Teil 4 – Anreicherung (Beispiele) und Fällung

Grundlagen und Anwendung moderner Trennverfahren

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Analytical Chemistry in Phytopharmacy

Steps for Quality Control

Selective materials for sample pretreatment:
- particles (spherical, irregular)
- monoliths
- SPE, columns, disks

Stationary phases for HPLC and μ-HPLC:
- particles (different mechanisms)
- monoliths

Screening: MALDI targets/MELDI materials:
- matrix free
- with matrix

Hyphenated techniques:
- μ-HPLC-ESI-MS
- μ-HPLC-MALDI-TOF MS

Infrared Spectroscopy:
- non-invasive quality control
Natural Products - Extraction and Purification - Strategies

Extractives
- Flavonoids
- Carbohydrates
- Vitamines
- Tocopherols
- Carotenoids
- Polyphenols
- Resveratrol
- Naphtodianthrone
- Hypericin, Pseudohypericin
- Hyperforin
- Lectines
- Proteins / Amino acids
- Phytoproteomics

Quality Control

Pharmacological studies of single and synergetic effects

Primula veris

Echinacea

Grapes
Extraction Scheme

**Plant material**
e.g. Scutellaria, St. John’s Wort etc.

**Extraction principle**
- Hydrothermolysis (flow through, p, T)
- Acidic hydrolysis
- Microwave
- Accelerated Solvent Extraction (ASE, Dionex)
- Mazeration

**Microbiologicale and pharmacological tests**
z.B. antimicrobial, anti-inflammatory (COX, LOX) properties

**Analysis**
- Solid-phase extraction (SPE)
- Separation (HPLC, μ-LC, LC-MS, CE, CE-MS, CEC)
- Vibrational spectroscopy (MIR, NIR, ATR, Raman)
- Antioxidative potential (DPPH, FRAP)

**Extracts**

**AIM**
- Increased extraction yield
- Increased selectivity and specificity
Highly Selective Solid-Phase Extraction of Plant Thionins Employing Metal Silicate based Sorbents

Highly Selective Solid-Phase Extraction of Plant Thionins Employing Metal Silicate based Sorbents

- Thionins are low molecular weight (~5 KDa) sulphur rich proteins
- Polypeptide chain of 45 to 48 amino acids
- Three to four internal disulphide bridges
- Customarily found in the endosperms of Gramineae
- Has the shape of the Greek capital letter gamma \( \gamma \)
- Isolation of thionins from complex samples has been a laborious excercise including several purification steps
- A selective SPE method for thionins enables a rapid and convenient isolation of these biologically active proteins
Highly Selective Solid-Phase Extraction of Plant Thionins Employing Metal Silicate based Sorbents

Pharmacological Importance of Thionins

• Antibacterial
• Antifungal
• Poisonous to yeast and insect larvae
• Anti-tumor
• In vitro protein synthesis inhibition
• Immunomodulatory effect on human granulocytes
• Induced apoptosis in lymphocytes
• Plant defence against pathogens
**SPE of Barley from Aluminum Silicate**

- **Activation:**
  Methanol/water (1/1, v/v)

- **Loading:**
  Gentle pipetting of extract

- **Washing:**
  Methanol/water (1/1, v/v)

- **Elution:**
  5% Formic Acid (in Methanol/water (1/1, v/v))
**SPE of Wheat from Aluminum Silicate**

- **Activation:**
  Methanol/water (1/1, v/v)

- **Loading:**
  Gentle pipetting of extract

- **Washing:**
  Methanol/water (1/1, v/v)

- **Elution:**
  5% Formic Acid (in Methanol/water (1/1, v/v))
SPE of Phenolic Acids using Bismuth Citrate and Zirconium Silicate Inside Micro Spin Columns

- Galloyl- and caffeoylquinic-acids are characterized by one free carboxylic group in the quinic acid moiety
- Caffeoylquinic acids are the esters of quinic acids with caffeic, ferulic or p-coumaric acids
- Galloylquinic acids are the esters of quinic acid with gallic acids
- Their biological activity includes anti-HIV, antiasthamatic, bronchial hyper reactivity, allergic reactions and anti-inflammatory properties
- Caffeoylquinic acids are antidiabetic, hypoglycemic, antihepatitis, hepatoprotective and anti cancer agents

1,3,4,5-tetra-o-galloylquinic acid

Chlorogenic acid

mono-caffeoylquinic-acid

Cynarin

di-caffeoylquinic-acid
Proposed binding mechanism of bismuth citrate and zirconium silicate with carboxylic group of phenolic acids
Schematic view of spin columns containing sorbents

Recovery study of standards

Comparison based upon nature of stationary phase/Type of interaction

Tetragalloyl-quinic acids

<table>
<thead>
<tr>
<th>Stationary Phase</th>
<th>TGQA % Recoveries</th>
<th>CYNARIN % Recoveries</th>
</tr>
</thead>
<tbody>
<tr>
<td>SILICA</td>
<td>9%</td>
<td>4%</td>
</tr>
<tr>
<td>C18</td>
<td>22%</td>
<td>39%</td>
</tr>
<tr>
<td>OASIS MAX</td>
<td>2%</td>
<td>70%</td>
</tr>
<tr>
<td>Zr-SILICATE</td>
<td>84%</td>
<td>94%</td>
</tr>
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</table>
Synthesis-Optimization by Capacity Measurements of Rosmarinic acid

<table>
<thead>
<tr>
<th>Name</th>
<th>M : CL Ratio</th>
<th>Capacity / mg/g(Polymer)</th>
<th>Solvent System</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 : 2</td>
<td>9.70</td>
<td>1-Prop/1,4-But.diol/water</td>
</tr>
<tr>
<td>2</td>
<td>1 : 3</td>
<td>9.03</td>
<td>1-Prop/1,4-But.diol/water</td>
</tr>
<tr>
<td>3</td>
<td>1 : 5</td>
<td>8.51</td>
<td>1-Prop/1,4-But.diol/water</td>
</tr>
<tr>
<td>4</td>
<td>1 : 3.5</td>
<td>9.45</td>
<td>1-Prop/1,4-But.diol/water</td>
</tr>
<tr>
<td>5</td>
<td>1 : 10</td>
<td>5.60</td>
<td>1-Prop/1,4-But.diol/water</td>
</tr>
<tr>
<td>9</td>
<td>1 : 4</td>
<td>8.51</td>
<td>1-Prop/1,4-But.diol/water</td>
</tr>
<tr>
<td>6</td>
<td>1 : 3.5</td>
<td>10.18</td>
<td>Dodecanol-Cyclohexanol (1:10/v/v)</td>
</tr>
<tr>
<td>7</td>
<td>1 : 3.5</td>
<td>n.a.</td>
<td>Isooctan:Toluol (1:1/v/v)</td>
</tr>
<tr>
<td>8</td>
<td>1 : 3.7</td>
<td>n.a.</td>
<td>Isooctan:Toluol (1:1/v/v)</td>
</tr>
</tbody>
</table>

Uptake of Rosmarinic Acid

Content of added Vinylimidazol / mol %
Loading capacities (mg g\(^{-1}\) Polymer) calculated by an iterative fitting of the Langmuir equation

\[ q = \frac{K(L) * q(m) * c}{1 + K(L) * c} \]
COMPARISON OF DIFFERENT SORBENTS

Normal phase Mode

Recovery in %

Polymer  SiO₂  Al₂O₃

Rosmarinic acid  Chlorogenic acid  DHHCA  Caffeic acid  2,3-DHB

COMPARISON OF DIFFERENT SORBENTS

Ion exchange phase Mode

- Elution I (50% PBS/ 50% MeOH)
- Elution II (2% TFA / MeOH)
- Oasis MAX- Elution II

Recovery in %

- Rosmarinic acid
- Chlorogenic acid
- DHHCA
- Caffeic acid
- 2,3-DHB

LC-MS/MS Experiments

Ethanolic extract ASE

Identification

Elution 50% PBS / 50% MeOH

### LC-MS/MS Experiments

- **LC-qTOF**
  - Ethanolic extract

- **Identification**
  - (Comparison with previous studies)

- **Elution method I**
  - 50% PBS / 50% MeOH

<table>
<thead>
<tr>
<th>Peak-Nr.</th>
<th>( t_r )</th>
<th>[M-H](^-)</th>
<th>Identification</th>
<th>Bonded</th>
<th>Elution</th>
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<tbody>
<tr>
<td>2</td>
<td>3.24</td>
<td>305.07</td>
<td>Gallocatechin</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.30</td>
<td>387.17</td>
<td>Medioresinol</td>
<td>+</td>
<td></td>
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<tr>
<td>4</td>
<td>3.35</td>
<td>431.19</td>
<td>Apigenin-7-O-glucoside</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.57</td>
<td>593.15</td>
<td>Luteolin-7-O-rutinoside</td>
<td>+</td>
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</tr>
<tr>
<td>6</td>
<td>3.72</td>
<td>477.11</td>
<td>Isorhamnetin-3-O-hexoside</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3.83</td>
<td>609.19</td>
<td>Unknown</td>
<td>+</td>
<td>+</td>
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<tr>
<td>9</td>
<td>3.88</td>
<td>461.11</td>
<td>Luteolin-3-O-glucuronide</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>3.91</td>
<td>197.05</td>
<td>Unknown</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>3.92</td>
<td>359.08</td>
<td>Rosmarinic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>4.43</td>
<td>283.06</td>
<td>Unknown</td>
<td>+</td>
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<tr>
<td>15</td>
<td>5.29</td>
<td>313.07</td>
<td>cirsimaritin</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>5.33</td>
<td>283.17</td>
<td>Genkwanin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>5.33</td>
<td>345.17</td>
<td>Rosmanol</td>
<td></td>
<td></td>
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<tr>
<td>21</td>
<td>5.71</td>
<td>283.06</td>
<td>Unknown</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>6.70</td>
<td>299.17</td>
<td>Hispidulin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>26</td>
<td>6.85</td>
<td>329.18</td>
<td>Carnosol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>7.77</td>
<td>331.19</td>
<td>Carnosic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>31</td>
<td>8.36</td>
<td>345.21</td>
<td>Methylcarnosate</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>
Desalting
Desalting of Proteins – Why?

**Spin Columns**

- C18-reversed phase

**Extraction Tips**

- ZipTips (Millipore)
Fullerene C60-Silica for Desalting Biological Samples

**Peptides**

**Proteines**

MALDI-MS spectra of the peptides and proteins which were eluted from Kovasil 300 Å C60-Silica

immobilization  washing  elution
Fullerene C60-amino silica for Desalting

Table 3. Comparison of Recovery Values Measured on C60-Aminosilica versus Sep-Pak and Oasis for Insulin and Bradykinin

<table>
<thead>
<tr>
<th>Substance Name</th>
<th>Conc'n (µg/mL) Loaded</th>
<th>C60-amino-silica</th>
<th>Sep-Pak C18</th>
<th>Oasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>insulin</td>
<td>10</td>
<td>72.3</td>
<td>79.6</td>
<td>72.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>95.5</td>
<td>87.4</td>
<td>92.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>97.2</td>
<td>91.8</td>
<td>91.7</td>
</tr>
<tr>
<td>bradykinin</td>
<td>30</td>
<td>85.0</td>
<td>—</td>
<td>84.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>88.4</td>
<td>—</td>
<td>88.6</td>
</tr>
</tbody>
</table>

Vallant Rainer M; Szabo Zoltan; Bachmann Stefan; Bakry Rania; Najam-ul-Haq Muhammad; Rainer Matthias; Heigl Nico; Petter Christine; Huck Christian W; Bonn Gunther K. Analytical chemistry (2007), 79(21), 8144-53.
Desalting of hydrophilic Phosphopeptides

C60-Amino Silica vs. ZipTip® C18 (Millipore)

- Phosphopeptide in supernatant
  No immobilization on commercial ZipTip® (Millipore)

- Phosphopeptide removed after washing
  No immobilization of phosphopeptide

- No Phosphopeptide in supernatant
  Quantitative binding of phosphopeptide on C60-Amino Silica

- Phosphopeptide eluted from C60-Amino Silica
Crystalline forms of Boron Nitride

β or cubic BN
- diamond analog
- stable
- very hard

γ or wurzite BN
- lonsdaleite analog
- only stable under high pressure

α or hexagonal BN
- graphite analog
- chemically inert
- nontoxic
Boron Nitride, a Novel Material for the Enrichment and Desalting of Protein Digests and the Protein Depletion

Incubation with tryptic digests

Washing and enrichment

Elution of peptides

Analysis of desalted and protein free peptide solutions

→ hexagonal structure
→ localized electrons

BN

60% ACN in 1% formic acid
60% ACN in 1% H3PO4 and 1% TFA

LC-ESI-MS
MALDI-MS
MALDI Spectra:
The identified peptides are labeled with asterisk. Sequence Coverage of desalted BSA > 68%
Boron Nitride, a Novel Material for the Enrichment and Desalting

MALDI spectra:

In total 26 phosphopeptides could be identified. All the identified phosphopeptides are labeled.
Boron Nitride, a Novel Material for the Enrichment and Desalting

MALDI MS spectra before and after BN enrichment. After SPE using BN the phosphopeptide could be enriched by a factor of 100.

Before enrichment
volume 2 ml
9.5 fmol/µl

After BN enrichment
elution volume 20 µl
100 fold enrichment

DS(P)EGRGS(P)GDPGK
Boron Nitride, a Novel Material for the Enrichment and Desalting

Recovery Study using different Phosphopeptides

<table>
<thead>
<tr>
<th>Sequence</th>
<th>[M+H]^+</th>
<th>pl</th>
<th>A 20 m2/g Recovery [%]</th>
<th>B 60 m2/g Recovery [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS(P)EGRGS(P)GDPGK</td>
<td>1321.44</td>
<td>2.96</td>
<td>102.78 %</td>
<td>99.74 %</td>
</tr>
<tr>
<td>VYGKTS(P)HLR</td>
<td>1140.55</td>
<td>7.22</td>
<td>102.05 %</td>
<td>101.85 %</td>
</tr>
<tr>
<td>KIGEGT(P)YGVVVK</td>
<td>1393.67</td>
<td>5.45</td>
<td>98.02 %</td>
<td>96.31 %</td>
</tr>
</tbody>
</table>

A = 60% ACN in 1% TFA and 1% H₃PO₄; B = 60% ACN in 1% formic acid
BN 20 m²/g

⇒ No recoveries for graphite under this conditions! DHB elution: problems with LC-MS
Boron Nitride, a Novel Material for the Protein Depletion

MALDI-MS analysis of a **human albumin** (**HSA**) protein spiked with a tryptic digest of HSA (50000:1) before (A) and after (B) the depletion of proteins with BN.

(A) Mixture before the protein depletion. **In source fragmentation of proteins.** (sequence coverage of 36%)

(B) MALDI spectra of the sample after SPE with BN and elution with ACN 1% FA (sequence coverage of 72%)

*asterisk: Mascot identified peptides*
Boron Nitride, a Novel Material for the Enrichment and Desalting

Langmuir adsorption isotherms of Bradykinin for BN 60, BN 20 and graphite 15

<table>
<thead>
<tr>
<th>Material</th>
<th>Max. Loading Capacity $m_{ads}$ [µg/mg]</th>
<th>$m_{ads}$/Surface Area [µg/m²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BN 20 m²/g</td>
<td>14.61</td>
<td>0.731</td>
</tr>
<tr>
<td>BN 60 m²/g</td>
<td>24.13</td>
<td>0.402</td>
</tr>
<tr>
<td>Graphite 15 m²/g</td>
<td>8.19</td>
<td>0.546</td>
</tr>
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</table>
Boron Nitride for the Isolation of Bisphenol Derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW</th>
<th>pKa</th>
<th>log K_{ow}</th>
<th>R^2</th>
<th>LOD [ng/ml]</th>
<th>LOQ [ng/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BADGE·2H_2O</td>
<td>376.44</td>
<td>14.7^b</td>
<td>2.05^b</td>
<td>0.999</td>
<td>27.0</td>
<td>82.0</td>
</tr>
<tr>
<td>BPF</td>
<td>200.23</td>
<td>7.5^a</td>
<td>2.91^a</td>
<td>0.999</td>
<td>27.0</td>
<td>82.0</td>
</tr>
<tr>
<td>BPA</td>
<td>228.29</td>
<td>9.6^a</td>
<td>3.32^a</td>
<td>0.999</td>
<td>33.0</td>
<td>99.0</td>
</tr>
<tr>
<td>BPZ</td>
<td>268.35</td>
<td>9.7^b</td>
<td>4.53^b</td>
<td>0.999</td>
<td>28.0</td>
<td>85.0</td>
</tr>
<tr>
<td>BADGE</td>
<td>340.41</td>
<td>-</td>
<td>4.02^a</td>
<td>0.999</td>
<td>30.0</td>
<td>90.0</td>
</tr>
</tbody>
</table>

BADGE·2H_2O
2,2-bis(4-glycidyloxyphenyl)propane

BPF
bisphenol F

BPZ
bisphenol Z

BPA
bisphenol A

BADGE
2,2-bis(4-glycidyloxyphenyl)propane
Risk associated with bisphenols

• The French Agency for Food, Environmental and Occupational Health & Safety showed that there are ‘recognized’ effects in animals (effects on reproduction, on the mammary gland, on metabolism, the brain and behaviour) and other ‘suspected’ effects in humans (on reproduction, metabolism and cardiovascular diseases). These effects could be observed, even at low levels of exposure, during sensitive phases of an individual’s development.

• The French Agency for Food, Environmental and Occupational Health & Safety endorses the conclusions of the Expert Committee on Assessment of the risks related to chemical substances relating to the risks associated with BPA for human health, and on toxicological data and data on the use of bisphenols M, S, B, AP, AF, F and BADGE.

• Endocrine Disruptors

Tolerable Daily Intake (TDI) of 50 µg BPA/kg body weight (b.w.)/day as set by EFSA in 2006.
The migration limit of BPA set by European Union of 600 µg/kg food.
Tolerable Daily Intake (TDI) of 150 µg BADGE and its hydrolytic products/kg body weight (b.w.)/day.
The migration limit of BADGE and its hydrolytic products 9000 µg/kg food.

Boron Nitride for the Isolation of Bisphenol Derivatives

Recovery for the enrichment of 5 Phenols

<table>
<thead>
<tr>
<th>Phenol</th>
<th>4-Nitrophenol</th>
<th>2-Chlorophenol</th>
<th>2-Nitrophenol</th>
<th>Dimethylphenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>[12.5 µg/ml]</td>
<td>[12.5 µg/ml]</td>
<td>[12.5 µg/ml]</td>
<td>[12.5 µg/ml]</td>
<td>[12.5 µg/ml]</td>
</tr>
<tr>
<td>87.51</td>
<td>94.74</td>
<td>108.08</td>
<td>91.29</td>
<td>100.71</td>
</tr>
<tr>
<td>93.97</td>
<td>102.95</td>
<td>114.70</td>
<td>95.20</td>
<td>111.82</td>
</tr>
<tr>
<td>91.36</td>
<td>101.91</td>
<td>113.24</td>
<td>96.88</td>
<td>105.84</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>90.95</strong></td>
<td><strong>99.87</strong></td>
<td><strong>112.01</strong></td>
<td><strong>94.46</strong></td>
</tr>
<tr>
<td>RSD</td>
<td>2.66</td>
<td>3.65</td>
<td>2.84</td>
<td>2.34</td>
</tr>
</tbody>
</table>

5 mg of BN was incubated with a phenol mixture and afterward eluted with 80% acetonitrile in water.
Analysis of 5 bisphenol derivatives after enrichment with BN by LC and fluorescence detector

HPLC-FLD chromatogramms of (A) standard solution containing 5 bisphenol derivatives [100 ng/mL] and (B) bisphenol leached from baby bottle after enrichment with h-BN-60.

Chromatographic conditions: Shimadzu LC-10 Acvp, Phenomenex Luna 5u C18 250*4.6 mm, isocratic 3 min 30% B, gradient 30-70% B in 15 min, flow rate 1mL/min, 50°C, inj.: 30 µL, FLD: 230/303 nm
Boron Nitride for the Isolation of Bisphenol Derivatives

Leaching of BPA from polycarbonate products

<table>
<thead>
<tr>
<th></th>
<th>leached BPA [ng/ml]</th>
<th>± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Babybottle 1. treatment</td>
<td>20.79</td>
<td>1.05</td>
</tr>
<tr>
<td>Babybottle 2. treatment</td>
<td>19.82</td>
<td>0.72</td>
</tr>
<tr>
<td>muffin form</td>
<td>2.26</td>
<td>0.07</td>
</tr>
<tr>
<td>Siringe</td>
<td>0.85</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Treatment conditions: boiling water for 1 h
### Determination of bisphenol derivatives

<table>
<thead>
<tr>
<th></th>
<th>BADGE·2H₂O</th>
<th>BPF</th>
<th>BPA</th>
<th>BPZ</th>
<th>BADGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>river water</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>cola</td>
<td>4.63 ± 0.14</td>
<td>2.23 ± 0.21</td>
<td>10.34 ± 0.41</td>
<td>nd</td>
<td>1.49 ± 0.04</td>
</tr>
<tr>
<td>apple juice</td>
<td>1.93 ± 0.15</td>
<td>3.11 ± 0.30</td>
<td>nd</td>
<td>nd</td>
<td>1.36 ± 0.22</td>
</tr>
<tr>
<td>lemon soda</td>
<td>8.14 ± 0.09</td>
<td>1.40 ± 0.07</td>
<td>nd</td>
<td>nd</td>
<td>5.70 ± 0.04</td>
</tr>
<tr>
<td>citrus soft drink</td>
<td>5.32 ± 0.71</td>
<td>nd</td>
<td>1.04 ± 0.31</td>
<td>nd</td>
<td>1.56 ± 0.042</td>
</tr>
<tr>
<td>herbal soda</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>energy drink</td>
<td>4.46 ± 0.07</td>
<td>1.87 ± 0.11</td>
<td>5.57 ± 0.81</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>canned mushroom liquid</td>
<td>20.42 ± 1.19</td>
<td>nd</td>
<td>8.60 ± 0.37</td>
<td>nd</td>
<td>1.81 ± 0.12</td>
</tr>
<tr>
<td>pickled cucumber liquid</td>
<td>1.81 ± 0.15</td>
<td>2.23 ± 0.21</td>
<td>125.00 ± 2.18</td>
<td>nd</td>
<td>1.44 ± 0.06</td>
</tr>
<tr>
<td>pickled onion liquid</td>
<td>2.98 ± 0.08</td>
<td>nd</td>
<td>13.45 ± 0.63</td>
<td>nd</td>
<td>1.74 ± 0.00</td>
</tr>
<tr>
<td>urine</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>
Boron Nitride for the Isolation of Bisphenol Derivatives

Langmuir adsorption isotherms of Bisphenol A on h-BN-60

max. loading capacity of Bisphenol A = 60 µg/mg
Aufgabe 1

Analyt: Disaccharide

Maltose (Malzzucker)
4-(α-D-Glucosido)-D-Glucose

Celllobiose
4-(β-D-Glucosido)-D-Glucose

Saccharose (Rohrzucker)
α-D-Glucosido-β-D-Fructosid

Lactose (Milchzucker)
4-(β-D-Galactosido)-D-Glucose

Welche Festphase? Warum?
Aufgabe 2

Analyt: Proteine

Welche Festphase? Warum?
Aufgabe 3

Analyt: Pestizide

Welche Festphase? Warum?
Table 1. Sorbents for solid phase extraction and separation mechanisms for solid phase separations.

<table>
<thead>
<tr>
<th>Sorbent</th>
<th>Structure</th>
<th>Analyte type</th>
<th>Dissolving solvents</th>
<th>Elution solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reversed Phase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octadeyl (C₁₈)</td>
<td>-(CH₃)ₑ(CH₃)</td>
<td>Nonpolar</td>
<td>methanol/water, acetonitrile/water</td>
<td>For nonpolar analytes: hexane, chloroform For polar analytes: methanol</td>
</tr>
<tr>
<td>Octyl (C₆)</td>
<td>-(C₅H₁₁)ₑ(CH₃)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl (C₂)</td>
<td>-(CH₃)₂(CH₃)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclohexyl</td>
<td>-(CH₄)₇(CH₃)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyl</td>
<td>-(CH₃)₇(CH₃)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Phase (bonded)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyano (CN)</td>
<td>-(CH₃)CN</td>
<td>Slightly - moderately polar - strongly polar</td>
<td>hexane, chloroform</td>
<td>methanol</td>
</tr>
<tr>
<td>Amino (NH₂)</td>
<td>-(CH₃)NH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diol (COH₂OH)</td>
<td>-(CH₃)₂OCH₂OCHOCH₂OH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Phase (adsorption)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kieselguhr (Diatomaceous Earth)</td>
<td>-SiOH</td>
<td>Slightly - moderately polar - strongly polar</td>
<td>hexane, chloroform</td>
<td>methanol (dependent on type of analyte)</td>
</tr>
<tr>
<td>Silica gel</td>
<td>-SiOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Florisil</td>
<td>Mg₅SiO₄</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alumina (neutral)</td>
<td>Al₂O₃</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion Exchangers (anion and cation Exchange)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino (NH₂)</td>
<td>-(CH₃)NH%H⁺</td>
<td>Anion exchange - Ionic Acid</td>
<td>Water or buffer (pH=pKa +2)</td>
<td>1) Buffer (pH=pKa +2)</td>
</tr>
<tr>
<td>1°, 2°- Amino (NH₂/NH₃)</td>
<td>-(CH₃)NH⁺</td>
<td></td>
<td></td>
<td>2) pH where sorbent or analyte is neutral</td>
</tr>
<tr>
<td>Quaternary Amine (N⁺)</td>
<td>-(CH₃)N⁺(CH₃)₂⁺</td>
<td></td>
<td></td>
<td>3) Solvent with high ionic strength</td>
</tr>
<tr>
<td>Carboxylic acid (COOH)</td>
<td>-(CH₃)COO⁻</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propyl Sulfonic Acid (SO₃₂OH)</td>
<td>-(CH₃)SO₃⁻</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aromatic Sulfonic Acid (ArSO₃₂OH)</td>
<td>-(CH₃)₂-C₆H₄SO₃⁻</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Solid Phase Extraction Technique – Trends, Opportunities and Applications

A. Žwir-Ferenc, M. Bizriuk*

Department of Analytical Chemistry, Chemical Faculty, Gdańsk University of Technology,
11/12 Gabriela Narutowicza Str., 80-952 Gdańsk, Poland
Teil 4- Fällung

Grundlagen und Anwendung moderner Trennverfahren

Günther K. Bonn
Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens University of Innsbruck, Innrain 80-82, A-6020 Innsbruck, Austria
Einleitung
Analytik der Biomoleküle

Komplexität von biologischen Proben

biologische Probe

→ Herausforderungen:

1. enorme Komplexität der Proben
2. geringe Konzentration der Zielanalyten
3. beschränkter dynamischer Bereich der analytischen Messgeräte
4. Störkomponenten (Detergenzien, Puffersysteme etc.)
Einleitung
Analytik der Biomoleküle

1. Enorme Komplexität von biologischen Proben

z.B. Blutserum

→ 22 Proteine machen 99% des gesamten Serumproteoms aus!
Einleitung
Analytik der Biomoleküle

2. Geringe Konzentration vieler Zielanalyten

Dynamischer Bereich von Blutplasma

Einleitung
Analytik der Biomoleküle

→ Effiziente Probenvorbereitung

- Hoher Probendurchsatz
- Verbesserte Detektion
- Gezielte Analyse

Effizienz

Selektivität
- Hoher Probendurchsatz

Empfindlichkeit
- Verbesserte Detektion

Geschwindigkeit

Matthias Rainer
Einleitung
Analytik der Biomoleküle

Zusätzliche Komplexität durch Variationen von Proteinen

• Bei einem Pentapeptid (Länge 5 Aminosäuren) gibt es $20^5 = 3.200.000$ Kombinationsmöglichkeiten
• Proteine haben eine Länge von 100 bis 1000 Aminosäuren, also $20^{100} - 20^{1000}$ Kombinationsmöglichkeiten
• Drehbarkeit der C-C Bindung in der Kette
• Peptidbindung normalerweise in trans-, kann aber auch in cis-Konformationen vorliegen
• Seitengruppen können modifiziert sein
• Posttranslationale Modifikationen (z.B. Glykosylierung, Phosphorylierung)
Fällung von Proteinen

Fällung von Proteinen

Proteine können auf verschiedene Art in ihrer nativen Form gefällt werden:

• Fällung durch Aussalzen

• isoelektrische Fällung (Fällung am IEP)

• Fällung mit organischen Lösungsmitteln

• Co-Präzipitation
Fällung von Proteinen

Proteine können auf verschiedene Art in ihrer nativen Form gefällt werden:

- **Fällung durch Aussalzen**

Fällung von Proteinen

Proteine können auf verschiedene Art in ihrer nativen Form gefällt werden:

- isoelektrische Fällung (Fällung am IEP)

Praktisch lässt sich der IEP auch zur Fällung von Proteinen aus einer Lösung nutzen. Die Löslichkeit eines Proteins wird sehr stark durch den pH-Wert der Umgebung beeinflusst und erreicht am isoelektrischen Punkt ein Minimum. Ober- oder unterhalb des IEP tragen alle Moleküle die gleiche Ladung (positiv oder negativ) und stoßen sich daher ab. Eine Zusammenballung zu unlöslichen Aggregaten ist durch die Abstoßung der Moleküle untereinander verhindert und das Protein bleibt in Lösung.
Fällung von Proteinen

Proteine können auf verschiedene Art in ihrer nativen Form gefällt werden:

- Fällung mit organischen Lösungsmitteln

→ Aceton Precipitation
→ TCA Precipitation
→ Wessel Flügge Precipitation
Fällung von Proteinen

Fällung mit organischen Lösungsmitteln

**Acetone Precipitation Protocol**

1. Cool the required volume of acetone to -20°C.
2. Place protein sample in acetone-compatible tube.
3. Add four times the sample volume of cold (-20°C) acetone to the tube.
4. Vortex tube and incubate for 60 minutes at -20°C.
5. Centrifuge 10 minutes at 13,000-15,000 x g.
6. Decant and properly dispose of the supernatant, being careful to not dislodge the protein pellet.
   **Optional:** If additional cycles of precipitation are necessary to completely remove the interfering substance, then repeat steps 2-5 before proceeding to step 7.
7. Allow the acetone to evaporate from the uncapped tube at room temperature for 30 minutes. Do not over-dry pellet, or it may not dissolve properly.
8. Resuspend in appropriate buffer.
Fällung von Proteinen

Fällung mit organischen Lösungsmitteln

**TCA Precipitation Protocol**

1. Add an equal volume of 20% TCA (trichloroacetic acid) to protein sample.
2. Incubate 30 min on ice.
3. Spin in microfuge at 4 deg. For 15 min.
4. Carefully remove all supernatant.
5. Add ~300 ul cold acetone and spin 5 min at 4 degrees.
6. Remove supernatant and dry pellet.
7. Resuspend samples in desired buffer.
Fällung von Proteinen

Fällung mit organischen Lösungsmitteln

Chloroform/Methanol Precipitation

1. To sample of starting volume 100 ul
2. Add 400 ul methanol
3. Vortex well
4. Add 100 ul chloroform
5. Vortex
6. Add 300 ul H₂O
7. Vortex
8. Spin 1 minute @ 14,0000 g
9. Remove top aqueous layer (protein is between layers)
10. Add 400 ul methanol
11. Vortex
12. Spin 2 minutes @ 14,000 g
13. Remove as much MeOH as possible without disturbing pellet
14. Speed-Vac to dryness
15. Bring up in 2X sample buffer for PAGE

Fällung von Proteinen

Proteine können auf verschiedene Art in ihrer nativen Form gefällt werden:

• Co-Präzipitation
Highly Efficient Phosphopeptide Enrichment by Calcium Phosphate Precipitation Combined with Subsequent IMAC Enrichment*

Xumin Zhang‡, Juanying Ye‡, Ole N. Jensen§, and Peter Roepstorff¶

A new method for enrichment of phosphopeptides in complex mixtures derived by proteolytic digestion of biological samples has been developed. The method is based on calcium phosphate precipitation of the phosphopeptides prior to further enrichment with established affinity enrichment methods. Calcium phosphate precipitation combined with phosphopeptide enrichment using Fe(III) IMAC provided highly selective enrichment of phosphopeptides. Application of the method to a complex peptide sample derived from rice embryo resulted in more than 90% phosphopeptides in the enriched sample as determined by mass spectrometry. Introduction of a two-step IMAC enrichment procedure after calcium phosphate precipitation resulted in observation of an increased number of phosphopeptides. Molecular & Cellular Proteomics; 6:2032–2042, 2007.

efficiencies are poor, selective enrichment is required. To date, numerous methods have been introduced for enrichment of phosphoproteins or phosphopeptides. The methods used for selective enrichment of phosphopeptides fall in two main categories: chemical derivatization (14–16) and affinity chromatography-based methods. Although the chemical derivatization methods are highly selective, they are not widely applied in the phosphoproteome studies most likely due to sample loss caused by the multiple reaction steps and increased sample complexity by unavoidable side reactions (15, 17, 18).

Chromatographic enrichment of the phosphopeptides has been widely used in phosphoproteome studies. This includes IMAC (17, 19–21), strong cation exchange chromatography (22, 23), strong anion exchange chromatography (24), and metal oxide chromatography (18, 25–27). In several studies
\[ 3\text{Ca}^{2+} + 2\text{PO}_4^{3-} \rightarrow \text{Ca}_3(\text{PO}_4)_2 \downarrow \]

A \[
\text{Ca}^{2+} + \text{P}–\text{O}–\text{PO}_3^{2-} \rightarrow \text{P}–\text{O}–\text{PO}_3\text{Ca}
\]

\[(3m+n) \text{Ca}^{2+} + 2m\text{PO}_4^{3-} + n \text{P}–\text{O}–\text{PO}_3^{2-} \rightarrow [\text{Ca}_3(\text{PO}_4)_2]_m[\text{P}–\text{O}–\text{PO}_3\text{Ca}]_n \downarrow \]

Peptide Mixture + Na$_2$HPO$_4$ + NH$_3$H$_2$O
+ CaCl$_2$

\[
\text{Centrifuge}
\]

\[
\text{Pellet} \quad \text{Supernatant (Collected)}
\]

\[
\text{Wash (CaCl$_2$)}
\]

\[
\text{Centrifuge}
\]

\[
\text{Wash (Collected)}
\]

\[
\text{Pellet (Dissolved by formic acid and then collected)}
\]
Phosphoproteomic Analysis of Human Brain by Calcium Phosphate Precipitation and Mass Spectrometry

Qiangwei Xia,† Dongmei Cheng,† Duc M. Duong,† Marla Gearing,‡ James J. Lah,§ Allan I. Levey,§ and Junmin Peng*,†

Department of Human Genetics, Department of Pathology and Laboratory Medicine, and Department of Neurology, The Center for Neurodegenerative Diseases, Emory University School of Medicine, Atlanta, Georgia 30322

Received January 21, 2008

Alzheimer’s disease (AD), the most common form of dementia, is manifested in the brain by the aggregation of amyloid plaques and neurofibrillary tangles. The tangles are primarily composed of microtubule-associated protein tau that is aberrantly hyperphosphorylated, suggesting that deregulated phosphorylation may contribute to AD pathogenesis. However, systematic analysis of the phosphoproteome in AD brain tissues has not been reported. We used calcium phosphate precipitation to analyze an AD postmortem brain, followed by liquid chromatography—tandem mass spectrometry. The protein sample was first resolved by one-dimensional polyacrylamide gel electrophoresis and subjected to gel excision and in-gel digestion. Phosphopeptides in the resulting peptide mixtures were enriched in a single step of calcium phosphate precipitation, and then analyzed by the LC-MS/MS approach. After database search, stringent filtering, and manual validation of neutral loss in the MS/MS spectra, a total of 466 phosphorylation sites on 185 proteins including tau were identified. A majority of sites were not described previously. This study demonstrates the feasibility of combining calcium phosphate precipitation with mass spectrometry for phosphoproteome analysis of postmortem human brain tissue.

Keywords: Alzheimer’s disease • protein phosphorylation • immobilized metal-affinity chromatography • calcium phosphate precipitation • proteomics
Postmortem AD brain

5 mg of proteins for SDS-PAGE

In-gel digestion

Calcium phosphate precipitation (within 30 minutes)

C18 desalting

LC-MS/MS (only accepting spectra with neutral loss)

551 phosphopeptides
466 phosphorylation sites
Motif-Specific Sampling of Phosphoproteomes

Cristian I. Ruse, Daniel B. McClatchy, Bingwen Lu, Daniel Cociorva, Akira Motoyama, Sung Kyu Park, and John R. Yates III*

Department of Chemical Physiology/Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, SR11, La Jolla, California 92037

Received February 22, 2008

Phosphoproteomics, the targeted study of a subfraction of the proteome which is modified by phosphorylation, has become an indispensable tool to study cell signaling dynamics. We described a methodology that linked phosphoproteome and proteome analysis based on Ba\(^{2+}\) binding properties of amino acids. This technology selected motif-specific phosphopeptides independent of the system under analysis. MudPIT (Multidimensional Identification Technology) identified 1037 precipitated phosphopeptides from as little as 250 µg of proteins. To extend coverage of the phosphoproteome, we sampled the nuclear extract of HeLa cells with three values of Ba\(^{2+}\) ions molarity. The presence of more than 70% of identified phosphoproteins was further substantiated by their nonmodified peptides. Upon isoproterenol stimulation of HEK cells, we identified an increasing number of phosphoproteins from MAPK cascades and AKAP signaling hubs. We quantified changes in both protein and phosphorylation levels of 197 phosphoproteins including a critical kinase, MAPK1. Integration of differential phosphorylation of MAPK1 with knowledge bases constructed modules that correlated well with its role as node in cross-talk of canonical pathways.

Keywords: Phosphoproteome • Barium • Protein Quantification • Beta adrenergic • Signal transduction
Total protein amount (250μg to 1000μg) → digested peptides

- Adjust pH to 3.5, add Ba^{2+}/acetone → dry down supernatant → MudPIT

- Adjust pH to 4.6, add Ba^{2+}/acetone → dry down supernatant → MudPIT

- Adjust pH to 8.0, add Ba^{2+}/acetone → dry down supernatant → MudPIT
Motif-Specific Sampling of Phosphoproteomes

Ba\textsuperscript{2+}/Acetone/pH Precipitation for Phosphopeptide Identification in HeLa Cells Nuclear Extract using MudPIT

Table 1. Identified Phosphopeptides from 1 mg of HeLa Cells Nuclear Extract for [Ba\textsuperscript{2+}] Sampling Conditions

<table>
<thead>
<tr>
<th>[Ba\textsuperscript{2+}] (\textmu mol)</th>
<th>pH 3.5</th>
<th>pH 4.6</th>
<th>pH 8.0</th>
<th>total\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>1644</td>
<td>801</td>
<td>153</td>
<td>2723</td>
</tr>
<tr>
<td>6</td>
<td>872</td>
<td>821</td>
<td>177</td>
<td>1868</td>
</tr>
<tr>
<td>1</td>
<td>1217</td>
<td>416</td>
<td>295</td>
<td>2215</td>
</tr>
</tbody>
</table>

\textsuperscript{a} It includes MS3 identified phosphopeptides.

Precipitation of Phosphoproteins by Trivalent Lanthanide Ions

A Top-Down Approach
Protein phosphorylation

Protein dephosphorylation

Berg JM et al. 2010
Protein separation and isolation

2. Chromatographic techniques

IMAC
Immobilized Metal ion Affinity Chromatography

MOAC
Metal Oxide Affinity Chromatography

Berg JM et al. 2010
According to: Leitner A. 2010
Proposed Precipitation Mechanism
Lanthanide Phoshates
(Solubility Products)

Scheme for Precipitation of Phosphoproteins by Trivalent Europium-, Terbium- and Erbium- Ions

Precipitant: $\text{KH}_2\text{PO}_4$

Precipitation

Washing

30% formic acid

Denaturation, tryptic digestion

10 min, 70W microwave-assisted digest

Proteins

Dissolution of pellet

Peptides

On-pellet digest

MALDI-MS

MALDI-MS

Methods and instruments

MALDI-TOF MS: linear mode
Methods and instruments

MALDI-TOF MS: reflector mode

\[ m_1 = m_2 \quad E_{\text{kin}, 1} > E_{\text{kin}, 2} \]
Bovine milk composition

- 87.1% water
- 4.9% carbohydrates
- 3.9% fats
- 3.4% proteins
- 0.7% minerals

α-S1-casein: 38%
α-S2-casein: 9%
β-casein: 29%
κ-casein: 9%
α-lactalbumin: 9%
β-lactoglobulin: 9%
others: 4%

according to Pavia DL. 1990 / Eigel WN et al. 1984
## Milk proteins

<table>
<thead>
<tr>
<th>protein (variant)</th>
<th>mol. weight / Da</th>
<th>amino acids</th>
<th>c / g·L(^{-1})</th>
<th>P per mol</th>
<th>SH</th>
<th>S-S per mol</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>α\textsubscript{s1}-casein (B 8P)</td>
<td>23,614</td>
<td>199</td>
<td>12 – 15</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>4.4 – 4.8</td>
</tr>
<tr>
<td>α\textsubscript{s2}-casein (A 11P)</td>
<td>25,230</td>
<td>207</td>
<td>3 – 4</td>
<td>11</td>
<td>2</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>β-casein (A2 5P)</td>
<td>23,983</td>
<td>209</td>
<td>9 – 11</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>4.8 – 5.1</td>
</tr>
<tr>
<td>κ-casein (B 1P)</td>
<td>19,023</td>
<td>169</td>
<td>2 – 4</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>5.3 – 5.8</td>
</tr>
<tr>
<td>α-lactalbumin (B)</td>
<td>14,176</td>
<td>123</td>
<td>0.6 – 1.7</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4.2 – 4.5</td>
</tr>
<tr>
<td>β-lactoglobulin (B)</td>
<td>18,363</td>
<td>162</td>
<td>2 – 4</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>5.1</td>
</tr>
<tr>
<td>serum albumin</td>
<td>66,267</td>
<td>582</td>
<td>0.4</td>
<td>0</td>
<td>1</td>
<td>17</td>
<td>4.7 – 4.9</td>
</tr>
</tbody>
</table>

according to: Eigel WN et al. 1984 / Fox PF et al. 1998
Precipitation of Phosphoproteins from Bovine Milk by Trivalent Europium-, Terbium- and Erbium- Ions

Europium

- [M+H]+
- [M+2H]2+

Terbium

- [M+H]+
- [M+2H]2+

Erbium

- [M+H]+
- [M+2H]2+

**Signal Intensity [a.u.]**

**m/z**

**Supernatent**

**Pellet**

**Wash 1**

**Wash 2**

**milk**

**non-phosphorylated milk-proteins**

- PPC-5 (m/z ~12–13 kDa)
- α-lactalbumin (m/z ~14.1 kDa)
- β-lactoglobulin (m/z ~18.3 kDa)

**phosphoproteins**

- α_{S1}-casein (m/z ~24.5 kDa)
- α_{S2}-casein (m/z ~24.5 kDa)
- β-casein (m/z ~25.1 kDa)
- κ-casein (m/z ~20.0 kDa)
On-Pellet Digest of precipitated Phosphoproteins from Bovine Milk by Trivalent Europium-, Terbium- and Erbium- Ions

Peptide Mass-Fingerprint-Analysis

<table>
<thead>
<tr>
<th></th>
<th>seq. Coverage [%]</th>
<th>Mascot Search Score</th>
<th>Error [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eu</strong></td>
<td></td>
<td></td>
<td></td>
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α1... αS1-Casein
α2... αS2-Casein
β ... β-Casein
κ ... κ-Casein

Terbium

Precipitation of Phosphoproteins from Egg-White by Trivalent Europium-, Terbium- and Erbium- Ions

**Precipitation of Phosphoproteins**

**Europium**

- Supernatent: [M+H]+
- Wash 1: [M+H]+
- Wash 2: [M+H]+
- Pellet: [M+3H]+

**Terbium**

- Supernatent: [M+H]+
- Wash 1: [M+H]+
- Wash 2: [M+H]+
- Pellet: [M+3H]+

**Erbium**

- Supernatent: [M+H]+
- Wash 1: [M+H]+
- Wash 2: [M+H]+
- Pellet: [M+3H]+

**Proteins**

- **Non phosphorylated egg-white proteins**
  - Lysozyme (m/z ~14 kDa)
  - Ovomucoid (m/z ~28 kDa)
  - Ovoglobulins G2+G3 (m/z ~30-45 kDa)
  - Ovotransferrin (m/z ~80 kDa)

- **Phosphoprotein**
  - Ovalbumin (m/z ~45 kDa)

On-Pellet Digest of precipitated Phosphoproteins from Egg-White by Trivalent Europium-, Terbium- and Erbium- Ions

Europium Peptide Mass-Fingerprint-Analysis

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Recovery Study of Phosphoprotein

2M Ln$^{3+}$-Chloride

take supernatant

bicinchoninic acid (BCA)

chelation of two bicinchoninic acid molecules with Cu$^{+1}$

reduction of Cu$^{+2}$ to Cu$^{+1}$ in the presence of proteins (alkaline conditions)

colorimetric detection 562 nm

UV/VIS
Methods and instruments

Colorimetric assays: Bradford and BCA

Bradford reagent: Coomassie Brilliant Blue G-250

BCA assay reaction

Protein + Cu^{2+} \xrightarrow{\text{OH}^-} \text{Cu}^+ + 2 \text{BCA}
Precipitation of Phosphoproteins by Trivalent Europium-, Terbium- and Erbium- Ions

Recovery Study

Recovery [%]

Volume Precipitant [µl]

Lanthanum
Europium
Terbium
Erbium

100%

$c_{\text{casein}} = 300\mu\text{g/ml}$
$c_{\text{precip.}} = 2\text{M}$
Precipitation of Phosphopeptides by Trivalent Lanthanide Ions

A Bottom-Up Approach
A Novel Strategy for Phosphopeptide Enrichment using Lanthanide Phosphate Precipitation

Workflow for the precipitation of phosphorylated peptides:

1. Digest proteins
2. Adjust pH to 3
3. Precipitate for 10 min
4. Wash pellet
5. Discard supernatant
6. Separate pellet
7. Dissolve pellet in 2% HCl
8. MALDI MS analysis

KH$_2$PO$_4$ → Ln$^{3+}$-Chloride
MALDI mass spectra taken from digested milk peptides after precipitation with trivalent lanthanide ions. A, phosphopeptide enriched by precipitation with Er$^{3+}$. B, phosphopeptide enriched by precipitation using Ho$^{3+}$. C, phosphopeptide enriched by precipitation using Ce$^{3+}$. α-S1 and β-S2 refers to first and second subunits of α-casein respectively. β-C refers to peptides form β-casein.
A Novel Strategy for Phosphopeptide Enrichment using Lanthanide Phosphate Precipitation

MALDI mass spectra taken from egg white peptides after precipitation with trivalent lanthanide ions. A, phosphopeptide enriched by precipitation with Er$^{3+}$. B, phosphopeptide enriched by precipitation using Ho$^{3+}$. C, phosphopeptide enriched by precipitation using Ce$^{3+}$. Only phosphorylated peptides are labeled.
A Novel Strategy for Phosphopeptide Enrichment using Lanthanide Phosphate Precipitation

MALDI mass spectra of a sensitivity study using two synthetic phosphopeptides. A, representing 500 fmol/μL; B, 10 fold dilution (50 fmol/μL) and C, 100 fold dilution (5 fmol/μL)
## Recovery of Phosphopeptides

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Development of New Bioanalytical Tools and Methods for the Enrichment of Phosphorylated Peptides and Proteins

Tryptic On-Pellet Digest of Precipitated Phosphoproteins

Workflow

3.6% HCl

Intensity

LaPO4

### Overview of recovered phosphopeptides from a protein mixture (lysozyme, cytochrome c, myoglobin, bovine serum albumin, a- and b-casein) and bovine milk

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<th>Protein</th>
<th>Phosphopeptide sequences</th>
<th>Phospho groups</th>
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Dephosphorylated HeLa cell lysate (1 mg/mL) with spiked α- and β-casein (5 μg/mL) after enzymatic on-pellet digestion using trivalent cerium cations. **α1, α2 and β correspond to the tryptic phosphopeptides deriving from αS1-, αS2, and β-casein, respectively.**
Development of New Bioanalytical Tools and Methods for the Enrichment of Phosphorylated Peptides and Proteins

On-pellet digest of precipitated proteins from Human Saliva

Highly efficient precipitation of phosphorylated peptides and proteins using trivalent lanthanide ions

Conclusion

- simple and fast method
- highly selective for phosphorylated peptides and proteins
- enables top-down and bottom-up phosphoproteomics
- no stationary phase or resin required (reduced unspecific binding)
- trypsin was observed to be not affected by the lanthanide ions
- the amount of precipitant can be adjusted to each application
- MS and LC-MS compatible
- allows automation using liquid handling robotics