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Research Article

Gel-free IEF in a membrane-sealed multicompartment cell for proteome prefractionation

A minidevice for performing gel-free proteome prefractionation *via* conventional IEF in soluble carrier ampholyte buffers is reported here. It consists of a compact block of polyoxymethylene in which eight samples and two electrode chambers are machined. Each of the eight sample chambers can be filled with up to 120 μL of sample and has the following size: 7 mm width, 3 mm depth and 10 mm height. The anodic and cathodic compartments have the same width and height as the sample chambers, but with a depth of 6 mm, thus accepting up to 250 μL of electrodic solutions. Focusing is in general accomplished in 2 h with a voltage gradient of up to 1000 V (7 cm electrode distance). Easy fractionation and collection of the content of the eight chambers is achieved by simply pressing a rubber diaphragm against the edges of the thin walls separating each well, this automatically breaking liquid continuity. The performance of this device has been tested by subfractionating total cell lysates of a human cancer cell line (U2Os) and of *Escherichia coli* bacterial cells, and by analysing the content of each chamber by mono-dimensional SDS-PAGE and 2-D maps.

Keywords:
Carrier ampholytes / IEF / Multichamber devices / Proteome prefractionation
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1 Introduction

In this third millennium, characterized by an exasperating march towards miniaturization at all costs (just to have a glimpse at the field, one could consult a number of Special Issues of *Electrophoresis* devoted to this topic, *e.g. Electrophoresis* 2000, *21*, pp. 1–254; *ibid.* 2001, *22*, pp. 185–370 and pp. 3843–4031; *ibid.* 2002, *23*, pp. 3459–3645, *ibid.* 2003, *24*, 3521–3833), scientists have forgotten (or perhaps they never knew) that IEF (still one of the leading techniques on today's separation science horizon) was born as a preparative technique in large-size columns (accommodating either 110 or 440 mL of sample volume) filled with a density gradient, supporting the pH gradient, for preventing electrodecantation phenomena (*i.e.* sedimentation of the denser, focused protein zones that would occur in free liquid) [1–3]. An entire experiment, including column setup, focusing, elution and the analysis of hundreds of fractions, required a minimum of 1 week of hard labor. Notwithstanding the intensive labor involved, the trend towards large-scale preparative fractionation devices continued over the years. Thus, in 1975, Rilbe and Pettersson described two additional types of columns, this time extremely short and thick, one with a column volume of 440 mL, the other accommodating 110 mL of sample volume. In such columns, more than 1 g of sperm whale myoglobin could be fractionated, the main band containing as much as 800 mg protein, an appreciable amount to be carried by a density gradient [4]. Abandoning vertical density gradient columns, Rilbe's group started developing multicompartment electrolyzers (MCEs) still based on the IEF fractionation principle. The first of such electrolyzers was built with 20 chambers and could be filled with up to 1000 mL of sample, with a load capacity of several grams of protein *per* day (separations were over in a 24-h period) [5]. As a last evolutionary step, a mammoth-size apparatus was described [6], containing 46 separation compartments, accommodating a total volume of 7.6 L and encompassing a length of 1 m. Fourteen grams of whey proteins could be completely separated into its main components (*i.e.* serum albumin, α-lactalbumin and β-lactoglobulin).

With the advent of IPGs [7], preparative separations were still implemented on a rather large scale. The first preparative attempts contemplated focusing on progressively

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Abbreviations: CF, continuous flow; **MCE,** multicompartment electrolyzer; **TBP,** tributylphosphine

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thicker IPG gels (5 mm thick), first in standard 5%T, 4%C matrices [8, 9] and then in progressively diluted polyacrylamide matrices, down to as low as 2.8%T, cast in horizontal troughs filled with 125 mL of total gel volume [10]. Upon realizing the severe drawbacks of preparative runs in gel matrices, Righetti's group reverted to the idea of MCE, exploiting the fine Immobiline chemistry. Such devices exploited the unique idea of isoelectric, buffering, zwitterionic membranes, able to confine groups of proteins, according to their p*I* values, into any compartment delimited by two membranes of precise p*I* value [11, 12]. Also these electrolyzers (six sample collection chambers plus two electrode reservoirs) were meant for processing large sample volumes and sizeable protein amounts, since they were connected to external reservoirs from which a continuous sample feed was guaranteed *via* recycling.

In recent years, however, due to the development of high-sensitivity protein analysis techniques, including mass spectrometers able to handle minute (of the order of picomole) sample levels, the trend has been towards miniaturization even in preparative instrumentation. Additionally, due to the extreme complexity of any proteome [13], prefractionation by any means (chromatographic and electrophoretic) has now become a common trend [14]. Aware of this new trend, the MCE with isoelectric membranes was miniaturized, so as to adapt it to proteome prefractionation with minute sample amounts [15]. The new instrument was shown to perform quite well in collecting proteome subfractions of very precise p*I* intervals, void of contamination from adjacent p*I* species [16–18]. An interesting variant of this approach is off-gel IEF in multicompartment devices [19–21]. If a series of chambers (up to 20), containing the proteome sample to be subfractionated, are placed directly on top of an IPG gel, in any desired pH interval, which is subjected to a voltage gradient, the sample proteins will move along the IPG migration path till reaching their p*I* value and thus collecting, at null surface charge, into the cup standing directly over the IPG gel segment titrating such species to their respective p*I* value. Just like the original MCE with isoelectric membranes, off-gel IEF permits collection of proteins in solution, a most desirable feature when proteins have to be further analyzed for ascertaining their identity. This instrument too was shown to perform quite well for fractionation not only of proteins, but also of their tryptic digests [22–24].

Notwithstanding the advantages of proteome prefractionation in IPG-based separation processes (high precision in pH gradient engineering, very high resolution, retrieval of sample uncontaminated by carrier ampholytes (CAs)), separations in conventional IEF in soluble amphoteric buffers have also been adopted recently, especially in the Rotofor system (and in the mini-Rotofor version) [25]. The Rotofor is assembled from 20 sample chambers, separated by liquid-permeable nylon screens, except at the extremities, where cation- and anion-exchange membranes are placed against the anodic and cathodic compartments, respectively, so as to prevent diffusion within the sample chambers of noxious electrodic products. At the end of the preparative run, 20 focused fractions are collected simultaneously by piercing a septum at the chambers' bottom *via* 20 needles connected to a vacuum source. The narrow-p*I* range fractions can then be used for generating conventional 2-D maps. In recent times, this methodology has taken another, unexpected turn: the Rotofor is used directly as the first dimension of a peculiar 2-D methodology, in which each fraction is further analyzed by hydrophobic interaction chromatography, using nonporous RP HPLC [26]. Each peak collected from the HPLC column is then digested with trypsin, subjected to MALDI-TOF MS analysis and MS-Fit database searching. More recently, Xiao *et al.* [27] have reported an unexpected application of the Rotofor, not just for fractionation of intact proteins in the presence of CAs, but for fractionation of peptide digests of an entire proteome (in this case, human serum) in an ampholyte-free environment. The peptides themselves would act as CA-buffers and create a pH gradient *via* an "autofocusing" process (with a caveat, though, the pH gradient will be quite poor, since only a few peptides have good buffering power and conductivity in the pH 5–8 range).

Due to the fact that the Rotofor is still a complex machine to operate and even in its mini-version it handles sizeable amounts of liquids in each chamber (at least 0.5 mL), we report here a static apparatus (in that no rotational stabilization is adopted), for proteome prefractionation, accommodating minute sample volumes (100 μL *per* chamber) based on a novel design in the chamber construction and in the fraction collection at the end of the IEF run.

2 Materials and methods

2.1 Chemicals and biologicals

Urea, SDS, thiourea, CHAPS, Tris, acetic acid, sodium hydroxide, Ampholines (pH 3–10), the visible stain Brilliant Blue G (for colloidal Coomassie blue preparation) and the *Escherichia coli* lyophilized cells were all from Sigma-Aldrich (St Louis, MO). Tributylphosphine (TBP) and acrylamide solution were purchased from Fluka (Buchs, Switzerland). IPG strips pH 3–10 linear range, Laemmli sample buffer and Whatman paper were provided by BioRad (Hercules, CA). Pharmalytes (pH 2.5–5.0 and 5.0–8.0) were purchased from GE Healthcare (Chalfont St. Giles, UK). The human cancer cells U2Os were a kind gift from Dr. S. C. Righetti, Istituto Nazionale dei Tumori, Milan.

2.2 Sample prefractionation by IEF in the static chamber

The human cancer cells U2Os as well as the *E. coli* lysates were directly solubilized in "2-D sample buffer" (7 M urea, 2 M thiourea, 3% CHAPS, 5 mM TBP and 10 mM acrylamide) and allowed to be alkylated at room tempera-

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ture for 60 min. To stop the alkylation reaction, 10 mM DTT was added to the solution, followed by 2.5% Ampholine pH interval 3–10 (for the U2Os lysate) or 3% Pharmalyte pH interval 2.5–8.0 (for *E. coli* proteins, obtained by mixing 1.5% Pharmalyte 2.5–5.0 and 1.5% Pharmalyte 5.0–80).

The eight-chambered device was loaded with 960 μL of cell lysate (120 μL *per* trough), whereas the anodic and cathodic chambers were filled with Whatman paper soaked with 250 μL of 50 mM free acetic acid (pH 3.0) at the anode and 50 mM free sodium hydroxide (pH 12.0) at the cathode, respectively. The two electrolytes were dissolved in the same solution as the one used for protein solubilization. The total amount of sample loaded was 1 mg. Focusing was continued for up to 3 h by setting a limiting power of 1 W, which allowed for a ramp voltage going from 300 to 1000 V at room temperature. At the end of the run, the eight fractions were collected and analyzed by SDS-PAGE and by 2-D mapping.

2.3 SDS-PAGE

Mono-dimensional SDS-PAGE of the samples collected from the present fractionation instrument was performed using 10-well, 1 mm thick, 13% polyacrylamide glycine gel plates. Fifteen microliters of each fraction was mixed with Laemmli sample buffer 2× and boiled for 5 min, after that 30 μL of the eight mixtures were loaded *per* lane and electrophoretic migration performed at 130 V until bromophenol blue, added as a running marker, reached the gel bottom. Staining and destaining were performed with colloidal Coomassie blue and a 7% acetic acid water solution, respectively.

2.4 2-D PAGE analysis

Seven-centimeter long IPG strips (BioRad) pH 3–10 were rehydrated with 150 μL of protein solution (60 μL of the content of each chamber as *per* Section 2.2, diluted to 150 μL with 2-D sample buffer), for 4 h. IEF was carried out with an initial voltage gradient from 100 up to 1000 V, followed by 1000 V constant for 5 h. The voltage was then increased again rapidly up to 5000 V in 30 min, and kept at such a value until reaching 30 kVh. For the second dimension, the IPG strips were laid on a 10–20% acrylamide gradient SDS-PAGE. The electrophoretic run was performed by setting a current of 5 mA/gel for 1 h, followed by 10 mA/gel for 1 h and 20 mA/gel until the dye front reached the bottom of the gel. Gels were then immediately stained in colloidal Coomassie blue. Destaining was performed in 7% acetic acid until the background became completely transparent. The 2-DE gels were scanned with a VersaDoc Imaging System (Model 3000, BioRad).

3 Results

3.1 Description of the instrument

Figure 1 gives drawings of the cell block (A and B) and a photograph (top view, C) of the assembled instrument. Basically, the instrument consists of three main acetal-polyoxymethylene (POM) blocks assembled onto an $8 \times 9\ cm^2$ base. In the fixed block (part A), eight sample wells are machined, having the following size: 7 mm width, 3 mm depth and 10 mm height, each accommodating 100–120 μL of sample volume. At the two extremities, anodic and cathodic compartments are carved into the block, having the same width and height as the sample chambers, but with a depth of 6 mm, thus accepting up to 250 μL of electrodic solutions. The wells are visible in Fig. 1B, in which the mobile block (part B) has been removed. The novel idea in this construction is how the content of the various chambers is isolated from the neighbouring ones at the end of the IEF run. This is obtained by acting on the mobile block (part B), that acts onto a rubber wall (Viton seal). During IEF operation, the rubber wall is withdrawn by approximately 1 mm, so that the liquid overflows from the diaphragms separating the various chambers, thus ensuring liquid continuity and current flow. At the end of the IEF run, by turning the black knob, the mobile block B is pressed against the rubber wall, automatically sealing all the chambers. The content of each chamber is then withdrawn with a syringe or directly with an eight-tip pipette.

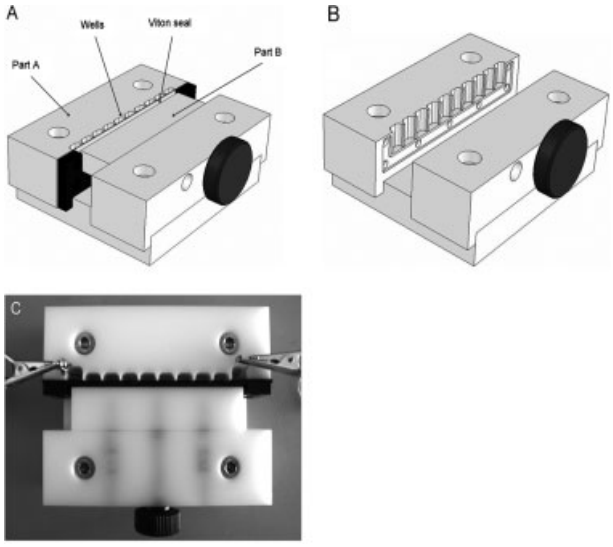

Figure 1. Drawings of the miniaturized free IEF instrument for proteome prefractionation (A and B) and photograph of the actual apparatus in operation (C). Automatic fractionation is achieved, at the end of focusing, by pressing the movable block B against the rubber wall (Viton seal). Panel B shows the profile of the ten chambers in the absence of the movable block B.

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3.2 Performance of the instrument

Figure 2 gives the evolution of current *versus* time, for two different applied voltages. It can be seen that, in both cases, focusing is obtained in *ca.* 20 min, not surprisingly, considering that the electrode distance is only 7 cm. Figure 3 gives the formation of pH gradient as a function of focusing time. It can be appreciated that the pH gradient is already formed after a 15 min run and is maintained (and fully developed) after 45 min of focusing. When running the multichamber device in presence of proteins, focusing is continued for up to 3 h, so as to ensure reaching a steady-state for all the proteins present in the sample. When measuring the conductivity profile of the liquid in the eight chambers, one obtains a U-shaped function, with a minimum at approx. pH 6–7, as well known in IEF (not shown) [28].

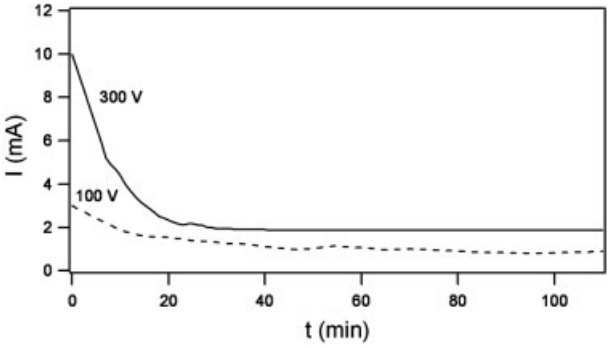

Figure 2. Evolution of current (*I*) *vs. time for two different voltages applied (300 V, continuous tracing and 100 V, dotted line).*

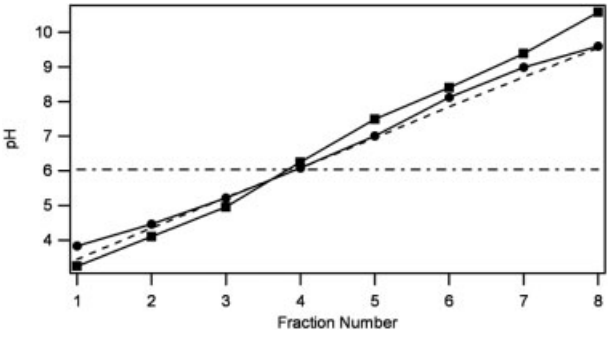

Figure 3. Time course of pH gradient formation. The horizontal line represents the pH prior to focusing. The slanted and broken line indicates the theoretical pH predicted for 3–10 ampholytes in an eight-chambered device. The circles and squares represent the pH measured after 15 and 45 min of focusing, respectively.

3.3 Biological results

In order to assess the performance in proteome prefractionation of this novel instrument, we have selected a total cell lysate of the human cancer cells U2Os and the water-soluble protein fraction of *E. coli*. Figures 4–6 display the results of these experiments. Panel (A) of Fig. 4 shows a 2-D map of a control, unfractionated total human cancer cell lysate, run in an IPG pH 3–10 in the first dimension. Panel (B) shows the mono-dimensional SDS-PAGE profiling of the contents of each chamber after fractionation on a 3–10 pH gradient (below the fraction nos., the pH value of each eluted fraction is reported). It can be appreciated that the SDS patterns are specific for each isoelectric fraction. In order to see how precise the p*I* cuts are, 2-D maps of some eluted fractions are displayed in Fig. 5. These maps are related to fraction nos. 1 (pH 4.33, upper right), 3 (pH 5.76, bottom)

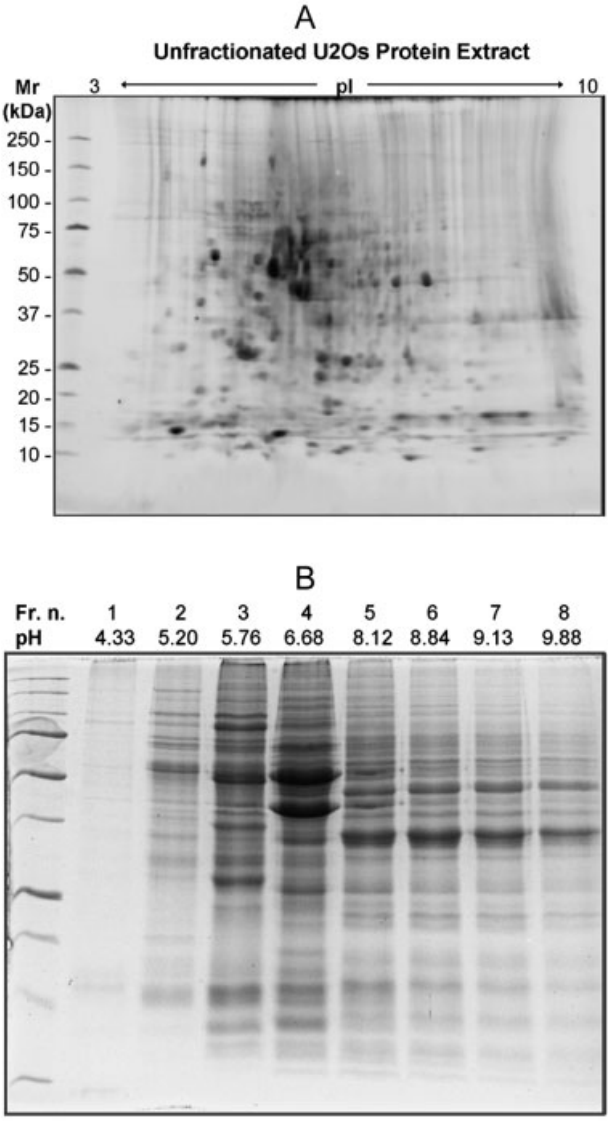

Figure 4. Analysis of a total cell lysate of human cancer cells U2Os. (A) Control 2-D map of the cell lysate in an IPG pH 3–10 interval. (B) Mono-dimensional SDS-PAGE of the content of each chamber after focusing in the minidevice shown in Fig. 1 using 3% Ampholine pH interval 3–10. Below the fraction no. the pH of each fraction is reported.

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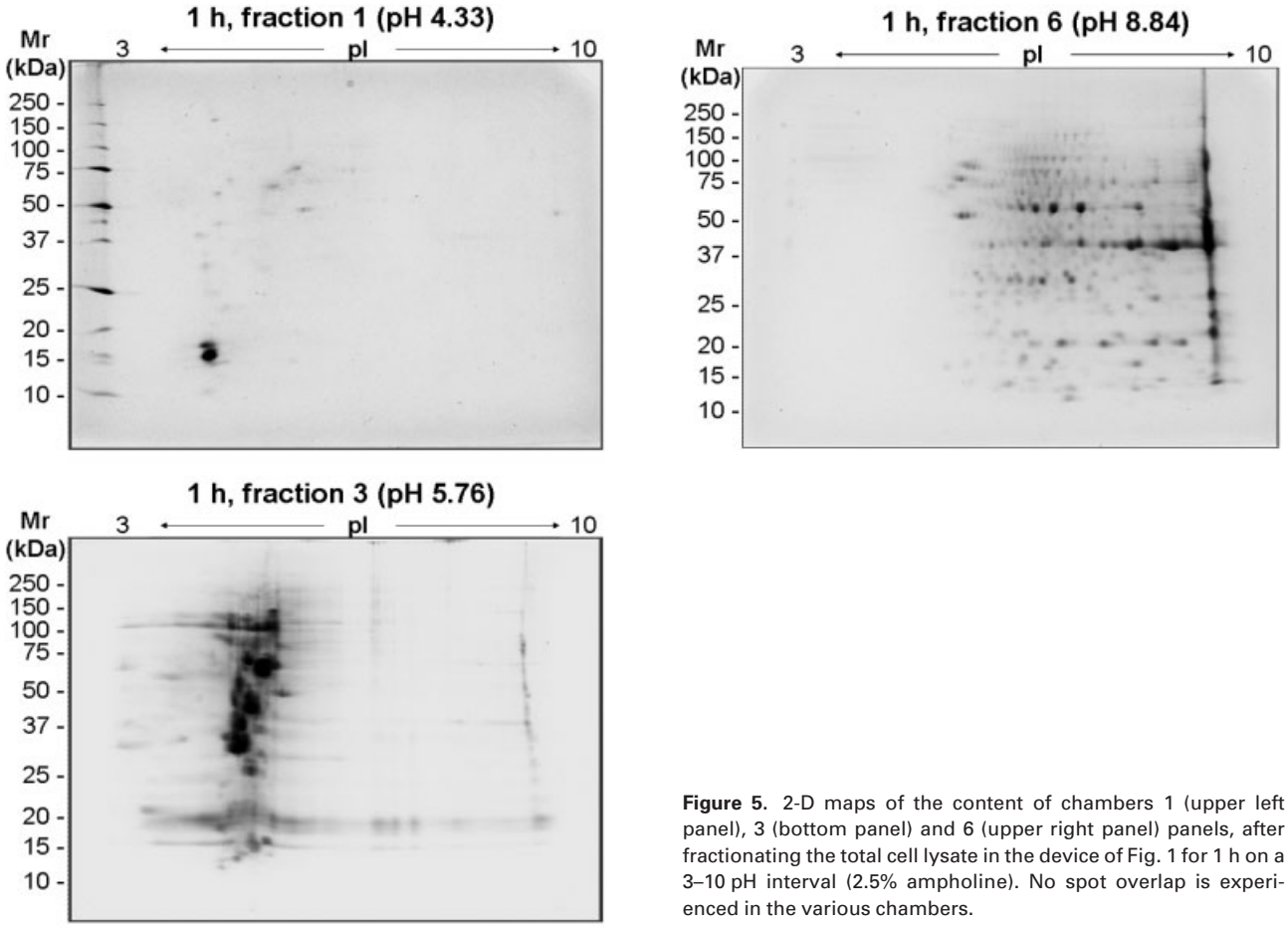

Figure 5. 2-D maps of the content of chambers 1 (upper left panel), 3 (bottom panel) and 6 (upper right panel) panels, after fractionating the total cell lysate in the device of Fig. 1 for 1 h on a 3–10 pH interval (2.5% ampholine). No spot overlap is experienced in the various chambers.

and 6 (pH 8.84, upper right). It can be appreciated that they display quite narrow p*I* cuts, with essentially no spot overlaps among the different fractions. In order to prove that steady-state conditions had been reached, the experiment was repeated with an *E. coli* total cell lysate, that was run for 1 and 3 h on a 2.5–8.0 pH interval. It can be appreciated (Fig. 6A–C) that the two 2-D profiles obtained from the same fractions after 1- or 3-h fractionation are quite similar, indicating that even the shorter focusing times are adequate for ensuring proper separations.

4 Discussion

As stated in Section 1, although prefractionation exploiting the IPG methodology has been preferred up to the present, more and more reports have appeared in the last few years dealing with prefractionation *via* conventional IEF in soluble CA buffers. Although we have reported only a few, selected applications based on the Rotofor, other instruments exist for performing this task, such as continuous flow (CF) IEF devices, as epitomized by the Octopus [29], allowing the collection of as many as 96 fractions. For instance, Hoffman *et al.* [30] have proposed CF-IEF as the first dimension of a 2-D map, the eluted fractions being directly analyzed by orthogonal SDS-PAGE. In turn, individual bands in the second SDS dimension were eluted and analyzed by ESI-IT-MS. By this approach, they could identify a number of cytosolic proteins of a human colon carcinoma cell line. One advantage of CF-IEF (and of course of all the focusing techniques in a gel-free environment) is immediately evident from their data: large proteins (*e.g.* vinculin, M_r 116.6 kDa) could be well recovered and easily identified; on the contrary, recovery of large M_r species has always been problematic in IPG gels. In addition to that, it is also known that IPG matrices tend to adsorb irreversibly hydrophobic and membrane proteins, rendering thus problematic their recovery and identification. All these phenomena do not occur when IEF is performed in a plain liquid phase, which probably accounts for the popularity of the Rotofor instrument. Our minidevice greatly simplifies the approach to gel-free IEF: it is compact, it allows for very small sample volumes (as little as 100 μL), for very simple fraction recovery and it disposes of the rotational stabilization implemented in the Rotofor. In fact, in our sys-

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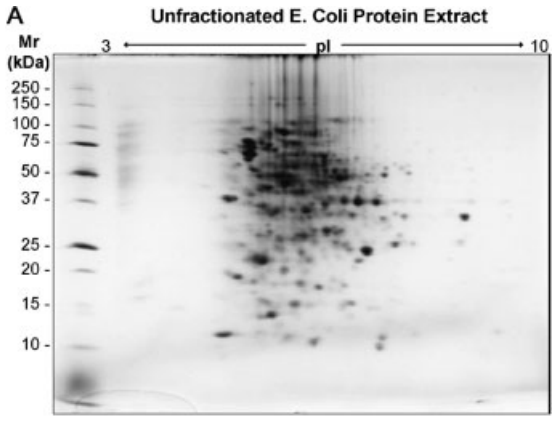
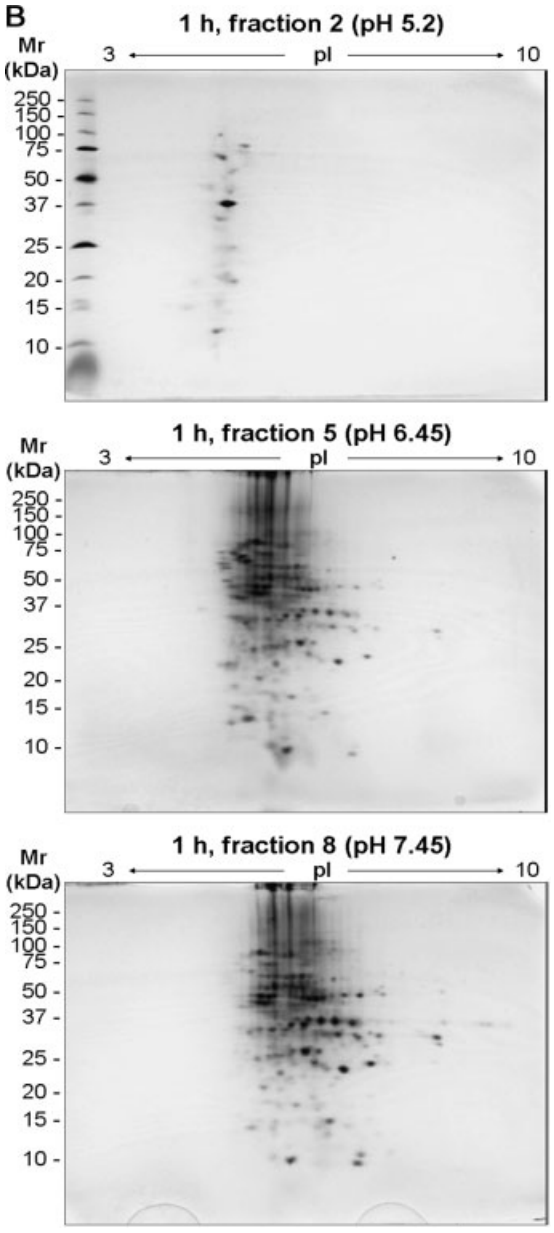
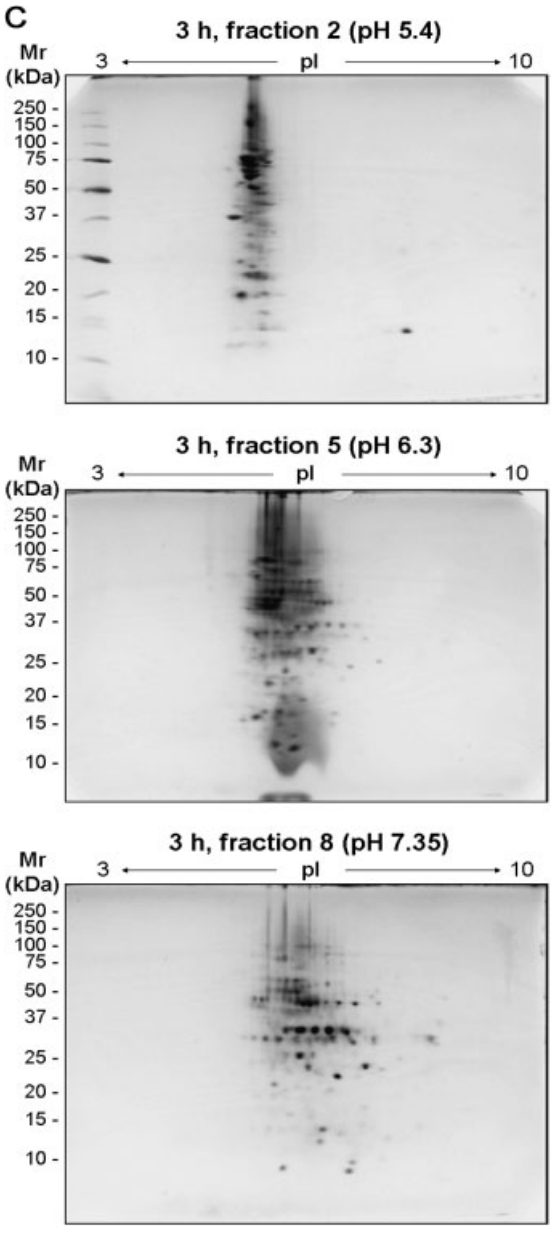


Figure 6. Time course of a fractionation of a total *E. coli* lysate in the present instrument on a 2.5–8.0 pH gradient (3% Pharmalyte 2.5–8.0). (A) 2-D map of a control, unfractionated lysate. (B) 2-D maps of the content of three chambers (2, 5 and 8) after 1 h of focusing. (C) 2-D maps of the content of three chambers (2, 5 and 8) after 3 h of focusing.





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tem, we do not experience any electrodecantation of proteins at (or in proximity of) their p*I* value, possibly because, since most proteome fractionation and analysis protocols call for a strongly denaturing mixture of urea and thiourea, the density of such solutions would prevent protein sedimentation in a free-liquid phase.

Another way of performing prefractionation for proteome analysis is the well-known "Radola technique" [31], already described in the early 1970s, consisting in focusing on a horizontal trough filled with Sephadex beads. This method has been recently re-introduced by Görg *et al.* [32]. However, this last approach again exploits a gel phase, which means scooping up segments of the Sephadex bed between anode and cathode and eluting the isoelectric fractions for further analysis.

Perhaps one of the major drawbacks of IEF in CA buffers is that the fractionation of alkaline proteins is not quite so good. This is not due to the short focusing times of 1 h (see Figs. 5 and 6B), since experiments run for longer times (see Fig. 6C) still show poor focusing in the alkaline region. In fact, while in the acidic region longer prefractionation times seem to produce slightly better p*I* cuts, as it can be appreciated in fraction 3 (Fig. 6C), which presents a slightly better resolution and a considerable protein enrichment with respect to the same fraction displayed in Fig. 6B, such an amelioration cannot be observed in the alkaline interval (see fraction 8, Figs. 6B and C, bottom panels). This could possibly be due to the onset of electroendoosmotic flow, an ever-present hazard in all IEF experiments in the presence of soluble CA buffers. Our group recently identified the major problem: essentially all the commercial brands of CAs, in the alkaline region, contain a majority of "poor" species, *i.e.* of CAs displaying rather large (p*I*–p*K*) values, thus unable to focus and properly buffer along the pH gradient [33]. Thus, an improvement in the synthesis of alkaline CA buffers is sorely needed.

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