Ternary Gradients for Packed Capillary Chromatography of Peptides by a Continuous Flow Syringe System

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Abstract

Packed capillary liquid chromatography has become increasingly important in the separation of peptides and proteins. This technique enhances sample sensitivity by increasing sample mass concentration due to the small ID columns. The low flow rates are also ideally suited for direct interface for LC-MS. This report describes a syringe based pumping system that delivers ternary gradients at flow rates down to 1µL/minute without the use of flow splitters. The pumping system can also be configured to operate at traditional microbore flow rates of up to 400µL/min with minimal hardware changes. An additional syringe module is added to create a continuous flow, syringe based, fluid delivery system which never subjects the capillary to substantial pressure changes and allows for rapid wash and system re-equilibration.

Ternary gradient chromatography is useful in the separation of complex peptide mixtures because of the selectivity differences imparted by the use of organic modifiers such as acetonitrile and methanol. The system described will generate ternary gradients using these and other modifiers. Data will be presented showing ternary gradient formations at flow rates of 2 to 5 µL/min. Run to run peptide map reproducibility data and peptide selectivity information based on organic modifier content will also be shown. Sample run turn-around times will be examined and compared to current syringe pump methods.

Introduction

Syringe pumps have long been considered very pressure stable fluid delivery systems; however, they must be refilled regularly. While this is not a problem if the analysis is completed by a single stroke of the syringe, there are applications in which continual delivery of solvent would be beneficial and which use of traditional syringe pumps might be impractical. This report examines the use of a ternary gradient syringe system coupled to a device which allows for continual fluid delivery while the gradient pumps refill. This device, termed the Transitional Liquid Delivery (TLD) slave unit, is designed for microflow gradient applications (flow rates of 1 to 500 µL/min) in which interrupted flow is not desirable. The complete pumping system allows the user to obtain excellent gradient formation at low flow rates while not having the system depressurize between runs, which is normally associated with use of syringe pumps. The result is a simple system similar to dual reciprocating syringes, yet allowing for gradient generation at the lowest flow rates of the syringe pump.

Because only a single fluid may be pumped the TLD device, it is best operated for wash or equilibration solvent delivery. Used in this manner, total run turnaround times can be shortened and therefore sample throughput increased.

This study incorporates a ternary gradient system for the separation of a complex peptide sample and examines the selectivity differences which can be generated using a three solvent gradient delivery system in conjuction with the continual flow, TLD, device.

Experimental

Data was collected using the following equipment:

Fluid Delivery: Eldex µPro Gradient Pump and µPro Slave Syringes, one for Ternary Gradient and one for Transitional Liquid Delivery (TLD) all fitted with 2 mL syringes

Injector: Valco Injection Valve; 1µL external loop

Column: Unimicro 200 x 0.32mm C-18 300Å 5mm packed capillary

Column Oven: Eldex CH-150 Column Oven

Detector: Shimadzu LC4 Variable Wavelength UV Detector with LC Packings UZ View 35nL flow cell

Data Acquisition: EZChrom Elite Data System

The slave syringe pumps were connected to a binary gradient syringe pump to create the ternary and TLD capability. A simple RS485 connection and pieces of 1/16" capillary tubing were required to connect the parts of this fluid delivery system.

Figure 1: Gradient and Transitional Liquid Delivery Schematic

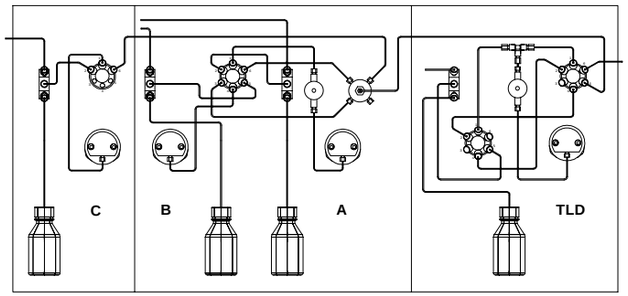

Figure 2: Transitional Liquid Delivery Pressure Trace

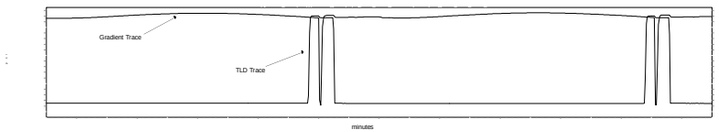

Figure 3: Eleven Replicate Injections (from Table 1)

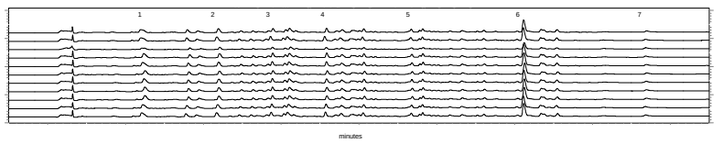

Figure 4: Selectivity Chromatograms

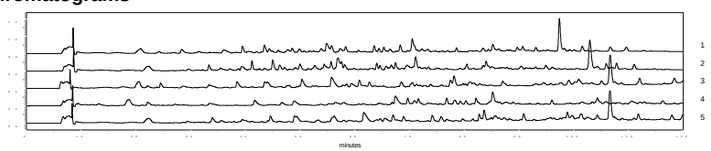

Table 1: Peptide Chromatography Using Continuous Microflow Syringe System; 11 Consecutive Runs

Conditions

Sample: 6.5pmole trypsinized a-chymotrypsinogen in 1µL
Flow Rate: 3µL/min
Equilibration: 15 minutes at 6%B
Gradient: 6%B to 76%B in 90 minutes
A: 0.05% TFA in water
B: 0.045% TFA in 60% acetonitrile and 40% water
Syringe and column temperature: 33ºC
Detection: UV210

TLD pressurizes and delivers B solvent for 5 minutes while gradient syringes refill and re-pressurize

		Peak Number						
	N	1	2	3	4	5	6	7
Av. Retention Time	11	16.82	26.33	33.51	40.6	51.69	66.19	81.89
Std. Dev (min.)		0.15	0.27	0.16	0.15	0.12	0.086	0.082
%RSD		**0.88**	**1.02**	**0.48**	**0.36**	**0.23**	**0.13**	**0.10**

Table 2: Ternary Gradient Conditions

Five multiple sets of runs were collected sequentially over a sixty hour period. Each run was repeated four times.

Sample: 65 pmoles of trypsinized a-chymotrypsinogen
Flow rate: 3 µL/min
Solvents: **A**: 0.05% TFA in water
B: 60% Acetonitrile / 40% 0.045% TFA in water
C: 60% Methanol / 40% 0.045% TFA in water
Equilibration: 20 min. at 2%**B** and 2%**C**
Gradient Shape & Time: linear; 120 min.

Gradient #	**1**	**2**	**3**	**4**	**5**
Gradient B	2%-46%	2%-60%	2%-4%	2%-40%	2%-22%
Gradient C	2%-46%	2%-2%	2%-95%	2%-25%	2%-75%

Results and Discussion

To create a system with continual flow for packed capillary chromatography, a ternary syringe HPLC gradient pump was connected to a single syringe with special valving, the transitional liquid delivery (TLD) slave pump (patent pending). The gradient syringes first prepressurize to operating pressures followed by an equilibration period. Following a sample injection and gradient generation, the TLD syringe is pressurized to the system pressure and a high pressure valve switches, taking the gradient system off line and putting the TLD syringe on line. The TLD then pumps solvent for a fixed amount of time. During the TLD solvent flow, the gradient syringes refill and subsequently repressurize to the current system pressure. The valve then switches back to place the gradient syringes on line with no loss of pressure during the transition. ***Figure 1*** is a schematic of the fluid path and system components of a binary gradient system with TLD. ***Figure 2*** is a superimposed pressure trace of two complete cross over processes. It consists of the gradient equilibration and generation followed by the TLD cycle. Pressure is maintained across the capillary column during this process with little or no changes during the transition.

Reproducibility

Chromatographic reproducibility under this mode of operation was examined by repeated injection of trypsinized a-chymotrypsinogen. Eleven consecutive chromatograms are shown in ***Figure 3*** and the associated %RSD data is shown in ***Table 1*** along with the chromatographic conditions. The continuous flow nature of this syringe system does not affect the typical reproducibility which is associated with syringe pumps at very low flow rates.

Selectivity Study

Repeated peptide injections were subjected to five different ternary gradient compositions containing TFA/water, acetonitrile and methanol. Conditions are described in ***Table 2.*** ***Figure 4*** shows a comparison of the five peptide separations and demonstrates the difference in selectivity associated with the gradient conditions described. Ternary gradients can enhance the separation of complex peptide mixtures and offer another variable to increase selectivity.

Conclusion

Complex peptide mapping offers a considerable challenge to the peptide chromatographer. This challenge is only heightened by the incorporation of micro flow rates associated with packed capillary chromatography. The importance of such techniques to LC-MS have also become increasingly clear. This report shows the capabilities of a continuous flow, syringe based pumping system which can deliver reproducible ternary gradients at the extremely low flow rates required by packed capillary columns. Such systems can offer true pulseless fluid delivery without the usual syringe refill process, and include the advantage of substantial selectivity enhancement which is associated with classic ternary solvent gradient systems.