Increase Sensitivity with Microbore Polymeric HPLC Columns from Agilent

Technical Overview

Introduction

All Agilent PLRP-S media are sufficiently robust and mechanically stable to pack into efficient microbore columns with internal diameters of 1.0 mm and 2.1 mm. PLRP-S microbore columns offer substantially increased sensitivity over standard bore columns, mainly due to the solvent content being much reduced, at comparative linear velocities. The improvement in sensitivity that can be gained by using microbore columns compared to traditional 4.6 mm analytical columns is clearly evident.

Agilent Technologies

Charateristics of microbore HPLC

As well as sensitivity improvements with microbore HPLC (Figure 1), the reduced solvent content has other beneficial effects, such as lower solvent consumption (Figure 2) and therefore lower chemical and disposal costs, especially valuable given environmental concerns. The increased sample content also provides reduced sample losses and 'carryover'. The lower surface area of the column itself provides reduced non-specific adsorption losses. Thus, for a sample peak eluting over one minute, in standard bore chromatography the analyte would be diluted in 1 mL of solvent, whilst for microbore analysis, the analyte would only be diluted in 50 µL of solvent. This gives a theoretical increase in sensitivity on concentration dependent detectors (such as UV-Vis and MS) of a factor of 20. The higher concentration of sample allows for better post column derivitization, either enzymatic or chemical. The flow rates utilized in microbore systems are also better optimized for LC/MS coupling, as it is unnecessary to remove the bulk solvent prior to entry into the MS.

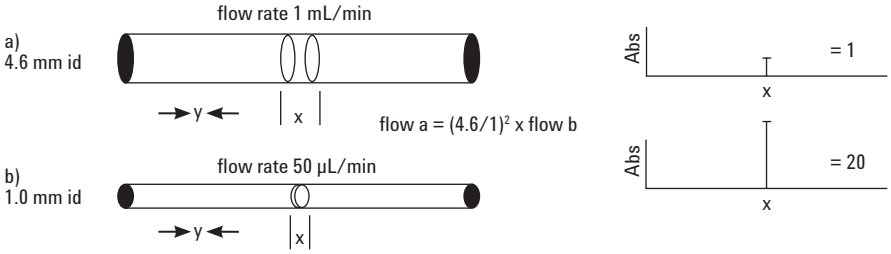

Figure 1. Increased sensitivity of a microbore system at comparative linear flow rates.

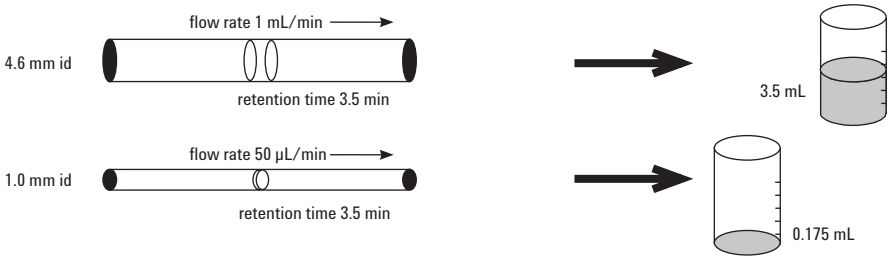

Figure 2. Solvent use in microbore systems.

Microbore HPLC does need to be optimized to obtain the best results. Low flow rates require reproducible, 'pulse free', low volume pumps. The system must have low dispersal characteristics and low dead volume to reduce dispersal and mixing which result in band broadening and poor peak shape. Connecting tubing must be of minimal id (50 µm) and short in length, with good, clean-cut end faces. High pressure gradient mixing is advisable due to the otherwise unacceptable time delay. At 50 µL/min, a 0.5 mL volume will take ten minutes to reach the column.

Another cause of poor peak shape can be in the detector flow cell where the standard bore flow cell's relatively high volume produces excessive mixing and poor peak shape. Changing to a specific microbore flow cell returns the good quality peak shape (Figure 3). The theoretical increases in sensitivity mentioned above can easily be achieved in an optimized system, as shown for a standard peptide separation (Figure 4). Where the microbore analysis increases in sensitivity, good peak shape is obtained.

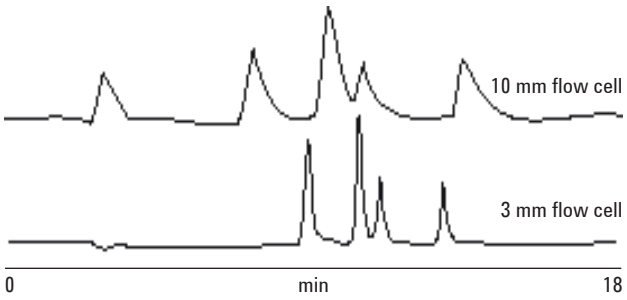

Figure 3. Pressure vs flow rate for two sizes of PLRP-S 300Å.

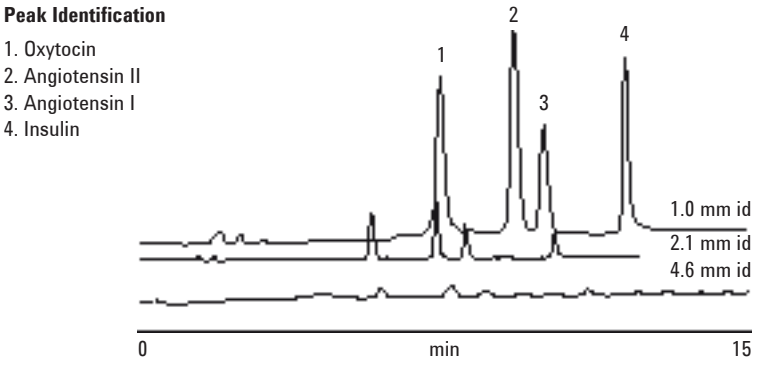

Figure 4. Peptide separation sensitivity on standard and microbore columns containing Agilent PLRP-S media.

Sensitive microbore HPLC of analgesics

The improvement in sensitivity that can be gained by using microbore columns compared to traditional 4.6 mm analytical columns is clearly evident in analgesic separations. Aspirin is a widely known analgesic receiving renewed interest due to its blood thinning properties that reduce the risk of heart attacks or thrombosis. Phenacetin is a similar pharmaceutical product, which has a much greater chromophore that affects the determination of the other samples at certain wavelengths. However, absorbance detection at a wavelength of 220 nm reduces this stronger absorbance of phenacetin, allowing detection of all three compounds in one analysis.

The increase in sensitivity of the microbore columns over the standard bore is clearly evident (Figure 5).

Peak Identification

1. Aspirin (comparative flow rate = 80 µL/min)
2. Phenacetin (comparative flow rate = 353 µL/min)
3. Salicylic acid actual flow rate = 1.5 mL/min) (comparative flow rate = 1.67 mL/min)

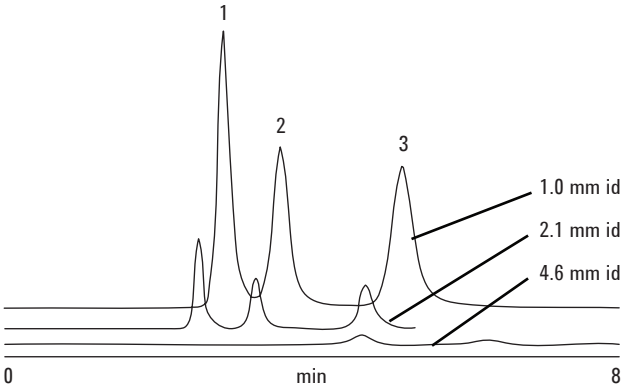

Figure 5. Analgesic separation comparison for 1.0 mm, 2.1 mm and 4.6 mm columns using Agilent PLRP-S media (isocratic system, 30% ACN, 0.1% TFA, 415 ng injection (per analyte), 0.5 µL injection valve, 0.8 µL flow cell).

The system was run under isocratic conditions, which were optimized for the 1.0 mm id column. The comparative linear flow rate of the 4.6 mm id column was 1.67 mL/min, but 1.5 mL/min was the highest flow obtainable with the HPLC equipment used. This is the main reason for the longer retention times observed for the 4.6 mm id column.

Standard curve plots were obtained for peak area versus concentration of analyte by serial dilution of known standards. The increased sensitivity of the 1.0 mm id column is clearly shown in Figures 6 to 8, as is on-scale linearity of the standard curve.

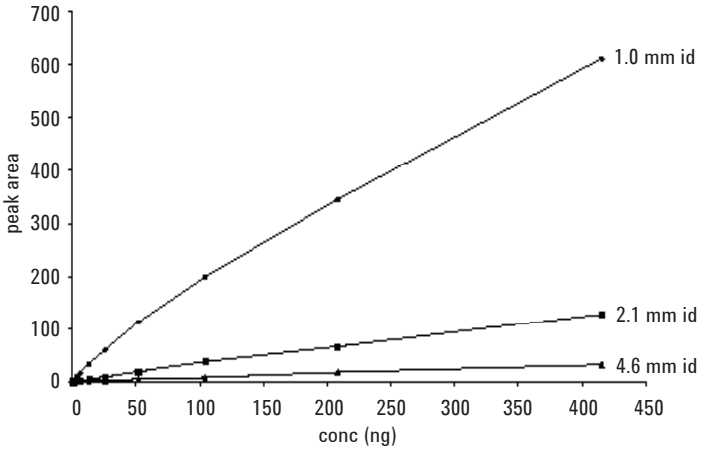

Figure 6. Phenacetin standard curve on Agilent PLRP-S.

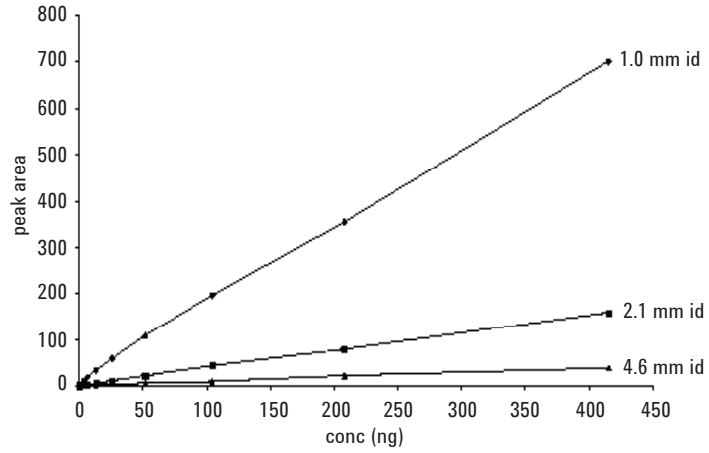

Figure 7. Salicylic acid standard curve on Agilent PLRP-S.

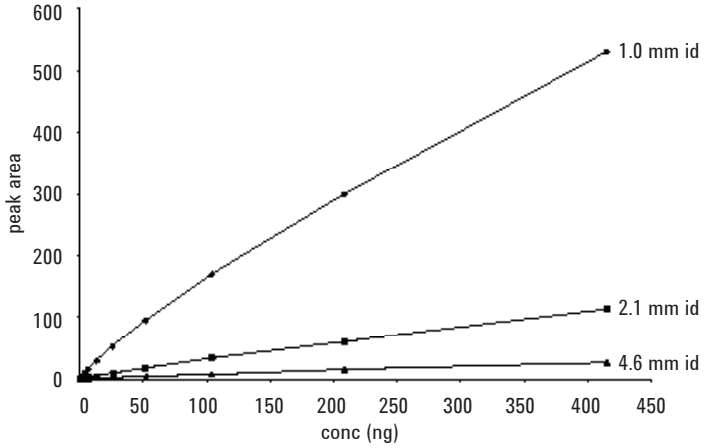

Figure 8. Aspirin standard curve on Agilent PLRP-S.

This linearity was observed throughout the range tested, producing a concurrent increase in lower detection limit (Figure 9). The detection limit was 25.33 pg for the 1.0 mm id column, 202 pg for the 2.1 mm id column but only 1.62 ng for the 4.6 mm id column.

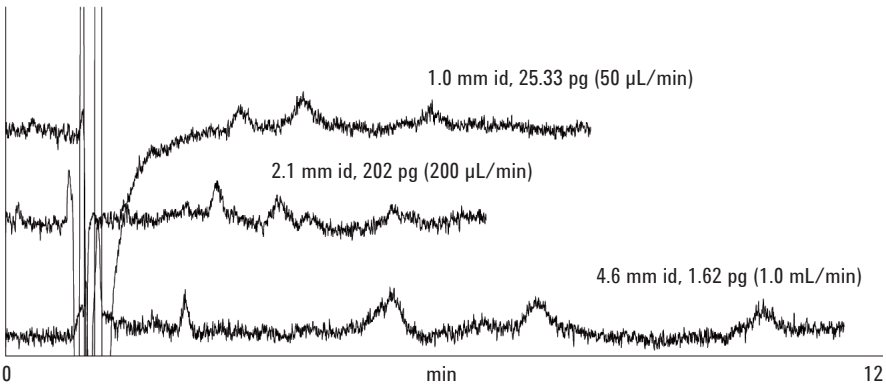

Figure 9. Analgesic detection limits on Agilent PLRP-S.

High resolution microbore HPLC of parabens

Parabens are esters of 4¬hydroxybenzoic acid commonly used as preservatives in a variety of products, including cosmetics. Parabens analyzed using PLRP-S media display much longer retention times than on alkyl bonded columns, and this allows greater resolution between the different paraben types. A mixture of methyl, ethyl and propylparaben was separated on PLRP-S 100Å columns of different id to compare sensitivity and peak shape. The 1 mm id microbore column showed very large improvements in sensitivity over the standard bore 4.6 mm id column at comparative flow rates (Figure 10).

Conditions

Columns:	PLRP-S 100Å 5 µm, 150 x 4.6 mm (p/n PL1111-3500) PLRP-S 100Å 5 µm, 150 x 2.1 mm (p/n PL1912-3500) PLRP-S 100Å 5 µm, 150 x 1 mm (p/n PL1312-3500)
Eluent:	50% ACN, 0.1% TFA
Inj Vol:	0.5 µL
Sample Conc:	1 µg per analyte
Detector:	UV, 254 nm

The 2.1 mm id column also showed improved sensitivity over the standard bore version. This increase in sensitivity was seen in all the standard curves (Figures 11 to 13). Good linearity in the detector response was also observed throughout the range of concentrations for every paraben. As a result of the increased sensitivity, the lower detection limit of the 1 mm id column was 0.975 ng compared to 1.95 ng for the 2.1 mm id column, and only 15.26 ng for the standard bore chromatography (Figure 14).

Peak Identification

1. Methyl paraben
2. Ethyl paraben
3. Propyl paraben

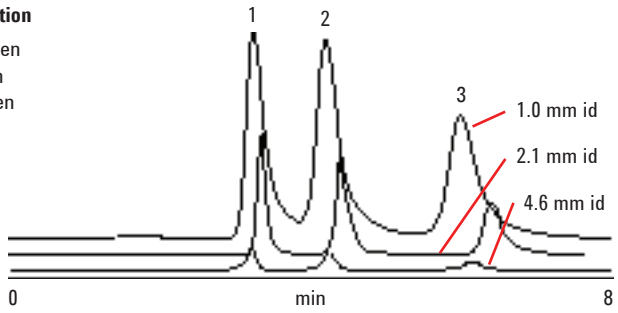

Figure 10. Comparison of Agilent PLRP-S columns of differing internal diameter in the analysis of parabens.

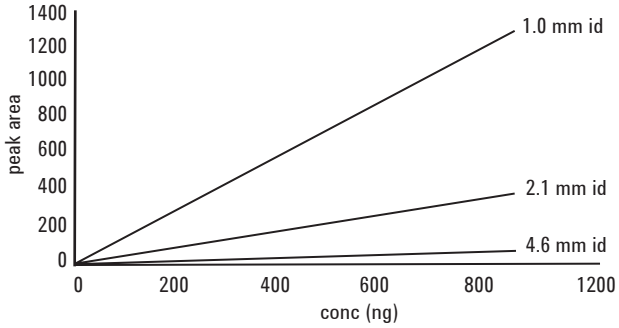

Figure 11. Methyl paraben standard curve on Agilent PLRP-S.

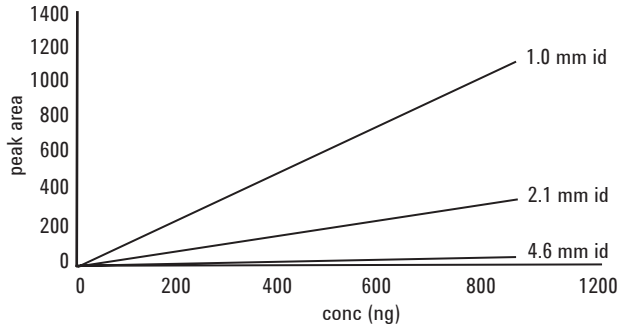

Figure 12. Ethyl paraben standard curve on Agilent PLRP-S.

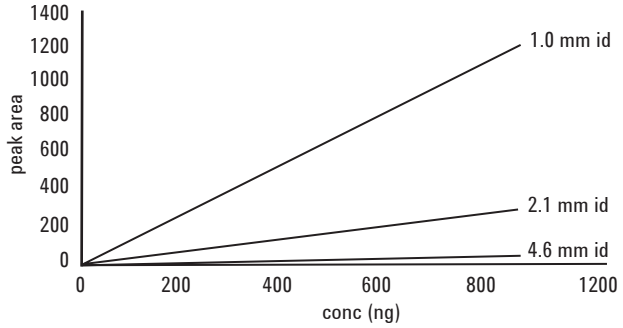

Figure 13. Propyl paraben standard curve on Agilent PLRP-S.

Peak Identification

1. Methyl paraben
2. Ethyl paraben
3. Propyl paraben

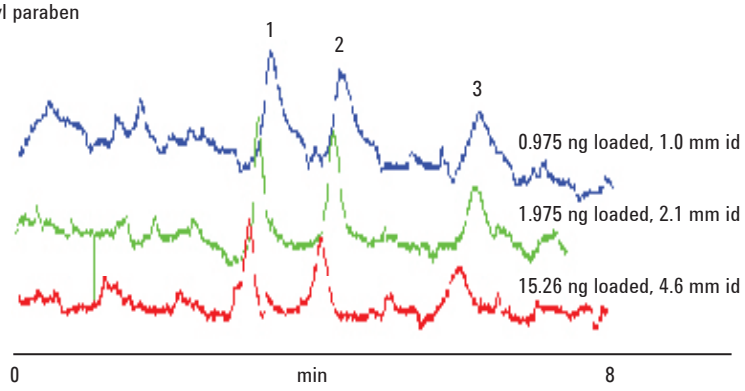

Figure 14. Detection limits of 1.0 mm, 2.1 mm and 4.6 mm id Agilent PLRP-S 5 µm, 150 mm columns.

Microbore analysis offers highly advantageous theoretical and practical increases in sensitivity and detection limits for all samples, especially where sample availability is at a minimum.

Microbore HPLC for sensitive peptide analysis

Most major advances in microbore technology have been driven by the protein and peptide separation field. This is due to the high K' displayed by peptides and proteins, which are ideally suited to high sensitivity gradient elution conditions. A perceived problem in microbore chromatography is the limited sample volume loading available, especially for trace sample analyses. However, this is much more a function of the analyte rather than the system. Samples with high K' values can be repeatedly loaded onto the column under binding conditions to allow 'on-column' sample enrichment. This can be particularly useful for protein and peptide analysis in dilute bulk biological media. The high sensitivity and lower detection limits are also ideally suited to applications where the amount of sample is the limiting factor. This is often the case in peptide and protein applications, such as peptide analysis, peptide mapping of proteins, amino acid analysis and terminal sequencing analysis. In all these cases, the microbore system's increased sensitivity has the potential to allow the user to probe further before the detection limits are reached.

The value of microbore PLRP-S is evident in an analysis of a standard peptide mix of oxytocin, angiotensins I and II and insulin. The increase in sensitivity for the same injection volume and concentration of the microbore columns over the standard bore 4.6 mm id column is clearly evident (Figure 15).

Conditions

Column:	PLRP-S 100Å 5 µm, 150 mm x various id
Eluent A:	0.01 M Tris HCl, pH 8
Eluent B:	A + 0.35 M NaCl, pH 8
Gradient:	Linear 20% ACN, 0.1% TFA to 50% ACN, 0.1% TFA over 15 min
Flow Rate:	1 mL/min
Inj Vol:	0.5 µL
Sample Conc:	0.25 mg/mL

Peak Identification

A. 1.0 mm if (flow rate 47 µL/min)
B. 2.1 mm if (flow rate 200 µL/min)
C. 4.6 mm if (flow rate 1 mL/min)

1. Oxytocin
2. Angiotensin II
3. Angiotensin I
4. Insulin

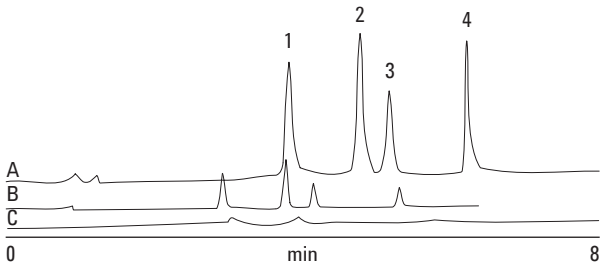

Figure 15. Peptide separation on Agilent PLRP-S 100Å 5 µm columns.

The increase in sensitivity was seen across the standard curve data points, all of which were linear and on scale (Figure 16).

Peak Identification

Diamond: 1.0 mm
Square: 2.1 mm
Triangle: 4.6 mm

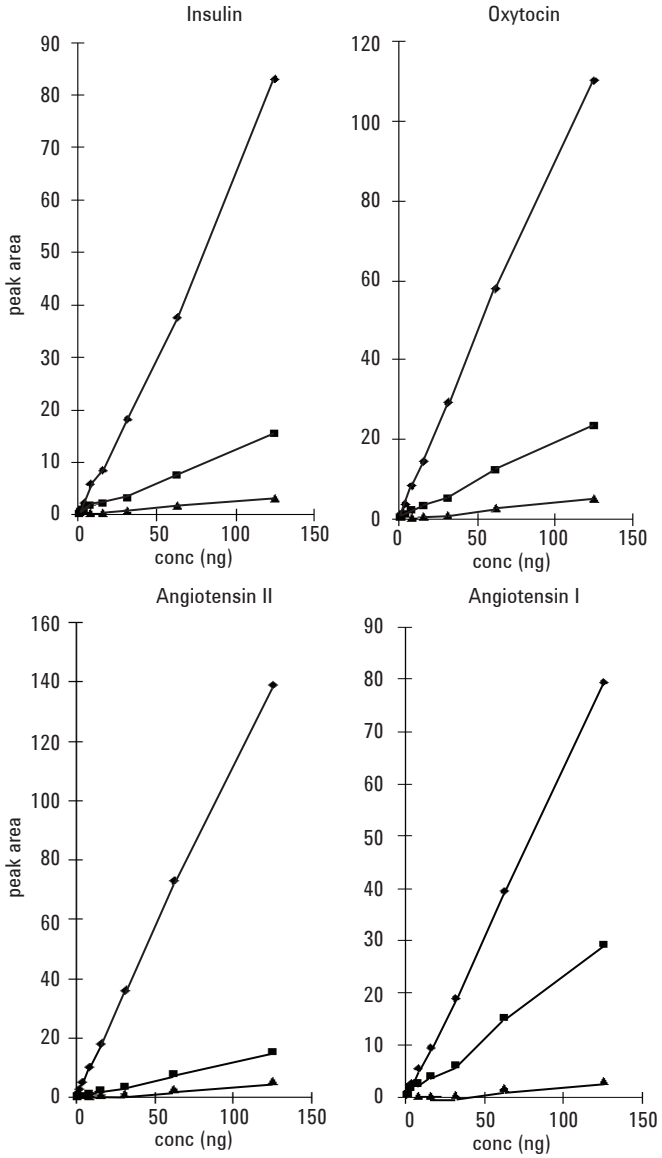

Figure 16. Standard curve data-point graphs on Agilent PLRP-S columns.

The increase in sensitivity was also displayed in the lower detection limits obtained where the 1.0 mm id column had a lower limit of 0.487 ng, and the 2.1 mm id column a limit of 0.975 ng, compared to the standard bore 4.6 mm id with a limit of 7.8 ng (Figure 17).

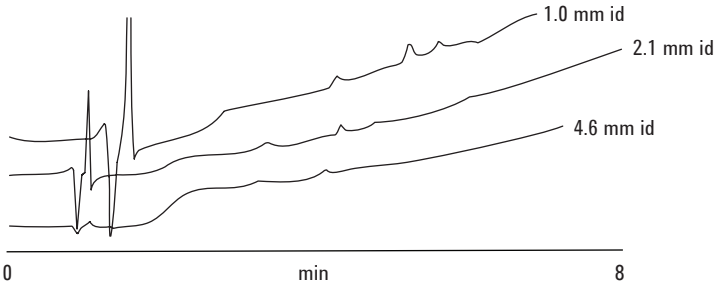

Figure 17. Detection limits of 1.0 mm, 2.1 mm and 4.6 mm id Agilent PLRP-S 100Å 5 µm, 150 mm media.

Thus, microbore LC analysis is well suited to, and has great potential for, investigation and analysis of protein and peptide biomolecules.

Unsurpassed stability of polymeric media

Agilent PLRP-S columns are designed for easy use across the pH spectrum from 1 to 14, with none of the restrictions associated with silica packings. Because of their stability and physical robustness, switching between organic modifiers such as ACN and THF is possible. PLRP-S HPLC media are inherently hydrophobic and reproducible, and do not require a bonded alkyl chain such as C8 or C18 to confer hydrophobicity. PLRP-S is widely used in separations of synthetic oligomers, synthetic polymer compositional analysis, gigaporous biomolecules, peptides, proteins, and oligonucleotides.

These data represent typical results. For further information, contact your local Agilent Sales Office.

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