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Extraction and characterisation of lemon, orange and grapefruit seeds press cake proteins

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RESEARCH ARTICLE

Abstract

The aim of this study was to characterise the proteins of lemon (*Citrus limon* var. Kütdiken), orange (*Citrus sinensis* var. Dörtyol) and grapefruit (*Citrus paradisi*var. Beyaz) seeds extracted from their defatted press cakes. The press cakes were collected from the cold oil pressing of the seeds which were pre-treated by different techniques. Ultrasound-assisted alkaline (pH=9.0) extraction and isoelectric point (pH=4.0) precipitation procedure yielded around 34.47-80.95% proteins. The extracted seed proteins were in good quality for colour and antioxidant capacity. The extracted-lyophilised proteins were more soluble at pH 11-12 and below 2. The viscosity of the protein dispersions ranged from 3.67 to 25.70 cP. Sixteen amino acids were quantified in the samples, while asparagine, cysteine, hydroxyproline and tryptophan were absent. Thermal denaturation temperature and enthalpy change of denaturation were reported for the first time. The gel electrophoresis indicated four distinct protein bands at around 10, 15-20, 20, and 25-37 kD. It was shown that while seed pretreatment prior to cold oil extraction caused protein yields to enhance, protein degradation especially, in naringinase and hesperidinase enzyme treated samples enhanced, as well. This study provides the evaluation of citrus seed proteins for the literature for the first time, and points out the possibility of utilisation of these proteins in food enrichments, animal feeding and non-food applications. More studies about applications of these seed proteins are expected.

Keywords: citrus seeds, press cakes, protein, extraction, thermal property

1. Introduction

The term *Citrus* refers to a common name of flowering plants belonging to *Rutaceae* family, which includes around 140 genera and 1,300 varieties. It originates from Southeast Asia, and is cultivated since ancient times almost in most warm climate areas of the world (Cemeroğlu, 2013; Matheyambath *et al.*, 2016). It was indicated that worldly cultivation areas for citrus trees account for around 9.7 million ha, and around 135 million tons fruits are produced in 2015 season. Orange is the first one with 57% of total production, followed by mandarin (22%), lemon (11%), grapefruit (9%) and others (http://tinyurl.com/jh7psmp). Turkey is the 9th producer in the world with 3.7 million tons annual production (TÜİK, 2014). According to the fruit juice sector report of Turkey (Akdağ, 2011), around 57,000 tons oranges, 43,000 tons lemons and 400 tons grapefruits are processed for juice or concentrates. After fruit processing, around 50% of the original fruit weight is separated as wastes including seeds, peels and pulps. These wastes are rich sources of biologically active components such as oil, protein, dietary fiber, flavonoids and phenolics (Bocco *et al.*, 1998; El-Adawy *et al.*, 1999a; Malacrida *et al.*, 2012; Nayak *et al.*, 2015; Rezzadori *et al.*, 2012; Russo *et al.*, 2014, 2015). Waste valorisation and biorefinery are essential issues in growing food process industries for global sustainability, bioeconomy, environmental protection and recycling in food supply chain (Matharu *et al.*, 2016).

There are some studies reporting the compositions of various citrus seeds (Anwar *et al.*, 2008; El-Adawy *et al.*, 1999a; El-Safy *et al.*, 2012; Habib *et al.*, 1986; Malacrida *et al.*, 2012; Saïdani *et al.*, 2004; Saloua *et al.*, 2009). In an early study (Akpata and Akubor, 1999), the proximate

composition of orange seed flour was found to contain 54.2% fat, 28.5% carbohydrate, 5.5% crude fibre, 3.1% crude protein and 2.5% ash (on dry weight basis). In another study, the protein contents of lemon, grapefruit, sweet orange and mandarin were reported as 6.43, 3.90, 5.56, and 9.56% on dry weight base, respectively. Also, the seeds contained 27.0-36.5% oil, 5.0-8.5% fibre and 4.6-5.6% ash (Anwar *et al.*, 2008). The composition of orange seeds were given as 17.01% protein, 2.01% non-protein nitrogen, 42.59% oil, 3.17% ash, 22.53% fibre, 14.70% total carbohydrate and 8.70% moisture (El-Adawy *et al.*, 1999a). Saloua *et al.* (2009) reported that 32.75% oil, 33.89% protein, 6.72% ash, 20.76% carbohydrate and 5.88% moisture were present in orange seeds.

In the present study, the proteins from lemon (*Citrus limon* var. Kütdiken), orange (*Citrus sinensis* var. Dörtyol) and grapefruit (*Citrus paradisi*var. Beyaz) seeds were investigated. The starting material for protein recovery was the de-fatted press cakes. The seeds were pre-treated and cold pressed for oil recovery. The remaining press cakes were the source of protein extraction in this study. Since cold pressing is a very clean, mild and easy process, it yields high quality press cakes for possible applications (Aydeniz *et al.*, 2014; Yılmaz *et al.*, 2015). In literature, many studies exist for protein isolation from various seed and kernel press cakes or flours (Achouri *et al.*, 2012; Hojilla-Evangelista *et al.*, 2015; Moure *et al.*, 2006; Rodrigues *et al.*, 2012; Sharma *et al.*, 2010; Yılmaz and Dündar Emir, 2016; Yılmaz and Huriyet, 2017; Yin *et al.*, 2011), while studies with citrus seed proteins are fairly limited (El-Adawy *et al.*, 1999a,b; El-Safy *et al.*, 2012).

Citron, orange and mandarin flours were analysed for chemical composition including amino acids, minerals, antinutritional factors, and for *in vitro* protein digestibility (El-Adawy *et al.*, 1999b). The total protein content of the raw seeds and defatted flours of citron, orange and mandarin were found to be 19.93 and 36.20%, 17.01 and 33.14% and 15.87 and 28.56%, respectively. The flours shown to include 18 amino acids, with glutamic acid, arginine and aspartic acid as the major components, and tryptophan, methionine and isoleucine as the limiting amino acids. Although there was no haemagglutinin activity, some trypsin inhibitor activity was measured in the flours (El-Adawy *et al.*, 1999b). In the other study of the same group (El-Adawy *et al.*, 1999a), citrus seed proteins were partially characterised for protein solubility and classification, electrophoresis and absorption spectrum. It was shown that protein solubility was around 13-16% in distilled water, and 72-76% in 0.1 M sodium hydroxide solution. The minimum solubilities were measured at around pH=4.0-4.5 region. Electrophoresis of the seed proteins indicated 3 major bands at around 37, 23 and 17 kD. A more recent study (El-Safy *et al.*, 2012) investigated the nutritional properties of eight different seed flours, including orange seed. Orange seed flour was shown to contain 3.06% protein on dry basis, and protein solubility was 23.11% in distilled water and 76.81% in 1.0 M sodium chloride solution. Seventeen amino acids were quantified in orange seed flour with glutamic acid, arginine and aspartic acid at the highest concentration. Although literature lacks about the uses and applications of citrus seed proteins, the functionality and possible uses of many edible oilseed proteins are reviewed (Moure *et al.*, 2006). More recently (Foegeding, 2015), protein functionality was discussed from molecular basis perspective, and these studies indicated that in future, proteins extracted from various plant sources may have diverse food applications.

In the present study, the effects of seed pre-treatments (hexane oil extraction, seed microwave roasting, seed enzyme treatment) prior to cold oil pressing on the protein yield and properties from the defatted press cakes of lemon, orange and grapefruit seeds were investigated. Protein extraction yields, pH-solubility properties, protein physicochemical properties, amino acid compositions, thermal properties and electrophoresis patterns were determined. This study provides the first data for the lemon, orange and grapefruit seeds proteins for the literature. The aim of this study was to characterize the lemon, orange and grapefruit seed proteins extracted from press cakes for possible food and non-food (feed, pharmaceutical, medical, material science, etc.) applications.

2. Materials and methods

Materials

In this study, the Kütdiken variety lemon seeds from Limkon Food Industry and Trade Inc. (Adana, Turkey), Dörtyol variety orange seeds from Anadolu Etap Penkon Co. (Mersin, Turkey) and Beyaz variety grapefruit seeds from Frigo-Pak Food Co. (Bursa, Turkey) were gifted from 2013-2014 harvest and processing season. The seeds were washed and cold pressed with lab scale machine (Koçmaksan ESM 3710, İzmir, Turkey; 12 kg seed/h capacity, single head, 2 hp, 1.5 kW power) in two separate batches for the 2 replicates of the study. The operational conditions were 30 rpm screw rotation speed, 10 mm exit die and max 40 °C exit oil temperature. The press cakes were collected, ground and frozen until analysis. The enzymes hesperidinase (Rham 143, 12 U/mg activities) and naringinase (Rham 142, 5 U/mg activities) were purchased from Prokazyme Ltd. (Reykjavik, Iceland). The electrophoresis materials, mini-protean precast gels (4-15%), precision plus protein all blue standards (10-250 kD), and all other electrophoresis chemicals were from Bio-Rad (Hercules, CA, USA) and purchased from Serra Ltd. Co. (Bursa, Turkey). All other chemicals and standards used in the analyses were purchased either from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA).

Seed pre-treatments

The seeds were pre-treated with different techniques prior to cold oil pressing to enhance oil yield as well as press cake quality. Brief descriptions of the procedures are below. The control group lemon seeds were roasted at 150 °C for 30 min in an oven (Inoksan PFE, Bursa, Turkey), then conditioned to 10% moisture, and cold pressed. The solvent extraction of the lemon seeds were carried out by drying the seeds until 5% moisture, finely grinding (Retsch Grindomix, Germany) and hexane extraction of oil (1:2.5 = seed:hexane, w/v) at 45 °C in water bath by mixing 12 h at 140 rpm, and repeating the process for 3 times to collect the micella. The wet press cake was then dried. The control group of the orange seeds was treated in the same way as the lemon seeds. The microwave treatment of the orange seeds were done by applying 360 Watt energy for 30 min total time in 3 min apply-3 min cease operational mode in a microwave oven (Beko MD 1505, Turkey). Finally, the moisture content of the seeds was arranged to 10% by water conditioning, and cold pressed. The control group of the grapefruit seeds were processed the same as lemon and orange seeds. The enzyme treatment to grapefruit seeds was completed by first grinding the seeds and then incubating them with 0.06 U/g seed naringinase (Rham 142) and 0.033 U/g seed hesperidinase (Rham 143) enzymes in 100 mM KH_2PO_4/K_2HPO_4 buffer solution (pH 7.5) at 65 °C for 4 h. After the incubation, the slurry was heated to 150 °C to inactivate the enzymes and to adjust the moisture level before cold oil pressing.

Defatting of the press cakes

Defatting of all citrus seed press cakes was accomplished according to the modified method of Manamperi *et al.* (2007). The ground press cakes were mixed with hexane (1:4, w/v) and stirred at room temperature for 2 h at 190 rpm speed. The same procedure was repeated 3 times, and the remaining hexane in the wet meal was removed in a forced-air oven at 60 °C for 1 h, and then under air hood overnight. The defatted press cakes were used immediately for protein extraction.

Protein extraction

The extraction procedure was modified from Wang *et al.* (2013). First, defatted press cakes and deionised distilled (DI) water were mixed at 1:20 (w/v) ratio, and mixed at 13,500 rpm for total 5 min with 1 min apply-1 min pause mode for heat control with an ultratorax (Yellow line D125 basic). Then, the pH was adjusted to 8.8 by 1 N NaOH. Another mixing operation at 13,500 rpm was applied under the same conditions and the pH was adjusted to 9.0. After applying the same final mixing operation, the slurry was sonicated with an ultrasound apparatus (Sonics VCX750, Newtown, CT, USA) at max 70% amplitude until max 40 °C heat reached by 5 sec apply-5 sec pause mode. The slurry was finally filtered through 0.053 mm mesh sieve to remove the insoluble particles, and centrifuged at 3,000×*g* for 20 min (Sigma 2-16K, Osterode, Germany) to collect the extracted proteins in the supernatant. The protein content of the supernatants was measured with the Bradford technique (Bradford, 1976) using bovine serum albumin as standard with a UV-visible spectrophotometer (Agilent 8453, Ratingen, Germany). To determine the best pH for protein precipitation, the isoelectric points (pI) of the protein extracts were determined according to Manamperi *et al.* (2007). First, the supernatant was aliquoted into tubes (10 ml) and their pH was adjusted by 1 N HCI in the range of 2.0 to 6.0 by 0.5 unit increments. After vortexing for 1 min, the solutions were centrifuged at 3,000×*g* for 20 min. Finally, the protein content of the supernatants were measured (Bradford, 1976), and the supernatants with the lowest protein content were selected as the pI value for each sample.

After selection of the optimum pH (9.0) and ultrasound application (70% amplitude until 40 °C temperature) for protein extraction, and determination of the pI (4.0) for protein precipitation, the protein extraction procedure was applied to all samples. Finally, the protein extracts were freeze-dried in a lyophiliser (Labfreeze FD-10 MR Bench-Top Freeze Dryer, Xiangtan city-Hunan, China), and placed into amber-coloured capped glass bottles. During the analyses, the protein extracts were kept at -20 °C. The extracted citrus seed protein lyophilisates could also be observed.

The protein yield values of the extraction process for the different seed press cakes were calculated by determining the total protein contents of the defatted press cakes and the extracted-lyophilised proteins by the Bradford (1976) method.

Protein solubility

The protein solubility as a function of pH was determined for each of the extracted proteins between pH = 2 and 12 by the modified method of Yin *et al.* (2011). The lyophilised protein extracts were first dissolved in DI water at 1% (w/v) ratio, and then 10 ml of the solutions were aliquoted into series of tubes to adjust their pH values by 1 unit increments using 1 N HCI or NaOH solutions. The tubes were vortexed for 2 min, and then the pH was measured and corrected again. Finally, the tubes were centrifuged at 2,291×*g* for 5 min, and the protein contents of supernatants were determined by the Bradford (1976) method. The protein solubility data were expressed as mg soluble protein/ml sample versus the pH values.

Protein physicochemical properties

The colour values of the extracted-lyophilised citrus seed proteins were measured with Minolta CR-400 Reflectance colorimeter (Osaka, Japan). The measurements were accomplished at least at five different points of the dry samples placed in a petri plate, and the parameters of L, a* and b* values were recorded with the previously calibrated (with white reference tile) instrument.

The antioxidant capacity values of the protein extracts was measured by the methods of Re *et al.* (1999), and Aydeniz and Yılmaz (2012). Each protein extracts (0.5 g) and 6 ml methanol: water (60:40) was mixed, vortexed and centrifuged (1,615×*g*, 15 min) to collect the phenolic extracts two times. The antioxidant capacity of the phenolic extracts were measured by the ABTS (the 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) radical cation) decolourisation assay. The results were expressed as TEAC (μmol Trolox equivalence/mg protein) value.

The apparent viscosity values of the protein extracts were determined according to Khalid *et al.* (2003) and Kanu *et al.* (2007). Dispersion of the proteins (5%, w/v) in DI water was prepared and the pH was adjusted to 7.0 using 1 N HCI or NaOH solutions. The viscosities of the dispersions were measured at 40 and 60 °C set by water bath circulating on Brookfield model DV II. Pro viscosimeter with Rheocalc software (Brookfield Eng. Lab., Inc., Middleborough, MA, USA) equipped with no: 18 spindle, and the values were recorded as cP values.

Protein amino acid composition

The methods of Dimova (2003), and Gheshlaghi *et al.* (2008) were modified for this analysis. The protein extracts were first hydrolysed for amino acid composition analysis. Around 0.1-1.0 g protein extracts were weighed into a Schoots glass and 20 ml of 6 N HCI was added. The glass was incubated in a vacuum incubator for 24 h at 110 °C for full hydrolysis. After cooling to room temperature, it was filtrated, and then 0.2 ml of the filtrate was dried under nitrogen gas at 50 °C. Then 0.5 ml of acetonitrile:methanol :triethylamine (100:50:20 ml) mixture and 0.1 ml of Edman reagent solution (1.2 ml phenylisothyocyanate in 100 ml acetonitrile) were added and derivatised at 40 °C in an incubator. Finally, it was dried under nitrogen gas at 40 °C, and 5 ml of 0.02 M ammonium acetate was added before filtration through 0.45 μm membrane filter. The amino acid composition was then determined through injection into an ultra-fast liquid chromatograph (Shimadzu, Tokyo, Japan) equipped with reverse phase Eclipse XDB-C18 column (5 μm, 4×6×150 mm). The mobile phase A was prepared by dissolving 0.78 g sodium dihydrogen phosphate and 0.88 g disodium hydrogen phosphate dihydrate in 1 L (pH=6.9). The mobile phase B was acetonitrile (HPLC grade). The injection volume was 10 μl, and flow rate was 1 ml/min. The column was held at 40 °C, and UV peak detection was read at 254 nm. The gradient programme was as follows: 100%A/0%B 0-0.01 min, 85%A/15%B 0.01-13 min, 75%A/25%B 13-22 min, 70%A/30% B 22-26 min, 40%A/60%B 26-28 min, 100%A/0%B 28-38 min. The amino acids were identified according to their retention times against those of commercially available standards.

Protein thermal properties

The method of Yin *et al.* (2011) was adapted for this analysis. A Perkin Elmer differential scanning calorimetry (DSC 400 Series, Groningen, the Netherlands) was used. Dispersions of protein extracts (10%, w/v) were prepared in 10 mM phosphate buffer (pH=7.0). Aliquots of the dispersions (10 μl) were hermetically sealed in aluminium sample pans, and analysed against an empty pan as the reference. Prior to analysis, calibration of DSC was done with indium and zinc standards. The temperature programme was heating samples from 30 °C to 120 °C by 10 °C/min heating rate. The thermograms were used by the Pyris 1 Manager software of the instrument to calculate the denaturation on-set temperature (T_o), peak of denaturation temperature (T_d) and enthalpy change of denaturation (ΔH).

Protein gel electrophoresis

Protein electrophoresis on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) was completed by the Laemmli method modified from Achouri *et al.* (2012). 10 mg protein extract, 0.950 μl Laemmli buffer (65.8 mM Tris-HCl, pH=6.8, 26.3% w/v glycerol, 2.1% sodium dodesyl sulfate-SDS, 0.01% bromophenol blue; Bio-Rad), and 0.50 μl 2-mercaptoethanol (Bio-Rad) were put into a test tube, and vortexed for 5 min. The proteins were denatured in a water bath at 100 °C for 5 min before immediate cooling on ice. Finally, the tubes were centrifuged at 1000×*g* at 10 °C in a refrigerated centrifuge (Sigma 2-16K, Osterode, Germany). 10 μl of the protein standard (precision plus protein all blue standards, 10-250 kD; Bio-Rad) and each of the protein samples were loaded on the mini-protean precast gels (4-15%; Bio-Rad). The gels were run at constant voltage (200 V) in buffer milieu (10xTris/glycine/SDS buffer; Bio-Rad). Finally, the gels were stained in 0.1% Coomassie brilliant blue R-250 solution for 4 h, before destaining and washing in the fixing solution. The gels were casted into cellophane membranes and dried at 45 °C for 2 h in a gel drying oven.

Statistical analysis

Protein extraction from the defatted seed press cakes was replicated twice, and the analyses in each replicate protein samples were done at least two times. The data were reported as mean ± standard deviation. Comparison of the

samples was with one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests with Minitab v. 16.1 (Minitab Inc., State College, PA, USA) and SPSS software v. 10.1 (SPSS Inc., Chicago, IL, USA) programmes. The level of confidence was at least 95% in all statistical analyses. Comparison of all data shown in the tables were done by seed pairs, namely the proteins extracted from lemon seeds compared with each other (its control and treatment group), and not with the proteins extracted from the orange seed or grapefruit seed, since the applied treatment groups prior to cold oil pressing of the three seeds were different.

3. Results and discussion

Extraction yield

In this study, the ultrasound assisted alkaline extraction and isoelectric point precipitation technique was used for protein extraction from the defatted citrus seeds press cakes. And the extracted-lyophilised proteins could be observed. Generally, colour and appearance of the protein extracts were alike. There was no significant darkening after extraction, since alkalinity was not above pH=9.0, as intentionally selected for better quality protein extracts. The isoelectric points (pI) of the seed proteins were determined (Figure 1), and shown to be very close to each other. Hence, the pI for all samples was accepted as 4.0 pH, and this value was used in all further protein extraction and precipitation procedures. Under the defined conditions of extraction and precipitation processes, the protein yield values (%) were calculated and given in Table 1 together with the determined protein contents of the defatted press cakes.

The yield values were based on the total protein contents measured in the defatted press cakes and protein contents of the extracted-lyophilised protein extracts. The whole seeds contained 19.41, 19.22, and 16.25% (by dry weight) proteins for the lemon, orange and grapefruit, respectively. Comparison of the protein yield values was done for each pair of the seeds separately. For example, cold pressed lemon seed is compared with solvent extracted lemon seed,

Table 1. The extraction yield values of the citrus seed proteins.[1]

Defatted presscake[2]	Protein content of presscake (% dw)	Protein yield (%)
CP-L	27.27±1.06^{A}	34.47±0.51^{B}
SE-L	20.98±0.93^{B}	53.28±1.04^{A}
CP-O	23.21±1.66^{B}	65.16±3.47^{A}
MR-O	29.41±0.61^{A}	74.05±4.00^{A}
CP-G	26.07±0.76^{B}	78.27±0.34^{B}
ET-G	28.49±0.28^{A}	80.95±0.35^{A}

[1] Capital letters within each column compare the statistical differences between each pairs of the seed samples by Tukey's test (P<0.05).
[2] CP-G = cold pressed grapefruit seed; CP-L = cold pressed lemon seed; CP-O = cold pressed orange seed; ET-G = enzyme treated grapefruit seed; MR-O = microwave roasted orange seed; SE-L = solvent extracted lemon seed.

and not with the rest of the samples, since each seed was treated with different techniques against its control prior to cold oil pressing. Hence, attention must be given during the examination of the data presented in the tables. The protein yield of cold pressed lemon seed protein (34.47%) was compared to its pair (solvent extracted lemon seed protein), and was found significantly lower (53.28%). Similar comparison for orange seed and grapefruit seed proteins could be observed from Table 1. Generally, enzyme treated grapefruit seeds yielded the highest protein (around 80.95%) among all samples. For all seeds, it can be perceived that treatments (solvent extraction, microwave roasting and enzyme treatment) resulted higher yields than that of their control samples. In literature (Achouri *et al.*, 2012; Hojilla-Evangelista *et al.*, 2015; Rodrigues *et al.*, 2012; Sharma *et al.*, 2010; Yılmaz and Dündar Emir, 2016; Yin *et al.*, 2011), diverse range of protein extraction yields or recovery rates were reported. Yield values between 10.6 and 27.4% were reported for proteins extracted from ten different edible oilseeds (Sharma *et al.*, 2010). Sesame proteins showed

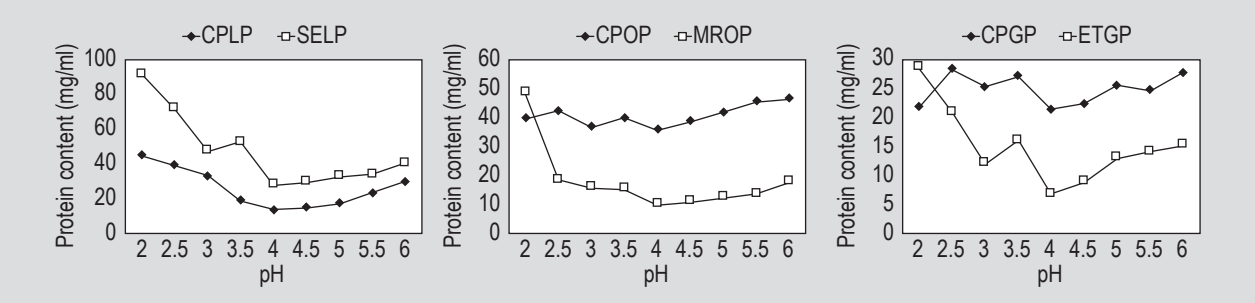

Figure 1. The isoelectric points (pI) of the citrus seed proteins. (CPLP = cold pressed lemon seed protein; SELP = solvent extracted lemon seed protein; CPOP = cold pressed orange seed protein; MROP = microwave roasted orange seed protein; CPGP = cold pressed grapefruit seed protein; ETGP = enzyme treated grapefruit seed protein).

different extraction yields depending on the extraction medium. The yield increased from 12.5 to 54.6% by addition of 1 M NaCI into the buffer (Achouri *et al.*, 2012). Similarly, the protein recovery rate (%) of saline extraction was 45.4%, and of acid precipitation was 23.0% for the pennycress press cake proteins (Hojilla-Evangelista *et al.*, 2015). Protein extraction yield values ranging from 10.72 to 61.34% were reported for poppy seed proteins from defatted press cakes, and indicated that preroasting and enzyme treatments have reduced the yields (Yılmaz and Dündar Emir, 2016).

Similarly, protein extraction yield values of 40, 33 and 15% for the control, roasted and enzyme treated capia pepper seed samples were reported (Yılmaz and Huriyet, 2017). Overall, protein extraction yield values found in this study are in good agreement with those reported in the literature. Since seed or kernel materials and applied extraction procedures varied, the protein yields varied, expectedly.

Protein solubility

The pH-solubility graphics of the extracted-lyophilised seed proteins are shown in Figure 2. In all samples, the protein solubilities were the lowest at around pH=4.0-5.0 range. Solubility gradually increased towards the basic end of pH scale, and reached the maximum at pH=11 or 12 for most of the samples. This solubility behaviour is similar to most seed and kernel proteins reported in the literature (Achouri *et al.*, 2012; Hojilla-Evangelista *et al.*, 2015; Sharma *et al.*, 2010; Yılmaz and Dündar Emir, 2016; Yin *et al.*, 2011).

In general, at higher pH values, the increased net negative charge or at lower pH values, the increased net positive charges on the protein surface contribute to the protein solubility, as could be observed from Figure 2. Likewise, at pI or near pI values, the protein solubilities are lowest due to unavailable net charges on protein surface (Achouri *et al.*, 2012; Yin *et al.*, 2011). There are very limited numbers of studies in literature for citrus seed proteins. In one study (El-Adawy *et al.*, 1999a), protein solubility index of citron, orange and mandarin flours were determined in distilled water, 0.1 M sodium chloride, potassium chloride and sodium hydroxide solutions. For all samples, solubilities were highest in the sodium hydroxide solution (around 72-75%). Also, they indicated that protein solubilities were lowest at around pH=4.5-5.5 range, and maximum at around pH=10-12 range. In another study (El-Safy *et al.*, 2012), protein solubilities of orange seed flours were determined to be 23.11% in distilled water, and 76.81% in 1.0 M sodium chloride solution. Very similar pH-protein solubility gradients were reported for poppy seed proteins extracted from defatted press cakes (Yılmaz and Dündar Emir, 2016). These findings concur with our results. Generally, the citrus seed proteins investigated in this study showed higher solubility values at acidic and basic pH ranges.

Physicochemical properties

The instrumental colour, viscosity and antioxidant capacity values of the extracted-lyophilised seed protein samples were determined as the main physicochemical properties, and summarised in Table 2. Comparisons of samples were done pair wise; cold pressed lemon seed protein (CPLP) is compared only with solvent extracted lemon seed protein, cold pressed orange seed protein compared only with microwave roasted orange seed protein, and cold pressed grapefruit seed protein (CPGP)compared only with enzyme treated grapefruit seed protein (ETGP).

The luminosity value ranged from 69.08 to 76.62, and the most luminous sample was CPGP. Similarly, the ranges of a* values (2.11-3.50) and b* values (16.65-20.42) were not large, but some statistical differences existed between each pair of seed samples. The protein samples could be observed visually as well. These colour values could be important in determining the food application areas of the protein extracts. While darker colours may create some problems for food formulations, lighter colours could be easily administrated in various food formulations like bakery and meat products, dry mixtures, soups, etc. It was indicated that the colour of the extracted nut proteins might

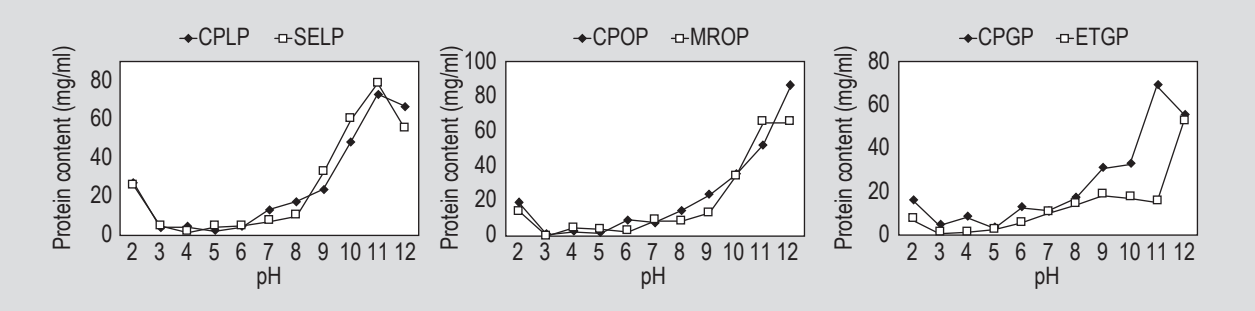

Figure 2. The pH-solubility properties of the citrus seed proteins. (CPLP = cold pressed lemon seed protein; SELP = solvent extracted lemon seed protein; CPOP = cold pressed orange seed protein; MROP = microwave roasted orange seed protein; CPGP = cold pressed grapefruit seed protein; ETGP = enzyme treated grapefruit seed protein).

Table 2. The physico-chemical properties of the citrus seed proteins.[1]

Sample[2]	Colour			Viscosity (cP)		TEAC (µmol Trolox/mg protein)
	L	a*	b*	40 °C	60 °C	
CPLP	70.80±0.68^{A}	3.18±0.23^{A}	16.65±0.12^{A}	5.18±0.99^{A}	4.78±0.77^{A}	2.33±0.17^{B}
SELP	71.59±1.17^{A}	2.29±0.06^{B}	16.74±0.12^{A}	3.82±0.31^{A}	3.67±0.11^{A}	2.81±0.07^{A}
CPOP	72.44±1.62^{A}	2.11±0.24^{B}	17.35±0.73^{A}	17.70±1.23^{A}	18.35±1.61^{A}	3.66±0.59^{A}
MROP	69.08±1.52^{A}	3.41±0.15^{A}	18.43±0.43^{A}	19.98±0.99^{A}	15.97±1.87^{A}	3.00±0.45^{A}
CPGP	76.62±0.45^{A}	3.50±0.10^{B}	19.35±0.26^{B}	22.38±0.59^{B}	22.18±0.23^{A}	3.48±0.16^{A}
ETGP	72.63±0.08^{B}	4.40±0.05^{A}	20.42±0.13^{A}	25.70±0.51^{A}	23.38±0.88^{A}	3.76±0.13^{A}

[1] Capital letters within each column compare the statistical differences between each pair of seed protein samples by Tukey's test (P<0.05).
[2] CPGP = cold pressed grapefruit seed protein; CPLP = cold pressed lemon seed protein; CPOP = cold pressed orange seed protein; ETGP = enzyme treated grapefruit seed protein; MROP = microwave roasted orange seed protein; SELP = solvent extracted lemon seed protein; TEAC = Trolox equivalance antioxidant capacity.

be due to the phenolic compounds, pigments, minerals present, and their reactions or interactions with other components (Sharma *et al.*, 2010). It was also indicated that highly basic extraction conditions (pH above 9.0) may yield more protein, but colour darkening due to some pigment reactions, and amino acid losses may occur; hence, extremes of pH values are not recommended for protein extraction (Foegeding and Davis, 2011; Moure *et al.*, 2006; Rodrigues *et al.*, 2012).

The viscosities of the protein dispersions (5%) at 40 and 60 °C were determined (Table 2). Generally, there was no difference between the treatments for each seed type, except for grapefruit seed proteins. The viscosity of ETGP was significantly higher than that of the control sample (CPGP) at 40 °C but not at 60 °C. Hence, it might be argued that preliminary enzyme (naringinase and hesperinidase) treatments of the grapefruit seeds may affect proteins to result in viscosity decreases. There was no significant difference for the viscosity values between the two measurement temperatures. On the other hand, Kanu *et al.* (2007) showed that the viscosity of sesame proteins enhanced significantly when measurement temperature increased from 40 to 70 °C. Unfortunately there is no data in literature to compare our findings with other citrus seed proteins. In different poppy seed protein extracts (Yılmaz and Dündar Emir, 2016), the viscosities measured at the same temperatures ranged between around 24 to 60 cP, which are much higher than the values measured for the citrus seed proteins in this study (Table 2). Hence, protein source can be a major determining factor for the viscosity values of the protein dispersions.

The Trolox equivalence antioxidant capacities of the protein extracts were also measured (Table 2). There was no significant difference between the sample pairs. The highest antioxidant capacity was measured in the ETGP extract (3.76 µmole Trolox/mg), while the lowest one was in the CPLP sample (2.33 µmole Trolox/mg). Unfortunately, there is no study in the literature for antioxidant capacity of citrus seed proteins to compare with our results; hence, this data is a valuable input. González-García *et al.* (2014) determined the antioxidant capacity of alcalase and thermolysin hydrolysed plum seed proteins to be around 0.460 and 0.772 µmole Trolox/mg sample, respectively. These values are significantly lower than those found in this study. But it must be kept in mind that both protein source and level of hydrolysation are different. In another study (Vaštag *et al.*, 2011), pumpkin oil cake protein isolate was hydrolysed with alcalase and alcalase + flovourzyme enzymes, and the antioxidant capacities were measured as 6.47 and 4.71-4.75 mM Trolox/mg sample, respectively. These studies indicate that antioxidant capacity could be an important factor in utilising extracted proteins from oilseed or kernels in various food applications. The presence of moderate level of antioxidant capacity compared to other sources in the citrus seed protein extracts may be important for their various applications.

Amino acid composition

The results of amino acid analysis are given in Table 3. Sixteen amino acids were quantified, and four amino acids (asparagine, cysteine, hydroxyproline, tryptophan) were absent in the protein samples. Aspartic acid and its amidated form asparagine, and glutamic acid and its amidated form glutamine were given as the sum of the respective forms. Since a prior disulphide bond reduction procedure was not done in this study, the fate of cysteine is not exact. Clearly, the standard deviation of amino acid results are high and this is due to the difficulty of the determination from such complex protein sources. Except a few amino acids, the

Table 3. The amino acid composition of the citrus seed proteins.[1]

Amino acid (mg/100 g)[2]	CPLP[3]	SELP[3]	CPOP[3]	MROP[3]	CPGP[3]	ETGP[3]
Ala	2,294.00±8.00^{A}	1,449.00±8.00^{B}	2,079.00±178.00^{A}	1,988.00±101.00^{A}	2,016.50±205.50^{A}	1,816.50±119.50^{B}
Arg	2,396.50±183.50^{A}	1,650.50±120.50^{B}	1,353.50±549.50^{A}	1,652.50±266.50^{A}	1,638.00±601.00^{A}	1,400.50±98.50^{B}
Asp+Asn	5,860.00±294.00^{A}	3,695.00±85.00^{B}	3,728.00±628.00^{A}	4,073.00±101.00^{A}	4,093.50±769.50^{A}	3,610.00±8.00^{B}
Glu+Gln	13,040.00±264.00^{A}	8,513.50±215.50^{B}	9,379.00±972.00^{A}	9,902.00±376.00^{A}	9,462.00±1,139.00^{A}	8,993.00±143.00^{B}
Gly	3,251.50±24.50^{A}	2,162.00±28.00^{B}	3,301.00±370.00^{A}	3,038.50±52.50^{A}	2,972.00±262.00^{A}	2,665.50±121.50^{B}
His	1,801.50±92.50^{A}	1,214.50±63.50^{B}	1,745.00±161.00^{A}	1,729.00±93.00^{A}	1,716.50±77.50^{A}	1,298.50±68.50^{B}
Ile	2,366.00±30.00^{A}	1,542.00±29.00^{B}	2,148.00±257.00^{A}	1,999.50±85.50^{B}	2,089.50±193.50^{A}	2,012.00±135.00^{A}
Leu	5,099.50±235.50^{A}	3,421.50±25.50^{B}	5,029.00±793.00^{A}	4,525.00±303.00^{B}	1,510.50±541.00^{B}	4,324.50±364.50^{A}
Lys	2,468.00±7.00^{A}	1,723.50±57.50^{B}	1,695.00±638.00^{A}	1,962.00±124.00^{A}	1,949.50±481.50^{A}	1,407.50±2.50^{B}
Met	434.00±3.00^{A}	329.00±29.00^{B}	909.50±358.50^{A}	525.00±133.00^{B}	461.50±185.50^{A}	267.00±21.00^{B}
Phe	3,129.50±165.50^{A}	2,092.50±1.50^{B}	3,067.00±483.00^{A}	2,672.00±230.00^{B}	2,775.00±352.00^{A}	2,465.00±238.00^{A}
Pro	2,582.50±146.50^{A}	1,770.50±15.50^{B}	2,765.00±592.00^{A}	2,466.00±67.00^{B}	2,500.00±398.00^{A}	2,371.50±180.50^{A}
Ser	2,960.50±131.50^{A}	1,927.00±49.00^{B}	2,722.00±128.00^{A}	2,636.50±106.50^{A}	2,653.00±227.00^{A}	2,004.00±106.00^{B}
Thr	2,143.50±200.50^{A}	1,402.00±95.00^{B}	2,120.50±200.50^{A}	2,054.50±224.50^{B}	2,164.50±444.50^{A}	1,442.00±115.00^{B}
Tyr	1,400.00±46.00^{A}	941.50±30.50^{B}	1,246.50±126.50^{A}	1,193.50±19.50^{A}	1,211.00±66.00^{A}	1,289.00±34.00^{A}
Val	2,964.00±65.00^{A}	1,814.00±20.00^{B}	2,615.00±234.00^{A}	2,315.00±236.00^{B}	2,549.50±266.50^{A}	2,150.50±210.50^{B}

[1] Capital letters within each row compare the statistical differences between each pairs of the seed samples (CPLP vs SELP, CPOP vs MROP, CPGP vs ETGP) by Tukey's test (P<0.05).
[2] Ala = alanine; Arg = arginine; Asp = aspartic acid; Asn = asparagine; Glu = glutamic acid; Gln = glutamine; Gly = glycine; His = histidin; Ile = isoleucine; Leu = leucine; Lys = lysine; Met = methionine; Phe = phenylalanine; Pro = proline; Ser = serine; Thr = threonine; Tyr = tyrosine; Val = valine.
[3] CPGP = cold pressed grapefruit seed protein; CPLP = cold pressed lemon seed protein; CPOP = cold pressed orange seed protein; ETGP = enzyme treated grapefruit seed protein; MROP = microwave roasted orange seed protein; SELP = solvent extracted lemon seed protein.

concentrations of all amino acids were decreased by the treatments applied to the seeds prior to oil extraction. Only in the grapefruit seeds, the microwave treatment enhanced the extractability of arginine, aspartic acid, glutamic acid and lysine. In all samples, the most abundant amino acids were glutamic acid + glutamine, aspartic acid + asparagine, leucine and glycine, while the lowest amounts were measured for methionine, tyrosine and histidin amino acids. The essential amino acids cysteine and tryptophan were not quantified in the samples, while other essential amino acids phenylalanine, isoleucine, leucine, lysine, treonine, and valine were present in all protein samples. El-Adawy *et al.* (1999b) determined the amino acid compositions of citron, orange and mandarin seed flours. They identified arginine (14.1-16.3%), glutamic acid (17.1-19.3%), and aspartic acid (8.2-11.0%) as the major amino acids, and threonine, isoleucine, sulphur amino acids, and lysine as the lower amino acids, respectively. Clearly our results generally concur with their findings, although methods and units of determination are different. More recently El-Safy *et al.* (2012) reported the amino acid composition of orange seed flour among seven other fruit seed flours. Glutamic acid, arginine, aspartic acid and leucine were the most abundant ones, while cystine, methionine, and histidine were the lowest available amino acids. Our results and the reports of El-Safy *et al.* (2012) mostly concur both for quantity and abundance data of the amino acids. Unfortunately, there is no other available data in literature for the lemon and grapefruit seed amino acids to compare; hence, this study provides this new information for the literature. Generally, citrus seed proteins are good in terms of essential amino acids availability and the amounts of other amino acids. Hence, these proteins could have potential to improve amino acid profiles of other plant protein sources such as oilseed flours, legumes, etc. Moreover, these proteins could be used in processed foods, non-food areas and as animal feed. Since this study provides some of the preliminary data about the amino acid composition and properties of the citrus seed proteins, more research needs for bioavailability, peptide allergenicity and *in vivo* studies are envisioned.

Thermal properties

Denaturation on-set temperature (T_o), denaturation temperature (T_d) and enthalpy change of denaturation (ΔH) were determined by DSC and the results are presented in Table 4. Usually, the values were close to each other for most samples, and only between cold pressed and solvent extracted lemon seed proteins, differences existed for the T_o and T_d values. Hence, it would be claimed out that oil

Table 4. The thermal properties of the citrus seed proteins.[1,2]

Sample[3]	T_0 (°C)	T_d (°C)	ΔH (J/g)
CPLP	49.45±0.08^A	72.24±0,00^A	1,104.70±10.70^A
SELP	37.85±0.06^B	62.80±0.04^B	1,064.90±04.90^A
CPOP	45.94±1.55^A	79.19±1,31^A	1,096.80±82.50^A
MROP	48.36±0.93^A	82.61±0.05^A	1,392.80±50.70^A
CPGP	45.56±3.39^A	75.28±4.77^A	1,332.00±61.00^A
ETGP	43.95±1.63^A	79.38±3.25^A	1,325.00±25.50^A

[1] Capital letters within each column compares the statistical differences between each pair of seed protein samples by Tukey's test (P<0.05).
[2] T_0 = denaturation on-set temperature; T_d = denaturation temperature; ΔH = denaturation enthalpy change.
[3] CPGP = cold pressed grapefruit seed protein; CPLP = cold pressed lemon seed protein; CPOP = cold pressed orange seed protein; ETGP = enzyme treated grapefruit seed protein; MROP = microwave roasted orange seed protein; SELP = solvent extracted lemon seed protein.

solvent extraction process may cause some changes in lemon seed proteins to denature at lower temperatures, while microwave treatment of the orange seeds and enzyme (naringinase and hesperinidase) treatment of grapefruit seeds prior to oil extraction had no effect on the thermal behaviour of the seed proteins compared with their control samples. It was stated that the T_d value shows the level of protein structural change based on hydrogen bonds breakage, and ΔH indicated that the protein portion remained unchanged or undenatured after the process. Higher T_o and T_d values indicate the presence of heat sensitive proteins in a sample denatured by the heat applied (Bukya and Vijayakumar, 2013; Yin *et al.*, 2011). There was no data reached in the literature for the thermal properties of citrus seed proteins. Hence, this study provides the first available data. In a previous study (Horax *et al.*, 2011), proteins isolated from bitter melon seeds, and the values of 106.2 °C for T_o, 113.10 °C for T_d and 8.10 J/g for ΔH were reported. Likewise, in the study of Yılmaz and Dündar Emir (2016), the seed proteins extracted from cold pressed poppy seed press cakes were evaluated, and ranges of 38.28-98.83 °C for T_o, 44.82-99.79 °C for T_d and 12.96-36.86 J/g for ΔH values were reported.

Overall, it can be concluded that thermal parameters mainly depend on the kind of protein, as well as the pretreatments applied to the materials before protein extraction. Compared to poppy seed and bitter melon seed proteins, citrus seed proteins seem more heat resistant due to their higher ΔH values.

Protein electrophoresis

The SDS-PAGE bands of citrus seed protein samples are presented in Figure 3. For lemon, orange and grapefruit seed proteins, four distinct bands were identified. Compared with the protein standard, the molecular weight of the identified protein bands were around 10, 15-20, 20 and 25-37 kD. There was no protein on the gel located above 50 to 250 kD range in comparison with standard. Generally, all treatments caused some decreases in band darkness and width, indicating some protein loss. While treatment effect was not so dominant for lemon seeds (solvent extraction of the oil), microwave treatment of orange seed prior to oil extraction decreased band darkness and density considerably. In the enzyme (naringinase and hesperinidase) treated grapefruit seeds, the proteins are almost totally absent on the gel band. These findings indicate that microwave treatment might have denatured the seed proteins to some extent, but most proteins still remained. Although the enzymes used were not protease type, the bands indicate heavy loss of proteins. Since no data was available about the presence of protease activity in the Rham 142 and 143 commercial enzymes used, it could be possible that the preparates could have some protease activities. Furthermore, during the enzyme slurry incubation of the crushed seeds, some proteases naturally present in the seeds might have been activated and they degraded the seed proteins. Citron, orange and mandarin seed proteins were determined by SDS-PAGE by El-Adawy *et al.* (1999a), and proteins with molecular weight at around 37.304, 23.516, and 16.740 daltons were identified. We identified 4 bands, but the molecular weight ranges are totally concurring in both studies. Since no data was available in the literature for lemon and grapefruit seed proteins, our study may have an important contribution to the literature.

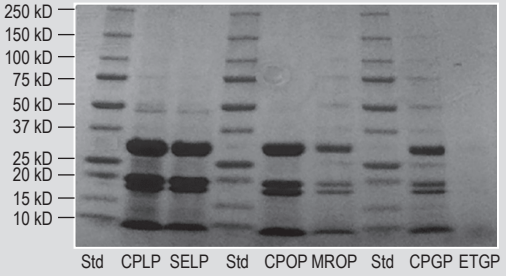

Figure 3. The electrophoresis bands of the citrus seed proteins. (Std = standard; CPLP = cold pressed lemon seed protein; SELP = solvent extracted lemon seed protein; CPOP = cold pressed orange seed protein; MROP = microwave roasted orange seed protein; CPGP = cold pressed grapefruit seed protein; ETGP = enzyme treated grapefruit seed protein).

4. Conclusions

This study reports some first data about lemon, orange and grapefruit seed proteins extracted from defatted press cakes. Ultrasound assisted alkaline (pH=9.0) extraction, and isoelectric point (pH=4.0) precipitation technique yielded around 34.47-80.95% protein. Solubility studies indicated that citrus seed proteins are most soluble at pH=11-12, and pH below 2. Under the defined extraction conditions, protein extracts with acceptable colour and some antioxidant activity were obtained. While sixteen amino acids were quantified, the most abundant amino acids were arginine, aspartic and glutamic acids with their amidate forms, leucine, and lysine in all samples. The thermal denaturation on-set temperature, denaturation temperature and enthalpy of denaturation were reported for the first time. The SDS-PAGE indicated four distinct bands at around 10, between 15 and 20, 20, and between 25 and 37 kD for all samples. Also, naringinase and hesperinidase enzymes incubation of crushed seeds prior to oil cold pressing were shown to reduce protein band intensity considerably. The findings of this study indicate that citrus seed proteins could be extracted by basic alkaline extraction-pI precipitation procedure with acceptable yields and physical quality parameters. The proteins could be credited nutritionally well due to available essential amino acids like lysine, leucine, isoleucine, methionine, phenylalanine, threonine, and valine at moderate quantities. This study may aid in utilisation of citrus seed proteins in food enrichment (functional foods, sports nutrition, special diets, etc.) area, as well as non-food applications (pharmaceutical, chemical synthesis, etc.) and animal feeding. Since citrus seeds are generally discarded as waste, this study may invoke valorisation of this mass bioresource. More studies about the functional properties and applications of the extracted proteins are underway.

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