JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Qualitative and Quantitative Evaluation of Protein Extraction Protocols for Apple and Strawberry Fruit Suitable for Two-Dimensional Electrophoresis and Mass Spectrometry Analysis

QIFA ZHENG,[†] JUN SONG,*[,†] KRISTEN DONCASTER,[†] ELDEN ROWLAND,[§] AND DAVID M. BYERS[§]

Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, 32 Main Street, Kentville, Nova Scotia B4N 1J5, Canada, and DalGEN Proteomics Core Facility, Atlantic Research Center, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

A modified phenol-based protocol and a phenol-free protocol that involves hot SDS extraction followed by TCA precipitation in acetone were qualitatively and quantitatively compared and evaluated on apple peel and strawberry fruit. The phenol protocol resulted in significantly higher protein yields of 2.35 ± 0.1 and 0.46 ± 0.06 mg/g of FW from apple and strawberry fruit, respectively, compared to the SDS protocol, which produced 0.74 ± 0.1 and 0.27 ± 0.02 mg/g of FW, respectively. 2-DE analysis of apple protein extracts revealed 1422 protein spots associated with the phenol protocol and 849 spots associated with the SDS protocol. Of these, 761 were present only in phenol gels, whereas 23 were exclusive to SDS samples. For strawberry, SDS extraction produced poor-quality spots with a high degree of streaking, indicating possible contamination. The application of a cleanup procedure resulted in a purified protein extract with high-quality spots. 2-DE analysis of strawberry protein extracts revealed 1368 spots for the phenol protocol and 956 spots for the SDS protocol accompanied by the cleanup procedure. Of these, 599 spots were present only in phenol gels, whlereas 109 were present only in SDS samples. Spots from each fruit tissue and extraction procedure were selected, and a total of 26 were identified by LC-MS/MS. Overall, this study demonstrates the complexity of protein extraction of fruit tissues and suggests that a phenol-based protein extraction protocol should be used as a standard procedure for recalcitrant fruit tissues, whereas a SDS protocol with or without a cleanup procedure may be used as an alternative protocol.

**KEYWORDS: Protein extraction; two-dimensional electrophoresis; phenol; sodium dodecyl sulfate; fruit tissue; LC-MS/MS**

## INTRODUCTION

Proteomics is a systematic approach to the study of global changes in proteins, providing an essential linkage between the transcriptome and metabolome (*1*). Among proteomic techniques, two-dimensional electrophoresis (2-DE) has been applied to resolve thousands of proteins to facilitate peptide composition analysis, peptide sequencing, and polypeptide identification using mass spectrometry (*2*). In plant tissues, 2-DE-based proteomic techniques have demonstrated the potential to investigate biochemical processes at the gene and protein levels (*3*−*8*). One of the primary advantages of proteomics research based on 2-DE is the ability to investigate hundreds or thousands of proteins simultaneously. The ability to precisely determine molecular weight by mass spectrometry (MS) and the development of genomic sequence databases for peptide mass matches make it possible to achieve a high throughput of plant protein identification (*1*, *2*).

Fruits and vegetables play important roles in the human diet. However, they are perishable due to natural ripening, senescence, and pathological decay. Biochemical processes related to quality changes occurring during ripening such as texture, appearance, flavor, and nutrition are not yet fully understood. Apple and strawberry are among the most consumed fruits in the world and are popular model systems for postharvest researchers to study ripening mechanism and fruit quality. Comparative 2-DE between pairs of samples has the potential in postharvest research of fruits and vegetables to investigate the physiological and biochemical changes related to fruit ripening and senescence. It can also be used to examine the effects of handling and storage treatments on the quality of stored fruits and vegetables. Protein extraction and sample preparation are two of the most critical steps in the 2-DE

* Corresponding author [e-mail songj@agr.gc.ca; fax (902) 679-2311].
[†] Agriculture and Agri-Food Canada.
[§] Dalhousie University.

10.1021/jf062850p CCC: $37.00 
Published on Web 02/13/2007

proteomic study of fruit. Fruits are considered to be recalcitrant plant tissues for proteomic analysis; it is often difficult to obtain high-quality protein suitable for 2-DE analysis. This is largely due to low protein content and the presence of interfering substances such as pigments, carbohydrates, polyphenols, polysaccharides, and starch (*9*). Although some protocols have been reported, optimum conditions for the preparation of protein extracts from plant tissues that are suitable for 2-DE have primarily been developed for young immature vegetative tissues (*9*−*11*). Establishing a reliable and effective protein extraction procedure is an essential step in conducting proteomic research. In a previous study, a protocol involving SDS extraction combined with TCA/acetone precipitation developed for apple and banana fruits was compared with two phenol-free protocols (*12*). Protein protocols utilizing phenol have also been reported to be suitable for the extraction of low concentrations of protein in vegetative plant tissue rich in contaminating components that interfere with electrophoresis (*13*). Phenol extraction of protein from tomato fruit was found to be comparable to protein precipitation with acetone (*14*), although in-depth quantitative data analysis was lacking. Recently, classical TCA/acetone precipitation and phenol extraction were compared with banana, apple, and potato plant tissues. The former was considered to be as useful as phenol as a standard protocol (*15*). An approach combining TCA/acetone and phenol extraction was evaluated qualitatively with aged leaves and apple flesh tissue (*16*). A systematic comparison between phenol and SDS/acetone precipitation is needed to improve the protein extraction procedure for fruit tissues.

In this study, we describe comparisons of a phenol-based protocol modified from that of Hurkman and Saravanan (*13*, *14*) and a SDS/TCA/acetone-based protocol established by Song et al. (*12*) in the extraction of protein from apple and strawberry fruit tissues. Differential results corresponding to these approaches including protein yield and 2-DE image are shown. Spots were selected for LC-MS/MS identification to reveal the difference between the two protocols; meanwhile, mass spectra of peptides obtained from spots present in both protocols are also illustrated to examine the spectra quality in relation to protocols. The application of these modified protocols for protein extraction will be beneficial for other proteomic studies on recalcitrant plant/fruit tissues.

## MATERIALS AND METHODS

**Plant Materials and Sample Preparation.** Apple peel and whole strawberry fruit were used as plant materials for protein extraction and analysis. Apples (*Malus domestica* cv. 'Red Delicious') were harvested at the commercial harvest stage and stored for 6 months in controlled atmosphere (CA) conditions (3.0 kPa of $N_2$ + 1.0 kPa of $CO_2$). Apple peels (2−5 mm thickness) were obtained with a commercial peeler, quickly frozen in liquid nitrogen, and stored at −85 °C. Strawberries (*Fragaria ananassa* cv. 'Mira') were harvested at the full red stage (2 days following the pink stage) at the Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, Kentville, NS, Canada. Harvested fruits were quickly frozen in liquid nitrogen and stored at −85 °C. Frozen samples were ground to a fine powder in liquid nitrogen using a stainless steel blender followed by a mortar and pestle. Ground samples were stored at −85 °C until used.

**Chemicals.** All chemicals used in this study were of the highest grade available from Sigma-Aldrich (Oakville, ON, Canada) and GE-Healthcare (formerly Amersham BioSciences, Baie-d'Urfié, QC, Canada). Milli-Q water (Millipore, Bedford, MA) with resistance greater than 18 MΩ was used throughout. All solutions were filtered through mixed cellulose ester membrane filters (0.45 μm) prior to use.

**Protein Extraction.** Protein was extracted and purified from ground samples using one of two methods: (1) SDS extraction followed by TCA−acetone precipitation; (2) phenol extraction followed by ammonium acetate−methanol precipitation. Extractions were completed as follows.

*SDS Extraction followed by TCA−Acetone Precipitation.* The standard SDS-based protocol was applied to both apple peel and whole strawberry samples, as described in ref *12*. Ground fruit tissue (2.0 g) was suspended in 12 mL of SDS extraction buffer [2% (w/v) SDS, 60 mM DTT, 20% (v/v) glycerol, 40 mM Tris-HCl, pH 8.5] and incubated for exactly 8 min at 90−94 °C. Incubated samples were centrifuged at 8000*g* for 15 min at 4 °C. The resulting supernatant was mixed with 25 mL of precipitation solution [(10% (w/v) TCA and 20 mM DTT in 100% ice-cold acetone], incubated at −20 °C for exactly 45 min, and then centrifuged at 18000*g* for 10 min at −4 °C. The resulting pellet was washed three times with 7.5 mL of washing solution (20 mM DTT in 100% ice-cold acetone), placed at −20 °C for 1 h in 35 mL of washing solution, and then centrifuged at 20000*g* for 10 min at −4 °C. Washed pellets were air-dried for 5 min and rehydrated in 700 μL of rehydration buffer [7 M urea, 2.0 M thiourea, 1.2% (w/v) CHAPS, 0.4% (w/v) ASB-14, 0.25% (v/v) IPG buffer, pH 3−11 NL, 30 mM Tris-HCl, pH 8.5, and 43 mM DTT] (*17*) for 1 h with continual shaking at room temperature (RT). Rehydrated samples were centrifuged at 12000*g* for 10 min at RT. The resultant supernatant corresponded to the total protein extract and was immediately stored at −85 °C until further analysis. For strawberry, protein extracts obtained from the standard SDS-based protocol were further purified using a 2-D Clean-Up Kit (GE Healthcare) following the manufacturer's instructions.

*Phenol Extraction followed by Ammonium Acetate−Methanol Precipitation.* The phenol-based protocol of Hurkman and Saravanan (*13*, *14*) was applied with minor changes. Ground fruit tissue (2.5 g) was suspended in 10 mL of ice-cold phenol extraction buffer [0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl, pH 8.5, 50 mM EDTA, pH 8.5, 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP), 40 mM DTT] and homogenized on ice at 6000 rpm for 60 s (Sentry Microprocessor with Cyclone I.Q.$^2$, Virtis, SP Industries, Gardiner, NY). The resulting homogenate was mixed well with an equal volume of Tris-HCl-saturated phenol (pH 8.0) and centrifuged at 10000*g* for 15 min at 4 °C. The upper phenol phase was collected, and the pellet was re-extracted once as above. The pooled phenol phases were mixed well with 5 volumes of ice-cold 0.1 M ammonium acetate in 100% methanol and incubated overnight at −20 °C. Incubated samples were centrifuged at 10000*g* for 15 min at 4 °C. The resulting pellets were washed twice with ice-cold methanol and then twice with ice-cold acetone containing 20 mM DTT. Washed pellets were partially dried and then redissolved in 500 μL (strawberry) or 700 μL (apple peel) of IEF buffer [7 M urea, 2.0 M thiourea, 4% (w/v) CHAPS, 10 mM DTT, 1% (v/v) IPG buffer, pH 3−11 NL)]. Samples were shaken continuously at RT for 20 min or until the pellet dissolved completely and then centrifuged at 12000*g* for 5 min at RT. The resultant supernatant corresponded to the total protein extract and was immediately stored at −85 °C until further analysis.

**Protein Quantification.** Protein concentration in all extracts was determined using the RC/DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) to compensate for interfering compounds according to the manufacturer's protocol. Bovine serum albumin (BSA) was employed as a standard. The protein yield was expressed as milligrams per gram of fresh weight. Three replicates were conducted, and protein yield is presented as mean ± standard deviation.

**2-D Electrophoresis.** Extracted proteins were separated by isoelectrofocusing (IEF) on a Multiphor II system (GE Healthcare) using 18 cm Immobiline DryStrip gels (GE Healthcare) with nonlinear pH gradients (pH 3−11 NL), according to the manufacturer's instructions. Protein (80.0 μg) and 10 μL of 2-D protein standard (Bio-Rad Laboratories) were mixed with DeStreak Rehydration Solution (GE Healthcare) to a final volume of 340 μL, which was loaded onto the DryStrip gel and incubated for a minimum of 10 h at RT. Following first-dimension separation for 30000 Vh at 20 °C (500 V for 1 h, 1000 V for 10 min, 2000 V for 10 min, then 3000 V for 9.66 h), strips were equilibrated with DTT equilibration buffer for 10 min, followed with equilibration in iodoacetamide buffer for 10 min at RT as described by the manufacturer (GE Healthcare). Second-dimension separation was conducted on large format 12.5% acrylamide SDS-PAGE gels (24 ×

**Table 1.** Yield and Concentration of Protein Recovered from Apple and Strawberry Fruit Using Phenol and SDS Extraction Protocols

| | apple | | strawberry | | |
|---|---|---|---|---|---|
| | phenol | SDS | phenol | SDS | SDS + cleanup |
| protein yield[a] (mg/g of FW) | 2.35 ± 0.10 | 0.74 ± 0.10 | 0.46 ± 0.06 | 0.27 ± 0.02 | 0.16 ± 0.02 |

[a] Mean ± standard deviation, $n = 3$.

21 cm) and run at 30 W for 30 min and at 100 W for the remaining time at 15 °C on an Ettan DaltSIX multigel system (GE Healthcare). Resultant gels were fixed in a solution containing 50% methanol and 10% acetic acid for 30 min and then visualized by staining with fluorescent dye (SYPRO Ruby, Bio-Rad Laboratories) at approximately 70 °C for 1 h and then at RT overnight. Stained gels were washed in a solution containing 10% methanol and 7% acetic acid for 30 min. Gels were exposed to UV light (310 nm), and gel images were captured by digital camera equipped with a Gelstar photographic filter (Wratten no. 9 filter, Cambrex Bioscience Rockland, Rockland, ME) under dark conditions.

**Image Analysis.** Digital images of 2-DE gels were processed and analyzed using PDQuest 2-D Image Analysis software (version 7.40, Bio-Rad Laboratories). Cropped images were processed with the following software settings for spot detection and background subtraction: for apple samples, sensitivity, 15; size scale, 5; minimum peak, 1000; background radius, 60; contra mean, 5 × 5; streak radius, 60 (vertical), 75 (horizontal); for strawberry samples, sensitivity, 15; size scale, 5; minimum peak, 1200; background radius, 55; contra mean, 5 × 5; streak radius, 55 (vertical), 55 (horizontal). To allow spot to spot comparisons across gels, a match set was created that included all gel images within an experiment (i.e., apple or strawberry). For each match set, a standard gel (master) was generated from the image within the match set that contained the greatest number of spots with an absence of streaking or other gel distortions. The software's automatic matching tool was used to match spots across gels within match sets. All spots matched by the software program were manually verified as follows. A spot was considered to be reproducibly present/absent when it was present/absent in at least two of the three replicate gels of a given treatment. Spots that were reproducibly present in a match set member but not in the master gel were manually added to the master image. For all gels, spot quantities (total pixel intensity within spot boundaries, calculated by image analysis software for Gaussian spots) were normalized to remove variations in spot intensity caused by nontreatment effects. Normalized spot quantity was equal to raw spot quantity expressed as a percentage of the total pixel quantity of all spots in a gel. Six landmarks (2-D SDS-PAGE standard, Bio-Rad Laboratories) were manually defined for accurate determination of $M_r$ and p*I*.

**MS Analysis and Protein Identification.** Selected polypeptide spots were manually excised from 2-DE gels under the exposure of UV. Spots then were treated with DTT to break disulfide linkages, alkylated with iodoacetamide, and then digested with trypsin. The resultant peptides were extracted in washes of ammonium bicarbonate solution, acetonitrile, and 10% formic acid. Extraction solvent was removed under vacuum, and the peptides were resuspended in 30 μL of 5% MeOH−0.5% formic acid. HPLC was performed on an LC Packings Ultimate nanoflow system (Dionex, Sunnyvale, CA). Samples (3 μL) were injected directly onto a monolithic reversed phase capillary column, 0.100 × 150 mm Chromolith CapRod RP-C18 endcapped (Merck KgaA, Darmstadt, Germany). The flow rate after splitting was 1.2 μL/min, and the sample was sprayed through a distal coated fused silica emitter tip, 75 μm i.d. with a 15 μm i.d. tip (New Objective, Woburn, MA). Mass spectrometry was performed on a hybrid triple-quadrupole linear ion trap (Q-TRAP LC-MS/MS, Applied Biosystems, Foster City, CA) equipped with a nanospray ion source. Spectra were acquired as follows using Information Dependent Acquisition mode in Analyst 1.4.1 software. For each MS scan, 375−1300, the two most intense ions were selected for an enhanced resolution (ER) scan to determine their charge states. Enhanced product ion scans, 100−1700, were initiated by the presence of doubly and triply charged ions with intensities exceeding $1 \times 10^5$ counts. Former target ions were excluded for 300 s after four occurrences. The ion spray voltage was 2100 V, the curtain gas was set to 20 (arbitrary units), and the declustering potential was 60 V. MS/MS peak lists were generated for database searching using Mascot version 1.6b9 or using Pro ID (Applied Biosystems) software. Product ion scans for precursors within a 1.0 Da range were summed if collected within 30 IDA cycles of each other. All spectra were centoided and deisotoped.

The raw MS/MS data were searched against NCBI viridiplantae entries, 278115 sequences, updated November 7, 2006 (NIH, Bethesda, MD), using the Mascot algorithm (Matrix Science, London, U.K.). The MS and MS/MS mass tolerances were 0.8 and 0.5 Da, respectively, and one missed cleavage was allowed. Carboxamidomethyl cysteines and oxidized methionines were set as variable modifications. Proteins with significant peptide matches were selected for error tolerant searching. The data were also searched against the SwissProt database, 234112 sequences, updated December 11, 2006 (Sprot version 50.8), to ensure no peptides from trypsin or keratin were present. Peptide ion scores of >41 indicate identity or extensive homology ($p < 0.05$) and are referred to as significant hits. Peptides below the significance threshold were reported only when other significant hits to the same protein were present. The Pro ID algorithm (Applied Biosystems) was used to search data against the EST database for apple, 195553 entries, updated December 19, 2005, and strawberry, 9213 entries, updated December 19, 2005 (Genome Database for Rosaceae, Washington State University, Pullman, WA; http://www.mainlab.clemson.edu/gdr). Search parameters for Pro ID were the same as for those used with Mascot. Peptides with a Pro ID confidence value of at least 95 were considered to be significant hits.

**Statistical Analysis.** Experimental design and data analysis were conducted using GenStat version 8.1 (VSN International Ltd., 2005, Herts, U.K.). Student's *t* test was conducted to determine the significance probability between the two protocols. Correlation coefficients between two protocols were obtained from PDQuest software.

## RESULTS

**Comparison of Protein Yield and Concentration Obtained with Phenol and SDS Extraction Protocols.** Total protein yields obtained by the two extraction protocols from apple peel and strawberry fruit were compared (**Table 1**). Total amounts of protein extracted depended on the protocol used. Overall, the phenol protocol gave higher protein yields than the SDS protocol for both apple peel and strawberry fruit with 2.35 ± 0.10 and 0.46 ± 0.06 mg/g of FW protein, respectively. Hot SDS yielded 0.74 ± 0.10 and 0.27 ± 0.06 mg/g of FW, respectively. Protein obtained with either extraction protocol from apple peel and strawberry fruit was well separated by SDS-PAGE and showed distinct polypeptide bands from 10 to 250 kDa with low background. More small polypeptide bands (10−15 kDa) were obtained with the phenol protocol than with SDS from both apple and strawberry (data not shown).

**2-DE Evaluation of Protein Extracted with Phenol and SDS Protocols.** *Apple Peel.* Qualitative analysis revealed that 1422 and 849 spots were extracted from apple peel using phenol and SDS protocols, respectively (**Figure 1A**,**B**). Of these, 761 spots were present only in phenol gels, whereas 23 spots were present only in SDS-extracted samples (**Table 2**). For matched spots, there was a strong correlation ($r = 0.75$) between the two extraction protocols in terms of relative intensity of matched spots. Further quantitative analysis revealed 201, 63, and 35

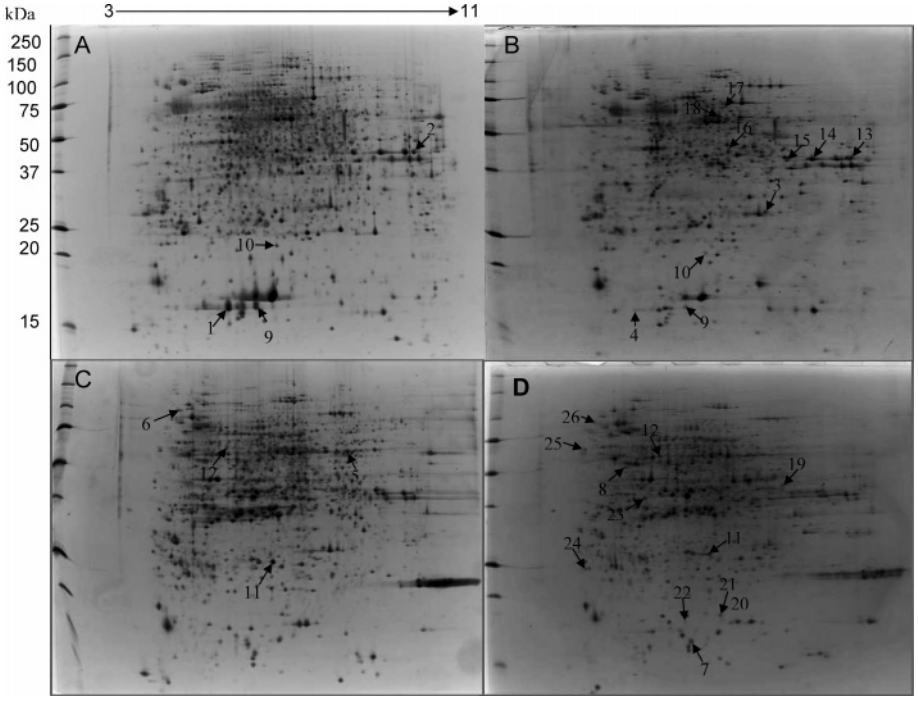


**Figure 1.** 2-DE analysis of apple and strawberry fruit protein extracts: (**A**) apple–phenol; (**B**) apple–SDS; (**C**) strawberry–phenol; (**D**) strawberry–SDS with cleanup. Eighty micrograms of protein was extracted from fruit tissue and dissolved in DeStreak solution. After isoelectrofocusing, proteins were further separated on SDS-PAGE (12.5%) polyacrylamide gels and visualized by Sypro Ruby staining. Arrows indicate proteins identified by LC-MS/MS with corresponding numbers listed in **Table 3**.

**Table 2.** Qualitative and Quantitative Analysis of 2-DE-Separated Proteins Obtained from Apple and Strawberry Fruit Using Phenol and SDS Extraction Protocols

| | apple | | strawberry | |
|---|---|---|---|---|
| | phenol | SDS | phenol | SDS + cleanup |
| total no. of spots[a] | 1422 | 849 | 1368 | 956 |
| CV[b] (%) | 15.2 | 2 | 5 | 1 |
| only in phenol or SDS | 761 (53.5%)[c] | 23 (2.7%) | 599 (43.8%) | 109 (11.4) |
| in phenol vs SDS | | | | |
| 2-fold > | 201 (13.9%) | | 344 (25.1%) | |
| 5-fold > | 63 (4.4%) | | 168 (12.3%) | |
| 10-fold > | 35 (2.5%) | | 124 (9.1%) | |
| in SDS vs phenol | | | | |
| 2-fold > | | 824 (97.1%) | | 678 (70.9%) |
| 5-fold > | | 577 (67.9%) | | 494 (51.7%) |
| 10-fold > | | 354 (41.7%) | | 337 (35.3%) |

[a] $n = 3$. [b] Coefficient of variation. [c] Percentage value.

spots with intensities 2-, 5-, and 10-fold greater, respectively, in phenol samples than in SDS. In comparison, 824, 577, and 354 proteins had spot intensities 2-, 5-, and 10-fold greater in SDS than in phenol (**Table 2**). To characterize differences between the two protocols, spot distribution by molecular weight and p*I* were compared from both phenol and SDS protocols (**Figure 2A**,**B**). The phenol protocol produced a higher percent in all categories except for 25−50 kDa and p*I* 3−6. No difference was found for p*I* 8−11. For either protocol, 95% of matched spots in apple gels had a $M_r$ between 25 and 200 kDa with p*I* from 3 to 8.

*Strawberry*. In strawberry, qualitative analysis revealed that 1368 and 956 spots were obtained from phenol and SDS protocols, respectively (**Figure 1C**,**D**). Of these, 599 spots were present only in phenol gels, whereas 109 spots were present only in SDS-extracted samples. For matched spots, the relationship between the two protocols was $r = 0.64$. Further qualitative analysis revealed 824, 577, and 354 spots with intensities 2-, 5-, and 10-fold greater, respectively, in phenol gels than in SDS. A total of 678, 494, and 337 proteins had spot intensities 2-, 5-, and 10-fold greater in SDS than in phenol (**Table 2**).

As in apple fruit, there was a significant difference in spot distribution on the basis of molecular weight and p*I* between the phenol and SDS protocols. The phenol protocol showed a higher percent of spots in categories of 100−200 kDa, p*I* 6−8 and 8−11, than SDS, whereas a lower percent with <25 kDa, 25−50 kDa, and p*I* 3−8.

No difference was found in the range of 50−100 kDa. For either protocol, >96% of the matched spots in strawberry gels had a $M_r$ between 25 and 200 kDa with p*I* from 3 to 8.

**Protein Identification by LC-MS/MS.** Following gel analysis, selected spots from each protocol and each fruit tissue were excised and identified by LC-MS/MS. To investigate differences between the two extraction protocols, we identified some of the proteins present only in phenol gels or only in SDS samples (**Table 2** and **Figure 1**). Apple peel proteins that were present in phenol gels but not in SDS were identified as major allergen Mal d 1.06A and fructose-bisphosphate aldolase (*Arabidopsis*

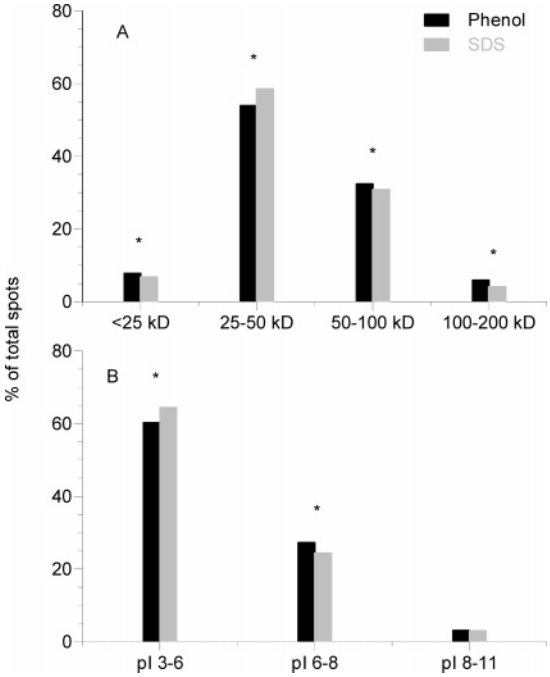


**Figure 2.** Characterization of abundant spots in apple fruit tissue obtained with phenol and SDS protocols within different categories of molecular weight (**A**) and p*I* (**B**). Eighty micrograms of protein from fruit tissue was dissolved in DeStreak solution. After isoelectrofocusing, the proteins were further separated on SDS-PAGE (12.5%) polyacrylamide gels and visualized by Sypro Ruby staining. * indicates $p < 0.05$.

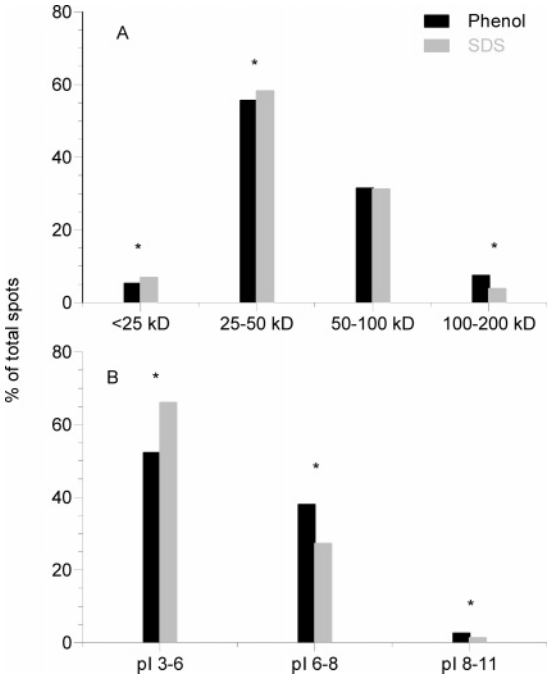


**Figure 3.** Characterization of abundant spots in strawberry fruit tissue obtained with phenol and SDS protocols within different categories of molecular weight (**A**) and p*I* (**B**). Eighty micrograms of protein from fruit tissue was dissolved in DeStreak solution. After isoelectrofocusing, the proteins were further separated on SDS-PAGE (8–16%) polyacrylamide gels and visualized by Sypro Ruby staining. * indicates $p < 0.05$.

*thaliana*) (**Table 3**; **Figure 1**, spots 1 and 2). Among the 23 spots present only in SDS gels, 6 were further identified as guanine nucleotide regulatory protein and major allergen Pru ar 1 (**Figure 1**, spots 3 and 4) as well as glyceraldehyde-3-phosphate dehydrogenase isoforms, aspartyl-tRNA synthetase (class IIb), adenosylhomocysteinase (*S*-adenosyl-L-homocysteine hydrolase), and a unknown protein (**Table 3**; **Figure 1**, spots 13−18).

Two strawberry proteins that were present in phenol gels but not in SDS gels were identified as 48 kDa glycoprotein precursor and a heat shock protein (**Table 3**; **Figure 1**, spots 5 and 6). A 17.7 kDa heat shock protein and a putative 26S proteasome regulatory particle were present only in SDS gels (**Table 3**; **Figure 1**, spots 7 and 8). In addition, eight more proteins present only in SDS protocol were identified (**Table 3**; **Figure 1**, spots 19−26).

To further examine the possible effects of extraction protocol on the identification of matched proteins (present in both phenol and SDS gels), 12 spots (six pairs) were selected from each fruit tissue. Each pair had the same $M_r$ and p*I* with similar spot densities in each extraction protocol (**Figure 1**). Among the 12 spots, 8 were identified as major allergen Mal d 1.03A and PBD1, peptidase/threonine, and endopeptidase from apple and cytosolic ascorbate peroxidase and ATP synthease $\beta$ subunit from strawberry (**Table 3**, spots 9−12), confirming that both extraction protocols gave similar identification results. Mass spectra from LC-MS/MS analysis of two peptides (SAEI-LEGDGGVGTIK) of the protein major allergen Mal d 1.03A from apple (spot 9) and (IGLFGGAGVGK) of the protein ATP synthease $\beta$ subunit (spot 12) from strawberry with two protocols are shown in **Figures 4** and **5**.

## DISCUSSION

2-DE-based proteomic techniques are an important research platform for investigating molecular mechanisms in plants. Fruit tissues are considered to be recalcitrant plant tissues for proteomic analysis, due to low protein content and the presence of interfering substances. Establishing a protein extraction procedure is thus a critical step when proteomic studies of fruit tissues are conducted. The application of proteomic technology in fruit research appears to be promising for exploring proteins/genes directly related to fruit development, ripening, and storage disorders. Unlike other plant tissue, fruit tissues contain significant amounts of polyphenolics, organic acids, lipids, pigments, terpenes, and polysaccharides that often interfere with protein extraction and 2-DE procedures (*9*). Thus, sample preparation becomes a critical step in proteomic research on fruit tissues.

A few papers have proposed specific methods for protein extraction from recalcitrant plants that contain high levels of interfering compounds including storage polysaccharides, lipids, polyphenolics, and some secondary metabolites (*11*, *13*, *14*). Our previous study demonstrated the benefits of using a simple protocol that involves a combination of hot SDS treatment and TCA/acetone precipitation on apple and banana fruit tissues (*12*). In the present study, an in-depth evaluation of the hot SDS and a modified phenol protocol was conducted on a large scale gel. Up to 1422 and 1368 proteins were shown in apple and strawberry, respectively. Protein and gel analysis revealed qualitative and quantitative differences between the two protocols. The quantitative differences seemed to be more profound for strawberry samples. Overall, both the phenol and hot SDS produced high numbers of protein spots for further 2-DE analysis, but the phenol protocol seemed to yield consistently higher numbers of protein spots. Protein protocols utilizing phenol have been reported as suitable for the extraction of low concentrations of protein in vegetative plant tissues rich in components that inhibit electrophoresis (*14*−*16*). Phenol dissolves proteins and lipids while leaving water-soluble substances

**Table 3.** Putative Identification of Protein Extracted from Apple and Strawberry Fruit with Phenol and SDS Protocols[a]

| | | | | | $M_r$ (kDa) | | p*I* | | |
|---|---|---|---|---|---|---|---|---|---|
| spot | protein name | Genbank EST accession | Mascot score | match % cov[b] | exptl | theor[c] | exptl | theor | matched sequences (Mascot score) |
| 1 | major allergen Mal d 1.06A (*Malus domestica*) | gi\|41323956 Malus_EB156894 | 687 | 69 | 17.5 | 17.4 | 5.06 | 5.22 | GVLTYETEYASVIPPAR (118)<br>LYNALVLDADNLIPK**[e] (60)<br>TVEILEGDGGVGTIKKVSF** (109)<br>KVSFGEGSEYSYVK* (54)<br>VSFGEGSEYSYVK** (97)<br>DNFDYSYSLIEGDAISDK** (99)<br>LVASGSGSIIK** (57)<br>LIENYLVANPDAYN (56)<br>ISYEIK* (37) |
| 2 | fructose-bisphosphate aldolase (*Arabidopsis thaliana*) | gi\|113624 Malus_CN948813 | 248 | 10 | 39.5 | 36.9 | 8.35 | 6.76 | GILAADESTGTIGK** (87)<br>ANSEATLGAYK, G →V (48)<br>L LASINVENVESNR, L→F** (113) |
| 3 | guanine nucleotide regulatory protein (*Vicia faba*) | gi\|395072 Malus_EB156499 | 139 | 15 | 29.9 | 25.3 | 6.41 | 6.39 | LIVIGDGGTGK** (63)<br>NLQYYEISAK** (47)<br>SNYNFEKPFLYLAR* (29) |
| 4 | major allergen Pru ar 1 (*Malus domestica*) | gi\|14423842 Malus_CV128571 | 45 | 5 | 17.7 | 17.4 | 4.9 | 5.22 | AFILDADNLIPK** (45) |
| 5 | 48 kDa glycoprotein precursor (*Fragaria* × *ananassa*) | gi\|54306583 | 153 | 27 | 51.4 | 13.3 | 6.56 | 9.23 | ELAFGVPVR (55)<br>SSSPSYQNVR (54)<br>NNNLQILCFEVNAK + carbamidomethyl (C) (44) |
| 6 | putative heat-shock protein [*Oryza sativa* (*japonica* cultivar group)] | gi\|1906830 Fragaria_DY 673585 | 156 | 3 | 96.6 | 88.3 | 4.68 | 5.02 | AIYYLATDSLK (36)<br>ELVSNASDALDK (29)<br>ELVSNASDALDKLR (13)<br>AQALGDTSSLEFMR + oxidation (M)** (78) |
| 7 | 17.7 kDa heat shock protein (*Carcia papaya*) | gi\|37933812 Fragaria_DY 667157 | 185 | 10 | 18.6 | 17.7 | 5.52 | 6.4 | EYPNSYVFVIDMPGLK + oxidation (M)** (76)<br>AMAATPADAK.E + oxidation (M)* (42)<br>LPPPEPK* (33)<br>SFNAPTR* (34) |
| 8 | putative 26S proteasome regulatory particle triple-A ATPase subunit 5a (*Oryza sativa*) | gi\|92882240 n/a | 115 | 12 | 50.7 | 47.8 | 4.92 | 4.94 | ADILDPALMR + oxidation (M) (15)<br>QTIFLPVVGLVDPDK (18)<br>GVLLYGPPGTGK (24)<br>LAGPQLVQMFIGDGAK + oxidation (M) (50)<br>TMLELLNQLDGFSSDDR + oxidation (M) (8) |
| 9 | major allergen Mal d 1.03A (*Malus domestica*) | gi\|60280841 DT042583 | 421 | 32 | 18.4 | 17.6 | 5.35 | 5.68 | LVASGSGSVIK (41)<br>SAEILEGDGGVGTIK** (92)<br>INFGEGSTYSYVK** (95)<br>SAEILEGDGGVGTIKK (52)<br>YSVIEGDAISETIEK** (94)<br>LFNAFVLDADNLIPK** (47) |
| 10 | PBD1, peptidase/threonine endopeptidase (*Arabidopsis thaliana*) | gi\|15228805 CN994849_MALUS | 119 | 19 | 25.0 | 22.5 | 5.54 | 5.95 | CILEIR + carbamidomethyl (C) (63)<br>LVVAPPNFVIK (36)<br>NGIPLTTAAAANFTR (20)<br>LILGDIITSVNGK** |
| 11 | cytosolic ascorbate peroxidase (*Fragaria* × *ananassa*) | gi\|5257546 Fragaria_CX 662161 | 339 | 36 | 27.7 | 27.3 | 5.72 | 5.69 | EGLLQLPTDKA** (54)<br>EDKPEPPPEGR* (43)<br>LAWHSAGTYDVK** (47)<br>ALLSDPVFRPLVEK (34)<br>QSAELAHGANNGLDIAVR (43)<br>YAADEDAFFADYALAHQR** (89)<br>NCAPLMLR + carbamidomethyl (C); oxidation (M) (22)<br>LFNAFVLDADNLIPK(7) |
| 12 | ATP synthease $\beta$ subunit (*Nicotiana plumbaginfolia*) | gi\|19685 Fragaria_CX661790 | 562 | 22 | 52.2 | 59.8 | 5.18 | 5.95 | IGLFGGAGVGK (57)<br>EMIESGVIK + oxidation (M) (35)<br>VVDLLAPYQR (58)<br>TIAMDGTEGLVR + oxidation (M) (51)<br>VLNTGSPITVPVGR (68)<br>FTQANSEVSALLGR (108)<br>LVLEVAQHLGENMVR + oxidation (M) (52)<br>EAPAFVEQATEQQILVTGIK (45)<br>IPSAVGYQPTLATDLGGLQER (88) |

**Table 3.** (Continued)

| spot | protein name | Genbank EST accession | Mascot score | match % cov[b] | $M_r$ (kDa) exptl | $M_r$ (kDa) theor[c] | p*I* exptl | p*I* theor | matched sequences (Mascot score) |
|---|---|---|---|---|---|---|---|---|---|
| 13 | glyceraldehyde-3-phosphate dehydrogenase (*Panax ginseng*) | gi\|34099812 DT040635 | 167 | 22 | 39.6 | 31.8 | –[d] | 6.4 | DAPMFVVGVNEK.E + oxidation (M)** (71) AASFNIIPSSTGAAK (28) FGIVEGLMTTVHSITATQK.T + oxidation (M) (29) GILGYTEDDVVSTDFLGDSR (39) AGIALNDTFVK** |
| 14 | glyceraldehyde-3-phosphate dehydrogenase (*Petunia* × *hybrida*) | gi\| 120673 DT040635 | 237 | 16 | 39.8 | 36.5 | 7.96 | 6.68 | DAPMFVVGVNEK + oxidation (M)** (74) TLLFGEKPVTVFG IR (96) AASFNIIP SSTGAAKA (23) VPTV DVSVVDLTVR** (44) |
| 15 | glyceraldehyde-3-phosphate dehydrogenase like protein (*Pyrus pyrifolia* var. *culta*) | gi\|120666 DT040635 | 199 | 23 | 38.3 | 36.7 | 8.3 | 7.96 | AGIALNDNFVK (63) DAPMFVVGVNEK + oxidation (M)** (59) AASFNIIPSSTGAAK (25) VPTVDVSVVDLTVR** (52) |
| 16 | unknown | gi\|51862498 CV126113 | 37 | 4 | 43.1 | 20.9 | 5.89 | 10.91 | GPTQEQLLSFLK** LGSESGFLDR** ISFGFEASNVLK** LIFTNALYFK* DLLPPGSLDSFTR* DGLPALVEK* ESIINQNDVAR* FSOHIFLPQEK* IGSKPIGRPR* |
| 17 | aspartyl-tRNA synthetase, class IIb; tRNA synthetase, class II (D, K, and N) (*Medicago truncatula*) | gi\|92897799 Malus_CN942434 | 144 | 2 | 58.9 | 60.5 | 5.71 | 6.04 | LIAGSSEGGAAVFR** (63) VFEIGPVFR + [+14.02 at N-term V]** (53) IQSQVGNVFR + [+14.02 at C-term R]* (28) |
| 18 | adenosylhomocysteinase (*S*-adenosyl-L-homocysteine hydrolase) (AdoHcyase) | gi\|417745 Malus_CN897833 | 105 | 6 | 52.9 | 53.4 | 5.71 | 5.65 | ATDVMIAGK.V + oxidation (M) (40) TEFGPSQPFK (26) LVGVSEETTTGVK**(39) |
| 19 | dihydroflavonol 4-reductase (*Fragaria* × *ananassa*) | gi\|2599562 Fragaria_CO382045 | 88 | 7 | 40.8 | 38.1 | 6.54 | 6.32 | GLLPLPQEEETEK** (43) GIEENLT NIHFSSK* (45) |
| 20 | LMW heat shock protein (*Fragaria* × *ananassa*) | gi\|2911276 Fragaria_DY671878 | 153 | 18 | 19.6 | 17.4 | 5.77 | 6.17 | AAME NGVLTVTVPK** (69) VELEEG NVLQISGER (84) |
| 21 | low molecular weight heat shock protein (*Malus* × *domestica*) | gi\|6969974 | 127 | 14 | 22.5 | 18.2 | 5.85 | 5.39 | ENSAF VNTR (29) AAMENGVLSVTVPK + oxidation (M)** (98) |
| 22 | peroxyredoxin (*Populus tremula* × *Populus tremuloides*) | gi\|19548660 | 52 | 5 | 20.3 | 17.4 | 5.48 | 5.56 | FALLVDDLK** (52) |
| 23 | spermidine synthase (*Malus* × *domestica*) | gi\|23495354 DY670196 | 129 | 7 | 35.5 | 36.4 | 4.62 | 4.79 | VLVLDGV IQLTER (69) VLVIGGGDGGVL R (60) GLTFTPESGFLPGR** GTVNPTTYNIVNK** GLDNNDFLAK** ISPYAVLTVK* VVSPPBKVVSLPR* |
| 24 | putative nascent polypeptide associated complex α chain [*Oryza sativa* (*japonica* cultivar group)] | gi\|115463539 CO817614 | 222 | 34 | 28.7 | 13.7 | – | 4.87 | NILF VISKPDVFK* (44) SPTSDTYVIFG EAK** (95) IEDLSSQ LQTQAAEQFK** (83) |
| 25 | concanavalin A-like lectin/ glucanase (*Medicago truncatula*) | gi\|92886357 DV440505 | 168 | 8 | 56.0 | 48.4 | – | 4.41 | LLS DNTLVFQFSVK (58) DNTLVFQF VK (42) FYAISAEFPEFSNK (68) |
| 26 | *hsp* 70-like protein (*Arabidopsis thaliana*) | gi\|7269278 Fragaria_DY671198 | 110 | 5 | 63.7 | 76.5 | – | 5.07 | IPAVQELVR* (59) DIDEVILVGGSTR*(30) QAVVNPEN TFFSVK (21) |

[a] Protein spots excised from gels stained with Sypro Ruby were subjected to digestion with trypsin and identified following mass spectrometry analysis (LC-MS/MS). [b] %Cov, percent of coverage. [c] Theoretical value found at NCBI. [d] Out of range of land markers. [e] **, sequences matched with EST databases with ProID confidence score of >95%. [f] *, sequences matched with EST databases with ProID confidence score of <95%.

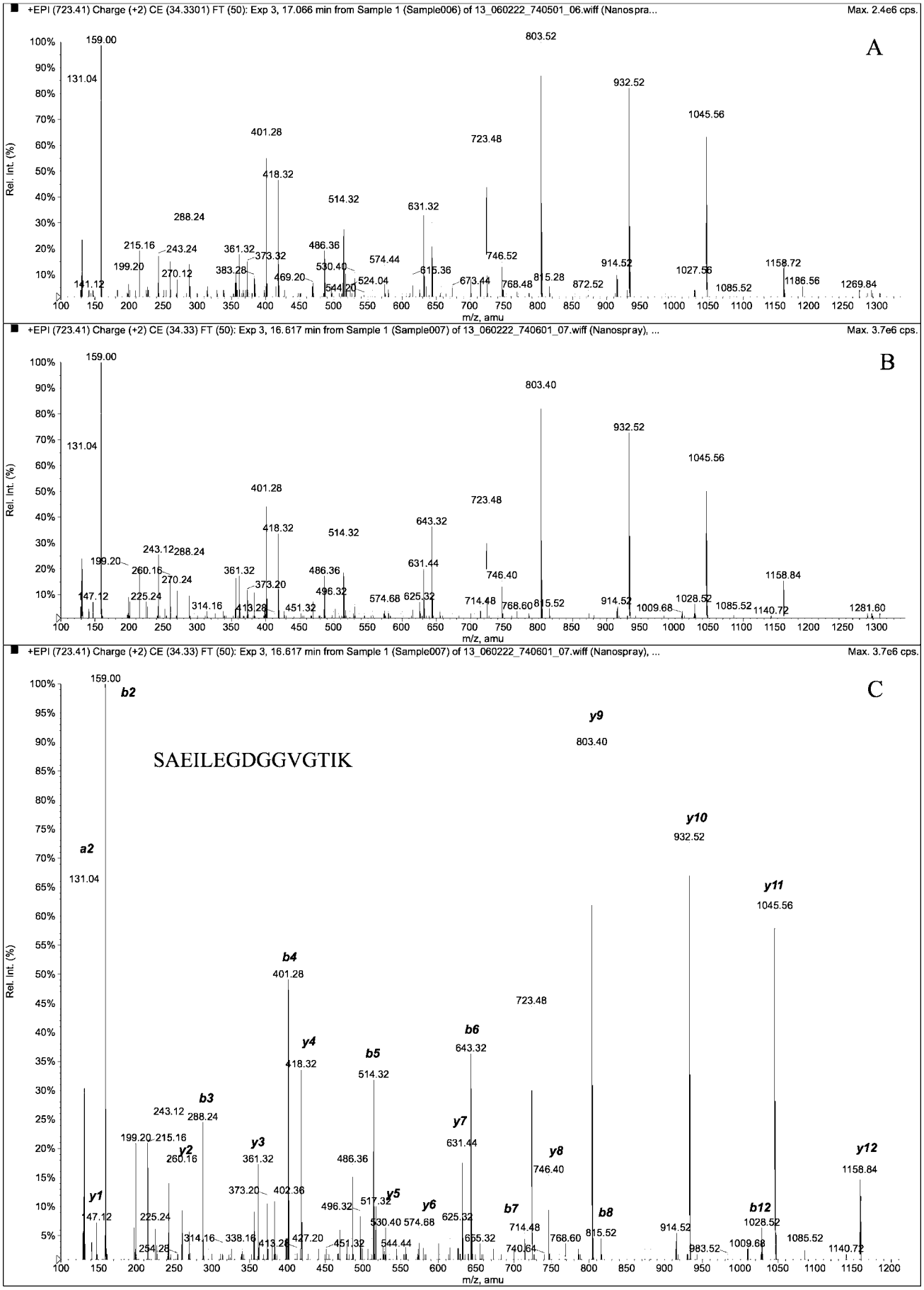


**Figure 4.** Product ion spectrum of peptide SAEILEGDGGVGTIK at *m*/*z* 803.4 from apple protein, major allergen Mal d 1.03A (spot 9 in **Table 3** and **Figure 1A**,**B**): (**A**) obtained with phenol protocol; (**B**) obtained with SDS protocol; (**C**) with major fragment ions labeled.

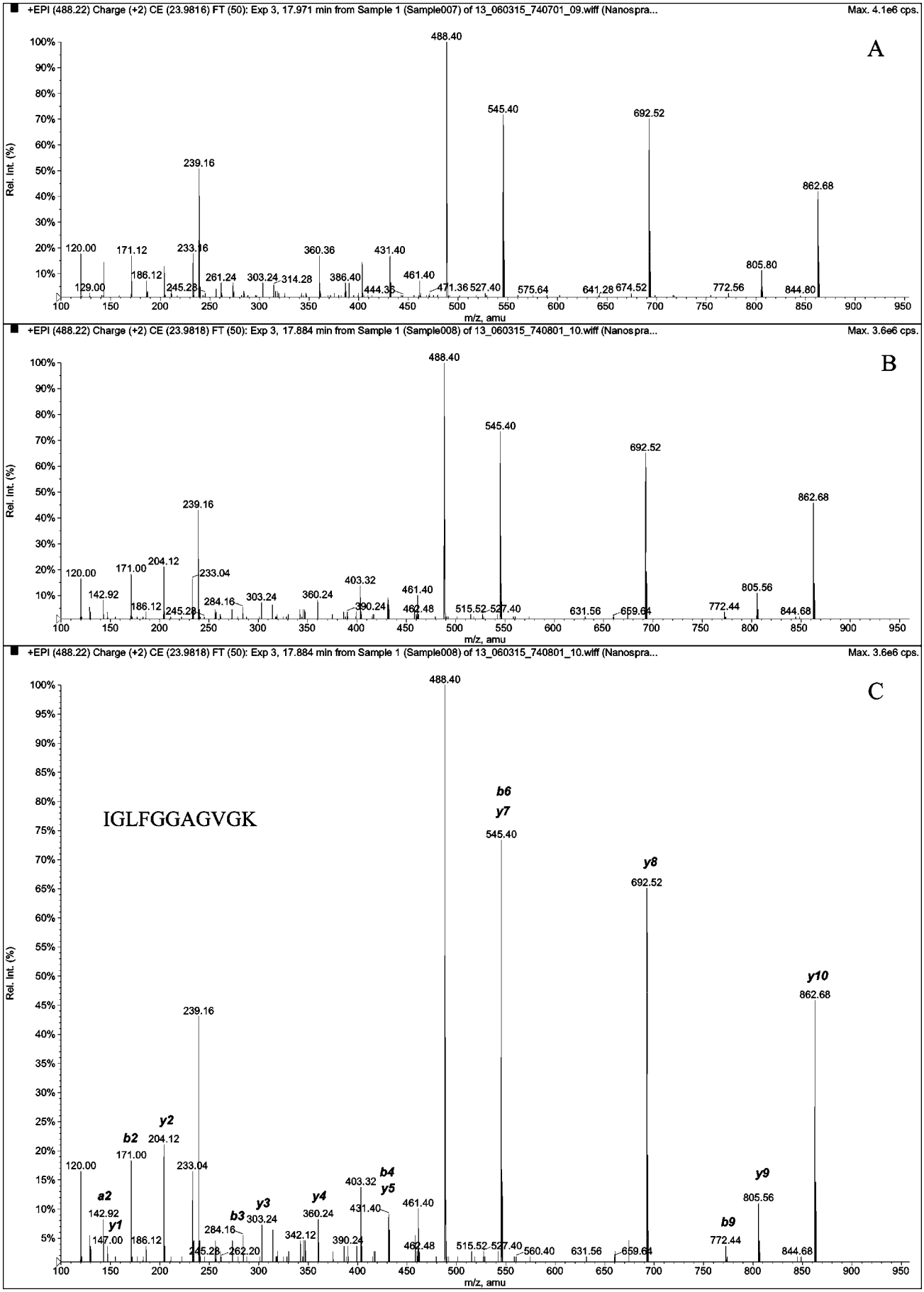


**Figure 5.** Product ion spectrum of peptide IGLFGGAGVGK at *m*/*z* 488.40 from strawberry protein, ATP synthease $\beta$ subunit (spot 12 in **Table 3** and **Figure 1A**,**B**): (**A**) obtained with phenol protocol; (**B**) obtained with SDS protocol; (**C**) with major fragment ions labeled.

(polysaccharides, nucleic acids, etc.) in the aqueous phase. Protein can then be separated from lipids with methanol precipitation in the presence of ammonium acetate. Phenol can also minimize protein degradation due to endogenous proteolytic activity (*18*). A phenol protocol gave satisfactory results in tomato tissues, including leaves and fruits. Proteins prepared in this manner from tomato and banana fruit were comparable to proteins precipitated with acetone (*12*). Recently, classic TCA/acetone precipitation and phenol extraction were quantitatively evaluated with banana, apple, and potato plant tissues, and both were considered to be useful as standard protocols (*15*). Another study conducted on mature grape berry cluster concluded that the phenol-based protocols were better than the TCA/acetone method with larger protein yield and greater spot resolution (*19*). In this study, we confirmed that the phenol protocol showed clear advantages for protein yields and spot numbers when apple and strawberry fruit tissues were used as protein sources. In our previous study, hot SDS preparation in combination with TCA/acetone precipitation was successfully developed for apple and banana fruits and was found to be an improved method over TCA/acetone precipitation alone (*12*). SDS is an excellent solubilizing reagent, which allows the recovery of membrane proteins (*20*, *21*), whereas heating in the presence of SDS can afford a faster inactivation of proteases (*22*, *23*) as well as enhance the solubility of membrane proteins (*24*). In this study, a large scale gel (24 × 21 cm) was applied to compare hot SDS with phenol for apple and other fruit tissues. Although a lower protein yield and fewer spots were obtained in apples, SDS showed a reasonable protein yield and distinct spots suitable for gel analysis as well as LC-MS/MS protein identification. The correlation coefficient between SDS and phenol protocols was approximately 75% for apple tissue. However, with strawberry sample preparation, interferences in protein samples remained even after TCA precipitation and resulted in poor resolution and considerable streaking in the 2-D PAGE. An additional cleanup procedure was applied to strawberry protein samples, which improved 2-D PAGE resolution significantly. It may be worthwhile to incorporate it in the sample preparation for strawberry fruit. A similar cleanup procedure was used by Hopkins et al. (*25*). Assessment of the two protocols using molecular weight and p*I* as criteria revealed a significant very similar distribution of spots both in phenol and in SDS preparations in most ranges of $M_r$ and p*I*; however, there appears to be relatively more variation in distribution in spot intensity in phenol than in SDS protocols.

Qualitative and quantitative analysis of 2-DE spots combined with LC-MS/MS identification revealed some important details of the two protocols. Although only a limited number of spots were selected for identification, two proteins from the phenol protocol were identified as the major allergen, Mal d 1.06A, and fructose-bisphosphate aldolase, whereas two proteins from the SDS protocol were identified as being guanine nucleotide regulatory protein and the major allergen Pru ar 1, from apple. Eighteen Mal d 1genes have been reported to be present in apples (*26*). In strawberry, a 48 kDa glycoprotein precursor and a putative heat shock protein were identified with the phenol protocol, whereas two other proteins from the SDS preparations were identified as a 17.7 kDa heat shock protein and a putative 26S proteasome regulatory particle. These proteins may support the findings that phenol may enhance accumulation of glycoproteins as reported by Saravanan and Ross (*14*), whereas SDS enhanced the recovery of membrane proteins (*19*, *23*). Although more protein identifications are needed to support these findings, these results might indicate that the proteins recovered using the two protocols are comparable yet complementary, with each method having specificity for certain groups of proteins. In addition, solubilization buffers applied in these two protocols may also contribute the presence of proteins (*17*).

Six pairs of proteins obtained from both apple and strawberry fruit were selected, which appeared to have similar spot intensities in the range of 300−5000 pixels (normalized spot intensities). These spots enabled an assessment of whether the different protocols affected LC-MS/MS identification on the same protein spots. Successful identification of four pairs of spots in both apple and strawberry tissue indicated that both protocols are valid for spot identification by LC-MS/MS. There is no significant difference in coverage of polypeptide identification between the two protocols (**Table 3**; **Figures 4** and **5**).

For differential comparison purposes, protein samples routinely should be extracted and purified by specifically designed procedures as different protocols can generate distinctly different patterns of protein spots on 2-D PAGE images (*14*). The SDS protocol is faster, safer, and easier to perform than the phenol-based protocol and can be used as a starting protocol for recalcitrant fruit tissues. Both methods are applicable to apple samples. However, proteins extracted from strawberries using the SDS method showed serious streaking in 2-D PAGE gels. A further cleanup procedure was applied, and although it may have caused protein loss (55−65% recovery), it was a necessary step to purify the protein extract before it was further analyzed by 2-DE. This cleanup procedure can be conducted with kits that are commercially available, and information about the recovery rate for protein cleanup should be collected under these circumstances. Overall, the phenol protocol is more proficient than the hot SDS, but its toxicity and time-consuming nature should be considered when a sample preparation procedure is being designed. To obtain the maximum protein recovery from fruit tissues with minimized time and cost, it may be helpful to combine protein extracts from the two protocols and then conduct further SDS-PAGE, IEF, 2-DE gel analysis, and spot identification using mass spectrometry. However, this application remains to be tested. This study characterized the qualitative and quantitative differences in protein preparation protocols and demonstrated the complexity of protein extraction procedures for recalcitrant fruit tissues. The qualitative and quantitative protein profiles obtained in this study provide evidence that there is no universal and simple sample preparation procedure for the recalcitrant plant/fruit tissue. The SDS protocol or SDS plus a cleanup could be used as a first approach for an unknown fruit tissue or as an alternative protocol to phenol extraction, especially if operator safety is a primary concern. Detection within the SDS extracts of 23 apple and 109 strawberry proteins that were not present in the phenol extracts suggests that it might be best to use at least two that would serve to complement each other. The combination of SDS and phenol in one extraction procedure demonstrated by Wang (*16*) may be beneficial to simplify the sample preparation procedures, but its full benefits as a universal approach need to be tested in more recalcitrant fruit tissues, especially fruit peels.

## ABBREVIATIONS USED

LC-MS/MS, liquid chromatography−tandem mass spectrometry; NL, nonlinear; $M_r$, molecular mass; p*I*, isoelectric point; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ASB-14, amidosulfobetaine-14; IPG, immobilized pH gradient; 2-DE, two-dimensional electrophoresis.

## ACKNOWLEDGMENT

We thank Drs. Greg Bezanson and John Delong for a critical review of the manuscript as well as Dr. Andrew R. Jamieson of AAFC for supplying strawberry fruit samples.

## LITERATURE CITED

(1) Jacobs, I. D.; van Der Heijden, R.; Verpoorte, R. Proteomics in plant biotechnology and secondary metabolism research. *Phytochem. Anal.* **2000**, *11*, 277−287.

(2) Roberts, J. K. M. Proteomics and a futures generation of plant molecular biologists. *Plant Mol. Biol.* **2002**, *48*, 143−154.

(3) Renaut, J.; Hausman, J. F.; Wisniewski, M. E. Proteomics and low-temperature studies: bridging the gap between gene expression and metabolism. *Physiol. Planta.* **2006**, *126*, 97−109.

(4) Gallardo, K.; Job, C.; Groot, S. P. C.; Puype, M.; Demol, H.; Vandekerckhove, J.; Job, D. Proteomic of *Arabidopsis* seed germination. A comparative study of wild-type and gibberellin-deficient seeds. *Plant Physiol.* **2002**, *129*, 823−837.

(5) Hoa, L. T.; Normura, M.; Kajiwara, H.; Day, D. A.; Tajima, S. Proteomic analysis on symbiotic differentiation of mitochondria in soybean nodules. *Plant Cell Physiol.* **2004**, *45*, 300−308.

(6) Kruft, V.; Eubel, H.; Jansch, L.; Werhahn, W.; Braun, H. Proteomic approach to identify novel mitochondrial proteins in *Arabidopsis*. *Plant Physiol.* **2001**, *127*, 1694−1710.

(7) Casado, J.; Sellés, S.; Martínez, R. B. Proteomic approach to blossom-end rot in tomato fruits (*Lycopersicon esculentum* M.): antioxidant enzymes and the pentose phosphate pathway. *Proteomics* **2005**, *5*, 2488−2496.

(8) Jacobs, D. J.; Gaspari, M.; van der Greef, J. Proteome analysis of the medicinal plant *Catharanthus roseus*. *Planta* **2005**, *221*, 690−704.

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

(10) Mechin, V.; Consoli, L.; Guilloux, M. L.; Damerval, C. An efficient solubilization buffer for plant proteins focused in immobilized pH gradients. *Proteomics* **2003**, *3*, 1299−1302.

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

(12) Song, J.; Braun, G.; Bevis, E.; Doncaster, K. A simple protocol for protein extraction of recalcitrant fruit tissues suitable for 2-DE and MS analysis. *Electrophoresis* **2006**, *27*, 3144−3151.

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

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

(15) Carpentier, S. C.; Witters, E.; Laukens, K.; Decker, P.; Swennen, R.; Panis, B. Preparation of protein extracts from recalcitrant plant tissues: An evaluation of different methods for two-dimensional gel electrophoresis analysis. *Proteomics* **2005**, *5*, 2497−2507.

(16) Wang, W.; Vignani, R.; Scali, M.; Cresti, M. A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. *Electrophoresis* **2006**, *27*, 2782−2786.

(17) Khoudoli, G. A.; Porter, I. M.; Blow, J. J.; Swedlow, J. R. Optimisation of the two-dimensional gel electrophoresis protocol using the Taguchi approach. *Proteome Sci.* **2004**, *2*, 6.

(18) Schuster, A. M.; Davies, E. Ribonucleic acid and protein metabolism in pea epicotyls. I. The aging process. *Plant Physiol.* **1983**, *73*, 809−816.

(19) Vincent, D.; Wheatley, M. D.; Cramer, G. Optimization of protein extraction and solubilization for mature grape berry clusters. *Electrophoresis* **2006**, *27*, 1853−1865.

(20) Molloy, M. P. Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. *Anal. Biochem.* **2000**, *280*, 1−10.

(21) Görg, A.; Weiss, W.; Dunn, M. J. Current two-dimensional electrophoresis technology for proteomics. *Proteomics* **2004**, *4*, 3665−3685.

(22) Rabilloud, T. Solubilization of proteins for electrophoretic analyses. *Electrophoresis* **1996**, *17*, 813−829.

(23) Jefferies, J. R.; Brophy, P. M.; Barrett, J. Investigation of *Fasciola hepatica* sample preparation for two-dimensional electrophoresis. *Electrophoresis* **2000**, *21*, 3724−3729.

(24) Grainer, F. Extraction of plant proteins for two-dimensional electrophoresis. *Electrophoresis* **1998**, *9*, 712−718.

(25) Hopkins, A.; McIntosh, R.; Layfield, R.; Keyte, J.; Dua, H.; Tighe, P. Optimised two-dimensional electrophoresis procedures for the protein characterization of structural tissues. *Proteomics* **2005**, *5*, 1967−1979.

(26) Gao, Z. S.; Schouten, H. J.; van der Meer, I. M.; van der Geest Kodde, J.; Breitenedier, H.; Hoffmann-Sommergruber, K.; Bosch, D.; Gilissen, L. J. W. J. Genomic cloning and linkage mapping of the Mad d1 (pr-10) gene family in apple (*Malus domestica*). *Theor. Appl. Genet.* **2005**, *111*, 171−183.

**Received for review October 4, 2006. Revised manuscript received December 17, 2006. Accepted January 4, 2007. This work is supported in part by funds from the Washington Tree Fruit Research Commission and from the Matching Industry Initiatives of Agriculture and Agri-Food Canada. Contribution 2334 of the Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada.**

JF062850P