MASARYKOVA UNIVERZITA

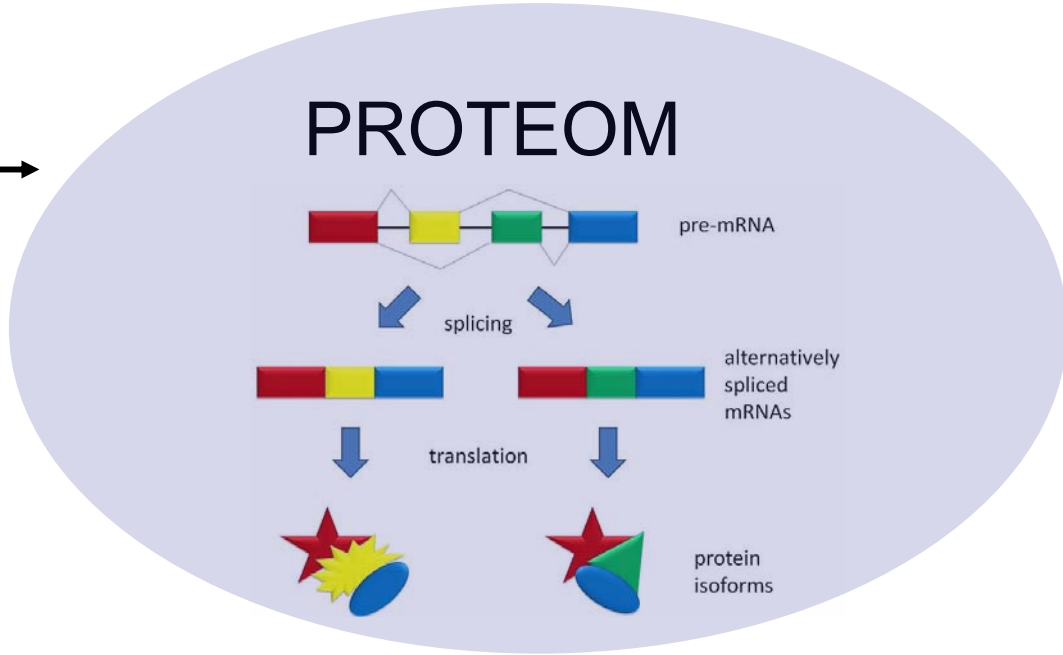
Středoevropský technologický institut CEITEC
Centrální laboratoř - Proteomika

PŘÍPRAVA PROTEINOVÉHO VZORKU PRO MS ANALÝZU

Hana Konečná

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

GENOM →

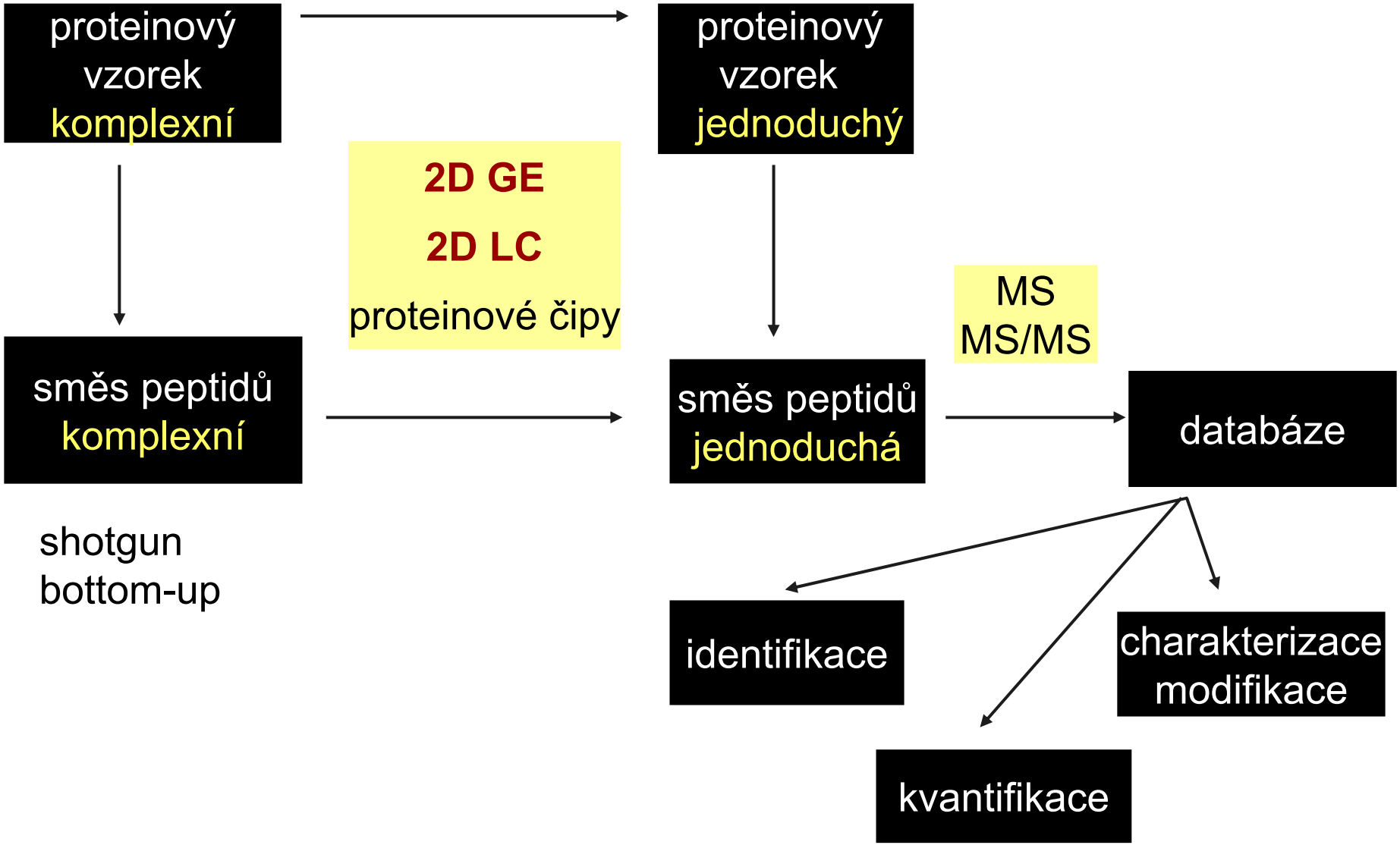


IZOFORMY

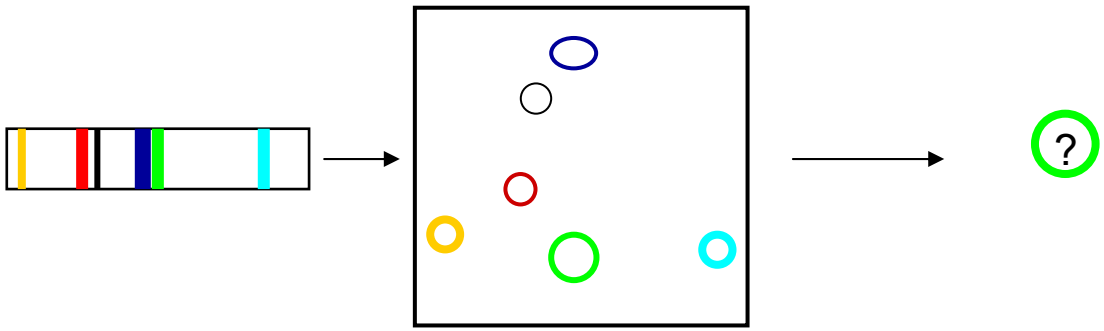
PTM asi 200 typů (fosforylace, glykosylace, acylace, methylace…)

KONCENTRAČNÍ ROZSAH asi deset řádů ↓

PREFRAKCIONACE → MS


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

I. Dvoudimenzionální elektroforéza 2D GE
II. Prefrakcionace
III. Multidimenzionální chromatografie

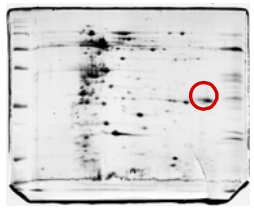


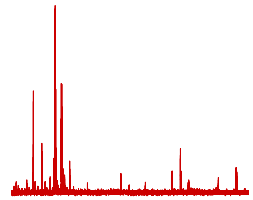
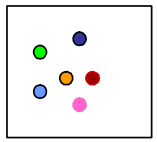
Proteomický experiment

extrakce **fokusace** **SDS-PAGE** **digest** **MS**

2DE

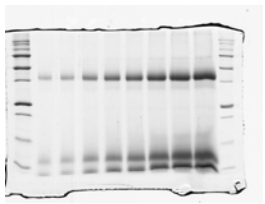




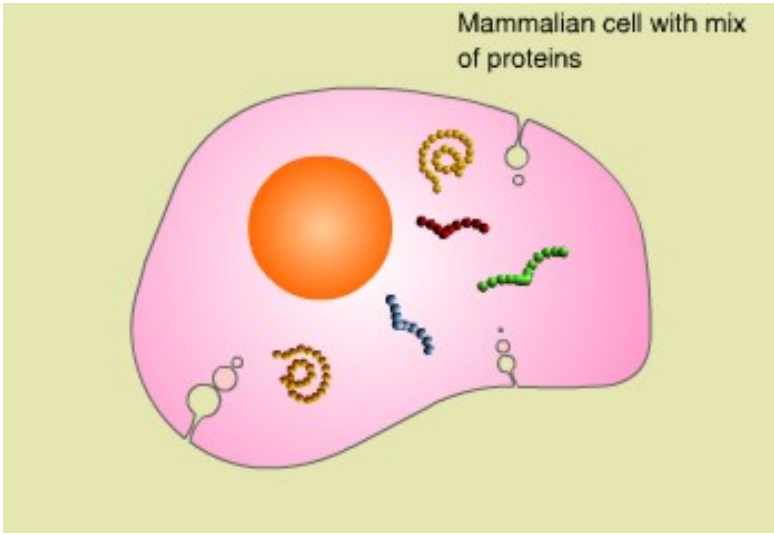
identifikace **neznámý protein**

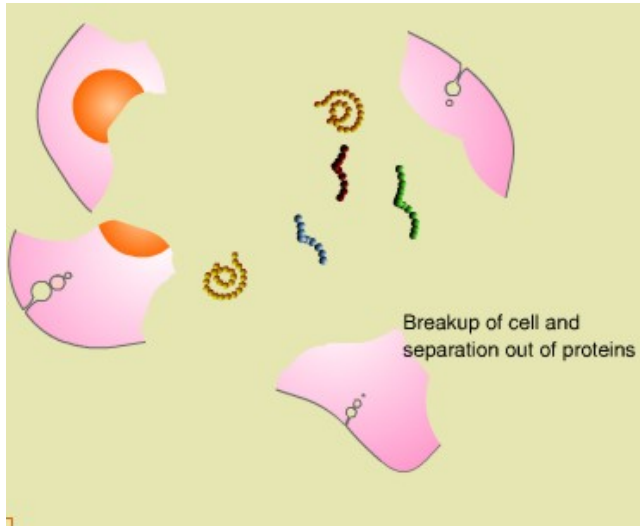
1DE



HOMOGENIZACE

- mechanicky
- ultrazvukem
- tlakem
- zmražením / rozmražením
- detergentovou lyzí





PŘÍPRAVA VZORKU

keratiny!

solubilizace močovina, thiomočovina, detergenty

redukce DTT, TBP

inhibice proteáz, fosfatáz, glykosyláz

odstranění kontaminant

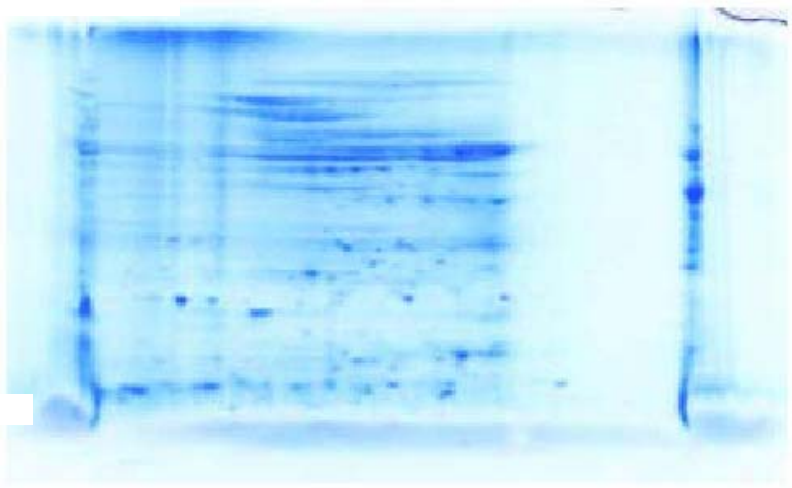
DETERGENTY

- žádný celkový náboj
- 0.5 – 4%
- použitelné ve vysokých koncentracích močoviny

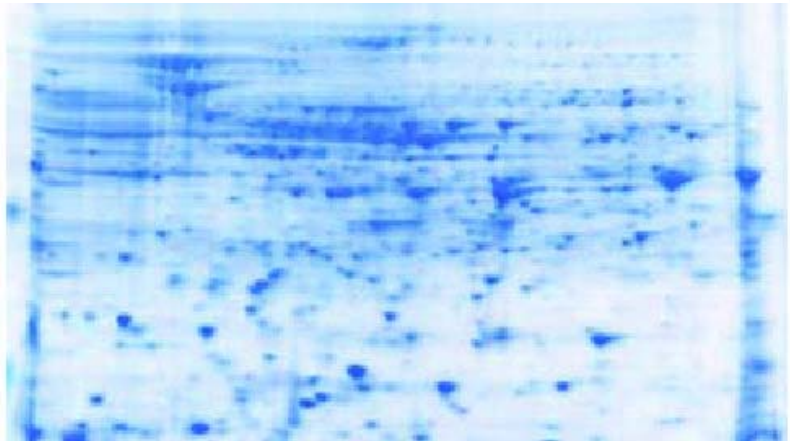
- neionogenní
- zwitterointové

- SDS jen v nízkých koncentracích (do 0.25%)

E.coli



CHAPS



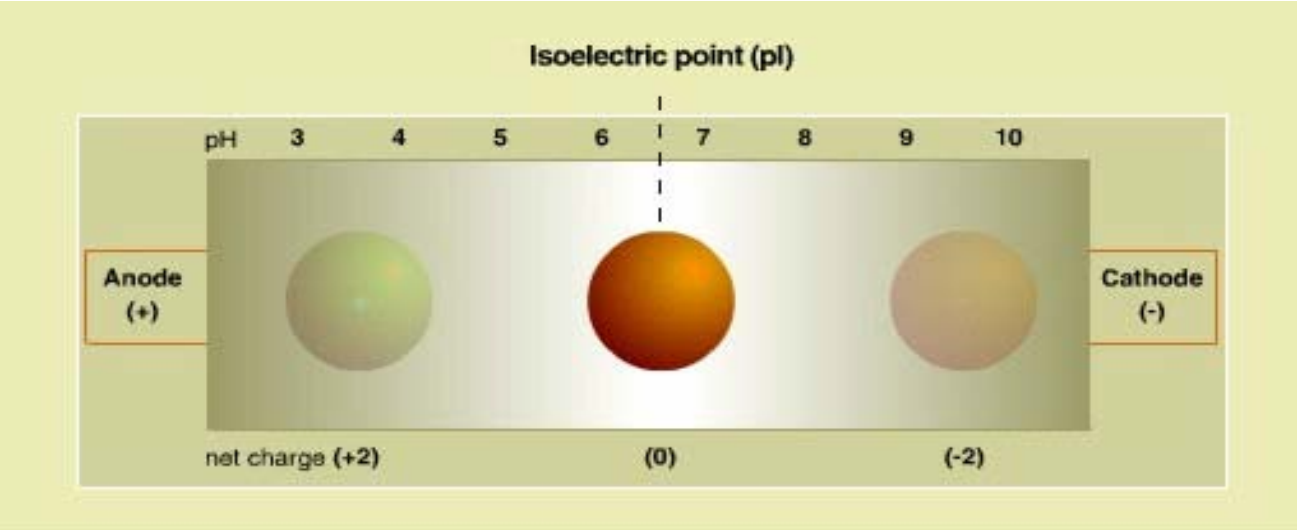
C7BzO

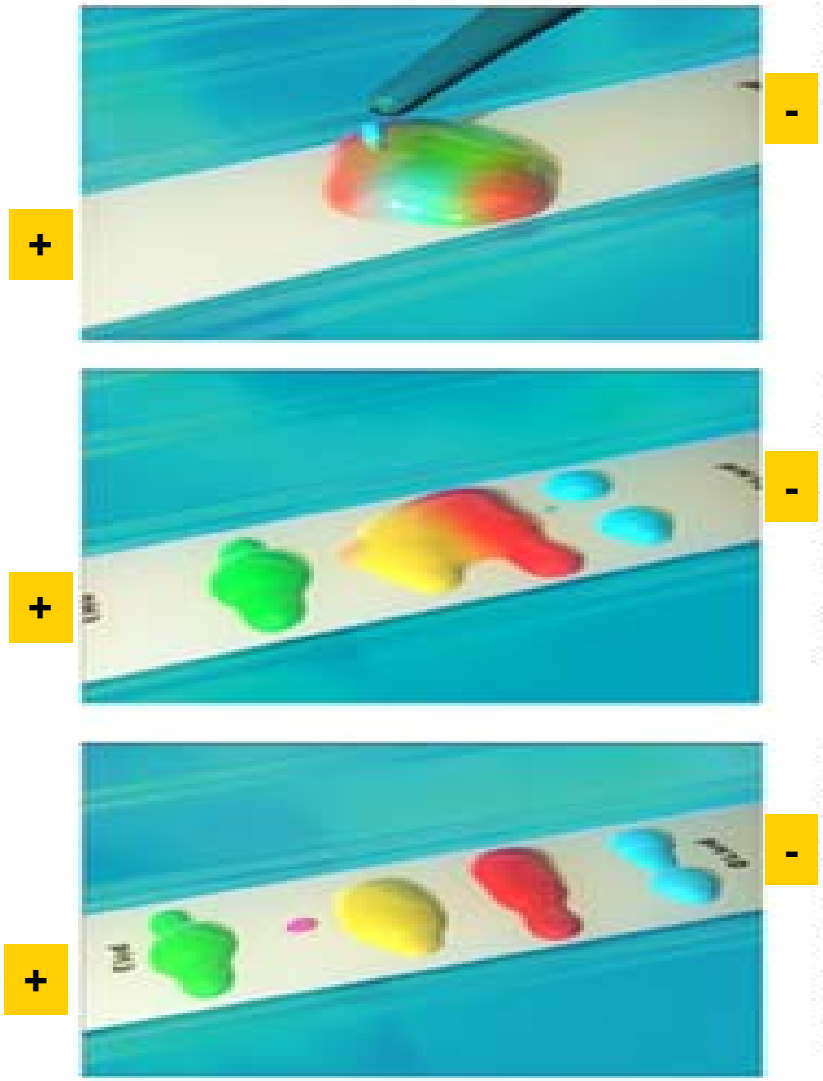
KONTAMINANTY

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

1. ROZMĚR IZOELEKTRICKÁ FOKUSACE

migrace nabitých částic v gradientu pH v elektrickém poli

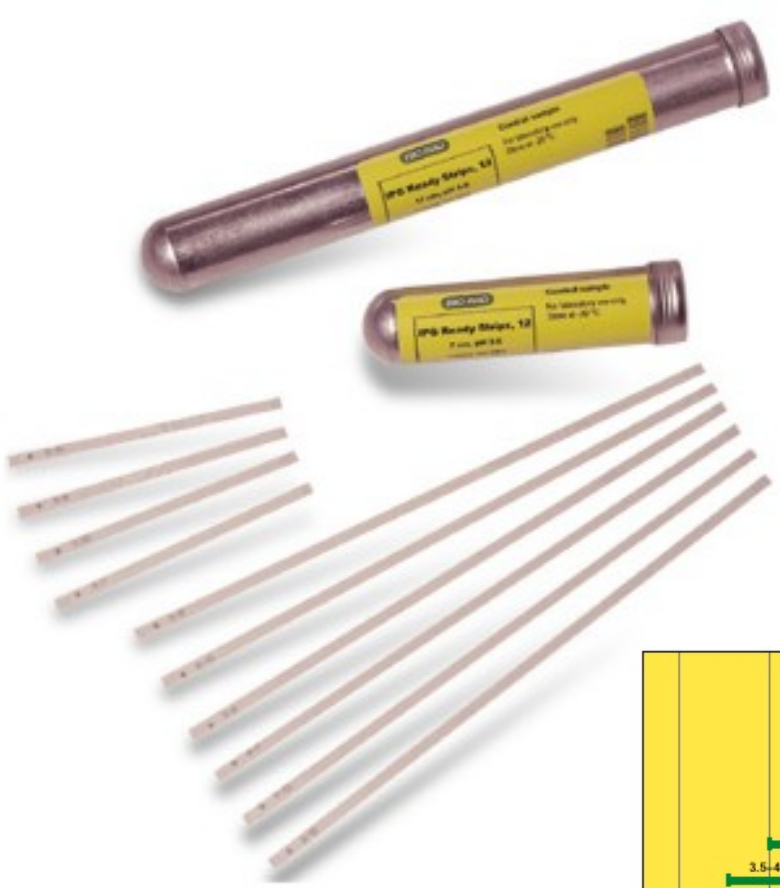


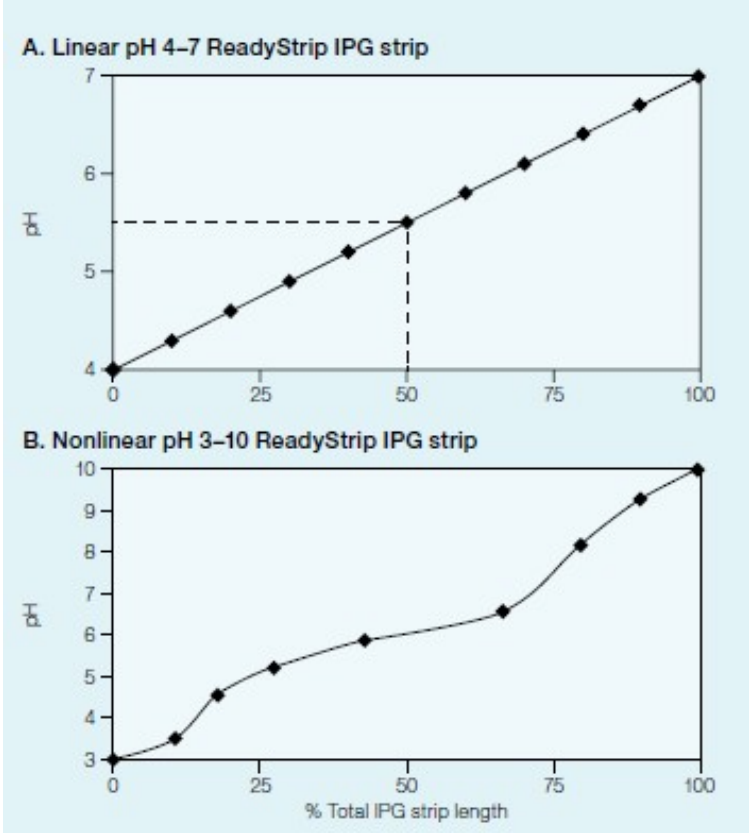


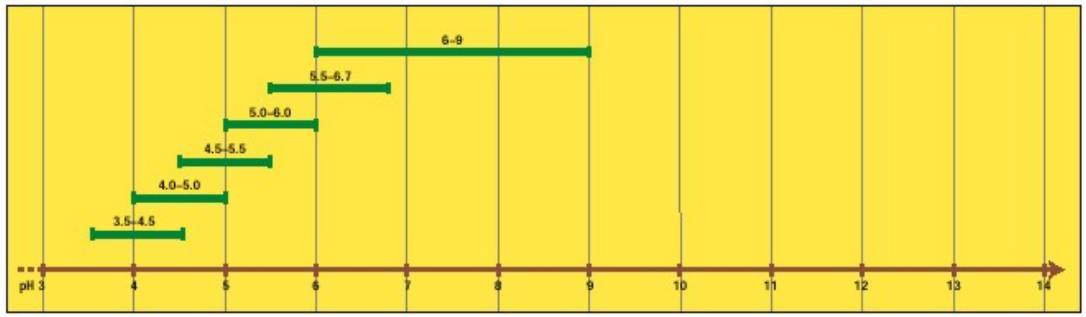
IZOELEKTRICKÁ FOKUSACE

- imobilizovaný pH gradient
- amfolyty

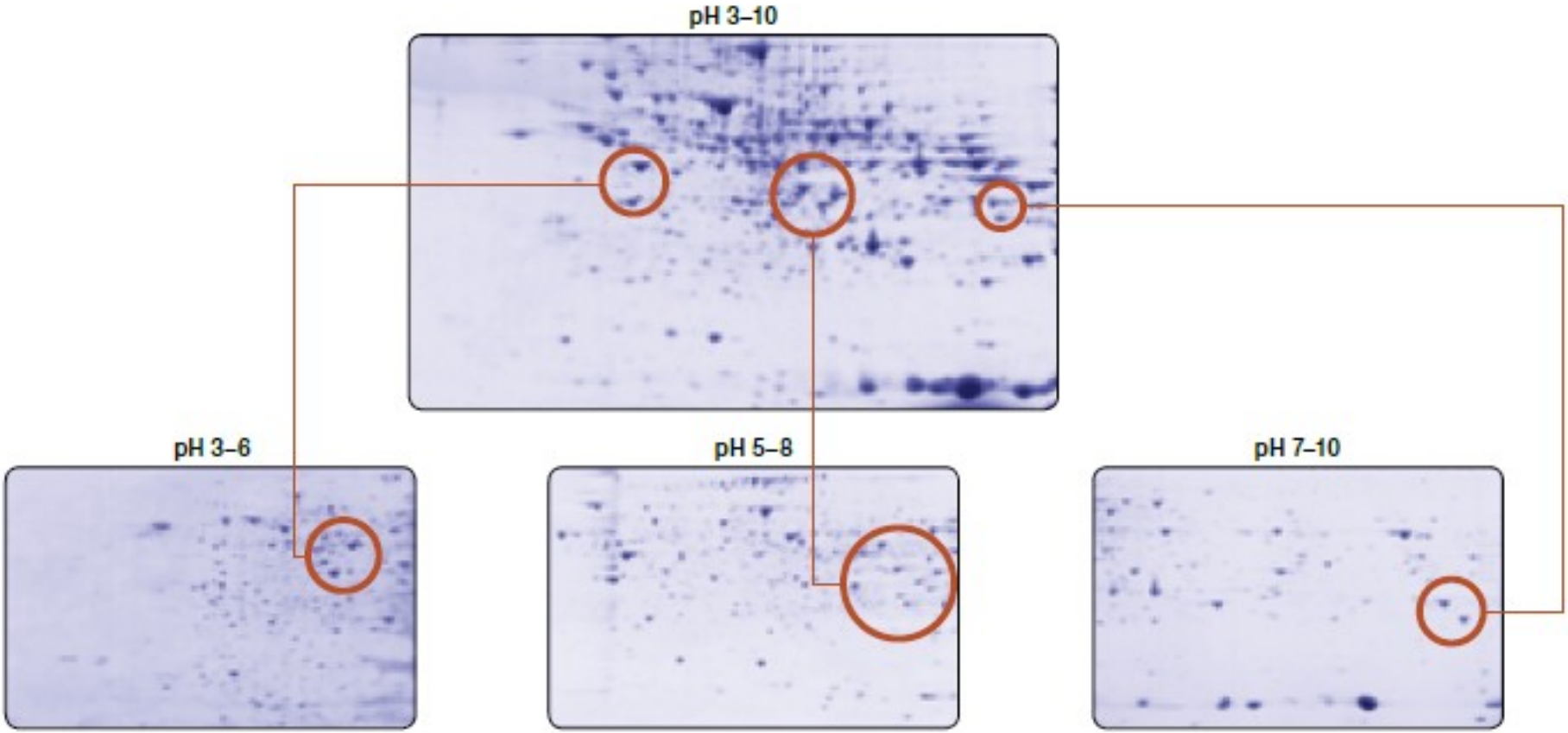
ROZSAH STRIPU
ROZMĚR STRIPU





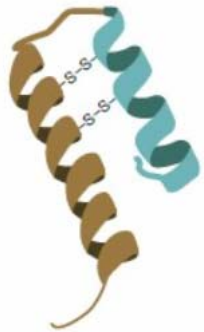


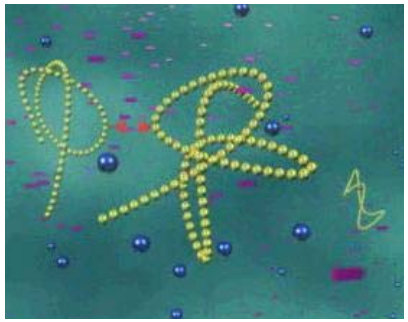
ROZSAH STRIPU

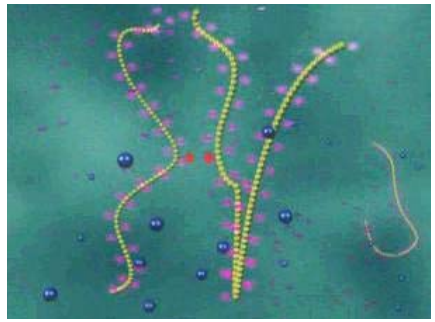


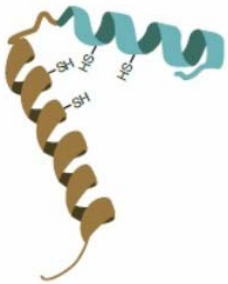
EKVILIBRACE STRIPU

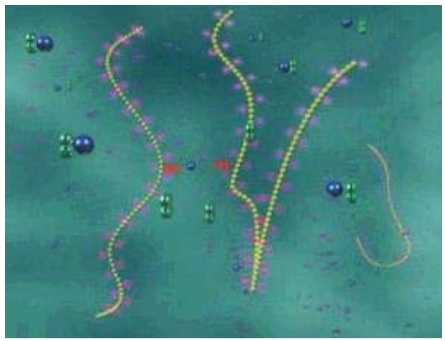
denaturace **SDS** •

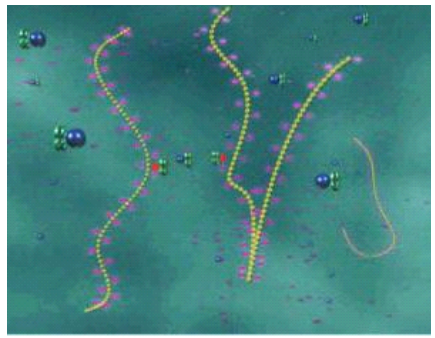


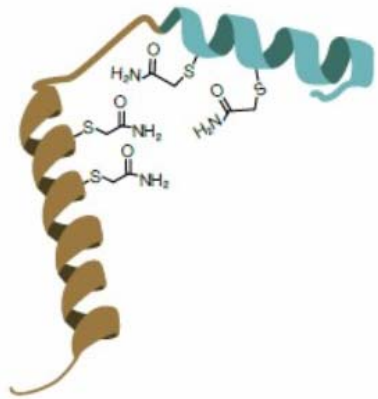








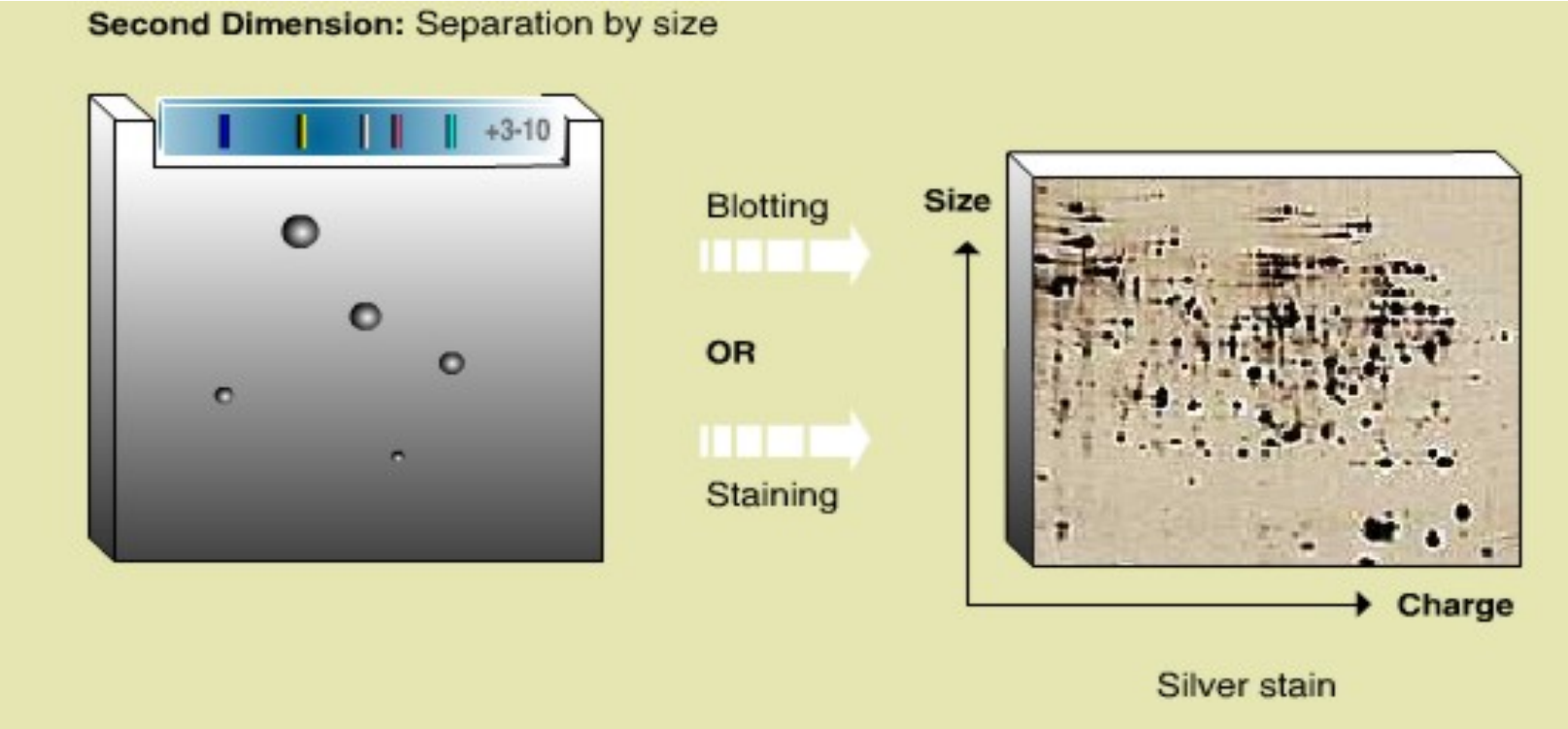




redukce **DTT** ● alkylace **IAA** •

2. ROZMĚR SDS-PAGE

migrace aniontů v elektrickém poli podle MW

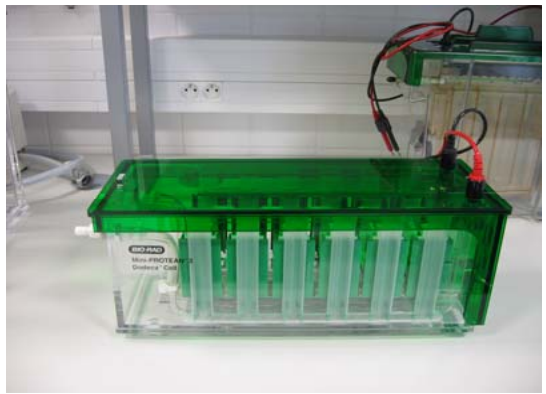


2-DGE INSTRUMENTACE

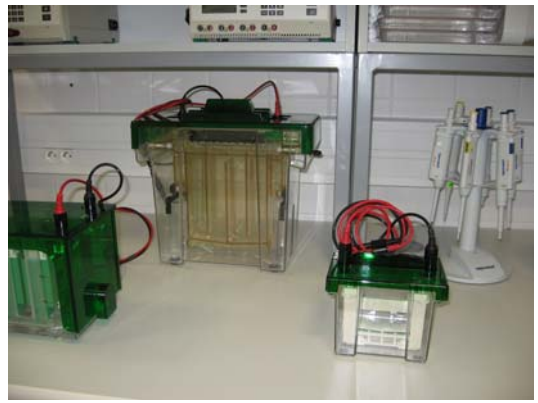
- Protean IEF
- Protean Dodeca Cell
- Densitometer GS-800
- FLA-7000

PDQuest, Quantity One

Protean Plus Dodeca Cell



Mini-Protean 3 Dodeca Cell

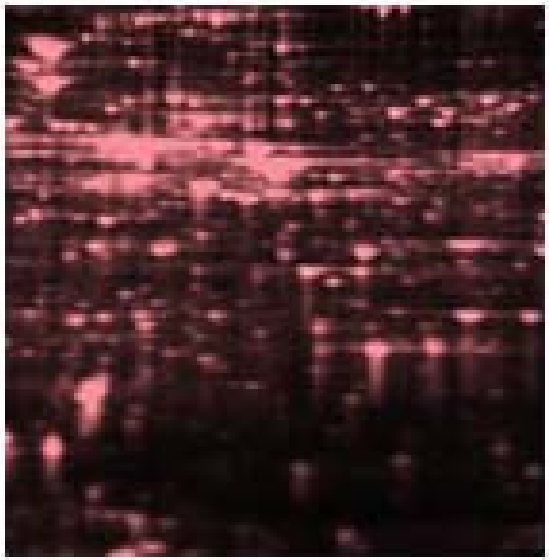


Protean II xi Cell

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

DETEKCE PROTEINU V GELU

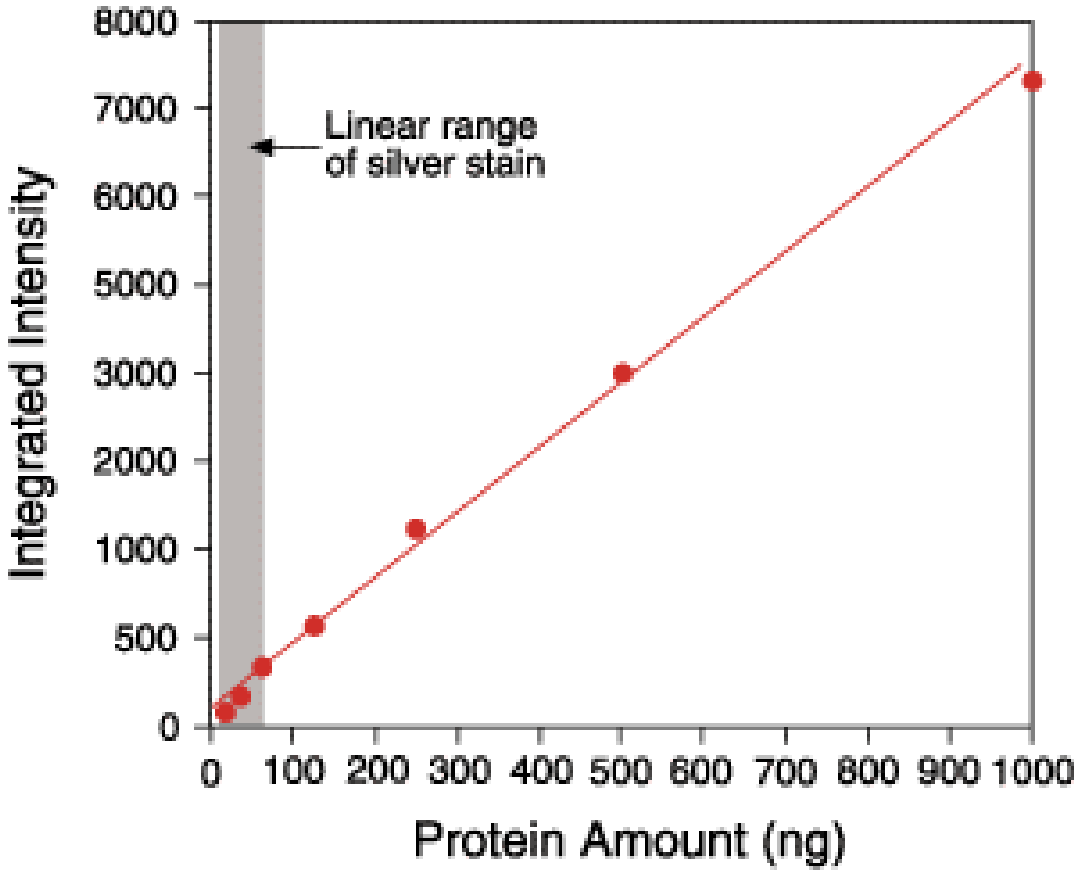


Sypro Ruby	Coomassie	silver
1.4 ng	36 ng	0.6 ng

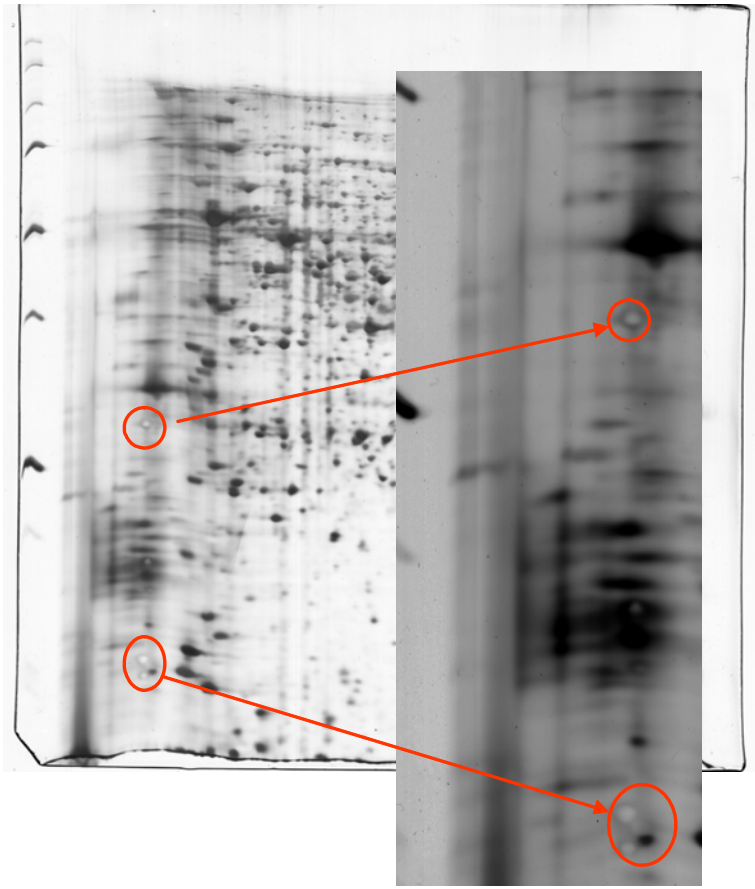
PTM specifická barvení *Pro-Q Diamond* *Pro-Q Emerald*

BARVENÍ PROTEINU – LINEARITA

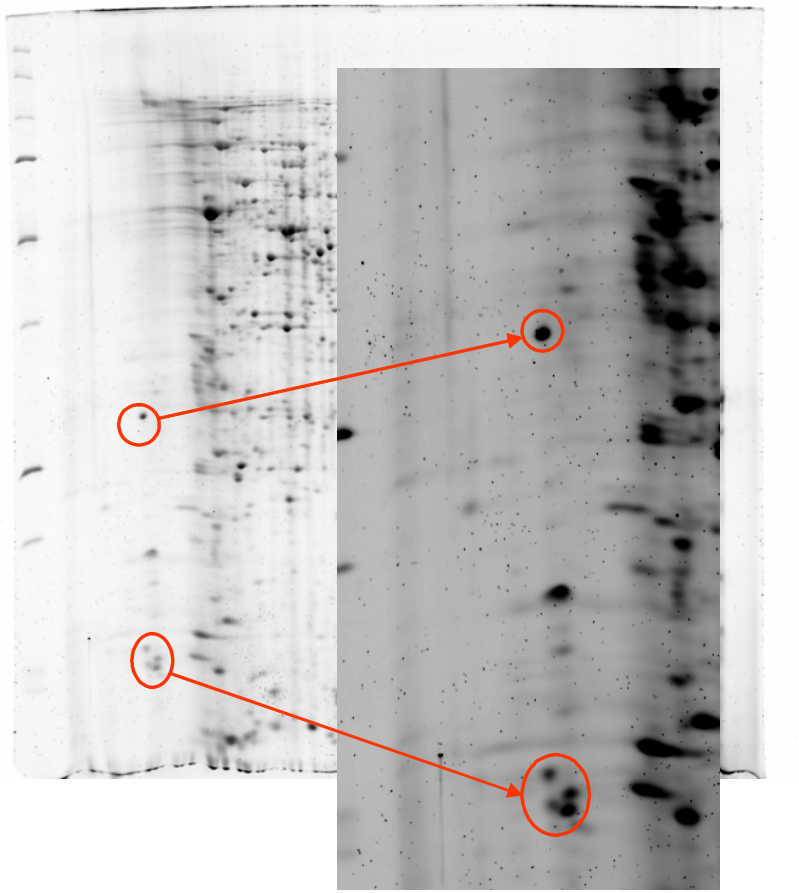
Sypro Ruby



Ag

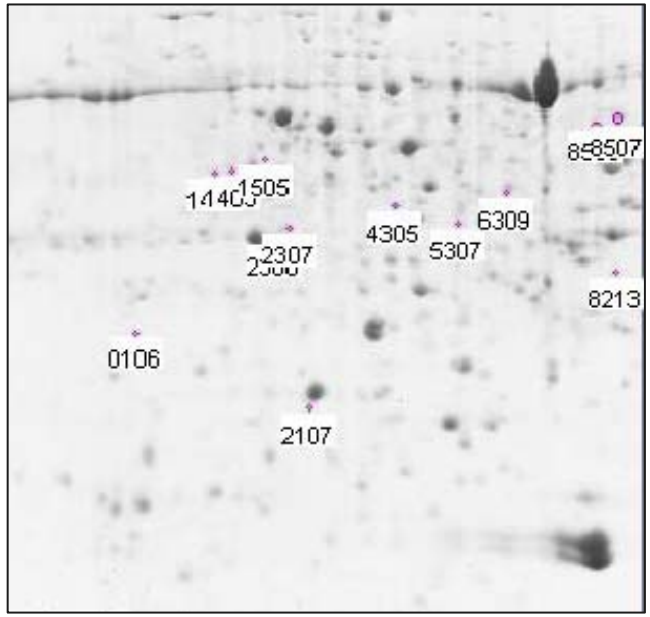


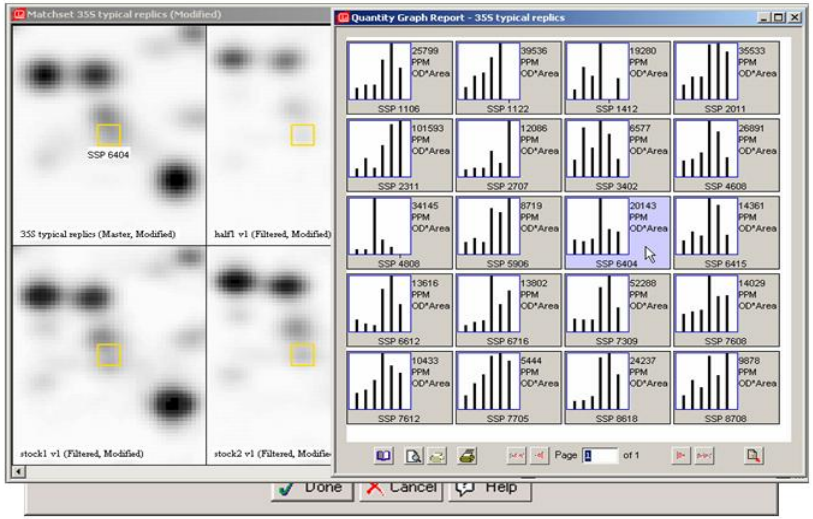
Sypro Ruby



ANALÝZA OBRAZU

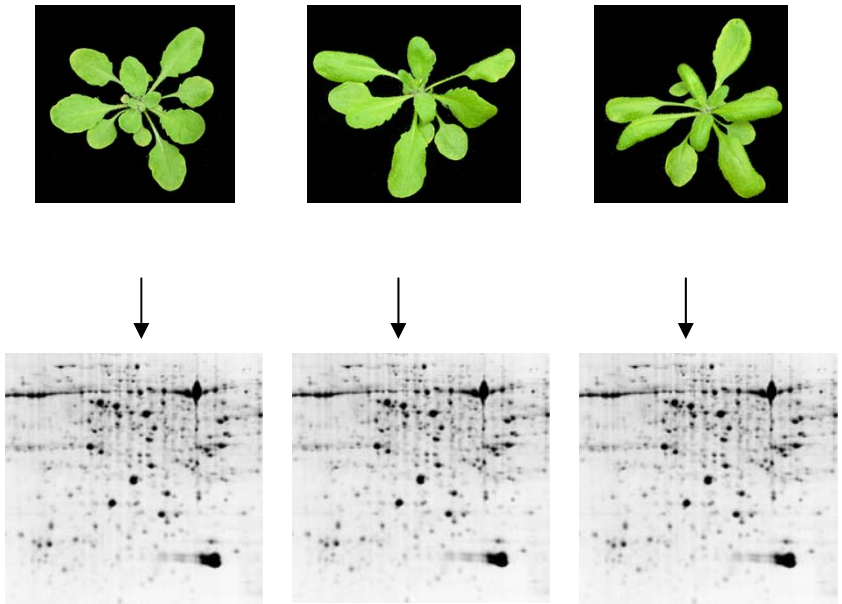
- kvalitativní
- kvantitativní





biologická variabilita

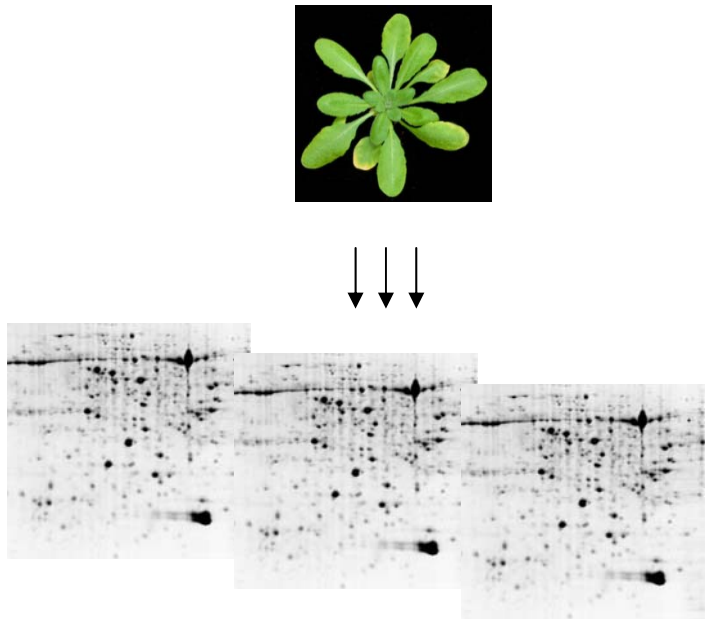
stejný organismus za stejných podmínek



biologické replikáty

technická variabilita

stejný vzorek stejně

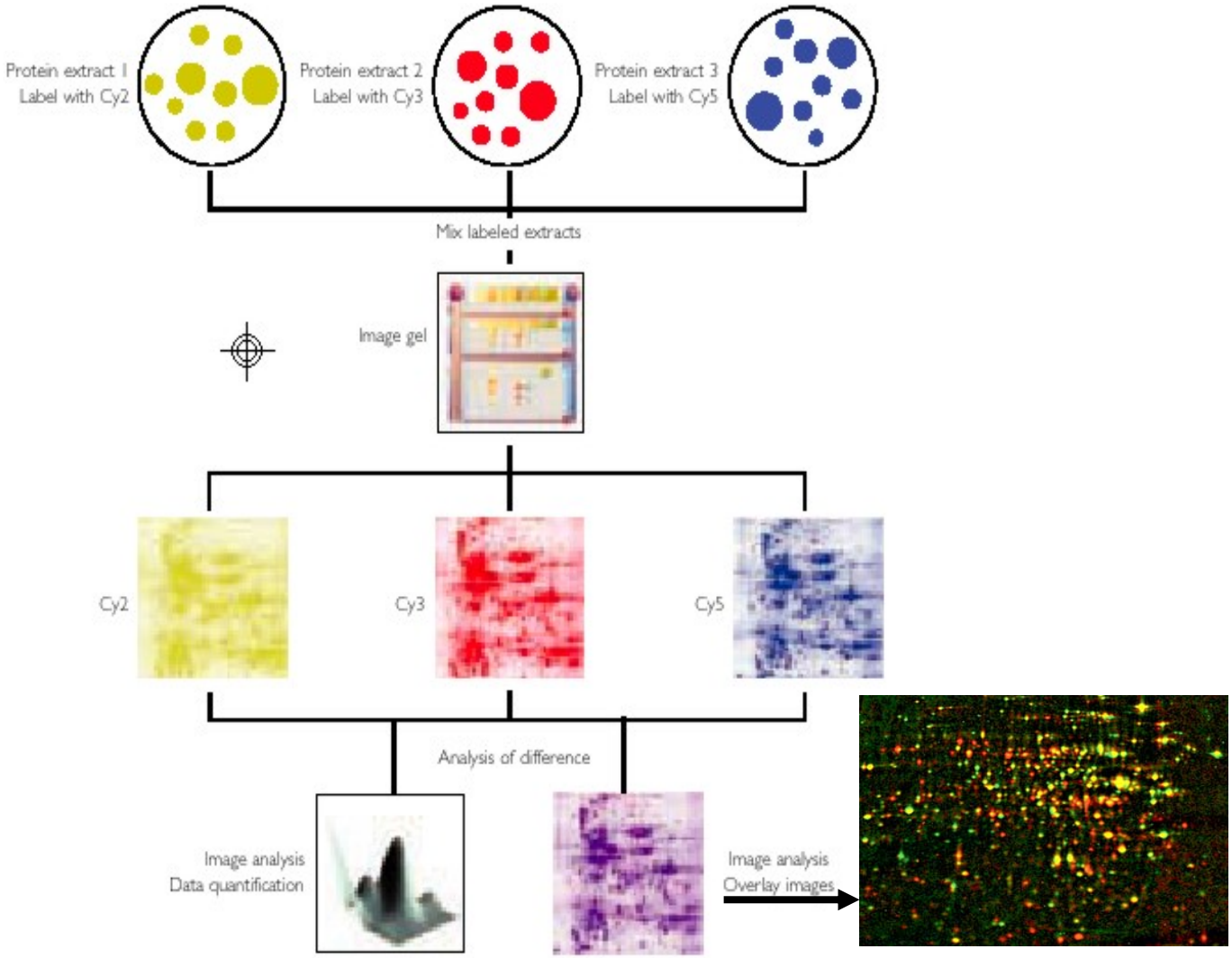


technické replikáty

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

Difference Gel Electrophoresis DIGE



BIOMARKERY

… jehly v kupce sena

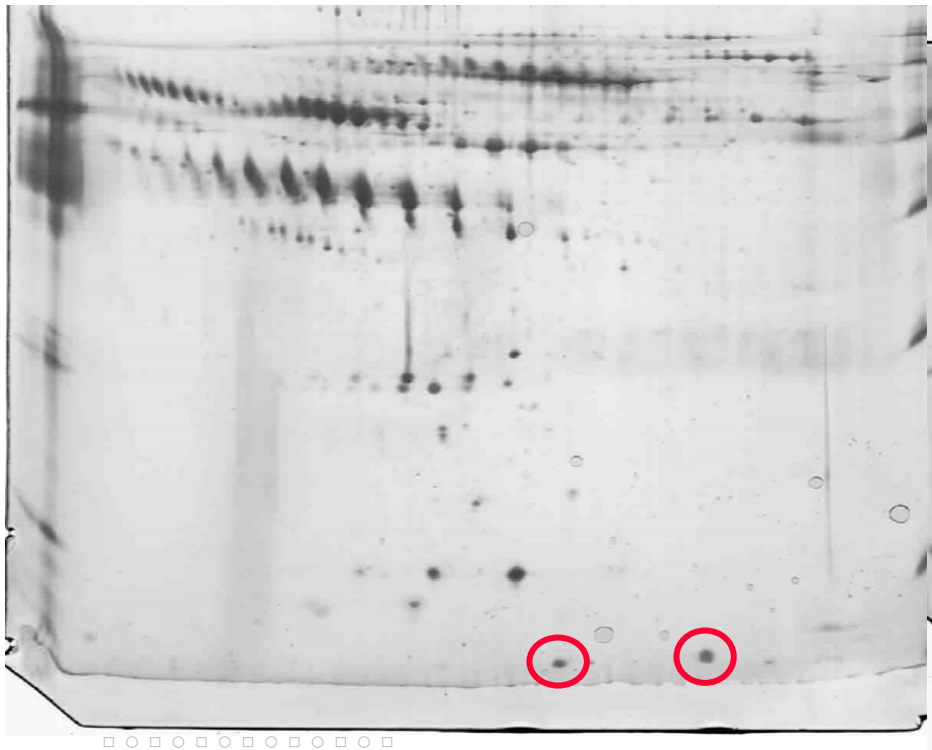
prefrakcionace . separace . identifikace . srovnání kontrola vs.vzorek

- **seno** - proteiny bez vztahu k onemocnění (pozadí)
- **jehly** - specifické proteiny pro onemocnění
- potenciální jehly jsou **obtížně validovatelné** biologická variabilita!
- nejsou pravidla, které jehly dále zkoumat
- jehly často **PTM,** nejsou identifikovány MS

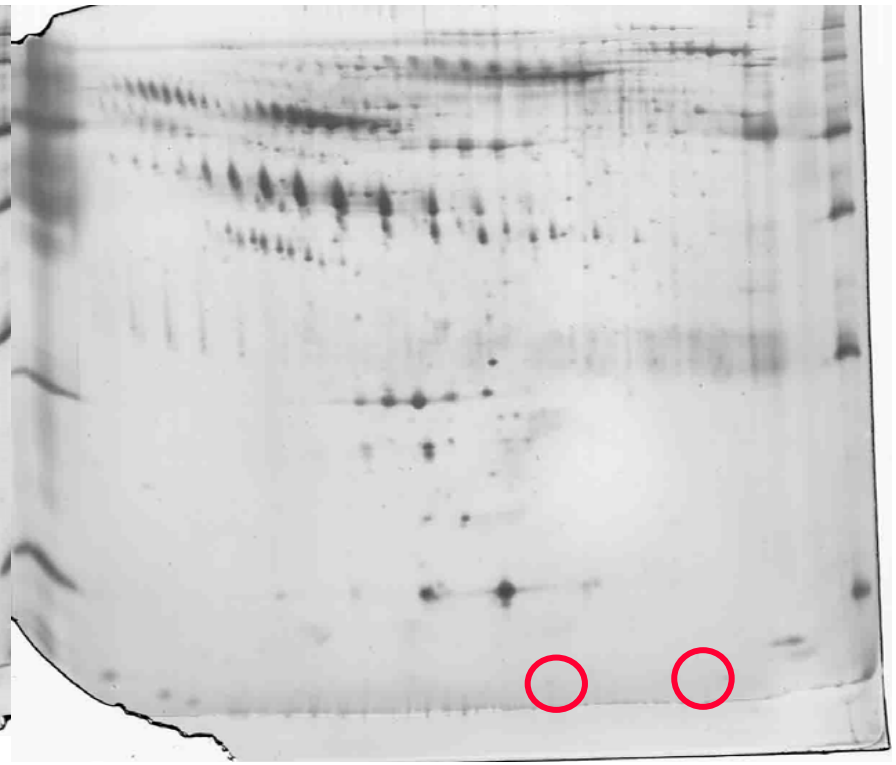
Biomarkery v lidské plasmě

SEPARACE → IDENTIFIKACE MS

den 21 – před klinickým projevem



den 44 – po klinickém projevu

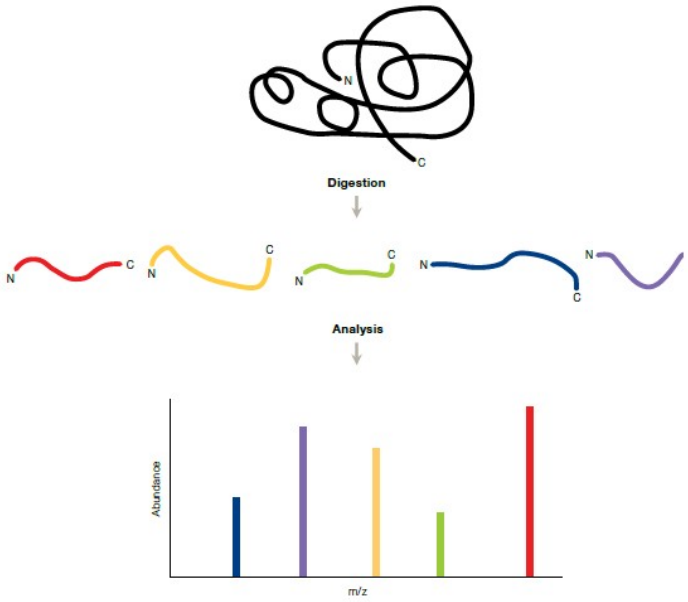


DIGESCE

trypsin Glu-C Asp-N thermolysin

MAVEPFPRRPITRPHASIEVDTSGTGGSAGSSE
KVFCLIGQAEGGEPNTVYELRNYAQAKRLFR
SGELLDAIELAWGSNPNYTAGRILAMRIEDAK
PASAEIGGLKITSKIYGNVANNIQVGLEKNTLS
DSLRLRVIFQDDRFNEVYDNIGNIFTIKYKGEE
ANATFSVEHDEETQKASRLVLKVGDQEVKSY
DLTGGAYDYTNAIITDINQLPDFEAKLSPFGD
KNLESSKLDKIENANIKDKAVYVKAVFGDLE
KQTAYNGIVSFEQLNAEGEVPSNVEVEAGEES
ATVTATSPIKTIEPFELTKLKGGTNGEPPATWA
DKLDKFAHEGGYYIVPLSSKQSVHAEVASFV
KERSDAGEPMRAIVGGGFNESKEQLFGRQAS
LSNPRVSLVANSGTFVMDDGRKNHVPAYMV
AVALGGLASGLEIGESITFKPLRVSSLDQIYESI
DLDELNENGIISIEFVRNRTNTFFRIVDDVTTFN
DKSDPVKAEMAVGEANDFLVSELKVQLEDQF
IGTRTINTSASIIKDFIQSYLGRKKRDNEIQDFP
AEDVQVIVEGNEARISMTVYPIRSFKKISVSLV
YKQQTLQA

- IN-GEL
- IN-SOLUTION



MS

G I G O

G I G O

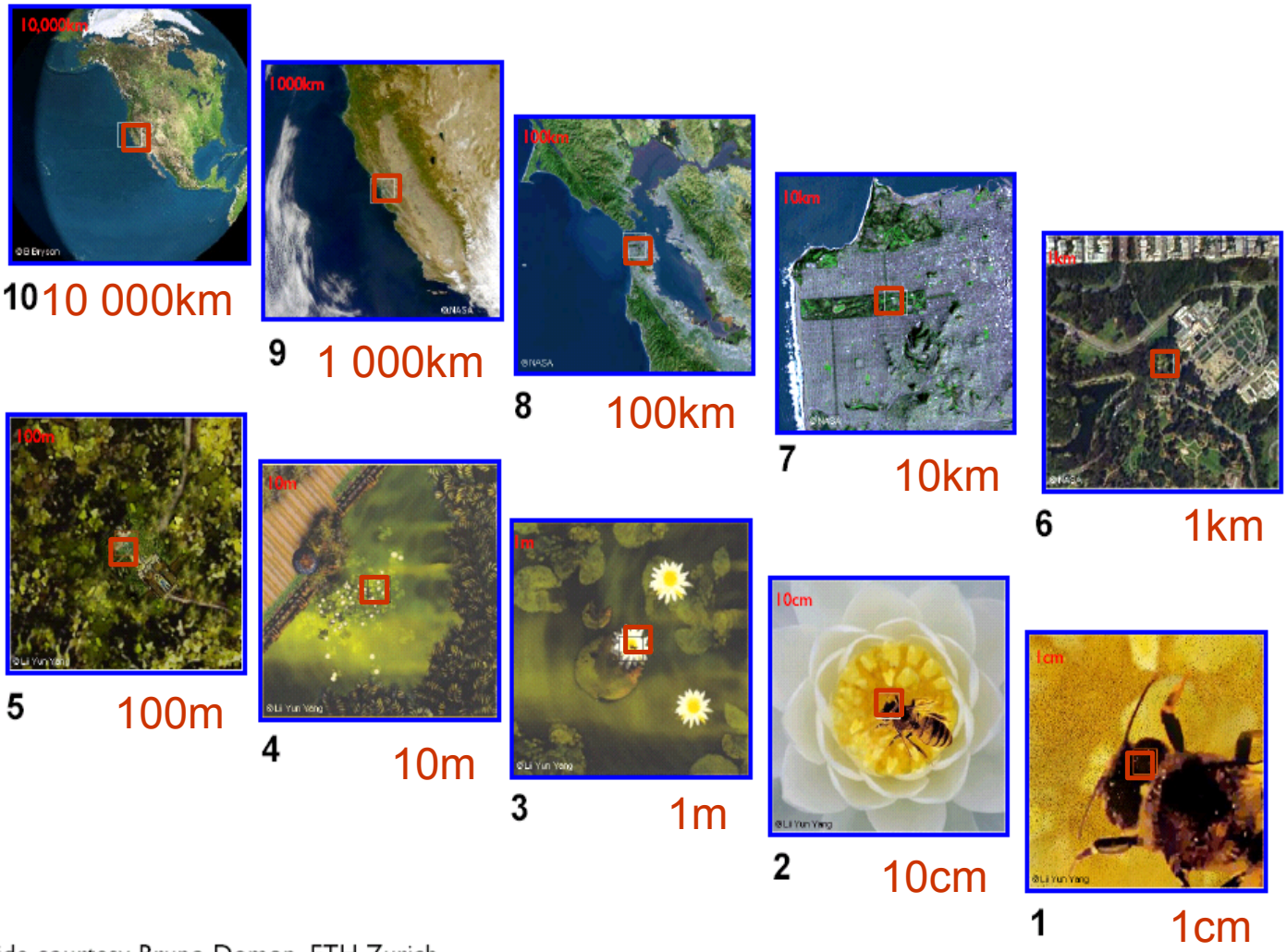
GARBAGE IN - GARBAGE OUT

LITERATURA

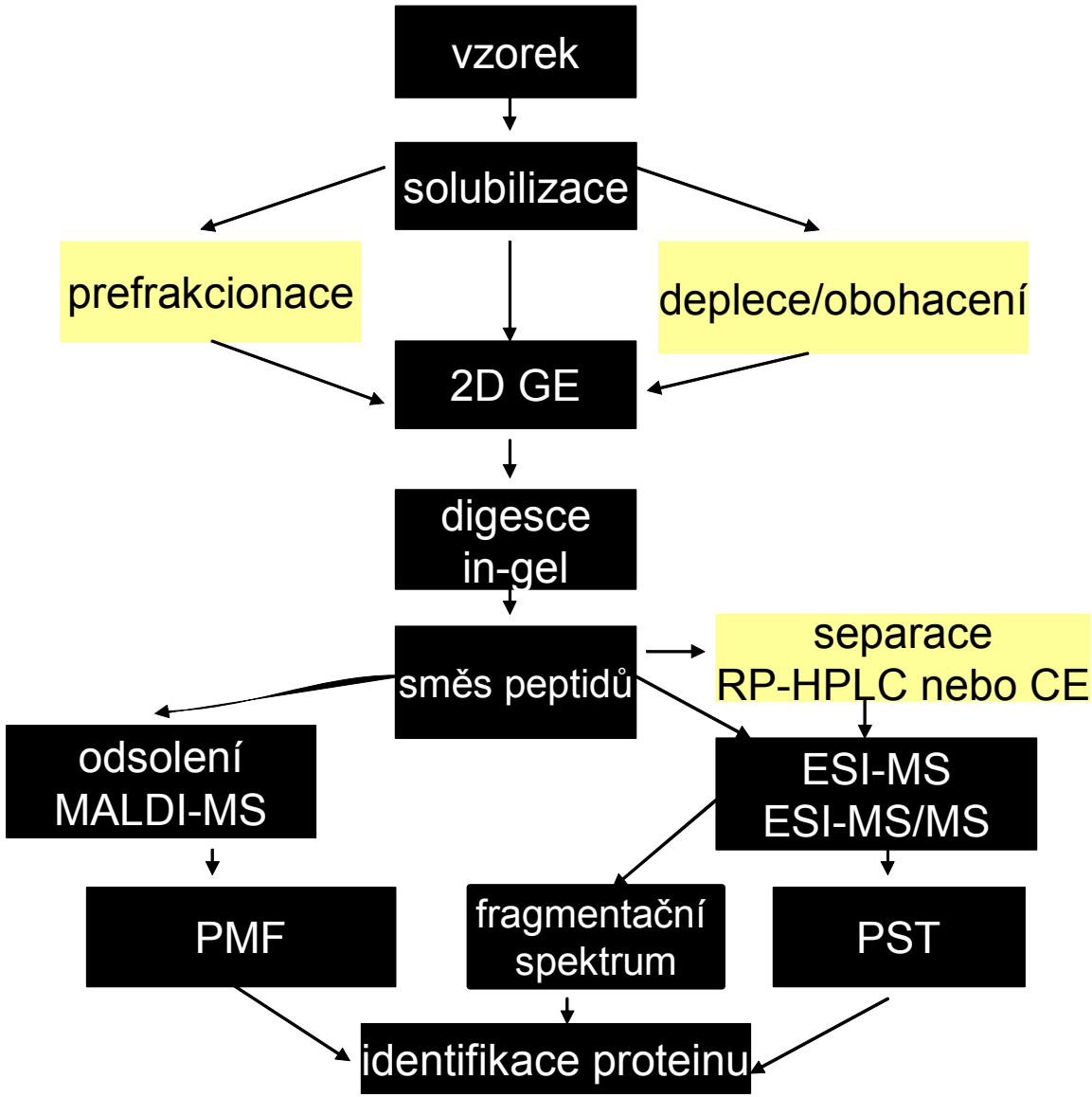
- R.M. Twyman: Principles of Proteomics
- R.Westermeier, T.Naven, H-R Höpker: Proteomics in Practice
- A.J.Link: 2D Proteome Analysis Protocols
- Current Protocols in Protein Science
- R.J.Simpson: Proteins and Proteomics
- T.Rabilloud: Proteome Research: Two-dimensional Gel
- Electrophoresis and Identification Methods
- A. Görg, W. Weiss, M.J.Dunn: Proteomics 2004, 4, 3665, rev.
- I. Miller, J. Crawford, E. Gianazza: Proteomics 2006, 6, rev.
- F.Chevalier: Proteome Science 2010, 8:23, review
- R. Burgess, M. Deutscher: Guide to Protein Purification

II. PREFRAKCIONACE

10^{10} Really Is Wide Dynamic Range

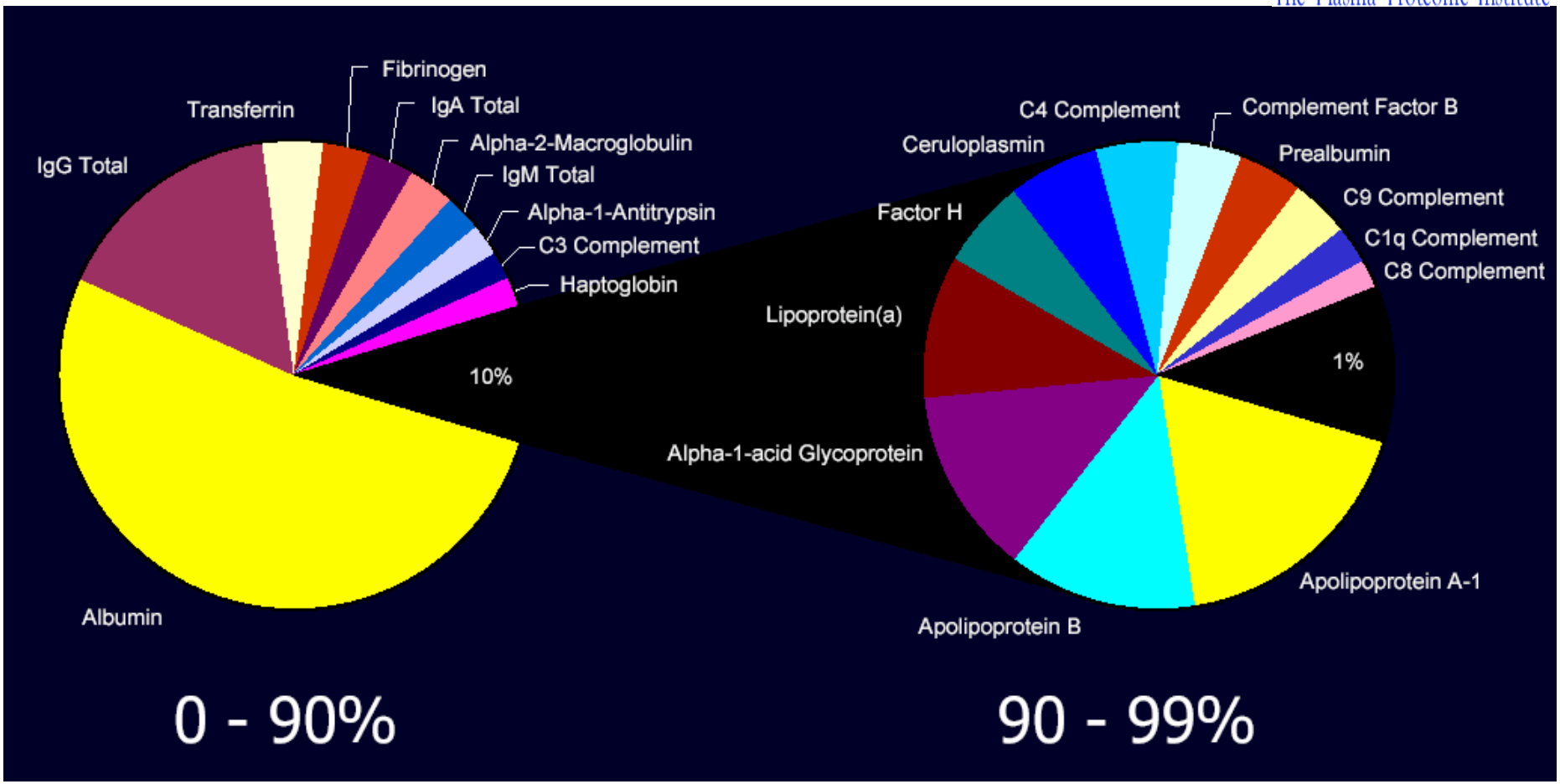


Slide courtesy Bruno Domon, ETH Zurich


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

Abundantní proteiny v lidské plazmě

The Plasma Proteome Institute

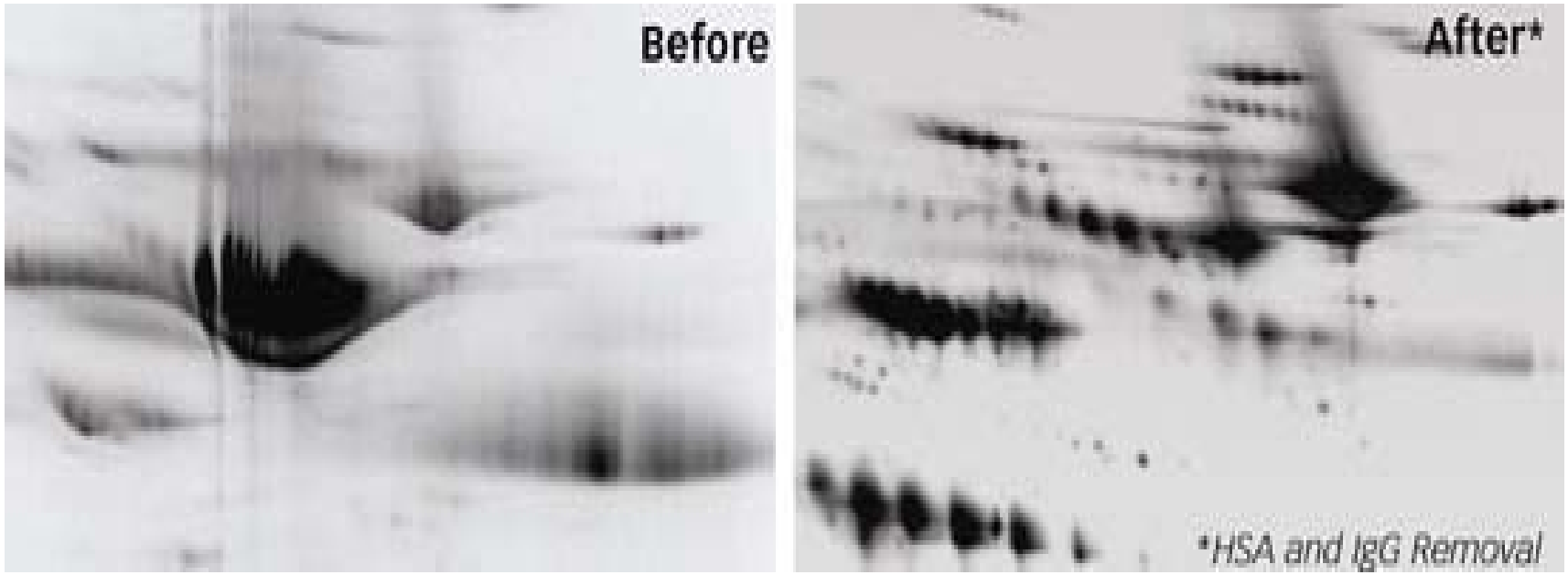


AFINITNÍ DEPLECE

odstranění abundantních proteinů afinitní chromatografií

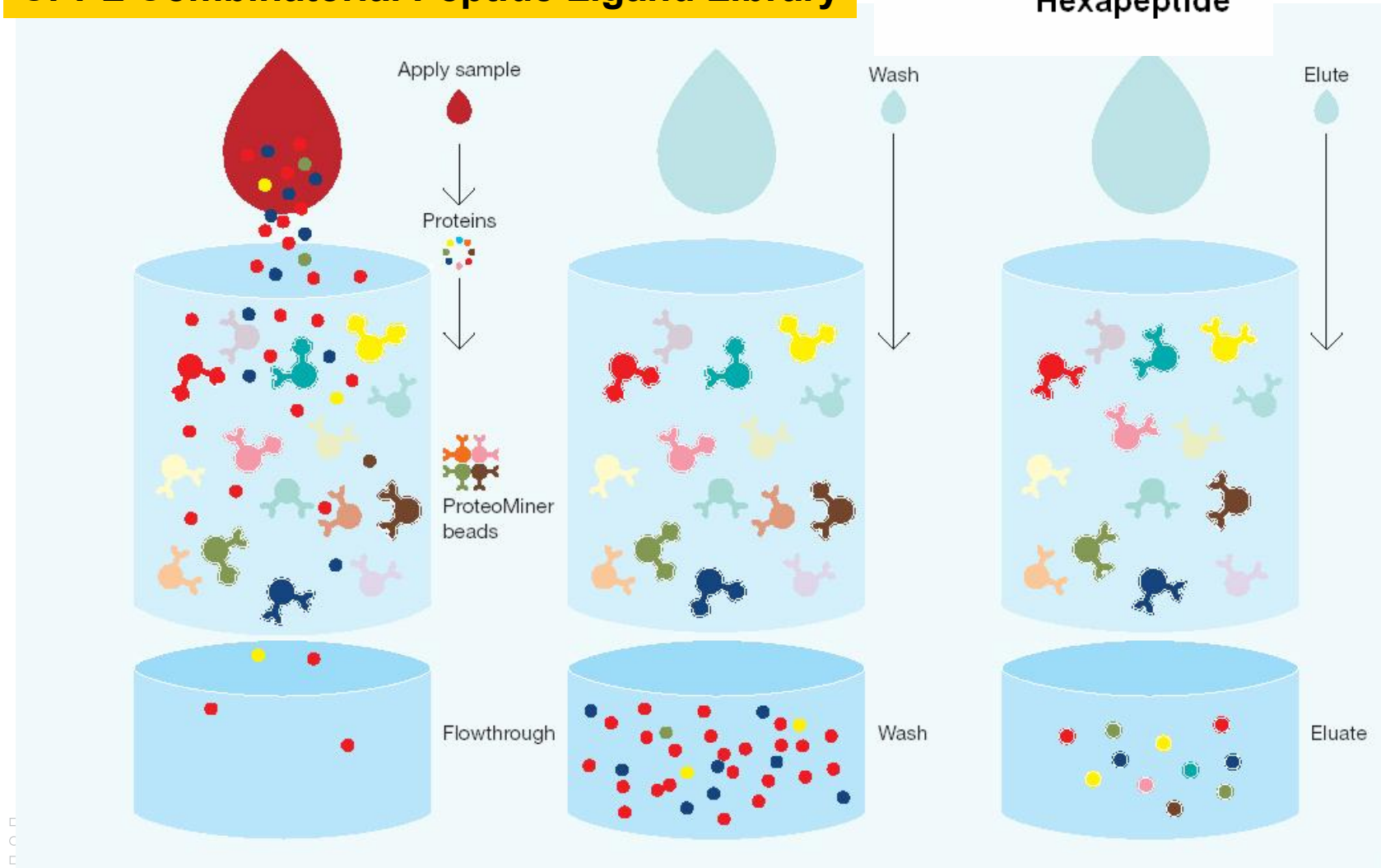
HSA IgG

interakce minoritních proteinů s abundantními ? ? ?

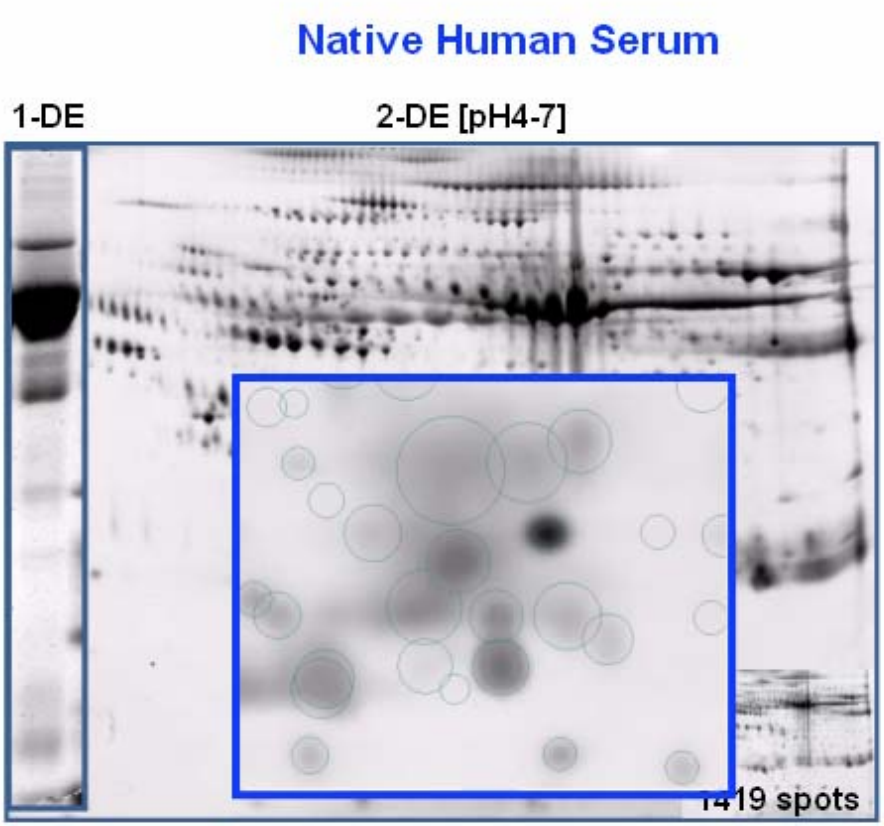


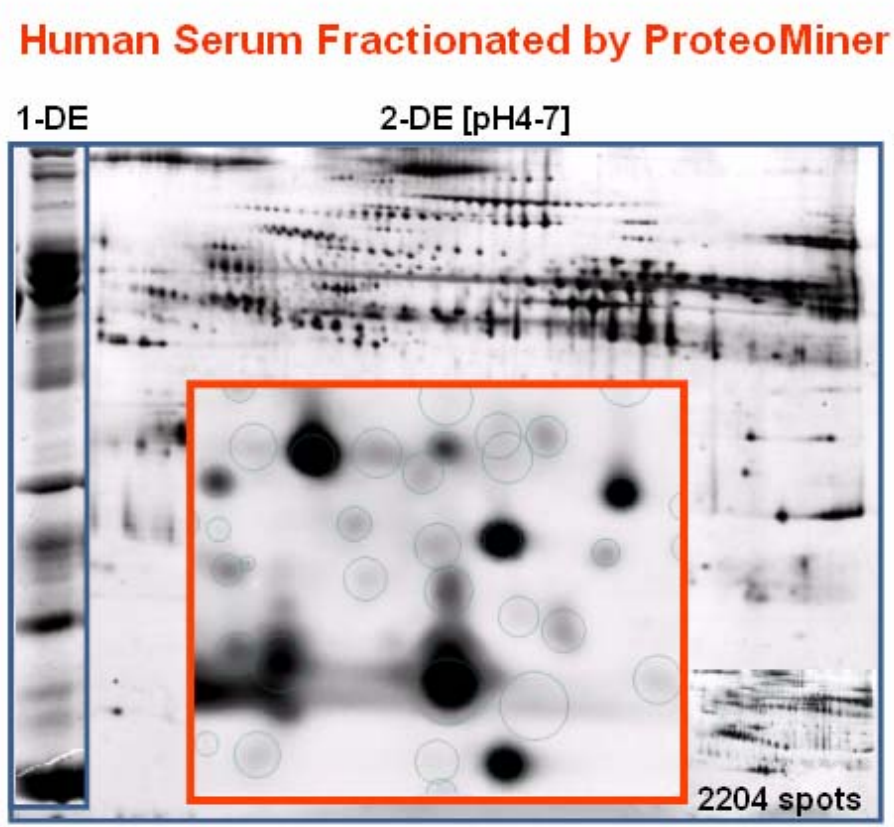
PREFRAKCIONACE

CPPL Combinatorial Peptide Ligand Library



PROTEOMINER





IEF PREFRAKCIONACE

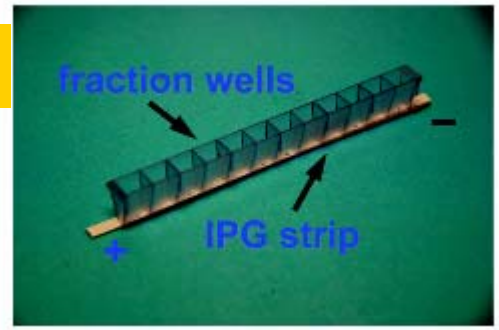
MicroRotofor

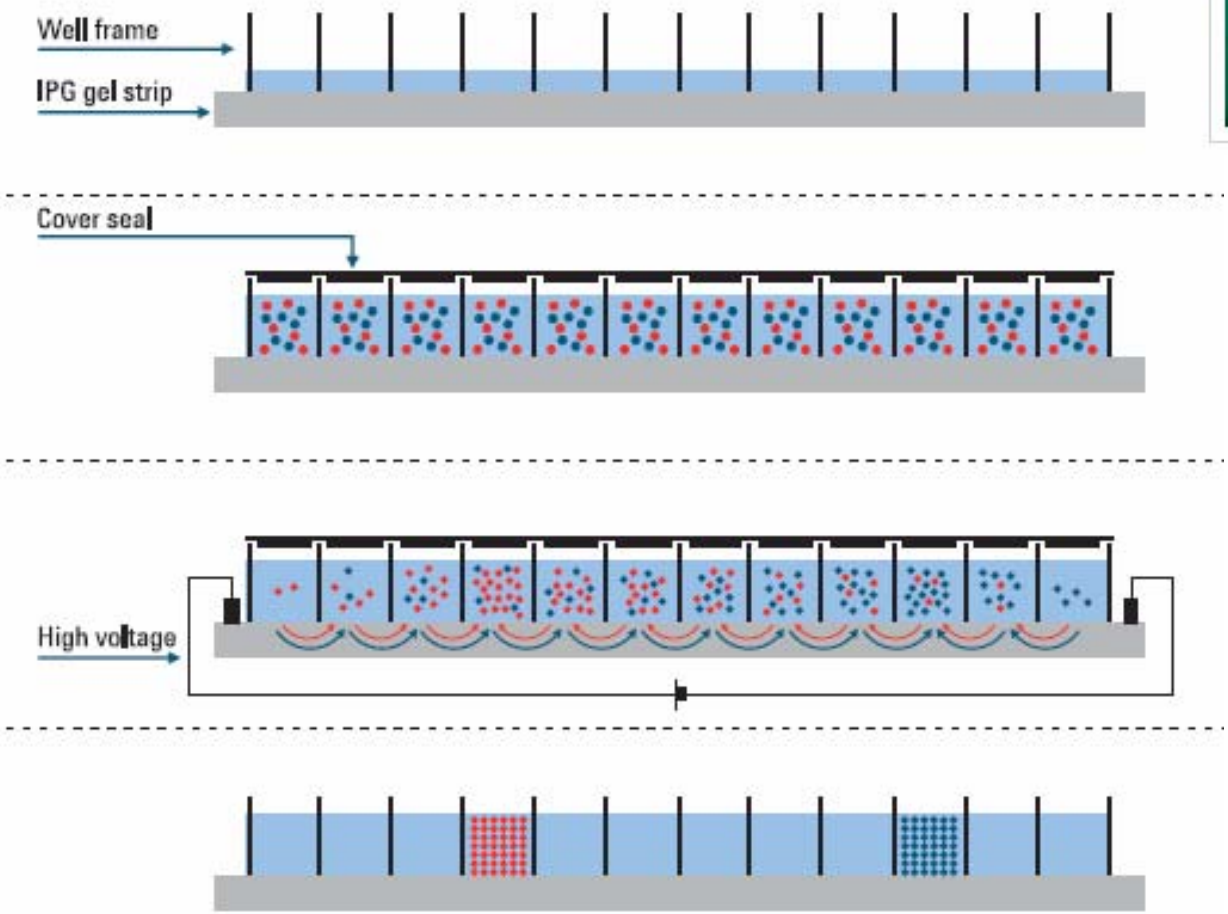
- prefrakcionace v roztoku

OffGel Fractionator

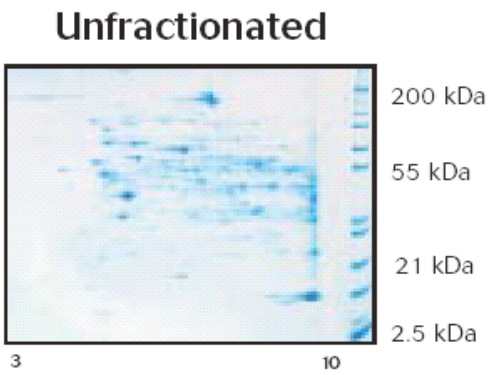
- prefrakcionace na IPG stripu

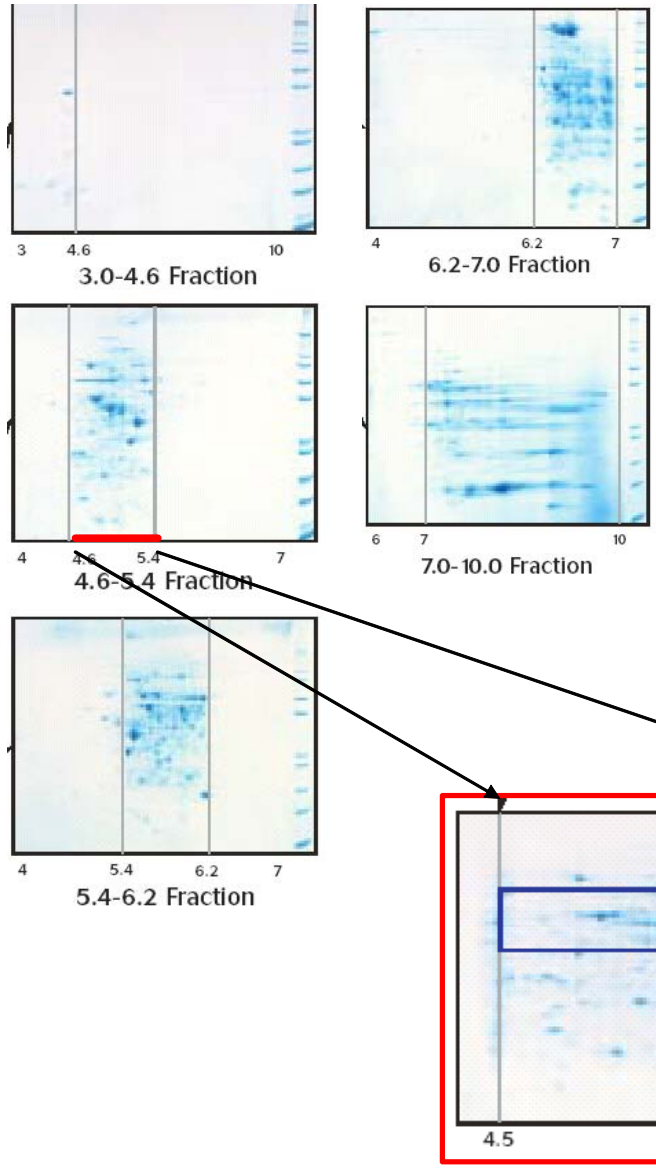
OFFGEL IEF prefrakcionace proteinů nebo peptidů



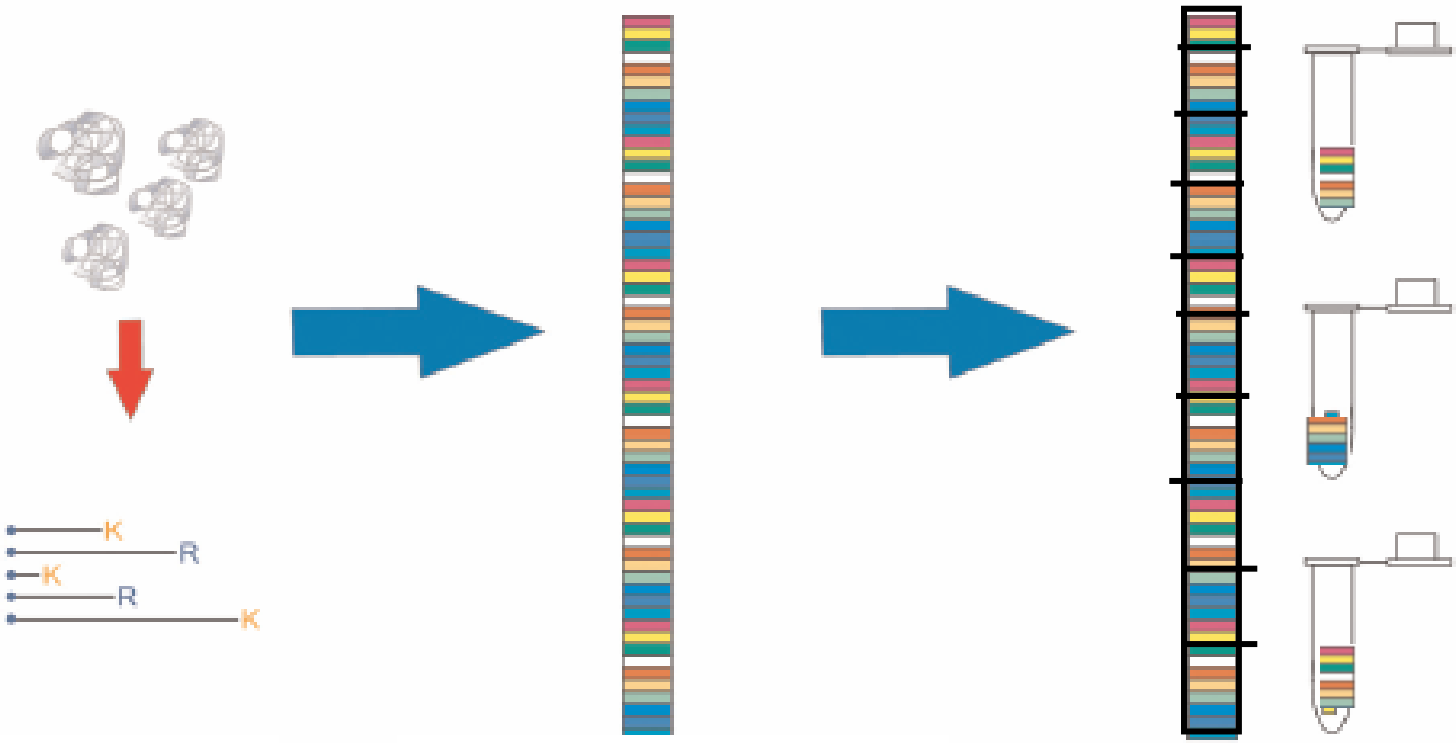


PREFRAKCIONACE MIKRO ROZSAHY





Prefrakcionace peptidů na stripu IPG-IEF



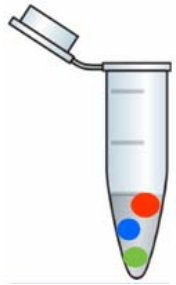
digest směsi proteinů

IEF směsi peptidů na IPG stripu

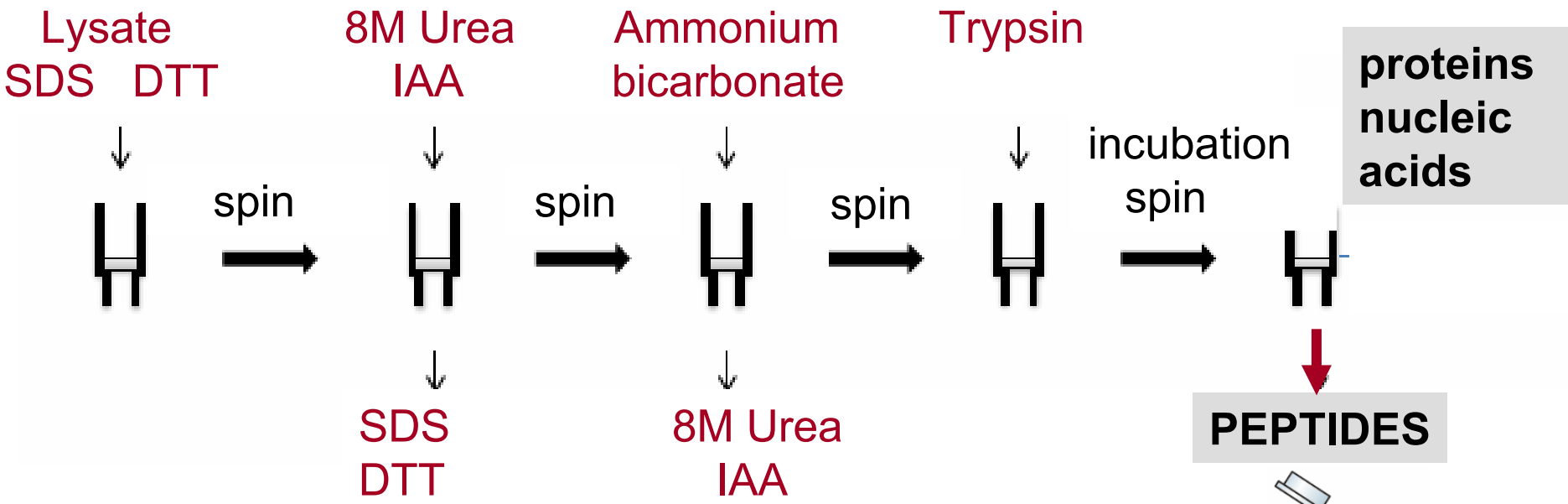
frakce stripu

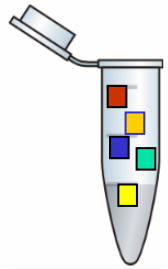
JOURNAL OF BIOMOLECULAR TECHNIQUES, VOLUME 16, ISSUE 3, SEPTEMBER 2005

FASP Filter Aided Sample Preparation



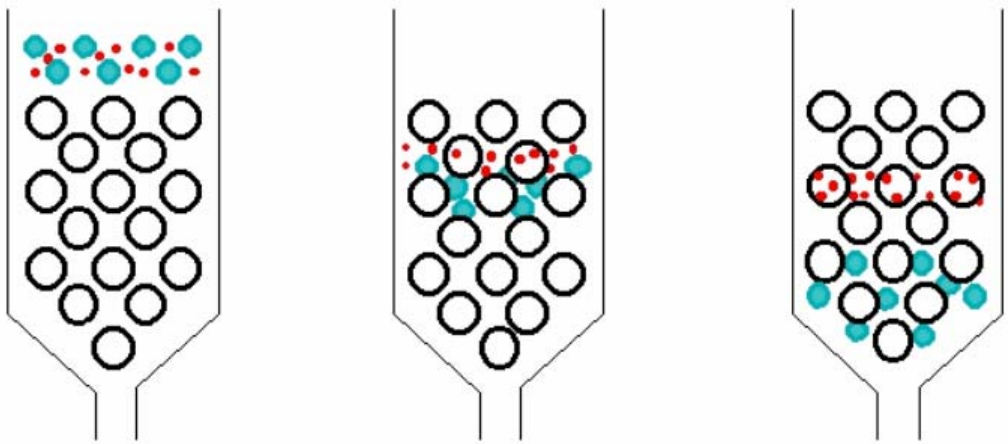
PROTEINS

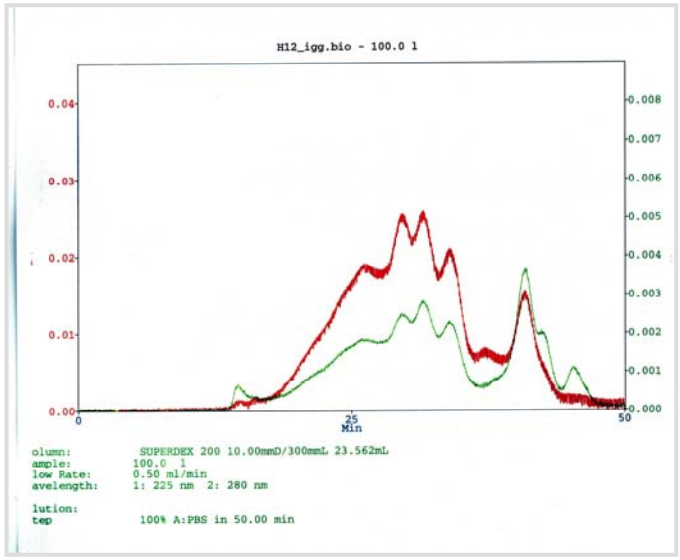




Nature Methods 6, 359 - 362 (2009)

GELOVÁ CHROMATOGRAFIE





INSPIRATIVNÍ LITERATURA PRO MÍRNĚ POKROČILÉ

Two-dimensional gel electrophoresis in proteomics: A tutorial
Thierry Rabilloud et al. *Journal of Proteomics* **2011**

Two-dimensional gel electrophoresis in proteomics: past, present and future
Thierry Rabilloud et al. *Journal of Proteomics* **2010**

Proteomic biomarker discovery: It's more than just mass spectrometry
Josip Blonder et al. *Electrophoresis* **2011**

III. KAPALINOVÁ CHROMATOGRAFIE LC
MULTIDIMENZIONÁLNÍ KAPALINOVÁ CHROMATOGRAFIE MDLC

SLOVNÍČEK

- hyphenated LC-MS on-line
- multidimensional LC-LC(-LC…)
- shotgun proteomics protein → digest → peptide → **HPLC** → MS

bottom-up

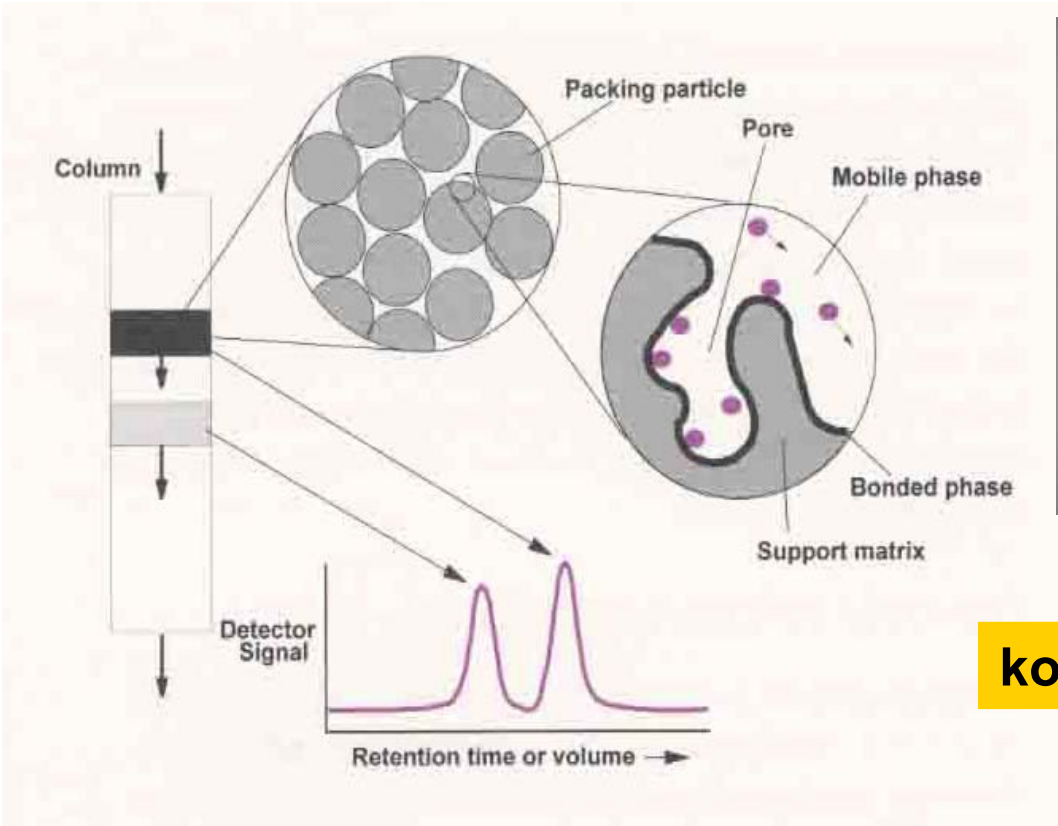
- (příliš) mnoho peptidů
- interference proteinů: stejná peptidová sekvence
- většina systémů MDLC

top-down

- intaktní proteinový iont
- limit velikosti - asi do 50 kDa

PROTEINY A PEPTIDY

PRINCIPY SEPARACE KAPALINOVOU CHROMATOGRAFIÍ



komplexnost

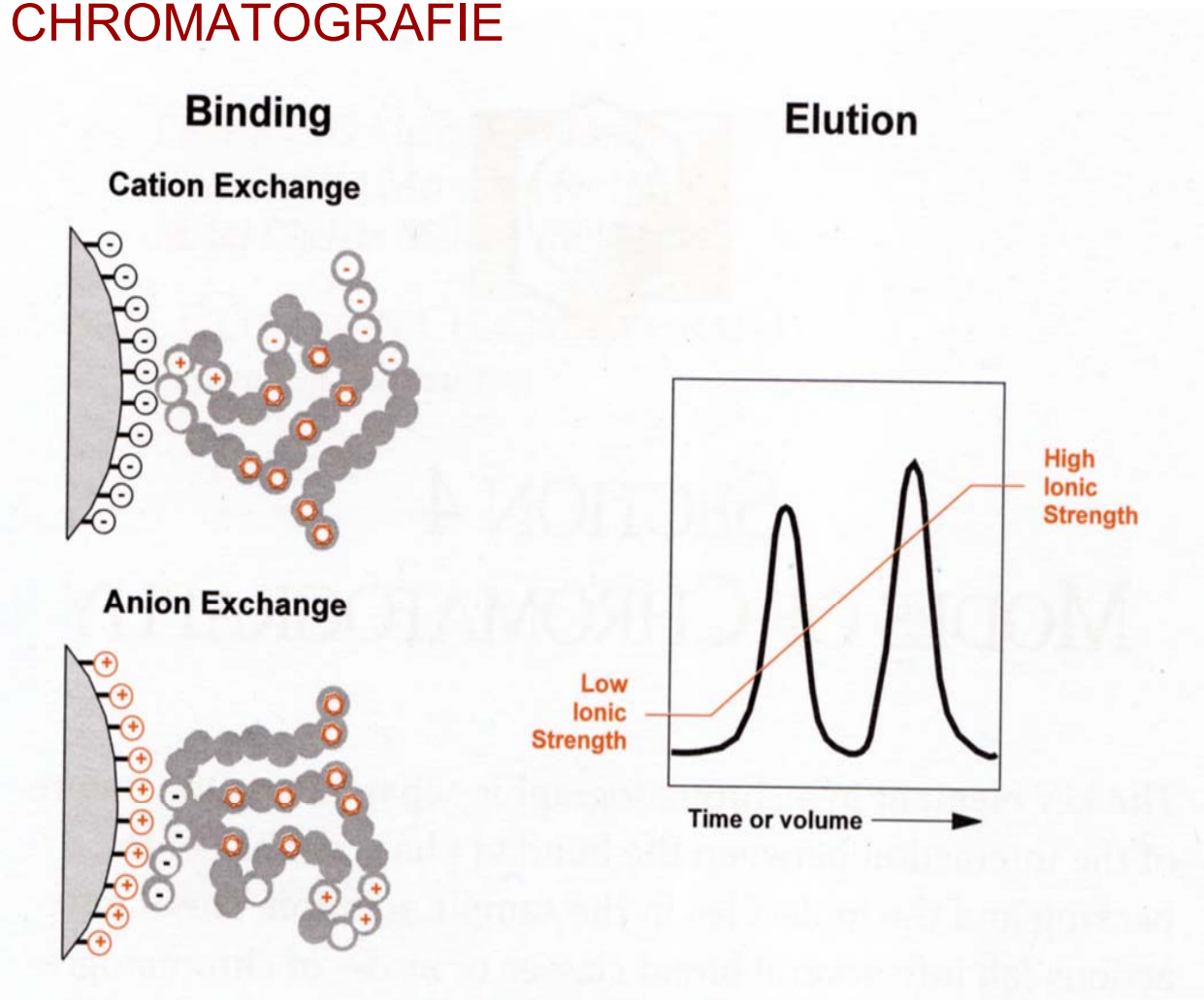
abundance

TYPY LC SEPARACE

CO ROZHODUJE →	KOLONA
▪ náboj	ionex
▪ hydrofobicita	reverzní fáze
▪ biospecifická afinita	afinitní
▪ velikost molekuly	gelová

… a kombinace

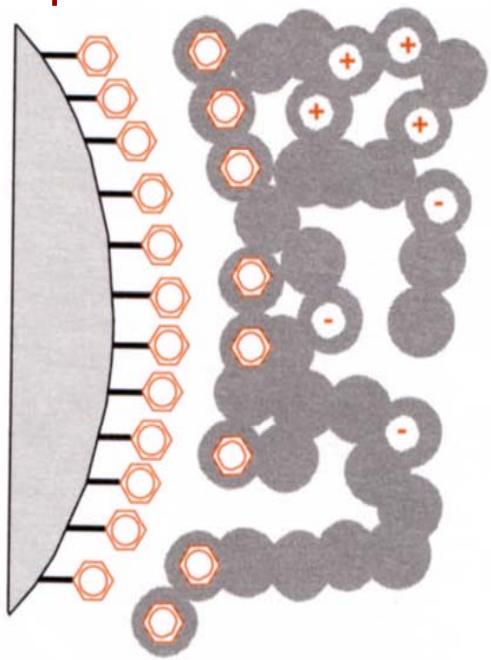
IONEXOVÁ CHROMATOGRAFIE



RP CHROMATOGRAFIE
reversed-phase chromatography

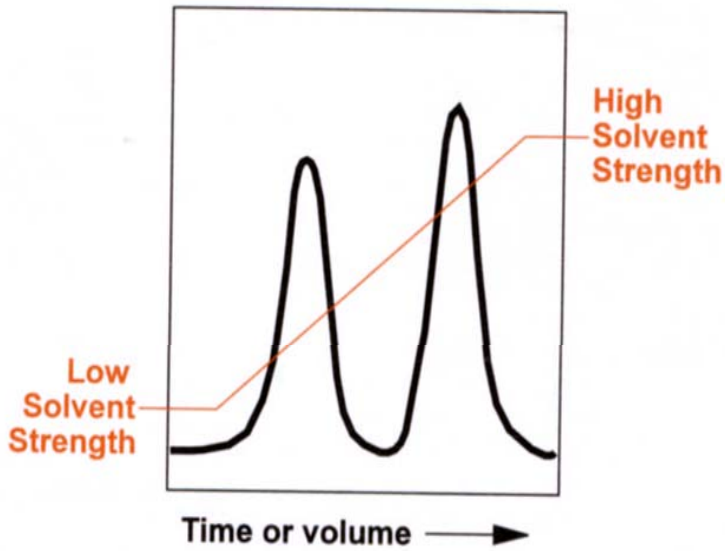
Binding

hydrophobic

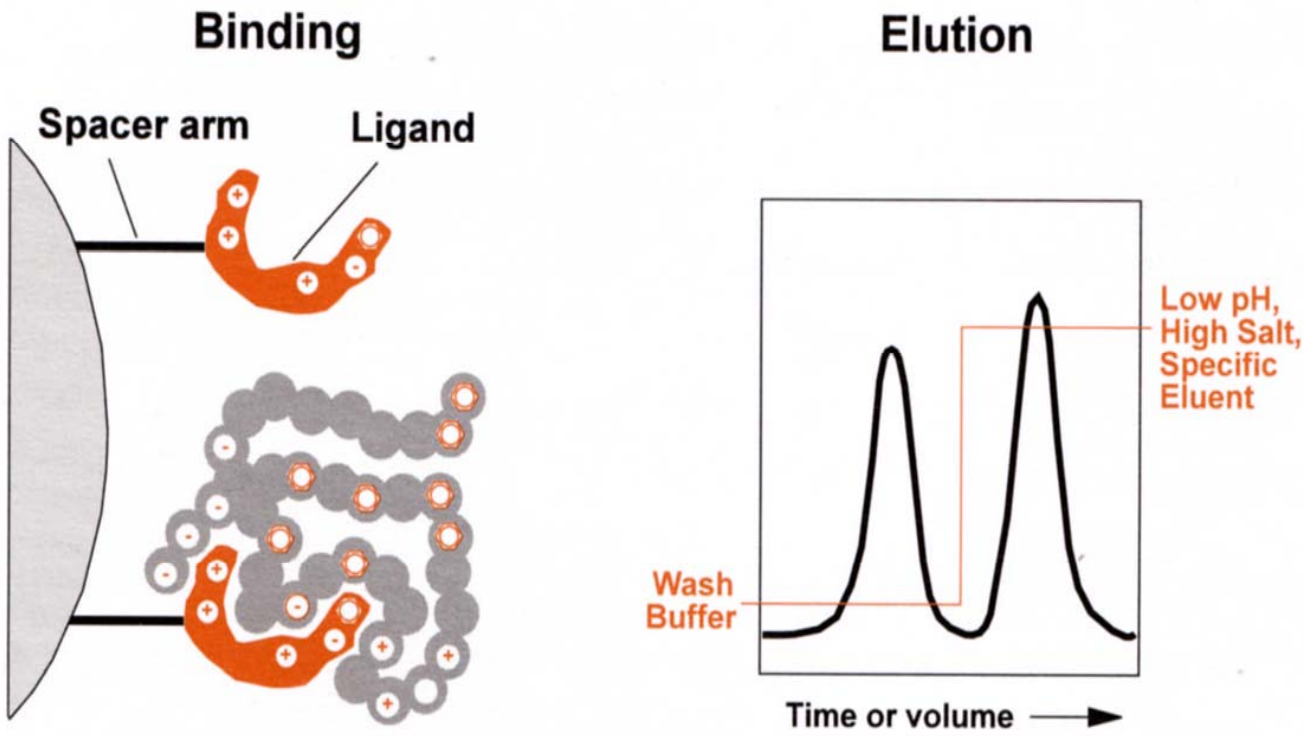


Elution

non-polar



AFINITNÍ CHROMATOGRAFIE

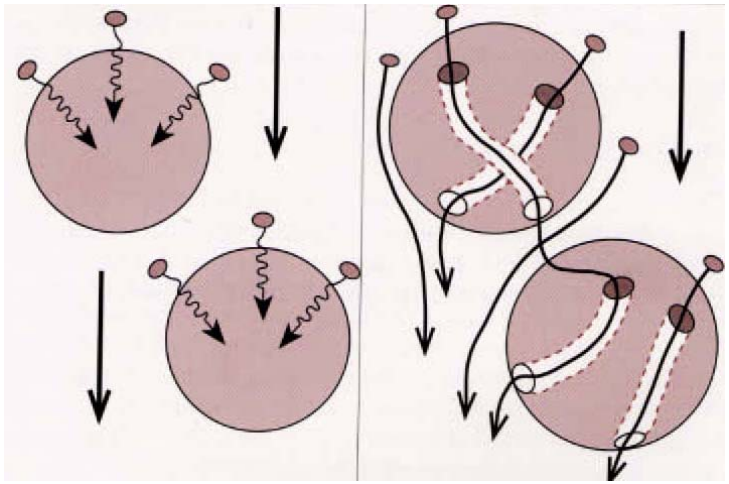


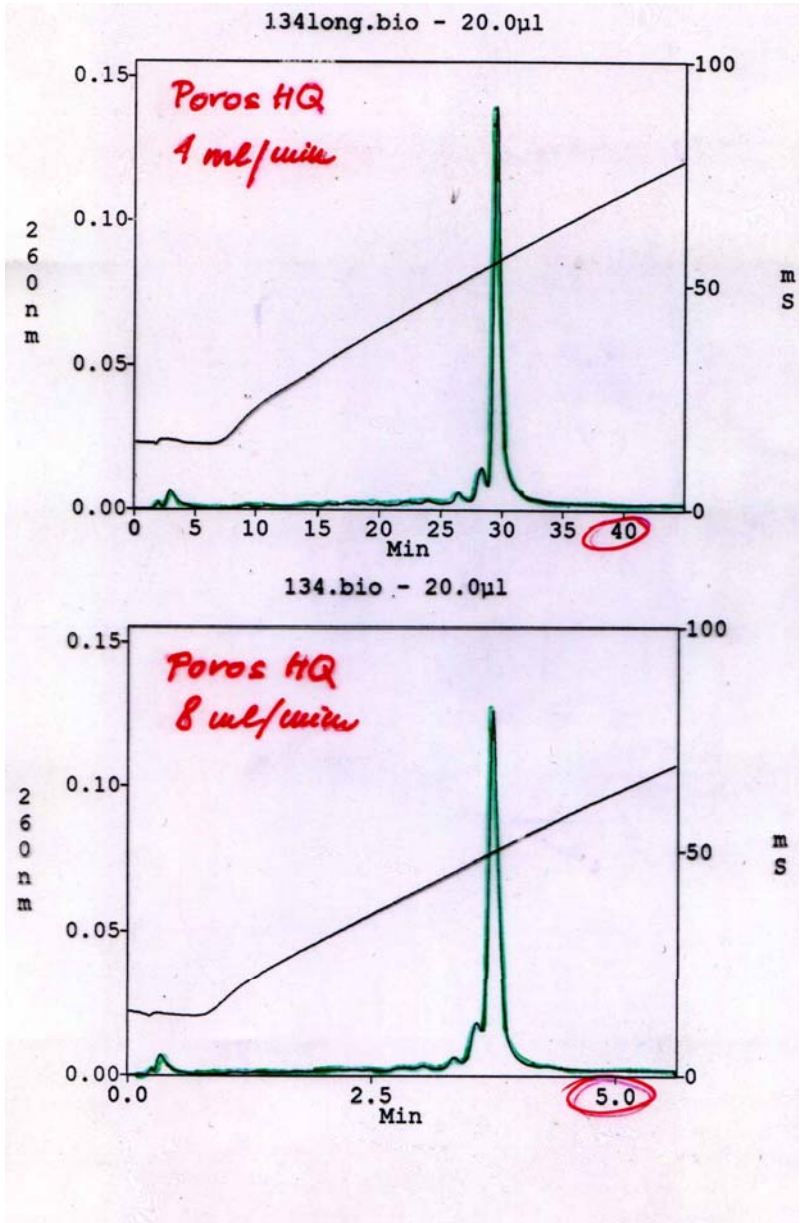
IMAC Immobilized Metal Affinity Chromatography

HPLC

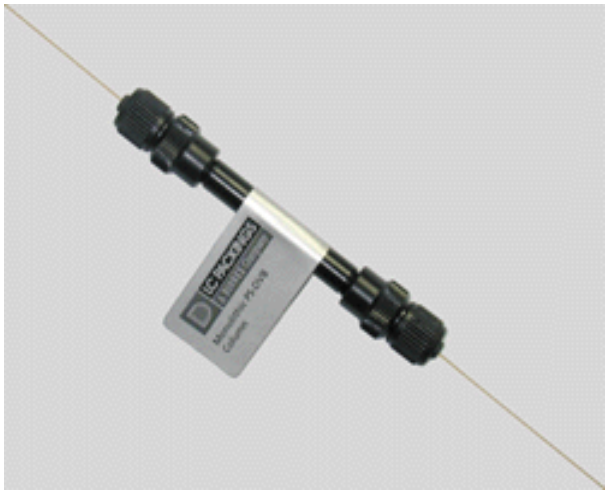
FPLC

PERFÚZNÍ LC





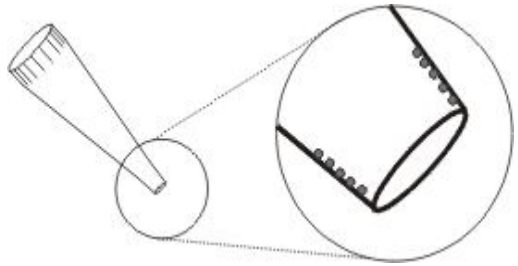
Kolona může vypadat různě . . .



TopTip

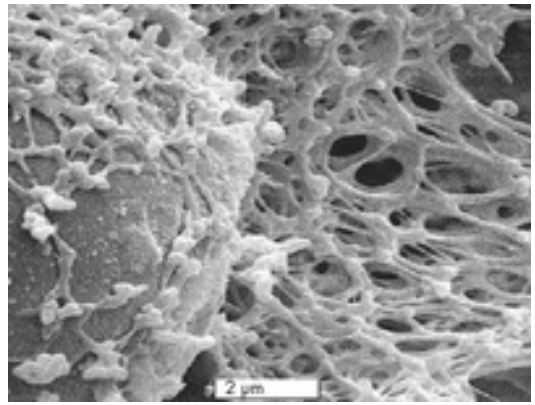
NuTip

Glygen



Zip Tip
Millipore

C18
C4
MC
SCX



MULTIDIMENZIONÁLNÍ CHROMATOGRAFIE

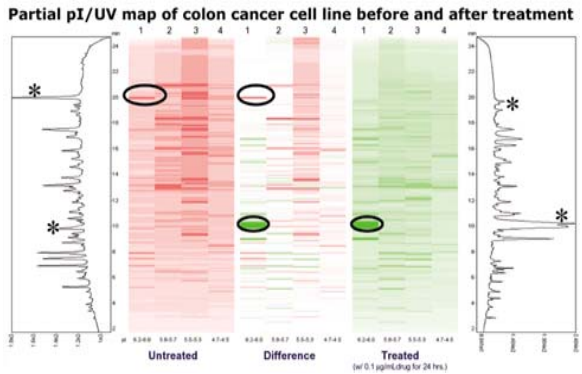
kombinace odlišných fyzikálních a chemických separačních principů

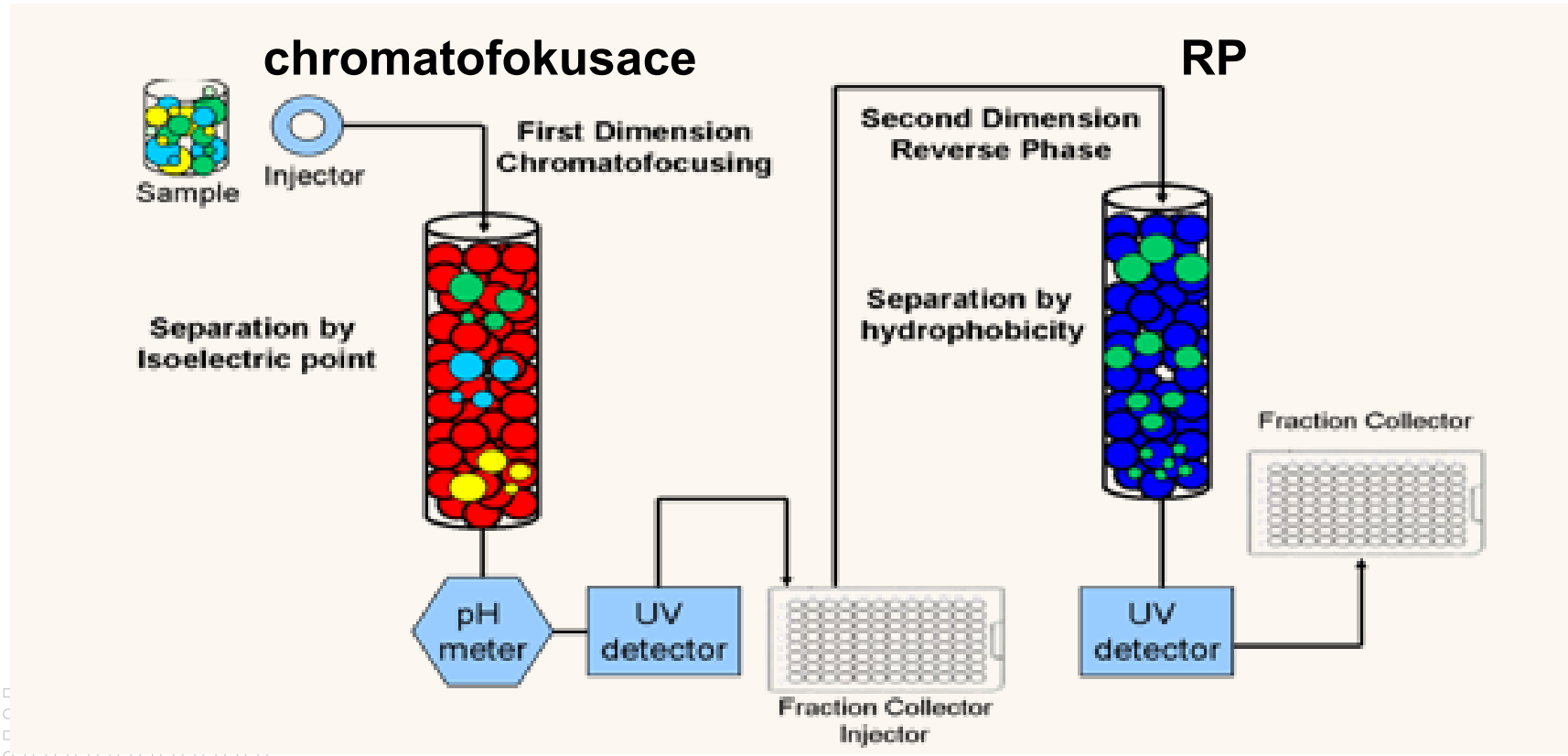
- **SCX/RP**
- **RP/RP**
- **HILIC/RP**
- **AC/MDLC** fosfoproteiny a fosfopeptidy, glykoproteiny a glykopeptidy

- diskontinuální off-line
- kontinuální on-line 1. kolona → 2.kolona
- dvoufázová kolona

optimalizace parametrů MDLC !

ProteomeLab PF 2D





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

LITERATURA - MULTIDIMENSIONÁLNÍ CHROMATOGRAFIE

Hyphenated dimensions in separation science
P.Q.Tranchida et al. *Journal of Chromatography* 2012

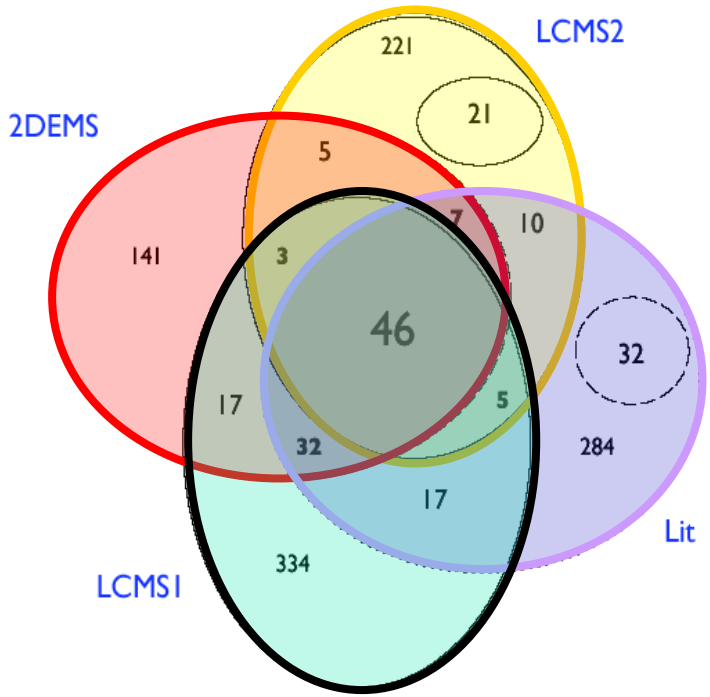
Multi-dimensional Liquid Chromatography in Proteomics
Xiang Zhang et al. *Anal Chim Acta* 2010

Multidimensional separation of peptides for effective proteomic analysis
H.J.Issaq et al. *Journal of Chromatography* 2005

Multi-dimensional liquid phase based separations in proteomics
Hong Wang, et al. *Journal of Chromatography* 2003

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

For all the complex problems and difficult questions there is always one simple, easily comprehensible **w r o n g** answer.

esf evropský sociální fond v ČR
EVROPSKÁ UNIE
MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ