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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AZOBENZENE DERIVATIVES WITH SPECTROPHOTOMETRIC AND ELECTROCHEMICAL DETECTION

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SUMMARY

The chromatographic behaviour of azobenzene and fourteen of its derivatives was studied by reversed-phase high-performance liquid chromatography with a C_{18} stationary phase. The optimal composition of the mobile phase is 9:1 methanol–0.01 *M* aqueous sodium dihydrogen phosphate which is 0.0002 *M* in ethylenediaminetetraacetic acid, with a pH of 4.5. The solutes can be detected spectrophotometrically, voltammetrically or polarographically. Spectrophotometric measurement in the visible range is more sensitive than in the UV range (detection limits of 0.04–0.1 ng at 410 nm compared with 0.3–0.5 ng at 265 nm). Voltammetric detection is highly sensitive for hydroxy and amino derivatives [detection limits 0.02–0.09 ng at +0.8 V (Ag–AgCl)], whereas for other substances the detection limits are a few nanograms. Polarographic detection is the least sensitive [detection limits 4–8 ng at −0.6 V (Ag–AgCl)]. All the calibration graphs exhibit good linearity, but spectrophotometric detection yields a wider linear dynamic range. Voltammetric detection is more precise at low solute concentrations (relative standard deviations of the peak heights 0.5–1.0% and 1.0–1.5% for voltammetric and spectrophotometric detection, respectively, with amounts of solute from 1 to 10 ng).

INTRODUCTION

Azobenzene and its derivatives are important in industry and many of them are carcinogenic[1–3], and reliable methods are therefore required for their determination. As titration, electrochemical and spectrophotometric methods are insufficiently selective for analyses of complex samples, effective separation is required. Thin-layer chromatography (TLC) has been used for checking the purity of azo dyes[4,5] and for their separation[6–10]. However, the separation efficiency in TLC is limited and quantitative evaluations are not sufficiently precise. Therefore, high-performance liquid chromatography (HPLC) has been used for the determination of azobenzene in phenylbutazone[11,12], dyes[13] and air[14] and as a metabolite of aniline[15]. Reversed-phase HPLC has also been used for the determination of some azobenzene derivatives[16–18].

As these substances absorb radiation strongly in both the UV and visible re-

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gions and can be electrochemically oxidized and reduced[19,20], both spectrophotometric and electrochemical detectors can be employed.

This paper deals with general conditions for the separation of azobenzene derivatives by reversed-phase HPLC and compares detection by spectrophotometry in the UV and visible regions, voltammetry at a carbon fibre electrode and polarography at a mercury drop electrode.

EXPERIMENTAL

Chemicals

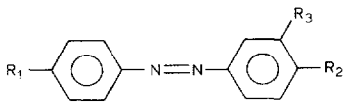
The substances studied are listed in Table I. Their purity was checked by TLC, reductometrically and by elemental analysis. The substances were dissolved either in methanol or in the mobile phase used. Stock solutions were prepared with a concentration of 0.001 mol l^{-1} and were appropriately diluted immediately before measurement. The other chemicals used were of analytical-reagent grade from Lachema (Brno, Czechoslovakia), except for sodium dihydrogen phosphate dodecahydrate, which was obtained from Merck (Darmstadt, F.R.G.).

Apparatus

The measurements were carried out using a Model 2150 HPLC pump (LKB, Bromma, Sweden), a Rheodyne valve with a 20 μl loop, a Separon SIX (7 μm) C_{18} glass column (150 × 3.2 mm I.D.) (Laboratorní přístroje, Prague, Czechoslovakia),

TABLE I

SUBSTANCES STUDIED



Except for azobenzene, which was obtained from Soyuzkhimexport (U.S.S.R.), all the test substances were synthesized in the Institute for Organic Syntheses, Pardubice, Czechoslovakia.

No.	*Abbreviation*	R_1	R_2	R_3	pK_a
1	4-NH_2-4'-OH-AB	NH_2	OH	–	
2	4-CH_3NH-4'-OH-AB	CH_3HN	OH	–	
3	DAHAB	$(CH_3)_2N$	OH	–	
4	DAAB	$(CH_3)_2N$	–	–	3.3
5	AB	–	–	–	
6	4'-SO_3Na-DAAB	$(CH_3)_2N$	SO_3Na	–	4.1
7	DAAAB	$(CH_3)_2N$	NH_2	–	
8	4'-$NHCOCH_3$-DAAB	$(CH_3)_2N$	$NHCOCH_3$	–	
9	4'-F-DAAB	$(CH_3)_2N$	F	–	
10	4'-Cl-DAAB	$(CH_3)_2N$	Cl	–	
11	4'-Br-DAAB	$(CH_3)_2N$	Br	–	
11$^+$	3'-Br-DAAB	$(CH_3)_2N$	–	Br	
12	4'-I-DAAB	$(CH_3)_2N$	I	–	
13	4'-COOH-DAAB	$(CH_3)_2N$	COOH	–	5.0
13$^+$	3'-COOH-DAAB	$(CH_3)_2N$	–	COOH	4.9

an LC-UV variable-wavelength spectrophotometric detector (Pye Unicam, Cambridge, U.K.), a 2151 UV–visible variable-wavelength monitor (LKB) and EDLC and ADLC 1 electrochemical detectors (Laboratorní přístroje). Carbon fibre[21] and mercury drop[22] detection cells were constructed in our laboratory. Electrochemical detection employed a three-electrode system with a saturated silver–silver chloride reference electrode and a platinum counter electrode. A TZ 4200 double-pen chart recorder was used. The spectrophotometric measurements were carried out on an SP 800 instrument (Pye Unicam) in 1-cm quartz cuvettes. Hydrodynamic voltammograms were measured point-by-point in the detection cells with a flow of mobile phase.

All the measurements were carried out at laboratory temperature (20 ± 2°C). The potentials are referred to the saturated Ag–AgCl reference electrode. In the polarographic measurements, the mobile phase was deaerated by the passage of purified nitrogen and a nitrogen atmosphere was maintained in the cell during the measurements.

RESULTS AND DISCUSSION

Separation conditions

In view of the character of the test substances, and the requirement for a sufficient electrical conductivity for voltammetric and polarographic detection, re-

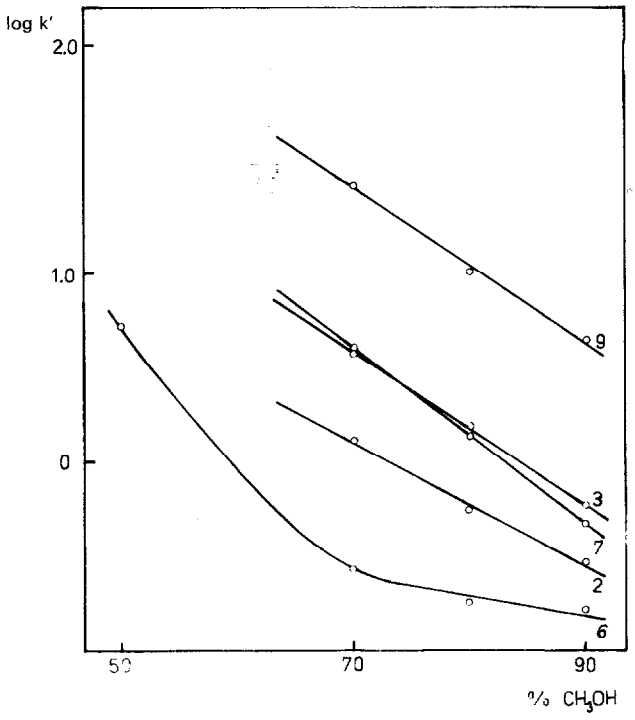


Fig. 1. Dependence of log k' on the methanol content in the mobile phase. Flow-rate, 0.3 ml min^{-1}; mobile phase, distilled water.

versed-phase HPLC with a C_{18} stationary phase was selected. The mobile phase consisted of methanol and an aqueous solution of sodium dihydrogen phosphate containing EDTA at a low concentration, to mask traces of metal ions. The dependences of the separation on the mobile phase flow-rate, methanol content, pH and on ionic strength of the mobile phase were studied. It was found that the measurements can be carried out at flow-rates from 0.3 to 0.5 ml min^{-1}, with a column efficiency of about $n = 1500$ and a resolution for isomers of, *e.g.*, $R_{1,2} = 6.4$ and 0.8 for 3'- and 4'-COOH-DAAB and 3'- and 4'-Br-DAAB, respectively.

The dependences of the capacity factors of the test substances on the methanol content in the mobile phase are shown in Fig. 1. The capacity factors decrease with increasing methanol content and the function $\log k' = f(CH_3OH, \%\ v/v)$ is linear. To accelerate the analysis, mobile phase containing 90% v/v methanol was subsequently used. As some derivatives are then poorly resolved, *e.g.*, 4-NH_2-4'-OH-AB and 4-CH_3NH-4'-OH-AB, or DAAAB and DAHAB, the methanol content in the mobile phase must be decreased for their separation. This is demonstrated in Fig. 2, in which examples of separations of five substances with similar capacity factors are given for methanol contents of 80 and 90%.

The dependences of the logarithms of the capacity factors on the mobile phase pH for several typical derivatives and at the optimal methanol content are shown in Fig. 3. As expected, these dependences are determined by the character of the substituents on the DAAB ring. Thus, the log k' values for 4'-F-DAAB, 4'-Cl-DAAB, 4'-Br-DAAB, 3'-Br-DAAB, 4'-I-DAAB, DAAB and AB are independent of the pH

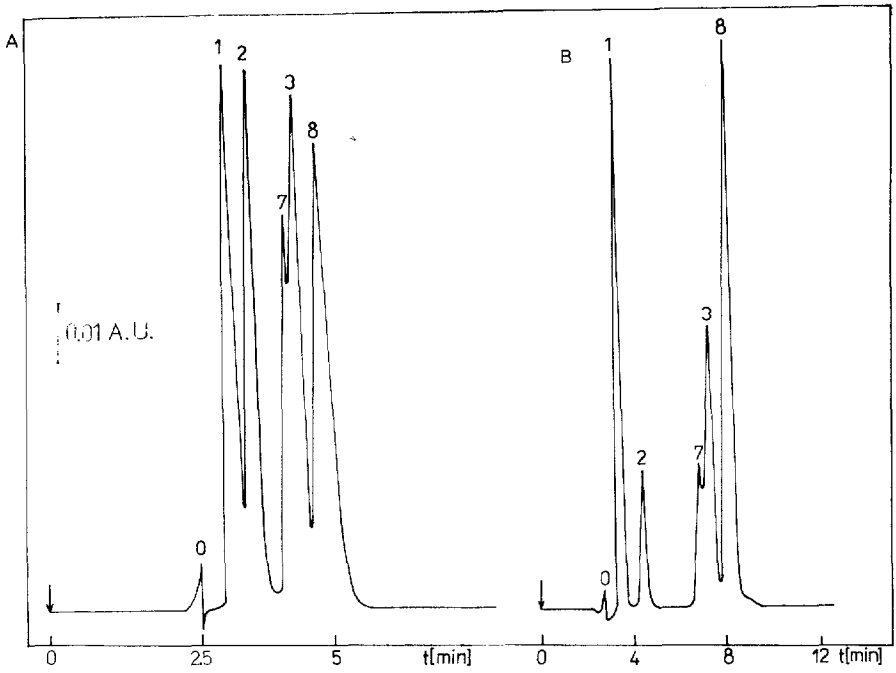


Fig. 2. Separation of five azobenzene derivatives in mobile phases containing (A) 90% and (B) 80% of methanol. Flow-rate, 0.3 ml min^{-1}; spectrophotometric detection at 410 nm.

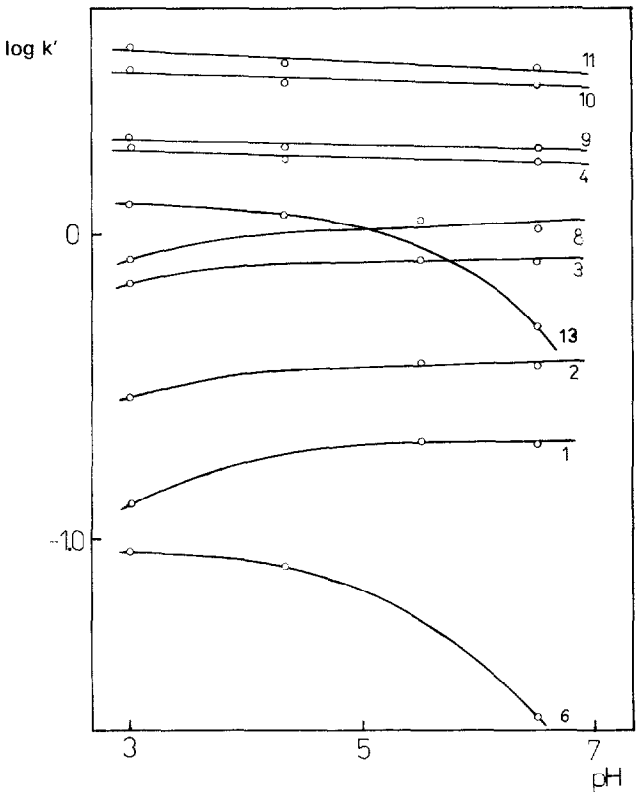


Fig. 3. Dependence of log k' on the mobile phase pH. Flow-rate, 0.3 ml min^{-1}; mobile phase, methanol–water (90:10).

within the range studied, *i.e.*, from 3.0 to 6.5. The retention of derivatives containing substituents that undergo protolytic reactions depends on the pH: the capacity factors of the substances with amino and hydroxy groups decrease with decreasing pH below 4.0, whereas those of substances containing groups with a dissociable hydrogen (4′-COOH-DAAB, 3′-COOH-DAAB and 4′-SO_3H-DAAB) decrease with increasing pH above 4.5. In view of these dependences, the pH of the mobile phase was adjusted to 4.5 for subsequent work.

As the electrochemical detectors require the mobile phase to be electrically conductive, a certain minimum amount of a salt must be present. It can be seen in Fig. 4 that the content of sodium dihydrogen phosphate has virtually no effect on the capacity factors of the test substances within the range studied (up to 0.01 *M* concentration), except for 4′-COOH-DAAB, 4′-SO_3H-DAAB and 3′-COOH-DAAB, for which the capacity factors increase with increasing ionic strength, owing to suppression of their dissociation.

The capacity factors for the optimal composition of the mobile phase, 90:10 methanol–aqueous 0.01 *M* NaH_2PO_4 with 0.0002 *M* EDTA (pH 4.5), are listed in Table II.

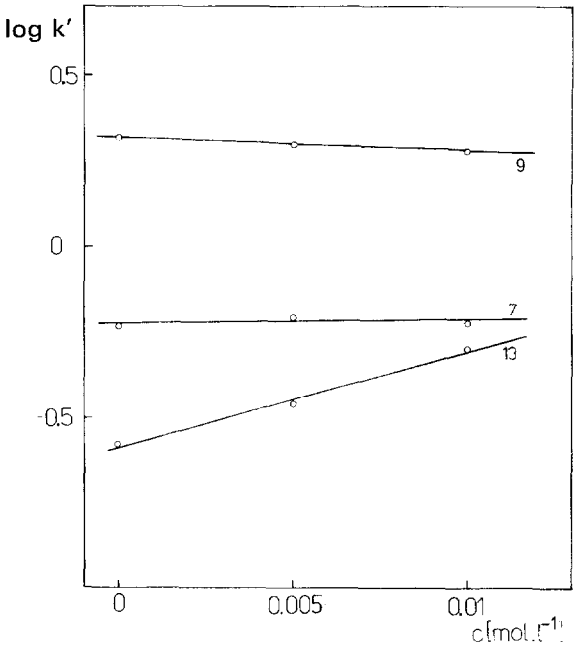


Fig. 4. Dependence of log k' on the salt concentration in the mobile phase. Flow-rate, 0.3 ml min^{-1}; mobile phase, methanol–water (90:10) + NaH_2PO_4.

Detection conditions

All the substances studied are oxidizable and thus can be detected voltammetrically by the carbon fibre detector; the amino and hydroxy derivatives are the most readily oxidized, as shown by the typical hydrodynamic voltammograms in the mobile phase given in Fig. 5. It can also be seen that the electrochemical reaction products are adsorbed on the electrode surface (depression of the limiting current). The aza group is reducible, *e.g.*, on mercury electrodes, and thus polarographic detection can also be used; a typical hydrodynamic polarogram in the mobile phase is

TABLE II

CAPACITY FACTORS (k') OF THE SUBSTANCES STUDIED

Mobile phase: 90:10 methanol–aqueous 0.01 *M* NaH_2PO_4 + 0.0002 *M* EDTA, pH 4.5.

Substance	k'	*Substance*	k'
4-NH_2-4'-OH-AB	0.16	4'-F-DAAB	1.80
4-CH_3NH-4'-OH-AB	0.29	4'-Cl-DAAB	3.11
DAHAB	0.58	4'-Br-DAAB	3.65
DAAB	1.62	3'-Br-DAAB	3.23
AB	0.34	4'-I-DAAB	4.30
4'-SO_3Na-DAAB	0.02	4'-COOH-DAAB	1.10
DAAAB	0.51	3'-COOH-DAAB	0.80
4'-$NHCOCH_3$-DAAB	0.74		

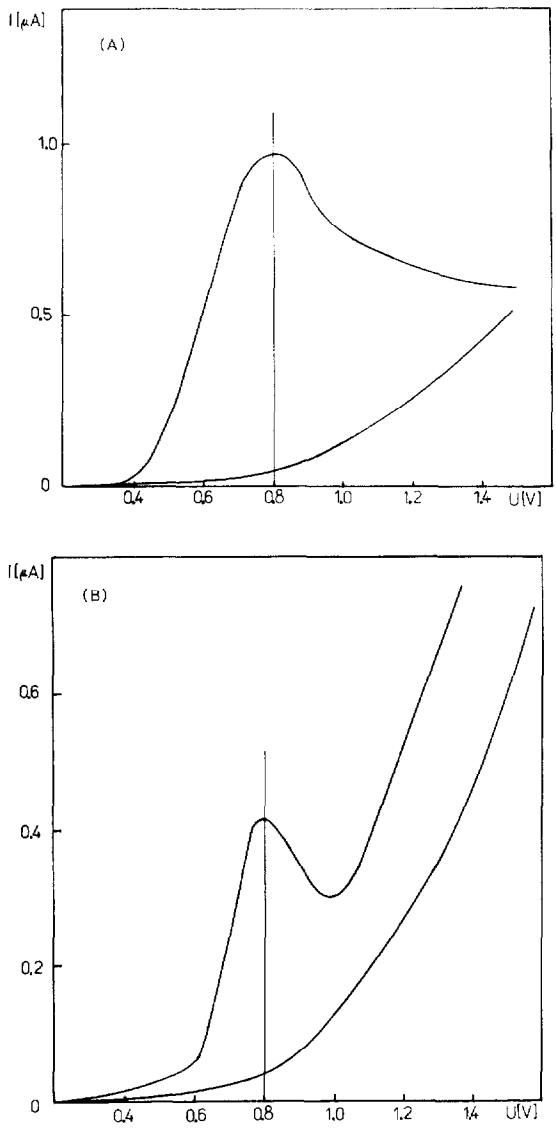


Fig. 5. Hydrodynamic voltammograms of (A) $NH_2C_6H_4N=NC_6H_4OH$ and (B) $(CH_3)_2NC_6H_4N=NC_6H_4SO_3Na$. Flow-rate, 0.5 ml min^{-1}; mobile phase, 90:10 methanol–aqueous 0.01 *M* NaH_2PO_4 + 0.0002 *M* EDTA; pH 4.5.

given in Fig. 6. The spectrophotometric absorption maxima of the substances in the mobile phase are summarized in Table III.

On the basis of these results, the following detection conditions were selected: UV spectrophotometry, 265 nm; spectrophotometry in the visible range, either the wavelengths of the absorption maxima for the individual substances or a wavelength of 410 nm for mixtures of substances; voltammetry, +0.8 V (Ag–AgCl); and polar-

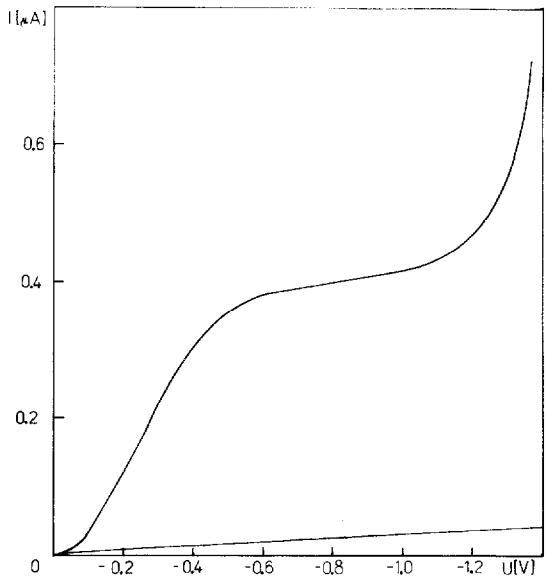


Fig. 6. Hydrodynamic polarogram of $CH_3NHC_6H_4N{=}NC_6H_4OH$. Conditions as in Fig. 5.

ography, −0.6 V (Ag–AgCl). Typical chromatograms obtained with detection by visible light spectrophotometry and voltammetry are given in Figs. 7 and 8, respectively.

TABLE III

ABSORPTION MAXIMA OF THE SUBSTANCES STUDIED IN THE UV AND VISIBLE REGIONS

Mobile phase as in Table II.

Substance	*Absorption maxima (nm)*	
	UV region	*Visible region*
4-NH_2-4′-OH-AB	203, 250	382
4-CH_3NH-4′-OH-AB	201, 252, 315	396
DAHAB	203, 235, 254, 316	404
DAAB	204, 255	408
AB	207, 229, 316	–
4′-SO_3Na-DAAB	203, 269	422
DAAAB	203, 254, 320	416, 444
4′-$NHCOCH_3$-DAAB	202, 235, 255, 315	415
4′-F-DAAB	203, 218, 259	408
4′-Cl-DAAB	203, 222, 265	416
4′-Br-DAAB	203, 223, 265	420
3′-Br-DAAB	204, 264	420
4′-I-DAAB	203, 226, 259	422
4′-COOH-DAAB	208, 286	494
3′-COOH-DAAB	208, 272	416

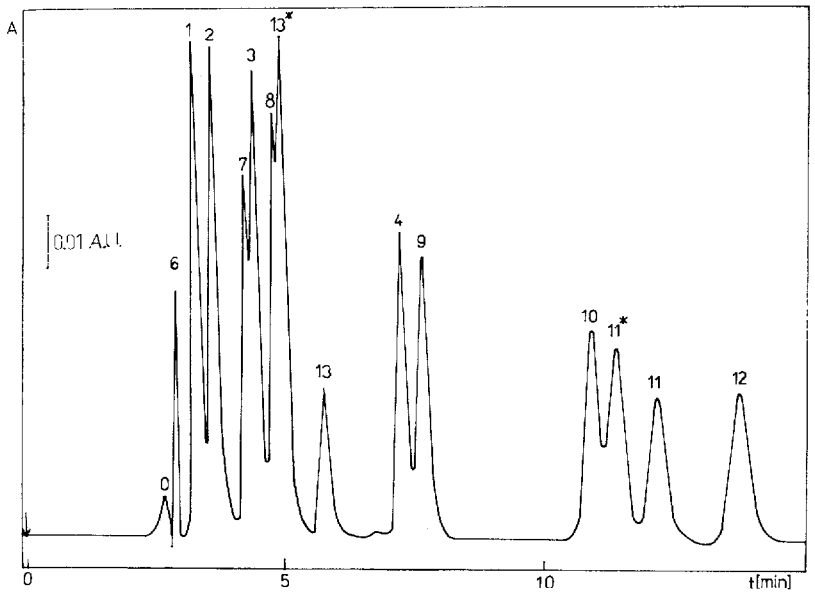


Fig. 7. Chromatograms of a mixture of 14 azobenzene derivatives. Flow-rate, 0.3 ml min^{-1}; mobile phase, as in Fig. 5; spectrophotometric detection at 410 nm.

Calibration data obtained by linear regression for four selected substances and spectrophotometric and voltammetric detection with measurement of peak heights are given in Tables IV and V. In all instances, good linearity is obtained (correlation coefficients from 0.9989 to 0.9999 for spectrophotometric detection and from 0.9975 to 0.9991 for voltammetric detection). The linear dynamic range is wider for spectrophotometric detection (the whole range studied); the calibration graph for voltam-

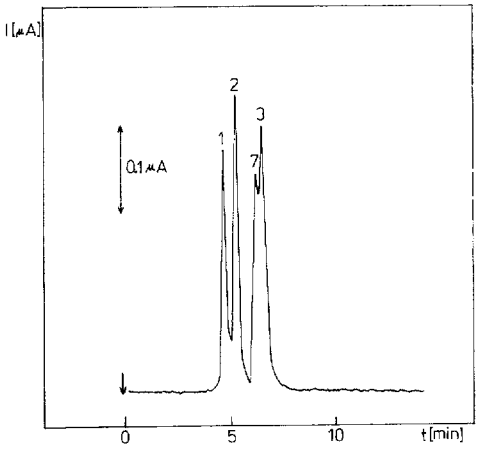


Fig. 8. Chromatogram of a mixture of four azobenzene derivatives. Flow-rate, 0.2 ml min^{-1}; mobile phase, as in Fig. 5; voltammetric detection at +0.8 V (Ag–AgCl).

TABLE IV

CALIBRATION DATA FOR VOLTAMMETRIC DETECTION AT +0.8 V (Ag–AgCl)

Calibration graph: $I = a + bm$ (I = current in nA; m = amount of analyte in ng).

Substance	*a (nA)*	*b (nA ng^{-1})*	*Correlation coefficient*	*Detection limit (ng)*
4-NH_2-4'-OH-AB	35.0	30.2	0.9984	0.02
4-CH_3NH-4'-OH-AB	16.1	10.6	0.9955	0.03
DAHAB	19.1	18.5	0.9975	0.03
DAAAB	7.2	18.5	0.9991	0.02

TABLE V

CALIBRATION DATA FOR SPECTROPHOTOMETRIC DETECTION AT THE WAVELENGTHS OF THE ABSORPTION MAXIMA OF THE INDIVIDUAL COMPOUNDS (SEE TABLE III)

Calibration graph: $h = a + bm$ (h = peak height in cm; m = amount of analyte in ng).

Substance	*Wavelength (nm)*	*a (cm)*	*b (cm ng^{-1})*	*Correlation coefficient*	*Detection limit (ng)*
4-NH_2-4'-OH-AB	392	2.36	8.02	0.9994	0.08
4-CH_3NH-4'-OH-AB	396	1.55	4.27	0.9994	0.10
DAHAB	404	0.29	8.74	0.9999	0.06
DAAAB	416	0.18	5.93	0.9993	0.07

TABLE VI

DETECTION LIMITS FOR THE SUBSTANCES STUDIED WITH DIFFERENT DETECTION METHODS

Substance	*Detection limit (ng)*			
	Visible region spectrometry	*UV spectrophotometry*	*Voltammetry*	*Polarography*
4-NH_2-4'-OH-AB	0.08	0.27	0.02	4.17
4-CH_3NH-4'-OH-AB	0.10	0.28	0.03	4.72
DAHAB	0.06	0.30	0.03	4.90
DAAAB	0.04	0.29	2.20	4.37
AB	0.30	0.35	2.66	4.18
4'-SO_3Na-DAAB	0.04	0.44	3.54	7.97
DAAAB	0.07	0.30	0.02	4.87
4'-$NHCOCH_3$-DAAB	0.08	0.36	0.09	5.82
4'-F-DAAB	0.09	0.32	2.46	4.59
4'-Cl-DAAB	0.07	0.35	3.39	5.97
4'-Br-DAAB	0.10	0.41	5.87	6.20
3'-Br-DAAB	0.12	0.39	7.54	6.72
4'-I-DAAB	0.11	0.41	2.48	6.37
4'-COOH-DAAB	0.05	0.45	4.41	6.25
3'-COOH-DAAB	0.04	0.36	3.81	6.67

metric detection ceases to be linear for amounts of the substances higher than about 50 ng in the volume injected.

The precision of the measurements (five measurements, $\alpha = 0.05$) is good for both spectrophotometric and voltammetric detection; the relative standard deviations were 0.5–1.0% for voltammetry and 1.0–1.5% for spectrophotometry for small amounts (1–10 ng) and *ca.* 3.5% for voltammetry and *ca.* 2.8% for spectrophotometry for larger amounts (50 ng). Hence voltammetric detection has a better precision at low analyte concentrations. The detection limits (twice the absolute noise value) are summarized in Table VI.

It is seen that polarographic detection, with detection limits between 4 to 8 ng, cannot compete in sensitivity with the other detection methods. Voltammetric detection is the most sensitive method for DAAB, DAHAB, 4-NH_2-4′-OH-AB and 4-CH_3-NH-4′-OH-AB, with detection limits of 0.02–0.03 ng, whereas for the other substances spectrophotometric detection in the visible region is more sensitive. The sensitivity of UV spectrophotometry is between those of visible-range spectrophotometry and voltammetry for these substances.

It can be concluded that UV spectrophotometric detection is satisfactory for common analyses; for trace analyses, spectrophotometry in the visible region is preferable, whereas voltammetric detection will find use in specialized analyses, where a high selectivity is required (especially trace analyses for metabolites of the test substances).

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