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Protein extraction from grape tissues by two-dimensional electrophoresis

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Summary

At the onset of proteomic studies protein samples have to be accurately separated by two dimensional electrophoresis (2-DE); subsequently polypeptides are identified. Grape tissues, in particular roots, can be very problematic due to their hardness and to the high content of compounds that interfere in classical protein extraction. We have used a phenol-based extraction method in the presence of a protease inhibitor and Polyvinylpolypyrrolidone (PVPP). In this paper we demonstrate that this extraction method gives satisfactory and reproducible protein separation allowing the identification of some proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

K e y w o r d s : proteomics, protein extraction.

Introduction

Systematic analysis of all protein expression patterns and protein sequences in different tissues, cells and subcellular fractions, the so-called proteomic, will play an important role in studying many different aspects of plant function. In fact, since much of the regulation of physiological processes occurs post-transcriptionally, the measurement of protein expression by post-genomic approaches is essential to give a more accurate and comprehensive picture of cellular activity.

Traditionally, two-dimensional electrophoresis (2-DE) is the basic technology for proteomic analysis (O'Farrel 1975). With this tool (coupled with image analysis, spot identification by mass spectrometry and database searches) complex patterns of gene expression can be studied at protein level (Celis *et al*. 1998, Gygi *et al.* 2000). Successful 2-DE separation is a critical step in studying proteome dynamics and characterizing relevant proteins but, for an accurate 2-DE analysis, protein extraction and purification are still a technical bottleneck. While for bacteria, yeast and animal tissues a high protein resolution is routinely reached, this is not the case for plant material, even if important advances in sample preparation have recently been reported (Damerval *et al.* 1986, Vienne *et al.* 1988, Tsugita *et al*. 1994, Kamo *et al.* 1995, Klose *et al*. 1995, Giavalisco *et al.* 2003). The difficulty in obtaining high quality protein extracts from plant material is mainly due to the low concentration of proteins over fresh weight, to the high activity of proteases and to high levels of interfering compounds such as pigments, polyphenols, terpenes, tannins, flavonoids, lignans, and anthocyanidines. These compounds are coextracted with proteins and affect 2-DE resolution. Phenolic compounds, for instance, interact with proteins, leading to their oxidation and degradation and cause distorted 2-DE patterns with horizontal and vertical streaks (Granier 1988, Tsugita and Kamo 1999, Wang *et al.* 2003).

Grape tissue is recalcitrant because of the high levels of phenols and polysaccharides; furthermore roots are very ligneous and hard (Lodhi *et al.* 1994; Hmamouchi *et al*. 1996).

Recently the effectiveness of a phenol-based extraction method has been shown to give a good protein yield with different recalcitrant plant tissues, such as olive leaves, tomato tissues, avocado and banana mesocarp as well as orange albedo and flavedo (Hurkman and Tanaka 1986, Wang *et al.* 2003, Rose *et al.* 2004, Saravanan and Rose 2004).

Here we demonstrate a phenol-based extraction method for grape roots and leaves, giving a high quality pattern of spots suitable for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

We also report root and leaf protein identification, since proteins from this grape material previously have not been identified by 2-DE analysis.

Material and Methods

P l a n t m a t e r i a l : *Vitis vinifera* cv. Cabernet Sauvignon plants were grown under controlled climatic conditions in a greenhouse at 25/20 °C day/night temperature and with 70 % relative humidity. 30 scions of this genotype were put into soil for rooting during 30 d, after which they were transferred into a hydroponic Hoagland solution (2 mM $Ca(No_3)_2$; 0.1 mM KH_2PO_4; 0.75 mM K_2SO_4; 0.65 mM $MgSO_4$; $1x10^{-4}$ mM $MnSO_4$; $5x10^{-5}$ mM $CuSO_4$; $5x10^{-5}$ mM $ZnSO_4$). After about 30 d in hydroponic culture, the roots and leaves were collected and frozen in liquid nitrogen. The samples were stored at -80 °C until use.

P r o t e i n e x t r a c t i o n : Phenol extraction of proteins is based on the protocol described by Hurkman and Tanaka (1986). We started from 1 g of frozen tissue ground to powder in liquid N_2 using a mortar and pestle. 5 % (w/w) of Polyvinylpolypyrrolidone (PVPP) were added to powdered samples. Total proteins were extracted in 4 volumes (w/v) of buffer containing 500 mM Tris-HCl pH 8, 700 mM sucrose, 10 mM EDTA, 4 mM ascorbic acid, 0.4 % β-mercaptoethanol, 0.2 % Triton X-100, 1 mM PMSF (Sigma, St. Louis, MO,

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USA), 1 µM Leupeptin (Fluka, Stenheim, Germany), 0.1 mg·ml^{-1} Pefabloc (Fluka), then stirred for 30 min at 4 °C. After centrifugation at 13,000 g for 20 min at 4 °C, the supernatant was transferred into a new tube and sonicated 3 times for 10 s at minimum power with intervals of 30 s. An equal volume of phenol saturated with Tris-HCl 0.1 M pH 8 (Sigma) was added to the samples and the proteins were incubated for 2 h at 4 °C. The phases were separated by centrifugation at 5,000 g for 20 min at 4 °C, and the upper phenol phase was pipetted into a new tube. The bottom aqueous phase was back-extracted with 2.5 ml of extraction buffer and 2.5 ml of phenol. Proteins were precipitated by adding 5 volumes of cold 0.1 M ammonium acetate in methanol to the phenol phase, vortexing and incubating at -20 °C overnight. Precipitated proteins were recovered at 13,000 g for 30 min, and then washed three times with cold methanolic ammonium acetate and twice with cold 80 % acetone. The final pellet was dried and dissolved in IEF solubilization buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 50 mg·ml^{-1} DTT), by vortexing, sonication, and incubation for 1 h at room temperature. Samples were centrifuged at 20,000 g for 15 min at 4 °C to clarify. If not analyzed immediately by 2-DE, the samples were stored at -80 °C. Before electrophoretic analysis, protein concentration was measured by the Bio-Rad protein assay (Hercules, CA, USA) (Bradford 1976). Bovine serum albumin was used as standard.

For comparison, the powder of tissues was extracted with trichloroacetic acid (TCA) precipitation (Damerval *et al*. 1986, Görg *et al.* 2000, Tsugita *et al*. 1996) and dense SDS buffer (60 mM Tris-HCl pH 6.8, 2 % (β-mercaptoethanol, 20 % glycerol, 2 % SDS). The SDS extracts were subjected to acetone precipitation (Halloway and Arundel 1988) and recovered proteins were dissolved as above.

Electrophoresis: Proteins were first separated by isoelectrofocusing (IEF). Each sample, root or leaf, was tested in tripled experiments from three independent extractions, TCA, SDS and phenol respectivly. Proteins were separated by using gel strips forming an immobilized nonlinear 3-10 pH gradient (Immobiline DryStrip, 13 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) and a 4-7 pH gradient for leaves. Strips were rehydrated in the IPGphor system (Amersham Biosciences) for 1 h at 0 V, 20 °C and 10 h at 30 V, 16 °C with the solubilization buffer containing 7 M urea, 2 M thiourea, 4 % CHAPS, 1 % DTT, 0.8 % of an appropriate carrier ampholyte (3-10 NL, or 4-7, IPG buffer; Amersham Biosciences), bromophenol blue 0.005 % and the protein extracts. IEF was performed at 16 °C in the IPGphor system (Amersham Biosciences) for 4 h at 200 V, from 200 to 3500 V in gradient during 30 min, 3 h at 3500 V, from 3500 to 8000 V in gradient during 30 min, after which the run was continued at 8000 V to give a total of 70 kVh. Before the second dimension gel, each focused strip was equilibrated for 30 min against 6 M urea, 30 % glycerol, 2 % SDS, 50 mM Tris-HCl pH 8.8, 2 % DTT in order to resolubilise proteins and reduce disulphur bonds. The SH groups were then blocked, for 15 min, by substituting the DTT with 2.5 % iodoacetamide in the equilibration buffer. Following the mentioned equilibration, strips were placed on top of vertical gels, 12.5 % constant concentration polyacrylamide gel (acrylamide/N,N'-bisacryloylpiperazine (PDA) 12.5 %T, 2,6 %C, 0.375 M Tris-HCl pH 8.8, 5 mM sodium thiosulphate, TEMED 0.05 v/v, APS 0.1 % p/v). As molecular weight marker (6-175 kDa), the Prestained Protein marker Broad range (New England BioLabs) was run on the acidic hand side of each gel. A denaturing solution (0.5 % agarose in running buffer) was loaded onto gel strips. Electrophoresis was performed at 4 °C in a Laemmli running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1 % SDS) for 30 min at 15 mA·gel^{-1} then at 45 mA·gel^{-1} until the dye front reached the bottom of the gel. Two gels, 16 cm x 20 cm x 1.5 mm, were run in parallel (Protean II, Bio-Rad).

Protein staining and analysis of 2-D gels: Analytical gels (100 µg of protein loaded) were stained with ammoniacal silver-staining according to (Hochstrasser *et al.* 1988), while preparative gels (600 µg of protein loaded) were stained with Coomassie Brilliant Blue G-250 (CBB G) (Sigma) in accordance with manufacturer's instructions. Gel images were digitalized by ImageScanner (Amersham Bioscience). Image analysis was carried out with the ImageMaster 2-D Elite version 2003.02 software (Amersham Biosciences). For MS analysis, 10 spots from each gel (root 3-10NL pH and leaf 4-7 pH) were selected by qualitatively comparing silver and CBB stained 2-D gels (Most of these spots are different in Mw and pH coordinates and they are present in tripled gels, so we used them as a reference spot).

MALDI-TOF-MS and database search: The spots of interest were carefully excised from blue-stained 2-D gels using a scalpel and digested overnight with bovine trypsin as described by (Shevchenko *et al.* 1996) using an automatic protein digester (DIGEST-PRO; Abimed, Langenfeld, Germany) and then lyophilized. Mass spectra of the tryptic digests were acquired by Voyager-DE-PRO MALDI-TOF mass spectrometer (Perseptive Biosystems, Framingham, MA, USA), using the dried droplet technique and alfa-cyano-4-hydroxycinnammic acid (CHCA) as a matrix. Immediately prior to use, the samples were re-dissolved in 10 ml 50 % acetonitrile, 0.1 % TFA, and 0.5 ml of the solution was loaded onto the MALDI target. The data were processed using the Data Explorer software.

Proteins were unambiguously identified by searching against a comprehensive non-redundant sequence database (DB) using the program ProFound (http://prowl.rockefeller.edu) (Zhang and Chait 2000) or the MASCOT search engine (http://www.matrixscience.com) (Perkins *et al.* 1999).

Results and Discussion

For a reliable proteomic analysis the first critical step is the preparation of protein samples compatible with 2-DE. Each extraction strategy should take into consideration the nature of sample tissue. For plant material this is particularly true due to the high level and great variability of non-proteic contaminants among plant species.

Recent results (Sarry *et al*. 2004) validated the TCA/acetone precipitation for protein extraction in phenolic material, *e.g.* grape berry. However, for other recalcitrant plant material containing high level of phenols and soluble polysaccharides (as tomato tissues, banana and avocado

mesocarp, orange peel), the phenol method showed a high efficiency for protein extraction and resolution (Rose *et al.* 2004, Saravanan and Rose 2004). We have tried to establish a method of protein extraction from grape roots and leaves.

Roots are critical organs for plant survival since they belong to the first organs of the plant which are vulnerable to different forms of stress such as drought, anoxia, and high concentrations of salts or heavy metals in the soil, with consequent effects on plants growth, production and quality characteristics of the fruit. Successful 2-DE separation of grape root proteins would permit to monitor global changes that occur in the root proteome in response to these physiological and/or environmental stimuli (Thiellement *et al.* 1999, Chang *et al.* 2000, Ouerghi *et al.* 2000, Bahrman *et al.* 2004).

The procedure carried out was the phenol-based method (Hurkman and Tanaka 1986, Granier 1988, Meyer *et al.* 1988) The final pellet obtained was easily solubilized in the IEF solubilization buffer and we obtained a protein yield of about 0.8 mg for 1 g of fresh weight. An example of a 2-DE pattern obtained by phenol is shown in Fig.1.

The 2-DE gels with samples extracted by phenol revealed more protein spots than the other two methods tested (Fig.1). In silver-stained gels, more than 600 sharply focused spots (estimated by ImageMaster 2-D Elite, Amersham) were revealed ranging in the pH interval 3-10 and the kDa interval ranging from 100 to 15. In the same intervals, in blue-stained gels it was possible to visualize 300 well defined spots.

In comparison with the other two protocols, the quality of the spots was high; even the basic polypeptides and those in the low molecular weight region of the gel appeared as round spots, well resolved and not diffused. Moreover, the gel patterns obtained by phenol extraction had a greater number of high molecular weight proteins.

The high spot resolution, the low background and the absence of vertical staining (streaking and smearing) indicate that phenol sufficiently removed interfering compounds by partitioning them in the aqueous phase. Also the addition of PVPP contributed to the purification by phenol compound scavenging. The increased number of protein spots coupled with high quality staining in the acidic region of the gel also indicates a more efficient disaggregation of protein complexes.

The reproducibility of the results was excellent, as evaluated on the basis of three independent extractions and, probably, it is also due to the protease inactivation by phenol extraction in the presence of protease inhibitors. The high quality of the spots permits their analysis by MALDI-TOF-MS. The major drawback of the protocol is that it is time consuming, it takes 2 d. The effectiveness of our protocol was also evaluated for leaf tissue.

Leaves are easier to grind and, in fact, the extraction yield is clearly higher than that of roots: about 3 mg of protein from 1g of fresh weight. The gels obtained (Fig. 2) were excellent in spot number (500) and resolution.

Once the extraction of proteins has been improved, the main obstacle in the plant proteome analysis is to apply the mass spectrometry-based identification method to plant species without complete genome data. Since a limited number of grape sequences is available in the database

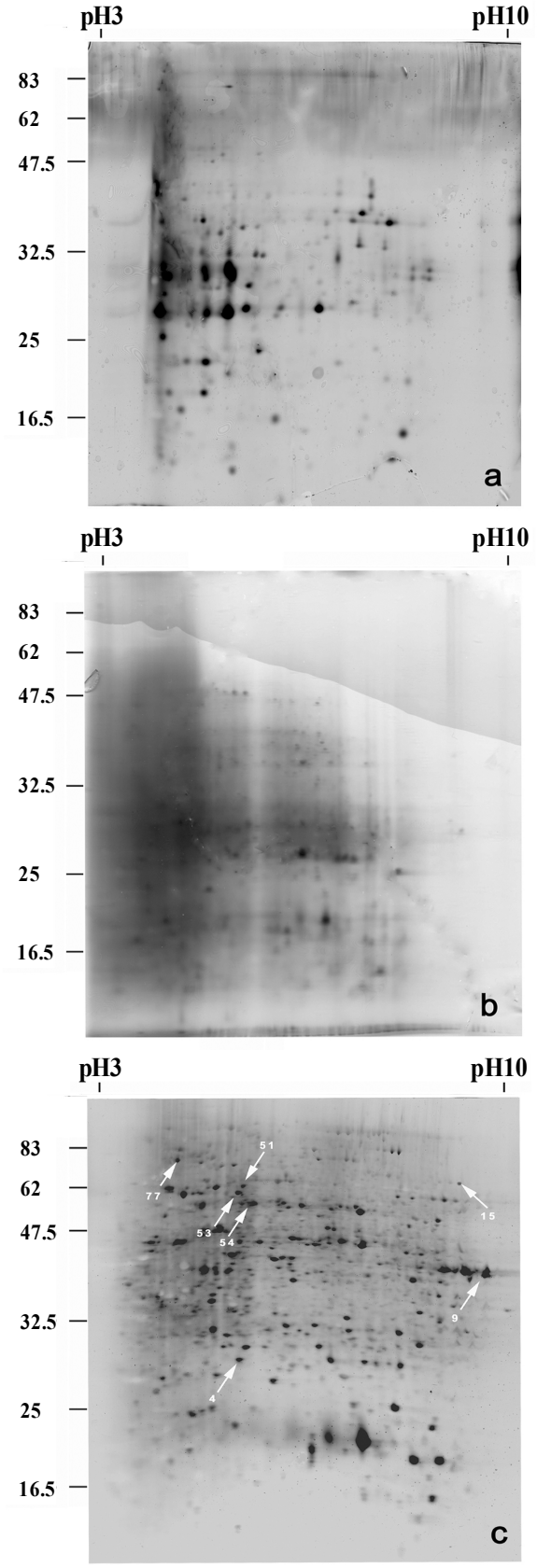

Fig. 1: 2-DE gels of grape root proteins extracted by different procedures: (a) TCA, (b) SDS buffer and (c) phenol. 100 µg of proteins are separated in the first dimension on an IPG strip (pH 3-10 NL) and in the second dimension on a 12.5 % acrylamide SDS-gel. The gels shown are silver-stained. The numbered spots were identified and the derived data are presented in Tab. 1. The size of protein standards are indicated on the left.

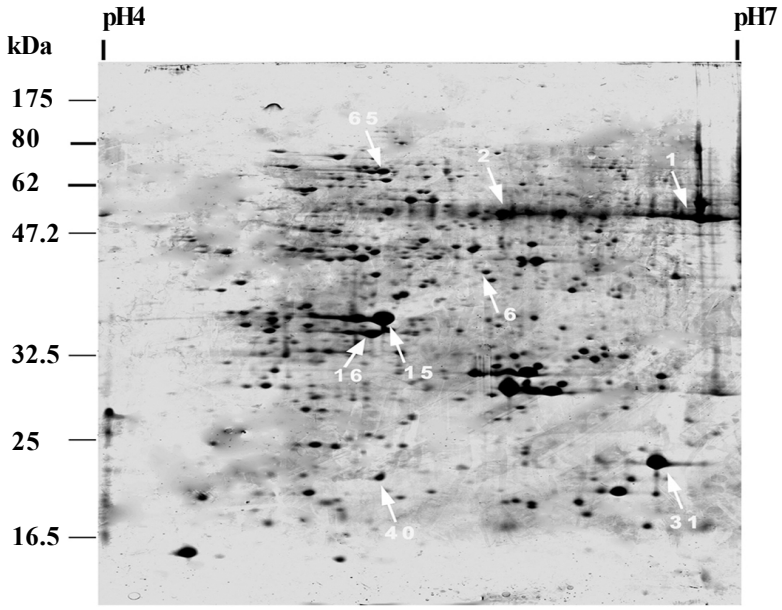

Fig. 2: 2-DE pattern of grape leaf proteins extracted using the phenol protocol. Proteins were separated in the first dimension on an IPG strip (pH 4-7). The gel was stained with CBB G. Other conditions as in Fig. 2. The numbered spots were identified and the derived data are presented in Tab. 2.

(about 500 hits in SWISS-PROT), protein identification was generally made by homology to proteins from other plants.

For protein identification by means of peptide mass fingerprints, we used Profound and MASCOT software using DB searches, including sequences from eukaryotic organisms or sequences from *viridiplantae* (green plants). In order to evaluate protein identification, we considered the percentage of sequence coverage (at least 15 %), the observation of distribution of matching peptides (authentic hit is often characterized by peptides that are adjacent to each other in the sequence and that overlap), the distribution of error (distributed around zero), the gap in probability and score distribution from the first to the other candidate. The unambiguously identified spots are listed in Tabs 1 and 2. The spots analyzed by MALDI-TOF-MS are a representative sample, with different Mw and pH coordinates, of proteins separated in our gels. It is evident from these preliminary investigations that the quality of proteins extracted by

Table 1

Root proteins identified on 2D gel (Fig. 1)

Spot No.	Protein	AC	Source of the maching protein	Peptides matched (n)	Sequence covered (%)	Apparent p/M_r	Calculated p/M_r
4	Peroxiredoxina antioxidant	AAF61460	*Brassica napus*	6	17	5.4/28.00	6.0/24.07
9	Kinesin related protein	CAA04956	*Arabidopsis thaliana*	7	21	9.0/44.00	8.0/44.21
15	Bifunctional aspartatokinase/ homoserine dehydrogenase precursor	P49080	*Zea mays*	13	38	8.5/66.00	6.8/100.40
51	Germacrene synthase short form	AAM21659	*Cichorium intybus*	18	43	5.5/64.00	5.0/64.52
53	S6	AAL87122	*Arabidopsis thaliana*	19	27	5.4/62.00	4.8/86.27
54	Terpene synthase	AAO18435	*Zea mays*	14	33	5.6/60.00	5.8/67.96
77	Heat shock protein	NP_200411	*Arabidopsis thaliana*	22	38	4.0/78.00	5.0/80.41

Table 2

Leaf proteins identified on 2D gel (Fig. 2)

Spot No.	Protein	AC	Source of the matching protein	Peptides matched (n)	Sequence covered (%)	Apparent p*I*/M_r	Calculated p*I*/M_r
1	Rubisco large subunit	AAL11894	*Prunus padus*	14	30	6.0/50.00	6.34/51.90
2	ATP synthase β subunit	CAB44232	*Muntingia calabura*	8	32	5.3/50.00	5.21/52.60
6	Rubisco Activase 1	AAG61120	*Gossypium hirsutum*	19	41	5.9/44.00	5.5/48.20
15	33kDa oxygen evolving protein of photosystem II	Q40459	*Tobacco*	12	40	5.3/35.00	5.9/35.38
16	33kDa oxygen evolving protein of photosystem II	NP_918587	*Oryza sativa*	13	38	5.2/33.00	6.0/35.0
31	Pathogenesis-related protein 10	CAC16165	*Vitis vinifera*	10	42	6.7/22.00	6.0/17.34
40	C2 domain containing protein	NP_565002	*Arabidopsis thaliana*	7	61	5.2/20.00	5.2/18.78
65	Heat shock cognate 70 kDa protein	P09189	*Petunia hybrida*	21	41	5.3/68.00	5.1/71.60

the phenol method is high for separation by 2-DE and subsequent MALDI identification of polypeptides. The proteins identified, in roots and leaves, prevalently are enzymes involved in the basic and secondary metabolism (spots 15, 51, 54 in roots, spots 1, 6, 15, 16 in leaves), energy metabolism (spots 2, 6, 15, 16 in leaves), regulation (spot 53 in roots, spot 40 in leaves). Other interesting polypeptides (spots 4, 77 in roots, spots 31, 65 in leaves) include protein classified as stress and defence proteins. As expected, the greater identified proteins were soluble proteins. But the buffer used is effective also for solubilization of some membrane associated proteins. In fact, an ATPase subunit and an oxygen-evolving complex were found. Instead, intrinsic membrane proteins are underrepresented on 2D gels due to their extreme hydrophobicity. Also low abundance proteins, like transcriptional factors, are not well recovered due to the intrinsic limits of the techniques.

Concluding Remarks

Phenol extraction allows optimal removal of phenolic compounds, nucleic acids and other contaminants, and prevents protease digestion, thus permitting a sufficient high protein yield. The resolution of these samples by 2-DE is of great quality. This is a good basis for characterizing the proteome in studies of remarkable applicative importance such as stress physiology of grape tissue.

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References

Bahrman, N.; Le Gouis, J.; Negroni, L.; Amilhat, L.; 2004: Wheat leaf proteome analysis using sequence data of proteins separated by two-dimensional electrophoresis. Proteomics **4**, 709-719.

Bradford, M.; 1976: Rapid and sensitive method for the quantisation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254.

Celis, J. E.; Ostergaard, M.; Jensen, N. A.; Gromova, I.; Rasmussen, H. H.; Gromov, P.; 1998: Human and mouse proteomic databases: Novel resources in the protein universe. FEBS Lett. **430**, 64-72.

Chang, W. W. P.; Huang, L.; Shen, M.; Webster, C.; Berlingame, A.L.; Roberts, J. K. M.; 2000: Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment, and identification of proteins by mass spectrometry. Plant Physiol. **122**, 295-317.

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

Giavalisco, P.; Nordhoff, E.; Lehrach, H.; Gobom, J.; Klose, J.; 2003: Extraction of proteins from plant tissues for 2-DE analysis. Electrophoresis **24**, 207-216.

Görg, A.; Obermaier, C.; Boguth, G.; Harder, A.; Scheibe, B.; Wildgruber, R.; Weiss, W.; 2000: The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis **21**, 1037-1053.

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

Gygi, S. P.; Corthals, G. L.; Zhang, Y.; Rochon, Y.; Aebersold, R.; 2000: Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. Proc. Natl. Acad. Sci. USA **97**, 9390-9395.

Halloway, P.; Arundel, P.; 1988: High-resolution two-dimensional electrophoresis of plant proteins. Anal. Biochem. **172**, 8-15.

Hmamouchi, M., Essafi, N., Lahrichi, M., Fruchier, A., Essassi, E. M.; 1996: Flavones and Flavonols in Leaves of Some Moroccan *Vitis vinifera* cultivars. Am. J. Enol. Vitic. **47**, 186-192.

Hochstrasser, D. F.; Harrington, M. G.; Hochstrasser, A. C.; Miller, M. J.; Merrill, C. R.; 1988: Improved separation and detection of proteins with retained background silver staining. Anal. Biochem. **173**, 424-435.

Hurkman, W. J.; Tanaka, C. K.; 1986: Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol. **81**, 802-806.

Kamo, M.; Kawakami, T.; Miyatake, M.; Tsugita, A.; 1995: Separation and characterization of *Arabidopsis thaliana* protein by two-dimensional gel electrophoresis. Electrophoresis **16**, 423-430.

Klose, J.; Kobalz, U.; 1995: Two-dimensional Electrophoresis of Proteins: An updated protocol and implications for a functional analysis of the genome. Electrophoresis **16**, 1034-1059.

Lodhi, M. A.; Guang-Ning, Y.; Weeden, N. F.; Reisch, B. I.; 1994: A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*. Plant Mol. Biol. Rep. **12**, 6-13.

Meyer, Y.; Grosset, J.; Chartier, Y.; Cleyet-Marel, J. C.; 1988: Preparation by two-dimensional electrophoresis of proteins for antibody production: Antibodies against proteins whose synthesis is reduced by auxin in tobacco mesophyll protoplasts. Electrophoresis **9**, 704-712.

O'Farrel, P. H.; 1975: High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**, 4007-4021.

Ouerghi, Z.; Rémi, R.; Ouelhazi, L.; Ayadi, A.; Brulfert, J.; 2000: Two-dimensional electrophoresis of soluble leaf proteins, isolated from two wheat species (*Triticum durum* and *Triticum aestivum*) differing in sensitivity towards NaCl. Electrophoresis **21**, 2487-2491.

Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S.; 1999: Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis **20**, 3551-3567.

Rose, J. K. C.; Bashir, S.; Giovannoni, J. J.; Jahn, M. M.; Saravanan, R. S.; 2004: Tackling the plant proteome: practical approaches, hurdles and experimental tools. Plant J. **39**, 715-733.

Saravanan, R. S.; Rose, J. K. C.; 2004: A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. Proteomics **4**, 2522-2532.

Sarry, J. E.; Sommerer, N.; Sauvage, F. X.; Bergoin, A.; Rossignol, M.; Albagnac, G.; Romieu, C.; 2004: Grape berry biochemistry revisited upon proteomic analysis of the mesocarp. Proteomics **4**, 201-215.

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

Thiellement, H.; Bahrman, N.; Damerval, C.; Rossignol, M.; Santoni, V.; De Vienne, D.; Zivy, M.; 1999: Proteomics for genetic and physiological studies in Plants. Electrophoresis **20**, 2013-2026.

Tsugita, A.; Kawakami, T.; Uchiyama, Y.; Kamo, M.; Miyatake, N.; Nozu, Y.; 1994: Separation and characterization of rice proteins. Electrophoresis **15**, 708-720.

Tsugita, A.; Kamo, M.; Kawakami, T.; Ohki, Y.; 1996: Two-dimensional electrophoresis of plant proteins and standardization of gel patterns. Electrophoresis **17**, 855-865.

Tsugita, A.; Kamo, M.; 1999: 2-D electrophoresis of plant proteins. Methods Mol. Biol. **112**, 95-97.

Vienne, de D.; Leonardi, A.; Damerval, C.; 1988: Genetic aspects of variation of protein amounts in maize and pea. Electrophoresis **9**, 742-750.

Wang, W.; Scali, M.; Vignani, R.; Spadafora, A.; Sensi, E., Mazzuca, S.; Cresti, M.; 2003: Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. Electrophoresis **24**, 2369-2375.

Zhang, W.; Chait, B. T.; 2000: Profound: An Expert System for Protein Identification Using Mass Spectrometric Peptide Mapping Information. Anal. Chem. **72**, 2482–2489.

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