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ORIGINAL ARTICLE

Comparative evaluation of extraction methods for total proteins from *Crocus sativus* L. (*Saffron*)

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Abstract Broadly speaking proteomic studies are one of the various techniques of utmost importance for understanding complex biological processes that occur under inductive conditions and revealing the multidimensional aspects of *Crocus sativus* in biological systems. In order to get an insight into the molecular changes and to characterize the variations in protein expression of *C. sativus*, a detailed proteomic analysis on one-dimensional gel electrophoresis is one of the basic steps to accomplish. We have compared total protein profiles of *C. sativus* extracted by three different recipes and analyzed on 10% sodium dodecyl sulfate polyacrylamide gels. Gels were subjected to densitometric analysis for further characterization. Among three different protocols NP-40 extraction buffer recipe resulted in the extraction of proteins most efficiently with minimum background and streaking. There was maximum solubilization of proteins with high efficiency. Such a profile can be used for high precision analysis of differential protein expression. This work is an attempt to assist researchers in effective extraction of proteins from *C. sativus*. As a researcher faces a perplexing array of choices as where to start we describe a method based on our collective analysis of the different protein protocols. This paper presents a method that could be applied at the outset of any proteomic study.
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1. Introduction

Crocus sativus commonly known as saffron is an autumn-flowering herbaceous perennial cormous plant from the family Iridaceae of order Asparagales, Monocotyledonae. The plant grows to a height of 20–30 cm, and sprouts 5–11 white and non-photosynthetic leaves known as cataphylls (Mehraj et al., 2015). *C. sativus* is believed to have evolved possibly from an eastern Mediterranean autumn-flowering *Crocus cartwrightianus*, also known as 'wild saffron'. *C. sativus* is believed to have

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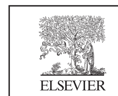

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been used initially as a spice. Persian is the first language in which using of saffron for cooking is recorded thousands of years ago. It is believed that word saffron is from the Latin word Safranum or French term Safran which itself is derived from the Persian word Za'feran. Saffron as it is commonly called, is the most precious and most expensive spice in the world derived from the stigma of the flower of *C. sativus* plant (Hill, 2004). Progressive research into biochemical properties of various components of plant has shown that it contains many plant derived chemical compounds with several biological activities. It is found to have antioxidative, anti-inflammatory, immune modulator, anti depressant and antiproliferative properties and many other medicinal uses. Constituents such as crocin found in saffron are found to have anti-cancer activities against leukemia, osteosarcoma, fibrosarcoma and ovarian carcinoma cells (Escribano et al., 1996; García-Olmo et al., 1999; Neghi, 1999; Abdullaev, 2002). It was recently found that saffron extract aids in maintaining body weight, hemoglobin levels and leukocyte counts during chemotherapy (Niar et al., 1991). Saffron is one of the most important plant species facing various constraints such as non-availability of good quality corms, poor soil fertility, anthropogenic pollution from brick kilns, automobile exhaust, cement factories etc, cement factories being the most important sources of pollution responsible for destroying saffron and reducing its yield (Jan and Bhat, 2006; Rafiq et al., 2008; Jan, 2009). Exposure to various pollutants for prolonged time leads to serious irreversible damages to plants causing progressive reduction in the photosynthetic ability, reduction in growth and productivity .The above facets of *C. sativus* has made it imperative to make various genomic and proteomic approaches for understanding and revealing its multidimensional biological system and to unveil the most probable target by which we can overcome the impact of environmental stress on its production and yield. Despite its biological significance and agricultural importance there isn't enough information regarding the mechanism of action of saffron as potent drug and how it works in multiple biological aberrations. Most saffron-related research has been carried out in relation to the stigmas, but this is often not fully and clearly expressed in research papers. Since genomic information available is limited in most cases proteomic studies provide a breakthrough for studying different aspects of organism, proteomic studies are deemed to be equivalent to a fully sequenced genomic study. If the quality and quantity of protein extracted is consistent and the samples are rich in protein diversity, relevant biological conclusions can be drawn from the data under study. Thus a highly reproducible protein extraction method is a fundamental requirement to ascertain differences in protein concentrations which are difficult to detect and to ascribe these changes to biological circumstances. In the backdrop of the potentiality of proteomics to bring out the elemental molecular features of saffron and unavailability of a protocol of significance for protein extraction, we begin with an earnest attempt to evaluate and compare various methods for their reliability in extracting saffron proteins. Proteomic study has many setbacks in comparison to genome based studies as there is no technique available for complete proteome analysis like PCR. There is no proteomic map available like a complete sequenced genomic map. It is indispensable to analyze proteins at the basal concentration and in the presence of many other proteins which are present in varying concentrations. Beside physiological issues experimental problems which pester incessantly proteomic approaches include gel-to-gel duplicability or similarity, biased approach toward identification of similar proteins in different studies, and most importantly reliability of various proteomic methods. Protein extraction in plants is technically challenging owing to high abundance of some proteins such as rubisco in plant extracts which interfere with the resolution of proteins of similar molecular weight as well as protein quantization. To address these remonstrations, we critically evaluated and analyzed protein extraction methods already made known in the literature. The virtues and pitfalls of each of these methods were determined using quantitative and qualitative gel electrophoresis methods and finally constituting an agreeable standardized way of a process that is repeatable and reproducible.

It is almost obligatory to assess the proteomic studies through an electrophoretic method. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) is the most ratiocinative method for proteomic analysis. SDS–PAGE exploits differences in a protein's molecular mass and correlates it with electrophoretic mobility resolving different proteins varying even by 1% in their electrophoretic mobility. Various modulations in the technique such as introduction of discontinuous buffer systems, Tris–HCl/Tris–Glycine, and pH, 6.8/8.3, respectively allows larger sample volumes to be loaded while proteins are being "stacked". SDS used in the technique binds strongly to proteins in the ratio of 1 SDS molecule per 2 amino acid residues thereby equating the negative charge/unit mass ratio. In addition employing sulfhydryl reagents and reducing agents to denature proteins, dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) are very effectual for reduction of disulfide bonds in proteins in SDS–PAGE.

2. Materials and methods

2.1. Plant material

Among the various saffron growing areas, Kashmir, India is believed to be one of the most important areas for producing saffron. Saffron plant is one of the most important species of Kashmir Karewas. Kashmir valley is known to produce one of the finest qualities of saffron as the environment is most suitable for growth and propagation of plant. Saffron plants were collected from one of the fields located in these Karewas in the flowering season (Mid October). Samples were thoroughly cleaned and then crushed immediately in liquid nitrogen into fine powder. Plant powder was stored in cryotubes at -70 °C till further use.

2.2. Protein extraction

We started with a common starting material i.e. 2 g of plant material was taken from liquid nitrogen crushed powder, transferred to a sterile tube to which respective extraction solutions were added. A simplified flow chart showing main steps in each process comparing the three different extraction protocols is shown in Fig. 1.

2.3. Acetate-urea buffer extraction

Acetate buffer (5 mM acetate with pH 5) constituted of 7.4 ml of .2 M acetic acid and 17.6 ml of .2 M sodium acetate, diluted to 100 m) was used with urea buffer (6 M urea, 1 M thiourea)

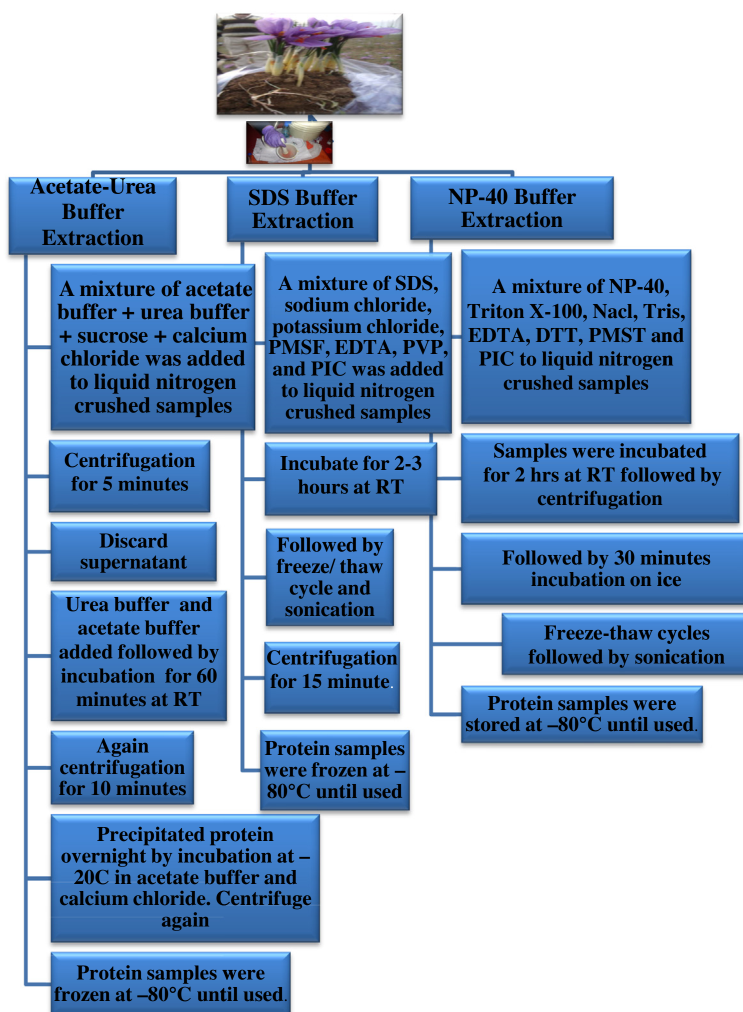


Figure 1 A simplified flow chart showing main steps in each process comparing the three different extraction protocols.

along with varying concentrations of sucrose and calcium chloride ($CaCl_2$). To liquid nitrogen crushed tissue, acetate buffer was added and mixed by inverting the tube 4–5 times. Sucrose (.4–1 M) was added followed by centrifugation at 5000 RPM for 5 min. Supernatant was discarded. Urea buffer and acetate buffer were added to samples followed by incubation at room temperature (RT) for 60 min. Again the samples were centrifuged at 1000 RPM for 10 min, and supernatant was discarded. Proteins were precipitated overnight by incubation at −20 °C in acetate buffer and $CaCl_2$ (.2–1 M). Precipitated sample was centrifuged at 14,000 RPM for 30 min. Protein samples were frozen at −80 °C until used.

2.4. SDS buffer extraction

SDS buffer was used with varying concentrations of SDS and sodium chloride (NaCl). SDS extraction buffer consisted of SDS (0.5–2%), NaCl (.3–1 M),100 mM potassium chloride (KCl), 0.1 mM phenylmethanesulfonylfluoride (PMSF), 2% 2-ME, 500 mM Tris (pH 7.5), 50 mM ethylenediaminetetraacetic acid (EDTA), 1% polyvinylpolypyrrolidone (PVP), and EDTA-free protease inhibitor cocktail (PIC). SDS buffer was added to liquid nitrogen crushed tissue sample. Samples were incubated for 2–3 h in buffer at RT followed by centrifugation at 10,000 RPM for 15 min. In order to evaluate the effect of different treatments as recommended in various protocols parallel samples were subjected to freeze–thaw cycles (20 min at −20 °C followed by 10 min incubation on ice) in one case and sonication (2 min,70-amplitude, Pulse 10 s on and 10 s off) in other. Samples were stored at −80 °C until used.

2.5. NP-40 buffer extraction

NP-40 buffer was used with varying concentrations of Triton X-100 (0.1–1%) and NaCl (0.1–0.3 M). NP-40 extraction buffer consisted of 1% NP-40, 1 M Tris (pH 7.4), 0.2% SDS, 1 mM EDTA, 50 mM DTT, 1 mM PMSF and EDTA-free protease inhibitor cocktail. Samples were incubated for 2 h in buffer at RT followed by centrifugation at 14,000 RPM for 25 min. In parallel samples were subjected to freeze–thaw cycles (15 min at −20 °C followed by 30 min incubation on ice) in one case and sonication (2 min, 70-amplitude, Pulse 10 s on and 10 s off) in other. Samples were stored at −80 °C until used.

2.6. Sample buffer preparation

The selection of a suitable sample buffer is a crucial step in proteomics survey as various parts of saffron plant reveal a high degree of biochemical as well as biophysical heterogeneity. Considering these biological realities we set at establishing a sample buffer preparation recipe most suitable for saffron. We compared protein sample buffers obtained using different protocols already described for different plant species. 1-D SDS–PAGE gels were used to evaluate the effect of different sample buffer compositions on various aspects of protein extraction, characterization, patterns, range of protein molecular weight and effect of interfering substances in the extracts. Table 1 shows the recipe of 5× sample buffer that showed best results. SDS is one of the important components strongly effecting protein resolution, so it needs to be maintained in enough concentration in buffer in order to maintain an adequate excess of it. SDS denatures proteins when heated and imparts a strong negative charge to all proteins. This role of SDS makes it a deciding component for quality resolution of proteins. Second important component in the sample buffer is a Thiol reagent which reduces disulfide bonds.

DTT and 2-ME seemed to be very effectual for reduction of disulfide bonds in proteins. Some proteins in plant samples seem to be resistant to solubilization by heating with SDS. Addition of a non-ionic detergent Triton X-100 facilitates solubilization of proteins such as histones and membrane proteins. One of the crucial steps effecting protein profile on SDS–PAGE is efficient loading of sample, in order to provide adequate density to samples, glycerol is added to sample buffer. Glycerol not only facilitates sample loading but also precludes convective migration of samples. The process of electrophoresis needs to be monitored in order to avoid running out of proteins, so a visual aid commonly known as a tracking dye is added to the sample buffer. Bromophenol blue helps to monitor the process polyacrylamide gel electrophoresis. Since bromophenol blue carries a slight negative charge at moderate pH, it migrates in the same direction as protein in a gel. The sample buffer once prepared can be stored at (−80 °C) in aliquots of 1 ml. Every time before use, sample buffer should be warmed at 37 °C and mixed thoroughly to completely dissolve the SDS.

2.7. Sample preparation

Sample preparation is another decisive step in SDS–PAGE that needs critical evaluation. Prior to loading protein lysates needs to be properly processed for complete denaturation and reduction of protein sample including resistant proteases. Sample buffer is to be added in adequate quantity to protein lysates as imbalanced sample to buffer ratio causes poor band resolution. Samples are to be mixed thoroughly and heated immediately at 100 °C for three minutes. Delayed heating of sample leads to degradation of protein causing electrophoretic artifacts. The most plausible reason for which seems to be proteolytic degradation by partially denatured proteases, which become more active as the polypeptides are exposed by SDS treatment. Immediate heating limits degradation by completely denaturing all proteins including resistant proteases. Heating for prolonged periods should be avoided as it would cleave the peptide bonds, cause degradation and smearing in profile. This treatment sufficiently reduces disulfide bonds, solubilizes and dissociates proteins without degradation. Before loading the sample, after heating step is complete samples should be centrifuged briefly in order to remove any insoluble plant residues. Failure to remove precipitated insoluble material causes streaking within the gel. Finally quantity of sample to be loaded in wells needs to be standardized as overloading of samples results in poorly resolved bands and distortion in gel patterns in adjacent lanes. Similarly underloading of sample prevents detection of proteins in minute quantities and most of the bands develop too faint for scanning. To prevent inadequate sample buffer-to-protein ratios, overloading, and underloading of samples, we determined concentration of the samples using a standard protein assay. For average gel thickness and well size, the amount of protein that we loaded for good band resolution and sharpness ranged from 40–60 μg for crude samples.

Table 1 Simplified outlay of concentrations of constituents of 5X sample buffer in stock and working solutions.

5X SDS–PAGE sample buffer	
Working solution	Stock solution
0.225 M Tris–Cl, pH 6.8	1 M Tris–Cl, pH 6.8
5% SDS	SDS
0.25 M DTT	1 M DTT
10% 2-ME	2-ME
1% Triton X-100	Triton X-100
25% Glycerol	Glycerol
0.01% Bromophenol blue	Bromophenol blue

2.8. One-D SDS–PAGE

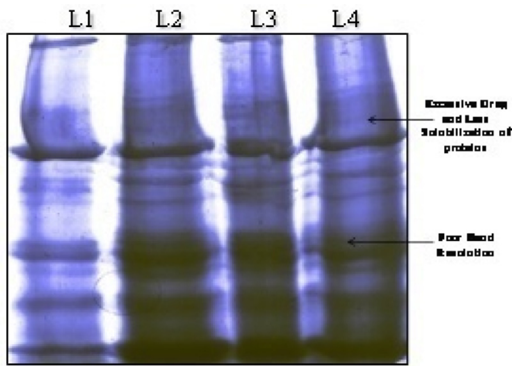
Protein from each extraction type was quantified using Bradford assay. Protein (40 μg) was boiled in SDS loading buffer and loaded onto 10% SDS–PAGE gel. Gels were run at 70-V till samples got stacked and then at constant 110-V for 2 h at room temperature. Following electrophoresis gel was removed from glass and placed in a suitable container with a lid not much larger or much smaller than the gel. Gel was rinsed in distilled water for 15 min decanting the water every 5 min with constant stirring on gel rocker. Enough Coomassie Stain (EzBlue^{R} stain reagent Sigma Aldrich) was added to cover the gel by 1/2 inch (~1.5 cm) and incubated for 1 h on gel rocker The solution was decanted and gel was washed with distilled-water for 2–3 times .To intensify protein bands gel was incubated in destaining solution (methanol −30%, acetic acid −10% in water) overnight.

2.9. Gel analysis

Gels were scanned on the GS-800 Calibrated Densitometer for qualitative and quantitative analysis of protein profile.

3. Results and discussion

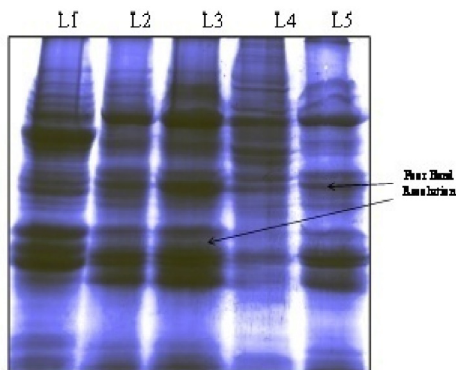
Quantitative analysis of the protein extracted by each extraction protocol was analyzed in the first instance by the Bradford assay. The most striking difference between the protocols tested was the protein yield obtained by each extraction



Lane 1: Total protein extraction using ALB + .4M Sucrose + .2M $CaCl_2$
Lane 2: Total protein extraction using ALB + .6M Sucrose + .2M $CaCl_2$
Lane 3: Total protein extraction using ALB + 1M Sucrose + .2M $CaCl_2$
Lane 4: Total protein extraction using ALB + 1M Sucrose + 1M $CaCl_2$

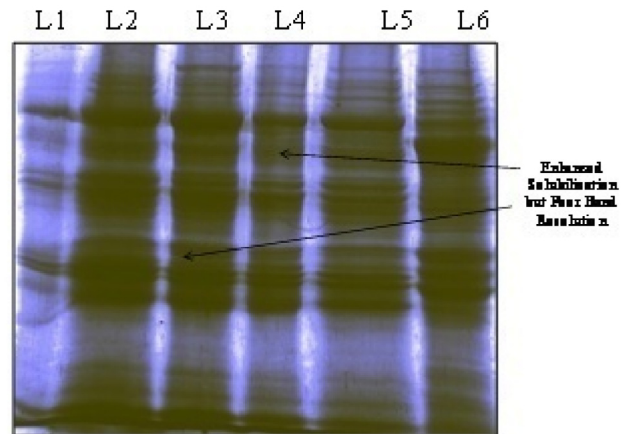
Figure 2 Comparison of relative amount and quality of protein extracted from saffron samples using ALB (acetate-urea lysis buffer).

protocol. The NP-40 lysis buffer extraction method resulted in a greater double higher yield in protein as compared with the acetate buffer and SDS buffer extraction methods. Beside there was prominent drag in the profiles which might be explained by the fact that these two extraction methods involve use of detergents at high concentration. Protein profile of all extraction protocols showed up proteins with a wide range of molecular weight from over 250 kDa to as low as 5 kDa. Comparison of relative amount and quality of protein extracted from samples using acetate lysis buffer with varying concentrations of sucrose and $CaCl_2$ showed conspicuous streaking, poor band resolution and poor protein solubilization. Changing concentration of sucrose and $CaCl_2$ showed up perceptible changes in profile however overall protein profile remained poor in all samples (Fig. 2). SDS buffer was used with varying concentrations of SDS and NaCl. Extraction with SDS lysis buffer led to increase in protein load and solubilization of proteins. Variations in SDS and NaCl concentrations



Lane 1: Total protein extraction using SDS-LB with 0.5% SDS + .3M NaCl
Lane 2: Total protein extraction using SDS-LB with 1% SDS + .3M NaCl
Lane 3: Total protein extraction using SDS-LB with 1% SDS + 1M NaCl
Lane 4: Total protein extraction using SDS-LB with 2% SDS + .3M NaCl
Lane 5: Total protein extraction using SDS-LB with 2% SDS + 1M NaCl

Figure 3 Comparison of relative amount and quality of protein extracted from saffron samples using SDS-LB (SDS lysis buffer).



Lane 1: Total protein extraction using NP-40 LB + Na .1M + Triton .1%
Lane 2: Total protein extraction using NP-40 LB + Na .1M + Triton .5%
Lane 3: Total protein extraction using NP-40 LB + Na .1M + Triton .1%
Lane 4: Total protein extraction using NP-40 LB + Na .3M + Triton .1%
Lane 5: Total protein extraction using NP-40 LB + Na .3M + Triton .5%
Lane 6: Total protein extraction using NP-40 LB + Na .3M + Triton .1%

Figure 4 Comparison of relative amount and quality of protein extracted from saffron samples using NP-40 LB (NP-40 lysis buffer).

led to streaking in some samples and poor band resolution in other, however overall SDS buffer failed to show reproducible 1-D extraction. The solubilization of proteins by SDS buffer showed higher extraction yield, however, the obtained protein concentrate showed low quality (Fig. 3). Henceforth, we worked on NP-40 lysis buffer with varying concentrations of NaCl and Triton X-100. Initially with slight changes in concentrations of NaCl and Triton X-100, protein profile improved over extraction but still profile showed poor band resolution and streaking (Fig. 4). The most plausible reason for which was incomplete lysis and high viscosity due to DNA contamination in samples respectively.

In order to enhance cell lysis to increase the proteome load of samples we treated the samples with NP-40 lysis buffer at

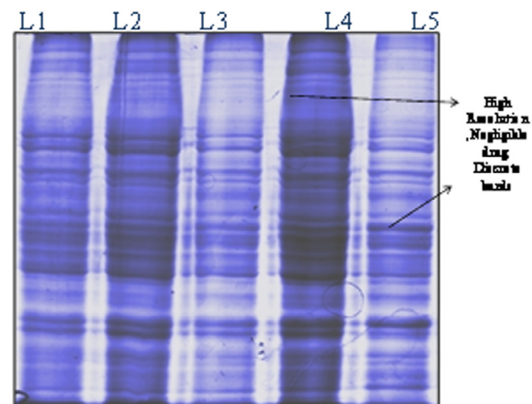


Figure 5 Relative amount and quality of protein extracted from saffron samples lysed with NP-40 LB + freeze/thaw cycle + sonication.

standardized concentration followed by alternate freeze thaw cycle at different time points. This treatment improved the quality of protein profile without adding detergent load to profile. Physical shearing through sonication of the samples reduces evident viscosity due to nucleic acids. So we further went to sonicate the samples for different durations and amplitude, along with standardized NP-40 lysis buffer recipe and freeze/thaw cycle duration. Sonication further improved the quality of proteome profile. There was no prominent streaking or background, gel seemed to be free of interfering substances. Highly reproducible banding profiles were observed using the NP-40 lysis buffer along with freeze/thaw cycles and sonication (Fig. 5). Overall acetate lysis buffer and SDS lysis buffer profiles lacked numerous bands present in the NP-40 multi-treatment extraction and in addition it also contained a unique set of bands not found in the other extraction methods.

4. Conclusion

The proteomic analysis has recently gained importance in basic and applied research, because identification of novel changes in protein expression helps to understand protein functions and plant responses to different environmental cues. In order to study proteomic profile of plants high quality proteomic profiles are preliminary requirements as inept extraction protocols replicate and amplify false positive in downstream processes which ultimately leads to wrong conclusions. Minor differences between SDS–PAGE gel-banding patterns are translated into major differences when plant extracts are examined using 2-DE., western blotting etc. These findings are now employed in comparative proteomic studies aiming at identifying proteins involved in abiotic and biotic stress responses. To study impact of various processes on total proteome, it is crucial to apply extraction methods selective for particular plant species. In this study, we compared three different methods already reported for the isolation of proteome from plants of cormous specie. The protein patterns resolved by 1-DE revealed clear differences between different preparations. Our results showed that various protocols used for protein extraction in many plant varieties failed to show good and reproducible results in saffron plant. We found the lowest number of protein contaminants with the highest number of extracted proteins obtained with NP-40 buffer. Also, the number of proteins exclusively found was highest for this buffer. Extraction by this method isolates a distinct “extractome”. The protocol described here allows enrichment of the protein extractome and reduction of contaminating fraction. It is suggested that the procedure described in this study may be widely applicable for protein extraction from leaves as well as floral parts.

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