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Potential application of pomegranate seed oil oleogels based on monoglycerides, beeswax and propolis wax as partial substitutes of palm oil in

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Title: Potential application of pomegranate seed oil oleogels based on monoglycerides, beeswax and propolis wax as partial substitutes of palm oil in functional chocolate spread

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Abstract: In this research, the effectiveness of pomegranate seed oil oleogel as a partial replacement of fat phase in chocolate spread was studied. Monoglyceride (MG), beeswax (BW) and propolis wax (PW) have been used as structuring agents at 5 g/100 g concentration to gel pomegranate seed oil. The oleogels were then combined with palm oil at 1:1 ratio. Different techniques, including polarized light microscopy, synchrotron XRD, mechanical analyses, and oil binding capacity were used to study the physical and mechanical properties of the palm oil-oleogel systems and produced chocolate spreads. Results highlighted that MG, BW and PW chemical nature led to the formation of different crystalline network in palm oil-oleogel systems. Chocolate spreads containing palm oil-oleogels showed an increase in the mechanical parameters in the order of $PW < BW < MG$. This trend might be attributed to the chemical composition of oleogelators and physical bonds formed in the samples. During storage, crystal transformation in MG and structural reorganization in waxes (PW and BW) samples showed a gradual decrease and increase in hardness, respectively. These findings could provide useful information in the application of pomegranate seed oil oleogel for novel confectionery products engineering.

Dear Editor,

I would like to submit to your attention the manuscript entitled “Potential application of pomegranate seed oil oleogels based on monoglycerides, beeswax and propolis wax as partial substitutes of palm oil in functional chocolate spread ” for consideration for publication on LWT Food science and Technology.

The aim of the present research was In this research, the effectiveness of pomegranate seed oil (PSO) oleogel as a partial replacement of fat phase in chocolate spread was studied. Monoglyceride (MG), beeswax (BW) and propolis wax (PW) have been used as structuring agents at 5.0 % (w/w) concentration to gel PSO. The oleogels (OG) were then combined with palm oil (PO) at 1:1 ratio (PO-OG). Different techniques, including polarized light microscopy, synchrotron XRD, rheological and mechanical analyses and oil binding capacity (OBC) were used to study the physical and mechanical properties of the PO-OG systems and produced chocolate spreads. Results highlighted that MG, BW and PW chemical nature led to the formation of different crystalline network in PO-OG systems. Chocolate spreads containing PO-OG showed an increase in the mechanical and rheological parameters in the order of $PW < BW < MG$. This trend might be attributed to the chemical composition of organogelators and physical bonds formed in the samples. During storage, crystal transformation in MG and structural reorganization in waxes (PW and BW) samples showed a gradual decrease and increase in hardness, respectively. These findings could provide useful information in the application of PSO oleogel for novel confectionery products engineering. We would greatly appreciate your comments on the paper.

Best regards

Sonia Calligaris

Dear Editor,

Please find the revised version of our manuscript LWT-D-17-01422. We have endeavoured to take into account or to respond to the Reviewer's comments as indicated below.

We hope that this response is satisfactory and that the manuscript will be suitable for publication in LWT- Food Science and Technology.

Best regards,
Sonia Calligaris

Reviewers' comments:

Reviewer #1: The manuscript "Potential application of pomegranate seed oil oleogels based on monoglycerides, beeswax and propolis wax as partial substitutes of palm oil in functional chocolate spread" study the use of pomegranate seed oil based oleogels as fat replacer in chocolate spread. The first section of the paper deals with preparation and characterization of pomegranate based oleogel using MAG and two types of Waxes. While the second sections deals with the preparation and characterization of chocolate spread using 50% oleogel and 50% palm oil. This is a very interesting topic with high possibility to be implemented in the industry.

One of the most important objectives in this study was to evaluate POG as oil phase for oleogel systems however the authors chose to compare between MAG and wax oleogel properties. The comparison of MAG or waxes using different oil phase could have been more appropriate in order to accomplish the research goal. In addition, the scientific discussion lacks some important features required to understand the results and conclusions.

Therefore, I would recommend accepting this paper after major revision. More specifically the authors should address the following issues:

We thank the reviewer for the appreciation of the manuscript. We have considered the revisions suggested to improve the quality of the manuscript.

Introduction:

The introduction section is written well however some information is missing. Please elaborate on the subjects below:

1. Why does saturated fats are bad? **Line 61-63**
2. The relation between fat crystals and texture. **Line 70-72**
3. What are oleogels? **Line 74-79**

4. what are MAGs and WAXS? Molecular structure, properties etc. **Line 83-88**
5. Why did you choose MAGs and waxes? **Line 80-83**
6. Why did you choose 5% oleogelator? **Line 104-106**

The above modifications have been inserted in introduction.

In line 65 the authors wrote: "... substitution of plastic fats with liquid..." what do you mean by plastic fats. Fats are not plastic at all!!!!

The sentence has been modified.

Material and methods:

In the material section, the type of the third FA component of MAG is missing (line 93: "38.8% C...")

Added in the text (line 114).

Oleogel preparation

The oleogel-PO preparation procedure involve heating and cooling twice (first for the oleogel and then with the PO). It is not necessary to heat twice. The oleogel-PO could be prepared just by mixing everything together heating and cooling. Same comment to the chocolate spread sample.

The heating/cooling cycles may affect the oil oxidation level, which can directly affect the oleogel strength (see Gravelle et al. Carbohyd Polym 135 (2016) 169-179 and Gravelle et al. Food Res Int 48 (2012) 578-583). This issue might be the reason for the different gel strength you obtained in the different formulations (with and without PO and in the chocolate form). Please check these issues with respect to your formulations. You can address this issue by producing oleogel-PO formulation directly (mixing everything together heating and cooling) and see if you receive different properties.

We agree with the reviewer that that heating/cooling cycles may affect the oil oxidation level. However, this kind of procedure was previously used also by other authors (Doan et al., 2017) and Manzocco et al., 2014) and allowed to simulate the possible usage of the oleogel-PO mixtures as solid fat substitute (i.e. palm oil and other shortenings) that have to be melted before usage in the formulation.

To reduce as much as possible oxidation we maintained samples at high temperature as less as possible, just the time needed to obtain a homogeneous sample. We add this information in the manuscript.

It should be noted that different authors showed that during oleogel preparation at high temperatures no significant changes in peroxide value were noted (Da Pieve et al. 2011. Food Research International. 44, 2978-2983) (Ogutcu et al. 2015. International Journal of Food Science and Technology. 50, 404-412). Moreover, Yilmaz and Öğütçü. (2014. Journal of the American Oil Chemists' Society. 91, 1007-1017) showed that oleogels containing hazelnut oil and monoglycerides and beeswax as structurant were very stable against oxidation.

In any case, we performed the test suggested by the reviewer and no significant differences in the rheological parameters were observed by preparing the samples in one step.

Chocolate spread preparation

In the chocolate spread preparation procedure: "...then dry ingredients were dispersed in the molten fat phase and manually stirred with a spatula until a homogeneous paste-like spread was obtained..." this is not a consistent procedure. How can you be sure you mixed the same each formulation? The mixing rate and time should be measurable.

We added the information in the text (line 131-139).

Rheology

How did you mount the sample in the Rheometer? It is well established that crystal based oleogels are shear sensitive thus spreading the oleogel on the rheometer plate and lowering the PP to specific gap could produce reasonable shear effects, which in turn affect your rheology results.

Information was added in the text (line 176-178).

Results and discussion

Oleogel-PO characterization

The authors discuss the different properties of different PSO/PO formulations with MAG, BW and PW. The author should have compared these formulations to other known oil oleogels. The main idea is the use of PSO which is unique, therefore the benefit of using this oil with respect to known oils is needed.

The benefit is mainly linked to its healthy properties of PSO, as evidence in the introduction and conclusions (line 64-65, 100-101 and 362-364). The approach applied in this paper to delivery PSO could be probably used also to delivery other oils.

In the XRD analysis (line 204-206) you refer to shorter and longer d spacing of beta form. What do you mean by that? Beta form has specific short spacing peaks. Please elaborate.

Sentence was modified (line 218-222).

In order to analyze the XRD data better the authors should discuss the neat MAG, PO, and Waxes peak footprint with respect to what you receive in the oleogel form (add the peak values to the text).

The sentence was modified accordingly (line 219-221 and 224-226).

In line 211-213 the authors refer to different molecular composition of PW which induce different short spacing peaks. What do you mean by different molecular composition? How does it affect the short spacing of XRD?

Sentence was modified and new reference was added (line 230-231).

The hardness analysis results are very low. The load cell used is 100N, which usually has sensitivity of <0.25% meaning the instrument can measure values with 0.25N accurately. Your values are in the range of the instrument sensitivity! Go to lower load cell to measure this kind of gels.

We agree with the reviewer. Since the rheological behavior describe better oleogel samples, results related to firmness were erased along with the relevant discussion in the text.

Line 224-226: very strong gels can be produced only be van der Waals interactions. Therefore the explanation referring the weaker gels of Waxes is not sufficient.

We modified the text accordingly to the different compatibility between wax and PO (line 240-242).

Line 235-236: the statement is referring to particle size, but you did not measure it. If you refer to the data from the images please specify it.

Sentence was modified (line 252).

Line 246-248: the palm oil can affect the PW crystallization as well...

Modifications have been inserted in the text (line 263-264).

Physical properties of chocolate spread

In the XRD analysis of the chocolate spread, the authors should present all the peaks obtained for the specific formulations. In addition, control results for chocolate spread without the oleogel should also be added to the data. Does the sugar content produces specific peaks (I guess yes)? Some of the peaks discussed in the text are not presented in the table (line 268-269 and line 259). Add the control sample in the table as well.

The XRD patterns related to sugar and MG-OG containing chocolate spread (chosen as an example) are shown in Figure S1 (see supporting information). We subtract sugar crystals peaks as well as those of PO containing chocolate spread from diffraction patterns of chocolate spread containing oleogels to highlight only signals generated by oleogelators. Text was modified to improve clarity (line 268-274). In Table 2, data are referred to the peaks attributed to the oleogelators.

The explanation for the MAGs sample decrease in hardness does not sound reasonable. The MAG lamellar structure age to a more stable structure, however it is not obvious that this stabilization process decreases the crystal network strength. Meaning the aging not necessarily

affect the network. More experimental work or literature review is required in order to verify this issue.

The new reference has been included in the text (line 314-318). According to these authors, (Bin Sintang et al. 2017. *European Journal of Lipid Science and Technology*, 119(3), 1–14), oleogels prepared with MGs suffer from storage stability issues owing to the slow polymorphic transition that leads to the formation of gritty β -crystals on aging.

In line 317-318 the authors refer to "... a good tolerance to the rate of deformation...". The reviewer is not familiar with such characteristic with respect to the G' higher than G'' . Please explain what you meant.

All the samples showed gel-like behavior as confirmed by G' values which were much higher than the corresponding G'' values (line 334).

Reviewer #2: Authors have evaluated the possibility of partially replacing palm oil food formulation with oleogels structured using 3 different structuring agents.

The formulation they have chosen is a chocolate paste which has been previously evaluated as a model formulation (authors need to discuss this paper in introduction, *Food Funct.*, 2014, 5, 645-652).

The reference has been included in the text (line 91-92).

What I find missing is the rationale behind selecting the 3 structuring agents. I advise authors to clearly justify why they were chosen.

In the introduction more details on the oleogelators selected have been included. The selection is based to the good performances in gelling oils of MG and waxes observed by other authors in literature and reported in the text.

The results suggests that MG oleogels gave the best results in terms of firms but the the polymorphic changes over storage seems to have compromised the structure (as is usually expected from MG oleogels, <http://onlinelibrary.wiley.com/doi/10.1002/ejlt.201500517/abstract>).

The reference has been included in the text (line 317-318).

EDITORIAL COMMENTS:

Please carefully check the author guidelines, some necessary changes are given below

- all text should be adjusted to the left margin

The modification has been done.

- avoid excessive use of abbreviations in the abstract. No need to define e.g. OBC (not used).

The modification has been done.

- L.24, elsewhere: do not use % as a concentration unit - replace here by 5.0 g/100 g. % may however be used as relative measure for example fatty acid composition.

The modification has been done.

- L.104 and elsewhere: space between number and °C

The modification has been done.

- L.157, elsewhere: give city of residence, country of instrument provider

The modification has been done.

- L.162 vs. L.171: check style consistency, either 10000 or 10,000 but do not use both styles

The modification has been done.

- L.184, elsewhere: Fig. not Figure

The modification has been done.

- Table footnotes: I suggest that you remove the a,b,c letters from sample identification. This may be a source of confusion (see statistical letters). Replace by e.g. "MG, Monoglyceride based oleogel-palm oil mixture (1:1); BW, Bees wax ..."

The modification has been done.

- Remove line numbers from figures

The modification has been done.

- Fig. 3: Symbol explanations must be removed from the figure and specified in the figure caption. Write out the abbreviations in the captions please.

The modification has been done.

- We evaluated oleogel as a partial replacement of palm oil in chocolate spread.
- The results showed that structuring agent influenced chocolate spread hardness.
- Chocolate spread containing monoglyceride was harder than beeswax and propolis wax.
- During storage, crystal transformation or reorganization changed the hardness.
- Oleogel-palm oil mixture displaced a good oil binding capacity in chocolate spread.

1 **Potential application of pomegranate seed oil oleogels based on monoglycerides, beeswax**
2 **and propolis wax as partial substitutes of palm oil in functional chocolate spread**

3

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21 **Abstract**

22 In this research, the effectiveness of pomegranate seed oil oleogel as a partial replacement of
23 fat phase in chocolate spread was studied. Monoglyceride (MG), beeswax (BW) and propolis
24 wax (PW) have been used as structuring agents at 5 g/100 g concentration to gel pomegranate
25 seed oil. The oleogels were then combined with palm oil at 1:1 ratio. Different techniques,
26 including polarized light microscopy, synchrotron XRD, mechanical analyses, and oil binding
27 capacity were used to study the physical and mechanical properties of the palm oil-oleogel
28 systems and produced chocolate spreads. Results highlighted that MG, BW and PW chemical
29 nature led to the formation of different crystalline network in palm oil-oleogel systems.
30 Chocolate spreads containing palm oil-oleogels showed an increase in the mechanical
31 parameters in the order of PW < BW < MG. This trend might be attributed to the chemical
32 composition of oleogelators and physical bonds formed in the samples. During storage,
33 crystal transformation in MG and structural reorganization in waxes (PW and BW) samples
34 showed a gradual decrease and increase in hardness, respectively. These findings could
35 provide useful information in the application of pomegranate seed oil oleogel for novel
36 confectionery products engineering.

37 **Keywords:** Pomegranate seed oil, Chocolate spread, Oleogel, Wax

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47 **1. Introduction**

48 The health benefits associated with the consumption of poly unsaturated fatty acid (PUFAs)
49 and especially conjugated linoleic acid (CLA) and conjugated linolenic acid (CLN) are well
50 demonstrated in the literature (Chojnacka et al., 2016). One of the most important oils
51 containing conjugated fatty acids is pomegranate seed oil (PSO). The specific trienoic fatty
52 acid found in PSO is puniceic acid (PA) which is a polyunsaturated fatty acid (18:3) also called
53 9-cis, 11-trans, 13-cis, octadecatrienoic acid. PA is referred as a "super CLA" whose effect is
54 even higher than that of an ordinary CLN (Aruna, Venkataramanamma, Singh, & Singh,
55 2016). This unique conjugated fatty acid presents several potential health benefits such as
56 cholesterol lowering, antidiabetic, anti-inflammatory and anticarcinogenic properties
57 (Kýralan, Gölükçü, & Tokgöz, 2009).

58 The introduction of PSO, and thus PA, in fat-based foods as substitute (partial or total) of
59 saturated fats could allow the development of new products with improved health properties,
60 not only thanks to the presence of essential fatty acids but also by reducing the total level of
61 saturated fats. As well known, consumption of saturated fatty acids at higher amount leads to
62 negative health implications, including obesity, cardiovascular diseases (CVD), high
63 cholesterol, cancer and type II diabetes (Micha & Mozaffarian, 2010).

64 Chocolate spreads could be a good candidate for the enrichment with PSO accompanied with
65 the reduction of saturated fats in the formulation. Chocolate spread is a suspension of solid
66 particles embedded into a fat crystal network (i.e. higher than 40 g/100 g) composed of
67 saturated fats such as palm oil, coconut oil and cocoa butter. This product is widely used
68 directly by consumers as delicious confectionary product or by the food industry as filling
69 ingredient in other formulations such as biscuits and cakes (Manzocco, Calligaris, Camerin,
70 Pizzale, & Nicoli, 2014). The sensory performances of chocolate spreads are strictly related to
71 the presence of a fat crystal network providing texture, mouthfeel and flavor to the product

72 (Marangoni et al., 2012). Thus, the partial substitution of solid hardstock fat with liquid oil
73 could greatly affect the chocolate spread performances and thus the product quality (Anese et
74 al., 2016; Doan et al., 2016). An emerging strategy is to substitute solid hardstock fat rich in
75 saturated fats with unsaturated oils solidified thanks to molecules forming self-assembly
76 networks. These systems are called oleogels that are self-standing, thermoreversible,
77 anhydrous and viscoelastic materials structured by a three-dimensional supramolecular
78 network of self-assembled molecules with limited solubility in an organic liquid (Co &
79 Marangoni, 2012; Patel & Dewettinck, 2016). A wide number of different oleogelators has
80 been proposed in the literature to gel oils. Among others, natural waxes and saturated
81 monoglycerides have been indicated as particularly promising for food applications (Da
82 Pieve, Calligaris, Co, Nicoli, & Marangoni, 2010; Doan, Van de Walle, Dewettinck, & Patel,
83 2015; Öğütçü & Yilmaz, 2015). Waxes deriving from different natural sources, such as
84 candelilla wax, carnauba wax, rice bran wax, beeswax and propolis wax, contain long-chain
85 fatty acid esters able to crystalize forming a three-dimensional network entrapping liquid oil
86 (Doan et al., 2015; Fayaz, Goli, Kadivar, et al., 2017). Similarly, monoglycerides (MGs) are
87 able to self-assemble into inverse bilayer nanostructures organized at micro-level into lamellar
88 platelets that finally interact immobilizing liquid oil (Da Pieve et al., 2010).

89 Recently, the application of oleogels in food products for the reduction of saturated fatty acids
90 as well as the delivery of essential polyunsaturated fatty acids has been studied in ice cream
91 (Zulim Botega, Marangoni, Smith, & Goff, 2013), chocolate containing products (Doan et al.,
92 2016), bakery products (Patel et al., 2014; Stortz & Marangoni, 2013; Anese et al., 2016),
93 frankfurters (Zetzl, Marangoni, & Barbut, 2012) and margarine (Hwang et al., 2013). Results
94 demonstrated that the novel structural approach could be a pursuable strategy even though a
95 careful re-examination of formulation and processing conditions should be done. In the
96 development of the new functional products, the knowledge of the structural behavior of the

97 oleogel in the formulation is fundamental to obtain a product with adequate textural and
98 sensory properties.

99 Based on these considerations, the aim of this research was to investigate the application of
100 pomegranate seed oil oleogels as co-structurants with palm oil (PO) in chocolate spreads **to**
101 **obtain a functional food enriched with PSO with a reduced saturated fat content.** To this
102 **purpose**, PSO oleogels containing 5 **g/100 g** of saturated monoglyceride, beeswax and
103 propolis wax were considered as partial replacers of palm oil (50% of replacement) in
104 chocolate spreads. It should be noted that the oil **gelling** properties of these three structurants
105 **at the selected concentration** have been already demonstrated by different authors (Da Pieve et
106 al., 2010; Fayaz, Goli, & Kadivar, 2017; Fayaz, Goli, Kadivar, et al., 2017; Yilmaz & Öğütçü,
107 2014).

108 **The PO-oleogel mixtures as well as** chocolate spreads containing **these** mixtures were
109 characterized by using different techniques, including polarized light microscopy, synchrotron
110 X-ray diffraction, mechanical and rheological analyses and oil binding capacity.

111 **2. Materials and methods**

112 *2.1. Materials*

113 MyverolTM saturated monoglyceride (MG) (fatty acid composition: 1.4% C_{14:0}, 59.8% C_{16:0},
114 38.8% C_{18:0}; melting point 68.05 ± 0.5 °C) was from Kerry Bioscience (Bristol, United
115 Kingdom), beeswax (BW) and propolis was from Espadana Mokamel Co. (Isfahan, Iran),
116 pomegranate seed oil (PSO) from Dastchinali Co. (Isfahan, Iran) and palm oil (PO) (saturated
117 fatty acid 48.45% w/w; melting point 26.89 ± 0.10 °C) was from Unigrà (Conselice, Italy).
118 Sugar and defatted cocoa powder were purchased in a local market. Propolis wax (PW) was
119 **extracted from** propolis according to Fayaz, Goly & Kadivar (2017) procedure.

120 *2.2. Oleogel preparation*

121 The oleogelators were dispersed in PSO at a concentration of 5 g/100 g. The mixture was
122 heated at 80 °C under magnetic stirring in a temperature controlled water bath. Just after
123 oleogelator melting, the mixtures were maintained at 80 °C for at least 10 min and
124 subsequently quiescently cooled at 20 °C to allow gel formation. Samples were stored at 20
125 °C for 24 h before analysis.

126 2.3. Oleogel-palm oil mixture preparation

127 The oleogels were mixed with PO (1:1 w/w) at 80 °C in a temperature controlled water bath
128 under magnetic stirring until the melting of all components and then cooled and stored at 20
129 °C for 24 h before analysis and usage in chocolate spreads.

130 2.4. Chocolate spread preparation

131 The chocolate spreads were prepared according to the methodology reported by Manzocco et
132 al. (2014) and Doan et al. (2016), with minor modifications. In particular, the samples
133 consisted of 40 g/100 g fat, 50 g/100 g sugar with fineness of 0.25 mm or lower (sugar
134 grounded and sifted with a 60-mesh sieve) and 10 g/100 g cocoa powder. Fat phase (oleogel-
135 palm oil mixture) was heated at 80 °C until complete melting in a temperature controlled
136 water bath, then dry ingredients were dispersed in the molten fat phase and manually stirred
137 with a spatula for 2 min until a homogeneous paste-like spread was obtained while
138 maintaining temperature at 80 °C. Chocolate spreads were cooled to 20 °C. Analyses were
139 carried out 24 h after preparation and during storage at 20 °C.

140 2.5. Analytical determinations

141 2.5.1. Polarized light microscopy

142 The microstructure of oleogels and OG-PO mixtures were studied using a polarized light (PL)
143 optical microscope (Leica DM 2000, Leica Microsystems, Heerburg, Switzerland) connected
144 with a Leica EC3 digital camera (Leica Microsystems). One drop of sample was placed in the
145 middle of a glass slide and a glass cover slip was centered above the drop. The samples were
146 analyzed at 20 °C using a 200× magnification. Micrographs were acquired and processed
147 using the application software Leica Suite LAS EZ (Leica Microsystems).

148 *2.5.2. Synchrotron XRD Analysis*

149 Synchrotron X-ray diffraction patterns were recorded at the X-ray diffraction beam-line 5.2 of
150 the Synchrotron Radiation Facility Elettra in Trieste (Italy). The X-ray beam emitted by the
151 wiggler source on the Elettra 2 GeV electron storage ring was monochromatized by a Si (111)
152 double crystal monochromator, collimated by a double set of slits giving a spot size of 0.2 ×
153 0.2 mm. A drop of sample was lodged into a nylon pre-mounted cryoloop 20 μm (0.7–1.0
154 mm) (Hampton Research HR4-965, AlisoVeiijo, CA, USA). Analyses were performed at 20
155 °C controlling the temperature by a 700 series cryocooler (Oxford Cryosystems, Oxford, UK).
156 Data were collected at a photon energy of 8.856 keV ($\lambda = 1.4 \text{ \AA}$), using a 2M Pilatus silicon
157 pixel X-ray detector (DECTRIS Ltd., Baden, Switzerland). Bidimensional patterns collected
158 with Pilatus were calibrated by means of a LaB₆ standard and integrated using the software
159 FIT2D (Hammersley, Svensson, Hanfland, Fitch, & Hausermann, 1996). The indexing of the
160 XRD patterns obtained by the crystalline phases was performed using the programs Winplotr
161 (Roisnel & Rodríguez-Carvajal, 2001) and Checkcell (Laugier & Bochu, 2000).

162 *2.5.3. Firmness*

163 Firmness was measured using an Instron 4301 (Instron International Ltd. High Wycombe,
164 UK). 20 g of sample were poured into cylindrical containers and penetration test was
165 performed by applying a cross-head speed of 25 mm/min. Samples were penetrated for 5 mm,

166 by using a 6.2 mm diameter cylinder mounted on a 100 N compression head. Force-distance
167 curves were obtained from the penetration tests and firmness was computed as the maximum
168 force applied to the samples by using the software Automated Materials Testing System
169 (version 5, Series IX, Instron Ltd., High Wycombe, UK).

170 *2.5.4. Rheological measurement*

171 Rheological properties of samples were determined using a Haake Rheostress 6000 (Thermo
172 Scientific, Rheostress, Haake, Germany) with application software Haake Rheowin
173 v.4.60.0001 (Thermo Fisher Scientific). The measurements were performed in a 40-mm
174 parallel-plate geometry system at 20 °C. Aliquots of about 4–5 g of sample were transferred
175 on the temperature-controlled measuring plate and the measuring gap was set at 1,000 µm.
176 These operations were conducted gently to minimize any possible damage of the crystalline
177 network. Samples were left to rest 5 min after loading before testing to relax and reach a
178 constant temperature. Stress sweeps measurement (stress 0.01–100 Pa for oleogel-palm oil
179 mixture and 10–10,000 Pa for chocolate spread) were carried out at a frequency of 1 Hz to
180 determine the linear viscoelastic region (LVR). The critical stress of chocolate spread was
181 determined as the stress where G' value decreased of more than 10% the values recorded in
182 the LVR. Frequency sweep with frequency scan from 0.1 to 10 Hz for OG-PO mixtures and
183 0.1 to 100 Hz for chocolate spreads were used with a fixed stress value included in the LVR.

184 *2.5.6. Oil binding capacity*

185 The oil binding capacity (OBC) of OG-PO mixtures and respective chocolate spreads were
186 determined following the methodology described by Manzocco et al. (2014). One gram of
187 molten sample was weighted into a microtube and centrifuged at 10,000 rpm for 15 min using
188 a microcentrifuge (Mikro 120, Hettich Zentrifugen, Andreas Hettich GmbH and Co,

189 Tuttligen, Germany). The released oil was computed as percentage ratio between the mass of
190 expressed oil over the total mass of sample.

191 *2.6. Statistical analysis*

192 All data were obtained from at least three measurements from two experiment replications
193 and reported as mean value \pm standard deviation. Data analysis was accomplished using the
194 Statistical Analysis System (SAS) (9.2 versions, SAS Institute, North Carolina, USA).
195 Analysis of Variance (ANOVA) was carried out and statistical differences among means were
196 determined by the least significant difference (LSD) method at significance level of 5%.

197 **3. Results and discussion**

198 *3.1. Physico-chemical properties of oleogel-palm oil mixtures*

199 The microstructure of PO, oleogels and their mixtures is reported in Fig. 1. Palm oil crystals
200 showed a spherulitic crystal morphology which is typical of PO crystallization (Doan et al.,
201 2016); whereas platelet-like crystals were observed in all oleogel samples, in good agreement
202 with literature data (Da Pieve et al., 2010; Fayaz, Goli, Kadivar, et al., 2017). The differences
203 in number and size of crystals among oleogels can be due to the different oleogelation process
204 of MG, BW and PW. In particular, the ability of MG to gel vegetable oils is associated to the
205 formation of lamellar phases. This organization is stabilized by hydrogen bonds between the
206 secondary and primary –OH groups of the MGs throughout the bilayers (Lopez-Martínez,
207 Charó-Alonso, Marangoni, & Toro-Vazquez, 2015). However, crystallization of beeswax and
208 propolis wax in liquid oils generally results in particulate gels where the network based on
209 van der Waals interactions of crystals and crystalline aggregates immobilize the liquid oil into
210 a three-dimensional structure (Doan et al., 2015).

211 The presence of palm oil affected the crystal morphology detected in oleogel-palm oil
212 mixtures (Doan et al., 2016). In MG-containing OG-PO mixture, both PO spherulitic crystals
213 and platelet-like crystals of MG were observed. On the other hand, platelet-like crystals
214 dominated the morphology of wax oleogel-PO mixtures.

215 To understand the crystal polymorphs formed in the samples, synchrotron XRD analyses were
216 performed (Fig. 2). In Fig. 2a, MG-based OG-PO mixture showed different peaks in the wide
217 angle X-ray diffraction region (WAXD) at 4.55, 4.35, 4.20, 3.86 and 3.73 Å along with two
218 peaks at 42.04 and 47.83 Å in the small angle X-ray diffraction region (SAXD). These results
219 suggest the presence of two β polymorphic forms, differing in lamellar thickness. In
220 particular, by comparing XRD patterns of neat MG and PO with those of mixtures containing
221 oleogels, it is possible to attribute the peak at 47.83 Å to MG crystals, whereas the peak at
222 42.04 Å to PO crystals. Wax-based OG-PO mixtures exhibited two peaks in the SAXD
223 region: one related to the PO crystalline phase (around 41.5 Å) and the other to the wax
224 crystals (65.78 and 65.39 Å for BW and PW, respectively) (Fig. 2b and c). The same
225 interplanar distances were noted in the XRD pattern of neat BW and PW, in agreement with
226 data reported by Fayaz, Goli, Kadivar, et al. (2017). In the WAXD region, besides the peaks
227 related to the β -crystalline phase of PO, the presence of two weak peaks at 4.14 and 3.73 Å
228 indicated the crystallization of waxes into β' form. Appearance of other peak at 3.84 Å in PW
229 based OG-PO mixtures compared to BW can be a direct consequence of different molecular
230 composition in PW, as highlighted in a previous work by our group (Fayaz, Goli, Kadivar, et
231 al., 2017).

232 Rheological parameters (storage G' and loss G'' moduli) of the systems were reported in Table
233 1. Results revealed that the rheological parameters of MG-based palm oil blend were higher
234 than those of wax-based mixtures. The different rheological behavior of the systems can be
235 attributed to the structure of the network formed in MG-PO blend in comparison to that

236 formed in waxes and PO systems. It can be hypothesized that in MG containing sample strong
237 reciprocal interactions between MG and PO crystals formed, leading to a close three-
238 dimensional network with strong gel property. Moreover, the formation of hydrogen bonds
239 expected between MG crystals could further improve the formation of a strong network. On
240 the contrary, in wax-palm oil blends the crystal interactions could be less strong due to the
241 lower chemical compatibility between wax and PO (Hwang, Kim, Singh, Winkler-Moser, &
242 Liu, 2012).

243 Despite the similar crystal morphology and polymorphism of beeswax and propolis wax,
244 beeswax-based palm blend showed higher rheological parameter values compared to propolis
245 wax. This difference can be attributed to the different chemical components of the waxes.
246 According to our previous work, propolis wax is expected to form a weaker gel in comparison
247 to beeswax. This has been attributed to the presence of different phenolic compounds in
248 propolis wax (pinostrobin, galagin, pinocembrin, and naringenin) that can disturb the
249 formation of the crystalline network (Fayaz, Goli, Kadivar, et al., 2017). As shown in Table 1,
250 interestingly, the OBC of MG and BW are not statistically different, which is not expected in
251 the light of rheological characteristics of the system. The obtained data again confirmed the
252 influence of crystal morphology on the oil binding capacity of systems. Blake, Co &
253 Marangoni (2014) compared the oil binding capacity of rice bran wax (RBW), candelilla wax
254 and carnauba wax oleogels. They found that candelilla wax oleogel exhibited higher oil
255 binding capacity compared to RBX, despite their spherical crystal morphology. They believed
256 that spherical crystals may adsorb higher oil quantities onto their surface. In addition, these
257 crystals dispersed throughout the oil phase, reduced the pore area and increased the tortuosity
258 of the network. Similarly, long needle-like structures of BW and PW formed good crystalline
259 matrices that mesh well at the inter-crystalline interfaces and enables to entrap a large volume
260 of liquid oil in the crystalline network (more than 90% OBC) (Doan et al., 2015; Fayaz, Goli,

261 Kadivar, et al., 2017). However, PW-oleogel-palm oil mixture exhibited a lower OBC. This
262 may be explained by the influence of PW on the crystallization of palm oil and vice versa. PW
263 and PO together may have effect on the formation, packing, and morphology of crystal
264 network leading to a reduced ability to entrap oils.

265 3.2. Physical properties of chocolate spread

266 In the second part of this research, OG– PO blends were used to formulate chocolate spreads.
267 The polymorphic structures of chocolate spread were evaluated during 23 days of storage at
268 20 °C. Diffraction patterns were rich of peaks due to the concurrent presence of fat crystals
269 (PO and oleogelators) as well as sugar crystals. As an example, the XRD pattern of MG-OG
270 containing chocolate spread sample after 1 day of storage at 20 °C is reported in Fig. S1 (see
271 supporting information) along with that of crystalline sugar. As expected, sugar showed a
272 high number of peaks in the 7.56 – 1.87 Å region due to the well-ordered structure of its
273 crystals. These peaks were thus subtracted within those of PO from chocolate spread
274 diffraction patterns to highlight only signals generated by the oleogelator crystals. Table 2
275 shows *d*-spacing related to gelator crystals in chocolate spreads. MG based chocolate spread
276 showed a peak at 46.83 Å (which appeared as a shoulder of the more defined PO related peak
277 at 42.14 Å) in the SAXD region that became more defined after 23 days of storage without
278 any significant change in *d*-spacing between the crystallographic reflecting planes (data not
279 shown). In the WAXD region a peak at 4.17 Å was observed corresponding to the presence of
280 MG α -form crystals. After 23 days, a series of new peaks related to MG (3.98, 3.89 and 3.78
281 Å) appeared in the WAXD region, highlighting the presence of a β -form. It can be inferred
282 that MG polymorphic transformation occurred during storage.
283 For BW and PW based chocolate spreads, a clear peak at 4.14 Å with a shoulder at 3.72 Å
284 was noted in the WAXD region. These signals could be related to the β' -form of waxes. These

285 peaks were still present after 23 days; however, the change in *d*-spacing of the wax lamellar
286 structure strongly suggested some important structural modifications or reorganization during
287 storage (Table 2).

288 These complex structural modifications had a significant effect on texture and rheological
289 properties of chocolate spreads. Table 3 shows the firmness of chocolate spread prepared with
290 OG-PO mixtures. As expected, samples resulted to be self-standing chocolate spreads with
291 different firmness depending on the oleogels used for their preparation. As can be seen from
292 Table 3, MG chocolate spread was harder than BW and PW. This is in agreement with the
293 aforementioned discussion on physical properties of MG based OG-PO mixture that showed
294 higher rheological parameters in comparison to wax based systems. The hardness of all
295 chocolate spreads was measured during storage at 20 °C. When comparing the different
296 samples, the firmness of both wax containing chocolate spreads gradually increased over
297 time; whereas, MG containing samples slightly decreased during storage at 20 °C.

298 The solid crystal network is a dynamic entity undergoing many changes during storage (Doan
299 et al., 2015). The increase in network hardness of wax-based chocolate spreads during storage
300 can be attributed to the reorganization of wax crystals in post-production isothermal
301 crystallization.

302 The aforementioned decrease of firmness in MG containing spread can be attributed to MG
303 structural changes during storage. According to Chen & Terentjve (2009), MG has different
304 phase behavior in hydrophobic solvents. Below the isotropic-lamellar transition temperature,
305 the inverse lamellar phase with hexagonal ordering is formed. In reverse lamellar ordering,
306 intramolecular hydrogen bonds were formed in a bulk state. By cooling down below the
307 crystallization point of MG, “sub-alpha” crystalline with orthorhombic chain packing in the
308 unit cell can be emerged with no visible change in hydrogen bonding. During aging,
309 intermolecular hydrogen bonds form between 1-hydroxy glycerol groups and causes the

310 segregation of chiral (D and L) isomers and the MG molecules rearrange into the most
311 thermodynamically stable structure of the β -crystal. Therefore, the highly ordered packing of
312 aged structures weakened the lamellar network scaffold and phase separation between solid
313 MG crystals and liquid oil takes place. These changes result in the collapse of the gel-network
314 with important modifications in system mechanical properties during aging. Other literature
315 also confirmed that, oleogels prepared with MGs could undergo polymorphic transformation
316 during storage from the sub- α or α -form (depending on storage temperature) to β polymorph
317 (Bin Sintang, Rimaux, Van de Walle, Dewettinck, & Patel, 2017; Lopez-Martínez et al.,
318 2015). This means that the decrease in hardness of MG chocolate spread over time may be
319 attributed to changes in hydrogen bonds and phase behavior of MG during storage time.

320 The stability of chocolate spreads under stress sweep is determined by the critical stress value
321 that is the first point where the curve of G' begins to vary (10%) from the G' value in linear
322 viscoelastic region (LVR). Critical stress represents the onset of nonlinearity which shows the
323 structure breakdown necessary for flowing to initiate (Doan et al., 2015). As shown in Fig. 3,
324 beyond the critical stress value which is 1419, 790.6 and 654.1 Pa for MG, BW and PW
325 respectively, a rapid decrease in G' values can be observed. That could be the result of
326 permanent deformation due to the breakage of intermolecular forces holding up the structure.

327 It can be concluded that MG chocolate spread showed a stronger structure than BW followed
328 by PW, in accordance with OG-PO results. Moreover, in chocolate spreads, the presence of
329 sugar and cocoa powder increased the amount of solids in the systems, and the interactions
330 between the -OH groups of MG crystals and the free -OH groups of sugar crystals led to a
331 stronger network in MG chocolate spread (Doan et al., 2016).

332 The frequency sweeps used to investigate the deformation behavior of chocolate spread
333 samples within the LVR is shown in Fig. 3b and Table 4.

334 All the samples showed a gel-like behavior as confirmed by G' values, which were much
335 higher than the corresponding G'' values (Fig. 3b). The chocolate spread formed by MG had
336 the highest G' value (49.09×10^5 Pa) as compared to the others (Table 4); however, BW and
337 PW oleogel containing chocolate spreads exhibited the same gel strength with G' values
338 higher than 22×10^5 Pa.

339 Moreover, the stored samples were also periodically tested using oscillatory rheological
340 measurements to evaluate any structural changes during 30 days of storage. MG sample
341 showed a slight decrease in both storage and loss moduli. On the other hand, BW sample had
342 significantly higher G' and G'' values compared to those recorded after 1 day of storage,
343 which confirmed firmness results. The rheological properties of PW also did not significantly
344 change over time.

345 Table 5 represents the OBC of MG, BW, and PW PO-OG containing chocolate spreads. All
346 samples showed a high OBC since oil release upon centrifugation was lower than 6%. This
347 result confirmed the high capability of OG-PO mixture to retain oil and their high physical
348 stability during storage in chocolate spread formulation. The relative OBC value differences
349 among samples highlight the importance of chemical composition of gelators on the
350 crystallization behavior and stability of the continuous fat mixture (Doan et al., 2016).

351 4. Conclusion

352 The results of the present study indicate that different MG, BW, and PW crystalline structures
353 in PSO resulted in different physico-chemical properties of oleogel-palm oil mixture and
354 deriving chocolate spreads. The interactions between the MG aggregated particles with PO
355 crystals had high influence on the formation of the strong oleogel-palm oil blend. However,
356 the lower chemical compatibility between wax and PO led to weaker systems (lower
357 rheological values). MG containing chocolate spread showed a higher hardness than that of

358 wax based chocolate spread. Changes in hydrogen bonds and polymorphism of MG led to a
359 reduction of chocolate spread firmness. However, the reorganization of BW and PW crystals
360 during storage increased the network hardness of wax-based chocolate spreads. In all cases,
361 OG-PO mixture displayed a good oil binding property and it is a good candidate for fat phase
362 substitute in chocolate spread formulation to lower the saturated fatty acid content. At the
363 same time, the presence of PSO in the formulation allowed to obtain a product with enhanced
364 functional properties.

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368

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449

450 **Tables**

451 Table 1. Storage (G') and loss (G'') moduli recorded at 1 Hz and oil binding capacity (OBC)
452 of MG, BW and PW based OG-PO mixture (1:1).

453

Sample	$G'(\text{Pa})\times 10^4$	$G''(\text{Pa})\times 10^4$	OBC (%)
454 MG	^A 30.57±0.33	^A 6.86±0.27	^A 93.07±0.15
455 BW	^B 6.33±0.09	^B 1.50±0.07	^A 96.34±3.47
456 PW	^C 4.44±0.15	^C 0.82±0.01	^B 70.73±1.97

457 Capital letters compare the three types of samples in the same column ($p \leq 0.05$).

458 Data are expressed as means \pm standard deviations of triplicate determinations.

459 MG, Monoglyceride based oleogel-palm oil mixture (1:1); BW, Beeswax based oleogel-palm
460 oil mixture (1:1); PW, Propolis wax based oleogel-palm oil mixture (1:1).

461

462 Table 2. *d*-spacing of XRD peaks related to gelators in chocolate spreads recorded in the
 463 SAXD and WAXD region after 1 and 23 days of storage at 20 °C.

Chocolate spread	1 day		23 days	
	<i>d</i> -spacing (Å) in SAXD region	<i>d</i> -spacing (Å) in WAXD region	<i>d</i> -spacing (Å) in SAXD region	<i>d</i> -spacing (Å) in WAXD region
MG-OG	46.83	4.17	47.31	3.98, 3.89, 3.78
BW-OG	53.92	4.14, 3.72	69.64	4.14, 3.72
PW-OG	54.21	4.14, 3.72	69.09	4.14, 3.72

464

465 MG-OG, Monoglyceride based oleogel containing chocolate spread; BW-OG, Beeswax based
 466 oleogel containing chocolate spread; PW-OG, Propolis wax based oleogel containing
 467 chocolate spread.

468

469 Table 3. Firmness (N) of MG, BW and PW oleogel containing chocolate spreads during 30
470 days of storage at 20 °C.

Chocolate spread	1day	15 days	30 days
MG-OG	^A 3.742±0.445 ^a	^{AB} 3.577±0.210 ^{ab}	^A 3.375±0.098 ^b
BW-OG	^B 2.388±0.162 ^b	^B 2.496±0.087 ^b	^B 2.749±0.162 ^a
PW-OG	^C 1.886±0.200 ^c	^C 2.141±0.079 ^b	^C 2.416±0.131 ^a

471

472 Capital letters compare the three types of samples in the same column ($p \leq 0.05$).

473 Lower case letters compare the three types of samples in the same row during storage ($p \leq$
474 0.05).

475 Data are expressed as means \pm standard deviations of triplicate determinations.

476 MG-OG, Monoglyceride based oleogel containing chocolate spread; BW-OG, Beeswax
477 based oleogel containing chocolate spread; PW-OG, Propolis wax based oleogel containing
478 chocolate spread.

479

480 Table 4. Storage (G') and loss (G'') moduli of MG, BW and PW oleogel containing chocolate
 481 spreads recorded at 1 Hz frequency during 30 days of storage.

Chocolate spread	1 day		15 days		30 days	
	$G' \times 10^5$	$G'' \times 10^5$	$G' \times 10^5$	$G'' \times 10^5$	$G' \times 10^5$	$G'' \times 10^5$
MG-OG	^A 49.09±0.41 ^a	^A 5.48±0.1 ^a	^A 32.02±1.22 ^b	^A 3.86±0.2 ^b	^A 32.48±1.46 ^b	^A 3.91±0.00 ^b
BW-OG	^B 22.08±0.43 ^b	^B 2.95±0.05 ^b	^B 26.71±0.69 ^a	^A 3.00±0.04 ^b	^A 29.38±1.32 ^a	^B 3.44±0.15 ^a
PW-OG	^B 22.41±0.68 ^a	^B 3.14±0.01 ^a	^C 22.89±0.28 ^a	^A 3.11±0.2 ^a	^B 23.73±0.02 ^a	^B 3.23±0.06 ^a

482

483 Capital letters compare the three types of samples in the same column ($p \leq 0.05$).

484 Lower case letters compare the three types of samples in the same row during storage ($p \leq$
 485 0.05).

486 Data are expressed as means \pm standard deviations of triplicate determinations.

487 MG-OG, Monoglyceride based oleogel containing chocolate spread; BW-OG, Beeswax
 488 based oleogel containing chocolate spread; PW-OG, Propolis wax based oleogel containing
 489 chocolate spread.

490

491 Table 5. OBC (%) of MG, BW and PW oleogel containing chocolate spreads during 30 days
492 of storage at 20°C.

Chocolate spread	1 day	15 days	30 days
MG-OG	^B 94.27±0.82 ^{ab}	^B 93.77±0.48 ^b	^B 95.05±0.17 ^a
BW-OG	^A 98.27±1.45 ^a	^A 98.22±1.52 ^a	^A 99.96±0.05 ^a
PW-OG	^B 93.20±0.97 ^b	^{AB} 95.96±1.53 ^a	^B 93.84±1.54 ^{ab}

494

495 Capital letters compare the three types of samples in the same column ($p \leq 0.05$).

496 Lower case letters compare the three types of samples in the same row during storage ($p \leq$
497 0.05).

498 Data are expressed as means \pm standard deviations of triplicate determinations.

499 MG-OG, Monoglyceride based oleogel containing chocolate spread; BW-OG, Beeswax based
500 oleogel containing chocolate spread; PW-OG, Propolis wax based oleogel containing
501 chocolate spread.

502

Figure Captions

Fig. 1. Polarized light microphotographs (PLM) of oleogels, palm oil and oleogel-palm oil mixture (1:1) samples.

Fig. 2. XRD patterns of MG (a), BW (b) and PW (c) based oleogel-palm oil mixture at 1:1 ratio.

Fig. 3. Stress (a) and frequency sweep (b) curves recorded at 20 °C for MG (square), BW (triangle) and PW (circle) oleogel containing chocolate spreads. G' is shown in solid and G'' is in open symbols.

Figures

Figure 1


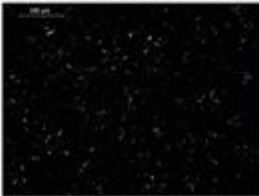
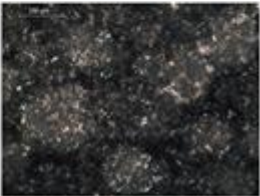




Sample	Oleogel or Palm oil	Oleogel-Palm oil mixture
Palm oil		
MG		
BW		
PW		

Figure 2

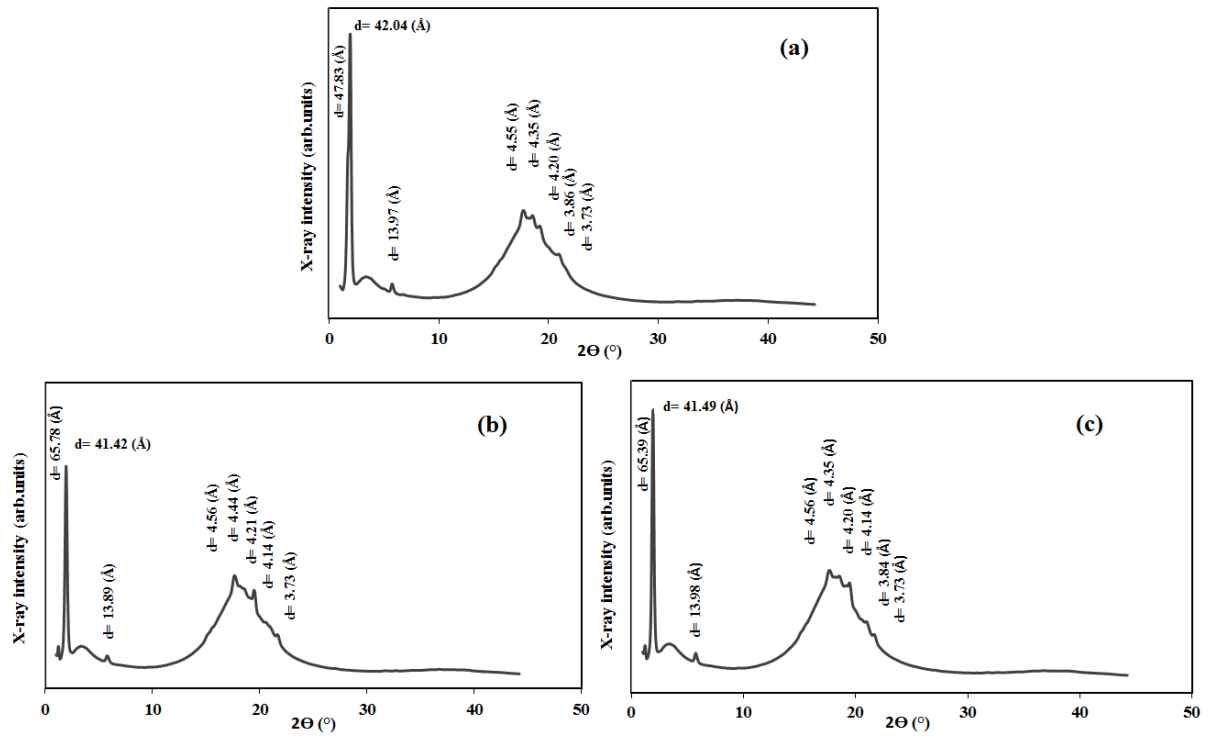
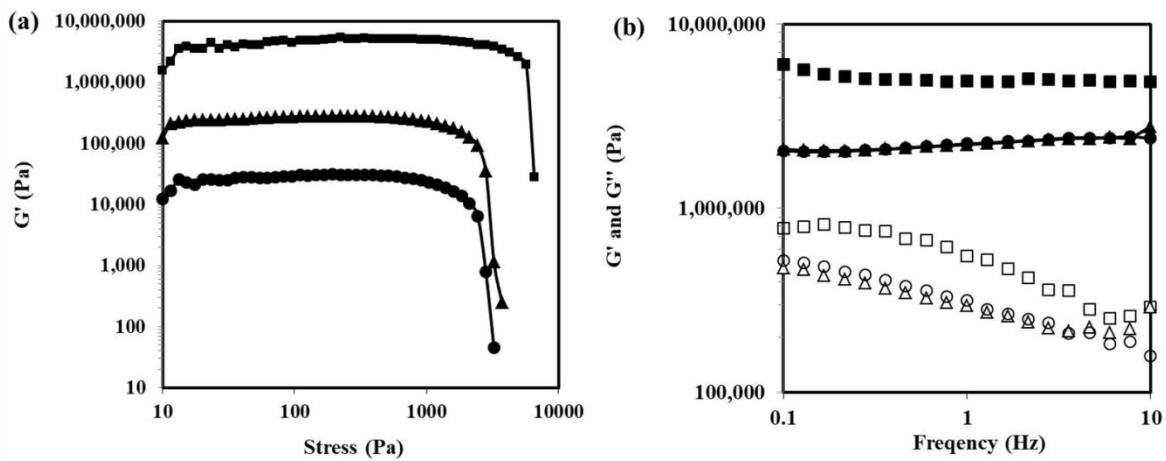
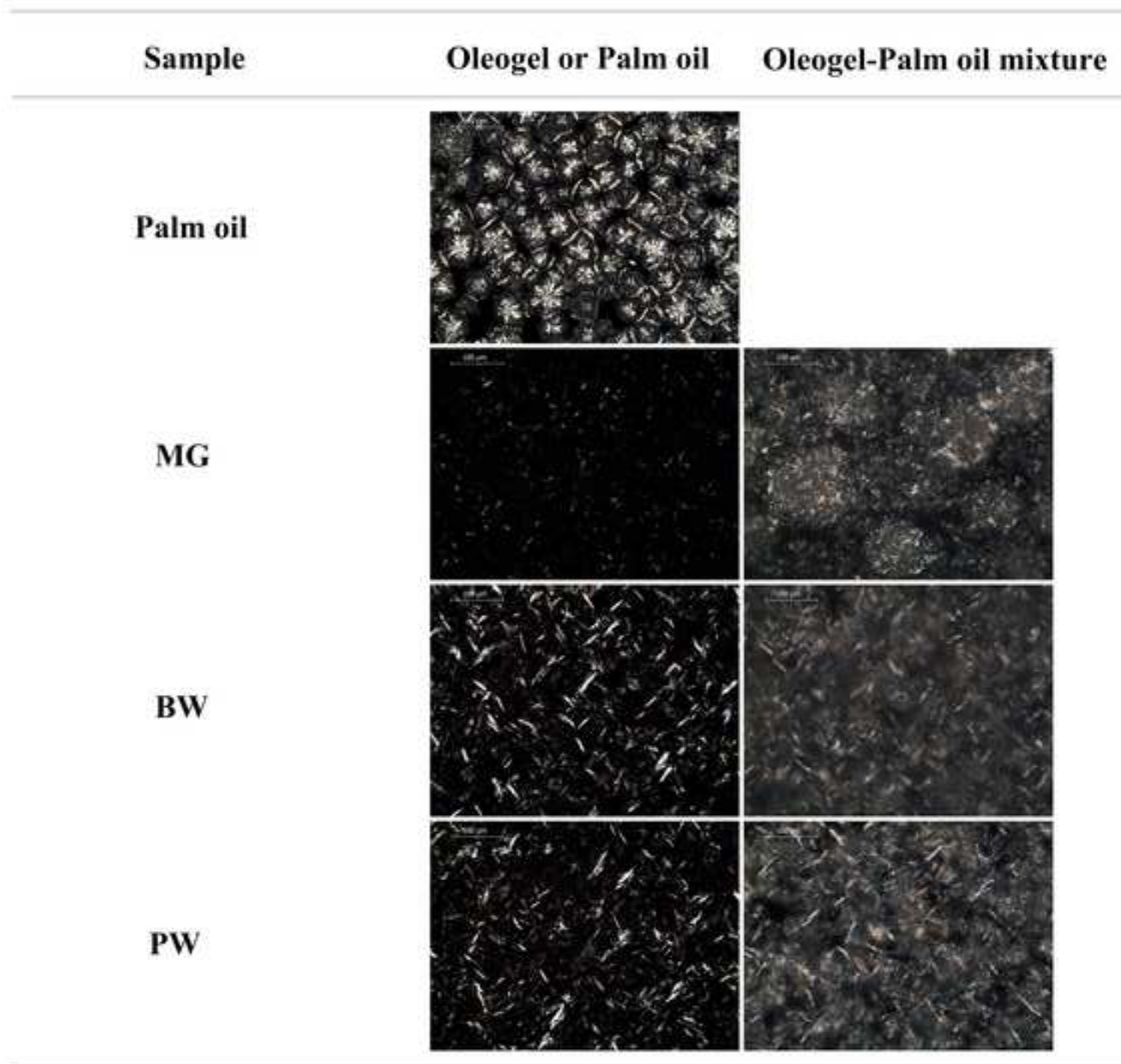


Figure 3



Figure

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Figure

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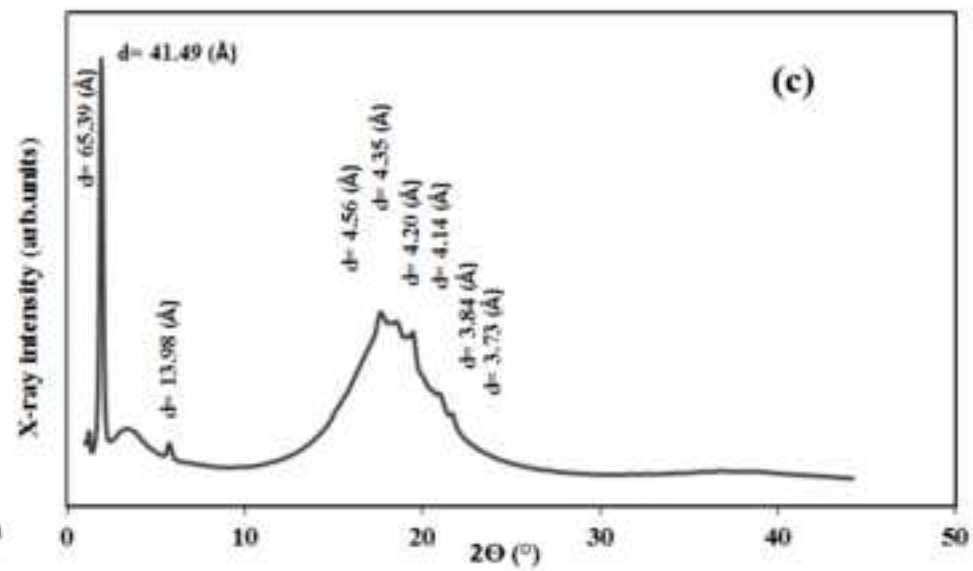
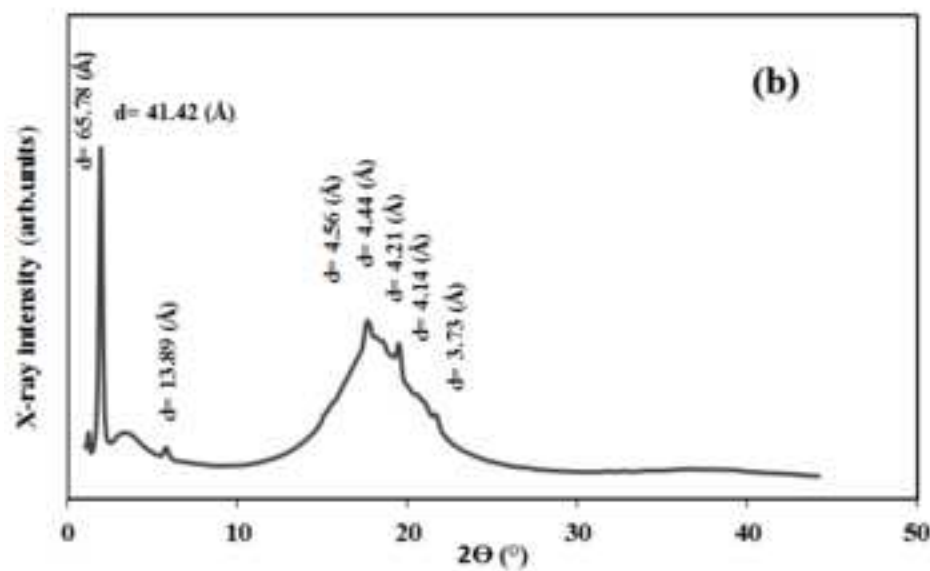
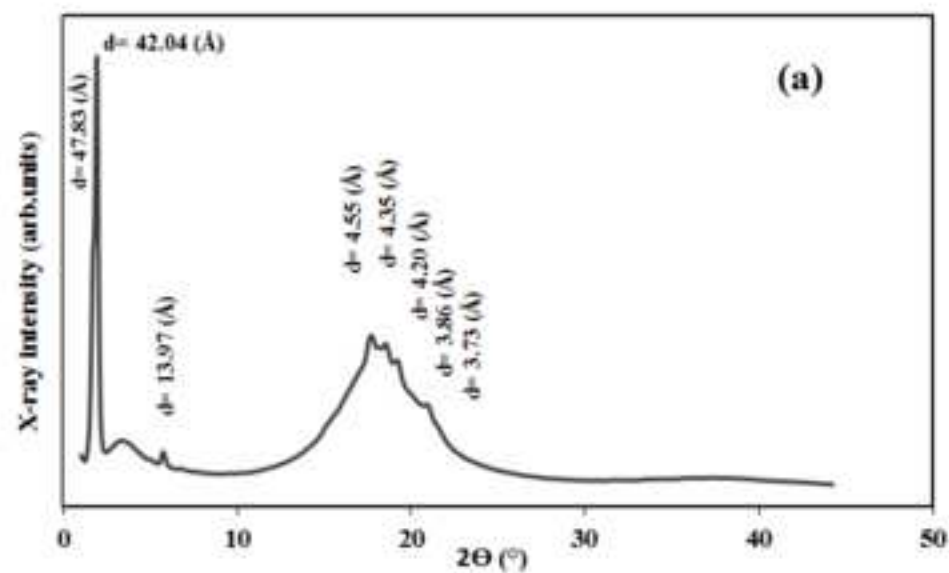
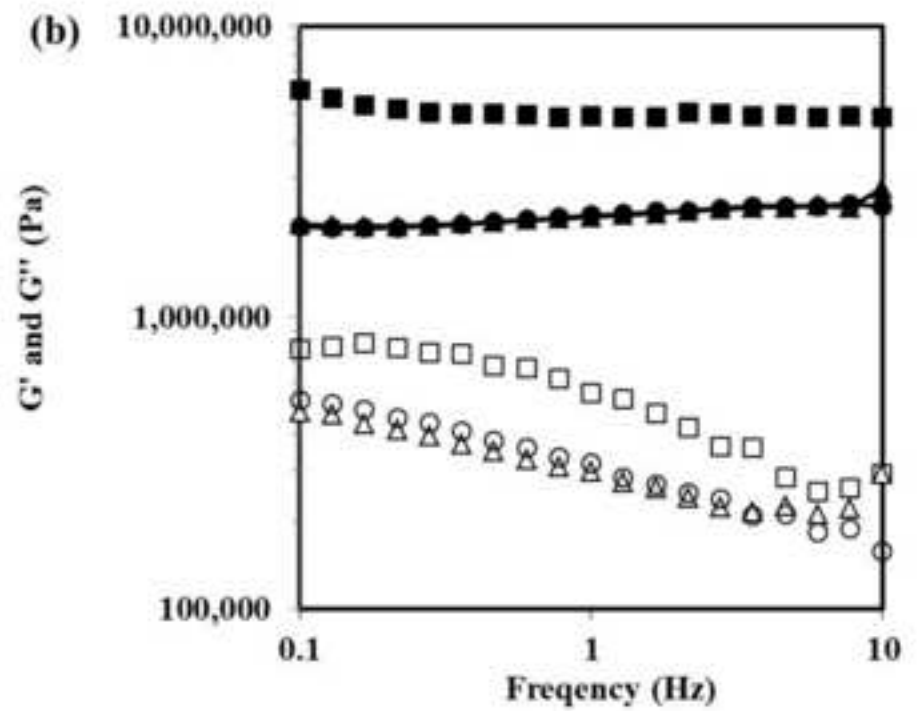
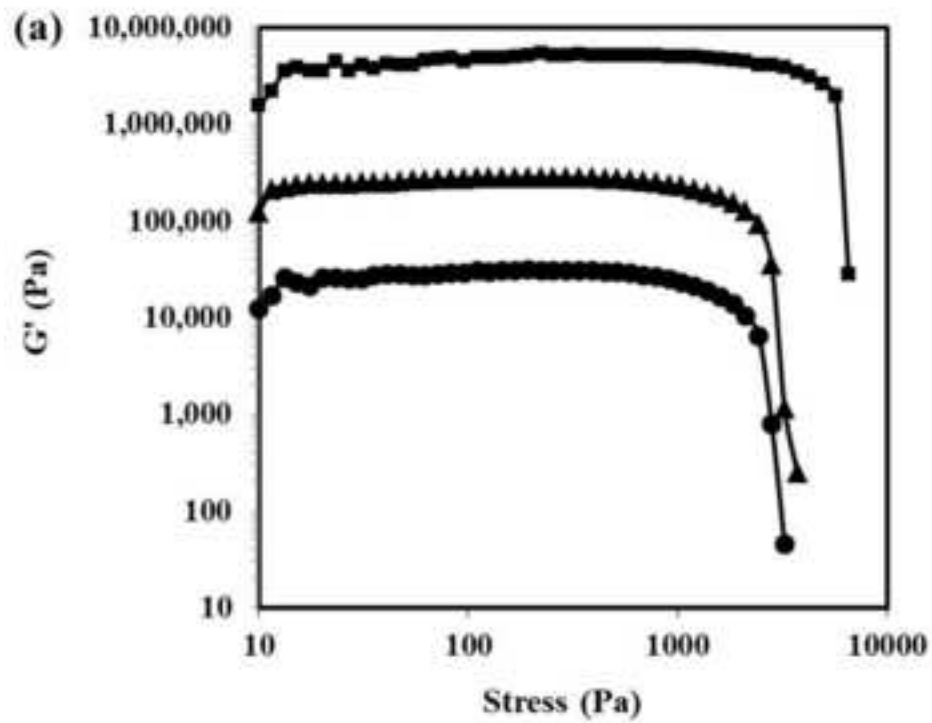


Figure
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Supplementary Material

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