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Application of trilobal capillary-channeled polymer (C-CP) fibers for reversed phase liquid chromatography and ESI-MS for the determination of proteins in different biological matrices

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ABSTRACT

Electrospray ionization mass spectrometry (ESI-MS) analysis provides a great deal of analytical information as a detection technique for biological species determinations. However, because of the common high ionic strength matrices and processing buffers, including salt and small molecules, additional sample preparation/desalting processes are necessary prior to introduction into the ESI source. The most typical form of processing is reversed phase liquid chromatography (RPLC). Limitations to low flow rates (<0.5 mL min^{-1}) for optimal spectral clarity and greatest sensitivity for conventional ESI generally imply use of low linear velocities and slow RPLC separations. Previous efforts in this laboratory have demonstrated the use of capillary-channeled polymer (C-CP) fibers for high throughput protein separations. More recently, a trilobal version of the fiber has proven to yield superior separations due to better packing quality. To further investigate the potential application of the trilobal-shaped C-CP fiber in LC-MS, trilobal polypropylene (PP) fiber columns were applied for RP-LC-ESI-MS protein determinations in highly-diverse biological matrices, including urine, saliva and HL5 cell culture media. Several parameters, including fiber packing density, mobile phase flow rate, and gradient rate were evaluated using a four-protein mixture (ribonuclease A, cytochrome c, lysozyme, and myoglobin). In addition, differences in ion-pairing performance between trifluoroacetic acid, formic acid, and acetic acid were evaluated. The effectiveness of the matrix removal is reflected in the near-identical qualitative and quantitative ESI-MS responses in all cases for a five-protein mixture. The results of this study demonstration the utility in the application of trilobal, polypropylene C-CP fibers in LC-MS analysis of biomacromolecules.

Introduction

The field of proteomics has greatly expanded over the last 30 years, providing insights into fundamental processes in highly diverse biological systems.[1] In the realm of clinical analyses, where proteins serve as biomarkers for disease, there is demand for quick, reliable, and accurate results to streamline the diagnosis process.[2] To this end, there has been much research in separating and analyzing proteins in biological media using liquid chromatography-mass spectrometry (LC-MS), predominately with the use of electrospray ionization (ESI).[3]

Even though there is a plethora of body fluids that can be used for protein analysis, such as blood, saliva, and tears, urine is especially attractive as a source as it is easily obtained, provides large sample volumes, can be obtained at frequent intervals, and the sampling is usually non-invasive.[4, 5] Similarly, saliva is also considered a favorable diagnostic medium.[6] Cell cultures are another important media in the field of disease diagnosis and basic cell metabolism studies.[7] In the case of urine, the glomerulus in the kidneys filters blood by extracting excess fluid and waste, including a complex mixture of proteins which is similar to proteins found in blood. This similarity makes urine an excellent medium to determine essential proteins.[8, 9]

Despite all of the advantages of using urine as a medium, there are a number of challenges when trying to analyze urine by ESI-MS. First, the total concentrations of proteins and peptides in urine are quite low when compared to blood plasma (20 mg L^{-1} vs. 60-85 g L^{-1} for healthy subjects)[10] . Second, the high concentration of salts in urine (e.g., NaCl and KCl at ~190 g L^{-1})[11] can suppress the number of protein ions created via the electrospray process; therefore, decreasing the utility of the approach.[12] Of course, the same sorts of challenges, along with others such as viscosity, occur to varying

degrees for proteins isolated from other biological fluids. Thus, for ESI-MS applications, the proteins must be isolated from inorganic salts and other small molecules and ions present in biological media,[12] indeed this impracticality exists in terms of determining proteins in the standard buffer media used across many bioprocessing steps.[10]

A variety of techniques are available to isolate proteins from biological media such as two-dimensional gel electrophoresis (SDS-PAGE) and solid phase extraction (SPE); however, these techniques usually increase the possibility of analyte loss, analysis time, and the possibility for contamination.[8] Beyond the isolation step, those methods require a separate method for determining the protein composition. In the case of SPE, most detection methods require that the proteins be further segregated from one another (e.g., chromatography) for successful analyses. Alternatively, an integrated liquid chromatography-mass spectrometry (LC-MS) method generally provides higher throughput, is fairly cost-effective, provides sensitivity that is suitable for many applications, and can analyze multiple analytes in one protocol. Furthermore, LC-MS can be applied across fundamentally different types of media.[13]

While LC-MS is very useful towards separating and analyzing proteins, it is usually not directly suitable for common biological fluid analyses due to the complexity of the matrices, thus necessitating the use of a matrix elimination step (i.e., SPE) prior to the LC separations.[14] Very often the challenges in LC-MS biomolecule analyses come from physico-chemical limitations in effectively combining the separation and ionization steps, particularly in the case of intact protein analysis.[15] Specifically, optimal solvent flow rates for some separations occur at volume flow rates that are too high for efficient ionization when using an ESI source.[2] In any case, the LC separation quality of

macromolecules on porous support phases is eventually compromised at high flow rates (throughput) due to mass transfer (van Deemter C-term) limitations. For this reason, core-shell types of stationary phases are receiving increased interest for intact protein LC.[16, 17] In terms of the separation chemistries, ion pairing agents used in RP-LC, such as trifluoroacetic acid (TFA), become ionization suppressants in ESI-MS,[18, 19] requiring post-separation remediation or compromises in the choice of mobile phase additives and the resultant chromatographic quality. Thus, the development of methods that are reliable, maintain high MS sensitivity and acceptable chromatographic throughput and quality is still a primary focus in the field of biomacromolecule LC-MS.

Capillary-channeled polymer (C-CP) fibers have been developed by Marcus and co-workers as stationary phases for biomacromolecule LC separations.[20] C-CP fibers are extruded from polypropylene (PP), polyester (PET), and nylon 6-based polymers in the form of 30-50 μm diameter fibers having eight channels running along their periphery. This diversity of base materials, along with facile surface modifications provides platforms for reversed phase (RP),[21] ion exchange (IEX),[22-25] hydrophobic interaction (HIC),[26, 27] and a variety of immunoaffinity[28-30] mode protein separations. The fiber structure and orientation in the column, along with essentially-zero porosity versus the size of proteins, allows for protein separations at high linear velocities (~100 mm s^{-1}) without significant van Deemter C-term broadening.[21, 31] In previous studies, this group has successfully applied a PP fiber column as a reversed-phase medium for LC-MS analysis of proteins in a urine matrix, without prior sample preparation.[32] Four proteins were separated and identified using ESI mass spectrometry, yielding a high signal-to-noise ratios.

More recently, a trilobal fiber structure (referred to as PPY) was described and its performance compared to the typical eight-channeled shape for reversed-phase protein separations.[33] van Deemter curves were generated to characterize column dynamics and the role of column interstitial fraction. Ultimately, the PPY columns yield elution peaks with greater symmetry and higher separation efficiency (resolution) than the previous eight-channel fiber geometry, due predominately to more uniform packing (lower A-term).[33] In the present study, the C-CP fibers having a trilobal shape are implemented for rapid protein separation followed by ESI-MS detection. Conflicts exist as the C-CP fiber separation provide the highest resolution (and throughput) under conditions of high linear velocity,[21] which on a first-principles basis conflicts with optimal ESI-MS performance, where lower solvent loading is preferred.[34] To adjust to these volume flow rate limitations, a smaller column diameter in comparison to the previous work (0.5 vs. 0.8 mm) was implemented. As such, separation parameters (including aspects of column packing) had to be optimized to affect high velocities at minimal volume flow rates. Likewise, investigations into alternative ion pairing agents and compositions, versus the typical 0.1% TFA, were undertaken. To illustrate the versatility of the method, test samples consisting of four- and five-protein suites (ribonuclease A (ribo A), cytochrome c (cyto c), lysozyme (lyso), myoglobin (myo) and bovine serum albumin (BSA)) were prepared in common phosphate-buffered saline (PBS), synthetic urine and saliva matrices, and commercial HL5 cell culture media. Based on the LC-MS chromatograms, the RP-LC-ESI using the trilobal C-CP fibers are demonstrated to be highly effective in affecting both matrix removal and rapid, high quality protein separations on column structures costing less than $5 each.

Experimental

Column construction - Polypropylene (PP) C-CP fibers that were melt-extruded in the trilobal geometry in the Clemson University, School of Material Science and Engineering (Clemson, SC, USA). The PP base material was chosen based on previous evaluations in terms of protein chromatography and SPE.[35, 36] In fact, the PP fibers have been shown to be effective in an in-line protein desalting protocol for ESI-MS.[37] The methods of assembling the microbore fiber columns, have been described previously in detail.[21, 38] Fibers were removed from the primary bobbin using a rotary counter to acquire the number of fibers required to achieve a given column interstitial fraction. The fibers were heat shrunk using boiling water and then cleaned sequentially using room temperature de-ionized water, acetonitrile and methanol to remove any latent anti-static spin-coating. Clean fibers were pulled by hand using a monofilament through lengths of PEEK tubing (ColeParmer, USA) having an inner diameter of 0.5 mm with the column lengths trimmed to 30 cm. The columns were then flushed with MilliQ water and ACN until a stable absorbance baseline at 216 nm was achieved, usually ~20 minutes. The interstitial fraction of each column was determined through the use of uracil injections under non-retaining conditions.[39]

Protein solution preparation - Proteins employed in these studies (ribonuclease A, lysozyme, myoglobin, BSA, and cytochrome c) were purchased from Sigma Aldrich (St. Louis, MO, USA). Stock solutions of each protein were prepared at concentrations of 2 mg mL^{-1} in 100 mM PBS and kept frozen between uses. For the initial studies to evaluate the role of interstitial fraction and the intra- and intercolumn separation reproducibility, mobile phase A was prepared with MilliQ water (conductivity 18.2 MΩ

cm^{-1}, Millipore Water System, Billerica, MA) and 0.1% TFA (Sigma Aldrich, Milwaukee, WI, USA), and mobile phase B was HPLC grade acetonitrile (ACN, Fisher Scientific, Pittsburgh, PA, USA) with 0.1% TFA. For the rest of the experiments, the TFA additive was replaced by formic acid (FA, Sigma Aldrich, Milwaukee, WI, USA) at 1.5% (volume) concentrations. In all cases, the full gradient from 100% aqueous solutions (with pairing agents) to 100% ACN (with pairing agents) was employed. For the studies involving different sample matrices, 100 μg mL^{-1} protein solutions were prepared by dissolving proteins in PBS, a synthetic urine matrix (194 g urea, 6 g calcium chloride, 11 g magnesium sulfate and 80 g sodium chloride dissolved in 4 L of MilliQ Water), a synthetic saliva matrix (2 g L^{-1} methyl-p-hydroxybenzoate, 10 g L^{-1} sodium carboxymethyl cellulose, 0.625 g L^{-1} KCl, 0.059 g L^{-1} $MgCl_2 \cdot 6H_2O$, 0.166 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.804 g L^{-1} K_2HPO_4, and 0.326 g L^{-1} KH_2PO_4 dissolved in MilliQ Water) and commercial HL5 cell culture media (0.5 % (w/v) proteose peptone, 1.3 mM $Na_2HPO_4 \cdot 7H_2O$, 0.5% (w/v) yeast extract, 0.5% (w/v) Thiotone E peptone, 55.5 mM glucose, and 2.57 mM KH_2PO4 in 1 liter of MilliQ water). The chromatographic gradient elution conditions were varied. For each of the separations presented here, the mixture injections were followed by a 1-minute hold time prior to the initiation of the solvent gradient. All data reported in graphic form are the results of triplicate separations, with the error bars representing ±1 s from the mean value.

Equipment - Basic characterization of the column hydrodynamics (e.g., interstitial fraction) and separation optimization was performed on a Dionex (Sunnyvale, CA) Ultimate 3000 HPLC system with a multi-wavelength VWD-3400 RS UV/ Vis detector controlled by Chromeleon 7 software. A constant sample injection volume of 5 μL was

applied throughout this work. While this volume is appreciable versus the column void volume (~50 μL), it is not a volume where overload effects have been seen on similar C-CP fiber columns.[40] The combined LC-MS experiments were performed on a ThermoScientific Surveyor+ LC-ESI-MS system. The system employs a Thermo Scientific (Waltham, MA, USA) LCQ Advantage Max™ ion trap mass analyzer (operating in the positive ion mode), with the accompanying Xcalibur™ data acquisition software used with the MS system. The instrument employs a universal Ion Max source that was operated here in the ESI mode. MS system performance was optimized using ribonuclease A as a standard compound, employing the Xcalibur™ auto-tune function. The system software also controls the coordinated LC and MS functionality.

Results and discussion

Role of interstitial fraction on linear velocity and separation characteristics - A consistent observation in all C-CP fiber-based separations of proteins, regardless of the chromatographic modality, is the improvement in resolution as the mobile phase velocity is increased.[21, 31, 38] For a given column packing density (interstitial fraction), higher velocities are affected by increasing the volume flow rate. This is, of course, disadvantageous in terms of ESI-MS detection. In practical terms, greater fiber numbers/densities (lower interstitial fractions) and smaller inner diameters lead to greater velocities at the same volume flow rate. As virtually all C-CP fiber protein separations occur in the "flat" region of the van Deemter plot, primary differences in terms of fiber number density are born out in A-term characteristics. Changing interstitial fractions present trade-offs in terms of chromatographic efficiencies, with "loose" packing resulting in very irregular paths and poor mass transfer characteristics

and “tight” packing introducing crimping along the column.[31] In both extremes, peak asymmetry is the primary victim regarding peak quality. There might be backpressure considerations in changing interstitial fraction, but for the C-CP fiber columns, pressures never exceed 1200 psi (~1.0 mL min^{-1} H_2O flow), regardless of the packing or flow rates in these typical analytical formats. Therefore, different from the previous effort using the trilobal fibers coupled with absorbance detection, the move to ESI-MS detection dictates a reduction in the column inner diameter (0.5 vs 0.8 mm) to affect higher velocities at lower volume flow rates, and thus a re-evaluation of column packing densities and their role in chromatographic performance is in order. On a first-principle’s basis, at the same interstitial fraction, equivalent linear velocities in the reduced-diameter columns are achieved at 62.5% lower volume flow rates.

To compare the separation characteristics of different interstitial fraction columns and identify the optimal packing density, three columns with varied numbers of fibers (with the determined interstitial fractions noted) were used in this study. The easiest way to affect different numbers of fibers is to change the number of rotations collected on the rotary counter. A four-protein mixture (lyso, myo, ribo A, and cyto c) prepared in phosphate-buffered saline (PBS) was separated using a constant 0.5 mL min^{-1} flow rate and a 15 min gradient 100% H_2O with 0.1% TFA to 100% ACN with 0.1% TFA. It is understood that operation of the different columns at the same volume flow rates does not impart the same resultant linear velocities, but in each case, the velocities are within the flat portions of the van Deemter diagrams for proteins. Columns with three, four, and five rotations (representing 210, 280, and 350 fibers per column) were used in the comparison. As seen in Fig. 1 the lowest-density fiber column (three rotations) does not

provide full resolution of the test proteins. As well, the peak shapes are not as sharp as those on the other two columns, with broadening on the front and trailing edges of the peaks. The higher fiber density columns provide improved peak shapes with clear improvements in the calculated resolution values ($R_s = 2\Delta t_R/(w_2 + w_1)$) seen for the cyto c:lyso critical pair. There is virtually no difference in the resolution between the four- and five-rotation columns, though the peak symmetry is better for the four-rotation separation. The minor tailing observed in the most-dense packing case is likely due to inter-channel crimping. Coupled with the greater ease of column packing (less fiber-tubing friction), we continued these studies using the four-rotation column format.

The same four-protein solution and chromatographic conditions were applied to assess the reproducibility of the column packing procedure and subsequent separation quality. As seen in Fig. 2, the peak shapes, protein elution times, and peak heights (recoveries) are quite consistent over four separately-packed trilobal fiber columns. To better quantify the separation quality, cytochrome c and lysozyme is again set as the critical pair for quantitative comparison. The metrics included in the embedded table represent triplicate (n=3) injections of the mixtures for each column. As can be seen, the respectively elution times are highly consistent for each column, and across the columns. Likewise, the RSD of recoveries (integrated absorbance) for each column vary by less than 10%, relative, with the vast majority being less than 5%. The variability across the columns was at the same level. Finally, the chromatographic quality, as determined by the resolution among four-protein peaks, was also consistent across the board. For example, the RSD of the computed resolution values for the critical pair is 1.66%.

Alternative mobile phase additive ---- formic acid - As has been employed in virtually all RP protein separations, regardless of the stationary phase medium,[41] trifluoroacetic acid (TFA) has routinely been applied as an ion pairing agent in C-CP fiber column separations to improve elution peak shapes and recoveries.[35] Its low UV (214 nm) molar absorptivity is advantageous for protein detection. However, TFA is also a well-known ion suppressant in ESI-MS, resulting in signal reduction and spray instability.[19] This suppression may be the result of TFA anions pairing with protonated cations leading neutralization in solution, thus preventing the electrospray droplet from ejecting the target analyte ion, and reducing the signal.[18] In order to erase the ion suppressant effects of TFA, a weaker acid, such a formic acid (FA), is often chosen as the ion pairing agent.[42] As might be expected, use of the weaker acid, has a counter—beneficial effect with respect to the performance of the chromatographic aspects of the LC-MS experiment. As such, FA concentrations are increased to achieve a pH value equivalent to 0.1% TFA (pH=2.04). However, high FA concentrations lead to sacrifices in terms of the product resolution of protein separations,[32] as much higher volumes of FA are needed versus TFA (approximately 10 times). Thus, for the target of ESI-MS detection, one must balance the need to use FA for the sake of good ESI performance with potential compromises relative to the quality of the LC step. To grasp the potential impacts of the FA pairing agent on protein separations on the PP C-CP fiber columns, a range of FA compositions were evaluated using the four-protein test mixture.

In this study, the performance six different percentages of FA in mobile phase of the were compared to that of 0.1% TFA; including 0.1%, 0.25%, 0.5%, 0.75%, 1.0% and 1.5% in both the aqueous (A) and organic (B) solvent phases. The RP gradient program

went from 100% aqueous FA (various % FA) to 100% ACN with the same percentage of FA in 15 min, at a constant flow rate of 0.5 mL min^{-1}. The solute absorbance was monitored at 280 nm as the UV cutoff of formic acid is 210 nm, which would contribute appreciable background absorbance at the 216 nm used with TFA as the pairing agent. Representative chromatograms are presented in Fig. 3, from 0.1% to 1.5% FA (with the pH values noted). As shown, cytochrome c and lysozyme solutes could not be separated at the lowest FA percentages (0.1% - 0.75%). As the FA percentage increased, the peak widths became narrower and better separation quality appears, as expected. The best separation quality occurs at 1.5% FA, the solvent of lowest pH. The dashed line drawn parallel to the injection peak to align the elution windows gives a qualitative indication that indeed, the lower-pH mobile phases result in greater protein retentivity. In all, the relationship between the TFA and FA concentrations required for efficient separation simply reflects the fact that FA is a weaker Brønsted acid.

A secondary consideration in moving from TFA to FA is the impact on column dynamics. Specifically, the viscosity of TFA is 1.8 mPa*s at 20°C and the viscosity of FA is 1.4 mPa*s at 20°C.[43, 44] Based on the 10X higher concentration though, the viscosity of mobile phase consisting of FA is higher than for TFA. The backpressure does indeed increase with higher FA percentage because of its high viscosity, from 550 psi (0.1% FA) to 650 psi (1.5% FA), in comparison to 300 psi (0.1% TFA). However, as a basic characteristic of the C-CP fiber columns is the low backpressures at these flow rates, there is no practical impact in terms of using FA. Ultimately, even at the 1.5% FA composition, the resolution value for the critical (cyto c:lyso) pair is still slightly lower than 0.1% TFA; 2.08 versus 2.33. Finally, as seen in the respective chromatograms

(Figs. 1 and 3), the detection sensitivity for the required 280 nm measurements represents a sacrifice; ofcourse if absorbance was the analytical detection mode, 0.1% TFA would be the natural solvent system. In conclusion, for the target of ESI-MS detection, further optimization regarding of the chromatographic step was undertaken using the 1.5% FA composition.

In order to assess any potential effects regarding protein loading, the linearity of responses/recoveries was determined for the four-protein mixture; covering a range of 5 to 40 μg mL^{-1} of each protein per injection, in order to optimize the balance of resolution and signal intensity. The same operation method of a 15 min gradient time at 0.5 mL min^{-1} was applied. Representative chromatograms and response curves are presented in Fig. 4. As shown, the peak height and peak area increase at a specific ratio as protein concentration increase. At the lower concentrations of 5 μg mL^{-1} and 7 μg mL^{-1}, the peaks were of very low absorbance values and hard to distinguish from the baseline. This concentration level would be readily seen in 216 nm absorbance measurements as used in TFA. The other extreme in concentration face consequences as well. Higher protein concentrations have high and sharper peaks but sacrifice resolution for the latter eluting solutes (lysozyme and myoglobin).

Previous studies using the eight-channeled C-CP fibers indicated that mass loadings of ~1 μg (ribonuclease A) led to the onset of overload effects including peak broadening, reduced recoveries (integrated peak areas) and column efficiency (N/N_0).[45] Those studies were performed on columns of ~64.2 mg fiber mass, thus overload effects occurred at solute injections of 1.56×10^{-2} μg mg^{-1}. The respective peak areas were plotted versus concentration (μg mL^{-1}) for the four proteins as shown in Fig. 4b,

The peak areas increase in a linear fashion with higher concentrations from 5 μg mL^{-1} to 20 μg mL^{-1}, however there is a distinct roll-over from 20 μg mL^{-1} to 40 μg mL^{-1}. Thus, it was determined that the concentration of injection should be around 20 μg mL^{-1} for the 5 μL injections due to the compromise between larger mass amounts (beneficial to MS detection) and better resolution (sacrificed by overloading effects).

These experiments were performed on columns of ~19.8 mg fiber mass, wherein overload effects began at solute injections of ~2 x 10^{-2} μg mg^{-1} which is ~30% higher than that of the eight-channeled C-CP fibers. Thus, the better resolution of the trilobal fiber geometry is complemented with greater binding capacities. Additionally, the response functions presented in Fig. 4b for first five concentration data points for each protein (omitting the 30 and 40 μg mL^{-1} points) show quite good agreement with linearity as represented by the R^2-values. The degradation from ideal behavior (R^2 = 1.000) is likely caused by the lower signal-to-noise (S/N) characteristics of the 280 nm absorbance in comparison to the 216 nm line used with TFA as the pairing agent.

Roles of gradient and volume flow rates on separation characteristics - Perhaps the most unique feature of C-CP fiber protein separations is the ability to operate at relatively high linear velocities, yielding higher throughput, while paying no penalties with respect to chromatographic quality.[31, 38] In order to study the role of mobile phase flow rate (linear velocity) on separation quality, flow rates were varied at a constant gradient rate of 5% mobile phase B min^{-1} (20 min gradient time) for the separation of the four-protein mixture, at a concentration of 20 μg mL^{-1}, each. The mobile phase flow rates of 0.2-0.6 mL min^{-1} equate to linear velocities of ~30-91 mm s^{-1}. As represented in Fig. 5 and quantified in Table 1, the previously noted effects are consistently seen for

the triobal fibers when using the 1.5% FA ion pairing agent. The improvement in throughput is obvious as the retention times of the proteins decreases with flow rate, reflecting the capture/release aspect of the protein gradient elution. The tabulated values reflect the fact that for each of the successive critical pairs, the resolution steadily improves with linear velocity as the peak widths narrow. This response clearly demonstrates a lack of mass transport limitations (van Deemter C-term). Essentially, higher linear velocities lessen the extent of longitudinal broadening (van Deemter B-term). As in the case of the previous evaluations, the precision of n=3 replicates vary from 1 - 3 %RSD in terms of retention time, resolution, and recoveries. The precision of the separations is not sacrificed at high linear velocities.

The primary penalty paid for running C-CP fiber protein separations at high linear velocities occurs in the apparent recoveries. The integrated peak areas are practically inversely related to the flow rates, reflecting clearly a case where the solutes are eluting in successively larger solvent volumes.[21, 38] Of course, this same situation occurs in ESI-MS of proteins in general,[46] where higher solvent loadings decrease the efficiency of the electrospray process. As the target operating flow rate for this LC-ESI-MS coupling was 0.5 mL min^{-1}, with even lower flow rates preferred, a flow rate of 0.4 mL min^{-1} was chosen moving forward, representing a very minor (~10%) sacrifice in chromatographic resolution versus the highest flow rate.

Gradient rate is another essential control parameter since the solvent composition directly affects the protein release from the nonpolar fiber surface. Proteins interact in a variety of ways with stationary phases (hydrophobicity, ionic, π-π, etc), thus, solvent gradients must be implemented for protein elution. The relationship between

gradient rate and separation quality was evaluated using varied gradient times, including 6, 8, 12, 18, 24, and 30 min from the 100% aqueous phase to the 100% organic phase (16.7%, 12.5%, 8.3%, 5. 6%, 4.2% and 3.3% B min^{-1}) at the volume flow rate of 0.4 mL min^{-1}. As would be predicted, ESI Fig. 1 reflects a case where faster solvent gradients result in higher overall throughput at the price of reduced retention time differences (Δt_R). At the same time, as the solvent gradient passes through each protein's elution window more quickly, the peak widths also decrease. The quantitative chromatographic figures of merit are presented in Table 2. These data clearly show an increase in resolution with decreasing gradient rates. However, the improvement in resolution is not in direct proportionally to the decrease in gradient rate, thus longer gradient times do not yield proportional improvements in resolution. At the same time, as expected, the peak heights for the elution bands are reduced as the proteins evolved across their relevant solvent windows. Finally, the total solvent consumption increases at the lowest gradient rate. Thus, there is little benefit for the lower gradient rates unless specific circumstances dictate the need for higher resolving powers. For the example protein suite here, a flow rate of 0.4 mL min^{-1}, and a gradient rate of 16.7 %B min^{-1} provides baseline resolution in a total analysis time of less than 6 minute, inclusive of the 1-minute hold time. The gradient time could be reduced to start closer to the start of the protein elution window, to yield even greater throughput.

RP-LC on trilobal C-CP fibers column with ESI-MS detection - Having derived operational parameters that allow for use of a less-suppressing ion pairing agent (FA) and low solvent burden for a conventional ESI source, while not compromising the inherent efficiency of protein separations on C-CP fibers, the final experimental point of

concern was the sample matrix-induced effects on the separation quality and ionization processes. In practice, the chromatographic process must first serve to render the sample matrix inconsequential to the isolation of the solutes from one another, and then deliver the analytes to the ionization source in a way that does not influence the spectral structure and responsivity. In this case, the three sample matrices were cell culture media, mock saliva, and mock urine. As can be gleaned from the solution compositions described above, these media differ greatly in both their chemical composition and physical traits. Urine, for example is salt-laden with solutes that severely suppress protein ionization. Saliva is very viscous, which could be problematic with respect to column hydrodynamics. Finally, cell culture media is an extremely complex matrix of highly diverse, and physically heterogeneous, composition.

The chromatograms presented in Fig. 6 demonstrate that the five-protein mixture (ribonuclease A, cytochrome c, lysozyme, myoglobin, and BSA, in order of elution), at concentrations of 20 μg mL^{-1} each (5 μL injection volume), can be effectively separated from the mock cell culture, saliva, and urine matrices, using both absorbance and ESI-MS detection. In this case, the chromatographic conditions represent a middle ground in terms of a gradient rate of 8.3 %B min^{-1} (across the 100 % aqueous/1.5 % FA to 100 % ACN/1.5 %FA), at a flow rate of 0.4 mL min^{-1}. In both chromatograms, integrating (non-specific) sorts of detection are used, absorbance at 280 nm (Fig. 6a) and the total ion chromatogram (TIC) (Fig. 6b) of the ESI-MS. Qualitatively, the chromatograms are very similar, with some degradation in signal-to-noise characteristics for the ESI-MS detection and slightly longer elution times (0.8 min) due to larger dead volumes in the transit to the ion source versus the absorbance cell. There is some loss in

chromatographic quality using the MS detection resulting from the fact that there is a mismatch between the diameters of the microbore column, the transfer capillary, and the ESI source inlet, leading to minor peak broadening. Very different from the case of the absorbance detection, the MS TIC shows an appreciable injection peak due to the elution of the matrix components, including salts, which are unretained on the polypropylene fibers, but are not detected by UV absorbance. A loss in S/N performance is seen in the TIC due to the very nature of the experiment (all signals contributing), but the extracted single ion chromatograms (SIMs) of the respective protein masses show very high S/N, reflecting the effectiveness of the on-column separations. As anticipated, all three chromatograms of the urine, saliva and cell culture media (both UV and TIC) look quite similar. The primary difference among three matrix samples is the injection peak since the salts and other concomitant species are very different.

Of course, the quality of the respective ESI-MS spectra are the ultimate points of comparison in this sort of testing. Each protein's mass spectrum was extracted from the TIC by averaging the spectra of the center of the elution peak, while subtracting the spectra of the neighboring chromatographic baselines for an equivalent time period. The resulting spectra are deconvolved within the Surveyor system software, charge states assigned, and molecular weights calculated. Representative ESI mass spectra extracted from the central portion of the respective protein elution peaks are presented in Fig. 7 for the case of the saliva samples. Each figure includes labels of the most prominent of the nH+ charge states for that protein. It should be noted that the predominate charge states are somewhat higher in these spectra in comparison to

those of the proteins introduced by direct infusion, a common occurrence as proteins may tend to unfold upon exposure to hydrophobic solvents and stationary phase surfaces.[15] Based on the individual separations, the molecular weights were determined to be: a) ribonuclease A = 13,698 ± 2 Da, b) cytochrome c = 12,384 ± 1 Da, c) lysozyme = 14,315 ± 4 Da, d) myoglobin = 16,974 ± 5 Da and e) BSA, 66,413 ± 40 Da across the 3 sample matrices. In each case, the relative differences in determined masses varies by 0.01 - 0.06 %RSD, a reflection of the high S/N quality. Due to the limited mass range by our Paul-type ion trap LC-MS instruments (100-2000 m/z), the mass spectrum of BSA protein, which has the highest molecular mass, has the highest level of errors. Even so, the calculated molecular weights and the narrow spreads support the efficacy of the methodology in terms of alleviating potential matrix effects.

Conclusions

This work has served to consolidate many past findings surrounding the use of C-CP fibers in protein analytics. In this study, the newly-developed trilobal fiber platform is shown to be an effective RPLC stationary phase for coupling with ESI-MS detection. Previous studies had quantitatively proven improved performance versus the eight-channeled version, principally through improved packing quality.[33] Because of the desire to operate at high linear velocities for optimum chromatographic performance, but low solvent load for ESI-MS detection, the column diameters were reduced appreciably (0.5 mm v. 0.8 mm) from previous studies. As the common TFA ion paring agent used in absorbance detection is not compatible with ESI-MS, the use of FA was evaluated. The influence of loading amount of proteins was studied using formic acid instead of TFA as additive. Finally, the elution conditions (flow and gradient rates) were

optimized for the eventual ESI-MS detection method. Those conditions yield high chromatographic quality in both the UV absorbance and MS modes, with no evidence of matrix (cell culture, saliva, and urine) effects observed in either the chromatographic or MS detection aspects at the 20 μg mL^{-1} protein level. Coupling of the PPY fiber column with ESI-MS provides a convenient method for desalting complex matrices, including urine, saliva and cell culture media, and separating proteins without prior sample preparation. Furthermore, separations can be performed on very short time scales (<10 mins) at high flow rates (0.4 mL min^{-1}) in comparison to conventional reversed-phase columns.

Having laid the groundwork and proving the basic lack of matrix effects relative to protein determinations in these diverse mock media, the full realization of suitability of the C-CP fiber LC-MS method must be validate using well-characterized reference materials and cell isolates. Potential advantages relative to overall throughput, reduced susceptibility to fouling, and low materials costs could be drivers towards greater acceptance. It is clear that comparisons with modern core-shell stationary phases are also in order. Recent attention has turned to the use of C-CP fibers in diverse ways in conjunction with two-dimensional (2D) protein separations, where rapid throughput and column regeneration suggests potential advantages for their use as the stationary phase in the second dimension of the experiment.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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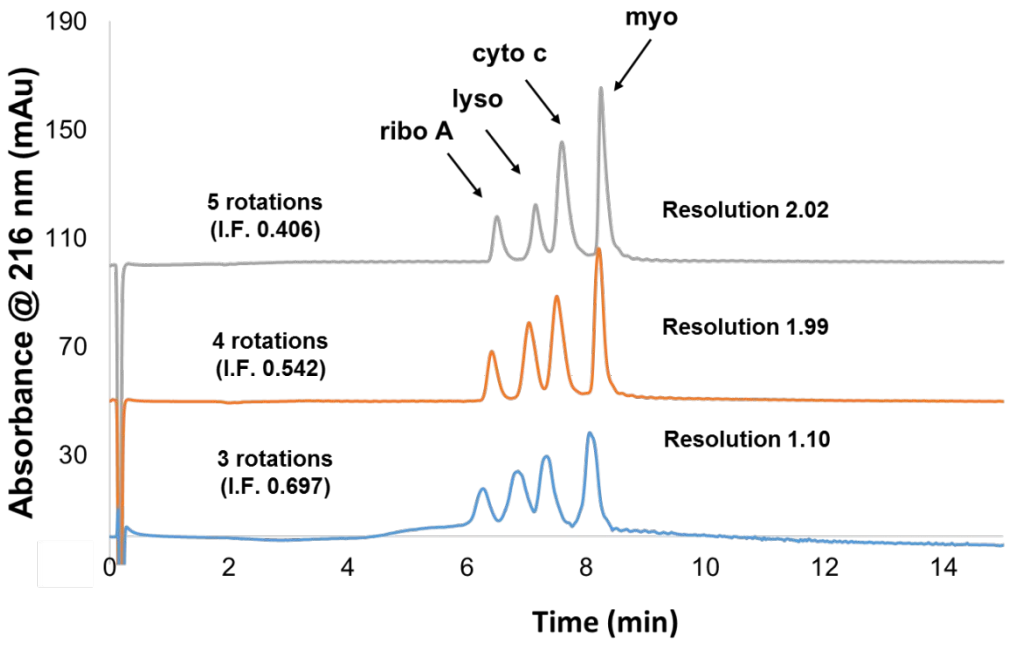


Fig. 1

Figure 1. Effects of column interstitial fraction (length = 30 cm) on the chromatographic performance of PPY C-CP fiber columns. Analytes: ribonuclease A, cytochrome c, lysozyme and myoglobin (50 μg mL^{-1} each, 5 μL injection), mobile phase: 0.1% aqueous TFA to 100% ACN + 0.1% TFA in 15 min, flow rate: 0.5 mL min^{-1}, 216 nm absorbance detection.

	Retention Time (min)		Peak Area (mAU*min)		Resolution
	Cytochrome c	Lysozyme	Cytochrome c	Lysozyme	
Column 1	7.20 ± 0.00	7.63 ± 0.01	3.09 ± 0.30	7.87 ± 0.24	2.01 ± 0.03
Column 2	7.20 ± 0.01	7.63 ± 0.02	3.26 ± 0.16	8.06 ± 0.16	2.01 ± 0.03
Column 3	7.20 ± 0.01	7.63 ± 0.01	3.40 ± 0.08	8.46 ± 0.10	1.99 ± 0.01
Column 4	7.17 ± 0.01	7.62 ± 0.01	3.55 ± 0.06	8.41 ± 0.03	1.94 ± 0.01

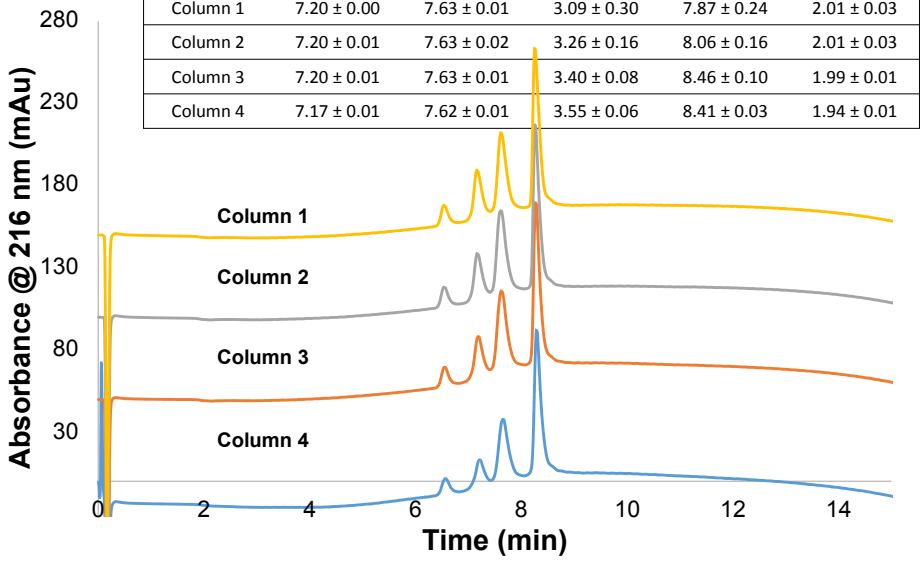


Fig. 2

Figure 2. Chromatographs of four-protein mixture RP separation on four different PPY C-CP fiber columns. Analytes: ribonuclease A, cytochrome c, lysozyme and myoglobin (50 μg mL^{-1} each, 5 μL injection), mobile phase: 0.1% aqueous TFA to 100% ACN + 0.1% TFA in 15 min, flow rate: 0.5 mL min^{-1}, 216 nm absorbance detection. Quantitative figures presented for the cyto c:lyso critical pair.

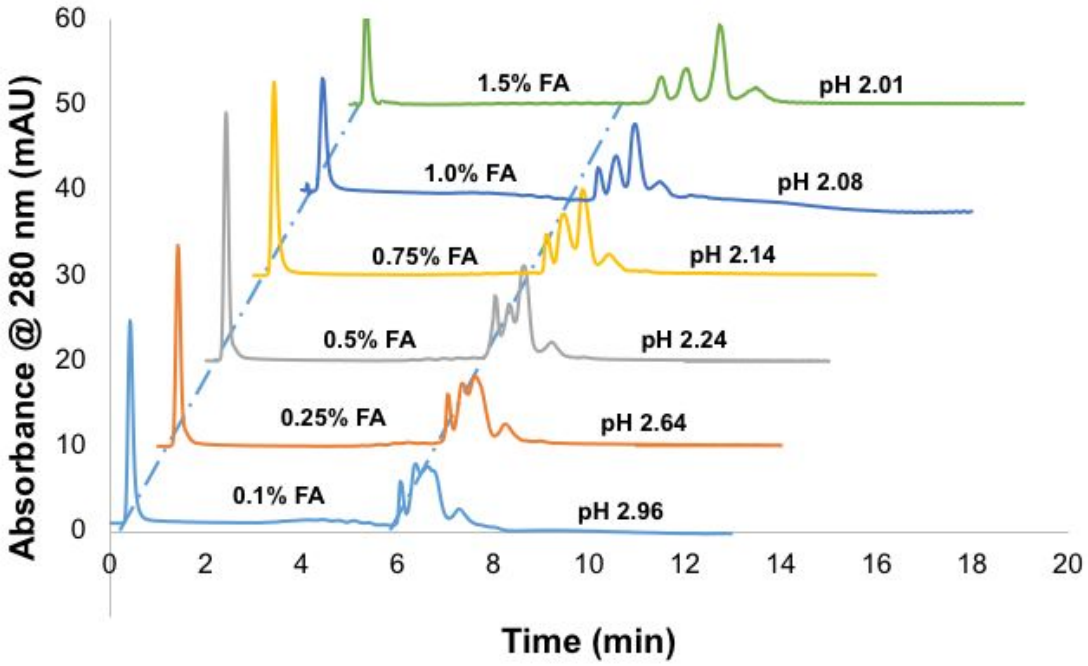


Fig. 3

Figure 3. Effects of formic acid percentage in the mobile phase on the chromatographic performance of PPY C-CP fiber columns. Analytes: ribonuclease A, cytochrome c, lysozyme and myoglobin (50 μg mL^{-1} each, 5 μL injection), mobile phase: 100% H_2O to 100% ACN (at the various FA percentages) in 15 min, flow rate: 0.5 mL min^{-1}, 280 nm absorbance detection.

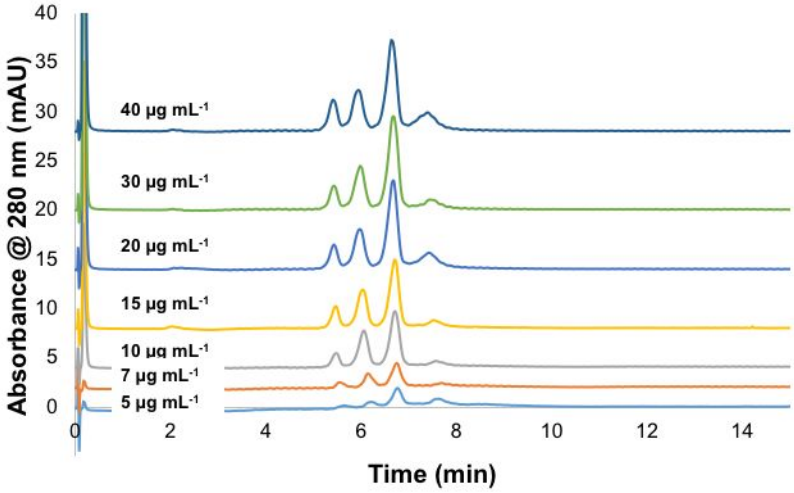


Fig. 4a

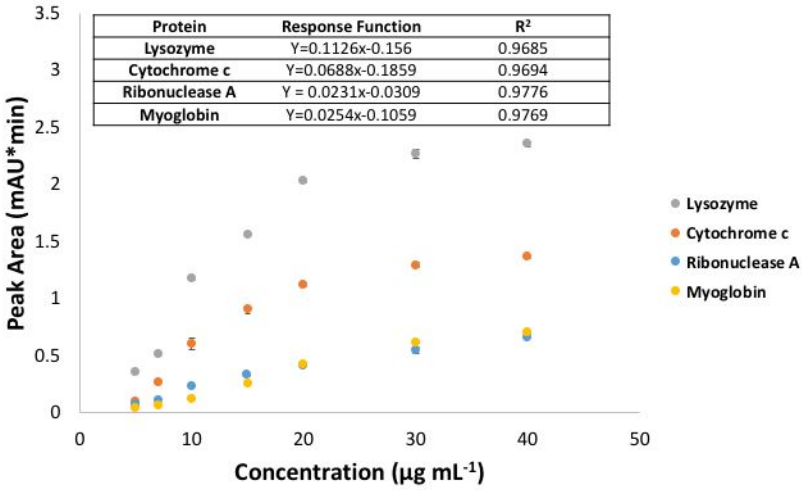


Fig. 4b

Figure 4. Influence of protein concentration on the chromatographic performance of PPY C-CP fiber columns. a) RP chromatogram and b) response curves. Analytes: ribonuclease A, cytochrome c, lysozyme and myoglobin (5 µL injection), mobile phase: 100% H_2O + 1.5% FA to 100% ACN + 1.5% FA in 15 min, flow rate: 0.5 mL min^{-1}, 280 nm absorbance detection.

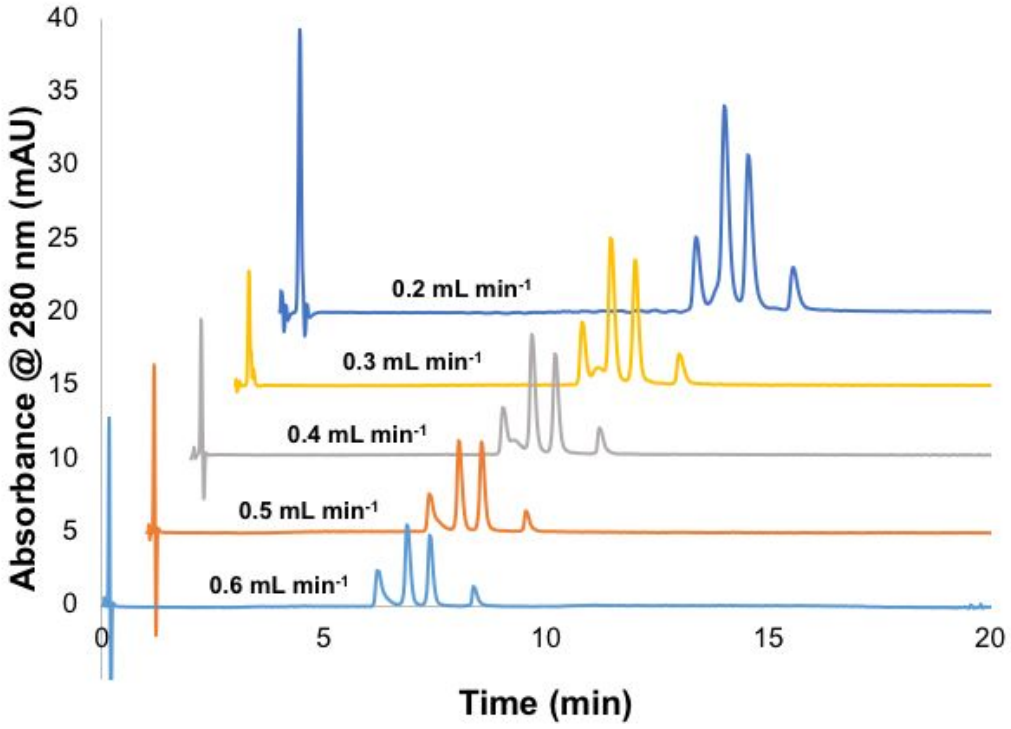


Fig. 5

Figure 5. Effects of flow rate on the chromatographic performance of PPY C-CP fiber column. Analytes: ribonuclease A, cytochrome c, lysozyme and myoglobin (20 μg mL^{-1} each, 5 μL injection), mobile phase: 100% H_2O + 1.5% FA to 100% ACN + 1.5% FA in 15 min, 280 nm absorbance detection.

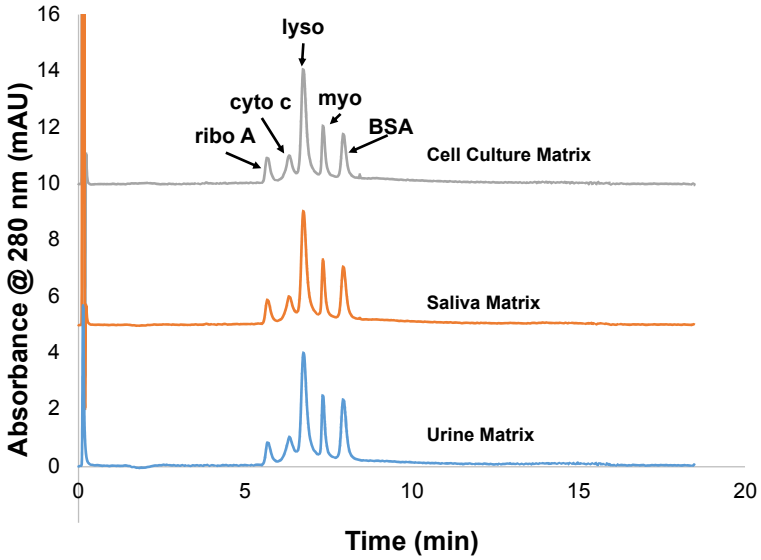


Fig. 6a

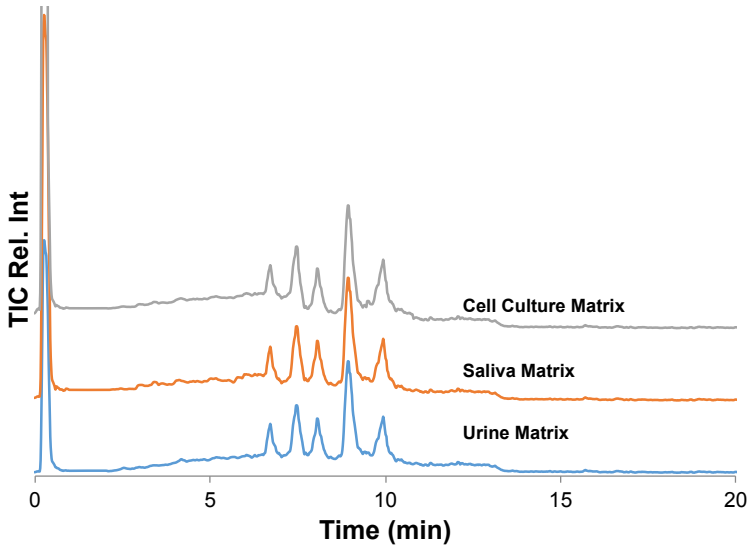
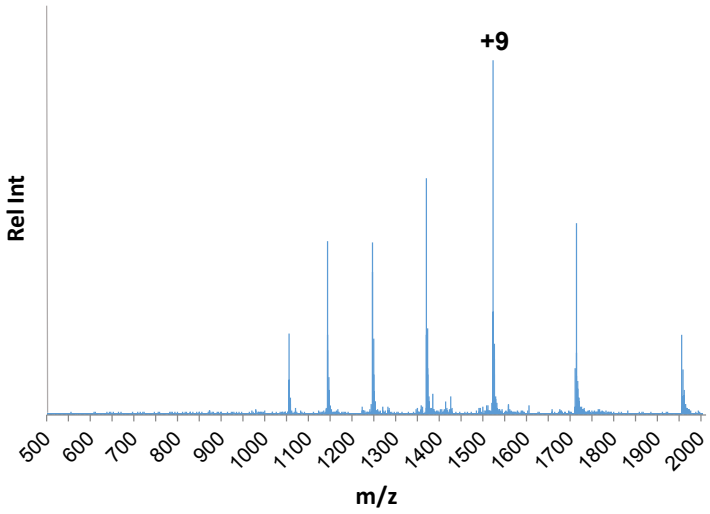


Fig. 6b

Figure 6. Chromatographic traces a) absorbance at 280 nm and b) ESI-MS total ion chromatogram (TIC) for the five-protein mixture in undiluted synthetic urine, saliva, and HL5 cell culture media matrices. Analytes: ribonuclease A, cytochrome c, lysozyme, myoglobin and BSA (20 µg mL^{-1} each, 5 µL injection), mobile phase: 100% H_2O + 1.5% FA to 100% ACN + 1.5% FA in 12 min (8.3% B min^{-1}), flow rate: 0.4 mL min^{-1}.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m/z

Fig. 7a

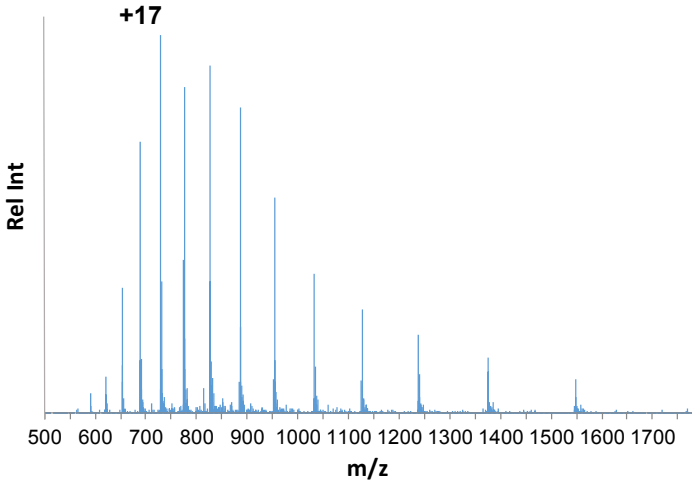
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Fig. 7b

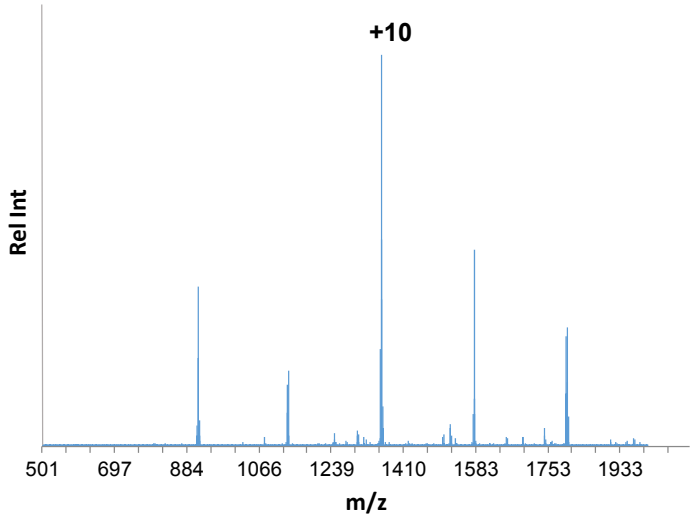
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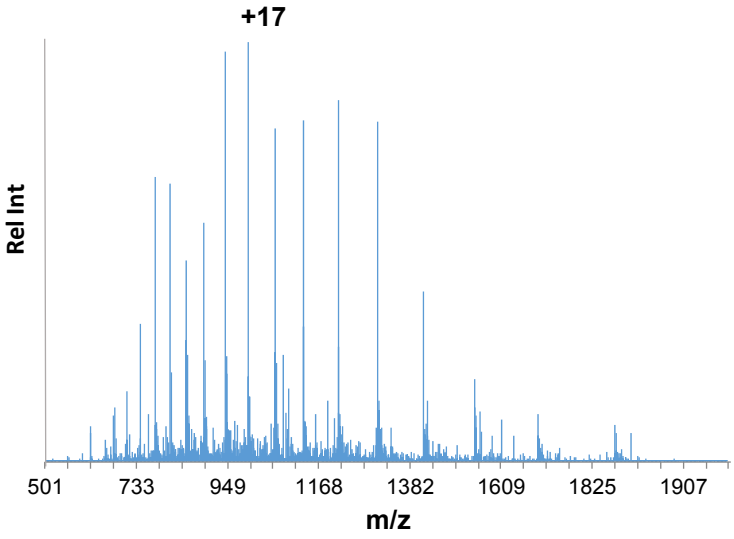
Fig. 7c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m/z

Fig. 7d

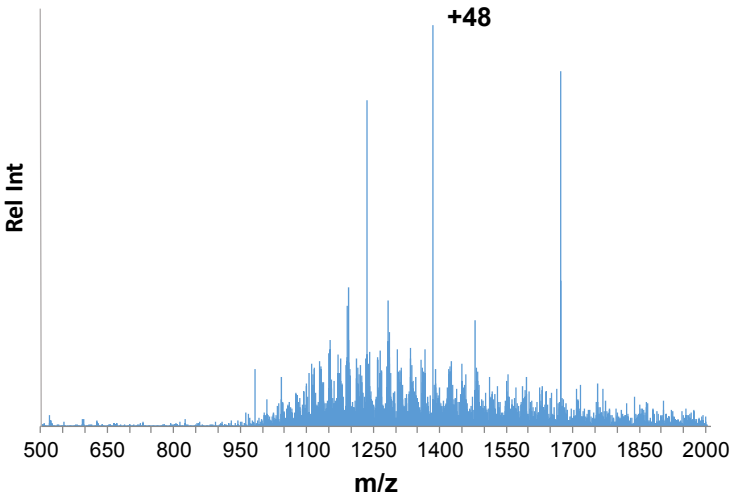


Fig. 7e

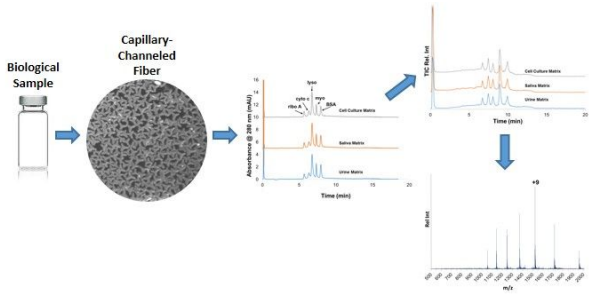
Figure 7. Extracted ESI-MS spectra representative of each of the five proteins for the separation presented in Fig. 6b for the mock saliva matrix. Identified base peaks a) ribonuclease A, $9H^+$ charge at 1522 Da; b) cytochrome c, $17H^+$ charge at 728 Da; c) lysozyme, $10H^+$ charge at 1356 Da; d) myoglobin, $17H^+$ charge at 999 Da; e) BSA, $48H^+$ charge at 1383 Da.

Table 1. Peak characteristics for the four-protein mixture separations as a function of mobile phase flow rate. Constant gradient rate of 6.67% ACN per minute (i.e., 15-min gradients).

Flow Rate	Retention Time (min)	Peak Area (mAU*min)	Peak width (50%, min)	Resolution
0.2 mL min^{-1} (Uo = 30.3 mm s^{-1})				
ribo A	9.37	19.57	0.165	
cyto c	10.01	20.78	0.168	2.28
lyso	10.55	14.25	0.160	1.96
myo	11.54	10.60	0.184	3.38
0.3 mL min^{-1} (Uo = 45.5 mm s^{-1})				
ribo A	7.82	13.30	0.135	
cyto c	8.46	14.01	0.146	2.71
lyso	9.01	10.25	0.140	2.26
myo	10.00	6.94	0.160	3.91
0.4 mL min^{-1} (Uo = 60.7 mm s^{-1})				
ribo A	7.03	10.64	0.136	
cyto c	7.68	10.59	0.129	2.88
lyso	8.21	7.86	0.130	2.40
myo	9.21	5.55	0.137	4.42
0.5 mL min^{-1} (Uo = 78.9 mm s^{-1})				
ribo A	6.55	8.51	0.124	
cyto c	7.21	8.46	0.122	3.19
lyso	7.73	6.04	0.122	2.52
myo	8.73	4.49	0.122	4.83
0.6 mL min^{-1} (Uo = 91.0 mm s^{-1})				
ribo A	6.22	7.06	0.126	
cyto c	6.89	4.04	0.119	3.21
lyso	7.40	5.06	0.110	2.63
myo	8.38	3.69	0.121	5.04

Table 2. Peak characteristics for the four-protein mixture separations as a function of gradient time/rate. Constant flow rate of 0.4 mL min^{-1}.

Gradient Time	Retention Time (min)	Area (mAU*min)	Width (50%) (min)	Resolution
6 min (16.7% B min^{-1})				
ribo A	4.40	5.39	0.061	
cyto c	4.67	10.97	0.058	2.65
lyso	4.89	11.13	0.063	2.08
myo	5.30	3.30	0.067	3.77
8 min (12.5% B min^{-1})				
ribo A	4.92	5.32	0.068	
cyto c	5.27	9.42	0.063	3.2
lyso	5.54	11.43	0.071	2.41
myo	6.10	2.36	0.075	4.49
12 min (8.3% B min^{-1})				
ribo A	5.92	5.25	0.088	
cyto c	6.46	9.69	0.083	3.73
lyso	6.86	10.78	0.096	2.64
myo	7.70	1.90	0.090	5.33
16 min (5.6% B min^{-1})				
ribo A	6.91	4.78	0.110	
cyto c	7.63	9.79	0.101	4.03
lyso	8.16	11.72	0.117	2.87
myo	9.26	1.98	0.106	5.81
20 min (4.2% B min^{-1})				
ribo A	7.89	6.12	0.014	
cyto c	8.81	9.98	0.115	4.29
lyso	9.44	10.18	0.129	3.09
myo	10.83	1.72	0.124	6.45
24 min (3.3% B min^{-1})				
ribo A	8.87	7.00	0.170	
cyto c	9.96	9.66	0.135	4.23
lyso	10.72	10.15	0.157	3.08
myo	12.36	1.98	0.139	6.51



Protein mixtures in biological matrices can be directly analyzed by RP-LC on trilobal C-CP fiber columns coupled with ESI-MS.