Guide to Reverse Phase SpinColumns Chromatography for Sample Prep

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Introduction

Fast Protein and Peptide Cleanup with C4, C8 or C18 SpinColumns

When working with protein and peptide samples some purification is usually required, therefore, to remove the salts, solvents, and protecting groups that can interfere with mass spectrometric or spectrophotometric analysis of the peptides, or to trace enrich small quantities of organism water or to isolate products in serum. The C4, C8 & C18 SpinColumns from Harvard Apparatus offer a rapid method for sample cleanup by isolating and/or purifying components of your sample.

The C4, C8 & C18 SpinColumns use reversed-phase chromatography for the separation of polypeptides and proteins. The type of column used is essential to achieving an optimized separation. The reverse phase columns SPE, are ideal for separation of proteins where high resolution of many closely sized components is needed, but it may require increasing non-polar solvents.

Introduction (cont.)

Wet Match

There is an easy acronym for determining polarity of your sample. It relies on samples dissolving in solvents they are like so polar compounds dissolve in polar solutions and non-polar compounds dissolve in non-polar solvents. So an easy way to determine the polarity of the sample is to dissolve it in different solvents.
Wet Match is an easy acroymn to assist you, and an easy way to remember is, you cannot start a fire with a “wetmatch” or “WMATCH”.

	Polar protic		**Polar aprotic**		**Nonpolar**		
	W	M	A	T	C	H	
	A	E	C	H	O	E	
	T	T	E	F	L	X	
	E	H	T		O	A	
	R	A	O		R	N	
		N	N		F	E	
		O	E		O		
		L			R		
					M		
Non Polar	C18	CH	C8	C4	CH	Silica	Polar

Modes of Separation

Step Gradient

Step 1

Step 2

Step 3

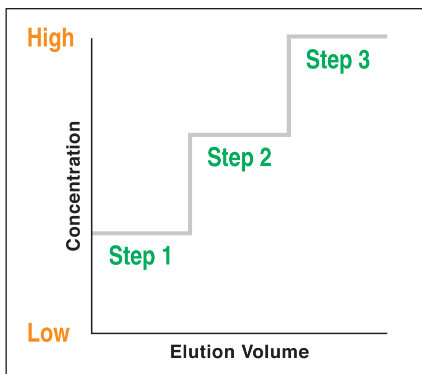
Waste

Collection

Prepare sample.

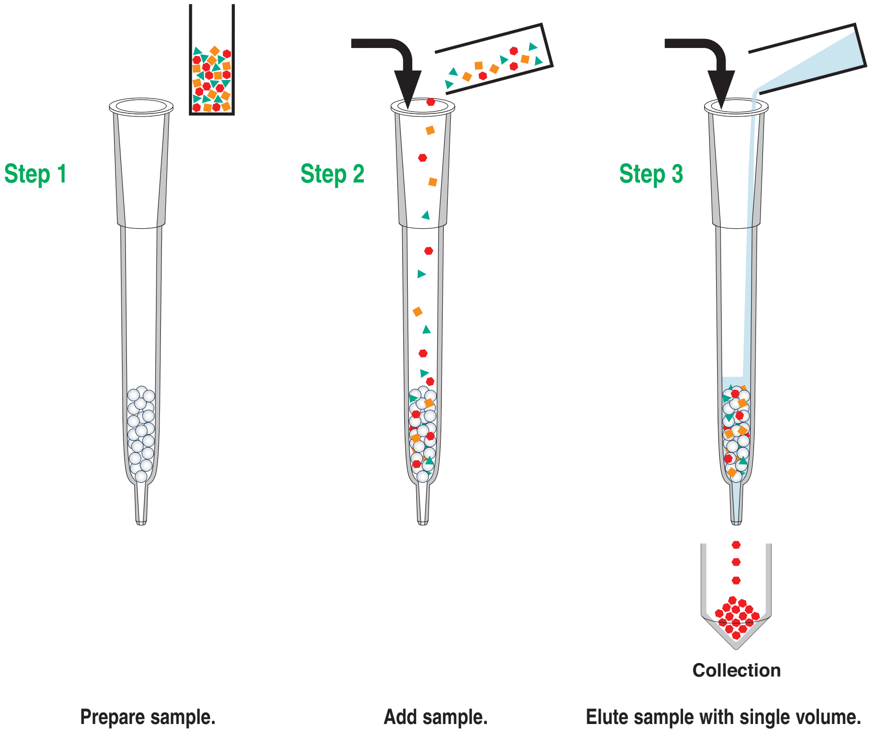
Add sample.
Sample remains in column.
Impurities pass through
the column.

Elute sample from column with
buffer. Low ionic strength or
solvent and increase the
concentration by steps.



Modes of Separation (cont.)

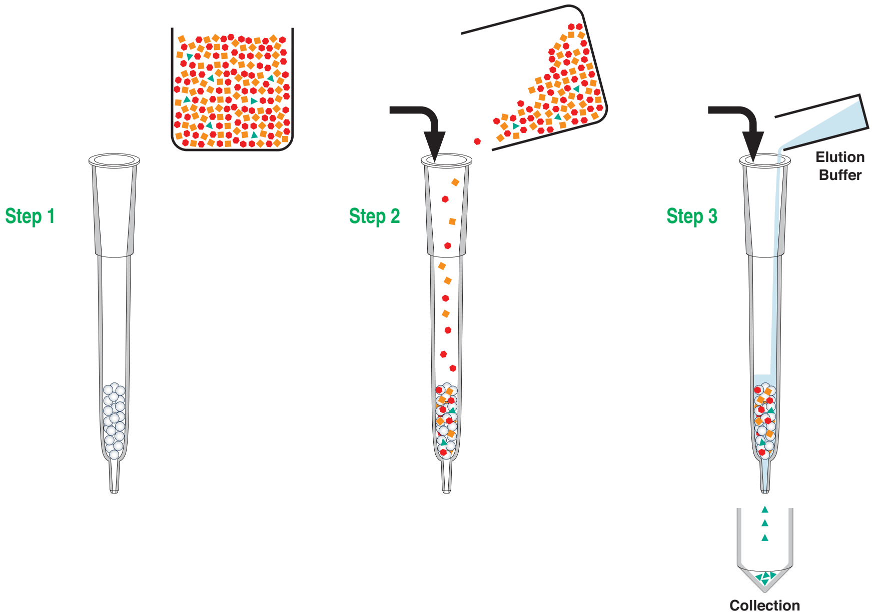
Direct Elution



separate By – Retaining/Retarding unwanted materials on the column and have the molecule of interest pass through the column with the void volume and collect.

Modes of Separation (cont.)

Trace Enrichment



Prepare sample.

Add sample.

Impurities remain in column, sample is eluted with buffer.

separate By – Retaining the molecule of interest to the column, then remove them with elution buffer.

SpinColumn Selectivity

Harvard Apparatus SpinColumns good selectivity, and feature:

- Faster protein, peptide separations than traditional column formats
- Macro SpinColumns in 200 µl packing
- Fast protein and peptide separations without conventional HPLC systems

The 150 µl, 300Å packing's are ideal for fast bioseparations in life science applications. The C18 packing's and short 5 mm column coupled with centrifugation give faster sample throughput.

SpinColumn Selectivity (cont.)

Description	Ultra-Micro	Micro	Macro	96-Well Micro	96-Well Macro
Bed Volume	37.96 µl	66.42 µl	191.45 µl	66.42 µl	191.45 µl
Sample Volume	10-25 µl	25-75 µl	75-150 µl	25-75 µl	75-150 µl
Sample Concentration	3-30 µg	5-60 µg	30-300 µg	5-60 µg	30-300 µg
Elution Ultra Volume	28.5 ml	50 ml	143 ml	50 ml	143 ml

Fast Protein Analysis

Optimization of Step Gradient Parameters for Fast Protein Analysis

SpinColumns are the perfect tool for fast reversed-phase protein separations. These are not columns so their plate count is low. In gradient selectivity come from exchange of polarities of solvent in and the volume. So selectivity in sample prep with prep columns is dependent on getting the sample to stick and use different polarities to elute off the sample or to have the molecule of interest to come off in a particular volume. Proteins adsorb at the head of the column and then desorbs and elute once the critical mobile phase concentration is reached. C18 SpinColumns are simple devices in which chromatographic functional groups have been packed. These SpinColumns have been modified with anion exchange, cation exchange, hydrophobic, hydrophilic, and affinity functional groups. For synthetic peptide cleanup, a C18 ligand is a very effective functional group. When used with appropriate buffers, the peptides are added to the column and will adsorb to the chromatographic ligands and are separated from other solutes and the solvent. Subsequent elution of the peptide with an appropriate buffer completes the purification. Using C18 SpinColumn, a peptide sample can be desalted for analysis in several minutes, supplying enough purified material for analysis by mass spectrometry. Multiple samples can easily be run in the same time period.

Reversed-Phase C18 Columns For Preparative purification of Proteins and Peptides

The reversed-phase adsorbent consists of porous divinylbenzene with a stable polar surface modification. C18 is very stable in the presence of both strong base and strong acid. It is a unique and versatile tool for protein and peptide chromatography. Its exceptional chemical resistance makes it ideal for separations of difficult proteins or peptides that require harsh conditions to maintain solubility, and also for more routine applications where it is desired to clean and sanitize columns between runs, for example by washing with strong alkali.

Specifications

C18 Specifications:

Particle Size	10 µm
Pore Size	300 À
Pore Volume	0.9 ml/gm
Surface Area	100 m^2/gm
%Carbon (w/w)	8%
Silica Class	Type B
Acid and alkali stable	pH 1.5-10

C8 Specifications:

Particle Size	5 µm
Pore Size	300 À
Pore Volume	0.9 ml/gm
Surface Area	100 m^2/gm
%Carbon (w/w)	5%
Silica Class	Type B
Acid and alkali stable	pH 1.5-10

C4 Specifications:

Particle Size	4.5 µm
Pore Size	300 À
Pore Volume	0.9 ml/gm
Surface Area	100 m^2/gm
%Carbon (w/w)	3%
Silica Class	Type B
Acid and alkali stable	pH 1.5-10

Chemical Stability

The C18 polymer has a 300 Å pore size to provide access to all adsorbent surfaces for large peptides and proteins. The chemistry of the 20 μm particles allows easy scale-up from Ultra-micro SpinColumns to Macro SpinColumns with minimal modifications to conditions.

The polymer-based reversed-phase SpinColumn is chemically stable and, more importantly, provides good performance for the separation of peptides and proteins. The packing material is based on porous (300 À pore diameter), highly crosslinked polystyrene-divinylbenzene spheres. Because of the high crosslinkage, the column gives excellent mechanical stability with a minimum degree of shrinking in aqueous and swelling in organic solvents. Buffers are required when the sample contains ionic or ionisable analytes. Without a buffer, poor peak shape and variable retention may result.

In general, for acids, increasing eluent pH leads to increased ionization and a decrease in retention. For bases, decreasing pH results in greater ionization and decreased retention. For robust methods, separations should be developed at a pH where retention is least affected by pH changes.

Protocol

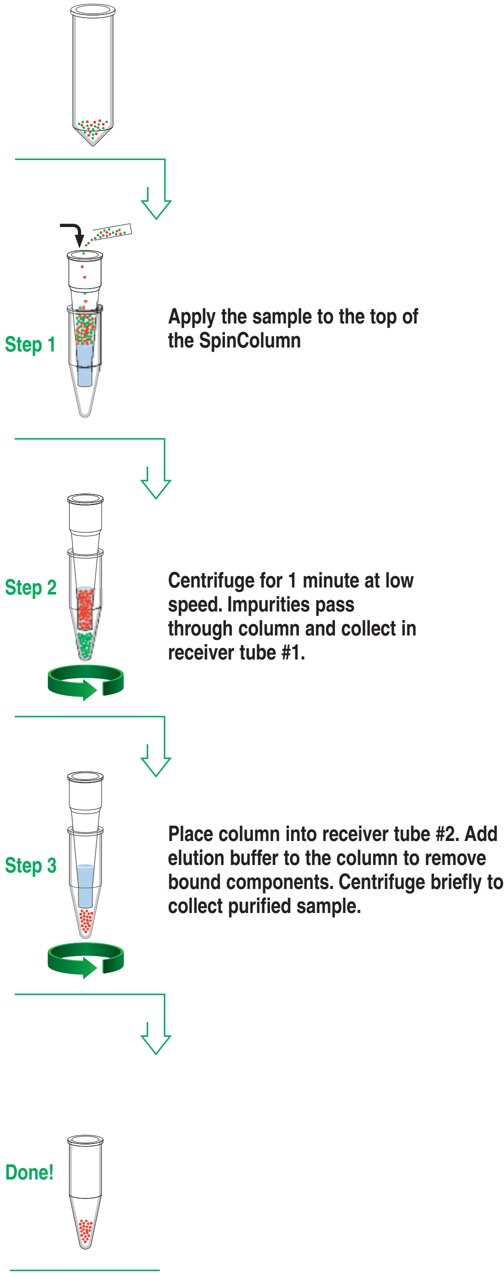
Peptides in aqueous buffers adsorb to C18 SpinColumns and can be subsequently eluted with an organic based buffer. As with reverse phase chromatography, the addition of trifluoroacetic acid as an ion-pairing agent to both the binding and elution buffers can greatly enhance adsorption and elution, respectively. Depending upon the nature of the peptide (i.e., composition and length), the elution buffer can be a combination of aqueous and organic solvents. This allows for washing of bound peptide at lower concentrations of organic solvent, and elution at a higher concentration.

Macro SpinColumn

1. Place the column into a centrifuge tube
2. Dissolve crude peptide in molecular biology grade water at 1 mg/ml.
3. Condition the column with 500 µl of 100% actonitrile or methanol in the column.
4. Centrifuge for 2 minutes at approximately 110x g.
5. Remove column from the tube and blot the exterior dry.
6. Rinse the column 2x with 500 µl molecular biology grade and Centrifuge for 2 minutes at approximately 110x g
7. Add between 75 µl and 325 µl of sample to the column (150 µg maximum sample).
8. Place the column in a new centrifuge tube and spin for 1 to 2 minutes at approximately 110x g.
9. The sample will be retained in the column and the salts, solvents will be in the collection tube.
10. Elute the sample from the column using 500 µl acetonitrile with 0.1% trifluoroacetic acid and place into a clean collection tube and spin at 110 x g for 1 minute. (depending on the polarity of the sample the above step may need to be repeated)

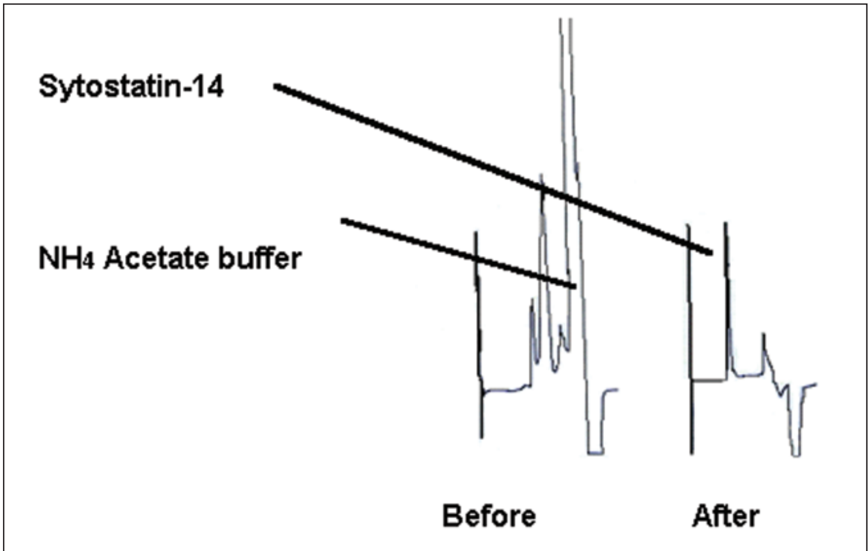
Protocol (cont.)

Solid Phase Extraction SpinColumns



Protocol (cont.)

For example:
Reverse Phase C-18, Somatostatin-14 binds in aqueous phase and after washing the SpinColumn. The peptide was eluted with 70% isopropanol (after) and injected to HPLC. The peptide is desalted as well concentrated by a factor of 2. The analysis was performed on HPLC Column (PolyHydroxyethyl A) manufactured by PolyLC, Inc



Discussion

Analysis of both the crude peptide solution and the cleaned peptide demonstrates that the C18 SpinColumn effectively and rapidly separates synthetic peptides from contaminating solutes. This ability to capture a desired peptide without purifying an entire sample and the compatibility of the SpinColumn with standard centrifuges can greatly speed the analysis of libraries stored in microwell plates.

Choice of Buffer pH

Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. In general, the effective pH range for a buffer is within ± 1 pH unit of the pKa. The buffer pH should be selected so that it is at least ± 1 pH units from the pKa of the Protein or peptide. This will ensure that the protein or peptide is 100% ionized or 100% non-ionized, for reproducible retention.

Buffer

For MS analyses, volatile buffers or additives are preferred, in order to minimize MS ion suppression and maintain sensitivity. In addition, non-volatile buffers such as phosphate may lead to contamination of the ion source. Buffer concentrations should be as low as possible e.g. 10 to 20mM. Ammonium salts are more volatile than those of Na+ or K+. TFA should be avoided with electrospray LC-MS as it reduces sensitivity. Formic acid or acetic acid (0.01 to 1% v/v) is preferred. For higher pH applications, ammonium hydroxide is recommended.

Discussion (cont.)

C18 SpinColumn Performance

The SpinColumn are packed with bonded silica, which allows exceptional high stability, The C18 is suitable for purification of various analytes. The unique mono-functional bonding chemistry for C18 avoids the formation of multiple C18 layers. This uniform phase allows the separation to achieve selectivity and efficiency.

Samples and Mobile Phases

Samples and solvents including buffers should be filtered through 0.45 µm or 0.2 µm filters before use. C18 bonded stationary phase is nonpolar in nature. It is recommended that the mobile phase be a mixture of organic solvent, such as methanol or acetonitrile and water. Even though C18 can tolerate aqueous buffers as mobile phases, pure aqueous mobile phase might effect separation.

Ordering Information

Order #	Product
74-7226	Ultra-Micro SpinColumn C-18, pkg. of 24
74-4607	Micro SpinColumn C-18, pkg. of 24
74-4107	Macro SpinColumn C-18, pkg. of 24
74-7206	Ultra-Micro SpinColumn C-18, pkg. of 96
74-4601	Micro SpinColumn C-18, pkg. of 96
74-4101	Macro SpinColumn C-18, pkg. of 96
74-7227	Ultra-Micro SpinColumn C-8, pkg. of 24
74-4608	Micro SpinColumn C-8, pkg. of 24
74-4108	Macro SpinColumn C-8, pkg. of 24
74-7207	Ultra-Micro SpinColumn C-8, pkg. of 96
74-4602	Micro SpinColumn C-8, pkg. of 96
74-4102	Macro SpinColumn C-8, pkg. of 96
74-7228	Ultra-Micro SpinColumn C-4, pkg. of 24
74-4609	Micro SpinColumn C-4, pkg. of 24
74-4109	Macro SpinColumn C-4, pkg. of 24
74-7208	Ultra-Micro SpinColumn C-4, pkg. of 96
74-4603	Micro SpinColumn C-4, pkg. of 96
74-4103	Macro SpinColumn C-4, pkg. of 96
74-5617	Micro SpinColumn C-18, 96-Well Plate, qty. 1
74-5657	Macro SpinColumn C-18, 96-Well Plate, qty. 1
74-5618	Micro SpinColumn C-8, 96-Well Plate, qty. 1
74-5658	Macro SpinColumn C-8, 96-Well Plate, qty. 1
74-5619	Micro SpinColumn C-4, 96-Well Plate, qty. 1
74-5659	Macro SpinColumn C4, 96-Well Plate, qty. 1

Contact Information

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