Scale Up of Free Flow Isoelectric focusing (FF-IEF) for the generation of ultra narrow pH-cuts from commercial ampholytes.

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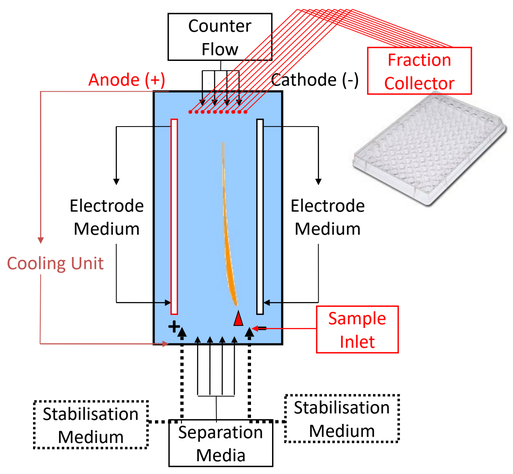
Overview: : Free-flow electrophoresis (FFE) is a matrix-free, very versatile electrophoretic separation technique. It can be used to fractionate mixtures of a great number of components including low molecular weight metal ions, organic substances, peptides, proteins, organelles or even whole cells. Separation is based on minimal differences in surface charge and/or isoelectric point (pI). Micro- to milligram quantities of samples can be processed within a reasonable time and components for research purposes are purified with excellent resolution. FFE is extremely versatile, because a variety of electrophoretic modes such as ZE, IEF, IZE can be applied. Therefore, it can be tailored to a customer's separation needs. In this way many different protocols have already been developed for the separation of diverse samples containing different peptides, protein isoforms, multiprotein complexes, ribosomes, liposomes, nanoparticles, cells, or DNA origami. Here we unveil another possibility of FFE application. We show its use for the fractionation of a raw product of commercial ampholytes, which leads to the generation of refined ampholytes with ultra narrow pH-cuts. This technique includes the regime of FF-IEF in a scaled up version. The step-wise approach to up-scaling is described below.

Principle of FF-IEF, continuous FF-IEF (Free Flow Isoelectric Focusing)



Introduction:

Commercially available ampholytes are synthetical products produced according to basic research, as published by H. Swenson in 1961. Many IEF-experiments using commercial ampholytes for the separation of proteins have been published. Unfortunately, users of different batches purchased from manufactures had to recognise, that slope and span of pH gradients often change depending on the batches of ampholytes obtained. Here we describe new FFE-protocols for the fractionation and purification of raw ampholyte mixtures at high throughput. It leads to refined ampholytes with defined ultra narrow pH-cuts.

Basic idea of scaling up for high throughput:

If a given pH gradient (see left picture, hatched blue area) is more narrow, the distance of deviation from the straight flow, i.e. the distance of electrophoretic migration is shorter until an amphoteric substance reaches the zone where the pH is equal to its pI. This results in shorter times needed for the final isoelectric focusing of each amphoteric substance. As final isoelectric focusing is desired to occur near the upper end of the separation chamber enhanced values of flow rates of media will be possible. Here we applied pH gradients, which were so narrow that CHIEF-processes could be run simultaneously in up to 3 independent areas of the separation chamber.

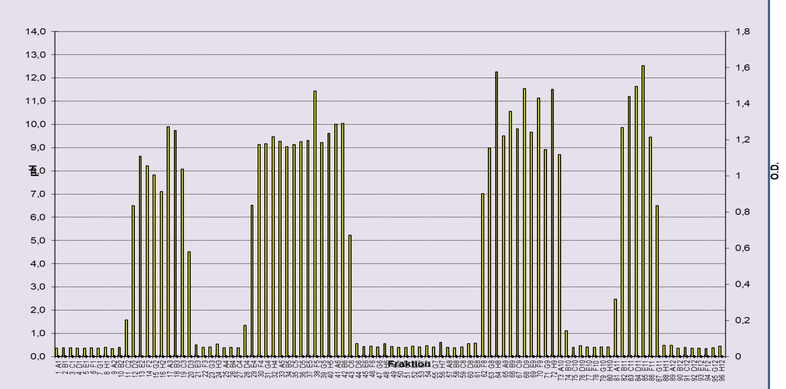
Free Flow Electrophoresis Instrument (standard version)

General Workflow of experiments below: Laminar fluid flow is the basis of each FFE experiment. Hence, also in this project each experiment was started with a „stripe test".
Dest water was injected via the inlets 1, 3, 5, 7 and 9 and dest. water with dye (SPADNS, dissolved at a conc. of 0.1 g/l), was injected via the inlets 2, 4, 6 and 8. These tests confirmed the basic requirement for any FFE-experiment:

- Uniform gap of separation cell ---- perfect operation of the fractionation device and ---- constant volume rates of all sections of the pump for the various media.

V 8642 FF-IEF-protocol 1: Single injection of ampholytes via inlet 5

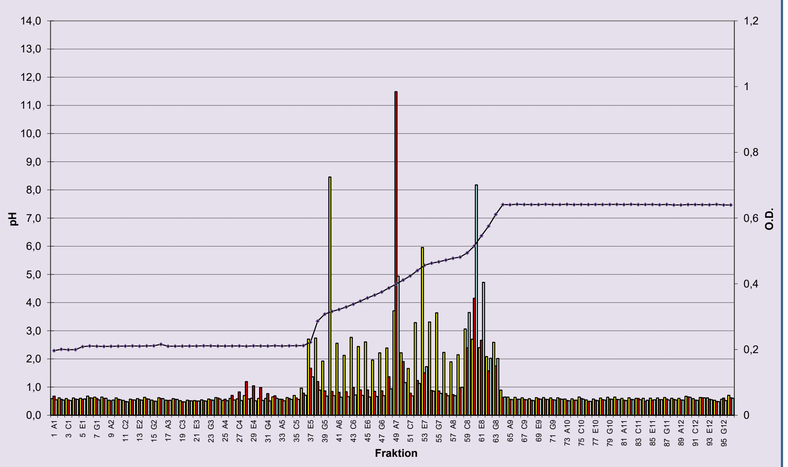
Stripe test



Injection of various media:
Medium for anodic stabilisation via inlets 1-4, 10% ampholyte solution via inlet 5 and cathodic stab. medium via inlets 6-9

Media inlet pipe	E1	E2	E3	E4	E5	E6	E7	E8	E9
Tube-Ø [mm]:	0.51	0.38	0.44	0.57	0.76	0.57	0.44	0.38	0.51
Media:	ARS				10 % Ampholyt	KRS			
pH					4,7				
Conductivity [µS]					685				

Pherograms of pH-values and of pI-marker concentrations determined for each well of the MTP-96 in which fractions were collected.
Electrophoretic data: 1000 V, 130 mA, total flow rate of media: 300 ml/h
Sample: SPADNS, pi markers: 3.9, 4.8 and 6.0



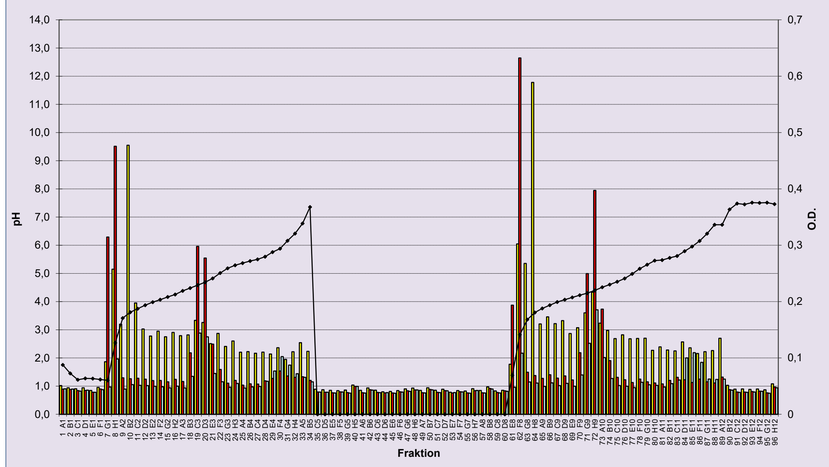
V 8647 FF-IEF-protocol 2: Two-fold simultaneous injection of ampholytes

Stripe test

Injection of various media:
Media for anodic stabilisation via inlets 1 and 6, 10% ampholyte solutions via inlets 2/3 and 7/8 and cathodic stab. medium via inlets 4/5 and 9

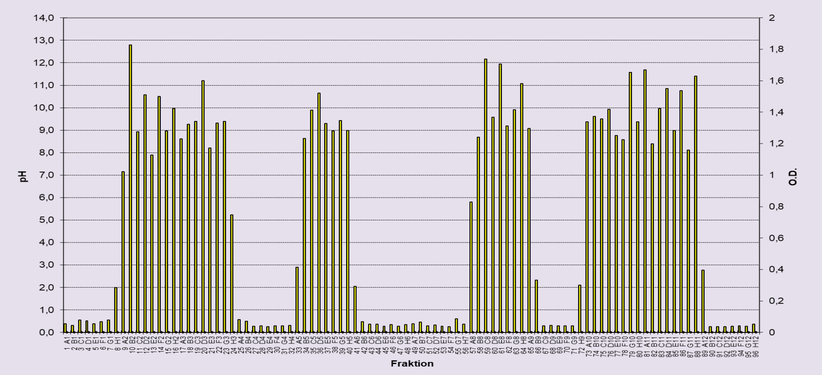
E1	E2	E3	E4	E5	E6	E7	E8	E9
0.57	0.57	0.57	0.64	0.44	0.64	0.57	0.57	0.57
ARS	10 % Ampholyte		KRS		ARS2	10 % Ampholyte		KRS
	4,7					4,7		
	690					690		

Pherograms of pH-values and of pI-marker concentrations determined for each well of the MTP-96 in which fractions were collected.
Electrophoretic data: 1000 V, 101 mA, total flow rate of media: 200 ml/h
Sample: SPADNS, pi markers: 3.9, 4.8 and 6.0



V 8681 FF-IEF-protocol 3: Three-fold simultaneous Injection of ampholytes

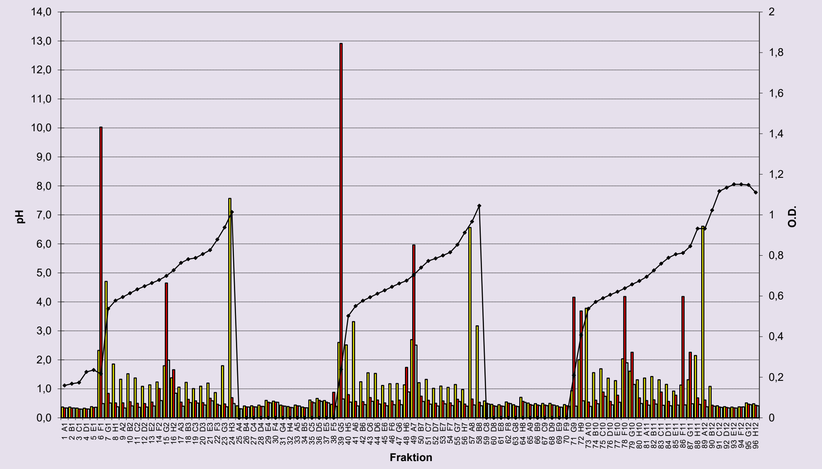
Stripe test



Injection of various media:
Media for anodic stabilisation via inlets 1, 4 and 7, 10% ampholyte solutions via inlets 2, 5 and 8,cathodic stab. medium via inlets 3, 6 and 9

E1	E2	E3	E4	E5	E6	E7	E8	E9
0.44	0.64	0.51	0.44	0.64	0.51	0.44	0.64	0.51
ARS	10 % Ampholyte	KRS	ARS	10 % Ampholyte	KRS	ARS	10 % Ampholyte	KRS
	4,76			4,76			4,76	
	689			689			689	

Pherograms of pH-values and of pI-marker concentrations determined for each well of the MTP-96 in which fractions were collected.
Electrophoretic data: 1200 V, 85 mA, total flow rate of media: 280 ml/h
Sample: SPADNS, pi markers: 3.9, 4.8 ,7.5 and 6.0 in region 3



Conclusion: It was demonstrated that the standard version of the FFE-instrument, type Nextgen, can be scaled up to fractionate samples of 10 % w/w raw ampholyte solution with throughput of 140 ml/h. This was achieved using a manifold (up to 3 fold) simultaneous CHIEF-(Continuous-High-performance-IsoElectric-Focusing)-process within the standard separation cell of Nextgen-instrument. FFE-fractions were obtained containing refined ampholytes with ultra-narrow spans of pH down to 0.2 ΔpH-units.

Key Features: FFE

- Up to three parallel pH gradients can flow adjacently and simultaneously through the separation chamber while current is applied between the electrodes.
- In each pH gradient region pI markers are separated equally.
- Excellent reproducibility from run to run and day to day
- Fast and sensitive detection of separation quality by subsequent photometrical evaluation of optical densities of fluids collected in each well of an MTP-96.
- Compatible with many other downstream techniques, e.g. SDS-gel, 2D-gel, HPLC, MS, and use of samples for further IEF.

Key Features: Fractionation of commercial ampholytes

- **Fine subfractionation of raw ampholine solution down to 0.2 Δ pH**
- **Max. concentration of amphoteric analyte: up to 10 % w/w ampholyte solution**
- **Throughput up to 140 ml/h enabled by simultaneous sample injection via three inlets and enhanced flow rates of media**

Outlook:

- **Use of FFE-fractions to remedy gaps of buffering capacity inside commercial batches**
- **Use of FFE-fractions as spikes into commercial batches of ampholytes, to generate areas of "ultraflat" slopes of pH-gradients**