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

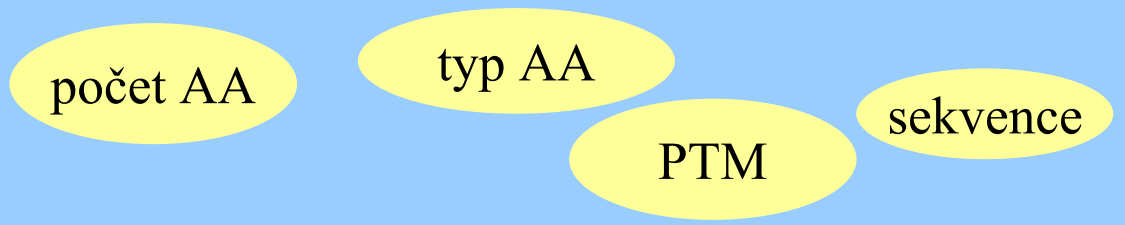
- komplexita 10^6 ?
- dynamika
- sekvence
- struktura
- abundance
- lokalizace
- modifikace
- interakce
- biochemická funkce

geny - nositelé instrukce

proteiny - vykonavatelé instrukce

STRATEGIE SEPARACE

- fyzikální rozdíly
- chemické rozdíly



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

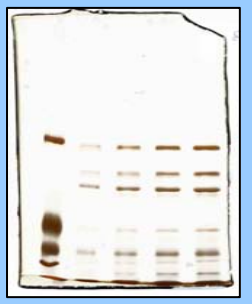
  MudPIT

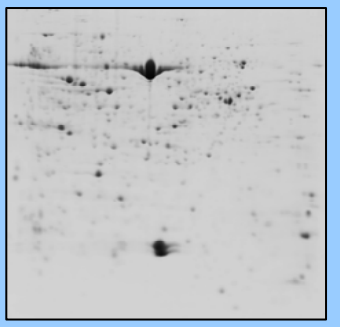
  ICAT...

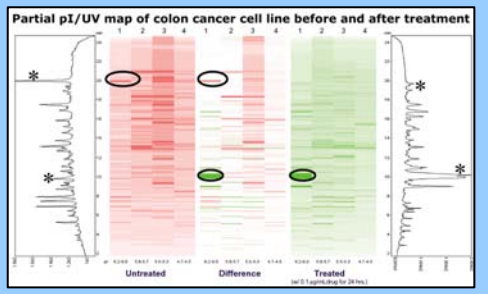
- proteinové čipy

  Functional protein arrays (protein proteinové interakce)

  Affinity arrays







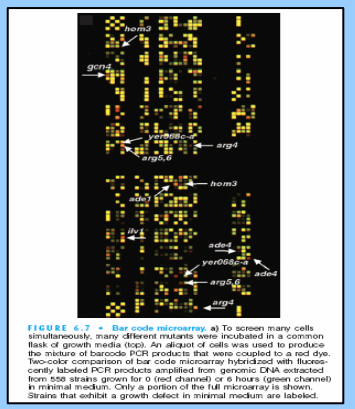
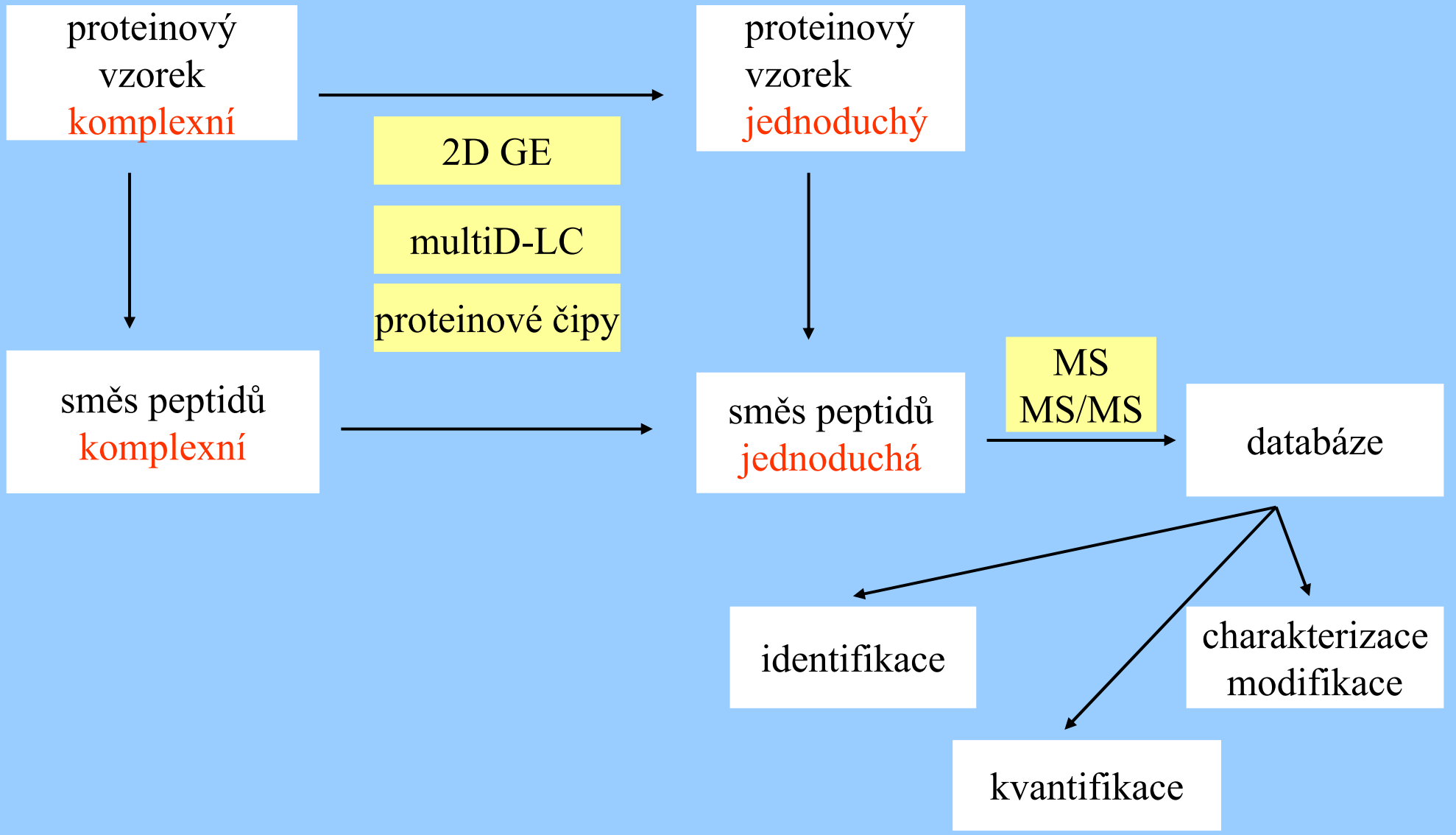


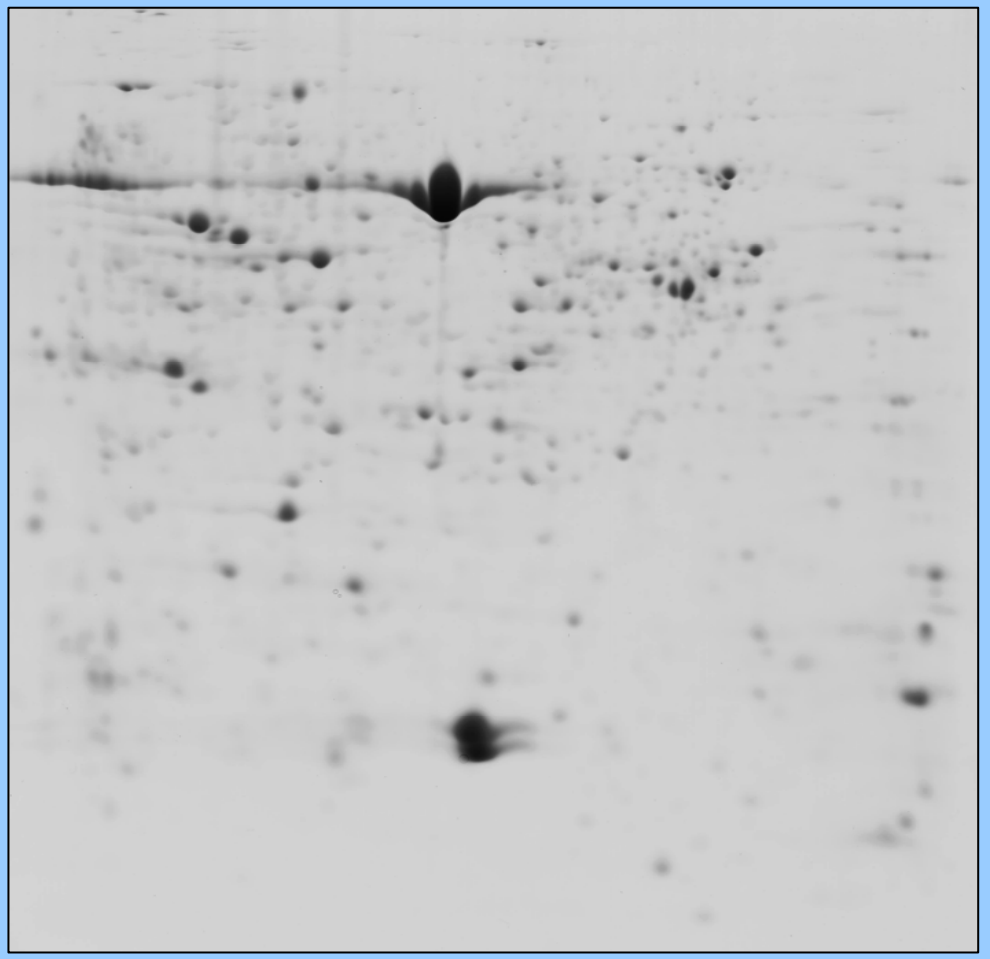
FIGURE 6.7 • Bar code microarray. a) To screen many cells simultaneously, many different mutants were incubated in a common flask of growth media (top). An aliquot of cells was used to produce the mixture of barcode PCR products that were coupled to a red dye. Two-color comparison of bar code microarray hybridized with fluorescently labeled PCR products amplified from genomic DNA extracted from 558 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.

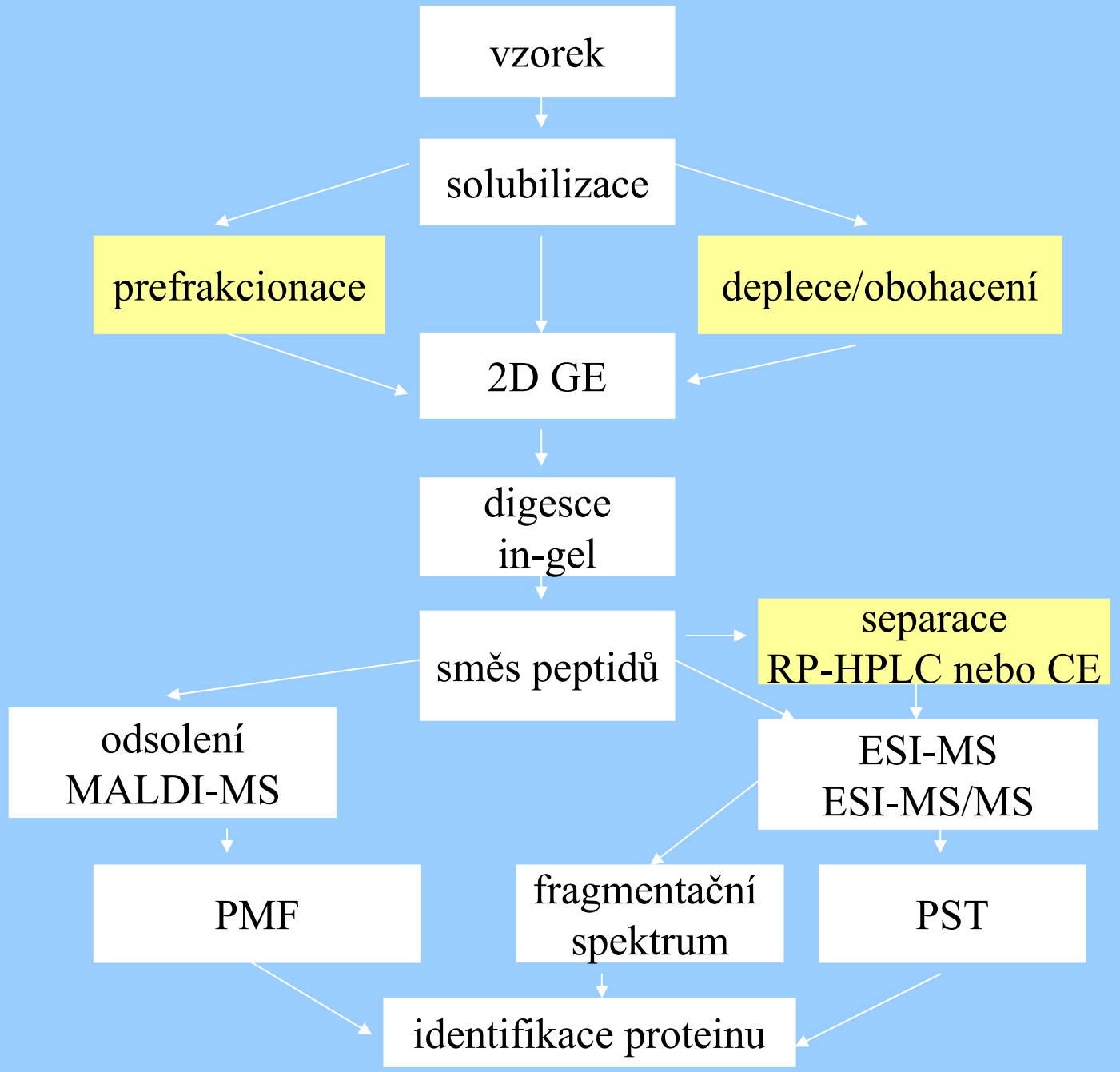

proteinový vzorek komplexní
2D GE
multiD-LC
proteinové čipy
proteinový vzorek jednoduchý
směs peptidů komplexní
směs peptidů jednoduchá
MS MS/MS
databáze
identifikace
charakterizace modifikace
kvantifikace

→ dvourozměrná gelová elektroforéza 2D GE

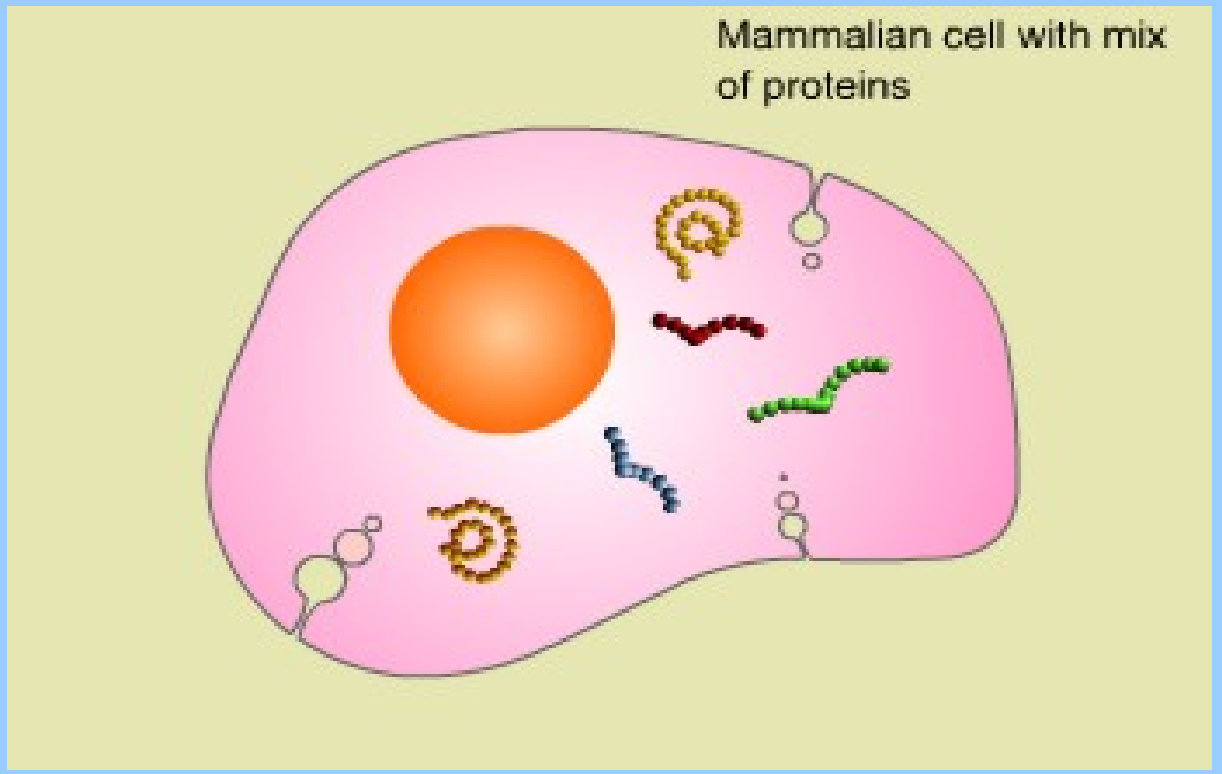
→ (multidimenzionální) kapalinová chromatografie

DVOUROZMĚRNÁ GELOVÁ ELEKTROFORÉZA 2D GE




vzorek
solubilizace
prefrakcionace
deplece/obohacení
2D GE
digesce
in-gel
směs peptidů
separace
RP-HPLC nebo CE
odsolení
MALDI-MS
ESI-MS
ESI-MS/MS
PMF
fragmentační
spektrum
PST
identifikace proteinu

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

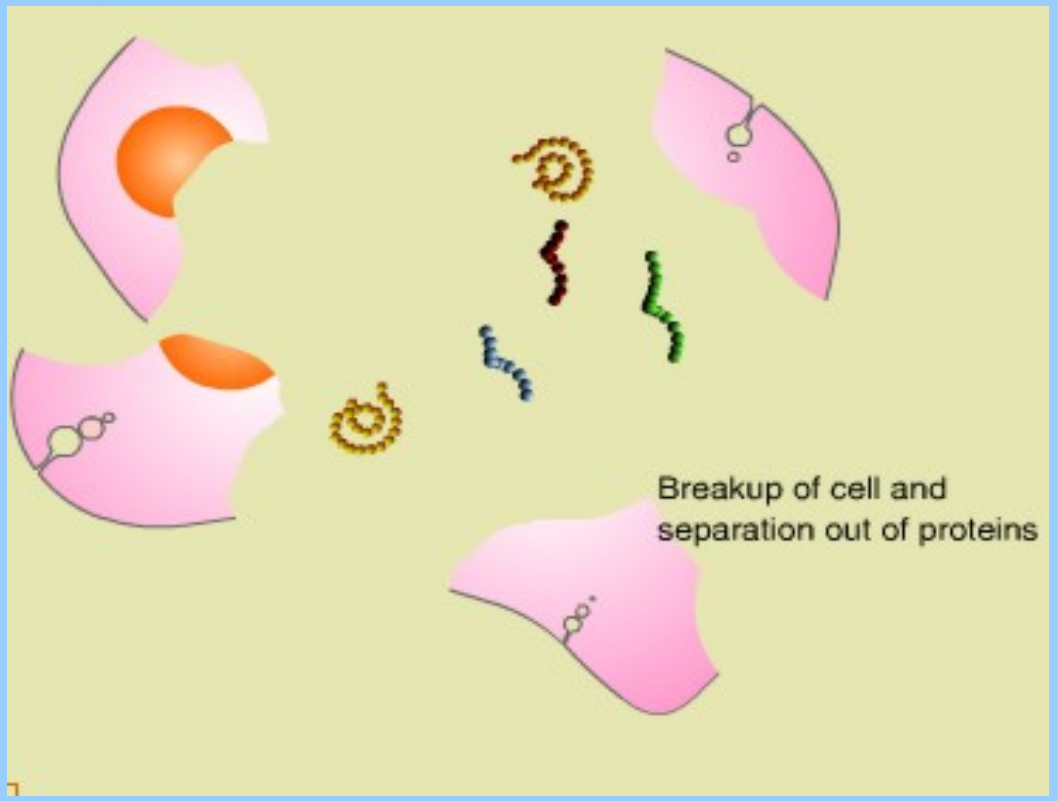
ZÁKLADNÍ PRAVIDLA

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

KONTAMINANTY

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

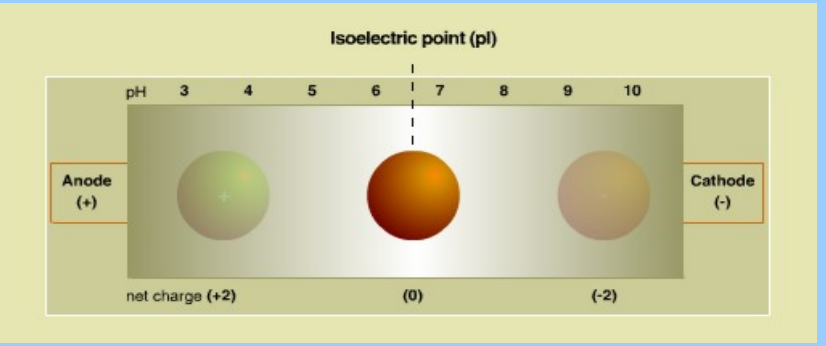
SOLUBILIZACE



2D GE

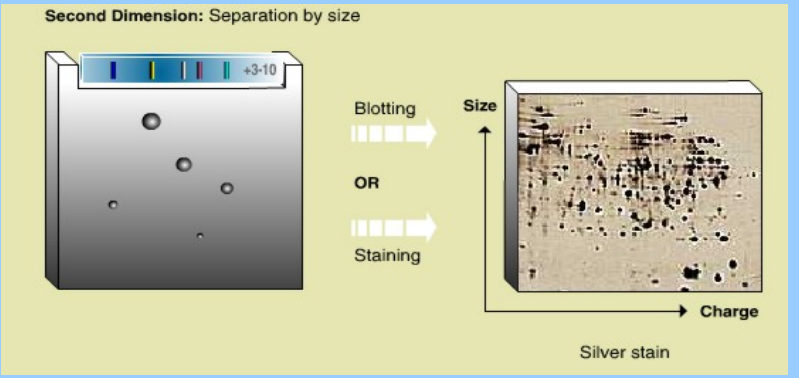
- první rozměr

IEF

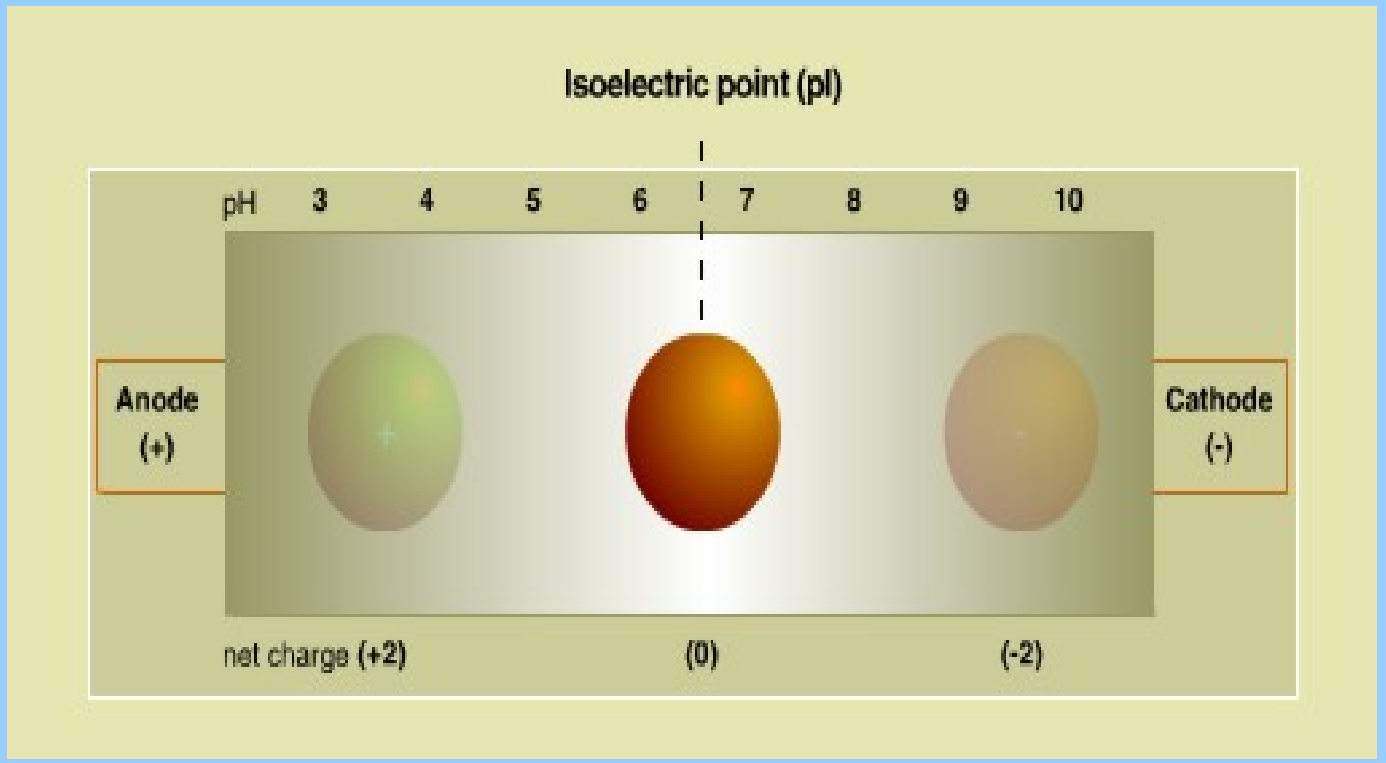


- druhý rozměr

SDS-PAGE

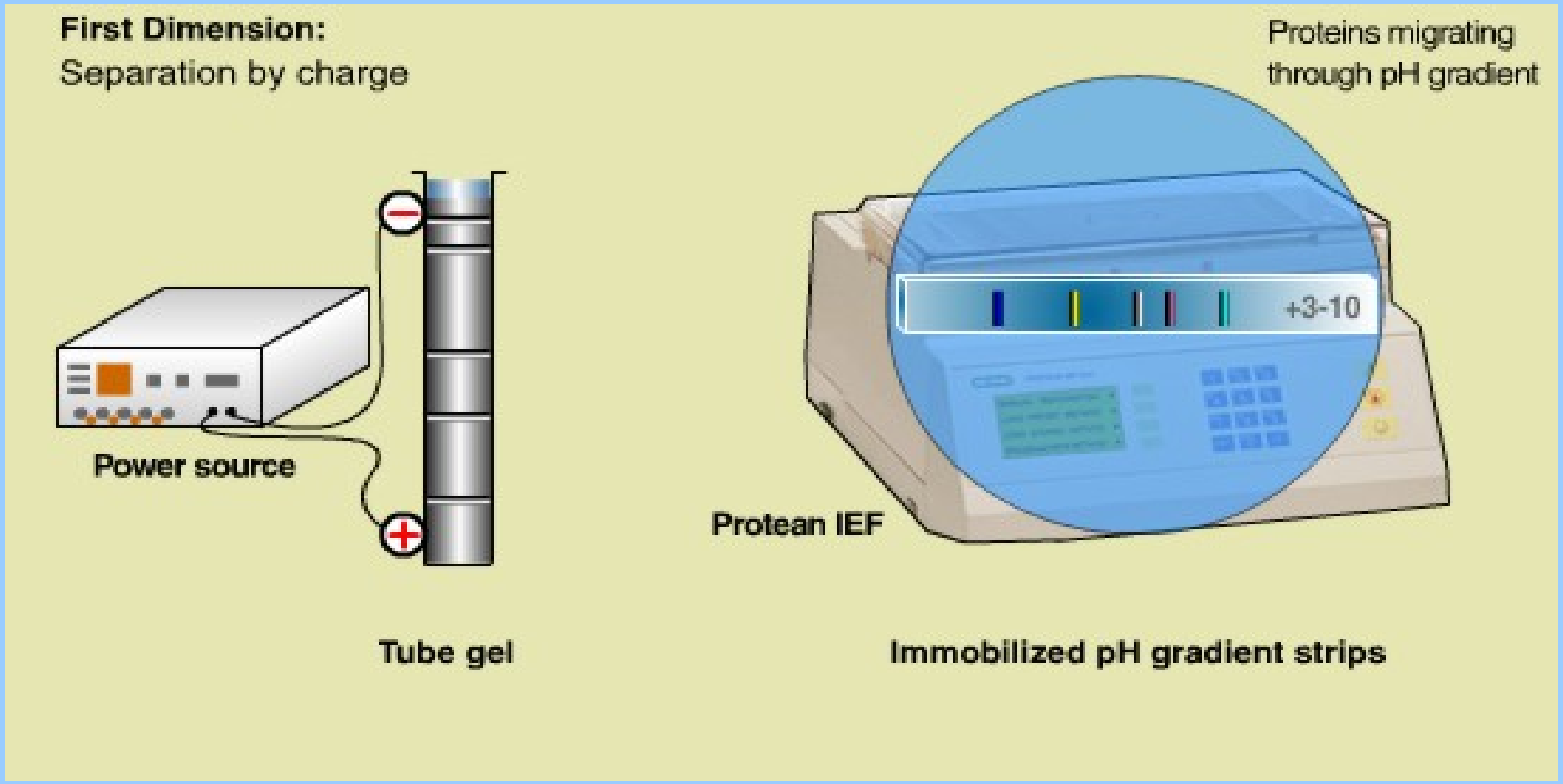


ISOELEKTRICKÝ BOD



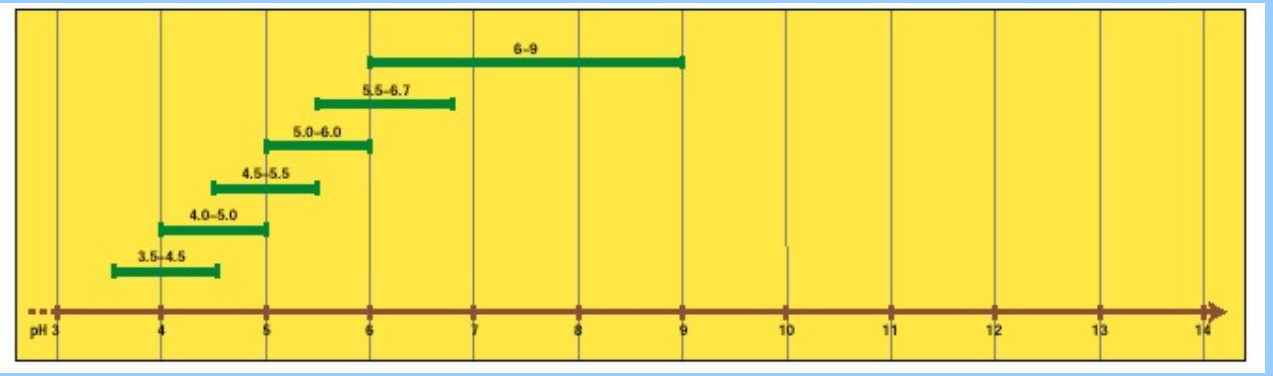
PRVNÍ ROZMĚR 2D GE

ISOELEKTRICKÁ FOKUSACE



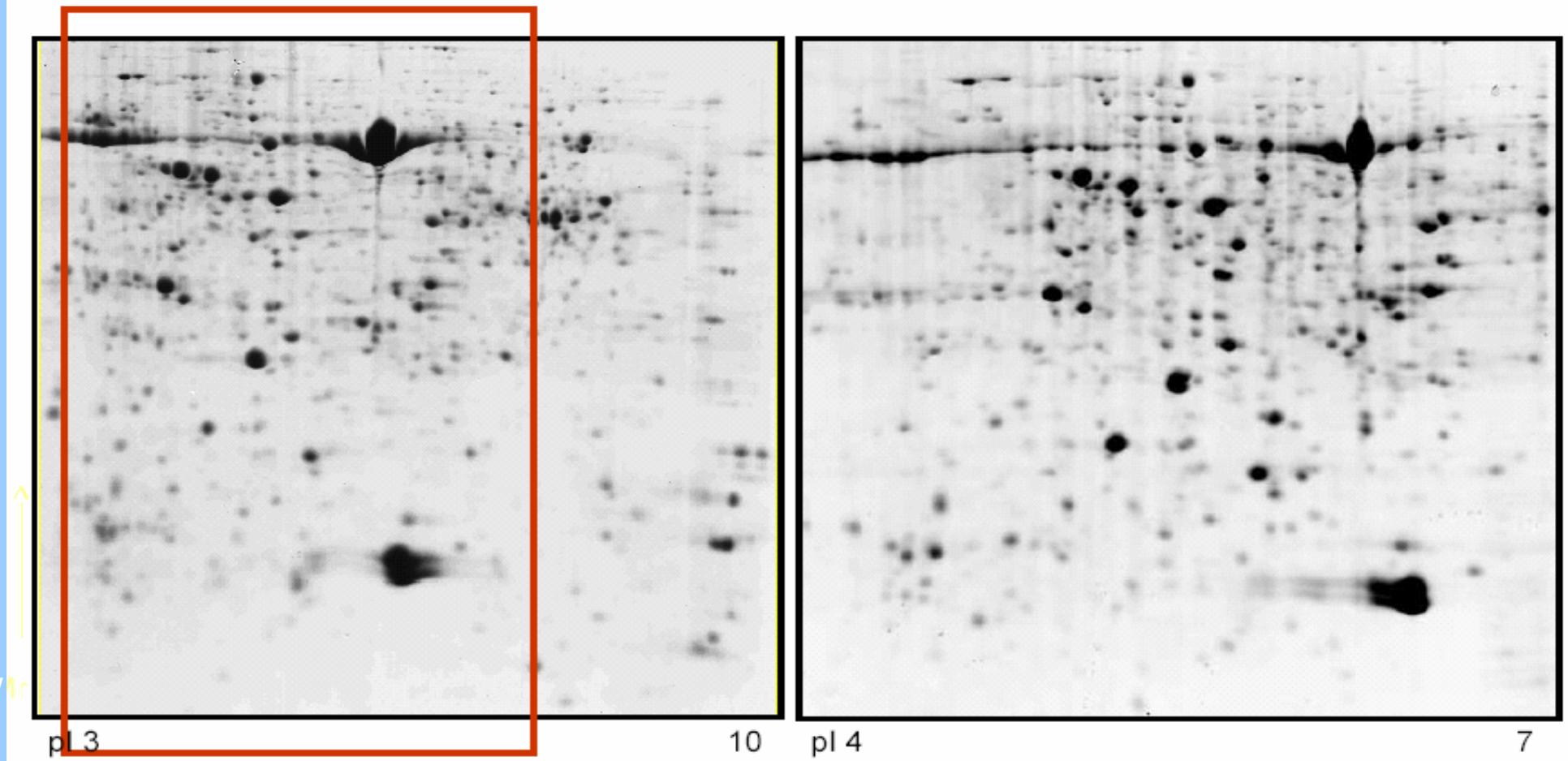
IPG STRIPY

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



překrývající se rozsahy

ROZSAH STRIPU



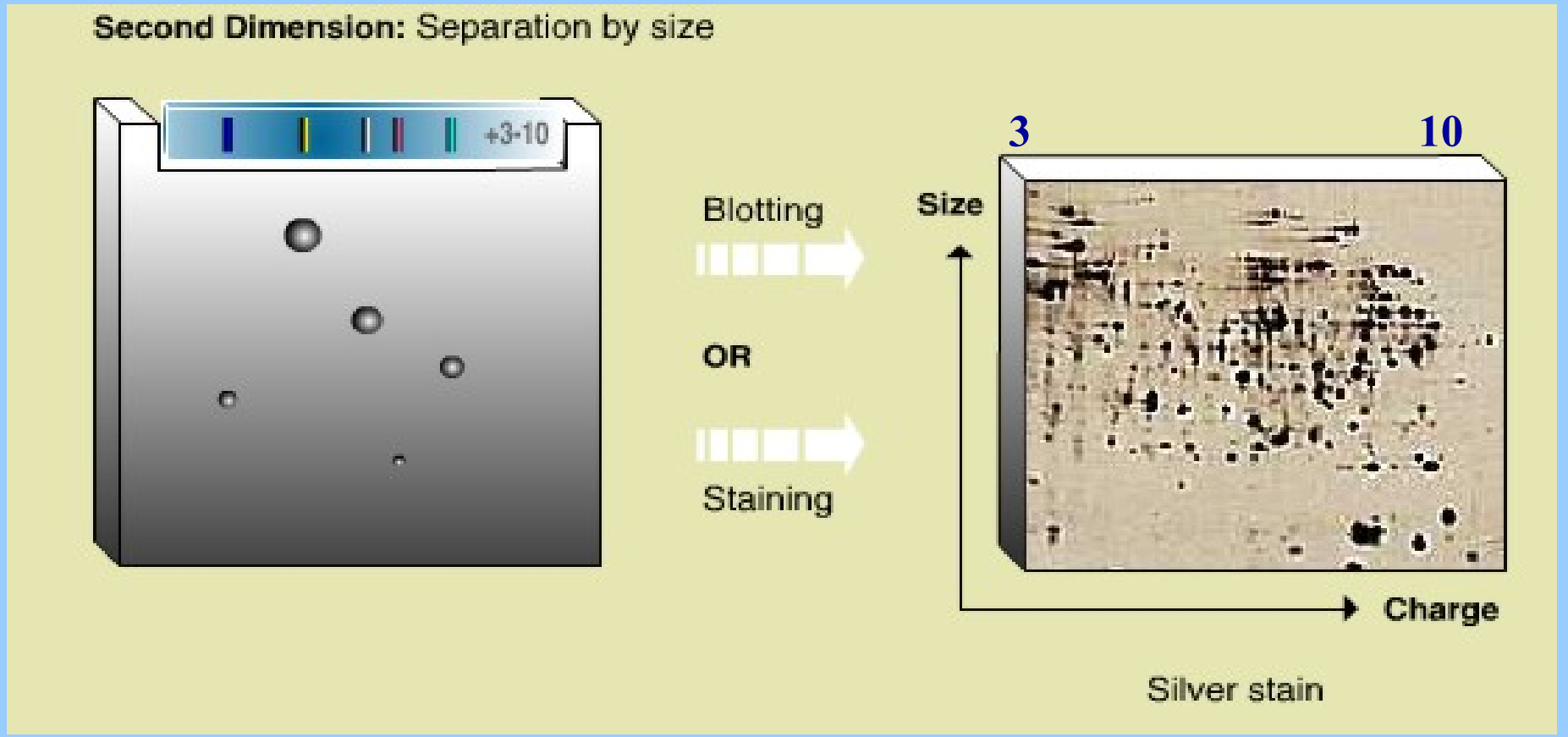
FOKUSAČNÍ PARAMETRY

- rehydratace
- aplikace vzorku
- ochrana cysteinu
- fokusační podmínky

Protean IEF Cell

DRUHÝ ROZMĚR 2 DE GE

SDS-PAGE ELEKTROFORÉZA

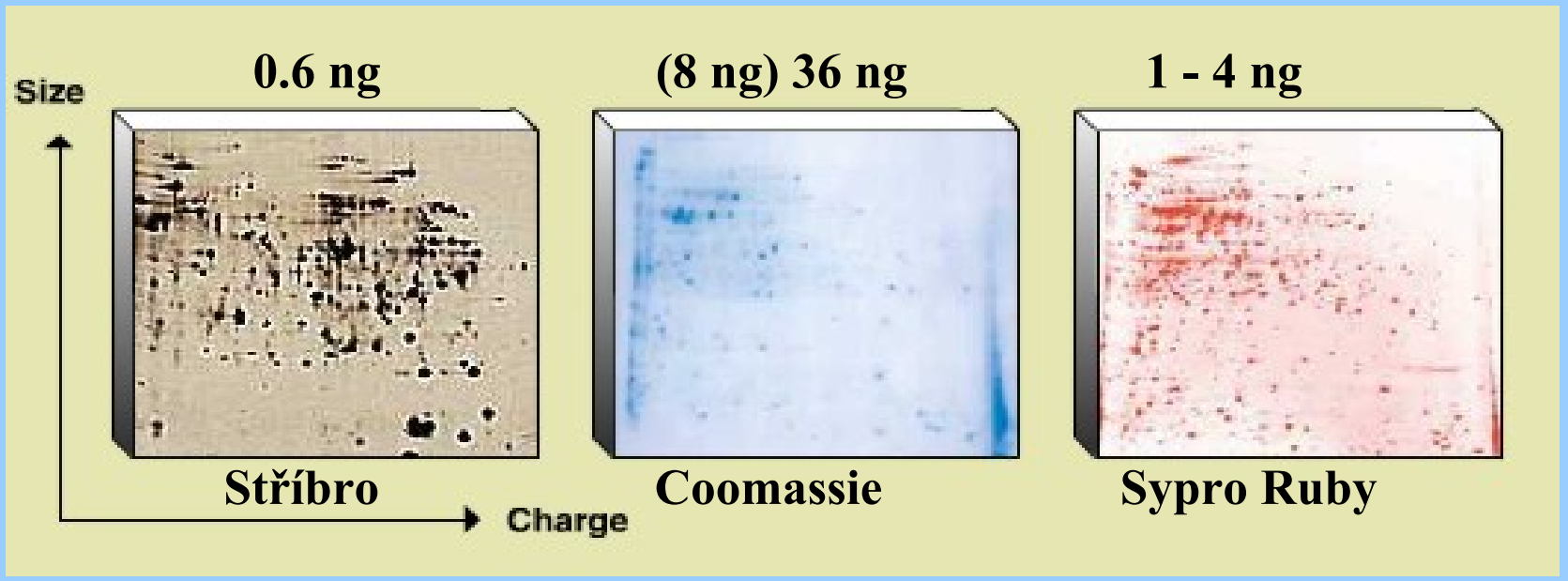


DETEKCE PROTEINU

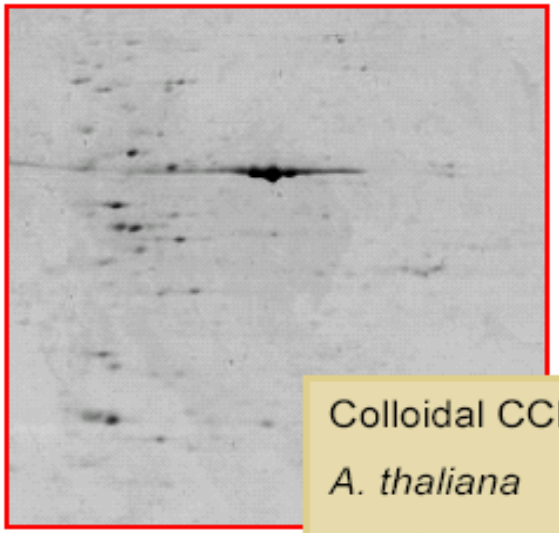
- gel x blot
- visualizace → barvení
  → radioaktivita
  → imunodetekce
- barvení v gelu
  - po elektroforéze
    před elektroforézou
  - specifické pro protein
    specifické pro PTM
  - viditelné spektrum
    fluorescence

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



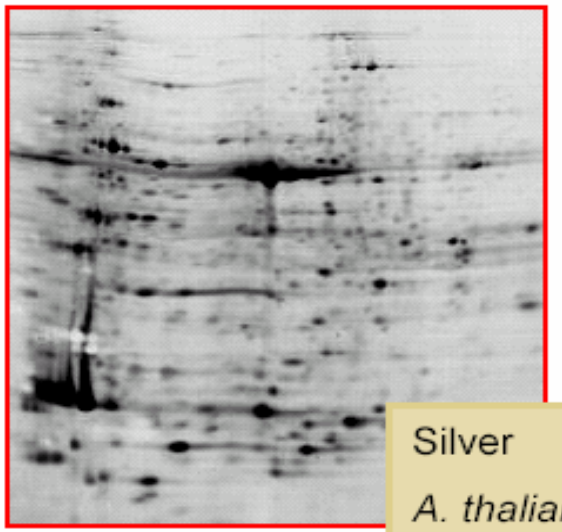
CITLIVOST BARVENÍ PROTEINU



Colloidal CCB

A. thaliana

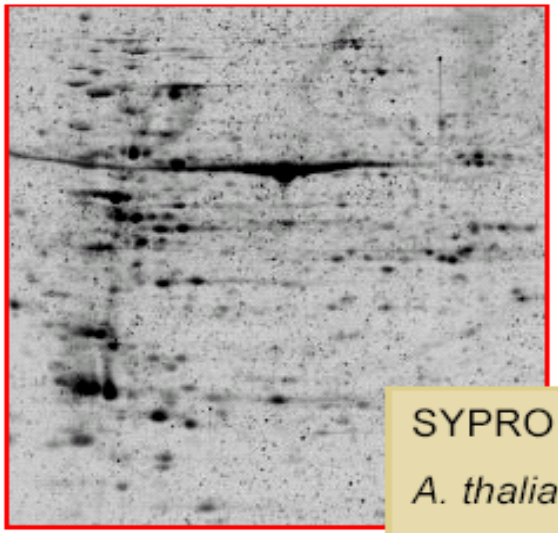
množství proteinu: 100 μg/gel



Silver

A. thaliana

množství proteinu: 20 μg/gel

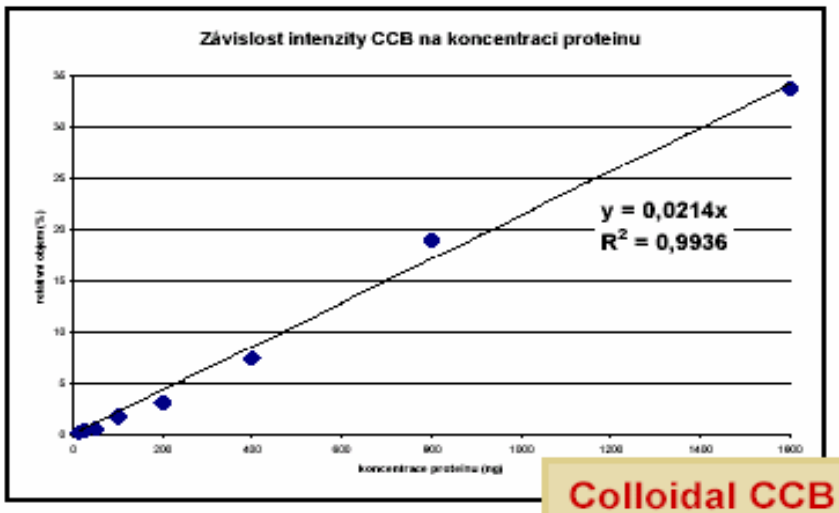


SYPRO Ruby

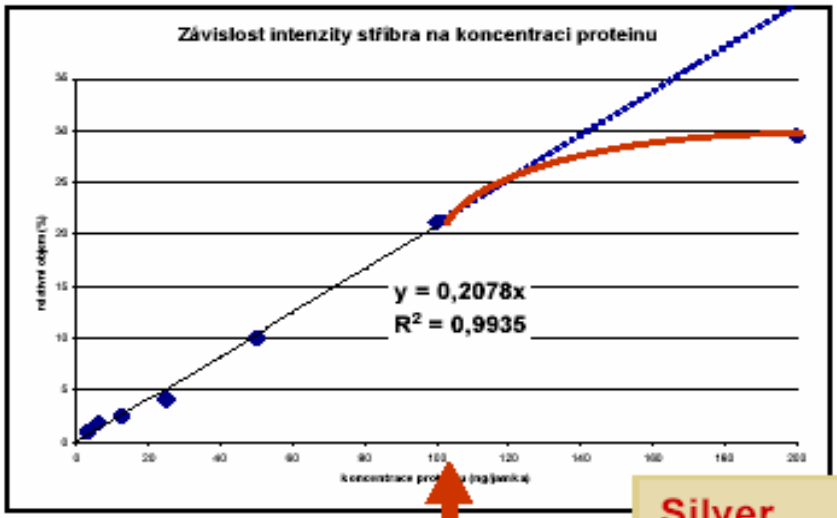
A. thaliana

množství proteinu: 20 μg/gel

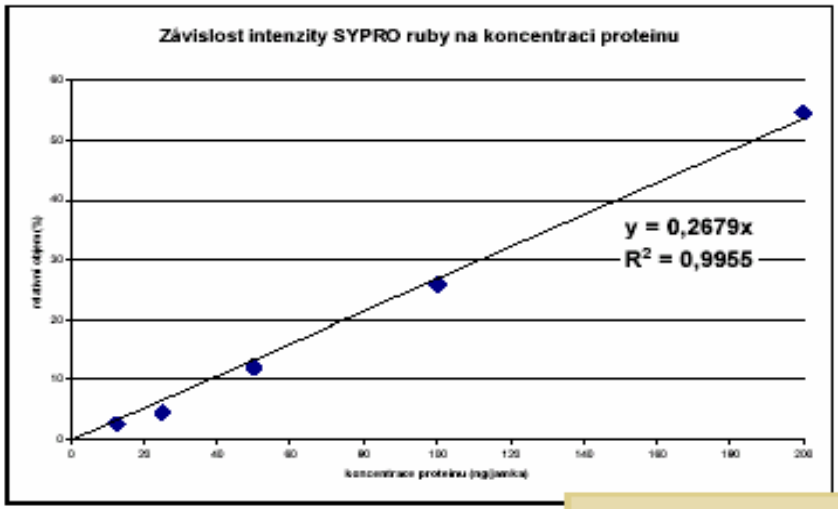
BARVENÍ PROTEINU - LINEARITA



Colloidal CCB

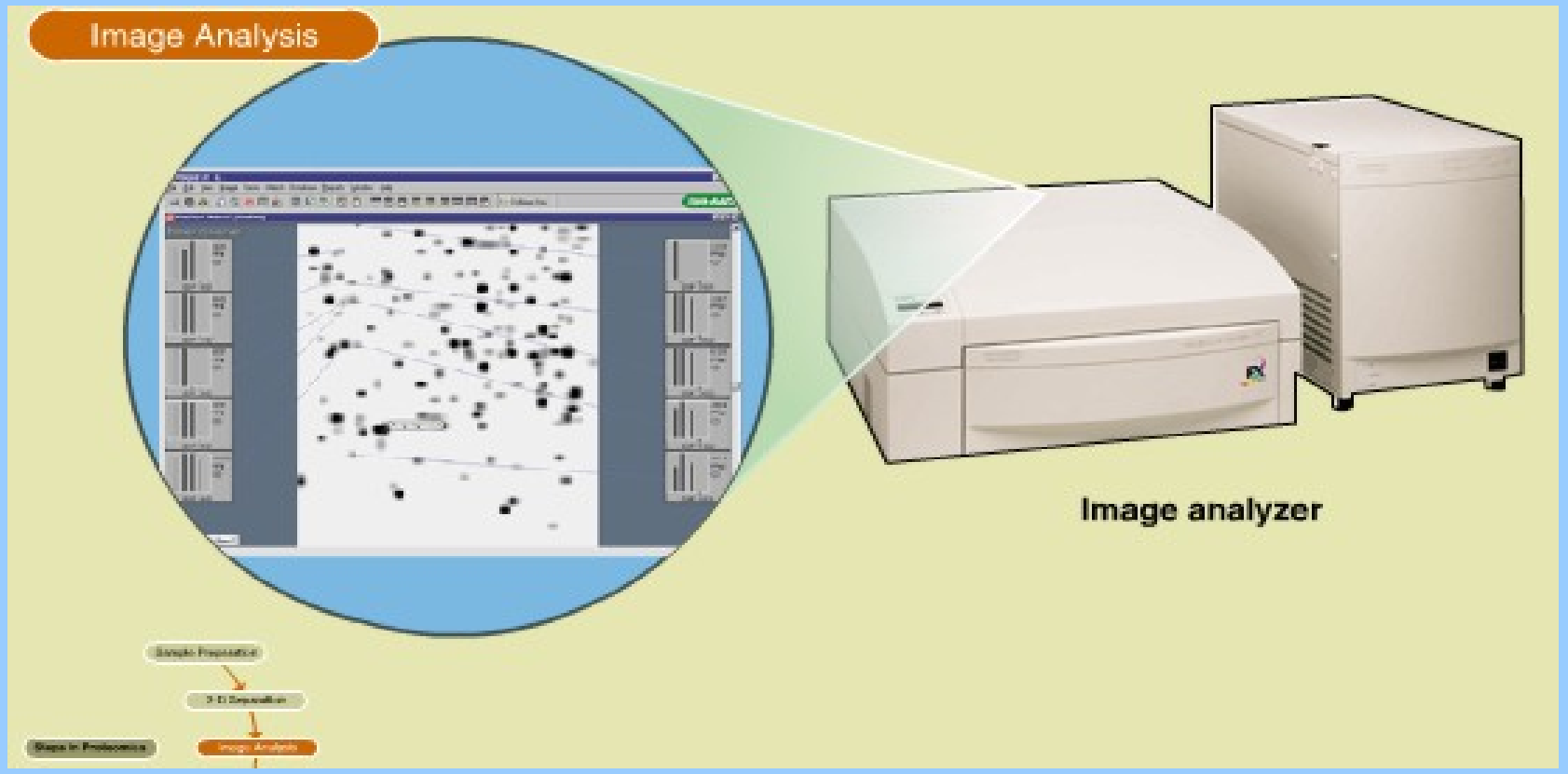


Silver



SYPRO Ruby

ANALÝZA OBRAZU

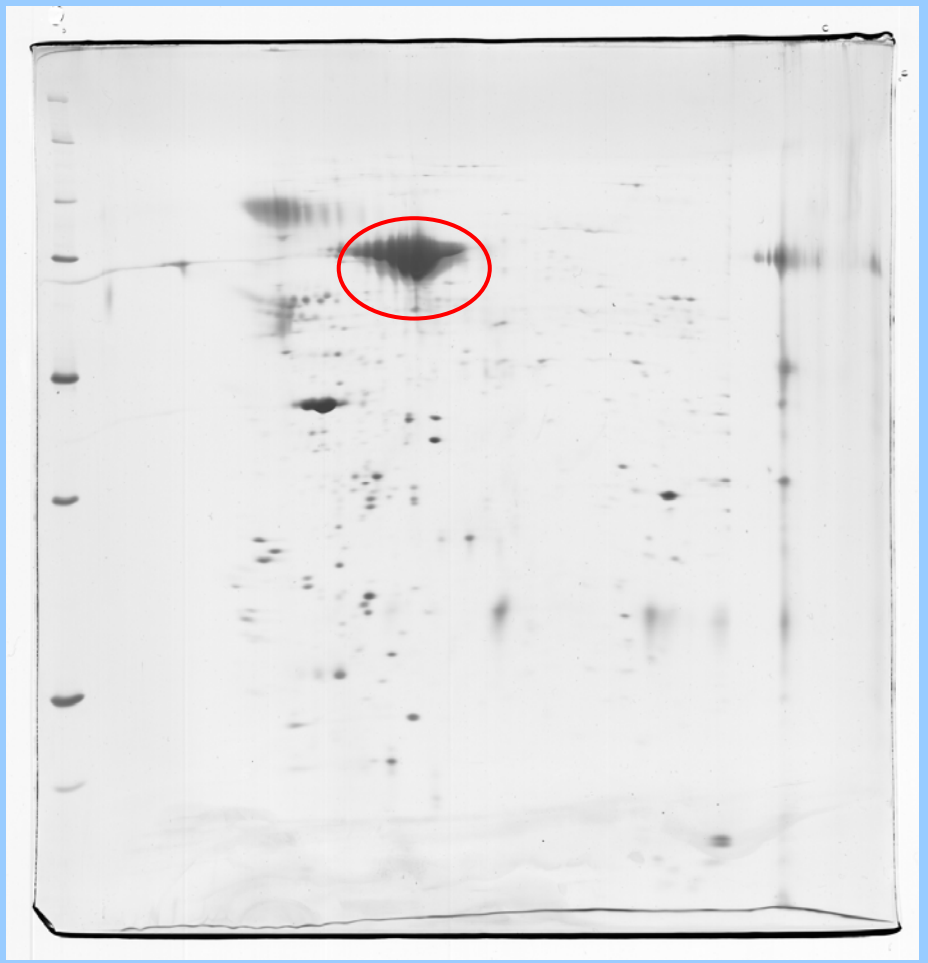


referenční mapa

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

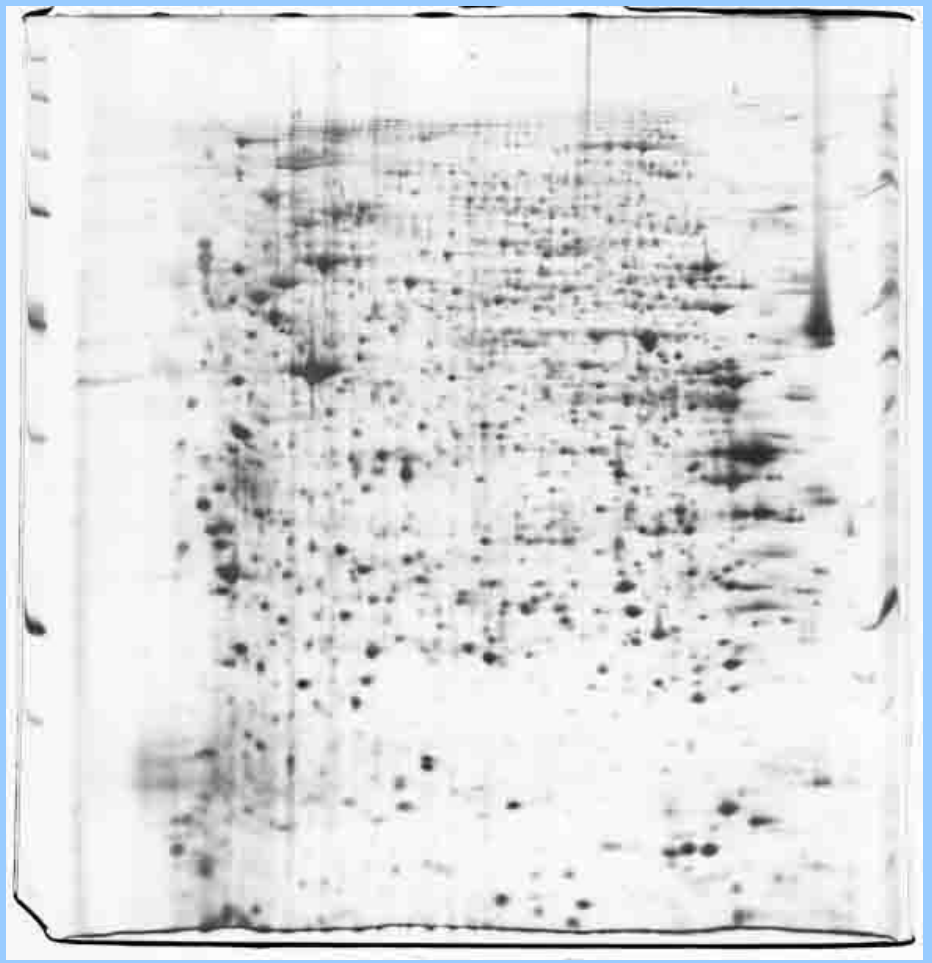
pI

Mr



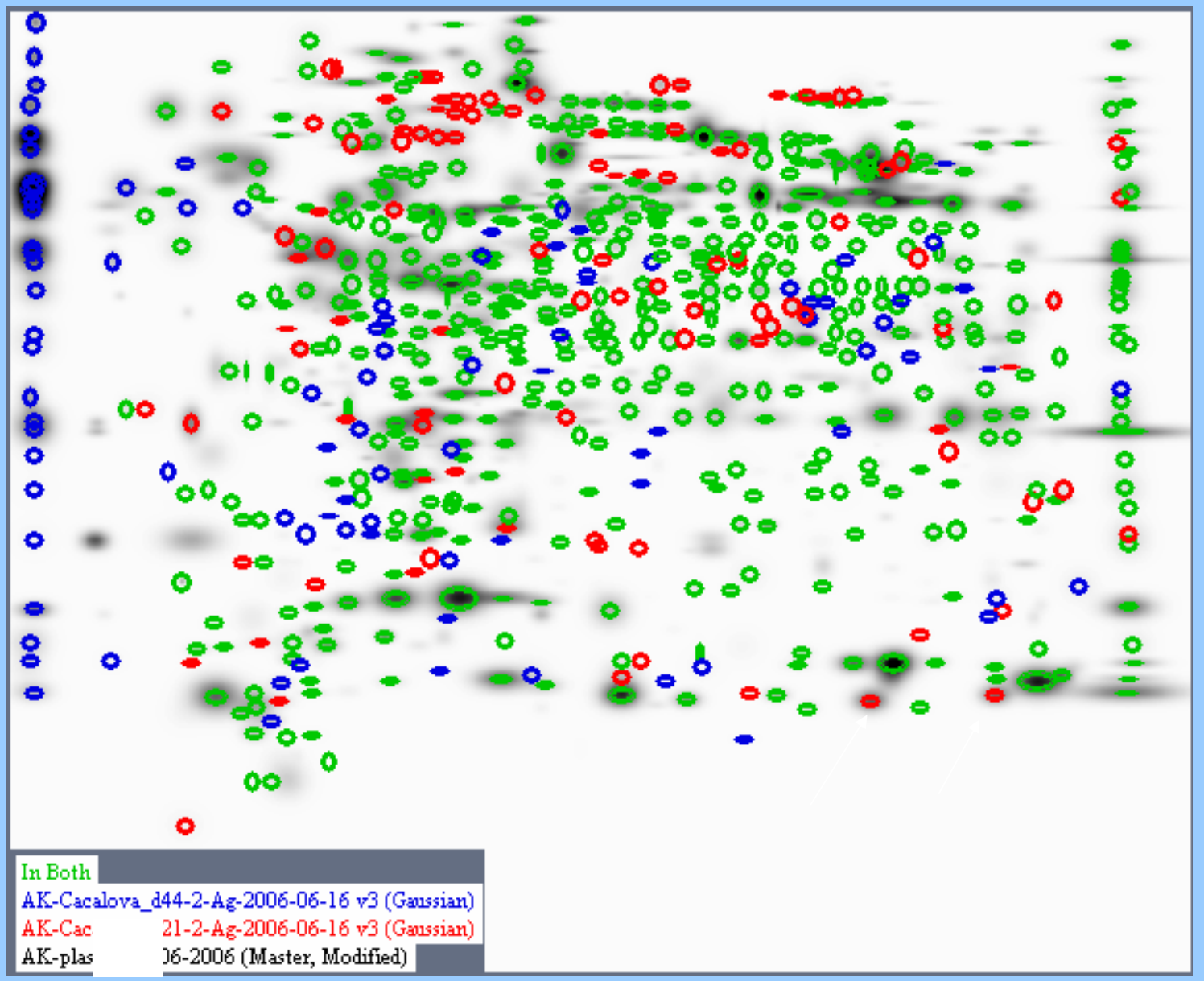
Sekretované vesikly kmenových buněk

pI



Lymfocyty

PDQuest


In Both
AK-Cacalova_d44-2-Ag-2006-06-16 v3 (Gaussian)
AK-Cac 21-2-Ag-2006-06-16 v3 (Gaussian)
AK-plas)6-2006 (Master, Modified)

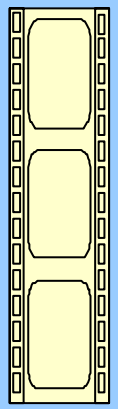
BIOMARKER

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

staré Řecko – sladká moč
dnes asi 150 proteinů klinicky relevantních

VALIDACE

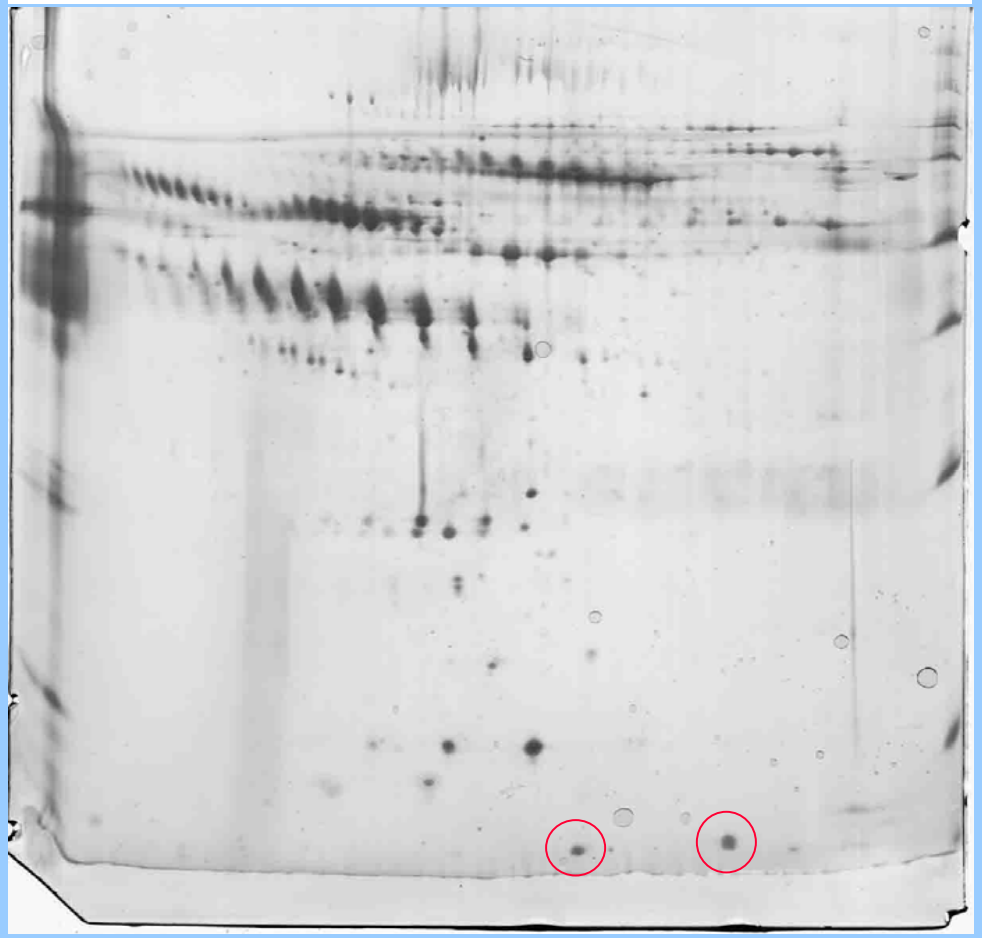
- analytická stabilní robustní metoda
- biologická biochemické dráhy a jejich změny, biologická variabilita u zdravých
- klinická prediktivní předpověd klinické změny, › než biologická variabilita



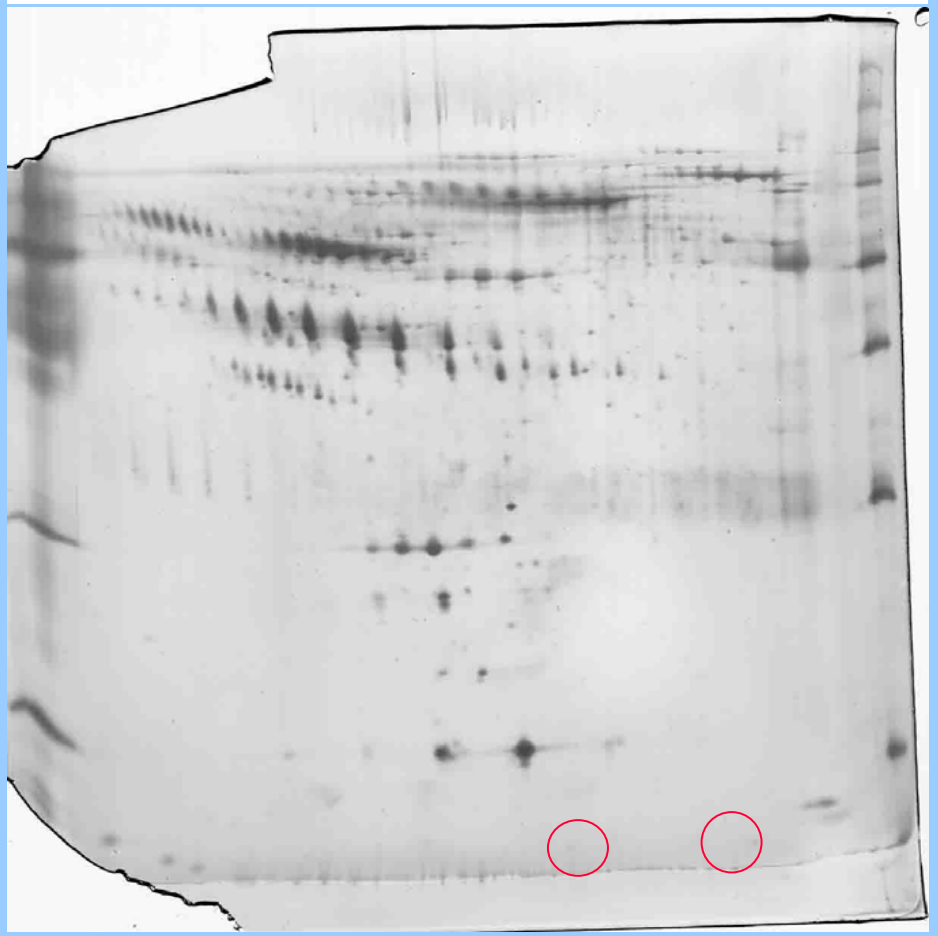
personalizovaná medicína
vlastní kontroly
etické problémy
kupka sena

Biomarkery onemocnění GvHD v lidské plasmě

Den 21 – před klinickým projevem GvHD

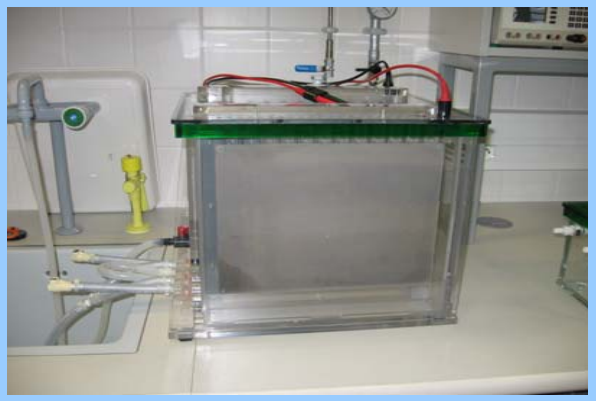


Den 44 – po klinickém projevu GvHD

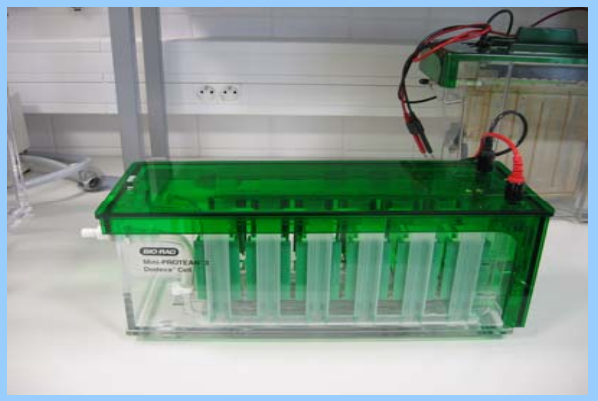


2D GE INSTRUMENTACE

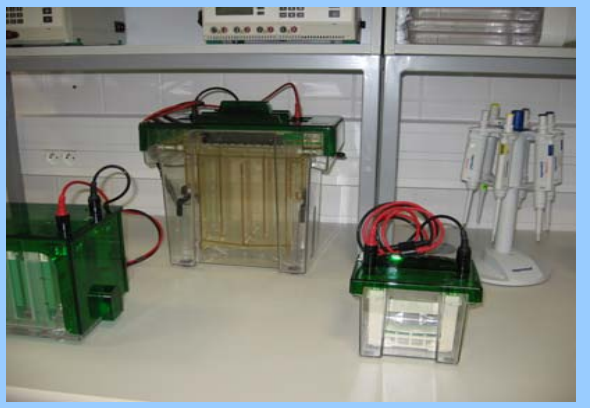
- Protean IEF
- Protean Dodeca Cell
- Densitometer GS-800
  PDQuest, Quantity One
- STORM



Protean Plus Dodeca Cell



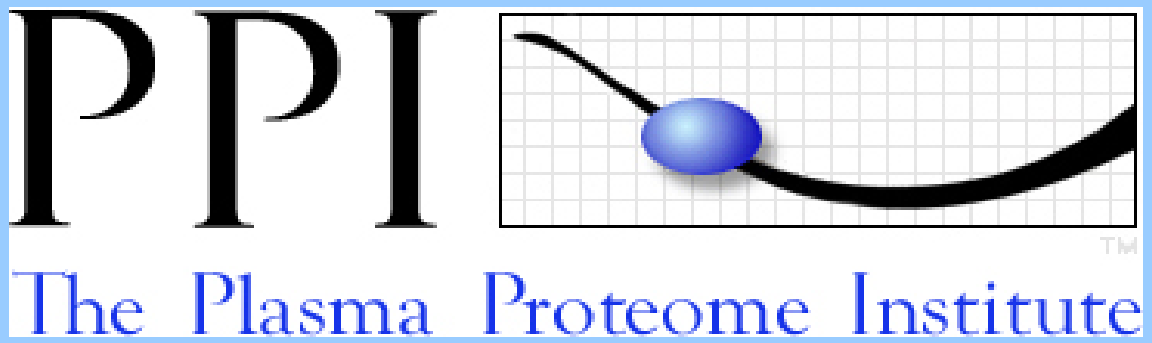
Mini-Protean 3 Dodeca Cell



Protean II xi Cell

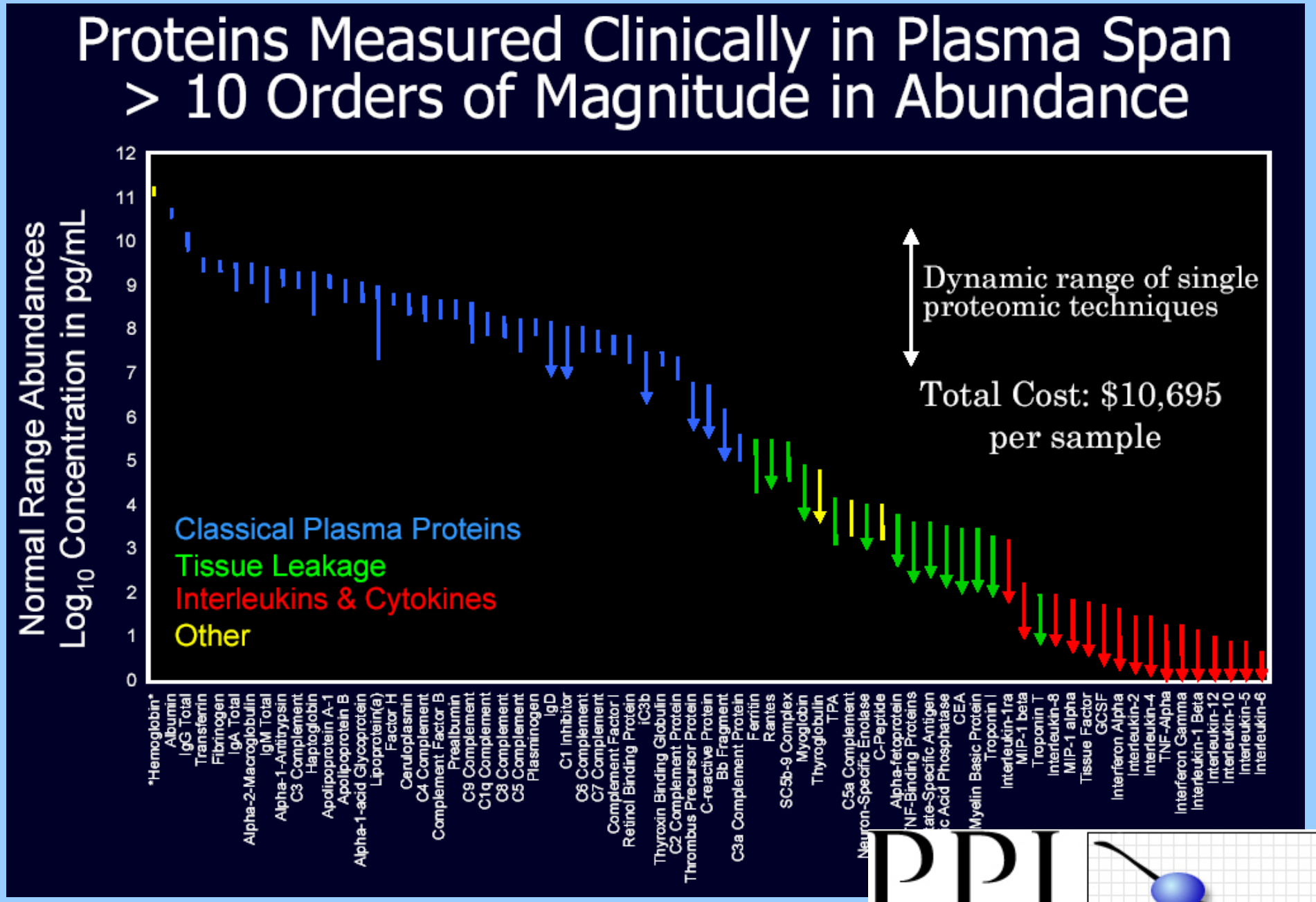
2D or not 2D ?

- 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



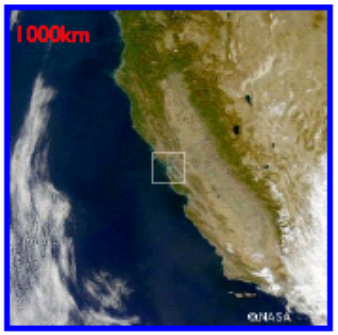
PPI

The Plasma Proteome Institute

10^{10} Really Is Wide Dynamic Range

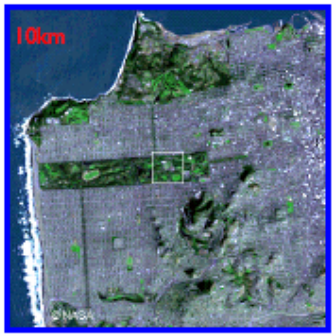
(Here on a linear scale)

10



9

8



7

6

5

4

3

2

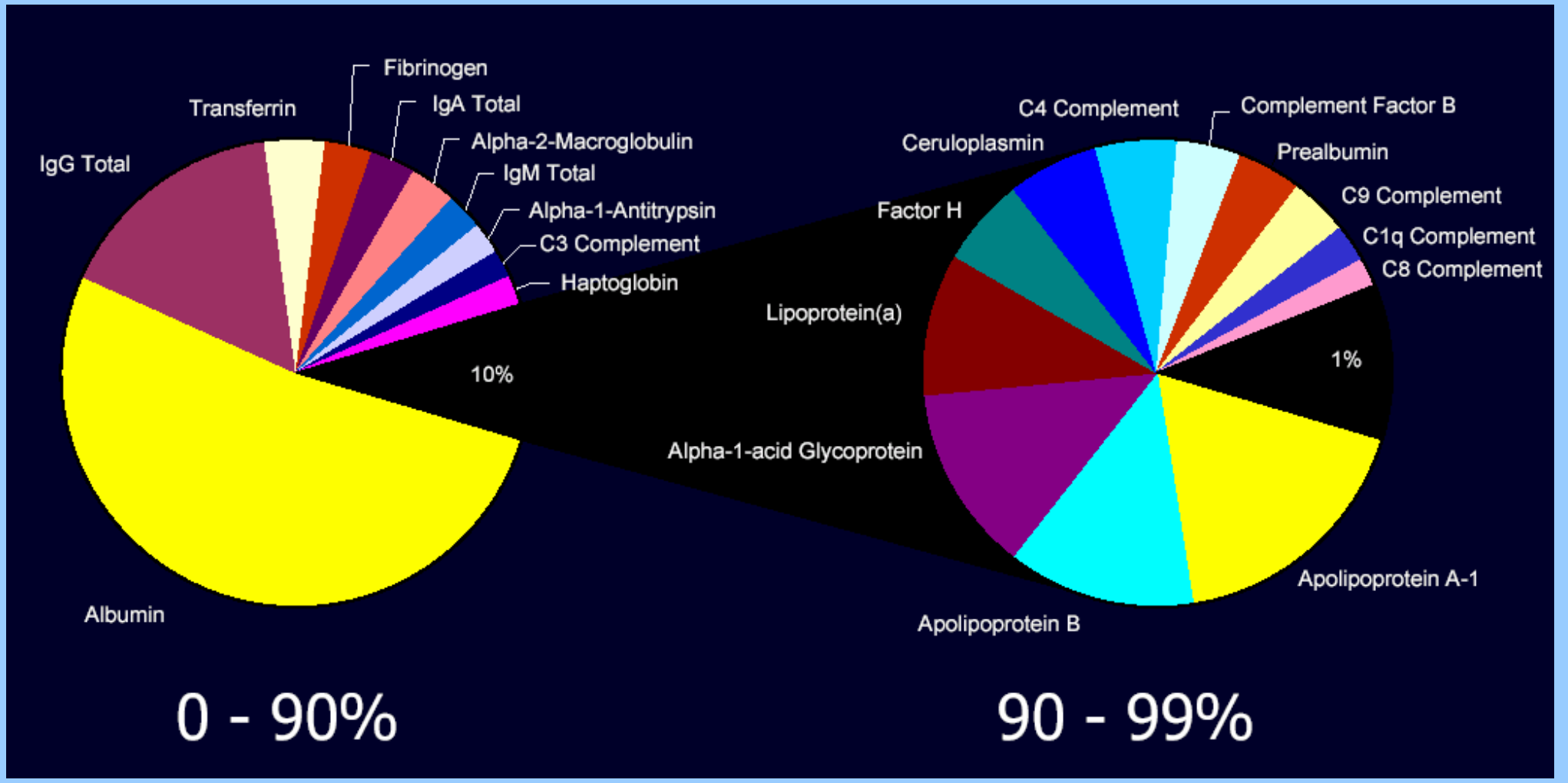
1

PPI

Slide courtesy Bruno Domon, ETH Zurich

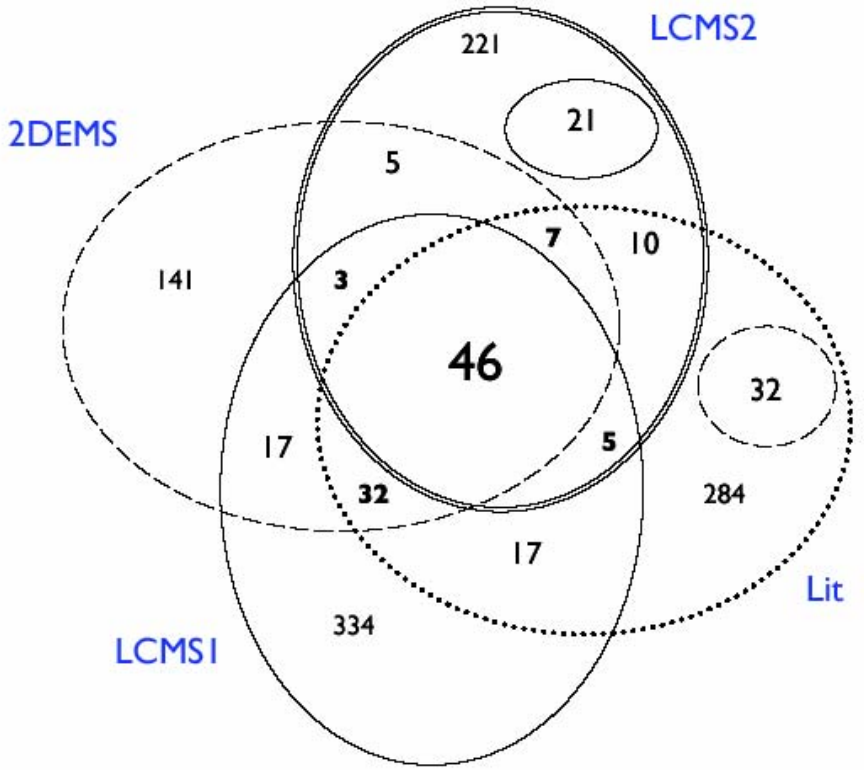
PPI

The Plasma Proteome Institute



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

From: The Human Plasma Proteome: A Non-Redundant List Developed by Combination of Four Separate Sources, N. L. Anderson et al, Molec. Cell Proteomics, 3: 311-326 (2004).

PPI

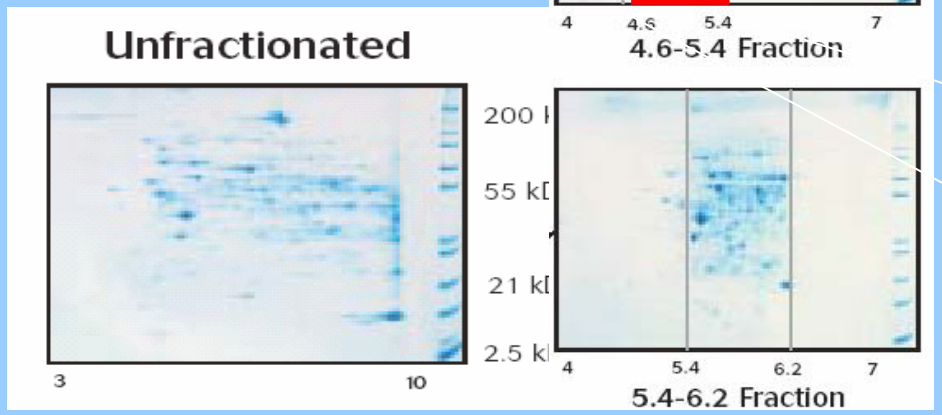
PREFRAKCIONACE

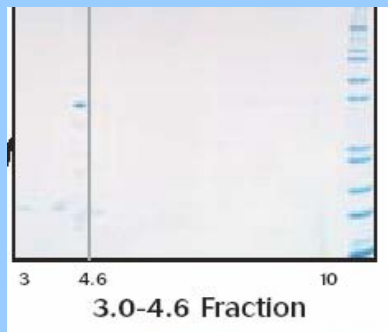
MicroRotofor

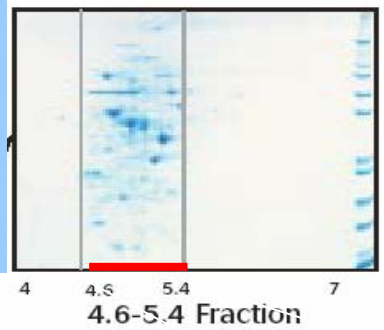
Zoom IEF

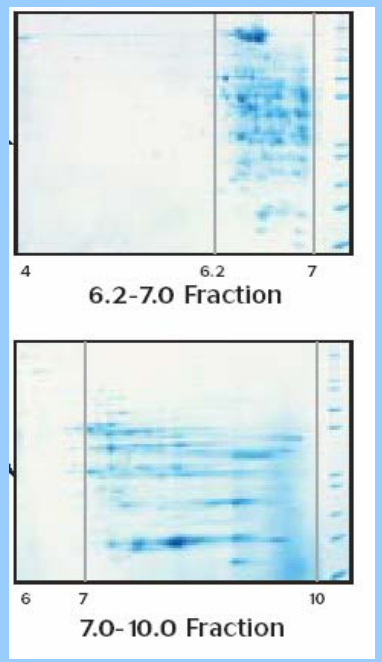
PREFRAKCIONACE pI

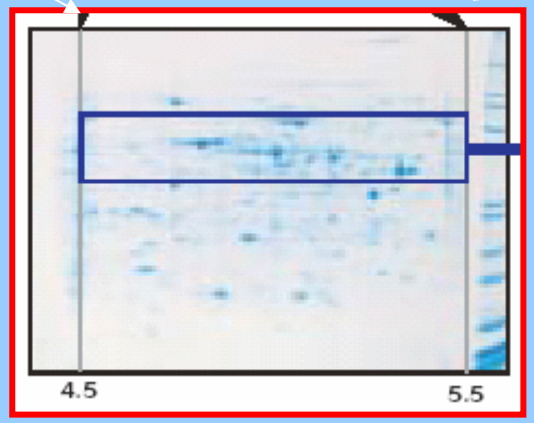
MIKRO ROZSAH pI







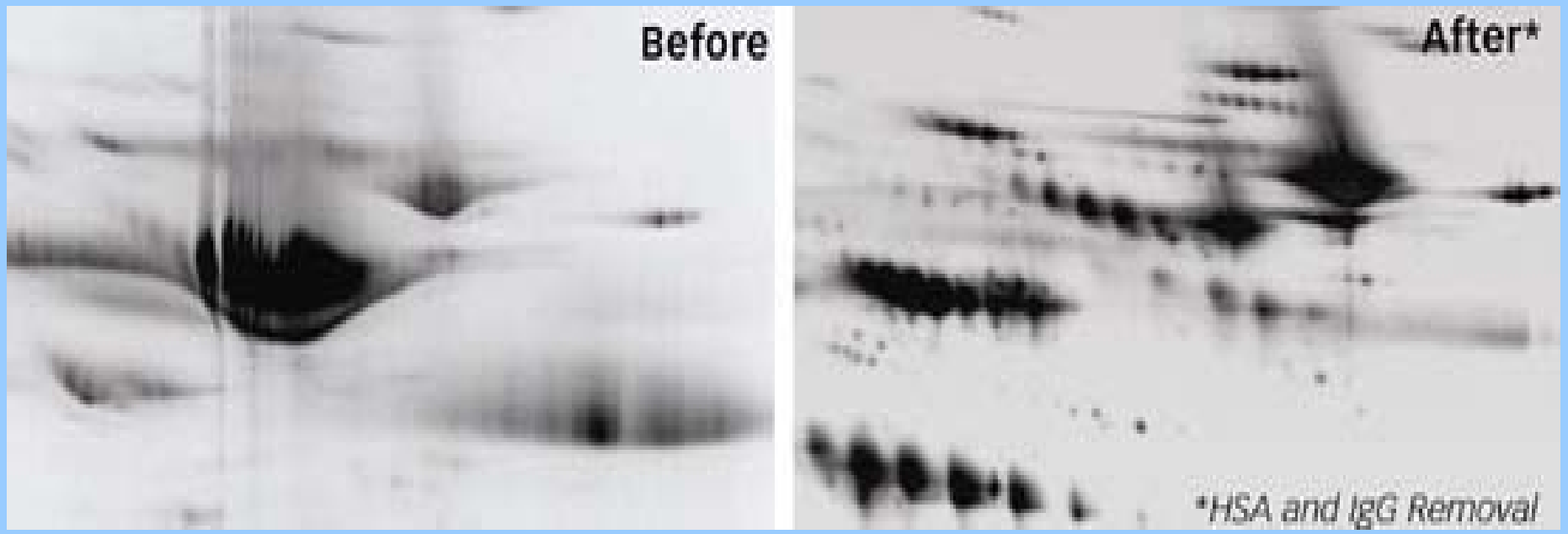




DEPLECE

odstranění abundantních proteinů

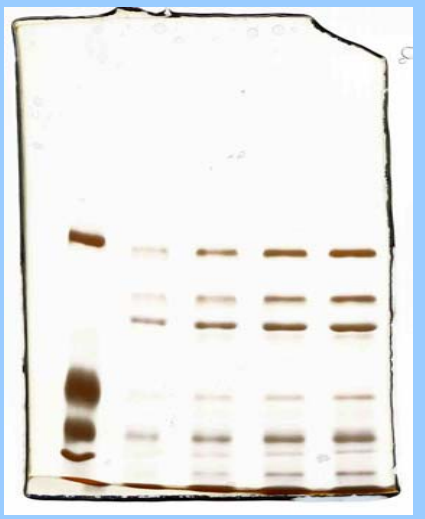
HSA
VivaPure
PROT20
...



Vivapure Kit

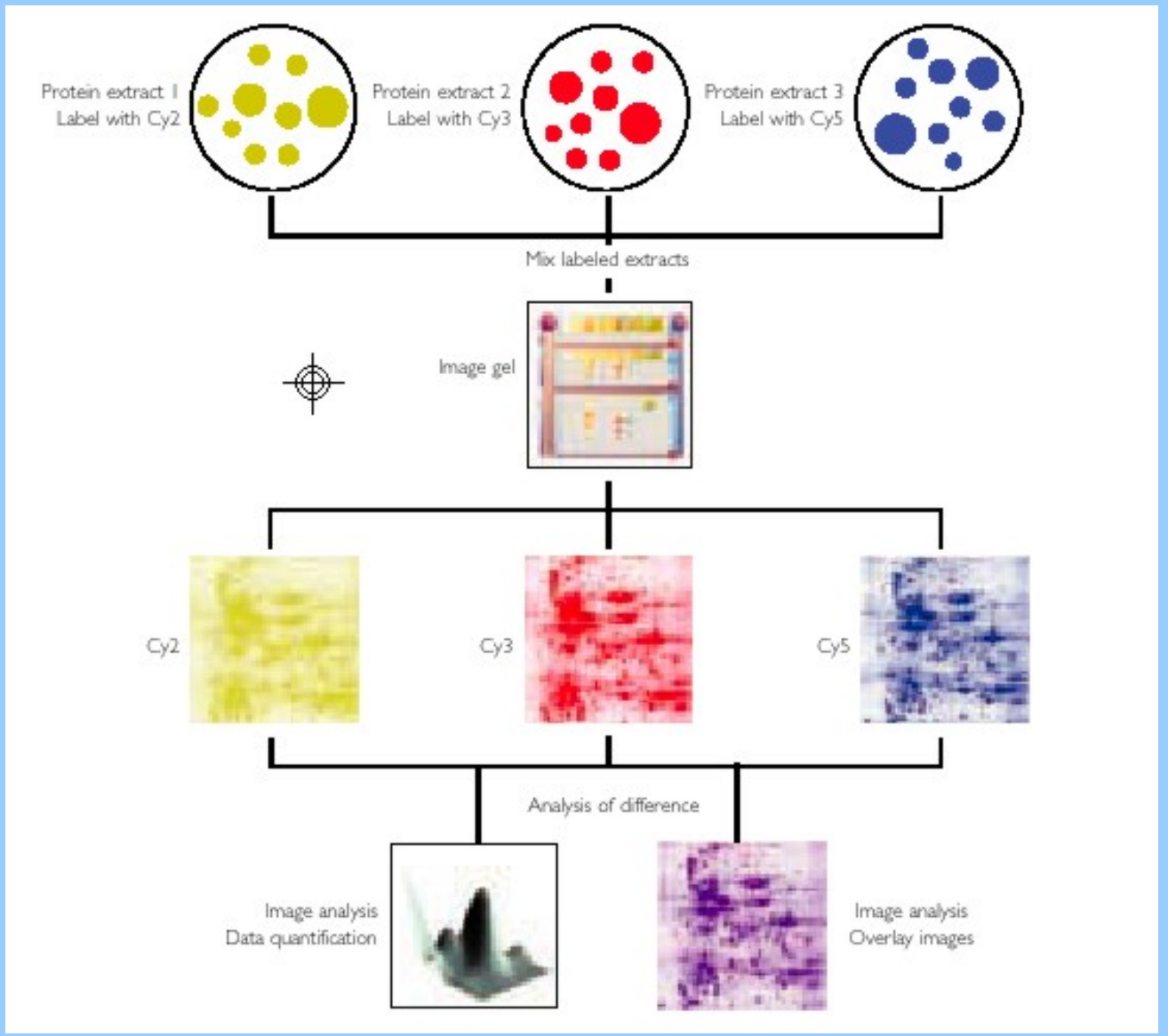
SDS PAGE

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



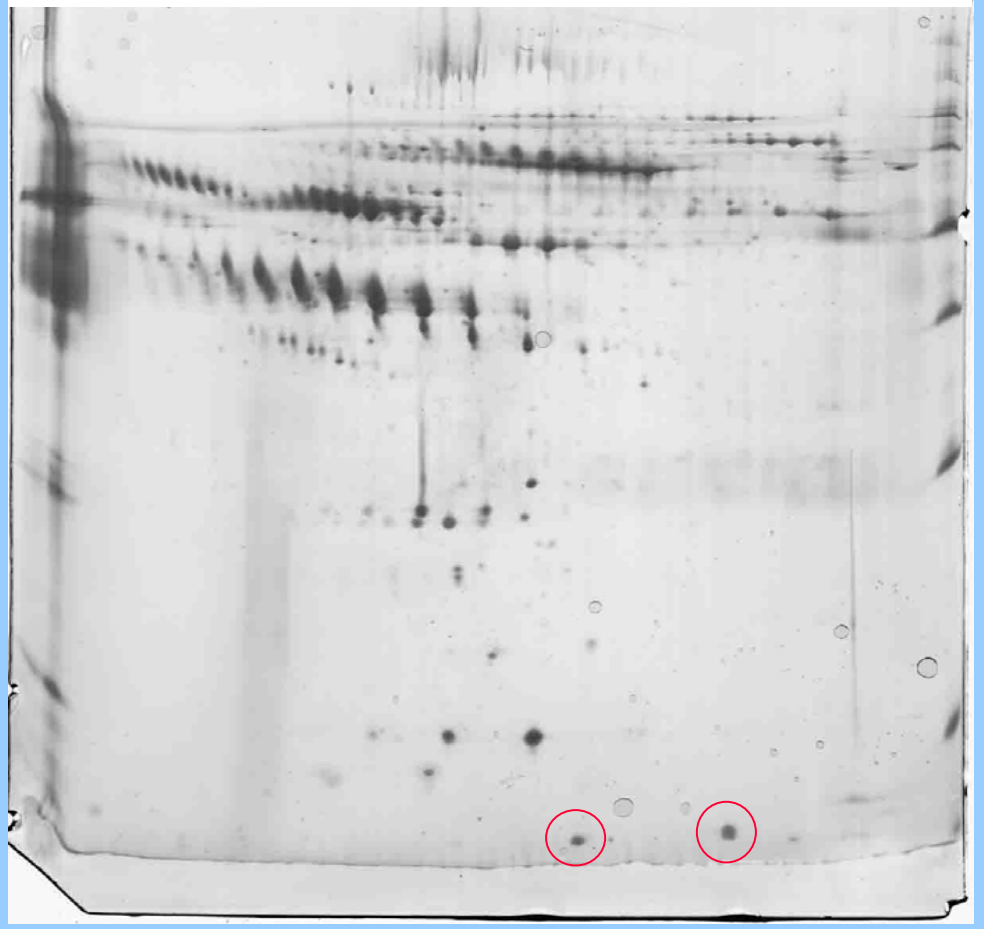
bakteriofág 812

Difference Gel Electrophoresis DIGE



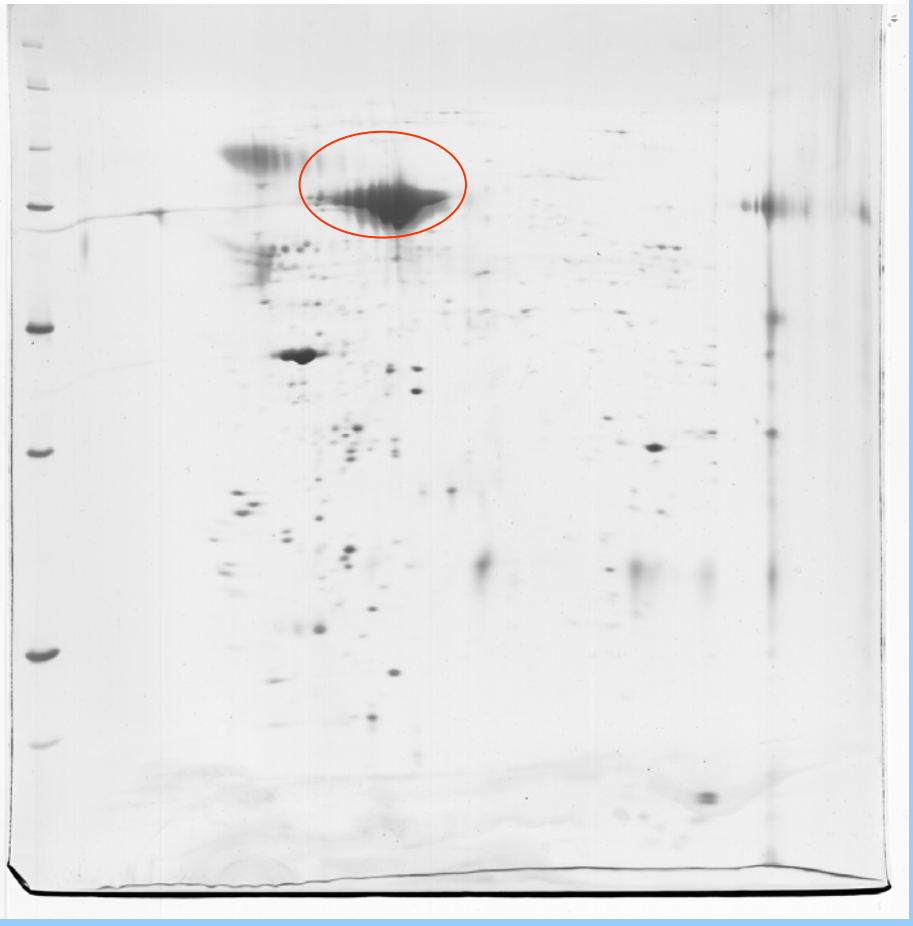
separace → identifikace

depletovaná plasma



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

vesikly kmenových buněk



BSA

DIGESCE

trypsin Glu-C Asp-N thermolysin

- IN-GEL
- IN-SOLUTION

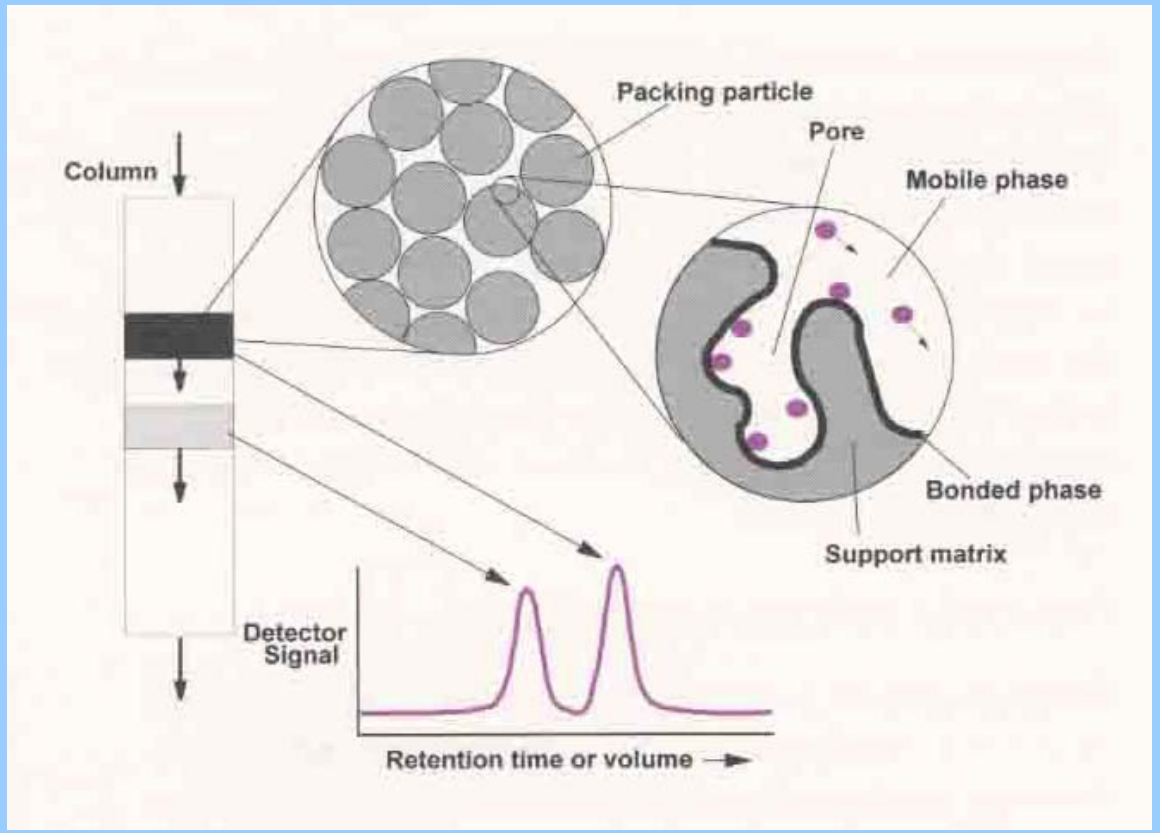
MAVEPFPRRPITRPHASIEVDTSGTGGSAGSSEKVF
CLIGQAEGGEPNTVYELRNYAQAKRLFRSGELLD
AIELAWGSNPNYTAGRILAMRIEDAKPASAEIGGL
KITSKIYGNVANNIQVGLEKNTLSDSLRLRVIFQD
DRFNEVYDNIGNIFTIKYKGEEANATFSVEHDEET
QKASRLVLKVGDQEVKSYDLTGGAYDYTNAIITD
INQLPDFEAKLSPFGDKNLESSKLDKIENANIKDK
AVYVKAVFGDLEKQTAYNGIVSFEQLNAEGEVPS
NVEVEAGEESATVTATSPIKTIEPFELTKLKGGTN
GEPPATWADKLDKFAHEGGYYIVPLSSKQSVHAE
VASFVKERSDAGEPMRAIVGGGFNESKEQLFGRQ
ASLSNPRVSLVANSGTFVMDDGRKNHVPAYMVA
VALGGLASGLEIGESITFKPLRVSSLDQIYESIDLDE
LNENGIISIEFVRNRTNTFFRIVDDVTTFNDKSDPV
KAEMAVGEANDFLVSELKVQLEDQFIGTRTINTS
ASIIKDFIQSYLGRKKRDNEIQDFPAEDVQVIVEGN
EARISMTVYPIRSFKKISVSLVYKQQTLQA

MS

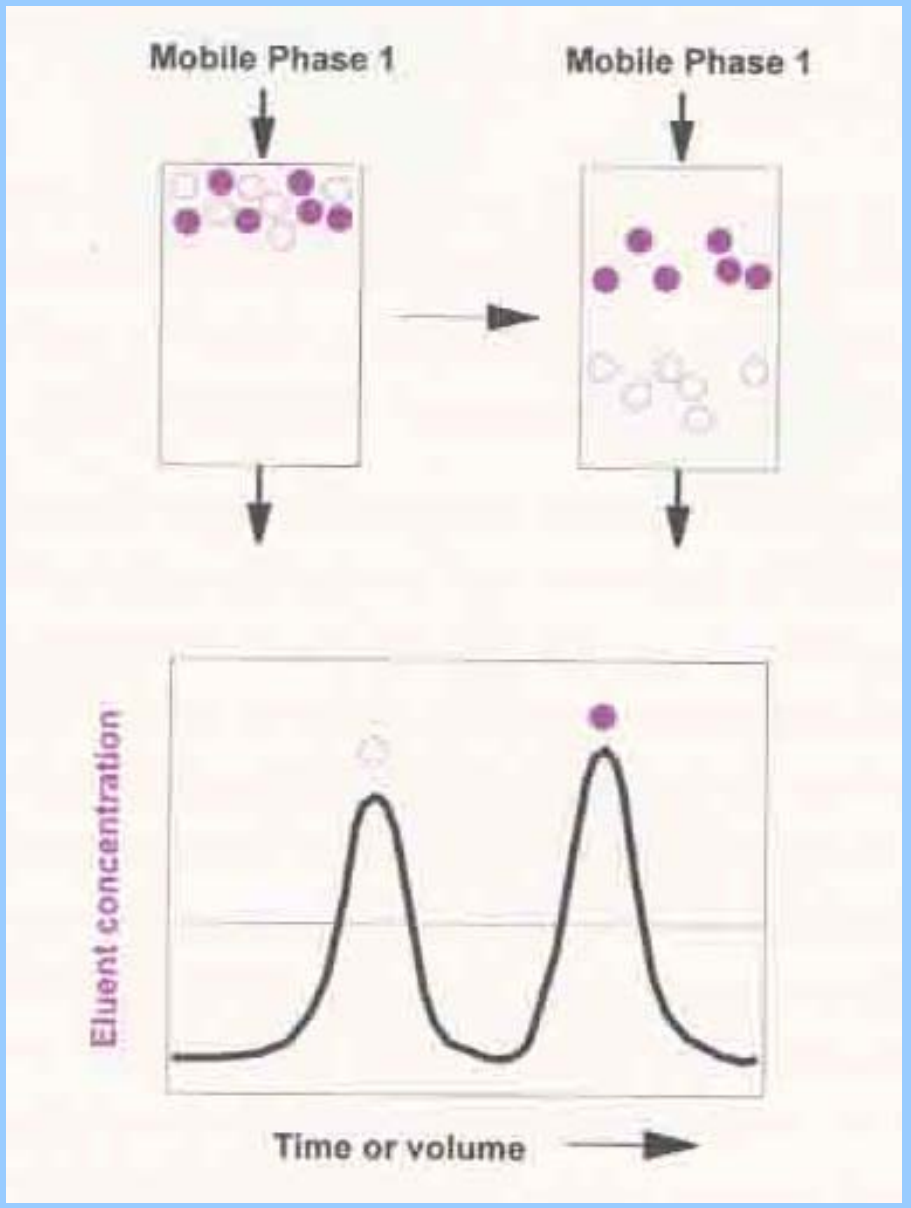
ALTERNATIVA 2D GE

MULTIDIMENZIONÁLNÍ CHROMATOGRAFIE

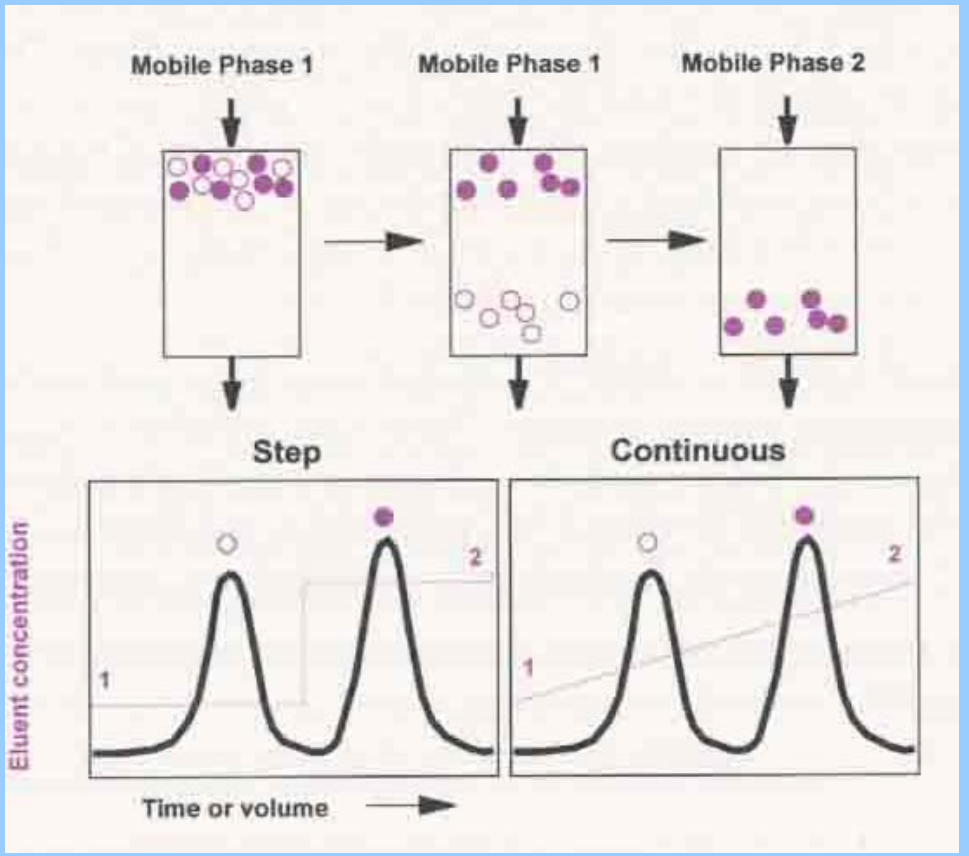
PROTEINY A PEPTIDY
PRINCIPY SEPARACE KAPALINOVOU CHROMATOGRAFIÍ



Isokratická eluce



Gradientová eluce

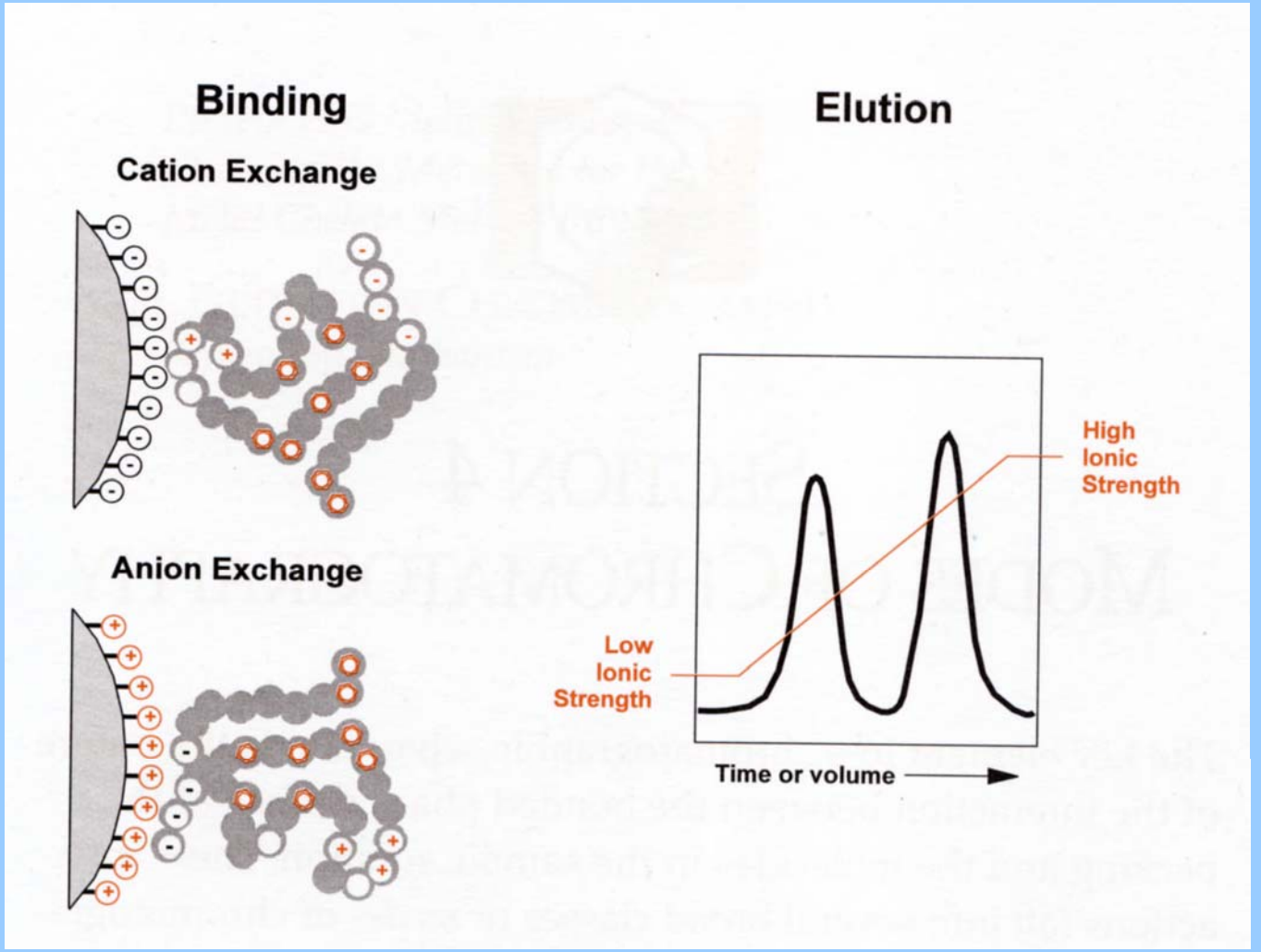


TYPY LC SEPARACE

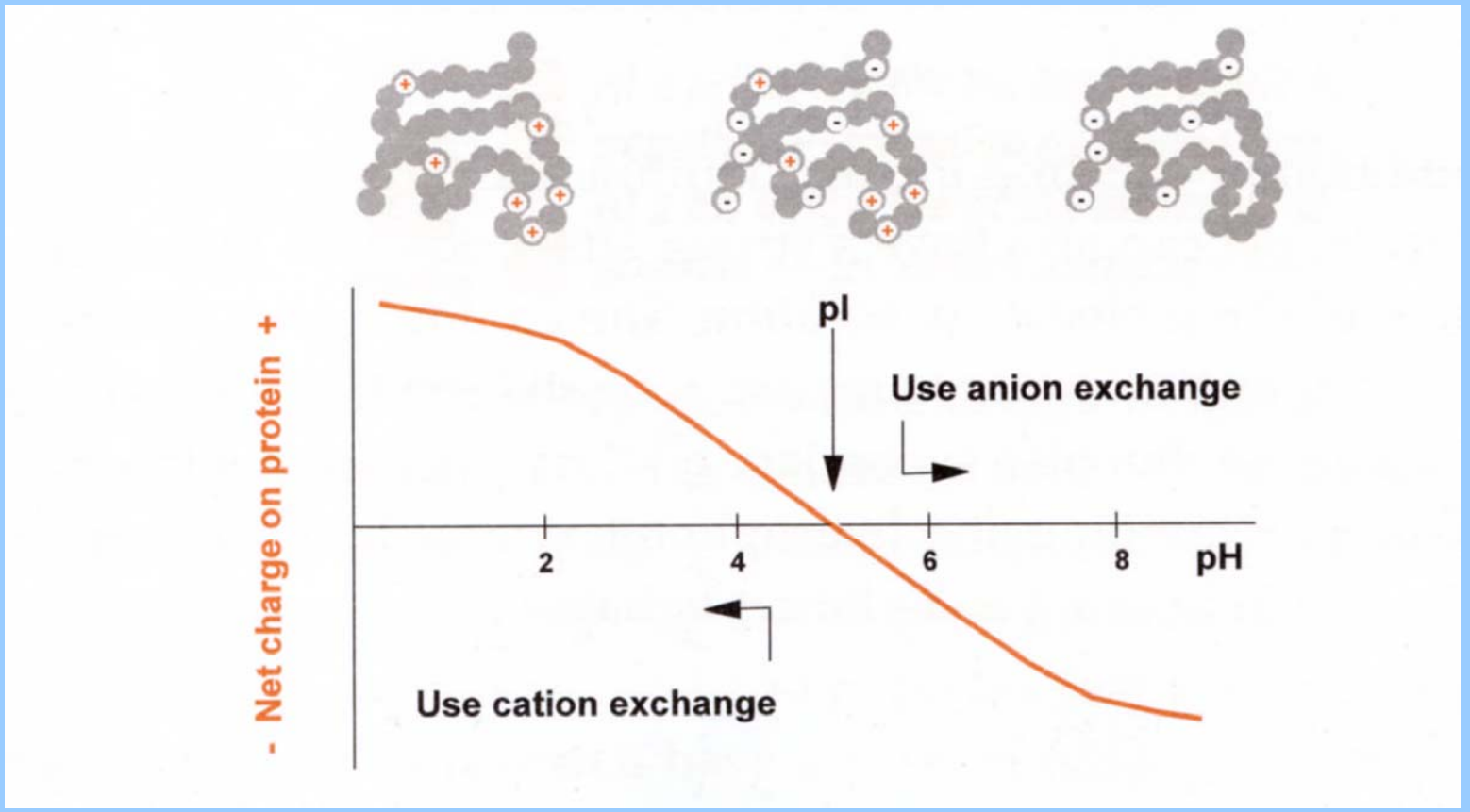
CO ROZHODUJE → KOLONA

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

IONEXOVÁ CHROMATOGRAFIE



Isoelektrický bod

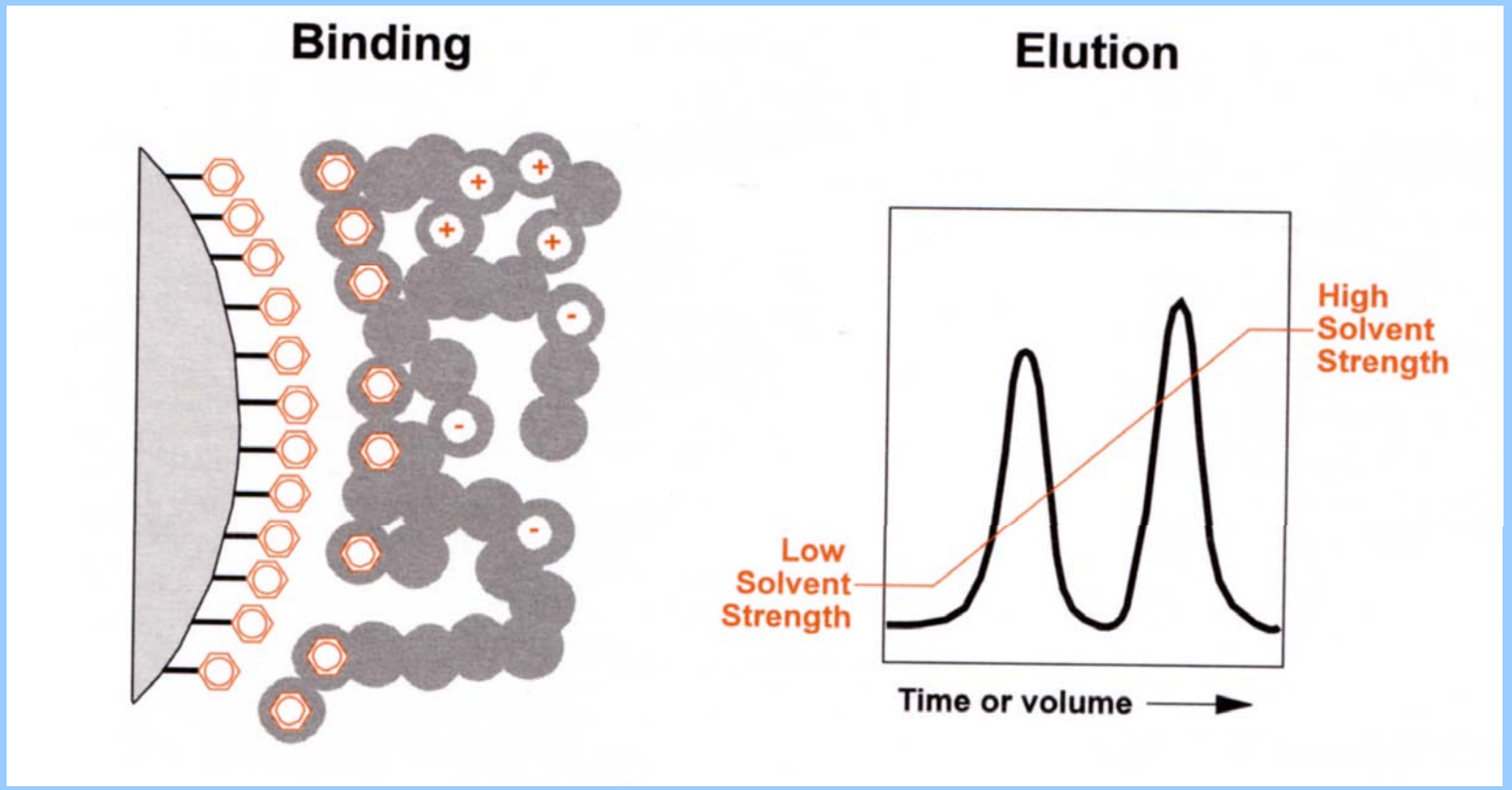


Funkční skupiny u ionexových matricí

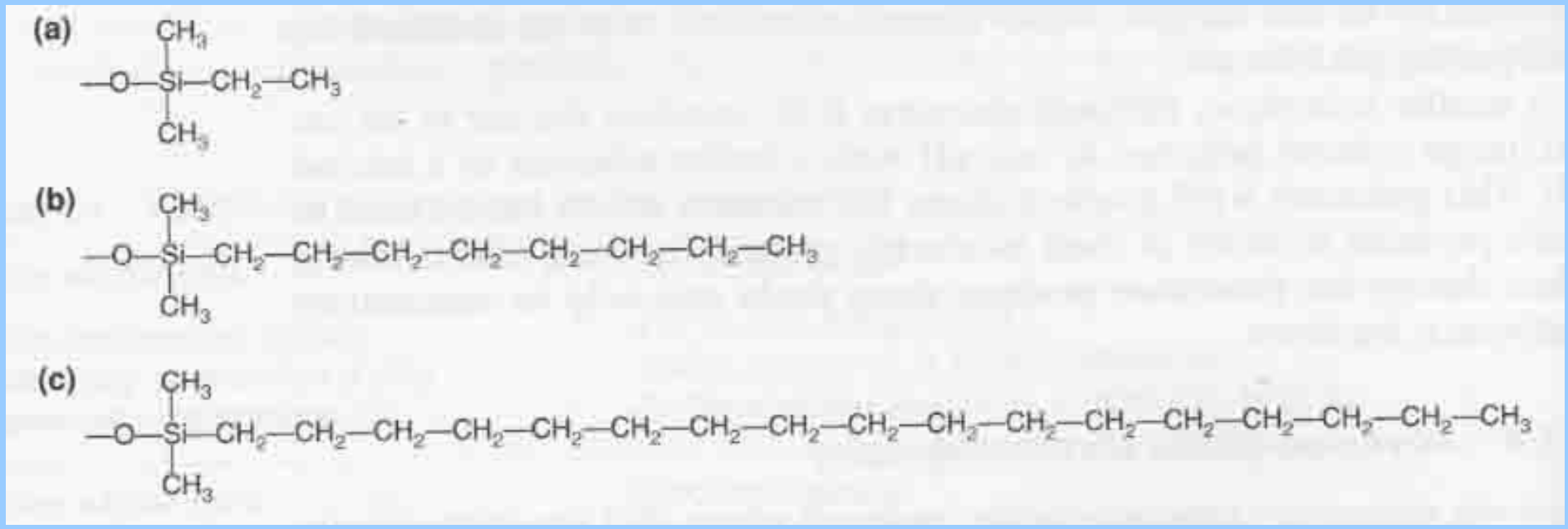
Table 2.1 Functional groups used on ion exchangers.

Anion exchangers	Functional group
Diethylaminoethyl (DEAE)	$-O-CH_2-CH_2-N^+H(CH_2CH_3)_2$
Quaternary aminoethyl (QAE)	$-O-CH_2-CH_2-N^+(C_2H_5)_2-CH_2CHOH-CH_3$
Quaternary ammonium (Q)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-N^+(CH_3)_3$
Cation exchangers	**Functional group**
Carboxymethyl (CM)	$-O-CH_2-COO^-$
Sulfopropyl (SP)	$-O-CH_2-CHOH-CH_2-O-CH_2-CH_2-CH_2SO_3^-$
Methylsulfonate (S)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2SO_3^-$

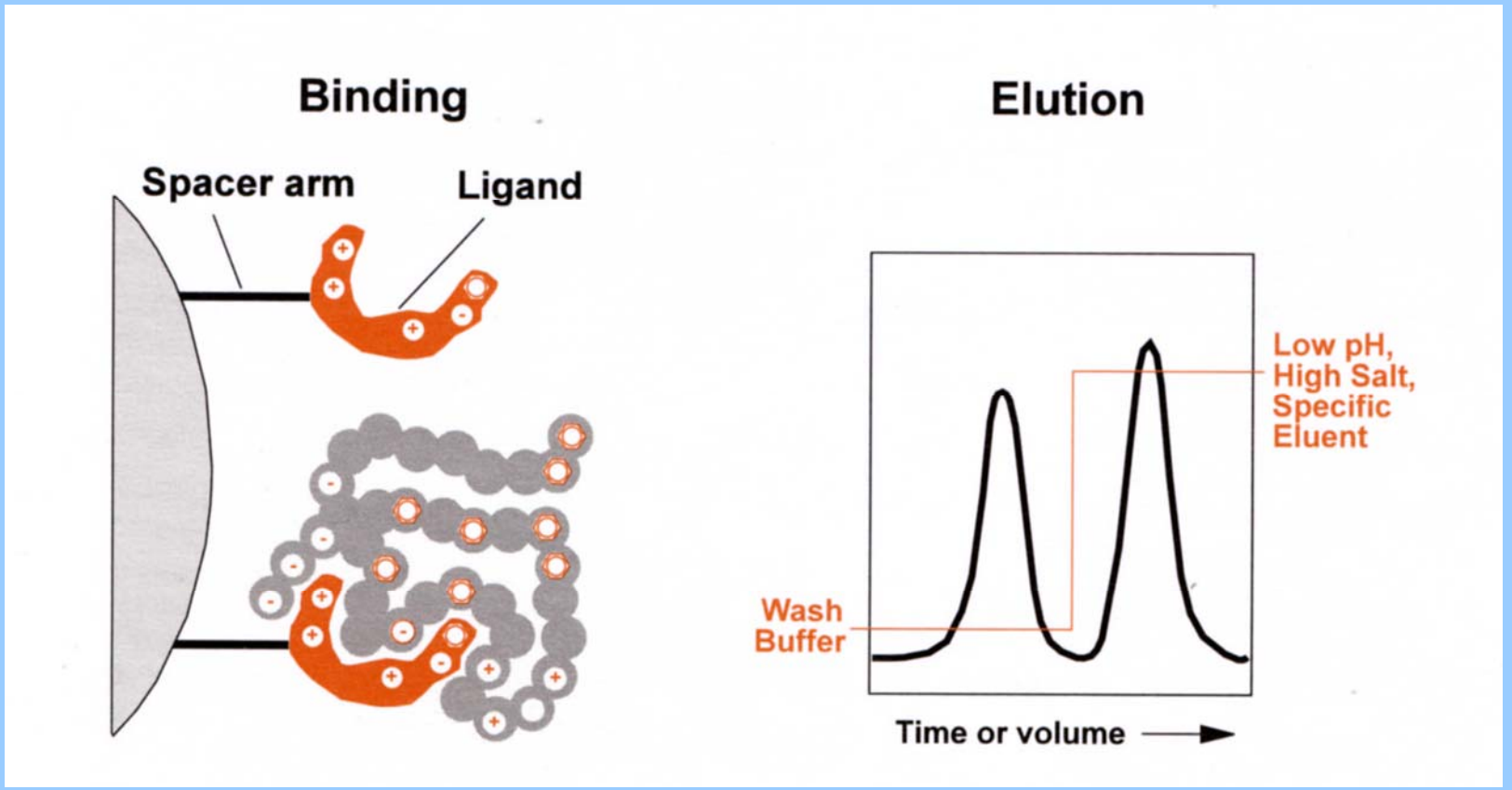
RP CHROMATOGRAFIE
reversed-phase chromatography



Funkční skupiny u RP matricí



AFINITNÍ CHROMATOGRAFIE



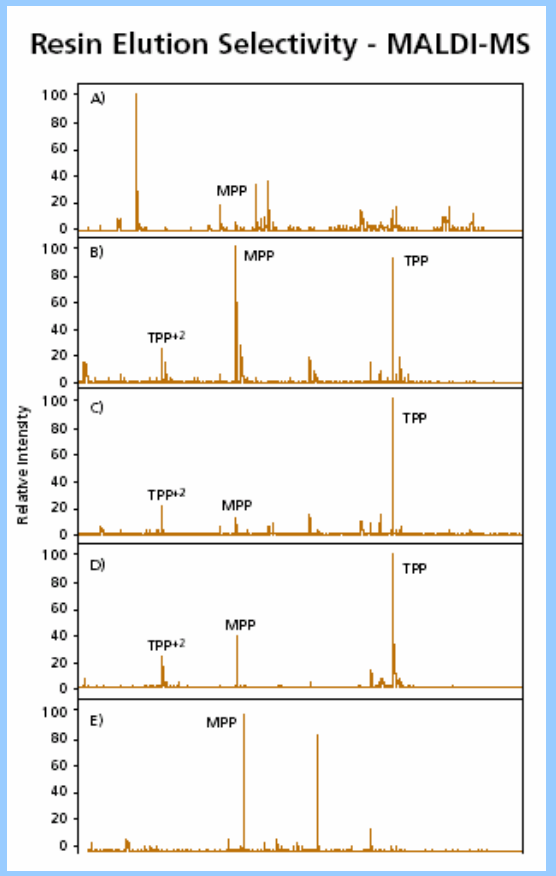
IMAC

Immobilized Metal Affinity Chromatography

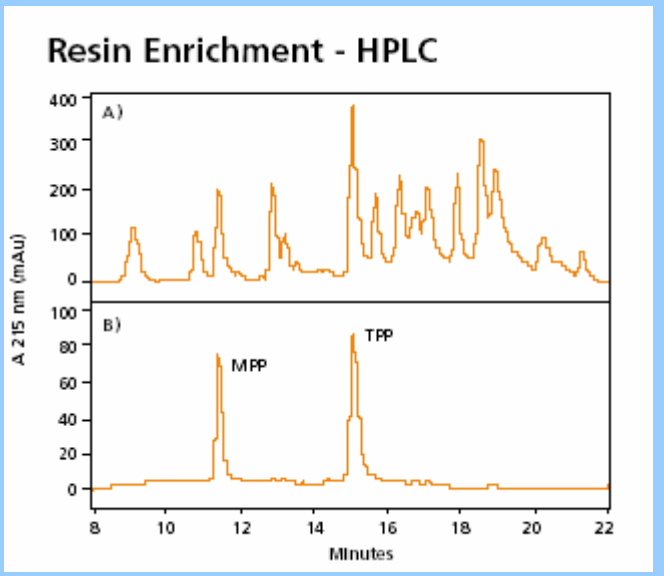
PHOS Select Iron Affinity gel

Surový

Po aplikaci



Kasein tryptic digest

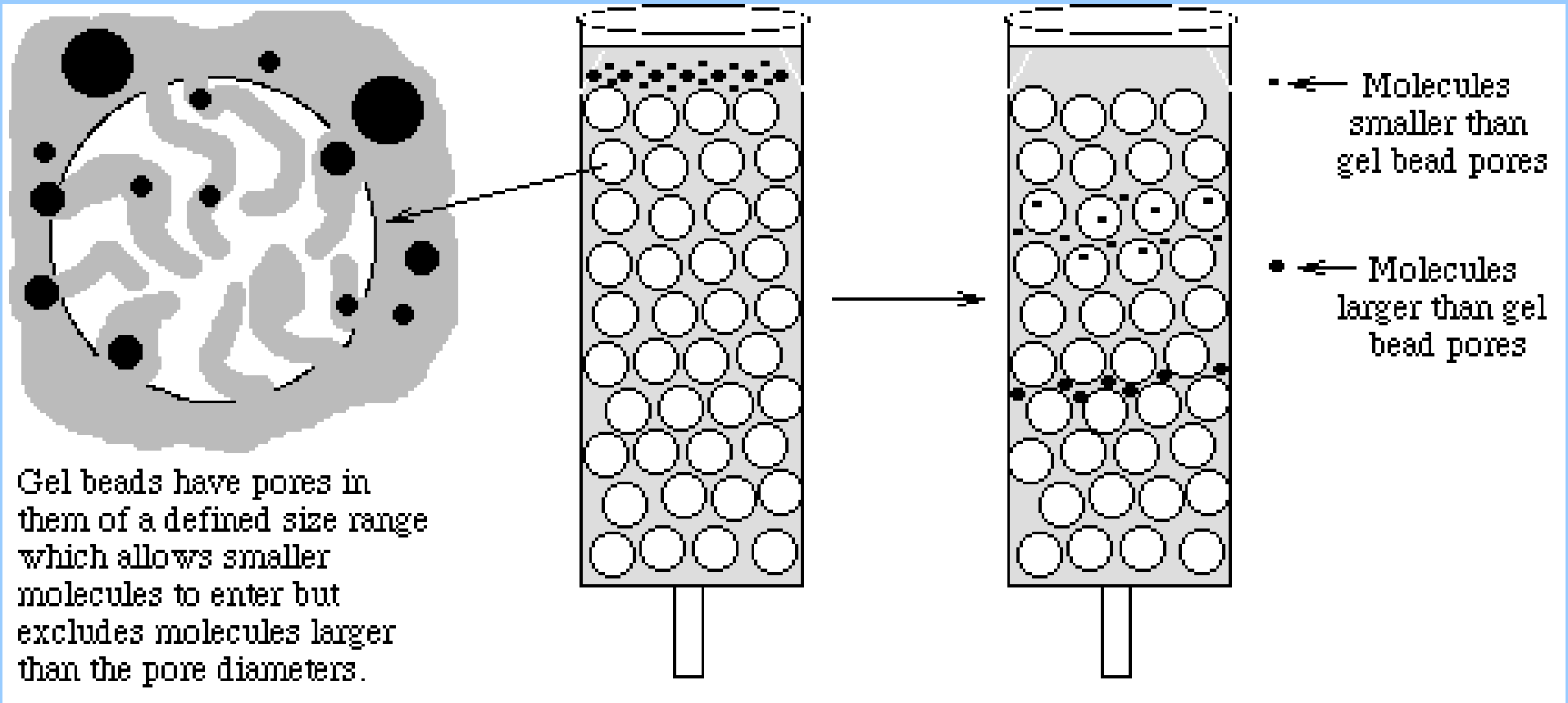


Surový

Po aplikaci

Kasein

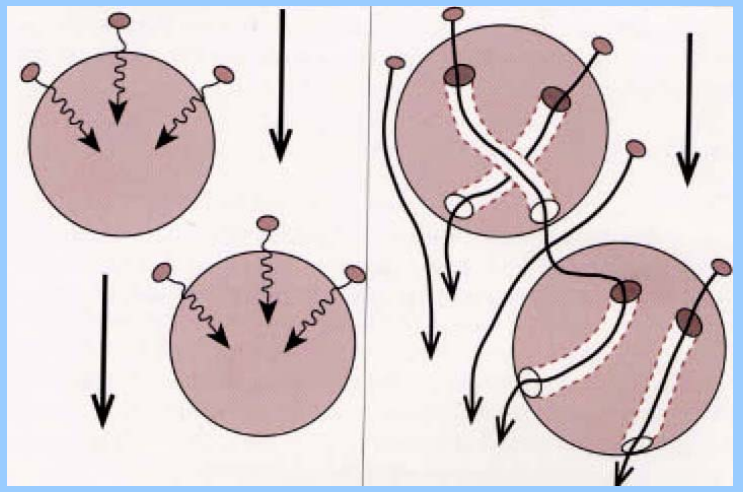
GELOVÁ CHROMATOGRAFIE



KAPALINOVÁ CHROMATOGRAFIE

- HPLC
- LC
- PERFÚZNÍ

POROS



klasický sorbent POROS

RP

SAX

WAX

SCX

WCX

HIC

Activated Affinity

Affinity

Application-Specific RP

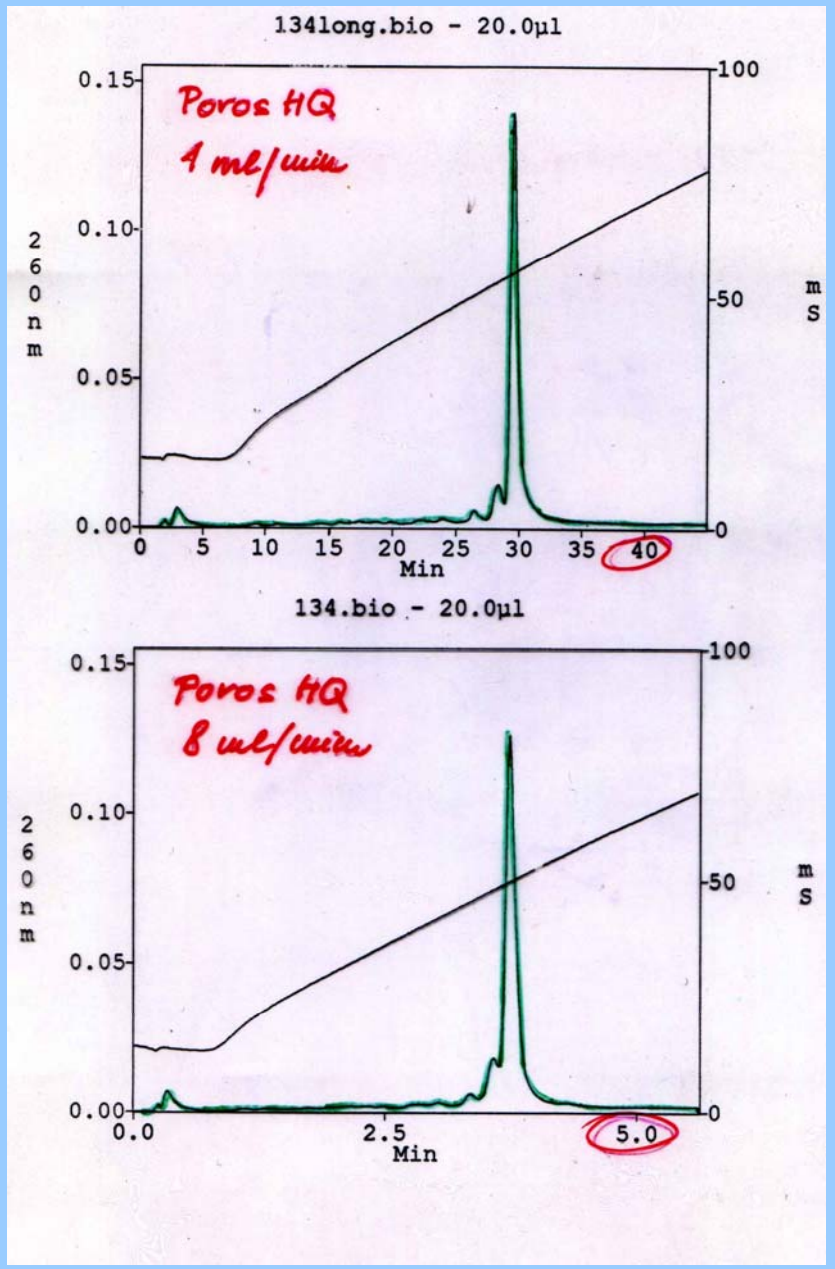
Choose the POROS Chemistry

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

	PRODUCT	FUNCTIONAL GROUP	FUNCTIONAL GROUP DENSITY	DYNAMIC CAPACITY*	R
REVERSED-PHASE CHROMATOGRAPHY					
	R1	Base Poly(styrene divinyl benzene)	Low phase ratio, providing low retentivity	5 mg/mL	Fo R1
	R2	Base Poly(styrene divinyl benzene)	High phase ratio, providing higher binding strength and retentivity	10 mg/mL	Ge of
ION-EXCHANGE CHROMATOGRAPHY					
Strong Anion Exchangers	HQ	Quaternized polyethyleneimine	High	55 mg/mL	Fo bu
	QE	Quaternized polyethyleneimine	Medium	30 mg/mL	Al
Weak Anion Exchangers	DEAE	Diethylaminoethyl	Medium	55 mg/mL	An
	PI	Polyethyleneimine	Medium	45 mg/mL	An
Strong Cation Exchangers	HS	Sulphopropyl	High	75 mg/mL (POROS 50 60 mg/mL)	Fo bu
	SP	Sulphopropyl	Medium	45 mg/mL	Al
	S	Sulphoethyl	Low	10 mg/mL	Sp ba
Weak Cation Exchanger	CM	Carboxymethyl	High	70 mg/mL	Ca
HYDROPHOBIC INTERACTION CHROMATOGRAPHY					
	HP2	High density phenyl	High	12 mg/mL	Fo pc
	PE	Phenyl ether	Medium	8 mg/mL	Ge m
	ET	Ethyl ether	Medium	4 mg/mL	Pr st
ACTIVATED AFFINITY CHROMATOGRAPHY					
For Activation	OH	Hydroxyl		———	Al di
Pre-Activated	AL	Aldehyde		———	Ca
	EP	Epoxide		———	Ca hy
	NH	Primary amine		———	Fo wi
	HY	Hydrazide		———	Fo m
AFFINITY CHROMATOGRAPHY					
	A	Recombinant Protein A		30 mg/mL	De ca Re
	G	Recombinant Protein G		15 mg/mL	Fo Br
	HE	Heparin		15 mg/mL	Fo lip
	MC	Imido-diacetate		15 mg/mL	Bi co
APPLICATION-SPECIFIC REVERSED-PHASED MEDIA					
	Oligo R3	Poly(styrene-divinyl benzene)	Very high phase ratio	30 mg/mL	Sp ph pr
	PepMap C18	Silica C18, end-capped	7% carbon loading	———	Pr ap

*Test protein

BioCAD 700E

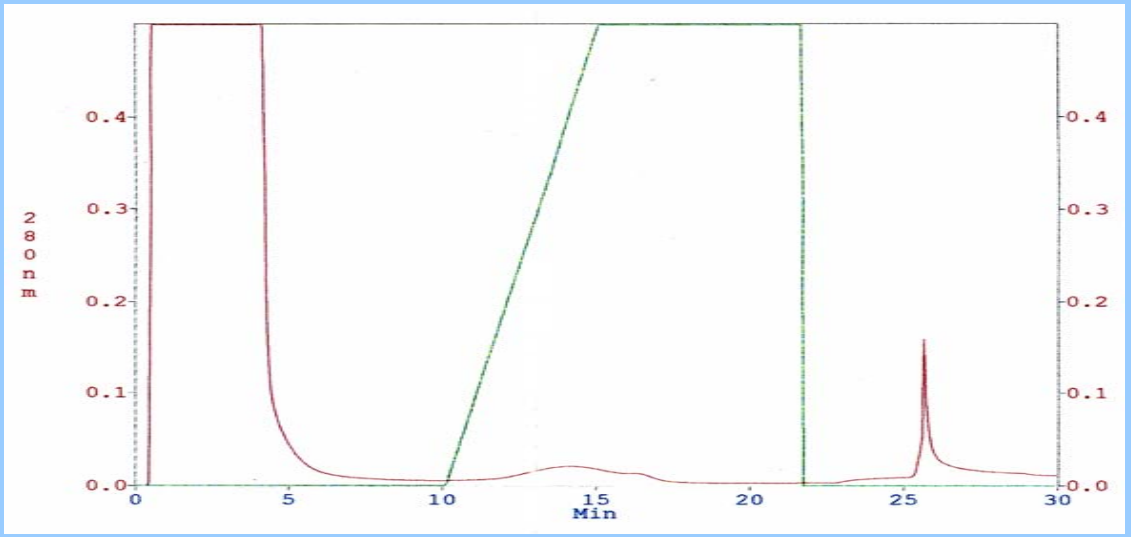

134long.bio - 20.0µl
Poros HQ
4 ml/min
260nm
0.15
0.10
0.05
0.00
100
50
0
ms
0 5 10 15 20 25 30 35 40
Min
134.bio - 20.0µl
Poros HQ
8 ml/min
260nm
0.15
0.10
0.05
0.00
100
50
0
ms
0.0 2.5 5.0
Min

MC Poros

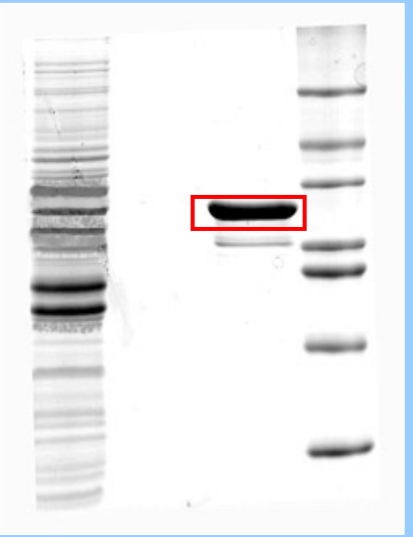
Metalochelatační purifikace
his-tag proteinu

Petra Borkovcová

Ni^{2+}MC POROS

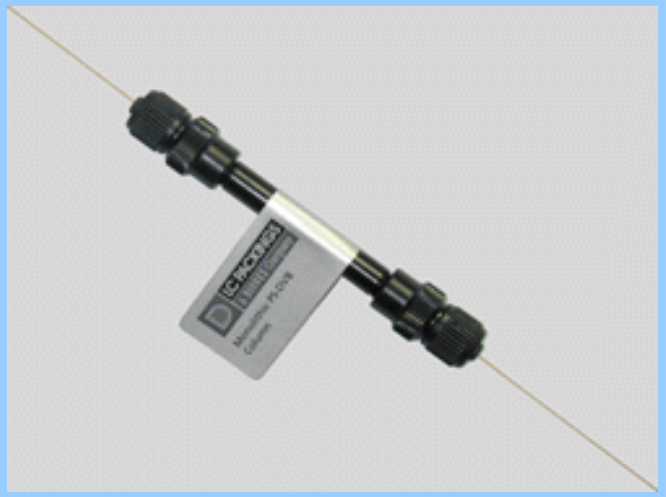


Čistota 95 %



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

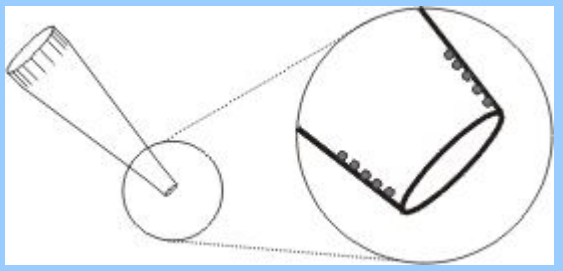
Kolona může vypadat různě . . .



Glygen

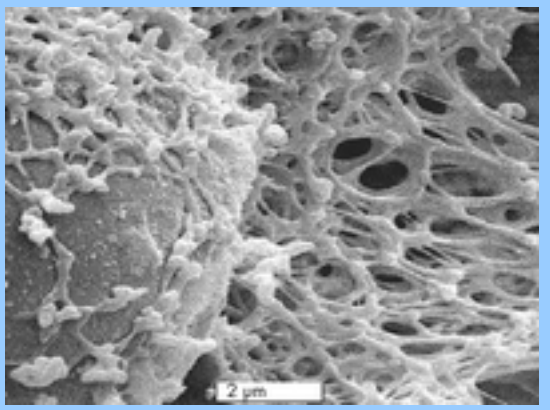
TopTip

NuTip



Zip Tip
Millipore

C18
C4
MC
SCX



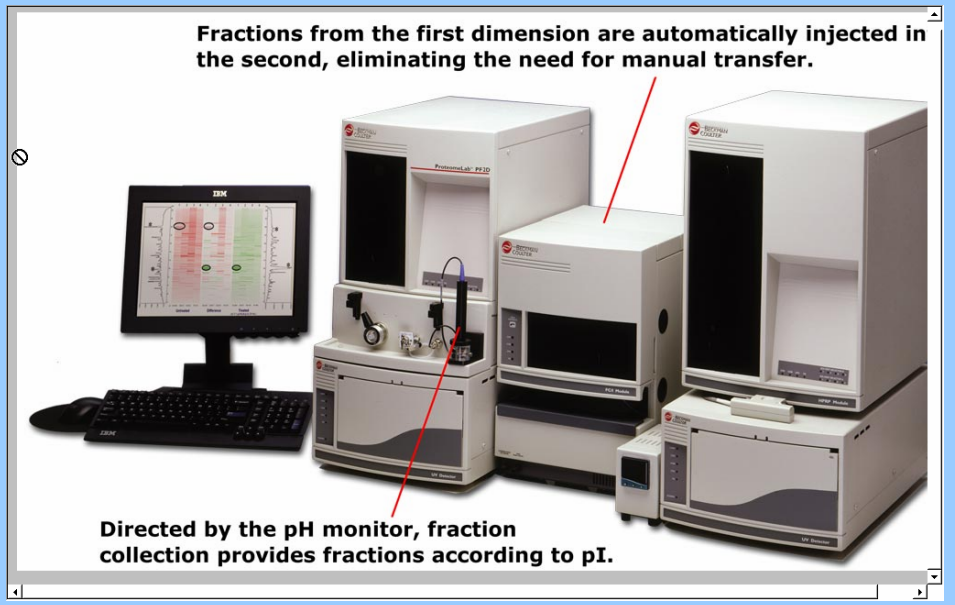
MULTIDIMENZIONÁLNÍ CHROMATOGRAFIE

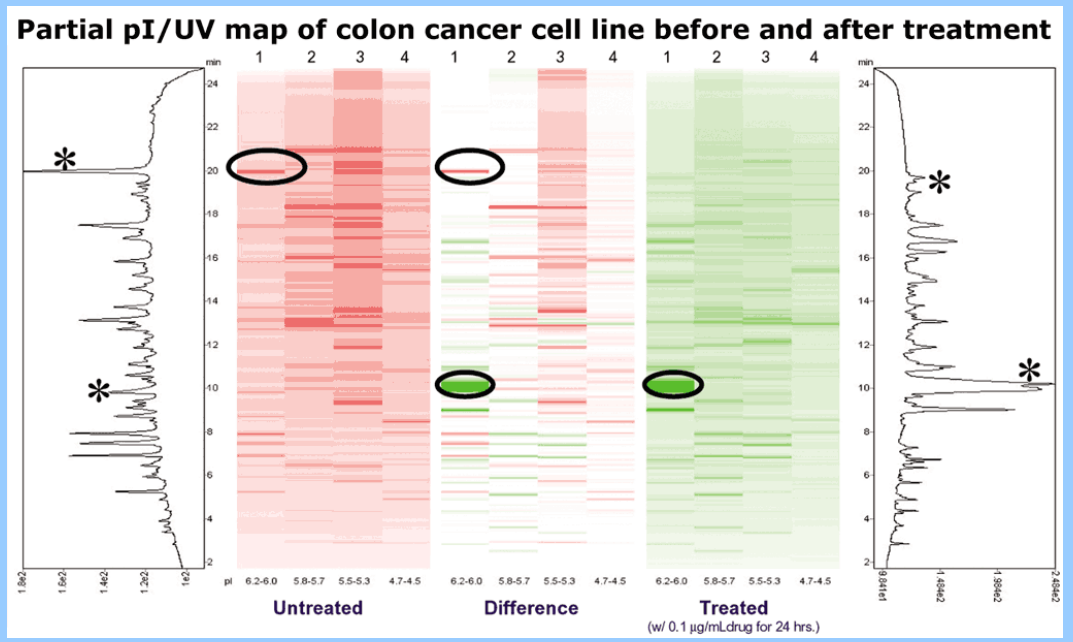
kombinace odlišných fyzikálních a chemických separačních principů

- diskontinuální
- kontinuální
- dvoufázová kolona

ProteomeLab PF 2D

- chromatofokusace
- RP





MULTIDIMENZIONÁLNÍ CHROMATOGRAFIE

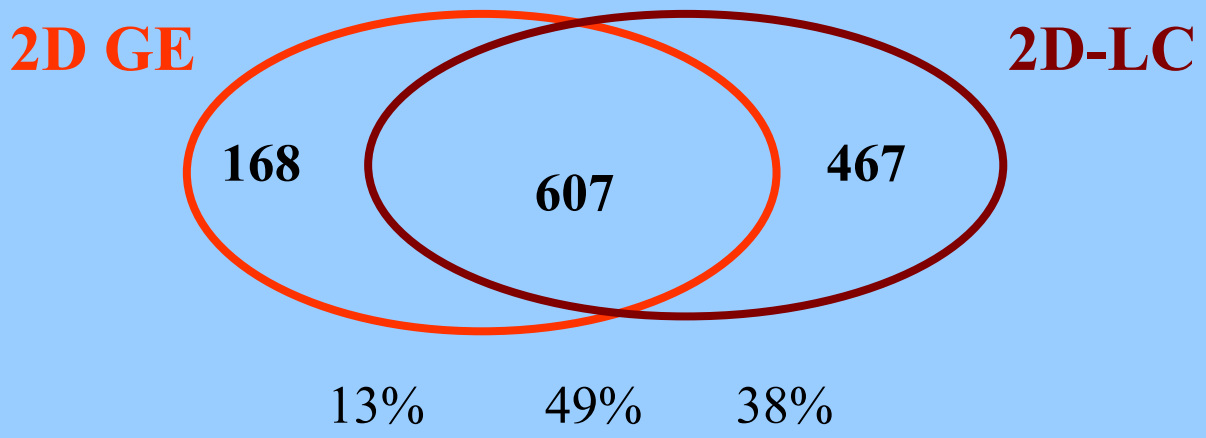
PRO

- 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



M. Hecker, University of Greisfwald, bakteriální proteom, HUPO 2005

LITERATURA

- 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