

Investigations on the chromatographic behavior of hybrid reversed-phase materials containing electron donor–acceptor systems

II. Contribution of π – π aromatic interactions

Jeannie Horak, Norbert M. Maier, Wolfgang Lindner*

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

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Abstract

The chromatographic behavior of three naphthalimide-type stationary phases were elucidated in terms of hydrophobic, silanophilic and π – π interaction properties, employing besides common chromatographic column test methods from Engelhardt and Tanaka, also new test mixtures of geometrical and functional aromatic isomers. It was found that the presence of electron donor/acceptor moieties within a reversed phase system did not only increase the overall retention times for aromatic solutes, but also lead to an enhanced shape selectivity of the hybrid stationary phase. In this context, shape discrimination is primarily based on the number of accessible π -electrons for π – π interaction with the embedded electron deficient ligand moieties. The most outstanding results were obtained for the 1,4,5,8-naphthalenediimidic selector with its horizontal arrangement on the silica surface, which enables a direct face-to-face π – π interaction with aromatic solutes, with only little hydrophobic contribution.

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1. Introduction

Besides the commonly used *n*-alkyl-type reversed-phase (RP) materials and columns based on the immobilization of *n*-alkyl-type ligands onto a silica support, alternative hybrid RP-type phases providing additional interaction sites and properties due to embedded functional groups become widely accepted and gain increased importance.

Recently, polar-endcapped [1,2] and polar-embedded RP phases with incorporated amide [2,3], carbamate [4,5] or urea groups [6,7] move in the center of interest. Due to their ability to undergo hydrogen bonding interactions with water molecules, such polar RP phases are claimed to be significantly more stable in highly aqueous and highly polar mobile phases than the conventional hydrophobic RP phases [1,8].

Alternatively, the introduction of hydrophobic π – π active aromatic moieties to the common *n*-alkyl chain RP-sites generates a concerted π – π -reversed-phase (π -RP) retention mechanism, which, as a consequence of the new func-

tionality, diversifies the common RP-interaction properties without altering the latter severely. As a result, solutes with π -systems will display a different retention behavior on π -RP-columns than on ordinary RP-columns, while solutes without π -electrons or with sterically hindered π -electron systems will be retained by the classical C8/C18 reversed-phase-like mode.

Due to the manifold possibilities to actually create π – π active moieties, numerous types of π – π active HPLC phases were already designed and investigated. Early and moderately π – π active phases were cyanopropyl phases [9] followed by non-polar selector types carrying a mono- [10] or poly-aromatic moiety [11–18]. Employing hetero-aromatic ring systems [19,20] and adding electron donating or withdrawing functional groups [15,18,21–23] that also provide additional interaction sites such as hydrogen bonding, increase tremendously the variability of π -RP-type selectors. This variability scenario is a general concept for selectivity tuning, which enables the design of tailor-made materials to solve separation problems that are beyond the capacity of ordinary C8/C18 RP-chromatography such as e.g. the separation of stereoisomers [22,24,25].

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

In this context, Sander et al. [26,27] describe the term “shape selectivity” as the property of monomeric and polymeric RP phases to separate constrained solutes, in particular polyaromatic hydrocarbons (PAHs) based on differences in their molecular shape, rather than differences in their physical or chemical properties. Wise et al. [28] introduced a slot model which describe the penetration of polyaromatic hydrocarbons into an imaginary slot in the ligand lattice depending on the length-to-breadth ratio (L/B) as well as the thickness of the solute molecule.

Beside this successful, but empirical approach by Sander and Wise [28], Yan and Martire [29] introduced the “molecular theory of selectivity enhancement”, which is based on statistical thermodynamics and include solute parameters such as the effective contact area, the van der Waals volume and the minimum cross-sectional area. Obviously this approach implicates a hydrophobic interaction between solute and stationary phase, which increases with increasing ligand length, ligand density and organic modifier concentration, since all these factors increase the effective contact area between solute and stationary phase.

A tentative understanding of the retention process in reversed-phase chromatography related to n -alkyl chain ligands is given also by the partitioning model [30]. This theory include the desolvation of solute and stationary phase, the creation of a cavity within the hydrophobic lattice of the stationary phase and the penetration of solute molecules into this cavity, which then enable the hydrophobic interaction of n -alkyl ligands and solute molecules. The ability of hydrophobic compounds to penetrate into such a hydrophobic environment increases with increased wetting of the phase, which is most effective with rather apolar and non-protic organic solvents. Although the formation of a cavity within the entirety of a RP-stationary phase is entropically expensive, the free energy gain that comes from the interaction between stationary phase and solute is the driving force in the retention process.

In C8/C18-type RP-chromatography the interaction between stationary phase and solute is primarily based on hydrophobic interaction with the alkyl chains of the n -alkyl chain ligands as well as silanophilic interactions with the remaining and respectively accessible surface silanol groups. It was proposed by Ohmacht et al. [31] that free silanols may also interact with aromatic solutes via $\text{OH}-\pi$ interaction providing thereby enhanced selectivity. Goto et al. reported that the carbonyl groups of poly(alkyl acrylate) grafted silica gels may also provide enhanced shape selective properties through carbonyl- π interaction reactions with aromatic solutes [32,33]. Evidently, the presence and availability of any $\pi-\pi$ active functionality within the entirety of a RP-phase will have a great impact on the overall chromatographic selectivity of compounds with extended π -electron systems. Whether this phenomena is called mixed mode selectivity or a somewhat affiliated “shape selectivity” becomes semantic, the emphasis is, however, to investigate the type of interactions, which cause structure related selectivities.

As known for a long time, the choice of organic modifier, even for isoelutotropic eluent mixtures, has already a major influence on the chromatographic behavior of conventional hydrophobic RP phases. This is in general a reflection of the physical and chemical properties of the immobilized ligands, their bonding chemistry as well as the surface properties of a modified silica support, concerning the residual silanol activity and their overall solvation status.

Under isoelutotropic conditions, the aqueous binary mobile phases of methanol, acetonitrile and tetrahydrofuran possess equal solvent strength and similar retention behavior on alkyl-based stationary phases [34]. The retention of hydrophobic compounds on n -alkyl phases depend strongly on the wettability and solvability properties of the organic mobile phase modifier to solvate both, the stationary phase and the analyte molecules. An increased solvation of the n -alkyl chains provides a better penetration of hydrophobic compounds into the hydrophobic ligand lattice and enables thereby better interaction possibilities between stationary phase and solute molecules, providing thereby increased retention times for the latter. Therefore, alkyl chain solvation increases with decreasing polarity of the organic solvent, in the order methanol (polar and protic), acetonitrile (less polar and non-protic) and tetrahydrofuran (non-polar and non-protic).

Once $\pi-\pi$ interaction sites are present, as it is for instance the case for polar embedded RP phases [2,4,35] and aromatic-RP phases, the differences between methanol and acetonitrile become even more significant, as acetonitrile shows $\pi-\pi$ solvent interactions. Acetonitrile as an electron-rich organic modifier, suppresses the $\pi-\pi$ interaction between the solute and the aromatic $\pi-\pi$ active moiety of the stationary phase through formation of electron donor-acceptor complexes with either the aromatic ligand or the solute, depending on whether the one or the other is the more electron-deficient counterpart. Under equal isoelutotropic strength for RP phases, a switch of the modifier from methanol to acetonitrile is expected to cause a strong decrease in retention for aromatic or multi-double bond solutes on π -RP-type phases. Increased retention can be achieved through an increased water content in the acetonitrile-containing mobile phase, which ought to reduce $\pi-\pi$ interactions between the π -electron systems of the organic modifier, the stationary phase and the solute, and encourage thereby the hydrophobic counterpart in this mixed mode retention mechanism [14,36,37].

Non-functional planar and non-planar polyaromatic hydrocarbons are commonly employed test solutes to demonstrate the presence of $\pi-\pi$ interaction, since they only allow hydrophobic and $\pi-\pi$ -type interactions, without further interaction possibilities [17,18,23,38]. Since shape selective separation is based on the arrangement of the molecular sub-units of the solute, π -RP phases should also be sensitive to the overall number as well as to the accessibility of interacting π -electrons within the solute molecule [14].

Another interesting feature is the enhanced retention of unsaturated compounds on polar embedded phases compared to standard *n*-alkyl-type RP-columns [5]. The inverse elution order for saturated and unsaturated solutes on shielded phases was explained by a slight π - π interaction between the carbonyl group of the shielding functionality, which acts as a weak π -electron source and the double bond of the solute. Similarly, carbonyl- π interactions were taken to account for the increased selectivity in the separation of *para*- and *meta*-terphenyl isomers on carboxylate shielded phases [32].

Recently, new hybrid RP-type phases with aryl groups as π - π interaction sites gained popularity [17,18,20,23]. Among those Nakashima et al investigated N-substituted aminopropyl modified stationary phases [39–41] with π - π active aromatic moieties, such as the 3-(1,8-naphthalimido)propyl modified silica gel (NAIP) [42,43]. Note that all described phases are immobilized in a brush-type manner onto the silica support.

In the present contribution the properties of three new hybrid naphthyl imide-type π -RP stationary phases (Fig. 1) are presented and their hydrophobicity, residual silanol ac-

tivity as well as their π - π interaction properties are systematically elucidated. Two of the three π -RP phases enclose a mono-imidic selector attached to alkyl chain spacers of differing length. The third π -RP phase carries a di-imidic selector that is bound to the silica surface, presumably in a loop-like fashion, allowing thereby face-to-face and face-to-edge π - π aromat stacking interactions, similar to the multi-legged bonded phases described by Saito et al. [44].

The aim of the present work was to investigate these novel π -RP-type stationary phases in terms of chromatographic behavior and selectivity in comparison to monomeric and polymeric C18-RP phases as well as to sulfur bearing RP-columns (S-RP) [52]. For this purpose we employed the chromatographic column tests described by Engelhardt [45–47] and Tanaka [13,48] as well as additional test mixtures containing polyaromatic hydrocarbons, functional isomers and geometrical isomers. Hence we tried to verify and elucidate the underlying retention mechanism for π - π active phases as well as to probe their scope of applicability.

2. Experimental

2.1. Chemicals

1,8-Naphthalene dicarboxylic anhydride and toluene were from ACROS ORGANICS (Merck, Vienna, Austria).

Allylamine, anthracene, benz[a]anthracene, benzene, biphenyl, butylbenzene, caffeine, chrysene, *N,N*-dimethylaniline, ethylbenzene, hydroquinone, naphthacene, naphthalene, 1,4,5,8-naphthalene tetracarboxylic dianhydride, *o*-nitrophenol, *p*-nitrophenol, pentylbenzene, phenanthrene, resveratrol, *cis*-stilbene, *trans*-stilbene, *cis*-stilbeneoxide, *trans*-stilbeneoxide, *o*-terphenyl, *m*-terphenyl, *p*-terphenyl, 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).

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

α,α -Azobutyronitrile, phenol, ethyl benzoate and resorcinol as well as HPLC-grade methanol, acetonitrile and chloroform were from Merck (Vienna, Austria).

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 μ L were injected at a flow-rate of 1 mL/min and an oven tem-

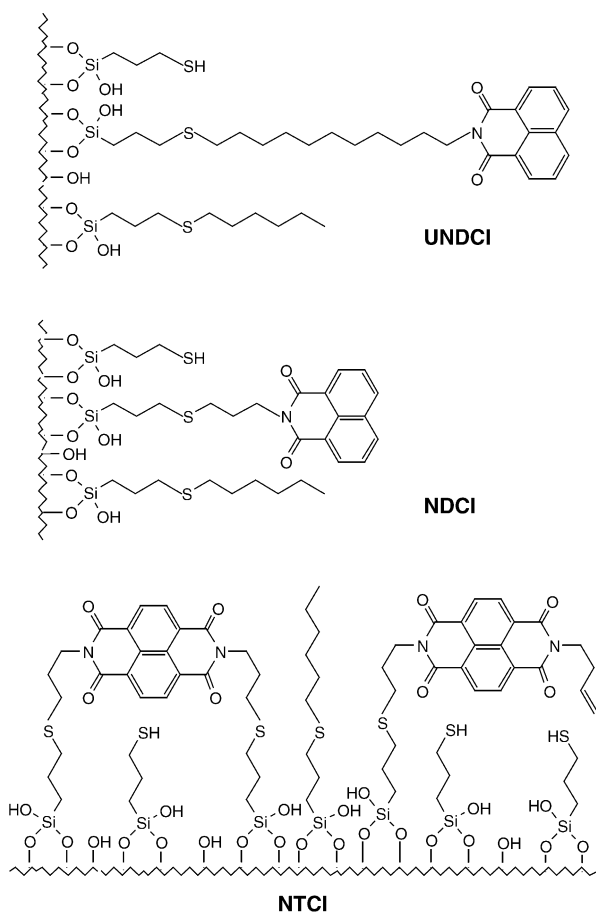


Fig. 1. Chemical structures of investigated silica based naphthalene imide-type stationary phases possessing *p*-RP-type retention behavior as well as S-RP-type stationary phases with C8 and C18 *n*-alkyl chains bound to the same 3-propylthiol silica gel support.

perature of 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 π -RP-type stationary phases and columns

N-(2-propenyl)-1,8-naphthalene dicarboxylic imide (C3–NDCI) and *N,N'*-bis (2-propenyl)-1,4,5,8-naphthalene tetracarboxylic diimide (C3–NTCI–C3) were synthesised through reaction of the corresponding mono- and di-anhydrides with allylamine as described in literature [49]. *N*-(10-undecenyl)-1,8-naphthalene dicarboxylic imide (C11–NDCI) was obtained through reaction of 10-undecenylamine [50,51] with 1,8-naphthalene dicarboxylic anhydride in chloroform employing a twenty percent excess of amine. Further purifications were performed for C3–NDCI through re-crystallization in methanol, for C3–NTCI–C3 through re-crystallization in chloroform and in case of C11–NDCI through flash-chromatography on silica using petrol ether:ethyl acetate 4:1 (v/v) as eluent. The outcome of synthesis and purification were verified and affirmed via NMR.

The obtained mono- and bis-alkenyl imides were immobilized onto 3-propylthiol silica gel (KROMASIL 100, particle size: 5 μm , pore diameter: 100 \AA , surface area: 314 m^2/g , 3-propylthiol-loading: 2.7 $\mu\text{mol}/\text{m}^2$, from AKZO NOBEL, Bohus, Switzerland through free radical addition in chloroform under nitrogen atmosphere, employing α,α -azobutyronitrile (AIBN) as a radical initiator. The end-capping of unreacted thiol groups was performed with *n*-hexene and AIBN as earlier described for the ligand molecules. The washing and drying procedure for the modified silica gels was consistent to the previously described protocol in reference [52].

For convenience, the selector abbreviations NDCI for C3–S–C3–NDCI, UNDCI for C3–S–C11–NDCI and NTCI for C3–S–C3–NTCI–C3–S–C3 are used as synonyms for the bonded π -RP stationary phases. The reproducibility of the immobilization reaction of the three π -RP selector types onto 3-mercaptopropyl silica gel are as follows: NDCI (1.90 $\mu\text{mol}/\text{m}^2$ with 0.08 R.S.D., $n = 3$); UNDCI (1.85 $\mu\text{mol}/\text{m}^2$ with 0.04 R.S.D., $n = 3$); NTCI (1.01 $\mu\text{mol}/\text{m}^2$ with 0.10 R.S.D., $n = 3$).

The elemental analysis of the modified silica materials showed the following results: NDCI silica gel (C: 13.49; H: 1.46; N: 0.76; S: 2.45%; NDCI coverage: 1.73 $\mu\text{mol}/\text{m}^2$ || C: 13.94; H: 1.56; N: 0.77; S: 2.41%; *n*-hexyl coverage: 0.20 $\mu\text{mol}/\text{m}^2$), UNDCI silica gel (C: 17.12; H: 2.16; N: 0.78; S: 2.37%; UNDCI coverage: 1.77 $\mu\text{mol}/\text{m}^2$ || C: 17.47; H: 2.22; N: 0.79; S: 2.35%; *n*-hexyl coverage: 0.16 $\mu\text{mol}/\text{m}^2$) and NTCI silica gel (C: 11.58; H: 1.34; N: 0.95; S: 2.56%; NTCI coverage: 1.08 $\mu\text{mol}/\text{m}^2$ || C: 12.19; H: 1.45; N: 0.95; S: 2.43%; *n*-hexyl coverage: 0.27 $\mu\text{mol}/\text{m}^2$).

All three naphthalene imide π -RP-type phases were packed in stainless steel columns of the dimension 150 mm \times 4 mm by the Austrian Research Center Seibersdorf. For comparison, a monomeric RP-C18 column Inertsil-C18 from GL Sciences Inc., Japan with the column dimension 150 mm \times 3 mm (particle size: 5 μm ; pore size: 100 \AA ; pore volume: 1.05 mL/g; surface area: 450 m^2/g) as well as a polymeric RP-C18 column Hypersil-green-PAH from Thermo Electron Corporation with the column dimension 150 \times 4 mm (particle size: 5 μm ; pore size: 120 \AA ; surface area: 170 m^2/g) were employed. Prior to use, all π -RP-columns were rinsed excessively with toluene to remove eventually remaining unbound selector molecules. If not in use, all columns were stored in methanol.

3. Results and discussion

3.1. Characterization of naphthalene imide-type hplc phases

The proposed structures of all three investigated silica based naphthalene imide-type π -RP phases are displayed in Fig. 1, their ligand loading as well as their number of residual thiol and silanol groups are summarized in Table 1.

As for the bonding density, the result of the elemental analysis provided practically the same surface coverage for the NDCI-material (1.73 $\mu\text{mol}/\text{m}^2$) as well as for the UNDCI-material (1.77 $\mu\text{mol}/\text{m}^2$). Surprisingly, the naphthalene diimide selector NTCI provided a surface coverage of only 1.08 $\mu\text{mol}/\text{m}^2$, though the immobilization reaction was performed in a five-fold excess of the selector. Due to the high molar excess of ligand in solution and the presence of two binding sites per molecule, the predominance for mono-diimidic binding was expected. Nevertheless, the result of the elementary analysis indicates a bis-diimidic binding scenario. Considering the fact that in the first step only one allyl group of the C3–NTCI–C3 selector is bound to the silica surface, positioning the second allyl group in closer proximity to other thiol groups at the surface than any remaining selector molecule in solution, a bis-diimidic binding seems possible.

This hypothesis was further confirmed by the elemental analysis for the endcapping of unbound thiol-groups with *n*-hexene. While the NDCI- and the UNDCI-phase provided the same total thiol-coverage of 1.93 $\mu\text{mol}/\text{m}^2$ with 0.77 $\mu\text{mol}/\text{m}^2$ residual thiol groups, the NTCI-material had a total thiol-coverage of only 1.35 $\mu\text{mol}/\text{m}^2$ with 1.25 $\mu\text{mol}/\text{m}^2$ residual thiol groups for a mono-imidic binding scenario (Table 1).

From the calculated surface coverage of 2.6 $\mu\text{mol}/\text{m}^2$ for 3-propylthiol silica, a mean distance between the immobilized thiol groups of about 8 \AA was calculated. In comparison, the NTCI-molecules are placed in a distance of approximately 12 \AA to each other. Considering that the rigid part of the NTCI selector molecule, the naphthalene

Table 1
Summary of investigated RP and hybrid π -RP columns plus their ligand loading and amount of residual thiol groups and silanol groups

Stationary phase ^a	Ligand ^b (Å)	Column labeling	Ligand-loading ($\mu\text{mol}/\text{m}^2$)	<i>n</i> -Hexene ($\mu\text{mol}/\text{m}^2$)	Free SH ($\mu\text{mol}/\text{m}^2$)	Free OH ^c ($\mu\text{mol}/\text{m}^2$)
C18 (polymeric)	32	Hypersil green PAH	3.20	–	–	e.c.
C18 (monomeric)	32	Inertsil ODS-3	1.54	–	–	e.c.
C3–S–C8/C3–SH/OH	23	C3–S–C8/SH	2.06	–	0.54	5.4
C3–S–C18/C3–SH/OH	38	C3–S–C18/SH	1.56	–	0.84	5.4
C3–S–C11–NDCI ^d	37	UNDCI	1.77	0.16	0.67	5.4
C3–S–C3–NDCI ^d	24	NDCI	1.73	0.20	0.67	5.4
C3–S–C3–NTCI–C3 (mono) ^d	32	NTCI	1.08	0.27	1.25	5.4
C3–S–C3–NTCI–C3–S–C3 (bis) ^d	44	NTCI	1.08	0.27	0.17	5.4

^a 3-Propylthiol loading: 2.6 $\mu\text{mol}/\text{m}^2$.

^b Maximum ligand length in the outstretching form, irrespective of bonding angles and bendings, but including siloxane and 3-propylthiol groups.

^c Maximum number of surface silanol groups: 8 $\mu\text{mol}/\text{m}^2$.

^d Also present: /C3–C₆/C3–SH/OH; “mono” and “bis” resemble the different calculation methods for the two binding-scenarios for the NTCI-phase.

diimide has a length of about 9–10 Å and each of the two freely movable allyl imide chains have a length of approximately 4.5 Å, the total length of a mono-bound NTCI selector would be about 30 Å.

With this in mind, every mono-bound NTCI-selector could bind its second allyl group spacer to the surface, until the unbound thiol-groups become inaccessible through sterical hindrance.

Moreover, the two electron withdrawing imide groups of the NTCI selector provide a strongly electron deficient naphthyl ring, making it susceptible to electron donor–acceptor interactions with the underlying 3-propylthiol groups of the silica gel [52]. A possible sulfur-aromatic interaction between residual thiol groups and the aromatic moiety of the mono-bound NTCI phase may force the aromatic ligand into a flat position, parallel to the silica surface and encourage thereby a covalent binding of the free allyl group to a neighboring thiol group. However, if free allyl-groups were to be present than they ought to bind to *n*-hexylthiol, which was not observed in our control experiment. It was also experimentally confirmed that a radical initiated polymerization of the NTCI-ligand could be excluded for the given calculations in Table 1.

This binding scenario leads to the assumption that the NTCI selector would be aligned parallel to the silica surface, thus enabling direct face-to-face and partial face-to-edge π – π interactions with aromatic solutes.

In contrast the two mono-imidic ligand types UNDCI and NDCI are stacked, but still in a brush-type fashion, having the aromatic moieties of the ligand chains most probably facing each other. These mono-imidic phases should therefore be prone to intercalation-type binding scenarios taking advantage of a possibly two-sided face-to-face interaction.

3.2. Chromatographic test methods

The following chapters will provide the results of some chromatographic column tests, which were already earlier discussed in literature [52] and will not be described in this context.

Although the column tests, designed by Engelhardt and Tanaka were originally developed to evaluate hydrophobic, silanophilic and shape discriminative properties of *n*-alkyl based RP-HPLC-columns, they may also be applicable to other column types. However, it can be expected that the complexity of information gained by such a large variety of test solutes will accelerate with increasing molecular interaction diversity of the novel phase.

Judged by the chemical structure of the investigated naphthalene imide-type stationary phases (Fig. 1), they ought to possess up to a certain extent reversed-phase characteristics combined with hydrogen bonding and more or less distinct π – π -type interaction properties. The π – π activity of the π -RP phases may comprise aromatic π – π stacking, carbonyl- π and sulfur-aromatic interactions [52]. While the hydrophobicity decreases from C18, UNDCI, NDCI to NTCI, the π – π activity of the tested phases increases in the same order.

A possible contribution of the sulfur atom from the underlying 3-propylthiol silica backbone [52] to the overall retention behavior of the π -RP phase can not be neglected and has to be considered. Note that the total number of bonds and therefore also the theoretical maximum length of the entire, fully extended ligands (Table 1) are approximately the same for C3–S–C3–NDCI (13 bonds) and C3–S–C8 (12 bonds) as well as for C3–S–C11–NDCI (21 bonds) and C3–S–C18 (22 bonds).

Furthermore the determined “silanol activity” ought to comprise the effect of the remaining surface silanol groups as well as the residual thiol groups. In case of the Tanaka test 2, the silanol activity is measured as a function of the hydrogen bonding capacity of the phase, which may also include hydrogen bonding from the carbonyl-functional imide groups of the here presented naphthalene imide-type π -RP phases.

3.2.1. Engelhardt test

Fig. 2 provides the chromatograms of the Engelhardt test specified by some relevant separation factors.

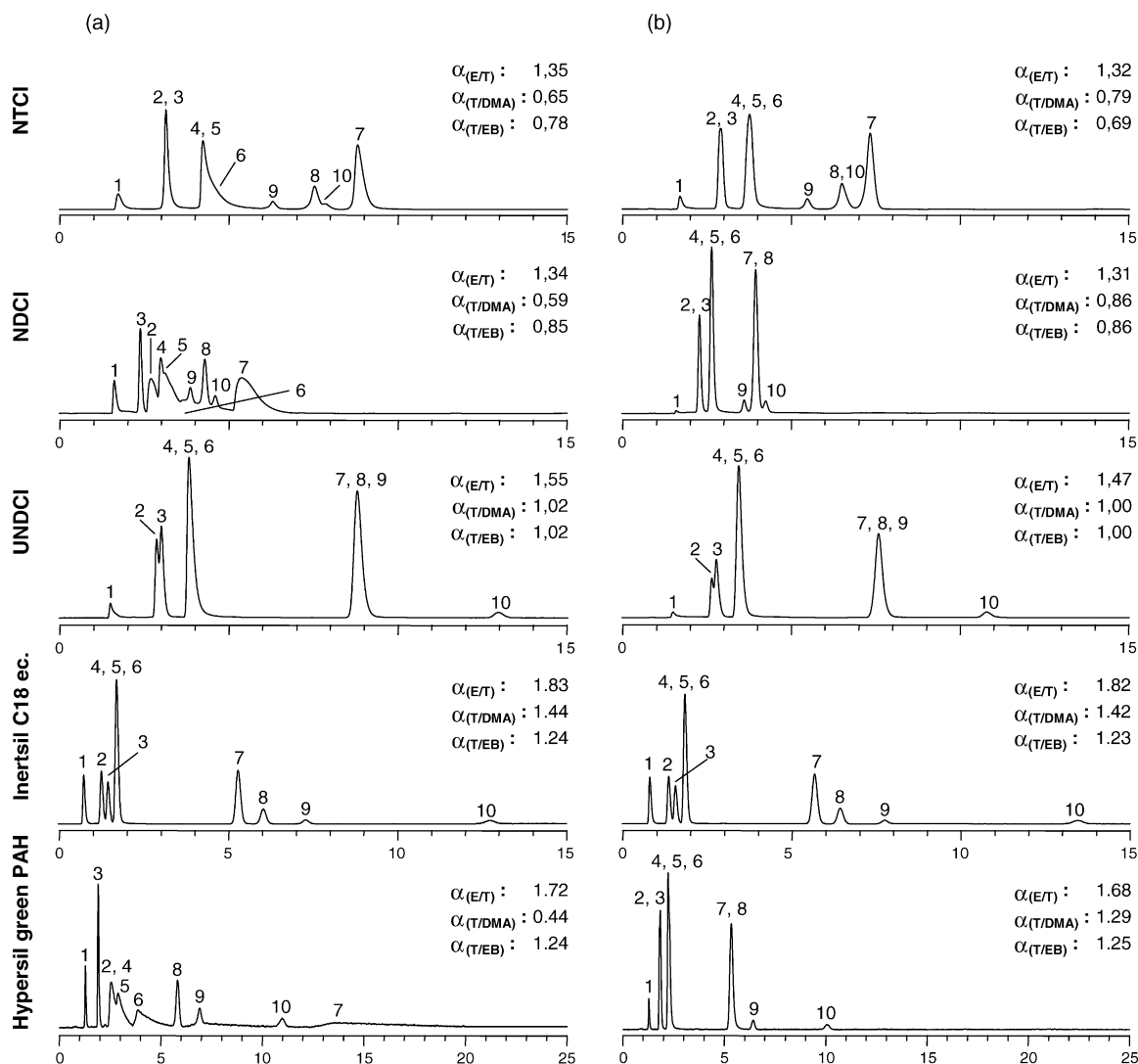


Fig. 2. Separation of the Engelhardt test set containing thiourea (1), aniline (2), phenol (3), *o*-toluidine (4), *m*-toluidine (5), *p*-toluidine (6), *N,N*-dimethylaniline (7), ethylbenzoate (8), toluene (9) and ethylbenzene (10), including the separation factors $\alpha_{(E/T)}$ for toluene (T) and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

It is apparent that the separation factor $\alpha_{(E/T)}$ for ethylbenzene (E) and toluene (T) decreases from the C18 column to the UNDCI and the NDCI-column. Note that the hydrophobicity of a reversed-phase material is directly correlated with the surface coverage as well as the ligand length of the selector, not considering in this context any solvation effects or conformational variations of the *n*-alkyl ligands in the stationary bulk phase. The surface coverage of the NDCI and the UNDCI-phase is about the same, but UNDCI is by nine ligand bonds longer than NDCI. Accordingly, the UNDCI-phase has the highest reversed-phase character of all three π -RP phases, which corresponds to its slightly higher $\alpha_{(E/T)}$ values compared to the other two π -RP phases in Fig. 2.

Surprisingly, the NDCI- and the NTCI-column provide the same $\alpha_{(E/T)}$ -values and possess therefore the same overall hydrophobicity, although the ligand density

of the NTCI-material is only two-thirds of that of the NDCI-material.

For the non-silanol-encapped π -RP columns, high silanol interaction properties were expected. The highest silanol activity was found for the NDCI phase. Besides a very strong peak tailing for all basic solutes and the separation of all three toluidine isomers, also a severe change in the elution order for all compounds was observed. Due to the relatively short spacer chain, the silanol groups on the NDCI-phase seem to be more accessible for interaction with the amino functional solute molecules and ethylbenzoate. In fact, the retention order of ethylbenzoate (EB) and toluene (T) can be used as a test to differentiate between C8 and C18 reversed-phase materials [46].

Although the NTCI-material had the same spacer length as the latter and a rather low surface coverage of 1.08 $\mu\text{mol}/\text{m}^2$, compared to NDCI and UNDCI (see Table 1), its silanophilic

activity was in between both mono naphthalene imide selectors. The peak shapes of the basic solutes were more symmetrical and, aniline and phenol co-eluted. It seems that less silanol groups are accessible for interaction. This result in combination with the same $\alpha(E/T)$ for NTCI as well as for NDCI supports the assumption that the NTCI–ligand may be bound in a loop like fashion to the surface.

In case of the UNDCI-selector, with its long alkyl chain spacer and its π -active domain at the chain end, less solute molecules may reach the silica surface and interact with the free silanol groups. Due to the high hydrophobicity of the long alkyl-chain, the UNDCI-material has relatively the lowest π – π activity of the three selector types. Therefore, the chromatograms of the UNDCI-column in Fig. 2(a) and (b) become similar to those of *n*-alkyl-type C18 columns [52], concerning peak shape as well as elution order. Surprisingly, the brand-new polymeric Hypersil-green-PAH-phase showed a severe peak-tailing for basic solutes, although it was described by the column-suppliers as being silanol-encapped.

An interesting finding of the Engelhardt test concerns the elution order of *N,N*-dimethylaniline (DMA) and ethylbenzoate (EB) and toluene (T). The *N,N*-dimethylaniline peak was the last eluting peak on the NDCI and the NTCI column in methanol–water, which could be explained by a high silanophilic interaction. But employing a buffered eluent that suppresses silanol activity, *N,N*-dimethylaniline is expected to elute before ethylbenzene. This was observed for the NDCI-column, but not for the NTCI-column, where *N,N*-dimethylaniline remains the last eluting peak. Considering that the strongest electron donor–acceptor interaction can be expected between the electron deficient NTCI-selector and the highly electron-rich *N,N*-dimethylaniline, the retention order of *N,N*-dimethylaniline and toluene may be used as an indicator for π – π interaction.

Also ethylbenzoate (EB) shows increased retention times on π – π active phases and even provide approximately the same separation factors of $\alpha = 1$ with toluene as *N,N*-dimethylaniline. The origin of the increased retention of ethylbenzoate lies probably in the polarization of the molecule due to the electron withdrawing ethylcarboxylate-group, which facilitates again a π – π stacking interaction with the polarized naphthalene diimide group of the π -RP-phase [53–55]. Therefore, both separation factors $\alpha(T/DMA)$ as well as $\alpha(T/EB)$ can be seen as possible indicators for pronounced π – π interaction.

3.2.2. Tanaka test

3.2.2.1. Tanaka test 1. As expected, the comparison of the separation factors $\alpha(A/B)$ for amylbenzene (A) and butylbenzene (B) in Fig. 3(a) clearly state the reduction of hydrophobic interactions with decreasing alkyl chain length. The hydrophobicity of the investigated phases decrease in the following order: Hyperil-green-PAH \sim Inertsil C18 e.c. > UNDCI > NDCI \sim NTCI. These results are consis-

tent with the results previously obtained by the Engelhardt test.

In the preliminary study in [52] it was already shown that the sulfur atom within the *n*-alkyl chain has a selectivity enhancing effect, which is illustrated by an increased separation factor $\alpha(T/O)$ for triphenylene (T) and *o*-terphenyl (O). Comparing the three π -RP phases, it becomes clear that shape selectivity increases with increasing π – π activity of the stationary phase and is most pronounced on the diimide NTCI phase, where triphenylene could not be eluted under the standard test conditions. Only with acetonitrile, a modifier that disrupts π – π interactions, triphenylene could be removed from the NTCI phase. As a result, shape selectivity increases gradually with increasing *n*-alkyl chain length of the spacer and increasing electron donor–acceptor interaction properties of the stationary phase, which are in the order of increased π – π interaction activity, S–aromatic, carbonyl– π and π – π aromatic stacking interactions.

An interesting finding of the Tanaka test concerns the reverse elution order of amyl benzene (A) and *o*-terphenyl (O) on π – π active phases. The separation factor $\alpha(A/O)$ is for *n*-alkyl-type RP-C18 materials approximately one or higher than one. For phases with increasing pre-dominance of the π – π interaction term, the more aromatic *o*-terphenyl is being stronger retained than amylbenzene, providing $\alpha(A/O)$ values below 1. Since $\alpha(A/O)$ decreases gradually in the order of increasing π – π activity, it can be seen as a possible indicator for the π – π activity of a hybrid π -RP-type stationary phase.

3.2.2.2. Tanaka test 2. Fig. 3(b) show the results of the Tanaka test 2. The intensity of the hydrogen bonding interaction is measured by the separation factor $\alpha(P/C)$ for phenol (P) and caffeine (C).

Generally short ligands provide increased accessibility to silanol as well as thiol groups. Therefore short chain phases e.g. the NDCI- as well as the NTCI-phase exhibit stronger hydrogen bonding properties than the corresponding long chain phases such as the UNDCI-phase, providing they possess similar ligand densities. However, the carbonyl groups of the naphthalimido moiety of the π -RP ligand, resemble additional hydrogen bond-acceptor sites and may contribute thereby to the strong retention of phenol on the three π -RP phases in Fig. 3(b). Nevertheless, the strong retention of caffeine can not be fully explained by hydrogen-bonding interaction with silanol-groups, especially since the ion exchange properties (see Tanaka test 3 and Tanaka test 4) of the investigated hybrid RP phases are too little to be responsible for such a strong effect. It is more likely that the strong π – π interaction properties of the hybrid π -RP phases are the origin.

In this context and as previously observed for the Engelhardt test, it is rather difficult to extract a possible contribution of an underlying π – π -type interaction, from a concerted interaction and retention mechanism implying hydrogen bonding, π – π and hydrophobic interaction.

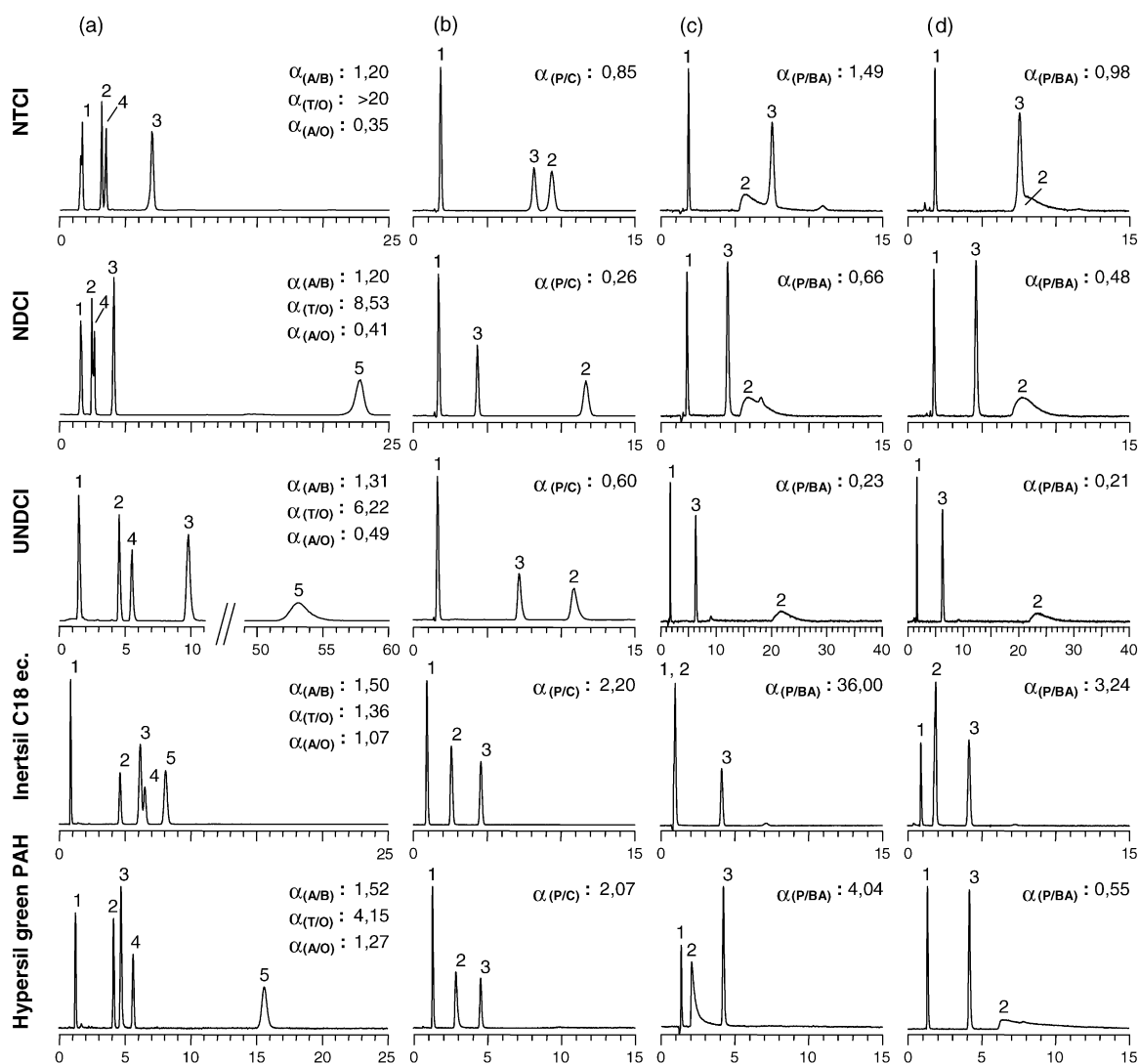


Fig. 3. (a) Separation of the Tanaka 1 test set containing uracil (1), butylbenzene (2), *o*-terphenyl (3), amylbenzene (4) and triphenylene (5), including the separation factors $\alpha(A/B)$ for butylbenzene (B) and amylbenzene (A), $\alpha(T/O)$ for triphenylene (T) and *o*-terphenyl (O) and $\alpha(A/O)$ for amylbenzene (A) and *o*-terphenyl (O) in methanol–water 632:200 (w/w)—1 mL/min, 30 °C, 254nm; (b) separation of the Tanaka 2 test set containing uracil (1), caffeine (2) and phenol (3), including separation factor $\alpha(P/C)$ for caffeine (C) and phenol (P) in methanol–water 237:700 (w/w)—1 mL/min, 30 °C, 254 nm; (c) and (d) Separation of the Tanaka 3 test and Tanaka 4 test, both containing uracil (1), benzylamine (2) and phenol (3), including the separation factor $\alpha(P/BA)$ 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.

The strong retention of phenol can be easily explained by the combination of π – π interaction and increased hydrophobicity due to the longer hydrophobic spacer.

3.2.2.3. Tanaka test 3 and Tanaka test 4. The results of the Tanaka test 3 and the Tanaka test 4 are displayed in Fig. 3(c) and (d). The different pH conditions of the two Tanaka tests enable the determination of the hydrogen-bond interaction properties (pH 2.7) as well as the ion-exchange properties (pH 7.6) of RP-type stationary phases. The test compounds are benzylamine (Ba) and phenol (P). While benzylamine (pK_a 9.4) is ionized and interact differently at the two mobile phase pH with the different types of silanols (pK_a 1–10), phenol is used as a reference, since it provides consistent retention times under both pH conditions.

However, comparing similar phases with differing *n*-alkyl chain length, the overall retention of phenol is only consistent on *n*-alkyl-type RP phases. On the S-RP phases in [52] as well as the here described π -RP phases, phenol seems to observe a stronger retention, which may be facilitated by additional hydrogen-bonding as well as sulfur-aromatic and π – π interaction. The absolute values of the separation factor $\alpha(P/Ba)$ for the two test solutes may be employed to compare the silanol/thiol-activity of similar *n*-alkyl-based RP phases, however, a direct comparison between different types of hybrid RP phases with more complex interaction properties seems very difficult.

Although the two short-chain π -RP phases NDCI and NTCI exhibit the same hydrophobicity ($\alpha(A/B)$ 1.20), phenol is retained stronger on the NTCI-phase, while benzyl-

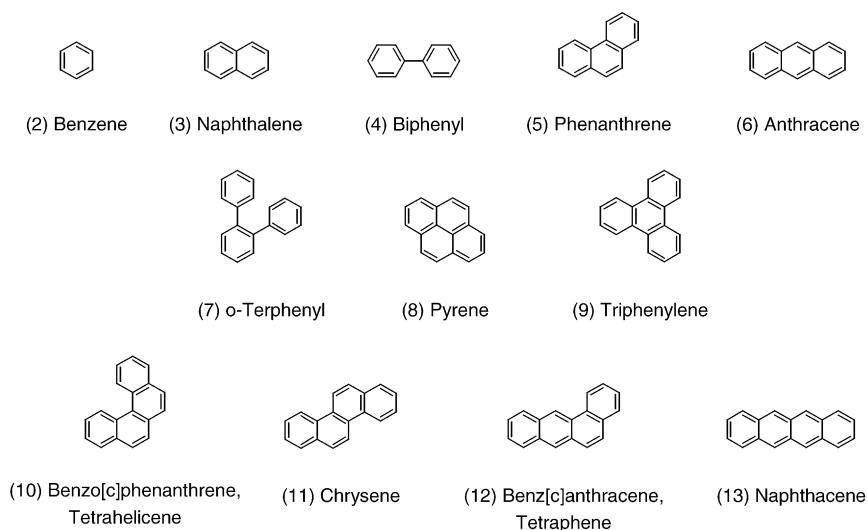


Fig. 4. Chemical structure of the investigated polyaromatic hydrocarbons.

amine retains the same on both phases. The corresponding chromatograms for the UNDCI-phase shows a very strong retention of benzylamine, while phenol is only slightly less retained compared to the NTCI-phase. Taking into account that the Engelhardt test of the UNDCI-phase shows only little interaction with weak bases (pK_a 4.4–5.1) this strong retention of benzylamin is rather surprising. Also the benzylamine-peak is broad but does not show the expected tailing, as observed for the C3–S–C18/SH phase in [52].

It seems that the increased spacer-length as well as the brush-like alignment of the UNDCI ligands may induce the formation of cavities within the phase. These imaginary cavities are facilitated with the 3-propylthiol- and/or silanol groups at the base, the *n*-alkyl chains at the flanks and with the naphthalimide carbonyl-groups at the orifice. The stronger retention of benzylamine on the UNDCI-phase compared to the short-chain NDCI-phase may be the result of a combined interplay of these diverse interaction properties, of which the dominating interaction is not definable.

3.3. Polycyclic aromatic hydrocarbons (PAHs)

The same two test assemblies of polyaromatic solutes will be investigated as previously employed to describe sulfur–aromatic interactions [52]. Note that these test analytes do not carrying any additional functional groups that might change their intrinsic π – π density or cause any additional interactions other than hydrophobic and electron donor–acceptor interactions (Fig. 4). A summary of all investigated PAHs including their molecular weight, number of π -electrons, length-to-breadth (L/B) values and their thickness is provided in Table 2.

3.3.1. PAH test set 1

The first test set contains eight PAHs of different size, shape and planarity. The smallest aromatic unit is benzene. Although both, naphthalene and biphenyl have two aromatic

benzene rings, naphthalene has only 10 π -electrons and is planar, while biphenyl has 12 π -electrons and is twisted out of plane. Phenanthrene and anthracene only differ in the position of one benzene unit. The four-ring PAHs, pyrene and triphenylene are also both planar, but differ in their number of π -electrons as well as shape. Due to their similar hydrophobic properties but different planarity, triphenylene and *o*-terphenyl are frequently used as a test set to determine the so-called “shape selectivity” of monomeric and polymeric *n*-alkyl-type RP-materials [48,56].

Fig. 5 shows the corresponding chromatograms including the separation factors for the planar/non-planar couples naphthalene (N) – biphenyl (B) and triphenylene (T) – *o*-terphenyl (O) as well as the separation factor of the isomers anthracene (A) and phenanthrene (P).

Hence the planarity of a compound is of major importance because it defines the number of π -electrons that are eventually accessible for π – π interaction with the aromatic domains of the π -RP-phase. For instance, the off-planar structure of biphenyl will allow less than 12 π -electrons to contribute simultaneously to the overall retention. Compar-

Table 2
Summary of investigated PAHs including some of their physical and chemical properties

Compound	MW	No. π -electrons	L/B	Thickness
(2) Benzene	78.1	6	1.099	3.883
(3) Naphthalene	128.2	10	1.238	3.884
(4) Biphenyl	154.2	12	–	–
(5) Phenanthrene	178.2	14	1.463	3.888
(6) Anthracene	178.2	14	1.566	3.882
(7) <i>o</i> -Terphenyl	230.3	18	1.110	–
(8) Pyrene	202.3	16	1.257	3.888
(9) Triphenylene	228.3	18	1.119	4.373
(10) Tetrhelicene	228.3	18	1.277	4.987
(11) Chrysene	228.3	18	1.734	3.922
(12) Tetrphene	228.3	18	1.599	3.887
(13) Naphthacene	228.3	18	1.896	3.885

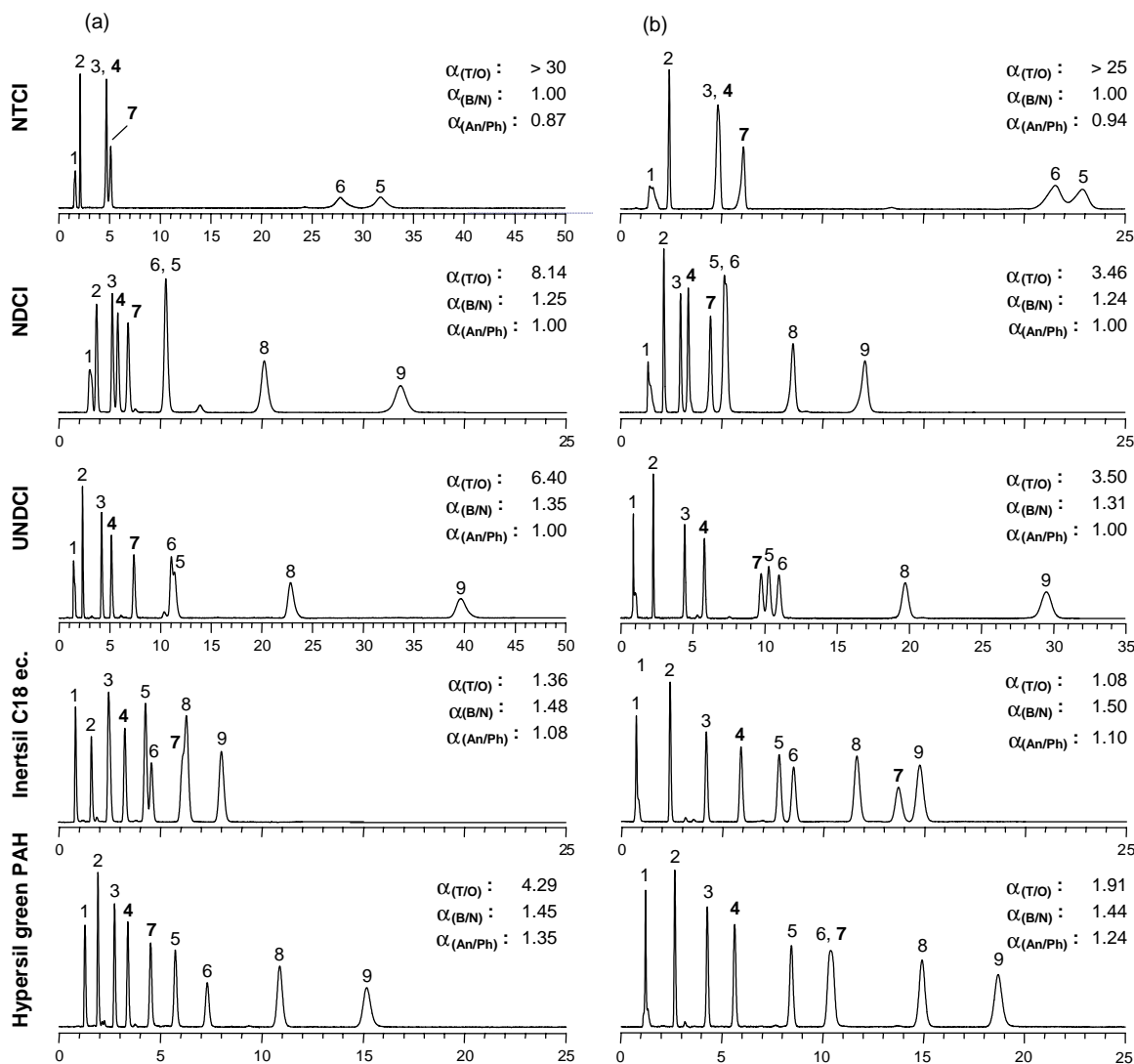


Fig. 5. Separation of PAH test set 1 containing uracil (1), benzene (2), naphthalene (3), biphenyl (4), phenanthrene (5), anthracene (6), *o*-terphenyl (7), pyrene (8) and triphenylene (9), including the separation factors $\alpha(T/O)$ for *o*-terphenyl (O) and triphenylene (T), $\alpha(B/N)$ for naphthalene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

ing the $\alpha(B/N)$ values of the three π -RP phases, it is apparent that $\alpha(B/N)$ decreases as the π - π interaction capacity of the phase increases. In case of the NDCI- and the UNDCI-phase, hydrophobic interaction is more dominant, while for the NTCI-phase π - π interaction is the superior counterpart. The disability of the planar NTCI-phase to distinguish between naphthalene and biphenyl may also lay in their small size. Their spatial areas are smaller than the molecular dimension of the naphthalene-diimide moiety of the NTCI-phase.

In contrast, *o*-terphenyl and triphenylene possess both the same number of π -electrons. However, since the two aryl groups of *ortho*-terphenyl are in very close proximity, a repulsion of their π -electron clouds seems obvious and provides thereby the off-planarity of the molecule in a low energy conformation. The number of π -electrons, which ef-

fectively contribute to retention is thereby reduced from 18 π -electrons to approximately 14 or even less. Consequently, *o*-terphenyl elutes on all three π -RP columns before anthracene and phenanthrene, while on RP- and S-RP phases [52] *o*-terphenyl always elutes after these two three-ring isomers.

As earlier discussed for the Tanaka test 1, the $\alpha(T/O)$ value is a significant measure to describe the shape selective property of a stationary phase. It increases in the order of increasing hydrophobic and π - π interaction properties of the hybrid RP phases: S-RP < Hypersil-green-PAH < UNDCI < NDCI \ll NTCI.

The π - π interaction of the NTCI-phase is so strong that planar PAHs with more than 14 π -electrons such as pyrene or triphenylene did not elute under the standardized methanolic mobile phase conditions, even when employing

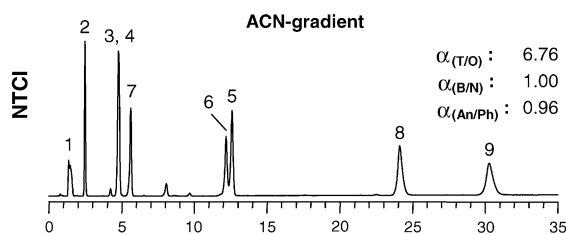


Fig. 6. Separation of the PAH test set 1 on the NTCI-column measured in the gradient mode employing acetonitrile–water 60–100% ACN in 15 min, then 100% ACN for 20 min at a flow rate of 1 mL/min, 30 °C and 260 nm.

pure methanol as eluent. Only with high percentages of acetonitrile, a modifier that suppresses π – π interaction, pyrene and triphenylene could be eluted at acceptable elution times (Fig. 6). Note that acetonitrile only reduces π – π interactions between solute and the aromatic moieties of the ligands, but does not completely eliminate them.

Since the organic modifiers methanol and acetonitrile exhibit different physical and chemical behavior, e.g. solvation and elution strength, their influence on solute retention can be very different on hybrid π -RP phases compared to conventional RP phases. Fig. 5b provides the results for an acetonitrile containing mobile phase with similar isoelutotropic strength as for the mobile phase using methanol in Fig. 5(a). Note that the isoelutotropic strength followed the concept reported in [34] for a C18 RP-phase.

It is apparent that the π – π interaction properties of acetonitrile is of no relevance for *n*-alkyl based RP phases. When extended π -systems are incorporated into the RP-lattice, acetonitrile may reduce π – π interaction between solute and selector through formation of electron donor–acceptor complexes with the π -systems. This leads to a reduced retention of aromatic solutes and provides a reduction of the α (T/O)-values on all three π -RP phases (Fig. 5(b)).

Another interesting finding was the reverse retention order of phenanthrene (peak 5) and anthracene (peak 6) on the investigated π -RP columns compared to the monomeric and polymeric C18-RP phases in Fig. 5 as well as the hybrid S-RP phases in [52]. Although phenanthrene and anthracene were almost co-eluted on the NDCI- and the UNDCI-column, the reversed retention order could be ascertained through individual injections. For the highly π – π active NTCI column the reversed retention order becomes clearly evident. This stands in accordance to the results obtained by Mifune et al. for π – π active metal phthalocyanines [19].

Retention due to π – π interaction has obviously a strong shape discriminative effect that is mainly based on the density and location of π -electrons within the molecular structure of the interacting aromatic species. Among isomers, ideal interaction and therefore highest retention can be expected for planar solutes with rather compact molecular structures, providing thereby small length-to-breadth (L/B) ratios (see later).

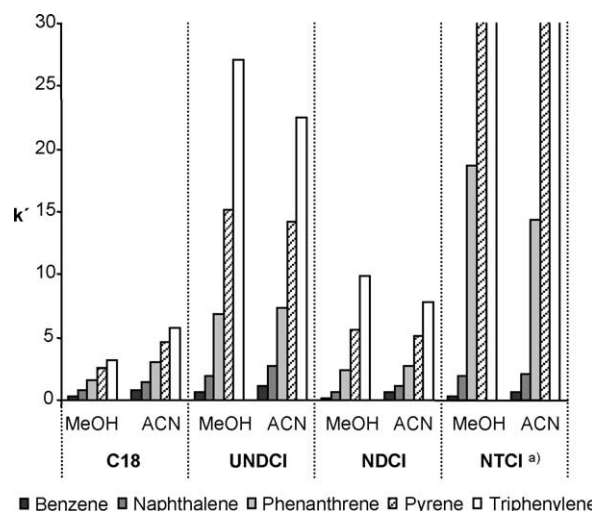


Fig. 7. The retention factors k' of five planar PAHs from Fig. 4 are plotted in order of increasing π -electron number against the investigated column-type and organic modifier used under aqueous isoelutotropic conditions. (a) For the NTCI column no elution was observed for pyrene and triphenylene under investigated mobile phase conditions.

In case of the NDCI-column and the UNDCI-column, the reversed-phase characteristics exhibited by the alkyl chain spacers with their comb-shape arrangement and the π – π interaction properties, serve apparently as opposing interaction properties that influence shape discriminative separation. The subtle balance between these two interaction terms may be responsible for the co-elution of phenanthrene and anthracene on the mentioned phases, but are probably also accountable for the high planar recognition ability, illustrated by the high α (T/O)-values in Fig. 5(a).

Comparing the retention characteristics for the different column types in Fig. 7, it becomes evident that for aromatic solutes an increase in the hydrophobicity of a stationary phase is by far not as effective as the increase in the π – π interaction properties. This is clearly illustrated by the strong, almost exponential increase in retention for large π -electron rich compounds on π -RP phases compared to a more linear increase in retention for a common RP-mode separation.

3.3.2. PAH-test set 2

The PAH test set 2 contains a set of four-ring PAH isomers with a molecular weight of 228. These isomers possess the same number of π -electrons, but differ in their spatial shape. Their L/B-values and their molecular thickness are listed in Table 2.

Fig. 8 illustrates the pronounced “shape selective” separation ability of a polymeric C18-RP phase compared to a monomeric C18-phase and compared to the π -RP phases. Among the π -RP phases, the NTCI-phase provided the best results, concerning the separation efficiency as well as the peak shape. Compared to the polymeric Hypersil-green-PAH column, only tetraphene (12) and naphthacene could not be resolved on the NTCI-phase. The most significant difference between these two phases is

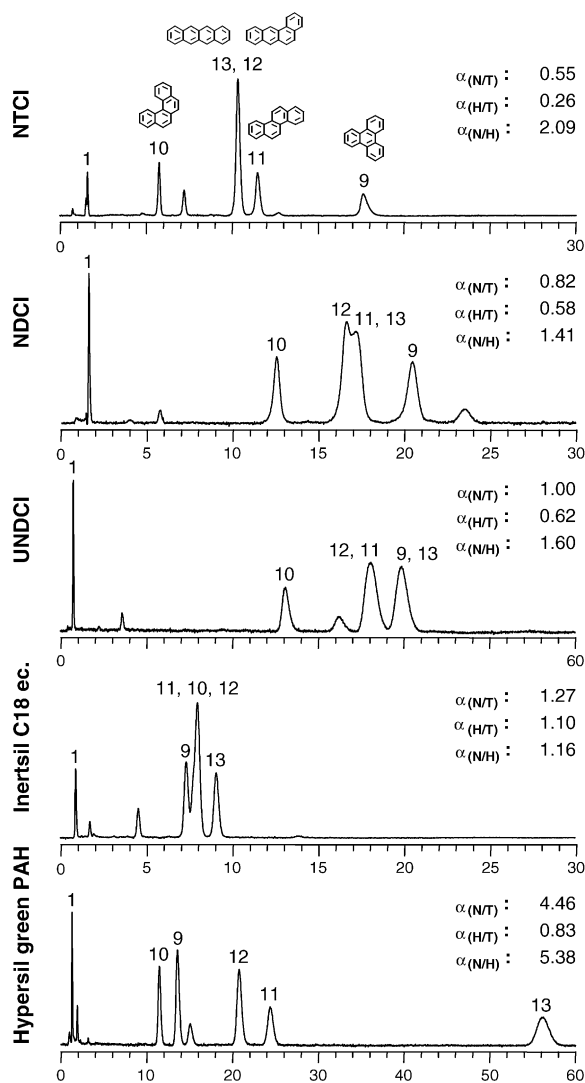


Fig. 8. Separation of the PAH test set 2 containing uracil (1), triphenylene (9), tetrahelicene (10), chrysene (11), tetraphene (12) and naphthacene (13) including the separation factors $\alpha(N/T)$ for naphthacene (N) and triphenylene (T), $\alpha(H/T)$ for tetrahelicene (H) and triphenylene (T) and $\alpha(N/H)$ for naphthacene and tetrahelicene in methanol–water 80:20 (v/v) and 100% acetonitrile (isocratic) for the NTCI-column—1 mL/min, 30 °C, 270 nm.

the reverse elution order of naphthacene and triphenylene. Apparently, the retention of naphthacene (N) is gradually declining while that of triphenylene (T) is increasing with the predominance of π – π interaction properties of the π -RP phases. The separation factor $\alpha(N/T)$ decreases in the order: polymeric C18 > monomeric C18 > UNDCI (=1) > NDCI > NTCI.

It can be concluded that on π -RP phases planar PAHs with condensed ring structures and small L/B-values observe a stronger retention than their linear analogues, which complies to the findings of Sanders et al in [57]. This tendency complies also with the reverse elution order of phenanthrene and anthracene on the NTCI-phase in Fig. 8 compared to the monomeric and polymeric *n*-alkyl-type phases.

Concerning the shape selective properties of polymeric RP phases, solute retention increases with increasing L/B-values [58], planarity and linearity compared to their corresponding non-planar [26] or bulky analogues [28]. This concept explains the stronger retention of phenanthrene and triphenylene compared to their highly linear analogues on *n*-alkyl-type RP phases.

Overall it can be stated that shape selectivity based on hydrophobic interaction and shape discrimination based on π – π interaction are completely different in origin and resemble two opposing parties in the separation of solutes with extended π -electron systems.

3.4. Separation of *cis/trans*-isomers

The following test set comprises the *cis*- and *trans*-isomers of three stilbene-derivatives, namely stilbene, stilbene oxide and resveratrol. Their *cis* and *trans*-isomers differ strongly in shape, providing thereby very differing π – π interaction properties. Furthermore these compounds differ strongly in their overall polarity, which increases from stilbene and stilbene oxide to resveratrol and provide in case of resveratrol and stilbene oxide additional hydrogen bonding sites.

In a general observation, the retention times of the *cis*- and *trans*-isomers increase with increasing π – π activity and increasing spacer length of the π -RP phases. Besides the previously described influence of the π -electron density, which is associated with the planarity of the solute, also the presence as well as the nature of additional functional groups may have a pronounced impact on the overall retention behavior.

The incorporation of imidic carbonyl-groups within the π -RP-type ligands, increases not only the π – π activity, but also increases the overall polarity of the phase and provide potential hydrogen bonding sites for polar compounds.

Due to steric hindrances and repulsion of the π -electron clouds, the two aryl groups of the *cis*-isomers of stilbene and resveratrol are twisted out of plane. In contrast, the non-hindered *trans*-isomers are planar and are therefore more prone to π – π interactions providing a stronger overall retention of *trans* isomers on π -RP phases compared to a conventional reversed-phase.

Thereby, the separation factor $\alpha(T/C)$ for the *cis* (C) and the *trans* (T) isomers depicted in Fig. 9 proved to be a useful measure to illustrate the influence of different stationary phase properties such as hydrophobicity, π – π activity, polarity and/or hydrogen-bond activity on solute–ligand interactions.

Comparing the brush-type ligands NDCI and the UNDCI in Fig. 9, it is evident that an increase in phase hydrophobicity has a stronger effect on the retention of the hydrophobic stilbene-isomers than for the more polar stilbene oxide and resveratrol isomers. In case of the planar aligned NTCI-selector, similar retention times as well as similar separation factors were obtained for the stilbene and resveratrol-isomers, while the stilbene oxide iso-

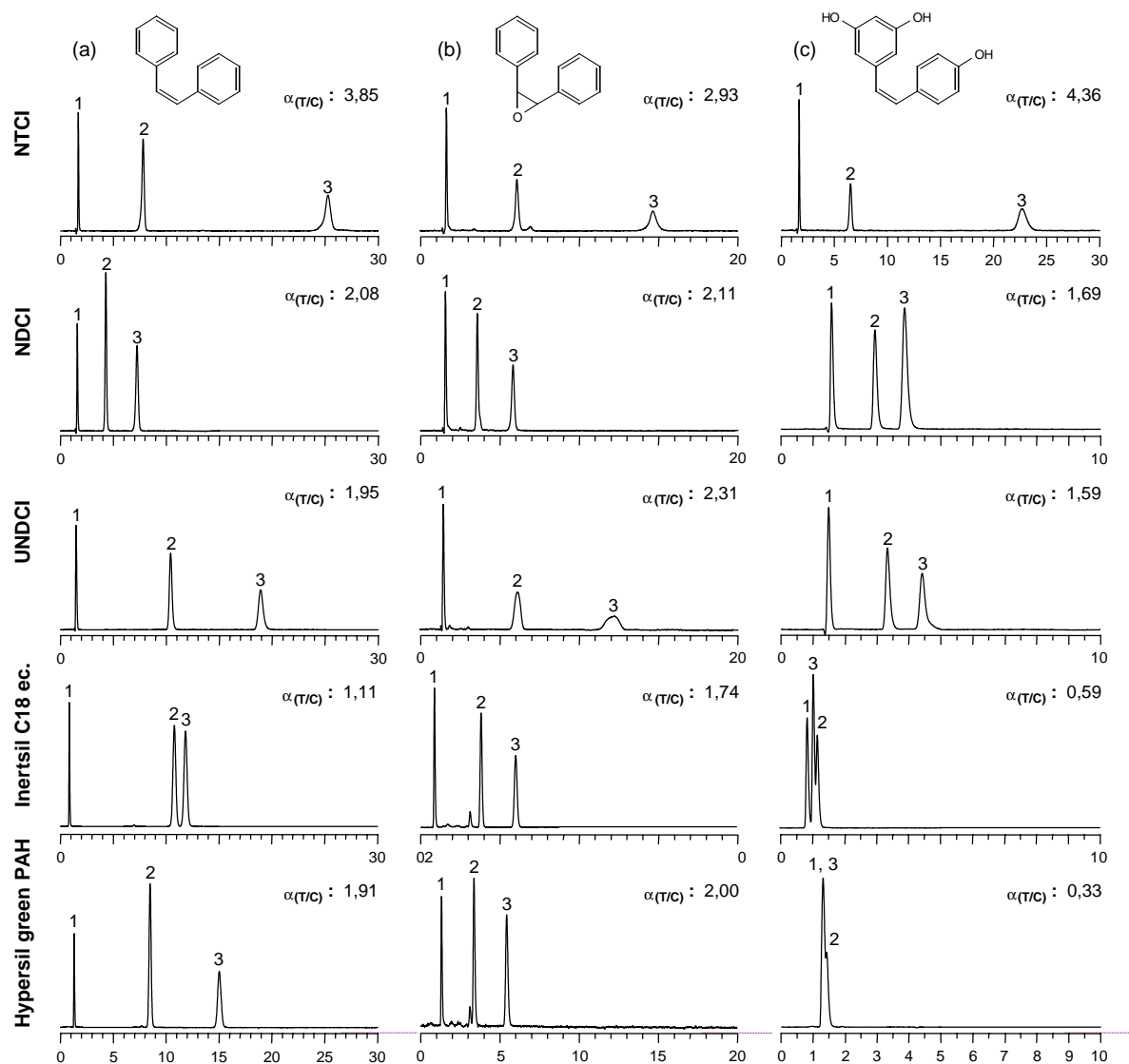


Fig. 9. Separation of *cis*- (2) and *trans*- (3) isomers of stilbene (a), stilbene oxide (b) and resveratrol (c) in methanol–water 70:30 (v/v) at a flow rate of 1 mL/min and a detection wavelength of 280 nm for stilbene and resveratrol and 254 nm for stilbeneoxide; including the separation factors $\alpha(T/C)$ for the *cis*- (C) and the *trans*-isomers (T).

mers provided similar results as obtained for the UNDCI-phase.

It may be proposed that for the brush-like aligned π -RP ligands more energy has to be invested to desolvate the solute molecules and insert them into the hydrophobic ligand lattice, providing thereby reduced retention for increasingly polar solutes, compared to the NTCI phase with its probably predominant face-to-face interaction mode.

Furthermore, the increased retention of the highly polar resveratrol-isomers may be caused by hydrogen bonding interaction with the carbonyl-groups as well as strong π - π interactions with the aromatic moiety of the naphthalimido-type ligands. Accordingly the similar retention characteristics of resveratrol on the two brush-type π -RP phases may suggest that it does not penetrate the UNDCI and NDCI phases as deeply as stilbene, but

prefers to interact mainly with their polar and π - π active domains.

Additionally, the increased polarity of the NDCI and UNDCI phase may also allow a slightly deeper penetration of the *trans*-resveratrol isomer between the π -RP ligands, compared to *n*-alkyl-type RP phases, where *trans*-resveratrol elutes always before its *cis*-analogue [52,59].

A comparison with the polymeric Hypersil-green-PAH column shows the pronounced planar recognition ability of all three π -RP phases, which provide $\alpha(T/C)$ values that are comparable or even higher than those obtained on the polymeric C18-RP-phase (Fig. 9). The major advantage of these π -RP phases is obviously their ability to strongly retain and separate even highly polar aromatic solutes, such as resveratrol, which are difficult to separate on hydrophobic *n*-alkyl-type RP phases.

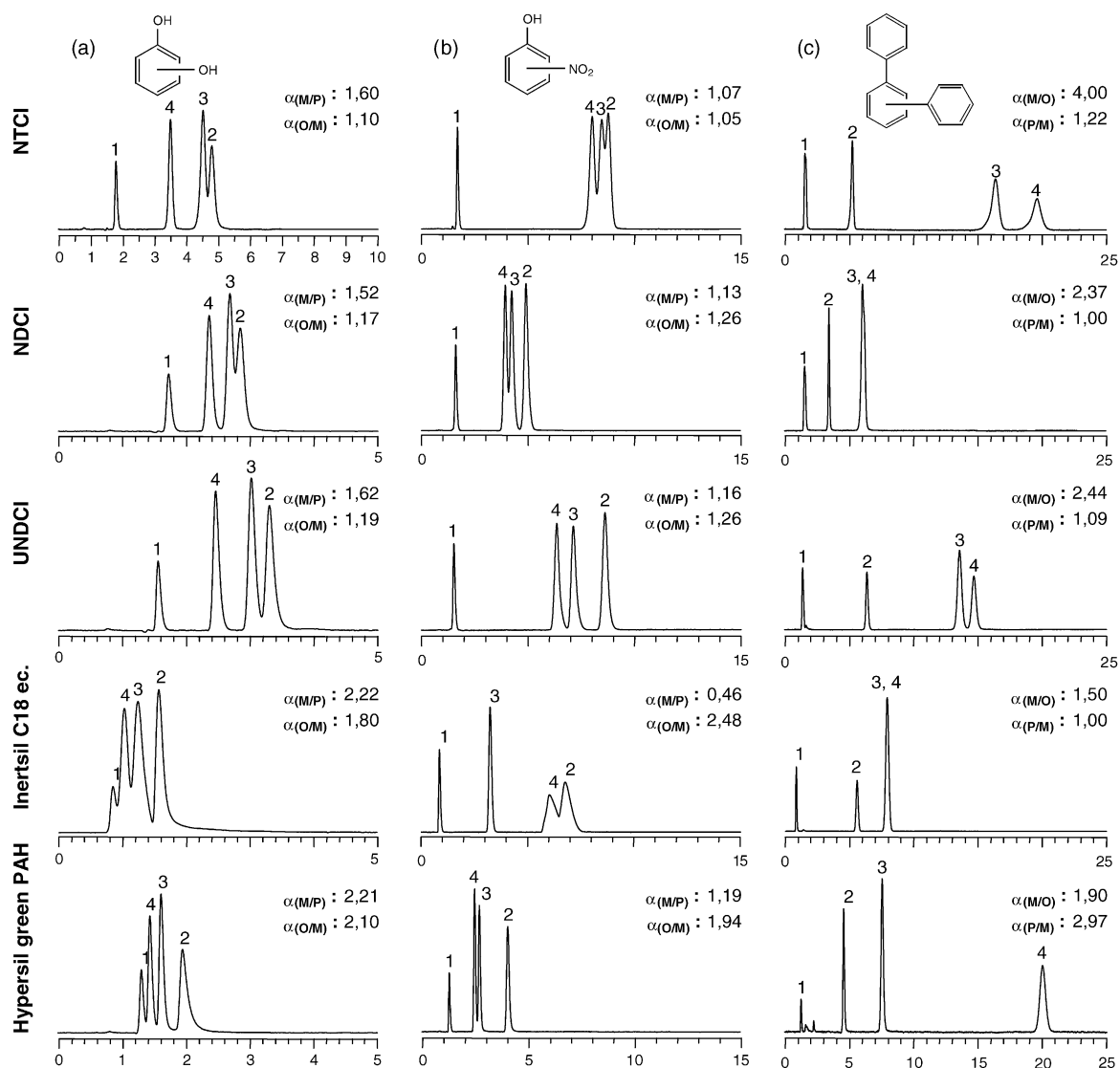


Fig. 10. Separation of the *ortho*- (2), *meta*- (3) and *para*- (4) isomers from (a) dihydroxybenzene in methanol–water 40:60 (v/v)—1 mL/min, 30 °C, 254 nm, (b) nitrophenol in methanol–water 50:50 (v/v)—1 mL/min, 30 °C, 280 nm and (c) terphenyl in methanol–water 80:20 (v/v)—1 mL/min, 30 °C, 254 nm using uracil (1) as the void volume marker.

3.5. Separation of functional and positional isomers

The *ortho*, *meta* and *para* isomers of dihydroxy benzene, nitrophenol and terphenyl were selected as test solutes to investigate the influence of different functional groups placed on an aromatic backbone with moderate π – π interaction capability.

Besides the π – π active aryl-groups of terphenyl also hydroxy-groups as well as nitro-groups are susceptible for electron donor–acceptor interactions, namely OH– π [60] and nitro– π [61,62] interactions. The intensity of their expected π – π activity increases in the order OH– π , nitro– π and π – π aromat stacking interactions.

As shown in Fig. 10, the terphenyl isomers exhibit an overall inverse elution order compared to the hydroxy- and the nitro-functional isomers. Similar to the earlier described

stilbene isomers, steric hindrance of closely positioned aryl groups in *ortho* position leads to an off-planarity of the aromatic systems reducing thereby the number of accessible π -electrons for π – π interaction. Therefore, *o*-terphenyl is least retained on π -RP phases, followed by *m*- and *p*-terphenyl, which elute close to each other and even co-elute in case of the NDCI-phase. The comparison of the chromatograms of the latter two columns as well as the C3–S–C18 and the UNDCI-column reveals the same behavior as obtained for the stilbene derivatives. Namely that the retention times of the *o,m,p*-terphenyl isomers increase with increasing hydrophobicity as well as increasing π – π activity of the investigated stationary phase and decreases with increasing polarity.

Hydroxy as well as nitro groups increase the polarity of the entire compound as well as the difference in po-

larity within the molecule. The rather moderate electron pushing hydroxy groups increases the electron density of the benzene ring in *ortho* and *para* position, while the strong electron pulling nitro group ought to provide an electron deficiency. Considering that the investigated π -RP selectors are rather electron deficient, a decrease in π - π interaction between nitro-containing solute and selector together with reduced elution times may be expected. Nevertheless, compared to the hydroxyphenolic isomers unexpectedly high retention times were observed for the nitrophenols, which could be the consequence of the π - π activity of the nitro-functionality, leading to strong π - π stacking interactions with the naphthalimido-moiety [54,55].

Nevertheless, the *ortho* isomers of the dihydroxybenzene and the nitrophenol compounds elute after the *meta* and *para* positioned isomers, which seems obvious, considering that the *ortho* position provides the highest accessibility for the remaining molecule to interact via hydrophobic as well as π - π aromatic interaction.

4. Conclusion

Attempts were made to interpret the retention characteristics of hybrid π -RP phases with diverse interaction properties employing RP-column tests, which were originally created to describe *n*-alkyl-type reversed phases. In this context we could show that chromatographic column tests such as those from Engelhardt and Tanaka could provide valuable information on molecular interaction and selectivities. This includes aspects concerning the presence and efficiency of hydrophobic, silanophilic as well as electron donor-acceptor interactions.

For the described hybrid RP phases with π - π active domains, the influence of the π - π interaction term on the overall retention behavior is best investigated with aromatic solutes with diversified planarity and π -electron density.

It was found that, for large polyaromatic compounds with extended π -systems, π - π interaction becomes more dominant, while for small aromatic compounds hydrophobic properties are more effective.

The retention of planar and non-planar PAHs on π -RP columns is based on a combination of their ability to penetrate between brush-like aligned *n*-alkyl-based ligands and the number of accessible π -electrons for interaromatic interaction between solute and stationary phase.

The retention behavior exhibited by the multi-legged NTCl-material is obviously strongly dominated by π - π interaction. Evidently, its loop-like construction facilitates a direct face-to-face and face-to-edge π - π stacking interaction between solute and aromatic ligand-domains with less hydrophobic contributions provided by the alkyl chain spacers, compared to the two comb-shape π -RP phases. However, hydrogen-bonding as well as carbonyl- π interaction may also be of some importance.

Concerning analytes with small π -electron systems (e.g. positional and functional isomers of benzene), hydrophobic interactions and polarity differences within and between such isomers are of higher importance to the overall retention and separation behavior than any increase in the π - π interaction properties of a hybrid π -RP-phase. Most probably, their differences in electron density are too little to be detected by the relatively large naphthalimido moieties of the investigated π -RP ligands.

Concerning the test set of stilbene derivatives, an increase in phase-hydrophobicity as well as in solute-polarity leads to overall reduced retention times, while strong π - π properties provide an increase in separation efficiency and even lead to changes in the elution order (e.g. for the isomers of resveratrol). Therefore, polar π -RP phases with a strong π - π interaction capacity are most suitable to retain and separate polar solutes with moderately large π -systems.

It was found that for solutes with extended π -electron systems, already small differences in planarity and shape can provide sufficient separation efficiencies on hybrid π -RP phases with strong electron donor/acceptor properties, which are even comparable to those observed on polymeric RP phases.

The reverse elution order of linear and condensed PAHs on polymeric RP phases compared to π -RP phases provides profound evidence that the underlying separation mechanism for these two phase-types are not only different in nature, but are also contrarily directed for strictly planar isomers. However, non-planar PAHs possessing a big dihedral angle behave similar on both phases.

It may be concluded, that the molecular shape of a solute dictates the effective contact area as well as the accessibility of its functionalities (e.g. functional groups or π -electrons) for interaction with the stationary phase, which of course can be very different in nature (e.g. monomeric, polymeric or multi-legged). Obviously, not only the overall shape of the solute is responsible for molecular recognition and chromatographic selectivity, but it is moreover the mode and intensity of the intermolecular interaction that provides shape selective separation.

In case of hybrid RP phases with diverse and multiple interaction sites, a concerted retention mechanism can be expected, which is significantly different from conventional RP-type systems. In other words, subtle effects can emerge, which may provide an interesting opportunity to develop and study such novel hybrid RP-systems more extensively, in order to expand the variety of hybrid reversed-phase materials to be used in liquid chromatography.

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