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Capillary ultrahigh-pressure liquid chromatography-mass spectrometry for fast and high resolution metabolomics separations

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ABSTRACT

LC-MS is an important tool for metabolomics due its high sensitivity and broad metabolite coverage. The goal of improving resolution and decreasing analysis time in HPLC has led to the use of 5 – 15 cm long columns packed with 1.7 – 1.9 µm particles requiring pressures of 8 – 12 kpsi. We report on the potential for capillary LC-MS based metabolomics utilizing porous C18 particles down to 1.1 µm diameter and columns up to 50 cm long with an operating pressure of 35 kpsi. Our experiments show that it is possible to pack columns with 1.1 µm porous particles to provide predicted improvements in separation time and efficiency. Using kinetic plots to guide the choice of column length and particle size, we packed 50 cm long columns with 1.7 µm particles and 20 cm long columns with 1.1 µm particles, which should produce equivalent performance in shorter times. Columns were tested by performing isocratic and gradient LC-MS analyses of small molecule metabolites and extracts from plasma. These columns provided approximately 100,000 theoretical plates for metabolite standards and peak capacities over 500 in 100 min for a complex plasma extract with robust interfacing to MS. To generate a given peak capacity, the 1.1 µm particles in 20 cm columns required roughly 75% of the time as 1.7 µm particles in 50 cm columns with both operated at 35 kpsi. The 1.1 µm particle packed columns generated a given peak capacity nearly 3 times faster than 1.7 µm particles in 15 cm columns operated at ~10 kpsi. This latter condition represents commercial state of the art for capillary LC. To consider practical benefits for metabolomics, the effect of different LC-MS variables on mass spectral feature detection was evaluated. Lower flow rates (down to 700 nL/min) and larger injection volumes (up to 1 µL) increased the features detected with modest loss in separation performance. The results demonstrate the potential for fast and high resolution separations for metabolomics using 1.1 µm particles operated at 35 kpsi for capillary LC-MS.

1. Introduction

Metabolomics utilizes measurements of a large number of metabolites from biological, environmental, or industrial sources [1,2]. Metabolomics has been applied in many areas, including food science, plant biology, biofuels, environmental studies, and biomarker and drug discovery for animal and human health [3–6]. Liquid chromatography-mass spectrometry (LC-MS) is a powerful tool for metabolomics due to its sensitivity and amenability towards different sample types and a broad range of compounds [7]. The current state of the art in LC-MS based metabolomics utilizes 1.7 µm particle diameter (d_p) stationary phase particles packed into analytical scale columns (e.g., 1 – 2.1 mm inner diameter (i.d.)) of 5 to 15 cm length [7–10]. Such columns provide peak capacities of ~100 – 200 in 5 – 20 min gradients at flow rates amenable to electrospray ionization (ESI) for fairly sensitive and information-rich analysis. The complexity and dynamic range of the metabolome however still exceeds the current state of the art, where typically thousands of compounds are present in a given sample. One single analytical technique is not yet sufficient to analyze an entire metabolome [2,7,8,11]. In this work, we evaluate use of both smaller d_p and longer capillary-scale columns for LC-MS based metabolomics.

Improvements in separation can often lead to better metabolomics data. For example, changing from columns packed with 3.5 µm to 1.7 µm stationary phase particles and utilizing a higher pressure system resulted in faster and more sensitive urine metabolomics analysis, cleaner mass spectra, and more confident multivariate metabolic profiling [12]. Further work involving reduction of the column i.d. from 2.1 to 1 mm allowed for better

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sensitivity due to lower flow rates while maintaining high linear velocities, enabling confident discrimination between two dose groups with less than 5 min analysis times per assay [13,14].

In principle, further improvements in separation efficiency or analysis time can be gained by employing even smaller stationary phase particles (e.g. ~1 µm). Widespread use of such particles has not been realized due to difficulties in synthesizing and efficiently packing small porous particles, the difficulty in maintaining separation efficiency while robustly interfacing to MS, and the increased pressure demand on instrument hardware [15]. Custom-built LC instrumentation capable of higher pressure (e.g. > 20 kpsi) has been developed by several research groups suggesting the potential to overcome the pressure limitations of working with smaller particles [16]. These systems have primarily been used with 30 – 200 cm long columns packed with 1.7 – 3 µm particles operated with long gradients of 400 – 2000 min. Such conditions yield high efficiency and peak capacity at the expense of analysis time. They have also mostly been demonstrated for peptide separations, and recently lipids and intact proteins [16–22]. A few reports have used ~1 µm porous particle packed columns with mixed results compared with larger particles [19,23]. These applications and demonstrations have only been limited to peptides [19,24,25]. Preparation and use of nonporous particle packed columns has been fairly successful; however, their relatively low loading capacity has limited their use for complex mixture analysis [26–31].

In principle, columns packed with micron-sized particles would benefit metabolomics assays where many complex samples must be analyzed and throughput is important. In this work, we used a custom-built gradient LC-MS system capable of 35 kpsi operating pressure with 1.1 µm particles packed in to 20 cm long capillaries and 1.7 µm particles packed in to 50 cm long capillaries for relatively fast (8 – 110 min, compared to prior reports of >1000 min [17,19,20,32]) and high resolution (peak capacity 150 – 500) separations. These experiments used capillary scale columns (75 – 150 µm i.d.) because of the ease of packing, compatibility with the ultrahigh pressure system, ease of making longer columns, and reduction of heating due to viscous friction [33]. Capillary LC columns can also provide better MS sensitivity with reduced ion suppression because of lower flow rates [34], utility for sample-limited analysis, and economical use of mobile and stationary phases [35]. Capillary columns are routinely used in proteomics but have not yet been widely implemented in metabolomics [36,37]. Benzoyl chloride (BzCl) derivatization was used to improve retention of polar metabolites on reversed phase columns, as such labeling strategies have shown to be useful for both targeted and untargeted workflows [38–40]. Finally, the effect of different LC-MS variables on MS feature detection was studied.

2. Materials and Methods

2.1. Chemicals and materials

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless specified otherwise. HPLC grade water, acetone, methanol, and acetonitrile were purchased from VWR (Radnor, PA). Potassium silicate (Kasil 2130) was purchased from PQ corporation (Valley Forge, IA). Particle size characterization was done using a Zeiss LEO 1455VP Scanning Electron Microscope (SEM) (Jena, Germany) for imaging and ImageJ software (NIH, Bethesda, MD) for d_p measurements. Pneumatic amplifier pump (Haskel, Burbank, CA) DSHF-300 was used for column packing and DSXHF-903 was used for column flushing and LC operation.

2.2. Standards and BzCl derivatization

A standard amino acid test mixture consisting of 10 µM acetylcholine and BzCl labeled proline, valine, tyrosine, and tryptophan was used for column evaluation. Each compound was dissolved in water and combined to make a 100 µM stock solution. The stock solution was derivatized by sequential addition of 100 mM sodium carbonate, 2% (v/v) BzCl in acetonitrile, and 1% (v/v) sulfuric acid in 20% (v/v) acetonitrile in water in a 2:1:1:1 ratio as previously described [38]. The BzCl derivatized standard mixture was diluted to a final concentration of 10 µM using water.

2.3. Human plasma extraction

Metabolites were extracted from pooled human plasma using a mixture of ice-cold methanol/acetone/acetonitrile (v/v/v 1:1:1) as the extraction solvent. To 100 µL of plasma, 400 µL of extraction solvent was added, vortexed, and centrifuged at 12,100 x g at 4°C for 10 min. The supernatant was removed to a glass HPLC vial, evaporated with nitrogen, and reconstituted with 100 µL of 90/10 (v/v) water/acetonitrile. The supernatant was then derivatized in the same manner described above.

2.4. Column packing

Polyimide-coated fused-silica capillaries with varying inner diameters and outer diameter of 360 µm were purchased from Polymicro Technologies, Inc. (Phoenix, AZ). Both the 1.7 µm and 1.1 µm particles were bridged ethyl hybrid (BEH) silica with C18 bonding (Waters Co; Milford, MA). Column frits were prepared by spotting an equal amount of potassium silicate and formamide on a glass microfiber filter paper (Reeve Angel; Clifton, NJ) and dabbing the end of the capillary ~ 5 times, and placed in a ~60°C oven overnight [41]. For isocratic separations, a pre-fritted, embedded spray tip (30 µm i.d.) (New Objective; Woburn, MA) was used instead of an outlet frit to limit post column dead volume. Acetone was used as the slurry solvent for all columns [18,42]. Slurry concentration for each particle size and the application of sonication while packing was chosen based on previous studies [23,43–45]. For 15 cm/1.7 µm columns representing commercial columns, packing was achieved using a 100 mg/mL slurry with a low-pressure packing apparatus at ~1000 psi and subsequently flushed at 15,000 psi in 50/50 (v/v) water/acetonitrile. For the 50 cm/1.7 µm columns and 20 cm/1.1 µm columns, slurry concentrations of 200 mg/mL and 30 mg/mL, respectively, were used. Low pressure (~1000 psi) was applied to form ~2 cm of packed bed, followed by immediate application of 30 kpsi while the column was submerged in a sonication bath as previously described [43]. The columns were subsequently flushed at 50 kpsi for 1 h. All columns were depressurized for 1 h, cut to the desired length, and an inlet frit was made as described above.

2.5. LC-MS operation

For isocratic separations, a split-flow injection system was employed as previously described with 50/50 (v/v) water/acetonitrile with 10 mM ammonium formate and 0.1 % formic acid [46,47]. A Thermo Finnigan LCQ Deca XP Plus (Thermo Fisher Scientific, San Jose, CA) using a nanospray ion source in positive ion mode was used for detection. A scheduled MRM method was employed using the following transitions: Acetylcholine (146-87), Bz-proline (220-174), Bz-valine (222-176), Bz-tryptophan (309-263), and Bz-tyrosine (390-240). The capillary voltage was 2 kV. Retention factors (k') were calculated by $k' = (t_r - t_0)/t_0$.

For all gradient separations, a modified UHPLC system capable of 35 kpsi operating pressure was used as previously described

[19,21]. Average flow rates at 35 kpsi were 2.5 µL/min and 1.8 µL/min on the 20 cm x 150 µm, 1.1 µm d_p and 50 cm x 100 µm, 1.7 µm dp_p columns, respectively. For the 15 cm columns representing commercial limits, a Waters NanoAcquity was used directly at 1 µL/min (~10 kpsi). Mobile phase A was water with 0.1 % formic acid. Mobile phase B was acetonitrile with 0.1 % formic acid. All injections were performed in partial loop mode on the NanoAcquity. Peak capacity was calculated by dividing the elution window by the average peak width (4σ – calculated by measuring $W_{1/2}$ and multiplying by 1.7) of metabolites and lipids eluting throughout the separation window. Column volume was calculated assuming a total column porosity of 0.8. The column oven was 60°C. Effluent from the column was connected to a Micromass Q-ToF Premier (Waters Co; Milford, MA) using a stainless-steel union and a fused silica spray needle with a 75 µm i.d. tapered to 30 µm tip (New Objective; Woburn, MA). The capillary voltage was 2.5 kV. 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*.

2.6. Feature detection

For the gradient separations with Q-TOF detection, mass spectra from 30 s (≤45 min analysis times) or 60 s (>45 min analysis times) windows of each chromatogram were extracted, baseline subtracted, and centered in Mass Lynx. A feature was defined as any signal with a unique *m/z* and retention time and above the average background signal from each separation.

3. Results and discussion

3.1. Kinetic plots for choice of particle size and column length

In this work, we investigated the potential of capillary LC-MS using 1.1 and 1.7 µm particles and ultrahigh pressure instrumentation for untargeted metabolomics separations. Kinetic plots [48] were used to find column lengths that could yield high efficiency while maintaining analysis times typical of metabolomics assays (e.g. ~5 – 30 min). For this kinetic analysis, columns are assumed to be equally well-packed, with the following reduced van Deemter coefficients: a = 0.4, b = 1.9, c = 0.18 [18]. Flow resistance and column dead times were estimated using the Kozeny-Carman equation, provided in more detail in the literature [33,48]. As shown in Fig. 1A, a 50 cm column with 1.7 µm particles at 35 kpsi should produce ~100,000 plates with a dead time just over 100 s. A 20 cm column with 1.1 µm particles should produce the same efficiency in approximately half the time. In contrast, if using current commercial capillary LC systems with 10 kpsi and 15 cm column length, only ~ 30,000 plates is achieved in a dead time of 50 s.

3.2. Isocratic column evaluation using amino acid standards

The limited reports on use of 1.1 µm porous particles is likely due to their low availability, high pressure requirement, and difficulties in packing [16,19,23]. We verified particle size and particle size distribution (PSD) of these particles using SEM (Figure S1). The average particle size was 1.3 µm, slightly larger than the 1.1 µm listing by the manufacturer. Similar discrepancies for 1.7 µm particles, which often appear as ~ 2 µm by SEM, have been shown [22,43]. (For consistency, we use the particle size listed by the manufacturer when referencing all particles.) The PSD for the 1.1 µm particles was 12% (120 particles counted), in good agreement with other porous particles indicating well-controlled sizing [49].

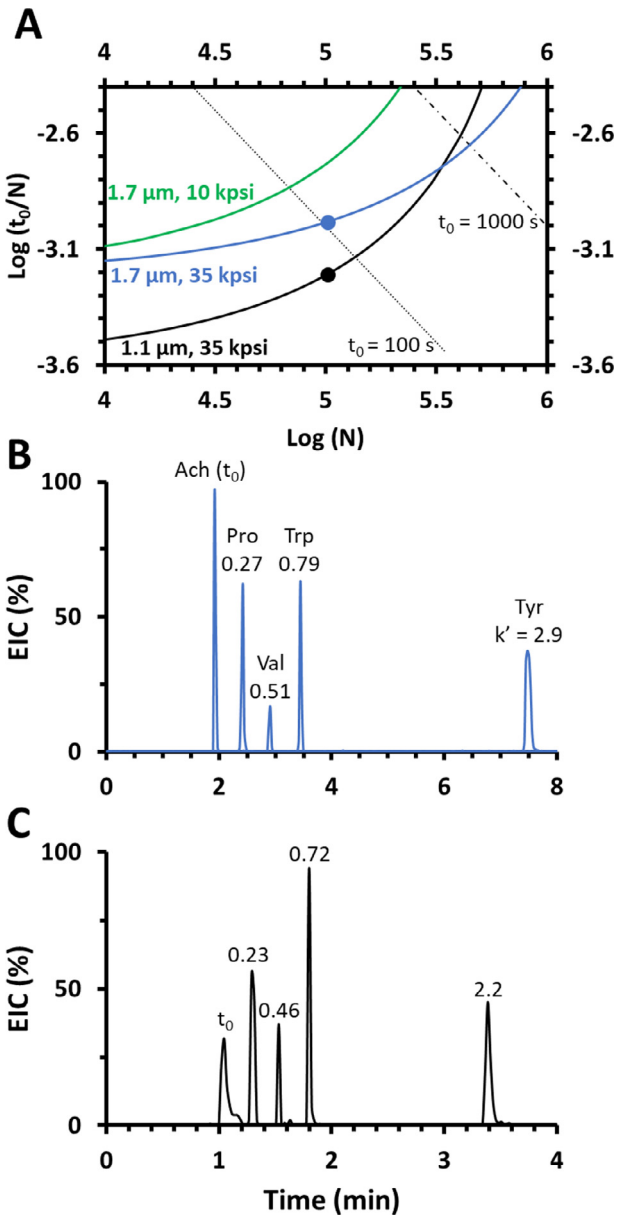

Fig. 1. (A) Kinetic plot illustrating theoretical improvements when moving from 1.7 µm to 1.1 µm particles with 35 or 10 kpsi instrument pressure. The two dots represent the column length that would produce ~100,000 plates with each particle size and the set pressure limit of 35 kpsi. Diagonal dashed lines representing column dead times of 100 s and 1000 s are shown for clarity. EICs for the isocratic separations using (B) a ~40 cm x 75 µm i.d., 1.7 µm d_p column and (C) ~20 cm x 150 µm i.d., 1.1 µm d_p column of the standard amino acid mixture with acetylcholine (Ach) as a dead time marker. Faster separation with the 1.1 µm particle packed column shows approximate agreement with theoretical expectations. Similar peak shapes and retention factors (k') for BzCl labeled metabolites were obtained with both particle types. Mobile phase was 50/50 (v/v) water/acetonitrile with 10 mM ammonium formate and 0.1% formic acid.

Initial chromatographic experiments were directed towards determining if the packing conditions and UHPLC system used here resulted in good performance for capillary columns. Fig. 1B & C shows results from isocratic separations of the standard amino acid mixture, along with acetylcholine (Ach) as the dead time marker, for 1.7 µm particles packed into ~40 cm long x 75 µm i.d. and 1.1 µm particles packed into ~20 cm x 150 µm i.d. columns. (We found little difference in column performance with i.d.'s of 75 – 150 µm for the isocratic conditions studied here, similar to recent studies using the same packing protocol employed here [21,22]). The

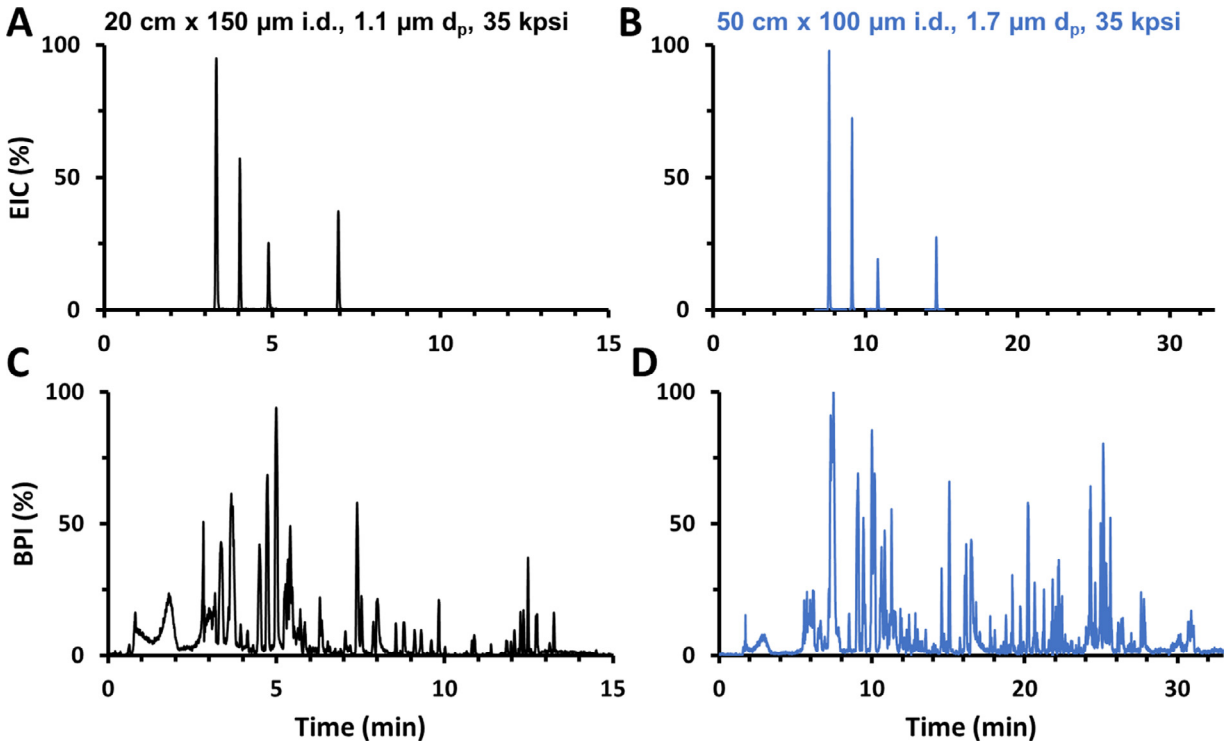

Fig. 2. Gradient separations of (A and B) a 0.2 µL injection of the standard amino acid mixture and (C and D) a 1 µL injection of a complex plasma extract. Black traces (A and C) are separations on a 20 cm x 150 µm i.d., 1.1 µm d_p column and blue traces (B and D) are on a 50 cm x 100 µm i.d., 1.7 µm d_p column. Other conditions: 20 – 100 %B gradient with a gradient volume of 10X column volume (8 %ΔB/column volume); 35 kpsi operating pressure; 60°C column oven. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid.

shorter length of 40 cm compared to the expected 50 cm from the kinetic plot was due to the limited length of the embedded spray tip capillary at the time. The dead time for the 1.1 µm column was ~50% faster as expected from the kinetic plot, and similar retention factors were achieved between the two columns for each amino acid. The 20 cm column with 1.1 µm particles generated 80,000 plates and the 50 cm column with 1.7 µm particles generated 85,000 plates, measured for Bz-valine (k' ~ 0.5) Additionally, these results showed that coupling to MS while maintaining chromatographic performance is possible, whereas many reports have shown deteriorated performance when coupling with MS compared to UV detection [50]. Peak shape was, however, slightly affected by MS scan rate for these separations due to the slow acquisition speed of the MS used, which could marginally affect plate count measurements (Figure S2).

3.3. Gradient column evaluation using amino acid standards

The isocratic analysis confirmed relatively good packing conditions; however, automated gradient separations are of interest for metabolomics due to the need to analyze many samples and separate a large range of compounds. Fig. 2A & B shows 35 kpsi gradient separations from a 0.2 µL injection of the standard amino acid mixture on a 20 cm x 150 µm i.d., 1.1 µm d_p and a 50 cm x 100 µm i.d., 1.7 µm d_p column, achieving a peak capacity of 118 ± 5 (n = 3 injections) and 153 ± 4 (n = 3 injections) in 8 and 15 min, respectively. A gradient of 10X the column volume (8 %ΔB/column volume) was used in both separations. These separations demonstrate that automated gradient formation with a commercial autosampler is possible while operating at 35 kpsi and maintaining the good performance of these columns. Importantly, the 1.1 µm particle packed column (20 cm x 150 µm i.d.) exhibited approximately similar peak capacity – 120 versus 150 – in about half the time compared to the 1.7 µm d_p column (50 cm x 100 µm i.d.). Interestingly, mixed results have previously been reported on the performance of such small particles (e.g. 0.8 – 1.3 µm) for peptide separations. In some instances, high peak capacities were achieved (~100 – 400) in 10 – 40 min. In other cases, longer columns packed with larger particles (e.g. 50 – 100 cm with 1.9 µm particles) provided higher peak capacity in the same amount of time than the shorter columns with smaller particles [19,24,25]. This disparity could be due to the difficulty in packing such small particles. Additionally, steeper gradients on longer columns could be more beneficial for peptides compared to shallower gradients on shorter columns due to the large solvent strength 'S' parameter of peptides [51,52].

3.4. Separations of complex plasma extract

3.4.1. Practical considerations for high efficiency capillary LC-MS metabolomics

Our studies with metabolite standards suggest agreement with the kinetic plots for both isocratic and gradient separations; however, practical constraints including injection volume and injection solvent can adversely affect separation performance and therefore negate the potential gains for practical metabolomics measurements. We chose to evaluate our columns with an extract of human plasma as an example of complex metabolomics samples [6]. We used BzCl derivatization to improve retention of polar metabolites on reversed phase columns; however, underivatized lipids and other metabolites were present and detected as well. Although an injection solvent of 100% mobile phase A provided the best peak shape and peak width for the standards (Fig. 2A & B), this solvent was not practical with the complex plasma extract as precipitation and loss of signal of the more nonpolar metabolites was observed. An injection solvent of ~70/30 H_2O/ACN provided better signal intensity for late eluting peaks. Moreover, injecting larger volumes (e.g., 0.5 and 1 µL compared to 0.2 µL for standards)

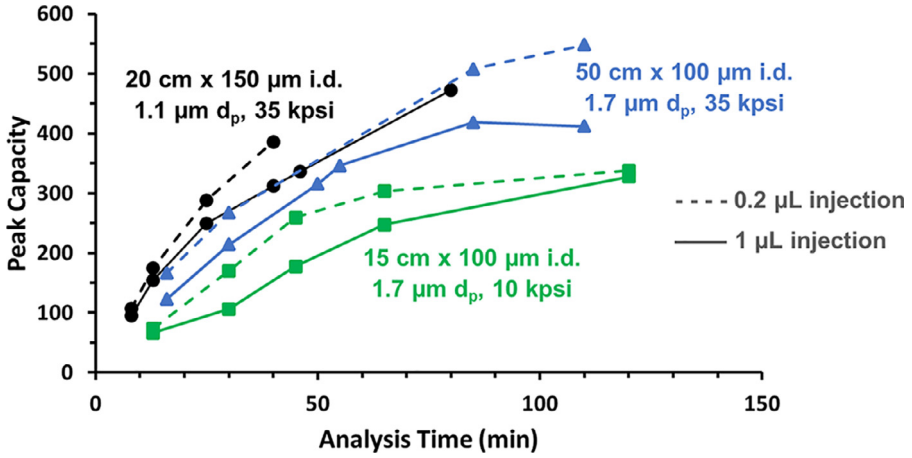

Fig. 3. Peak capacity plotted as a function of analysis time for various columns investigated in this work. Dashed lines represent separations from a 0.2 µL injection and solid lines represent a 1 µL injection. The 20 cm and 50 cm columns were operated at 35 kpsi, and the 15 cm column operated at ~10 kpsi (1 µL/min), all with varying gradient times. Other conditions are the same as in figure 2.

provided better signal for most metabolites and a higher number of features detected (see section 3.5). Example base peak intensity (BPI) chromatograms of a 1 µL injection of the plasma extract on a 20 cm x 150 µm i.d., 1.1 µm d_p and 50 cm x 100 µm i.d., 1.7 µm d_p column using a gradient volume of 10X column volume (8 %ΔB/column volume) at 35 kpsi show good signal response across the separation space and similar peak shapes compared to the standards (Fig. 2C & D). The changes in solvent and injection volume discussed above led to ~32%, ~12%, and ~0% increase in peak width for early, middle, and late eluting compounds, respectively, for a 13 min separation on the 20 cm x 150 µm i.d., 1.1 µm d_p column (Figure S3).

3.4.2. Potential for fast separations using 1.1 µm particles

We evaluated the peak capacity from separations of the plasma extract at different analysis times and injection volumes for 1.7 µm d_p columns (15 and 50 cm long x 100 µm i.d.) and the 1.1 µm d_p column (20 cm x 150 µm i.d.) (Fig. 3). The 15 cm column packed with 1.7 µm particles was operated at ~10 kpsi (constant 1 µL/min), representing current state-of-the-art capillary UHPLC. The 1.1 µm particle packed column consistently outperformed both the 50 cm and 15 cm x 100 µm i.d. columns with 1.7 µm particles in terms of peak capacity per analysis time.

We further examined the results from Fig. 3 in two ways: potential for fast separations and potential for high resolution separations. Choosing a relatively short analysis time of 13 min, we compared peak capacities of the shorter columns due to their smaller void times amenable for fast separations. The 1.1 µm d_p column (20 cm x 150 µm i.d.) generated a peak capacity of 153 ± 3 (n = 2 injections) and 183 ± 9 (n = 3 injections) for a 1 µL and 0.2 µL injection, respectively. These values were noticeably higher than the 1.7 µm d_p column (15 cm x 100 µm i.d. at 10 kpsi, representing commercial limits), which were 59 ± 5 (n = 3 injections) and 73 ± 6 (n = 3 injections) for a 1 µL and 0.2 µL injection, respectively. The good peak capacity in short times should be of benefit for metabolomics studies that require many samples to be analyzed. Our results for the larger particles appear to be reasonable as similar peak capacities, ~60 in 15 min, were recently reported with commercial capillary columns (15 cm with 1.7 µm particles) for small molecule separations with UV detection and comparable flow rates to those studied here [53].

BPI chromatograms of a 1 µL injection for the 13 min gradient separation of the complex plasma extract on the 1.1 µm d_p (20 cm x 150 µm i.d.) and 1.7 µm d_p (15 cm x 100 µm i.d. at 10 kpsi) columns show the enhanced peak shape and peak capacity of the 1.1 µm column for relatively fast separations (Fig. 4). Extracted ion chromatograms (EICs) of *m/z* 222 (Bz-valine) and *m/z* 496 (lysophosphatidyl choline (LPC) 16:0 (sn-1 and sn-2 isomer)) illustrate the improved peak width and resolution of the 1.1 µm d_p x 20 cm column (35 kpsi) compared to the 1.7 µm d_p x 15 cm column (10 kpsi). Early eluting peaks such as valine were particularly broad on the 15 cm x 100 µm i.d., 1.7 µm d_p column compared to the 20 cm x 150 µm, 1.1 µm d_p column for this 1 µL injection. This disparity could be due to the larger column volume of the 20 cm x 150 µm i.d. (~2.8 µL) vs. the 15 cm x 100 µm i.d. (~0.94 µL); however, decreasing the injection volume to 0.2 µL resulted in little improvement on the 15 cm x 100 µm i.d., 1.7 µm d_p column at 13 min (dashed vs. solid green lines (square symbols) in Fig. 3). We originally chose 150 µm i.d. for the short (20 cm) 1.1 µm particle packed column to approximately match the column volume of the 50 cm x 100 µm, 1.7 µm columns. To further investigate the column volume difference and better compare with the 15 cm x 100 µm i.d., 1.7 µm d_p column, we packed 20 cm x 100 µm i.d. capillaries with the 1.1 µm particles (column volume ~1.3 µL) and found only a slight decrease in peak capacity by ~18 % for a 0.2 µL injection relative to the 20 cm x 150 µm i.d., 1.1 µm d_p column (Figure S4). This change is a marginal decrease in peak capacity compared to the 60% lower peak capacity with the 15 cm x 100 µm i.d., 1.7 µm d_p column in Figs. 3 and 4. The improvement in peak capacity with the smaller particles is therefore likely a combination of increased efficiency, larger column volume, higher packing pressure [54], and higher operating pressure, as higher pressure has been shown to increase retention of small molecules [46,55]. Further studies should be focused on individually assessing the impact of these variables on injection volume for metabolite separations.

3.4.3. Potential for high resolution separations

Our second examination of Fig. 3 investigated the potential for achieving higher peak capacity using 1.1 µm particles. As shown in Fig. 3, for a 1 µL injection, a target peak capacity of ~350 can be achieved in 45 min with the 1.1 µm d_p column (20 cm x 150 µm i.d.), compared to 60 min and 120 min for the 1.7 µm d_p columns (50 cm and 15 cm/10 kpsi x 100 µm i.d., respectively). BPI chromatograms of these three separations are shown in Fig. 5, with EICs of *m/z* 496 (LPC 16:0) and *m/z* 391 displayed to compare peak shape and resolution between the three columns. The 1.1 µm particles can thus be used for relatively quick yet high peak capacity separations relative to the other columns if 35 kpsi is available.

Even higher resolution separations are of interest for isomers or isobaric compounds and for providing broader metabolome

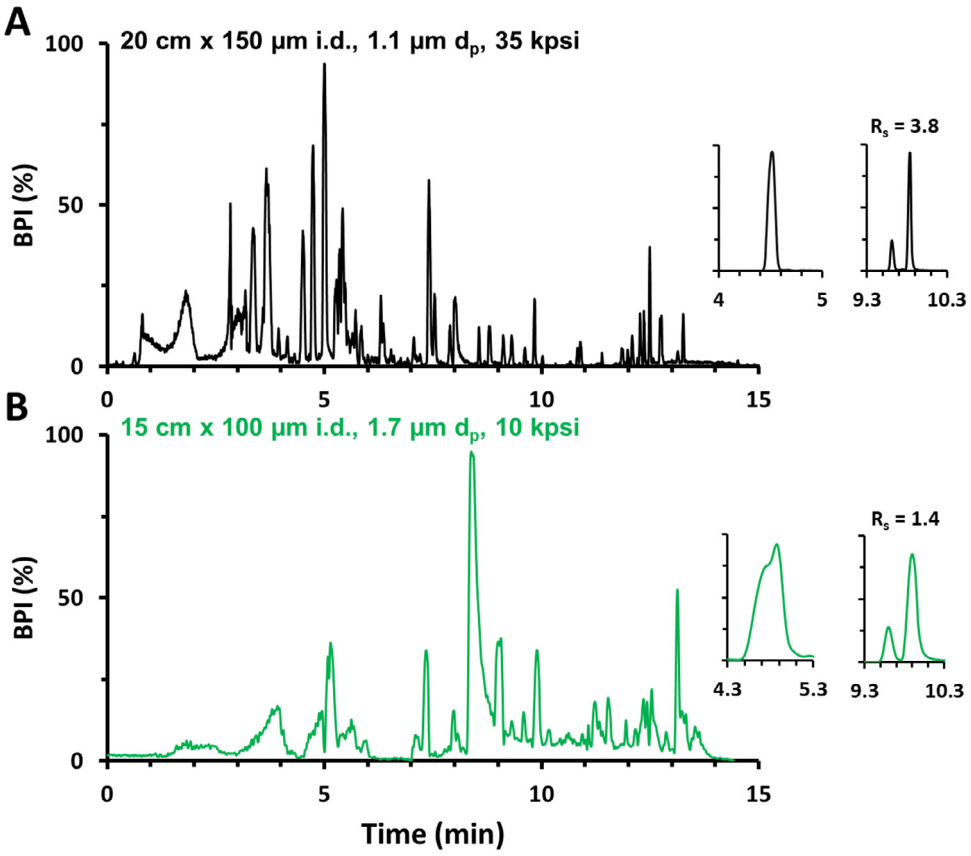

Fig. 4. Comparison of (A) a 20 cm x 150 µm i.d., 1.1 µm d_p column at 35 kpsi and (B) a 15 cm x 100 µm i.d., 1.7 µm d_p at 10 kpsi (commercial limitations for capillary LC) for a relatively fast 13 min gradient. BPI chromatograms of BzCl labeled plasma extract for a 13 min gradient are shown for a 1 µL injection of a BzCl labeled metabolite extract. Representative EICs of an early eluting compound, Bz-valine (*m/z* 222), and a late eluting compound, lysophosphatidyl choline (LPC) 16:0 (*m/z* 496), are shown for comparison of peak shape and peak widths. The resolution (R_s) of LPC 16:0 sn-1 and sn-2 isomers is shown.

coverage. High resolution separations of small molecules and metabolites have been demonstrated with peak capacities ranging from 1500 – 1800 using long (e.g. >100 cm) columns and ~33 h analysis times [17,32]. Such long separation times are useful in some conditions, but many metabolomics studies require numerous samples and such times can become prohibitive. The potential for high resolution separations in relatively short analysis times is an attractive feature of sub-2 µm columns operated at 35 kpsi. For a 0.2 µL injection, a peak capacity of ~400 is achieved with a 40 min separation on the 20 cm x 150 µm i.d., 1.1 µm d_p column, and a peak capacity of ~550 was achieved on the 50 cm x 100 µm i.d., 1.7 µm d_p column in 110 min (Fig. 3). Smaller injection volumes than those studied here could lead to even higher peak capacities in the same time; however, MS sensitivity and identification of low level metabolites could suffer (discussed more in section 3.5). Furthermore, higher peak capacities or shorter analysis times could be attained with moving to even smaller particles and/or longer columns with higher instrument pressures.

3.5. Feature detection in human plasma

The previous sections discussed preparation and use of capillary columns that can provide higher separation efficiency and peak capacities than current commercially available particle sizes and column lengths in shorter analysis times. Real metabolomics assays however rely on confident and in-depth metabolome coverage and annotation. While higher peak capacity separations have provided broader metabolome and proteome coverage [12,17,56], a number of LC-MS variables can affect MS response and metabolomics metrics. For example, steeper gradients have shown to provide higher MS signal due to the narrower peaks compared to shallower gradients [57]. Furthermore, while higher flow rates have shown to provide higher peak capacities for the same analysis time [58], the ionization may suffer and hinder metabolite coverage. We therefore evaluated several variables using full-scan (MS1) feature detection as a proxy for the information content possible from the metabolomics assay (Fig. 6). A feature was defined as any signal with a unique *m/z* and retention time and above the average background signal from each separation.

Increasing the injection volume from 0.2 to 1 µL consistently provided a much higher number of features detected from BzCl-labeled plasma extracts across the three column types investigated in this work (Fig. 6A), despite the slight losses in peak capacity that were observed with these larger injection volumes (see Fig. 3 and section 3.4.1). The effect of increasing peak capacity by varying the gradient time (e.g. Fig. 3) on feature detection was also investigated (Fig. 6B). For each column type, a general increase in the number of features detected was seen as the peak capacity increased. This trend is likely due to resolution of isobaric compounds and alleviation of ionization suppression from co-eluting species compared with shorter and lower resolution separations [56,59]. Higher feature counts at longer analysis times could be over-inflated, however, as feature count is often biased compared to high confidence metabolite annotation and identification [60]. Interestingly, the lowest efficiency column – the 15 cm/1.7 µm at 10 kpsi – provided higher feature counts than the longer columns at higher pressure for the same peak capacity (albeit at longer analysis times to achieve the same peak capacity). This observation is likely due to the lower flow rate on the 15 cm x 100 µm i.d., 1.7 µm d_p column at 10 kpsi (1 µL/min

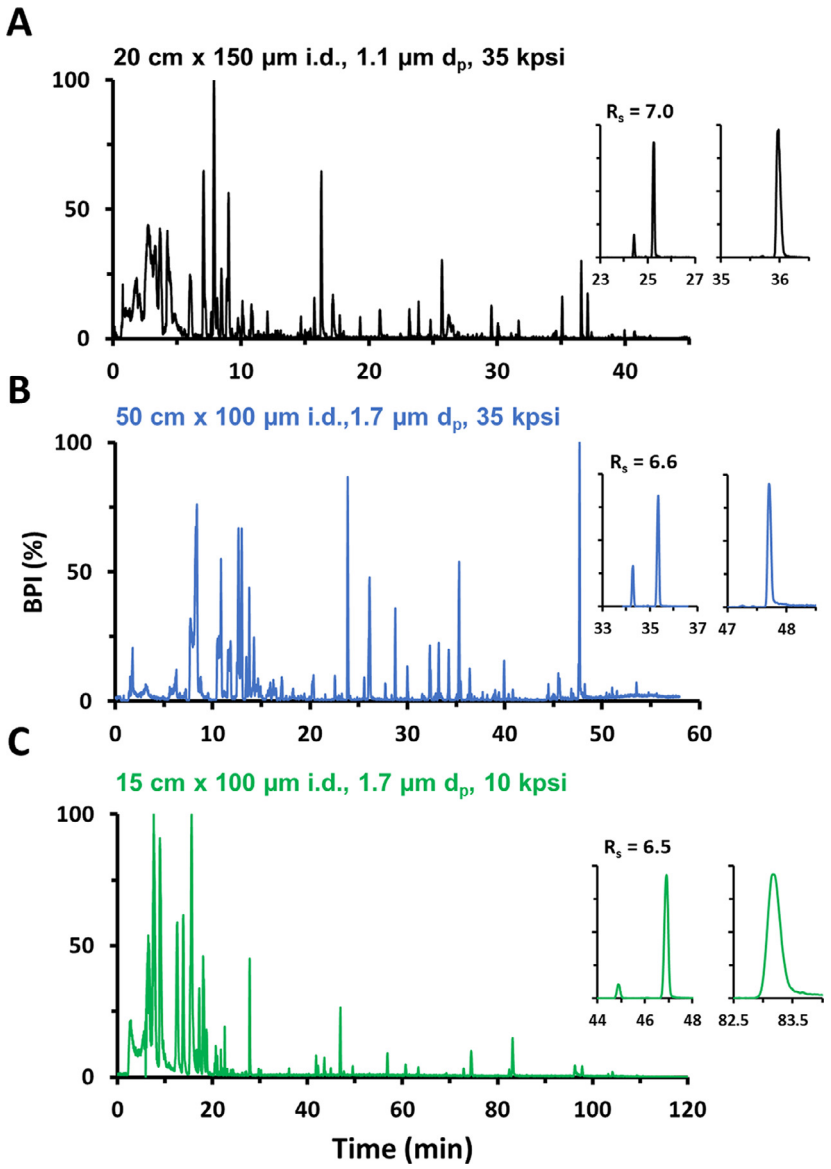

Fig. 5. Example BPI chromatograms from a 1 µL injection of BzCl labeled plasma extract illustrating the time required to achieve a peak capacity of ~350 with the different columns, particle sizes, and pressure limits investigated in this work. EICs of *m/z* 496 (LPC 16:0) and 391 are displayed, with the resolution (R_s) of LPC 16:0 isomers shown.

versus ~1.8 – 2.5 µL/min on the columns run at 35 kpsi). Lower flow rates can provide increased ionization efficiency due to smaller initial droplet sizes and easier desolvation; this improvement in ionization can lead to less ionization suppression and interference from matrix effects [34,61]. To further investigate this hypothesis and attempt to provide similar results with the higher efficiency columns, we operated the 20 cm x 150 µm i.d., 1.1 µm d_p column at lower flow rates (by operating the pneumatic pump at 10 and 15 kpsi) with the same gradient slope of 8 %ΔB/column volume (Fig. 6C). Reducing the flow rate down to 700 nL/min resulted in over double the number of features detected. Selected EICs of Bz-Trp and Bz-Tyr detected from the plasma extract showed higher peak intensities and larger peak areas with the lower flow rate separations (Figure S5). These data corroborate that lower flow rates indeed give higher MS response and thus more features detected for the conditions studied here.

Taken together, a higher injection volume combined with lower flow rates provided the highest number of features detected for a given analysis time. Increasing separation peak capacity through extending the gradient time also increased feature counts; however, this approach decreased analysis throughput. Further work involving reduction in column i.d. and flow rate to the low nL/min range may further increase metabolite coverage while maintaining the chromatographic advantages of combining smaller particles with higher pressure. Reduction of column i.d. and use of nanoflow/capillary LC-MS has been heavily utilized in proteomics studies and has provided large increases in proteome coverage, and in some cases has been extended to metabolomics [37,62–64]. Use of trap columns may be needed to mitigate the gradient delay when going to such low flow rates and alleviate band broadening from injecting on such narrow columns. Thus, in order to combine the benefits of larger injection volumes and high resolution separations for capillary LC-MS based metabolomics, pre-column focusing strategies likely need to be implemented [65–67]. Given the complexity of factors influencing feature count and MS signal, optimization not only in chromatography but of these other factors on metabolite identification is worth a more in-depth study. Future studies should also employ a more rigorous evaluation of

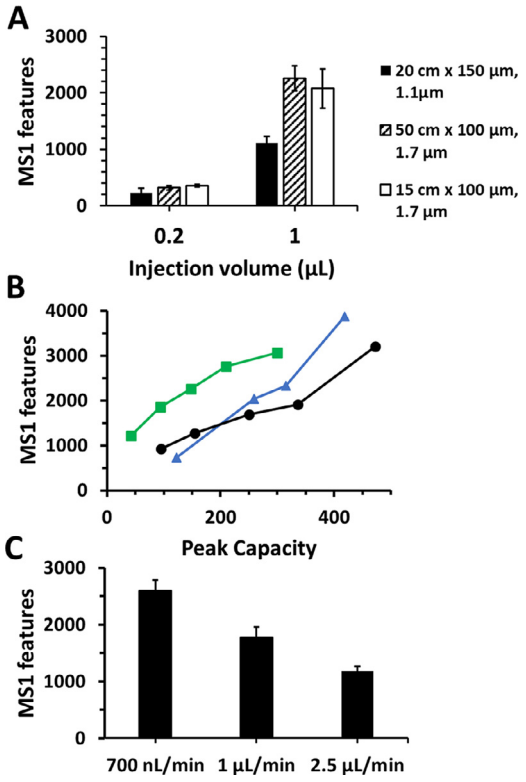

Fig. 6. Effect of different LC-MS variables on MS1 feature detection from BzCl labeled plasma extract. (A) Effect of injection volume on feature count and peak capacity for the three columns shown in Figure 3. All separations used a gradient volume of 10X the column volume (8 %ΔB/column volume). (B) Effect of increasing peak capacity through longer gradient times on feature count for a 1 µL injection on the same three columns. (C) Effect of flow rate (adjusted by changing inlet pressure) on feature count for a 1 µL injection on the 20 cm x 150 µm i.d., 1.1 µm d_p column using a gradient volume of 10X the column volume. Error bars represent standard error from duplicate injections.

metabolite annotation and the effect of these variables on high confidence identification rather than features [60,68,69].

3.6. System robustness and repeatability

The repeatability of retention times and peak widths of the analytes are important for routine, long term metabolomics assays. We performed column repeatability tests for the 15 cm x 100 µm i.d., 1.7 µm d_p, 20 cm x 150 µm i.d., 1.1 µm d_p, and 50 cm x 100 µm i.d., 1.7 µm d_p columns discussed above. Average RSDs in retention time and peak width for the amino acid mixture for all columns were below 4% and 8%, respectively (Table S1). Additionally, the long-term use and repeatability of the 20 cm x 150 µm i.d., 1.1 µm d_p column was assessed over a 5 month period (Table S2). No signs of column degradation or clogging were observed, with average retention time and peak width RSDs of 6% and 11%, respectively, for the amino acid mixture. These deviations are similar to previously reported packed capillary C18 columns [19,21,54].

4. Conclusions

This study illustrates the feasibility and potential impact of using 1.1 µm particles in 20 cm long columns paired with a gradient capillary LC system capable of 35 kpsi for metabolomics assays. We have found packing conditions and instrumentation that allow approximate agreement with theory for using such columns with ultrahigh pressure instrumentation, while demonstrating routine use with practical considerations for metabolomics samples. The 1.1 µm particle packed columns enable higher peak capacity at relatively short, and practical, analysis times of 13 min compared to columns packed with larger particles as commonly used currently. The columns also allowed relatively high peak capacities (e.g. peak capacity 300 – 500) to be reached at a ~30% faster time compared to 50 cm x 100 µm i.d., 1.7 µm d_p columns, and nearly 3x faster time compared with 15 cm x 100 µm i.d., 1.7 µm d_p columns at 10 kpsi. For metabolomics assays, interplaying variables such as flow rate, peak capacity, and injection volume can all be manipulated to increase the number of features identified in the human plasma extract.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Matthew J. Sorensen: Data curation, Formal analysis, Validation, Writing - original draft. **Robert T. Kennedy:** Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461706.

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