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Research Article
Comparison of Protein Isolation Methods from *Brassica napus* subsp. *oleifera* Seeds Growing in Turkey

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Abstract

Background and Objective: Canola (*Brassica napus* L.) is an important source of vegetable oil and is the second largest oil seed crop after soybean. Many analytical techniques have been reported for the identification and quantification of proteins. In this study, the main objective was to evaluate the most effective protein isolation method from canola seeds. **Materials and Methods:** Four different winter canola types from *Brassica napus* subsp. *oleifera* seeds were cultured in Turkey. Proteins were isolated using four methods from these types of canola and were analyzed by SDS-PAGE method. Phenylalanine Ammonia-Lyase (PAL) enzyme activities of canola types were measured. Besides, high performance liquid chromatography (HPLC) chromatographic method was performed in order to obtain the cinnamic acid amount in the methanol extracts of the canola seeds. **Results:** The TCA method yielded the highest protein contents among four protein isolation methods. These methods showed that *Orkan* type contains higher protein. The highest PAL enzyme activity was found at *Eurol* and *California* types. According to HPLC results, the highest amount of cinnamic acid was determined in *Eurol* type. **Conclusion:** Protein isolation and identification from canola types containing such active specific proteins is of great importance both economically and scientifically. For protein isolation from canola seeds, TCA method yielded was the highest protein contents.

Key words: Canola, protein isolation, cinnamic acid, PAL, SDS-PAGE, HPLC

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Canola (*Brassica napus* L.) is one of the most important members of the Cruciferae. *Brassica napus* is an important source of vegetable oil and is the second largest oil seed crop after soybean[1]. Evaluation of genetic diversity among wild and crop plant population is necessary for the protection, conservation and useful application of germ plasms, identification of suitable parents for high-quality crosses and characterization of the genetic content of important breeding traits[2-5].

In Turkey, rapiska, rapitsa and kolza are known names of canola. There are two physiological periods of canola as winter and summer types. Generally winter canola cultivation is performed. Canola, which is produced in Canada, is also used for feeding of poultry[6].

The removal of unwanted components provides 34-40% protein-containing edible canola. The importance of the canola protein for human diet began[7-9] in 1970s. Unlike other herbal proteins, canola proteins were found to be rich in lysin and methionine aminoacids[10,11]. Canola seeds includes essential amino acids at high rates[12].

Since canola is widely available, using canola proteins to compete with rooted protein products such as soybean provides an alternative for future of the food sector. The canola proteins are used in feed and fertilizer industry, not for food production. In order to consume canola seed proteins as food, the chemical and physicochemical properties of proteins need to be clarified. The previous studies showed that the SDS-PAGE analysis in protein determination is used as a successful method[12]. Canola seeds are also rich in phenolic compounds. For the quantification of phenolic compounds, there are many techniques. High performance liquid chromatography (HPLC) is one of the most widely used quantification method[13-15].

In this study, the main objective was to evaluate the most effective protein isolation method from canola seeds. Proteins isolated from four different winter canola types from *Brassica napus* subsp. *oleifera* seeds cultured in Turkey were analyzed by SDS-PAGE method and PAL enzyme activities of canola types were measured.

MATERIALS AND METHODS

This study was performed for 6 months.

Plant material: *Brassica napus* subsp. *oleifera* winter types *Orkan*, *Bristol*, *California* and *Eurol* seed were determined from Selçuk University, Faculty of Agriculture.

Protein isolation: Four different protein isolation buffers/methods were used to extract proteins from canola seeds. These protein isolation methods were adapted from the literature[16]. For all methods, 0.1 g canola seeds were powdered in liquid nitrogen using mortar and pestle.

Protein isolation method 1: Canola seed powder was mixed with 500 µL buffer solution 1 (1 M Tris-HCl (pH: 6,8), 10% SDS (w/v), 1M DTT, 10% glycerol). Mixture was vortexed, incubated at 70°C for 15 min and centrifuged at 6500 rpm for 10 min at 4°C. About 30 µL supernatant was mixed with loading dye and incubated at 100°C for 5 min. After centrifugation for 1 min, supernatant was used for SDS-PAGE.

Protein isolation method 2: Canola seed powder was mixed with 500 µL buffer solution 2 (0,05 M Na_2CO_3, 0,05 M EDTA, 1,2 M sucrose, 2% SDS, 1 M DTT). Mixture was vortexed, incubated at 70°C for 15 min and centrifuged at 6500 rpm for 10 min at 4°C. About 30 µL supernatant was mixed with loading dye and incubated at 100°C for 5 min. After centrifugation for 1 min, supernatant was used for SDS-PAGE.

Protein isolation method 3: Canola seed powder was mixed with 500 µL phosphate buffer (0,2 M, pH: 6,8). Mixture was vortexed, incubated at 70°C for 15 min and centrifuged at 6500 rpm for 10 min at 4°C. About 30 µL supernatant was mixed with loading dye and incubated at 100°C for 5 min. After centrifugation for 1 min, supernatant was used for SDS-PAGE.

Protein isolation method 4 (TCA method): Canola seed powder was homogenized with 100% (w/v) cold trichloroacetic acid (TCA) was added onto seeds as final concentration would be 20% TCA. The mixture was mixed on ice for 20 min and centrifuged at 14000 rpm for 15 min at 4°C. Pellet was dried at room temperature to discard TCA and washed with 0.2 mL cold acetone. Mixture was vortexed and centrifuged at 14000 rpm for 2 min at 4°C. Pellet was dried at room temperature.

Phenylalanine ammonia-lyase (PAL) activity assay: The PAL enzyme converts phenylalanine amino acid into cinnamic acid. For PAL enzyme activity measurement, 0.25 g seed was powdered using mortar and pestle with liquid nitrogen. Canola seed powder was mixed with 1 mL buffer solution 3 (20 mM (pH:7,0) phosphate buffer, 5% PVP, 0,1% Triton X-100, 50 mM ascorbic acid, 18 mM mercaptoethanol). The mixture was centrifuged at 16500 rpm for 10 min. Ammonium

sulphate was added as final concentration would be 50%. After centrifugation at 16500 rpm for 10 min, supernatant was discarded. Pellet was resuspended with 1 mL 20 mM phosphate buffer. About 250 µL mixture was mixed with 875 µL 0,06 M borate buffer (pH: 8.2) and 10 mg mL^{-1} phenylalanine. Mixture was incubated at 30°C for 30 min and its absorbance value was measured at 290 nm (Fig. 2).

Bradford assay: Protein amounts of canola seeds after isolation were calculated with Bradford assay using BSA calibration graph.

SDS-PAGE: Protein samples isolated from canola seeds were analyzed with SDS-PAGE. About 12 µL protein sample was mixed with 3 µL 3X SDS loading buffer and loaded into gel. Samples were run at 200 V and 100 A. Gel was stained with staining solution.

Seed methanol extraction: Canola seeds were incubated in methanol for 6 h at 30°C. Methanol extracts of canola seeds were incubated at 40°C in evaporator in order to discard solvent. Lyophilized samples were kept at 4°C until use.

High performance liquid chromatography (HPLC): Cinnamic acid analysis of canola seed methanol extracts were performed with HPLC at Süleyman Demirel University Student Experimental and Observational Research and Application Center, Isparta. About 20 mg of methanol extract was dissolved in 1 mL methanol and 20 µL of this solution was injected to HPLC. The standards for HPLC chromatograms are 1: Gallic acid, 1: Chlorogenic acid, 3: Vanillic acid, 4: Ferulic acid and 5: Cinnamic acid.

RESULTS

Protein amounts of canola types: Protein quantities of the samples were found using Bradford Assay by calculating using the equation obtained from BSA calibration graph. Protein quantities obtained from different protein isolation methods are shown in Table 1. According to the results, *Orkan* type contains higher protein. In addition, as a result of protein isolation by TCA method, higher protein amounts were obtained for all types. The amount of protein extracts were not directly proportional to the quality.

Quality of protein samples: The quality of protein extracts was examined by SDS-PAGE. TCA method yielded the highest protein contents, however smeared bands in SDS-PAGE were obtained. As a result, the best band image was obtained with method 2 and the protein bands on the gel were clearly visible (Fig. 1). For the other protein isolation methods, band images were not clear.

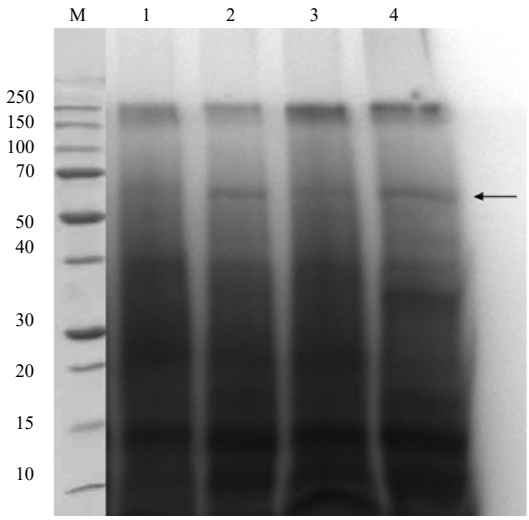

Fig. 1: SDS-PAGE gel image of protein samples isolated with method 2
M: Marker, 1: *Bristol* type, 2: *Orkan* type, 3: *Eurol* type, 4: *California* type. (PAL protein: 65 kDa, arrow shows PAL protein)

Table 1: Protein amounts of different protein isolation methods

	Methods (mg g^{-1})			
Parameters	1	2	3	4 (TCA method)
Bristol	0.52	0.85	0.76	0.92
Orkan	0.86	1.09	0.82	1.21
Eurol	0.45	0.63	0.75	0.87
California	0.40	0.67	0.54	0.83

Table 2: The PAL enzyme activities and cinnamic acid amounts in methanol extracts of different canola types

Parameters	*Bristol*	*Orkan*	*Eurol*	*California*
PAL activity (U mL^{-1})	24.68	49.77	87.76	85.70
Protein (mg mL^{-1})	0.14	0.14	0.11	0.10
Protein (mg g^{-1}) canola	0.67	0.72	0.57	0.51
Cinnamic acid (µg g^{-1})	10.70	15.40	23.80	14.90

PAL enzyme activities: The PAL enzyme converts phenylalanine amino acid into cinnamic acid. In order to evaluate the PAL enzyme activity trans-cinnamic acid calibration graph was drawn (Fig. 2) (R^2 value = 0.9994). The PAL enzyme activity (unit) was determined as the amount of enzyme converting 1 µmol phenylalanine to cinnamic acid per minute. The two types with the highest PAL activity are *Eurol* and *California* types as seen in Table 2.

PAL presence analysis with HPLC: Methanol extraction from canola seeds was performed to determine the presence of

PAL. HPLC analysis was performed as an alternative to PAL enzyme activity assay. Cinnamic acid, a product of PAL enzyme, was analyzed in methanol extracts obtained from each type of canola. Chromatograms obtained from HPLC analysis are shown in Fig. 3-6. The data obtained are shown in Table 2. The highest amount of cinnamic acid was detected in *Eurol* type.

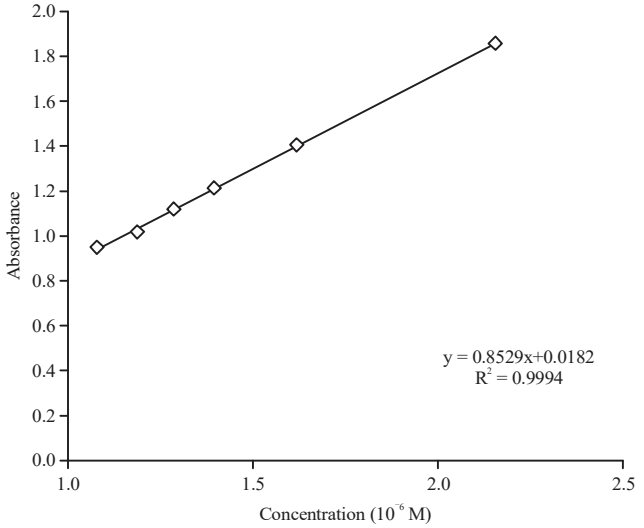

Fig. 2: Cinnamic acid calibration graph: PAL enzyme converts phenylalanine amino acid into cinnamic acid. So, cinnamic acid amount also gives the PAL enzyme activity. R^2 is very close to 1, which shows the absorbance rate is almost accurate

DISCUSSION

In this study, four different protein isolation methods from canola seeds were performed and compared. For protein isolation from canola seeds, the most effective method was modified TCA method. The two types with the highest PAL activity are *Eurol* and *California* types of canola and the highest amount of cinnamic acid was detected in *Eurol* type.

The increase in consumer awareness about healthy foods, has encouraged researchers to identify natural components in different products[17]. It has been suggested that the bioactive components of canola protein are an attractive source. Protein isolation and identification from canola types containing such active specific proteins is of great importance both economically and scientifically. Therefore protein isolation from this oil seed is important.

Phenylalanine ammonia lyase (PAL) is the key enzyme for the synthesis of phenolics. The PAL is one of the main products in flavonoid biosynthesis in plant biochemistry. It was shown that PAL activity and phenolic compounds and was also found that there is a correlation between the

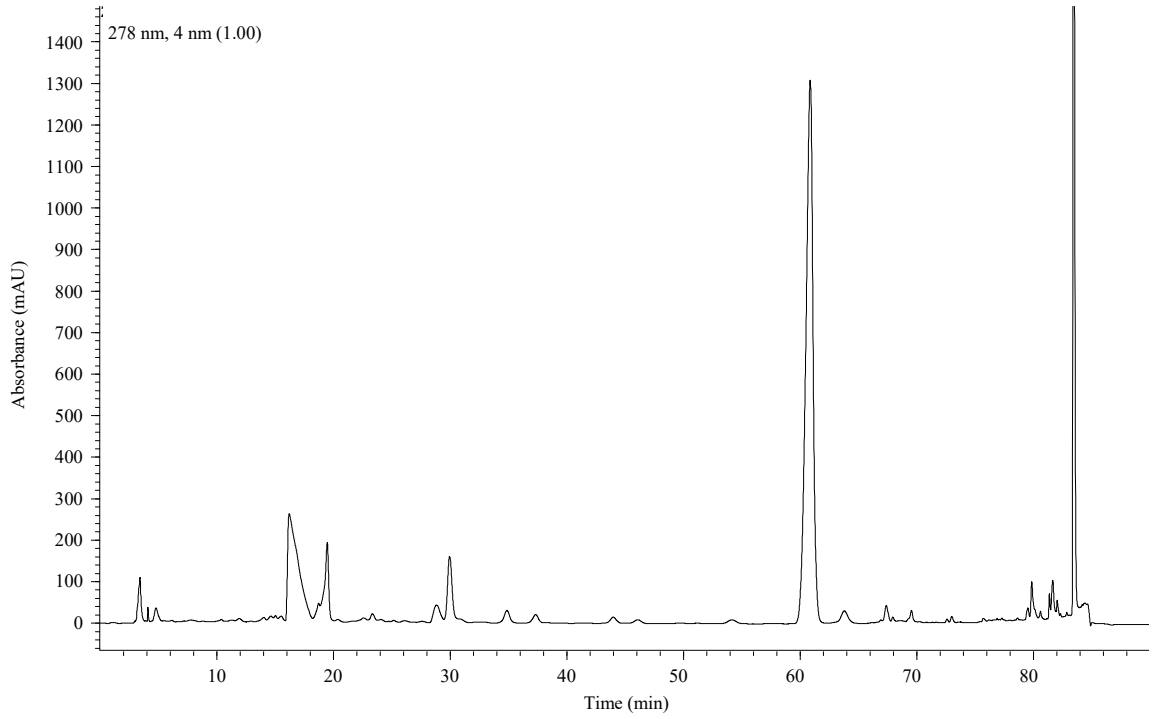

Fig. 3: HPLC chromatogram of methanol extract of *Bristol* type canola

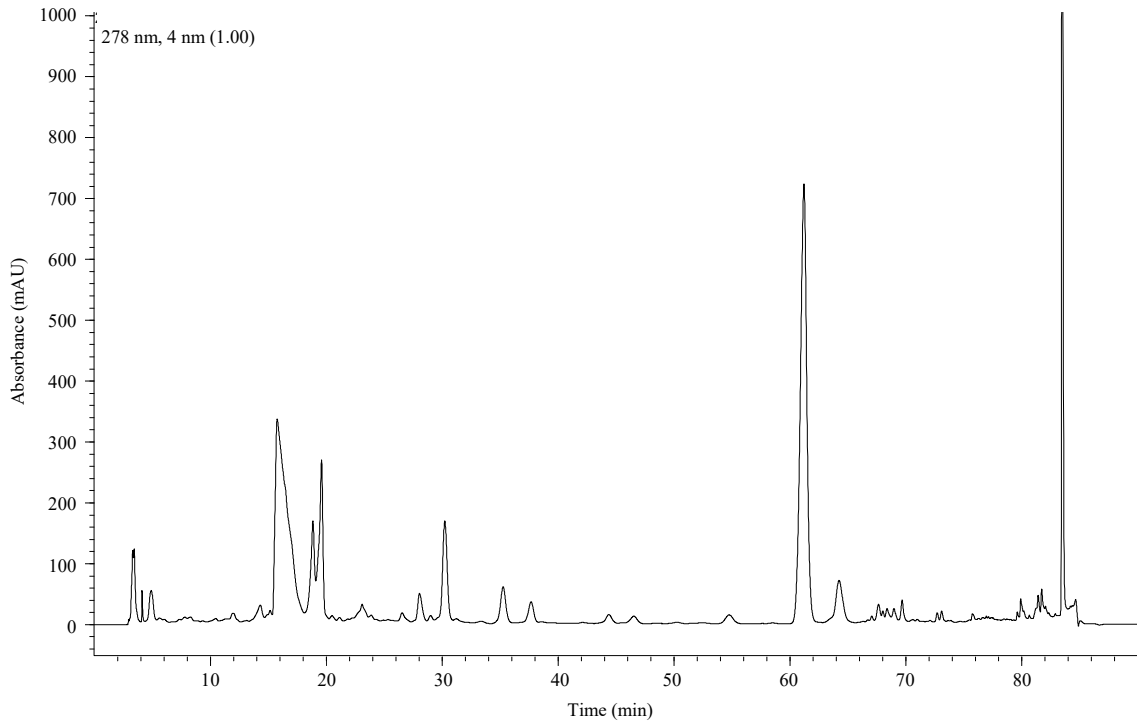

Fig. 4: HPLC chromatogram of methanol extract of *Orkan* type canola

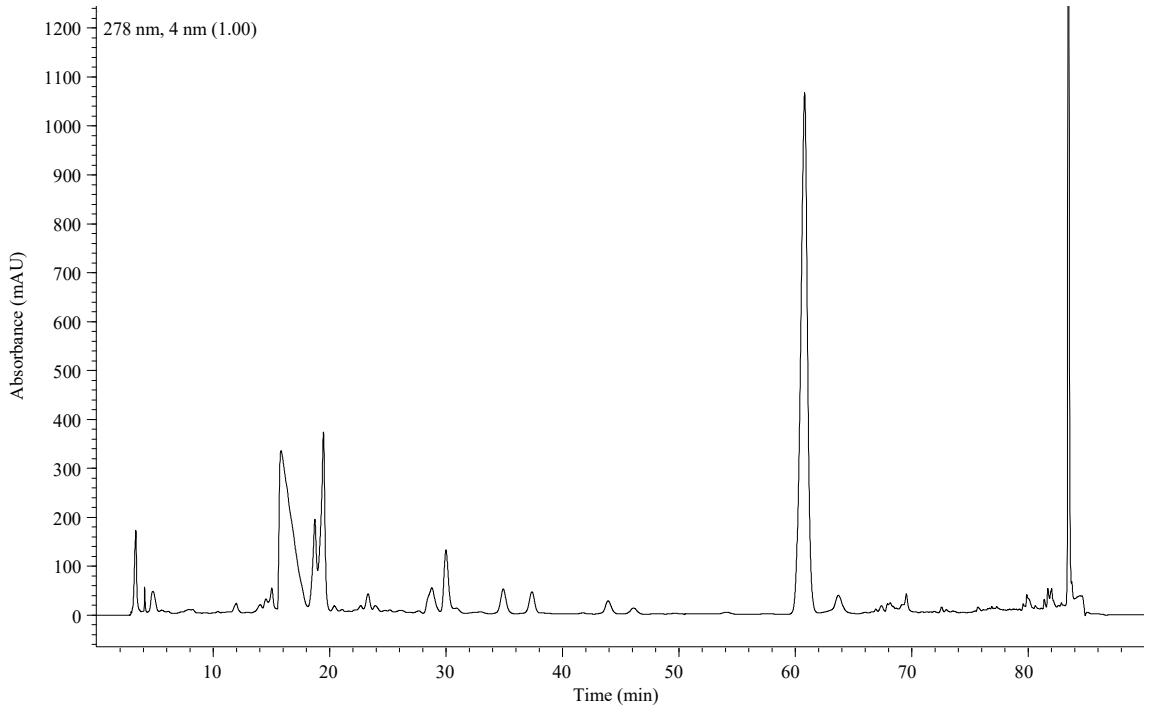

Fig. 5: HPLC chromatogram of methanol extract of *Eurol* type canola

formation of phenolic compounds. The most common secondary phenolic compounds in plants are derived from phenylalanine. In particular, flavonoid biosynthesis begins by converting phenylalanine amino acid into cinnamic acid by PAL enzyme. Therefore, cinnamic acid production serves as PAL enzyme activity[18].

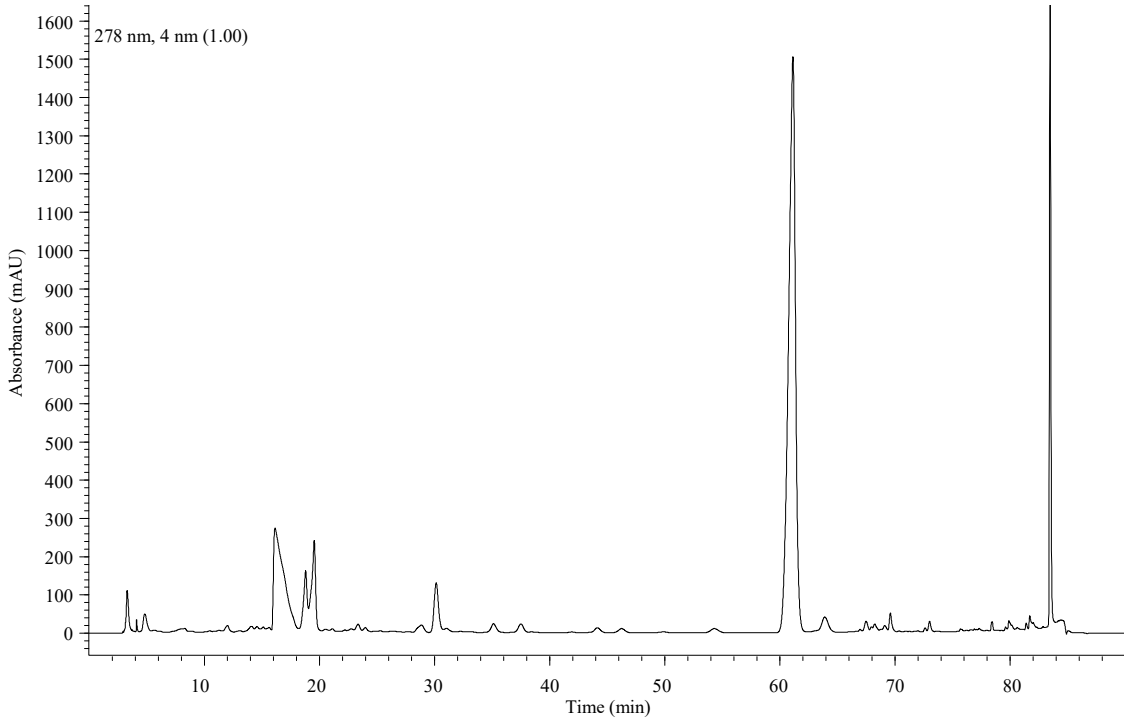

Fig. 6: HPLC chromatogram of methanol extract of *California* type canola

Flavonoids and cinnamic acids are known to be the most important anti-oxidant and free radical scavenger and chain breakers. The PAL enzyme catalyzes primary and secondary metabolism to complete separation point, an important regulatory step catalyzes the formation of a variety of phenolic compounds[18].

The protein extraction from oil seeds is very important case. There are many critical points because plant tissues are difficult for protein extraction especially for further protein analysis. Tissue disruption has an importance because plants have complex polysaccharides. Mostly used method for tissue disruption is using mortar and pestle with liquid nitrogen[19]. The same method was used for this study for disruption. It also minimizes the proteolysis which is very critical for protein studies. Wang *et al.*[19] reviewed different TCA methods, however there is no information about canola seeds. The most preferred method for precipitation and concentration of total proteins is TCA method[19,20]. The most effective method for canola seeds was TCA method.

Canola is an important industrial plant because of oil and protein contents. In the future, for canola seeds, improved protein extraction methods have to be modified. According to the protein properties such as molecular weight, solubility and isoelectric points, many different modifications are possible. These should be studied more comprehensively in order to get high quality proteins.

CONCLUSION

Protein isolation and identification from canola types containing such active specific proteins is of great importance both economically and scientifically. In this study, different protein isolation methods from canola seeds were compared and the most effective method was modified TCA method. For protein isolation from canola seeds, TCA method yielded the highest protein contents.

SIGNIFICANCE STATEMENT

This study evaluated the comparison of protein isolation methods from canola seeds and it will help the researchers to choose more suitable methods for higher yield of protein from oil seeds.

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