Solvent-Strength Selectivity in Reversed-Phase HPLC

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Key Words

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Solvent-strength selectivity

Summary

Solvent-strength selectivity refers to the variation of band spacing by changing the %-organic in the mobile phase (ion-pair or reversed-phase HPLC). A review of the literature has been combined with new experimental data to illustrate the general potential of this approach for HPLC optimization. It appears that most samples exhibit significant changes in band spacing method development based on solvent-strength optimization plus computer simulation (DryLab software) are given for illustration. For relatively simple mixtures (10 or fewer components), it appears that solvent-strength optimization compares favorably with other methods such as mapping the organic-solvent selectivity of methanol, acetonitrile, tetrahydrofuran, and water.

Introduction

Method development is of broad interest for separations by reversed-phase HPLC. Several different approaches have been described for optimizing such separations [1–4], most of which emphasize the control of band spacing or selectivity. Separation selectivity can be systematically varied in several ways, but the most popular procedure is some form of optimization based on the use of different solvents or secondary-equilibria. Solvent-type optimization refers to the mapping of retention as a function of the composition of mobile phases composed of methanol, acetonitrile, tetrahydrofuran and water [5]. The optimization of secondary-equilibria can be carried out in similar fashion, by varying pH and/or the concentration of an ion-pair reagent [6]. Either approach is widely accepted as a powerful technique for improving the resolution of most samples.

A change in solvent strength (% organic in an aqueous-organic mobile phase) while other variables are held constant has not been considered useful for varying band spacing (e.g., discussion of [7]). Nevertheless a few workers have successfully used this approach for developing an isocratic HPLC method [8–10]. For the purpose of varying band spacing, a change in %-organic in isocratic HPLC is equivalent to changing the steepness of the gradient in gradient elution. Several recent papers have reported significant changes in band spacing as a result of changing gradient steepness [11–13]. In addition to these examples where changes in solvent strength (or gradient steepness) have been deliberately used to vary selectivity, the literature shows numerous "accidental" examples where changes in solvent strength lead to changes in band spacing and reversals of elution order ([14, 15] for ion-pair HPLC, [16–20] for reversed-phase separation).

We will refer to the intentional use of changes in %-organic to alter band spacing as solvent-strength optimization (SSO). This paper explores this alternative to traditional solvent-type optimization for HPLC method development, and attempts to show when it is most promising. We also present a substantial data base – gathered from both the literature and our own laboratories – to substantiate our belief that SSO is a broadly applicable and often preferable means for carrying out HPLC method development. A few comparisons of the use of SSO vs solvent-type optimization are presented, as well as the combined use of solvent-strength plus solvent-type optimization. Finally, the most effective application of SSO and related procedures is favored by the use of computer simulation for minimizing experimental runs and presenting results in an easily interpreted format. We will illustrate this here, using commercially-available software (DryLab I from LC Resources).

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Experimental

HPLC Equipment and Conditions

Du Pont 8800 liquid chromatographs were used to collect the data reported here. Experimental details and a description of samples and materials have been given elsewhere [9]. For further information on various procedures and calculations, see Refs. [7, 21, 22].

Samples

The various hydrocarbon and nitro-compound samples were the gift of M. Rigney (Dept. of Chemistry, University of Minnesota). The steroid samples have been described previously [7]. The herbicide samples consisted of both common herbicides and proprietary compounds obtained from the Agricultural Chemicals Department of Du Pont (Wilmington, DE, USA); the exact chemical structures of these latter compounds were not disclosed in all cases.

Computer Simulations

DryLab I and DryLab G software (LC Resources, San Jose, CA, 95125, USA) was used with an IBM-XT personal computer for performing calculations related to solvent-strength optimization. Previously described software [23] was used for the solvent-type optimization studies reported here.

Precision of Measured S-values

Previous reports [12, 22] have shown that values of $S = d(\log k')/d\Phi$ can be measured from gradient elution runs with an accuracy of ± 5–10% (see Glossary of terms at end of paper). In the present paper we are primarily concerned with the determination of differences in S-values for adjacent bands in a typical sample, using DryLab I or DryLab G software (the algorithm for measuring S-values is the same in each program). The accuracy required in such S-value differences is ± 1–2%. The approach used here mimics what a chromatographer would do during method development based on computer simulations with this software; values of S were obtained from two experimental runs (different gradient times) for a total sample. Differences in these S-values will be more accurate than individual S-values, because of the cancellation of certain measurement errors [24, 25]. We attempted to assess the reliability of data reported here as follows.

A sample of 22 o-phthaldehyde derivatives of the common amino acids had been separated [26] using gradient elution with different gradient times (25cm, 5μm C18 column, acetonitrile/water gradients). Raw experimental data from these runs were kindly supplied to us by the authors. DryLab I was used to measure S-values for each compound, using two different run-pairs: 14 and 46min gradient times, and 11 and 27min gradient times. The agreement between S-value differences for the same adjacent band-pairs was equivalent to ± 0.8% in S (1σ).

In a second comparison, we determined values of S for 16 polyaromatic hydrocarbons as described here – using gradient times of 20 and 60min. This determination was

Table 1. Summary of S-value Measurements from the Literature Plus Data Reported Here. Average values of $(\delta S/\bar{S})$ are reported and are corrected for any correlation of S with solute retention (see text).

Compound type	Ref.	n*	Organic solvent**	Avg. $(\delta S/\bar{S})$	Comment ***
Alkyl benzenes	27	11	MeOH	±0.1%	correlates
Polyaromatic hydrocarbons	34	16	MeOH	±2 %	correlates
Polyaromatic hydrocarbons****	*****	16	ACN	±4 %	correlates
(Table V)		16	MeOH	±4 %	correlates
Chlorobenzenes	27	12	MeOH	±0.5%	correlates
Chlorotoluenes	27	9	MeOH	±0.4%	correlates
Chloronaphthalenes	27	16	MeOH	±1.9%	correlates
Chlorobiphenyls	27	65	MeOH	±1.4%	correlates
o-Phthaldehyde derivatives of amino acids	26	22	ACN	±1.5%	correlates
Miscellaneous substituted organics	30	31	MeOH	±6 %	correlates
			ACN	±14 %	
			THF	±12 %	correlates
Nitrocompounds	*****	10	MeOH	±9 %	correlates
(Table II)			ACN	±9	
			THF	±8	
Steroids	*****	6	MeOH	±5	
(Table III)			ACN	±4	correlates
			THF	±3	
	38	12	MeOH	±3	
			ACN	±5	
			THF	±3	
			PrOH	±4	
			MeEtOH	±5	
			MeEtOAc	±4	
	39	5	MeOH	±8	
			ACN	±2	
Herbicides	*****	15	MeOH	±10	
(Table IV)			ACN	±10	
			THF	±4	correlates
Herbicides	31				
phenylureas		10	MeOH	±2 %	correlates
		10	ACN	±5 %	
s-triazines		6	MeOH	±1 %	correlates
phenoxycarbonic acids		7	MeOH	±2 %	correlates
phenoxycarbonic esters		7	MeOH	±2 %	correlates
Peptides, proteins					
IL2-muteins	12	30	ACN	±7 %	correlates
nuclease muteins	40	13	ACN	±2 %	
lysozyme digest	11	17	ACN	±10	
myoglobin digest	11	–	ACN	±12	
Dansyl amides	41	18	MeOH	±3	correlates
			EtOH	±6	

* number of solutes

** MeOH, methanol; ACN, acetonitrile; THF, tetrahydrofuran; PrOH, propanol; MeEtOH, 2-methoxyethanol; MeEtOAc, 2-methoxy ethylacetate

*** correlates indicates dependence of S on sample retention; values of $(\delta S/\bar{S})$ are corrected for this dependence – that is, values of δS are measured vs the best-fit correlation curve; see text for discussion

**** a polymeric bonded phase was used in these studies; such columns give different selectivity vs monomeric phases

***** present study

Table II. Summary of retention parameters for reversed-phase separation of nitro-compound sample. Column, 25 X 0.46cm, 5μm Zorbax C8; mobile phase, 5–100% organic/water in 15 and 45 min, at 2ml/min; 35°C.

Compound	values of S*			values of log k_w		
	MeOH	ACN	THF	MeOH	ACN	THF
nitrobenzene	3.02	3.12	4.28	2.05	1.86	2.57
2,6-dinitro-toluene	3.62	3.65	4.70	2.51	2.28	2.34
benzene	2.72	2.87	3.91	2.03	1.85	2.81
2-nitrotoluene	3.51	3.37	4.71**	2.58	2.19	2.33**
4-nitrotoluene	3.50	3.35	4.71**	2.60	2.20	2.33**
3-nitrotoluene	3.51	3.35	4.68	2.64	2.22	2.35
toluene	3.34	3.12	4.99	2.67	2.21	2.20
2-nitro-1,3-xylene	4.03	3.49**	4.82	3.09	2.46**	2.28
4-nitro-1,3-xylene	3.85	3.49**	4.40	3.02	2.46**	2.50
1,3-xylene	3.79	3.21	4.62	3.18	2.47	2.38

* for different organic solvents; MeOH, methanol; ACN, acetonitrile; THF, tetrahydrofuran; averages for duplicate determinations

** approximate values because two bands are overlapped in each gradient run

repeated with a different column, and it was found that S-value differences agreed between the two determinations within ± 1.2% (1σ).

In a final comparison, four repeat gradients each (8 runs) were carried out (15 and 45min, 5–100% methanol/water) for the nitro-compound sample of Table II. The repeatability of values of S and differences in S (adjacent bands) was as follows: S, ± 4% (1σ); δS, ± 1.2% (1σ). We have also previously shown [9] that resolution as a function of mobile-phase %-organic (which depends on these differences in S) can be accurately and reproducibly predicted when appropriate procedures are followed.

Background and Theory

An example of changes in band spacing that result from a change in solvent strength is shown in Fig. 1, for a 10-component mixture of some aromatic compounds and their nitrated derivatives. All 10 bands are resolved in the chromatogram of Fig. 1a, for 30% methanol/water as mobile phase, but the run time is an unacceptable 160min. Upon changing to 50% methanol/water, bands #2 and 3 coalesce (arrow), and bands #8 and 9 reverse positions (arrows). With 70% methanol/water bands #8 and 9 have moved further apart, band #6 has merged with #8 (arrow), and bands #3 and 4 have also combined (arrow). The most practical separation of this sample occurs for 55% methanol/water (Fig. 2), where adequate resolution (R_S = 1.3) is achieved within a reasonable run time (17min).

The effect of solvent strength on band spacing is further illustrated in the hypothetical example of Fig. 3. Here log k′ is plotted vs the volume-fraction Φ of organic in the mobile phase. This example assumes the relationship

$$\log k' = \log k_w - S\Phi , \qquad (1)$$

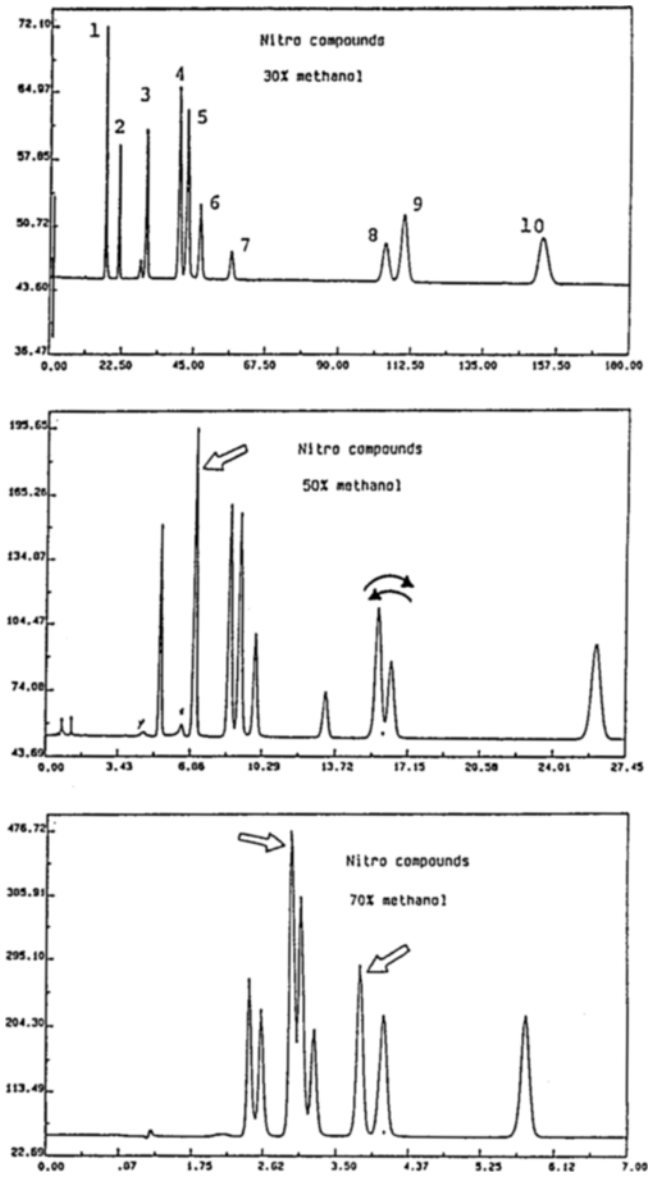


Fig. 1

Isocratic separation of nitro-compound sample. Conditions as in Table II except for mobile-phase composition. (a) 30% methanol/water; (b) 50% methanol/water; (c) 70% methanol/water.

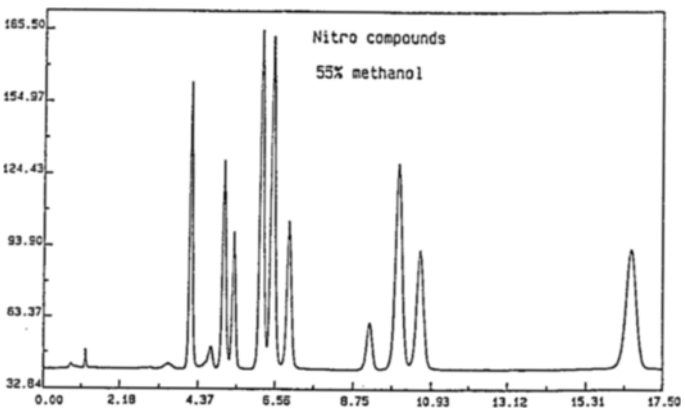


Fig. 2

Best isocratic separation of nitro-compound sample. Conditions as in Table II except mobile phase is 55% methanol/water.

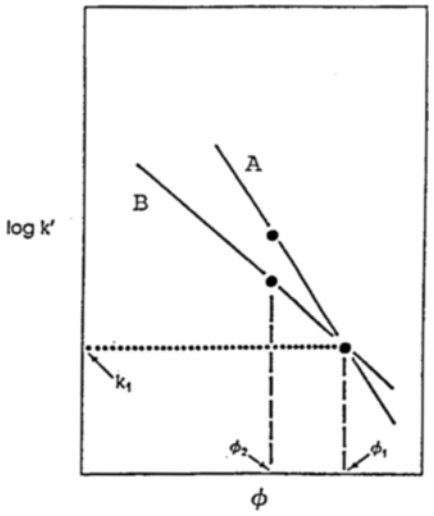


Fig. 3

Theoretical basis of solvent-strength optimization. Sample composed of compounds A and B; see text for details.

where k' refers to the solute capacity factor for a mobile phase of composition Φ, k_w is the (extrapolated) value of k' for water as mobile phase ($\Phi = 0$), and S is the slope of plots as in Fig. 3. A large number of experimental studies have documented the approximate validity of eq. 1, several of which are referred to in the subsequent discussion [27]. Values of S are approximately constant as Φ is varied, but each compound will have a characteristic value of S; values of S may also differ for changes in the organic solvent and column.

Resolution vs Solvent Strength as a Function of the Sample

For a mobile phase of composition $\Phi = \Phi_1$, compounds A and B of Fig. 3 co-elute with no separation. Changing Φ to a value Φ_2, however, results in different values of k' for each compound. That is, a change in solvent strength (Φ) leads to a change in the separation factor α. These changes in band spacing require that the S-values for the two compounds differ. It is therefore of interest to know the relative probability of such differences in S; this is one goal of the present paper.

It is not sufficient that values of S differ for two compounds; this difference must be large enough so that a change in Φ will result in their resolution. The required difference in S can be derived as in the Appendix:

$$(\delta S/\bar{S}) = (8/2.3)\,\Delta R_s\, N^{-0.5} \left[(1+k')/k'\right]/\left[\log(20\, k_a/k_z)\right] . \qquad (2)$$

Here δS is the difference in S-values for two adjacent bands, $\bar{S}$ is their average S-value, ΔR_s refers to a necessary increase in resolution R_s, k_a refers to k' for the first-eluted band and k_z equals k' for the last band. Eq. 2 provides insight into the use of SSO for HPLC method development. First, we see that it is not the absolute difference in solute S-values that is important, but their relative difference ($\delta S/\bar{S}$ rather than δS).

Second, the applicability of SSO is limited by the retention range of the sample (k_a/k_z). Acceptable solvent strengths (values of Φ) will position all sample bands so that $1 < k' < 20$. When the ratio of k'-values for the last band vs the first (k_z/k_a) approaches a value of 20, the choice of Φ becomes fixed by the need to avoid $k' < 1$ or $k' > 20$; the required value of $\delta S/S$ therefore becomes large. Consequently, for this case Φ cannot be varied to improve band spacing (SSO). SSO becomes more useful as the retention range of the sample becomes smaller, because then a larger range of Φ-values can be explored. This limitation does not apply to gradient elution, which suggests the use of gradient elution as an alternative to isocratic separation in solvent-strength optimization, for samples with a wide spread in k'-values. For gradient elution, eq. 2 applies with $k_z/k_a = 1$.

As an example of the application of eq. 2, assume an improvement in resolution such that $\Delta R_s = 1.0$ is required. This might be the case for two bands that are initially totally overlapped ($R_s = 0$). Further assume a value of N = 10,000, $k' = 5$ (average case), and $k_z/k_a = 10$. Then, from eq. 10 $(\delta S/S) = 0.14$. That is, about a 14% difference in S-values will be required for the resolution of the two bands using SSO.

The latter example is somewhat pessimistic, however. First, k_z/k_a is often less than 10, and for gradient elution (discussed later) it equals about one for every sample. Second, the initial band-pair will rarely have $R_s = 0$; in general, R_s will lie between 0 and .6 for a pair of apparently overlapping bands. In many cases, an increase in R_s of $\Delta R_s = 0.5$ will make the difference between poor and adequate resolution. Inserting the latter values into eq. 10 gives a (best case) value of $(\delta S/\bar{S}) = 0.02$. So if SSO is to prove useful, the required difference in S-values for two bands will vary from about 2 to 15%, depending on the particular situation. This means that $(\delta S/\bar{S})$ values of as little as 2% can be useful in some cases, while for values of $(\delta S/\bar{S}) = 5\%$ the sample can usually be separated by means of solvent-strength optimization.

S as a Function of Solute Structure

The first detailed discussion of S as a function of solute structure was that of Geng and Regnier [28, 29], who showed that values of S can vary widely with the structure of the solute molecule; e.g., $2 < S < 150$. These authors postulated a displacement model for reversed-phase retention, in which a sorbing solute molecule releases some number Z previously retained solute molecules from the stationary phase. Z is proportional to the contact area of the solute molecule, and is therefore often proportional to solute molecular weight. It can be shown [12] that S and Z are related as

$$S = 2.3\Phi Z , \qquad (3)$$

where Φ refers to an average value (Φ) during the elution of the sample from the column. We have seen (eq. 1) that relative rather than absolute values of S are of interest in SSO: we can therefore (approximately) equate values of S and Z in the following discussion.

S vs solute molecular weight and retention. The preceding discussion suggests that values of S will increase regularly with the size of the solute molecule, and this is generally observed [27–29]. Additionally it has been found in several (but not all) studies that S tends to increase with solute retention [27, 30, 31]. Usually this relationship is reported in the form

$$S = a\,(\log k_w) + b\,, \qquad (4)$$

where a and b are constants for a given HPLC system. Since there is a general trend of increasing retention in reversed-phase HPLC as solute molecular size increases, an increase in S with retention is not unexpected. However other factors also affect solute retention, which explains the frequent exceptions to this relationship. One study [30] found that S correlated strongly with solute retention for methanol/water as mobile phase but not for acetonitrile/water; other workers [31] have found (for different solutes) comparable correlations of S with retention for both organic solvents. A previous discussion [9] has shown that changes in S (δS) that correlate with retention are not very useful for carrying out SSO. To be of value in assessing the general applicability of SSO, values of ($\delta S/\bar{S}$) must be corrected (reduced) for any dependence on solute retention [4]. Values of ($\delta S/\bar{S}$) reported here are so corrected (see discussion of Fig. 4).

S vs solute functionality. Values of S for solutes of similar size and/or retention are observed to vary somewhat with the nature (and number) of polar substituent groups on the solute molecule. Thus one study [9] showed S increasing for the following compound types (benzene derivatives):

anilines < alkyl benzenes < chlorobenzenes < ethers < aldehydes, ketones < nitriles < unsubstituted polyaromatics < nitro compounds < phthalates < phenyl alkanols.

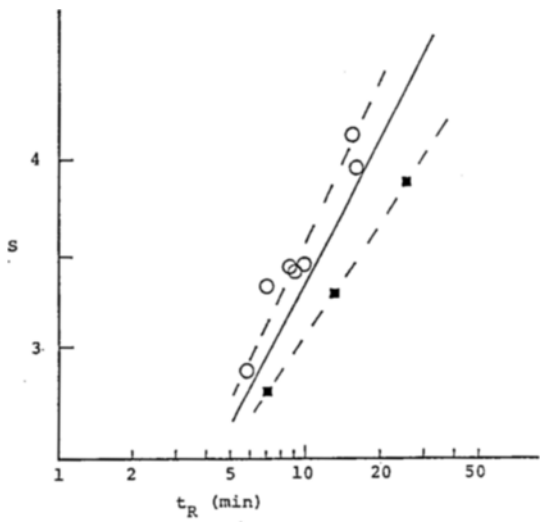


Fig. 4

Correlation of solute S-values with retention for nitro-compound sample of Table II; methanol as organic solvent. ○ = nitro-compounds; ■ = parent hydrocarbons.

When these changes in S are corrected for the contact area of the molecule, it appears that there is a tendency for more polar molecules to exhibit smaller values of S, other factors equal. Likewise another study [31] showed different values of S for herbicides of different functionality, after correcting for the dependence of S on retention.

S vs solute molecular shape and conformation. If the displacement model for reversed-phase retention [28, 29] is correct, S should be determined by the contact area required for adsorption of the solute molecule. Therefore S should vary with molecular shape and conformation. By analogy with related effects that determine the temperature-dependence of retention in reversed-phase HPLC, it might also be expected that "more compact" molecules will exhibit differences in S that arise from corresponding changes in entropy upon retention [32].

Consider first the shape of small, nonpolar solute molecules, specifically aliphatic and aromatic hydrocarbons. The molecular shape of these compounds can be characterized as either "extended" or "compact". Generally it is found (other factors equal) that compact molecules have smaller S-values. One study [33] shows this for the reversed-phase separation of several hydrocarbons with methanol/water mobile-phases: cyclo-octane and hexamethylbenzene (compact) have smaller S-values than extended molecules such as p-terphenyl, n-octane, various primary alkynes, phenylhexane and 1,4-diphenyl-butane. As a result, these compact molecules become more retained vs extended molecules, as the %-organic in the mobile phase increases.

Another study reports S-values for various fused-ring aromatic hydrocarbons with methanol/water as mobile phase [34]. It can be shown for these data that S-values correlate with sample retention (as in other studies):

$$S = 1.109\,(\log k_w) - 0.0265\,(\log k_w)^2\,. \qquad (5)$$

The quantity k_w refers to the value of k' for water as mobile phase. After correcting for the dependence of S on sample retention (eq. 5), it is found that "extended" benzologs such as naphthalene, phenanthrene, chrysene, etc. have larger S-values by an average of 0.8%, while more highly condensed ("compact") structures such as acenanphthylene, pyrene and perylene have smaller S-values by an average of 0.8%. These S-value differences are small, but we will see in a later section that they can play a significant role in solvent-strength optimization.

The conformation of large molecules such as synthetic polymers and proteins would be expected to have an important effect on values of S. Thus native proteins in aqueous mobile phases, or synthetic polymers in "poor" solvents, will have a compact, globular conformation and smaller contact areas. Denatured proteins or synthetic polymers that are well solvated by the mobile phase will have more extended conformations and larger contact areas. Therefore we expect values of S to be smaller (for the same compound) in the former case (compact conformation) vs the latter (extended, random-coil conformation). Likewise the dependence of S on solute molecular weight should vary for these two cases. Compounds having an extended conformation should show S increasing in propor-

tion to solute molecular weight. Compounds with a more compact conformation should show S increasing with some fractional power of molecular weight (e.g., roughly 2/3 power for spherical shapes, such as native, globular proteins).

In agreement with this picture, it was found for several proteins that Z (and S) increase in proportion to solute molecular weight, for a mobile phase (propanol/formic acid/water) that is highly denaturing [28]. For less denaturing mobile phases (acetonitrile/water) and other proteins, values of S were found to increase approximately as the half-power of molecular weight [35], suggesting some retention of 3-dimensional structure in this case. Likewise S-values for polystyrenes of varying molecular weight [36] show a one-half-power dependence on molecular weight in the poor-solvent system THF/water. Finally, another study found 3-fold differences in S for proteins of the same molecular weight (interleukin-2 muteins), and showed that higher S-values appear to correlate with easier (more complete) denaturation of the protein.

Values of S from the Literature

A review of the literature reveals a number of studies that describe the measurement of S-values for various compounds in the usual reversed-phase systems. Several such compilations have been summarized and extended by Johnson et al. [37] and by Opperhuizen et al. [27]. To be useful for the purpose of inferring values of $(\delta S/\bar{S})$ for different sample types, it is necessary that special care be taken in the measurement of these data (see discussion of Experimental section). Many of these studies involve compounds of similar structure – generally less-polar hydrocarbons and/or simple mono-functional derivatives based on common functional groups (-NO_2, -OH, -CHO, etc.). Table I summarizes some results from selected studies. Since the systems of Table I cover a wide range in solute structure and solvent type, and include data for about 600 individual S-values, these results can be used to draw general conclusions about the variability of S and the prospects for solvent-strength optimization. In many cases (Table I) it is apparent that the average values of $\delta S/\bar{S}$ is large enough to make SSO quite promising. In other examples (e.g., certain of the herbicides of ref. [31]), the sample appears to exhibit neglible changes in band spacing as Φ is varied.

Results and Discussion

Values of S were measured for several groups of related compounds (nitro-compounds, steroids, herbicides, polyaromatic hydrocarbons), using three different solvent systems (methanol, acetonitrile or THF plus water). These studies were expected to answer some questions concerning solvent-strength optimization, and are summarized in Table I.

Nitro-Compounds

Gradient elution runs were used to derive values of S for the compounds of Table II, using the DryLab I program based on the treatment of Ref. [22] and further described in Ref. [9]. Resulting values of S are tabulated in Table II for each solvent system: methanol, acetonitrile and THF. Significant band-to-band variations in S are noted, as illustrated in Fig. 4 where S for each compound is plotted vs isocratic retention time (50% methanol/water, predicted from DryLab I). Deviations of the data-points in Fig. 4 from the best straight line (solid curve) are an indication of potential changes in band-spacing during SSO. The average deviation of all points equals 0.22 units, which is equivalent to 6% in S[1]. This suggests that SSO should be promising for this sample. We note also in Fig. 4 that individual compound classes (aromatic hydrocarbons, nitrobenzene derivatives) show less deviation of S-values from the best curves (dashed lines) through these data. However variations in S for the nitrobenzene derivatives are still large enough to permit some band-spacing changes using SSO. Fig. 4 also shows a clear correlation between S and solute retention for this system.

Values of S for the nitro-compound sample show a somewhat similar dependence on solute structure for each of the three solvent-systems. This is seen in Fig. 5, where values of S for acetonitrile (a) and tetrahydrofuran (b) as organic solvent are plotted vs corresponding values for methanol. Values of S for the three nitrotoluene isomers of Table I are identical within experimental error for all three solvents. This suggests that SSO will be less advantageous for compounds of closely similar structure. However the differences in S for the two nitroxylene isomers (bands #8 and 9 of Fig. 1a) are large enough to allow an SSO procedure for their resolution. This can be seen in the examples (previously discussed) of Fig. 1.

Steroids

Values of S were obtained for the 6 steroids of Table III, for each of the three organic solvents (same procedure as for Table I). There is no significant correlation of S with retention for these compounds, except with acetonitrile as solvent (the latter data show S decreasing with increasing retention). The band-to-band variations in S for these steroids (δS) are somewhat smaller than for the nitrocompounds: 0.15 to 0.19 units for the different solvents, or an average $(\delta S/\bar{S}) = 4\%$. Nevertheless we will see that these small differences in S can play a significant role in SSO. The structures of these six compounds are fairly similar, differing only in one or two hydroxyl or carbonyl groups in the steroid ring-system. There is little correlation of S-values between the various solvent systems.

Herbicides

Values of S were obtained for the 15 herbicides of Table IV, for each of the three organic solvents (same procedure as in Table I). There is no significant correlation of S with retention for methanol or acetonitrile, but S tends to in-

1) We can also estimate the average difference in values of S from the band-to-band values, equal to ± 0.33 units (uncorrected for the correlation of S with retention). However the latter value overstates the significance of variations in S for this sample, so far as solvent-strength optimization is concerned.

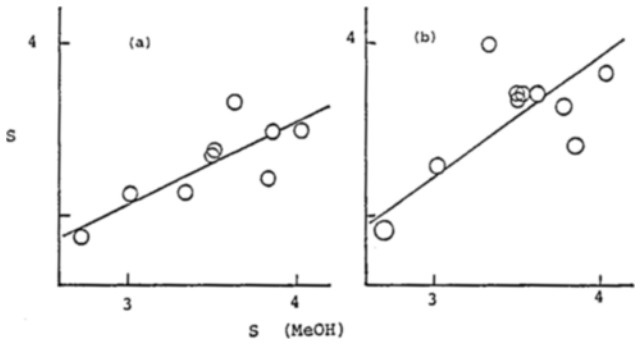


Fig. 5

Correlation of solute S-values for nitro-compound sample; (a) S-values for acetonitrile vs methanol; (b) S-values for THF vs methanol; data of Table II.

Table III. Summary of retention parameters for reversed-phase separation of steroid sample. Column, 25 X 46cm, 5μm Zorbax C8; mobile phase, 5–100% organic/water in 15 and 45min, at 2ml/min; 35°C.

Compound	values of S*			values of log k_w		
	MeOH	ACN	THF	MeOH	ACN	THF
prednisone	5.24	6.30**	6.10	3.17	2.41**	1.98
cortisone	5.22	6.19	5.93	3.20	2.43	2.00
hydrocortisone	4.88	6.30**	6.15	3.13	2.41**	2.14
dexamethasone	5.15	5.95	6.39	3.51	2.59	2.52
corticosterone	4.83	5.24	6.20	3.40	2.51	2.32
cortexolone	5.03	5.27	6.11	3.57	2.57	2.40

* for different organic solvents, as in Table II.

** approximate values because two bands are overlapped in each gradient run

Table IV. Summary of retention parameters for reversed-phase separation of herbicide sample. Column, 15 X 0.46cm, 5μm Zorbax C8; mobile phase, 10–80% organic/water in 15 and 30min, at 1.5ml/min; 40°C.

Compound*	values of S**			values of log k_w		
	MeOH	ACN	THF	MeOH	ACN	THF
N-methyl benzyl amine	4.35	***	***	1.38	***	***
A	3.64	***	***	1.25	***	***
B	3.29	***	***	1.21	***	***
C	3.75	***	***	1.48	***	***
D	4.02	4.03	3.60	2.13	1.93	1.51
E	3.30	3.53	3.43	2.28	1.94	1.79
F	4.65	4.61	4.58	2.89	2.30	1.95
G	4.39	4.35	4.90	2.84	2.29	2.11
benzaniline	3.76	3.74	4.81	2.61	2.30	2.43
H	4.38	4.21	4.81	2.88	2.30	2.00
phenyl sulfone	4.18	3.71	4.84	2.86	2.42	2.29
I	4.37	4.36	4.89	2.95	2.39	2.27
J	3.85	4.11	5.02	2.71	2.22	1.78
K	3.88	3.67	5.43	3.09	2.56	2.81
L	–	3.55	5.51	–	3.11	3.54

* These are in most cases proprietary compounds (see Experimental section)

** For different organic solvents, as in Table II.

*** Compounds elute too early in the gradient for reliable S-value determinations

Table V. Summary of retention parameters for reversed-phase separation of polyaromatic-hydrocarbon sample. Column, 15 X 0.46cm, 5μm Supelcosil LC-PAH; mobile phase 5–100% organic/water in 20 and 60min, at 2ml/min; 35°C.

Compound	values of S*		values of log k_w	
	MeOH	ACN	MeOH	ACN
naphthalene	4.07	5.33	3.03	2.60
acenaphthylene	4.39	5.51	3.36	2.84
acenaphthene	4.33	5.19	3.57	2.94
fluorene	4.46	5.40	3.74	2.80
phenanthrene	4.65	5.37	3.95	3.17
anthracene	4.61	5.19	4.05	3.21
fluoranthene	4.73	5.15	4.25	3.31
pyrene	4.64	4.89	4.25	3.25
benz(a)anthracene	4.85	4.78	4.74	3.50
chrysene	4.70	4.60	4.69	3.44
benzo(b)fluoranthene	4.82	4.42	4.97	3.55
benzo(k)fluoranthene	4.73	4.27	5.01	3.57
benzo(a)pyrene	4.56	4.04	4.91	3.49
dibenz(a,h)anthracene	4.44	4.20	4.96	3.77
benzo(g,h,i)perylene	4.69	3.92	5.24	3.59
indeno(1,2,3-cd)pyrene	4.29	3.64	4.94	3.73

* for different organic solvents, as in Talbe II

crease with retention for THF. The band-to-band values of $(\delta S/\bar{S})$ average about 10% for methanol and acetonitrile, and 4% for THF. Therefore there are excellent prospects for the use of SSO to achieve resolution of various mixtures of these compounds, especially for methanol and acetonitrile. Values of S show a rough correlation between methanol and THF.

Polyaromatic Hydrocarbons

The 16 compounds of Table V were separated on a polymeric alkyl-phase column (Supelcosil LC-PAH) using 5–100% gradients of both acetonitrile/water and methanol/water. Values of S and log k_w for each compound and organic solvent are listed in Table V. There is a moderate correlation of values of S with retention (ACN data); interestingly, S decreases with increasing retention and molecular weight (the opposite of the behavior of these compounds on monomeric alkyl-phase columns; [34]). Separations of this sample using THF/water gradients gave badly bunched chromatograms with much poorer resolution than for the other two organic solvents. No attempt was made to derive values of S from these latter runs (which would have been difficult).

Solvent-Strength Optimization for Achieving the Separation of the Samples of Tables II–V

The compounds of Tables II–V allow us to examine the practical application of SSO to typical samples composed of mixtures of related compounds. We have used the entire compound-set of Tables II or III, since each of these samples can be separated isocratically. We have divided the compounds of Table IV into two 6-component samples, since the retention range of this compound-set is somewhat wide for the isocratic separation of the entire mixture: group #1, compounds D, E, F, G, benzaniline and H;

group 2, compounds G, H, phenyl sulfone, I, J and K. We have also examined SSO for the entire sample of Tables IV and V, using gradient elution for the final procedure. This is described in an accompanying paper [21].

Acetonitrile/water mobile phases. It is generally recommended when using SSO for the development of an HPLC method to begin with acetonitrile as organic solvent. The UV-transparency of this solvent extends to lower wavelengths, and acetonitrile/water mobile phases are less viscous (lower column pressures). Two experimental gradient runs were carried out initially (with two different values of t_G, as in Tables II–V). The resulting retention data were entered into the DryLab I program, allowing trial-and-error computer simulations to be carried out in place of further experimental runs. A more efficient use of the DryLab I software, however, is to (a) request the k'-range as a function of %-organic, then (b) ask for a relative resolution map (RRM) over the %-organic range that corresponds to $1 < k' < 20$ for the sample in question (a relative-resolution map is a plot of minimum or worst resolution as a function of %-organic in the mobile phase, for a column of specified plate-number). This procedure was followed in the present case for those samples that could be separated isocratically (see Ref. [21] for a similar discussion of the gradient separation of the herbicide and polyaromatic hydrocarbon samples).

In the case of the nitro-compound sample (Table II, ACN column), it was found that the same two compounds (#8, 9) overlapped in each experimental gradient run. This was also observed for bands #1 and 3 of the steroid sample (Table III, ACN column). This means that SSO cannot provide the resolution of these two samples, using acetonitrile/water mixtures as mobile phase. This was not the case for the two herbicide samples (Table IV) or the polyaromatic hydrocarbon sample (PAH, Table V). The RRM's for the two herbicide samples are shown in Fig. 6. Here it is seen (for a 10,000-plate column) that a resolution of $R_s > 2$ can be achieved for each sample, using an appropriate value of %-acetonitrile in the mobile phase. "Best" values of %-organic for each sample are summarized in Table VI, along with other pertinent information.

Combination of SSO with solvent-type optimization. When SSO is found not to work with one organic solvent (acetonitrile in the above examples), two additional gradient runs can be obtained with a second organic solvent (methanol or THF), and SSO can be repeated using the DryLab I software. Likewise, if it is found that separation is possible with the first organic solvent, but greater resolution is desired, SSO can also be repeated with a second organic solvent. This approach was carried out for the four "isocratic" samples of Tables II–IV (methanol as organic solvent), with the resulting resolution maps shown in Fig. 7. It is seen that separation can be achieved for each of these four samples. It was also found that methanol provides better resolution for the PAH sample, compared with acetonitrile.

Best values of %-methanol for each sample are summarized in Table VI as before. For the two herbicide samples, we see that the maximum resolution attained with acetontrile as organic solvent (Figs. 6a, b) is greater than with methanol as solvent (Figs. 7c, d). Therefore there is no advantage to be gained by using methanol instead of acetonitrile. In the case of the nitro-compound and steroid samples, a maximum resolution of $R_s > 1.5$ is possible for each sample with methanol (but not acetonitrile) as organic solvent.

The latter approach was continued, using THF as organic solvent. The nitro-compound sample was found to be inseparable by SSO with this solvent (the same two compounds – #4 and 5 – overlap in each experimental gradient run), but RRM's for the remaining three samples are shown

Table VI. Summary of conditions for optimum separation of the samples of Tables II–IV using different organic solvents.

Solvent	Sample	Optimum %-organic	Minimum R_s	k'-range
ACN	nitro	–	0.0	–
	steroid	–	0.0	–
	herb #1	27	3.1	7–20
	herb #2	35	1.8	6–19
MeOH	nitro	55	1.3	2–13
	steroid	50	2.2	3–11
	herb #1	48*	1.5	2–6
	herb #2	66%	0.8	1–3
THF	nitro	–	0.0	–
	steroids	24	2.9	3–10
	herb #1	34	1.1	2–6
	herb #2	32	1.6	1–12

* other %-organic values give higher R_s, but k'-values are not in the range of 1–20

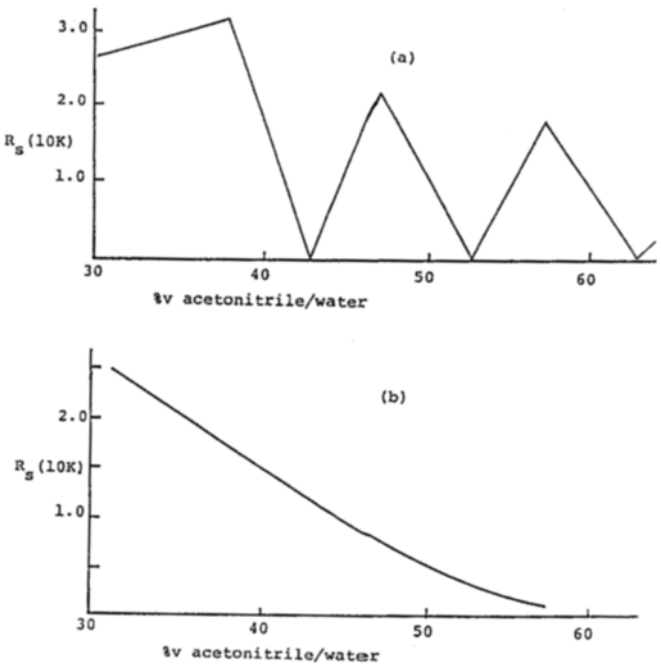


Fig. 6

Relative resolution maps (minimum resolution for 10,000-plate column) for acetonitrile/water mobile phases and isocratic separation. (a) herbicide sample #1; (b) herbicide sample #2.

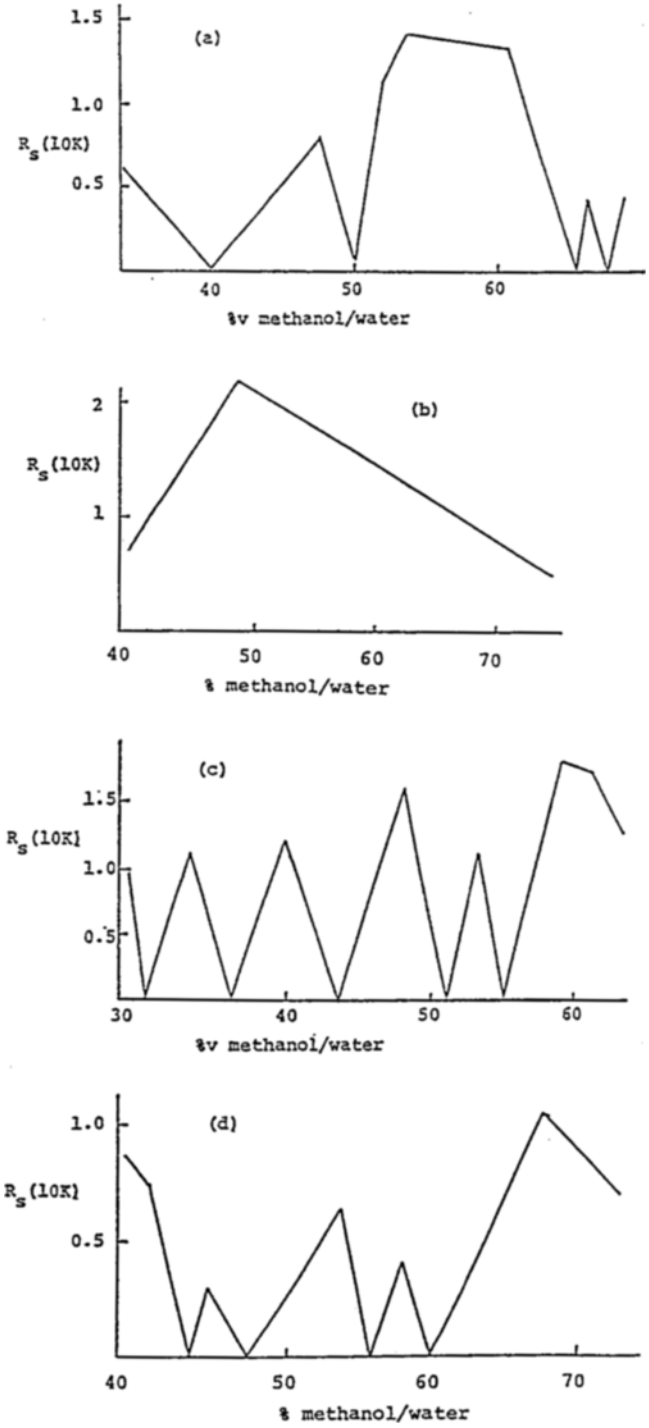


Fig. 7

Relative resolution maps for methanol/water mobile phases and isocratic separation. (a) nitro-compound sample; (b), steroid sample; (c) herbicide sample #1; (d) herbicide sample #2.

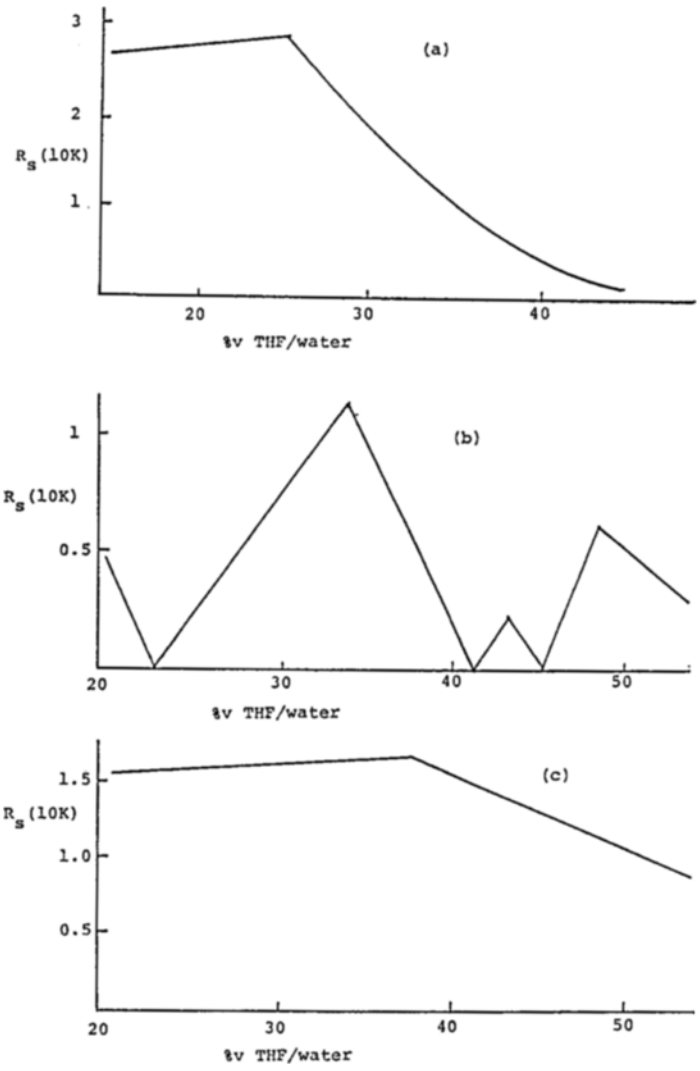


Fig. 8

Relative resolution maps for THF/water mobile phases and isocratic separation. (a) steroid sample; (b) herbicide sample #1; (c) herbicide sample #2.

in Fig. 8. The maximum resolution for the steroid sample (Fig. 8a) exceeds that obtainable using methanol as organic solvent (Fig. 7b), but the maximum resolution for each herbicide sample (Figs. 8b, c) is less than can be obtained with acetonitrile as organic solvent (Figs. 6a, b). The conditions for maximum sample resolution with THF as organic solvent are summarized in Table VI.

Experimental isocratic separations. The information of Table VI for each sample was next used to predict optimum isocratic separation, and some of these runs were confirmed experimentally, as shown in Fig. 2 and 9. Fig. 2 shows the best separation of the nitro-compound sample, with 55% methanol/water. Figs. 9a, b show the optimized isocratic separations of the steroid sample, using either methanol or THF respectively. The THF-separation (Fig. 9b) is clearly better, as predicted by the resolution maps of Fig. 7b and 8a. These results could have been further improved by optimizing column length, particle size and flowrate, however we chose not to do this.

Comparisons of Solvent-Strength Optimization (SSO) vs Solvent-Type Optimization

The present examples have demonstrated the potential power of SSO. It is possible to combine the advantages of SSO and solvent-type optimization in various ways, and to compare the results with either individual approach. The

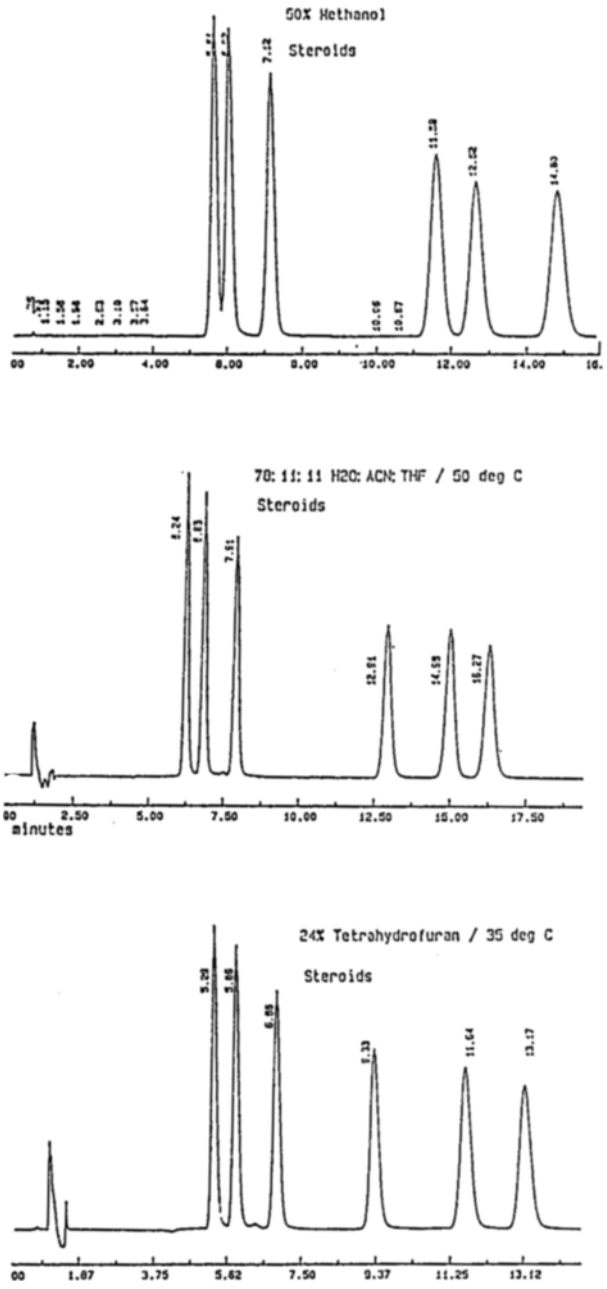


Fig. 9

Optimized isocratic separations of steroid sample. Conditions of Table III except as noted. (a) 50% methanol/water; (b) 24%/THF water; (c) 11% acetonitrile plus 12% THF/water (solvent optimization result).

simplest procedure, in our opinion, is to use SSO as illustrated in the examples of Fig. 6–8. This means starting with acetonitrile as organic solvent, carrying out SSO with that solvent, and then continuing with other solvents (repeating SSO each time) if previous separations are inadequate. This procedure works for each of the samples of Tables II–V (shown here or in Ref. [21]).

An alternative is to use solvent-type optimization from the start, as described by Glajch et al. [5]. We carried out this approach for the nitro-compound and steroid samples of Tables II and III. For the nitro-compound sample, solvent-type optimization showed that methanol/water was the optimum mobile phase, giving the same separation as shown in Fig. 2. Solvent-type optimization in the case of the steroid sample provided the separation of Fig. 9c. This is seen to be similar to that of Fig. 9b (24% THF, from SSO), and the mobile phase is also somewhat similar (11% acetonitrile plus 12% THF). Thus in these two cases, SSO gives essentially the same results as from solvent-type optimization. However, the number of experimental runs for SSO will generally be less. We do not expect that SSO will always provide comparable resolution vs solvent-type optimization. For more complex samples, having a larger number of components (e.g., 10 or more compounds), full solvent-type optimization (mapping of all methanol/acetonitrile/THF mixtures) will probably be required in most cases.

Solvent-type optimization can be combined with SSO in other ways, as illustrated by the approach of Smilde et al. [39]. These workers use a scheme based on the optimization of mixtures of methanol, acetonitrile and water – where solvent strength (and run time) is allowed to vary. We have compared their separation of a 5-component steroid sample by this procedure with SSO, using data reported by them [39]. Their predicted optimum separation has a resolution of $R_s = 2.4$ (vs 2.3 experimental), based on a mobile phase of 4% acetonitrile plus 67% methanol. Using SSO with methanol as solvent, we predict a resolution of $R_s = 2.2$, in about the same run time. Thus again SSO appears comparable to various alternatives based on solvent-type optimization, when the number of compounds in the sample is not too large.

Conclusions

A number of conclusions can be drawn from the present study. First, it appears that solvent-strength optimization (SSO) is a generally viable option for initial HPLC method development under reversed-phase conditions. This approach to HPLC method development seeks to optimize the spacing of bands within the chromatogram by varying the %-organic in the mobile phase.

The success of SSO depends on differences in the S-values ($S = d[\log k']/d\Phi$) of adjacent solute bands within the chromatogram. If a change in solvent strength (% organic in the mobile phase) is to lead to the resolution of an initially-overlapped bandpair, the S-values of the two bands must differ by at least 2% (most favorable case), but preferably by 5% or more. The present study (47 solutes of widely varying structure, 3 mobile-phase systems) shows an average difference in S between adjacent bands of 6% for samples of "related" compounds; i.e., typical samples for HPLC separation. Other studies involving a wide range of sample types (Table I) show comparable variability in S. We therefore conclude that there is a general tendency toward variation in S for adjacent chromatographic bands, sufficient to make SSO a broadly useful technique. However some samples show negligible variations in S and therefore require another method-development approach.

Second, the applicability of SSO depends on the retention range of the sample. SSO for isocratic HPLC methods will be most promising for samples whose components have generally similar k′-values; e.g., a k′-range of 2 to 4-fold. Samples with a k′-range of 10–20-fold will be less likely to undergo substantial changes in band spacing for allowable changes in solvent strength, because the possible change in %-organic will be less, if all sample bands are to elute with $1 < k' < 20$. However, SSO can be applied to samples of wider retention range if gradient elution is used for the final method. This also applies to samples where $1 < k' < 20$ for all sample bands; that is, such samples are normally separated isocratically, but their separation by gradient elution allows the more effective use of SSO.

Third, the present study provides further insight into the effect of solute molecular structure on S. It appears that S varies in a major way with differences in solute molecular weight, functionality (or polarity), and conformation (in the case of peptides and proteins). Smaller differences in S can arise from subtle variations in the shape of the solute molecule, as illustrated here by various polycyclic aromatic hydrocarbons. Small but sometimes significant differences in S are found also for isomeric compounds (e.g., nitroxylenes of Table II). Finally, S-values for a particular solute vary significantly from one solvent system to another; e.g., methanol, acetonitrile or THF as organic solvent in reversed-phase mobile phases. Little useful variation in values of S was observed for non-polar compounds of closely related structure (e.g., alkyl or chlorobenzenes, Table I).

Fourth, it is recommended that the SSO approach to HPLC method development be started with acetonitrile/water as mobile phase. However if SSO is unsuccessful with acetonitrile as organic solvent, the same procedure can be repeated with either methanol or THF. In the present study several different samples were used to evaluate this approach. Initial method development using SSO with acetonitrile resulted in the successful separation of two herbicide samples, but was unsuccessful for the case of a nitro-compound sample and a steroid mixture. Repeating SSO with methanol as solvent gave good separations of the steroid and nitro-compound mixtures, but did not improve the herbicide separations obtained with acetonitrile. Finally, the use of SSO with THF improved the steroid separation, but did not enhance the separation of the other three samples.

In the latter example it was found that acetonitrile was best for two samples, methanol was best for one sample, and THF was best for the remaining sample. This general approach to HPLC method development (repeated use of SSO with different organic solvents) represents a combination of SSO and a simplified form of solvent-type optimization as introduced by Glajch, Kirkland et al. [4]. A comparison of SSO with solvent-type optimization for two samples did not show a significantly better separation using either approach. However solvent-type optimization is expected to be preferable for more complex samples (containing more than 10 components), but the number of experimental runs will be larger.

Finally, the use of SSO for HPLC method development is considerably enhanced by the use of commercially available computer programs for both isocratic (DryLab I) and gradient (DryLab G) procedures. Some illustrations of this are described here.

Glossary of Terms

a, b	constants (eq. 12)
F	mobile-phase flowrate (ml/min)
k'	capacity factor (Ref. [7])
k_a, k_z	k′-values for first and last bands in chromatogram
k_w	value of k' for water as mobile phase
k_{wa}, k_{wb}	values of k_w for solutes A and B
k_1, k_2	k′-values for solute in mobile phases with $\Phi \approx \Phi_1$ and Φ_2 (Fig. 3)
N	column plate number (Ref. [7])
P	see eq. i-4
R_s	resolution (Ref. [7])
S	$d(\log k')/d\Phi$ (eq. 1)
$\bar{S}$	average value of S for two adjacent bands
S_a, S_b	value of S for compounds A and B
SSO	solvent-strength optimization
t_G	gradient time (min)
Z	number of organic-solvent molecules displaced by solute molecule upon retention (eq. 11)
α	separation factor (Ref. [7])
α_1, α_2	α-values in mobile phases 1 and 2 for bands A and B; $(\alpha = k_b/k_a)$
δS	difference in S-values for two adjacent bands A and B
ΔR_s	some required increase in R_s, to achieve adequate final resolution of adjacent bands A and B
Φ	volume-fraction of organic solvent in mobile phase
Φ_1, Φ_2	values of Φ for mobile phases 1 and 2 (Fig. 3)

Appendix. Derivation of eq. 2.

We will assume solutes A and B plus mobile phases 1 and 2 as in Fig. 3. Let S_a and S_b refer to S-values for solutes A and B, with subscripts a, b, 1, 2 referring to solutes A and B and mobile phases 1 and 2, respectively. Values of α can be defined for each mobile phase, equal to (k_b/k_a), where k_a and k_b refer to k' for A and B, respectively. Then from eq. 1

$$\log\alpha_1 = \log(k_{wb}/k_{wa}) - (S_b - S_a)\,\Phi_1 \qquad \text{(i-1)}$$

and

$$\log\alpha_2 = \log(k_{wb}/k_{wa}) - (S_b - S_a)\,\Phi_2\ . \qquad \text{(i-2)}$$

We are interested in the change in α ($\alpha_2-\alpha_1$) that will result from some change in Φ equal to ($\Phi_2-\Phi_1$). From eqs. i-1, 2 this is

$$\log\alpha_2-\log\alpha_1 = -(S_a-S_b)(\Phi_2-\Phi_1). \qquad \text{(i-3)}$$

The allowable change in Φ is determined by the k′-range of the sample and the average value of S for the two compounds. Thus if the k′-values of the first and last bands in the sample are k_a and k_z, and if $1<k'<20$ for all bands, then the maximum allowable change in k′ for the sample is a factor P equal to

$$P = 20/(k_z/k_a) = 20\,k_a/k_z\,. \qquad \text{(i-4)}$$

For example, if k′-values range from 2 to 10 for mobile phase #1, the average value of P is 20 (2/10) = 4. Thus a k′ range of either 1 to 5 or 4 to 20 is allowable (for P = 4). This assumes that a given change in Φ will affect all k′-values by the same factor, which is the case if values of S for the initial and final bands are roughly the same. We will see that this is generally a good approximation. For a given sample, values of P can vary from about 1 to 20, assuming that the sample can be separated isocratically. The average (as opposed to the maximum) change in k′ is then $\sqrt{P}$.

The average allowable change in Φ, given a value of P, is

$$\Phi_2-\Phi_1 = (\log P)/2\bar{S}\,, \qquad \text{(i-5)}$$

where $\bar{S}$ is the average value for the sample. This value of ($\Phi_2-\Phi_1$) assumes either $k_a = 1$ or $k_z = 20$. Now the change in α from eqs. 4 and 6 is given by

$$\log\alpha_2-\log\alpha_1 = [(S_a-S_b)(\log P)]/2\bar{S}$$

$$\log(\alpha_2/\alpha_1) = (1/2)\,\delta S/\bar{S}\,(\log P)\,. \qquad \text{(1-6)}$$

Here ($\delta S/\bar{S}$) is the difference in solute S-values for A and B expressed as a fraction of the average S-value $\bar{S}$ for the two bands.

Eq. 7 can be used to estimate the required difference in S-values for two adjacent bands that will allow SSO to achieve their resolution, assuming that the two bands are initially totally overlapped ($\alpha = 1$). From the resolution eq. (7)

$$R_s = (1/4)(\alpha-1)\,N^{0.5}\,[k'/(1+k')]\,, \qquad \text{(i-7)}$$

we can write that a change in resolution ΔR_s resulting from a change in α (equal to $\alpha_2-\alpha_1$) is equal to

$$\Delta R_s = (1/4)(\alpha_2-\alpha_1)\,N^{0.5}\,[k'/(1+k,)\,.^* \qquad \text{(i-8)}$$

The required change in α for a separation with some necessary change in resolution ΔR_s can be written for the case of α_2 and α_1 small (the case of usual interest) as

$$(\alpha_2-\alpha_1) = 2.3\log(\alpha_2/\alpha_1)$$

$$= 4\,R_s\,N^{-0.5}\,[(k'+1)/k']\,. \qquad \text{(i-9)}$$

Eqs. i-5, i-7, i-9 give eq. 2.

* R_s will change also as a result of a change in k′ that is associated with this change in α; however, the change in k′ can be either favorable (larger) or unfavorable (smaller). In the general case we will not know whether k′ must be increased or decreased in order to increase α and resolution.

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