Journal of Chromatography B, 871 (2008) 125–129

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

High reproducibility of two-dimensional liquid chromatography using pH-driven fractionation with a pressure-resistant electrode

Elsa Suberbielle [a,b], Daniel Gonzalez-Dunia [a,b], Frédéric Pont [c,*]

[a] INSERM, U563, Toulouse, France
[b] Université Paul-Sabatier, Toulouse, France
[c] INSERM, Plateau technique Interactions et Profils d'expression Protéiques, Institut Claude de Préval (IFR30), Toulouse, France

ARTICLE INFO

Article history:
Received 3 April 2008
Accepted 23 June 2008
Available online 1 July 2008

Keywords:
Two-dimensional liquid chromatography
HPLC
Proteomics
Reproducibility
Peak concordance
Chromatogram comparison
PF2D
Chromatofocusing
Reverse phase high-performance liquid chromatography
Non-porous silica

ABSTRACT

Automated two-dimensional liquid chromatography using the PF2D system from Beckman Coulter provides a fractionation platform well suited for differential proteomic studies. To date, the reliability and reproducibility of PF2D has not been accurately tested. Here, we used an optimized software and a pressure-resistant pH electrode, allowing a precise and reproducible control of the pH limits for each fraction during PF2D. We tested the reliability of this improved system by performing several rounds of fractionation using the same protein extract. Three UV maps were generated, leading to 54 chromatograms and more than 3000 protein peaks. Using semi-automated software for peak-to-peak comparison between 2D-LC chromatograms, we demonstrate that the peak concordance is very high. The rates of concordance were higher in the second dimension repeatability tests, indicating that the limiting factors for 2D-LC reproducibility rely on the p*I* fractionation and sample preparation steps. The reproducibility between maps was closely related to pH curves similarities, further stressing the need of careful pH adjustment and precise electrode calibration.

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1. Introduction

One of the main challenges in proteomics relies on the ability to generate a reproducible fractionation of the protein samples. Indeed, the discovery of novel biomarkers, together with studies of disease pathogenesis, such as for cancer, rely on differential proteomics, i.e., on the accurate comparison between control and pathological situations [1]. To date, protein fractionation still represents a limiting step in a proteomic study [2] and no single fractionation strategy has demonstrated the capacity to cover the whole proteome. In addition to 2-DE, alternative fractionation methods are available, including automated liquid-based 2D liquid chromatographic (2D-LC) systems [3], such as that provided by the Proteome-Lab™ PF2D from Beckman Coulter. The PF2D separates proteins in the first dimension according to their p*I* using chromatofocusing, followed by a fractionation according to hydrophobicity, using reversed phase chromatography in the second dimension. Several recent proteomic studies have shown that the different fractionation technologies are highly complementary, with a remarkably low number of common proteins identified in all cases [4,5]. In addition, it seems clear that 2D-LC allows the identification of a large set of proteins and is adapted for novel protein discovery [4,5].

The PF2D system is relatively new and the reliability and reproducibility of this fractionation procedure has not been formally assessed or quantified accurately. It was shown that PF2D could generate relatively reproducible UV profiles, based on the visual examination of the chromatograms [6–8]. However, a certain level of variability was due to the dynamic nature of the p*I* fractionation in the first dimension fractionation. As a result, the pH limits of the fractionation gradient could not be fixed, leading sometimes to shifts in fractionation. In this work, we present a systematic assessment of the reproducibility of PF2D fractionation, using an improved methodology for the first dimension fractionation. We

Abbreviations: 2D-LC, two-dimensional liquid chromatography; 2-DE, two-dimensional gel electrophoresis; HPLC, high-performance liquid chromatography; MS, mass spectrometry; HPCF, high-performance chromatofocusing; RPHPLC, reverse phase high-performance liquid chromatography; NPS, non-porous silica; CAN, acetonitrile; TFA, tri-fluoro-acetic acid.

* Corresponding author at: Plateau technique Interactions et Profils d'expression Protéiques, Bât B Pavillon Lefèbvre, CHU Purpan, BP 3028, 31024 Toulouse Cedex 3, France. Tel.: +33 5 6274 8398; fax: +33 5 6274 8384.
E-mail address: frederic.pont@inserm.fr (F. Pont).

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doi:10.1016/j.jchromb.2008.06.043

used a software patch, ensuring that all fractionation experiments start at the exact same pH value. In addition, we used a prototype pH electrode allowing the accurate pH measurement under pressure. We tested the reliability of this improved system by performing several rounds of PF2D fractionation using aliquots of the same protein extract. We used a semi-automated software tool for peak-to-peak comparison between 2D-LC fractionation experiments, facilitating the systematic analysis of more than 3000 peaks. We provide herein the first formal demonstration of the high level of reproducibility of PF2D fractionation, which underscores the interest of this fractionation method for studies of differential proteomics.

2. Experimental

2.1. Sample preparation

Vero E6 cells (ATCC CRL-1586) were grown in DMEM (Invitrogen, Cergy Pontoise, France), supplemented with 10% heat-inactivated fetal calf serum (PAA laboratories, Les Mureaux, France) and 1× Bufferall (Sigma–Aldrich, Lyon, France). Cells (140×10^6) were harvested by scraping, washed once in phosphate buffered saline (Invitrogen) and recovered by centrifugation for 5 min at $430 \times g$ at room temperature. Cell pellets were resuspended in lysis buffer prepared according to the ProteomeLab™ PF2D procedure (ProteomeLab™ PF2D, Beckman Coulter, Fullerton, CA, USA) containing a cocktail of protease inhibitors (Sigma–Aldrich) and lysis was performed according to the ProteomeLab™ PF2D human cell lysis protocol. Protein concentration was determined using the 2D-quant protein assay (Amersham, Les Ulis, France). Aliquots of cell extracts were stored at −80 °C until use.

2.2. Liquid chromatography

Prior to chromatofocusing, an aliquot of the cell extract was thawed, desalted on a PD-10 Sephadex™ G-25 gel filtration column with a 5 kDa cut-off (Amersham) and eluted using the chromatofocusing Start Buffer. 2D-LC was performed using the ProteomeLab™ PF2D Protein Fractionation System (Beckman Coulter), which consists of two HPLCs, two UV detectors, an auto-sampler and a fraction collector. The first dimension fractionation of PF2D consists in chromatofocusing, based on charge. After collection of the fractions from the first dimension in the collector module, each of them is automatically introduced into the second dimension reversed phase chromatography column, which separates proteins based on their hydrophobicity.

Chromatofocusing was performed on an HPCF 1-D column (250 mm × 2.1 mm, Beckman Coulter). This first dimension HPLC module was equipped with a 5 ml sample loop. The signal was recorded at 280 nm. The pH gradient was generated using Start Buffer (pH 8.5) and Eluent Buffer (pH 4), both included in the ProteomeLab™ PF2D kit. The chromatofocusing column was first equilibrated for 130 min with Start Buffer at pH 8.5 at a flow rate of 0.2 ml/min, before being loaded with 1.5 mg of the desalted protein extract. The flow-through was collected and after a stable baseline was established (35 min), a linear pH gradient was initiated by infusing the Eluent Buffer for 95 min with a constant flow rate of 0.2 ml/min. The proteins with a p*I* < 4 were finally eluted by washing the column with 1 M NaCl. A software patch (SP1 Beckman Coulter) was used to ensure that each fraction collection experiment would start at a pH value of 8.3. Fraction collection from the first dimension was controlled with an in-line pH meter equipped with a prototypic pressure-resistant pH electrode (replacement kit p/n A48657). Fractions were collected every 10 min, except during the pH gradient portion of the run, from pH 8.5–4.0, when fractions were collected at 0.3 pH unit-intervals.

The second dimension analysis used a non-porous RPHPLC using a C18 column (4.6 mm × 33 mm, Beckman Coulter) packed with 1.5 μm non-porous silica and kept at 50 °C in a heated column jacket. Eighteen fractions from the first dimension were injected in the NPS-C18 column and eluted using a water/acetonitrile gradient at 0.75 ml/min. The injection volume was between 50 and 500 μl depending on the protein concentration in the first dimension fraction. Solvent A was 0.1% TFA in water and solvent B was 0.08% TFA in ACN. The gradient consisted in 100% solvent A for 2 min, 0–100% solvent B for 30 min and 100% solvent B for 4 min. The UV signal was recorded a 214 nm.

2.3. Analysis of chromatograms

Chromatograms were integrated using the 32Karat software (Beckman Coulter). Data containing the surfaces and retention times of the peaks were exported as a text file and a peak-to-peak analysis was performed using the 4.020 version of the GC–LC-concordance software (Spectrochrom, Bouc Bel Air, France. http://www.spectrochrom.com/). Briefly, this software converts the chromatograms into histograms and performs pairwise comparisons (Fig. 1). The software automatically finds the best polynomial equation to model the peak positions using the retention time tolerance and the equation order specified by the operator. The retention time tolerance was set to 5% and the surface of the peaks analyzed was set between 0.1% and 100% of the largest peak area. The percentage of concordance between the chromatogram A and B was calculated as follows:

% concordance = (number of peaks in chromatogram A concordant with chromatogram B × 100)/(total number of peaks in chromatogram B).

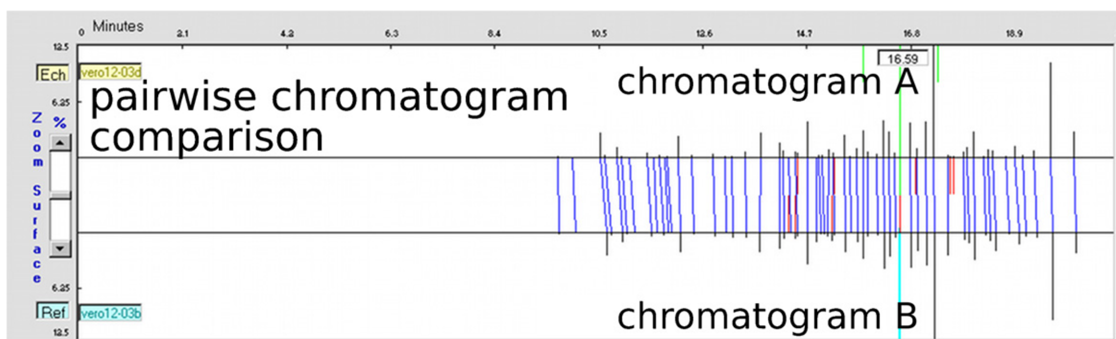

Fig. 1. Comparative chromatogram analysis using the GC–LC-concordance software. Screenshot of one analysis. The chromatograms were integrated and converted into histograms, with bar lengths being proportional to the original peak surface. Two mirroring histograms are represented in black. Concording peaks are linked together by blue lines, while non-concording peaks are tagged with a red line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In addition to the peak-to-peak analysis using the GC–LC-concordance software, each concordant peak was visually examined and validated. When required, some peaks were manually integrated, in particular when a shoulder was not correctly detected by the automated analysis.

3. Results and discussion

3.1. Control of sample homogeneity

The procedure used for sample preparation is an important parameter that can drastically affect reproducibility and is particularly important during the design of a differential proteomic study. For our repetitive 2D-LC maps, we prepared aliquots of the same cellular extract that were stored in the lysis buffer at −80 °C, to limit the risks of protein degradation. The simultaneous preparation of the cellular extracts for all repetitive PF2D rounds allowed us to ensure that any residual proteolysis occurring during the lysis procedure would be similar for all experiments, thereby limiting the addition of another variable in the analysis. However, the last steps of desalting/gel filtration of the sample were performed prior to each fractionation experiment, to match the conditions of a differential proteomic study, in which these steps are mandatory and could be responsible for a decrease in reproducibility.

3.2. Assessment of the repeatability of chromatofocusing fractionation

One of the limitations of the chromatofocusing step in the PF2D system is the difficulty to precisely control the pH gradient from one run to another. Although the fraction collector is triggered by an online pH meter, there is still a significant level of variability, due to the difficulty to measure accurately a pH value under pressure. Indeed, the pH electrode has been designed to accurately detect pH changes, but not necessarily to record precise pH values. As a result, the repeatability of this fractionation step is completely dependent on the repeatability of the pH gradients. In addition, the pH limits of the fractions are not similar between maps because the pH limits of the fractions are not fixed before the run. In this work, we have implemented two significant improvements to the PF2D chromatofusing step. First, we used a novel prototypic electrode (p/n A48657) that has been designed not only to accurately measure changes in pH, but also to give accurate pH measurements under pressure. With this new electrode, the pH value under pressure was 8.51 ± 0.01 at the beginning of the gradient for all three experiments (Fig. 2). This pH value measured at a calibrated bench pH meter was 8.55, indicating that the accuracy of the measurements under pressure is in the range of 0.04 pH units. Second, we used a software patch, which enables the fractions to start precisely at pH 8.30 for each experiment. Consequently, the pH limits for each fraction are exactly the same between experiments (Fig. 2), provided that the collection volume is not limiting. As a matter of fact, we increased the collecting time to 10 min, because we observed that the volume could be limiting for some fractions of the gradient with the usually recommended collecting time of 8.5 min. Indeed, reproducibility was improved by 3.4% when a collecting time of 10 min was used instead of 8.5 min.

3.3. Analysis of the repeatability of the second dimension fractionation

To test for the second dimension repeatability, the same series of first dimension fractions were used several times for a second dimension fractionation. The use of a C18 NPS column in association to a UV detector at 214 nm enables the detection of proteins in the nanogram up to the microgram range [9]. For this reason the chromatograms were analyzed with a dynamic range of 10^3.

When testing the reproducibility of a simple chromatogram containing about 30 peaks, the concordance between chromatograms was 100% (Fig. 3A).

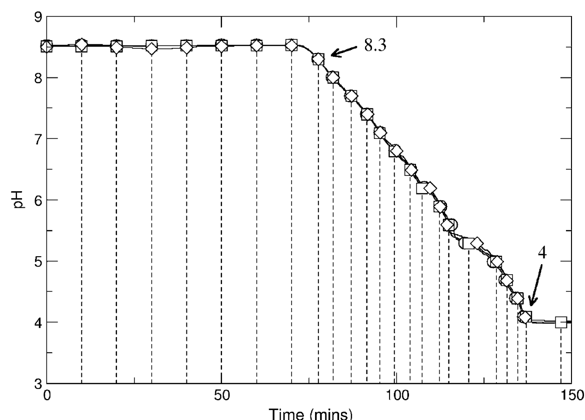

Fig. 2. Reproducibility of the pH gradients using the optimized PF2D system. pH gradients from experiments #1 (circles), #2 (squares), #3 (diamonds) generated during the chromatofocusing step. Vertical dotted lines represent the fractions limits, which start at pH 8.3 and end at pH 4.0. The software patch SP1 enables fractions from each experiment to have exactly the same pH limits.

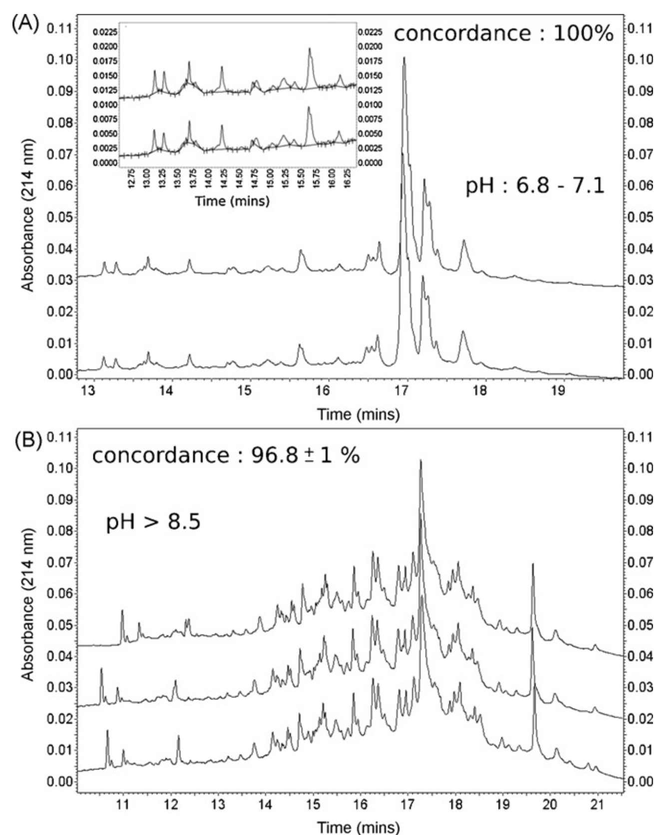

Fig. 3. Representative examples of the repeatability of 2D-LC second dimension fractionation. Top: protein UV (214 nm) profiles obtained after two injections of the same first dimension fraction (pH 6.8–7.1) on a C18 NPS column. Inset: detail of the peak integration performed by the 32karat software. The resulting % of peak concordance is indicated on the figure. Bottom: protein UV (214 nm) profiles obtained after three injections of the same first dimension fraction (pH > 8.5) on a C18 NPS column. The % of peak concordance is indicated on the figure.

Table 1
Analysis of peak concordance between 2D maps (%)

Peak concordance	Map #2	Map #3
Map #1		
% concordance	91.59 ± 3.99	89.24 ± 3.16
# of concording peaks	1062	1067
Map #3		
% concordance	89.76 ± 5.24	NA[a]
# of concording peaks	1074	NA[a]

Results are expressed as means ± S.D. (n = 18).
[a] Not applicable.

The most complex chromatogram of a 2D-LC experiment is generally obtained with the fraction of basic proteins (pI > 8.5) not retained by the column. We injected this fraction in triplicate onto the NPS column and we obtained chromatograms of about 80 peaks (Fig. 3B). With these complex chromatograms, the concordance between chromatograms was 96.76 ± 0.98% (n = 3).

The lower concordance rate for complex chromatograms may be due to different factors. First, it is important to note that most of the peaks are not well resolved in these complex chromatograms. Indeed, MS analysis of 2D-LC peaks has shown that most of the peaks are in fact composed of a mixture of proteins. A large peak can contain 10, 20 or even more proteins. Therefore, the peak integration of such chromatograms is a critical step, since a small change in a shoulder slope can be responsible for a different integration of the same peak between two chromatograms. As a result, the same peak will be split into two peaks in one case and not in the other, creating a mismatch immediately detected during the software processing of the chromatograms. Second, this decreased concordance rate may be the result of local deformations of the chromatograms, due to slight variations in the ACN gradient between experiments. An example of these local deformations is shown in Fig. 3B, where we observed that the first peaks of the top chromatogram had a slightly higher retention time than the two other chromatograms. While not globally affecting the rest of the chromatogram, this nevertheless impacted the resolution of peaks located at around 12 min (Fig. 3B).

In any event, the concordance rates remained remarkably high, and our results indicate that the second dimension is repeatable enough to detect small changes in peak intensities.

3.4. Analysis of the 2D proteins maps

Three 2D maps were obtained after subjecting aliquots of the same protein extract to the whole PF2D fractionation procedure, including sample preparation. A visual inspection of the maps revealed good similarities and the differential maps displayed peaks of small intensities, indicating a good reproducibility of the experiments (data not shown).

A peak-to-peak analysis of all chromatograms was performed, leading to an accurate measurement of the reproducibility. Results of the map-to-map reproducibility are displayed in Table 1.

When performing the overall analysis of the reproducibility between all three experiments, we concluded that the mean concordance was 90.19 ± 4.26% (n = 54). It should be stressed that these values reflect faithfully the reproducibility of the whole fractionation procedure, including not only the two HPLC steps of 2D-LC but also the last steps of sample preparation, in particular the gel filtration step, and the potential impact of sample storage.

In order to explain the slight variations in reproducibility observed between the three performed experiments, we calculated the residual sum of squares (RSS) for each couple of curves of the pH gradients. The RSS is an evaluation of the distance between two pH curves and we found that the RSS (#1,#2) = 6.7, the RSS (#2,#3) = 8.19 and the RSS (#1,#3) = 13.62. Therefore, it appears that the most reproducible maps have also the most reproducible pH curves, further stressing the importance of this initial fractionation step and the interest of the improvements tested herein. In any event, it remains clear that the reproducibility of a fractionation experiment using PF2D depends on several, non-exclusive parameters: the reproducibility of the sample preparation and the effect of sample storage, the reproducibility of the pH curves during the fractionation and the reproducibility of the second dimension gradient. Our results indicate that the reliability of fractionation using PF2D is well suited to perform an accurate differential proteomic study. It should nevertheless be kept in mind that this two-dimensional fractionation method will not permit the complete fractionation of all the proteins from a global cell extract and that the UV intensity of a peak reflects the signal of a mixture of proteins. However, the high level of reproducibility of the 2D maps, as demonstrated in this study, enables a quick identification of the fractions in which an abundant protein is responsible for a UV change. In addition, when a subproteome is analyzed by 2D-LC, the resulting peaks are sharper and less coelution is observed [10]. In most cases, a subsequent quantification strategy by MS will nevertheless be required to evaluate the amount of each protein individually, either at the level of the intact protein [11] or at the peptide level after tryptic digestion [12].

4. Conclusions

Here, we provide the first comprehensive assessment of protein fractionation using PF2D, by performing a systematic peak-to-peak measurement of 2D-LC reproducibility. We took advantage of recent hardware and software improvements in the chromatofocusing step of 2D-LC, enabling a fractionation based on fixed pH values. Our results demonstrate the high level of reproducibility of the PF2D system, indicating its suitability for differential proteomic studies.

Acknowledgements

This work was supported by grants from INSERM, from Région Midi-Pyrénées, from the Genopôle and Canceropôle Grand-Sud-Ouest and from an ANR-06-MIME-005-01 grant to DGD. ES is a recipient of a “poste d’accueil” from INSERM. DGD is supported by the CNRS.

The authors would like to acknowledge Beckman Coulter and especially Jerald S. Feitelson, Michael Simonian and Jeff Chapman for their support and for providing the prototype electrode and software patches. Raymond Loyer, from Spectrochrom, is acknowledged for providing the GC–LC-concordance software and for his useful collaboration in the tuning of the mathematical parameters.

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