Ternary Gradient Chromatography of Peptides on Packed Capillaries

Bruce Black, Eldex Laboratories, Inc. Napa, CA 94558

Introduction

Syringe pumps have long been shown to be useful for low flow rate HPLC applications. They have been successfully used in such techniques as LC-MS and micro-preparative packed capillary chromatography This report examines the use of a gradient syringe system which has been modified to incorporate a third syringe to make a ternary HPLC system capable of operating routinely at flow rates between 1 and 10μL/ min., specifically for packed capillaries with a diameter of 0.320mm

Ternary gradient chromatography has been shown to be useful in the separations of small molecules and offers another tool for the separations of complex peptide mixtures. Increased interest in enhancing sensitivity has promoted advances in packed capillary technology. These columns require precise fluid delivering devices which are capable of delivering low flow rates with enhanced gradient precision. The instrumentation described here has the precision and accuracy required for just such applications and offers the chromatographer reproducibility comparable to traditional flow rates without the necessity flow splitting.

Experimental

Data was collected using the following equipment:

Fluid Delivery:	Eldex MicroPro Gradient Pump and a MicroPro Slave Syringe, all fitted with 2 mL syringes
Injector:	Valco Injection Valve; 1μL external loop
Column:	Unimicro 200 x 0.32mm C-18 300Å 5μm 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 pump was connected to a stand alone binary gradient syringe pump to create the ternary capability. A simple RS485 connection and one piece of 1/16" capillary tubing was required to connect the two parts of this fluid delivery system.

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 α-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% →90%	2% →2%	2% →68%	2% →24%
C	2% →46%	2% →2%	2% →90%	2% →24%	2% →68%

Figure 1: Ternary Gradient Schematic

C   B   A

Ternary Slave   Binary Gradient

Table 1: Ternary Gradient Reproducibility Data

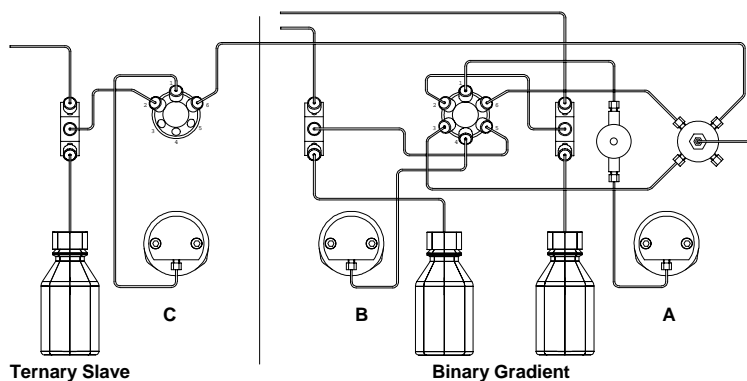| Gradient		N	1	2	3	4	5	6
1	Av. Retention times (min)	4	14.01	31.75	51.63	74.89	95.95	108.43
	Std dev (min)		0.29	0.39	0.49	0.26	0.30	0.18
	%RSD		**2.13**	**1.24**	**0.95**	**0.30**	**0.29**	**0.18**
2	Av. Retention times (min)	4	18.61	27.55	40.76	57.91	73.64	78.54
	Std dev (min)		0.18	0.17	0.14	0.13	0.09	0.09
	%RSD		**0.96**	**0.62**	**0.35**	**0.22**	**0.13**	**0.12**
3	Av. Retention times (min)	4	18.98	33.96	56.86	78.19	112.04	118.36
	Std dev (min)		0.18	0.40	0.17	0.25	0.54	0.54
	%RSD		**0.97**	**1.18**	**0.29**	**0.32**	**0.48**	**0.46**
4	Av. Retention times (min)	4	18.64	29.69	45.62	66.27	84.11	92.24
	Std dev (min)		0.47	0.52	0.62	0.81	0.09	0.17
	%RSD		**2.53**	**1.76**	**1.36**	**1.23**	**0.11**	**0.18**
5	Av. Retention times (min)	4	19.10	37.33	57.87	86.36	108.75	114.03
	Std dev (min)		0.05	0.15	0.23	0.35	0.30	0.25
	%RSD		**0.28**	**0.41**	**0.39**	**0.41**	**0.27**	**0.21**

Peak Numbers

Figure 2: Four Replicate Injections Using Ternary Gradient Condition 3
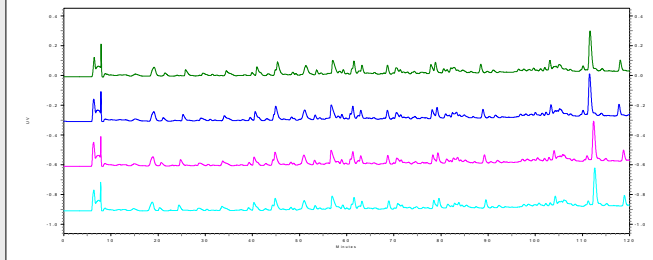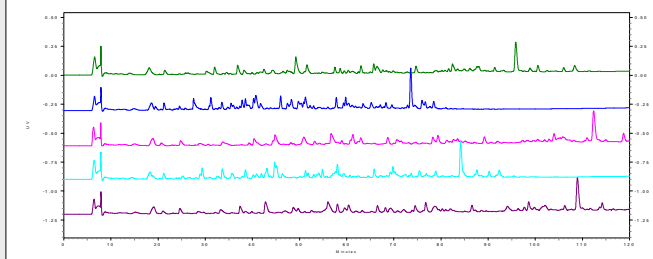

Figure 3: Comparative Ternary Gradient Traces
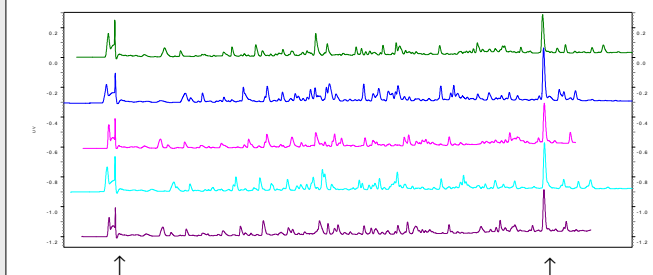

Figure 4: Normalized Traces

Results and Discussion

A diagram of the ternary gradient system used in this study is shown in *Figure 1*. It consists of a standard dual piston syringe pump coupled with a single slave syringe. Besides the electronic connection, the only connection required between the two parts of this system is the capillary tube connecting the output of the slave pump to the appropriate mixer port of the binary gradient device.

Repeated peptide injections were subjected to 5 different gradient compositions containing TFA/water, acetonitrile and methanol. Conditions are described under *Gradient Conditions*. Each set of conditions was repeated 4 times and a representative set of these results is shown in *Figure 2*. *Figure 3* shows a comparison of all five sets of ternary gradient conditions The statistical reproducibility of this data is shown in *Table 1*. *Figure 4* displays an enhancement of the selectivity differences between the five sets of conditions by normalizing each chromatogram to the solvent front and a late eluting peptide.

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 and its importance to such techniques as LC-MS. This report shows the capabilities of a 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 a substantial selectivity enhancement compared to traditional binary systems without a loss of resolution which might be associated with the added hardware.