Selective Isolation of Phosphotyrosine Containing Peptides

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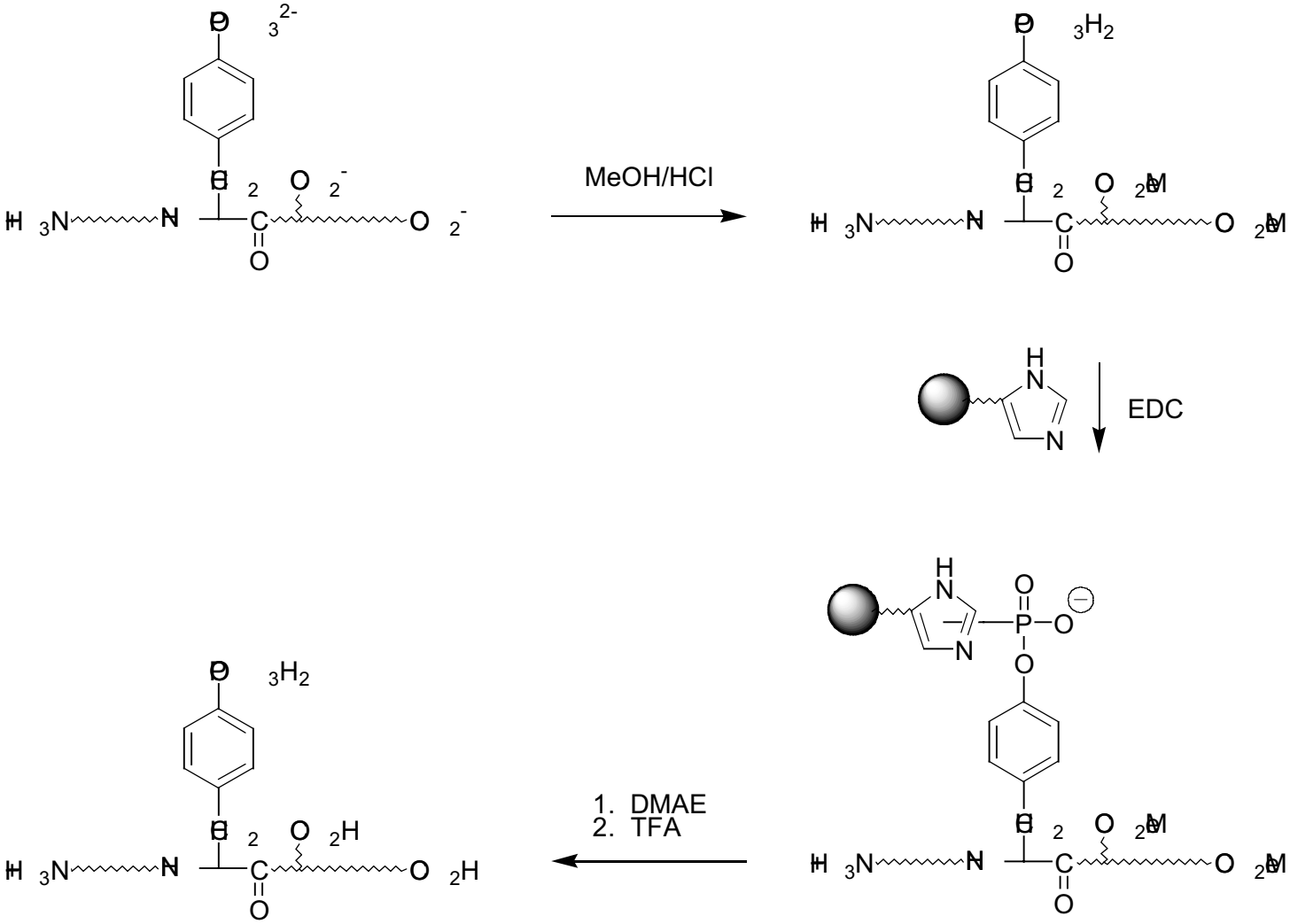
Abstract

Post-translational modifications of proteins have long been of great interest in the field of proteomics. Of these modifications, phosphorylation has always been of particular interest. Isolation of phosphopeptides from protein digest mixtures would allow further identification and characterization of these phosphorylation sites.

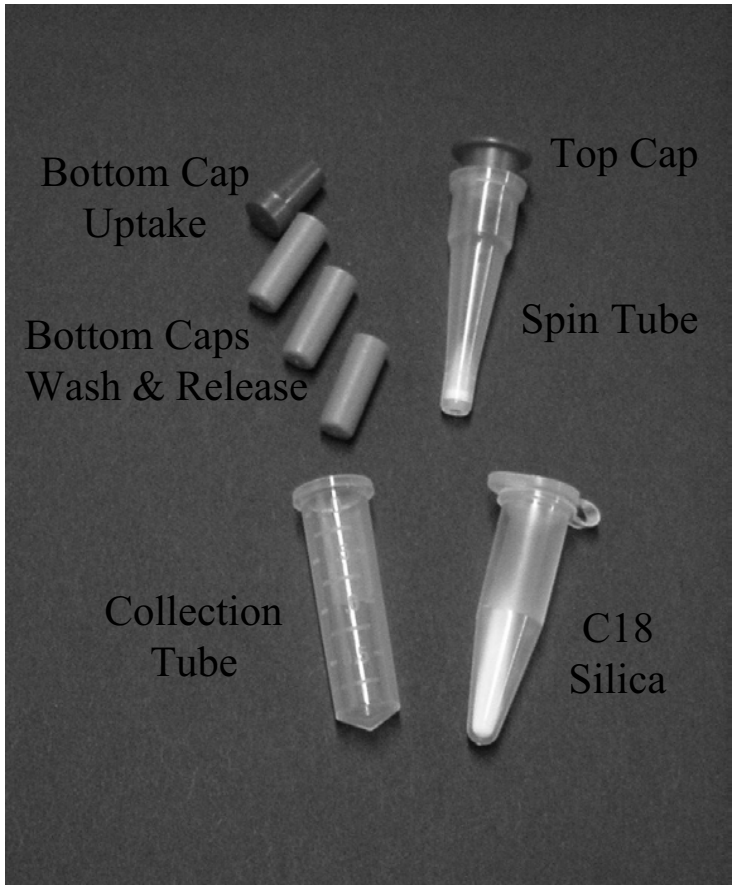
An alternative to the standard immobilized metal affinity chromatography (IMAC) procedures has been developed for the selective isolation of phosphotyrosine peptides using a solid support (Pi^{3}™-pTyr). Binding conditions insure the selective covalent binding of the phosphopeptides from a mixture of peptides and proteins. Release of the phosphopeptides from the solid support then allows further analysis of the peptides by LC, MS, etc.

Pi3™-pTyrosine Chemistry



The Methodology

Simple Tools



Reagents

- CH_3OH
- HCl
- MES
- EDC
- Dimethylamino-ethanol
- CH_3CN
- Aqueous TFA

The Method

Seven Easy Steps

- Step 1: Esterify free carboxyl groups
- Step 2: Capture phosphotyrosine peptides
- Step 3: Deesterify carboxyl groups
- Step 4: Wash to remove non-bound peptides
- Step 5: Release bound phosphotyrosine peptides
- Step 6: Collect released peptides
- Step 7: Concentrate peptide solution

Step 1: Peptide Esterification

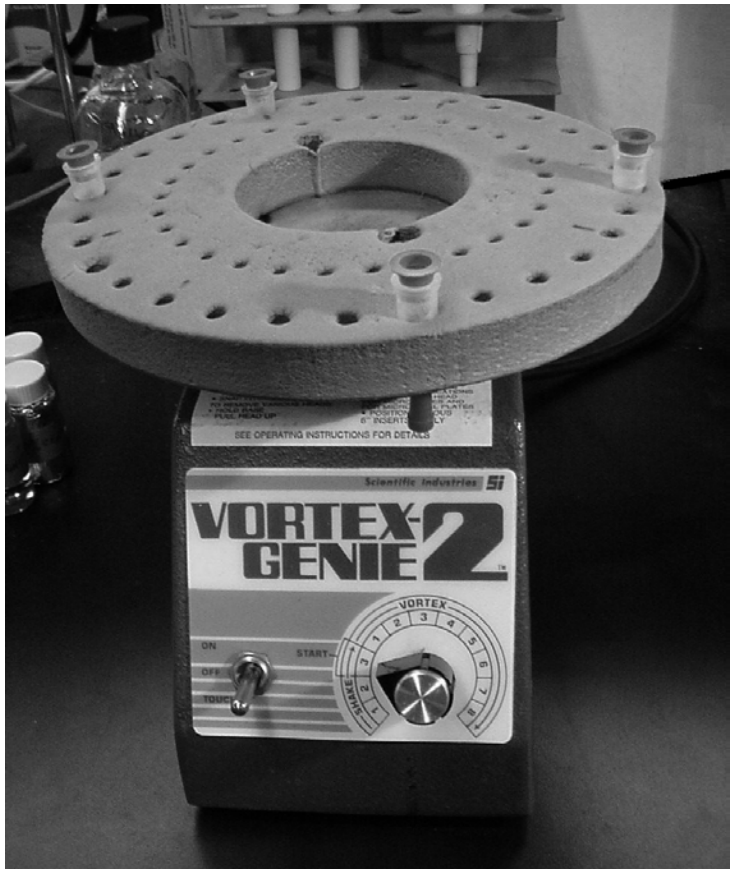
Free Carboxyl Groups

- Dry sample in microcentrifuge tube.
- Add 1 volume CH_3OH/HCL.
- Incubate at 50° C.
- Add 9 volumes 0.2 M MES, pH 6.0.

Step 2: Peptide Capture

Phosphotyrosine Peptides

- Wash Pi^3-pTyr with CH_3OH. Remove wash by centrifugation. Repeat with 0.2 M MES, pH 6.0.
- To the Pi^3-pTyr in the spin-column add peptide ester solution. Add an equal volume of 60 mg/ml EDC, 0.2 M MES, pH 6.0.
- Vortex for 2 hr at ambient temperature.



Steps 3 & 4: Deesterify and Wash After Capture

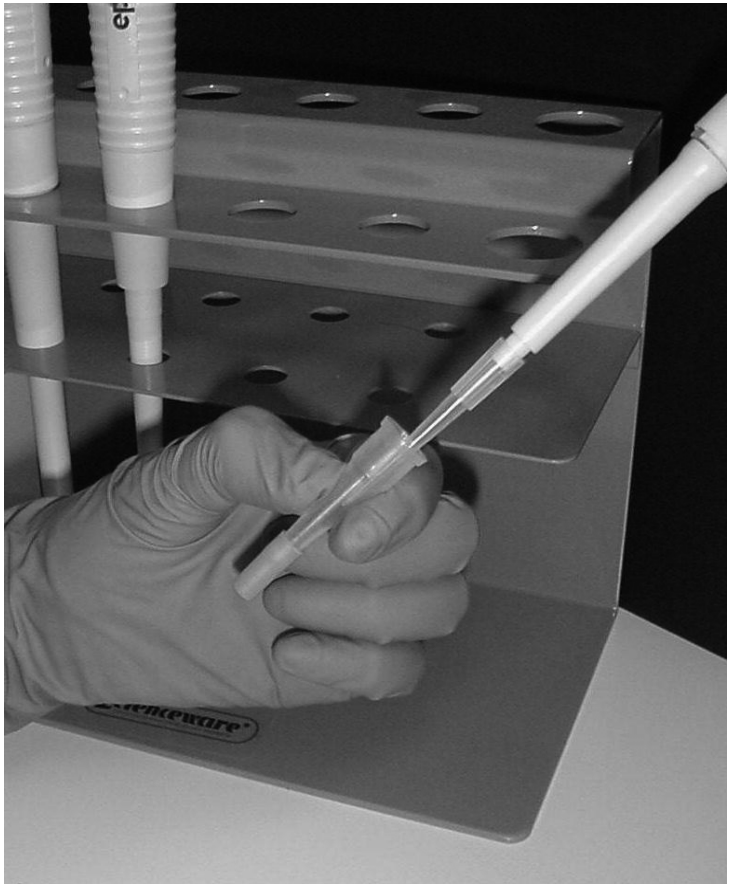
Cleave Esters and Remove Non-Bound and Adsorbed Peptides

- Remove uptake solution by centrifugation.
- Add 10% DMAE to the Pi^3-pTyr and vortex for 2 hr to deesterify.
- Add 70% CH_3CN/0.1% TFA and vortex to release non-bound peptides.
- Centrifuge to remove solution after each vortexing step.

Step 5: Peptide Release

Phosphotyrosine Peptides

- Add 4% TFA to the Pi^3-pTyr.
- Vortex for 2 hr at ambient temperature.



Step 6: Sample Collection

Isolate Peptide Solution and Prepare for Clean-up

- Spin down and collect the ~100 μL releasing solution.
- Wash the spin-column with 100 μL of 4% TFA.
- Collect this wash and combine with the saved releasing solution and dilute with 200 μL H_2O.

Step 7: Sample Clean-up

Desalt and Concentrate

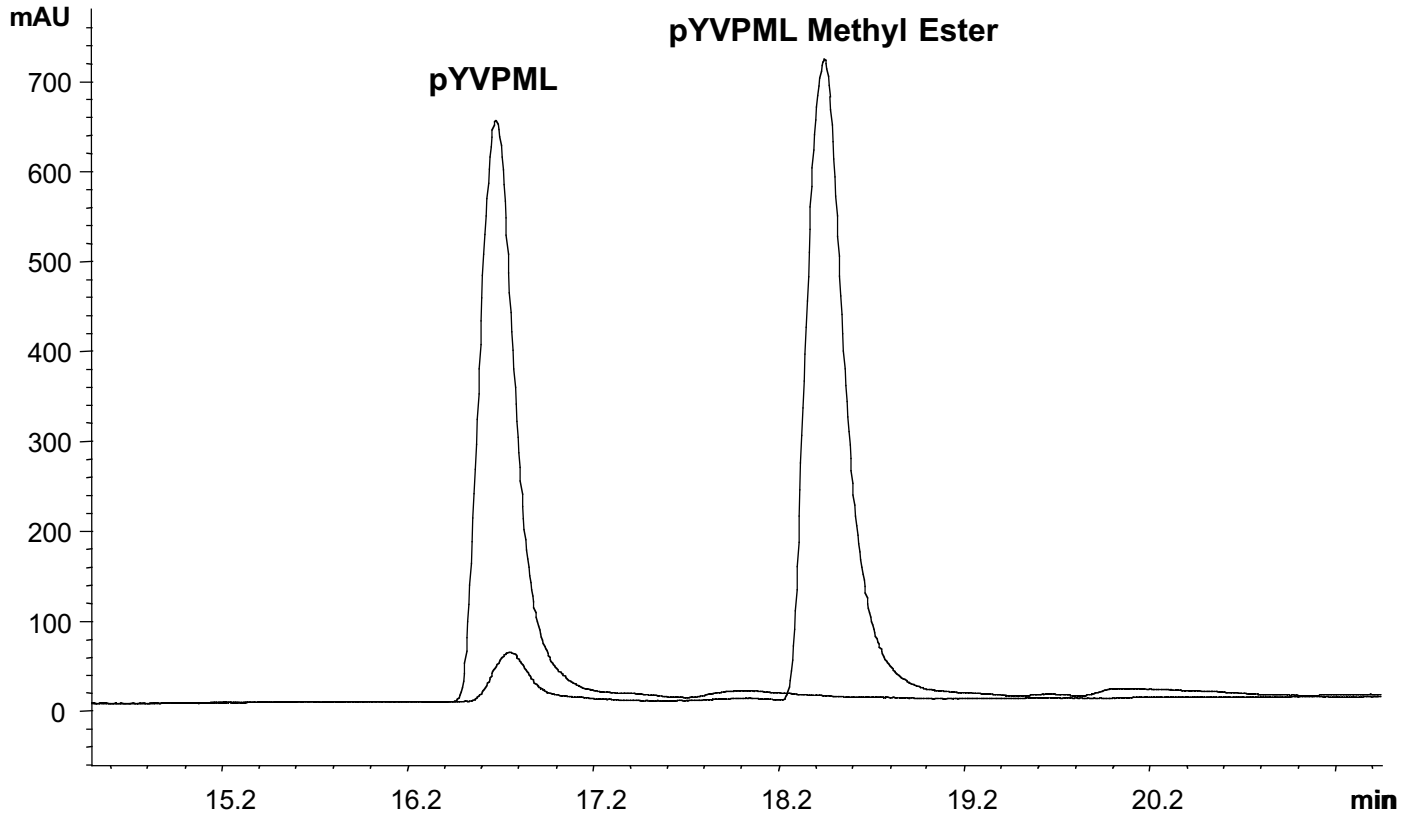
- Place 100 mg of C_{18} silica in a clean spin-column, and wash with 400 μL each of CH_3OH and 2% TFA.
- Load the diluted released solution onto the silica column and wash with 2% TFA.
- Elute the sample with 70% CH_3CN/0.1% TFA.
- Collected peptides are ready for analysis.

Results

- The esterification procedure used to modify free carboxyls is mild enough to minimize modification of amides.
- A phosphotyrosine containing peptide was isolated selectively by use of Pi^3-pTyr from a mixture of phosphothreonine, phosphoserine, and phosphotyrosine peptides.
- A phosphotyrosine containing peptide was isolated from a mixture of a phosphotyrosine peptide and apomyoglobin tryptic digest.
- Pi^3-pTyr isolated phosphotyrosine peptides from as little as 250 pmol of starting peptides using HPLC for analysis.

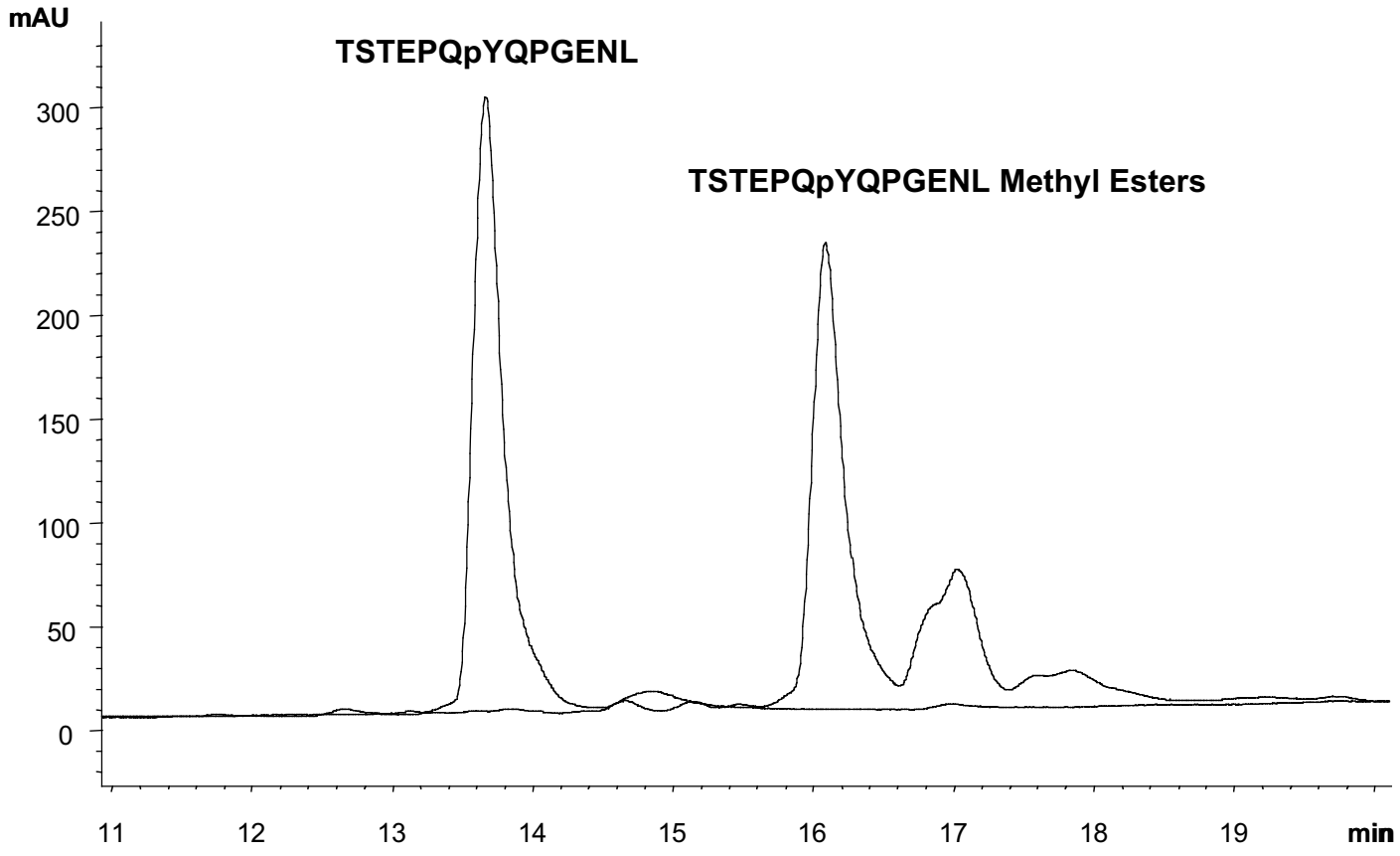
HPLC of Phosphotyrosine Peptides

pYVPML and its Methyl Ester - Single Esterification Site



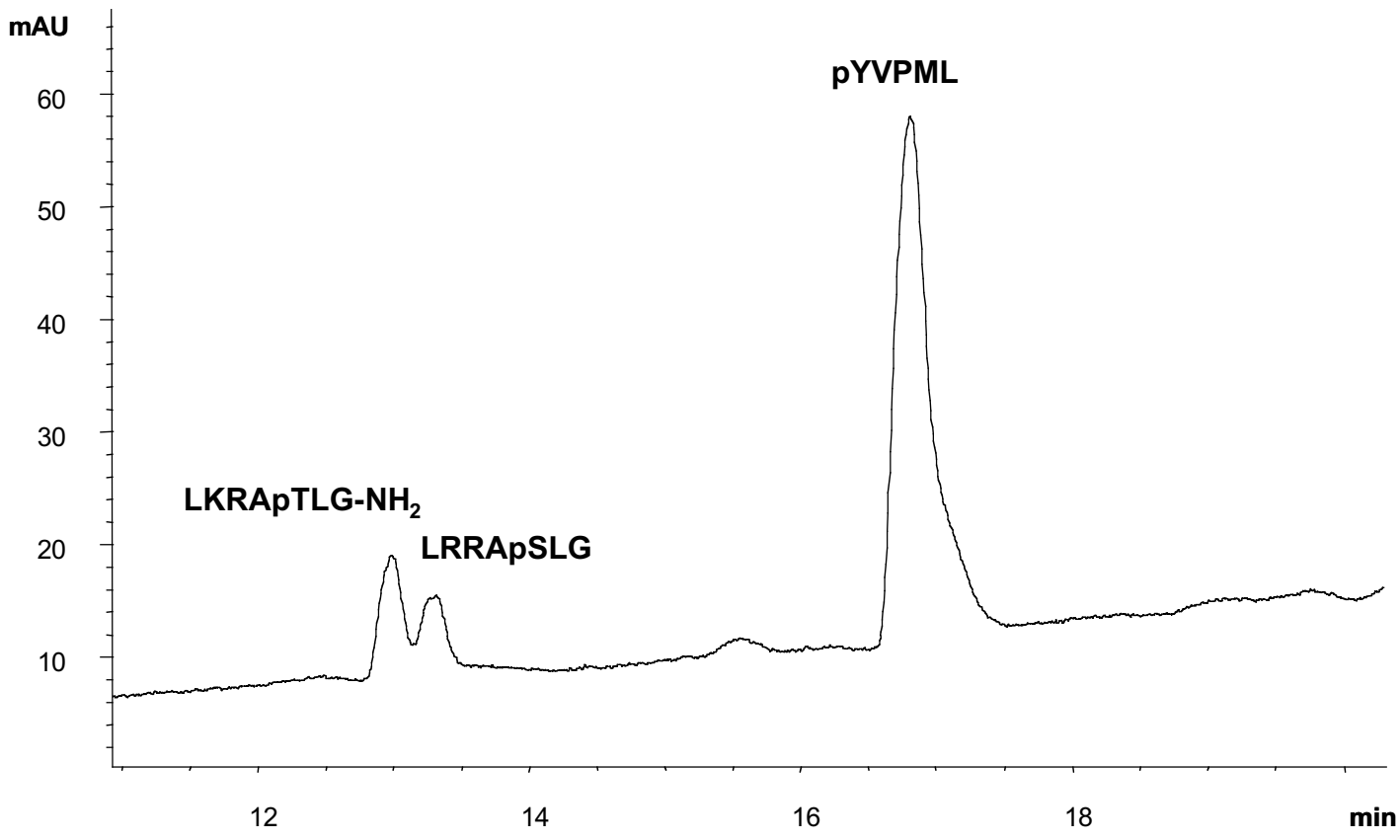
HPLC of Phosphotyrosine Peptides

TSTEPQpYQPGENL and its Methyl Ester - Multiple Esterification Sites



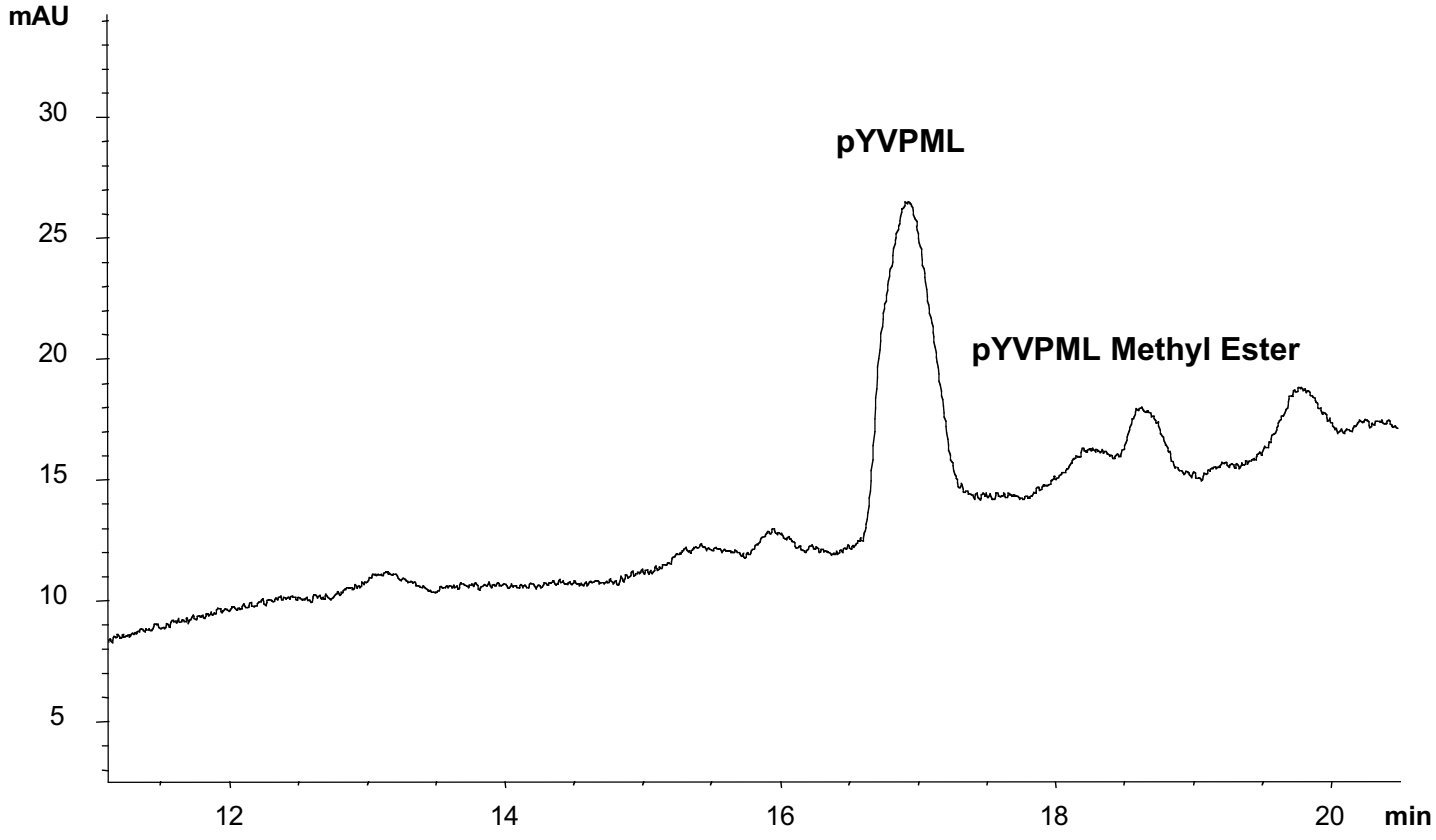
HPLC of Phosphopeptide Mixture "A"

LKRApTLG-NH_2, LRRApSLG, and pYVPML



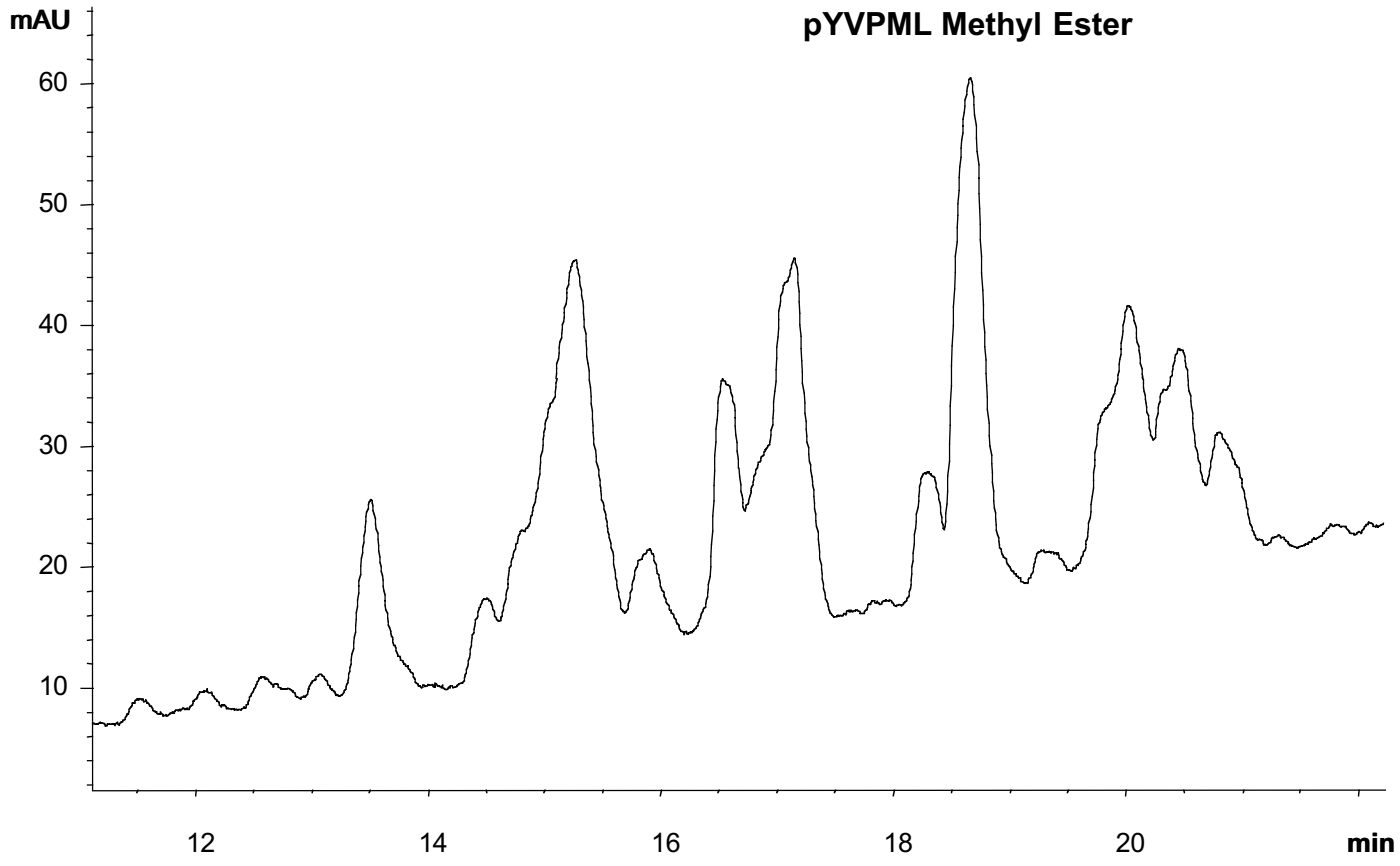
HPLC of Release from Pi^3-pTyr

Example of 250 pmol of Each Peptide in Mixture "A"



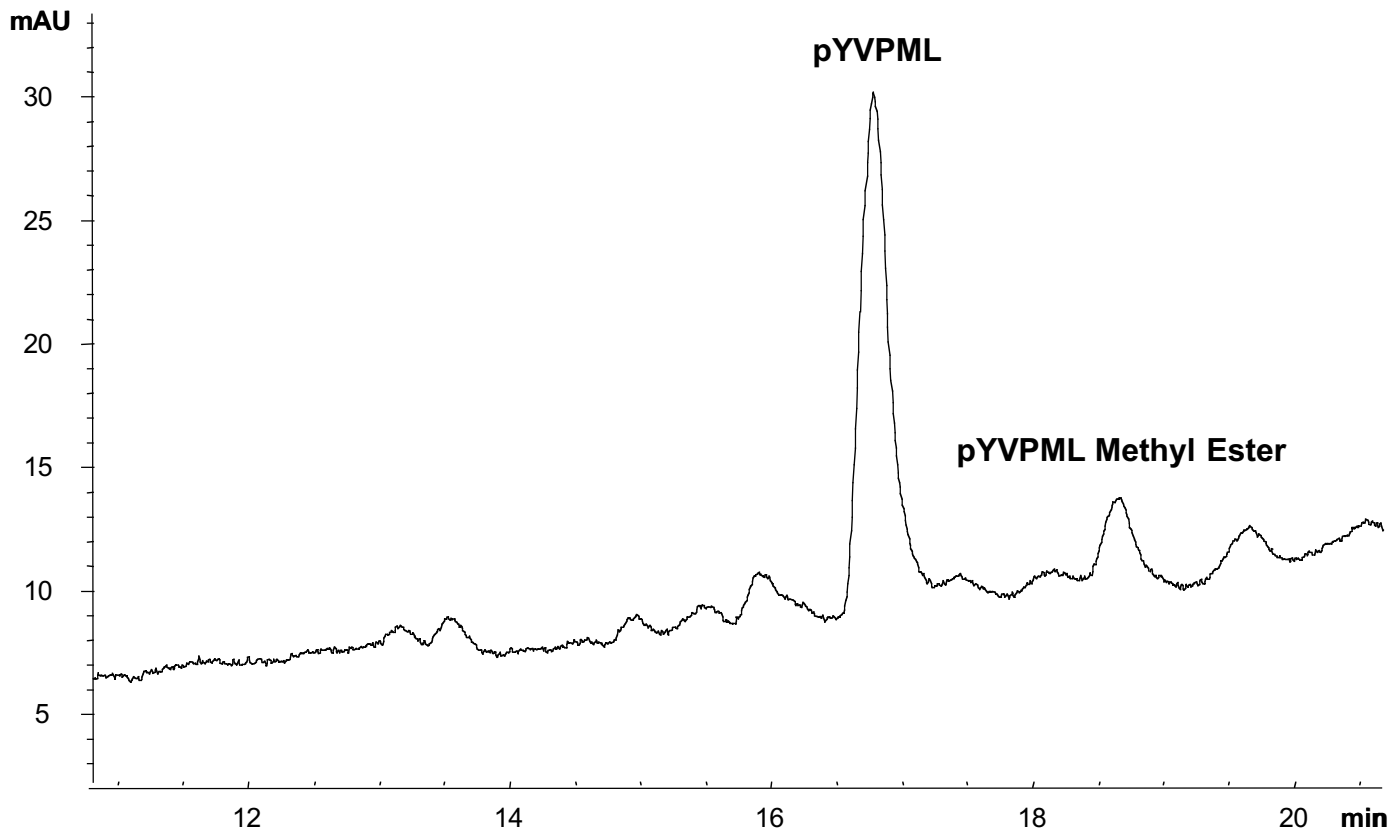
HPLC of Peptide Mixture "B"

Methyl Esters of Apomyoglobin Tryptic Digest and pYVPML



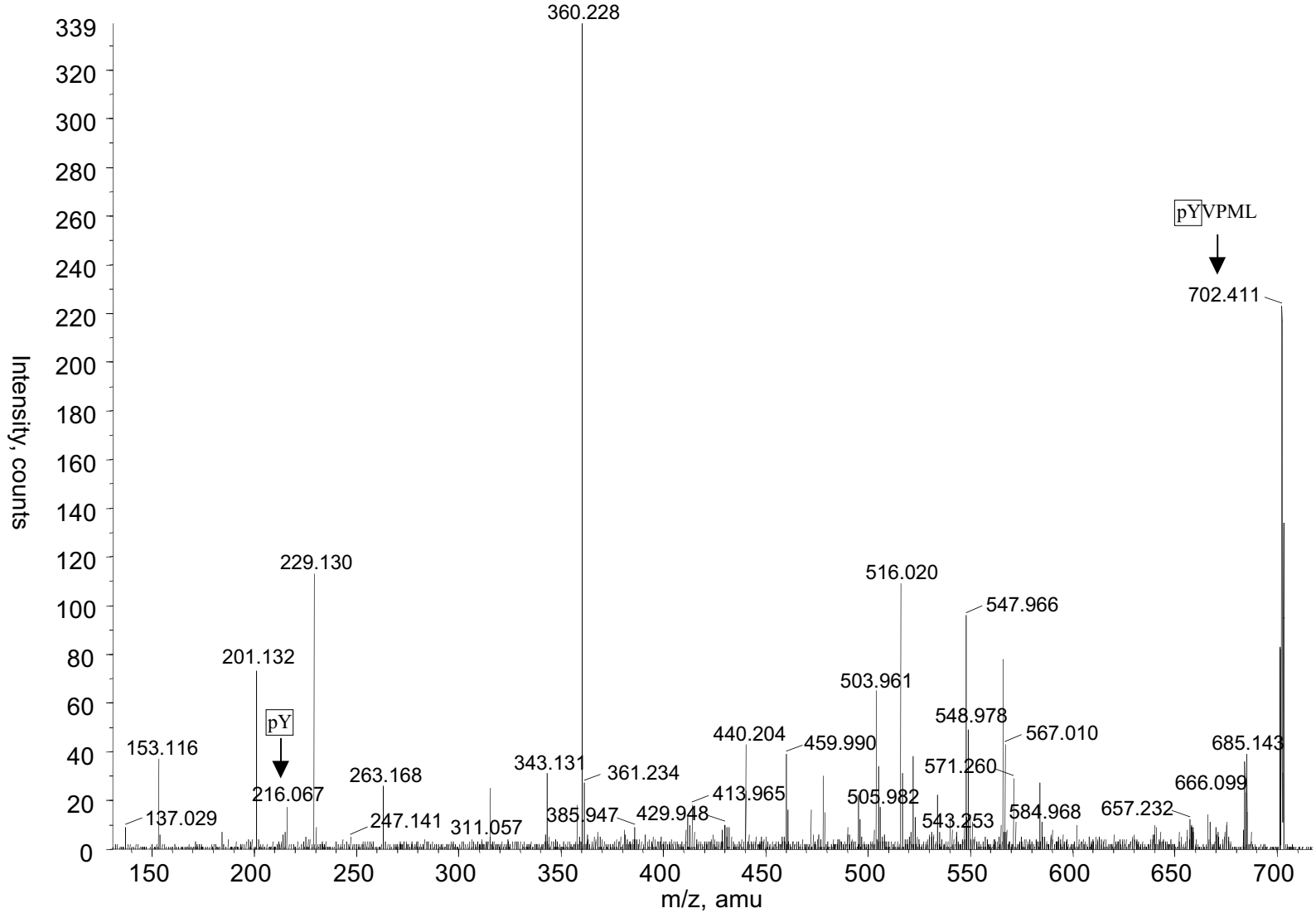
HPLC of Release from Pi^3-pTyr

Example of 250 pmole of each Component in Mixture "B"



MS/MS of Release from Pi^3-pTyr

Peptide Mixture "B" - Methyl Esters of Apomyoglobin Tryptic Digest and pYVPML



Sample run on o-Maldi QSTAR qq TOF
Courtesy of David Hawke, Applied Biosystems

Conclusions

- A phosphotyrosine specific linker (Pi^3-pTyr) has been prepared which exclusively isolates phosphotyrosine containing peptides from mixtures of peptides.
- With a mixture of phosphopeptides the Pi^3-pTyr method was specific for phosphotyrosine in the presence of phosphoserine or phosphothreonine.
- A straight-forward esterification of free peptide carboxyl groups prevents side-reactions and non-specific covalent binding of peptides.
- Deesterification of the bound peptides returns them to their original state.
- The Pi^3-pTyr method has been demonstrated to be effective in the mid-picomole through nanomole range.