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THE BEHAVIOUR OF HYDROPHYLIC SUBSTANCES IN REVERSED-PHASE CHROMATOGRAPHY

A CONTRIBUTION TO THE MECHANISM OF REVERSED-PHASE PAPER CHROMATOGRAPHY

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SUMMARY

The chromatographic behaviour of both lipophylic and hydrophylic benzeneazo-2-naphthol derivatives was studied using untreated papers and papers impregnated with nonpolar or weakly polar organic stationary liquids and the same mobile phase. It was observed that on reversed-phase chromatograms dyes containing sulpho groups behaved as if the papers did not contain the organic stationary phase, whereas the lipophylic dyes followed normal reversed-phase partition mechanism. Thus, two stationary phases must be considered to be present in the reversed-phase chromatograms: the organic liquid and the cellulose–water complex. The choice of the stationary phase involved in the separation process is dependent on the character of the compound chromatographed. In some cases both stationary phases can be involved.

INTRODUCTION

Chromatography on papers impregnated with a nonpolar or weakly polar stationary phase using a polar mobile phase is called reversed-phase paper chromatography. The stationary phase usually contains paraffin oil, 1-bromonaphthalene, silicone oil, olive oil, lauryl alcohol, etc., and the mobile phase consists mostly of aqueous mixtures of lower aliphatic alcohols, acetone, acetic acid, ammonia, etc. saturated with the stationary phase. The chromatographic paper itself is supposed to function as an inert support of the stationary phase[1]. Partition of the chromatographed compounds between the nonpolar stationary and the more polar mobile phase is considered to be the mechanism of the separation process[2]. This type of chromatography is especially suitable for compounds of lipophylic character, *i.e.* compounds very soluble in nonpolar solvents and fats.

In a recent work on paper chromatography of acid anthraquinone dyes[3] we made observations with papers impregnated with lauryl alcohol. These typical water-soluble dyes behaved on the reversed-phase chromatograms as if the paper had not been impregnated. This observation made us carry out a more detailed study of the behaviour of hydrophylic substances on reversed-phase chromatograms and of the role of cellulose in reversed-phase chromatography of hydrophylic substances. The results of this study have been summarised in this communication.

EXPERIMENTAL

All azo dyes were chromatographically purified compounds from our collection of model dyes. They were applied in the form of 1% solutions in pyridine or aqueous pyridine. Whatman No. 3MM paper was used throughout all experiments. 10% paraffin oil in hexane, 10% 1-bromonaphthalene in chloroform and 5% lauryl alcohol in ethanol were used for impregnation. The impregnation procedure was carried out in the usual manner.

The following series of chromatographic experiments was always carried out at the same time.

Tank I: untreated paper/mobile phase.

Tank II: (a) untreated paper/mobile phase saturated with paraffin oil;
(b) paper impregnated with paraffin oil/mobile phase saturated with paraffin oil.

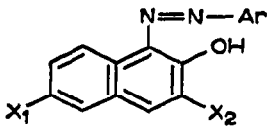
Tank III: (a) untreated paper/mobile phase saturated with 1-bromonaphthalene;
(b) paper impregnated with 1-bromonaphthalene/mobile phase saturated with 1-bromonaphthalene.

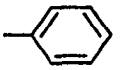
Tank IV: (a) untreated paper/mobile phase saturated with lauryl alcohol;
(b) paper impregnated with lauryl alcohol/mobile phase saturated with lauryl alcohol.

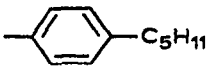
All compounds are intensely coloured, and therefore no special method for visualisation of the spots was necessary.

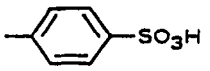
RESULTS AND DISCUSSION

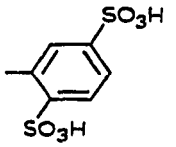
We intended to study the problem by comparing the chromatographic behaviour of the typically hydrophylic and lipophylic substances on both the normal and the reversed-phase chromatograms under the same conditions. The general, well-known relationships between the chromatographic behaviour and the chemical structure and between the chromatographic behaviour and the composition of the mobile phase were chosen to indicate the mechanism of the chromatographic process. It was therefore necessary to find a suitable series of model substances that would involve both extreme types of compounds under consideration. The following series of azo dyes was found to meet our requirements.

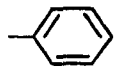


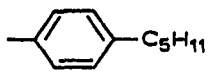
I $X_1 = X_2 = H$; Ar = 

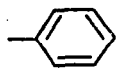
II $X_1 = X_2 = H$; Ar = 

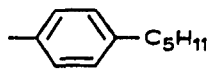
III $X_1 = X_2 = H$; Ar = 

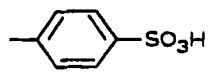
IV $X_1 = X_2 = H$; Ar = 

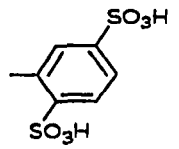
V $X_1 = SO_3H$; $X_2 = H$; Ar = 

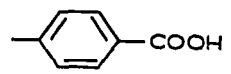
VI $X_1 = SO_3H$; $X_2 = H$; Ar = 

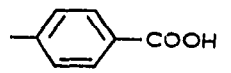
VII $X_1 = X_2 = SO_3H$; Ar = 

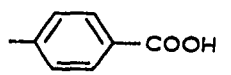
VIII $X_1 = X_2 = SO_3H$; Ar = 

IX $X_1 = X_2 = SO_3H$; Ar = 

X $X_1 = X_2 = SO_3H$; Ar = 

XI $X_1 = X_2 = H$; Ar = 

XII $X_1 = SO_3H$; $X_2 = H$; Ar = 

XIII $X_1 = X_2 = SO_3H$; Ar = 

All model compounds are derivatives of benzeneazo-2-naphthol, compound I. The lipophylic character of this compound is increased by the presence of an aliphatic C_5 chain in compound II. These two dyes are representatives of typical fat-soluble

dyes usually chromatographed in reversed-phase systems[4,5]. Derivatives of compound I containing one, two, three or four sulpho groups (III–X) are typical acid dyes for which normal aqueous or ammoniacal solvent systems are suitable[6]. Their hydrophylic character is slightly decreased by introduction of C_6 aliphatic chains (VI and VIII). The series has been completed by compounds with carboxy groups (XI–XIII).

The scheme of experiments was arranged so that the same model compounds could be run simultaneously on chromatograms with and without impregnation using the same mobile phase saturated with the stationary liquid. A control chromatogram was always run on untreated paper without saturation of the mobile phase. It was necessary to control the influence of the presence of the stationary phase in the mobile phase. Mixtures of lower aliphatic alcohols with ammonia, aqueous acetic acid or hydrochloric acid were used as mobile phases. The results obtained will be discussed separately for each pair of solvents forming the mobile phase.

1-Propanol–ammonia

The mobile phase 1-propanol–ammonia (2:1), using untreated paper, represents a typical solvent for hydrophylic substances. It has been recommended for the separation of acid dyes[6], sulphonic acids[7], etc. The same separation mechanism working for the solvent systems with 1-butanol and water or ammonia, *i.e.* predominantly the partition mechanism, is thought to exist though it has not yet been possible to decide definitely between adsorption or partition. This is, however, not decisive for our discussion, and we shall call the separation mechanism for this type of solvent system the "normal mechanism". Water or the cellulose–water complex is the stationary and the propanol–ammonia mixture the mobile phase involved in this separation process.

The sulpho group has the greatest influence on the chromatographic behaviour of the benzeneazo-2-naphthol derivatives in this solvent system (Table I). The R_F values decrease with the increasing number of sulpho groups, and thus the following sequence of spots is obtained according to the decreasing R_F values: I > III > IV > IX > X. An adequate increase in R_F values is caused by the presence of the C_6 aliphatic chain (I < II; V < VI; VII < VIII).

When the original ratio of 1-propanol–ammonia (2:1) is changed to 1:1 or 1:2, respectively, the chromatographic behaviour of the compounds under investigation is as follows. The sequence of the spots remains unchanged but the R_F values of all compounds increase with the increasing water content in the mobile phase. Using untreated paper this is also characteristic for the "normal mechanism".

If all the three mobile phases are saturated with paraffin oil and the model compounds are chromatographed on untreated paper, the results obtained are just the same as in the case of the unsaturated mobile phase. Even the absolute R_F values are practically identical. The amount of paraffin oil necessary to saturate the mobile phase seems to be so small that it does not affect the solubility of the chromatographed compounds.

Very interesting results, however, are obtained when papers impregnated with paraffin oil and the mobile phases saturated with the stationary liquid are used. These systems represent typical reversed-phase systems in which the sequence of the spots should be reversed. From the results summarised in Table I it is evident that dyes I and II, containing no sulpho groups, and designated above as typically lipophylic, do

TABLE I

R_F VALUES OF BENZENEAZO-2-NAPHTHOL DERIVATIVES

A = untreated paper/mobile phase saturated with paraffin oil;
B = paper impregnated with paraffin oil/mobile phase saturated with paraffin oil;
C = untreated paper/mobile phase saturated with 1-bromonaphthalene;
D = paper impregnated with 1-bromonaphthalene/mobile phase saturated with 1-bromonaphthalene;
E = untreated paper/mobile phase saturated with lauryl alcohol;
F = paper impregnated with lauryl alcohol/mobile phase saturated with lauryl alcohol.

Compound	*Mobile phases*									
	1-Propanol–ammonia						*Ethanol–ammonia*			
	2:1		*1:1*		*1:2*		*1:1*			
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*C*	*D*
I	0.96	0.78	0.98	0.70	0.99	0.37	0.47s[a]	0.28	0.68	0.03
II	0.97	0.38	0.99	0.16	0.99	0.10	—	—	0.60s	0.00
III	0.83	0.83	0.93	0.93	0.99	0.99	0.95	0.94	0.96	0.96
IV	0.50	0.49	0.81	0.80	0.96	0.95	0.92	0.91	0.90	0.90
V	0.80	0.79	0.90	0.89	0.98	0.98	0.96	0.95	0.95	0.96
VI	0.90	0.90	0.95	0.95	0.99	0.99	0.99	0.98	0.99	0.99
VII	0.42	0.41	0.72	0.71	0.93	0.92	0.85	0.84	0.80	0.80
VIII	0.65	0.64	0.89	0.88	0.99	0.99	0.97	0.96	0.93	0.93
IX	0.11	0.10	0.52	0.50	0.85	0.85	0.77	0.76	0.76	0.76
X	0.02	0.02	0.41	0.41	0.85	0.85	0.74	0.74	0.69	0.69
XI	0.80	0.79	0.92	0.92	0.98	0.98	0.86	0.85	0.94	0.94
XII	0.42	0.41	0.76	0.75	0.94	0.93	0.80	0.79	0.79	0.78
XIII	0.10	0.09	0.52	0.51	0.84	0.83	0.67	0.66	0.72	0.72

Compound	*Mobile phases*													
	1-Propanol–25% acetic acid								*Methanol–1 N HCl*					
	2:1		*1:1*		*1:2*				*1:1*					
	A	*B*	*A*	*B*	*A*	*B*	*C*	*D*	*A*	*B*	*C*	*D*	*E*	*F*
I	0.96	0.80	0.98	0.67	0.99	0.40	0.94	0.11	0.00s	0.00	0.00s	0.00	0.00s	0.01
II	0.98	0.51	0.99	0.19	0.99	0.02	0.90	0.05	0.00	0.00	0.00	0.00	0.00	0.00
III	0.72	0.70	0.85	0.84	0.95	0.95	0.95	0.95	0.67	0.67	0.64	0.63	0.68	*0.52*[b]
IV	0.30	0.28	0.60	0.60	0.83	0.82	0.81	0.80	0.77	0.77	0.74	0.74	0.77	0.78
V	0.72	0.70	0.85	0.84	0.94	0.94	0.94	0.94	0.64	0.64	0.64	0.62	0.65	*0.50*[b]
VI	0.85	0.85	0.93	0.93	0.99	0.99	0.99	0.99	0.35	0.35	0.30s	0.22	0.36	*0.03*[b]
VII	0.27	0.26	0.57	0.56	0.82	0.83	0.80	0.80	0.71	0.71	0.68	0.69	0.71	0.67
VIII	0.50	0.49	0.80	0.79	0.95	0.94	0.93	0.92	0.67	0.67	0.54	0.54	0.68	*0.53*[b]
IX	0.03	0.03	0.32	0.33	0.72	0.73	0.67	0.66	0.79	0.79	0.78	0.78	—	—
X	0.01	0.01	0.21	0.23	0.67	0.66	0.61	0.60	0.81	0.82	0.80	0.80	—	—
XI	0.90	0.90	0.90	0.90	0.89s	s	0.98s	0.34	0.00	0.00	0.00	0.00	0.00	0.00
XII	0.54	0.53	0.70	0.70	0.80	0.80	0.86	0.87	0.33	0.33	0.33	0.34	0.35	0.35
XIII	0.12	0.11	0.50	0.49	0.69	0.70	0.70	0.70	0.48	0.48	0.49	0.49	0.51	0.51

[a] s = streak.
[b] The values printed in italics are discussed in the text.

change their R_F values considerably and that the sequence of their spots is reversed. Evidence thus suggests that the solvent system is, in fact, a reversed-phase system suitable for fat-soluble substances. On the contrary, the R_F values and the sequence of spots of compounds containing sulpho groups remain unchanged as if the paper did not contain the organic stationary phase, *i.e.* paraffin oil. This results in a quite

unusual sequence of spots according to their decreasing R_F values: III > I > IV > IX > X. The presence of the C_5 aliphatic chain causes a decrease in the R_F value of the fat-soluble dye (II–I) but an increase in the R_F values of the sulpho group-containing dyes (VI–V, VIII–VII). There is also a considerable difference in the influence of the 1-propanol–ammonia ratio in the mobile phase upon the R_F values. The R_F values of the lipophylic dyes I and II are decreased considerably with an increasing water content in the mobile phase, whereas the R_F values of the sulphonated compounds are increased under the same conditions. This is illustrated by the two schemes in Figs. 1a and b. The influence of the carboxylic group is very similar to that of the sulpho group. The carboxylic group is dissociated in the ammoniacal medium and the compounds containing the carboxylic group have practically identical R_F values with the corresponding sulphonated compounds (III–XI, VII–XII, IX–XIII).

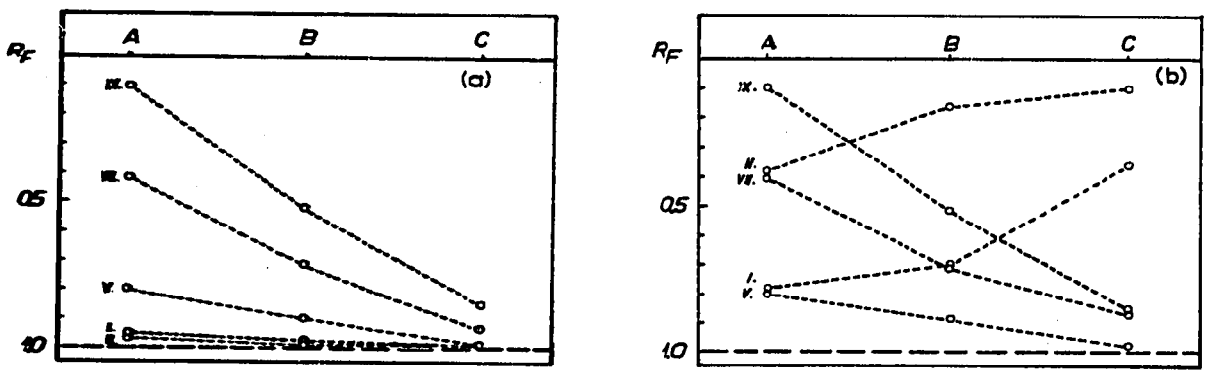


Fig. 1. Chromatographic behaviour of compounds I, II, V, VII and IX on (a) untreated paper and (b) paper impregnated with paraffin oil. Mobile phase, 1-propanol–ammonia in different proportions: (A) 2:1, (B) 1:1, (C) 1:2.

Ethanol–ammonia

The same sequence of spots and relations between the chemical structure and the chromatographic behaviour are observed when ethanol–ammonia (1:1) is used instead of 1-propanol–ammonia (1:1) as the mobile phase on untreated paper. This suggests that the same mechanism for the separation process is involved. The only difference is that dyes I and II begin to form streaks and all sulpho- and carboxylic group-containing dyes have higher R_F values. This has been expected because the R_F values will increase with the decreasing length of the chain of the aliphatic alcohol in such normal systems.

The impregnation of the paper with paraffin oil or 1-bromonaphthalene has no influence on the absolute R_F values and the sequence of the spots of the sulphonated dyes but causes a considerable decrease in the R_F values of compounds I and II in comparison with 1-propanol–ammonia (1:1) as mobile phase. The absolute R_F values of compounds I and II are dependent on the stationary phase used. The R_F value of compound I is higher when 1-propanol–ammonia (1:1) instead of ethanol–ammonia (1:1) is used on paper impregnated with paraffin oil. This is in accordance with the usual experience in reversed-phase chromatography.

1-Propanol–25% acetic acid

When 25% ammonia is replaced by 25% acetic acid in the mixtures with 1-propanol, the same relations between the chromatographic behaviour and the

chemical structure as well as the water content of the mobile phase can be observed. The presence of the organic stationary phase in the paper does not influence the behaviour of the hydrophylic substances but causes a change in the partition of lipophylic dyes I and II in the same way as when the 1-propanol–ammonia systems are used. The decrease in the R_F values of dyes I and II is dependent on the type of the stationary phase (Fig. 2). The carboxylic group is not dissociated under these conditions, and therefore the dyes containing this group have higher R_F values than the corresponding dyes with sulpho groups. The contribution of the undissociated carboxylic group in XI, in which no other water solubilising groups are present, with 1-bromonaphthalene-impregnated paper is like that of a lipophylic group; the interaction of XI with the stationary phase results in a considerable decrease in the R_F value in comparison with the system with untreated paper.

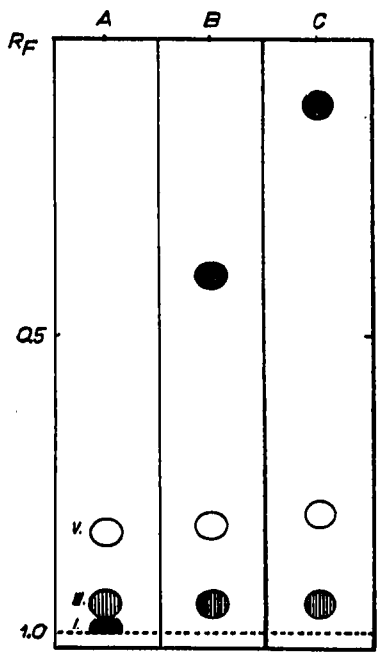


Fig. 2. Chromatographic behaviour of compounds I, III and V in solvent system 1-propanol–25% acetic acid (1:2). (A) untreated paper; (B) paper impregnated with paraffin oil; (C) paper impregnated with 1-bromonaphthalene.

Methanol–1 N HCl

The results obtained with methanol in mixtures with both ammonia and 1 *N* HCl indicate that the separation mechanism is influenced by other factors. In the system methanol–1 *N* HCl (1:1) using untreated paper, the R_F values increase with the increasing number of sulpho groups and the sequence of spots is the following: X > IX > IV > III > I. Thus, the influence of the sulpho group upon the chromatographic behaviour is the reverse of that in the normal systems. The same relation between the number of sulpho groups and the chromatographic mobility has been observed by LATINÁK[8] in the case of naphthalenesulphonic acids chromatographed with electrolyte solutions. The reversed sequence of spots has been explained by a partition between the cellulose–water complex (as stationary phase) and the electrolyte solution (as mobile phase) influenced by the salting-out effect of the electrolyte

by which the solubility of the chromatographed compounds in the mobile phase is decreased.

The effect of the C_5 aliphatic chain is also reversed in comparison with the normal systems. Compounds VI and VIII have lower R_F values than the corresponding dyes V and VII. The lipophilic dyes I and II remain on the start, obviously because of low solubility in the mobile phase.

The saturation of the mobile phase by paraffin oil, 1-bromonaphthalene or lauryl alcohol does not affect the absolute R_F values significantly when untreated paper is used.

When the paper is impregnated with paraffin oil, no difference can be noticed on the chromatograms. The R_F values of all sulphonated compounds are exactly the same as on untreated paper, indicating that the organic stationary phase does not take part in the separation process of those compounds. When 1-bromonaphthalene instead of paraffin oil is used as the stationary phase, the same results are obtained with one very important exception. Dye VI containing one sulpho group and the C_5 aliphatic chain has a considerably lower R_F value on impregnated paper in comparison with the untreated paper. This compound has the least hydrophilic character of all the sulphonated dyes chromatographed, and thus some interaction with the stationary organic liquid might be suspected.

The impregnation with lauryl alcohol causes significant changes in the chromatographic behaviour of compounds II, V, VI and VIII, and it has no effect on the behaviour of compounds IV, VII, XII and XIII. The differences in R_M values obtained from the R_F values of these compounds using untreated and impregnated paper (E and F in Table I) are very interesting (Table II). It is evident that compounds with one sulpho group (III, V) show interaction with the organic stationary liquid.

TABLE II

ΔR_M VALUES INDICATING THE CHANGES IN R_F VALUES ON UNTREATED AND IMPREGNATED PAPER
Mobile phase: methanol–1 *N* HCl, impregnation with lauryl alcohol.

Compound	*Number of sulpho groups*	*Number of C_5 chains*	ΔR_M
III	1	—	−0.293
V	1	—	−0.269
VI	1	1	−1.260
VIII	2	1	−0.275
IV	2	—	+0.026
VII	2	—	−0.081

In compound VI this interaction is increased by the presence of the C_5 aliphatic chain. Compound VIII contains two sulpho groups but shows affinity to the organic stationary phase due to the presence of the C_5 chain (the C_5 chain seems to be reversely equivalent to one sulpho group) whereas no interaction with the organic stationary phase can be observed in the case of compounds IV and VII containing two sulpho groups.

CONCLUSION

The results obtained in this study have shown that two stationary phases must be considered to be present in reversed-phase paper chromatograms prepared in the usual manner: the organic nonvolatile liquid and the cellulose–water complex.

Lipophylic compounds are partitioned between the organic stationary phase and the mobile phase. Hydrophylic compounds behave as if the paper did not contain the organic stationary phase and the separation process involves only the cellulose–water complex and the mobile phase. Such cases can be found, however, in which both stationary phases are simultaneously involved in the separation process. This was observed when sulphonated dyes, of which the polarity had been decreased by the presence of an aliphatic chain in their molecules, were chromatographed on papers impregnated with lauryl alcohol instead of paraffin oil.

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