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A RATIONAL METHOD OF MOBILE PHASE OPTIMIZATION BASED ON THE USE OF ISOHYDRIC SOLVENTS

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SUMMARY

In order to facilitate the use and automation of liquid column chromatography in control laboratories, we have made a classification and a rational combination of solvents. By using an experimental determination of polarity, based on the water content of previously defined isohydric solvents, we have elaborated a method for the optimization of separations that considerably reduces the operating time, gives a more accurate description of processes and greatly limits the number of systems able to solve most of the problems.

Practical separations of drug formulations, barbiturates, aromatic bases and phenothiazines are considered.

INTRODUCTION

For some years, high-performance liquid chromatography (HPLC) has been used increasingly to control the purity of pharmaceuticals. In comparison with gas chromatography (GC), it permits the separation of heat-labile and non-volatile products. It tends to complement or replace thin-layer chromatography (TLC) because of the high speed now attainable, its ability to deal with quantitative problems and the possibilities for its automation.

As far as pharmaceutical analysis is concerned, HPLC is of great interest owing to its flexibility and the variety of adsorbent–mobile phase systems that can be used. However, in control laboratories, the analyst often finds himself in a quandary. It is true that, for practical reasons, certain parameters are not modified as far as possible. There is firstly the equipment, on which depend the flow-rate and pressure available, the dimensions of the column and the characteristics of the detection, and secondly the chromatographic support itself (nature, particle size, specific surface area, pore volume, etc.). In most instances the same assembly must be used for several analyses and transfer from one to another must be achieved quickly. The composition of the mobile phase is, therefore, the most versatile parameter. This com-

position determines the activation state of the support, the selectivity of the mobile phase and its eluting power.

The mobile phase is of prime importance and can be subjected to many variations, so that the determination of its optimal composition makes the choice difficult for the analyst.

A first choice must be made when a separation is being elaborated. Whether the operator relies on bibliographic data or previous experience (TLC, for example), he is often faced with various solutions which are not easy to put into effect. In fact, a problem may sometimes be solved by means of several different systems.

A second choice is imperative when the separation method elaborated must be applied in a control laboratory. It may be asked whether the first choice will not result in an increase in the number of solvents used in the laboratory, which will entail supply and handling expenses and a considerable waste of time (preparation of the mobile phases, rinsing of the equipment, equilibration of the columns).

Moreover, substantial diversification of chromatographic systems restricts the automation of control laboratories, prevents standardization of methods (for pharmacopoeias, for instance) and interferes with the communication of results and the comparison of procedures between different laboratories.

According to our personal experience, which is based on a large number of examples, we think that, in view of the efficacy of available supports, it is possible to restrict the number of solvents used and to solve most of the outstanding problems under standardized conditions.

To establish such conditions, we first made a selection among the available solvents, taking into account their physico-chemical and toxicological properties. We then tried to define a rational method to develop separations by means of retained solvents. The method recommended is based principally on the use of isohydric solvents, which permit perfect reproducibility to be obtained and allow retention times to be predicted from a restricted number of trials.

EXPERIMENTAL

Chromatographic equipment

The chromatograph used was a Varian Aerograph Model 8520 liquid chromatograph; it is fitted with two pumps and enables mixtures of two solvents in variable proportions to be produced very easily. Products were detected with a 254-nm UV detector. Separations were carried out on columns constructed from stainless-steel tubing (length 5–15 cm, I.D. 6 mm). Injections were made using a high-pressure sampling valve (Hamilton, ref. 77,503) feeding the column by means of a special connection equipped with an adjustable flow-rate side-tube for solvents[1,2].

The separations elaborated were repeated on a simple apparatus at low pressure (Chromaflux LC 50; Prolabo, Paris, France) equipped with experimental columns without fittings (I.D. 6 mm). Injections were then performed by means of a sampling valve.

Adsorbents

The experiments were carried out on Spherosil XOA 600 and XOA 800, which are two grades of spherical porous silica from Rhône-Poulenc having specific surface

areas of about 600 and 800 m^2/g, mean pore diameters of about 90 and 40 Å and pore volumes of about 0.9 and 0.5 ml/g, respectively. Experimental supports with mean particle diameters of 5 and 7 μm were used. Spherosil Normatom XOA 600 and 800 are now commercially available from Prolabo (Cat. No. 28, 271, 106 and 28, 272, 100).

The separating ability of Spherosil as a packing material in HPLC has been previously described, together with its use in moderate-pressure chromatography[3–5].

Reagents and samples

Methanol, 1,2-dichloroethane, 2,2,4-trimethylpentane, ethyl acetate and acetonitrile were of spectrophotometric grade, diisopropyl oxide was of analytical-reagent grade and triethylamine was of synthesis grade (obtained from Prolabo and Merck, Darmstadt, G.F.R.).

The solutes used are indicated in the figure legends and are pharmaceutical compounds from Rhône-Poulenc.

Plotting of graphs

A Hewlett-Packard 9825A computer and a Hewlett-Packard 9872A plotter were used in order to plot retention times *versus* the mobile phase polarity.

We elaborated a program that allowed us to plot the relative differences in retention times, the theoretical plate number necessary to carry out a separation and the resolution obtained for a given column as functions of solvent polarity.

SELECTION OF A LIMITED NUMBER OF SOLVENTS

Usually, the solvents with which the first liquid chromatographic separation trial of a given mixture is performed are chosen empirically. As far as we know, there are no simple and accurate rules for choosing the ideal mobile phase.

The first choice can be based on previous experience with the separation of closely related products, on a literature survey, on thin-layer chromatographic results or on personal practice.

From the results obtained in this first trial, various solutions may be considered to improve the resolution and to achieve a convenient separation under satisfactory conditions of pressure, duration, flow-rate, etc. For a given chromatographic column we shall firstly examine variations in the composition of the mobile phase.

These variations in composition are first carried out by the analyst to define the polarity of the mobile phase leading to the required capacity factors (k') (ref. 6). However, to obtain a given polarity, it is possible to use various mixtures the selectivities of which are different, according to a principle developed by Neher[7]. This may be convenient for the performance of certain separations, but makes the choice of solvents difficult, as the result is often unexpected.

If there are many peaks to be separated, the increase in the resolution between two peaks due to the use of a more "selective" mobile phase is often detrimental to the resolution between two other peaks.

The search for a selective mobile phase is therefore uncertain without a rational method, which is why we tried to simplify this approach and made a choice among the main available solvents.

A first selection was achieved among closely related solvents by eliminating those which showed the poorest chromatographic qualities (high viscosity), too low boiling and flash points (risk of fire, explosion, bubbles in the detectors) or too high a UV cut-off (ketones). Other solvents, such as tetrahydrofuran and dioxan, were discarded because of the particular behaviour of many solutes in these solvents (elution speeds not logically related to the predicted polarities, exclusion phenomena, etc.).

It must be pointed out that the polarity of certain commercial solvents is not reliable because of the impurities or stabilizers they may contain, the effect of which is not negligible (ethanol in chloroform, aromatics in absolute ethanol, water in spectrophotometric-grade ethanol, etc.). The high toxicity of certain solvents must also be taken into consideration.

The different selection criteria are listed in Table I. The principal physico-chemical characteristics in which the analyst is interested are presented in the first four columns of values. On the basis of these values, some solvents can be discarded.

In the other columns, we have indicated the principal reasons for discarding

TABLE I

CRITERIA FOR SELECTION OF SOLVENTS

Values in italics indicate undesirable properties.

Solvent	*Viscosity (cP)*	*Boiling point (°C)*	*Flash point (°C)*	*UV cut-off (nm)*	*Anomalous behaviour*	*Impurities, stabilizers present*	*Toxic*	*Solvents selected*
n-Pentane	0.23	*36.1*	10	210				
n-Hexane	0.32	*69*	*−28*	210				
Cyclohexane	*1.00*	80.7	2.5	210				
n-Heptane	0.41	98.4	*− 0.5*	210				
2,2,4-Trimethylpentane	0.50	99.2	16	210				*
Benzene	0.65	80.1	*−11*	*280*			yes	
Toluene	0.59	110.6	4	*285*			yes	
Carbon tetrachloride	0.97	76.8	—	*265*			yes	
Chloroform	0.57	61.2	—	245		yes	yes	
Methyl chloride	0.44	*39.8*	—	235				
1,2-Dichloroethane	0.79	83.5	—	230				*
Diethyl oxide	0.23	*34.6*	*−41*	220				
Diisopropyl oxide	0.37	68.3	−22	220				*
Tetrahydrofuran	0.55	66.0	*−14.5*	220	yes			
Dioxan	*1.54*	101.3	11	220	yes			
Ethyl acetate	0.45	77.1	7	260				*
Acetonitrile	0.37	81.6	5.6	210				*
Acetone	0.56	56.5	*− 9*	*330*				
Isopropanol	*2.3*	82.3	12	210				
Ethanol	*1.20*	78.3	16	210		yes		
Methanol	0.60	64.7	15.6	210				*
Dimethylformamide	0.63	153	67	*270*				
Ethylamine		*16.6*						
Diethylamine		55.5						
Triethylamine		90						*
Acetic acid		118		250				*

the other solvents. In the last column, we have indicated the few solvents finally selected. This limited number of solvents allow a large range of polarity to be covered. Among the solvents selected, acetic acid and triethylamine act as additives intended to fix the mobile phase acidity, as will be demonstrated further.

Saunders[8], by using the same criteria (apart from the boiling point), came to similar conclusions when selecting five solvents (a hydrocarbon, an ether, a chlorined solvent, acetonitrile and methanol).

The solvents selected can be classified into three groups according to their polarity. Methanol and acetonitrile are considered to be polar solvents, and are readily miscible with water. Ethyl acetate, 1,2-dichloroethane and diisopropyl oxide are solvents of medium polarity. They are not miscible with water but are miscible with all of the other solvents. 2,2,4-Trimethylpentane (isooctane) and *n*-heptane are not very polar solvents, with similar properties. They are not miscible with water or the solvents classified as polar solvents. For simplification, only isooctane was selected, but it could have been replaced with *n*-heptane.

It may be noticed that the polar and non-polar solvents selected exhibit a very low absorbance at the operating wavelength (254–280 nm) and similar spectra below 250 nm. Unfortunately, their mixtures cannot be prepared directly because of their immiscibility. To obviate this difficulty, a sufficient amount of a medium-polarity solvent can be added, which makes every mixture miscible. Unfortunately, medium-polarity solvents show a higher UV absorption than those belonging to the two other groups. However, if the same amount of medium-polarity solvent is always introduced, mixtures of constant UV absorption are obtained, which allows the mobile phases to be modified or gradients to be effected more easily.

Fig. 1 summarizes how to use the different mixtures.

In mixing medium-polarity solvents on the one hand with polar solvents and on the other with the non-polar solvent selected (isooctane or *n*-heptane), six polar mixtures and three not very polar mixtures are obtained. These mixtures (A and B) are then mixed in order to create ternary mixtures having the same medium-polarity solvent, thus enabling wide ranges of selectivity and polarity to be covered. The six ternary mixtures achievable by this method allow, in most instances, an adequate selectivity to be chosen.

In our opinion, the use of other solvents to make such ternary mixtures should be an exception, because the expected increase in selectivity rarely compensates for the decrease in efficiency due to the less suitable physico-chemical properties for liquid chromatography.

Some exceptions can be made, for instance, when the solutes have a particular solubility in a solvent or when there is some incompatibility between the solvents and the solutes (degradation, methylation, etc.).

A wider diversity might be obtained by replacing the medium-polarity solvent with a combination of two or three other solvents; this would give much more complex mixtures that could be useful for certain separation problems. However, the use of five- or four-component mobile phases is seldom necessary.

Our method of choosing the mobile phase composition therefore leads to mixtures of at least three solvents and these complex mixtures, apart from the properties discussed above, are often suitable for adsorption chromatography. In fact, separations are better carried out with a solvent mixture than with a pure solvent, a well

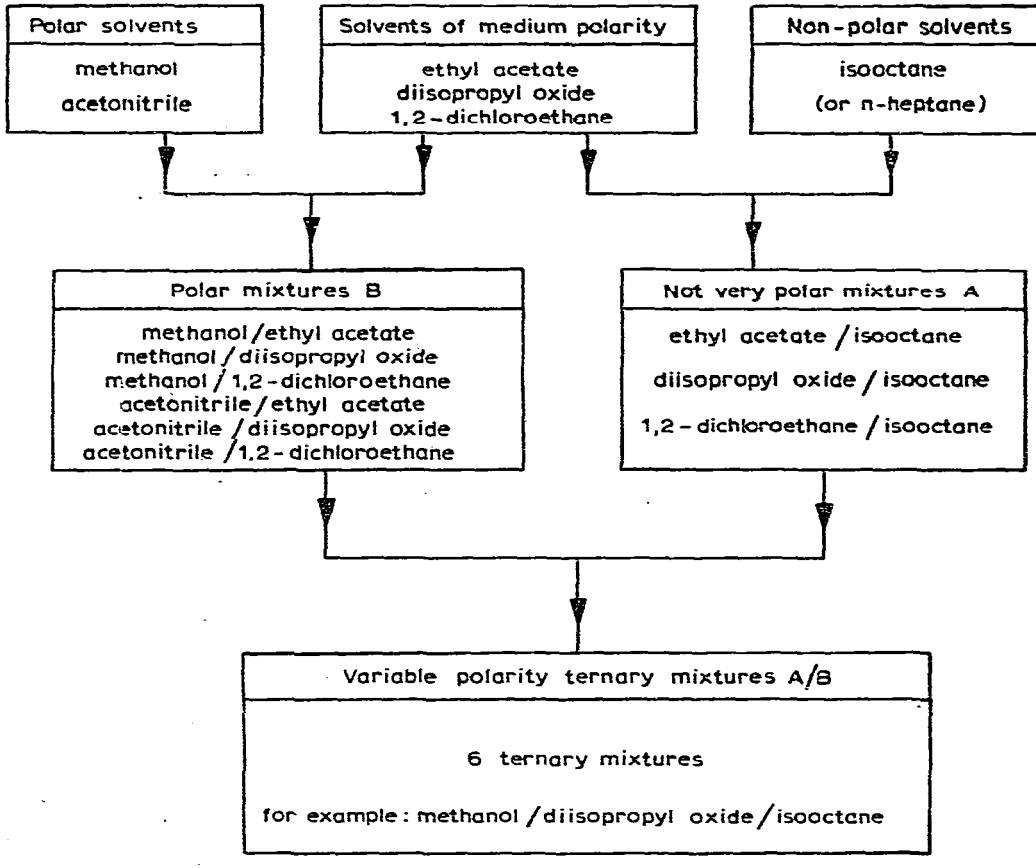

Fig. 1. Classification and mixtures of solvents.

known phenomenon in thin-layer chromatography. This can be explained by the heterogeneity of the adsorbent surface, which shows more or less strong "active sites"[6,9]. The strong sites are partly responsible for peak tailing, owing to excessive retention of part of the solute. This phenomenon is decreased by levelling the activation of the support by means of adsorption of water[6,9,10]. A polar solvent can act in the same way on the strongest sites, so that a mixture containing a small amount of polar solvent instead of a pure solvent would be preferable.

Finally, the selection of a certain number of ternary mixtures has the following main advantages: miscibility of every mixture, virtually constant UV absorption, wide polarity range, large choice of selectivity, good efficiency, limitation of solvent supply and storage, the possibility of preparing large amounts of mixtures A and B, limitation of water content adjustments, the possibility of carrying out numerous different separations on the same apparatus with the same mixtures A and B and standardization of a restricted number of chromatographic systems. Moreover, it permits a rationalization of the process of optimization of a chromatographic separation.

OPTIMIZATION OF SEPARATION

Our study was concerned principally with the optimization of separations by means of the mobile phase. Therefore, it consists essentially in choosing its selectivity and in optimizing its polarity, its acidity and its water content, on which the activation state of the adsorbent depends[10,11].

Statement of a problem in liquid–solid chromatography

Choice of the mobile phase solvents. An orientation trial is firstly made by choosing one of the six mixtures shown in Fig. 1.

Among the medium-polarity solvents selected, diisopropyl oxide is the least polar, the least UV-absorbing and has the lowest viscosity, which makes it the most interesting. Among the polar solvents, methanol is preferred to acetonitrile, because of the more restricted range of polarity covered by acetonitrile and its higher cost. As the differences between isooctane and *n*-heptane are insignificant, the choice is made on the basis of commercial quality and availability.

The following two mixtures of solvents were selected for the first trial, according to objective criteria: mixture A (not very polar), 2,2,4-trimethylpentane–diisopropyl oxide (1:1, v/v); and mixture B (polar), diisopropyl oxide–methanol (1:1, v/v).

Water content of solvents. The control of the water content of the mobile phase is essential in order to maintain the activity of the adsorbent and to ensure the reproducibility of the separation, as has been shown previously[10,11]. We have thus defined isohydric solvents[10,11] as solvents which correspond to the same hydration level of the adsorbent. The water contents of isohydric solvents for Spherosil XOA 600 equilibrated with ethyl acetate containing 0.06% of water are given in Table II. Table II also gives values for an adsorbent with a greater specific surface area, Spherosil XOA 800. The two ranges are comparable, but this does not mean that the amounts of water adsorbed are the same.

TABLE II

WATER CONTENTS OF PURE ISOHYDRIC SOLVENTS

Standardization solvent: ethyl acetate containing 0.060% of water.

Solvent	*Water content of Spherosil XOA 600 (%, v/v)*	*Water content of Spherosil XOA 800 (%, v/v)*
Methanol	5.2	5.2
Dimethylformamide	4.7	
Isopropanol	0.7	0.3
Acetonitrile	0.22	0.18
Dioxan	0.14	
Tetrahydrofuran	0.13	
Ethyl acetate	0.060	0.060
Diisopropyl oxide	0.008	0.010
1,2-Dichloroethane	0.007	0.009
Chloroform (0.5% ethanol)	0.006	0.006
Cyclohexane	<0.0005	
n-Heptane	<0.0005	<0.0002
2,2,4-Trimethylpentane	<0.0005	<0.0002

TABLE III

WATER CONTENTS OF THE SIX TERNARY MIXTURES OF ISOHYDRIC SOLVENTS

Corresponding values of $1/N_{H_2O}^{isoh}$. Adsorbent: Spherosil XOA 600.

B (%)	*SYSTEM 1: a: Isooctane b: Ethyl acetate c: Methanol*		*SYSTEM 2: Isooctane Diisopropyl oxide Methanol*		*SYSTEM 3: Isooctane Dichloroethane Methanol*		*SYSTEM 4: Isooctane Ethyl acetate Acetonitrile*		*SYSTEM 5: Isooctane Diisopropyl oxide Acetonitrile*		*SYSTEM 6: Isooctane Dichloroethane Acetonitrile*	
	Water (%)	*I/N*	*Water (%)*	*I/N*	*Water (%)*	*I/N*	*Water (%)*	*I/N*	*Water (%)*	*I/N*	*Water (%)*	*I/N*
0	0.0300	488.5	0.0041	2873.6	0.0036	4668.5	0.0310	472.8	0.0041	2873.6	0.0036	4668.5
1	0.0560	265.0	0.0301	398.1	0.0296	574.7	0.0321	460.4	0.0052	2288.8	0.0047	3597.8
2	0.0820	183.2	0.0560	217.0	0.0555	309.3	0.0332	448.8	0.0063	1908.0	0.0058	2934.1
3	0.1080	140.8	0.0820	150.5	0.0815	213.0	0.0343	437.9	0.0074	1640.4	0.0069	2482.5
4	0.1340	114.9	0.1079	116.1	0.1075	163.3	0.0354	427.8	0.0085	1442.1	0.0080	2155.2
5	0.1600	97.4	0.1339	94.9	0.1334	132.9	0.0365	418.2	0.0096	1289.1	0.0091	1907.2
6	0.1860	84.8	0.1599	80.7	0.1594	112.4	0.0375	409.2	0.0107	1167.6	0.0102	1712.7
7	0.2120	75.2	0.1858	70.4	0.1853	97.7	0.0386	400.7	0.0118	1068.8	0.0113	1556.2
8	0.2380	67.8	0.2118	62.7	0.2113	86.5	0.0397	392.6	0.0129	986.8	0.0124	1427.4
9	0.2640	61.8	0.2377	56.6	0.2373	77.8	0.0408	385.0	0.0140	917.7	0.0135	1319.7
10	0.2900	56.9	0.2637	51.7	0.2632	70.9	0.0419	377.8	0.0151	858.6	0.0146	1228.2
15	0.4200	41.5	0.3935	37.0	0.3931	49.8	0.0474	346.7	0.0206	658.0	0.0202	921.0
20	0.5500	33.4	0.5233	29.6	0.5229	39.2	0.0528	322.0	0.0261	541.8	0.0257	745.9
25	0.6800	28.4	0.6531	25.2	0.6527	32.8	0.0583	302.0	0.0316	466.2	0.0312	632.8
30	0.8100	25.0	0.7829	22.2	0.7825	28.6	0.0637	285.4	0.0371	412.9	0.0367	553.6
35	0.9400	22.5	0.9127	20.0	0.9123	25.5	0.0692	271.4	0.0426	373.4	0.0422	495.2
40	1.0700	20.6	1.0425	18.4	1.0422	23.2	0.0746	259.4	0.0481	342.9	0.0478	450.2
45	1.2000	19.2	1.1723	17.2	1.1720	21.4	0.0801	249.1	0.0536	318.7	0.0533	414.6
50	1.3300	18.0	1.3021	16.2	1.3018	20.0	0.0855	240.1	0.0591	299.0	0.0588	385.7
60	1.5900	16.2	1.5616	14.7	1.5614	17.9	0.0964	225.1	0.0700	268.9	0.0698	341.5
70	1.8500	14.9	1.8212	13.6	1.8211	16.3	0.1073	213.2	0.0810	246.9	0.0809	309.4
80	2.1100	14.0	2.0808	12.8	2.0807	15.2	0.1182	203.5	0.0920	230.2	0.0919	285.0
90	2.3700	13.2	2.3404	12.2	2.3404	14.3	0.1291	195.4	0.1030	217.0	0.1030	265.9
100	2.6300	12.6	2.6000	11.7	2.6000	13.6	0.1400	188.6	0.1140	206.4	0.1140	250.4

From these values, the water contents of mixtures can be calculated[10,11]. For the limited number of systems selected, values have been tabulated (Table III), which makes their routine use in the laboratory easier.

Table III shows how much water the basic mixtures A and B must contain, and the water content of each mixture *versus* the percentage of polar solvent B. For example, in system 2, mixture A (isooctane–diisopropyl oxide, 1:1) must be adjusted to 0.0041% of water and mixture B (diisopropyl oxide–methanol, 1:1) to 2.6% of water.

However, in practice, good quality solvents contain only a few tens of parts per million of water, and the accurate adjustment of the water content of solvent A is not necessary if more than 5–10% of solvent B is used, because the water content of solvent B is much higher than that of solvent A. In the same way, it is only necessary to add 2.6% of water to solvent B, the trace amounts of water present in diisopropyl oxide and methanol being negligible.

On the other hand, if a non-polar mixture is used (less than 5% of solvent B),

it is essential to control the water content and to adjust it in order to obtain reproducible and predictable results[10,11].

The water contents listed in Tables II and III define for the adsorbent an activation state a_a to which we have assigned an arbitrary value of 1 (refs. 10 and 11). These water contents are generally used for the first trial.

The activation state can be modified and optimized, as will be seen below, by adjusting the water contents of the solvents. However, this requires longer equilibration times and the results are less predictible than those obtained by optimization of the mobile phase polarity. Consequently, we tried to keep this factor constant in order to facilitate the use of the same column for different problems.

Mobile phase acidity. The separation of non-ionic organic compounds such as aromatic hydrocarbons eluted by non-polar solvents (such as *n*-hexane) can be considered as "pure adsorption chromatography", the interference of acid–base phenomena being excluded by the non-dissociating character of the solvents used. Coinversely, in the partition chromatography of very polar solutes, an important role is played by these phenomena because of the large amount of water present in the system. In the pharmaceutical industry, solutes to be separated often have medium polarity, but methods using solvent mixtures with non-negligible dielectric constants are still referred to as adsorption chromatography.

Even if the amount is small, the addition of a proton donor or acceptor to the mobile phase will have important consequences for the chromatographic system, more especially as the dielectric constant of the solvent mixture is high. At the adsorbent level, the addition of a small amount of acid or base will lead to neutralization of the most active sites[12] and "peak tailing" will be reduced as a consequence.

The acidic or basic character of the mobile phase also acts directly on the solutes by fixing the form in which they are chromatographed. Even in solvents with a low dielectric constant, all of the acidic or basic solutes can be chromatographed in the form of either an acid (AH-type acid) or a base (B-type base), or even in the form of an ion pair (ABH undissociated salt, for example).

If the acidity of the mobile phase is not fixed for these types of solutes, peak tailing might occur with solvents considered as neutral (action of acidic or basic impurities in the solvents).

The elution of the solute in its molecular form is generally used in adsorption chromatography, by acidification of the mobile phase with a AH-type acid[13,14] or by alkalinization with a B-type base[4,10,11,15–18].

When the adsorbent is silica, the levelling in the activity of the stationary phase is more efficient if a base is added when chromatographing neutral or amphoteric solutes, the latter being eluted as ion pairs.

As the acids or bases to be separated by adsorption chromatography are weak, formic or acetic acid can be used to fix the acidic solutes in their molecular form; in the same way, ammonia or mono-, di- or triethylamine can be used to fix the basic solutes in that form.

The amount of acid or base added to the mobile phase is generally small, in order not to deactivate the adsorbent completely. However, this amount must be accurately controlled in order to obtain reproducible results. Fig. 2 shows an adsorption isotherm of ethylamine on Spherosil XOA 600. For the low values generally used ($<0.5\%$), the amount of ethylamine adsorbed is 10–20-fold higher than the

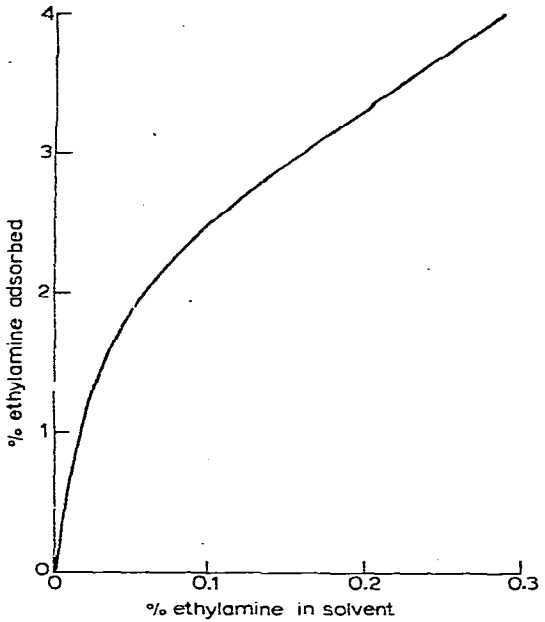

Fig. 2. Adsorption isotherm of ethylamine. Adsorbent: Spherosil XOA 600, initial activation by heating to 140°. Solvent: ethyl acetate–methanol–water (79.2:20:0.8).

amount in the mobile phase. Small absolute variations of the amount of base in the mobile phase may cause large variations in solute retention times.

The control of the acidity of the mobile phase not only affects the reproducibility, but is also a means of improving a separation, because it allows one to modify the polarity of the solutes and of the mobile phase, and also the activity of the adsorbent. Separations with respect to the solute pK_a can be performed in some instances, and the separation of neutral solutes from ionic compounds can be achieved.

An amount of 0.2% (v/v) is generally used in a first trial. Variations in this amount of acid or base have a less predictible influence on the chromatographic system than variation of the polarity and, consequently, are used only when the optimization of the polarity of the mobile phase does not permit the desired separation to be obtained.

Initial operating conditions. From the above considerations, a first trial can be carried out with a mixture of two solvents (A and B) with the following compositions: A, 2,2,4-trimethylpentane–diisopropyl oxide–acetic acid or triethylamine–water (50:50:0.2:0.0041); and B, diisopropyl oxide–methanol–acetic acid or triethylamine–water (50:50:0.2:2.6).

The proportions of solvents A and B depend on the polarity of the solute. In order to elute all the solutes, a polar mixture is desirable for the first trials, but it is also desirable to obtain at least a beginning of a separation between the solutes.

Preliminary trials can be made by TLC, but the use of TLC to determine exactly the mobile phase composition for subsequent use in HPLC[9,19,20] seems unsuitable in terms of the desired rapidity. A rigorous transposition from one technique to the other needs the same adsorbents with the same activity state[19], requires trans-

position coefficients to be established and operating conditions to be strictly maintained. At present, owing to the rapidity of HPLC, it is possible to optimize the separation on column directly.

We consider that TLC, either from previous experience, as bibliographic data or as specially performed trials, is a useful technique for estimating approximately the necessary polarity of the mobile phase. With an unknown mixture, it also enables one to estimate the number of peaks to be eluted. At the same time, one can establish whether very polar substances are present (not eluted). A qualitative estimation of these data is a good basis for choosing the polarity of the initial solvent mixture for HPLC.

With this first preliminary trial, capacity factors (k') of every solute are rapidly obtained with accuracy ($1 < k' < 20$).

Optimization of the mobile phase composition

Plotting curves $k' = f(1/N_{H_2O}^{isoh})$. A linear relationship between the capacity factors (k') of solutes and the inverse of the molar fraction of water in isohydric solvents ($1/N_{H_2O}^{isoh}$) has been shown previously[10,11].

This simple relationship can be used to optimize a separation. The capacity factors of the solutes need to be measured in two or three mixtures only of solvents A and B and the straight lines representing k' *versus* $1/N_{H_2O}^{isoh}$ can be plotted. This is achieved by using Table III, giving $1/N_{H_2O}^{isoh}$ *versus* the percentage of polar solvent B. The limited number of systems selected allows graph paper already marked up with $1/N_{H_2O}^{isoh}$ and %B scales to be used.

When the straight lines have been drawn, a first qualitative estimation can be made regarding the validity of the system. If there is some peak tailing, the acidity of the system may be incorrect and therefore must be modified. This may occur, for example, if, in an unknown mixture, acidic compounds are chromatographed with a mobile phase that contains a basic component.

The slopes of the straight lines $k' = f(1/N_{H_2O}^{isoh})$ allow one to establish whether the activation state of the adsorbent is suitable. If the slopes are too small, variations in the polarity of the mobile phase will give small variations in retention times and optimization will not be possible. If the slopes are very high, small variations in polarity will lead to large variations in retention times, and therefore it will be difficult to maintain good reproducibility. The slopes of the curves can be modified by varying the activation state of the adsorbent[11]. In practice, the activation state, α_a (refs. 10 and 11), is varied by changing the water content of solvent B within reasonable limits (not close to saturation, for example) for mixtures containing more than 5% of B.

However, variation of the water content has the disadvantage of leading to long equilibration times. This optimization possibility should be used only if the following method of optimization of the mobile phase composition has proved unsuccessful.

Resolution versus the mobile phase polarity. The linear relationship between the retention times (T) and the polarity of solvent mixtures ($1/N_{H_2O}^{isoh}$) allows the retention times (or capacity factors, k') in the whole polarity range of solvents A and B to be predicted from a few trials. The straight lines obtained can be used to optimize the resolution according to the following methods.

(a) General method. This first method of optimization is general and suitable for complex problems. An expression for the resolution is used that permits the resolution due to the efficiency of the column to be optimized separately from the resolution due to the relative interval of retention times obtained with different mobile phase polarities.

From the standard equations $R_s = 2\left(\frac{T_2 - T_1}{W_2 + W_1}\right)$, $N_1 = 16\left(\frac{T_1}{W_1}\right)^2$ and $N_2 = 16\left(\frac{T_2}{W_2}\right)^2$, assuming $N_2 \approx N_1 = N$, we used the derived equation

$$R_s = \frac{\sqrt{N}}{2} \cdot \frac{T_2 - T_1}{T_2 + T_1}$$

With this equation it is not necessary to measure the peak width, and incomplete separations can be used to predict the relationship between resolution and the polarity of the mobile phase.

Curves of $T = f(1/N^{isoh}_{H_2O})$ are used to predict, from a limited number of trials, retention times for other mixtures of solvents A and B. With experimental and calculated values, curves of $T_2 - T_1/T_2 + T_1$ for each pair of peaks are plotted against the polarity of the mobile phase, $1/N^{isoh}_{H_2O}$.

Calculations can be carried out with a computer, as has already been done in GC[21–24] for choosing the composition of stationary phase mixtures. In the same way, a semi-empirical optimization strategy using "window diagrams" has been developed for optimizing the pH of aqueous solvents in reversed-phase chromatography of weak organic acids[25].

The optimal solvent composition can be chosen by using the curves obtained, and then the necessary theoretical plate number (N) can be calculated for achieving the desired resolution:

$$N = \left[2\, R_s\left(\frac{T_2 + T_1}{T_2 - T_1}\right)\right]^2$$

The conditions of column length, flow-rate, etc., can be chosen independently of the composition of the mobile phase.

The various possibilities will be shown in the following examples, which are of increasing complexity.

Example 1: separation of barbiturates. A column of good efficiency is used (Spherosil Normatom XOA 600, $d_p = 6\ \mu m$, 15×0.6 cm) at a flow-rate close to the minimum of the Van Deemter curves (1 ml/min). For barbiturates with acidic properties (see the list of solutes in the legend of Fig. 5), the mobile phase is acidified with 0.2% of acetic acid. A standard activation state is used ($a_a = 1$). The retention times are measured by injecting separately the solutes, in mixtures of 30% and 10% of B (system No. 2). The values obtained allow one to draw the straight lines $T = f(1/N^{isoh}_{H_2O})$, as shown in Fig. 3. From these straight lines, the retention times of the solutes are predicted for other mixtures of solvents A and B and curves of $T_2 - T_1/T_2 + T_1$ *versus* $1/N^{isoh}_{H_2O}$ are plotted, giving Fig. 4.

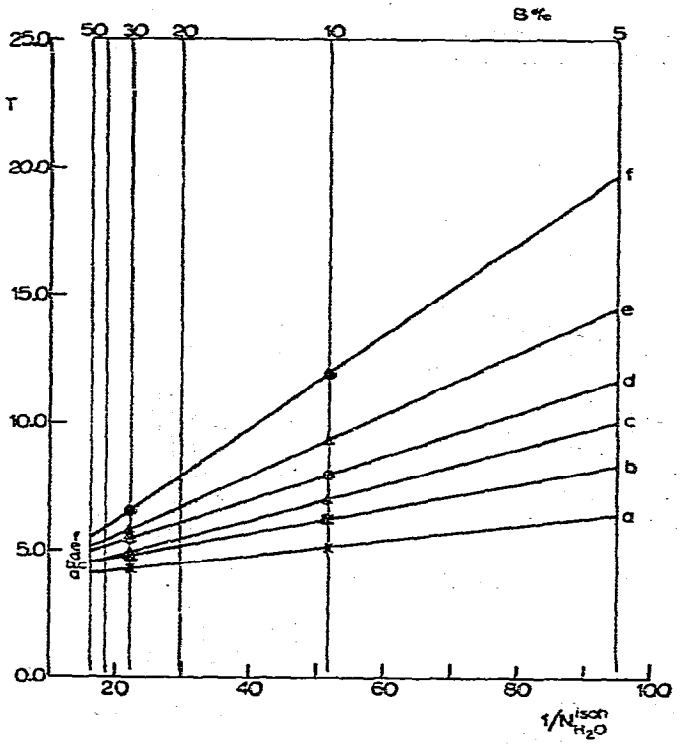

Fig. 3. Relationship between retention times of barbiturates and the mobile phase polarity. Column: 15 × 0.6 cm Spherosil XOA 600, 6 μm. Mobile phase A: isooctane–diisopropyl oxide (1:1). Mobile: phase B: diisopropyl oxide–methanol (1:1). Acidity: 0.2% of acetic acid in A and B. Adsorbent activation state: $\alpha_e = 1$ (see corresponding water contents in Table III). Flow-rate: 1 ml/min. Solutes: see legend of Fig. 5.

It can be seen that the resolution increases when $1/N_{H_2O}^{isoh}$ increases or when the polarity of the mixtures of A and B decreases. For the poorest separated peaks b and c, the number of theoretical plates necessary to give a resolution (R_s) of 1.7 can be calculated. More than 10,000 plates are necessary at 20% of B and about 4000 plates at 10% of B. With the solutes to be separated, only the latter value can be achieved. The conditions selected ($L = 15$ cm, flow-rate = 1 ml/min) give the predicted separation shown in Fig. 5.

Example 2: separation of aromatic bases. On a 15 × 0.6 cm column, the retention times of aromatic bases (see the legend of Fig. 9) eluted at 2 ml/min in solvent system No. 2 (see Table III) containing 0.2% of triethylamine are measured and plotted against the polarity of the mixtures of A and B. The straight lines obtained (Fig. 6) allow the term $T_2 - T_1/T_2 + T_1$ to be calculated for each pair of peaks against the polarity of the mobile phase (Fig. 7). Most of the peaks are separated better when the polarity decreases, except for peaks e and f. The point of intersection of the curves for the poorest separated peaks (d–e and e–f) determines an optimal polarity of the mobile phase for 5% of B.

For this polarity, a theoretical plate number of 5800 is necessary to obtain a resolution (R_s) of 2, as shown in Fig. 8. The separation corresponding to the optimal

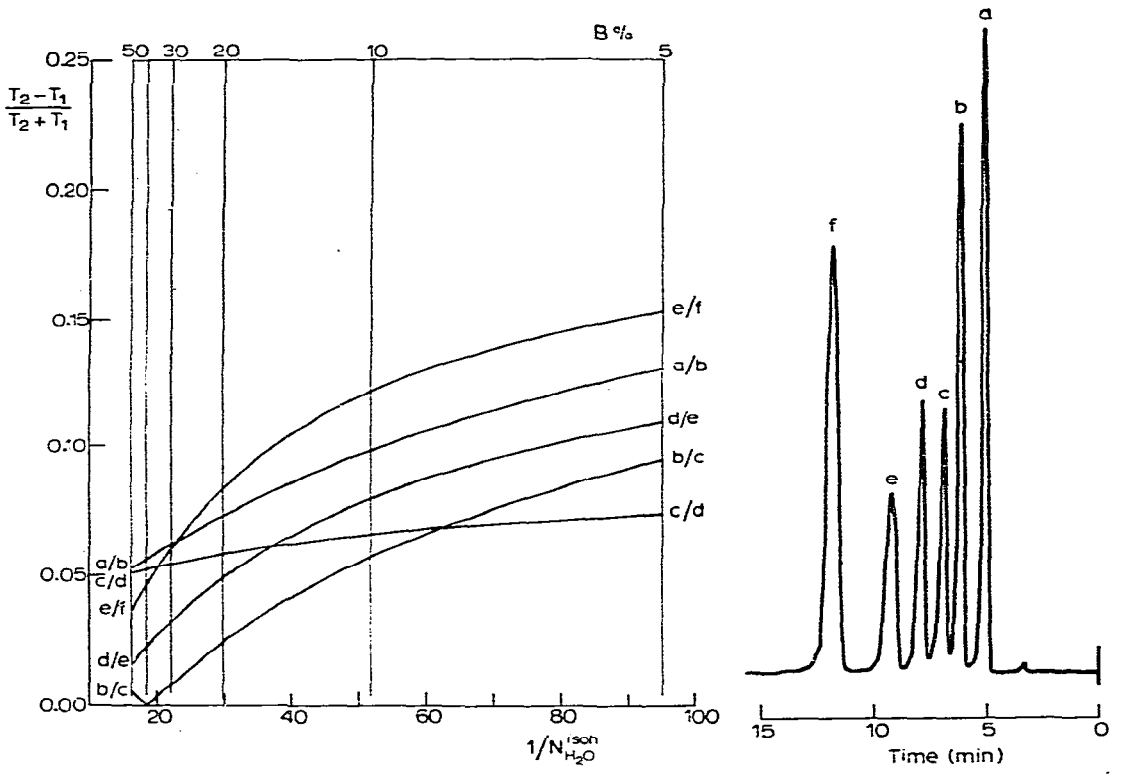

Fig. 4. Optimization of separation of barbiturates. Expression of T_2-T_1/T_2+T_1 *versus* the polarity, according to the values obtained from the straight lines plotted in Fig. 3.

Fig. 5. Optimized separation of barbiturates. Column: 15 × 0.6 cm Spherosil XOA 600, 6 μm. Mobile phase: 10% of B, *i.e.*, isooctane–diisopropyl oxide–methanol (45:50:5), containing 0.2% of acetic acid and 0.26% of water (see legend of Fig. 3). Flow-rate: 1 ml/min. ΔP: 25 bar. Detection: UV, 254 nm. Solutes: (a) penthiobarbital; (b) methylphenobarbital; (c) amobarbital; (d) allobarbital; (e) phenobarbital; (f) mephebarbital.

conditions is shown in Fig. 9, the six aromatic bases being separated in about 7 min, as predicted by Fig. 6.

Example 3: separation of phenothiazines. Using the same method, straight lines of $T = f(1/N_{H_2O}^{isoh})$ are drawn for preliminary trials with 50 and 20% of B containing 0.2% of triethylamine, and for an activity state $\alpha_a = 1$. Retention times are obtained rapidly on a short column (5 × 0.6 cm) and give the straight lines represented in Fig. 10, showing a retention time inversion between peaks f and g for about $1/N_{H_2O}^{isoh} = 25$.

In Fig. 11, it can be seen that there are two possibilities for separating all of the peaks. The first takes place when $1/N_{H_2O}^{isoh} \approx 38$ (about 15% of B) and the second when $1/N_{H_2O}^{isoh} \approx 20$ (about 35% of B).

Fig. 12 shows that the corresponding plate numbers necessary for achieving a resolution (R_s) of 2 are about 1500 and 2500, respectively. This latter efficiency is easy to obtain and the most polar mixture (35% of B) is chosen, allowing the separation to be performed in the shortest time (Fig. 13).

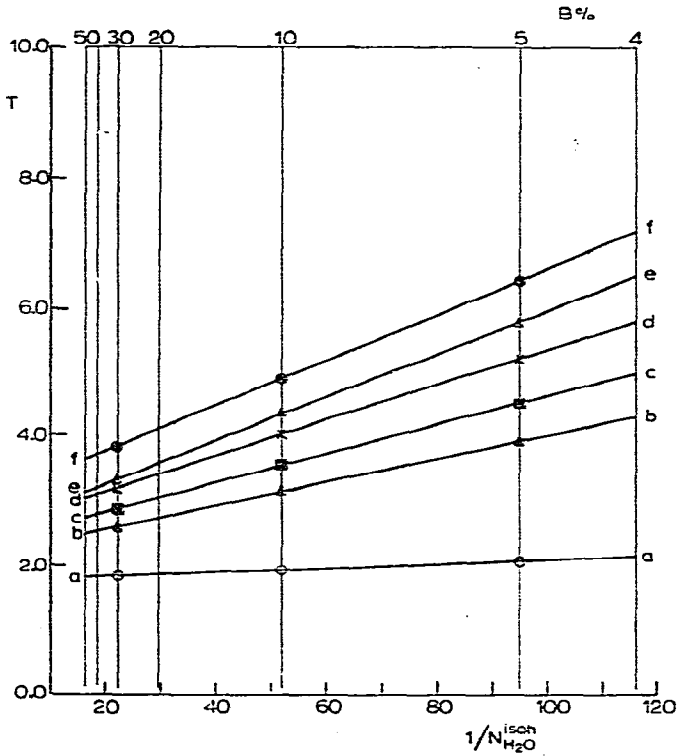

Fig. 6. Retention times of aromatic bases *versus* the polarity of the mobile phase. Column: 15 × 0.6 cm Spherosil XOA 600, 6 μm. Mobile phase A: isooctane–diisopropyl oxide (1:1). Mobile phase B: diisopropyl oxide–methanol (1:1). Acidity: 0.2% of triethylamine in A and B. Adsorbent activation state: $\alpha_s = 1$ (see Table III). Flow-rate: 2 ml/min. Solutes: see legend of Fig. 9.

(b) Simplified method. The second method, which does not need any calculation, allows simple problems to be solved when the number of peaks is not very important.

Chromatograms of the solutes are obtained in two mixtures of solvents A and B, enabling the retention times to be measured. The peak width is also measured, and the straight lines for retention times are enclosed by the straight lines corresponding to the start and the end of the peak[10]. The composition of the solvent that allows the separation to be performed in the shortest possible time can readily be seen.

An example is illustrated by Fig. 14, which shows the retention times of the active constituents of "Théralène Pectoral" (Théraplix, Paris, France). It can be seen that these polar substances are easily separated on a short column with the pure polar solvent B. The corresponding chromatogram is shown in Fig. 15. The conditions selected permit the use of simple apparatus at low pressure (Chromaflux LC 50; Prolabo).

Fig. 16 shows the optimization of the separation of the active constituents of a veterinary injection solution (soluté injectable 357 V; Specia, Paris, France) and an internal standard. The retention times and peak width were measured by injecting separately the solutes in mixtures of 30 and 5% of B. It can be seen that a mixture containing 10% of B allows the components to be separated in the shortest possible time.

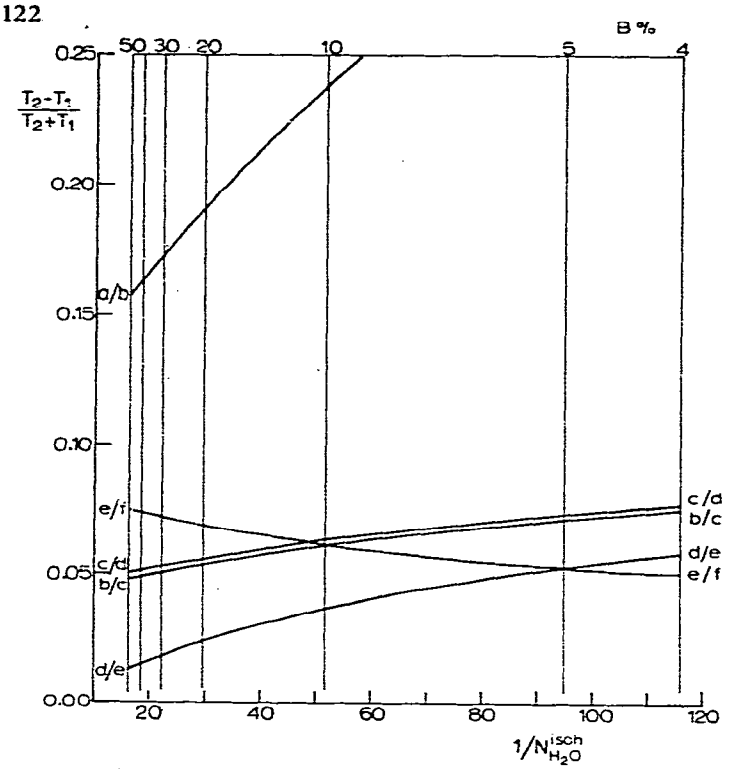

Fig. 7. Optimization of separation of aromatic bases. Expression of T_2-T_1/T_2+T_1 *versus* the polarity, according to the values obtained from the straight lines plotted in Fig. 6.

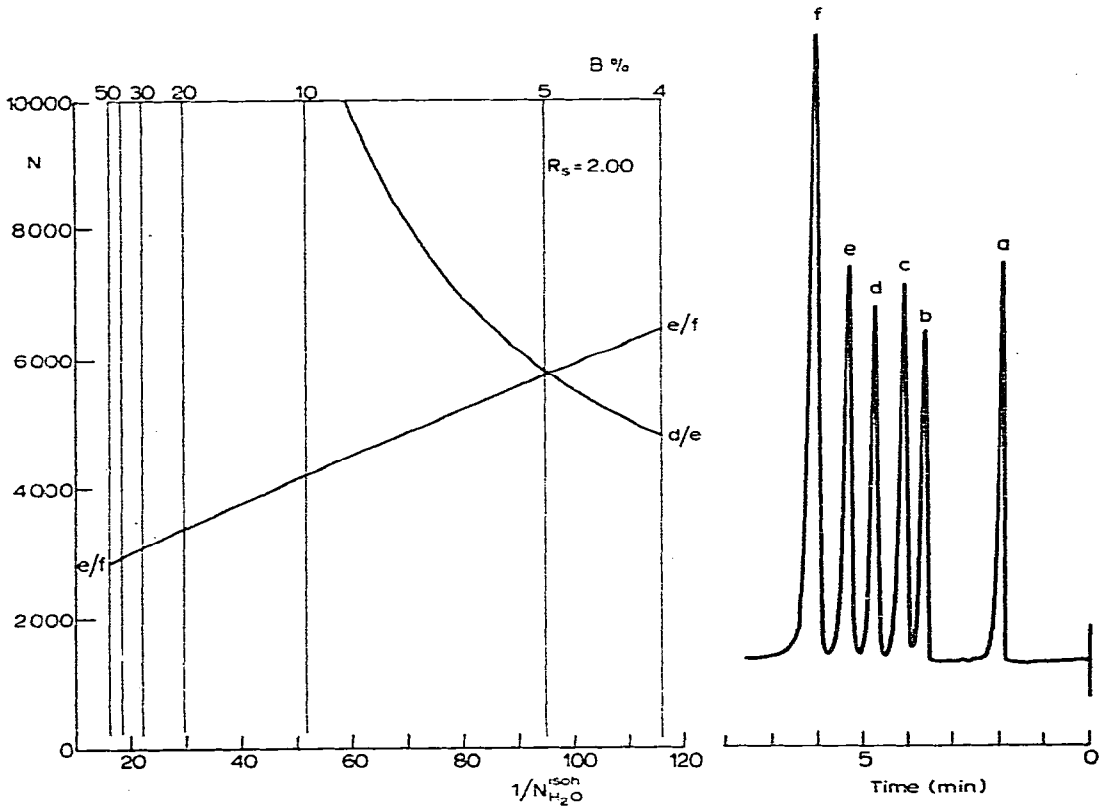

Fig. 8. Theoretical plate number necessary for the separation of aromatic bases with a resolution of $R_s = 2$, *versus* the mobile phase composition.

Fig. 9. Optimized separation of aromatic bases. Column: 15 × 0.6 cm Spherosil XOA 600, 6 μm. Mobile phase: 5% of B, *i.e.*, isooctane–diisopropyl oxide–methanol (47.5:50:2.5), containing 0.2% of triethylamine and 0.13% of water (see Figs. 6, 7 and 8). Flow-rate: 2 ml/min. ΔP: 42 bar. Detection: UV, 254 nm. Solutes: (a) dimethylaniline; (b) *o*-toluidine; (c) aniline; (d) quinoline; (e) isoquinoline; (f) pyridine.

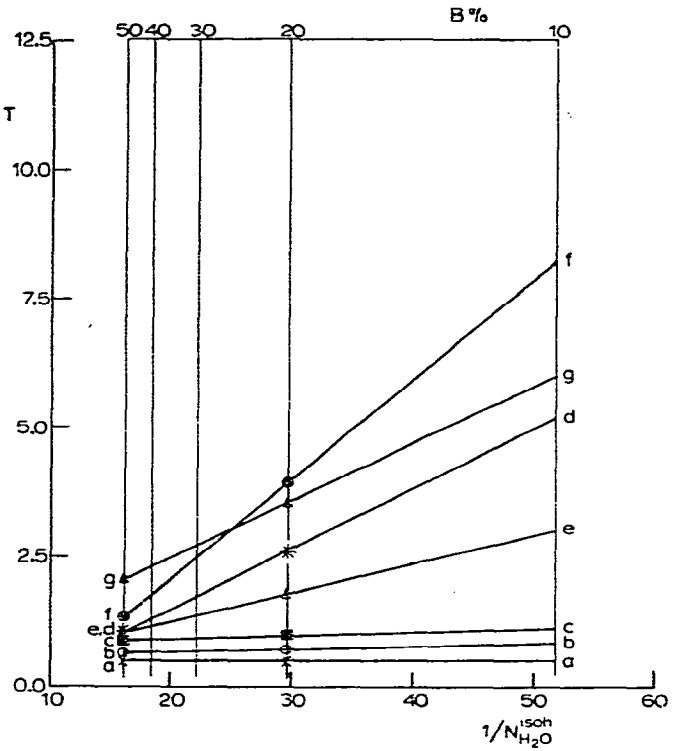

Fig. 10. Retention times of phenothiazines *versus* the polarity of the mobile phase. Column: 5 × 0.6 cm Spherosil XOA 600, 5 μm. Mobile phase A: isooctane–diisopropyl oxide (1:1). Mobile phase B: diisopropyl oxide–methanol (1:1). Acidity: 0.2% of triethylamine in A and B. Adsorbent activation state: $a_a = 1$. Flow-rate: 3 ml/min. Solutes: see legend of Fig. 13.

Fig. 17 shows the chromatogram obtained under these conditions. The retention times predicted by the curves in Fig. 16 agree with those actually obtained: peak a (internal standard), expected $T = 1.55$, measured $T = 1.59$ min; peak b (chlorpromazine), expected $T = 4.25$, measured $T = 4.27$ min; and peak c (promethazine), expected $T = 5.20$, measured $T = 5.10$ min.

DISCUSSION

For complex problems, our general method is helpful and avoids the need for a long trial-and-error study owing to the decrease in resolution between certain peaks and the increase in resolution between others. Tedious work is also avoided when the separation cannot be performed in the system used. Only two trials are necessary to establish whether another system exhibiting a different selectivity must be employed. By using isohydric solvents, the column will be quickly equilibrated with the new mixture of solvents[10,11].

If these variations do not improve the separation, the activity state of the adsorbent can be changed by modifying the water content of the solvents[10,11]. For

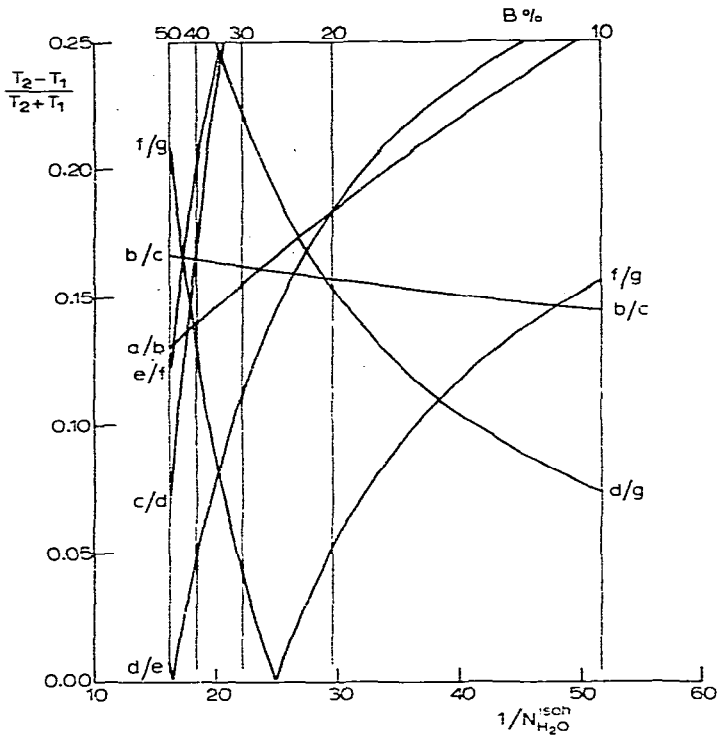

Fig. 11. Optimization of separation of phenothiazines. Expression of T_2-T_1/T_2+T_1 *versus* the polarity, according to the values obtained from the straight lines plotted in Fig. 10.

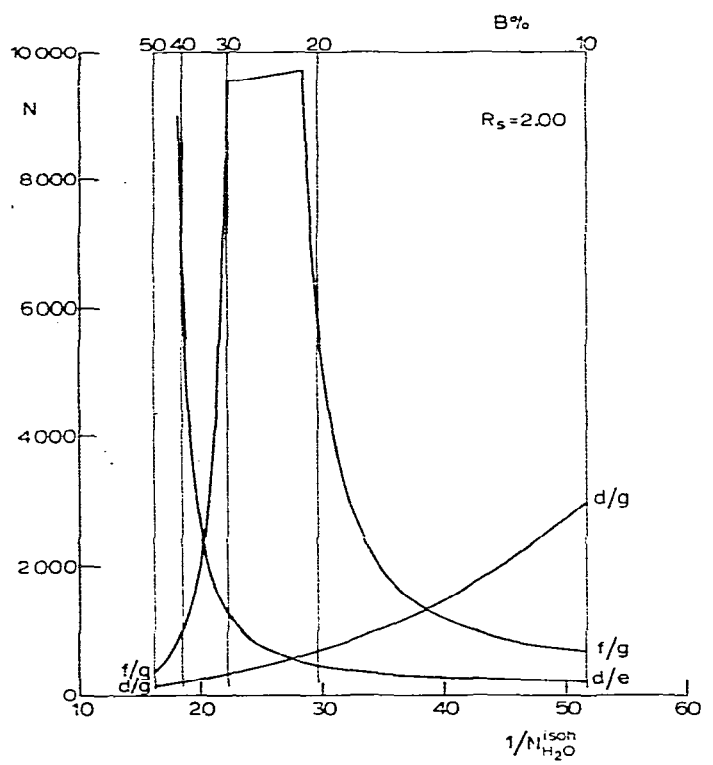

Fig. 12. Theoretical plate number necessary for the separation of the most difficult pairs of peaks with a resolution of $R_s = 2$ *versus* the mobile phase composition.

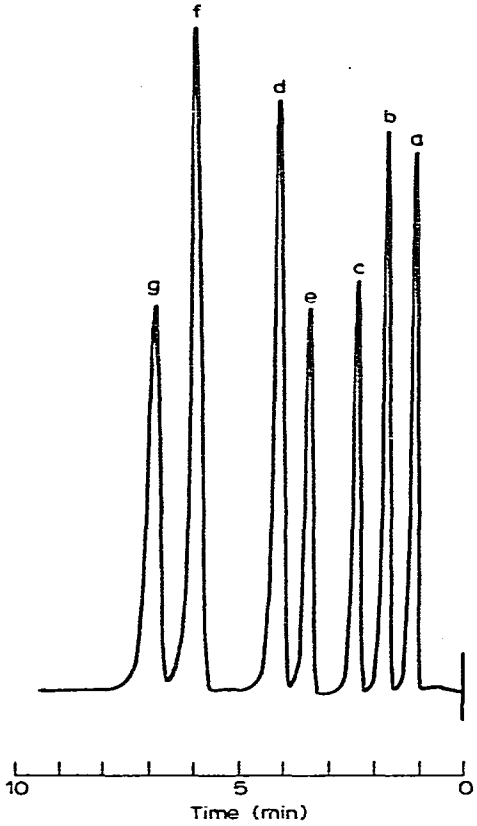

Fig. 13. Optimized separation of phenothiazines. Column: 15 × 0.6 cm Spherosil XOA 600, 6 μm. Mobile phase: 35% of B, *i.e.*, isooctane–diisopropyl oxide–methanol (32.5:50:17.5), containing 0.2% of triethylamine and 0.91% of water (see Figs. 10, 11 and 12). Flow-rate: 3 ml/min. ΔP: 80 bar. Detection: UV, 254 nm. Solutes: (a) 3-chlorophenothiazine; (b) levomepromazine; (c) chlorpromazine; (d) dimethothiazine; (e) propericiazine; (f) pipothiazine; (g) oxomemazine.

proton donors or acceptors, variations in the acidity of the mobile phase can also be used to improve a separation.

However, if a rational method of optimization is employed, most of the problems can be solved by means of the two mixtures of solvents A and B used in the examples described (Table III, system No. 2). This method is summarized in the scheme in Fig. 18.

From this method, we can ascertain that in adsorption chromatography more than 90% of the separations can be achieved with the same mixture of solvents A (isooctane–diisopropyl oxide) and B (diisopropyl oxide–methanol) and that a variation in the amount of base or acid added to the solvent is required in less than 10% of the cases, and a variation in the activity state in less than 20% of the cases.

These results are particularly interesting for control laboratories because they avoid the proliferation of different systems and permit automation owing to the limited number of systems involved and the reproducibility of the separations.

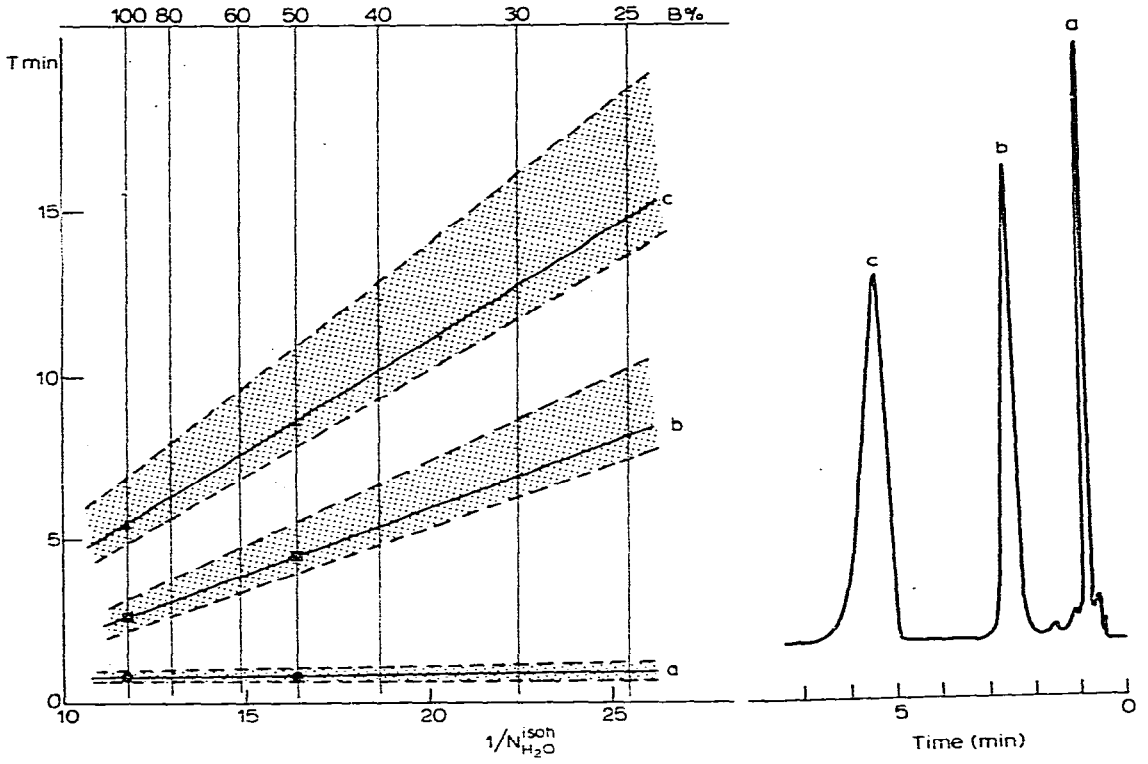

Fig. 14. Optimization of separation of the active constituents of "Théralène Pectoral". Column: 5 × 0.6 cm Spherosil XOA 600, 6 μm. Isohydric mobile phases: A, isooctane–diisopropyl oxide (1:1); B, diisopropyl oxide–methanol (1:1). Activation state: $\alpha_a = 1$ (water content of solvent B: 2.6%). Acidity: 0.2% of triethylamine. Flow-rate: 2 ml/min. ΔP: 35 bar.

Fig. 15. Separation of the active constituents of "Théralène Pectoral". a, Alimemazine; b, codethyline; c, ephedrine. Detection: UV, 254 nm. Conditions: 100% of solvent B (see legend of Fig. 14).

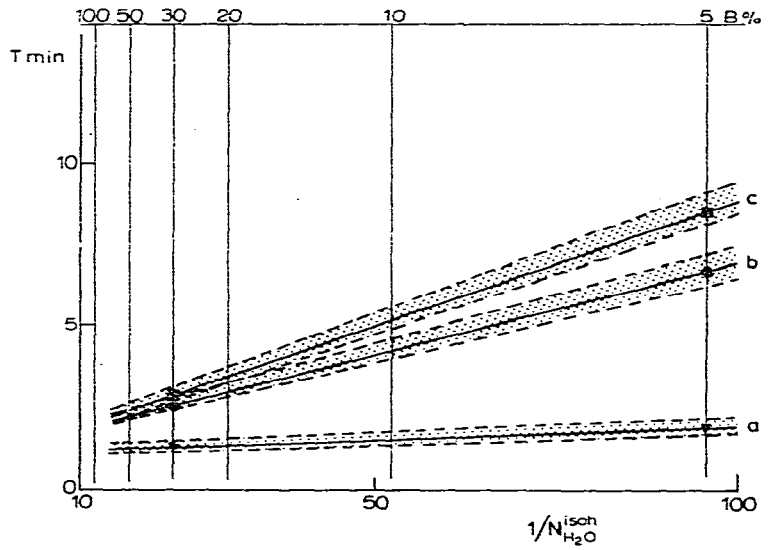

Fig. 16. Optimization of separation of the active constituents of the injection solution 357 V. Column: 15 × 0.6 cm Spherosil XOA 600, 6 μm. Isohydric mobile phases: A, isooctane–diisopropyl oxide (1:1); B, diisopropyl oxide–methanol (1:1). Activation state: $\alpha_a = 1$ (water content of solvent B: 2.6%). Acidity: 0.2% of triethylamine. Flow-rate: 3 ml/min. ΔP: 70 bar.

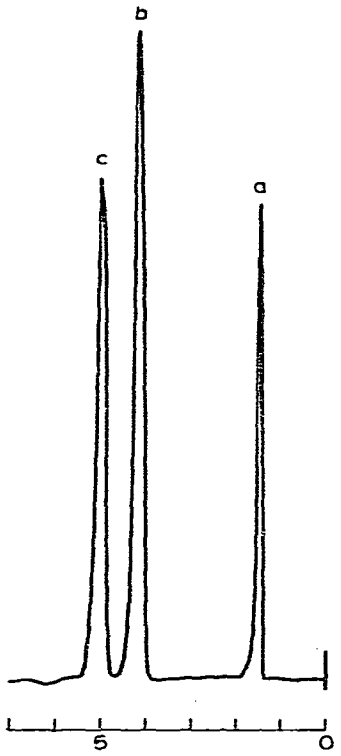

Fig. 17. Separation of the active constituents of the injection solution 357 V. a, Internal standard (3-chlorophenothiazine); b, chlorpromazine; c, promethazine. Detection: UV, 254 nm. Conditions: 10% of solvent B (see legend of Fig. 16).

Our method of optimization can be applied with other polarity scales, but linear relationships are preferable, as they permit accurate predictions from a few experimental data.

We have already pointed out some analogies between $1/N_{H_2O}^{isoh}$ and Snyder's eluent strength parameter, ε^0, and compared the linear relationships k' *versus* $1/N_{H_2O}^{isoh}$ and log k' *versus* log X_B, where X_B is the molar fraction of the more polar solvent[11]. However, we think that there are some advantages in using $1/N_{H_2O}^{isoh}$ as a polarity scale.

Let us consider Snyder's relationship[6]:

$$\log k^0 = \log V_a + \alpha(S^0 - A_s\varepsilon^0) \tag{1}$$

where

k^0 = sample adsorption distribution coefficient;
V_a = adsorbent surface volume;
α = adsorbent surface activity function;
S^0 = free energy of adsorption of a sample compound;
A_s = molecular area of adsorbed sample molecules;
ε^0 = solvent strength parameter.

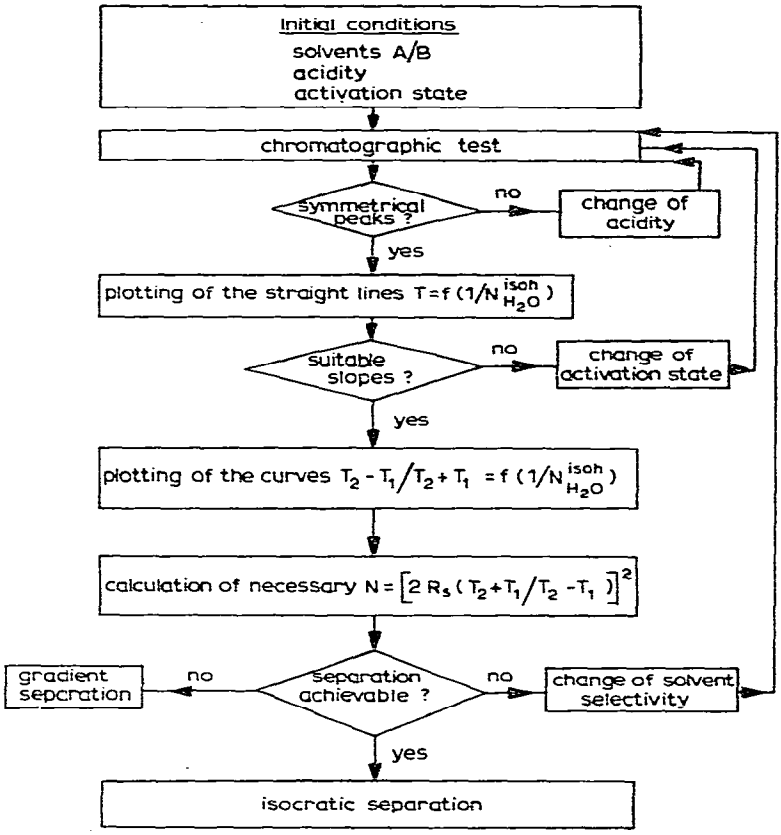

Fig. 18. Scheme for optimization of the mobile phase composition.

For a binary mixture of solvents, the parameter ε^0 is given by the expression

$$\varepsilon_{AB} = \varepsilon_A + \frac{\log\left[N_B 10^{\alpha n_B(\varepsilon_B-\varepsilon_A)} + 1 - N_B\right]}{\alpha n_B} \tag{2}$$

where N_B is the molar fraction of solvent B and n_B is the effective molecular area of an adsorbed molecule of solvent B (where B is the strongest solvent component).

For ternary mixtures, a similar expression can be derived[6,26], assuming that the two strongest solvent components cover the whole adsorbent surface.

By simplification of Snyder's equation, many workers[26–40] have used the equation

$$\log k' = a - n \log X_B \tag{3}$$

where a and n are constants and X_B is the molar fraction of the strongest solvent in a binary mixture.

With regard to the above equations, we can make the following remarks.

(1) They use logarithmic scales, which do not permit values for k' and X_B equal or very near to zero to be plotted. For example, the first peaks (a) in Figs. 10 and 16 are not plottable because $k' \approx 0$.

(2) These equations are more complex than ours, which needs only a calculation of molar fractions and gives a direct linear relationship between k' (or T) and $1/N_{H_2O}^{isoh}$, without any logarithmic conversion.

(3) These equations should give linear relationships between $\log k'$ and ε^0 or $\log X_B$; linear curves over restricted polarity ranges have often been verified, but many deviations have also been noted[26,27]. When plotting our own data against ε^0 or $\log X_B$, in general we do not obtain linear curves. In some instances, this is due to the fact that k' is nearly zero or $X_B = 0$ (Fig. 17 in ref. 11). Regarding the simplified relationship between $\log k'$ and $\log X_B$, deviations may occur because the polarity of solvent A is neglected.

(4) The most important problem in the use of ε^0 or X_B is that the water content of the solvents is not taken into account. In the simplified relationship between $\log k'$ and $\log X_B$, the water content of the solvents is neglected, and also the activation state of the adsorbent. When X_B is varied without the water content being adjusted, the activation state of the adsorbent is also modified and this interferes with the retention times, as shown previously.

Binary mixtures of solvents A and B are in fact ternary; they always contain water and this third, neglected, component is the most important. The importance of water can also be seen by considering Snyder's eqns. 1 and 2 for $\log k^0$ and ε_{AB}, which depend on the adsorbent surface activity function, α. Theoretically, ε^0 values are determined for $\alpha = 1$ by adding a known amount of water to an activated adsorbent. However, the water contents of the solvents are not adjusted to the level necessary to maintain this amount of adsorbed water. Consequently, α cannot be maintained at the correct value and the determination and the use of ε^0 are not likely to be very accurate. Further, it is not clear from Snyder's work whether the ε^0 values have to be calculated for pure solvents or for the necessary solvent–water binary mixtures.

We think that the deviations from linearity of the relationship between $\log k'$ and ε^0 are due mainly to uncontrolled variations in the adsorbent activity. For further development, measurements of ε^0 for isohydric solvents would be of great interest.

At present, the use of $1/N_{H_2O}^{isoh}$ as a polarity scale seems to be a good compromise between a complex theoretical relationship which in practice shows some failures and a too simplified expression which does not represent the properties of all of the mobile phase components (particularly the weakest solvent and water) and of the adsorbent. The inverse of the molar fraction of water in isohydric solvent mixtures takes into account the polarity of each component, the mobile phase composition and the adsorbent activity.

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