet frit was applied using the Kasil method [32].

2.4. Instrument operation

A constant pressure pump was used to perform separations at 35 kpsi as previously described [23]. Mobile phase A was 60/40 (v/v) water/acetonitrile with 10 mM ammonium

formate and 0.1% (v/v) formic acid. Mobile phase B was 85/10/5 (v/v/v) 2-propanol/ acetonitrile/water with 10 mM ammonium formate and 0.1% (v/v) formic acid. One μL was injected on column using a Waters NanoAcquity UPLC. The column oven was set at 60 °C. A 50-100% B gradient was used in all separations, with a 100% B hold for 10 column volumes. Gradients were loaded on to a gradient storage loop using a binary solvent manager of the NanoAcquity [23]. Constant pressure separations were performed using a pneumatic amplifier pump (DSXHF-903 Haskel pump (Burbank, CA). Column volumes were calculated assuming a column porosity of 0.8. The peak capacity was calculated by dividing the elution window by the average peak width (4σ) of 12 lipid standards that eluted throughout the separation window. Plate heights were measured using the peak width at half maximum. Effluent from the column was connected to a Micromass Q-ToF Premier using a stainless-steel union connected to fused silica spray needle with a 75 μm inner diameter tapered to 30 μm . The scan rate was set to 0.3 s with a 0.1 s inter-delay. The MS was operated in full scan, positive ion mode with a mass window of 150-1000 m/z . External calibration was performed regularly using sodium formate. Source parameters were tuned by directly infusing 5 μM PC 18:1(9Z)/18:1(9Z)). The spray voltage was 1.75 kV, the source temperature was 100 °C, and sheath gas was 0.3 bar.

2.5. Lipid identification

Lipids were putatively identified at the lipid class level using LipidBlast software [8,34]. Mass spectra from 1 min elution windows were baseline subtracted and centered. The corresponding m/z values that were above 100 counts were input into the software. The mass accuracy was set at 10 mDa. Following *in-silico* identification, redundant species and salt adducts that corresponded to the same lipid were removed. If multiple lipids were matched for one m/z value (e.g. within 10 mDa mass units), only one lipid was considered an identification.

3. Results and discussion

In this work, we investigated the potential benefits of packing and operating capillary columns at 35 kpsi on lipid separations and lipidome coverage. We first compared the separation performance of a 25 cm and 50 cm column, packed under identical conditions as described in section 2.3, to assess the impact that column length has on gradient separations of complex lipid extracts from human plasma. We then compared two 50 cm columns, one that was packed using sonication and high slurry concentrations, which was recently shown to improve column efficiency, particularly for longer columns [27], and one packed without the use of sonication. Lastly, we compared these results with a 15 cm column operated at 15 kpsi, the current limits in commercial instrumentation. Altogether, for a given analysis time, longer columns packed with high slurry concentration and sonication and operated at 35 kpsi provided higher peak capacities, more lipid identifications and cleaner mass spectra from complex mixtures of lipids compared with shorter columns.

We found good repeatability for column preparation and performance. Supplemental table 2 summarizes the retention time and peak capacity variation across three separate columns for each column length that was used in this work. In each condition, a gradient slope of 5%

change in mobile phase (ΔB)/column volume was programmed and the instrument was operated at 15 kpsi for the 15 cm columns and 35 kpsi for the 25 and 50 cm columns. The results showed good repeatability in retention time and peak capacity for gradient separations of the standard lipid mixture. Retention time variation between columns was 1 – 3 % RSD and peak capacity variation was 3 – 6 % RSD. The sonicated columns were evaluated using hydroquinone as previously described with reduced plate heights of three columns shown in Supplemental Figure S1 [27]. Based on these results, we performed more detailed comparisons of individual columns under different condition.

3.1. Effect of column length on lipid separations

In the first evaluation, we compared 25 cm and 50 cm columns that were prepared under identical packing conditions. Due to the shorter analysis times possible on the 25 cm column, method development was first carried out on this column using a standard lipid mixture to obtain a general understanding of lipid separations at 35 kpsi. Separation of the standard lipid mixture on the 25 cm column operated at 35 kpsi and 60 °C is shown in Figure 1a. A 10X column volume was used as the gradient volume at 50-100% B, corresponding to a 5 % ΔB per column volume. This gradient slope has previously provided good separation space and peak capacity for peptides [23,35]. Previous ultrahigh pressure work has shown that higher pressures in HPLC can alter separation selectivity and retention [31,36]; however, no significant differences between 15 kpsi and 35 kpsi operating pressure was seen in regard to retention or selectivity (discussed in more detail in section 3.3). Similar to previous reports for RP-LC of lipids, separation is primarily based on the number and length of carbon chains attached to the glycerol backbone. For example, monoglycerides elute in the first portion of the chromatogram, whereas the more non-polar triglycerides and cholesteryl esters elute at the end [37]. The peak capacity for this 30 min separation was 110 ± 5 ($n = 3$ injections). Figure 1b shows the separation of a lipid plasma extract on the 25 cm column with the same conditions as Figure 1a. The peak capacity was 115 ± 7 ($n = 3$ injections). Using LipidBlast to analyze the chromatogram, 189 ± 29 ($n = 3$ injections) lipids were putatively identified at the lipid class level in full scan mode [8,29]. The higher number of identifications than peak capacity confirms that some lipids co-elute but are resolved by the mass spectrometer.

We sought to assess the impact that doubling the column length with columns packed under identical packing conditions has on peak capacity of complex lipid extracts with a variety of analysis times operated at 35 kpsi. A plot of the peak capacity as a function of the analysis time for the 25 cm and non-sonicated 50 cm columns is shown in blue and green traces, respectively, in Figure 2. For the 25 cm column, the gradient slopes ranged from 10 % to 0.5 % ΔB /column volume. For the non-sonicated 50 cm column, the gradient slopes ranged from 5% to 1% ΔB /column volume. Again, all separations were performed at 35 kpsi for these two columns. Figure 3 shows the base peak chromatogram of a lipid plasma extract on the 25 and non-sonicated 50 cm columns with 2 h analysis time and 35 kpsi inlet pressure. The peak capacity on the non-sonicated 50 cm column was 265 ± 5 ($n = 3$ injection), which was only slightly higher than the 25 cm column of 237 ± 10 ($n = 3$ injection). For visual clarity, extracted ion chromatograms for three lipids eluting across the separation space are shown in Figure 3c to illustrate the similar peak widths between the two columns.

The modest improvement in peak capacity with length for columns packed under identical packing conditions may be related to packing quality for the longer column. Reduced van Deemter plots using phosphatidyl choline (PC) 18:1/18:1 as a test analyte showed worse performance for the 50 cm column compared to the 25 cm column (Supplemental Figure S2). The reduced plate height minimum ($h_{\min}$) on the 25 cm column was 1.6, while on the 50 cm column $h_{\min}$ was 5.2. At 35 kpsi, the reduced plate heights were 4.0 and 6.6 on the 25 cm and 50 cm columns, respectively. One explanation for this worse kinetic performance of the 50 cm column is the difficulty to pack longer columns. In particular, axial heterogeneities in the packing bed have been attributed to poor chromatographic efficiency for longer columns [38].

3.2. Effect of column packing procedures on lipid separations

The above findings indicated that the conditions used for packing the columns used did not generate equivalent performance per unit length in the tested columns, i.e. the conditions used were not ideal for longer columns. It was recently shown that sonication during column packing improves column efficiency, with reduced plate heights of 1.05 being reported for meter-long capillary columns [27]. Therefore, we compared two 50 cm long columns: one packed without sonication and one packed with sonication. Other slight differences between packing methods were employed as described in section 2.3 based on previously reported packing methods. For simplicity we refer to the two methods as “sonicated” and “not sonicated”. The black and green traces in figure 2 show the peak capacity as a function of analysis time for the sonicated and not sonicated columns, respectively. It is clear that the sonicated column outperformed the non-sonicated column for gradient separations of complex lipid extracts. At short and steep gradients, the improvement is not as drastic. Improvements in peak capacity at longer analysis times for longer columns is consistent with gradient elution theory and has previously been reported for peptide separations using 50-200 cm long columns [23,39,40].

Example base peak chromatograms from the two 50 cm columns shows the improved separation for the sonicated column for a 4 h separation (Figure 4). The sonicated column provides narrower peaks and more baseline resolved peaks than the non-sonicated column. For the 240 min separation shown in Figure 4, the peak capacity of the non-sonicated column was 306 ± 8 ($n = 3$ injections) and 407 ± 5 ($n = 3$ injections) for the sonicated column. Extracted ion chromatograms of three lipids from the plasma extract eluting across the separation space are shown in figure 4c to help visually see the improvement in peak width and shape between the two columns.

3.3. Comparison with commercial pressures

We also compared the performance of the system described here with what could be achieved with pressure limits of current state-of-the-art commercial instrumentation. For this study, we packed a 15 cm long column up to 20 kpsi and performed gradient separations at 15 kpsi. The separation performance of this column consistently under-performed the higher-pressure columns discussed previously. Figure 5 shows base peak chromatograms of a lipid extract from plasma on the 15 cm column at 15 kpsi and a sonicated 50 cm column at 35 kpsi with a constant gradient slope of 2.5 % B/column volume. At these conditions, the

peak capacity was 93 ± 2 ($n = 3$) for the 15 kpsi case and 265 ± 5 ($n = 3$) for the 35 kpsi case, albeit at a longer analysis time. Extracted ion chromatograms of three lipids from the plasma extract are shown in panel c with elution time windows of 8% of the total analysis time. A comparison of the 15 cm and 50 cm columns at a constant analysis time for a lipid extract from plasma is shown in Supplemental Figure S3. The peak capacity of the standard lipid mixture as a function of analysis time for the 15 cm column operated at 15 kpsi in comparison to the higher pressure columns is shown in Figure 2. The peak capacity on the 15 cm column plateaued at a maximum of about 200, with no further gain achieved with increased analysis time. Longer columns and higher pressures are therefore particularly advantages at longer analysis times, offering up to 95% increase in peak capacity for the same analysis time. Improved performance with higher pressure limits is due to both being able to operate longer columns and possibly better packing. A recent study on peptide separations revealed that columns packed at 30 kpsi resulted in a 17% increase in peak capacity and a 16% increase in peptide identifications compared to those packed at 10 kpsi for 30 cm long columns despite both separations being performed with the same commercial UHPLC system at ~11 kpsi [41].

3.4. Relationship between peak capacity and lipid identifications

The above experiments illustrate that use of long, well-packed columns provides substantial increases in peak capacity for lipid separations. A critical goal of a lipidomic experiment is to enable identification or detection of large numbers of lipids. To evaluate the effect of using ultra-high pressure on ability to detect or identify discrete lipids in complex samples, we used LipidBlast software [8] to putatively identify lipids in the samples at the class level [29], e.g. fatty acid tails or double bond positioning are not differentiated. (All experiments were performed in positive ion mode only because the only instrument we had available for these experiments was not functional in negative ion mode. In principle, use of negative mode would reveal even more identification from lipid classes, such as fatty acids, that are better detected that way.)

The results here show a roughly linear correlation between the chromatographic peak capacity and the number of lipids identified, independent of analysis time (Figure 6). For example, comparing the 15 cm and 50 cm sonicated column, with a constant 130 min analysis time, 206 ± 18 lipids ($n = 2$ injections) versus 480 ± 85 ($n = 2$ injections) lipids were identified, respectively. The peak capacities on the 15 cm and the 50 cm sonicated columns for those separations were 190 ± 10 ($n = 3$ injections) and 315 ± 5 ($n = 3$ injections), respectively (Supplemental Figure S3). The lower number of identifications found with the shorter column may be mostly attributed to the lower peak capacity. It is also possible that other factors contributed as well. For example, to obtain a 130 min separation on the 15 cm column, a gradient slope of 0.2 % B/column volume was required. This shallow of a gradient may reduce signal intensity as peaks become broader, which could potentially limit the number of lipids identified.

Representative mass spectra from the beginning, middle, and end portions of the chromatogram of a lipid extract from plasma showed much cleaner spectra on a 100 min analysis on the sonicated 50 cm column compared to a 30 min analysis on the 15 cm column

(Figure 7). Importantly, cleaner mass spectra can allow easier interpretation of data, leading to more identifications [42].

The linear relationship between the chromatographic peak capacity and the number of lipids identified by mass spectrometry illustrates the importance of high resolution separations for lipidomics. The linear relationship between the chromatographic peak capacity and the number of lipids identified by mass spectrometry is in agreement with a previous study on peptide and protein separations [43]. A primary reason for this effect is likely reduction of ionization suppression. Ionization suppression has been well documented for lipids and it is likely that suppression due to co-elution is alleviated with higher peak capacity separations, leading to better signal for more lipids and therefore more lipid identifications [6]. This conclusion is supported by the observation that mass spectra from individual retention times were cleaner (Figure 7). While finding that improved peak capacity with longer improves number of identifications is possibly not surprising, there have been few reports of the effect of longer columns for LC-MS based lipidomics to demonstrate this effect. Further, several factors may prevent this effect from being realized. When employing long, shallow gradients, signal intensity can diminish due to dilution, potentially offsetting the benefits of higher resolution separation for identification. It is also possible that while thousands of lipids may be present in a sample, a relatively small number is detectable in which case better peak capacity would not improve numbers identified; however, our result shows that further gains in peak capacity are likely desirable to further improve lipidome coverage. Also, since we used low flow rates, which can also reduce ionization suppression, it is possible that better chromatographic resolution would not further increase identification [19]. Our results here show that higher peak capacity is still beneficial when employing nanoESI.

The mass spectrometry method employed here does not allow for distinguishing of isomers as two or more unique identification. The increase in lipid identifications observed here could potentially be an under-estimate because more isomers are resolved. For example, PC 18:1 (9-cis) and PC18:1 (9-trans) are baseline resolved (see section 3.5); however, because they have the same mass, they are identified as only one unique feature with the current MS conditions and software used in this work. Further work can be done using lipid identification software and MS/MS capabilities that allow full identification of different isomers to better understand the impact that higher resolution separations have in untargeted LC-MS based lipidomics. This was recently done for example using online ozonolysis to study the impact of ion mobility separation on lipid isomer analysis [44].

3.5. Lipid isomer separations

In the last set of experiments, we evaluated the effect that longer columns operated at 35 kpsi had on the resolution of certain lipid isomer pairs. A number of lipid isomers can exist for one lipid species, adding complexity to a lipidomics analysis [7]. Separation or partial separation of three sets of lipid isomers on the 25 cm and sonicated 50 cm column is shown in Supplemental Figure S4. Panel A is PC 16:0/2:0 vs PC 2:0/16:0, panel B is PC 14:0/18:0 vs PC 18:0/14:0, and panel C is PC 18:1 (9-cis) vs PC 18:1 (9-trans). Panels A and B are examples of regioisomers in which bonding to the sn-1 and sn-2 positions on the glycerol

backbone are switched. The subtle differences between PC 14:0/18:0 and PC 18:0/14:0 allowed only partial separation, while the larger difference between PC 2:0/16:0 and PC 16:0/2:0 allowed baseline resolution on both columns. Resolution increased in panels A and C on the 50 cm column. However, for the PC 14:0/18:0 pair, an increase in resolution was not seen between the 25 and 50 cm columns. Alternative stationary or mobile phases are likely required for separation of this more difficult lipid isomer pair.

4. Conclusions

An ultra-high performance liquid chromatography-mass spectrometry system operable up to 35 kpsi was evaluated for the separation of lipids from complex extracts from human plasma. Longer columns of 25 and 50 cm packed and operated at ultrahigh pressure outperformed 15 cm columns operated at 15 kpsi, with peak capacity improvements ranging from 20 – 95% at the same analysis time. Sonication while packing 50 cm columns was necessary to take full advantage of the longer column length. Use of 35 kpsi inlet pressure allowed for reasonable analysis times using 50 cm long columns and avoids excessive band broadening due to longitudinal diffusion (B-term). A linear increase in the number of lipid species identified was observed with an increase in the chromatographic peak capacity. Lastly, the resolution of both regional and geometrical isomers increased with longer columns and shallow gradients. These results demonstrate the benefits of using longer columns packed and operated at ultrahigh pressure for improving lipid separations and lipidome coverage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- 15 – 50 cm columns operated up to 35 kpsi for lipid separations
- Chromatographic peak capacities over 400 for lipid extract from human plasma
- Putative lipid identification of nearly 500 lipids from human plasma extract
- Resolution of regional and geometrical lipid isomers

Figure 1.

Chromatograms of a) the lipid standard mixture displayed as overlaid extracted chromatograms and b) lipid extracts from human plasma displayed as a base peak chromatogram on a 25 cm x 100 μ m column operated at 35 kpsi with a 50-100% B gradient at 60°C. The gradient slope was 5 % B/column volume. Mobile phase A consisted of 60/40 ACN/water with 10 mM ammonium formate and 0.1% formic acid and mobile phase B was 85/10/5 IPA/ACN/water with 10 mM ammonium formate and 0.1% formic acid. See table S1 for lipid abbreviations.

Figure 2.

Peak capacity plotted as a function of analysis time for lipid separations on the different columns studied in this work. Analysis time was varied by changing the amount of mobile phase loaded on the storage loop effectively giving a longer, shallower gradient. Peak capacity was calculated by dividing the gradient time by the peak width at base of 12 lipid standards eluting throughout the separation window. Other conditions are the same as in Figure 1.

Figure 3.

Chromatograms of a lipid extract from human plasma on a) 50 cm column and b) 25 cm column packed under identical conditions at a constant analysis time of 120 min. The gradient slope was 0.6% B/column volume for the 25 cm column and 2% B/column volume for the 50 cm column. For clarity, the y-axis is zoomed to 50% height to focus on the peak widths at the base. Other conditions are the same as in Figure 1. Extracted ion chromatograms for three lipids eluting across the separation space are displayed in panel c

showing similar peak widths between the 25 and 50 cm non-sonicated columns packed under identical packing conditions.

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Figure 4.

Representative chromatograms of a lipid extract from human plasma on the 50 cm columns studied in this work showing the influence of sonication during column packing of longer columns. Panel a is from the sonicated column and panel b is from the non-sonicated column. The 240 min gradient corresponded to a 1% B/column volume. For clarity, the y-axis is zoomed to 30% height to focus on the peak widths at the base. Other conditions are the same as in Figure 1. Extracted ion chromatograms for three lipids eluting across the

separation space displayed in panel c show improved peak width and peak shape for the sonicated column.

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Figure 5.

Chromatograms of a lipid extract from plasma on a) 15 cm column operated at 15 kpsi and b) 50 cm column operated at 35 kpsi, both with a gradient slope of 2.5 % B/column volume. Other conditions are the same as in Figure 1. Extracted ion chromatograms in panel c for three lipids eluting across the separation space show improved peak width and peak shape for the 50 cm sonicated column compared to the 15 cm column. Retention windows are 8% of the total analysis time.

Figure 6. Lipid identifications from LipidBlast software is plotted as a function of the chromatographic peak capacity for various columns and conditions studied in this work. Error bars are mean standard error from duplicate injections for each condition.

Figure 7.

Example mass spectra of eluted lipids from a lipid extract from human plasma on a 15 cm (A, B, C) and 50 cm column (D, E, F). Averaged mass spectra are from a 0.2 minute elution window of the base peak corresponding to LPC 18:1 (A and D), PC 36:3 (B and E), and CE 18:2 (C and F). Other LC-MS conditions are the same as in Figure 1.