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UNIVERSAL LIQUID CHROMATOGRAPHY METHODS

IV. USE OF 190-nm FULL GRADIENTS BY ADJUSTMENT OF ELUENT ULTRAVIOLET ABSORBANCE AND pH WITH GASES

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SUMMARY

The full-gradient "universal liquid chromatography" methods are extended to 190 nm detection for even greater sensitivity and detection of more components than shown previously at 210 nm. Universal liquid chromatography offers major time/costs savings by increasing problem solving throughout. Further, at 190 nm, simple molecules such as aliphatic alcohols are detected and 254-nm detectable species are quantified to much lower levels.

Universal liquid chromatography "elutes-all" in a first wide-polarity, full gradient run and "detects-all" with low wavelength (less than 214 nm) UV and permits easy resolution changes. A single universal method replaces many different tailored isocratic runs for first-run-problem-solving of most mixtures. These methods eliminate method development for each new problem and also permit unattended runs of totally different separation problems. Universal methods permit rapid product, mix, or raw material comparisons of good *vs.* bad, new *vs.* old, or starting *vs.* purified materials while signaling new components that might not be seen in the narrow polarity window of isocratic runs.

"Injection loading" of ion-pairing agent is also extended to 190 nm. This allows rapid run-to-run change in selectivity and exploration of ion-pairing effects plus determination of charges of ionic species. A new "gas baseline adjustment" method allows very simple eluent preparation to fix pH and give flat, ghost-peak-free baselines. A new method is given to calibrate detectors at all wavelengths and a simple "linear mixer" eliminates short-term baseline noise.

INTRODUCTION

"Universal" liquid chromatography (LC) methods offer major laboratory and customer time saving since they often (1) permit first-run-problem-solving, (2) eliminate method development time, and (3) allow different separation problems to be run in sequence with no method or solvent changes.

The key elements of a universal method are (1) most components are eluted, (2) most components are detected, and (3) resolution can be modified in a simple,

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automated manner. Ideally, universal methods should give rapid, accurate, and precise results while quantifying both main and trace components. They should be automated, have high resolution, and cover a wide range of polarity. "General detection" is approached as the wavelength of UV detection is lowered to 214 nm and below.

Previous universal methods include "sequential isocratic step" (SIS) LC for several LC modes[1] and full gradients at 210 nm UV[2] for the reversed-phase LC mode. A new simple method of injection loading ion pairing agents allows cation pairing, anion pairing, and reversed-phase modes to be explored in consecutive runs[3]. This often allows resolution to be quickly optimized and the charge of the individual components to be determined.

Recently gradients have been run at low wavelengths (185–214 nm) using reversed-phase LC. However these applications used limited gradient ranges of strong solvent. Van der Wal and Snyder[4] quantified poly(ethylene glycol) oligomers at 185 nm with water to *ca.* 30% acetonitrile gradients but higher acetonitrile concentrations led to a large mid-gradient "bump". Haeffner-Gormley *et al.*[5] analyzed lysozyme tryptic peptide pools at 205 nm with 0.1 *M* ammonium chloride (pH 4.1) to 36% acetonitrile gradients but they found an *ca.* 80% baseline shift at 0.1 a.u.f.s. With microbore columns, Schwartz *et al.*[6] analyzed a peptide mixture at 214 nm using a 0.1% phosphoric acid–10 m*M* potassium dihydrogen phosphate buffer (pH 2.3) to 50% acetonitrile gradient with a nearly flat baseline at *ca.* 0.1 a.u.f.s.

This paper extends the range of UV detection, previously limited to 210 nm[2,3], down to 190 nm. With 190-nm UV detection, even simpler species, such as aliphatic alcohols, can be detected and most other species can be quantified to much lower levels[7]. Extending full gradients to 190 nm required some new approaches to preparing LC eluents and methods for dealing with detection. Modeling experiments with an LC systems, using no column, revealed a baseline "bump" problem that may be caused by molecular association of the water and acetonitrile. The triethylamine additive was found to eliminate this problem. Carbon dioxide (CO_2) was found to be an ultra-clean counter-ion for the protonated triethylamine, so the aqueous eluent is "gassed" with this very soluble gas. A partial pressure of nitrous oxide (N_2O) in the CO_2 is used to raise the 190-nm UV absorbance of the aqueous eluent to match that of the acetonitrile to obtain a flat baseline. Special methods are used to prevent bubbles from affecting the pump reproducibility and detector signal.

EXPERIMENTAL

Apparatus

For the "gas baseline adjustment" of eluent, the following "gassing" system was used: the gas is a mixture of CO_2 to titrate triethylamine (TEA) and N_2O to achieve a flat baseline. Cylinders of "bone dry grade" CO_2 (liquid, Linde Div., Union Carbide, New York, NY, U.S.A.) and U.S.P. N_2O (Linde) were used. Both cylinders were fitted with two-stage regulators (No. 8-320, 100 p.s.i. gauge, Matheson, Glouces-ter, MA, U.S.A.). In-line restrictors made of 3 × 0.46 cm I.D. guard columns (Brownlee, Santa Clara, CA, U.S.A., particle size 5 μm, OD-GU for N_2O, and 10 μm, 18-GU for CO_2) were fit into the system with two brass Swagelock fittings (No. 140-200, Cambridge Valve and Fitting, Billerica, MA, U.S.A.), as at the gas regu-

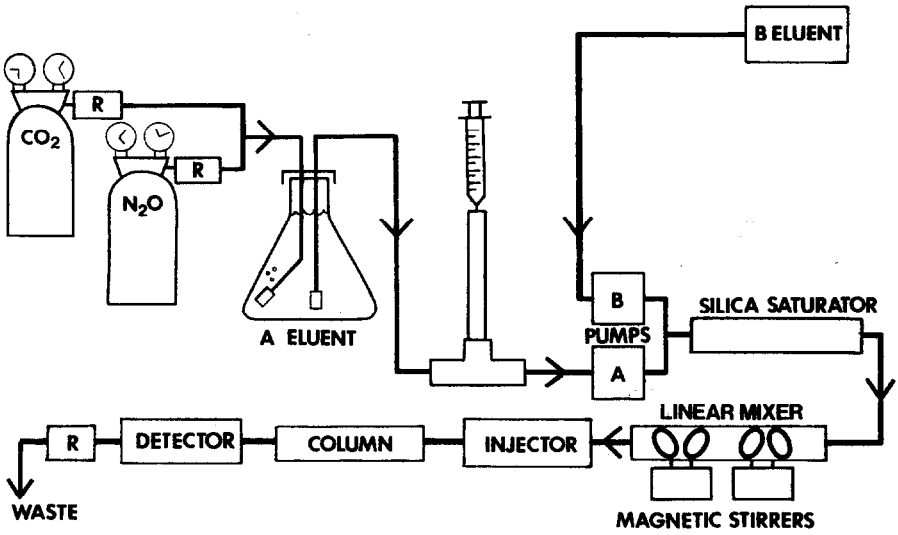

Fig. 1. Schematic diagram of the instrument components for universal liquid chromatography showing the resistors in the N_2O and CO_2 gas sources for the gas baseline adjustment of the aqueous eluent, the gas ballast/bubble trap inverted "T", the linear mixer, and the outlet resistor.

lators shown in Fig. 1. Transparent Teflon™ PFA tubing, (No. TK-6375-01, 1/8 in. O.D., 1/16 in. I.D., Cole-Parmer, Chicago, IL, U.S.A.) with Altex-type fittings (No. 200-42 and No. 200-42 Rainin Instruments, Woburn, MA, U.S.A.) were screwed into Brownlee guard column holders (with Teflon™ tape). Since flow through the restrictors is proportional to pressure, the partial pressure of N_2O is adjusted by varying the cylinder pressure. The gases are bubbled through the aqueous eluent using a metal frit (No. 05-0141, Rainin).

The aqueous flow stream uses the transparent PFA tubing (above) so that any gas bubbles in the inlet stream can be readily seen. A gas ballast/bubble trap inverted "T" is necessary at the inlet of the pump for pump flow reproducibility. This consists of a 1/4 in. stainless Swage "T" (No. 400-3, Rainin) with a vertical side-arm of 1/4 in. O.D. transparent PFA tubing (No. TK-6375-02, Cole-Parmer) and the top is fit with a 25-ml plastic syringe. Gas in the syringe acts as a "gas ballast" to minimize pump inlet pressure pulsations and to eliminate bubble formation at the pump inlet[8]. Also, any gas bubbles in the flow stream are removed by the vertical arm (partially filled with eluent).

A detector back-pressure restrictor (a No. 18-MP Brownlee guard column) after the UV detector prevents bubbles from coming out of solution and affecting the UV signal.

The Hewlett-Packard LC uses a 0.25-ml in-line mixer which is adequate for most work at detector wavelengths of 210 nm or above. However, at 190 nm (or with the refractive index or conductimetric detector), a much larger in-line high-pressure "linear-mixer" was found necessary to eliminate short-term noise (*ca.* 2–6 sec frequency at 2–4 ml/min flow). The column for the 4-ml linear mixer is 25 cm × 0.62 cm I.D. (880984804, DuPont, Wilmington, DE, U.S.A.) and the column for the 10-ml linear mixer is 25 × 0.94 cm I.D. (880984906, DuPont). Four narrow magnetic stir

bars (No. F-37120, 7/8 × 3/16 in., Bel-Art, Pequannock, NJ, U.S.A.) with their poles opposing, oscillate with the ends of the stir bars describing circles. This creates at least eight zones of opposing mixing elements. Each of two narrow magnetic mixers drives two stir bars.

Materials

RP-18, 5-μm particle size, guard columns (OD-GU, 3 × 0.46 cm I.D., Brownlee) preceded analytical columns (OD-MP, 10 × 0.46 cm I.D., Brownlee).

LC-grade water for eluent was prepared from 4 l of distilled water (Belmont Springs, Belmont, MA, U.S.A.) directly UV irradiated for 3 h with stirring in a 4-l Erlenmeyer flask with the UV lamp from a No. 16 HPLC Reservoir from Photronix (Medway, MA, U.S.A.)[9]. Triethylamine (TEA) was purified through alumina as described previously[1]. Some purified TEA was purchased (No. 25108, Pierce, Rockford, IL, U.S.A.). TEA was stored at −10°C and, when sampling, condensation of atmospheric water in the cold TEA was minimized by rapid sampling and by breaking the main fraction into smaller 50-ml portions.

The gas baseline adjustment method was used with a new simple and fast method to prepare eluent. Purified TEA, 2.069 ml, was rapidly injected with a 5-ml repeating pipet into 4 l of LC-grade water previously sparged with the CO_2–N_2O gas mix for *ca.* 15 min. No titration or other attention was necessary. The pH of the final solution is 5.3 for this 3.6 m*M* TEA solution, and pH varied from 5.2 to 5.95 as TEA concentration was varied from 1.8 to 14 m*M*. Different concentrations of TEA required different partial pressures of N_2O to match the initial and final baselines. Gas pressures that give flat baselines were *ca.* 30 p.s.i. for CO_2 and 56 p.s.i. for N_2O. Other pressures may be required depending on restrictor permeability, acetonitrile purity, etc. Once the system was set-up to hold a flat baseline, it was only necessary to change the pressure of the N_2O a few p.s.i. as batches of acetonitrile or aqueous eluent were changed.

In some work, eluents of pH's other than the "natural" pH of TEA carbonate, 5.3, were desired. "TEA (2 m*M*), phosphate (4.2 m*M*), carbonate pH 3" was prepared from 3.81 l of LC-grade water with 0.70 ml of phosphoric acid (selected for low UV)[2], 1.10 ml of purified TEA, and CO_2 sparging. The final eluent pH of 3 was adjusted with 1:10 dilute phosphoric acid and the baseline adjusted for flatness by changing the N_2O level. Some work used a potassium phosphate pH 2.3 buffer made by mixing 2 l of LC-grade water with 2 ml of concentrated phosphoric acid and 2.76 g of potassium dihydrogen phosphate[6].

Procedures

Detector response vs. wavelength. Perforated screens with punched slotted holes (Perforated Products, Brookline, MA, U.S.A.) of known transmittance (by area measurements) were used to calibrate the detector at different wavelengths, with the cell in place. The screens were 0.3 absorbance (No. 1060, 0.026, × 0.157 in. slots), 0.6 a.u. (No. 1050F, 0.020 × 0.157 in. slots), and 0.9 a.u. (No. 1040F, 0.016 × 0.157 in. slots). Zero absorbance was obtained with no screens in place and an opaque card gave infinite absorbance.

Fig. 2a shows how simply and quickly (*ca.* 10 min) this five-point calibration can be made from 190 nm to 600 nm at *ca.* 50-nm increments. With the Hewlett-

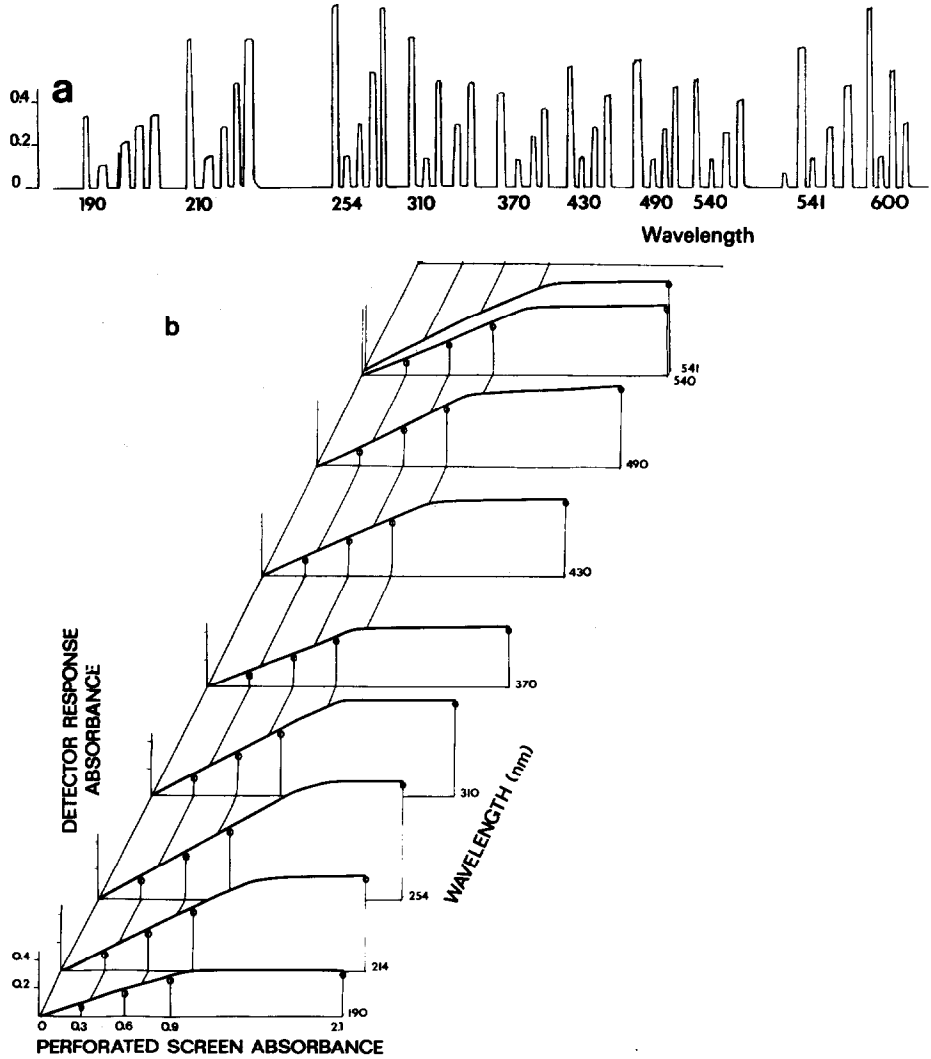

Fig. 2. Calibration of the Hewlett-Packard variable-wavelength detector using perforated screens of known absorbance (transmittance). (a) Recorder trace when wavelength is set, recorder is re-zeroed, and screens of infinite (opaque), 0.3, 0.6, and 0.9 absorbance are sequentially inserted into the light path when the detector cell is in place. (b) Three-dimensional plot of detector response (*y* axis) *vs.* screen absorbance (*x* axis) for different wavelengths (*z* axis). See text for explanation of anomalies.

Packard variable-wavelength detector, the wavelength was first entered and the detector auto-zeroed by entering "zero enter, zero enter". This recorded the 0% absorbance. The opaque card was then inserted into the light path to record the response, the wavelength then changed, the detector re-zeroed, and the first screen inserted, etc. with each additional screen. A black cloth over the cell reduced stray light with the screens in place. The Fig. 2a output can be used directly to compare the change in absorbance at different wavelengths as the lamp ages, or to compare the response, for example, at 190 nm *vs.* 254 nm.

RESULTS AND DISCUSSION

Typical simple reversed-phase 190 nm baselines

Fig. 3 shows the type of clean and flat baseline that can be obtained with high sensitivity (0.1 a.u.f.s.) and very general detection (192 nm UV) while covering the polarity range from full aqueous to full acetonitrile gradients. Chromatogram a shows a typical baseline found with 3.6 m*M* TEA treated with CO_2 to the "natural" pH of 5.3, named "TEA (3.6 m*M*), carbonate pH 5.3".

The clean-up of TEA through alumina was time consuming, and it was possible to obtain a clean and flat baseline with one source of commercially available purified TEA (Pierce, chromatogram b). However, some lots did not give a clean baseline.

The above systems used the "natural" pH (5.3) found when 3.6 m*M* TEA was titrated with CO_2 gas. A pH 3 full gradient run at 192 nm gave a flat baseline as shown in chromatogram c using the buffer called "TEA (2 m*M*), phosphate (4.2 m*M*), carbonate pH 3.0", prepared as described in the Experimental section. Other pH buffers could be prepared in a similar way.

Technique for comparing sensitivities at different wavelengths

The techniques for comparing sensitivities at different wavelengths are illustrated in Fig. 4 with a peptide mixture made from an insulin protein, hydrochloric acid hydrolysate. The improvement in detectability at 190 *vs.* 214 nm was *ca.* 1.5 to 1.0 (chromatograms b *vs.* a). The nominal setting of 0.1 a.u.f.s. gave a lowered re-

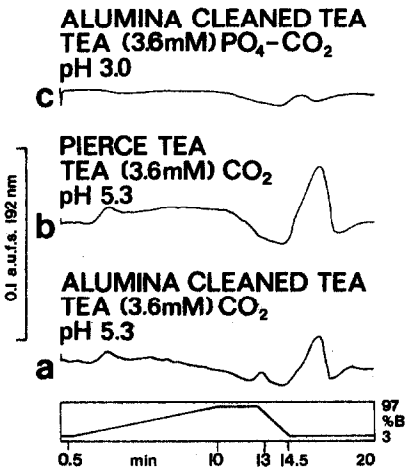

Fig. 3. Typical low noise baseline for sensitive, 190-nm UV runs using triethylamine (TEA) eluents. (a) Alumina-purified TEA (3.6 m*M*) carbonate at the "natural" pH of 5.2; (b) commercially available (Pierce) TEA (3.6 m*M*) carbonate, pH 5.2; (c) alumina-purified TEA (3.5 m*M*) phosphate (4.2 m*M*) carbonate, pH 3.0. Injections of 16 μl are used with a flow of 4.5 ml/min and a C_{18} 5-μm dp Spheri-5 Brownlee 3 × 0.46 cm I.D. guard column with a 10 × 0.46 cm I.D. column. The 20-min runs are from 3 to 97% acetonitrile in a pH 5.3 TEA carbonate buffer prepared as described in the text and using the gradient shape shown at the bottom. The baseline portions past 13 min are in the return gradient and are used to monitor the cleanliness of the system. The "gas baseline adjustment" uses a CO_2 to N_2O pressure ratio as shown for each curve with gas resistors and the 4 ml "linear mixer" as described in the text.

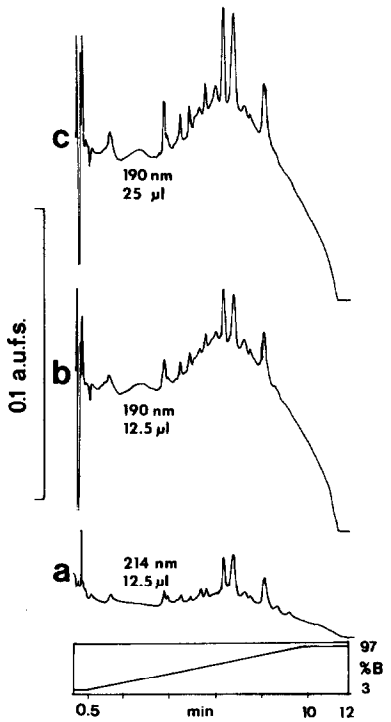

Fig. 4. Example of technique for comparing sensitivity at different wavelengths using an insulin hydrochloric acid protein hydrolysate. The peak heights at 190 nm (b) *vs.* 214 nm (a) are 1.5 to 1.0, but when corrected for the difference in true absorbance ratio (Fig. 2b of 1.40 to 1.00) give a final improvement in sensitivity of 2.1 to 1.0 for 190 *vs.* 214 nm. The doubling in peak height with doubling in sample size, (c) *vs.* (b), at 190 nm, shows that the detector is operating in the system linear range. A 15 × 0.46 cm I.D. Zorbax ODS 7.5-µm particle size column was used. Note the wide baseline shift with the gradient to acetonitrile from potassium dihydrogen phosphate buffer pH 2.3. UV absorbance adjusted to match acetonitrile using the "gas baseline adjustment" of helium and N_2O.

sponse as the wavelength went down. If a correction were made for the "true" absorbance ratio (1.4 to 1.0) as determined with neutral density screens (as explained below), the ratio of peak heights would change to 2.1 to 1.0 for the peptide mixture at 190 *vs.* 214 nm. Note that this buffer system, without TEA, shows considerable baseline shift at 190 nm (chromatograms b and c).

With this unknown mixture, the linearity of the 190 nm system was easily established since injecting twice the sample size gave peaks twice the height (and area) (Fig. 4, chromatograms b *vs.* c).

System linearity at 190 nm

The linearity of the system at 190 nm for alcohols is shown in Fig. 5. For TEA (3.6 m*M*), carbonate pH 5.3, notice the very flat baseline at 0.1 a.u.f.s. sensitivity (chromatogram a) and the usable baseline found at 0.014 a.u.f.s. (chromatogram b). The calibration plot of the areas of butyl alcohol (C_4) through C_7 alcohols was linear over a sample size range greater than 0.18–2.9 µg. It was critical to check the calibration linearity when using these low wavelengths for detection since lamp aging

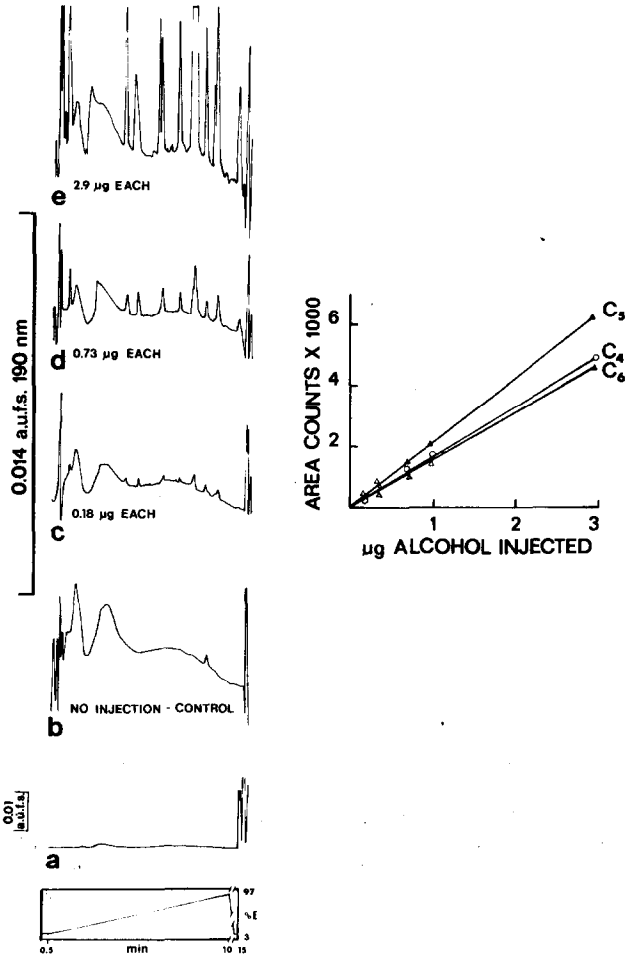

Fig. 5. System linearity for aliphatic normal alcohols with 190-nm detection showing a very flat baseline with 0.1 a.u.f.s. and no sample (a), and alcohol peaks with very high sensitivity of 0.014 a.u.f.s. (b–e), and linearity of calibration curve (right). Sample is a mix of C_2–C_{12} alcohols. In (d) the seven adjacent sharp peaks in the middle of the chromatogram are the C_4–C_{10} alcohols. Chromatography conditions as in Fig. 3.

and eluent contamination made it easier to exceed the system linear limit[2,4]. Recent variable-wavelength detectors that allow the absolute absorbance to be directly monitored, help to determine if the detector were stable; however, absorbance readings may be only approximate at low wavelengths, as discussed next.

Detector response vs. wavelength

As described in a previous paper[2], detection at low wavelengths (185–210 nm) brings a new realm of problems. The absorbance below which the detector is linear is called the "detector linear limit". This is not expected to be constant *vs.* wave length because other detector parameters vary with wavelength, *e.g.* the output of the light source and the response of the detector. Previously, it was pointed out that

the "system linear limit", in absorbance units, is the difference left between the eluent absorbance and the detector linear limit. If operated within the system linear limit, peak height (and area) will be in proportion to sample size injected, provided the column is not overloaded.

One problem is that it has not been possible to estimate the detector linear limit. In the visible region, spectrophotometers are calibrated with sodium chromate solution. However, at low wavelengths, no such standards exist. Chemical standards for low wavelength calibration would have to take into account the impurities in the standards and the solvents used to dissolve them. Also, it is probable that lamps and detectors age in different ways in the low wavelength and high wavelength regions. Thus, calibration at 500 nm may not reflect calibration at 190 nm.

A method for multi-point calibration of detectors at all wavelengths is proposed here. Screens with known transmittance (determined by area measurements) approximate neutral density filters at all wavelengths. (One error in this method comes from lower than expected responses because defraction disperses lower wavelength light more than higher wavelength light.) Fig. 2a illustrates how fast and easy it was to do a five-point calibration curve (at 0, 0.3, 0.6, 0.9 and infinite absorbance) at any wavelength (as described in the Experimental section).

Note in Fig. 2b the detector output (y axis, absorbance read off the printer) for any given perforated screen absorbance (x axis) changes with wavelength (z axis). Even with a new lamp, at 190 nm the response was nearly half the value it was at 214 nm (which was nearly identical to the 210 nm response, not shown). Also at 190 nm, there was a reduction in the maximum absorbance for which the system was linear (the intercept of the calibration curve with the infinite absorbance line). With eluent in the detector cell, this system linear limit at 190 nm would be reduced further due to the absorbance of the eluent.

By 254 nm the response was nearly constant all the way up to 540 nm. There was a discontinuity at 540 and 541 nm peculiar to this Hewlett-Packard detector. This represented the difference in response of the sample and reference photo-detectors which switch functions at this wavelength owing to geometric design of the detector.

This perforated screen calibration technique provides a simple new method to (1) compare detector response at different wavelengths, (2) follow the aging of lamps, and (3) determine the effect of the cell and eluents on the system linear limit at different wavelengths. These kinds of comparison do not require that the absorbances of the screens be accurately known.

"Gas baseline adjustment" for simple, clean eluent preparation

Originally, degassing of eluents was used to prevent bubbles from causing spikes in the detector signal or to prevent bubbles formed by cavitation on the pump inlet from affecting flow regularity. Recent uses for degassing include protecting eluents from contaminants from the laboratory atmosphere, preventing oxidation of samples, eluent, or stationary phases, and preventing oxygen interference with fluorescence, electrochemical or low wavelength UV detection. Classically eluents are "degassed" of nitrogen and oxygen by boiling via heat and/or vacuum, sonifying, vacuum/membrane degassing or by bubbling (sparging) with helium.

In contrast to these degassing methods, the eluent made here involved "gas-

sing" the aqueous mobile phase with the highly soluble CO_2 containing some N_2O. This gassing system required some precautions (see Experimental) to prevent bubble problems from affecting pump reproducibility (the gas ballast/bubble trap "T") and detector signal (the detector back-pressure restrictor)[8].

The two gases had different functions. The CO_2 was found to be a clean and convenient counter-ion for TEA, and it titrates the TEA to a fixed pH of 5.3. When water and acetonitrile are mixed there appeared an "eluent-association" bump or hump in the middle of a gradient that was eliminated by the TEA (described below). The CO_2 also served as a diluent to control the partial pressure of the N_2O. The N_2O raised the 190 nm UV absorbance of the aqueous eluent to match that of the acetonitrile. Since the N_2O is un-retained in this reversed-phase LC system, a flat baseline is obtained with the gradient. The partial pressure of the N_2O in the CO_2 determined the final UV absorbance of the aqueous eluent, and this was proportional to the gauge pressure of the gas tanks. Thus, matching the UV absorbance of the initial and final eluent was simply a matter of making minor adjustments on the tank outlet pressure.

Using different levels of N_2O, the gas baseline adjustment method was used to achieve flat baseline from *ca.* 185–230 nm (the UV absorption of N_2O). N_2O diluted with helium has been used to obtain flat baselines with TEA–phosphate buf-

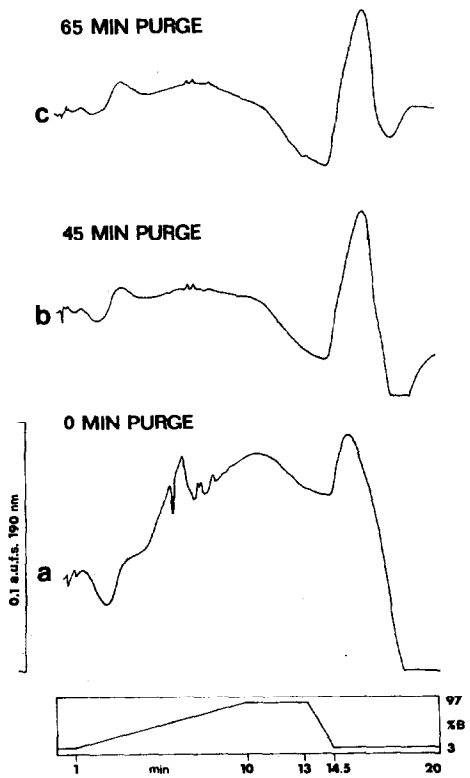

Fig. 6. Improvement of the baseline with purge time of the CO_2–N_2O mixture through TEA. Conditions as in Fig. 3.

system described above). With the SIS-LC steps at 5, 20, 40, 60, 80, and 95% acetonitrile (AN), (Fig. 10, chromatogram b), it became apparent that 95% AN had about the same UV absorbance as *ca.* 50% AN, and that the absorbance reading between 50 and 95% AN was higher. These same results were found also at 2 ml/min and 10-min long steps, indicating that the UV shift was not flow dependent. These results indicate that this bump at 190 nm is not caused by some kinetic or mixing problem.

The 5–80% AN portion of the steps in chromatogram b was nearly linear. This

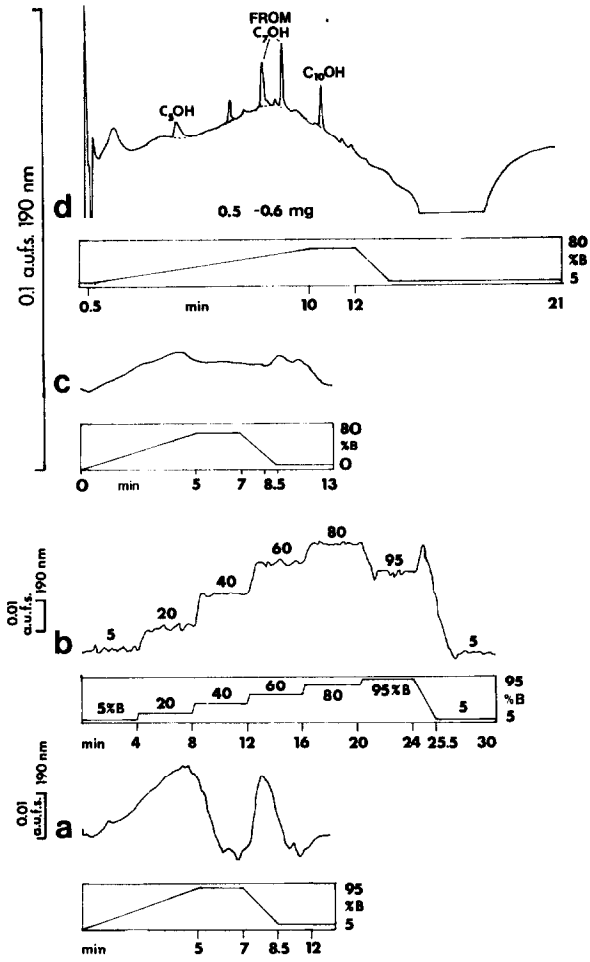

Fig. 10. Experiments to elucidate source of midgradient bump in which no columns are used at 5 ml/min for a 5–95% AN gradient with pure water adjusted to the same UV absorbance as pure acetonitrile using the gas baseline adjustment method with N_2O in helium (giving the carbonated water a pH of 5.2. (b) Sequential isocratic step runs showing that water and acetonitrile matched in UV absorbance give higher UV signals at the 80% isocratic step than at the 50 or 95% steps. (c) A 5–80% gradient showing a nearly flat baseline when the water UV absorbance is matched to that of 80% acetonitrile using the gas baseline adjustment. (d) With a column in place, and the eluents matched as in (c), an improved baseline with a 3–80% acetonitrile gradient in pure water showing aliphatic alcohols. Other conditions are shown in Fig. 3, except a 10-ml linear mixer is used.

indicated that if the 190-nm UV absorbance of water (with no additives) were matched to the UV absorbance of 80% acetonitrile, a gradient should give a flat baseline, as can be seen in chromatogram c, in this case with no column in place. When a column was used, the baseline between 5 and 80% AN is usable (chromatogram d).

This bump appeared to be caused by some property of the mixed eluent that went up to a maximum near 50–60% AN and then fell again. This parallels other phenomena observed with mixtures of water and acetonitrile. Viscosity reaches a maximum at *ca.* 50% AN[10] and gas solubility reaches a minimum at *ca.* 50% AN[13]. These phenomena may be thought of as manifestations of molecular interaction. It is possible that initially AN pairs with water on a molar basis, and later in the gradient, these "associated species" are diluted by the additional AN. With this hypothesis, refractive index (RI) (and density) is expected to not be additive. In the prospect that the baseline bump might be associated with RI effects in the UV detector causing the 190-nm light level or pattern hitting the detector to shift, some RI experiments were tried.

The bottom of Fig. 11 shows that the refractive index of AN is close to water,

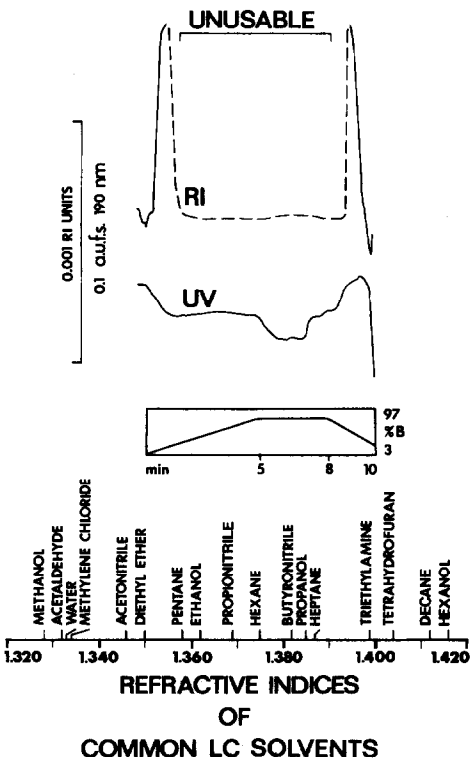

Fig. 11. The RI trace (upper curve) is off-scale for most of the gradient run with Waters RI-401 detector at the least sensitive setting when the UV trace at 190 nm shows a close match (lower curve). The 3–97% gradient is from pure water matched in UV absorbance to purge acetonitrile with the gas baseline adjustment technique. The bottom is a linear scale showing the relative refractive indices of common LC solvents.

fers for gradients at 210 nm[3]. Oxygen diluted with helium was also successfully used to adjust the baseline at 210 nm, but, like air in the mobile phase, this may oxidize samples, solvent components, and columns.

Fig. 6, chromatogram a, shows that on initial preparation of 3.6 m*M* TEA–CO_2 with only CO_2 (no N_2O) and a column in place, the baseline shifted upward and showed several broad ghost peaks. However, on sparging for 1 h with the gas mixture the ghost peaks disappeared (reason unknown) and the baseline mismatch was corrected by the N_2O.

There were several advantages of the gas baseline adjustment technique, besides the simplicity of preparing eluent. The same gas ratio brings any volume of eluent to the same pH and UV absorbance; no titration was necessary. Further, since the level of N_2O in the aqueous eluent was rapidly reversible (in *ca.* 5–10 min), overshooting the UV absorbance was easily corrected simply by lowering the N_2O gauge pressure (unlike the case of adding non-volatile UV absorbers like sodium azide). Since TEA and water were readily purified and the two gases were very pure, the baseline was very flat with practically no ghost peaks (see Figs. 3 and 5a). The protonated TEA cation has the important property of reducing tailings of basic substances[11] and aiding the elution of polymers[12] and injection loaded ion pair reagents[3].

An advantage for preparative LC is that TEA–carbonate is totally volatile (room temperature nitrogen drying of 300 ml of 3.6 m*M* TEA–CO_2 eluent showed less than 3 mg residue when 161 mg were expected). This volatility would aid in isolating non-volatile components without buffer salts. However, TEA–carbonate is stable and does not readily vaporize out of gas-sparged solution. Chromatograms at the end of a 4-l batch of eluent (at 16 h at 4 ml/min) were identical with those at the beginning of the run.

Mixer to eliminate short-term noise

The Hewlett-Packard LC has a high pressure 0.25-ml magnetic mixer in-line where the flow streams from the two pumps are mixed. While the pumping rate of

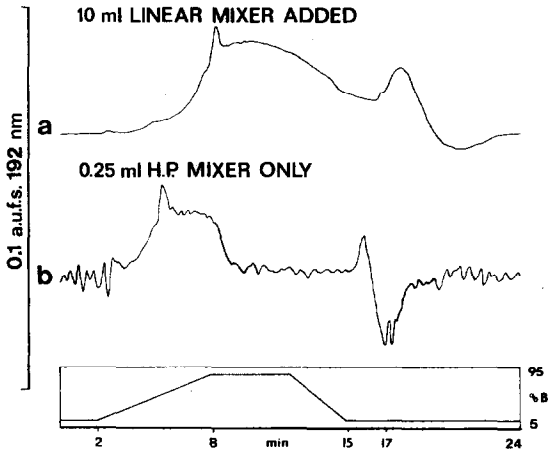

Fig. 7. The reduction in short-term noise when the mixing volume is increased from 0.25 ml (b) to *ca.* 10 ml (a) using the "linear mixer" described in the Experimental section.

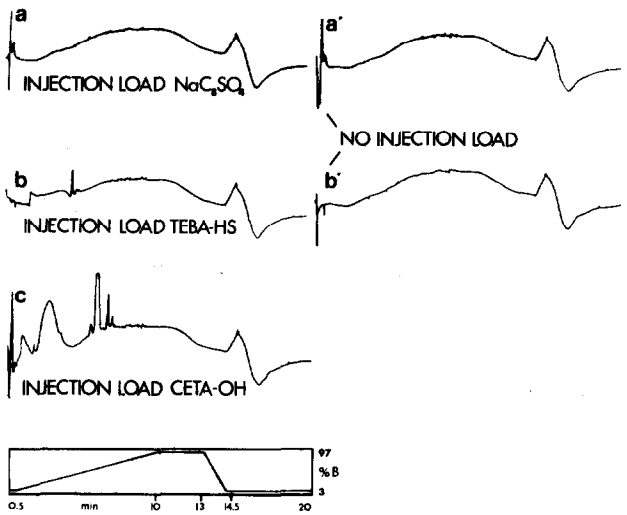

Fig. 8. Typical low-noise baselines for "injection loading" ion-pairing agents (left side) followed by simple reversed-phase runs (right side). Injection loading of sodium octanesulfonate (a) and tetrabutylammonium hydrogen sulfate (b), give baselines with few ghost peaks, and cetyltrimethylammonium hydroxide (c) gives a baseline with many ghost peaks. Injection loading of ion pairing agent from vial 1 of two 16-μl samples of 1 *M* ion-pairing agent followed by a wait of 0.5 min and then a sample injection of 16 μl. Other chromatography conditions are shown in Fig. 3.

these LC pumps is very fast (4.7 Hz), the flow feedback circuit causes the output of the two pump heads to compensate with flow adjustments every 1–3 sec. This puts slight short-term solvent concentration pulses into the flow stream that are generally not time-averaged out by the hold-up volumes of the system (*ca.* 3 ml) or the mixer. This short-term noise is generally no problem with usual detectors (as 210 nm UV or above). However, with the high background absorbance when the UV detector was operated at 190 nm, the baseline showed severe short-term noise (as seen in Fig. 7, chromatogram b). This short-term noise was eliminated effectively by inserting a much larger volume "linear mixer" of either 10-ml volume (chromatogram a) or 4 ml. The construction of this mixer is described in the Experimental section. The 4-ml linear mixer was used in most work since this eliminated most short-term noise and put a minimum delay into the gradient (*ca.* 1 min at 4.5 ml/min).

The 10-ml mixer was found also to reduce the short term noise when using two Waters 6000 pumps to "dial-a-mix" solvent with a Perkin-Elmer LC-21 electrical conductivity detector.

Typical injection loading ion-pair 190-nm baselines

A previous publication showed the advantages of injection loading ion-pairing agents[3]. Adjacent runs can rapidly explore the effects of different ion-pair agents, or the level of different ion-pair agents to optimize the resolution of a complex mix, if sufficient resolution is not found in a first simple reversed-phase run. Further, the ionic charge at the pH of the eluent can be determined by the movement of peaks without ever having to isolate individual components. This is done with a simple series of three adjacent cationic, anionic and reversed phase runs.

The injection loading technique involves injecting different levels of ion-pair agents a few seconds before injecting the sample. The level can be varied by the volume injected (10–50 μl), the concentration of ion-pair agent (0.25–2 *M*), or the number of injection loads (1–7). Usually 16 μl of 1 *M* ion-pair agent is loaded in one or two injections. Since the eluent contains a low level of TEA as the carbonate, it was found that the gradient not only elutes sample, but also washes excess ion-pair agent from the column so that reproducible chromatograms were obtained. Weak sodium propionate solutions were also found capable of eluting injection loaded ion-pair agents sufficiently slowly to allow the ion-pairing effect, yet sufficiently thoroughly, with full gradients, to allow reproducible runs.

Fig. 8 shows that injection loading of several ion-pairing agents is possible with the 190-nm full gradient system. The injection loading of 1 *M* tetrabutylammonium phosphate (chromatogram b) shows a nearly flat baseline compared to the second run (chromatogram b') which was in the simple reversed-phase mode. Similar injection loads of sodium octanesulfonate (chromatogram a) showed an even cleaner baseline. However, injection loading of sodium cetyltrimethylammonium hydroxide (chromatogram c) showed a number of ghost peaks that obscured a sizeable portion of the run, as found previously with 210-nm detection[3]. Further, the TEA in the mobile phase was not sufficient to remove this material in a single gradient and runs were not reproducible.

Development of techniques for 190-nm full-gradient runs

In first efforts to develop a 190-nm full-gradient run, it was found that the LC systems developed previously for flat baseline gradients with 210-nm detection[2] could not be extended to 190-nm detection. The final 190-nm system required discovery that (1) TEA eliminated the bump problem, (2) CO_2 was a clean (ghost-peak-free) source of counter-ion for the TEA, and (3) a partial pressure of N_2O in CO_2 was a clean, un-retained UV absorber that could adjust the aqueous eluent UV absorbance to match the acetonitrile. A number of modeling experiments led to these discoveries and an understanding of problems to be solved to achieve flat baseline 190-nm full gradients. The key experiments that lead to the 190-nm full-gradient system used a simplified LC with pure water and acetonitrile, no linear mixture, and *no silica saturator or analytical column in place.*

The advantage of 190 nm of near universal detection lead to the exaggerated anomalous baseline problems. These took the form of baseline shifts with the gradient (baseline mismatch), ghost peaks, and broad "bumps".

Baseline mismatch readily occurred because clean water had an absorbance at 190 nm less than acetonitrile and this causes a 0.5–0.8 a.u. ramp-shaped shift in the baseline during a gradient. One way to obtain a flat baseline during a run was to use electronic compensation to subtract the baseline shift of a control (with no sample injection) from the baseline shift with the sample. Without precise matching of flow, gradient shape, and the start of integration, anomalous dips occur before and after peaks (see Fig. 9, bottom). The second method for eliminating baseline shift problems, as shown above, was to add a non-retained (ghost-peak-free) UV-absorbing component, as N_2O, to the aqueous eluent to raise the absorption to match that of the acetonitrile.

The second anomalous baseline problem was ghost peaks. Common sources

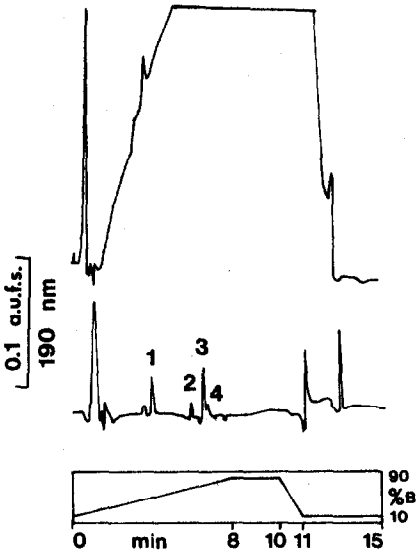

Fig. 9. Examples of the use of electronic compensation to subtract the baseline of a control run from a sample run for a sensitive, 190-nm UV detection with a full gradient. The upper curve is the baseline shift without correction and the lower curve is the corrected baseline with a Perkin-Elmer LC-75 detector. Flow is 4 ml/min and a C_{18} 5-μm particle size Spheri-Brownlee 3-cm guard column with a 25-cm Whatman 10-μm particle size column. The 15-min runs are from 10% to 90% acetonitrile in pH 3, 15 m*M* triethylamine phosphate with 0.00004% sodium azide anti-microbial.

of ghost peaks in LC are trace contaminants either in the water or acetonitrile, in the buffer components, or in additives to aid elution, as TEA. Methods described previously for eliminating sources of ghost peaks included using UV-irradiated distilled water, alumina-cleaned TEA, and lots of phosphoric acid selected for low UV absorbance[2]. Preventing problems from microbial growth is achieved by using fresh eluents, refrigerating eluents, or adding anti-microbials, such as sodium azide. Also "eluent conditioner columns" of poly(styrenedivinylbenzene) porous polymers in the high pressure aqueous line eliminated all but the most polar ghost peaks[9].

A third anomalous baseline problem was the baseline "bump". With 210-nm full gradients, one cause of this bump was amine-like contaminants in older lots of LC-grade acetonitrile collecting on the column early in the gradient run and eluting later. This problem could be eliminated by using fresh (less than two months old) LC-grade acetonitrile, or by installing a high-pressure active acid-alumina "eluent conditioner column" in the (dry) acetonitrile line.

The work at 190 nm reported here showed another source of a baseline bump problem. The 190-nm UV absorbance of water and acetonitrile was matched using the gas baseline adjustment method. When a gradient was run, a bump was found to appear in the middle of the run (Fig. 10, chromatogram a). *Since no column was used,* the cause of the bump could not have been the pick-up of materials by the column early in the run and the elution of them later.

To determine if the bump were caused by some kinetic effect of mixing, a 5 ml/min sequential isocratic step (SIS) LC run[1] was used to establish 4-min steps each of which reached isocratic equilibrium (using the same eluents and the no-column

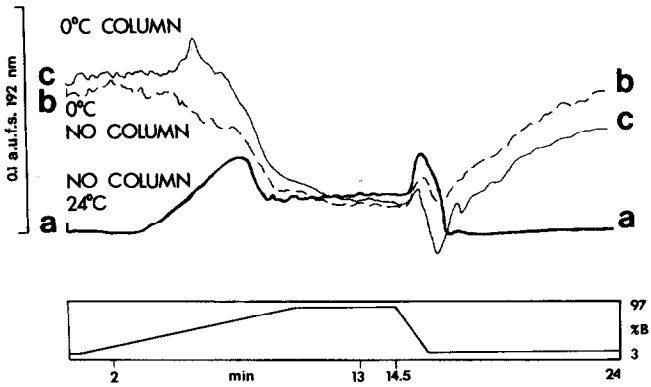

Fig. 12. Effect of detector cell and eluent temperature on baseline shift and bump. With no column in place, when changing from 24°C (a) to 0°C (b) the bump at 5 min is less apparent and the absorbance of the initial aqueous eluent shifts upward by 0.06 a.u. (60% of scale) and the acetonitrile absorbance shifts downward by only 0.004 a.u. (4% of scale). At 0°C when a column is added (as in Fig. 3), a bump appears at 3 min. The eluent is pure water matched in UV absorbance to purge acetonitrile with CO_2–N_2O. The 0°C temperature is achieved by passing the eluent through tubing (5 m × 0.020 in. I.D.) in an ice bath and insulating the detector cell with foam rubber. A dry nitrogen purge prevents moisture condensation on the cell.

1.344 *vs.* 1.333, when compared with common LC solvents[14]. However, with the least sensitive range on the Waters RI-401 refractive index detector (0.001 RI units full scale), Fig. 11 (top) shows that the signal rapidly went off scale in only the first part of the gradient. The RI detector indicated only that a considerable change in RI was taking place and this RI detector could not be used to investigate the bump found with water to AN gradients.

Another approach was attempted to determine if RI changes could be associated with the bump shown by the 190-nm UV detector. Different UV detectors having different cell designs to minimize RI effects were expected to change the size of the bump. However, the Beckman-Altex Model 165 detector and the Waters Model 481 variable-wavelength detector (with cell designs to minimize RI effects) both gave the same bump problem as found with the Hewlett-Packard variable-wavelength detector. These results indicated that either the bump found with 190-nm UV detection did not correlate with RI effects, or the present designs of UV detector cells were not able to eliminate the magnitude of the RI effect observed at 190 nm.

Since temperature changes are expected to alter molecular association, and thus change the magnitude of the bump, the effect of cooling the system was investigated. With no column in place, when the temperature of the eluent was changed from 24°C (Fig. 12, solid heavy line, curve a) to 0°C, (dashed light line, curve b), the 192-nm UV absorbance of the initial (aqueous) eluent increased by 0.06 a.u. or 60% of full scale. However, the UV absorbance at the end of the gradient (AN, at 14 min) changed only a little (decreased by 0.004 a.u. or 4%). The bump at 5 min was less apparent with the low temperature conditions without a column (chromatogram b) and with a column in place (chromatogram c). However, the bump may have been disguised under the baseline shift. Further investigation of the temperature effect on the bump, with proper matching of initial and final baselines was abandoned with the discovery that the addition of TEA eliminated the bump.

These temperature experiments revealed some other characteristics about the 190-nm system with no TEA in the eluent. The apparent size of the bump is affected by temperature, and, more importantly, the baseline shift at low wavelength UV is strongly temperature dependent due to increased UV absorption by the water. If detector temperature is not controlled, the baseline can shift with ambient temperature changes. Further, it would be possible to thermostat the column to different temperatures, as long as the temperature of the eluent in the detector cell is controlled. Also, the baseline can be adjusted partially by changing the temperature of the eluent in the detector cell, perhaps by thermostating the detector cell to one temperature and having the column at another temperature.

CONCLUSION

This paper has shown some simple and practical methods for achieving detection with 190 nm UV using full gradients. UV at 190 nm allows a greater variety of compounds to be detected and gives greater sensitivity for quantifying most compounds. The full gradient capability gives the time saving first-run-problem-solving advantage of a universal LC method. The techniques for eliminating anomalous baseline problems with UV detection should be useful for eliminating similar problems with other kinds of detector. The specific methods for confirming that a detector was operating in the linear region, and the simple perforated screen method for calibrating UV–visible detectors at all wavelengths may allow more objective comparisons of different UV detectors. The "linear mixer" eliminated short term baseline noise with 190-nm UV detection when solvents were blended from two pumps. This points out the need for better mixing for some instruments if low wavelength, electrochemical, or refractive index detection are to be used.

The technology for preparing and pumping eluents saturated with the highly soluble gas, CO_2, offers new possibilities for rapid preparation of ultra-clean eluents. The technique for raising the aqueous baseline UV absorbance to match that of the acetonitrile via the partial pressure of the UV absorbing gas N_2O has the advantages of being simple, clean, and over compensations can be readily reversed.

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