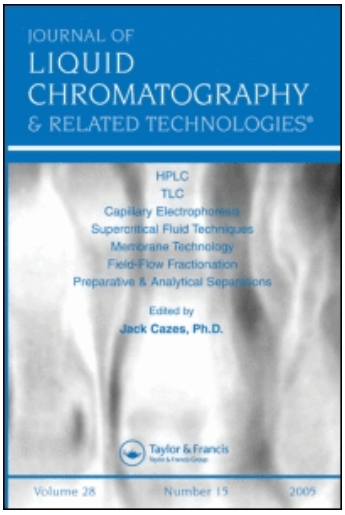

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Low-Cost Liquid Chromatography (LC-LC). IV. "Pulsed Open Tube Gradient Generation", A New Approach for Generating Nanoliter Volume Linear and Tailored Gradients for Capillary Electrophoresis and Micro-LC

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LOW-COST LIQUID CHROMATOGRAPHY (LC-LC). IV. "PULSED OPEN TUBE GRADIENT GENERATION", A NEW APPROACH FOR GENERATING NANOLITER VOLUME LINEAR AND TAILORED GRADIENTS FOR CAPILLARY ELECTROPHORESIS AND MICRO-LC

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ABSTRACT

There is a need to generate gradients of nanoliter volumes compatible with 25-100 micron i.d. capillaries used in capillary electrophoresis (CE). Balchunas and Sapaniak [1] showed that manually produced step gradients of increasing propanol and surfactant concentration with micellar electrokinetic chromatography gave an improved separation. And while SDS-PAGE has shown remarkable results in capillaries e.g. [2], gradients in gel crosslinking to extend the molecular weight range that can be resolved has not yet been shown in capillaries. Gradients in pH in polyelectrolytes for isoelectric focusing show some of the highest resolution known. These have been generated in-place by

electromigration. Small gradients are also needed in micro-liquid chromatography, a field receiving renewed interest today because of the high costs of solvent disposal.

Previous papers on "low cost liquid chromatography" (LC-LC) described how to eliminate the injection valve for "weak-eluent sample-loading" [3, 4] and 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 [5, 6].

Our previous paper on LC-LC described a simple-gradient method [7, 8, 9] in which an abrupt discontinuous interface between the weak and strong eluent can be spread into an S-shaped gradient by passing the interface through an open tube. Volumes were in the microliter range (20 to 110 uL), determined by the open tube dimensions (0.53 mmm i.d.) and the flow rate through the open tube. Here we extend this simple-gradient approach to the high nanoliter range using 0.10 mm i.d. tubing.

This paper describes a new, very flexible method for producing shaped nanoliter gradients ("nano-gradients") og a broad volume range, independent of tube dimensions and flow rates. In the pulsed open tube generator, (1) the gradient can be changed in volume over a broad range, and (2) the gradient shape can be tailored. The new approach tailors the shape of the gradient by using a multiport valve to pulse in alternating slugs of weak and strong eluent; the timing determines the composition; and the open tube mixes the segments together.

The pulsed open tube generator described here should permit nanoliter and lower (picoliter) gradient generation when narrower bore open tubes are used (e.g. 50 to 5 i.d. micron open tubes).

INTRODUCTION

Future needs exists for small gradients far below 1 ml, as outlined previously [7, 8]. 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 small gradients from 0.07 to 0.28 microliters (70 - 280 nanoliters!) are required. Instead of using 6 to 24 packed column volumes to elute samples as in LC, in

capillary electrophoresis (CE), there is a need for filling a single empty column volume of a open tube 10-100 cm long and 0.050 and 0.020 mm i.d. with various small gradients. For CE, micro-gradients in acrylamide levels (for molecular weight sieving), pH buffers (for isoelectric focusing), surfactant and organic levels (for micellar separation of neutral and charged molecules), require micro-gradients ranging from 0.08 to 78 nanoliters.

The open tubular gradient generator described below has the possibility of producing linear gradients for the small volume applications described above. This paper shows that gradients to 880 nanoliters can be produced and how the shapes and volumes can be tailored. Since this open tube gradient generator uses 100 micron i.d. open tubes, narrower open tubes (to 50 to 5 micron) using the same pumping and injection techniques should produce gradients to picoliter size.

Gradients for columns smaller than the conventional diameter (4-5 mm) often cannot be directly generated with conventional liquid chromatography (LC) equipment, and the approaches used for producing gradients near 1 ml were reviewed recently by Berry and Schwartz [10, 11].

The pulsed open tube gradient generator differs from the two common methods used for generating conventional milliliter volume gradients, illustrated in Figure 1 for two-eluent gradient generation. With high pressure gradient generation (Figure 1A),

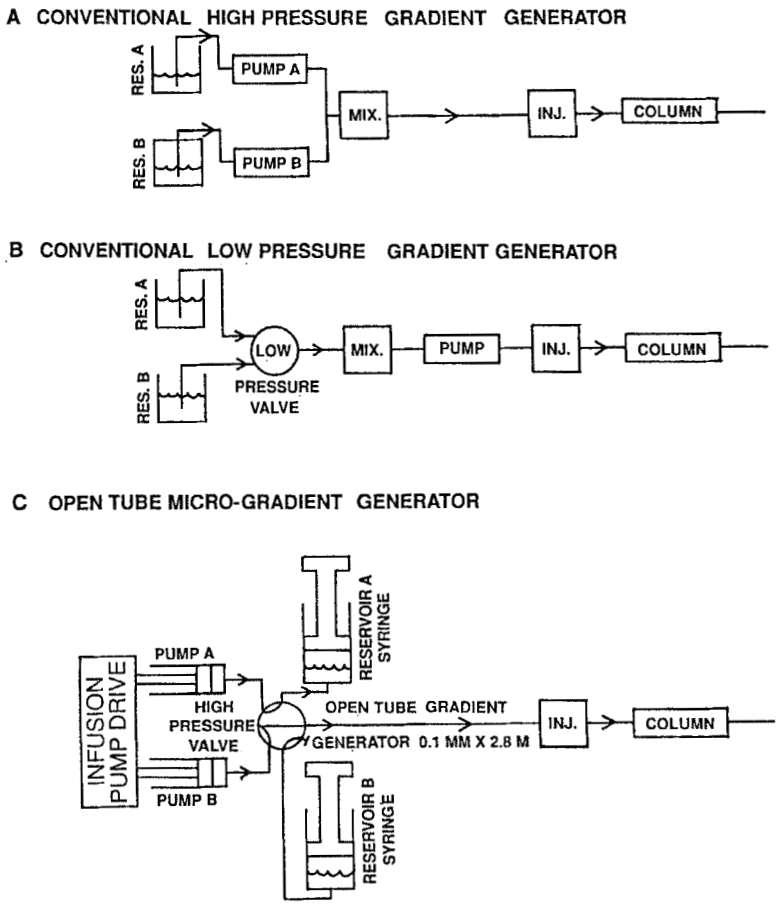

Figure 1. Comparison of different conventional gradient generation approaches (high pressure mixing, A; low pressure mixing, B); and the pulsed open tube gradient generation approach shown here, C. Details of C are in the text.

dual high pressure pumps provide separate eluents which are deposited as streams side by side in connecting tubing (i.e. not as sequential slugs)[12]. A pump-controller determines both the composition of the gradient and the flow rate. With low pressure gradient generation (Figure 1B), the single high pressure pump draws by suction solvent from an eluent selection valve, producing sequential slugs of eluent-A, than eluent-B, then eluent-A, etc. [12]. A valve-controller determines the composition of the gradient, and a pump-controller determines the flow rate.

Pulsed open tube gradient generation (Figure 1C) has elements of both of the previously described systems. Dual high pressure pumps (syringe displacment pumps here) provide a fixed flow continuously, and a high pressure valve samples these flows to form sequences of slugs of eluents which are mixed as they pass through a fused silica tube. A valve-controller determines the composition of the gradient and a pump-controller determines the flow rate.

There are several advantage in the pulsed open tube generator approach: The timing valve can be relatively slow (from ca. 2 cycles per second) compared to that required for low pressure timing (sometimes 10-50 cycles per second). Also, the need for complex electronics and motors to continuously vary the flow of the pump is not required, as with high pressure gradient generation. Instead, each pump is set at the desired flow, and

the valve is programmed to select slugs of solvent to produce the desired gradient shapes or isocratic solvent compositions. Since high speed valves are not used, there is no need to link the valves activation to the pump piston motion. Indeed, with pulsed open tube gradient generation, the system can be built from off the shelf components.

These nano-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.

MATERIALS AND METHODS

The pulsed open tube gradient generator also uses an infusion pump and two syringes (as before) [7, 8], but several equipment changes permit gradient volume and gradient shape to be controlled. First, to obtain lower flows, the infusion pump was used with 1,000 microliter syringes (1001, Hamilton, Reno, NV) instead of the 10,000 uL syringes used previously. Second, for spreading of the interface into a gradient, a 0.100 millimeter i.d. fused silica open tube (2.8 meters long) was used instead of the 0.530 mm i.d. (180 cm long) open tube used previously. Third, the 12-port valve (EQ27, SN 4799, Valco, Houston, TX) was controlled by the timer of a Gilson robotic autosampler (401, Gilson, Middleton, WI; the minimum time is 0.01 minutes).

Figure 1C 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. The only syringes that worked correctly were smooth glass syringe barrels with Teflon-tipped pistons. With these 1,000 uL syringes and the possible infusion pump drive rates, flows from 0.056 to 500 uL/min could be obtained, vs. 0.56 to 5,000 uL/min obtained previously.

"On-tube" detection directly in the 0.100 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), modified as described by us elsewhere [7, 8, 13]. Usual settings were at 0.1 AUFS with a 10 mv recorder at 5 cm/min.

Operation to Produce Simple-Gradients

Figure 1C shows how gradients are produced. The weak eluent pumping-syringe (Fig. 1C, labeled "Pump-A") and the strong eluent syringe (Fig. 1C, labeled "Pump-B") were initially manually filled and all air bubbles removed. The acetonitrile contained 10% v/v acetone for 254 nm UV visualization of the gradient shape. The syringes were then inserted into the infusion pump

drive, and both syringes operated continuously. To make a simple-gradient run, the pump was operated until aqueous eluent filled the open tube, and then the run was begun by manually activating the electrical valve so that acetonitrile was abruptly switched to the inlet of the open tube (the valve position shown in Fig. 1C). Note that the flow from aqueous Pump-A continues but delivery is now diverted to the reservoir-A syringe. This single interface is spread as it passes through the open tube to make a "simple-gradient" like those described previously and shown in Figure 2. The gradient volumes are fixed by the flow rate and open tube dimensions.

Operation for Producing Extended-Gradients and "Tailoring" Gradient Shape

"Tailoring" a gradient shape can involve combinations of a number of possible changes at any point: flat plateaus can be added; gradient slope can be changed; non-linear concave or convex segments can be added; or even reverse gradients can be introduced. Various time programs can be used for generating tailored gradients.

With extended-gradients, the gradient volume is extended to much larger volumes than produced by the simple-gradient method, by having a timer activate a valve back and forth many times to select portions of eluent-A, then eluent-B, then eluent-A, then eluent-B, etc. for various proportions of time. Table 1 shows an

TABLE 1

Program to pulse a valve with an 8 segment gradient (0.08 min long) pulsed N-times per segment. In segment 1, the "*" gives a permanent record of the timing on the chart recording by having event contact 3 make a momentary contact shorting the detector signal to ground. If this is inserted in each segment, the ratio of the distances between the 3 marks as well as by the number of repeat marks provides a record on the recording of the number of segments (8), pulse number (N) per segment, and number of runs. The Gilson 401 output connections are: 8 to recorder case and Valco valve green wire; 23 to valve blue wire, and 1 to valve red wire.

```
ESTABLISHES THE GRADIENT VOLUME BY THE NUMBER OF SEGMENTS AS
WELL AS THE NUMBER OF REPEATS OF THE ENTIRE GRADIENT
                INPUT C5/1      ENTER "N", SEGMENT REPEATS
                A0=0
                INPUT A1/14     ENTER NUMBER REPEAT RUNS
                FOR A = 1/50
                A0 = A0 + 1
                IF A0 > A1
                HOME
GIVES AN INITIAL MARK AND ALARM SIGNAL 0.08 MIN LONG
                AUX 8/1   TURNS ON ALARM
                AUX 3/1   MARK CLOSED
                WAIT 8
                AUX 3/1   MARK OPEN
                AUX 8/0   TURNS OFF ALARM
                PRINT A0
GIVES GRAD. VOL. BY SEGMENT NUMBER & REPEATS OF ENTIRE GRAD.
    SEGMENT 1: FOR B2 = 1/C5
                AUX  0/2        VALVE: WATER TO ACETONITRILE
               *AUX  3/2        MARKS THE SEGMENT BEGINNING
                WAIT 1
                AUX  1/2        VALVE: ACETONITRILE TO WATER
               *AUX  3/2        MARKS THE SEGMENT MIDDLE
                WAIT 7
               *AUX 3/2         MARKS THE SEGMENT END
                NEXT B2
    SEGMENT 2   FOR B2 = 1/C5
                AUX  0/2        VALVE: WATER TO ACETONITRILE
                WAIT 2
                AUX  1/2        VALVE: ACETONITRILE TO WATER
                WAIT 6
                NEXT B2
SEGMENT 3 TO SEGMENT 7, SAME STRUCTURE AS SEGMENT 2,
    WITH WAIT TIMES CHANGED TO 3/5; 4/4, 5/3, 6/2, AND 7/2.
LAST SEGMENT:3 MIN ACETONITRILE, 3 MIN WATER THEN NEXT RUN
                AUX  0/2        VALVE: WATER TO ACETONITRILE
                WAIT 300        HOLD FOR 3 MINUTES
                AUX  1/2        VALVE: ACETONITRILE TO WATER
                WAIT 300        HOLD FOR 3 MINUTES
                NEXT A
```

8-segment gradient. A short gradient might directly chain together single pulses of these 8-segments (or fractions). Segment-1 (12.5% B) would be 0.07 min aqueous/0.01 min organic; segment-2 (25% B) would be 0.06 min aqueous/0.02 min organic; and so forth, all the way to segment-7 (87.5% B), that would be 0.01 min aqueus/0.07 min organic. Before being spread in the open tube to a smooth gradient, these pulses would appear as perfect steps, with the baseline rising to the full organic level and then falling to the pure aqueous level many times; the time it spends in the organic position continually increasing.

For extending the volume of the gradient, N, the number of repeats pulses of each segment, can be readily changed in the gradient in Table 1. For example, if N is entered as 2, a longer 1.28 min gradient is produced (8-sements X 0.08 min/seg X 2 repeat pulses). Each segment would repeat twice giving this time sequence: segment-1 (12.5% B) 0.07 min aqueous/0.01 min organic; segment-2 (a repeat of 12.5%) 0.07 min aqueous/0.01 min organic; segment-3 (25% B) 0.06 min aqueous/0.02 min organic; segment-4 (also 25% B) 0.06 min aqueous/0.02 min organic; etc.

Operation for Creating Isocratic Plateaus

Table 2 shows a program in which the ratio of the valve in the eluent-A position and in the eluent-B position can be changed to a fixed level to give isocratic plateaus at any percent, or for any duration. Flow is determined by the syringe speed and the

TABLE 2

Program to pulse a valve to insert in a simple-gradient plateaus with an aqueous time (B1) and an acetonitrile time (B2), the segments being repeated A1 times.

```
ESTABLISHES THE GRADIENT VOLUME BY THE NUMBER OF SEGMENTS AS WELL
AS THE NUMBER OF REPEATS OF THE ENTIRE GRADIENT

     A0 = 0
     INPUT B1/41     ENTER "TIME A", THE HUNDREDTHS OF
                     MINUTES IN AQUEOUS ELUENT
     INPUT B2/42     ENTER "TIME A", THE HUNDREDTHS OF
                     MINUTES IN AQUEOUS ELUENT
     INPUT A1/14     ENTER "N", THE NUMBER OF REPEATS OF
                     SEGMENT
     FOR A = 1/50
     A0 = A0 + 1
     IF A0 > A1
     HOME
     PRINT A0/14     PUTS ON THE DISPLAY THE CYCLE NUMBER

[GIVES AN INITIAL MARK AND ALARM SIGNAL 0.08 MIN LONG
     AUX 3/2         MARK CLOSED
     AUX 1/2         VALVE: ACETONITRILE TO AQUEOUS
     AUX 8/0         TURNS OFF ALARM
     WAIT B1         WAIT THE AQUEOUS TIME
     AUX 3/2         MARK OPEN
     AUX 0/2         VALVE AQUEOUS TO ACETONITRILE
     WAIT B2         WAIT THE ACETONITRILE TIME
     NEXT
```


gradient shape is determine by the valve timing. For example, for a plateau of 25% B (theoretical) that is 2.56 min long (8 segments X 0.08 min/seg X 4 repeat pulses), the valve is programmed for the following time sequence (all the times sequences are the same and repeated 4 times): segment-1, (25% B) 0.06 min aqueous/0.02 min organic; segment-2, (also 25% B) 0.06 min aqueous/0.02 min organic; etc.

Refilling the Pumping-syringe

The 1,000 uL pumping-syringes had to be refilled by manually pressurizing the reservoir-syringes, vs. previously where the pumping-syringes were refilled by suction. Also, stainless steel lines (40 cm X 0.03 inch i.d.) had to be used to replace the plastic lines (both Teflon and Tefzel) connecting the pumping-syringes to the valve. Plastic permitted too much air permeability through the tubing, so air bubbles appeared in the gradients (even though the eluents were vacuum degassed just before filling).

RESULTS AND DISCUSSION

Our previous publication on generating "simple-gradients" required switching only once from eluent-A to eluent-B [7, 8]. However, simple-gradients have certain disadvantages: (1) to change gradient volume it is necessary to change the flow between the "gradient generation time" of the run and the "use-time" of the run (when the gradient passes into an LC or CE column), and (2) the range of the gradient is limited as a function of the flow during the gradient generation time and the length and diameter of the open tube.

The new pulsed open tube extended gradient system described here overcomes these two problems of simple-gradients and involves several improvements: (1) gradients can be tailored to any shape; (2) a broad range of gradient volumes from 0.88 to 12

uL (over 25-fold) can be obtained by "pulsing" the valve; and (3) the flow during the "gradient generation time" can be the same (or different) from the flow for the "use-time" of the gradient.

Simple-Gradients of Nanoliter Volume

Figures 2 and 3 show a simple-gradient down to the high nanoliter volume (880 nL). With the simple-gradient approach, the valve is used only to switch one time from eluent-A to eluent-B.

Note in Figure 2 that volumes from 880 nanoliter to 5,440 nanoliter (0.88-5.44 uL) are produced when the gradient generation flow is changed from 0.67 to 33.89 uL/min (linear velocity of 0.86 to 43.2 cm/min). These gradients are S-shaped as shown in Figure 2. There is no reason to doubt that this principle of using lower flows and narrower open tubes can be used to make low nanoliter and picoliter size gradients in a dedicated LC or capillary electrophoresis system.

In Figure 2, 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. One anomaly of unknown origin is the small blip at the top of each gradient. Since 10% v/v acetone is added to the acetonitrile (eluent-B) for UV visualization, the blip may be due to the acetone being sorbed to

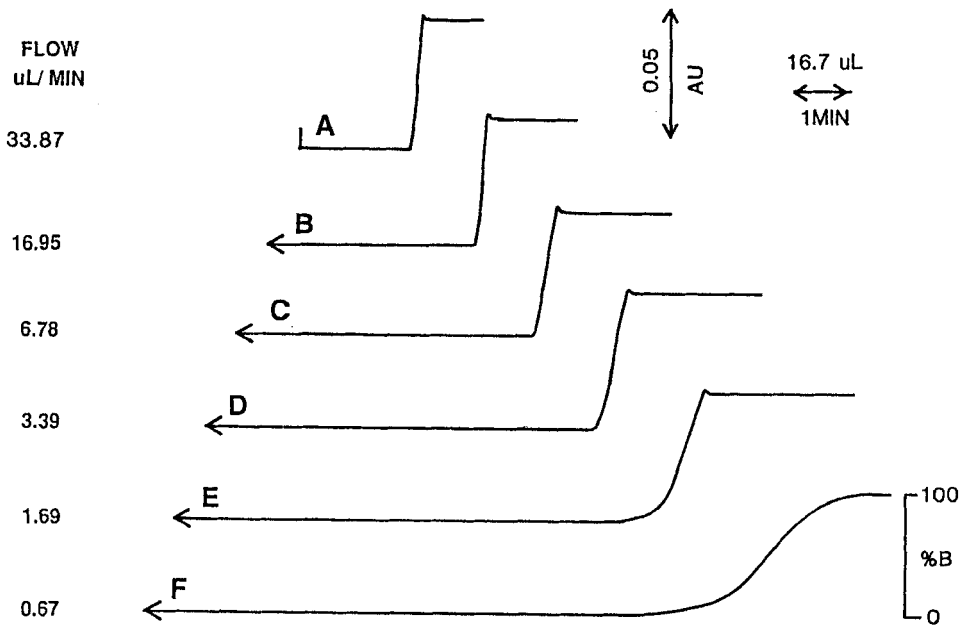

Figure 2. Symmetrical, linear "simple-gradients" produced by having the valve switch one-time from eluent-A to eluent-B, feeding the interface through a 0.100 mm X 280 centimeter open tube gradient generator operated at the flows shown to the left in the figure. The point at which the valve was activated is only shown for baseline A (33.87 uL/min); the other start positions are proportionately distant from the gradient and are too far to show on the figure. For each, the rise represents the baseline movement from water to acetonitrile with 10% v/v acetone to give UV detectable gradients. Chart speed was 5 cm/min.

the inside of the open tube at low %B and being displaced later at higher acetonitrile concentrations.

As before, with the volumes of these simple-gradient ranging from 0.88 to 5.44 microliters, and a open tube with a volume of 22 microliters, several forward and reverse gradients

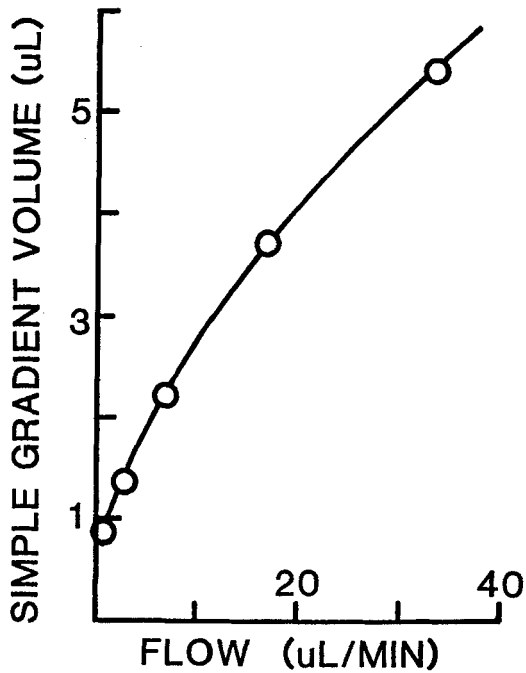

Figure 3. Simple-gradients to the nanoliter volume vs. flow. Other conditions as in Figure 2.

could be contained in the open tube gradient generator at any one time, without waiting for one to elute before starting the second.

Extended-Gradients Using Pulsing

Figures 4 and 5 show how pulsing can be used to greatly extend the volume of a gradient, while keeping the flow fixed. The timing program is shown to the right of Figure 5-A. Note that the simple-gradient (Figure 4-F) (produced by a single valve

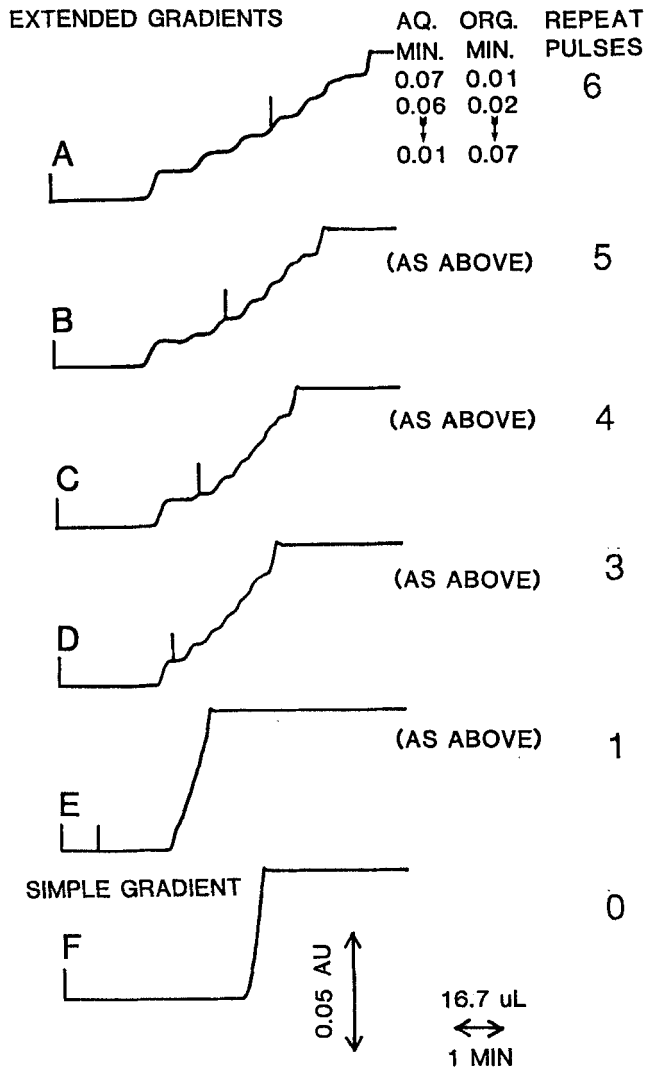

Figure 4. "Extended-gradients" produced by having the valve switch many times from eluent-A to eluent-B (different "repeat pulses"), and feeding the many interfaces into a open tube generator. The beginning and ending activations of the valve are shown by the vertical marks on the baselines. An 8-segment gradient used 0.08 min segments created by using different "Aqueous Minutes" and "Organic Minutes" for a "repeat pulses", as shown in the figure. Other condlitions as in Figure 2.

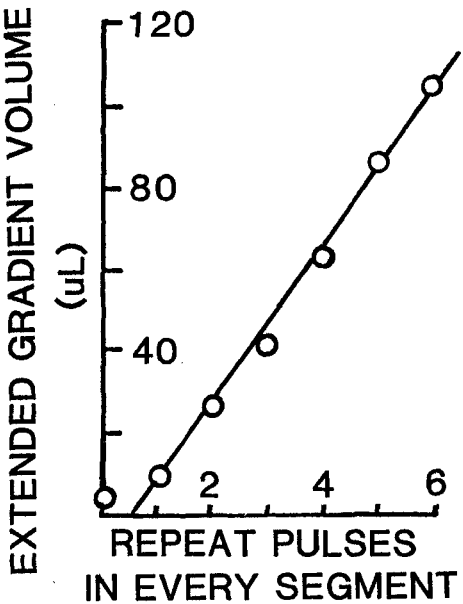

Figure 5. Linearity of the extended-gradient volume vs. the number of repeat pulses in every segment. Other conditions as in Figure 4.

activation from eluent-A to eluent-B) is much smaller (3.73 uL at 16.7 uL/min) than the gradient of ca. 10 uL produced even with a single pulse with an 0.64 min gradient (8-segment gradient X 0.08 min/segment X 1 pulse) (Figure 4E). The nominal gradient times vary from 12.8 min for the 20-pulse case down to 0.64 min for the 1-pulse case (Figure 4A down to 4E). Figure 5 shows that the extended gradient volumes decrease from ca. 110,000 nL down to ca. 10,000 nL (110-10 uL). A disadvantage of this extended gradient method is that the shape is not perfectly linear with an 8-segment gradient, in that small ripples are produced on the baseline, but these are highly repeatable.

Extended-Gradients with Increased Flow and More Repeat Pulses

A larger volume gradient can be seen in baselines with more segments since the gradient time is increased e.g. the 8-segment gradient is 0.64 minutes long (8-segments X 0.08 min/seg X 1 repeat pulse) (Fig. 4E) and the 12-segment gradient is 1.44 min long (12-segments X 0.12 min/seg X 1 repeat pulse) (Fig. 6B).

Increasing the flow rate by ca. 5-fold (from 6.7 to 32 uL/min), while keeping the number of repeat pulses constant, increases the volume of a gradient but increases the ripple in the baseline (e.g. Figure 6, compare baselines B, D and F or C, E, and G.).

At high flows, increasing the number of repeat pulses of each segments (i.e. from 1 to 2 to 6 pulses; Fig. 6, compare F vs. G. vs. H) does not importantly change the MAGNITUDE of the ripple on each segment. However, with more segments, the ripples are more apparent (e.g. compare G at 2-pulses to H at 6-pulses).

Extended-Gradients By Increasing Segment-Time

The effect of three gradient programs that increase the time per segment by 10-fold (from 0.16 to 1.60 min/segment) is shown in Figures 7-B to 7-D). The pulsing times are shown to the right of each. These programs use an 8-segment gradient with only one repeat of each pulse. Note that segment-times of 0.16 min/segment gives a smooth gradient, but segments twice as long (0.32 min/segment, Figure 8-C) gives very noticeable baseline ripple.

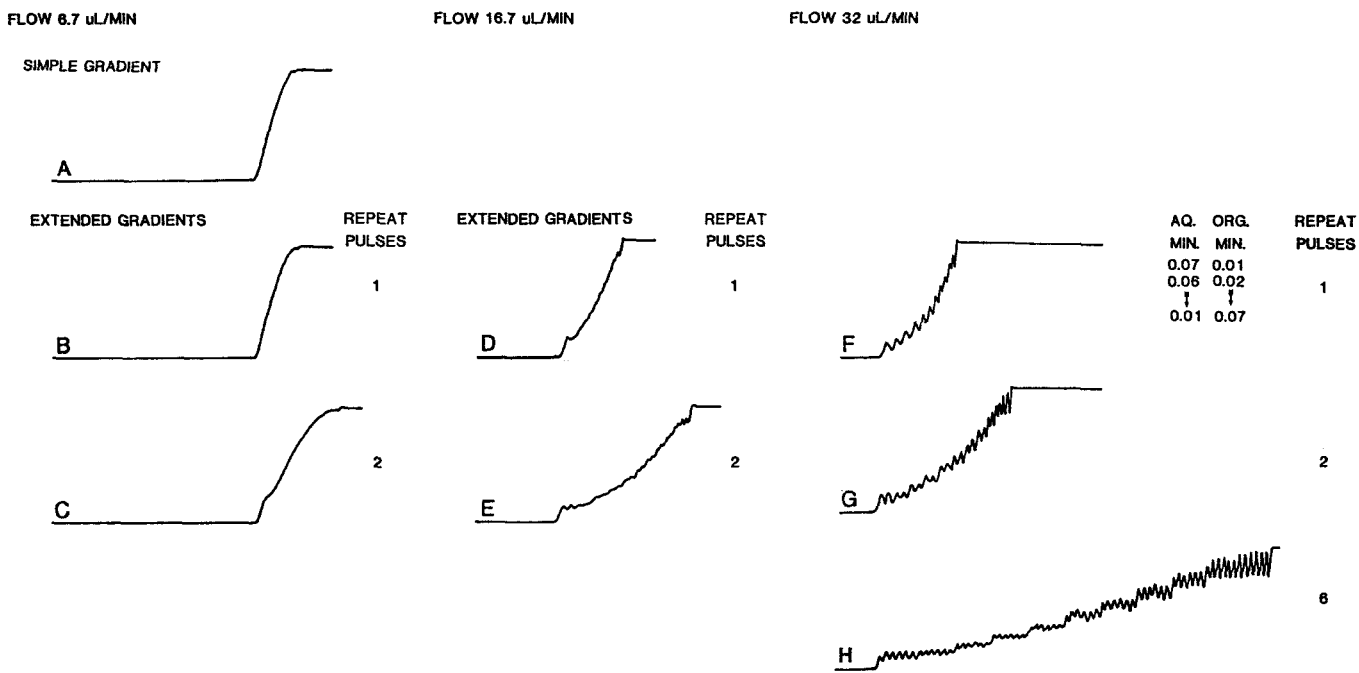

Figure 6. Effect of increasing flows (from left to right) and pulse numbers (top to bottom) on extended-gradient volumes and gradient shapes. A 12-segment gradient is used with a 1,000 uL syringe (60 mm barrel length) run at 0.4 mm/min (6.6 uL/min) (left), 1 mm per minute (16.6 uL/min) (center), and 2 mm/min (32 uL/min) (right). Other conditions as in Fig. 2.

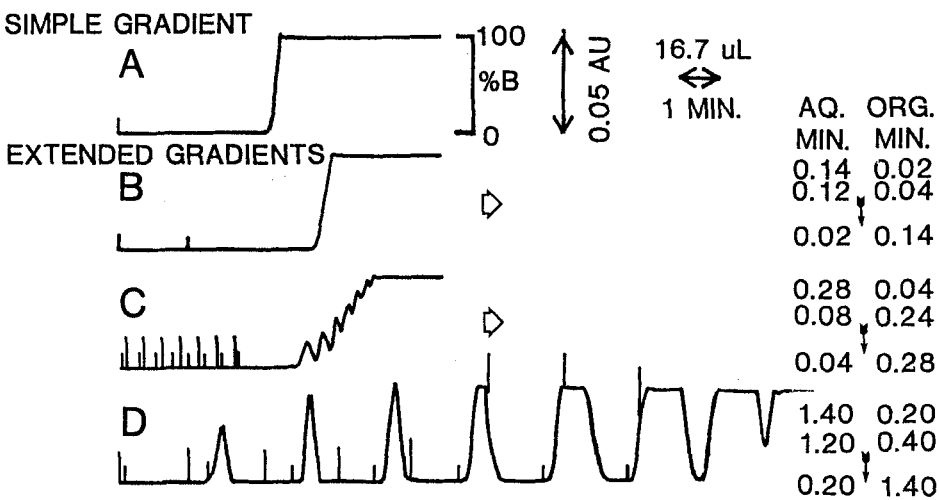

Figure 7. The effect of increasing the segment-time on creating extended-gradients (always 12.5% per segment with each of the 8-segments repeated only one pulse). Segment timing for all runs are shown to the right. A: Simple-gradient control run of a single valve activation from eluent-A to eluent-B. B: Extended-gradient of 0.16 min segment-time, created by 0.14 min aqueous to 0.02 min organic (1-pulse); then 0.12 min aqueous to 0.04 min organic (1-pulses), etc. C: Extended-gradient of 0.32 min segment-time created by 0.28 min aqueous to 0.04 min organic (1-pulses), etc. D: Extended-gradient of 1.60 min segment (1.40 min aqueous to 0.20 min organic (1-pulses) then 1.20 min aqueous to 0.40 min organic (1-pulses), etc. In C and D, the tall marks on the baseline are the valve activating from acetonitrile to water, and the short marks are the valve activating from water to acetonitrile; (these marks are too close together to show in B, where only the first and last marks are shown). Flow is 16.7 uL/min. Other conditions as in Figure 2.

Figure 7-D shows that with very long segment-times (1.6 min/segment), the transition from elution chromatography to frontal chromatography becomes apparent. Initial short "injections" of eluent-B (i.e. 0.2 min organic) gives an elution chromatography type peak of the strong eluent. However, later, long "injections" (i.e. 1.40 min organic segments) give a frontal chromatography (breakthrough) type baseline.

Changing Baseline Ripples

For a given set of eluents (i.e. of fixed viscosity, miscibility, etc.) and given open tube generator, combining the previous discussions, baseline ripples can be reduced by using shorter segment times (below 0.32 min/segment) (Fig. 7) or lower flows (below ca. 32 uL/min) (Fig. 6). Increasing the open tube capillary length would also decrease baseline ripple by increasing the mixing of zones. Also more viscous eluents or eluenst whose mixtures are more viscous would also decrease the baseline ripple.

The ability to produce ripples on the baseline at high flows may prove useful for the "pulsing-optimization" we described some years ago [14]. Pulsing-optimization (1) permits testing a peak for purity; (2) allows separation of un-resolved peaks; and (3) may permit precise tailoring of a gradient to produce any desired resolution between every peak pair. Open tube gradient generation described here may be a means to conveniently and reproducibly generate these weak eluent pulses for pulsing-optimization.

Extended-Gradient Reproducibility

Figure 8 shows typical gradient reproducibility found with a open tube gradient generation, in which 32.06 uL gradients varied by 2.75% average deviation. Reproducible results were only found if absolutely all air bubbles and fluid leaks were removed from the high pressure part of the system.

Simple-Gradients Tailored by Inserting Plateaus

Simple-gradients (Figure 9A), in which the valve is abruptly switched from eluent-A to eluent-B, can be modified by having single or multiple plateaus of constant composition inserted at nearly any %B level. Figures 9B to 9H show plateaus of a "theoretical % organic" from 87.5 %B down to 12.5 %B (in 12.5% segments) as described in Table 2. Figure 9J shows two plateaus inserted in a simple gradient.

The "actual % organic" (%B) is determined as 100 X (the plateau height in cm)/(total baseline shift in cm). The "theoretical % organic" is simply the percent of time the valve samples the organic eluent (e.g. 0.07 min in eluent-A and 0.01 min in eluent-B gives a 12.5% B theoretical % organic composition). A problem with the current system is that the theoretical % organic does not match the actual % organic (Figure 10). The difference is linear with the actual %B being 27.5 %B higher when set at 12.5 %B theoretical, but showing nearly no deviation at 87.5 %B theoretical.

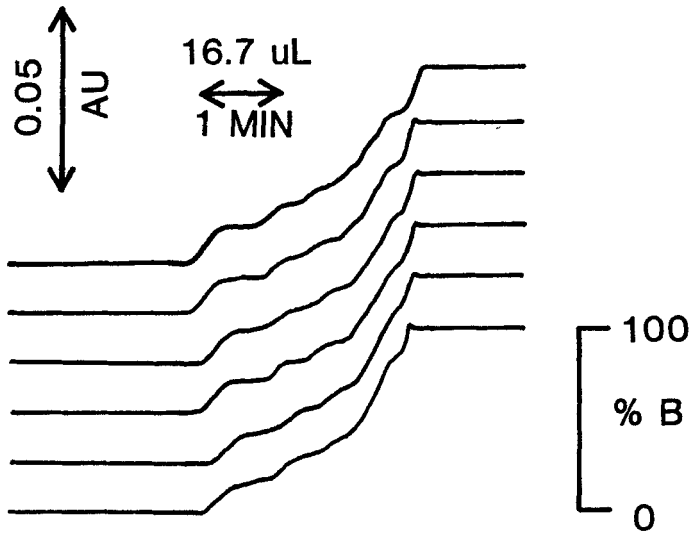

Figure 8. Typical reproducibility of repeat extended-gradients (an 8-segment gradient of 12.5 %B steps), repeating each segment three times each, "3-pulses". Flow is 16.7 uL/min with other conditions as in Figure 2. by a

This lowest actual % organic is reflected in extended-gradients (1) having a steep onset and (2) having deviations from perfect linearity. One method that improves gradient linearity is to increase the number of segments in a gradient. A12-segment gradient (0.11 min aqueous and 0.01 min organic) was found to give a 21.7% actual % organic plateau. The theoretical % organic is 8.33 % organic; considerably below the 40 actual % found with the 8-segment gradient. Gradient linearity might also be improved by making the back pressure in the syringes constant, independent of valve position, by using the same narrow 0.100 mm

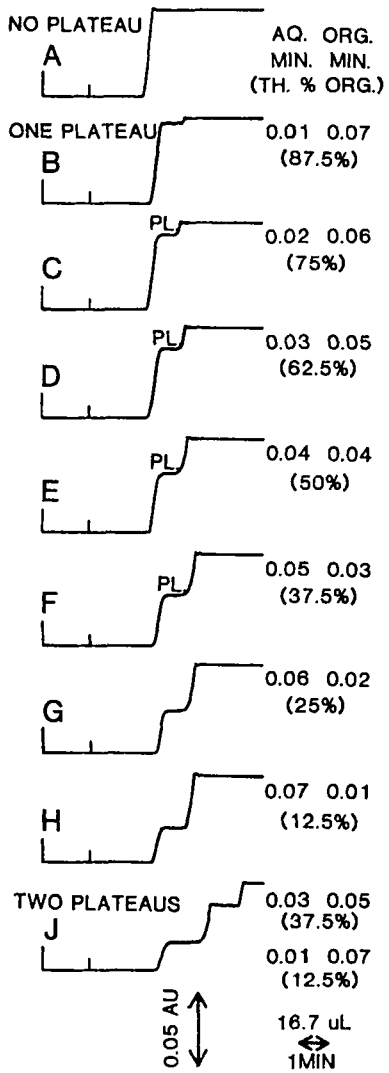

Figure 9. Simple-gradient (A) tailored by inserting plateaus ("PL.")(B to J). Plateaus at any of 8 "theoretical % organics" levels (in 12.5% segments) uses 0.08 min segments at 10 repeat pulses each. For example, baseline B is created with 0.01 min eluent-A followed by 0.07 min eluent-B, and this sequence is repeated 10 times (10-pulses). "I" shows a double plateau created by Aqueous Min/Organic Min of 0.01/0.07 followed immediately by 0.03/0.05. On each baseline, the 10-cycles activate between the tall initial mark and the shorter mark on the baseline (individual valve-activation marks are too close together to show). Flow is 16.7 uL/min with other conditions as in Figure 2.

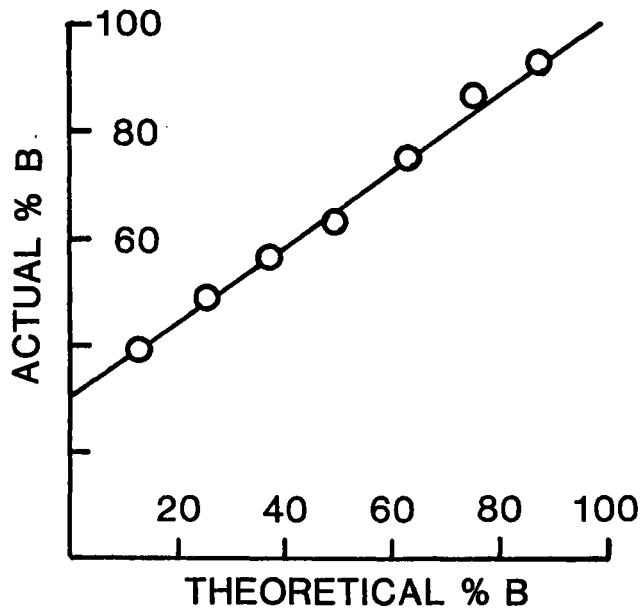

Figure 10. Actual percent organic (%B) vs. theoretical percent organic for a simple-gradient modified by inserting plateaus, as shown in Figure 9.

i.d. 2.8 meter long open tubes for the two "return lines" connecting the valves to the two reservoir-syringes (Figure 1C) as is used for the gradient generation.

The deviation between the theoretical and actual % organic may be: (1) solvent compressiblity or compliance differences between the two syringes [12]; volume contractions of the mixed solvents; or some constant error in the valve timing. Differences in the precise dimensions of the syringes is not a cause since the same deviations were found when the solvents in each syringe were switched.

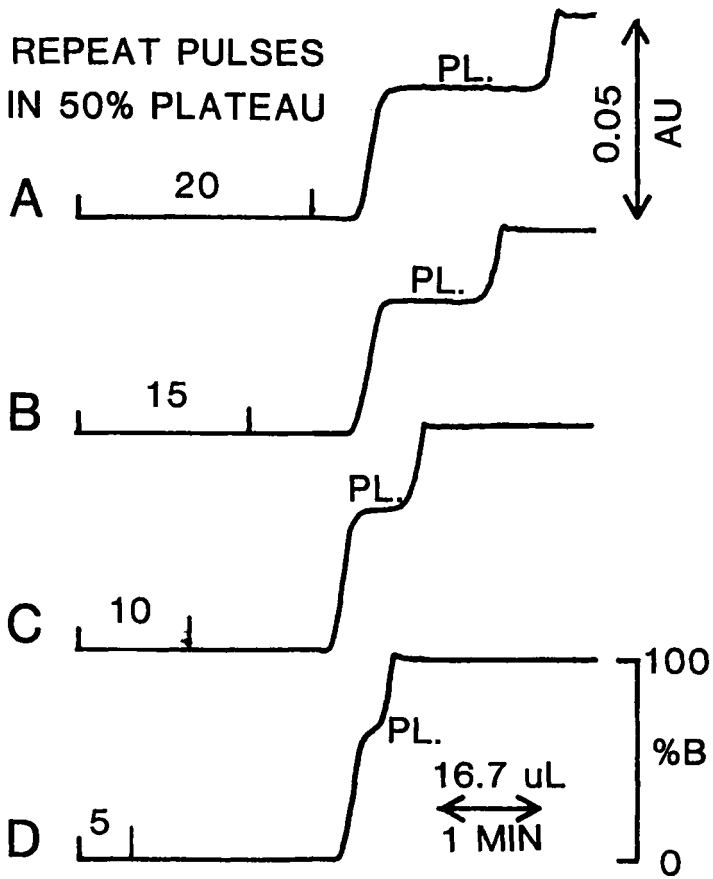

Figure 11. Increasing the plateau volume ("PL." by increasing the number of repeat pulses at the 50% composition segment, from 5 repeats (D) up to 20 repeats (A). The beginning and ending activations of the valve are shown by the vertical marks on the baselines. Other conditions as in Figure 2.

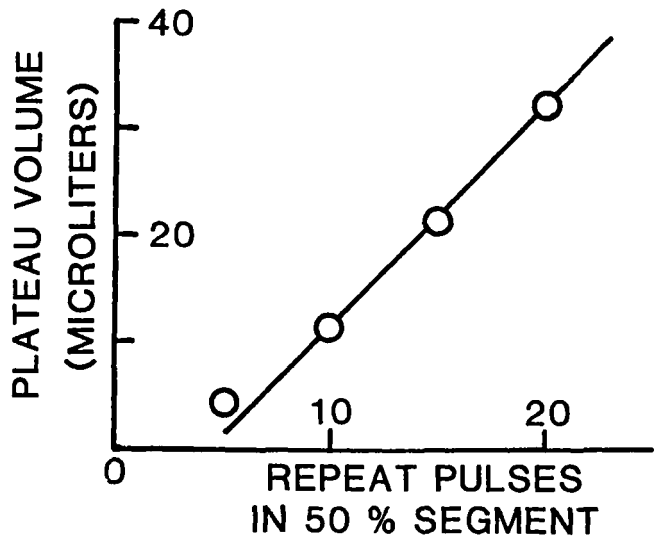

Figure 12. Linearity of the plateau volume vs. the number of repeat pulses in the 50% segment composition ratio. Data taken from Figure 11. Other conditions as in Figure 2.

Figure 11 and 12 show that increasing the repeat pulses directly increases the plateau volume in direct proportion, as expected. The deviation in the 5 repeat-pulse figure is due to the difficulty in measuring the small gradient volume in Figure 11D.

Sample Injection with Small Gradients

Sample injection with small gradients can introduce important mixing problems, such as gradient volume changes, gradient shape changes, and gradient delay. Instead of conventional injection, samples might best be loaded by "weak-

eluent-sample-loading", such as described by Berry previously [3, 4]. With this method, with conventional size columns (4.6 X 100 mm), very dilute sample is dissolved in the weak eluent, peaks are accumulated (focussed) on the column, and finally the peaks are eluted by the gradient. The system was automated with a robotic autosampler used only to place the weak eluent inlet tube to pump A into different 23 mL vials of weak eluent (each containing a different dissolved sample). Future work will bring these novel approaches to injection and gradient generation together in new low cost liquid chromatography (LC-LC) methods.

CONCLUSIONS

The pulsed open-tube gradient generation approach uses a valve to switch between the flow from two syringes with a 0.100 mm open tube to spread the interface into an S-shaped gradient. With a simple-gradient approach, as shown by us previously, a single activation of the valve from eluent-A to eluent-B produces a single eluent interface. This interface produces gradients down to the high nanoliter range (880 nL). As before, this gradient volume can be changed an order of magnitude by varying the flow during the gradient generation time. More importantly, by having the valve switch back and forth between the two syringes, "pulsing" is achieved, and extended-gradients can be made in the 1-100 uL range, without having to vary the flow. In addition, one or more plateaus can be inserted in a gradient at

any concentration, and the length of these plateaus can be extended to any volume, permitting "tailoring" the shape of microliter and nanoliter gradients. Tubes more narrow than the 0.100 mm i.d. open tubes used here should permit low nanoliter and picoliter gradients to be produced.

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