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UNIVERSAL LIQUID CHROMATOGRAPHY METHODS

V. "PULSING" WITH WEAK ELUENT TO RESOLVE PEAKS*

VERN V. BERRY
Chemistry Department, Salem State College, Salem, MA 01970 (U.S.A.)
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SUMMARY

With liquid chromatography "pulsing" is a new approach for resolving peaks and resolution optimization with fluorescence detection. A slug of an additive (*e.g.* 500 μl of weak eluent) is injected into the gradient stream while a run is in progress. Pulsing injections are timed so that pulsing agent arrives at the unresolved-peaks to slow abruptly the gradient, resulting in improved resolution. Pulsing leaves in place the separated peaks and focuses separation efforts only on unresolved-peaks.

With pulsing, only partial resolution of all peaks need be achieved by using simple gradients or high plate counts. It is only necessary to provide space in the chromatogram for ultimately moving the unresolved-peaks. Pulsing with the proper volume and time intervals is used to adjust the resolution between each set of adjacent unresolved-peaks.

A chromatogram with a single properly timed pulsing is identical to the unpulsed chromatogram except that an unseparated peak pair is split into two resolved peaks. Thus pulsing has a minimum affect on adjacent peaks so the movement and identities of peaks can be readily followed. Since pulsing does not split pure peaks, pulsing can be used to test each single peak to determine if it is a single component.

INTRODUCTION

The needs exist to extend the advantages of modern liquid chromatography (LC) to ever more complex biochemical mixtures (> 50 peaks) and to obtain the costs savings found with optimized analyses (optimized to achieve sufficient separation in a minumum time). Separations of complex mixtures classically has been approached by using optimization routines that change such variables as pH, temperature, eluent composition, etc., (reviewed in ref. 1) or by using columns with more separating capacity (available plates). However, Davis and Giddings recently pointed out that

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achieving full resolution of very complex mixtures by using more plates alone is limited[2].

Pulsing involves injecting a large plug (200–500 μl) of an additive (weak eluent, here) during the run so as to slow locally the gradient near poorly resolved peaks and improve resolution. Francois *et al.*[3] showed with their steroid analyzer (a liquid–liquid system) that gradients of decreasing elution strength could be used to improve separation between poorly resolved peak pairs and tailor the separation.

Pulsing is "injection loading" of weak eluent at various times during a run. This, like our previous method of modifying resolution by injection loading of ion pair agents before a run[4–6] allows easy modification of selectivity with universal LC methods. Universal LC methods typically use full gradients (to elute most components) and low wavelength 190 nm[7] or 210 nm[8] UV detection (to detect most substances). Universal LC methods can speed problem solving since they often (1) allow on-line "first-run-problem-solving", (2) reduce or eliminate method development time and costs for each new problem, and (3) allow different separation problems to be run un-attended with no system changes (see review, ref. 9). Pulsing provides a simple new approach to improving resolution of mixtures that fail to be resolved on a first universal LC run.

This paper will demonstrate the effect of pulsing with fluorescence detection for improving resolution and will show equipment for achieving pulsing.

EXPERIMENTAL

Apparatus

Fig. 1 shows the schematic apparatus for pulsing weak eluent into the flow stream. The arrow-line shows the usual flow stream of eluent, and the dashed-line shows the flow stream for automated valve pulsing of weak eluent. For automated valve pulsing, the Hewlett-Packard (Avondale, PA, U.S.A.) syringe-type injector (No. 79841A) was preceded with a 10-port valve (only 8 ports are shown) (No. EQC 104, Valco, Houston, TX, U.S.A.). The contacts were controlled by the "external events" contacts supplied with the instrument. In the valve, the eluent passes through one or the other of two 500-μl pulsing loops (39.5 cm × 0.050 in. I.D. stainless-steel tubing) and then on to the injector, column, and detector.

For pulsing weak eluent, a "loop refill pump" (No. 6000, Waters Assoc., Milford, MA, U.S.A.) continuously recycles eluent from the A reservoir through one or the other of the 500-μl pulsing loops. (A less expensive low pressure pump could be used as the loop refill pump.)

Since multiple pulsing as fast as 0.1 min apart are sometimes used, the loop refill pump run at 9 ml/min is fast enough to displace the 0.5 ml of gradient solution in the loop back to a 4-l reservoir A so as to be ready for a second pulse. The valve substitutes a slug of pulsing solvent for a small portion of the gradient and causes only a minor pressure pulse.

The regular chromatography flow stream (arrow-line, Fig. 1) was maintained in the "highly protected LC" mode[10]. The detector was a Waters 420 AC fluorescence detector equipped with a 360 nm excitation filter and 450 emission filter using a 1F4T5BL (No. 78245) lamp with the 1-V output to the Hewlett-Packard liquid chromatograph on the No. 1 sensitivity setting and attenuation 2 ex 7.

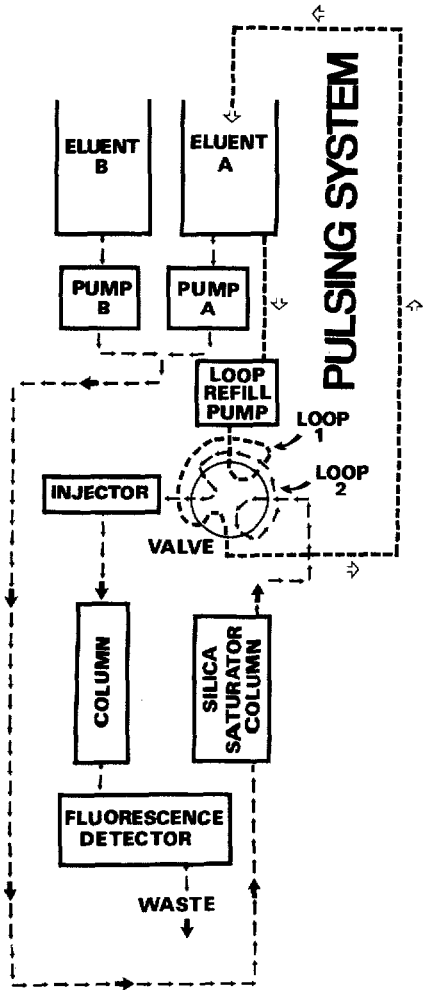


Fig. 1. Schematic diagram of the instrument components for pulsing weak eluent (aqueous eluent A). The dashed-line shows the flow of the pulsing agent and the arrow-line shows the flow of the LC eluent. The Valco valve with two 500-μl loops for pulsing precedes the syringe-type injector in the Hewlett-Packard 1084 liquid chromatograph. Loop 1 and loop 2 are used alternately for pulsing in weak eluent under the control of the event-timer of the liquid chromatograph.

Materials

Columns and run conditions are given in the figure captions. Low-wavelength-compatible triethylamine (16 m*M*) phosphate pH 4.3 and solvents are as described previously[8].

Sample preparation and dansylation used the Bongiovanni method[11] with sample preparation being made directly in the crimp-seal 2-ml autosampler vials (No. 12894, Pierce Chemical, Rockford, IL, U.S.A.). Sample (0.5 ml) was mixed with 0.2 ml of 0.5 *M* sodium carbonate (pH 11) and 0.2 ml of 3 mg/ml Dns chloride (No. 21751, Pierce Chemical) was carefully layered on top and the vials sealed. The re-

action was started by mixing the vials and setting them for 10 min in a boiling water bath with subdued lighting. No quenching of un-reacted Dns chloride with amine was used. Amino acid mixtures were made from the 27 acidic and neutral (No. 20086, Pierce) and 12 basic (No. 20087, Pierce) amino acids, supplemented with the amines analyzed by Seiler *et al.*[12] including putrescine, cadaverine, histamine, spermidine, spermine, ethanolamine, carnosine, creatinine, tryptamine, and the additional amino acids cysteine, cysteic acid, homocystine, homoserine, cystathionine, norleucine, norvaline, aminocaproic acid, methionine sulfoxide, and isovaleric acid. All were generally diluted to *ca.* 0.1 μmoles/ml and dansylated as above.

Injection loading of 15–25 μl of the ion pairing agent sodium octanesulfonate (1 *M*) was used in some experiments to improve the resolution in the front part of the chromatogram[4,6].

Pulsing methods

Manual pulsing was used in the first exploratory efforts with pulsing. Weak eluent was manually injected using the Hewlett-Packard 1084 syringe-type injector itself (here called the "syringe"). After sample was injected with the syringe set at 25 μl and the run was in progress, the injector was manually set to its maximum volume of 200 μl (slowly so as not to disturb flow). By manually operating the pneumatic valves with a screwdriver[13], the syringe was filled with 200 μl of water and prepared to inject. At the exact moment during the run when pulsing was to be applied, the bypass solenoid was manually operated to inject the 200 μl of water into the flow stream.

For automated pulsing with the Valco valve (Fig. 1), the program shown in Table I was used. This was part of a complex set of 6 pulsing in a single run.

RESULTS AND DISCUSSION

Manual pulsing with water

Pulsing was first investigated with a manual approach by hand-manipulating the syringe-type injector as described above. Precisely when the retention time of the dansyl acid peak was printed-out (7.71 min), the bypass valve was activated for pulsing in the water. The shaded peak in the un-pulsed run (Fig. 2, chromatogram A and replicate A′) was split by the pulsing into two (cross-hatched) peaks (Fig. 2, chromatogram B and replicate B′).

The timing of the pulsing is indicated by means of a bracket under the chromatogram. The left vertical arm of the bracket below chromatogram B in Fig. 2 shows the time when the pulse was inserted, and the right vertical arm shows the approximate arrival time when the pulse affects the peak. The extreme and abrupt affect of pulsing on a gradient is suggested by showing a notch (width exaggerated) in the gradient profiles at approximately the elution position of a pulse. These gradient-profile are shown above each chromatogram in Fig. 2 and 3.

The vertical lines a–d in Fig. 2 connecting chromatograms A′ and B, C and D, etc. highlight specific resolution changes as each set of peaks is tested with pulsing, and are called the resolution-change-lines. The large single peak at 10.98 min in chromatograms A and A′ (shaded) is seen to split into two fully resolved peaks after pulsing, as shown by the (shaded) peaks in chromatograms B and B′. This change

TABLE I

EXAMPLE OF THE "EXTERNAL CONTACT" (EXT CONT) IN THE "TIME PROGRAM" FOR AUTOMATED VALVE PULSING WITH THE HEWLETT-PACKARD 1084 LIQUID CHROMATOGRAPH

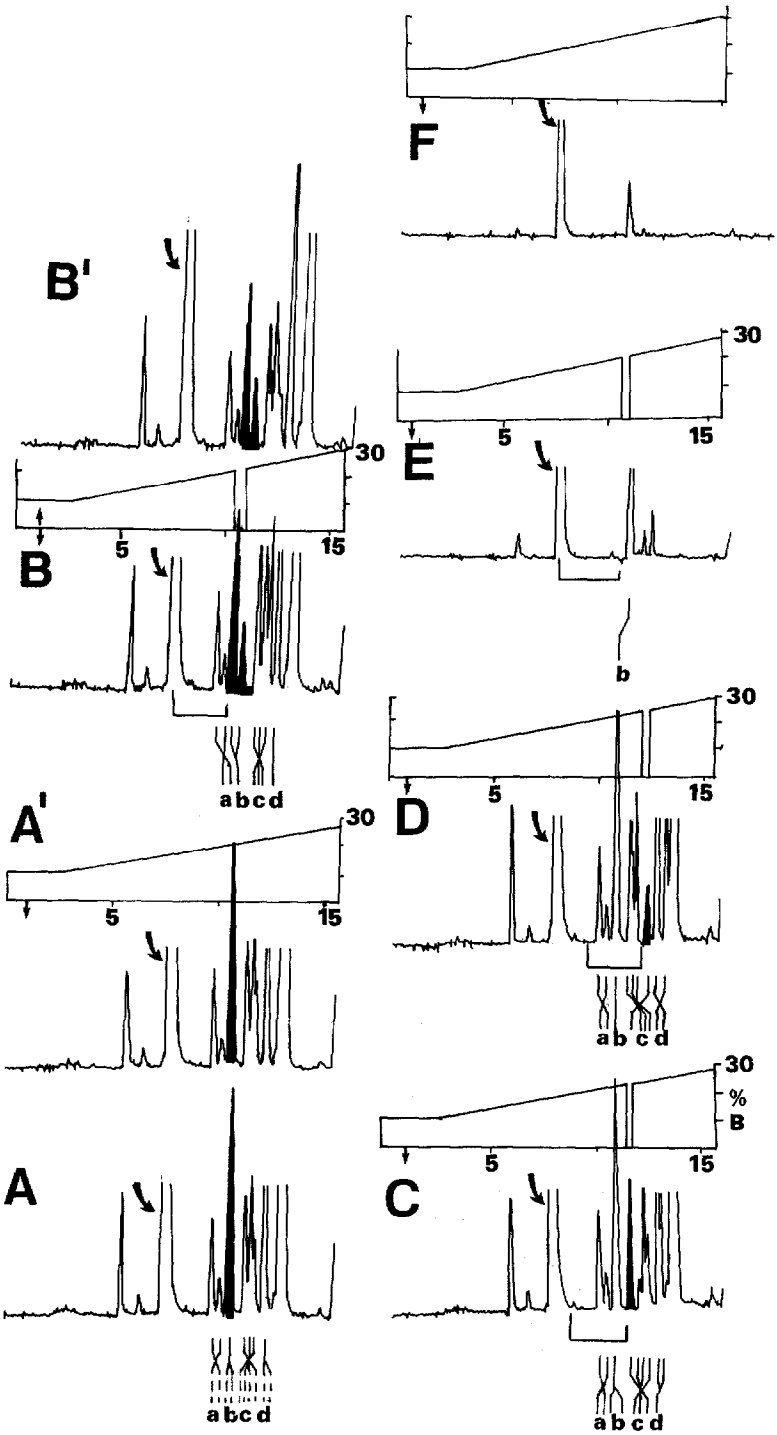
The 19.11 EXT CONT signal that closes the contact and activates the valve is opened 0.01 min later by the 19.12 EXT CONT (−) (minus) command. The 19.11 and 19.22 commands separated by 0.1 min produce a "double pulse".

```
MESSAGE 26   2   1
FLOW          3.00    0.00
%B           12.0     0.0
COLUMN P            237
MAX P       400
MIN P         0
OVEN TEMP   75       26
EXT SGNL
WAVL S:R   254 :      0

CHT SPD       0.50
ZERO         15.0
ATTN 2↑       7
AREA REJ           1000
SLP SENS      1.00

10.00   FLOW          3.00
11.00   FLOW          2.00
12.00   %B           12.0
19.11   EXT CONT      5
19.12   EXT CONT      5
19.21   EXT CONT      4
19.22   EXT CONT      4
22.54   EXT CONT      5
22.55   EXT CONT      5
22.64   EXT CONT      4
22.65   EXT CONT      4
24.00   %B           18.0
31.76   EXT CONT      5
31.77   EXT CONT      5
39.16   EXT CONT      4
39.17   EXT CONT      4
44.00   %B           40.0
52.00   %B           75.0
52.00   STOP
```

in peak number is highlighted by the resolution-change-line b between chromatogram A′ and B. For another example, of the set of four unresolved peaks in chromatogram B′ (peak set c), the first peak is resolved out in chromatogram C (shaded peak). Simultaneously this pulse resolves peak set d, a single peak in chromatogram B′, into two peaks in chromatogram C. With pulsing at a later time, the last (shaded) peak in peak set "c" in chromatogram D, is resolved out.

The dotted resolution-change-lines under chromatogram A in Fig. 2 (and under chromatogram A in Fig. 3) summarizes the composite results found from checking all pulsing experiments on a given sample. Thus, the single peak at b can be resolved into two peaks; the triple peak at c can be resolved into at least four peaks, etc.

The time between inserting a pulse and observing the effect should be close to


F
B'
30
5
15
E
B
b
a b c d
30
5
15
A'
30
5
15
D
a b c d
30
%
B
5
15
A
C
a b c d
a b c d

the elution time for an unretained component, or the void time (t_0). With the automated system described next, an additional time increment equal to the hold-up volume of *ca.* 300 μl between the pulsing valve and the column injector must be taken into account. The elution time for an unretained peak is estimated to be 2.67 min (8.01 ml) for the column (91 × 0.46 cm) and flow (3 ml/min) used here. Fig. 4 shows direct observation of this pulse arrival time and shape (plug flow) by poulsing in weak eluent containing acetone for UV detection. Arrival times estimated in this way show that the hold-up times varied from 2.68 to 2.87 min (8.04–8.61 ml) for the 91-cm long column. The assumpotion that the acetone behaves like the components in the weak eluent may be incorrect and more direct detection methods might be better.

The most useful method to estimate the arrival time of a pulse is to follow the effect of the pulse on the retention and resolution of peaks. The pulse-arrival-time as determined by the effect of the pulse ranged from 4.8 to 6.2 min.

With manual pulsing, reproducibility was found acceptable only when the print-out of some peak on the chromatogram was used as a marker to signal the pulsing time. This minimizes run-to-run variations and provides a precise point for pulsing. However, a marker peak will usually not appear at the pulsing time required to separate a particular set of unresolved-peaks. The automated valve method, described next, proved to be more reproducible (to 0.02 min) and a much less laborious method for pulsing.

Automated valve pulsing with weak eluent

The chromatograms in Fig. 3 show the exploration of various pulsing intervals, numbers of pulses, and locations of pulses (*i.e.* early or late in the gradient) to achieve separation of the amino acid–amine mix.

To use pulsing, the chromatogram is examined for "suspect-peaks", *i.e.* evidence of un-resolved peaks. Evidence can range from the obvious (*e.g.* slightly overlapping peaks, or peaks with clear inflection points), to the obscure (*e.g.* single peaks abnormally broad vs. neighbor peaks, or sharp single peaks in which a slope is seen in the ratio from the signals from two different detectors). Nine sets of suspect-peaks

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Fig. 2. Manual pulsing with the Hewlett-Packard syringe-type injector of 200 μl of water to affect resolution of dansyl amino acids. The outlet gradient profile (between chromatograms A and B, C and D, etc.) has notches to show the arrival of pulses. The left side of the brackets under the chromatograms show insertion times and the right side of the brackets shows arrival times of pulses. The vertical resolution-change-lines between chromatograms (*e.g.* between A′ and B, B′ and C, C and D, etc.) show specific resolution changes *e.g.* pulsing peak set b in run B resolves into two peaks (shaded) what was a single peak in A′ (shaded). Reproducibility is shown by replicates A and A′, B and B′. The final peak content, as revealed by pulsing, is shown by the dotted resolution-change-lines under chromatogram A. Run E shows that the single component, homoserine is not split by pulsing compared to the unpulsed run (run F). Pulsing was achieved by manually operating the Hewlett-Packard syringe-type injector to inject 200 μl of water precisely at the indicated times as explained in the text. A C_8 10 μm d_p Aquapore RP-300 Brownlee 3-cm guard column and 44-cm column (all 0.46 cm I.D.) was used with a gradient from 10% acetonitrile (at time zero) to 28% acetonitrile (at 14 min) in triethylamine (16 m*M*) phosphate buffer, pH 4.5. A flow of 2 ml/min was used with a column temperature of 50°C and silica saturator columns as described under experimental. Sample was 25 μl of the 60 component Dns-amino acid and amine mix. The arrow indicates the Dns-acid peak. Peak set a is an unknown and phosphoserine, set b is an unknown and homocysteine, set c is a phosphoserine component, arginine and two unknowns, and peak set d is homoserine and an unknown.

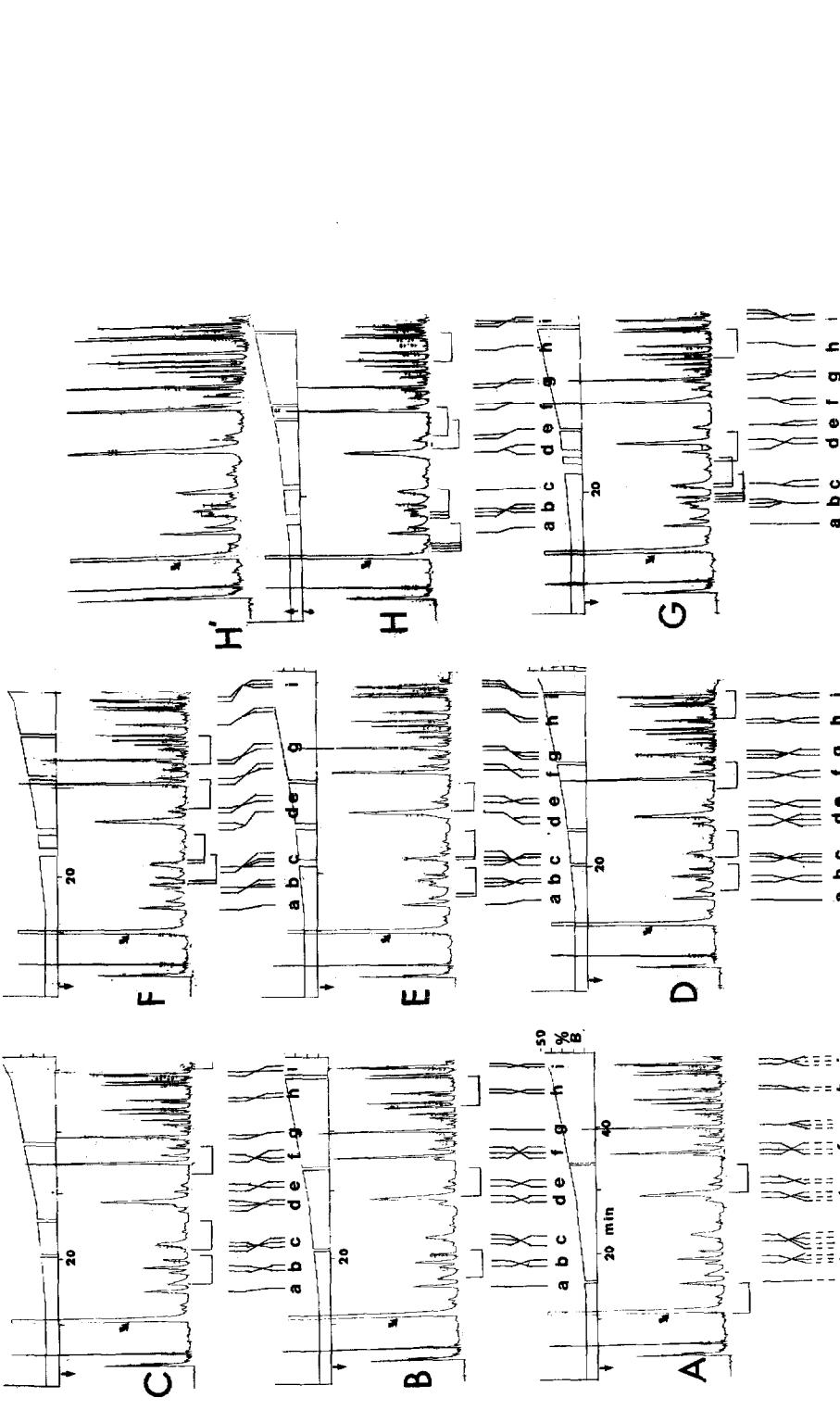

Fig. 3. Automated pulsing with the Valco valve (as in Fig. 1) of 500 μl of weak eluent A (triethylamine 16 m*M*, phosphate buffer pH 4.5) to affect resolution of Dns-amino acids–amines. The dotted resolution-change-lines under chromatogram A show the final peak content to be 24 peaks in 9 partially resolved peak sets (a–i) containing initially 17 partially resolved peaks. Eight chromatography runs (A to H) and the 28 pulsing experiments were used. Reproducibility of automated valve pulsing is good (compare H to replicate H' at 2-fold higher sensitivity). The flow was 3 ml/min until 10 min, and then 2 ml/min with an 91-cm long column at 75°C with silica saturator columns. Injection loading of 15 μl of 1 *M* sodium octanesulfonate was made immediately before sample injection of 15 μl of the Dns-amino acid-amine sample. The gradient program was isocratic at 12% acetonitrile for 12 min, to 44% (at 40 min), and then to 75% at 52 min.

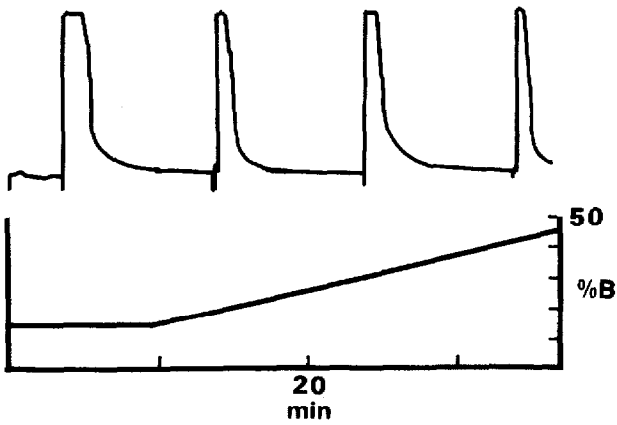

Fig. 4. Pulsing profile of a 500-μl from the Valco valve preceding the syringe-type injector of the Hewlett-Packard liquid chromatograph at various times during a gradient from 12 to 50% acetonitrile in aqueous eluent. The pulsing agent is 10% acetone in water detected by 254 nm UV (0.64 a.u.f.s.). Other conditions as in Fig. 3.

(peak sets a–i) were examined in the 9 chromatograms in Fig. 3 with 28 different pulsing experiments. The dotted resolution-change-lines under chromatogram A show that the 17 suspect peaks gave an final peak count of 24 peaks.

The time of pulsing before suspect peaks, *i.e.* the "pulsing-interval" is critical. The pulsing-interval is close to the void time, t_0, as mentioned above. The effect of different pulsing-intervals on suspect-peak f, for example, is shown by those chromatograms in which the right leg of the bracket is close to peak-set f in Fig. 3. Peak set f shows three peaks that are mostly resolved by pulses at 5.94 min (chromatogram A) and 5.99 min (chromatogram B) before the peaks. For peak-set f only two peaks are seen when the pulse precedes the suspect-peaks by a shorter time: 4.23 min (chromatogram H), 4.13 min (chromatogram F), 1.93 min (chromatogram C) or 1.71 min (chromatogram D). They are also unresolved when the pulse precedes the suspect-peaks f by a longer time: 6.42 min (chromatogram H) or 7.05 min (chromatogram E). Similar affects can be seen for suspect-peak set c.

Secondary pulsing effect

Multiple pulsing early in the run can have a secondary effect to sharpen peaks much later in a gradient run. Suspect-peaks "g" splits when 5 pulsings are used early in the run and when the nearest pulsing is 9.58 min (chromatogram E) or 7.78 min (chromatogram F), much too early to cause direct effects. Peak-set g is resolved even more when it is preceded by 7 pulsings (chromatogram G) or 9 pulsings (chromatogram H) much earlier in the run. The primary pulsing effect to produce partial resolution, as observed above, is found when pulsing precedes the suspect-peak set g by 5.70 min (chromatogram C, to give two peaks) and 5.53 min (chromatogram D, to give three peaks). In both of these cases only two other pulsings were used much earlier in the runs.

Another example of the secondary pulsing effect is seen with suspect peak i. The two sharp partially resolved peaks (chromatograms A–D) are resolved into 3

peaks (chromatogram G) when the run is preceded by 7 pulses (the nearest pulse is 6.40 min). However, only two peaks are seen when only two pulsings are used earlier in the run (chromatogram B) (the nearest pulse is 6.19 min). Note that a smaller third peak appears on the tail of the two main peaks when pulsing precedes the suspect by 5.56 min (chromatograms H and H'). The extent and cause of the secondary effect to pulsing needs to be investigated further.

CONCLUSIONS

The experiments reported here indicate only the beginning potential of pulsing. It was shown that with fluorescence detection, pulsing can be used to go into a chromatogram and increase resolution between specific poorly resolved peak pairs and that single peaks can be tested to determine if they are single components. Pulsing is compatible with both gradient or isocratic separations and can be used to optimize the separation of simple and complex mixtures.

Pulsing may often be faster than conventional optimization methods since peaks in the whole chromatogram generally retain their order and identities are more readily followed. In contrast, isocratic-4-solvent optimizations cause the simultaneous movement of all peaks, resolution of some peaks may diminish, and peaks may switch order as the resolution of other peaks improves. Sometimes it is necessary to apply up to 7 runs with each individual component, and individual components are often not available. The ability to follow peak identities is a time saving for one-time-only separations where a single pulsing run (after the initial run) potentially can separate several unresolved-peaks to solve the problem.

The observations shown here were made with pulsing only weak eluent and using fluorescence detection for which the baseline upset with the valve operation are minimal. In initial work with UV detection, the baseline upset obscured weak-eluent pulsing, but pulsing in other materials could be followed. Pulsing ion pair agents at different points during a run was modified resolution through broad sections of the chromatogram. Single pulsings of strong eluent, acetonitrile, eluted early peaks and improved resolution of the first part of the chromatogram but multiple acetonitrile pulsings washed off all sample. Pulsing acids, bases and buffers may also allow whole sections of complex chromatograms to be reproducibly modified.

Much remains to be learned about the use of pulsing. However, pulsing points to a new approach to dynamically affecting separations while they are taking place.

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