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A 2-D liquid-phase chromatography for proteomic analysis in plant tissues ☆

Andrea Pirondini, Giovanna Visioli, Aliosha Malcevschi, Nelson Marmiroli *

Division of Environmental Biotechnology, Department of Environmental Science, University of Parma, Parco Area delle Scienze, 11/a, 43100 Parma, Italy

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Abstract

Two-dimensional liquid chromatography based on a high-performance chromatofocusing in the first dimension followed by high-resolution reversed-phase chromatography in the second dimension can be used as a complementary approach to protein separation with two-dimensional gel electrophoresis. In this work, *Arabidopsis thaliana* proteins obtained from different tissue extracts were resolved by using a new automated system, ProteomeLab PF 2D commercialized by Beckman Coulter (Fullerton, CA, USA). In particular, protein patterns obtained after two different extraction procedures ($MgSO_4$ and urea buffer) were compared. Reproducibility of the protein patterns was also confirmed in different injections of the same sample and in the comparative analyses of some proteins by MALDI-TOF/MS. Computer analysis of the chromatograms revealed that with this two-dimensional liquid phase technique, hundreds of "virtual bands" can be identified and compared in crude plant protein lysates.

Keywords: *Arabidopsis thaliana*; Comparative proteomics; Two-dimensional liquid chromatography; Protein crude extracts

1. Introduction

Plant comparative proteomics is becoming increasingly attractive as the rapidly expanding plant genomic and expressed sequence tags (EST) databases provide new opportunities for protein identification. The evaluation of the proteome, the protein status of a cell type, tissue, organ and whole organism, is an alternative strategy to address complex biological questions like the link existing between genotype and phenotype [1]. In particular, comparative proteomic analysis aims to characterise the differences between protein profiles in relation to genetic diversity [2].

So far, plant proteins have been analysed by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) [3]. A partial automation of this procedure consisting of a robotic lift of protein spots embedded in the gel, followed by extraction, destaining and protein digestion, has been finalised with reasonable success to the further protein characterisation and identification by mass spectrometry (MS) [4]. Despite these technical innovations, a bottleneck in proteome analysis remains the entire procedure of protein extraction. The heterogeneity of polypeptide molecular size, charge, hydrophobicity, complexation and cellular distribution makes it almost impossible to capture and solubilise the entire complement of proteins in a given sample [4,5]. Particular problems arise with hydrophobic proteins that can escape detection with 2-D PAGE [6,7] and with proteins that are expressed at very low levels, as most of regulatory proteins do [8]. Recently multi-step extraction methods have been developed to obtain an improved level of purification and solubilisation of proteins for 2-D PAGE analysis [9,10]. Specialised protocols have also been developed for the analysis of specific sub-set of proteins, such as membrane proteins [11,12], secreted or cell wall-associated proteins [13,14], glycosylated proteins [15] and proteins of organelles [16]. These sequential extractions, however, may lead to a reduction in protein concentration and to a decrease in the reproducibility of the protein patterns, which are both particularly relevant in order to compare whole proteome profiles. In fact, comparative proteome analysis typically involves "one step" chemical extraction procedures with a stringent solvent cocktail that is capable of disrupting protein aggregates and solubilise significant amounts of proteins [4].

Recently proteome analyses have also been performed in a "gel less" condition by using protein fractionation procedures based entirely on liquid chromatography (LC). The main

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* Corresponding author.
E-mail address: nelson.marmiroli@unipr.it (N. Marmiroli).

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advantage of LC is that crude protein extracts can be analysed after few purification steps thus achieving a higher level of reproducibility than most of the chemical procedures, allowing a better comparison of protein patterns [10,17]. The use of LC or two-dimensional liquid chromatography (2-D LC) separations, instead of gel mapping, have also proven to be robust methods for characterizing large numbers of total plant protein samples and proteins from plant organelles or subcellular compartments, followed by selective intact-protein analysis by MS [18–24].

Multidimensional protein identification technology (MudPIT) has also been developed for the analysis of peptide mixtures generated from complex plant protein samples, in parallel with 2-D PAGE [25,26]. Moreover, isotope coded affinity tag (ICAT) technique in the LC–MS/MS system has also emerged as a tool for plant proteins to improve quantitative comparisons among plant proteins in the absence of 2-D PAGE [27].

Among the different LC approaches a 2-D LC separation technique, based on chromatofocusing (CF) in the first dimension and high performance reversed phase (HPRP) liquid chromatography in the second dimension, was recently developed and tested with success on different protein samples from humans [28,29] and bacteria [30]. The CF technique, developed by Sluyterman and co-workers [31,32], combines elements of both ion exchange liquid chromatography (IEC) and isoelectric focusing (IEF) and has shown to be useful for preparative-scale applications: it is fast, has a high resolving power, and combines unique selectivity with the ability to retain protein native state [33]. In particular, CF employs a retained, internally produced pH gradient formed at low ionic strength that propagates like a front of the adsorption behaviour of molecular species in the elution buffer. Separation is based on charge differences among proteins, which are eluted from the column at a pH termed the "apparent" isoelectric point (pI_{app}) which is generally near the true p*I* of a protein. CF can be conducted over ranges of several pH units, in which case it is possible to separate proteins with resolution of about 0.1 p*I* units. The combination of all these characteristics allows for a fine separation of high amount of heterogeneous proteins [32,34,35].

In this paper, a 2-D LC automated system, ProteomeLab^{TM} PF 2D Protein Fractionation System (Beckman Coulter), that coupled high performance chromatofocusing (HPCF) and HPRP liquid chromatography was tested for the first time for the fractionation and for quantitative comparison of different *Arabidopsis thaliana* protein extracts, which is the model plant for which most of the proteomic and genomic information are available (http://www.arabidopsis.org/). Crude leaf and root protein extracts were obtained by using two extraction procedures which differed in the buffer composition ($MgSO_4$ and urea). The extracts were than analysed. HPCF produces liquid pH fractions in the first dimension separation, followed by HPRP liquid chromatography of each of the pH fractions in the second dimension. A dedicated software then converts complex chromatograms of a large number of fractions into easily visualized 2-D maps, "virtual gels", in which pH is plotted against the retention time. In silico analysis of different "virtual gels" provided a complete catalogue of the qualitative and quantitative differences existing between leaf and root protein patterns obtained with the two extractions methods. Reproducibility of the protein patterns was also tested. Some liquid fractions obtained were collected in a 96-microwell plate and further characterised by MALDI-TOF/MS in order to verify the consistency of the procedure and to have more information on the biological proprieties of the proteins isolated.

2. Experimental

2.1. Chemicals

Ethanol, $Ca(ClO)_2$, Tris [tris (hydroxymethyl) aminomethane], glycerol were purchased from Merck (Darmstadt, Germany). Triton X-100, Sucrose, Agargel, $MgSO_4$, β-mercaptoethanol, phenylmethylsulphonilfluoride (PMSF), urea, thiourea, *n*-octylglucoside, Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), protease inhibitor cocktail, iminodiacetic acidic, NH_4OH, NH_4HCO_3, DTT, iodoacetamide, Trypsin, α-cyano-4-hydrixycinnamic acid, were purchased from Sigma (St. Louis, MO, USA). Murashige and Skoog salts mixtures were obtained from Duchefa Biochemie B.V. (Haarlem, The Netherlands). BCA Protein Assay Kit was purchased by Novagen, Merck KGaA (Darmstadt, Germany). PD-10 desalting disposable columns were purchased from Amersham Biosciences (Uppsala, Sweden). Trifluoracetic acidic (TFA), acetonitrile (ACN) and water of HPLC quality were obtained from J.T. Baker (Deventer, Holland). ZipTipC18 were purchased from Millipore (Billerica, MA, USA). CF start buffer (SB), CF eluent buffer (EB) were commercialized by Beckman Coulter (Fullerton, CA, USA).

2.2. Apparatus

2-D LC chromatography was performed by using ProteomeLab PF 2D instrument commercialized by Beckman Coulter (Fullerton, CA, USA). HPCF-1D column (250 mm × 2.1 mm internal diameter, 300 Å pore size) and HPRP C18 column (4.66 mm length × 3.3 mm internal diameter, 1.5 μm particle size) were patented by Beckman Coulter (Fullerton, CA, USA).

Mass Spectrometry analyses were performed by using MALDI-LR in TOF/MS mode instrument commercialised by Micromass Waters Corporation (Milford Massachussetts, USA).

2.3. Plant material

A. thaliana seeds, ecotype Ws-1, were kindly provided by NASC (http://nasc.nott.ac.uk). Plants were grown in aseptic conditions for 14 days before harvest. In brief, seeds were surface-sterilized in 70% (v/v) ethanol for 2 min, followed by 5% (w/v) $Ca(ClO)_2$ and 0.02% (v/v) Triton X-100 for 15 min, then rinsed in sterile water. The seeds were germinated in 82 mm diameter Petri dishes containing Murashige and Skoog salts mixtures [36] supplemented with 1% (w/v) sucrose and 0.8% (w/v) agargel. Petri dishes were placed at 22 °C with a 16 h photoperiod in a growth chamber. The fluorescence lamps provided

an illumination of approximately 120 μE/m^2. Leaves and roots were collected separately, frozen in liquid nitrogen and stored at −20 °C before protein extractions.

2.4. Protein samples preparation

A. thaliana crude protein extracts were obtained by using: (i) $MgSO_4$-based extraction buffer [37] or (ii) urea-based extraction buffer.

For each method, total amounts of 1 g of roots and leaves were ground in liquid nitrogen with addition of SiO_2, to favour breakage of the cell walls.

In $MgSO_4$-based extraction protocol [37], the fine powder was resuspended in 50 mM Tris [tris (hydroxymethyl) aminomethane] HCl pH 7.8, 10 mM $MgSO_4$, 0.1% (v/v) β-mercaptoethanol and 2 mM phenylmethylsulphonilfluoride (PMSF). The solution was then centrifuged in a pre-cooled rotor spin at 16,000 × *g* for 5 min at 4 °C. The pellet, containing the larger cellular residues and SiO_2, was discarded; the supernatant was finally centrifuged at 16,000 × *g* for 30 min at 4 °C. The upper phase was stored at −20 ° C for the following steps.

With the urea extraction buffer, the fine powder was transferred to a 15-ml polypropylene tube. For each 0.5 ml of cell pellet, 2 ml of lysis buffer was added, containing 7.5 M urea, 2.5 M thiourea, 12.5% (v/v) glycerol (Merck), 62.5 mM Tris [tris (hydroxymethyl) aminomethane] HCl pH 7.8, 2.5% (w/v) *n*-octylglucoside, 6.25 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), 1.25 mM protease inhibitor cocktail. The solution was vortexed for 1 min, sonicated for 5 min and finally centrifuged at 18,000 × *g* for 60 min; temperature was maintained at 20 °C to prevent precipitation of the lysis buffer. The supernatant was removed and stored in a no-frost freezer at −20°, until use.

In each case, prior to injection into the CF column, the plant protein extract was desalted and equilibrated to the column environment by PD-10 desalting workmade disposable columns containing prepacked Sephadex G-25 Medium with exclusion limit of 5000 Da. Column equilibration was performed by using approximately 25 ml of CF start buffer and the sample was then eluted with 3.5 ml of CF start buffer. The capacity of the system allows the loading of up to 2 ml of sample, with a range of loading capacity between 0.5 and 5 mg of protein sample.

2.5. Protein quantification

Protein quantification was performed by using BCA Protein Assay Kit. This method is based on a biuret reaction which implies the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline solution and a concentration-dependent detection of the monovalent copper ions produced. Each samples was analysed with a UV–vis spectrophotometer at 562 nm with different dilutions of BSA, from a standard of 2 mg/ml, in the range of 20–2000 μg/ml. The BSA dilutions were prepared in CF start buffer. For comparison, equal amounts (1 mg) of total protein from different extraction were loaded into the first dimension of the 2-D LC system. Reproducibility was evaluated by analysing different samples of the same extract (1 and 2 mg).

2.6. First dimension: chromatofocusing

Proteins were separated in the first dimension by CF, performed on an HPCF-1D column. With this technique, proteins bonded to a strong anion exchanger followed by elution with a continuously decreasing pH (8.5–4.0) gradient. The pH gradient was generated on the column by two buffers: start buffer (SB) and eluent buffer (EB). The calibration of both buffers was an important step: SB and EB were sonicated for 5 min and then their pH was adjusted to 8.5 and 4.0, respectively, using either a saturated solution (50 mg/ml) of iminodiacetic acidic if the buffer was too basic or 1 M NH_4OH if the buffer was too acidic. The column was first equilibrated to the initial pH 8.5 using CF start buffer at a flow rate of 0.2 ml/min. After this step, 2 ml of sample were introduced with a manual injector into the column for the first dimension CF. Twenty minutes after sample injection, the first dimension pump switches to the CF eluent buffer (pH 4) at a flow rate of 0.2 ml/min. The interaction of the column filling with the CF eluent buffer produced a gradually decreasing pH gradient that travelled through the column as a retained front. The pH gradient affected the proteins net charge and their adsorption/desorption to the positively-charged matrix of the column, causing protein separation in the effluent. The pH of the mobile phase was monitored on-line by a post-detector pH flow cell. The proteins were eluted based on their isoeletric point (p*I*), measured for absorbance at 280 nm, and collected in a 96 deep-well plate by a fraction collector according to predetermined pH decrements of 0.3 pH units during the gradient, or in 1 ml volumes when the pH did not change. At the 115th min the most acidic proteins were recovered by washing the column with 1 M NaCl for 15 min. The column was finally washed with water for 45 min, therefore the CF separation took of total of approximately 185 min.

2.7. Second-dimension: high performance reversed-phase chromatography

HPRP was carried out in a C18 column. The mobile phase consisted of A: 0.1% TFA in water and B: 0.08% TFA in acetonitrile. Separation was performed at 0.75 ml/min with an increasing gradient of B. During the first 2 min 100% of solvent A was pumped into the column; in the next 35 min the gradient was created in the column by switching the flow from 0 to 100% solvent B; this is followed by 100% B for 4 min and 100% A for 9 min. In order to obtain a better resolution, the separation was done at 50 °C.

The eluent from the second dimension was monitored by a second high performance UV–vis detector at 214 nm, that provided a more universal and sensitive detection of proteins via peptide bonds. Fractions were immediately collected in a 96-well plate for MS analysis by using an automated fraction collector.

2.8. MALDI TOF analysis

Eluted fractions were evaporated to a final individual volume of 10 μl, using a Speed Vac. Protein digestion was

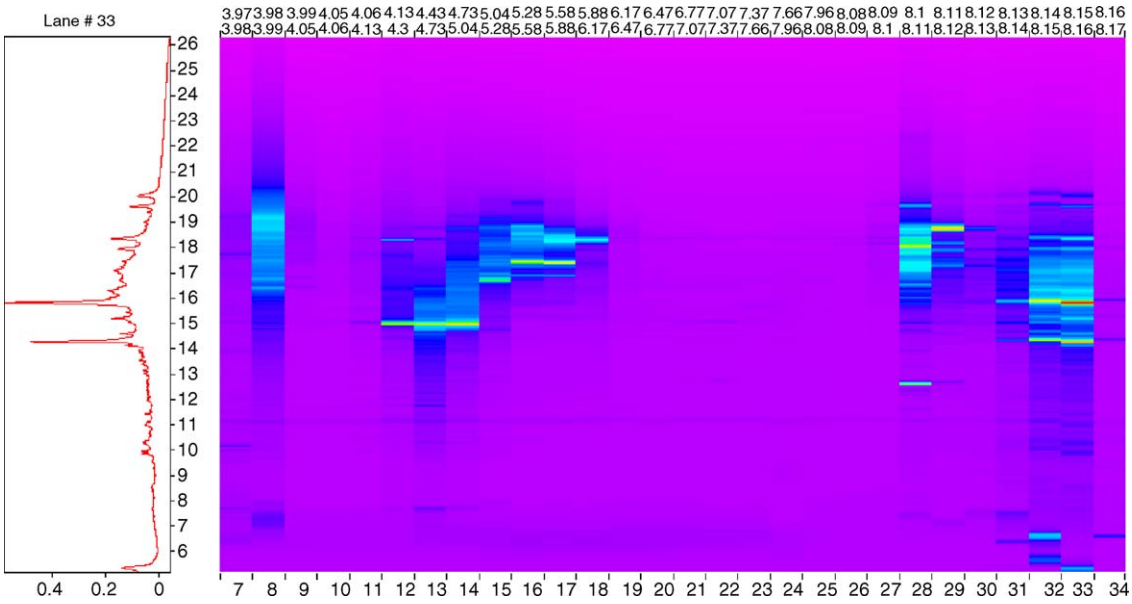


Fig. 1. ProteoVue 2-D expression map of an *Arabidopsis thaliana* root protein extract (urea buffer). The *x*-axis is in isoeletric point (pI) units from 4.0 to 8.0. The *y*-axis displays increasing hydrophobicity. The colour scale of the bands represents the relative intensity of each band by UV detection at 214 nm. The chromatogram of a single lane (lane 33) is evidenced on the left.

performed by incubating each fraction in 25 mM NH_4HCO_3 and 2 mM DTT in a water bath at 60 °C for 1 h. The alkylation of the reduced sulfhydryl groups was carried on by adding 1 mM iodoacetamide, at 25 °C, for 30 min in the dark. 1.5 μl of Trypsin (125 μg/ml) in 50 mM NH_4HCO_3 was added. Digestion was carried out putting the fractions on a medium speed at 37 °C for 24 h. The digested samples were then purified with a ZipTipC18 using the procedure recommended by the manufacturer. One microliter of each purified peptide was spotted directly onto a stainless steel MALDI target plate with 1 μl of a saturated solution of α-cyano-4-hydrixycinnamic acid in 0.1% TFA:ACN (2:1, v/v). The solution was allowed to dry at room temperature and a spot was produced. Positively charged ions were analysed in reflectron mode. External calibration was performed by using ProteomMass™ peptide and protein MALDI/MS calibration kit (Sigma, St. Louis, MO, USA). In particular, calibration peptide fragments were: angiotensin II (human) *m/z* 1.046, 542 ACTH fragment 18–39 (human) *m/z* 2.465, 198 insulin-oxidized B chain (bovine) *m/z* 3.494, 651. The spectra were obtained by randomly scanning the

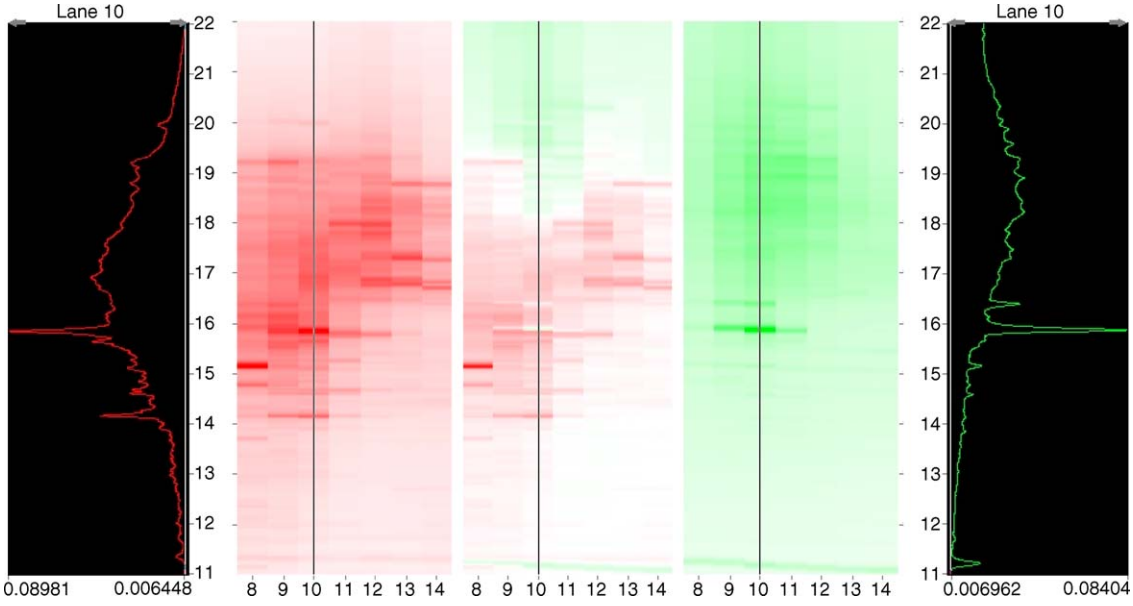


Fig. 2. DeltaVue 2-D differential map of $MgSO_4$ vs. urea leaf protein gradient fractions. The $MgSO_4$ protein extract is shown in shades of red and the urea protein extract is shown in shades of green. The central lane is a differential map of the protein expression of $MgSO_4$ vs. urea protein samples. The lane has been obtained by subtracting the area of peak in the green lane from the corresponding peak area in the red lane, evidencing qualitative and quantitative differences. The different chromatograms of a single lane (lane 10) are evidenced on both sides.

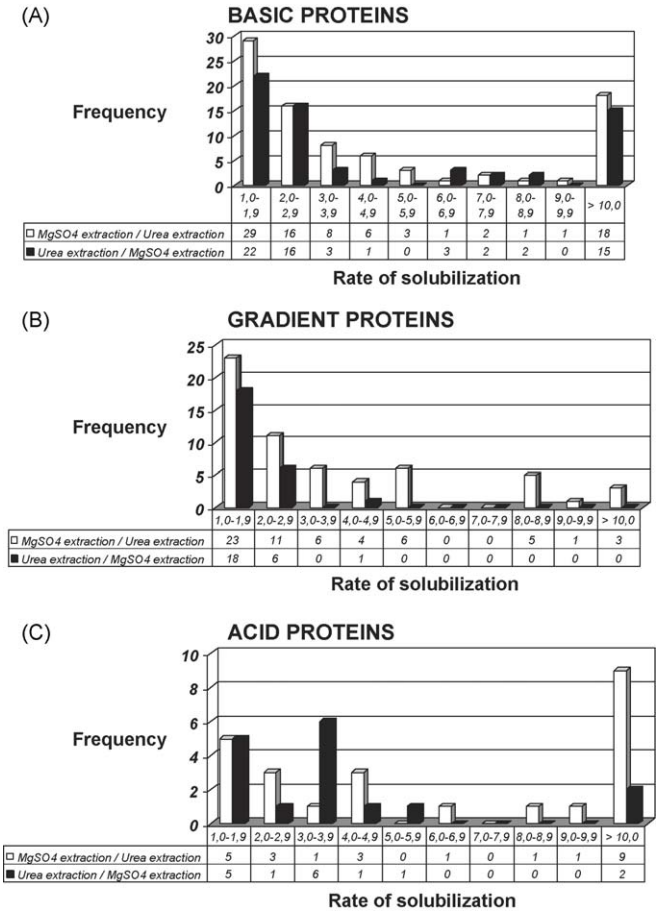


Fig. 3. Quantitative distribution in leaf protein concentration, expressed as a ratio between $MgSO_4$ vs. urea (white bars) and between urea vs. $MgSO_4$ samples (black bars). (A) Basic proteins; (B) gradient proteins; (C) acid proteins. *x*-Axis displays increasing rate of solubilisation of different proteins; *y*-axis displays the number of protein with the different ratio of solubilisation.

sample surface. Typically 100 shots were averaged to improve the signal-to-noise ratio. All spectra were analysed using the MassLynx 4.0 software (Micromass Waters Corporation).

2.9. Softwares and statistical analysis

ProteoVue software (Eprogen, Darien, IL, USA) was utilised to convert chromatographic intensities from the 2-D LC of each pH fraction into a band intensity format. This produced a highly detailed map with the dimensions of hydrophobicity and p*I*. The 2-D LC maps could be viewed in several coloured formats where the colour intensity was proportional to the relative intensity of each chromatographic peak. DeltaVue software (Eprogen, Darien, IL, USA) was utilised for the differential analysis of corresponding fractions from two different sample sets. This software compared chromatogram peaks corresponding to the same protein in the two samples, allowing quantification by a subtraction analysis. A differential map was achieved by point-to-point subtraction and it is viewed between the two original sample sets.

Wilcoxon signed ranks test for two related samples was used to determine differences between groups of paired data when the data did not meet the criteria required for a parametric test: in this case it was used to identify significant differences between the chromatograms obtained using the two methods. For all the fractions absorbance values produced by the same proteins were compared to discover which extraction method was, in general, better to extract acidic, basic and proteins with p*I* between 4 and 8 (referred to as "gradient proteins").

The peptide mass fingerprints deriving from MS were analysed with the Mascot program (http://www.matrixscience.com) using Swiss-Prot database limited to *A. thaliana* plant specie. The following parameters were used for database searches: mass accuracy below 100 ppm, maximum of one missed cleavages by trypsin, carbamidomethilation of cysteine as fixed modification.

3. Results and discussion

In this work, a multidimensional chromatography technique was investigated for the first time using plant protein crude extracts to test its loading capacity, protein separation and reproducibility.

This automated system ProteomeLab^{TM} PF 2D Protein Fractionation System consists of a first dimension HPCF, followed by a second dimension HPRP liquid chromatography. In particular CF was chosen for the preparative analysis of protein samples as an effective alternative to conventional isoelectric focusing (IEF) techniques [32,34,35].

To obtain *A. thaliana* root and leaf crude protein extracts, a number of extraction buffers were tested, based on different reagents: $MgSO_4$ [37], urea (Beckman Coulter), phenol-tricloroacetic [38], tricloroacetic acidic-acetone [39].

$MgSO_4$ and urea buffers revealed the best performance in terms of protein amounts and purity assessed by

Table 1
Wilcoxon signed ranks test for two related samples (urea–$MgSO_4$) of leaf protein extracts

Urea–$MgSO_4$ (leaf samples)	Total proteins	Basic proteins	Gradient proteins	Acid proteins
Number of ranks	277	138	95	44
Z (based on positive ranks)	−2.145	−0.957	−1.988	−2.100
Asymp. Sig. (two-tailed)	0.032	0.338	0.047	0.036
Monte Carlo Sig. (two-tailed)				
Sig.	0.031*	0.339	0.045*	0.035*
Lower bound	0.026	0.327	0.039	0.031
Upper bound	0.035	0.351	0.050	0.040

Asterisks correspond to the level of significance: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

one-dimensional SDS-PAGE (data not shown), and were utilised for the subsequent protein separation analyses. In literature, the urea buffer demonstrated to give optimal results using ProteomeLab PF 2D [29,40]. Ionic detergents, highly sulfonated or sodium bearing material, Triton X-100 are in fact not recommended for the chemistry of PF 2D (Beckman Coulter PF-2D Protocol). However, this extraction procedure has been previously performed only in bacteria [30] and mammalian cell [28,29,40] and therefore required an adaptation for use in plant protein extraction. $MgSO_4$ buffer was previously utilised in preparation of plant protein crude extracts [37]. In particular, it was suitable for this type of analysis because it did not contain any interfering substances for the chemistry of the PF 2D system.

Starting from the same plant material, 1 g of leaves and roots, a different efficiency was observed between the $MgSO_4$ and urea extraction procedures in the total amount of proteins extracted. In particular, $MgSO_4$ buffer seemed to solubilise a higher amount of leaf (1 mg with $MgSO_4$ and 0.7 mg with urea) and root (1.9 mg with $MgSO_4$ and 1.5 mg with urea) proteins.

Equal amounts of $MgSO_4$ and urea protein samples were subsequently injected into PF 2D for 2-D LC analysis. Single chromatograms obtained were then converted by the ProteoVue software (Eprogen, Darien, IL, USA) in a "virtual gel" in which every single band corresponds to a protein with a specific p*I*, and hydrophobicity. The relative intensities of the colour are directly proportional to the differences in protein concentration. As an example, in Fig. 1 the ProteoVue profile of an *A. thaliana* root protein extract is shown (urea buffer). Different protein sets were evidenced according to the separation in the pH range between 4 and 8 (gradient fraction). In particular, ProteoVue software generated maps containing about 300 resolved proteins for leaf samples, and about 400 for root samples, for both extraction methods.

Comparison of roots and leaves of the *Arabidopsis* proteome obtained with $MgSO_4$ and urea buffers was then performed using DeltaVue software (Eprogen, Darien, IL, USA).

Leaf samples, extracted with $MgSO_4$ or urea buffers, evidenced several qualitative differences in the protein patterns. In fact, from the graphic representation of Fig. 2 (central lane) it is possible to appreciate a differential map of the $MgSO_4$ and urea protein samples evidenced in gradient fraction. This map was obtained by subtracting the absorbance value of the same protein solubilised in $MgSO_4$ and urea buffers.

The data obtained were also elaborated by using the DeltaVue software. This software was specifically developed to detect quantitative differences among protein fractions, allowing for the comparison of low concentrated proteins. The diagrams in Fig. 3A–C show rates of solubilisation of more than 200 of basic and acidic proteins as well as proteins separated in the pH gradient. In these diagrams it was evidenced which condition favoured solubilisation of each protein specie and the rate of its solubilisation. There were ranges of protein concentration according to the different grade of solubilisation in relation to the different buffers used. As shown in Fig. 2, in the gradient fraction, a higher amount of proteins was detected when using the $MgSO_4$ buffer as compared with the urea buffer. Wilcoxon signed ranks test for two related samples confirmed that these differences were significant ($p < 0.05$). This test was also applied for the acidic protein fractions and the significance of the data was also confirmed ($p < 0.05$). Differently, no significant differences between the two extraction methods were found for the basic proteins. Analyzing all the protein extracted, it was possible to demonstrate that the extraction with $MgSO_4$ buffer worked significantly better, at least for leaf samples, than extraction with urea buffer ($p < 0.05$) (Table 1).

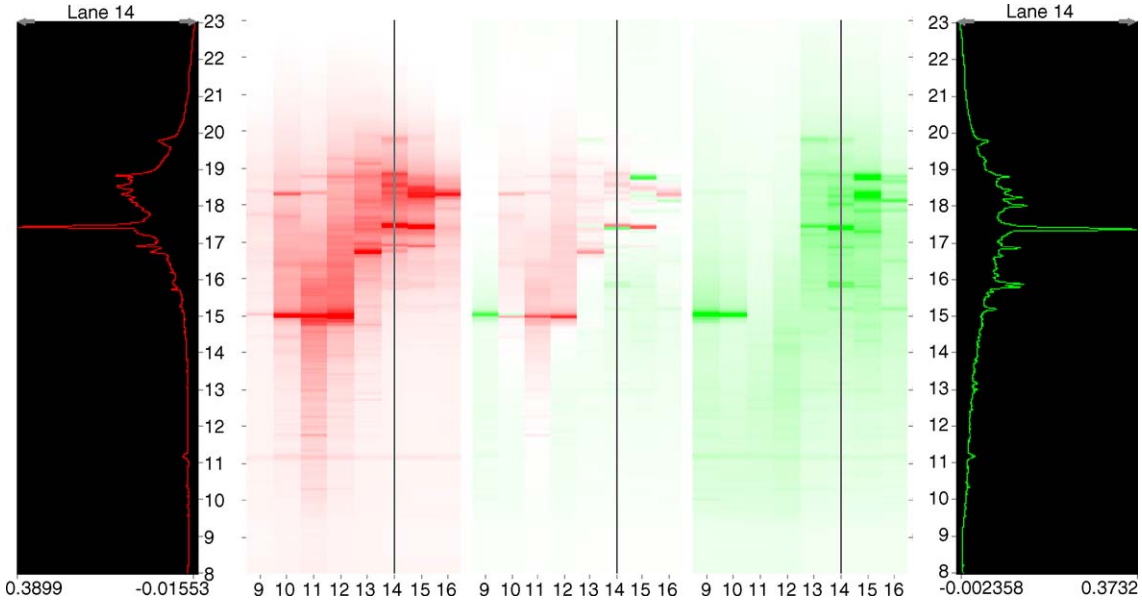


Fig. 4. DeltaVue 2-D differential map of $MgSO_4$ vs. urea root protein gradient fractions. The $MgSO_4$ protein extract is shown in shades of red and the urea protein extract is shown in shades of green. The central lane is a differential map of the protein expression of $MgSO_4$ vs. urea protein samples. The lane has been obtained by subtracting the area of peak in the green lane from the corresponding peak area in the red lane, evidencing qualitative and quantitative differences. The different chromatograms of a single lane (lane 14) are evidenced on both sides.

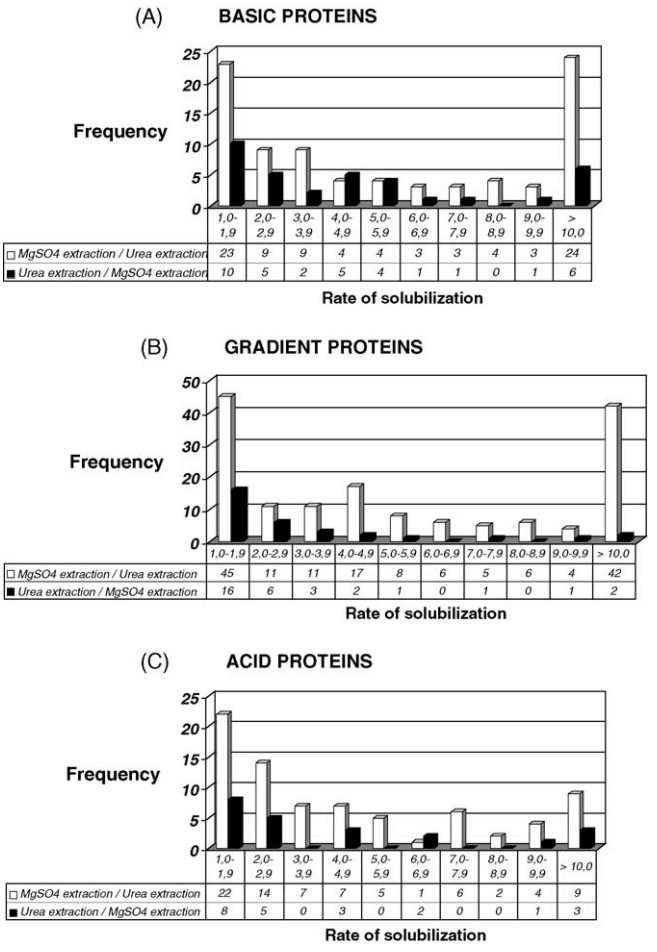


Fig. 5. Quantitative distribution in root protein concentration, expressed as a ratio between $MgSO_4$ vs. urea (white bars) and between urea vs. $MgSO_4$ samples (black bars). (A) Basic proteins; (B) gradient proteins; (C) acid proteins. *x*-Axis displays increasing rate of solubilisation of different proteins; *y*-axis displays the number of protein with the different ratio of solubilisation.

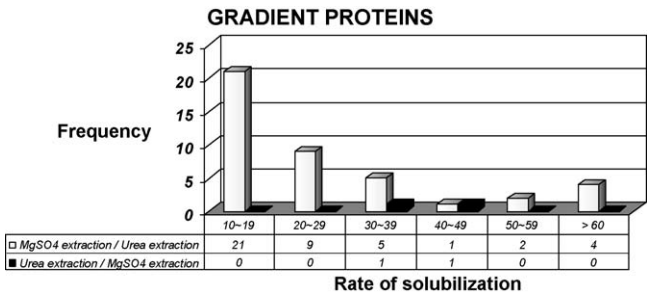
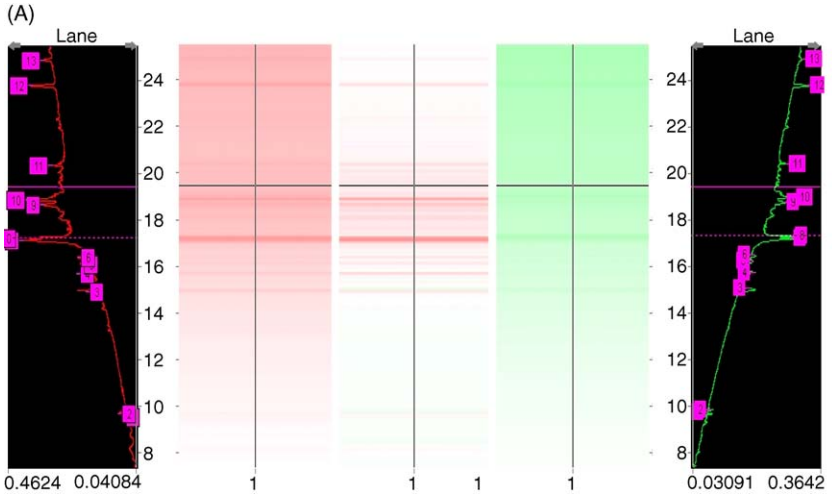


Fig. 6. Quantitative distribution of a sub-set (concentration level >10 times) of root proteins of the gradient fraction, expressed as a ratio between $MgSO_4$ vs. urea (white bars) and between urea vs. $MgSO_4$ samples (black bars). *x*-Axis displays increasing rate of solubilisation of different proteins; *y*-axis displays the number of protein with the different ratio of solubilisation.

As far as the root proteins, differences between protein patterns after $MgSO_4$ and urea extraction were observed. $MgSO_4$ root extracts gave a more defined chromatogram in different pH fractions (data not shown), and a higher amount of solubilised proteins than urea buffer extracts as shown for the gradient fraction in Fig. 4. In particular, the analysis performed by using DeltaVue software on the more abundant proteins revealed about 160 proteins more concentrated using the $MgSO_4$ buffer than using the urea buffer (Fig. 5A–C). Further out of these 160 proteins, 40 proteins were 10 times more abundant when extracted with $MgSO_4$ buffer (Fig. 6). The quantitative differences were estimated statistically significant by using Wilcoxon signed ranks test for two related samples when total patterns were compared ($p < 0.05$). A particular significance were found for the gradient fraction protein (pH 4–8) ($p < 0.05$) (Table 2).

Overall these data seem to confirm that extraction with $MgSO_4$ buffer could be utilised to obtain plant crude protein extracts adapted for chromatographic separation.

Another task in comparative proteomic studies by 2-D PAGE is to obtain the reproducibility of data [4,10].



Peak	Left peak / Right peak
1	2.032
2	1.92
3	2.016
4	2.025
5	1.952
6	1.936
7	1.978
8	2.011
9	2.016
10	1.977
11	1.911
12	2.004
13	2.001

Fig. 7. (A) 2-D differential maps of a selected pH fraction (p*I* 5.6–5.9) showing a comparison between two independent injections (shades of red, 2 mg and shades of green, 1 mg) of the same leaf protein extract ($MgSO_4$ buffer). Reproducibility of the protein pattern resulting from PF 2D separation is evident in the central map. (B) Quantitative data related to the most abundant proteins in the fraction obtained by subtracting the area of peak in the green lane (1 mg of proteins) from the corresponding peak area in the red lane (2 mg of proteins).

Table 2
Wilcoxon signed ranks test for two related samples (urea–$MgSO_4$) of root protein extracts

Urea–$MgSO_4$ (root samples)	Total proteins	Basic proteins	Gradient proteins	Acid proteins
Number of ranks	277	148	89	41
Z (based on positive ranks)	−2.031	−1.196	−2.209	−0.867
Asymp. Sig. (two-tailed)	0.042	0.232	0.027	0.386
Monte Carlo Sig. (two-tailed)				
Sig.	0.043*	0.239	0.026*	0.392
Lower bound	0.037	0.228	0.022	0.379
Upper bound	0.048	0.250	0.030	0.404

Asterisks correspond to the level of significance: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Table 3
Putative proteins separated by PF 2D, from *Arabidopsis* root extracts by $MgSO_4$ and urea buffers identified by MALDI-TOF/MS and "in silico" analysis

Fraction	Peak	Protein name	p*I* Fraction interval	p*I*	Mr	Percentage coverage (%)	Accession number
6	1	Hypothetical protein SCRL24	8.01–8.04	8.64	10726	57	P82643
6	2[a]	Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial precursor	8.01–8.04	8.78	64599	17	Q9LDD8
6	3	Ribosome biogenesis regolatory protein homolog	8.01–8.04	9.81	35509	25	Q9SH88
6	4	Putative low molecular weight cysteine-rich protein LCR17	8.01–8.04	9.11	10626	30	Q9T0E3
6	5[b]	RING-H2 finger protein ATL40 precursor	8.01–8.04	8.95	35838	33	Q8W571
14	1[b]	SNF1-related protein kinase catalititic alfa subunit	6.89–7.19	7.02	58689	22	P92516
14	2	Probable *trans*-2-enoyl-CoA reductasee, mitochondrial precursor	6.89–7.19	6.77	40823	33	Q8LCU7
18	1[a]	Probable coenzyme A diphosphatase NUDT11	5.71–5.99	5.65	25679	40	Q8LET2
18	2	Kinesin-3	5.71–5.99	5.90	85030	20	P46875
23	1	Calmodulin 2/3/5	4.13–4.43	4.11	16735	25	P25069
23	2	Calnexin homolog 2 precursor	4.13–4.43	4.77	60490	35	Q38798
23	3	Putative clathrin assembly protein	4.13–4.43	4.94	72171	40	Q9ZVN6

Columns report fractions, progressive peak number, protein names, first dimension p*I* interval, theoretical p*I*, percentage of coverage resulting by MASCOT algorithm and accession number of Swiss-Prot database.
[a] Proteins undetectable in urea samples (peak height $<0.05A_{214}$).
[b] Proteins differentially expressed in root tissues.

The sensitivity and the reproducibility of the protein patterns with PF 2D was tested by performing different injections of the same extracts (leaf protein lysates), obtained by using $MgSO_4$ buffer. A subtractive map of the different fractions produced by DeltaVue software in shown in Fig. 7A for the most concentrated proteins (pH fraction 5.6–5.9). The rate of solubilisation in the two different injections of the same sample was confirmed by the quantitative data showed in Fig. 7B. The same analysis in

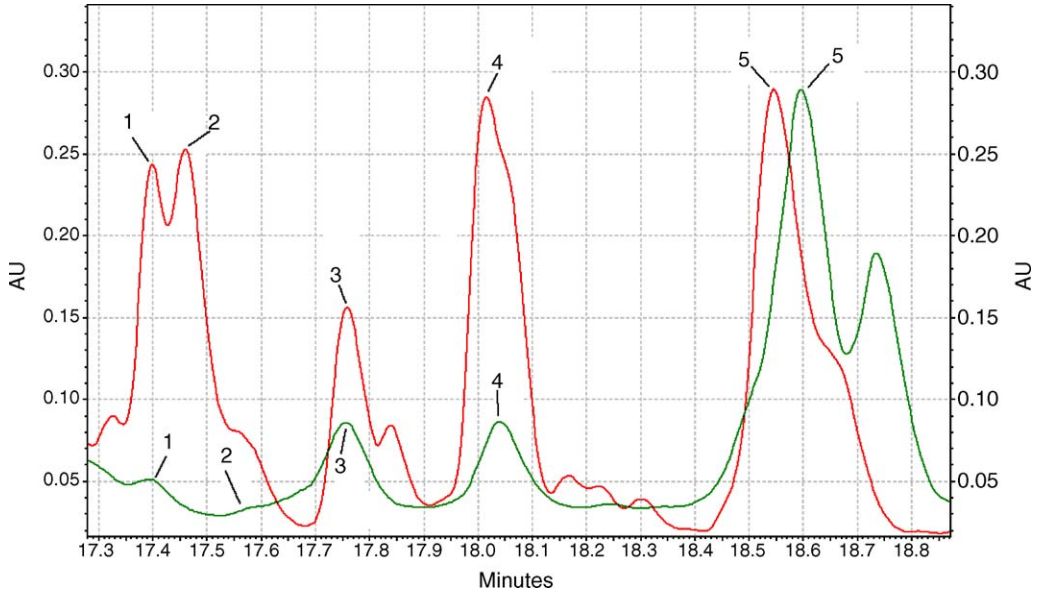


Fig. 8. Second dimension absorbance profile at 214 nm of fraction 6 of $MgSO_4$ (red) and urea (green) root protein extracts. Peaks, corresponding to the same retention time, were eluted and numbered progressively. The corresponding proteins were indicated in Table 3.

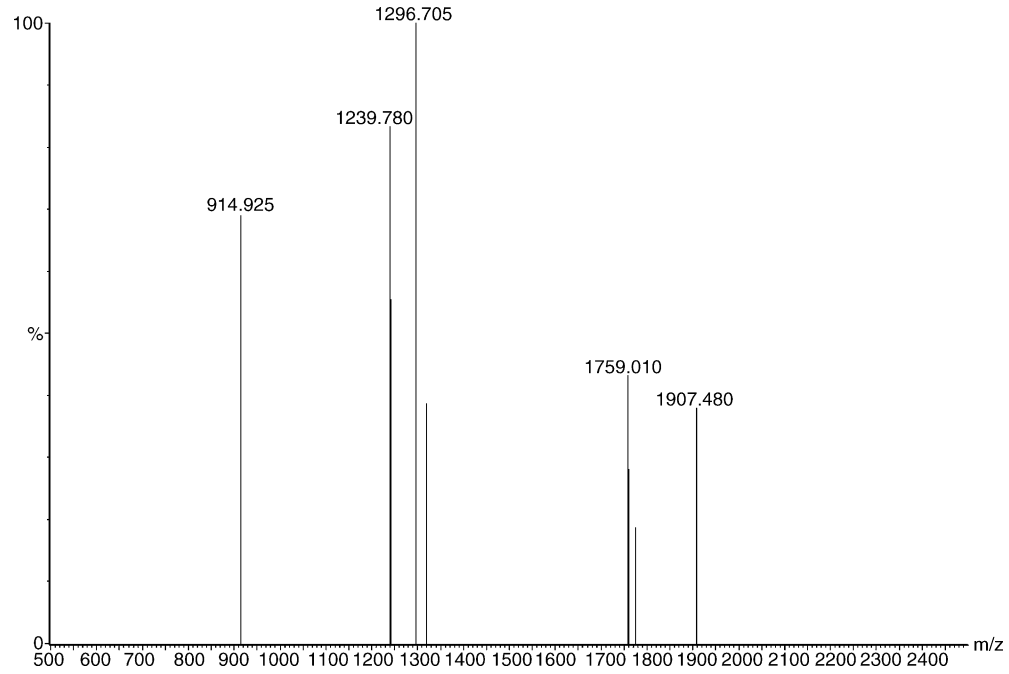


Fig. 9. Mass spectrum of one identified protein RING-FL2 finger protein ATL40 precursor (accession number Q8W571).

root samples gave comparable results (data not shown). The data obtained fit quite well with what recently reported by Soldi and collaborators [29] analysing the reproducibility of PF-2D system applied to the analysis of human urine proteins.

The accuracy and the reproducibility of the chromatographic separation in the second dimension was also tested by MALDI-TOF/MS. Twelve peaks, from root protein crude extracts, deriving from four fractions in the 4–8.5 pH range (6, 14, 18, 23) (Table 3, Fig. 8), showing different absorbance intensities between $MgSO_4$ and urea extraction methods were analysed. The MALDI Spectra obtained (Fig. 9) were used to enter *A. thaliana* database in order to access information on the biological identity of proteins present in the peaks considered. The MS combined with "in silico" analysis gave identity information on the putative proteins present in the peaks including the exact Mr and a confirmation of the pI_{app}. For some of the proteins the experimental p*I* value showed only a partial correspondence with the absolute p*I* value, but for most of the proteins identified the correspondence was acceptable. Similar inconsistencies have been described by other authors [29] and have been attributed to the fact that in liquid chromatography systems the pI_{app} is influenced also by the concentrations of ions present in the liquid and adsorbed phase and by the binding of a small part of the charged protein to the ion exchanger [41,42].

4. Conclusion

In this work, a multidimensional chromatographic technique has been utilised for the separation and comparison of different plant protein crude extracts.

The method provided a convenient 2-D map of protein profiles when proteins were categorized by both p*I* and hydrophobicity. The use of 2-D LC mapping for analysing protein expression had many advantages over chemical 2-D PAGE: (i) the high loading capacity, that greatly enhances the detection of low abundance proteins; (ii) the generation of an user-friendly map of protein expression that is easy to interpret and manipulate "in silico" by ProteoVue software; (iii) the possibility to analyse differential expression between two samples by DeltaVue software. Automation of the entire 2-D procedure allowed for minimal user interference, producing a good reproducibility of the data set. In addition liquid phase separations support the collection of protein peaks/bands of interest that can be for further characterisation by MS.

The combination of 2-D LC and MS analyses was fundamental to: (i) confirm the data of p*I*, (ii) obtain precise indication of Mr and (iii) find out from protein databases possible candidate proteins.

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