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PolyETHYL A™, PolyMETHYL A™, & PolyPROPYL A™ Columns

Initial Use: PolyETHYL A™, PolyMETHYL A™, & PolyPROPYL A™ columns are silica-based materials that operate through hydrophobic interactions (HIC). Columns are shipped in methanol. Prepare the column for use in the following manner (200 x 4.6mm ID columns):

Flush with 15 column volumes of water (30 minutes @ 1 mL/min)
Flush with 30 column volumes of conditioning buffer (1 hour @ 1 mL/min) ← This can be any buffered solution of high ionic strength between pH 3 – 6.5. i.e. 300mM KH_2PO_4.
Flush with 15 column volumes of water (30 minutes @ 1 mL/min)
Flush with 30 column volumes of mobile phase (1 hour @ 1 mL/min)

It's a good idea to run at least one gradient cycle with a new column before injecting samples. Changes in the topography of the polymeric coating may lead to modest changes in the retention times during the first few following exposure to aqueous mobile phases.

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 before use. Failure to do so may cause the inlet frit to plug. If a salt gradient is being used, flush the column with 15 column volumes of the low-salt buffer before equilibrating with the high-salt buffer. At the end of the day, flush the column with 15 column volumes of water and plug the ends.

Sample Preparation: A hydrophobic protein or peptide will generally bind well if the sample buffer contains as least 25% v/v of the high salt mobile phase buffer. Good binding of a hydrophilic species may require the presence of 50% v/v of the high salt mobile phase buffer. Fractions from reversed phase columns have been applied directly to HIC columns; it is necessary to dilute such samples with at least 1 equal volume of the high salt buffer mobile phase to overcome the effects of organic solvents and trifluoroacetic acid, both which promote elution. Chaotropic agents such as guanidinium hydrochloride also promote elution and may necessitate the addition of more salt to promote binding.

Loading Capacity: The loading capacity of a 4.6mm ID column is about 4-5 mg of protein/injection, depending on the strength of the protein's binding to the support.

Storage: 1) Overnight: 100% Mobile Phase B. 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.**

HIC of Proteins and Peptides: Salts that promote "salting out" will also promote absorption in HIC columns. A commonly used gradient is a decreasing salt gradient from 2M ammonium sulfate + 25mM potassium phosphate, pH 6.5, to 100% 25mM potassium phosphate. Proteins and peptides will elute in order of increasing surface hydrophobic character. Proteins that are sensitive to ammonium sulfate can be bound with 1.2M citrate instead; however, this precludes monitoring at 200nm. Sensitive proteins can also be eluted with gradients of increasing concentrations of detergents or ethylene glycol. HIC is not very sensitive to pH unless there is a marked change in the surface charge of the protein in question. Prolonged exposure to a pH above 8 should be avoided. Organic solvents do not harm the column.

Effect of Secondary and Tertiary Structure: HIC is sensitive to the presence of glycosidic side chains. A single gene product may elute in several peaks, reflecting differing degrees of glycosylation. Tertiary structure also affects HIC. When a protein is conformationally labile, it tends to elute in a broad peak. However, the concentration of hydrophobic residues in a particular domain on a protein surface constrains the configuration of binding and leads to elution in sharp peaks.

Problems with HIC:

1) Desalting: Unfortunately the salts used in HIC are not lyophilizable. They may be eliminated from a sample using chromatography (steric exclusion, reversed phase, etc.) or dialysis.

2) Baseline: The marked gradients in salt concentration used in HIC lead to great changes in the refractive index of the mobile phase. This may lead to artificial peaks in the baseline. For example, the use of an ammonium sulfate gradient produces a rising baseline with a broad maximum when the effluent is monitored at 220nm. Rising or falling baselines may be observed, depending on the salt and wavelength used. The problem is less severe at 254nm and 280nm. The change in baseline imposes a lower limit on the amount of protein that can reliably be detected on-line. The problem is eliminated entirely by monitoring fluorescence instead of absorbance if the protein or peptide contains tryptophan.

3) Denaturation: HIC promotes retention of tertiary structure, and most proteins are recovered with 80-100% retention of biological activity. However, quaternary structures may be affected; the high salt concentrations used cause some subunit containing proteins to fall apart. This effect is generally independent of the column, and must be assessed in each individual case.

4) Aggregation: Generally, it is not a significant problem. Mixtures that tend to aggregate in solution sometimes elute from HIC columns in discrete peaks. Aggregation usually involves hydrophobic interactions, but it appears that the hydrophobic surface of the column packing out-competes the tendency of individual protein monomers to bind to each other.