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High-speed and high-resolution UPLC separation at zero degrees Celsius

Thomas E. Wales[1], **Keith E. Fadgen**[2], **Geoff C. Gerhardt**[2], and **John R. Engen**[1,3*]

1*The Barnett Institute, Northeastern University, Boston, MA 02115*

2*Waters Corporation, Milford, MA 01757*

3*Department of Chemistry & Chemical Biology, Northeastern University, Boston, MA 02115*

Abstract

The conformational properties of proteins can be probed with hydrogen/deuterium exchange mass spectrometry (HXMS). In order to maintain the deuterium label during LC/MS analyses, chromatographic separation must be done rapidly (usually in under 8–10 minutes) and at zero degrees Celsius. Traditional RP-HPLC with ~3 micron particles has shown generally poor chromatographic performance under these conditions and thereby has been prohibitive for HXMS analyses of larger proteins and many protein complexes. Ultra performance liquid chromatography (UPLC) employs particles smaller than 2 microns in diameter to achieve superior resolution, speed, and sensitivity as compared to HPLC. UPLC has previously been shown to be compatible with the fast separation and low temperature requirements of HXMS. Here we present construction and validation of a custom UPLC system for HXMS. The system is based on the Waters nanoACQUITY platform and contains a Peltier-cooled module that houses the injection and switching valves, online pepsin digestion column, and C-18 analytical separation column. Single proteins in excess of 95 kDa and a four-protein mixture in excess of 250 kDa have been used to validate the performance of this new system. Near baseline resolution was achieved in 6 minute separations at 0 °C and displayed a median chromatographic peak width of ~2.7 sec at half height. Deuterium recovery was similar to that obtained using a conventional HPLC and icebath. This new system represents a significant advancement in HXMS technology that is expected to make the technique more accessible and mainstream in the near future.

INTRODUCTION

Hydrogen/deuterium exchange mass spectrometry (HXMS) is now an established analytical technique for the analysis of protein conformation and dynamics[1–3]. Proteins are labeled under physiological conditions and the deuterium that has exchanged into the protein is measured with mass spectrometry. Despite the wealth of knowledge that can be gleaned from such experiments, there have been certain technical obstacles that have prevented wide-spread use of the method[1]. The main obstacles come from the constraints of the hydrogen exchange reaction itself. In order to retain the deuterium that exchanged into the protein, the pH must be held at 2.5 and the temperature at 0 °C for all analysis steps prior to sample introduction into the mass spectrometer[4]. Under these “quench conditions”, the half-life for deuterium reversion to hydrogen in the 100% H_2O environment of the HPLC solvents is on the order of 30 to 120 minutes, depending on amino acid sequence[4]. Therefore, while maintaining the quench conditions, chromatographic separation prior to mass analysis must be done as rapidly as

*Address correspondence to: John R. Engen, 341 Mugar Life Sciences, Northeastern University, Boston, MA 02115-5000, 617.373.6046 (tel); 617.373.2855 (fax), j.engen@neu.edu.

possible to minimize the loss of the deuterium label. Unfortunately, traditional RP-HPLC with particles in the 3.0–5.0 micron size range gives quite poor chromatographic performance under these conditions. As a result, there can be ion suppression effects, peptides may overlap in both elution and in the mass spectra, data analysis becomes more complex, and automation of data analysis becomes more challenging.

In an attempt to address the limitations of traditional HPLC, we have previously[5] explored the utility of separations involving the smaller particles (sub 2 micron) found in ultra performance liquid chromatography (UPLC)[6] separations. It was shown that superior resolution, chromatographic speed, and better MS response could be obtained by performing the separations with 1.7 μm particles at approximately 10,000 psi in an ice bath. A subsequent publication has also shown the advantages of smaller particles[7]. In our previous report, the chromatographic separation was not optimized, and due to certain plumbing constraints with the commercially available ACQUITY UPLC system we had employed, online digestion was not used and the temperature was not rigorously held at 0 °C[5]. Here we present the validation of a custom UPLC system that corrects these problems. This new system utilizes Waters nanoACQUITY UPLC pumps interfaced with a custom-built, temperature-regulated module. Deuterium loss is comparable to that which is observed in experiments that use an ice bath, online pepsin digestion is integrated into the system, and chromatographic performance has been optimized. This system vastly improves the analytical performance that can be achieved in HXMS experiments.

EXPERIMENTAL

Chemicals

Formic acid, HPLC-grade acetonitrile and water, and full length proteins were purchased from Sigma-Aldrich (St. Louis, MO). MassPrep peptide standards were obtained from Waters Corporation (Milford, MA).

Cooled UPLC module

The cooled UPLC module was designed and fabricated at Waters (Milford, MA). Whenever possible, existing Waters components were utilized in the construction of the custom cooled chamber. This includes, but is not limited to, the power supply, control printed circuit boards, valves, and wiring harnesses. Multi-stage thermoelectric cooling modules were purchased from Melcor (Trenton, NJ). A liquid cooling system including pump, copper heat exchangers, and a radiator were purchased from Thermaltake (City of Industry, CA). Semi-rigid closed cell PVC insulation was purchased from McMaster-Carr (Chicago, IL).

Deuterium exchange reactions

Highly deuterated peptides (Waters MassPREP Peptide Standard containing RASG-1, bradykinin, and angiotensin I and II) were prepared by dissolving the lyophilized peptides into D_2O that was adjusted to pD 2.5 with DCl. Peptides were allowed to deuterate at 20 °C for two hours before infusion directly into the instrument in 50:50 D_2O:acetonitrile using a syringe pump.

Labeled cytochrome c (462 μM stock solution in 20 mM Tris, 100 mM NaCl and 3 mM DTT) was diluted to usable concentrations of 64 and 12.8 μM for HPLC and UPLC, respectively. Deuterium exchange was initiated by adding a 15-fold excess of 99% deuterium oxide buffer (20 mM Tris, 100 mM NaCl and 3 mM DTT) at 21 °C. At each exchange-in time point an aliquot (100 picomoles for HPLC, 20 picomoles for UPLC) from the exchange reaction was transferred to a separate tube containing an equal volume of quench buffer (300 mM potassium phosphate, pH 2.6, H_2O). Quenched samples were immediately analyzed. Highly deuterated

cytochrome c was prepared by diluting the stock solution 15-fold into D_2O pD 2.5, incubating at 37 °C for 6 hours and quenching as described above.

Online digestion, separation, and mass analysis

Unlabeled proteins, highly deuterated peptides and cytochrome c were analyzed using the UPLC system and conventional HPLC. In both LC-systems, labeled samples (50 μLs) were injected at a flow rate of 100 μL/min into a 2.1 mm × 50 mm stainless steel column that was packed with pepsin immobilized on POROS-20AL beads [prepared as described in[8, 9]]. Under these conditions, the digestion time was approximately 30 seconds.

In the HPLC experiments, a Shimadzu HPLC (LC-10ADvp) system was used. Peptic peptides eluting from the online pepsin digestion step were trapped and desalted on a 1 mm × 8 mm C-18 peptide trap (Michrom Biosciences) and desalted for 3 min. The trap was placed inline with the analytical column, a Zorbax C-18, 3.5 μm 300 Å, 1.0 mm × 50 mm column (Agilent Technologies), and eluted into the mass spectrometer with a gradient of 15 to 30% acetonitrile in 6 min at a flow rate of 40 μL/min. HPLC mobile phases contained 0.05 % trifluoroacetic acid. The C-18 peptide trap and analytical column, as well as the injection and switching valves were placed in an ice-bath to maintain the required 0 °C. The mobile phases were kept in a separate ice-bath and then flowed through pre-cooling stainless steel loops (located before the gradient mixing tee) in the main ice-bath to ensure that they were cool prior to meeting deuterated sample. The pepsin column was held above the ice bath at approximately 15 °C[9].

In the UPLC experiments, peptic peptides from online pepsin digestion were trapped and desalted on a VanGuard Pre-Column (2.1 mm × 5 mm, ACQUITY UPLC BEH C18, 1.7 μm) for 3 min. The trap was placed in-line with an ACQUITY UPLC BEH C18 1.7 μm 1.0 × 100 mm column (Waters Corp.) and eluted into the mass spectrometer with a 8–40 % gradient of acetonitrile over 6 min at a flow rate of 40 μL/min. The volume of the system from the mixer to the head of the analytical column was ~ 30 μL which includes ~ 8 μL volume of the trap column in line. All mobile phases for the UPLC system contained 0.1 % formic acid.

Mass spectral analyses were carried out on a Waters LCT classic or QToF Premier. The LCT was used for initial validation of the cooled UPLC module chromatography and not for any analyses of deuterium incorporation. LCT classic instrument settings were: 3.2kV cone and 40 V capillary voltages. The LCT source and desolvation temperatures were 150 and 175 °C, respectively with a desolvation gas flow of 1024 L/hour and a cone gas flow of 99 L/hour. LCT mass spectra were acquired using a 0.50 sec scan time and 0.1 sec interscan delay time. QTof instrument settings were: 3.5kV cone and 40 V capillary voltages. The QTof source and desolvation temperatures were 80 and 175 °C, respectively with a desolvation gas flow of 600 L/hour. QTof mass spectra were acquired using a 0.450 sec scan time and 0.050 sec interscan time. All QTof data were collected in ESI (+) and V mode. Deuteration levels were calculated by subtracting the centroid of the isotopic distribution for peptide ions of undeuterated sample from the centroid of the isotopic distribution for peptide ions from the deuterium labeled sample. Deuterium levels were not corrected for back-exchange and are therefore reported as relative [1].

RESULTS AND DISCUSSION

Cooled UPLC module construction

Our previous work in this area made use of an instrument with an autosampler and only cooled the separations column with ice after it emerged from the ACQUITY housing[5]. While not ideal, it clearly demonstrated the promise of UPLC for HXMS analysis. When developing the 2nd generation system for this work we decided to utilize the nanoACQUITY architecture in

place of ACQUITY. The reason for this was two-fold. First, the standard nanoACQUITY system utilizes both an injection and trapping valve. This minimized software and fluidic requirements for the incorporation of online digestion and trapping. Second, when necessary, the chromatography can be scaled down from the typical 1mm ID columns used here to 150μm ID columns as the nanoACQUITY is capable of pumping at much lower flow rates than utilized in the current work. Other technical obstacles in terms of cooling such low flow rates exist and have not yet been surmounted but it is obvious that separations at lower flow rates in the future will further enhance sensitivity and reduce sample consumption.

The cooled UPLC module and the control unit housing all necessary electronics were designed to replace the sample manager that is standard on the nanoACQUITY platform. Figure 1 shows the overall layout of the system as well as the construction of the module. The temperature controlled chamber is accessed through a removable cover that once closed, insulates the interior for effective cooling. The injection and trapping valves project into the cooled chamber with the majority of their thermal mass either housed inside the cooled chamber or within the insulated walls. An injection port compatible with a standard needle syringe is exposed on the top of the cooled chamber housing. The system has four Peltier elements on either side that are in contact with the 3/16” aluminum walls. Liquid heat exchangers are used to remove heat from the Peltiers and ethylene glycol is circulated through the exchangers and a radiator located at the back of the cooling module. Rigid insulation surrounds the inner chamber and ranges from 0.75” to 1.5” thick providing an R-value of 4 to 8. A resistance temperature detector (RTD) is used for feedback control of the desired temperature. The temperature within the chamber is maintained to within 0.2 °C of the set temperature.

Within the cooled chamber, a secondary chamber is positioned to accommodate the pepsin digestion column (see Figure 1B,C). A small resistive heater is used to warm the digestion column to a user controllable value, typically near 15 °C where digestion is slightly more efficient[8]. Dry nitrogen is piped into the cooled chamber to create a slight positive pressure inside; the nitrogen is exhausted via a tube that sheaths the transfer capillary to the mass spectrometer. This nitrogen serves two purposes: it prevents the buildup of condensation inside the chamber and also supplies cool air to the transfer capillary to limit the amount of deuterium loss experienced in this region of the flow stream. The solvents from the gradient pump and isocratic pump (used for online digestion and trapping) enter the cooled module at the front. Inside the chamber, each solvent line is connected to a 1.0 × 50 mm column cartridge which contains 100 μm zirconia particles. These cartridges are used to cool the solvent to a low temperature before it meets the sample at either the pepsin or separation columns. Control of the injection and switching valves, which dictates the amount of time spent in the pepsin digestion flowpath, is integrated into the control software for the nanoACQUITY system.

Chromatography with the cooled UPLC module

Validation of the new design for sample introduction began by ensuring that control and chromatographic performance of this modified nanoACQUITY was similar to that of an off-the-shelf nanoACQUITY. Separation efficiency and reproducibility were tested using digests of phosphorylase B and a protein mixture containing enolase, alcohol dehydrogenase (ADH), bovine serum albumin (BSA), and phosphorylase B. The first tests were performed at room temperature because, generally, reducing the temperature in an HPLC system with typical 3–5 micron particles significantly compromises the chromatographic efficiency and we wanted to validate the chromatography before introducing the temperature variable. Online digestion was performed at room temperature and separation of the resulting peptides was completed in six minutes with an instrument cycle time of 10 minutes from injection to system re-equilibration. An overlay of six successive separations of the peptic peptides from digestion of 15 pmoles of phosphorylase B (a 97 kDa protein) was made and a sample of that data is

shown in Figure 2A for a selected region of the chromatogram. Near baseline resolution was achieved with a median chromatographic peak width of approximately 2.7 seconds at half height. Not only was the separation highly reproducible but the quality of this separation is extremely high given the short amount of time allowed for gradient elution. Based on the number of unique ions observed, we estimate that there are more than 250 peptides produced during the online digestion of phosphorylase B.

Confident that the chromatographic performance of the newly-designed system was similar to any other nanoACQUITY UPLC system, we proceeded to test the effect of lowering the temperature. Figure 2B shows that as the temperature of the cooled chamber was lowered from room temperature (~ 21 °C) to near 0 °C (pepsin digestion remained at 15 °C, see above), the chromatographic performance was not greatly altered. The back pressure increased from 5300 psi to 8500 psi during the temperature change, but was still within the capability of the nanoACQUITY pumps. While there are slight differences in the BPI chromatograms between the separation at room temperature and the separation near 0 °C, the overall quality of the separation was maintained. High-quality chromatographic separations at 0 °C in six minutes for peptic digestions of a 97 kDa protein were mostly unheard of prior to this analysis.

Not only did the excellent chromatography display itself in the chromatograms, but it did so in the mass spectra as well. Each peak in the chromatographic separation was rich with information and there was very little overlap of ions in the mass spectra. Chromatographic focusing due to the high resolution of the separation only further improved the signal in the mass spectrometer. The ion intensity ranged from 20 to over 100 counts (0.5 sec scan time, see Experimental section for details) during the heart of the chromatographic separation of phosphorylase B peptic peptides at ~1 °C (Figure 2C; the two marked peaks on the Figure 2B chromatogram show where the peptides in Figure 2C elute). The average mass spectra shown here had an MS S/N of 93 (12.25 scans average) after UPLC separation of only 20 pmoles of material whereas typical mass spectra obtained after HPLC separation of 100 pmoles of material had an average mass spectral S/N of 124 (14.75 scan average) (data not shown).

The protein consumption in Figure 2B–C (15 pmoles phosphorylase B, 1.45 μg) was approximately 10- to 20-fold less than what we typically use for non-UPLC separations. Another example of the reduced sample requirements is shown in Figure 2D, along with a comparison of the chromatography observed during separation of a 52 kDa protein (Abl tyrosine kinase) using HPLC or UPLC. The UPLC conditions here are identical to those shown above in Figure 2B–C. The significant improvement in separation efficiency with sub-2 μm particles is evident.

The chromatographic potential of the cooled UPLC module was further tested with a mixture of four proteins with a sum total molecular weight of 249.8 kDa. Figure 2E shows the base peak intensity (BPI) chromatogram for the online digestion of enolase, ADH, BSA, and phosphorylase B. The chromatographic resolution did not suffer too dramatically even in the presence of this large number of peptides, which we estimate to be in excess of 500 based on other MS/MS experiments (data not shown). Being able to chromatographically resolve this many peptides in a separation of ~6 minutes at 0 °C has generally not been possible before. These data illustrate the potential of this system for the analysis of large proteins and protein complexes.

Deuterium recovery

An essential gauge of the performance of this new UPLC module is the recovery of deuterium during analysis. As described in the introduction, to maximize the information obtained from a hydrogen/deuterium exchange experiment, it is necessary to maintain as much of the label as possible during the analysis steps. In our first report on the combination of UPLC with

HXMS, there was a 13–20% increase in deuterium loss as a result of the sample injection configuration[5]. In the new UPLC module reported here, such elevated levels of deuterium loss have been eliminated, as described below.

A mixture of heavily deuterated peptides was used to quantify the deuterium recovery in the UPLC module versus recovery with the traditional ice-bath cooled HPLC system. Direct infusion of heavily deuterated peptides into the QTof Premier mass spectrometer yielded an average deuterium loss of 5.5% (data not shown). This value is in agreement with previous measurements of instrument-derived deuterium loss in mass spectrometers with the same source geometry[5] and must be taken into account when interpreting the deuterium losses seen in the analyses as a whole. Deuterium losses as a result of the HPLC or UPLC steps were measured in two ways. First, a mix of peptides were heavily deuterated, adjusted to the HX quench conditions (just as a protein sample whose labeling reaction were being quenched and then injected immediately) and injected into each LC system independently but coupled to the same mass spectrometer. The average deuterium loss for the peptides was 27.3% and 30.0% for the HPLC and UPLC systems, respectively (data not shown). The 2.7% increase in deuterium loss with the UPLC module is most likely a measurable difference for these peptides because the deuterium loss in peptides can be quantified to about ±1.0–1.5%. The increase represents a tolerable amount of deuterium loss given the significant improvements in separation efficiency and mass spectral quality, but probably is not particularly deleterious to HXMS experiments, see next section. It is also a significant improvement on the losses reported in the initial HXMS work with UPLC[5], primarily as a result of the improved cooling afforded by the new UPLC module over that obtainable in an unmodified ACQUITY UPLC. It should be noted that the deuterium losses in this work can not be directly compared with those in the previous publication[5] because the analysis conditions are not the same. The inclusion of online digestion and optimization of the separation time and gradient lead to more deuterium loss than was reported previously. Concurrent with that, however, is an improvement in the deuterium recovery for the UPLC system here over what was done with UPLC previously as a result of much improved cooling.

Deuterium recovery from labeled proteins

Although the analysis of deuterium recovery from heavily deuterated model peptides is an essential experiment to perform when testing any HXMS system, a more informative test of deuterium recovery comes in the analysis of peptides derived from deuterated proteins. The main reason for this is that losses of deuterium from peptides that are nearly 100% deuterated (what is being called “heavily deuterated” here) will always be higher than losses observed with partially deuterated peptides because heavily deuterated peptides have more deuterium to lose. Most peptides from well-folded globular proteins are partially deuterated as a result of protection from exchange by tertiary structure. Such peptides will therefore, have less deuterium to lose and are more representative of the real samples one might encounter with any analysis system. To further validate the deuterium recovery in the UPLC module, a folded protein (in this case cytochrome c) was deuterated in solution for various amounts of time (see Experimental section), the deuterium exchange quenched and the protein injected into the HPLC and UPLC systems. Figure 3A shows a typical BPI chromatogram for the UPLC separation of peptides resulting from online digestion of deuterated cytochrome c. All measurable peptides were contained within a 3.5 minute elution window at 0 °C and the separation was highly reproducible. Similar separations were performed with the HPLC system and the chromatography was not nearly as good but reliable enough to process the deuterium incorporation for many peptides (data not shown). Deuterium incorporation into a number of peptides from cytochrome c digestion was compared for the HPLC and UPLC systems. Figure 3B–E illustrates the deuterium incorporation for four representative peptides from 4 distinct parts of cytochrome c. It is fairly clear that the deuterium levels measured are by most accounts

extremely similar whether the separation was done with the HPLC system and an ice bath or with the new UPLC module. We conclude that the analysis of deuterium levels in the UPLC module provides the same deuterium incorporation information one would obtain with the traditional ice-bath cooled HPLC system.

CONCLUSIONS

Overall, this new UPLC system represents a considerable evolution in HXMS technology in terms of chromatographic performance. Due to the improved chromatography, less material needs to be injected for digestion (now 10–20 pmoles/injection), larger proteins/protein complexes become more accessible (because chromatographic separation is still good for peptides from proteins with a combined molecular weight of 250 kD), online digestion is combined with UPLC and ice is no longer required. All of this occurs with a drastic improvement in separation efficiency (see Ref. 5 and Figure 2A) and an average increase in deuterium loss of <3%. With almost all parameters under computer control, which now includes the temperature of the chromatographic step, analyses should be much more reproducible and have even greater potential for automation (see also[10]) both in terms of injection and analyses as well as in terms of data processing. As such, this UPLC module development will hopefully further advance the utilization of HXMS methodology.

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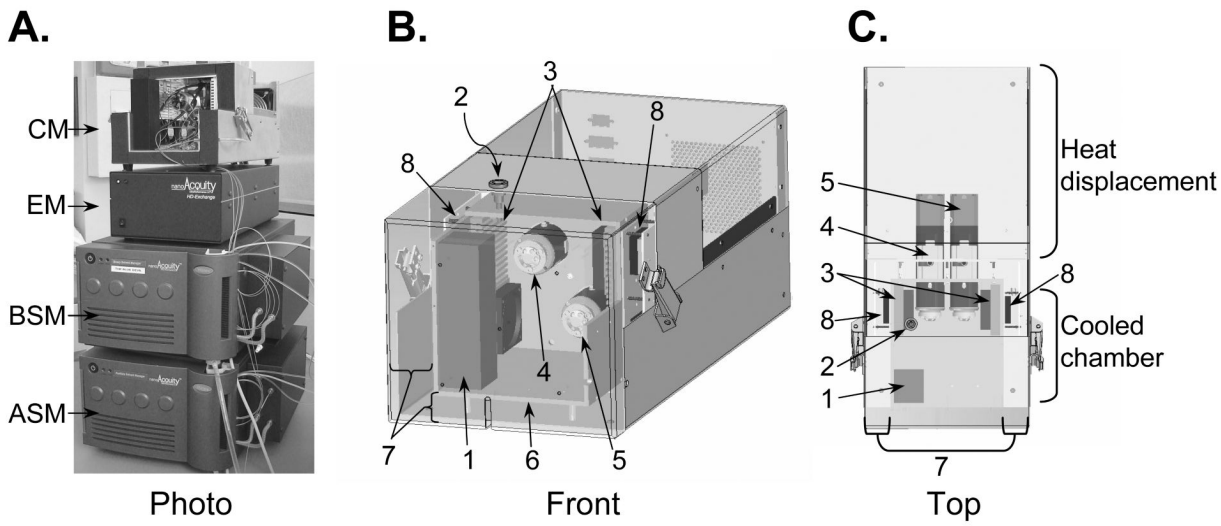

Figure 1. The custom HXMS nanoACQUITY

(**A**). Photograph of the modified ACQUITY system. The pumps, cooling module and control electronics are illustrated: CM-cooling module; EM-electronics module; BSM-binary solvent manager; ASM-auxiliary solvent manager. (**B**). Schematic of the cooling module from the front. Key components: 1-pepsin column housing; 2-injection port; 3-heat sinks; 4-injection valve; 5-switching valve; 6-0.25" aluminum plate for thermal mass; 7-insulation; 8-Peltier unit. The flow path is the same as has been published previously[9]. (**C**). Top-down view of the cooling module. Heat generated by the Peltiers is displaced via circulating coolant at the back of the unit. The cooled chamber is in the front of the unit. The key components are as in panel B.

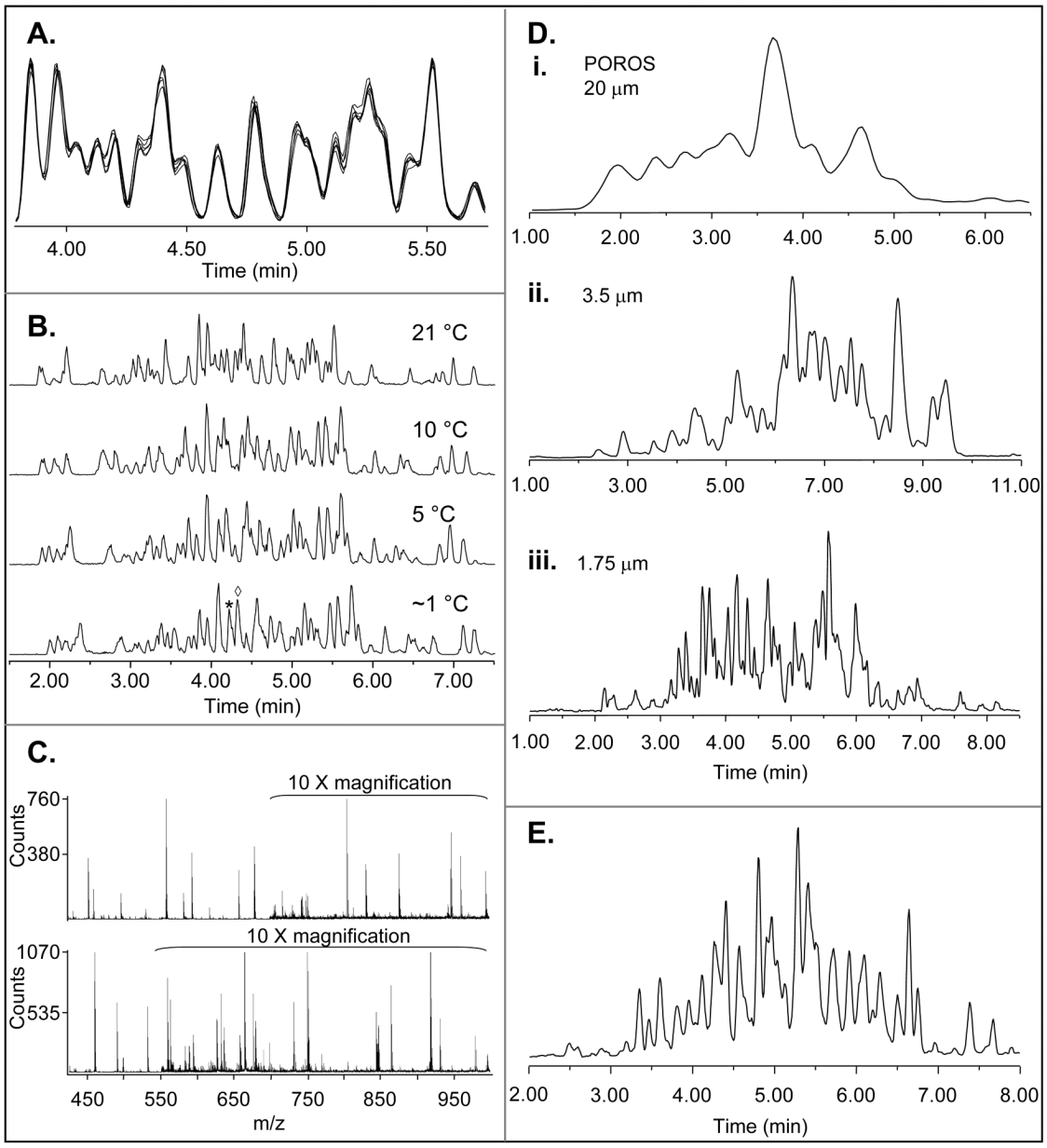

Figure 2. Reproducibility and separation efficiency using the UPLC module

(**A**). BPI chromatograms of the online digestion of 15 pmoles of phosphorylase B and separation at 20–21 °C (see Experimental section for exact gradient information). Six independent experiments have been overlaid. The region of the chromatogram (elution times 3.75–5.75 minutes) with the highest density of peaks is shown. (**B**). Separation as a function of temperature. BPI chromatograms of 15 pmoles phosphorylase B digested online and separated at the following chamber temperatures: 21 °C, 10°C, 5°C, 1°C. The (*) and (◊) indicate the positions at which the example mass spectra in panel (C) were obtained. The bar above the top trace indicates the region of the data shown in panel A. (**C**). Cumulative mass spectra between elution times 4.2 and 4.4 minutes. The top spectrum is the sum of 12 scans taken between elution times 4.2 and 4.3 minutes (* in panel B) and the bottom spectrum is the sum of 12 scans taken between elution times of 4.3 and 4.4 minutes (◊ in panel B). Data are

magnified 10x for m/z range 700–1000 and 550–1000 for the top and bottom spectra, respectively. (**D**). Demonstration of the improvement in separation at 0 °C with different chromatographic media. In each case, the same 52 kDa protein was digested with pepsin and separated. BPI chromatograms of digested protein are shown. (**i**). 200 pmoles of digested protein desalted using a POROS 20-R2 trap and separated using a 0.5 × 100 mm perfusion column packed with POROS 20-R2 (20 μm particle size), 2–55% ACN in 9 mins. (**ii**). 90 pmoles of digested protein desalted on a 1 mm × 8 mm C-18 peptide trap and separated using a Zorbax C-18, 3.5 μm 300 Å, 1.0 mm × 50 mm column, 8–40% ACN in 6 mins. (**iii**). 10 pmoles of digested protein desalted using a 1 mm × 5 mm, ACQUITY UPLC BEH C18, 1.7 μm pre-column and separated using an ACQUITY UPLC BEH C18 1.7 μm 1.0 × 100 mm column, 8–40% ACN in 11 mins. (**E**). BPI chromatogram obtained during separation of a mixture of proteins totaling 249.8 kDa. The proteins were: 20 pmoles yeast enolase, 20 pmoles yeast alcohol dehydrogenase, 30 pmoles bovine serum albumin, and 60 pmoles rabbit phosphorylase B. This separation was performed at an average chamber temperature of −0.5° C.

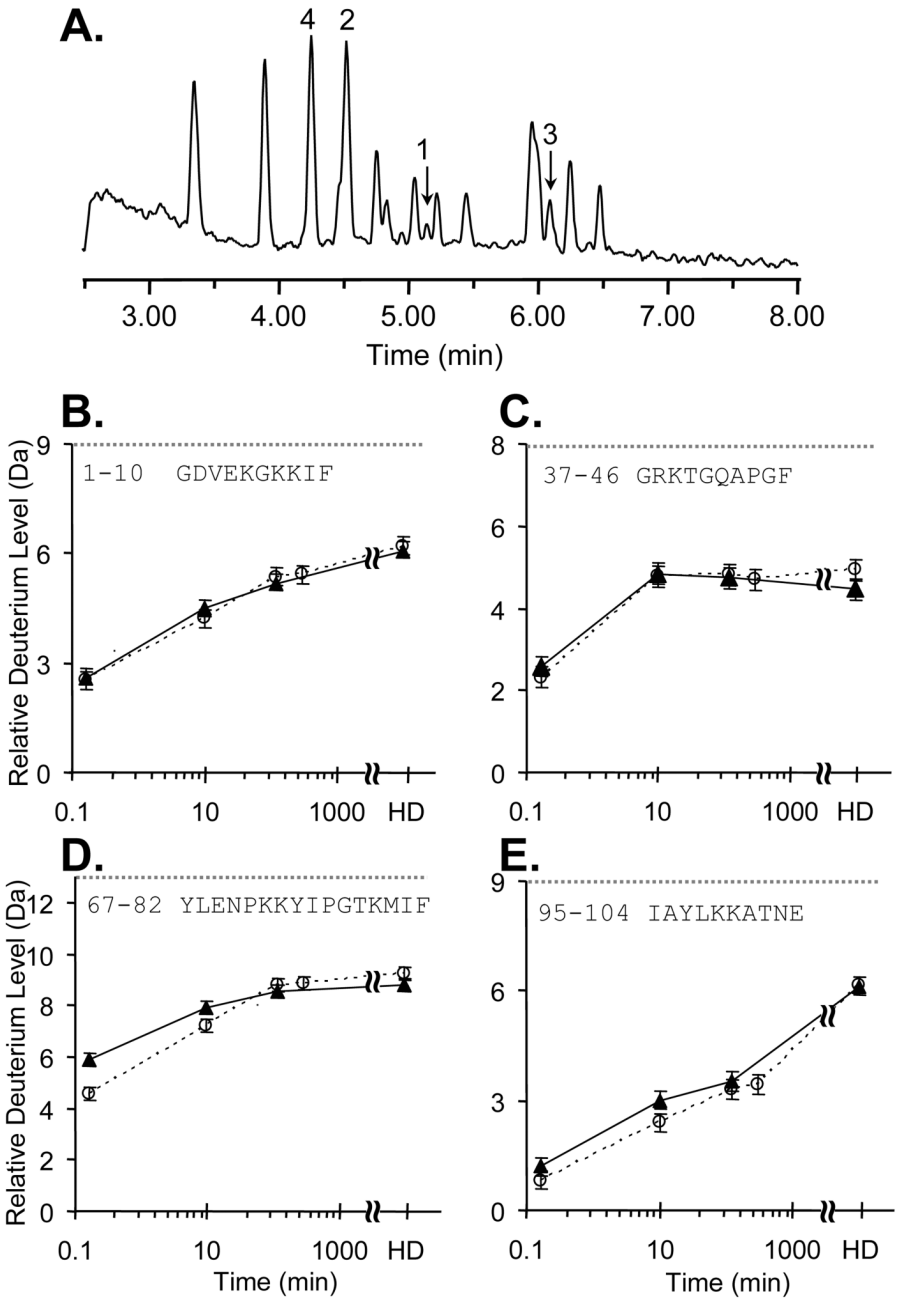

Figure 3. Deuterium incorporation in peptic peptides of cytochrome c analyzed using HPLC or UPLC systems

(**A**). BPI chromatogram of the UPLC separation of cytochrome c peptides at 0 °C. The chromatographic peaks representing four peptides used to validate the instrument are indicated on the chromatogram. 1: peptide 1–10; 2: peptide 37–46; 3: peptide 67–82; and 4: peptide 95–104. (**B–E**). Deuterium incorporation with time (log scale) for four peptic peptides of cytochrome c acquired using the HPLC system (▵) or the UPLC module (▴). The final data point on each plot is for the heavily deuterated form (HD) of each peptide (see Experimental section). The error bars represent the worst error seen for these data points, ± 0.25 Da over the course of three replicates.