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CHARACTERISATION OF ACID DYES IN FORENSIC FIBRE ANALYSIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING NARROW-BORE COLUMNS AND DIODE ARRAY DETECTION

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SUMMARY

A gradient elution high-performance liquid chromatographic (HPLC) system with a diode array detector and a short narrow-bore (40 × 1 mm I.D.) column has been used to characterise a number of acid dyes. The resolution and reproducibility of the HPLC system have been evaluated and the detection limits for various dyes have been estimated. Comparisons are made with current methods of fibre dyestuff examination used in forensic science. The system has been applied to the analysis of dye extracted from single fibres. Using diode array detection, both chromatographic and spectral data can be produced in a single operation from casework sized samples.

INTRODUCTION

An important part of forensic fibre examination involves the comparison of textile dyestuffs. Two techniques are routinely used for this purpose, thin-layer chromatography (TLC)[1–4] and visible microspectrophotometry[1,5]. While both are highly discriminating, they have limitations in their usage. Microspectrophotometry is applicable to the smallest fibres recovered but only considers the spectral characteristics of the mixed dyestuffs. While there is considerable variation in this attribute, chemical and structural differences between dyes are not exploited. TLC requires a relatively large amount of extracted dye if all components are to be recognised and maximum discrimination achieved. For a short length of fibre or where longer lengths are lightly dyed, sufficient material may not be available. In addition, TLC is essentially qualitative, the colours and intensities of separated dye components being assessed subjectively. The reproducibility of dye separation in TLC can also in some circumstances be poor[6]. High-performance liquid chromatography (HPLC) would appear to offer a viable approach for the separation of fibre dyes overcoming many of the problems associated with TLC.

HPLC has been applied widely to characterise acid dyes used as food colourants. Here reversed-phase ion-pair chromatography has often been the preferred method[7–13] although reversed-phase systems without ion-pairing[14] and ion-exchange

chromatography[15,16] have been investigated. In addition, acid dyes in waste water have been separated using reversed-phase ion-pair systems[17,18]. Disperse dyes[17,19,20] and direct dyes[21] have also been examined using various HPLC systems.

Forensic applications of HPLC for characterising colourants have been limited. Dyes from ball-point pen inks have been separated using unmodified silica[22], several different dye classes being separated. The analysis of some writing inks has also been described[23]. HPLC has proved to be useful in the characterisation of colourants used in illicit drug manufacture. Acid food dyes in heroin[24] and in other drug preparations[25,26] have been identified by reversed-phase ion-pair chromatography. In addition, home dyeing products found as colourants in illicit tablets have been separated on similar systems[27]. Recently, the use of HPLC with multiple-wavelength detection to characterise disperse dyes extracted from polyester fibres has been reported[28]. In this case the detection limits were estimated to be 200 pg of injected dye.

The use of HPLC for characterising dyes extracted from single fibres has been limited by a number of practical problems. With conventional HPLC columns, typically of 4–5 mm internal diameter and 10–25 cm in length, considerable dilution of the sample by eluent occurs during chromatography. In consequence large amounts of material are needed to provide an effluent concentration sufficient for detection. A possible solution is to use columns of shorter length and with smaller internal diameters[29,30]. With the smaller column volume, less dilution occurs and hence sensitivity is increased. However, narrow-bore columns require special packing techniques and small volume connections[31].

A further consideration is that the dyes used on fibres differ widely in their chemical structures within each application class. As a consequence some will be very strongly retained on an HPLC column while others have no retention. The problem can be overcome by the use of gradient elution which enables mixtures of such compounds to be chromatographed within a single run[27].

An additional feature of fibre colourants is they may consist of a mixture of several dyes of different colours. This makes the selection of a single wavelength for the detection of all components difficult if not impossible: to obtain maximum sensitivity and selectivity with the limited amount of dye available, it is important to monitor at a strongly absorbing wavelength for each component. A detector is therefore required which can monitor a number of wavelengths simultaneously. The diode array detector is such a device and is now commercially available[33,34]. Such a detector also allows the complete ultraviolet (UV)–visible (VIS) spectrum of the components to be recorded as they emerge from the column. In this way, HPLC with diode array detection can produce both chromatographic and spectral information from a single sample.

In this paper gradient elution HPLC with diode array detection and narrow-bore columns for the characterisation of small quantities of acid dyes is investigated. The reproducibility, sensitivity and specificity of this method compared to TLC is investigated and spectral information examined.

EXPERIMENTAL

Materials

Methanol (HPLC grade), tetramethyl ammonium chloride (HPLC grade) and

water (HPLC grade) were obtained from Fisons (Loughborough, U.K.). Ammonium dihydrogenphosphate was AnalaR grade (BDH, Poole, U.K.).

Eluent preparation

Two eluents (eluents A and B) were mixed to form the mobile phase. Eluent A consisted of methanol (200 ml), water (1800 ml), tetramethylammonium chloride (2.19 g, 0.01 *M*) and ammonium dihydrogenphosphate (0.23 g, 0.001 *M*). Eluent B contained methanol (1600 ml), water (400 ml), tetramethylammonium chloride (2.19 g, 0.01 *M*) and ammonium dihydrogenphosphate (0.23 g, 0.001 *M*). Dissolved gases were removed by purging with helium for 10 min. The pH values were determined as 5.7 and 6.7 for eluents A and B, respectively.

HPLC equipment

Two pumps (Model 6000A, Waters Assoc., Harrow, U.K.) were used to deliver the eluents from 500 ml glass reservoirs to a stainless-steel T-piece (1/16 in.), the mixture then passing through 50 mm of 0.02 in. I.D. stainless-steel tubing into a Gilson dynamic mixer (Model 811, Anachem, Luton, U.K.) fitted with a 65-μl mixing chamber. The well-mixed mobile phase then flowed through 180 mm of 0.006 in. I.D. stainless-steel tubing to the sample injection valve (Rheodyne Model 7125 fitted with a 5-μl loop). The pulse dampers were removed from both HPLC pumps. A Brownlee MPLC cartridge holder with a polymer resin cartridge (PRP-GU, Anachem, Luton, U.K.) was installed in the stainless-steel pipe between the pump delivering eluent A and the mixing T-piece to remove possible contaminants.

The proportions of eluents A and B in the mobile phase were varied during chromatography using a solvent programmer (Model 660, Waters Assoc.). The gradient profile used consisted of a 600-s linear change from the initial eluent composition (95% eluent A and 5% of eluent B) to the final composition (5% of eluent A and 95% of eluent B). This final eluent composition was then maintained for a further 300 s. The two HPLC pumps were each fitted with microflow control units (Part No. WAP060421, Waters Assoc.) to achieve low flow-rates. The flow-rate of the mobile phase in the column was set at 0.05 ml/min. The column was conditioned by pumping eluent A for at least 10 min before injection of the sample. After chromatography, the mobile phase was returned to initial conditions using a 2-min linear reverse gradient.

HPLC column

The column was fabricated from 40 mm × 1/8 in. diameter stainless-steel rod by drilling a 1-mm hole down the centre. The ends of the resulting tube were then ground flat and the central hole polished with 600 mesh carborundum powder. The column was packed with ODS-Hypersil (5-μm particle size, Shandon Southern Products, Runcorn, U.K.) using a balanced density packing procedure[31] in which the packing material (35 mg) was dispersed in tetrabromoethane–methanol (4:1, w/w) and pressurised with hexane. The material was held in place between 1/8 in. stainless-steel mesh discs (Part No. 206, HETP, Macclesfield, U.K.) using drilled out (zero dead volume) 1/8–1/16 in. reducing unions. The mesh discs themselves were sandwiched between two 1/8-in. washers of 1 mm I.D. made from PTFE sheet (0.005 in. thick) to eliminate dead volume. One end of the column was connected to the injector

using 25 mm of 0.006 in. I.D. tubing and the other to the spectrophotometer flow cell having reduced the length of its inlet tube to 25 mm in length. The outlet pipe of the flow cell was fitted with an adjustable back pressure device (Part No. 900435, Phase Separations, Queensferry, U.K.).

Diode array spectrophotometer

The detector system consisted of a diode array spectrophotometer (Model 8450A, Hewlett-Packard, Winnersh, U.K.) fitted with an 8-μl quartz flow cell (Model 178-32QS, Hellma, Muhlheim-Baden, F.R.G.) using an adjustable cell holder (Model HP89008A, Hewlett-Packard). The spectrophotometer was interfaced to a microcomputer (Model 85, Hewlett-Packard), dual disk drive (model 9895A, Hewlett-Packard) and a printer/plotter (Model 7225B, Hewlett-Packard). A suite of programs was available to monitor the flow cell absorbance at one or more UV–VIS wavelengths and to capture UV–VIS spectra when eluted components were detected[35]. Parameters defined by software included the start, end time and scaling for a chromatogram, the wavelengths to be monitored (up to 8), and the time interval between measurements. The mode for spectral capture (*e.g.*, when absorbance exceeds a defined limit at a spectific monitor wavelength), the number of spectra captured and their wavelength range could also be defined. Programs were also available to evaluate and plot all the chromatographic and spectral data. The HPLC system is shown diagramatically in Fig. 1.

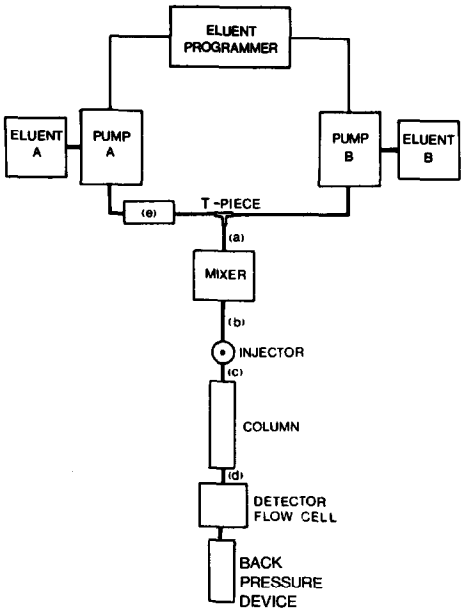


Fig. 1. HPLC system for acid dye separation. (a) Stainless-steel tubing, 50 mm × 0.02 in. I.D.; (b) stainless-steel tubing, 180 mm × 0.006 in. I.D.; (c) stainless-steel tubing, 25 mm × 0.006 in. I.D.; (d) stainless-steel tubing, 25 mm × 0.006 in. I.D.; (e) Brownlee MPLC polymer resin cartridge, type PRP-GU.

Investigation of the gradient profile

To investigate the gradient profile operating during chromatography, acetone (1 ml) was added to eluent B (125 ml) in its reservoir[36]. The gradient run was initiated and the absorbance monitored at 255 nm.

Chromatographic baseline

The stability of the chromatographic baseline was assessed by initiation of the gradient run without injection of material onto the HPLC column. Absorbance was monitored at 255, 398, 510 and 610 nm.

System test standards

To monitor the performance of the chromatographic system, a test solution containing phenol (0.01 g, BDH chromatography grade), *p*-cresol (0.01 g, Aldrich gold label purity) and anisole (0.01 g, Aldrich, 99% purity) in eluent A (100 ml) was used. The mixture (5 μl) was injected onto the column, the elution scheme initiated and the absorbance monitored at 255 nm.

Separation of dyes by HPLC

The solid dyes listed in Table I were used to investigate the retention properties shown by compounds with a wide range of different chemical structures. The dye (0.01 g) was dissolved in eluent A (100 ml) and 5 μl of this solution (500 ng) injected onto the column. The gradient elution run was initiated and absorbance monitored

TABLE I

ACID DYES INVESTIGATED WITH A NARROW-BORE HPLC SYSTEM USING GRADIENT ELUTION

Commercial name	*CI* designation*	*Chemical class*
Tartrazine	Acid Yellow 23	Monoazo
Orange G	Acid Orange 10	Monoazo
Orange II	Acid Orange 7	Monoazo
	Acid Red 37	Monoazo
	Acid Red 88	Monoazo
New Crocein	Acid Red 18	Monoazo
Amarath	Acid Red 27	Monoazo
Lissamine Fast Yellow 2G	Acid Yellow 17	Monoazo
Brilliant Crocein MOO	Acid Red 73	Disazo
Ponceau SS	Acid Red 150	Disazo
Patent Blue VF	Acid Blue 1	Triphenylmethane
Acid Fuchsin	Acid Violet 19	Triphenylmethane
	Acid Green 27	Anthraquinone
Solway Blue RNS	Acid Blue 47	Anthraquinone
Solway Green G	Acid Green 25	Anthraquinone
Nigrosine	Acid Black 2	Azine
Napthol Yellow S	Acid Yellow 1	Nitro
Napthol Green B	Acid Green 1	Nitroso
Indigo Carmine	Acid Blue 74	Indigoid
Erythrosin B	Acid Red 51	Xanthene

* Colour Index, Society of Dyers and Colourists.

at 255, 398, 510 and 610 nm. Spectra of components, between 250 and 700 nm, were captured if the absorption at the 255 nm wavelength increased by more than 0.005 absorbance units (a.u.) within a 1-s measurement period. Duplicate chromatographic separations were made for all dye solutions. All dyes were of commercial quality and contained between 50 and 90% dye by weight.

Detection limits of dyes separated by HPLC

The acid dyes Violet 19, Red 27, Green 27, Yellow 17, Black 2, Orange 10 and Red 73 (Table I) were dissolved in eluent A to produce solutions containing 500, 50 and 5 ng in 5 μl of solution. The dyes were injected onto the column (5 μl of solution), the gradient run initiated and absorbance monitored at 255, 398, 510 and 610 nm.

Detection limits of dyes separated by TLC

The seven dyes separated previously by HPLC (Acid Violet 19, Black 2, Red 27, Green 27, Yellow 17, Orange 10 and Red 73) were examined by TLC. Dyes were dissolved in eluent A and volumes containing 500, 100, 50, 10 and 5 ng of dye were applied to DC-Alufolien Kieselgel 60 F254 plates (Merck, Darmstadt, F.R.G.) in small portions, drying in a stream of cold air between additions. The dyes were developed for 2–4 mm in methanol to produce a concentrated line of dye, then chromatographed in a Camag twin trough tank[6], allowing the plates to equilibrate for 10 min before elution. Plates were developed for 40–60 mm using pyridine–amyl alcohol–10% ammonia solution (4:3:3), chloroform–water–methanol–ammonia (11:1:7:1) and *sec.*-butanol–acetone–water–4% ammonia solution (5:5:1:2) as eluents[1]. The colours and R_F values of components together with their appearance when illuminated with 255 and 350 nm light were recorded. Replicate separations were made for all dyes in the three eluents.

Reproducibility of separations by HPLC

The long and short term reproducibility of the narrow-bore gradient elution system was investigated. To evaluate long term stability, the solution used as a system test standard was chromatographed at approximately weekly intervals over a four-month period and the retention times of phenol, *p*-cresol and anisole recorded. The day to day stability of the system was investigated by obtaining four successive replicate chromatograms for the six solutions used previously to investigate detection limits (Acid Violet 19, Red 27, Green 27, Yellow 17, Orange 10 and Red 73, solutions containing 500 ng/5 μl of solution). The mean retention time and absorbance of the major component as well as the standard deviation were calculated in each case.

Extraction and HPLC of dye from wool fibre

A single blue wool fibre approximately 10 mm long was placed in a capillary tube together with 5–10 μl of pyridine–water (4:3, v/v). The sealed tube was heated for 20 min at 90°C and then the contents transferred with a drawn out pasteur pipette to a 100-μl Reacti-Vial (Pierce & Warriner, Chester, U.K.). The pyridine–water was removed by directing a stream of dry nitrogen onto the solution through a pasteur pipette, the flow being maintained for 5 min after the residue first appeared dry to ensure that all traces of pyridine were removed. The coloured residue was dissolved in about 5–6 μl of eluent A with the aid of a vortex mixer. This solution (5 μl) was

then injected onto the HPLC column and the gradient run initiated, monitoring the absorbance at 255, 398, 510, 560 and 610 nm. Spectral capture (250 to 700 nm) was initiated if the absorbance measured at 255 nm increased by more than 0.005 a.u. over a 1-s measurement period.

RESULTS AND DISCUSSION

The acid dyes are colourants that contain ionisable acid groups and hence ion-pair chromatography was necessary to obtain satisfactory retention on an ODS-silica column. Tetramethylammonium chloride was used as the ion-pairing reagent. Initial experiments indicated that the acid dyes investigated could not be chromatographed satisfactorily within a single isocratic system. Gradient elution was therefore adopted to accommodate the very different retention properties of the dyes. Buffering the mobile phase was necessary to ensure that acid groups in the dyes were ionised and so capable of ion pairing with the tetramethylammonium ion. A pH of near 6 was selected to ensure complete ionisation of dyes without the eluents damaging the silica packing material. Ammonium dihydrogenphosphate was used for buffering.

Thorough degassing of eluents was necessary to prevent the formation of bubbles in the flow cell. The use of the backpressure device helped to eliminate this outgassing. The polymer resin cartridge in the pipe delivering eluent A guarded against the build up of UV absorbing material on the analytical column which might otherwise be eluted during a gradient run.

Work with the gradient elution system showed that the removal of the HPLC pump pulse dampers was essential[30]. Without this modification, the eluent from one HPLC pump was forced into the supply pipe of the other against the elasticity of its pulse damper. When maximum compression was reached, the flow reversed resulting in a mobile phase consisting of unmixed "slugs" of each eluent. Differences in the optical properties of these "slugs" produced large absorbance bands in chromatograms. It was also found that if one pump was stationary at the beginning or end of a gradient run, its volume acted as a pulse damper with similar effects to those previously described. Thus, gradients were always formed with both HPLC pumps operating throughout.

The use of a simple T-piece for mixing was inadequate. The addition of the dynamic mixer was necessary to produce smooth changes in the gradient profile[30,36,37]. The small volume of the dynamic mixing chamber ensured that the delay between the gradient profile being formed and its arrival at the column was minimised. For a similar reason the volume of pipework between mixer and injection valve was as small as possible. In addition, the volume of tubing between the sample injector and flow cell was minimised to avoid dilution of the sample by eluent and so a loss of mass sensitivity.

Alignment of the flow cell in the spectrophotometer beam was critical. With the small cross section of the 8-μl flow cell, correct positioning ensured that as much energy as possible was directed through the cell giving maximum stability and sensitivity.

Investigation of the gradient profile

Fig. 2 shows the response of the 255-nm detector after the gradient elution run

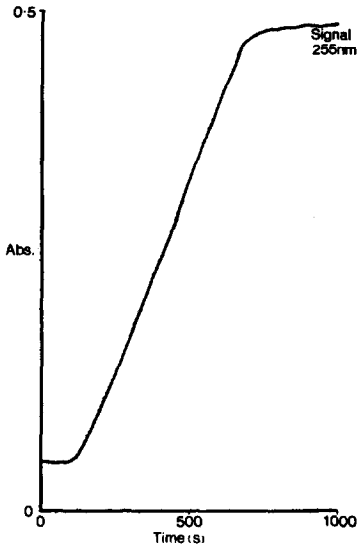


Fig. 2. Gradient profile obtained by mixing two eluents A and B. Eluent A: methanol–water (10:90, v/v); eluent B: methanol–water (80:20, v/v) spiked with acetone (0.8%); mobile phase: linear methanol–water gradient (13.5% to 76.5% methanol, 0.01 *M* tetramethylammonium chloride, 0.001 *M* ammonium dihydrogenphosphate); flow-rate: 0.05 ml/min; column: ODS-silica, 40 × 1 mm I.D.; detection: 255 nm.

is started with eluent B containing acetone. After an initial constant period (approximately 120 s), absorbance rises linearly for 600 s and then stabilises again at the higher level. Absorbance changes reflect the amount of acetone present in the effluent and as acetone is unretained by the column, the amount of eluent B in the mobile phase during elution. The delay of about 120 s corresponds to the time needed for the gradient profile to move from the mixing T-piece through the dynamic mixer, the injection valve and column to the flow cell. At a flow-rate of 0.05 ml/min, this indicates that this volume is about 100 μl. After the initial delay, the rise in absorbance is virtually linear, closely following the programmed gradient. Overall therefore, the elution scheme consists of an initial isocratic period (*ca.* 120 s) using the initial eluent mixture, a linear gradient increase for 600 s and then a second isocratic period (*ca.* 180 s) using the final eluent composition. Pumps A and B are primed with 10% and 80% methanolic eluents respectively. It should be remembered that neither pump is stationary at the extremes of the gradient profile (95% eluent A and 5% B under initial conditions, 5% A and 95% B under final conditions). Thus methanol concentration varies from 13.5% to 76.5% during the run. Experience showed that the gradient profile was very reproducible over many runs.

Chromatographic baseline

Fig. 3 shows the chromatograms obtained during a gradient run without injection of material onto the column. The chromatograms corresponding to detection at the selected monitor wavelengths of 255, 398, 510 and 610 nm are illustrated.

While the absorbance at the beginning and the end of the gradient run were very similar, excursions from a smooth baseline were evident, and these were subjected to further investigations. It was noted that the baseline disturbances were very

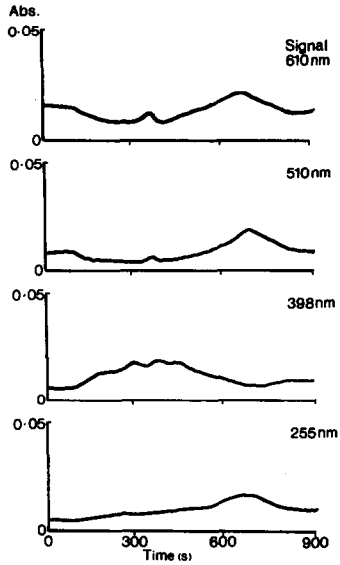


Fig. 3. Chromatograms obtained for gradient elution without injection of sample. Mobile phase: 10 min linear methanol–water gradient (13.5% to 76.5% methanol, 0.01 *M* tetramethylammonium chloride, 0.001 *M* ammonium dihydrogenphosphate); flow-rate: 0.05 ml/min; column: ODS-silica, 40 × 1 mm I.D.; detection: standard monitor wavelengths (255, 398, 510, 610 nm).

reproducible and occurred over a very wide wavelength range in both the VIS and UV regions. On this evidence it seemed unlikely that the disturbances were arising from eluent impurities observed previously as a problem in gradient elution HPLC[38–40]. Bubble formation or refractive index (RI) disturbances in the flow-cell, were more likely explanations. Bubble formation seemed less likely as the eluents were thoroughly degassed with helium while a back-pressure regulator had been fitted to the flow-cell outlet. Furthermore, a narrow-bore glass tube was connected to the outlet of the dynamic mixer and even without any backpressure applied no bubble formation was observed.

If the liquid in an optical flow-cell does not have uniform RI, the beam of light may be focused or de-focused altering the energy falling on the detector and hence causing an apparent change in absorbance. The presence of such unstable states in the flow-cell was investigated by a series of experiments in which the eluent flow was stopped abruptly by turning the handle of the injection valve into a position mid-way between load and inject thus blocking the flow-path. When the experiment was conducted during a blank gradient run at a point where the baseline showed a maximum excursion the apparent absorbance of the stationary liquid trapped in the flow-cell was observed to fall rapidly to a value similar to that of initial or final conditions. This result strongly supported the view that the disturbances arose from transient RI effects.

Incomplete mixing of eluents A and B could explain RI disturbances in the flow cell but the experiment in which a glass tube was connected to the dynamic mixer showed no signs of poor mixing. A further possibility was the presence of a "thermal lens" in the flow cell arising from a difference in temperature between the

effluent from the HPLC column and the inner walls of the flow cell. Under such circumstances it has been shown that the liquid in contact with the walls of a cylindrical flow cell can have a different temperature (and hence different RI) from that flowing down the centre of the cell. It is this phenomenon which accounts for the flow-sensitivity of some UV detectors[41]. This hypothesis was eventually discounted as the detector showed no change in apparent absorbance on starting or stopping the flow of 100% eluent A pumped through the system.

The true explanation of the baseline disturbances lies in the relationship between the eluent flow-rate with the narrow-bore HPLC column, the rate of gradient formation and the flow-cell volume. The passage of eluent through the flow cell (50 μl/min) is relatively slow when compared to the rate of gradient formation (13.5% to 76.5% methanol in 10 min). Thus, the liquids entering and leaving the 8-μl flow-cell will have slightly different RI values leading to the observed disturbances. In effect, the flow-cell acts as a static mixing chamber as the percentage of organic modifier in the eluent changes. The problem does not occur with conventional HPLC columns as the flow-rates used (1–2 ml/min) ensure that the rate of change of eluent composition in an 8-μl flow cell is very low.

Experiments in which the 8-μl flow cell was replaced by a 0.5-μl flow-cell with an identical gradient run demonstrated dramatic reductions in the chromatographic disturbances with virtually flat baselines. Unfortunately the reduction in cell path-length from 10 to 1 mm with the new flow cell caused a large fall in the detector sensitivity. Consequently the present work has been conducted using the 8-μl flow cell where baselines were adequate for chromatography with absorbance ranges of about 0.05 a.u.f.s.

Separation of dyes by HPLC

The retention times of dye components separated by HPLC are shown in Table II. It is immediately apparent that some dyes are very complex mixtures, CI Acid Black 2 for example containing at least nineteen constituents. The chromatograms for this dye, shown in Fig. 4, contain small individual peaks superimposed on a broad absorbance band. It is apparent that the dye contains numerous compounds, with those present in high concentration visible above an envelope of unresolved components. Fig. 5 shows the UV–VIS spectra obtained for some of the detected components labelled in Fig. 4. It is interesting to note that the major constituent of Acid Black 2 is yellow (Fig. 4, peak 1), the majority of other components being dull blue or green. It should be remembered that all of these coloured components may not become attached to the fibre during dyeing. Hence the results shown here represent the maximum number of components that could be detected from fibres on extraction.

The importance of multiple-wavelength detection is well illustrated by Acid Blue 74. Chromatograms produced by detection at 255 and 610 nm are significantly different. As can be seen in Fig. 6, at 610 nm there are two major peaks (peaks 1 and 3) while at 255 nm an additional component is detected (peak 2). Inspection of the UV–VIS spectra, also shown in Fig. 6, shows that the components eluted as peaks 1 and 3 have a strong absorbance band at 610 nm and another near 255 nm. For peak 2, its only major absorbance is at 255 nm, absorption at 610 nm being relatively small.

TABLE II

RETENTION DATA FOR DYES SEPARATED BY HPLC

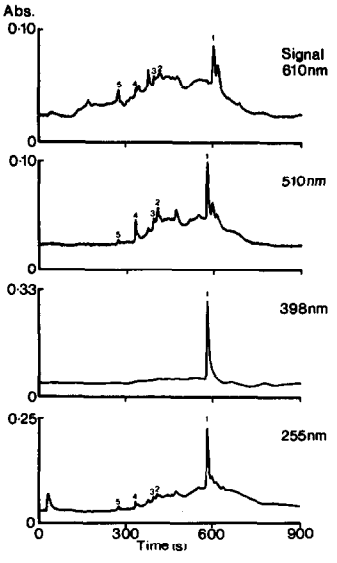
Acid dye	*Band No.*	*Detection wavelength (nm)*	*Retention time* (s)*	*Acid dye*	*Band No.*	*Detection wavelength (nm)*	*Retention time* (s)*
Yellow 1	1	398	116	Red 73	1	510	560
	2	255	378				
				Red 88	1	510	648
Yellow 17	1	398	305				
	2	398	568	Red 150	1	510	308
					2	510	332
Yellow 23	1	255	56		3	510	390
					4	510	436
Orange 7	1	510	388		5	510	476
	2	510	476		6	510	532
	3	510	485		7	510	548
	4	510	560		8	510	588
	5	510	616		9	510	610
					10	510	720
Orange 10	1	255	202				
	2	255	282	Green 1	1	255	36
	3	255	317		2	255	44
	4	255	472		3	255	55
	5	255	563		4	255	191
					5	255	214
Red 18	1	510	76		6	255	333
	2	510	219		7	255	409
	3	510	276		8	255	552
	4	510	421				
	5	510	578	Green 25	1	255	592
					2	255	606
Red 27	1	255	63		3	255	810
	2	255	163				
	3	255	298	Green 27	1	255	381
	4	255	429		2	255	536
					3	255	587
Red 37	1	255	232		4	255	607
	2	255	264		5	255	631
	3	255	304		6	255	655
	4	255	340		7	255	667
	5	510	420		8	255	675
	6	510	456		9	255	687
					10	255	750
Red 51	1	510	678		11	255	778
Blue 1	1	610	400	Blue 47	1	255	552
	2	610	452		2	255	600
	3	610	512		3	255	622
	4	610	524		4	255	646
	5	610	544		5	255	664
	6	610	589				
	7	610	592				
	8	610	606				
	9	610	664				

(Continued on p. 198)

TABLE II (*continued*)

Acid dye	*Band No.*	*Detection wavelength (nm)*	*Retention time* (s)*	*Acid dye*	*Band No.*	*Detection wavelength (nm)*	*Retention time* (s)*
Blue 74	1	610	96	Black 2	1	610	274
	2	255	180		2	510	333
	3	610	212		3	610	341
					4	610	377
Violet 19	1	255	22		5	610	385
	2	510	30		6	510	397
	3	510	65		7	510	409
	4	510	118		8	510	417
	5	510	142		9	510	433
	6	510	197		10	510	444
	7	510	242		11	510	460
	8	510	287		12	510	472
	9	510	304		13	510	520
	10	510	344		14	510	548
					15	398	580
					16	510	595
					17	510	611
					18	255	639
					19	610	696

* Mean of duplicate determinations.



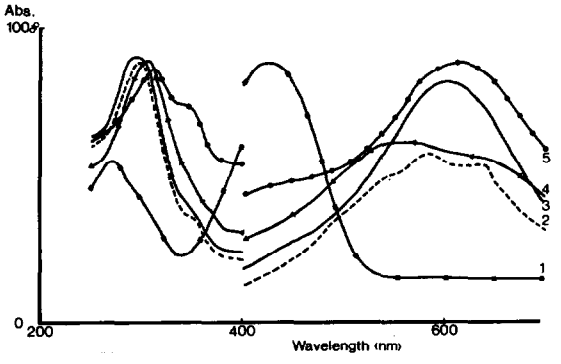


Fig. 4. Chromatograms obtained from Acid Black 2. Mobile phase: 10 min linear methanol–water gradient (13.5% to 76.5% methanol, 0.01 *M* tetramethylammonium chloride, 0.001 *M* ammonium dihydrogenphosphate); flow-rate: 0.05 ml/min; column: ODS-silica, 40 mm × 1 mm I.D.; detection: standard monitor wavelengths (255, 398, 510, 610 nm). Spectra of components 1–5, see Fig. 5.

Fig. 5. UV–VIS spectra of components separated by HPLC from Acid Black 2. Component 1, 0.66 a.u.f.s.; component 2, 0.21 a.u.f.s.; component 3, 0.13 a.u.f.s.; component 4, 0.14 a.u.f.s.; component 5, 0.07 a.u.f.s. For chromatograms and component identification, see Fig. 4.

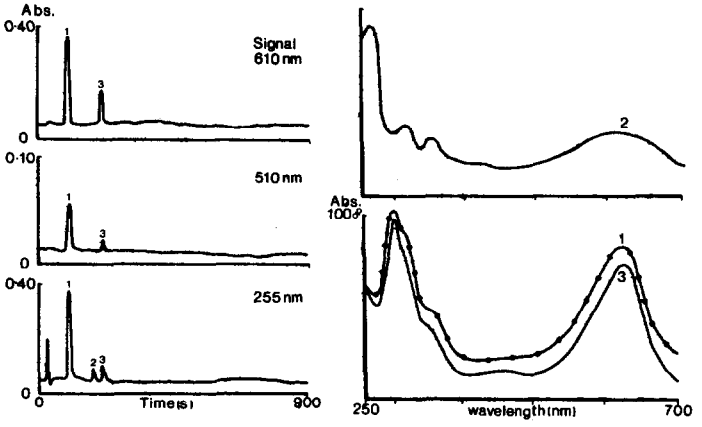


Fig. 6. Chromatograms and spectra obtained from Acid Blue 74. Chromatograms; mobile phase: 10 min linear methanol–water gradient (13.5% to 76.5% methanol, 0.01 *M* tetramethylammonium chloride, 0.01 *M* ammonium dihydrogenphosphate); flow-rate: 0.05 ml/min; column: ODS-silica, 40 mm × 1 mm I.D.; detection: 255, 510 and 610 nm. Spectra; component 1, 0.60 a.u.f.s.; component 2, 0.07 a.u.f.s.; component 3, 0.15 a.u.f.s.

The spectra of dyestuffs consist of broad absorbance bands across the UV–VIS region. The four wavelengths selected for routine screening of dyes (255, 398, 510 and 610 nm) were chosen to give an even spread across the spectroscopic range. At least one detection wavelength is then likely to lie close to an absorbance maximum of a dyestuff. In the casework context, a preliminary experiment may be performed with the four specified wavelengths using fibres from the control garment. Further monitor wavelengths can then be selected, in addition to the original four, based on the UV–VIS absorption maxima of the components detected. In this way the sensitivity of the system can be maximised.

As can be seen in Fig. 7, the retention times of the dyes studied are fairly evenly

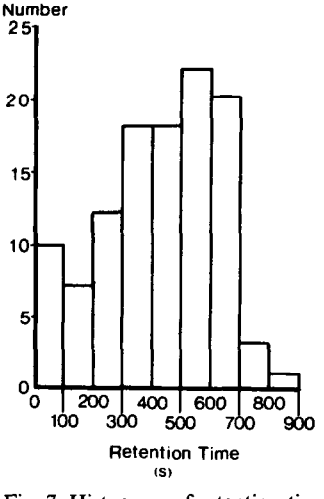


Fig. 7. Histogram of retention times for components separated by HPLC from acid dyes.

TABLE III

DETECTION OF DYE COMPONENTS BY HPLC

Dye	*Peak No.*	*Detection wavelength (nm)*	*Absorbance at peak maximum**		
			500 ng	*50 ng*	*5 ng*
Acid Violet 19	1	255	0.59	0.070	N**
	2	510	0.11	0.009	N
	3	510	0.04	0.003	N
	4	510	0.01	N	N
	5	510	0.10	0.011	N
	6	510	0.22	0.022	N
	7	510	0.12	0.011	N
	8	510	0.03	0.002	N
	9	510	0.01	N	N
	10	510	0.02	N	N
Acid Red 27	1	255	1.10	0.16	0.017
	2	255	0.005	N	N
	3	255	0.005	N	N
	4	255	0.005	N	N
Acid Green 27	1	255	0.006	N	N
	2	255	0.007	N	N
	3	255	0.006	N	N
	4	255	0.017	N	N
	5	255	0.020	N	N
	6	255	0.005	N	N
	7	255	0.005	N	N
	8	255	0.005	N	N
	9	255	0.071	0.007	N
	10	255	0.121	0.015	N
	11	255	0.647	0.088	0.011
Acid Yellow 17	1	398	1.31	0.215	0.028
	2	398	0.005	N	N
Acid Orange 10	1	255	1.42	0.231	0.03
	2	255	0.03	N	N
	3	255	0.01	N	N
	4	255	0.01	N	N
	5	255	0.01	N	N
Acid Red 73	1	510	2.22	0.407	0.060
Acid Black 2	1	610	0.01	N	N
	2	510	0.020	0.005	N
	3	610	0.008	N	N
	4	610	0.005	N	N
	5	610	0.005	N	N
	6	510	0.008	N	N
	7	510	0.017	N	N
	8	510	0.008	N	N
	9	510	0.005	N	N
	10	510	0.005	N	N
	11	510	0.005	N	N
	12	510	0.012	N	N
	13	510	0.005	N	N
	14	510	0.005	N	N
	15	398	0.25	0.03	N
	16	510	0.02	0.005	N
	17	510	0.009	N	N
	18	255	0.008	N	N
	19	610	0.005	N	N

* Mean of four determinations.

** N = Not detected.

distributed across the chromatographic range up to 700 s. Thus most species are eluted during the gradient profile producing maximum resolution of the components. The final isocratic period in the gradient profile is necessary to remove a few strongly retained compounds. Considering the different chemical types, molecular weights and functional groups represented in the dyes examined, it is likely that the elution system will accommodate most acid dyes which might be encountered. Although the short, narrow bore column (40 × 1 mm I.D.) used in the present study does not have a high efficiency, the acid dyes are well separated on the HPLC system by using a gradient elution system.

Detection limit of dyes separated by HPLC

The components detected at the 500, 50 and 5 ng levels together with their measured absorbances are shown in Table III. It is apparent that when a colourant contains principally one constituent, the detection of 5 ng is readily achieved although at this level any minor constituents are generally not detected. Examples here are Acid Red 73 (single component), Acid Orange 10 and Acid Yellow 17 (one major component). Fig. 8 shows the chromatograms obtained for one of these dyes, Acid Orange 10, at the 500, 50 and 5 ng levels. At the 500-ng level, four minor components are detected at retention times greater than the major component. In contrast, with dyes such as Acid Black 2 and Acid Violet 19, which contain at least two major and numerous minor components, detection is only possible around the 50-ng level with the HPLC system described. In these cases a considerable total weight of dye is needed to include detectable quantities of any single component.

Detection limits of dyes separated by TLC

The components separated by TLC using three different eluent systems and the specified dye quantities are shown in Tables IV, V and VI. In nearly all cases, HPLC reveals many more components for each dye than TLC. For example, sepa-

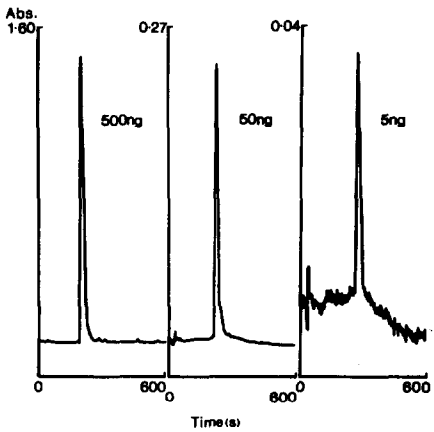


Fig. 8. Chromatograms obtained with 500, 50 and 5 ng of Acid Orange 10. Mobile phase: 10 min linear methanol–water gradient (13.5% to 76.5% methanol, 0.01 *M* tetramethylammonium chloride, 0.001 *M* ammonium dihydrogenphosphate); flow-rate: 0.05 ml/min; column: ODS-silica, 40 mm × 1 mm I.D.; detection: 255 nm.

TABLE IV

DETECTION OF DYE COMPONENTS BY TLC

Eluent: pyridine–amyl alcohol–10% aq. ammonia (4:3:3, v/v/v).

Dye	*Band No.*	*Visible colour*	*Fluorescent colour*		R_F*	*Weight of dye (ng)***				
			255 nm	*350 nm*		*500*	*100*	*50*	*10*	*5*
Acid Violet 19	1	Blue/red			0.02	D	N	N	N	N
	2	Blue/red			0.10	D	D	D	N	N
	3	Blue/red	Blue		0.17	D	N	N	N	N
	4	Blue/red	Blue		0.27	D	N	N	N	N
Acid Red 27	1	Red			0.40	D	D	D	D	D
Acid Green 27	1	Green			0.67	D	D	D	N	N
	2	Green			0.69	D	D	N	N	N
	3	Green			0.85	D	N	N	N	N
Acid Yellow 17	1	Yellow			0.60	D	D	D	N	N
Acid Orange 10	1	Orange			0.29	D	D	D	N	N
Acid Red 73	1	Red			0.67	D	D	D	D	D
Acid Black 2	1	Blue			0.02	D	D	D	N	N
	2	Grey			0.10	D	D	D	D	D
	3	Purple			0.63	D	D	D	N	N
	4	None		Pink	0.65	D	N	N	N	N
	5	Yellow			0.67	D	D	D	N	N

* Mean of duplicate determinations using 500 ng of dye.

** D = Component detected; N = component not detected.

ration of Acid Violet 19 by HPLC revealed ten components at the 500-ng level (Fig. 9) while the best TLC system revealed seven and the worst only three. The HPLC system was also more sensitive than TLC in detecting Acid Orange 10, Green 27 and Yellow 17. Both HPLC and TLC detected components of Acid Red 27 and Acid Red 73 to the limit of 5 ng. In the TLC of the red dyestuffs, the good contrast of the dye against the white plate and the sharpness of the bands clearly aids their visualisation. For the orange, green and especially the yellow dyes, contrast on the TLC plate was less favourable and hence the detection of bands is more difficult. With Acid Violet 19, both chromatographic methods were usable below 50 ng of dye although at this level HPLC reveals seven components while TLC shows at best three.

With Acid Black 2, TLC is superior to the present HPLC system in detecting material at low levels, TLC being usable with 5 ng of dye. In addition, at the 50-ng level, TLC can reveal up to four components while the HPLC system shows three. Here the poor resolution of TLC aids detection. In TLC, with just a few resolved bands, there is sufficient material in each for detection on the plate. In HPLC, with its high resolution, the numerous individual components are each below the detection limit. An additional factor contributing to the sensitivity of TLC in this case may well be the strong contrast of the dark grey bands against the white plate. Fortunately such complex mixturese are usually encountered with black dyestuffs where a considerable amount of dye (up to 8% of the fibre weight) is present. In a typical casework wool fibre (5 mm long, 25 μm diameter) such a dyeing would produce about 250 ng of material, sufficient for the HPLC system to detect the multiplicity of in-

TABLE V

DETECTION OF DYE COMPONENTS BY TLC

Eluent: chloroform–water–methanol–ammonia (11:1:7:1, v/v).

Dye	*Band No.*	*Visible colour*	*Fluorescent colour*		R_F*	*Weight of dye (ng)***				
			255 nm	*350 nm*		*500*	*100*	*50*	*10*	*5*
Acid Violet 19	1	None		Purple	0.04	D	D	D	N	N
	2	Pink		Purple	0.09	D	D	D	N	N
	3	Pink		Purple	0.13	D	D	N	N	N
	4	Pink		Purple	0.28	D	N	N	N	N
	5	Pink		Purple	0.30	D	D	D	N	N
	6	Pink		Purple	0.35	D	D	N	N	N
	7	Pink		Purple	0.46	D	D	N	N	N
Acid Red 27	1	Pink			0.15	D	D	D	D	D
Acid Green 27	1	Blue/green			0.28	D	N	N	N	N
	2	Blue/green			0.56	D	D	D	N	N
	3	Blue/green			0.63	D	N	N	N	N
	4	Blue/green			0.86	D	D	D	N	N
Acid Yellow 17	1	Yellow			0.35	D	D	D	N	N
Acid Orange 10	1	Orange			0.31	D	D	D	N	N
Acid Red 73	1	Red			0.39	D	D	D	D	D
Acid Black 2	1	Grey			0.03	D	D	D	N	N
	2	Grey			0.09	D	N	N	N	N
	3	Grey			0.29	D	N	N	N	N
	4	Grey			0.35	D	N	N	N	N
	5	Grey			0.36	D	D	D	N	N
	6	Yellow			0.78	D	D	D	N	N
	7	Pink		Pink	0.81	D	D	D	N	N

* Mean of duplicate determinations using 500 ng of dye.
** D = Component detected; N = component not detected.

TABLE VI

DETECTION OF DYE COMPONENTS BY TLC

Eluent: *sec.*-butanol–acetone–water–4% aq. ammonia (5:5:1:2, v/v).

Dye	*Band No.*	*Visible colour*	*Fluorescent colour*		R_F*	*Weight of dye (ng)***				
			255 nm	*350 nm*		*500*	*100*	*50*	*10*	*5*
Acid Violet 19	1	Pink		Purple	0.36	D	D	D	N	N
	2	Pink		Purple	0.52	D	D	D	N	N
	3	Pink		Purple	0.64	D	N	N	N	N
Acid Red 27	1	Pink/red			0.60	D	D	D	D	D
Acid Green 27	1	Blue/green			0.75	D	D	D	N	N
	2	Blue/green			0.87	D	N	N	N	N
	3	Blue/green			0.95	D	N	N	N	N
Acid Yellow 17	1	Yellow			0.77	D	D	D	N	N
Acid Orange 10	1	Orange			0.30	D	D	D	N	N
Acid Red 73	1	Red			0.87	D	D	D	D	D
Acid Black 2	1	Grey			0.03	D	D	D	N	N
	2	Purple			0.49	D	N	N	N	N
	3	Pink			0.88	D	D	D	N	N
	4	Yellow			0.96	D	D	D	N	N

* Mean of duplicate determinations using 500 ng of dye.
** D = Component detected; N = component not detected.

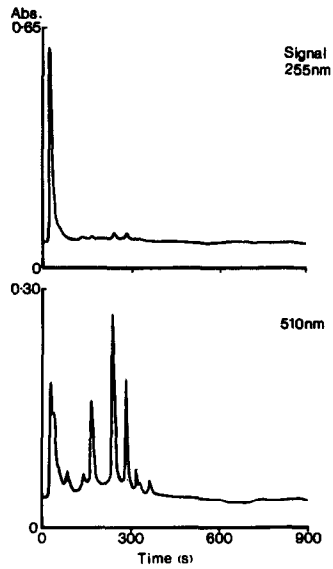


Fig. 9. Chromatograms obtained for Acid Violet 19. Mobile phase: 10 min linear methanol–water gradient (13.5% to 76.5% methanol, 0.01 *M* tetramethylammonium chloride, 0.001 *M* ammonium dihydrogenphosphate); flow-rate: 0.05 ml/min; column: ODS-silica, 40 mm × 1 mm I.D.; detection: 255 nm and 510 nm.

dividual components. It must also be remembered that the dyes used in this study were of commercial quality and contained only 50 to 90% of colourant by weight. Hence the actual detection limits of both HPLC and TLC will be somewhat better than reported here.

A restraint on the sensitivity of the gradient HPLC system is the stability of the chromatographic baseline. RI changes within the cell limit the sensitivity of the system to about 0.05 a.u.f.s. However, it is important to realise that the system is capable of simple isocratic separations. Under these conditions a more stable baseline is produced and so sensitivity is enhanced. Suitable isocratic conditions are conveniently deduced from the proportions of eluent A and B present when peaks elute in the gradient run: a composition with slightly less eluent B is used so that peaks are eluted close to, but separated from, the injection disturbance. In this position maximum sensitivity is achieved for the isocratic run. Fig. 10 shows the results obtained by injection of 2000, 1000 and 500 pg of Acid Orange 10 using isocratic conditions of 70% eluent A and 30% eluent B. Comparing these results with those obtained with the gradient elution technique (Fig. 8), it is apparent the baseline is flatter and so detection of small amounts of dye is possible.

System standard and reproducibility

Fig. 11 shows the chromatogram resulting from injection of the test mixture of phenol, *p*-cresol and anisole and detecting at 255 nm. All the bands tail to some extent, bands having widths at half height of between 10 and 14 s. Such values are useful for monitoring the column performance, large increases in band width indicating a need to repack.

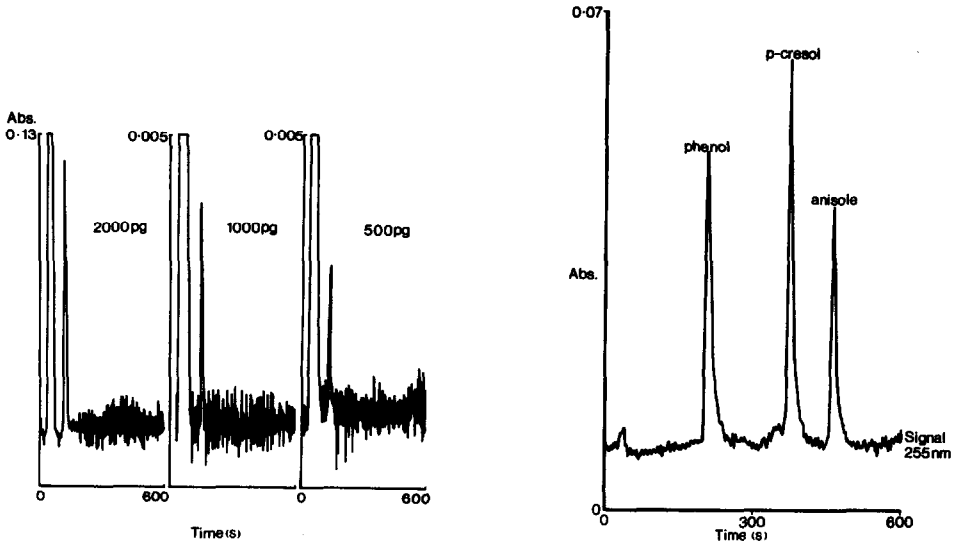


Fig. 10. Chromatograms obtained with 2000, 1000 and 500 pg of Acid Orange 10. Mobile phase: isocratic methanol–water [70% eluent A and 30% eluent B, (31% methanol), 0.1 *M* tetramethylammonium chloride, 0.001 *M* ammonium dihydrogenphosphate]; flow-rate: 0.05 ml/min; column: ODS-silica, 40 mm × 1 mm I.D.; detection: 255 nm.

Fig. 11. Chromatogram obtained with test mixture of phenol (500 ng), *p*-cresol (500 ng), anisole (500 ng). Mobile phase: 10 min linear methanol–water gradient (13.5% to 76.5% methanol, 0.01 *M* tetramethylammonium chloride, 0.001 *M* ammonium dihydrogenphosphate); flow-rate: 0.05 ml/min; column: ODS-silica 40 mm × 1 mm I.D.; detection: 255 nm.

TABLE VII

REPRODUCIBILITY OF RETENTION TIMES AND PEAK HEIGHTS IN HPLC

Identification	*Detection wavelength (nm)*	*Peak identity*	*Retention time (s)*	*Mean standard deviation (s)*	*Peak height (a.u.)*	*Mean standard deviation (a.u.)*
Standard*	255	Phenol	240	26		
	255	*p*-Cresol	410	24		
	255	Anisole	507	24		
Acid Violet 19**	255		22	3	0.59	0.01
Acid Red 27**	255		62	6	1.10	0.10
Acid Green 27**	255		777	12	0.65	0.07
Acid Yellow 17**	398		310	7	1.31	0.03
Acid Orange 10**	255		215	10	1.40	0.10
Acid Red 73**	510		565	6	2.22	0.05
Acid Black 2**	398		587	14	0.25	0.02

* Mean of 24 measurements over a 4-month period.

** Within day replication.

The mean retention times and standard deviations for the constituents of the test mixture are shown in Table VII. These are the results of 24 separate determinations spread over a four-month period involving several repackings of the HPLC column. The reproducibility is only moderate over this time but this is not unexpected for an HPLC system of this complexity. The test mixture was found to be particularly useful for monitoring changes in the performance of the system.

Of greater significance is the within day reproducibility since this will determine the usefulness of the system in comparing samples in casework. The variation in retention times and peak heights of dyes for replicate successive measurements are illustrated in Table VII. It can be seen that the short term reproducibility of retention times is very acceptable and comparable with that found for simple isocratic systems. Furthermore, the reproducibility of peak heights indicates that quantitative comparisons between samples are feasible.

Extraction and HPLC of dyes from wool fibres

The current HPLC system has been successfully applied to the examination of acid dyestuffs extracted from single wool fibres. A typical example of the chromatograms obtained for a blue wool fibre is shown in Fig. 12. The UV–VIS spectra of the seven major components detected are also shown. It is clear that the extracted material contains a mixture of blue, blue/red and red dyes. Minor components were also observed for which spectra were not captured. For this particular blue wool the four standard monitor wavelengths (255, 398, 510 and 610 nm) were used with the addition of a fifth wavelength (560 nm) selected to correspond more closely with the absorbance maxima of two separated components (Fig. 12, peaks 5 and 6).

To obtain chromatograms of comparable quality to those obtained with solid dyes it was found that all traces of pyridine must be removed from the extracts.

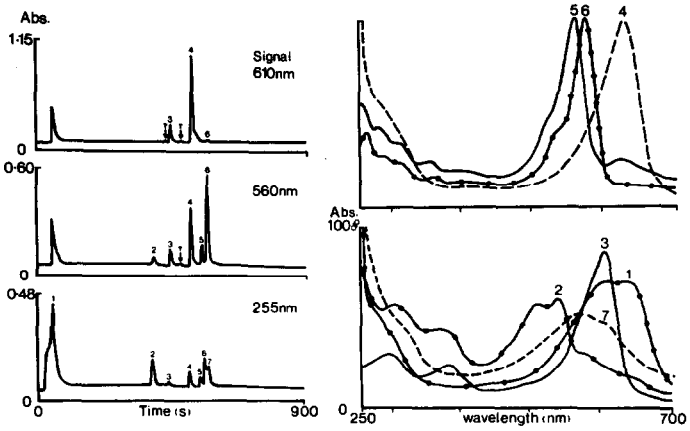


Fig. 12. Chromatograms and spectra obtained for dye extracted from a blue wool fibre. Chromatograms; mobile phase: 10 min linear methanol–water gradient (13.5% to 76.5% methanol, 0.01 *M* tetramethylammonium chloride, 0.001 *M* ammonium dihydrogenphosphate); flow-rate: 0.05 ml/min; column: ODS-silica, 40 mm × 1 mm I.D.; detection: 255, 560 and 610 nm. T = Minor components. Spectra; component 1, 0.65 a.u.f.s.; component 2, 0.10 a.u.f.s.; component 3, 0.30 a.u.f.s.; component 4, 1.18 a.u.f.s.; component 5, 0.18 a.u.f.s.; component 6, 0.55 a.u.f.s.; component 7, 0.12 a.u.f.s.

Pyridine produces a broad band starting at about 200 s which precludes detection at 255 nm. Prolonged or excessive heating of fibres during extraction was also found to be inadvisable. Where extraction was prolonged, UV absorbing material was produced, presumably from degradation of the wool fibre, making detection of dyestuff at 255 nm difficult. The dissolution of extract residues in eluent A for injection is recommended as the use of solvents of higher eluting strength gave poor peak shapes and caused large disturbances to the chromatographic baseline.

The blue wool has been examined previously by TLC[1]. In that investigation the extracted dye was separated into just three components, two blue and one red. The sample has also been examined by microspectrophotometry[1] when two absorbance maxima were reported at 624 and 578 nm. Using HPLC, the principal constituents are peaks 1, 4 and 6 (Fig. 12) being the most strongly absorbing components in the extract. The first two have their major visible absorbances near 620 nm and the latter *ca.* 575 nm. Thus HPLC with diode array detection can elucidate both chromatographic and spectral information in one analytical operation.

CONCLUSIONS

HPLC with a narrow-bore column can be used to separate the small amounts of dye obtained from single fibres. The resolution of dye mixtures is considerably better by HPLC than TLC and it is the more sensitive technique for all except the most complex mixtures.

The gradient elution scheme can accommodate dyes of very different structure, providing adequate resolution in a reasonable time. The system is also flexible allowing different isocratic compositions to be used when enhanced sensitivity is required.

The reproducibility of chromatographic peaks for replicate determinations is sufficient to allow the quantification of dye components. An estimate of the relative amounts of components in a mixture may be a useful factor in forensic dye comparisons.

The diode array spectrophotometer provides variable multiple-wavelength detection. The selection of four detection wavelengths across the spectral range can facilitate the analysis of dye mixtures where individual components have very different absorbance spectra. Additional detector wavelengths can then be added to maximise sensitivity when dealing with small amounts of dye. The UV–VIS spectra obtained by the spectrophotometer are of great value when selecting these additional detector wavelengths. They may also have a role in discriminating and identifying dyestuffs with similar chromatographic properties.

Overall, the system would appear to have potential for the forensic examination of dyestuffs. It is envisaged that an initial experiment using the control sample and the standard gradient elution system would provide information on the sensitivity and detector wavelengths required. Based on this the HPLC system may then be configured to provide maximum information in the casework comparison if required.

Further work is in progress to examine the discrimination which can be achieved amongst single wool fibres of the same colour. In addition, the potential of HPLC with diode array detection in forensic casework for the analysis of small quantities of dyestuffs belonging to other dye classes will be explored.

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