GRASAS Y ACEITES 73 (2)
April-June 2022, e462
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0324211

Acidic and enzymatic pre-treatment effects on cold-pressed pumpkin, terebinth and flaxseed oils

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Submitted: 09 March 2021; Accepted: 07 June 2021; Published online: 13 June 2022

SUMMARY: Oil yield and the properties of oil can be improved with various enzymatic pre-treatments before obtaining oil from oilseeds by cold-press extraction. A commercial mixture of pectolytic enzymes was used in this study. In addition, apple seed meal as a source of β-glucosidase enzyme and citric acid were applied to oilseeds (pumpkin, terebinth and flaxseed) as pre-treatments. The results were evaluated by comparing the effects of the pre-treatments on oil yield and properties. Enzyme preparate could increase the oil yield of pumpkin seeds (~300%) and flaxseed (151%). Significant increases in the phenolic contents of terebinth (from 91.67 to 319.33 mg GAE/kg) and flaxseed oils (from 12.03 to 40.47 mg GAE/kg) were achieved by citric acid and enzymatic pre-treatments. These two pre-treatments were also effective in terms of peroxide formation and oxidative stability in terebinth oil. With the help of the pre-treatments applied to oilseeds it was possible to increase the transition of phenolics from seeds to oil for terebinth oil with increase ratios of 245% for citric acid, 248% for the enzymatic process compared to the control.

KEYWORDS: *β-glucosidase; Cold-pressed oil; Enzymatic pre-treatment; Flaxseed; Pumpkin seed; Terebinth*

RESUMEN: ***Efectos de pretratamientos ácido y enzimático sobre los aceites de calabaza, terebinto y linaza prensados en frío***. El rendimiento y las propiedades del aceite se pueden mejorar con varios pretratamientos enzimáticos antes de obtener el aceite de las semillas oleaginosas mediante extracción por prensado en frío. En este estudio se utilizó una mezcla comercial de enzimas pectolíticas. Además, se aplicó, como pretratamientos, harina de semilla de manzana, como fuente de enzima β-glucosidasa y ácido cítrico, a semillas oleaginosas (calabaza, terebinto y linaza). Los resultados se evaluaron comparando los efectos de los pretratamientos sobre el rendimiento y las propiedades del aceite. El preparado enzimático puede aumentar el rendimiento de los aceite de las semillas de calabaza (~ 300%) y la linaza (151%). Se lograron aumentos significativos en los contenidos fenólicos de terebinto (de 91,67 a 319,33 mg GAE/kg) y aceites de lino (de 12,03 a 40,47 mg GAE/kg) mediante pretratamientos con ácido cítrico y enzimas. Estos dos pretratamientos también fueron efectivos en términos de formación de peróxido y estabilidad oxidativa en aceite de terebinto. Con la ayuda de los pretratamientos aplicados a las semillas oleaginosas, fue posible aumentar el traspaso de fenoles de las semillas al aceite, para el caso del aceite de terebinto, con porcentajes de aumento del 245% con el tratamiento con ácido cítrico y 248% para el proceso enzimático, en comparación con el control.

PALABRAS CLAVE: *Aceite prensado en frío; β-glucosidasa; Pretratamiento enzimático; Semilla de calabaza; Semilla de lino; Terebintos*

Citation/Cómo citar este artículo: Özkılıç SY, Arslan D. 2022. Acidic and enzymatic pre-treatment effects on cold-pressed pumpkin, terebinth and flaxseed oils. *Grasas Aceites* **73** (2), e462. https://doi.org/10.3989/gya.0324211

1. INTRODUCTION

Oilseeds are a rich source of bioactive components. Their direct consumption is widespread and the oils of these seeds have recently attracted attention due to their protective, restorative effects and flavors. The processes used to obtain oil from oilseeds affect oil quality (Siger and Józefiak, 2016). Various methods are used for oil extraction. These usually include distillation, solvent-supercritical fluid extraction and the pressing method. In the pressing method, the yield is lower than other methods, but the quality of the oil obtained is high (Çalıkoğlu *et al*., 2006). Advantages to this method are the small amount of energy consumed in oil production from oilseeds, easy application, no chemical or heat treatment required and high oil quality. This method is quite valuable in terms of obtaining oil quality; however, the yield is much lower than the solvent extraction method (Maier *et al*., 2009). High polyphenol content, which is one of the important quality parameters of oil, can pass from seed to oil by a cold-press method without deterioration. The press method has different applications such as mechanical pressing, hydraulic pressing or pressing with gear grinders for oil extraction (Sevindik and Selli, 2016).

To increase the yield, pre-treatments of oil samples before extraction have been evaluated in many studies (Anwar *et al*., 2013; Karataş, 2015; Koubaa *et al*., 2016; Mazaheri *et al*., 2019). The most common among them is the heat treatment. However, in some oils, heat treatment decreases the quality of the oil and even causes the oil unsuitable to be defined in the cold-pressed oil range. Other pre-treatments are roasting, ultrasound or microwave application, maceration and irradiation (Şeran, 2011). Another pre-treatment that is thought to be effective in increasing oil quality is enzyme addition. Enzymes are used in the food industry to increase yields in the production of fruit juice, oil and sugar by affecting polymers on the cell wall. However, using enzymes was a major disadvantage that leads to a high priced process. But now, thanks to advances in biotechnology, low price and high-quality enzyme formulations can be obtained.

In this paper, pumpkin seed, flaxseed and terebinth were used. Pumpkin seed is a widely produced oilseed with high commercial value. In particular, the raw form of pumpkin seeds without a shell has the highest bioactive component (tocopherols, polyphenols, squalene, piperazine) among the pumpkin species (Nakić *et al*., 2006). The range of oil varies from 40 to 60% depending on the type of pumpkin. Pumpkin seeds are rich in unsaturated fatty acids, especially oleic (30.35%–42.07%) and linoleic acid (43.68%–52.15%) (Akin *et al*., 2018). This oil is preferred in salads (Jafari *et al*., 2012). The use of terebinth seed spreads over a wide range of applications, from cosmetics to food. The reasons for selecting the terebinth seed in this study are the high oil content of the terebinth and its richness in oleic acid, high number of phenolic compounds and its potential as a commercial product. The highest fatty acid content in the terebinth seeds was found as monounsaturated oleic acid with a range of 51.2–67.5 g/100 g (Ertaş *et al*., 2013). Oleic acid (45.8%), linoleic acid (23.93%) and palmitic acid (24.27%) have been identified as the main fatty acids of terebinth oil (Kaya and Özer, 2015). Flaxseed, which is rich in quality protein, is a natural source of phytochemicals such as flavonoids, lignans and phenolic acids. Cold-pressed flaxseed oil contains about 50% α-linolenic acid (ALA), which is one of the omega-3 fatty acids. A low temperature operation should be preferred for long shelf-life and high quality due to its high ALA content (Wiesenborn *et al*., 2005)**.**

Phenolic compounds found in oilseeds are available in free, soluble conjugates and insoluble-bound forms. The distribution of phenolic compounds varies in different parts of the seeds (Rahman *et al*., 2018). These plant-derived phenolic compounds are in conjugated form with sugar functional groups. During the process or metabolism, these glycoside forms are converted to aglycones by enzymatic or chemical means. This transformation can increase the concentration and bioactivity of phenolic compounds in free form (Küçükhüseyin, 2012). Few studies have reported the application of enzymes to increase phenolic production in processes using organic solvents or water in the extraction of phenolic compounds from vegetable samples (Laroze *et al*., 2010). The use of enzymes in oil extraction processes provides a high number of antioxidant compounds and an increase in the yield by disrupting the plant cell wall (García *et al*., 2001). β-glucosidases are enzymes capable of hydrolyzing β-glucoside bonds in oligosaccharides or phenolics with glycosidic compounds (Ergöçen, 2013). The β-glucosidase enzyme is commonly found in many fruit seeds (fig, apricot,

grape, papaya) and bread yeast (Sirilun *et al*., 2016). The study by Yu *et al.* (2007) revealed that the β-glucosidase enzyme obtained from apple and peach kernels was more suitable than that of commercially supplied β-glucosidase. The dry matter of apple and peach kernels was determined as the most suitable enzyme source due to their high activity, broad substrate specificity and strong stability. In addition, in the same study, the total activity and specific activity of β-glucosidase in the crude extract of apple seed were given as 54.8 U and 0.38 U per mg, respectively. Besides, the half-life of apple seed β-glucosidase at 50 °C was 42.9 h. It was stated that this result showed that the glucosidase obtained from apple seeds had higher thermal stability than commercial almond glucosidase. These two enzymes with similar pH preference (apple seed β-glucosidase and commercial almond β-glucosidase) were most active at pH 6.0 and remained stable up to 24 hours in the pH range of 5.0-9.0. It was concluded that apple seed glucosidase is an easily accessible natural catalyst and can be reused for about for one month without immobilization. The crude meal from apple seed was used for the first time in the study by Tong *et al*., (2004) as an easily available and inexpensive biocatalyst in the large-scale synthesis of alkyl-β-glucopyranosides. The world yield of apples was approximately 76 million metric tons in the 2019/20 season, and the majority of these are used for juice production, which generates a large amount of waste including seeds and pomace. If these fruit wastes can be used as enzyme sources, the waste will be valued and the price of the enzyme can also be reduced greatly. Hence, in this study, apple seed meal was used as a simple and cost-effective source of β-glucosidase enzyme. Alternatively, the acid hydrolysis of glycosides and phenolics has also been a common practice. Usually, HCl has been used for this procedure (Watson *et al*., 2014). Here, in our study, citric acid was preferred for acid pre-treatment since it is not harmful, easy to access, economical and widely used in the food industry.

The current study investigates the effect of a commercial enzyme mixture, apple seed meal and citric acid pre-treatments applied to oilseeds on oil yield and oil properties (peroxide value, FFA, free radical scavenging activity). With the commercial enzyme mixture, which is a pectinase complex, it is aimed to degrade the cell wall and hydrolyze glycosidic aglycons with apple seed meal; while the objectives of the citric acid pre-treatment are cell wall disruption and the hydrolysis of aglycones.

The effect of enzyme pre-treatment on oil yield and properties in oil extraction has been investigated in many studies (Latif *et al.,* 2007; Latif and Anwar, 2009; Latif *et al.,* 2011; Liu *et al.,* 2016). However, the number of studies using enzyme treatment before cold press oil extraction is limited (Soto *et al.,* 2008). No studies which utilize apple seed β-glucosidase and citric acid as pre-treatments are available. Citric acid and apple seed β-glucosidase provide an easily accessible and inexpensive source for industrial applications. In addition, the different effects of pre-treatments originating from the seeds were revealed by using three different oil seeds (pumpkin, terebinth and flaxseed) in the study.

2. MATERIALS AND METHODS

2.1. Materials

Terebinth and flaxseed were obtained from local markets in Konya, Turkey. Hulled pumpkin seeds harvested in Ürgüp (Turkey) were purchased from a local market. All chemicals and standards were of analytical grade and purchased either from Merck (Darmstadt, Germany) or Sigma- Aldrich (Germany).

2.2. Preparation of oilseeds for cold pressing

The oilseeds were divided into equal batches (500 g) and stored in a cool, dry place till pressing. The seeds were milled to a particle size of approximately 2–4 mm to increase surface area before cold pressing. The grinding process was conducted with an Arsel industrial kitchen grinder (220 V, 0.8 kg capacity, 0.3 kW, 9000 rpm/9 min motor power). The milled seeds were evenly distributed on a tray (40x60x5 cm, 0.2 cm thick). 0.1 M (pH=6) Na_2HPO_4 buffer solution was used in all pre-treatments.

Citric acid pre-treatment*:* a citric acid pre-treatment was performed to oilseeds by spraying with a ratio of 1% (by seed weight). For this pre-treatment, 5 g of citric acid were dissolved in 25 mL purified water. The resulting citric acid solution was sprayed into the ground seeds homogeneously in the presence of 100 mL buffer solution.

Commercial enzyme*:* Olivex (Advanced Enzyme Technologies Ltd. India), a pectinase complex produced by the fungus *Aspergillus aculeatus* was

added to the batches at a ratio of 1%. For this purpose, 5 g enzyme were weighed for 500 g sample and dissolved in 100 mL buffer solution. The solution was sprayed onto the seeds.

Apple seed meal: To produce a meal rich in the β-glucosidase enzyme, 'Golden Delicious' apples (*Malus domestica* Borkh.) which were harvested in 2016–2017. The fresh apple seeds were powdered via a coffee grinder, washed three times with ethyl acetate and two times with acetone and filtered under vacuum (Yu *et al*., 2007). The aim of this procedure was to remove some phenolic compounds and lipids from the apple seeds with the help of acetone. For a convenient and low-cost material in industrial application, only the isolation stage was used to produce β-glucosidase-rich meal from apple seeds. After the process, the solid extract remaining on the filter paper was left at room temperature for 24 h to remove the residual acetone (Ergöçen, 2013). The dry residue was stored in the refrigerator at +4 °C until use. 50 g of apple seed meal was used for 500 g oilseeds (10%) in the presence of 100 mL buffer solution.

The samples were individually incubated with each of the three pre-treatment methods for 3 h, at 60 °C (Emir *et al*., 2015). The samples were mixed at regular intervals during incubation. A control oil sample was also prepared by pressing the seed material under the specified conditions without any pre-treatment.

2.3. Cold pressing of oil seeds

A manual screw-press extractor (Karaerler NF 500, Turkey, 50 kg seed/h capacity, single head, 1.5 kW power) was used for the cold-pressed oil extraction. The screw rotation speed in a cold-press extraction was determined as 20 Hz for flaxseed, and 10–15 Hz for pumpkin and terebinth seeds. During the cold-pressing process, the temperature did not exceed 50 °C. The oils and press cake samples were taken and weighed, the oil phases were filtered and placed in dark colored glass bottles and kept at +4 °C. Defatted meal samples were tightly packed in plastic bags and stored in the dark at +4 °C.

2.4. Determination of oil content and yield

Oil seeds which were cleaned and brought to dry weight were ground using a mill. The total oil content of the ground samples was determined in the oil analyzer (Velp Scientifica Ser 148/6 Series) with n-hexane. According to the Randall method (Medvedevskikh *et al.,* 2021), 2.5 g ground seeds were weighed into the device cartridges and extracted with n-hexane for 1 h. At the end of the period, the solvent was removed from the extraction vessels and weighed and the oil content in the seeds was determined as a percentage. The analyses were conducted twice in parallel.

Oil yield was calculated as the ratio of the weight of the oil obtained at the end of the extraction to the initial weight of the seed. The extraction yield is the amount of oil obtained from the cold pressing of 100 kg oilseeds. The yield was calculated using the following equation:

$$\text{Extraction yield} = (A_{oil} / A_{seed}) \cdot 100$$

A_{oil} is the mass of the extracted oil (kg) and A_{seed} is the mass of the oilseed (kg).

2.5. Quality indices

The determination of oil quality indices was performed according to Regulation EEC/2568/91 (EU, 1991) by determing free fatty acid (FFA) (mg KOH/g) and peroxide value (PV) (meqO_2/kg).

2.6. Determination of total phenolic compounds (TPC) and DPPH radical scavenging capacity

The concentration of TPC was determined colorimetrically using FCR (Folin-Ciocalteu reagent) (Singleton *et al*., 1999). 6 g of oil sample were weighed, dissolved in 3 mL n-hexane and vortexed for 2 min with the addition of 6 mL methanol/water (80:20 v/v) for extracting phenolic substances. The n-hexane and methanol/water phases were separated via centrifuge (at 3000 rpm for 5 min). This process was repeated three times, and the methanolic phases were evaporated using a nitrogen evaporator. The methanolic extracts were stored in the refrigerator until analysis. To obtain the methanolic extract from press cake samples, 10-g samples were weighed into a 100 mL Erlenmeyer flask and adjusted to volume with methanol/water (80:20 v/v). The flasks were shaken at 250 rpm for 2 h. After shaking, the supernatants were taken and centrifuged at 10000 rpm for 10 min. This process was repeated twice and the supernatants were filtered and concentrated up to 15 mL in a rotary evaporator at 40 °C. Methanol extracts obtained from oil and press

cake were also used to calculate the DPPH free radical scavenging capacity.

For the determination of the total number of phenolics, the extracts were taken to the test tube at appropriate concentrations and their volumes were made up to 5 mL with ultra-pure water. 0.5 mL of FCR/distilled water (1: 3 v/v) was added and stirred for 30 min in the dark. At the end of this period, 1 mL of a sodium carbonate solution (35%) and 3.5 mL of purified water were added and the mixture was kept in the dark again for 2 h. Absorbance values were measured at 725 nm in a spectrophotometer (Biochrom Libra S22, UK) against a blank sample.

For the determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of oil and press cake extracts prepared at proper concentrations were placed in test tubes and the volume was completed to 1 mL with buffer solution (Tris-HCl). 2 mL of a methanolic solution of DPPH were added and left to react in the dark at room temperature. The decrease in absorbance at 517 nm was recorded in a spectrophotometer (Biochrom Libra S22, UK) after 30 min. Results were calculated based on the percent inhibition of the DPPH radical (Blois, 1958).

2.7. Oxidation stability of cold-pressed oils

892 Professional Rancimat apparatus and StabNet 1.0 software were used for induction time measurements. Oil samples (3 g) were weighed into reaction vessels and heated at 120 °C under a dry airflow of 20 L/h. The volatile compounds released during oxidation were collected into a cell containing distilled water, and the increasing water conductivity was continually measured. The time taken to reach the conductivity inflection point was recorded as the induction period (IP), and expressed in hours (h). All determinations were conducted in triplicate.

2.8. Statistical analysis

The data obtained as a result of the research were subjected to analysis of variance "General linear model multivariate analysis"; significant differences between the mean values of the main sources were compared with Duncan's multiple comparison tests (Zolman, 1993). SPSS 10.0 for Windows (v.10) was used for statistical analysis and the significance level was given as $P \leq 0.05$. This research was performed in three duplicates with a replicate.

3. RESULTS AND DISCUSSION

3.1. Oil yield and quality

The total oil contents of the oilseeds were 46.97, 43.86, and 37.80% on a dry weight basis for pumpkin seed, terebinth and flaxseed, respectively, which were determined by the rapid oil extraction method. The lowest oil yield was obtained by cold pressing untreated pumpkin seeds (around 16% of the initial oil content). The low oil yield of hulled pumpkin seed was known to us according to previously published data. For instance, in the study conducted by Neđeral and coworkers (2012), it was reported that the oil yield of untreated hulled pumpkin seed from the cold-press process was 48% lower than the initial oil content for husked pumpkin seeds. On the other hand, the highest increase in oil yield due to the pre-treatments was obtained for pumpkin seeds. For all three pre-treatment (apple seed meal, citric acid and enzyme), the increase was around 300%. Although not at the levels seen in pumpkin seeds, pre-treatments also caused increases in oil yield for flaxseed. The highest oil yield increase in this oily seed was achieved by enzymatic pre-treatment (an increase from 16 to 24.23% , which corresponds to a 151% increase). Anwar *et al*. (2013) evaluated the effect of three enzymes on the flaxseed cold-press extraction and reported a remarkable effect on oil recovery with 8,12, and16% increase rates. Ranalli *et al*. (2005) and Latif *et al*. (2011) explained the enhancement in oil yield with the help of enzymatic treatment by using rupturing cell walls and expelling some more free oil. Moreover, Ezeh and coworkers (2016) reported that they achieved the highest oil recovery of 90% by applying enzymatic pre-treatment on tiger nut tubers. The average moisture content in hulled pumpkin seeds and terebinth were 7.2 (Neđeral *et al.,* 2012) and 6.17% (Dalgıç *et al.,* 2011), respectively. Oil yields were different as a result of cold-press extraction of these two oilseeds, whose total oil contents were close to each other. The physical and chemical properties of the seeds, such as moisture, crude oil, crude fiber and diameter-to-length ratio may be effective in the formation of this difference. The pre-treatments for terebinth seed negatively affected the oil yield and even caused decreases of 22-27% compared to the control. The emulsion formation caused by the mechanical effect of pre-treatments and pressing was

responsible for the decrease in oil yield from terebinth seeds. The negative effect of pre-treatments in this case could be attributed to the specific physical structure of the mass. A slurry texture was formed after mechanical pressing due to its rich content in resinous substances and this phenomenon triggered the emulsion formation in terebinth paste. Particularly, when seeds with high oil content are ground into very small particle sizes, the particles can easily stick together (Liu *et al*., 2016).

Although enzymatic pre-treatment led to higher values and apple seed meal and citric acid resulted in lower values, these changes in FFA were not significant in the pumpkin seed oil (Table 1). Because of the apple seed meal pre-treatment (1.77 mg KOH/g oil), FFA in flaxseed oil significantly decreased compared to that of the control (2.14 mg KOH/g oil). The application of apple seed meal was thought to have inactivated the lipase enzyme. This inhibition effect was not statistically significant for pumpkin seed oil, but a significant effect was determined for the flaxseed oil. On the other hand, the pre-treatments showed the most spectacular changes in the FFA of terebinth seed oils. The highest increase was determined in the samples treated with enzyme preparate, as the average FFA values were 11.98 mg KOH/g oil. Note that the FFA of untreated terebinth oil (control sample) was also higher compared to untreated flaxseed and pumpkin seed oils. The relatively higher moisture content and specific cell structure of terebinth seeds were probably responsible for these high FFA levels. The incubation step in the pre-treatment triggers lipolytic activity due to moisture and impurities results in the hydrolysis and formation of free fatty acids. The cellulolytic effect of the enzyme mixture should also be taken into account. Similar findings were reported by Emir *et al*. (2015) that enzymatic pre-treatment increased the FFA of cold-pressed poppy seed oil. Codex standards indicate that the acid value of cold-pressed oils and edible fats should be less than 4.0 mg KOH/g.

The PV of the oil samples ranged from 1.51–5.79 meqO_2/kg (Table 1). The PVs of pumpkin and flaxseed oils were higher in pre-treated samples. Among

Table 1. Oil yields and quality indices of pre-treated oil samples obtained by cold press extraction.

Pre-treatments	**Oil seeds**		
	Pumpkin seed	**Flaxseed**	**Terebinth**
Oil yield (%)			
Control	7.52 ± 0.34†c C	16 ± 0.49 c** B	26.3 ± 0.69 a A*
Citric acid	22.58 ± 0.80 a A	16.4 ± 0.33 c C	20.46 ± 0.44 b B
Commercial enzyme	20.07 ± 1.07 b B	24.23 ± 0.20 a A	20.73 ± 0.92 b B
Apple seed meal	22.92 ± 0.80 a A	20.44 ± 0.36 b AB	19.39 ± 1.80 b B
Free fatty acid (mg KOH/g)			
Control	1.95 ± 0.46 B	2.14 ± 0.13 ab[ii] B	3.91 ± 1.00 b A
Citric acid	1.58 ± 0.27 B	2.13 ± 0.52 ab B	7.05 ± 0.47 b A
Commercial enzyme	2.04 ± 0.58 B	2.88 ± 0.26 a B	11.98 ± 2.77 a A
Apple seed meal	1.58 ± 0.35 B	1.77 ± 0.35 b B	5.47 ± 0.36 b A
Peroxide value (meq O_2/kg)			
Control	2.35 ± 0.26 c B	1.51 ± 0.79 b B	4.79 ± 0.23 a A
Citric acid	5.79 ± 0.67 a A	1.99 ± 0.59 ab C	3.68 ± 0.39 b B
Commercial enzyme	3.77 ± 0.92 b	2.53 ± 0.23 ab	3.49 ± 0.24 b
Apple seed meal	4.8 ± 0.20 ab A	3.27 ± 0.63 a B	4.61 ± 0.41 a A
Induction Period (h)			
Control	6.05 ± 1.22 B	0.44 ± 0.31 C	8.27 ± 0.57 b A
Citric acid	5.34 ± 0.53 B	0.46 ± 0.26 C	9.12 ± 0.39 b A
Commercial enzyme	4.85 ± 0.30 B	0.50 ± 0.31 C	11.12 ± 0.79 a A
Apple seed meal	5.00 ± 0.37 B	0.75 ± 0.38 C	6.18 ± 0.32 c A

Note: †Data represent mean ± SD of 3 replicates in duplicate. *Lowercase letters show the significant differences between pre-treatment applied and non-applied samples ($P \leq 0.05$) and **Uppercase letters show the significant differences among oilseeds ($P \leq 0.05$) using Duncan's multiple comparison test.

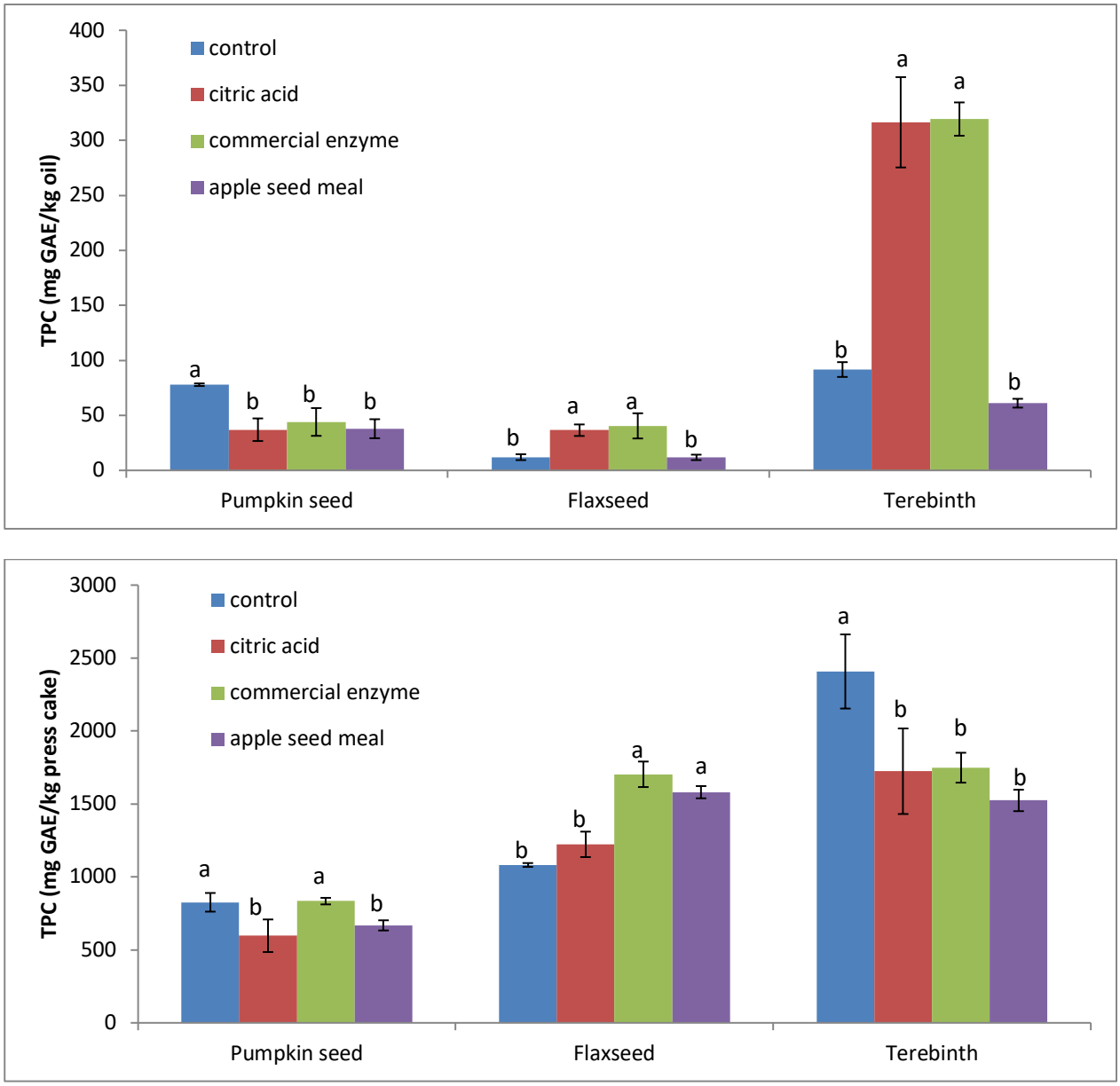

FIGURE 1. Total phenolic contents in oil and press cake samples obtained by cold-press extraction. Data represent mean ± SD of 3 replicates in duplicate. Lowercase letters show the significant differences among pre-treatments ($P \leq 0.05$) using Duncan's multiple comparison test.

the treatments, the increase in PV was more evident for apple seed meal than treated flaxseed oil. However, it was citric acid which considerably increased the PV of pumpkin seed oil. It was obvious that the incubation process performed within the scope of pre-treatments triggered the oxidation of these oils. In contrast, citric acid and commercial enzyme pre-treatments provided a significant reduction in the PV of terebinth oil compared to that of the control sample ($P \leq 0.05$). It might be possible for the polar phenolic components in the natural structure of the terebinth seed to better exert their antioxidant activity thanks to the pre-treatments. In addition, the transition of apolar phenolics or other antioxidants to oil occurred at a higher rate thanks to the citric acid and enzymatic pre-treatments (Figure 1). The fact that this was not the case for flaxseed may be attributed to its rich α-linolenic acid (ω-3) content. Pumpkin seed oil, on the other hand, did not show resistance to oxidation as much as terebinth oil due to its fatty acid composition and its relatively low phenolics and antioxidant content. Considering the

oxidative stability of the oil, the presence of some natural compounds with antioxidative capacity is a crucial factor (Vujasinovic *et al*., 2010). Tuberoso *et al*. (2007) found that the correlation between antioxidant activity and oilseed composition was variable and that this variability was caused by squalene, chlorophyl, carotenoid, phenolics and their mutual interactions. In addition, the oxidative stability of oil relies on the fatty-acid profile. The correlation between fatty acids and oxidative stability correlates positively with oleic acid with a negative correlation with linolenic and linoleic acid content (Tańska *et al*., 2016).

3.2. TPC and DPPH radical scavenging capacity

The use of acid or alkaline for hydrolysis is a general approach to the cleavage of glycosides from phenolic acid esters, phenolic acids and flavonoids. Enzymatic hydrolysis is another method applied as an alternative to acid hydrolysis. Glucosidases, pectinases, cellulases and amylases are some of the types of enzymes used for enzymatic hydrolysis (Adlard *et al*., 2011). This pre-treatment is a suitable alternative for increasing the recovery of polyphenols (Laroze *et al*., 2010). Moroever, the enzymatic hydrolysis of glycosides increases the hydrophobicity and bioactivity of the extracts since aglycones have a strong hydrophobicity (Do *et al.,* 2009).

Citric acid and commercial enzyme pretreatments led to significant increases in the number of TPC in terebinth and flaxseed oils ($p \leq 0.05$). This enhancing effect has been noted for terebinth seed oil with a much more significant difference between untreated and treated samples. Apple seed meal pre-treatment did not show any positive effect on the transition of phenolics into the oil (Figure 1).

However, regarding pumpkin seed oil, all the pre-treatments showed negative effects on the recovery of phenolic compounds, causing a ~76% reduction. The physical structure, moisture content and the character of the phenolic compounds in the ground seed may be effective in the reduction in the amount of phenolic components transferred to pumpkin seed oil as a result of the pre-treatments applied. Due to its unique tissue and cell structure, each oilseed is shredded to different sizes even if the same conditions are applied. Therefore, the penetration of the applied acids and enzymes may have remained at different levels. Since citric acid, enzyme preparation and apple seed enzyme extract were applied to the ground seed in a buffer solution, the moisture content of the seed mass was increased. During the subsequent incubation, the humidity remained at relatively high levels compared to the control, although there was moisture loss. Besides, the phenolic components in these oilseeds have different properties. Due to their features, such as containing more or less glycosidic bonds, having a large polyphenolic molecular structure or a simple phenolic acid structure, the amount of phenolics that pass into the oil and that remain in the paste may vary. In summary, the physical structure of the ground seed, its moisture content and the molecular structures of the phenolic components contained in the seed were effective on hydrolysis. Andjelkovic *et al.* (2010) reported the total phenol content in raw pumpkin seed oil to be between 24.71–50.93 mg GAE/kg, which was lower than the values determined in our study. They also stated that this difference in phenolic concentrations might be caused by variations in the harvesting and production conditions of the seeds. The higher values determined in our study may be due to cold pressing and lack of roasting step. Flaxseed is a rich source of flavonoids, lignans (especially secoisolariciresinol diglucoside (SDG)) and phenolic acids. The lignans are conjugated with carbohydrates and mostly located in the fiber part of flaxseed. Alkaline treatment breaks the link and releases free SDG, which is also used to obtain the aglycone form of lignans (Sainvitu *et al*., 2012). The increase in the total phenolic content of flaxseed oil samples (from 12.03 mg GAE/kg to 40.47 mg GAE/kg with commercial enzyme pre-treatment) was due to the release of phenolics from conjugated to free form via acidic and enzymatic hydrolysis.

In the study of Yıldız (2013), the total flavonoid content of terebinth fruit was reported as 76.01 mg-CE/g extract. As a result of the HPLC analysis, to evaluate the flavonoid composition, luteolin, luteolin-7-glucoside and apigenin-7-glucoside compounds were determined to be the major compounds (Kavak *et al*., 2010). These compounds exist in the form of glycosides in plants. These flavonoids in the glycoside form can be released by separating the conjugated aglycones with acid and enzyme application. However, some glycosides show strong resistance to acid hydrolysis. For example, 7-*O*-glucuronides and 7-*O*-glucosides are glycosides that show

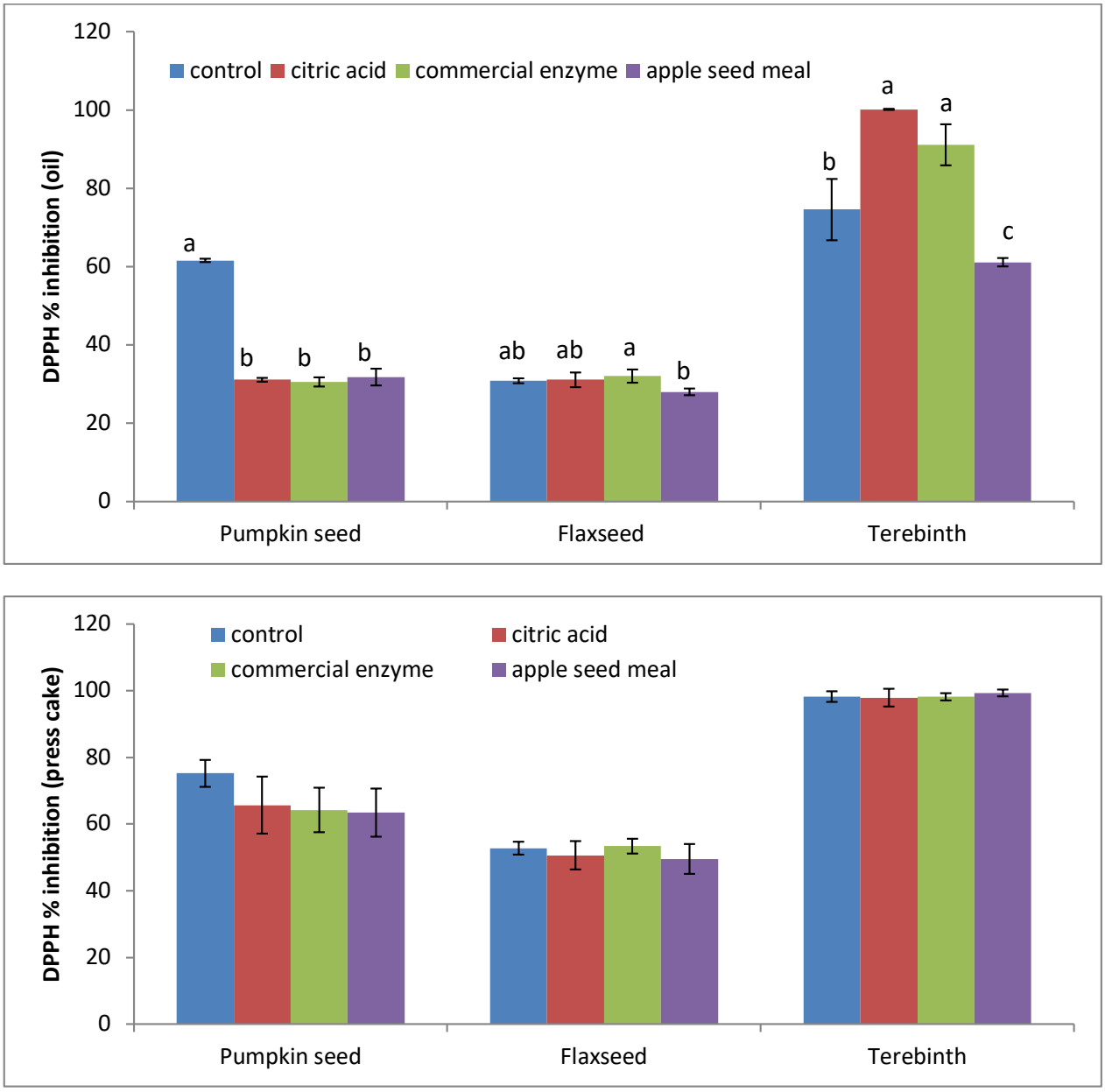

FIGURE 2. DPPH radical scavenging activity values in oil and press-cake samples obtained as a result of cold-press extraction. Data represent mean ± SD of 3 replicates in duplicate. Lowercase letters show the significant differences among pre-treatments ($P \leq 0.05$) using Duncan's multiple comparison test.

the most resistance to acid hydrolysis. Alternatively, these glucosides are rapidly hydrolyzed with suitable enzymes. Some *O*-glycosides have been reported to require prolonged heating (4–6 h) as they are difficult to dissolve with the effect of aqueous acid. For instance, luteolin 7-glucoside requires 6 h of heating with 30% H_2SO_4 for complete hydrolysis (Harborne, 1965). Considering this information, the increase in the number of TPC in terebinth oil due to enzyme and acid pre-treatments can be explained (from 91.67 mg GAE/kg to 319.33 and 316.4 mg GAE/kg, respectively). In order for apple seed meal to be effective in increasing the level of TPC as well, pre-treatment conditions could be rearranged (e.g., long incubation time or high incubation temperature).

Preceding works on oils such as flaxseed, olive, hemp seed and cottonseed pointed out that an enzyme pre-treatment notably enhanced the recovery

of polyphenolic compounds and improved antioxidant activities in oils (Ranalli *et al*., 2005; Latif *et al*., 2007; Latif and Anwar, 2009; Anwar *et al*., 2013). Moreover, Soto *et al*. (2008) demonstrated that enzyme-aided cold-pressed borage seed oil had three times more antioxidant content than the values obtained from a non-enzyme-aided process. In our study, apple seed meal pre-treatment did not show any positive effect on the transition of phenolics to the oil. Apparently, beta glucosidase enzyme obtained from apple seed pulp was at a level to increase the oil yield of the processing steps, but not at a level to allow phenolic transition.

It could be said that the relationship between the TPC remaining in the press cake and the amount passing into the oil was meaningful for terebinth seeds. That is, press cakes from citric acid and enzyme-treated oil samples containing much higher amounts of TPC than the control also contained significantly lower amounts of TPC compared to the control. Acid and enzyme pre-treatments provided the formation of aglycones in the terebinth seed, thus increasing the amount of phenolic components transferred to the oil. Regarding the flaxseed samples, the opposite relationship, although expected, was not seen. As could be seen from the much lower number of flaxseed phenolics that transferred to oil compared to terebinth oil, this might be because most of the phenolics contained in flaxseeds were polar. As mentioned above, the release of phenolics from conjugated to free form and the formation of aglycones via acidic and enzymatic hydrolysis was more evident for terebinth oil due to higher transition ratios (245% for citric acid, 248% for enzyme pre-treatment compared to the control).

The DPPH inhibition ratios of the oil samples showed a direct correlation with their TPC contents. Commercial enzyme and citric acid pre-treatments for flaxseed and terebinth oil improved DPPH radical scavenging activity. The antioxidant effect of citric acid addition against hydrolytic and oxidative rancidity (Arawande *et al.,* 2011) or its ability to chelate metal ions (Hraš *et al.,* 2000) may explain the increase in antioxidant activity due to citric acid pre-treatment. In addition, an increase in pH due to the addition of citric acid may have created a pro-oxidant effect by enabling peroxides to be decomposed more easily.

DPPH analysis results performed on press cakes did not reflect the effect of the pre-treatments applied (Figure 2). After the oil was extracted, the number of phenolic components in the press cake increased proportionally. The phenolic content of press cake could not reveal the differences between pre-treatments, probably due to the small number of phenolics transferred to the oil. That is, phenolics transformed into oil could be considered significant for oil, but these were not significant amounts for press cake. Fruehwirth *et al*. (2020) reported that the phenolic content in flaxseed oil as 0.27 mg GAE/100 g seeds and only 0.1% of polyphenols were transferred from seeds into the cold pressed oil, which was explained by the hydrophilic properties of most polyphenols (Tsao, 2010).

3.3. Oxidative stability of cold-pressed oils

Pumpkin seed oil is a member of the oleic-linoleic-type oils. The oils with a high degree of unsaturation are more susceptible to oxidative degradation. Squalene is considered important because of the antioxidant activity in pumpkin seed oil. The antioxidant activity of squalene is against polyunsaturated fatty acids (Dessi *et al*., 2002). The oxidative stability of cold-pressed pumpkin seed oils determined by the rancimat test was 4–4.5 h (IP) (Gorjanović *et al*., 2011), which was lower than the values of the present study (6.05 h). Vujasinovic *et al*., (2010) reported the oxidative stability index of cold-pressed pumpkin seed oil at 100 °C to be 18.4 ± 0.3 h (at an airflow of 18 L/h). The study by Murkovic and Pfannhauser (2000) revealed that the induction time of pumpkin seed oils, measured at 120 °C, was 6.83 h, similar to the result in our study.

The differences in the induction times of the pre-treated pumpkin seed and flaxseed samples compared to untreated samples were not statistically significant. On the other hand, in accordance with PV values, enzyme and citric acid pre-treatments gave significantly higher induction times for terebinth oil (11.2 h). As mentioned above, being rich in monounsaturated oleic acid and its endogenous antioxidant compounds gave terebinth oil more stability against oxidation. The remarkably low oxidative stability of flaxseed (0.44 h for the untreated sample) is not surprising due to its high content in α-linolenic acid, which is more prone to oxidation.

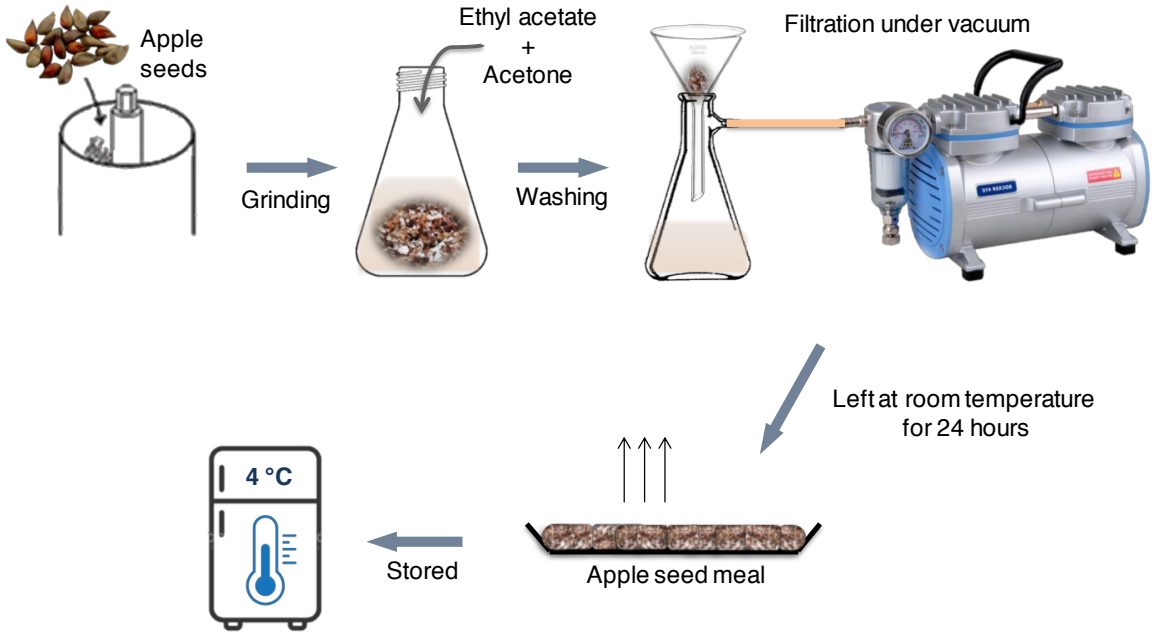

FIGURE 3. Flow diagram of the preparation of apple seed meal.

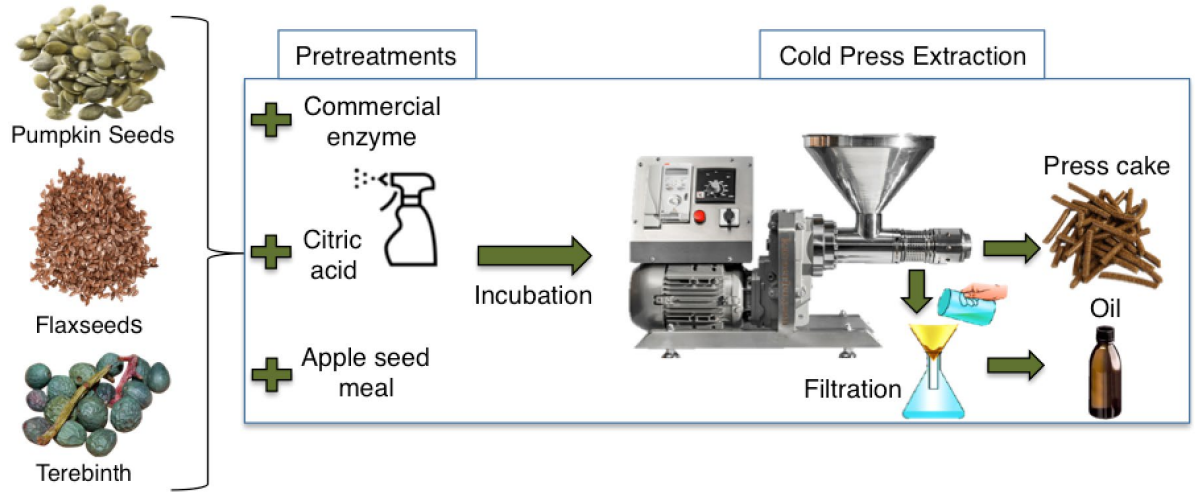

FIGURE 4. Scheme of seed oil extraction line.

4. CONCLUSIONS

Pre-treatments have provided an oil yield increase of up to 300% in pumpkin seeds, oil yield increased by 151% in flaxseeds with the aid of enzyme pre-treatment. With the mechanical effect of pre-treatment and pressing, the yield diminished due to the emulsion formation in terebinth seed paste.

Along with no significant effect on induction time, pre-treatments increased the development of peroxide values in pumpkin and flaxseed oils, probably due to the effect of the incubation process. In the case of terebinth oil, pre-treatments had a positive effect on peroxide values. The antioxidant compound contents and fatty acid compositions of the oils were the influencing factors on peroxide value.

The expected opposite relationship between the press cake and the phenolic content in the oil was not observed for flaxseed oil, since most of the phenolics it contained were polar. The effect of pre-treatments

on the release of phenolics from the conjugated form was through acidic and enzymatic hydrolysis and the formation of aglycone oil. The observation of this relationship in terebinth oil can be attributed to the increase in the transition of apolar aglycones to this oil.

Citric acid and enzyme pre-treatments are recommended for higher transfer of phenolics to oil. These two applications are also effective in terms of peroxide and oxidative stability in terebinth oil. The application of apple seed meal helped reduce free acidity in oil. In addition, it was as successful as citric acid in increasing the oil yield from pumpkin seeds.

This study demonstrated that enzyme and acid pre-treatments can be explored as a feasible alternative to conventional cold pressing for improving not only the oil yield but also the nutritive and functional quality of oils. Future studies can be conducted to get more effective results by modifying the process conditions, particularly incubation, and merging them with different treatments. Citric acid and enzyme pre-treatment methods can also be modified to have positive effects in terms of yield, so that it would be possible to produce improved terebinth oil in terms of yield, oxidative stability and functionality.

5. ACKNOWLEDGMENTS

The authors want to thank Necmettin Erbakan University and The Coordinatorship of Scientific Research Projects, Konya, Turkey (Project number, BAP–171319003) for financially supporting this research. This manuscript is prepared from the master science thesis of Sıddıka Yusra Özkılıç.

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