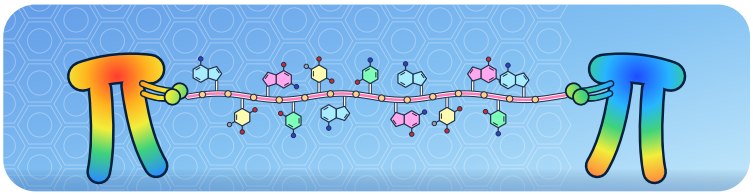
Science with Passion

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# No Pairing Required: Oligonucleotide Analysis on Phenyl Stationary Phases

J. Kramer, M. Prüfer , J. Wesolowski; kramer@knauer.net

KNAUER Wissenschaftliche Geräte GmbH, Hegauer Weg 38, 14163 Berlin; www.knauer.net



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## SUMMARY

Oligonucleotide analysis is challenging because of the molecules charged phosphate backbone, hydrophilicity, structural variability, and chemical modifications. Phenyl stationary phases provide an ion-pair-free, MS-compatible alternative, retaining oligonucleotides through hydrophobic, dispersive, and π–π interactions with exposed nucleobases. This application note compares a common phenyl, a pentafluoro phenyl and a phenyl-hexyl phase and shows how length, modification, secondary structure, and phase chemistry affect retention and selectivity.

## INTRODUCTION

Oligonucleotides are an increasingly important class of therapeutic compounds, but their chromatographic analysis remains challenging because of their highly charged phosphate backbone, pronounced hydrophilicity, sequence-dependent conformation, and frequent chemical modifications. Relevant impurities, including shortmers, sequence variants, and degradation products, often differ only slightly from the full-length product, placing high demands on chromatographic selectivity and efficiency [1-3].

Ion-pair reversed-phase liquid chromatography (IP-RPLC) is widely used for oligonucleotide analysis because alkylammonium reagents mask the negative charges of the phosphate backbone and facilitate retention on hydrophobic stationary phases [1,3]. However, these reagents may contaminate chromatographic and mass-spectrometric systems, reduce column lifetime, and cause ion suppression [1,2]. Ion-pair-free reversed-phase chromatography using aromatic stationary phases is therefore an attractive, potentially MS-compatible alternative [1,2].

Phenyl-bonded stationary phases can retain oligonucleotides through hydrophobic, dispersive, and aromatic interactions, particularly π–π stacking with exposed nucleobases [1,2]. Retention depends on oligonucleotide length, sequence, chemical modifications, and

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secondary structure [1-3]. Folded structures may limit nucleobase accessibility and reduce retention, whereas linear oligonucleotides can interact more extensively with the aromatic surface [1].

A conventional phenyl phase and phenyl-hexyl phase provide π–π and hydrophobic interactions, whereas the electron-deficient ring of a pentafluoro phenyl phase (PFP) can additionally promote directional donor-acceptor π, dipolar, and polar interactions [1] **(Fig. 1/Fig. 2)**.

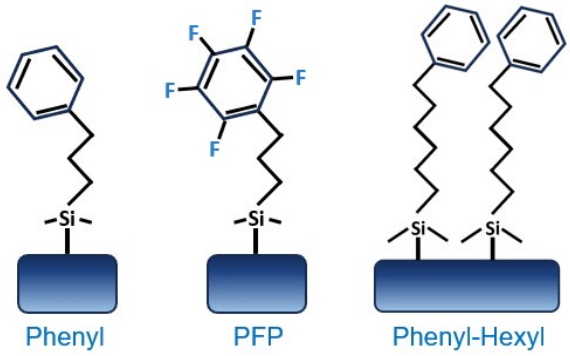



**Fig. 1** Schematic illustration of different phenyl based stationary phases

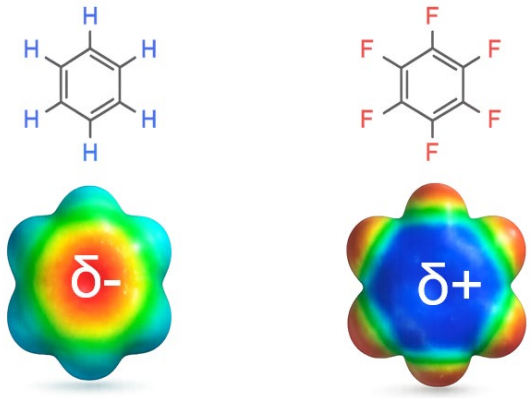



**Fig. 2** Schematic illustration of stationary phase modification; left – phenyl, right – PFP

The phases may therefore produce different retention patterns and elution orders for closely related oligonucleotides. Chromatographic performance can also be influenced by ligand density, spacer structure, particle morphology, pore size, endcapping, silica properties, and column hardware [1,2].

This application note compares a PFP, phenyl-hexyl and a conventional phenyl stationary phase for ion-pair-free reversed-phase analysis of oligonucleotides.

## SAMPLE PREPARATION

All samples were already available in a purified, desalted and dissolved form after synthesis. The samples were filtered through a 0.45 µm nylon filter and if necessary, they were diluted with water.

## RESULTS

Four different exemplary oligonucleotide samples were used throughout the whole method development process **(Tab. 1)**.

**Tab. 1** Sample information

| Sample | Type | Length | Molar Mass [Da] | Modifications |
|---|---|---|---|---|
| 64mer DNAss | DNA oxidized | 64 | 19488.71 | n/a |
| dT24 C12 | DNA with C12 linker | 25 | 7502.06 | Amino C12 Linker at end of 5' |
| SCR Antisense | RNA | 21 | 6568.06 | RNA's 2dTs at 3' with 1er thioate |
| 20mer | DNA semi thioate | 20 | 6141.07 + 302 | 10 thiolations with a DMT protecting group |

The first tested column was a Eurospher II 100-3 PFP stationary phase. According to literature [1] the following method starting conditions were chosen **(Tab. 2, G1)**. The 20mer sample showed the best behavior with the chosen starting conditions. The 64mer sample was retained but the peak shape showed tailing as well as fronting. Furthermore, the dT24 C12 oligonucleotide sample was barely detected with a very small peak. The SCR Antisense sample eluted within the dead time **(Fig. 3)**.

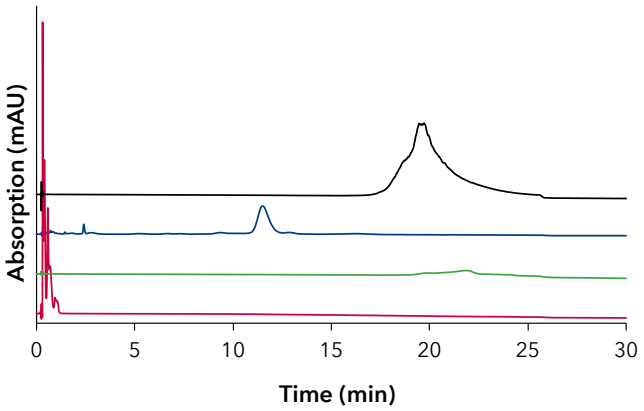



**Fig. 3** Starting conditions according to Tab. 2 G1, red – SCR Antisense, green – dT24 C12, blue – 20mer, black – 64mer; Eurospher II 100-3 PFP column


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The amount of eluent B was reduced to 10 %, to see whether the SCR sample can be retained more effectively. Furthermore, the gradient slope was increased to see if there will be an effect on the peak shape. These changes and new parameters are summarized in **Tab. 2, G2**.

**Tab. 2** Different gradient conditions; Eurospher II 100-3 PFP column

| Parameter | Description | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Eluent A | 50 mM ammonium acetate, pH 8.0 | | | | | | | | |
| Eluent B | 100 mM ammonium acetate:methanol 50:50 (v/v), pH 8.0 | | | | | | | | |
| Temperature | 50 °C | | | | | | | | |
| Detection | UV@260 nm | | | | | | | | |
| Flow rate | 0.5 ml/min | | | | | | | | |
| Gradient | G1 | | | G2 | | | G3 | | |
| | Time [min] | % A | % B | Time [min] | % A | % B | Time [min] | % A | % B |
| | 0.00 | 80 | 20 | 0.00 | 90 | 10 | 0.00 | 90 | 10 |
| | 2.00 | 80 | 20 | 2.00 | 90 | 10 | 2.00 | 90 | 10 |
| | 22.00 | 60 | 40 | 15.00 | 60 | 40 | 15.00 | 50 | 50 |
| | 25.00 | 60 | 40 | 18.00 | 60 | 40 | 18.00 | 50 | 50 |
| | 25.02 | 80 | 20 | 18.02 | 90 | 10 | 18.02 | 90 | 10 |
| | 30.00 | 80 | 20 | 22.00 | 90 | 10 | 22.00 | 90 | 10 |

**Fig. 4** shows the obtained chromatograms of the ON samples under the new gradient conditions **(Tab. 2, G2)**. The peak shape of the 20mer and 64mer was improved. Furthermore, due to the reduction of organic amount at the beginning, the SCR Antisense ON is no longer eluted within the dead time. The dT24 C12 sample now shows a visible peak.

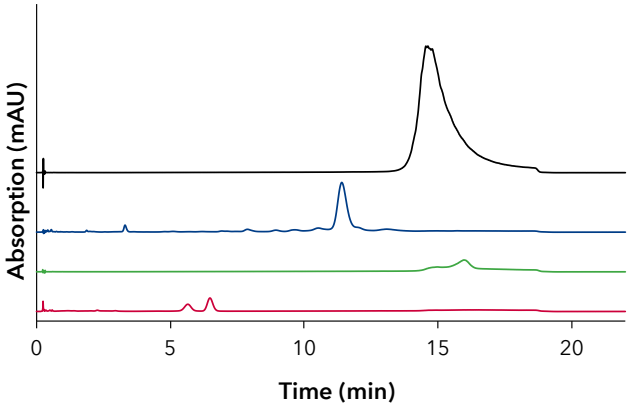



**Fig. 4** Adjusted gradient according to Tab. 2 G2, red – SCR Antisense , green – dT24 C12, blue – 20mer, black – 64mer; Eurospher II 100-3 PFP column

To see if further improvement is possible, the gradient times were kept the same, but the maximum organic amount was increased to 50 % B **(Tab. 2, G3)**. The increase of eluent B to 50 % leads to complete elution of the dT24 C12 sample. The detected peaks at a retention time of around 15 minutes **(Fig. 4)** were not the main component but might correspond to earlier eluting impurities. The other samples only showed minor changes in retention time as an effect of the steeper gradient slope **(Fig. 5)**.

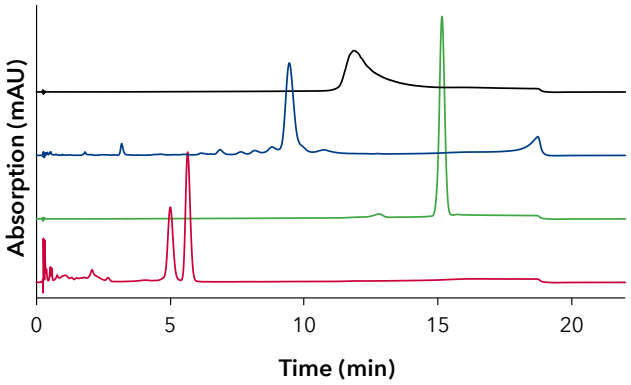



**Fig. 5** Final gradient according to Tab. 2 G3, red – SCR Antisense, green – dT24 C12, blue – 20mer, black – 64mer; Eurospher II 100-3 PFP column

Using the final settings from **G3**, all samples could be retained sufficiently on the pentafluoro phenyl stationary phase. For comparison and to see the effect of different interactions, the samples were next measured on a common phenyl stationary phase using mainly the π-π interactions. The gradient conditions from **Tab. 2**, **G3** were directly transferred to a Eurospher II 100-3 Phenyl column **(Fig. 6)** in the same column dimension as the used PFP stationary phase.

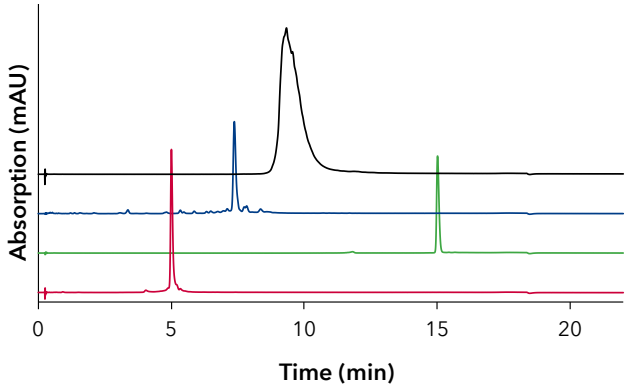



**Fig. 6** Gradient according to Tab. 2 G3, red – SCR Antisense, green – dT24 C12, blue – 20mer, black – 64mer; Eurospher II 100-3 Phenyl column

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The next investigated column was the common phenyl stationary phase. To see if further improvement is possible, the gradient times were kept the same, but the maximum organic amount was increased to 60 % B **(Tab. 3, G4)**. The result of thew adjustment of the gradient to the phenyl stationary phase is displayed in **Fig. 7**. The adaptation of the gradient only showed minor impact on the SCR and 20mer ON sample. Due to the steeper gradient slope, the symmetry of the 64mer sample was improved. For the dT24 C12 sample a similar distribution compared to **G3** was observed, regarding the peak shape. The increase to 60 % B resulted in an earlier elution of the dT24 C12 oligonucleotide.

**Tab. 3** Gradient conditions; Eurospher II 100-3 Phenyl, Eurospher II 100-3 Phenyl-Hexyl

| Parameter | Description | | |
|---|---|---|---|
| Eluent A | 50 mM ammonium acetate, pH 8.0 | | |
| Eluent B | 100 mM ammonium acetate:methanol 50:50 (v/v), pH 8.0 | | |
| Temperature | 50 °C | | |
| Detection | UV@260 nm | | |
| Flow rate | 0.5 ml/min | | |
| Gradient | **G4** | | |
| | Time [min] | % A | % B |
| | 0.00 | 90 | 10 |
| | 2.00 | 90 | 10 |
| | 15.00 | 40 | 60 |
| | 18.00 | 40 | 60 |
| | 18.02 | 90 | 10 |
| | 22.00 | 90 | 10 |

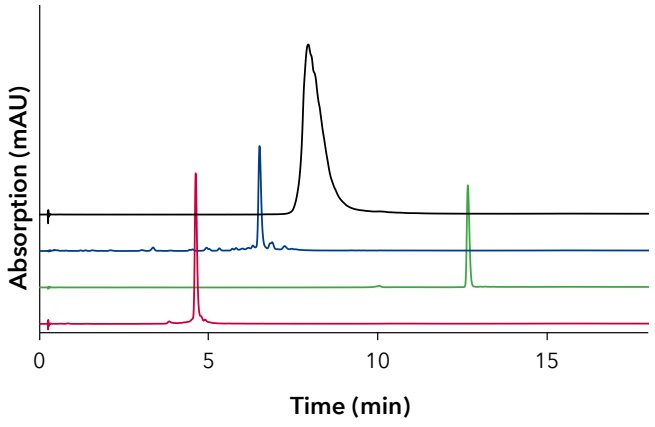



**Fig. 7** Gradient according to Tab. 3 G4, red – SCR Antisense, green – dT24 C12, blue – 20mer, black – 64mer; Eurospher II 100-3 Phenyl column

The last tested column was a phenyl-hexyl stationary phase. It has a phenyl (aromatic) group attached via a six-carbon alkyl linker to the silica surface. Therefore, it provides a different selectivity than traditional C18 columns because it combines hydrophobic interactions with π-π interactions and other aromatic recognition mechanisms. The gradient settings from **Tab. 3, G4** were directly transferred to the phenyl-hexyl column. The resulting chromatograms are displayed in **Fig. 8**.

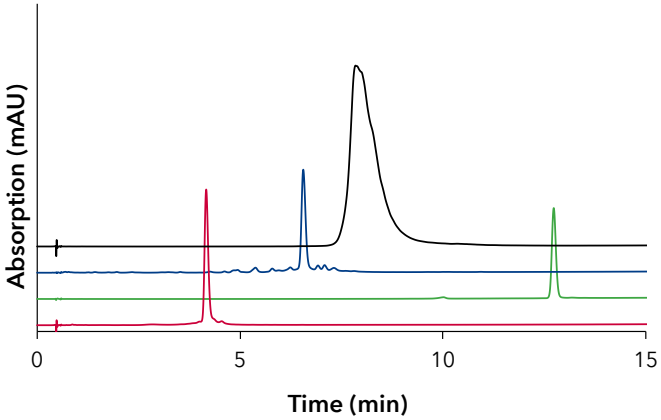



**Fig. 8** Gradient according to Tab. 3 G4, red – SCR Antisense, green – dT24 C12, blue – 20mer, black – 64mer; Eurospher II 100-3 Phenyl-Hexyl column

The direct method transfer **(Tab. 3, G4)** from the common phenyl stationary phase to the phenyl-hexyl column was performed successfully. The same retention pattern was observed for the selected oligonucleotide samples.

**Tab. 4** Gradient conditions; Eurospher II 100-3 Phenyl-Hexyl column

| Parameter | Description | | |
|---|---|---|---|
| Eluent A | 50 mM ammonium acetate, pH 8.0 | | |
| Eluent B | 100 mM ammonium acetate:methanol 50:50 (v/v), pH 8.0 | | |
| Temperature | 50 °C | | |
| Detection | UV@260 nm | | |
| Flow rate | 0.5 ml/min | | |
| Gradient | **G5** | | |
| | Time [min] | % A | % B |
| | 0.00 | 90 | 10 |
| | 0.50 | 90 | 10 |
| | 10.00 | 40 | 60 |
| | 12.00 | 40 | 60 |
| | 12.02 | 90 | 10 |
| | 17.00 | 90 | 10 |


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The last adjustments from **G4** to **G5** on the phenyl-hexyl column showed no significant improvement regarding the peak shape. As expected, all oligonucleotide samples eluted earlier due to the change in gradient slope.

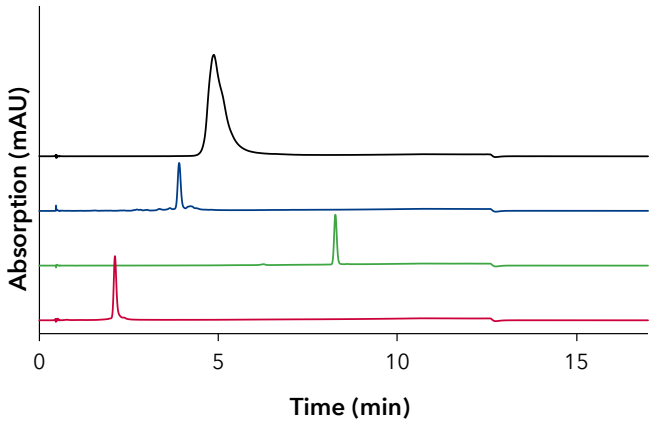



**Fig. 9** Gradient according to Tab. 4 G5, red – SCR Antisense, green – dT24 C12, blue – 20mer, black – 64mer; Eurospher II 100-3 Phenyl-Hexyl column

In summary, the direct comparison of the three different phenyl based stationary phases shows a similar retention time pattern. The elution order was the same on all columns.

## CONCLUSION

The results demonstrate that the pentafluoro phenyl (PFP), conventional phenyl, and phenyl-hexyl stationary phases are suitable for the ion-pair-free RP-HPLC analysis of the investigated oligonucleotides. On the PFP phase, reducing the initial amount of eluent B from 20 to 10 % improved retention of the less-retained samples, particularly SCR Antisense. Increasing the maximum amount of eluent B to 50 % enabled complete elution of the dT24 C12 sample and improved the overall method performance. The transfer of the optimized gradient to the conventional phenyl phase resulted in comparable retention behavior and the same elution order as observed on the PFP phase. Increasing the maximum amount of eluent B to 60 % caused earlier elution of dT24 C12 and improved the peak symmetry of the 64mer, while only minor changes were observed for the SCR Antisense and 20mer samples.

The phenyl-hexyl phase also showed the same general retention pattern and elution order as the PFP and conventional phenyl phases. Because of the different ligand structure, combining an aromatic phenyl group with a six-carbon alkyl linker, it provides a distinct balance of hydrophobic and aromatic interactions. Direct transfer of the phenyl gradient resulted in earlier elution of all oligonucleotides after adjustment of the gradient slope. However, the modified gradient did not produce a significant improvement in peak shape. Under the investigated conditions, the phenyl-hexyl phase therefore did not show a clear performance advantage over the conventional phenyl or PFP phase. The comparable elution order observed on all three columns indicates that the retention behavior was governed not only by the stationary-phase chemistry but also by oligonucleotide length, chemical modification, secondary structure, and nucleobase accessibility. A clear superiority of one phase could not be established for the selected samples.

Compared with the zwitterionic HILIC method described in **VBS0090**, the phenyl-based phases provide complementary selectivity through hydrophobic, dispersive, and π–π interactions with exposed nucleobases. By avoiding ion-pairing reagents, the phenyl, PFP, and phenyl-hexyl phases offer potentially improved compatibility with downstream mass spectrometry and reduced risk of system contamination or ion suppression. Overall, IP-RPLC, HILIC, and ion-pair-free RP-HPLC on aromatic stationary phases provide complementary options with respect to analysis speed, selectivity, and MS compatibility. The choice between conventional phenyl, PFP, and phenyl-hexyl phases should therefore be based on the oligonucleotide sequence, length, chemical modifications, secondary structure, and the required analytical selectivity.

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## MATERIAL AND METHODS



**Fig. 10** System configuration

**Tab. 5** System configuration

| Instrument | Description | Article No. |
|---|---|---|
| Pump | AZURA® P 6.1L Pump LPG, Stainless Steel, 10 ml/min | APH34EA |
| Autosampler | LH 8.1 Liquid Handler, 557 mm version with Robotic Cooler, including injection valve and fast wash station | A51001 |
| Sample Loop | Sample loop 20 µl, flexible stainless steel tubing with 1/16" ends, ID 0.25 mm | A05646 |
| LH Syringe | Hamilton X-Type 25µL PTFE Tip, fixed Nadel 22s gauge | A510542 |
| Detector | AZURA® DAD 2.1L Diode Array Detector, 190-700 nm | ADC01 |
| Flow Cell | UV Flow Cell Cartridge, LightGuide, Analytical, 10 mm, 2 µl, SSt, 1/16'', 50 bar, up to 5 ml/min | AMC19XA |
| Thermostat | AZURA® CT 2.1 Column Thermostat for up to 8 HPLC columns with temperature range between 5-85 °C | ATC00 |
| Column I | Eurospher II 100-3 PFP, 50 x 2 mm ID | 05BE071E2G |
| Column II | Eurospher II 100-2 Phenyl, 50 x 2 mm ID | 05BE050E2F |
| Column III | Eurospher II 100-3 Phenyl-Hexyl, 100 x 2 mm ID | 10BE161E2G |
| Software | ClarityChrom 10.1 station single instrument license one time base Workstation, autosampler control included | A1670 |
| Software | ClarityChrom 10.1 PDA license for PDA data processing | A1676 |

**Tab. 6** LH 8.1 cleaning settings used for all measurements

| Parameter | Description |
|---|---|
| Solvent 1 | Methanol |
| Solvent 2 | Methanol:water 10:90 (v/v) |
| Injection Mode | Sandwich |
| Sandwich Solvent | Solvent 1 |
| Pre-clean Syringe | 3 cycles Solvent 1 (25 µl), Stay |
| Post-clean Syringe | 1 cycle Solvent 2 (1 µl), Stay<br>2 cycles Solvent 1 (25 µl), Stay |
| Sample Wash | - |
| Valve Wash | 3 cycles Solvent 1 (25 µl)<br>Execute: After post-clean syringe |

## REFERENCES


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