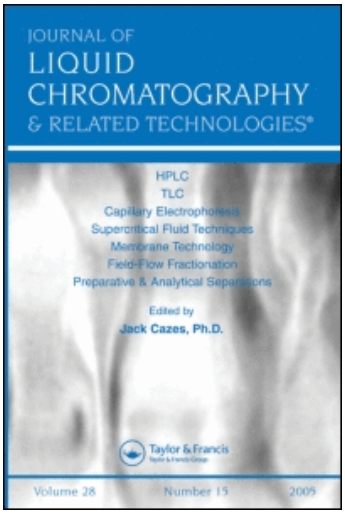

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Influence of Coverage Density of Chemically Bonded C_{18} Phases on the Retention Data of Substances Eluted in Dead Volume During RP HPLC Analysis

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INFLUENCE OF COVERAGE DENSITY OF CHEMICALLY BONDED C_{18} PHASES ON THE RETENTION DATA OF SUBSTANCES ELUTED IN DEAD VOLUME DURING RP HPLC ANALYSIS

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Dedicated to Prof. Dr. Ernst BAYER, in honour of his 65th birthday

ABSTRACT

In this paper there are discussed the results of studies relating to the effect of controlled coverage density (α_{RP}) of silica gel surface with C_{18} chemically bonded phase (CBP) on the retention of some test substances (H_2O, D_2O, CH_3OH, CH_3OD) most often used to the determination of dead volume (V_o). In the consideration it has regarded the influence of many effects accompanying with RP HPLC elution *e.g.* specific and unspecific interactions, solvatation, displacement, local concentration changes, diffusion and exclusion. Special attention was paid to the influence of physico-chemical properties of prepared packings. Therefore the packing materials surface before and after chemical modification were characterized by different instrumental methods *e.g.*: porosimetry, pH-metry, elemental analysis, CP/MAS NMR, GC and HPLC.

INTRODUCTION

During HPLC analysis, realized on the columns with chemically bonded phases (CBP), it often appears problem in precise determination of capacity factors (k') for the substances characterized by too rapid elution (low values of retention volume). In consequence, determination of dead volume (V_o) of the reverse-phase (RP) HPLC columns is difficult and/or may often be saddled with a significant error [1-3].

There are known different methods of V_o determination. Some of these methods are based on gravimetric measurements [4,5], other on linearization of logarithmic dependence of reduced retention time and the number of carbon atoms for homologous series of alkylbenzenes [6,7] or alcohols [8]. Moreover, different salts [6] and other organic species [9] were also used for this purpose. However, the most reliable results were obtained by Riedo and Kováts [10], who have determined V_o values using labelled substances (np. D_2O). The application of labelled substances was described also by Knox and Kaliszan [11], Engelhardt et al. [12] and other authors [13]. Above mentioned authors have considered the elution mechanism of unretained substance in correlation with the column bed porosity taking into account the interactions between stationary phase, mobile phase and injected substance (marker). This problem was described in details by Smith et al. [3]. In most cases a dead volume was determined on the basis of the peak localized near the staring point of eluted substances, and before first basic separated peak.

It is known that during RP HPLC elution using of mixed solvents as a mobile phase the extra peaks appear on

the chromatogram, near peaks corresponding to individual components of analyzed samples. These peaks can not be identified with any separated substances. This phenomena is observed particularly in the case of binary mobile phase and/or contaminated eluent [3,14]. These peaks are usually registrated either by nonselective *e.g.* UV photometer or refrectometer, as well as by specific, *e.g.* electrochemical detectors [15]. In the literature these peaks are called as: "*ghost peaks*", "*solvent peaks*", "*system peaks*", "*eigen peaks*" and/or "*vacant peaks*" [3,9-14,16-22].

Slais and Krejci [14] suppose that the presence of these peaks are connected with the local change concentration of the mobile phase components after sample injection or even with the fact that composition of eluent differs slightly from the mobile phase concentration in equilibrium. In opinion of McCormick and Karger [16] formation of these peaks is due to displacement of organic solvent from stationary phase after injection of analyzed sample. Berek et al. [17,21,22] suggest the existence of strict relation between formation of solvent peaks and the changes in solvatation of stationary phase by the solvent molecules. Such effect was confirmed by Melander et al. [18]. Riedo and Kováts [10] have shown that for eluent consisting of (N+1) components it obtains N solvent peaks after injection of trace amount of one of the mobile phase component. Elution volume of solvent peak is constant independently on the fact which component is injected if the disturbations in mobile phase concentration are relatively low. Knox and Kaliszan [11] have presented the relationship between the magnitude of baseline disturbation and retention volume of solvent peaks. Erkekenes et al. [23] have noticed

that injection of pure water causes the formation of the peak generated by dilution of mobile phase. Moreover, in their earlier papers Buszewski et al. shown that scattering of UV light in detector cell [24] as well as the mode of sample injection by means of different injectors can influence also perturbation in baseline and in consequence can cause the generation of extra peaks during RP HPLC elution.

Considering the reasons of solvent peaks formation many authors have tried to utilize them to determination of dead volume (V_o). However, determination of this parameter presents many difficulties either theoretical and/or practical nature. This is connected with numerous effects taking place during RP HPLC elution and influencing the retention, or in the difficulties connected with in precise determination of parameters characterizing of the column packing. It should be noted that the results obtained by different research workers are are difficult to comparison because of very different origin of packings and columns used in these investigation. Hence, having the packing with controlled coverage density and complete surface characteristics we have tried to utilize of generated solvent peaks for explanation of the effects accompanying the RP HPLC elution of standard substances used to the determination of dead volume.

EXPERIMENTAL

Apparatus and physico-chemical investigation

The porosity parameters of the silica-gels (S_{BET} - specific surface area; V_p - pore volume; D - mean pore diameter) were determined by low temperature

adsorption-desorption of nitrogen using a Sorptomatic instrument, Model 1800 (Carlo Erba, Milano, Italy).

The concentration of surface silanol groups (α_{SiOH}) was determined by the method proposed by Nondek and Vyskočíl [26], based on the determination of methane formed during the reaction of the $(CH_3)_2Zn...THF$ complex with silanol groups.

Solid-state NMR measurements before and after chemical modification were performed on a Bruker MSL 200 spectrometer (Rheinstetten, Germany) with samples of 200 - 300 mg in double-bearing rotors of zirconia. Magic-angle-spinning (MAS) was carried out at a spinning rate of 4 kHz. ^{29}Si cross-polarization (CP/MAS NMR) spectra were recorded with a pulse length of 5 μs together with a contact time of 5 ms and a pulse repetition time of 2 s. For ^{13}C CP/MAS NMR spectra a contact time of 12 ms was used. All NMR spectra were externally referenced to liquid tetra-methylsilane (TMS) and the chemical shifts (δ) were given in parts per million (ppm).

The degree of alkylsilyl ligand coverage on the packing surface was calculated from the carbon content, determined with a CHN analyzer, Model 240 (Perkin Elmer, Norwolk, CT, USA).

Chromatographic measurements were carried out on a liquid chromatograph HP-1050 (Hewlett Packard, Waldbronn, FRG) equipped with a 7121 sampling valve (Rheodyne Co., Berkeley, CA, USA) and a Vectra 16/QS (Hewlett Packard) computer system.

Materials and reagents

The spherical SG-7/G silica-gel prepared by Drs. I. Novák & D. Berek of the Polymer Institute, Slovak

Table I.
Physico-chemical characteristics of the used packing materials; α_{RP} = concentration of chemically bonded C_{18} groups [μmol/m^2], α_{SiOH} = concentration of accessible silanol groups [μmol/m^2], D = mean pore diameter [nm], V_p = pore volume [cm^3/g], S_{BET} = specific surface area [m^2/g].

No. of Porosity packing	Type of packing	Surface coverage C(%)	α_{RP}	α_{SiOH}	D	V_p	S_{BET}
1	*Bare silica gel*	-	-	5.21	20.0	2.1	361
2		5.08	0.72	4.53	18.28	1.92	338
3	*Silica gel C_{18}*	11.69	1.61	3.45	17.37	1.81	323
4	*monomeric*	17.20	2.60	2.52	15.71	1.65	314
5	*structure*	21.20	3.46	1.58	15.24	1.60	203
6		24.50	4.24	0.42	14.28	1.55	175

Academy of Sciences (Bratislava, Czecho-Slovakia) was used as a support of chemically bonded phases. The physico-chemical characteristics of bare and modified materials are given in Table I.

For chemical modification, octadecyldimethylchlorosilane (MC_{18}) (Wacker GmbH, München, Germany) and special prepared morpholine as an reaction activator [28] (Reachim, Moscow, Russia) were used.

Methanol and water (*Polskie Odczynniki Chemiczne* - POCh, Gliwice, Poland and J.T. Baker, Gros Gerau, FRG) were used for eluents preparation and as markers in chromatographic investigations. All the other solvents, *i.g.* toluene, benzene, n-hexane, propan-2-ol were of analytical grade purity (POCh, Gliwice and/or Merck, Darmastadt, FRG). Used as other markers D_2O and CH_3OD purchased from the Nuclear Research Institute (Swierk/near Warsaw, Poland).

Stainless-steel tubes (100 x 4.0 mm I.D.) were purchased from *Chemical Reagent Factory* LPPH-OCh (Lublin, Poland).

Surface chemical modification

Chemical modification of the surface of silica-gel supports was carried out under vacuum (10^{-3} Pa) in a glass reactor, made without contact of the reagents with the environment [29], using only monofunctional silanes. This method of mechanism and reaction conditions have been published previously [28,29].

HPLC column packing procedure

The slurry, 1.8 g of the prepared stationary phases in 35 ml propan-2-ol, was placed in an ultrasonic bath for 5 minutes and then filled into the column using 150 ml methanol as a packing solvent. All columns were packed under a pressure of 50 MPa using a Haskel D-122 packing pump (Haskel Inc., Burbank, CA, USA) according to the procedure described earlier [27,28].

RESULTS AND DISCUSSION

Surface characterization

In Table I are listed the important data characterized material packings with controlled coverage density of C_{18} CBP used in the investigations. From these data results that with the increase in percentage the carbon deposited on the surface (% C and α_{RP}) the decrease of surface concentration of accessible silanol groups (α_{SiOH}) is observed. Moreover, with the increase of α_{RP} values the parameters characterized the porosity (D, V_p

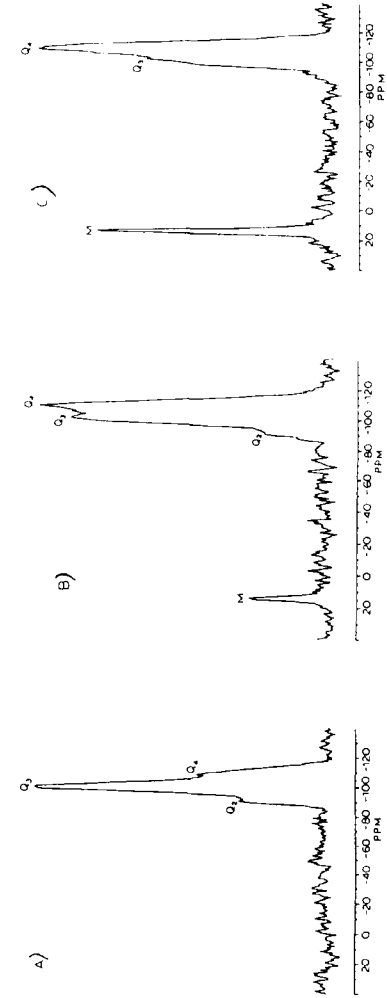

Fig. 1. ^{29}Si CP/MAS NMR spectra of bare # 1 (a) and modified silica gel (b) - packing # 2, (c) - packing # 6.

and S_{BET}) decrease. For example, regarding the differences between the above parameters observed for the packings # 1 and # 6 (Table I) it observes the reduction of D values by 28.6%, V_p values by 26.2 % whereas the reduction of S_{BET} and α_{SiOH} are very large (51.6 % and 92 %, respectively). On the basis of the above observations it can expect that the packing prepared according to the procedure described earlier should be dense and homogenous [28-30]. However, more reliable information relating to surface structure of formed CBP film may be obtained only by CP/MAS NMR investigations [30-32].

Fig. 1a - c present the ^{29}Si CP/MAS NMR spectra obtained for unmodified silica gel (packing # 1, Table I) and for these materials where accessible silanol groups were blocked by monofunctional alkylsilyl ligands with differentiated coverage density (packings # 3 and # 6, Table I). From the presented spectra as well as from the comparison of chemical shifts [30-32] results that with increase number of alkylsilyl groups present on the support surface (peak M; δ = + 14.0 ppm) there decrease the contributions of geminal Q_2 (δ = - 91 ppm) and free Q_3 (δ = - 100 ppm) silanol groups. The contribution of siloxan groups Q_4 (δ = - 109 ppm) increases proportionally. In the case of packing # 6 (α_{RP} = 4.24 μmlo/m^2, Tabe I) complete, practically, elimination of useless geminal silanols was observed. ^{13}C CP/MAS NMR measurements permit to state whether all alkylsilyl ligands have been covalently bonded with the surface of silica support [31-33].

Fig. 2 presents an exemplar ^{13}C CP/MAS NMR spectrum registrated for the packing # 6 (α_{RP} = 4.24 μmol/m^2, Table I). The bande situated in δ = + 12 ÷ + 33 ppm

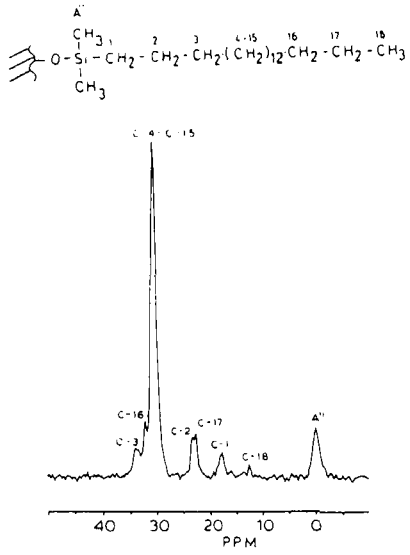

Fig. 2. ^{13}C CP/MAS NMR spectra of monofunctionally modified silica gel (packing # 6)

corresponds to alkyl part of bonded C_{18} ligand, whereas the peak A'' (δ = +2.3 ppm) corresponding $\equiv Si-O-Si(CH_3)_2-$ segment indicates the formation of *"pure monomeric"* structure of CBP [31-33]. This last information has great importance during the considerations of the effects accompanying the RP HPLC elution.

Chromatographic investigation and dead volume determination

Fig 3. presents a typical RP HPLC chromatogram of the separation of aromatic hydrocarbons derivatives obtained on the column with the packing # 6 (Table I). The methanol and water (80 - 20 % v/v) mixture was used as a mobile phase. On the chromatogram, a part from

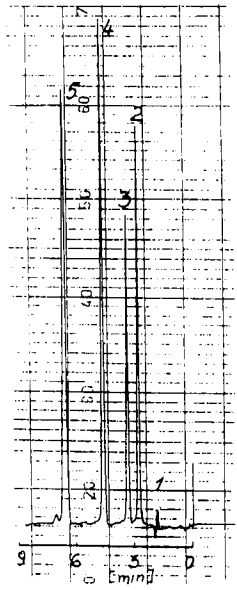

Fig. 3. RP HPLC separation of test mixture: 1) solvent peaks, 2) phenol, 3) cresol, 4) benzene, 5) toluene.
Chromatographic conditions:
column: C_{18} phase, 250 x 4 mm I.D., packing # 6,
mobile phase: 70 - 30 % MeOH - H2O
detector: UV - 254 nm.

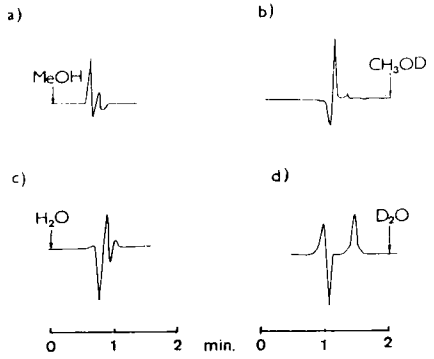

Fig. 4. Chromatograms of solvent peaks for four markers obtained on the columns with packing # 6. Chromatgraphic conditions see Fig. 3.

Table II.
V_{Rs} and R_o values for different markers using 70 - 30% v/v MeOH - H_2O composition as the mobile phase.

No of column and packing	V_{Rs}				V_{Ro}
	CH_3OH	CH_3OD	H_2O	D_2O	D_2O
# 2	1.20	1.18	1.23	1.20	0.57
# 3	1.16	1.16	1.22	1.13	0.56
# 4	1.09	1.09	1.15	1.06	0.59
# 5	0.93	0.95	1.00	0.97	0.56
# 6	0.84	0.86	0.94	0.99	0.58

peaks corresponding to five components of test mixture it can observe also other peaks so called "*solvent peaks*" (Fig 3.). From different authors and our detailled investigations [5,6,8-11,14, 16-20,24,25,34] results that these peaks are generated during RP HPLC elution and are due to by many following factors: intermolecular interactions, solvatation, diffusion, local change of concentration and displacement.

Fig. 4 a - d present the exemples chromatograms containing solvent peaks which are utilized to determination of "dead volume" (V_{Rs}). For this purpose, the methanol (Fig. 4 a), water (Fig.4 c) and their deutered derivatives *i.e.* CH_3OD (Fig. 4 b) and D_2O (Fig. 4 d) were injected onto individual columns. From the comparison of the chromatograms it can be seen that in all considered cases the profiles of solvent peaks differ significantly in spite of the same measuring conditions. The retention volume values (V_R) corresponding to these peaks, although in small degree, are different (Table II). These differences relate to H_2O and D_2O, because retention volumes of CH_3OH and

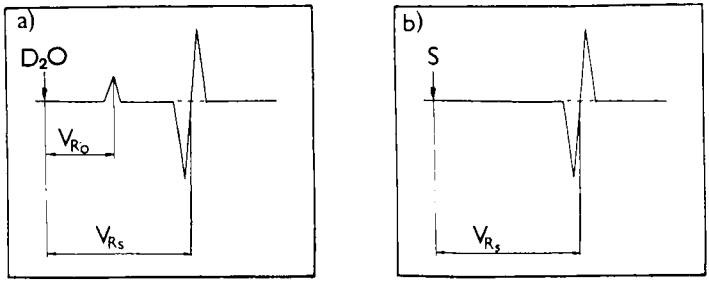

Fig. 5. Graphycal method of dead volume determination for RP HPLC column using D_2O (a) and MeOH, CH_3OD and H_2O (b) as markers.

CH_3OD are nearly the same. Moreover, in the case when D_2O is used as the marker on the chromatograms an additional peak (V_{Ro}) appeared. This peak was utilized by us to detrmine the dead volume. Our results [34] completed by results obtained by Kaliszan and Knox [11] permit to suppose that dead volume of D_2O (V_{Ro}) and other markers (V_{Rs}) may be determined graphically in the way presented in Fig. 5 a & b.

Based on such graphical model of dead volume determinaton it has considered the effect of coverage density of C_{18} CBP on the changes of retention volumes of individual markers. For this purpose the dependences of retention volume (V_{Rs} and V_{Ro}) and/or porosity of packing bed (ϵ) *versus* the composition of binary (methanol-water) mobile phase (ϕ) were utilized (Fig. 6 a - e).

Regarding the presented relationships it can be seen that in four (Fig. 6 a-c & e) among five cases it cannot obtain the significantly marked plateau region permitting to univocal determination of dead volume.

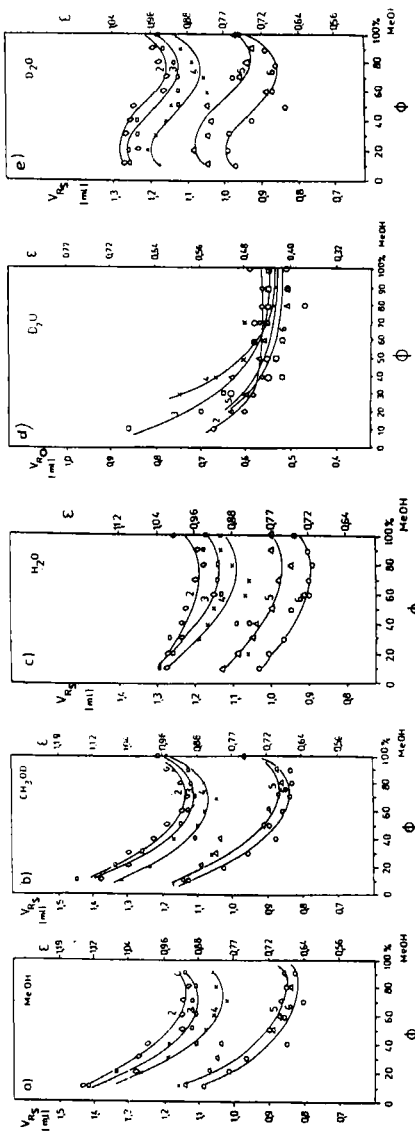

Fig. 6. Relationship of V_{Rs} and V_{Rg} and/or ε *vs* MeOH-H_2O mobile phase composition (ϕ) for MeOH (a), CH_3OD (b), H_2O (c) and D_2O (d & e) used as solvent peak markers.

Only in this case when D_2O is injected into individual columns (Table I) and when content of methanol in mobile phase changes from 50 to 100 % of MeOH the linear run of these dependence can be obtained. Moreover for each curve there is kept the sequence of the increase of retention volumes (V_{Rs} and V_{Ro}) with the decrease of coverage density of packing surface with alkylsilyl ligands (α_{RP}). This can indicate a greate participation of interactions between the marker and accessible unblocked silanol groups (Fig. 6 a-c & e). Hence, the migration of the marker through hydrophobic chains becomes more easy. From the presented relationship the next conclusion may be drawn *i.e.* it can distinguish two types of the courses of the curves corresponding to two different types of preferred interactions. For first type it can expect the greater sorption of the marker on the surface sparsely covered with C_{18} (packings # 2 - 4) due to silanophobic interactions. For second type, there predominate mainly hydrophobic interactions (packings # 5 & # 6) connected with preferential interactions such: chain - chain and chain - marker. This causes a significant decrease of chains mobility which causes of turn that the diffusing effect are less remarkable. The curves corresponding to the packing # 6 (Fig 6 a - e) are situated in lower parts of the diagram in spite of convex profile of the silica gel support pores [35].

The other problem is connected with precise determination of retention volume for D_2O. In this connection the dependence considered by us must be related to two generated peaks (Fig. 6 d & e). The volume expressed by V_{Ro} is practically constant (for the range of 55 to 100 % MeOH in H_2O), and for this reason there was possible the utilization of this

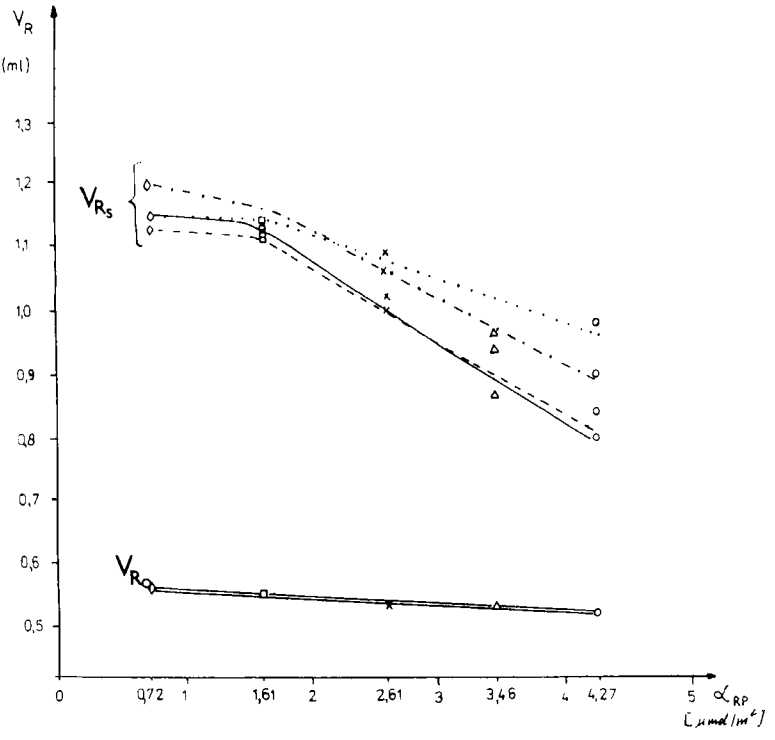

Fig. 7. Dependence between V_R of solvent peaks *vs* α_{RP} for different markers: (·····) D_2O; (·-·-·-·) H_2O; (———) CH_3OH; (- - - -) CH_3OD and (═══) first peak of D_2O.

volume to determination of dead volume. The V_{Ro} values obtained by us are comparable to those obtained by Berendsen et al. [6]. Moreover, from the course of above dependences there result two facts:

(i) lack of interactions between D_2O molecules and alkylsilyl chains; these molecules interact only with residual silanol groups deactivated earlier with water molecules,

(ii) D_2O may be used as the marker in determination of kinetic and thermodynamic dead volume.

This last supposition can be confirmed by the numerical values of retention volume of first solvent peak (Fig. 5 a, Table II). These data are constant for all tested columns when the content of methanol in the binary mobile phase changes from 50 to 100 % v/v (Fig. 6d).
The comparison of dependence V_R (including V_{Rs} and V_{Ro}) *versus* coverage density of alkylsilyl ligands (α_{RP}) at constant composition of mobile phase and constant other measurement conditions is especially interesting (Fig.7). In the case of D_2O (for first peak) the course of this dependence in whole α_{RP} range is practically linear. The slope of the line towards lower V_R (V_{Ro}) value is due probably to greater mobility of alkyl chains as well as to changes in their conformation, especially at low α_{RP} values [30,34]. Individual V_{Ro} values obtained for the columns containing the packings of controlled coverage density correspond probably to kinetic values of dead volume [6]. In this case, other markers the significant deviations from considered course are observed. In the case of other markers are useless to determination of kinetic dead volume according to earlier suggestions of Knox i Kaliszan [11]. However, this not does exclude the possibility of their application to determination of thermodynamic dead volume of the column for HPLC.

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