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Purification of Squalene and Tocopherols from Olive Oil Deodorized Distillate

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Abstract

Olive oil deodorized distillates are rich in 3-5% tocopherol and 8-10% squalene. This study used the saponification technique, one of the pre-separation techniques, to purify tocopherol and squalene, which are found in high amounts in deodorized distillates. The saponification process removed 70% of free fatty acids, and 9-10% tocopherol and 17-18% squalene were purified. Tocopherol and squalene obtained after the saponification process were enriched using semi-preparative chromatography. 6 different fractions were obtained. While the second fraction contained high amounts of tocopherol and squalene, it was seen that the third fraction contained squalene and other compounds separated from tocopherols. As a result, 31-32% tocopherol and 36-37% squalene were obtained.

Keywords: Deodorized distillate, tocopherols, squalene, semi-preparative chromatography

1. Introduction

Olive oil is a highly valuable product due to its organoleptic properties and known benefits to human health. The size of the olive oil industry is of great importance for Türkiye, which is among the leading countries in olive oil production, both economically and socially. Millions of tons of olive oil, unsuitable for direct use in our country, are made suitable for consumption through physical or chemical refining methods. During these processes, important by-products are formed at various stages. Therefore, technological advancements are needed to re-evaluate these by-products obtained during refining [1].

Due to the high temperatures reached during the deodorisation stage of the refining process for olive oil, deodorised distillates containing rich by-products are separated from the oil. These products contain valuable bioactive compounds such as tocopherol derivatives, sterol derivatives, and squalene, which are widely used in the food, cosmetic, and health industries. These important bioactive components are significant for their ease of identification regarding type and quantity within the distillate and for improving recycling methods and converting them into high-value-added products [1-4].

The most important bioactive compounds with high antioxidant capacity in olive oil deodorized distillate are tocopherols. Tocopherols protect cell membranes, provide vessel fluidity, and help prevent atherosclerosis. The tocopherol content consists of two main groups: the chromanol ring and the hydrophobic side chain. Tocopherol has a saturated isoprenoid side chain, while tocotrienol contains isoprenyl side chains with three double bonds. Each group has structures (α-, β-, γ-, and δ-) which differ in the number and position of methyl substituents in the chromanol ring. α-Tocopherol is the first homolog exhibiting biological activity, in contrast to the other homologs (β,

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γ, δ), which show reduced activity. γ-Tocopherol has only 10% of the activity of α-tocopherol and 1% of the activity of α-tocopherol. Due to having more hydroxyl groups in its structure than other tocopherols, α-tocopherol is extremely sensitive to various oxidative effects and is reported to be superior to other derivatives regarding antioxidant activity [5-8].

With these antioxidant properties, tocopherols are an important preventative measure against some chronic diseases, such as cardiovascular diseases and cancer. In a clinical study, it was found that a high dose (<67 mg of α-tocopherol) of vitamin E, used continuously for at least two years, reduced the risk of coronary heart disease by approximately 31-65% [9].

In a study aimed at determining the amount of tocopherol in olive oil deodorized distillate, the total tocopherol content was identified as 32,000 ppm (3.2%), with the quantity of alpha-tocopherol measured at 15,000 ppm (1.5%) [10].

The most important hydrocarbon derivatives found in olive oil are squalene and β-carotene. Squalene is the main component of the unsaponifiable part of olive oil and accounts for more than 90% of the total hydrocarbon content. During the refining of olive oil, squalene is primarily removed from the oil, especially during the deodorization stage, and ends up in the deodorised distillate (DD). Squalene, which is an important element of the immune system, is used as an immune booster in vaccines. Its commercial production is primarily derived from shark liver, a valuable resource [1, 2].

The presence of squalene in olive oil significantly contributes to its health effects. Studies have indicated that olive oil consumption reduces the risk of pancreatic cancer and lowers the risk of developing breast cancer in women who consume olive oil. Research suggests that the protective effects of extra virgin olive oil are due to its high squalene content, supported by numerous animal studies. The most comprehensive among these studies investigated the effects of squalene on skin, colon, and lung cancer in mice. Considering all this, the daily intake of squalene has a distinctly anti-carcinogenic effect. In addition to its anti-carcinogenic properties, squalene has been found to offer other health benefits [11, 12]. In another study, the squalene levels in olive oil deodorized distillate were reported as 85,000 ppm (8.5%) [10].

One study involving saponification reported that the unsaponifiable substances contained high amounts of tocopherols (19,600 ppm), phytosterols (5,500 ppm), and squalene (323,000 ppm) [17].

The preparative chromatography technique, which is widely used in many sectors, particularly in the food, cosmetics, and pharmaceutical industries, is frequently employed in recovery studies to separate and purify materials with high economic value. In one study, tocopherols and tocotrienols were separated from deodorized distillate using a semi-preparative HPLC technique. From the results obtained from the experiments, fractions α-tocopherol and α-tocotrienol were successfully purified without saponification in the examination made in corn oil deodorized distillate [24].

As a result, a new technique has been developed for purifying valuable bioactive compounds, specifically tocopherols and squalene, in deodorized distillates. The saponification method separated squalene and tocopherols in DD from free fatty acids. The extract obtained was processed using the semi-preparative chromatography technique to achieve a high-purity product. Additionally, bioavailability studies will be conducted to convert the purified bioactive compounds into commercial products, resulting in a high-added-value final product.

2. Materials and Methods

This study used deodorized distillates obtained from the chemical refining of olive oils by Agricultural Credit Oil Industry and Trade.

2.1. Determination of Type of Glyceride

The International Union of Pure and Applied Chemistry (IUPAC) technical report was used to determine the type of glyceride in deodorised distillate. 1 g of sample was dissolved by adding 10 mL of tetrahydrofuran (THF), passed through a 25 mm PTFE syringe filter, filled into capped glass vials with a volume of 1.5 mL and made ready for analysis [1].

Table 1. Chromatographic conditions for the determination of polar and non-polar substances.

Stationary Phase (Column)	One guard column and two serially connected 30x7.7 mm gel columns: 500Å-100Å
Mobile Phase	THF
Mobile Phase Flow Rate (mL/min)	1.0
Detection	RID 35 °C
Analysis Time (min)	20
Injection Amount (µL)	20
Column Oven Temperature (°C)	30

2.2. Determination of Free Fatty Acids Composition

In determining deodorized distillate fatty acid compositions, fatty acids found in the triglyceride structure and not volatile were subjected to esterification pretreatment according to EC method 14105 to be analyzed by GC technique. According to the relevant method, approximately 0.1 g of the sample to be analysed was weighed into a suitable and clean tube and 0.1 mL of 2 M methanolic KOH solution was added. In this way, KOH decomposed TGs into fatty acids. Fatty acids that became potassium salts combined with methanol and formed methyl esters. 10 mL of hexane was added, shaken for 30 minutes, passed through a 25 mm polytetrafluoroethylene (PTFE) syringe filter, and filled into capped glass vials with a volume of 1.5 mL and made ready for analysis. Fatty acid composition analyses of samples converted to methyl ester derivatives were performed on the Agilent 7890 GC device, which can perform automatic analysis and has an FID detector [1].

Table 2. Chromatographic conditions for the determination of FAME (Kara 2008).

Stationary Phase (Column)	Agilent HP-88 (100m, 0.25mm, 0.25 µm)		
Mobile Phase	He		
Mobile Phase Flow Rate (mL/min)	1.3		
Detection	FID Temperature: 250 ºC, Dry air: 300 mL/min, Hydrogen: 30 mL/min, Make up (He): 10 mL/min		
Analysis Time (min)	59.50		
Split	Splitless		
Injection Amount (µL)	1		
Column Oven Temperature	ºC/min	ºC	min
		50	2
	4	240	10

2.3. Determination of Tocopherol and Tocotrienol

The analysis was performed to determine the amounts of α, β, γ, and δ tocopherol and tocotrienol (tocopherols) in the deodorized distillate including the determination of 1 g of oil sample dissolved in 10 mL of hexane by HPLC technique. Tocopherol-tocotrienol analyses were performed on the diol column in the Agilent 1200 HPLC device using crude pomegranate seed oil dissolved in hexane. In chromatographic separations, β- and γ tocopherol separations, which were very close to each other, were particularly considered [1].

Table 3. Chromatographic conditions for the determination of tocopherol and tocotrienol.

Stationary Phase (Column)	Lichrospher 100 5µm 25x0.4
Mobile Phase	%99 Hekzan-%1 İzopropil alkol
Mobile Phase Flow Rate (mL/min)	1.0
Detection	FLD 30 ºC
Analysis Time (min)	30
Injection Amount (µL)	20
Column Oven Temperature	30

2.4. Determination of Volatile Components

Improvements were made to the method by considering the methods in the literature to determine volatile components with GC-MS. According to the relevant process, 100 mg of the sample was taken and dissolved in 2 mL of acetone. The prepared solutions were mixed with a vortex for 1 min, passed through a 0.45 µm PTFE syringe filter, filled into capped glass vials with a volume of 1.5 mL, and made ready for analysis [27].

Table 4. Gas Chromatography Mass Spectrometry (GC-MS) Volatile Component Method Conditions

Injector		**Carrier Gas**		**Column Oven**			**MS Temperature**	
Mod	Temperature	Type	Flow Rate	°C/min	°C	min	Source	Quadropole
1µL Pulsed Split (50:1)	180 °C	He	0.7 mL/min	 10 20	50 200 280	2 10 2	230 °C	150 °C
Stationary Phase (Column)		HP-5 MS (5%-Phenyl methylpolysiloxan)						

2.5. Saponification

DD samples (100 g) were saponified with 50 mL of ethanol and excess NaOH and calculated according to the saponification number. The solution was heated at 80 °C for 60 min. After saponification, the samples were allowed to cool to room temperature and transferred to a separatory funnel. The unsaponifiable fraction was collected by adding 100 mL of distilled water and 200 mL of n-hexane [1, 25-28].

2.6. Purification of Squalene and Alpha Tocopherol by Semi-Preparative Chromatography

A semi-preparative chromatographic system separated valuable bioactive compounds in the deodorised distillate. In line with the optimum method determined by the chemometric design for analytical HPLC analyses, some optimisation experiments were performed on the deodorised distillate sample to find the new optimum parameter for scaling to the semi-preparative HPLC system. The parameters used in the experiments are given in Table 5 [24].

Table 5. Optimization parameters for the Semi-Preparative column

Stationary Phase (Column)	Semi-Preparative column Silicycle CN 120Å 10 µm (250x10)
Mobile Phase	%99.18 Hekzan-%0.82 İzopropil alkol
Mobile Phase Flow Rate (mL/min)	1.5
Detection	FLD 30°C
Analysis Time (min)	30
Injection Amount (µL)	100
Column Oven Temperature	15

3. Results

Free fatty acids were removed using saponification, and the product was extracted with an extraction system. The obtained extract was separated and recovered from tocopherol and squalene in deodorized distillates using a semi-preparative HPLC technique.

3.1. Determination of Type of Glyceride

Within the scope of the study, triglyceride analyses of deodorised distillate sample (blue line), stearic acid (red line) and sunflower oil samples (green line) for comparison were performed using a standard liquid chromatography method. In the triglyceride analyses performed, it was observed

that there was a small amount of triglyceride in the deodorized distillate sample. A small amount of triglyceride is expected to be carried into the distillates as a requirement of the deodorisation process. Monoglyceride and diglyceride structures were not encountered in the analysis.

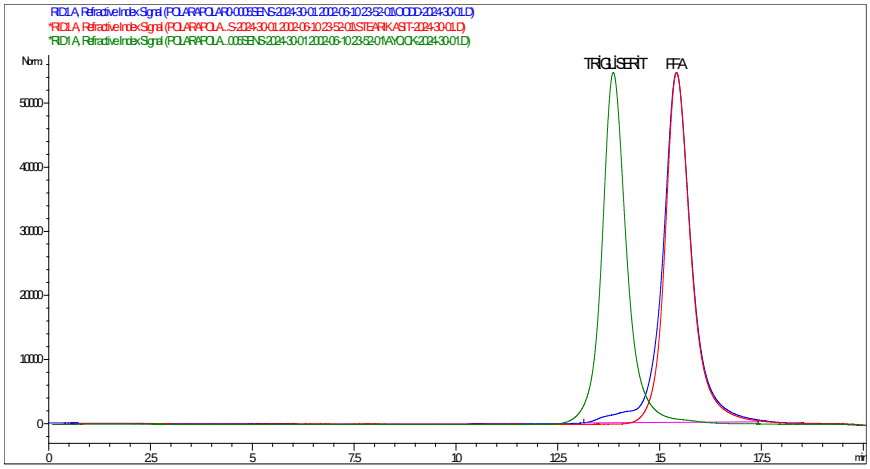

Figure 2. Comparison chromatograms of polar and non-polar substance analysis of deodorized distillate.

3.2 Determination of Free Fatty Acids Composition

The fatty acid composition analyses of the deodoriser distillate samples were examined according to the Kara.M method, and the chromatogram of the fatty acid composition was given.

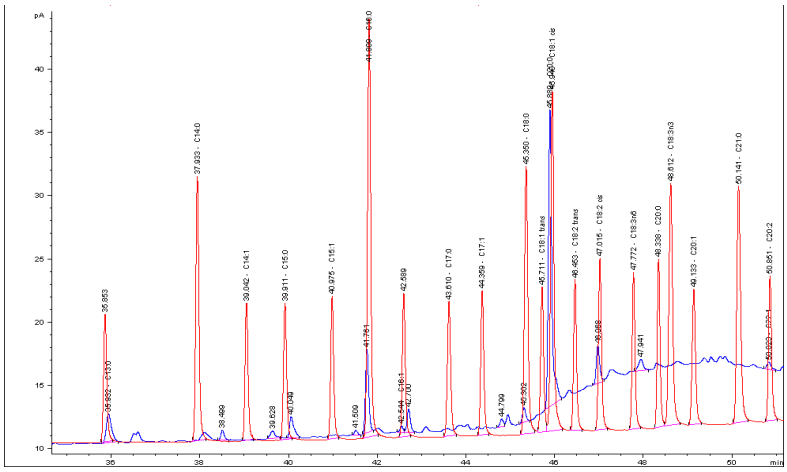

Figure 3. Fatty acid composition analysis comparison chromatography

3.3 Determination of Tocopherol and Tocotrienol

The standard liquid chromatography method was used in the studies conducted to determine the amount of tocopherol-tocotrienol in deodorised distillate samples. Deodorised distillates of edible oils containing high tocopherol content are especially rich in tocopherol. The total tocopherol amount of deodorized distillate samples used in the study was 32,306 mg/kg. The highest value was from α-tocopherol, known for its vitamin E activity at 15,328 mg/kg and β+γ-tocopherol at 16,733 mg/kg.

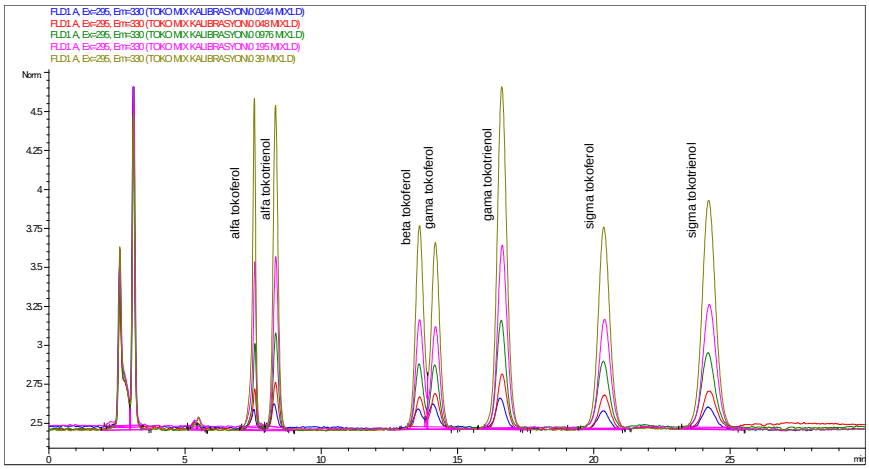

Figure 4. Chromatography analysis of tocopherol and tocotrienol of deodorized distillate.

3.4. Determination of Volatile Components

Valuable bioactive compounds such as squalene also pass into the deodorized distillate. The squalene content was determined by GC-MS.

3.5. Saponification

DD samples (100 g) were saponified, and the amount of free fatty acids in the distillate was found to be approximately 65%. The saponification process removed more than 70% of the free fatty acids.

3.6. Purification of Squalene and Alpha-Tocopherol by Semi-Preparative Chromatography

A semi-preparative chromatography system separated valuable bioactive compounds in the deodorised distillate. 6 different fractions were collected and analysed. Tocopherol and squalene were found to be relatively pure in the second fraction. High levels of squalene were detected in the third fraction.

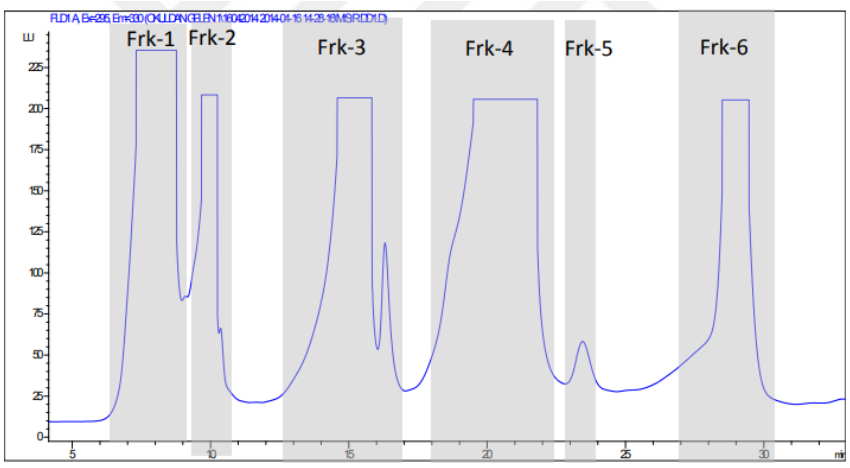

Figure 5. Fraction chromatogram of deodorized distillate

4. Discussion

The most important and completely unpreventable losses in the vegetable oil sector are the bioactive components that occur during the deodorization process. Unfortunately, it is impossible to completely prevent these losses, which can be reduced to some extent by improving the deodorization process. For this reason, deodorized distillates, which are considered by-products of the deodorization step, must be evaluated effectively. Tocopherols and squalene are valuable natural antioxidants. Natural vitamin E is a structure consisting of α, β, γ, and δ tocopherols and their tocotrienols. Each of these eight vitamin E and squalene components has different antioxidant and biological activities.

Before saponification, olive oil DD samples were analyzed for glycerides, FAME, tocopherol, and squalene content. Afterwards, saponification was performed to remove free fatty acids (FFA), resulting in a product rich in squalene and tocopherol. The final product was obtained through semi-preparative chromatography to recover squalene and tocopherol at high purity, which can be utilised in the cosmetics, food, and pharmaceutical industries (see Table 6). In a study aimed at determining the amount of OODD tocopherol, the total content was 3.2%, with alpha-tocopherol measured at 1.5%. The amounts detected before the saponification process were found to be similar. Additionally, the squalene content was 8.5% [10].

Among tocopherols, the maximum concentration of alpha-tocopherol (1.7-3.9%) was found in all analysed DD samples in the results obtained after the saponification process. The results obtained for tocopherols (8.5-8.9%) [14, 16] and squalene (15.2-15.7%) [16, 28] were quite similar to the literature data.

Table 6. Comparison of bioactive species of the OODD sample (%)

	OODD	After the saponification process	After semi-preparative chromatography
Tocopherols	3-4%	9-10%	31-32%
Squalen	8-9%	17-18%	36-37%

Conclusions

As can be understood from these results, tocopherols types and squalene, which have a high market share and can be used as natural antioxidants and vitamins in the cosmetics, pharmaceutical, and food sectors, can be extracted from deodorized distillates, which are refining wastes, by enriching them with saponification process and by supercritical fluid extraction system. Their recovery can be achieved in larger amounts by semi-preparative HPLC.

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