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

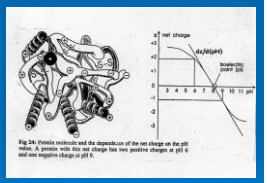
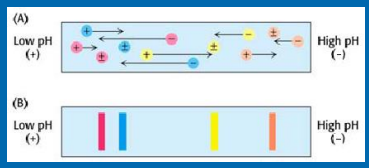


*Table 5-2.* **Isoelectric Points of Several Common Proteins**

| *Protein* | pI |
|---|---|
| Pepsin | <1.0 |
| Ovalbumin (hen) | 4.6 |
| Serum albumin (human) | 4.9 |
| Tropomyosin | 5.1 |
| Insulin (bovine) | 5.4 |
| Fibrinogen (human) | 5.8 |
| γ-Globulin (human) | 6.6 |
| Collagen | 6.6 |
| Myoglobin (horse) | 7.0 |
| Hemoglobin (human) | 7.1 |
| Ribonuclease A (bovine) | 9.4 |
| Cytochrome *c* (horse) | 10.6 |
| Histone (bovine) | 10.8 |
| Lysozyme (hen) | 11.0 |
| Salmine (salmon) | 12.1 |






## Simul 5



## 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)



## Typický výsledek gelové IEF

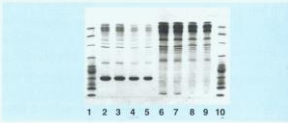
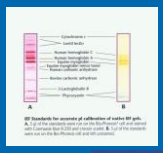
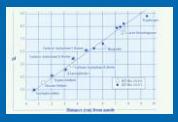



**Fig. 2. Typical separation in the Model 111 Mini IEF Cell (5% polyacrylamide gel with 2% Bio-Lyte 3/10 ampholytes).** Focusing was carried out in a stepped fashion (100 V for 15 minutes, 200 V for 15 minutes, 450 V for 1 hour). Samples are: lanes 1 & 10: Bio-Rad's IEF Standards; lanes 2-5: Dilutions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



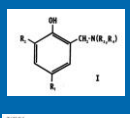


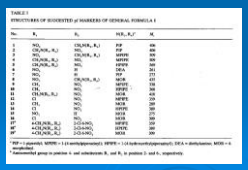
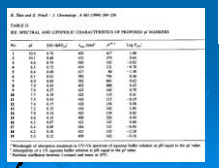
## Nízkomolekulární barevné pI markery


- 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, $\lambda_{max}$ > 400 $A_{1\%}$ > 100
  - Čistota, > 99 %,
- Dostupnost, cena markeru
  - Stabilita - hydrolýza, oxidace, fotodegradace, mikroorganismy



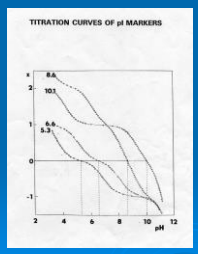
## Aminomethylované nitrofenoly



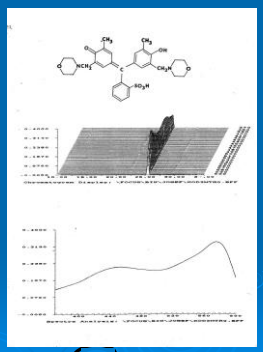


## Žluté pI markery



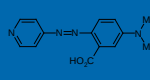



## Příklad barevného pI markeru





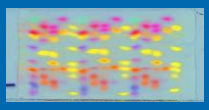
## Spektrofotometrické určení pI

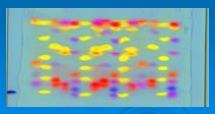

$pK_1$ = 3.50 ± 0.02 | pI = 4.71 ± 0.02 | $pK_2$ = 5.92 ± 0.02

## Určení pI interpolací v gelové IEF

Gradient pH



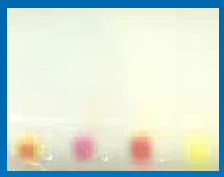
Směs 30 jednoduchých pufrů



Biolyt 3 – 10

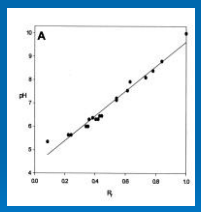


## Dynamika fokusace v gelové IEF

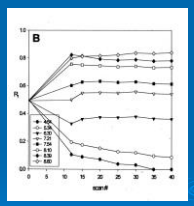




## Dynamika pH gradientu Biolyt 3-10




**Lineární gradient pH 4 - 10**




Po ½ hod malé změny pH gradientu



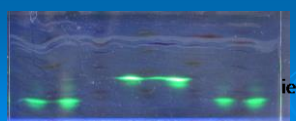
## Dynamika fokusace v gelové IEF



## Vývoj fluorescenčních pI markerů

Vis

fluorescence



ief446_INDEX.exe



yellow markers

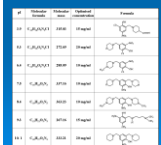
Pardubice 2005

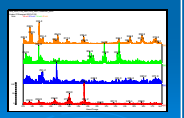
**Mass spectrometric characterization of low-molecular-mass color pI markers and their use for direct determination of pI value of proteins**

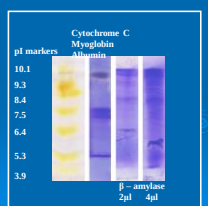
Mazanec, K, Slais, K., Chmelik, J.

J. Mass Spectrom. 41 **2006** 1570-1577

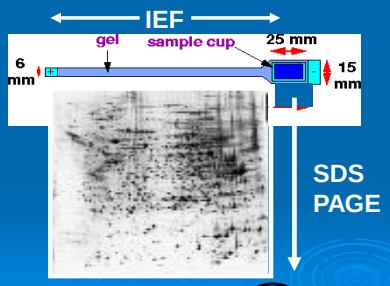
Mass spectra of nitro-substituted pI markers





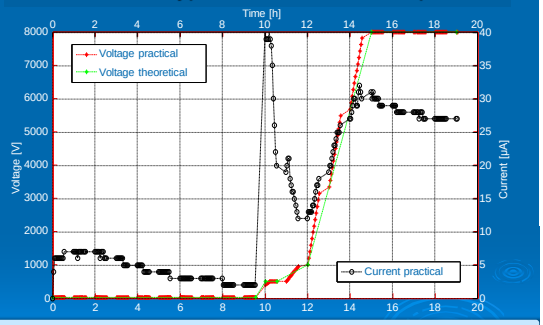


## 2D Gel electrophoresis



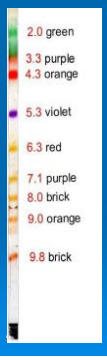



## Záznam typické IEF na IPG stripu




Credit:
Deng, X., Hahne, T., Schröder, S., Redweik, S., Nebija, D., Schmidt, H., Janssen, O., Lachmann, B., Wätzig, H., (2012). The challenge to quantify proteins with charge trains due to isoforms or conformers. *Electrophoresis*, *33*(2), 263–9. doi:10.1002/elps.201100321

## Test of color pI markers - LM ladder




Hanspeter Schickle,

ETC Elektrophorese-Technik GmbH, Kirchentellinsfurt, Germany

Strips rehydrated 2 hours under Kerosene

run native 7 hours with Nitrogen

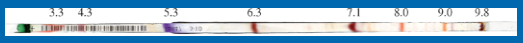
Amersham Multiphor.

Courtesy of Dr. Hanspeter Schickle



## 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



## 2D - typical result – silver staining

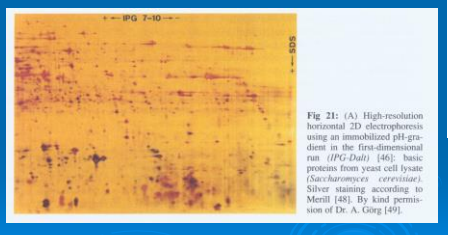
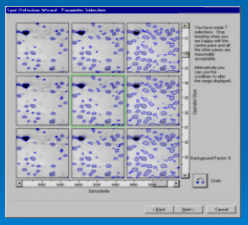
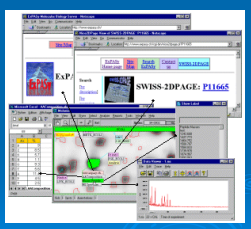



Fig 21: (A) High-resolution horizontal 2D electrophoresis using an immobilized pH-gradient in the first-dimensional run (*IPG-Dalt*) [46]: basic proteins from yeast cell lysate (*Saccharomyces cerevisiae*). Silver staining according to Merill [48]. By kind permission of Dr. A. Görg [49].

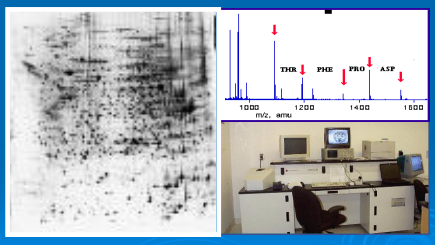


## 2D Gel electrophoresis - Software





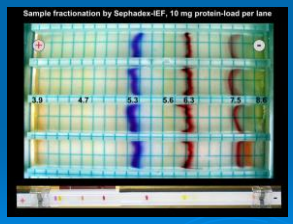
## Protein identification by 2D gel electrophoresis -MS






## 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.,

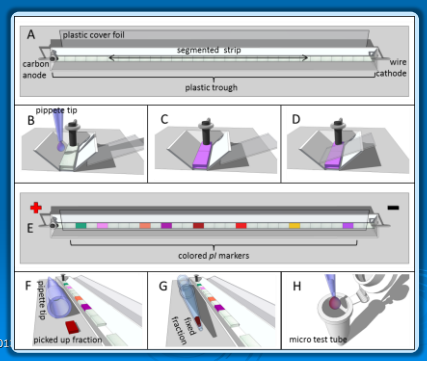
Institute of Experimental Medicine AV ČR, v.v.i., Prague

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

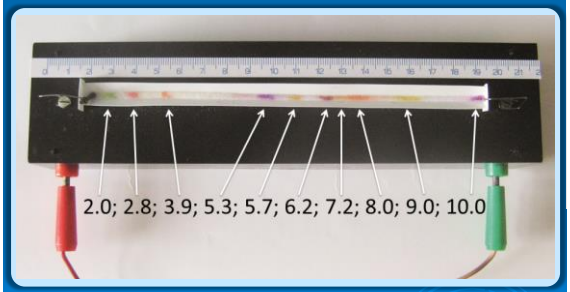


## Mikropreparativní sIEF v proužku netkané textilie






## sIEF nosných pufrů s barevnými pI markery

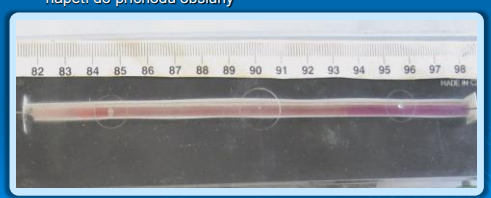



**fokusovaná směs:**
0,15 ml zásobního roztoku 12ti nosných pufrů v hydroxidu sodném
0,05 ml zásobního roztoku barevných pI markerů
0,15 ml ethylen glykolu; 0,05 ml butanolu; 0,1 ml vody



## Animace průběhu separace

- separace probíhá většinou cca 12 hodin
  - večer nadávkování vzorku a zapnutí zdroje
  - ráno možno sbírat frakcionovaný vzorek
  - pokud není možnost extrahovat frakce, zdroj udržuje nejvyšší napětí do příchodu obsluhy

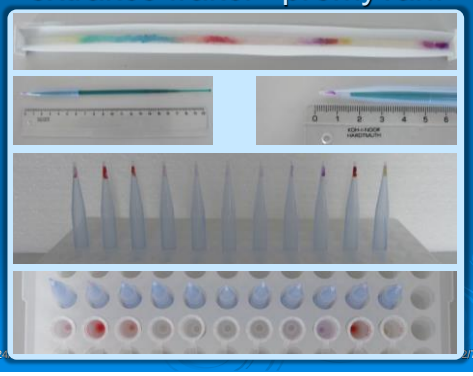


# Odběr frakcí

- dvě základní konfigurace pro odběr frakcí:
  1. proužek při separaci v celku – frakce vybrány po IEF na základě polohy markerů a vystřihnuty
  2. proužek dopředu nařezaný na kousky definované délky a kousky jsou před separací vyrovnány do fokusačního korýtka a přilepeny roztokem sacharózy příp. jiné fixační látky
     - při lepení možnost přidat i směs nosných pufrů a barevných markerů →„instantní proužek“ → stačí přidat vodu a vzorek a zapnout zdroj



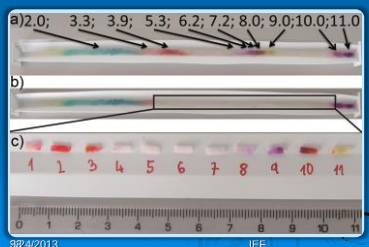
# extrakce frakcí - promývání





# Frakcionace syrovátky


- optimalizace pro dosažení co nejvyšší čistoty caseinomacropeptidu
  - separovaný vzorek – 1% (m/V) roztok surové syrovátky
  - přidání spacerů – IMAC (imidazol-1-yl-octové kyseliny) a Tris (tris(hydroxymethyl)aminometanu)




program ext. zdroje:

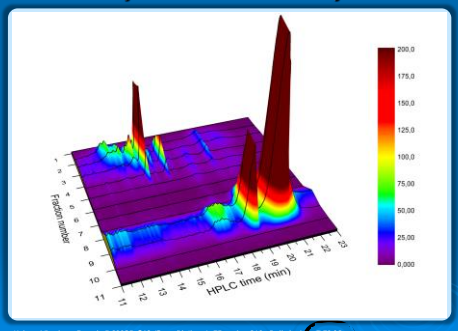
100 V – 200 V 4 hod.
200 V – 1000 V 4 hod.
1000 V – 3000 V 4 hod.
3000 V → do sběru frakcí

vzorek:
0,375 ml 1% (w/v) syrovátky;
0,05 ml zásobního roztoku;
barevných *pI* markerů;
0,1 ml ethylen glykolu;
0,05 ml butanolu;
0,025 ml 0,1 mol·l$^{-1}$ IMAC;
0,1 ml of 0,1 mol·l$^{-1}$ Tris;

extrakce: 6mm proužek do 100 µl vody



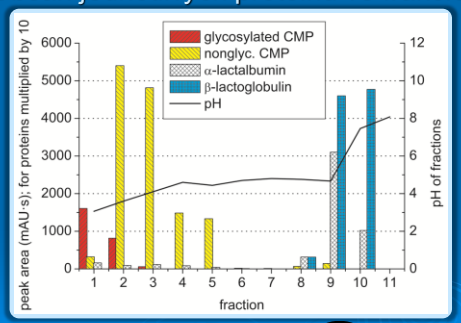
# HPLC analýza frakcí získaných z sIEF




Kolonia Microbore Poroshell 300SB-C18 (5 µm částice, 1×75 mm) + C18 předkolonka, při 70 °C
Průtok 20 µl·min$^{-1}$
A: H$_2$O/ACN s 0.1% (v/v) TFA lineární gradient od 5 do 80 % B v 30 min B: ACN (30 min)
dávkovaný objem: 4 µl



# Obsah jednotlivých proteinů ve frakcích



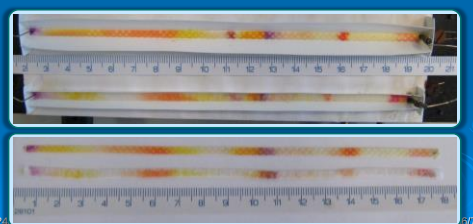

Výtěžek:

| | | | |
|---|---|---|---|
| glykosylovaný CMP | 44 % | neglykosylovaný CMP | 80 % |
| α-laktalbumin | 77 % | β-laktoglobulin | 101 % |



# Testování dalších typů netkaných textilií

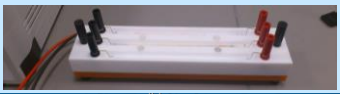
- různé materiály ze vzorkovníku firmy Polytex s.r.o. (Malé Svatoňovice)
- nejlepší materiál (Novolin, 100 g·m$^{-2}$) podobné charakteristiky jako původní netkaná textilie ale vyšší pevnost v tahu a menší třepivost – jednodušší manipulace a omezení odpadávání krátkých vláken



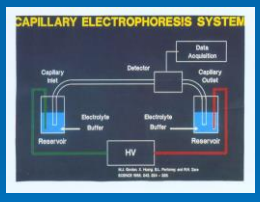
## sIEF na PřF MU

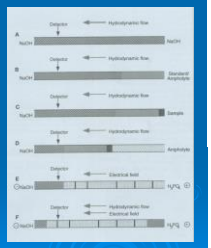
- Diplomová práce
  - Bc. Petr Švéda, školitel doc. RNDr. Přemysl Lubal, Ph.D.
  - vytvoření prototypu společně se separační metodou
  - následně převést metodu do výuky na ústavu analytické chemie PřF MU





## Kapilární IEF



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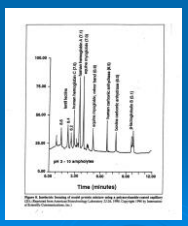
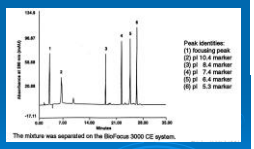
## Kapilární IEF standardů

proteiny



pI markery

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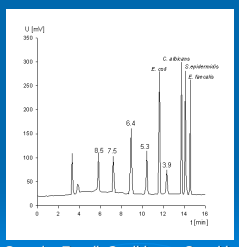
## CIEF mikroorganismů





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

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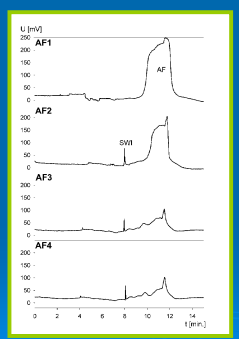
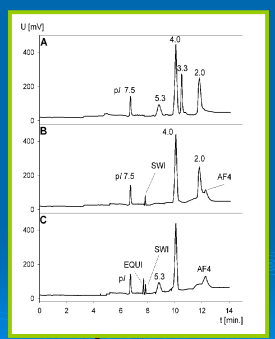
## CIEF virů s UV detekcí

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## Patogeny z různých zdrojů

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 % |

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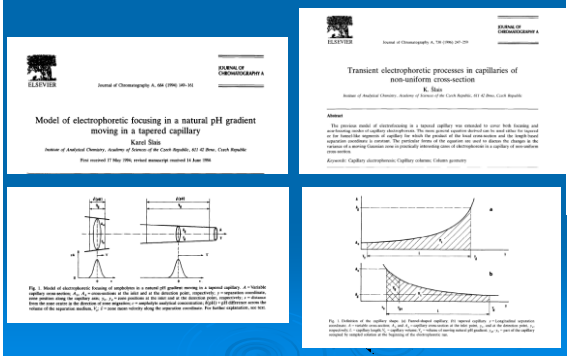
## Tapered capillary in cIEF

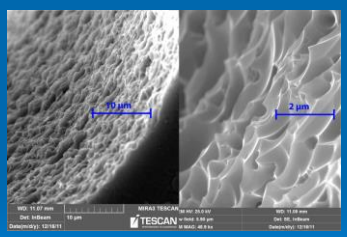


Model of electrophoretic focusing in a natural pH gradient moving in a tapered capillary
Karel Slais

Transient electrophoretic processes in capillaries of non-uniform cross-section
K. Šlais



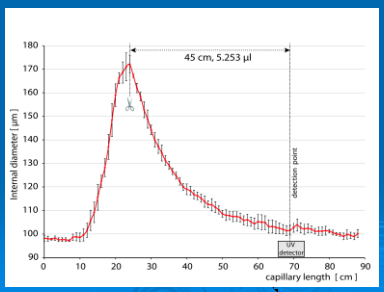
## Supercritical water in preparation of tapered fused silica capillaries

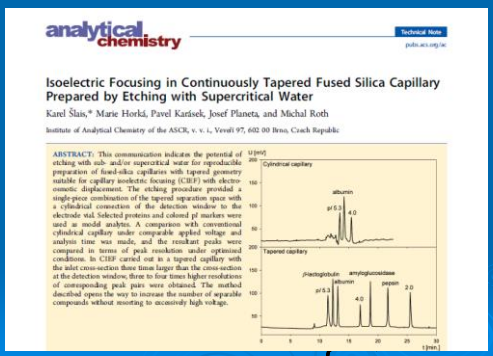

Effect of treatment of 100 µm i.d. fused silica capillary with supercritical water in semi-dynamic mode. Experimental conditions: 400 °C, 32 MPa, 20 replacements of supercritical water.



Dependence the local internal diameter of etched fused silica capillary on the capillary length.
The cutout of the segment used as the tapered capillary in cIEF and the detection window are indicated.





analytical chemistry — Technical Note

Isoelectric Focusing in Continuously Tapered Fused Silica Capillary Prepared by Etching with Supercritical Water

Karel Šlais,* Marie Horká, Pavel Karásek, Josef Planeta, and Michal Roth

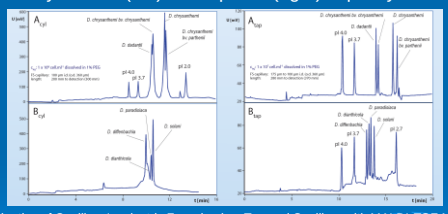
Institute of Analytical Chemistry of the ASCR, v. v. i., Veveří 97, 602 00 Brno, Czech Republic

ABSTRACT: This communication indicates the potential of etching with sub- and/or supercritical water for reproducible preparation of fused-silica capillaries with tapered geometry suitable for capillary isoelectric focusing (CIEF) with electro-osmotic displacement. The etching procedure provided a single-piece combination of the tapered separation space with a cylindrical connection of the detection window to the electrode vial. Selected proteins and colored pI markers were used as model analytes. A comparison with conventional cylindrical capillary under comparable applied voltage and analysis time was made, and the resultant peaks were compared in terms of peak resolution under optimized conditions. In CIEF carried out in a tapered capillary with the inlet cross-section three times larger than the cross-section at the detection window, three to four times higher resolutions of corresponding peak pairs were obtained. The method described opens the way to increase the number of separable compounds without resorting to excessively high voltage.



Resolution of several *Dickeya* bacterium species with similar isoelectric points by capillary isoelectric focusing employing

cylindrical (left) and tapered (right) capillary.



Combination of Capillary Isoelectric Focusing in a Tapered Capillary with MALDI-TOF MS for Rapid and Reliable Identification of Dickeya Species from Plant Samples
Horka, M; Salplachta, J; Karasek, P; Kubesova, A; Horky, J; Matouskova, H; Slais, K ; Roth, M
ANALYTICAL CHEMISTRY Volume: 85 Pages: 6806-6812 JUL 16 2013



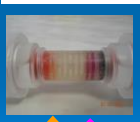
## Preparative Liquid phase IEF

BIO-RAD

**Rotofor**

MicroRotofor

Total 2.5 ml sample
Ten 0.25 ml fractions
2 hours run time

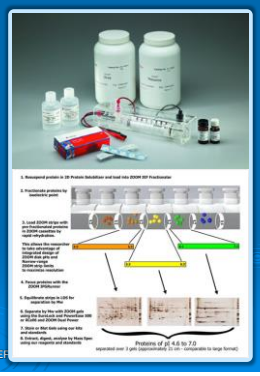


Preparativní autofokusace peptidů + pI markerů


Tomas, R; Yan, LS; Krenkova, J; Foret, F.
ELECTROPHORESIS, 28 (13): 2283-2290 2007

## ZOOM® IEF Fractionator (Invitrogen)

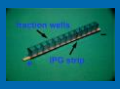
- komůrka pro fokusaci je oddělena polyakrylamidovými disky s definovaným pH
- proteiny musí procházet přes tyto disky dokud nedorazí do izoelektrického bodu
- objem každé části 650 µl
- vysoká reprodukovatelnost
- příprava vzorku: rozpustit, denaturovat, alkylovat, bez zákalu a částic

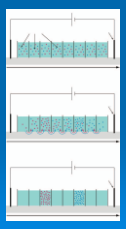




Agilent Technologies

## OFF - GEL electrophoresis





P.E. Michel, F. Reymond, I. L. Arnaud, J. Josser, H. H. Girault, J. S. Rossier, *Electrophoresis* 2003, *24*, 3–11

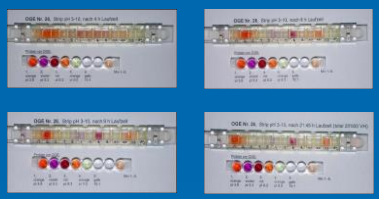
Agilent 3100 OFFGEL Fractionator

pI-based fractionation of proteins and peptides with liquid-phase recovery. introduced *May 30, 2006*, Co-developed with DiagnoSwiss S.A.

Use of pI-dye markers as on-line trackers for the focusing of peptides during electrophoresis on the OFFGEL fractionator device (OGE). 22 hours runtime

Courtesy of M. Heller DKF, University of Bern, Switzerland

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



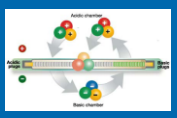
- **pI-marker dyes** were added at 10 ug (dark orange, pI 3.9; violet, pI 5.2; red, pI 6.2; bright orange, pI 8.0) or 30 ug (yellow, pI 10.1), respectively.
- Peptide/dye solution was distributed into the 13 wells of the OGE.
- IPG strips pH 3-10 from BioRad re-hydrated in OGE buffer were used.
- Focusing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

Courtesy of M. Heller, DKF, University of Bern, Switzerland

## Parallel isoelectric focusing.

protein forest

Charged proteins/peptides migrate through the dPC™ chip between the acidic and basic sides until they encounter a gel plug whose pH is at or near their pI. The uncharged protein/peptide will no longer migrate and become "trapped" in these plugs



G. Zilbersteinet al, Parallel isoelectric focusing chip; Proteomics2004, 4, 2533–2540.

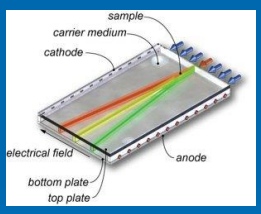
S. Buhskpan et al, Matrixes, Arrays, Systems and Methods; US PT 7,166,202, 2007.

dPC™ Chip Specifications

| | |
|---|---|
| Fractionation Time | 30 -45 minutes |
| pH Ranges* | 4.2-6.2, 6.0-8.0, 7.2-9.2 |
| Max. Resolution | 0.05 pH |
| Number of plugs per chip | 41 |
| Throughput | 1-6 samples/run |
| | 96 samples/8 hr |
| Loading Capacity | 20-100 µg Typical Load |
| | 2.0 mg Maximum Load |



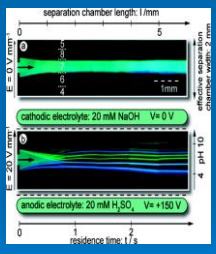
## Free-Flow Electrophoresis




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.



## Microfluidic high-resolution free-flow isoelectric focusing




**FFIEF of seven fluorescent IEF markers**

Voltage = 150 V, current = 50 mA

Markers (pI 4, 5.1, 6.2, 7.2, 8.1, 9, and 10.3) are fully separated within less than 2 s.

The sample flow rate was 0.4 mL/min ($v$ = 2 mm/s).

The apparent kinks in the fluorescent tracer paths are caused by merging multiple photographs.

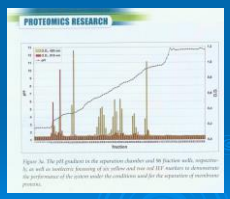


Kohlheyer, D., Eijkel, J. C. T., Schlautmann, S., van den Berg, A., Schasfoort, R. B. M., *Anal. Chem.* 2007, *79*, 8190–8198.

## Preparative Free Flow Electrophoresis

FFE Service GmbH
Dr. Gerhard Weber
D-85551 Kirchheim
Germany

WEBER, Gerhard; Margeritenweg 23 85551 Kirchheim (DE).
WO/2002/050524, 07.12.2001,






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

Základní idea

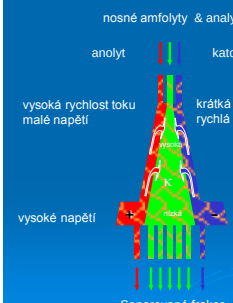
- Fluidika – kontinuální rozšiřování plochého kanálu při toku kapaliny od vstupu k výstupu při čemž je generován rozbíhavý tok

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

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

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

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

1 W constant power load

switched off at 11 hod 30 min

switched on at 11 hod 40 min

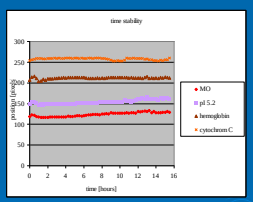
Flow inputs:
Anolyte: 0.05 M $H_3PO_4$ , 5.2 mS/cm,1 mL/h
Catholyte: 0.05 M NaOH, 11mS/cm, 1 mL/h
Carriers and pI markers: 0.75 mS/cm, 4 mL/h,

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



## Časová stabilita polohy linií IEF v rozbíhavém toku




**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, 2008, 29, 4503-4507

## Preparativní DF IEF piva

Mazanec K., Bobalova J., Slais K. Anal Bioanal Chem 2009, 393, 1769-1778



## DF IEF extraktů ječmene, sladu a piva

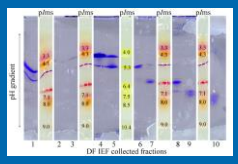
Kontinuálně dávkované pI markery: **1 kapka roztoku směsi pI markerů**
oranžová – marker pI 2.5
fialová - marker pI 11

Vstup - Extrakt ječmene (neodsolený) + pufry + markery pI 2.5 a 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., Bobalova J., Slais K.
Anal Bioanal Chem 2009, 393, 1769-1778

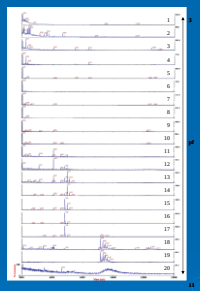


## Kombinovaný sken IEF gelu frakcí z DF IEF piva



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

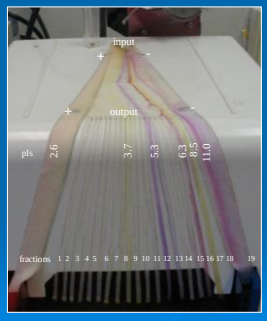
Mazanec K., Bobalova J., Slais K.
Anal Bioanal Chem 2009, 393, 1769-1778

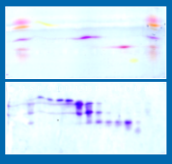


MALDI-MS spektra 20ti DF IEF frakcí proteinů ze surového extraktu ječmenného sladu.



## Preparative divergent flow IEF without carrier ampholytes for separation of complex biological samples



Separation of proteins in individual yeast lysate DF IEF fractions by polyacrylamide gel IEF.

DF IEF **without carrier ampholytes** with yeast lysate sample and colored pI markers.

Desalting, preconcentration, preseparation

M. Stastna, K. Slais, Electrophoresis,31, 2010,433-439



Projekt FRVŠ 2012

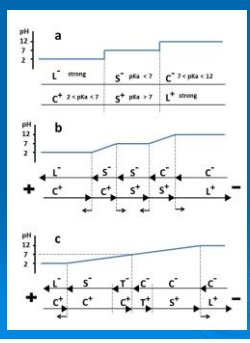
Vypracování laboratorní úlohy na zařízení pro izoelektrickou fokusaci v rozbíhavém toku

- laboratorní úloha vyučována v rámci předmětu C9320 Metody biochemického výzkumu na PřF MU
- výuky se zúčastnilo 45 studentů
- projekt byl úspěšně obhájen před komisí PřF MU 20. února 2013

- Použití chladicího boxu:
- Chlazení
- Odpařování
- Kontaminace
- Bezpečnost



## Electrolyte system for fast preparative focusing in wide pH range based on bidirectional isotachophoresis (BITP)




$L^-$ leading anion of strong acid
$L^+$ - leading cation of strong base,
$C^-$ - anionic counter ions,
$C^+$ - cationic counter ions,
$S^-$ - anionic spacers,
$S^+$ - cationic spacers,
$T^-$ - the fastest $C^-$ in LB in anionic ITP part and
$T^+$ - the fastest $C^+$ in LA in cationic ITP part.

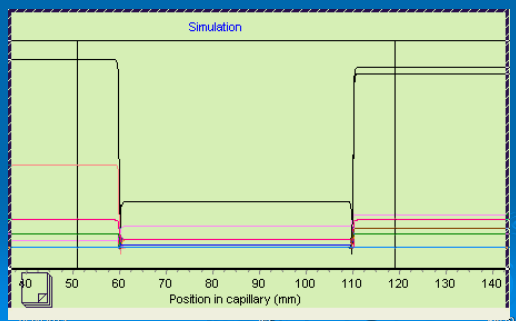
## The composition of LB, LA and spacer electrolytes used for simulation and in the experiment verification

| catholyte | pK | $\mu \times 10^{-9}$ [$m^2 V^{-1} s^{-1}$] | $M_r$ | simulated conc [mM] | conc [mgL$^{-1}$] |
|---|---|---|---|---|---|
| lysine | 10.79 | -26.4 | 146.19 | 7 | 1023.3 |
| ACA | 10.80 | -28.8 | 131.20 | 10 | 1312.0 |
| GABA | 10.56 | -29.0 | 103.10 | 10 | 1031.0 |
| β-alanine | 10.24 | -30.8 | 89.10 | 10 | 891.0 |
| glycine | 9.78 | -37.4 | 75.00 | 7 | 525.0 |
| asparagine | 9.02 | -31.6 | 130.00 | 5 | 650.0 |
| TAPS | 8.30 | -25.0 | 243.28 | 5 | 1216.4 |
| TAPSO | 7.70 | -26.0 | 259.28 | 5 | 1296.4 |
| NaOH | 13.70 | +51.9 | 40.00 | 60 | 2400.0 |
| **analyte** | | | | | |
| glutamic acid | 2.16, 4.32 | +27.4, +27.6 | 147.13 | 5 | 735.7 |
| β-alanine | 3.55 | +38.5 | 89.10 | 10 | 891.0 |
| GABA | 4.03 | +28.8 | 103.10 | 10 | 1031.0 |
| ACA | 4.37 | +29.8 | 131.20 | 10 | 1312.0 |
| creatinine | 4.83, 9.20 | +37.0, 37.2 | 113.10 | 5 | 565.5 |
| HPN | 5.29 | +30.0 | 123.20 | 5 | 616.0 |
| BisTris | 6.40 | +26.0 | 209.20 | 5 | 1046.0 |
| HEMorf | 6.80 | +30.2 | 131.20 | 5 | 656.0 |
| $H_2O_2$ | - | -82.9 | 86.00 | 25 | 2450.0 |

| | pK | $\mu \times 10^{-9}$ [$m^2 V^{-1} s^{-1}$] | $M_r$ | simulated conc [mM] | conc [mgL$^{-1}$] |
|---|---|---|---|---|---|
| **spacers** | | | | | |
| MOPS | 7.20 | -26.9 | 209.30 | 3 | 627.9 |
| ACES | 6.70 | -31.3 | 182.20 | 3 | 546.6 |
| MES | 6.09 | -28.0 | 213.25 | 3 | 639.8 |
| picolinic acid | 5.30 | -29.6 | 123.11 | 3 | 369.3 |
| ammonium acetate | 4.76 | -42.7,-39.4 | 77.09 | 3 | 231.3 |
| glycolic acid | 3.89 | -42.4 | 118.10 | 3 | 354.3 |
| phosphoric acid | 2.16,7.21,12.67 | 344,61.4,71.5 | 192.12 | 3 | 576.4 |
| imidazol | 7.15 | +52.0 | 68.10 | 3 | 204.3 |
| EtMorf | 7.70 | +30.0 | 115.20 | 3 | 345.6 |
| Tris | 8.08 | +29.5 | 121.10 | 3 | 363.3 |
| morpholine | 8.35 | +41.3 | 87.00 | 3 | 261.0 |
| aemediol | 8.70 | +33.5 | 105.10 | 3 | 315.3 |
| aminomethylpropanol | 9.60 | +30.0 | 89.14 | 3 | 267.4 |
| ethylaminoethanol | 10.00 | +30.0 | 89.14 | 3 | 267.4 |
| piperidin | 11.12 | +39.8 | 85.10 | 3 | 255.3 |



## Computer simulation of dynamics in newly suggested electrolyte system based on bidirectional ITP

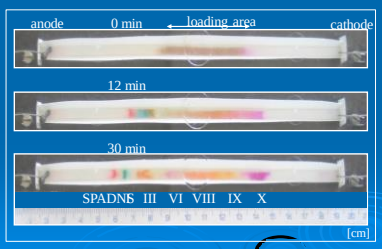





## The animation of the experiment with colored indicators subjected to BITP electrofocusing in newly suggested electrolyte system and carried out on nonwoven strip in V-shape trough during 30 min.



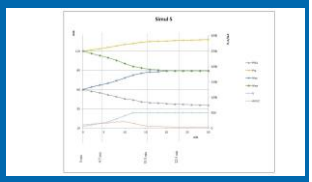
## The examples of representative images displaying bidirectional ITP electrofocusing process in nonwoven strip in V-shape trough with colored pH indicators taken at 0, 12 and 30 minutes.



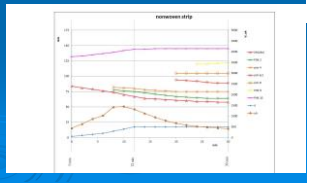



## The electrofocusing dynamics shown as dependence of zone position on analysis time

simulation

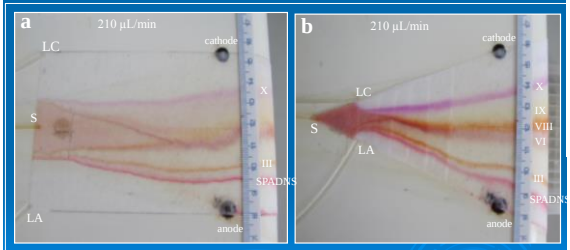


experiment carried out on linear nonwoven strip in the V-shape trough

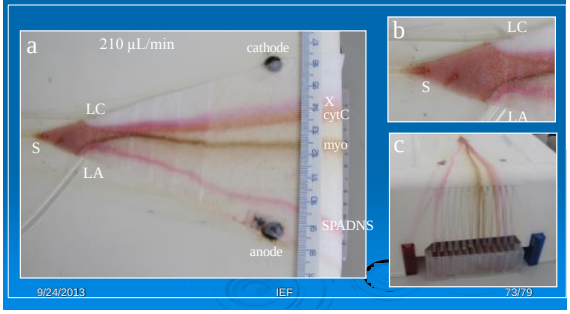




## The images of bidirectional ITP electrofocusing with continuous flow in rectangular (a) and trapezoidal (b) separation beds under the same experimental conditions



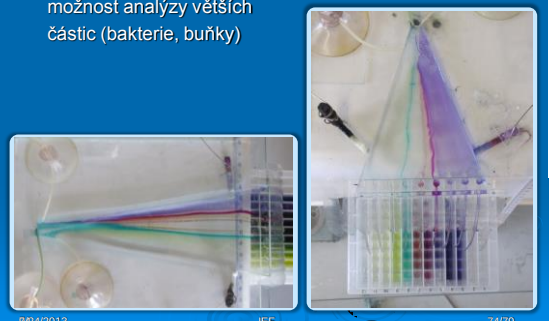
**The example of bidirectional ITP separation and electrofocusing in continuous flow of cytochrome C (cytC) and myoglobin (myo)**






- FF- BITP v rozbíhavém kanále

možnost analýzy větších částic (bakterie, buňky)





## Conclusions

The examined electrolyte system is suggested mainly for preparative sample treatments with relatively low (reasonable) number of eluted collected separated fractions, typically between 10 to 20 fractions. Thus, the total sum of spacers and counter ions does not need to substantially exceed number of 20,

compounds needed for preparation of the running solutions can be chosen from available cheap components,

The buffers are typically low molecular organic molecules which can contribute to compatibility with downstream fraction processing.



Running solutions can be optimized in advance and, *vice versa*, observed experimental results can be directly used for simulation and further solution modification.

By nature of ITP, the described method of focusing by bidirectional ITP with use of multiple counter ions is directly applicable for the separation and enrichment of ampholytes as well as both weak and strong electrolytes.

When the suggested electrolyte system is used in continuous flow devices the properties of leading electrolytes (mainly their pH and conductivity) make them simultaneously the electrode electrolytes. This strongly simplifies both the device construction and the operation.



The main advantage of the use of the suggested electrolyte system is seen in the possibility to use high current densities at the initial stages of focusing without danger of the local overheating. This fact strongly reduces the time needed for analysis completion and it applies to both modes of suggested bidirectional ITP including linear mode and planar arrangement with continuous flow.

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