Some remarks on characterization and application of stationary phases for RP-HPLC determination of biologically important compounds

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Received 11 April 2005; revised 10 May 2005; accepted 19 May 2005

ABSTRACT: Biologically active compounds such as vitamins, steroids, nucleosides, peptides and proteins play a very important role in coordinating living organism functions. Determination of those substances is indispensable in pathogenesis. Their complex structure and physico-chemical properties cause many analytical problems. Chromatography is the most common technique used in pharmaceutical and biomedical analysis. The interaction between analyte and stationary phase plays a major role in the separation process. The structure of the packing has a significant influence on the results of the separation process. Various types of spectroscopic techniques, such as nuclear magnetic resonance spectroscopy, infrared spectroscopy, fluorescence spectroscopy and photoacoustic spectroscopy can be useful tools for the characterization of packings. Surface area measurements, elemental analysis, thermal analysis and microcalorimetric measurements are also helpful in this field. Part of the paper contains a description of chromatographic tests used for the determination of column properties. The description of the possibilities of surface characterization is not complete, but is focused on the most popular techniques and practical chromatographic tests. All the presented methods made possible the design and quality control of a new generation stationary phases, which are the future of high-performance liquid chromatography. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: liquid chromatography; stationary phase characterization; vitamins; steroids; peptides; nucleosides

INTRODUCTION

High-performance liquid chromatography (HPLC) is the most popular technique for the qualitative and quantitative analysis of a wide range of analytes. The determination of biologically active substances, often responsible for the proper function of natural living systems, is particularly important in the pharmaceutical field. All substances can be divided into groups with regard to their structures, properties or biological function, among which are drugs. Physicochemical properties play an important role in governing the biological performance of drugs. However these properties influence not only the biological activity of a compound, but also its chromatographic behaviour. They are the main deciding factors in the possible interactions between the analyte and the stationary phase surface, and consequently the separation process (Pietrzyk, 1989; Buszewski and Szumski, 2002; Kowalska et al., 2005).

The development of chemically bonded stationary phases and column preparation techniques guarantees the high precision and efficiency of analysis. In spite of new materials introduced to chromatography and related techniques, such as polymers (Pietrzyk, 1989; Unger, 1990), carbons (Unger, 1990), complex adsorbents (carbosiles) (Eltekowa et al., 2000), pure and modified aluminium and zirconium oxides (Unger, 1990; Nawrocki et al., 2004), silica-based stationary phases with chemically bonded ligands are the most popular (Buszewski et al., 1998; Unger, 1990; Poole and Poole, 1991; Neue, 1997). Recently the popularity of silica-based monolithic reversed-phase columns has increased (Motokawa et al., 2002). Stationary phase structure is not a homogenous material. Chemically bonded ligands are distributed on the surface uniformly and even after the best modification residual silanols are present. Physico-chemical properties such as matrix type and structure, porosity, type and concentration of hydroxyl groups and metal impurities and the structure of chemically bonded ligands on the silica surface have been the subject of many publications (Nawrocki and Buszewski,
This has made it possible to explain many phenomena and effects which influence the synthesis reaction mechanism and also the separation process. Determination of the structure and physicochemical characteristics of adsorbents allowed the pattern of analyte molecule behavior during the chromatographic process to be predicted, and provided some information about its quality. The arrangement of chemically bonded film depends on the ligand properties, mobile phase composition and temperature. Very often analyte structure may change under the above conditions and interact with the stationary phase (Fig. 1). This can particularly be observed for big molecules. Knowledge of stationary phase architecture and its influence on the separation process allows optimal choice of a column for analysis.

The chromatographic column should not be like a black box where we know that there is separation but we do not know why. Inside the column there is a kind of small laboratory where physical and chemical processes can be investigated, and it is the basis for modeling, for example, interactions on the border analyte ⇔ biological barrier. Inside the chromatographic column various interactions take place: physical, e.g. ion–ion, ion–dipole or dipole–dipole; chemical, e.g. hydrogen bonding, electron pair donor–electron pair acceptor or solvophobic. Determining which is predominant is very difficult and detailed studies are necessary. Each interaction determines selectivity (αij) and resolution (Rsi; Poole and Poole, 1991; Buszewski et al., 1998), expressed as:

\[
R_{sij} = \sqrt{N} \frac{\alpha_{ij} - 1}{\alpha_{ij}} \frac{k_j}{k_i^*} + 1
\]

where \(N\) is the number of theoretical plates, \(\alpha\) the selectivity (\(\alpha_{ij} = k_j/k_i\)) and \(k_j^*\) the capacity factor.

There is also still the question of which is the most important for chromatographers: efficiency represented by the number of theoretical plates (\(N\)) or selectivity? It is connected with qualification of the selectivity influence on the retention.

Selectivity of the chromatographic system is a consequence of the enthalpy and entropy changes, which depend on the stationary phase structure and coverage density as well as mobile phase composition.

\[
\ln k_j = -\Delta H^0/RT + \Delta S^0/R + \ln \varphi
\]

where \(H\) is the enthalpy, \(S\) the entropy, \(R\) the gas constant, \(T\) the temperature and \(\varphi\) the percentage of organic modifier in mobile phase.

Figure 2 shows the selectivity differences for various stationary phases using phenol, benzene and aniline as test compounds. The effect of the column type is significant and much more apparent in methanol–water than in acetonitrile–water mobile phases (Jandera et al., 2004a,b).
Here is a question which is very current: what composition of mobile phase should be applied, only water–organic or with salt addition or with acidic pH? We suggest using pure water–organic mobile phase. Salts or buffers in mobile phase cause residual silanol dissociation, which very often is not profitable for analysis. However, in quantitative structure–retention relationship (QSRR) studies, which allow the processes taking place on the border of phases, e.g. blood–brain barrier or external–internal cell environment, to be followed, it is easier to operate the hydro-organic mobile phase in pure water.

The aim of this paper is to review the theoretical and practical characterization and evaluation of the most interesting columns and packings with specific properties. Investigation of interactions between analyte and stationary phase surfaces, which allow retention mechanism determination, is also the purpose of this review.

**CHARACTERIZATION OF SOME SELECTED STATIONARY PHASES FOR HPLC**

There are several methods of adsorbent surface chemical modification. The first comprises hydro-silylation of silicon atoms incorporated in siloxane bonds (Unger, 1990). The second, more traditional, method proposes chemical bonding of various organosilanes with active groups such as metoxy-, etoxy-, amino- or chloro- (Sander and Wise, 1987; Unger, 1990; Buszewski, 1992). The most popular are monomer- and polymer-type stationary phases. Hydrophobic stationary phases (e.g. C2, C6, C18, C30) are very popular in pharmaceutical, clinical, biochemical and environmental routine analysis (Poole and Poole, 1991; Claessens, 1999). Very often application of commercial columns does not give satisfactory results of basic, acidic or other polar compound separation. Bad resolution and peak asymmetry are the consequences. The peak is labeled as tailing when it deviates from the ideal, symmetrical shape of a Gaussian peak (asymmetry factor 0.95 ≥ fAS ≤ 1.25). Peaks tail because more than one retention mechanism causes separation and chemically bonded ligands are heterogeneously distributed on the support surface (Nawrocki and Buszewski, 1988). Triethylamine is a popular mobile-phase additive to reduced peak tailing, but high-coverage stationary phases are better for this purpose. A thin film should better shield residual silanols, which determine selectivity (α) and of course resolution (R50). The thin film, as a hydrolytic pillow, forms on the alkylamide stationary phase (SG-AP) surface in reversed-phase mode with high water concentration in mobile phase. Figure 3 presents amine mixture separation on SG-AP. For these polar compounds a long retention time usually causes peak tailing. The hydrolytic pillow shields residual silanols hence the long retention time for diethylaniline (k = 45), and the peak shape is close to the Gaussian curve (fAS = 1.52).

Huge evolution in column testing and characterization and the precise control of the modification process permit the design of new, intelligent stationary phases. Traditional hydrophobic stationary phases are popular and useful, but the new challenges of analytical chemistry require more selective materials. Recently, materials with a diffusion barrier (e.g. polar groups in hydrophobic chains) and mixed phases containing various functional groups have become the newest solutions. Such stationary phases are derivatives of amino materials and are used for chromatographic separation, but also as specific support for bonding biological compounds like cholesterol and phospholipids (Pidgeon et al., 1994; Buszewski et al., 1999, 2001). Among analytical chemists there is still an intensive search for packings with properties closer to biological membranes, to enable modeling of the processes on the border of two phases (e.g. liquid ⇔ solid or liquid ⇔ liquid), two environments (internal an exterior cell environment) or biological barriers (blood ⇔ brain). The use of these stationary phases is in imitating biological membranes (Fig. 4) to make it possible to follow, simulate and describe the processes in natural systems. The generation of retention changes in analytes may be treated as a precise instrument in the control of selectivity and, as a consequence, in the modelling of processes taking place in live organisms.

Stationary phases with physically or chemically bonded components of natural membranes are suitable not only for compound separation but also for
compound lipophilicity (hydrophobicity) determination (Nasal et al., 2003). For years the octadecyl silica stationary phases were the most popular in the lipophilicity study of compounds. To obtain a universal chromatographic lipophilicity scale, application of various types of stationary phases is required. The chromatographic column can also be a very useful tool for studying drug–protein interactions.

Cholesterol moieties are powerful tools in chromatography because they form liquid crystal structures, contain chiral atoms and are a major constituent of the biological membrane lipid phase. The cholesterolic column is useful in pharmacokinetic studies, especially in modeling the penetration of xenobiotics and drugs through biological membranes. Chiral centers make possible the separation of natural isomers, e.g. estradiol or tocopherol (Buszewski et al., 2000, 2003b, 2004). Cholesterolic stationary phase can be used under non-aqueous conditions for separation of racemic mixtures of 1-methyl-2-piperidinoethylesters of 3- and 4-alkoxyphenylcarbamic acid, which is a drug potentially employed in local anesthesia (Fig. 5).

Immobilization of phospholipids, the building materials of biological cell membranes, is also a step in the creation of artificial membranes inside the chromatographic column. Phospholipid density on the support surface is similar to the density in biological membranes. Such stationary phases provide an environment for the study of many interactions between the lipid bilayer and molecules like peptides, sugars, nucleic acids, amine acids, ions, drugs and xenobiotics. Unfortunately, immobilized artificial membranes (IAM) (Fig. 4) have no lateral mobility of the bonded molecules of phospholipids (Pidgeon et al., 1994).

The stationary phases containing peptides such as bovine serum albumin (BSA), human serum albumin (HSA) or keratin were very promising as alternative systems for separation of optically active compound and modelling of natural biological systems (Buszewski et al., 1994; Aubry et al., 1994; Gilpin et al., 1991; Tittelbach and Gilpin, 1995). Albumins as serum proteins play an important role in drug action because the free, unbound fraction of a drug in blood undergoes distribution (Nasal et al., 2003). Keratin as a constituent of the epidermis, after physical immobilization on the silica surface, was proposed for modelling xenobiotic permeation (Turowski and Kaliszan, 1997). Another interesting biomolecule chemically and physically immobilized is melanin (Knörle et al., 1998). Because of high affinity of xenobiotics to melanin and its presence in internal and external tissues, e.g. skin, hair, eye and brain, the binding mechanism is very interesting.

The major permeability barrier in human skin is provided by the outer layer. This layer consists of dead cells surrounded by an extracellular matrix containing lipids, e.g. ceramids. The stationary phase with ceramids covalently bonded on the silica surface is useful for the prediction of skin permeability constants of compounds (Yin et al., 1998).

In chiral Pirkle’s stationary phases (Pirkle and Finn, 1981; Pirkle and Pochapski, 1989; Pirkle and Burke, 1991; Fig. 6), donor–acceptor and $\pi$–$\pi$ interactions are

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**Figure 3.** The amine (aniline, methylaniline, dimethylaniline, diethylaniline) mixture separation obtained on the SG-AP phase. Column, 125 × 4.6 mm i.d.; mobile phase, 35-65% v/v MeOH–H$_2$O; flow rate, 1 mL/min; detector, UV, 254 nm.
predominant and cyclodextrins materials play important role in separation techniques (HPLC, CZE, GC; Unger, 1990; Menges and Armstrong, 1991). These materials permit preparative separation of optically active compounds, which is important in the pharmaceutical and cosmetics industries.

Chromatographic separation with another interesting stationary phase type, zwitterionic, is one of liquid chromatography separation modes much studied in recent years. This packing material consists of zwitterionic functionality. The schematic illustration shown in Fig. 6 shows one of the typical zwitterionic groups, which belong to the strong/strong category, and both the negative and positive groups retain their charge over the entire operational pH range. Applications of these types of stationary phases include separation of inorganic ions and biological macromolecules (Jonsson, 2003; Jiang, 2003; Jonsson and Appelbald, 2004).

Analyte varieties and retention mechanisms based on hydrophobic, hydrophilic, size exclusion and ion-pairing interactions resulted in many specific and selective chromatographic columns, but no ideal one (Buszewski, 1995). The selection of a proper column for solving a separation problem is sometimes very difficult. The column quality depends on: (i) homogeneity of particle packing in the column bed; and (ii) homogeneity of the chemically bonded stationary phase surface. High
quality and physico-chemical properties of the supports and substrates are necessary for reproducible synthesis of new packing and homogenous arrangement of chemically bonded ligands (Dorsey and Dill, 1989). Sometimes data analysis is impossible, because even nominally identical columns show different chromatographic properties (Neue, 1997). There is no ideal and no one uniform procedure for column evaluation and selection. Many tests for the determination of quality of HPLC columns have been described in the literature (Claessens et al., 1998, Claessens, 1999, 2001; Stella et al., 2001; Krupczyńska et al., 2004a,b). All are based on statistical, empirical and thermodynamics methods.

PHYSICO-CHEMICAL METHODS FOR STATIONARY PHASE SURFACE CHARACTERIZATION

Advanced physicochemical techniques such as infrared (IR) absorption spectroscopy, cross polarization/magic angle spinning solid-state nuclear magnetic resonance (CP/MAS NMR), fluorescence spectroscopy, porosimetry, elemental analysis, atomic, electron and tunnel microscopies and differential scanning calorimetry can be applied for surface characterization and better understanding of retention mechanisms (Berendsen and de Galan, 1980; Murthy et al., 1985; Bayer et al., 1986; Snyder et al., 1988; Albert, 2003; Hansen and Callis, 1983; Buszewski and Suprynowicz, 1987; Kasturi et al., 1994; Table 1). Sorption of gases and vapors on a solid is a powerful tool for determining surface pore-size distribution and packing porosity (Sander and Wise, 1987). Measuring the molecular weight distribution by gel permeation chromatography using a series of polystyrene standards is another possibility for estimation of the pore-size distribution. The retention volume of non-retained solutes decreases for chemically bonded stationary phases (Buszewski and Suprynowicz, 1987). Elemental analysis provides information about the quantity of carbon, nitrogen and hydrogen by burning the sample in oxygen. The carbon content allows

Figure 5. Separation of a racemic mixture of 1-methyl-2-piperidinoethyl esters of 3-alkoxyphenylcarbamic acid. Column, cholesterolic (250 × 4.5 i.d.); mobile phase, hexane–isopropanol–methanol, 60:5:35 v/v/v.
Figure 6. Schematic structures of the most frequently used stationary phases for biologically active compound analysis.
calculation of surface coverage according to the Berendsen equation (Berendsen and de Galan, 1980). For the case of end capping phases, Buszewski et al. (1994) modified the mentioned equation (Kasturi et al., 1994).

Thermal gravimetric analysis is one of the simplest methods for surface characterization. The weight loss during sample burning in oxygen is equal to that of the chemically bonded phase (Sander and Wise, 1987). First applications of infrared spectroscopy prove only the presence of isolated, bonded or associated silanol groups on silica and also bonded ligands on the support surface. This method became more popular and useful in the investigation of the silica surface after the introduction of modern sampling techniques such as diffuse reflection in Fourier transform (Kasturi et al., 1994). Improvement of the methods allows investigation of the kinetics of modification and bonded moieties dynamics (Sander and Wise, 1987).

With nuclear magnetic spectroscopy more detailed information can be obtained on the nature of ligand bonding to the surface. Suspended-state and two-dimensional NMR is useful to observe the dynamics of chemically bonded ligands for various solvents and temperatures (Albert, 2003). Cross-polarization, high-power enhanced sensitivity, resolution of solid-state measurements and MAS at rates higher than 10 kHz overcame problems with line broadening because of the mobility of bonded ligands, chemical shift anisotropy, long spine-lattice relaxation times and proton–carbon dipolar interactions (Pines et al., 1973).

### CHROMATOGRAPHIC METHODS FOR COLUMN CHARACTERIZATION

All of the above techniques are useful for proper reaction control during synthesis of chemically bonded
stationary phases. Problems arise when we have to evaluate a chromatographic column and choose the best one for laboratory practice. Spectroscopic techniques are not sufficient and require a pure stationary phase, not a whole column.

Statistical methods, e.g. principal component analysis and cluster analysis, are one of the possibilities for selection and classification of a large number of columns (Sander and Wise, 1995). Also, for column estimation, thermodynamic measurements are interesting. The Van’t Hoff plot (log retention factor of a compound vs the reciprocal absolute temperature) provides information on the enthalpy and entropy contributions to retention. Van’t Hoff correlation can also show whether the retention mechanism may change over the studied temperature range (Sander and Wise, 1987, 1995).

It is obvious that chromatographic methods are the most popular and very useful for evaluation of chromatographic columns. They provide fast and practical information on the column properties. Test procedures should characterize not only the column but also the chromatographic system and especially the properties of the mobile phase. Hydrophobicity, residual silanol groups activity, shape selectivity and metal impurities are the most often considered parameters in characterizing stationary phases. Bristow and Knox (1997) published the first fundamental work on retention parameters and column kinetic properties characterization. Since that time many tests based on empirical, thermodynamic and statistical methods have been published (Table 2).

Empirical tests are based on the retention parameters of various compounds, which should reflect all, good and bad, sites of the column (Buszewski et al., 1999; Claessens, 2001). Below the most popular and useful tests, in our opinion, are given. Tanaka proposed uracil \( t_0 \), thiourea \( t_{th} \), amylbenzene, butylbenzene, triphenylene, \( o \)-terphenyl, caffeine, phenol and benzylamine for determining the number of alkyl chains, hydrophobicity, steric selectivity, hydrogen bonding capacity and ion-exchange capacity at pH > 7 and pH < 3. The results were visualized as a hexagon whose symmetry depended on column quality and properties (Kimata et al., 1989).

Engelhardt and co-workers (Engelhardt and Junghem, 1990; Engelhardt et al., 1997), for hydrophobicity, silanol activity and shape selectivity determination proposed aniline, phenol, 4,4’-dimethyl-aniline, p-ethylaniline, toluene, ethylbenzene, triphenylene, \( o \)-, \( m \)-, p-toluidine and \( o \)-terphenyl as test compounds. They established that the retention of neutral compounds depends on the carbon loading on the support surface and length of chemically bonded organic ligands. If aniline is eluted before phenol and toluidine isomers are not separated, silanol activity is low and the column is suitable for separation basic compounds.

Walters (1987) suggested benzene and anthracene for the determination of hydrophobicity and also anthracene and \( N,N \)-dietylo-\( m \)-toluidamid for silanol activity measurement.

Usually test compounds are neutral compounds, but evaluation of HPLC columns just for basic compounds is very important. McCalley (2000) proposed measuring retention factors, asymmetry factors and column efficiency for a set of analytes (nortriptyline, nicotine, amphetamine, pyridine, benzylamine, codeine, quinine, procainamide and diphenhydramine) using organic modifiers such as acetonitrile, methanol and tetrahydrofuran at pH 3.0 and neutral pH.

Rohrschneider (2001) introduced a new approach, combining the retention data with the solvation parameter model. He proposed using naphthalene, acetanilidine, phenol and benzonitrile to characterize C18 columns in a reversed-phase system.

Neue et al. (2003) suggested that propranolol and amitriptyline can be used for the estimation of surface silanols and naphthalene and acetylnaphthalene as markers of packing hydrophobicity, dipropylphthalate and butylparaben for determination of the polar selectivity.


Jinno (1997) used big molecules such as fullerenes \( \text{C}_{60} \) and \( \text{C}_{70} \) to study retention, which explains molecular shape recognition on the packing surface.

Macromolecules such as polymers were used to investigate silanophilic interactions and the extent to which the probes can penetrate the chemically bonded ligands (Berek and Novák 1994; Berek and Tarbajovska, 2002).

Mobile phase composition has a significant influence on stationary phase structure and selectivity, hence tests in solvents other than water–organic system are desirable. Pyridine and 2,6-dimethylypyridine were applied as test compounds to determine residual silanols on the modified surface using normal-phase (non-aqueous) conditions (Nondek et al., 1986).

Bronopol (2-bromo-2-nitropropane-1,3-diol) was suggested for column evaluation under total aqueous conditions, especially as regards the homogeneity of packing surface coverage by chemically bonded phase and spacing of residual silanols (Buszewski et al., 2003a). This is also useful marker for determination column lifetime.

Claessens (1999) studied the influence of mobile phase pH on column stability and life-time. Using better quality silica gel as support extended the usable mobile phase pH range in experiments. A very low
### Table 2. Chromatographic tests for columns evaluation

<table>
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<tr>
<th>Test</th>
<th>Test characteristic</th>
<th>Principle</th>
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<tbody>
<tr>
<td>Knox (Bristow and Knox, 1977)</td>
<td>Specific adsorption chromatography and reversed-phase conditions to column tests</td>
<td>Standardization of test conditions for HPLC columns</td>
</tr>
</tbody>
</table>
| Tanaka (Kimata et al., 1989) | Test analytes: uracil ($t_0$), thiourea ($t_0$), amyllobenzene, butyllobenzene, triphenylene, $o$-terphenylene, caffeine, phenol, benzylamine | Hydrophobicity = $k_{amylobenzene}/k_{butyllobenzene}$  
Alkyl ligands amount = $k_{amylobenzene}$  
Steric selectivity = $k_{triphenylene}/k_{terphenylene}$  
Hydrogen bond capacity = $k_{caffeine}/k_{phenol}$  
Ion exchange capacity (IEC): pH > 7 = $k_{benzylamine}/k_{phenol}$  
IEC pH < 3 = $k_{benzylamine}/k_{phenol}$ |
| Engelhardt (Engelhardt and Junghem, 1990; Engelhardt et al., 1997) | Test analytes: uracil ($t_0$), aniline, phenol, N,N-dimethylamline, $p$-ethylaniline, toluene, ethylbenzene | Hydrophobicity = $k_{ethylaniline}/k_{toluene}$  
silanophilic activity = $p$-ethylaniline peak asymmetry (5% of peak high) |
| Walters (1987) | Test analytes: uracil ($t_0$), benzene, anthracene | Hydrophobicity = $k_{anthracene}/k_{benzene}$ |
| McCally (2000, 2003) | Test analytes: nortriptyline, nicotine, amphetamine, pyridine, benzylamine, codeine, quinine, procainamide, diphenhydramine | Retention factors, asymmetry factors and column efficiency |
| Neue (Neue et al., 2003) | Test analytes: amitriptyline, naphthalene, acetylnaphthalene, dipropylphthalate and butylparabene | Estimation of the surface silanols  
Markers of packing hydrophobicity  
Determination of the packing polar selectivity |
| Sander and Wise (1987, 1995) | Test analytes: benzo[a]pyrene (BaP), phenantren[3,4-c]phenantrene (PhPh), tetrabenznaphtalene (TBN) | Column selectivity:  
monomer column $\alpha_{TBN/BaP} > 1.7$  
polymer column $\alpha_{TBN/BaP} < 1$  
oligomer column $1 < \alpha_{TBN/BaP} < 1.7$ |
| Jinno (1997) | Test analytes: big molecules such as fullerenes | Molecular shape recognition |
| Berek (Berek and Novák, 1994; Berek and Tarbajovska 2002) | Test analytes: macromolecules such as polymers | Silanolophilic interactions |
| Buszewski (Buszewski et al., 2003a) | Test analytes: bronopol, acetophenon, benzene, toluene | Column classification measured according to selectivity to bronopol as an indicator of coverage density, resolution and column lifetime |
| Kaliszam (Kaliszam, 1987; Buszewski et al., 1997) | 25 test analytes with different properties | QSRR method based on structure–retention relationships |
| Jandera (Jandera et al., 1996; Krupczyńska et al., 2004a,b) | Test analytes: naphthalene sulfonic acids | Surface coverage homogenity silanol activity |
concentration of heteroatoms (e.g. metals) enables analysis at pH > 8.5.

Evaluation methods are also based on the same model. Horváth’s silanol scavenging model (Horváth et al., 1976) explains the suppression of residual silanol activity when triethylamine is added to the mobile phase (Philipson et al., 1998). The amine is a blocker that binds residual silanol groups and N,N-dimethylamine is the test compound.

Galushko (1991, 1993) recommended a method based on the solvatic computational model. According to this idea, retention is a consequence of analyte and stationary phase structure, and also mobile phase composition. In fact, this is a function of solvation free energy differences for the analyte molecules partly solvated by mobile and stationary phase.

Kaliszan (Kaliszan, 1987; Buszewski et al., 1997) proposed a QSRR model based on the linear free energy relationship (LFER) and Soczewiński’s equation (Soczewiński and Wachtmeister, 1962):

$$
\log k = \log k_w - s \varphi
$$

where $k$ is the retention factor, $k_w$ the retention factor extrapolated to pure water as the mobile phase, and $\varphi$ the percentage of organic modifier in mobile phase. The Abraham equation (Abraham and McGowan, 1987) is:

$$
\log SP = c + rR_2 + p\pi^H + a\Sigma\alpha^H + b\Sigma\beta^H + vV_s
$$

where $R_2$ is the excess molar refraction, $\pi^H$ the solute dipolarity/polarizability, $\alpha^H$ and $\beta^H$ the solute overall hydrogen bond acidity and hydrogen bond basicity, and $V_s$ the solute characteristic volume of McGowan (Abraham and McGowan, 1987). The constants $c$, $r$, $s$, $a$, $b$ and $v$ reflect the corresponding properties of the considered HPLC system.

QSRRs provide information about the dependence of analyte structure and chromatographic parameters. QSRRs seem to be a very promising and objective tool for investigation of retention mechanism, retention prediction and design of new stationary phases, but unfortunately large set of solutes must be measured to obtain valuable data (Kaliszan, 1987).

QSRR equations present stationary phases specific retention properties and they are the basis for column comparison and prediction of retention mechanism (Buszewski et al., 1997).

The correlation between log $k$ corresponding to pure aqueous mobile phase ($\log k_w$) and the partition coefficient between n-octanol and water ($\log P$) for organic compounds is used as the reference hydrophobicity parameter in chemometric tests of stationary phases. For a pseudo-membrane cholesterolic stationary phase, such correlation is linear and very similar to the results obtained in shake-flask experiments, so it can be applied for partition coefficient ($\log P$) determination.

The QSRR equations well illustrate the specific retention properties of particular stationary phases. A multiparameter regression analysis of statistically correct equations allows conclusions to be drawn concerning the mechanisms governing separation in particular chromatographic systems. On the basis of the above equations we can make a comparative analysis of columns. An example may be a comparison between a column containing a cholesterol molecule (SG-CHOL), an immobilized artificial membrane and an octadecyl column (SG-C18). The higher coefficients of correlation, $\log k_{wSG-C18} = f(\log k_{wSG-CHOL})$, $r = 0.962$, than for $\log k_{wIAM} = f(\log k_{wSG-CHOL})$, $r = 0.926$, indicate higher cholesterolic stationary phase hydrophobicity than for IAM (Fig. 7). It could be concluded that SG-CHOL has similar hydrophobic properties to SG-C18. Because cholesterol is one of the components of the natural membrane, hydrophobicity should be determined better for this stationary phase than for octadecyl or IAM packings.

Jandera proposed a semi-empirical description by interaction indices for characterization and prediction of retention selectivity in reversed-phase liquid chromatography (Jandera, 1995). The interaction indices are related to the unit volume of the space where interactions analyte $\equiv$ mobile phase $\equiv$ stationary phase occur. Non-polar interactions are proportional to the analyte molecule size ($V_s$). Specific retention factors ($k^*$) were introduced for the effects of the solute size correction.

$$
\log k^* = \frac{\log k - \log \Phi}{V_s} = A - BI
$$

The phase ratio ($\Phi = V_s/V_M$) is the volume ratio of the stationary ($V_s$) and mobile phase ($V_M$) in the column. The parameters $A$ and $B$ depend on the stationary phase, mobile phase and temperature and can be determined by measuring the retention in the terms of $\log k^*$ using a set of suitable selected standard test compounds to provide an idea of the effect of the solute polarity on the retention on a specific chromatographic column (Jandera, 1995). The presence of polar groups in the

![Figure 7. Correlation between log $k_{wSG-C18}$ vs log $k_{wSG-CHOL}$ and between log $k_{wIAM}$ vs log $k_{wSG-CHOL}$](image-url)
stationary phase may cause deviations from the linearity of the log $k^*$ vs $I_s$ plots.

On the basis of a linear free energy relationship, column hydrophobicity can be characterized by methylene group selectivity ($\alpha$), usually in the homologous $n$-alkylbenzene series. The selectivity ($\alpha$) and the end group (phenyl) retention ($\beta$) increase with increasing column hydrophobicity:

$$\log k = \log \beta + n_{C} \log \alpha = a_0 + a_n n_{C} - m_0 + m_n n_{C} \varphi$$

$$= (a_0 + a_n n_{C})(1 - p \varphi) - q \varphi$$  \hspace{1cm} (6)

where $k$ is the retention and $\varphi$ the percentage of organic modifier in mobile phase.

The parameters $a_0$, $a_n$, $m_0$, $m_n$, $p$ and $q$ can be used for characterisation of reversed-phase retention and selectivity. The contributions of the repeat methylene group to the retention in water ($a_n$) and to the decrease in retention caused by the organic solvent ($m_n$) are almost identical for various chemically bonded alkyl stationary phases (Jandera et al., 2004a,b). The phenyl end groups' contribution to the retention in water, $a_0$, and to the decrease in retention per unit concentration of the organic solvent ($m_0$) and the parameter $q$ depend on the chemistry of the stationary phase and on the possible presence of polar groups on the stationary surface. Low (sometimes even negative) $q$ values indicate increased end group retention (phenyl) with respect to the methylene retention, extrapolated to water as the eluent.

The same author proposed gradient lipophilic selectivity and gradient oligomer selectivity ($P_o$) as new criteria characterising the selectivity for oligomer compounds with non-polar or polar repeat monomer units, assuming the validity of eq. (6), where the parameters characterize the end-group ($a_0$) and the repeat oligomer unit ($a_i$) retention in pure water and the effects of the methanol concentration (volume fraction, $q$) on the change in the repeat group selectivity ($m_i$) on the end-group retention ($m_0$) (Jandera et al., 2003):

$$P_o = \frac{\Delta R}{V^0_{\text{G}}} = \frac{\sqrt{N}}{4} \frac{1}{V^0_{\text{m}}} \frac{1}{1 + k_{c}}$$

$$= \sqrt{N} \frac{1}{V^0_{\text{m}}} \frac{\log a_0}{m(A - \varphi_0)(1 + 10^{[a_0 - mA - m\Delta R_{\text{m-1}}]})}$$

$V_G$ in eq. (7) is the gradient volume, i.e. the volume of the eluate from the start to the end of gradient elution, $A$ and $\varphi_0$ are the initial and the final concentrations of the organic solvent, respectively, $N$ is the column plate number (under isocratic conditions), $V_m$ is the column hold-up volume and $n$ is the number of monomer units in the particular oligomer.

$P_o$ can be used to evaluate the column ability to resolve compounds with constant differences in polarities in a certain polarity or molecular weight range, for instance oligomers according to the molar mass distribution (Krupczyńska et al., 2004a,b). The gradient oligomer capacity is affected by the chemistry of the bonded ligands, end capping and other stationary phase properties and a higher amount of bonded carbon increases the gradient lipophilic capacity.

The behavior of ionic compounds in reversed-phase chromatography can be used as a useful marker of the properties of the stationary phases (Jandera et al., 1996; Krupczyńska et al., 2004a,b). In aqueous mobile phases, even the sorbents without ionizable chemically bonded groups, are able to form an electrical charge on the surface in contact with electrolyte solutions. Ions with an opposite charge to that of the packing surface, contained in a diffusion double layer close to the surface, interact by repulsive or attractive forces with ionizable compounds and affect their retention. The retention of the acids strongly depends on the packing type and aromatic di- and tri-sulfonic acids are sensitive markers of the stationary phase properties, silanophilic activity and other possible polar interactions. Molecules can be negatively charged and excluded from the stationary phase by repulsive interactions with residual silanols and elute close to the column dead volume in pure aqueous–organic mobile phase, often as strongly asymmetrical peaks. A combination of various attractive and repulsive interactions with different types of the adsorption centers may result in significant band tailing and was also employed to explain the experimental behavior of enantiomers on some chiral stationary phases (Jandera et al., 2002). This is also possible reason for strong peak tailing on some bonded alkylsilica columns in contrast to organic polymer columns, which can be used as an additional column performance criterion.

The retention increases when electrolytes (salts) are added to the mobile phase in concentrations 0.1–1 mol/l (Jandera et al., 1996). Increasing ionic strength decreases the thickness of the electrical double layer on the adsorbent surface, thus changing the entropy and enhancing the enthalpy arising from dispersion and dipole–dipole interactions of the non-ionic parts of the solute with the non-polar bonded alkyls (Sharp et al., 1991).

**APPLICATION OF STATIONARY PHASES AND COLUMN**

Stationary phases for HPLC and the evolution of related techniques permit qualitative and quantitative determination of biologically active compounds such as vitamins, steroids, nucleosides and peptides. Lack of vitamins can lead to abnormal body function and diseases such as rupturing of blood cells, cancer, blood coagulation, night blindness and enzyme disturbance.
Steroid hormones are essential in controlling human body functions as part of the endocrine system together with neuronal systems and the immune system. They are important in protecting against stress, shock and inflammation, act to maintain blood pressure and control water and electrolytes (sodium and potassium). Sex hormones are responsible for a virilization effect and increase muscle volume, promote growth of prostate and breast cancer cells, make the ovulatory phase of the menstrual cycle and development of breast and bone occur (Zejca and Gorczyca, 1998). They have been analyzed for medical diagnosis of stress, hypertension, amenorrhea, infertility, etc. Steroids and other compounds occur in the organism and pharmaceutics are often optical isomers (enantiomers) which possess widely different biological activity. Methods for the isolation of diastereoisomers in both pharmaceutical formulations and biological materials are of great importance. Nucleotides and nucleosides are essential constituents of nucleic acids and enzyme cofactors required for the proper functioning of cells, tissues and organs. The importance of nucleosides is demonstrated by severe symptoms, which result from defects in transfer ribonucleic acid (tRNA) structure. tRNA has a very heterogeneous structure, resulting from modifications in the nucleosides. The control of the formation of these altered nucleosides is the key to cancer prevention and treatment (Davis et al., 1979). The role of proteins in living system is very important, e.g. for enzymatic catalysis, transportation and storage, basic components of muscle, neurotransmission, immunological protection and growth control (Stryer, 1999). Saccharides are also essential for living organisms. They are energy sources and intermediates in metabolic processes. Polysaccharides are elements of membranes structure and ribose and deoxyribose are DNA and RNA components (Stryer, 1999). Thus control of the content of all these compounds in the human diet and in pharmaceutical products is necessary. Chromatography and related techniques are very helpful for this purpose. The most popular stationary phases for the analysis of the discussed compounds are listed in Table 3 and their structures are presented in Fig. 6.

**MINIATURIZATION AS A FUTURE SOLUTION**

There is generally no problem with any simple determination, but the situation is drastically complicated when the determination of substances in complex sample matrices, for example, in plant or animal tissue extracts, is required. Then it is necessary to apply more developed systems, which is associated with miniaturization techniques. Nowadays considerable progress in miniaturization may be observed in liquid chromatography. Two major trends can be distinguished here, as presented in Fig. 8. The first one concerns column miniaturization, especially decreasing their inner diameters (narrow-bore columns) and the possibility of their application to supercritical liquid chromatography, gas chromatography and electromigration techniques (Lord et al., 1997) such as electrochromatography (CEC). Table 4 presents the parameters which support the thesis that micro-columns should be applied instead...
Table 3. The most popular stationary phases for HPLC analysis of biologically active compounds

<table>
<thead>
<tr>
<th>Group of analytes</th>
<th>The stationary phase type</th>
<th>Abbreviation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamins</td>
<td>Octadecyl</td>
<td>SG-C_{18}</td>
<td>Sadlej-Sosnowska et al., 1986; Sau et al., 1997; Ake et al., 1998; Rentel et al., 1998; Cho et al., 2000; Li et al., 2000; Salo-Väinänen et al., 2000; Buszewski and Zbanyszek, 2002; Markopoulou et al., 2002; Gadzala-Kopciuch et al., 2003; Kall, 2003; Wakabayashi et al., 2003; Vinas et al., 2003; Fanali et al., 2004; Kleidus et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Triacontyl</td>
<td>SG-C_{30}</td>
<td>Sander et al., 1994; Pursch et al., 1996; Cook et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Cholesterolic</td>
<td>SG-CHOL</td>
<td>Buszewski et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Amide-C_{16}</td>
<td>—</td>
<td>Vinas et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Octyl</td>
<td>LC-8-DB</td>
<td>Ivanovic et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Cyclodextrine</td>
<td>—</td>
<td>Spencer et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Octyl</td>
<td>SG-C_{8}</td>
<td>Hill et al., 1997</td>
</tr>
<tr>
<td>Steroids</td>
<td>Octadecyl</td>
<td>SG-C_{18}</td>
<td>Bowers and Sanaullah, 1996; Chang, 1997; Kuronen et al., 1998; Gonzalez-Rumbreras and Izquierdo-Hornillos, 2000; Kelly, 2000; Nozaki, 2001; Appelbald and Irgum, 2002; Szécsi et al., 2004; Nithipatikom et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Cholesterolic in</td>
<td>SG-CHOL</td>
<td>Buszewski et al., 2001; 2003b</td>
</tr>
<tr>
<td></td>
<td>case of an optical</td>
<td>SG-10-CHOL</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>isomers separation</td>
<td>SG-MIX</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Nucleosides</td>
<td>Octadecyl</td>
<td>SG-C_{18}</td>
<td>Hartwick and Brown, 1976; Hartwick et al., 1979; Davis et al., 1979; Zakaria and Brown, 1981; Liebich et al., 1997; Uesugi et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Cholesterolic</td>
<td>SG-CHOL</td>
<td>Kowalska et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Alkylamide</td>
<td>SG-AP</td>
<td>Kowalska et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Zwitterionic</td>
<td>—</td>
<td>Jonsson and Appelbald, 2004</td>
</tr>
<tr>
<td></td>
<td>Butyl</td>
<td>SG-C_{4}</td>
<td>Falick et al., 1989; Boyes and Walker, 1995; Fujinari et al., 1996; Dichtler et al., 1998; Carr, 2002; Hamada et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Octyl</td>
<td>SG-C_{8}</td>
<td>Falick et al., 1989; Boyes and Walker, 1995; Dichtler et al., 1998; Carr, 2002</td>
</tr>
<tr>
<td>Peptides and</td>
<td>Octadecyl</td>
<td>SG-C_{18}</td>
<td>Kunitani and Johnson, 1986; Tempst et al., 1986; Garnick et al., 1988; Falick et al., 1989; Boyes and Walker, 1995; Fujinari et al., 1996; Dichtler et al., 1998; Wagner et al., 2000; DePhillips and Lenhoff, 2001; Carr, 2002;</td>
</tr>
<tr>
<td>proteins</td>
<td>Triacontyl</td>
<td>SG-C_{30}</td>
<td>Dichtler et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Cyclodextrine</td>
<td>—</td>
<td>Tang et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Bovine serum</td>
<td>BSA</td>
<td>Harada et al., 1996</td>
</tr>
<tr>
<td>Saccharides</td>
<td>Ethyl, butyl, phenyl</td>
<td>SG-C_{5}, SG-C_{4}, SG-Ph</td>
<td>Staby and Mollerup, 1996; Perkins et al., 1997; Berna et al., 1998; Machold et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Zwitterionic</td>
<td>—</td>
<td>Jiang, 2003</td>
</tr>
<tr>
<td></td>
<td>Aminopropyl</td>
<td>SG-NH_{2}</td>
<td>Indyk et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Amide</td>
<td>—</td>
<td>Churms, 1996; Kakita et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Protein-based</td>
<td>—</td>
<td>Yamauchi et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Cyclodextrine</td>
<td>—</td>
<td>Schumacher and Kroh, 1995</td>
</tr>
<tr>
<td>Diastereoisomers</td>
<td>Cyclodextrine</td>
<td>—</td>
<td>Kawaguchi et al., 1983; Armstrong et al., 1990</td>
</tr>
<tr>
<td></td>
<td>Pirkle phases</td>
<td>—</td>
<td>Pirkle and Finn, 1981; Pirkle and Pochapski, 1989; Pirkle and Burke, 1991</td>
</tr>
<tr>
<td></td>
<td>Protein-based</td>
<td>—</td>
<td>Auel and Rogers, 1987; Gilpin et al., 1991; Tittelbach and Gilpin, 1994</td>
</tr>
<tr>
<td></td>
<td>Chiral crown ethers</td>
<td>—</td>
<td>Walbroehl and Wagner, 1994; Hanksins et al., 1996; Steff Eck et al., 2002; Hyun et al, 2001</td>
</tr>
<tr>
<td></td>
<td>Cholestrolic</td>
<td>SG-CHOL</td>
<td>Delaurent et al., 1997; Buszewski et al., 1999; 2003b, 2004</td>
</tr>
<tr>
<td></td>
<td>Bovine serum albumin</td>
<td>—</td>
<td>Zhang et al., 2000</td>
</tr>
</tbody>
</table>

SG, silica gel.
Table 4. Comparison of the characteristic parameters of HPLC columns

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Conventional (250 × 4.6)</th>
<th>Narrow bore (250 × 1.5)</th>
<th>Micro (250 × 0.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear velocity of the mobile phase (u)</td>
<td>mm/s</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Flow rate (F)</td>
<td>mL/min</td>
<td>1.0</td>
<td>0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Peak volume (Vₚ)</td>
<td>µL</td>
<td>116</td>
<td>12</td>
<td>1.4</td>
</tr>
<tr>
<td>Peak height (Cₚₚₚ)</td>
<td>%</td>
<td>1.2</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Mobile phase consumption during 7 h</td>
<td>mL</td>
<td>420</td>
<td>42</td>
<td>4.2</td>
</tr>
</tbody>
</table>

of columns with reduced diameters and conventional ones. Table 4 shows that, with laminar flow of the mobile phase in all cases (u = 1.4 mm/s), the characteristic factor is 100. This concerns important parameters such as peak volume and peak height, and those connected with column efficiency represented by the number of theoretical plates. This approach allowed performance close to h = 2.2 to be achieved for columns packed with 5 µm particles.

Certainly the bottleneck here is frits responsible for packing bed stability and permeability, depending on grain arrangement homogeneity, i.e. the use of appropriate procedures of column packing (Dorsey and Cooper, 1994; Buszewski and Szumski, 2002). That is why recent research has been aimed at obtaining the so-called ‘monolithic-rod’ columns (Fig. 8), allowing elimination of the above inconveniences (Buszewski and Szumski, 2002).

The second trend in liquid chromatography miniaturization is planar systems, such as chips (quartz plates with formed, modified channels; Fig. 8). The literature provides descriptions of chip application to such separation techniques as capillary electrophoresis (Armstrong and He, 2001; Buszewski et al., 2003c), capillary gel electrophoresis (Manz et al., 1992), micellar electrokinetic chromatography (Kaniansky et al., 2000), capillary electrochromatography (Hoffmann et al., 1999), isotachphoresis (Kaniansky et al., 2002) and liquid chromatography (Jacobson et al., 1994; von Heeren et al., 1996). These systems were successfully employed for the separation of amino acids and proteins, and there is hope that they can be also useful in the work on the human genetic code (genomica, proteomica).

To sum up, the major advantages of micro-systems are:

- great reduction in chemical consumption;
- high efficiencies due to the utilization of the electroosmotic flow (flat flow profile) and long capillary columns used;
- high mass sensitivity because of low chromatographic dilution;
- the possibility of coupling LC, CZE and CEC with the different spectrometers, e.g. NMR, ICP and/or MS (hyphenated techniques);
- several modes of operation (CE, CGE, MEKC, CEC, etc.); and
- unification (the same column for several techniques, e.g. HPLC, SFC, CEC).

CONCLUSIONS

Chromatography is 100 years old and widescale HPLC application is possible owing to various types of stationary phases. Determination of the structures and properties of packing is very important for quality control and understanding retention mechanisms. Since Bristow and Knox (1977) published the fundamental paper, the evolution of methods for testing stationary phases has been rapid. Despite major possibilities in chromatographic packing testing, there is still no test which can be regarded as ‘the best’. Presented advanced techniques make possible stationary phase architecture determination and also the design of new materials.

A new generation of stationary phases are useful in modeling the processes in living cells and also more selective determination of xenobiotics.

Acknowledgments

This work was supported by the Polish Committee of Scientific Research (KBN, Warsaw, Poland, grant no. 3 T09A 150 28).

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