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Pomegranate Oil Analysis with Emphasis on MALDI-TOF/MS Triacylglycerol Fingerprinting

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Pomegranate oil (PGO) is a unique and quite rare edible oil produced from *Punica granatum* L. seeds. It is considered to be a powerful health-benefiting agent, due to its antioxidative, anticancer, and antilipidemic properties. The aim of this study was to achieve a comprehensive and detailed profile of the different components of PGO. The fatty acid profile and phytosterol composition were determined by GC-MS; the triacylglycerol (TAG) compositions were profiled by the mass spectrometry tool of MALDI-TOF/MS. Results showed linolenic acid (18:3) to be the predominant fatty acid in the PGO (64–83%), as previously reported. The linolenic acid fraction was composed of four different chromatographically separated peaks that are assumed, according to MS data (based on both FAME and DMOX derivatization), to be attributed to different geometric isomers of conjugated linolenic acid (CLNA), punicic acid (18:3: 9-*cis*,11-*trans*,13-*cis*) being the major isomer. The MALDI-TOF/MS finger printing results showed the different TAG compositions present in the PGO, the major ones being LnLnLn and LnLnP. This unique PGO TAG fingerprint enables it to be differentiated from most other common edible oils. Phytosterols were found in quite a high concentration in the PGO (4089–6205 mg/kg), about 3−4-fold higher than in soybean oil. A detailed profile of the phytosterols in the PGO showed a wide variety, the major phytosterols being β-sitosterol, campesterol, and stigmasterol. This study depicts a new detailed analysis of PGO, showing great potential for further research regarding the physiological effects of specific valuable components in pomegranate oil.

KEYWORDS: Pomegranate oil; GC-MS; MALDI-TOF/MS; triacylglycerol (TAG); conjugated linolenic acid (CLNA); punicic acid; phytosterols

INTRODUCTION

Edible oil is one of the most commonly consumed products in the food industry. Recent studies make it clear that consumed oils have a tremendous effect on human physiology, including lipid metabolism, development of chronic disease, and overall well-being (*1*). Although conventional edible oils such as soybean, corn, and canola have their own importance, there are more rare and unfamiliar oils having unique characteristics and health-promoting traits. Pomegranate oil (PGO), produced from the seeds of the plant species *Punica granatum* L., is such an oil. It has been studied to some extent and is known as an antioxidant and anticancer agent (*2, 3*).

Conjugated fatty acids, having a number of conjugated double bonds, are known to have favorable physiological effects (*4*). The main fatty acids included in this category that have been widely researched are conjugated linoleic acid (CLA) and conjugated linolenic acid (CLNA), with two and three double bonds, respectively. These fatty acids have various effects such as antitumor activity and body fat reduction (*5, 6*). CLNA is present in only minor quantities in most vegetable oils (up to 0.2% by weight), yet there are several seed oils, such as tung oil, catalpa seed oil, and pomegranate seed oil, that contain 40–80% of CLNA (*7, 8*). Different positional and geometric isomers of CLNA have been reported in seed oils, including α-eleostearic (9-*cis*,11-*trans*,13-*trans*), calendic (8-*trans*,10-*trans*,12-*cis*), jacaric (8-*cis*,10-*trans*,12-*cis*), catalpic (9-*trans*,11-*trans*,13-*cis*), punicic (9-*cis*,11-*trans*,13-*cis*), and β-eleostearic (9-*trans*,11-*trans*,13-*trans*) acids (*6, 9*).

Most of the analytical studies regarding PGO present its fatty acid profile (*1*), which is characterized by a dominant percentage of punicic acid (*7, 10*). Punicic acid is considered to be an anticancer agent, as demonstrated by its inhibition of human prostate cancer cell invasion (*11*). It has also been reported to reduce apolipoprotein B100 secretion, which is correlated with the incidence of coronary heart disease and atherosclerosis (*5*).

There is a rising interest in the composition of fatty acids within the oil, namely, as triacylglycerol derivatives. Triacylglycerols (TAGs) are the main components of edible oils. They consist of a glycerol backbone attached to three esterified fatty acids (*12*). It is suggested that TAG structure, and not only its fatty acid profile, is of special importance regarding its physiological effect (*13*). Many studies concerning lipid metabolism and effect within the human body emphasize the importance of

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the structure–activity relationship (*8, 14, 15*). Therefore, analytical methods enabling determination of the TAG composition are in great need.

Different methods have been applied for the analysis of intact TAGs. Chromatographic methods are most commonly in use for this purpose, including gas–liquid chromatography (GLC), high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and supercritical fluid chromatography (SFC) (*12*). These methods have some disadvantages, for example, difficulties in eluting high molecular mass TAGs from HPLC columns and GLC detector response factors necessary for reliable quantification, especially for the analysis of polyunsaturated TAGs. Thus, attaining a complete separation of TAG species requires a combination of chromatographic methods (*12*). Recently, soft ionization mass spectrometry (MS) techniques such as atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) have been applied to analyze nonvolatile TAGs (*16*). These methods do not require the time-consuming chromatography step.

MALDI-TOF/MS has become a powerful and important tool for the analysis of large biopolymers. It creates a minimal degree of analyte fragmentation and thus enables the examination of rather complex structures, rather than the most basic "building blocks" of the analyte (i.e., TAGs as opposed to fatty acids) (*17*). It was originally used mainly for the analysis of proteins and peptides, synthetic polymers, and also carbohydrates to some extent, yet recent studies have also shown this tool to be applicable for the analysis of lipids (*17, 18*). Lipids are, in fact, highly suitable for MALDI because both the sample and most common matrices in use are soluble in organic solvents, forming a single organic phase. This creates an extremely homogeneous matrix/analyte mixture, which is an important criterion for credible MALDI results (*19*). MALDI-TOF/MS is considered to be a fast and accurate method, not requiring any prior derivatization stage and supplying results within minutes (*17*).

Another important aspect of oil analysis, beyond the major components of the oil (the TAGs and the fatty acids composing them), is the determination of its minor components profile, for example, its phytosterol composition. Phytosterols are compounds originating in the plant kingdom, structurally and functionally similar to the animal-derived cholesterol molecule (*20*). The term phytosterols is usually used to refer to both sterols (having a double bond at position 5 of the sterol ring structure) and stanols (the double bond at position 5 reduced) (*21*). These compounds have been shown to possess both anticancer activity (*22*) and LDL-lowering properties, resulting in the reduction of risk for coronary heart disease (*21*).

The present study had two objectives. The first goal was to achieve a comprehensive profile of major and some minor components of the PGO, that is, the fatty acids, with special attention given to the CLNAs, and the phytosterol composition. The second goal was the application of the powerful tool of MALDI-TOF/MS to the profiling of TAGs in the PGO. Achieving these objectives would supply important data for researchers trying to isolate specific components relevant to the physiological effects of PGO and help establish a structure–activity correlation. Moreover, it would highlight the potential of MALDI-TOF/MS technology and demonstrate a detailed method of analyzing oil.

MATERIALS AND METHODS

Plant Material. Fruit samples from trees of four different varieties of *P. granatum* L. including common Israeli pomegranate, Hershkowitz (H); common Israeli pomegranate, Mule-Head (MH); common California pomegranate, Wonderful (W); and new Ben-Gurion University (BGU)-selected pomegranates (types 1 and 2, BG1 and BG2, respectively, and some other types generally referred to as BGU-selected) were cut open, thus releasing the seeds from within the fruit compartments. The grains were cleaned of pulp and juice by hand, carefully washed, and then dried for 48 h in an oven heated to 70 °C. The resulting dried seeds were used as raw material for the oil extraction.

Oil Extraction. The PGO was extracted by a pressurized solvent extraction (PSE) technique, using the onePSE machine (Applied Separation, Inc.).

The samples were prepared according to the onePSE user's manual, version 1.91.

Dried seeds (1.5 g) were crushed by a mortar and pestle with an added 3 g of *onePSE* matrix.

Extraction conditions were as follows: Operating temperature was set to 110 °C, pressure was set to 100 bar. onePSE was programmed to perform the extraction in two consecutive cycles of 25 min, using hexane as a solvent. After these cycles, the hexane was evaporated in a rotary evaporator under reduced temperature (below 50 °C).

The following analytical tests were performed mostly on four PGO samples (BG1, W, H, and BG2, numbered 1–4, respectively), some on other BGU-selected pomegranate samples or on MH pomegranate samples, and additionally on a food-grade commercial soybean oil control (Shemen Ltd.). Soybean oil was selected as a representative of commonly consumed oils to compare and thus highlight the uniqueness of the PGO profile.

Fatty Acid Profile Determination by GC-MS. Fatty acid methyl esters (FAMEs) of the oil were prepared by placing 30 μL of oil in an Eppendorf tube, diluting with 600 μL of heptane, and adding 150 μL of 2 N methanolic potassium hydroxide solution (*23*). The combined solution was shaken vigorously twice for 30 s and left to stratify until the upper solution became clear. This solution was collected and evaporated to dryness under N_2 gas flow; the methyl esters were resuspended in 60 μL of heptane and left to stratify at −20 °C for a few hours. A 1 μL sample from the upper solution was then collected and injected into the GC for determination.

To support and clarify the data regarding the linolenic acid fraction of the oil achieved by FAMEs, another derivatization method was applied. 4,4-Dimethyloxazoline (DMOX) derivatives of the oil's fatty acids were prepared by heating them in excess 2-amino-2-propanol at 190 °C overnight and performing a separation and drying process (*37*). One microliter of the *n*-hexane dissolved sample was injected into the GC for determination.

The model 6890N gas chromatograph (Agilent Technologies) was equipped with a 30 m × 0.25 mm i.d., 0.25 μm, HP-SMS column. Operating conditions were as follows: column temperature was increased from 50 to 186 °C at a rate of 4 °C/min, then from 186 to 210 °C at 2 °C/min, and then at 4 °C/min to 300 °C; injector temperature was set to 250 °C, and detector temperature was set to 300 °C; linear velocity of the helium carrier gas was 30 cm/s. Fatty acids were identified both by their retention time and by mass spectra obtained with a 5973 Network Mass Selective Detector (Agilent) operating at 70 eV according to the GC-MS library. The percentage of each acid was calculated according to the following formula: % X = (area X × 100)/total area (weight percentage).

Phytosterol Composition Determination by GC-MS. Phytosterol composition determination has been previously described (*24*) and was performed here with some adjustments.

Preparation of the Unsaponifiables. Five grams of oil was put into a 250 mL flask, and 500 μL of 0.2% α-cholestanol solution was added. The combined solution was evaporated to dryness with nitrogen, and 5 g of this dry filtered material was returned to the same flask. A 2 N ethanolic potassium hydroxide solution (50 mL) was added, and the saponification was carried out by boiling and stirring the sample. The sample was heated for 20 min, then 50 mL of distilled water added, and the sample was cooled to approximately 30 °C. The contents were transferred to a separating funnel using distilled water rinses, about 50 mL total, approximately 80 mL of ethyl ether was added, and the sample was shaken vigorously for 30 s and allowed to settle. The lower aqueous

Table 1. Calculated Molecular Masses of Possible TAG Compositions

mass[a]	TAG[b]	CN:DB[c]
830.329	PPP	51:0
852.335	PPLn	53:3
854.351	PPL	53:2
856.367	PPO	53:1
858.383	PPS	53:0
874.341	LnLnP	55:6
876.357	PLLn	55:5
878.373	LLP	55:4
878.373	POLn	55:4
880.389	PSLn	55:3
880.389	POL	55:3
882.405	OOP	55:2
882.405	PSL	55:2
884.421	PSO	55:1
886.437	SSP	55:0
896.347	LnLnLn	57:9
898.363	LnLnL	57:8
900.379	LLLn	57:7
900.379	LnLnO	57:7
902.395	LLL	57:6
902.395	LnLnS	57:6
902.395	OLLn	57:6
904.411	OOLn	57:5
904.411	LLO	57:5
904.411	SLLn	57:5
906.427	OOL	57:4
906.427	LLS	57:4
906.427	SOLn	57:4
908.443	SSLn	57:3
908.443	OOO	57:3
908.443	SOL	57:3
910.459	SSL	57:2
910.459	OOS	57:2
912.475	SSO	57:1
914.491	SSS	57:0

[a] Masses are for sodium adduct ions. [b] P, palmitic acid, 16:0; S, stearic acid, 18:0; O, oleic acid, 18:1; L, linoleic acid, 18:2; Ln, linolenic acid, 18:3. [c] CN:DB, carbon number:number of double bonds.

phase was separated and collected into a second separating funnel. Two more extractions were done from the water/alcohol phase using 65 mL of ethyl ether each time.

The ether extracts were pooled into a single separating funnel and washed with distilled water (50 mL each time), until the wash gave a neutral reaction. Then the wash water was removed, and the sample was dried with anhydrous sodium sulfate and filtered on anhydrous sodium sulfate into a previously weighed 250 mL flask; the funnel and filter were washed with small aliquots of ethyl ether.

The ether was evaporated to a few milliliters and then dried with nitrogen; drying was completed in a stove at 100 °C for approximately 15 min; the sample was weighed after cooling in a desiccator.

Separation of the Sterol Fraction. An approximately 5% solution of the unsaponifiables in chloroform was prepared, and a 0.25 mm silica gel TLC plate (Merck, Darmstadt, Germany) was streaked with 0.3 mL of this solution. At the same time, 2−3 μL of the sterol reference solution were streaked to aid identification of the sterol band after developing.

The plate was placed into the developing chamber and allowed to elute until the solvent (toluene/acetone, 95:5 v/v) reached 1 cm from the upper edge of the plate; the plate was left inside the hood to evaporate the solvent. The plate was sprayed with 2,7-dichlorofluorescein solution, and the sterol band was identified under ultraviolet light. This band was scraped from the silica gel, and the final comminuted material was placed into the filter funnel; 10 mL of hot chloroform was added and filtered under vacuum, and the filtrate was collected in a conical flask attached to the filter funnel.

The collected residue was washed three times with ethyl ether (10 mL each time), and the filtrate was collected in the same flask attached to the funnel; this filtrate was evaporated to a volume of 4–5 mL and the residual solution was transferred into a 10 mL test tube; this solution was dried by heating in a gentle flow of nitrogen, dissolved again with acetone (a few drops), and evaporated to dryness again. The sample was placed in a stove at 105 °C for approximately 10 min and then allowed to cool in a desiccator and weighed. The residue contained in the tube consists of the sterol fraction.

Table 2. Fatty Acid Profile (Percent) of Pomegranate Oil, Samples 1–4, and a Soybean Oil Control As Determined by GC-MS

	oil type				
fatty acid	PGO 1 (BG1)	PGO 2 (W)	PGO 3 (H)	PGO 4 (BG2)	soybean
8:0		0.02			
14:0	0.02	0.03	0.05	0.02	0.10
15:0	0.02	0.03	0.03	0.02	0.02
15:1					0.01
16:0	3.15	3.89	14.51	3.56	15.00
16:1	0.03	0.06	0.10	0.02	0.08
17:0	0.07	0.03	0.07	0.07	0.10
17:1					0.07
18:0	2.37	3.09	2.94	2.66	7.10
18:1	5.21	10.11	12.02	7.17	31.87
18:2	3.58	2.14	4.38	4.67	43.40
18:3, total	**83.46**	**78.06**	**64.05**	**79.94**	
A	**61.61**	**61.75**	**52.77**	**73.55**	
B	**13.44**	**9.39**	**6.93**	**3.98**	
C	**0.19**	**0.18**	**0.11**	**0.18**	
D	**8.22**	**6.74**	**4.23**	**2.23**	
19:0					0.02
19:1	0.07		0.14	0.07	
20:0	0.57	0.85	0.56	0.55	0.82
20:1	0.96	1.06	0.77	0.78	0.40
20:2	0.11	0.06	0.06	0.06	
21:0	0.05	0.05	0.04	0.03	
22:0	0.15	0.25	0.13	0.13	0.75
23:0		0.04	0.03	0.03	0.05
24:0	0.09	0.14	0.07	0.08	0.21
25:0	0.07		0.02	0.06	
26:0	0.05	0.07	0.05	0.05	
28:0		0.03			

Preparation of the Trimethylsilyl Ethers. Silylation reagent consisting of a 9:3:1 (v/v/v) mixture of pyridine/hexamethyldisilazane/trimethylchlorosilane in the ratio of 50 μL for every milligram of sterol was added to the test tube containing the sterol fraction, and the tube was shaken until the sterols were completely dissolved. The sample was left for at least 15 min at ambient temperature and then centrifuged for a few minutes. The obtained clear solution is ready for gas chromatography.

The gas chromatography (details given previously) operating conditions were as follows: column temperature was set to 260 °C, injector temperature was set to 280 °C, and detector temperature was set to 290 °C. Linear velocity of the helium carrier gas was 35 cm/s; split ratio was 25:1. One microliter of substance was injected.

Peak Identification. Individual peaks were identified on the basis of retention times and by comparison with a mixture of sterol TMSE analyzed under the same conditions.

The sterols were eluted in the following order: cholesterol, brassicasterol, 24-methylenecholesterol, campesterol, campestanol, stigmasterol, Δ-7-campesterol, Δ-5,23-stigmastadienol, clerosterol, β-sitosterol, sitostanol, Δ-5-avenasterol, Δ-5,24 stigmastadienol, Δ-7-stigmastenol, Δ-7-avenasterol. The concentration of each individual sterol, expressed in milligrams per kilogram of oil, was calculated as follows: sterol concentration (mg/kg) = $A_x m_s \times 1000/A_s m$, where A_x = peak area for sterol x, A_s = area of the α-cholestanol peak, m_s = mass of α-cholestanol added (in milligrams), and m = mass of the samples used for determination (in grams).

Expression of the Results. Individual sterol concentration was recorded as milligrams per kilogram of oil and summed as "total sterols".

MALDI-TOF Analysis. Samples were dissolved in hexane to 0.1–1 mg/mL. A 2,5- MALDI matrix solution was prepared by dissolving 2,5-dihydroxybenzoic acid in 90% methanol to about 20 mg/mL. For

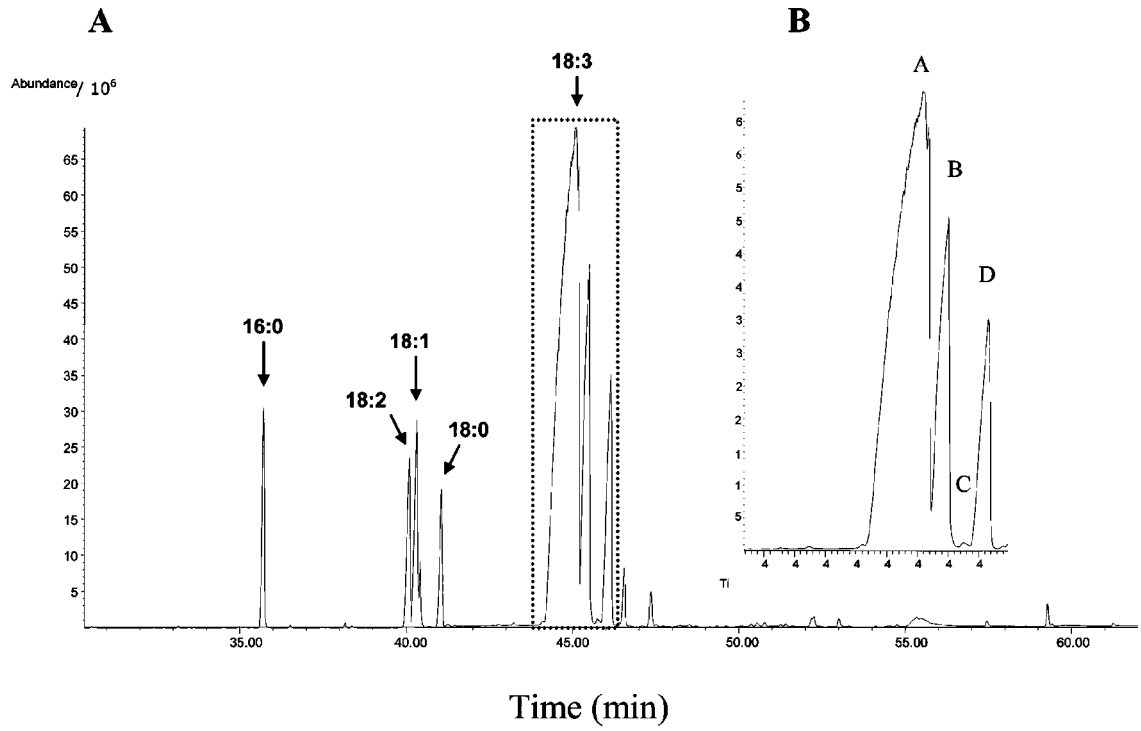

Figure 1. Gas chromatography results for pomegranate oil, sample 1 (BG1): (**A**) full chromatogram; (**B**) detail of linolenic acid (18:3) region of the chromatogram, showing four separate peaks.

MALDI analysis, small volumes of samples and matrix were mixed together in a ratio of 1:2, and 1 μL was then applied directly to a stainless steel MALDI target. Samples were analyzed on a Reflex IV (Bruker Daltonik GMBH, Bremen, Germany) MALDI-TOF mass spectrometer using 337 nm radiation from a nitrogen laser. An accelerating voltage of 20 kV was used. The spectra were recorded in reflectron mode within a mass range of *m*/*z* 450−2400 (*25*).

Data Processing. Some calculations were made to determine the theoretical molecular mass of the different optional TAGs (*25*). The five major fatty acids, according to previous paper (*1*, *8*), constituting TAGs in the PGO (and also in soybean oil, with different proportions) are palmitic acid (P, 16:0), stearic acid (S, 18:0), oleic acid (O, 18:1), linoleic acid (L, 18:2), and linolenic acid (Ln, 18:3). All possible combinations for TAGs composed of these fatty acids were put together and their molecular weights calculated. The molecular weight of a sodium ion (Na^+) was added to the TAG mass because this is the predominant ion in the matrix used for this experiment. It is assumed that during the ionization process this ion is added to the TAGs, in most cases one ion per TAG molecule. This calculation is displayed in the following equation:

$$\mathrm{Mw(TAG)} = \mathrm{Mw[glycerol} - 3(\mathrm{OH})] + \mathrm{Mw}[\mathrm{FA_1} + \mathrm{FA_2} + \mathrm{FA_3}) - 3(\mathrm{H})] + \mathrm{Mw}[\mathrm{Na^+}] \quad (1)$$

The results of the possible TAG combinations and their expected molecular masses are shown in **Table 1**. The last column of this table shows the number of carbons (carbon number, CN) in each TAG composition and also the number of double bonds (DB) in the molecule.

RESULTS AND DISCUSSION

Fatty Acid Profile. The fatty acid profile of PGO samples 1–4 and a soybean oil control was determined by GC-MS (**Table 2**). The profile is of FAME derivatives, although a very similar chromatographic separation was achieved by DMOX derivatization as well. All PGO samples demonstrated a similar distribution of fatty acids, ranging from myristic acid (14:0) to very long chain fatty acids such as cerotic (26:0). Sample 2 differed from the rest by having a wider range of fatty acid chain length, having unusual caprylic (8:0) and montanic acids (28:0). The most dominant peak in the chromatogram of all these samples was the one representing the linolenic acid (18:3) (**Figure 1A**, data shown for only sample 1). This peak is followed by three other, smaller peaks (one being almost unseen) (**Figure 1B**) that have also been identified by the MS as conjugated linolenic acids, all having a molecular ion at *m*/*z* 292 or 331 for FAME and DMOX derivatization, respectively. In agreement with previous paper (*1*, *8*, *10*), the integral sum of these four peaks, meaning the total percentage of the linolenic acid of all other fatty acids in the pomegranate oil, ranged between 64 and 83%, the lowest value attributed to sample 3 and the highest to sample 1. Another difference between the four PGO samples was the proportion between the different linolenic acid peaks as shown in the integration (**Table 2**). For example, in sample 4, the ratio between the first and second peaks is approximately 18, whereas the same ratio for sample 3 is approximately 8.

The soybean oil control contained no detectable linolenic acid. Previous studies (*28*) have reported that soybean oil contains a small quantity of linolenic acid (5.6%), yet even this small percent has been reported to decrease with an increase in the level of hydrogenation (*27*). The dominant fatty acids in this oil were linoleic acid (18:2, 43.4%), oleic acid (18:1, 31.9%), palmitic acid (16:0, 15.0%), and stearic acid (18:0, 7.1%), in accordance with previous data (*28*). These four fatty acids were also present in the PGO samples, each constituting only a few percent of the total fatty acid profile. Sample 3 was exceptional in its palmitic acid percentage (14.5%), being the main component after linolenic acid, as opposed to the profile of the other PGO samples.

Linolenic Acid Peaks. The linolenic acid peaks in the GC-MS chromatogram were analyzed and identified by their chromatographic separation and mass spectrum (MS), both of FAME and DMOX derivatives. In each sample there were four peaks identified as linolenic acid, all having quite a similar FAME MS pattern, differing mainly in the intensities of the

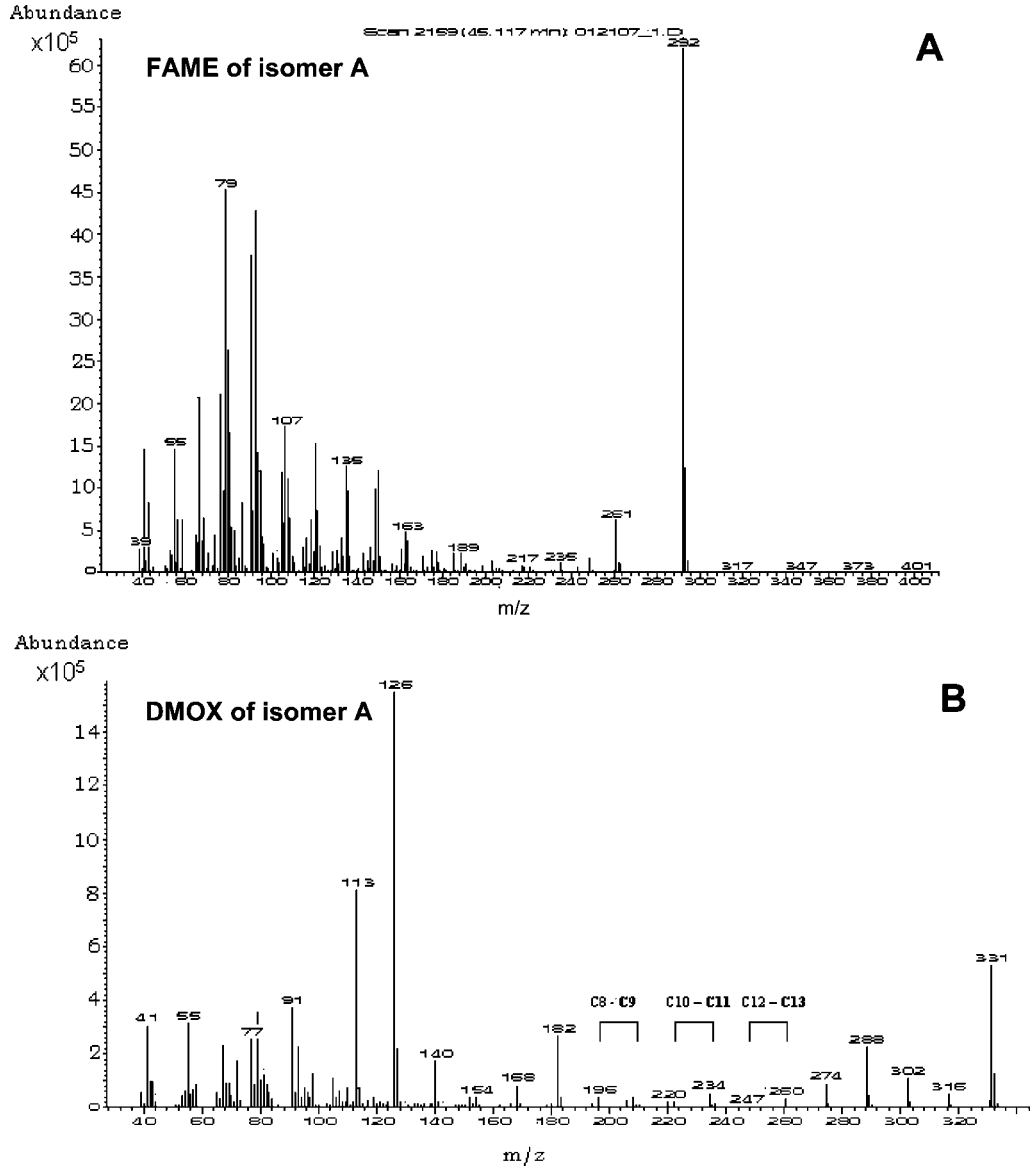

Figure 2. Linolenic acid isomers, sample 1 (BG1): (**A**) mass spectrum of isomer A with FAME derivatization; (**B**) mass spectrum of isomer A with DMOX derivatiztion. Double bonds at positions 9, 11, and 13 are represented by peaks at *m*/*z* 196 (C8), *m*/*z* 208 (C9), *m*/*z* 222 (C10), *m*/*z* 234 (C11), *m*/*z* 248 (C12), and *m*/*z* 260 (C13). A difference of 12 mass units between C_{n-1} and C_n implies a double bond at position C_n.

Table 3. MS Peak Intensities of the Four Linolenic Acid Isomers in Sample 1[a]

isomer	*m*/*z* 55	*m*/*z* 67	*m*/*z* 79	*m*/*z* 91	*m*/*z* 93	*m*/*z* 107	*m*/*z* 121	*m*/*z* 135	*m*/*z* 150	*m*/*z* 261	*m*/*z* 292
A	23.3	32.8	71.7	59.5	67.9	27.3	24.2	19.8	19.3	9.8	100.0
B	26.1	35.7	79.9	64.8	75.5	30.5	25.8	21.1	19.1	9.1	100.0
C	40.4	47.1	100.0	75.3	89.3	35.3	31.7	27.1	16.8	11.0	84.6
D	28.6	38.9	85.5	69.1	83.9	32.9	27.8	22.7	22.4	9.3	100.0

[a] Values are given as percent of the base peak intensity.

fragmentation ion peaks (**Figure 2A** and **Table 3**). Data are shown for sample 1, yet were very similar for other PGO samples. The major ion peaks in all FAME spectra were *m*/*z* at 79, 91, 93 and the molecular ion at 292. These ion peaks are known to characterize the MS of α-eleostearic acid, the 9-*cis*,11-*trans*,13-*trans*-CLNA isomer (*29*). The different intensities of these marker ions existing in the FAME MS of the four linolenic acid peaks, combined with the separation achieved by the chromatography, suggest that these peaks represent four different geometric (and not positional) isomers of 9,11,13-CLNA (referred to as isomers A, B, C, and D, according to elution order). Another reinforcement of this conclusion was given by the DMOX spectra of the four linolenic acid peaks in sample 1 (**Figure 2B**, data shown for isomer A). These spectra all exhibited the characteristic fragmentation of 9,11,13-CLNA, with a molecular ion at *m*/*z* 331, the predominant ions at *m*/*z* 113 and 126, and a mass separation of 12 units between C8 and C9 (*m*/*z* 196, 208), between C10 and C11 (*m*/*z* 222, 234), and between C12 and C13 (*m*/*z* 248, 260), indicating the 9, and 11, and 13-positions of the double bonds (*38*).

Previous studies have reported the predominance of linolenic acid in the pomegranate oil fatty acid profile; some have regarded only the total percentage of this fatty acid (*1*), some briefly mentioned that this total percentage included

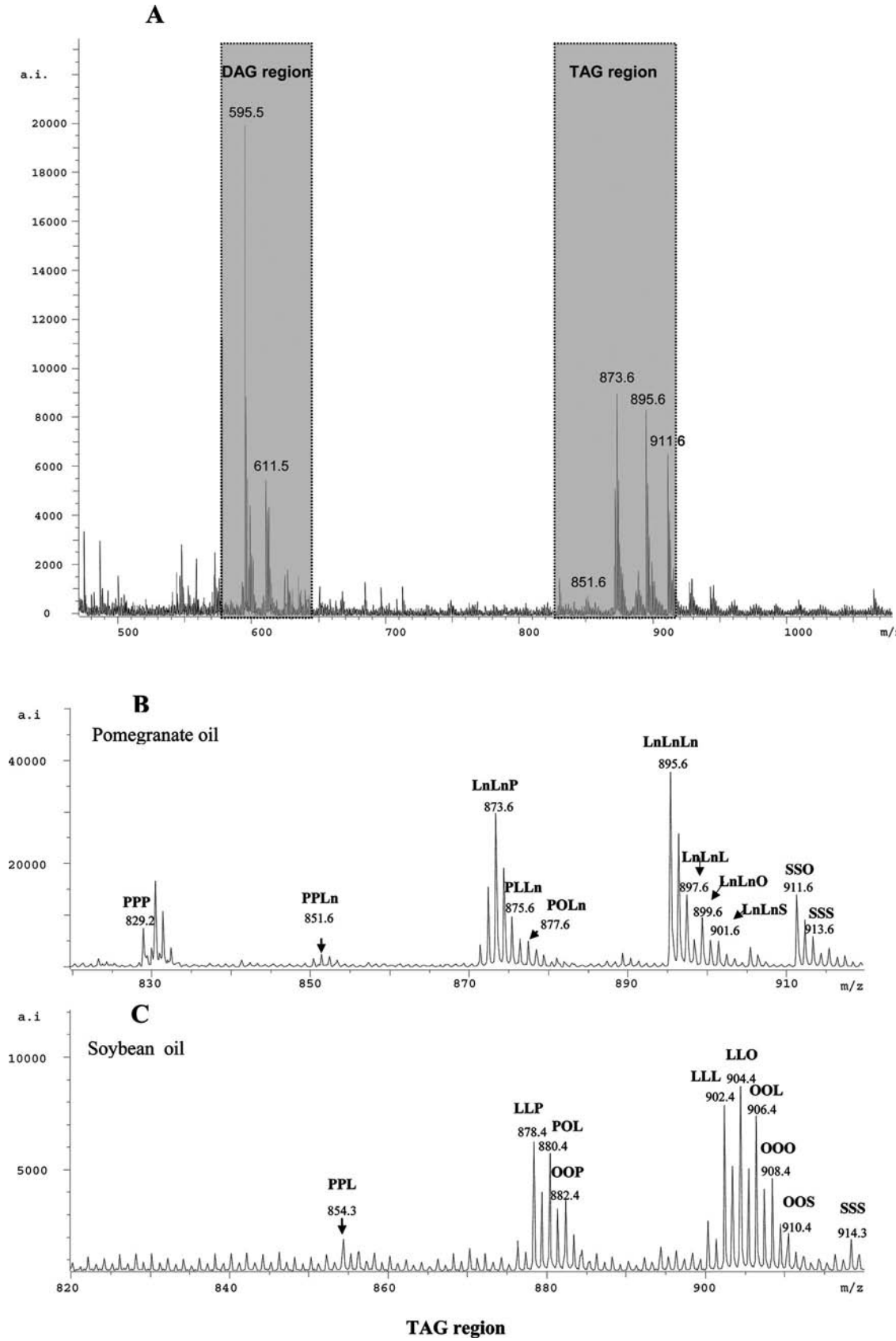

Figure 3. (**A**) *m*/*z* 470–1070 region of MALDI-TOF/MS mass spectra of pomegranate oil, sample 4. The spectra includes both diglyceride (DAG) and triacylglycerol (TAG) regions. The spectra were recorded in positive ion mode; the ions are sodium ion adducts. (**B**) TAG region of pomegranate oil, sample 4. (**C**) TAG region of soybean oil sample.

different isomers with double bond positions at 9,11, and 13 (*30*), and others have differentiated these isomers (*7*, *8*, *10*). The studies that differentiated the isomers separated them by a chromatographic method, and the identification was made by comparing the retention time to a standard or isolated isomers. In our study, conclusions regarding the

Table 4. Phytosterol Profile of Pomegranate Oil Samples and a Soybean Oil Control As Determined by GC-MS

	oil type			
phytosterol (mg/kg)	PGO (W)	PGO (MH)	PGO (BGU-selected)	soybean
cholesterol	0.11	8.83	8.07	5.63
brassicasterol	0.30	1.12	0.91	5.16
24-methylenecholesterol	0.53	2.12	2.95	2.14
campesterol	424.56	357.84	527.91	216.64
campestanol	3.55	1.96	4.99	7.32
stigmasterol	182.22	195.43	288.44	267.92
Δ-7-campesterol	11.45	4.36	8.17	6.02
Δ-5,23-stigmastadienol	10.33	5.55	6.29	6.01
clerosterol	45.30	33.37	29.45	9.57
β-sitosterol	3726.84	3205.04	5039.22	884.62
sitostanol	24.06	3.83	6.51	25.59
Δ-5-avenasterol	116.82	169.61	128.12	8.15
Δ-5,24-stigmastadienol	20.50	7.53	10.43	1.97
Δ-7-stigmastenol	27.41	71.19	105.74	43.92
Δ-7-avenasterol	49.18	21.19	38.15	13.67
total	4643.16	4088.97	6205.35	1504.33

structure of the isomers were based on the analysis of MS from GC-separated peaks and on previous results.

The first isomer (isomer A) in all of the PGO samples constituted the largest fraction of the total CLNA isomers by far (**Table 2**). It can therefore be most probably identified as the punicic acid isomer (9-*cis*,11*trans*,13-*cis*), in accordance with the well-established data reporting this acid to be the lead component of pomegranate oil (*7*, *8*, *10*). Tagaki et al. (*7*) reported the presence of α-eleostearic (9-*cis*,11-*trans*,13-*trans*) and catalpic (9-*trans*,11-*trans*,13-*cis*) acids in PGO in addition to punicic acid. Özgül-Yücel (*10*) also reported only three isomers, adding β-eleostearic (9-*trans*, 11-*trans*, 13-*trans*) and leaving out the α-eleostearic isomer, whereas Suzuki et al. (*8*) reported all four isomers discussed herein. These differences demonstrate some ambiguity in the literature regarding the less familiar CLNA isomers in the PGO, although part of the reason might be different origins of the pomegranate fruit, affecting the oil's composition.

Although elucidation of the complete structure of isomers B, C and D cannot be decisively determined by these data, there is some indication implying the possible structure of one isomer. Isomer C, the least abundant of the four isomers, showed a FAME peak intensity pattern somewhat different from that of the other isomers, having a base peak at *m*/*z* 79, exceeding the intensity of the molecular ion peak at *m*/*z* 292 (**Table 3**). Interestingly, this has previously been reported to indicate a cis configuration at the central double bond (*31*). This would imply that isomer C is neither of the possible isomers mentioned above, all having trans configurations in their centered double bond. To the best of our knowledge, no CLNA isomer with double bonds at positions 9, 11, and 13 and a cis configuration at position 11 has previously been reported. In the case of PGO, this is possibly due to the low concentration of this isomer (0.1–0.2%) (**Table 2**), requiring a very accurate method of analysis.

Further research, including FTIR and NMR methodologies (*38*), is needed to determine the exact configurations of the pomegranate oil isomers, but the data shown here are an important starting point. The importance of conjugated fatty acids for health benefits is becoming more and more obvious (*4*), and the specific relevance of the group of isomers discussed here (9,11,13-CLNA) is demonstrated in their much stronger cytotoxic effect on cancerous cells than the effect of an 8,10,12-CLNA isomer (*8*).

Triacylglycerol Analysis by MALDI-TOF. Figure 3A shows a broad MALDI-TOF/MS spectrum of pomegranate oil, sample 4, including the diacylglycerol (DAG) region (ranging between *m*/*z* ∼580 and 650) and the TAG region (ranging between *m*/*z* ∼830 and 915). In **Figure 3** the focus is on the TAG region of the PGO, sample 4 (**Figure 3B**), in comparison to the same region of the MALDI-TOF spectrum of soybean oil (**Figure 3C**). The two most dominant peaks identified in the PGO TAG region were attributed to the TAG compositions LnLnLn (at *m*/*z* 895.6) and LnLnP (at *m*/*z* 873.6). The other main peaks identified were attributed to the compositions PPP, PPLn, PLLn, POLn, LnLnL, LnLnO, LnLnS, OOL, SSO, and SSS. For the soybean oil sample, the two most dominant peaks identified in the TAG region were LLO (at *m*/*z* 904.4) and LLL (at *m*/*z* 902.4). The other main peaks identified were attributed to the compositions PPL, LLP, POL, OOP, OOL, OOO, OOS, and SSS. An isotopic correction was performed for both samples to ensure that minor peaks were not artifacts, that is, did not result from the isotopic distribution of adjacent, more dominant, TAG peaks.

There were some minor differences in the molecular weights of the pomegranate and soybean oils' TAGs; for example, the SSS was observed at *m*/*z* 913.6 for the pomegranate oil and at *m*/*z* 914.3 for the soybean oil. This was probably due to a slightly different calibration point taken for the different samples. Differences between the expected, theoretically calculated mass (**Table 1**) and the observed one were negligent and probably derived from a calibration error.

In addition to these qualitative results, an attempt to establish the percentage of each of the TAG compositions was made (data not shown). The fatty acid profile was then deduced according to the distribution of each fatty acid in the TAGs, yet these data differed from the GC-MS results quite significantly. This difficulty in utilizing the MALDI tool for quantitative results has been previously reported (*26*, *32*), indicating the need for an internal standard (*33*).

Determination of TAG composition has been shown to have some important implications. Natural oils are known to have a characteristic TAG profile (*12*). The MALDI-TOF/MS enables receiving a rather unique peak pattern in the spectrum, a "fingerprint" of the pomegranate oil, discriminating it from other oils, such as soybean oil (**Figure 3**). This information is important to confirm the authenticity of the oil, a crucial issue in the food industry (*34*).

Another implication of profiling the TAG compositions is a better understanding of the metabolism and physiological effects of the oil. There is accumulating evidence that TAG composition and structure influence lipid metabolism and, thus, the risk of atherosclerosis (*14*) and coronary heart disease. To achieve a full understanding of lipid metabolism, the stereospecific structure of the TAGs is needed, meaning the *sn* position (1, 2, or 3) of each fatty acid on the glycerol backbone. Unfortunately, these data are not achievable by MALDI-TOF/MS, using the procedure that was carried out, and yet in some cases this does not have an effect, that is, homogeneous compositions such as LnLnLn (the most prominent TAG in the PGO), PPP, etc. Another possibility for speculating on the positions of each fatty acid, without the need for further investigation, is the well-known typical TAG structure. Saturated fatty acids tend to be situated in primary positions (*sn*-1 and *sn*-3), whereas polyunsaturated fatty acids are mainly positioned at *sn*-2 (*12*). As a consequence, it is fairly reasonable to assume that PLnP is the correct structure for this composition, rather than PPLn. Another interesting question raised, regarding the structure of the TAGs specifically in the PGO and in correlation with the previously discussed linolenic acid isomers, is the composition of isomers constituting an Ln-containing TAG, for example, LnLnLn. Following the conclusion that the linolenic acid fraction includes different isomers, the LnLnLn TAG complexity rises, and it is important to take into consideration that a different composition or position of the isomers could affect the structure–activity relationship.

The data given here are an important foundation for further research, including the use of stereospecific methods that are needed to get decisive conclusions regarding the TAG structure.

Phytosterol Profile. The phytosterol profiles of three PGO samples (varieties W, MH, and BGU-selected) and a soybean oil control were determined by GC-MS (**Table 4**). The total concentration of phytosterols in the PGO samples was approximately 3−4-fold higher (ranging between 4089 and 6205 mg/kg) than that of the soybean oil (1504 mg/kg), which was in quite good agreement with Ostlund (*20*) and Phillips et al. (*35*). The most prominent phytosterol in both the PGO samples and the soybean oil was β-sitosterol, constituting about 80% of the total phytosterols in the pomegranate oil and only 59% in the soybean oil. The next two most dominant phytosterols for the pomegranate and the soybean oil were campesterol and stigmasterol. Another phytosterol found in quite a significant concentration in the PGO, but to a lesser extent in the soybean oil, is Δ-5-avenasterol, ranging between 117 and 170 mg/kg in the different PGO samples.

Phytosterols are found in all plant food products, yet they are found in the highest concentrations in vegetable oils (*20*). Phytosterols are hydrophobic and tend to form stable crystals when purified. Thus, they need to be specially adapted, dissolved in fat, or formulated to become bioavailable when given as supplement products (*36*). Foods highly enriched with phytosterols, such as pomegranate oil, can replace such supplements. Moreover, most clinical trials test phytosterols as mixtures, as it is difficult to separate a sufficient quantity of an individual phytosterol. This study details a wide range of different, naturally occurring, phytosterols, and their quantities in PGO. The relatively high concentrations, combined with the exact identification given here, may allow isolation and further research regarding the biological effects of specific phytosterols. The results shown here apply both to the health-benefiting potential lying in the complete composition of PGO and to the importance of further analysis of specific components of the oil that can be isolated and used for pharmaceutical purposes in the future.

ABBREVIATIONS USED

CLNA, conjugated linolenic acid; DAG, diacylglycerol; FAME, fatty acid methyl etser; L, linoleic acid; Ln, linolenic acid; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time of flight/mass spectrometry; O, oleic acid; P, palmitic acid; PGO, pomegranate oil; S, stearic acid; TAG, triacylglycerol.

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