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SOLVENT PROPERTIES AND THEIR EFFECTS ON GRADIENT ELUTION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

III. EXPERIMENTAL FINDINGS FOR WATER AND ACETONITRILE

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SUMMARY

The use of gradient elution high-performance liquid chromatography (HPLC) has imposed more stringent requirements for purity upon the solvents which will be used in these experiments. It has been made clear that acetonitrile has a greater native absorbance than water in the short-wavelength UV region (190–260 nm), but this alone cannot account for the baseline disturbances which many chromatographers observe when using this solvent. Furthermore, this phenomenon could not account for the baseline disturbances encountered when acetonitrile is used with fluorescence detection or with some electrometric detectors. We investigated the behaviour of several lots of acetonitrile by UV–HPLC UV absorbance spectroscopy and pH, as well as by classical means. We found that two of the impurities responsible for the chromatographic behaviour of acetonitrile are acetamide and ethyleneimine or its oligomers.

INTRODUCTION

In Parts I and II, the bulk and molecular properties of water and acetonitrile were reviewed. Part III compares the performance of several lots of acetonitrile under conditions likely to influence the performance of the solvent under both isocratic and gradient elution conditions. We evaluated:
    (1) spectral performance (including spectral effects of gas re-dissolution);
    (2) pH and pH*, indicators of the tendency of the solvent to skew the peaks of polar and ionisable materials;
    (3) clean-up; and
    (4) gradient performance comparisons, in which a series of blank gradients compares the tendencies of each lot to produce interfering peaks.

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EXPERIMENTAL

Graphs of experimental results were prepared using a Hewlett-Packard 7220C X–Y Graphics Plotter interfaced to a DEC-System 20 through a Beckman Phoenix 80 Intelligent Data Terminal. The plotting routines were called from Fortran 77 programs.

The UV–VIS spectrophotometer used in this work was a Beckman DU-7 equipped with high-resolution graphics accessory, motorised cell mount drive, graphics printer/plotter, kinetics accessory, and Peltier-effect thermostatted cell mount. Spectra were usually obtained in the 350–195 nm wavelength range, but a preliminary set of spectra in the 700–195 nm range was obtained. Absorbance ranges were varied as necessary to suit the individual case.

Quartz UV cells used in re-dissolution experiments were 1.0 cm square from a matched set of six. All spectra were obtained under temperature control. The temperatures chosen for this set of experiments were 10°C, 15°C, 25°C and 37°C, to include the usual range of temperatures found in a chromatography laboratory. The 37°C observation was made to include the conditions likely to be used in the separation of biogenic molecules.

Where helium sparging was used, the solvent was placed in an all-glass continuous purge apparatus. This apparatus was washed with HPLC-grade methanol and dried for 3 h at 125°C between uses. The helium was Linde (Union Carbide), 99.9999% pure. The regulator was a Linde UPE 3-150 high purity regulator, and PTFE tubing was used to deliver the gas. Stainless-steel metering and shut-off valves were placed in line to allow adjustment of flow-rates.

Non-aqueous solvents used in this study were provided, gratis, by J. T. Baker, Mallinckrodt, Fisher Scientific, MCB (OmniSolv), Ashland Specialty Chemicals and Burdick and Jackson. The water was HPLC-grade, supplied by J. T. Baker or Burdick and Jackson. The solvents comprising the mixtures were used as received.

Glassware was washed with hot chromate–sulfuric acid solution, rinsed five times with purified water (22 MΩ resistivity) and dried for 1 h at 125°C in a forced draft oven between uses.

Fig. 1 shows the spectra of the various lots of acetonitrile in the 195–350 nm range. The region above 350 nm is of limited interest in liquid chromatography and impurities which absorb in this region would be visible.

pH AND pH* MEASUREMENTS

pH control is important in many separations. The possibility that an amide or an imine could be present as an impurity in acetonitrile suggested that pH measurements be made. The presence of significant quantities of either species would lead to elevated pH's, so the solvent would be less suitable for the separation of acidic analytes. Furthermore, high pH's shorten the lives of silica-based columns. The presence of primary or secondary amines also produces a high background in fluorescence experiments using post-column derivatisation.

The pH meter used here was an Orion 811 with glass-sheathed temperature compensator and a gel-filled combination electrode. Calibration buffers were Beckman pH 4.01 (phthalate) and Beckman pH 7.00 (phosphate). The ion activities of
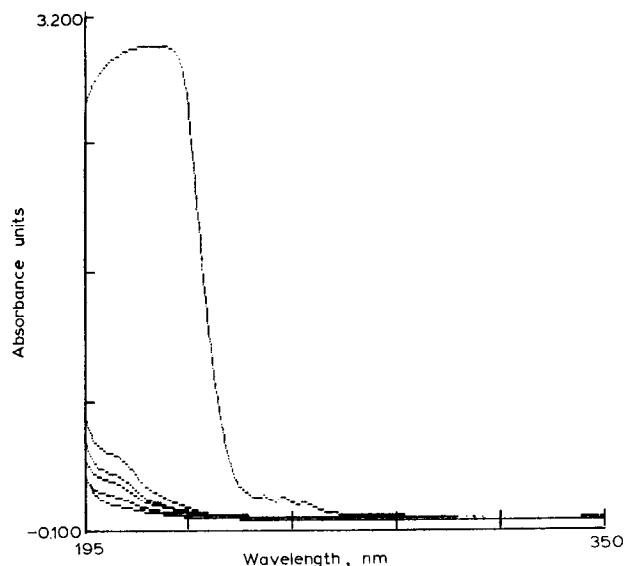
Fig. 1. Spectra of six lots of acetonitrile in the 195–350 nm region.

the buffers were adjusted, where necessary, by the addition of appropriate amounts of neutral acetonitrile. Neutral acetonitrile was made by titrating acetonitrile with aqueous NaH_2PO_4 or aqueous $NaOH$ to either a Congo Red (pH 3.0–5.0) or Brilliant Yellow (pH 6.6–7.8) endpoint. Volumes of buffer and acetonitrile were independently measured, and a nominal total volume of 200 ml was produced. The water–acetonitrile mixtures were made in a similar way, adding the acetonitrile to the water in a 250-ml beaker with magnetic stirring. The mixtures thus produced were allowed to reach room temperature before spectra and pH were recorded.

The electrode was re-calibrated between all measurements using a mixture of buffer and neutral acetonitrile mixture of the same composition as the unknown mixture. The pH of the unknown mixture, determined against a buffer whose ion activity has been adjusted, is termed pH* (ref. 1). When the temperatures of the mixtures had returned to ambient, their pH* values were measured. Fig. 2 shows the pH* values of the mixtures. Table I shows the electrode error inherent in water-scale pH measurements of water–acetonitrile mixtures.

Pronounced quenching occurred in a colleague's laboratory when the composition of his mobile phase changed from 50 to 60% acetonitrile. Quenching manifested itself in a decrease in noise (from about 15 mV to about 8 mV crest height) together with greatly reduced peak heights. These circumstances occurred at the first use of acetonitrile from a new source. The UV spectrum of the new material was identical to that shown in Fig. 1. There was a pronounced maximum around 226 nm. The UV cut-off of the material was approximately 260 nm. Further investigation produced results consistent with the presence of an amide. Direct liquid introduction and thermospray liquid chromatography–mass spectrometry experiments showed that at least two compounds were present. One was subsequently identified as ace-
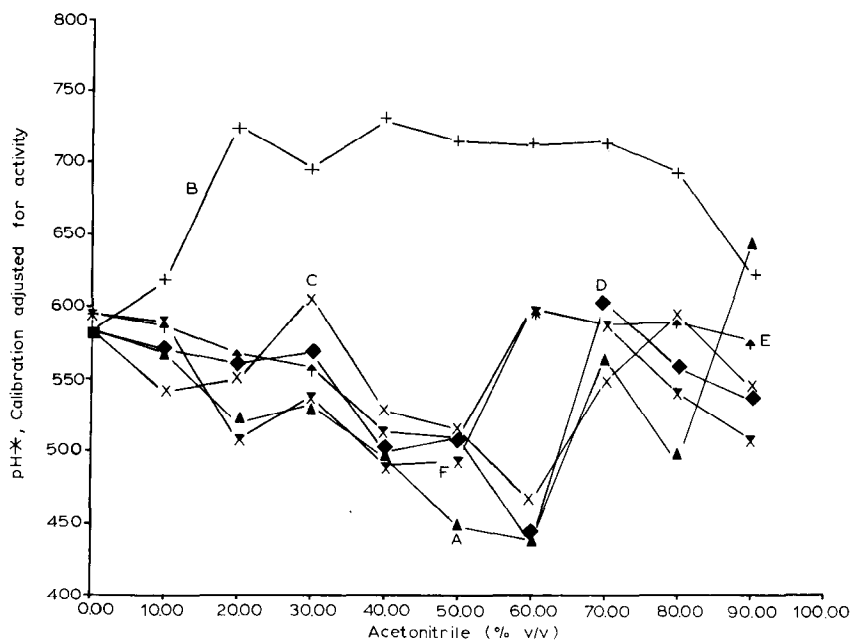
Fig. 2. pH* values of water–acetonitrile mixtures made from various lots of acetonitrile.

tamide, the other is probably an oligomer of ethyleneimine. Reaction with *p*-dimethylaminobenzaldehyde produced a yellow Schiff base[2], and reaction with *o*-phthaldialdehyde produced a fluorescent product. The water-scale pH of a 50% (v/v) mixture of the material in water was 9.2.

GRADIENT CHROMATOGRAM COMPARISONS

The chromatograph used was a Beckman Model 332, consisting of two Model

TABLE I

APPARENT pH AND ELECTRODE POTENTIALS OF WATER–ACETONITRILE MIXTURES

Acetonitrile (%)	pH	Electrode potential (mV)		Potential change
		Buffer	Mixture	
0	4.00	164.0	164.0	0
10.0	4.24	164.0	150.4	13.6
20.0	4.54	164.0	131.7	32.3
30.0	4.83	164.0	117.1	46.9
40.0	5.16	164.0	95.5	68.5
50.0	5.41	161.4	80.6	80.8
60.0	5.76	168.8	65.9	102.9
70.0	5.93	168.9	56.4	112.5
80.0	6.06	164.4	42.5	121.9
90.0	6.77	178.0	1.2	176.8

100A pumps, a Model 420 controller, a 2.8 ml dynamic mixer, a Model 165 varia-ble-wavelength detector and two BD-41 strip-chart recorders. The detector wave-lengths were 254 nm and 205 nm. The output ranges were adjusted to suit the needs of the chromatogram, but automatic ranging was always used. The spans of the recorders were 10 mV, and the chart speeds were 5 mm/min. The column was Beck-man Ultrasphere ODS (5 μm), 250 mm × 4.6 mm I.D. The gradient used for solvent comparisons was linear from 0 to 100% acetonitrile in 30 min, with a 2-min regen-eration period and a 10-min re-equilibration hold at 0% acetonitrile.

Each manufacturer's acetonitrile was used to obtain three gradient chromato-grams. The Auto-Range feature of the detector was used to make sure none of the chromatogram was lost, even though the resulting chromatograms were more dfficult to interpret. Typical chromatograms from all but one of the submissions are shown in Figs. 3–7. One lot was omitted because the spectral evidence against it indicated that the chromatogram could not have been recorded due to detector saturation. The omitted material was later used as the subject of a "clean-up" experiment (vide infra).
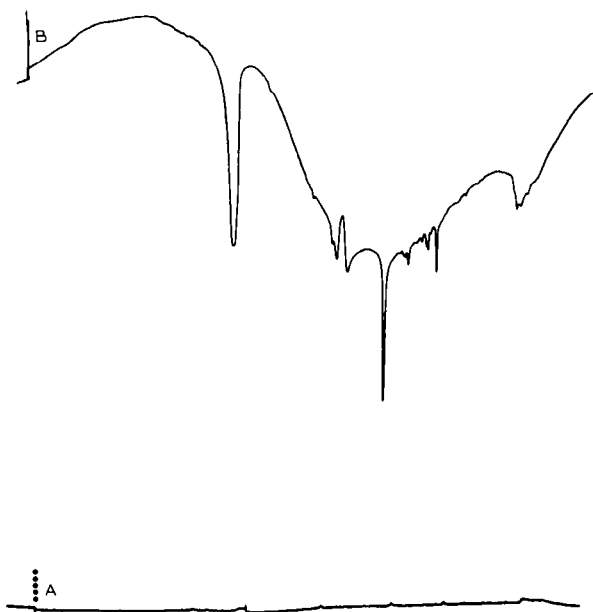

Fig. 3. Gradient chromatogram using acetonitrile from manufacturer A. Pumps: Beckman Model 100A. Controller: Beckman Model 420. Detector: Beckman Model 165; wavelength B = 254 nm, range B: 0.1 a.u. = 10 mV; wavelength A = 205 nm, range A: 0.1 a.u. = 10 mV. Column: Beckman Ultrasphere ODS 5 μm, 250 mm × 4.6 mm I.D. Solvent A: water; solvent B: acetonitrile; starting composition: 0% ace-tonitrile, initial hold: 0.01 min, ending composition: 100% acetonitrile, gradient rate: 5%/min, final hold: 15 min.

Another series of gradient chromatograms was obtained in an effort to at-tribute the peaks shown in Figs. 3–7 to one or the other of the solvents. An isocratic hold time of varying length was inserted into the 0–100% gradient at 10% acetoni-trile. The concentration of acetonitrile was high enough to ensure the elution of water-borne impurities, but not high enough to elute the suspected acetonitrile im-
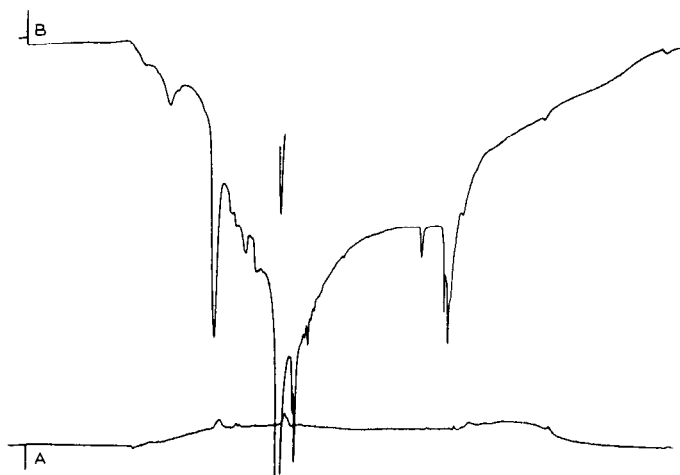
Fig. 4. Gradient chromatogram using acetonitrile from manufacturer B. Conditions as in Fig. 3.

purities. The gradient was allowed to continue after the hold time had elapsed, and the heights of the eluted peaks were measured. The resulting heights were subjected to linear regression against the length of the hold (or the volume of acetonitrile pumped). The correlation coefficient was greater than 0.95 for all peaks which eluted at acetonitrile concentrations at or above 20% (nominal). The heights of peaks which eluted before 20% acetonitrile followed the volume of water pumped more closely.
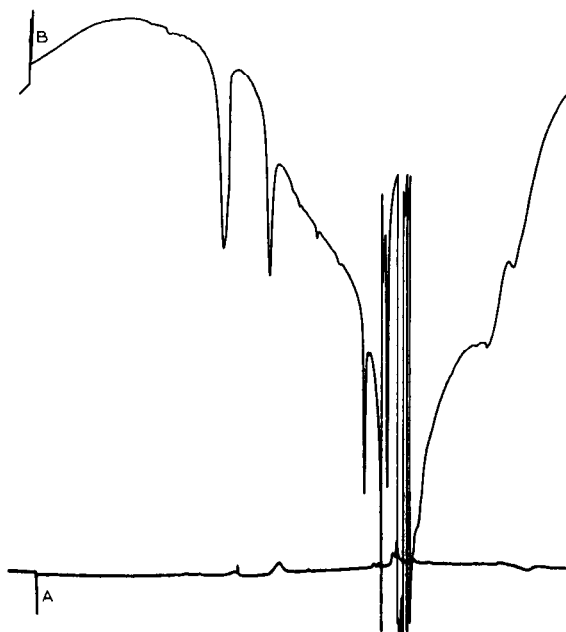
Fig. 5. Gradient chromatogram using acetonitrile from manufacturer C. Conditions as in Fig. 3.
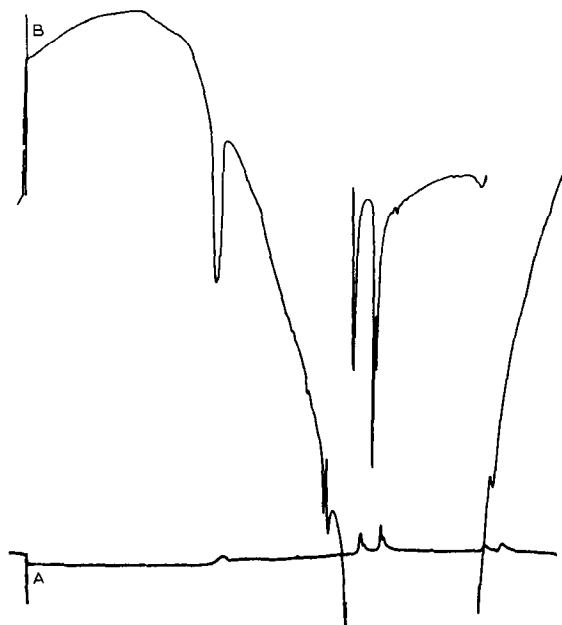
Fig. 6. Gradient chromatogram using acetonitrile from manufacturer D. Conditions as in Fig. 3.

PURIFICATION EXPERIMENTS

Dolan and Berry[3] reported the use of alumina to purify acetonitrile "on-line" between the pump and the mixer. Two lots of acetonitrile were subjected to treatment with alumina in order to test their conclusions. One lot was the lot whose UV spec-
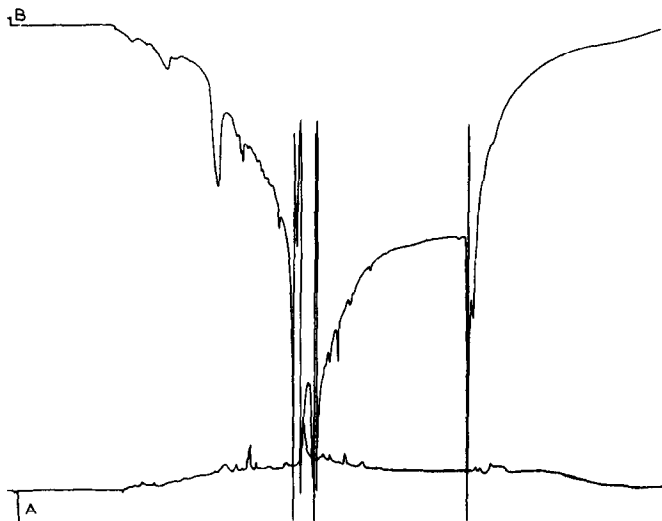

Fig. 7. Gradient chromatogram using acetonitrile from manufacturer E. Conditions as in Fig. 3.
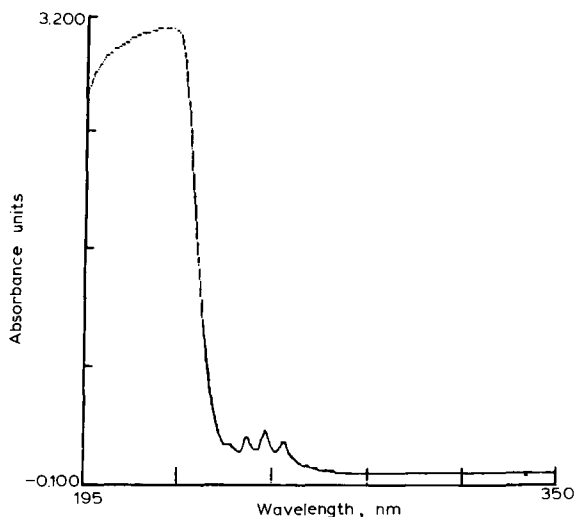
Fig. 8. Spectrum of "impure" acetonitrile before purification. This lot of acetonitrile was used as a "worst case" feedstock for the alumina and silica purification experiments. Pumped through 250 g of alumina or silica, the spectra of the first three catches are shown in Figs. 9, 10 and 11, and Figs. 16, 17 and 18, respectively.

trum showed the greatest absorbance, and the other was that whose spectrum showed the least absorbance. Alumina used in these experiments was Woelm Neutral, Brockman Super 0, obtained from ICN (Cleveland, OH, U.S.A.). The alumina was stored in an oven at 130°C at all times, and was dispensed hot. Silica used in another attempt to purify acetonitrile was Woelm 18, stored at 130°C until used, and dispensed hot.

Each sorbent was poured into a glass column, 300 mm × 25 mm I.D., with adjustable bed supports. The bed supports were inserted and adjusted so that there
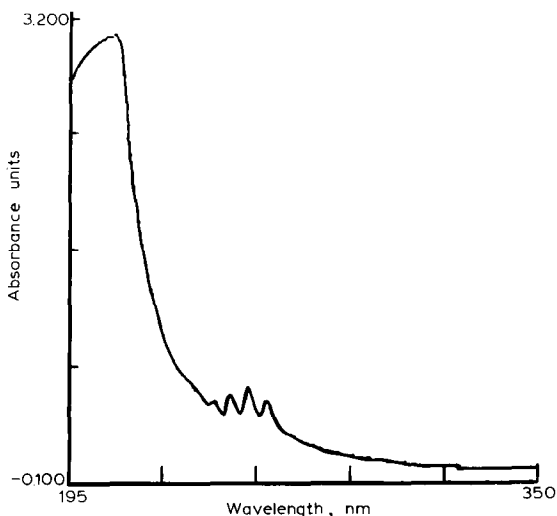

Fig. 9. First catch: impure acetonitrile from alumina column (see Fig. 8). Pump: Beckman 100A. Flow-rate: 2.0 ml/min. Column: 300 mm × 25 mm glass, packed with 250 g alumina.
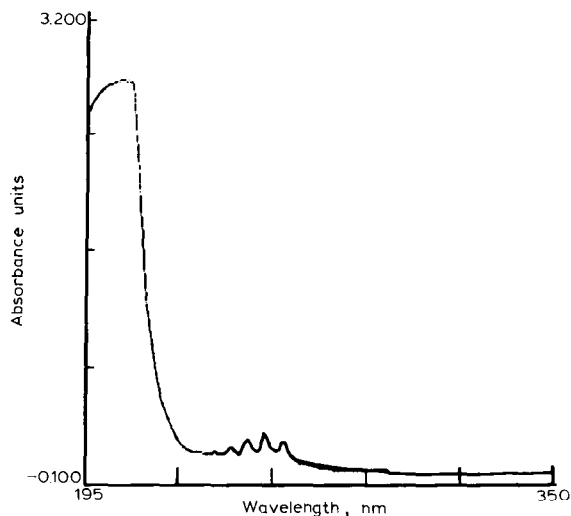
Fig. 10. Second catch: impure acetonitrile from alumina column (see Fig. 8). Conditions as in Fig. 9.

was no open volume in the column, and the column was connected to a Beckman Model 100A pump. The acetonitrile to be purified was pumped at 9.9 ml/min until the column was wetted. The flow-rate was then reduced to 2.0 ml/min, and several fractions were collected. The first catch was approximately 10 ml. Subsequent fractions were of approximately 25 ml. The fractions were taken for UV/Vis spectroscopy. Spectra in the 195–350 nm range were obtained at 10°C. Figs. 8–11 show the spectra of the impure acetonitrile treated with alumina. Figs. 12–15 are the spectra of purer acetonitrile before and after treatment with alumina. The alumina clean-up was repeated, using acidic and basic alumina, with the same results as above. Figs.
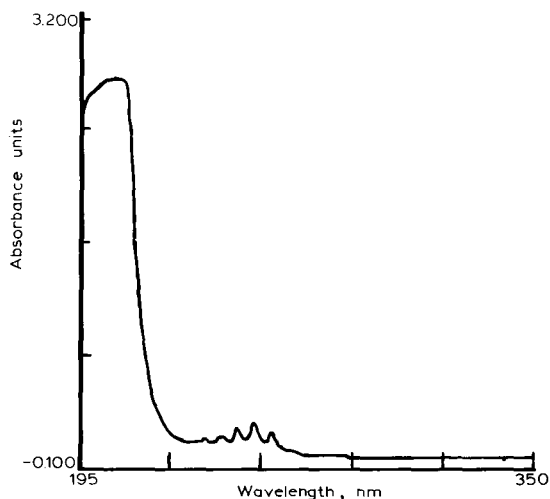

Fig. 11. Third catch: impure acetonitrile from alumina column (see Fig. 8). Conditions as in Fig. 9.
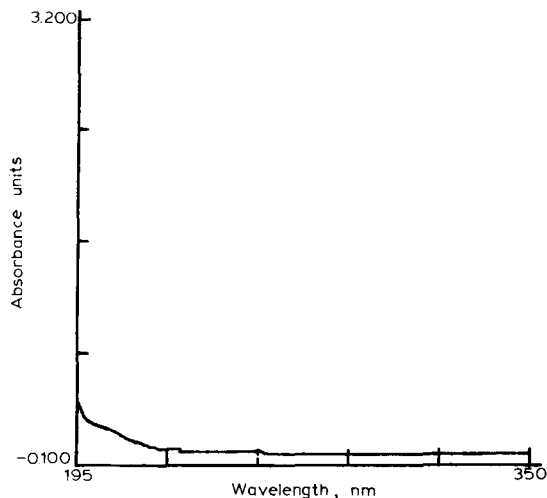
Fig. 12. Spectrum of "pure" acetonitrile before purification. This lot of acetonitrile was used as a "best case" feedstock for the alumina purification experiments. Pumped through 250 g of alumina, the spectra of the first three catches are shown in Figs. 13, 14 and 15.

16–18 are the spectra of the impure acetonitrile after treatment with silica. The first catch from the silica column was yellow. Later catches were less discolored. Break-through volumes were approximately 250 ml acetonitrile/100 g alumina.

DE-GASSING AND GAS RE-DISSOLUTION

The effects of dissolved gases upon absorbance at short wavelength were mea-sured by de-gassing the solvents and allowing them to sit in the open air. The first step was to determine the length of time necessary to de-gas water and acetonitrile
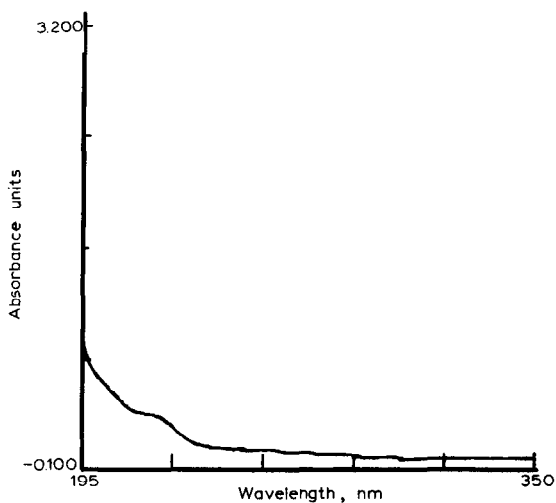

Fig. 13. First catch: pure acetonitrile from alumina column (see Fig. 12). Conditions as in Fig. 9.
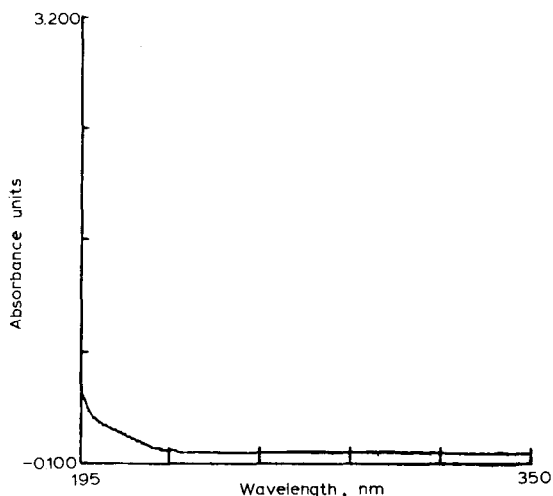
Fig. 14. Second catch: pure acetonitrile from alumina column (see Fig. 12). Conditions as in Fig. 9.

completely. This was accomplished by extended purging of both solvents with helium (48 h at 25°C). About 50% of the acetonitrile and 35% of the water was lost to evaporation in this way, but the absorbance of both solvents was quite low at 195 nm. The spectra of the extensively purged solvents were taken to be the zero point (at which no air was dissolved in either solvent), and another purging run was started. Aliquots were removed at 4-h intervals, and the run was allowed to continue until the spectra of the materials were identical to the zero-point spectra. This occurred between 4 and 8 h after starting for water, and between 12 and 16 h after starting for acetonitrile. Boiling achieved the same results in about 3 h for water, and in about 4 h for acetonitrile. Vacuum de-gassing was abandoned after 96 h, without matching the zero-point spectra.
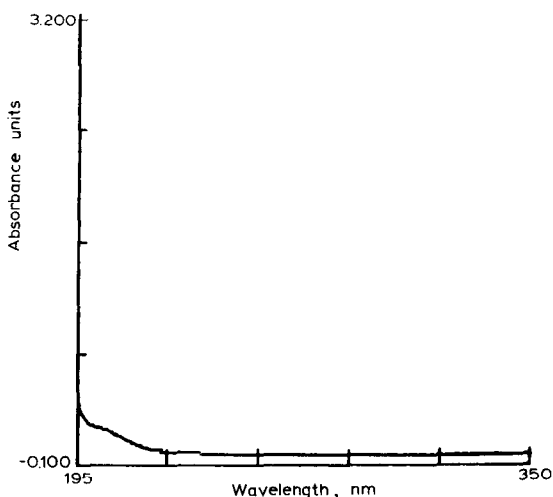

Fig. 15. Third catch: pure acetonitrile from alumina column (see Fig. 12). Conditions as in Fig. 9.
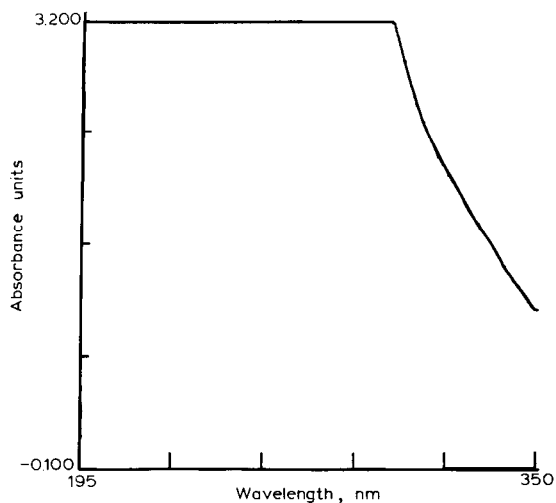
Fig. 16. First catch: impure acetonitrile from silica column (see Fig. 8). Column packed with 250 g silica; other conditions as in Fig. 9.

Aliquots of the completely de-gassed solvents were placed in open, standard 1-cm quartz cells, and were allowed to re-dissolve air at controlled temperatures. Absorbance was measured at 1-s intervals for 240 min and plotted. The change in absorbance was taken as an indication of the extent of air re-dissolution. The spectral data show that dissolved air contributes more to the absorbance of acetonitrile than to water (air is more soluble in acetonitrile than in water). Furthermore, there is an initial period of rapid re-dissolution, as evidenced by the slope of the absorbance–time curve in the first 30 min of the experiment. The remaining 210 min of the experiment showed steady increases in absorbance in both water and acetonitrile, but
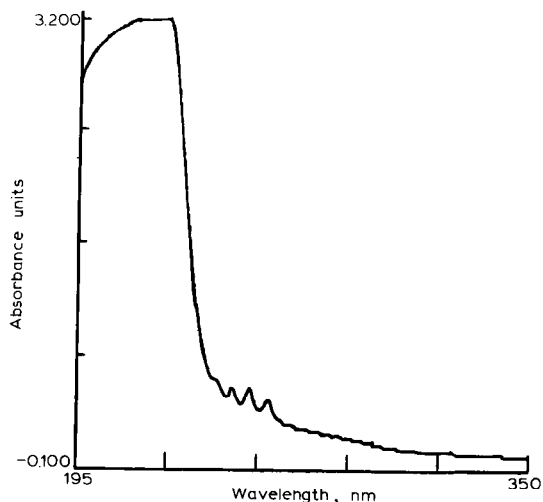

Fig. 17. Second catch: impure acetonitrile from silica column (see Fig. 8). Conditions as in Fig. 16.
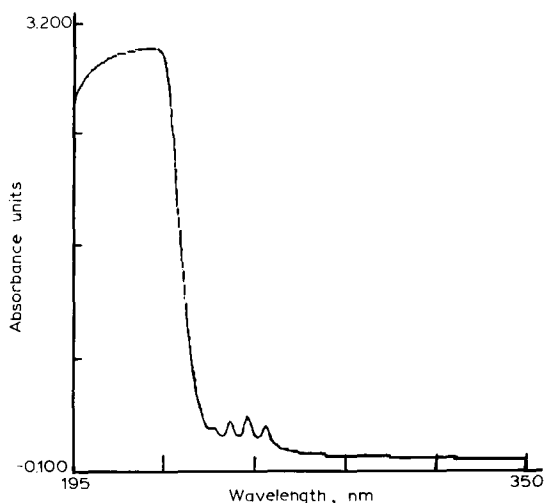
Fig. 18. Third catch: impure acetonitrile from silica column (see Fig. 8). Conditions as in Fig. 16.

the slope of the acetonitrile curve was greater at all times during the experiment. The solubility of air in both solvents was inversely proportional to temperature, as evidenced by both the lower absorbance (after accounting for density changes) and the lower slope of the absorbance–time curve. Fig. 19 shows the absorbance–time curves for acetonitrile (upper plot) and water (lower plot) at one temperature.

Fig. 19. Absorbance vs. time for water (A) and acetonitrile (B) at 10°C.

RESULTS AND DISCUSSION

Acetonitrile and water are both difficult to obtain in purity adequate to the demands of gradient high-performance liquid chromatography (HPLC) using short-wavelength detection. Purification efforts made in the ch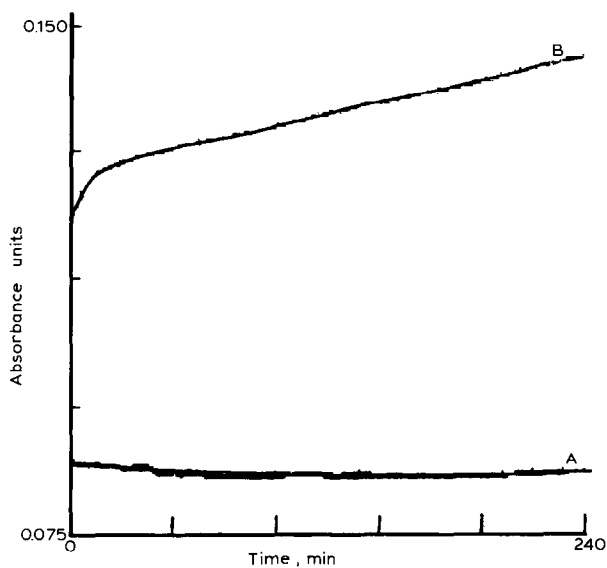emical laboratory meet with varying degrees of success, as the conflict of Dolan and Berry's results with our own demonstrates. Any attempt to purify reagents within a chromatograph must be viewed with some skepticism, because of the inevitable break-through of the impurities. When this transpires, the usual result is contamination of the entire chromatograph. The results shown here indicate that a bad lot of acetonitrile can be marginally improved by treatment with alumina, but a good lot of acetonitrile can suffer harm (as evidenced by higher absorbance at short wavelength) by such treatment, applied indiscriminately. The results of silica clean-ups were uniformly bad. Absorbances in the 195–230 nm region increased from 3.2 to 4.2 for the bad lot, and from 0.001 to 0.5 in the case of the good lot.

Some of the impurities are either primary or secondary amines as shown by their reactions to form Schiff bases as well as their reactions to form cupric dithio-carbamates. Primary and secondary amines will react by both paths; tertiary amines and nitriles will not. There is reason to believe that acetonitrile is subject to decomposition, or to the formation of an equilibrium complex in which air (probably oxygen) participates. Acetonitrile which has been exposed to air for an extended period shows an increased tendency to react with o-phthalaldehyde to form fluorescent products. The same material, upon extended treatment with helium, shows no further tendency to react. Quieter detector signals result from the use of continuous sparging and inert gas blankets.

Water is as difficult to purify as acetonitrile. Re-distillation is of no benefit, as the impurities do not assort themselves into either high-boiling or low-boiling categories. Sparging with helium, especially at elevated temperatures, is the most convenient treatment for the removal of low-boiling impurities. The high-boiling matter may be removed by pumping the water over a clean reversed-phase column. This process, although it could be adapted to "on-line" use, is not generally suitable for such use, as break-through volumes are not very high (250–1000 ml). Purified water was stored in small bottles with PTFE-faced septa, which proved useful for about 24 h. Longer shelf life was attained by storing the small bottles inside larger bottles containing freshly-activated carbon, but the gain was only a further 48 h, and that only if no chlorinated solvents, benzene, toluene, aldehydes or ketones were open in the laboratory. None of the water received in our laboratories has ever been found free of detectable traces of phthalate esters or compounds which emulate their retention behaviour. Dibutyl phthalate and bis(2-ethylhexyl)phthalate were the principal culprits.

Mixing water and acetonitrile causes some unusual problems inside the liquid chromatograph. The process usually generates a significant endotherm, which must be compensated before the mobile phase reaches the column if no deterioration of the chromatogram is to take place. The combination of relatively high flow-rates, short columns and small volumes between the mixer and the column head is one which is most susceptible to the effects of enthalpic cooling. Flow-rates which exceed one-third of the mixing volume per minute generally produce poor mixing, with the

result that some of the mixing enthalpy is liberated closer to or within the column.

Thermostatting the column is a well-known precaution, taken when the need for retention time reproducibility is great. The procedure also improves the shape of the eluting bands. A pre-column can serve as a heat exchanger, in order to pre-heat the mobile phase before it arrives in the column. The effects of a temperature difference between the wall and the center of the column have been modelled, and the shape of the peaks eluting from the column under such circumstances has been shown to depend mainly upon the gradient (the difference in temperature divided by the column radius) and the column efficiency. The composition of the mobile phase was less important in this model, which explicitly excluded a model of the effects of frictional heating. The temperature gradient thus modelled had a cross-sectional shape which was asymptotic (at the column axis) to the wall temperature.

The effects of solvent temperature on the mobile phase composition were also calculated. The temperature of the incoming solvents has a significant effect on the composition of the mixed mobile phase. This will have consequences in retention time precision, as well as in resolution.

The secondary effects of temperature differences between the mobile phase and the column will be seen at the detector as baseline anomalies. Changes in the density of the mobile phase produce, first, a true absorbance effect, changing the number of moles of an absorbing species in the light path in proportion to the change in density. The detector signal will also be affected by the change in refractive index of the mobile phase. In the water–acetonitrile system, by far the most significant part of this change occurs in the first part of the gradient, in the change from 0 to about 30% (v/v) acetonitrile.

Refractive indices are affected by the wavelength of observation. We found no experimental values for the refractive indices of water, acetonitrile, or their mixtures at wavelengths in the blue and UV end of the spectrum, but the effect of wavelength upon refractive index was modelled from the appropriate equations by fitting the inverse fourth-order dependence to experimental values of the refractive index of acetonitrile. This equation was used to operate on the experimental values of the refractive index of water–acetonitrile mixtures to produce a solid (or a series of surfaces) which describes the refractive index behavior of water and acetonitrile at reasonable temperatures, compositions and wavelengths.

The work of Hobbs[4] and that of Campbell et al.[5], showing the effects of dissolved gases and temperature on absorbance, is extended to shorter wavelengths and other temperatures. De-gassing has been shown to be useful in preventing the formation of strongly-absorbing species in acetonitrile, and in reversing the formation of the species. Sparging with helium was shown to be the most convenient means of continuous de-gassing. The re-dissolution of air into water and acetonitrile was observed. The effect of gas re-dissolution was much more pronounced in acetonitrile than in water at all temperatures. Saturation of water with air was virtually complete after 4 h, but acetonitrile continued to absorb air at 4 h. The most rapid and dramatic spectral changes occurred in the first 45 min.

The quality of an acetonitrile lot can be assessed by recording a UV spectrum between 195 and 350 nm. Generally, the lower the optical density in the 195–260 nm region, the more suitable the material will be for gradient HPLC. The higher the absorbance in the 195–230 nm region, the higher will be both the short-wavelength

UV background and the fluorescent background in post-column *o*-phthalaldehyde methods. The latter is particularly important in the determination of amino acids, biological polyamines and carbamate insecticides. Furthermore, the pH* of acetonitrile–water mixtures seems to vary directly with the optical density in the 195–230 nm region. The pH* of the mixtures is important in the separation of all polar compounds, and is particularly important in the separation of ionisable compounds, where the impurities may act as counter-ions or as competing bases, thus introducing another cause of peak distortion.

Solvents for gradient HPLC may deteriorate on prolonged storage. Transportation can introduce unexpected impurities into a solvent lot between the time when it was produced and the time it is used. Storage of HPLC solvents with other reagent chemicals, especially aromatics and chlorinated solvents, should be avoided. Continuous de-gassing is necessary, especially when using short-wavelength UV detection.

Finally, it is false economy to use solvents which are not especially purified for HPLC. One manufacturer's label stated that his acetonitrile was suitable for HPLC and pesticide residue analysis, but was not transparent in the UV. This material was found to be unsuitable for any HPLC separation with which we are familiar, and detrimental to most of them. The same would be true if the material were used for clean-up of pesticide residues. We have found that "on-line" clean-up of acetonitrile is not always effective, especially with bad lots.

AUTHORS' NOTE

We do not wish to pillory a manufacturer, but, rather, to point out the variety of factors which affect gradient elution HPLC. Consequently, none of the results will be traced to a specific company. One must use care in the selection of columns and mobile phase reagents, and care in their storage and handling. All one's caution may still come to naught for reasons beyond the control of the manufacturer. The label "HPLC-grade" guarantees only that the solvent was suitable for such a use by the manufacturer's definition when the manufacturer examined it. The solvent may have changed between the time that it was manufactured and the time it is used. Furthermore, the manufacturer's definition may not be the same as the user's. There is also lot-to-lot variation within a process, which applies to the production of both solvents and columns, and the prudent chromatographer will, therefore, not rely on his ability to obtain another of "the best I've ever seen". Indeed, as the demands of chromatographers become more extreme, the effects of lot variation and the effects of the properties of mixed solvents will become more visible. The responsibility of dealing with these effects, and identifying the offending member cannot be entirely thrust off on the manufacturers. The chromatographer owes it to himself to be familiar with the properties of the system(s) with which he is dealing, so that he can minimise the effects of a change in any one of its parts.

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