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Review

Apparent simplicity of reversed stationary phases for high-performance liquid chromatography

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ABSTRACT

The effect of pretreatment and the pore structure of the support, the type of silicon–carbon anchor group and the chain length of the alkyl modifier, the chemical modification procedures and the operating conditions on the characteristics and the structure of the bonded layer of reversed stationary phases for high-performance liquid chromatography have been studied.

CONTENTS

1. INTRODUCTION

Stationary phases based on porous silicas chemically modified with organic compounds are the most widely used type in modern high-performance liquid chromatography (HPLC). The leading position is held by stationary phases with bonded alkyl groups, the so-called reversed phases. Changes in the content of the organic component of a mobile phase and the introduction of special additives allow variations over wide ranges of the selectivity of separation of diverse mixtures, from the simplest ions [1] to biopolymers [2]. Even some special problems such as analyses of enantiomers can be solved by means of chromatographic systems that make use of reversed stationary phases [3,4].
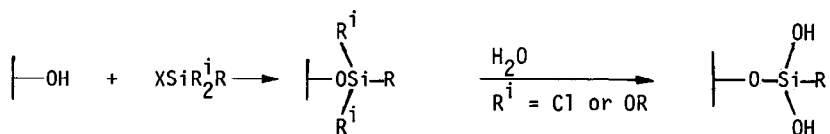
It is due to this versatile character of reversed phases and their apparent simplicity that more than half of all HPLC-based analyses [5] have been performed in the reversed-phase mode. In fact, reversed stationary phases do not contain functional

groups, and the bonded layer can be regarded, in a first approximation, as a thin hydrocarbon film whose thickness and total content of bonded organic material can be varied by changing the alkyl group length and the specific surface area of the silica.

However, by the end of 1970s, it became clear that this simplified approach does not account for the different properties of similar reversed phases. Thus, with the most widely used ODS phases with attached octadecyl radicals, which are produced by numerous manufacturers, not only brands but even batches can differ from each other [6,7]. Reproducibility of the properties and synthesis of reversed phases with preselected characteristics became problematic in connection with the development of preparative and industrial-scale versions of chromatography. Numerous studies carried out in the last decade have widened our knowledge of the peculiarities of the molecular organization of the bonded layer of reversed stationary phases. The aim of this review is to consider briefly the main reasons for the diversity of properties of similar stationary phases and means of solving this problem.

2. MODIFICATION PROCESS AND ACCESSIBILITY OF SILANOL GROUPS

The general and the most widespread method for the synthesis of reversed stationary phases is the interaction of hydroxyl-containing silica with organosilicon compounds of the formula $R_{4-n}SiX_n$, where X is Cl, OCH_3, OC_2H_5, $N(CH_3)_2$ etc. [8]. Owing to steric hindrance, the modification affects only about half of the surface silanol groups and, with bi- or trifunctional modifiers ($n = 2$ or 3), a portion of reactive Si–X groups remains unreacted, thereby producing on hydrolysis additional hydroxyls at the anchor silicon atom:

$$\left|-OH \;+\; XSiR_2^iR \longrightarrow \left|-OSi-R \begin{array}{c} R^i \\ \backslash \\ \diagup \\ R^i \end{array} \xrightarrow[R^i = Cl \text{ or } OR]{H_2O} \left|-O-Si-R \begin{array}{c} OH \\ \diagup \\ \backslash \\ OH \end{array}\right.$$

With trifunctional modifiers this can result in an increase in the surface concentration of silanol groups in comparison with their content in the starting support [9].

Silanol groups can interact with the components of a mobile phase, thereby influencing the structure of the bonded layer, and also with solutes, creating non-uniformity of adsorption centres. Hence the idea of the absence of functional groups in reversed stationary phases is erroneous. The silanol groups represent the "functional" groups; interactions with these groups in the separation of basic compounds can become predominant [10].

It has been shown that the selectivity of silica modified with octadecyltrichloro-silane with respect to the aniline–benzene pair is 33. Capping of silanol groups by additional treatment with trimethylchlorosilane ("end-capping") or the use of octadecyldimethylchlorosilane as a monofunctional modifier results in a change in elution order, the selectivity being equal to 0.4–0.5 (9:1 methanol–water as eluent) [10]. It therefore follows that the content of accessible silanol groups in "genuine" reversed stationary phases should be as low as possible. To achieve this goal, "end-capping" is usually performed, which involves additional treatment with small-size silanizing

agents (trimethylchlorosilane, hexamethyldisilazane, etc.). The efficiency of such a process is usually not reported, although the accessibility of silanol groups can be analysed by existing procedures [5]. The absence of data on the amount and accessibility of the surface silanol groups in many instances does not allow the logical selection of a reversed stationary phase to meet the demands of a particular problem and often produces incorrect conclusions on the irreproducibility of chemical modification of silica with alkylsilanes. It is obvious also that the number of silanol groups accessible and the contribution to retention of interactions therewith depend on the size and chemical character of compounds to be analysed.

To characterize chromatographic phases in more detail, it is desirable to have additional information on the accessibility of silanol groups expressed as selectivity coefficients in the separation of a specific pair of compounds, e.g., aniline–benzene [10].

When considering reversed stationary phases, it was first assumed that the starting silica was chemically pure with identical silanol groups. Unfortunately, real supports often contain appreciable amounts of admixtures that can create, provided they are accessible to the solute molecules, a broad range of adsorption centres with different properties. What is essential is that silanol groups at a silicon atom linked to an extraneous atom can differ markedly from "pure" silanol groups. This can be illustrated by a study [11] on the adsorption activity of accessible silanol groups of reversed stationary phases by a paramagnetic probe. The dynamic behaviour was examined of nitroxyl radicals adsorbed on samples of silica modified with alkyltrichloro- and alkyldimethylchlorosilanes and on samples of some silica with a zirconium oxide film applied and then modified with alkyltrichlorosilanes. In the case of phases of the type
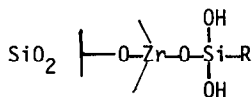
the behaviour of the probe differs from that for the pure silica modified with alkyltrichlorosilane and is close to that for silica modified with alkyldimethylchlorosilane. It therefore follows that the adsorption activity of silanols at silicon linked to a zirconium atom is considerably lower.

Chemically pure silica also contains a set of silanol groups with different properties, which, provided these groups are accessible, results in a set of adsorption centres of different activity in a modified silica. Special treatment can alter the ratio of silanols of different types and this affects markedly the chromatographic properties of reversed stationary phases [12,13].

3. BONDING DENSITY AND DISTRIBUTION OF BONDED MOLECULES THROUGHOUT THE SURFACE

It is seen that it is necessary to achieve maximum shielding of the surface of the original support in order to obtain reproducible reversed stationary phases. There are two approaches that which are usually employed, viz.. the use of optimum

modification conditions which ensure a bonding density of 2.4–2.9 groups/nm^2 (or 4.0–4.8 μmol/m^2) [14,15], which is close to the theoretical limit, or additional silanization ("end-capping"), which permits variations of the bonding density of a long-chain modifier over a broad range.

An alternative approach advocated by Kirkland *et al.* [16] involves the use of modifiers with bulky alkyl substituents in an anchor group, *e.g.*, ClSi(i-C$_3$H$_7$)$_2$R; such sterically protected bonded layers effectively shield the original silica surface and are more stable towards hydrolysis than conventional alkyldimethylsilyl modifiers.

Methods have been elaborated that ensure the theoretically possible extent of bonding. The first method involved the use of modifiers of the formula (CH$_3$)$_2$NSi(CH$_3$)$_2$R, which were introduced by Kováts and co-workers [15,17], and subsequently the use of chlorosilanes in the presence of base with optimization of the parameters of the modification process and the ratio of reagents [18,19].

Table 1 lists data [17–23] that demonstrate that bonding densities close to the theoretical limit (entries 1–11) can be achieved when sufficiently wide-pore silicas and optimum conditions of the process are employed. Modification with alkylchlorosilanes in the absence of organic bases (entries 12 and 17) or with alkoxysilanes (entries 21 and 22) substantially decreases the bonding density to 2.3–2.9 μmol/m^2, which is equal to 54–69% of the maximum value. This decrease cannot be accounted for only by the decrease in the reactivity of the modifiers, as even prolonged (over 24 h) treatment with an excess of modifier does not result in an increase in bonding density [18,19].

We consider that this is connected with different mechanisms of coverage of the surface with modifier molecules and, in general, three variants are possible: (a) statistical (random) coverage, when modifiers bond independently of each other (Fig. 1A); (b) island-like coverage, where a bonded molecule favours fixation of the next molecule in the close neighbourhood ("positive neighbour effect") (Fig. 1B); and (c) uniform (homogeneous) coverage, which is characterized by attachment of a new modifier molecule to the surface preferably some distance away from the already bonded molecules (Fig. 1C).

As can be seen from Fig. 1, the properties of the modified surface will differ depending on the type of distribution, both at low and high degrees of surface coverage. Indeed, at low levels the bonded layer consists of aggregates or "islands" of bonded molecules for island-like coverage of separate bonded molecules for uniform coverage, and of aggregates together with separate molecules for random coverage.

It is seen that only with island-like coverage is the theoretically highest bonding density attainable (Fig. 1). The bonded molecules are unable to undergo lateral motion and in the case of random coverage this leads to the appearance of non-uniform coverage with numerous defects, *i.e.*, areas of "naked" surface of the silica whose dimensions can reach those of an organosilicon portion of a modifier (*ca.* 0.4 nm^2).

Mathematical modelling of random coverage of a surface with disks [24] demonstrates that the extent of coverage in this instance is only 51–56% of that theoretically possible for a close-packed monolayer. This value is in a good agreement with those obtainable on modification of silica with alkoxysilanes or chlorosilanes in the absence of organic bases.

It therefore appears that the above-mentioned modification proceeds with a random type of surface coverage, whereas the use N,N-dimethylaminosilanes or chlorosilanes together with organic bases results in island-like coverage.

TABLE 1

EFFECT OF THE CHAIN LENGTH, PORE STRUCTURE OF THE SUPPORT AND TYPE OF MODIFIER ON THE BONDING DENSITY [17–23]

| No. | Modifier (activator[a]) | Support | | Bonding density | Ref. |
		S (m²/g)	D_{pore} (nm)	(μmol/m²)	
1	$(CH_3)_2NSi(CH_3)_3$	179	—	4.77	17
2	$(CH_3)_2NSi(CH_3)_2C_3H_7$	179	—	4.31	17
3	$(CH_3)_2NSi(CH_3)_2C_{10}H_{21}$	179	—	4.30	17
4	$(CH_3)_2NSi(CH_3)_2C_{18}H_{37}$	179	—	4.20	17
5	$(CH_3)_2NSi(CH_3)_2C_{22}H_{45}$	179	—	4.16	17
6	$(CH_3)_2NSi(CH_3)_3$	28	130	5.23	20
7	$(CH_3)_2NSi(CH_3)_2C_6H_{13}$	28	130	4.36	20
8	$(CH_3)_2NSi(CH_3)_2C_{10}H_{21}$	28	130	4.16	20
9	$(CH_3)_2NSi(CH_3)_2C_{18}H_{37}$	28	130	4.13	20
10	$(CH_3)_2NSi(CH_3)_2C_{22}H_{45}$	28	130	4.40	20
11	$ClSi(CH_3)_2C_{18}H_{37}$ (morpholine)	360	20	4.21	19
12	$ClSi(CH_3)_2C_{18}H_{37}$ (without activator)	360	20	2.28	19
13	$ClSi(CH_3)_2C_{18}H_{37}$ (pyridine)	360	20	3.68	19
14	$ClSi(CH_3)_2C_8H_{17}$ (imidazole)	296	25	3.53	18
15	$ClSi(CH_3)_2C_8H_{17}$ (2,6-lutidine)	296	25	3.48	18
16	$CF_3OCOSi(CH_3)_2$ (2,6-lutidine)	296	25	3.45	18
17	$ClSi(CH_3)_2C_8H_{17}$ (without activator)	320	15	2.90	18
18	$(CH_3)_2NSi(CH_3)_2C_8H_{17}$	297	25	4.10	21
19	$CF_3OCOSi(CH_3)_2C_8H_{17}$ (2,4-lutidine)	297	25	3.80	21
20	$ClSi(CH_3)_2C_8H_{17}$ (2,4-lutidine)	297	25	3.57	21
21	$CH_3OSi(CH_3)_2C_8H_{17}$ (2,4-lutidine)	297	25	2.55	21
22	$C_2H_5OSi(CH_3)_2C_8H_{17}$ (2,4-lutidine)	297	25	2.39	21
23	$ClSi(CH_3)_2C_{16}H_{33}$ (pyridine)	67	50	3.66	22
24	As 23	66	25	3.50	22
25	As 23	186	13.5	3.33	22
26	As 23	283	11.5	3.17	22
27	As 23	213	10.5	2.83	22
28	As 23	476	5.4	2.17	22
29	$Cl_3SiC_{16}H_{33}$ (pyridine)	90	45	3.67	23
30	As 29	115	40	3.67	23
31	As 29	250	14	3.50	23
32	As 29	274	13	3.50	23
33	As 29	420	9	3.00	23
34	As 29	527	7	2.33	23

[a] Dimethylaminosilanes are bonded without activator in all instances.

The above theoretical considerations were recently confirmed in direct experiments.

A method was developed [25,26] for studying the relative positions of molecules fixed on silica, based on electron spin resonance (ESR) analysis of ion-radical tetracyanoquinodimethane salts, which are used as a paramagnetics probe. Its application to alkylsilanes revealed that the coverage of the surface with alkylchlorosilanes is of the island-like type in the presence of pyridine and of the random type in its
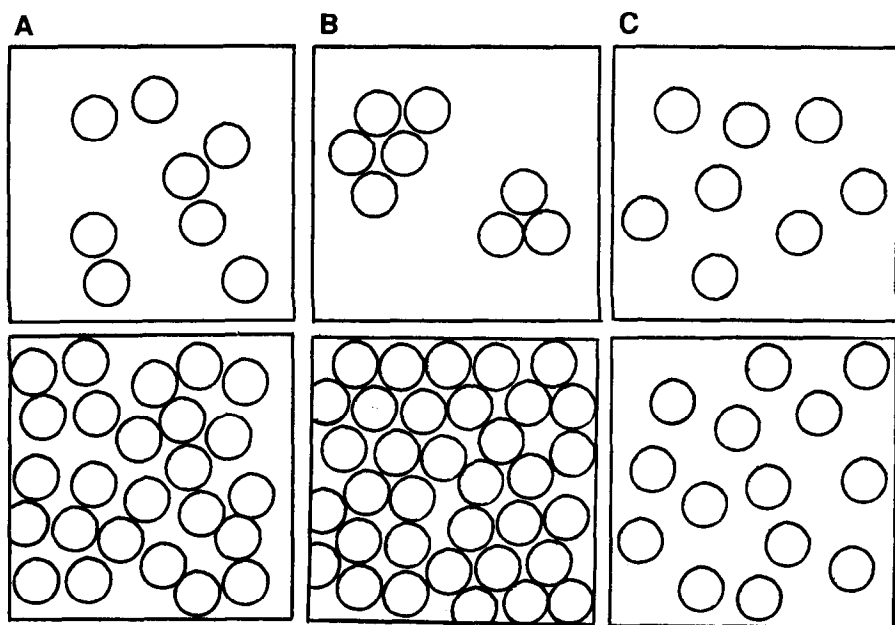
Fig. 1. Variants of the distribution of bonded molecules along the surface at low (top row) and close to the limiting (bottom row) degree of coverage: (A) random; (B) island-like; (C) uniform.

absence [27]. Alkoxysilanes were found to cover the surface in a random or uniform mode.

Rationalization of the reasons for a particular type of coverage is not possible owing to a lack of experimental data. However, some suggestions can be made. For example, the island-like mechanism of coverage with N,N-dimethylaminosilanes can be rationalized as follows. The reaction of a modifier with a silanol group results in liberation of dimethylamine. The latter is adsorbed on an adjacent silanol group to ensure its higher reactivity in comparison with other, free, silanol groups:
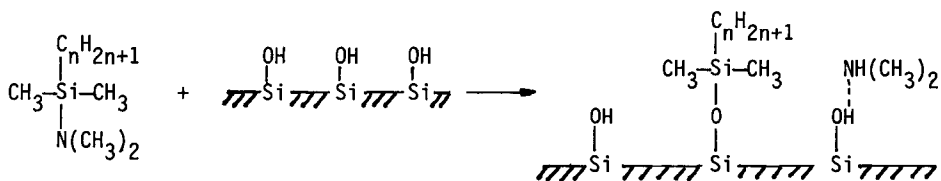
Attachment of the next modifier molecules liberates the adsorbed dimethylamine with the formation of one more molecule of the base. It thus results in a permanent increase in concentration of a base in proximity to the emerging island of the bonded molecules.

Activation of the adjacent position on modification with alkylchlorosilanes in the presence of an organic base is probably connected with disturbance of the adsorption layer close to the bonded molecule caused by the hydrochloride of the base

used. It is this temporary defect in the adsorption layer that can be the preferred site for the reaction with the next molecule of the modifier.

What is essential with modification in the presence of organic bases is the order of introduction of reagents: the base should precede the modifier [27], otherwise a portion of the latter will have time to cover the surface randomly and subsequent addition of an organic base will not repair defects in the bonded layer already formed. Hence the role of the base is not reduced simply to binding of hydrogen chloride evolved [18,19]. Realization of one or other type of coverage depends on the type of organic base used.

One more reason for different coverages of a surface with a modifier can be associated with energetic non-uniformity of the surface or variations of the concentration of silanol groups in different regions of the surface. The distribution of silanol groups is believed to be uniform [8] so that their surface concentration is almost the same for any region. A model of non-uniform distribution of silanol groups has been suggested [28,29] on the basis of experiments with pyrene derivatives that predetermines the character of scattering of the modifier. Non-uniformity can also be connected [30] with geometric irregularities of the surface itself, the effective concentration of silanol groups being higher at sites of contacts of primary globes and lower at their tops.

Experimental data also cannot be neglected that show that several types of silanol groups differing in acidity and reactivity exist on the surface of silica [8,12,13,31]. Nevertheless, it is primarily the chemical nature of the modifier and the modification conditions that determine the character of coverage of the surface and bonding density in the case of wide-pore hydroxylated silicas. The same support may be covered in both an island-like and a random manner [27].

Bearing in mind that the bonding density does not depend on the alkyl group length in the case of non-porous or wide-pore silicas (Table 1), it is worth mentioning again the peculiarity of trimethylsilyl derivatives, which afford higher bonding densities. This feature allows these derivatives to be used to correct the majority of defects of reversed stationary phases, provided that bonding of a long-chain modifier has been performed under conditions that did not ensure maximum bonding density. Additional silanization ("end-capping") results in a higher total concentration of bonded alkyl groups up to the level of closest bonding [19]. Nevertheless, the accessibility of silanol groups seems to remain higher than on the closest bonding of long-chain alkylsilanes [19].

4. ROLE OF THE PORE STRUCTURE OF THE SUPPORT

Consideration of the characteristics of reversed stationary phases did not take into account the effect of geometric structural properties of the support. At the same time, even the simplest analysis of a bonded layer demonstrates that its structure depends to a large extent on the pore structure of silica [14,23,32]. Studies of the effect of the pore structure of the support on bonding density have been reported [14,23,32]. Bonding density was shown [14] to decrease linearly with increase in the chain length of the alkyl radical bonded for a fixed pore size, and this fits well the theoretical models.

The data in Table 1 clearly show that the bonding density of alkylsilyl radicals is much lower than the maximum bonding density for a support with an average pore

diameter of 10–14 nm and it decreases with decreasing average pore diameter of silica. This is accounted for by the appearance of steric hindrance, which prevents a free arrangement of long alkyl chains in pores of small diameter.

With a flat surface and wide-pore supports, the maximum bonding density is determined by geometric parameters of the organosilicon moiety (anchor group) of the modifier. The bonded hydrocarbon chain in this instance possesses high conformational flexibility. Depending on temperature and solvent, they may form almost any conformation, from a "brush" structure with straight bonded molecules extended perpendicular to the surface, to a "liquid-like" structure with bonded chains folded so as to fill in the closest way the space adjoining the support surface (Fig. 2). The structure with high conformational mobility has been termed flexible [22].
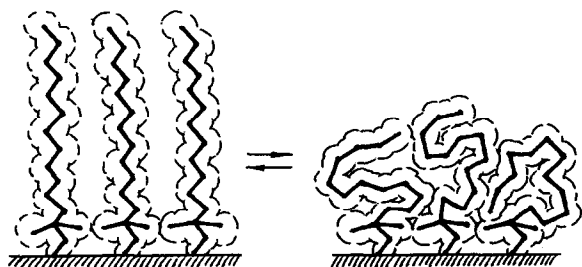

Fig. 2. Flexible structure of bonded layer. The possible conformational transition between "brush" and "liquid-like" structures is shown.

A decrease in the pore diameter of the support will result inevitably in a situation where the bonded long-chain molecules undergo mutual steric hindrance. The bonding density in this instance depends on the ratio of the average pore diameter or, more exactly, on the pore-size distribution and the length of the modifier alkyl chain [14,22,23,32,33].

Consideration of the bonded layer of reversed stationary phases within the framework of the model of cylindrical pores demonstrates that on passing to pores of smaller diameter there is a diameter D^* stating from which the bonding density of alkyl chains decreases linearly with decrease in the pore diameter [22,23]. In small pores, in conformity with the principle of maximum bonding density [22] which follows from the irreversibility of the chemical modification, long-chain alkyl chains have to adopt a conformation that provides for their most compact packing on a surface (otherwise they just cannot fit the small pores) (Fig. 3). Calculations show [22] that the bonded layer thickness may be of a strictly definite value for a small pore with a diameter $D < D^*$ regardless of the length of a hydrocarbon radical. The bonded layer thickness also decreases linearly with decrease in pore diameter and such a layer with a strongly restricted conformational mobility of the attached chains possesses a "rigid" structure [22].

The value of D^* depends on the modifier alkyl chain length and is 4.9 for C_8, 9.8 for C_{16} and 10.8 nm for C_{18} chains [22]. Hence the use of small-pore supports sets limits for attaining the maximum bonding density.

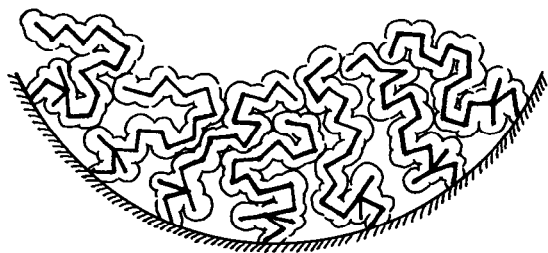The situation is more complicated because each support is characterized by

Fig. 3. Rigid structure of bonded layer in narrow pores (cylindrical model).

a pore-size distribution. Hence a portion of the pores is accessible for free arrangement of a modifier whereas the remaining part is too narrow. Thus, for example, sample No. 25 (Table 1) is characterized by an unsymmetrical pore-size distribution. The starting support contains a considerable number of pores with diameter less than 9.8 nm, so that about 60% of the whole surface is concentrated in pores with diameter $D < D^*$ for the hexadecyl radical. It is this circumstance that determines the low value of the bonding density despite the fact that the average pore diameter of this support considerably exceeds D^* for the hexadecyl radical.

According to the classification given previously [22], the bonded layer possesses a "rigid" structure in pores with diameter $D < D^*$ and a "flexible" structure for $D > D^*$, taking into account the pore-size distribution, and the purely rigid structure will exist for samples the pore-size distribution of which includes values up to D^* (i.e., the diameter of all the pores is less than D^*), and a purely flexible structure will be characteristic of samples the pore-size distribution of which starts from D^*, i.e., the diameter of all the pores is greater than D^*. Thus, for instance, ODS phases ($D^* = 10.8$ nm) will be rigid for silica supports with an average pore diameter of 4–8 nm and flexible if the average pore diameter exceeds 15 nm. Should the average pore diameter be close to D^* the bonded layer possesses an intermediate structure, a portion of the modified surface being rigid and the remainder being flexible.

Analysis of a more real globular structure of silica makes the situation more complex. In fact, the rigid structure is realized at regions close to the sites of contact of primary globes whereas the labile structure is realized at their tops (Fig. 4). Nevertheless, the main conclusions drawn from consideration of a cylindrical model also hold for the globular pore model. Thus, in pores with a diameter of 6 nm about 100% of the bonded layer of octadecyl radicals is rigid, whereas in pores with a diameter of 15 nm the major part of the attached chains does not undergo mutual steric hindrance and may change their conformation easily. The broken line in Fig. 4 represents the space that can be occupied by totally extended octadecyl chains.

Previous calculations [34] demonstrate the effect of the geometric structural properties of the support, viz., the average pore diameter D_0 (the maximum pore-size distribution), the dispersion of the pore-size distribution (σ) and the bonded chain length, on such properties of the bonded layer of reversed stationary phases as carbon content, bonding density (ρ) and the percentage of rigid structure (R) (Figs. 5–8).

As follows from these calculations, it is unreasonable to use supports with an average pore diameter of 4–6 nm and octadecylsilyl modifiers in an attempt to increase
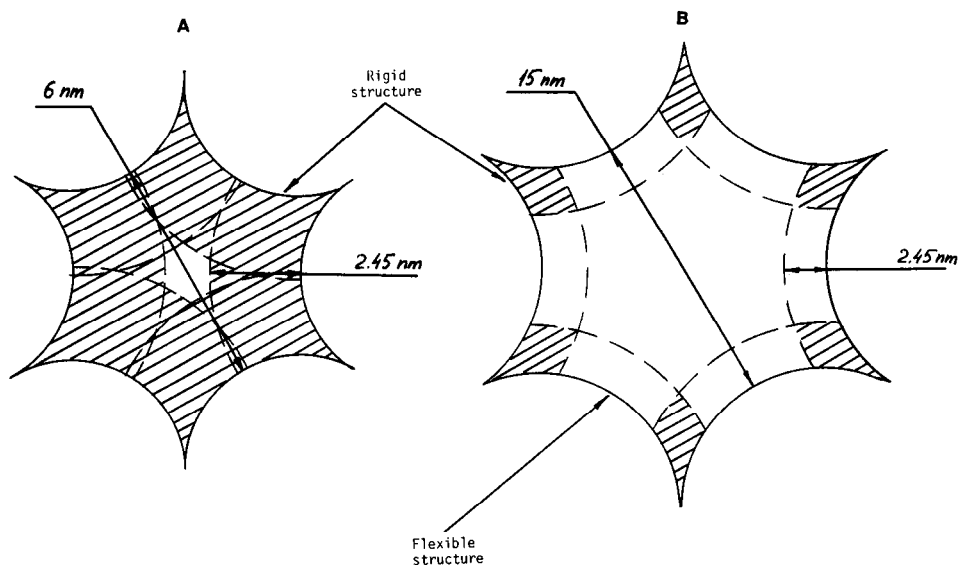
Fig. 4. Rigid and flexible structures within pores of a support modified with octadecylsilyl groups (globular model). (A) Pore diameter 6 nm; (B) pore diameter 15 nm. Broken line corresponds to the length of totally straightened C_{18} chains (2.45 nm).

the carbon content owing to the increase in specific surface area. The increase in the latter is accompanied by a decrease in bonding density (Fig. 7) and consequently the carbon content is not greater than those of supports of average pore diameter 8–10 nm (Fig. 5).

These calculations show that a decrease in the average pore diameter to below 12–14 nm results, for ODS phases, in a sharp increase in the proportion of rigid structure (Fig. 8). The adsorptive and chromatographic properties of reversed-phase supports based on small-pore silicas should differ markedly from those of stationary

Fig. 5. Effect of the average pore diameter and dispersion of the pore size distribution, σ, on the carbon content for C_{18} reversed stationary phases. Pore volume = 0.75 ml/g. 1, σ = 1 nm; 2, σ = 3 nm.

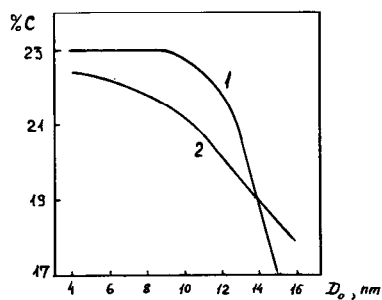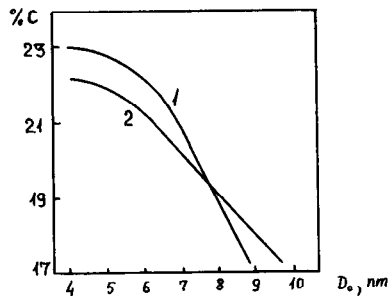Fig. 6. Effect of the average pore diameter and dispersion of the pore size distribution, σ, on the carbon content for C_8 reversed stationary phases. Other details as in Fig. 5.
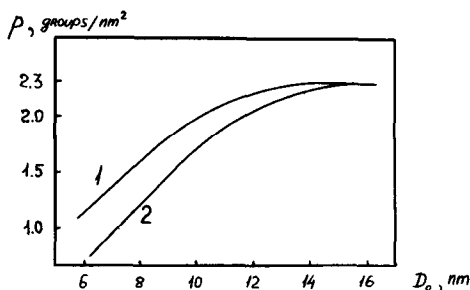
Fig. 7. Effect of the average pore diameter and dispersion of the pore size distribution, σ, on the bonding density for C_{18} reversed stationary phases. 1, $\sigma = 1$ nm; 2, $\sigma = 3$ nm.
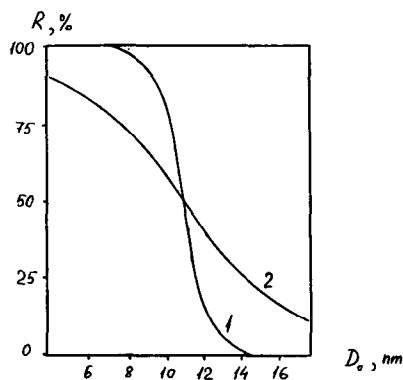
Fig. 8. Effect of the average pore diameter and dispersion of the pore size distribution, σ, on the percentage of rigid structure for C_{18} reversed stationary phases. Other details as in Fig. 5.

phases with a flexible structure, provided that theoretical considerations on rigid and flexible structures are in good agreement with the real situation. This difference has found direct proof in studies of the behaviour of fluorescent [35] and paramagnetic [11] probes adsorbed in a bonded layer of alkylsilane-modified silicas.

The effective viscosity of the bonded alkyl layer for wide-pore ($D_0 = 40$–50 nm) modified silicas increases regularly from 1.7 cP for a trimethylsilyl-modified material to 231.7 cP for an octadecylsilyl-modified material, pyrene being the fluorescent probe [35]. It was shown that the activation energy for diffusion of pyrene into the bonded layer increases linearly with increase in the bonded alkyl chain length. The bonded layer is therefore totally permeable to pyrene, interacting with the layer throughout its thickness. A decrease in the average pore diameter of a support modified with hexadecyldimethylchlorosilane results in a decrease in the effective viscosity from 162.5 cP ($D_0 = 45$ nm) to 36.0 cP ($D_0 = 5.4$ nm). It therefore follows that the behaviour of the bonded layer of hexadecyl chains resembles that of the layer with C_{10}–C_{12} alkyl groups. Pyrene molecules behave as if they slip along the surface, being unable to penetrate the densely woven rigid structure.

Analogous results were obtained with stable nitroxyl radical as a paramagnetic probe [11]. ESR spectra of nitroxyl radicals adsorbed on a C_{16} phase bonded to supports with average pore diameters of 5.6, 10.5 and 11.5 nm are the superposition of signals of two types, which correspond to rapid and slow rotation of the radicals. The latter is associated with the molecules that have penetrated the bonded layer whereas the former is manifested by molecules adsorbed on the surface of the layer. Rapidly rotating radicals behave like radicals adsorbed on silica modified by trimethylsilyl groups.

With respect to chromatographic processes, rigid and flexible structures should appear different. In the latter instance the separation mechanism for liquid chromatography should be closer to partitioning whereas an adsorption mechanism should be realised for supports with a rigid structure. These supports appear as stationary phases with bonded chains of smaller length.

Fig. 9 shows the dependences obtained by Karch *et al.* [36]. On passing from a wide-pore silica (Si-500) with an average pore diameter of 50 nm modified with octadecyl radicals (6.5% C) to a support based on silica Si-100 (D_o = 10 nm) with a carbon content of 22%, an increase is observed, as expected, in the capacity factors for aliphatic alcohols in reversed-phase chromatography (Fig. 9A). An analogous support based on silica Si-60 (D_o = 6 nm) with the same content of bonded octadecylsilyl modifier (22% C) retains alcohols much more weakly. This effect becomes more pronounced with water as the eluent (Fig. 9B) and is greatest for bulkier molecules such as phenols (Fig. 9C). This can be accounted for if it is kept in mind that a rigid structure is realized for a support with a pore diameter of 6 nm.

The above discussion leads to the conclusion that the use of supports with a rigid structure is hardly recommendable. Indeed, only a part of the bonded organic material participates effectively in chromatographic processes for samples of this kind.
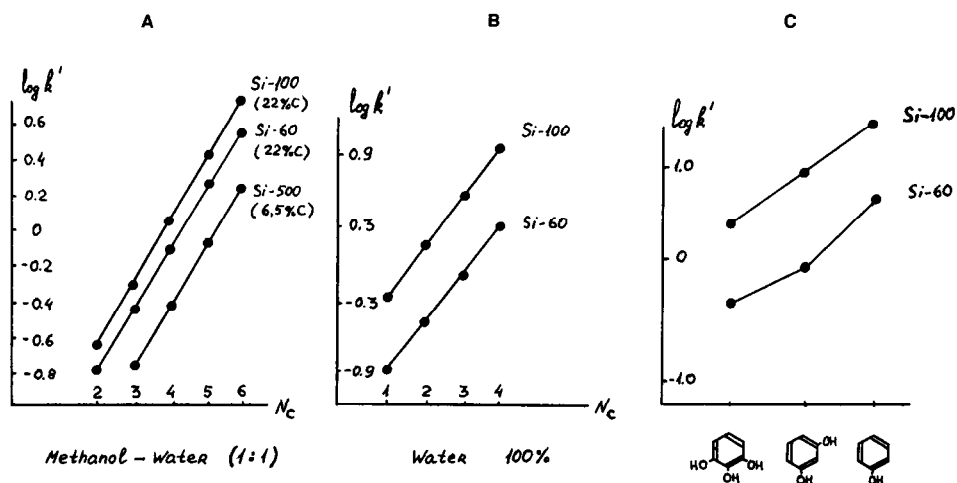

Fig. 9. Effect of the average pore diameter of the initial support (6, 10 and 50 nm) of C_{18} phases on retention of C_1–C_6 aliphatic alcohols (A and B) and phenols (C) in reversed-phase chromatography. Eluent: (A) water–methanol (1:1); B and C, water. Taken from ref. 36.

Supports with an intermediate (mixed) structure of a bonded layer should possess the least reproducible (batch to batch) properties. This can be exemplified as follows. Fig. 8 shows the calculated dependence of the percentage of rigid structure (ODS phase) on the average pore diameter. Most commercial ODS phases are based on supports with an average pore diameter of 8–12 nm. The composition of two specimens of ODS phase with D_o = 10 and 11 nm, which hardly differ in carbon content and bonding density, reveals that they can differ four-fold with regards to the percentage of rigid structure in a bonded layer (Fig. 8, curve 1). Uncontrollable variations of average pore diameter from batch to batch may change the ratio of the surface area with a rigid structure to that with a flexible structure and hence may affect the chromatographic properties of the stationary phases. This example, in our opinion, is an obvious demonstration of how unfortunate is the choice of the octadecylsilyl

modifier–silica Si-100 pair, which is used on a wide scale by the majority of manufacturers.

It is supports based on wide-pore silicas with a flexible structure of the bonded layer that best meet the demands of being "good" reversed stationary phases in which all the organic material attached takes part in the chromatographic process.

Fig. 10 shows a diagram of the bonded layer state for reversed-phase supports which combines the results of calculations [22,34]. It allows the kind of the bonded layer structure (rigid, flexible or mixed) to be determined from the data on the starting support and the alkyl chain length of the modifier and to select the optimum support–modifier pair to prepare a stationary phase with a given structure of the bonded layer. Fig. 11 analogously refers to silica with a wide pore-size distribution; comparison of these two diagrams clearly demonstrates the advantage of a support with a narrow pore-size distribution (small σ). The "broad" distribution increases the irreproducible area of the mixed structure strongly.
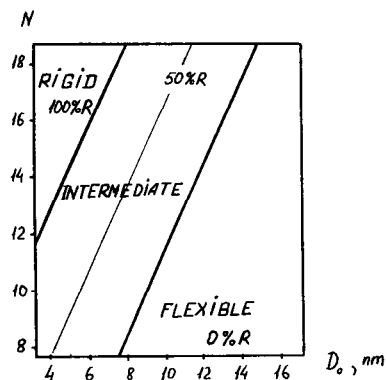

Fig. 10. Diagram of the bonded layer state for reversed stationary phases. Pore volume = 0.75 ml/g; σ = 1 nm.

It was not the aim of this short survey to analyse in detail numerous literature data on physico-chemical studies on the bonded layer of reversed-phase supports. This is covered, for instance, in an excellent review [5]. What is of note, however, is that some authors excessively generalize limited experimental data to all reversed stationary phases irrespective of the relationships between the pore structure of a support and the attached alkyl chain length, which causes the apparent dispersion of data. In this section, an attempt was made to demonstrate that the so-called ordinary bonded ODS phase cannot exist.

In accordance with the principle of geometric structural conformity [34], similar properties are inherent only in samples with bonded layer structures of the same type that is unambiguously determined by a combination of characteristics, viz., pore structure of the support, the type and alkyl chain length of the modifier, bonding density, the type of distribution of the modifier, carbon content and percentage of rigid structure, and not solely by carbon content and chain length of the attached modifier. Results obtained for supports with flexible structures cannot be transferred to
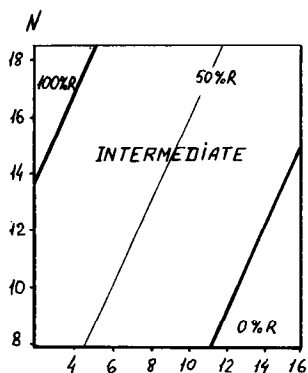
Fig. 11. Diagram of the bonded layer state for reversed stationary phases. Pore volume = 0.75 ml/g; σ = 3 nm.

supports with rigid structures. This means that the very putting of a question on whether the bonded C_{18} layer is "brush-" or "liquid-like" is incorrect. Everything depends on the test conditions and particular sample, as will be illustrated below.

5. EFFECT OF TEMPERATURE ON BONDED LAYER STRUCTURE

Temperature-induced changes in the structural organization of the bonded layer, that is, phase transitions in the bonded layer, can be one of the reasons for alterations in the chromatographic parameters of reversed stationary phases. Of special interest are phase transitions revealed by means of gas chromatography [20,37], liquid chromatography [38,39], differential scanning calorimetry [20,37,39,40] and IR [41] and NMR spectroscopy [37,42].

The temperatures of phase transitions in the "dry" state of the long-chain alkylsilanes with 16, 17, 18, 19, 20 and 22 carbon atoms bonded with maximum density to the surface of wide-pore silica are 7.5, 17.5, 26.5, 31.5, 36 and 45°C, respectively [20]. A phase transition in the bonded layer is manifested by the usual linear plot of ln k' vs. $1/T$ becoming more complex, and even an increase in retention with temperature is observed in the region of phase transition [20,37]. The dependence profile is then determined not only by the properties of the support but also by the nature of the solute and, as a consequence, the selectivity of separation can be changed with temperature, up to inversion of the elution order to sample compounds [39].

Every factor that disturbs the uniformity of the bonded layer lowers the phase transition temperature. According to the data by André (taken from ref. 37), the enthalpy of phase transition in a bonded layer depends largely on the average pore diameter for a wide range of reversed-phase supports with different pore structures. Phase transition is not observed at all with pore diameters less than 5 nm [38,43].

With wide-pore silicas, phase transition is realized over a narrow temperature interval which is essentially broadened as the average pore diameter is decreased owing to non-uniformity of the pore structure [39,40].

The temperature and enthalpy of phase transition decrease as the bonding density is lowered and at bonding densities of less than 2.5 μmol/m^2 no phase

transitions are observed at all [37]. Passage from mono- to bi- and trifunctional modifiers also leads to a decrease in phase transition temperature [37,39].

The absence of a diffuse character of phase transitions with small-pore supports is in accord with the concept of the existence, in narrow pores, of a rigid structure with a limited conformational mobility of the attached chains.

Phase transitions seem to be strongly pronounced only for flexible structures. The physical essence of these phase transitions consists in "melting–solidification" of the bonded hydrocarbon layer and is accompanied by considerable changes in adsorptive properties [20,37,39,40,44].

A sharp increase in the mobility of the bonded alkyl chains at temperatures above the phase transition temperature was demonstrated by NMR spectroscopy [37]. The enthalpy and entropy values of phase transitions in a bonded layer are about four times smaller than the corresponding values for n-alkanes [37,40]. This difference is believed [20,37] to be accounted for by the fact that it is only fragments of bonded chains that participate in surface phase transitions.

A clearly manifested phase transition was shown by GC and DSC methods [20] to be specific only for specimens with sufficiently long ($n \geqslant 16$) alkyl chains. Reversed phases with short hydrocarbon chains ($n < 10$) do not exhibit phase transitions, and they are weak for C_{10}–C_{14} chains.

The above-mentioned phase transitions occur in both the absence and the presence of a solvent. It was shown [39,45] that the temperature of phase transition is higher for aqueous eluents owing to hydrophobic interactions which stabilize the bonded layer structure.

Analysis of the available data allows the conclusion that solutes adsorb apparently at the outer surface of the bonded layer under LC conditions at temperatures below the phase transition temperature. At temperatures above this point the solute molecules can penetrate the bonded layer, which should result in the realization of a partition mechanism of retention.

The most important practical conclusion that can be drawn is that it is necessary to perform separations at temperatures above the phase transition temperature in order to achieve reproducible results. This temperature may vary, depending on the type of silane, bonding density, application (or not) of end-capping within the temperature range 15–30°C for ODS phases, that is, at temperatures close to ambient and which are the most often employed in HPLC. Hence the use of ODS phases is hardly advantageous from this point of view. Insignificant variations in the ambient temperature may result in dramatic alterations of the chromatographic properties of the phase. In this respect we agree with the suggestion [20] that the use of shorter alkylsilanes (C_{16}, C_{14}) is more favourable, which is illustrated by Fig. 12.

Hence the drawbacks inherent to octadecylsilyl modifiers are associated both with the formation of a rigid structure and with irreproducibility as regards phase conditions of the bonded layer. What can be done to alleviate this problem?

Provided that the conventional chain length of the modifier with eighteen methylene units is retained, the bonding density should be decreased. Then the temperature of the phase transition will be below ambient, and the pore diameter at which the rigid structure is realized will be lower. Thus, with a bonding density of 2.5 μmol/m^2 the critical diameter D^* for a C_{18} phase will be 9 nm rather than 10.8 nm [22]. It is important to note that recent experimental results [46] demonstrated that the
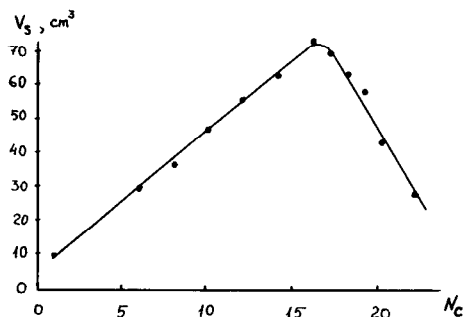
Fig. 12. Dependence of the GC retention volume of heptane at 25°C on the bonded alkyl chain length. Taken from ref. 20.

capacity factors with a C_{18} support remained constant at bonding densities of 2.5 $\mu mol/m^2$ and more. These data confirm previous theoretical calculations [47,48]. The constancy of capacity factors has been established for dimethylaniline and butyl benzoate [46] and it means that the selectivity with respect to the above pair of compounds is also retained over a wide range of bonding densities. Unfortunately, the above data can hardly be considered to be general. For example, the selectivity is markedly changed as a function of bonding density of octadecyl groups with poly-nuclear aromatic compounds [49]. Thus, the selectivity of separation of benzo[e]phen-anthrene and naphthacene is permanently increased from 1.2 to 1.8 within the bonding density range 1.5–4.0 $\mu mol/m^2$ [49].

Hence reproducible reversed-phase silicas can be hardly obtained by decreasing of the bonding density. When decreasing the bonding density, one should also bear in mind that it is possible to obtain different supports with respect to the distribution of bonded molecules depending on the type of modifier and modification procedure used, as was mentioned above. Hence modification under conditions of "island-type" filling, even with a shortage of modifier, does not permit to phase transitions be avoided.

The second way of preventing phase transitions and reducing the contribution of a rigid structure consists in decreasing the alkyl chain length of the modifier. For a C_{16} support the phase transition temperature is below ambient even at the highest bonding density [20]. On the other hand, with silicas of average pore diameter 10–25 nm only part of the bonded methylene units (from nine up to fourteen) interact with the solute, as was shown above in connection with pyrene diffusion [35] and elsewhere [46,47,50]. Considering the chromatographic process, it means that a further increase in the chain length has no effect on capacity factors. Hence, it is possible to suggest that to attain a higher reproducibility one should discard the general use of C_{18} supports and to apply bonded phases with chain lengths of 14–16 carbon atoms. In order to provide a flexible structure of the bonded layer the initial support should not have pores smaller than 10 nm [22].

So far we have mainly considered reversed phases modified by long-chain alkylsilanes. However, supports with bonded C_4–C_8 alkyl chains are also widely used. In this instance there are no problems connected with phase transitions and rigid structures and therefore such reversed phases should be more reproducible. However,

some problems should be taken into account here also. As was shown recently, the conformation of the bonded alkyl chains does not change monotonically with increase in the chain length in the range C_1–C_{10}. For example, it was shown [51] using NMR spectroscopy that C_5–C_8 bonded alkyl groups exhibit the highest mobility. In this instance the mobility of methylene units is increased towards the terminal methyl groups. The methylene units within butyl radicals are almost identical in mobility and are present in a more restrained state than those in C_5–C_8 groups. Differences in conformation and mobility of bonded alkyl groups may lead to variations in interactions with highly organized molecules, e.g., with proteins and peptides [52,53]. It is for such substances that reversed phases with short alkyl groups are useful. There is a sharp maximum in the dependence of retention of paracelcine peptides on the bonded alkyl chain length when using C_2 and C_4 phases; the capacity factors on these phases are 2–4 times greater than those on C_1 and C_5–C_{20} phases [53].

Hence one should been in mind that even slight variations in the structure of the modifier may result in significant changes in the chromatographic properties of reversed-phase packings with respect to chromatography of biopolymers. Therefore, in order to obtain reproducible reversed phases, it is important to use individual compounds, e.g., n-alkylsilanes, that do not contain admixtures of structural isomers.

6. EFFECT OF SOLVENT ON BONDED LAYER STRUCTURE

The nature and composition of the eluent are two of the basic variables in HPLC separation processes. When analysing the behaviour of reversed stationary phases, one should take into account the possible interactions of the solvent molecules with bonded hydrocarbon chains and accessible silanol groups on the surface.

The penetration of eluent molecules into the bonded layer leads to an increase in its volume. As the bonded chains are attached to the surface, the only way to increase the layer volume is to alter their conformation and, as a last resort, to effect a transition from a "liquid-like" to a "brush-type" structure.

The alteration of conformations of bonded alkyl chains that occurs on permeation of the eluent components into the bonded layer and additional structuring of the layer in comparison with "dry" samples have been observed by IR [41] and NMR spectroscopy [54–57].

The change in state of the bonded layer due to penetration of solvent molecules may be the reason for the alteration of the retention mechanism on variation of the eluent composition [58].

Direct experiments with pyrene as a fluorescent probe demonstrated that the diffusion of solute molecules within the bonded layer is markedly increased in the presence of solvent [59]. It was shown that the environment of pyrene sorbed on an ODS support in the absence of a solvent is very non-polar and it corresponds to that in liquid hydrocarbons. In the presence of solvent the environment of pyrene depends both on the eluent composition and on the properties of the ODS support.

On application of binary mixtures, e.g., alcohol–water, acetonitrile–water, tetrahydrofuran–water, the organic molecules having a high affinity to bonded hydrocarbon chains permeate mainly into the bonded layer. However, in relation to the structure of the bonded layer (rigid, flexible), the nature of the modifier and the method of modification, the resulting effect of the solvent on the bonded layer can be

different. Two papers [60,61] dealing with the effects of the eluent composition on the polarity of the bonded layer as studied by analysis of the fine structure of the fluorescence spectra of pyrene sorbed on reversed stationary phases are considered as an example. In these papers LiChrosorb RP-18 based on silica Si-100 modified by octadecylmethyldichlorosilane [60] and Partisil ODS-2 [61] based on silica with a pore diameter of 5 nm [62] modified by octadecyltrichlorosilane and end-capped were studied.

It was demonstrated [60] that when the content of organic modifier in the mobile phase is increased (up to 30% with methanol and up to 14% with acetonitrile), the polarity of the environment of sorbed pyrene is decreased in comparison with an aqueous eluent. In the authors' opinion, this is caused by penetration of organic components of the eluent into the bonded layer and blocking of accessible silanol groups. A further increase of the content of, e.g., acetonitrile (from 14 to 28%) leads an increase in the polarity of the pyrene environment owing to saturation of the bonded layer by the molecules of the organic component.

In contrast, an increase in the polarity of the environment was observed [61] at a low content of organic component in the eluent in comparison with an eluent containing a high concentration of organic component (methanol). This effect was explained by "displacement" of pyrene from the bonded layer at low contents of methanol in the eluent.

In our opinion, these apparently contradictory results [60,61] may be explained by the effect of the pore structure on the state of the bonded layer. Thus, the stationary phase [61] consists in a 100% rigid structure and the penetration of pyrene into the bonded layer is hampered. This is related first to eluents containing a small amount of organic component, which hardly wet the bonded phase. An increase in the organic component content favours wetting of the bonded layer and the partial penetration of pyrene into the layer of attached hydrocarbons. Thus the polarity of the pyrene environment is decreased. The rigid bonded layer causes a low accessibility of silanol groups for both pyrene and methanol. The difficulties in penetration of methanol into the rigid bonded layer were confirmed [63].

According to the models considered above, part of the bonded layer in case of a support based on LiChrosorb Si-100 [60] has a flexible structure. It is difficult to estimate the proportion of flexible structure as some batches of LiChrosorb Si-100 may have an average pore diameter of even 16–17 nm [14], i.e., may contain about 100% flexible structure in bonded layer.

The flexible bonded layer is permeable both to pyrene and solvent molecules. In an aqueous eluent pyrene present in the bonded layer is able to interact with silanol groups. Methanol or acetonitrile molecules at a low concentration in the eluent penetrate into the bonded layer and decrease the polarity of the pyrene environment owing to competing adsorption on accessible silanol groups by the polar part. A further increase in the content of organic modifier results in saturation of the bonded layer and an increase in the polarity of the pyrene environment.

Hence the variations in the structure of the bonded layer of ODS stationary phases may lead to basically different mechanisms of interaction between the support and a solute and the support and a mobile phase.

In contrast to organic components of the mobile phase, the penetration of water molecules into the bonded layer is unfavourable. This process becomes easier with
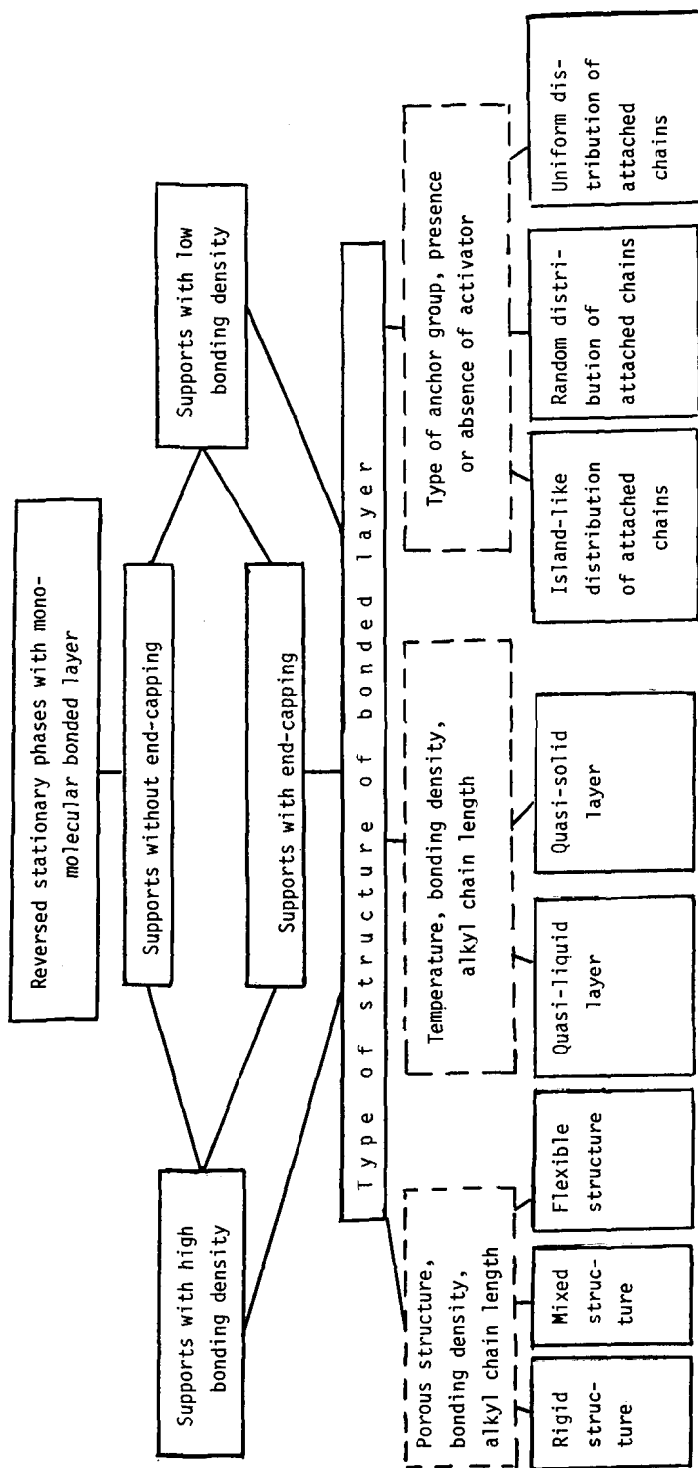
Fig. 13. Types of reversed stationary phases.

increase in temperature and it results in alterations of the conformation of the bonded chains. A series of reversed-phase supports with bonded octyl-, nonyl- and decylsilyl groups were studied [64] and, on the basis of the results obtained, the authors suggested a model of the bonded layer in which bonded chains might be present in a straightening or "collapsed" state. The bonded chains are favourable for association in water, whereas on heating the transfer from an associated into a straightening state occurs. Penetrating between chains at elevated temperature, water molecules prevent collapse of the bonded layer on cooling. The collapsed state can be restored by treatment with an organic solvent (*e.g.*, acetonitrile), which removes water from the bonded layer, and it is also removed with the subsequent conditioning of the column with water. The temperature of the above conformational transition depends on the chain length of the bonded alkyl radical, and is 40.7, 51.8 and 60.1°C for C_8, C_9 and C_{10} phases, respectively [64].

Further investigations of the effect of the nature of the solvent on the conformational transitions in the bonded layer demonstrated that removal of the organic solvent from the bonded layer by water and the consequent rearrangement of the bonded layer structure proceed very slowly and depend on the solvent type. These effects define the dependence of the properties of a reversed stationary phase on its prehistory and it should always be borne in mind in order to obtain reproducible results.

CONCLUSIONS

The specific features of the molecular organization of bonded layers of reversed stationary phases considered in this review demonstrate that the properties of apparently simple supports based on porous silica with bonded alkyl groups depend on a series of factors: pretreatment and structural peculiarities of the support, type and alkyl chain length of the modifier, the conditions of chemical modification and further maintenance. Among the available reversed-phase supports we have only considered those most commonly used, with a monomolecular layer of fixed organosilicon modifiers. The practical variety of reversed stationary phases is great. Recently, much interest has been shown in so-called "polymeric" reversed phases with a condensed layer of alkyltrichlorosilanes having a high selectivity to polynuclear aromatic hydrocarbons [65]. The variety (certainly not complete) of reversed-phase supports with a monomolecular layer of bonded alkylsilanes and states of the bonded layer are shown schematically in Fig. 13. This variety of reversed stationary phases should be taken into account when considering their synthesis in the laboratory or when choosing from the large number of available industrial materials.

Finally, we end by quoting the expression of Pauli: "a surface is created by the devil". The apparently "simple" reversed stationary phases are a good illustration of this point.

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