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# UNIVERSAL LIQUID CHROMATOGRAPHIC METHODS

## III. SENSITIVE, LOW-WAVELENGTH GRADIENTS WITH A NOVEL INJECTION-LOADING ION-PAIRING TECHNIQUE*

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SUMMARY

This paper extends to the ion-pairing mode the "universal methods" previously reported for the reversed-phase mode. These methods offer savings of laboratory and customer time and cost as they often allow "first run problem solving", reduce method development for each new problem and allow different separation problems to be run in sequence with no system changes. Major and minor components of unknown wide-polarity mixtures are analyzed with the near-universal detection possible at 210 nm.

A new technique of injection loading an ion-pairing (IP) agent with an automated liquid chromatograph into simple buffered eluents is introduced. Full gradients from aqueous buffers to acetonitrile are achieved at 0.1 a.u.f.s. and 210 nm without ghost peaks or baseline shifts (less than 5%). Additionally, in three consecutive 12-min runs, the three liquid chromatographic modes (with different selectivities), *viz.*, cation-pairing, anion-pairing and simple reversed-phase, can be explored in order to optimize the separation of a particular sample. The charge of the individual components at the eluent pH can be determined by the movement of peaks in consecutive runs. Other advantages of injection loading are that the pump and column life are preserved, only small amounts of clean ion-pairing agents are required and the effect of new ion-pairing agents on a sample can be investigated in one run. We also present a new technique for achieving flat 210-nm UV baselines. This is accomplished by bubbling a mixture of nitrous oxide (UV absorbing) with helium of a composition that varies the aqueous background UV absorption to match the second gradient eluent, acetonitrile.

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## INTRODUCTION

The two key elements of a universal method are that most components can be eluted and most components can be detected in a single run (elute and detect most components). Ideally, universal methods should quantitate both the main components and trace impurities of almost any sample while covering wide polarity ranges of elution automatically. Sensitive "general detection" of most organic compounds at low levels (detectable with 0.1 a.u.f.s.) can be obtained with low-wavelength 210-nm UV detection using either the "sequential isocratic step" approach[1] for many chromatographic modes, or the universal gradient systems developed recently[2] for the reversed-phase (RP) mode.

One problem with these previous reversed-phase universal full gradient (aqueous to acetonitrile) 210-nm systems is that the selectivity is not easily changed. If the eluent–column combination used does not give an adequate resolution of the peaks of interest in a sample, changing the selectivity requires a change of columns and/or eluents, and even then the selectivity changes often are not predictable. The three solvent systems compatible with 210-nm detection previously developed[2] (a pH 3, a pH 9 and an unbuffered aqueous system) have the disadvantage that the change-over in order to vary the selectivity is time consuming.

This paper extends the universal chromatography concept to the ion-pair mode and introduces injection loading of an ion-pairing agent with full gradients to explore automatically different modes of separation in rapid succession using one column and one mobile phase. Instead of putting an ion-pairing agent in the aqueous eluent as is done classically, one or more injections of very concentrated ion-pairing agent (1 *M*) are made a few seconds before the sample is injected. A full aqueous to acetonitrile gradient is then run to elute the sample. As the aqueous eluent containing a low level of triethylamine phosphate (5 m*M*) also elutes the ion-pairing agent, the retentions and peak areas are reproducible from run to run. The ion-pairing agents and eluents are pre-selected or purified so as to give a clean, flat baseline at 210 nm.

One useful set of full gradient runs at 210 nm is the sequence "cation-pairing/anion-pairing/reversed-phase". The reversed-phase mode gives qualitative information about the relative polarities of the peaks with the least polar peaks eluting last. Additional qualitative information about the charge (positive or negative) on individual components at the eluent pH is developed in the cation- and anion-pairing runs. Thus in *ca.* 40 min, three different modes of chromatography each with different selectivities are carried out to improve the chance of achieving full resolution, and qualitative information on the charge and relative polarity of the components is obtained.

The effects of the number of injection loads, the nature of the ion-pairing agent and other characteristics of the injection-loading mode were studied.

Qualitative information on the charge of a component at the eluent pH has been obtained previously. This followed from earlier observations on peak retention change *versus* charge and concentration of ion-pairing agent by Bidlingmeyer *et al.*[3]. It was found that if the ion-pairing agent and sample are of opposite charge, the retention increases with ion-pairing concentration; an ion-pairing agent and sample for of the same charge, the retention decreases with increasing concentration of ion-pairing agent; for neutral samples, the retention changes very little with concentration

of ion-pairing agent. However, these observations gave no simple, practical way of investigating the component charge as very long times are required to change eluents containing an ion-pairing agent. A second method of identifying the charge of components was demonstrated by Denkert *et al.*[4] using a single-run technique. The work dealt with non-UV-absorbing sample ions and an UV-absorbing ion-pairing agent in the mobile phase, the charge of a particular peak being deduced from whether it was positive or negative (vacancy-like). While having the advantage of detecting non-UV-absorbing samples, quantitation is difficult with UV-absorbing samples and only isocratic runs of limited polarity range were run.

Normally ion-pair chromatography is performed under isocratic conditions[5] or gradients over short polarity ranges[6,7]. A wider polarity range ion-pairing run with 5 m*M* butanesulfonic acid from 40% tetrahydrofuran in water to 40% tetrahydrofuran in acetonitrile was demonstrated for ion-pair chromatography by Folonari and Garlasco[8]. The limited use of full gradients in ion-pair chromatography was pointed out by Jandera and Churacek[9], who used wide gradients from 0 to 40% methanol in water to separate highly polar aromatic sulfonic acids but employed strong inorganic electrolytes instead of ion-pairing agents. Brown *et al.*[10] used a limited polarity gradient scheme with ion pairing to separate dansylated polyamine derivatives in blood and urine. Their system consisted of 50% acetonitrile in 0.02 *M* 1-heptanesulfonic acid (Waters PIC-B7) and a gradient of up to 80% acetonitrile. Citti *et al.*[11] developed an isocratic ion-pairing separation of the five DNA bases but found it necessary to add a limited gradient at the end of the run in order to elute adenine. The isocratic portion used 0.7% methanol in 20 m*M* potassium phosphate buffer containing 5 m*M* sodium heptanesulfonate and the gradient was run up to 14% methanol.

The concept of "injection loading" an ion-pairing agent into a system is new. Under these conditions, no ion-pairing agent is present in the eluent and a full gradient containing quaternized triethylamine removes the injection-loaded ion-pairing agent to return the column to a reproducible state for the next run. Injection loading, however, has been used for other purposes. Crommen *et al.*[12] used injection loading of an ion-pairing agent to shorten the long equilibration time usually required for isocratic chromatography with an ion-pairing agent in the mobile phase. Euston and Baker[13] used injection loading to load large amounts of sample on to a column for trace analysis. The first "injection loading" was that by Huber *et al.*[14] (see also Snyder and Kirkland[15] and Berry and Engelhardt[16]), in which injection loading of the immiscible water-rich phase was used to speed up the equilibrium loading with normal-phase partition liquid chromatography (LC).

## EXPERIMENTAL

### *Materials*

The "eluent conditioner columns", triethylamine phosphate (TEAP) buffer and solvents that are necessary to obtain the flat 210-nm, full-gradient baselines were prepared as described previously[2].

Brownlee Spheri 5, particle diameter, $d_p$ = 5 $\mu$m, RP-18, guard columns (3 × 0.46 cm) preceded analytical columns (10 × 0.46 cm) (American Lab Supply, Babson Park, MA, U.S.A.). Acceptable ion-pairing agents for injection loading were tetrabutylammonium hydrogen sulfate (TEBA-HS) from Aldrich (Milwaukee, WI,

U.S.A.) and the anionic agents such as sodium pentanesulfonate ($NaC_5S$) through dodecane sulfonate ($NaC_{12}S$) from Regis (Morton Grove, IL, U.S.A.).

At the end of the paper, a preparative ion-exchange technique for cleaning up the ion-pairing agent is reported. Tetrabutylammonium (TEBA) hydroxide was cleaned up sufficiently to be added to the aqueous mobile phase in the classical manner used for ion-pair LC. The low-pressure ion-exchange approach used the following acid-resistant system. A ceramic and Teflon pump (No. 5550-1296, Fluid Metering, Oyster Bay, NY, U.S.A.) was used with a 25 × 0.9 cm all-glass and Teflon column with a plunger (Rainin, Woburn, MA, U.S.A.) packed to 15 cm with Dowex 50-X8 ($H^+$), 100–200 mesh (Fisher Scientific, Fair Lawn, NJ, U.S.A.).

*Programming the Hewlett-Packard LC system for sequential cation-pairing, anion-pairing, reversed-phase runs by injection loading*

Fig. 1 shows a typical chromatogram for carrying out sequential cation-pairing, anion-pairing and simple reversed-phase runs. With each new vial the new "change–run, stop–run" time is inserted into the time program, displacing the old run time. Thus the programming is very simple (*e.g.*, 1-0, TIME 0.5, STOP RUN; 2-0, TIME 10, STOP RUN; 3-0, TIME 0.5, STOP RUN; 4-0, TIME 10, STOP RUN, etc.). The injection sequence is explained in Fig. 1. Water was the sample in this control run. Identical reversed-phase runs (runs 2 and 4) after cation and anion

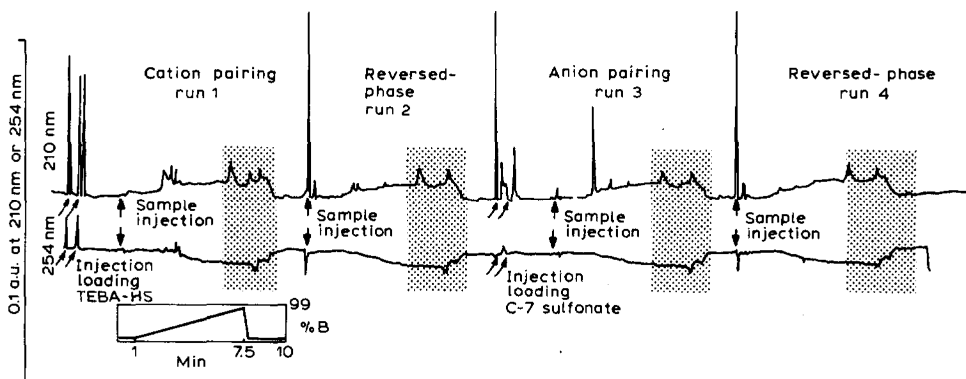


Fig. 1. Typical low-noise baselines for the run sequence showing injection-loading cation-pairing/anion-pairing/reversed-phase "universal LC" with the advantages of sensitive low-wavelength (210 and 254 nm) full gradients. Injection loading of ion-pairing agent (curved arrows) from vial 1 of two 8-$\mu$l samples of 1 *M* cation-pairing agent, tetrabutylammonium hydrogen sulfate (TEBAHS), after a wait of 0.5 min followed by "cation-pairing run 1" with a first 8 $\mu$l injection of sample (straight arrow) from vial 2 and a full gradient. A second injection of sample from vial 2 and a full gradient gives "reversed-phase run 2", as the gradient of run 1 elutes ion-pairing agent from the column. Injection loading of two 8-$\mu$l portions of 1 *M* anion-pairing agent, sodium heptanesulfonate ($NaC_7S$), from vial 3 is followed by a sample injection from vial 4 to give "anion-pairing run 3" with a gradient. A second injection of sample from vial 4 and a gradient gives "reversed-phase run 4", which may be omitted as it repeats reversed-phase run 2. Injections of 8 $\mu$l are used with a flow-rate of 6 ml/min and a C-18 5 $\mu$m $d_p$ Spheri-5 Brownlee 3 cm guard column with a 10-cm column. The 10-min gradients are from 1 to 99% acetonitrile (eluent B) in pH 3, 5 m*M* triethylamine phosphate buffer using the gradient shape shown at the bottom. This "clean" baseline requires eluent components and "eluent conditioner columns" as described under Experimental. The shaded baseline portions are in the return gradient and are used to monitor the cleanliness of the system. The "gas baseline adjustment" uses a nitrous oxide to helium pressure ratio of 10:30 p.s.i.

pairing is evidence that these middle-sized ion-pairing agents are indeed washed from the column by a single gradient with triethylamine phosphate. Other experiments in which three reversed-phase runs were made after a single ion-pairing run showed identical second to fourth chromatograms, giving further evidence that these ion-pairing agents are washed from the column by a single aqueous triethylamine phosphate to acetonitrile gradient. Large ion-pairing agents (cetyltrimethylammonium or dodecane sulfonate) are not immediately removed by a single gradient[17], and this will be discussed later.

Most automated instruments can perform injection loading of ion-pairing agents. The key requirement is that two different time programs be used on alternate runs. The Waters LC system with the Wisp Autosampler has been used with the advantage over the Hewlett-Packard 1080 that only one vial need be used for each of the two ion-pairing agents compared with placing ion-pairing agents in alternative vials, which leaves twice as many positions for samples. The new Hewlett-Packard 1090 LC system and the new IBM LC system also have this advantage.

*Matching initial and final baselines by the "gas baseline adjustment" technique*

For some runs a new "gas baseline adjustment" technique is used for increasing the 210-nm UV absorbance of the aqueous eluent to match the absorbance of the acetonitrile. By continuously flow-mixing nitrous oxide with helium, one can adjust the UV absorbance of any aqueous eluent. Resistors consisting of (old) Brownlee $C_{18}$ pre-columns (3 × 0.46 cm) ($d_p$ = 10 μm for helium and $d_p$ = 5 μm for nitrous oxide) precede a mixing tee for the gas sparging the aqueous eluent. Baseline matching at 210 nm is accomplished by adjusting the pressures of the helium and nitrous oxide (initially 30 and 10 p.s.i., respectively). Inserting the gas bubbler is simpler than the method of adding increments of an unretained UV absorber[2] to each new batch of eluent and is reversible; too high a UV baseline can be decreased by simply lowering the nitrous oxide pressure. No detection or pump bubble problems or chemical changes in the eluents are observed in the systems studied. Fig. 10 (chromatograms c and d) shows the extreme baseline shifts if no gas baseline adjustment is used. Other chromatograms use the gas baseline adjustment technique.

## RESULTS AND DISCUSSION

*Examples of separations using sequential cation-pairing, anion-pairing, reversed-phase runs by injection loading*

The clean baselines possible with injection loading and the run sequence described above are shown in Fig. 2 for anionic samples (chromatograms a-1 to a-4), cationic samples (b-1 to b-4), neutral samples (c-1 to c-4) and a complex sample mixture containing all of the above (d-1 to d-4). All are universal runs (detection at 210 nm, full gradients). The retention of 2-naphthalene sulfonate (peak 7) is 3.64 min (chromatogram a-1) in the first run compared with 2.94 min for the second run (reversed phase) (a-2) with injection loading of the cation-pairing agent tetrabutylammonium hydrogen sulfate (TEBA-HS). With injection loading of the anion-pairing agent sodium heptanesulfonate ($NaC_7S$), the retention of peak 7 decreases to 2.84 min (a-3). A reduced retention for species of the same charge as the ion-pairing agent is expected from work by others[3,17]. Note that chromatograms a-2 and a-4,

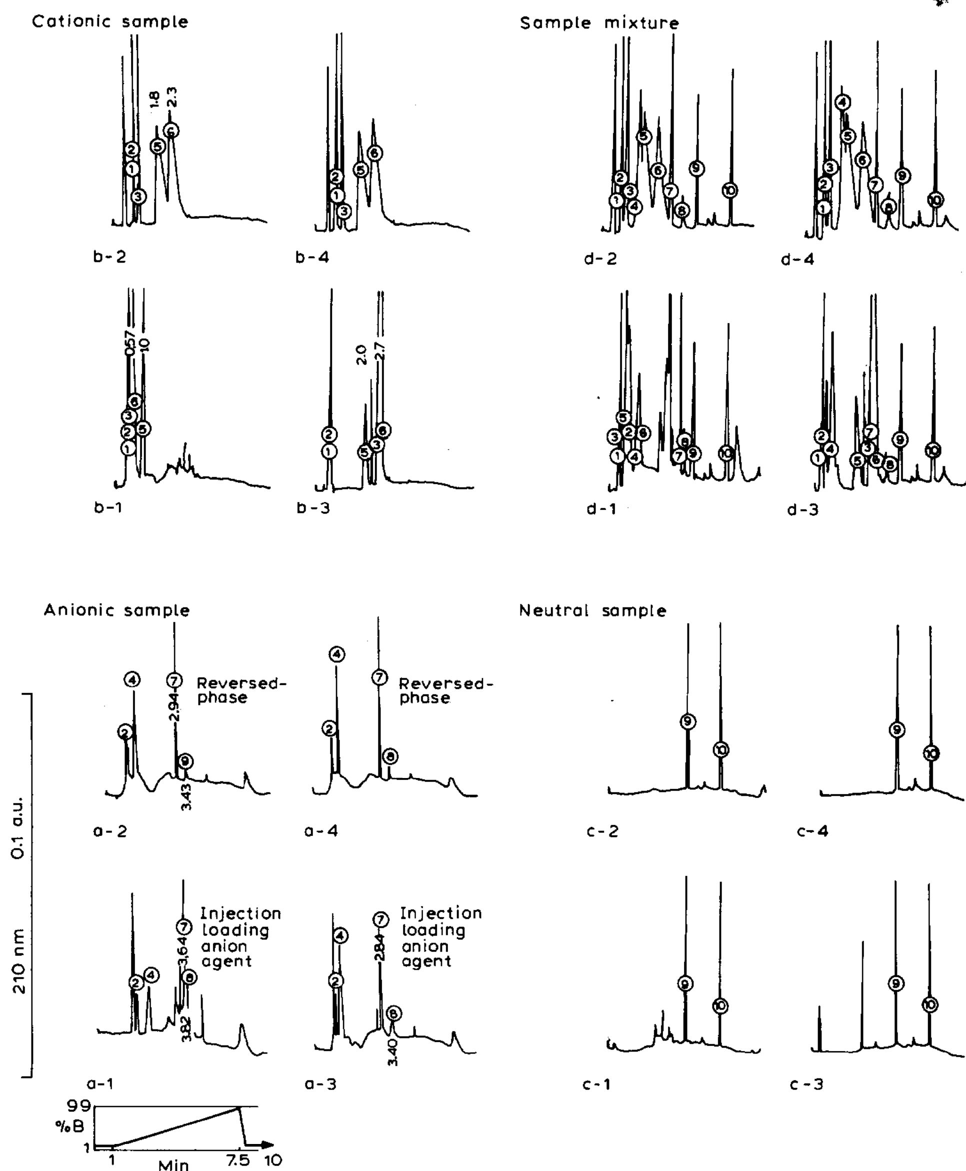


Fig. 2. Typical elution patterns for anionic samples (a-1 to a-4), cationic samples (b-1 to b-4), neutral samples (c-1 to c-4) and a mixture of all of the above (d-1 to d-4) with cation-pairing/anion-pairing/reversed-phase injection-loading runs. The first run (-1) is always injection loading of cation-pairing agent, the second run (-2) is reversed phase, the third run (-3) is injection loading of anion-pairing agent and the fourth run (-4) is reversed phase. Conditions as in Fig. 1. Peak identities and (1) tetrahydrofuran, 10,000 μmoles to dissolve sample; (2) bromide, 260 μmoles; (3) tyramine · HCl, 100 μmoles; (4) iodide, 60 μmoles, (5) trimethylphenylammonium, 200 μmoles, (6) 2,4,6-trimethylpyridinium, 300 μmoles; (7) 2-naphthalenesulfonate, 20 μmoles; (8) 3,4-dinitrobenzoate, 10 μmoles, (9) anthracene impurity; and (10) anthracene, 80 μmoles.

both expected to be in the reversed-phase mode, show identical retentions and peak patterns. As these runs follow the corresponding opposite ion-pairing run, this is evidence that these moderate sized ion-pairing agents are eluted from the column by a single gradient run with triethylamine phosphate in the mobile phase. The retention of a second anionic compound, 3,4-dinitrobenzoate (peak 8) shows a parallel effect for change in retention.

It should be noted that even small changes in retention with this fast-flow full-gradient run represent the equivalent of very large retention changes in the isocratic mode frequently used with ion-pair LC.

For cationic samples alone, the retention time of trimethylphenylammonium (peak 5) of 1 min in the first run with cation pairing (b-1) is less than the retention of 1.8 min for the second run (reversed phase) (b-2). Again, as expected, the retention time is reduced by an ion-pairing agent of the same charge as the sample. With injection loading of an anion-pairing agent, the retention of peak 5 increases to 2.0 min (b-3), indicating that ion pairing is taking place. The other two cationic samples present, tyramine (peak 3) and trimethylpyridinium (peak 6), show similar behavior.

An important observation is the extreme changes in selectivity, which vary from the peak sequence 3, 6 and then 5 for cation pairing, to 3, 5 and 6 for the reversed-phase mode and to 5, 3 and 6 for anion pairing. Further, the tailing of the poorly shaped peaks found with the simple reversed-phase mode (b-2 and b-4) are considerably reduced by either ion-pairing modes (b-1 or b-3) and the peak heights, and hence sensitivity, correspondingly increase.

As expected, the neutral samples anthracene (peak 10) and the anthracene impurity (peak 9) show no change in retention in any of the modes (*cf.*, chromatograms c-1 to c-4)[3,17]. The complex sample mixture of all of the above anionic, cationic and neutral species is shown in chromatograms d-1 to d-4. Note that the retention of each component is independent of the other components of opposite charge. No ion pairing between compounds appears to be important here as 16 $\mu$mole of ion-pairing agents are injection loaded compared with less than 0.0024 $\mu$mole of each sample.

*Effect of number of injection loads on retention*

Figs. 1 and 2 show the injection loading of 16 $\mu$mole of ion-pairing agent (two 8-$\mu$l injections of 1 *M*). Fig. 3 shows the effect of varying the number of loading injections from zero, *i.e.*, the simple reversed-phase mode, then 1, 2, 4 and 7 injections (equivalent to 0, 8, 16, 32 and 56 $\mu$mole of ion-pairing agent) for both the cationic tetrabutylammonium hydrogen sulfate and the anionic heptanesulfonate. In each instance the trends noted above for changes in retention *versus* charge of sample and ion-pairing agent are followed.

With injection loading of the cation-pairing agent tetrabutylammonium hydrogen sulfonate, the anionic sample 2-naphthalenesulfonic acid (peak 7) increases in retention and forms a plateau after only one injection (O—O). With injection loading of the anion-pairing agent heptanesulfonate, one injection load causes a large decrease in the retention of the sample (O---O). A plateau in graphs of retention *versus* ion-pairing agent concentration is well recognized in isocratic ion-pair chromatography[19,20].

For a cationic sample, *e.g.*, tyramine, injection loading of the opposite-charge

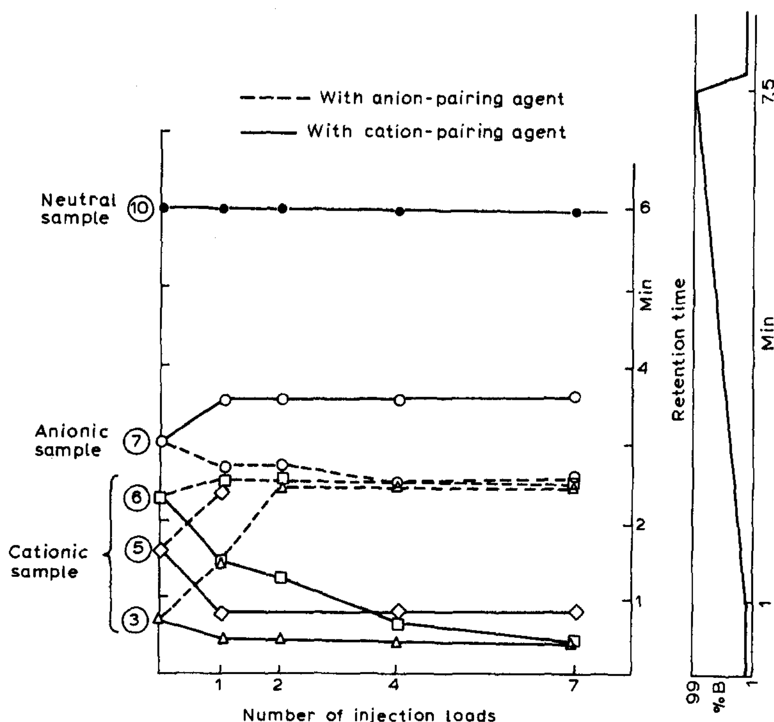


Fig. 3. Effect of number of injection loads on retention time with injection loading of cation-pairing agent, TEBA-HS (solid lines), and anion-pairing agent, $NaC_7S$ (broken lines), for a neutral sample (anthracene, peak 10), an anionic sample (2-naphthalenesulfonic acid, peak 7) and cationic samples (tyramine, peak 3; trimethylphenylammonium, peak 5; and 2,4,6-trimethylpyridinium, peak 6). Zero injection loads are simple reversed phase. Other conditions as in Fig. 1.

anion-pairing agent increases the retention but two injections are required before a plateau in retention is reached (△---△). Note that all three cationic samples, trimethylphenylammonium, trimethylpyridinium and tyramine ions, show the same plateau in retention as the anionic sample, at 2.3 min. A possible reason why so many compounds elute at the same point may be that the anion-pairing agent, heptanesulfonate, is eluted from the column at this point. If this is true, an anion-pairing agent that is retained longer, such as octanesulfonate or dodecanesulfonate, should eliminate this effect of identical plateau regions for different compounds. With injection loading of a cation-pairing agent of the same charge, tetrabutylammonium hydrogen sulfonate, two cationic species, tyramine (peak 3) and trimethylphenylammonium (peak 5), show a large decrease in retention with only one injection of ion-pairing agent. However, the longer retained cationic sample, trimethylpyridinium ions, shows a decrease in retention that continues up to seven injections (□—□). Hence the selectivity can easily be changed by varying the number of injection loads. A separation of a complex mixture with the optimal resolution of the most peaks is obtained by programming the LC system to try 1–7 injection loads.

*Effect of chain length of ion-pairing agent on retention*

The effect on retention of the chain length of the anion-pairing agent with injection loading can be seen by comparing Fig. 4, using sodium dodecanesulfonate and sodium pentanesulfate ($NaC_5S$), with Fig. 2 (chromatograms d-3 and d-4), using sodium heptanesulfonate ($NaC_7S$). Note that with the longer retained dodecanesulfonate, the cationic components are retained considerably longer (peaks 5 and 6, Fig. 4, chromatogram a) compared with Fig. 2 (chromatogram d-3).

The reversed-phase run with dodecanesulfonate (Fig. 4, chromatogram b), even when using TEAP as the eluent, shows evidence of carry-over to a second run in the retention of the cationic peak 5 (3.2 min with dodecanesulfonate compared with 1.6 min with pentanesulfonate). Carry-over of ion-pairing agent could be a problem in that a continuous build-up during a series of runs would result in changed retention times[17].

Injection loading of pentanesulfonate gives no ion-pairing effect (Fig. 4, chromatograms e and f). Only sulfonates with a $C_7$ or longer tail have the potential of giving differing peak patterns when TEAP is used in the eluent. Ion-pairing agents with different tail lengths can be used to optimize the separation.

Testing ion-pairing agents for cleanliness of the baseline is as simple as adding a sample of water. Note that pentanesulfonate shows a clean baseline (chromatograms g-h) but that dodecanesulfonate shows some ghost peaks (c and d).

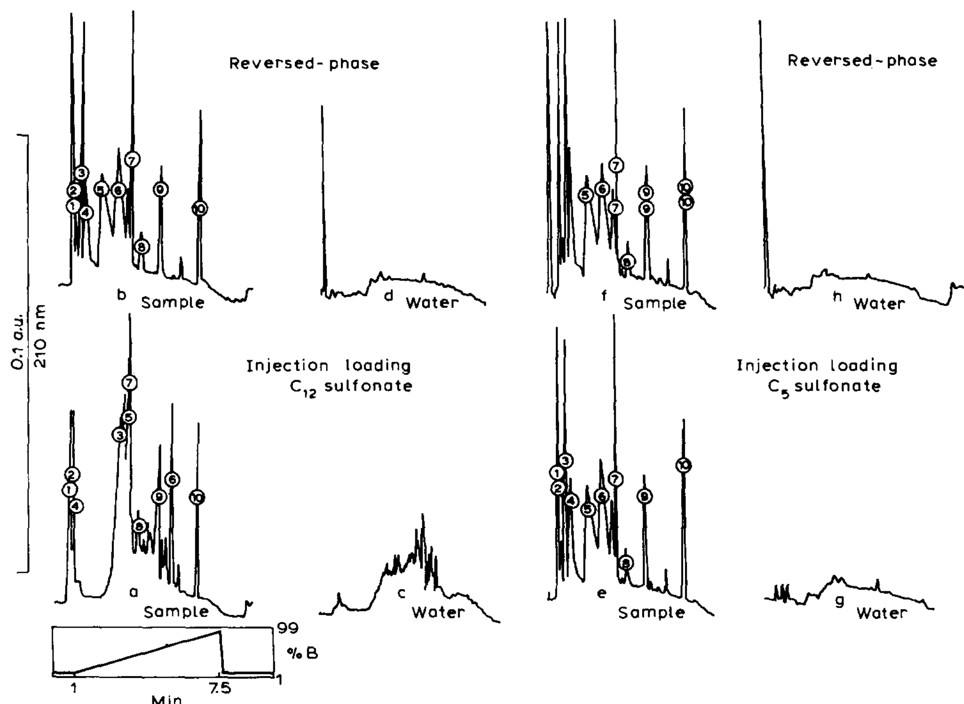


Fig. 4. Effect of chain length of injection-loaded anion-pairing agent. With no sample injection, $NaC_{12}S$ shows some dirt (c) and $NaC_5S$ a clean baseline (g) compared with the simple reversed-phase control runs [(d) and (h)]. The $-C_{12}S$ increased the retention of cationic components (a) (peaks 3, 5 and 6) compared with $-C_7S$ (Fig. 2, chromatogram d-1) as the $-C_5S$ shows no retention changes (e) compared with the control, the run with no ion-pairing agent (f). Peak identities and run conditions as in Fig. 2.

*Removing long-chain ion-pairing agents from columns*

Two techniques were found to elute most levels of long-chain ion-pairing agents from columns. For large cation-pairing agents, such as cetyltrimethylammonium hydroxide, the acetonitrile concentration is held at 99% and a short-chain ion-pairing agent of opposite charge, pentanesulfonate ($NaC_5S$), is injected at the end of the gradient run. The column retention returns to that found for the simple reversed-phase mode. A cetyltrimethylammonium pentanesulfonate ion pair forms, which is eluted by the acetonitrile.

Another method was found for removing large anion pairing agents from the column, such as methylene blue, used in Gnanasambandan and Freiser's technique[21] for the ion-pairing separation of neutral species. The progressive plug-like loading of the blue dye from the inlet on a Waters radial compression separation system $C_{18}$

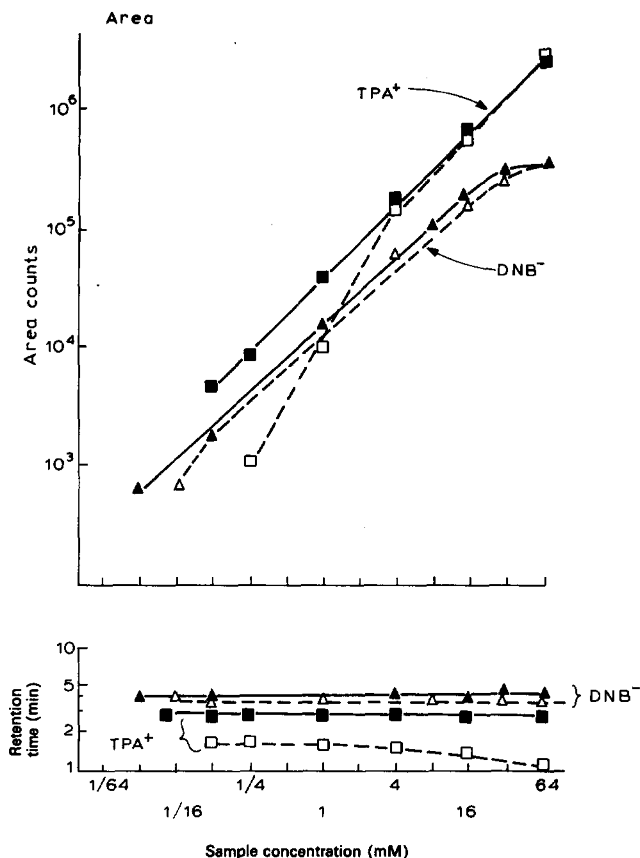


Fig. 5. Sample linear range with injection loading of cationic and anionic ion-pairing agents. The area (upper plot) for cationic trimethylpyridinium ($TPA^+$) with $-C_7S$ ion-pairing agent (■) shows greater linearity than the $TPA^+$ with reversed-phase runs (□), as does the area for anionic dinitrobenzoic acid ($DNB^-$) with TEBA-P ion-pairing agent (▲) *vs.* DNB with reversed-phase runs (△). The lowest area counts indicate the detection limits (lower for ion pairing). The retention times (lower plot) for samples remain constant except for $TPA^+$ with the reversed-phase mode, which shows decreased retention at high loadings. Injection volume 8 $\mu$l. Conditions as in Fig. 2.

column could be followed visually as the column is translucent. Acetonitrile alone does not remove the dye. However, the column is returned to a clean, white state by an acetonitrile eluent containing tetrabutylammonium phosphate. These examples suggest that injection loading of low-molecular-weight ion-pairing agents will elute high-molecular-weight ion-pairing agents from a column when a strong eluent is used, such as acetonitrile.

*Linearity of plot of peak area versus retention time with injection loading of ion-pairing agents*

With injection loading of an ion-pairing agent, the calibration graph is more linear than with simple reversed-phase runs (Fig. 5) and shows linearity over three orders of magnitude for both trimethylphenylammonium and 3,4-dinitrobenzoate.

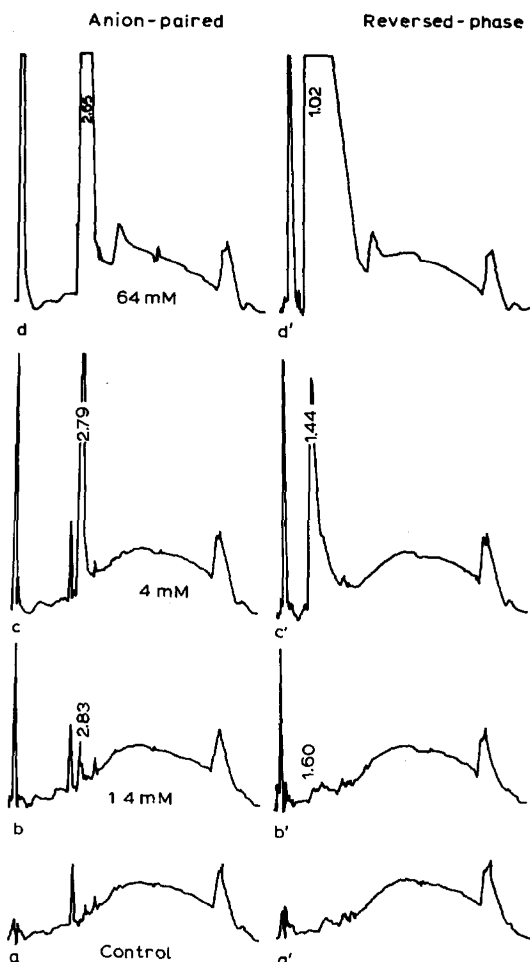


Fig. 6. Typical calibration chromatograms for trimethylphenylammonium bromide by ion pairing with injection loading (2 × 8 $\mu$l) of $NaC_7S$ (1 *M*) (left chromatograms) and by the simple reversed-phase mode (right chromatograms). Conditions as in Fig. 2.

Fig. 5 also shows that the retention times of these components do not change over this sample range. The observed non-linearity of the DNB curve at a loading of 64 m$M$ solution (*i.e.* 0.5 $\mu$mol in 8 $\mu$l) is due to overloading of the detector at 254 nm. The retention with the simple reversed-phase mode decreases from 1.6 to *ca.* 1.2 min as the sample load increases (Fig. 5, lower plot, broken lines). For a gradient run, this is a large change. This represents column overloading, which is not seen in the ion-pairing run.

The sharper peaks with the ion-pairing *versus* the reversed-phase mode (Fig. 6, chromatogram b *versus* b', c *versus* c') show a factor of two lower detection limit for both $TPA^+$ and $DNB^-$ (Fig. 5). Further, there is no evidence with the system shown here of depletion of the ion-pairing agents when 16 $\mu$mol of ion-pairing agent were injection-loaded compared with 0.5 $\mu$mol for the most concentrated sample.

*Purification of ion-pairing agents for injection loading at 210 nm*

At 254 nm, clean and flat baselines[2] can usually be obtained with many readily available ion-pairing agents. At 210 nm, fewer ion-pairing agents can be used directly (see Experimental section). Chloride, hydroxide, hydrogen sulfate or phosphate counter-ions are used directly as they do not absorb at 210 nm. Cationic tetrabutylammonium salts with counter ions (or buffers) containing acetate, bromide, iodide, nitrate, chlorate, etc., cannot be used as these counter ions will obscure a section of the chromatogram after injection loading. The Waters Assoc. $C_6$ sulfonate (Low UV PIC B-6) gives acceptable baselines even with four 8 $\mu$l injections of the 0.25 $M$ undiluted concentrates.

The injection-loading method can easily be used to rapidly evaluate purification procedures for ion-pairing agents. The injection-loading method requires only about 10 min for an injection-loading control run to show impurities, and 10 min for a sample run to confirm ion pairing. Moreover, as only about 16 $\mu$mole are required (*ca.* 2 mg of tetrabutylammonium hydrogen sulfate) even analytical LC columns can be used for their preparation.

*Elimination of impurity ghost peaks with a cation-pairing agent in the eluent*

Fig. 7 (right) shows that the areas and heights of the ghost peaks increase in direct proportion with the "loading" time between runs. When an ion-pairing agent is present in the mobile phase, in the conventional manner, it is necessary to reproduce the time between runs in order to fix the areas of the ghost peaks contributed by any ion-pairing agent in the eluent. When short times are used between runs, the ghost peak interferences will be less. Enough time must be provided to "load" sufficient ion-pairing agent for ion pairing to take place. With injection loading it is not so critical to keep constant the time between runs as the ghost peak pattern is dependent only on the purity of the injection-loaded ion-pairing agent and not the time between runs.

Fig. 7 (chromatogram a) shows a typical impurity pattern with a cation-pairing agent in the eluent, prepared from Eastman tetrabutylammonium hydroxide titrated with phosphoric acid to pH 3. Even though this TEBA-OH is one of the cleanest eluents available, the 210-nm peak pattern was too large to permit the impurity peaks to be distinguished easily from sample peaks.

Fig. 8 compares the 210- and 254-nm impurity levels of some other sources of

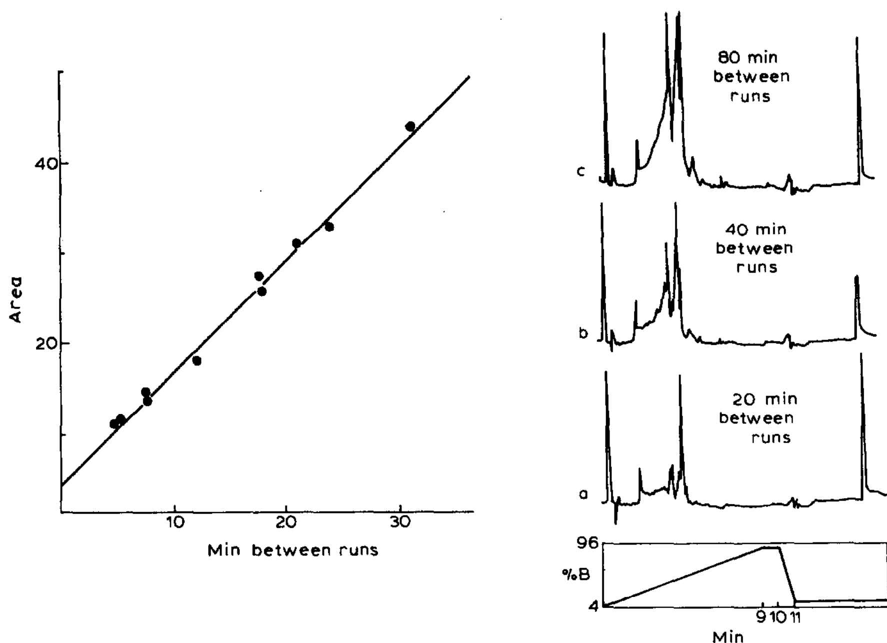


Fig. 7. Chromatograms (right) showing increase in ghost peaks found as the time between runs is increased with 2 m*M* TEBA-P in the mobile phase. The plot shows that the area of the largest peak (4.6 min) increases in proportion to the time between runs. A flow-rate of 4 ml/min was used with a 4–96% gradient and no aqueous eluent conditioner column.

tetrabutylammonium hydroxide. Fisher tetrabutylammonium hydroxide (chromatogram a) gave such a high 210-nm absorption, even at 2 a.u.f.s., that the 210-nm signal was completely blocked. Chromatogram b shows the peak pattern for Waters Assoc. Low UV PIC A, for which only one major peak occurs in the center of the run at pH 2.8. This Low UV PIC A at its "natural" pH of 7.5 (but at 2 m*M* compared with the

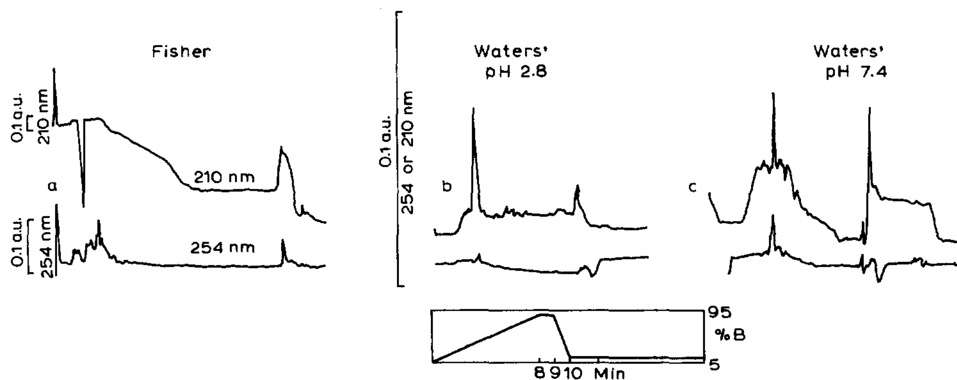


Fig. 8. Illustration of the very high impurity level with Fisher TEBA-P in the mobile phase, obtained by titrating 2 m*M* TEBA-OH to pH 7 with phosphoric acid (a). Note the short 0.1 a.u. marker and the fall in the 210-nm baseline during the gradient. Impurity peak pattern found with Waters Assoc. TEBA made from "Low UV PIC A" at 2 m*M* when the pH is reduced from the natural value of 7.4 (c) to 2.8 (b) by titrating with phosphoric acid. Conditions as in Fig. 7.

5 m*M* intended) showed a considerable "mid-gradient" hump at 210 nm compared with the same agent at pH 2.8 (cf., chromatograms b and c).

The Fisher tetrabutylammonium hydroxide (0.4 *M* in water) is an example of a dirty starting material that would readily show successful clean-up (see Experimental) using the following approach. The $TEBA^+$ displaces the $H^+$ from the strongly acidic ($RSO_3^-$) cation-exchange resin. With alkaline tetrabutylammonium hydroxide (pH *ca.* 11), primary, secondary and tertiary amines are in the free base form and can be eluted from the cation exchanger with acetonitrile (AN) (Fig. 9, at *ca.* 15 min). The purified TEBA is then eluted with 1 *M* hydrochloric acid. The second 2-ml fraction of hydrochloric acid contained the cleanest tetrabutylammonium chloride, as can be seen in Fig. 10, chromatogram b. This run (at 0.1 a.u.f.s.) compared with the initial material (Fig. 8, chromatogram a, 210 nm at 2 a.u.f.s.) shows that the ion-exchange approach reduces the absorbance by over 1000-fold and gives material usable at 210 nm.

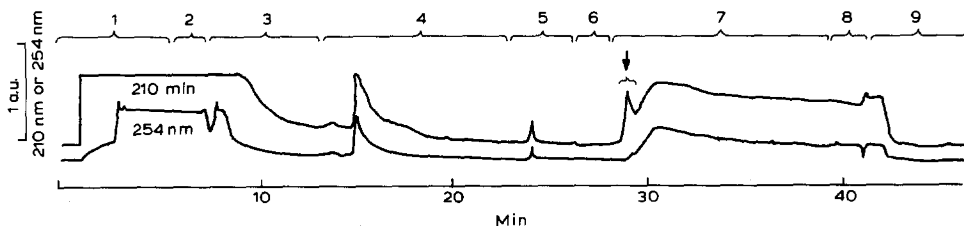


Fig. 9. The 254- and 210-nm chromatograms of the low-pressure preparative cation-exchange purification of Fisher TEBA-OH. The second 2-ml fraction eluted by hydrochloric acid (arrow) is the purest. Steps in the clean-up: (1) load the column with 0.4 *M* Fisher TEBA-OH, 25 ml; (2) flush the lines with water; (3) flush the column with water, 25 ml; (4) wash the column with acetonitrile, 40 ml; (5) flush the column with water, 15 ml; (6) flush the line with 1 *M* HCl; (7) wash the column with 1 *M* HCl, 40 ml (the 2–4-ml fraction is the cleanest); (8) flush the line with water; (9) the wash column with water, 25 ml. Flow-rate: *ca.* 3.6 ml/min.

This preparation of cleaned-up ion-pairing agent showed that it is indeed possible to obtain a relatively clean baseline with 210-nm detection and full gradients with the concentional technique of mixing ion-pairing agents in the mobile phase. The ion-pairing agent itself gives no major peaks at 210 nm.

## CONCLUSION

Injection loading of ion-pairing agents offers a number of advantages besides just extending the "universal LC" approach from the simple reversed-phase to the ion-pairing mode (elute and detect most components with 210-nm full gradients). With an ion-pairing agent in the eluent, ion pairing can be used in place of ion exchange to give the advantages of sharper peaks, more stable and reproducible methods and more varied selectivity by changing the ion-pairing agents[22], and the ability to separate both ionic and non-ionic materials in the same run. Injection loading of ion-pairing agents with gradients allows fast, systematic variation of selectivity, identification of the charges of peaks, decreased tailing and improved sen-

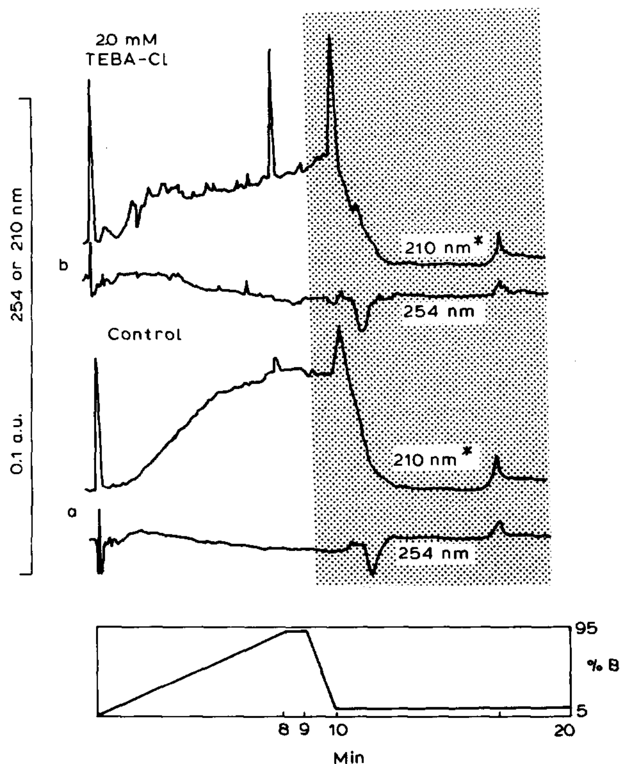


Fig. 10. Successful clean-up of Fisher TEBA-OH by over 1000-fold (b) compared with the starting impurity level (Fig. 8, chromatogram a) using the preparative ion-exchange clean-up of Fig. 8. The control run (a) is water with no ion-pairing agent. Chromatogram a shows the extreme shift in baseline where no "gas baseline adjustment" with nitrous oxide–helium mixtures is used to match the initial and final baselines (see Experimental). Conditions as in Fig. 7.

sitivity, high confidence in identification if the cation-pairing/anion-pairing/reversed-phase sequence shows the same retentions for the known and unknown peaks and confidence that a specific peak is a single component if the peak shifts in the sequence but no new peaks appear. Different ion-pairing agents with differing charges and hydrophobicities can be investigated. The corrosive and clogging effects of ion-pairing agents on pumps is eliminated. Recent work with anionic polymers indicates that injection loading of the small cationic tetrabutylammonium phosphate during the intermediate (control) runs with water prevents carry-over of polymer.

Two limitations of the injection-loading technique have been pointed out: a fully automated LC system is required to make practical the complex injection sequence and "clean" ion-pairing agents must be selected if runs are to be made at 210 nm.

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