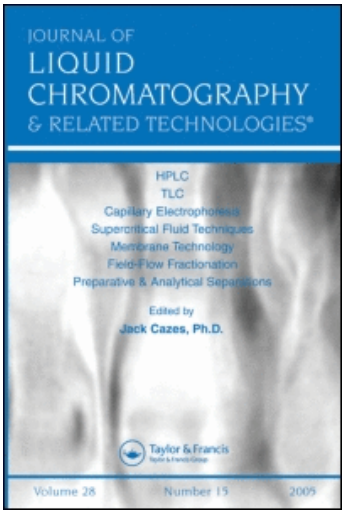

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LOW-COST LIQUID CHROMATOGRAPHY (LC-LC). III. OPEN TUBE GRADIENT GENERATION FOR MICRO-LC

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ABSTRACT

Previous papers on low cost liquid chromatography (LC-LC) described how an on-line robotic autosampler, required to change samples, can be used to eliminate the injection valve for "weak-eluent sample-loading" [1, 2]. Another LC-LC paper described how a flow-through system can be used to take eluents to a fixed and reproducible pH, eliminating the time and effort to prepare eluents [3].

This paper on LC-LC describes how an fused silica open tube can be used to reproducibly generate symetrical linear S-shaped "micro-gradients" that can be varied in volume in the 20 to 110 microliter range. Several of these micro-gradients can be contained in the open tube generator at one time so that runs can be made as quickly as with conventional gradient generation

systems. The apparatus and the wide bore (0.53 mm i.d.) fused silica open tube gradient generator described here should permit sub-microliter (nanoliter) gradient generation when narrower bore open tubes are used (e.g. 0.25 to 0.01 mm). Open tube gradient generation eliminates the conventional dynamic stirred mixer and substitutes a single high pressure pump for dual high pressure pumps and a gradient controller.

Future work will show that when the three LC-LC methods are combined one autosampler can operated many LC instruments, each being run by only a single high pressure pump, detector, and data system. This combined LC-LC approach will eliminate one pump, a valve injector, the gradient/pump controller, and the gradient mixer (as well as eluent preparation).

INTRODUCTION

Gradients for columns smaller than the conventional diameter (4-5 mm) often cannot be directly generated with conventional liquid chromatography (LC) equipment. The several dozen approaches that have been used for producing small "micro-gradients" with volumes below 1 ml were reviewed recently by Berry and Schwartz [4, 5]. Some methods use conventional equipment with part of the gradient split out for micro-LC and some methods use scaled-down equipment.

Several other gradient generator approaches have been shown to produce the desired linear micro-gradients in the microliter range, but not to the nanoliter range. These approaches usually start with the discontinuous interface produced when the weak eluent is abruptly switched to the strong eluent. This interface can be modified in several ways. When passed through a series of

"connected exponential mixers" [6] or small columns (3 X 0.46 cm) packed with porous or solid particles [7], Berry, Takeuchi, and Ishii showed that linear micro-gradients in the 90-600 uL range are produced. Slais showed a "spiral-helical generator" using a spiral open tube to induce "secondary flow" across the open tube to produce variable linear micro-gradients from 60-700 uL for 1 mm LC columns run at 60 uL/min [7]. It is difficult to see how these devices can be further miniaturized for gradients in the nanoliter range, as can the open tube gradient generator described here.

The common problem found when trying to obtain gradients in the microliter and nanoliter range by producing a discontinuous interface between two eluents is that mixing volumes in the system will often produced an "exponential gradient". Exponential gradients have several disadvantages [4]: (1) the gradient beginning is sharp (often displacing some peaks), (2) the intermediate zone is curved exponentially (frequently not giving good spacing of peaks), and (3) the final composition is reached in only a very long time. A more desirable micro-gradient shape is "linear", with the advantages: (1) the gradient beginning slopes gently (often separating early peaks), (2) the intermediate zone has a fixed rate of composition change (e.g. %B per ml of eluent), and (3) the final composition is reached in a short and predictable time. Another criteria of good micro-gradients, not yet addressed by anyone, is the need for tailoring

the micro-gradient shape at any point during the run so that more gentle composition changes might be used in areas in which more LC resolution is needed. A possible solution to tailoring micro-gradients is described here.

Future needs exist for micro-gradients far below 1 ml. A conventional 4.6 mm i.d. column uses for a gradient 15 to 60 mL (15,000 - 60,000 uL, 6 - 24 packed column liquid volumes)[4]. The ratios of cross-sectional area of the conventional column (4.6 mm i.d.) to the area of the micro-column allows the volumes of micro-gradients to be calculated. For micro-LC with packed columns from 0.52 mm to 0.10 mm, there is a need for generating gradients in the 190 to 760 uL range. For open tubular LC using capillaries at 0.010 millimeter i.d., solvent micro-gradients from 0.07 to 0.28 microliters (70 - 280 nanoliters!) are required. For open tubular LC using capillaries at 0.001 millimeter (1 micron) i.d., solvent micro-gradients from 0.000708 to 0.0028 microliters (0.7 - 2.8 nanoliters!) are required.

Capillary zone electrophoresis (CZE) has a different use for micro-gradients. Instead of using 6 to 24 packed column volumes to elute samples as in LC [4], there is a need for filling an empty single column volume of a capillary 10-100 cm long and 0.050 and 0.020 mm i.d. with various micro-gradients. For CZE, micro-gradients in acrylamide levels (for molecular weight sieving), pH buffers (for isoelectric focusing), surfactant levels (for micellar separation of neutral and charged

molecules), and organic composition require micro-gradients ranging from 0.08 to 78 nanoliters. These nanoliter volume gradients are a challenge to future technology.

The open tubular gradient generator described below has the possibility of producing linear micro-gradients for some of the applications described above. This paper shows that gradients to 20 microliters can be produced with such a system and shows possible practical approaches in which many gradients may be contained at one time in the open tube gradient generator. Since this open tube gradient generator uses wide bore open tubes (0.53 mm i.d.) for 20 uL gradients, narrower open tubes (to 0.01 mm) using the same pumping and injection techniques should produced nanoliter size gradients. Instead of conventional injection, samples might best be loaded by "weak eluent sample loading" by having an autosampler automatically load each separate sample in weak eluent in a zone in the fused silica capillary separated by the stong eluent, such as described by Berry, Fay, and Pretorius previously [1,2]. Mixing problems from conventional injectors are thus eliminated. Future work will bring these novel approaches to injection and gradient generation together in new low cost liquid chromatography (LC-LC) methods.

These micro-gradient systems are likely to be needed in the future of biotechnology since protein separations by LC typically require a gradient and often only very small amounts of gene-engineered proteins are available.

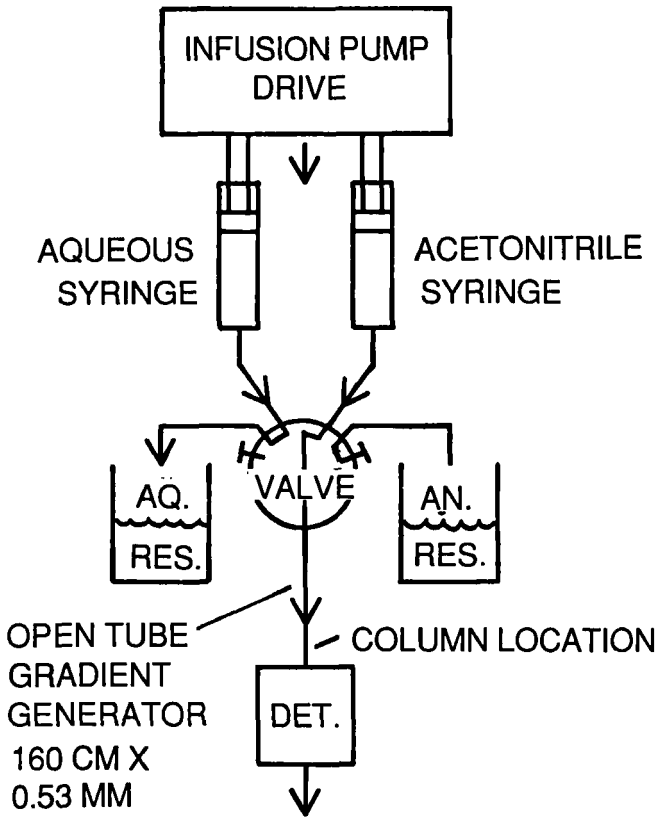

FIGURE 1. Apparatus for modeling gradient generation with open tubes (see text).

MATERIALS AND METHODS

Figure 1 shows the schematic diagram of the apparatus used for gradient generation with open tubes. The infusion pump (#5200, Scientific and Research Instruments Limited, 335 Whitehorse Rd, Croyden, Surrey, England) moves a driving bar forward at speeds settable between 0.2 and 30 mm per minute or

per hour. The resulting flows depend on the volume of the syringes used in the infusion pump; smaller diameter syringes give lower flows. The only syringes that worked correctly were smooth glass syringe barrels with Teflon-tipped pistons. For this work syringes were used of 10 mL volume contained in 60 mm of barrel length (#1010, Hamilton, Reno, NV, USA). With these 10 ml syringes and the infusion pump drive rates possible, flows from 0.55 to 5,000 uL/min could be obtained.

Figure 1 shows how gradients are produced. The weak eluent syringe (Fig. 1, labeled "Aqueous Syringe") and the strong eluent syringe (Fig. 1, labeled "Acetonitrile Syringe") were manually filled and all air bubbles removed. The acetonitrile containing ca. 10% v/v acetone for UV visualization of the gradient shape. The syringes were then inserted into the infusion pump drive, and both syringes operated continuously. To make a run, the pump was operated until aqueous eluent filled the open tube, and then the run was begun by activating the electrical valve so that acetonitrile was abruptly switched to the inlet of the open tube (the valve position shown in Fig. 1). A delay volume was found equal to the volume of the open tube (350 microliters). Note that eluent from the pump that was not connected to the open tube (the "Aqueous Syringe" in Fig. 1) was passed back to a reservoir ("Aq. res.") for re-use. Advantages of this arrangement are: (1) flows are un-interrupted when the gradient is begun (i.e. when the valve is activated); (2) eluents are not wasted; and (3) flows

can be measured directly (gravimetrically) from one pump when the other pump is connected to the open tube gradient generator.

The open tube gradient generator was made from a 0.53 mm i.d. fused silica open tube capillary (170 cm long, 160 cm from valve to detection) (Anatech, Johannesburg, S. Africa). Connections of the open tube to the center port of the 12-port electrically operated valve (Valco, Houston, TX, USA) used carbon ferrules (Scientific Glass Engineering, Austin, TX, USA). (The valve must have more than 8-ports.) The two connections from the syringes to the valve were made with 30 cm lengths of high pressure 1/16 inch o.d., 0.030 inch i.d. Tefzel tubing (#1528, Chrom Tech, Apple Valley, MN, USA). The tubing connections to the syringes used 1/4-28 syringe couplings (P604, Chrom Tech), unions (P603, Chrom Tech), and flangeless fittings (P200, Chrom Tech). The tubing connections to the valve used stainless steel 1/16 ferrules and male nuts. The fused silica, connectors, and Tefzel tubing have the capability of operating to several hundred atmospheres pressure, although pressures were low in this model system in which a packed separation column was not used.

"On-tube" detection directly in the 0.53 mm i.d fused-silca open tube capillary at 254 nm was made by inserting the open tube directly in a modified 254 nm UV detector (441, Waters/Millipore, 2018 Bramley S. Africa). For detector modification, the 1 cm thick steel detector cell body was cut with a 1.5 mm deep, 1.5 mm wide slit from the top to the bottom in the photodiode face over

the reference cell (described elsewhere [10]). This leaves the sample cell of the detector intact for conventional LC. A hole drilled in the steel base of the detector just below this point permits 10 cm of fused silica to pass below the detector for collecting the waste eluent.

Ultraviolet light passes through fused silica with the imide coating removed. A window in the center of the fused silica open tube was made by using a sharp razor blade under a microscope to shave off two 0.6 mm wide imide zones on opposite sides of the open tube. The open tube was positioned vertically and rotated to permit the most light to strike the photodiode. The most light was determined by comparing the signal with the "light blocked" (by positioning fused silica with imide intersecting the light path) vs. the signal with "light through" (by positioning the fused silica with the shaved slit interecting the light path). When positioned, the open tube was firmly held in place and stray light blocked from the slit with a large piece of modeling clay at the top of the steel detector cell body. Typically the 254 nm detector was operated at the 0.1 absorbance setting using a 10 mv recorder running at chart speed of 3 cm per min. Changing the vertical and rotational position of the open tube to center the slit in the light path did change the lowest detector absorbancy setting that could be reached by using the detector "zeroing" control for bringing the signal on-scale. With a front and rear open tube slit of ca. 0.6 mm, and proper positioning, enough

light passed through the cell so the signal could be zeroed on scale down to the setting of 0.02 AU.

RESULTS AND DISCUSSION

Gradient shapes found with the 0.53 mm X 160 cm open tube gradient generator are shown for various flows from 332 uL per min (Figure 2, top) down to 6.7 uL per min (Figure 2, bottom). The gradient volumes are also indicated. Note the good symmetry and linearity of the gradients. High flows (top) give large gradient volumes (not visually evident because of the constant chart speed used for all runs). At high flows viscous drag in the open tube dominates and the gradients are broad. At low flows diffusional mixing is expected to determined gradient volumes. The long gentle bend of the 1.6 meter length of the open tube into a half circle ca. 1 meter lin diameter is expected to induce little secondary flow mixing across the open tubes since little "coiling" is produced in the system [8].

The gradient volumes were calculated as the volume between the intercepts of the asymptotic line to the linear (center) part of the gradient zone and the intersection of the initial and final base lines, as illustrated in the bottom gradient (Figure 2, E).

Figure 3 summarizes the gradient volumes vs. flow rate as generated in Figure 2. Note that gradient volumes from 20 to above 110 uL are possible.

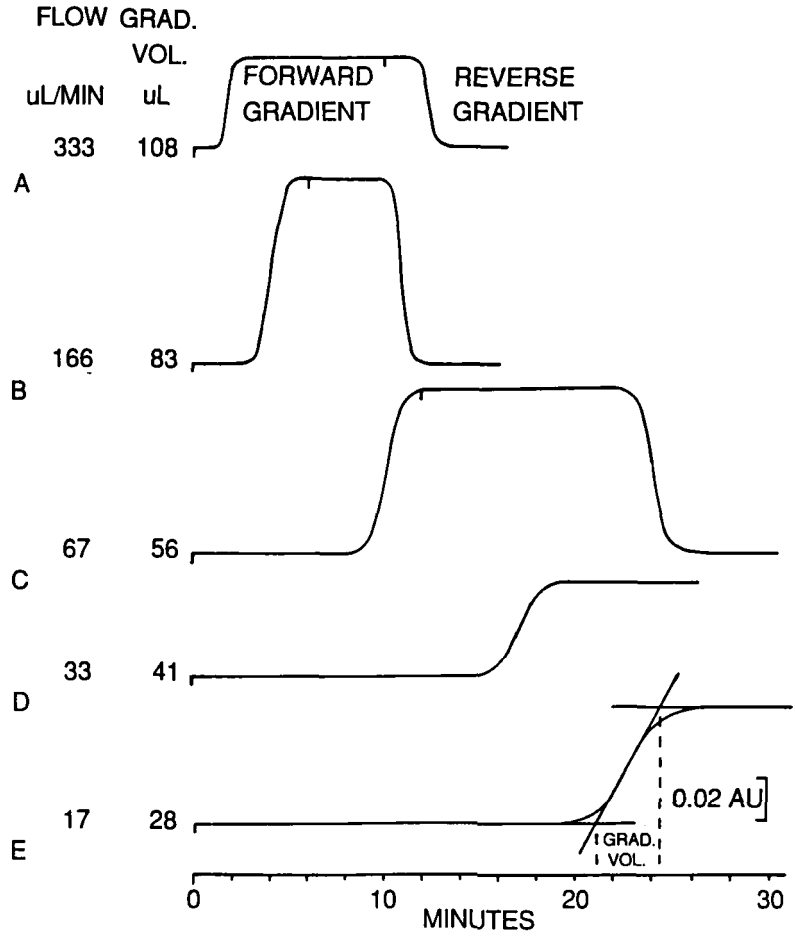

FIGURE 2. Symmetrical linear gradients produced by a 0.53 mm X 160 centimeter open tube gradient generator operated at the flows shown in the figure. For each, the first rise represents the baseline movement from water to acetonitrile (with 10% v/v acetone to give UV absorbance). Vertical marks on the gradient traces indicate when the valve was activated. Baseline shifts were ca. 50% of full scale with a detector setting of 0.1 AU and a 10 millivolt recorder. Chart speed was 3 cm/min. The fall in baselines in runs A-C shows the reverse gradient from acetonitrile to water. The method for determining the gradient volume is shown in E. Different rises in the baselines from runs A to E are due to small variations from day to day in the (UV-absorbing) acetone content of the acetonitrile.

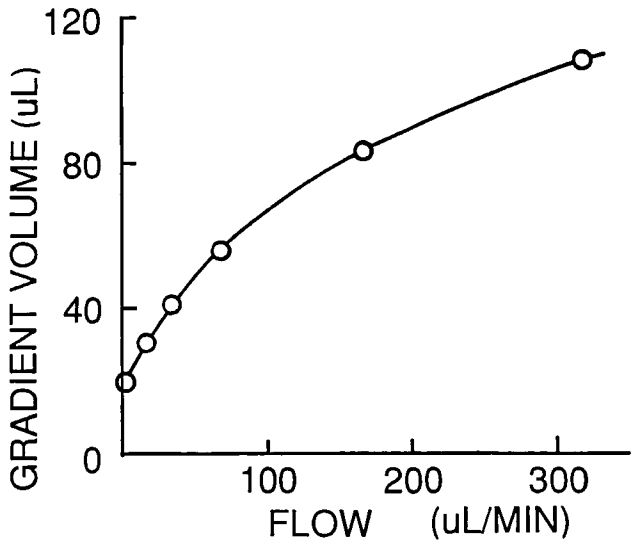

FIGURE 3. Summary of gradient volumes vs. flows for the systems described in Fig. 2.

Gradient volume reproduciblities are good. Gradient volumes are the average of three gradient runs. The average of five sets of these average deviations was 1.8%, with individual average deviations ranging from 0.1% to 5.3%.

The forward gradients (water to acetonitrile) and reverse gradients (acetonitrile to water) showed similar symmetrical linear shapes. However, for flows ranging from 16 to 166 uL/min, the reverse gradient volumes were typically 15 to 30% larger than the forward gradient volumes. This could be due to slight differences in the syringes. Reverse and forward gradient volumes showed similar average deviations (1.8%).

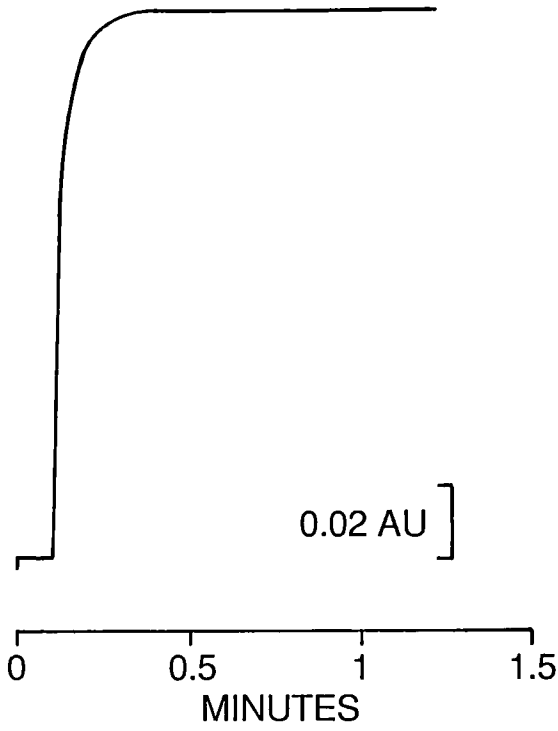

FIGURE 4. Non-symmetric exponential-type gradient produced when the 0.53 mm i.d. open tube gradient generator is too short (8.5 cm). Flow is 100 ul/min. The rise represents the baseline movement from water to water with 10% v/v acetone to give UV absorbance. Baseline shifts were ca. 80% for full scale with a detector setting of 0.2 AU and a 10 millivolt recorder. Chart speed was 12 cm/min.

Figure 4 shows the less linear, more "exponential" gradient produced when too short a tube (only 8.5 cm long) is used. Compare this to the much more symmetical gradients for the 160 cm long tube shown in Figure 2B and 2C (at flows of 166 and 67 uL/min, respectively).

The volume of eluent required to elute the center of the gradient (middle of the S-portion) was determined for flows ranging from 333 down to 6.7 uL per minute. This volume ranged from 308 uL (at fast flows) up to 347 uL (at slow flows). As expected, the latter volume (347 uL) corresonds closely to the open tube generator volume of 350 uL calculed as a cylinder of 0.53 mm i.d. and 160 cm long.

Gradients can be generated rapidly. At a flow of 166 microliters per minute, Figure 5 shows that 2 full forward gradients (and three reverse gradients) can be generated in less than 12 minutes.

With gradient volumes ranging from 20 to 100 microliters, and a open tube with a volume of 350 microliters, several forward and reverse gradients could be contained in the open tube gradient generator at any one time, without waiting for one to elute before starting the second. For example, a flow of 16 uL per minute gives a gradient volume of 28 microliters (ca. 64 microliters from the very onset of the gradient to the final composition). Such a flow and gradient volume is compatible with 0.32 mm i.d. fused silica columns [4] such as available from Alltech (Deerfield, IL, USA)) or LC Packings (San Francisco, CA, USA). The valve could be activated as frequently as every 4 minutes (64 microliters). Thus, two forward and two reverse gradients could be contained on the open tube gradient generator at one time. Once the first gradient has traversed the open tube

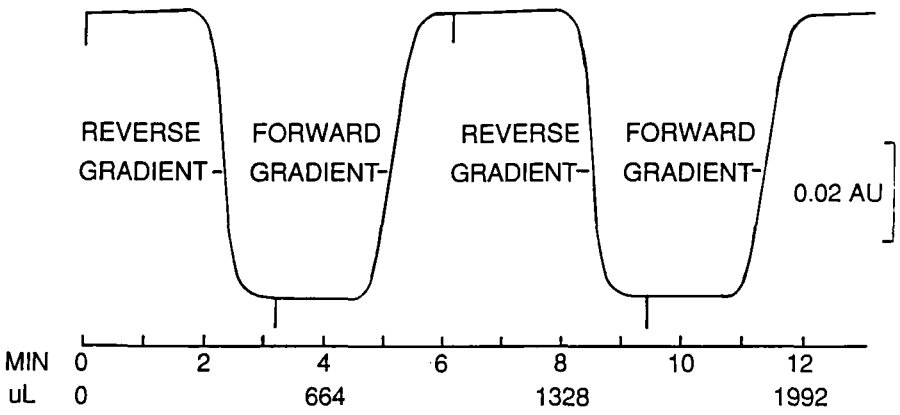

FIGURE 5. Examples of two fast gradients (83 uL each) in less than 12 minutes beginning with a reverse gradient. The four numbered vertical marks on the gradient traces indicate when the valve was activated. Other conditions as in Figure 2.

generator in ca. 22 minutes, separations could be obtained every 8 minutes.

An anomalous blip in the gradient was produced when syringes other than the glass barrel with Teflon piston is used to generate gradients. Unsuccessful 10-ml syringes were: all-glass syringes, all plastic syringes with elastomer pistons, combinations of glass and plastic syringes, and combinations with heavy silicone grease on the pistons. It is believed the anomalies are due to variations of flow due either to the piston occasionally "sticking" with subsequent slippage of the clutch of the infusion pump or a quick "catching up" giving a faster movement of the piston. Further investigation of the cause of

these anomalies may permit tailoring a micro-gradient shape for a repeated routine analysis in micro-LC.

CONCLUSIONS

This paper has shown that symmetrical linear gradients down to 20 microliters in volume can be generated with long (160 cm) fused silica open tubes of 0.53 mm i.d. Smaller gradient volumes should be possible by using long fused silica tubes of smaller diameters. While this system was modeled with an infusion pump using glass syringes, other low flow pumps could be substituted, such as the high pressure steel piston displacment pumps available from Applied Biosystems, Isco, or Milton Roy. The fused silica, switching valve, and other fittings can be used to many hundreds of atmospheres pressure. The possiblity of dissolving sample in the weak eluent (weak-eluent sample-loading) [1, 2] combined with having many such micro-gradients in a open tube at one time gives the possibility of some new approaches to fast low cost liquid chromatography (LC-LC) that will be pursued in future publications.

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REFERENCES

1. Berry, V., Fay, J., and Pretorius, V., in P. Sandra and W. Bertsch (Ed.), "Proceeding of the Ninth International Symposium on Capillary Chromatography,", Dr. Alfred Huethig Verlag, Heidelberg (1988) p387.

2. Berry, V., Fay, J., and Pretorius, V., J. Liq. Chromatogr., 12, 1043, 1989.

3. Berry, V., Pretorius, V.,(to be published).

4. Berry, V. and Schwartz, H., in F. Yang (Ed.), "Microbore Column Chromatography, A Unified Approach to Chromatography", Marcel Dekker, New York (1989), p67.

5. Schwartz, H. and Berry, V., LC Mag. 2, 110 1985.

6. Berry, V., Takeuchi, T. and Ishii, D., J. Liq. Chromatogr., July, 1986.

7. Berry, V., Takeuchi, T. and Ishii, D., J. High Resolut. Chromatogr. Chromatogr. Commun. 10, 659, 1985.

8. Slais, K. and Frei, R., Anal Chem., 59, 376, 1987.

9. Snyder, L. and Dolan, J., "DryLab G, A PC-Video Program to Simulate Experimental Gradient-Elution LC Runs", LC Resources, Lafayette, CA, Section 2, 1987.

10. Berry, V., vanRossen, P., and Pretorius, V.(to be published).