Biphenyl Modified Silica Stabilised by Bulky Substituents - A New Stationary Phase for HPLC

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Biphenyl modified HPLC phases are, due to their orthogonal selectivity, an interesting alternative to conventional reversed phase silica. However, the use of UV detection in combination with aromatic phases can result in spurious peaks which make interpretation of the chromatogram considerably more difficult. The reason is the hydrolysis of biphenyl ligands. The introduction of bulky isobutyl substituents at the silicon atom of the ligand improves the stability. Therefore, the noise due to phase bleed can be almost completely avoided. In addition, these bulky substituents also influence the selectivity of this new biphenylpropyldiisobutylsilyl modified silica. The chromatography of forty-five organic substances is studied comparatively on octadecyl, octyl, and biphenyl sorbents.

Introduction

Octadecyl and octyl phases are probably the most widely used sorbents in reversed phase (RP) chromatography. Their ability to separate substance mixtures is enormous [1]. In addition, the use of mass spectrometry or diode array detectors is increasing. These detectors also provide information for substance identification and can detect coelutions. Nevertheless, the quantification of coeluting substances can still be challenging. There are numerous compounds that cannot be distinguished by MS or whose UV spectra are very similar. Therefore, stationary phases with orthogonal selectivity are still of major interest. Ligands with aromatic groups are alternatives in RP chromatography [2]. They are chemically inert and the selectivity of the stationary phase is additionally influenced by the possibility of π-π interactions [3, 4]. However, the frequently used phenylpropyl or phenylhexyl groups show less retention compared to octadecyl ligands [5]. As an alternative, biphenyl groups can be utilised, which exhibit stronger interactions through a larger conjugated π-system and a higher carbon number. Biphenyl modified silica shows comparable retention times to octadecyl phases [5].

Experimental

Stainless steel columns (100 mm length x 3 mm ID and 50 mm length x 4 mm ID) with the stationary phases NUCLEOSHELL® Biphenyl, 2.7 µm and NUCLEOSHELL® RP 18plus, 2.7 µm both based on core shell silica are commercially available (MACHEREY-NAGEL, Germany). NUCLEOSHELL® π² and NUCLEOSHELL® RP 8plus are not commercially available. For comparison, they were produced according to the confidential procedures of NUCLEODUR® π² and NUCLEOSHELL® RP 18plus. These sorbents were filled in stainless steel columns by a slurry packing method. The structures of the ligands are shown in *Table 1*. The HPLC columns Kinetex® Biphenyl, 2.6 µm (Phenomenex, Germany) and Raptor® Biphenyl, 2.7 µm (Restek, USA) were purchased as comparison columns (100 mm length x 3 mm ID).

HPLC grade solvents were used for the preparation of the eluents. Water was prepared by an ultra clear GP UV UF purifier (Evoqua, Germany). The various test compounds were of reagent grade or higher purity and were purchased from several sources.

The HPLC equipment used in this work were a Nexera XR (Shimadzu, Germany) and a Vanquish UHPLC system (Thermo Scientific, Germany). Solutions (c = 1 mg/mL) were prepared in methanol. The void volume was determined by injection of uracil solution.

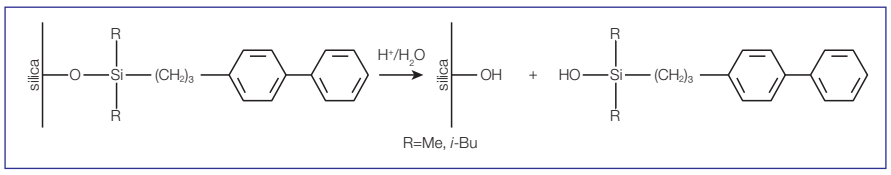
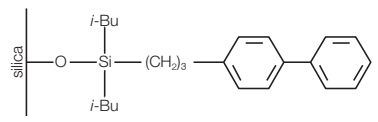
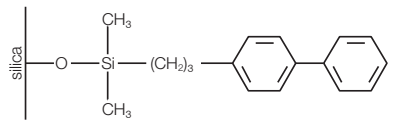
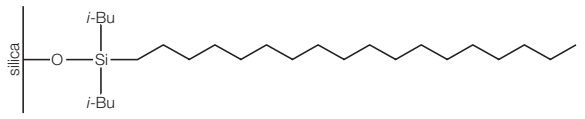
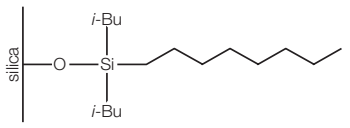


Figure 1. Hydrolysis of biphenyl ligands.

Table 1. Surface modifications of the investigated core-shell silicas.

Stationary Phase	Ligand structure
NUCLEOSHELL® Biphenyl	
*NUCLEOSHELL® π² **	
NUCLEOSHELL® RP 18plus	
*NUCLEOSHELL® RP 8plus **	
** This stationary phase is not available commercially*	

Results and Discussion

Ligands are usually attached to silica via a siloxane bond. Partial hydrolysis of this siloxane bond can occur under acidic conditions [6]. In the case of a biphenyl containing modification, the hydrolysis leads to compounds with biphenyl containing groups *(Figure 1)*. These compounds show a strong absorption of UV light in the wavelength range of 220 to 300 nm and can cause rising baselines in the chromatogram *(Figure 2)*. This can complicate the quantification of the analytes. The similarity of the UV spectra of eluate and the pure substance allylbiphenyl demonstrates the biphenyl containing structure of the molecules that cause this disturbance. This malfunction is only visible in gradient mode. At high water contents of the eluent hydrolysis of the siloxane bond occurs. Due to their hydrophobic character, the compounds accumulate on the stationary phase. The enriched impurities elute at higher acetonitrile levels. This gradient method was used to compare the bleed behaviour of four biphenyl modified core shell silica *(Figure 2)*. Biphenylpropyldimethylsilyl modified NUCLEOSHELL® π² shows the most intense spurious peak followed by Kinetex® Biphenyl and Raptor® Biphenyl (both biphenyldimethylsilyl-modified). Obviously, the direct attachment of the biphenyl group to the silicon has a stabilising effect, most likely due to steric shielding of the siloxane bond. In addition, the attachment of the biphenyl group via a propyl spacer leads to stronger hydrophobic interactions [5], which indicate a higher occupancy density. This would also explain the

increased bleeding behaviour of the NUCLEODUR® π². Following Kirkland [7], an addition of two bulky isobutyl substituents to the biphenylpropyl should minimise hydrolysis of the surface modification. Therefore, NUCLEOSHELL® Biphenyl does not show any appreciable spike *(Figure 2)* and thus can be used under acidic conditions in gradient mode.

For selectivity testing, forty-five organic compounds specified in *Table 2* were chromatographed isocratically with acetonitrile / water (50:50, v/v) and methanol / water (60:40, v/v respectively). These compositions were chosen because they have a similar elution power [1]. 1 µL of a methanolic solution (about 1%) of the compounds were injected at a flowrate of 1 mL/min. The column temperature was 25 °C. A diode array detector (200-300 nm) was used to detect and to identify the substances. The determined retention factors are summarised in *Table 2*.

The comparison of two sorbents can be visualised by plotting the retention factors in a dot diagram. If two HPLC columns have equal retention factors, all points are on a straight line with a slope of one. This line is marked with a red line in *Figures 3-8*. Points above this line are for compounds that retain more strongly on the NUCLEOSHELL® Biphenyl phase than on the second phase, points below the line for compounds with lower interactions and thus smaller retention factors.

Table 2. Determined retention factors.

	retention factor		retention factor		retention factor		retention factor	
	NUCLEOSHELL® Biphenyl		NUCLEOSHELL® π²		NUCLEOSHELL® RP 18plus		NUCLEOSHELL® RP 8plus	
	ACN[1)]	MeOH[1)]	ACN[1)]	MeOH[1)]	ACN[1)]	MeOH[1)]	ACN[1)]	MeOH[1)]
Acenaphthylene	7.97	15.02	6.08	13.87	7.43	9.06	4.24	3.75
Pentylbenzene	15.63	33.47	12.86	38.44	32.83	56.82	14.88	21.80
Anisole	1.96	1.92	1.79	2.58	2.16	1.84	1.71	1.16
Fluorene	8.45	17.14	9.06	31.97	11.25	17.80	5.77	6.42
Chlorobenzene	3.03	3.12	2.79	4.04	3.87	3.77	2.66	2.06
Fluoranthene	13.98	33.69	16.59	69.62	19.42	33.96	7.99	9.64
Phenol	0..63	0.50	0.54	0.53	0.61	0.52	0.63	0.42
Naphthaline	4.60	5.79	4.48	8.54	5.73	6.51	3.53	2.96
Anthracene	11.04	23.70	13.20	50.92	14.83	23.68	6.77	7.62
Toluene	2.93	2.94	2.58	3.49	3.92	3.96	2.70	2.10
Propylbenzene	6.74	9.53	5.75	11.25	11.07	14.38	6.28	6.55
Butylbenzene	10.25	17.90	8.60	20.88	19.06	28.63	9.63	11.89
Acetophenone	1.17	1.36	1.05	1.89	1.13	0.85	1.00	0.68
Butylbenzoate	6.00	12.17	5.01	14.69	9.31	11.89	5.45	6.55
Biphenyl	7.50	12.88	7.47	20.61	9.63	13.42	5.44	5.65
4-Nitrotoluene	2.72	3.57	2.76	6.85	2.79	2.20	2.15	1.46
Ethylbenzoate	2.53	3.61	2.20	4.53	3.15	3.15	2.27	2.06
Ethylbenzene	4.38	5.14	3.79	6.11	6.39	7.20	4.01	3.60
N.N-Dimethylaniline	2.89	2.98	2.53	3.99	3.22	2.76	2.26	1.58
m-Cresol	0.86	0.84	0.73	0.93	0.90	0.92	0.85	0.68
o-Cresol	0.97	0.92	0.82	1.03	1.02	1.03	0.95	0.74
p-Cresol	0.86	0.85	0.73	0.96	0.89	0.92	0.84	0.66
2.6-Dimethylphenol	1.47	1.50	1.25	1.72	1.66	1.70	1.40	1.12
2.6-Dimethylaniline	1.43	1.36	1.25	1.69	1.47	1.19	1.23	0.81
Nitrobenzene	1.81	1.85	1.73	2.99	1.73	1.19	1.49	0.87
Dimethylphthalate	1.30	1.61	1.15	2.33	1.23	0.83	1.12	0.69
Diethylphthalate	2.58	4.15	2.20	5.61	2.89	2.43	2.28	1.82
1-Phenylethanol	0.63	0.84	0.53	0.89	0.64	0.84	0.62	0.67
Pyrene	14.54	35.36	17.50	71.25	20.98	37.65	8.10	10.10
Chrysene	22.29	77.73	31.41	209.02	32.54	70.54	11.18	16.36
Propiophenone	1.99	2.62	1.83	3.76	2.10	1.70	1.65	1.21
Diethyl adipate	1.90	3.72	1.51	4.23	2.19	2.25	1.81	1.83
Diisobutylphthalate	11.81	35.07	8.84	39.60	20.51	26.22	11.75	16.25
Triphenylene	21.14	68.82	27.33	152.00	28.49	58.27	9.98	13.83
o-Terphenyl	21.88	68.69	20.74	104.20	31.00	59.94	15.00	24.29
Prometryn	3.42	8.22	2.56	9.33	4.13	6.95	2.76	4.40
Desoxycorticosterone	3.83	39.14	4.37	117.48	2.05	5.32	1.58	4.12
Estrone	2.79	13.11	2.72	25.33	2.45	4.85	1.87	3.23
Progesterone	10.64	110.73	12.42	301.80	6.93	14.58	4.44	11.33
Propylparabene	1.29	2.37	1.08	2.73	1.49	2.33	1.25	1.56
p-Terphenyl	27.80	116.86	32.71	255.89	40.95	103.16	16.14	30.05
Benzofluoranthene	33.24	154.51	49.33	444.67	54.50	139.42	15.78	27.49
5-(p-Methylphenyl)-5-phenylhydantoin	1.14	2.95	0.98	4.09	1.07	1.99	0.98	1.28
Dibutylphthalate	12.85	42.94	10.09	51.50	21.50	29.70	11.81	17.00
Benzopyrene	45.07	232.28	51.38	413.73	54.53	139.39	14.91	26.48

[1)] Chromatographic conditions: Column 50 mm x 4 mm, acetonitrile/water 50:50 (v/v) or methanol/water 60:40 (v/v), 1 mL/min, 25°C, DAD 220-300 nm.

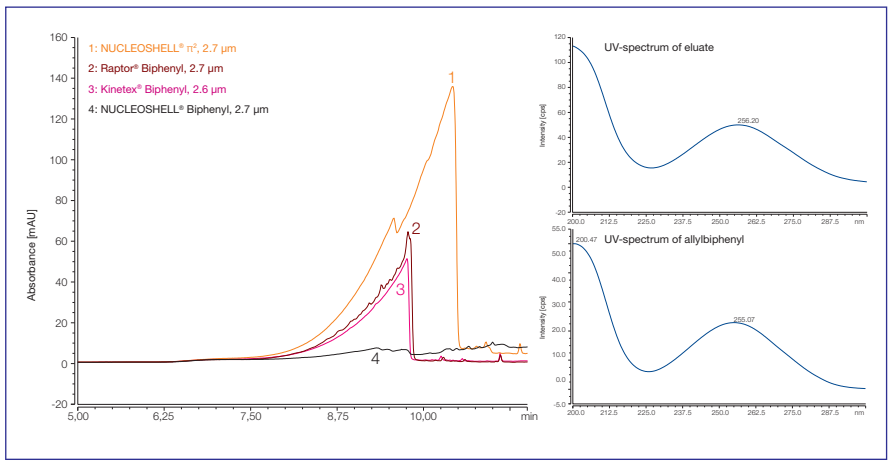


Figure 2. pH stability of different biphenyl sorbents using acidic gradients

Chromatographic conditions: column: 100 mm x 3 mm, eluent A: 1 % H3PO4, Eluent B: ACN, 40 °C, UV, 254 nm, 0.56 mL/min, gradient: equilibration 10 min 10 % B, hold 10 % B for 5 min, from 10 % to 90 % B in 5 min, hold 90 % B for 3 min, in 1.0 min to 10 % B, 0.56 mL/min.

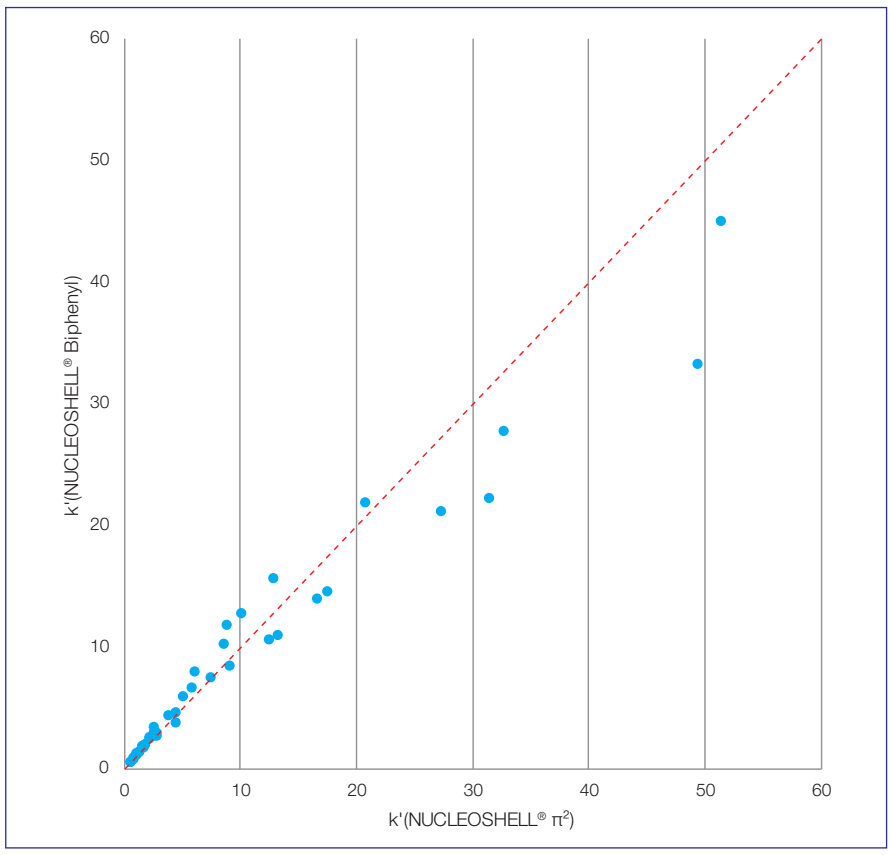


Figure 3. Comparison of NUCLEOSHELL® π² and NUCLEOSHELL® Biphenyl (acetonitrile/water).

The comparison of the two aryl phases NUCLEOSHELL® Biphenyl and NUCLEOSHELL® π² in *Figure 3* shows that for compounds with lower retention factors, NUCLEOSHELL® Biphenyl often exhibits stronger interactions. This reverses at higher values. Here the retention factors and thus the interactions of NUCLEOSHELL® π² are stronger. This certainly depends on the choice of the analytes. Most of the mononuclear aromatic compounds studied here are located in the lower part of the retention factors. They show relatively weak interactions with the biphenyl group [5]. The isobutyl groups of NUCLEOSHELL® Biphenyl increase the hydrophobic interactions of the phase, so that these molecules retain more. At higher k' values, compounds like polynuclear aromatics and steroids are located. These substances undergo stronger interactions with biphenyl ligands. Therefore, NUCLEOSHELL® π² compared to NUCLEOSHELL® Biphenyl shows longer retention times due to the higher proportion of π-π interactions.

The comparisons with octadecyl and octyl ligands are shown in *Figures 4 and 5*. With a few exceptions, the octadecyl phase shows longer retention times. The exceptions are mainly steroids. The retention factor of progesterone shows a value increased by a factor of 1.53, for desoxycorticosterone even a factor of 1.86 could be determined. On the other hand, alkylaromatics like pentylbenzene are more strongly retained on the RP 18 phase. The lower hydrophobicity of the octyl group means that all compounds tested on NUCLEOSHELL® Biphenyl have longer retention times. The largest differences between the two phases can be found for steroids and some polynuclear aromatics.

In *Figures 6 - 8*, the sorbents are compared in the eluent system methanol / water. Under these conditions, NUCLEOSHELL® π² retains all compounds more strongly than NUCLEOSHELL® Biphenyl *(Figure 6)*. For example, a factor of 3.0 could be determined for desoxycorticosterone. In contrast to the octadecyl column, NUCLEOSHELL® Biphenyl shows on average stronger interactions. The retention factors of desoxycorticosterone and progesterone are more than seven times greater on the biphenyl phase. In the case of pentylbenzene the retention factor decreases by a factor of 0.59. A similar behaviour is shown by the comparison with the octyl phase *(Figure 8)*. While the retention factors of desoxycorticosterone, progesterone, and benzopyrene increase by factors of 8.8 to 9.8, phenol for example, is only increased by 19%.

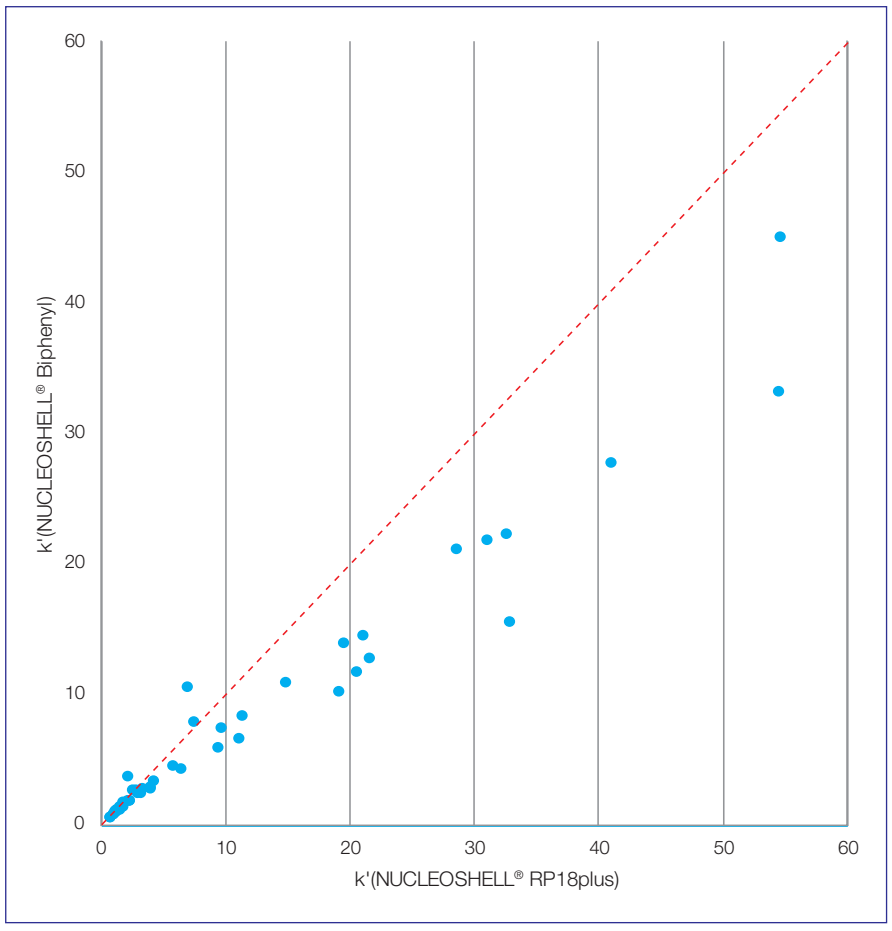


Figure 4. Comparison of NUCLEOSHELL® RP 18plus and NUCLEOSHELL® Biphenyl (acetonitrile/water).

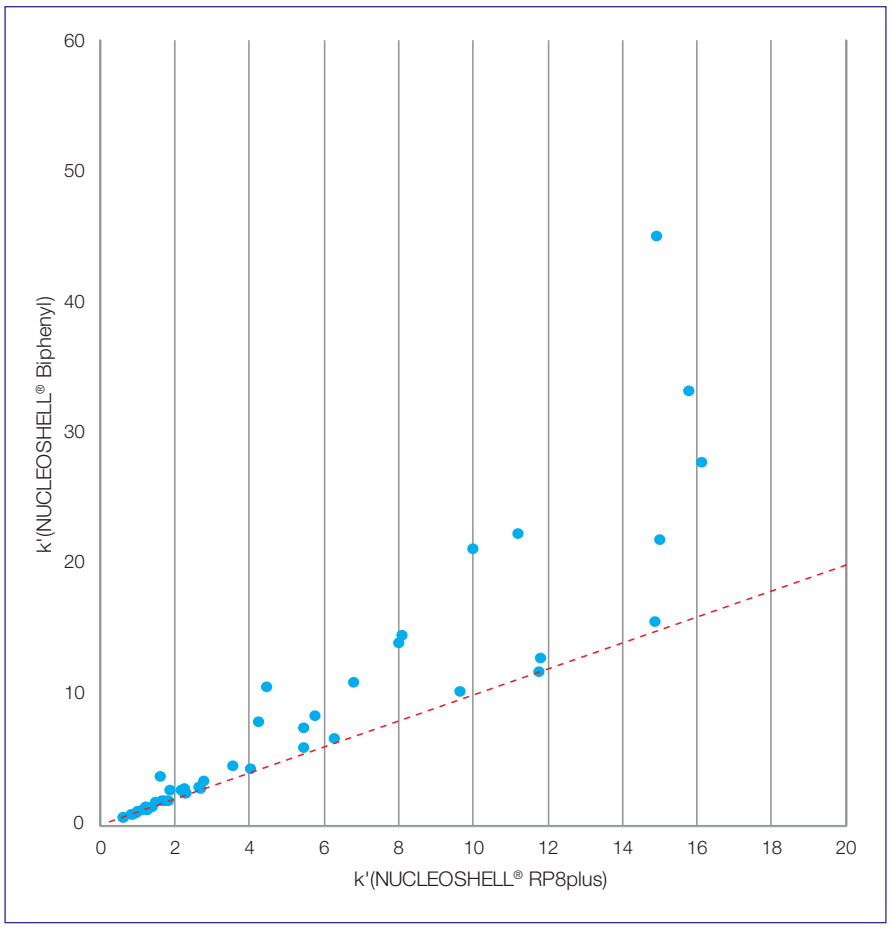


Figure 5. Comparison of NUCLEOSHELL® RP 8plus and NUCLEOSHELL® Biphenyl (acetonitrile/water)

The different behaviour of acetonitrile and methanol can be explained by the triple bond of the nitrile function. This allows acetonitrile to compete with the analytes for the π-π interactions of the stationary phase. This explains the moderate differences between the phases when using acetonitrile as organic modifier. Methanol, on the other hand, does not reduce the π-π interactions between stationary phase and analyte. The influence of the biphenyl group is therefore much more pronounced.

Table 3. Description of the formulas used.

formula	*description*
$k'_{rel}(i, c)=\frac{k'(i, c)}{k'(i, ref)}$	*Relative retention factor of compound i on column c in comparison to the reference column (NUCLEOSHELL® Biphenyl)*
$\overline{k'_{rel}(c)}$	*Averaged relative retention factor over all compounds on column c as a measure of the mean interaction strength*
$RSD=\frac{\sqrt{\frac{1}{n}\sum[k'_{rel}(i, c)-\overline{k'_{rel}(c)}]^2}}{\overline{k'_{rel}(c)}}$	*Relative standard deviation as a measure of the dispersion of the relative capacity factors*

For quantitative evaluation, the quotient of the retention factor of the respective column and that of the biphenyl column is formed for each compound *(Table 3)*. The mean of the values of a column is a measure of the average interaction strength of this phase compared to NUCLEOSHELL® Biphenyl. The relative standard deviation (RSD) of the quotient describes the selectivity differences of the two stationary phases. The larger this value, the more the phases differ in their interactions and thus in their selectivity. When acetonitrile is used as the organic modifier, NUCLEOSHELL® π² shows an average interaction strength of 95% compared to NUCLEOSHELL® Biphenyl *(Table 4)*. This is surprising to the extent that due to the bulky isobutyl groups a lower occupation density and thus smaller retention factors were expected. The higher average interaction strength is mainly caused by the increase in retention of mononuclear aromatics. These compounds are only weakly retained by biphenyl ligands. For the retention of NUCLEOSHELL® Biphenyl the interactions with the additional isobutyl groups are responsible. Compared to the alkyl modified silica, the interaction strength of NUCLEOSHELL® Biphenyl is between the octadecyl (123%) and octyl phases (78%). The relative standard deviation is 0.24 in both cases, but a value of 0.18 could be determined between NUCLEOSHELL® Biphenyl and π². Thus, the two biphenyl phases also show different selectivities.

In contrast to methanol, acetonitrile has a nitrile group with a triple bond. The solvent competes with the analytes for the π-π-interactions of the stationary phase. This explains the moderate differences between the phases when using acetonitrile as organic modifier. With methanol the mean interaction strength of NUCLEOSHELL® π² increases significantly with 154%. In contrast, the interaction strengths of the pure alkyl phases decrease to 91% (octadecyl) and 48% (octyl) compared to NUCLEOSHELL® Biphenyl. The relative standard deviations and thus the selectivity differences between the phases also increase significantly if the organic modifier is switched to methanol.

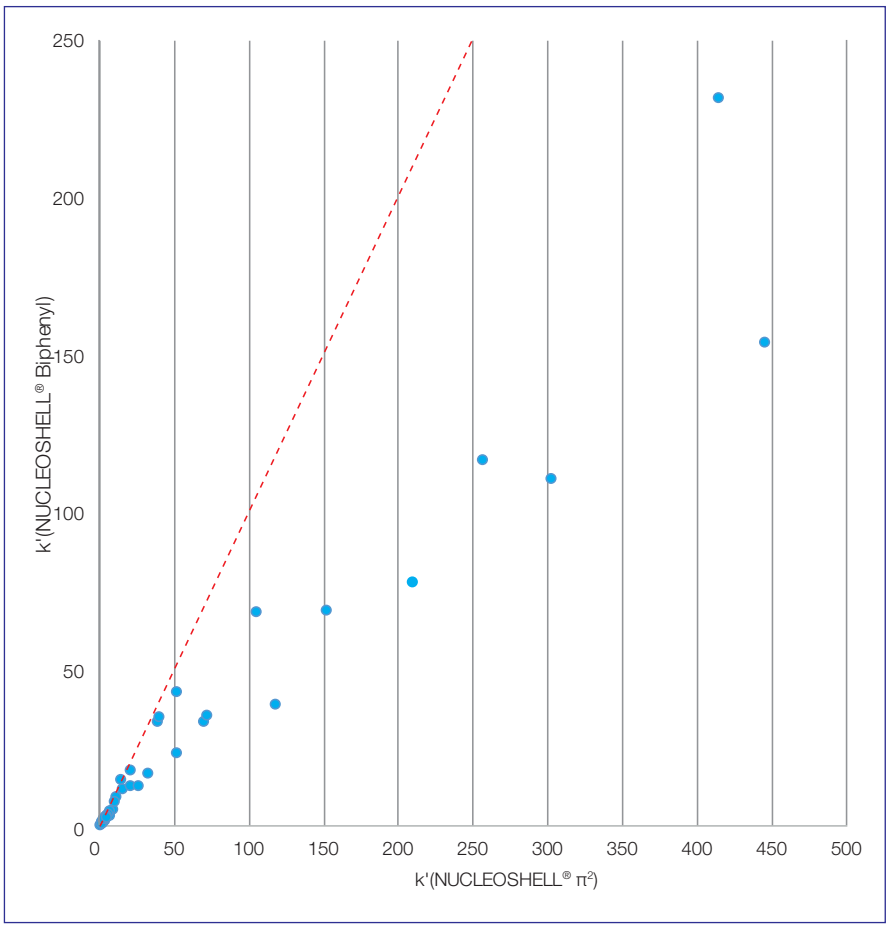


Figure 6. Comparison of NUCLEOSHELL® π² and NUCLEOSHELL® Biphenyl (methanol/water)

Figures 9 - 11 show the great difference for the chromatograms of the investigated sorbents. NUCLEOSHELL® RP 8plus is compared to NUCLEOSHELL® biphenyl in *Figure 9*. The higher interaction strength leads to the longer retention times of the aryl phase. This and the stronger interactions between biphenyl and steroidal structures lead to the better resolution of the first three compounds. The next chromatogram *(Figure 10)* shows a comparison of NUCLEOSHELL® RP 18plus and NUCLEOSHELL® Biphenyl for the separation of a further test mixture. Desoxycorticosterone is clearly separated from butyl paraben and propiophenone due to its steroidal structure on the biphenyl phase. The separation of the diisobutyl phthalate and pyrene peak pairs is probably based on stronger π-π interactions with the conjugated aromatic system. This is also the reason for the elution reversal in pentylbenzene and chrysene.

In *Figure 11* the aryl phases NUCLEOSHELL® Biphenyl and NUCLEOSHELL® π² are compared. Under the same conditions NUCLEOSHELL® Biphenyl needs more time for the separation. This is because the sorbents in the lower k'-region often show stronger retention *(Figure 3)*.

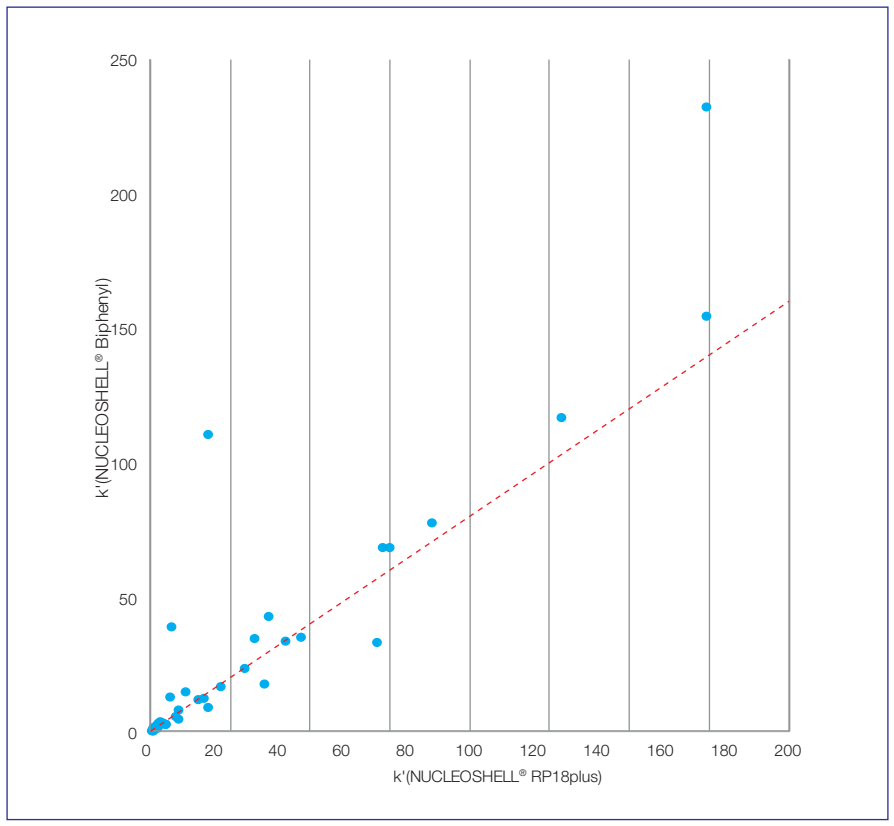


Figure 7. Comparison of NUCLEOSHELL® RP 18plus and NUCLEOSHELL® Biphenyl (methanol/water)

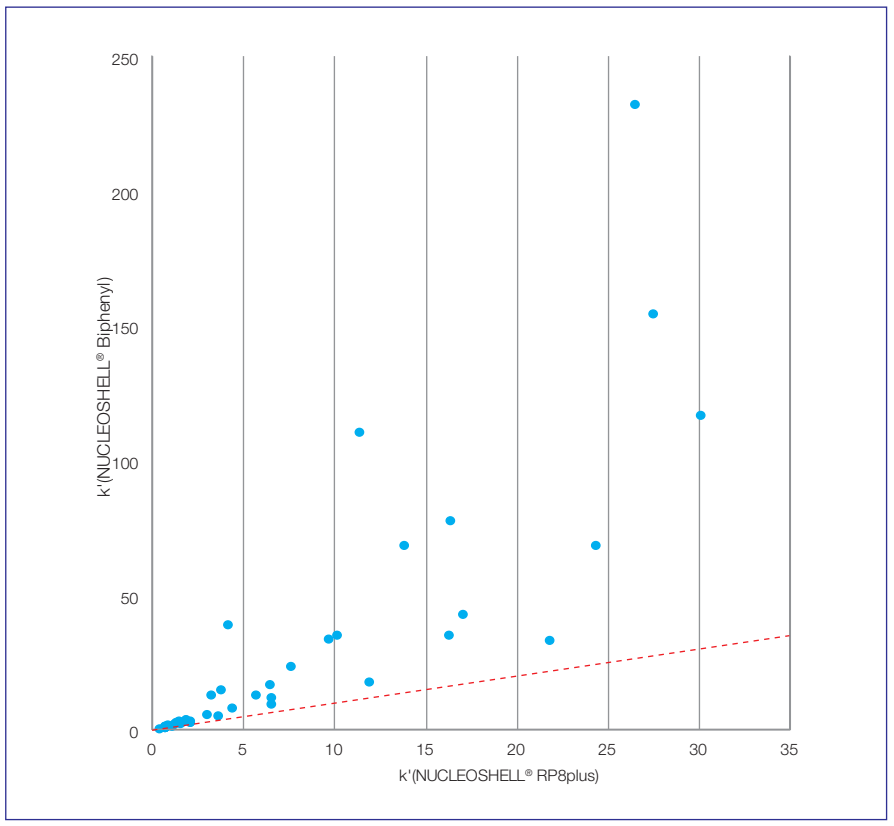


Figure 8. Comparison of NUCLEOSHELL® RP 8plus and NUCLEOSHELL® Biphenyl (methanol/water)

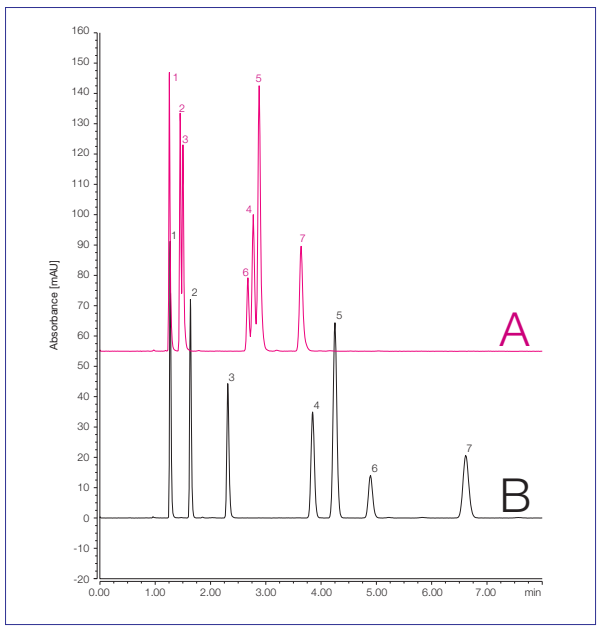


Figure 9: Separation of a test mixture on NUCLEOSHELL® RP 8plus and NUCLEOSHELL® Biphenyl

Chromatographic conditions: column A NUCLEOSHELL® RP 8plus, column B NUCLEOSHELL® Biphenyl, 100 mm x 3 mm, acetonitrile/water 55:45 (v/v), 0.56 mL/min, 40 °C, UV, 254 nm, 1. propiophenone, 2. propylparaben, 3. desoxycorticosterone, 4. biphenyl, 5. fluorene, 6. progesterone, 7. pyrene.

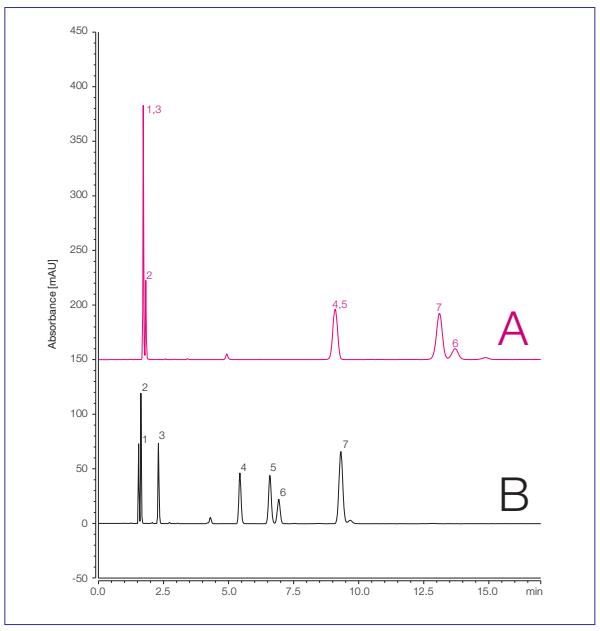


Figure 10: Separation of a test mixture on NUCLEOSHELL® RP 18plus and NUCLEOSHELL® Biphenyl

Chromatographic conditions: column A NUCLEOSHELL® RP 18plus, column B NUCLEOSHELL® Biphenyl, 100 mm x 3 mm, acetonitrile/water 55:45 (v/v), 0.56 mL/min, 40 °C, UV, 254 nm, 1. propiophenone, 2. butylparaben, 3. desoxycorticosterone, 4. biphenyldiisobutylphthalate, 5. pyrene, 6. pentylbenzene, 7. chrysene.

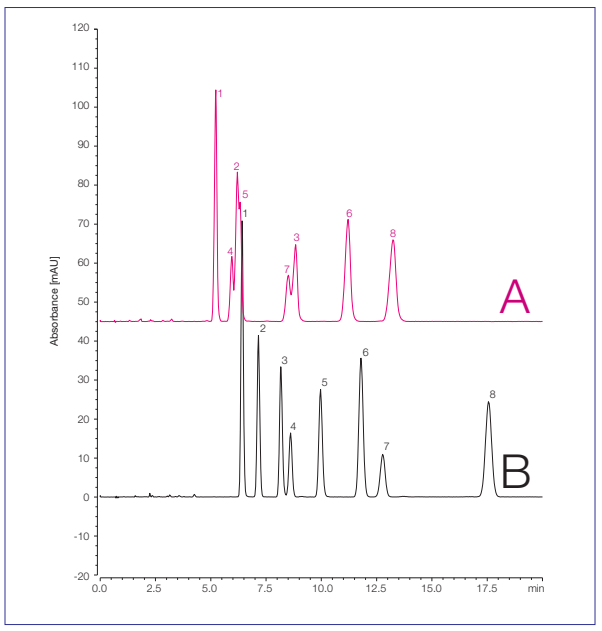


Figure 11: Separation of a test mixture on NUCLEOSHELL® π² and NUCLEOSHELL® Biphenyl

Chromatographic conditions: column A NUCLEOSHELL® RP 18plus, column B NUCLEOSHELL® Biphenyl, 100 mm x 3 mm, acetonitrile/water 50:50 (v/v), 0.50 mL/min, 35 °C, UV, 254 nm, 1. biphenyl, 2. fluorene, 3. progesterone, 4. butylbenzene, 5. diisobutylphthalate, 6. pyrene, 7. pentylbenzene, 8. o-terphenyl.

Although both phases have biphenyl containing modifications, several reversals of the order of elution occur. The reason for this is the dilution of the π-π interactions by the two isobutyl groups. Mononuclear aromatics, which show rather less interactions with the biphenyl groups, gain in retention in comparison to the polynuclear aromatics and the steroid progesterone.

Table 4. Comparison of interaction strength and dispersion.

Stationary phase	***results acetonitrile***		***results methanol***	
	$k'_{rel}(c)$	*RSD*	$k'_{rel}(c)$	*RSD*
NUCLEOSHELL® Biphenyl, 2.7µm (reference column)	*1.00*	*---*	*1.00*	*---*
NUCLEOSHELL® π², 2.7µm	*0.96*	*0.18*	*1.54*	*0.34*
NUCLEOSHELL® RP18plus, 2.7µm	*1.23*	*0.24*	*0.91*	*0.36*
NUCLEOSHELL® RP8plus, 2.7µm	*0.78*	*0.24*	*0.48*	*0.43*

Conclusion

NUCLEOSHELL® Biphenyl is a new biphenyl modified core shell silica. The introduction of bulky substituents to the silicon atom improves the stability of this stationary phase against acidic conditions. The selectivity is significantly different from octadecyl and octyl sorbents. The biphenyl ligand plays a crucial role in the interactions of this phase. Due to the influence of the two isobutylgroups, significant differences in the selectivities between NUCLEOSHELL® Biphenyl and NUCLEOSHELL® π² can be observed. The change of acetonitrile and methanol makes it possible to influence the strength of the π-π interactions and is therefore another exciting tool for optimising the separation.

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