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Comparison of Different Methods of Extraction for Pomegranate Seeds †

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Abstract: Pomegranate seed oil (PSO) has attracted considerable attention because of its potentially beneficial biological effects. This oil consists of the high content of polyunsaturated fatty acids mainly conjugated type, punicic acid. Punicic acid has antioxidant and anticancer activity. Aim of this research was to compare the properties of PSO obtained by cold extraction, the Soxhlet extraction and Accelerated Solvent Extraction (ASE). Oxidative stability of oils from pomegranate was determined by using the calorimetric method. The determination of fatty acid composition was carried out by gas chromatographic analysis of fatty acid methyl esters. The positional distribution of fatty acids in the sn-2 and sn-1,3 positions of triacylglycerols (TAG) was based on the ability of the pancreatic lipase to selectively hydrolyse ester bonds in the sn-1,3 positions. Sterols composition was determined with GC-MS. The greatest amount of oil can be obtained using the Soxhlet method (12–15%) and the least the ASE method (10–11%), but the ASE oil is more diverse in terms of sterol content. All the extracted oils were rich in punicic acid (about 80%). In the external positions of TAG there is mainly punicic acid, while in the internal positions there are oleic and linoleic acids.

Keywords: pomegranate seed oil; accelerated solvent extraction; soxhlet extraction; cold extraction; punicic acid; sterols composition; oxidative stability; fatty acid composition

1. Introduction

The pomegranate is called the fruit of life, the elixir of love, the heavenly fruit, and happen to be a symbol of longevity and fertility. It is often recognized as the earliest and sacred fruit that belongs to the Punicaceae family. It is the source of many medicinal raw materials and functional foods, such as fresh and processed pomegranate juice and pomegranate cortex [1,2]. Pomegranate juice has antioxidant and anti-inflammatory effects due to the presence of anthocyanins, flavonoids and phenolic acids, which inhibit the activity of inflammation activators. Pomegranate cortex is a rich source of alkaloids and tannins, which have anti-parasitic effects [2]. The oil from pomegranate seed has also attracted considerable attention because of its potentially beneficial health effects: lipid fraction extracted from pomegranate seeds can improve immune function in vivo, reduce hepatic triacylglycerols (TAG) accumulation and act as a chemopreventive agent against hormone-related human cancers [3]. The hypoglycemic effect of pomegranate seed oil (PSO) may be due to the

presence of one of the conjugated linolenic acids - punicic acid. The interest in the conjugated fatty acids has been growing because of their anti-diabetic and anti-cancerous properties [2].

There are about 500 varieties of pomegranate known worldwide, which have different quality characteristics of the fruit, such as size, shape, color, flavor and seed hardness [1]. Pomegranate is not only consumed as a fresh fruit, but is used as a raw material in the production of various products such as juices, syrup, jams and wine. Pomegranate seeds, which make up 10% of the weight of the fruit, are a waste product of the food industry. The oil content in pomegranate seeds is ranged from 12 to 20% of dry weight [3–6].

A number of techniques have been reported for extraction and quantification of oil from seeds. For analytical purposes the lipid fraction is usually isolated from seeds in a Soxhlet apparatus with non-polar organic solvents [7]. The conventional Soxhlet method and other shaking or stirring methods require long extraction periods, large sample sizes, and large amounts of toxic solvents that are expensive and can cause environmental problems [8]. Accelerated (pressurized) solvent extraction (ASE) is the alternative method for the extraction of oil from seeds. This method which uses organic solvents at high temperatures and pressures above the boiling point for a short time can increase the solubility of the compound, solvent diffusion rate and mass transfer [9]. Extraction with ASE is gaining more and more attention nowadays due to the lower amount of solvents and the lower process time required compared to other methods of extraction [10].

The aim of this work was to compare the properties of PSO (from two different regions of Croatia) obtained by cold extraction, the Soxhlet extraction and Accelerated Solvent Extraction.

2. Materials and Methods

2.1. Materials

Pomegranate seeds were obtained from the Neretva river region of Croatia—south Dalmatia (pomegranate dark—PD) and Šibenik region of Croatia—north Dalmatia (pomegranate light—PL). The seeds have been ground to prepare the test sample.

2.2. Extraction of Oil from Seeds

Oil and moisture contents of the seeds were determined by using ISO methods 665 [11]. The data obtained were used to calculate oil yield, defined as the percentage of oil on dry basis.

2.2.1. Soxhlet Extraction

The Soxhlet extraction for the determination of the hexane extract called the "oil content" was in accordance with reference method ISO 659 [12].

2.2.2. Cold Extraction

Cold extraction was performed by hexane. Sample mass of 25 g was extracted with 100 mL of hexane at room temperature using the magnetic stirrer. After 20 min, the sample was centrifuged for 20 min at 5,000 rpm. Supernatant was decanted and the pellet was returned to the flask and extracted one more times with 100 mL of hexane for 20 min. Extracts were combined and evaporated to dryness at 60 °C on a rotary evaporator and afterwards purged under the stream of nitrogen to remove any residual solvent.

2.2.3. Accelerated Solvent Extraction

ASE was applied for oil extraction from pomegranate seeds. The procedure was conducted on Dionex™ ASE™ 350 Accelerated Solvent Extractor (Thermo Fisher Scientific Inc., Sunnyvale, CA, USA) using n-hexane as the extraction solvent. For extraction purposes, a mixture of sample (8 g) and diatomaceous earth (0.5 g) was placed into 34 mL stainless steel cells fitted with 2 cellulose filters at the bottom of the cells. Extraction conditions were set according to the method described by Lohani et al. [13], slightly modified: temperature 100 °C, static extraction time 10 min and 6 extraction cycles,

constant pressure of 10.34 MPa, 30 s of purge with nitrogen and 50% of flushing. Obtained extracts were collected in 250 mL glass vessel with Teflon septa, evaporated at 60 °C under vacuum and afterwards purged under the stream of nitrogen to remove any residual solvent.

2.2. Fatty Acid Composition

The determination of fatty acid composition was carried out by gas chromatographic analysis of fatty acid methyl esters (FAME). FAME were prepared according to the standard ISO method 5509 [14] and injected into a gas chromatograph equipped with an FID detector according to ISO method 5508 [15].

2.3. Positional Distribution of Fatty Acids in the sn-2 and sn-1,3 Positions of TAG

Method of determination of positional distribution of fatty acids in the sn-2 and sn-1,3 positions of TAG has been described by Bryś et al. [16].

2.4. Determination of Sterols

Sterols were determined with accordance with reference method ISO 12228 [17].

2.5. PDSC Measurements

The oxidative stability of oils was carried out by a differential scanning calorimeter (DSC Q20 TA) coupled with a high-pressure cell (PDSC). Oil samples of 3–4 mg were weighed into an aluminum pan and placed in the sample chamber in the isothermal temperature 120 °C and under oxygen atmosphere with an initial pressure of 1400 kPa. Obtained curves were analyzed using TA Universal Analysis 2000 software (City, Country). For each sample, the output was automatically recalculated and presented as the amount of energy per one gram.

2.6. Statistical Analysis

The results are expressed as the mean value with the standard deviation. Relative standard deviation was calculated, where appropriate, for all data collected using Microsoft Excel Software. One-way analysis of variance ANOVA was performed using the Statgraphics Plus, version 5.1 (City, Country). The value of $p \leq 0.05$ was set as a statistical significance limit. Differences were considered to be significant at a p-value of 0.05, according to Tukey's Multiple Range Test All experiments were carried out at least in duplicate, each with at least two analytical measurements.

3. Results

Extraction yield (Table 1) was determined to assess the overall extraction efficiency. Extracts obtained after cold extraction and Soxhlet extraction are characterized by the highest yield followed by ASE. The PSO from south Dalmatia it is richer in oil (12.1–16.9%) followed by north Dalmatia (11.0–14.0%). The results of PDSC measurements, expressed as the oxidation induction times are shown in Table 1. The PDSC tests for PSO samples performed at isothermal temperature of 120 °C showed that their induction times were short and ranged from 0.32 to 4.55 min.

The results of the determination of the fatty acid composition of the PSO are presented in Figure 1a. PSO showed from 4.1 to 7.4% of polyunsaturated fatty acids (PUFA). The main representative of PUFA is linoleic acid. PSO contains also from 4.1 to 5.9% of monounsaturated fatty acids (MUFA) including oleic acid and from 4.4 to 5.4% of saturated fatty acids (SFA) including palmitic and stearic acid. The most abundant fatty acid in this oil is punicic acid, belonging also to PUFA. The content of this conjugated linolenic acid in PSO is from 77.4 to 85.3%. ASE and cold extraction methods made it possible to obtain PSO richer in PUFA acids compared to the Soxhlet method. The PSO from North Dalmatia it is richer in PUFA followed by south Dalmatia.

Table 1. Extraction yields (%, mean ± SD) and induction time (min, mean ± SD) of light and dark pomegranate seed oil (PL and PD) after using different methods of extraction (cold extraction-CE, Accelerated Solvent Extraction-ASE, Soxhlet extraction-SOX).

Type of sample	Extraction yield [1]	Induction time [1]
PD_CE	16.95 ± 0.59 d	4.55 ± 0.15 e
PD_ASE	12.05 ± 0.13 b	3.75 ± 0.06 d
PD_SOX	16.31 ± 0.67 d	0.71 ± 0.03 b
PL_CE	13.34 ± 0.30 c	3.81 ± 0.25 d
PL_ASE	10.99 ± 0.22 a	2.63 ± 0.08 c
PL_SOX	14.02 ± 0.17 c	0.32 ± 0.11 a

[1] The different lower case letters in the same column indicate significantly different values ($p < 0.05$).

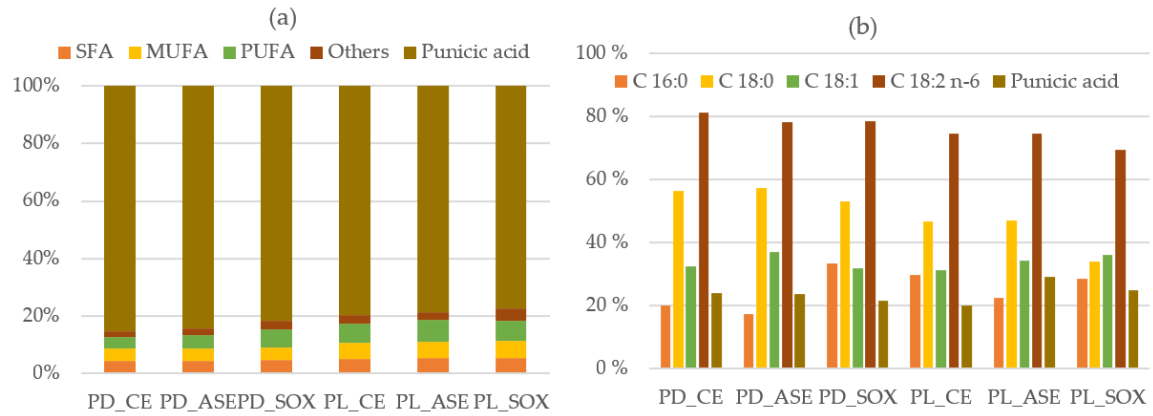

Figure 1. Fatty acid composition (**a**) and distribution in TAG (**b**) for dark and light pomegranate seed oil (PD and PL) after using different methods of extraction (cold extraction-CE, Accelerated Solvent Extraction-ASE, Soxhlet extraction-SOX). PUFA—polyunsaturated fatty acids, MUFA—monounsaturated fatty acids, SFA— saturated fatty acids.

The results of the percentage of fatty acids in the sn-2 position are presented in Figure 1b. PSO contains from 69.3% to 81.2% of linoleic acid in the sn-2 position, whereas the percentage of punicic acid in this position is from 20.0% to 29.3%, which means that it is mainly located in the sn-1 and sn-3 positions. Percentage of the stearic acids in sn-2 position of TAG in all PSO exceeded 33%, which means that it is located mainly in the internal position of TAG, whereas percentage of palmitic acid in sn-2 position of TAG in PSO did not exceed 33%, which means that it is mainly in the external positions of TAG. The distribution of oleic acid in TAG of PSO is close to the statistical one.

The sterols composition in PSO is shown in Figure 2. Seven compounds were postulated for wherein the sterol marker was β-sitosterol constituting from 62.7% to 71.9% of the total sterols content. The next major components were campesterol (9.4–11.7%), Δ5-avenasterol (5.6–9.7%) and stigmasterol (3.9–4.5%). The type of PSO and the extraction method did have a significant effect on the phytosterol content. In the cold extraction method the highest amount of β-sitosterol was produced.

4. Discussion

The oil content in PSO, according to the literature data, is ranged from 12 to 20% [5,6]. The obtained results were consistent with the literature data. The extraction method, among other, affects the content of the extracted oil. The results of other researchers confirm that the fatty acid present in the largest amount in the pomegranate seed oil is geometric and positional isomers of unsaturated octadecatrienoic acid-punicic acid (cis-9, trans-11, cis-13 C18:3) [18,19]. Silva et al. [20] investigated the influence of the extraction techniques expeller pressing, alcohol-extraction and supercritical CO_2 on the chemical composition PSO. According to scientists, the method with the application of CO_2 allowed to obtain an oil containing over 80% punicic acid. The ASE methods and cold extraction also

make it possible to obtain PSO containing above 80% punicic acid. The results of other researchers suggest that stigmasterol, Δ5-avenasterol, campesterol, and β-sitosterol, in order of increasing abundance, were the most common sterols in PSO [3]. The results obtained in this study are consistent with the literature data.

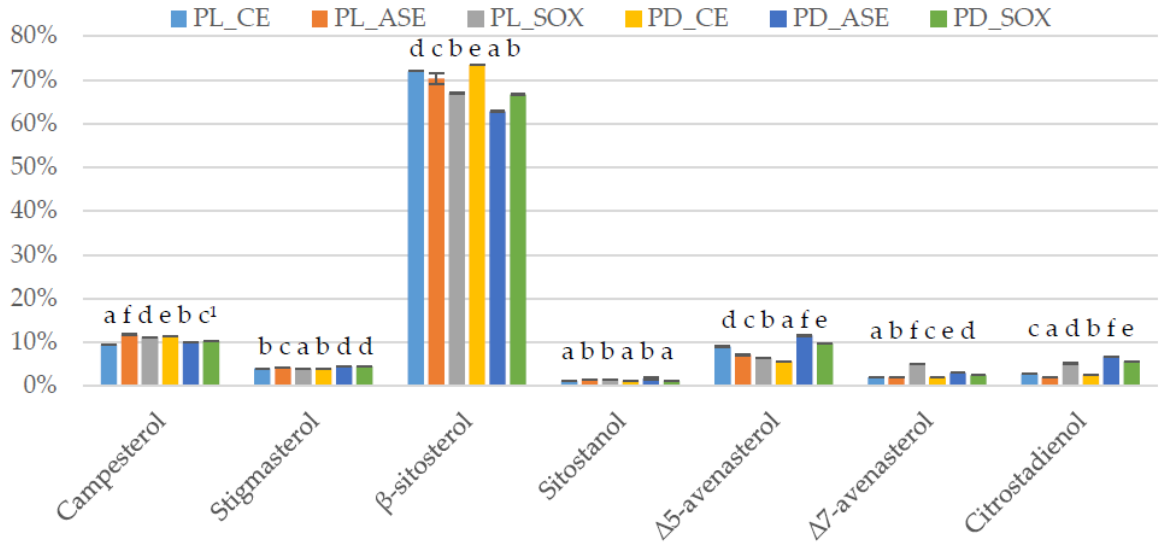

Figure 2. Individual phytosterols (%, mean ± SD) in light and dark pomegranate seed oil (PL and PD) after using different methods of extraction (cold extraction-CE, Accelerated Solvent Extraction-ASE, Soxhlet extraction-SOX). Different letters indicate that the samples are significantly different at $p < 0.05$ for each type of phytosterols.

Due to the high content of unsaturated fatty acid, PSO exhibits desirable nutritional and medical properties, although it would be vulnerable to oxidation [5].

The results obtained with PDSC confirm the low oxidative stability of PSO. Microencapsulation techniques are the methods that scientists believe can be used to increase the oxidative stability of the oil [5]. The addition of pomegranate peel extract to the oil can also have a significant positive impact on improvement of the quality and stability parameters of pomegranate seed oil [6].

5. Conclusions

The type of method used to extract the PSO has an effect on yield, sterol content and fatty acid composition. The ASE method produces an oil containing a lot of unsaturated fatty acids, but the amount of extracted oil is lower compared to other methods.

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