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Isolation of Tryptic Phosphopeptides by ERLIC (Electrostatic Repulsion-Hydrophilic Interaction Chromatography)

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ABSTRACT

Anion-exchange chromatography is not able to cleanly separate tryptic peptides with 1 phosphate from peptides with no phosphate, owing to the electrostatic repulsion of the positively charged termini. When the column is operated in the HILIC mode, though, then the hydrophilicity of the phosphate group plus its electrostatic attraction to the stationary phase accomplishes this separation despite the electrostatic repulsion of the termini. This combination is called ERLIC: Electrostatic Repulsion-Hydrophilic Interaction Chromatography. Selectivity for phosphate groups is ensured by operation at pH 2. A gradient to 0.2 M phosphate elutes peptides with 1-4 phosphate groups. Retention is much greater using the volatile salt ammonium formate, to the point that it is practical to isolate phosphopeptides via solid-phase extraction in the ERLIC mode. Unlike high-affinity methods involving titania or IMAC, ERLIC is sensitive to aspects of peptide composition besides the phosphate group; the selective isolation and fractionation of phosphopeptides can be accomplished in one run. This makes it suitable as a high-resolution mode for samples containing thousands of phosphopeptides, as was demonstrated with the analysis of the digest of a HeLa cell lysate.

Selectivity is strongly influenced by orientation and sequence effects. Tryptic peptides are oriented with the C-terminus facing the stationary phase, so the closer the phosphate group is to the C-terminus, the more it influences retention and the later the peptide elutes.

INTRODUCTION

Anion-exchange chromatography (AEX) has been examined as a method for enrichment of phosphopeptides. However, at a pH low enough to uncharge Asp- and Glu- residues (so as to distinguish them from phosphate groups), the electrostatic repulsion of the positively-charged termini causes the elution of tryptic fragments prior to the void volume [**1**], and attachment of a single phosphate group does not suffice to overcome this repulsion. Such phosphopeptides aren't well-resolved from nonphosphopeptides in AEX [**2**]. When the column is operated in the HILIC (Hydrophilic Interaction Chromatography) mode, though, then the considerable hydrophilicity of the phosphate group plus its electrostatic attraction accomplishes this separation despite the repulsion from the termini. This combination is called ERLIC (Electrostatic Repulsion-Hydrophilic Interaction Chromatography) [**3**]. A gradient from 20 mM sodium methylphosphonate to 0.2 M triethylamine phosphate elutes peptides with 1-4 phosphate groups [**2**]. Volatile salts such as ammonium formate are much weaker displacing agents than phosphate or methylphosphonate salts. Retention of phosphopeptides is much greater with them, to the point that it is practical to isolate phosphopeptides via solid-phase extraction (SPE). This poster explores the use of ERLIC with ammonium formate mobile phases for isolation of phosphopeptides, either via gradient elution of a column or via SPE.

MATERIALS AND METHODS

The HPLC system was a model Essence® from Scientific Systems Inc. (State College, PA). Control and data collection was via EZStart® (Scientific Software). The HPLC column used for AEX and ERLIC was PolyWAX LP™, 5-µm, 300-Å (PolyLC Inc., Columbia, MD); 100x4.6-mm unless indicated otherwise. The SPE cartridges were TopTips (item# TT200WAX) packed with 20-µm PolyWAX LP (PolyLC).

Reagents: Phosphoric acid was HPLC-grade from Fisher Scientific while all other reagents were from Fluka. Methylphosphonic acid was *purum*-grade. Formic acid was *puriss./HPLC* grade. Triethylamine was *ultra* grade. All other reagents were HPLC-grade. A 1 M stock solution of ammonium formate was prepared by weighing formic acid into a beaker, adding a magnetic stir bar and water, adjusting the pH to 2.2 with ammonium hydroxide, then diluting to the mark in a volumetric flask. The pH of the final mobile phases was not measured after addition of acetonitrile (ACN). Sodium methylphosphonate (Na-MePO4) stock solution, pH 2.0, was prepared by addition of NaOH to a solution of methylphosphonic acid in water. Triethylammonium phosphate (TEAP) stock solution, pH 2.0, was prepared by addition of triethylamine to a solution of phosphoric acid in water.

Peptides: The following peptides were synthesized as described [**4**] using standard Fmoc solid-phase chemistry on an ABI 433A peptide synthesizer (Applied BioSystems, Foster City, CA): a) The peptide GGAAGLGY(p)LGK; b) The set of peptides with the sequence WWGSGPSGSGGSGGGK, with phosphate groups on 0-4 Ser- residues; c) The sequence variant peptides NAAAAAAWK, AAANAAAWK, AAAAAAWNK and their amidated analogs. Peptides SLYSSSPGGAYVTR (Vimentin(51-64)), SLYSSS(p)PGGAYVTR, SVNFSLTPNEIK (MAP 1B(1271-1282)), and SVNFSLT(p)PNEIK were a gift of Ken Jackson (Molec. Biol.-Proteomics Facility, Univ. of Oklahoma Health Sciences Center). Peptide TRDIYETDYYRK (Insulin Receptor (1142-1153)) and its phosphorylated analogue were purchased from Quality Controlled Biochemicals (Hopkinton, MA).

Elution with a gradient from Na-MePO4 to TEAP: Mobile phase A was 20 mM Na-MePO4, pH 2.0, with 70% ACN. Mobile phase B was 0.2 M TEAP, pH 2.0, with 60% ACN. After 5' at 100% A, there was a 20' linear gradient to 100% B followed by a 10' hold at 100% B. Flow rate: 1 ml/min.

Phosphate vs. Formate in Gradient Elution

Column: PolyWAX LP, 100x4.6-mm; 5-µm, 300-Å
Detection: A220 (blue), or A280 (red) 1 ml/min
Gradient: See Methods.

P0: WWGSGPSGSGGSGGGK
P1: WWGSGPSGSGGS(p)GGGK
P2: WWGSGPSGS(p)GGS(p)GGGK
P3: WWGSGPS(p)GS(p)GGS(p)GGGK
P4: WWGS(p)GPS(p)GS(p)GGS(p)GGGK

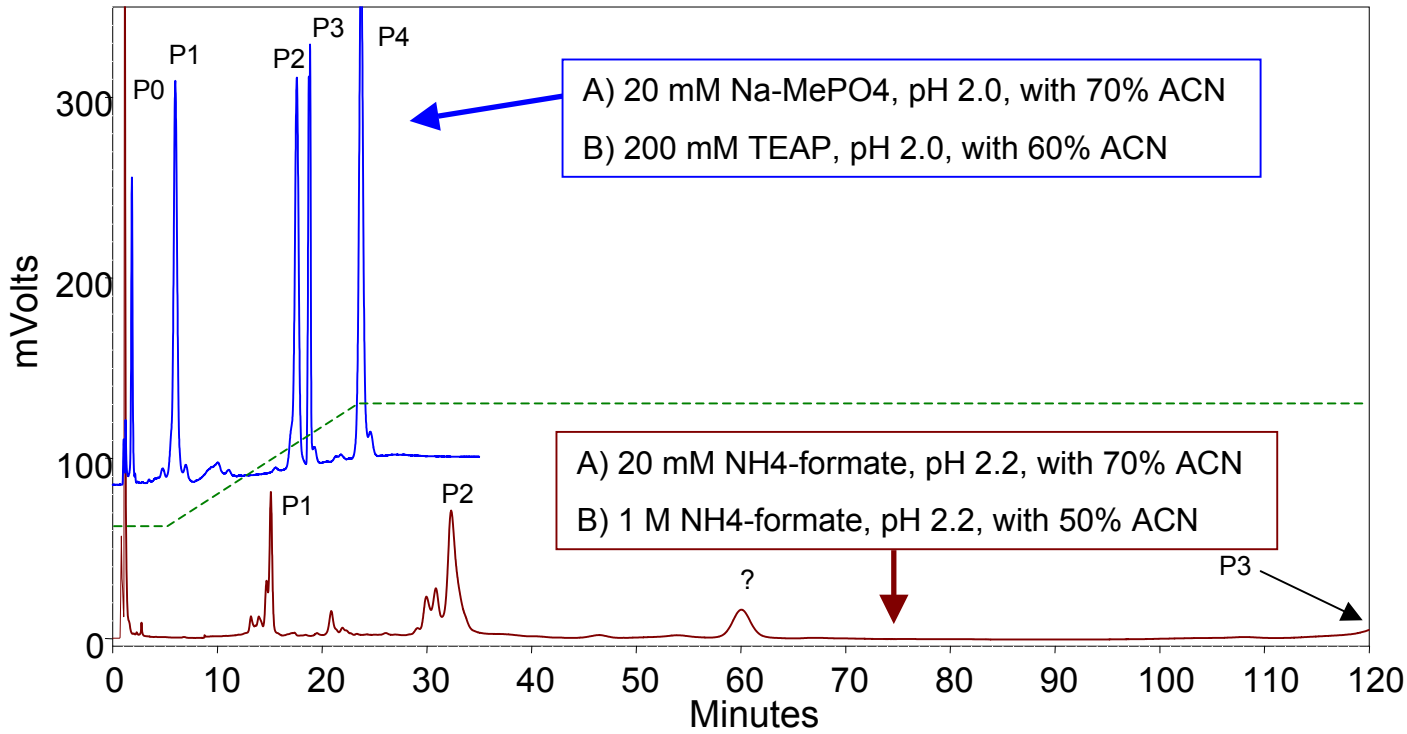


Fig. 1. Selective retention of phosphopeptides. At pH 2.2, the formate is ~ 97% in the form of the unbuffered acid. Unbuffered formic and acetic acids are extremely weak eluting agents; far weaker here than is TEAP. **RESULT: Far stronger retention of phosphopeptides**. Presumably less ammonium formate would be needed for elution at a higher pH, but then Asp- and Glu- residues would become charged and the selectivity for phosphopeptides would be lost. Under the present conditions, the electrostatic repulsion still causes the nonphosphorylated peptide to elute in the void volume.

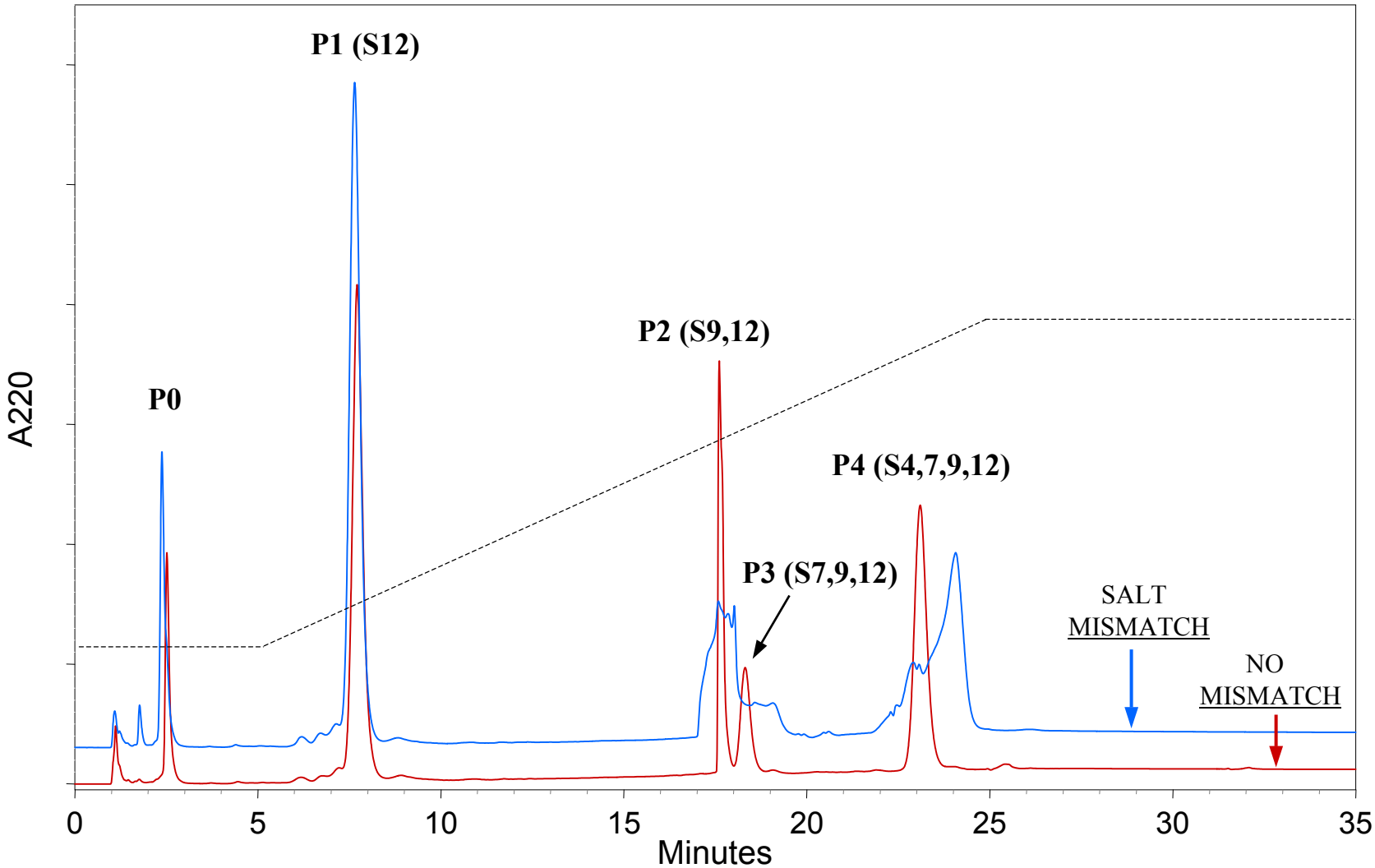


Fig. 2. ERLIC of Phosphopeptides: Effect of Salt Mismatch

Sample: WWG**S**GP**S**G**S**GG**S**GGGK with 0-4 phosphates, dissolved in 20 mM NH4-formate, pH 2.2, w. 70% ACN

HPLC analysis: 104WX0503 column with Na-MePO4 – TEAP gradient per standard ERLIC method

RED: Sample freeze-dried and redissolved in Na-MePO4 mobile phase (MP A) before analysis (no mismatch)

BLUE: 5 µl of sample in NH4-formate solvent mixed with 15 µl of Na-MePO4 (MP A) for injection (salt mismatch)

∴ **Mismatch between counterions severely affects the peak shape of multiphosphorylated peptides**

Elution of Ideal Tryptic Monophosphopeptide Standards: ↑ [Salt] vs. ↓ [ACN]

Column: PolyWAX LP Flow rate: 1 ml/min. Detection: As noted
Gradient: 0-5': 0%B; 5-45': 0-100% B; 45-50': 100%B
MP A: 20 mM NH_4-Formate, pH 2.2, with 70% ACN
MP B: [TOP] 100 mM NH_4-Formate, pH 2.2, w. 64% ACN;
[BOTTOM] 20 mM NH_4-Formate, pH 2.2, w. 10% ACN

KEY

A:	SLYSSSPGGAYVTR (Vimentin(51-64))
B:	SVNFSLTPNEIK (MAP 1B(1271-1282))
C:	WWGSGPSGSGGSGGGK (#1644)
A+P:	SLYSSS(p)PGGAYVTR
B+P:	SVNFSLT(p)PNEIK
C+P:	WWGSGPSGSGGS(p)GGGK (#1645)
D+P:	GGAAGLGY(p)LGK (#1876)

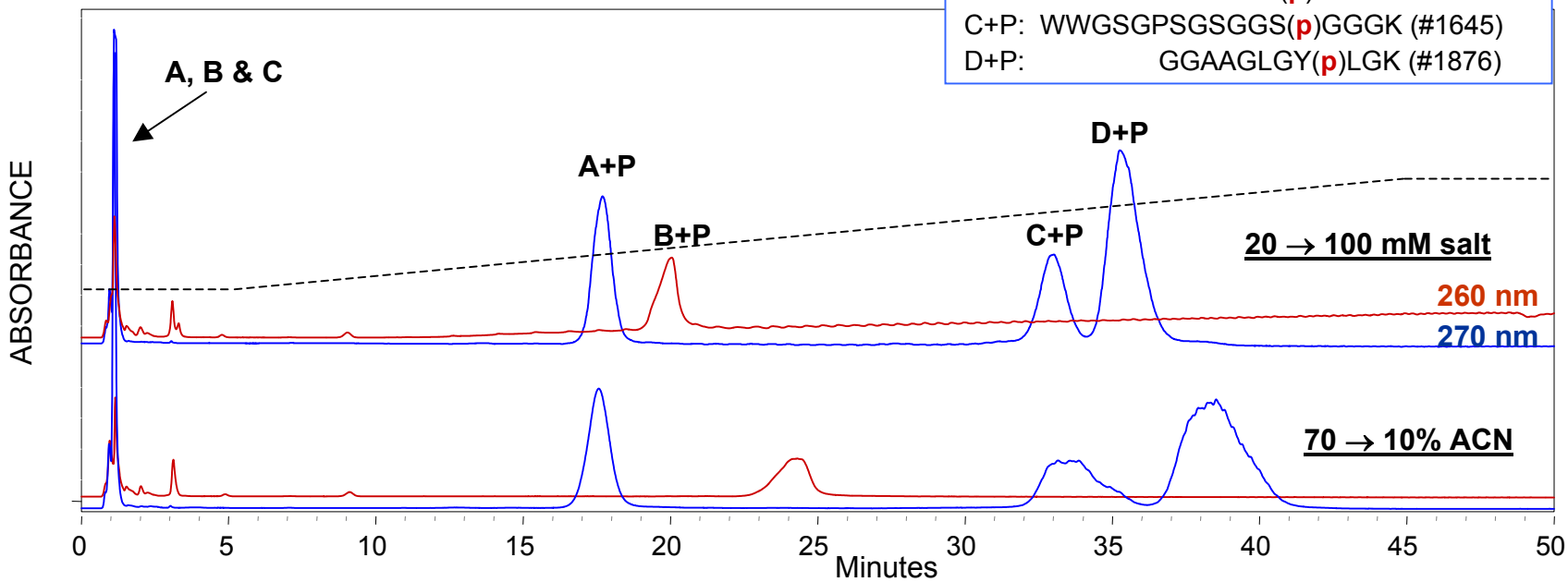


Fig. 3. Elution with formate buffer; alternative gradients. Increasing the salt content of the mobile phase is a standard way to elute solutes in ion-exchange, and works well here [*TOP*]. Selectivity and peak shapes are quite good. An alternative gradient of decreasing [ACN] was also tried, switching the mode from ERLIC to AEX, since tryptic monophosphopeptides are not well-retained in the AEX mode [*BOTTOM*]. The selectivity was retained although peak shapes deteriorated to some extent. The ACN gradient has two significant advantages: 1) Since the salt concentration doesn't vary, the absorbance baseline is steady. This suggests the possibility of monitoring ~ 220 nm for peptides that lack aromatic residues; the baseline would be ~ 0.4 AU but steady. 2) The use of only 20 mM ammonium formate would be quite convenient for direct flow to a mass spectrometer. Of course, a salt gradient would be necessary to elute peptides with more than one phosphate.

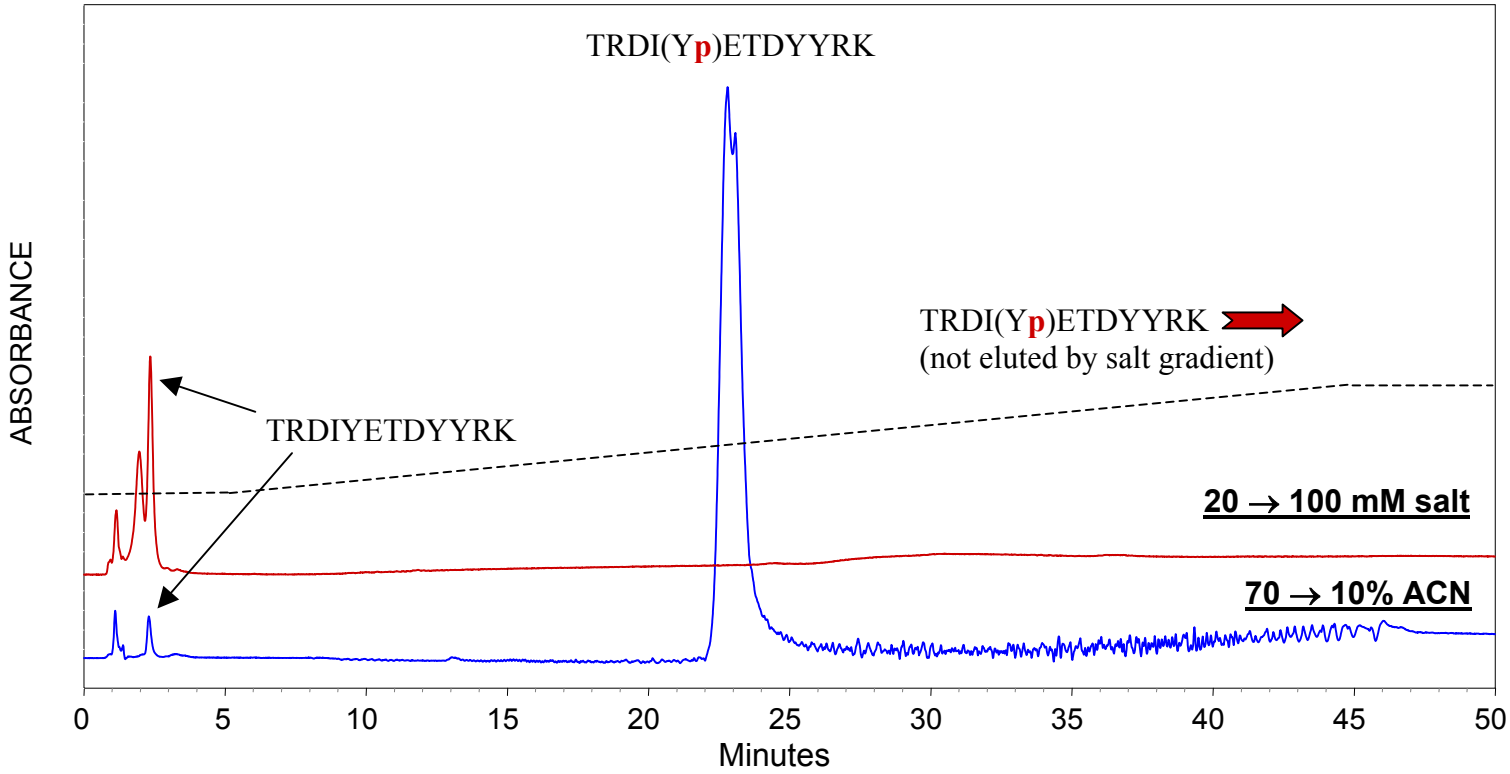


Fig. 4. Elution of Nonideal Tryptic Phosphopeptide Standards: ↑ [Salt] vs. ↓ [ACN]

CONDITIONS: Per Fig. 4. SAMPLE: Variants of Insulin Receptor (1142-1153)

These peptides have an extra basic residue at the C-terminus, a "ragged end", as well as one near the N-terminus. Basic residues are quite hydrophilic. This additional hydrophilic interaction led to the failure of the monophosphopeptide to be eluted by the gradient to 100 mM salt that eluted the standards in Fig. 4. The alternative gradient of decreasing [ACN] eliminated the hydrophilic interaction. The electrostatic repulsion of the basic residues now led to elution in the same time frame as the standards in Fig. 4.

Peptides with ragged ends and missed cleavages are commonly encountered in complex tryptic digests. CONCLUSION: **A decreasing [ACN] gradient is preferable to increasing [salt] for elution of singly phosphorylated tryptic fragments**.

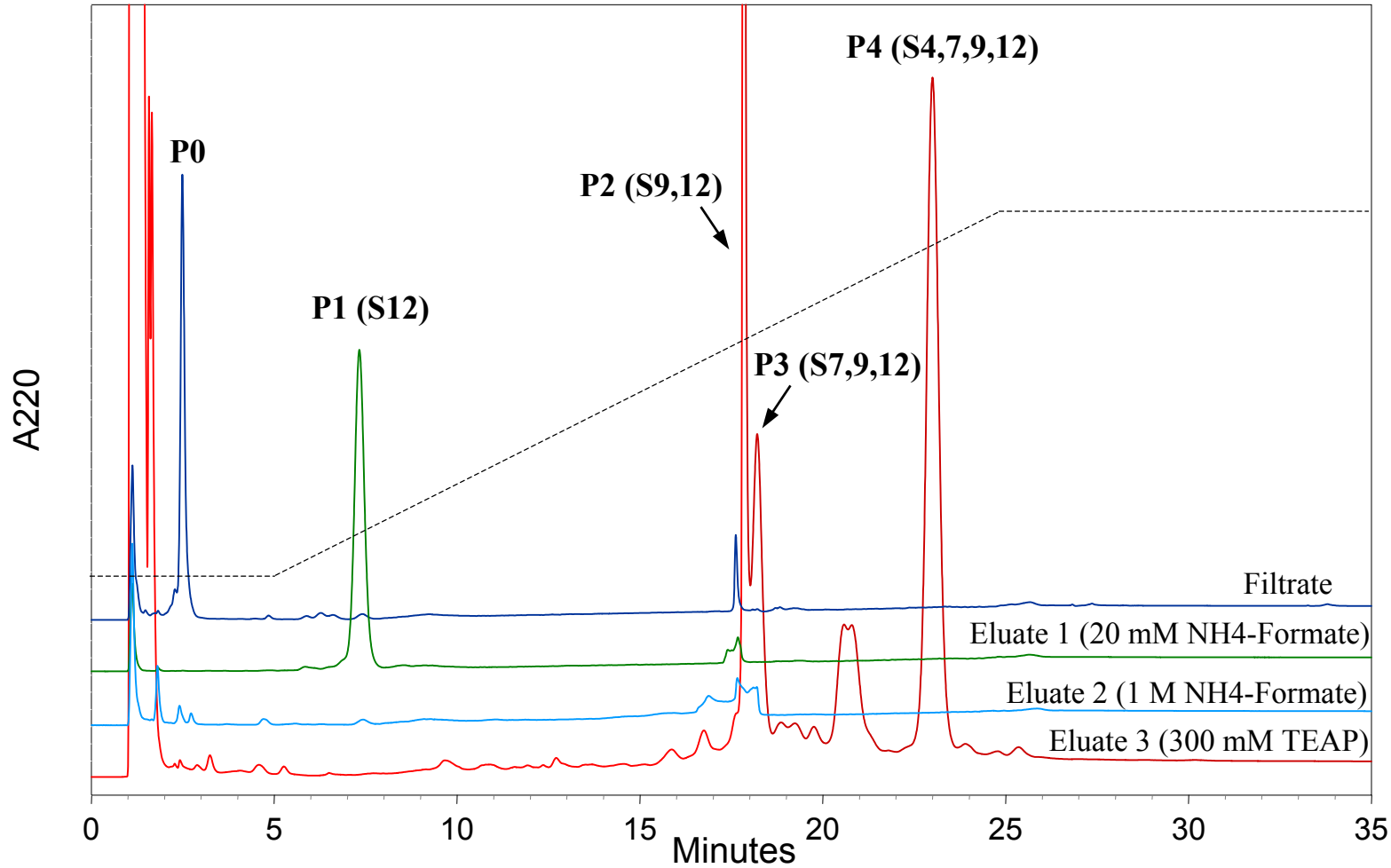


Fig. 5. ERLIC-SPE Fractionation of a Mixture of Phosphopeptides

SPE cartridge: Item# TT200WAX Sample: WWG**S**GP**S**G**S**GG**S**GGGK with 0-4 phosphates

Binding solvent: 20 mM NH4-formate, pH 2.2, w. 70% ACN. Eluting solvents (all 10% ACN): 1) 20 mM NH4-formate, pH 2.2; 2) 1 M NH4-formate, pH 2.2; 3) 300 mM TEAP, pH 2.0 (desalted for HPLC)

HPLC analysis: PolyWAX LP column with Na-MePO4 – TEAP gradient per standard ERLIC method

∴ Singly phosphorylated peptides will elute with a step to 10% ACN in 20 mM NH4-formate. Some doubly phosphorylated peptides might elute with a step to 1 M NH4-formate. However, elution of peptides with 3 or 4 phosphates and some with 2 phosphates requires a stronger eluting salt like TEAP (or possibly KH2PO4).

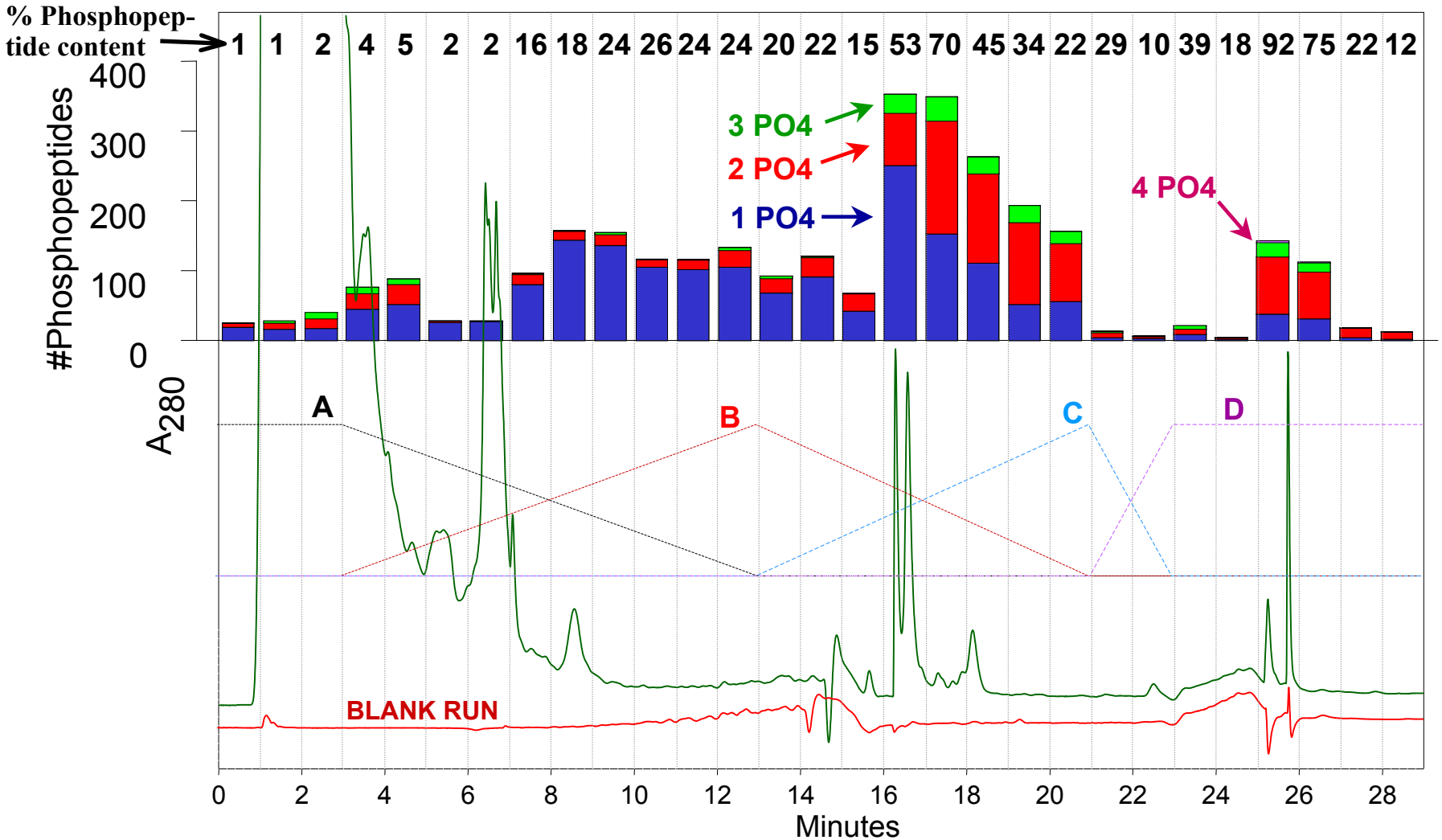


Fig. 6. ERLIC of HeLa Cell Lysate Tryptic Digest

Sample: 1.5 mg. HeLa digest/200 µl MP A; fractions collected at 1' intervals
Column: PolyWAX LP, 100x4.6-mm (104WX0503) DETECTION: 280 nm Flow: 1 ml/min
Gradient: A) 20 mM NH4-formate, p H 2.2, w. 70% ACN; B) Same but 10% ACN; C) 1 M NH4-formate, pH 2.2, w. 10% ACN; D) 0.3 M TEAP, pH 2.0, w. 10% ACN

The column was eluted with linear gradients of the 4 solvents used in Fig. 5 and 29 fractions were collected. These were analyzed via RPC-MS using a LTQ-FT MS [**4**]. Over 3000 phosphopeptides were identified with little effort, since the solvent in fractions 1-21 was volatile. This profile serves as a guide to the composition and abundance of phosphopeptides to be expected at various points in the gradients.

Orientation Probes for HILIC and ERLIC

NAAAAAAWK	Tryptic sequence with N-terminal asparagine
AAANAAAWK	Asparagine in middle
AAAAAAWNK	Asparagine near C-terminus

Fig. 7. OBSERVATION: The elution order of the phosphopeptide standards in Fig. 4 seems to correlate with how close the phosphate group is to the C-terminus. That suggests that it's the preferred contact region. This speculation was tested with the above sequence variants.

CONCEPT: Asparagine is polar and contributes to retention in HILIC (and ERLIC is a form of HILIC). Here it serves as a reporter group on the orientation of the peptide during chromatography; the peptide that's best-retained will be the one that's oriented with the Asn- closest to the stationary phase, where it can interact most effectively and promote retention.

RESULTS: See Fig. 8

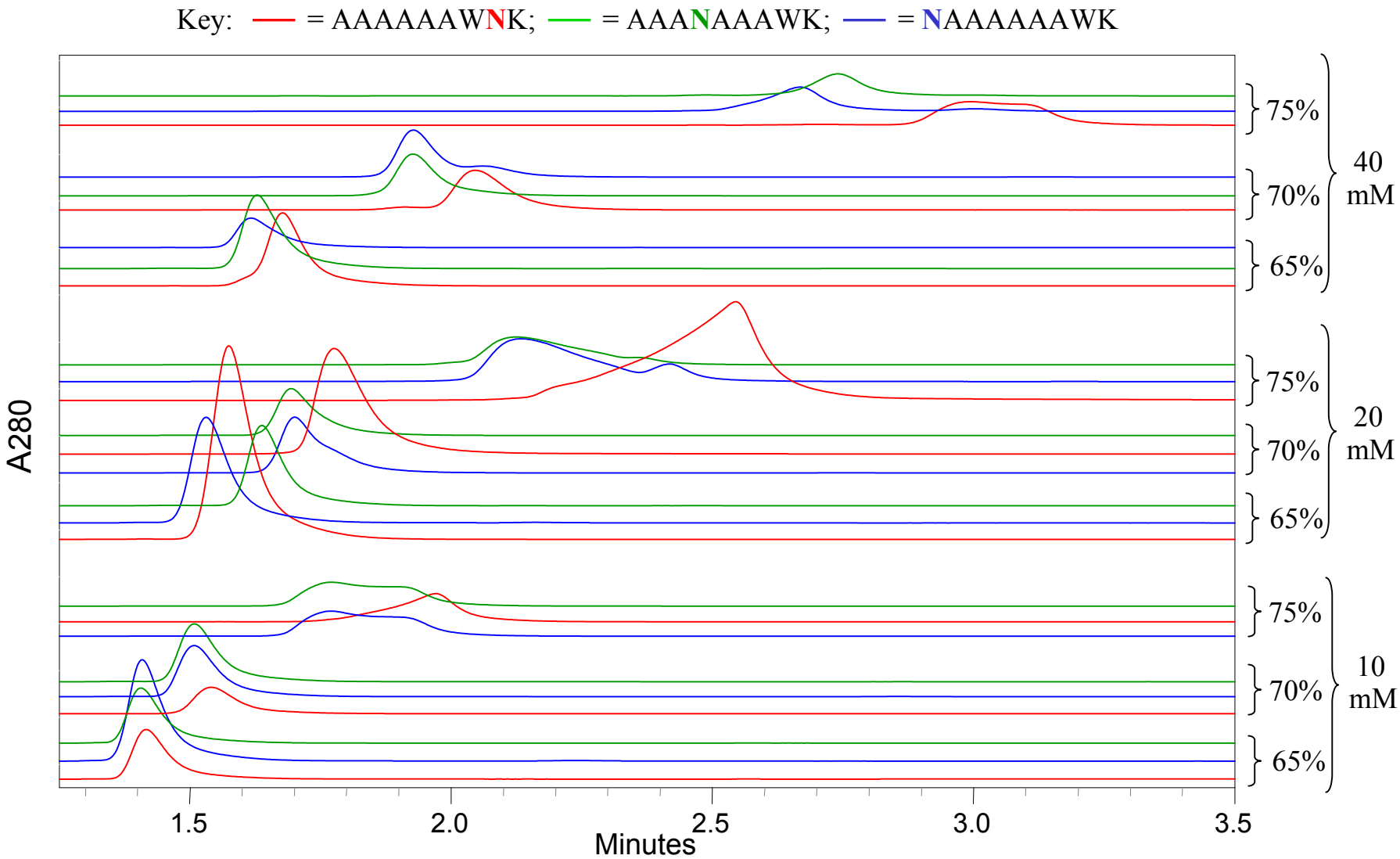


Fig. 8. Orientation Study of Nonphosphorylated Peptide Sequence Variants. Column: PolyWAX LP, 200x4.6-mm; 5-µm, 300-Å 1 ml/min A_{280} Mobile Phase: 10, 20 or 40 mM ammonium formate, pH 2.2, with ACN (% as indicated)
In nearly every case, the variant with Asn- at the C-terminal end elutes last. This indicates that **these peptides are oriented with the C-terminal end facing the stationary phase**.
NOTE: Salt shields electrostatic effects, repulsive in this case, so retention increases with [salt].

SPECULATION:

At low salt concentration, the sidechain of the Arg- or Lys- group of a tryptic peptide could form a zwitterion with the C-terminus. This zwitterion would be less basic than the N-terminus and would be less repelled in ERLIC, hence the orientation with the C-terminus down. At higher salt concentration, the concentration of counterions would be high enough to outcompete the internal charged groups for interaction, and the zwitterionic behavior would be reduced.

This speculation was tested by removing the ability to form a zwitterion; the peptide orientation standards were resynthesized with the C-terminus amidated.

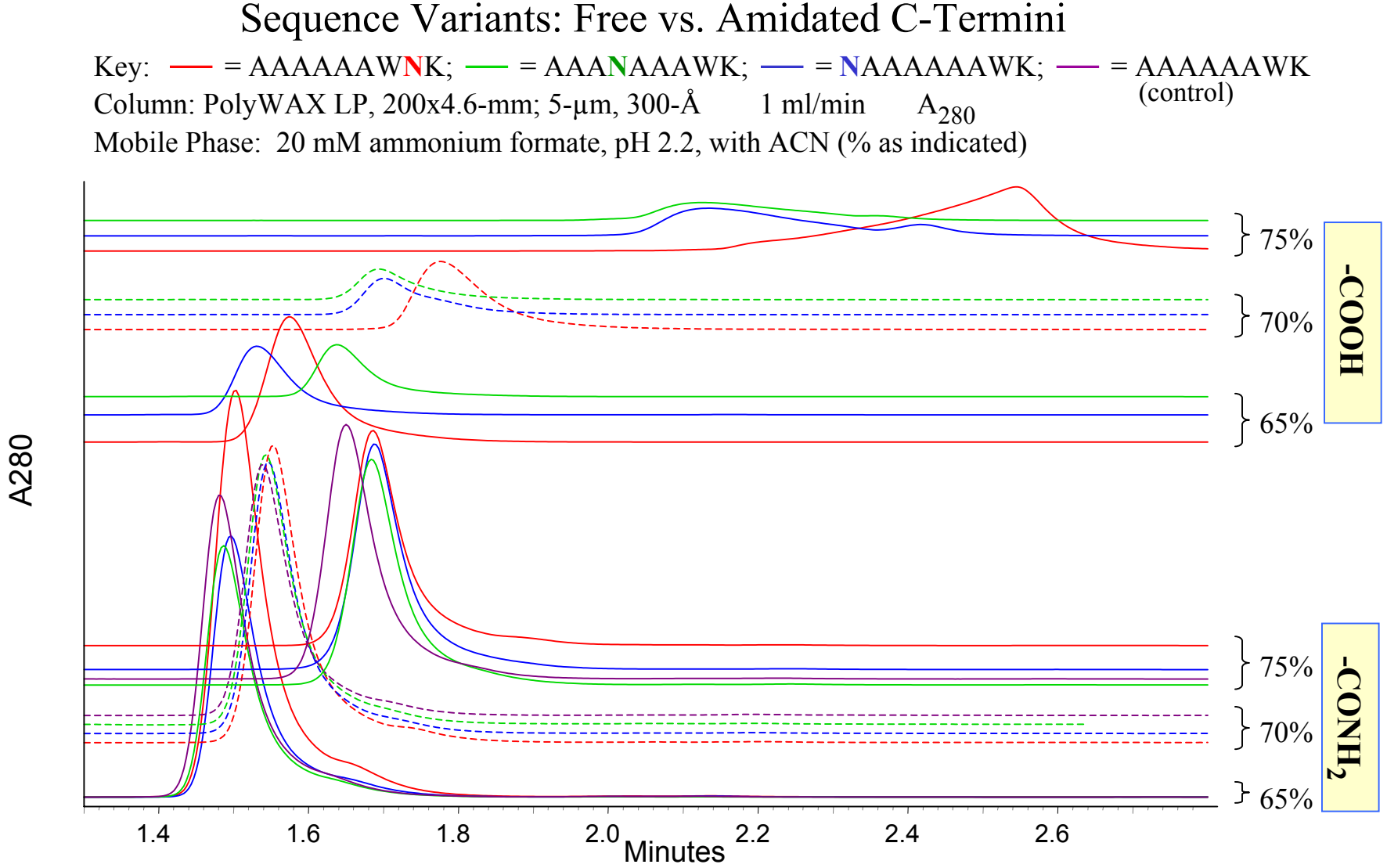


Fig. 9. Observations: 1) Amidation of the C-terminus leads to earlier elution in every case.

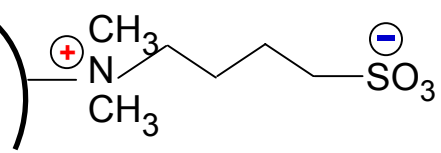
2) When the C-terminus is free, the variant with Asn- at the C-terminal end elutes last in nearly every case. Amidation of the C-terminus abolishes the C-terminal orientation; all variants practically coelute except for the control missing the Asn- residue.

These data support the hypothesis that the C-terminus of a tryptic peptide is in a zwitterionic bond to some extent with the basic C-terminal residue's side chain. The side chain then repels the stationary phase less, so that end is the preferred contact region and retention is increased.

OBSERVATIONS AND PREDICTIONS:

1) **C-terminal fragments from a tryptic digest should elute later than blocked N-terminal fragments as a class in either anion- or cation-exchange chromatography or HILIC**. With blocked N-terminal peptides the two charged groups are both at the C-terminus and might form a zwitterion that doesn't interact well with any ion-exchange material. With a C-terminal fragment the two charged groups are on opposite ends of the molecule. The peptide can assume the orientation necessary to bring a group into proximity with a stationary phase of the opposite charge. This would also permit the amino- terminus, a polar group, to approach a HILIC surface in isolation rather than in a less polar zwitterion.

2) ZIC-HILIC material (Sequant AB): This material has the following structure, and is ostensibly a neutral zwitterionic stationary phase for HILIC. It's actually a good analogue of a tryptic C-terminal end. It acts like a zwitterion < 6 mM salt, a cation-exchanger between 6-20 mM salt, and a neutral material > 20 mM salt (high enough to shield the electrostatic effects). This is evident in chromatography of sialylated glycans [**5**] and charged pharmaceuticals [**6,7**]. Also, in general, C-terminal fragments do elute later from this material than do blocked N-terminal fragments [**8**].



DISCUSSION

Using volatile solvents in ERLIC, separation of phosphopeptides from nonphosphopeptides is easy. Phosphopeptides can also be separated from each other with high resolution, an important consideration in analysis of complex mixtures via mass spectrometry. The number of phosphopeptides identified from the HeLa lysate digest compares favorably with the best results reported using titania or IMAC, with appreciably less effort expended. One could prospectively perform these separations using a capillary in the ERLIC mode with direct elution to a mass spectrometer.

Observations of note:

1) ERLIC can handle peptides with > 2 phosphate groups with no difficulty. The fractions eluted with 0.3 M TEAP are likely to contain more peptides with > 3 phosphates than the four that were identified. The limiting factor in their identification at present is not the chromatography but the capabilities of mass spectrometry.

2) The ratio of phosphorylated S, T and Y residues in the HeLa lysate fractions was 81:15:4. This is a higher percentage of phosphorylated Y (pY) than has commonly been reported in the literature [**9**]. That may reflect one of the following two explanations: a) ERLIC handles multiphosphorylated peptides better than other enrichment methods; 76% of the pY residues were on such peptides (1P:2P:3P peptides in quantities of 46:88:58); b) Peptides with pY residues tend to be of low abundance, and the ERLIC method identified many such peptides.

2) Tryptic peptides (ideal ones, anyway) appear to be oriented with the C-terminus facing the stationary phase. The closer the phosphate group is to the C-terminus, the more it promotes retention. This appears to be a major factor governing selectivity in this mode. Orientation effects are likely to be important in determining selectivity in other modes of chromatography as well.

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