Proteomics

SCX-RPC of Tryptic Digests: No single analytical method suffices to identify every protein in a very large collection. A widely-used approach ("shotgun" or "bottom-up" proteomics) is to digest all the proteins in a mixture with trypsin and divide the resulting peptides into sets small enough to permit identification via MS/MS or QTOF-MS. For samples with < 200 peptides, reversed-phase HPLC usually provides adequate resolution, but larger samples must first be divided into subsets via a complementary method. The best way to accomplish this is to use **Strong Cation-Exchange (SCX)** to separate the digest into fractions by differences in charge-to-mass ratio. Each SCX fraction can then be analyzed separately via RPC-MS. This sequence has been used to identify as many as 12,000 peptides in a sample. Our **PolySULFOETHYL Aspartamide™** material is the best for the purpose and is used by most proteomics labs. It is best performed with a salt gradient at pH 2.7-3.0, where carboxyl- groups have lost their negative charge and nearly all peptides have a net + charge. About 65% of the peptides in a typical complex tryptic digest have a net charge of +2 (due to the basic N-terminus and the Lys- or Arg- at the C-terminus). A linear salt gradient would cause these to elute bunched up in a few fractions, defeating the purpose of a second dimension of chromatography. Instead, elute the column with a 2-segment linear gradient; a shallow gradient (to about 180 mM salt) over most of the gradient time to spread out the +1 and +2 peptides and a steep gradient to ~ 0.5 M salt to elute the +3 and +4 peptides. The following is a good example of uniform distribution of the peptides in a tryptic digest.

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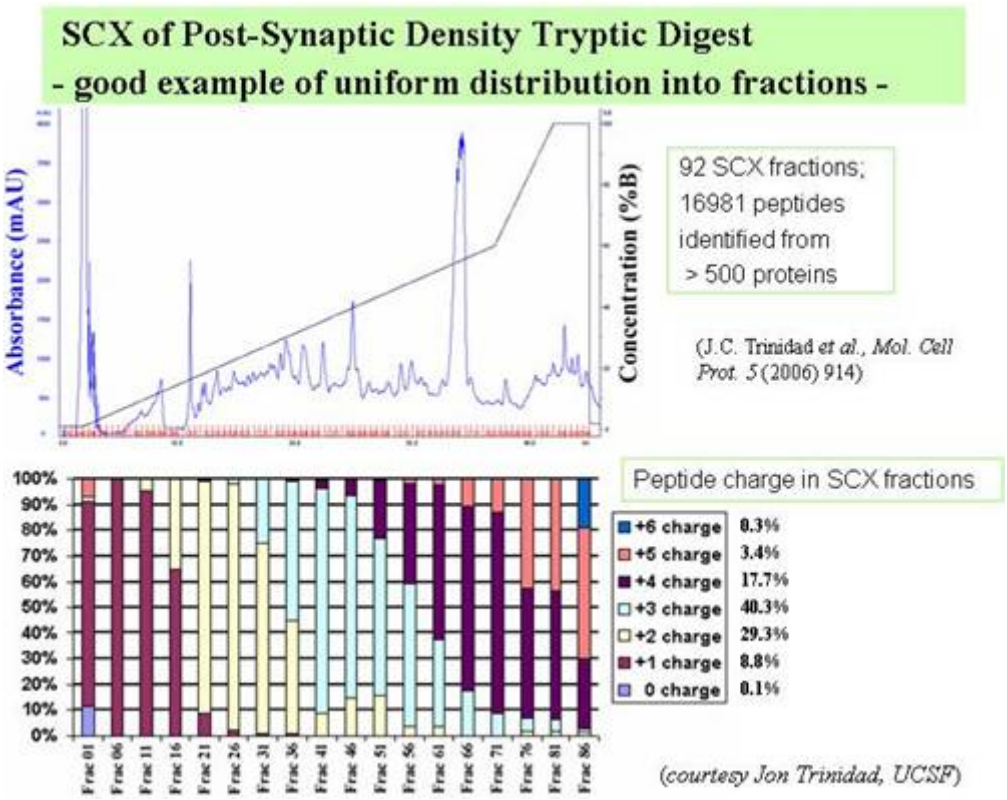

Online vs. Offline SCX Fractionation: Packed capillaries of **PolySULFOETHYL A™** can be eluted via steps of increasing salt concentration, with each fraction going to a desalting RPC trap cartridge or directly to a RPC capillary. This arrangement is commonly called **MuDPIT**. The alternative is to collect fractions offline and then inject them separately onto the RPC capillary ("**divorced MuDPIT**").

Online advantages: Easier to handle extremely small samples and to automate.

Offline advantages:

1) Can be performed with simpler equipment.

2) The mobile phase can contain 20-25% ACN, which can be removed prior to the RPC step. This affords sharper peaks. Result: A higher percentage of the peptides elute within a single collected fraction instead of being split between two adjacent fractions (in favorable cases, >75% of the total peptides in a really complex tryptic digest). This increases success in peptide identification, since you are more likely to get a detectable quantity (~ 15 fmol) of a low-abundance peptide within a single fraction and it is less likely to be sharing the fraction with a peptide from a high-abundance protein (which could suppress its ionization at the MS step).

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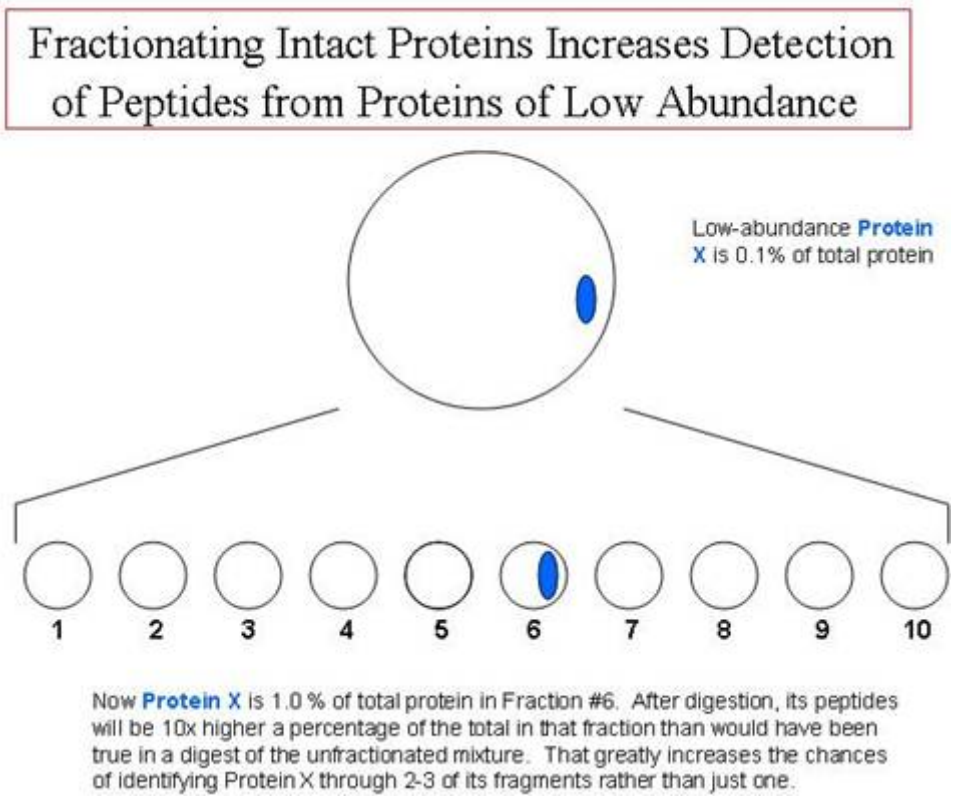
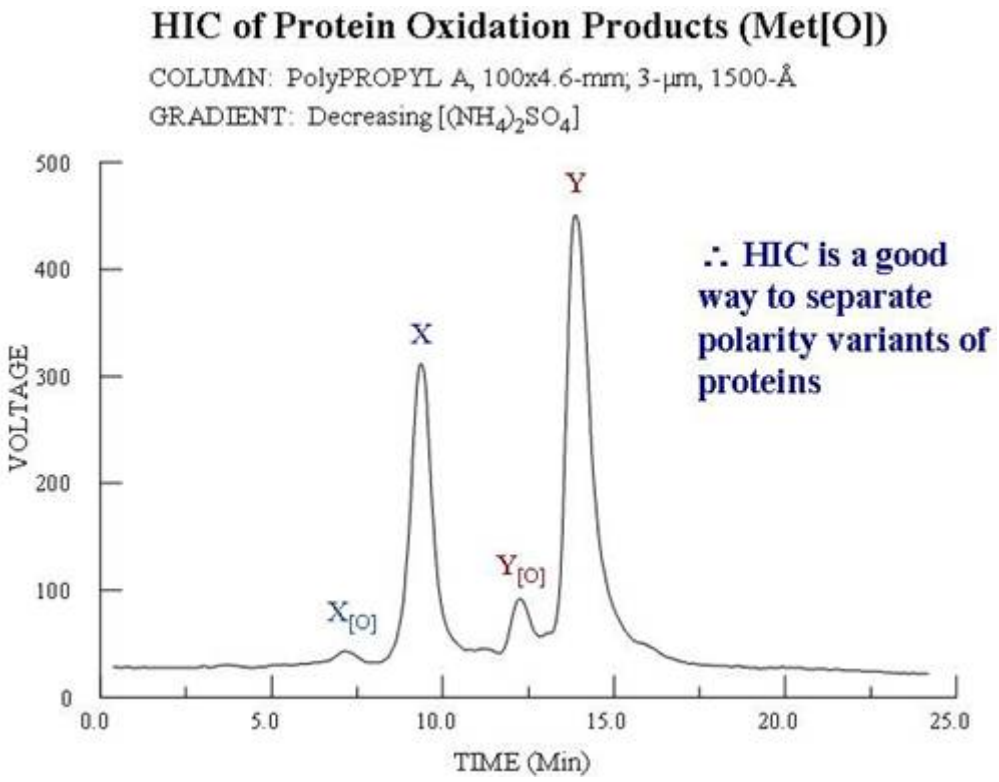
3) The SCX column can be larger than the RPC capillary. This permits loading of more sample, making it more likely that you will have > 15 fmol of each peptide to detect. It also permits use of a faster flow rate. This facilitates collection of numerous fractions (as many as 100 at some labs). That, in turn, increases the chances of identifying the peptides of low abundance, since it is less likely that they will be sharing a fraction with a peptide of high abundance. For example, Gan et al. (*Proteomics 5* (2005) 2468) identified 2.8x more proteins by collecting 40 SCX fractions than they did with 25 SCX fractions.
4) It is possible to use linear gradients instead of step gradients. This increases resolution.

CONCLUSION: Offline fraction collection is superior . Kevin Blackburn (NC St. U) states* that he identifies 3x more peptides with offline fraction collection than with online, with all of the increase representing peptides of low abundance.
*(ASMS '00 and '01).

Predigest Fractionation of Intact Proteins : This can be very useful in several cases:
1) There are so many proteins in the sample that even the 2-D combination of SCX-RPC does not suffice to fractionate the tryptic digest sufficiently to identify low-abundance peptides.
2) The sample contains a few proteins of much higher abundance than the others. Collecting them in their own fractions will preclude their tryptic fragments masking peptides from proteins of low abundance. See the example below of THP-1 monocytes.
3) While the number of proteins is limited, the ones of interest are of low abundance and would be masked if not separated from the high-abundance proteins prior to digestion. See examples of this with Histones H4 and H1.5.
4) Distributing proteins into fractions prior to digestion increases the chances of identifying a specific protein by more than one peptide. See the following schematic:



General-purpose methods for this include Hydrophobic Interaction (HIC) and ion-exchange (IEX) chromatography. HIC is a good method for fractionating water-soluble proteins by differences in their hydrophobicity [BELOW]. Unlike reversed-phase chromatography, it is a nondenaturing mode. However, hydrophobic proteins would be difficult to keep in solution in HIC.



By contrast, IEX can be performed with organic solvents in the mobile phase, making it prospectively compatible with all proteins. See Ion Exchange of Proteins with Organic Solvents.

Using an anion- and a cation-exchange column in series yields a mixed-bed arrangement that retains all proteins. The example below is a crude lysate of THP-1 monocyte cell pellet, obtained with a **PolyCAT A™** and a **PolyWAX LP™** column. Gradients with volatile salts are possible.

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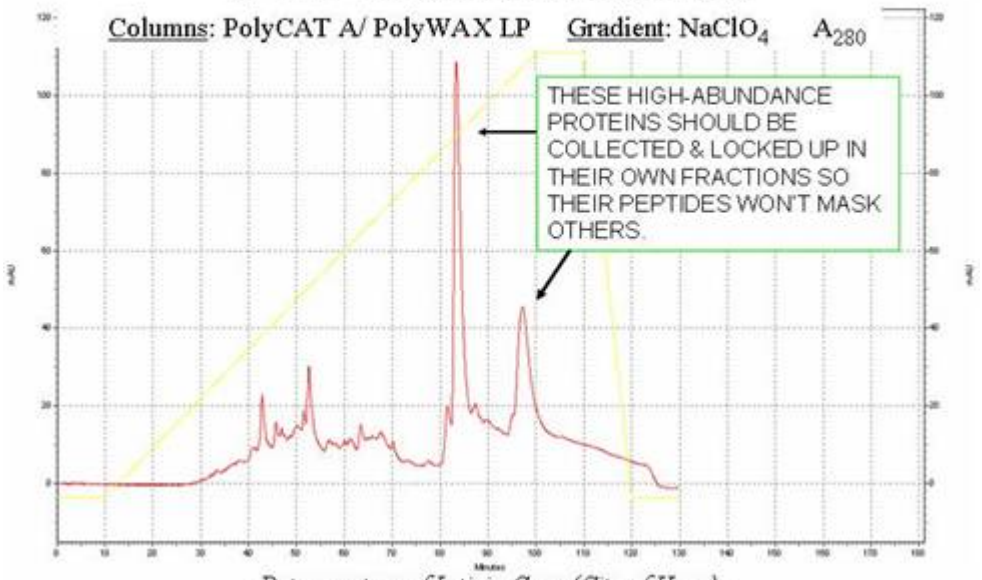
Example of HPLC of Intact Proteins:

Mixed-bed IEX of THP-1 Monocyte Cell Lysate

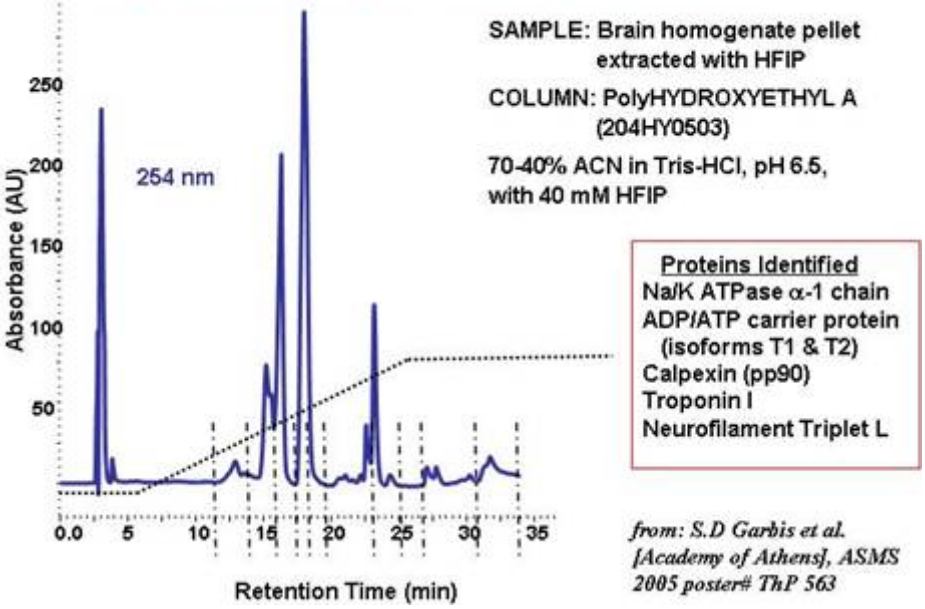
(~ $6x10^6$ cells; water-soluble fraction)



- Data courtesy of Leticia Cano (City of Hope) -

HILIC of membrane proteins: HILIC works well for membrane proteins, as per the examples below. Volatile solvents can sometimes be used for this. Histones are best resolved with **PolyCAT A™** columns in the HILIC mode.

- HILIC of Mouse Brain Membrane Proteins -



from: S.D Garbis et al. [Academy of Athens], ASMS 2005 poster# ThP 563

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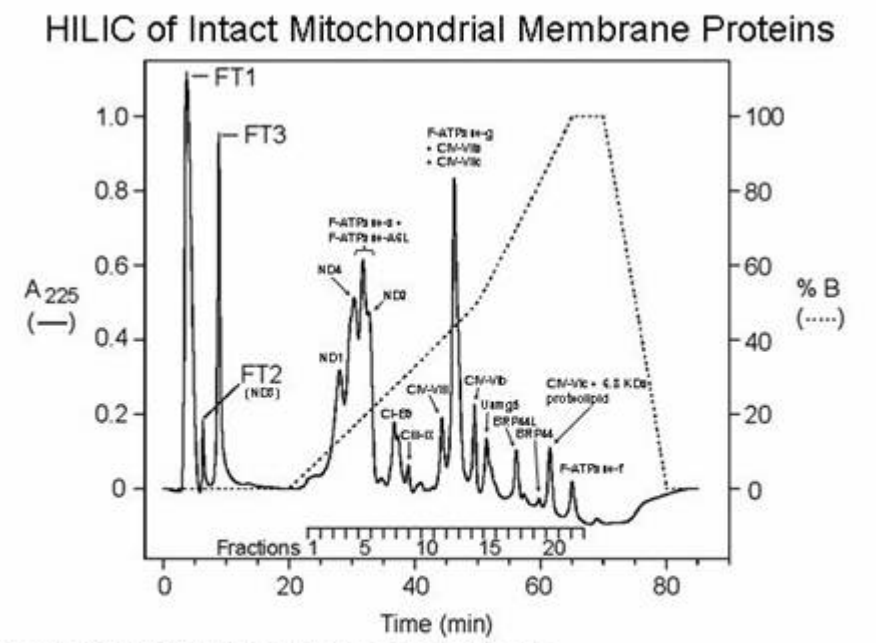
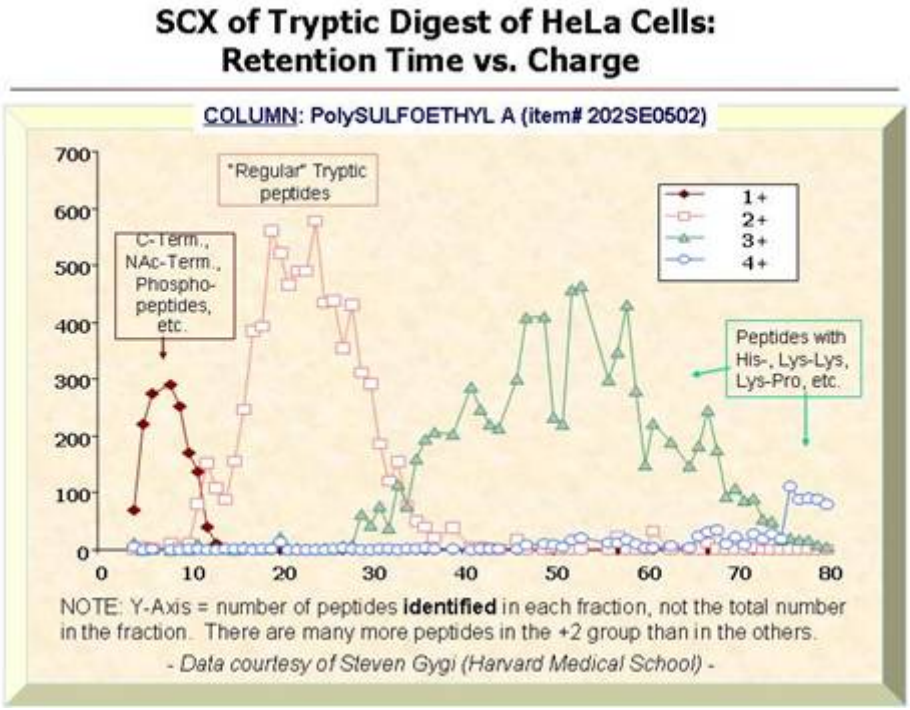


Sample: 2-PrOH/ACN (pH 3.7) extract of bovine heart mitochondria
Column: PolyHYDROXYETHYL A (100 x 2.1 mm)
Gradient: 20 mM ammonium formate, pH 3.7, + 0.5% HFIP; (63% 2-PrOH + 22.5% ACN) to 30% 2-PrOH

From: Carroll et al (2006) PNAS 103, 16170-16175.

Courtesy of John Walker, Medical Research Council Dunn Human Nutrition Unit, Cambridge, U. K.

Isolation of **Phosphopeptides** and Other Classes of Peptides from Tryptic Digests: The typical tryptic peptide has a net charge of +2 at pH 2.7-3.0, due to the N-terminus and the Lys or Arg residue at the C-terminus. Attachment of a phosphate group lowers the net charge of the peptide to +1. Thus, the earliest-eluting SCX fractions are enriched in phosphopeptides, as well as C-terminal and blocked N-terminal fragments. The 200-Å pore version of **PolySULFOETHYL A™** has a higher surface area than the 300-Å material normally used for proteomics and can pull the +2 peptides away from the +1 peptides reasonably completely [BELOW]. Beausoleil et al. (*PNAS 101* (2004) 12130) have used this approach to identity over 2000 phosphopeptides from the tryptic digest of HeLa cell lysate. In various studies, about 20-30% of the phosphopeptides in a really complex tryptic digest eluted in this +1 window. A good recent examination of this approach is: J.C. Trinidad *et al., Mol. Cell. Proteomics* 5 (2006) 914.



At the other extreme, crosslinking two +2 peptides results in a peptide with a net charge of +4. Thus, in a nonreduced tryptic digest, the disulfide-linked peptides elute appreciably later than most of the other peptides. This method has been used to isolate them selectively.

Problems with Membranes and Whole Cell Pellets:
1) Dissolving the sample.
2) Eliminating lipids from the resulting solution.
3) Maintaining membrane and structural proteins in solution during subsequent fractionation.

Such samples can be dissolved within minutes, at room temp., with a 4:1 solution of HFIP [*neat*] and conc. formic acid (96-98%). Membrane proteins can also be extracted with propanol or acetonitrile containing HFIP (ref.: J. Carroll et al., *PNAS 103* (2006) 16170). These solvents avoid the interference that detergents impose with downstream analytical methods. The lipids in the resulting solution can be eliminated by passing it through an SPE cartridge in the HILIC mode; *e.g.*, item# SPEHY1203, TT200HEA, etc. Lipids (and salts and detergents) are not retained but proteins and peptides are. The proteins and peptides can then be eluted with steps to increasingly aqueous solution. To maintain the proteins in solution during IEX-HPLC, use $NaClO_4$ for the gradient and include

the following in the mobile phases: 50 mM HFIP, 20% ACN and 20% PrOH. In extreme cases, use 35% ACN and 35% PrOH. Alternatively, use **PolyHYDROXYETHYL A™** in the HILIC mode, per the examples above with membrane proteins.

ICAT®; iTRAQ®; MuDPIT :
ICAT: If you can ascertain a protein's presence by identification of 2 or 3 of its tryptic fragments, then identification of the rest is redundant. The "AT" part of ICAT eliminates most of the tryptic fragments from a protein, making it possible to identify more proteins from complex mixtures. The complete digest is usually fractionated by SCX on **PolySULFOETHYL A™** prior to affinity purification on an avidin column. This cleans up the sample and eliminates excess ICAT reagent, which might otherwise saturate the binding capacity of the avidin.
iTRAQ: This facilitates measurements of relative abundance but does not necessarily simplify the mixture in the manner of ICAT. After reaction with the iTRAQ reagents, be sure to drop the pH to 3.0 or less and desalt the mixture in order to get good retention in SCX.
MuDPIT: See discussion above about online vs. offline methods.

Common Pitfalls in Proteomics Strategies :
1) Failure to eliminate lipids: These clog RPC columns and interfere in general. Eliminate them with a SPE-HILIC cartridge or else perform ion-exchange with a mobile phase containing > 40% organic solvent.
2) Using urea, SDS or Gd.HCl during trypsinization: These are incompatible with RPC and/or MS. Use 30% PrOH instead. Ref: W.K. Russell *et al., Anal. Chem. 73* (2001) 2682.
3) Failure to adjust pH after trypsinization or iTRAQ reaction: Trypsinization and iTRAC derivatization is performed ~ pH 8 while subsequent SCX is performed at pH 2.7-3.0. Failure to adjust the pH will lead to elution of a sample in the void volume. If possible, use NH_4HCO_3 as the trypsinization buffer instead of Tris, since it can be eliminated via lyophilization.
4) Sample too salty: If using a SpeedVac® to get rid of NH_4HCO_3, take the sample to dryness three times in succession. Desalting iTRAQ reaction products is better than merely diluting them with the SCX mobile phase.
5) Use of TFA or HFBA: Some authors have recommended their use in the sample solvent or the starting mobile phase for SCX. DON'T! Tryptic peptides are frequently not retained with such solvents. Use

either 5 mM KH_2PO_4 or else 0.1% formic acid or acetic acid.
6) Overloading the SCX column or capillary: Recommended maximum loads of peptide per injection are as follows for **PolySULFOETHYL A™** columns: 0.30-mm i.d., 25µg; 1.0-mm i.d., 250µg; 2.1-mm i.d., 1.0 mg; 4.6-mm i.d., 5 mg. Some groups exceed these levels by up to 6x. This saves labor and yields more concentrated fractions. Problems:

a) The columns usually last 1/6 th as long.
b) Peaks are broader. This means there will be more peptides in each collected fraction. The RPC capillary might be overloaded, resulting in a smaller percentage of peptides being identified.

CONCLUSION: Use a SCX column large enough for your sample.