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Investigations on the chromatographic behavior of hybrid reversed-phase materials containing electron donor–acceptor systems I. Contribution of sulfur–aromatic interactions

Jeannie Horak, Wolfgang Lindner*

Institute of Analytical Chemistry, University of Vienna, Währingerstrasse 38, 1090 Vienna, Austria

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Abstract

The influence of sulfur–aromatic interactions on the chromatographic separation behavior of hybrid RP phases, containing thiol-groups and/or embedded sulfide-groups (S-RP) has been investigated. To allow a precise outline of this new interaction mode, a wide variety of S-RP phases with different alkyl chain length, with and without residual thiol-groups and silanol-endcapping were prepared and tested in comparison to some conventional monomerical as well as polymerical *n*-alkyl type RP phases. The solute test sets employed in this study comprised the classical chromatographic column tests from Engelhardt and Tanaka as well as test assemblies containing polycyclic aromatic hydrocarbons, stilbene-based *cis*/*trans* isomers and functional isomers of benzene. In general, a pronounced strong planar recognition ability as well as a strong increase in the retention for aromatic compounds have been noticed. It was furthermore found that not only the sulfur atom incorporated into the alkyl chain, but also the residual thiol-groups of the 3-propylthiol silica backbone contribute to the overall retention behavior of these novel S-RP type phases.

Keywords: Stationary phases, LC; Sulfur–aromatic interactions; Polar embedded phases; Shape selectivity; Propylthiol silica gel; Polynuclear aromatic hydrocarbons

1. Introduction

Although the number of new types of stationary phases increases annually, the majority of bonded phases employed in reversed-phase high-performance liquid chromatography (RP-HPLC) are still of the reversed-phase *n*-alkyl type, mostly C_{18} and C_8. The choice of employing C_{18} columns for analysis is mostly based on their well-documented and wide-ranging applicability as well as their reliability concerning high selectivity, reproducibility, and stability (ruggedness). Considering that minor changes in preparation conditions of the crude silica or the bonding reaction can already provide major variations in selectivity, the impact of another reagent type or reaction technique is vast. Besides the actual outcome of the immobilization reaction, also the alkyl chain length and the bonding density of the ligands, the surface concentration of residual surface silanols have a great impact on the retention behavior of a column. It is common knowledge that due to the ion-exchange character of surface silanol groups especially basic solutes show a strong peak-broadening, peak-tailing, and some retention abnormalities on old or non-silanol-endcapped RP-columns. As can be seen, there are diverse dependent variables in RP-type stationary phase synthesis and many more that are not yet described or classified as such. Obviously, the aim of manufacturers must be to optimize and control the process of preparation in order to provide their customers with columns of enhanced reproducibility, consistent selectivity, and improved stability.

For this purpose, numerous chromatographic column tests were designed for both manufacturers and customers to trace the amount of variation between different *n*-alkyl based columns [1]. These column tests mostly contain a set of different substances, which are commonly available in every standard laboratory. The best known and widely used column tests are those from Engelhardt and coworkers [2–4], Tanaka

* Corresponding author. Tel.: +43-1-4277-52300; fax: +43-1-315-1826.
E-mail address: wolfgang.lindner@univie.ac.at (W. Lindner).

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and coworkers [5,6], Neue et al. [7,8], Walters [9], and Galushko [10]. All mentioned chromatographic test methods have in common, that they provide information about the rest silanol activity as well as the hydrophobicity of the tested RP-materials. Additional information about the so-called "shape selective" properties as well as information about a possible acidic pretreatment of the basic silica can be obtained by the Tanaka test [1,5].

For conventional brush type *n*-alkyl based stationary phases the term shape selectivity is mainly used to describe enhanced separation selectivity of polyaromatic hydrocarbons (PAHs) based on differences in their molecular shape [11–13], rather than differences in their physical or chemical properties. Nevertheless, parameters that influence shape selective separation of polyaromatic hydrocarbons include the effective contact area, the van der Waals volume and the minimum cross-sectional area of the solute molecule [14].

Mostly, non-functionalized PAHs are employed to describe shape selectivity, because as a consequence of the global structure of the stationary phase only their difference in shape contributes to retention. In actual fact also residual silanol groups [15] as well as the carbonyl groups [16,17] of polar embedded phases were reported to enhance selectivity via OH–π interaction and carbonyl–π interaction with aromatic solutes.

Kirkland and Henderson [18] introduced an alternative approach to reduce the silanol activity of a stationary phase by employing monofunctional alkyl silanes with bulky side chains, such as dimethyl, diisobutyl or diisopropyl groups. Such "sterically protecting" groups provide a kind of sterical as well as hydrophobic shielding of the surface silanol groups, making them less accessible to polar solutes and water molecules. This results in an increased hydrolysis stability as well as increased hydrophobicity of the bonded RP phases.

Besides that, there is also the possibility of introducing polar groups into the hydrophobic lattice of a stationary phase. In recent years polar endcapped [19,20] as well as a large variety of novel polar embedded RP phases were created and discussed in the literature, such as ether groups [21], acrylate [16,22], amide [23–26], carbamate [23,24,27], and urea [28]. Similar to the sterically protecting groups, polar embedded groups incorporated near the silica surface also provide a kind of sterical shielding of the surface silanol groups, making them less accessible to interaction reactions. The major advantage of these polar groups is their ability to interact with water molecules via hydrogen bonding. This increases the polarity near the silica surface and ensures a high solvatability of the entire hydrophobic phase even at low percentages of organic modifier [21]. The hydrophobic ligand lattice is more stable in terms of phase collapse [29] at highly aqueous mobile phase conditions and provides improved chromatographic performance for basic solutes.

Principally, there are two ways to prepare a polar embedded silica phase. The original method is a two-step surface modification reaction [25,26]. In this context, in the first stage an aminopropyl silica support was prepared followed by the transformation of the amino group into an amide or urea group by a second reaction with acid chloride or isocyanate. The major disadvantage of such a two-step approach is the remaining unreacted amino groups, which add some unwanted anion exchange characteristics to the reversed-phase retention behavior of such polar embedded phases. Note that in a two step-approach the derivatisation reaction of the silica bound functional groups is hardly ever 100%, thus the resultant modified material will always bear a certain amount of the original functionality, may it be amino groups in case of 3-aminopropyl silica gel or thiol groups when choosing a 3-propylthiol silica backbone instead (see later). This problem can be overcome by employing a one-step approach. Thereby a pre-prepared polar embedded alkyl silane is bound to the silica surface, providing a modified polar embedded phase free from residual amino-propyl groups [27]. Obviously, the more elaborate one-step approach is more reasonable for any investigation concerning the influence of polar groups incorporated into a hydrophobic lattice, but requires more efforts regarding the synthesis of the desired silane.

Generally, it can be noted that every change in the molecular structure of a stationary phase either by incorporation of hetero-atoms or through introduction of functional groups goes hand in hand with additional and often very diversified interaction modes with different solute types. One aim in RP-type stationary phase design is often simply to create novel hybrid RP phases to solve specific separation problems for target analytes that cannot be resolved by the common *n*-alkyl type RP phases. In many cases a possible contribution of small variations in stationary phase design are simply left aside or ignored, assuming that they may only have a minor influence on the retention behavior of the novel phase. But the truth is that every change in the construction of the stationary phase, may it even be the absence of a single methylene group or its replacement by a hetero-atom, changes the physical and chemical properties of the entire stationary phase and thus of its overall RP type selectivity.

Note that *n*-alkyl chains with even numbers of carbon atoms posses slightly higher capacity factors than such with odd numbers. This strange appearing affect may have its origin in the different crystalline states of odd *n*-alkanes, which crystallize in orthorhombic symmetry and even alkanes, which crystallize in triclinic symmetry [30].

Presently, short thiol-bearing ligands silanized onto silica gel are a common support for further modification reactions of allyl-containing selector molecules via free radical addition employing a radical initiator such as azo-isobutyro nitrile (AIBN) [16,31,32]. A possible contribution of the incorporated sulfur atom, concerning an active interaction in separation was so far never discussed in this context, which is rather surprising, considering that the outcome of such a modification protocol is a polar sulfur-embedded and polar endcapped stationary phase, having a large number of silanol groups replaced by 3-propylthiol groups.

However, in proteomics, stacking interactions between sulfur bearing and aromat-ring bearing amino acids were observed from the crystallographic data from protein structure investigations [33,34]. Deduced from these observations it was assumed that sulfur–aromatic interactions are of pronounced importance for a directed folding of polypeptide chains as well as for the maintenance of protein stability [35]. NMR- and CD-studies showed side-chain–side-chain interactions between the sulfur-containing amino acids cystein and methionine and the aromatic moiety of phenylalanine within an α-helical conformation [36]. Since there is enough evidence for the presence and the significance of sulfur–aromatic interactions in biological systems, they may also have some influence on the chromatographic selectivity of certain analytes, particularly those possessing aromatic moieties, chromatographed on sulfur containing RP-type stationary phases.

However, and in another context, Porath et al. [37] introduced already in 1985 the importance of the so-called thiophilic adsorption chromatography [38–40] for the selective binding of immunoglobulins from human serum. Although these so-called T-gels are already in use since two decades, the mode of interaction between such sulfur-bearing adsorbents and proteins was so far not discussed and may be subject of profound interest for future studies.

The aim of the present work was to investigate sulfur–aromatic interactions from a chromatographic point of view and to determine their contribution to the overall RP type selectivity. Hence, we denominate these new hybrid materials as S-RP phases. In order to determine the influence of this polar sulfide linkage, which is positioned rather close to the silica surface, we have prepared and tested a variety of *n*-alkyl based S-RP phases with sulfur bound *n*-alkyl chains of different number of C atoms ($n = 0, 8, 14, 18$) (Fig. 1). These S-RP columns were also compared with monomerical C_8/C_{18} RP phases as well as with a polymerical C_{18} PAH-phase of commercial origin.

To elucidate the difference between residual silanol groups, residual thiol groups, and the actual incorporated sulfide sulfur in regards to their contribution to retention and selectivity, we compare a silanol-endcapped S-RP phase from a one-step approach to a corresponding phase prepared by a two-step modification reaction. For a deeper understanding of the retention mechanism of S-RP phases we also investigated a non-modified, non-endcapped 3-propylthiol phase, quasi the backbone of the *n*-alkyl derivatized S-RP phases as a control experiment.

The column tests from Engelhardt and Tanaka were employed for a first tentative classification of the interaction properties exhibited by these novel S-RP phases, followed by more specific investigations concerning polarity, shape selectivity, and separation efficiency. For this purpose, we assembled solute test mixtures containing different functional isomers and geometrical isomers as well as a collection of polyaromatic hydrocarbons, which differ in size and shape.

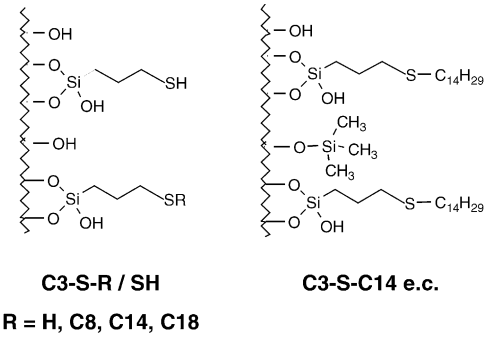

Fig. 1. Chemical structures of investigated silica based polar embedded S-RP type stationary phases of differing *n*-alkyl chain length, with and without silanol-endcapping (ec).

2. Experimental

2.1. Chemicals

Toluene was purchased from ACROS ORGANICS (Merck, Vienna, Austria). For synthesis freshly distilled technical chloroform and technical toluene were used.

Anthracene, benzene, benz[a]anthracene, biphenyl, butylbenzene, caffeine, chrysene, 4-dimethylaminopyridine, *N*,*N*-dimethylaniline, ethylbenzene, hydrochinone, naphthacene, naphthalene, *o*-nitrophenol, *p*-nitrophenol, 1-octene, 1-octadecene, pentylbenzene, phenanthrene, phloroglucin dihydrate, resveratrol, *cis*-stilbene, *trans*-stilbene, *cis*-stilbeneoxide, *trans*-stilbeneoxide, *o*-terphenyl, *m*-terphenyl, *p*-terphenyl, 1-tetradecene, thiourea, *o*-toluidine, *m*-toluidine, triphenylene, and uracil were purchased from Sigma-Aldrich (Vienna, Austria).

Benzo[c]phenanthrene was from Dr. Ehrendorfer GmbH (Augsburg, Germany).

3-Mercaptopropyltrimethoxysilane and hexamethyldisilazane were from ABCR, Karlsruhe, Germany.

m-Nitrophenol was from Hoechst and aniline, brenzcatechine, dichloromethane, and *p*-toluidine were from LOBA Feinchemie (Fischamend, Austria).

α,α-Azoisobutyronitrile, phenol, ethyl benzoate, pyrogallol, and resorcin as well as HPLC-grade methanol, acetonitrile, and chloroform were from Merck.

Silica gels were KROMASIL-100 with a particle size of 5 μm, a pore size of 100 Å and a surface area of 314 m^2/g from AKZO NOBEL, Bohus, Switzerland and Prontosil-120 with a particle size of 5 μm, a pore size of 120 Å, and a surface area of 300 m^2/g from Bischoff, Leonberg, Germany.

With exception of the C_3–SH column all stationary phases were prepared with Prontosil-120.

2.2. Equipment

Throughout all measurements a HPLC-system consisting of a L-6200A Intelligent Pump, a D-6000A Interface and a

L-7200 Autosampler from Merck-Hitachi, Darmstadt, Germany was employed. The detection device was an UV-975 Intelligent UV–vis single wavelength detector from Jasco, Biolab, Vienna, Austria.

If not otherwise stated, the following conditions were maintained for all measurements: sample aliquots of 10 μL were injected at a flow-rate of 1 mL/min. If not otherwise stated, the columns were thermostated at 30 °C. The void volumes of the tested columns were determined with void volume markers, using uracil or thiourea. The composition of the individual test mixtures are described in the corresponding chapters.

2.3. Preparation of S-RP-type stationary phases

2.3.1. C_3–S–C_8/SH and C_3–S–C_{14}/SH

1-Tetradecene (13 mmol) and 1-octene (13 mmol) were each immobilized onto 3 g 3-propylthiol silica gel [41] (silane-loading: 2.6 μmol/m^2) through a radical induced addition in 50 mL freshly distilled chloroform under nitrogen atmosphere, employing 50 mg α,α-azo isobutyro nitrile (AIBN) as the radical initiator. The reaction vessel was refluxed under stirring over night.

2.3.2. C_3–S–C_{18}/SH

Five grams of 3-propylthiol silica gel, 1-octadecene (21.8 mmol), 50 mg AIBN, and 50 mL *n*-heptane were evaporated on a rotary-evaporator at 40 °C to complete dryness. The octadecene and AIBN coated 3-propylthiol silica gel was refluxed in 50 mL dry methanol under nitrogen atmosphere for 6 h.

2.3.3. C_3–S–C_{14} ec

1-Tetradecene (52.2 mmol) was coupled onto 3-propylthioltrimethoxysilane (26.4 mmol) through a radical induced addition in distilled toluene (10 mL) under nitrogen using AIBN (5 mol%) as a radical starter. Although the completeness of the reaction was determined after 6 h refluxing through TLC (diethylether:ethylacetate 9:1), the reaction time was prolonged by 2 h to ensure a complete reaction to 1-tetradecylsulfanylpropyl-trimethoxysilane.

The 1-tetradecylsulfanylpropyl-trimethoxysilane solution and 4-dimethylaminopyridine as a catalyst were added to a slurry of 5.5 g Prontosil silica gel in 20 mL dry toluene and stirred for 15 h under reflux under nitrogen atmosphere. The so-obtained 1-tetradecylsulfanylpropyl-silica gel was end-capped with hexamethyldisilazan (8.4 mmol) in distilled toluene by refluxing the slurry over night under nitrogen flow.

All modified silica gels were filtered under vacuum and washed successively with 4 × 75 mL of reaction solvent, with 4 × 75 mL of hot methanol (techn. grade), with 2 × 25 mL of hot methanol (HPLC-grade) and with 50 mL petrolether. The silica gels for column-packing were then dried at 60 °C over night, whereas the samples for elemental analysis were dried at 60 °C under vacuum for at least 12 h.

2.3.4. HPLC-columns

The elemental analysis of the modified S-RP type silica materials showed the following results: C_3–SH silica gel (C: 3.96; H: 0.75; S: 2.38%; C_3–SH coverage: 2.36 μmol/m^2), C_3–S–C_8/SH silica gel (C: 11.30; H: 2.22; S: 2.57%; C_3–S–C_8 coverage: 2.06 μmol/m^2), C_3–S–C_{14}/SH silica gel (C: 14.68; H: 2.84; S: 2.46%; C_3–S–C_{14} coverage: 1.82 μmol/m^2), C_3–S–C_{14} ec silica gel (C: 13.55; H: 2.61; S: 2.17%; C_3–S–C_{14} coverage: 2.16 μmol/m^2; C: 14.11; H: 2.74; S: 2.12%; trimethylsilyl-coverage: 0.50 μmol/m^2), C_3–S–C_{18}/SH silica gel (C: 15.70; H: 2.85; S: 2.42%; C_3–S–C_{18} coverage: 1.56 μmol/m^2). The abbreviation SH stands for free non-end capped residual thiol groups and ec stands for silanol end-capping.

All S-RP type stationary phases were packed in stainless steal columns of the dimension 150 × 4 mm by the Austrian Research Center Seibersdorf. Four commercial fully endcapped RP-columns, an Inertsil ODS-3 and an Inertsil C_8-3 column from GL Sciences Inc., Japan with the column dimension 150 × 3 mm, a Hypersil-BDS-C_{18} from Hewlett Packard with the column dimension 125 × 4 mm and a Hypersil-green-PAH from Thermo Electron Corporation with the column dimension 150 × 4 mm were also tested. Particulars about the RP-packing materials as stated as such by the manufacturer. The Inertsil silica gel has a particle size of 5 μm, a pore size of 100 Å, a pore volume of 1.05 mL/g, and a surface area of 450 m^2/g. The Hypersil BDS silica gel has a particle size of 5 μm, a pore size of 130 Å, and a surface area of 170 m^2/g. The Hypersil-green-PAH silica gel has a particle size of 5 μm, a pore size of 120 Å, and a surface area of 170 m^2/g.

3. Results and discussion

3.1. Characterization of S-RP type HPLC-phases

Table 1 gives an overview of all investigated S-RP type as well as some commercially available *n*-alkyl type C_8 and C_{18} columns. All loading densities were calculated from the data derived from the elemental analysis and the surface area of the crude silica gel. For the commercial phases, the data were provided by the corresponding companies.

As shown in Table 1, the ligand loading of the S-RP phases decreases with increasing ligand length for the sulfur bound *n*-alkyl chains. In the same order, the number of residual thiol-groups increases, whereas the number of free silanol groups remains the same. In contrast the 1-tetradecenylsulfonpropyl silica gel was prepared in a one-step procedure by immobilization of the corresponding pre-modified trimethoxy-silane onto the silica surface. The ligand density gained by this synthesis protocol was even higher than that of the C_3–S–C_8/SH phase, synthesized in a two-step approach. An additional endcapping reaction of this phase leads to a C_3–S–C_{14} phase that possess a reduced number of remaining thiol

Table 1
Summary of investigated *n*-alkyl type RP phases and sulfur incorporated S-RP phases

Stationary phase[a]	Column labelling	*n*-Alkyl chain (μmol/m^2)	Free SH (μmol/m^2)	Free OH[b] (μmol/m^2)
C_{18} (polymeric)	Hypersil green PAH	3.20	–	ec
C_8 (monomeric)	Inertsil C_8-3	2.08[c]	–	ec
C_{18} (monomeric)	Inertsil ODS-3	1.54[c]	–	ec
C_{18} (monomeric)	Hypersil-BDS-C_{18}	2.50	–	ec
C_3–S–C_{14} ec	C_3–S–C_{14} ec	2.16	–	ec
C_3–SH/OH	C_3–SH	2.36	2.36	5.6
C_3–S–C_8/C_3–SH/OH	C_3–S–C_8/SH	2.06	0.49	5.4
C_3–S–C_{14}/C_3–SH/OH	C_3–S–C_{14}/SH	1.82	0.62	5.4
C_3–S–C_{18}/C_3–SH/OH	C_3–S–C_{18}/SH	1.56	0.84	5.4

[a] 3-Propylthiol loading: 2.6 μmol/m^2.
[b] Maximum number of surface silanol groups: 8 μmol/m^2; in case of silanol-endcapping: ec.
[c] Approximate values calculated for 9 and 15% carbon loading for Inertsil-C_{18} and Inertsil-C_8 materials.

groups as well as a reduced number of accessible silanol groups.

3.2. Chromatographic test methods

The chromatographic column tests from Engelhardt and coworkers [3,4] and Tanaka and coworkers [5,6] were employed to evaluate the properties of our hybrid S-RP type columns in comparison to three silanol-endcapped RP-C_8 and RP-C_{18} columns.

Basically, both column tests provide information about the hydrophobic and silanophilic properties of tested reversed-phase columns. The Tanaka test, which actually comprises four tests is also equipped to describe the shape selective as well as the hydrogen-bond activity and ion exchange properties of a stationary phase.

Despite the fact that such chromatographic column tests were originally designed to evaluate *n*-alkyl based RP-HPLC-columns, mainly those of the C_8 and C_{18} type, they may also provide valuable information about hybrid RP-materials carrying additional interaction sites, such as polar embedded groups.

Also note that the determined “silanol activity” ought to comprise not only the effect of the residual silanol groups but possibly also that of the remaining 3-propylthiol groups.

3.2.1. Engelhardt test

The Engelhardt test used in this study comprised a set of 10 solutes with differing structure and chemical properties, employing the standardized mobile phase compositions methanol–water 49:51 (w/w) and methanol–1 mM phosphate buffer (pH 7) 49:51 (w/w).

According to Engelhardt et al., the retention factor α(E/T) of ethylbenzene (E) and toluene (T) describes the hydrophobicity of a tested column, while a comparison of peak symmetry and retention times for the basic solutes in methanol–water and methanol–buffer provides information about its residual-silanol activity. Generally, a separation of *o*-, *m*-, and *p*-toluidine and a change in the elution orders of phenol and aniline as well as *N*,*N*-dimethylaniline, ethylbenzoate, and toluene is directly correlated to an increased silanol activity. Strong peak tailing and increased retention times for basic solutes in methanol–water is suppressed by using 1 mM phosphate buffer instead of pure water. A comparison of the chromatograms for methanol–water and methanol–buffer gives therefore an approximate measure for the intensity of the silanol and/or thiol activity of a stationary phase.

The results of the Engelhardt test for both mobile phase compositions is shown in Fig. 2 for some S-RP phases with residual thiol groups, while Fig. 3 provides the comparative results for commercial C_8 and C_{18} phases plus a silanol-endcapped and “thiol-free” 1-tetradecenylsulfanylpropyl phase. The main differences between the latter and the comparative monomeric C_{18} phases lies in the replacement of one methylene group by a sulfanyl group compared to C_{18} and a slightly higher ligand density for the C_3–S–C_{14} ec phase (Table 1).

Taking the different ligand loading, listed in Table 1 for the S-RP phases in Fig. 2a in account, then it becomes clear that the silanophilicity of the S-RP phases ($n = 0, 8, 14, 18$) declines with increasing ligand density, which is reportedly higher for short ligands.

Naturally, a higher ligand density as well as a higher ligand length reduce both the accessibility of surface silanols and propyl bound thiols for interaction with basic solutes. But the comparison of the chromatograms of the C_8 and the C_{18} S-RP phase in Fig. 2a reveals the strong influence of the close proximity of aligned ligands, which seems to reduce the penetrability of solute molecules into the ligand lattice most effectively. However, the strong peak-tailing of basic test solutes on the polymeric Hypersil-green-PAH phase is rather exceptional, especially since it is claimed to be fully silanol-endcapped and the results were generated on a newly purchased column.

The C_3–S–C_{14} ec provides the same elution order for the Engelhardt test-mix as observed on the Inertsil-C_{18} phase with overall increased retention times for all test solutes. The co-elution of the three toluidine isomers in a sharp symmetrical peak as well the nicely resolved elution of the

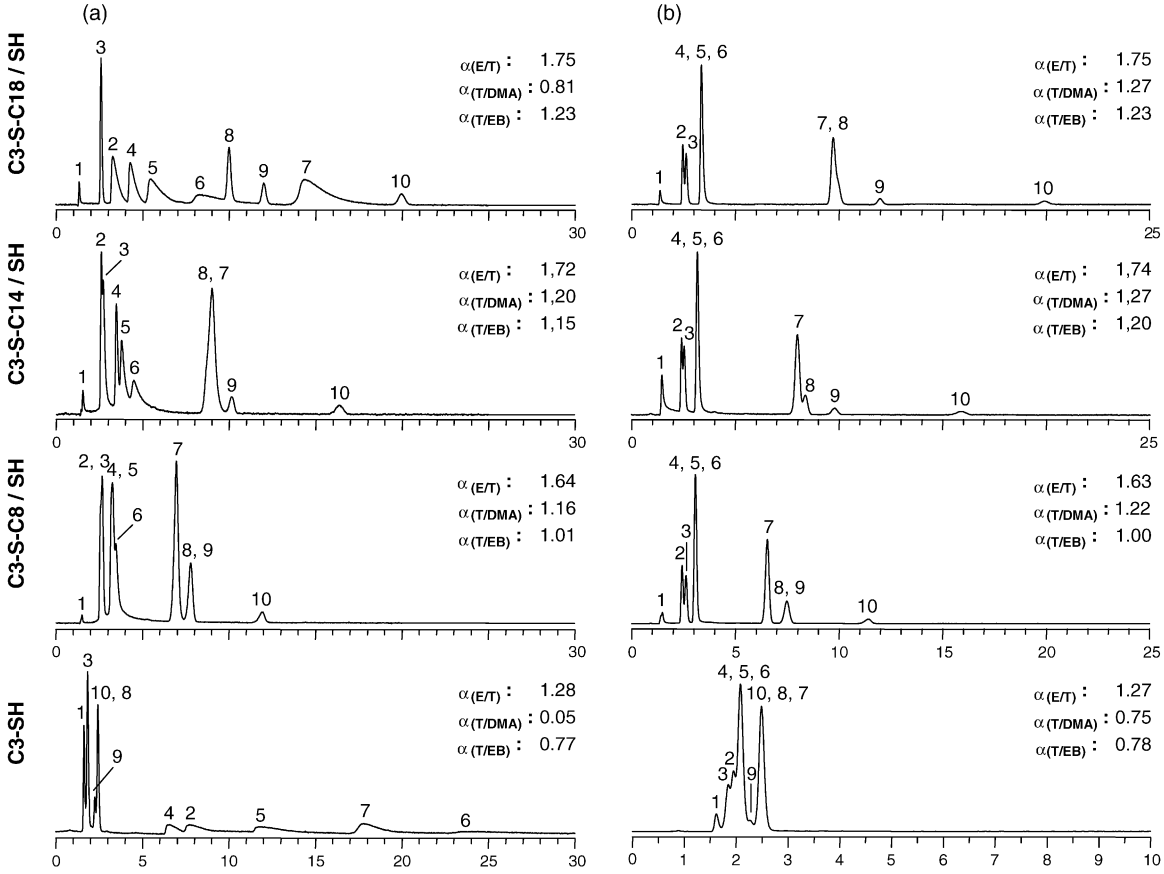

Fig. 2. Separation of the Engelhardt test set containing thiourea (**1**), aniline (**2**), phenol (**3**), *o*-toluidine (**4**), *m*-toluidine (**5**), *p*-toluidine (**6**), *N*,*N*-dimethylaniline (**7**), ethylbenzoate (**8**), toluene (**9**), and ethylbenzene (**10**), including separation factors α(E/T) for toluene (T) and ethylbenzene (E), α(T/DMA) for *N*,*N*-dimethylaniline (DMA) and toluene (T) and α(T/EB) for ethylbenzoate (EB) and toluene (T) in (a) methanol–water 49:51 (w/w) and (b) methanol–1 mM phosphate buffer (pH 7.0) 49:51 (w/w)—1 mL/min, 40 °C, 254 nm on *n*-alkyl-type sulfur incorporated S-RP phases.

aniline peak prior to the phenol peaks are clear indicators for a successful silanol-endcapping of the C_3–S–C_{14} ec phase.

The hydrophobicity term α(E/T) reflects the hydrophobicity increase with increasing ligand length without an obvious effect of any variation in the ligand density. The C_3–S–C_{14}/SH phase and the corresponding endcapped C_3–S–C_{14} ec phase provide thereby approximately the same hydrophobicity values, which are within the lower hydrophobicity range observed for commercial C_{18}-type RP phases.

The comparison of the separation factors α(T/EB) for ethylbenzoate (EB) and toluene (T) and α(T/DMA) for *N*,*N*-dimethylaniline (DMA) and toluene between different phases (Figs. 2 and 3) and for both mobile phase compositions ((a) and (b)) enables an estimation of the silanol activity as well as the thiol activity of these novel stationary phases. Stationary phases that provide approximately the same α(T/EB) and α(T/DMA) values for mobile phase (a) and (b) are mostly silanol endcapped. A decline of α(T/EB) and α(T/DMA) for methanol–water in comparison to a high value for methanol–buffer is an indicator for a silanophilic and thiophilic activity of a stationary phase. The general decline of the stated separation factors, especially that of α(T/DMA) for the buffered mobile phase with decreasing ligand length, in particular that of the sulfur-bonded alkyl chains may be an indicator for electron–donor/acceptor interactions between the extended electron cloud of the sulfur and the aromatic moiety of the test solutes [42]. The lowest values were observed for the non-endcapped 3-propylthiol silica phase having the highest thiophilic activity.

3.2.2. Tanaka test

3.2.2.1. Tanaka test 1. The Tanaka test 1 in Figs. 4a and 5a contains five solutes, uracil, butylbenzene, amylbenzene, *o*-terphenyl, and triphenylene. The separation factor α(A/B) of the two alkylbenzenes, butylbenzene (B), and amylbenzene (A) describes the hydrophobicity of a column. It is often also stated as α(CH2), because the increment of retention of alkylbenzenes that is caused by each additional methylene group depends on the surface coverage as well as the alkyl chain length of the ligand [5]. The steric selectivity is being measured by the separation factor α(T/O) of the non-planar *o*-terphenyl (O) and its highly planar analogue triphenylene (T).

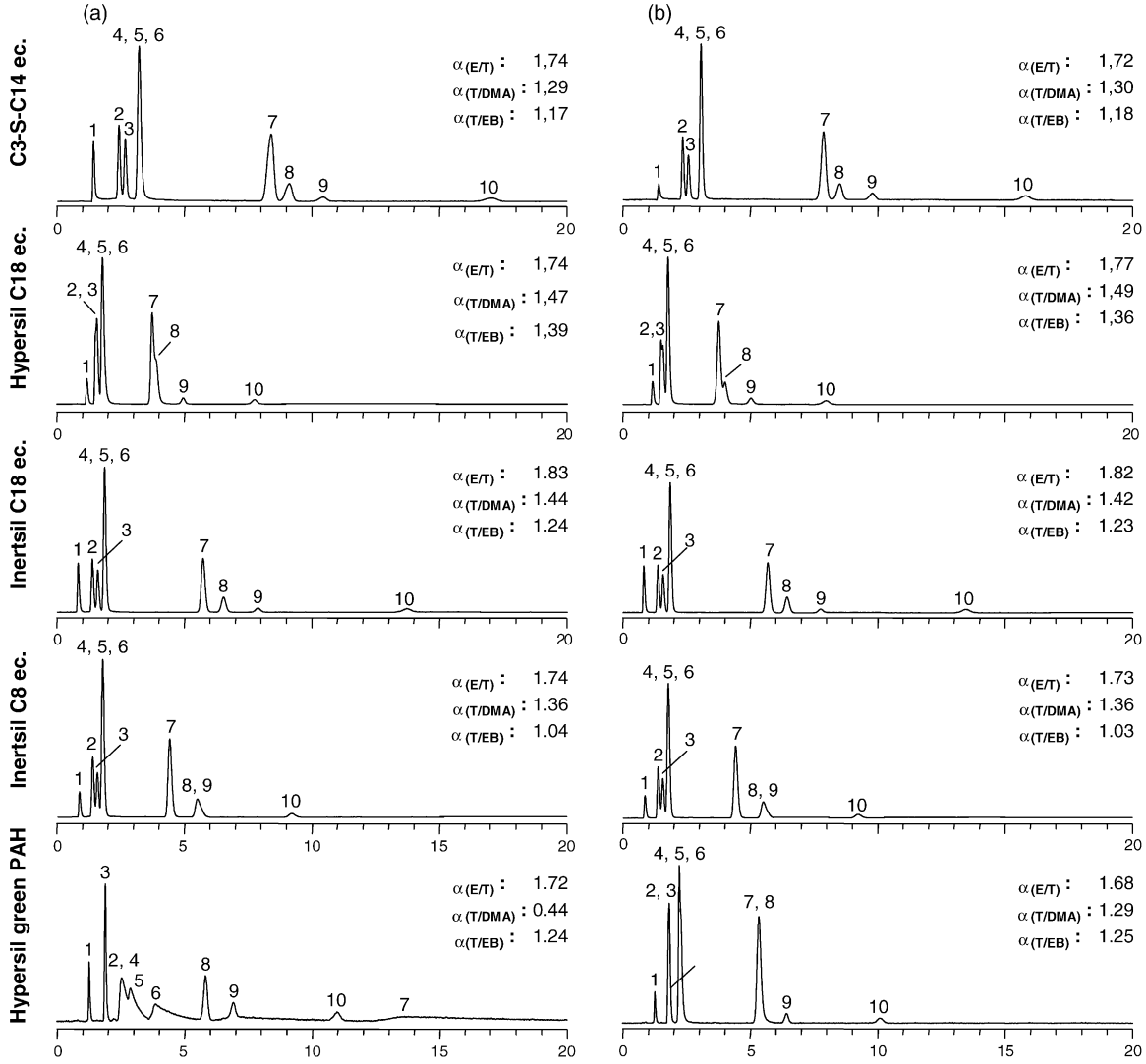

Fig. 3. Separation of the Engelhardt test set containing thiourea (**1**), aniline (**2**), phenol (**3**), *o*-toluidine (**4**), *m*-toluidine (**5**), *p*-toluidine (**6**), *N*,*N*-dimethylaniline (**7**), ethylbenzoate (**8**), toluene (**9**), and ethylbenzene (**10**), including separation factors α(E/T) for toluene (T) and ethylbenzene (E), α(T/DMA) for *N*,*N*-dimethylaniline (DMA) and toluene (T) and α(T/EB) for ethylbenzoate (EB) and toluene (T) in (a) methanol–water 49:51 (w/w) and (b) methanol–1 mM phosphate buffer (pH 7.0) 49:51 (w/w)—1 mL/min, 40 °C, 254 nm on silanol endcapped RP and S-RP phases.

In accordance to the previously stated hydrophobicity term α(E/T) from the Engelhardt test, the corresponding α(A/B) values of the Tanaka test also increase with increasing *n*-alkyl chain length and do not depend on the actual ligand density. Surprisingly, the α(A/B) values for the commercial C_8 and C_{18} phases are approximately the same as observed for the corresponding 3-propylthiol bound C_8- and C_{18}-chain phases. Also the C_3–S–C_{14}/SH and the C_3–S–C_{14} ec phases possess comparable hydrophobicity values, which are slightly lower then those obtained for the conventional C_{18} phases with the same number of overall bonds. It seems obvious that the alkyl sulfide- and thiol-groups act as a kind of weak polarity barrier, which mainly emits the sulfur bound, mobile phase directed alkyl chain to effectively contribute to the overall hydrophobicity of the phase. The propyl-chains below this sulfur barrier, including the residual propylthiol chains do not seem to contribute to the hydrophobic retention.

A rather unexpected result was the strong increase in shape selectivity for sulfur incorporated RP phases in Fig. 4 compared to the monomeric *n*-alkyl type C_{18}-phases. The separation factor α(T/O) in Fig. 4a increases gradually with increasing alkyl chain length of the sulfur bound ligand. The highest values were obtained for the two thiol bearing phases C_3–S–C_{14}/SH and C_3–S–C_{18}/SH. Surprisingly, the C_3–S–C_{14} ec (Fig. 5a) exhibited a shape selectivity index that is by 0.3 lower then its thiol group bearing analogue and by 0.3–0.6 higher than for the two investigated C_{18} RP phases. Note that already the 3-propylthiol backbone provides comparable and in some cases even higher shape selective properties then the investigated monomeric *n*-alkyl based RP-materials, although the retention is much reduced.

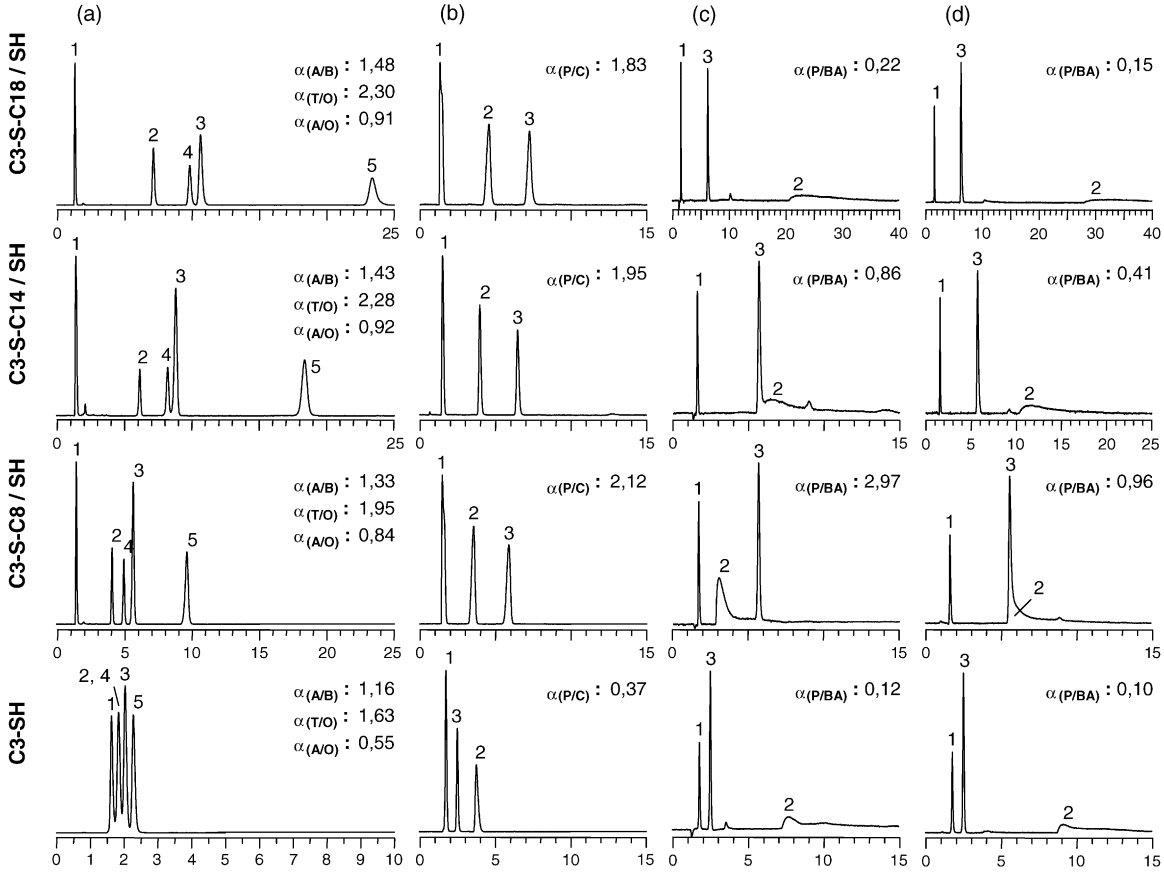

Fig. 4. (a) Separation of the Tanaka test 1 set containing uracil (**1**), butylbenzene (**2**), *o*-terphenyl (**3**), amylbenzene (**4**), and triphenylene (**5**), including separation factors α(A/B) for butylbenzene (B) and amylbenzene (A), α(T/O) for triphenylene (T) and *o*-terphenyl (O), and α(A/O) for amylbenzene (A) and *o*-terphenyl (O) in methanol–water 632:200 (w/w)—1 mL/min, 30 °C, 254 nm; (b) separation of the Tanaka test 2 set containing uracil (**1**), caffeine (**2**), and phenol (**3**), including separation factor α(P/C) for caffeine (C) and phenol (P) in methanol–water 237:700 (w/w)—1 mL/min, 30 °C, 254 nm on *n*-alkyl type sulfur incorporated S-RP phases; (c) and (d) separation of the Tanaka test 3 and Tanaka test 4, both containing uracil (**1**), benzylamine (**2**), and phenol (**3**), including the separation factor α(P/BA) for benzylamine (BA) and phenol (P) in (c) methanol–20 mM phosphate buffer (pH 2.7) 30:70 and in (d) methanol–20 mM phosphate buffer (pH 7.6) 30:70.

Overall, it can be stated that the shape selective properties of sulfur-bearing RP phases are slightly enhanced to the corresponding monomeric *n*-alkly-type phases and can be generally placed between the two extremes of the monomerically and the polymerically modified RP phases.

Taking a close look on the chromatograms in Fig. 4a, it is apparent that the elution order of amylbenzene (A) and *o*-terphenyl (O) is reversed on S-RP phases. As illustrated in Fig. 5a, the separation factors α(A/O) for conventional *n*-alkyl-type RP phases are approximately 1 or even higher than 1. Whereat for S-RP phases, the α(A/O) values decrease with decreasing hydrophobicity of the S-RP phase and is lowest for the bare 3-propyl-thiol silica support. Judged by the low hydrophobicity and the relatively high shape selectivity of the C_3–SH phase, it can be proposed that this phase possesses high sulfur–aromatic interaction properties. Hence, α(A/O) may be an useful indicator for the electron–donor/acceptor activity of hybrid RP phases, such as S-RP type and π-RP type materials, which will be discussed in reference [42].

Consequently, it can be stated that the incorporation of a sulfide group within the alkyl chain has a higher influence on shape selectivity then any further extension of the sulfur bound alkyl chain length, above a C_{14}-chain. Besides the sulfur atom in the alkylsulfide, also the thiol-sulfur provides an important electron donor source for electron–donor/acceptor interaction with aromatic solutes, providing thereby increased shape discriminating properties for hybrid S-RP phases and increased retention times for solutes with aromatic moieties.

3.2.2.2. Tanaka test 2. The Tanaka test 2 in Figs. 4b and 5b comprises the compounds phenol (P) and caffeine (C). It is claimed to trace hydrogen bonding interactions between surface silanol groups and caffeine. On stationary phases with high silanol activity, caffeine will observe a stronger retention and may even change the elution order with phenol. Hence, for non-endcapped RP phases, α(P/C) will show values below 1.

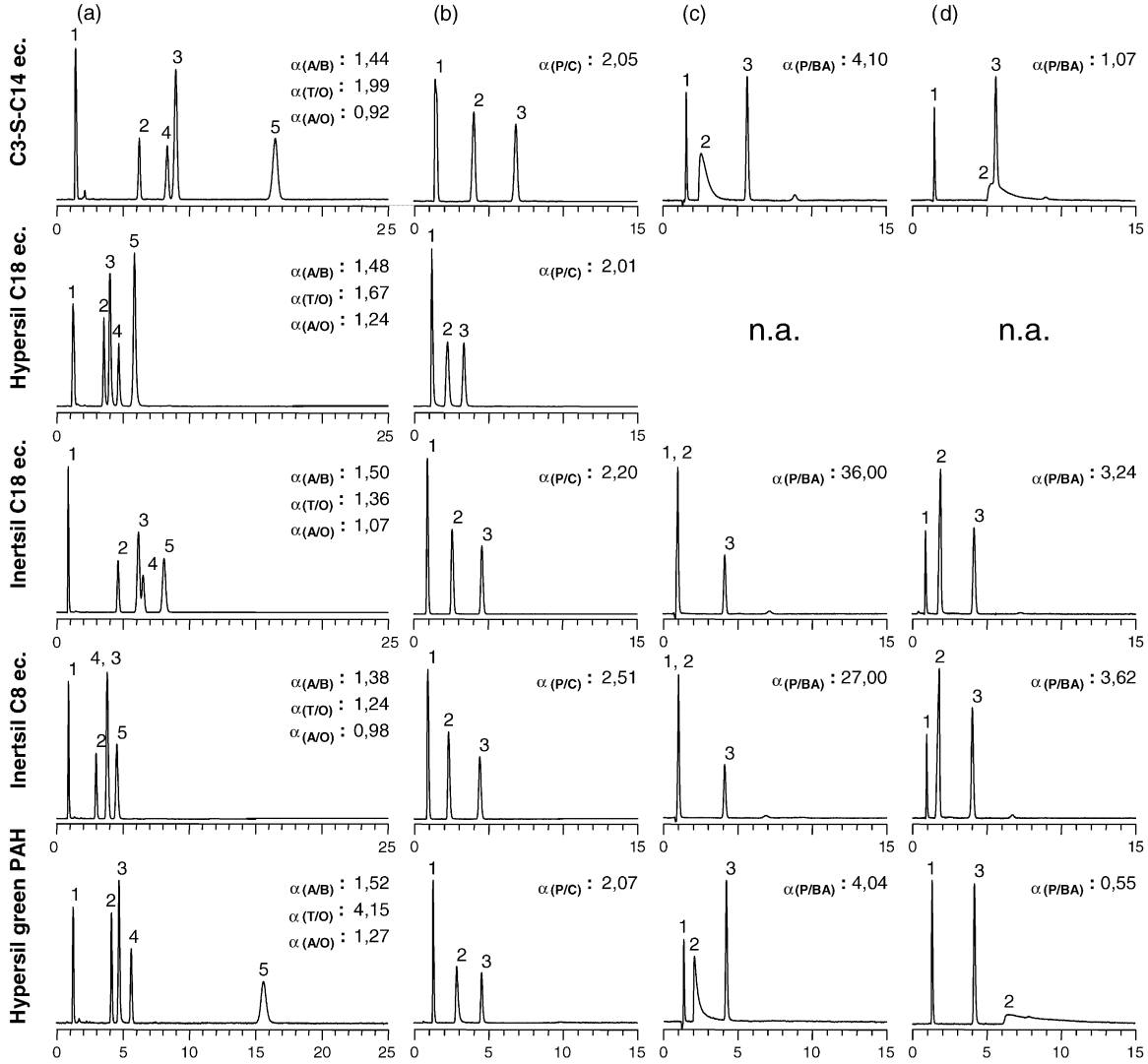

Fig. 5. (a) Separation of the Tanaka test 1 set containing uracil (**1**), butylbenzene (**2**), *o*-terphenyl (**3**), amylbenzene (**4**), and triphenylene (**5**), including separation factors α(A/B) for butylbenzene (B) and amylbenzene (A), α(T/O) for triphenylene (T) and *o*-terphenyl (O), and α(A/O) for amylbenzene (A) and *o*-terphenyl (O) in methanol–water 632:200 (w/w)—1 mL/min, 30 °C, 254 nm; (b) separation of the Tanaka test 2 set containing uracil (**1**), caffeine (**2**), and phenol (**3**), including separation factor α(P/C) for caffeine (C) and phenol (P) in methanol–water 237:700 (w/w)—1 mL/min, 30 °C, 254 nm on silanol-endcapped RP and S-RP phases; (c) and (d) separation of the Tanaka test 3 and Tanaka test 4, both containing uracil (**1**), benzylamine (**2**), and phenol (**3**), including the separation factor α(P/BA) for benzylamine (BA) and phenol (P) in (c) methanol–20 mM phosphate buffer (pH 2.7) 30:70 and in (d) methanol–20 mM phosphate buffer (pH 7.6) 30:70; abbreviation n.a. for not applicable.

Concerning the hydrogen bonding term α(P/C) in Fig. 4b, it is obvious that the C_3–SH phase possess the highest hydrogen bonding activity. The values obtained by the other thiol bearing phases decrease slightly with increasing ligand length, because that implicates an increase of the thiophilic and silanophilic activity due to an enhanced accessibility, provided by the reduced ligand densities.

Comparing again the thiol-bearing C_3–S–C_{14} phase with its endcapped analogue and with the RP type C18 phases, then the α(P/C) values decline in the following order, which is also the order of increasing hydrogen bonding activity: Inertsil-C_{18} ≫ Hypersil-green-PAH > C_3–S–C_{14} ec > HP-BDS-C_{18} > C_3–S–C_{14}/SH. The fairly new Inertsil-C_{18} phase provide higher values then the HP-BDS-C_{18} column, which was already frequently in use and surely possesses a certain amount of accessible silanol groups. The low α(P/C) value of the Hypersil-green-PAH column and the considerable tailing of the benzylamine peak in Fig. 5c and d clearly indicate the presence of active sites for ion-pairing and hydrogen-bonding interactions.

3.2.2.3. Tanaka test 3 and Tanaka test 4. In the Tanaka tests 3 and 4, which are shown in Figs. 4c and d and 5c and d caffeine is replaced by the strong base benzylamine ((B); pK_a 9.4). The different pH of the buffered mobile phases of the Tanaka test 3 (pH 2.7) and the Tanaka test 4 (pH 7.6), indicates the presence as well as the acidity of the accessible silanol groups. While under both pH conditions,

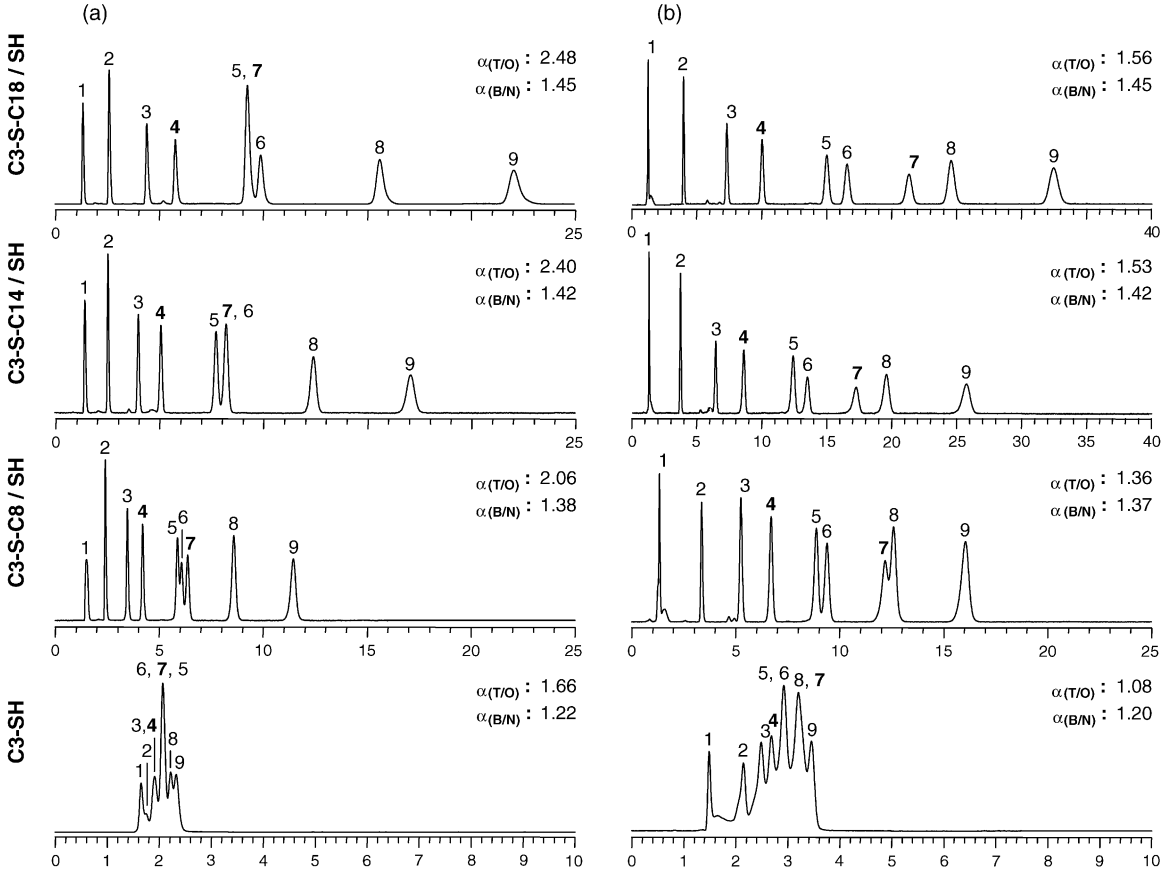

Fig. 6. Separation of PAH test set 1 containing uracil (**1**), benzene (**2**), naphthalene (**3**), biphenyl (**4**), phenanthrene (**5**), anthracene (**6**), *o*-terphenyl (**7**), pyrene (**8**), and triphenylene (**9**), including separation factors α(T/O) for *o*-terphenyl (O) and triphenylene (T) and α(B/N) for naphthalene (N) and biphenyl (B) in (a) methanol–water 80:20 (v/v) and (b) acetonitrile–water 60:40 (v/v)—1 mL/min, 30 °C, 260 nm on *n*-alkyl based sulfur incorporated S-RP phases.

benzylamine is protonated and therefore ionized, the extent of deprotonation for silanol groups depend highly on their pK_a-value, which may vary between 1 and 10 [1].

It can be expected that at a pH of 2.7 most silanol groups are undissoziated and undergo hydrogen-bonding interaction with benzylamine. However, although low in their average amount (∼less than 1%), those silanol groups with pK_a-values below 2.7 are nevertheless highly acidic and therefore highly reactive. These acidic silanol groups will obviously interact with benzylamine in an ion-exchange reaction, providing the strong peak tailing of benzylamine observed in Figs. 4c and 5c. At pH 7.6, however, most silanol groups will be dissoziated and fully account for an ion-exchange interaction with benzylamine, which is clearly illustrated by its extreme peak tailing and by its strong and even irreproducible retention of benzylamine on non-silanol-endcapped phases in Figs. 4d and 5d.

The chromatograms of the C_3–S–C_{14} ec column in Fig. 5c and d clearly demonstrate the presence of residual thiol-groups (pK_a 10.61), which may have their origin in the formation of disulfide bonds between the 3-propyl-(trimethoxy)-silane molecules during the one-pot radical addition of the *n*-alkene. The breakage of the disulfide-bonds may then lead to trace amounts of free 3-propylthiol groups on the silica surface, which can also be considered as weakly acidic sites. The interference of residual silanol-groups may be excluded, since the phase is fully endcapped.

3.3. Polycyclic aromatic hydrocarbons (PAHs)

In order to investigate the influence of sulfur–aromatic interactions on the shape selectivity of hybrid-RP phases compared to conventional brush-type and polymeric RP phases, two different sets comprising polycyclic aromatic hydrocarbons were assembled and separately investigated. An extended summary of all investigated PAHs, including their molecular weight, number of π-electrons, length-to-breadth (*L*/*B*) values and their thickness [43] is provided in [42].

3.3.1. PAH test set 1

The first test set contains eight polycyclic aromatic hydrocarbons, which differ strongly in their size and shape. Note that with exception of biphenyl and *o*-terphenyl all polyaromatic test solutes are planar. The separation factors for the planar/non-planar couples α(T/O) for triphenylene (T) and *o*-terphenyl (O) as well as α(B/N) for biphenyl (B) and naphthalene (N) are investigated as possible measures for shape selectivity. Their values are listed beside the chromatograms

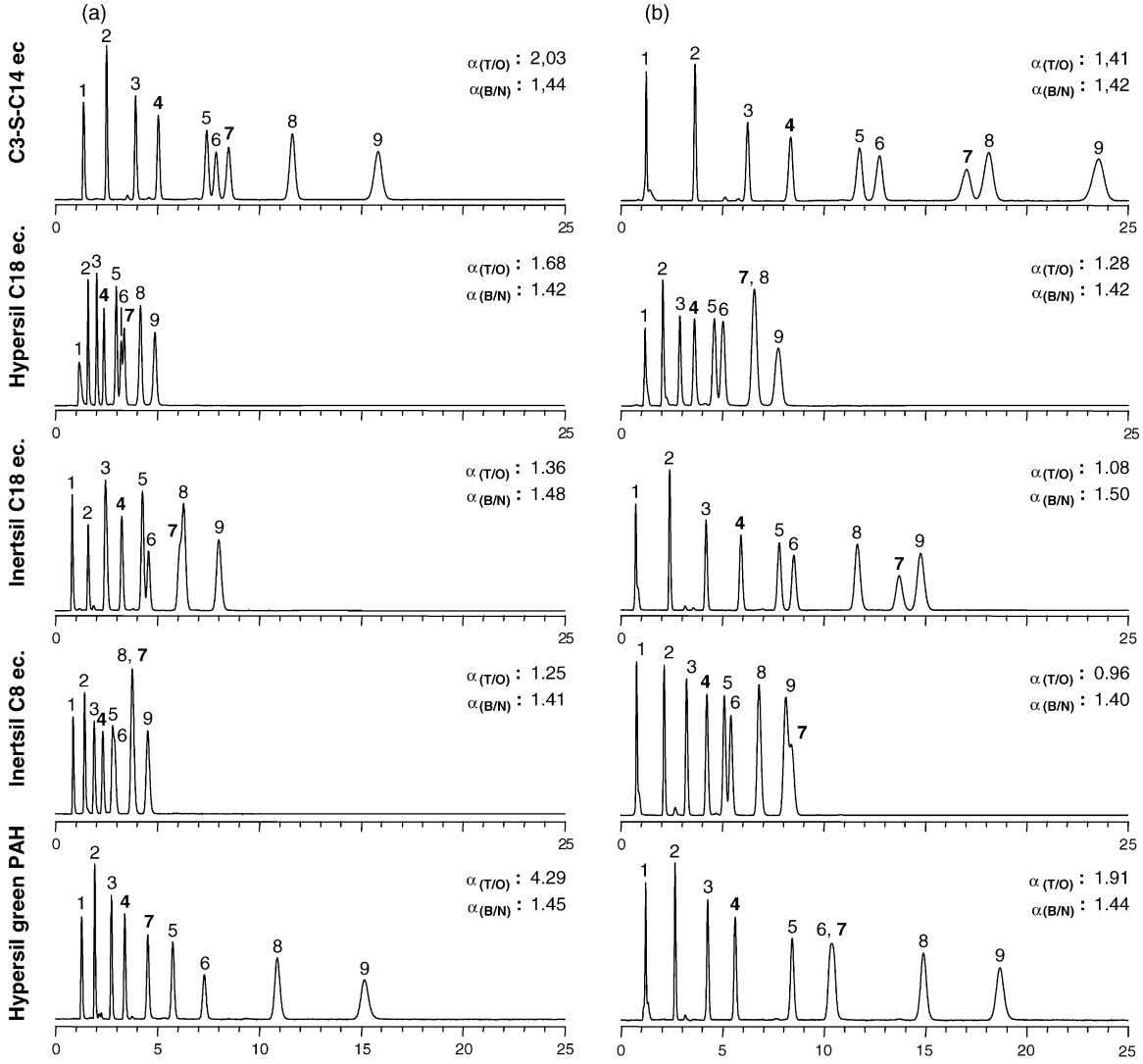

Fig. 7. Separation of PAH test set 1 containing uracil (**1**), benzene (**2**), naphthalene (**3**), biphenyl (**4**), phenanthrene (**5**), anthracene (**6**), *o*-terphenyl (**7**), pyrene (**8**), and triphenylene (**9**), including separation factors α(T/O) for *o*-terphenyl (O) and triphenylene (T) and α(B/N) for naphthalene (N) and biphenyl (B) in (a) methanol–water 80:20 (v/v) and (b) acetonitrile–water 60:40 (v/v)—1 mL/min, 30 °C, 260 nm on silanol-endcapped RP and S-RP phases.

in Figs. 6 and 7. Note that triphenylene and *o*-terphenyl were already earlier discussed as essential compounds of the chromatographic column test from Tanaka in Section 3.2.2 and shall therefore not be discussed too widely in this context.

In order to investigate the influence of different organic modifiers on the overall retention behavior of these new polar embedded and quasi also “polar endcapped” phases, all measurements were performed with aqueous mobile phases employing methanol and acetonitrile under isoeluotropic conditions [44]. The use of isoeluotropic eluent mixtures shall provide equal solvent strength for the two binary mixtures and enable thereby an adequate illustration of the different properties and influences of the two organic modifiers on solute retention. Since the selected PAHs do not carry any functional groups, differences in mobile phase composition were assumed to only influence the absolute retention times, but ought not to change the order of elution.

However, the different elution order of *o*-terphenyl (**7**) compared to the other PAHs in Fig. 6 and Fig. 7 clearly illustrates the influence of the organic modifier on the shape selective properties of a stationary phase. It is also apparent that the retention times of all solutes increase with increasing *n*-alkyl chain length and also increase with the change of organic modifier from methanol (a) to acetonitrile (b) under isoeluotropic conditions, which may be the result of a better wetting of the hydrophobic ligands by acetonitrile.

A comparison of the α(T/O) values shows clearly the contribution of the sulfur groups incorporated into the RP phase as well as the influence of the increasing hydrophobicity with increasing alkyl chain length. Although the bare 3-propylthiol phase provides α(T/O) values that are comparable or even higher than those obtained for the investigated monomeric RP phases, the overall retention times on the C_3–SH phase are rather low. This shortcoming

is counteracted by extending these short surface bound 3-propylthiol-ligands with hydrophobic alkyl chains. The additional hydrophobicity increment provided by the *n*-alkyl chain ligands that are directed towards the mobile phase, not only increases the overall retention times, but also amplifies their shape selective properties due to hydrophobic interactions as well as sulfur–aromatic interactions. Also the small planar/non-planar couple naphthalene and biphenyl exhibits a slight selectivity enhancement with increasing ligand length (Fig. 6). This occurrence may have its origin in the higher hydrophobicity as well as the higher π-electron density of the biphenyl molecule, which is by two C-atoms and two π-electrons larger than naphthalene.

A comparison of the C_3–S–C_{14}/SH phase in Fig. 6 with the endcapped analogue in Fig. 7 reveals slightly higher retention times and a by 0.45 higher α(T/O) value for the thiol-bearing phase. It may be assumed that the residual thiol-groups generated by the two step-approach contribute to some extend to the shape selective properties of S-RP phases.

At this point it can be concluded, that hydrophobic interaction is the dominant interaction mode for S-RP type stationary phases. For the investigated set of small PAHs, the contribution of a S-aromatic interaction provide only a slight increase in planar recognition compared to the conventional brush-type RP phases, but leads to a large increase in retention. The overall selectivity of the investigated S-RP phases become, however, comparable to that of the polymeric Hypersil-green-PAH column.

3.3.2. PAH test set 2

In contrast to the first PAH test set, this following selection of PAHs comprise a set of isomeric four-ring PAHs (compounds **9**–**13**), which differ only in their spatial shape, which in actual fact defines their contact and interaction area with the stationary phase.

As shown in Fig. 8, the best separation of all four-ring isomers was obtained on the polymeric C_{18}-phase, while no separation was achieved on the C_3–SH phase. However, with the increase in hydrophobicity, provided by the *n*-alkyl chain ligands that are bound to the 3-propylthiol phase, the shape selective properties of the S-RP phase increases with increasing length of their *n*-alkyl ligand. The C_3–S–C_{18}/SH phase enables the separation of tetrahelicene, triphenylene and naphthacene. The isomers chrysene and tetraphene are not separated on the C_3–S–C_{18}/SH phase, while the Hypersil-green-PAH column separates all isomers at double the elution time compared to the latter. Note, that all chromatograms were taken under standardized mobile phase conditions and do not resemble the optimum separation parameters of the respective phases.

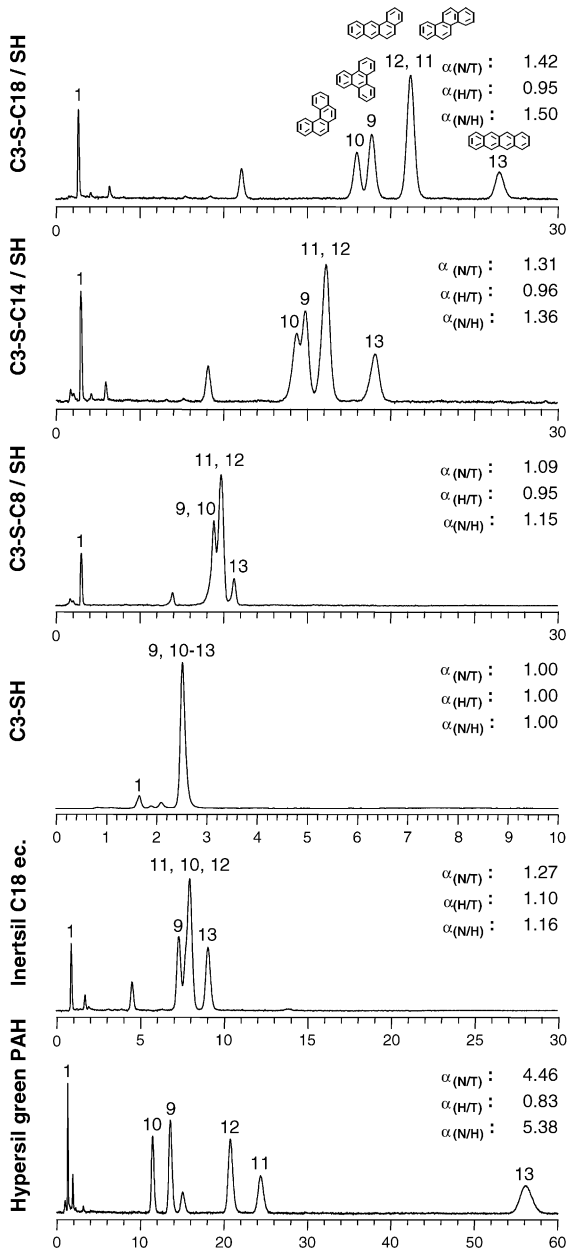

Fig. 8. Separation of the PAH test set 2 containing uracil (**1**), triphenylene (**9**), tetrahelicene (**10**), chrysene (**11**), tetraphene (**12**), and naphthacene (**13**) including the separation factors α(N/T) for naphthacene (N) and triphenylene (T), α(H/T) for tetrahelicene (H) and triphenylene and α(N/H) for naphthacene and tetrahelicene in methanol–water 80:20 (v/v)—1 mL/min, 30 °C, 270 nm.

3.4. Separation of cis/trans-isomers

The *cis*- and *trans*-isomers of three stilbene-derivatives, namely stilbene, stilbene oxide and resveratrol comprise the same aromatic stilbene backbone, but differ in their overall polarity and shape. The polarity increases from stilbene and stilbene oxide to resveratrol, and provide in case of stilbene oxide and resveratrol additional hydrogen bonding sites for interaction with the sulfide groups as well as the remaining thiol and silanol groups of the S-RP phases. Furthermore the *cis*- and *trans*-isomers differ strongly in shape and provide thereby a separation due to the different shape selective properties of the investigated hybrid S-RP

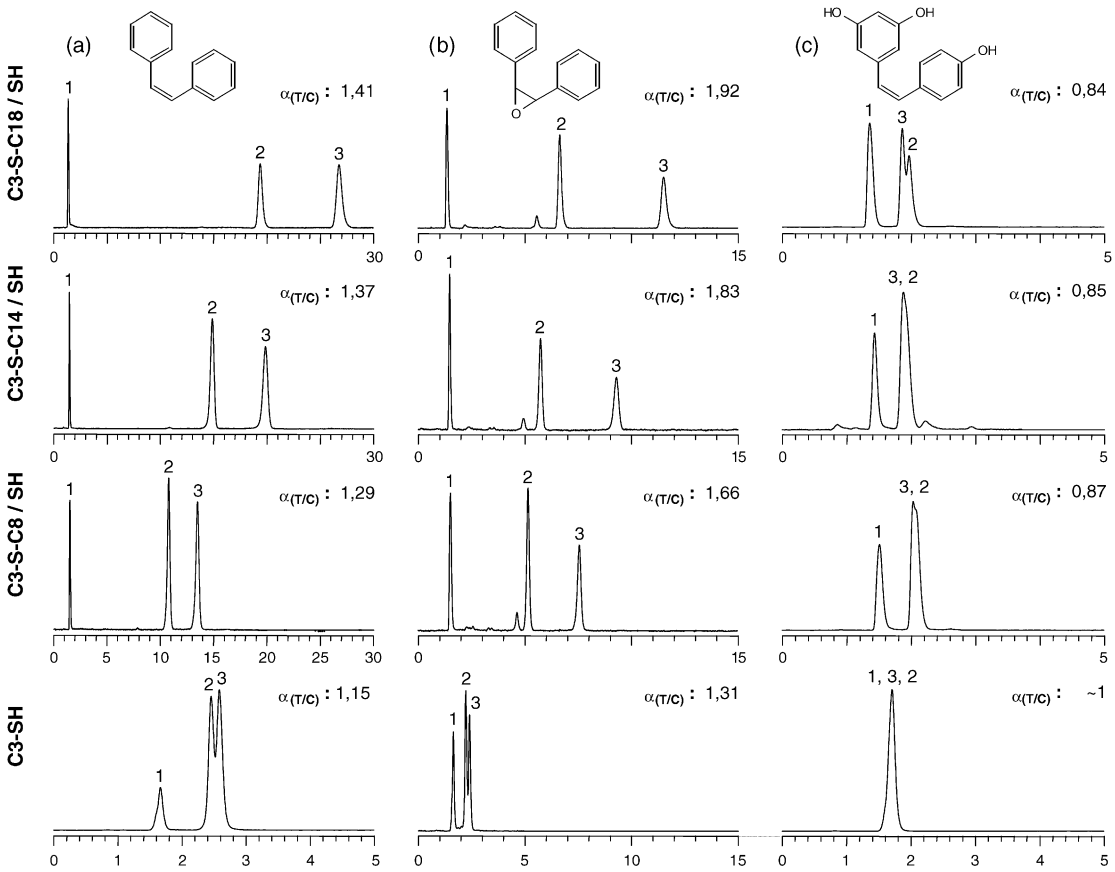

Fig. 9. Separation of uracil (**1**) and *cis* (**2**)/*trans* (**3**) isomers of stilbene (a), stilbene oxide (b), and resveratrol (c) in methanol–water 70:30 (v/v) at a flow rate of 1 mL/min and a detection wavelength of 280 nm for stilbene and resveratrol and 254 nm for stilbeneoxide for *n*-alkyl-type sulfur incorporated S-RP phases; including the separation factors α(T/C) for the *cis*- (C) and the *trans*-isomers (T).

phases. The separation factors α(T/C) for the *cis* (C) and the *trans* (T) isomers of the three test sets are listed *next* the corresponding chromatograms in Figs. 9 and 10.

In accordance with the previously obtained results, the retention times as well as the shape selectivity marker α(T/C) of the test solutes stilbene and stilbene oxide increase with increasing ligand length (Fig. 9) of the S-RP phases. Surprisingly, already the C_3–SH phase provide a nice separation of the void volume marker uracil from the isomer couples of stilbene and stilbene oxide. For resveratrol under the standardized mobile phase condition with 70% methanol, all three peaks co-eluted on the C_3–SH phase, whereas for the other S-RP phases at least a baseline separation from uracil could be achieved. A comparison of the thiol-bearing and the endcapped C_3–S–C_{14} ec phase leads to comparable separation factors for the isomers. The chromatograms in Fig. 10 illustrate clearly the enhanced shape selectivity of the C_3–S–C_{14} ec phase compared to the other monomerically modified non-sulfur bearing *n*-alkyl phases. However, a comparison of the C_3–S–C_{14} ec phase with the polymeric Hypersil-green-PAH phase results in a stronger retention of stilbene and stilbene oxide at reduced α(T/C) values for the S-RP phase.

Note, that on common RP phases *trans*-resveratrol always elute before its *cis*-analogue, even on the polymeric Hypersil-green-PAH phase. The reason for this phenomenon is the more accessible hydrophobic area of the *cis*-form, which enables better hydrophobic interaction possibilities with the reversed-phase material. On the other hand all three hydroxy-groups are concentrated on the other side of the molecule, enabling thereby a “half-way” penetration of the molecule into the hydrophobic lattice of the stationary phase and providing thus a slightly better retention of the *cis*-isomer compared to its *trans*-analogue.

3.5. Separation of functional and positional isomers

In the following study different functional and positional isomers were chosen as test solutes to investigate the interaction properties of different functional groups on sulfur incorporated RP phases. Hence, the *ortho*, *meta*, and *para* isomers of dihydroxy benzene, nitrophenol, and terphenyl were selected as possible test compounds. While every additional electron-donating hydroxy group increases the electron density of the benzene ring, the electron-withdrawing nitro group provides an electron deficiency.

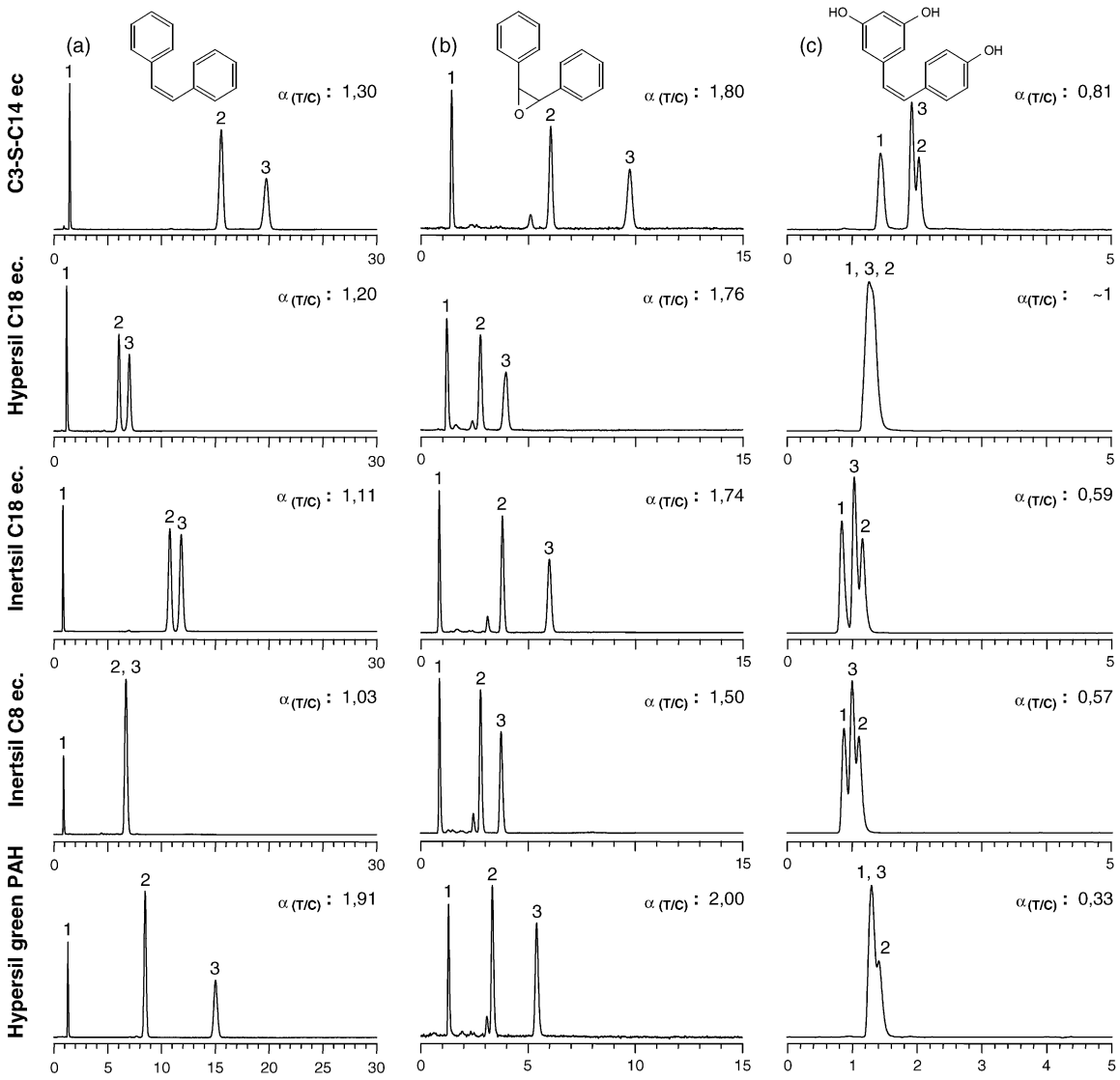

Fig. 10. Separation of uracil (**1**) and the *cis* (**2**)/*trans* (**3**) isomers of stilbene (a), stilbene oxide (b), and resveratrol (c) in methanol–water 70:30 (v/v) at a flow rate of 1 mL/min and a detection wavelength of 280 nm for stilbene and resveratrol and 254 nm for stilbeneoxide on *n*-alkyl-type RP phases and a sulfur incorporated S-RP phase with silanol-endcapping (ec); including the separation factors α(T/C) for the *cis*- (C) and the *trans*-isomers (T).

3.5.1. Isomers of terphenyl

As previously shown for the Tanaka test and the PAH test set 1, *o*-terphenyl is a non-planar polyaromatic compound that is successfully employed as an indicator for the planar recognition ability of a stationary phase. Due to the bulkiness of the two aryl-groups that are attached to the benzene ring and the rotational freedom of the attaching C–C bond, the three terphenyl-isomers differ strongly in the extent of their off-planarity. The close proximity of the two π-electron clouds of the aryl groups in *ortho*-position provides a strong sterical hindrance that is amplified by the repulsion of the two aromatic π-electron clouds. Hence, the *o*-terphenyl isomer posses the highest deviation from planarity, followed by *m*-terphenyl and *p*-terphenyl. Note that this *ortho–meta–para* elution order is reversed to the one observed for the nitrophenols and the di-hydroxybenzenes.

The increased retention times as well as the higher separation factors for the *ortho* and the *meta* isomers in Fig. 11c compared to the monomeric C_{18} phases in Fig. 12c, illustrate clearly the high planar recognition ability of these novel sulfur incorporated RP phases. However, for the C_3–S–C_8/SH phase as well as the Inertsil C_{18}-RP phase no separation for the *meta* and *para o*-terphenyl isomers was observed.

In case of the S-RP phases, the addition of hydrophobicity based shape selectivity, provided by the increase in the *n*-alkyl chain length, leads to the baseline separation of the *meta* and *para*-isomers in Fig. 11c. This effect stands in accordance to the separation behavior of the polymeric Hypersil-green-PAH phase, where the separation of the *meta* and the *para* isomers is strongly favored. The better separation efficiency of the *meta* and *para o*-terphenyl isomers

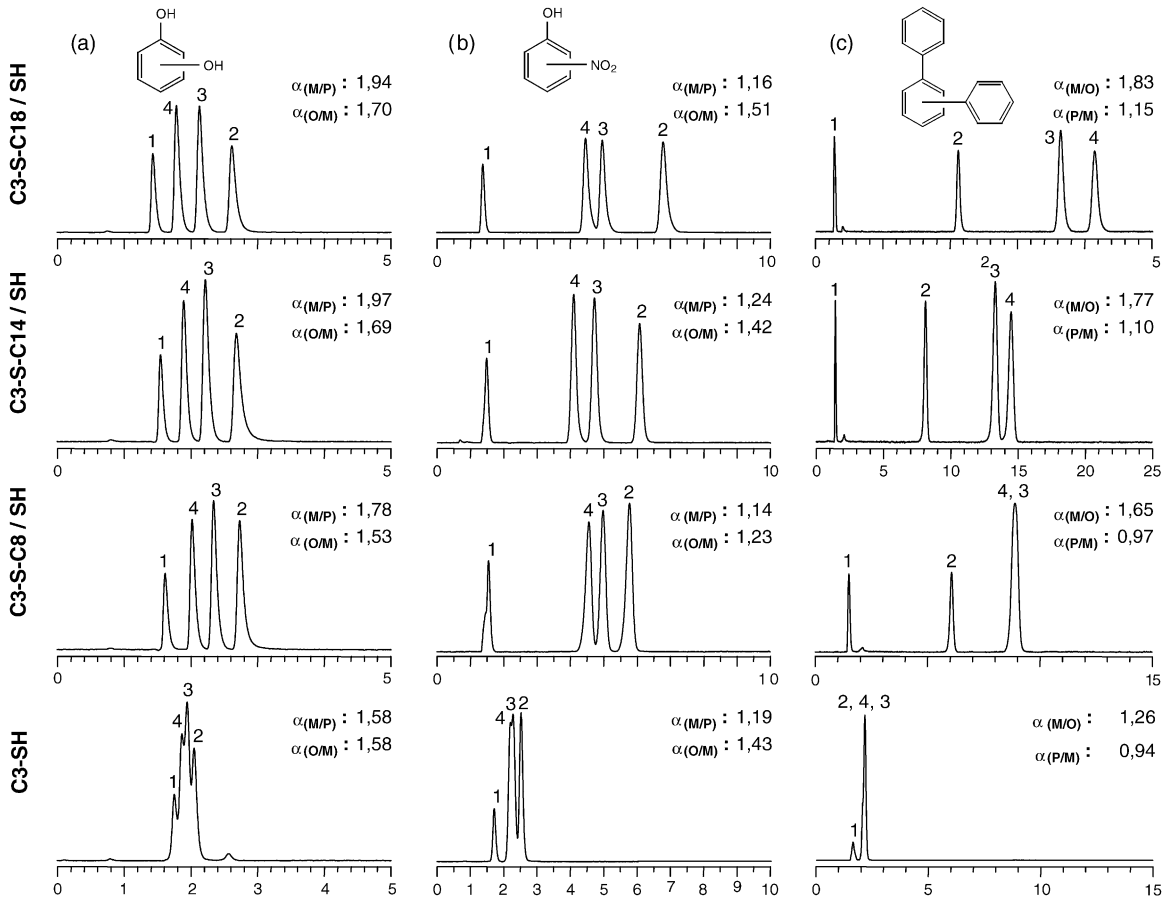

Fig. 11. Separation of uracil (**1**) and the *ortho*- (**2**), *meta*- (**3**), and *para*- (**4**) isomers of: (a) dihydroxybenzene in methanol–water 40:60 (v/v)—1 mL/min, 30 °C, 254 nm; (b) nitrophenol in methanol–water 50:50 (v/v)—1 mL/min, 30 °C, 280 nm; (c) terphenyl in methanol–water 80:20 (v/v)—1 mL/min, 30 °C, 254 nm on *n*-alkyl-type sulfur incorporated S-RP phases.

on the 3-propylthiol-bearing C_3–S–C_{14}/SH phase compared to the "thiol-free" C_3–S–C_{14} ec phase is most probably the result of additional sulfur–aromatic interaction and is consistent to our previous results.

The different retention behavior of the novel S-RP phases and the polymeric C_{18} phase provides clear evidence that the underlying interaction-mechanisms, which lead to shape selective discrimination is very different for these two phase types.

3.5.2. Isomers of dihydroxybenzene and nitrophenol

The elution order *para–meta–ortho* for dihydroxybenzene and nitrophenol in Fig. 11a and b corresponds to the accessibility of the benzene ring to hydrophobic interactions as well as electron donor–acceptor interactions between the sulfur group and the aromatic moiety of the solutes. Although the *o*-, *m*-, *p*-dihydroxybenzene isomers as well as the *o*-, *m*-, *p*-nitrophenol isomers are already nicely separated on the C_3–S–C_8/SH column, the additional alkyl-chain length of the corresponding C_{14} and C_{18} phase provides increased separation factors for the *ortho*-isomer especially for the nitro-group containing compounds. The separability of the *meta* and the *para* isomers, however, do not seem the be much influenced. The comparison of the thiol-bearing and the silanol-endcapped C_3–S–C_{14} ec phases in Figs. 11 and 12 provides once again slightly higher α-values for the thiol-bearing phase.

For these small positional and functional isomers the polymeric Hypersil-green-PAH phase exhibits the same general separation behavior as observed for the Hypersil-BDS and the S-RP phases. However, concerning the separation efficiency for the *m*- and *p*-nitrophenol isomers, the S-RP phases perform even better than the polymeric phase.

Surprisingly, on the two Inertsil C_8- and C_{18}-columns the *meta*- and *para*-nitrophenol isomers elute in reverse order (*meta–para–ortho*), compared to the Hypersil-BDS C18 column (*para–meta–ortho*) and the novel S-RP-columns (Fig. 12b). Furthermore, the mentioned peaks are exceptionally broad and distorted.

All three RP-columns have in common that they are silanol endcapped. The only feasible difference lies in their age and their extent of usage, respectively. While the two Inertsil columns are both new, the Hypersil-BDS column was already in use for some time and might pos-

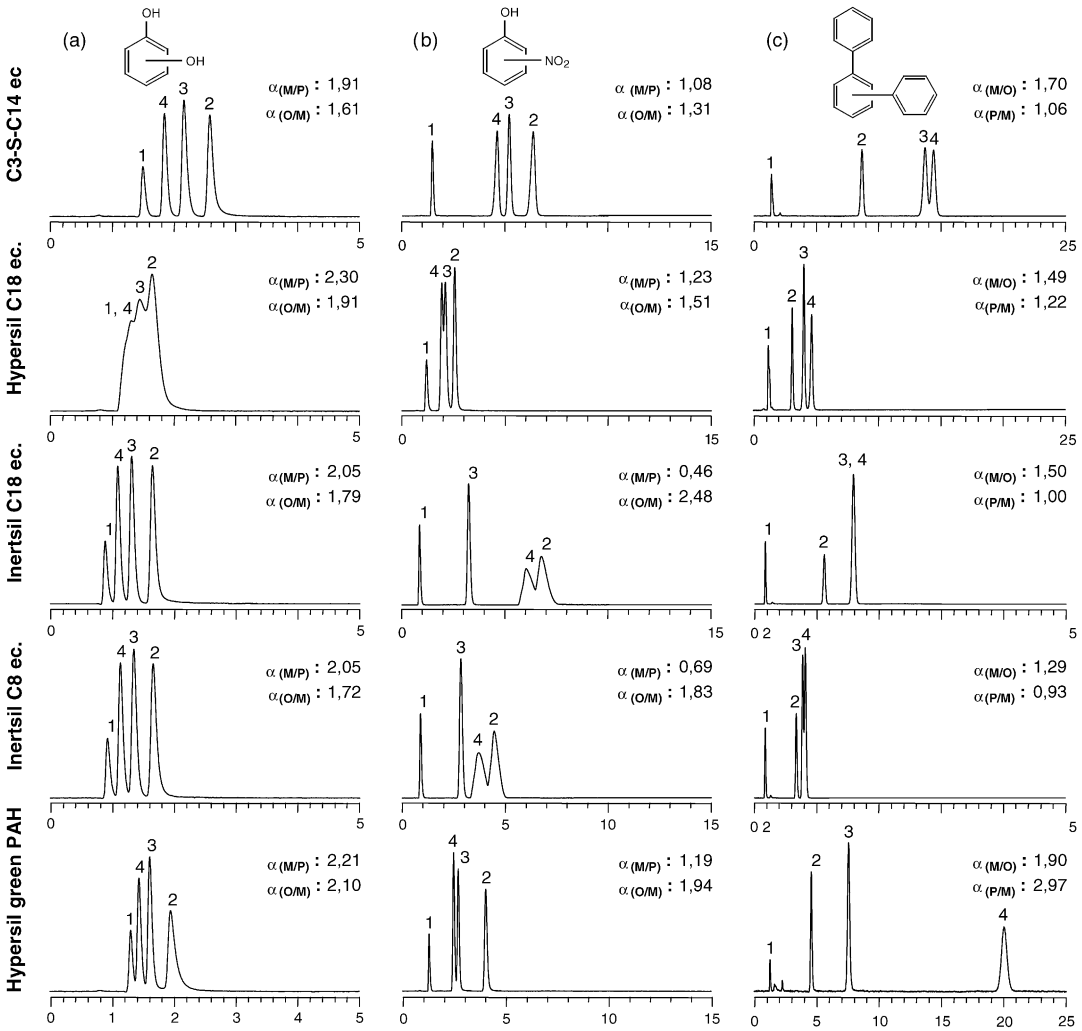

Fig. 12. Separation of uracil (**1**) and the *ortho*- (**2**), *meta*- (**3**), and *para*- (**4**) isomers of: (a) dihydroxybenzene in methanol–water 40:60 (v/v)—1 mL/min, 30 °C, 254 nm; (b) nitrophenol in methanol–water 50:50 (v/v)—1 mL/min, 30 °C, 280 nm; (c) terphenyl in methanol–water 80:20 (v/v)—1 mL/min, 30 °C, 254 nm on silanol endcapped RP and S-RP phases.

sess small amounts of free and accessible silanols due to phase hydrolysis. These few active sites may not be traceable with basic solutes, but may be sufficient to provide a slight increase in phase polarity and stability, which ought to provide an increased penetrability of polar compounds into the hydrophobic lattice of the stationary phase.

Due to the electron withdrawing nitro-group on one side and the electron pushing hydroxy group on the other side, the *para*-nitrophenol molecule ought to be highly polarized. This makes them prone to π–π stacking-interaction. Since the fully endcapped Inertsil phases are highly hydrophobic, the polarized *p*-nitrophenol isomers may presumably prefer to interact with other hydrophobically retained *p*-nitrophenols or *o*-nitrophenol isomers then to be resolvatized and to dissociate back in solution. The improved peak shapes of the *ortho*- and the *para*-nitrophenols for diluted injection solutions may confirm this ‘’stacking”-proposition (Fig. 13).

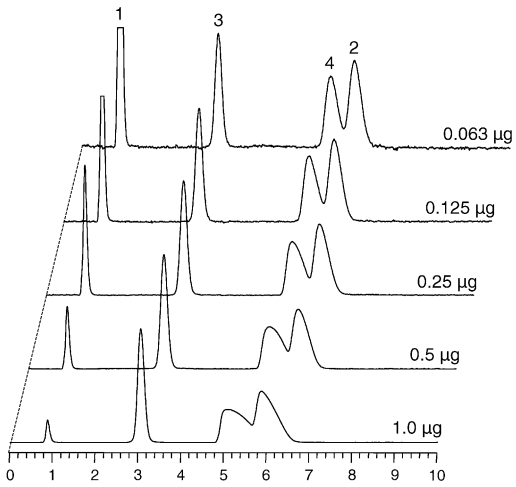

Fig. 13. Comparison of the peak performance of *ortho*- (**2**), *meta*- (**3**), and *para*- (**4**) nitrophenol when decreasing their concentration in the injection solution.

4. Conclusions

The novel sulfide and thiol-groups incorporated RP phases (S-RP) described in this paper possess hydrophobic, as well as sulfur–aromatic interaction sites. It was shown that sulfur–aromatic interaction has not only an enhancing effect on retention, but also provides some contribution to shape selective separation especially for solutes with extended π-electron systems. In some cases already the non-modified 3-propylthiol phase, the linking-support for our sulfur-alkyl phases provides a planar recognition ability that is higher or at least comparable to values obtained for commercially available brush-type RP-materials.

The increase in hydrophobicity by increasing the *n*-alkyl chain length combined with the presence of sulfidic groups and residual 3-propylthiol groups provided somewhat comparable results to the shape selective properties of the here investigated polymeric modified Hypersil-green-PAH phase. Although one must consider that the underlying retention mechanisms, which provide shape discriminative separation is very different for the two types of RP phases, it can be generally stated that the separation properties of S-RP phases may be placed between that of conventional brush-type RP phases and polymeric shape-selectivity-PAH-phases.

Another interesting feature of these novel S-RP phases is the fact that the sulfidic-groups as well as the residual 3-propylthiol groups introduce some surface-near polarity into the hydrophobic lattice of conventional RP phases. Consequently, the here-described phases may also be classified as polar-embedded and polar-endcapped RP phases. The comparable α(A/B)-values from the Tanaka test 1 for the corresponding RP and S-RP phases with the same *n*-alkyl chain length confirm our proposition of a surface-near polar sulfur-boundary, which acts like a polar penetration-barrier for hydrophobic compounds. However, a more detailed description of the modified and slightly enhanced planar recognition abilities of polar-endcapped and polar-embedded RP phases as well as their performance under highly aqueous media, which is frequently described by analysts and manufacturers alike will be discussed in a subsequent article [45].

The separation factor α(A/O) for *o*-terphenyl and amylbenzene generated by the Tanaka test 1 was found to be a possible indicator for the presence of electron donor–acceptor properties of a stationary phase. This aspect will be described in more detail in a subsequent article for highly π–π active π-RP phases [42].

Obviously, a possible contribution of the incorporated sulfur groups, may it be in form of a sulfide linkage or residual thiol-groups on the overall retention characteristics of a hybrid RP phase need to be considered during stationary phase design, when employing a 3-propylthiol silica gel support as the basis for a subsequent immobilization reaction.

As shown in this study, it is most important and even necessary to take a closer look on the entirety of a silica based stationary phase, especially when investigating the properties of novel ligands that are immobilized onto a pre-functionalized silica support. Every incorporated functional group, may it be the remaining silanol groups on the silica surface or incorporated thiol- and sulfide-groups imported by the linking support of the pre-modified silica gel, will have a pronounced impact on the overall retention characteristics of a novel stationary phase.

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