# Fatty acids, sterols and triglycerides composition of cold pressed oil from pomegranate seeds

**Osman KOLA**[1,*]
**Erva PARILDI** [1]
**Murat Reis AKKAYA** [1]
**Bahri Devrim ÖZCAN** [2]
**Emrah MAMUR** [3]
**Burçak TÜRKER** [1]

[1] Department of Food Engineering, Faculty of Engineering, Adana Alparslan Türkeş Science and Technology University Sarıçam/Adana-Turkey

[2] Department of Animal Science, Faculty of Agriculture, Çukurova University, Sarıçam/Adana-Turkey

[3] T.C. Trade Ministry, Middle Mediterranean Management, Mersin Laboratory Management Akdeniz/Mersin-Turkey

*CORRESPONDING AUTHOR*
*Email: okola@atu.edu.tr*
*Tel.: +905333953603*

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Pomegranate seed oil (PSO) was obtained by cold pressing of seeds of pomegranate (*Punica granatum* L.) grown in Turkey. Total crude oil was obtained from pomegranate seeds on a 14.62% dry matter (DM) and it was determined to having a high content of polyunsaturated fatty acids (87.82%). The dominant fatty acid in pomegranate seeds was determined to be punicic acid (C18:3n-5; 9c, 11t, 13c) and it was found that it constitutes about 76.14% of the total fat content. It was observed that the total tocopherol content of PSO was 258.40 mg/100g and the main tocopherol was α-tocotrienol and the others were α-tocopherol (189.89 mg/100g) and γ-tocopherol (53.14 mg/100g), respectively. The total sterol content of PSO was 930 mg/100g with a predominance of β-sitosterol in our study and the β-sterol content was 87.32%. The major triacylglycerols (TAG) in pomegranate seed oil were determined to be Stearic-Punicic-Punicic acid (SPuPu; 4.34%), Oleic-Punicic-Linoleic acid (OPuL; 1.10%) and Palmitic-Punicic-Linoleic acid (PPuL; 0.71%), respectively.

**Keywords**: Cold press, Fatty acid composition, Pomegranate, Punicic acid, Sterol, Tocopherol

## 1. INTRODUCTION



Pomegranate is one of the most known and most consumed fruits due to its high antioxidant properties. Pomegranate (*Punica granatum* L.) is a perennial herb relating to the genus *Punica* of *Punicoideae* sub family of the *Lythraceae* family. There are two varieties of *Punica* family. These are *Punica protopunica* and *Punica granatum*. The origin of pomegranate comes from a large region extending from India to Iran and the fruit has been grown for centuries especially in the Mediterranean region [1].
The pomegranate fruit consists of three main parts as peel, juicy testa (arils) and kernel. Pomegranate arils are the edible part of the fruit and these arils are covered with a thin membrane consist of the juice and seed. Pomegranate arils amount to about 52% of the whole fruit and a single pomegranate aril consists of 78% fruit meat and 22% seed. Pomegranate is rich in soluble solids, sugars, organic acids, phenolics, pigments, dietary fibre, and lipids. As it can be used in the production of pomegranate juice, sour sauce and molasses, oil is also produced from the wastes of these products and from the seeds [2, 3].
Pomegranate seed oil contains between 12% and 20% oil on a dry matter basis [3]. The antioxidant capacity of pomegranate seed oil is lower than the peel, but it is higher than the fruit juice [4]. Pomegranate seed oil contains significant amounts of polyunsaturated fatty acids (PUFA), including conjugated linolenic acid (CLnA) isomers, also known as Punicic acid [3, 5, 6]. Punicic acid (C18:3n-5; 9c, 11t, 13c), also called trichosanic acid, is an omega-5 (ω-5, n-5) long chain polyunsaturated fatty acid and conjugated α-linolenic acid (CLnA)

isomer [3]. Conjugated linolenic acid (CLnA) has positive biological effects on human health. Triglycerides and fatty acids are the richest energy sources in nutrition [1].
Pomegranate is a fruit of which production and consumption is constantly increasing due to its antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal and antiproliferative effects. In many of the previous studies, pomegranate's antioxidant capacity was reported as higher than many fruits. Some of the components responsible for the anticancer and anti-inflammatory effect of pomegranate seeds are tocols (tocopherols and tocotrienols), phytosterols (ergosterol, campesterol, stigmasterol, β-sitosterol), hydroxybenzoic acids (gallic, elagic acid), hydroxycinnamic acids (e.g., caffeic acid). Phytosterols are substances effective on human health, especially due to their cholesterol absorption inhibitory effects. These substances act in the intestines and improve the HDL/LDL ratio [7].
Several studies have been previously published on tocopherols [2, 8-11], sterols [2, 7, 10-12] and triacylglycerols [1, 13, 14] of pomegranate seed and especially pomegranate seed oil. The aim of the present work is to determine the tocols, fatty acid compositions, sterols and triacylglycerols of pomegranate seed oil. The fatty acid compositions and sterols were analysed by gas chromatography (GC), tocopherols and glyceride content were analysed by High Performance Liquid Chromatography (HPLC) on a reversed phase column and some physicochemical properties (oil yield, refractive index and colour) were studied.



## 2. MATERIALS AND METHODS

### 2.1. MATERIALS

Pomegranate seeds were obtained from Göknur Gıda A.Ş. (Adana, Turkey). The seeds were separated from the fruits during the pomegranate juice production and dried in a belt dryer continuously working at about 60°C after cleaning the surfaces in a brushing machine.
All analysis were repeated three times with the same pomegranate seed oil. Data analysis statistics were performed using Microsoft Excel 2016 and the values were given as a mean and standard deviation.

### 2.2. CHEMICALS AND REAGENTS

A standard mixture of 37 fatty acid methyl esters (FAMEs) was bought from Sigma-Aldrich (St. Louis, MO, USA). Betulin (Lup-20(29)-ene-3β, 28-diol), an internal standard with a purity of >98%, was purchased from Sigma Chemical Co. (St. Louis, MO, USA).
All organic solvents were HPLC-grade and purchased from Delta Chemicals (Adana, Turkey). All other chemicals were used as an analytical quality reagent and purchased from Delta Chemicals (Adana, Turkey). The research utilised the Elga Purelab OptionQ water system (Anamed Analytical Team, Turkey).

### 2.3. OIL EXTRACTION

Pomegranate seed oil extraction was performed by a cold press oil machine (Karaerler brand NF500 model, Turkey). The cold press oil machine was preheated, and the temperature was set to 100°C. The pomegranate seeds were pressed at a frequency of 15 Hz at 45°C. The temperature of the pomegranate seed oil obtained from the cold press machine was provided to be below 45°C by means of a recycling cooling system (4°C).

### 2.4. REFRACTIVE INDEX

The refractive index of the pomegranate seed oil was determined using a Digital Abbe refractometer (Krüss AR2008, Germany), and given as nD 20°C.

### 2.5. TOCOPHEROLS

Tocopherol analyses of pomegranate seed oil were performed by the method of Beyhan et al. 2017. Tocopherol analysis, alpha (α) tocopherol, gamma (γ) tocopherol and beta (β) + delta (δ) tocopherol, were performed on HPLC equipped with 3 µm C18 reversed-phase column (15 cm × 4.6 mm, Spherisorb ODS2, Phase Separation, Clwyd, UK). A mixture of methanol/water (97:3 v/v; 1.05 mL/min) was used as mobile phase. During the first 5 minutes, an excitation of 325 nm and an emission of 490 nm of retinol were followed by a stimulation of 295 nm and an emission of 330 nm with fluorescence detector [15].

### 2.6. FATTY ACID COMPOSITION

Fatty acid composition of the pomegranate seed oil samples was determined by modifying the analytical method described by TS EN ISO 12966-2 and Method 4 [16, 17]. Esterification of fatty acids was performed before being analysed by vortexing a solution of 0.1 g of oil and 10 mL of n-heptane with 0.5 ml of methanolic KOH solution. The test tube was shaken with vortex for 30 seconds and then centrifuged at 4000 rpm for 10 minutes. The clear phase was filtered with a 0.22 mm polyamide nylon syringe and injected into 2 mL vials. Gas chromatography analysis was carried out using an Agilent 7820A GC with a flame ionisation detector (FID). The fatty acid methyl esters (FAMEs) analysis was performed on an HP-88 Column (100m × 250µm × 0.25µm). The initial oven temperature was gradually increased starting from 50°C. It was increased at a ratio of 10°C/min to 175°C and preserved at 175°C for 10 min. Then the temperature was raised to a ratio of 2°C/min to 210°C and preserved at 210°C for 5 min. It was raised to a ratio of 10°C/min to 230°C and kept at 230°C for 2 min. Lastly, the temperature was raised to a ratio

of 5°C/min to 240°C and kept at 240°C for 8 min. For the analysis, 1.0 µL of diluted samples [n-heptane 1/100 (v/v)] were injected in split mode (30:1; 250°C; 25 psi). Hydrogen gas was used as a carrier gas at a flow rate of 35 mL/min. The composition of fatty acids (%) was determined by defining individual fatty acids by checking with retention time of known standards and denoted as a percentage of the total fatty acids.

### 2.7. GLYCERIDIC COMPOSITION

The triacylglycerol (TAGs) analysis was performed by modifying the COI methods (2017) on Agilent Infinity II 1260 HPLC device equipped with a reversed phase column and a refractive index detector (RID). The triacylglycerols were separated from other oil components with column chromatography. The oil sample dissolved in petroleum ether was placed on a chromatography column containing a silica gel absorber previously conditioned. ACE 5 C18 column (250 mm × 4.6 mm × 5 µm) was used for separation. Chromatography conditions were as follows: The mixture of acetonitrile/acetone (36.5:63.5) was used as mobile phase, the column temperature was 35°C, flow rate was 1.0 mL/min and the injection volume of samples was 20 µL. The triacylglycerols were defined by a comparison with a reference chromatogram [18–21]. TAGs with equivalent carbon number 42 as theoretically ($ECN42_{theoretical}$) were determined with the computer program of the EC Regulation 2568/91 method using the fatty acid profile [21].

### 2.8. STEROL ANALYSIS

A procedure, TS EN ISO 12228-1: "Determination of individual and total sterols contents - Gas chromatographic method - Part 1: Animal and vegetable fats and oils", described by Turkish Standards Institutions for the gas chromatographic determination of the content and composition of sterols in animal and vegetable fats and oils was performed [22]. The lipid with added betulin as internal standard was saponified and the unsaponifiable matter was extracted as mentioned above. On a simple silica gel plate, the bands corresponding to the sterols and triterpenic fractions of alcohol were isolated from the extract by thin layer chromatography (TLC). The sterols recovered from the plate were transformed into a mixture of ethanol and diethyl ether, and the mixture was analysed by GC using an Agilent 6850 GC equipped with FID detector and a HP-5 (30 m × 320 µm × 0.25 µm) column. The chromatographic conditions: injector at 280°C at 7.9 psi, HP-5 column at 260°C, and detector at 290°C. Injection volume was 1.0 µL and the split ratio was 10:1. Hydrogen as a carrier gas was used at a flow rate of 35 mL/min.

### 2.9. COLOUR

The colour of pomegranate seed oil was detected with the help of a Bench-top Minolta Spectrophotometer CM-5 (Konica Minolta, Tokyo, Japan). Colour values were given in the CIELAB D65 illuminant system and a 10° angle of vision. L* (lightness), a* (redness), b* (yellowness), Chroma (C*), and Hue (*h*), were registered.

## 3. RESULTS WITH DISCUSSION

### 3.1. DETERMINATION OF SOME QUALITY CHARACTERISTICS

Some physical and chemical properties of pomegranate seed oil (PSO) obtained by cold press are given in Table I.

As seen in Table I, it was determined that the total amount of crude oil obtained from pomegranate seeds by cold press method was 14.62% on a dry matter basis. The refractive index (1.5748 at 20°C) of the PSO obtained in our study was found to be slightly higher than the refractive index compared to previous studies [23, 24]. In previous studies conducted by Khoddami & Roberts (2015) and Dadashi et al. (2013), the refractive index of PSOs at 25°C ranges between 1.4977-1.4983 and 1.461-1.527, respectively. These differences could be attributed to the variety, ecological conditions, and the temperature at which the measurement can affect the refractive index of the PSO [23, 24].

As seen in Table I, the main tocopherol in PSO was determined to be α-tocotrienol (1898.90 µg/g) followed by γ-tocopherol (531.38 µg/g) and α-tocopherol (153.67 µg/g), respectively. Other tocopherols (β- and δ- tocopherol) and tocotrienols (β-, γ- and



**Table I -** Some quality characteristics of cold pressed pomegranate seed oil

| **Characteristics** | | |
|---|---|---|
| Total Crude Oil | g/100 g | 14.620 ± 1.517 |
| Refractive Index | nD20 | 1.5748 ± 0.0000 |
| **Tocopherol** | | |
| α-tocopherol | µg/g | 153.671 ± 15.863 |
| α-tocotrienol | µg/g | 1898.987 ± 73.166 |
| γ- tocopherol | µg/g | 531.383 ± 32.098 |
| Total sterol content | mg/100 g | 930.008 ± 0.115 |
| **Colour** | | |
| L* | D65 | 59.840 ± 0.242 |
| a* | D65 | 14.646 ± 0.114 |
| b* | D65 | 80.361 ± 0.130 |
| C* | | 81.704 ± 0.123 |
| h | | 79.670 ± 0.091 |

[1]All analyzes were repeated 3 times in the same pomegranate seed oil

δ- tocotrienol) could not be detected. On the other hand, α-tocopherol, γ-tocopherol and α-tocotrienol values in our study were lower than those obtained in the previous studies [2, 8–11]. These differences may be the result of different chromatographic separation conditions and sample preparation methods that allow complete separation of all tocopherols and tocotrienol compounds. Pomegranate seed oil contains high concentrations of tocopherols. Of these tocopherols, γ-tocopherol has a strong antioxidant capacity and α-tocopherol has high vitamin E activity. Pomegranate seed oil is extremely high in unsaturation and is easily exposed to oxidation, however it can be used as a healthy food and can be consumed or used as a food ingredient in formulations.

The values listed in Table I for Hunter L*- (lightness), a*- (redness), and b* (yellowness) reflect the colour spectrum of the PSO. As can be seen in Table I, cold pressed PSO was dark yellow. The a* values of cold pressed pomegranate seed oils were between dark yellow and orange. When the b* values were examined, it was understood that the PSO had a dark yellowness value.

## 3.2 DETERMINATION OF FATTY ACID CONTENT

The fatty acid composition of PSO is given in Table II. It was determined that the monounsaturated fatty acid (MUFA) content was 7.5%, polyunsaturated fatty acid (PUFA) content was 80.3% and the total

**Table II -** Fatty acid composition (%) of the oil of pomegranate seed [1]

| Fatty Acid (FA) | Fatty Acid abbreviation | Fatty acid quantity (%) [2] |
|---|---|---|
| Lauric acid | C12:0 | 0.004 ± 0.000 |
| Myristic acid | C14:0 | 0.017 ± 0.004 |
| Palmitic acid | C16:0 | 2.351 ± 0.120 |
| Palmitoleic acid | C16:1 | 0.017 ± 0.001 |
| Heptadecanoic acid | C17:0 | 0.053 ± 0.006 |
| Stearic acid | C18:0 | 1.737 ± 0.008 |
| Oleic acid | C18:1 | 4.098 ± 0.002 |
| Linoleic acid | C18:2 | 4.172 ± 0.056 |
| Punicic acid | C18:3 | 76.144 ± 1.012 |
| Arachidic acid | C20:0 | 0.400 ± 0.042 |
| Eicosenoic acid | C20:1 | 0.661 ± 0.052 |
| Eicosadienoic acid | C20:2 | 0.052 ± 0.001 |
| Eicosatrienoic acid | C20:3 | 0.088 ± 0.002 |
| Docosadienoic acid | C22:2 | 0.279 ± 0.008 |
| Lignoceric acid | C24:0 | 4.611 ± 0.048 |
| Nervonic acid | C24:1 | 3.401 ± 0.036 |
| **Σ SFA** | | 9.173 ± 0.034 |
| **Σ MUFA** | | 8.177 ± 0.027 |
| **Σ PUFA** | | 80.735 ± 0.194 |
| **Σ UFA** | | 88.912 ± 0.112 |

[1] All analyzes were repeated 3 times in the same pomegranate seed oil.
[2] SFA, MUFA and PUFA stand for saturated, monounsaturated, polyunsaturated and unsaturated fatty acids, respectively.



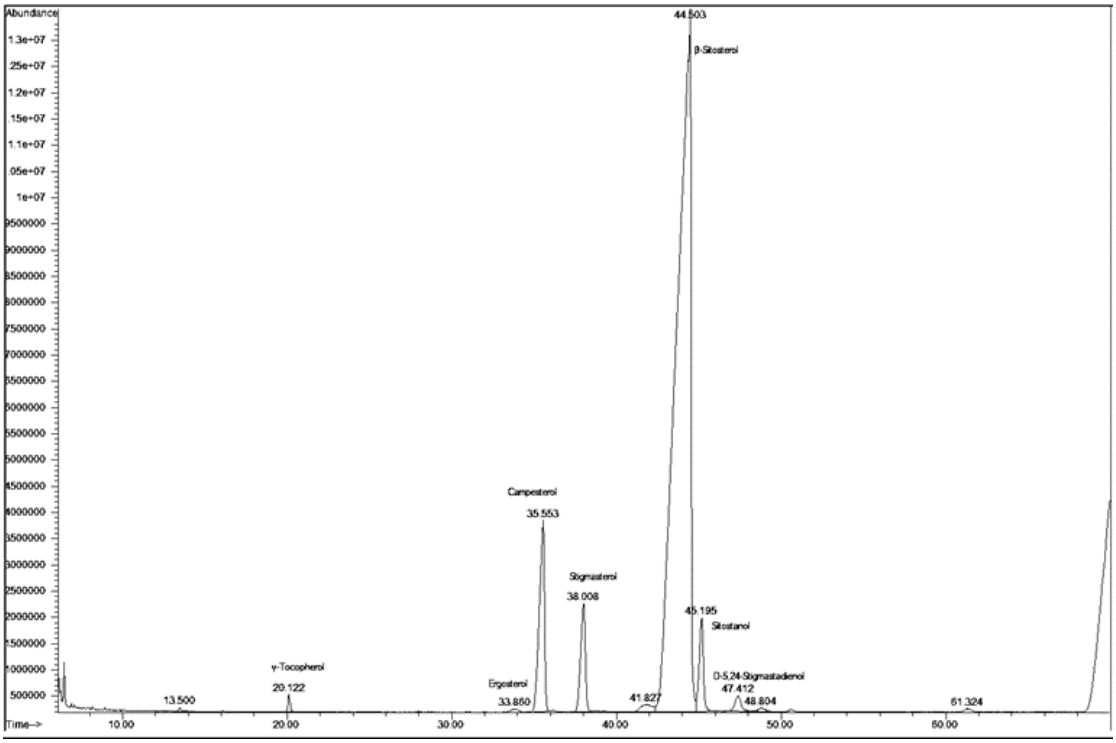


**Figure 1 -** GC chromatogram of the sterols composition of pomegranate seed oil

unsaturated fatty acid (UFA) content was 87.8%. The main unsaturated fatty acids in PSO were reported as being Punicic acid (Pu; 76.1%), Lignoceric acid (4.6%), Linoleic acid (L; 4.2), Oleic acid (O; 4.1%) and Nervonic acid (3.4%), respectively. As seen from the results, it was determined that the dominant fatty acid was Punicic acid (Pu; trichosanic acid; C18:3n-5; 9c, 11t, 13c). The total saturated fatty acid content was 8.7% and the main saturated fatty acid (SFA) is palmitic acid (2.4%).

The high content of punicic acid (trichosanic acid; C18n-5: 9c, 11t, 13c) in pomegranate seed oil can be used to provide essential fatty acids balance in the daily diet and care products.

## 3.3. DETERMINATION OF PHYTOSTEROLS COMPOSITION

Table III shows the sterol composition and total sterol content of pomegranate seed oil (PSO), and the sterol composition chromatogram was shown in Figure 1.

Although more peaks were detected in the chromatogram (Fig. 1), only β-sitosterol (83.20%), Campesterol (8.22%), Stigmasterol (4.24%), Sitostanol (3.31%), Δ5-D24 stigmastadionol (0.81%) and Ergosterol (0.22%) was defined separately and quantified based on retention time comparison with sterol standards (Tab. III).

Sterol composition (%) of the pomegranate seed oil in our study were different from the results announced by Caligiani et al. (2010); Fernandes et al. (2015), (2015b); Garima & Akoh (2009); Habibnia et al. (2012) [2, 7, 8, 10, 11]. However, as reported in the literature, β-sitosterol was the main phytosterol in PSO [7, 10, 12].

Regarding the total sterol content, it was determined that the total sterol content of PSO was 930 mg/100 g in our study (Table I) and the β-sterol content was 87.32% (Tab. III). Verardo et al. (2014) determined that the total sterol content varied between 746-1642 mg/100 g, depending on the varieties [12]. In previous studies, the total sterol content of PSO generally ranged from 363.6 [2, 21] to 575.8 mg/100g [11].

In several studies by Caligiani et al. (2010), Fernandes et al. (2015a), Habibnia et al. (2012) and Verardo et al. (2014) on the sterol content of pomegranate seed oil, it was determined that β-sitosterol content ranged between 414-806.9 mg/100g, 220.1-354.2 mg/100g, 459.6-500.3 mg/100g and 513-1142 mg/100g, respectively [2, 7, 11, 12]. In comparison, the findings (773.76 mg/100g) obtained in our study on β-sitosterol were among the values (220-1142 mg/100g) specified in the literature. The reason for these differences in sterol content and distribution in pomegranate seed oil is deemed to be due to the different pomegranate varieties due to the regions in which they were grown and the growing conditions.

**Table III -** Sterol composition (%) of the pomegranate seed oil [1]

| Sterols | Sterols quantity (%) |
|---|---|
| Cholesterol | 0.000 ± 0.000 |
| Cholestanol | 0.000 ± 0.000 |
| Brassicasterol | 0.000 ± 0.000 |
| 24-metien cholesterol | 0.000 ± 0.000 |
| Ergosterol | 0.221 ± 0.003 |
| Campesterol | 8.218 ± 0.084 |
| Campestanol | 0.000 ± 0.000 |
| Stigmasterol | 4.243 ± 0.132 |
| Δ7-campesterol | 0.000 ± 0.000 |
| β-sterol | 0.000 ± 0.000 |
| Δ5-23 stigmastadianol | 0.000 ± 0.000 |
| Clerosterol | 0.000 ± 0.000 |
| β-sitosterol | 83.196 ± 1.218 |
| Sitostanol | 3.310 ± 0.081 |
| Δ5-avenastenol | 0.000 ± 0.000 |
| Δ5-D24 stigmastadionol | 0.810 ± 0.102 |
| Δ7 stigmastenol | 0.000 ± 0.000 |
| Δ7-avenasterol | 0.000 ± 0.000 |

[1]All analyzes were repeated 3 times in the same pomegranate seed oil

**Table IV -** Triglyceride compositions and $ECN42_{theoretical}$ values of pomegranate seed oil

| Triacylglycerols | TAG quantity (%) |
|---|---|
| LLL | 0.01020 |
| PoLL | 0.00014 |
| PoPoL | 0.00000 |
| PoPoPo | 0.00000 |
| OPuL | 1.09720 |
| PoOPu | 0.00493 |
| PPuL | 0.70940 |
| PPoPu | 0.00319 |
| SPuPu | 4.34154 |
| $ECN42_{theoretical}$ (ΔECN42) | 6.16660 |

P: Palmitic acid, S: Stearic acid, Po:Palmitoleic acid, Pu:Punicic acid, O: Oleic acid, L: Linoleic acid

## 3.4. DETERMINATION OF THE TRIACYLGLYCEROLS (TAG) CONTENT

The composition analysis results of the triacylglycerols (TAG) of pomegranate seed oil (PSO) obtained in our study are given in Table IV and compared with olive oil (Fig. 2).

The fatty acids are esterified in the native TAG molecule to three stereospecific positions on the backbone of glycerol. The dominant TAG molecular species in olive oil are OOO, OOP OLO [25]. However, as

can be seen from the findings obtained in our study (Tab. IV), the dominant TAG molecules in pomegranate seed oil were determined to be SPuPu (4.34%), OPuL (1.10%) and PPuL (0.71%), respectively (O= oleic acid; L= linoleic acid; P= palmitic acid; Pu= punicic acid; S= stearic acid).

In many previous studies, researchers have found that the main fatty acid in PSO is punicic acid and varies between 70-85% [2, 3, 8, 22, 23]. It has been reported in several studies that punicic acid has positive effects on diseases such as cardiovascular, obesity, diabetes (type 2), cancer, inflammation and metabolic syndromes [9, 12, 23].

Kaufman & Wiesman (2007), reported that the main TAG compositions in PSO are LnLnLn and LnLnP [13]. Turtygin et al. (2013) reported that they detected six different triglyceride structures, namely PuPuPu, PuPuβEl (βEl: β-eleostearic acid), PuPuL, PuPuO, PuPuP and PuPuS in the TAG content of pomegranate seed oil [14]. However, in a study by Topkafa et al. (2015), it was reported that they separated, identified and quantified 19 triglycerides in pomegranate seed oil. In this research, PuPuPu (32.99%) was identified to be the primary triglyceride in pomegranate seed oil. The researchers reported that it was followed by PuPuCa and PuCaCa, and constituted 27.72% and 10.11% of total triglyceride, respectively [1].

To find out the presence of a small amount of seed oil (rich in linoleic acid) in olive oil and pomace oil, real and theoretical ECN42 triglyceride content is applied. As seen in our study, pomegranate seed oil has an ECN42 value of 6.17% and is rich in linoleic and punicic acids. In addition, as seen in Figure 2; it has been determined that among the 40-46 TAG species (ECN40-ECN46) in pomegranate seed oil, it contains monoglycerides, diglycerides and equivalent carbon number. However, olive oil has mostly TAG types between ECN44-ECN50.

Pomegranate seed is a waste by-product in the juice industry; however, it is also used as a source of seed oil with beneficial health properties, physiological activities, and rich nutritional value. TAG and fatty acid content analysis are the most important analytical methods used for identifying the nature and properties of vegetable oils.

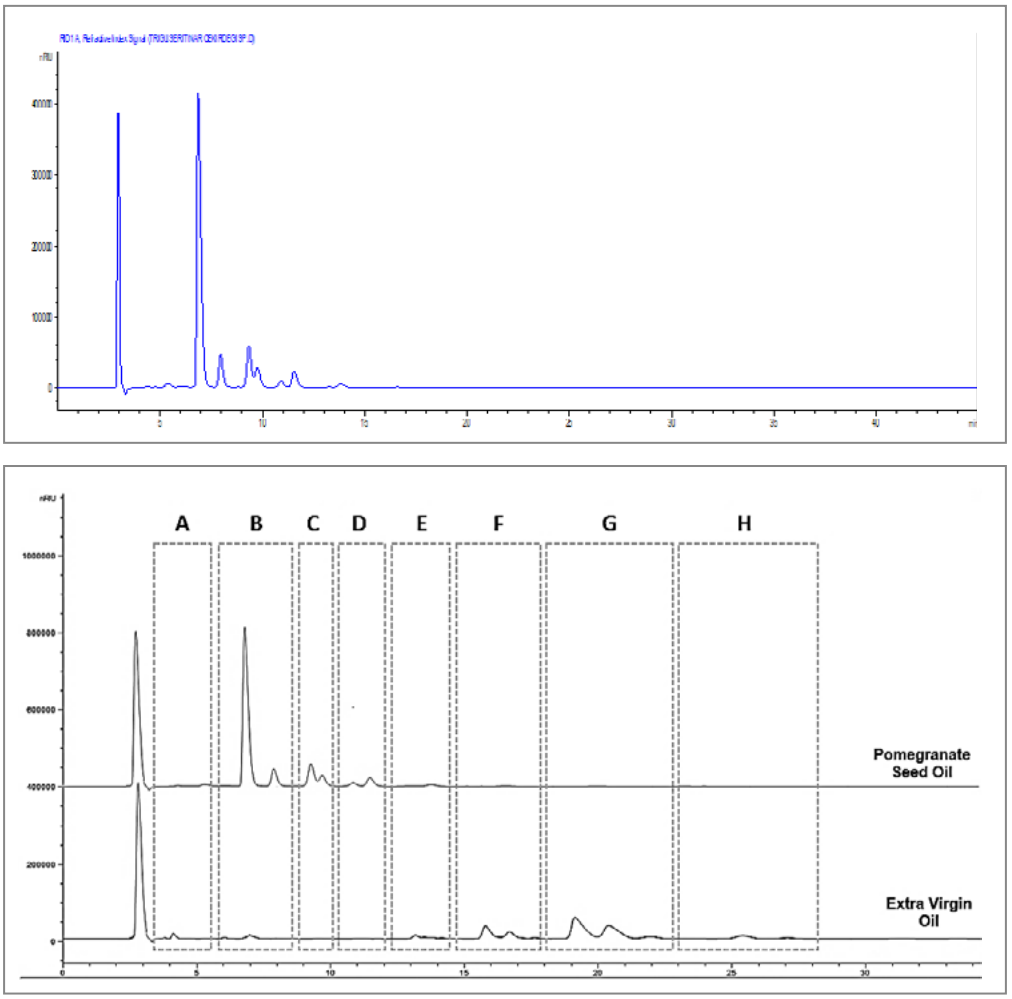


**Figure 2 -** Triacylglycerols (TAG) chromatogram of pomegranate seed oil (I) and comparison with olive oil (II) (A: Monoglycerides, B: Diglycerides, C: ECN40, D: ECN42, E: ECN44, F: ECN46, G: ECN48, H: ECN50)

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