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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

Article Type: Research paper

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

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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.

Cover Letter

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

***Detailed Response to Reviewers**

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 Öğütcü. (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 then 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 abbreviatioons 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.

***Highlights (for review)**

- 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.

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.

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

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Materials

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

2.2. Oleogel preparation

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

2.3. Oleogel-palm oil mixture preparation

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

2.4. Chocolate spread preparation

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

2.5. Analytical determinations

2.5.1. Polarized light microscopy

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

2.5.2. Synchrotron XRD Analysis

Synchrotron X-ray diffraction patterns were recorded at the X-ray diffraction beam-line 5.2 of the Synchrotron Radiation Facility Elettra in Trieste (Italy). The X-ray beam emitted by the wiggler source on the Elettra 2 GeV electron storage ring was monochromatized by a Si (111) double crystal monochromator, collimated by a double set of slits giving a spot size of 0.2 × 0.2 mm. A drop of sample was lodged into a nylon pre-mounted cryoloop 20 μm (0.7–1.0 mm) (Hampton Research HR4-965, AlisoVeijo, CA, USA). Analyses were performed at 20 °C controlling the temperature by a 700 series cryocooler (Oxford Cryosystems, Oxford, UK). Data were collected at a photon energy of 8.856 keV (λ = 1.4 Å), using a 2M Pilatus silicon pixel X-ray detector (DECTRIS Ltd., Baden, Switzerland). Bidimensional patterns collected with Pilatus were calibrated by means of a LaB_6 standard and integrated using the software FIT2D (Hammersley, Svensson, Hanfland, Fitch, & Hausermann, 1996). The indexing of the XRD patterns obtained by the crystalline phases was performed using the programs Winplotr (Roisnel & Rodríquez-Carvajal, 2001) and Checkcell (Laugier & Bochu, 2000).

2.5.3. Firmness

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

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

2.5.4. Rheological measurement

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

2.5.6. Oil binding capacity

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

189 Tuttlingen, 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 ± 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).

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

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

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

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

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

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

3.2. Physical properties of chocolate spread

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

For BW and PW based chocolate spreads, a clear peak at 4.14 Å with a shoulder at 3.72 Å was noted in the WAXD region. These signals could be related to the β′-form of waxes. These

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

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

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

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

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

The stability of chocolate spreads under stress sweep is determined by the critical stress value that is the first point where the curve of G′ begins to vary (10%) from the G′ value in linear viscoelastic region (LVR). Critical stress represents the onset of nonlinearity which shows the structure breakdown necessary for flowing to initiate (Doan et al., 2015). As shown in Fig. 3, beyond the critical stress value which is 1419, 790.6 and 654.1 Pa for MG, BW and PW respectively, a rapid decrease in G′ values can be observed. That could be the result of permanent deformation due to the breakage of intermolecular forces holding up the structure. It can be concluded that MG chocolate spread showed a stronger structure than BW followed by PW, in accordance with OG-PO results. Moreover, in chocolate spreads, the presence of sugar and cocoa powder increased the amount of solids in the systems, and the interactions between the -OH groups of MG crystals and the free -OH groups of sugar crystals led to a stronger network in MG chocolate spread (Doan et al., 2016).

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

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

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

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

4. Conclusion

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

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

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Tables

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

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

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

Data are expressed as means ± standard deviations of triplicate determinations.

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

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	
	d-spacing (Å) in SAXD region	*d*-spacing (Å) in WAXD region	*d*-spacing (Å) in SAXD region	*d*-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

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

Chocolate spread	1day	15 days	30 days
MG-OG	$^{A}3.742\pm0.445^{a}$	$^{AB}3.577\pm0.210^{ab}$	$^{A}3.375\pm0.098^{b}$
BW-OG	$^{B}2.388\pm0.162^{b}$	$^{B}2.496\pm0.087^{b}$	$^{B}2.749\pm0.162^{a}$
PW-OG	$^{C}1.886\pm0.200^{c}$	$^{C}2.141\pm0.079^{b}$	$^{C}2.416\pm0.131^{a}$

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

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

Data are expressed as means ± standard deviations of triplicate determinations.

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

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'×10^5	G"×10^5	G'×10^5	G"×10^5	G'×10^5	G"×10^5
MG-OG	$^{A}49.09\pm0.41^{a}$	$^{A}5.48\pm0.1^{a}$	$^{A}32.02\pm1.22^{b}$	$^{A}3.86\pm0.2^{b}$	$^{A}32.48\pm1.46^{b}$	$^{A}3.91\pm0.00^{b}$
BW-OG	$^{B}22.08\pm0.43^{b}$	$^{B}2.95\pm0.05^{b}$	$^{B}26.71\pm0.69^{a}$	$^{A}3.00\pm0.04^{b}$	$^{A}29.38\pm1.32^{a}$	$^{B}3.44\pm0.15^{a}$
PW-OG	$^{B}22.41\pm0.68^{a}$	$^{B}3.14\pm0.01^{a}$	$^{C}22.89\pm0.28^{a}$	$^{A}3.11\pm0.2^{a}$	$^{B}23.73\pm0.02^{a}$	$^{B}3.23\pm0.06^{a}$

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 ± 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

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

Chocolate spread	1day	15 days	30 days
MG-OG	$^{B}94.27\pm0.82^{ab}$	$^{B}93.77\pm0.48^{b}$	$^{B}95.05\pm0.17^{a}$
BW-OG	$^{A}98.27\pm1.45^{a}$	$^{A}98.22\pm1.52^{a}$	$^{A}99.96\pm0.05^{a}$
PW-OG	$^{B}93.20\pm0.97^{b}$	$^{AB}95.96\pm1.53^{a}$	$^{B}93.84\pm1.54^{ab}$

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

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

Data are expressed as means ± standard deviations of triplicate determinations.

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

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

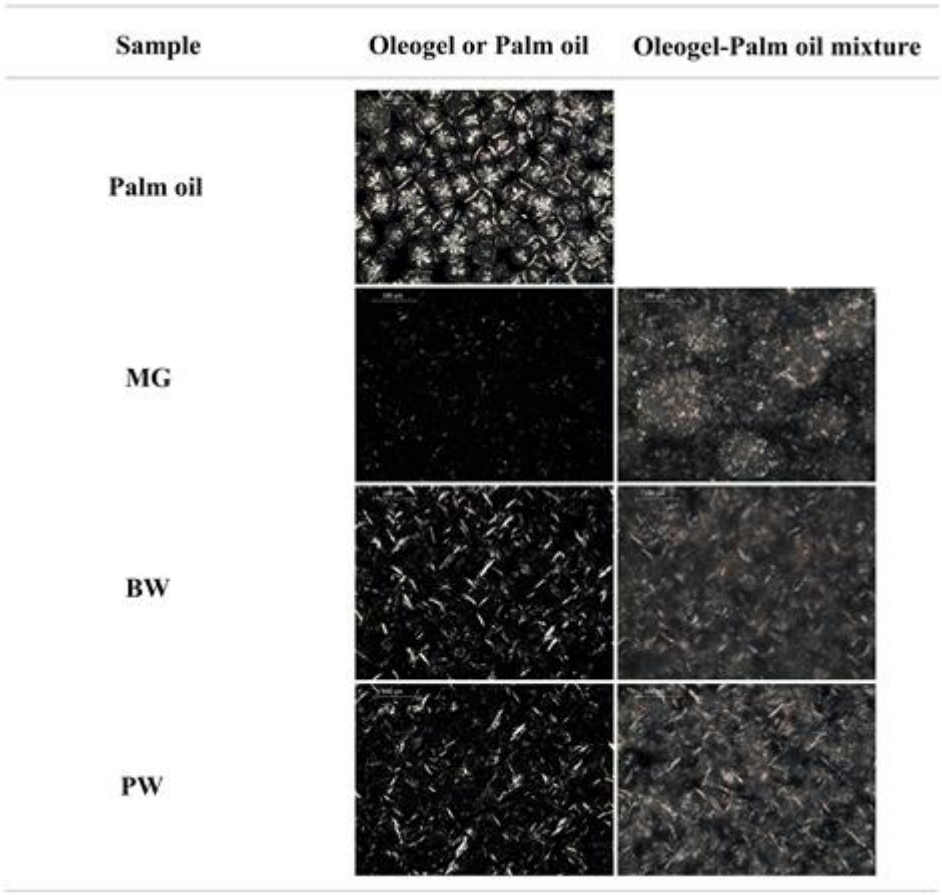
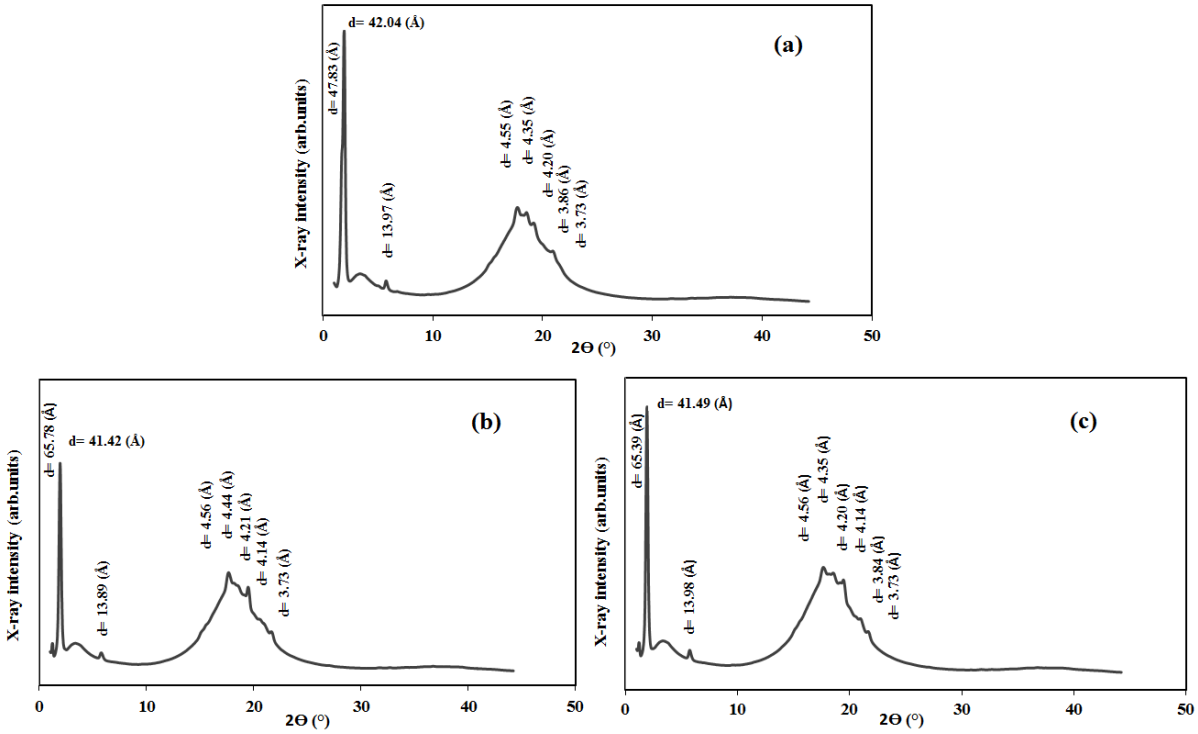


Figure 2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d= 47.83 (Å)
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d= 4.35 (Å)
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d= 3.86 (Å)
d= 3.73 (Å)
X-ray intensity (arb.units)
2Θ (°)
(b)
d= 65.78 (Å)
d= 41.42 (Å)
d= 13.89 (Å)
d= 4.56 (Å)
d= 4.44 (Å)
d= 4.21 (Å)
d= 4.14 (Å)
d= 3.73 (Å)
X-ray intensity (arb.units)
2Θ (°)
(c)
d= 41.49 (Å)
d= 65.39 (Å)
d= 13.98 (Å)
d= 4.56 (Å)
d= 4.35 (Å)
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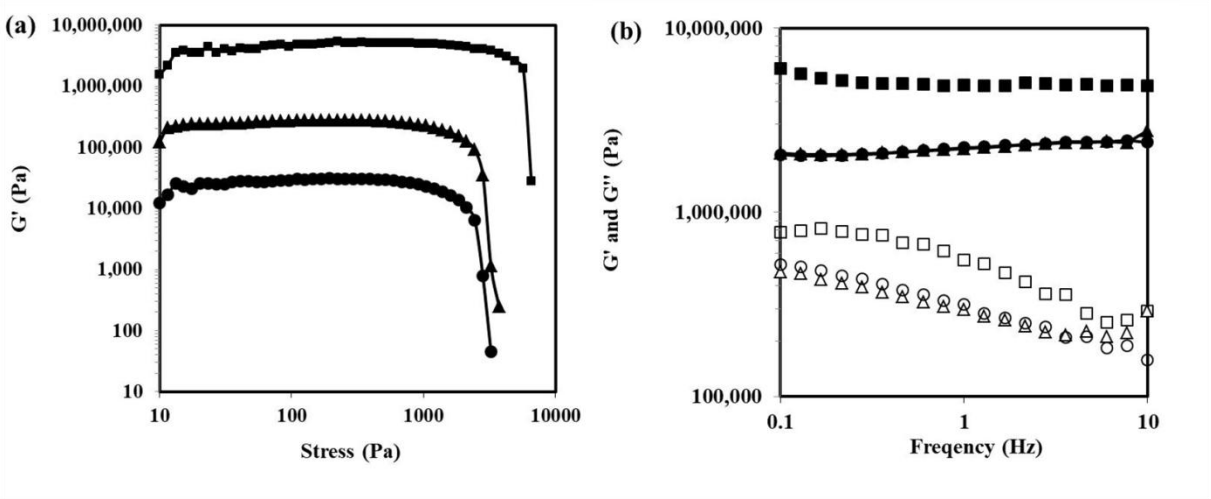
d= 3.73 (Å)
X-ray intensity (arb.units)
2Θ (°)

Figure 3


(a)
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G' (Pa)
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Stress (Pa)
(b)
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Freqency (Hz)

Figure
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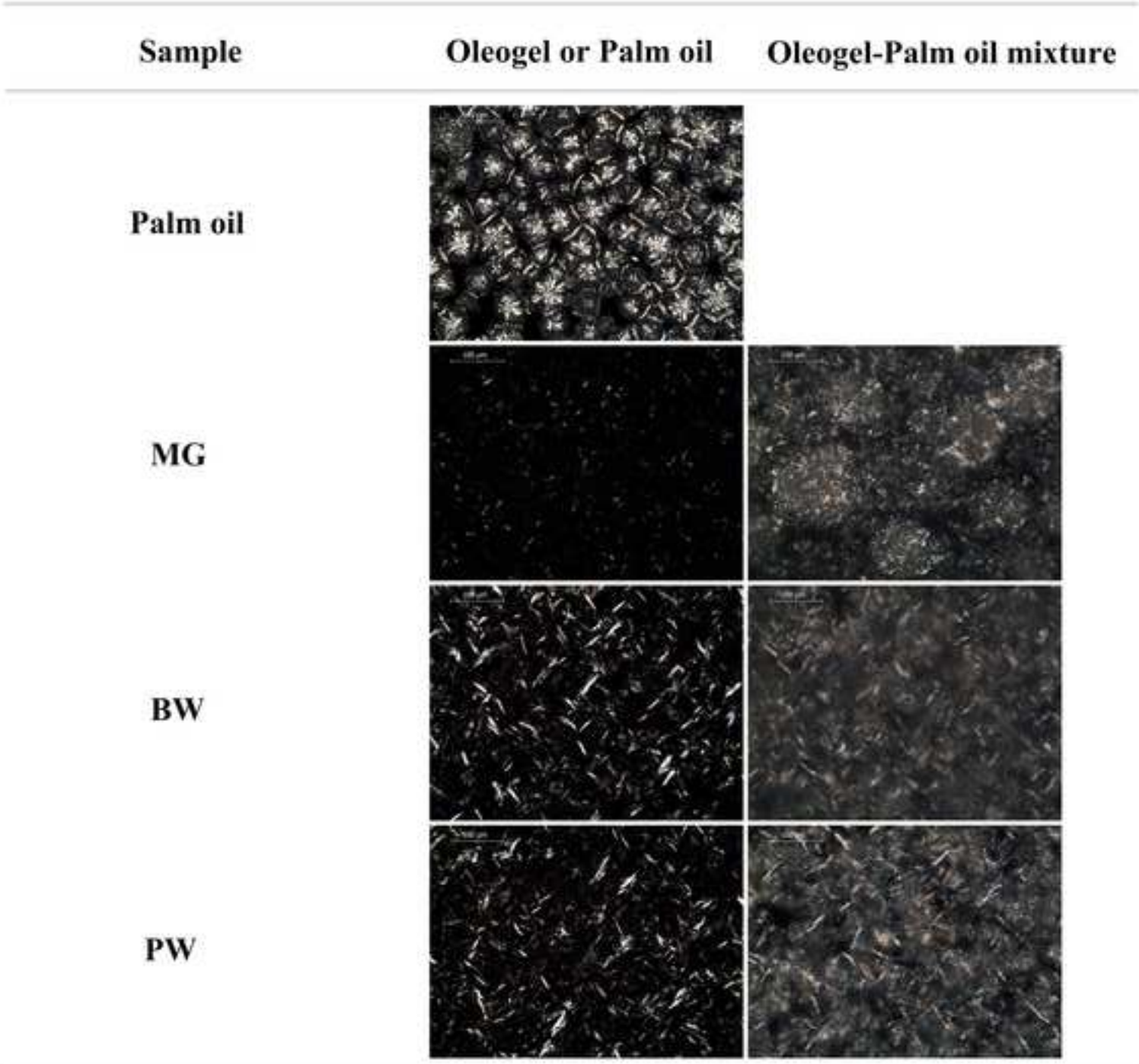

Figure

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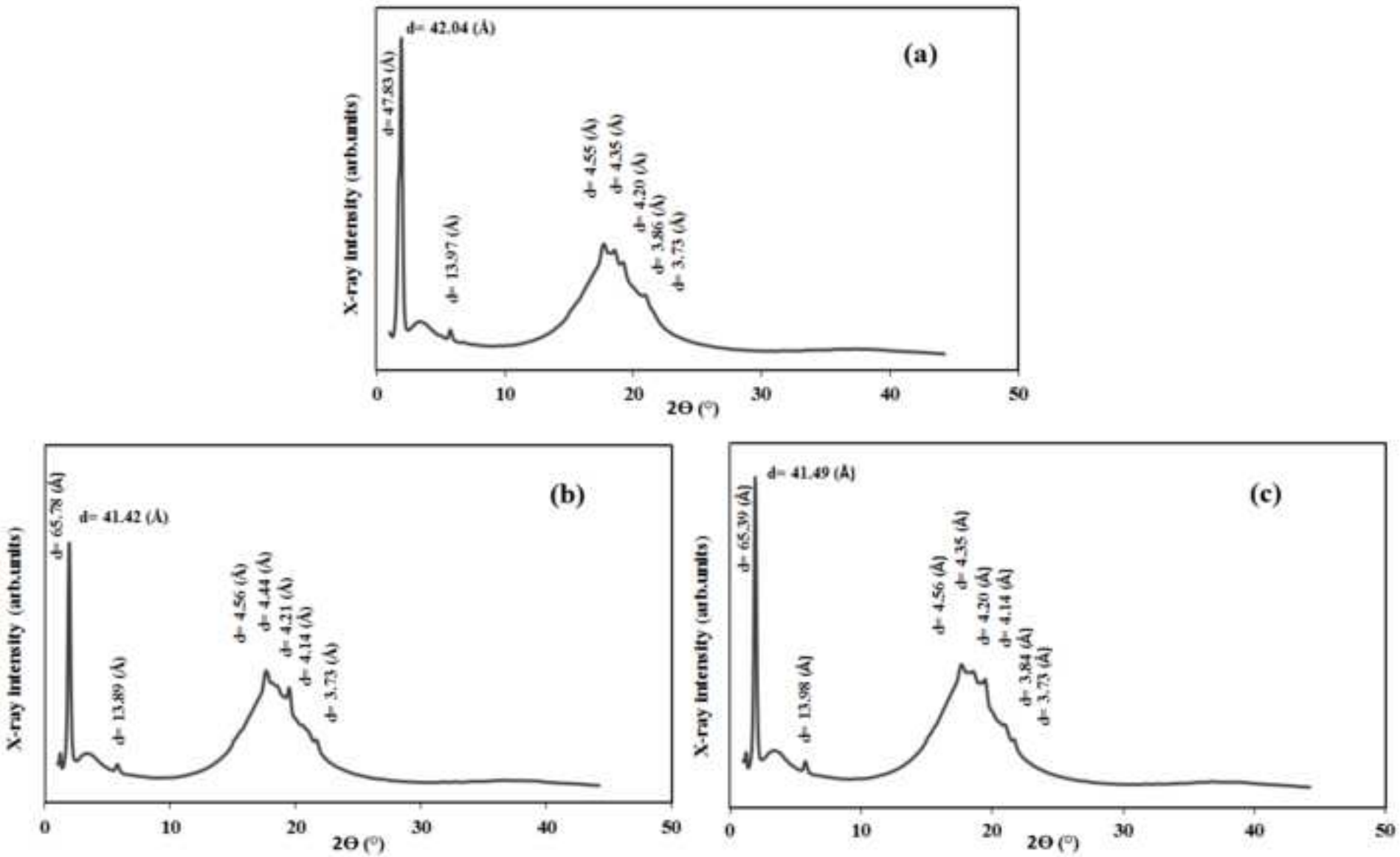

Figure
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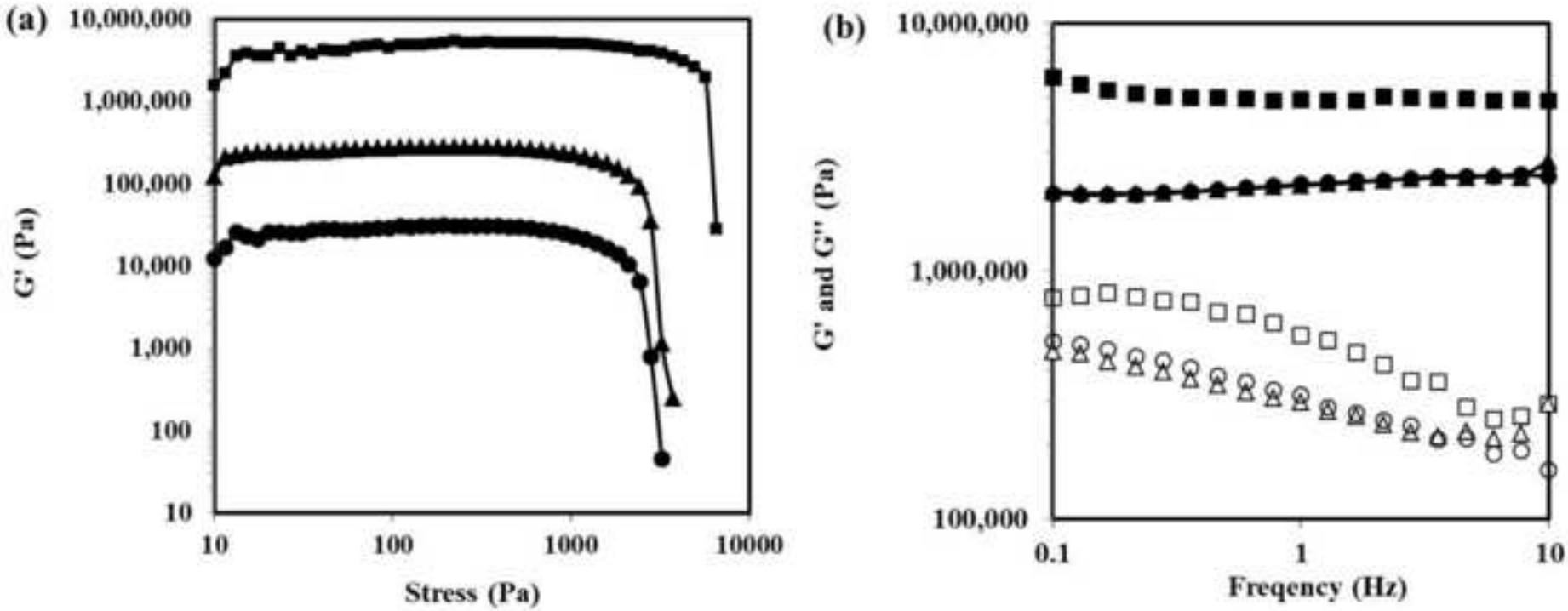

Supplementary Material

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