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Journal of Chromatography A, 946 (2002) 83–90

JOURNAL OF CHROMATOGRAPHY A

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Enhancement of selectivity in reversed-phase liquid chromatography

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Received 16 July 2001; received in revised form 8 November 2001; accepted 14 November 2001

Abstract

In an effort to gain insight into the relationship between stationary phase solvation and selectivity, the use of short- and medium-chained-length alcohols (methanol, *n*-propanol, *n*-butanol, and *n*-pentanol) as mobile phase modifiers in reversed-phase liquid chromatography (RPLC) was investigated to determine their impact on chromatographic selectivity. A wide range of mobile phase compositions was evaluated because of the large effect exerted by solvent strength on selectivity. Employing a set of six vanillin compounds as retention probes, evidence is presented to support the view that an increase in the hydrophobicity of the organic modifier used in RPLC can increase the selectivity of the C_{18} alkyl bonded phase while simultaneously decreasing the retention time of the eluting solutes. Thus, we are presented with an interesting paradox: higher selectivity and shorter retention times, which can be attributed to changes in either solvent selectivity and/or stationary phase solvation by the organic modifier.

Keywords: Mobile phase composition; Stationary phases, LC; Retention mechanisms; Selectivity; Bonded phase wetting; Vanillins

1. Introduction

Reversed-phase liquid chromatography (RPLC) is the method of choice for the analysis and purification of many chemical and biological molecules because of the selectivity, efficiency, and the broad range of substances that can be chromatographed by this technique. Over two thirds of all analytical separations are performed by RPLC. The popularity of RPLC can be attributed to the development of chemically stable, micro-particulate-bonded phases that provide rapid mass transfer and a high degree of reproducibility [1].

Although significant advances continue to be made in applications of RPLC, there has been far less progress in the development of mechanistic models, which describe in detailed molecular terms the RPLC separation process. Control and manipulation of the separation process is crucial and requires an understanding of retention and knowledge of the role played by the composition of the mobile phase in that process.

Retention in RPLC is more than solute interaction with the *n*-alkyl chains of the bonded phase. The stationary phase is enriched in organic modifier, with the extracted solvent interacting with the solute. The nature of this interaction is not well understood. Some workers [2–8] hold the view that organic modifier when sorbed or partitioned into the bonded stationary phase changes its sorbent properties. They believe that an understanding of stationary phase solvation is important because changes in selectivity

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0021-9673/02/$ – see front matter
PII: S0021-9673(01)01520-5

are known to occur as a result of changes in the concentration of the organic modifier in the mobile phase or from changes in the type of organic modifier used in the mobile phase, which in turn influences the concentration of organic modifier dissolved in the stationary phase. Other workers [9,10] argue that the organic modifier affects only the solvent properties of the mobile phase and has little effect on the properties of the stationary phase.

In an effort to gain insight into the relationship between stationary phase solvation and selectivity, the use of short and medium-chained-length alcohols (methanol, *n*-propanol, *n*-butanol, and *n*-pentanol) as mobile phase modifiers in RPLC was investigated to determine their impact on chromatographic selectivity. A wide range of mobile phase compositions was evaluated because of the large effect exerted by solvent strength on selectivity. Employing a set of six vanillin compounds as retention probes, evidence is presented to support the view that an increase in the hydrophobicity of the organic modifier used in RPLC can increase the selectivity of the C_{18} alkyl bonded phase while simultaneously decreasing the retention time of the eluting solutes. We are, therefore, presented with an interesting paradox: higher selectivity and shorter retention times, which can be attributed to changes in either solvent selectivity and/or stationary phase solvation by the organic modifier.

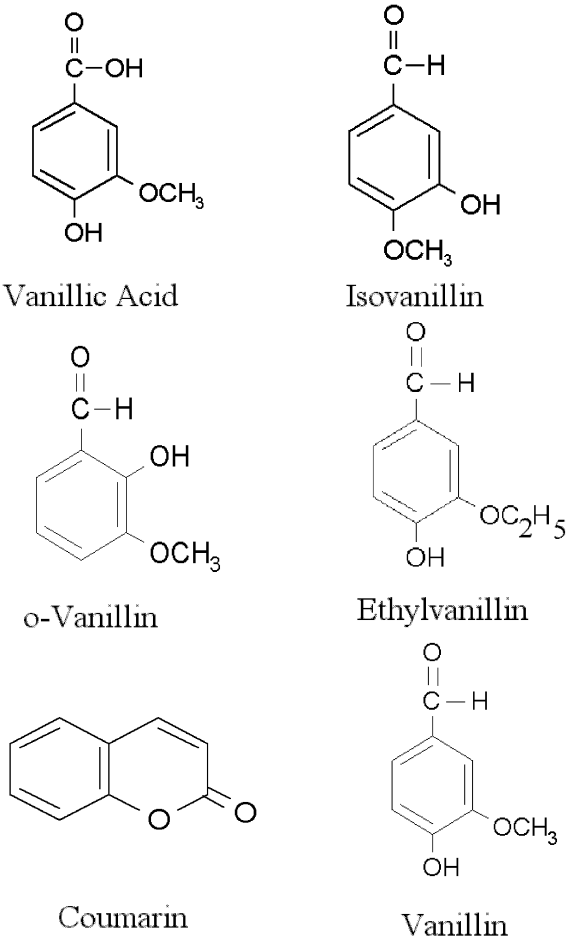

Fig. 1. Vanillin compounds.

2. Experimental

The retention probes used in this study, the vanillin compounds (see Fig. 1), were obtained from Aldrich and were used as received. Stock solutions of the vanillin compounds ($1 \cdot 10^{-2}$ *M*) were prepared with methanol (Fisher, HPLC grade), and then diluted to the appropriate working concentration ($5 \cdot 10^{-4}$ *M*) using doubly distilled water.

The organic modifiers used in this study, methanol, propanol, butanol, and pentanol, were purchased from Fisher. All mobile phases were prepared using doubly distilled water filtered with 0.45 μm pore size Varian nylon-66 filters to remove particulate matter. Mobile phases containing propanol, butanol, or pentanol were prepared via transfer pipette because of the small volume of organic modifier that was needed to prepare these mobile phases. Each mobile phase solution was degassed prior to use. All mobile phases were percolated through the column at a flow-rate of 1 ml/min for approximately 120 min to ensure reproducible solvation of the stationary phase by the mobile phase.

The separation of the vanillin test mixture was performed using a Perkin-Elmer Tridet HPLC system equipped with a 254 nm ultraviolet detector. The analytical column used was a BDS-Hypersil C_{18} (100×4.6 mm) purchased from Keystone Scientific. All HPLC measurements were performed at a flow-rate of 1 ml/min.

The dead volume of the system was determined by injecting different solutions such as methanol,

methanol–water, or water onto the BDS column. This volume, approximately 1.0 ml, was used for all k' calculations. k' values determined in this study were averages of at least triplicate determinations. Deviations in individual capacity factor values were never greater than 5%. All k' values were measured at ambient temperature.

3. Results and discussion

A series of chromatograms were run to illustrate the advantages of using hydrophobic alcohols as organic modifiers in RPLC. The test mixture consisted of six compounds: vanillin, the principal flavor component in vanilla extract, and isomers and analogues of vanillin (see Fig. 1). Food chemists [11] have long been interested in isolating and quantifying these compounds in a variety of sample matrices, which, in part, was our motivation in choosing these compounds to study via RPLC. The interaction of these compounds with the alkyl-bonded phase can also provide additional information about the solvation of the stationary phase since these compounds are more hydrophilic than benzene, toluene, and other commonly used retention probes.

Because the vanillin compounds are weakly retained by the BDS C_{18} column, it was necessary to use water as the primary solvent to prepare the vanillin test mixture. If a stronger solvent such as methanol were used to prepare the vanillin sample, the test mixture would not have been deposited onto the head of the column as a thin plug during sample injection with the result being increased band broadening.

Fig. 2 shows a chromatogram of the vanillin test mixture using a mobile phase consisting of methanol–water (25:75), which is the methanol–water mobile phase that yielded the best separation of the vanillin compounds on the BDS C_{18} column. Interestingly enough, this is the same mobile phase recommended by Supelco for isolating vanillin from a variety of sample matrices with a C_{18} alkyl bonded phase [11]. The number of plates generated by the column for each compound (with the exception of vanillic acid) was computed using the Foley–Dorsey method [12] and is shown in Table 1.

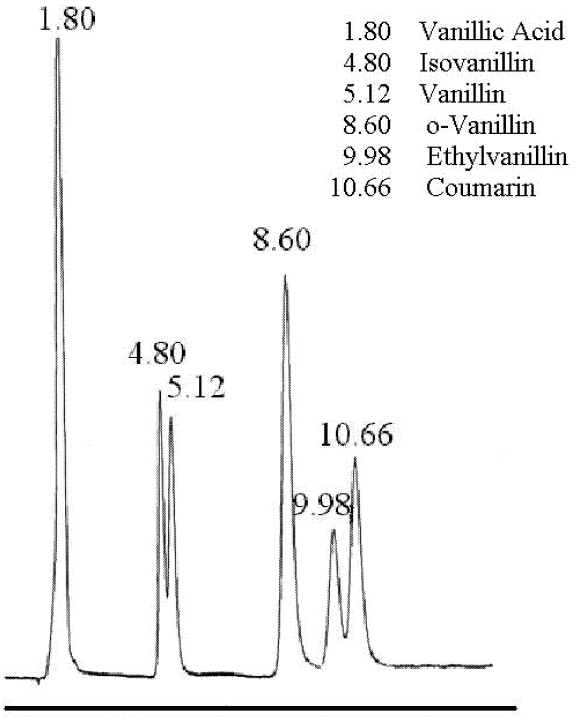

Fig. 2. Separation of the vanillin test mixture using a methanol–water (25:75) mobile phase.

In Fig. 3 a chromatogram of the same test mixture using a mobile phase consisting of butanol–water (2.25:97.75) is shown. The number of plates generated by the column for each compound (with the exception of vanillic acid) is listed in Table 1. Three things are apparent from an examination of these two chromatograms (see Figs. 2 and 3) and the data listed in Table 1. First, the test mixture is completely separated by the butanol–water mobile phase but not by the methanol–water mobile phase. Second, the efficiency of the C_{18} column is approximately the same for these two mobile phases, which implies that differences in the resolution of the test mixture for these two mobile phases is due to differences in chromatographic selectivity. (Actually, excessive tailing of the solutes is observed for the butanol mobile phase, which we attribute to extra column band broadening from the tri-det system and possibly slow detector electronics. Evidence to support our belief is that B/A values are significantly worse for the butanol mobile phase, which gives much shorter retention times for the solutes). Third, the retention

Table 1
Variation of efficiency and asymmetry with mobile phase

Compound	Number of plates	
	Methanol–water (25:75)	Butanol–water (2.25:97.75)
Isovanillin	3070 (asymmetry = 1.38)	2940 (asymmetry = 1.57)
Vanillin	3130 (asymmetry = 1.40)	3040 (asymmetry = 1.52)
o-Vanillin	3190 (asymmetry = 1.28)	2770 (asymmetry = 1.66)
Ethylvanillin	3700 (asymmetry = 1.33)	2850 (asymmetry = 1.24)
Coumarin	4650 (asymmetry = 1.33)	3660 (asymmetry = 1.58)

time of each vanillin compound is greater when methanol–water (25:75) is used as the mobile phase.

To elucidate the role of the organic modifier in the separation of the vanillin test mixture on the BDS C_{18} column, it was necessary to examine retention data for each vanillin compound in a systematic manner. In RPLC, insight into the factors that influence the separation process can sometimes be gained by a thorough analysis of retention data obtained for a set of congeners using Eq. (1), where Φ is the volume percentage of organic modifier in the mobile phase, B is a measure of the interaction of the solute with the mobile phase and is a constant for a given solute and $\ln k_w$ is the logarithm of the capacity factor for the compound in a purely aqueous medium which is determined from the regression. Eq. (1) can also be used to predict selectivity and resolution for a separation over a narrow range of Φ [13]:

$$\ln k' = \ln k_w - B\Phi \quad (1)$$

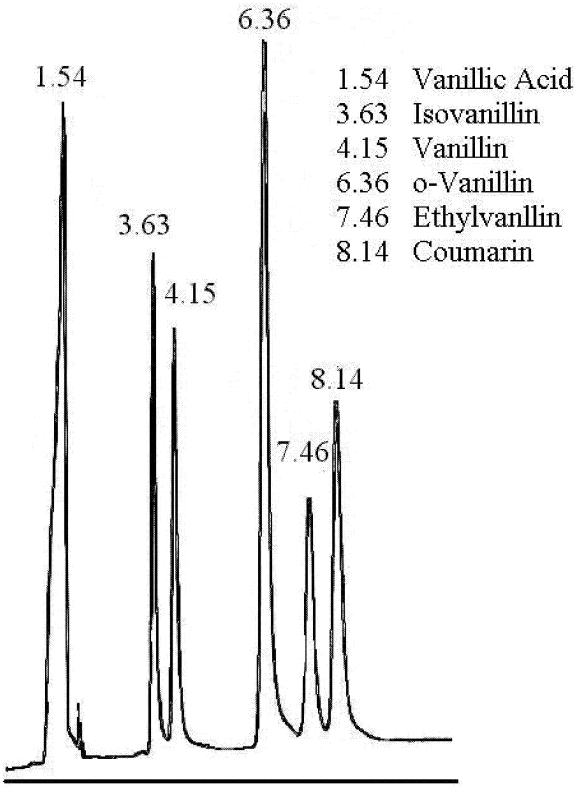

Fig. 3. Separation of the vanillin test mixture using butanol–water (2.25:97.75) mobile phase.

Fig. 4 shows a plot of $\ln k'$ versus Φ for each vanillin compound. Five methanol–water mobile phases were used to generate the $\ln k_w$ plots: 20%, 22.5%, 25%, 27.5%, and 30% (v/v) methanol in water. Each compound in the text mixture exhibited the classical RPLC hydrophobic behavior, that is, retention time decreased linearly as the concentration of the organic modifier increased because the stationary phase was saturated by organic modifier over the mobile phase composition region investigated. (For this study, it was not possible to generate retention data using methanol–water mobile phases with less than 20% methanol because of difficulties in eluting these compounds off the column. Furthermore, we did not generate an $\ln k'$ plot for vanillic acid because the compound co-eluted with the dead marker).

Fig. 5 shows a plot of $\ln k'$ versus Φ for each vanillin compound which was generated using 12 butanol in water mobile phases: 1%, 1.25%, 1.5%, 1.75% 2%, 2.25%, 2.5%, 3%, 3.5%, 4%, 4.5%, and 5% (v/v) butanol in water. Every $\ln k'$ plot was bilinear, with the break occurring at the same mobile

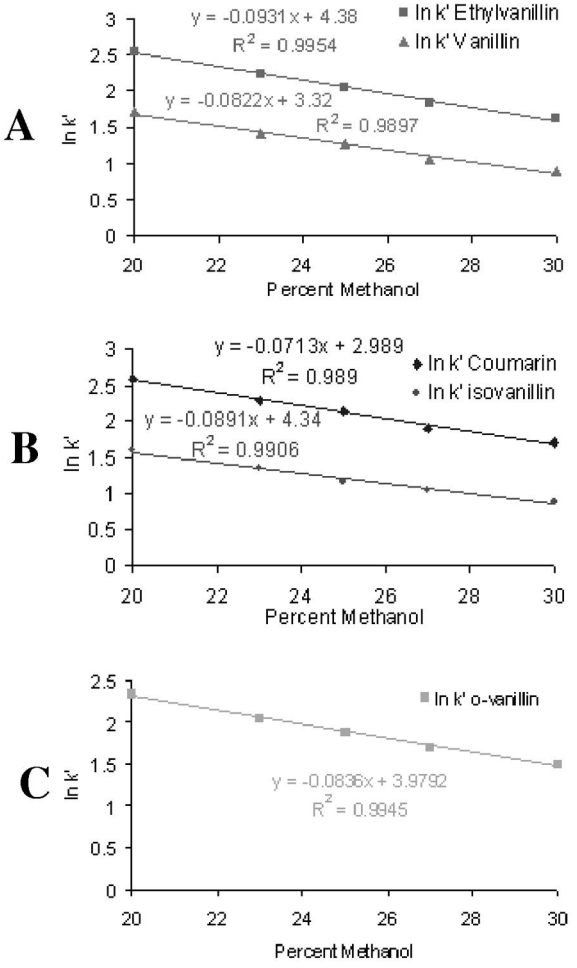

Fig. 4. Ln k' plots with methanol–water mobile phases for (A) ethylvanillin and vanillin, (B) coumarin and isovanillin, and (C) *o*-vanillin.

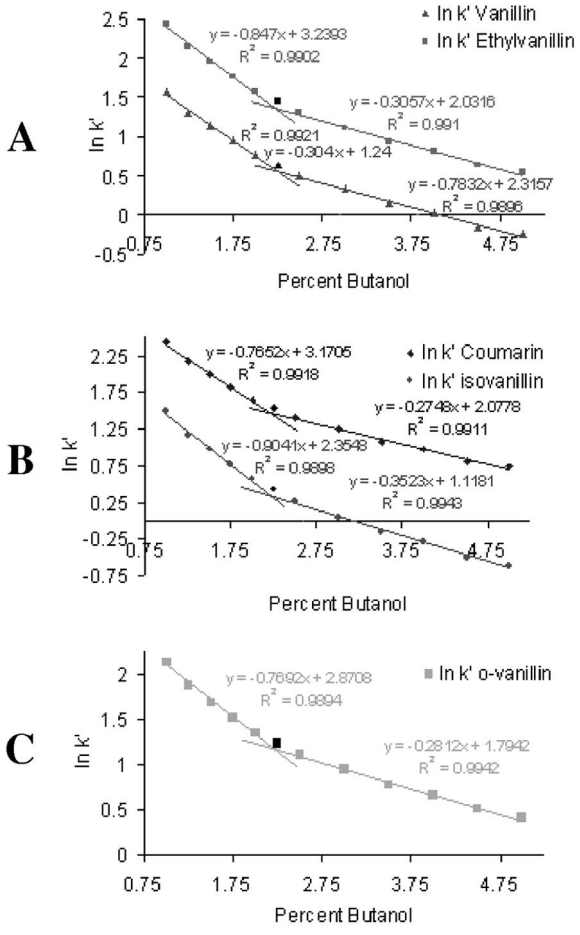

Fig. 5. Ln k' plots with butanol–water mobile phases, for (A) ethylvanillin and vanillin, (B) coumarin and isovanillin, and (C) *o*-vanillin.

phase composition (butanol–water, 2.25:97.75). The break in each plot indicates that a change in the structure of the stationary phase has occurred [14]. The first line (''low alcohol concentration'') probably corresponds to a simultaneous change in both mobile and stationary phase whereas the second line (''high alcohol concentration'') corresponds to classical RPLC behavior, which is a change in the mobile phase, with the stationary phase remaining unchanged because the organic modifier has saturated it.

If our interpretation of the ln k' plot data is correct, then the concentration of butanol in the mobile phase necessary to ensure complete saturation of the C_{18} alkyl bonded phase should be approximately 2.5% (v/v) [15], which turns out to be the case. Furthermore, the break in the ln k' plots should appear at higher organic modifier concentration for *n*-propanol and at lower organic modifier concentration for *n*-pentanol. This is confirmed by the ln k' data shown in Figs. 6 and 7.

All the ln k' plots were reproducible, that is, whether we started at higher organic modifier concentration and moved towards lower concentration or vice-versa, the same results were obtained. Thus, the

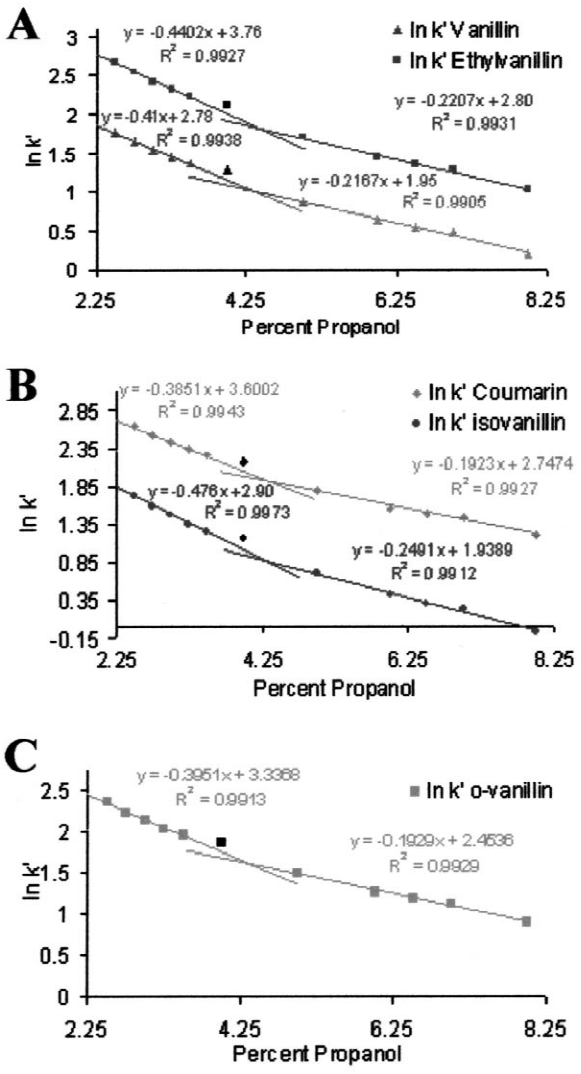

Fig. 6. Ln k' plots with propanol–water mobile phases for (A) ethylvanillin and vanillin, (B) coumarin and isovanillin, and (C) *o*-vanillin.

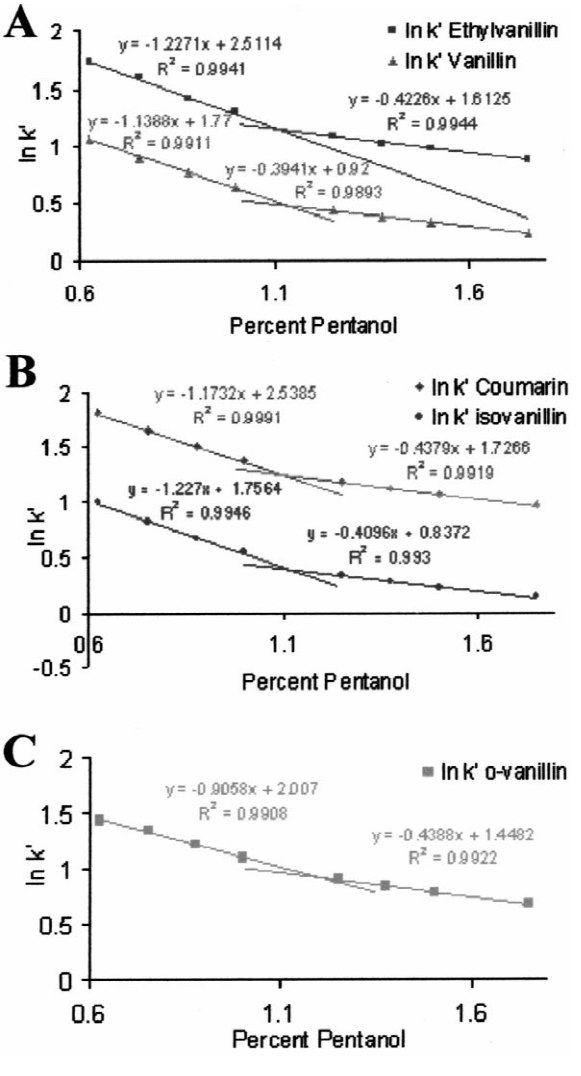

Fig. 7. Ln k' plots with pentanol–water mobile phases for (A) ethylvanillin and vanillin, (B) coumarin and isovanillin, and (C) *o*-vanillin.

break in the ln k' plots cannot be attributed to a conformational effect involving the folding of the C_{18} chains.

For each vanillin compound, the computed k_w value in the regression equation developed from the ''high alcohol concentration'' butanol data (see Fig. 5) is approximately two orders of magnitude larger than the corresponding k_w value in the regression equation developed from the methanol data (see Fig. 4). This result is not surprising because these k_w values do not represent true k_w values. Rather, each represents what the capacity factor would be, if the conformation and composition of the stationary phase in pure water were the same as in organic aqueous mixtures. Therefore, the differences in these k_w values probably reflect differences in the solvation of the bonded phase by methanol and butanol. These differences cannot be explained by uncertainties in the least-squares fitting of the data, which can be as high as 80% [16].

The computed k_w values for each vanillin compound in the regression equations developed from the ''low alcohol concentration'' data also differed by two orders of magnitude for the propanol, butanol and pentanol mobile phases. We also attributed differences in these values to how the alcohols interacted with the C_{18} alkyl bonded phase. Felitsyn and Cantwell [5,6] reported that butanol exhibited Langmuir behavior on C_{18} alkyl bonded phases, whereas propanol did not exhibit this type of behavior. Felitsyn and Cantwell hypothesized that propanol caused the C_{18} chains to undergo some type of rearrangement, where as butanol's behavior was quite unremarkable. Although studies of this nature have not yet been performed with pentanol, it may be that pentanol interacts and thereby wets the bonded phase in a manner dissimilar to both propanol and butanol. For each vanillin compound, it is possible that differences in k_w values in the low alcohol concentration region for the propanol, butanol, and pentanol mobile phases can be correlated to the ability of these alcohols to wet the bonded phase.

Therefore, differences in the solvation of the stationary phase by methanol and butanol may be a plausible explanation for the observed difference in selectivity exhibited by the two mobile phases (methanol–water and butanol–water). Butanol, when it partitions into the C_{18} bonded phase, may act as a co-solvent. If that were the case, specific solute–modifier interactions would occur in the stationary phase that would explain the observed differences in selectivity between the methanol–water and butanol–water mobile phases.

Another plausible explanation is that butanol partitions into the bonded phase providing a more extended ordered surface thereby increasing chromatographic selectivity [17,18]. When the alkyl-bonded phase is not well solvated, the result is a stationary phase with low contact surface area. By increasing the hydrophobicity of the organic modifier, the contact surface area of the bonded phase will increase due to greater solvation by the organic solvent. In turn, this will increase its selectivity while decreasing the retention volume due to a decrease in the void volume. Thus, the improved resolution of the vanillin compounds when switching from methanol to butanol as the mobile phase modifier may be attributed in some measure to an increase in the contact surface area of the bonded phase due to the superior wetting of butanol, which is a result of its greater hydrophobicity.

Felitsyn and Cantwell [6] postulated that butanol might be adsorbed at the C_{18}–mobile phase interface. When organic modifier is adsorbed at the stationary phase–mobile phase interface, its primary contribution to retention (assuming that its concentration in the mobile phase is low) is a competition for space with the solute. Under these circumstances, the organic modifier will profoundly influence retention if the solute is also adsorbed at the interface. As the hydrophobicity of the organic modifier is increased, it is then more difficult for the analyte to displace the organic modifier. This mechanism could explain the decrease in the retention time that occurs when butanol–water (2.25:97.75) is used as the mobile phase in lieu of methanol–water (25:75). Since the vanillin compounds are weakly retained by the C_{18} column, they probably do not penetrate very far within the C_{18} alkyl bonded phase and may in fact lie at the interface for the aqueous mobile phases that we have investigated as part of this study.

A third plausible explanation for differences in selectivity exhibited by the two mobile phases is selective solute–modifier interactions in the mobile phase. The enhanced separation of the solute pairs, isovanillin/vanillin and coumarin/ethylvanillin, in butanol–water could be the result of solvent selectivity differences between methanol and butanol. Although methanol and butanol are from the same solvent family, it has been reported that changes in selectivity between lower and higher homologues are sometimes significant for solvents that undergo strong self-hydrogen bonding such as alcohols [18]. This would explain the greater selectivity and shorter retention times obtained for the vanillin compounds when butanol is used as the organic modifier in lieu of methanol.

Acknowledgements

B.K.L. acknowledges helpful discussions with Professor Josip Kratohvil and Stig Friberg. Financial support for this work from DeGussa is gratefully acknowledged.

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