

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

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15 Running title: two-dimensional liquid chromatography reproducibility in proteomics

The abbreviations used are: 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; ACN, acetonitrile; TFA, tri-fluoro-acetic acid.

Abstract

Two-dimensional liquid chromatography using the PF2D system from Beckman Coulter is increasingly used in proteomics to provide an automated fractionation platform and to circumvent some limitations of fractionation using 2-D electrophoresis. To date, the reliability and reproducibility of the PF2D fractionation procedure has not been formally 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 fractions. We tested the reliability of this improved system by performing several rounds of PF2D using aliquots of the same protein extract. Three UV maps were generated, leading to 54 chromatograms and more than 3000 protein peaks. We used a semi-automated software tool for peak-to-peak comparison between 2D-LC fractionation experiments to provide an accurate measurement of the reproducibility. The mean peak concordance was very high. The rates of concordance were higher in the second dimension repeatability tests, indicating that the limiting factors in 2D-LC reproducibility rely in the pI 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. Combined with good laboratory practice, 2D-LC using the PF2D system is a reproducible fractionation platform well suited for differential proteomics.

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Introduction

One of the main challenges in proteomics centers 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). Although the resolution and speed of mass spectrometers have been significantly improved these recent years, protein fractionation still represents a limiting step in a proteomic study. Indeed, it has been shown that the improvement of the fractionation step significantly increases the number of proteins that can be subsequently analyzed by a mass spectrometer, thereby resulting in a dramatic increase of the dynamic range of an analysis (2). To date, there is no single fractionation strategy that has demonstrated the capacity to cover the whole proteome, but several complementary approaches are now available. Protein fractionation has been traditionally performed using 2-DE. However, this fractionation method exhibits several limitations. It often restricts the analysis to the most abundant proteins, has demonstrated a relative lack of reproducibility and has trouble resolving proteins of extreme hydrophobicity, mass or isoelectric point (pI) (3, 4). To circumvent these limitations and to provide an alternative to 2-DE, liquid-based

65 2D liquid chromatographic (2D-LC) systems have been developed (5). The recent
availability of an automated system based on 2D-LC, the Proteome-LabTM PF2D from
Beckman Coulter, facilitates the effective implementation of a 2D-LC experiment. The
PF2D system separates proteins in the first dimension according to their pI using
chromatofocusing, followed by a fractionation according to hydrophobicity, using
70 reversed phase chromatography in the second dimension. The pI-based
fractionation, as performed during the first dimension of the PF2D (and in 2-DE), has
been shown to be especially useful to study proteins post-translational modifications,
such as phosphorylation (6). Several recent proteomic studies comparing the
different available fractionation strategies have shown that the different technologies
75 are highly complementary, with a remarkably low number of common proteins
identified by all technologies (7, 8). However, it seems clear that 2D-LC allows the
identification of more proteins than 2D-gels and may be more suited for novel protein
discovery (7, 8).

Automated 2D-LC fractionation, such as that provided by the PF2D system, is
80 relatively new and the reliability and reproducibility of this fractionation procedure
remains to be formally assessed and quantified accurately. It was shown that PF2D
could generate relatively reproducible UV profiles, based on the visual examination of
the chromatograms (9-11). However, a certain level of variability was due to the

dynamic nature of the pI fractionation in the first dimension fractionation. As a result,

85 the pH limits of the fractionation gradient could not be fixed, leading sometimes to small 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 used a software patch, ensuring that all fractionation experiments would start at the exact same pH value. In addition, we used a prototype
90 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
95 demonstration of the high level of reproducibility of PF2D fractionation, which underscores the interest of this fractionation method for studies of differential proteomics.

100 **Experimental**

Cell cultures - Vero E6 cells (ATCC CRL-1586) were grown to confluence in DMEM (Gibco-Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (PAA) and 1X Bufferall (Sigma-Aldrich). Cells (140×10^6) were harvested by scraping, washed once in Phosphate Buffered Saline (Invitrogen) and recovered by

105 centrifugation for 5 min. at 430 x g at room temperature. Cell pellets were then resuspended in lysis buffer (ProteomeLabTM PF2D, Beckman) containing a cocktail of protease inhibitors (Sigma-Aldrich) and lysis was performed according to the ProteomeLabTM PF2D human cell lysis protocol. Protein concentration was determined using the 2D quant protein assay (Amersham, Biosciences, Piscataway,

110 NJ, USA). Aliquots of cell extracts were stored at -80°C until ready for use.

Liquid chromatography - Prior to chromatofocusing, an aliquot of the cell extract was thawed, desalted on a PD-10 SephadexTM G-25 gel filtration column with a 5 kDa cut-off (Amersham Biosciences, Piscataway, NJ, USA) and eluted using the chromatofocusing Start Buffer. 1.5 mg of protein extract was used in each 2D-LC

115 experiment. 2D-LC was performed using the ProteomeLabTM PF2D Protein Fractionation System (Beckman Coulter, Fullerton, CA, USA), 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. The fractions can be finally collected into 96-deep-well plates.

Chromatofocusing was performed on an HPCF 1-D column (250 x 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 Elution Buffer (pH 4), both included in the ProteomeLabTM PF2D kit. The chromatofocusing column was first equilibrated for 130 minutes 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 minutes), a linear pH gradient was initiated by infusing the elution buffer for 95 minutes with a constant flow rate of 0.2 ml/min. The proteins with a pI < 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 to 4.0, when fractions were collected at 0.3 pH unit-intervals.

The second dimension analysis used a nonporous RPHPLC using a C18 column (4.6 x 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 minutes, 0-100% solvent B for 30 min and 100% solvent B for 4 minutes. The UV signal was recorded at 214 nm.

Chromatogram analysis - Chromatograms were integrated using the 32Karat software (Beckman Coulter, Fullerton, CA, USA). Data containing the 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 pair-wise comparisons. The software automatically finds the best quadratic equation to model

155 the peak positions using the retention time tolerance and the equation order specified
by the operator. A surface ratio between concordant peaks can also be calculated
and used as a constraint to exclude peaks. 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
160 B was calculated as follows:

$$\% \text{ concordance} = (\text{number of peaks in chromatogram A concordant with} \\ \text{chromatogram B} \times 100) / (\text{total number of peaks in chromatogram B})$$

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
165 peaks were manually integrated, in particular when a shoulder was not correctly
detected by the automated analysis.

Results and discussion

170 *Sample preparation:* 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
175 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
180 which these steps are mandatory and could be responsible for a decrease in
reproducibility.

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
185 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 detect accurately 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 exactly similar
190 between maps, which further complicates the analysis. 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 $8.51 \pm$
195 0.01 at the beginning of the gradient for all three experiments (Figure 1). 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
200 the same between experiments (Figure 1), provided that the collection volume is not limiting. As a matter of fact, we increased the collecting time to 10 minutes, 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 when comparing a collecting time of 10 minutes and 8.5 minutes (data not

205 shown).

Chromatogram concordance analysis: A total of 54 chromatograms were generated, representing more than 3000 peaks. In order to assess accurately the reproducibility of the chromatograms with such a large amount of data, we performed a peak-to-peak analysis using a modified version of the GC-LC-concordance software. This software enables the comparison of two histograms obtained after peak integration of the chromatograms (Figure 2). The software uses a mathematical model and the retention time error to determine the theoretical position of a peak (see methods).

Second dimension repeatability: the repeatability of the second dimension is an important parameter to test because it gives the theoretical limit of the repeatability of the 2D-LC experiment. 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 (13). For this reason the chromatograms were analyzed with a dynamic range of 10^3 .

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

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 (Figure 3B). With these complex chromatograms, the peak concordance between chromatograms was $96.76 \pm 0.98 \%$ ($n = 3$).

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 on Fig 3B, where we observed that the first peaks of the top chromatogram had a slightly higher retention time than the 2 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
245 peak intensities.

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. The maps were obtained with a starting protein amount of 1.5 mg. In some fractions, the protein amount was barely sufficient to obtain a good UV signal,
250 stressing the necessity of using at least 1.5 mg of protein to obtain a satisfactory map when working with a whole proteome. 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
255 measurement of the reproducibility. Results of the map-to-map reproducibility are displayed in Table I.

When performing the overall analysis of the reproducibility between all three experiments, we concluded that the mean peak concordance was 90.19 ± 4.26 % (n=54). It should be stressed that these values reflect faithfully the reproducibility of the

260 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
265 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
270 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
275 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
280 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 (14). 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 (15) or at the peptide
285 level after tryptic digestion (12).

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
290 the chromatofocusing step of 2D-LC, in particular the use of a new pH electrode
giving accurate measurements under pressure combined with a software modification
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.

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REFERENCES

- 310 1. Monteoliva, L. and Albar, J.P. (2004) Differential proteomics: an overview of gel and non-gel based approaches. *Brief Funct Genomic Proteomic* **3**, 220-239
2. Sheng, S., Chen, D. and Van Eyk, J.E. (2006) Multidimensional liquid chromatography separation of intact proteins by chromatographic focusing and reversed phase of the human serum proteome: optimization and protein database. *Mol. Cell Proteomics* **5**, 26-34
- 315 3. Issaq, H.J. and Veenstra, T.D. (2007) The role of electrophoresis in disease biomarker discovery. *Electrophoresis* **28**, 1980-1988
4. Skalnikova, H., Halada, P., Dzubak, P., Hajduch, M. and Kovarova, H. (2005) Protein fingerprints of anti-cancer effects of cyclin-dependent kinase inhibition: identification of candidate biomarkers using 2-d liquid phase separation coupled to mass spectrometry. *Technol. Cancer Res. Treat.* **4**, 447-454
- 320 5. Lubman, D.M., Kachman, M.T., Wang, H., Gong, S., Yan, F., Hamler, R.L., O'Neil, K.A., Zhu, K., Buchanan, N.S. and Barder, T.J. (2002) Two-dimensional liquid separations-mass mapping of proteins from human cancer cell lysates. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **782**, 183-196
- 325 6. Zhu, K., Zhao, J., Lubman, D.M., Miller, F.R. and Barder, T.J. (2005) Protein pI shifts due to posttranslational modifications in the separation and characterization of proteins. *Anal. Chem.* **77**, 2745-2755
7. McDonald, T., Sheng, S., Stanley, B., Chen, D., Ko, Y., Cole, R.N., Pedersen,

- 330 P. and Van Eyk, J.E. (2006) Expanding the subproteome of the inner mitochondria
using protein separation technologies: one- and two-dimensional liquid
chromatography and two-dimensional gel electrophoresis. *Mol. Cell Proteomics* **5**,
2392-2411
8. Li, X., Gong, Y., Wang, Y., Wu, S., Cai, Y., He, P., Lu, Z., Ying, W., Zhang, Y.,
335 Jiao, L., He, H., Zhang, Z., He, F., Zhao, X. and Qian, X. (2005) Comparison of
alternative analytical techniques for the characterisation of the human serum
proteome in hupo plasma proteome project. *Proteomics* **5**, 3423-3441
9. Park, K.H., Lipuma, J.J. and Lubman, D.M. (2007) Comparative proteomic
analysis of b. cenocepacia using two-dimensional liquid separations coupled with
340 mass spectrometry. *Anal. Chim. Acta* **592**, 91-100
10. Soldi, M., Sarto, C., Valsecchi, C., Magni, F., Proserpio, V., Ticozzi, D. and
Mocarelli, P. (2005) Proteome profile of human urine with two-dimensional liquid
phase fractionation. *Proteomics* **5**, 2641-2647
11. Yan, F., Subramanian, B., Nakeff, A., Barder, T.J., Parus, S.J. and Lubman,
345 D.M. (2003) A comparison of drug-treated and untreated hct-116 human colon
adenocarcinoma cells using a 2-d liquid separation mapping method based upon
chromatofocusing pl fractionation. *Anal. Chem.* **75**, 2299-2308
12. Old, W.M., Meyer-Arendt, K., Aveline-Wolf, L., Pierce, K.G., Mendoza, A.,
Sevinsky, J.R., Resing, K.A. and Ahn, N.G. (2005) Comparison of label-free methods
350 for quantifying human proteins by shotgun proteomics. *Mol. Cell Proteomics* **4**,
1487-1502
13. Daully, C., Perlman, D.H., Costello, C.E. and McComb, M.E. (2006) Protein
separation and characterization by np-rp-hplc followed by intact maldi-tof mass

spectrometry and peptide mass mapping analyses. *J. Proteome Res.* **5**, 1688-1700

- 355 14. Cussac, D., Pichereaux, C., Colomba, A., Capilla, F., Pont, F., Gaits-Iacovoni, F., Lamant, L., Espinos, E., Burlet-Schiltz, O., Monsarrat, B., Delsol, G. and Payrastre, B. (2006) Proteomic analysis of anaplastic lymphoma cell lines: identification of potential tumour markers. *Proteomics* **6**, 3210-3222
15. Zhao, J., Chang, A.C., Li, C., Shedden, K.A., Thomas, D.G., Misek, D.E.,
360 Giordano, T.J., Beer, D.G. and Lubman, D.M. (2007) Comparative proteomic analysis of barrett's metaplasia and esophageal adenocarcinomas using 2-d liquid mass mapping. *Mol. Cell Proteomics* **6**, 987-999

Figure Legends

365 **Figure 1. 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. The gradients were generated inside the chromatofocusing column by injecting an eluent buffer at pH 4.0 in the column, previously equilibrated with the start buffer at pH 8.5. Vertical dotted lines represent

370 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.

Figure 2. Comparative chromatogram analysis using the GC-LC-concordance

software. Screenshot of one analysis output. The chromatograms were integrated

375 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.

380 **Figure 3. Representative examples of the repeatability of 2D-LC fractionation.**

Top: protein UV (214nm) 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 for the analysis. The inset table shows the results of peak concordance.

385 Bottom: protein UV (214nm) profiles obtained after three injections of the same first dimension fraction (pH>8.5) on a C18 NPS column. The inset table shows the results of peak concordance.

Tables

TABLE 1: Mean peak concordance between 2D maps (%)

	Peak concordance	Map #2	Map #3
Map #1	w/o	91.59 ± 3.99	89.24 ± 3.16
	# of concording peaks	1062	1067
Map #3	w/o	89.76 ± 5.24	NA ¹
	# of concording peaks	1074	NA ¹

390 Results are expressed as mean ± SD (n = 18). ¹ not applicable

Figures

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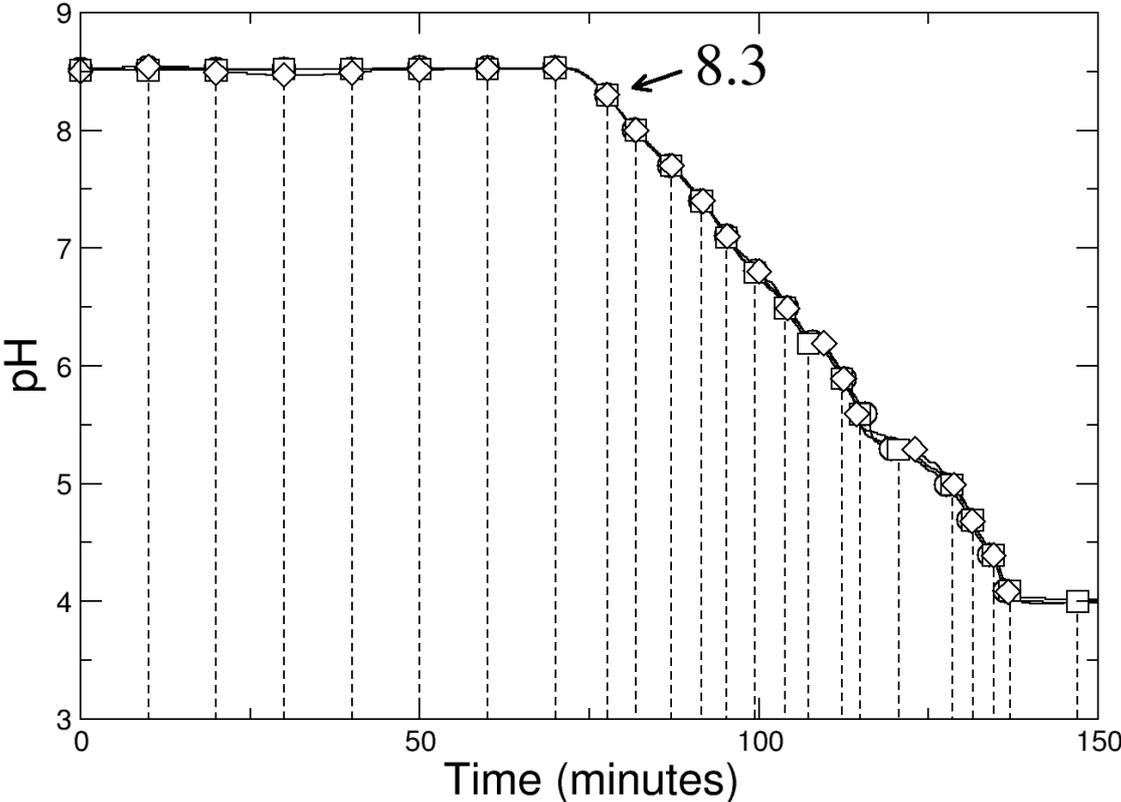


Figure 1.

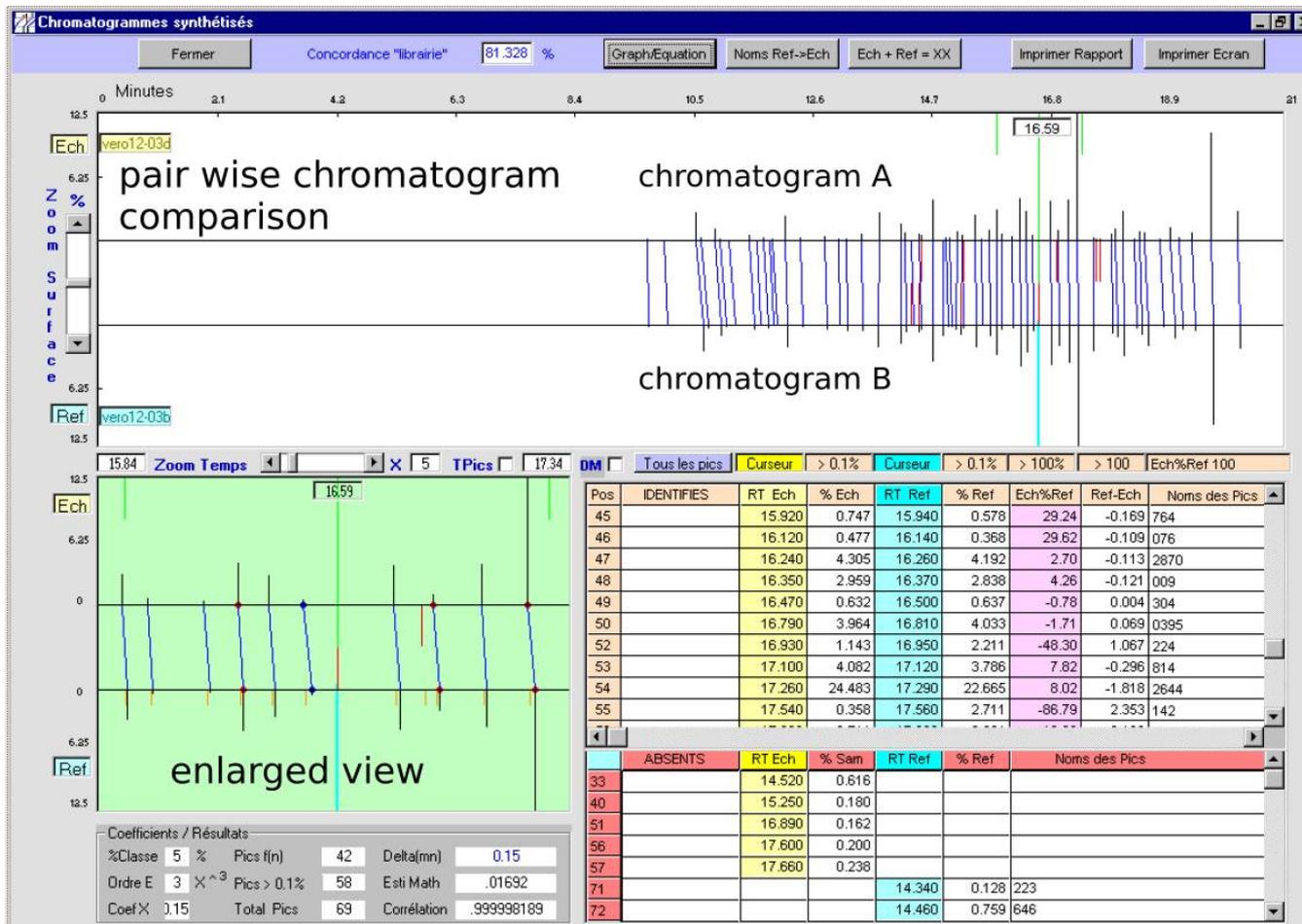


Figure 2.

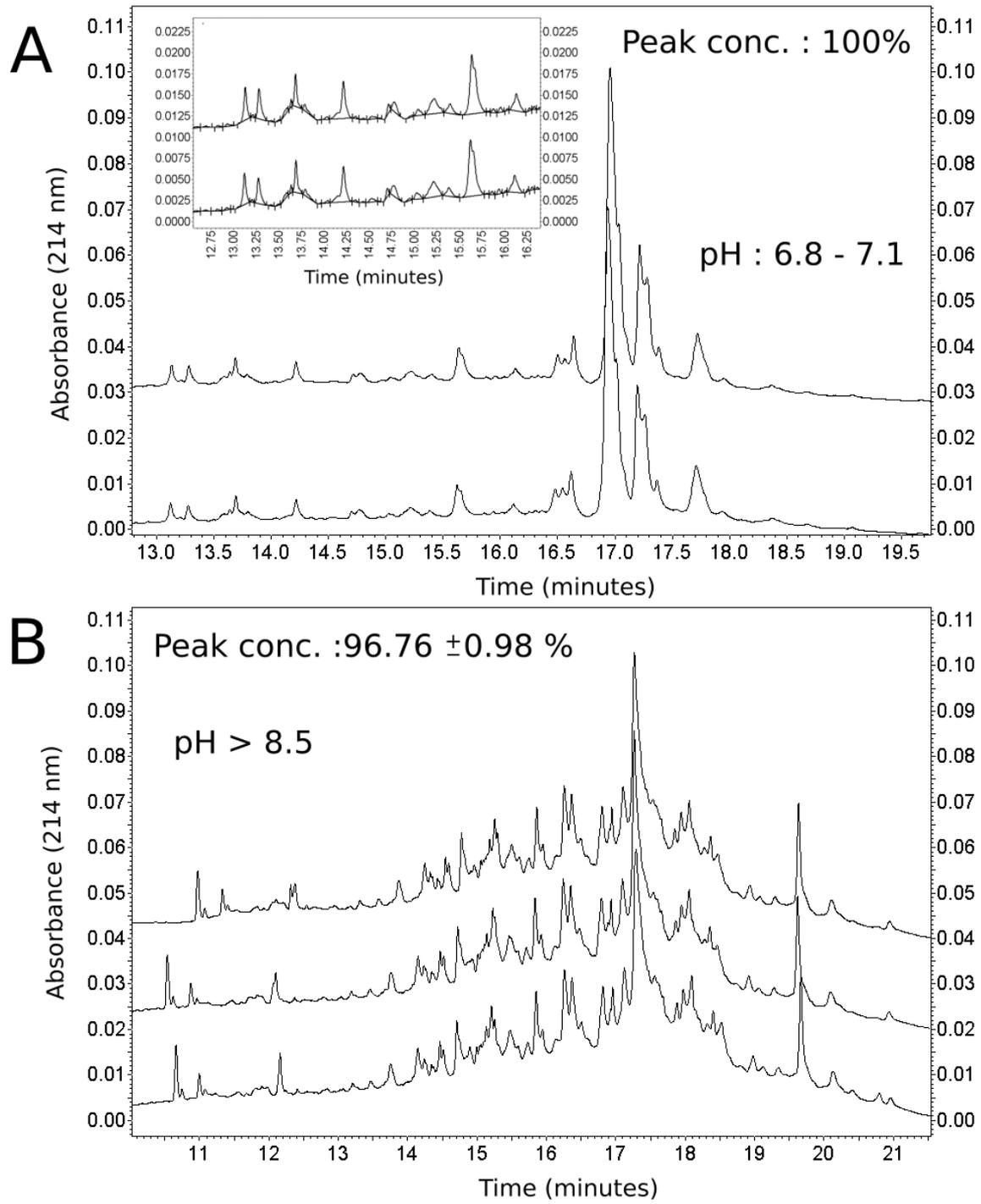


Figure 3.