Comparison of Different Methods of Extraction for Pomegranate Seeds †

Joanna Bryś 1,*, Marko Obranović 2, Maja Repajić 2, Klara Kraljić 2, Dubravka Škevin 2, Andrzej Bryś 3, Agata Górska 1, Ewa Ostrowska-Ligeza 1 and Magdalena Wirkowska-Wojdyła 1

1 Department of Chemistry, Institute of Food Sciences, Warsaw University of Life Sciences, 02-787 Warsaw, Poland; agata_gorska@sggw.edu.pl (A.G.); ewa_ostrowska_ligeza@sggw.edu.pl (E.O.-L.); magdalena_wirkowska@sggw.edu.pl (M.W.-W.)
2 Department of Food Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, 10000 Zagreb, Croatia; mohran@pfb.hr (M.O.); maja.repajic@pfb.unizg.hr (M.R.); kkraljic@pfb.hr (K.K.); dskevin@pfb.hr (D.Š.)
3 Department of Fundamental Engineering, Institute of Mechanical Engineering and Energetics, Warsaw University of Life Sciences, 02-776 Warsaw, Poland; andrzej_brys@sggw.edu.pl

* Correspondence: joanna_brys@sggw.edu.pl; Tel.: +48-22-593-7615
† Presented at the 1st International Electronic Conference on Food Science and Functional Foods, 10–25 November 2020; Available online: https://foods_2020.sciforum.net/.

Abstract: Pomegranate seed oil (PSO) has attracted considerable attention because of its potentially beneficial biological effects. This oil consists of a high content of polyunsaturated fatty acids, mainly conjugated type, punicic acid. Punicic acid has antioxidant and anticancer activity. The aim of this research was to compare the properties of PSO obtained by cold extraction, Soxhlet extraction and Accelerated Solvent Extraction (ASE). The 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 hydrolyze 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 by using 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 happens to be a symbol of longevity and fertility. It is often recognized as the earliest and most 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 seeds has also attracted considerable attention because of its potentially beneficial health effects: the 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 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 ranges from 12 to 20% of dry weight [3–6].

A number of techniques have been reported for the 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, 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 were 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 method 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

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 5000 rpm. Supernatant was decanted and the pellet was returned to the flask and extracted one more time 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.

2.3. 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].

2.4. 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 Bryš et al. [16].

2.5. Determination of Sterols

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

2.6. 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. For each sample, the output was automatically recalculated and presented as the amount of energy per gram.

2.7. 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 (Statistical Graphics Corporation, Warrenton, VA, USA). 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 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 an 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 also contains 4.1 to 5.9% of mono-unsaturated fatty acids (MUFA) including oleic acid, and 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, also belonging 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 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 a     | 0.71 ± 0.03 b   |
| PL_CE         | 13.34 ± 0.30 c     | 3.81 ± 0.25 a   |
| 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 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. The percentage of the stearic acids in the sn-2 position of TAG in all PSO exceeded 33%, which means that it is located mainly in the internal position of TAG, whereas the percentage of palmitic acid in the 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, 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.
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 phytosterol.

4. Discussion

The oil content in PSO, according to the literature data, ranges from 12 to 20% [5,6]. The obtained results were consistent with the literature data. The extraction method, among others, 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 of PSO. According to scientists, the method with the application of CO$_2$ allowed the acquisition of 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.

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 the 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.

Author Contributions: Conceptualization, J.B., M.O.; methodology, J.B., M.O., K.K., D.Š., M.R., A.G., E.O.-L. and M.W.-W.; investigation, J.B., A.B., M.O., and M.R.; formal analysis, J.B., M.O., K.K., D.Š. and M.R.; writing—original draft preparation, J.B., M.O., K.K., D.Š., A.G., A.B.; writing—review and editing, J.B., A.G., M.O., D.Š.; supervision, D.Š., A.G.; funding acquisition, J.B. All authors have read and agreed to the published version of the manuscript.
Funding: This research was funded by the Scholarship Fund of Warsaw University of Life Sciences for PhD students and young academic teachers (Własny Fundusz Stypendialny Szkoły Głównej Gospodarstwa Wiejskiego w Warszawie).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Melisa Trputec for technical support in the laboratory.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mahesar, S.A.; Kori, A.H.; Sherazi, S.T.H.; Kandhro, A.A.; Laghari, Z.H. Pomegranate (Punica granatum) Seed Oil. In Fruit Oils: Chemistry and Functionality: Ramadan, M., Ed.; Springer: Cham, Switzerland, 2019; pp. 691–710, doi:10.1007/978-3-030-12473-1_37.

2. Białełk, A.; Stawarska, A.; Bodecka, J.; Białełk, M.; Tokarz, A. Pomegranate seed oil influences the fatty acids profile and reduces the activity of desaturases in livers of Sprague-Dawley rats. Prostaglandins Other Lipid Mediat. 2017, 131, 9–16, doi:10.1016/j.prostaglandins.2017.05.004.

3. Caligiani, A.; Bonzanini, F.; Palla, G.; Martina, C.; Renato, B. Characterization of a Potential Nutraceutical Ingredient: Pomegranate (Punica granatum L.) Seed Oil Unsaponifiable Fraction. Plant Foods Hum. Nutr. 2010, 65, 277–283, doi:10.1007/s11130-010-0173-5.

4. Natolino, A.; Da Porto, C. Supercritical carbon dioxide extraction of pomegranate (Punica granatum L.) seed oil: Kinetic modeling and solubility evaluation. J. Supercrit. Fluids 2019, 151, 30–39, doi:10.1016/j.supflu.2019.05.002.

5. Yekdane, N.; Goli, S.A.H. Effect of Pomegranate Juice on Characteristics and Oxidative Stability of Microencapsulated Pomegranate Seed Oil Using Spray Drying. Food Bioprocess. Technol. 2019, 12, 1614–1625, doi:10.1007/s11746-019-02325-8.

6. Drinić, Z.; Mudrić, J.; Ždunić, G.; Bigović, D.; Menković, N.; Savinik, K. Effect of pomegranate peel extract on the oxidative stability of pomegranate seed oil. Food Chem. 2020, 333, 127501, doi:10.1016/j.foodchem.2020.127501.

7. Krauljalis, P.; Venskutonis, R.P.; Pukaliskas, A.; Kazernavicut, R. Accelerated solvent extraction of lipids from Amaranthus spp. seeds and characterization of their composition. J. Food Sci. Technol. 2013, 54, 528–534, doi:10.1016/j.jfsw.2013.06.014.

8. Chena, W.; Liu, Y.; Song, L.; Sommerfeld, M.; Hu, Q. Automated accelerated solvent extraction method for total lipid analysis of microalgae. Algal Res. 2020, 51, 102080, doi:10.1016/j.algal.2020.102080.

9. Liu, X.; Fan, K.; Song, W.; Wang, Z. Optimization of accelerated solvent extraction of fatty acids from Coix seeds using chemometrics methods. Food Meas. 2019, 13, 1773–1780, doi:10.1016/j.foodmeas.2019-0095-7.

10. Ahmad, R.; Ahmad, N.; Aljamea, A.; Abuthayn, S.; Aqeel, M. Evaluation of solvent and temperature effect on green accelerated solvent extraction (ASE) and UHPLC quantification of phenolics in fresh olive fruit (Olea europaea). Food Chem. 2020, 128248, doi:10.1016/j.foodchem.2020.128248.

11. International Organization for Standardization. ISO 665: Oilseeds—Determination of Moisture and Volatile Matter Content; International Organization for Standardization: Geneva, Switzerland, 2000.

12. International Organization for Standardization. ISO 659: Oilseeds—Determination of Oil Content (Reference Method); International Organization for Standardization: Geneva, Switzerland, 2009.

13. Lohani, U.C.; Fallahi, P.; Muthukumarappan, K. Comparison of Ethyl Acetate with Hexane for Oil Extraction from Various Oilseeds. J. Am. Oil Chem. Soc. 2015, 92, 743–754, doi:10.1007/s11774-015-2644-1.

14. International Organization for Standardization. ISO 5509: Animal and Vegetable Fats and Oils—Preparation of Methyl Esters of Fatty Acids; International Organization for Standardization: Geneva, Switzerland, 2001.

15. International Organization for Standardization. ISO 5508: Animal and Vegetable Fats and Oils—Analysis by Gas Chromatography of Methyl Esters of Fatty Acids; International Organization for Standardization: Geneva, Switzerland, 1996.

16. Bryš, J.; Vaz Flores, I.F.; Górska, A.; Wirkowska-Wojdyłowa, M.; Ostrowska-Ligęza, E.; Bryš, A. Use of GC and PDSC methods to characterize human milk fat substitutes obtained from lard and milk whey oil mixtures. J. Ther. Anal. Calorim. 2017, 130, 319–327, doi:10.1007/s10973-017-6452-8.

17. International Organization for Standardization. ISO 12228-1: Determination of Individual and Total Sterols Contents—Gas Chromatographic Method—Part 1: Animal and Vegetable Fats and Oils; International Organization for Standardization: Geneva, Switzerland, 2014.

18. Habibnia, M.; Ghavami, M.; Ansaripour, M.; Vosough, S. Chemical evaluation of oils extracted from five different varieties of iranian pomegranate seeds. J. Food Biosci. Technol. 2012, 2, 35–40.

19. Fernandes, L.; Pereira, J.A.; Lope‘z-Corte, I.; Salazar, D.M.; Ramalhosa, E.; Casal, S. Fatty acid, vitamin E and sterols composition of seed oils from nine different pomegranate (Punica granatum L.) cultivars grown in Spain. J. Food Compos. Anal. 2015, 39, 13–22.

20. Silva, L.O.; Ranquine, L.G.; Monteiro, M.; Torresa, A.G. Pomegranate (Punica granatum L.) seed oil enriched with conjugated linolenic acid (cLNA), phenolic compounds and tocopherols: Improved extraction of a specialty oil by supercritical CO2. J. Supercrit. Fluids 2019, 147, 126–137, doi:10.1016/j.supflu.2019.02.019.