Exploiting RRHT Columns with Different C18 Selectivities to Quickly Develop Methods for Endocannabinoids

Application

Pharmaceutical

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Abstract

Robust HPLC methods are developed systematically by manipulating three key factors controlling resolution: selectivity, efficiency, and retention. Agilent Rapid-Resolution High-Throughput (RRHT) columns significantly reduce method development time because they offer 1) smaller particles packed in shorter columns, 2) a variety of bonded phases on ZORBAX 1.8 µm packing, and 3) a variety of phases for large potential selectivity differences. This application describes how RRHT columns allowed for the rapid development of methods for selected endocannabinoids.

Introduction

Short rapid-resolution high-throughput (RRHT) columns are ideal for HPLC method development. The smaller particles packed in short (30 mm to 150 mm) columns provide about the same resolution as standard analytical columns (150 mm to 250 mm, 5 µm particles), but in a fraction of the time [1]. The column characteristics selectivity (α), efficiency (N) and retention (k) control the separation of compounds of interest. Separation or resolution is defined by these characteristics:

$$\text{Resolution} = (¼)\,(\alpha\text{-}1)\,\sqrt{N}\,(k/k+1)$$

In this application, these three factors controlling resolution will be systematically examined to develop an HPLC method quickly. Faster method development is achieved by using a variety of RRHT columns instead of standard analytical sized columns.

Endogenous cannabinoids are neurotransmitters that naturally occur in animal organs, especially the brain, and have a role as intercellular messengers similar to the well-known acetylcholine, gamma aminobutyric acid (GABA), or dopamine. They are quite different, however, endocannabinoids being lipophilic and found in cell membranes, whereas acetylcholine, GABA, and dopamine are highly water soluble and are found in vesicles inside of cells.

Anandamide or arachidonoylethanolamide (AEA) was the first endocannabinoid discovered, in 1992. Since then, research has shown that these neurotransmitters play significant roles in many life functions, including memory, sleeping and eating patterns, and even implantation of the blastocyst (embryonic stage) in the uterus. Structures of anandamide and other endocannabinoids analyzed in this application are represented in Figure 1.

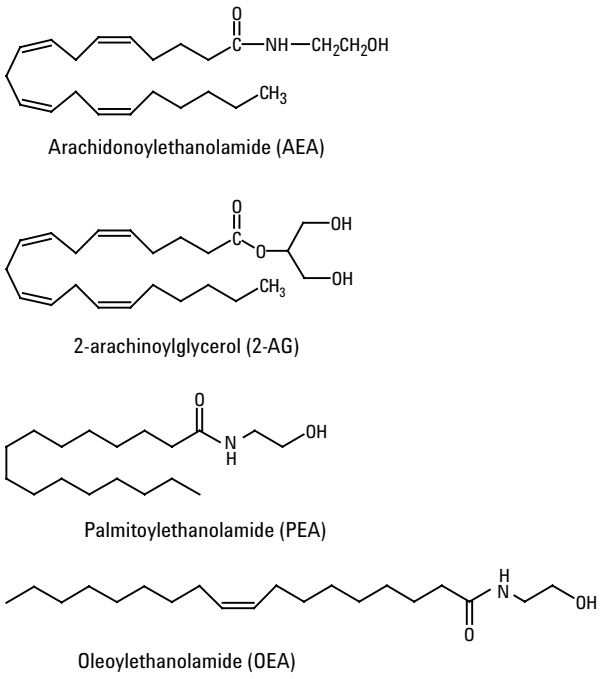

Figure 1. Endocannabinoid-related compounds.

Experimental

Four endocannabinoid fatty amides were obtained from Sigma-Aldrich (Bellefonte, PA, USA):

- Arachidonoylethanolamide (AEA)
- 2-arachinoylglycerol (2-AG)
- Palmitoylethanolamide (PEA)
- Oleoylethanolamide (OEA)

These were diluted in methanol to a concentration of about 1 to 5 mg /mL each component, then diluted 1:100 in 50% methanol/water for a final sample concentration of 0.01 to 0.05 mg/mL.

While it is common to use 2.1-mm id columns for electrospray ionization-mass spectrometry (ESI-MS) because low flow rates (>1 mL/min) allow for optimal electrospray ionization and introduction to the high-vacuum mass spectrometer, 4.6-mm id columns work quite well in many instances, including this one, and furthermore allow easier method transfer to conventional HPLC systems using UV detectors. The following Agilent Technologies ZORBAX RRHT columns were used:

Column	Size	Part number
Eclipse Plus-C18	4.6 mm × 50 mm, 1.8 µm	959941-902
	4.6 mm × 100 mm, 1.8 µm	959964-902
	4.6 mm × 150 mm, 1.8 µm	959994-902
Eclipse XDB-C18	4.6 mm × 50 mm, 1.8 µm	927975-902
StableBond-C18	4.6 mm × 50 mm, 1.8 µm	827975-902
	4.6 mm × 100 mm, 1.8 µm	828975-902
	4.6 mm × 150 mm, 1.8 µm	829975-902
Extend-C18	4.6 mm × 50 mm, 1.8 µm	727975-902

The HPLC system was an Agilent 1200 RRLC with a G1956B Single Quad Mass Spectrometer Detector (MSD). LC parameters include: a binary pump with mobile phase A: 0.1 % formic acid in water (v/v), B: 0.1% formic acid in acetonitrile (v/v), flow rate: 1.5 mL/min., column temperature 30 °C. Relevant MSD parameters include: scan mode (total ion chromatogram), with a scan range of 100 to 500 Daltons, positive mode electrospray ionization (ESI), drying gas: 12 L/min., drying gas temperature 325 °C, nebulizer pressure: 35 psig, capillary voltage: 3000, and a detector peak width setting of 0.05 min.

Results and Discussion

Examining Selectivity (α) for Best Resolution

The variety of stationary phases available on ZORBAX Rapid Resolution (RR), 3.5 µm and RRHT 1.8 µm columns makes them useful for method development, especially for changing selectivity. The short column lengths and consequently short equilibration followed by short analysis times allows for straightforward and quick investigations into selectivity.

Having a variety of bonded phases (columns) at hand to sequentially try in method development analyses demonstrates the different selectivity easily gained from the columns. Figure 2 is an overlay of four different C18 bonded phases available on ZORBAX 1.8 µm particles. We were able to generate the data in less than a half hour because the columns can be quickly equilibrated, run, and substituted for the next column. All have a symmetrical peak shape and similar retention. Notice however, that although only four compounds comprise the endocannabinoid sample, a smaller fifth

peak is detected. It is seen as a shoulder or front on the PEA peak in the Eclipse Plus-C18 chromatogram, and as a resolved peak when Eclipse XDB-C18 is used. This impurity is believed to be 1,3-arachidonolyglycerol, a rearrangement of 2-AG. This is based on extracted ion MSD data. Another interesting point is that the peak elution order is different on the ZORBAX SB-C18 compared to the other C18s.

The differences in selectivity between the four columns are due to the subtle, yet important differences in bonding, such as the type of bonding, the endcapping, or even the amount and type of silanols on the silica. Other factors that influence selectivity: mobile phase composition, temperature and pH are identical.

These four C18 bonded phases differ slightly, all based on 1.8-µm ZORBAX Rx-SIL silica. Each column has its strengths. The Eclipse Plus is the most inert of the four. The Eclipse XDB and the Extend have slightly more silanols and the StableBond has the most exposed silanols. Silanols are often thought to be deleterious but can be taken advantage of to provide selectivity.

Another way to alter selectivity is to change the mobile phase. In MSD applications, volatile mobile phases (MS friendly) are preferred. Here, only a popular aqueous acetonitrile, formic acid mixture was used. Additionally, making different mobile phases for experimentation followed by flushing/equilibrating the HPLC system consumes more time than simply substituting columns. The goal here was rapid method development.

Eclipse Plus-C18 and SB-C18 columns, because of their different analyte elution order under identical mobile phase conditions, were chosen for further method development of the endocannabinoids, highlighting the positive effects that exposed silanols can have on a separation. Eclipse XDB-C18 would likely be a successful choice, too.

Examining Retention (k) for Best Resolution

With two stationary phases chosen that offer promising selectivity for the endocannabinoids, the retention factor of the resolution equation could now be quickly addressed. The mobile phase strength (percent organic) was changed in 4% increments to alter the retention factor, and hopefully improve resolution. Again, the entire sequence of runs to determine the best organic strength took less than an hour by using RRHT columns. Figure 3 shows the effect of changing the organic solvent concentration on resolution. Typi-

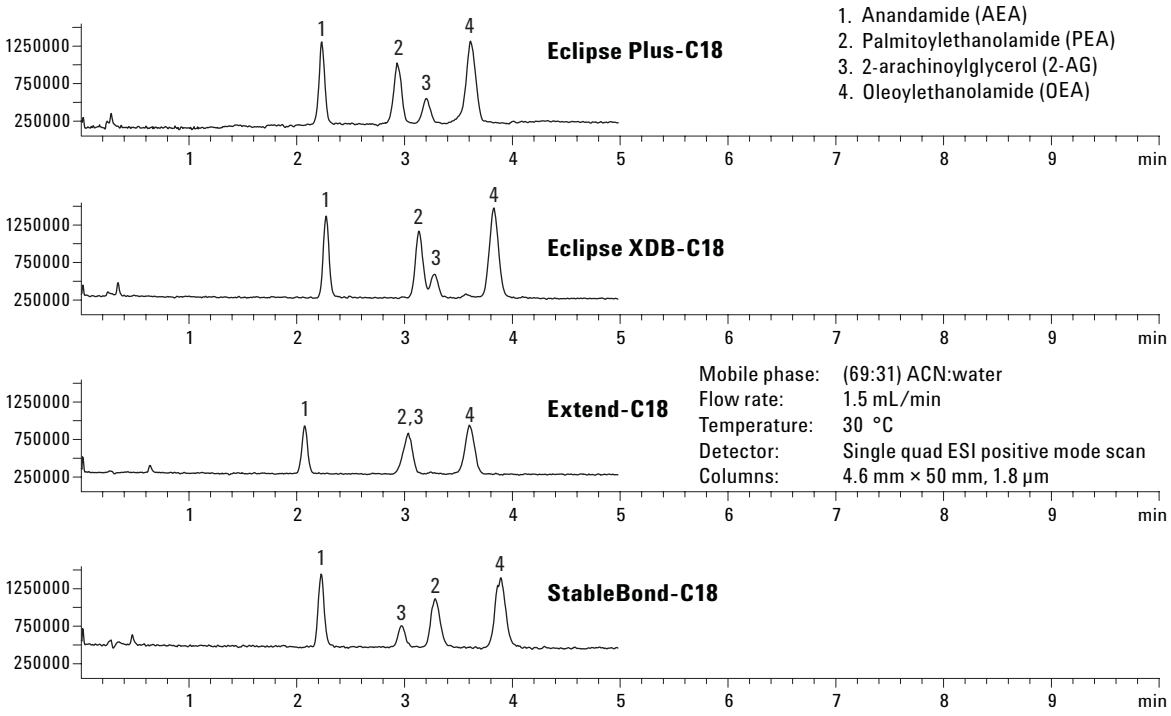

Figure 2. Short RRHT columns quickly highlight different C18 selectivity.

cally, in reversed phase chromatography, k increases as the organic strength weakens. The 2-AG and 1,3-AG peaks are retained at a different rate (move faster than the other peaks). At 77% ACN, the 2-AG coelutes with the PEA, and the 1,3-AG is resolved. At 65% ACN, the two peaks have shifted so the 2-AG is completely resolved, but the 1,3-AG coelutes with the OEA peak. None of the mobile phases completely resolved the cannabinoids on the 4.6 mm x 50 mm RRHT column; however, the 73% and 69% mobile phases partially resolved the five peaks.

Generally, when k is less than 1, reproducibility is poor. Problems with injection solvent or sample matrix are more likely to occur. When k is between 1 and 10, resolution and time can be optimized effectively. When k is greater than 20, improvement in resolution is negligible, and analysis time is too long.

Examining Efficiency (N) for Best Resolution

Longer RRHT columns (100 mm, 150 mm) were substituted for the shorter 50-mm columns to investigate the third factor controlling resolution, efficiency. Using longer 100-mm and 150-mm 1.8-µm columns incrementally improved resolution, ultimately resolving the four analytes in the sample plus the additional 1,3-AG peak (Figures 4 and 5).

Efficiency is improved by increasing column length, decreasing particle size, and minimizing extra-column volume. Because particle size and extra column volume is the same for Figures 5 and 6, efficiency gains are due to column length.

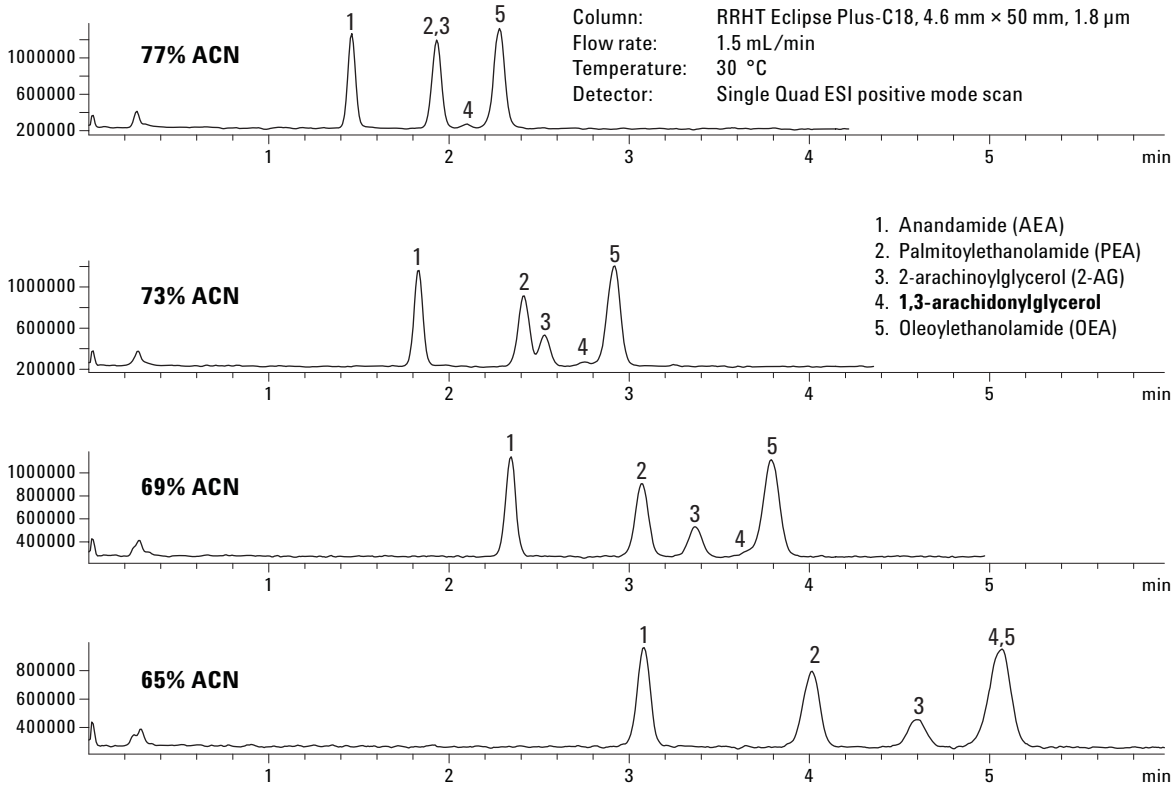

Figure 3. Short RRHT columns quickly highlight mobile phase strength's influence on k.

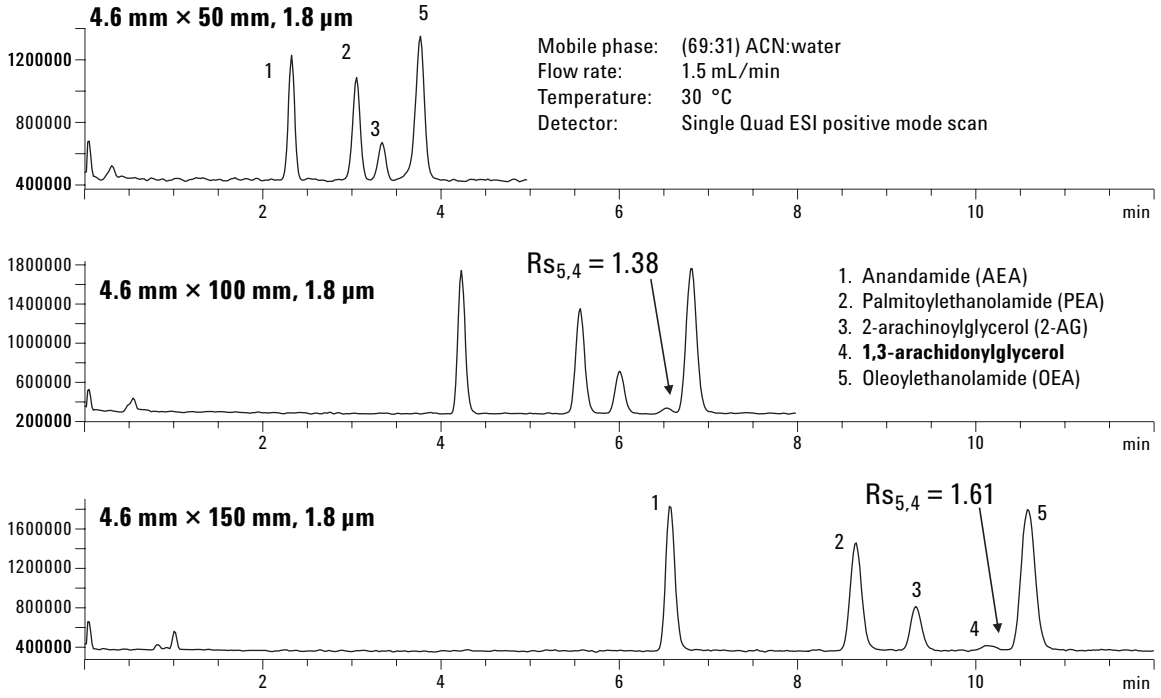

Figure 4. Longer Eclipse Plus RRHT columns improve efficiency to resolve 1(3)-arachidonylglycerol.

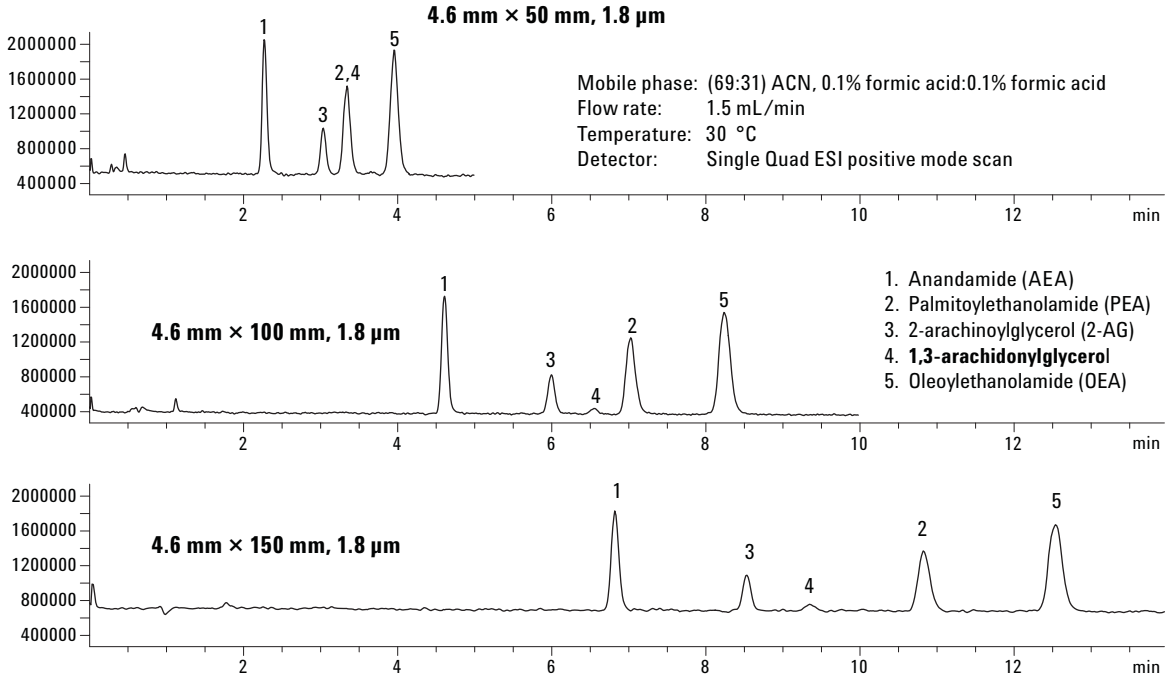

Figure 5. Longer SB-C18 RRHT columns improve efficiency to resolve 1,3-arachidonylglycerol.

The Final Methods

Employing a variety of RRHT columns in different lengths and different selectivities (stationary phases) made it possible to quickly develop a working method. In this case, more than one method was developed. Two RRHT columns, Eclipse Plus-C18 and SB-C18, 4.6 mm × 150 mm, were shown to separate the endocannabinoids with sufficient resolution (Figure 6). In fact, the 100 mm lengths were also successful in an even shorter amount of time (Figures 4 and 5, middle chromatograms). Thus four options are available for the chromatographer. Selectivity options are advantageous, for example multiple sample matrices may have different interfering peaks, requiring different selectivities to accurately quantify analytical peaks.

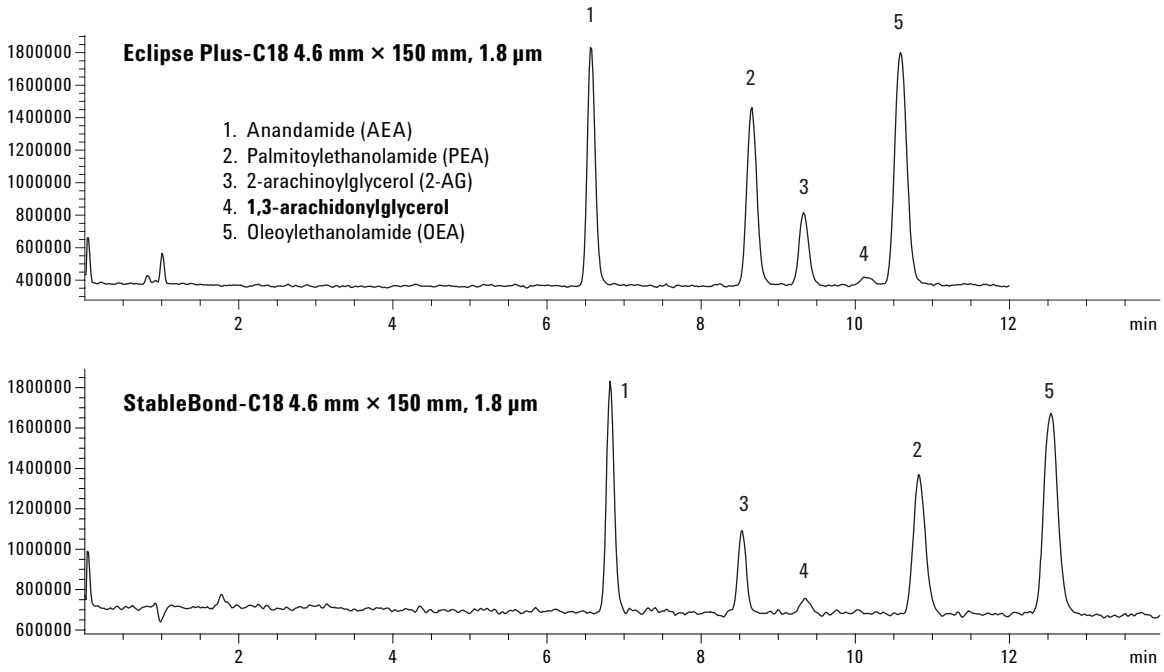

Figure 6. Two options for endocannabinoid analysis highlighting selectivity and resolution of long RRHT columns.

Conclusions

Robust HPLC methods can be developed systematically by manipulating the three factors that control resolution: selectivity, efficiency, and retention. Many chromatographic runs are performed to examine each factor, which is time consuming. RRHT columns can significantly cut the time required for method development.

One reason for the time savings is that smaller particles packed in shorter columns provide about the same resolution as larger particles packed in longer columns but in less time. Another reason is the variety of bonded phases (selectivities) available on ZORBAX 1.8 µm packing. In addition to the four C18 phases described in this work, a variety of other phases, such as C8, cyano (CN), phenyl, and SB-Aq (proprietary high aqueous phase) are available for even greater potential selectivity differences. Over 90 RRHT column configurations (bonded phases and column dimensions) allow the chromatographer to quickly tackle the three factors that control resolution and develop robust methods in less time compared to standard analytical columns.

Reference

1. John W. Henderson, Jr., "'Plug & Play' Fast and Ultra Fast Separations Using 3.5-µm Rapid Resolution and 1.8-µm Rapid Resolution High Throughput Columns," 2005, Agilent Technologies publication 5989-2908EN.

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