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EPITACHOPHORESIS – METHOD FOR EXTRACTION OF BIOMACROMOLECULES FROM COMPLEX MATRICES

*New perspectives on analytical chemistry**

Helena Hrušková[a,b] , Ivona Voráčová[a] , Markéta Laštovičková[a] , Michael Killinger[a,b], and František Foret[a]

[a] *Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veveří 967/97, 602 00 Brno*
[b] *Department of Chemistry, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic*
HelenaHruskova@email.cz

Epitachophoresis is a new separation preparative technique based on discontinuous electrolyte systems enabling the preconcentration of biomacromolecules from complex biological matrices. Moreover, it allows the transfer of these compounds from biological liquids, such as urine or growth medium, to a simpler solution, suitable for subsequent analyses. In our recently published papers, we focused on the use of epitachophoresis to pretreat the samples containing proteins and DNA. The technique enabled more than seventyfold preconcentration with recoveries reaching more than 90%. The preparative nature of epitachophoresis enables one to subsequently analyse the isolated biomacromolecules by various techniques including mass spectrometry, capillary electrophoresis, gel electrophoresis, ELISA analysis, or new-generation sequencing.

Keywords: DNA, proteins, epitachophoresis, concentration, matrix

Introduction

Body fluids (such as blood and urine) contain many compounds whose abundance reflects the organism's state. DNA and proteins are among the most important molecules occurring in body fluids. Both have a variety of functions in the body and show their potential as biomarkers for diseases. Therefore their analysis can be used for diagnostic purposes[1,2]. However, direct detection of these biomarkers in body fluids is difficult as their analytical signal may interfere with the signals of other compounds present in the matrix.

Moreover, biomacromolecules are commonly present in samples at low concentrations, further complicating their analysis[3]. For this reason, it is necessary to develop new tools to preconcentrate and purify biomarkers from biological matrices. This article introduces a newly designed modern extraction and separation technique – epitachophoresis (ETP).

Mgr. Helena Hrušková won the Metrohm 2023 Award for the best paper in the field of liquid chromatography for the separation of ionic and polar compounds. *In 2014–2017 she obtained her Bachelor's degree in Chemistry at Masaryk University, Faculty of Science, Department of Chemistry, Brno and in 2017–2019 her Master's degree in Analytical Chemistry at the Faculty of Science, Charles University in Prague and she is currently a PhD student at Masaryk University, Faculty of Science, Department of Chemistry, Brno. Her PhD thesis focused on micro- and meso- fluidic instrumentation for the enrichment of biological samples is being prepared at the Department of Bioanalytical Instrumentation, Institute of Analytical Chemistry of the CAS in Brno under the supervision of Ing. František Foret, CSc. Among other things, she received a CASSS student travel grant to the prestigious international conference HPLC 2022 symposium in San Diego (USA).*

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The concentration of biomacromolecules by epitachophoresis

ETP has already been described in our previous studies[4–7]. ETP is a preparative separation technique based on discontinuous electrophoresis that allows to preconcentrate and purify solutions of biomacromolecules from large volumes (up to tens of mL) of biological matrices (see Fig. 1).

The ETP is based on two circular vessels connected in the center by a collection well with a dialysis membrane. Each vessel also contains circular electrodes whose polarity is selected according to the type of analyte. The upper electrode always repels the analyte toward the center of the device, and the electrode in the lower vessel attracts it. Before the experiment, the lower vessel and the collection well are filled with the leading electrolyte (LE). A gel containing LE ions is placed in the upper vessel. A biological fluid (e.g., urine or cell medium) mixed with terminating electrolyte (TE) ions is poured between the gel and the wall of the larger vessel.

Once the electrodes are connected to the voltage source, DNA/proteins are repelled by the upper electrode towards the center of the device. Since the electrolyte system is chosen to perform discontinuous electrophoresis, the analyte molecules will focus into a narrow circular zone migrating to the center of the device. Once the analyte zone enters the collection well, the DNA/proteins can be collected using a pipette. The analytes are preconcentrated from the total volume injected into the device to the LE volume in the collection well, usually tens to hundreds of microlitres. In our publications, we have achieved a preconcentration factor of 75.

Another effect ETP provides is the purification of biomacromolecules from the matrix components. This purification enables the analysis of the sample using techniques sensitive to the content of interfering substances (salts, albumin, etc.). Several processes achieve the extraction of the analyte into a suitable solution. DNA carries a negative charge due to phosphate groups and is concentrated in the anionic mode. Proteins can carry both a positive or a negative charge depending on the pH of the solution. We have chosen a positive charge and low pH (described more in Chapter 3) for our purposes. Ions that are oppositely charged to those of the analyte migrate toward the electrode that repels the analyte, resulting in their exclusion from the sample. Another effect of this technique is reducing the content of salts and small molecules. Dialysis takes place in a collection well placed at the center of the device. Compounds reaching the well by migration that are smaller than the cut-off of the dialysis membrane are not captured by the membrane. Unlike macromolecules, they pass through the membrane into the second vessel and are eliminated from the sample.

Epitachophoresis of proteins

In our work[7], that was a part of the Metrohm 2023 Prize competition, we focused on using ETP to concentrate proteins. The pH of the solution determines the charge of proteins. To concentrate proteins using ETP, we chose a low pH corresponding to their positive charge. The obtained fraction was dissolved in an acidic volatile buffer, allowing the possibility of developing online coupling of ETP with mass spectrometry. Method development was focused on the optimization of several parameters. Firstly, the electrolyte system was chosen, followed by the electrical power and the type of separation gel. Cytochrome c was used as a model protein in a sufficient concentration (0.1 mg mL^{-1}) to make the developed zone observable by the naked eye. Essential criteria were the total experimental time, the heating of the center of the ETP device due to the high current density, and the shape of the developed protein zone. The optimization process introduced an electrolyte system consisting of 40 mM ammonium acetate pH 4 (LE) and 20 mM acetic acid

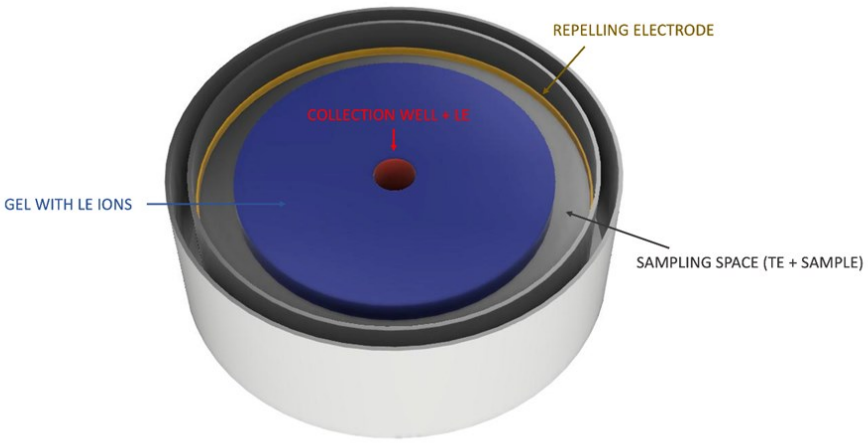

Fig. 1. **Schematic representation of the ETP device**. Adapted and modified with permission from ref.[7].

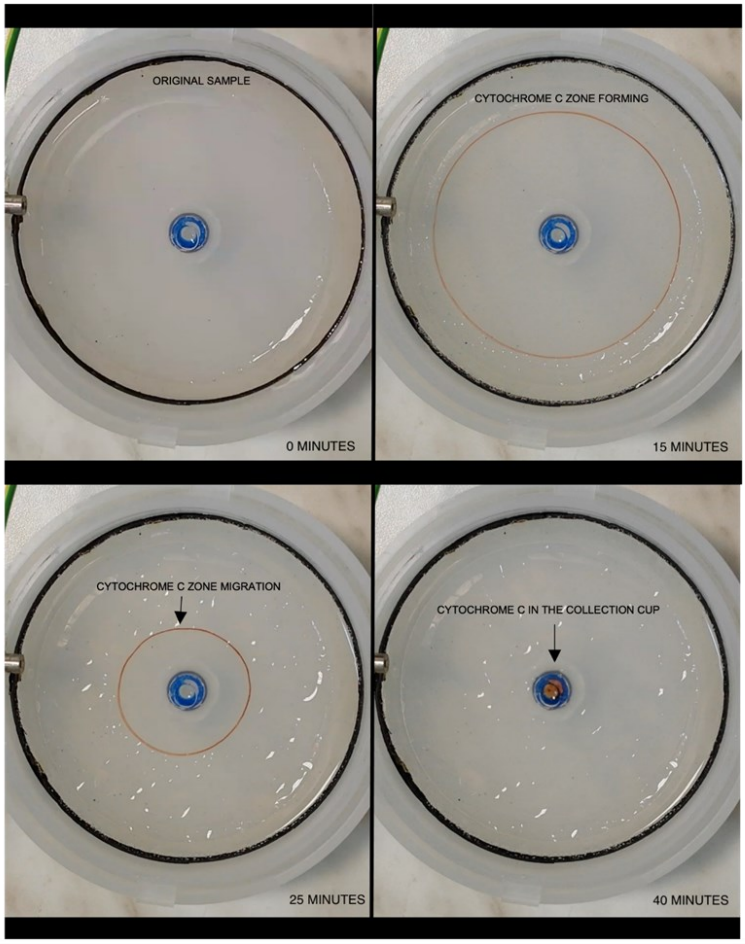

Fig. 2. **Optimized ETP process**. The protein concentration in 10 mL TE was 0.1 mg mL^{-1}. In the experiment, 0.5% agarose gel with a diameter of 7.5 cm, 40 mM ammonium acetate pH 4 as LE, and 20 mM acetic acid as TE were used. The process was captured at the 0th, 15th, 25th, and 40th minute. Adapted and modified with permission from ref.[7].

(TE). This electrolyte system provided low concentration time, low temperature of the device center, and a symmetric protein zone (see Figure 2).

In the experiments, an electric power of 5 W was used, and after 25 minutes from the start of the experiment, it was reduced to 1 W. The power decrease prevented possible denaturation or degradation of the protein due to overheating of the center of the device. Another important parameter was the gel type, serving mainly as an anti-convection and separation medium. Agarose gels could be used to concentrate cytochrome c. However, in experiments with myoglobin and hemoglobin, agarose gel showed strong protein adsorption to the gel edge visible by the naked eye.

For this reason, polyacrylamide was tested as a separation medium. With this type of gel, adsorption was eliminated. However, the concentration time was prolonged due to the higher density of polyacrylamide. Therefore, their diameter was reduced to 3 cm or 3.5 cm, so the experiment time did not exceed 45 minutes (see the detailed description in article[7]).

We reached the preconcentration factor of 29-67 and up to 98% recovery. Similar recovery values were obtained when the method was applied to a biological matrix (urine) with the addition of protein (see Figure 3). The concentration factor, in this case, was 32. In the work's last phase, we detected cytochrome c by concentrating the culture medium of HeLa cells. Without the ETP step, detection was not possible[7].

Conclusion

Our previous results published in papers[4–7] demonstrate that ETP could be an important technique for extracting proteins and DNA from biological matrices. ETP

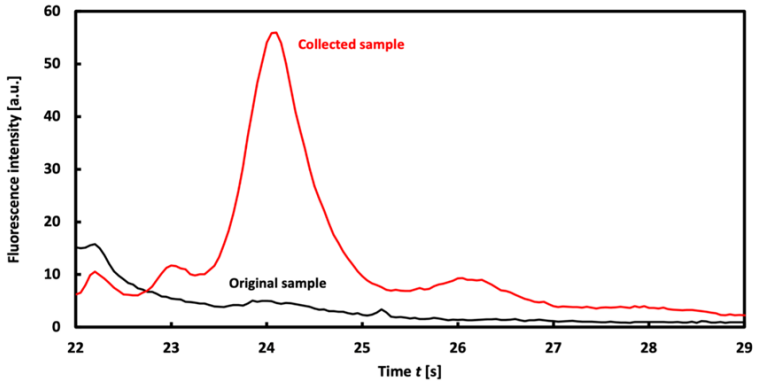

Fig. 3. **CGE-LIF of the original sample** (5 mL of urine with the spike of 1 mL of 1 mg mL^{-1} solution of cytochrome c standard – (black curve) and the fraction after the ETP process (red curve). The concentration of standard cytochrome c in TE was 50 µg mL^{-1}. 5 mL of urine was added to a total volume of 20 mL TE. A 6% (37.5:1 - acrylamide:bisacrylamide) polyacrylamide gel with a diameter of 3.5 cm, 40 mM ammonium acetate pH 4 (LE), and 20 mM acetic acid (TE) were used in the experiment. Adapted and modified with permission from ref.[7].

allows the preconcentration of target biomolecules and enables the transfer of the analyte into a solution suitable for subsequent analysis. Another advantage of ETP is recovery reaching over 90%.

In conclusion, several characteristics of ETP make the technique advantageous over other preparative techniques. For protein processing by ETP, surfactants and other additives are unnecessary, and the concentration time is less than one hour. Purification and concentration of proteins by affinity and microextraction methods are usually multi-step. ETP is a one-step process, and the final fraction is dissolved in a low-concentrated buffer, allowing future combination with mass spectrometry.

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