Shodex™

HPLC Columns

The advantages of pressure-resistant, high-performance polymer columns in HPLC and LC-MS

State-of-the-art polymer columns can achieve more than you think, breaking the limitations of silica gel columns

Contents

What to expect from this whitepaper

There are many myths about polymer phases in high-performance liquid chromatography (HPLC). Unlike silica gel based columns, they have not yet established themselves as the standard choice for many chromatographic applications – neither in laboratories nor in the minds of users. However, despite being so popular, silica gel cannot do it all. On the contrary: silica-based phases are severely restricted in their application range due to the material used. Many separations are much easier to do by instead using polymer phases.

This whitepaper will demonstrate to you the advantages of state-of-the-art high-performance polymeric stationary phases compared to classic silica gel phases in HPLC and LC-MS. Eleven examples of applications, ranging from food and bioanalytics to the quality control of pharmacological active ingredients and therapeutics, will shed light on the vast range of exciting application areas.

The whitepaper will help you to complement your own portfolio of analytical techniques with polymer-based separation columns for new approaches to your chromatographic challenges.

1. Popular, but no all-rounders: Silica gel columns and their limitations in liquid chromatography

Hydrophobic surface-modified silica gels of different selectivities and particle size distributions are by far the most important stationary phases for HPLC and U(H)PLC. Despite its popularity, silica gel has the disadvantage that a high pH mobile phase can dissolve the silica gel matrix. As a consequence, it is recommended to stay within a limited pH range of between 2 and 7 for the mobile phase when using a silica gel based separation column.

Conversely, this means that the selectivity of an alkaline eluent cannot be used when optimizing a separation. Furthermore, some synthetically produced silica gels are not perfectly pure with respect to their metal ion content. In chromatography of alkaline analytes, this results in inferior peak shapes and often poorly reproducible retention times. In the case of surface-modified phases, there is the added risk that unreacted (free) silanol groups cause retention of alkaline compounds due to their acidity. Here, too, peaktailing and poorly reproducible retention times often occur due to strong adsorptive interactions between the free silanol groups of the stationary phase and the analyte.

For these reasons scientists have repeatedly requested that a pH-stable phase be developed that requires only short analysis times. When silica gel is used, only rather specially bound HPLC phases or hybrid materials with an optimized particle size distribution meet these requirements.

> „What options are there to meet analytical challenges when using pressure-resistant, high-performance polymer columns instead of silica gel based separation columns?“

2. Robust, versatile and long-lasting: The advantages of polymer-based LC columns?

Polymeric stationary phases are designed to meet the following requirements: an extended pH range from 2 to 13 for applications, no unwanted silanol interactions and high chemical purity with respect to metal ions. In addition, bleeding is low compared to surface-modified reversed phases (RP), enabling sensitive detection, especially when using particle-sensitive techniques such as multiangle light scattering (MALS). Their column lifetime is also two to three times longer than that of silica gel based separation columns (Fig. 1). Due to their pronounced chemical stability in all aqueous and organic solvents, polymeric phases allow the separation of acids and bases with the corresponding acidic or alkaline eluents.

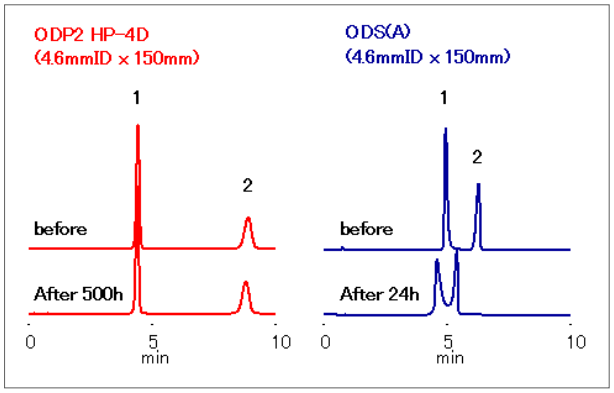


Fig. 1 Comparison: polymer column and silica column

Advantages of polymer-based versus silica-based stationary phases:

- Extended application range from pH 2 to 13
- No unwanted silanol interactions
- High chemical purity with respect to metal ions
- Low column bleeding
- Sensitive detection particularly with particle-sensitive detectors
- Two to three times longer column lifetime
- Separation of acids and bases thanks to high chemical stability
- Pressure-resistant even at 150 bar to 200 bar

The polymer support determines selectivity

The selectivity of polymer phases is determined by the choice of the appropriate polymer type. For example, polystyrene-divinylbenzene-based phases are very unpolar and are thus often used as stationary phases in gel permeation chromatography with purely organic solvent mixtures or functionalized for ion or ligand exchange chromatography.

Polar polymethacrylate and polyhydroxymethacrylate can be used in bioanalytics for (aqueous) size exclusion and affinity chromatography. Separation columns based on hydrophilic or hydrophilic-modified polymers are particularly interesting for polar analytes that are not sufficiently retained on conventional reversed phases. In such a case, an aqueous normal-phase system, also known as hydrophilic interaction liquid chromatography (HILIC), can be used.

Functionalized polyvinyl alcohol (PVA) is an ideal base material for HILIC and RP applications. For a HILIC separation, the functional groups can be primary or tertiary amines or, in the reversed phase, C18, C8 or C4 groups. Alternatively, ion exchange chromatography on mixed-mode phases showing both RP and ion exchange mechanisms should be considered.

Versatile and pressure-resistant

In short, polymer phases are extremely versatile. In addition, the narrower particle size distribution of many polymer columns ensures better separation efficiency. The pressure stability of most polymer phases is sufficiently high; most reversed phases can be used in a pressure range between 150 bar and 200 bar.

3. Practical applications – these are the ones you should know about

The following applications characterize a new generation of pressure-resistant, efficient polymer phases with their impressive range of analytical applications.

3.1. Small molecule and larger biomolecule analytics using reversed phase HPLC

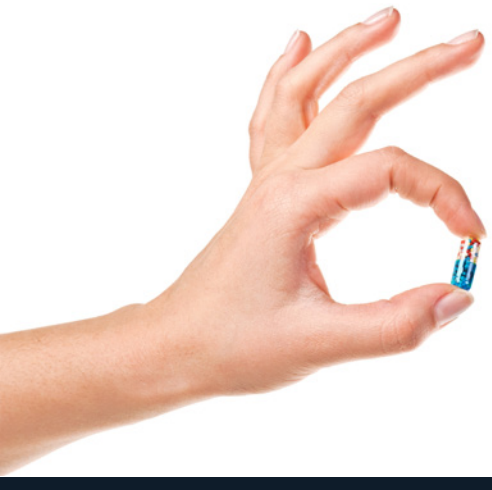
Depending on the pore size, reversed phases can be suitable for analyzing so-called "small molecules" as well as larger biomolecules such as proteins and peptides.

3.1.1 Analysis of small molecules using polyhydroxymethacrylate columns

The most commonly used reversed phases are based on silica gel. Turning to high-performance polymer phases, **the Shodex™ ODP2 HP** family of separation columns is a new alternative based on polyhydroxymethacrylate particles with 40 Å pore size.

The columns are pressure-resistant up to 150 bar and available with a standard inner diameter (ID) of 4.6 mm as well as 2 mm for LC/MS.

The resolution is high: ODP2 HP has almost twice as many theoretical plates as typical polymer-based reverse phase columns and, due to its smaller pore size, is predestined for RP separations of small molecules such as pharmaceutical compounds in the presence of protein matrix.



The column shows better retention of polar substances than the silica-based ODS equivalent.

It is ideal for LC/MS analysis of polar compounds; its pH stability from 3 to 12 is extremely useful to optimize the mobile phase (corresponds to USP L39; no functional group on base material).

The polar interaction between the stationary phase and the polar analyte does not require a functional group such as C18, since the polymer stationary phase (polyhydroxymethacrylate) itself is highly polar and can therefore interact.

> „The Shodex™ ODP2 HP is predestined for separating small molecules such as pharmaceutical compounds in the presence of protein matrix.“

3.1.2 The all-rounder not only for small molecules

The **Shodex™ Asahipak ODP-50** or **ODP-40** is another separation column showing a similar behavior as the equivalent, conventional ODS phase.

The phases consist of polyvinyl alcohol (PVA) as the stationary phase with C18 as the functional group. The column is available with 5 µm or 4 µm particle sizes and in a variety of lengths and inner diameters. Its excellent robustness allows even difficult matrices to be separated.

The column is particularly suitable for pharmaceutical "small molecules", but also to separate amino acids, peptides and proteins. Thanks to its exceptionally wide range of applications, the Shodex™ Asahipak ODP-50 or ODP-40 can effectively replace many silica C18 columns available on the market.

The column has a working pH range from 2 to 13 and is 100% polymer-based and thus silanol-free. There are far less adsorptive interactions due to the absence of (free) silanol groups, which reduces the loss of protein on the column. The column can be operated with 100% water and is predestined for the analysis of alkaline substances. It also meets the requirements of USP L67.

> Thanks to its extremely wide range of applications, ranging from small molecules to amino acids, peptides and proteins, the Shodex™ Asahipak ODP-50 and ODP-40 can effectively replace many different silica C18 columns on the market.

An application for antibiotics

This application sheds light on the analysis of ampicillin using the reverse phase column Asahipak ODP-50 4D.

▸ Click here to find out more

3.1.3 Quantitative determination of water-soluble vitamins in multivitamin products

The **Shodex™ RSpak DE-413L** (4.6 mm ID x 250 mm, 4 µm) is ideally suited for separating water-soluble vitamins. RSpak DE are polymer-based RP separation columns. They can be used universally over a wide pH range. The pore size is 25 Å, the particle size distribution 4 µm and it is pressure resistant up to 180 bar.

A remarkable application of this separation column is the quantitative determination of **water-soluble vitamins** in commercially available multivitamin products.

The sample preparation of the multivitamins is simple: a dilution step using diluted phosphoric acid and ultrasonic treatment, with only folic acid being prepared differently. Because this HPLC method was designed for routine analyses, robustness and reproducibility are particularly important. The polar properties of water-soluble vitamins necessitate adding ion pair reagents to the mobile phase under RP conditions in order to achieve sufficient retention on the separation column.

Using ion pair reagents places special demands on the method to ensure high reproducibility and robustness. If the concentration of the reagent is unsuitable or the equilibration times are insufficient, problems often occur during method validation. For less robust HPLC separations it is problematic to use ion pair reagents to make a hydrophobic stationary phase suitable for the analysis of polar substances.

It is better to select a suitable separation column, e.g. from the polymer-based RSpak DE series with similar hydrophobicities as ODS columns, but which can be used universally over a wide pH range even under highly aqueous elution conditions.

There is also no dewetting effect with purely aqueous eluents and thus no loss of retention compared to hydrophobically modified C18 phases.

> While the polymer-based RSpak DE RP separation column's hydrophobicity is similar to that of ODS columns it can be used universally across a wide pH range, allowing stable analyses even under highly aqueous elution conditions.

Figure 2 shows the chromatograms of the vitamin standard solutions during UV detection. Within 20 minutes (including equilibration time) the ten vitamins are baseline separated in a phosphoric acid/acetonitrile gradient according to the developed method. Detection is performed at 254 nm and 210 nm respectively. The method achieves good linearity for all vitamins and can therefore be used to quantitatively determine water-soluble vitamins in commercial multivitamin products.

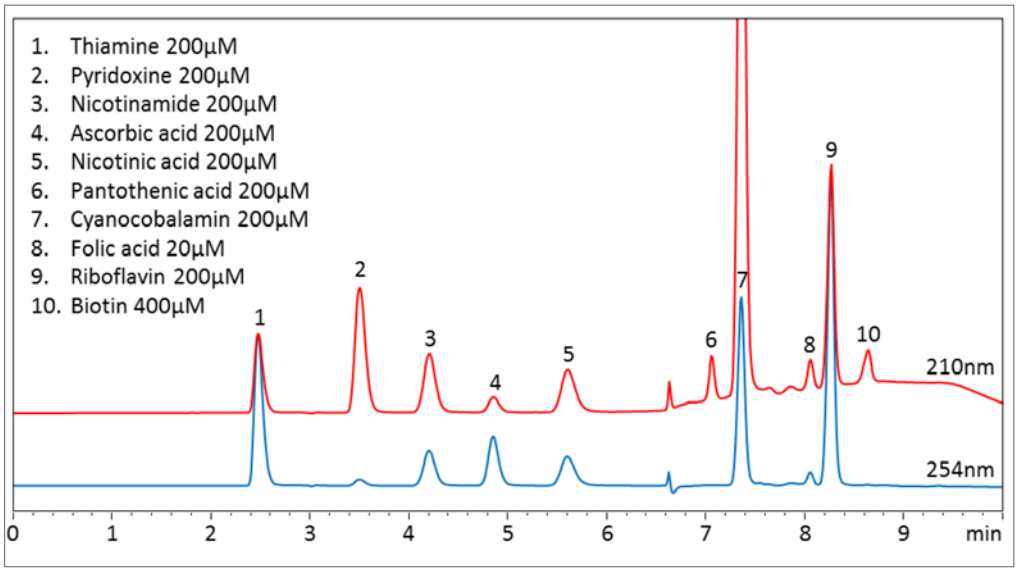


Fig. 2 Analysis of a multivitamin product by UV detection

3.1.4 Amino acids, peptides and proteins – how to succeed with large biomolecule analytics

For separating biomolecules, C4 or C8 polymer columns with a significantly larger pore size and lower hydrophobicity than C18 columns are suitable. The **Shodex™ Asahipak C4P-50** and **Asahipak C8P-50** columns, whose functional groups are C4 and C8 on a purely polymeric particle, are worth mentioning here. The relatively large pore size is suitable for the analysis of amino acids, peptides and proteins. These PVA-based polymeric separation columns can be used with purely aqueous eluents or buffer solutions in a wide pH range from 2 to 13.

If typical C18 reversed phases are to be used for the analysis of amino acids, peptides, proteins and small molecules, the **Asahipak ODP-50** or **ODP-40** (described above) with a relatively large pore size of 250 Å and available in two different particle size distributions of 5 µm or 4 µm can also be used. ODP-40, with its 4 µm, provides the better separation performance. Both column materials are based on PVA and can be used in a wide pH range from acidic to alkaline (pH 2 to 13). Under acidic elu-

tion conditions, peptides can be separated particularly well. Since the base polymer is free of silanol, the peak shape and thus also the separation of bases is improved (corresponds to USP L67).

For analyzing larger biomolecules such as proteins and peptides, a reversed phase based on PS-DVB is also suitable: the **Shodex™ RSpak RP18-415** with its pore size of 450 Å and pressure resistance up to about 220 bar (corresponds to USP L21).

3.2 When reversed phases are not an option: Analyzing highly polar substances by hydrophilic interaction liquid chromatography (HILIC)

Hydrophilic interaction liquid chromatography has become established for separating polar compounds, especially in food, pharmaceutical and biotechnological analytics.

This technique, which is orthogonal to RP chromatography, provides marked retention of all substances that are hardly or not at all retained on reversed phases, i.e. which elute near the column's dead time.

The elution order is typically the reverse of that in RP chromatography, in which the polar substances are eluted after the non-polar ones.

Here are some polymeric HILIC phases and their applications.

Application for sugar analysis

In two applications we compare the polymer column Asahipak NH2P-50 with a conventional silica column for separating different sugar molecules.

▸ To find out more, click here and here

3.2.1 The ideal column for sugar analytics and particle-sensitive detectors

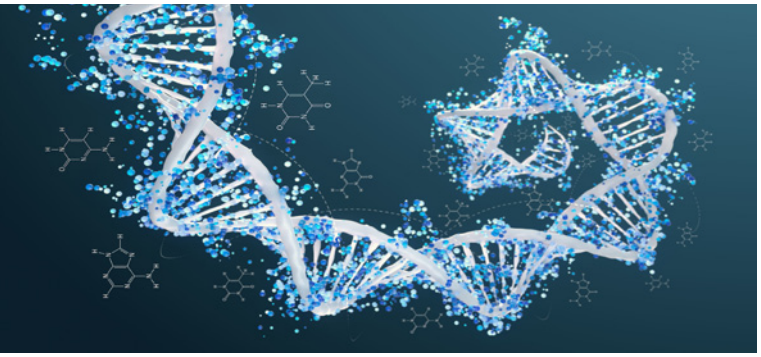
Conventional HILIC separations are currently performed using silica-based columns with primary amino or amide groups as the functional groups.

Particularly in sugar analytics, the separation of anomeric forms of sugars (e.g. alpha and beta glucose) causes considerable problems with pH stability. To suppress the equilibrium of the anomeric sugar forms, higher pH values and/or higher temperatures are required. Neither is possible over a longer period with silica-based amino columns.

The **Shodex™ Asahipak NH2P-50** column does not have this limitation. Due to the phase stability pointed out above, also regarding the column's low tendency to bleed, it is ideally suited for particle-sensitive detections such as evaporative light scattering. This method is much more sensitive than the usual refractive index detection.

The columns are also suitable for LC/MS.

„The Asahipak NH2P-50 is ideally suited for particle-sensitive detections such as evaporative light scattering due to its phase stability, and with the column's low tendency to bleed.“



3.2.2 Analyzing saccharides, organic acids and amino acids in parallel

The PVA-based separation column **Shodex™ HILICpak VG-50 2D** offers advantages for the analysis of monosaccharides, disaccharides and oligosaccharides as well as other polar compounds, organic acids and amino acids under alkaline gradient conditions with ELSD, Corona-CAD as well as LC/MS.

Because the base polymer is functionalized with tertiary amino groups, that are sterically protected, the formation of Schiff bases is avoided during analyses of reducing sugars. This results in high recoveries during elution. A further advantage is reduced column bleeding and thus the long working life of the separation column.

Regeneration using alkaline mobile phases is possible. The main advantage of the **HILICpak VG-50 2D** over silica-based amino columns is that its selectivity changes under alkaline conditions. Anionic compounds, such as organic acids, would usually be retained, but by using an alkaline mobile phase they are eluted and can be analyzed.

The polymer-based amino column **HILICpak VG-50 2D** offers a number of separation advantages under alkaline elution conditions. Saccharides, organic acids and amino acids can be analyzed simultaneously, which was previously only possible with precolumn derivatization or the addition of ion pair reagents to the mobile phase.

LC/MS with ammonia-water/acetonitrile gradient elution achieves satisfactory separations of different hydrophilic compounds and high sensitivity in ESI/MS detection (Fig. 3). The column temperature of 40°C allows the viscosity of the eluent to be lowered and reduces pressure fluctuations in the course of the gradient profile.

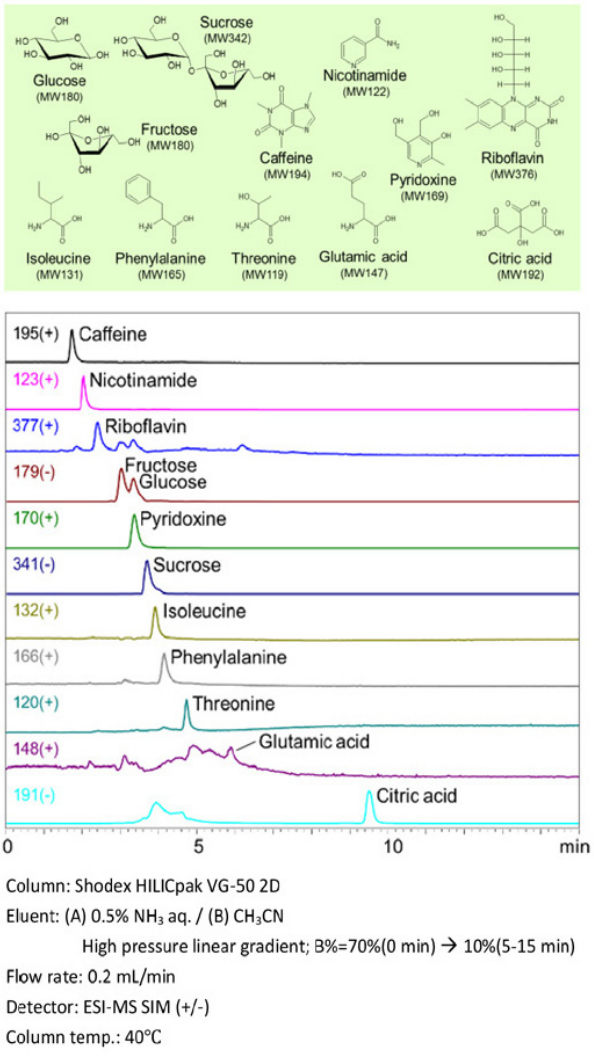


Fig. 3 LC/MS analysis of an energy drink

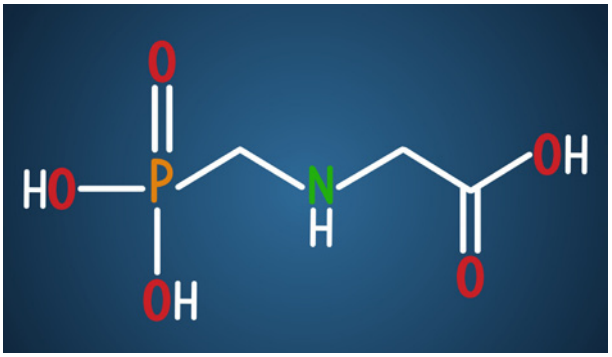
> „The HILICpak™ VG-50 2D is also suitable for analyzing saccharides, citric acid, amino acids, caffeine and water-soluble vitamins in energy drinks“

The method described here is also suitable to test **commercial energy drinks** for saccharides, citric acid, amino acids, caffeine and water-soluble vitamins.

3.2.3 Simultaneous analysis of glyphosate and five other herbicides or metabolites using LC-MS/MS

The **Shodex™ HILICpak VT-50 2D** column (2.0 mm ID x 150 mm, 5 µm), based on a PVA polymer, is suitable for analyzing anionic substances using HILIC. Depending on the selected eluent, it also allows the functional quaternary ammonium groups to work in anion exchange mode. The PVA polymer base provides excellent chemical stability and a long column life. The column can be used with LC/MS-compatible buffers and is also suitable for separating phosphorylated sugars.

An extremely interesting application, given the public scrutiny that glyphosate/glufosinate have come under, is using the separation column HILICpak VT-50 2D for highly sensitive LC/MS-MS and simultaneous analysis of six organophosphate herbicides or their metabolites under alkaline elution conditions.



This method makes it possible to determine glyphosate and its metabolite aminomethylphosphonic acid (AMPA), glufosinate and its metabolite 3-methylphosphinicopropionic acid (MPPA) as well as ethephon and fosetyl in parallel.

The PVA-based column is functionalized with quaternary ammonium groups. Because glyphosate forms metal complexes that can lead to peaktailing, PEEK is used for the column hardware instead of steel. The inner diameter of 2 mm ensures high LC/MS sensitivity.

With only small amounts of organic solvents in the eluent the column can operate in the RP/ion exchange mode, with large amounts in the HILIC mode.

> This method allows the fast and robust analysis of organophosphate herbicides and related compounds within 20 minutes and without any need for precolumn derivatization, ion pair reagents or gradient elution.

Click here to find out more

3.2.4 Simplified analysis of cationic, low-molecular substances such as amino acids, neurotransmitters and various pharmaceuticals

Hydrophilic, positively charged analytes with low molecular weight, such as choline, acetylcholine and other neurotransmitters, oral antidiabetics and aminoglycoside antibiotics are best separated using the **Shodex™ HILICpak VC-50 2D** separation column based on a PVA polymer with carboxyl group functionalization.

This results in simpler analysis conditions than with other methods. Figure 4 shows a schematic representation of the polymer used for VC-50 2D.

Fig. 4 Functional carboxyl group

Due to its hydrophilicity, the acetonitrile content in the eluent can be increased to enhance the effectiveness of the HILIC mode, leading to increased retention of hydrophilic compounds. Cation exchange mechanisms also provide additional retention of positively charged analytes on the stationary phase.

Amino acids: In addition to neurotransmitters, successful baseline separation is also possible for amino acids.

Figure 5 shows the LC/MS chromatograms of cysteine and 20 other amino acids. The alkaline amino acids histidine, lysine and arginine are strongly retained on the column due to cationic ion exchange effects.

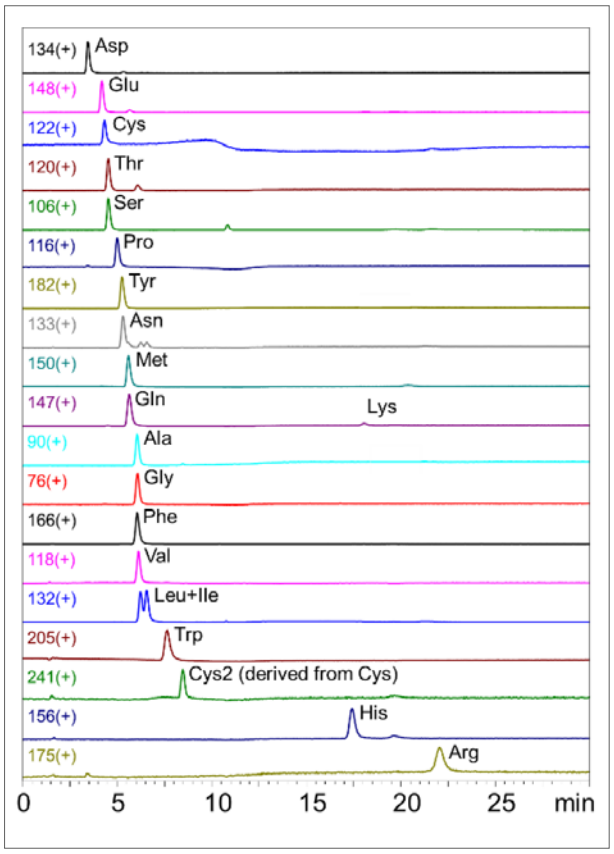


Fig. 5 Separation on the HILICpak VC-50 2D

The elution of these compounds is achieved by increasing the proportion of formic acid in the gradient elution, thereby weakening the cationic ion exchange bonds. In addition, the two isomeric compounds leucine and isoleucine are separated.

Small molecule peptides: Potential is currently being discovered in the functionalities of small molecule peptides in medicine, pharmaceuticals and food. For example, such peptides can serve as interesting labeling precursors in radiochemistry. Figure 6 shows the LC/MS chromatograms of five dipeptides and three tripeptides.

The results clearly demonstrate that HILICpak VC-50 2D is suitable also for the analysis of small molecule peptides.

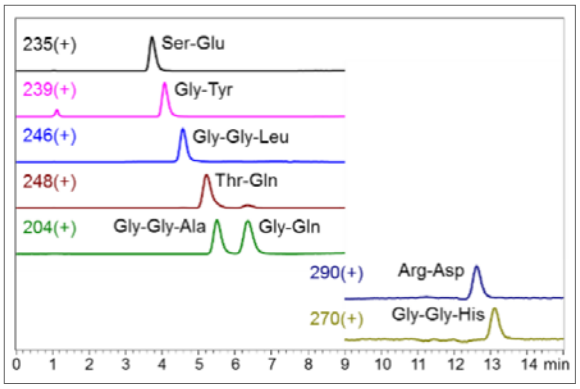


Fig. 6 LC/MS separation of small molecule peptides

Aminoglycoside antibiotics: Streptomycin and its derivative dihydrostreptomycin are aminoglycoside antibiotics. They are separated under acidic, formic acid containing elution conditions. The LC/MS chromatograms of streptomycin and dihydrostreptomycin are shown in Figure 7.

Other aminoglycosides such as kanamycin, tobramycin, gentamicin, neomycin and amikacin are retained on the column even if the formic acid concentration is increased to 500 mM.

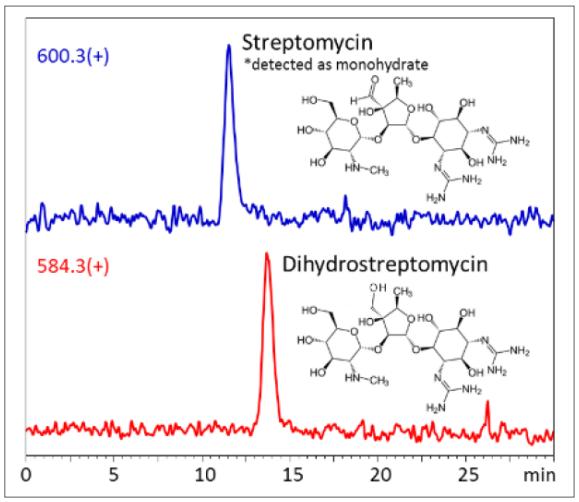


Fig. 7 Antibiotics analysis without ion pair reagent

These aminoglycoside antibiotics possess more than four functional amino groups, making them more cationic than streptomycin and dihydrostreptomycin.

If ammonia is now added to the eluent, these five compounds elute.

Figure 8 shows the LC/MS chromatograms of aminoglycoside antibiotics. One possible explanation is that using the alkaline eluent suppresses the dissociation of the amino groups in the aminoglycosides and thus also the ionic interactions between the analytes and the polymeric support, resulting in elution.

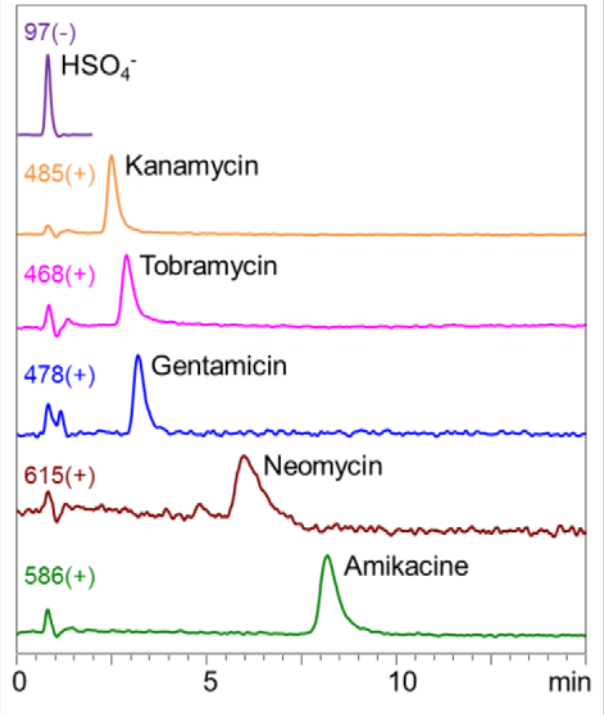


Fig. 8 HILIC separation under alkaline conditions

In the above application simple LC/ESI-MS separations to analyze low molecular weight cationic compounds with formic acid/acetonitrile elution are carried out using the polymeric carboxyl HILIC column Shodex™ HILICpak VC-50 2D.

Alkaline elution conditions are also recommended for analyzing cationic compounds, which under acidic conditions are usually strongly retained on the column.

> Using gradient elution offers another advantage if several compounds with considerable differences in their cationic strength are to be separated simultaneously.

3.2.5 Highly selective separation of oligonucleotide therapeutics using a conventional HILIC column

The column used for this application, **Shodex™ HILICpak VN-50 2D**, is a conventional HILIC column based on PVA with diol group functionalization. Again, a separation column with an inner diameter of 2 mm is used to enable sensitive LC/ESI-MS detection. Not only oligosaccharides, which cannot be separated by size exclusion chromatography or with conventional HILIC phases, but also oligonucleotides can be separated with gradient elution.

This application describes the analysis of up to 30-mer oligonucleotides with the polymer-based HILICpak VN-50 2D diol HILIC column in combination with LC/UV/ESI MS. The method separates the oligos depending on their degree of polymerization, thereby achieving a high level of selectivity. Gradient elution with 50 mM ammonium formate/acetonitrile is straightforward, especially when compared with the commonly used RP chromatography, where an ion pair reagent must be added, or with ion exchange chromatography, which requires the addition of highly concentrated salts.

The volatility of the eluent used here also simplifies the desalting process during purification.

In addition, the polymer-based column material allows the use of alkaline wash solutions that prevent unspecific adsorption on the column and minimize any associated difficulties during analysis or in preparative scale set-ups. This is why the HILICpak VN-50 2D series columns presented in this application are predestined for use in the development, quality control and purification of oligonucleotide therapeutics.

Click here to find out more

3.3 Analysis of protein aggregates and additives in antibody therapeutics

3.3.1 Separation of monomers and dimers in antibody therapeutics

Antibody therapeutics are highly effective biopharmaceuticals and play an important role particularly in immunology and cancer therapy.

However, it is known that during the manufacturing process and/or storage they can interact to form dimers, trimers and other larger aggregates.

> In addition to the loss of efficacy, it is suspected that there is a particular immunogenicity of these protein aggregates with potential clinical effects in patients. Monitoring of such aggregates is therefore extremely important in quality control.

To separate protein aggregates, silica-based SEC columns are generally used. The **Shodex™ PROTEIN LW-803** is a comparable column with a high level of separation efficiency when compared with the columns of other manufacturers. To compare columns, IgG with a molecular weight (MW) of approx. 150,000 is analyzed.

The following size exclusion chromatography (SEC or GFC) columns, all of which are recommended for antibody analysis, were compared:

- **Shodex™ PROTEIN LW-803** (8.0 mm I.D. x 300 mm, 3 µm)
- **Column A, different manufacturer** (7.8 mm I.D. x 300 mm, 4µm)
- **Column B, different manufacturer** (7.8 mm I.D. x 300 mm, 2.7 µm)

The comparison in Figure 9 shows the PROTEIN LW-803 column to be more efficient at separating IgG monomers (4), dimers (3), trimers (2) and aggregates (1).

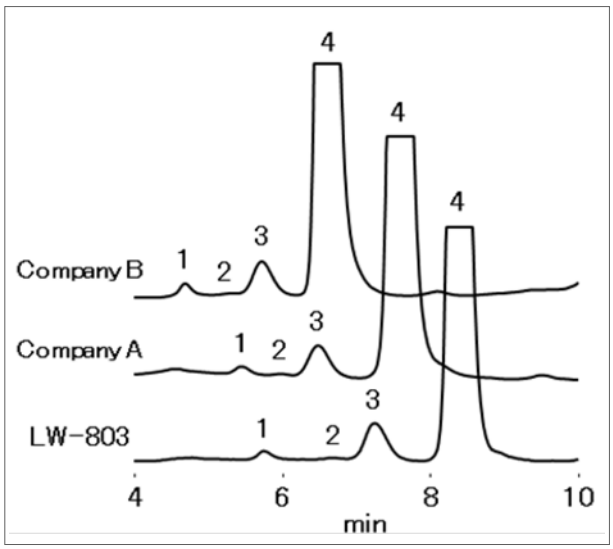


Fig. 9 Separation of IgG aggregates

The column can also be used to separate lower molecular weight compounds, such as antibody metabolites or other proteins, making it suitable for a wide range of applications.

3.3.2 LC/MS analysis of surfactants in antibody drugs

The **Shodex™ ODP2 HP-2D** – a polyhydroxymethacrylate-based reversed phase column with a high separation efficiency – is extremely hydrophilic in its properties, so proteins can be separated from low molecular weight compounds.

In addition, its small pore size of 40 Å enables size exclusion, so proteins elute in the dead volume. Therefore IgG can be completely separated from surfactants, which are retained and separated on the column by the RP mechanism. This is what is investigated in this application.

To prevent IgG from entering the mass spectrometer, a switching valve is placed behind the UV detector. The column's eluate obtained between 0 and 5 minutes is collected in a waste bottle and the eluate obtained after 5 minutes is injected into the mass spectrometer.

Tween was selected as the test surfactant in this application because it is often added to antibody pharmaceuticals. Tween con-

sists of a sorbitol backbone, polymers of ethylene oxide (EO) and long-chained fatty acids linked by esters.

Because Tween is not a defined single-molecule compound, the ion suppression experiments were performed with Tween 20 and Tween 80 standard and IgG in NaCl solutions.

„It was shown that the Shodex™ ODP2 HP-2D column rapidly separates non-ionic surfactants like Tween in the presence of IgG and NaCl and enables highly sensitive mass spectrometric detection with minimal ion suppression. It is unnecessary to deproto-nate or desalt the sample.“

Click here to find out more

4. Summary

High-performance polymer reversed phases have basically reinvented RP chromatography. They do not have the drawbacks of silica gel modified phases and allow the selectivity of an alkaline eluent to be effectively used for method development. In routine analysis it is therefore now possible to reduce the use of inflexible ion exchange methods or methods that require adding ion pair reagents and make MS detection difficult or impossible due to non-volatile buffer solutions.

Hydrophilic interaction liquid chromatography is the ideal method for the retention and separation of hydrophilic/polar compounds in carbohydrate and amino acid analytics. In addition to this, using polymeric HILIC phases with diol functional groups to separate oligonucleotides is becoming a new field of application with great potential. This is also the case for the quality control of antibody-based drugs where there is the need to test for protein aggregates or dimers during the manufacturing process.

There are many myths about polymer phases. This whitepaper, however, has provided you with in-depth knowledge of the true performance capabilities of polymer phases and with many different applications that state-of-the-art high-performance polymers support. Polymer-based HPLC columns no longer stand in the shadow of conventional silica gel columns. Rather, they are able to enrich your analytics by offering new applications to meet your chromatographic challenges. Give the Shodex™ columns a try.

You will see: many separations can be done with much simpler mobile phases.

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