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"SEQUENTIAL ISOCRATIC STEP" LIQUID CHROMATOGRAPHY: A HIGH-THROUGHPUT, PROBLEM-SOLVING APPROACH WITH SENSITIVE, NEAR-UNIVERSAL DETECTION FOR WIDE POLARITY MIXTURES

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SUMMARY

A technique called "sequential isocratic step" (SIS) liquid chromatography (LC) is introduced that provides a significant advantage for industrial laboratories of substituting automation for manpower to potentially triple the problem solving time available to a laboratory by systematically running "pilot separations" at nights and over weekends. Separations of completely unknown, wide-polarity mixtures are obtained with high sensitivity and with the near-universal detection possible with 190 nm UV absorption. Conventionally, for samples showing good 254 nm UV absorption, as aromatics, gradients from full aqueous buffer to full acetonitrile frequently give good separations and can serve as a "pilot-run" for isocratic conditions for faster repetative analyses. However, many compounds show only poor detectability at 254 nm but 190 nm allows sensitive detection for all but a few functional groups. The SIS chromatography approach involves having a microprocessor LC sequentially create a series of isocratic eluent steps, 20% apart, of acetonitrile in aqueous buffer. The sample is injected for each of the steps with unattended operation during the night, and the resulting series of 6 chromatograms compared the next day. While each segment spans only a short polarity range of *ca.* 10 k' units, the 6 segments taken together cover the polarity range from full acetonitrile to full aqueous elution in less than 45 min. Since no gradient is used, SIS-LC overcomes two limitations of gradient elution using 190 nm detection, which are: (1) the large shift in the background gradient baseline makes it impossible to detect sample peaks and (2) "ghost" peaks collected from the impure water at the beginning of the gradient elute later in the gradient and obscure sample peaks. The SIS-LC approach eliminates the effects of both ghost peaks and baseline shifts. Further, we demonstrate that with SIS-LC the "detection window" between the solvent background UV absorbance and the upper linear limit of the detector is usually large enough so that inexpensive commercial distilled water and water with amine additivies can be used at 190 nm for general detection. Examples show how SIS-LC can be used to quantify components or quickly establish log k' *vs.* % acetonitrile plots, or compare lots of chemicals.

INTRODUCTION

Some of the problems of separating mixtures of widely differing polarity

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range, *e.g.* in residues left from biological extracts, were recently discussed by Martin *et al.*[1] with their work on "flip-flop" chromatography. Snyder and Kirkland[2] review how the "general elution problem" of eluting wide polarity mixtures with optimum capacity factor, k', for fast and sensitive analyses is solved in various ways, including solvent gradients, column switching techniques, and flow and temperature programming. These authors point out that while gradient elution can cover a wide polarity range, some cases may require other approaches to give advantages of faster separations and lower detection limits.

The increasing prevalence of complex mixtures and the need to analyze quickly varied unknowns in production or troubleshooting situations, gives chromatography methods covering a wide polarity range a decided advantage, since little time is spent searching for the right system. Another advantage of wide polarity range liquid chromatographic (LC) methods is that contaminants of widely different polarity compared to the main components, such as surfactants, colloids, or insoluble components, are not overlooked if they are properly solubilized before LC.

At 254 nm UV detection, a favorable LC gradient analysis covering a wide polarity range can detect aromatics or samples with conjugated bonds having molar absorptivities (ε) of 10,000 or more[3] to a detection limit of 1 ng or lower. Typical gradients use highly purified water to acetonitrile and give nearly flat baselines at detector sensitivities of 0.013 a.u.f.s.

However, in more practical gradient separations, it is desirable to use less expensive solvents such as methanol or lower quality acetonitrile and water, or solubilizing amines[4,5], ion-suppression buffers, or ion-pair reagents. In other practical situations it is desirable to detect in the presence of UV absorbers, the "non-UV absorbers" such as fatty acids[6,7], glycerides[8], sugars, amino acids or simple aliphatic alcohols or carboxy compounds, without the need to derivatize, while spanning a wide polarity range. With these more practical situations, the baseline shift from full aqueous to full organic solvent can be 0.5 a.u. or more, requiring that the detector sensitivity be set so low to keep the baseline on scale that only major components can be detected. At lower wavelengths, this background UV absorbance of the solvent usually grows much larger[9]: at 190 nm water to acetonitrile gradients show a baseline shift of 0.2 to 0.5 a.u. or more, and water to methanol gradients show baseline shifts of 1 to 3 a.u.

Despite these problems with practical systems, the rewards of using 190 nm UV detection can be great. Most substances are expected to be detected and thus derivatization can be avoided. The chromophore molar absorptivities for absorption bands from 185–210 nm is above 1000 for most common materials, including ethers, thioethers, amines, disulfides, oximes, ethylenes, ketones, aldehydes, and nitro chromophores[3], suggesting limits of detection of 1 μg to 1 ng (ref. 10). Aromatic and conjugated systems have a 1 to 3 order of magnitude lower limit of detection. In fact, even many common inorganic anions show enough useful UV absorption to be detected at a level of 1 nM and below with ion-pairing chromatography.

Another problem with gradient elution is that contaminants collected from either the water or the strong solvent, such as methanol[11], in the early part of a gradient chromatogram can elute later in the chromatogram and obscure peaks from the sample.

What is desired is a fast, reliable chromatographic system that is fully auto-

mated for unattended operation. It should allow the full aqueous to full organic elution to be covered, but show no effect from impurities found with inexpensive common LC solvents or LC additives, while allowing the detector to operate at high sensitivity (0.02 a.u.f.s.) at 190 nm UV detection so that most organic species can be detected to low levels.

To achieve these aims a new technique is introduced for covering a wide polarity range while achieving high sensitivity with near-universal detection: "sequential isocratic step" (SIS) LC. SIS-LC involves having a microprocessor LC sequentially create a series of isocratic eluent steps of 100, 80, 60, 40, 20, and 0% acetonitrile in aqueous buffer. After each step the column is allowed to reach equilibrium, the microprocessor prints out the results of the previous step, re-zeros the baseline and re-injects from the same vial. After the series of steps from 100 to 0% acetonitrile is completed and the 6 injections of the sample are made, the microprocessor returns to the original 100% acetonitrile level, changes to a new sample, and starts on a second SIS-LC run with the new sample. All of this is done with 190 nm UV detection if near-universal detection is desired, or at any other wavelength if more detection specificity is desired.

Some earlier work with automated step gradients has been reported. In Parris'[12] review of automated methods development, the step gradient approach is presented with the 254-nm detector and the 5.75-μm infrared detector. The power of automated methods development is illustrated by some short isocratic step programming from 65 to 40% strong solvent at 254 nm, followed by wavelength programming in a publication by Schrenker[13]. To illustrate the application of the ternary gradient capability of the Spectra-Physics liquid chromatograph, a 40 to 100% water–methanol gradient was systematically modified with 10% isocratic increments of acetonitrile to show that this "programmed run" technique could allow a difficult separation to be achieved[14,15].

These previous step gradient investigations did not use general detection as with 190 nm UV, nor operate over wide polarity ranges. Further, the ability of microprocessor instruments to re-zero baselines and thus eliminate the effects of significant solvent background signals was overlooked. Thus the advantages of SIS-LC of high sensitivity, near-universal 190 nm detection with non-critical solvent requirements while covering the full polarity range from full aqueous to full organic elution with unattended operation were not recognized previously.

The SIS-LC method proposed here substitutes automation for expensive manpower while frequently producing successful separations of completely unknown mixtures with high sensitivity and the near-universal detection possible with 190 nm UV absorption. Twenty or more separation problems can be investigated per night, vastly increasing the problem solving throughput of a laboratory.

The 20% acetonitrile steps coupled with the high flows of 5 ml/min and 5.8-min steps gives a k' range of 10 per step and this usually has provided nicely resolved peaks for most sample problems studied so far. However, the advantages of universal detection and unattended problem solving for narrower steps, if needed, can be obtained with the Hewlett-Packard LC by methods described later.

It must be cautioned that the SIS-LC approach is an adjunct to gradient elution but allowing the general elution problem to be solved with a near-universal 190 nm detector. The SIS-LC approach does not substitute for a wise choise of

chromatography modes based on some knowledge of the sample. For example, inorganic anions will not be retained or resolved by a reversed-phase system with only water and acetonitrile. Amines to form ion-pairs are required. However, we will show that the SIS-LC approach will allow ion-pair separations of inorganic anions to be very quickly characterized, so good column, pH, and % acetonitrile combinations can be selected.

EXPERIMENTAL

Materials

Zorbax C_{18} octadecyl columns or Zorbax C_8 octyl columns (25 × 0.46 cm; DuPont, Wilmington, DE, U.S.A.) preceded by Brownlee Labs. RP-18 or RP-8 precolumns (3 × 0.46 cm; Rheodyne, Berkeley, CA, U.S.A.) were used in this work. A column (25 × 0.46 cm) of Whatman (Clifton, NJ, U.S.A.) 37–53-μm silica was used after the pump and before the mixer for the aqueous solvent line to saturate the aqueous phase with silica. The silica-saturator column and precolumn allow Zorbax columns to be used in excess of 3 months at 24 h/day, 6 days a week. The usual 5 ml/min flow does not give optimum plate counts, but the need is for scanning wide k' ranges on each step and covering a wide polarity range in *ca.* 45 min.

Unless otherwise specified, the solvents were Burdick & Jackson Labs. for HPLC (Muskegon, MI, U.S.A.) and "commercial distilled water" in 5-gallon polyethylene bags from Belmont Springs (Belmont, MA, U.S.A.). HPLC grade water from Baker (Phillipsburg, NJ, U.S.A.) was used for some work. UV irradiated water was prepared by irradiating 6 l of distilled water for at least 3 h in a No. 816 HPLC Reservoir from Photronix (Medway, MA, U.S.A.).

The 0.15 *M* triethyl amine phosphate solutions of various pHs were prepared from Fisher Scientific (Fair Lawn, NJ, U.S.A.) reagent-grade triethyl amine purified by passing it through a column of Fisher basic alumina, Brockman activity I. A rapidly stirred aqueous suspension of the amine was titrated to the desired pH with a Mallinckrodt (St. Louis, MO, U.S.A.) reagent-grade 85% phosphoric acid, selected from several lots so as to have less than 0.35 a.u./cm at 190 nm on a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 340 spectrophotometer when run against air.

Tetrabutyl ammonium phosphate solutions of the desired pH and concentrations were prepared by titrating solutions of Eastman tetrabutyl ammonium hydroxide titrant supplied as a 0.4 *M* solution in water (Fisher) with the 85% phosphoric acid described above.

A 0.01 *M* potassium dihydrogen phosphate buffer containing 0.004% (0.004 g/100 ml) of sodium azide is conveniently made by dispensing 50 ml of a stock solution into a 3.81-l jug of UV irradiated water and titrating to the desired pH with either the above 85% phosphoric acid or sodium hydroxide. A 500-ml batch of stock solution is made by dissolving 52.53 g of reagent-grade Baker potassium dihydrogen phosphate and 1.544 g of Fisher reagent-grade sodium azide in 500 ml of UV irradiated water.

Chem Service (Media, PA, U.S.A.) phthalate standards and Eastman alcohols and alkanes were used to prepare standards.

Equipment

A Hewlett-Packard (Avondale, PA, U.S.A.) 1084 liquid chromatograph was used having a 190 to 600 nm variable-wavelength detector (No. 79875A), a 0–200 μl variable-volume injector (No. 79841A), and a 60-position automatic sampling system (No. 79842A). This instrument allows up to 7 programmable repeat injections from the same vial and 60 different vials to be run. The 1084 instrument was equipped with the B software, however, some work was done with the C software. The SIS program given below is compatable with either the B or C software.

For some work, a Waters Assoc. (Milford, MA, U.S.A.) R401 refractive index (RI) detector was attached after the UV detector. An attenuation setting of 1× gave a full scale signal of $6 \cdot 10^{-6}$ RI units. The flow from the pump was split just before the injector through a second Zorbax C_{18} column so both the reference and sample sides of the RI detector always contained the same solvent. A Cannon Instrument (State College, PA, U.S.A.) 5-gallon constant temperature bath was used to thermostat the RI detector at 38°C.

Attenuations for the Hewlett-Packard UV detector are in powers of two, with the following correspondence for a 16.8-cm full scale deflection: an attenuation of 2^3 is 0.0008 a.u./cm or 0.013 a.u.f.s.

Programming the liquid chromatograph

The effect of a "SIS run" is that the full gradient is approximated by a series of 6 steps going from the strong to the weak eluent. Thus a SIS run consists of a set of six short isocratic chromatograms as shown in Fig. 1. Six injections of the same sample are made, and the areas can be printed between each short step, however, the area printout is frequently suppressed as in this paper. There are no baseline shift effects because the microprocessor allows each step to equilibrate before it re-zeros the baseline as the injection is made. By going in the direction of strong to weak solvent, no components from an earlier step can obscure peaks from a later injection.

Table I shows a typical Hewlett-Packard software program which takes about 2 to 3 min to enter. Since the programming is critical to a successful SIS run, it is worthwhile to briefly discuss the important steps. The zero preceding every step of the "Printout" in Table I indicates that for each of the 60 possible samples, the entire SIS program shown under Printout will be repeated. The three lines labeled (0–3), for example, indicate that for the third injection, the isocratic solvent composition begins at 80% B and this is held constant until 5 min, and by 5.1 min the isocratic composition changes to 60% B, where it remains until 5.8 min, the time under command (0–0). At 5.7 min, also command (0–0), the area counts are printed out and the run stops. The Hewlett-Packard liquid chromatograph inserts a 1-min equilibration pause after the 5.8-min command. The two 100% B commands (0–1, 0–2) gives an added time for returning the column to equilibration from the previous 0% B composition, and also flushes the bypass valve of 0% B solvent, remaining from the seventh injection of the previous sample.

A convenient nomenclature to describe the SIS conditions outlined above and in Table I is: "SIS conditions: 5 min isocratic, 5.8 min per step".

The Hewlett-Packard liquid chromatograph can fail to generate the desired SIS run if the following program commands are violated. The "area %" printout command must be entered explicitly to prevent the software from momentarily

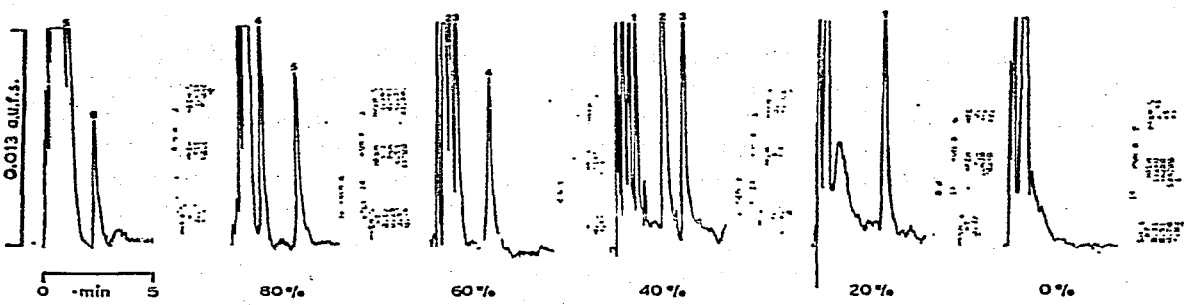

Fig. 1. Typical SIS liquid chromatogram set of six short isocratic steps using the step sequence shown in Table I and showing the position of the area printouts. The steps are 100, 100, 80, 60, 40, 20 and 0% acetonitrile in water. Step 1 is not shown. Phthalate sample, 10 μl of acetonitrile containing 60 μg each of (1) potassium hydrogen phthalate, (2) dimethyl phthalate, (3) diethyl phthalate, (4) dibutyl phthalate, (5) diphenyl phthalate, (6) dioctyl phthalate, (7) diundecyl phthalate.

ABLE I

SEQUENTIAL ISOCRATIC STEP PROGRAM FOR THE HEWLETT-PACKARD 1084 B OR 1084 C LIQUID CHROMATOGRAPH

Input

```
CHG RUN DELETE

CHG RUN - 1 %B    1 0 0 @
CHG RUN - 2 %B    1 0 0 @
CHG RUN - 3 %B    8 0 @
CHG RUN - 4 %B    6 0 @
CHG RUN - 5 %B    4 0 @
CHG RUN - 6 %B    2 0 @
CHG RUN - 7 %B    0 @

CHG RUN - 1 TIME 5 %B    1 0 0 @
CHG RUN - 2 TIME 5 %B    1 0 0 @
CHG RUN - 3 TIME 5 %B    8 0 @
CHG RUN - 4 TIME 5 %B    6 0 @
CHG RUN - 5 TIME 5 %B    4 0 @
CHG RUN - 6 TIME 5 %B    2 0 @
CHG RUN - 7 TIME 5 %B    0 @

CHG RUN - 1 TIME 5 . 1 %B    1 0 0 @
CHG RUN - 2 TIME 5 . 1 %B    8 0 @
CHG RUN - 3 TIME 5 . 1 %B    6 0 @
CHG RUN - 4 TIME 5 . 1 %B    4 0 @
CHG RUN - 5 TIME 5 . 1 %B    2 0 @
CHG RUN - 6 TIME 5 . 1 %B    0 @
CHG RUN - 7 TIME 5 . 1 %B    1 0 0 @

CHG RUN - TIME 5 . 7 AREA% @
CHG RUN - TIME 5 . 8 STOP @

CHG RUN 3 0 STOP

CHG RUN 3 0 FLOW 0 @

OPTN  2 INJ/BTL: 7 @
```

Printout

```
0 -1 %B          100.0
0 -1   5.00 %B         100.0
0 -1   5.10 %B         100.0
0 -2 %B          100.0
0 -2   5.00 %B         100.0
0 -2   5.10 %B          80.0
0 -3 %B           80.0
0 -3   5.00 %B          80.0
0 -3   5.10 %B          60.0
0 -4 %B           60.0
0 -4   5.00 %B          60.0
0 -4   5.10 %B          40.0
0 -5 %B           40.0
0 -5   5.00 %B          40.0
0 -5   5.10 %B          20.0
0 -6 %B           20.0
0 -6   5.00 %B          20.0
0 -6   5.10 %B           0.0
0 -7 %B            0.0
0 -7   5.00 %B           0.0
0 -7   5.10 %B         100.0
30 -0 STOP
30 -0 FLOW             0.00
 0 -0   5.70 AREA%
 0 -0   5.80 STOP

 FLOW          5.00    4.97
 %B          100.0    98.8
 COLUMN P              168
 MAX P       400
 MIN P         0
 VW SGNL
 WAVL S:R    190 :       0

 CHT SPD       2.54
 ZERO         20.0
 ATTN 2↑       3
 AREA REJ                500
 SLP SENS      0.10

  5.00  %B          100.0
  5.10  %B          100.0
  5.70  AREA%
  5.80  STOP
```

Other considerations, unless specified:
Solvents: A, Belmont distilled water; B, acetonitrile.
Sample size: 10 μl.
Column: Zorbax C_{18}, 25 × 0.46 cm.
Precolumn: Brownlee C_{18}, 3 × 0.46 cm.

trying to return the solvent composition to that of the previous step, even though it is normal for the program to print "area %" after the "stop" function. This "area %" command must preceed the "stop" function by at least 0.1 min. Commands to list composition, pressure, etc. (as "0–0" list column pressure) must not be used. On rare occasions at high sensitivities for some solvents, the small plug of solvent from the previous step that is injected when the bypass valve is activated, may elute just as the baseline is being re-zeroed. If this plug of solvent produces a peak, then the baseline of the chromatogram can be below the scale of the paper. Programming a re-zero function or changing the step % will eliminate this problem.

Most modern microprocessor based liquid chromatographs can do automated SIS-LC. The six basic requirements to achieve the main benefits of problem solving for *ca.* 20 different samples overnight are: (1) 190 nm UV is used as the detector; (2) a sequence of 5 to 7 isocratic steps ranging from 100% to 0% B can be repeatedly generated automatically; (3) 5 to 7 repetitive injections of the same sample can be made automatically some minutes after the isocratic steps have been generated; (4) the chromatogram display automatically re-zeros the baseline after each injection; (5) 20 or more different samples can be run in sequence; and (6) an integrator–printer generates the areas of peaks displayed. Given the above instrument requirements, most of the components can be quantified in 20 or more totally different samples in one overnight run.

The "different samples" can be 20 samples from totally different problems and the SIS approach would allow the best isocratic run conditions to be selected the next day for each problem. Alternatively, the "different samples" can be 20 different lots of the same sample if a comparison is required immediately, without need for selecting the best isocratic conditions.

RESULTS AND DISCUSSION

SIS-LC advantages over gradient elution

The three advantages of SIS-LC over conventional gradient LC are illustrated in Fig. 2. Both gradient and SIS-LC cover the wide polarity range from full aqueous to full organic elution. SIS-LC offers in addition (1) general detection of most compounds with 190 nm UV; (2) sensitive detection at 190 nm UV; and (3) freedom from artifact "ghost" peaks from impure solvents.

The first SIS-LC advantage, general detection of most compounds with 190 nm UV, can be seen by comparing all the chromatograms of the alcohols to the control runs in Fig. 2. Only the SIS run at 190 nm (chromatogram k) shows the alcohols detectable *vs.* the control run (chromatogram l). Note that the high sensitivity (2^3 attenuation, 0.013 a.u.f.s.) 254 nm gradient run (chromatogram b) and 254 nm SIS run (chromatogram e) of the alcohols do not allow the alcohols to be detected, since alcohols do not show significant 254 nm UV absorption.

The second SIS-LC advantage, high-sensitivity detection at 190 nm UV, can be seen by comparing the alcohol runs at 190 nm detection for the SIS run (chromatogram k) to the gradient run (chromatogram h). The shift in the baseline due to the difference in absorption at 190 nm of the water to acetonitrile gradient requires that the minimum sensitivity be 2^8 attenuation (0.430 a.u.f.s.) in order to keep the

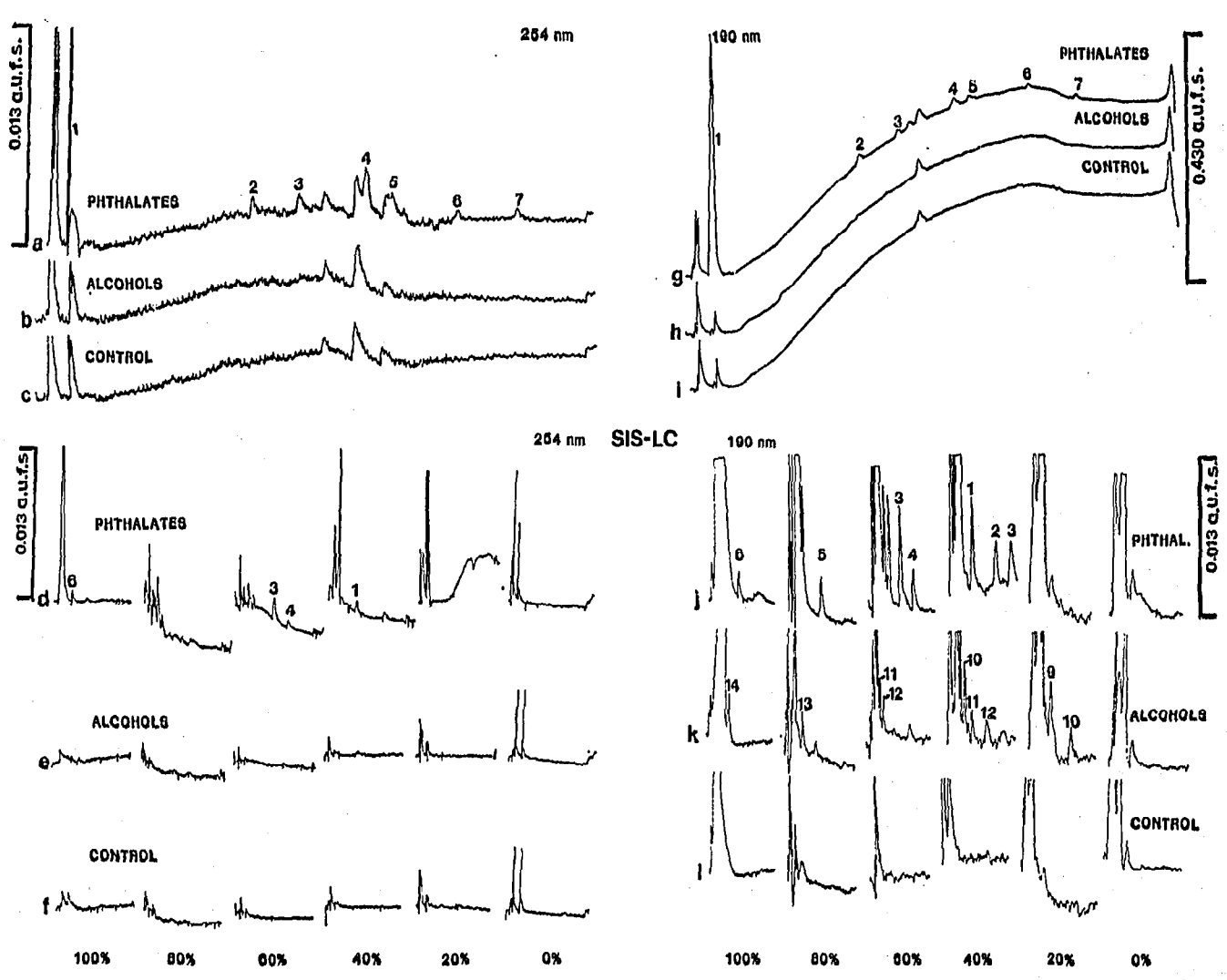

Fig. 2. Comparison of gradient LC and SIS-LC at 254 and 190 nm UV detection (see text). Gradient is from 5 to 95% in 9 min, isocratic at 95% for 3 min, re-equilibrate at 5% for 3 min. SIS conditions: 5 min isocratic, 5.8 min per step. All other conditions as in Table I. Phthalate sample, numbered as in Fig. 1, except 10 μl of acetonitrile containing 2–4 μg of each. Alcohol sample, 10 μl of acetonitrile containing 60 μg each of (8) ethanol, (9) *n*-butanol, (10) *n*-pentanol, (11) *n*-hexanol, (12) *n*-heptanol, (13) *n*-decanol, (14) *n*-undecanol.

baseline on scale. At this low detector sensitivity, the alcohol gradient run (chromatogram h) cannot be distinguished from the control (chromatogram i).

The greatly increased sensitivity advantage can also be seen for compounds absorbing strongly at 254 nm, but showing increased UV absorption at lower wavelengths, as typically found with aromatic compounds. Note that with high sensitivity detection of 0.013 a.u.f.s., the phthalate peaks can be readily detected at 254 nm with the gradient (chromatogram a) but the peaks are a factor of 10 to 50 times higher with the SIS run at the same sensitivity but at 190 nm (chromatogram j).

For phthalates, the SIS run at 254 nm is expected to have slightly lower detection limits than a gradient because the sharpening effect of gradients is missing[16]. However, the SIS run at 254 nm might be used with favorable UV absorbing samples if isocratic separation conditions are desired.

Fig. 3 graphically shows the greatly improved sensitivity of 190 nm UV detection over 254 nm detection. The SIS run at 254 nm (chromatogram b) shows much smaller peaks than the SIS run at 190 nm (chromatogram a) for the high UV absorber diphenyl phthalate. Note that the components visible at 254 nm show peaks 10 to 50 times higher at 190 nm. The advantage of more general detection with 190 nm is graphically illustrated by the presence of *ca.* 10 new components in this nominally "pure" sample of diphenyl phthalate. This variation in sensitivity with detection wavelength, in fact, can be used to "tune in" some specificity to the SIS approach. For example, this comparison of 190 and 254 nm detection shown in Fig. 3 allows the main component to be readily distinguished from the impurities, since diphenyl phthalate is known to have a very high 254 nm UV absorption.

The SIS-LC approach with 190 nm general detection would be particularly useful for comparing lots of aromatics, such as this phthalate, since very sensitive detection of the aromatic impurities is expected.

The third SIS-LC advantage is freedom from artifact or ghost peaks from impure solvents. Three to four of these ghost peaks can be seen in the middle of the control gradient runs both at 254 nm and 190 nm (chromatograms c and i, Fig. 2). Ghost peaks originate from impurities collected on the column from the water or organic phase being eluted later in the gradient[11]. These ghost peaks can obscure legitimate sample peaks as can be seen by comparing the phthalate run (chromatogram

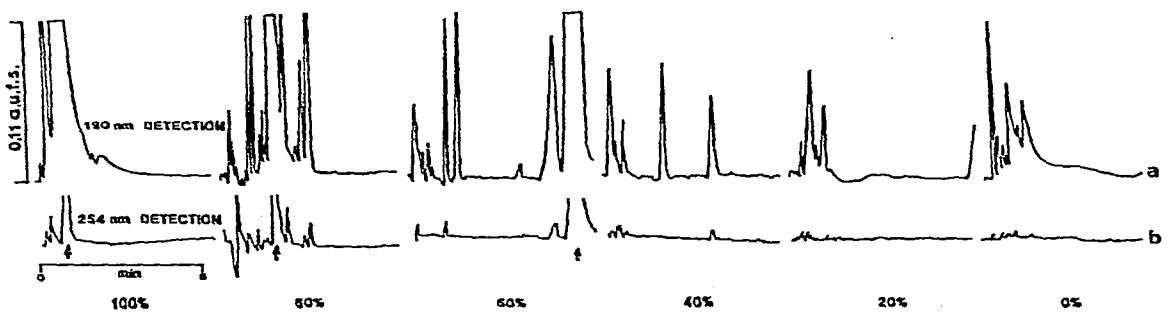

Fig. 3. SIS-LC of diphenyl phthalate comparing 190 nm UV detection (a) to 254 nm UV detection (b). Arrows indicate elution position of diphenyl phthalate. Sample, 10 μl of acetonitrile containing 2 μg; flow-rate, 3 ml/min. Other conditions as in Table I.

a) to the control run (chromatogram c), Fig. 2. Costly solvents specially purified for LC can minimize this problem with gradients, but can rarely completely eliminate the problem, especially if solvent additives are used.

Chromatograms f and l of Fig. 2 of the SIS runs at 254 and 190 nm detection show that no peaks occur beyond the initial part of each SIS step. No ghost peaks from compounds picked up early in the run and eluting later in the run can occur with SIS-LC because each SIS step is isocratic and at equilibrium before samples are injected. Later it will be shown that relatively impure solvents can be used with SIS-LC.

The "initial peaks" at the front of each SIS step are generally limited to a k' of less than 2 and arise from three sources. The bypass valve in the Hewlett-Packard injector holds solvent from the previous step which enters the column when the auto-injector begins to take in the sample. This causes some initial peaks. The solvent used to dissolve the sample is generally impure by the criterion of 190 nm detection and this causes some initial peaks. Finally, components of the sample only partially retained on a particular step contribute also to the initial peaks. These initial peaks from all three sources elute quickly and provide a peak-free baseline on each SIS step from a k' of 0.3–2 up to *ca.* 10 or more for sensitive detection.

Application of SIS-LC to compare lots of a production solvent

For comparing raw material old to new lots, aged to preserved lots, and acceptable to unacceptable, problem, lots, the SIS-LC approach is particulary useful because of the sensitive and very general detection possible. Frequently a comparison of two SIS runs will highlight differences that can flag problems. Fig. 4 shows the results of SIS-LC to compare acceptable to unacceptable lots of ethyl acetate. The 190 nm detection showed 7 trace level peaks where only 3 could be seen with gradient elution at 254 nm. The trace level peaks varied in ratio from 1.5 to 20 fold, and, as discussed below, UV spectra obtained with the SIS "wavelength scan" mode, aided in identification of the problem impurity and elimination of the problem.

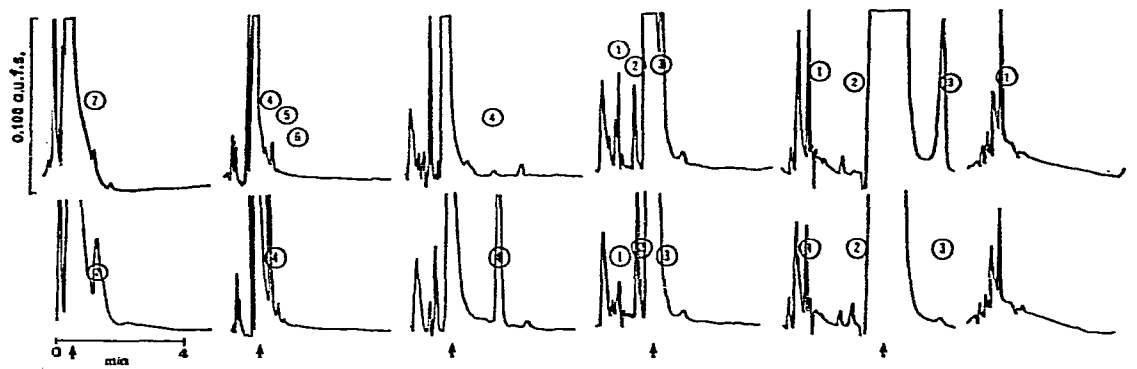

Fig. 4. SIS-LC with 190 nm detection showing impurity level differences in two lots of ethyl acetate. Arrows indicate elution position of ethyl acetate. Sample, 20 μl; flow-rate, 3 ml/min; column, Zorbax C_8 with no precolumn. Other conditions as in Table I.

Application of SIS-LC to determine spectra of unknowns in complex mixtures

With the Hewlett-Packard 1084 liquid chromatograph equipped with the programmable UV detector, the SIS-LC method was used to determine the UV absorption spectra in a completely unattended manner of the seven ethyl acetate impurities shown in Fig. 4. Seven vials of the same ethyl acetate lot were loaded in the auto-sampler and the SIS runs such as shown in Fig. 4 were repeated overnight at 10-nm increments from 190 to 280 nm UV detection. The next day, the area counts were plotted for each of the impurity peaks *versus* the wavelength of the particular SIS run to give the UV spectra of all contaminants simultaneously. The results of this SIS "wavelength scan" approach for three ethyl acetate components are shown in Fig. 5, the solid lines. The dashed lines in Fig. 5 show that the spectra obtained by the usual approach compares closely to that obtained by the SIS wavelength scan approach. The tedious manual steps of memorizing baselines, trapping peaks, and rescanning are eliminated.

The advantages of the SIS wavelength scan approach is that the absorbance spectra of any number of contaminants can be obtained in only a few automated SIS runs and the resulting composite spectra of all components show the relative absorption contributed by each component to the final UV absorption of the mixture.

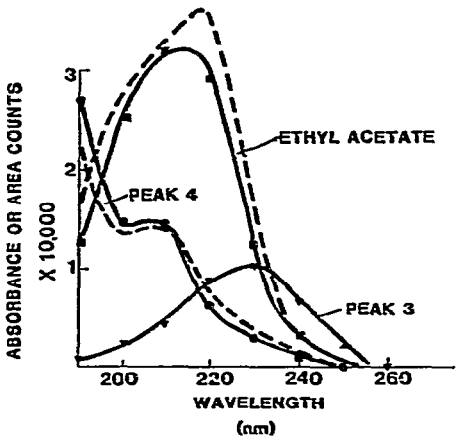

Fig. 5. UV absorption spectra of ethyl acetate components. The solid lines are spectra produced by the SIS "wavelength scan" approach described in the text. At any wavelength, the absorbance of each component shows the relative contribution to the total absorbance of the impure ethyl acetate. The dashed lines are spectra produced by manually scanning eluted peaks trapped in the Hewlett-Packard LC detector. Peak identities correspond to those in Fig. 4.

SIS-LC to characterize sample behaviour through log k' vs. % B plots

The orderly progression of retention of individual alcohols by SIS-LC can be seen in Fig. 6. With 20% acetonitrile increments in the SIS steps, two or more steps show isolated peaks for every alcohol from methanol to decanol.

SIS-LC allows the plot of log k' *vs.* %B solvent to be generated in only one unattended night run with only 30 min effort for constructing the plot, for even non-UV absorbing materials, as is illustrated by the data of Fig. 6 being plotted in Fig. 7. These curves are expected to be linear[17,18] or have slight curvature[19,20].

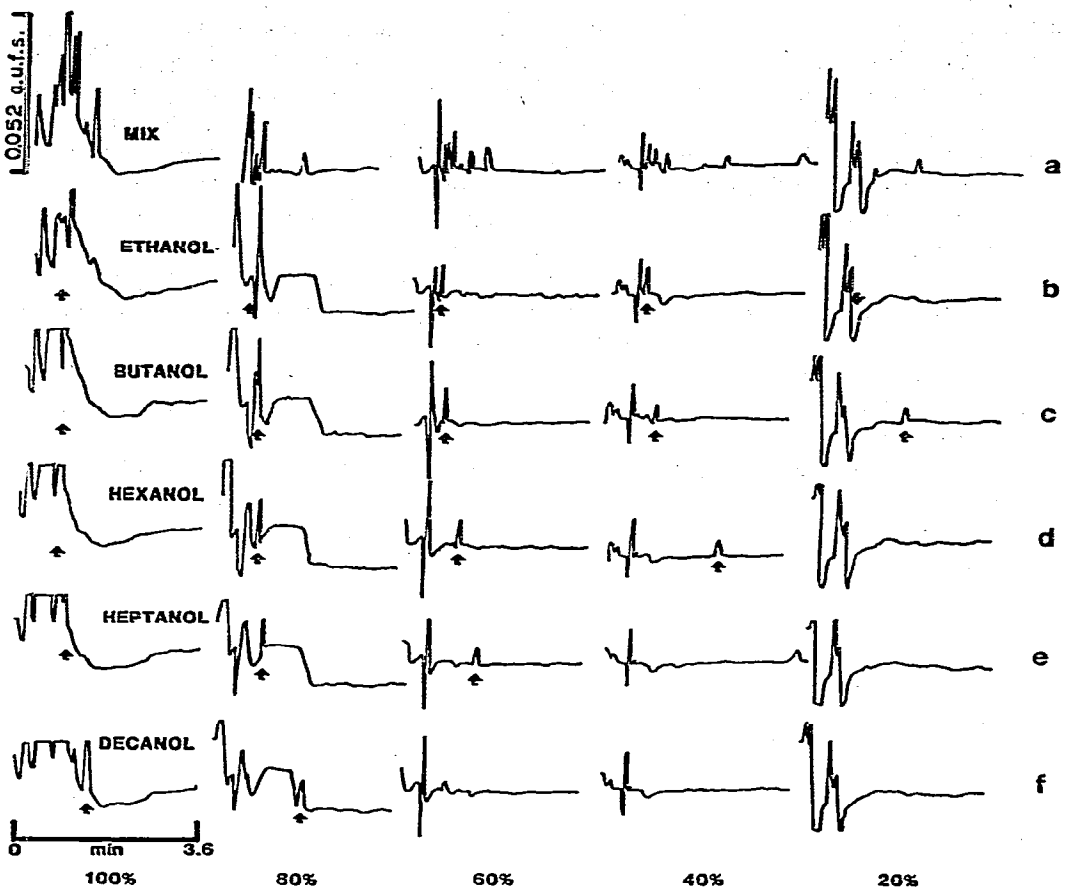

Fig. 6. SIS-LC with 190 nm detection of normal aliphatic alcohols. SIS conditions: 3 min isocratic, 4 min per step. Sample, 20 μl of acetonitrile containing 60 μg of each alcohol. Eluent A: 0.15 *M* triethyl amine phosphate, pH 3. Other conditions as in Table I.

When the Hewlett-Packard liquid chromatograph was allowed to inject air, the baseline upsets found with 190 nm UV detection were discovered to come, in part, from the oxygen in the injected air. The plot of log k' *vs.* %B for oxygen in air was determined by SIS-LC and is shown in Fig. 7, the dashed line. Note that oxygen shows only a slight decrease in retention from a k' of 2.5 to 0.1 in going from full aqueous to full organic elution.

The plot of alcohol log k' or phthalate log k' *vs.* %B (Figs. 7 and 8) shows that the more polar the component, the greater the number of 20% SIS steps in which the components can be seen. The polar material, potassium acid phthalate, elutes with a k' of less than 10 in all steps (Fig. 8) as does oxygen, butanol, and lower alcohols (Fig. 9). The larger, less polar phthalates, as diundecyl and dioctyl phthalates, show k' values less than 10 only in isocratic compositions of 90% acetonitrile or greater and these components are seen only in the strongest acetonitrile SIS steps.

The regular progression of the phthalate and alcohol plots of log k' *vs.* % B shown in Figs. 7 and 8 can be used to deduce structural data since homologues often

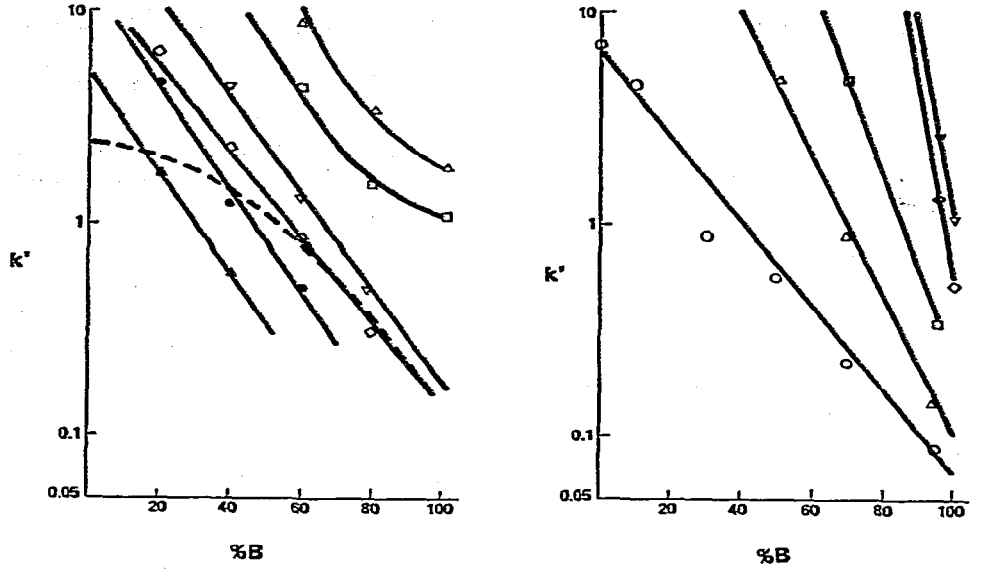

Fig. 7. Relationship between the capacity factor, k', and the mobile phase composition for oxygen and various normal aliphatic alcohols. Alcohols are in the 0.15 *M* triethyl amine phosphate, pH 3 aqueous buffer–acetonitrile system and oxygen in in the water–acetonitrile system. ▲, *n*-butanol; ●, *n*-pentanol; ◇, *n*-hexanol; ▽, *n*-heptanol; □, *n*-decanol; △, *n*-dodecanol. Dashed line is oxygen. Alcohol conditions as in Fig. 6, oxygen conditions as in Fig. 16.

Fig. 8. Relationship between the capacity factor, k', and the mobile phase composition for various phthalates in a water–methanol system. ○, Potassium hydrogen phthalate; △, diethyl phthalate; □, dibutyl phthalate; ◊, dioctyl phthalate; ▽, diundecyl phthalate. SIS conditions: 10 min isocratic, 12 min per step. Eluent A, commercial distilled water; eluent B, methanol; flow-rate 2.6 ml/min; detector wavelength, 254 nm. Other conditions as in Table I.

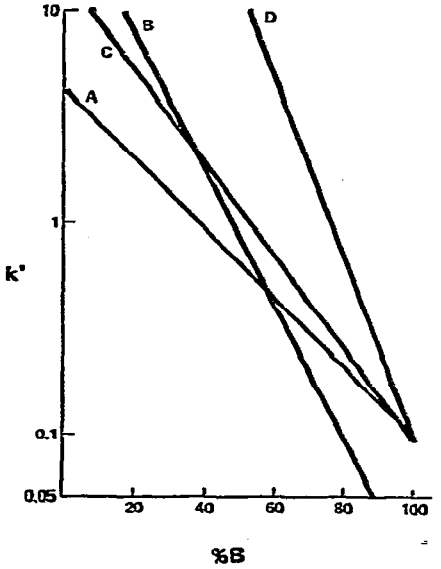

Fig. 9. Relationship between the capacity factor and the mobile phase composition for four monomers, A, B, C and D. Eluent A, 0.15 *M* triethyl amine phosphate, pH 3; eluent B, methanol. All other conditions as in Fig. 8.

give straight line plots for a given % B composition, when log k' *vs.* carbon number is plotted, for a given % B composition[17].

The regular behaviour of samples displayed by log k' *vs.* % B plots and the plot described above allows SIS-LC to provide improved confidence that known and unknown peaks are identical if the retention times match in multiple SIS steps. The probability that different substances will have identical log k' *vs.* % B plots is low since that would require identical solution and adsorption behaviour.

Choice of width of %B steps and elution volume of a step

The plots of the log k' *vs.* %B curves in Figs. 7 and 8 illustrate the relationship between the choice of the width of the %B steps (usually 20%) and the volume of solvent pumped for each step, the elution volume. For the columns used here, the void volume, V_0, is *ca.* 2.6 ml and thus, to see components with k' values up to 10, 29 ml of eluent is pumped on each step (flow of 5 ml/min for 5.7 min per step). For the variety of compounds investigated so far, including homologous series showing changes of 1 carbon (aliphatic alcohols) and 2 carbons (phthalates); the 20% steps would not miss any material, *i.e.* k' values are between 0.05 and 10 for every homolog on at least one step.

Examining Fig. 8 allows one to envision a mixture of materials as non-polar as dioctyl to diundecyl phthalates that would not be resolved in a first SIS-LC run using 20% steps. However, these substances would show a peak between the void volume and a k' of 10 on the final step in the first run. In this case, the first SIS-LC run (1) would serve as a "pilot run" to suggest a first isocratic composition assuming nothing was known about the mixture; (2) would allow many such totally unknown samples to be explored in an unattended night run; and (3) would highlight the presence of any more polar materials. Figs. 7 and 8 show that samples with the more usual higher polarity range between the polar end of ethanol and potassium hydrogen phthalate to the less polar end of *n*-decanol and dibutyl phthalate would be eluted on a step and would be separated from their neighbor homologs.

The use of the SIS-LC method for the group of more polar materials, the inorganic anions by ion-pair separation, is illustrated in a later section in which 5% B steps are used over the limited range to 25% acetonitrile.

If desired, the advantages of universal detection and unattended problem solving with steps narrower than 20% over the full range of polarity from 0 to 100% acetonitrile can be obtained with the Hewlett-Packard liquid chromatograph in the following manner. The Hewlett-Packard limit of 7 injections per bottle may be overcome by using one set of samples from tubes 1 to 30 using 7.7% steps from 0 to 46.2% and a second set of samples from tubes 31 to 60 from 53.9% to 100%.

Application of SIS-LC to find the "best" isocratic separation conditions

Fig. 9 illustrates the use of SIS-LC to rapidly find the "best" isocratic separation conditions both for favorable UV-absorbing materials, for which gradients could be used, and for samples showing low 254 nm UV absorbancy, where gradient elution fails. Isocratic LC is often preferred to gradient LC because usually separations are faster, reproducibility is better, and instrumentation is cheaper.

Fig. 9 illustrates the use of SIS-LC to find the best single isocratic step for mixtures of four monomers so they can be quantified rapidly in a routine manner. SIS runs of the four monomers alone and the mixture were made in one night run and

the plot of log k' *vs.* %B constructed in only 30 min, as shown in Fig. 9. An optimum separation for this monomer mixture can be seen from the graph as follows. An upper and lower "k' window" is set to represent the longest retention to be accepted and the shortest retention that can be distinguished from an unretained peak. If 0.1 is the lowest acceptable k', then %B above 80% cannot be used since monomer B would be un-resolved from t_0. In fact, 70% B gives monomer B a retention k' of 0.2, separates A and C, and gives monomer D the short retention k' of 1.8 for a fast analysis. Usual systems are less complex than this example and the best isocratic separation can be seen by just examining the SIS runs of the mixture.

SIS-LC with the RI detector

The RI detector is often considered a nearly universal detector but with a sensitivity of *ca.* 10^3 fold less than the 254 nm UV detector when used with favorable samples. The RI detector has been used with gradients, but with great experimental difficulties and with sensitivities even more limited (to 16×) because of the problems in matching the refractive indices of the two solvents used to generate the gradient[21]. Since SIS-LC eliminates the baseline drift problem, and achieves the full polarity range between full aqueous and full organic elution with a series of isocratic steps, we investigated using SIS-LC with the RI detector.

Two experimental difficulties limited using the Waters Assoc. R401 RI detector in a fully automated mode as done with the 190 nm UV detector. The flow from the pump was split just before the injector through a second reversed-phase column so both the reference and sample sides of the RI detector always contained the same solvent. Despite this, the first problem was that the "optical zero" adjustment on the detector still had to be manually reset after each new step of solvent had equilibrated.

The second problem was that the "%B" microprocessor command to mix the 90, 70, 50 and 10% B isocratic compositions for the intermediate SIS steps did not give a sufficiently flat baseline for the RI detector to be operated below the 8× setting. Thus solvents were manually changed by switching reservoirs to the A pump, and after equilibration, the RI detector was manually re-zeroed.

The SIS-LC run in Fig. 10 compares the RI detector to the 190 nm UV detector for simple alkanes. Alkanes are expected to be among the hardest compounds

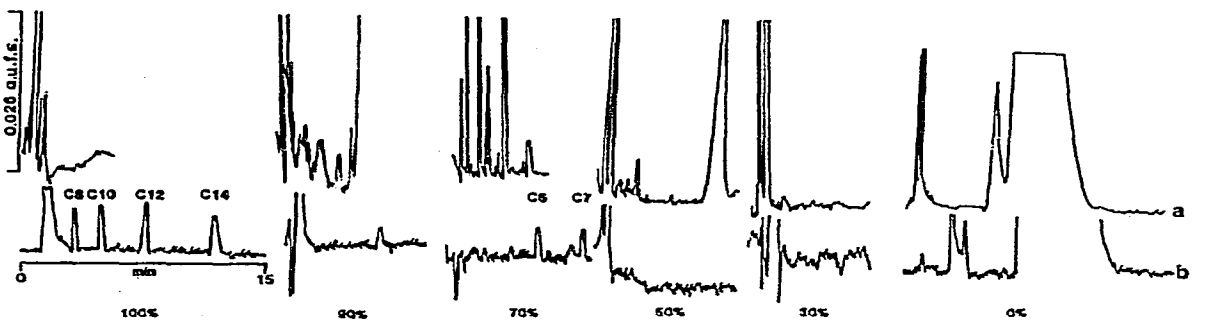

Fig. 10. Manual modeling of the SIS-LC for alkanes comparing the 190 nm detector (a) to the RI detector (b). RI detector at full, 1×, sensitivity; 190 nm UV detector at 2^4 attenuation or 0.026 a.u.f.s. SIS steps 5–10 min long; flow-through sample and splitter column *ca.* 4.5 ml/min, each. Sample, 10 μl acetonitrile containing 10 μg each of C6 (*n*-hexane), C7 (*n*-heptane), C8, C10, C12, C14.

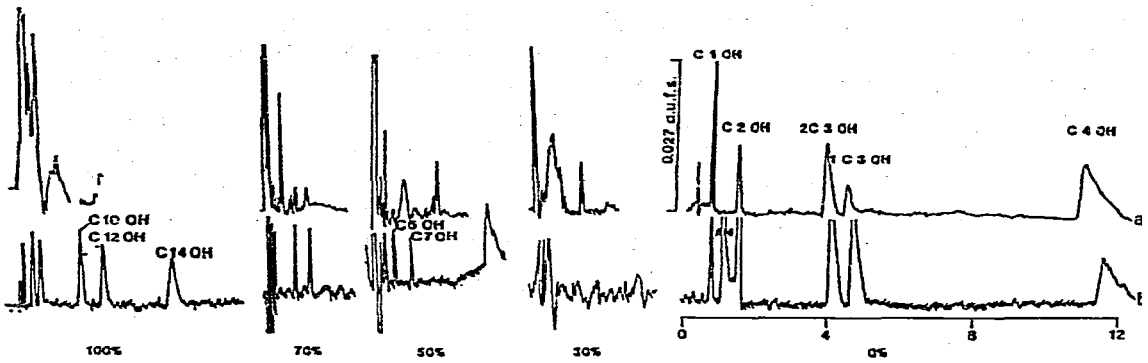

Fig. 11. Manual modeling of the SIS-LC for normal aliphatic alcohols comparing the 190 nm UV detector (a) to the RI detector (b). Other conditions as in Fig. 10. Sample, 10 μl acetonitrile containing 10 μg of C1 OH (methanol), C2 OH (ethanol), 2C3 OH (isopropanol), 1C3 OH (*n*-propanol), C4 OH (*n*-butanol), C5 OH, C6 OH, C7 OH, C10 OH, C12 OH, C14 OH.

to detect by UV absorption at 190 nm and mostly only trace UV absorbers can be seen with the 190 nm chromatogram (chromatogram a). The RI chromatogram (chromatogram b) allows the C_6 to C_{14} alkanes to be distinguished readily from trace UV impurities. Note that this detector can be run at full sensitivity (1×) while using the SIS approach to cover the full polarity range from aqueous to organic elution.

The SIS-LC run in Fig. 11 shows that the RI detector at full sensitivity (1 ×) shows similar sensitivity for the alcohols compared to the 190 nm UV detector operated at 2^4 attenuation (0.027 a.u.f.s.), as was found recently for monosaccharides[22]. It is important to notice that with the system used here, the C_1 to C_4 alcohols can be fully resolved and detected with either detector. In addition, the acetonitrile used as solvent for the alcohol standards can be seen to elute between the methanol and ethanol by RI detection, but not by 190 nm UV detection.

SIS-LC for volatile substances

There are some situations in which LC may be performed to analyze samples that could be run by GC. For example, the excellent separation shown in Fig. 11 of the simple aliphatic alcohols was applied to determine the residual butanol content in a reaction mixture containing a heat-labile butanol ester. Fig. 12 shows that the butanol in the ester mix was readily quantified with a 20% acetonitrile step, using the 190 nm detector. GC could not be used readily for this analysis since the heated injection port was expected to decompose the ester and generate additional butanol. Potentially a single SIS-LC run can quantify both the volatile and non-volatile or heat-labile components in a mixture, eliminating multiple methods, sample preparations, etc.

Effect of water quality on SIS-LC runs

Earlier we showed that the ghost peaks found with gradient elution is no problem with SIS-LC, since equilibrium is established on each isocratic step before sample is injected. However, the finite 190 nm UV absorption of contaminants in the water could affect the height of the "detection window" between the UV absorption of the solvent and the linear range of the detector (1.0 a.u.f.s. for the Hewlett-Packard used here). Since the 190 nm UV absorption of water available to us was

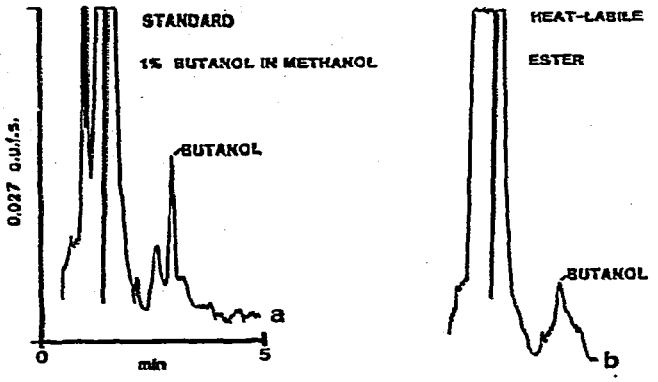

Fig. 12. LC with 190 nm UV detector to determine free *n*-butanol in a heat-labile butanol ester (b). a, A standard of 1% butanol in methanol. Sample, 10 μl of a 15% (w/w) ester solution in methanol; flow-rate, 3 ml/min; eluent composition, 20% acetonitrile in water. All other conditions as in Table I.

always below 0.1 a.u., the detection window remaining is *ca.* 0.9 a.u., and thus no effect on peak areas was anticipated. To confirm this hypothesis and to detect any other unforeseen problems, we selected both the purest water and the least pure distilled water available to us by methods described by Bristol[23] and ran the phthalate ester standards by SIS-LC. These runs were like those shown in Fig. 2, chromatogram j, and no differences in area counts or other parameters were seen between our purest water (Baker water and UV-irradiated distilled water) and the least pure water (commercial distilled water).

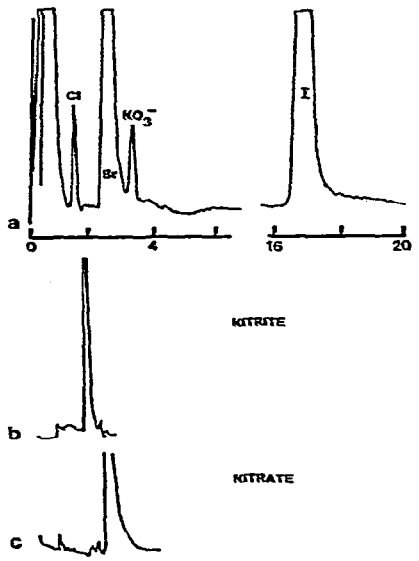

Fig. 13. LC with 190 nm UV detection of anions showing long retention with ion-pairing agent. a, Chloride, bromide, nitrate and iodide in tetrabutyl ammonium phosphate (0.003 *M*, pH 7); b, 30 nM potassium nitrite; c, 30 nM potassium nitrate, both in tetrabutyl ammonium phosphate (0.003 *M*, pH 7) in 5% acetonitrile.

Application of SIS-LC to characterize ion-pair inorganic anion separations

The usefulness of the SIS-LC approach to characterize quickly a separation mechanism with little operator attention is illustrated with the ion-pair, inorganic anion separation first described by Reeve[23]. This anion separation using 0.1% cetyl trimethyl ammonium phosphate and 215 nm UV detection was found to occur also with our 0.004 *M* tetrabutyl ammonium phosphate (TEBAP) and 190 nm detection scheme. The TEBAP buffer gave a faster equilibration (*ca.* 20 column volumes, *vs.* over 100 for cetrimide) and left sufficient UV detection window to allow our system to quantitate chloride to 1 nM whereas it had not been detected previously.

Plots of log *k'* *vs.* % acetonitrile such as shown in Fig. 14 are generated with about only 30 min operator effort, the time to construct the plot. The method simply involves making unattended SIS-LC runs for each anion of interest, using the column

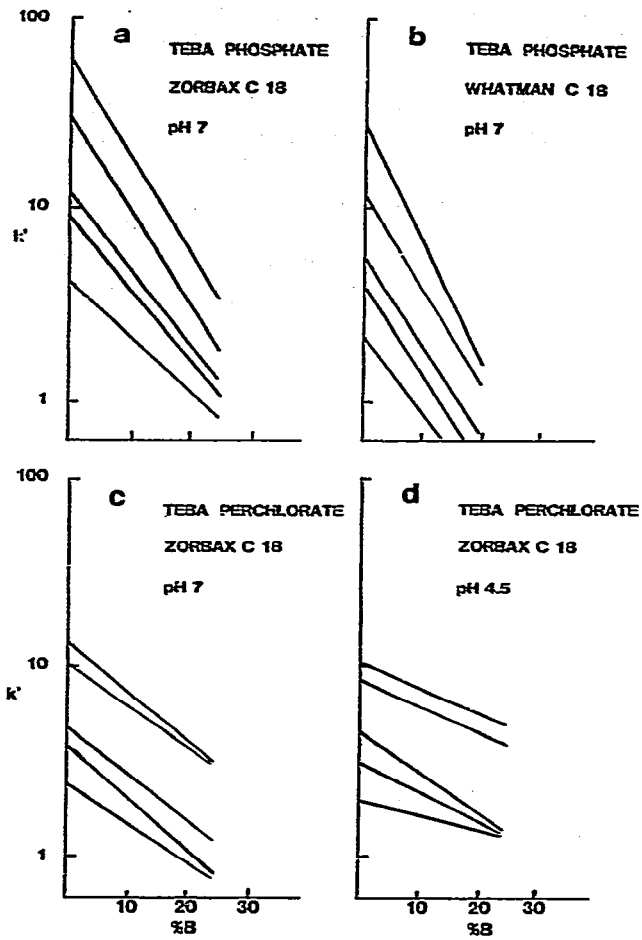

Fig. 14. Typical log *k'* *vs.* % B curves for inorganic anions Cl, Br, NO_3^-, I, $S_2O_3^{2-}$ (from bottom to top) for various columns, ion-pair agents, and pHs that can be determined with unattended overnight SIS-LC runs. Tetrabutyl ammonium (TEBA) is the ion-pairing cation. Other conditions as in Table I.

and solvent conditions for the plot desired. Fig. 14 illustrates plots for determining the effect on the log k' *vs.* % acetonitrile for various column types (plots a and b), different tetrabutyl ammonium (TEBA) counter ions (phosphate is in plot a *vs.* perchlorate in plot c) and different pHs (pH 7 is plot c and pH 4.5 is plot d). For these experiments, the TEBA ion-pair agent was placed in the "A reservoir" and the TEBA ion-pair agent with 25% acetonitrile was placed in the "B reservoir". The 20% increments in the % B program of Table I, resulted in 5% increments in acetonitrile steps. A series of SIS runs as in plots c and d when plotted for different pHs allowed the effect of pH on k' to be quickly assessed.

CONCLUSION

The SIS-LC approach allows sensitive detection of most substances with the 190 nm UV absorption, where gradient elution pilot runs fail because of baseline shifts and ghost peaks. Certain amine additive systems and inexpensive distilled water were shown to be compatable with the 190 nm detection. The unattended operation substitutes automation for expensive manpower and allows 20 or more separations to be investigated per night, for problems amenable to the reversed phase or ion-pairing mode. However, the method should extend to normal phase or ion exchange modes.

The SIS-LC method potentially can quantify in the same run both volatile components normally run by GC, such as O_2 and C_1 to C_{12} aliphatic alcohols, as well as heat-labile or non-volatile materials including Cl and other inorganic anions. Other advantages are that the UV–visible spectra of all components in a complex mixture can be obtained simultaneously and automatically and coincidence of elution in several SIS steps corroborates proof-of-identity of unknowns.

Finally, the "best" isocratic separations as well as comparisons of acceptable to unacceptable lots, and new to control lots is possible with sensitive general detection over the wide polarity range from full aqueous to full acetonitrile elution.

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