DEPARTMENT OF LIQUID PHASE SEPARATIONS

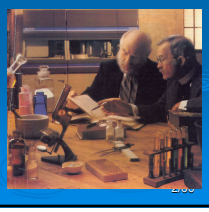
jac brno Ústav analytické chemie Akademie věd ČR Veveří 97, 611 42 BRNO

Izoelektrická fokusace

K. Šlais

Izoelektrická fokusace - IEF

- elektromigrační separační analytická metoda využívající existence izoelektrického stavu amfolytů, kdy efektivní náboj je nulový.
- pH = pI
- Analyty - proteiny



- Separace - ΔpI < 0.01
- Fokusace – zakoncentrování
- Charakterizace - pI

Protein jako amfolyt

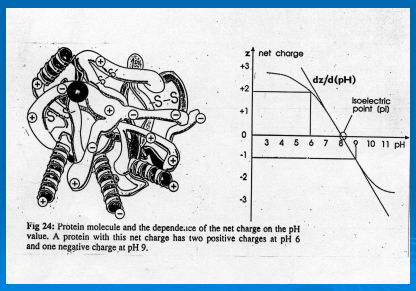
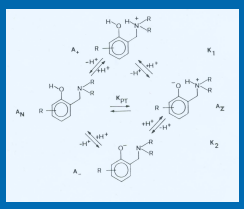
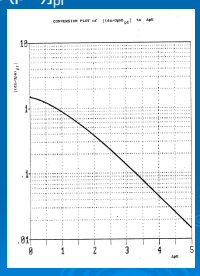


Fig 24: Protein molecule and the dependence of the net charge on the pH value. A protein with this net charge has two positive charges at pH 6 and one negative charge at pH 9.

Biprotický amfolyt

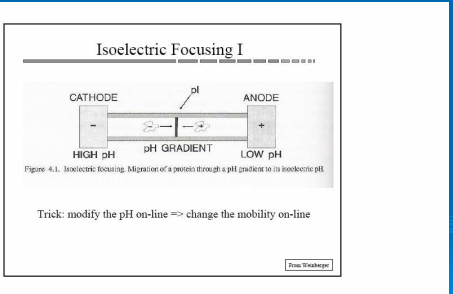
$[-dz/d(pH)]_{pI}$





$pI = (pK_1 + pK_2)/2$

ΔpK



Vznik pH gradientu

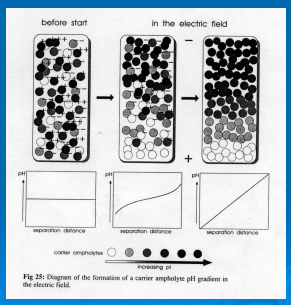


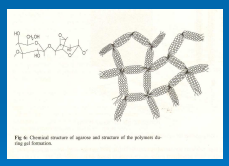
Fig 25: Diagram of the formation of a carrier ampholyte pH gradient in the electric field.

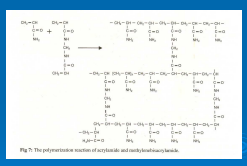
Druhy IEF

- Gelová IEF
  - S nosnými amfolyty
  - S imobilizovaným gradientem (IPG)
  - Dvourozměrná elektroforéza 2D = IEF + SDS PAGE
- Kapilární IEF
- Preparativní IEF
  - Free flow
  - Komorová (např. Rotofor)

Struktura gelu

agarosa polyakrylamid





Varianty gelové IEF

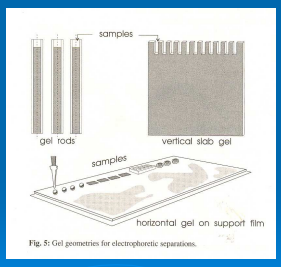
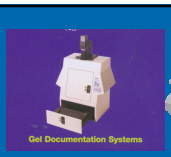
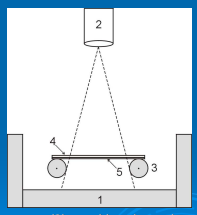


Fig. 5: Gel geometries for electrophoretic separations.

Instrumentace





(1) outer chamber, (2) CCD camera, (3) graphite electrodes, 50 mm distance between contact points, (4) glass plate, (5) polyacrylamide gel of size 125 x 54 x 0.4 mm.

Vertical gel IEF

Typický výsledek gelové IEF

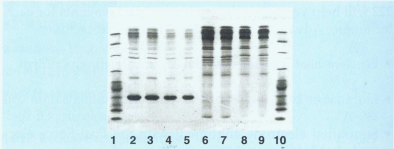
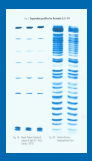
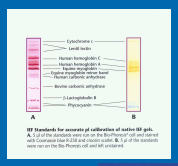
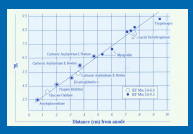


Fig. 2. Typical separation in the Model 111 Mini IEF Cell (5% polyacrylamide gel with 2% Bio-Lyte 3/10 ampholytes). Focusing was carried out in a stepped fashion (100 V for 15 minutes, 200 V for 15 minutes, 450 V for 1 hour). Samples are: lanes 1 & 10: Bio-Rad's IEF Standards; lanes 2-5: Dilutions of horseradish peroxidase; lanes 6-9: Dilutions of Japanese water moccasin snake venom.

Standardy pI - proteiny





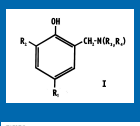


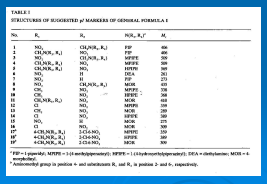
nestabilní,
nečisté,
drahé,
málo barevné,
málo rozpustné při pI

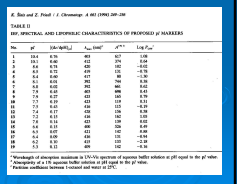
Nízkomolekulární barevné pI markery

- Požadavky na pI markery
  - Škála pI od ~ 2 do 11, po ~ 0.5 pI
  - dobré amfolyty, -dz/dpH ~0.5 > 0.05, ΔpK ~2 < 4
  - rozpustnost ve vodě při pH = pI, > 1 mg/ml
  - různé barvy, λ_{max} > 400 $A_{1\%}$ > 100
  - Čistota, > 99 % ,
- Dostupnost, cena markeru
- Stabilita - hydrolýza, oxidace, fotodegradace, mikroorganismy

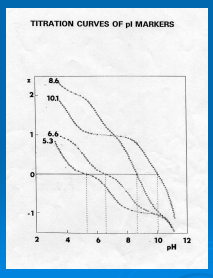
Aminomethylované nitrofenoly

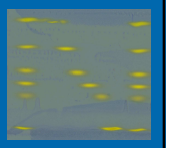




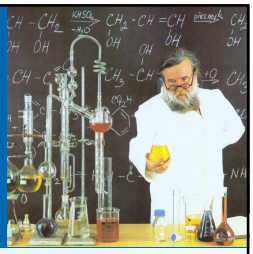


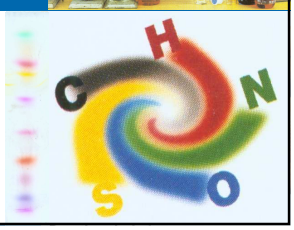
Žluté pI markery



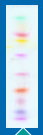


? Barevné pI markery ?



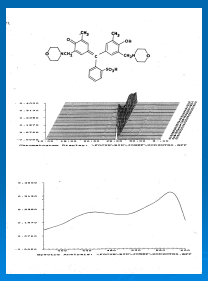


Chemie → nízkomolekulární barevné pI markery

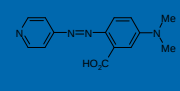


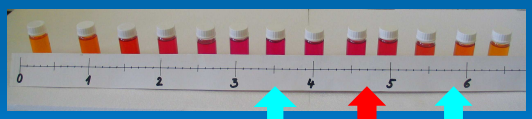
- Škála pI od 2 do 11
- dobré amfolyty,ΔpK~2<4
- rozpustnost > 1 mg/ml
- různé barvy
- Čistota
- Dostupnost, cena
- Stabilita
  - hydrolýza,
  - oxidace,
  - fotodegradace,
  - mikroorganismy
  - separační prostředí dithiothreitol,

Příklad barevného pI markeru



Spektrofotometrické určení pI





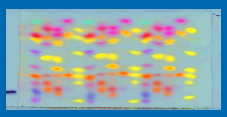
pK_1 = 3.50 ± 0.02

pI = 4.71 ± 0.02

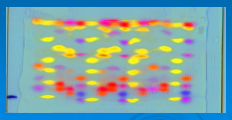
pK_2 = 5.92 ± 0.02

Určení pI interpolací v gelové IEF

Gradient pH

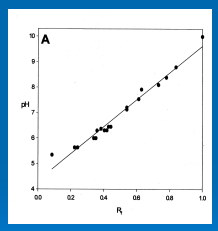


Směs 30 jednoduchých pufrů

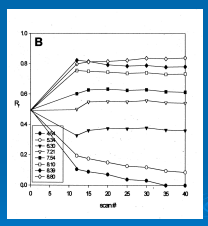


Biolyt 3 – 10

Dynamika pH gradientu Biolyt 3-10



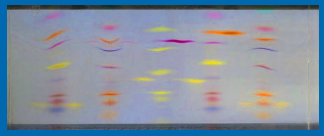
Lineární gradient pH 4 - 10



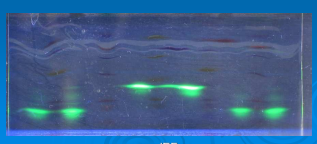
Po ½ hod malé změny pH gradientu

Vývoj fluorescenčních pI markerů

Vis



fluorescence

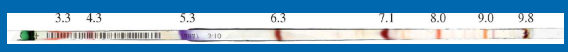


ief446_INDEX.exe

colored markers

IEF of mixture of chosen pI markers in the first dimension strip of 2D gel electrophoresis

in Clinical Proteomics. From Diagnosis to Therapy. J. Van Eyk and M.J. Dunn (Eds.),
Chapter 2, Protein Separation by Two-Dimensional Electrophoresis
Pamela M. Donoghue, Miroslava Stastna, Michael J. Dunn, p 13,
2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim



Immobiline Dry Strip (Amersham Biosciences) pH 3–10, 18 cm.
Apparatus: Protean IEF Cell (BioRad).
Sample: 10 ml of pI markers mixture diluted with 340ml of IEF buffer (8M urea, 2M thiourea, 4% CHAPS, 1% DTT, 0.01% bromophenol blue, 1.5% (v/v) hydroxyethyl disulfide, 0.2% (v/v) IPG buffer pH 3–10).
The acidic end is on the left and the basic end on the right side of the strip.
The pI values of individual pI markers are marked in the picture

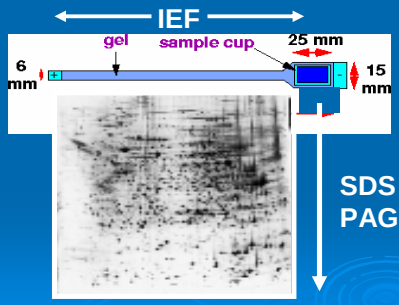
7 cm IPG strip pH 3-10 NL

pI 3.3 – červená	1 µg
pI 4.3 – oranžová	3 µg
pI 5.3 – levandulová	3 µg
pI 5.7 – žlutá	5 µg
pI 6.2 – červená	3 µg
pI 7.6 – červená	3 µg
pI 9.0 – žlutá	5 µg

7 cm IPG strip pH 4-7

pI 4.3 – oranžová	3 µg
pI 5.3 – levandulová	3 µg
pI 5.7 – žlutá	5 µg
pI 6.2 – červená	3 µg

2D Gel electrophoresis



2D - typical result – silver staining

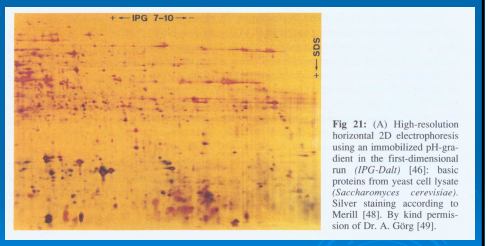
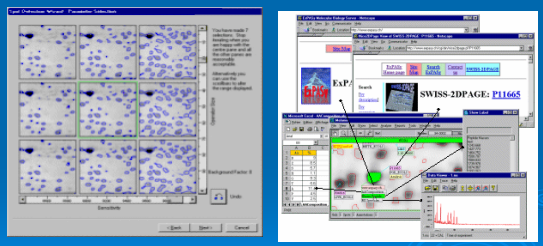
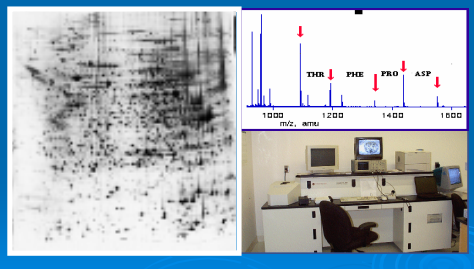


Fig 21: (A) High-resolution horizontal 2D electrophoresis using an immobilized pH-gradient in the first-dimensional run (*IPG-Dalt*) [46]; basic proteins from yeast cell lysate (*Saccharomyces cerevisiae*). Silver staining according to Merill [48]. By kind permission of Dr. A. Görg [49].

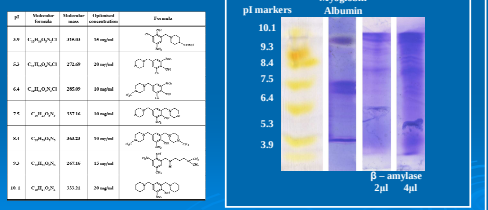
2D Gel electrophoresis - Software

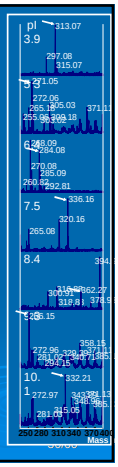


Protein identification by 2D gel electrophoresis -MS



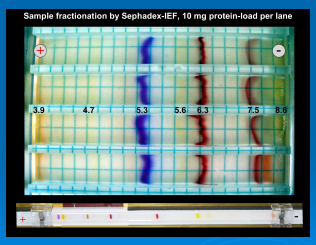
LOW-MOLECULAR MASS PI MARKERS IN COMBINATION OF GEL IEF WITH MALDI-TOF MS





IEF in Granulated Sephadex Gels

Methods in Molecular Biology, vol. 424: Volume 1: Sample Preparation and Pre-Fractionation, Edited by: A. Posch ,
Chapter 22, **Sample Prefractionation in Granulated Sephadex IEF Gels**
Angelika Görg, Carsten Lück, and Walter Weiss, p 277,
Humana Press Inc., 2007, Totowa, NJ



Use of coloured **pI - markers** to determine the slope of the pH gradient and the position where to ‚cut' and remove the individual Sephadex fractions in order to fit to the corresponding narrow pH range IPGs

Courtesy of Carsten Lück

IEF in Sephadex gels and IPG strips

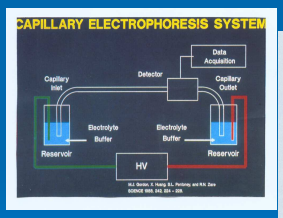
Hodný Z., Přidalová J.,

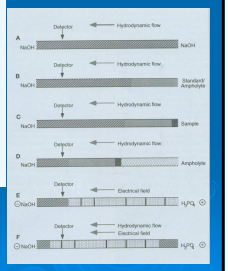
Institute of Experimental Medicine AV ČR, v.v.i., Prague

Courtesy of Z. Hodný

pI markers - LM ladder
Home made strip,
linear gradient pH 4-10,
11cm,
1 min 30V,
50 min 30V -> 3500V,
2 hours 3500V.
Courtesy of J. Přidalová

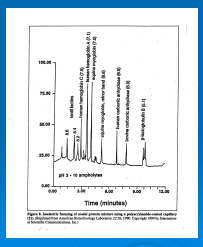
Kapilární IEF



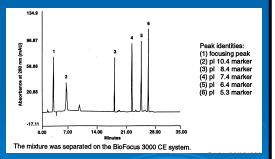


Kapilární IEF standardů

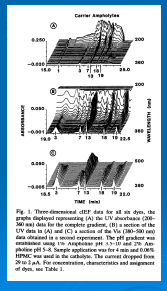
proteiny

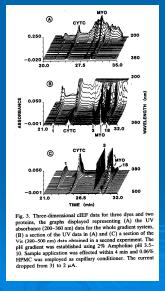


pI markery



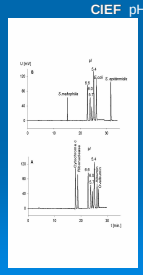
Kapilární IEF s DAD detekcí

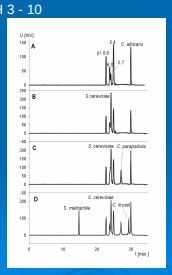




Capillary isoelectric focusing and fluorometric detection of proteins and microorganisms dynamically modified by poly(ethylene glycol) pyrenebutanoate
Horká, M., Růžička, F., Horký, J., Holá, V., Šlais, K.

Anal. Chem., 78 **2006** 8438-8444
CIEF pH 3 - 10





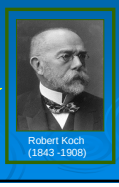
Mikroorganismy (MO) = bakterie, kvasinky, viry, paraziti

Normální flóra každého jedince = bakterie, event. plísně, ale **nikdy** viry.

Mikroorganismy, význam = výrobní prostředek v biotechnologiích koloběh látek, symbiotické organismy

Patogenní mikroorganismus = živé biologické agens schopné vyvolat masové infekční onemocnění nebo otravu lidí, zvířat či rostlin

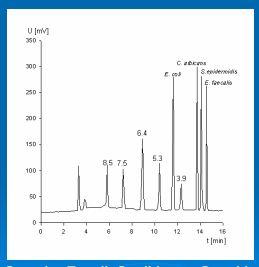
16.stol. - mor



Robert Koch (1843 -1908)

Mycobacterium tuberculosis

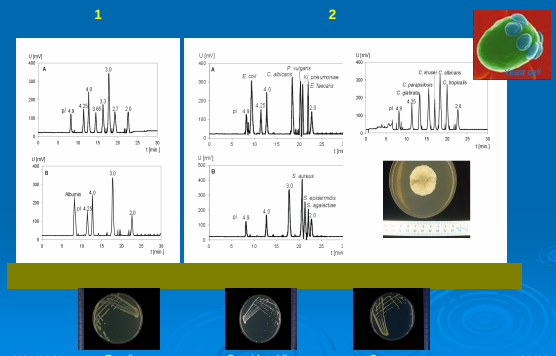
CIEF mikroorganismů



Sample: *E. coli, C. albicans, S. epidermidis, E. faecalis* in physiological saline solution, 4 x 10^8 cell ml^{-1}.

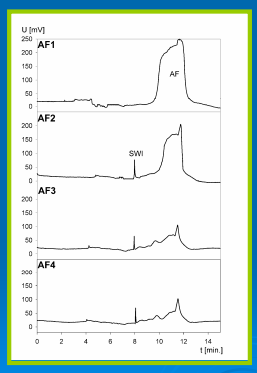
280 nm, pH gradient 2 - 5

1 2

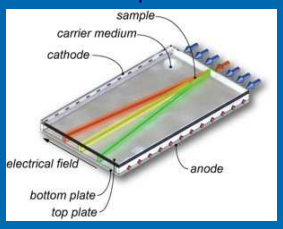


E. coli *S. epidermidis* *S. aureus*

CIEF virů s UV detekcí



Microfluidic Free-Flow Electrophoresis for Proteomics on a chip



The miniaturization of FFE implies several advantages especially considering sample volume and separation speed. In contrast to the tens of milliliters of sample consumed by conventional large scale FFE devices, microfluidic FFE systems require only tens of nanoliters up to hundreds of microliters of sample. This is especially interesting in clinical analysis where often only low sample volumes are available. Furthermore, instead of residence times of up to tens of minutes, microfluidic FFE (μ-FFE) devices separate within several seconds.

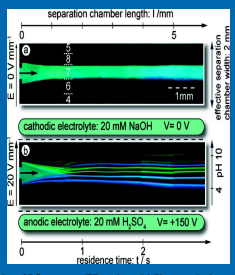


Figure 4 Free-flow isoelectric focusing of 7 fluorescent IEF markers: (a) Photograph shows the device, when no voltage was applied. The white numbers directly correspond to the stream numbers given in Figure 2. (b) When 150 V (*I* = 50 A) was applied, the markers (pI 4, 5.1, 6.2, 7.2, 8.1, 9, and 10.3) fully separated within less than 2 s. The sample flow rate was 0.4 L min^{-1} (*v* = 2 mm s^{-1}) The apparent kinks in the fluorescent tracer paths are caused by merging multiple photographs.

Published in: Dietrich Kohlheyer; Jan C. T. Eijkel; Stefan Schlautmann; Albert van den Berg; Richard B. M. Schasfoort; *Anal. Chem.* **2007**, 79, 8190-8198.
DOI: 10.1021/ac071419b

Preparativní IEF proteinů

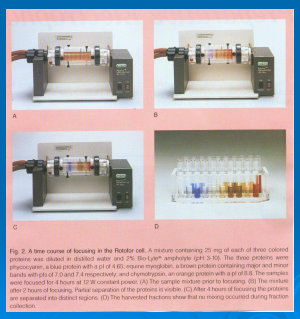


Fig. 2. A time course of focusing in the Rotofor cell. A mixture containing 25 mg of each of three colored proteins was diluted in distilled water and 2% Bio-Lyte® ampholyte (pH 3-10). The three proteins were phycocyanin, a blue protein with a pI of 4.65; equine myoglobin, a brown protein containing major and minor bands with pIs of 7.0 and 7.4 respectively; and chymotrypsin, an orange protein with a pI of 8.8. The samples were focused for 4 hours at 12 W constant power. (A) The sample mixture prior to focusing. (B) The mixture after 2 hours of focusing. Partial separation of the proteins is visible. (C) After 4 hours of focusing the proteins are separated into distinct regions. (D) The harvested fractions show that no mixing occurred during fraction collection.

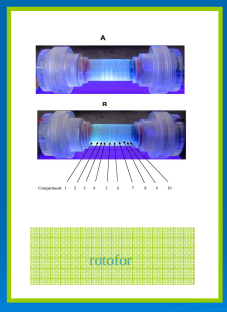
Preparativní autofokusace peptidů

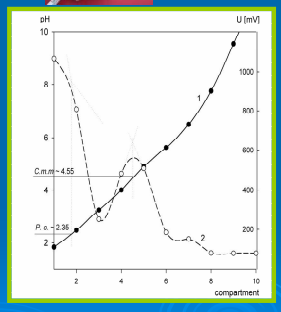
pI = 3.9 pI = 5.3

Table 1. The strains of the plant pathogens included in this study; comparison of their isoelectric points, pI, and RSDs from three measurement of the migration times, t, for each from the strains.

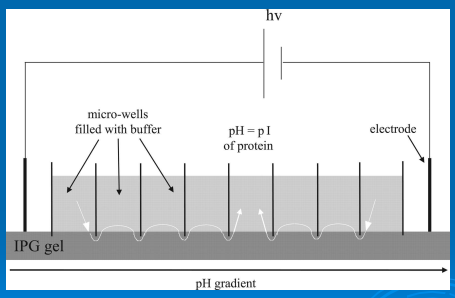
Abbreviation in Figs.	Strain	pI
C. michiganensis	*Clavibacter michiganensis* subsp. *michiganensis* CCM 1635	4.6
	Clavibacter michiganensis subsp. *michiganensis* VURV C254	4.6
	Clavibacter michiganensis subsp. *michiganensis* VURV 2/4/99	4.7
	Clavibacter michiganensis subsp. *michiganensis* VURV 5090	4.6
	Clavibacter michiganensis subsp. *michiganensis* VURV 5059	4.7
	Clavibacter michiganensis subsp. *michiganensis* VURV 7008	4.7
	Clavibacter michiganensis subsp. *michiganensis* VURV 7018	4.6
	Clavibacter michiganensis subsp. *michiganensis* VURV 7030	4.7
	C. michiganensis	pI = 4.7, RSD = 1.9 %
X. vesicatoria	*Xanthomonas vesicatoria* CCM 2101	4.0
	Xanthomonas vesicatoria CCM 2102	4.1
	Xanthomonas vesicatoria VURV P-1-1	4.0
	Xanthomonas vesicatoria VURV P-6-1	4.1
	Xanthomonas vesicatoria LMG 2804	4.1
	Xanthomonas vesicatoria LMG 667	4.1
	X. vesicatoria	pI = 4.1, RSD = 0.7 %
P. syringae	*Pseudomonas syringae pv. tomato* CFBP 5422	4.0
	Pseudomonas syringae pv. tomato CFBP 2212	4.0
	Pseudomonas syringae pv. tomato IVIA 1733.3	4.0
	P. syringae	pI = 4.0, RSD =1.9 %
P. corrugata	*Pseudomonas corrugata* CFBP 4901	2.4
	Pseudomonas corrugata CFBP 5465	2.4
	Pseudomonas corrugata CFBP 6663	2.4
	Pseudomonas corrugata IVIA 614.5.3	2.4
	P. corrugata	pI = 2.4, RSD = 0.9 %

FF IEF

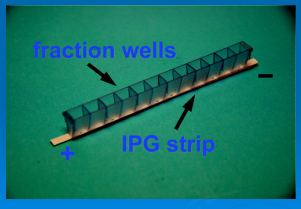


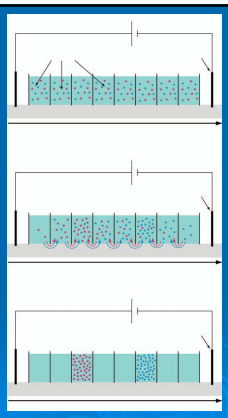


Schematic presentation of the setup used for OFFGEL electrophoresis



Horth, P. (2006) Mol. Cell. Proteomics 5: 1968-1974

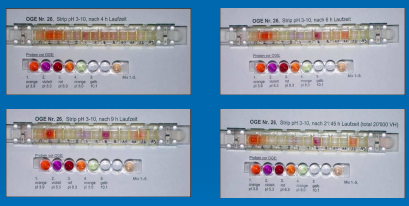




Use of pI-dye markers as on-line trackers for the focusing of peptides during electrophoresis on the OFFGEL fractionator device (OGE).

Heller, M.

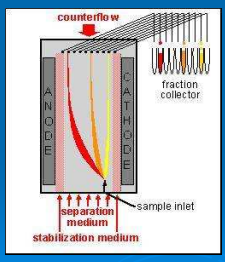
DKF, University of Bern, Switzerland

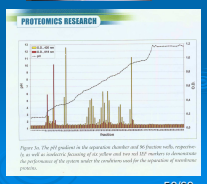


- **pI-marker dyes** were added at 10 ug (dark orange, pI 3.9; violet, pI 5.2; red, pI 6.2; bright orange, pI 8.0) or 30 ug (yellow, pI 10.1), respectively.
- Peptide/dye solution was distributed into the 13 wells of the OGE.
- IPG strips pH 3-10 from BioRad re-hydrated in OGE buffer were used.
- Focusing was done by setting a maximal potential (1250 or 1500 V) and a current limit of 50 uA.

Courtesy of M. Heller

Preparativní free flow IEF





IEF v rozbíhavém toku (divergent flow IEF, DF IEF)

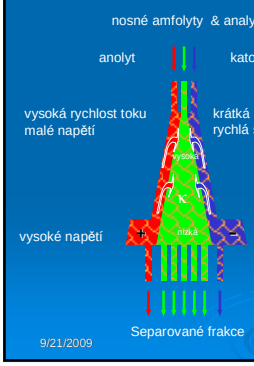
Základní idea

- Fluidika – kontinuální rozšiřování plochého kanálu při toku kapaliny od vstupu k výstupu při čemž je generován rozbíhavý tok

a současně,

- IEF - malé příčné napětí na vstupu kanálu a vysoké příčné napětí na výstupu kanálu

IEF v rozbíhavém toku



Fluidika – rozbíhavý tok

IEF – řízení elektrického proudu vodivostí kapaliny κ

Jednoduché zařízení:

Membrány eliminovány použitím porézního lože

Separační plocha a vstupy a výstupy kapaliny tvořeny netkanou textilií

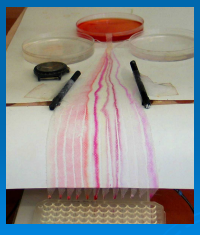
Kontakty k elektrodám tvořeny netkanou textilií

Tok generovaný hydrostaticky

Divergent flow IEF

K. Slais

Electrophoresis 2008



The polypropylene nonwoven web 0.1 mm thick lies on white polyvinylchloride flexible sheet

input strips dipped in Petri dishes containing:
above left – anolyte
above middle - solution of carriers and pI markers
above right – catholyte

middle left - carbon rod anode
middle right – carbon rod cathode

output strips - bottom - microplate

Streamlines of red pI markers from left - pI = 3.3, 4.7, 6.2, 7.6, 11.0

Flow due to hydrostatics and capillary elevation

Constant power load 1 W

No cooling

Dynamics of divergent flow IEF

K. Slais
Electrophoresis 2008

1 W constant power load

switched off at 11 hod 30 min

switched on at 11 hod 40 min

Flow inputs:
Anolyte: 0.05 M H_3PO_4 , 5.2 mS/cm,1 mL/h
Catholyte: 0.05 M NaOH, 11mS/cm, 1 mL/h
Carriers and pI markers: 0.75 mS/cm, 4 mL/h,

Holdup volume: 1 ml
Separation area: 71 cm^2

Streamlines of red pI markers from left
pI = 3.3, 4.7, 6.2, 7.6, 11.0

IEF v rozbíhavém toku IEF v rovnoběžném toku

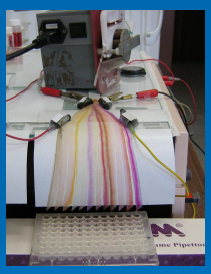
730 V

380 V

Materiál separačního prostoru, délka 15 cm, výstupní šířka 8 cm, výkon 1 W
Pracovní roztoky
Výstupní tok 6 mL/h = 0.9 cm/min
jsou stejné u obou geometrií

Šlais K. Electrophoresis 29 **2008** 2451-2457

IEF v rozbíhavém toku s jedním vstupem



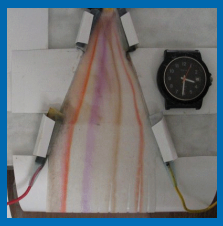
Linie červených pI markerů a proteinů od leva
oranžová - methyl oranž,
levandulová - marker pI 5.2
červená – marker pI 6.2
hnědá – hemoglobin, 0.5 mg/ml
cihlová – cytochrom C, 0.5 mg/ml
fialová – marker pI 11

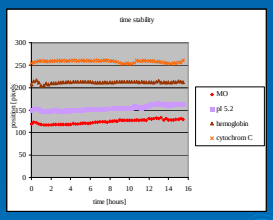
Vstupní roztok s pI markery a proteiny má vodivost 1.05 mS.cm-1.
průtok 0.18 mL.min-1
Vstupní elektrody – 75 V, 1.5 mA
Výstupní elektrody – 380 V, 1. mA

Šťastná M., Šlais K., Electrophoresis, přijato -00293

IEF v rozbíhavém toku s jedním vstupem

Časová stabilita polohy linií





Linie od leva
oranžová - methyl oranž, levandulová - marker pI 5.2
hnědá – hemoglobin, 0.5 mg/ml, cihlová – cytochrom C, 0.5 mg/ml, průtok 0.18 mL.min-1

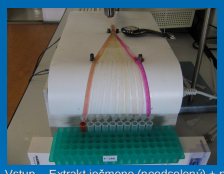
Kolísání linií 3.96 %, 3.94 %, 1.26 % a 1.88 %

Šťastná M., Šlais K., Electrophoresis, přijato -00293

Použití barevných pI markerů při preparativní SI DF IEF aplikované na extrakt ječmene

1 kapka roztoku směsi pI markerů LM

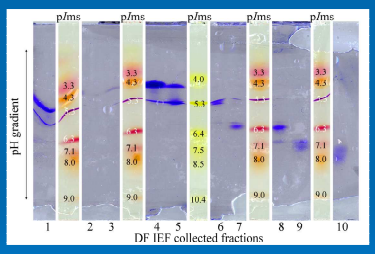
Kontinuálně dávkované pI markery:
oranžová - MO,
fialová - marker pI 11



Vstup - Extrakt ječmene (neodsolený) + pufry + MO + marker pI 11
průtok - 0.23 ml/ min,
vodivost - 1.0 mS/cm
Vstupní elektrody: 4 mA, 20 V
Výstupní elektrody: 6 mA, 800 V

Mazanec K., Bobálová J., Šlais K., Anal. Bioanal. Chem, odesláno

Kombinovaný sken IEF gelu frakcí z DF IEF piva



Barevné pI markery skenované ihned po gel IEF
Proteiny skenované po vybarvení Commassie

Mazanec K., Bobálová J., Šlais K., Anal. Bioanal. Chem, odesláno