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# ОСНОВНІ ПУБЛІКАЦІЇ

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## *SELENIUM-CONTAINING PEPTIDES AS SPECIFIC ANALYTES FOR HPLC STATIONARY PHASES EVALUATION*

*Висока якість хроматографічних роділень забезпечується високою однорідністю і щільністю покриття поверхні стаціонарної фази. Взаємодія між стаціонарною фазою, аналітом і мобільною фазою упродовж хроматографічного процесу визначається хімічними характеристиками і структурою хімічно утвореного шару на поверхні гелю кремнезему. Механізми утримування (розподіл, адсорбція, обмін іонів, стеричні особливості), селективність і розділення є результатом властивостей стаціонарної фази.*

*Мета даної роботи полягала у визначенні різнорідності поверхні носія на основі фізико-хімічних досліджень та хроматографічних випробовувань. Для цього були одержані серії насадкових матеріалів з хімічно прищепленими до кремнієвого гелю октадецильними ланцюгами. На основі результатів ядерно-магнітної спектроскопії $^{29}Si$ та хроматографічних вимірювань побудована проста топографічна модель хімічно модифікованої стаціонарної поверхні. Для оцінки одержаних в лабораторії октадецильних стаціонарних фаз з високою і низькою щільністю покриття перед захистом і після захисту кінцевих груп, використовувались водорозчинні селен вмістні пептиди (833 і 2607 дальтон). Завдання роботи полягало у вивченні відмінностей у щільності та однорідності покриття поверхні і конформаційних змін хімічно зв'язаної частини, а також впливу цих параметрів на розділення суміші селен вмісних пептидів.*

*Высокое качество хроматографических разделений обеспечивается высокой однородностью и плотностью покрытия поверхности стационарной фазы. Взаимодействие между стационарной фазой, аналитом и мобильной фазой на протяжении хроматографического процесса определяется химическими характеристиками и структурой химически образованного слоя на поверхности геля кремнезема. Механизмы удерживания (распределение, адсорбция, обмен ионов, стерические особенности), селективность и разделение являются результатом свойств стационарной фазы.*

*Цель данной работы заключалась в определении разнородности поверхности носителя на основе физико-химических исследований и хроматографических измерений. Для этого были получены серии насадочных материалов с химически привитыми к кремниевому гелю октадецильными цепями. На основании результатов ядерно-магнитной спектроскопии $^{29}Si$ и хроматографических измерений построена*

*простая топографическая модель химически модифицированной стационарной поверхности. Для оценки полученных в лаборатории октадецильних стационарных фаз с высокой и низкой плотностью покрытия перед защитой и после защиты концевых групп, использовались водорастворимые селен-содержащие пептиды (833 и 2607 дальтон). Задание работы заключалось в изучении отличий в плотности и однородности покрытия поверхности и конформационных изменений химически связанной части, а также влияния этих параметров на разделение смеси селен-содержащих пептидов.*

*Stationary phases surface overage homogeneity and density assure high quality of chromatographic separation. Chemical character and structure of chemically formed layer on the silica gel surface determine interactions between stationary phase, analyte and mobile phase during chromatographic process. Retention mechanisms (partitioning, adsorption, ion exchange, steric exclusion), selectivity and resolution are consequence of the stationary phases properties.*

*The aim of the present studies was to determine the heterogeneity of the packing surface on the basis of physicochemical investigations and chromatographic tests. A series of packings with octadecyl chains chemically bonded into a silica gel were prepared for this purpose. $^{29}$Si CP/MAS NMR spectras and chromatographic measurements results were the base for simple topographical model of the chemically modified stationary surface. Water-soluble selenium-containing peptides (833 Da and 2607 Da) were used for the evaluation of laboratory-prepared octadecyl stationary phases with high and low coverage density before and after end-capping. The aim of this work was to study the differences in the surface coverage density and homogeneity and conformation changes of chemically bonded moieties and the influence of these parameters on the separation of selenopeptides mixture.*

## *Inroduction*

Dynamic development of chromatography and related techniques is possible thanks to a wide variety of chromatographic column packings and detection methods. Polymers, carbons, pure and modified aluminium and zirconia oxides are the newest stationary phases` types but still silica based materials with octadecyl chemically bonded groups are the most popular [1,2]. Progress in the columns and packings development made high performance liquid chromatography (HPLC) the most popular method for the separation of biologically active compounds, responsible for the proper function of living systems. Physico-chemical properties influence on compound biological activity, chromatographic behavior and analysis result. It is deciding factor in the possible interactions between the analyte, the stationary phase surface and mobile phase. Stationary phase structure characterization and retention mechanism prediction are a key to choose the best column for a given application. A whole group of physico-chemical techniques such as: porosimetry, elemental analysis, nuclear magnetic resonance spectroscopy ($^{29}$Si and $^{13}$C CP/MAS NMR), infrared spectroscopy, diversity scanning calorimetry, microcalorymetry, *etc.* were used to characterize chromatographic packings [3-6]. Column performance is evaluated by chromatographic tests characterizing the retention, the separation selectivity and the peak asymmetry for selected test compounds [6-11]. Computer calculations for molecular modeling are also used in stationary phase and solute structures description [12-15]. The alkyl chain length, attachment chemistry, bonding density of organic groups to the silica and also heterogeneity of

the support (silica gel), solute configuration, mobile phase composition and temperature influence on the configuration of chemically immobilized chains [1,4,15].

During the modification reaction, there is no possibility of blocking all superficial hydroxyl groups on the silica support by organic modifiers molecules due to the steric effects [16,17]. This materials contain three types of reactive centers: bonded organic moieties (C18 or other), residual silanols and sometimes impurities (e.g. metal ions) [1,3,4,18]. Energetic differences on the support surface and structural properties of the analyte influence which type of interactions and retention mechanism (adsorption, partition, ion exchange or steric exclusions) is predominant. Residual, non-modified silanols can also react with the solvent molecules by the specific and non-specific interactions [1,15,18].

In this paper polar selenopeptides (MW 833 Da and 2607 Da) molecules were used as very specific chromatographic test analytes in the study of density and homogeneity of laboratory prepared chemically bonded stationary phases with different surface coverage density. The optimization of the separation conditions of selenopeptides with regard to stationary phase structure is very important for LC-ICP/MS or LC-MS/MS analysis of complex mixtures of these compounds. Selenopeptides were purchased from selenized yeast food supplements. The structure of used selenopeptides is not fully known. Probably they are *ball-like* molecules with external hydrophilic and internal hydrophobic groups and they can change shape in hydro-organic mobile phases. Such structure allows an easier penetration among the stationary phase chemically bonded groups, especially there where surface coverage is heterogeneous and if the size of analyte molecule is lower than the space between bonded moieties.

Hyphenated techniques based on the combination of chromatography with mass spectrometry play dominate role for the analysis of species present in biological samples. Coupling liquid chromatography (high performance, ion-exchange, size-exclusion), electrophoresis, gas chromatography with atomic spectrometry are the most frequently applied systems. More than 75 elements can be determined, most of them at detection limits less than 1 part per billion, using inductively coupled plasma atomic emission spectrometry (ICP--AES) and inductively coupled plasma mass spectrometry (ICP-MS) as detection method [19].

## *Expermental*

### Reagents and materials

The support of laboratory prepared chromatographic C18 stationary phases was silica gel Kromasil® 100 AT 0191 (Akzo Nobel, Bohus, Sweden). Physico-chemical characteristic of the bare silica gel is presented in Table 1.

The following reagents were used for the stationary phases preparation: octadecyldimethylchlorosilane (Johnson Matthey ALF Products, Karlsruhe, Germany), trimethylchlorosilane (Sigma-Aldrich, Steinheim, Germany), morpholine (Reachim, Moscow, Russia). HPLC grade organic solvents were purchased from Scharlau Chemie S.A. (Barcelona, Spain).

Selenopeptides were prepared from selenized yeast (Alltech, Lexington) according to Ref [20]. Their molecular masses, structures and sequences are listed in table 2.

**Table 1. Physico-chemical characteristic of the bare Kromasil® 100 AT 0191**

| Parameter | Value |
|---|---|
| Particle shape | Spherical |
| Mean particle size, $d_p$ | 5 μm |
| Specific surface area, $S_{BET}$ | 295 $m^2/g$ |
| Pore volume, $V_p$ | 0.92 $cm^3/g$ |
| Mean pore diameter, D | 113 E |
| Concentration of OH groups, $\alpha_{OH}$ | 7.1 $\mu mol/m^2$ |
| Trace amount of metals, $C_M$ | <20 ppm |

**Table 2. Test analytes properties**

| Analyte symbol | Structure/diameter | Mass |
|---|---|---|
| P | ERDDXNXDXGXGHDQSEGGXK | 2607.152 Da |
| E | TYENXKK | 833.22 Da |

## *Instrumentation*

Laboratory prepared stationary phases were packed into 125 mm x 4.6 mm i.d. stainless steel columns under a pressure of 50 MPa a using laboratory-made set based on a DSF-122 packing pump (Haskel INC, Burbanck, CA, USA).

Selenopeptides reversed-phase HPLC analysis were performed using an 1100 series pump (Agilent Technologies, Palo Alto, USA). ICP/MS (Elan 6000, PE-SCIEX, ON, Canada) fitted with a cross-flow nebulizer and double-pass Scott spray chamber was used as a detector, 82Se isotope was monitored. For on-line HPLC-ICP/MS analysis, the interface consisted of a cooled low-volume cyclonic spray chamber (Glass Expansion, Romainmotier, Switzerland), a 0.85 mm I.D. alumina torch injector, a set of platinum cones. The use of an auxiliary oxygen flow of 15 mL/min made possible the introduction into the plasma of 60% methanol at 0.75 mL/min without sensitivity losing.

## *Results and discussion*

Octadecyl stationary phases were prepared according procedures described previously [21,22]. As a result of the silica gel modification, packings with high ($C_{18}^{H}$) and low ($C_{18}^{L}$) coverage density before and after end-capping (EC) have been prepared. The surface characteristics of the octadecyl stationary phases is given in Table 3.

**Table 3. Surface characteristics of the octadecyl stationary phase**

| Packing | Type of the phase | Carbon contents $P_C$ [%] | Coverage density [μm/m$^2$] | Percent of surface coverage [%] |
|---|---|---|---|---|
| $MC^{L}_{18}$ | monomer | 10.66 | 1.75 | 23.3 |
| $MC^{L}_{18}$ + EC | monomer | 12.68 | 2.13 | 28.5 |
| $MC^{H}_{18}$ | monomer | 17.10 | 3.10 | 41.5 |
| $MC^{H}_{18}$ + EC | monomer | 17.45 | 3.17 | 42.4 |

where: EC - end-capping, H - high coverage density, L - low coverage density.

$^{29}$Si CP/MAS NMR spectras give important information about surface coverage density (Fig. 1).

For bare silica gel three characteristic signals are observed for: geminal ($Q^2$, δ=-92 ppm) and free silanols groups ($Q^3$, δ?=-100 ppm) and oxosilanes ($Q^4$, δ=-108 ppm). Band M (δ=12.5-13.00 ppm) corresponds to the monomeric structure with one-point ligand-silanol group bond [5,23]. The synthesis of low coverage density stationary phase causes a decrease in $Q^2$ and $Q^3$ signals intensity (*ca.* 50%) with an increase in M signal intensity (*ca.* 50%). Geminal groups are not completely reduced for low coverage materials and even end-capping does not block these centers. Due to geometry and dimension of modifier molecules (octadecylsilane) not all surface silanols are blocked in modification reaction and heterogenous surface coverage is obtained. Unblocked -OH groups may have a negative influence on the separation, particularly of polar compounds.

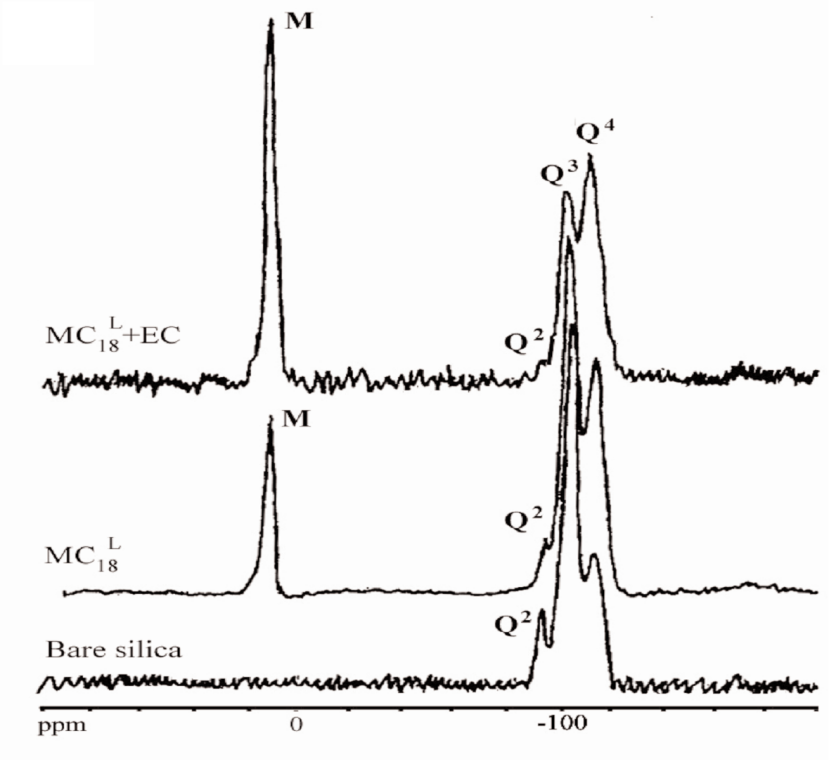


**Figure 1. $^{29}$Si CP/MAS NMR spectra for the bare silica gel and low coverage density octadecyl stationary phase.**

Selenopeptides are *ball-like* hydrophobic-hydrophilic molecules with hydrophobic part inside *ball* and external hydrophilic groups. The elution of peptides in a reversed-phase chromatographic system is performed with water-organic solvents, in this study methanol, containing an ion-pair reagent or buffer. The organic modifier dissolves sample and assures elution while an ion-pair agent or buffer sets the eluent pH and stationary phase charge to enhance the retention. Trifluoroacetic acid (TFA) is the most popular ion-pairing regent as also modifies residual silanols on the stationary phase surface. A TFA molecule has a less-polar and a more-polar end. The polar part interacts with basic chains and the less polar end penetrates the stationary phase, thus facilitating interaction of the basic side chains with the reversed-phase surface [24]. TFA may cause a different orientation of a peptide relative to the adsorbent surface and its charge.

Both selenopeptides have law retention and elute near the column dead time (Fig. 2).

Partition retention mechanism is less probable because of molecules size and to small distance between chemically bonded groups. Peptide conformation makes impossible an interaction of *hydrophobic foot* with chemically bonded groups into silica gel surface. If partition takes place it is possible that analytes *slide* on the stationary phase surface is expected because of the molecule size and steric barriers. Larger molecule P (M=2607.152 Da) is better retained on the law coverage stationary phase. This fact suggests higher concentration of adsorption centers in law coverage packing and heterogeneous coverage of the silica surface by chemically bonded ligands than for high coverage stationary phase (Table 3).

Therefore adsorption retention mechanism is foreseen and also size-exclusion effects should be considered. Octadecyl, octyl and butyl stationary phases, the most popular in peptides separations, provide a hydrophobic adsorption centers. Peptides retention may be considered as *sitting* on the stationary phase, with most moiety part exposed to the mobile

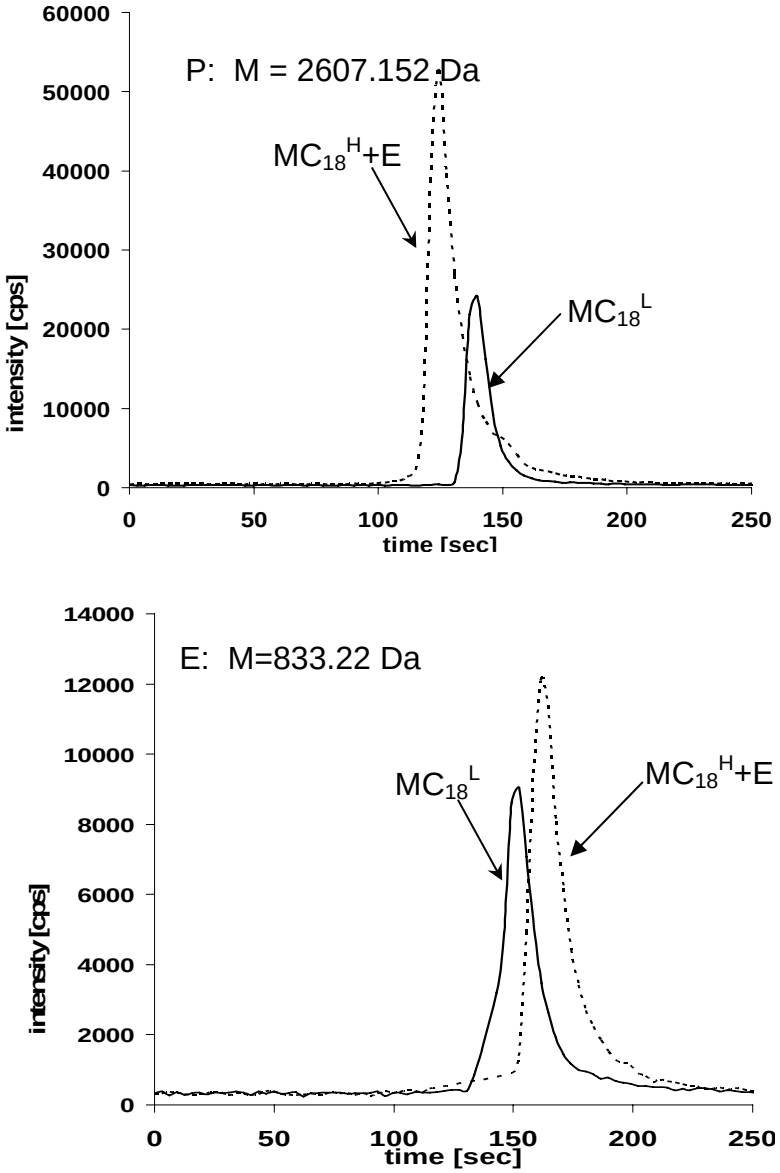


**Figure 2. Differences in retention for various structures of seleno-peptides. Mobile phase: methanol/water 40/60, flow rate: 0.6 mL/min, detection ICP-MS ($^{82}$Se). X – corresponds to selenomethionine. (.....) $C_{18}^{H}$+EC stationary phase, (_____) $C_{18}^{L}$ stationary phase.**

phase and only a small of it in contact with a stationary phase. Separation is based on the *active centers* energetic differences in the in peptide molecule which results from amino acid sequence and molecule conformation.

Smaller analyte E (M=833.22 Da) with regard of molecule shape easier penetrate space among octadecyl groups so retention on the stationary phase with lower carbon concentration on the surface (10.66%) (Fig.2). Better retention on the high coverage packing indicates hydrophobic interactions between analyte and stationary phase whereas hydrophilic interactions are probable for selenopeptide P.

Study of mobile phase composition influence on elution confirms above considerations. Simple Soczewinski-Wachtmeister dependence [25] capacity factor vs. percentage of organic solvent in mobile phase provides information about hydrophobicity of chromatographic system:

$$\log k = \log k_w - s\varphi \quad (1)$$

Where: k – capacity factor, $k_w$ – value of the coefficient of retention extrapolated to the reference value in pure water, φ – organic modifier content in mobile phase, s – constant value.

Linear correlation log k vs organic modifier content in mobile phase indicates that for selenopeptide E interactions with stationary phase are stronger and retention prediction is easier than for the higher molecular weight analyte (M=2607.152 Da) of selenopeptide P (Fig. 3). High regression coefficients (r2=0.972-0.997) confirmed this.

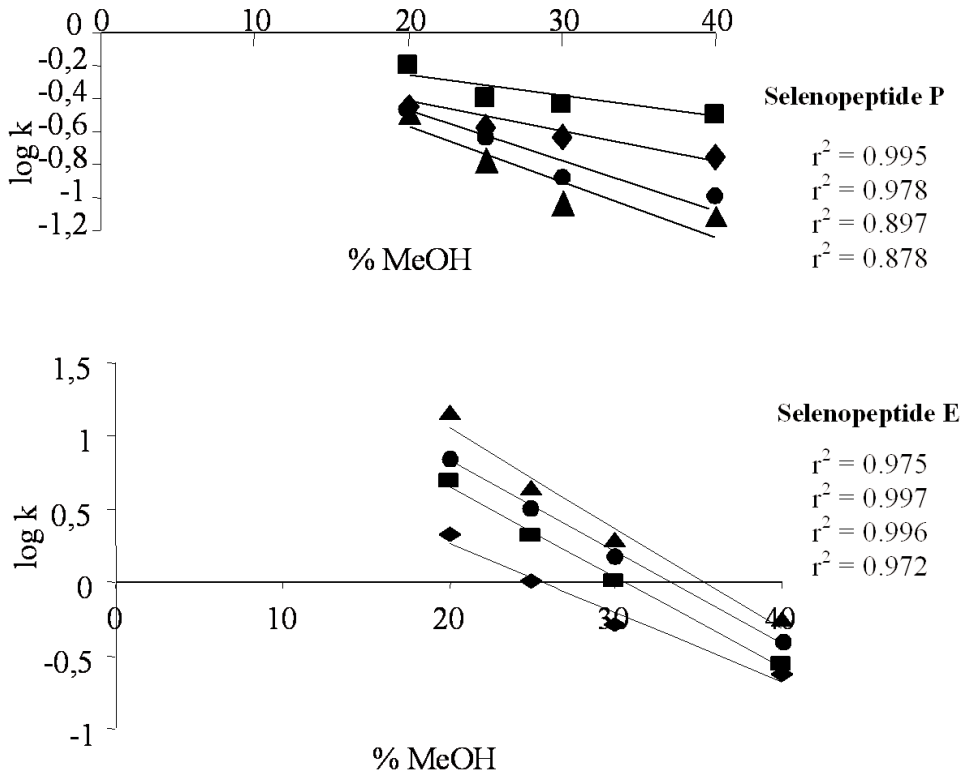


**Figure 3. Correlation between selenopeptides retention and organic modifier contents in mobile phase. $MC_{18}^{L}$, $MC_{18}^{L}$ + EC, $MC_{18}^{H}$, $MC_{18}^{H}$ + EC.**

For the elution of molecule P, mobile phase elution strength does not play so significant role as for analyte E. Solute E contact time with low coverage stationary phase is shorter which shows that molecules easier enter between hydrophobic chemically bonded C18 ligands. Selenopeptide P retention is the weakest on high coverage stationary phase surface what confirms possible sliding of analyte on the top of chemically bonded groups.

Taking into account the above considerations a simple model of big and small analyte molecules behavior on the stationary phase could be proposed (Fig. 4).

Distance between silanol groups equal 5A and their concentration on the surface (*ca.* 8 μmol/$m^2$ ≈ 4.5 groups -OH/$nm^2$) was first, basic assumption [26,27]. Assume that octadecyl moiety bonded into silica gel surface has cylindrical shape (diameter *ca.* 3.2 A) extended *near head* (diameter *ca.* 6 A) spaces between chains are accessible for analytes and mobile phase components molecules. The composition of eluent influences the retention time and also the stationary phase conformation while the solvation of the surface depends on

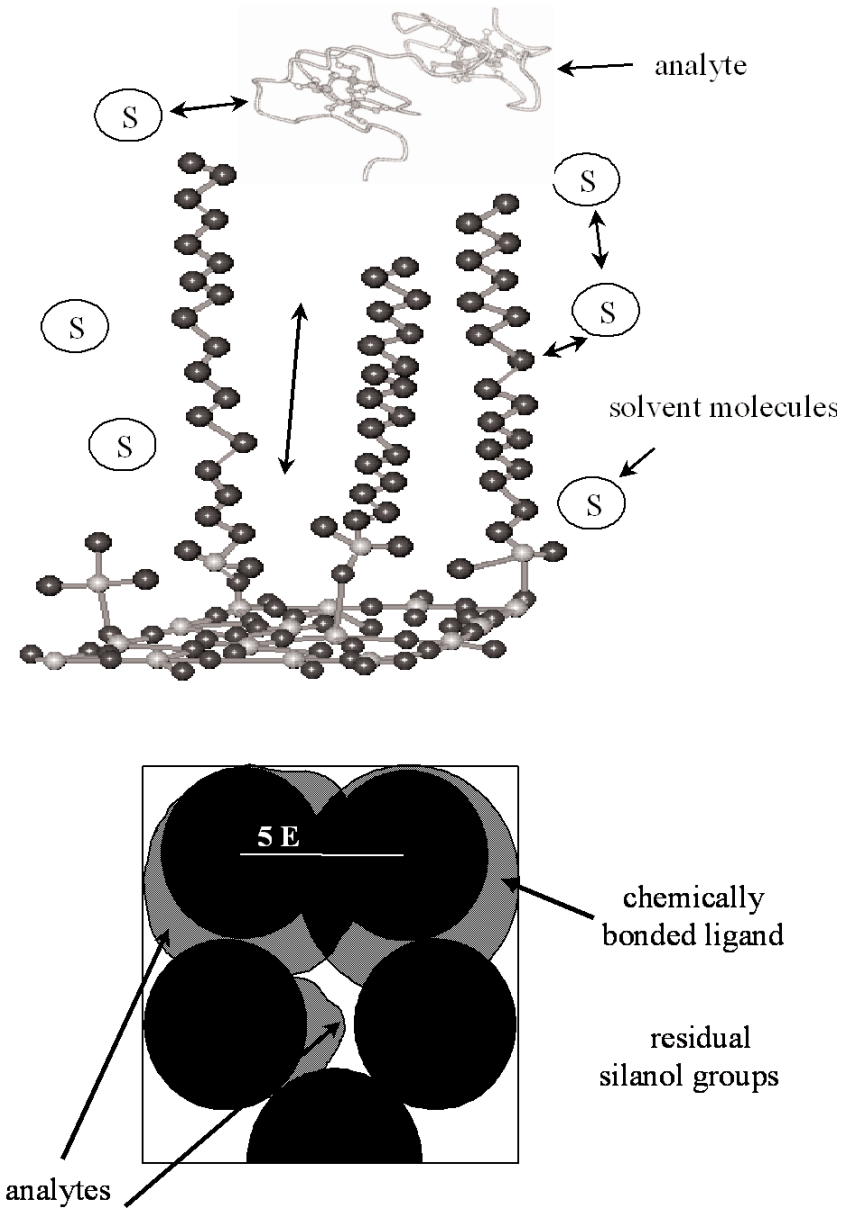


**Figure 4. Simple model of small and big molecules behavior on the stationary phase surface (vertical and horizontal projection).**

the coverage density and also solvent type. Bonded ligands are also able of mutual hydrophobic interactions (chain ⇔ chain). High and homogenous coverage of support surface prevent analyte migration to silica gel surface and among bonded groups.

## *Conclusions*

Chemical and physical modification of the silica surface by organosilanes permit the synthesis of stationary phases for liquid chromatography and related techniques. Physico-chemical techniques such as CP/MAS NMR, FT-IR enable defined chemically bonded film structure. Chromatographic measurements are also good tool for the stationary phases surface evaluation. Test analytes such as selenopeptides helped to design simple model of the packing surface. Presented silica gel surface with bonded organic moieties schema is simple but helpful in understanding and imagine structure as also interactions responsible for retention.

Synthesis conditions leads to receive homogenous and dense surface coverage effectively screens residual silanols and makes it impossible for solvent and analytes molecules to penetrate among chemically bonded moieties. It has significant influence on selectivity and resolution.

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