Historically, researchers have been unable to fully realize the benefits of mass spectrometry as an analysis method for phosphopeptides because isolation of the molecules from non-phosphorylated peptides is frequently required before examination of the complex samples can proceed (1). Previously, immobilized metal affinity chromatography (IMAC) was the most widely utilized technique for phosphopeptide enrichment by mass spectroscopy. IMAC methods can vary widely in effectiveness depending on the type of metal ion and loading/elution procedure. The technique also uses valuable research time for the required metal ion loading and washing steps and is difficult to incorporate into an on-line application (6). As non-specific binding of non-phosphorylated peptides further hampers the technique, researchers using mass spectroscopy needed a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectroscopy (1, 2). Recently, several papers and posters have been published demonstrating the unique ability of titanium dioxide and zirconium dioxide to selectively retain phosphopeptides contained in complex biological mixtures (1, 2).

**Titania Pre-Columns**

For example, Pinkse et al. report an innovative approach to automate the method for the enrichment of phosphopeptides using a 2D technique with titanium dioxide particles as the first dimension and a reversed phase silica C18 column as the second dimension (1). The complex proteolytic digests were loaded onto the titanium dioxide pre-column using acidic conditions; retaining the phosphorylated peptides and allowing the rest of the digest to concentrate on the reversed phase column. After the analysis of the digest peptides on the reversed phase column is complete the phosphopeptides are eluted, under alkaline conditions, from the titanium dioxide for analysis. The authors report the method has a recovery above 90% and allows for the identification of previously uncharacterized phosphorylation sites (1). Additionally, they report the titanium dioxide pre-columns could be used for over 200 runs without reduced performance (1). However, using this method, the titanium dioxide does appear to have non-specific binding issues especially of non-phosphorylated peptides with acidic residues.

Larsen et al. took the Pinkse research one step further and dramatically improved the selectivity of the Pinkse method by loading the peptide samples onto the titanium dioxide in 2,5-dihydrobenzoic acid (DHB) (2). In a direct comparison of the titanium dioxide and IMAC methods for semi-complex samples the titanium dioxide pre-columns had a greater yield of phosphorylated peptides and fewer contaminating non-phosphorylated peptides (2). This effect was enhanced as the complexity of the samples increased (2).

**Zirconia Microtips**

At the recent ASMS 2005 conference Kweon et al. report the successful use of a zirconium dioxide microtip for the enrichment of phosphopeptides (3). Phosphopeptides from proteolytic peptide mixtures were selectively isolated and enriched by binding to zirconia microtips. For this application, the zirconia phosphopeptide enrichment proved superior to titanium dioxide and IMAC methods.

Figure 1. Glygen’s Lab-in-a-tip™ SPE pipette tips

Sachtopore-NP (titanium dioxide) and ZirChrom-PHASE (zirconium dioxide) are available as bulk particles or packed analytical, semi-prep or prep sized HPLC columns. In addition, both materials are available as packed or embedded particle SPE pipette tips (Glygen’s Lab-in-a-tip™ SPE pipette tips, Figure 1). More information is available on our website at www.zirchrom.com or by contacting a ZirChrom technical specialist by phone at 1-866-STABLE-1 or by e-mail at support@zirchrom.com.

**References**


**ZirChrom Separations, Inc.**

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Visit www.zirchrom.com for more application notes using ultra-stable, high efficiency ZirChrom columns.
We report a rapid highly selective enrichment procedure utilizing 2,5-dihydroxybenzoic acid (DHB) to enhance the selective enrichment of phosphorylated peptides on a titanium dioxide micro-column. This unique technique dramatically increases the selectivity, and thus sensitivity, of enrichment purification of phosphorylated peptides from complex mixtures of non-phosphorylated and phosphorylated peptides.

Introduction
Historically, researchers have been unable to fully realize the benefits of mass spectrometry as an analysis method for phosphopeptides because isolation of the molecules from non-phosphorylated peptides is frequently required before examination of the complex samples can proceed (1). Previously, immobilized metal affinity chromatography (IMAC) was the most widely utilized technique for phosphopeptide enrichment by mass spectroscopy. However, IMAC methods can vary widely in effectiveness, use valuable research time for the required metal ion loading/washing steps and are difficult to incorporate into on-line applications (1). As non-specific binding of non-phosphorylated peptides further hampers the technique, researchers using mass spectroscopy needed a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectroscopy (1, 2).

The following rapid (less than 5 min/sample) highly selective enrichment procedure, developed by the Department of Biochemistry and Molecular Biology, University of Southern Denmark (Odense, Denmark), dramatically increases the selectivity of enrichment in comparison to traditional IMAC methods.

Experimental
A complex mixture of phosphorylated and non-phosphorylated peptides, diluted 1:5 in loading buffer, was enriched using titanium dioxide bulk particles packed into a 3mm long micro-column. The enrichment procedure was as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Sachtopore®-NP titanium dioxide micro-column [Part# TI02-0310-5(100)]</td>
</tr>
<tr>
<td>Loading Buffer</td>
<td>10uL-300 mg/mL DHB in 80/20 ACN/1% TFA, pH 1.9</td>
</tr>
</tbody>
</table>
| Wash Buffer        | 1. 10uL-300 mg/ml DHB in 80/20 ACN/0.1% TFA pH 1.9  
                    | 2. 20uL-80/20 ACN/0.1% TFA, pH 1.9 |
| Elution Buffer     | 20uL - NH4OH, pH 10.5               |

Figure 1 demonstrates performance of the material with a relatively simple mixture (1:1 ratio) of non-phosphorylated and phosphorylated peptides. At this level of complexity the titania based method compares favorably with traditional techniques, enabling detection of equal numbers of phosphopeptides and reducing the number of non-phosphorylated peptides retained. As sample complexity increases so does the selectivity of the binding for the phosphorylated versus the non-phosphorylated peptides (see reference 2 for additional data).

This method can be tailored to your specific application needs. ZirChrom technical support can help to optimize and transfer this method to your site. Please contact ZirChrom technical support at 1-866-STABLE-1 or support@zirchrom.com for details about Sachtopore®-NP bulk or Sachtopore®-NP guard inserts.

Acknowledgements
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References


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Visit www.zirchrom.com for more application notes using ultra-stable, high efficiency ZirChrom columns.
We report a rapid, highly selective enrichment procedure for phosphopeptides utilizing titanium dioxide (TiO$_2$) & zirconium dioxide (ZrO$_2$) SPE tips. β-casein digest samples purified via NuTip™ SPE tips, manufactured by Glygen Corporation (Columbia, MD), display exceptional signal to noise ratios for phosphopeptide analysis and eliminate many difficulties present in traditional IMAC methods.

Introduction

Historically, researchers have been unable to fully realize the benefits of mass spectrometry as an analysis method for phosphopeptides because isolation of the molecules from non-phosphorylated peptides is frequently required before examination of the complex samples can proceed (1). Previously, immobilized metal affinity chromatography (IMAC) was the most widely utilized technique for phosphopeptide enrichment by mass spectroscopy. However, IMAC methods can vary widely in effectiveness, use valuable research time for the required metal ion loading/washing steps and are difficult to incorporate into on-line applications (1). As non-specific binding of non-phosphorylated peptides further hampers the technique, researchers using mass spectroscopy needed a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectroscopy (1).

The following rapid enrichment procedure, developed by Glygen Corporation and New Objective, Inc. (Woburn, MA), maintains high enrichment selectivity without the complications and irreproducibility inherent in traditional IMAC methods (2).

Experimental

An overnight trypic β-casein digest was performed and the sample was then diluted with a 0.1% formic acid solution to generate a 1 pmol/µL solution. The enrichment procedure was as follows:

- **Product:** Titanium Dioxide & Zirconium Dioxide NuTip™ (part # NT1TIO & NT1ZRO)
- **Conditioning:** Tips conditioned with 5 aspiration/expulsion (A/E) cycles of HPLC grade water
- **Loading:** 10 µL of sample loaded in 10 A/E cycles
- **Wash:** 10 µL of HPLC grade water for 10 A/E cycles
- **Elution:** 2 µL of 50/50 50mM NH$_4$HCO$_3$/50mM TEA in 5 A/E cycles
- **Post Elution:** Addition of 2 µL of a 50mM TEA in methanol solution followed by immediate mixing and centrifugation.
- **Detection:** All samples were analyzed via ESI-MS in negative-ion mode.

Figure 1 demonstrates performance of TiO$_2$ and ZrO$_2$ Trap’nTip™ (Trap’nTip™ is a miniaturized form of NuTip™, manufactured exclusively by Glygen Corporation for New Objective, Inc.). A phosphopeptide control set was used for tuning purposes and to confirm the identity of peaks in Figure 1. The results obtained on both TiO$_2$ and ZrO$_2$ compare favorably with traditional techniques, successfully enriching the phosphopeptides and thus greatly improving the signal-to-noise ratio for phosphopeptide analysis (2).

References


NuTip™ and Trap’nTip™ are trademarks of Glygen Corporation and New Objective, Inc., respectively.
The following text investigates the effect of sample loading buffer on a rapid, highly selective enrichment procedure for phosphopeptides utilizing zirconium dioxide (ZrO₂) SPE tips. For α-casein digest samples enriched using ZrO₂ NuTip™ SPE tips, manufactured by Glygen Corporation (Columbia, MD), a low pH formic acid loading buffer enabled the most effective and specific enrichment of phosphopeptides.

Introduction

Historically, researchers have been unable to fully realize the benefits of mass spectrometry as an analysis method for phosphopeptides because isolation of the molecules from non-phosphorylated peptides is frequently required before examination of the complex samples can proceed (1). Immobilized metal affinity chromatography (IMAC) techniques, the most widely utilized technique for phosphopeptide enrichment, can vary widely in effectiveness, use valuable research time for the required metal ion loading/washing steps and are difficult to incorporate into online applications (1). As non-specific binding further hampers the technique, researchers using mass spectrometry needed a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectrometry (1).

This rapid enrichment procedure, developed by Kweon and Hakansson at the University of Michigan (Ann Arbor, MI), optimizes sample loading conditions for the enrichment of phosphopeptides using zirconium dioxide SPE tips (2).

Experimental

An overnight tryptic α-casein digest was performed and the sample was then diluted with loading solution (see figure 1) to generate a 100 pmol solution. The enrichment procedure was as follows:

- **Product:** 50 µg Zirconium Dioxide NuTip™ (part # NT1ZRO)
- **Conditioning:** Tips conditioned with 10 µL loading solution (see figure 1) for 3 aspiration/expulsion (A/E) cycles.
- **Loading:** 10 µL of sample loaded in 10-20 A/E cycles
- **Wash:** 10 µL of HPLC grade water for 2 A/E cycles
- **Elution:** 10 µL of 0.5% piperidine solution for 2 A/E cycles
- **Post Elution:** Eluted samples were dried and reconstituted in 2-propanol/ACN/water (1:1:2) with 0.25% piperidine.
- **Detection:** All samples were analyzed via ESI FT-ICR in negative-ion mode.

Figure one compares the spectra of five different sample loading buffer conditions. The superior loading buffer is the 2.4% formic acid buffer (pH 2.0). Even raising the pH to 3.0 makes a large difference in the number of contaminating non-phosphopeptides (2). To achieve maximum recovery of the bound analytes the washing and elution solutions were also optimized. The highest phosphopeptide recovery was achieved with water washing solution and a 0.5% piperidine (pH 11.5) elution solution (2).

This method can be tailored to your specific application needs. Please contact ZirChrom technical support at 1-866-STABLE-1 or support@zirchrom.com for details.

References

The following compares and contrasts zirconium dioxide (ZrO₂) and titanium dioxide (TiO₂) SPE tips for rapid enrichment of phosphopeptides. Although, for the α-casein digest samples tested, either technique proves more effective than traditional methods, interestingly, the ZrO₂ tips enriched singly phosphorylated peptides in greater abundance.

**Introduction**

Historically, researchers have been unable to fully realize the benefits of mass spectrometry as an analysis method for phosphopeptides because isolation of the molecules from non-phosphorylated peptides is frequently required before examination of the complex samples can proceed (1). Immobilized metal affinity chromatography (IMAC) techniques, the most widely utilized technique for phosphopeptide enrichment, can vary widely in effectiveness, use valuable research time for the required metal ion loading/washing steps and are difficult to incorporate into online applications (1). As non-specific binding further hampers the technique, researchers using mass spectrometry needed a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectrometry (1).

The following rapid enrichment procedure, developed by Kweon and Hakansson at the University of Michigan (Ann Arbor, MI), was applied to both the ZrO₂ and the TiO₂ NuTip™ SPE tips, manufactured by Glygen Corporation (Columbia, MD), to compare and contrast the enrichment of phosphopeptides from a trypic α-casein digest (2). The ZrO₂ and TiO₂ materials used in this study were manufactured by ZirChrom Separations and Sachtleben Chemie GmbH, (Duisburg, Germany), respectively.

**Experimental**

An overnight tryptic α-casein digest was performed and the sample was then diluted with 3.3% formic acid (pH 2) to generate a 100 pmol solution. The enrichment procedure was as follows:

- **Product:** 50 μg Zirconium Dioxide NuTip™ (part # NT1ZRO) & 50 μg Titanium Dioxide NuTip™ (part # NT1TIO)
- **Conditioning:** Tips conditioned with 10 μL 3.3% formic acid (pH 2) for 3 aspiration/expulsion (A/E) cycles.
- **Loading:** 10 μL of sample loaded in 10-20 A/E cycles
- **Wash:** 10 μL of HPLC grade water for 2 A/E cycles
- **Elution:** 10 μL of 0.5% piperidine (pH 11.5) for 2 A/E cycles
- **Post Elution:** Eluted samples were dried and reconstituted in 2-propanol/ACN/water (1:1:2) with 0.25% piperidine.
- **Detection:** All samples were analyzed via ESI FT-ICR in negative-ion mode.

Figure 1 compares three mass spectra; (a) before enrichment, (b) after enrichment using ZrO₂ SPE tips, and (c) after enrichment using TiO₂ SPE tips. Phosphopeptides are numbered and non-phosphorylated peptides are labeled with their corresponding amino acid residue numbers.

**Reference**


NuTip™ is a trademark of Glygen Corporation.
The following compares and contrasts the selectivity of zirconium dioxide (ZrO₂) and titanium dioxide (TiO₂) SPE tips with varying concentrations of α-casein for rapid enrichment of phosphopeptides. The results demonstrate that phosphopeptide selectivity for α-casein was not compromised when sample amount was decreased to 25 pmol. Below 25 pmol, a smaller (25 µg) SPE tip is required to obtain acceptable signal to noise ratios.

Introduction

Immobilized metal affinity chromatography (IMAC), the most widely utilized technique for phosphopeptide enrichment, can vary widely in effectiveness, uses valuable research time for the required metal ion loading/washing steps and is difficult to incorporate into on-line applications (1). As non-specific binding further hampers the technique, researchers need a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectroscopy (1).

The following rapid enrichment procedure, developed by Kweon and Hakansson at the University of Michigan (Ann Arbor, MI), was applied to various sized ZrO₂ and the TiO₂ NuTip™ SPE tips, manufactured by Glygen Corporation (Columbia, MD), to analyze the sensitivity of the enrichment of phosphopeptides from several concentrations of trypic α-casein digest (2). The ZrO₂ and TiO₂ materials used in this study were manufactured by ZirChrom Separations and Sachtleben Chemie GmbH, (Duisburg, Germany), respectively.

Experimental

An overnight trypic α-casein digest was performed and the sample was then diluted with 3.3% formic acid (pH 2) to generate a 100 pmol solution. Samples of decreasing concentration were created from this solution, and the sensitivity of the enrichment protocol was tested. The enrichment procedure was as follows:

<table>
<thead>
<tr>
<th>Product</th>
<th>Conditioning</th>
<th>Loading</th>
<th>Wash</th>
<th>Elution</th>
<th>Post Elution</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 &amp; 25 µg ZrO₂ NuTip™ (part # NT1ZRO)</td>
<td>Tips conditioned with 10 µL 3.3% formic acid (pH 2) for 3 aspiration/expulsion (A/E) cycles.</td>
<td>10 µL of sample loaded in 10-20 A/E cycles</td>
<td>10 µL of HPLC grade water for 2 A/E cycles</td>
<td>10 µL of 0.5% piperidine (pH 11.5) for 2 A/E cycles</td>
<td>Eluted samples were dried and reconstituted in 2-propanol/ACN/water (1:1:2) with 0.25% piperidine.</td>
<td>All samples were analyzed via ESI FT-ICR in negative-ion mode.</td>
</tr>
<tr>
<td>50 &amp; 25 µg TiO₂ NuTip™ (part # NT1TIO)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 demonstrates clearly that phosphopeptide selectivity is not compromised when sample amount is decreased from 100 pmol to 50 and then finally to 25 pmol. As the MS analysis system was not optimized for high sensitivity, poor signal to noise values were obtained with samples lower than 25 pmol in concentration. Halving the size of the SPE tips (refer to Figure 1) dramatically improves the signal to noise ratios for a 1 pmol sample however selectivity is still compromised when compared to higher concentration samples.

Table 1. Selectivity (%) of 50-ug ZrO₂ and TiO₂ Microtips for Phosphopeptide Enrichment of a Tryptic Digest of α-casein as a Function of Sample Amount

<table>
<thead>
<tr>
<th>Concentration</th>
<th>ZrO₂ Enrichment</th>
<th>TiO₂ Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 pmol</td>
<td>67</td>
<td>62</td>
</tr>
<tr>
<td>50 pmol</td>
<td>85</td>
<td>77</td>
</tr>
<tr>
<td>25 pmol</td>
<td>83</td>
<td>74</td>
</tr>
</tbody>
</table>

* Defined as relative phosphopeptide signal

Figure 1: Negative mode ESI FT-ICR mass spectra (8 scans) from 1pmol of a trypsin digest of α-casein obtained following phosphopeptide enrichment with a 50-ug ZrO₂ (a) and a 25-ug ZrO₂ NuTip™ SPE tip (b). Identified nonphosphorylated peptides are labeled with their corresponding amino acid residue numbers and α-casein isoform.

NuTip™ is a trademark of Glygen Corporation.

References