Ultra narrow pH-cuts obtained from ampholytes by Scaled Up Free Flow Isoelectric focusing (FF-IEF). Part 2: Their use to flatten pH gradients formed by commercial ampholytes within a pH span of interest.

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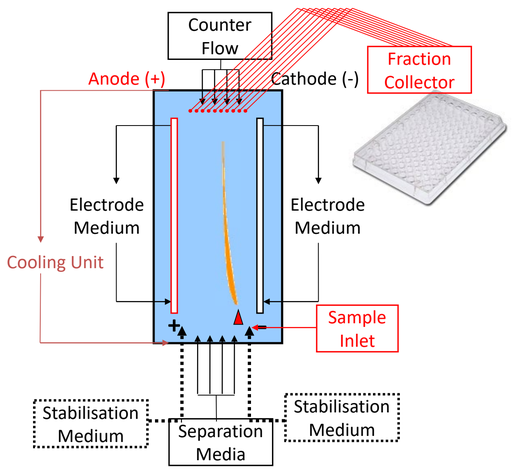
Overview: **Free-flow electrophoresis (FFE) is a matrix-free electrophoretic separation technique that separates analytes ranging in size from metal ions to whole cells according to minimal differences in charge and/or isoelectric point (pI). It can be used to obtain micro- to milligram quantities of purified samples for research purposes with excellent resolution. The technique is compatible with a variety of electrophoretic modes such as ZE, IEF, IZE etc. Since FFE is extremely versatile, it can be tailored to separation needs of individual customers. A wide range of protocols already exists for the subfractionation of analytes such as peptides, protein isoforms, multiprotein complexes, ribosomes, liposomes, nanoparticles, cells, and DNA origami. Recently we applied the regime of FF-IEF in a scaled up version and demonstrated its use for the fractionation of commercial raw ampholytes. By the new procedure we obtained considerable quantities of refined ampholytes with ultra narrow pH-cuts. Here we show that ampholyte fractions obtained in a first run can be used to flatten the slopes of pH gradients formed by Pharmalytes within pH spans of interest.**

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



1. Introduction: Many IEF-experiments have been published which were performed using commercial ampholytes for the separation of proteins. Unfortunately, users of different batches purchased from manufactures had to recognize that slope and span of pH gradients often vary depending on the batches of ampholytes obtained.
In each kind of electrophoresis ampholytes form pH gradients as the electrical current applied triggers different migration of different ampholytes' components. By the means of FFE the components forming the pH gradients can be collected in up to 70 fractions when they leave the separation chamber. Each of these fractions shows different pH values declining from the cathodal to the anodal side of the chamber.
Recently we developed a separation protocol for a scaled up version of FF-IEF, to process up to 140 mL/h raw ampholytes (see part 1). In this way fractions of ampholyte cuts with small span of pH-values were obtainrd in quantities sufficient for further use.
Now we present scaled up- FF-IEF experiments, which show that commercioally available raw ampholines with pH-values ranging from 5.5 till 9 (graphs 3.1 and 3.2) can be separated in fractions with very narrow pH spans. When the fractions were pooled, ampholytic pH cuts were obtained with pH spans of e.g. 0.7 or 1.2 units. In subsequent FF-IEF experiments selected pools were added to Pharmalytes and Pharmalytes together with a selected pool of ampholytic pH cut were processed. The pH gradients obtained now were flattened as demonstrated by the resulting pH/pI pherograms (3.4 ; 3.6).

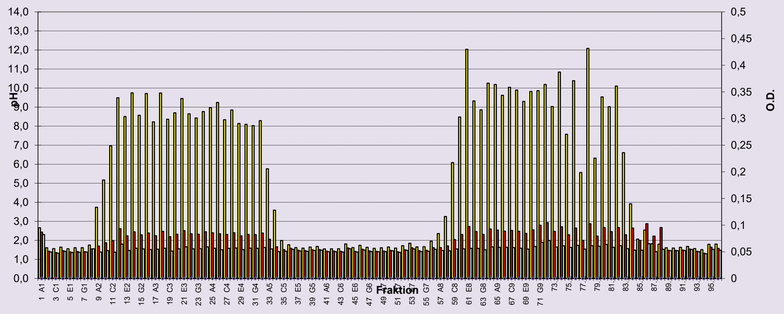
Free Flow Electrophoresis Instrument (standard version)

2. General Workflow of FFE-experiments: First, FFE quality control (QC) procedures were carried out to ensure proper FFE instrument set up and media composition. There are three types of QC procedures (QC 1-3), which have to be passed as described in earlier presentations such as P05.

3. Details of FFE-experiments:

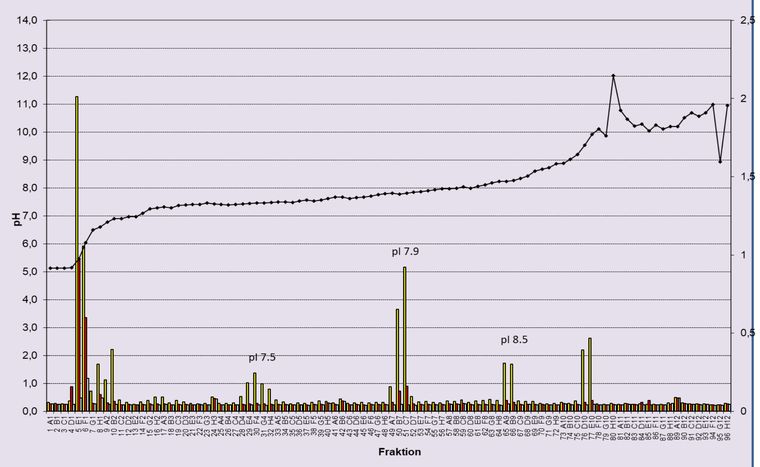
All media flowing adjacently through the separation cell were composed as described in part 1 for the V 8647 FF-IEF-protocol 2: Two-fold simultaneous injection of ampholytes.

3.1 V 8697p3: experiment without voltage applied.
Pherogram displays absorbance of UV-280 nm and fluorescence of 280/360 nm of 10 % ampholytes leaving FFE separation cell through FFE-fractions 9-35 and 53-86, which lay opposite to inlets E2/E3 and E6/E7, respectively.



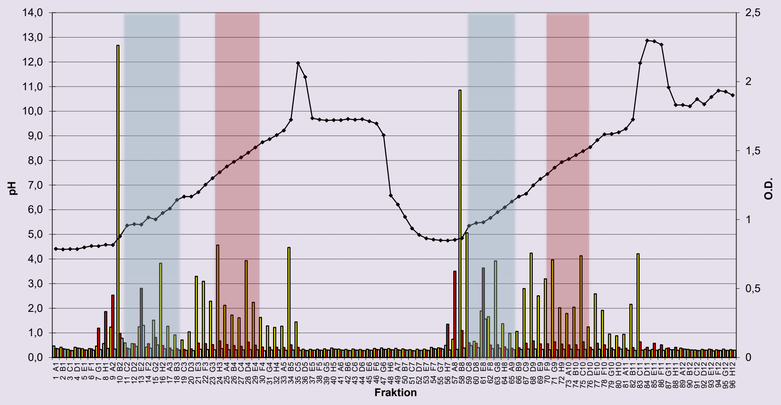
Pharmalytes with pooled ampholyte cuts

3.4 V8700 p14: FF-IEF-process of media with 1.5 % Pharmalytes 8-10.5, spiked with pool of FFE-fractions (pH 7.5 – 8.2=read area in 3.2)
6 inlets for 1.5 % ampholytes in E2-E7
Voltage: 1800 V, Current: 28 mA, flow rate of media: 56 ml/h
Sample: special pI-Mix for CHIEF-separations of analytes with pI>7:
SPADNS and pI-arkers: 3.9, 4.8, 5.3, 6.7, 7.5, 7.9, 8.5 and 10



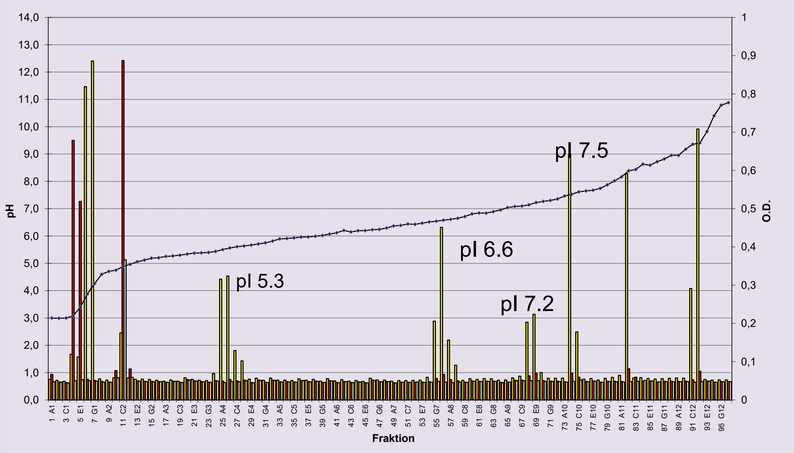
Fractionation of 10% ampholytes in fractions with ultra narrow spans of pH.

3.2 8697p10: FFE-experiment with voltage applied.
10 % ampholytes was injected through inlets E2/E3 and E6/E7 adjacent to additional media as in 3.1. Voltage: 900 V; Current: 118 mA;
Flow rate of all media: 125 ml/h; Throughput of 10 % ampholtes: 80 ml/h;
Sample: pI-Mix: SPADNS and pI-arkers: 3.9, 4.8, 5.3, 6.7, 7.5, 8.5 and 10.



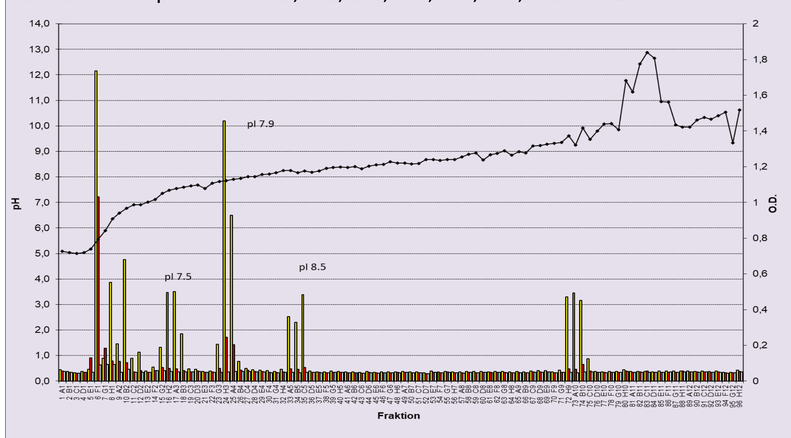
Pure Pharmalytes 5 - 8

3.5 V8703 p6: FF-IEF-process of media with 1.5 % Pharmalytes 5 - 8 injected through inlets 2-7;
Voltage: 1800 V; Current: 15 mA; Fflow rate of media: 45 ml/h;
Sample: special pI-Mix for CHIEF-separations of analytes with pI>5;
SPADNS and pI-arkers: 3.9, 4.8, 5.3, 6.7, 7.2, 7.5, 8.5 and 10.



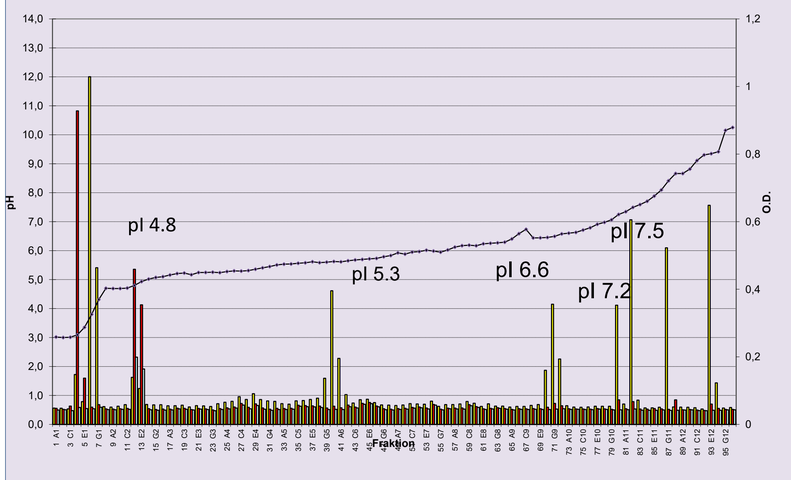
Pure Pharmalytes 8 – 10.5.

3.3 V8700 p6: FF-IEF-process of media with 1.5 % Pharmalytes 8-10.5 injected through inlets 2-7
Voltage: 1800 V; Current: 28 mA; Flow rate of media: 53 ml/h;
Sample: special pI-Mix for CHIEF-separations of analytes with pI>7;
SPADNS and pI-arkers: 3.9, 4.8, 5.3, 6.7, 7.5, 7.9, 8.5 and 10.



Pharmalytes with pooled ampholyte cuts

3.6 V8703 p 14: FF-IEF-process of 1.5 % Parmalytes 5-8, spiked with pool of FFE-fractions (pH 5.2-6.4= blue area in 3.2).
Voltage: 1500 V, Current: 28 mA, flow rate of media: 40 ml/h
Sample: special pI-Mix for CHIEF-separations of analytes with pI>5:
SPADNS and pI-arkers: 3.9, 4.8, 5.3, 6.7, 7.2, 7.5, 8.5 and 10



Conclusion: By the means of the standard version of the FFE-instrument, type Nextgen, 80 ml/h of 10 %w/w raw ampholyte solution were processed (3.2). FFE-fractions were obtained with an ultra-narrow span of pH down to 0.35 ΔpH-units. FF-IEF-fractions of 3.2 with pH spans 7.5 to 8.2 and 5.2 to 6.4 were pooled, respectively. The pools were mixed with commercial batches of Pharmalytes (Pharmalytes 8-10.5; see 3.4 and Pharmalytes 5-8; see 3.6). The added pools taken from 3.2 flattened the slopes of the pH-gradients of Pharmalytes 8-10.5 (3.4) and of Pharmalytes 5-8 (3.6). Please compare the pherograms 3.3 versus 3.4 and 3.5 versus 3.6.

Key Features of FFE-techniques

- Use of two separation parameters:
  difference of charge density and difference of pI-values
- Wide range of sample volumes: from 10 µl up to liters
- Matrix free separation, ideal for separation of particulate material and for conserving protein activity/protein complexes
- Excellent reproducibility from run to run and day to day
- Compatible with many other downstream techniques,
  e.g. SDS-, IEF- and 2D-GE, HPLC, MS,

Key Features of presented FF-IEF-protocols

Large-scale-FF-IEF-fractionation of 10 % ampholyte solution
- **Fine subfractionation of ampholine solution down to 0.35 ΔpH**
- **Sample throughput (10 % w/w) ampholyte solution up to 80 ml/h**

Finest FF-IEF-separation of 1.5 % commercial ampholytes
- **Using batches of commercial ampholytes ultra flat regions of pH gradients can be forme, if selected pools of raw ampholytes preseparated by FF-IEF are added.**
- **pH-values of neighboring FFE-fractions differ by 0.025 ΔpH in 3.4 and 3.6**

Outlook:

- **Use of FFE-fractions, to remedy gaps of buffering capacity**
- **Use of FFE-fractions, to generate ultraflat slopes of pH-gradients**