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Evaluation of protein extraction methods for enhanced proteomic analysis of tomato leaves and roots

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ABSTRACT

Proteomics is an outstanding area in science whose increasing application has advanced to distinct purposes. A crucial aspect to achieve a good proteome resolution is the establishment of a methodology that results in the best quality and wide range representation of total proteins. Another important aspect is that in many studies, limited amounts of tissue and total protein in the tissue to be studied are found, making difficult the analysis. In order to test different parameters, combinations using minimum amount of tissue with 4 protocols for protein extraction from tomato (*Solanum lycopersicum* L.) leaves and roots were evaluated with special attention to their capacity for removing interferents and achieving suitable resolution in bidimensional gel electrophoresis, as well as satisfactory protein yield. Evaluation of the extraction protocols revealed large protein yield differences obtained for each one. TCA/acetone was shown to be the most efficient protocol, which allowed detection of 211 spots for leaves and 336 for roots using 500 µg of leaf protein and 800 µg of root protein per gel.

Key words: bidimensional electrophoresis, proteins, protocol optimization, tomato.

INTRODUCTION

The amplification of gene action in an eukaryotic cell is mostly due to the existent variety of translational and post-translational processes, which can form a great number of proteins (isoforms) from a single gene (Wouters et al. 2013). The final result is a highly specific pool of proteins differing among cell types and conditions to which they are exposed. In order to study the translational results of any given condition, proteomic techniques are being widely used (Rose et al. 2012, Arruda et al. 2013). However, two-dimensional electrophoresis (2-DE) is not a simple technique and according to the sample preparation, the

results can be quite different indicating the need for well optimized procedures that include repeatability and reproducibility (Barbosa et al. 2013, Tabb 2013).

With regard to the plant material, each tissue has its own peculiarities, for instance, when targeting the root tissue, protein concentration is a crucial problem when compared to leaf tissue, whereas the latter presents large amounts of nonprotein components or interferents, which can compromise two-dimensional gel electrophoresis (2-DE) separation (da Silva et al. 2009).

Sample preparation, which is crucial for proteomic analysis, consists of 3 major steps: protein extraction, removal of interferents and protein resolubilization in a buffer solution (Tabb 2013).

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Although there are several sample preparation protocols available in the literature (Palma et al. 2011, Rodrigues et al. 2012), protocols specifically designed for tomato species (*Solanum lycopersicum* L.) are not well defined.

Tomato is an economically relevant crop of great importance in the human diet, and is a major crop plant and a model system for fruit development (Gratão et al. 2012, TGC 2012). The tomato cultivar Micro-Tom (MT) has been successfully used as a model plant to explore several aspects of plant development due to important characteristics such as a short developmental cycle (Meissner et al. 1997). Additionally, a MT mutant collection with other tomato lines are available at “HCPD-Lab Micro-Tom Mutants”, which has been explored for different features, hormonal (Monteiro et al. 2011, Carvalho et al. 2013) and metal (Gratão et al. 2009, Dourado et al. 2013, 2014, Nogueirol et al. 2015) responses and biochemical and histological characterization (Monteiro et al. 2012). However, since the complete tomato genome sequence was published (TGC 2012), reports have focused on genetic and genome features and little information with a focus on proteome is available.

Due to the importance of comprehensive proteomic studies, this study reports the evaluation of 4 main protocols available for tomato leaves and roots protein extraction for optimal two-dimensional electrophoresis. In spite of the fact that we selected total protein extraction protocols, to our knowledge, this methodological study may help other researchers to use the established method for different purposes not only for tomato species, but also for pepper, eggplant and so forth.

MATERIALS AND METHODS

PLANT MATERIAL

Tomato plants (*Solanum lycopersicum* L.) – MT cultivar - were cultivated in a greenhouse (Piracicaba, Departamento de Genética – ESALQ/USP) in trays with vermiculite at average temperatures of 18 to

24 °C approximately (night and day, respectively). Irrigation was carried out daily using tap water until germination (15 days approximately). Thereafter, the plants were transferred to a hydroponic system using Hoagland and Arnon (1950) solution, initially at a low concentration (10%), which was increased daily to 50% so that the plants would best adapt to this salt solution. A total of 5 plants were harvested (30 days of age approximately), pooled and immediately frozen in liquid nitrogen and stored at -80 °C.

Reagents used in electrophoretic separation (acrylamide, DTT, iodoacetamide, urea, thiourea, ampholytes, strips for isoelectric focusing and mineral oil) were from Bio-Rad (Hercules, CA, USA) and GE healthcare (Freiburg, Germany). All other chemicals (including mineral acids, reagents for buffers preparation and gel stain) were obtained from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany). All solutions were prepared with deionized-autoclaved water (≥ 18.2 M Ω cm) using a Milli-Q water purification system (Millipore, Bedford, USA).

PROTEIN EXTRACTION

The plant tissues (roots and leaves) were grounded in a mortar with liquid nitrogen added with 10% PVPP. Five hundred mg of fresh leaf and 1.5 g of fresh root were weighed in ice-cold centrifuge tubes (3 replicates) to which the extraction buffer solution (Table I) was added and homogenized (each protocol used a different period and method). As an alternative procedure, although we did not employ it in this study, the extractor buffer can be directly added to the mortar containing the sample, which might result in better protein extraction efficiency. The 4 different protocols were tested as follows:

Protocol 1 – TCA/Acetone method: Ten mL of ice-cold precipitation solution (Table I) were added to the plant tissue in centrifuge tubes immersed in ice (40 mL capacity) and briefly homogenized in vortex. Tubes were placed in the freezer at -20 °C for

2h. The remaining insoluble material was removed by centrifugation at 21,000 x g at 4 °C for 30 min. The pellet obtained was washed twice with 10 mL of ice-cold solution (0.07% β -mercaptoethanol (β -ME) in acetone) with 2h intervals at -20 °C and

then centrifuged at 21,000 x g at 4 °C for 30 min. The supernatant was gently discarded in order to maintain the pellet integrity. The sample was then placed in a vacuum desiccator at 4 °C for 2 days for complete drying.

TABLE I
Description of buffer composition and volume used.

Extraction Buffer	Composition	Volume used
TCA/Acetone (P1) Xu et al. (2008)	10% Trichloroacetic acid (TCA); 0.07% β -ME in Acetone P.A.	10 mL
Phenol (P2) Amalraj et al. (2010)	0.7 M Sucrose; 0.5 M Tris; 50 mM EDTA; 1 mM PMSF ⁽¹⁾ ; 2% β -mercaptoethanol (β -ME); phenol ⁽²⁾ , pH 7.5	15 mL
Tris /TCA (P3) Lee et al. (2011)	100 mM Tris; 5 mM DTT; 1 mM EDTA; 1 mM PMSF ⁽¹⁾ ; TCA in Acetone ⁽³⁾ , pH 8.5	8 mL
Tris-base (P4) Xu et al. (2008)	40 mM Tris; 5 M Urea; 2M Thiourea; 2% CHAPS; 5% PVP; 2% β -ME	4 mL

⁽¹⁾ Diluted in 0.5 mL isopropanol; ⁽²⁾ 15 mL per tube added after 30 minutes allowing soluble proteins to migrate from the aqueous buffer to phenolic phase; ⁽³⁾ 30 mL per tube added afterwards to the supernatant in order to wash extraction residues and precipitate the proteins.

Protocol 2 – Phenol method: Tubes with sample and extraction solution (Table I) immersed in ice were homogenized in a shaker at 70 rpm for 30 min. Buffered phenol (15 mL) was added to each tube immersed in ice and left for 30 min in a shaker. Samples were centrifuged at 10,000 x g at 4 °C for 30 min and the supernatant transferred to another tube. Two mL of phenol and 15 mL of extraction solution were added to the tubes and homogenized in a shaker for 30 min inside foam with ice. Another step of centrifugation was performed. The phenol extract was transferred and divided into 2 new tubes and the tube volume was completed with 0.1 M ammonium acetate (in 100% methanol), mixed and incubated at -20 °C overnight. The mixture was centrifuged at 16,000 x g for 20 min at 4 °C and the supernatant discarded. Pellets were covered with 40 mL 0.1 M ammonium acetate in 100% methanol. The samples were left to precipitate for 2 to 3h at -20 °C and then centrifuged at 16,000 x g for 20 min at 4 °C.

This step was repeated twice and then the pellet was left to dry in a vacuum desiccator at 4 °C for 2 days.

Protocol 3 – Tris/TCA method: Extraction solution (Table I) was added to the samples and the tubes were agitated for 15 min in a shaker with ice. The mixture was centrifuged at 12,000 x g for 30 min at 4 °C. The supernatant was divided into 2 new tubes and to each tube 30 mL of 20% TCA in acetone was added, mixed and incubated overnight at -20 °C. The samples were centrifuged at 21,000 x g for 30 min at 4 °C and the supernatant discarded. Pellets were washed with an ice-cold solution of 0.07% β -ME in acetone and then left to dry in a vacuum desiccator at 4 °C for 2 days.

Protocol 4 – Tris-base method: Four mL of extraction solution (Table I) were added to the plant tissue, briefly homogenized in vortex and centrifuged at 12,000 x g for 15 min at 4 °C. The supernatant was recovered and 16 mL of washing solution (0.07% β -ME in acetone) was added. The

mixture was incubated for 2h minimum at -20 °C and then centrifuged at 12,000 x g for 15 min at 4 °C. This step was repeated twice to properly wash the pellet, and then the tubes were left to dry in a vacuum desiccator at 4 °C for 2 days. Regardless of the protocol, in order to speed up the drying process we suggest drying the spots using nitrogen gas (ca. 1 min).

To guide the reader, a schematic representation of the 3 methodologies is presented in Figure 1.

PROTEIN SOLUBILIZATION AND QUANTIFICATION

After the pellets were completely dried, proteins were solubilized according to Amalraj et al. (2010) with a solubilization buffer (SB) consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG

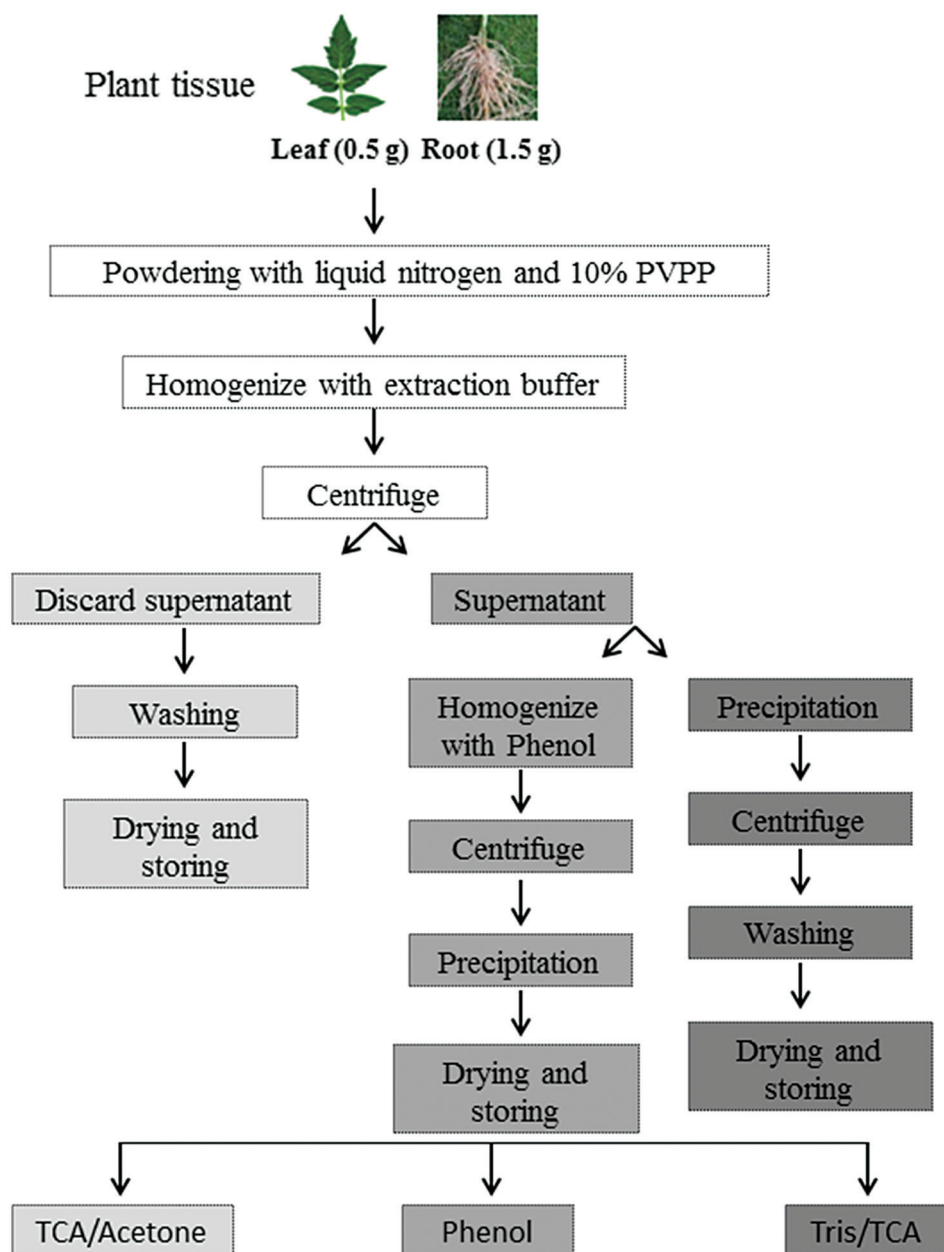


Figure 1 - Schematic outline of the 3 protein extraction methods tested.

buffer and 0.3% DTT. SB solution (2.0 mL – TCA; 450 μ L – Phenol; 300 μ L – Tris/TCA; and 300 μ L – Tris-base for 0.5 g of leaves, and 2.5 mL – TCA; 450 μ L – Phenol; 300 μ L – Tris/TCA; and 400 μ L – Tris-base for 1.5 g of roots) was added to the pellets and the mixture was vigorously stirred for 3 min and sonicated in cold water for 20 min for homogenization. The samples were centrifuged for 15 min at 21,000 \times g at 4 °C, and the supernatant was recovered and transferred to a microtube, where it was additionally centrifuged for 10 min at 14,000 rpm at 4 °C to separate cell debris. After this second centrifugation the supernatant was transferred to a new microtube and stored at -80 °C for further analysis. Total soluble protein concentration for all samples was determined by the Bradford method (1976) using bovine serum albumin as standard.

TWO-DIMENSIONAL GEL ELECTROPHORESIS

First-dimension separation was carried out using immobilized pH gradient IPG strips for isoelectric focusing – IEF (18 cm length, pH 3-10 NL, Immobiline DryStrip, GE Healthcare, Freiburg, Germany), rehydrated overnight with 2-DE buffer (7 M Urea, 2 M Thiourea, 4% Chaps, 0.5% Triton X-100, 50 mM DTT, 2% IPG and 0.5% Bromophenol blue) containing 500 μ g and 800 μ g of leaf and root protein, respectively, resulting in a final volume of 375 μ L. IEF was performed at 20 °C using an Ettan IPGphor 3 IEF system (GE Healthcare, Freiburg, Germany) testing 2 protocols:

- a) Amalraj et al. (2010) modified nonlinear increase – 4h rehydration, step-*n*-hold at 30 V for 12h (step 1), step-*n*-hold at 100 V for 1h (step 2), step-*n*-hold at 200 V for 1h (step 3), step-*n*-hold at 400 V for 1h (step 4), step-*n*-hold at 700 V for 1h (step 5), step-*n*-hold at 1000 V for 1h (step 6), step-*n*-hold at 5000 V for 10h (step 7), step-*n*-hold at 8000 V for 4h (step 8), step-*n*-hold at 100 V for 3h (step 9);
- b) Saravanan and Rose (2004) modified nonlinear increase – 1h rehydration, 0 to 30 V over 12h (step 1), 30 to 150 V over 1h (grad 2), 150 to 300 V over 1h (grad 3), 300 to 500 V over 1h (grad 4), 500 to 3000 V over 2h (grad 5), 3000 to 6000 V over 2h (grad 6), 6000 to 10000 V over 2h (grad 7), and then held at 10000 V for 7h (step 8).

After isoelectric focusing, the proteins were reduced with 6 mL per strip of 0.05 M Tris-HCl (pH 8.8) containing 6 M Urea, 30% Glycerol, 2% SDS and 1% DTT. The proteins were then alkylated with 6 mL per strip of 0.05 M Tris-HCl (pH 8.8) containing 6 M Urea, 30% Glycerol, 2% SDS, 2.5% Iodoacetamide and 0.002% Bromophenol blue, both with gently agitation at room temperature for 15 min.

The second dimension separation was performed in a 12.5% polyacrylamide gel (GE Healthcare). Strips already equilibrated were horizontally accommodated at the top of the gels, sealed with 0.5% agarose solution and then placed in a gel cell with buffer solution (0.025 M Tris, 0.192 M glycine, 0.1% SDS – pH 8.3). Electrophoresis was conducted at 4 °C using 15 mA per gel for 30 min, followed by an amperage increase to 30 mA per gel for 7h (Laemmli 1970).

Subsequently, proteins were fixed for 1h in a solution containing 40% ethanol and 10% acetic acid prior to staining with 5% (w/v) Coomassie blue G-250 for 12h (Candiano et al. 2004) under gentle agitation. Gels were then washed 3 times with Milli-Q water and immediately digitalized.

IMAGE ANALYSES

Images were digitalized in a scanner with *LabScan* 5.0 version software (Amersham Biosciences) and analyzed with the *Image Master 2D Platinum* 7 (GE Healthcare) software. All images were subjected to automatic detection of spots and to contrast settings, smooth (value: 7 – leaves; 6 – roots), saliency (value: 100 – leaves; 120 – roots)

and minimum area (value: 5 – leaves; 4 – roots). Subsequently, a manual curation was performed to confirm the veracity of spots, and to improve alignment between gel replicates for subsequent comparison between treatments.

RESULTS

The results (Fig. 2) showed that the Amalraj et al. (2010) IEF protocol was not suitable for the tomato

species. The gels obtained with the Saravanan and Rose (2004) IEF protocol exhibited a higher number of spots, which were also better defined and more intense in comparison to the other IEF protocol.

Among the 4 protein extraction methods evaluated, TCA/Acetone resulted in the highest protein yields for both leaves and roots (Table II). Although presenting lower protein concentrations (mg mL^{-1}) in relation to the other 3 methods, the

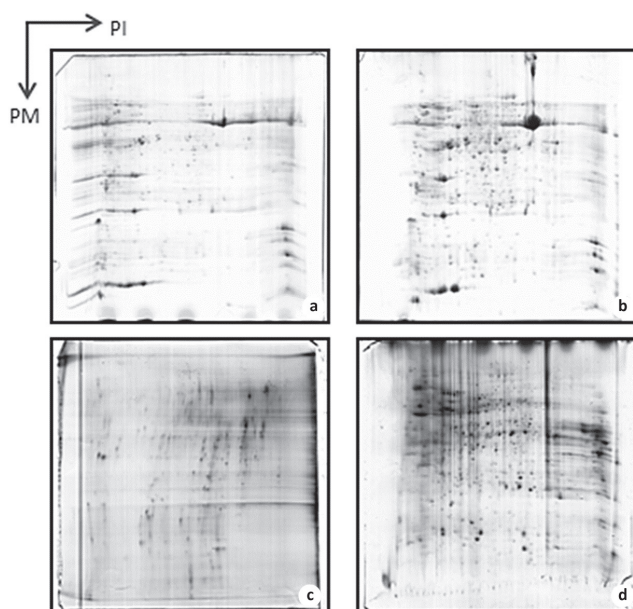


Figure 2 - 2-DE gel electrophoresis profiles of tomato cv. MT leaves (**a** and **b**) and roots (**c** and **d**) obtained with TCA/Acetone resolved within an IEF pH range of 3-10. (**a**) and (**c**) were focused with Amalraj et al. (2010) IEF protocol; (**b**) and (**d**) were focused with Saravanan and Rose (2004) IEF protocol.

TABLE II
Mean quantification of total soluble proteins and protein yield using different extraction methods.

Extraction method	Total soluble protein (mg mL^{-1})		Protein yield (mg gfw^{-1})	
	Leaves	Roots	Leaves	Roots
TCA/Acetone	4.24 a	1.84 b	16.96	3.06
Phenol	10.60 ab	4.90 a	9.54	1.47
Tris/TCA	14.24 a	2.73 b	8.55	0.55
Tris-base	8.07 b	2.84 b	4.84	0.76

Means followed by the same letter in the same column did not differ statistically by Tukey test at 5% probability of error.

higher values of protein yield (mg fw^{-1}) were considered far more significant.

The Tris-base protocol (protocol 4) was tested several times and yielded suitable values of protein concentration, however, the extracts presented a large viscosity (gelatinous dark mass), which impaired sample isofocalization and gel separation. Such result did not allow any further use of this protocol, which was discarded from this study.

Gel images (Fig. 3, Fig. 4) enabled the observation of most proteins extracted with TCA/Acetone

and Tris/TCA methods, as well as spot abundance and intensity. For both tissues, the majority of protein spots were present mainly in the 4-7 pH range of 2-DE.

There was no significant difference between numbers of protein spots (Table III) found for each extraction method.

TABLE III
Mean number of spots obtained from three replicates \pm standard deviation for each extraction method.

Extraction method	Spots	
	Leaves	Roots
TCA/Acetone	211 \pm 06	336 \pm 28
Phenol	214 \pm 22	329 \pm 00
Tris/TCA	248 \pm 33	356 \pm 41

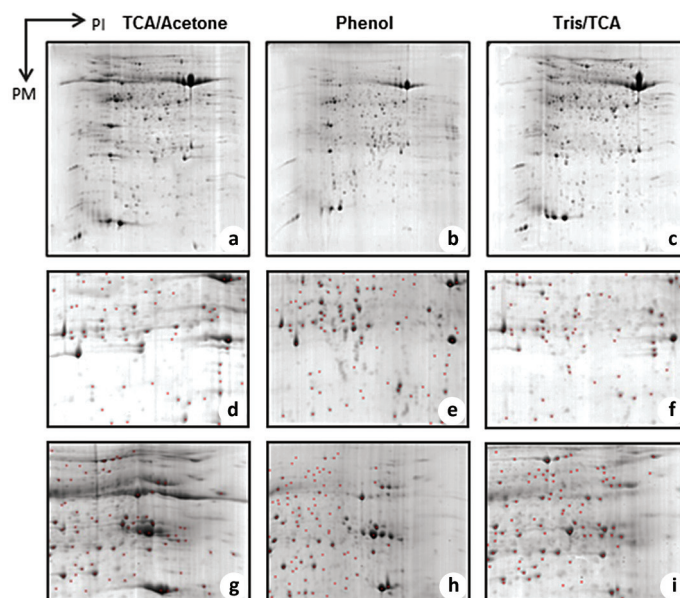


Figure 3 - 2-DE gel electrophoresis profiles of tomato cv. MT leaves obtained with TCA/Acetone, Phenol and Tris/TCA protocols (a, b and c) respectively – resolved within an IEF pH range of 3-10. Representative gel regions zoomed to compare more closely differences in the extraction protocols (d to i).

DISCUSSION

The amount of fresh tissue to be used depends on the type of experiment to be carried out and may be a major obstacle in the reproducibility of a proteomic analysis. The most critical steps are protein extraction and sample preparation, especially in plants, which may present low protein content and produce several types of secondary metabolites (Luque-Garcia et al. 2011). Moreover, in a recent editorial paper published by Azevedo (2012), the “omics” procedures that are now available and largely employed by Brazilian research groups for a number of organisms, and other emerging

techniques and their integration with different analytical techniques (metabolite measurements, gene expression analyses, enzymatic studies, etc) are essential to address key biological issues.

In previous studies extraction protocols have been tested (Sheoran et al. 2009, Pavoković et al. 2012, Rose et al. 2012). In this work we used the minimum plant material required (0.5 g leaf and 1.5 g root) in all extractions tested to achieve a certain standard level, whereas in other papers higher amounts of material to perform the same technique were normally used (Saravanan and Rose 2004, Persia et al. 2008, da Silva et al. 2009,

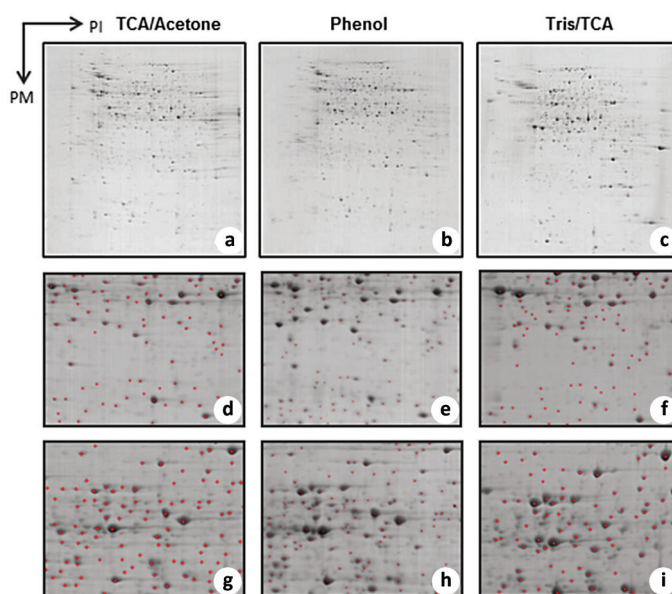


Figure 4 - 2-DE gel electrophoresis profiles of tomato cv. MT roots obtained with TCA/Acetone, Phenol and Tris/TCA (**a**, **b** and **c**) respectively – resolved within an IEF pH range of 3-10. Representative gel regions zoomed to compare more closely differences in the extraction protocols (**d** to **i**).

Kim et al. 2013). We developed a standardized 2-DE protocol to obtain accurate protein maps for tomato leaves and roots, starting from a minimum amount of plant tissue sufficient to perform gels in triplicates or quadruplicates, in association with a relatively simple method of extraction.

According to the gel images (Fig. 2), the Saravanan and Rose (2004) IEF protocol was much more efficient in separating tomato proteins in relation to that reported by Amalraj (2010), exhibiting a higher number of spots, which were also well defined and more intense, therefore, it was chosen to be used in all protein extractions henceforth. Perhaps this observed difference is due to the first protocol being based on a more smooth changing of voltage, which caused the tomato proteins to be more conserved.

Some modifications were applied to the IEF protocol described by Saravanan and Rose (2004), which resulted in approximately 200-400 well-resolved spots. It was also possible to notice that

most spots were resolved within the 4-7 pH range. Our results are supported by Roan et al. (2013), who also found increased resolution of 2D gels by using pH 4-7 isoelectric focusing gels for papaya seeds. Therefore, for further analysis of tomato proteome we recommend the use of strips within this range for isofocalization.

In a primary analysis, by visually comparing the gels obtained for the 3 extraction methods, it is possible to infer that the Phenol method (Protocol 2) resulted in a smaller diversity of spots for both leaves and roots in relation to the TCA/Acetone and Tris/TCA methods (Protocols 1 and 3, respectively). However, according to the statistical analysis applied (Tukey test – 5% probability of error) the number of spots did not differ significantly among the 3 protocols.

It is known that the acetone based methods efficiently concentrate proteins while removing salts, sugars and some lipids (Pavličević et al. 2013) and is also relatively faster than the Phenol method.

However, a few lipids, such as phenolic compounds, remained as contaminants, interfering with the isoelectric focusing and causing some horizontal streaking (Fig. 3a, c). An important alteration in the TCA/Acetone protocol was to maintain the extraction cold during all steps, in order to prevent hydrophobic interactions and formation of complexes, thus enhancing the sample solubilization. We believe this to be one of the reasons why this method exhibited a wider range of protein types.

Although the Phenol method maximizes the elimination of interferents rendering sharply defined spots (Sheoran et al. 2009), it is quite time consuming and exhibited a smaller range of spots for tomato leaves and roots (Fig. 3, Fig. 4). The lower number of spots (except compared to TCA/Acetone for leaves) could be explained by lower detection of less abundant proteins (Lee et al. 2010).

The Tris/TCA method exhibited higher number of spots, but its yield was far from satisfactory, especially when taken into account the reproducibility with limited amount of material. With this method, we observed high values of total soluble proteins (Table II), but according to Sheoran et al. (2009) this value may be altered by the presence of contaminants and little peptides.

The analysis of protein yield showed that the TCA/Acetone method was the most efficient for both tomato leaves ($16.96 \text{ mg gfw}^{-1}$) and roots (3.06 mg gfw^{-1}). In a comparison study of 4 extraction methods for soybean, Natarajan et al. (2005) reported that the TCA/Acetone method exhibited the highest protein resolution and spot intensity of all proteins, corroborating our results. Additionally, several less abundant and high molecular weight proteins were clearly resolved and strongly detected using the TCA methods. These results led us to elect this as the most suitable protocol for performing 2-DE analysis in tomato. Although the TCA/Acetone method was found to be the most efficient method, we suggest further downstream analysis, such as protein identification

by mass spectrometry to confirm the efficiency of the protein extraction method, once Sheoran et al. (2009) reported MOWSE score variation, which occasionally results in non-significant protein identification for the same spot according to the protein extraction method employed.

Based on results obtained under the conditions tested, we concluded that the protocol using TCA/Acetone extraction resulted in the best extraction efficiency and greater protein separation using two-dimensional gel electrophoresis. Another advantage is that this protocol is the less time consuming among the ones tested and is now standardized for limited amounts of tomato plant tissue. Therefore, the use of TCA/Acetone extraction protocol combined with the IEF protocol described here for extraction and separation of tomato cv MT proteins may facilitate tomato proteomic research and guide protocols for other complex plant materials. Additionally, the suggested proteomic method approach may be used as a starting point for proteomic studies of other related plant species.

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RESUMO

A proteômica é uma área incrível da ciência cujas aplicações vêm avançando para diversos propósitos. Um aspecto crucial para se alcançar boa resolução proteômica é o estabelecimento de uma metodologia que resulte na melhor qualidade e ampla representação das proteínas totais. Outro aspecto importante é que em muitos estudos quantidades limitadas de tecido e de proteínas totais no tecido a ser estudado são encontradas, tornando difícil a análise. Com o objetivo de testar diferentes parâmetros, combinações usando mínimas quantidades

de tecido com 4 protocolos para extração protéica de folhas e raízes de tomateiro (*Solanum lycopersicum* L.) foram avaliadas com atenção especial para a capacidade de remover interferentes e atingir resolução adequada em eletroforese bidimensional, bem como rendimento satisfatório de proteínas. A avaliação dos protocolos de extração revelou grandes diferenças na quantidade de proteínas obtidas por cada um. O protocolo TCA/acetona mostrou ser o protocolo mais eficiente, que permitiu a detecção de 211 “spots” para folhas e 336 para raízes utilizando 500 µg de proteína de folhas e 800 µg de proteínas de raiz por gel.

Palavras-chave: eletroforese bidimensional, proteínas, otimização de protocolo, tomateiro.

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