Teil 2 – SPE, Fällung

Grundlagen und Anwendung moderner Trennverfahren

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Festphasenextraktion - Prinzip

Das Prinzip basiert wie bei allen chromatographischen Methoden auf der *Wechselwirkung* des Analyten *mit stationärer und mobiler Phase*.
Es findet ein Adsorptions- bzw. Extraktionsprozess zwischen fester (stationärer) und flüssiger (mobiler) Phase statt, wobei die Wechselwirkung des Analyten mit der festen Phase stärker (oder schwächer) ist als mit dem Elutionsmittel.
Dadurch können nun

- Zielanalyten zurückgehalten und Interferenten eluiert oder
- Zielanalyten eluiert und Interferenten zurückgehalten werden,

wodurch eine Reinigung (clean-up) und Anreicherung der Analyten erreicht werden kann.
Es stehen grundsätzlich alle in der Chromatographie verwendeten Trägermaterialien zur Verfügung, es kann mit allen Trennmechanismen (Normalphasen, Umkehrphasen, Ionenaustausch, Größenausschluss, Affinitätschromatographie etc.) gearbeitet werden.

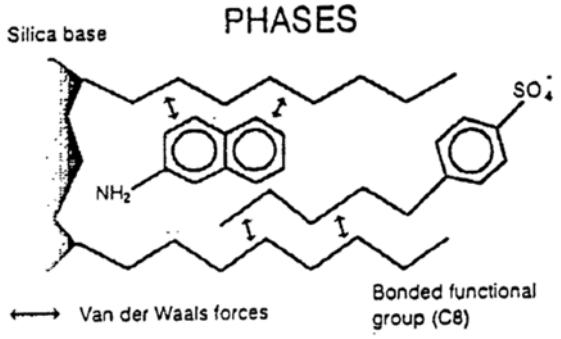
Festphasenextraktion - Prinzip

Bindungskräfte zwischenmolekularer Wechselwirkungen:

Wechselwirkung	zu beobachten zwischen z.B.	Bereich der Energie
Van der Waals Kräfte	Alkylketten, aromatische Ringe	2 – 10 KJ/Mol
Dipolare Anziehung	Heterocyklen, Carbonyle	5 – 15 KJ/Mol
Wasserstoffbrücken	Alkohole, Amine, Säuren	20 – 50 KJ/Mol
Coulomb´sche Anziehung	alle ionischen Verbindungen	100 – 400 KJ/Mol
Bildung kovalenter Bindungen	nicht-metallische Verbindungen	> 300 KJ/Mol

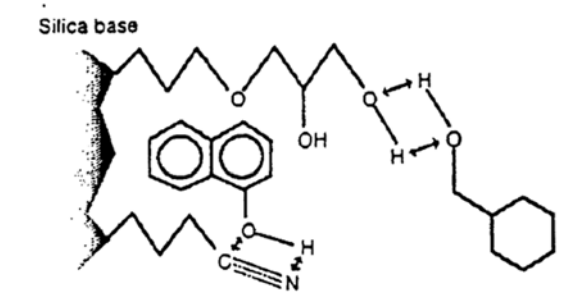
Einzelne Wechselwirkungen oder Kombinationen aus diesen können zur Retention oder Elution der gewünschten Zielsubstanzen dienen.
Gemeinsam mit einer großen Auswahl an Elutionsmitteln und ihrer Gemische (gemäß der eluotropen Reihe) kann somit jedes nur erdenkliche Trennproblem gemeistert werden.

INTERACTIONS ON NON-POLAR PHASES



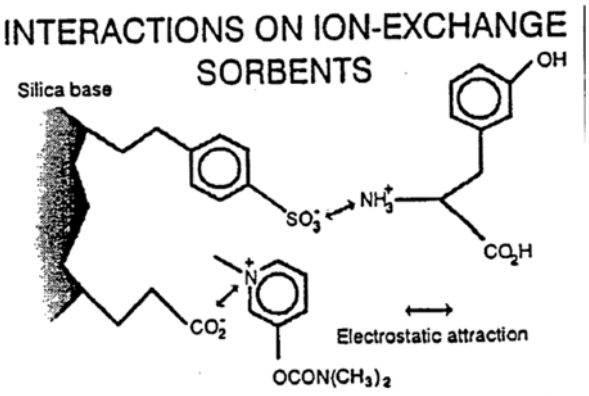
Examples: BETX from groundwater (M752); TCAs from biological fluids (M325); Xanthines from beverages (M204)

INTERACTIONS ON POLAR PHASES



Examples: Anti-inflammatories from pharmaceutical preparations (M1008); Fungicides from citrus fruit (M2); Lipid classes from serum (M269)

INTERACTIONS ON ION-EXCHANGE SORBENTS

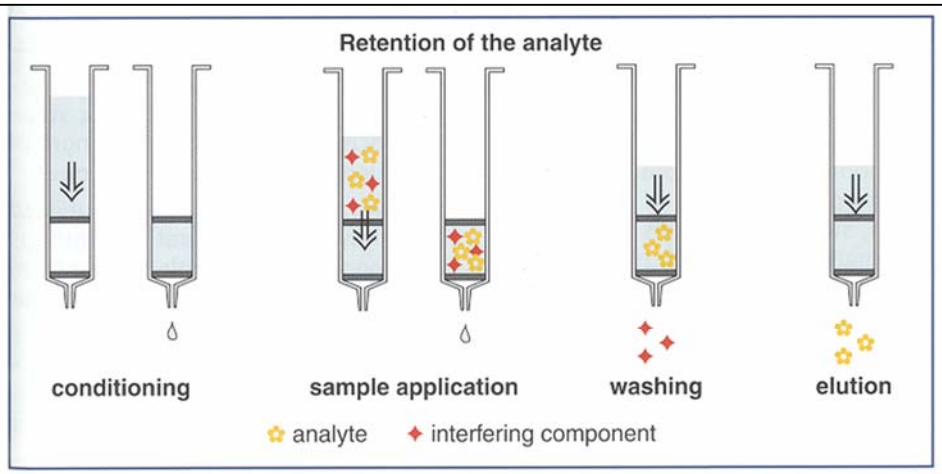


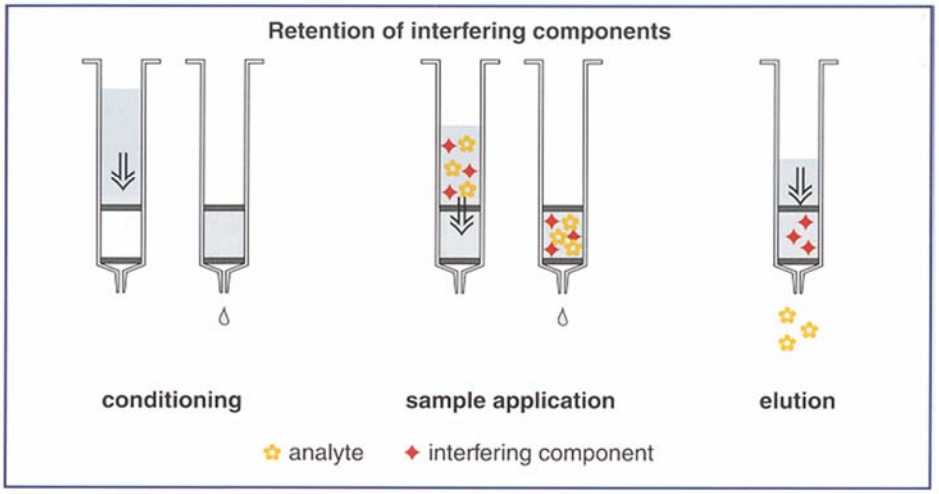
Examples: Amino acids and their metabolites from urine (M1209); Anticholinergics from plasma (M813); Polychlorinated pollutants in fly-ash (M1056)

Festphasenextraktion - Prinzip

Durchführung der Festphasenextraktion:

- Konditionieren: Vorbereitung des Trägermateriales (Solvatisierung) für eine reproduzierbare Interaktion mit der Probenmatrix.
- Probenaufgabe: Langsames Aufgeben des Extraktes in möglichst kleinem Volumen. Das Extraktionsmittel soll derart beschaffen sein, dass die Analyte maximale Retention am Trägermaterial zeigen. Manchmal haben die Matrixverunreinigungen keine Retention, sodass eine Trennung von Analyt und Matrix schon durch die ersten beiden Schritte erreicht ist.
- Waschen: Elution interferierender Verbindungen
- Elution der Zielanalyte: Das Durchleiten eines geeigneten Elutionsmittels, das speziell ausgewählt wird, um die Analyt/Sorbens-Wechselwirkung zu brechen, führt zu einer (möglichst) selektiven Elution der Zielanalyte.

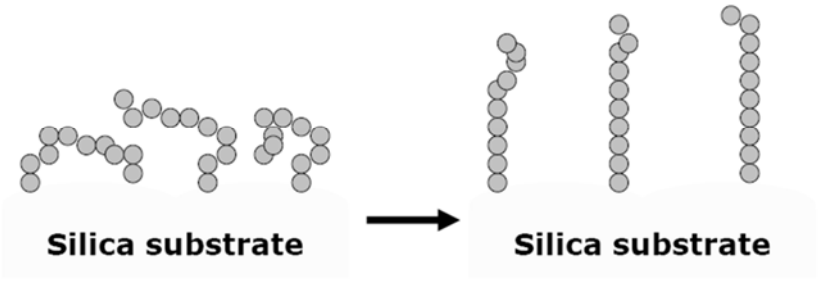




Macherey-Nagel Chromatography Catalog

Conditioning

- Make the sorbent compatible with sample solution for close contact in small channels
- The sorbent should not be dry at any stage



C-18 activated with n-hexane

Loading/Adsorption

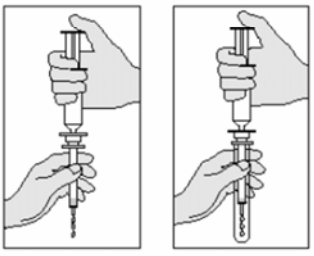
- Gentle vacuum, or pump
- At reasonable rate, depend on column dimension, particle size
  - Small particles, more efficient, permit faster flow rate
- The sorbent should not be allow to go dry at any point
  - Air in the column prevent efficient interfacial contact between liquid and solid phase

Washing

- Remove interferences coadsorbed from the SPE column
- The wash solution must not be too strong to partially eluted the analyte of interest

Elution

- Eluting solvent should be strong enough to completely removed adsorbed analytes from the sorbent as small a volume as possible (small *k*)
- Compatible with the analytical method (i.e., Low BP – GC)
- Free from impurity
- Low cost and non toxic



Kapazitätsfaktor

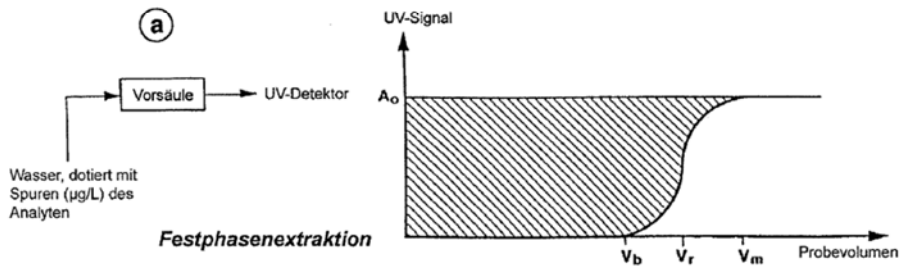
Je länger ein Stoff in der stationären Phase verbleibt, desto größer wird der Kapazitätsfaktor und damit auch die Retentionszeit des Analyten. Der Kapazitätsfaktor gibt an, um wieviel länger sich Moleküle an der stationären Phase im Vergleich zur mobilen aufhalten. Mit Bruttoretentionszeit (tR) und Totzeit (t0) gilt:

$$k = \frac{t_s}{t_m} = \frac{t_R - t_0}{t_0}$$

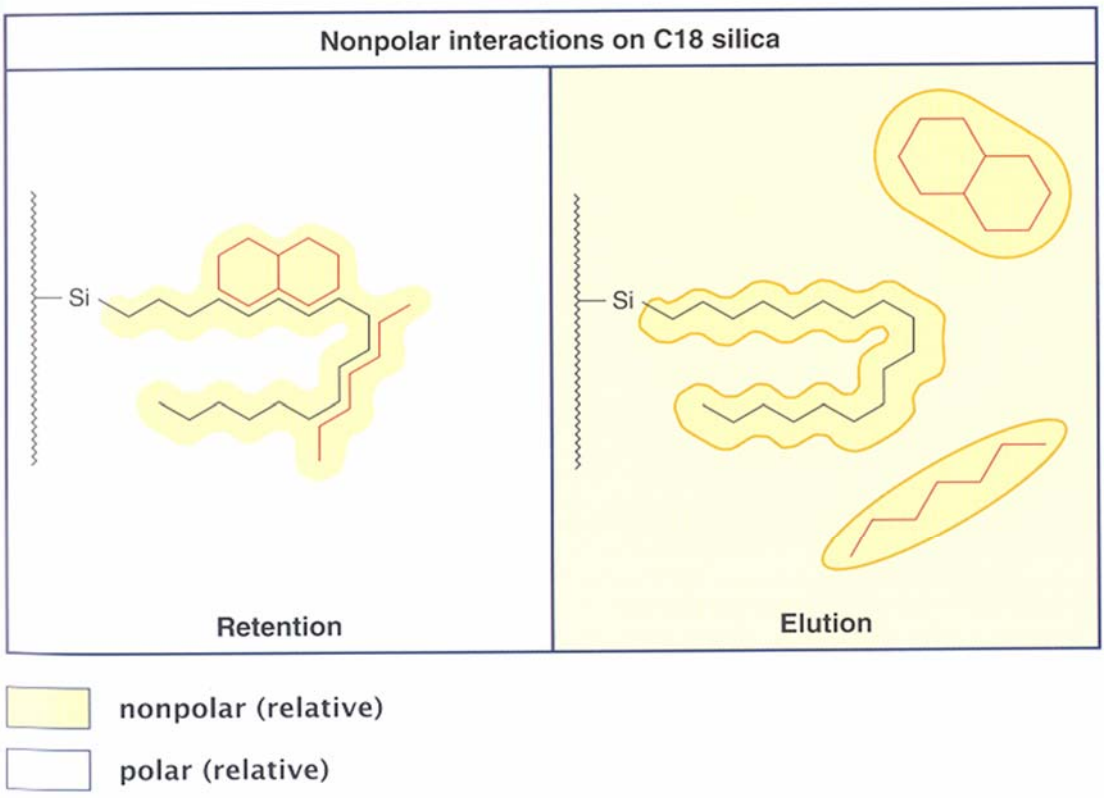
Ein hoher Kapazitätsfaktor beschreibt ein hohes Retentionsverhalten!

Festphasenextraktion
Durchbruchvolumen

Eine wichtige Kenngröße der Festphasenextraktion ist das Durchbruchsvolumen V_b. Wird ein großes Volumen einer verdünnten wässrigen Analytlösung über eine SPE-Kartusche angereichert und gleichzeitig das UV-Signal aufgezeichnet, so wird ab einem bestimmten Probevolumen der Analyt von der nachfolgenden wässrigen Probenmatrix bereits wieder eluiert werden; 1 Prozent des maximalen UV-Signals A_0 definiert das Durchbruchsvolumen V_b (siehe auch Abb.). Die gemessene UV-Absorption wird so lange ansteigen bis die eluierende Lösung die gleiche Konzentration wie die Probelösung aufweist; V_m ist bei 99 Prozent von A_0 definiert. Da die Theorie der Flüssigchromatographie auch auf die Festphasenextraktion angewandt werden kann, wie Abbildung 7 verdeutlicht, können Durchbruchsvolumina über die chromatographischen Kenngrößen bestimmt werden. Dazu müssen anstelle der HPLC-Säulen die jeweiligen SPE-Adsorbentien und anstelle der sonst üblichen Elutionsmittel reines Wasser verwendet werden.



Festphasenextraktion



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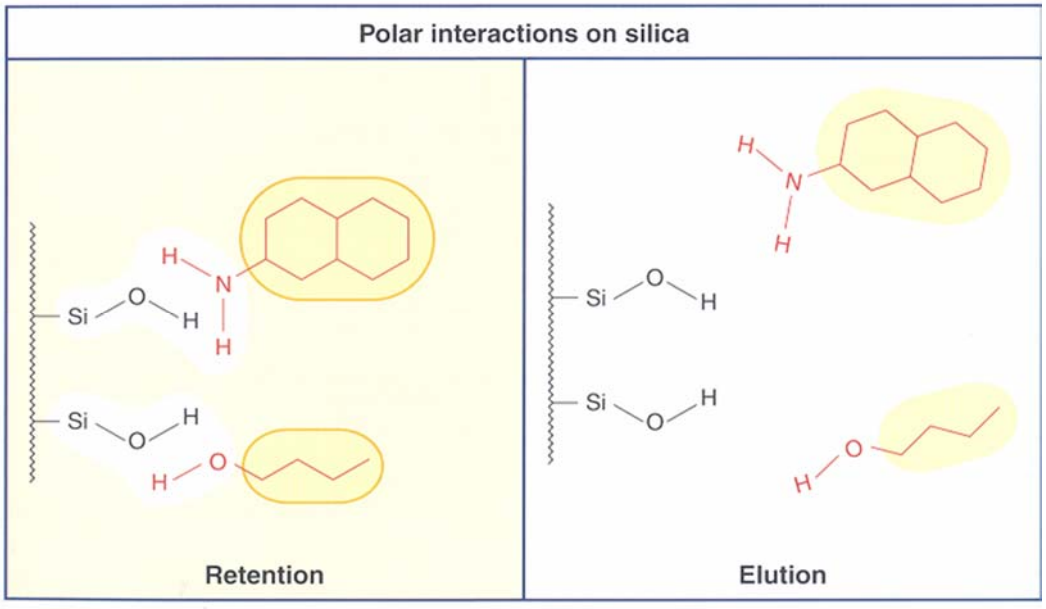

In general, polar compounds are easily adsorbed to a polar adsorbent from a nonpolar environment and are eluted with a polar solvent. The opposite holds true for nonpolar compounds. They are easily adsorbed from a polar environment onto nonpolar surfaces. Elution is achieved by solvents of lower polarity.

nonpolar (relative)

polar (relative)

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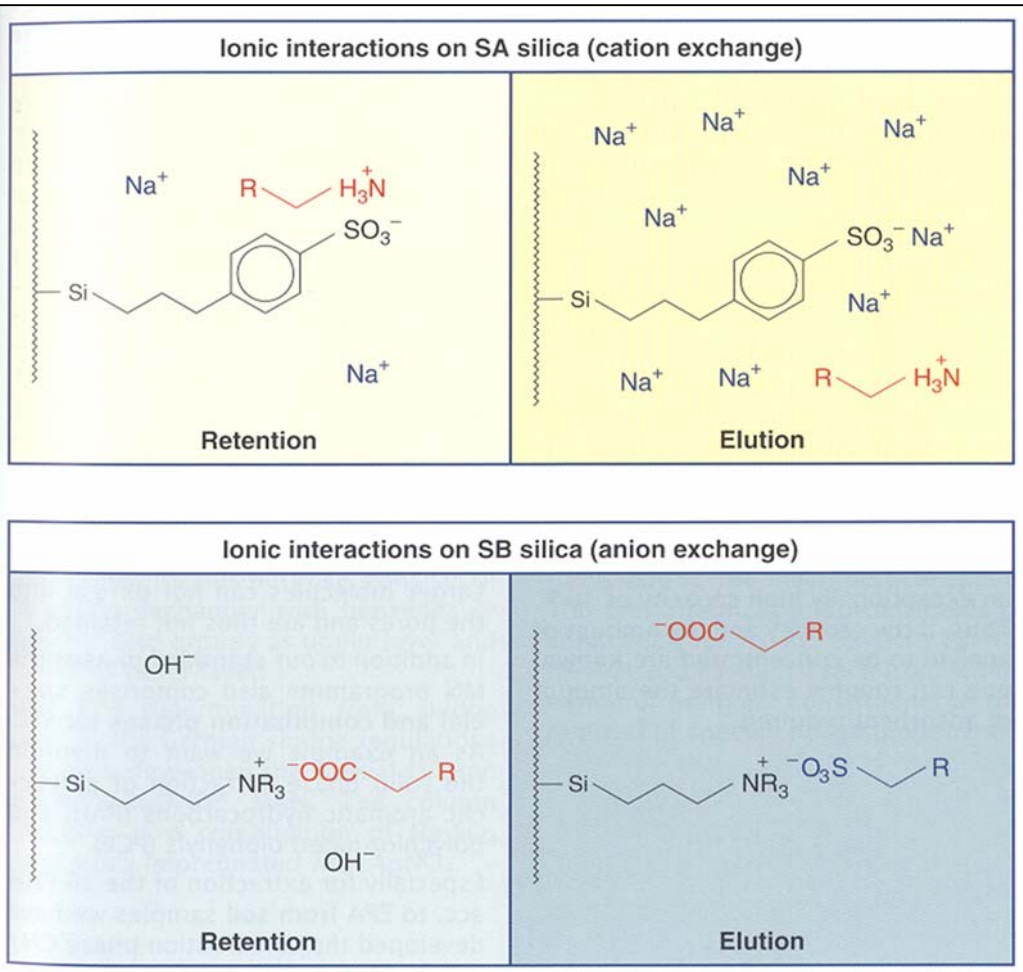

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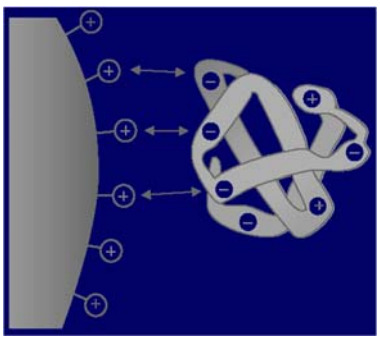
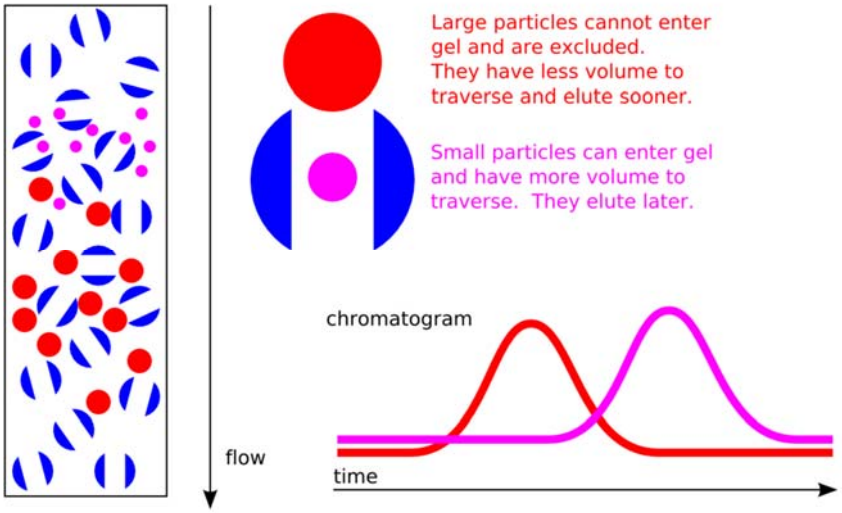
Ion Exchange Chromatography

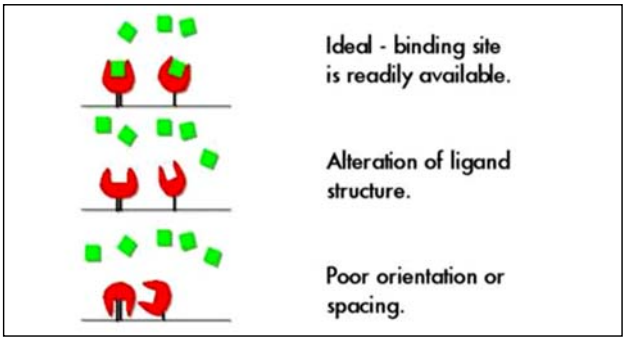


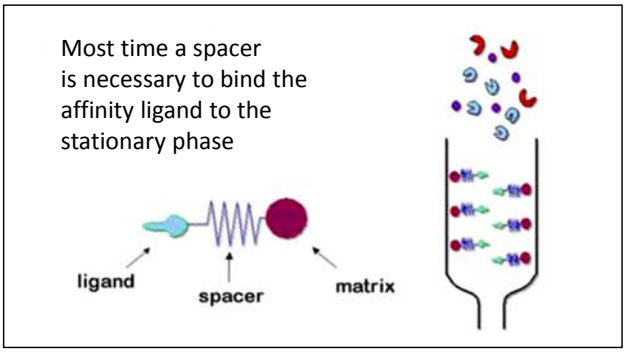
		Operating
Type of Exchanger	**Functional group**	**Type**
Strong anion	—N^+(—CH_3)	Quaternary Amine
Weak anion	—NH_2	Primary amine
	—NH	Secondary amine
	—N	Tertiary amine
Strong cation	—SO_3^-	Sulfonic acid
Weak cation	—COO^-	Carboxylic acid

Size Exclusion Chromatography



Affinity Chromatography





Affinity ligands and applications

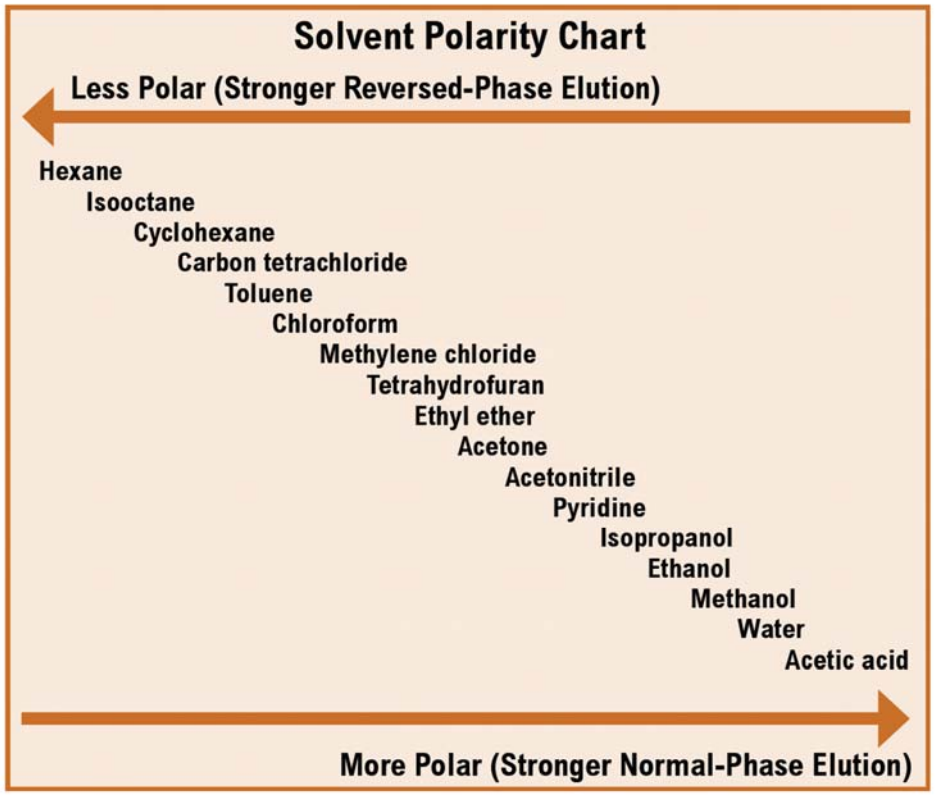
LIGAND ➔	APPLICATIONS
• Avidin	• Biotin derivatives
• Aprotinin	• Serine proteases
• Biotin	• Avidin
• Concanavalin A	• Glycoproteins, Oligosaccharides
• Gelatin	• Fibornectine enzymes
• Glutathione	• Enzymes related to glutathione
• Heparin	• Blood coagulation factors
• Iminoacetic acid	• Interferon, serum proteins
• Lysine	• Plasminogen, polysaccharides
• Protein A	• Human IgG
• Phophorylethanolamine	• C-reactive protein
• Protein G	• IgG immune complex
• Protamine	• IgM

Curtesy of Dr. R. Bishoff

Choice of Eluting Solvent

- Reversed Phase SPE
  - Nonpolar solvent
  - Methanol
  - Acetonitrile
  - Ethyl acetate
  - Acetone
  - Methylene chloride
- Normal Phase SPE
  - A function of the eluotropic strength

Auswahl des richtigen Elutionsmittels



Polarity	Solvent	Miscibility with water
nonpolar	Hexane	no
	Isooctane	no
	Petroleum ether	no
	Cyclohexane	no
	Carbon tetrachloride	no
	Chloroform	no
	Methylene chloride	no
	Tetrahydrofuran	yes
	Diethyl ether	no
	Ethyl acetate	poor
	Acetone	yes
	Acetonitrile	yes
	Isopropanol	yes
	Methanol	yes
	Water	yes
polar	Acetic acid	yes

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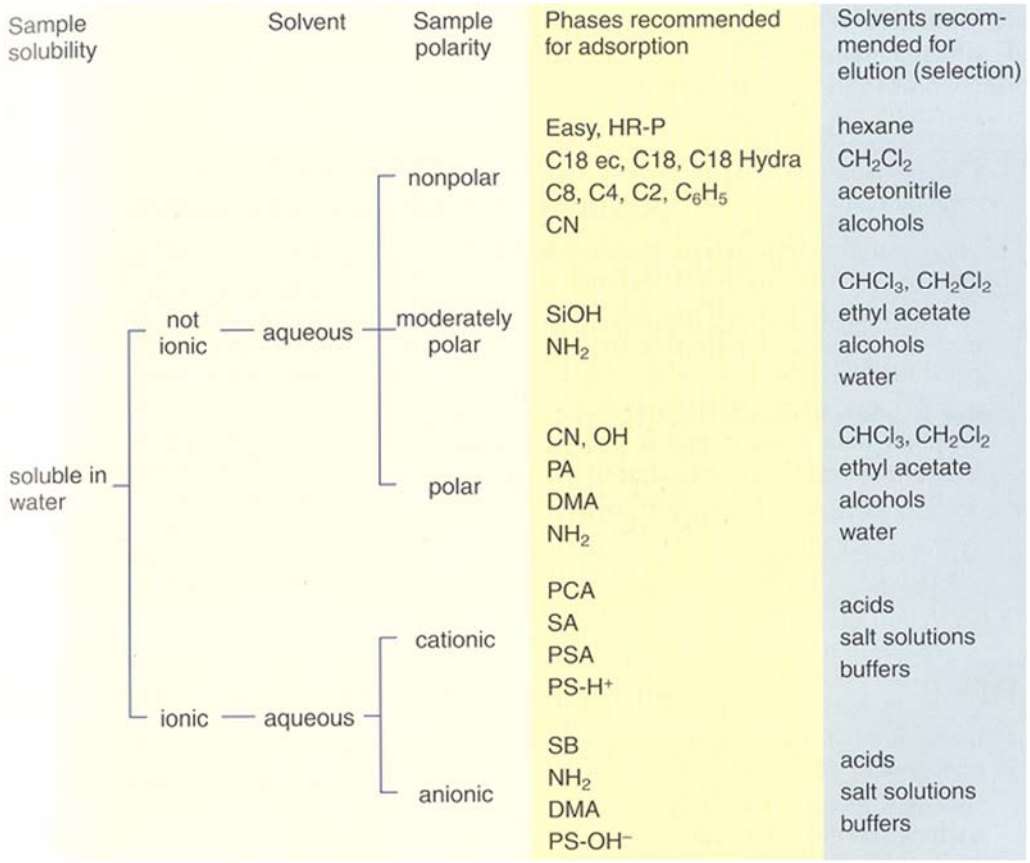
Auswahl der richtigen stationären Phase



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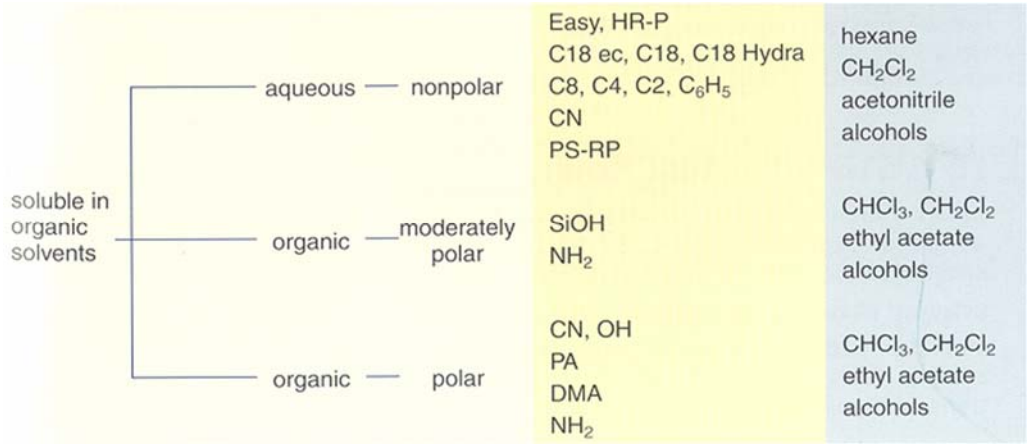
Auswahl der richtigen stationären Phase



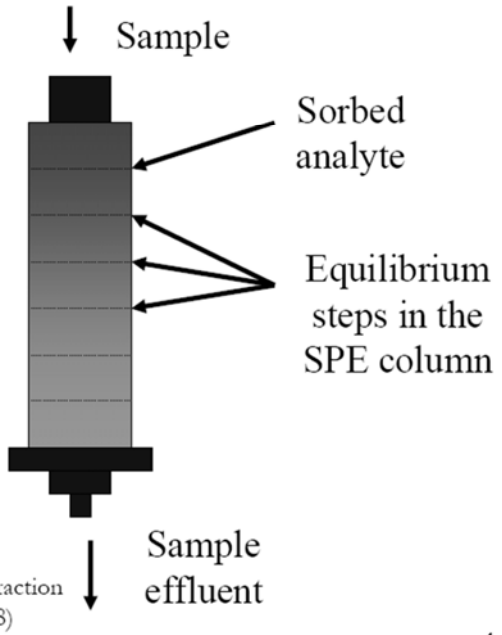
DMA - Dimethylamphetamine

Background of chromatographic methods

Technique	Parameter for separation	Based on
Gel filtration	Size/Shape	Form of the molecule
Ion exchange	Charge	Asp, Glu, Lys, Arg, His
Hydrophobic interaction	Hydrophobic sites	Trp, Phe, Ile, Leu, Tyr, Pro Met, Val, Ala
Reversed phase	Hydrophobic sites	Trp, Phe, Ile, Leu, Tyr, Pro Met, Val, Ala
Metal chelate	Affinity for metals	His, Trp, Cys
Affinity	Biological function	eg: antibody – antigen
Covalent	Covalent interaction	Uses SH groups (Cys)

Plate Theory and RP-SPE

- Series of equilibrations occur (batches), K_d is very large
- Typical SPE contains only 20 plates or less (10,000 plates for HPLC)
- Not for separation, but for simple isolation of analytes in an on/off mode



Ref.: E.M. Thurman, and M.S. Mills, "Solid-Phase Extraction Principles and Practice," John Wiley & Sons, Inc. (1998)

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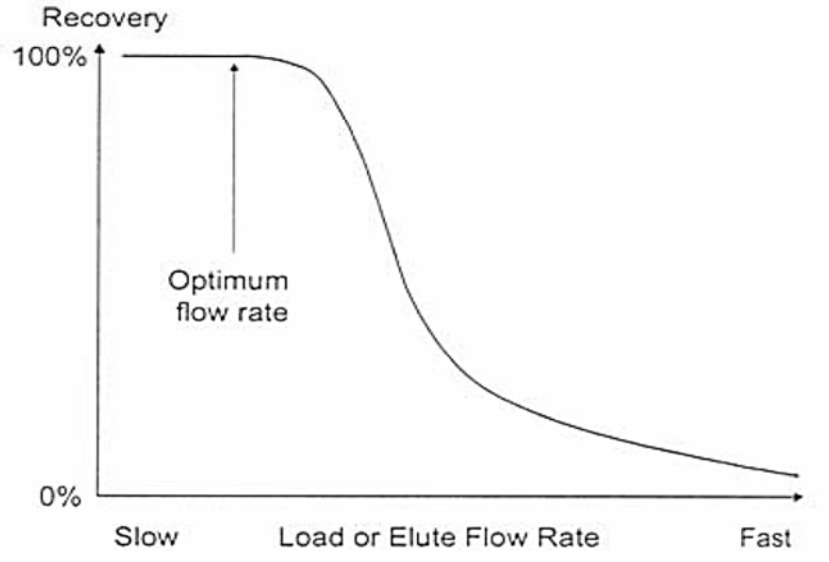
Parameters Affecting N

- Flow rate
- Particle size and plate number
- Column length
- Temperature

Relationship Between Sample Recovery and Flow Rate in SPE



Vorteile von SPE

- Einfache Handhabung
- Schnelle Extraktion
- Geringe Mengen an mobiler Phase
- Möglichkeit zur Automatisierung
- Relative billige Trennmethode

→ Limitationen

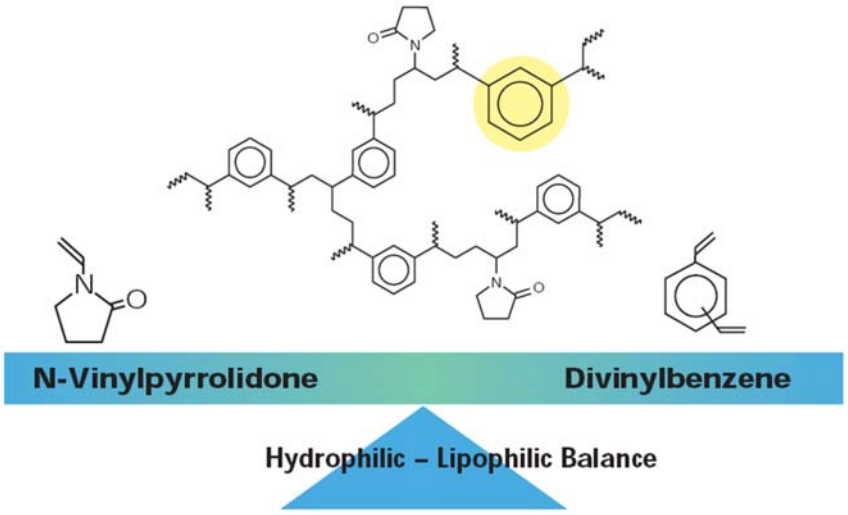
- Für extrem polare (gut wasserlösliche) Analyten eher ungeeignet
- Problem der Überladung (richte Auswahl der Dimension)
- Kopplung nur beschränkt möglich (z.B. HPLC)

Troubleshooting

- Incomplete elution
  - Increase eluting solvent, change solvent, pH adjustment, back elution
- Breakthrough of analyte
  - Increase the amount of sorbent, change the sorbent, reduce flow rate
- Interfering substances
  - Wash with solvent selectively elute the interferences, clean up the eluent with another sorbent
  - SPE bleed, wash the sorbent, change the solvent

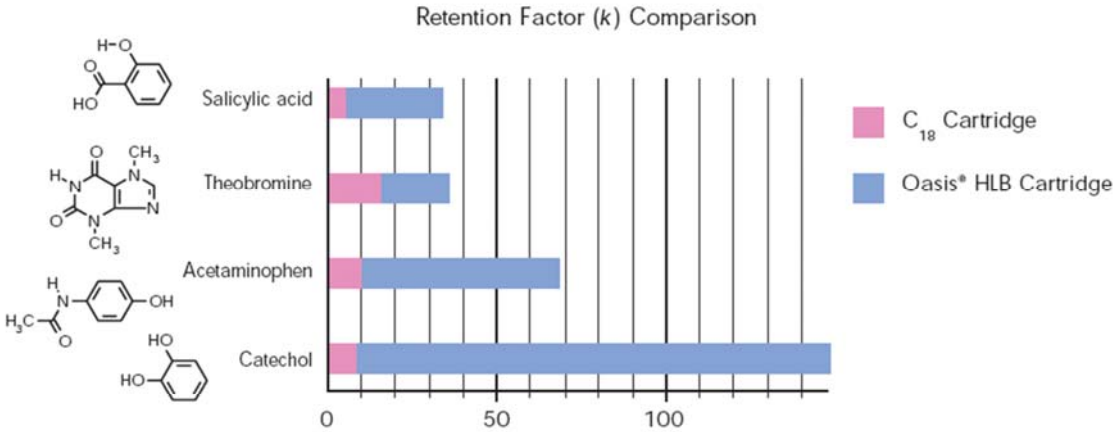
Example

Oasis Material (Waters)



Optimal Properties for Reversed-Phase SPE
Specific Surface Area: 810 m^2/g
Average Pore Diameter: 80 Å
Total Pore Volume: 1.3 cm^3/g
Average Particle Diameter: 30 µm or 60 µm

Oasis Material (Waters)
Alternative to C18



Data shown were obtained with two 3.9 mm x 150 mm columns, each packed with one of the sorbents, operated under the same conditions: mobile phase: 20 mM potassium phosphate, pH 7.0/methanol (95/5 v/v); temperature: 30 °C; flow rate: 1.0 mL/min; detection: UV @ 254 nm.

Example

IMAC

Immobilized metal-ion chromatography

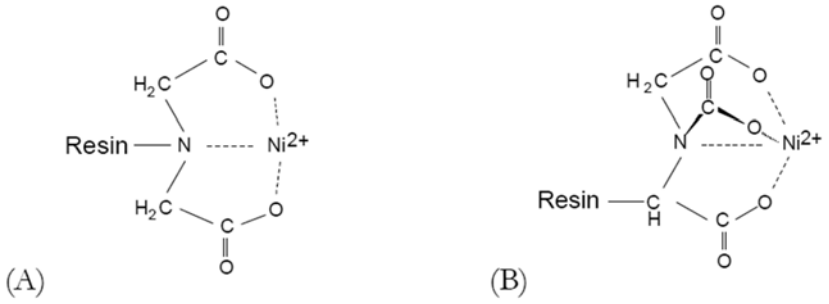
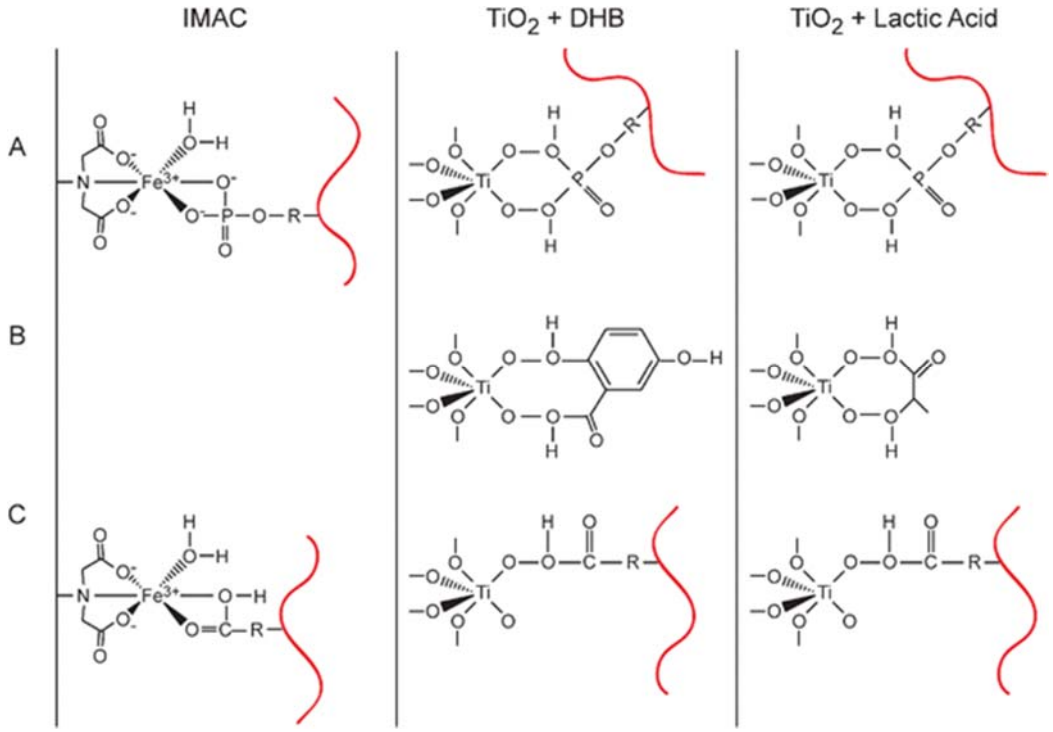


Figure 3-1: *Ni^{2+}-IMAC based on iminodiacetic acid (IDA), forming a tri-dentate complex (A) and nitrilotriacetic acid (NTA), showing a tetra-dentate metal ion complex (B).*

for specific binding of phosphopeptides!

Immobilized metal ion/Metal Chelate affinity chromatography is separation technique that is based on coordinate covalent binding between proteins and metal ions. Proteins have a wide variety of amino acids composition which, in effect, generates a range of different affinities towards metal ions. However, not many naturally occurring proteins have affinity for metal ions, so the technique is mainly used to purify recombinant proteins. For example proteins can be engineered to contain a poly-histidine tail (histidine can generally act as a ligand towards divalent metal cations). If the stationary phase is immobilized with divalent metal cations, a mixture of proteins can be separated based on their ability to interact with the metal ions. Those proteins containing a higher number of histidine residues would be able to bind to the column more tightly than those with fewer histidine residues.

Several different types of immobilized metal ion column have been developed to separate various proteins (e.g. Fe, Co, Cd, Ni, or Zn). Protein separation in IMAC generally depends on the strength of the metal ion-protein bond. Thus, choosing the type of immobilized ion is crucial to the success protein separation. By far the most widely-used technique is to use an immobilized nickel column, and to engineer poly-histidine tags of six or more residues onto the recombinant proteins of interest. One thing to keep in mind is that the binding between metal ion and protein must be reversible, allowing elution of bounded protein at later steps. Three different elution strategies can be applied to IMAC competitive elution, stripping elution and pH Adjustment.



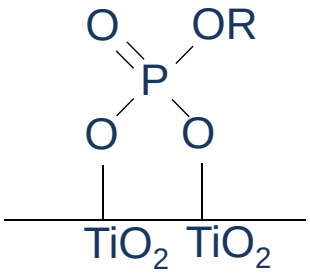
Rogers et al., Mol. BioSyst. , 2009, 5, 1122-1129

Example

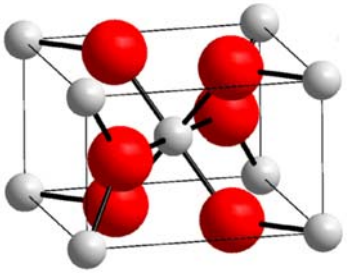
MOAC

Metal Oxide Affinity Chromatography

for specific binding of phosphopeptides!

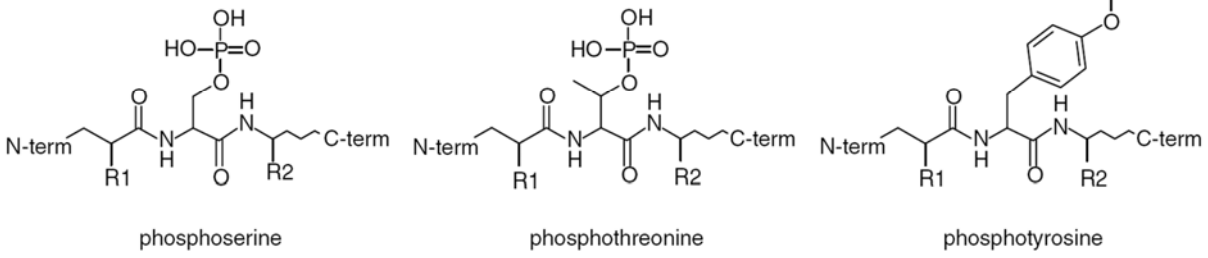


Mechanism: Bridging Bidentate



Rutil, Anatas, Brookit

Major challenges in phosphopeptide analysis



phosphoserine phosphothreonine phosphotyrosine

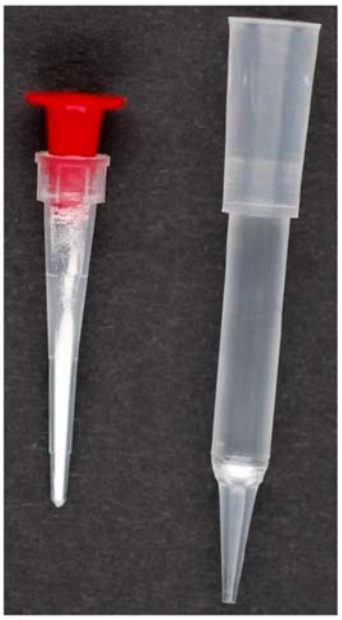
pSer : pThr : pTyr = 1800 : 200 : 1

~ 30% of all proteins in eukaryotic cells are phosphorylated

- low abundant proteins
- Phosphorylation can occur at multiple residues within a protein
- Dynamic regulation of phosphoproteins
- weak ionization in positive ionization mode
- ion suppression
- lability of the phosphoesterbond during fragmentation by CID

Titania Tips

Glygen
Bare titanium dioxide formed to 10 µm spheres.



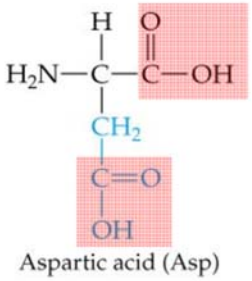
GL-Science
Monolithic solid phase, coated with titanium dioxide.

Sample treatment

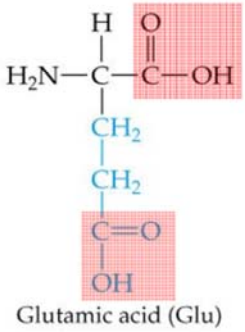
- Conditioning
- Loading
- Washing
- Eluting
- Acidification

TiO_2 loading solvent - pH

Usual loading buffer has a pH of 2.7 – 2.9 (acetic acid)

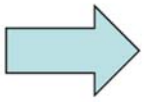


Aspartic acid (Asp)



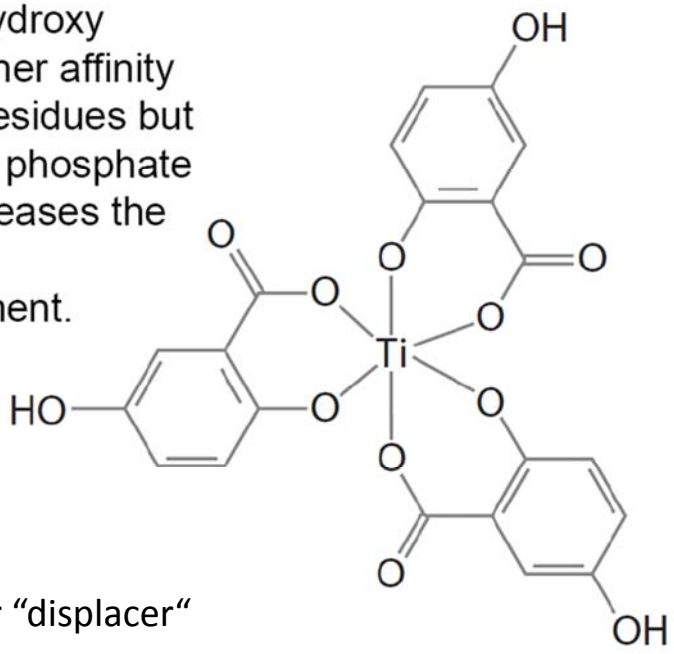
Glutamic acid (Glu)

pK_{a1} ($CH_3OPO(OH_2)$) = 1.1


Loading buffer with very low pH (TFA, HFBA)

Loading solvent containing DHB

TiO2 adsorbs DHB (Dihydroxy Benzoic Acid) with a higher affinity than acidic amino acid residues but with a lower affinity than phosphate groups, hence DHB increases the specificity of TiO2 in phosphopeptide enrichment.



DHB as “excluder“ or “displacer“

Proposed by Mann and Olsen

Sample treatment

- **Conditioning**
  - 1 x 40µL AcN 80%; 1 x 40µL loading solvent
- **Load**
  - Loading solvent: 420mM OSA, 50 mg/mL DHB, 0.1% HFBA, 20% HAc
- **Wash**
  - 1 x 40µL loading solvent; 2 x 40 µL AcN 80%/TFA 0.1%
- **Elute**
  - 1 x 40µL Ammonium dihydrogene phosphate (50 mM) adjusted to pH 10.5 with ammonium hydroxide.

Karl Mechtler et. al

Enrichment of *in vitro* phosphorylated ERK1 digest

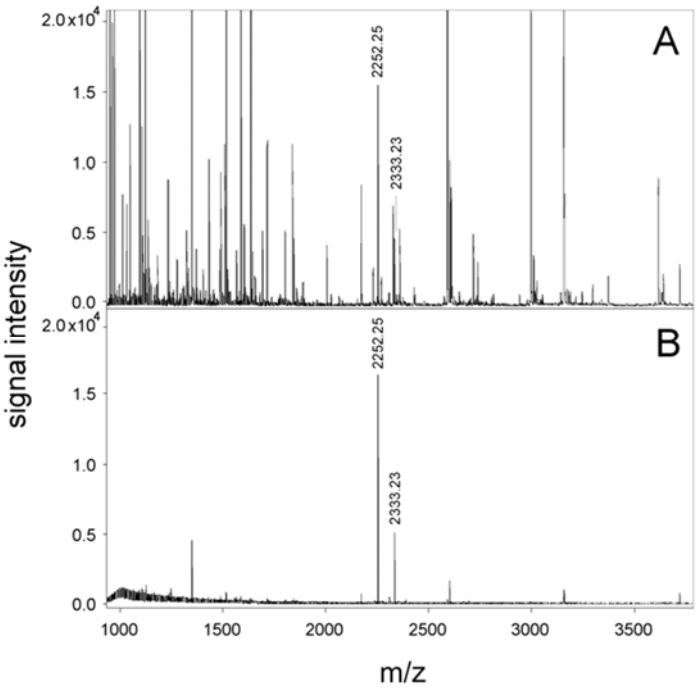
MALDI MS spectra:

1.) before enrichment (A)

2.) after enrichment with poly(DVB)-TiO_2/ZrO_2 tips (B)

Signals at *m/z* 2252.25 and *m/z* 2332.23 correspond to phosphorylated peptides

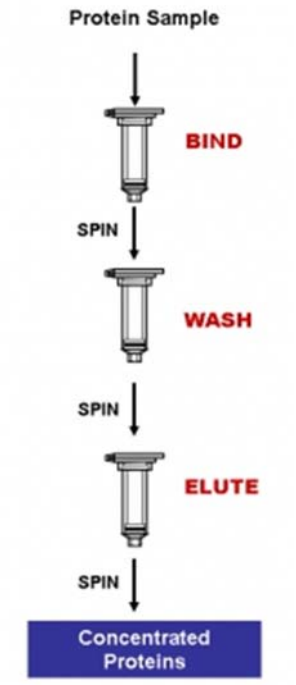
Collaboration with Prof. Lukas Huber, Biocenter - Innsbruck



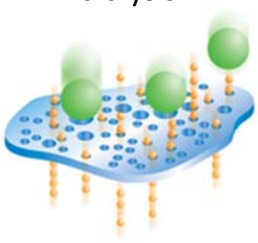
Desalting of Proteins – Why?

Spin Columns

C18-reversed phase



dialysis



Spin Columns

Extraction Tips

ZipTips (Millipore)

Zip-tip protocol:

1. Use a P10 pipette set to 10µl.
2. Wash the Zip-Tip with 0.1% trifluroacetic acid (TFA) in acetonitrile
3. Wash the Zip-Tip with 0.1% TFA in 1:1 acetonitrile:water
4. Equilibrate the Zip-Tip twice with 0.1% TFA in water
5. The sample, dissolved in 10 µl of water or 0.1% TFA water, is passed through the Zip-Tips repeatedly by pipeting in and out to bind the sample to the resin.
6. Wash the Zip-Tip three times with 0.1% TFA, 5% methanol in water
7. Elute the sample from the Zip-Tip in 2µl in 0.1% TFA 50% acetonitrile.
   * For electrospray, elute with 1% formic acid/50% methanol.

e. Ultrafiltration:

An ultra filter is a molecular sieve with extremely small pores. Most ultra filtration units are made of a sample cup with a membrane sealed to its bottom. The cup sits inside a centrifuge tube. The membranes have different molecular weight cut-off.

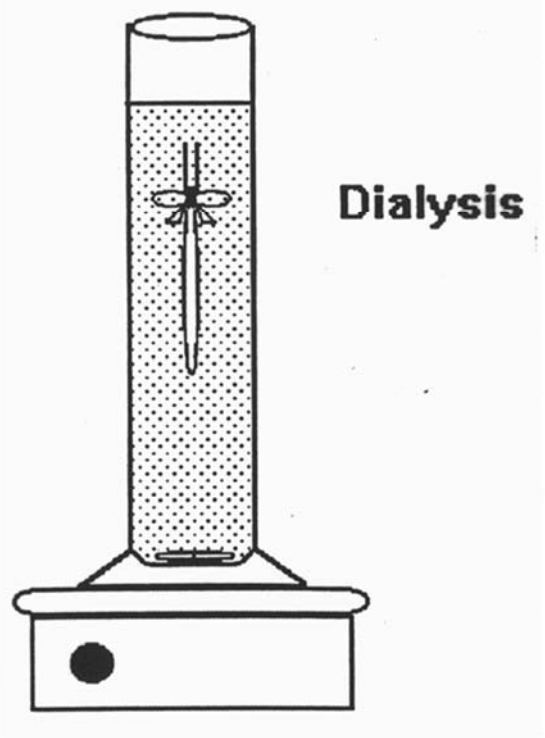
Method:

Determine what size particles are to be removed from or concentrated in a solution, and be sure that the filter is chemically compatible with any fluids that it may be exposed to.

Determine flow rate of filtration process by taking into consideration the pore size of the filter, the viscosity, volume, the particulate load of the sample, before choosing centrifugation speed and duration.

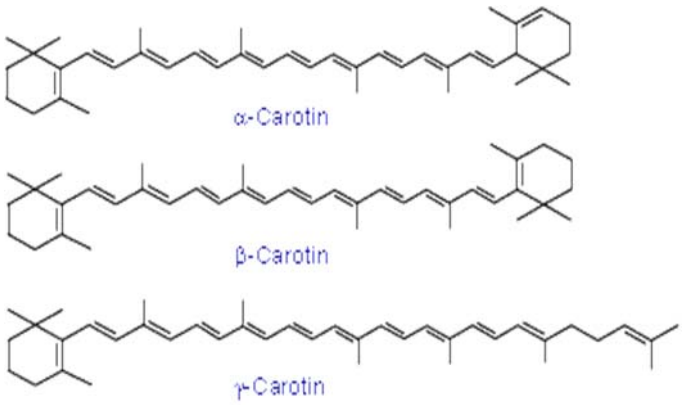
g. Dialysis:

Frequently it is necessary to remove salts or change the buffer in which a protein is dissolved. This can be achieved by dialysis. The protein solution is placed in a bag made of a semi-permeable membrane and placed in an appropriate buffer. Small molecules pass across the membrane while large ones are retained. The membrane is made of cellulose acetate with pores of 1-20 nm in diameter. The size of the pores determines the molecular weight of the molecules that will be retained.



Aufgabe 1

Analyt: Carotinoide



Welche Festphase? Warum?

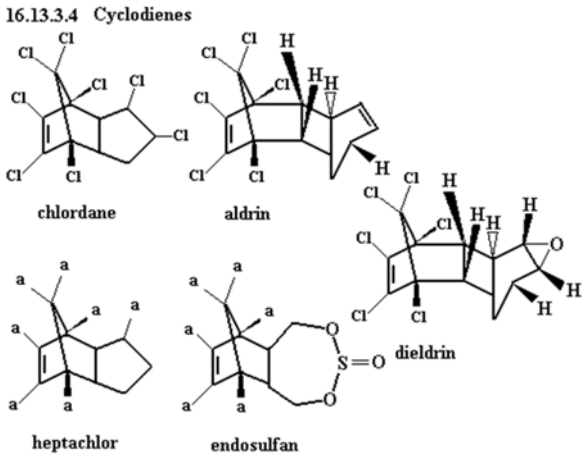
Aufgabe 2

Analyt: Proteine

Welche Festphase? Warum?

Aufgabe 3

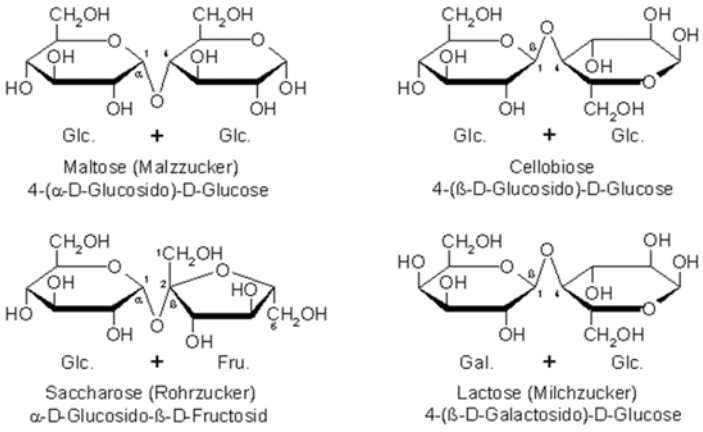
Analyt: Pestizide



Welche Festphase? Warum?

Aufgabe 4

Analyt: Disaccharide



Welche Festphase? Warum?

Table 1. Sorbents for solid phase extraction and separation mechanisms for solid phase separations.

Sorbent	Structure	Analyte type	Dissolving solvents	Elution solvents
Reversed Phase				
Octadecyl (C_{18}) Octyl (C_8) Ethyl (C_2) Cyclohexyl Phenyl	$-(CH_2)_{17}CH_3$ $-(CH_2)_7CH_3$ $-CH_2CH_3$ $-CH_2CH_2-C_6H_{12}$ $-CH_2CH_2-C_6H_6$	Nonpolar	methanol/water, acetonitrile/water	For nonpolar analytes: hexane, chloroform For polar analytes: methanol
Normal Phase (bonded)				
Cyano (CN) Amino (NH_2) Diol (COHCOH)	$-(CH_2)_3CN$ $-(CH_2)_3NH_2$ $(CH_2)_3OCH_2CHOHCH_2OH$	Slightly- moderately polar - strongly polar	hexane, chloroform	methanol
Normal Phase (adsorption)				
Kieselguhr (Diatomaceous Earth) Silica gel Florisil Alumina (neutral)	-SiOH $-SiOH$ Mg_2SiO_3 Al_2O_3	Slightly- moderately polar - strongly polar	hexane, chloroform	methanol (dependent on type of analyte)
Ion Exchangers (anion and cation Exchange)				
Amino (NH_2) 1°, 2°- Amino (NH/NH_2) Quaternary Amine (N^+) Carboxylic acid (COOH) Propyl Sulfonic Acid (SO_2OH) Aromatic Sulfonic Acid ($ArSO_2OH$)	$-(CH_2)_3NH_3^+$ $^-(CH_2)_3NH^+CH_2CH_2NH_2$ $^-(CH_2)_3N^+(CH_3)_3$ $-(CH_2)_2COO^-$ $^-(CH_2)_3SO_2O^-$ $^-(CH_2)_3-C_6H_6-SO_2O^-$	Anion exchange - Ionic Acid Cation exchange - Ionic Base	Water or buffer (pH=pKa +2) Water or buffer (pH=pKa-2)	1) Buffer (pH=pKa +2) 2) pH where sorbent or analyte is neutral 3) Solvent with high ionic strength 1) Buffer (pH=pKa-2) 2) pH where sorbent or analyte is neutral 3) Solvent with high ionic strength

Solid Phase Extraction Technique – Trends, Opportunities and Applications

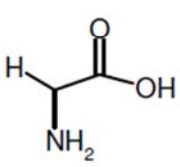
A. Żwir-Ferenc, M. Biziuk*

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

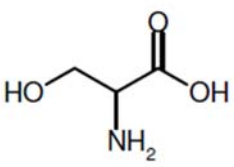
Anreicherung von Peptiden und Proteinen

Proteinogene Aminosäuren I

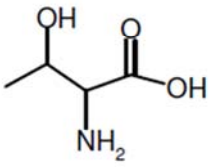
Polare α-Aminosäuren



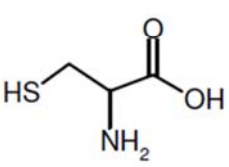
Glycin
Gly, G



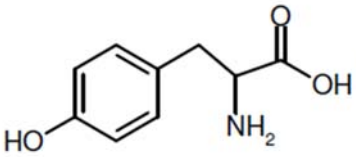
Serin
Ser, S



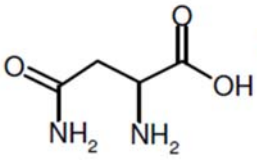
Threonin
Thr, T



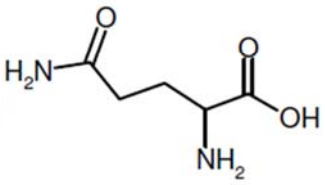
Cystein
Cys, C



Tyrosin
Tyr, Y

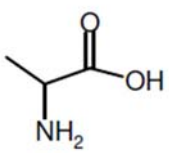


Asparagin
Asn, N

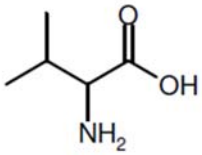


Glutamin
Gln, Q

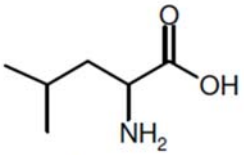
Hydrophobe α-Aminosäuren



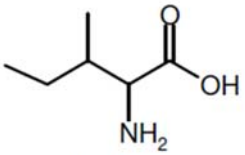
Alanin
Ala, A



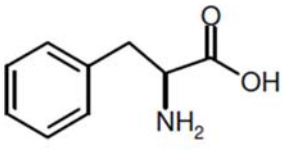
Valin
Val, V



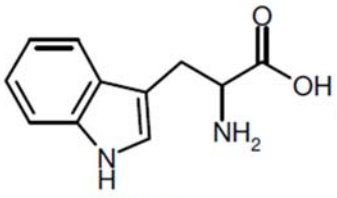
Leucin
Leu, L



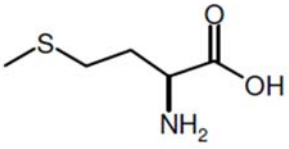
Isoleucin
Ile, I



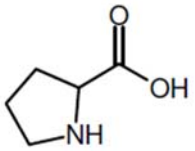
Phenylalanin
Phe, F



Tryptophan
Trp, W



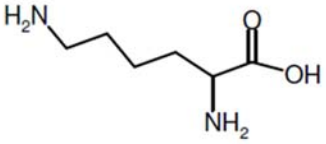
Methionin
Met, M



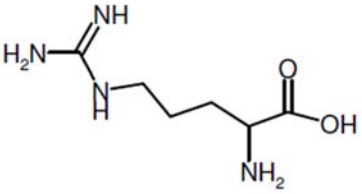
Prolin
Pro, P

(Iminosäure)

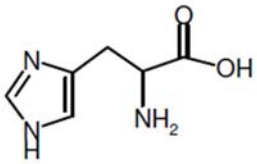
Basische α-Aminosäuren



Lysin
Lys, K

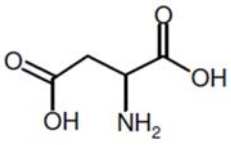


Arginin
Arg, R

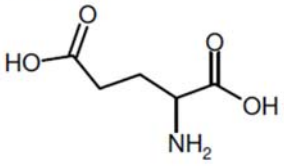


Histidin
His, H

Saure α-Aminosäuren



Asparaginsäure
Asp, D



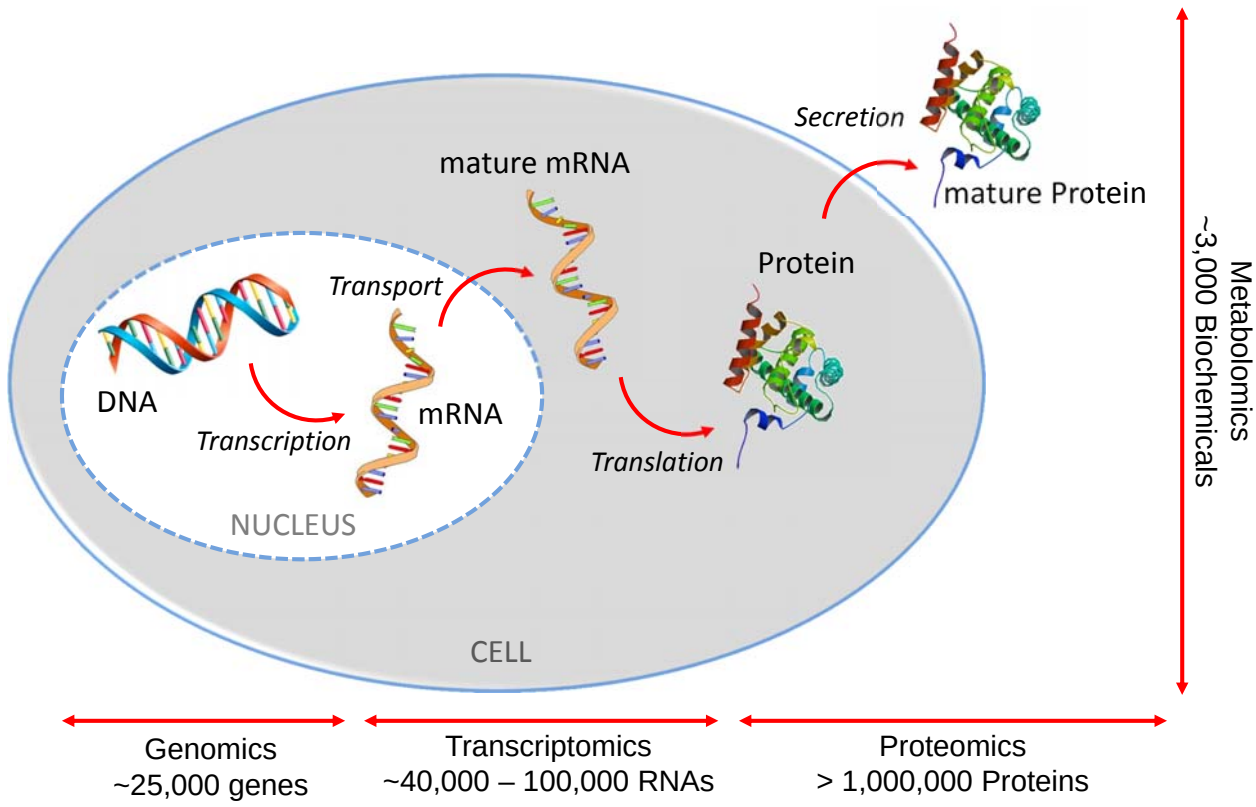
Glutaminsäure
Glu, E

Warum das Proteom untersuchen ?

Das Genom sagt, was potentiell in einer Zelle passieren könnte,
das Proteom sagt uns, was tatsächlich passiert.

Ein Genom – zwei Proteome

Proteom = Gesamtheit aller Proteine



Proteintrennung - Herausforderungen

Proteine sind sehr vielfältig bezüglich ihrer Eigenschaften wie
Größe, Ladung, Polarität, Struktur usw.

Proteinpool

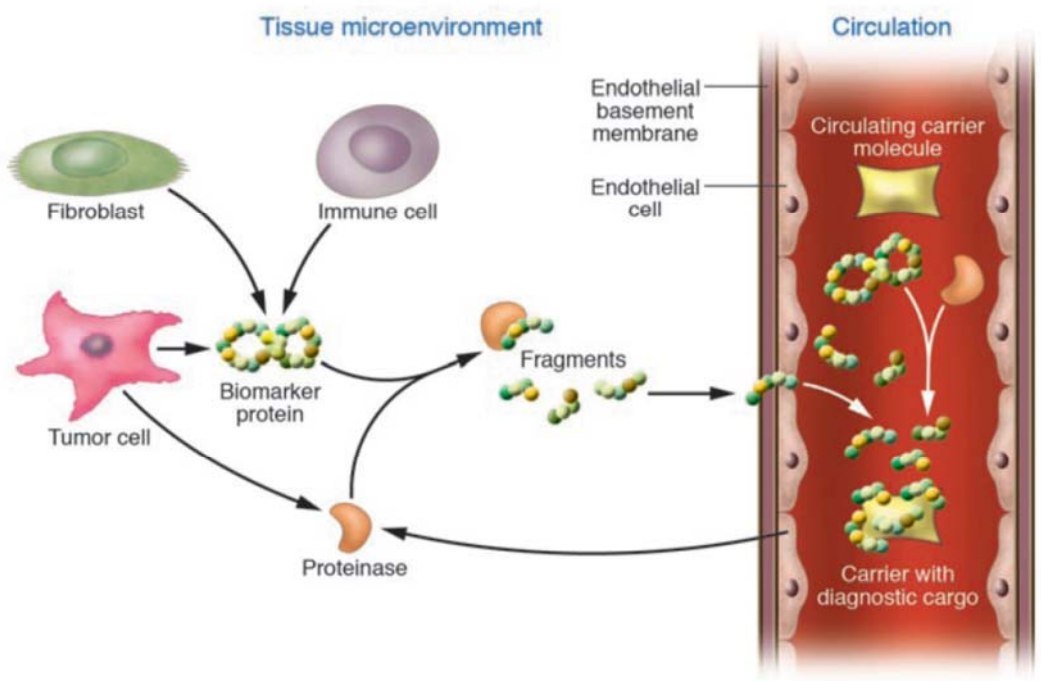
Theorie: Proteinlänge: 100 Reste: 20^{100} mögliche Proteine!

Humangenom

	ca. 25.000 Gene	
Pro Zelle aktiviert:	ca. 10.000 Gene	☛ 10.000 Proteine
Splicing:	x5	☛ 50.000 Proteine
Modifikationen:	x10-20	☛ 1 Mio. Proteine (?)
Gewebe:	Mehrere Zelltypen	☛ X Mio. Proteine

Konzentration: sehr unterschiedlich (Differenz bis Faktor 10^{12})

Bioanalysis - Serum - Biomarker



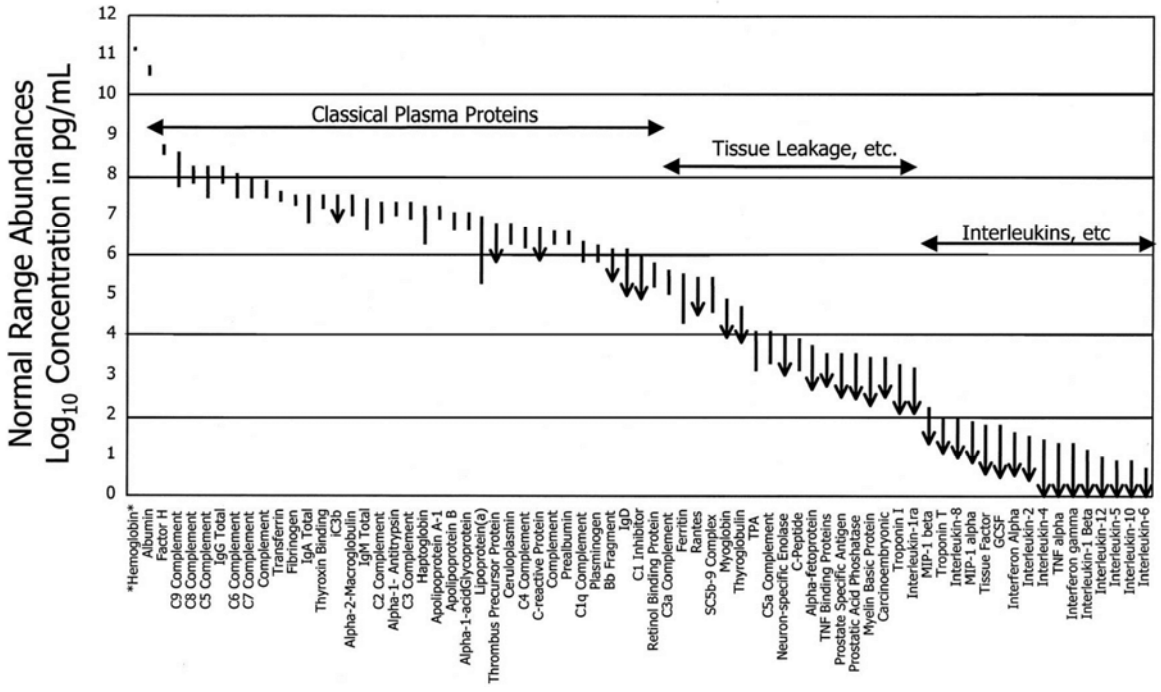
Proteomics contributes to our understanding of health and disease

- Up- and/or downregulation of protein abundance
- Protein-protein interactions
- Post-translational modifications
- Leakage of proteins from diseased cells or tissues

→ Biomarker discovery

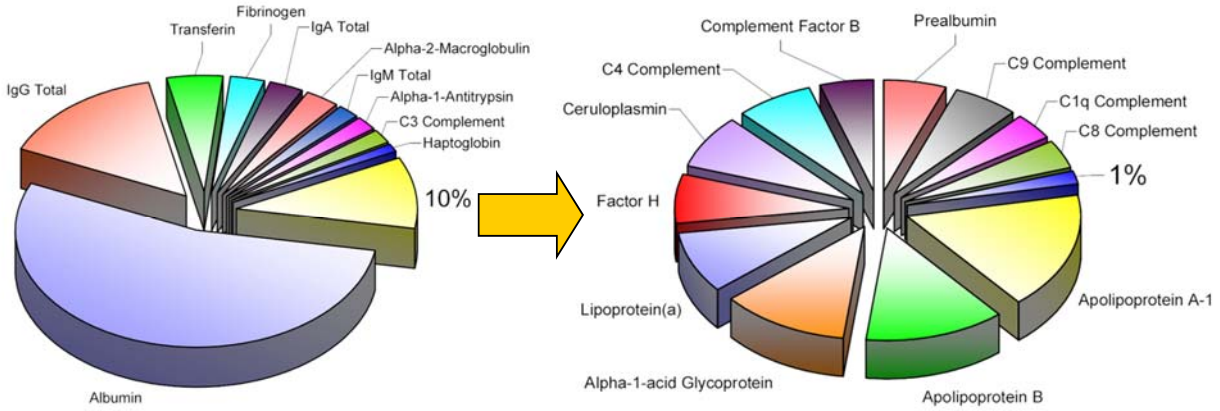
Protein abundance in human plasma

Dynamic Range



Anderson, N. L. (2002) Mol. Cell. Proteomics 1: 845-867

Bioanalysis - Complexity of Human Serum



90% 10%

22 proteins are approx. 99% of the whole serum proteome

Proteine

Größe

Peptide: von 200 - ca. 10.000 g/mol (2 - 100 AS)

Proteine: ca. 10.000 g/mol bis >500.000 g/mol (100 - 5000 AS)

Mittleres Molekulargewicht eines Aminosäurerests

ca. 110 g/mol (100mer ≈ 11.000 g/mol = 11 kDa)

Saure Proteine

hoher Anteil an Asparagin- und Glutaminsäure (pI < 4)

Basische Proteine

hoher Anteil an Lysin, Arginin und Histidin (pI > 10)

"Typische" Proteine

Molekulargewicht: 25-40 kDa und pI: 4-9

ca. 80% aller (humanen) Proteine

Proteinstruktur

- Primärstruktur (Sequenz)
- Sekundärstruktur (α-Helix, β-Faltblatt, Turn, usw.)
- Tertiärstruktur (räumliche Anordnung aller Atome, Raumstruktur)
- Quartärstruktur (Komplex mehrerer Proteine)

Viele Proteine haben eine stabile Tertiärstruktur (z.B. globulär)

Einige Proteine sind flexibel (ungeordnet)

Proteinketten oft über Disulfidbrücken verknüpft

intra- oder intermolekular

gegebenenfalls reduzierten (Thiole) und alkylieren

Proteinstruktur

nativ

korrekte Struktur des Proteins (aus natürlicher Umgebung) bleibt erhalten (biologisch aktive Form)

Denaturierung

Struktur des Proteins (Tertiär- und Quartärstruktur) wird "zerstört" ☛ ungefaltet oder partiell entfaltet

Renaturierung (Rückfaltung)

Protein nimmt wieder seine native Struktur an

Fällung von Proteinen

Das Verhalten von Proteinen in wässriger Lösung hängt von der Art des Lösungsmittels ab, denn in Gegenwart von Salzen, Säuren oder Laugen ändert sich das Löslichkeitsverhalten der Proteine stark. Viele Methoden zur Trennung von Proteinen machen sich diese Eigenschaft zunutze, so z.B. bei der Fällung von Proteinen aus einer Lösung.

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:

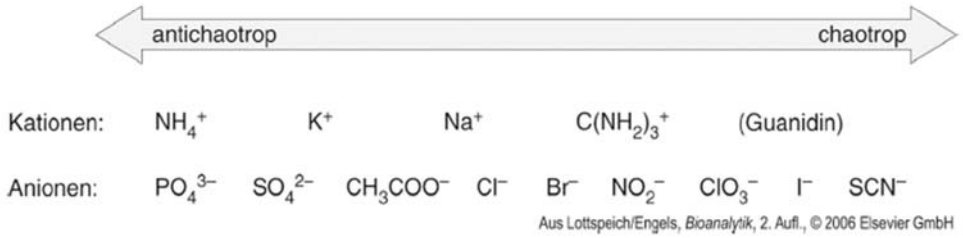
- Fällung durch Aussalzen

Das Aussalzen von Proteinen ist ein Spezialfall einer Fällungsreaktion, die als Trennverfahren bei der Reinigung von Proteinen eingesetzt wird. Dabei nutzt man aus, dass Proteine nur dann in Lösung vorliegen, wenn sie über eine ausreichende Hydrathülle aus Wassermolekülen verfügen. Werden der Lösung Salze zugesetzt, so binden die dissoziierten Ionen ihrerseits Wassermoleküle in ihrer Hydrathülle und entziehen diese den Proteinmolekülen. Ab einer bestimmten Salzkonzentration, die von der Art des Salzes und dem Protein abhängt, wird dieser Effekt so stark, dass das Protein nicht mehr in Lösung gehalten werden kann, d.h. es fällt aus der Lösung aus und bildet einen Niederschlag.

Fällung von Proteinen

Hofmeister-Reihe

- antichaotrope Salze besonders gute und schonende Fällungsmittel
- chaotrope Salze: halten Proteine in Lösung



Aus Lottspeich/Engels, *Bioanalytik*, 2. Aufl., © 2006 Elsevier GmbH

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 isoelektischen 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*

Precipitation of Proteins

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.

Precipitation of Proteins

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.

Precipitation of Proteins

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 H_2O
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

Reference: Wessel, D. and Flugge, U. I. Anal. Biochem. (1984) 138, 141-143

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*[S]

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

Co-precipitation of phosphorylated peptides by calcium ions

Idea!

→ calcium phosphate precipitation and co-precipitation
of phosphorylated peptides/proteins with calcium phosphate.

$$3Ca^{2+} + 2PO_4^{3-} \longrightarrow Ca_3(PO_4)_2\downarrow$$

$$Ca^{2+} + (R) - O - PO_3^{2-} \longrightarrow (R) - O - PO_3Ca$$

$$(3m+n)\ Ca^{2+} + 2mPO_4^{3-} + n(R) - O - PO_3^{2-} \longrightarrow [Ca_3(PO_4)_2]_m \bullet [(R) - O - PO_3Ca]_n\downarrow$$

(R)… peptide residue

Ca^{2+}… calcium ions

*X. Zhang, J. Ye, O. N. Jensen, P. Roepstorff. Highly efficient phosphopeptide enrichment by calcium phosphate precipitation combined with subsequent IMAC enrichment.Molecular&Cellular Proteomics **2007**, 6, 2032.*

research articles Journal of proteome research

Phosphoproteomic Analysis of Human Brain by Calcium Phosphate Precipitation and Mass Spectrometry

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Received January 21, 2008

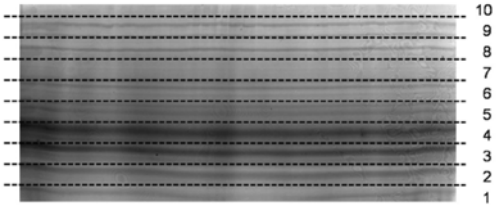
Alzheimer's disease (AD), the most common form of dementia, is manifested in the brain by the aggregation of amyloid plaques and neurofibrillary tangles. The tangles are primarily composed of microtubule-associated protein tau that is aberrantly hyperphosphorylated, suggesting that deregulated phosphorylation 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)

↓

C_{18} desalting

↓

LC-MS/MS (only accepting spectra with neutral loss)
551 phosphopeptides
466 phosphorylation sites

research articles Journal of proteome research

Motif-Specific Sampling of Phosphoproteomes

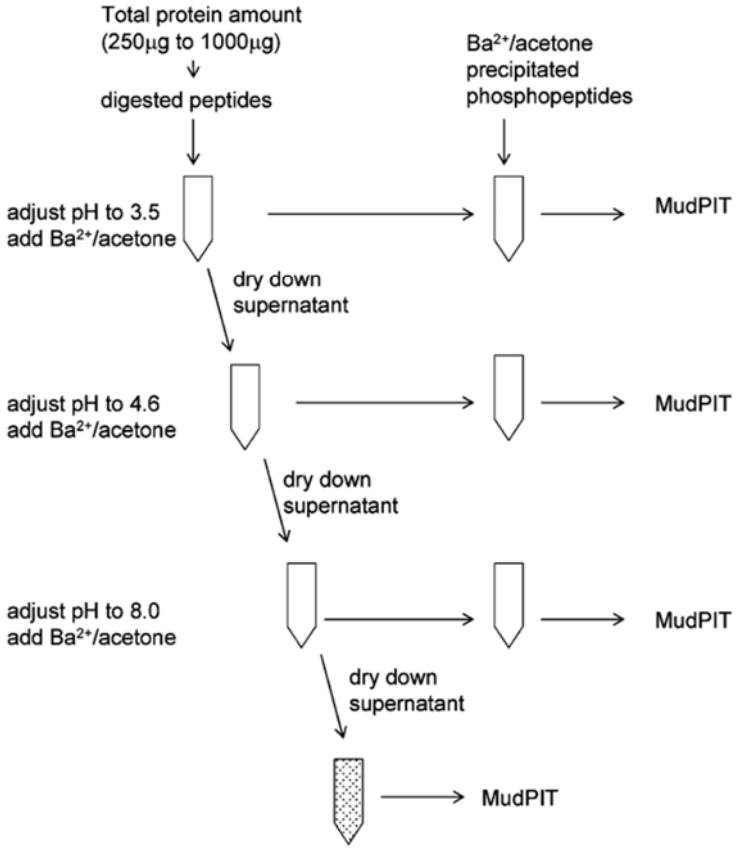
Cristian I. Ruse, Daniel B. McClatchy, Bingwen Lu, Daniel Cociorva, Akira Motoyama, Sung Kyu Park, and John R. Yates III*

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



Motif-Specific Sampling of Phosphoproteomes

Ba^{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^{2+}] Sampling Conditions

[Ba^{2+}] (μmol)	pH 3.5	pH 4.6	pH 8.0	total[a]
7.5	1644	801	153	2723
6	872	821	177	1868
1	1217	416	295	2215

[a] It includes MS3 identified phosphopeptides.

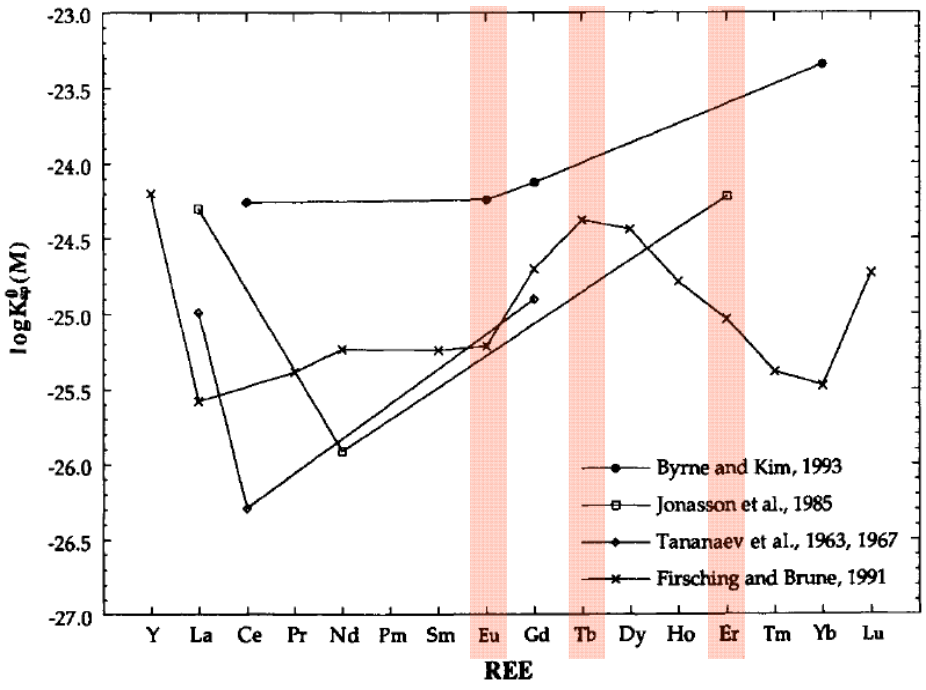
Ruse CI, McClatchy DB, Lu B, Cociorva D, Motoyama A, et al. 2008. Motif-specific sampling of phosphoproteomes. J. Proteome Res. 7:2140–50

Precipitation of Phosphoproteins by Trivalent Lanthanide Ions

A Top-Down Approach

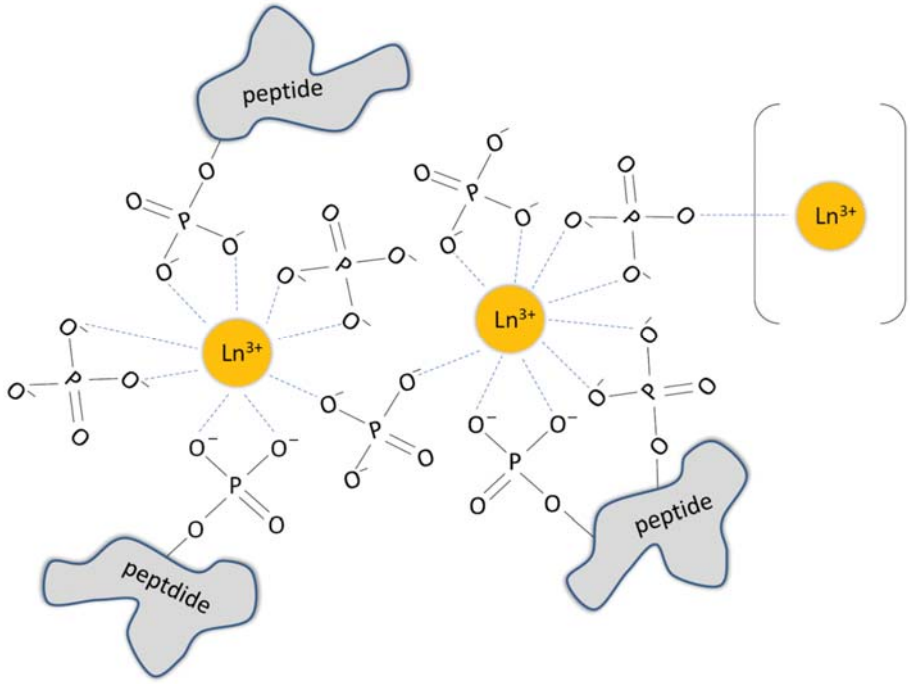
Lanthanide Phoshates

(Solubility Products)

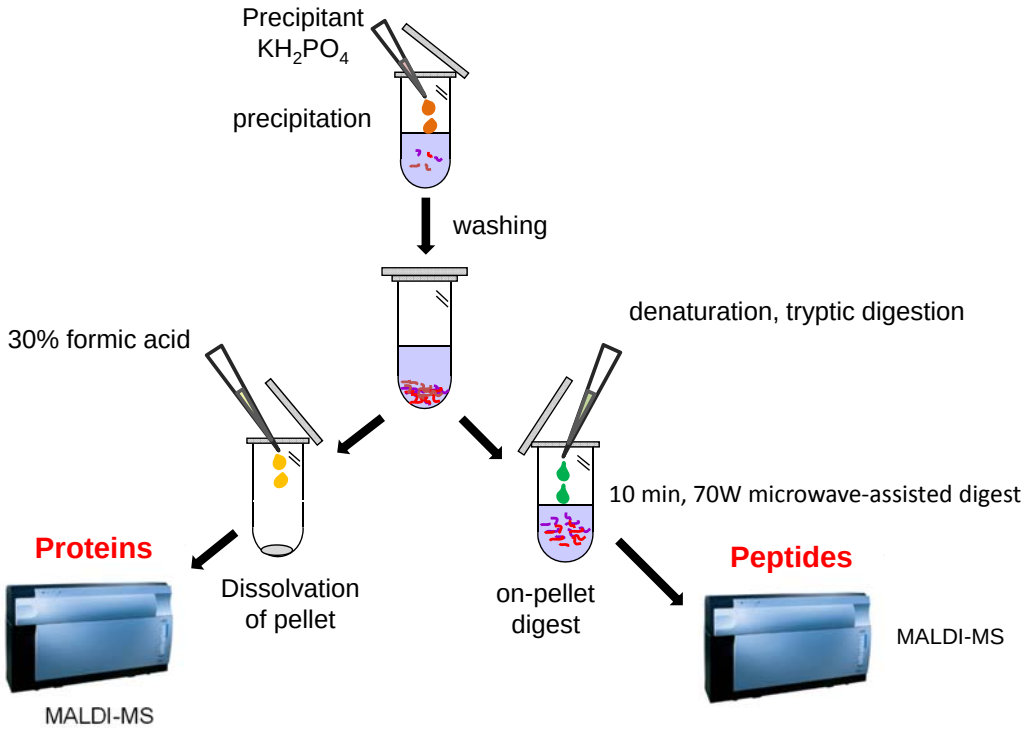


Li, X. et al. Geochimica et cosmochimica acta,1997.61(8):p.1625-1633

Proposed Precipitation Mechanism

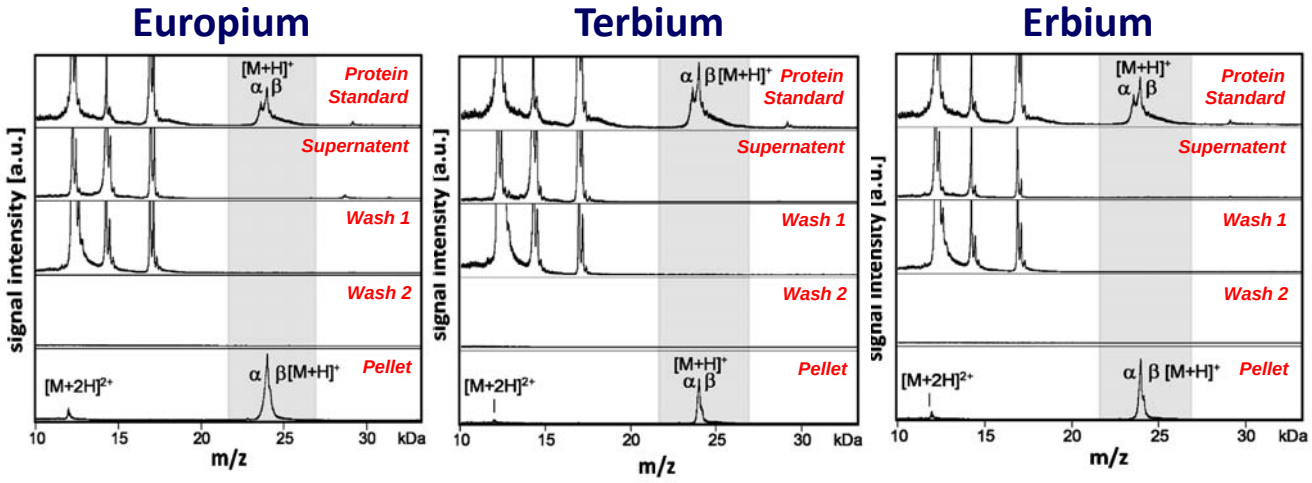


Scheme for Precipitation of Phosphoproteins by Trivalent Europium-, Terbium- and Erbium- Ions



Yüksel et al. Anal Bioanal Chem (2012) 403:1323–1331

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



Standardproteins

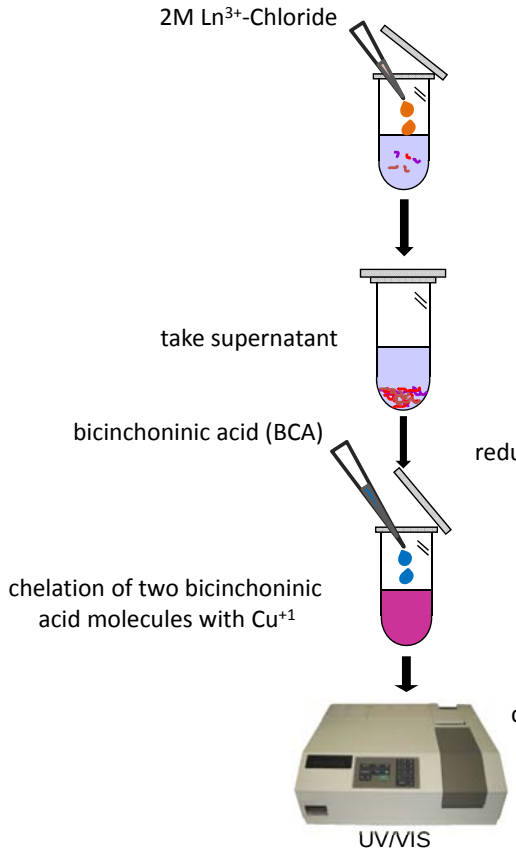
cytochrom c (m/z ~11.7 kDa)
lysozyme (m/z ~14.4 kDa)
myoglobin (m/z ~17 kDa)
BSA (m/z ~ 69 kDa)

Phosphoproteins

α_{S1}-casein (m/z ~24.5 kDa)
β-casein (m/z ~25.1 kDa)

Yüksel et al. Anal Bioanal Chem (2012) 403:1323–1331

Recovery Study of Phosphoprotein

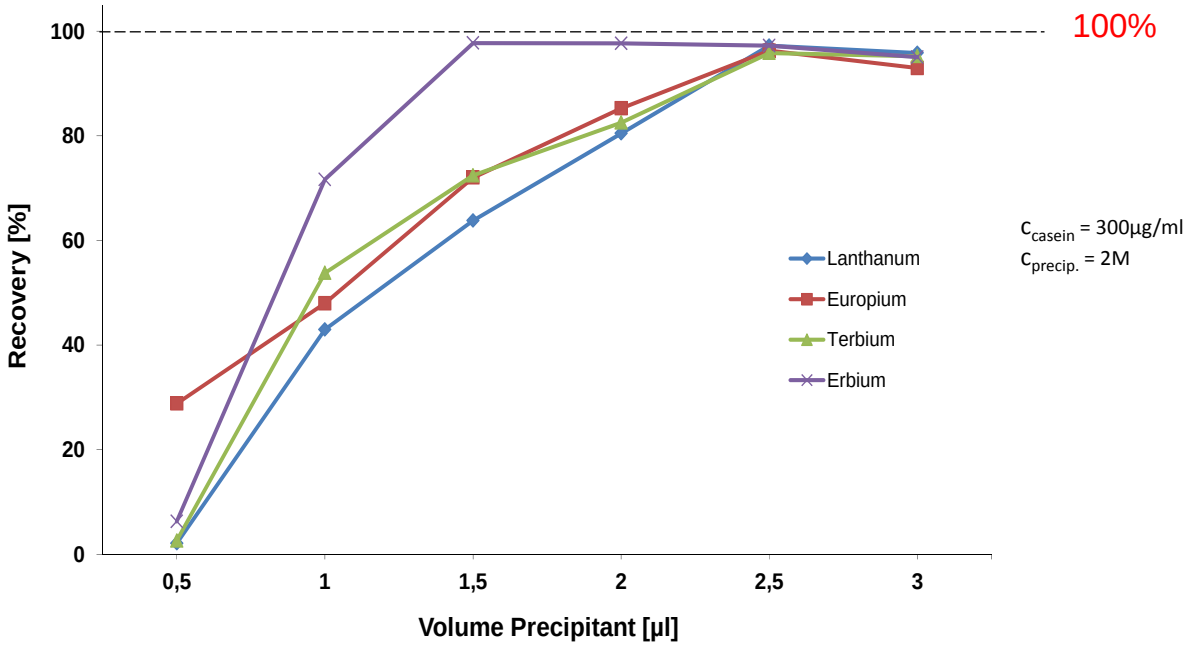


reduction of Cu^{+2} to Cu^{+1} in the presence of proteins (alkaline conditions)

colorimetric detection 562 nm

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

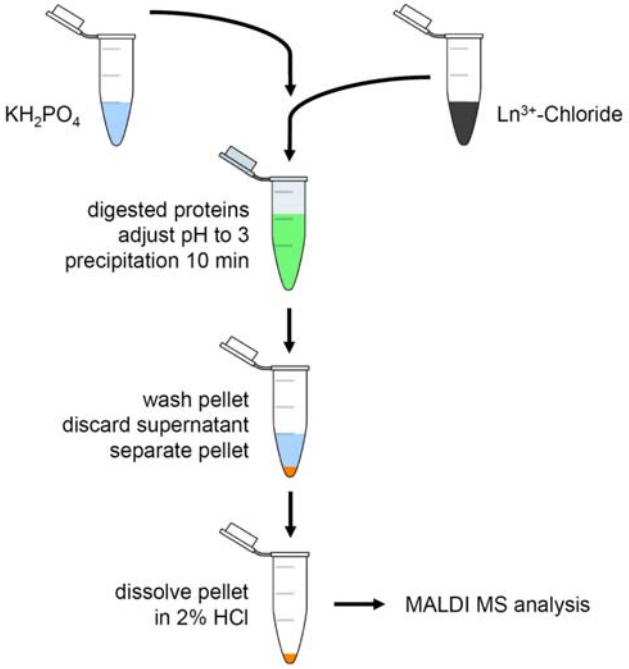
Recovery Study



Precipitation of Phosphopeptides by Trivalent Lanthanide Ions

A Bottum-Up Approach

A Novel Strategy for Phosphopeptide Enrichment using Lanthanide Phosphate Precipitation



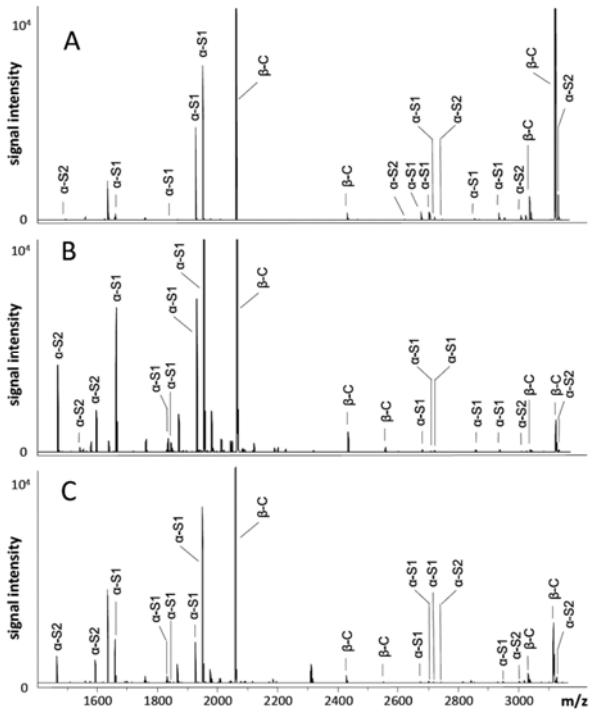
Workflow for the precipitation of phosphorylated peptides

A Novel Strategy for Phosphopeptide Enrichment using Lanthanide Phosphate Precipitation

Erbium

Holmium

Cer



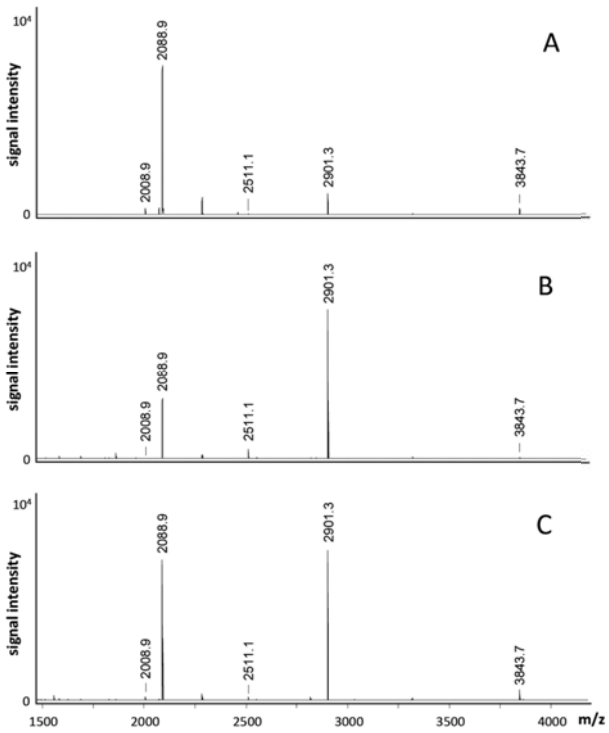
MALDI mass spectra taken from digested milk peptides after precipitation with trivalent lanthanide ions. A, phosphopeptide enriched by precipitation with Er^{3+}. B, phosphopeptide enriched by precipitation using Ho^{3+}. C, phosphopeptide enriched by precipitation using Ce^{3+}. α-S1 and β-S2 refers 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

Erbium

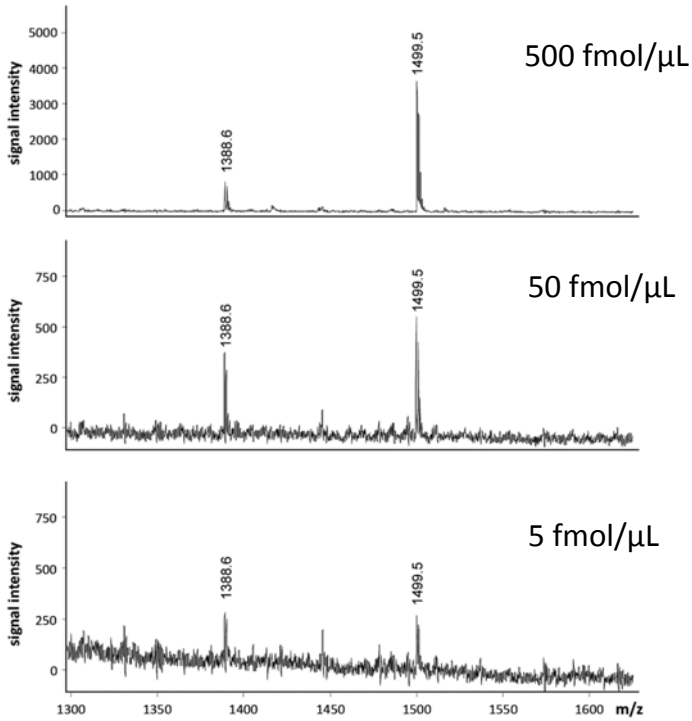
Holmium

Cer



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

$[M+H]^+$ Da	Phosphopeptide Sequences[a]	Phospho-groups	$ErCl_3$	$HoCl_3$	$CeCl_3$	$LaCl_3$	$EuCl_3$	$TmCl_3$	$TbCl_3$	TiO_2
1254.52	EVVGSpAEAGVDAA (Ov-(340–352))	Mono	-	-	-	-	-	-	-	-
1331.53	EQLSpTSpEENSK (α-S2-(141–151))	Mono	-	-	-	-	-	-	-	-
1411.50	EQLSpTSpEENSK (α-S2-(141–151))	Di	-	-	-	-	-	-	-	-
1466.61	TVDMESpTEVFTK (α-S2-(153–164))	Mono	+	+	+	+	+	+	+	+
1594.70	TVDMESpTEVFTKK (α-S2-(153–165))	Mono	+	+	+	+	+	+	+	+
1660.79	VPQLEIVPNSpAEER α(-S1-(121–134))	Mono	+	+	+	+	+	+	+	+
1832.83	YLGEYLIVPNSpAEER (α-S1)	Mono	+	+	+	+	+	+	+	+
1847.69	DIGSESpTEDQAMEDIK (α-S1-(58–73))	Mono	-	+	+	-	+	+	-	-
1927.69	DIGSESpTEDQAMEDIK (α-S1-(58–73))	Di	+	+	+	+	+	+	+	+
1951.95	YKVPQLEIVPNSpAEER (α-S1-(119–134))	Mono	+	+	+	+	+	+	+	+
2061.83	FQSpEEQQQTEDELQDK (β-C-(33–48))	Mono	+	+	+	+	+	+	+	+
2088.89	EVVGSpAEAGVDAASVSEEFR (Ov-(340–359))	Mono	+	+	+	+	+	+	+	+
2432.05	IEKFQSpEEQQQTEDELQDK (β-C-(33–48))	Mono	+	+	+	+	+	+	-	-
2511.13	LPGFGDSpIEAQCGTSVNVHSSLR (Ov-(62–84))	Mono	-	-	-	-	-	-	-	-
2556.10	FQSpEEQQQTEDELQDKIHPF (β-C-(48-67))	Mono	+	+	+	+	+	+	-	+
2619.04	NTMEHVSpSpSpEESpIISQETYK (α-S2-(17–36))	Tetra	+	+	+	+	+	-	-	-
2678.01	VNELSpKDIGSpESpTEDQAMEDIK (α-S1-(52–73))	Tri	+	-	-	-	-	-	-	+
2703.50	LRLKKYKVPQLEIVPNSpAEERL(α-S1-(114–135))	Mono	+	+	+	+	+	-	+	+
2720.91	QMEAESpISpSpSpEEIVPNSVEAQK (α-S1-(74–94))	Penta	+	-	-	+	+	-	+	+
2747.10	NTMEHVSpSpSpEESpIISQETYKQ (α-S2-(17–37))	Tetra	+	-	-	-	-	-	-	-
2856.50	EKVNELSpKDIGSpESTEDQAMEDIK (α-S1-(50–73))	Di	+	+	+	-	+	-	-	+
2901.32	FDKLPGFGDSpIEAQCGTSVNVHSSLR (Ov-(59–84))	Mono	+	-	+	+	-	+	-	-
2935.15	EKVNELSpKDIGSpESpTEDQAMEDIK (α-S1-(50–73))	Tri	+	+	-	+	+	-	-	+
2966.16	ELEELNVPGEIVESpLSpSpSpEESITR (β-C-(17–40))	Tetra	-	-	+	-	-	-	-	-
3008.01	NANEEEYSIGSpSpSpEESpAEVATEEVK (α-S2-(61–85))	Tetra	+	+	+	+	+	-	+	+
3042.27	RELEELNVPGEIVESLSpSpSpEESITR (β-C-(16–40))	Tetra	+	+	+	+	+	-	+	-
3087.99	NANEEEYSIGSpSpSpEESpAEVATEEVK (α-S2-(61–85))	Penta	+	+	-	+	+	-	-	+
3122.27	RELEELNVPGEIVESpLSpSpSpEESITR (β-C-(16–40))	Tetra	+	+	+	+	+	-	+	+
3132.20	KNTMEHVSpSpSpEESpIISQETYKQEK (α-S2-(16–39))	Tetra	+	+	+	+	+	-	+	+
			23	20	19	20	21	12	14	18