Pak. J. Bot., 46(5): 1549-1560, 2014.

2-DE PROTOCOL OPTIMIZATION AND EVALUATION FOR PROTEOME ANALYSIS OF GENUS *CLEMATIS* TAXA (RANUNCULACEAE)

MUHAMMAD ISHTIAQ*, MEHWISH MAQBOOL, TANVEER HUSSAIN AND SHEHZAD AZAM

Department of Botany, Mirpur University of Science & Technology (MUST) Bhimber Campus, Bhimber, Azad Kashmir, Pakistan
**Corresponding author e-mail: drishtiaqajk@gmail.com; Tel. & Fax: +92582892035*

Abstract

An approach was conducted to optimize two-dimensional gel electrophoresis (2-DE) method for leaf proteome analysis of genus *Clematis* species, as a molecular approach to explore its taxonomy and differentially expressed genome patterns. During establishment and optimization of protocol we extracted proteins by three extraction protocols, *viz*., phenol-SDS (PS) method, TCA/acetone (TA) method and lysis buffer (LB) method, and PS was the best one with 2.35±0.05 µg protein yield. For protein solubilization two lysis buffers (LB-1 & LB-2) were prepared, used and comparatively LB1 depicted better resolution. Proteins were by quantified by the Bio-Rad protein assay (Hercules, CA, USA) with bovine serum albumin as standard and purified by 2-D clean-up Kit (Amersham Biosciences). 2-DE analysis was conducted on pH 3~10, non-linear gradient strips (24cm) as first step, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 13% polyacrylamide gels as the second phase. For spot visualization gels were stained for with silver stain. The gels were scanned using Powerlook 2100XL scanner and gel images were analyzed by ImageMaster 2-D Platinum. Validation of experiment was performed by measuring analytical variance (AV) and biological variance (BV) for replicate spots. AV was calculated for 60 protein spots present in three replicate 2-DE gels of the same protein extract and BV was determined for the same protein spots from independent tissue extracts corresponding to leaves from different plants, or the same tree at different orientations or sampling times during a day. Values of 26% for the analytical variance and 58.6% for the biological variance among independent sampled species were obtained. This provided a threshold values for the evaluation of protein expression changes in comparative proteomic investigations with this species. Some spots were selected and subjected to liquid chromatography mass spectrometry (LC-MS) for identification purpose. Due to absence of *Clematis* DNA or protein sequences databases, FASTA and BLAST similarity searches were performed against other plant species databases were used for protein identification. The significance of 2-DE proteome analysis in predicting evolutionary trend of *Clematis* (liana) species and its potential significance in taxonomic identification for Traditional Chinese Medicine (TCM) pharmacopeia is described.

Key words: Proteomics; Leaf proteome; Traditional Chinese medicines; C*lematis chinensis*; Two-dimensional electrophoresis; LC-MS; Quality control

Introduction

Plants have been used by man as source of food, shelter and medicines since time immemorial. The genus *Clematis* (Tie Xie Lian) is systematic unit of Ranunculaceae with ± 300 species worldwide, including 147 (93 endemic) in China (Wang, 1998; Ishtiaq *et al*., 2010a, 2010b). *Clematis* was assigned a separate taxon first time by Linnaeus (Tamura, 1967). It is distinguished from other genera of the family by woody climbing stems (for most *Clematis* species) and opposite leaves. The genus comprises of perennial herbs and low shrubby bushes to all climbing coarse woody vines (liana) with worldwide distribution, growing not only in temperate and cold regions of both hemispheres but also in tropics as well (Tamura, 1967). Many species of the genus are extracted to prepare traditional Chinese medicines (TCMs) to cure various ailments in China and other parts of globe since long time (Geng, 1985; Wen *et al*., 1993; Ishtiaq *et al.,* 2006, 2010a). *Clematis* originated TCMs have been used as anti-cancer, anti-inflammatory, anti-allergy, antithrombotic, anti-mutagenic (Ishtiaq *et al.,* 2010b), to cure gonorrhoea (Armando *et al*., 1995), HIV syndrome (Yung, *et al.*,2001), different tumors (Qiu *et al*., 1999), hepatic disorders (Chiu *et al*., 1988), fungal infections (Heinrich, 2000), rheumatism, fever, viral infections, edemas and other diseases (Ishtiaq *et al.,* 2010a).

As TCMs are mainly botanic in nature and their raw materials are being exploited for drug discovery and development (Wang *et al*., 1986) but for their safe for health drug quality control parameters should be set and monitored properly. In this context authentic identification of herb or its raw-product is prerequisite by approved rules of FDA and WHO. Traditionally, herbalists rely on morphological parameters but it is dubious and can't be trusted blindly always for identification purpose (Ishtiaq *et al.,* 2007a). In some cases, poor identification of medicinal plants may lead to use wrong botanic materials in ethnomedicines (TCMs), causing loss of human lives (Zhu, 2002; Banaras *et al.,* 2012). It is very important to remove this discrepancy and solve the plethora of mis-identification of botanic herbs or products which culminates into loss of life, finance and labour. Another chemical marker based method is also of great significant but it demands procedural expertise to develop and optimize it. Different analytical procedures have been developed, optimized and applied for these purpose in TCMs of *Clematis* and other genera (Ishtiaq *et al*., 2007b, 2010a).). In previous studies, our research group has developed protocols using high throughput technologies for quality control of TCMs originating from *Clematis* and other allied taxa (Ishtiaq *et al*., 2006, 2007a, 2007b).

Recent, a revolutionary shift in molecular biology has been switched many imminent researchers to use life controlling factors (DNA genome) for identification and phylogenetic analysis of species ((Mehmood *et al*., 2013; Zada *et al.,* 2013); hence this has been applied for *Clematis* (DNA study) taxonomic exploration. Other branch of molecular markers called expressed genome (proteins) are also used in many plant taxa to explore their taxonomic status (Ishtiaq *et al.,* 2007, 2010; Ndimba *et al.,* 2003; Maria *et al.,* 2005; Rogers *et al.,* 1999). Some

researchers have conducted proteomics study on many plants but hitherto nothing is available for this genus by using 2-DE approach. The purpose of this study was to develop and optimize a 2-DE protocol for leaf protein analysis of *Clematis*. Second theme was to develop expressed genome patterns for proper identification and phylogenetic revision of selected species of the genus. Thirdly, to obtain species specific protein finger printings for its prompt and authentic identification that might help in quality control of TCMs and other botanic drugs raising reliability and trust of public masses.

Materials and Methods

Plant specimens: Plant samples of different species were collected in triplicate from different localities of same or different habitats i.e., TMSBR and Hangzhou botanical garden (HBG), listed in Table 1. The selected plants were devoid of any environmental stress symptom and each sample was thoroughly washed with dd H2O and stored at –80ºC until use. The samples were collected of same age, from same direction of each plant specimen with the view to minimize biological variance. Some collected specimens were authenticated and herbaria were prepared and placed in College of Pharmaceutical Sciences (CPS), Zhejiang University, Hangzhou, P.R. China.

Chemicals: Ammonium persulfate (APS), Glycerol, Bisacrylamide(bis), Tris(hydroxymethyl) aminomethane (Tris), Sodium dodecyl sulfate (SDS), Glycine, N,N,NU,NU-tetramethylethyldiamide (TEMED), ultra pure Urea, protease inhibitor cocktail, 2-D cleanup kit, 2-D Quant Kit were purchased from Amersham Pharmacia Biotech. Acrylamide, Dithiothreitol (DTT), 3-3-1-propane-sulfonate (CHAPS), Mineral oil, Coomassie G-250 (ultra pure grade) and Agarose were obtained from Shanghai Biotech. Iodoacetamide (IAA) was purchased from Fluka BioChemika. HPLC-grade acetonitrile was purchased from Merck, Germany. HPLC-grade trifluroacetic acid (TFA) was purchased from Tedia, USA. Formic acid (FA) was purchased from Acros Organics. Bromophenol blue, carrier ampholytes, linear immobiline dry strips pH gradient 3-10 (24cm long) from (Amersham Biosciences) and agarose were purchased from Pharmacia Diagnostics (Uppsala, Sweden). Silver nitrate, formaldehyde, EtOH, acetic acid and HCl were of high purity grade. Molecular weight markers were purchased from Amersham Biosciences.

Instrumentation & Softwares: IPG-strips (24cm length, 0.5mm thickness) with NL gradient pH 3~10 and 3~7 (Amersham Biosciences). IPGPhor (Amersham Biosciences), Bio-Rad Mini Protean II system (Bio-Rad, Hercules, CA, USA). Ettan DALT Π system (Amersham Biosciences). Eppendorf Centrifuge (5810 R). Densitometer. Powerlook 2100XL (Umax) scanner. Applied Biosystems QSTAR PULSAR^{TM} quadruple TOF mass spectrometer coupled with LC Packings Ultimate nano HPLC workstation (Amsterdam, The Netherlands). 2-D gel pictures were processed by ImageMaster 2D Platinum software (Amersham Biosciences).

Protein extraction procedure: In 2-DE analysis in order to obtain high resolution and good results multifarious factors are involved affecting its results' precision directly or indirectly. The optimization of leaf protein extraction, its purification, quantification and solubilization is inevitable for better gel snaps and subsequent analysis. To analyze the leaf proteome of *Clematis* species, we have used three different methods and selected the one with better protein spot resolutions and fair back ground.

Table 1. Clematis plant species included in 2-DE analysis, voucher number & geographical distribution.

S. No.	Species codes	Species	Herbaria number	Classification (Wang W.T.2005)	Geographical distribution and habitat information
1.	A	*Clematis peterae* (var) *trichocarpa* W.T. Wang	W, Zh.712112	(*Clematis*: *Clematis*)	TMSBR
2.	D	*C. finetiana* Level. et. Vant.	W, Zh.712111	(*Clematis*: *Rectae*)	TMSBR
3.	G	*C. heraclefolia* DC.	W, Zh.71213	(Clematis: Tubulosae)	TMSBR
4.	N	*C. chinensis* Osbeck	W, Zh.71211	(Clematis: Rectae)	TMSBR
5.	Q	*C. armandii* Franch.	W, Zh.71216	(Clematis: Rectae)	TMSBR
6.	L	*C. puberula* var. *ganpiniana* (Lévl. et Vant.) Wang	W, Zh.71217	(Clematis: Clematis)	TMSBR
7.	I	*C. apiifolia* DC.	W, Zh 71214	(Clematis: Clematis)	TMSBR
8.	R	*C. henryi* Oliv.	W, Zh.71219	(Viorna: Connatae)	TMSBR
9.	C	*C. intricata* Bung.	W, Zh 712126	(Clematis: Clematis)	Hebei Province
10.	T	*C. terniflora* DC.	W, Zh 712127	(Clematis: Rectae)	TMSBR
11.	U	*C. huchouensis* Tamura	W, Zh.71212	(Clematis: Viticella)	Hangzhou & Jiangsu
12.	P	*C. grandidentata* (Level. et. Vant) W.T. Wang	W, Zh.712113	(Clematis: Clematis)	TMSBR
13.	V (1)	*Ranunculus muricatus* L.	W, Zh. 712115	Ranunculus	Hangzhou
14.	V (2)	*Ranunculus japonicus* Thunb.	W, Zh. 712116	Ranunculus	TMSBR
15.	V (3)	*Ranunculus japonicus* Thunb.	W, Zh. 712121	Ranunculus	East, TMSBR
16.	V (4)	*Ranunculus muricatus* L.	W, Zh. 712120	Ranunculus	Lin an
17.	W (1)	*Paeonia suffruticosa* Andr.	W, Zh. 712117	Paeonia	Lin an, TMSBR
18.	W (2)	*Paeonia suffruticosa* Andr.	W, Zh. 712122	Paeonia	TMSBR
19.	W (3)	*Paeonia obovata* Maxim.	W, Zh. 712118	Paeonia	Hangzhou
20.	Y	*Houttuynia cordata* Thunb.	W, Zh. 712119	Houttuynia:(Saururaceae)	TMSBR

Abbreviations used above: TMSBR: Tian Mu Shan Biosphere Reserve; W: Wild; C: Cultivated; Zh: Zhejiang University Herbarium, species (of genus Clematis) are arranged according to classification system of Wang W.T. 2005

Preparation of dry tissue powder: Stored leaves (5g) were cut with neat scissors and ground in mortal and pestle in liq N @ ice bath. Obtained powder was re-suspended (0.2–0.3g) in 1.0–2.0mL cold acetone in 2.0mL microtubes and vortexed thoroughly for 1min and centrifuged at 10,000 rpm in Eppendorf (Centrifuge 5810 R) for 5min (4ºC). The process was repeated twice and pellet was placed in mortar and dried at room temperature (20min). This powder was fine ground into finer form by aid of quartz and stored in microtubes. It was sequentially rinsed with cold 10% TCA in acetone until the supernatant became colorless, then it was washed with cold aqueous 10% TCA twice, and finally with cold 80% acetone twice. Each time the pellet was re-suspended completely by vortexing, and then centrifuged. The final pellet was dried at room temperature and used for protein extraction, or stored @ –80ºC for future use.

Phenol-SDS method (PS): Proteins were extracted according to the protocol described by Wang *et al.,* (2003) with few modifications. Briefly, about 0.05–0.1g of the dry powder of leaf tissue was re-suspended in 0.7 mL phenol (Tris-buffered, pH 8.0; Sigma St. Louis, MO, USA) and 0.7 mL dense SDS buffer (30%sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% β-mercaptoethanol) in a 2.0 mL microtube. The mixture was vortexed thoroughly for 3 min and phenol phase was partitioned by centrifugation at 10,000 rpm for 10 min. The upper phase (phenol) was pipetted to fresh microtubes (0.2 mL for 1.5 mL tube, 0.4 mL for 2.0 mL tube). Extraction process was repeated and phenol fractions were mixed. To precipitate proteins about 5 volumes of cold methanol plus 0.1M ammonium acetate was added to phenol phase and mixture was stored at –20ºC for over night. Precipitated proteins were recovered at 10,000 rpm for 10 min (4ºC), and washed with cold methanolic ammonium acetate and cold 80% acetone twice (each). The final pellet was dried and stored at -20ºC until use. Prior to 2-DE run, proteins were dissolved in 100 µL of lysis buffer containing 7 M urea, 2 M thiourea (w/v), 2% CHAPS, 1% Ampholytes pH 3-10 (v/v), (Biorad), 40mM Tris, 10 mM Acrylamide.

TCA-acetone method (TA): Leaf powder was prepared as mentioned above. Dry powder ca. 5.00g was homogenized in four volumes of cold acetone (–20ºC). Extraction buffer containing 10% TCA (w/v) and 0.07% β-mercaptoethanol (v/v) in acetone was used in first extraction and, 10% TCA (w/v) in acetone was used for second extraction (Damerval *et al.,* 1986). The sample was kept at –20ºC over night and centrifuged at 14,000 rpm for 15 min and pellet was recovered. The obtained pellet was rinsed with cold (–20ºC) acetone containing 0.07% (w/v) DTT and centrifuged again. The washing was performed twice. The pellet was vacuum dried and solubilized in lysis buffer one by carefully sonicating/vortexing. The obtained slurry was centrifuged again and supernatant was stored at –20ºC until use.

Lysis buffer method (LB): Dry powder of leaves' was obtained by the foresaid method. Leaf powder was directly solubilized in the lysis buffer (0.75 mL/200mg) containing 7M urea, 2M thiourea, 3% CHAPS, 1% ampholytes (pH3-10), 40mM Tris, 10mM acryl amide (Herbert *et al*., 2001). Extraction was allowed for two hours at room temperature under continuous shaking while alkylation was blocked by adding 10mM DDT. Extracts were centrifuged at 10, 000 rpm for 20 min using Eppendorf (Centrifuge 5810 R) at 4ºC and supernatants were stored at –20ºC until further use.

Protein quantification and purification: The obtained proteins were quantified by the Bio-Rad protein assay (Hercules, CA, USA) with bovine serum albumin as standard (Bradford, 1976) and purified by 2-D clean-up Kit (Amersham Biosciences). Prior to first run, protein extracts were supplemented with trace of bromophenol blue and resolved by two-dimensional electrophoresis.

Optimization of different 2-DE parameters: Various parameters regarding the optimization of protocol for proteome analysis by 2-DE technique were evaluated and impact of each was studied in the subsequent analysis. In the analysis we had considered the effects of pH, concentration of sample load, voltage applied in IEF and SDS-PAGE steps, solubilization buffers and effects of storage time on sample.

Two-dimensional Gel Electrophoresis (2-DE)

IEF Phase: The first dimension was performed on IPG-strips (24cm length, 0.5mm thickness) with non linear gradient from pH 3~10 (Amersham Biosciences) (Righetti, 1990). The rehydration solution contained 7 M urea, 2 M thiourea, 3% CHAPS, 1% ampholytes (pH3-10), 40mM Tris, 10 mM acryl amide. Purified protein samples were dissolved in rehydration solution supplemented with 0.02% Bromophenol blue and DTT (2.8mg/ml) was added just prior to use. For analytical run (to visualize common and differential proteins) 80µg and for preparative runs (to obtain spots for identification with LC-MS/MS) 200µg proteins of each sample were loaded onto dry IEF strips, using the overnight in-gel re-swelling method (Berkelman *et al.,* 1998). The re-swelled IPG strips were subjected to IEF at 20ºC with first rehydration step for 12 hours at 30 V, followed by focusing for 1 hour at 100 V, 1 hour at 200 V, 1 hour at 500 V, 1 hour at 1000 V, 30 min for voltage increasing to 8000V, and remaining 8000V for 66 kVh on an IPGPhor (Amersham Biosciences).

SDS-PAGE phase: Focused strips were equilibrated using a first incubation step in equilibration solution (6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl, pH 8.8), containing 1% w/v DTT for 15 min, followed by a second incubation step in 2.5% w/v iodoacetamide in the same equilibration solution for 15 min as suggested by Roh (2004). Equilibrated strips were gently rinsed with SDS electrophoresis buffer and loaded on top of 12.5% w/v vertical SDS-polyacrylamide gels (26×20 cm), prepared using a Bio-Rad Mini Protean II system (Bio-Rad, Hercules, CA, USA), according to Laemmli (1970). The second dimension separation was performed sequentially with a constant voltage of 5W/gel for 1h, 10W/gel for 1 h, followed by 20W/gel for 6h using the Ettan DALT Π system (Amersham Biosciences). A

molecular weight calibration kit for SDS electrophoresis (Amersham Biosciences) was used as a molecular size marker on all gels.

Staining of gel images: All gels used for analytical purpose were fixed with solution containing 40% ethanol and 10% acetic acid for over night and stained with silver stain for spot visualization and matching (Shevchenko *et al.,* 1996; Heukeshoven and Dernick, 1985) while preparative gels were fixed with solution containing 40% ethanol and 10% acetic acid for 60 min, and stained with CBB R250 over night, cropped and used to determine their polypeptide sequences by LC-MS-MS approach (Neuhoff *et al*., 1988).

Analytical and biological variability (repeatability of experiment): Repeatability of an optimized protocol is the most important and critical step in good experiment design. In order to check repeatability of the established protocol, three samples extracted individually at different days were resolved by 2-DE at same and different days. The obtained 2-D gels predicted that analytical conditions were appropriate for the subsequent runs and method predicted good reproducibility and reliability and can be used for proteome analysis of *Clematis* taxa as well as for other plants. These observations can be viewed in results section below. Proteins samples extracted from three different accessions of one species were also analyzed to visualize the biological variance and consistency of presence or absence of protein polypeptides as gel spots and only polypeptides with constant spot distribution patterns were conducted for subsequent analysis.

Protein gel images and statistical analysis: Stained gels were scanned using Powerlook 2100XL (Umax) scanner and gel images were analyzed using ImageMaster 2-D Platinum software (Amersham Biosciences) (Apple, 1999). For comparative study of protein expression, gels containing consistent protein profiles with reproducible and highest number of common spots from three replicas were selected. In order to minimize the contribution of experimental variations, three separate gels were analyzed for each accession. Those spots displaying the same distribution patterns in three replicas were selected for further analysis. A standard gel was constructed with highest number of spots. All subsequent spot matching and analysis was performed by comparing all gel maps with the standard gel image by 2-D Platinum software (Amersham Biosciences) and manually. Gel patterns from each independent analysis were matched together and the relative abundances (%V) of each spot in gel were compared, using student's *t*-test ($p<0.05$). The percent value (%V) represents the pixel density of each spot normalized for the total pixel density from all spots in the same gel. This internal calibration can make the data independent of gel variations, as recommended by software manufactures (Ndimba *et al.,* 2003). It has been shown that for a large proportion of proteins, the integrated optical density is linearly related to the protein amount (Damerval, 1994; Jensen *et al.,* 1999). The 2-DE approach thus affords a unique opportunity to investigate the genetic determination of protein amount for several gene products at a time, whether their function is known or not. The Mr of proteins was determined by co-electrophoresis of standard marker proteins on the gels (Sigma, St. Louis, Mo, USA). The p*I* of the proteins was calculated by migration of protein spots on 24 cm IPG (pH 3-10, non-linear; Amersham Biosciences) strips.

Gels comparison and experimental repeatability: The obtained three gels of each species were matched by Melanie 3.0 software package (Appel *et al.,* 1991) and generated a reference gel image for each accession. For more validity and reliability, we performed two types of parameters: one was co-electrophoresis by mixing equal amounts of sample from each accession and species N (as an internal reference marker-*IRM*), and obtained gels were used to elucidate the position of each spot by comparison with IRM. Then mixed gels were matched and compared with each other and a global gel with 1800 reproducible spots was created. Secondly, for validity of these experimental results another comparison technique was also used, in which those spots which were permanently present in each gel image were marked by box and used as reference marker spots/regions for location of common and differential spots in gels of each accession (Chutipong *et al.,* 2007). The data about presence or absence of spots were formulated in a matrix form which was used to construct a phenetic tree for identification and differentiation of species. The criteria of spots comparison was based on counting the number of spots commonly (nxy) or absent (noo) and specifically present in one (nxo) or the other (noy) of the two considered accessions. Each spot considered for comparative analysis was attributed %Vol and its values were formulated as matrix form. Those spots which were present commonly in at two species were used for comparative taxonomic analysis. More than 300 protein spots were overall differentially present among the different accessions of the genus but for construction of phenetic tree only those spots were used which predicted fair volume with discriminative boundaries and good separations.

In-gel digestion and extraction of peptides: Some differentially expressed proteins in different accessions were harvested from CBB stained gels manually for protein identification process. Identification of protein was conducted by liquid chromatography mass spectrometry (LC-MS) analysis. Prior to LC-MS run, in-gel digestion of spots was performed according to the method by Jensen with some modifications (Jehsen *et al.,* 1999). Briefly, protein spots were excised from the CBB-stained gels and were cut into small pieces. Extracted pieces were destained twice using 60µL 200 mMol/L NH4HCO3/ACN (50:50 v/v), shrunk by dehydration in 60µL ACN twice, then completely dried at 37ºC for about 20 min. Cystine bonds were reduced with DTT (10 mM in 50 mM ammonium bicarbonate, 56ºC, 60 min) and alkylated with iodacetamide (55 mM in 50 mM ammonium bicarbonate, room temperature, 45 min). The reagents were washed out with 50 mM ammonium bicarbonate and gel pieces dried in a SpeedVac. The samples were then swollen in a digestion buffer containing 100 mM NH4HCO3 and 12.5 ng/µL trypsin (sequencing grade, Roche Diagnostics, USA) at

37ºC for overnight. The supernatant was removed, and the peptides were extracted with 5% FA in 50% ACN by sonication at room temperature. The extracts were combined and concentrated in presence of N2 and, stored at 4ºC until analysis.

Protein identification using LC-MS/MS technique: Mass spectrometric peptide separation and sequencing was performed on an Applied Biosystems QSTAR PULSAR™ quadruple TOF mass spectrometer coupled with LC Packings Ultimate nano HPLC workstation (Amsterdam, The Netherlands). After digestion process, ca. 3.0µL of each concentrated peptide digest was loaded onto an LC Packings C18, 5um, PepMap™ nano-precolumn, washed free of salts with 450 µL of 0.5% (v/v) ACN, 0.05% (v/v) TFA and eluted with a 6 mL linear gradient of 5% (v/v) ACN, 0.05% (v/v) TFA to 65% (v/v) ACN, 0.05% (v/v) TFA through a filter splitter onto an LC Packings C18, 3 µm, PepMap™ nano-column for direct infusion at 200 *n*Lmin-1 through a nano-spray tip into the mass spectrometer. TOF-MS spectra were collected between mass range of 100-2000 amu throughout the gradient elution and precursor ion selection and product ion spectra were generated using Applied Biosystems BioAnalyst software's fully automated switching and acquisition procedures. The spectra were internally calibrated using two trypsin autolysis peaks at m/z 842.510 and 2211.105. Only multiply charged precursor ions species were selected for fragmentation and peptide sequencing. For protein identification all MS/MS product ion spectra generated from each sample were used in MASCOT (http://www.matrixscience.com) database search of NCBInr database of all *Viridiplantae* sequences available. For protein identification these parameters were considered: maximum of one missed cleavage peptide was allowed, a mass tolerance of 0.3 Da, and MS/MS tolerance of 0.4 Da were used, and variable modifications such as ribulose biphasphate and other proteins were taken into account. Tryptic autolytic fragments and contaminations were removed from the data set used for database search. The Mr of each protein computed by the p*I*/Mr tool with (http://www.matrixscience.com) was compared to the Mr calculated in 2-D gel, contributing additional proof of identity of the analyzed protein spot.

Results and Discussion

The species of *Clematis* are very important because they are potential source of TCMs and other botanics in China and around the globe. The proper identification is main issue for the botanic drugs obtained from genus *Clematis*. Although different techniques are used for authentication purpose but expressed genome pattern is employed first time for the genus. Leaf protein profile of *Clematis* species was preliminary analyzed by 2-DE technique by optimizing its different parameters. Hence, in order to optimize a protocol for leaf proteome analysis different extraction and solubilization methods were evaluated and optimized under different analytical conditions. Particularly, protein extraction from plant tissue is often complicated due to non-protein contaminants indigenous to the plant, such as organic acids, lipids, polyphones, pigments, terpenes, etc (Granier, 1999). This study revealed the optimization of different analytical steps such as extraction, solubilization, purification, quantification, sample loading quantity, sample storing time, pH, IEF, SDS voltage steps, staining, image analysis and spot identification by LC-MS. The repeatability of experiment was evaluated by extracting same sample at different times and then running the sample on different/same days, and importance of each parameter with its results is presented.

In first step proteins were extracted with three different protocols *viz*., phenol-SDS (PS), TCA/acetone (TA) and lysis buffer (LB). Starting from ca. 1.0 g of fresh weight (FW) of leaf produced 0.2-3.0 g of dry powder; PS method produced 2.35 mg whilst TA and LB yielded 1.8 mg and 1.2 mg of proteins, respectively (Table 2; Fig. 1). The PS protocol not only predicted better yield but also presented fair gel snaps because PS removes out non-protein contaminants indigenous to the plant, such as organic acids, lipids, polyphones, pigments, terpenes, etc (Granier, 1998; Ishtiaq *et al.,* 2010b). The run analysis predicted that the gel images of TA and LB extracted samples had vertical streaking and high dark back background that is due to contaminants amalgam (Fig. 2).

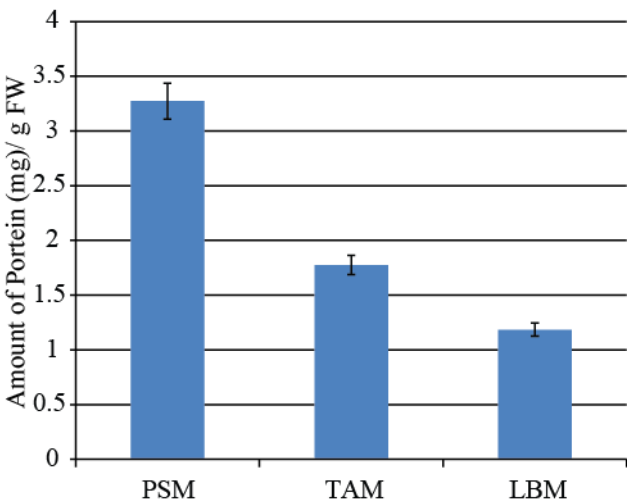

Fig. 1. Amount of leaf protein of Clematis chinensis extracted by three different methods PSM: Phenol-SDS method, TAM: TCA acetone method, LBM: Lysis buffer method.

Table 2. Proteins yield extracted from leaf of *Clematis chinensis* by three different protocols.

Method name	Protein yield exp. # 1	Protein yield exp. # 2	Protein yield exp. # 3	Mean (X)	RSD (%)
PSM	3.235	3.312	3.269	3.272	0.0385
TAM	1.787	1.721	1.821	1.776	0.0508
LBM	1.185	1.1256	1.243	1.184	0.0587

PSM: Phenol-SDS method, TAM: TCA acetone method, LBM: Lysis buffer method

PS method depicted good spot resolution and with no streaking, even basic polypeptides appeared as round shaped spots up to pH 10.0 and it produced more number of spots (850 spots) with fair gel images and high reproducibility (Fig. 2). That might be due to prompt dissolving process in phenol phase and separation as upper layer leaving below contaminants in aqueous phase and, phenol also prevents protein degradation due to endogenous proteolytic process (Rujin, 1998). Hence, PS was chosen in this phylogenetic and biosystematic study of genus *Clematis* to identify different botanics/TCMs. Moreover, this method can be used for further detailed proteome analysis of genus *Clematis* taxa as well as for other taxa of different genera for inter-genera differentiation of family Ranunculaceae.

Protein solubilization: In order to see the effects of different lysis buffers (LBs) on solubilization of proteins, two LBs were applied. The lysis buffer one (LB.1) contained 7 M urea (w/v), 2 M thiourea (w/v), 2% CHAPS, 1% Ampholytes pH 3-10 (v/v), (Biorad), 40mM Tris, 10mM Acrylamide. While lysis buffer two (LB.2) consisted of 9 M Urea (w/v), 2% CHAPS, 1% Ampholytes pH 3-10 (v/v), (Biorad). It was concluded that proteins were better solubilized in the LB.1 than LB.2. The sample solubilized in former buffer medium produced more number of (840) spots with fair back ground than in latter one (Fig. 3) that is due to activity of thiourea (Herbert, 2001).

Effect of pH: Samples were resolved by different pH IPG strips with 24 cm and NL features. The obtained gels showed that samples resolved by IPG strip pH 7~10 has low separation and few spots number (620). The gels produced by resolving proteins on IPGstrip pH 3~10 has better results with 840 spots and good separation of maximum polypeptides **(**Fig. 4).

Effects of storage time on extracted protein samples: The effects of storage time on the extracted protein samples were evaluated by analyzing the two samples extracted from the same accession but at different time intervals. It was observed that proteins were degraded with passage of time; no matter they were stored at -20ºC or -80ºC (Fig. 5).

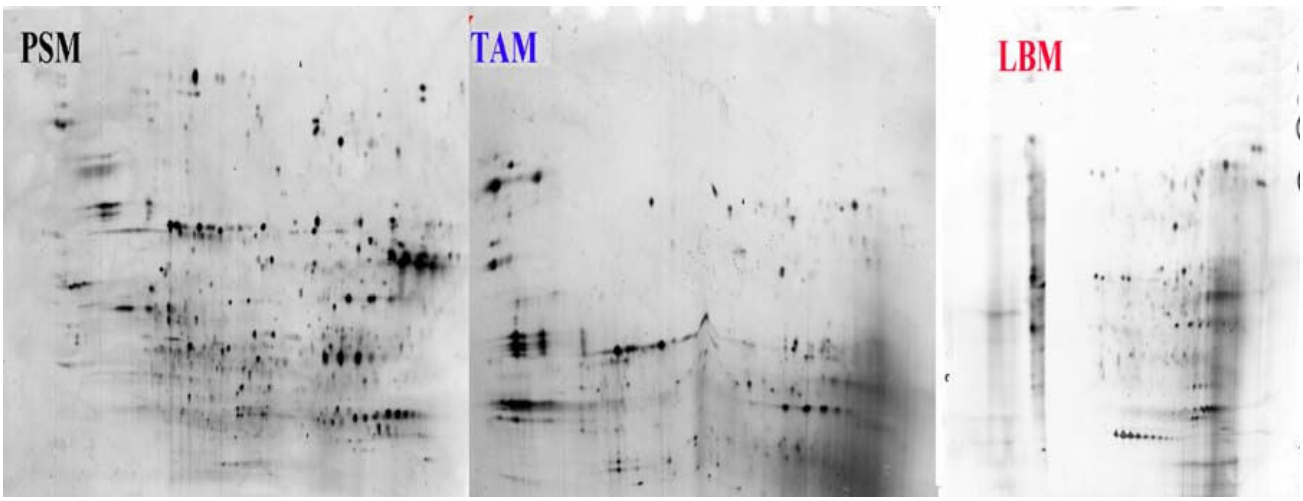

Fig. 2. Three maps of Clematis chinensis obtained by three different protocols; PSM: Phenol-SDS method, TAM: TCA acetone method, LBM: Lysis buffer method. The gels were stained with silver stain.

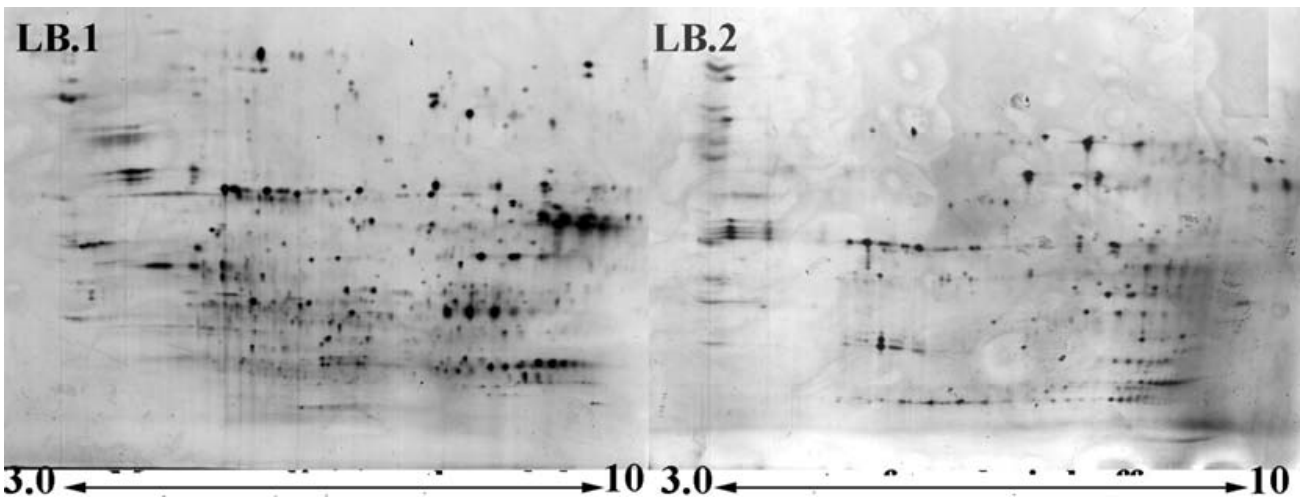

Fig. 3. Effect of solubilization buffers on Clematis chinensis leaf protein spots distribution in 2-DE gel analysis.

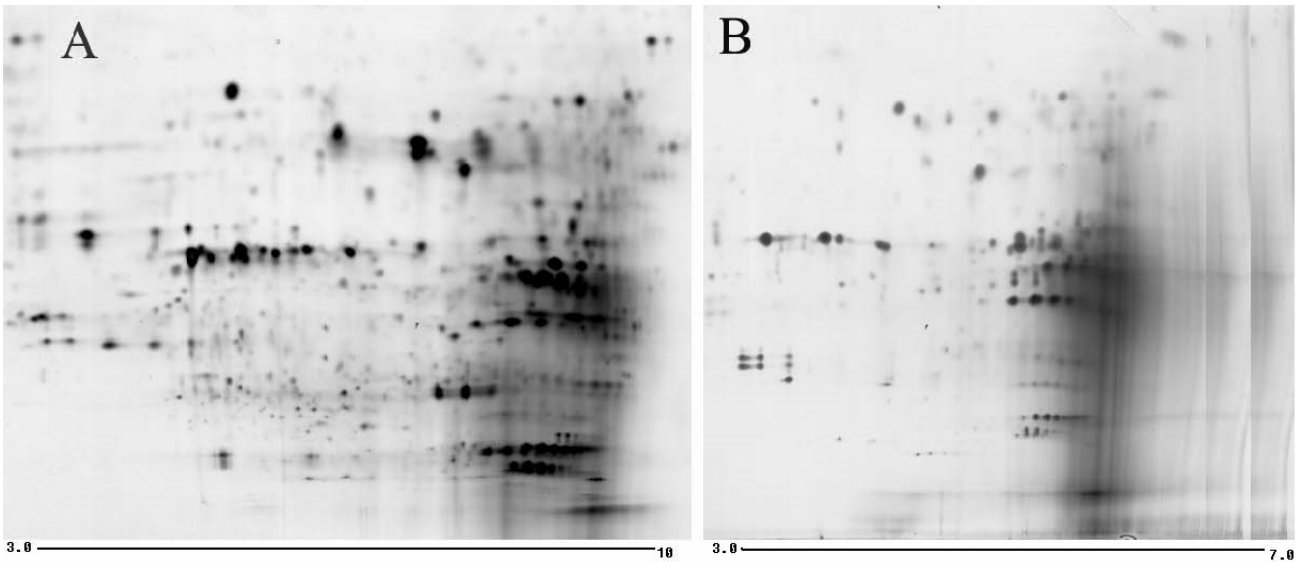

Fig. 4. Effect of pH on sample analysis of Clematis chinensis leaf proteins during 2-DE run.

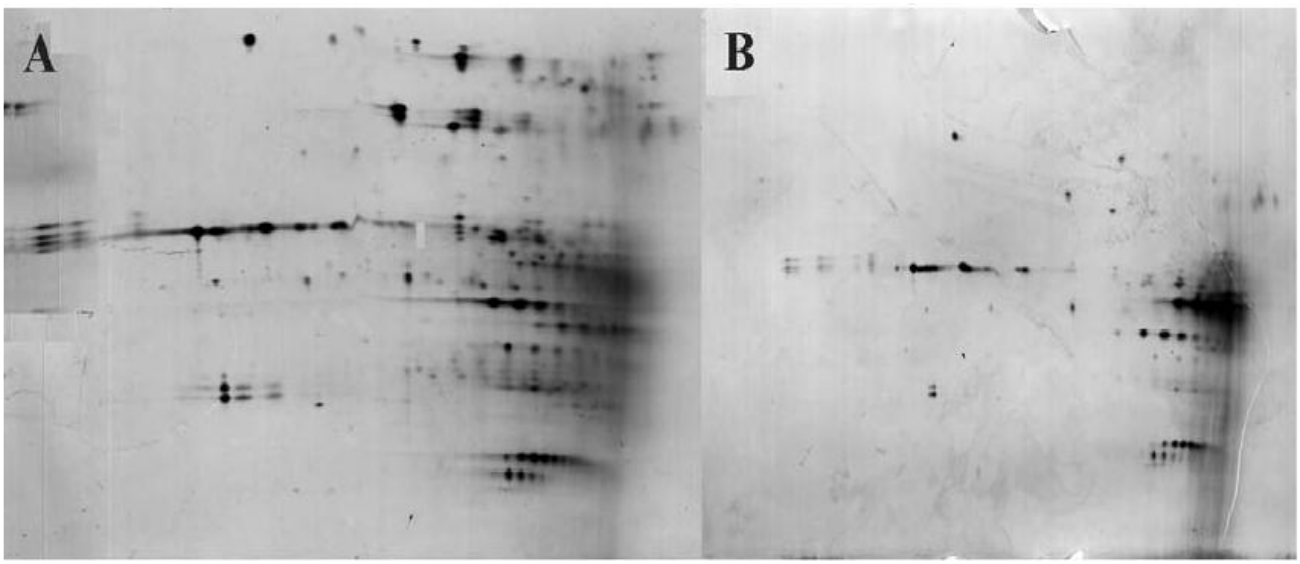

Fig. 5. Effect of storage time on Clematis chinensis leaf proteins during 2-DE analysis.

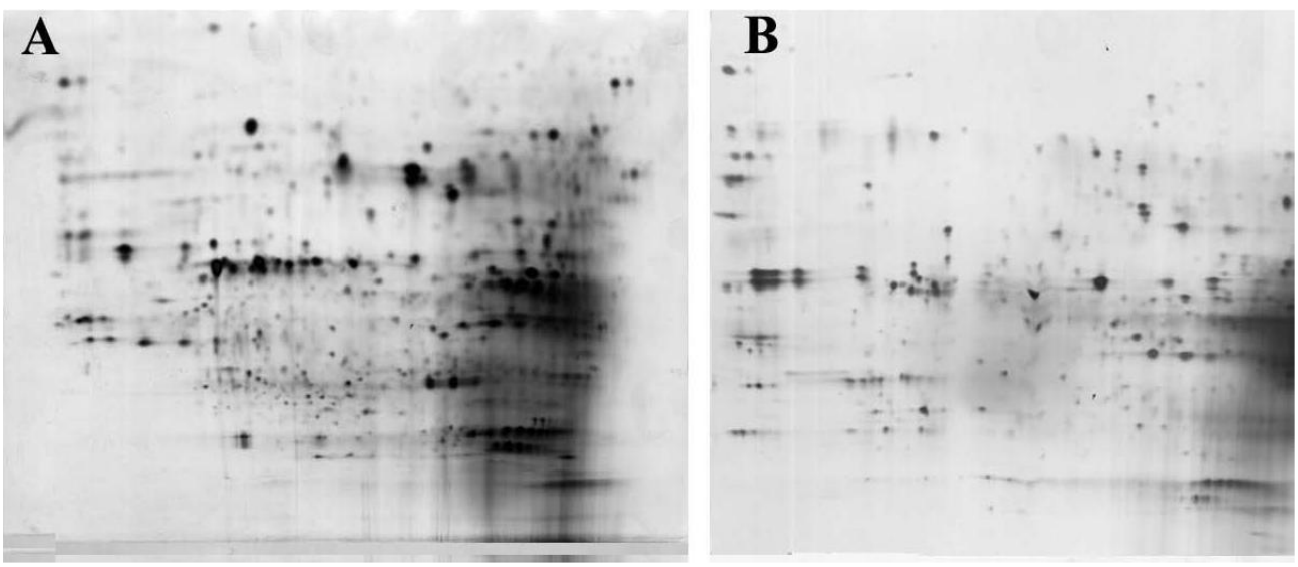

Fig. 6. Effect of load quantity of leaf proteins of Clematis chinensis on gel images during 2-DE analysis.

Effect of sample quantity load on spot resolution on gels: During the protocol optimization, we also tried different protein loads during various 2-DE runs. We used different protein sample concentrations 50µg, 75µg, 100µg and 120µg and analyzed under similar experimental conditions and obtained results depicted that sample loadings 100~120µg produced good gel images with more number of spots in analytical runs (Fig. 6).

Effects of voltage applied during IEF step: In order to observe what effects are exerted when different voltages were employed during IEF run step, we applied different voltage programmes i.e., 55, 66, and 75 kVhs to obtain the most appropriate one for this protocol. Different voltages produced variable number of spots and back grounds on the gels. From results depicted in the Fig. 7, it is seen that that 66 kVhs produced good gel image pictures with more and fair spot distribution.

Effects of run steps and current applied during SDS-PAGE step: Whether number of steps and current applied during the SDS-PAGE has more or less effects on protein resolution of the samples, we tried only two types of programmes i.e., 2nd phase run with two (10 and 20W) or three (5, 10 and 20 W) steps per strip during each run. And results obtained showed that three steps run programme is better than two steps and images are presented in Fig. 8.

Repeatability of experiment: The optimized protocol for proteome analysis of *Clematis* taxa showed good experimental repeatability. The results exposed by analyzing same protein sample in different analytical runs exhibited good reproducibility conditions. Hence, the established protocol can be used not only to analyze leaf proteome of this genus species but also it can serve as modal for proteome research in other medicinal plants too (Figs. 9 & 10).

Protein identification by LC-MS technique: Leaf protein extracts were obtained by PSM protocol and analyzed by two-dimensional electrophoresis. The marked spots (Fig. 11) were analyzed by LC-MS for identification and detailed results will be published in another article. As a demonstration, mass spectrum of ATP synthase alpha chain (spot 12) protein is shown (Fig. 12).

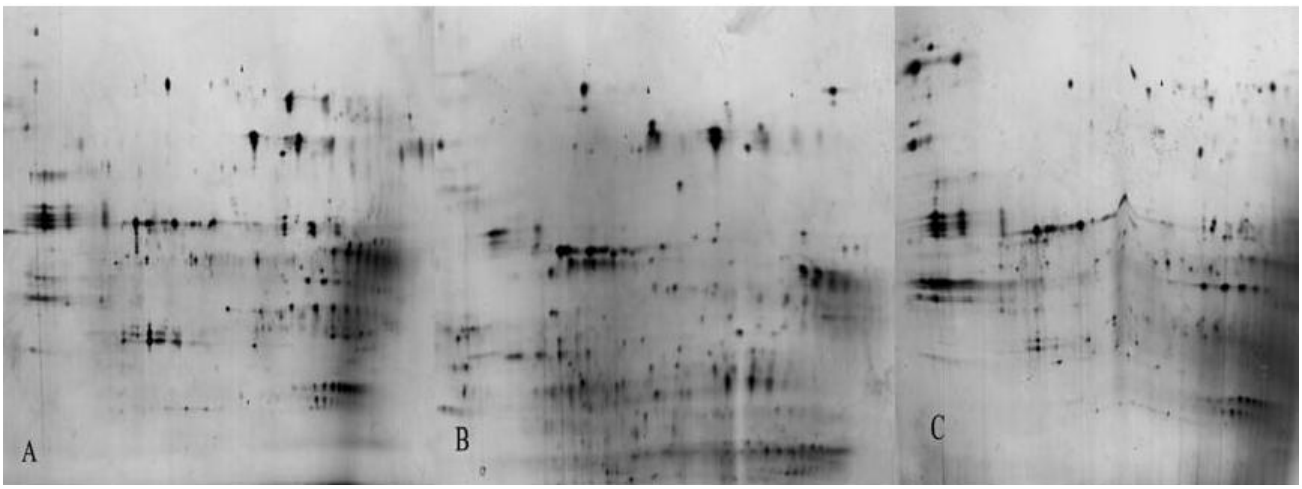

Fig. 7. Effect of different voltage steps on leaf proteins analysis of Clematis chinensis during 2-DE run.

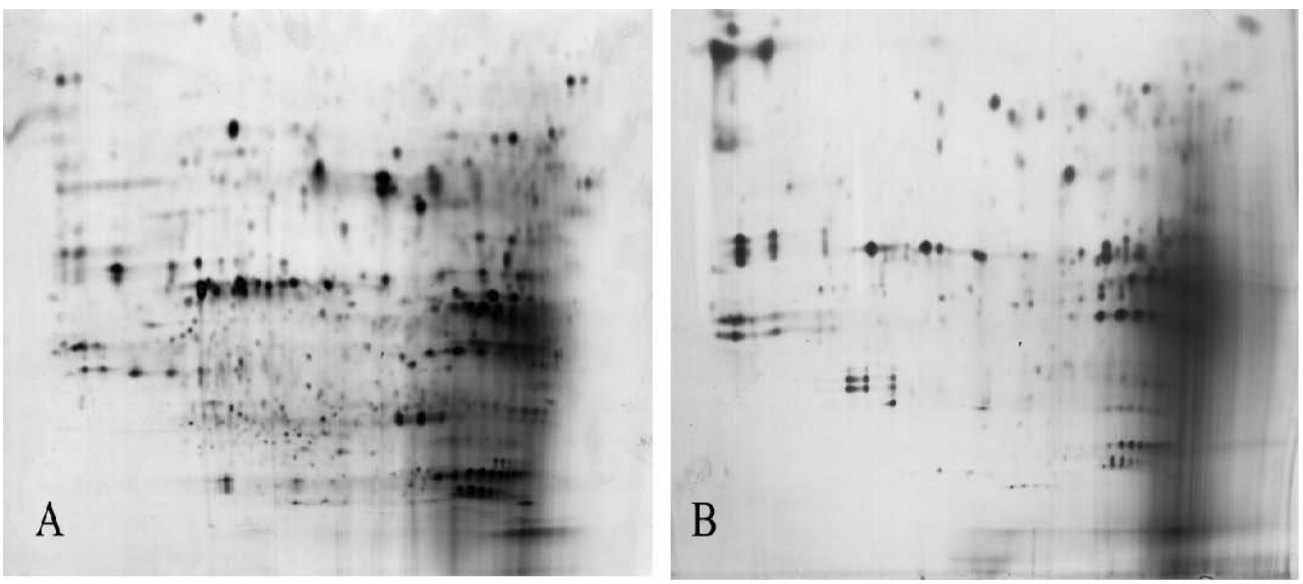

Fig. 8. Effect of different voltage steps on leaf proteins analysis of Clematis chinensis during SDS-PAGE Step; A: three steps; B: two steps.

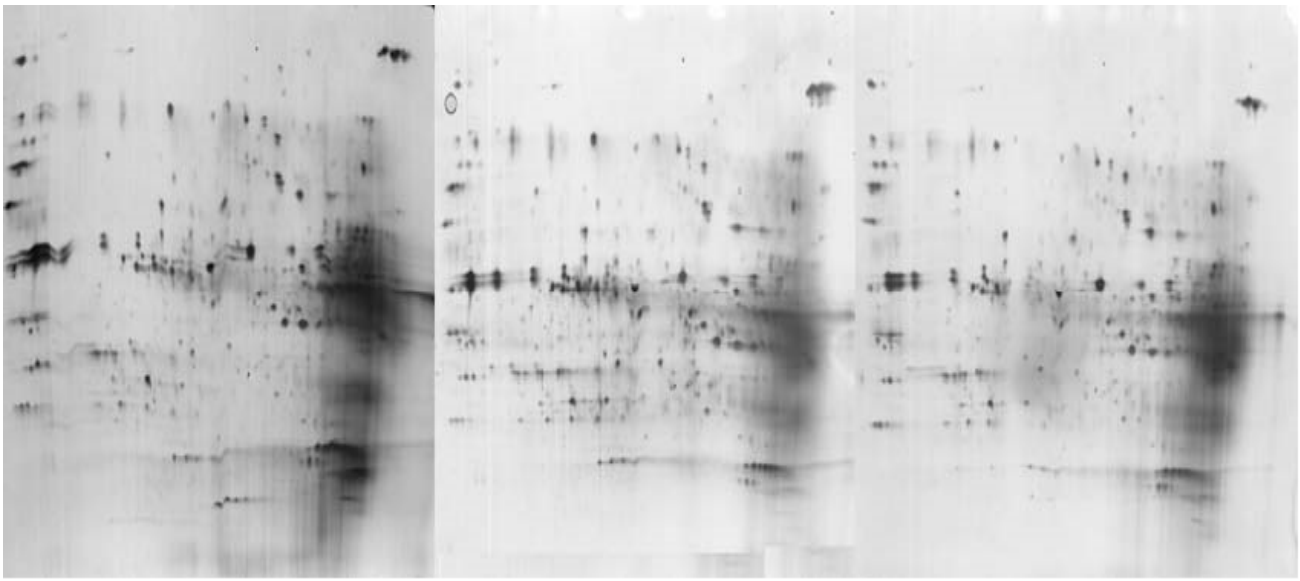

Fig. 9. Three maps representing repeatability of protocol, same sample was extracted at different time and run under same experimental conditions of 2-DE analysis

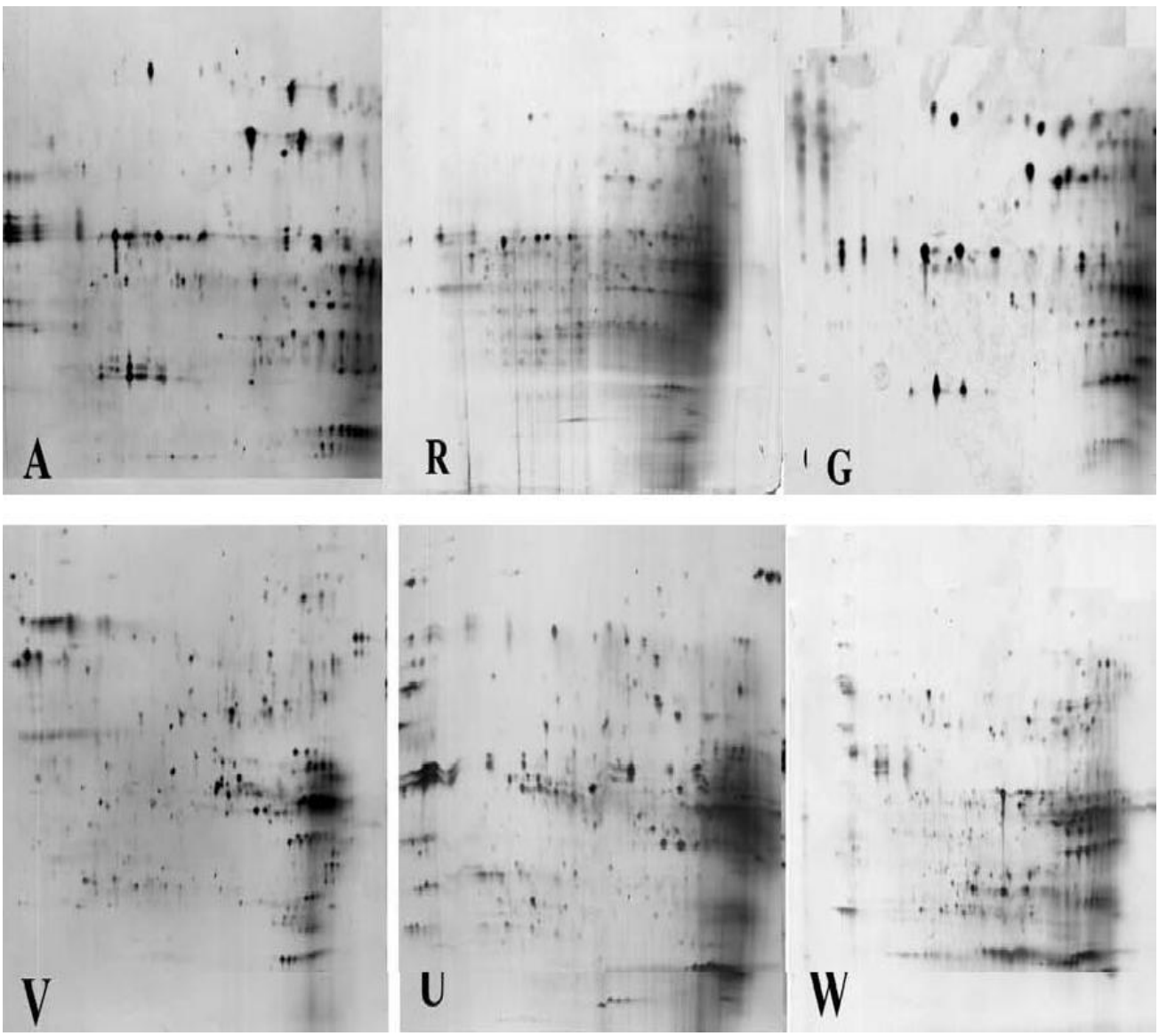

Fig. 10. Representative gel maps of different Clematis species run at 3~10 pH and 13% gel with SDS during 2-DE analysis.

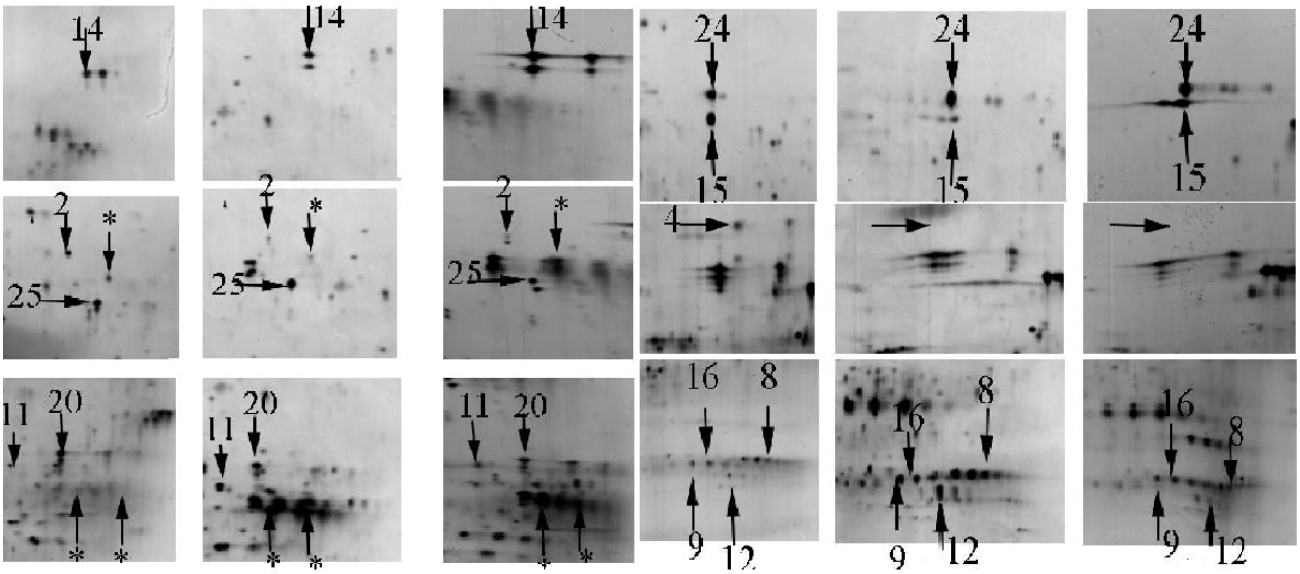

Fig. 11. Enlargement of some regions of Clematis species (D, N, Q) gels to highlight some of the differentially expressed protein spots. Arrows in each region point to proteins that were regulated and numbered points indicate proteins identified.

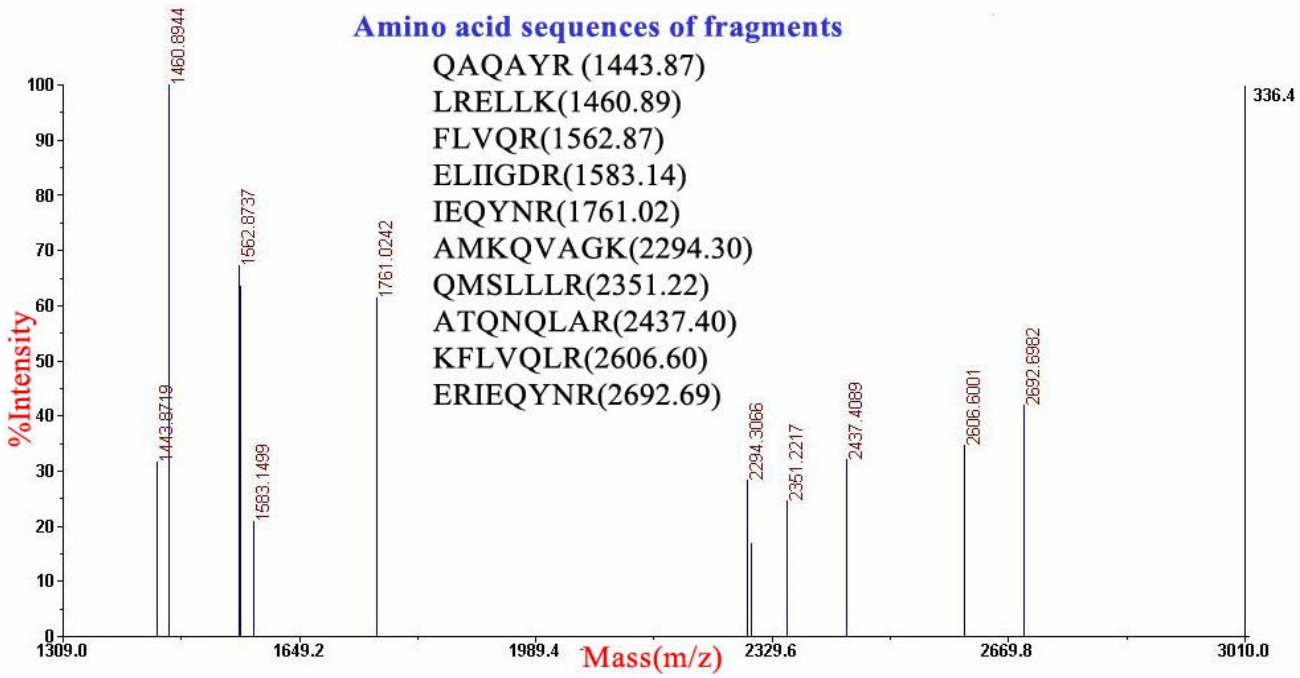

Fig. 12. LC-MS spectrum of ATP synthase alpha chain protein isolated from 2-D gel. Out of 14 representative spectra, ten peptides were matched and listed.

References

Appel, R.D., D.F. Hochstrasser, M. Funk, J.R. Vargas, C. Pellegrini, A.F. Muller and J.R. Scherrer. 1991. The MELANIE project: from a biopsy to automatic protein map interpretation by computer. *Electrophoresis*, 12: 722-735.

Armando, C.M.H., M. Emilia, C. Ericka, E.S. Blanka, J. Elsa, P. Eduardo, C. Guillermo. 1995. Antigonorrhoeal activity of plants used in Guatemala for treatment of sexually transmitted diseases. *Journal of Ethnopharmacology*, 48: 85-88.

Bahrman, N., M. Zivy and H. Theillement. 1988. Genetic relationships in the Sitopsis section of Triticum and the origin of the B genome of polyploid wheats, *Heredity*, 61: 473-480.

Bahrman, N., M. Zivy and H. Thiellement. 1988. Genetic relationships in the *Sitopsis* section of *Triticum* and the origin of the B genome of polyploid wheats. *Heredity*, 61: 473-480.

Banaras, S., S. Aman, M. Zafar, M. Khan, S. Abbas, Z.K. Shinwari and S.N Shakeel. 2012. Molecular identification and comparative analysis of novel 18s ribosomal RNA genomic sequences of wide range of medicinal plants. *Pak. J. Bot*., 44(6): 2021-2026.

Berkelman, T. and T. Stenstedt. 1998. *2-D Electrophoresis using immobilized pH gradients*, Amersham Biosciences Instruction Manual, Amersham Pharmacia Biotech, Piscataway, N J, USA, pp. 35-36.

Bradford, M.M. 1976. Protein assay by dye binding. *Anal. Biochem*., 72: 248-254.

Canovas, F.M., E. Dumas-Gaudot, G. Recorbet and J. Jorrin. 2004. Plant proteome analysis. *Proteomics*, 4: 285-298.

Chiu, H.F., C.C. Lin, C.C. Yang and F. Yang. 1988. The pharmacological and pathological studies on several hepatic protective crude drugs from Taiwan (I). *Am. J. Chin. Med*., 16(3-4): 127-137.

Chutipong, A.N.T., Issariya Chairam, Narumon Prakrongrak, Sanun Jogloy, Aran Pathanothai, 2007. Isolation and identification of peanut leaf proteins regulated by water stress. *Pak. J. Biol. Sci*., 10(10): 1611-1617.

Damerval, C. 1994. Quantification of silver-stained proteins resolved by two-dimensional electrophoresis: Genetic variability as related to abundance and solubility in two maize lines. *Electrophoresis*, 15: 1573-1579.

Damerval, C., D. De Vienne, M. Zivy and H. Thiellement. 1986. Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. *Electrophoresis*, 7: 52-54.

Dennis, I. Jacobs, Margriet, S. van Rijissen, Robert van der Heiden and Robert Verpoote. 2001. Quantification of silver-stained proteins resolved by two-dimensional electrophoresis: Genetic variability as related to abundance and solubility in two maize lines. *Proteomics*, 1: 1345-1350.

Geng, B.Q. 1995. Experimental studies on the effect of *Clematis chinensis* on the biliary tract. *Zhong Yao tong bao* (Beijing, China: 1981), 10(9): 37-39.

George Mohr and Alan M. Lambowitz. 2003. Physiological and proteomic approaches to address the active role of ozone in kiwifruit post-harvest ripening. *Nucleic Acids Research*, 31(2): 647-652.

Gong, F., Y.Z. Liang, P.S. Xie and F.T. Chau. 2003. Information theory applied to chromatographic fingerprint of herbal medicine for quality control. *J. Chromatogr*., A 10(02): 25.

Granier, F. 1988. Extraction of plant proteins for two-dimensional electrophoresis. *Electrophoresis*, 9: 712-718.

Herbert, B., M. Galvani, M. Hamdan and E. Olivieri. 2001. eduction and alkylation of proteins in preparation of two-dimensional map analysis: why, when, and how? *Electrophoresis*, 22: 2046.

Heukeshoven, J. and R. Denrick. 1985. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis*, 6: 103-112.

Ho, C.S., Y.H. Wong and K.W. Chiu. 1989. The hypotensive action of Desmodium styracifolium and Clematis chinensis. *American Journal of Chinese Medicine*, 17(3-4): 189-202.

Ishtiaq, M., A.S. Mumtaz, Y. Wang, Y.Y. Cheng, T. Mehmood and M. Ashraf. 2010a. Proteins as biomarkers for taxonomic identification of traditional chinese medicines (TCMs) from subsection rectae genus *Clematis* from China. *World Applied Sciences Journal* 8 (Special Issue of Biotechnology & Genetic Engineering): 62-70.

Ishtiaq, M., He Qing, P.G. Xiao and Y.Y. Cheng. 2007a. *Clematis huchouensis* Tamura: A traditional chinese herbal medicine and its quality control using a high performance liquid chromatography technique. *Biol. Pharm. Bull*., 30(1): 165-168.

Ishtiaq, M., P.G. Xiao, Q. He and Y.Y. Cheng. 2010b. Biosystematics and plant proteomics: Role of proteomics in plant phylogenetics analysis. *Pak. J. Biol. Sci*., 10(20): 3487-3496.

Ishtiaq, M., Q. He, Y.Y. Cheng and P.G. Xiao. 2006. Ethnobotany of medicinal plants from Tian Mu Shan biosphere reserve, Zhejiang Province, China. *Asian J. of Pl. Sci*., 5(4): 646-653.

Jensen, O.N., M. Wilm, A. Shevchenko and M. Mann. 1999. Proteomic analysis of differentially expressed *Chlamydia pneumoniae* genes during persistent infection of HEp-2 cells. *Methods Mol. Biol*., 112: 513-530.

Jonathan, M.S., R.G. James and B.E. Frederick. 2004. The Actin Intron-A Phylogenetically Informative DNA region in *Clematis* (Ranunculaceae), *J. SIDA*., 21(2): 879-886.

Karin, M., S. Benoit, C.F. Lydie, M. Zivy and H. Thiellement. 2001. Comparative proteomics to establish genetic relationships in the Brassicaceae family. *J. Proteomics*, 1: 1457-1462.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.

Li, R.W., G. David Lin, S.P. Myers and D.N. Leach. 2003. Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. In: d-D ProteomeAnalysis protocols. Aj. J. Link (ed) Methods. *J. Ethnopharmacology*, 85(1): 61-67.

Lum, H., K.L. Fung, P.Y. Cheung and M.S. Wong. 2002. Proteome of oriental ginseng Panax ginseng C. A. Meyer and the potential to use it as an identification tool. *Proteomics*, 2(9): 1123-1130.

Maria, T.G., N. Campostrini, Z. Lello, C. Corrado, I. Carlo and G.R. Pier. 2005. Two dimensional mapping as a tool for classification of green coffee bean species. *Proteomics*, 5: 710-718.

Mehmood, A., M.J. Jaskani, S. Ahmad and R. Ahmad. 2013. Evaluation of genetic diversity in open pollinated guava by iPBS primers. *Pak. J. Agri. Sci*., 50: 591-597.

Ndimba, B.K., S. Chivasa, J.M. Hamilton and W.J. Simon. 2003. Proteomic analysis of changes in the extracellular matrix of Arabidopsis cell suspension cultures induced by fungal elicitors. *Proteomics*, 3: 1047-1059.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.*, 250: 4004-4021.

Qiu, G., M. Zhang and Y. Yang. 1999. The antitumour activity of total saponin of clematis chinensis. *Journal of Chinese Medicinal Materials*, 22(7): 351-353.

Rightii, P.G. 1990. Immobilized pH gradients: *Theory and Methodology*, Elsevier, Amsterdam. *The Netherland*, pp. 206-220.

Rogers, W.J., G. Bezard, A. Deshayes and I. Meyer.1999. Biochemical and molecular characterization and expression of the 11S-type storage protein from *Coffea arabica* endosperm. *Plant Physiology and Biochemistry*, 37(4): 261-272.

Roh, G.S., Y. Shin, S.W. Seo and B.R. Yoon. 2004. Proteome analysis of differential protein expression in allergen induced asthmatic mice lung after dexamethasone treatment. *Proteomics*, 4: 3318-3327.

Rujin Chen, Pierre Hilson, John Sedbrook, Elizabeth Rosen, Timothy Caspar, A.M. Schuster and E. Davies. 1983. Ribonucleic Acid and protein metabolism in pea epicotyls : I. The aging process. *Plant Physiol.*, 73: 809-816.

Shevchenko, A., M. Wilm, O.Vorm and M. Mann. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem*., 68: 850-858.

Tamura, M. 1967. Morphology, ecology and phylogeny of the Ranunculaceae VII. *Sci. Rep. Osaka University*, 16-2: 21-43.

Theillement, H., M. Seguin, N. Bahrman and M. Zivy. 1989. Homoeology and phylogeny of the A, S, and D genomes of the triticinae, *J. Mol. Evol*., 29: 89-94.

Wang W., M.S., V. Rita, S. Antonia, S. Elisabetta, M. Silvia and C. Mauro. 2003. Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. *Electrophoresis*, 24: 2369-2375.

Wang W.T. 1998. Notulae de Ranunculaceis Sinensibus (ＸＸⅡ),Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds *Acta Phytotaxonomica Sinica*, 36(2) 2369-2375.

Wang, L.C., Y.H. Cao, X.P. Xing and J.N. Ye. 2005. Simultaneous chromatographic fingerprinting and quantitative analysis of flavonoids in Pollen Typhae by high-performance capillary electrophoresis. *Chromatographia*, 62: 283.

Wang, R.J. 1999. Flora of China, family Ranunculaceae. *J. Trop. Subtrop. Bot*., 7: 28.

Wang, W.T. 2003. A revision of *Clematis* sect. *Clematis* (Ranunculaceae). *Acta Phytotaxonomica Sinica*, 41: 1-62.

Wen, K.C., C.Y. Huang and F.L. Lu. 1993. Determination of baicalein and puerarin in traditional Chiense Medicines preparation by High performance Liquid Chromatography. *J. Chromatogr*., 631: 241.

Xu, R., W. Zhao, J. Xu, B. Shao and G. Qin. 1996. Studies on bioactive saponins from Chinese medicinal plants. *J. Adv. Exp. Med. Biol*., 404: 371-82.

Yung, B., S.M.Y., H.K. Miyuki, T. Norio, N. Hirotsugu, M. Toru, O.H. Masao. 2001. Inhibitory effects of Korean plants on HIV-1, phytotherapy research. 15: 481-486.

Zada, M., N. Zakir, M.A. Rabbani and Z.K. Shinwari. 2013. Assessment of genetic variation in Ethiopian mustard (*Brassica carinata* A. Braun) germplasm using multivariate techniques. *Pak. J. Bot*., 45(S1): 583-593.

Zhu, R.C. 1995. A report on medicinal plants used by Yao people in hunan. Hunanense. *Journal of Herbal Medicine*, 11: 26-28.

Zivy, M., E.M. Said and H. Thiellement. 1995. Distance indices in a comparison between the A, D, I and R genomes of the Triticeae tribe. *Electrophoresis*, 16: 1295-1300.

(Received for publication 30 November 2012)