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Eluotropic strength in non-aqueous liquid chromatography with porous graphitic carbon

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Abstract

Porous graphitic carbon is an attractive packing for the chromatographic analysis of highly hydrocarbonaceous compounds with non-aqueous mobile phase. An eluotropic-strength scale of 10 pure organic solvents was established using the methylene selectivity from the fatty acid methyl ester homologous series (chain length between 18 and 31 carbon atoms). Eight binary mobile phases combining a weak solvent: methanol or acetonitrile with a strong solvent: toluene, chloroform, dichloromethane or tetrahydrofuran at different volume fractions φ of strong solvents (ranging from 0.3 to 1.0) were tested and their eluotropic strengths were then compared with those of pure solvents. The curves of the eluotropic strength versus the volume fraction of the strong solvent followed two different trends: linear or curved. The knowledge of the pure solvent strength is not sufficient to predict the eluotropic strength of solvent in the mixture. Then modelling of the eluotropic strength for binary mobile phases was envisaged in order to provide a prediction tool. This model was assessed for the establishment of the composition of eight iso-eluotropic mobile phases. Good assessment was found except in the case of toluene with acetonitrile where the difference between the predicted and the real value was the highest.

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1. Introduction

For the heterogeneity study of lipid class, a recommended chromatographic approach is to combine a hydrophobic stationary phase with non-aqueous mobile phases [1] since molecules are insoluble in water. As one of the main structural variations presents in lipids is the chain length, the driving

force of the separation should be the methylene selectivity. Therefore porous graphitic carbon (PGC) is an interesting packing material [2] for these applications because of its property of greater discrimination for methylene group than octadecyl silica (ODS) [3,4].

However, there are only a few, recent applications of PGC in the field of lipid [5–7] or high molecular mass compound [8,9] analysis in spite of noteworthy improvement of the separation compared with ODS [10,11]. One of the reasons for this limitation was the lack of knowledge of the retention in this

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particular field. Therefore these applications with PGC generally required an important development study. Consequently, ODS remains the more practical stationary phase for the development of new methods for lipid class analysis.

This work was devoted to enrich the knowledge about the retention on PGC by studying the eluotropic strength (ε°). The objective of this work is to provide an appropriate and general ε° scale for PGC in non-aqueous HPLC for such compounds. The strategy was first to establish an eluotropic strength scale of pure solvent although other solvent strength scales already exist [12,13]. However as the authors had demonstrated that solvent scales are solute-dependent, the ε° scale developed herein was based only on dispersive interaction (i.e. non-specific interaction) in order to achieve a general eluotropic scale always usable except when the polar retention effect of graphite (PREG) occurs. Therefore, the methylene selectivity from non-polar compounds with high hydrocarbonaceous volume was used. As the composition of mobile phase often contained more than one solvent in order to benefit a modulation of selectivity, ε° was also studied for binary mixtures and attempted to be modelled. Such a model would be very useful for mobile phase optimisation since it can allow the establishment of isoeluotropic mobile phases. As the structural variation in lipids is not limited to the number of methylene groups, the selectivity can be modulated independently of the ε° with such an approach.

2. Experimental

2.1. Chemicals

The homologous series of fatty acid methyl esters ($16 \leq n \leq 31$, except 29) and wax esters ($24 \leq n \leq 34$) with the acidic part containing 12 carbon atoms were purchased from Sigma (St. Quentin Fallavier, France). All solvents were HPLC-grade from Fisher (Elancourt, France), and all the mobile phases contained 0.1% of triethylamine (TEA) and an equimolar amount of formic acid (HCOOH) (Prolabo, Nogent sur Marne), which increase the detection response without significantly modifying the retention [14,15].

2.2. Apparatus

Chromatographic measurements were carried out with a Thermo Separation Products P1000 XR gradient pump with a TSP SCM1000 (Thermo Separation Products, San Jose, CA, USA) vacuum membrane degasser connected to a Kontron autosampler 360 (Bio-Tek Kontron Instruments, Milan, Italy) equipped with a 5- μ l sample loop injection valve. Detection was carried out with a Cunow DDL 11 evaporative light scattering detector (Eurosep, Cergy, France) where the drift tube temperature was set at 35 °C and nitrogen pressure at 1 bar. Homologues with a chain length shorter than 18 carbon atoms cannot be detected. The chromatograms were recorded with a PC-integrator KromaSystem 2000 1.60 (Bio-Tek Kontron Instruments, Milan, Italy). The flow-rate was set at 0.4 ml min⁻¹. The column was Hypercarb[®] (5 μ m), 100 \times 2.1 mm I.D. (Hyper-sil, Runcorn, UK) and was thermostated at 50 °C with a Jetstream 2 temperature controller (Thermotecnics Products, Austria).

2.3. Calculations

Statistical tests were performed using Unscrambler 6.11 (Camo AS, Trondheim, Norway).

3. Results and discussion

With PGC two different retention mechanisms were reported: non-polar adsorption [16,17] and PREG [2]. This latter appears to be an effect which is additional to the normal dispersive effect found with conventional reversed-phase materials. However non-aqueous HPLC is devoted to the separation of high molecular mass compounds within homologous series thus the ε° is mainly governed by non-specific interactions. The increase in the chain length of homologous series is then suitable to assess the ε° of organic mobile phase.

The retention mechanism with carbon material is often compared to classical adsorption chromatography as the surface contact between the solute and the carbon surface determines the strength of the interaction [2]. Therefore the basic principle of adsorption chromatography as described by Snyder,

which is the exchange process between solute and solvent molecules at the surface of the adsorbent [18], can be applied to the PGC adsorbent. And thus the notion of ε° is associated with adsorption energy. Combining the ε° definition of Snyder and the use of the methylene selectivity, ε° with PGC can be measured experimentally with the equation [12]:

$$\varepsilon^\circ_{\text{solvent}} = (\log \alpha_{\text{CH}_2, \text{reference}} - \log \alpha_{\text{CH}_2, \text{solvent}}) / A_{\text{CH}_2} \quad (1)$$

where $\alpha_{\text{CH}_2, \text{reference}}$ and $\alpha_{\text{CH}_2, \text{solvent}}$ are, respectively, the methylene selectivity value in the eluent of reference and in the studied mobile phase: pure or solvent mixture, and $A_{\text{CH}_2} = 1$, is the surface area of one methylene group in contact with the surface (i.e. 1 unit = 8.5 \AA^2) [18]. Therefore this definition takes into account only the contribution of non-specific interaction to the retention. In non-aqueous HPLC, methanol was commonly found to be the weakest eluent and thus was selected as the reference for the ε° scale, i.e. $\varepsilon^\circ_{\text{MeOH}} = 0.00$ [19].

The methylene selectivity is defined by:

$$\log \alpha_{\text{CH}_2} = \log k_{n+1} - \log k_n \quad (2)$$

As reported with PGC, the logarithms of retention factors k of members of the homologous series with n or $n+1$ carbon atom(s), vary linearly with the number of methylene groups [4,20]. Therefore, $\log \alpha_{\text{CH}_2}$ is more practically obtained by calculating the slope of this relationship:

$$\log k(n) = \log \alpha_{\text{CH}_2} n + \log \beta \quad (3)$$

where n is the number of carbon atoms in the molecule and $\log \beta$ represents the specific interaction of the residue of the molecule isolated from the alkyl chain.

3.1. Eluotropic strength scale of pure solvents

The first step in method development is the selection of solvents with the appropriate ε° for the elution of the solutes. The knowledge of pure solvent ε° is then required. The ε° scale of pure solvents for the lipid analysis on PGC was thus established using methylene selectivity from the homologous series constituted by fatty acid methyl esters (FAMES). These lipids were selected since they were available with a large range of chain length (between 18 and 31 methylene units) which constituted an important hydrocarbonaceous volume.

The ε° of 10 commonly used solvents was calculated using Eqs. (1) and (3) (Table 1). The weakest solvents were methanol (CH_3OH) and acetonitrile (CH_3CN), intermediary solvents: acetone, ethyl acetate, propanol-1; and strong solvents: heptane, tetrahydrofuran (THF), dichloromethane (CH_2Cl_2), chloroform (CHCl_3) and toluene.

This classification followed roughly the polarity of the solvent. However, it was suggested that the size, the geometry and polarizability of the solvent were important criteria [23] that govern ε° . Hence, toluene with its planar and aromatic structure led to a greater

Table 1
Eluotropic strength scales of pure solvents

Mobile phase	Range of n	Log α_{CH_2}	Present work ε°	Other studies	
				$\varepsilon^{\circ a}$	$\varepsilon^{\circ b}$
CH_3OH	16–19	0.23	0.00	0.00	0.00
CH_3CN	16–21	0.22	0.01	0.04	–0.01
Acetone	16–22	0.17	0.06	–	–
Propanol-1	16–22	0.16	0.07	0.08	–
Ethyl acetate	20–28	0.14	0.08	0.10	0.04
Heptane	24–31	0.11	0.11	–	–
THF	19–31	0.09	0.13	0.14	0.08
CH_2Cl_2	18–31	0.10	0.13	0.14	0.06
CHCl_3	23–31	0.08	0.15	0.20	0.11
Toluene	24–31	0.06	0.17	–	–

^a Ref. [12].

^b Ref. [13].

interaction with the PGC surface than heptane, and thus a greater ε° . Furthermore, CH_3CN used as pure mobile phase was found slightly stronger than CH_3OH , perhaps due to its higher interaction with the carbon surface.

Furthermore, the ε° values established for PGC were shown to be solute-dependent [13]. Compared to previous scales (Table 1) [12,13], ours was qualitatively comparable although the measurement conditions were all different. Only one qualitative difference was observed: Kaur's study using phenyl-alkanes with short chain ($n=2$) as the homologous series showed CH_3OH stronger than CH_3CN [13] whereas we found the reverse. Otherwise we found the same order of solvent strength as Colin et al. [12] although their packing material was not Hypercarb[®] and the homologous series were short phenyl-alkanes. However, the difference can be related to the range of chain length used for the determination of the slope of the relationship $\log k$ versus n curve (Eq. (3)). The closer the methylene group was to the phenyl group, the more its retention with the carbon surface was influenced. Therefore shorter homologues should induce a deviation in the linear trend. An overall conclusion indicated that both CH_3OH and CH_3CN were very weak solvents with regard to PGC.

Overall quantitative differences were encountered: the smallest ε° values were found with Kaur's experimentation conditions [13], then ours were slightly higher and those from Colin and Guiochon the highest [12]. That can be mainly due to the nature of the packing (which was not a Hypercarb[®] in the case of the study of Colin and Guiochon) and the different homologous series used and range while the column temperature should be of less influence on the ε° scales. Using CH_3OH , Möckel et al. [4] have demonstrated with nine different homologous series that $\log \alpha_{\text{CH}_2}$ values were found approximately identical and had calculated the free sorption enthalpy of -1.5 kJ mol^{-1} per CH_2 group. This result can be used to determine the difference in $\log \alpha_{\text{CH}_2}$ values induced by a temperature variation between 25 and 50 °C. This variation was then assessed at 0.02 which is sufficiently weak to explain the qualitative differences between ε° scales. Consequently, that validates the scale proposed in Table 1

which is qualitatively relevant for solvent development with PGC for lipids other than FAMES.

However the development of mobile phases involves typically more than one solvent. But the resulting ε° of the solvent mixture is not necessarily the proportional contribution of each solvent involved. In order to establish and to be able to predict the ε° of solvent mixtures, binary mobile phases were then tested.

3.2. Eluotropic strength scale of binary mobile phases and its modelling

In order to reach the ε° of binary mobile phases, Eq. (1) was applied with solvent mixtures. A systematic study was then developed for studying binary mobile phases composed of one weak and one strong solvent. From the established ε° scale of pure solvents, CH_3OH and CH_3CN were selected as weak solvents and THF, CH_2Cl_2 , CHCl_3 and toluene as strong solvents. The selection of these solvents was guided by their solvation property of lipids and by the solvents found suitable for a previous molecular species analysis with PGC [10]. The retention data were obtained using 10 FAMES in different compositions of each binary mobile phase with volume fractions φ of strong solvent ranging from 0.3 to 1.0 (increment=0.1). Three measurements of retention factors were realised at different days to account for day-to-day precision.

Although the relationship between $\log \alpha_{\text{CH}_2}$ and the volume fraction of strong solvent of the mobile phase was extensively studied with grafted silica, studies using PGC in NARP-LC were limited to pure strong solvents. Therefore the development of modelling of binary mobile phase ε° would be of great interest in order to be able to describe the behaviour of the solvent mixture and thus predict ε° .

As PGC is an adsorbent, the eluotropic strength of a binary solvent mixture should be described by the model developed by Snyder for polar adsorption with silica [18]:

$$\varepsilon_{ab}^\circ = \varepsilon_a^\circ + \frac{\log(N_b 10^{\beta_* A_b (\varepsilon_b^\circ - \varepsilon_a^\circ)} + 1 - N_b)}{\beta_* A_b} \quad (4)$$

where the subscript "a" refers to the weak solvent, "b" to the strong solvent and "ab" to the solvent

mixture. Consequently ε_a° , ε_b° , and ε_{ab}° are the strengths of those solvents or mixture, N_b is the molecular fraction of solvent b, β^* is the surface activity function of the support, and A_b the molecular area of an adsorbed molecule of solvent b.

Colin et al. [21] attempted to apply this equation for the prediction of the eluotropic strength of binary mobile phases with pyrocarbon material for two pairs of solvent: CH₃OH–CHCl₃ and CH₃CN–xylen. Their assumptions were that β^* surface activity value was arbitrarily equal to unity and they used A_b values determined with silica material. The solvent molecular surface A_b in contact with the stationary phase also plays a major role in the shape of Eq. (4). This latter point is a theoretical limitation of the use of Eq. (4) with PGC. From its calculation, Eq. (4) supposes that one molecule of strong solvent displaces one molecule of weak solvent from the stationary phase surface [18]. In the case of a difference in A_b values between the two solvents, this assumption may hold with silica if we also assume a 1:1 ratio between interacting solvent molecules and silanols. PGC is a flat planar surface composed of sheets of hexagonally arranged carbon atoms with sp² hybridization [22]. Thus a stoichiometry higher than 1 may exist (and even non integer stoichiometry) that hinders the development of an equation similar to Eq. (4) on the basis of the respective size of the weak and strong solvent. Consequently, Colin et al. [21] found a rather poor agreement with the experimental values with non-aqueous mixtures with this approach.

Therefore, the rather empirical model developed by Jandera for grafted silica in RP-HPLC [23] was then envisaged which allows to simply express the variation of $\log \alpha_{\text{CH}_2}$ and $\log \beta$ with the volume fraction φ of strong solvent in the mobile phase:

$$\log \alpha_{\text{CH}_2} = a_0 + a_1\varphi + a_2\varphi^2 \quad (5)$$

$$\log \beta = b_0 + b_1\varphi + b_2\varphi^2 \quad (6)$$

where $\log \alpha_{\text{CH}_2}$ is related to the non-specific interaction of the methylene groups, $\log \beta$ to the specific interaction from the residue of the invariant part of the homologues and φ is the volume fraction of the strong solvent. Thereby, ε° can be related to φ though Eq. (1).

The objective of Jandera was the establishment of a model which requires few physico-chemical data in order to be used. Consequently, assumptions were necessary for its simplification. The main one was based on a predominant contribution of the interaction between the solute and the mobile phase to the retention [24]. Thus the model is a descriptive function of $\log \alpha_{\text{CH}_2}$ and $\log \beta$ which has the advantage of being readily usable.

Then the ε° via $\log \alpha_{\text{CH}_2}$ was attempted to be related to the volume fraction of the strong solvent through the equations of Jandera et al. (Eqs. (5) and (6)) that accounts for the change in $\log \alpha_{\text{CH}_2}$ and $\log \beta$ parameters of Eq. (3) with the volume fraction φ of stronger solvent in the mobile phase. Table 2 summarises the coefficients of Eqs. (5) and (6) which were fitted against data with significance for each parameter a_i or b_i tested by a Student's *t*-test at 5% risk. In the case where the a_2 or b_2 parameter was not significantly different from zero, Eq. (5) or (6) was reduced to a linear model. And if a_1 or b_1 was also not significantly different from zero, $\log \alpha_{\text{CH}_2}$ and $\log \beta$ could be considered to be independent of the variation of φ .

Attempts to fit $\log \beta$ versus φ to quadratic or linear equations were unsuccessful. The intercept was the only significant parameter, $\log \beta$ was thus found independent of the variation of the strong solvent. There was no significant difference between $\log \beta$ calculated for mobile phases involving CH₃CN and CH₃OH. The mean value of b_0 was then calculated for the eight groups of binary mobile phases.

The examination of values of Table 2 shows that,

Table 2
Parameters of the terms $\log \alpha_{\text{CH}_2}$ and $\log \beta$ according to Eqs. (5) and (6)

	Log α_{CH_2}				log β b_0
	a_0	a_1	a_2	r	
CH ₂ Cl ₂ –CH ₃ CN	0.209	–0.111	–	0.997	–2.30
THF–CH ₃ CN	0.213	–0.119	–	0.999	–2.28
CHCl ₃ –CH ₃ CN	0.210	–0.135	–	0.998	–2.30
Toluene–CH ₃ CN	0.183	–0.129	–	0.994	–2.14
CH ₂ Cl ₂ –CH ₃ OH	0.223	–0.177	0.051	0.997	–2.24
THF–CH ₃ OH	0.226	–0.133	–	0.993	–2.27
CHCl ₃ –CH ₃ OH	0.226	–0.202	0.050	0.998	–2.21
Toluene–CH ₃ OH	0.217	–0.287	0.135	0.990	–2.22

except for toluene-based mobile phases, the a_0 values (that reflect $\log \alpha_{\text{CH}_2}$ in pure weak solvent) were found effectively similar for all CH_3CN solvent systems. The same observation was true for mobile phases involving CH_3OH . Therefore Eq. (5) described properly the $\log \alpha_{\text{CH}_2}$ variations over the entire range of φ , but was not appropriate in the case of mobile phases involving toluene especially associated with CH_3CN . However, as underlined by Jandera because of the approximation for the development of this model, such restriction often occurred and did not constitute a main limitation of its use in a specific range of φ .

Considering the variation of $\log \alpha_{\text{CH}_2}$ with φ by the model of Jandera (Eq. (5)), two trends were underlined—linear and quadratic: toluene and chlorinated solvents led to linear and quadratic relationship, respectively, when they were associated with CH_3CN and CH_3OH , whereas THF only exhibited a linear trend whatever the associated weak solvent.

From the model presented in Fig. 1, the ε° behaviour of binary mobile phases can be summarized as follows:

- using CH_3CN with an identical volume fraction φ of THF or CH_2Cl_2 led to mobile phases with equivalent ε° ,
- CH_2Cl_2 – CH_3OH mobile phases are markedly

stronger than THF– CH_3OH mobile phases of equivalent composition until a φ value of 0.8,

- the ε° values of toluene– CH_3OH or CHCl_3 – CH_3OH and, to a lesser extent CH_2Cl_2 – CH_3OH mobile phases are higher than their CH_3CN counterpart between φ values from 0.4 to 0.9.

However for toluene-based mixtures as underlined by the a_0 value, the curves are not linear and clearly exhibit an important curved $\log k$ versus φ relationship whatever is the weak solvent. These behaviours seem to underline the fact that the greater the difference in polarities of both solvents, the greater the curvature of the relationship.

3.3. Assessment of the model

The interest of this model was to understand the modification in ε° with solvent mixtures. However its use can be envisaged for the establishment of iso-elutropic mobile phases which could constitute an important tool during mobile phase optimisation. Therefore the model was assessed for the achievement of iso-elutropic binary mobile phases. Other compounds were then selected than those used for the establishment of the model: wax esters as they are available with higher hydrocarbonated volume than FAMES. Otherwise the homologues contained

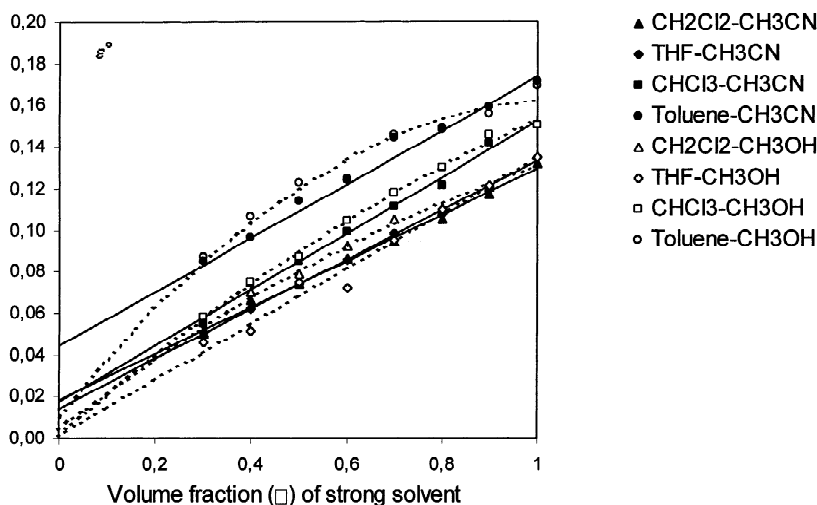


Fig. 1. Eluotropic strength of binary mobile phase versus volume fraction φ of strong solvents. Points from Eq. (1) with $\log \alpha_{\text{CH}_2}$ fitted with Eq. (5). Dotted lines: CH_3OH -based mobile phases. Solid line: CH_3CN -based mobile phases.

between 24 and 34 carbon atoms which constituted a range more hydrophobic than those with FAMES.

Arbitrarily, a composition of CHCl_3 – CH_3CN 70:30 was selected which had the advantage to involve intermediate values of φ . The ε° of this system using Eq. (5) was found to be 0.115. Using the homologous series of waxes and Eqs. (1) and (2), a value of 0.114 was found which was very close to the calculated value. The φ values for all other binary mobile phases were calculated with Eq. (5) of the model and are summarised in Table 3.

Experimentally iso-elutotropic mobile phases with ε° approximately equal to 0.115 were determined which corresponded to a retention factor of 17 ± 0.5 of the last eluted compound (wax at 34 carbon atoms) with CHCl_3 – CH_3CN 70:30. From these experiments, ε° was calculated with Eq. (1) and the φ values compared to those proposed by the model (Table 3).

The model provided mobile phase compositions close to the real iso-elutotropic mobile phases, and all trends underlined by the model of binary ε° were verified, i.e. the same compositions of mobile phase were expected when THF and CH_2Cl_2 were used with CH_3CN ; smaller values of φ for CH_2Cl_2 – CH_3OH mobile phases than THF– CH_3OH ; and using toluene– CH_3OH , CHCl_3 – CH_3OH and CH_2Cl_2 – CH_3OH , the φ values were smaller than those involving CH_3CN .

The most important difference between the predicted and experimental φ values was observed for the mobile phase containing toluene– CH_3CN . This binary mobile phase clearly exhibits an adsorption-

shaped curve which is not correctly described by the model. This underlines the limit of this model to describe ε° along the full range of φ . In cases of mobile phase with CH_3CN (except with toluene), the curves can be extrapolated until φ equals zero regarding the close values of a_0 and the correct correlation between the predicted and real values of φ obtained. Therefore the model provides a useful guideline: the description of the behaviour of solvents in a mixture which cannot be afforded with the elutotropic strength scale of pure solvents, and the calculation of isoelutotropic mobile phases in a large range of φ .

4. Conclusion

The relative ε° found herein for binary mobile phases is consistent with the solvent classification in our previous ceramide molecular species study [10], i.e. toluene and CHCl_3 were the strongest solvents especially when associated with CH_3OH . THF and CH_2Cl_2 were close together when associated with CH_3CN , whereas with CH_3OH , CH_2Cl_2 became stronger than THF.

An ε° scale of organic solvents on PGC was made available for separation of compounds with high hydrocarbon volume. Their relative ε° values in mixtures were described under two types of behaviour—linear and quadratic. This latter behaviour is encountered preferentially when the difference in the relative affinity of the solvents for the stationary phase is more marked. Although theoretically Jan-

Table 3
Isoelutotropic mobile phases

Binary mobile phase	φ of strong solvent			ε° obtained from φ experimental
	Calculated values from the model (Table 2)	Experimental values	$\Delta\varphi$ between calculated/experimental	
Toluene– CH_3CN	0.43	0.52	0.09	0.116
Toluene– CH_3OH	0.48	0.44	0.04	0.117
CHCl_3 – CH_3CN	0.70	0.70	0.00	0.114
CHCl_3 – CH_3OH	0.66	0.65	0.01	0.115
CH_2Cl_2 – CH_3CN	0.85	0.84	0.01	0.113
CH_2Cl_2 – CH_3OH	0.78	0.74	0.04	0.112
THF– CH_3CN	0.82	0.84	0.02	0.111
THF– CH_3OH	0.83	0.85	0.02	0.111

dera's equations are not properly representative of the retention mechanism of the PGC, the model has been shown to be usable for the prediction of mobile phase compositions in a domain of medium solvent strength and presents the advantage of being practical.

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