PolySULFOETHYL A™
- for Cation-Exchange of Peptides

This strong cation-exchange (SCX) material was developed specifically for HPLC of peptides. At pH 2.7-3.0, peptides lose their (-) charges and have net (+) charge. Thus, PolySULFOETHYL A™ is a general-purpose alternative to reversed-phase (RPC), fractionating peptides by differences in charge rather than polarity. Compared to other SCX materials based on sulfopropyl- (SP-) groups, PolySULFOETHYL A™ is unusually hydrophilic. This minimizes hydrophobic interactions with peptides, with high recovery and less peak tailing. Capacity is also high, permitting better retention and fractionation of the weakly basic peptides from tryptic digests. The capacity of an ion-exchange material like this is 4x that of a comparable RPC column. Therefore, ion-exchange should be the initial step of a multi-step purification. Use PolySULFOETHYL A™ for:

1) Multidimensional HPLC of peptide mixtures, such as tryptic
digests in proteomics analyses (including iTRAQ® and ICAT®* reaction products).

2) Isolation of peptides from natural products.

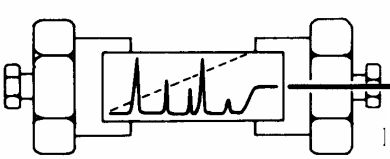
3) QC and purification of synthetic peptides.

4) Selective isolation of disulfide-linked peptides, phosphopeptides and C-terminal fragments from tryptic digests.

5) Mapping of peptide digests (tryptic, V8, CNBr, etc.).

Proteins can be run on PolySULFOETHYL A™ columns too; at pH 3, retention is all but guaranteed. An example is the analysis of Lung Surfactant Protein on a PolySULFOETHYL A column operated in the Hydrophilic Interaction (HILIC) mode.

Standard material for peptide applications is 300-Å, in either 3- or 5-µm. The 200-Å material has about 25% greater capacity and is preferred for phosphopeptide isolation and fractionation of iTRAQ® reaction mixtures. For proteins, use 1000-Å material or the 3-µm, 1500-Å material.



PolyLC INC.

PolySULFOETHYL A™ Columns

Initial Use: PolySULFOETHYL A is a silica-based material with a bonded coating of a hydrophilic, anionic polymer: poly(2-sulfoethyl aspartamide). Thus, it is a strong cation-exchange (SCX) material. Columns are shipped in methanol. Flush new columns with at least 15 column volumes of water (30 mL for a 200x4.6-mm column). Next, condition the column with 3 gradient cycles of the buffers to be used for SCX. Run a gradient from 0-100% B over 20', remain at 100% B for 5', then return to 100% A and equilibrate for 25' before starting the second gradient cycle, etc. After the third cycle, the column is ready to use.

New HPLC columns sometimes absorb small quantities of proteins or phosphorylated peptides in a nonspecific manner. The sintered metal frits have been implicated in this. Eluting the column for 20-24 hr. at a low flow rate with 40mM EDTA.2Na usually solves the problem. This passivates all metal surfaces in the HPLC system, as well as the column [CAUTION: This treatment can affect the integrity of the frits in some cases, and should probably be avoided with columns packed with 3-μm material. In some cases this has also caused the collapse of 5-μm, 200-Å column packings].

Routine Use: Columns should be used at ambient temperatures. Filter mobile phases and samples use. Failure to do so may cause the inlet frit to plug. This frit is replaceable. At the beginning of the day, flush the column with 15 column volumes of the high-salt buffer before equilibrating with the low-salt buffer. At the end of the day, flush the column with 15 column volumes of water and plug the ends.

Loading Capacity: The loading capacity of a 4.6mm ID column is about 4mg of peptide/injection. However, the best shapes in proteomics applications are obtained at loading levels ~40% of this amount.

Storage: 1) Overnight: 100% mobile phase A. 2) Several days: Store in water. 3) Longer periods: Store in water in the refrigerator, with the ends plugged. **ACN can be added to the storage solvent (*e.g.*, ACN:Water = 80:20) to retard microbial growth.**

Column maintenance: After every 250 runs, invert the column and run it backwards. Continue using the column in this inverted direction for the next 250 samples, then repeat this treatment. If possible, open the inlet and fill in any voids with bulk PolySULFOETHYL A™ after running 500 samples.

Minimize Iron in the System: This coating chelates Fe^{+3}, which ruins its performance. If chloride-containing mobile phases are used regularly, passivate the HPLC system every 4 weeks with 40mM EDTA.2Na solution, run through the system overnight at a low flow rate. NOTE: If the HPLC system has not been used for several days (e.g., over a weekend), then Fe^{+3} ions tend to accumulate in the fluid in the lines. When restarting the system, flush this fluid to waste offline before diverting flow through the column.

Cation-Exchange of Peptides: This material has been developed specifically for cation-exchange of peptides in the pH range 2.7-3.0. It will function as a cation-exchanger above pH 4, but in that range it has no particular advantage over weak cation-exchangers such as PolyCAT A™. At pH 3, basic residues in peptides (His, Arg, Lys) are positively charged, as are N-termini. Acidic residues (Asp, Glu) are uncharged, and the C-termini are predominantly uncharged. Thus, most peptides with free N-termini will have net charges of at least +1 and will bind to PolySULFOETHYL A. They can then be eluted with a salt gradient. A good general-purpose buffer system is 10 mM KH_2PO_4, pH 2.7-3.0, with 20% ACN, with a linear gradient to the same buffer but with 0.5 M KCl. Peptides elute in order of increasing net positive charge. Selectivity can be manipulated by varying the amount of organic solvent, but peaks are broader if it's omitted entirely. Recovery is generally high or quantitative; however, it may be advisable to use guard cartridges with especially troublesome mixtures such as CNBr cleavage digests or crude tissue extracts. If a volatile mobile phase is desired, then ammonium formate can be used as both the buffering and gradient salt; however, its use precludes monitoring absorbance at 220nm.

Specific Isolation of Crosslinked Peptides: A typical tryptic peptide has a charge of +2 at pH 3, due to the N-terminus at one end and the Lys or Arg at the other. Linking two such peptides with a disulfide or other bridge results in a peptide with a charge of +4. Such peptides are eluted later than the typical tryptic peptide, permitting their convenient isolation and identification.

Peptide Purification and 2-D Chromatography: Retention on PolySULFOETHYL A is complementary to that on reversed-phase HPLC (RPC). Thus, an SCX step is an excellent way to break up a large set of peptides, like a tryptic digest, into smaller subsets to be further resolved by RPC. Use RPC columns last, since they have several times less capacity than SCX columns and yield products in volatile mobile phases.

Tryptic digests: 60-70% of the peptides (+1 or +2 charge) elute with a gradient to 0.25M KCl. The rest typically have charges of +3 or +4. It may be necessary to use a gradient to 0.6 M salt to elute those. In order to distribute tryptic peptides as uniformly as

possible in the collected fractions, a linear gradient with two segments is best; one segment to 0.25M salt that involves ~ 75% of the gradient and a second segment from 0.25-0.6 M salt with pH 4.5-6 over the remaining 25% of the gradient.

Isolation of Phosphopeptides from Tryptic Digest: Many phosphopeptides elute in the fractions containing peptides with net charge of +1. These are < 3% of a complex tryptic digest, which makes it much easier to identify them. Use PolySULFOETHYL A™ with a 200Å pore diameter; due to its higher surface area, it is better able to separate the +1 and +2 peptides cleanly than is the 300Å version. Use mobile phases with pH 2.7, not 3.0.

Cation-Exchange of Proteins: Regardless of pI value, all proteins have a net positive charge at pH 3 and will be retained by PolySULFOETHYL A unless they are hyperphosphorylated. This permits ion-exchange separation of all proteins on a single column. Since proteins are large molecules, PolySULFOETHYL A with pores of 1000 Å or wider should be used. Furthermore, many proteins are denatured at pH 3, exposing hydrophobic residues in the core and leading to aggregation. This can be prevented by inclusion of a high level of organic solvent in the mobile phases and the use of $NaClO_4$ as the gradient salt. For ordinary proteins, try 10% ACN + 10% PrOH. Levels as high as 35% + 35% have yielded good results, especially with hydrophobic proteins. With such high levels, selectivity is affected by the superimposition of hydrophilic interactions on top of the electrostatic effects. Solubility can also be maintained by adding 50mM hexafluoro-2-propanol to the sample solvent and the mobile phases. The sodium salt of methylphosphonic acid is a good buffering salt if $NaClO_4$ is used for the gradient.

Column Leaching: All chromatography packings bleed minute amounts of stationary phase when in use. In the case of PolySULFOETHYL A, this could potentially result in small amounts of aspartic acid, taurine, and other coating components leaching from the column. If this is a concern, then elute a new column with 50mM formic acid at a low flow rate for 20-24 hours. This accelerates the leaching of coating components that aren't covalently attached to the silica. Thereafter, flush out the formic acid with water, equilibrate, and use as usual.