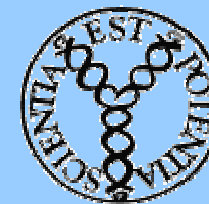




**Oddělení funkční genomiky a proteomiky**  
Přírodovědecká fakulta Masarykovy university



Charakterizace proteinů hmotnostní spektrometrií  
Bi 7050

# **PŘÍPRAVA PROTEINOVÉHO VZORKU PRO MS ANALÝZU**

**Hana Konečná**

Centrální laboratoř

# PROTEOM

---

- komplexita       $10^6$  ?
- dynamika
- sekvence
- struktura
- abundance
- lokalizace
- modifikace
- interakce
- biochemická funkce

**geny** - nositelé instrukce

**proteiny** - vykonavatelé instrukce

## STRATEGIE SEPARACE

---

- fyzikální rozdíly
- chemické rozdíly

počet AA

typ AA

PTM

sekvence

*lokalizace v buňce, solubilita, velikost, náboj, pI*

- vysoké rozlišení
- jednoduché směsi proteinů
- vysoká kapacita
- automatizace

*centrifugace, postupná extrakce, chromatografie, elektroforéza*

# ALTERNATIVY SEPARACE

- 2D gelová elektroforéza

IEF + SDS

- 1D gelová elektroforéza

SDS PAGE

BN PAGE...

- multidimenzionální LC

MudPIT

ICAT...

- proteinové čipy

Functional protein arrays (protein proteinové interakce)

Affinity arrays

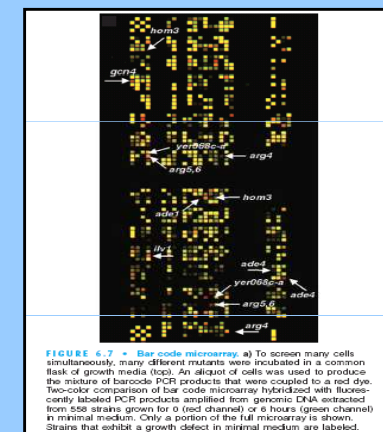
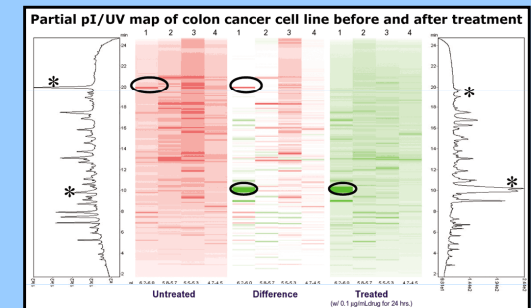
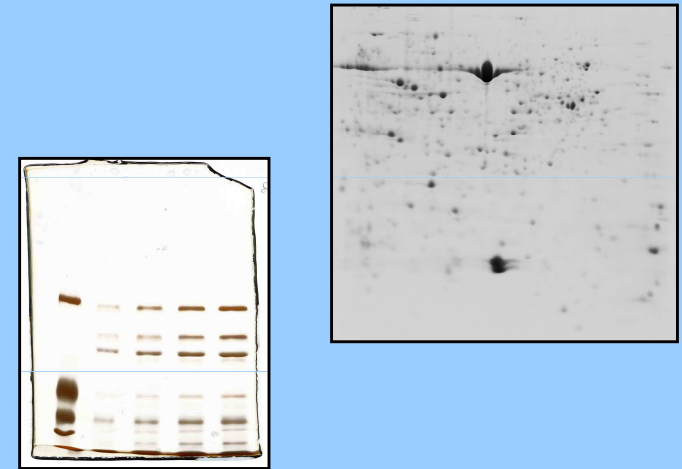
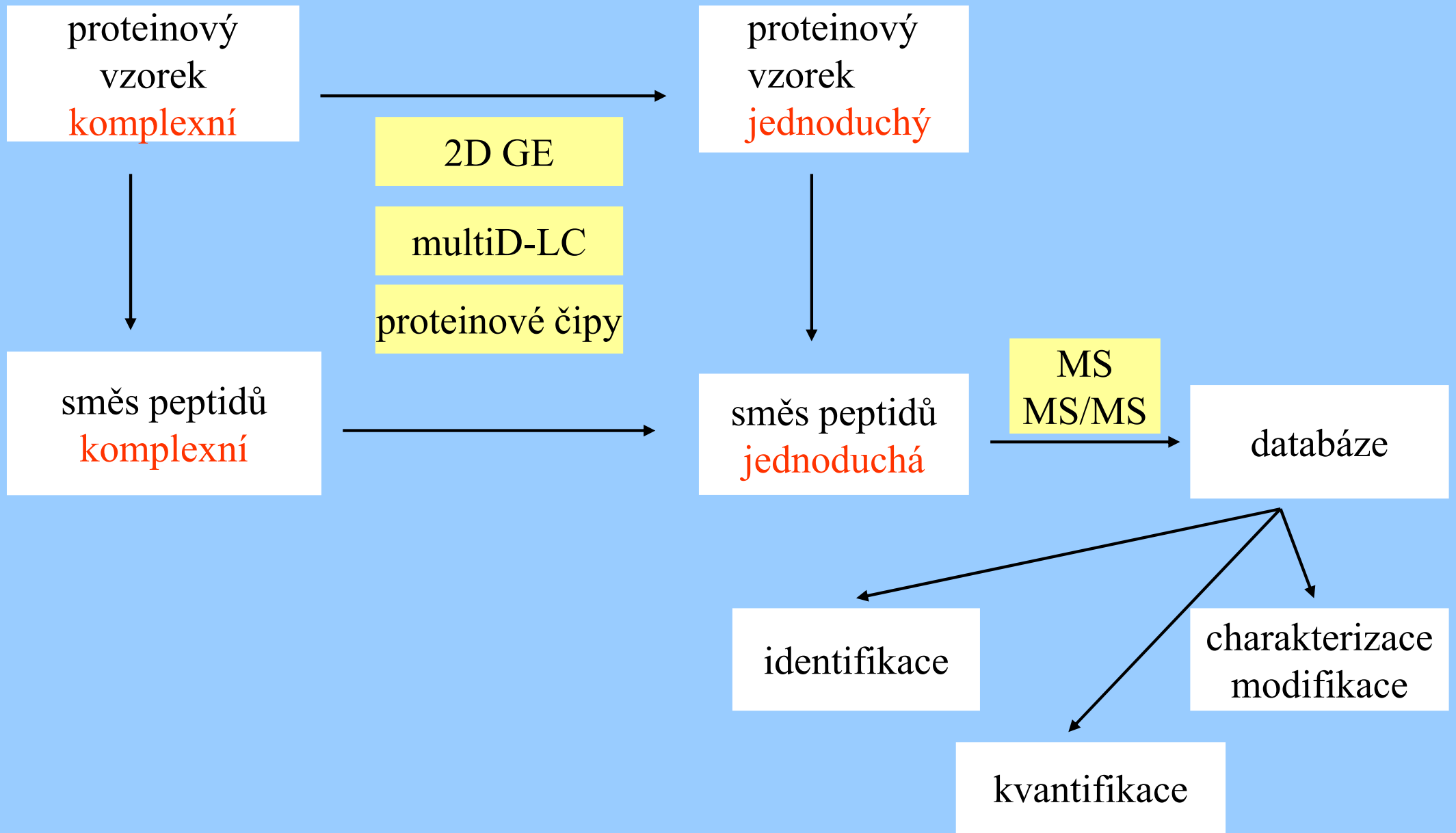


FIGURE 6.7 • Bar code microarray. a) To screen many cells simultaneously, many different mutants were incubated in a common flask of growth media (top). An aliquot of cells was used to produce the mixture of barcode PCR products that were coupled to a red dye. Two-color comparison of bar code microarray hybridized with fluorescently labeled PCR products amplified from genomic DNA extracted from 556 strains grown for 0 (red channel) or 6 hours (green channel) in minimal medium. Only a portion of the full microarray is shown. Strains that exhibit a growth defect in minimal medium are labeled.

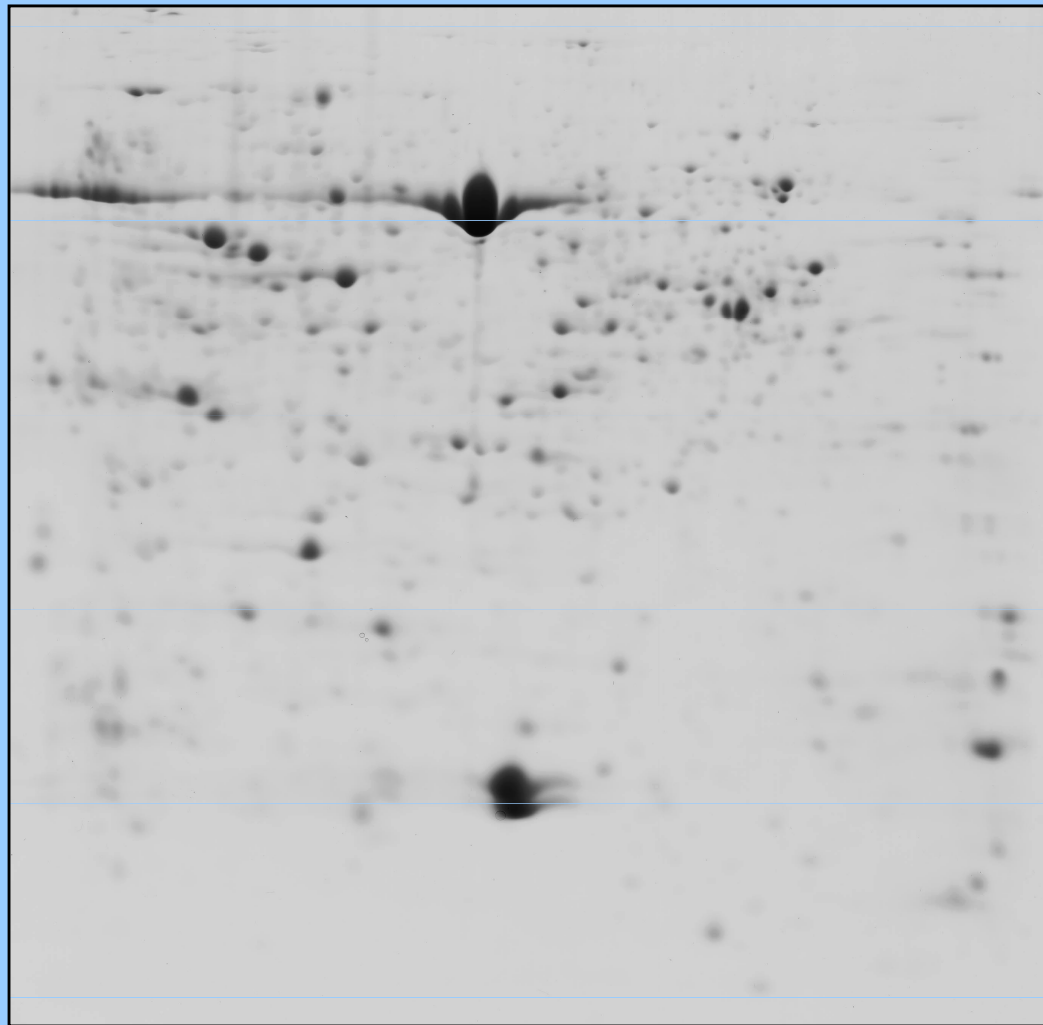


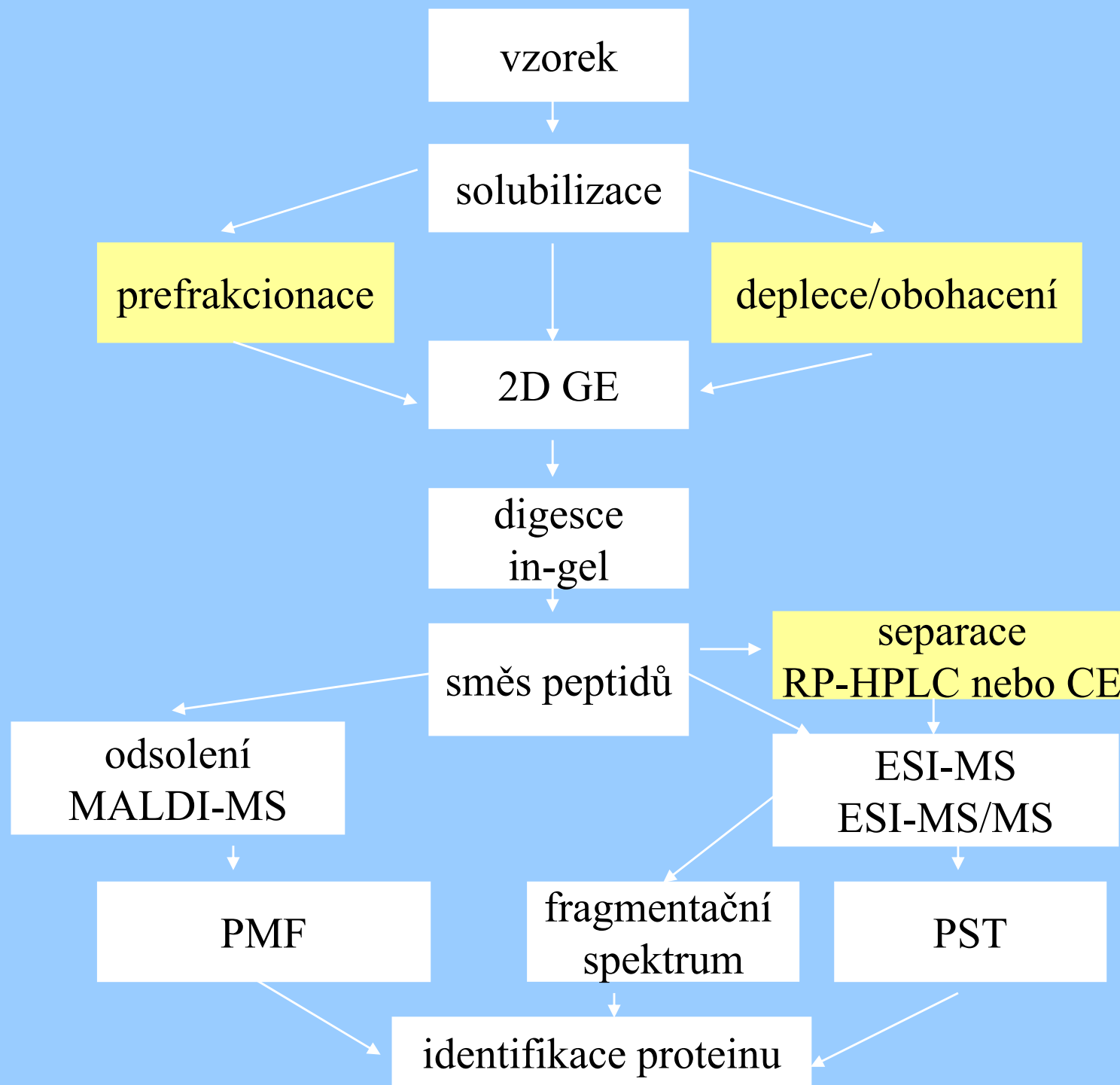
→ dvourozměrná gelová elektroforéza 2D GE

→ (multidimenzionální) kapalinová chromatografie

# DVOUROZMĚRNÁ GELOVÁ ELEKTROFORÉZA 2D GE

---

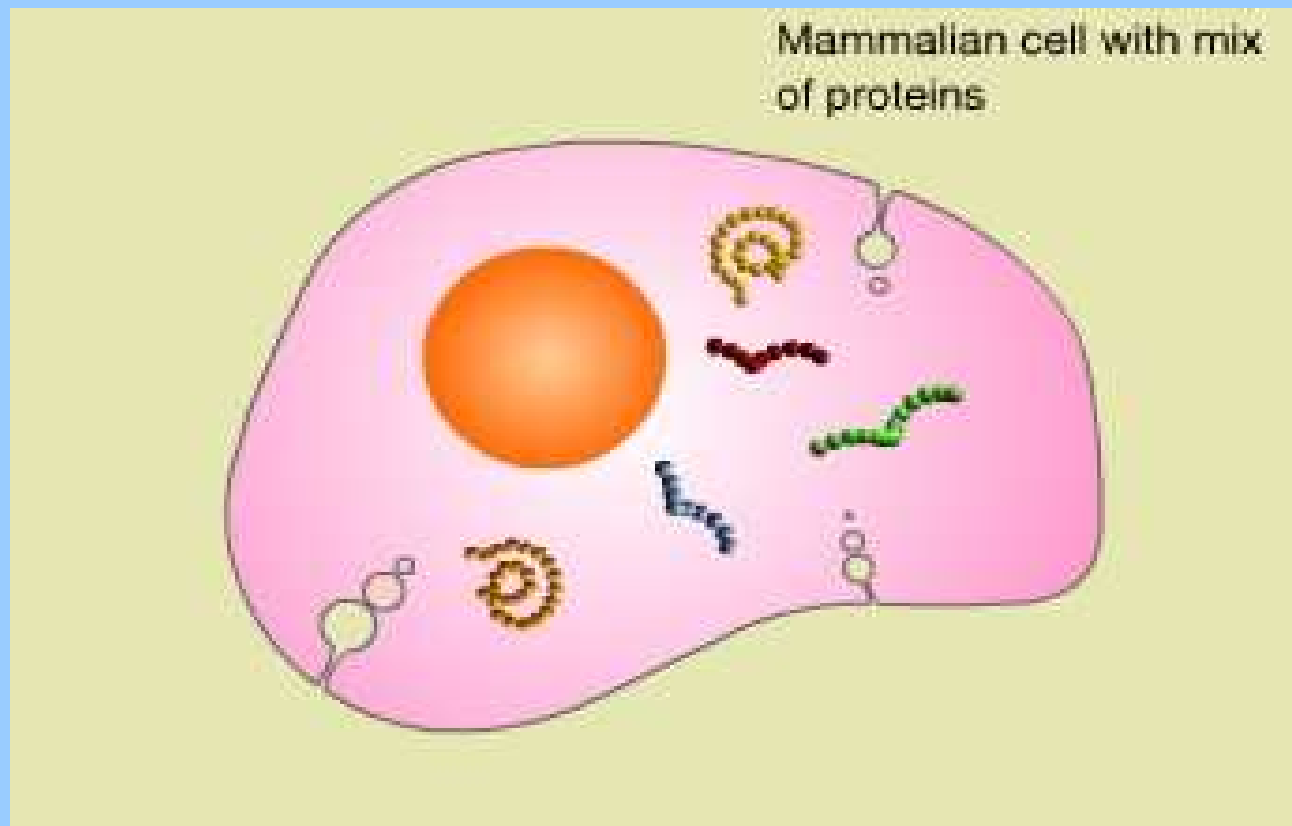






# ANALYZOVANÝ VZOREK

---



## SOLUBILIZACE VZORKU

---

- nekovalentní interakce – denaturace **detergenty**  
CHAPS, Triton, Nonidet  
**chaotropy**  
močovina, thiomočovina
- disulfidické můstky – redukce
- inhibice proteáz, fosfatáz, glykozidáz
- odstranění solí, lipidů, polysacharidů, NA

## ZÁKLADNÍ PRAVIDLA

---

- zabránit proteolýze
- jednoduchý postup
- čerstvé reagensy
- čerstvý vzorek
- odstranit pevné částice - centrifugace
- odstranit kontaminanty

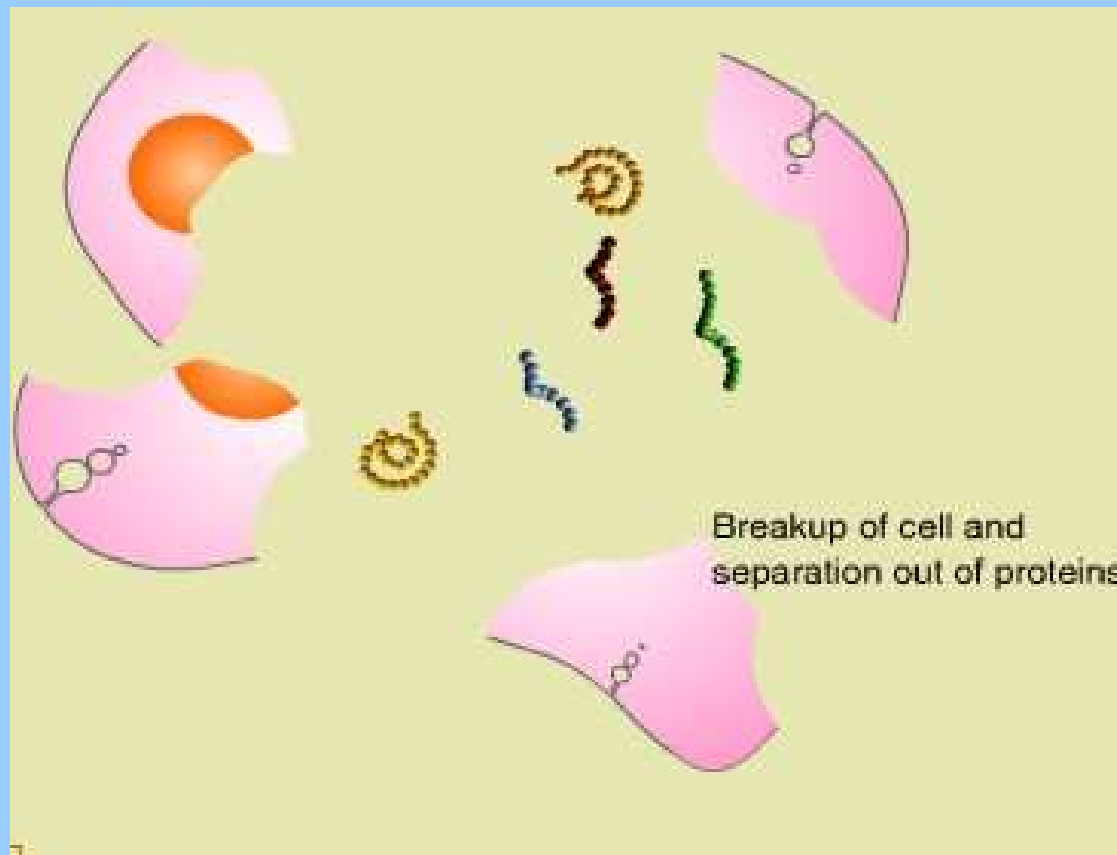
## KONTAMINANTY

---

- soli, zbytky pufrů
- malé endogenní molekuly
- iontové detergenty
- nukleové kyseliny
- polysacharidy
- lipidy
- fenolické látky

# SOLUBILIZACE

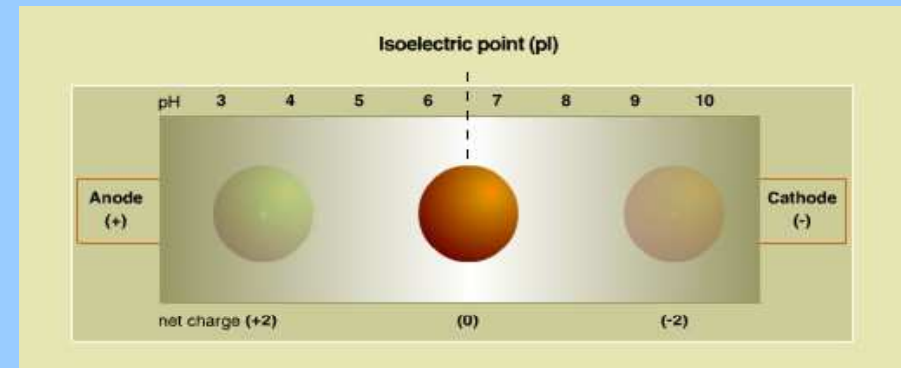
---



## 2D GE

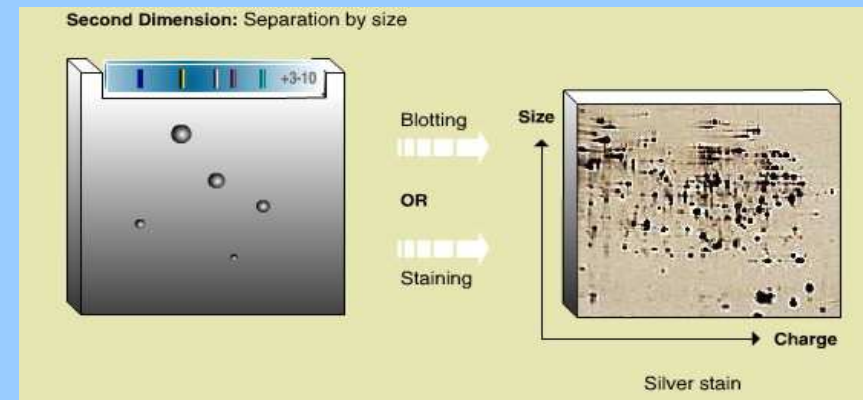
- první rozměr

## IEF



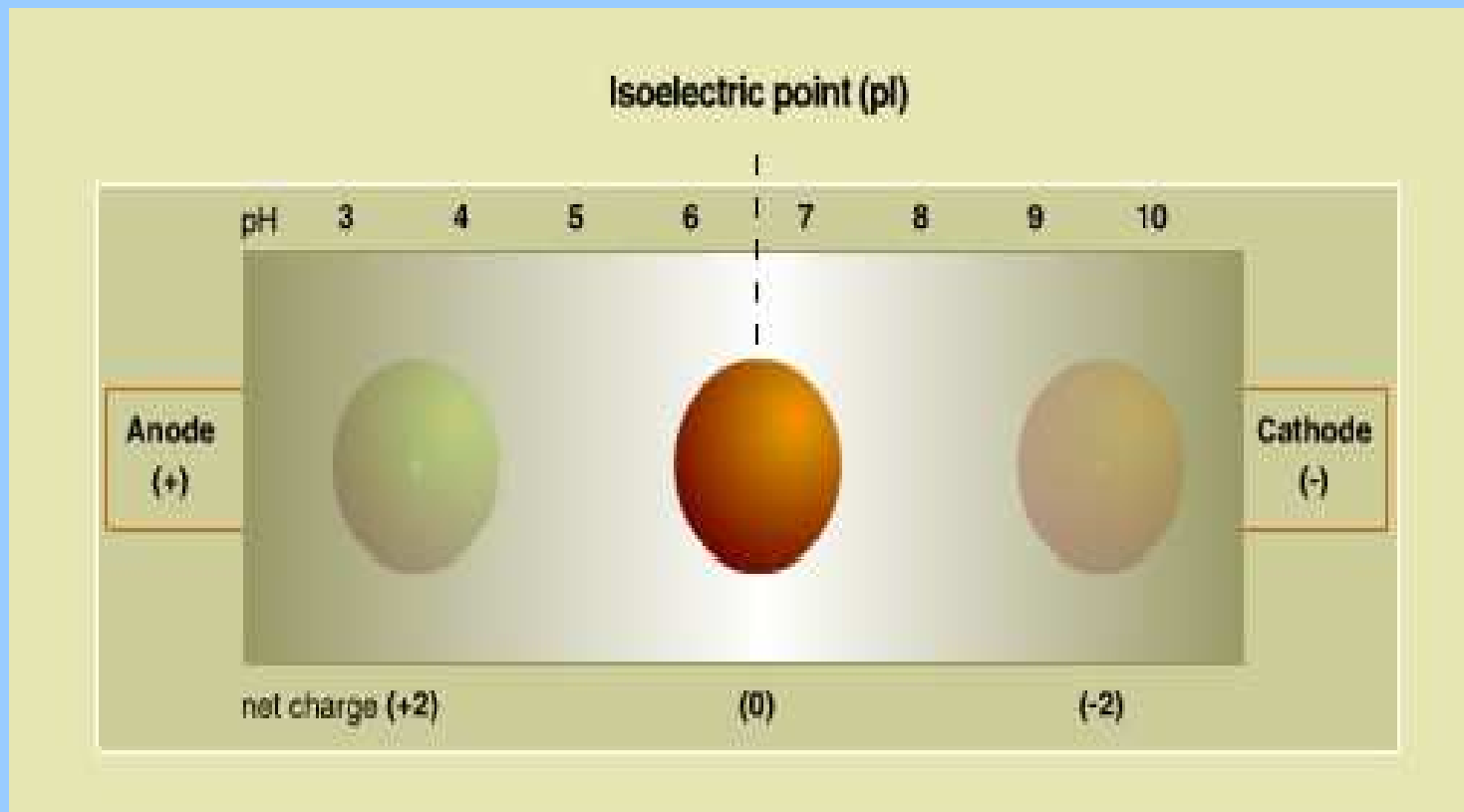
- druhý rozměr

## SDS-PAGE



# ISOELEKTRICKÝ BOD

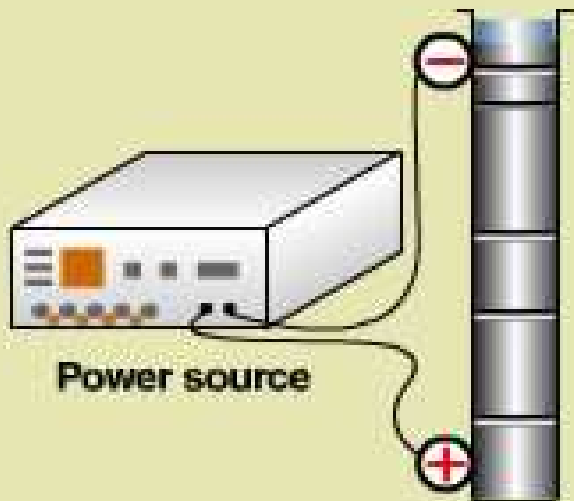
---



# PRVNÍ ROZMĚR 2D GE

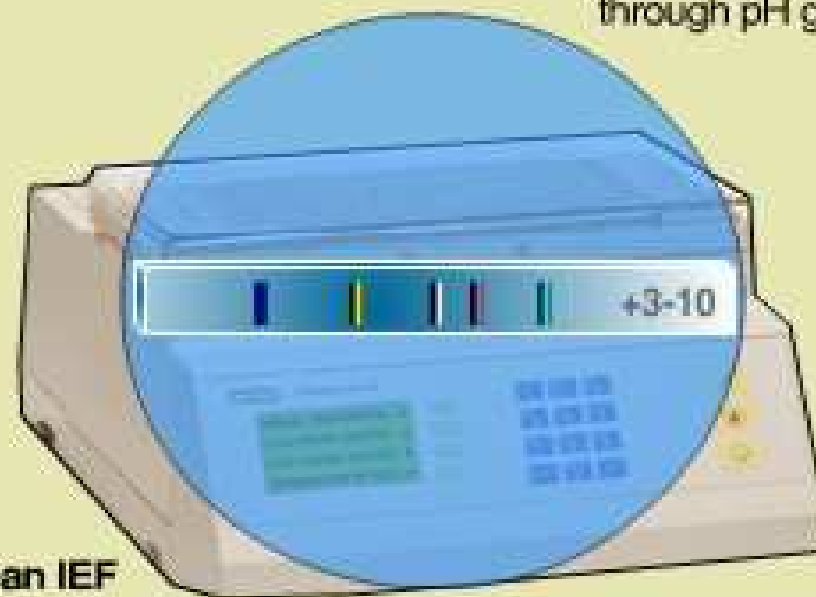
## ISOELEKTRICKÁ FOKUSACE

**First Dimension:**  
Separation by charge



Tube gel

Proteins migrating  
through pH gradient



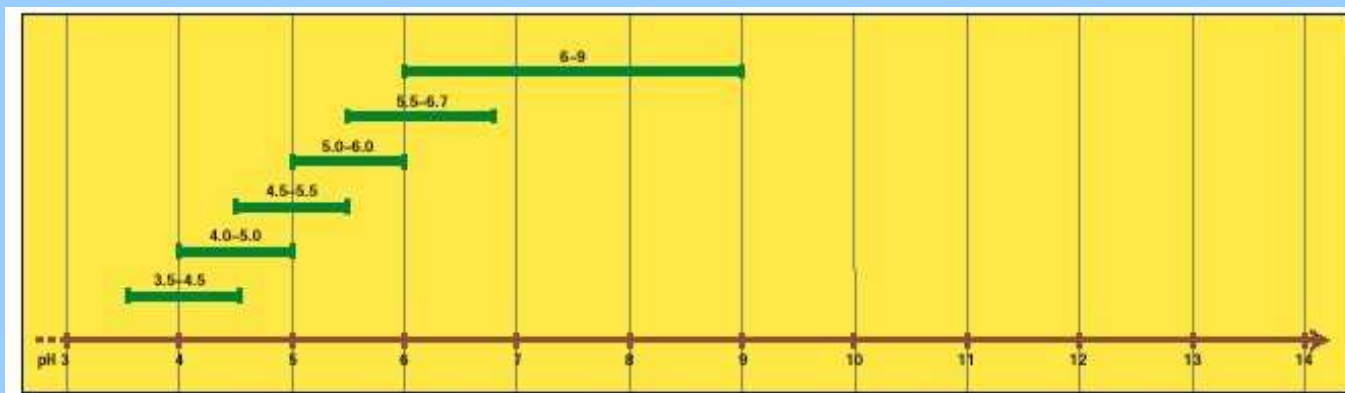
Immobilized pH gradient strips



## IPG STRIPY

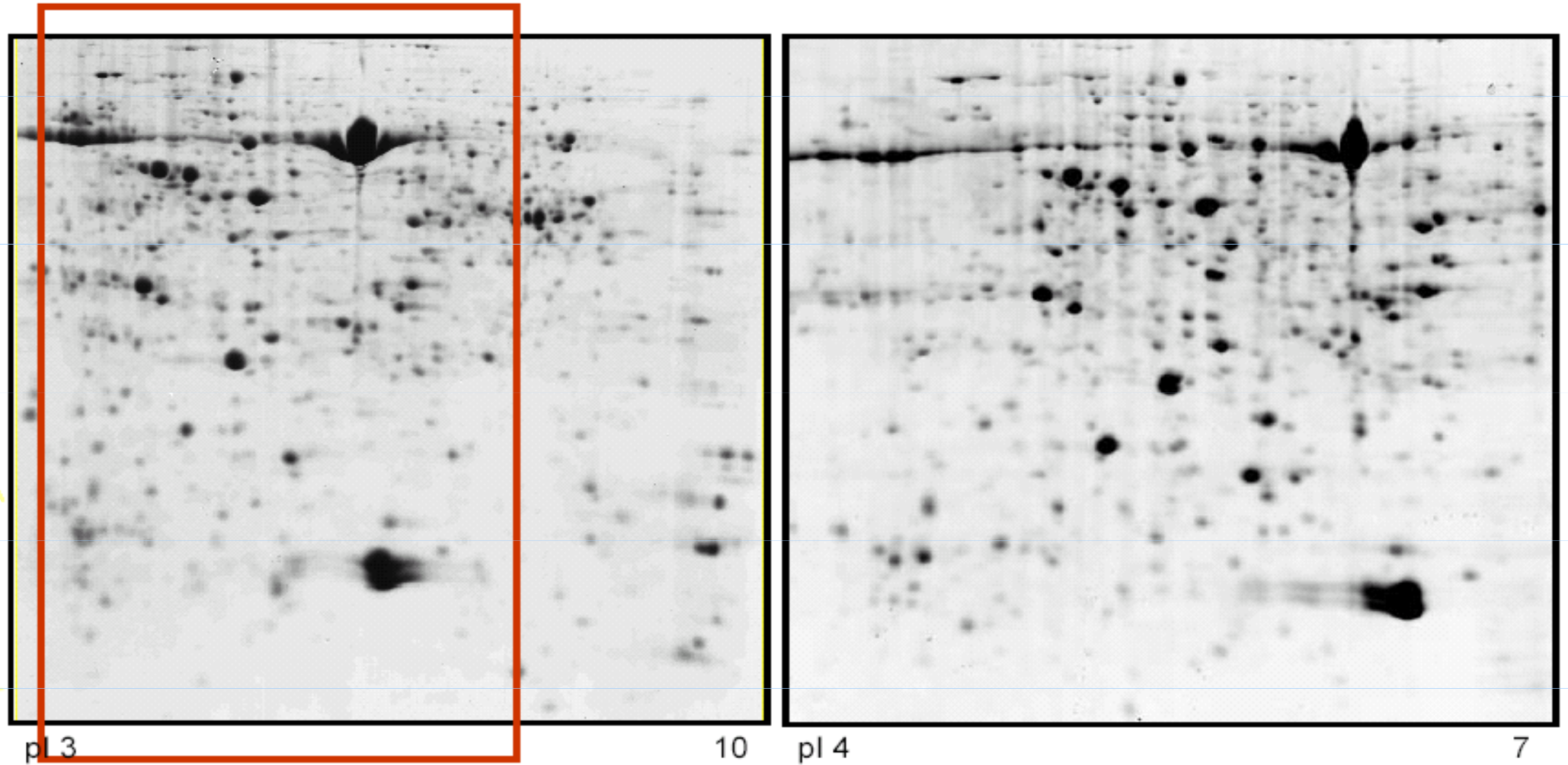


- široký rozsah 3-10, 3-10NL
- úzký rozsah
- mikro rozsah



překrývající se rozsahy

# ROZSAH STRIPU



# FOKUSAČNÍ PARAMETRY

---

- rehydratace
- aplikace vzorku
- ochrana cysteinu
- fokusační podmínky

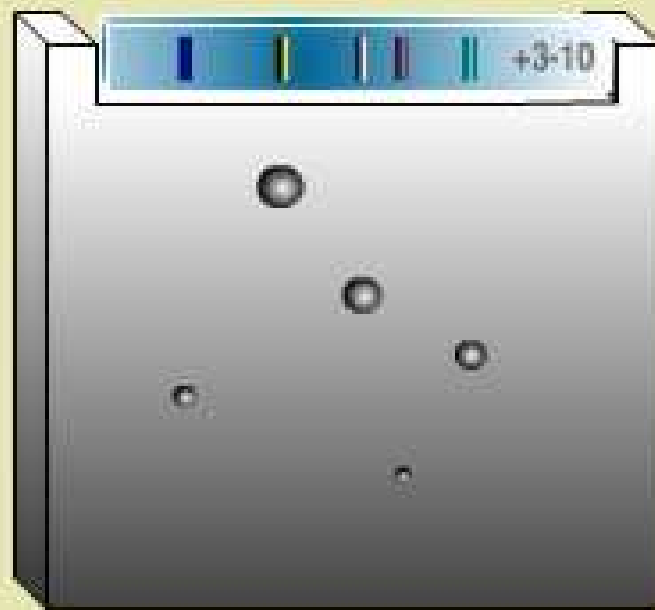


Protean IEF Cell

# DRUHÝ ROZMĚR 2 DE GE

## SDS-PAGE ELEKTROFORÉZA

Second Dimension: Separation by size



Blotting



OR



Staining

3

10

Size



Charge

Silver stain

# DETEKCE PROTEINU

---

- gel x blot
- visualizace
  - barvení
  - radioaktivita
  - imunodetekce
- barvení v gelu
  - po elektroforéze  
před elektroforézou
  - specifické pro protein  
specifické pro PTM
  - viditelné spektrum  
fluorescence

## BARVENÍ PROTEINU V GELU

Coomassie Blue R-250

Coomassie Blue G-250

Stříbro: kompatibilní s MS

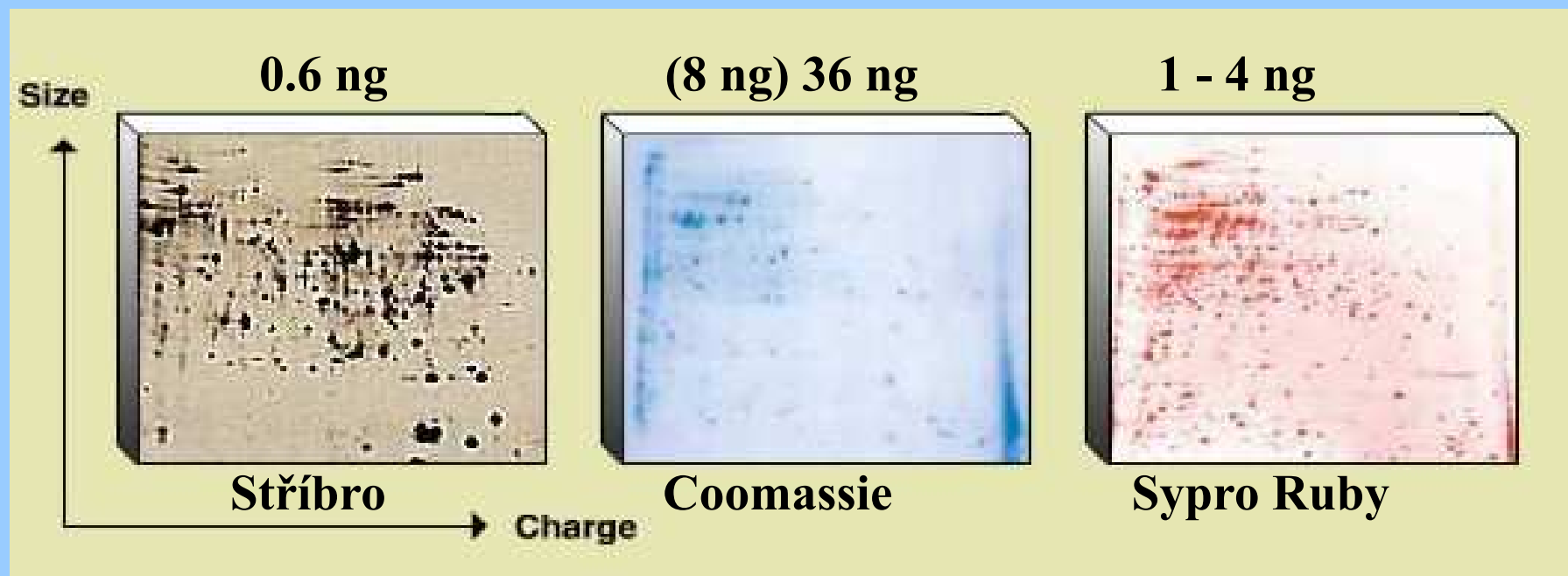
nekompatibilní s MS

Sypro Ruby

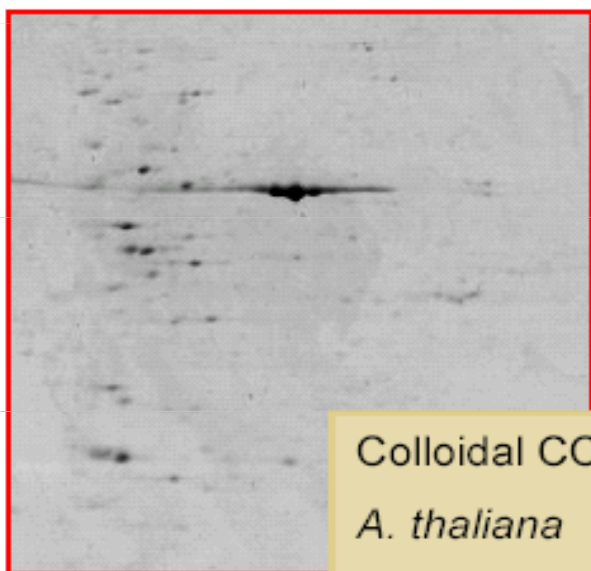
Flamingo Pink

Pro-Q Diamond

Pro-Q Emerald



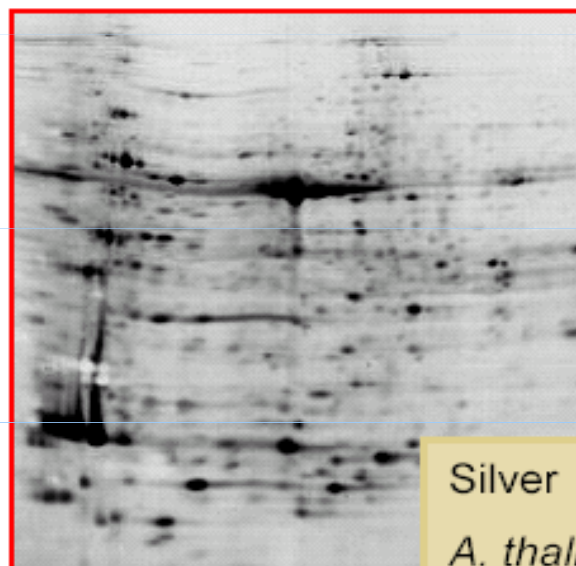
# CITLIVOST BARVENÍ PROTEINU



Colloidal CCB

*A. thaliana*

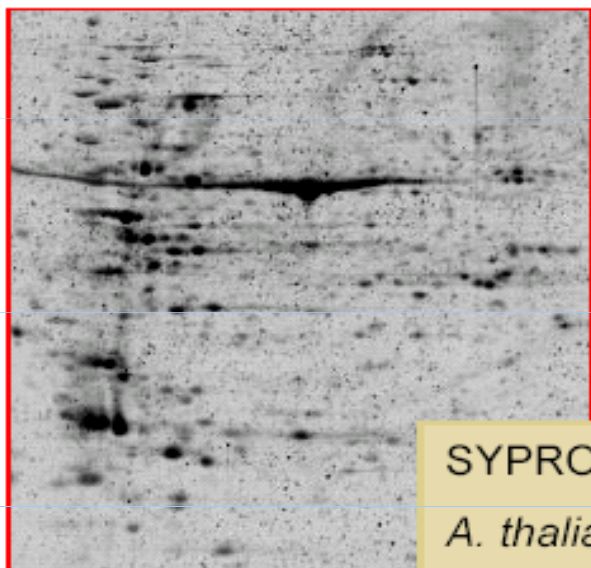
množství proteinu: 100  $\mu\text{g/gel}$



Silver

*A. thaliana*

množství proteinu: 20  $\mu\text{g/gel}$

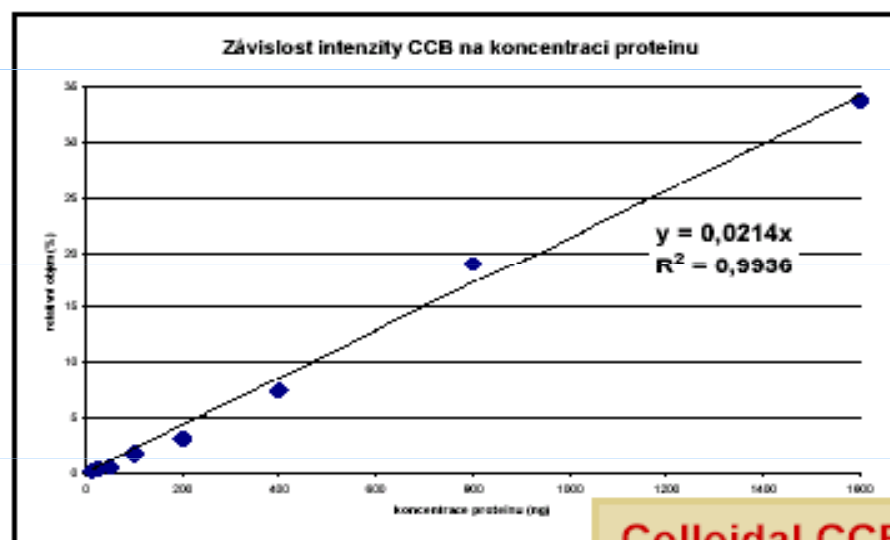


SYPRO Ruby

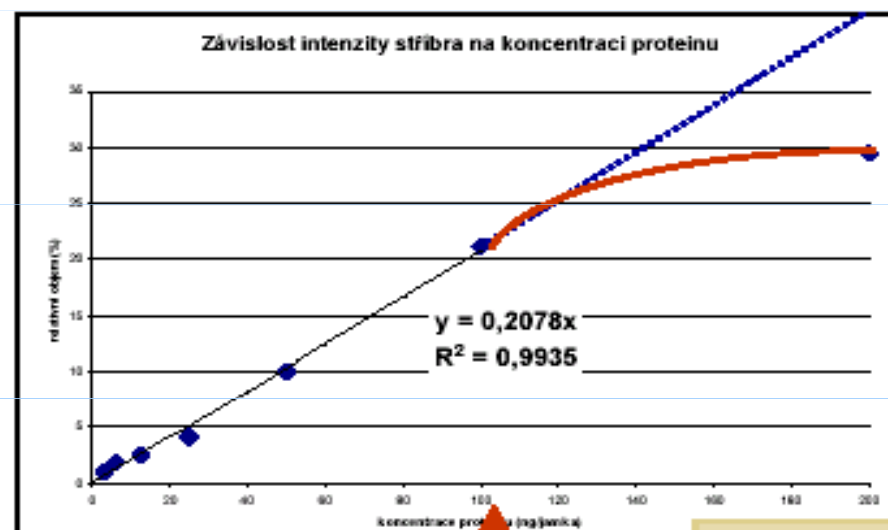
*A. thaliana*

množství proteinu: 20  $\mu\text{g/gel}$

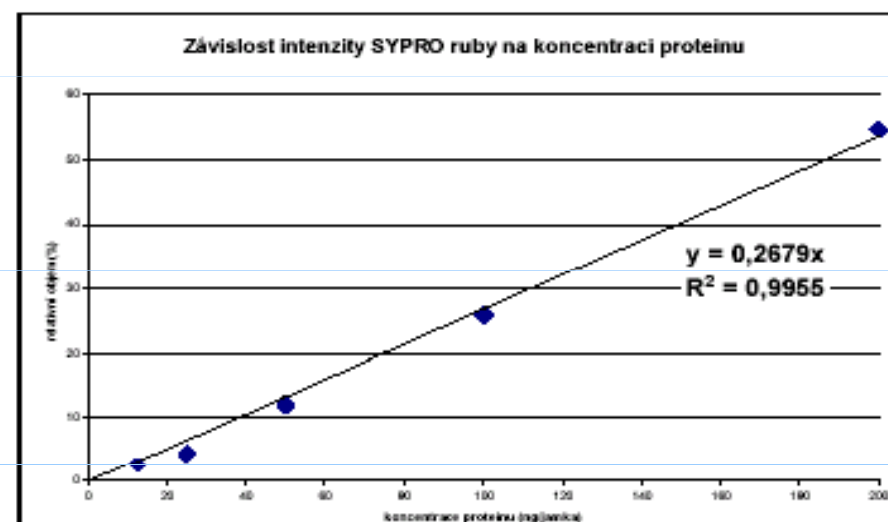
# BARVENÍ PROTEINU - LINEARITA



Colloidal CCB



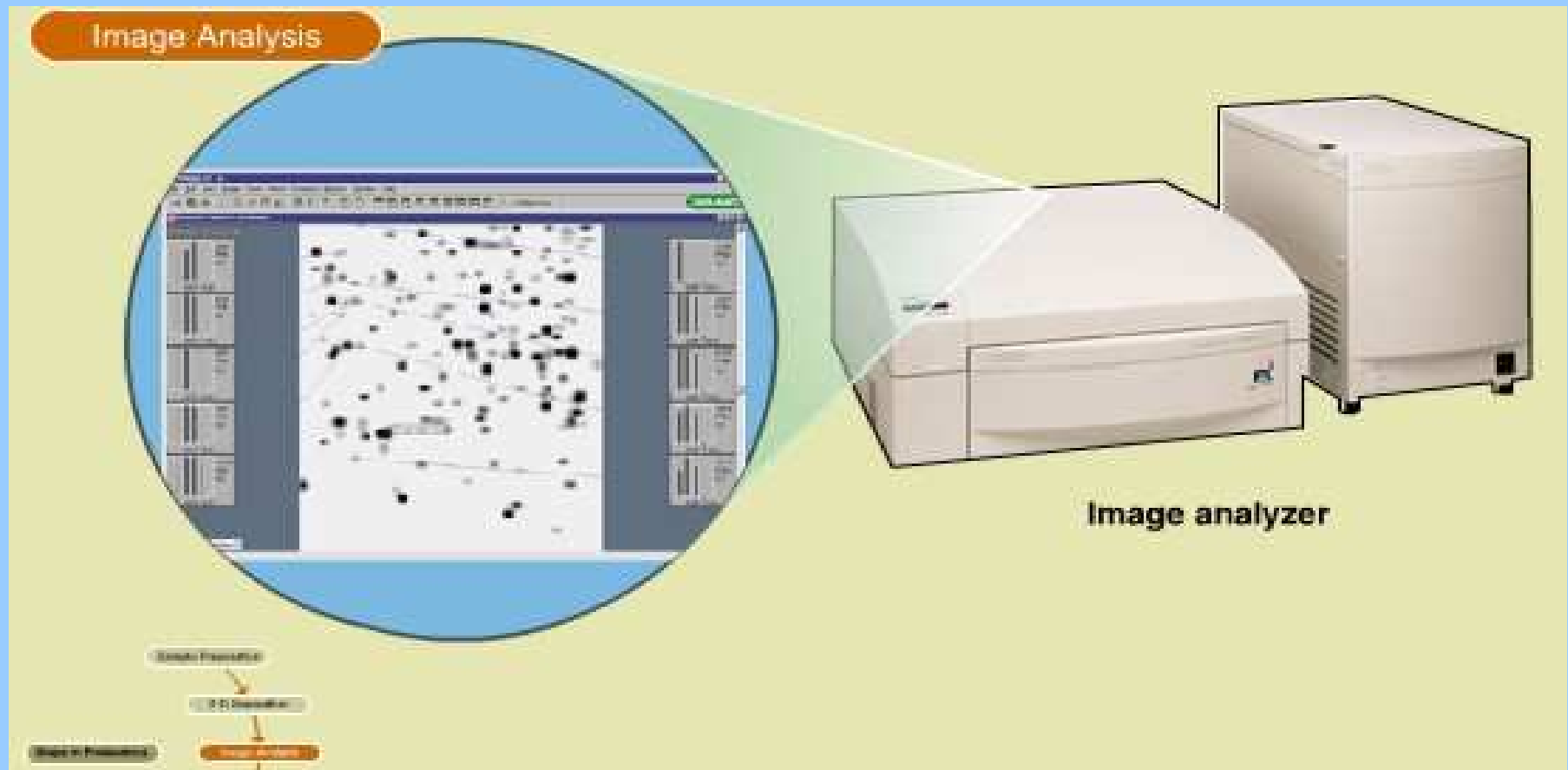
Silver



SYPRO Ruby



# ANALÝZA OBRAZU

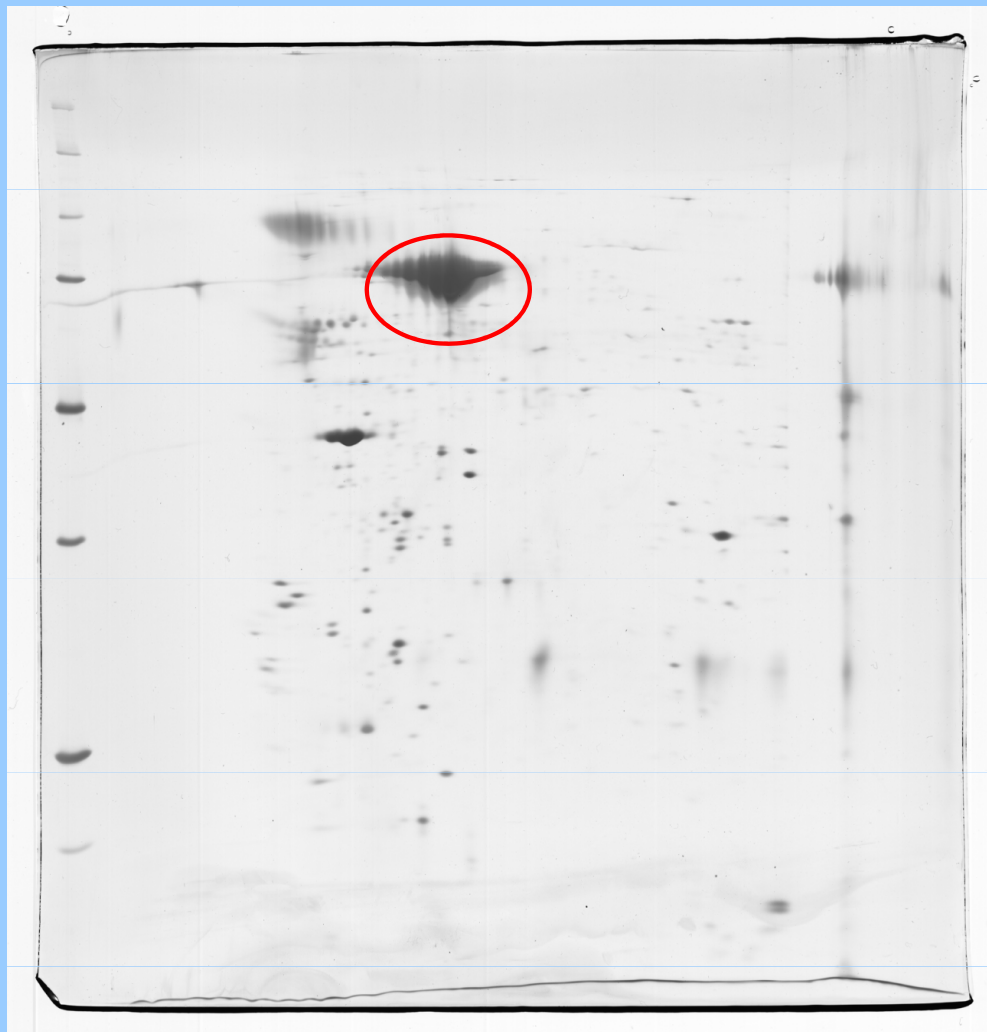


referenční mapa

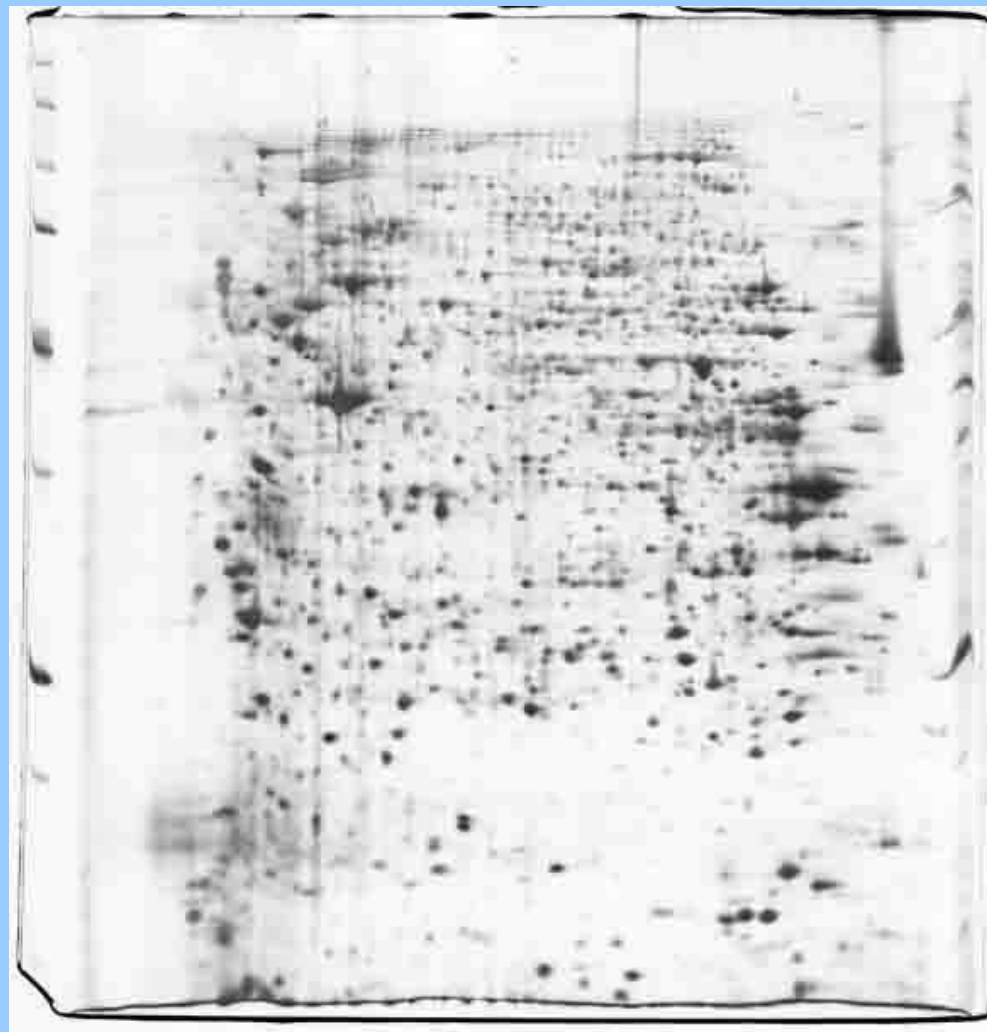
paralelní vzorky • průměrný gel • kvalita • kvantita • porovnání referenčních map

pI

Mr



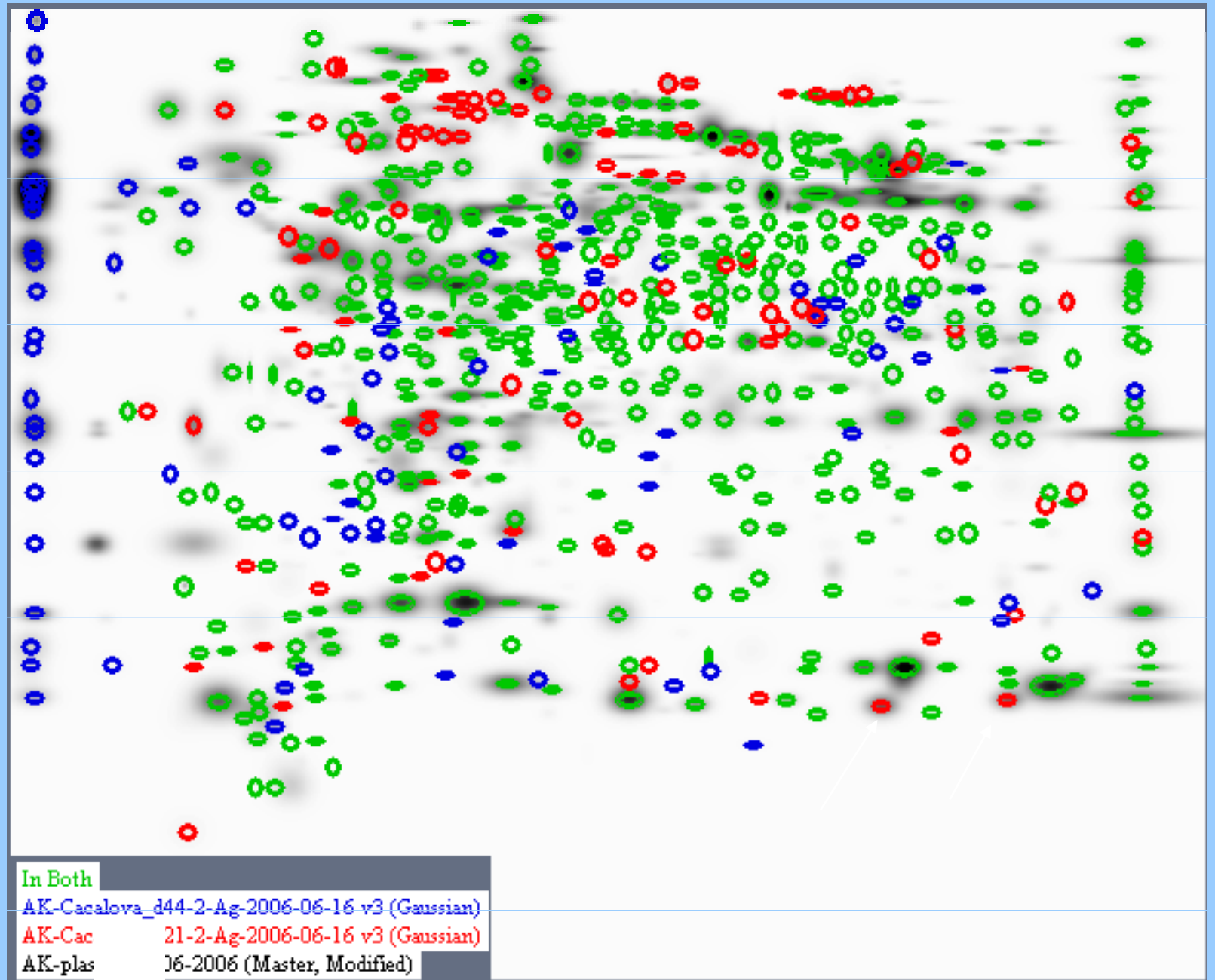
pI



Sekretované vesikly kmenových buněk

Lymfocyty

# PDQuest



## BIOMARKER

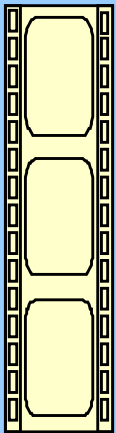
---

- detekuje přítomnost onemocnění
- přispívá k zvládnutí nemoci - předpověď a potvrzení účinku léku

staré Řecko – sladká moč  
dnes asi 150 proteinů klinicky relevantních

## VALIDACE

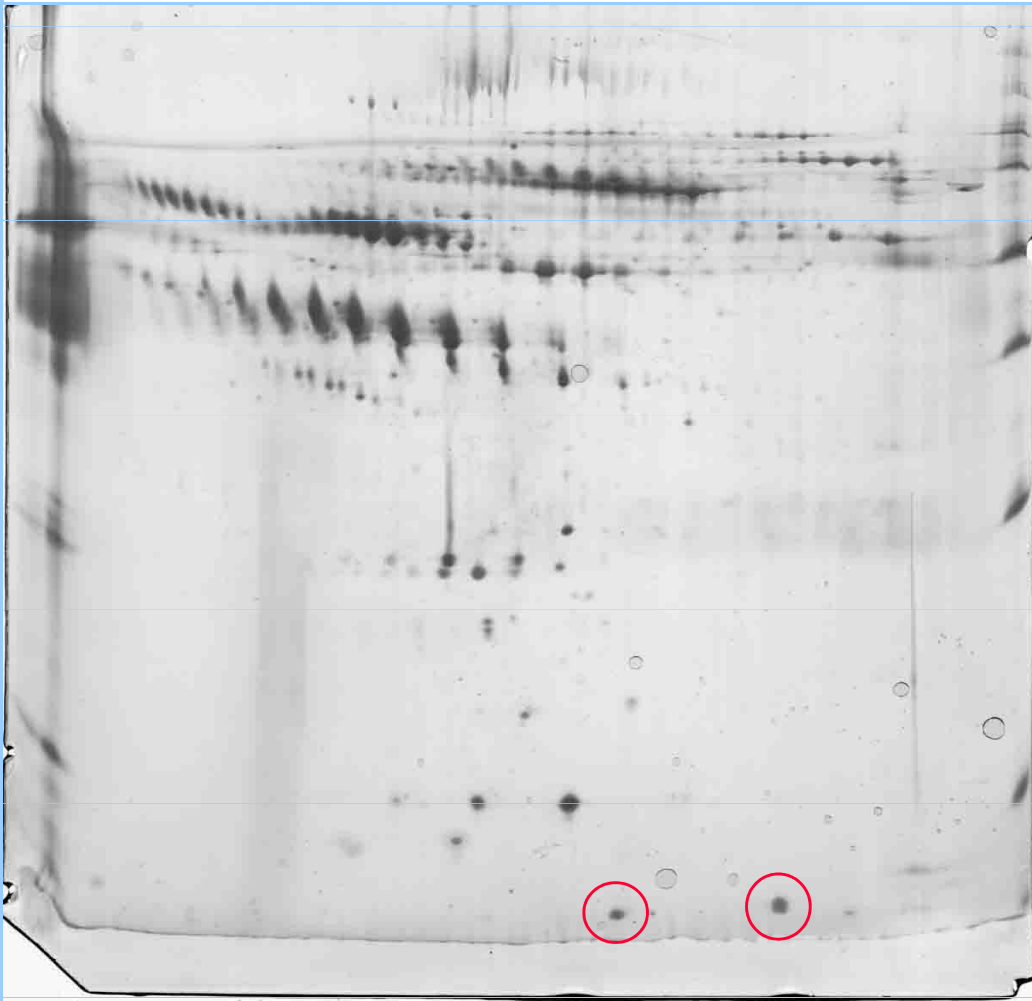
- **analytická** stabilní robustní metoda
- **biologická** biochemické dráhy a jejich změny, biologická variabilita u zdravých
- **klinická** prediktivní předpověď klinické změny,  $\uparrow$  > než biologická variabilita



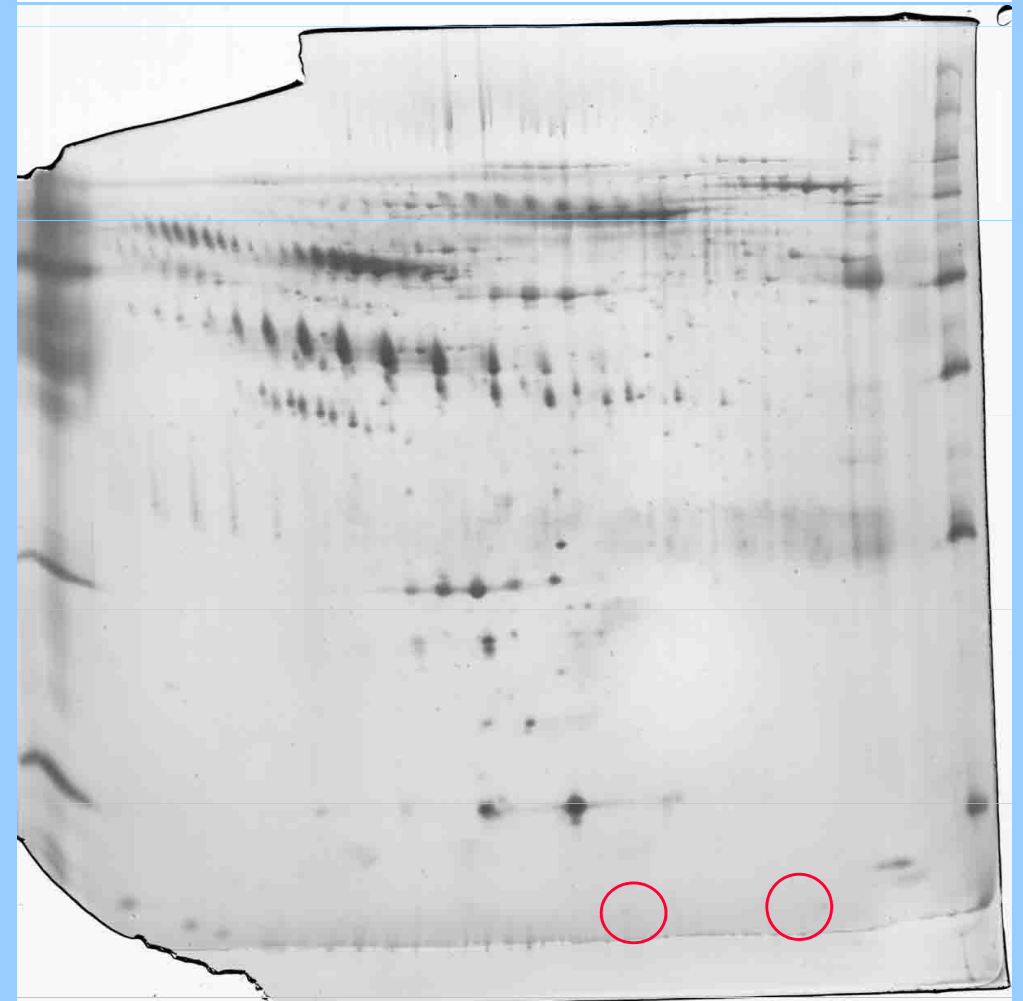
personalizovaná medicína  
vlastní kontroly  
etické problémy  
kupka sena

# Biomarkery onemocnění GvHD v lidské plasmě

Den 21 – před klinickým projevem GvHD

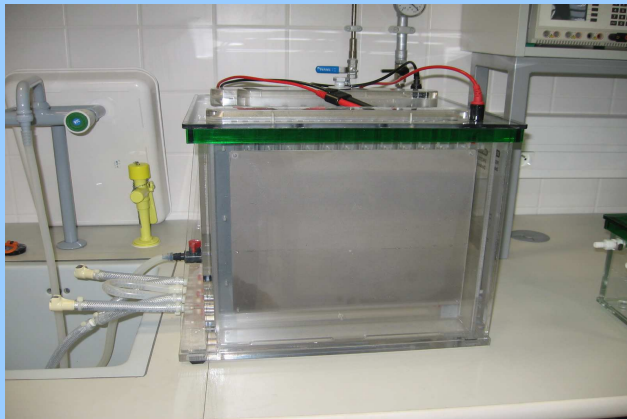


Den 44 – po klinickém projevu GvHD

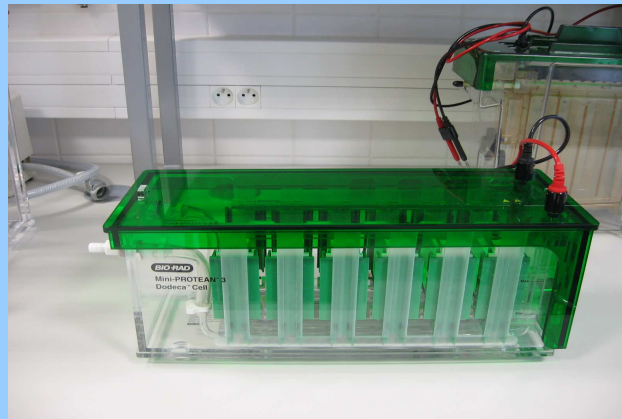


## 2D GE INSTRUMENTACE

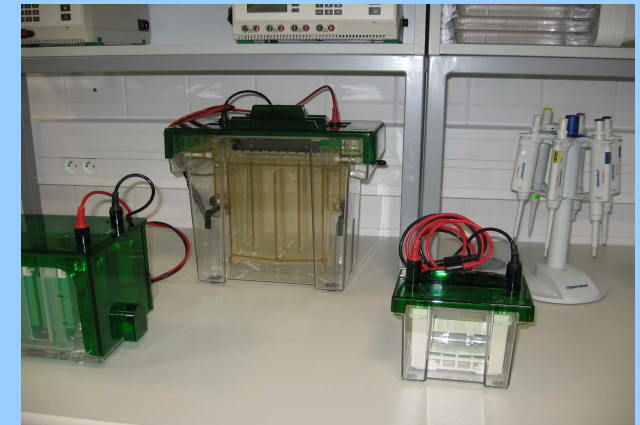
- Protean IEF
- Protean Dodeca Cell
- Densitometer GS-800  
*PDQuest, Quantity One*
- STORM



Protean Plus Dodeca Cell



Mini-Protean 3 Dodeca Cell

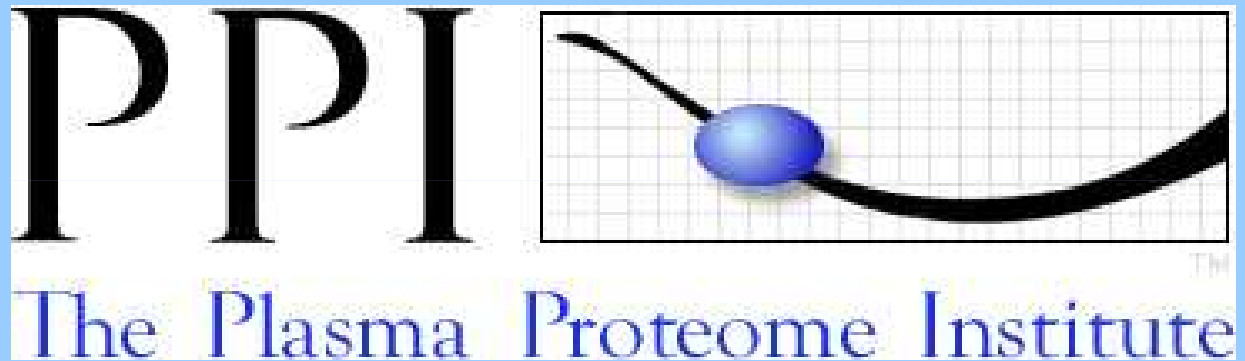


Protean II xi Cell

## 2D or not 2D ?

---

- rozlišení
- vizuální aspekty
- multigelové jednotky
- dynamický rozsah
- extrémní proteiny (membránové, basické...)
- reprodukovatelnost, image analýza
- citlivost barvení
- pracnost
- nesnadná automatizace
- postdigesční extrakce

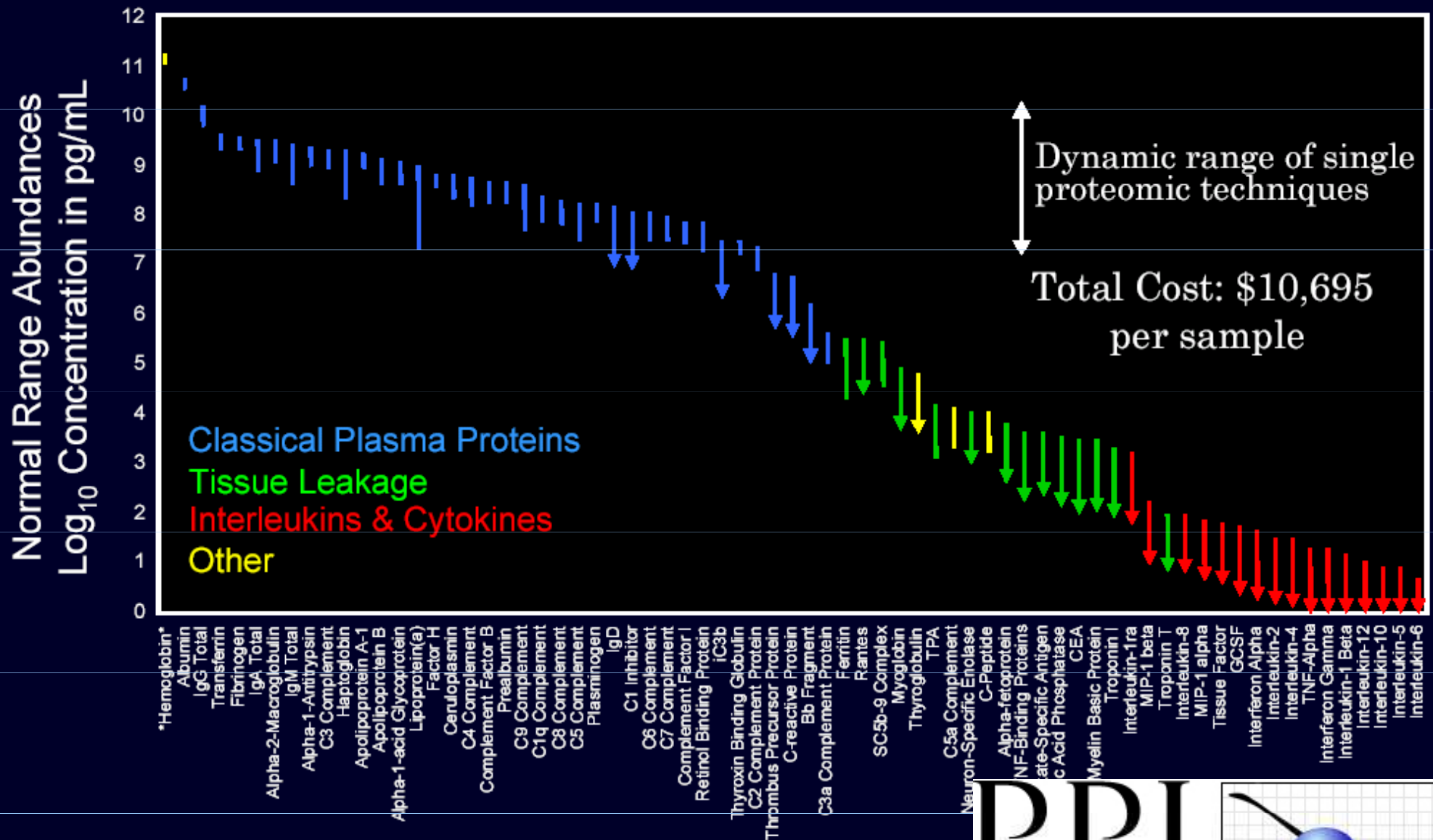


Washington, DC

Dr. Leigh Anderson, 2002



# Proteins Measured Clinically in Plasma Span > 10 Orders of Magnitude in Abundance

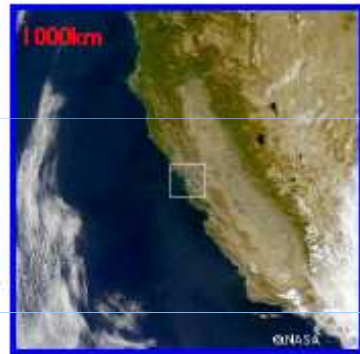


# $10^{10}$ Really Is Wide Dynamic Range

(Here on a linear scale)



10



9



8



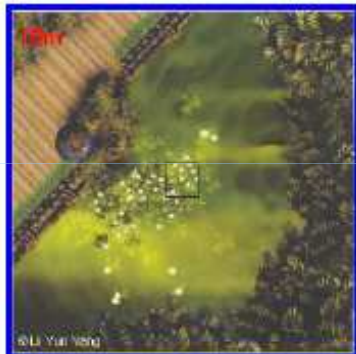
7



6



5



4



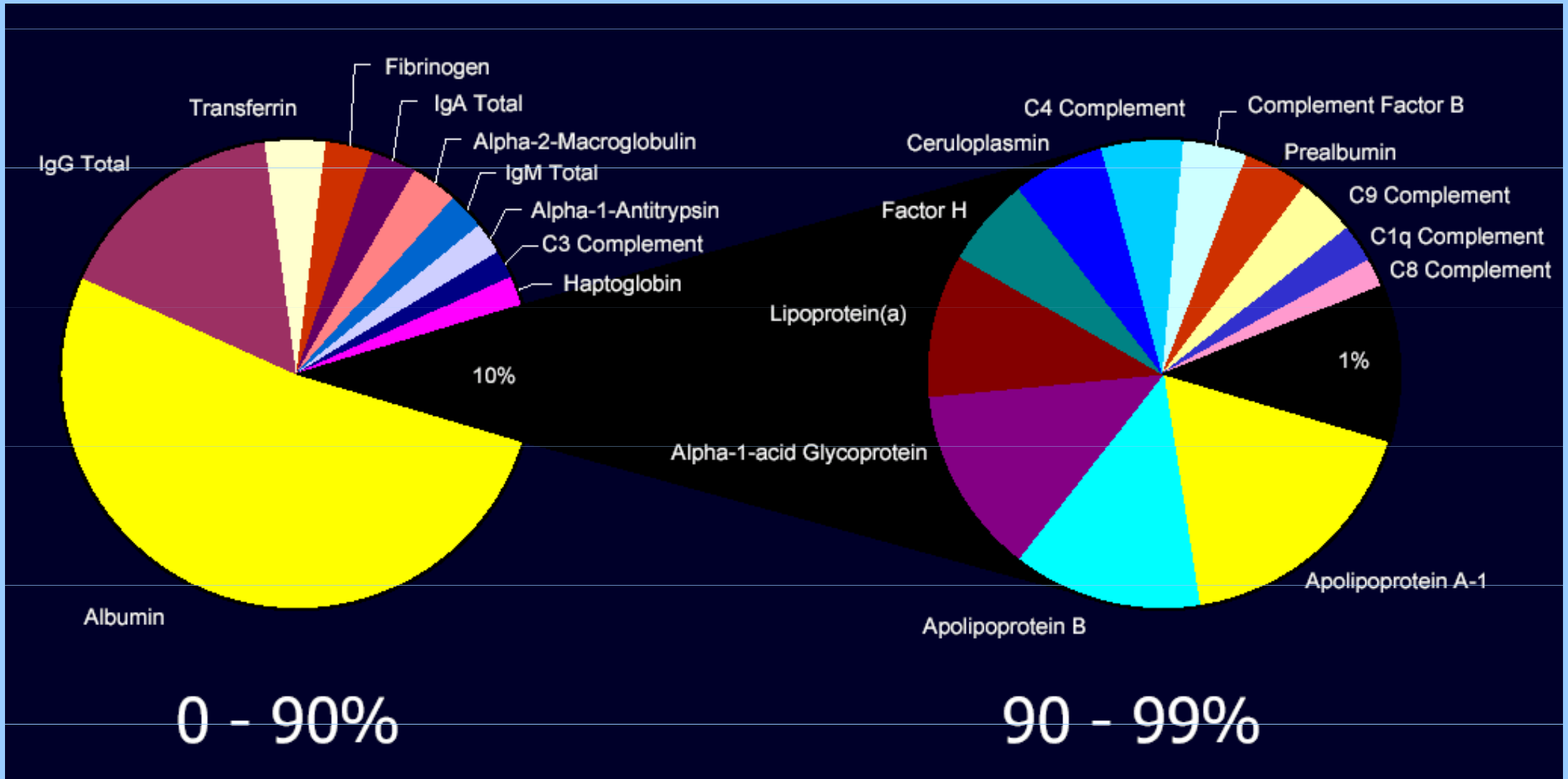
3



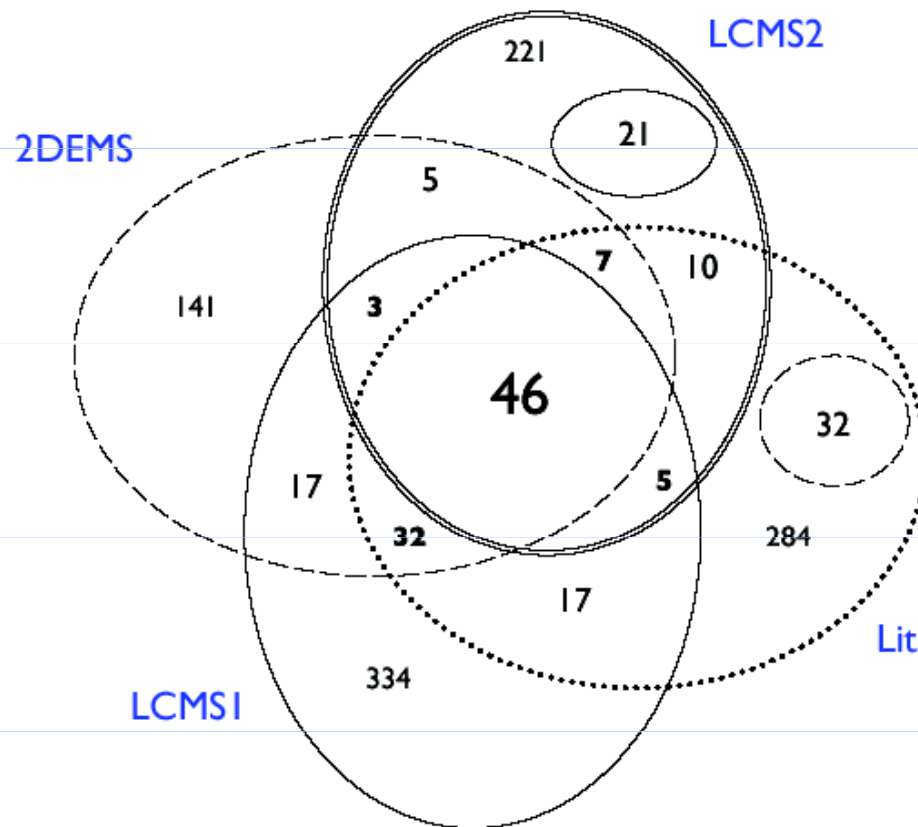
2



1



# Different Platforms See Different Plasma Proteomes: Small Overlap of Four Plasma Proteome Datasets (Number of NR proteins)



- 46 proteins in all four lists
- 195 proteins in 2 or more lists
- 1175 NR proteins total

# PREFRAKCIÓNACE

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MicroRotor



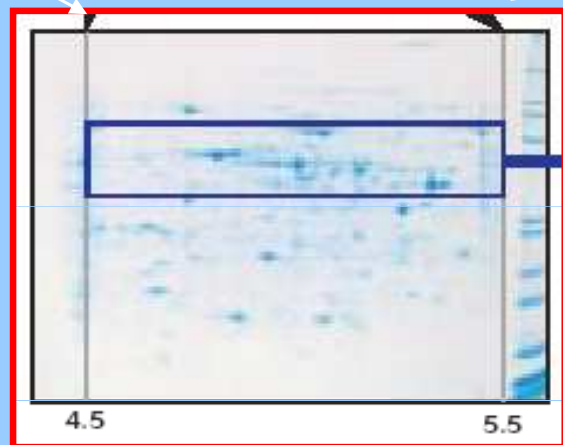
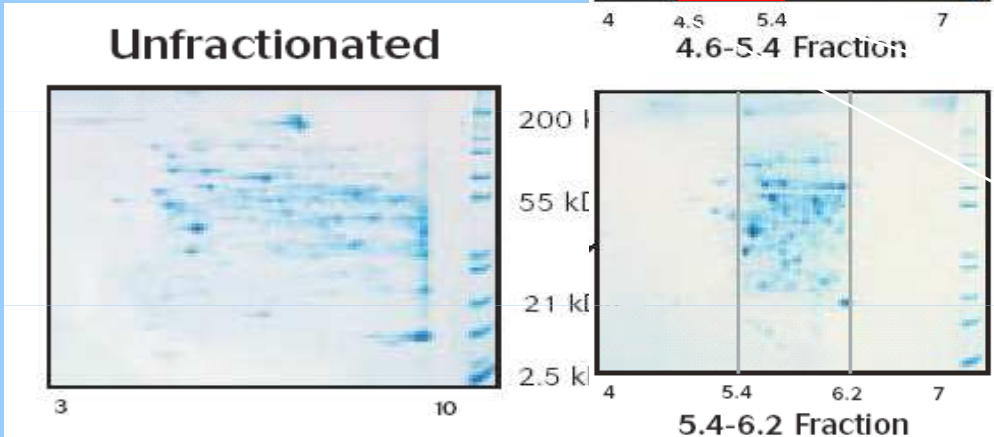
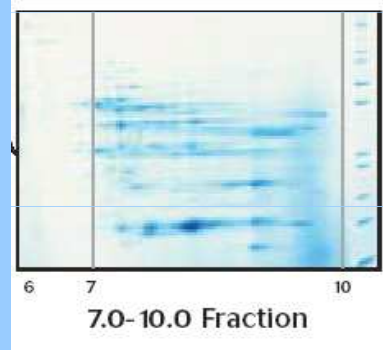
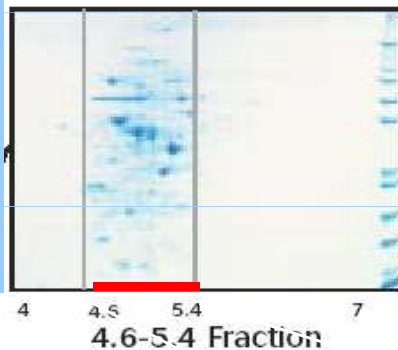
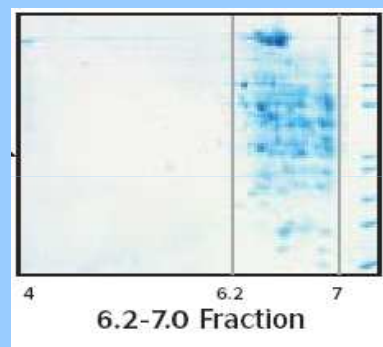
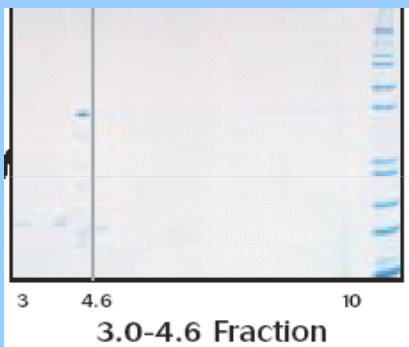
Zoom IEF

PREFRAKCIÓNACE

pI

MIKRO ROZSAH

pI



# DEPLECE

---

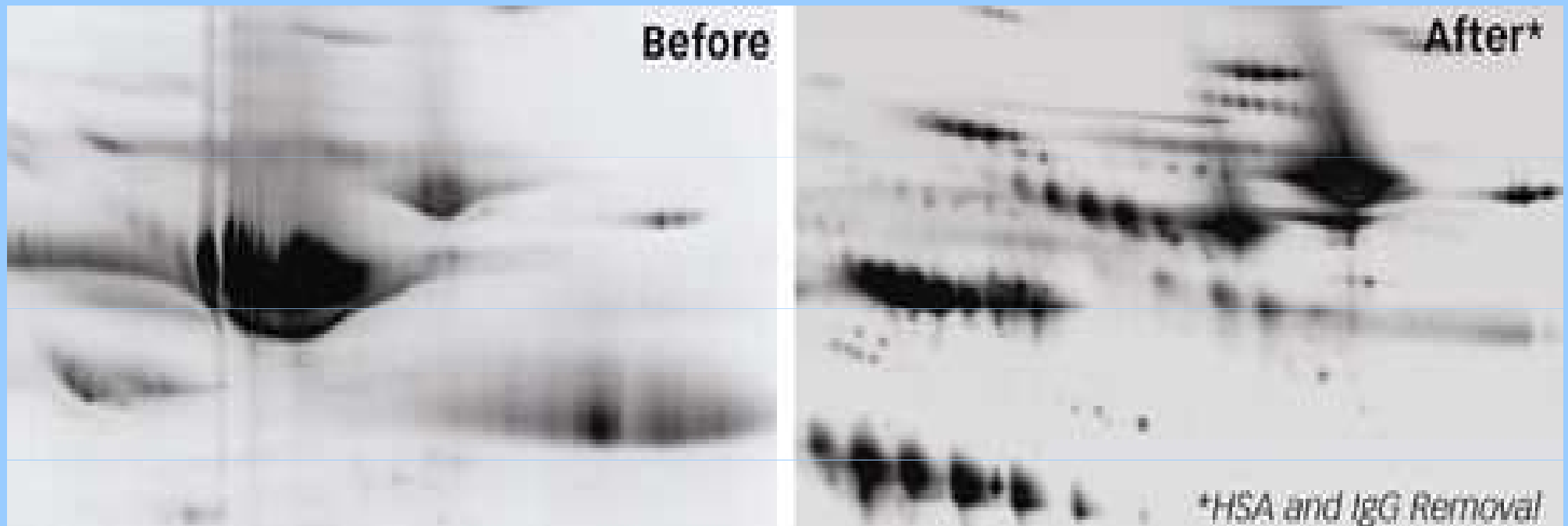
odstranění abundantních proteinů

HSA

VivaPure

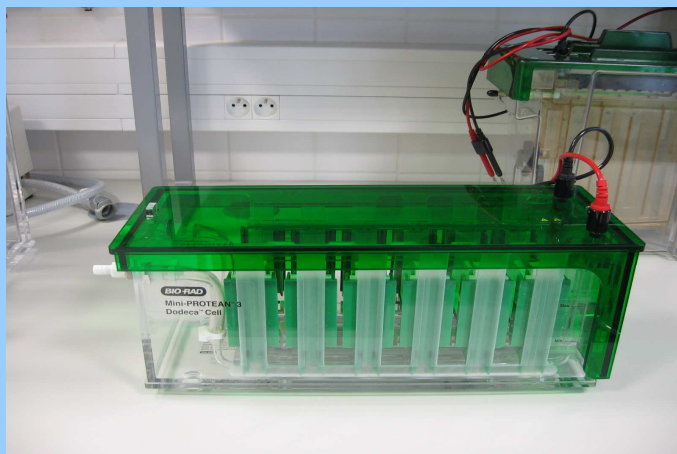
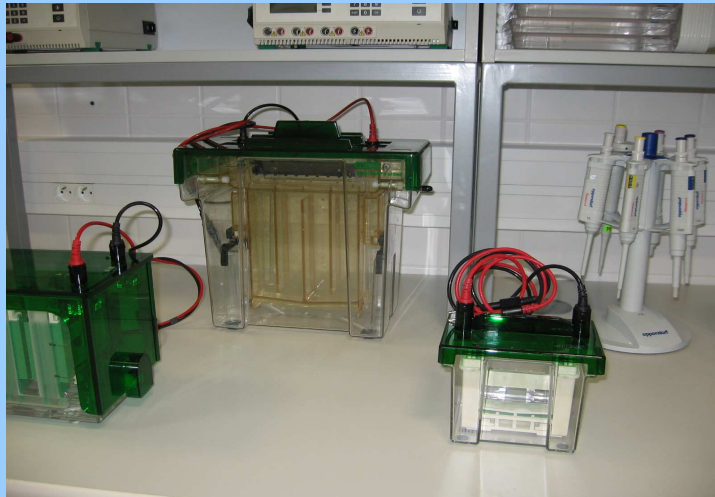
PROT20

...

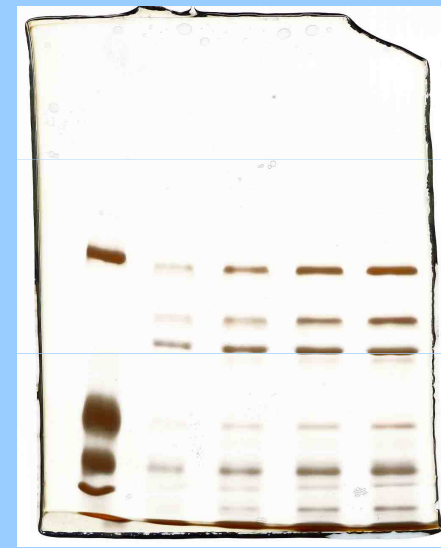


Vivapure Kit

# SDS PAGE



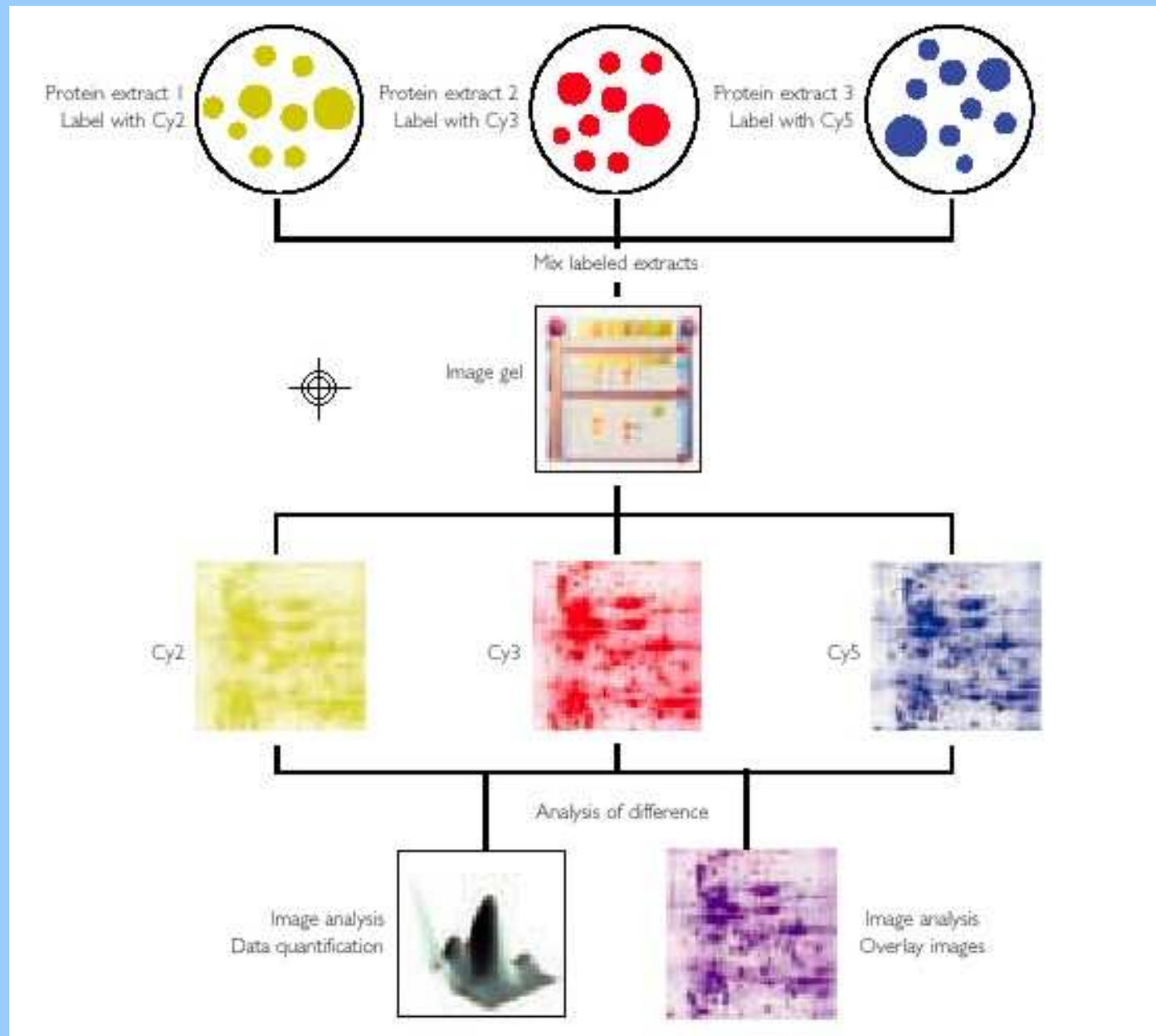
Protean II  
Mini-Protean 3  
Mini-Protean 3 Dodeca Cell



bakteriofág 812



# Difference Gel Electrophoresis DIGE

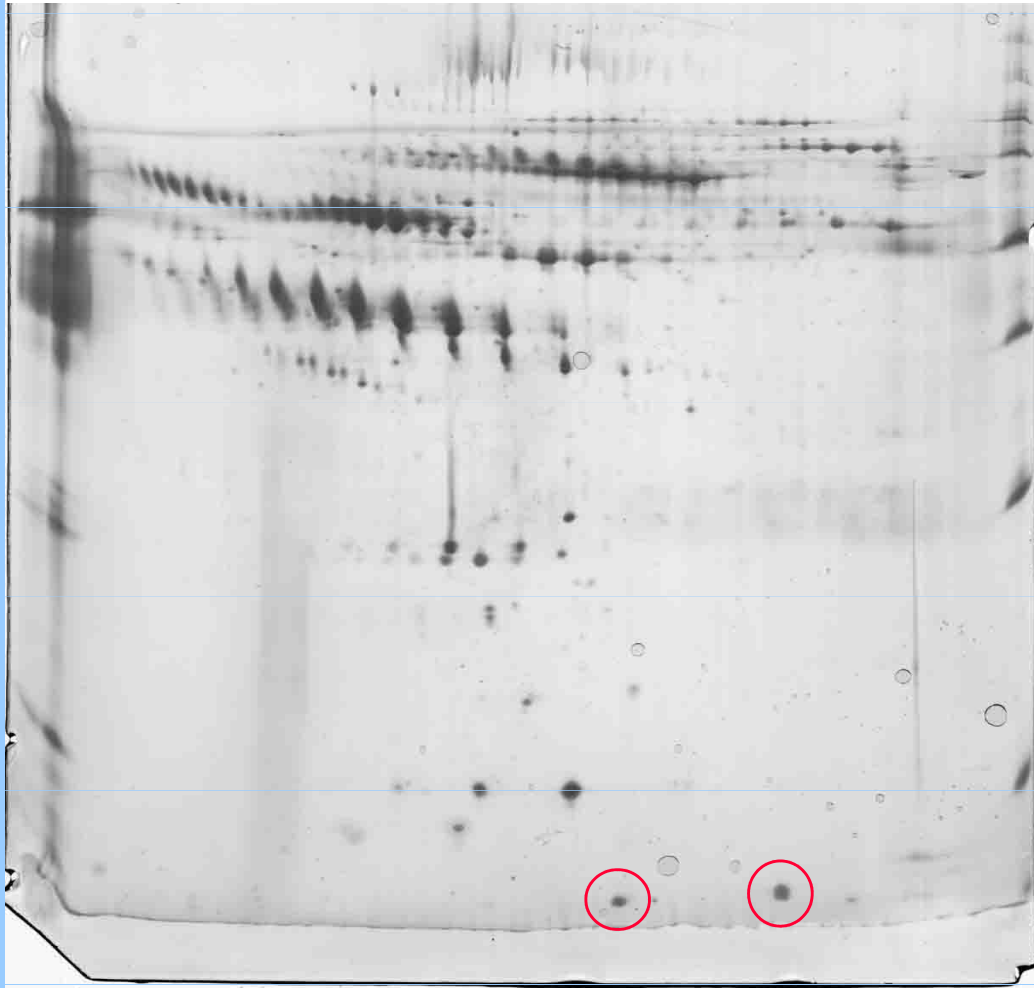


**separace**



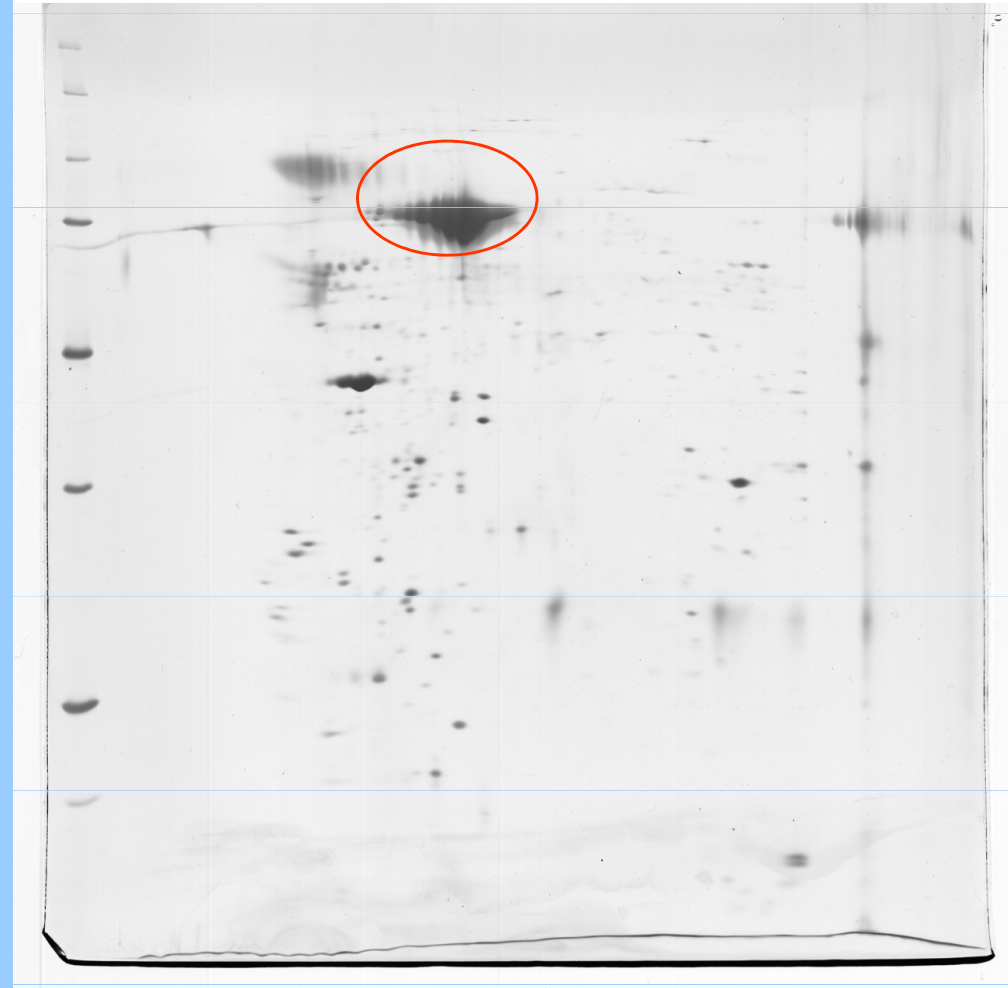
**identifikace**

**depletovaná plasma**



Calcium-Depleted Human C-Reactive Protein  
Amyloid related serum protein

**vesikly kmenových buněk**



BSA

# DIGESCE

trypsin   Glu-C   Asp-N   thermolysin



MAVEPFRRPITRPHASIEVDTS GTGG SAGSSEKVF  
CLIGQAEGGEPNTVYELR NYAQAKRLFRSGELLD  
AIELAWGSPNYTAGRILAMRIEDAKPASAEIGGL  
KITSKIYGNVANNIQV GLEKNTLSDSLRLRVIFQDD  
RFNEVYDNIGNIFTIKYKGEEANATFSVEHDEETQ  
KASRLVLKVG DQEVKSYDLTGGAYDYTNAITDIN  
QLPDFEAKLSPFGDKNLESSKLDKIENANIKDKAV  
YVKAVFGDLEKQTAYNGIVSFEQLNAEGEVPSNV  
EVEAGEESATVTATSPIK TIEPFELTKLKG GTNGEPP  
ATWADKLDKFAHEGGYYIVPLSSKQSVHAEVASF  
VKERSDAGEPMRAIVGGGFNESKEQLFGRQASLS  
NPRVSLVANS GTFVMDDGRKNHVPAYMVAVALGG  
LASGLEIGESITFKPLRVSSLDQIYESIDLDELNENG  
IISIEFVRNRTNTFFRIVDDVTTFN DKSDPVKAEMA  
VGEANDFLVSELKVQLEDQFIGTRTINTSASI KDFI  
QSYLGRKKRDNEIQDFPAEDVQVIVEGNEARISMT  
VYPIRSFKKISVSLVYKQQT LQA

- IN-GEL
- IN-SOLUTION



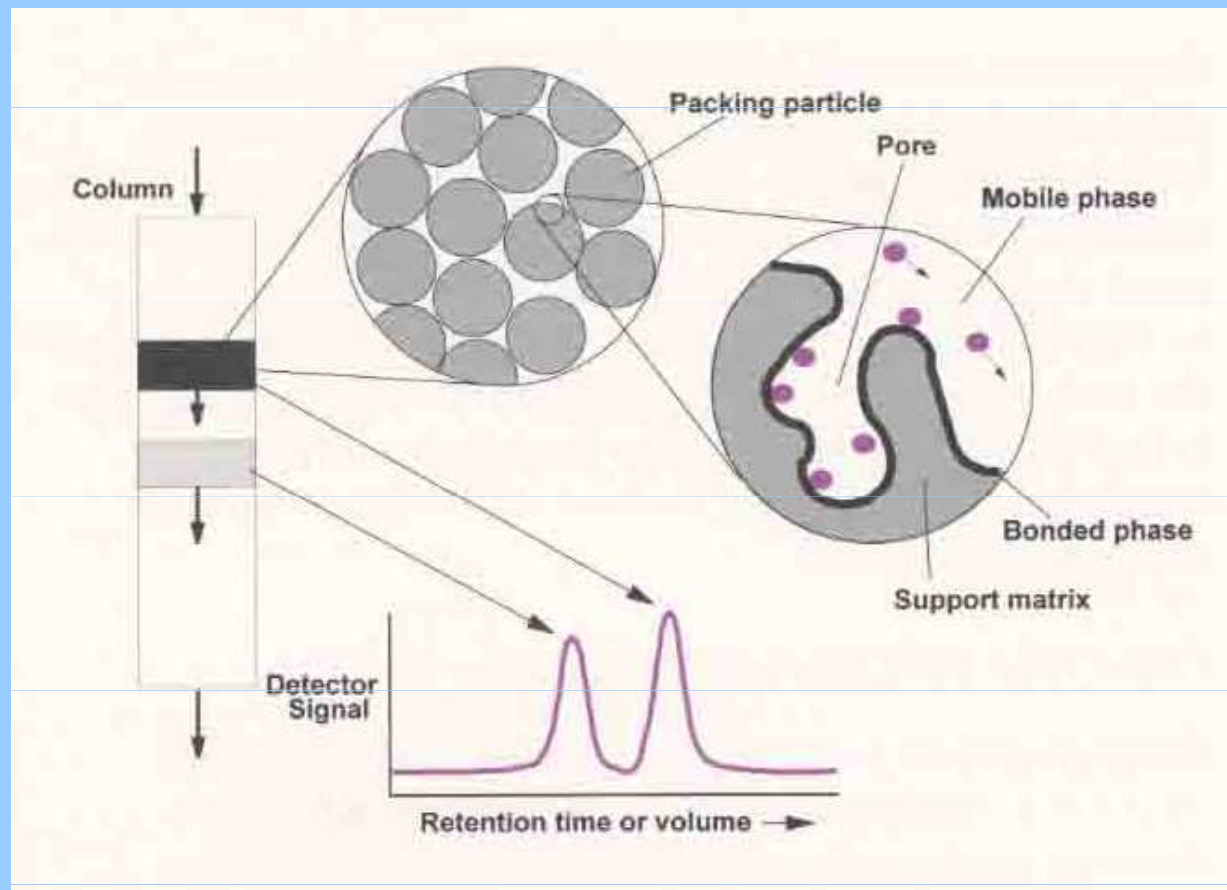
MS

ALTERNATIVA 2D GE

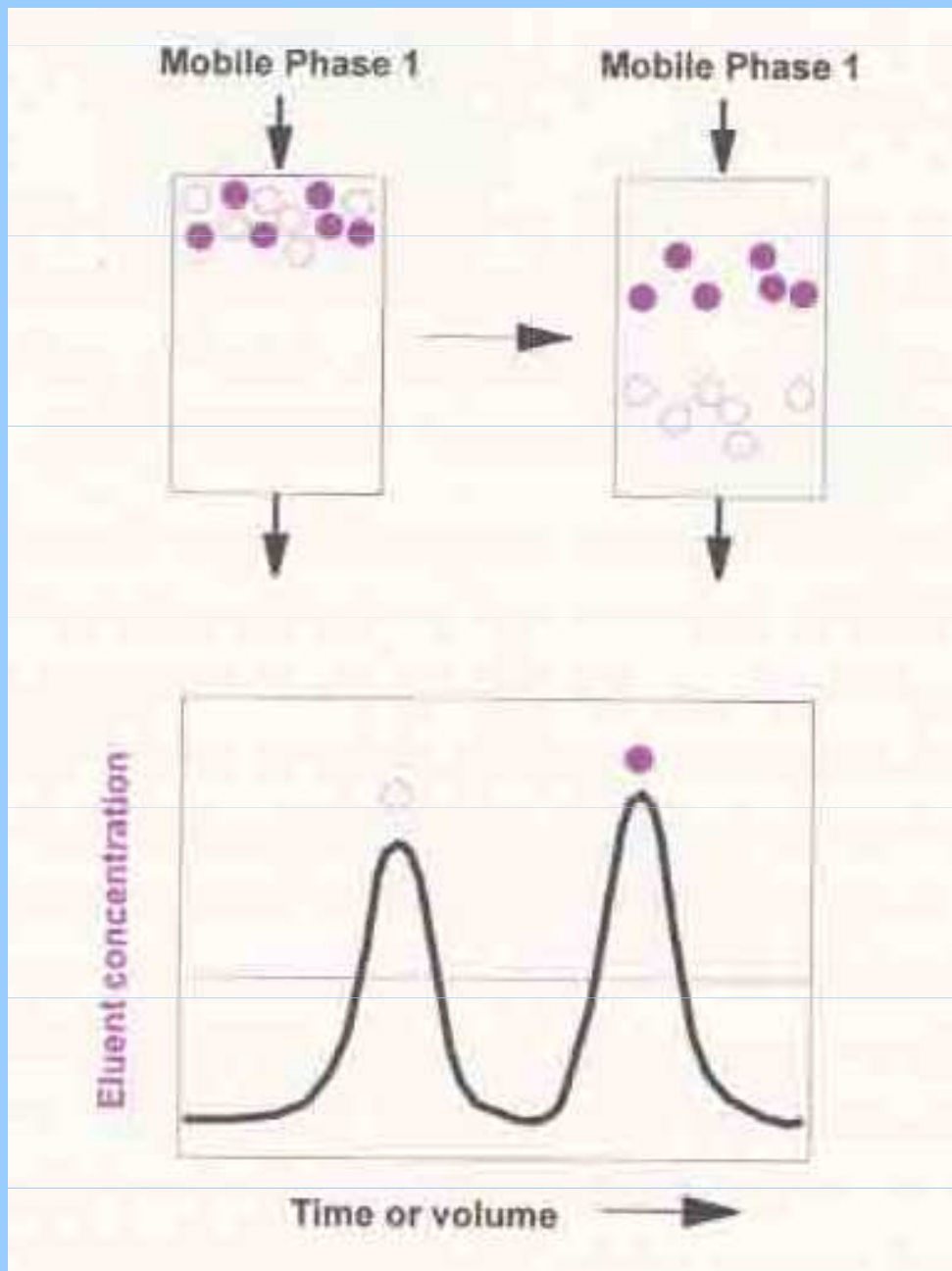
---

# MULTIDIMENZIONÁLNÍ CHROMATOGRRAFIE

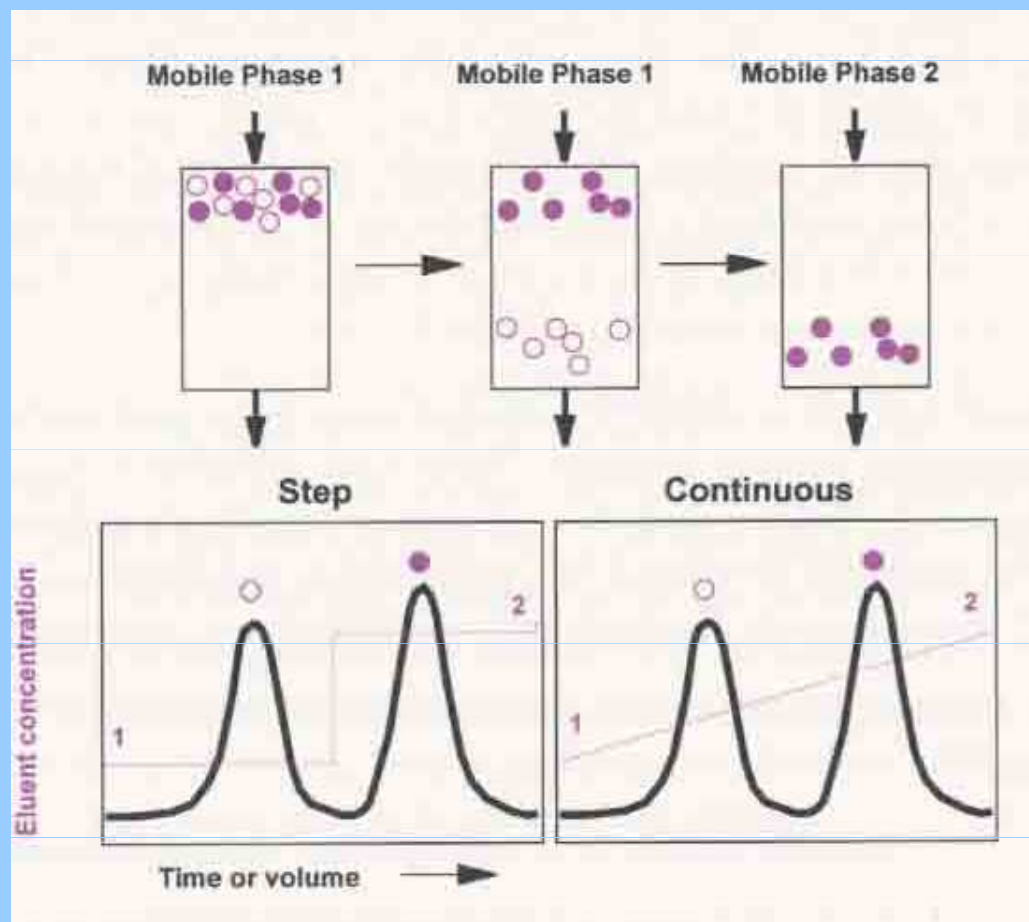
# PROTEINY A PEPTIDY PRINCIPY SEPARACE KAPALINOVOU CHROMATOGRafiÍ



# Isokratická eluce



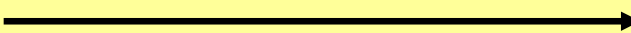
# Gradientová eluce



# TYPY LC SEPARACE

---

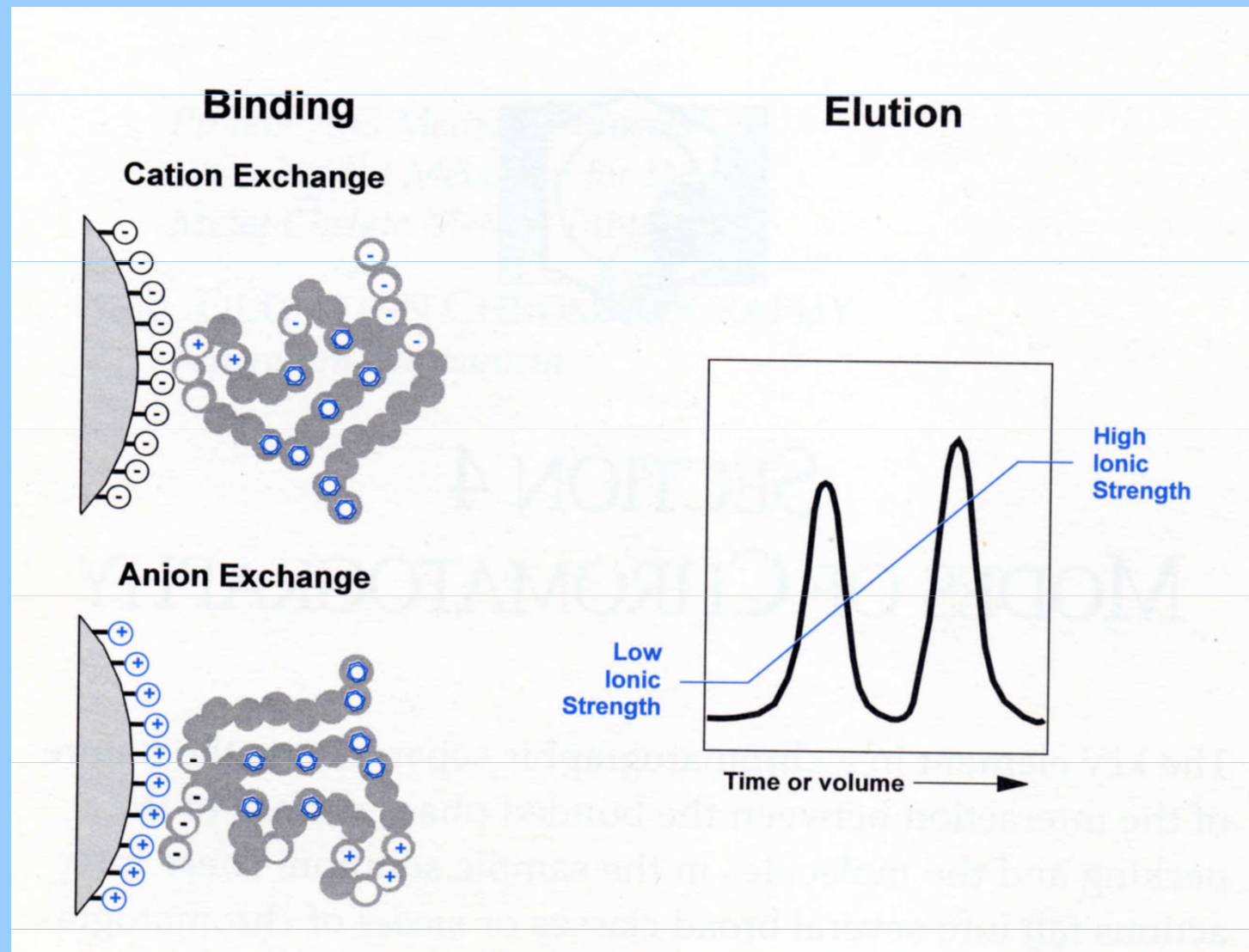
CO ROZHODUJE



KOLONA

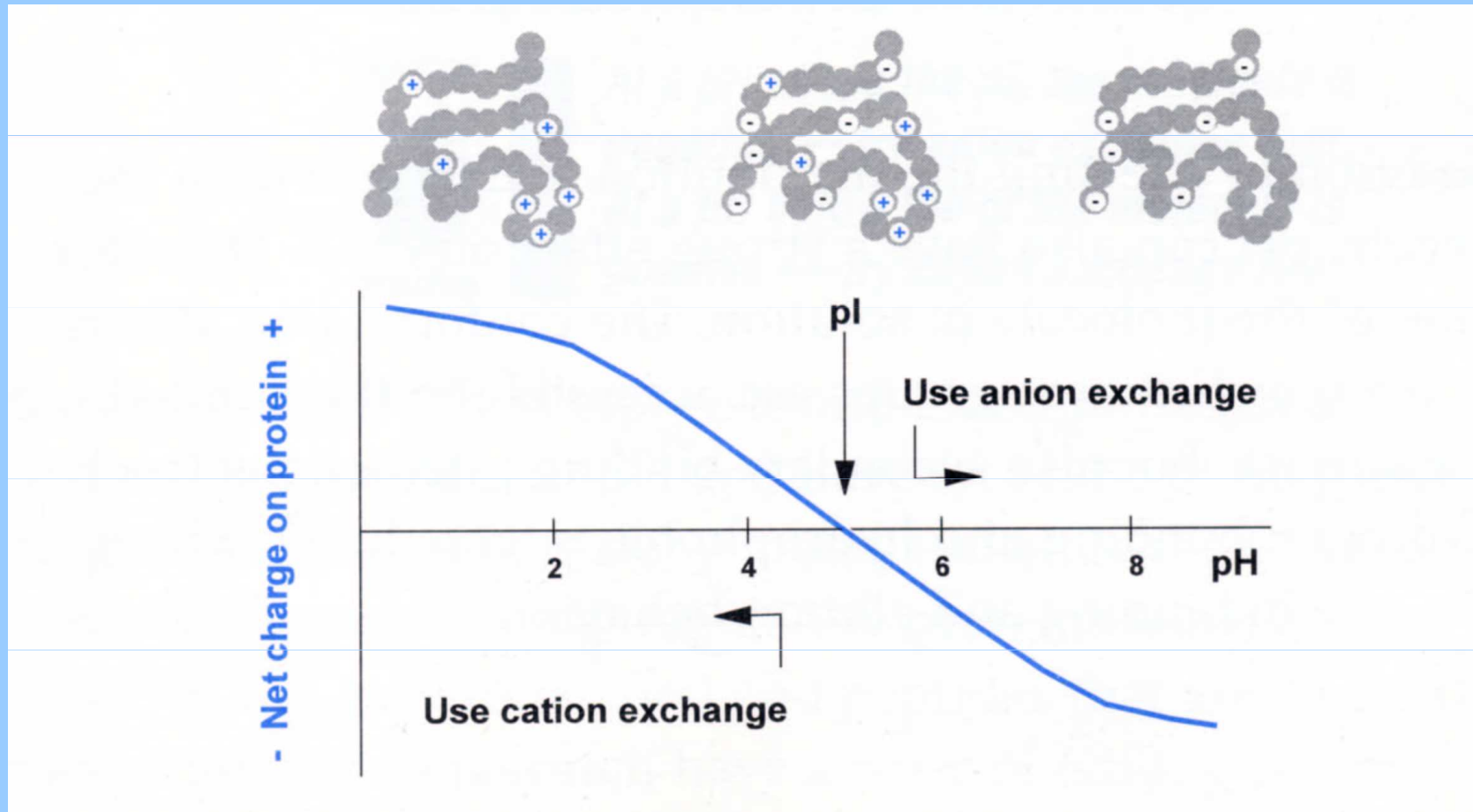
- |                         |               |
|-------------------------|---------------|
| ▪ náboj                 | ionex         |
| ▪ hydrofobicita         | reverzní fáze |
| ▪ biospecifická afinita | afinitní      |
| ▪ velikost molekuly     | gelová        |

# IONEXOVÁ CHROMATOGRAFIE





# Isoelektrický bod



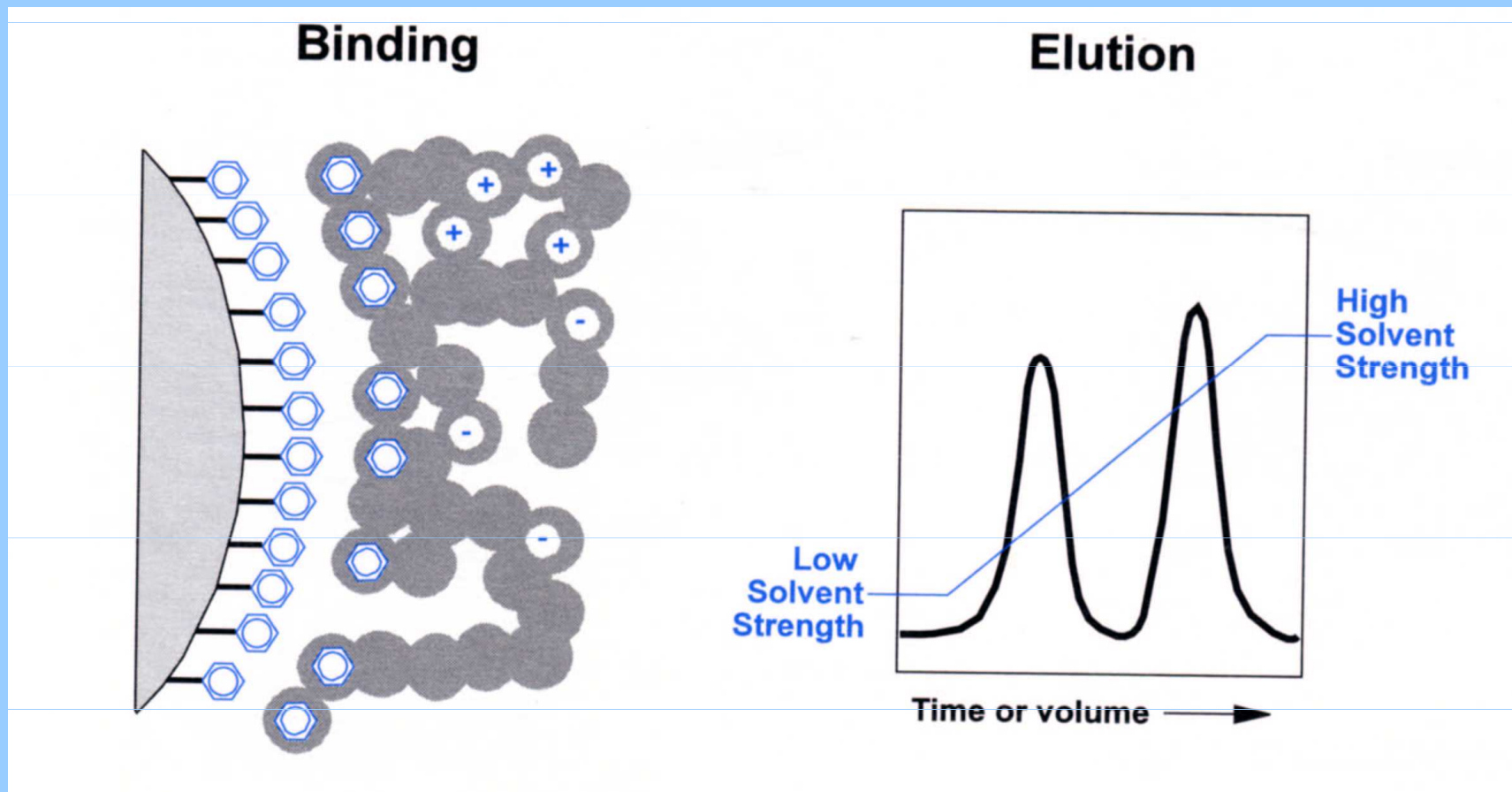
## Funkční skupiny u ionexových maticí

**Table 2.1** Functional groups used on ion exchangers.

Anion exchangers	Functional group
Diethylaminoethyl (DEAE)	$-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}^+\text{H}(\text{CH}_2\text{CH}_3)_2$
Quaternary aminoethyl (QAE)	$-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}^+(\text{C}_2\text{H}_5)_2-\text{CH}_2\text{CHOH}-\text{CH}_3$
Quaternary ammonium (Q)	$-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$
Cation exchangers	Functional group
Carboxymethyl (CM)	$-\text{O}-\text{CH}_2-\text{COO}^-$
Sulfopropyl (SP)	$-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{CH}_2\text{SO}_3^-$
Methylsulfonate (S)	$-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2\text{SO}_3^-$

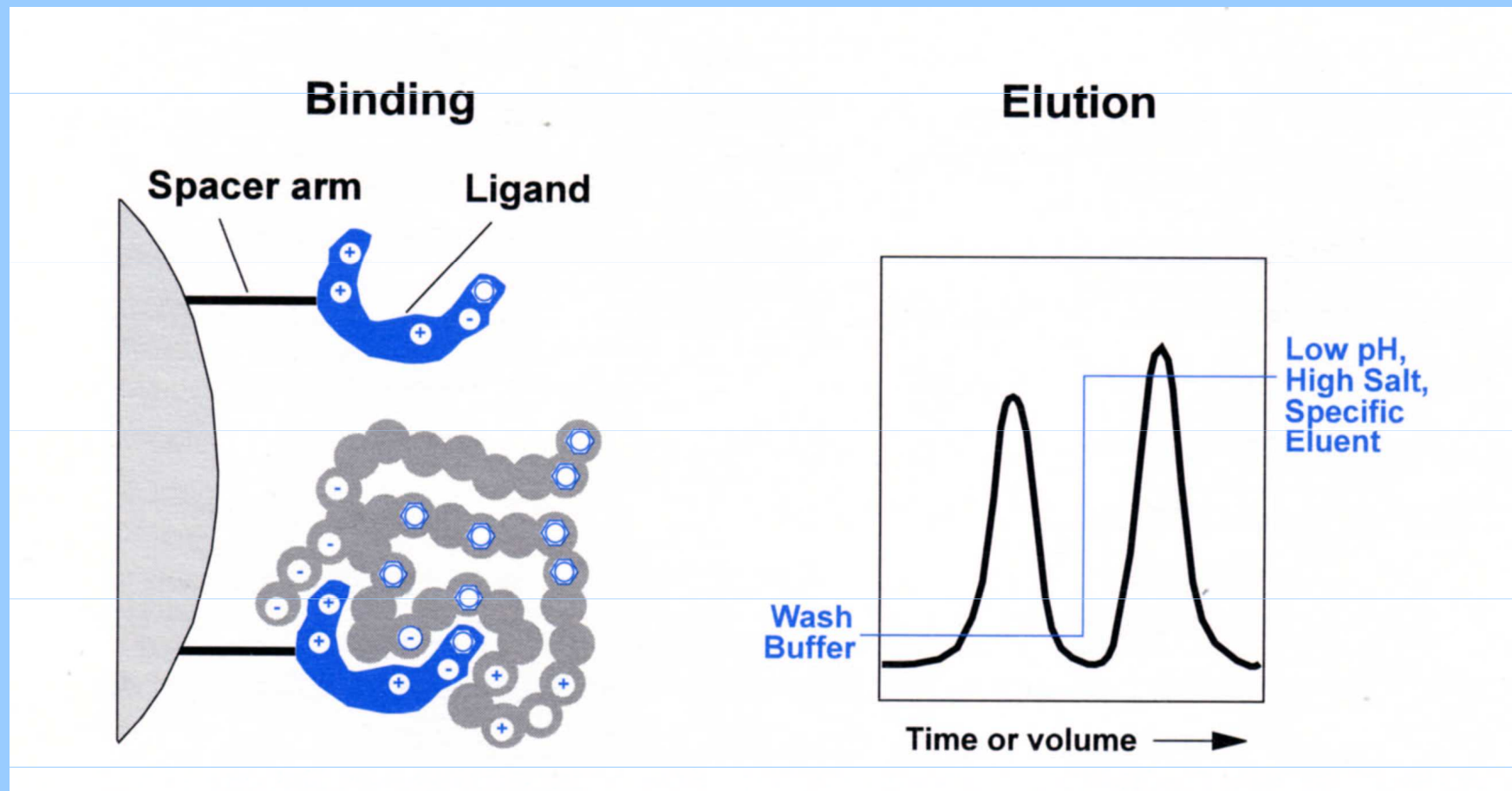
# RP CHROMATOGRAFIE

reversed-phase chromatography





# AFINITNÍ CHROMATOGRAFIE

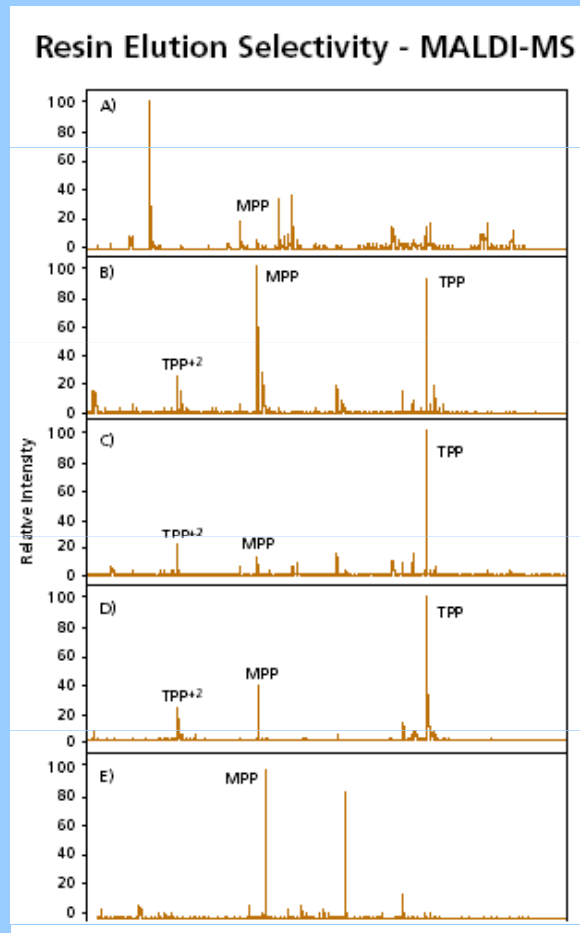


# IMAC

## Immobilized Metal Affinity Chromatography

### PHOS Select Iron Affinity gel

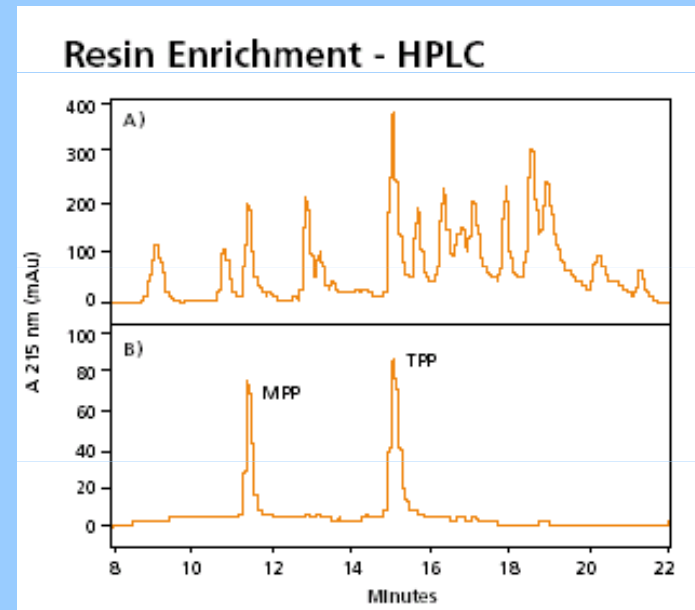
Surový



Po aplikaci

Kasein tryptic digest

### Resin Enrichment - HPLC

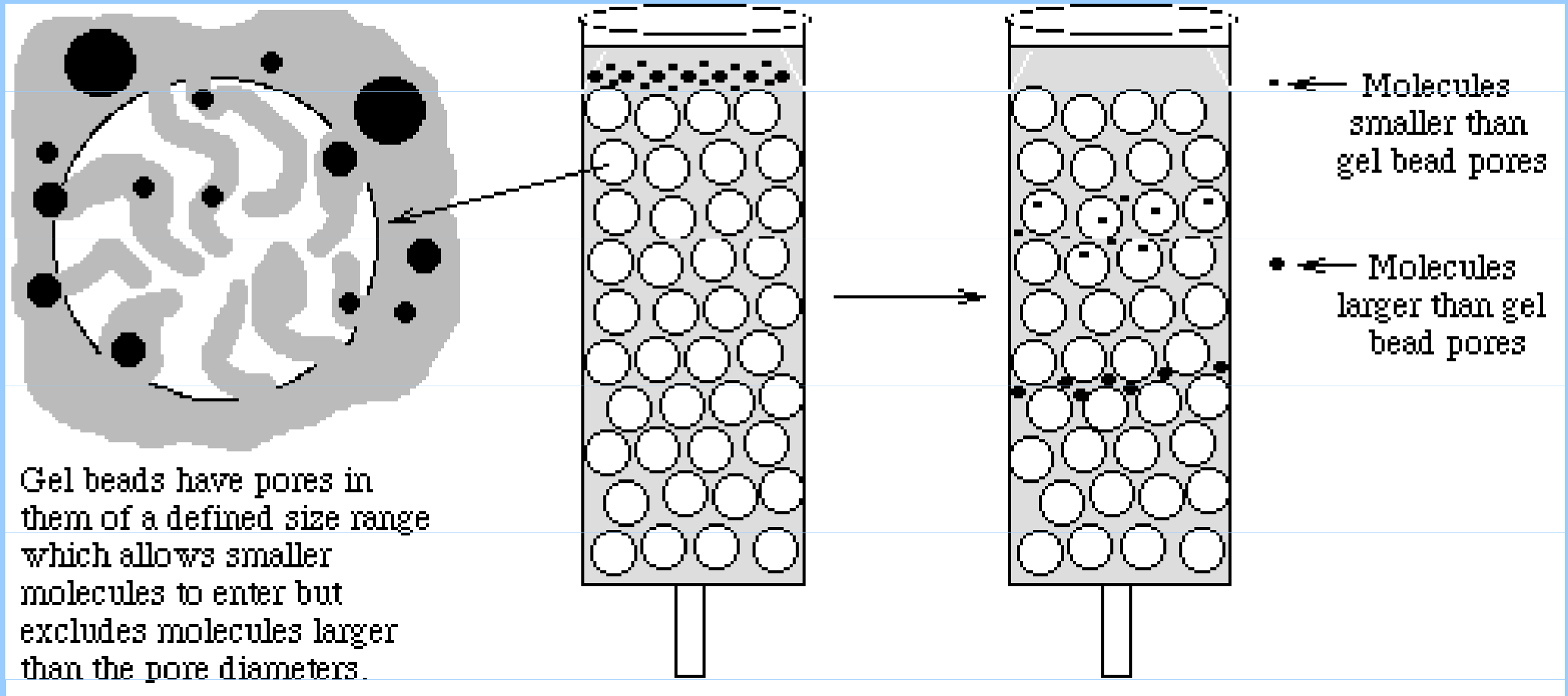


Surový

Po aplikaci

Kasein

# GELOVÁ CHROMATOGRAFIE



# KAPALINOVÁ CHROMATOGRRAFIE

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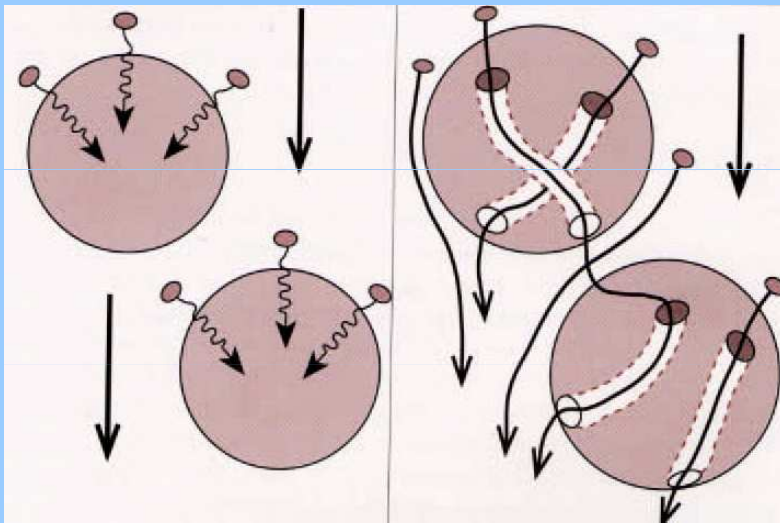
→ HPLC

→ LC

→ **PERFÚZNÍ**



# POROS



klasický sorbent

POROS

- RP
- SAX**
- WAX
- SCX
- WCX
- HIC

Activated Affinity

**Affinity**

**Application-Specific RP**

## Choose the POROS Chemistry

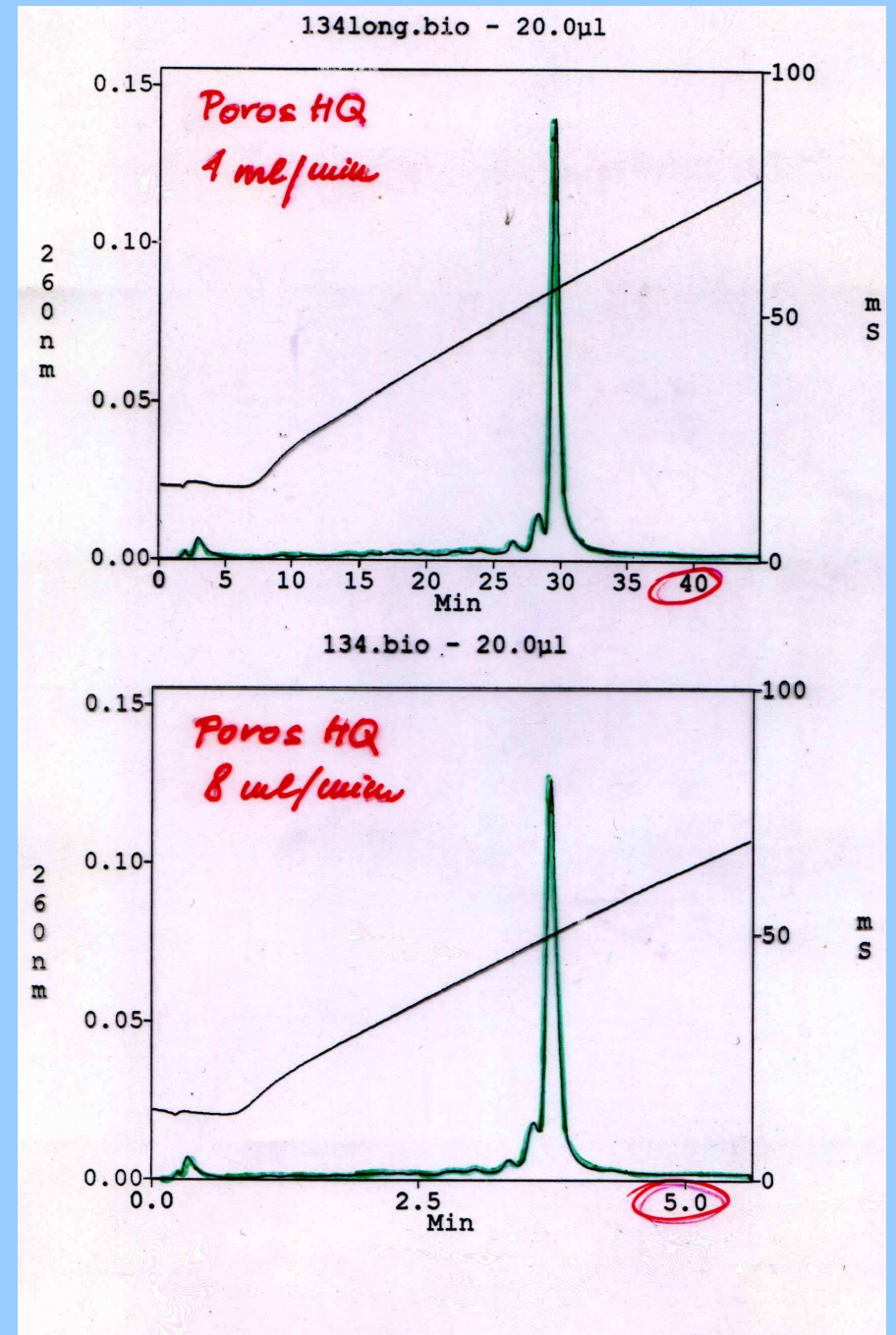
Once you've selected a particular mode of chromatography, you will have a range of POROS surface selectivity, resulting from differences in functional group and/or functional group density. While it may not be possible to select the ideal chemistry, it can be advantageous to explore alternatives. With the range of POROS chemistries and their dynamic capacities, it becomes a powerful new variable to exploit when optimizing your separation.

	PRODUCT	FUNCTIONAL GROUP	FUNCTIONAL GROUP DENSITY	DYNAMIC CAPACITY*	REMARKS
<b>REVERSED-PHASE CHROMATOGRAPHY</b>					
	<b>R1</b>	Base Poly(styrene divinyl benzene)	Low phase ratio, providing low retentivity	5 mg/mL	For RP1
	<b>R2</b>	Base Poly(styrene divinyl benzene)	High phase ratio, providing higher binding strength and retentivity	10 mg/mL	Good for RP2
<b>ION-EXCHANGE CHROMATOGRAPHY</b>					
<b>STRONG ANION EXCHANGERS</b>	<b>HQ</b>	Quaternized polyethyleneimine	High	55 mg/mL	For basic proteins
	<b>QE</b>	Quaternized polyethyleneimine	Medium	30 mg/mL	Alternative for basic proteins
<b>WEAK ANION EXCHANGERS</b>	<b>DEAE</b>	Diethylaminoethyl	Medium	55 mg/mL	For basic proteins
	<b>PI</b>	Polyethyleneimine	Medium	45 mg/mL	Alternative for basic proteins
<b>STRONG CATION EXCHANGERS</b>	<b>HS</b>	Sulphopropyl	High	75 mg/mL (POROS 50 60 mg/mL)	For acidic proteins
	<b>SP</b>	Sulphopropyl	Medium	45 mg/mL	Alternative for acidic proteins
	<b>S</b>	Sulphoethyl	Low	10 mg/mL	Specialized for acidic proteins
<b>WEAK CATION EXCHANGER</b>	<b>CM</b>	Carboxymethyl	High	70 mg/mL	For acidic proteins
<b>HYDROPHOBIC INTERACTION CHROMATOGRAPHY</b>					
	<b>HP2</b>	High density phenyl	High	12 mg/mL	For hydrophobic proteins
	<b>PE</b>	Phenyl ether	Medium	8 mg/mL	Alternative for hydrophobic proteins
	<b>ET</b>	Ethyl ether	Medium	4 mg/mL	Specialized for hydrophobic proteins
<b>ACTIVATED AFFINITY CHROMATOGRAPHY</b>					
<b>FOR ACTIVATION</b>	<b>OH</b>	Hydroxyl			Alternative for affinity chromatography
	<b>AL</b>	Aldehyde			Alternative for affinity chromatography
<b>PRE-ACTIVATED</b>	<b>EP</b>	Epoxide			Alternative for affinity chromatography
	<b>NH</b>	Primary amine			Alternative for affinity chromatography
	<b>HY</b>	Hydrazide			Alternative for affinity chromatography
<b>AFFINITY CHROMATOGRAPHY</b>					
	<b>A</b>	Recombinant Protein A		30 mg/mL	For antibodies
	<b>G</b>	Recombinant Protein G		15 mg/mL	Alternative for antibodies
	<b>HE</b>	Heparin		15 mg/mL	For glycoproteins
	<b>MC</b>	Imido-diacetate		15 mg/mL	Alternative for glycoproteins
<b>APPLICATION-SPECIFIC REVERSED-PHASE MEDIA</b>					
	<b>Oligo R3</b>	Poly(styrene-divinyl benzene)	Very high phase ratio	30 mg/mL	Specialized for oligonucleotides
	<b>PepMap C18</b>	Silica C18, end-capped	7% carbon loading		Specialized for peptides

\*Test protein



BioCAD 700E

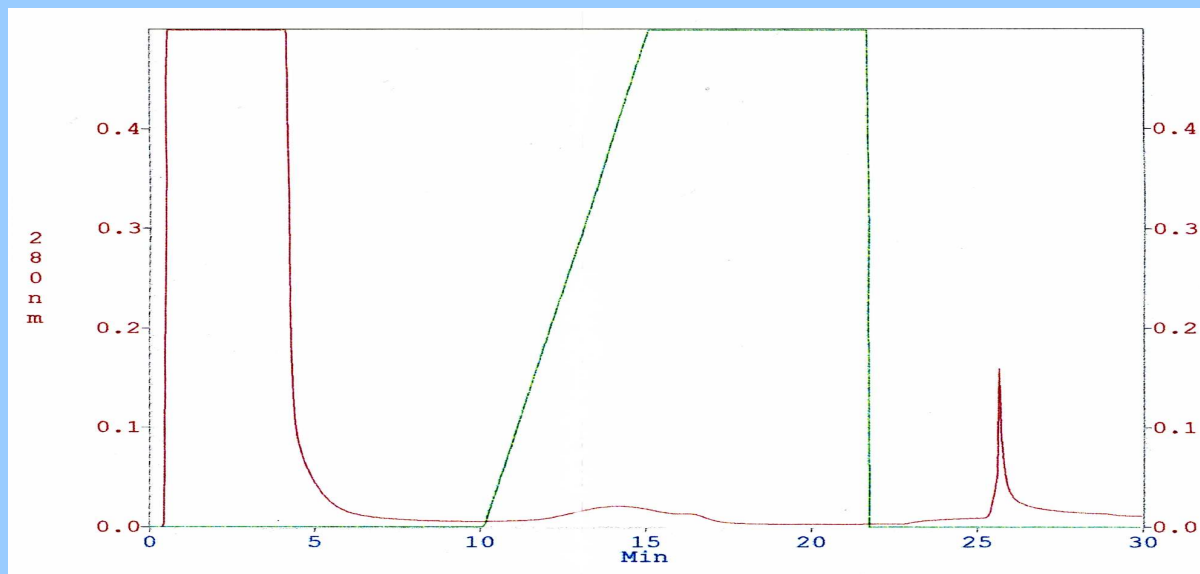


# MC Poros

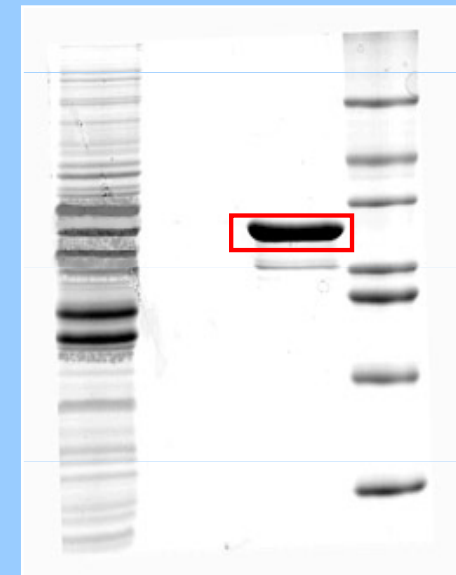
Metalochelatační purifikace  
his-tag proteinu

Petra Borkovcová

## Ni<sup>2+</sup>MC POROS

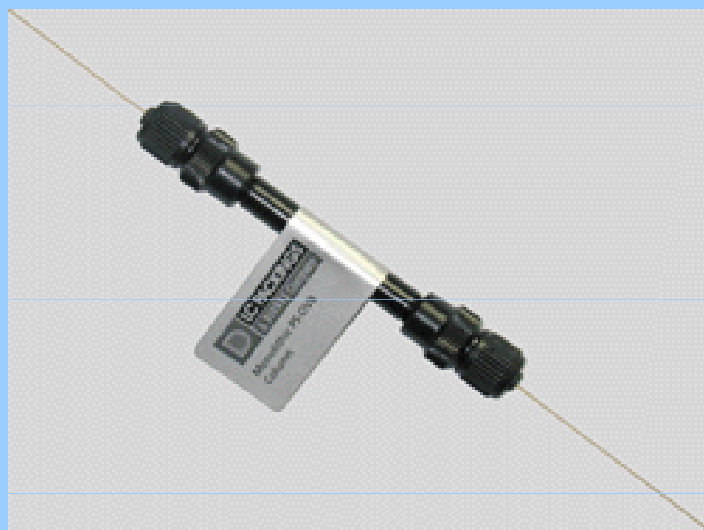


Čistota 95 %



Perfúzní MC chromatografie	1 hodina
Agaróza MC chromatografie	2 hodiny
Gelová chromatografie	10 hodin

# Kolona může vypadat různě . . .

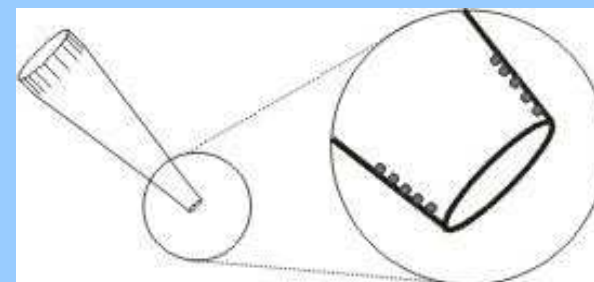
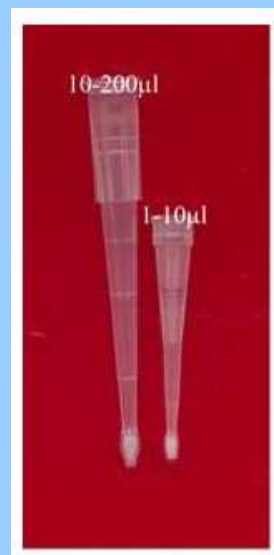


Glygen

TopTip

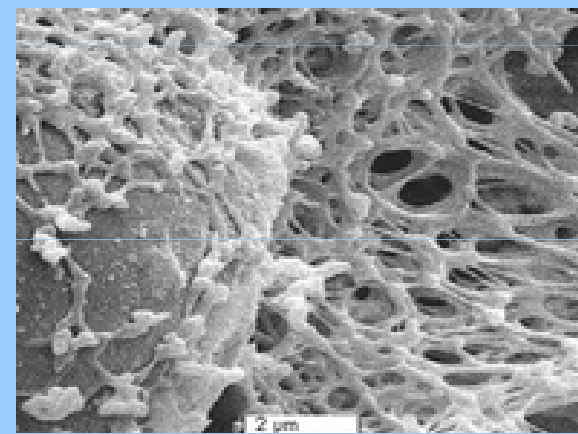


NuTip



Zip Tip  
Millipore

C18  
C4  
MC  
SCX



# MULTIDIMENZIONÁLNÍ CHROMATOGRAFIE

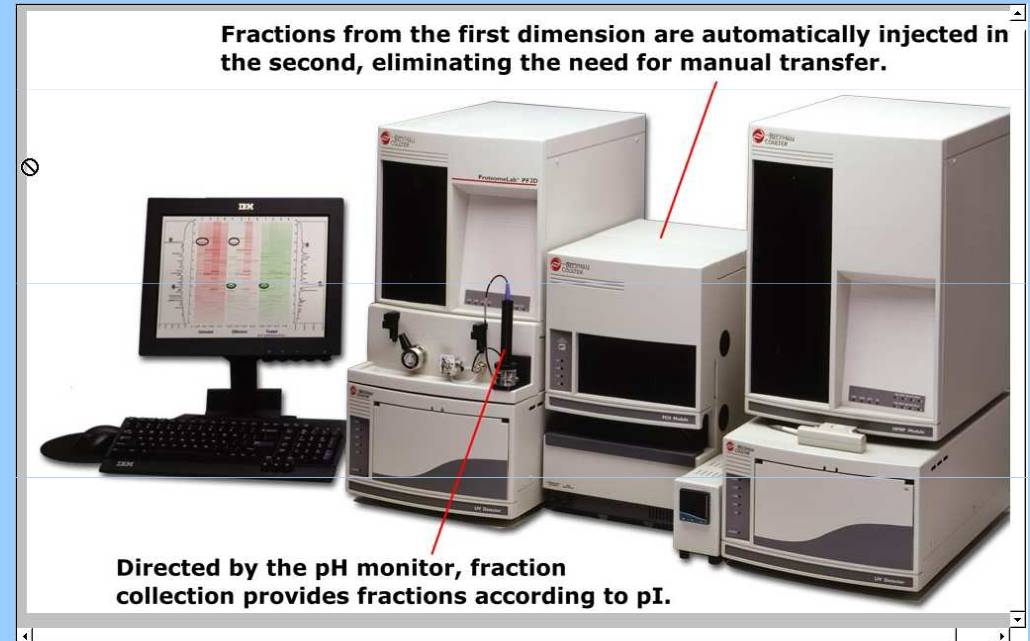
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kombinace odlišných fyzikálních a chemických separačních principů

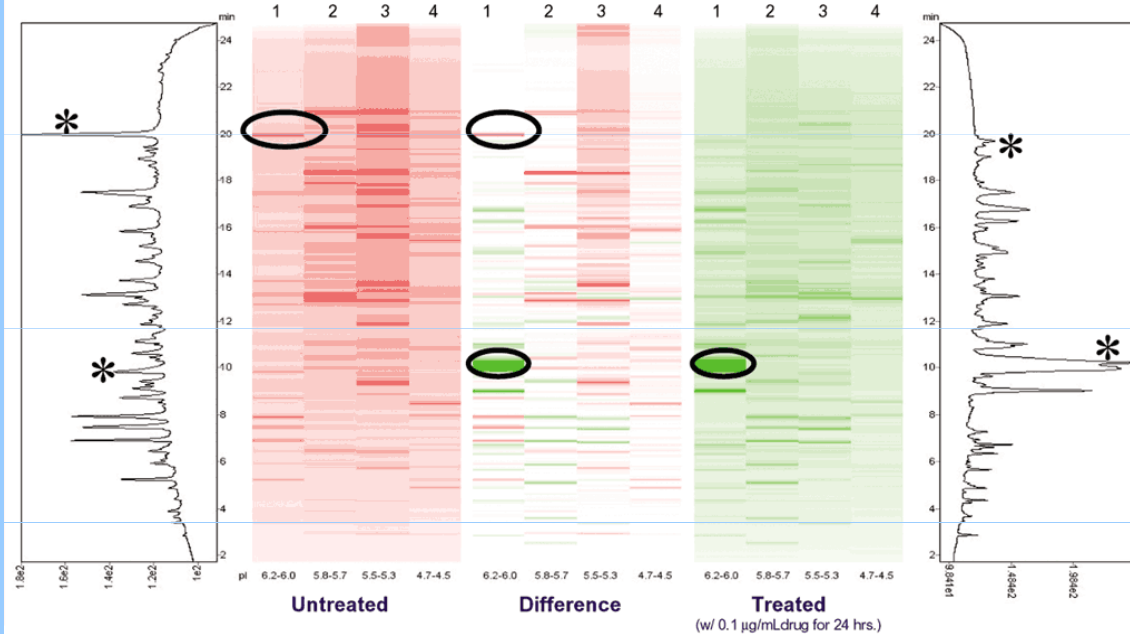
- diskontinuální
- kontinuální
- dvoufázová kolona

# ProteomeLab PF 2D

- chromatofokusace
- RP



Partial pI/UV map of colon cancer cell line before and after treatment



# MULTIDIMENZIONÁLNÍ CHROMATOGRRAFIE

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## PRO

- velké objemy vzorku
- možnost koncentrace na koloně
- membránové proteiny, basické proteiny
- není nutno barvit
- peptidy – přímé napojení na MS
- automatizace

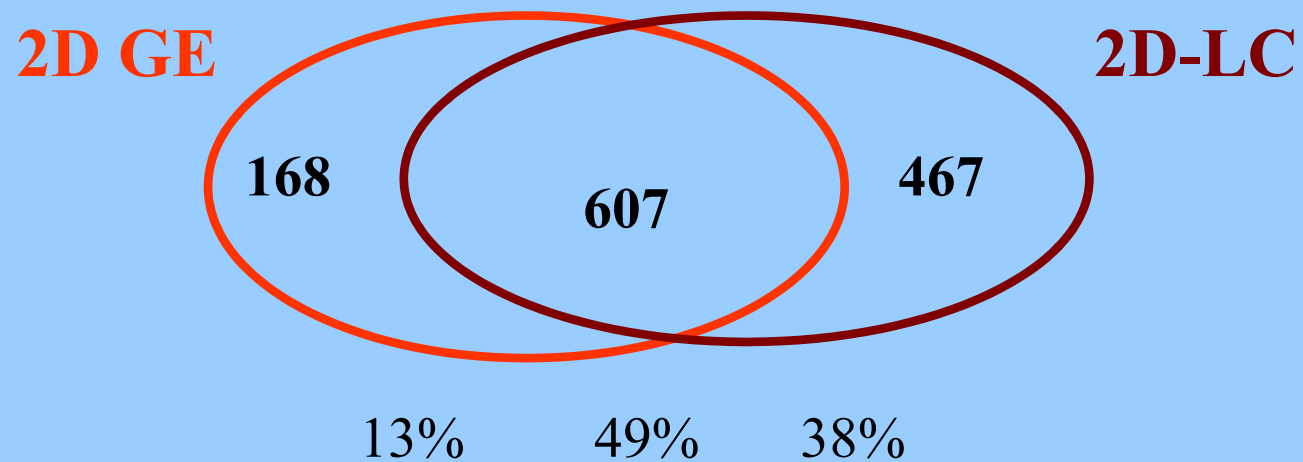
## PROTI

- vizuální aspekty ztraceny: pI a Mr
- LC je sériová analýza
- GE může běžet současně pro více vzorků



## KOMPLEMENTARITA METOD

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## LITERATURA

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- R.M. Twyman: Principles of Proteomics
- R.Westermeier, T.Naven: Proteomics in Practice
- A.J.Link: 2D Proteome Analysis Protocols
- T.Rabilloud: Proteome Research: Two-dimensional Gel Electrophoresis and Identification Methods
- Busy Researcher's Guide to Biomolecule Chromatography
- Current Protocols in Protein Science

**G I G O**

**G I G O**

**GARBAGE IN - GARBAGE OUT**