GRASAS Y ACEITES 70 (3)
July–September 2019, e316
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0709182

Column chromatography as a method for minor components removal from rapeseed oil

E. Rokosik[a], K. Dwiecki[a,✉], M. Rudzińska[b], A. Siger[a] and K. Polewski[c]

[a]Department of Food Biochemistry and Analysis, Poznań University of Life Sciences, Mazowiecka 48, 60-623 Poznań, Poland
[b]Institute of Food Technology of Plant Origin, Poznań University of Life Sciences, Wojska Polskiego 31, 60-624 Poznań, Poland
[c]Department of Physics and Biophysics, Poznań University of Life Sciences Wojska Polskiego 38/42, 60-637 Poznań, Poland

✉Corresponding author: krzysztof.dwiecki@up.poznan.pl

Submitted: 19 July 2018; Accepted: 05 December 2018. Published online: 7 May 2019

SUMMARY: The purpose of this study was to verify the influence of different chromatographic column beds (silicic acid, activated charcoal, aluminum oxide, silica gel) on the concentration of individual minor components (sterols, tocopherols, carotenoids and chlorophyll) in rapeseed oil. With the use of a combination of these beds, a three-stage optimized method for removing minor components from rapeseed oil was developed. It was demonstrated that the combination of silicic acid and activated charcoal removed about half of the sterols present from the oil. Aluminum oxide turned out to be the most effective bed in removing tocopherols, purifying the oil to their minimum level (2.6 mg/kg). All adsorbents used had similar capacity to purify oil from pigments (carotenoids and chlorophyll). In the three-stage purification process free sterols were almost completely removed (to the level 90.0 mg/kg). Purification of β-carotene and chlorophyll from the oil was also very effective. Tocopherols were completely removed with this method, except for a small amount of α-tocopherol (0.4 mg/kg), which results from its relatively weak interaction with a hydrophilic bed. The developed method may be used in studies on the effect of association colloids on bulk oil autoxidation processes.

KEYWORDS: *Association colloids; Column chromatography; Oil autoxidation; Rapeseed oil; Sterols; Tocopherols*

RESUMEN: ***Cromatografía en columna como método para la eliminación de componentes menores del aceite de colza.*** El propósito de este estudio fue verificar la influencia de diferentes rellenos de columnas cromatográficas (ácido silícico, carbón activo, óxido de aluminio, gel de sílice) sobre la concentración de componentes menores individuales (esteroles, tocoferoles, carotenoides y clorofila) en aceite de colza. Gracias a esto, se desarrolló un método optimizado de tres etapas para eliminar los componentes secundarios del aceite de colza (utilizando una combinación de todos los rellenos descritos anteriormente). Se ha demostrado que con la combinación de ácido silícico y carbón activo se elimina del aceite alrededor de la mitad de los esteroles presentes. El óxido de aluminio resultó ser el relleno más eficaz para eliminar los tocoferoles, purificando el aceite hasta su nivel mínimo (2,6 mg/kg). Todos los adsorbentes utilizados tenían una capacidad similar para purificar el aceite de pigmentos (carotenoides y clorofila). En el proceso de purificación en tres etapas, los esteroles libres se eliminaron casi por completo (hasta el nivel de 90,0 mg/kg). La purificación de aceite de β-caroteno y clorofila también fue muy efectiva. En este método, los tocoferoles se eliminaron completamente, excepto pequeñas cantidades de α-tocoferol (0,4 mg/kg), lo que resulta de su interacción relativamente débil con un relleno hidrófilo. El método desarrollado se puede usar en los estudios sobre el efecto de los coloides de asociación en los procesos de autooxidación de aceites a granel.

PALABRAS CLAVE: *Aceite de colza; Asociación de coloides; Autooxidación del aceite; Cromatografía de columna; Esteroles; Tocoferoles*

ORCID ID: Rokosik E https://orcid.org/0000-0002-3604-4414, Dwiecki K https://orcid.org/0000-0002-1438-1217, Rudzinska M https://orcid.org/0000-0001-6343-332X, Siger A https://orcid.org/0000-0002-3681-153X, Polewski K https://orcid.org/0000-0003-0445-7721

Citation/Cómo citar este artículo: Rokosik E, Dwiecki K, Rudzińska M, Siger A, Polewski K. 2019. Column chromatography as a method for minor components removal from rapeseed oil. *Grasas Aceites* **70** (3), e316. https://doi.org/10.3989/gya.0709182

1. INTRODUCTION

The autoxidation of lipids results in the deterioration of food quality because it leads to a loss in the nutritional value of plant oil, the generation of off-flavor and color alterations. Oils enriched in polyunsaturated fatty acids are increasingly used on the market. Due to the fact that these products are very sensitive to oxidation, a better understanding of the mechanisms of lipid oxidation and the development of new antioxidant technologies is necessary (Homma *et al*., 2015). The oxidation of lipids is a very complex process, based on a series of chemical reactions. Further knowledge on the mechanisms of lipid oxidation underlines the very important influence of microemulsions in oil, mainly on its stability and structure in autoxidation processes. In plant oil small amounts of water are present. However, water plays an essential role in the oxidation of lipids in bulk oil, mainly due to its activity with respect to hydroperoxides and reactive metal ions (Budilarto and Kamal-Eldin, 2015). Plant oils, aside from the predominant amount of triacylglycerols and low water content, also contain many minor components such as phospholipids, sterols, tocopherols, free fatty acids, pigments etc. These substances are surface-active compounds. In the presence of a small amount of water and at a concentration above their critical micelle concentrations (CMC) they self-aggregate and form association colloids (Chen *et al.,* 2011b). These structures are considered oil oxidation sites because they can affect the physical location of antioxidants, prooxidants and oxidation substrates that are able to accumulate at the oil-water interface. More studies are still needed to confirm that association colloids are the main site for lipid oxidation (especially in the initiation phase). This knowledge would allow for changing colloid structures and effective antioxidant supply, thus improving the oxidation stability of oils containing polyunsaturated fatty acids (Chaiyasit *et al*., 2007). The main components of edible vegetable oils are triacylglycerols (95–98% by mass of oil). After the refining process, oil contains only about 1% of minor components but those substances play a significant role in shaping the quality of the oil (Xenakis *et al*., 2010). A refining process which removes most of minor components is often necessary to maintain a high-quality product (Kachel-Jakubowska *et al*., 2015; Ghazani and Marangoni 2013; Čmolík and Pokorný, 2000). There are several studies on the effect of individual minor components on oil oxidation, but there is still not much research concerning the effects of the combination of them. Research on the influence of minor components on the formation of association colloids and their effect on oil oxidation will allow for developing new antioxidant technologies and products with higher stability (Chaiyasit *et al*., 2007). Understanding of the resulting colloidal structures can also be used to enrich the oil with additional components, which will contribute to the creation of products with better functional and health properties (Xenakis *et al*., 2010). In the studies on the effect of association colloids on oxidation processes, it is very important to remove minor components from the oil (especially amphiphilic substances able to form reverse micelles as well as antioxidants and prooxidants like chlorophyll). Defined amounts of minor components are then added to the purified bulk oil, and thus their influence on the oxidation process is tested under controlled conditions. In this way, artifacts resulting from the presence of unidentified amphiphilic compounds and native antioxidants are avoided. So far mostly soybean and corn oil are used in studies on the formation of reverse micelles in oils (Kittipongpittaya *et al*., 2014; Chen *et al*., 2010; Chen *et al*., 2011b). Stripped oil (purified of minor components) is prepared mainly using column chromatography filled with adsorbents like silicic acid, activated charcoal or aluminum oxide (Kittipongpittaya *et al*., 2014; Cui *et al*., 2014; Khuwijitjaru *et al*., 2009; Nyström *et al*., 2007) and the content of minor components is determined very selectively (the content of polar components assayed according to Association of Official Analytical Chemists (AOAC) official method 982.27, silica gel G thin layer chromatography (TLC) with detection by iodine vapor, determination of tocopherol content using NP-HPLC) or no information is provided on the removal of minor components (Laguerre *et al*., 2011). There are also no reports concerning the effect of different sorbents (beds of chromatographic column) on the individual compound concentration in rapeseed oil after purification. This type of data would allow for the development of an optimal method for the removal of minor components from this vegetable oil. In the area of our interest there is rapeseed oil, which is one of the three most commonly produced oils worldwide with a global production of over 23.5 million tons (Siger *et al*., 2015). Therefore, the aim of this study was to verify the influence of different chromatographic column beds (adsorbents: silicic acid, activated charcoal, aluminum oxide, silica gel) on the concentration of individual minor components (amphiphilic compounds, native antioxidants, pigments) in rapeseed oil after purification. The determination of the effectiveness of different beds in relation to individual minor components allowed for the development of a three-stage optimized method of minor component removal from rapeseed oil. This method is based on the use of a combination of analyzed beds, able to effectively remove individual components (sterols, tocopherols, chlorophyll, β-carotene, etc.). Oil which is purified according to this method will be suitable for studies on the influence of minor components on the formation of association colloids and their effect on oil oxidation.

2. MATERIALS AND METHODS

2.1. Materials and chemicals

The material used for the tests was 2090 mL refined rapeseed oil, "Kujawski" (per one replicate) in a plastic bottle (ZT Kruszwica S.A., Poland) purchased from a local market. Aluminum oxide 90 standardized, silicic acid (precipitated, extra-pure, light), activated charcoal powder (extra-pure), 1,4-dioxane (99.8%), starch and methyl *tert*-butyl ether were purchased from Merck (Darmstadt, Germany). All tocopherol homologues (purity > 95% by HPLC) were from Calbiochem-Merck Biosciences (Darmstadt, Germany). Chloroform (98.5%), methanol (99.8%), acetic acid (99.5-99.9%), potassium iodide, sodium thiosulfate solution 0.1 mol/L, diethyl ether, ethyl alcohol (96%), *n*-hexane (99% HPLC-grade) and potassium hydroxide were purchased from POCh (Poland). Ethyl acetate (≥ 99.7%) was from Honeywell (Poland). Silicic gel (high-purity grade), 5α-cholestane and sterol standards were from Sigma-Aldrich (Germany). All chemicals used in this study were of analytical grade.

2.2. Preparation of purified rapeseed oil

Purified (stripped) rapeseed oil was prepared using column chromatography in three different ways (variants). Briefly, silicic acid (100 g) was washed three times with a total volume of 3 L of distilled water and dried at 110 °C for 20 h (Boon *et al*., 2008). Aluminum oxide (180 g) was activated at 100 °C for 16 h and at 200 °C for 8 h (Nyström *et al*., 2007; Yoshida *et al*., 1992). Silica gel (180 g) was activated at 180 °C for 18 h. In variant I, a chromatographic column (2.0 cm internal diameter × 42 cm height) was packed sequentially with 10 g of silicic acid, followed by 3 g of activated charcoal and another 10 g of silicic acid. In a second variant (II), an aluminum oxide column was packed with 60 g of this adsorbent. A third variant of purification (III) was applied with a chromatographic column packed with 25 g of silica gel. Prior to the purification process, 30 mL of *n*-hexane were applied to each column. Next, 30 mL of rapeseed oil were dissolved in 30 mL of *n*-hexane and passed through the column. The whole process was carried out using a vacuum pump. The container used to collect the purified oil was held in an ice bath and covered with aluminum foil to retard lipid oxidation during stripping. The solvent present in the stripped oil was removed with a vacuum rotary evaporator (vacuum evaporator R-215, Büchi Labortechnik AG, Flawil, Switzerland) at 30 °C and traces of the remaining solvent were evaporated under a nitrogen stream.

In the three-stage method of minor component removal, a chromatographic column (10.0 cm internal diameter × 52.0 cm height) was packed sequentially with 300 g of silicic acid, followed by 90 g of activated charcoal and again 300 g of silicic acid. Next, 2000 mL of rapeseed oil were dissolved in 2000 mL of *n*-hexane and passed through the column. The partially purified oil was then passed through a column with activated charcoal (100 g) and aluminum oxide (1250 g). In the last step, oil which was purified in two stages was passed through the column packed with silica gel (540 g). The solvent present in the stripped oil was removed with a vacuum rotary evaporator and traces of solvent were evaporated under a nitrogen stream. All experiments were carried out in triplicate.

2.3. Total chlorophyll and β-carotene determination

A measurement of the content of chlorophyll was performed according to the AOCS Official Method Cc 13d-55. The β-Carotene content in oil was determined by applying a calibration curve of β-carotene (Fluka, Germany) (0.1–5 μg) dissolved in *n*-hexane and recording the absorbance at 450 nm. *n*-hexane was used for oil dilution to obtain the absorbance in a range of the calibration curve.

2.4. Determination of tocopherols by NP-HPLC

An analysis of tocopherol content was performed as described by Siger *et al*., (2017). An oil sample (1 g) was dissolved in *n*-hexane in a 10 mL flask. The solution was transferred to vials for analysis. Tocopherols were quantitatively and qualitatively determined using a Waters HPLC system (Waters, Milford, MA) consisting of a pump (Waters 600), autosampler (Waters 2707), fluorimetric detector (Waters 474), photodiode array detector (Waters 2998 PDA), column oven (Waters Jetstream 2 Plus), and LiChrosorb Si 60 column (4.6 mm x 250 mm, 5 μm) by Merck (Darmstadt, Germany). The mobile phase in this analysis was a mixture of *n*-hexane with 1,4-dioxane (96:4 v/v). The flow rate was 1.0 mL/min. To detect the fluorescence of tocopherols, the excitation wavelength was set at $\lambda = 295$ nm and the emission wavelength at $\lambda = 330$ nm.

2.5. Separation of esterified sterols in oil

The separation process followed the procedure described by Verleyen *et al*., (2002). In the process of separation of esterified and free sterols the polarity difference between them was used. The column was packed with 15 g silica gel. Oil samples (1.5 g) were weighed into a beaker and transferred using *n*-hexane (2 × 5 mL), and rinsed into a chromatography column which was packed with silica gel. A mixture of solvents (75 mL *n*-hexane/ethyl acetate (90:10 v/v)) was used to elute the esterified sterol fraction. After evaporation of the solvent

assays were performed according to the procedure described for the sterol analysis. The content of free sterols was determined by subtracting the esterified sterols from the total sterols.

2.6. Sterol determination

Total and esterified sterol (phytosterols) determination was performed as described by Siger *et al*., (2017). The sterol content was determined by GC. First, lipids (50 mg) were saponified with 1 M methanolic KOH for 18 h at room temperature. Next, water was added and the unsaponifiables were extracted three times using *n*-hexane/methyl *tert*-butyl ether (1:1 v/v). A nitrogen stream was used to evaporate the solvent. Dry residues were dissolved in 0.2 mL pyridine and silylated with 0.8 mL of Sylon BTZ. The sample prepared in this way (1.0 µL) was injected in the splitless mode. Derivatives of the sterols were separated on a HP 6890 series II Plus (Hewlett Packard, Palo Alto, USA) with a DB-35MS capillary column (25 m x 0.20 mm, 0.33 µm; J&W Scientific, Folsom, CA). The column temperature was held at 100 ˚C for 5 min, and then programmed to 250 ˚C at 25 ˚C/min, held for 1 min, and further programmed to 290 ˚C at 3 ˚C/min and held for 20 min. The detector temperature was set at 300 ˚C. The carrier gas used was hydrogen at a flow rate of 1.5 mL/min. In order to quantify the sterols, internal standard 5α-cholestanol was used. Phytosterols were identified by comparing the obtained retention data with the relevant standards.

2.7. Determination of oil quality parameters

The quality parameters of purified rapeseed oil were determined according to ISO standard methods: peroxide value (PV), ISO-3960:2007 and acid value (AV), ISO-660:2009.

2.8. Water content in oil determination

The water content in rapeseed oil was determined using the Karl Fischer method (ISO-8534:2008).

2.9. Statistical analysis

Results are presented as means ± standard deviation from three replicates of each experiment. For statistical evaluation, the Student's t-test and ANOVA with Tukey's test were applied. All tests were considered significant at $p < 0.05$. The statistical analysis was performed using Statistica 10.0 software (StatSoft, Inc., Tulsa, OK).

3. RESULTS AND DISCUSSION

During the oil purification, different beds (column deposits) were used in three variants: I - three layers packed into the column sequentially: silicic acid on the bottom, activated charcoal in the middle layer and silicic acid on the top; II - aluminum oxide; and III -silica gel. In each variant, refined rapeseed oil dissolved in *n*-hexane was applied to the column filled with the bed. After the purification process, the contents of sterols, tocopherols, chlorophyll and β-carotene were determined. The results are summarized in table 1. The most commonly used method for purifying oil of its minor components is the use of silicic acid and activated charcoal (Homma *et al*., 2015; Kittipongpittaya *et al*., 2016; Chen *et al*., 2011a). This method (variant I) purifies the oil of only a part of the sterol contents (4130.0 mg/kg relative to 5840.0 mg/kg in refined oil before purification; Table 1). The bed was more effective in relation to tocopherols, adsorbing about half of their contents (336.2 mg/kg relative to 630.2 mg/kg in refined oil). Chlorophyll was not detected in the oil after variant I of purification; whereas the content of pigments before this process was 0.04 mg/kg. In the case of β-carotene, a decrease from 2.02 mg/

Table 1. Contents of sterols, tocopherols, chlorophyll and β-carotene in purified oil using various types of beds and in refined oil before purification.

	Determined compounds			
Type of bed	**Sterols (mg/kg)**	**Tocopherols (mg/kg)**	**Chlorophyll (mg/kg)**	**β-Carotene (mg/kg)**
variant I silicic acid /activated charcoal /silicic acid	4130.0 ±310.0^{a}	336.2 ± 4.4^{c}	n.d.	1.39 ± 0.17^{b}
variant II aluminum oxide	4900.0 ± 510.0^{a}	2.6 ± 0.4^{a}	n.d.	1.68 ± 0.12^{b}
variant III silica gel	7860.0 ± 120.0^{c}	85.2 ± 1.1^{b}	n.d.	0.96 ± 0.09^{a}
refined rapeseed oil	5840.0 ± 160.0^{b}	630.2 ± 7.4^{d}	0.04 ± 0.01	2.02 ± 0.11^{c}

Values (means ± SD; n=3) with different index letters are statistically different at the significance level ($p < 0.05$). ANOVA with Tukey's test was applied.
n.d. – non detected

kg to 1.39 mg/kg was observed in this substance after purification in variant I (Table 1). The removal of chlorophyll, β-carotene and tocopherols from the oil used in autoxidation studies is important due to the prooxidant and antioxidant activities of these compounds. The content of pigments in refined oil is at a low level due to the bleaching treatment which is normally performed during refining. In this process carotenoids and chlorophyll, as well as soaps, residual phosphatides, oxidation products and other minor compounds are adsorbed in bleaching earth or bleaching clays (activated or natural) (Ghazani and Marangoni, 2013). The most important part of the bleaching process is the removal of chlorophyll because it acts as a photo-sensitizer, catalyzing the oxidation of oil in light (Rotkiewicz *et al*., 2002). In addition, activated charcoal is used in the process of purifying frying oil of pigments and thermal degradation products (Buczek and Chwiałkowski, 2005; Chwiałkowski, 2013). Activated charcoal is the most commonly used adsorbent not only due to its strong adsorption properties but also to its low production cost, obtained as a result of the thermal processing of graphite and soot. According to literature data the biggest drawback of this bed is the lack of selectivity of the removed compounds, so when more selective purification is expected, other beds are selected, such as aluminum oxide (Rajczykowski and Loska, 2016). Aluminum oxide was used in variant II of purification. It is very often used in water treatment technology due to its strong adsorption properties and large surface area (Ravindhranath and Ramamoorty, 2017). During the purification of oil, aluminum oxide turned out to be a very effective bed that retained almost all of the tocopherols, leaving only 2.6 mg/kg in the purified fraction compared to 630.2 mg/kg in refined oil. The high adsorption capacity of aluminum oxide, coming from its high affinity to tocopherols, was confirmed by Chu *et al*., (2005). These authors confirmed the higher tocopherol adsorption capacity of aluminum oxide in comparison to such adsorbents as synthetic brominated polyaromatic SP 207 and functionalized polyaromatic Dowex Optipore L-285. However, the content of sterols after purification (variant II) was higher (4900.0 mg/kg) compared to the use of variant I with silicic acid and activated charcoal (4130.0 mg/kg). The use of aluminum oxide was also effective in the case of β-carotene (decrease in concentration from 2.02 mg/kg to 1.68 mg/kg) and chlorophyll removal (not detected after purification process).

The third variant (III) used to remove antioxidants and other minor components from refined rapeseed oil was silica gel. Surprisingly, the concentration of sterols after elution from the silica gel column (7860.0 mg/kg) was higher than in the oil before purification (5840.0 mg/kg; table 1). This may be the result of the competition between triacylglycerols and long-chain phytosterol esters for binding to silica. According to Verleyen *et al*., (2002) more than 60% of the sterols present in refined rapeseed oil are in the esterified form. Ostlund *et al*., (2002) removed sterols from corn oil in their studies using column chromatography in 2-step purification on silica gel. In the first step, more polar than triacylglycerols, free sterols and ferulic acid esters of sterols were removed by diluting oil in hexane and passing it through a silica gel bed. In the second step, using a separate column packed with silica gel, the partially purified oil was passed through and purified from long chain sterol esters. The bed was saturated with triacylglycerols and long-chain sterol esters competing for binding to silica. Triacylglycerols were then eluted with a mixture of isopropanol and hexane. This may explain the high content of sterols in the purified oil in our experiment. During the purification on silica gel, the sterol esters were retained in the column as a result of affinity for the bed similar to the affinity of triacylglycerols. Concentrated in this way, the esters were then eluted with triacylglycerols, thereby their final concentration in the oil after purification was increased. Silica gel was more effective than silicic acid and activated charcoal in removing tocopherols. The content of those antioxidants after purification was determined at 85.2 mg/kg. Variant III also purified the oil of its β-carotene content to the greatest extent (0.96 mg/kg relative to 2.02 mg/kg in refined oil). As in the case of aluminum oxide no chlorophyll was detected in the oil after passing it through the column filled with silica gel (table 1).

The research conducted shows that none of the applied variants purified the oil of all amphiphilic compounds, antioxidants and pigments. Therefore, it was decided to use a combination of previously described types of beds, in order to obtain an optimized method for the removal of minor components. Therefore, a three-stage purification process was applied. First, oil dissolved in hexane was passed through columns filled with a combination of silicic acid and activated charcoal (as in variant I). The partially purified oil was then passed through a column with activated charcoal and aluminum oxide, which was used in variant II. Activated charcoal was added to remove the remains of pigments. The last step was to pass the purified oil from the two stages through a column filled with silica gel (as in variant III). After the three-stage purification process, the oil was subjected to a series of analyses, the results of which are presented in Table 2. The obtained results show that after the three-stage purification process, the oil contained traces of β-carotene (0.08 mg/kg compared to 2.02 mg/kg in refined oil), exceeding the purification efficiency of each of the three variants (1.39 – 0.96 mg of β-carotene/kg of the oil). As in variant I, II and III oil subjected to the three-stage process did not contain chlorophyll (Table 2).

Table 2. Contents of sterols, tocopherols, chlorophyll and β-carotene in purified oil using the three-stage purification method and in refined oil.

	Sample	
Determined compounds	**Purified rapeseed oil**	**Refined rapeseed oil**
Sterols (mg/kg)	4520.0 ± 120.0[a]	5840.0 ± 160.0[b]
Tocopherols (mg/kg)	0.4 ± 0.1[a]	630.2 ± 7.4[b]
Chlorophyll (mg/kg)	n.d.	0.04 ± 0.01
β-Carotene (mg/kg)	0.08 ± 0.02[a]	2.02 ± 0.11[b]

Values (means ± SD; n=3) with different index letters are statistically different at the significance level ($p < 0.05$). Student's t-test was applied.
n.d. – non detected

Table 3. Contents of tocopherol homologues in purified oil using the three-stage purification method and in refined oil.

Tocopherol homologues (mg/kg)	**Sample**	
	Purified rapeseed oil	**Refined rapeseed oil**
α-T	0.4 ± 0.1[a]	266.0 ± 1.4[b]
β-T	n.d.	n.d.
γ-T	n.d.	311.6 ± 3.5
δ-T	n.d.	52.6 ± 1.5
Sum of tocopherols	0.4 ± 0.1[a]	630.2 ± 7.4[b]

Values (means ± SD; n=3) with different index letters are statistically different at the significance level ($p < 0.05$). Student's t-test was applied.
n.d. – non detected

Similarly, the tocopherol content decreased significantly to 0.4 mg/kg compared to 630.2 mg/kg in refined oil. Two homologues of tocopherol present in refined rapeseed oil (γ-tocopherol (311.6 mg/kg) and δ-tocopherol (52.6 mg/kg)) were removed completely (table 3), while the content of α-tocopherol was reduced from 266.0 mg/kg to a very low level 0.4 mg/kg (figure 2). Interactions between tocopherols and the hydrophilic stationary phase may be explained using a normal phase HPLC chromatogram. In the chromatographic system consisting of a Si60 column (silica gel stationary phase) and hydrophobic mobile phase (a mixture of *n*-hexane with 1,4-dioxane (96:4 v/v)) during flaxseed oil tocochromanol analysis, the retention time of α-tocopherol was the shortest among all homologues of tocopherols (9.36 min compared to 14.29 min, 15.78 min and 23.39 min in the case of β-, γ- and δ-homologues, respectively (Siger *et al*., 2014)). This means that the interaction of α-tocopherol with silica gel was the weakest (among all homologues of tocopherols). Therefore, in the three-stage purification system analyzed, α-tocopherol, which interacted with the bed in a relatively weak manner, was not completely removed.

Table 4. Contents of free, esterified and total sterols in purified oil using the three-stage purification method and in refined oil.

	Sample	
Sterols in oil [mg/kg]	**Purified rapeseed oil**	**Refined rapeseed oil**
Free sterols	90.0 ±20.0[a]	2320.0 ± 210.0[b]
Esterified sterols	4430.0 ± 110.0[b]	3520.0 ± 140.0[a]
Total sterols	4520.0 ± 140.0[a]	5840.0 ± 130.0[b]

Values (means ± SD; n=3) with different index letters are statistically different at the significance level ($p < 0.05$). Student's t-test was applied.

The research shows that sterols are still the most problematic compound to remove from rapeseed oil. The content of sterols was decreased after the three-stage purification process to 4520.0 mg/kg (from 5840.0 mg/kg in refined oil). As stated above, sterols occur in free and esterified form in rapeseed oil. The majority of sterols are esters (4847.0 mg/kg), while the content of free forms is at the level of 2783.0 mg/kg in relation to their total content of 7671.0 mg/kg, which slightly changes depending on the type of raw material (Verleyen *et al*., 2002). From the point of view of the study of association colloids in bulk oils and their role in autoxidation, it is crucial to remove free sterols, which are amphiphilic compounds. That is why in our study free and esterified sterols were isolated from the oil after the three-stage purification process and determined according to the methodology proposed by Verleyen *et al.*, (2002). The results of the assays are shown in table 4. In the three-stage purification process only a small amount of sterols was removed (4520.0 mg/kg compared to 5840.0 mg/kg in refined oil; table 4). However, when analyzing the compositions of different forms of sterols in purified oil, esterified sterols predominated (4430.0 mg/kg), while the content of free sterols significantly decreased (90.0 mg/kg) compared to their content in refined oil (2320.0 mg/kg). As in the case of variant III (silica gel) the content of esterified sterols in oil after purification (4430.0 mg/kg) was higher than in refined oil (3520.0 mg/kg). This confirms the mechanism by which the sterol esters are retained and concentrated onto the column. In the interactions between sterols and chromatographic polar stationary phase (silica gel, aluminum oxide) hydrogen bonding and dipole-dipole interactions are involved (Akoh and Min, 2008). That is why amphiphilic free sterols with free hydroxyl groups (a hydrophilic moiety) are more strongly bound by the polar stationary phase than more hydrophobic esters (see Figure 1). Such interactions result in a significantly more effective removal of free sterols from the oil in the described process. Esterified sterols have hydrophobic properties similar to triacylglycerols (Figure 1) and most likely remain in

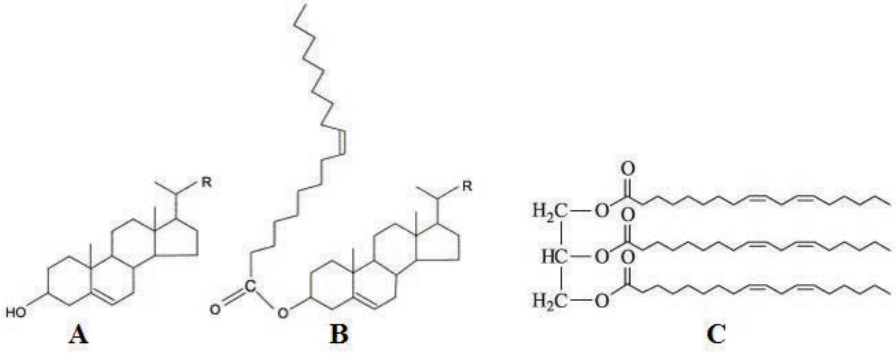

FIGURE 1. Basic structures of (A) free alcoholic sterol, (B) esterified form of sterol and (C) triacylglycerol (Toivo *et al*., 2000; Gomez *et al*., 2011).

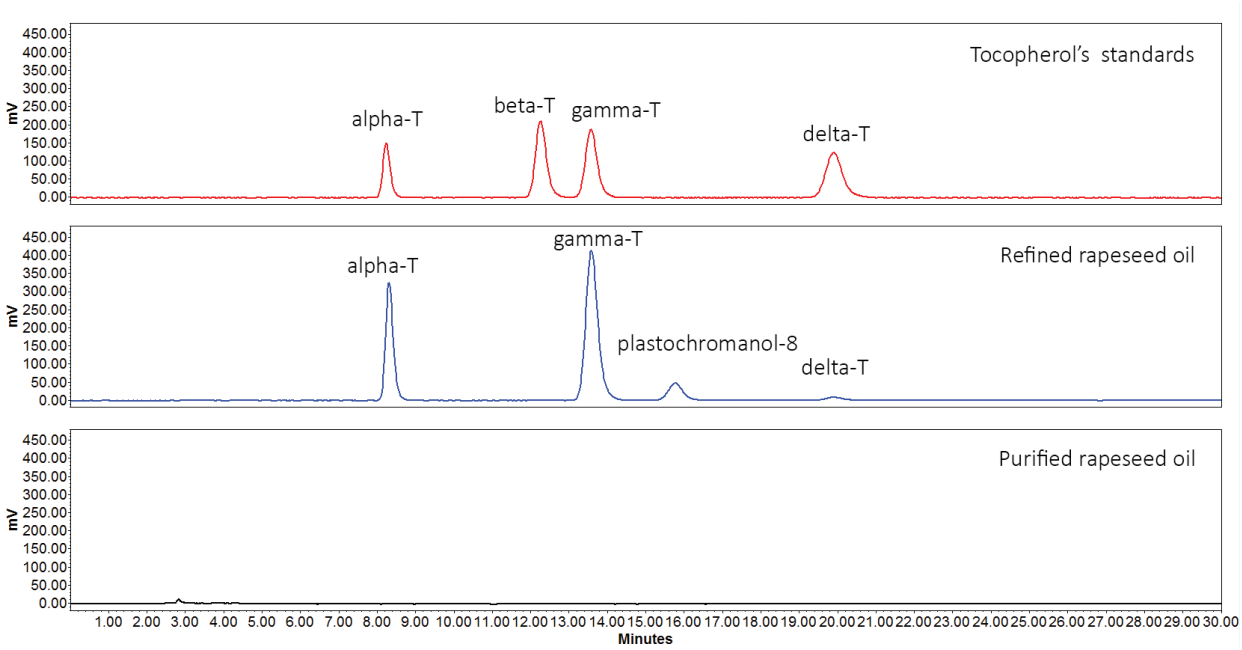

FIGURE 2. Chromatogram of tocopherol standards, and tocopherols in refined rapeseed oil and purified rapeseed oil.

the non-polar (lipid) phase of bulk oil containing small amounts of water and reverse micelles (or other association colloids). On the contrary, free sterols, which due to their amphiphilic nature accumulate on the interface between oil and water, take part in the formation of association colloids. For this reason, the removal of free sterols from the oil is crucial in model studies on the effects of association colloids on the autoxidation of lipids. The three-stage purification process developed in this work is therefore useful in removing sterols from bulk oil in this type of research.

In order to check whether the purification process affected the quality of the oil, peroxide value and acid value were determined after the three-stage process. These parameters are a measure of oxidative and hydrolytic rancidity. The peroxide value and acid value of the purified oil were 0.62 mEq O_2/kg and 0.09 mg KOH/g, respectively. These parameters are much lower than the values accepted for refined oil by the international food standard Codex Alimentarius (10 mEq O_2/kg and 0.6 mg KOH/g, respectively). The low contents of peroxides and hydroperoxides as well as free fatty acids in the analyzed oil are beneficial from the point of view of its use in research concerning association colloids because these compounds are amphiphilic substances which affect the structure and properties of reversed micelles. Another important factor from the point of view of the formation of association colloids is the water content in oil. Using the Karl Fischer method, its content in oil after purification was found to be 322 ppm. This value did not differ substantially from the average value recorded in refined vegetable oils (300 ppm); (Budilarto and Kamal-Eldin, 2015).

4. CONCLUSIONS

There is no single universal bed able to remove all the amphiphilic substances present in rapeseed oil. In this study different chromatographic adsorbents (silicic acid, activated charcoal, aluminum oxide, silica gel) were used to purify oil of minor components including amphiphilic compounds, antioxidants and pigments. Silica gel proved to be the most effective bed during the removal of β-carotene, while chlorophyll was completely removed by all types of adsorbents. Rapeseed oil was almost fully purified of tocopherols (to the level 2.6 mg/kg) using aluminum oxide. All the analyzed beds adsorbed the sterol esters relatively weakly, and in the case of silica gel, the concentration of these substances on the bed was observed, which resulted in an increase in their content in the oil. Finally, the three-stage purification process, with the combination of all the beds described above was applied. In this process free sterols were almost completely removed (to the level 90.0 mg/kg). The removal of these compounds from the oil is crucial in model studies on the effects of association colloids on the autoxidation of lipids. This is due to their amphiphilic nature resulting in their accumulation on the interface between oil and water and the formation of reversed micelles. Three-stage purification was also very effective in removing β-carotene and chlorophyll. Tocopherols were completely removed with this method, except for small amounts of α-tocopherol (0.4 mg/kg), which resulted from its relatively weak interaction with a hydrophilic bed.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support from the National Science Centre, Poland, under grant NCN 2016/23/B/NZ9/02793.

REFERENCES

Akoh CC, Min DB. 2008. Food Lipids: Chemistry, Nutrition, and Biotechnology, Third Edition (Food Science and Technology), 3rd ed. Taylor & Francis Group, Boca Raton, London, New York, pp. 119-120

Boon CS, Xu Z, Yue X, McClements DJ, Weiss J, Decker EA. 2008. Factors affecting lycopene oxidation in oil-in-water emulsions. *J. Agric. Food Chem*. **56**, 1408-1414. https://doi.org/10.1021/jf072929+

Buczek B, Chwiałkowski W. 2005. Oczyszczanie oleju po smażeniu potraw z ryb za pomocą utlenionego węgla aktywnego oraz krzemianu magnezu. *Zeszyty Naukowe / Akademii Ekonomicznej w Krakowie* **689**, 17-36.

Budilarto ES, Kamal-Eldin A. 2015. The supramolecular chemistry of lipid oxidation and antioxidation in bulk oils. *Eur. J. Lipid Sci. Technol*. **117**, 1095-1137. https://doi.org/10.1002/ejlt.201400200

Chaiyasit W, Elias RJ, McClements DJ, Decker EA. 2007. Role of physical structures in bulk oils on lipid oxidation. *Crit. Rev. Food Sci.* **47**, 299-317. https://doi.org/10.1080/10408390600754248

Chen B, Han A, Laguerre M, McClements DJ, Decker EA. 2011a. Role of reverse micelles on lipid oxidation in bulk oils: impact of phospholipids on antioxidant activity of α-tocopherol and trolox. *Food Funct.* **2**, 302–309. https://doi.org/10.1039/c1fo10046g

Chen B, Han A, Mcclements DJ, Decker EA. 2010. Physical structures in soybean oil and their impact on lipid oxidation. *J. Agric. Food Chem*. **58**, 11993-11999. https://doi.org/10.1021/jf102763p

Chen B, McClements DJ, Decker EA. 2011b. Minor components in food oils: a critical review of their roles on lipid oxidation chemistry in bulk oils and emulsions. *Crit. Rev. Food Sci.* **51**, 901–916. https://doi.org/10.1080/10408398.2011.606379

Chu BS, Baharin BS, Che Man YB, Quek SY. 2005. Comparison of selected adsorbents for adsorption and desorption of vitamin E from palm fatty acid distillate. *J. Food Lipids*. **12**, 23-33. https://doi.org/10.1111/j.1745-4522.2005.00003.x

Chwiałkowski W. 2013. Zastosowanie mieszaniny węgli aktywnych o różnym charakterze powierzchni do oczyszczania oleju posmażalniczego. *Inżynieria i Ochrona Srodowiska* **16**, 361-371.

Čmolík J, Pokorný J. 2000. Physical refining of edible oils. *Eur. J. Lipid Sci. Tech.* **102**, 472–486. https://doi.org/10.1002/1438-9312(200008)102:7<472::AID-EJLT472>3.0.CO;2-Z

Cui L, Kittipongpittaya K, McClements DJ, Decker EA. 2014. Impact of phosphoethanolamine reverse micelles on lipid oxidation in bulk oils. *J. Am. Oil Chem. Soc.* **91**, 1931-1937. https://doi.org/10.1007/s11746-014-2544-9

Ghazani SM, Marangoni AG. 2013. Minor components in canola oil and effects of refining on these constituents: a review. *J. Am. Oil Chem. Soc.* **90**, 923-932. https://doi.org/10.1007/s11746-013-2254-8

Gomez NA, Abonia R, Cadavid H, Vargas IH. 2011. Chemical and spectroscopic characterization of a vegetable oil used as dielectric coolant in distribution transformers. *J. Brazil. Chem. Soc*. **22**, 2292-2303. https://doi.org/10.1590/S0103-50532011001200009

Homma R, Suzuki K, Cui L, McClements DJ, Decker EA. 2015. Impact of association colloids on lipid oxidation in triacylglycerols and fatty acid ethyl esters. *J. Agric. Food Chem*. **63**, 10161-10169. https://doi.org/10.1021/acs.jafc.5b03807

Kachel-Jakubowska M, Kraszkiewicz A, Lorencowicz E, Koszel M, Przywara A. 2015. Effects of thermal treatment of seeds on quality and oxidative stability of oils. *Agric. Agric. Sci. Proc.* **7**, 255-259. https://doi.org/10.1016/j.aaspro.2015.12.041

Khuwijitjaru P, Yuenyong T, Pongsawatmanit R, Adachi S. 2009. Degradation kinetics of gamma-oryzanol in antioxidant-stripped rice bran oil during thermal oxidation. *J. Oleo Sci*. **58**, 491-497. https://doi.org/10.5650/jos.58.491

Kittipongpittaya K, Panya A, Cui L, McClements DJ, Decker EA. 2014. Association colloids formed by multiple surface active minor components and their effect on lipid oxidation in bulk oil. *J. Am. Oil Chem. Soc.* **91**, 1955-1965. https://doi.org/10.1007/s11746-014-2541-z

Kittipongpittaya K, Panya A, Decker EA. 2016. Role of water and selected minor components on association colloid formation and lipid oxidation in bulk oil. *J. Am. Oil Chem. Soc*. **93**, 83–91. https://doi.org/10.1007/s11746-015-2752-y

Laguerre M, Chen B, Lecomte J, Villeneuve P, McClements DJ, Decker EA. 2011. Antioxidant properties of chlorogenic acid and its alkyl esters in stripped corn oil in combination with phospholipids and/or water. *J. Agric. Food Chem*. **59**, 10361-10366. https://doi.org/10.1021/jf2026742

Nyström L, Achrenius T, Lampi A-M, Moreau RA, Piironen V. 2007. A comparison of the antioxidant properties of steryl ferulates with tocopherol at high temperatures. *Food Chem*. **101**, 947-954. https://doi.org/10.1016/j.foodchem.2006.02.046

Ostlund RE, Racette SB, Okeke A, Stenson WF. 2002. Phytosterols that are naturally present in commercial corn oil significantly reduce cholesterol absorption in humans. *Am. J. Clin. Nutr*. **75**, 1000-1004. https://doi.org/10.1093/ajcn/75.6.1000

Rajczykowski K, Loska K. 2016. Heavy metals adsorption on aluminium oxide and stabilization of spent adsorbent using a SULSTAR technology. *Archives Waste Manag. Envir. Protect.* **18**, 67-72.

Ravindhranath K, Ramamoorty M. 2017. Nano aluminum oxides as adsorbents in waterremediation methods: a review. *Rasayan J. Chem*. **10**, 716-722. https://doi.org/10.7324/RJC.2017.1031762

Rotkiewicz D, Konopka I, Tańska M. 2002. Barwniki karotenoidowe i chlorofilowe olejów roślinnych oraz ich funkcje. *Rośliny Oleiste -Oilseed Crops* **XXIII**, 563-579.

Siger A, Dwiecki K, Borzyszkowski W, Turski M, Rudzińska M, Nogala-Kałucka M. 2017. Physicochemical characteristics of the cold-pressed oil obtained from seeds of *Fagus sylvatica* L. *Food Chem.* **225**, 239-245. https://doi.org/10.1016/j.foodchem.2017.01.022

Siger A, Kachlicki P, Czubiński J, Polcyn D, Dwiecki K, Nogala-Kałucka M. 2014. Isolation and purification of plastochromanol-8 for HPLC quantitative determinations. *Eur. J. Lipid Sci. Tech*. **116**, 413-422. https://doi.org/10.1002/ejlt.201300297

Siger A, Kaczmarek A, Rudzińska M. 2015. Antioxidant activity and phytochemical content of cold-pressed rapeseed oil obtained from roasted seeds. *Eur. J. Lipid Sci. Tech*. **117**, 1225-1237. https://doi.org/10.1002/ejlt.201400378

Toivo J, Lampi A-M, Aalto S, Piironen V. 2000. Factors affecting sample preparation in the gas chromatographic determination of plant sterols in whole wheat flour. *Food Chem.* **68**, 239-245. https://doi.org/10.1016/S0308-8146(99)00201-0

Verleyen T, Forcades M, Verhe R, Dewettinck K, Huyghebaert A, De Greyt W. 2002. Analysis of free and esterified sterols in vegetable oils. *J. Am. Oil Chem. Soc*. **79**, 117-122. https://doi.org/10.1007/s11746-002-0444-3

Xenakis A, Papadimitriou V, Sotiroudis TG. 2010. Colloidal structures in natural oils. *Curr. Opin. Colloid In. Sc*. **15**, 55-60. https://doi.org/10.1016/j.cocis.2009.11.007

Yoshida H, Kondo I, Kajimoto G. 1992. Participation of free fatty acids in the oxidation of purified soybean oil during microwave heating. *J. Am. Oil Chem. Soc*. **69**, 1136-1140. https://doi.org/10.1007/BF02541050