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# Separation and analysis of proteins by perfusion liquid chromatography and electrospray ionization mass spectrometry

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**Abstract**

Perfusion LC has been interfaced with electrospray ionization mass spectrometry (ESI-MS) for the rapid separation and molecular mass determination of proteins. The combination of this unique chromatographic system and the multiply charged envelope of the ESI-MS data allows this analysis to be accomplished in just a few minutes when using the optimum conditions determined. The performance of the perfusion system has been compared to both 1 mm I.D. and 320 $\mu$m I.D. columns packed with conventional $C_{18}$ media.

## 1. Introduction

The use of electrospray ionization (ESI) for the interfacing of mass spectrometry (MS) and liquid chromatography (LC) as developed by Fenn and co-workers [1–3] has been proven to both desolvate and ionize fragile chemical species, such as proteins, peptides, nucleic acids and pharmacologically active compounds, from solution for transport to a mass analyzer.

One of the characteristic and unique features of this particular "soft" ionization technique when used for the analysis of proteins is the generation of a distribution of ions with a varying large number of charges. The number of charges can be as high as 1 per every 500 u of the protein's molecular mass and results in the production of a complex mass spectrum with a multiply charged envelope which has peaks appearing at many masses. Because the number of charges appearing on each ion population differs by an integral amount from any other population, these individual contributions to the total spectra can be "deconvoluted" to calculate the molecular mass of the entire protein molecule [4,5]. Since there is a great quantity of information contained within the multiply charged envelope, the accuracy of this calculation can be very high, such that a protein having a molecular mass of tens of thousands can be identified to within 1 u.

A second consequence of the appearance of highly charged ions, is that these species appear at a substantially reduced range on the $m/z$ scale, and therefore fall within the range of most conventional mass analyzers. This result is also largely responsible for ESI's proven ability to determine accurately the molecular mass of proteins weighing over 100 000 u.

The promising combination of using on-line protein LC separations coupled with ESI-MS detection has been hampered, however, by an incompatibility between the LC mobile phase and solvent which can be electrosprayed. Specifically, the inclusion of 0.1% trifluoroacetic acid (TFA) in the LC mobile phase, a practice commonly used in protein and peptide separations, inhibits the electrospray process re-

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sulting in complete loss of ion signal. Also, the flow-rates used for LC are usually considerably higher than the optimum flow-rate of 1 μl/min or less required for optimum ESI-MS. Fortunately, this difficulty has been solved through the development of a number of assisted-ESI techniques, such as pneumatically assisted nebulization [6–8] and ultrasonically assisted nebulization [9].

Perfusion LC [10–13] is rapidly becoming a useful technique for the analysis of peptides and proteins. The unique chromatographic media used is characterized by both a primary liquid flow path through large internal pores (6000–8000 Å) and a secondary diffusive path through smaller pores (300 Å). Because the mobile phase carrying the analytes flows through the interior of the particles, instead of mostly around them, this medium is particularly beneficial when working with larger species which have smaller diffusion constants. The result of employing this chromatographic medium in a column format is the reduction by 5- to 10-fold of the total analysis time, as compared with a conventional column, required to successfully complete a peptide or protein separation. A related effect is the reduction of chromatographic peak widths so that under optimum conditions, proteins or peptides completely elute in 5–10 s.

This paper explores the performance obtained from the coupling of perfusion LC, a new type of high-performance liquid chromatography, with ESI-MS for the analysis of proteins. The investigations presented here focus on several areas. The first is the effect of LC conditions, i.e. mobile phase flow-rate, on performance followed by deconvolution of a protein LC peak and a sensitivity study. The second is a comparison of performance for the separation and detection of a protein mixture by ESI-MS between the perfusion LC column and 1 mm I.D. and 320 μm I.D. columns packed with conventional media. This particular comparison is interesting since the 320 μm I.D. column has the same physical dimensions as the perfusion column, while the 1 mm I.D. column operates at a similar flow-rate to the perfusion column.

## 2. Experimental

### 2.1. Apparatus

The pump used for mobile phase delivery in all cases was a dual-syringe type, Model ABI 140A from Applied Biosystems (Foster City, CA, USA). For the 320 μm I.D. perfusion column and the 1 mm I.D. "microbore" column, the pumping system was used without modification. However, in order to deliver linear gradients at low flow-rates suitable for the 320 μm I.D. "packed-capillary" column, a flow-splitting device consisting of a simple tee with a fused-silica restrictor was constructed. With this system, the pump was operated at 50 μl/min, and the resulting flow was measured at 4.7 μl/min optimum for this column. This system was rugged and provided reproducible results, so it was used for all these particular studies. Sample injection was accomplished for all columns with a Valco Instruments (Houston, TX, USA) CI4W valve having a 1-μl internal loop.

The microbore or 15 cm × 1 mm I.D. column was purchased from Vydak (Hesperia, CA, USA) and was packed with 5-μm $C_{18}$ particles. The perfusion LC column was purchased from LC Packings (San Francisco, CA, USA) and was 15 cm × 320 μm I.D. This column was packed with 10-μm Poros II medium from Perceptive Biosystems (Cambridge, MA, USA). The packed-capillary column was fabricated from 320 μm I.D. fused-silica from Polymicro Technologies (Phoenix, AZ, USA) and was 15 cm in length as well. The reversed-phase packing material used was again the $C_{18}$ 5-μm particle size from Vydak.

The electrospray ionization source was similar to that developed at Yale [1–3] except that an ultrasonic nebulizer [9] was substituted for the original needle assembly. This particular source design allowed independent control of the source potentials on the needle ($V_{need}$), cylindrical electrode ($V_{cyl}$), nosepiece ($V_{nose}$) and capillary entrance ($V_{ent}$). With this configuration, the system was always operated with $V_{need}$ = ground in order to protect the user from electrical shock and isolate the LC system from high voltage. Drying-

gas temperature was always set to 300°C. The mass spectrometer (HP89A) was from Hewlett-Packard (Palo Alto, CA, USA).

### 2.2. Materials

Protein samples were obtained from Sigma (St. Louis, MO, USA). At the time of injection, these samples were dissolved at the appropriate concentration in a mobile phase composition which was identical to the beginning of the LC gradient used. All water was obtained from a Barnstead (Boston, MA, USA) NANOpure II system. Acetonitrile (ACN) was purchased from Mallinckrodt (Paris, KY, USA). All solvents were filtered through nylon 66 membranes from Anspec (Ann Arbor, MI, USA). TFA was also obtained from Mallinckrodt.

## 3. Results and discussion

### 3.1. Effect of mobile phase flow-rate on performance of a perfusion column

In order to determine the optimum mobile phase flow-rate for this system, a mixture of proteins containing ubiquitin, cytochrome *c* and myoglobin (30 pmol each) was injected on the column in a series of experiments where the flow-rate was varied from 25 to 125 $\mu$l/min in increments of 25 $\mu$l/min. While the flow-rate was increased, the identical gradient conditions were maintained as a linear ramp from 20 to 90% ACN in water (with 0.1% TFA) in 5 min. Total ion current (TIC) was recorded as the quadrupole mass analyzer was scanned from 500 to 1300 *m/z* units. This resulting series of TIC traces at increasing mobile phase flow-rates is shown in Fig. 1. Of the trends seen here, perhaps the most important and significant is the reduction in total analysis time required as the flow-rate was increased. At the maximum value used, 125 $\mu$l/min, the time required to complete this separation was just over 3 min, considerably less than the time required at 25 $\mu$l/min. Furthermore, the resolution between the first two eluting species (ubiquitin and cytochrome *c*)

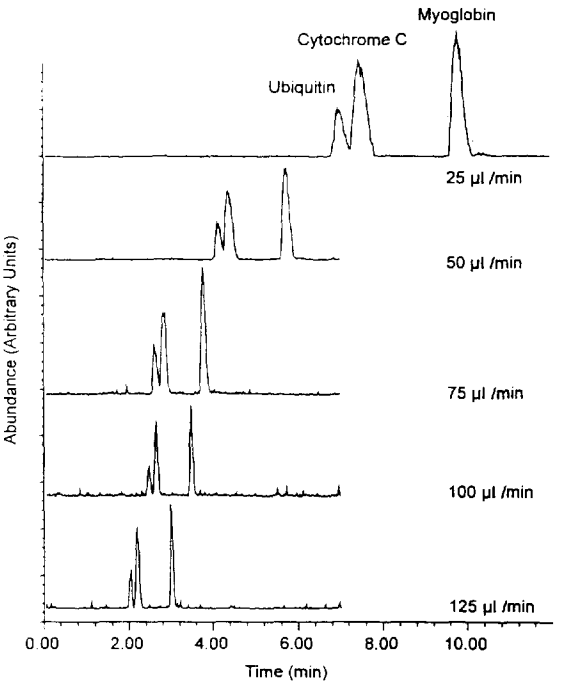


Fig. 1. Effect of mobile phase flow-rate on the TIC from a separation of a protein mixture. Gradient: 20–90% ACN in water (0.1% TFA) in 5 min.

and column efficiency was maintained at nearly the same value as the flow-rate was increased. This relatively "flat" Van Deemter response is one of the characteristics which makes perfusion LC so attractive and allows much higher than normal flow-rates to be employed. Proteins have especially low diffusion constants so these species are expected to benefit greatly from the large pore structure of the perfusion media.

Another notable effect of employing the perfusion material was a considerable reduction in chromatographic peak widths, which ranged from 5–10 s, depending on the protein, for the 125 $\mu$l/min separation. This and the overall analysis time might be improved with even higher flow-rates still, but the back pressure on this particular system was already precariously high at 2600 p.s.i. (1 p.s.i. = 6894.76 Pa), so no further increases in flow-rate were attempted. At a peak width of just 5 s, only five data points could be taken as a protein eluted when operating the mass analyzer at its maximum scan

speed, approximately 1 scan/s. Increasing the scan speed much beyond this point begins to degrade the mass spectral resolution due to ion statistics or ultimately, the flight time of an ion through the quadrupole rods. Scanning too fast causes the ion population being analyzed to be exposed to a changing radio frequency (rf) field. Because the quality of individual scans is good, five data points when averaged together seem sufficient to give good results. This is demonstrated in Fig. 2, which shows an average of the scans taken during the elution of the 5-s cytochrome *c* peak in the 125 μl/min separation from Fig. 1. It is important to note that further reduction in peak widths will mandate the use of a mass analyzer with faster scan rate capabilities, such as a time-of-flight mass spectrometer. Finally, the averaged spectra in Fig. 2 could be deconvoluted (Fig. 3) via a simple algorithm [4] to give the molecular mass of the protein. The value determined here, 12 359.51 u, is within 0.004% of the accepted molecular mass of cytochrome *c* at 12 360 u, thus differing by approximately 0.5 u.

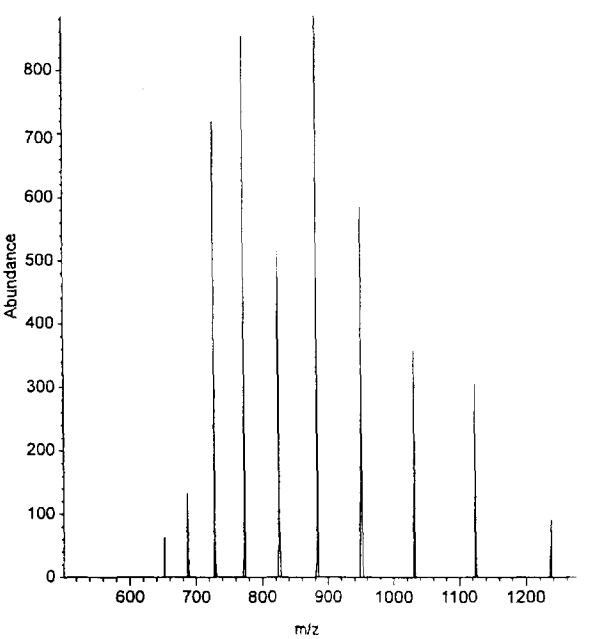


Fig. 2. Average of scans during the elution of cytochrome *c* at a mobile phase flow-rate of 125 μl/min on the perfusion column.

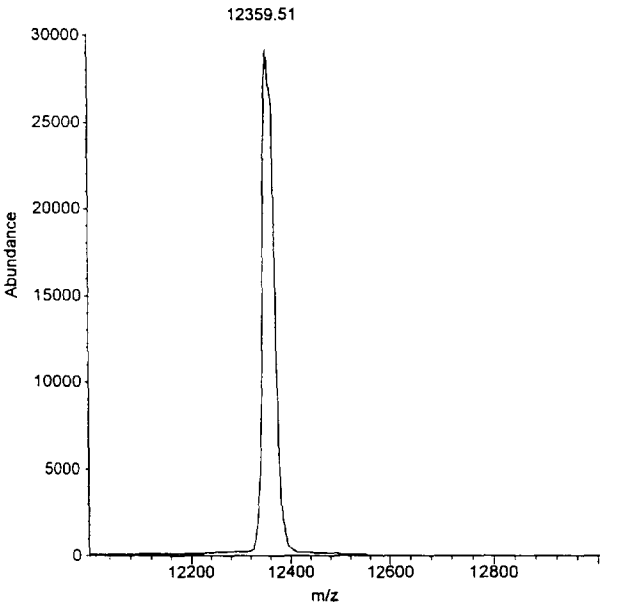


Fig. 3. Deconvolution of cytochrome *c* from MS data in Fig. 2.

### 3.2. Linear dynamic range and limit of detection of a perfusion column

Using the optimized flow-rate above, 125 μl/min, the linear dynamic range (LDR) for injections of cytochrome *c* with this system was determined both in scan mode (500–1300 *m/z*) and selected ion monitoring (SIM) mode (825 *m/z*). These data are shown in Fig. 4. The response in both scan and SIM were both linear until approximately 200 pmol, were injected. The limit of detection (LOD) in SIM mode was found to be 64 fmol while the LOD in scan mode was found to be 1600 fmol. The difference here is as expected and similar to other LC methods. The much higher LOD in scan mode is the result of both increased background noise, normally seen while scanning many more *m/z* channels, and greatly decreased signal averaging, also encountered in the scan mode of operation.

Future work will concentrate on the prepara-

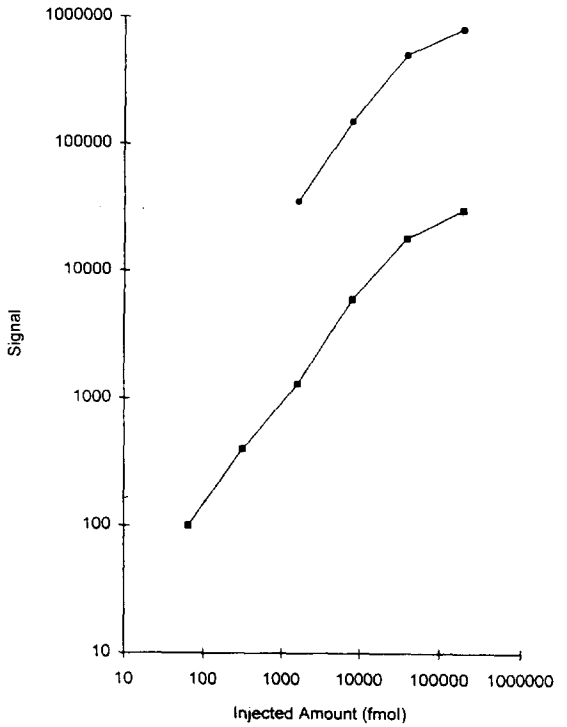


Fig. 4. Signal vs. concentration of cytochrome *c* from scan (●) and SIM (■) modes of MS operation using perfusion column.

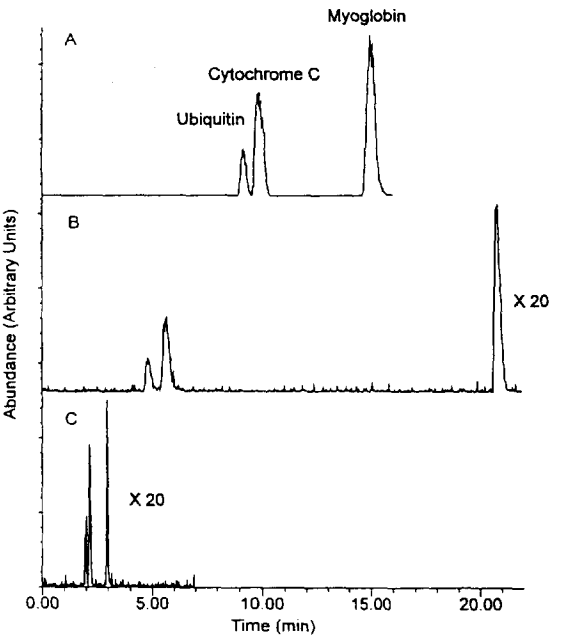


Fig. 5. Comparison of TIC from a protein mixture with different columns using best conditions for each: (A) 320 $\mu$m I.D. capillary column packed with conventional media, gradient: 30–90% ACN in water (0.1% TFA) in 30 min, (B) 1.0 mm I.D. microbore column also packed with conventional media, gradient: 25–90% ACN in water (0.1% TFA) in 15 min, (C) perfusion column, 20–90% ACN in water (0.1% TFA) in 5 min.

tion and use of even smaller column diameters and more sensitive detection in order to increase the linear dynamic range at the lower end.

### *3.3. Comparison of perfusion to packed-capillary and microbore LC with conventional packing media*

In a final set of experiments, performance of this system was compared with both packed-capillary (320 $\mu$m I.D.) and microbore (1.0 mm I.D.) columns packed with conventional $C_{18}$ media. This comparison is interesting, since the packed-capillary column has the same diameter as the perfusion column, while the 1 mm I.D. column has a similar optimum flow-rate as the perfusion LC column. Fig. 5 summarizes the results of these experiments where the same mixture of proteins (30 pmol each) was injected onto the columns under their respective ideal operating conditions. This figure shows the resulting TICs obtained when the quadrupole mass analyzer was scanned from 500–1300 *m/z* units.

Fig. 5A is the TIC from the 15 cm × 320 $\mu$m I.D. capillary column packed with the conventional Vydak $C_{18}$ 5-$\mu$m particles. The total analysis time was approximately 15 min. Fig. 5B is the TIC from the 15 cm × 1 mm I.D. microbore column, also packed with the conventional Vydak $C_{18}$ 5-$\mu$m particles. The analysis time, being approximately 21 min, does not differ markedly from the 320 $\mu$m I.D. microcolumn. However, the sensitivity of the 1.0 mm column is nearly 20-fold less than the 320 $\mu$m capillary column. This result is as expected, since the relative sensitivity of two LC columns of otherwise equal character, is known to vary by the

inverse of the square of the two radii. In this case, that calculation gives a gain of at least a factor of 10. Finally, Fig. 5C shows the TIC obtained from the 320 μm I.D. perfusion column. The sensitivity here is again approximately 20-fold less than the packed-capillary column yields yet is essentially equal to the sensitivity of the microbore column. Dramatically different, however, was the total analysis time required. With the perfusion column, the entire separation was completed in just 3 min. This is nearly 7 times faster than the 1 mm I.D. column and 5 times faster than the 320 μm I.D. columns packed with conventional media. These results are similar to those reported by Kassel et al. [14] where the performance of peptides on perfusion, packed-capillary and microbore LC–MS systems was evaluated.

## 4. Conclusions

In these studies, it has been shown that the use of the perfusion media in an LC column format allows for a reduction in analysis time for a protein mixture of at least a factor of 5, as compared to either a 1.0 mm I.D. column or a 320 μm I.D. column packed with conventional media. Care was taken in this study to maintain the chromatographic resolution between two of the eluting species across the range of columns used so that the comparisons would be fair. The optimum mobile phase flow-rate for the perfusion column was found to be 125 μl/min, which generated peak widths of between 5 and 10 s. This flow-rate is approximately 25-fold higher than would be expected from a column of the same diameter but packed with conventional media. Using the perfusion column, an average of five scans recorded during the elution of cytochrome *c* was sufficient to allow the deconvolution of the multiply charged envelope appearing in the mass spectrum and determine the molecular mass of this protein to within 1 u.

The results also show that both the 1 mm I.D. column packed with conventional media and the perfusion column suffer a signal loss of approximately 20-fold, compared to the 320 μm I.D. column packed with conventional media. For this reason, future work will focus on the development and use of even smaller-I.D. columns with the perfusion media in order to recover some of this sensitivity.

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