Agilent

Agilent PROTEEMA GPC/SEC Columns

User Manual

Notices

Document Information

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Contents

1 Introduction

Thank you for choosing an Agilent PROTEEMA column, a high-quality, high-performance product ready for immediate use. To prolong the lifetime and to optimize its performance, please read this user manual thoroughly, paying special attention to the warnings, hints, and tips sections.

Each column has a unique serial number and is quality checked for efficiency in the eluent in which the column is delivered. The test conditions and results are shown on the certificate of analysis (CoA), which is available on our website at:

www.agilent.com/en/ecertificates-of-performance

For additional information about Agilent GPC/SEC products, see

www.agilent.com/en/product/gpc-sec-columns-standards

1.1 Specifications

PROTEEMA columns are packed with highly porous particles of modified silica and are supplied as standard in water with 7.5 mM sodium azide to prevent microbial growth and tested for efficiency with ethylene glycol (EG).

Table 1. Operational specifications for Agilent PROTEEMA columns.

Column Type	Maximum Operating Pressure per Column (bar/psi)	Maximum Operating Pressure for Three Column Set (bar/psi)	Maximum Operating Temperature	Efficiency Plates/m Ethylene Glycol in Water (7.5 mM NaN_3)
PROTEEMA 3 µ	100/1,450	200/2,901	70 °C	>75,000
PROTEEMA 5 µ	60/870	120/1,740	70 °C	>50,000

1.2 Solvent compatibility

PROTEEMA is compatible with a wide range of aqueous eluents. The columns have an optimum performance, when used with eluents that have a pH in the range 2.0 to 7.5, for the analysis of natural and synthetic peptides, proteins, and enzymes. The transfer to compatible eluents is easy provided the guidelines in this user manual are followed.

For solvents outside this pH range, other Agilent column materials may be more appropriate to maintain a balance between sample ionic character, eluent pH, and packing material polarity for a size-only based separation based on the "magic triangle" concept (Figure 1).

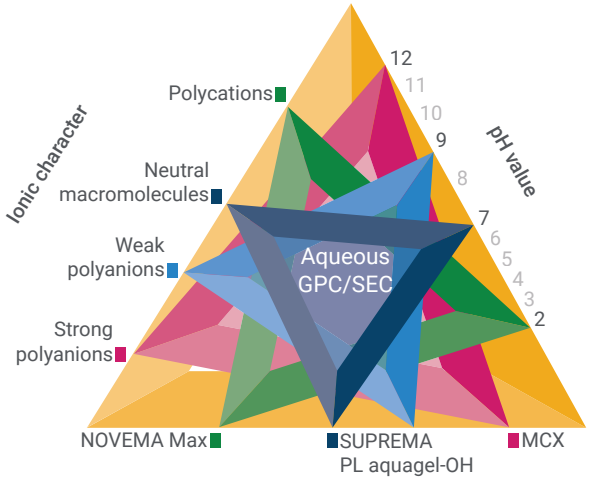

Figure 1. "Magic triangle" illustrating the fact that optimal aqueous GPC/SEC performance requires a balance between sample ionic character, stationary phase, and mobile phase pH.

Table 2. Experimental characteristics of Agilent PROTEEMA columns.

Parameter	Range	Comment
pH Stability	2 to 8	Chemical stability
Optimum pH Range	2 to 7.5	Column lifetime shortened at higher pHs
Temperature	<70 °C	Operate at 10 to 35 °C for optimum column lifetime
Organic Modifiers	<100% v/v	e.g., methanol, acetonitrile, tetrahydrofuran
Salts	< 0.5 M	e.g., NaN_3, NaCl, $NaNO_3$
Buffers		Compatible with most commonly used buffers

1.3 Example conditions

Table 3. Example experimental conditions when using Agilent PROTEEMA columns.

Eluent	Analyte
0.2 M Na_2SO_4, pH 5.0	Low molecular weight heparin
Phosphate buffer pH 6.6 + 0.5 M NaCl	Peptides, proteins, glycoproteins, gelatin

NOTE

It is recommended that 2 to 10 mmol/L NaN_3 is added to the eluent to prevent microbial growth.

2 Column Installation

When using the column for the first time, it is recommended to use the eluent that the column was originally supplied in and to perform a system plate count noting the backpressure due to the column. However, this is not essential.

2.1 Preparing the GPC/SEC system

Remove any existing columns and store according to the manufacturer's instructions. Connect the injector directly to the detector with appropriate tubing and connectors (Section 2.5). Transfer the GPC/SEC system from the current eluent to that required, paying attention to the miscibility and compatibility with the hardware as per the manufacturer instructions.

2.2 Eluent flow rate

The optimum eluent flow rates, as well as the volume of the solvent contained in the column (the "column volume") depend on the column dimensions (Table 4).

Table 4. The optimum eluent flow rates as well as the volume of the solvent contained in the column (the "column volume") depend on the column dimensions (Table 4).

Column Dimensions	Typical Flow Rate (mL/min)	Optimum Flow Rate (mL/min)	Column Volume (mL)
30 mm x 4.6 mm	0.1 to 0.7	0.33	0.5
250 mm x 4.6 mm	0.1 to 0.7	0.33	4.2
50 mm x 8.0 mm	0.3 to 2.0	1.0	2.5
300 mm x 8.0 mm	0.3 to 2.0	1.0	15

Avoid subjecting the column to any sudden shocks, such as sudden changes in flow rate. When applying flow to the column, set the flow rate to 0.0 mL/min and turn on the pump. Always increase the flow rate in small increments (e.g., 25% optimum flow rate every 15 seconds until the desired operating flow rate is reached).

Even if the flow is in the allowed range, do not exceed the maximum pressure recommended for the column.

2.3 Temperature

When using viscous eluents, increasing the temperature decreases the eluent viscosity and therefore the column pressure, and is also beneficial to the separation. In such cases, use a low flow rate initially and heat the column at a rate no greater than 2 °C/min, ensuring that the final operating temperature is at least 10 °C below the boiling point of the eluent. When at temperature, the flow can be increased to the required rate. To cool the columns down, set a low flow rate and turn off the heating, allowing the columns to cool down naturally. The maximum operational temperature is 70 °C.

2.4 Tubing

For best results, use 1/16 in OD stainless steel tubing for connections between columns, injectors, and detectors. The ID of the tubing should be 0.007 in (0.18 mm) for microcolumns (4.6 mm ID) and 0.010 in (0.25 mm) for analytical columns (8.0 mm ID). For optimum performance, use short lengths of machine-cut capillary tubing thus ensuring that the tubing fits flush inside the fitting, minimizing dead volume and system dispersion.

Plastic tubing, such as PEEK, should be used with caution, checking the temperature and pressure ratings.

2.5 Connectors

Different column types use similar, but subtly different, fittings. The performance of the system can be negatively influenced, or, in the worst case, damage can occur if care is not taken. For best results, use standard stainless steel 1/16 in nuts and one-piece ferrule compression fittings.

WARNING

The distance from the ferrule to the end of the capillary (stop depth or seating depth) must be 1 mm (Figure 2). Different column types have differing lengths of tubing protruding from the ferrule.

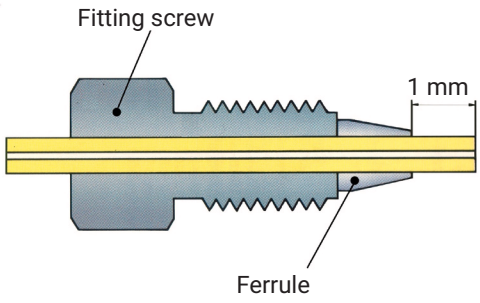

Figure 2. Schematic of the capillary connection.

Check the compatibility of the two connectors between the column and the injector and detector. For best practice, use new connectors with the Agilent column. The old connectors, together with the columns being replaced, can be put into storage if these columns will be reused.

Plastic connectors and ferrules, such as PEEK, should be used with caution, checking the temperature and pressure ratings. However, these connectors offer some practical advantages in that the ferrule does not permanently grip the tubing. The connector can also be easily adjusted to accommodate the different lengths of protruding tubing used by the different manufacturers forming a leak-tight connection with little dead volume.

2.6 Connecting the column to the injector or the preceding column in a set

Please take care when you install and test your new column. Remove the end plugs from the column and set aside for use when storing the column. When making the connections, place the wrenches on the fitting screw and column end fitting. Never use the flats that are machined on the column body.

Noting the direction of flow displayed on the column, connect the inlet fitting with the injector or preceding column using the appropriate nuts and new ferrules at each end of the tubing. Ensure that the capillary is as far as possible into the injector or column head before tightening the nut. Do not overtighten. The nut should be tightened so that the ferrule bites into the capillary tubing and no leak occurs. Excess force should not be required.

To prevent trapped air being pumped into the column, unscrew the nut at the column inlet and slowly pump a few drops of eluent to the waste before reconnecting the column.

2.7 Connecting columns in series

For connections between columns use the column connector delivered with each Agilent column. The precolumn should be connected to the injector and the remaining pore sizes connected in sequence of increasing pore size so that the largest porosity is closest to the detector. Only combine columns of the same particle size. To avoid porosity mismatch, we advise using the recommended column combinations shown in Table 5. Do not combine linear/mixed bed/multipore columns with single porosity columns. Each time a column is added, the procedure “Connecting the column to the injector or the preceding column in a set” should be followed.

Table 5. Recommended analytical column sets.

Separation Range [Da]	Description
100 to 150,000	1 x PROTEEMA precolumn, 5 µm, 8 x 50 mm (part number PRA080505) 3 x PROTEEMA analytical column, 5 µm, 100 Å, 8 x 300 mm (part number PRA0830051E2)
100 to 1,200,000	1 x PROTEEMA precolumn, 5 µm, 8 x 50 mm (part number PRA080505) 3 x analytical PROTEEMA column, 5 µm, 300 Å, 8 x 300 mm (part number PRA0830053E2)

2.8 Connecting the column to the detector

Connect the outlet fitting of the column to a long piece of 1/16 in tubing to take the eluent to the waste. Pump three to four column volumes of eluent through the column to the waste at the recommended operational flow rate. Check for any leaks. Stop the flow, remove the waste tubing from the outlet and connect to the detector using the appropriate nuts and new ferrules at each end of the tubing. Apply the desired flow rate, again checking for leaks. When the detector has stabilized, the column is ready for use.

2.9 Testing column performance

To verify correct installation, the column can be tested by measuring the plate count/meter and the resolution and comparing with the CoA, noting that the measurements are a reflection of the dispersion in the whole system and not just the column alone. See Sections 2.2 and 3.3 for optimum flow rates, concentrations, and injection volumes.

To measure plate count, inject a suitable low molecular test probe under test conditions as detailed on the CoA.

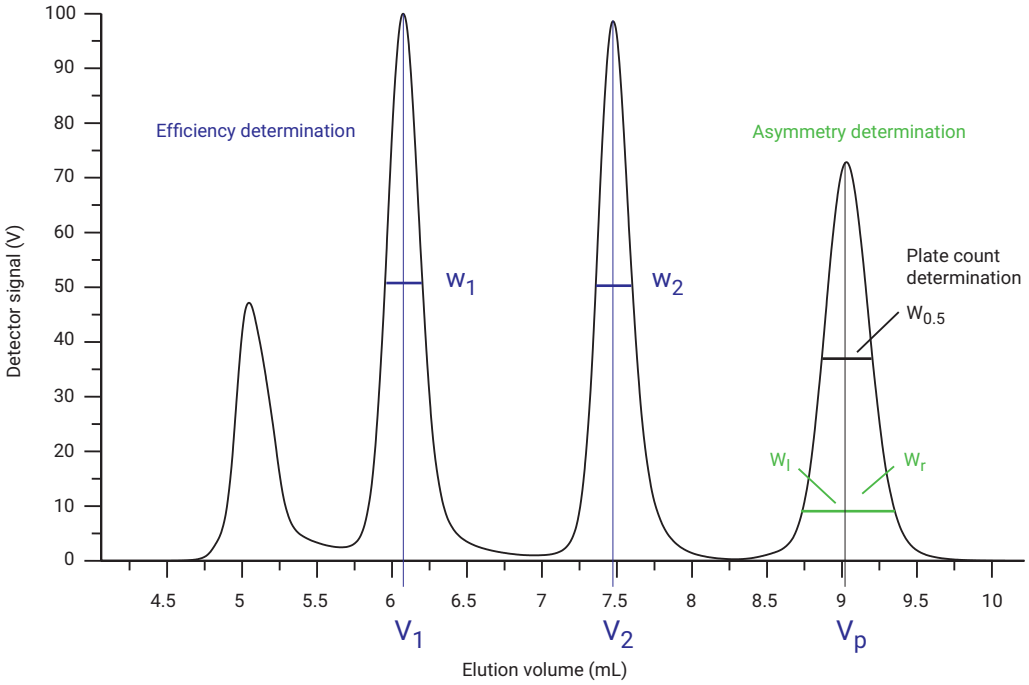

Figure 3. Variables used for determination of plate count, asymmetry, and resolution.

Determination of theoretical plates N:

$$N = \left(\frac{V_p}{\sigma}\right)^2 = 5.54\left(\frac{V_p}{w_{0.5}}\right)^2$$

Where:
V_p = Elution volume of test probe in mL
σ = Peak dispersion
$w_{0.5}$ = Width of peak at ½ height in mL = 2.35 σ

L = Column length in meters

$$\frac{\text{Plates}}{\text{Meter}} = \frac{N}{L}$$

Determination of peak asymmetry A:

$$A = \frac{W_l}{W_r}$$

NOTE

The definition of asymmetry is as per ISO 13885-1. Sometimes, asymmetry is defined as the inverse of this formula.

A better way to determine the separation power for polymers is the calculation of the resolution factor. To do this, a mixture of polymer standards with narrow distribution of molar masses is injected:

$$\text{Resolution} = R_s = \frac{V_2 - V_1}{2(\sigma_1 + \sigma_2)} = \frac{V_2 - V_1}{0.86(w_1 + w_2)}$$

For comparison, it is better and easier to calculate the specific resolution:

$$\text{Specific resolution} = R_{sp} = \frac{0.579}{\sigma \cdot D} = \frac{Rs}{\lg\left(\frac{M_1}{M_2}\right)}$$

Where:

M_1, V_1 and w_1 are the molecular weight, elution volume, and peak width at ½ height of peak 1.

M_2, V_2 and w_2 are the molecular weight, elution volume, and peak width at ½ height of peak 2.

$$D = -\frac{d\lg M}{dV} = \text{slope of the calibration curve}$$

σ = Peak dispersion obtained from plate count measurements

NOTE

When first installing a column set, it is extremely useful to perform plate count/meter and resolution measurements as well as noting the backpressure in the chosen eluent. These parameters can be used to monitor ongoing column performance and to diagnose problems.

3 Usage Guidelines

3.1 Eluent preparation

Use high-quality HPLC grade eluents, free from large particles and filtered using a 0.45 µm filter if required. Degas the eluent thoroughly, preferably using an online degasser.

NOTE

When using Agilent PROTEEMA columns with laser light scattering detectors, it is recommended that they be purchased pre-equilibrated for light scattering (PROTEENA Lux, see part numbers in Table 6). Extra steps must be taken to obtain optimum low noise baselines. Filtering solvents under vacuum through a 0.2 µm filter and avoiding the use of salts and other additives in the eluent can improve performance.

Table 6. PROTEEMA Lux columns pre-equilibrated for use with light scattering detectors.

Part Number	Description
PRA080505LS	PROTEEMA Lux precolumn, 8 x 50 mm, particle size 5 µm
PRA0830051E2LS	PROTEEMA Lux analytical column, 8 x 300 mm, particle size 5 µm, 100Å, molecular weight separation range 100 to 150,000 Da (based on protein molecular weights)
PRA0830051E3LS	PROTEEMA Lux analytical column, 8 x 300 mm, particle size 5 µm, 1000Å, molecular weight separation range 1,000 to 7,500,000 Da (based on protein molecular weights)
PRA0830053E2LS	PROTEEMA Lux analytical column, 8 x 300 mm, particle size 5 µm, 300Å, molecular weight separation range 1,000 to 1,200,000 Da (based on protein molecular weights)

3.2 Transferring to different solvents

Only use PROTEEMA columns with compatible eluents. If the column must be used with another eluent, it can be changed by careful solvent exchange without loss of resolution. Differences in plate counts that can occur in these cases are often due to viscosity differences of the eluents.

Before changing eluents, ensure that both the initial eluent and the desired eluent are completely miscible. If they are not miscible, seek an intermediate that is miscible with both.

3.2.1 Changing eluents

Do not exceed the recommended maximum column pressure while changing solvents.

Frequent transfer of columns between eluents decreases column lifetime.

1. Prepare the GPC/SEC system with the new eluent. Connect the column/column set to the injector and attach some 1/16 in tubing to the outlet directed to the waste.
2. Pump approx. two column volumes of the eluent at a tenth of the optimum flow rate through the column(s) and then slowly increase the flow rate to half the optimum flow rate and pump a further eight column volumes, e.g., for 300 mm x 8.0 mm columns, start at 0.1 mL/min and increase to 0.5 mL/min.
3. Stop flow and connect the column(s) to the detector.
4. Start flow at 0.1 mL/min. Adjust column temperature to the required value (Section 2.3) and when at temperature, increase the flow rate step by step up to the desired value.
5. Ensure that all detector lines are completely purged, and that the system is thoroughly equilibrated before making measurements.

3.3 Sample preparation

Samples and polymer standards used for column calibration should be prepared in the same eluent as the GPC/SEC system. To minimize so called “solvent peaks” when using RI detection, use eluent taken directly from the solvent reservoir of the GPC/SEC system.

The total amount that can be injected without deterioration in separation performance depends on the total column length and is calculated from the product of injection volume and the sample concentration. A modern approach is to keep the injection volume small to minimize dispersion caused by the loop itself. If required, to improve detection, vary the injected mass. The information in Table 7 is for guidance only. Large pressure surges related to the injection must be avoided. It may be that for viscous solvents, viscous samples (typically very high molecular weights) and smaller particle size GPC/SEC packings that steps must be taken to reduce the pressure surge. These steps include but are not restricted to, using lower concentrations, larger injection volumes, lower flow rates, and increasing column temperature.

Table 7. Sample quantity recommendations for optimal GPC/SEC separation performance.

Sample, Molecular Weight	Concentration (g/L)	Flow Rate	Injection Volume (µL) Column Set	
			Column Dimensions mm (L × ID)	
			250 × 4.6	300 × 8.0
Plate Count	1	Optimum	5	20
Narrow Stds. < 1 M Da	1	Optimum	5	20
Narrow Stds. > 1 M < 3 M Da	<0.5	Optimum	5	20
Narrow Stds. > 3 M Da	<0.5	50% optimum	5	20
Broad Stds., Samples < 1 M Da	1 to 3	Optimum	10	50
Broad Stds., Samples > 1 M Da	<1	50% optimum	10	50

Always ensure that samples are completely dissolved before injecting on the GPC/SEC system. High molecular weight materials require several hours for complete dissolution and are best left overnight before injection.

Sample filtration (0.5 µm or, for samples above 1 M Da, 2.0 µm) should be used after complete dissolution of the sample, to prevent blockage of the columns. However, it should be noted that filtration may potentially remove some of the sample as well as unwanted particulates.

To avoid damage of the primary column system through adsorption of sample impurities or contaminants within your samples, we strongly recommend the use of a precolumn.

4 Care and Maintenance

4.1 Storage

If the columns are to be used again within one week, then it is not necessary to put the columns into storage provided the eluent used does not degrade and there is no chance of salt precipitation. If feasible, the GPC system should either be put into recycle mode or be run at a very low flow rate.

Never leave the columns at elevated temperatures without flow or allow the columns to dry out. If recycle mode is used, replace with fresh eluent when the system is used again.

Replace any eluent that contains salt or acids before storage. Store the columns in water/NaN3 to prevent microbial growth. When removing the column(s) from the system, the end plugs must be replaced to prevent the column(s) drying out. Store in a cool place but never let the eluent freeze.

4.2 Troubleshooting

Table 6. Troubleshooting guidance.

Problem	Potential Cause	Corrective Action	Recommendation
Increase in operating pressure	Blockage of solvent filter between pump and injector	Replace filter	Check pump seals and solvent quality
	Blocked or overtightened connecting tubing	Replace connector	Check sample solubility and filter samples
	Blocked inlet frit of column	Replace frit or replace column	Check solvent quality and sample solubility
	Adsorption of sample on column	Replace precolumn; undertake column regeneration	Alter analysis conditions to prevent adsorption
Decrease in operating pressure	Air in the system; leak	Check degassing of solvent; check connectors	Check if degasser is working; replace worn connectors
Loss of plate count or resolution	Worn or badly made connections	Check and replace connectors if required	
	Problem with injector	Check injector	
	Column frits blocked	Undertake column maintenance	
	Precolumn blocked/saturated	Replace precolumn	
	Analytical column damaged	Replace column	
Changing peak shape	Column damaged	Check plate counts	Replace or repair damaged column
	Sample adsorbing	Clean columns; replace precolumns	Find analysis conditions to prevent adsorption
No peak	Problem with injector	Check injector	
	Problem with detector	Check detector	
	Sample adsorbing	Clean columns; replace precolumns	Alter analysis conditions to prevent adsorption

When diagnosing problems, it is recommended to perform a series of logical experiments by removing components such as columns or connectors from the system one by one to identify which component is the cause of the problem.

4.3 Column repair

For best results, perform repairs on columns individually using pure eluents with no salts or acids. Always flush the columns to the waste.

The performance of a column may deteriorate due to blockage of the frits, sample adsorption or damage to the packing bed causing an increase in backpressure.

4.3.1 Partially dried-out columns

During storage, some of the solvent may evaporate through the threads (this typically happens during long storage times and with high storage temperatures and volatile eluents). It is good practice to keep columns with volatile eluents in a refrigerator when not in use, to prevent solvent evaporation. It is an indication of a partially evaporated column solvent if the expected pressure does not build up or the pump constantly readjusts the flow. In this case, immediately reduce the flow down to 0.1 mL/min (to prevent damage to the packing) and fill the column until no more bubbles appear at the column outlet and column backpressure is stable. Then add the next column using the previously mentioned installation procedure until each column is installed in your system.

NOTE

If you want to install a complete set of columns where the solvent has been partially evaporated, you can connect all columns when solvent appears from the previous column. The flow rate can then be set to 0.1 mL/min unattended (for example overnight) with no negative effects.

4.3.2 Partially blocked columns

Columns become partially blocked when particulates get trapped in the frits or in the packing bed and/or sample components adsorb on the frits or packing material. Due to the varying chemical nature of possible samples and column history, there is no single treatment that is successful in all cases. It is often not possible to recover a column. Certain steps can be taken either individually or together to try to at least partially recover performance. These steps should be undertaken on individual columns to avoid potential contamination problems.

Reverse flush the column to the waste at a low flow rate (25% of the optimum flow rate) for at least four column volumes. Afterwards, reconnect the column in the correct direction. Slowly increase the flow rate to see if the backpressure generated on the column has reduced and if it can be operated under normal conditions. If the pressure is now reasonable, perform a plate count test and also, if desired, a resolution test. If the pressure remains high, try the following steps, or directly change the frits.

1. Increase the temperature of the column in combination with Section 4.3.2 following the guidelines in Section 2.3. Flush for 10 column volumes.
2. Change the eluent or use modifiers to try to dissolve any adsorbed components in combination with Section 4.3.2 and, if appropriate, step 1 in this section. Flush for 10 column volumes.

4.4 Changing frits

This procedure can be used for changing both the inlet and outlet column frit. The inlet frit is particularly susceptible to blocking. The column should only be opened to change frits if a blockage could not be removed by the previously mentioned measures.

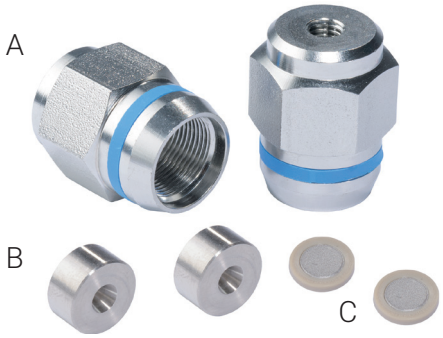

Figure 4. Components of the column head
A) End fittings
B) Fitting adapters
C) Frits

1. Remove the column from the system and replace the end plug in the end that is not being changed.
2. Using two wrenches, one placed on the flat of the column body nearest the end fitting (Figure 4) to be changed and the end fitting itself, slowly unscrew and remove the end fitting, the end fitting adapter, and finally slide off the frit and seal from the top of the column.
3. Inspect the top of the gel packing to ensure that there are no holes in the gel packing.
4. Inspect the end fitting adapter to ensure it is not damaged.
5. Rinse end fitting and fitting adapter to ensure that they are entirely clean and free of particles. Re-assemble the fitting adapter (flat end towards the frit) and a new frit in the end fitting.
6. Holding them at an angle of 45° to each other, screw the end fitting, the adapter, and new frit back onto the column until finger tight. Then use the wrenches to tighten by a further 60° turn.
7. Connect the column in reverse direction and flush for three column volumes to the waste, ensuring that gel particles do not elute due to a poorly seated frit and seal.
8. Connect the column in the correct direction and check the pressure and plate count.

5. Ordering Information

Table 8. Ordering information for spare parts.

Part Number	Description
299-2045	PEEK/titanium replacement frits, 8.0 mm ID column (pk of 2)
299-2046	PEEK/titanium replacement frits, 4.6 mm ID column (pk of 2)
PL1310-0008	Tubing ferrules, 1/16 in, 5/pk
PL1310-0007	Column connecting nuts 1/16 in, 5/pk
PL1310-0048	Connecting tubing for inter-column connection, 100 mm length, ID 0.25 mm, 10/pk, for 8.0 mm and 20 mm ID columns, combine with ferrules (PL1310-0008) and nuts (PL1310-0007)
5021-1816	Connecting tubing for inter-column connection, 105 mm length, ID 0.17 mm, for 4.6 mm ID columns combine with ferrules (PL1310-0008) and nuts (PL1310-0007)
5021-1818	Connector from column to injector/detector, 280 mm length, ID 0.17 mm, for 4.6 mm ID columns, combine with ferrules (PL1310-0008) and nuts (PL1310-0007)
5022-6508	Connector from column to injector/detector, 280 mm length, ID 0.25 mm, for 8.0 mm ID columns, combine with ferrules (PL1310-0008) and nuts (PL1310-0007)

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