CHROM. 14,430

UNIVERSAL LIQUID CHROMATOGRAPHY METHODS

II. SENSITIVE, LOW-WAVELENGTH, GRADIENT REVERSED-PHASE METHODS*

VERN V. BERRY

Central Analytical Research, Polaroid Corporation, 750 Main Street, Cambridge, MA 02139 (U.S.A.)
(First received April 21st, 1981; revised manuscript received September 29th, 1981)

SUMMARY

A reversed-phase liquid chromatography "universal method" is presented that reduces expensive manpower costs for industrial laboratories by substituting automation for operator-attended runs and by eliminating method development for each new separation problem. Successful, fast (15–30 min), "first-run problem-solving" and quantitation of even trace components of completely unknown wide-polarity mixtures are obtained with the near universal detection possible with 210 nm UV detection. The reversed-phase method uses gradients at both acid and alkaline pH values (3–9) that allow low-wavelength detection at 210 nm with high detector sensitivity (0.1 a.u.f.s.) and baseline drift of less than 5% and no ghost peaks. The techniques that make possible this new realm of gradient work are presented.

Finally, a discussion is presented of the interaction of detector components, UV absorbance of eluents, and sample absorption spectra; factors that become critical at 190–210 nm detection.

INTRODUCTION

For industrial analytical laboratories that are faced with heavy daily loads of many different heat-labile or non-volatile materials that must be analyzed for main components or impurities, there is a real need for "universal" liquid chromatography (LC) methods. Such universal methods should be able, rapidly, accurately, precisely, and automatically, to quantitate both main components and trace impurities of almost any organic compound, while covering wide polarity ranges of elution. "General detection" of most organic compounds can be obtained to low levels (detectable with 0.1 a.u.f.s.) with low-wavelength UV detection (210 nm). Separation of components of widely different polarity is classically accomplished by gradients, column

---

switching, flow programming, and temperature programming. The use of sequential isocratic step (SIS) LC to cover as wide a polarity range as gradients but without the problems of baseline drift, ghost peaks, and the need for clean eluents was presented recently[1].

This paper describes a new approach to "universal" LC that fulfills the above criteria: sensitive, low-wavelength reversed-phase gradient elution. This method covers the full polarity range from full aqueous to full organic elution, without baseline problems, and allows general detection of most organic compounds to low levels.

Some earlier work with sensitive, low-wavelength gradients has been reported, although gradients were not as "universal" in that they did not cover as wide a polarity range, did not have as low a detection wavelength, or did not use as high a detector sensitivity. Also, previous work did not systematically study the cause of gradient baseline problems or methods for eliminating them.

The most universal gradients have been in the field of LC of amino acids, peptides, and proteins. Rivier first introduced to high-performance liquid chromatography (HPLC) the advantages of trialkylamine phosphate buffers[2] that give high recoveries and high UV transparency to less than 200 nm for peptide bond detection (vs. acetate and formate buffers[3]).

Hearn and Grego[4,5] show gradients at 210 nm with 0.2 a.u.f.s. and detection using gradients from water with 0.1 % phosphoric acid to 50 % acetonitrile for protein digests. Bennett et al.[6] show gradients at 210 nm with 0.4 a.u.f.s. detection using linear gradients from 0.01 M perfluorinated carboxylic acids to the acid and 58 % acetonitrile with good baselines and high protein recoveries.

Majors and co-workers[7,8] have used an approach to "universal" LC separation systems for aqueous samples in which the emphasis was on separating the lower-molecular-weight (MW) components from higher-MW complex matrices by the techniques of multi-dimensional LC using coupled columns.

Some early approaches to "universal" LC methods used normal-phase silica LC. Building on earlier suggestions by Snyder[9], Rabel[10] showed that the 254-nm UV detector could be used with medium sensitivity to limit baseline drift when used with a series of eluents covering the relatively wide polarity range from hexane to methanol. The "incremental gradient elution" approach of Scott and Kucera[11] used a moving wire with a flame ionization detector and a clever system to generate a gradient from heptane through eleven intermediate polarity solvents up to water with a normal-phase silica column. However, this system did not detect materials with modest volatility, the detector and gradient system were difficult to use (and are no longer commercially available), and the normal-phase chromatography did not give good separation of ionic or polar components.

This paper presents an approach to universal LC methods that can rapidly, accurately, and precisely quantitate most of the organic components that can be detected with low-wavelength UV (210 nm) and can be eluted with acetonitrile from a reversed-phase LC system. Three sensitive, low-wavelength (0.1 a.u.f.s., 210 nm) gradient methods are described that give little baseline drift (less than 0.005 a.u.) and no detectable ghost peaks: one buffered at pH 3 with triethylamine phosphate solubilizing agent for acidic samples; one at pH 9 for basic samples; and one unbuffered system. The techniques used to eliminate and determine the source of ghost peaks and baseline shifts (the "mid-gradient hump" and UV mismatch between initial and final

eluent) are applicable to other eluent systems that may be required for other separation problems. In a last section, precautions with 210-nm low-wavelength universal detection that are necessary for selecting the right wavelength to optimize sensitivity and give linear detection are presented.

EXPERIMENTAL

The "eluent conditioner column" sequence in the "acid" and "alkaline" systems that proved to give such low drift and ghost-free baselines were made from 25 × 0.46 cm I.D. DuPont columns (Wilmington, DE, U.S.A.). An 80–200 mesh acid alumina Brockman activity I eluent conditioner column was installed in this line to remove amine-like materials from the acetonitrile that cause the "mid-gradient hump", as explained later. This column had to be repacked when the mid-gradient hump re-appeared (after *ca.* 2–3 gallons of acetonitrile). A porous polymer eluent conditioner column was installed in the aqueous eluent line to remove impurities that cause ghost peaks, as explained later. This column was dry tap-packed with 80–100 mesh Chromosorb 101 styrene–divinylbenzene porous polymer (Supelco, Bellefonte, PA, U.S.A.). Before the first use and each morning, this column was cleaned by turning a valve arrangement so that *ca.* 15–25 ml (5 min) of acetonitrile flushed the aqueous line. A silica eluent conditioner column of 37–53 μm Whatman silica was installed after the gradient mixer and before the in-line filter in front of the injector to diminish silica dissolving from the analytical columns, especially a problem with the pH 9 alkaline system[12]. The 2-μm outlet frit of this column required periodic changing because of pressure build-up, presumably because of silica "fines" released from dissolved silica.

A 1084B Hewlett-Packard (Avondale, PA, U.S.A.) LC was used with Zorbax C_{18} octadecyl columns (25 × 0.46 cm I.D.; DuPont) preceded by Brownlee Labs "Spheri 5" RP-18 guard column (3 × 0.46 cm I.D.; Rheodyne, Berkeley, CA, U.S.A.). The best system used recent 6-μm d_p Zorbax columns (of serial number 8000 or higher) which have *ca.* 30–40% reduced permeability compared to earlier columns of *ca.* 7-μm d_p particles[13] (d_p = particle diameter).

Typical solvents were Burdick & Jackson acetonitrile for HPLC (Muskegon, MI, U.S.A.) (absorbance at 190 nm was 0.8 a.u. and at 210 nm was 0.01 a.u.) and water, UV irradiated for at least 3 h in a No. 816 HPLC Reservoir from Photronix Co. (Medway, MA, U.S.A.) just before use.

Gallon (3.81 l) lots of the "acid" buffer of 7.5 mM triethylamine phosphate (TEAP), pH 3, were prepared by titrating 2.891 g of purified triethylamine in irradiated water with *ca.* 20 ml of phosphoric acid diluted to *ca.* 8.5% (1 in 10). The final acid eluent typically had a UV absorbance of 0.34 a.u. at 190 nm, 0.06 a.u. at 200 nm, and 0.05 a.u. at 210 nm. The dilute phosphoric acid (Mallinckrodt, St. Louis, MO, U.S.A.) was made from reagent-grade 85% phosphoric acid selected from several lots so as to have less than 2.5 a.u. at 190 nm. The triethylamine (Fisher, Fair Lawn, NJ, U.S.A.) was purified by passing *ca.* 1 l through a 50 × 2.5 cm I.D. column of Fisher basic alumina, Brockman Activity I. Triethylamine so purified had the 190 nm absorbancy reduced from 1.2 a.u. to 0.64 a.u. and the 210 nm absorbancy reduced from 2.5 a.u. to 1.2 a.u. Gallon lots of the "alkaline buffer", 2.5 mM ammonium borate, pH 9, were prepared by titrating 0.589 g of Mallinckrodt (reagent-grade)

boric acid with *ca.* 12 ml of a reagent-grade ammonium hydroxide (Baker, Phillipsburg, NJ, U.S.A.) diluted to *ca.* 2.8% (1 in 10). The final alkaline buffer typically has a UV absorbance of 1.0 a.u. at 190 nm, 0.17 a.u. at 200 nm, and 0.05 a.u. at 210 nm.

PROCEDURES

Experiments were done with acrylamide monomer to determine the "highest linear sample size" (in micrograms) at each detector wavelength for the TEAP eluent system as follows. Serial dilutions of acrylamide were prepared from 0.06 to 60 mg/ml. For each wavelength from 190 to 260 nm in 10 nm increments, a sample solution was selected so that for the 2–200 μl range of the Hewlett-Packard injector, that concentration would show a "response curve" with linear and non-linear portions in a "best fit" plot of area counts *vs.* amount injected. For the monomer example, 254 nm required a concentration of 60 mg/ml; 240–220 nm required 0.6 mg/ml; and 200–190 nm required 0.06 mg/ml. From the response curve at each wavelength, the highest linear load point (in micrograms) is the intersect between the response curve and a line drawn through the origin and any point 10% lower than the linear part of the response curve, paralleling the ASTM method[14].

Experiments such as that described above indicate that peaks not going off the printer-plotter paper at a detector sensitivity of 2^9 (*i.e.* less that 1 a.u. high) are within the linear range of the Hewlett-Packard variable-wavelength detector when using the acid (pH 3) 7.5 mM TEAP buffer at all wavelengths from 190 nm up. For the alkaline (pH 9) 2.5 mM ammonium borate buffer, peaks are within the linear range of the detector if they are below 0.25 a.u. at 210 nm.

RESULTS AND DISCUSSION

Achieving sensitive, low-wavelength gradients

Introduction. Fig. 1 shows the type of clean and flat baseline that can be obtained with high sensitivity detection (0.1 a.u.f.s.) and very general detection (210 nm) while covering the polarity range from full aqueous to acetonitrile elution, and using additives to control the buffer pH or to elute strongly retained components. Note that one system uses a triethylamine additive, which is well documented as a good eluent[2], and the second system is alkaline at pH 9. The power of these high- and low-pH systems together, to separate acidic and basic compounds, will be illustrated below. The gradient shape is shown in the lower part of the figure.

A practical "acceptable baseline" is illustrated by the dashed curve between the aqueous and acetonitrile (Fig. 2). With this definition, the highest sensitivity that can be achieved readily would come from a 50% shift in baseline (up or down), since this is the lowest sensitivity setting in which the whole chromatogram will be on scale.

A number of problems can cause the acceptable baseline to deviate from the experimental gradient baseline, as illustrated in Fig. 2. The three primary problems are (1) the "mid-gradient hump", (2) UV absorbance mismatch between initial and final eluents, and (3) aqueous-eluent-derived ghost peaks. The source of these different types of problem, methods for determining their causes, and methods to prepare eluents with flat gradient baselines will now be presented.

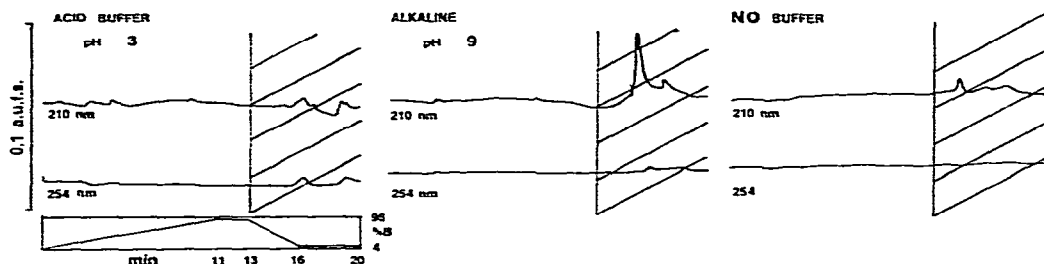While Fig. 1 illustrates the "clean" noise-free baseline that was ultimately

Fig. 1. Typical low-noise baselines for sensitive, low-wavelength, full gradients using an "acid buffer" of pH 3, 7.5 mM triethylamine phosphate (left); an "alkaline buffer" of pH 9, 2.5 mM ammonium borate (middle); and a "no buffer" system of irradiated distilled water. A flow-rate of 3 ml/min is used with a reversed-phase, long column of a 3-cm Brownlee C_{18} guard column followed by a 25-cm 6-μm Zorbax C_{18} column. The 20-min gradients are from 4 to 96% acetonitrile using the gradient shape shown at the bottom. This "clean" baseline system uses eluent components selected or purified by methods described in the text plus the following 0.46-cm diameter "eluent conditioner columns": a 25-cm 150–200-μm Chromosorb 101 porous polymer column in the aqueous eluent line; a 25-cm 75–200-μm acid alumina column in the acetonitrile line; and a 15-cm 37–53-μm Whatman "silica saturator" column after the gradient mixer and before the injector. The hatched portions of the baselines are in the return gradient and are used only to monitor the cleanliness of the system.

achieved in a routine fashion, the other chromatograms shown in this section generally evolved over a period of time, before the methods for eliminating the various baseline problems were developed. To evaluate the three problems about to be discussed, the time between runs must be reproducible, as is possible with a microprocessor-controlled LC, and replicate runs must be made until reproducible chromatograms are obtained. Thus, the first chromatogram must usually be discarded.

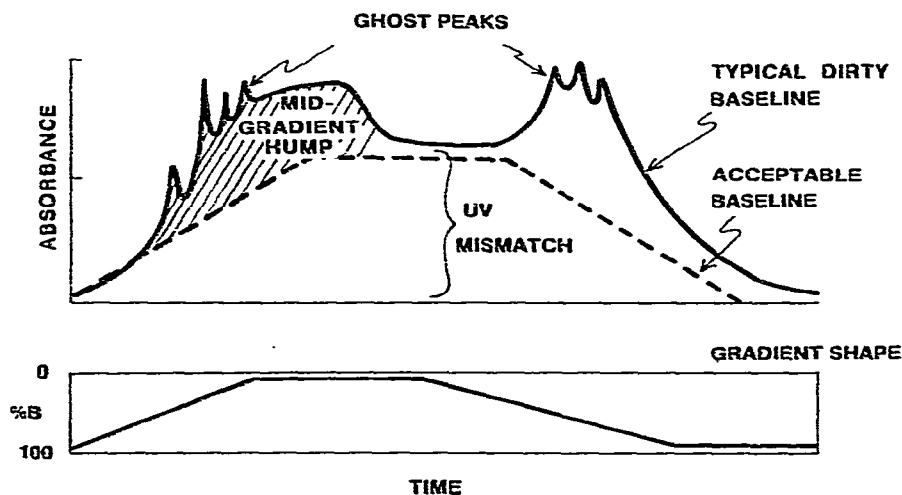*The "mid-gradient hump" problem.* The shaded zone between the acceptable

Fig. 2. Illustration of a typical "dirty" gradient baseline showing (1) the "mid-gradient hump" problem, (2) the problem of UV absorbance mismatch between initial and final eluents, and (3) the ghost peak problem. The "acceptable baseline" (dashed line) results if the mid-gradient hump and ghost peak problems are eliminated.

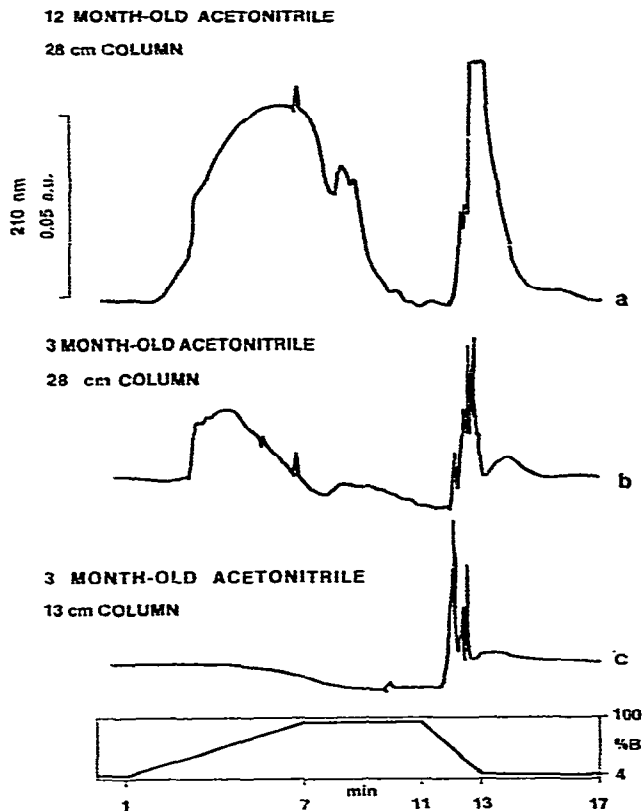**12 MONTH-OLD ACETONITRILE**

28 cm COLUMN

Fig. 3. Sensitive, low-wavelength gradients showing that the "mid-gradient hump" diminishes with newer lots of HPLC grade acetonitrile [(a) vs. (b)] and with shorter columns [(b) vs. (c)]. The gradient is from irradiated and degassed water to acetonitrile at 4 ml/min with a 3-cm Brownlee C_{18} guard column and 25-cm Zorbax C_{18} column (a) and (b) or a 3-cm Brownlee C_{18} guard column and a 10-cm Brownlee C_{18} column (c).

baseline and the experimental baseline in Fig. 2 is the "mid-gradient hump" that was found to originate from the acetonitrile. A large mid-gradient hump can be seen in Fig. 3, chromatogram a, originating from a lot of acetonitrile distilled *ca.* 12 months earlier. A more recently distilled lot of acetonitrile, 3 months old, gives a reduced mid-gradient hump (compare chromatograms a and b).

The source and elimination of the mid-gradient hump was discovered by modeling experiments. Rabel[15] has suggested that contaminants in the acetonitrile will cause sharp ghost peaks during a gradient. The possibility was investigated that such acetonitrile contaminants might give broad peaks and be the cause of the mid-gradient hump. Fig. 4 shows that the initial mid-gradient hump (the shaded portion) remains unchanged as the acetonitrile is made 6 and 12 μM with the uncharged additive benzene (chromatograms b and c). As expected, the mismatch of initial and final eluent UV absorbance from *ca.* 7 to 9 min is seen, and this increases linearly with concentration of benzene in the acetonitrile.

With this modeling experiment, as benzene is added, two sharp peaks appear at
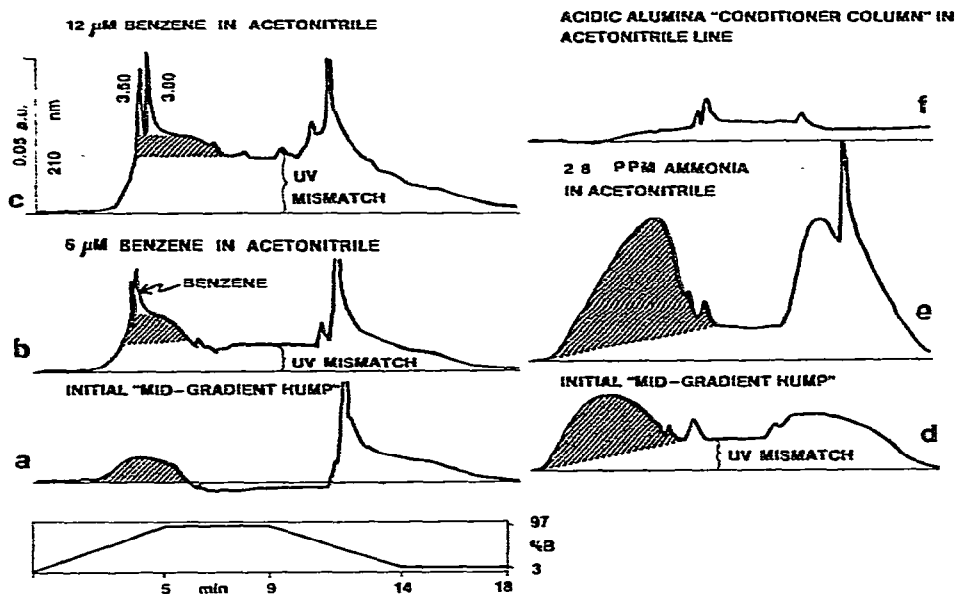
Fig. 4. Modeling experiments that led to the elimination of the "mid-gradient hump" (hatched areas). The initial hump (a) is constant with increasing benzene from 6 to 12 μM in acetonitrile (b) and (c) giving sharp peaks at 3.8 min (benzene elution position) and 3.5 min. In another experiment, the initial hump in (d) increases with the addition of 28 ppm ammonium hydroxide in acetonitrile (e). The mid-gradient hump is eliminated by an acidic alumina "conditioner column" in the acetonitrile line (f). The gradient is from 12-month-old acetonitrile at 4 ml/min with a 3-cm Brownlee C_{18} column and 25-cm Zorbax C_{18} column with an aqueous eluent conditioner column of porous polymer added in (f).

3.5 and 3.8 min, and the second peak is at the elution position of benzene. The area of the second peak increases linearly with benzene concentration in acetonitrile. This is evidence that a contamination of a single uncharged pure component in the acetonitrile will not cause a mid-gradient hump. It is possible that oligomeric contaminants can cause a series of fused peaks to produce a hump, under some conditions.

Other work using pyridine and picoline as model samples showed that these amines give peaks 20–40 times broader than uncharged molecules on an unbuffered water-to-acetonitrile reversed-phase gradient. However, they give sharp peaks with good retention when the aqueous eluent is 0.1% in ammonia, and sharp but un-retained peaks when the eluent is acid (pH 3, 15 mM TEAP). Also, early amino acid analyzer work was plagued with a mid-run hump that proved to be ammonia[16]. The addition of ammonia to the acetonitrile (28 ppm) doubles the area of the mid-gradient hump (chromatogram e). Under the possibility that amines such as ammonia in the acetonitrile can cause the mid-gradient hump, an "acetonitrile conditioner column" of acidic alumina was placed in the acetonitrile line. This column eliminates the mid-gradient hump (chromatogram f) and potentially allows even older lots of acetonitrile to be used.

Before discovering that the acetonitrile conditioner column of acidic alumina could eliminate the mid-gradient hump, other methods were found that reduced the size of the hump. Fig. 3 shows that reducing the column length from 28 cm (chromato-

gram b) to 13 cm (chromatogram c) nearly eliminates the hump at the cost of reduced separation. Note that the C_{18} loading is reduced to a greater extent than the proportion to column length since the Brownlee column has a lower C_{18} loading than Zorbax C_{18} (7 *vs.* 15%). In other work, for identical columns, with an acetonitrile gradient from 3 to 70%, the mid-gradient hump was reduced to *ca.* one-half the 3–97% gradient level. However, a shortened gradient moves away from universal LC in not utilizing the fullest extent of the gradient so as to separate and elute as wide a sample polarity as possible.

UV absorbance mismatch between initial and final gradient eluents. The second type of baseline problem with gradients is the mismatch of UV absorbance between the initial aqueous eluent and the final acetonitrile eluent. The gradient illustrated in Fig. 3 gives a sufficiently long final isocratic run at 100%-acetonitrile so that the baseline shift due to UV mismatch between the aqueous and the acetonitrile eluents can be seen and can be distinguished from the mid-gradient hump problem. By the 11-min point, the column has reached equilibrium with acetonitrile. Note that for the lot of acetonitrile giving even a very bad hump (chromatogram a), the UV absorption mismatch is very low, below 0.005 a.u., using these very clean eluents. Allowing the
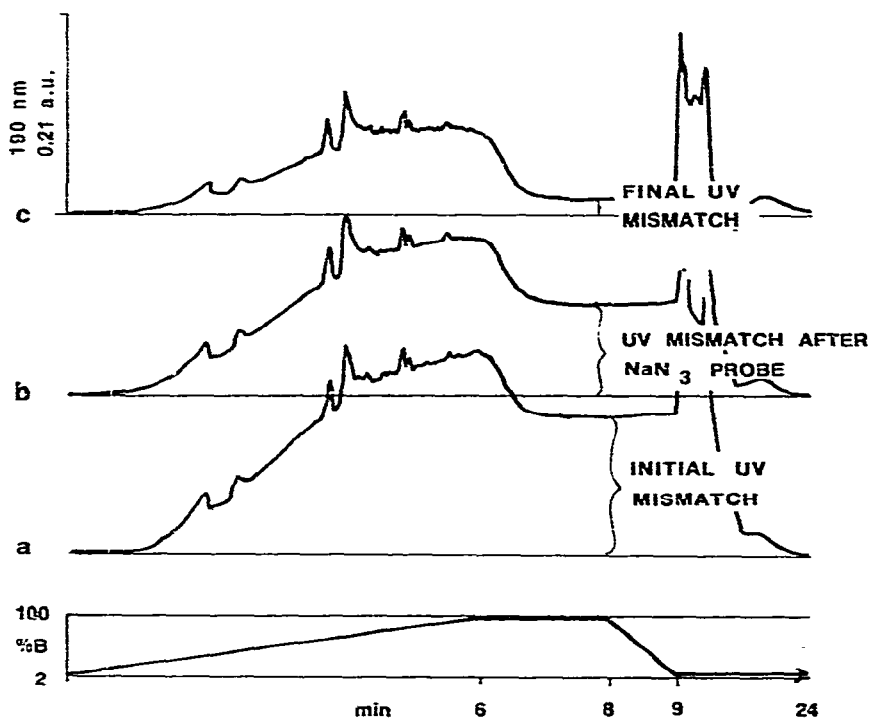

Fig. 5. Example of reducing the UV mismatch between the initial and final gradient eluents by adding an unretained component, 0.2% sodium azide, to the lower UV absorbing aqueous eluent. The 6-cm UV mismatch in (a) is reduced to 4 cm with a "probe" addition of 10 ml of sodium azide per liter of aqueous eluent in (b) and to 0.4 cm with a final addition of 20 ml/l in (c). The pH 2 aqueous uses 10 mM sodium phosphate buffer to acetonitrile gradient at 5 ml/min using a 25-cm Zorbax C_{18} column with no eluent conditioner columns.

column to come to equilibrium with the large volume of acetonitrile allows the mid-gradient hump problem and UV mismatch problem to be distinguished and each clearly assessed.

Were the acid alumina column not used to eliminate the mid-gradient hump as described in the previous section, a practical fast cost-effective gradient might end at some point at the top of the gradient hump, perhaps at 7 min in Fig. 3, chromatogram a. This would suggest a UV mismatch between initial and final eluents that could not be confirmed in a static spectrophotometer measurement of the eluent UV absorbances.

Using a gradient in which the final composition is held at 100 % will allow the UV mismatch between the initial and final eluents to be readily assessed. If the UV mismatch is such that the baseline shifts down, then a cleaner initial eluent must be selected; perhaps by decreasing the concentration of additives. Alternatively, adding an unretained UV absorber to the acetonitrile might be used to raise the UV absorption of the final eluent so as to give a flat baseline. The technique for using additives is illustrated in the following situation.

If the UV mismatch between the initial and final eluent is such that the baseline shifts up with the gradient, the UV absorption of the initial solvent can be increased so as to give a nearly flat baseline by adding an unretained UV absorber to the aqueous eluent, as described in Fig. 5. This example is for an aqueous (pH 2, 10 mM sodium phosphate) to acetonitrile gradient (3–100 %) in which the techniques were not used to minimize the mid-gradient hump problem or eliminate ghost peaks. Note that the sodium azide did not change the retention of any of the ghost peaks or contribute any ghost peaks itself. Additionally, sodium azide will protect the aqueous system from microbial growth.

Several other approaches can be used to minimize the UV mismatch between the initial and final eluents in a gradient. Usually going to higher wavelengths will minimize the UV mismatch. Fig. 6, chromatograms a and b, show a nearly flat baseline at 220 nm and 210 nm compared to the 200 and 190 nm baselines. While higher wavelength detection can minimize the UV mismatch, this works against the general detection possible at the lower 190–210 nm wavelengths. An alternative approach to match initial and final UV absorbance is to increase the UV absorbance of the aqueous eluent by increasing the concentration of the usual additives as TEAP or ammonium borate.

Eliminating aqueous derived ghost peaks. The third problem found with 210 nm, 0.1 a.u. sensitivity gradients is the presence of ghost peaks in going from the aqueous to non-aqueous eluents. The source of these peaks is from the initial or final eluent in reversed-phase chromatography[15]. A method for determining the ghost peak levels is illustrated in Fig. 7, and has been discussed by Bristol et al.[17]. Gradients show the ghost peaks, and the sizes of the ghost peaks diminish as the volume of aqueous phase is reduced. Thus, a first and classical method for reducing ghost peaks from the aqueous eluent is to shorten the equilibration time between runs.

A second and recently popular method for eliminating ghost peaks from the aqueous eluents is to use UV irradiation to photo-oxidize organics in water. The principle of photo-oxidation is established as an LC halide photoconductivity detector (Model 965; Tracor Instruments, Austin, TX, U.S.A.)[18], as an organic carbon analyzer[19], and as a means to prepare water for LC[20,21].
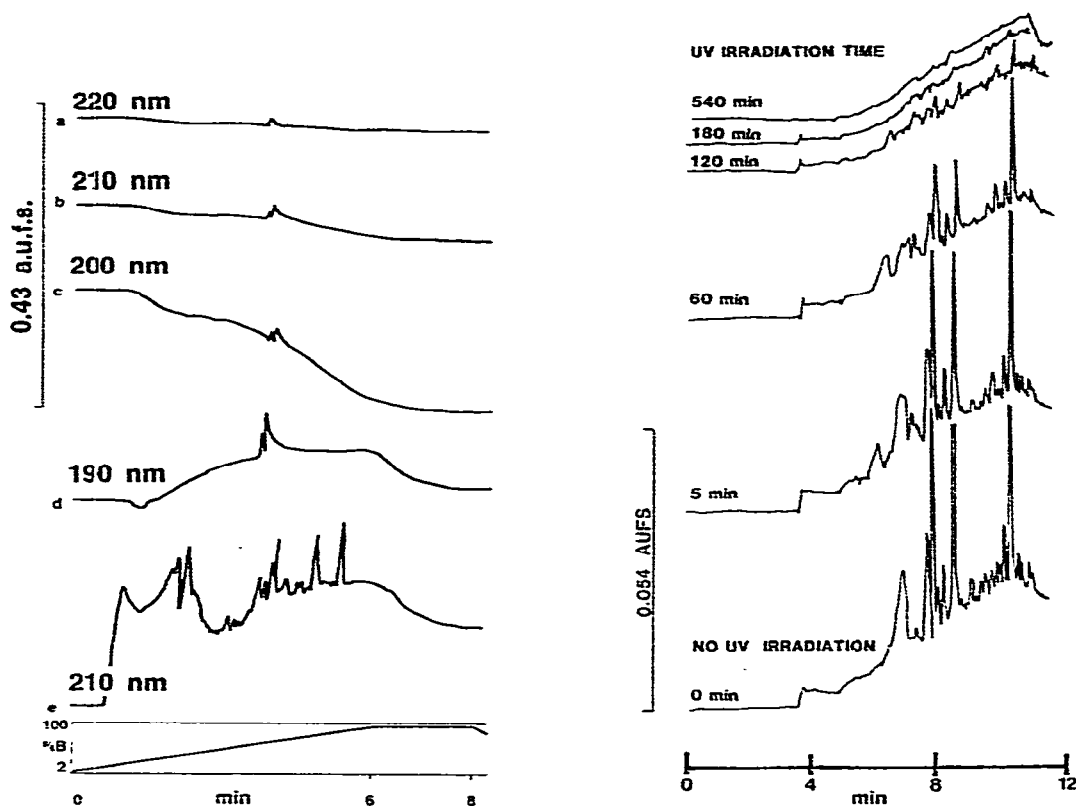
Fig. 6. Gradient chromatograms showing that higher wavelength detection at 220 nm (a) and 210 nm (b) give flatter baselines than lower wavelengths, as 200 nm (c) and 190 nm (d). The effect of additives to increase ghost peaks, as the ion-pairing tetrabutylammonium phosphate (pH 2, 4 mM) is determined by comparing chromatograms with no additive [(e) vs. (b)]. A 3-cm Brownlee C_{18} guard column and a 25-cm Zorbax C_{18} column is used at 5 ml/min with a gradient from water with $2.8 \cdot 10^{-4}$ g per liter of sodium azide to acetonitrile for (a) to (d) to minimize the UV mismatch between initial and final eluent. Sensitivity is low and no eluent conditioner columns are used.

Fig. 7. Gradient liquid chromatograms showing the UV irradiation method for preparing LC-grade water. The initial level of impurities in commercial distilled water is eliminated by UV irradiation from 0 to 540 min (bottom to top chromatograms) in a Photronix HPLC reservoir. The gradient is from 0 to 95% in 9 min; isocratic at 95% for 3 min; return to 3% in 1 min; and initial isocratic water for 28 min (73 ml) to concentrate impurities. A flow-rate of 5 ml/min is used with a 25-cm Zorbax C_{18} column.

The effect of a Model 816 UV irradiator LC reservoir (Photronix, Medway, MA, U.S.A.) for removing ghost peaks originating from the water can be seen in Fig. 7. After 3 h the ghost peaks from the water are undetectable.

Besides water, the second major source of ghost peaks is the additives used to buffer pH, form ion-pairs, or solubilize and elute samples (as trialkylamines). The effect of impurities in additives can be determined by comparing the chromatogram of water to that of water with additives. For example, Fig. 6 shows that the ion-pairing agent tetrabutylammonium phosphate, pH 2, 4 mM (chromatogram e), shows many peaks at 210 nm compared to the water (chromatogram b).

UV irradiation of water containing additives is not useful. Irradiation of 10 mM potassium phosphate buffer produced high UV-absorbing materials that broke through the column after *ca.* 3 l of irradiated buffer were pumped. Thus the water should be purified with UV irradiation before adding buffers, amines, etc.

The third major method of reducing ghost peaks from additives is to clean and/or select components. For example, phosphoric acid lots with minimum UV absorption were selected simply by comparing their UV spectra, and triethylamine lots were cleaned through a column of basic alumina. Both methods are described in the Experimental section.

Despite alumina cleaning of triethylamine and selecting phosphoric acid lots, ghost peaks from the TEAP were still evident, as can be seen in Fig. 8, chromatogram a. A novel and fourth approach was successfully used to eliminate the mid-polar ghost peaks arising from the aqueous TEAP. An "eluent conditioner column" of styrene–divinylbenzene polymer (Chromosorb 101) was used in the aqueous line before the mixing chamber, as described in the Experimental section. Chromatogram b shows that the mid-polar ghost peaks are completely eliminated by the aqueous eluent conditioner column. The aqueous conditioner column cleans more than 1 gallon of the TEAP buffer, and is regenerated by a 5 min (15–25 ml) purge with acetonitrile each morning.

Octadecyl and octyl silica columns have been used off-line[22] to remove ghost peaks from water, but the porous polymer columns offer the advantages of on-line
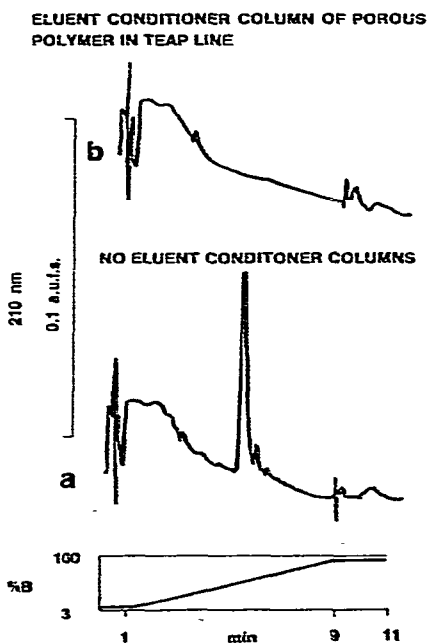

Fig. 8. Elimination of ghost peaks at 5 min (a) by an "eluent conditioner column" of styrene–divinylbenzene "porous polymer" in the aqueous TEAP (pH 3, 15 mM) line (b). A 3-cm Brownlee C$_{18}$ guard column and 10-cm Brownlee C$_{18}$ column are used at 5 ml/min with no acetonitrile eluent conditioner column to eliminate the initial hump from 0 to 3 min.
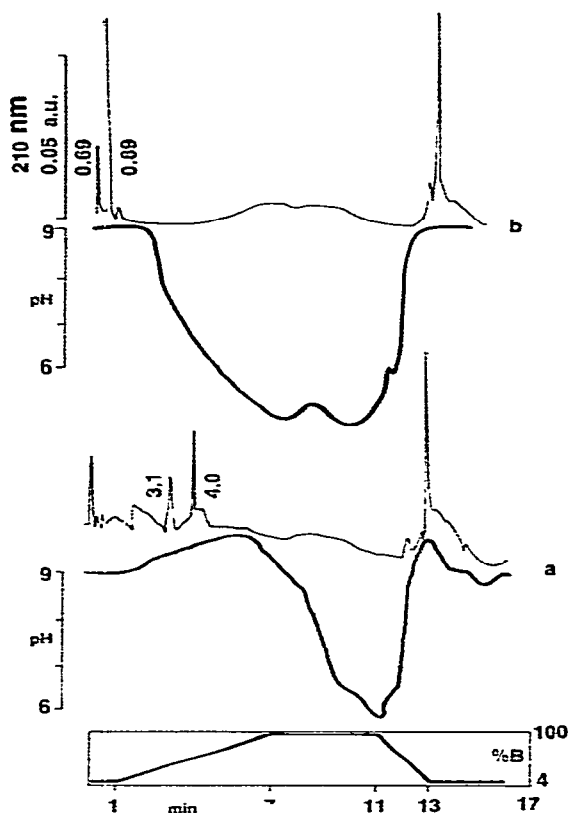
Fig. 9. Reduction in retention of model amine, 2-methylthiazolidine in 0.4 M KOH in pH 9 alkaline system frcm 4.0 min (a) to 0.89 min (b) when an open lot of 2.5 mM ammonium borate buffer absorbed atmospheric carbon dioxide over a weekend. The heavy line shows the delayed fall in pH in the fresh (a) *versus* the older eluent (b). A 25-cm Zorbax C_{18} column at 4 ml/min is used with an aqueous eluent conditioner column.

conditioning of the solvent just before use as well as much increased capacity and convenience.

Precautions with alkaline buffers for gradients

With alkaline buffers in LC systems some special precautions were found necessary for successful long-term chromatography. Fig. 9 shows that retention of samples can be affected by absorbed atmospheric carbon dioxide. Chromatogram a shows that the model amine, 2-methylthiazolidine, had a retention of 4.0 min in a freshly prepared pH 9 buffer. The corresponding pH trace as determined by the eluent flowing over a pH electrode is shown as the heavy line. Chromatogram b shows that the retention of the amine diminishes to 0.89 min with the same 1 gallon lot of buffer sitting open to the atmosphere over the weekend. Note that the pH trace begins to fall at *ca.* 2 min in this run, as against 6 min in the run with the fresh eluent. A fresh lot of eluent restored the retention and pH trace to that shown in chromatogram a. Purging the head of the eluents with a slow stream of helium or simply capping the buffer and
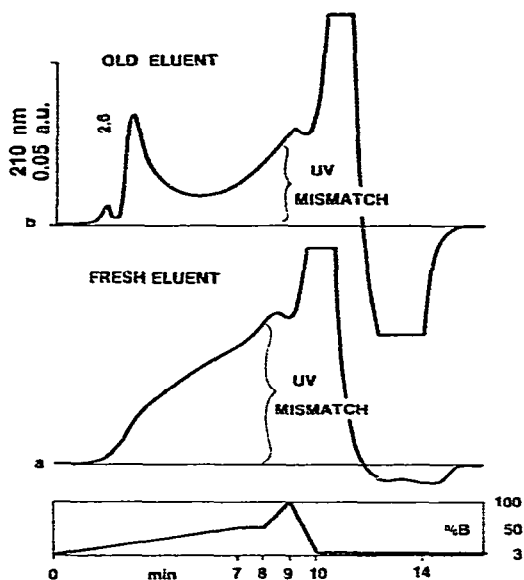
Fig. 10. Fresh eluent (a) *versus* "old" eluent stored at 6°C for 4 days (b) showing broad microbial contaminants at 2.6 min and a decrease in UV mismatch between the initial and final eluent due to build-up of microbial products in the aqueous pH 9, 2.5 mM ammonium borate eluent. A 3-cm Brownlee C_{18} guard column and a 25-cm Zorbax C_{18} column are used at 5 ml/min with a silica-saturator column.

acetonitrile tightly with aluminum foil eliminated amine retention changes with buffer age.
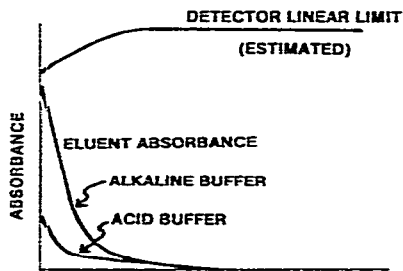
A second precaution with the alkaline buffer system is that a silica-saturator column of large particle, high-permeability silica must be used for successful long-term chromatography[12]. With alkaline buffers, the silica-saturator column must be after the mixer and before the injector and thus in the gradient stream. Initial experiments with pH 9 buffers made of ammonium borate and ammonium phosphate showed that if the silica-saturator column is used on the aqueous eluent line alone, after 10–20 gradients, sufficient gelatinous silica would precipitate on the filter after the mixer and before the injector to overpressure the system.

Fig. 10 illustrates another precaution necessary with all aqueous buffers when using sensitive, low-wavelength detection. Microbial growth can cause ghost peaks. In Fig. 10 a broad peak is seen at 2.6 min that appeared in a 6°C refrigerated ammonium borate buffer after 4 days storage. Ghost peaks from microbial growth are often of such low molecular weight materials that they cannot be removed by the porous polymer aqueous eluent conditioner column. Similar levels of ghost peaks were noticed eventually in all buffers except those in which 0.04% sodium azide was added. Thus buffers must be made fresh and usually cannot be stored for more than 2 to 3 days even with refrigeration.

Precautions with sensitive, low-wavelength detection and universal LC system

The sensitive reversed-phase gradient "universal method" described above makes it easy to detect and quantitate all components to low levels on a first run by using low-wavelength detection (at 210 nm). However, this low-wavelength detection

PLOT I

DETECTOR LINEAR LIMIT

(ESTIMATED)

ABSORBANCE

ELUENT ABSORBANCE

ALKALINE BUFFER

ACID BUFFER

PLOT II

SYSTEM LINEAR LIMIT

ACID SYSTEM

ALKALINE SYSTEM
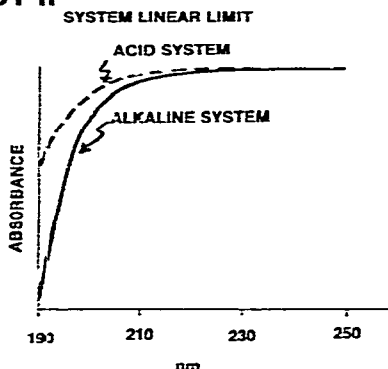
ABSORBANCE

190   210   230   250

nm

Fig. 11. Plot I shows the "detector linear limit" (lamp, detector, optics and sample cell) and the absorbance of the eluents. Plot II shows that the difference between the "detector linear limit" and the eluent absorbances gives a "system linear limit" that is higher for the acid system than for the alkaline system.

requires some special precautions compared to the usual 254-nm detection in order to be useful. Compounds with moderate absorptivities at 254 nm frequently have very high absorptivities at low-wavelength detection, and low concentrations can often exceed the maximum system linear limit.

Fig. 11 illustrates some important relationships between the detection system, the eluent, and the sample. Note that the "detector linear limit" is actually the entire detection system linear limit which is determined by the lamp output and age, the detector characteristics and age, stray light, detector bandwidth, characteristics and cleanliness of the optics and detector cell, as well as the electronic conditioning that may be used to make linear the response of the detection system *versus* wavelength. The upper line shown in Plot I is an estimate of the detector linear limit, which can vary from instrument to instrument and with time. The relative eluent absorbance spectra of the acid and alkaline buffers are correct, with the alkaline buffer giving 1.0 a.u.f.s. at 190 nm, in a 1-cm cell as mentioned in the Experimental section.

For the alkaline system, the "system linear limit" is the difference between the detector linear limit and the eluent absorbances (Fig. 11, Plot I). This system linear limit is shown in Plot II. Note that the system linear limit is usually important only at low wavelengths where the eluent absorbance and detector non-linearity may become significant. The system linear limit is wider for the acid buffer system *versus* the alkaline system, since the acid eluent has the lower UV absorbance. Thus, if 190-nm

detection is used (and zero percent acetonitrile) any peak eluting with an absorbance between the curves in Plot II would be in the non-linear zone of the detection system for the alkaline buffer, but in the linear zone for the acid buffer. Decreasing the sample size would give a proportional decrease in area counts for the acid system.

Accurate specific absorbances of materials are generally not known in the low UV range since low-wavelength detectors and clean materials are only now available with the advent of the LC coupled with sensitive spectrophotometers. Thus little can be done, at present, to predict concentrations of samples that fall in the system linear limit for a specific wavelength. In fact, the next section will show the inverse approach, in which the system linear limit for a particular detection system and eluent is estimated experimentally from the "highest linear sample size".

The wide range in absorptivities of different components in a mixture (leading to poor proportionality to weight-percent); the high absolute level of absorptivities (leading to sensitive detection but possible detector overload); and the nearly universal detectability of all components (leading to complex chromatograms) when used with the universal chromatography methods described above requires that some special precautions be used, as will now be presented.

Sensitive, low-wavelength detector precautions: wavelength selection for sample size

In classical spectrophotometry, it is good practice when developing a colorimetric analysis to select a photometer wavelength in the middle of a broad absorption band, provided no interferences absorb at that point. This minimizes errors in re-selecting the detector wavelength, or changes during a test due to the photometer heating, vibrating, etc. A typical method then adjusts the concentrations of samples so they are below the "highest linear concentration" for the spectrophotometer, although quantitation is possible in the response curve zone where the detection system is non-linear but still not saturated.

In LC analyses using variable-wavelength detectors, other considerations may make it desirable in some cases to select detection wavelengths far from the peak absorption band maxima. For example, to quantitate both the trace level components and major components in the same sample, wavelengths of high absorption might be
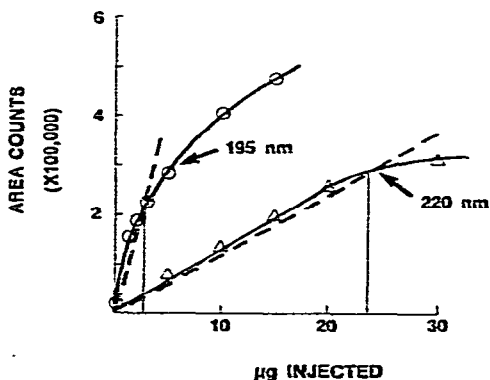

Fig. 12. Peak area counts *versus* sample size injected for methacrylamide showing the "highest linear sample size" of *ca.* 24 μg at 220-nm detection but less than 2 μg at 195-nm detection.

necessary to detect the trace components. However, the major component might grossly overload the detector at this wavelength. With the new generation of rapid-scanning UV-visible detectors now appearing, as well as the microprocessor-controlled Hewlett-Packard detector used in this work, it is possible to quantitate both major and minor components by changing the detection wavelength. The relationship between the UV absorption spectra of compounds and the "highest linear sample size" is illustrated below.

The highest linear sample size at a particular detection wavelength is defined here as the amount of sample that causes a 10% lower value than expected from a plot (the response curve) of area counts *versus* amount of sample injected. Details for determining the highest linear sample size are described in the Experimental section. Fig. 12 shows a typical plot of area counts *versus* amount injected using both 195- and 220-nm detection for methacrylamide monomer. Curve a shows that for this monomer the highest linear sample size is only 2 μg at 195 nm, but it is 24 μg at 220 nm.

Data from plots similar to this were used to determine the highest linear sample size at wavelengths between 190 and 254 nm. This is shown along with the UV absorption spectra for acrylamide in Fig. 13. The UV absorption spectra was determined by the automated "gradient wavelength scan" mode[1] by plotting the area counts *versus* wavelength resulting from a 0.2 μg sample injected at each wavelength. This sample size is below the highest linear sample size for all wavelengths.

The plot of wavelength *versus* highest linear sample size shows the experimentally determined relationship between the UV absorption of acrylamide monomer and the highest linear sample size at each wavelength. The highest linear sample size decreases at lower wavelengths for three reasons: (1) the UV absorption of the sample increases; (2) the eluent UV absorption increases; and (3) the detection system has lower linearity. Points (2) and (3) determine the *system* linear limit, as described above.
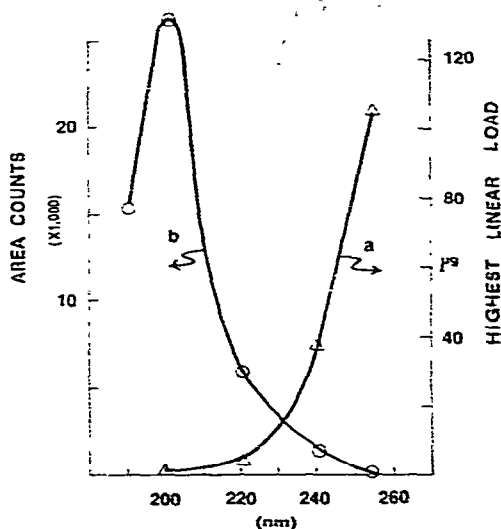

Fig. 13. Experimentally determined "highest linear sample size" as amount injected *versus* detector wavelength (a), and UV absorption spectra as area counts *versus* detector wavelength (b) for acrylamide monomer. Methods are described in the Experimental section.

A figure such as Fig. 13 suggests an important advantage of variable-wavelength detectors over fixed-wavelength detectors, such as those now offered with 214-nm and 229-nm detection. If the situation exists where the largest sample volume is being injected to maximize detector sensitivity for the samples available, then it will be desirable to change the detection wavelength depending on the problem. In the example shown in Fig. 13, if the problem were to quantitate trace levels of acrylamide below 0.5 μg (in the sample volume injected), then the best detection wavelength is the absorption maximum, near 200 nm. On the other hand, if the problem were to quantitate larger levels of monomer, ranging from trace levels to a maximum of 35 μg, then the side of the absorption band at a wavelength of 240 nm is best, since trace level samples give the largest peaks and the highest level samples just fall on the sample linear range. A fixed-wavelength detector at 254 nm would give no detectable levels for many of the trace level samples, and a fixed-wavelength detector at 214 nm would quantitate the trace level samples but require dilution or a reduction in injected sample volume to quantitate samples with higher monomer levels.

CONCLUSION

Industrial laboratories can realize considerable costs savings with "universal LC methods" that substitute automation for expensive manpower and allow "first-run problem-solving". The universal method of full-gradient reversed-phase LC is described that gives sensitive, low-wavelength detection (0.1 a.u.f.s. at 210 nm) with less than 5% baseline drift and no ghost peaks. Techniques for minimizing baseline problems by using aqueous and acetonitrile "eluent conditioner columns", solvent and additive selection and cleanings, reduced column length or column loading, reduced gradients or equilibration times, UV irradiation of water, and addition of components to eliminate UV mismatch, potentially can be applied to other modes of chromatography.

The low-wavelength detection (190–210 nm) that gives the above universal methods the power to quantitate most all components to low levels brings a new realm of problems. As rapid-scanning UV detectors become common in LC, one can anticipate extensions of the discussions begun here of the interaction of (1) the detector components linear limit, (2) the UV absorbance of the eluents, and (3) the absorption spectra of the sample. The combined effect of points (1) and (2) at low wavelengths give a "system linear limit" that determines a wavelength-dependent "highest linear sample size" that must be considered in order to obtain the benefits of nearly universal detection of even trace level components.

REFERENCES

1 V. V. Berry, *J. Chromatogr.*, 199 (1980) 219.
2 J. E. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 343.
3 T. Greibrokk, E. Jensen and G. Ostvold, *J. Liquid Chromatogr.*, 3 (1980) 1277.
4 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 203 (1981) 349.
5 M. T. W. Hearn, *J. Liquid Chromatogr.*, 3 (1980) 1255.
6 H. P. J. Bennett, C. A. Browne and S. Solomon, *J. Liquid Chromatogr.*, 3 (1980) 1355.
7 J. A. Apffel, T. V. Alfredson and R. E. Majors, *J. Chromatogr.*, 206 (1981) 43.
8 E. L. Johnson, R. Gloor and R. E. Majors, *J. Chromatogr.*, 149 (1978) 571.
9 L. R. Snyder, *Anal. Chem.*, 46 (1974) 1384.
10 F. M. Rabel, *Amer. Lab.*, 6, No. 1 (1974) 331.
11 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 83 (1973) 257.
12 J. G. Atwood, G. J. Schmidt and W. Slavin, *J. Chromatogr.*, 171 (1979) 109.
13 J. Larman, Column Product Manager, DuPont, Wilmington, DE, personal communication, March, 1981.
14 *Annual Book of ASTM Standards*, Standard ANSI/ASTM E 685-79, American Society for Testing and Materials, Philadelphia, PA, May, 1979.
15 F. M. Rabel, in J. Q. Walker (Editor), *Chromatographic Systems, Problems and Solutions*, Preston Publishing, Niles, IL, 1979, p. E4.
16 J. R. Benson, in R. N. Perham (Editor), *Instrumentation in Amino Acid Sequence Analysis*, Academic Press, New York, 1975, pp. 25–28.
17 D. W. Bristol, *J. Chromatogr.*, 188 (1980) 193.
18 D. J. Popovich, J. B. Dixon and B. J. Ehrlich, *J. Chromatogr. Sci.*, 17 (1979) 643.
19 S. J. Poirier, *Amer. Lab.*, 10, No. 12 (1978) 78.
20 G. N. Peterson and J. R. Montgomer, *Anal. Chim. Acta*, 113 (1980) 395.
21 S. J. Poirier and P. M. Sienkiewicz, *Amer. Lab.*, 12, No. 12 (1980) 69.
22 M. Gurkin and J. Ripphahn, *Amer. Lab.*, 12, No. 5 (1980) 99.