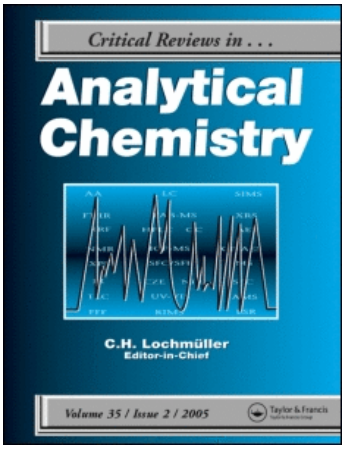

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New Generation of Chromatographic Packings and Columns for Determination of Biologically Active Compounds

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Analysis of biologically active substances is particularly important in the pharmaceutical and biomedical area. For separation of polar compounds or complex mixtures by normal (NP) or reversed phase liquid chromatography (RP-HPLC) and/or electromigration techniques, it is necessary to apply a new generation of packings and columns with strictly defined properties. It is connected to the definition of chromatographic behavior and determination of compounds that are described by its structure, as well as chemical and physical properties. One of the factors playing a predominant role in the separation process is the interaction between analyte and stationary phases. During recent years a large variety of stationary phases have become available and have been also applied in routine and research chromatographic separations. Bonded phases are in widespread use and popular because of the great number of available packing materials, allowing the solution of a scale of different separation problems. At the present time analytical methods cannot be restricted only to the so-called "black box." To meet the requirements of modern analytical techniques, strong demands are put on further, deeper understanding of the essence of the separation process, among other things, on the basis of conclusions about interactions between solute and stationary phase surfaces. For surface characterization different physicochemical methods such as CP/MAS, NMR, FT IR, DCS, chromatography, etc., have been described. The resolution, as reflected by efficiency, selectivity, and retention patterns on these materials, has been demonstrated. The effect of structure of stationary phases on retention of a model series of test analytes has been proved and numerically expressed by means of the Quantitative Structure-Retention Relationship (QSSR). The main aim of this paper is to present possibilities of determining different biologically active compounds (e.g., vitamins, steroids, nucleosides, peptides) in complex chromatographic methods (sample preparation, final analysis, validation) using new generation of stationary phases, columns, and chips divides. Special attention is dedicated to the advantage of packing materials imitating natural membranes because of the possible examination of the interaction between drug ⇔ membrane. They can permit to the design of new pharmaceuticals and observation of processes taking place on the border of phases without interfering in natural systems.

Keywords vitamins, steroids, nucleosides, peptides and proteins, separation techniques, stationary phase, sample preparation

INTRODUCTION

The determination of biologically active substances, often responsible for the proper function of the natural systems, is particularly important in the biomedical and pharmaceutical area. The development of modern analytical methods based on physicochemical effects and processes proceed in natural environments and live organisms has progressed to the possibility of quality and quantity determinations, to smaller detection level (ppb, ppt, and even ppq levels), decreases in the determination levels and also increases in precise measurements. The range of applicability of different analytical techniques e.g., electrochemical, spectral, and spectroscopic or separation (chromatography and electromigration techniques) is connected to not only properties and type of substances determined but also to selectivity and method reproducibility, making it possible to isolate and enrich the analytes. Such a complex approach to analytics,

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together with a decrease in detection limit of determined analytes, and drastic reduction of quantity of material subjected to analytical processing the sample, traces trends that predominate in analytics. This example shows significantly that these activities require development of new, unconventional procedures of sample preparation and also coupling them into hyphenated, hybrid systems limiting operator interference (automation, robotics) (1).

Modern analytical chemistry cannot limit itself only to so-called *black boxes*, which make possible only a limited number of determinations. This restriction arises from operator-analytical chemist's consciousnesses, who thanks to their knowledge will be able to use civilization's achievments. How relevant becomes G. V. Iyengary's formulation that: "The analyst is the most important component of any analytical system." He should ask why determination of given compound gives better results with this type of column than with another one. Analysts should wonder what is happening inside the *black box*—inside the separation system (2, 3). One of the factors playing a predominant role in the separation process is the interaction between analyte, stationary phase, and mobile phase (Figure 1), which influences the retention process.

For the separation of polar compounds or complex mixtures by normal (NP) or reversed phase high performance chromatography (RP-HPLC) and/or electromigration techniques, it is necessary to apply a new generation of packings and columns with strictly defined properties. It is connected with the definition of chromatographic behavior and determination of compounds, which are described by its structure, as well as chemical and physical properties. Depending on the nature of analyzed biologically active substance, the proper column and its working conditions are selected for the purpose of obtaining good both selectivity and resolution.

SELECTIVITY OR RESOLUTION AS A REMAINING QUESTION

Retention expressed as capacity factor (k), as efficiency (N), and selectivity (α) in HPLC determines resolution (R_s) effectiveness for complicated mixtures Equation 1:

$$R_s = \frac{1}{4}\sqrt{N}\frac{(\alpha - 1)}{\alpha}\frac{k_2}{(1 + k_2)} \quad [1]$$

For two peaks, α can be expressed as Equation 2:

$$\alpha = k_2/k_1 \quad [2]$$

α is a thermodynamic parameter determined by the free enthalpy, Equation 3

$$\Delta(\Delta G^o) = -RT \ln \alpha = -RT \ln \frac{k_2}{k_1} \quad [3]$$

where k_1 and k_2 are the capacity factors for peaks 1 and 2, respectively; α-selectivity; R–gas constant; T–temperature; and ΔG^o–free enthalpy.

Selectivity is one of the initiators, which in a significant way determines resolution effectiveness of complicated mixtures. This parameter is a result of the stationary phase structure, mobile phase composition, chemical properties of analytes and experimental conditions. It defines specific and nonspecific interactions between the mobile phase (MP), stationary phase (SP), and analyte (A). As presented in Figure 1, each element of

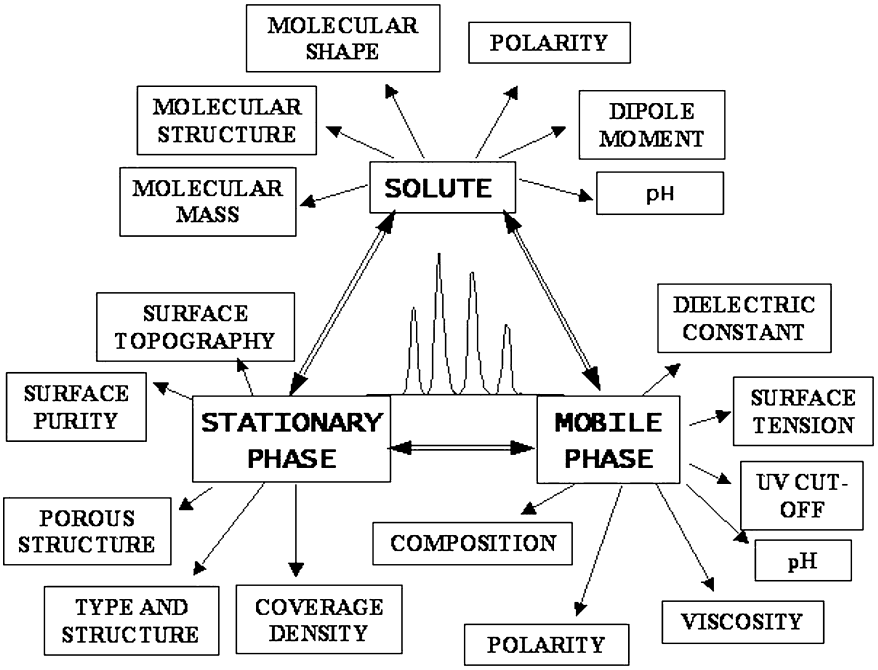

FIG. 1. Chromatographic selectivity as a scale of interaction analyte ⇔ mobile phase ⇔ stationary phase.

the considered systems is described by another parameter. From the quantitative point of view, the thermodynamic state of the considered system is described by Equation 4:

$$\ln \alpha = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \varphi \quad [4]$$

where ΔH^0 and ΔS^0 correspond to enthalpy and entropy; parameters describing of mass exchange in temperature function (T); R-gas constant; and φ-organic modifier percent in mobile phase.

The comparison of analyte retention on the various stationary phases is given by a specific selectivity $S_{2,1}$ as in Equation 5:

$$S_{2,1} = \ln \alpha = \ln k_2 - \ln k_1 \quad [5]$$

This selectivity could be measured as differences in the retention of analyte containing various functional groups in relation to nonsubstituted compounds (e.g., monosubstituted benzenes in relation to benzene) (4).

The functional groups' contribution in retention could be also determined according to Tomlinson and Smith (5, 6) using the functional groups' contribution parameter (τ_x). Parameter τ_x is determined by differences in the retention of two compounds with various functional groups Equation 6.

$$\tau_x = \log(k_{R-X}/k_{R-H}) \quad [6]$$

where k_{R-X}, k_{R-H} - capacity factor for the analyte without substituent and the compound with functional group.

If we compare Equation 2 and 6, the relationship to selectivity is obvious. On the other hand τ relates to Hansch partition coefficient Equation 7, which is based on the solvophobic theory (7).

$$\pi_x = \log(P_{R-X}/P_{R-H}) \quad [7]$$

where P_{R-H}, P_{R-X}—n-octanol-water coefficient for compound without functional group and analyte with substituent.

Comparison of those two constants for different test solutes is one of the methods for predicting retention on stationary phases.

The selectivity in chromatography and related techniques may be controlled by changes in the mobile and stationary phases (3). That state can be achieved by:

- choice and optimization of components and their participation in creation of mobile phase;
- optimization of pH;
- choice and optimization of ionogenic salt concentration;
- successive introduction of packing with controlled coverage density by chemically bonded stationary phases;
- introduction of packing with differential structural and topographic properties; and
- implementing of a wide range of packing with different functional groups.

To be up to the requirements of modern analytical techniques, strong demands are placed on a deeper understanding of the essence of separation processes, among others on basis of the conclusions about interactions between solute and stationary phase surface. This is the reason why, before starting the analysis, we should know as much as we can about analyte and packing material.

Stationary Phase Surface Characterization

Selectivity explanation and knowledge of all factors that cause retention is not possible without full characterization of the stationary phase. Retention ⇔ analyte and retention ⇔ stationary phase surface relationships are the basis of the separation process. To estimate the stationary phase properties and to describe mechanisms controlling retention, the Quantitative Structure–Chromatographic Retention Relationships (QSRR) model, proposed by Kaliszan, is applied (8, 9). QSRRs define dependencies between chromatographic retention and structures of compounds, and determines them quantitatively by numerical values. By the means of this model, it is possible to predict and determine the retention mechanism through the descriptors' examinations of different compounds (10, 11). The test proposed by Kaliszan is based on specially designed test analytes (12). Depper investigations into the molecular mechanism of the RP HPLC separation, a general solvation model developed by Soczewinski-Snyder expressed by Equation 8 (13), and by Abraham as formulated by Equation 9, were tested for many columns (14):

$$\log k = \log k_w - S\varphi \quad [8]$$

where k—the capacity factor; k_W—the capacity factor extrapolated to pure water as a mobile phase; and φ—the percentage of organic modifier in the mobile phase.

$$\log k = \log k_0 + r R_2 + v V_X + s\pi_2^H + a \sum \alpha_2^H + b \sum \beta_2^H \quad [9]$$

where analyte descriptors were used as follows: R_2—the excess molar refraction; π_2^H—analyte dipolarity/polarizability; $\sum \alpha_2^H$ and $\sum \beta_2^H$—the analyte overall hydrogen bond basicity; and V_X—the McGowan characteristic volume of analyte.

We can distinguish three basic types of relationships for a series of well-defined test compounds with different properties (14):

- $\log k = f$ (log P),
- $\log k = f\ (R_2, \pi_2^H, \sum \alpha_2^H, \sum \beta_2^H, V_x) \leftrightarrow$ retention vs. Abraham solvatochromic parameters; and
- $\log k = f\ (\delta_{\min}, \mu^2, \text{SAS}) \leftrightarrow$ retention vs. structural descriptors calculated using molecular modeling.

where: log P—logarithm of the coefficient of partition into n-octanol and water, applied as a referential parameter of compound hydrophobicity; $\delta_{\min}$—electron overcharge on the atom; μ—dipole moment; and SAS—solvent-accessible surface.

TABLE 1
Physicochemical characterization of adsorbents for HPLC and related techniques

Characteristic value	Technique	Units	"Theoretically optimal" criteria	References
Specific surface area	Low temperature adsorption-desorption of nitrogen or helium	$m^2\ g^{-1}$	320	(15, 16)
Mean pore diameter		nm	10	
Pore volume		$cm^3\ g^{-1}$	1.2	
Interparticle column porosity	Chromatography	—	$\leq$0.84	(15)
Trace amounts of metals	AAS, ICP	ppm	$\leq$500	(15, 17)
Particle shape	Microscopy		Accurately spheroidal	(15, 18)
Surface pH	pH-metry of suspensions	—	$3 \leq pH \leq 7$	(15)
Concentration and type of OH groups	NMR, FT-IR, chromatography	$\mu mol\ m^{-2}$	$5 \leq \alpha_{OH} \leq 8$	(15, 19–27)
Percentage of carbon, nitrogen, and hydrogen content	Elemental analysis	% δ[ppm]		(15, 16, 28)
Structure of chemically bonded film	NMR, FT-IR	$\gamma[cm^{-1}]$		(19–27)
Hydrophobicity	Chromatography			(22, 24, 29, 30)
Shape and steric selectivity	Chromatography			(22, 29)

For better understanding of processes taking place on the borders of phases, it is necessary to define surface properties of stationary phases. A wide range of physicochemical methods is applied to determine the support surface properties (Table 1). The most common technique, which supplies valuable information, is elemental analysis. The quantity of carbon, nitrogen, and hydrogen can be measured directly by burning the sample in the oxygen. This method allows the calculation of the surface coverage density (α_{RP}) according to the Berendsen equation (15, 26). α_{RP} value permits the calculation of surface modification degree, Equation 10:

$$\chi = \frac{\alpha_{RP}}{\alpha_{\mathrm{SiOH}}} \qquad [10]$$

where α_{RP} is the surface coverage density [$\mu mol/m^2$]; and α_{SiOH} is silanols concentration in the silica surface [$\mu mol/m^2$].

Thermogravimetry (TGA) and differential scanning calorimetry (DSC) were also applied to determine various surface characteristics of the chemically bonded phases (31–34). The first of those methods is a useful technique for the determination of organic ligands on the support surface. DSC allows the study of enthalpy effects related to the changes in water/silica gel systems with temperature. It is also possible to monitor the phase and structural transitions of adsorbent films on microporous solid surface (35–40).

The nuclear magnetic resonance (NMR) technique using cross-polarization (CP) and magic-angle spinning (MAS) is extremely useful for surface characterization of modified silica surfaces. The analysis of the NMR spectra for ^{13}C and ^{29}Si supplied much precise information about structures, which can be obtained as a result of mono- or multistep surface modification (19–21). In some of the first work in this field, the existence of various kinds of silanols on the silica surface has been proved by ^{29}Si CP/MAS NMR (21). Another spectroscopic method, which provides complementary information on the presence of functional groups on the surface of modified adsorbent, is infrared spectroscopy. FT-IR spectroscopy has been used for monitoring of progress of chemical modification of silica and its surface (17, 26, 27, 40). Except for NMR and IR spectroscopy, fluorescence is must often used (40–44). X-ray fluorescence was applied for depth analysis and X-ray photoelectron spectroscopy for surface analysis to characterize adsorbents. Comparing the results of these two methods gives a picture of the differences between the inner pore network and the outer particle surface.

Characterizing stationary phases by elemental, thermal, microcalorimetric analysis, as well as by all the spectroscopic methods, has one disadvantage. Usage of those techniques requires the destruction of the chromatographic column in order to get to chemically modified support. To avoid damaging the column, chromatographic methods can be applied. Chromatographic tests evaluate quality and analytical suitability on the basis of intermolecular interactions between the analyte, the stationary phase, and the mobile phase, so that any test based on retention characterizes not just the chromatographic column but the entire chromatographic system. The first and fundamental work in this area was done by Knox and Bristow (45), who used a mixture of components with ideal thermodynamic behavior to test the kinetic properties of HPLC columns. Later, many tests based on empirical, statistical, and thermodynamic methods were proposed (46). Table 2 presents some of them adopted

TABLE 2
Chromatographic tests for columns evaluation

Test	Test characteristic	Principle
Bristow and Knox (45)	Specific adsorption chromatography and reversed phase conditions to columns' tests	Standardization of test conditions for HPLC columns
Tanaka (29)	Test analytes: uracil (t_0), thiourea (t_0), amylobenzene, butylobenzene, triphenylene, *o*-terphenylene, caffeine, phenol, benzylamine	Hydrophobicity = $k_{\text{amylobenzene}}/k_{\text{butylobenzene}}$ Alkyl ligands amount = $k_{\text{amylobenzene}}$ Steric selectivity = $k_{\text{tripfhnylene}}/k_{\text{o-terphenylene}}$
	Eluent: MeOH/H_2O 80/20 v/v	
	Eluent: MeOH/H_2O 30/70 v/v	Hydrogen bond capacity = $k_{\text{caffeine}}k_{\text{phenol}}$
	Eluent: MeOH/0.02M phosphoric buffer (pH = 7.6) 30/70 v/v	Ion exchange capacity-IEC pH > 7 = $k_{\text{benzylamine}}/k_{\text{phenol}}$
	Eluent: MeOH/0.02M phosphoric buffer (pH = 2.7) 30/70 v/v	IEC pH < 3 = $k_{\text{benzylamine}}/k_{\text{phenol.}}$
Engelhardt (22)	Test analytes: uracil (t_0), aniline, phenol, *N*,*N*-dimethylaniline, *p*-ethyloaniline, toluene, ethylobenzene	Hydrophobicity = $k_{\text{ethylobenzene}}/k_{\text{toluene}}$; Silanol activity = p-ethylaniline peak asymetry (5% of peak high)
	Eluent: MeOH/H_2O 55/45 v/v; T = 40°C λ = 254 nm	Shape selectivity = $k_{\text{triphenylene}}/k_{\text{o-terphenyl}}$
	Test analytes: uracil (t_0), triphenylene, o-tertphenylene	
	Eluent: MeOH/H_2O 75/25 w/w, T = 40°C. λ = 254 nm;	
Kardel and Daldrup (23)	Test analytes: 5-(p-methylphenyl)-5-phenylhydantoine, diazepam, diphenylhydramine	Silanol activity: Low: $k_{\text{diphenylhydramine}} > k_{\text{MPPH}}$ High: $k_{\text{diphenylhydramine}} > k_{\text{MPPH}}$
	Eluent: 156 g ACN + 340 g phosphate buffer pH = 2.3. λ = 221 nm	Testing of columns ability to clinical and toxicological analysis
Walters (47)	Test analytes: uracil (t_0), benzene, anthracene	Hydrophobicity = $k_{\text{anthracene}}/k_{\text{benzene}}$
	Eluent: ACN/H_2O 65/35v/v; T = 40°C λ = 254 nm	
	Test analytes: uracil (t_0), anthracene *N*,*N*-diethyl-*m*-toluamide (DETA)	Silanol activity = $k_{\text{DETA}}/k_{\text{anthracene}}$
	Eluent: 100% ACN, temp. 40°C	
McCally (24)	Test analytes: nortriptyline, nicotine, amphetamine, pyridine, benzylamine, codeine, quinine, procainamide, diphenhydramine	Retention factors, asymmetry factors and column efficiency
	Eluent: ACN, MeOH THF at pH 3.0 and neutral pH	
Rohrschneider (48)	Test analytes: naphtalene, acetonilidine, phenol, benzonitrile	Characterization of columns for RP HPLC
	Eluent: MeOH/H_2O and ACN/H_2O	
Neue (25)	Test analytes: amitriptyline, naphthalene, acenaphtalene, dipropylphtalate and butylparaben	Estimation of the surface silanols Markers of packing hydrophobicity
	Eluent: neutral pH: 35% 20 mM K_2HPO_4-KH_2PO_4buffer pH = 7.0 and 65% MeOH, acidic pH: 20% 50 mM H_3PO_4 - KH_2PO_4buffer pH = 3.0 and 80% ACN	Determination of the packing polar selectivity

(Continued on next page)

TABLE 2
Chromatographic tests for columns evaluation *(Continued)*

Test	Test characteristic	Principle
Sander and Wise (16, 49)	Test analytes: benzo[a]pirene (BaP), phenantro[3,4-c]phenantrene (PhPh), tetrabenznaphtalene (TBN). Eluent: ACN/H_2O 85/15, flow rate 2 ml/min $\lambda = 254$ nm	Column selectivity, Monomer column $\alpha_{TBN/BaP} > 1.7$, polymer column $\alpha_{TBN/BaP} < 1$, oligomer column $1 < \alpha_{TBN/BaP} < 1.7$
Jinno (50)	Test analytes: big molecules such as fullerenes Eluent: n-hexane	Molecular shape recognition
Berek (51)	Test analytes: Macromolecules such as polymers	Silanophilic interactions
Jandera Krupczyńska, and Buszewski (52)	Test analytes: naphtalenesulphonic acids Eluent: 0.4M Na_2SO_4, $\lambda = 254$ nm	Surface coverage homogeneity, silanol activity
Nondek, Buszewski, and Berek (53)	Test analytes: pyridine, 2,6-dimethylpiridine, acetone, nitrobenzene Eluent: n-heptane/chloroform 30/10, flow rate: 1.0 ml/min, temp. 20°C, $\lambda = 254$ nm	Silanol activity
Galushko (30)	Test analytes: aniline phenol, benzene, toluene	Hydrophobicity and silanophobicity
Kaliszan (8)	25 test analytes with different properties	QSRR method based on structure—retention relationships

for quantitative description of chromatographic columns quality in RP HPLC (22–25, 29, 30, 45, 47–53).

Full stationary phases characterization allows for their classification proposed, for instance, by Unger (54) (Figure 2). He distinguished phases with *mono-layer* structure—"monomers" (Figure 2a), obtained by means of a monofunctional modifier ($F = 1$), "polymers" (Figure 2b) ($F = 1$–3) or those with *sandwich* structure (Figure 2c), where the "bed" is formed as the result of multistage polymerization, and then modified. The latest solutions are oriented at the preparation of packings with the so-called "diffusion barrier" (e.g., polar units are "built" into the hydrophobic chain) (Figure 2d), or various kinds of mixed phases (Figures 2e and 2f) containing different specific functional groups.

Analyte Properties

All substances can be divided into groups with regard to their structure, proprieties, or biological functions. Among others are drugs, of which analysis is especially important to the pharmaceutical industry. Scientifically, a drug is "any substance, other than food, which is taken to change the way the body or the mind functions." It is generally recognized that physicochemical properties play an important role in governing the biological performance of drugs. The most important physicochemical properties related to biological performance are: lipophility, molecular size, pK_a of the weak acid or weak base, chemical stability, solubility, and molecular interactions (55–57). However those properties influence not only the biological activity of a compound, but also its chromatographic behavior. They are the main factor related to possible interactions between the analyte and the stationary phase surface, and consequently about separation processes (12).

Vitamins are organic molecules that function in a wide variety of capacities within the body. The most prominent function is as cofactors for enzymatic reactions. The vitamins are of two types distinct in their solubility in water: water-soluble vitamins, such as thiamin (B_1), biotin (H), and ascorbic acid (C); and fat-soluble vitamins such as vitamin A, and E (58). The molecular structure of the compound uniquely defines all its physical, chemical, and biological properties. The structures of some of the vitamins are presented at Figure 3. In their structure polar functional groups occur (hydroxyl, ionic, carboxylic), able to hydrogen bond and capable of donor—acceptor interactions, as well as aromatic rings, responsible for the $\pi \ldots \pi$ interactions, and alkyl ligands, able to have van der Waals interactions.

Steroids, like vitamins, have a variety of uses in the human body. Those substances are fat-soluble hormones. Steroids in human body fluids are present in free and conjugated forms. Free steroids are slightly hydrophobic, and the conjugated forms (glucuronide and sulfate) are hydrophilic. The solubility of steroids in water influences their extraction methods from adrenospecimens. Steroids have molecular sizes ranging form about 200–1000, and are nonvolatile. All of steroids share the same basic four-ring (three cyclohexane rings and one cyclopentane) carbon structure, but differ in the number of carbon atoms attached to the number-17 carbon atom in the structure, and in the manner in which hydrogen, oxygen, and the hydroxyl groups are attached to the carbon atoms (Figure 3) (59). In their structure, except for hydroxyl groups, aromatic rings, alkyl ligands, specific interaction sites—chiral centers—are also present.

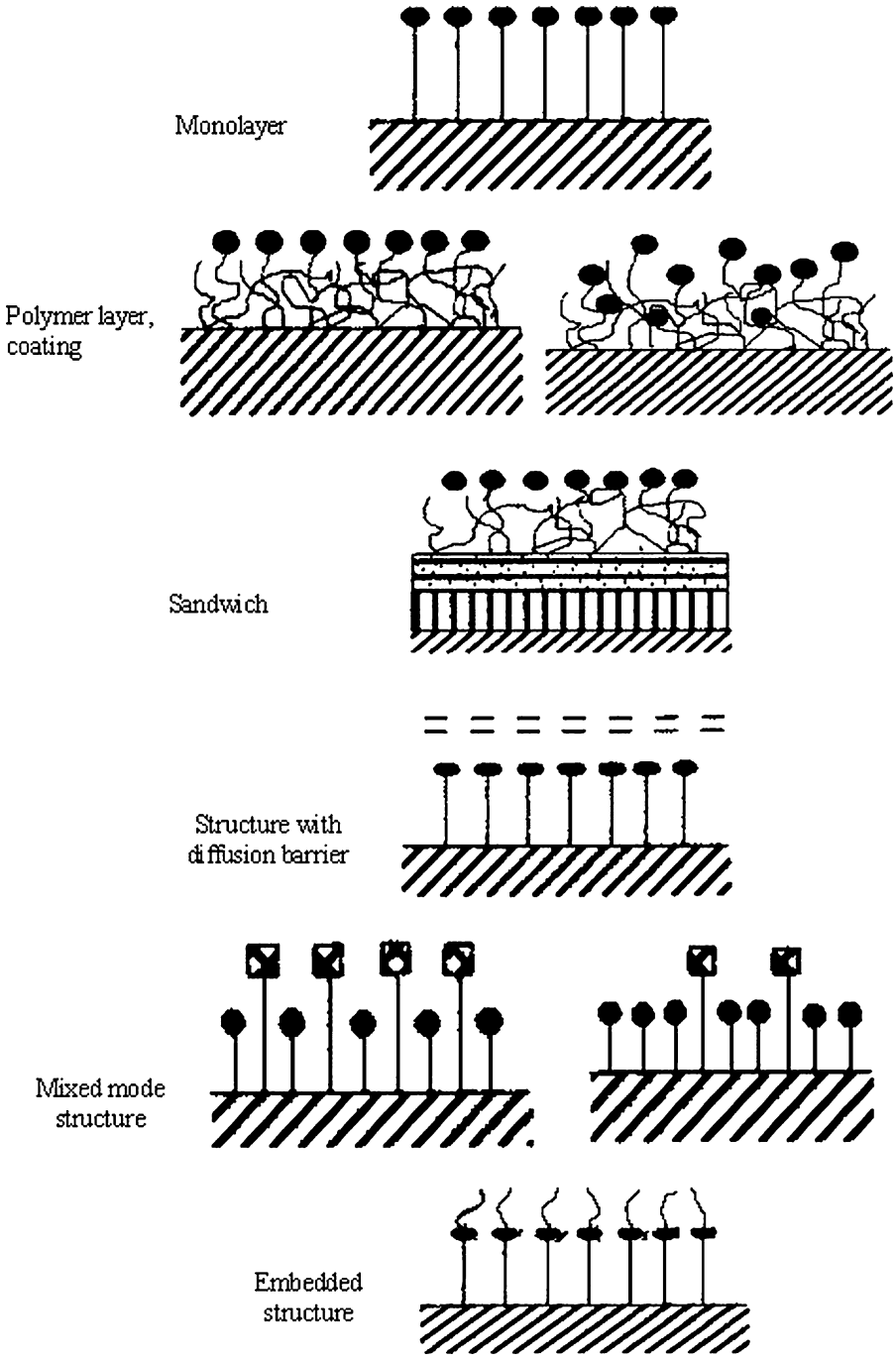

FIG. 2. Chemically bonded stationary phases classification according to (54).

Substances essential for the proper function of organism are likewise of interest to researchers. To this group of compounds belong, among others, nucleosides, nucleotides, peptides, and proteins (Figure 3). Nucleosides are both weak acids and bases (60, 61). Above a characteristic pK_b value, they are negatively charged and below pK_a-positively charged. The acid-base character of given molecules plays an important role in their separation mechanism in a chromatographic column. In the case of peptide and protein analysis, the most important properties of this compounds are their size, charge, hydrophobicity, and biological interaction or affinity (62). The properties of small peptides are similar to those of individual amino acids. Therefore, the character of their side chains and substituent groups determines their chromatographic behavior, i.e., the strength of the basic and acidic ionizable groups present, and also by the degree of hydrophobicity or hydrophility. With increasing

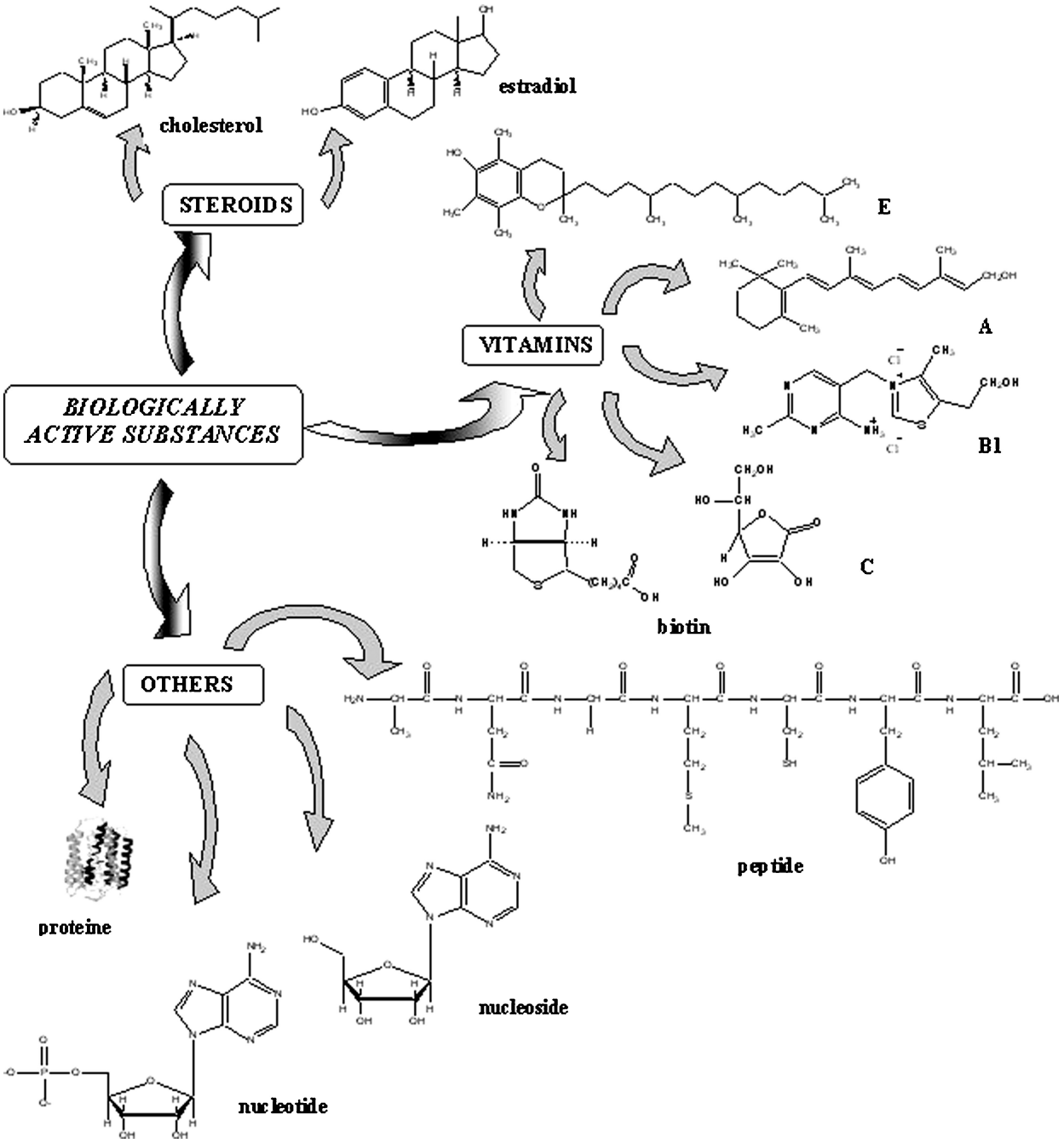

FIG. 3. Schematic structures of example groups of biologically active compounds.

number of amino acid residues, the importance of the peptide primary (and progressively secondary, tertiary, and quaternary) increases. As a consequence the conformation of molecules begins to play a role and can decisively affect their retention (63, 64).

The similar variety of a functional group characterizes also the stationary phases, on which surfaces might be localized the hydrophilic and hydrophobic ligands. Both functional groups on the packing surface and in the analyte structure will determine the possible interactions between them (Table 3). As it seems one of the most important matters is knowledge of exactly what kind of ligand and how much of which one is situated on the modified support surface. This knowledge facilitates suggestions about retention.

Evolution that took place in the stationary phase characterization permits better control of packing synthesis process and the design of new, more selective systems. Determination of low concentrations of biologically active substances present in the

TABLE 3
Interactions in chromatographic separations

Intermolecular interactions
Physical
Ion – ion
Ion – dipole
Dipole – dipole
Dipole – induced dipole
Instantaneous dipole – induced dipole (dispersive interactions)
Chemical
Hydrogen bonding
Electron pair donor – Electron pair acceptor (EPD – EPA interactions)
Solvophobic (hydrophobic)
General term incorporating both physical and chemical interactions

complex matrix requires the advantage of more sensitive methods. Miniaturization of separation and detection modes fulfill those requirements and thus is the future of analytical methods (Figure 4). The scientists paid attention to the development of micro separation systems, which allow high efficiency, good resolution, and short analysis time, high mass sensitivity because of low sample dilution, connection possibilities to mass spectrometry (NMR, ICP, MS), and unification. Thanks to those advantages, all of these methods have become very popular in the sample preparation as well as in the final analyte determination.

SAMPLE PREPARATION

Among the entire scale of sample preparation methods, based on the oldest theoretical descriptions (distillation, extraction, adsorption, filtration, freezing out) and their modifications, are interesting to analytical chemists. It is important so these methods could be miniaturized and adapted for chromatographic and related techniques determinations (predominant in routine laboratory practice) in a simple way. It is true the choice of isolation methods ⇔ determination dominates the old chemical rule "like dissolves like." The scheme of complex analytical proceeding presented in the Figure 5 shows a wide spectrum of methods, which guarantee result at the high level (65).

There is a simple coupling of sample preparation methods and final determination (off-line system) in many cases of routine determinations. There are applied to on-line systems within automation and robotics, which gives more precision in determinations and the possibility of coupling of systems in the series. They are recommended especially for routine analysis. An example may be coupling of extraction and derivatization techniques including enzymatic cutting or selectivity sorption and membrane exclusion. These all are for meeting the requirements of QC/QA (quality control/quality assurance); the basic requirement of validation (stage of final proceeding). Yet, to reach that requires attention to systematic implementation of exploiting new materials as a new generation stationary silica phase and adsorbents with differential architecture (e.g., carbons, diatomites, zeolites, or porosic polymers). These phases containing different groups (aminopropyl, aryl, diol, octadecyl, and octyl) may be used successfully to isolate such biologically important substances as vitamins, hormones, amino acids,

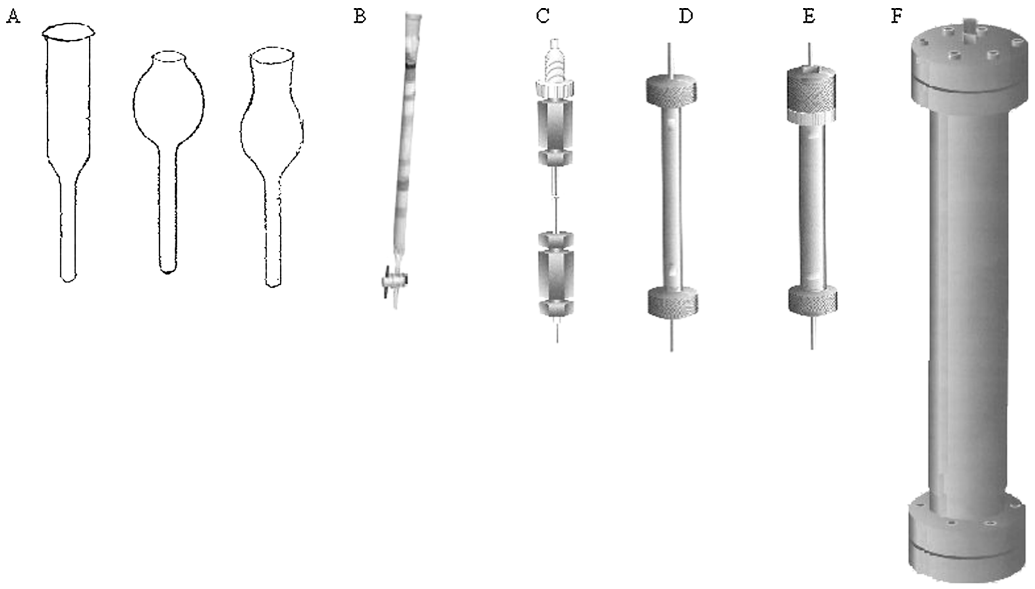

FIG. 4. Schematic illustration of high performance liquid chromatography column evolution process (A) according Tswiet, (B) according Sthal'a, (C) conventional analytical column (250 × 4.6 mm ID), (D) narrow bore column (250 × 1.0 mm ID), (E) microcolumn for micro-HPLC and/or CZE (100 μm × 250 mm ID), and (F) preparative column (250 × 45 mm ID).

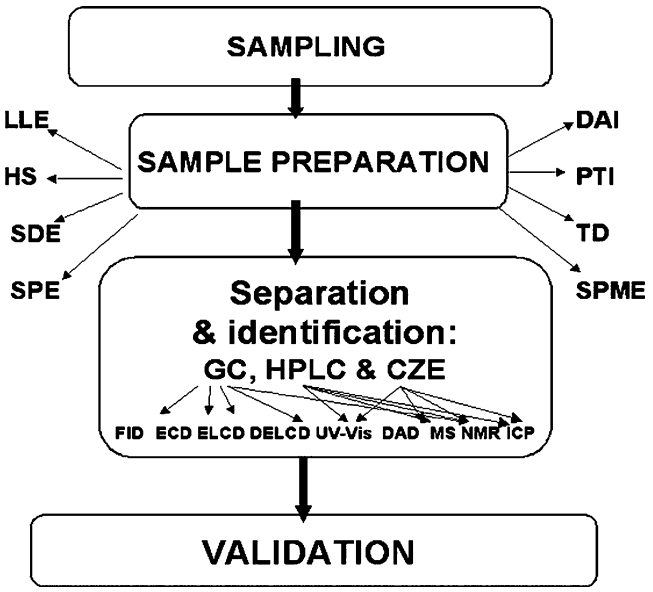

FIG. 5. The schema of complex analytical proceeding for trace analysis.

alkaloids, sample enrichment, or their purification from interfering substances (e.g., proteins) (65).

Sample extraction procedures and the selection of extraction solvents and additives are determined by the source of the material, the stability of the molecules, the chromatographic and detection techniques that will be used (66).

Vitamins

The conventional method for the isolation of fat-soluble vitamins includes liquid-liquid extraction using solvents such as hexane, acetone, tetrahydrofuran, methanol, heptane, acetonitrile, and xylene (67–70). Some samples e.g., urine, should be hydrolyzed before analysis as also proteins from serum and plasma are precipitated before vitamin extraction by adding ethanol (71), acetonitrile (72), and an inorganic salt such as sodium sulfate (73). Extraction from complex matrices such as animal feeds is not easy, and the sample should be first saponified to extraction in order to disrupt the matrix and degrade triacylglycerols and produce soaps of the free fatty acids (65). Solid phase extraction (SPE) has been employed in some applications as sample preparation method for vitamin analysis (67, 75–80). SPE is rapid, selective, provides good preconcentration and recovery of analyte. The most popular sorbent is octadecyl (77, 81–83) (Figure 6), but also pure silica (84), combination of C_{18} and silica (85), Florisil (86), polar aminopropyl silica (87). Miniaturization of extraction is very important in clinical and pharmaceutical analysis thus solid-phase microextraction (SPME) procedures were developed (78, 88).

Some vitamins, especially fat-soluble, are sensitive to light, temperature, and oxygen. Supercritical fluid extraction (SFE) is an alternative possibility for fast sample preparation. It offers minimal solvent consumption, the exclusion of oxygen, and higher selectivity (68). SFE was used for vitamin A extraction from liver (89) and cereal products (90), vitamin D from pharmaceuticals (91). Vitamins are also extracted by SFE from such complicated matrices as food (92), serum (93), creams (94), ointments (95), and powder infant formulas (96).

Steroids

Specimens used for clinical analysis of steroids include urine, serum, and saliva. Different behavior of steroids in human specimens requires different considerations for developing sample preparation. Conjugated steroids are often hydrolyzed with a strong inorganic acid (sulfuric acid, hydrochloric acid, nitric acid), or treated with sulfatase or glucuronidase enzymes to produce the free form prior to analysis. Free forms of steroids are often at too low a level for analysis. Therefore, enrichment of free urinary steroids are carried out by extraction with octadecyl silica or bovine serum albumin-bound ODS gel-packed minicolumns (97, 98). To obtain information on serum-free steroids, serum specimens are ultra-filtrated by dialysis (99) or membrane filtration. Albumin-bound steroids are released easily from albumin with organic solvents (dichloromethane, diethyl ether) and solid phase extraction on the C_{18} packings (Figure 6) (100).

The steroids in hair specimens were extracted after alkaline digestion (101). Preparation of human urine requires dilution with ammonium acetate, ultrasonication, centrifugation, and solid-phase extraction (102).

Nucleosides

To study content of the free nucleoside form cells, the most important first step is to extract compounds into a liquid medium. The reagents used for this purpose should: lyse the cell, precipitate the protein, and also provide a neutral environment. The most commonly used is perchloric acid, and the resulting acidic supernatant is neutralized with potassium hydroxide (103, 104). Protein removal is probably the most important step in the analysis of nucleosides and bases in biological matrices. For that purpose, perchloric acid or trichloroacetic acid have also been used (105). In the case of extraction of nucleosides from serum, plasma and urine samples have been ultrafiltrated through membrane cones with retention of high-molecular weight proteins (106, 107). This method is preferred since it does not alter the pH of medium, dilute the sample, or interfere with the UV absorbance of sample constituents. tRNA samples require the enzymatic hydrolysis with specific enzymes, e.g., different types of nuclease, to obtain ribonucleosides (108, 109). According to Weisseman procedure urine samples are diluted, acidified, run on ion-exchange chromatograph column. The eluate was treated with silver nitrate, resuspended with hydrochloric acid, heated to 100°C and centrifuged (110, 111). Urines are extracted also by affinity chromatography using a phenylboronic acid gel (112).

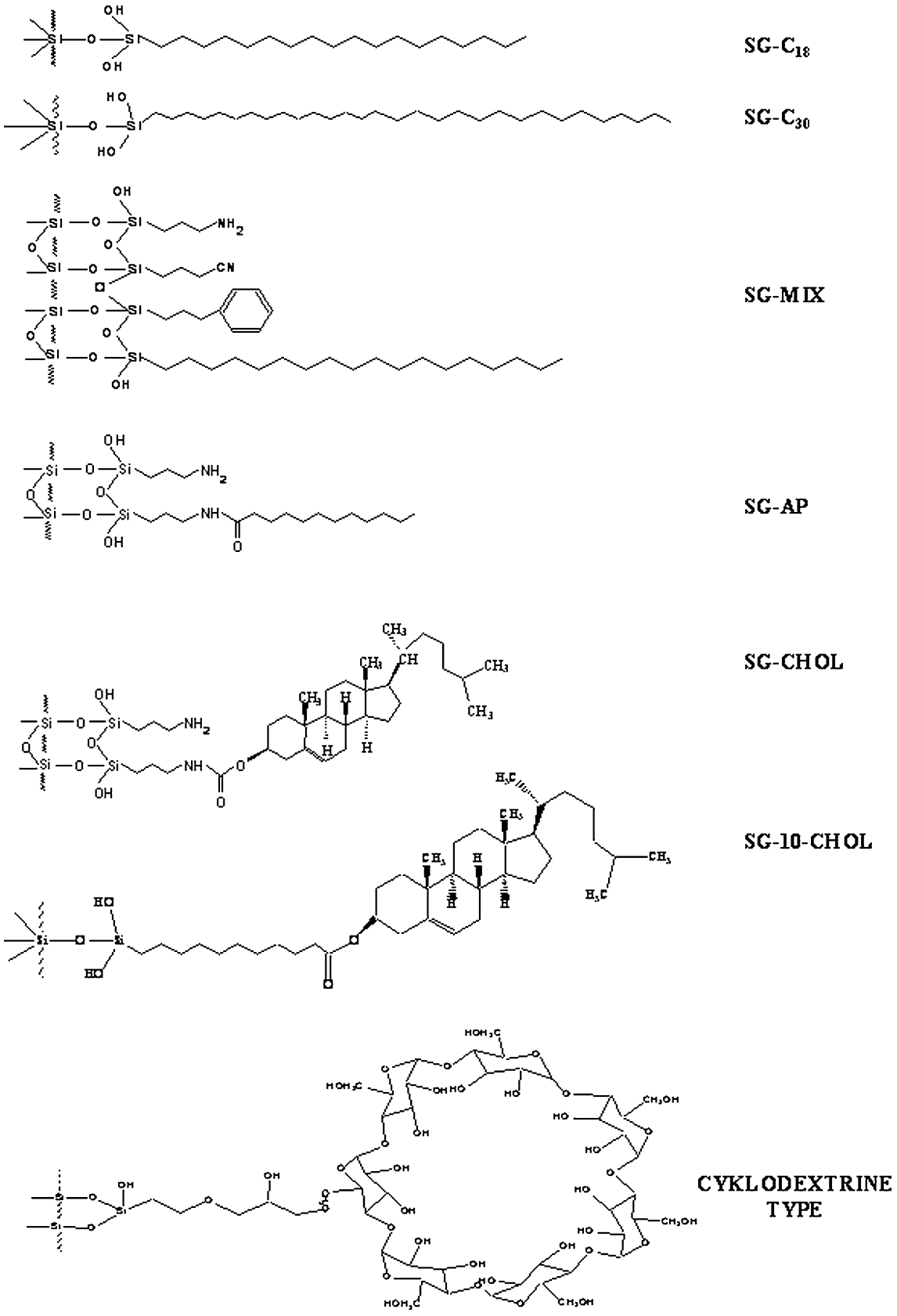

FIG. 6. Schematic structure of the stationary phases presented in the paper.

DETERMINATION POSSIBILITIES OF SOME BIOLOGICALLY ACTIVE SUBSTANCES

There is no problem with any simple determination of biologically active compounds; however, the situation is drastically complicated when the determination of the substance occurs in a sample with complex structure of matrices, e.g., plant or animal tissues. Then, it is necessary to apply more developed and automatized systems such as hyphenated and coupled techniques. These techniques allow the determination of substances at a low concentration level and makes possibile speciation analysis with the confirmation of the analyte identity. Such high-resolution systems are often miniaturized, requiring the use of specific packing, columns, or (now) chip characterization with specific, repeated, dedicated as "to measure" called "tailor-made" properties. Those properties should guarantee high shelf efficiency, selectivity and reproducibility of retention data (2, 3).

Vitamins

Vitamins play an important role in healthy life of the human organism. There are many pharmaceuticals and multivitamin products to fulfill an organism's need. The vitamins are divided into fat- and water-soluble. Lack of vitamins can lead to abnormal organism function and disease such as: rupturing of blood cells and cancer, blood coagulation, night blindness, and enzyme function disturbance. This is why the control of vitamin content in human diet and pharmaceutical products is necessary. Chromatography and related techniques are very helpful for this purpose. It is not possible to determine all vitamins at the same time. Hence, methods for group analysis were developed.

Planar (113), thin-layer (114), and high-performance liquid chromatography (115–120) capillary electrophoresis (121–122), micellar electrokinetic chromatography (122, 124, 126), reversed-phase ion-pair chromatography (126) were used for the qualitative and quantitative simultaneous analysis of B-vitamins. The RP-HPLC method was used for the investigation of ascorbic acid (127) as were micellar electrokinetic chromatography (124) and capillary electrophoresis (128). Vitamins A and E are anti-oxidants, which play very meaningful roles in appropriate human functions, especially in counteracting cancer. There are many methods for determination of the above substances. Usually, reversed phase liquid chromatography is applied (116, 117, 120, 129–134). Isomer separation of compounds generically called vitamins A and E is very difficult. Some isomers are biologically active, some are not. Thus far, satisfactory separation has been achieved using normal-phase chromatography (135), supercritical fluid chromatography with fast atom bombardment mass spectrometry (136), and normal phase chromatography with a moving belt using electron impact and chemical ionization (137). The application of "traditional" C_{18} stationary phases is not sufficient for separation of β and γ isomers. Albert and coworkers obtained the best results for reversed-phase C_{30} material with on-line coupled mass spectrometry (MS) and nuclear magnetic resonance (NMR) for compound identification (132, 133). Buszewski and coworkers proposed the cholesterolic stationary phase for isomer separation (Figure 7). Resolution for C_{18} stationary phase is very poor while for cholesterolic column isomers separation is good. It is also a very interesting study of potential interactions between analyte (biologically active compound) $\Leftrightarrow$ stationary phase, which contains components of biological membranes (134).

Other vitamins (PP, K, D, H) could also be determined using liquid chromatography and related techniques (116, 117, 120, 128, 138, 139). Of course, various detection possibilities are possible. UV-Vis is the most popular (115, 117, 126, 134, 140, 141) but also electrochemical, fluorescence, evaporative light scattering, and NMR detectors are powerful (118, 132, 133, 142–144).

Steroids

Determination of steroids has become important in the medical and pharmaceutical fields. Their analysis has been carried out for medicinal diagnosis of stress, hypertension, amenorrhea, infertility, etc. Methods for steroid analysis must respond to the clinical demands for rapid and high sensitive assays. Because of their close structural similarity, metabolic versatility, and occurrence at low concentrations in body fluids and tissues, the development of reliable analytical methods for steroids is a challenging subject for analytical chemists (145).

Some of the procedures used in the control of pharmaceutical formulations containing one anabolic steroid are based on the UV spectrophotometric measurements of the organic extracts of the compound (146). Other techniques showing better selectivity have being introduced, such as thin layer and column chromatography, but they are time consuming. Finally reversed-phase liquid chromatography (RP HPLC) methods with UV-Vis and DAD detection with isocratic (147) and gradient elution (148) were developed in steroid separation. The most frequently used stationary phase is octadecyl SG-C_{18}. One of the difficulties is the separation of optical isomers, which often possess a widely different biological activity. Methods for their isolation are very important. Estradiols are the most active among all the estrogens in the human body, responsible for stimulating growth of the female reproductive system. The hormone 17-α-estradiol is the most active of all well-known female sexual hormones, while the isomer 17-β-estradiol is almost not active.

The work of Buszewski et. al. (149) describes results of application of the new types of stationary phases, imitating biological membranes, for the separation of estradiols using the RP HPLC system. Results from chromatographic investigations of steroids are presented in Figure 8.

The most effective separation of 17-α-estradiol from this diastereoisomer was obtained for the SG-CHOL (Figure 6) packing with an acetonitrile-water mixture (60/40 v/v) as a mobile phase. The better resolution for this stationary phase is a consequence of the specific structural properties of cholesterol-based materials. Both estradiols are different in the configuration at the chiral center of carbon-17. The molecule of cholesterol possesses 8 asymmetric carbons, which makes cholesteric

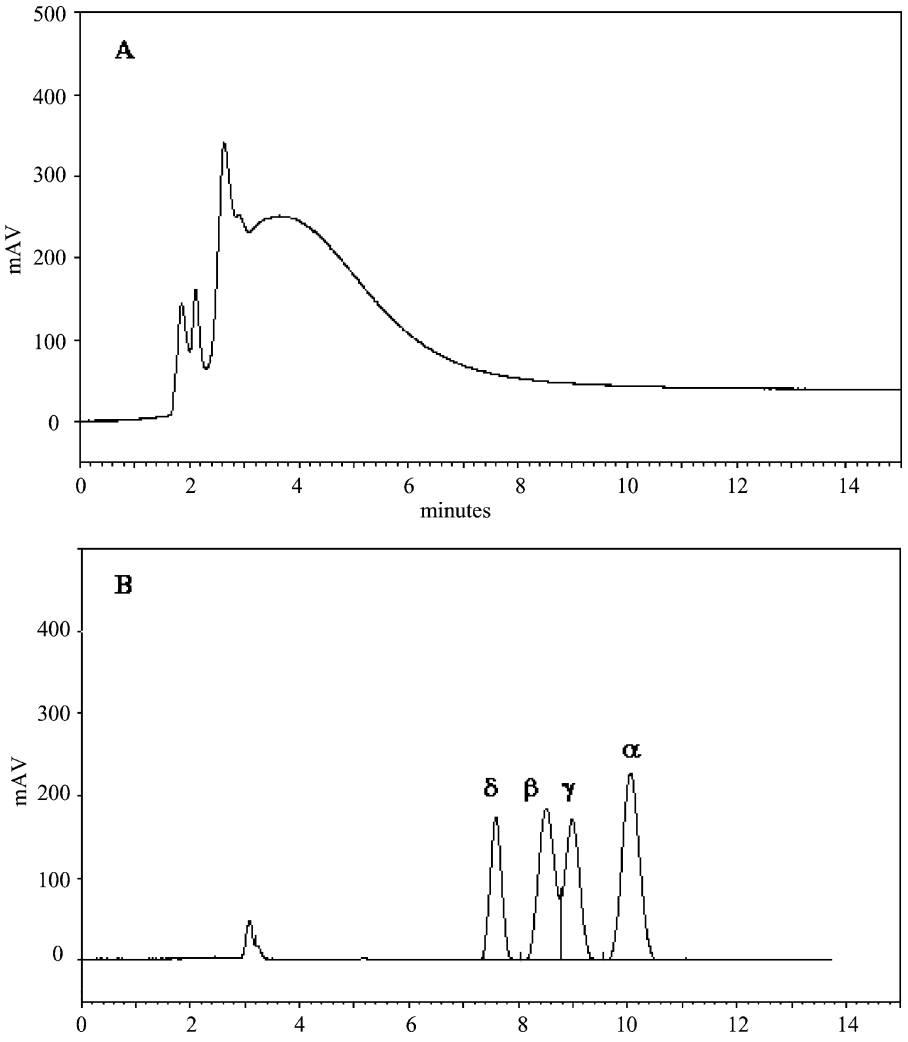

FIG. 7. Chromatograms of tocopherol isomers separation for various columns: SG-C_{18} (A), SG-Cholesterolic (B); mobile phase: 100% methanol, flow rate: 1 ml/min, detection: UV-Vis DAD.

stationary phases a promising material for the separation of enantiomers (150, 151). However, better shape recognition capabilities for SG-CHOL material could be also a consequence of liquid crystalline properties of the stationary phase (152, 153). Hydrophilic groups localized in the steroid analyzed determine the better separation on those stationary phases, where the surface has more polar properties. For the hydrocarbonaceous SG-C_{18} material, resolution equals zero, which is in agreement with the hydrophobic character of the bonded organic chains, without specific groups in its structure.

Similarly, good resolution and selectivity were obtained during separation of the drug—budesonide (22-S and 22-R epimers) on SG-10-CHOL packing material (150). Budesonide epimers differs in the configuration of 1 carbon atom and 22-R is more or less 3 times more biologically active than 22-S epimer. Also important in the case of pharmaceutical analysis is the separation of finasteride from its metabolite *N*-methylfinasteride; it is impossible to separate them on the octadecyl stationary phase. For that purpose, packing with polar and nonpolar properties has been used. SG-MIX consists of hydrophilic ligands such as: hydroxyl (−OH), amino ($-NH_2$), cyano (−CN), and also hydrophobic octadecyl ($-C_{18}$), octyl ($-C_8$), and phenyl (−Ph) groups. Application of this stationary phase gave good results and good resolution (154).

The mobile phase in HPLC consists most frequently of high concentrations of acetonitrile/methanol and water. Nevertheless in the case of the ion-pairing mode, which also has been used, the mobile phase contains counterion (155). Micellar liquid chromatography (MLC) also require additives to the mobile phase e.g., sodium dodecyl sulfate (SDS). Amin and coworkers used a micellar SDS mobile phase to determine mixtures of anabolic steroids (156). He demonstrated that this method has few advantages in comparison to reverse-phase HPLC: sensitivity, and speed, and simplicity (149, 155).

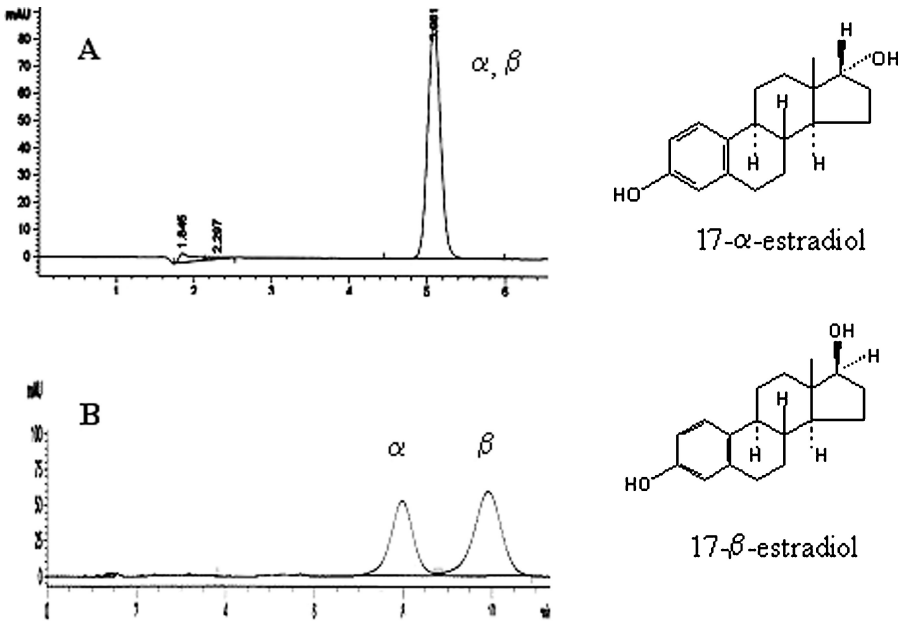

FIG. 8. Separation of 17-α-estradiol (α) and 17-β-estradiol (β) on (A) SG-C18, (B) SG-CHOL. Separation conditions—mobile phase: ACN/water 60/40 v/v, flow rate—1 ml/min, detection—UV, $\lambda = 254$ nm.

Numerous other methods have been described for characterization and determination of the steroids; for example immunoassay, receptor binding assay, and gas chromatography (GC) mass spectrometry (MS) capillary electrophoresis (CE) (157–159). Separation of steroids by all these methods takes a long time. Sample preparation often makes the method longer, e.g., in the case of LC-MS utilization, which has recently been used because of its specificity and versatility. The problem is, however, efficiencies of the ionization, because most steroids are ionized relatively low. Derivatization enhances the ionization efficiencies of steroids, leading to high sensitivity and specific detection. For electrospray ionization MS, the introduction of permanently charged moieties or easily ionizable moieties effectively increases the sensitivity of detection of steroids. Among others Shimada and coworkers (158) have introduced several methods of neutral steroid ionization for LC-MS. Ma and coworkers (160) have also studied the steroid separation possibilities by LC-MS. Tandem ion-spray LC-MS-MS was used by Bean for the sulfate and glucuronide conjugates of testosterone and epitestosterone (99).

Due to low volatility and thermal instability, drug conjugates are not directly suitable for gas chromatography mass spectrometry, so these compounds are commonly hydrolyzed enzymatically for further characterization. Following isolation and derivatization, the released steroids are then confirmed by GC–MS (99). The majority of analytical methods for endogenous and exogenous androgens use GC–MS (or GC), which allows the identification of these steroids and metabolites with low detection limits; however, they require the previous derivatization. The derivatization forms most commonly used for GC or GC–MS have been the trimethylsilyl (TMS) and other alkylsilyl ethers for hydroxy functions and the *O*-methyloxime (MO) for ketonic functions. Juricskay and Telegdy reported (161) the determination of 28 different urinary steroids. Clinical applications of GC and GC–MS of steroids were reviewed in 1999 (162).

Steroids have UV absorption (254 nm) but no native fluorescence except for estrogens. The steroids in human fluids are often at levels too low to detect; therefore, highly sensitive determination methods, better than UV, are indispensable. For that purpose fluorescence and chemiluminescence detection methods were developed. However, steroids have no native fluorescence except for testoestrogens, and derivatization of those compounds was required (145). The fluorescence methods for steroids analysis and detection introduced were native fluorescence (163), photochemical derivatization (164), and sulfuric acid–ethanol fluorescence (165), and fluorescent derivatization with labeling reagents (166). Chemiluminescent determination of steroid enables for the detection limit estimation is the same or superior to the limit of radioimmunoassay (167, 168).

Due to long analysis times for conventional chromatographic methods, ultraminiaturization of a separation system has been a recent research direction. Advantages of such systems in the clinical laboratory include primarily faster analysis. Fused-silica capillary in microchip application is the major tool for miniaturization in GC, HPLC, and CE (145, 169–172).

Fused silica capillaries, which have narrow bore and long length, were used for GC and GC-MS. Capillary GC is characteristic for high resolution in the analysis of steroids in clinical specimens. Capillary GC coupled with MS became a more powerful tool to identify unknown peaks of steroid for doping tests,

etc., because MS analyzes the chemical structures of the volatile steroids after separation by capillary GC on-line (169, 170).

The fused-silica capillary is also used for HPLC separation of anabolic steroids. To both: the inner face of a capillary fused-silica tube and on gels packed into the capillary, functional groups such as octadecyl were bonded. Capillary HPLC is very suitable for MS detection, especially capillary HPLC coupled to MS with an electrospray ionization source. The main advantage of capillary HPLC coupled to MS for steroid analysis is that there is no need for their derivatization (173–175).

Miniaturization in micellar electrokinetic capillary chromatography (MEKC) is possible thanks to the use of fused-silica capillaries (171). Steroids form micelles with a cationic surfactant as a buffer component, and migrate by electricity. Steroids are detectable with UV detection (254 nm), and photodiode array detection after separation by CE on-line.

A microchip with a microfabricated channel has been tested for assay of steroids in a serum specimen. Separation of free, fluorescein-labeled cortisol was performed using a microchip electrophoretic system. The microchannel enables fast separation within a few minutes, and the sample volume required is very small [172].

Capillary electrochromatography as a separation technique in pharmaceutical analysis offers highly efficient, fast, high peak capacity, so it is an interesting method for the steroid analysis. Thiam et al. has studied the effect of acidic modifier, buffer concentration, mobile phase composition etc., on cholesterol and derivatives separation (176). Stead et al. (177) and Seifar et al. (178) introduced the utilization of CEC in the study of endogenous steroids. Que et al. used a macroporous acrylic monolithic stationary phase for isocratic and gradient endogenous steroids analysis by capillary electrochromatography with laser-induced fluorescence (LIF) and electrospray ionization (ESI) mass spectrometry (MS) detection (179). Lord et al. demonstrated utilization on-line CEC-MS in steroids separation (180). Jiskra et al. developed a method for the separation of 5 closely related pharmaceuticaly active steroids by capillary electrochromatography (181).

Nucleosides

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 the giving symptoms, which result form defects in transfer ribonucleic acid (tRNA) structure. tRNA that has the most heterogeneous structure results from modifications in the nucleosides. Borek and Kerr (182) have discussed atypical tRNAs. Modifications of the major nucleosides occur by the addition of methyl groups by methyltransfrease enzymes. In the tumor cells, tRNA methyltransferases are very active, and the levels of modified nucleosides are thus the measure of the turnover rate of tRNA. There is no incorporation mechanism of modified nucelosides into tRNA, and they are eliminated from the organism in the urine. The control of the formation of these altered nucleosides is the key to cancer prevention and treatment (105, 180, 183).

Early methods of nucleoside analysis included separations from urine using cation-exchange isolation followed by thin-layer chromatography (TLC). However, this method does not have a good selectivity, efficiency of separation, or reliability of quantitative measurement (105, 183, 184).

Because of TLC low sensitivity another technique was applied for modified nucleoside analysis. Among others Gehrke and coworkers have used gas-liquid chromatography (GLC). This method has good sensitivity and resolution compared to TLC. The disadvantage is, however, extensive cleanup of the samples and derivatization of the nucleosides before chromatography (105, 185–187).

More recent methods of modified nucleoside analysis includes various types of high-performance liquid chromatography (HPLC) (102, 105, 106, 108, 181, 183, 188–191). The most popular are: ion-exchange chromatography (IEC), reversed phase (RP HPLC), and ion-pairing (IPC). Nucleosides are relatively weak bases and acids (102, 189). These compounds are positively charged below their pK_b and thus they can be separated by cation exchange. Between the pK_b and pK_a, nucleosides are neutral, so they can be analyzed by the reversed phase, and above their pK_a, they can be separated by the anion exchange (because the nucleosides are negatively charged).

In cation exchange the stationary phase consists of fixed anionic sites. These interact electrostatically with cationic solutes, which are thus retained. In the anionic exchange mode, the situation is opposite (102, 189). Ribo- and deoxyribonucleosides have been separated on the cation exchange column (192); however, mixtures of bases and ribonucleosides have been resolved by anion exchange (193). Characteristic for ion-exchange techniques is good separation of nucleosides, but they lack the sensitivity necessary for analysis at low levels and often require long analysis times (102, 105, 188).

In the reversed phase HPLC the extent of the retention is determined by the hydrophobic interactions; therefore, the more polar and ionic compounds elute earlier than nonpolar substances. RP HPLC is presently the most widespread technique used in modified nucleoside analysis because of many possible factors that can be changed to yield good separation and selectivity. Hartwick and coworkers studied UV-absorption of nucleosides in serum and were the first to optimize conditions for their analysis using RP HPLC (188, 191). They also examined the influence for ionic strength of the mobile phase buffer on a nucleoside separation (188).

Gehrke and coworkers appointed HPLC UV spectra for almost 67 modified nucleosides (106). They likewise established the effect of flowrate, sample volume injected, methanol concentration, type of buffer salts, and temperature on the elution of the nucleosides, as well as the effect of pH of the mobile phase (106, 183). The last (pH) effect offers interesting possibilities in modified nucleoside retention control, through the changes in the given compound structure with the pH of mobile phase

TABLE 4
Results of the nucleoside mixture separation

SG-C18				SG-AP				SG-CHOL			
Compound	k	f_{AS}	α	Compound	k	f_{AS}	α	Compound	k	f_{AS}	α
Pseudouridine	0.24	1.03		7-Methylguanosine	0.25	1.21		Pseudouridine	0.48	1.00	
7-Methylguanosine	2.83	1.23	1.35	1-Methyladenosine	2.21	—	1.19	1-Methyladenosine	2.19	1.17	1.32
1-Methyladenosine	3.81	1.20		1-Methylinosine	2.62	—		7-Methylguanosine	2.90	1.19	
1-Methylinosine	5.34	1.15	1.21	Pseudouridine	3.92	—	1.40	1-Methylinosine	6.22	1.18	1.63
1-Methylguanosine	6.46	1.25		1-Methylguanosine	5.43	1.52		1-Methylguanosine	10.42	1.27	
Adenosine	9.25	1.35		Adenosine	7.93	1.54		Adenosine	12.77	1.31	
8-Bromoguanosine	19.41	1.53		8-Bromoguanosine	18.08	1.59		8-Bromoguanosine	33.71	1.26	

Retention k, selectivity α, and asymmetry f_{AS} values, appointed in 10% of peak height in isocratic elution (composition of the mobile phase: 96% v/v 10 mM $NH_4H_2PO_4$, 4% v/v MeOH) with the pH buffer equal 7.5 for the octadecyl (SG-C18), alkylamide (SG-AP), cholesterolic (SG-CHOL) stationary phases.

do change. An increase in pH reduces the positive charge on the basic nucleosides and increases the negative charge on the acidic nucleosides. The basic nucleosides become less ionic; therefore, the retention time is increased. Acidic nucleosides become more ionic, thus the retention is decreased. Neutral nucleosides will eluate in the same retention time (106). All of these effects were studied on the same, most popular, in the nucleoside analysis, stationary phase type – octadecyl. Recently another type of stationary phase was used in the separation of nucleosides: containing cholesterol molecules bonded to the silica surface, alkylamide, and amine ligands (194). Results obtained indicated that the stationary phase surface properties influence also the nucleoside separation (Table 4, Figure 9).

Liebich and coworkers realized the quantitation of urinary nucleosides by HPLC (112). This method using immobilized enzyme reactors was introduced by Kito and coworkers in modified nucleoside analysis (195). Some of them have attempted to develop an immunoassay for the case of quantification (196).

Among IEC and RP HPLC the ion-pairing mode of liquid chromatography in nucleoside analysis is used. In this technique compounds, that contain both liphophilic and ionic moieties, are added to the mobile phase. The ionic moiety can pair with ionic analytes such as nucleosides of an opposite charge; therefore, greater retention of charged solutes can be achieved on reversed-phase systems (102, 197). Very good results are obtained with RP HPLC because of limited application of IPC in nucleoside studies. This method could be useful while modified nucleosides are eluted close to the void volume, and thus cannot be resolved in reversed-phase system. Ehrlich and coworkers had added to the mobile phase heptanesulfonate in case of

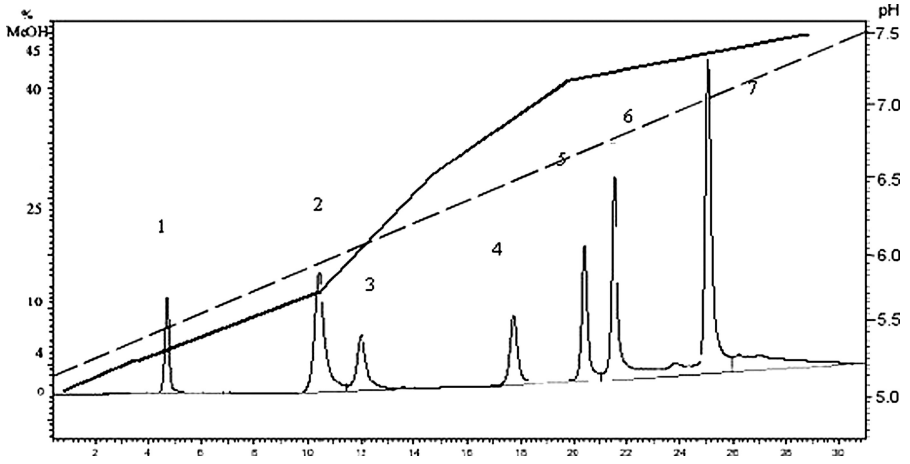

FIG. 9. Separation of the nucleosides mixture for the cholesterolic stationary phase in gradient elution (gradient program 10 mM $NH_4H_2PO_4$pH = 7.5 and increasing part of methanol: 3 minutes—4% MeOH, 10 minutes—10% MeOH, 15 minutes—25% MeOH, 20 minutes—40% MeOH, 30 minutes—45% MeOH). Notation: 1—pseudouridine, 2—1-methyladenosine, 3—7-methylguanosine, 4—1-methylinosine, 5—1-methylguanosine, 6—adenosine, 7—8-bromoguanosine.

5-methylcytosine analysis, so substances were retained in octadecyl column and separated from the rest of the compounds in the mixture (198). Uesugi and coworkers have used triethylamine as a counterion in the separation of adenosine nucleoside and nucleotides (199).

Capillary electrochromatography (CEC) gives interesting possibilities in nucleoside separation, combining HPLC and CE by applying to a packed column typical for HPLC high-voltage. CEC offers a larger number of parameters to adjust to obtain an optimal separation. In nucleoside analysis it allows are to avoid the use of gradient elution typical in HPLC and ion-pairing agents. Helobe and coworkers have used capillary electrochromatography in nucleoside separation (200). Results indicated that conventional HPLC methods require about twice the time to analyze nucleosides in comparison to CEC, whereas capillary electrochromatography shows good retention time reproducibility.

Peptides and Proteins

Peptides are amino acids polymers. Amide linkages between amino acids are known as peptide bonds; the product of peptide bond formation between two amino acids is called a dipeptide. Polypeptides contain many amino acid units. Proteins are natural polypeptides containing more than 50 amino acid units. Most proteins are polymers of 100–300 amino acids (201). The role of proteins in living system is very important: enzymatic catalysis, transportation and storage, basic component of muscle, neurotransmissions, immunological protection, and growth control (202). Peptide chromatographic behavior is determined by the character of the side chains and substituent groups—the strength of the basic and acidic groups and also hydrophobicity or hydrophilicity.

Size-exclusion, ion-exchange, and reverse-phase chromatography are widely used tools for the analysis and purification of biomolecules such as peptides and proteins. Liquid chromatography is able to separate polypeptides of nearly identical sequences (203, 204), those obtained from trypsin digestion and for much larger molecules (205–212). Preparative liquid chromatography is used to purifiy natural and synthetic peptides, in microquantities for sequencing (213, 214). Liquid chromatography and related techniques are applied in the analysis of protein therapeutic products, products of enzymatic digestion, and in monitoring of genetic changes (215–217).

Liquid chromatography use is not possible without a stationary phase. It makes separation and good resolution possible, differentiating types of liquid chromatography that are used for peptides and protein purification and analysis. Understanding the retention mechanism for peptides is important in predicting their separation and selection of proper columns for analysis. Peptides as large molecules are adsorbed on the hydrophobic surface and only the hydrophobic part of peptide is able to interact. More hydrophobic peptides are retained longer than hydrophilic. Differences in peptide hydrophobicity are consequences of amino acid sequences and conformation.

The length of the hydrocarbon chain grafted into the stationary phase surface determines its hydrophobicity and selection of column. C_{18} stationary phases are preferred for peptides and small proteins less than 5,000 Da; proteins larger than 5,000 Da and small polypeptides are usually analyzed on C_4 column (203). C_8 packing is also used despite its similarity to C_{18}, but selectivity sometimes is much better than for octadecyl columns. Columns vary in properties because of the chemically bonded ligand types but also in the way this moiety is grafted into the support surface. Monomer stationary phases are prepared by bonding the hydrocarbon chlorosilane with one reactive chlorine to the silica gel. Chlorosilanes with multiple reactive chlorines (two or three) create polymer stationary phases (54). Silica support is better screened by silicon polymer and hydrogen interactions analyte $\Leftrightarrow$ residual silanols on the surface are not as strong as for monomer stationary phases. This effect as also support for surface coverage density and end-capping of silanols can influence selectivity and peak shape (Figure 10). Mass transfer for high coverage density stationary is worse, so peak asymmetry is a consequence of this phenomenon. Low coverage surface density permits better penetration of molecules, good selectivity, and better peak shape.

Silica-based stationary phases are porous materials, and the interactive surface for analytes is inside the pores. For small molecules, particles with pores of 100 Å diameter are useful. Because of sterical reasons large pore diameters (300 Å) are required for satisfactory separation of big peptides (>2000 MW).

Chip technology is a powerful tool for on-line peptide digestion and separation (218). Slentz and coworkers described fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) trypsin digestion, affinity selection, and separation on a column of collocated monolithic support structures (COMOSS) in an integrated, electro-osmotically driven, microfabricated system. Microfabricated frits were used to retain immobilized trypsin and a Cu(II) loaded IMAC sorbent (for affinity selection). This allowed protein digestion and the capture of histidine-containing peptides on the same chip (Figure 11).

Peptide retention depends very much on the eluent pH because of protonation/deprotonation of molecules. Synthetic polymer-based reversed phase materials that are stable in wild range of pH are alternative for traditional silica based columns are sensitive to mobile phase pH (63, 203).

Peptides as compounds able for ionization could be purified and analyzed by ion-exchange chromatography however separation efficiency is not so good as for reverse-phase chromatography, and large peptides may be strongly sorbed. This technique is based on electrostatic interactions of the ionic compound with the charged functional groups of the stationary phase. Ion-exchange chromatography separates proteins by charges under near-physiological and nondenaturing conditions (63, 203).

Anion-exchange chromatography retains peptides by the interaction of amine groups on the ion-exchange stationary phase with aspartic or glutamic acid side chains, which have pKs $\sim$ 4.4. So, in the mobile phase buffered at pH > 4.4, retention decreases

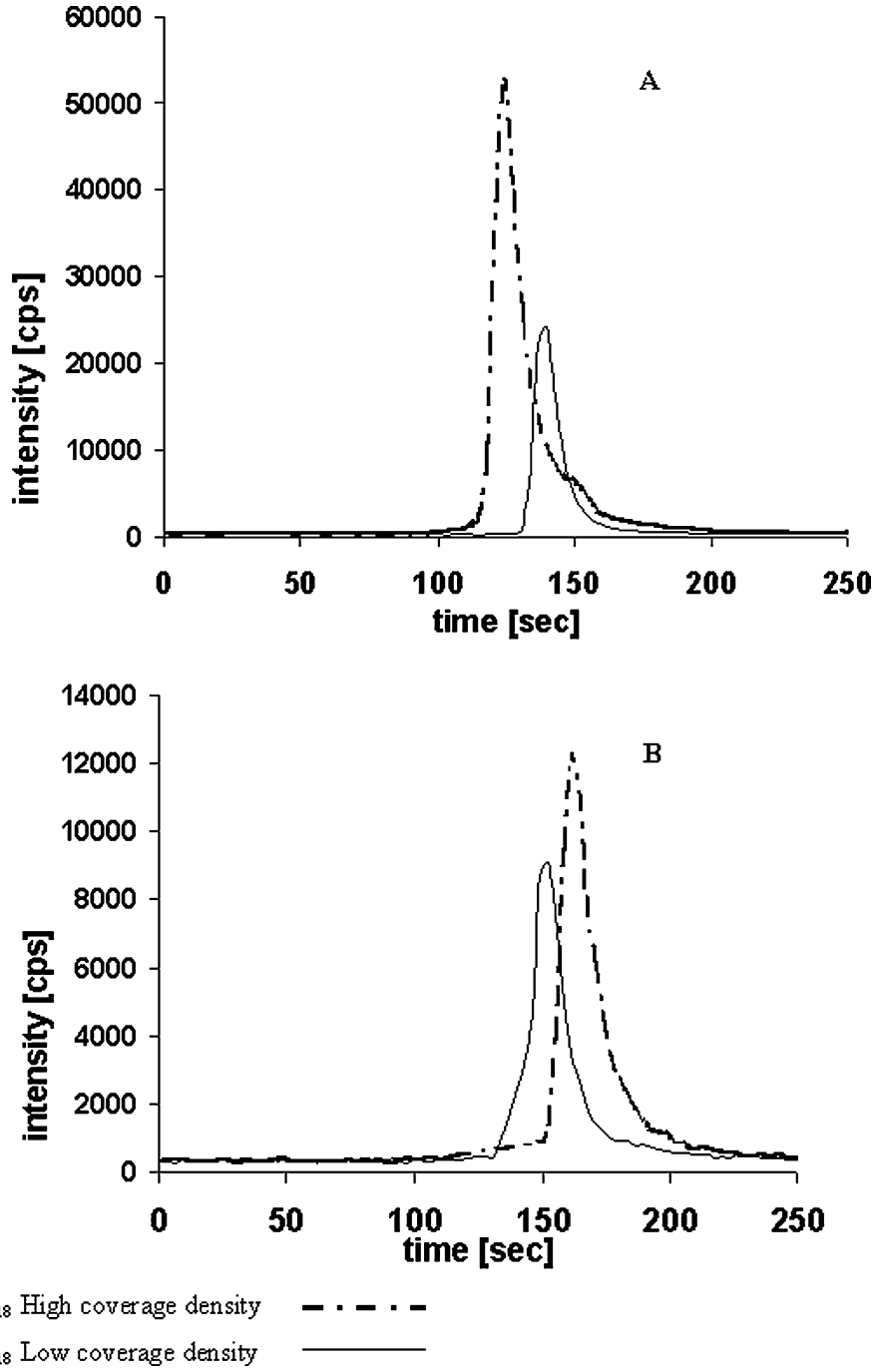

FIG. 10. Differences in retention and peak asymmetry for selenopeptides (A) XGHDQSGTK; (B) TYENXKK; mobile phase methanol/water 40/60 % v/v, flow rate 0.6 ml/min, detection: ICP/MS ^{82}Se; C_{18} high coverage density stationary phase (–·–), C_{18} low coverage density stationary phase (—).

because of protonation of carboxyl groups. Proteins containing the same number of anionic side chains can often be separated at pHs between 7 and 10, where histidine is not protonated and lysine begins to deprotonate (219).

Cation-exchange chromatography is based on the interactions of sulfonic acid groups on the stationary phase surface with histidine (pK∼6.5), lysine (pK∼10), and arginine (pK∼12), the mobile phase pH is adjusted to 6 or 7 to keep the basic side chains protonated. Compounds with the same number of basic moieties could be separated at mobile phase pHs between 3 and 5, where aspartic acid and glutamic acid are partially protonated (219, 220). Silica-based stationary phases are not recommended for

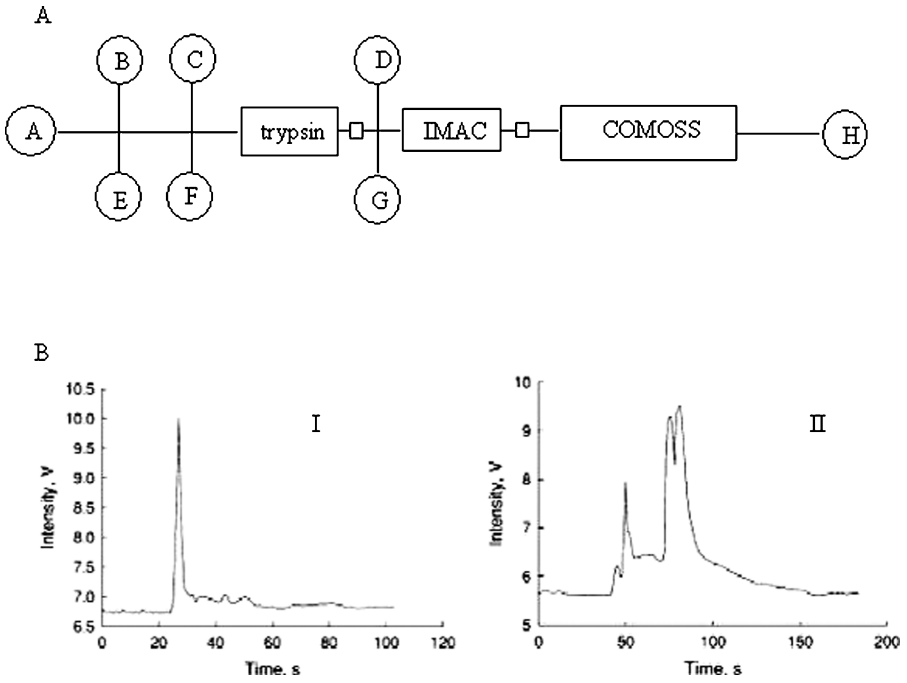

FIG. 11. (A) Scheme of microfabricated system: IMAC—immobilized metal affinity chromatography, COMOSS—column of collocated monolithic support structures; (B) Chromatogram of FITC-BSA without (I) and after (II) on chip digestion (1 mM phosphate buffer pH 7.0, 500 V/cm) (From (218) With permission).

ion chromatography because drastic pH changes (particularly pH $\sim$ 9) could lead to degradation of silica support (221).

Size-exclusion chromatography (SEC), also called gel filtration, is useful for purification and preseparation of peptide mixtures. Gel filtration separates molecules according to differences in size and is the simplest and mildest of the chromatographic techniques (217). SEC is proper for molecules that are sensitive to changes in pH, concentration of metal ions, and environmental conditions. Stationary phases for SEC minimized nonspecific interactions; however, there are still weakly anionic and hydrophobic columns. A low pH is recommended for peptide separation with gel filtration, except strong acidic peptides, which are insoluble at low pH values. The addition of an organic modifier decreases nonspecific hydrophobic interactions between peptide $\Leftrightarrow$ SEC stationary phases (63). Various packings e.g., polyvinyl pyrrolidone-coated silica, polyhydroxyethyl aspartamide, spherical silica particles of various pore sizes coated with hydrophilic film and hydroxypropylated dextran beads are used in SEC of proteins and peptides (63).

Sometimes chiral separation is necessary for full purification of peptide mixtures. This task could be performed in three modes: (1) separation on achiral column with chiral agents in mobile phase (223), (2) separation on chiral columns, and (3) precolumn derivatization with chiral reagents (224). Cyclodextrins are the most popular for the synthesis of chiral stationary phase and as additives to mobile phases (Figure 6). Also, macrocyclic antibiotics such as vancomycin, ristocetin, and teicoplanin are used as stationary phases for peptides enantioseparations (225, 226). Antibiotics are utilized as chiral additives to mobile phase (227).

Hydrophobic chromatography has been also used for peptide isolation and separation. Contrary to reverse-phase chromatography, mild hydrophobicity of the stationary phase for hydrophobic chromatography and high ionic strength of the mobile phase maintains peptides in their native states (228, 229).

High performance affinity chromatography (HPAC) is a very selective method for peptide analysis, and experimental conditions are mild for the solute. Retention is based on the specific biological interactions. HPAC could be applied only for peptides or proteins that are capable of specific bonding to the immobilized affinity counterpart (63, 230, 231).

While high-performance liquid chromatography is a well-established method in peptide and protein research, the relatively new technique, capillary electrochromatography (CEC), is expected to have great potential in the separation of peptides and proteins as well (232–235).

New generation of monolithic columns for CEC is applied in peptide and protein separations and for peptides mapping (234–238). Surface-alkylated polystyrene monolithic columns are proper for peptides analysis by hyphenated techniques such as LC-ESI-MS (237). Composition of the mobile phase depends on the peptide and the stationary phase type. Peptide elution from chromatograph is caused by aqueous solvent containing organic modifier and buffer or an ion-pairing reagent. The ion-pairing reagent or buffer maintains the mobile phase pH and interacts with peptide to make retention possible. Trifluoroacetic

acid is the most popular ion-pairing reagent but also hydrochloric acid, heptafluorobutyric acid (239), triehylamine phosphate (240), and formic acid are useful in LC/MS separation of hydrophobic peptides. Anionic, rather than cationic, ion-pairing reagents are preferred for peptide analysis due to better selectivity obtained (241).

Because the peptide retention mechanism is based on adsorption/desorption phenomenon, mobile phase composition is very important in desorption moments. Sometimes 1% of organic modifier in the mobile phase can change retention clearly. This is why gradient elution is usually recommended for peptides separation.

Technical development permits the application of very sensitive and selective detection methods including low-wavelength UV (242), evaporative light scattering detection (ELSD), (243, 244), and nuclear magnetic resonance (245, 246). Analytical approaches based on the use of mass spectrometry (MS) are also widely used in peptide and protein analysis. In particular, matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) with time of flight (TOF) analyzers and electrospray-mass spectrometry (ESI-MS) with quadrupole mass spectrometry became major tools in measuring molecular masses of proteins. ESI-MS provides valuable information about compound structure. It is not possible to cite all publications concerning application MALDI-MS and ESI-MS in peptide and protein investigations. We would like to suggest just a few (247–250). The combination of mass spectrometry (MS and MS–MS) with HPLC reduces the need for chromatographic resolution because of the resolution capacity of the mass spectrometer (251–254). Detection sensitivity is better than with UV detection.

FUTURE RESEARCH

The authors realize the extent of the analytical field for naturally active compounds. This paper is not about stationary phases and recommendation of techniques. We would like to suggest that the octadecyl stationary phase is the most popular, but because of analyte properties it is not a universal material. New packing technology, miniaturization, especially chip technology, separation system unification, and new, selective detection systems are challenges for analysts.

At the beginning of the 21st century we have to respond to the challenges concerning:

- technology of packing, columns, and other separation systems, such as: chips, multiplates, membranes and the problem of selectivity;
- searches for new types of specific detectors and identification systems,
- miniaturization of separation and detection systems;
- unification of separation systems (the same column is recommended for several different techniques);
- new methodologies of sample preparations and combining them into on-line systems;
- modern and simple analytical methods and procedures;
- standard samples and reference materials, spectrum and retention data libraries, etc.; and
- automation and robotics of the separation process by the application of multi-dimensional coupled systems or building of hybrid units.

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