Capillary-Nano-HPLC for Proteomics I

- Size Exclusion Chromatography (SEC) -

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Electrophoresis and liquid chromatography are two highly efficient and complementary methods in biochemical research. Thanks to its flexibility, rapidity and high level of sensitivity, size exclusion chromatography can be used for the analysis and characterisation of biopolymers such as proteins or nucleic acids.

Many of the results and major advances recently achieved in gene technology and biotechnology would not have been analytically/technically feasible without 2-dimensional gel electrophoresis. This applies to proteomics in particular. Proteomics is the analysis of the proteins found at specific times in a living cell, i.e. almost snapshots in time of the entire protein pattern. 2-dimensional gel electrophoresis is still the most suitable method for this purpose due to its unique, high resolution and relatively high sensitivity. Over 1000 spots and proteins can thus be analysed in a single experiment [1].

The disadvantages of electrophoretic techniques, however, include the relatively extensive amount of work involved, the length of the experiments, the non-detectability of peptides and smaller proteins (< 10 000 D) [2], the only induced reproducibility of results and, often, the loss of biological activity, especially as far as enzymes are concerned. These disadvantages must, therefore, be offset by the benefits of HPLC, such as flexibility, rapidity and simple quantifiable methodology with even greater sensitivity where possible. Multi-dimensional capillary HPLC appears to be a useful, efficient, complement to 2-dimensional gel electrophoresis, and not only for proteomics. This is actually a cascade of various chromatographic methods, the sequence of which is adapted to suit the biochemical problem in question. This technique includes size exclusion chromatography (SEC), ion-exchange chromatography (IEX), hydrophobic interaction chromatography (HIC) and reversed-phase chro-matography (RPC).

Thus, the following experiments confirm the flexibility, rapidity and, in particular, the superior sensitivity of SEC in purification, re- and desalting techniques and in determining the apparent molecular weights of proteins [3] and polynucleic acids [4-6].

Materials and instruments

The buffer substances (analytical grade) and solvents (LiChrosolv) used were provided by Merck (Darmstadt). All the marker proteins provided by Merck (Darmstadt), Fluka (Buchs), Roche Diagnostics (Mannheim) and Serva (Heidelberg) were purified in advance on a conventional LC column (2.6 x 60 cm), packed with Sephacryl S-200 HR from Amersham-Pharmacia (Freiburg). The eluents were sterile-filtered and continuously degassed with helium. The NovoGROM capillary columns used were packed with Super SW 2000 or Super SW 3000 by GROM (Rottenburg) in conjunction with TOSOH BIOSEP (Stuttgart). The HPLC system comprised a Microflow Pump MicroPro (Sunchrom, Friedrichsdorf), a nano-in-jector with 30 or 300 nL loops (Upchurch, Oak Harbor, WA, USA) and a Spectroflow Spectra Focus UV detector with PC 1000 integration software and Intel 486/66 PC.

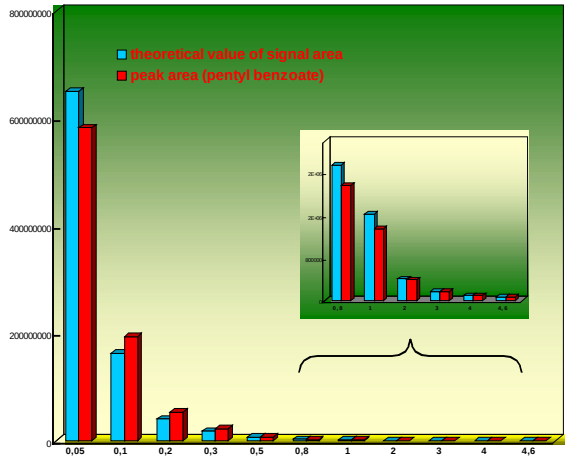
Results and discussion

Earlier studies reported that the replacement of a conventional HPLC column with an internal diameter of 4.6 mm by a capillary column with an ID of 50 µm ID (for the analysis of pharmaceuticals or benzoates, for example), increased the sensitivity by more than 8.000-fold [7]. This would equate to an 83- to 232-fold increase in sensitivity with capillary columns with IDs of 500 µm and 300 µm (Fig. 1). Since the chromatograms used to plot a calibration

Fig 1

≥ 8 000-fold Increase in Sensitivity

peak area vs. column i.d.

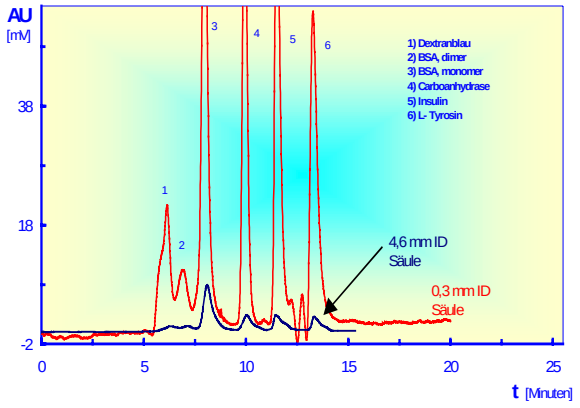


Stationary phase: GROM Sapphire 110 C18 - 5 µm, **Column size:** 125 mm x 0.05mm - 4.6mm i.d., **Eluent:** A 45% H2O, B 55% ACN (v/v), **Flow** (lin. vel.)**:** 0.80 mm/s, **Temperature:** RT, **Detector** (UV)**:** 254 nm, **Flow cell:** 0.2mm /50nL, **Injection:** 30 -300 nL, **Sample:** Alkylbenzoates (20-75 mg/mL)

line to determine the molecular weight of proteins were initially carried out on a column measuring 300 x 4.6 mm connected by a 150 x 0.1 mm capillary to a 15 µL flow-through cell (10 mm path length), only a fused silica flow-through cell of 4 nL with 0.2 mm path length was, however, available for the corresponding experiments employing capillary columns. Thus, sensitivity could only be increased 50-fold in these experiments (Figs. 2, 3). Sensitivity would have increased approximately 230-fold even in these experiments if a so-called nano-LC flow-through cell with a path length of 10 mm had been available. This once again confirms that sensitivity depends not only on the internal diameter of the column, but also corroborates the Lambert-Beer law regarding the path length of the flow cell of the detector used.

Fig. 2

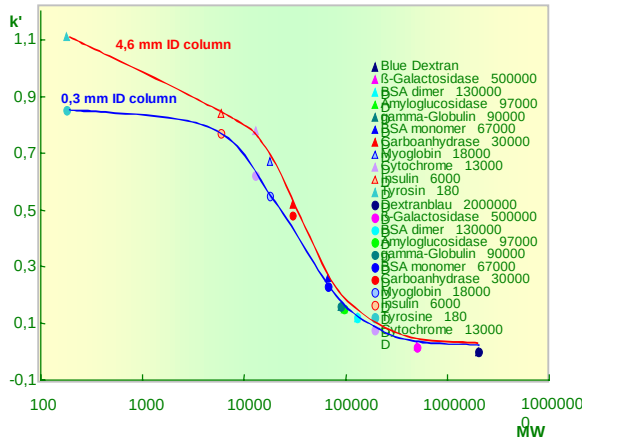
SEC of a protein mixture



Stationary phase: TOSOH Super SW 2000; **Column size**: 300 mm x 0.5 mm; **Eluent**: 0.1 M Na-Phosphat, pH 6.8, 0.1 M NaCl; **Flow Rate**: 0.35 mm/s; **Temperature**: RT; **Detection**(UV): 206 nm; **Flow Cell**: 4 nL / 0.2 mm; **Injection**: 300 nl of a protein mixture (0.1 – 0.4 mg/mL)

Fig. 3

MW-determination of proteins via SEC



Stationary Phase: TOSOH Super SW 2000, 4 µm **Column dimensions:** 300 mm x 4,6 mm or 0.3 mm; **Eluent:** 0.1 M Na-Phosphat, pH 6.8, 0.1M NaCl; **Flow rate:** 0.35 mm/s; **Temperature:** RT; **Detector:** 206 nm (UV); **Flow cell:** 15 µl / 10 mm or 4 nl / 0.2 mm; **Injection:** 10 µl or 300 nl protein solution (~ 0.1-0.5 mg/mL)

It should, however, be noted that, in general, at least 2 ng per protein spot must be applied in order to detect a protein by "silver staining" on a 2-dimensional gel [8]. Only 1.5 ng in 300 nL was, however, injected on the 300 x 0.3 mm capillary column, corresponding to a limit of detection of less than 0.02 ng (> 3-fold signal/noise ratio). Therefore, the limit of detection of proteins determined by capillary HPLC is basically lower than that obtained with the corresponding 2D-electrophoresis techniques. Despite the relatively low separation efficiency compared with other separation techniques, one advantage of size exclusion chromatography (SEC) is that the proteins of a cell or urine extract can easily be re-, respectively desalted in a single chromatographic procedure. They are thus not only purified or enriched but their molecular weight can also be determined at the same time if the capillary column has been previously "calibrated" with corresponding marker proteins. A further advantage of liquid chromatography (capillary SEC in this instance) is that, unlike electrophoresis in polyacrylamide gels, biological activity per se of even relatively unstable enzymes is very often maintained (Fig. 4).

In this case, "resalting" is synonymous with the fact that the protein-containing eluate, for instance, is accurately adjusted in line with the ion strength and –species required for the next separation stage in a chromatographic cascade, e.g. for ion-exchange or hydrophobic interaction chromatography. "Desalting", on the other hand, implies that the SEC column has been previously conditioned in a volatile buffer of lower ion strength and interesting proteins can easily be submitted by RP capillary HPLC to sequence analysis and investigation of protein structure [9].

Figure 5 clearly shows that besides for protein analysis capillary SEC is also suitable for screening and rapid, highly sensitive controls performed on product receipt, e.g. of insulins or commercially available interferons.

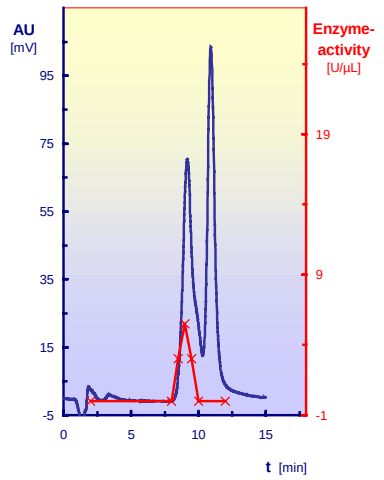
Outcome

The advantages and strengths of conventional size exclusion chromatography can easily be transferred to capillary SEC, resulting in considerably greater sensitivity than that obtained to date. The aim of past and present experiments was and still is to show that the benefits of conventional chromatographic techniques carried out to date in biochemical laboratories, such as SEC, IEX, HIC,

RPC etc., can easily be combined with the advantages of the faster, highly sensitive and modern capillary HPLC techniques for the purposes of proteomics [10].

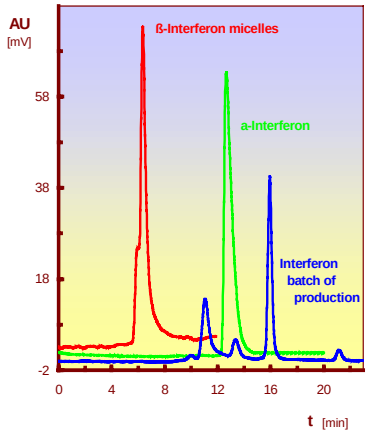
However, in order to analyse over 1000 proteins in a single procedure like in the case of proteom analysis by 2-dimensional gel electrophoresis, the individual chromatographic stages must, without fail, be connected by fully automated procedures with user-friendly, i.e. straightforward software to produce a cascade of chromatographical steps, often referred to in full as “multidimensional HPLC”. The individual components and the software required to this end are produced by corresponding manufacturers, but, regrettably, an integrated, automated combination of these modules is not yet commercially available.

Fig. 4 ***Maintenance of enzyme activity - product control -***



Stationary Phase: *TOSOH* Super SW 2000 - 4 µm; **column:** 300 mm x 0,3 mm; **Eluent:** 0,1 M Na-phosphate pH 6,8 - 0,1M NaCl; **Flow** (lin. vel.)**:** 0,35 mm/s; **Temperature:** RT; **Detection:** 206 nm (UV); **Flow cell:** 4 nL / 0,2 mm; **Sample:** 300 nL ß-Galactosidase (0,45 mg/mL)

Fig. 5 ***Rapid protein screening, sample from production***



Stationary phase: *TOSOH* Super SW 2000; **column size:** 300 mm x 0,3 mm**; Eluent:** 0,1 M Na-phosphate pH 6,8 - 0,1M NaCl; **Flow rate:** 1,48 µL/min; **Temperature:** ~ 21° C; **Detection** (UV)**:** 206 nm; **Flow cell:** 4 nL / 0,2 mm; **Injekction:** 300 nl protein mixture.

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