Crystallization behavior and structural build-up of palm stearin wax hybrid fat blends

Kato Rondou¹, Marie Romanus^{1,3}, Ellen Verwee^{1,2}, Ivana A. Penagos^{1,3}, Fien De Witte¹, Andre G. Skirtach², Koen Dewettinck^{1,3}, <u>Filip Van Bockstaele^{1,3}</u>

¹ Faculty of Bioscience Engineering, Department of Food Technology, Safety and Health, Food Structure and Function (FSF) Research Group, Ghent University, Ghent, Belgium

² Faculty of Bioscience Engineering, Department of Biotechnology, Nano-Biotechnology Laboratory, Ghent University, Ghent, Belgium

³ Faculty of Bioscience Engineering, Vandemoortele Centre "Lipid Science and Technology", Ghent University, Ghent, Belgium

Email corresponding author: filip.vanbockstaele@ugent.be

Abstract

Hybrid fat blends have emerged as a promising approach to improve the properties of wax-based oleogels which can be used as healthier alternatives for saturated fat products such as butter and margarine. The application of wax-based oleogels is limited because of their sensitivity to shear and waxy mouthfeel. This research investigates the interaction between waxes and palm stearin in hybrid fat blends. For the oleogels, 150% of the critical gelling concentration of the waxes was used, specifically 6% berry wax (BEW), 1.5% candelilla wax (CLW) and 6% carnauba wax (CRW), all incorporated in rapeseed oil. The concentration of palm stearin (PS) in the reference system (30PS) and the hybrid fat blends was 30% (PS-BEW, PS-CLW, PS-CRW). It was found that the addition of PS to the wax-based oleogels largely affected the properties on the nano- to macroscale. On the nanoscale, the polymorphic transition from α to β of palm stearin was delayed when adding BEW, while it did not change with the other waxes. On microscale, the high melting CRW acted as a seeding template for the palm stearin in PS-CRW, resulting in the formation of fat crystals that were rich in CRW in the center. For PS-BEW and PS-CLW, it was hypothesized that the fat crystals were the result of co-crystallization. All three hybrid fat blends showed improved rigidity and shear sensitivity compared to the PS reference and the wax- based oleogels on a macroscale. These results demonstrate that the interaction between palm stearin and waxes can have a positive effect on the final structure.

Keywords: Oleogelation, Hybrid fat blends, Fat crystallization, Synchrotron X-ray scattering, Raman spectroscopy, Natural waxes

Acknowledgments

We acknowledge the European Synchrotron Radiation Facility (ESRF) for provision of beamtime at the DUBBLE beamline (BM26) and we would like to thank Martin Rosenthal for his assistance during the experiments. BOF-UGent (Bijzonder Onderzoeksfonds) is acknowledged for its financial support (BOF/STA/202009/049). The FWO (Fonds Wetenschappelijk Onderzoek) is acknowledged for its financial support in the acquisition of the Xenocs Xeuss 3.0 X-ray Scattering (XRS) equipment (FWO Hercules Grant AUGE/17/29), the PhD fellowships (1128923N and 1SA5321N), infrastructure

support I002620N and the DUBBLE travel grant. Thanks to Vandemoortele Lipids NV for providing the samples and its financial support to the Vandemoortele Centre "Lipid Science and Technology".

1 Introduction

Oleogelation is a common way to structure liquid oil into solid-like fats while reducing the amount of saturated fatty acids. Within the field of oleogelation, different gelators can be used depending on the production method and applications. One of the promising oleogelators are natural waxes given their high availability and the low critical gelling concentrations. Chemically, a wax is defined as an ester of a long chain fatty acid and a monohydric long chain alcohol [1]. However, depending on their origin, waxes can be a complex mixture of wax esters, hydrocarbons, free fatty acids, free fatty and free fatty alcohols. The final composition will largely affect the physical properties of the wax and its oleogel. In general, research shows that stable oleogels can be formed with different waxes at (very) low concentrations (0.5-7%) [2-4]. However, based on European regulations, natural waxes cannot (yet) be applied in food applications as structurants [5,6].

Even though wax based oleogels are widely reported in literature to be stable, some properties such as shear sensitivity and mouthfeel can still be improved. As a result, the formulation of hybrid fat blends is gaining interest to improve these properties. Hereby, other hardstock fats or gelators can be used to create a more stable fat crystal network. Ramirez-Gomez et al. (2016) investigated the use of both candelilla wax and fully hydrogenated soybean oil in safflower oil and demonstrated that the hybrid fat blend had a higher elasticity and a higher ability to recover after applying shear. Similar findings were found for other hybrid systems [7-9]. The effect of adding another hardstock to wax based oleogels is largely affected by the crystallization behavior of the different components and the interactions. Generally, edible fats crystallize in three different polymorphs, namely α , β ' and β , which is analyzed with X-ray scattering techniques (XRS). The α -polymorph is the least stable polymorph and is characterized with a d-spacing of 4.15 Å, indicating a hexagonal packing. The intermediate β ' is characterized with d-spacings 3.8 Å and 4.2 Å, corresponding to an orthorhombic packing [10-12]. The most stable β -polymorph has a triclinic packing with a characteristic d-spacing of 4.6 Å. A different crystallization behavior was reported for waxes. Neat waxes tend to crystallize into an orthorhombic sub-cell structure (β), which is characterized by the d-spacings 4.15 Å and 3.73 Å. However, depending on the composition, waxes such as berry wax can also crystallize into a hexagonal sub-cell (α), characterized with one small peak at 4.15 Å [3]. Next to polymorphism, other important characteristics of edible fats are the crystallization and melting temperature and the microstructure since they largely affect the mouthfeel. These properties are commonly investigated with differential scanning calorimetry and polarized light microscopy. Recently, Raman spectroscopy has also gained significant interest for studying the distribution of different components in fat-rich food products [11, 13-15]. The main advantage of using Raman spectroscopy is the ability to visualize a fat-rich product based on its chemical composition.

Hybrid fat systems seem to be promising to improve the structure of wax based oleogels. In this research, the main aim is to better understand the interaction between natural waxes (berry wax; BEW, candelilla wax; CLW, carnauba wax; CRW) and a hardstock fat (palm stearin; PS). Hereby, wax based oleogels and a 30% PS reference system were produced and compared towards their hybrid fat blend containing the same concentrations of wax (150% of their critical gelling concentration) and

palm stearin (30%). Combining synchrotron X-ray scattering, polarized light microscopy, Raman spectroscopy and rheology resulted in a multiscale characterization of the hybrid fat blends to elucidate the interaction between the wax and PS. However, further research is necessary in lowsolid hybrid fat blends to mimic traditional fats while reducing the amount of saturated fatty acids.

2 Materials and methods

2.1 Samples

Berry wax (BEW), candelilla wax (CLW) and carnauba wax (CRW) were provided by Lambert Chemicals SPRL (Herstal, Belgium), produced by C.E. Roeper GmbH (Hamburg, Germany). Palm stearin (PS) and rapeseed oil (RSO) were provided by Vandemoortele Lipids NV (Izegem, Belgium). The main components and fatty acids are summarized in Table 1. Hereby, the composition of the waxes is based on literature [16] and the composition of the palm stearin was provided by Vandemoortele Lipids NV.

| Sample | Main components | Main fatty acids | | |
|-------------------------------------------------------------------------|--------------------|----------------------------|--|--|
| Berry wax ^a | Free fatty acids | C16:0 | | |
| Candelilla wax ^a | Hydrocarbons | C31:0 | | |
| Carnauba wax ^a | Wax esters | FA - FAL C16-C24:0 - C32:0 | | |
| Palm stearin | PPO, POP, PPP, POO | C16:0, C18:1c, C18:2c | | |
| ^a Doop at al. (2017) [16] EA - fatty acid EAL - fatty alabal | | | | |

Table 1 Composition of the different gelators

Doan et al. (2017) [16], FA = fatty acid, FAL= fatty alchol

2.2 Production of the reference and (hybrid) oleogels

First, the critical gelling concentration (CGC) of the waxes was determined by increasing the concentration in rapeseed oil from 0.5% to 3% in steps of 0.5% (w/w). The maximum concentration was increased to 5% (w/w) in a similar way when needed. Based on these results, 150% of this CGC was further applied to ensure that the oleogels were solid-like and to have sufficient sensitivity. This resulted in the concentrations of 6%, 1.5% and 6% (w/w) for BEW, CLW and CRW respectively. For the PS reference and the hybrid fat blends, 30% (w/w) of PS in rapeseed oil was used. Rapeseed oil was mixed with the corresponding hardstock(s) and heated till 90°C for 10 min while stirring. Next, the mixtures were placed in a -23°C freezer (in containers with a diameter and height of 30 mm) to statically crystallize the samples (in the absence of shear). When the temperature reached 15°C, the samples were stored overnight at 15°C prior to analysis. The reference and wax oleogels will further be referred to as 30PS, 6BEW, 1.5CLW, 6CRW and the hybrid fat blends as PS-BEW, PS-CLW, PS-CRW.

2.3 Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to investigate the crystallization behavior of the samples (DSC Q1000 - TA Instruments, New Castle, USA). Aluminum Tzero pans were filled with 4-10 mg of sample and hermetically sealed. The sample was heated till 100°C for 10 min, followed by a cooling ramp at 5°C/min till 5°C and kept isothermal for 30 min. This was done in triplicate and the results were analyzed with TRIOS (TA Instruments). The melting temperature was defined as the temperature where the area percentages of the endothermic peak reached 99%

2.4 Synchrotron X-ray scattering

Simultaneous SAXS and WAXS measurements were performed at the DUBBLE beamline BM26 at the European Synchrotron Radiation Facility (ESRF; Grenoble, France) for 30PS, PS-BEW, PS-CLW and PS-CRW. X-rays with a wavelength of 1.033 Å at 12 keV were generated. SAXS patterns were collected with a Pilatus 1M detector for 0.06 Å⁻¹ < q < 0.6 Å⁻¹ and WAXS with a 300K-W linear Pilatus detector for 0.6 Å⁻¹ < q < 2.6 Å⁻¹. Quartz capillaries were filled with a small amount of sample and heated till 80°C for PS, PS-BEW and PS-CLW and 100°C for PS-CRW. After melting for 10 minutes, the samples were statically crystallized at 10°C/min till 15°C in the capillary while simultaneous SAXS and WAXS measurements were performed every 3 s. These measurements continued during an isothermal time of 10 min at 15°C. The temperature was controlled with a Linkam stage (THMS600). SAXS and WAXS spectra were corrected by subtracting the intensity of an empty capillary with the same diameter. The thickness of the crystal nanoplatelets (CNP) was calculated by using (1) the Scherrer equation $\left(\frac{2\cdot\pi\cdot K}{FWHM}\right)$ based on the full width at half maximum (FWHM) and a shape factor K of 0.9 [17,18] and (2) the BWA method to create a volume-weighted distribution of the CNP thickness as described by Rondou et al. (2022) [19].

2.5 Polarized light microscopy

The microstructure of the oleogels and the hybrid fat blends was visualized with polarized light microscopy (Leica DM2500, Diegem, Belgium). The temperature was kept at 15°C with a Linkam cooling system. A 20x magnification (HCX PL Fluotar 20x/0.5) was used and the images were acquired with the Leica Application Suite (LAS) software. Three images were taken for each sample. Afterwards, the contrast of the images was adapted and the size of the crystals related to palm stearin were analyzed (20 crystals for each sample) with ImageJ [20].

2.6 Raman spectroscopy

Raman microscopy mapping was used to visualize the distribution of the different components within the microstructure of the hybrid fat blends. A confocal Raman microscope (Alpha 300R+, WITec GmbH, Ulm, Germany) with a 785 nm excitation diode laser with a power before objective of 100-130 mW (Toptica XTRA II, Germany), a UHTS 300 spectrometer cooled at -70°C and a CCD camera (iDus 401 BR-DD, ANDOR, UK) was used. Acquisitions were taken with a 40x / 0.6 NA objective (Nikon, Japan) at 300 gratings/mm (spectral range 500-3200 cm⁻¹). Raman spectra of neat PS and the different waxes were taken as references. A mapping of 100x100 µm with a pixel size of 1 µm and an acquisition time of 0.5 s was performed. The control FIVE software (WITec) was used to acquire the spectra and to correct for cosmic rays if necessary. Afterwards, Rstudio (version 2023.12.1) was used for background correction, normalization and visualization of the microstructure. Two different methodologies were applied. First, a k-means clustering [13] was performed in which the reference spectra were added to verify the clustering. The initial amount of clusters was put at 4 to be able to separate the palm stearin, wax and rapeseed oil. If the palm stearin and wax (added reference spectra) were grouped in the same cluster, the amount of clusters was reduced to 3. For the clustering images, an additional component "X" was added to indicate the interaction of the crystals with the rapeseed oil. In the second method, the Euclidean distance (d) between the spectra of the hybrid fat blend at each position of the mapping and the references spectrum was calculated. This was repeated for the two references (palm stearin and wax) with the full spectrum (500-3100 cm⁻¹). Afterwards, the Euclidean distance d was transformed towards

similarity $\left(\frac{1}{1+d}\right)$. Finally, the similarity was scaled within the sample to avoid incorrect interpretations related to the concentration and visualized as a heatmap. This allowed to have a more detailed estimation of the distribution of the different components.

2.7 Texture analysis

The texture was analyzed with a 5942 Instron TA 500 Texture Analyzer (Lloyd Instruments, Bognor Regis, UK). A container with a diameter of 3 cm was filled with 10 g of sample after which a 4 mm diameter stainless steel cylindrical probe was used to penetrate the sample. After reaching the trigger value of 0.03 N, the measurement started with a penetration depth of 10 mm at 1 mm/s. The measurement was performed 1 day after the production of the samples and five replicates were used.

2.8 Amplitude sweep

An amplitude sweep was performed using a rheometer (MCR 302; Anton-Paar, Germany) with a sand blasted plate-plate geometry (25 mm) of which the temperature was set at 15°C with a Peltier system. The sample was kept isothermal for 5 min at 15°C with a gap size of 1 mm after which a strain sweep was done from 0.001 to 2. In total 44 data points were collected and three replicates were performed one day after production. The linear viscoelastic region (LVR) was determined by a storage modulus (G') offset of 10% from the average of the first five G' datapoints. Within the LVR, the average complex modulus (G*) and the phase shift angle (δ) were calculated and the end of the LVR is characterized by the yield stress. Upon further deformations, the flow point is determined by the intersection between the storage modulus (G') and the loss modulus (G") [21].

2.9 Statistics

Statistical analysis was done in RStudio (version 2023.12.1). Homogeneity and normality were verified with respectively the Levene and Shapiro-Wilk test. Based on these results, the Kruskal-Wallis test and DunnettT3 tests were used to compare the means. All tests were performed with a significance level of 0.05.

3 Results and discussion

0

20

40

Temperature (°C)

60

80



3.1 Crystallization and melting behavior

Fig. 1 Crystallization (left) and melting (right) curves of the reference (30PS), oleogels (6BEW, 1.5CLW, 6CRW) and the hybrid fat blends (PS-BEW, PS-CLW and PS-CRW) when cooled/heated at 5°C/min.

100

0

20

40

Temperature (°C)

60

Fig. 1 shows the crystallization and melting curves of the different oleogels and hybrid fat blends. In addition, Table 2 summarizes the onset temperature of crystallization (T_c) and the melting temperature (T_m) for the PS reference, the oleogels and the hybrid fat blends. Due to the presence of wax and PS in the hybrid fat blends, the T_c and T_m are separated into two events. When a clear distinction could be made between the crystallization/melting of the wax and the PS, event 1 corresponds to the wax and event 2 to PS. For the PS reference and the oleogels, only one crystallization peak is visible with an increasing onset temperature in the order of 6BEW<30PS<1.5CLW<6CRW. Regarding the hybrid fat blends, multiple peaks can be distinguished for PS-CLW and PS-CRW. Especially for PS-CRW, two distinct peaks can be observed. First the CRW starts to crystallize (event 1), which will act as a seeding template for the PS (event 2), resulting in an increase in the crystallization temperature of the PS fraction. Both BEW and CLW have a similar effect on the PS crystallization, however, no separate crystallization peak for BEW in PS-BEW was found. Perhaps because BEW is rich in free fatty acids, which will crystallize after the triglycerides present in PS. Given the lower concentration of BEW, this crystallization peak can be obscured by the bigger peak related to PS. On the other hand, it might also be possible that the BEW does not start to crystallize within the applied cooling protocol. The addition of wax to 30PS resulted in a significant increase in the onset temperature of crystallization of the PS fraction, which is in line with literature [22, 23]. This can be the result of the seeding effect of the wax or due to molecular interactions between the wax and the PS. The presence of waxes in the hybrid fat blends also had an impact on the melting behaviour. The melting peak is shifted towards lower temperatures for the hybrid fat blends compared to 30PS. Even though these results illustrate a decrease in melting temperature, the melting temperature remains relatively high so that the impact on the organoleptic properties should be investigated.

6BEW 1.5CLW

6CRW

30PS

PS-BEW

PS-CLW

PS-CRW

80

100

Table 2. Onset temperature of crystallization (T_c) and the melting temperature (T_m) of palm stearin reference (30PS), the oleogels (6BEW, 1.5CLW, 6CRW) and hybrid fat blends (PS-BEW, PS-CLW, PS-CRW). Letters a-h indicate significant differences within T_c and T_m (p<0.05).

| | 6BEW | 1.5CLW | 6CRW | 30PS | PS-BEW | PS-CLW | PS-CRW |
|------------------------------|----------------------------|------------------------------------------|-------------------------|-------------------------|----------------------------------------|---------------------------|-------------------------------|
| T _{c, event 1} (°C) | 17.2 ± 0.1 ^a | 36.1 ± 1.9 ^{b,c,d} | 58.8 ±0.3 ^f | 21.7 ± 0.1 ^g | ^g 26.52 ± 0.03 ^b | 34.6 ± 0.2 ° | 55.8 ± 0.1 ^h |
| $T_{c, event 2}$ (°C) | - | - | - | - | - | 24.9 ± 0.3 ^{b,e} | 25.8 ± 0.1 ^{d,e} |
| T _{m, event 1} (°C) | 43.7 ± 4.2 ^{a,c,} | ^d 56.5 ± 1.9 ^{a,c,d} | 86.5 ± 0.5 ^b | 55.9 ± 0.4 ª | a 53.4 ± 0.4 ° | 52.4 ± 0.4 ° | 83.6 ± 0.1 ^b |
| T _{m, event 2} (°C) | - | - | - | - | - | - | 49.0 ± 0.1 ^d |

3.2 Polymorphism





Fig. 2 shows the WAXS profiles during crystallization from 80°C (30PS, PS-BEW, PS-CLW) or 100°C (PS-CRW) till 15°C. For 30PS, a first peak appears at 4.13 Å when cooling to 25.4°C, indicating the apolymorph. During the isothermal time at 15°C, a shift from 4.13 Å toward 4.55 Å can be found. This indicates the polymorphic transition from a directly into β , which was also observed by Penagos et al. (2023) [12]. According to Kellens et al. (1990), the transition from a to β occurs for even saturated monoacid triglycerides with a fatty acid chain length below 20 carbon atoms due to faster kinetics [24]. When adding BEW to 30PS, similar d-spacings can be observed, nonetheless, the a-peak remains present for a longer duration, indicating a slower polymorphic transition. Based on literature, it is known that the intensity of the a-polymorph of BEW is very weak when diluted in oil so that this peak is most likely originating mainly from the a-polymorph of PS [3]. Additionally, as shown in Table 2, the crystallization temperature of 6BEW is only around 17°C. Therefore, it might be possible that the BEW is not yet fully crystalline, hampering the transition of PS form a to β . Contrarily to the hybrid PS-BEW fat blend, PS-CLW showed an additional peak at 3.78 Å next to the one at 4.14 Å. This is characteristic for the orthorhombic sub-cell packing of CLW. After the polymorphic transition of the

 α -PS into β -PS, the two peaks at 4.14 and 3.78 Å coming from the CLW (β ') remain. Similar d-spacings were found for CRW. Given the high onset temperature of crystallization for 6CRW, the first peaks at d-spacings 4.14 Å and 3.76 Å are from the crystallization of CRW in PS-CRW. Upon further cooling, the peak at 4.14 Å broadens, indicating the crystallization of PS into α , followed by a polymorphic transition to β . During the polymorphic transition of PS, the CRW still appears in the orthorhombic packing.

Regarding the waxes, a strong anisotropy in crystal growth has been reported [25, 26]. The interactions within the lamellar plane are stronger compared to the interactions between the methyl end groups. This results in a lower growth rate in the thickness of the crystal nanoplatelet and therefore weaker short spacings. Additionally, the lamellar thickness of BEW and CLW is expected to be similar to the 2L packing of the TAG present in PS (mostly C16, C18). BEW is rich in C16:0 free fatty acids which tend to crystallize in a head-to-head orientation [27] and CLW is rich in C31:0 hydrocarbons [16]. This does not apply for carnauba wax, which mostly contains wax esters with a total carbon length of 46 to 54 carbon atoms. As a result, the lamellar thickness of CRW is expected to be much bigger, namely in the order of a 3L packing of C16, C18 TAG.



Fig 3. 3D plots (top) and heatmaps (bottom) of time-resolved SAXS of the reference (30PS) and PSwax hybrid fat blends PS-BEW, PS-CLW, PS-CRW when cooled at 10°C/min. The dotted line indicates the start of the isotheral time at 15°C.

The time-resolved SAXS profiles during crystallization are displayed in Fig. 3. Starting with 30PS, the polymorphic transition from α to β corresponds to a shift towards a broader peak at higher q-values (0.14 Å⁻¹ to 0.15 Å⁻¹), indicating thinner lamellae and thinner crystal nanoplatelets. Hereby, the crystal nanoplatelet of the α -polymorph (final spectrum) consists of 11.6 lamellae with a lamellar thickness of 45.9 Å and 5.7 lamellae of 41.7 Å for the β -polymorph when applying the Scherrer equation (Table 3). For PS-BEW, a prolonged existence of the α -PS and no additional peaks related to the BEW can be observed. The absence of both SAXS and WAXS peaks for BEW might indicate that there are no unique peaks when combining with PS and/or the absence of crystalline BEW. The BEW components will most likely be incorporated into the lamellae of the α -PS crystals [28], hindering the polymorphic transition. The effect of adding CLW to PS was not visible in the SAXS profiles, both the position and the shape of the peak remained the same. However, the WAXS profiles of PS-CLW illustrated the

orthorhombic packing of CLW without hampering the polymorphic transition of PS. This could be the result of the relatively low concentration or the similarity between the ordered aliphatic chains of CLW and PS. When adding CRW, a SAXS peak with a d-spacing of 81.6 Å appeared before the PS-crystallization due to the high crystallization onset temperature of CRW. Upon further cooling, the presence of CRW crystals did not affect the polymorphic transitions of PS.

| | α-polymorph palm stearin | | β-polymorph palm stearin | | |
|--------|--------------------------|------------------|--------------------------|------------------|--|
| | n _{Scherrer} | n _{BWA} | n _{Scherrer} | n _{BWA} | |
| 30PS | 11.7 | 9.7 | 5.6 | 6.0 | |
| PS-BEW | 11.5 | 9.4 | 5.0 | 5.5 | |
| PS-CLW | 11.8 | 9.6 | 5.8 | 5.8 | |
| PS-CRW | 11.2 | 9.0 | 5.7 | 5.5 | |

Table 3 Average CNP thickness of the palm stearin fraction obtained with the Scherrer equation $(n_{Scherrer})$ and the BWA method (n_{BWA}) .

Detailed information on the heterogeneity of the CNP thickness of the palm stearin fraction was obtained by applying the BWA method [29]. Figure 4 shows the distribution of the CNP thickness of the samples containing PS in the α -polymorph and the β -polymorph, both the final spectrum. The average thickness is summarized in Table 3. Generally, it can be observed that the CNP thickness of PS within the different samples remained similar. Only for PS-BEW, a slight shift in the distribution of the β -polymorph could be established. However, the difference in the average CNP thickness is minimal. Additionally, it can be seen that the less stable α -polymorph has a broader distribution which is more shifted towards more lamellae compared to the more stable β -polymorph. In other words, the CNP thickness of the β -polymorph of palm stearin is more homogeneous and smaller compared to the α -polymorph.



Fig. 4 Normalized volume weighted distribution of the CNP thickness obtained with the BWA method for the samples containing palm stearin. The left graph shows the distribution of the α-polymorph of the palm stearin fraction and the right graph shows the β-polymorph.

3.3 Crystal morphology



Fig. 5 Polarized light microscopy images of the 30PS reference (A), the oleogels: 6BEW (B), 1.5CLW (D), 6CRW (F) and the hybrid fat blends: PS-BEW (C), PS-CLW (E), PS-CRW (G). The scale bar is 50 μm.

The microstructure of the oleogels and the hybrid systems is visualized in Fig. 5. Contrarily to the results obtained at the nanoscale (polymorphism, CNP thickness), big differences were visible at the microscale. The smallest crystals were found for 6BEW and 1.5CLW, intermediate ones for 30PS and big crystals for the 6CRW. For the hybrid systems, it can be seen that the overall crystal size of PS has decreased when compared to 30PS. For 30PS, an average size of $18.4 \pm 4.2 \,\mu\text{m}$ was found while the size decreased to $11.5 \pm 3.3 \,\mu\text{m}$, $6.4 \pm 1.2 \,\mu\text{m}$ and $5.7 \pm 1.4 \,\mu\text{m}$ for respectively PS-BEW, PS-CLW and PS-CRW. This was also reported in literature [23, 28]. The decrease in crystal size can result in a product with a smoother mouthfeel [30]. For PS-CRW, a clear distinction can be made between the CRW crystals and the PS crystals.



Fig. 6 Raman mappings obtained with k-means clustering (Clustering) and the Euclidean distance (Similarity) of the hybrid fat blends: PS-BEW, PS-CLW, PS-CRW. Component 'X' represents the interaction between the crystals and the oil. The scale bar is 20 μm.

Raman microscopy is a versatile technique that provides label-free characterization and has proven to be useful in many applications [31,32]. In research on food materials, Raman microscopy mapping had been used amongst others to investigate synergistic interactions between lecithin and fruit wax [33]. Here, it is used to visualize components of PS-BEW, PS-CLW, PS-CRW fat hybrid blends (Fig. 6). Hereby, both the clustering analysis and the similarity based on the Euclidean distance is shown. The number of clusters was defined based on the ability to make a distinction between the wax and PS reference (see 2.6) and the 'X' represent the interaction between the liquid oil and the crystals. Regarding the similarity plots based on the Euclidean distance, the bright color indicates a higher similarity with a certain reference material at that specific location. It is important to note that the wax concentration is lower compared to the palm stearin concentration, resulting in lower similarity values in the scaled plots. Only for PS-CRW, a clear distinction can be made between the center of the crystals and the outer layer (Fig. 6). The clustering showed a CRW center surrounded by PS within the rapeseed oil (RSO). This was also visualized more into detail with the similarity plots where a brighter color was found for the PS reference in the center of the crystals. Taking into account the DSC and XRS results, it can be hypothesized that the carnauba wax crystallizes first, acting as a template for the PS crystallization. For PS-BEW and PS-CLW, no distinction could be made between the wax and palm stearin, indicating the presence of too small crystals to detect or co-crystal formation. The occurrence of co-crystal formation was also hypothesized by Toro-Vazquez et al. (2009) for tripalmitin-candelilla wax oleogels [22]. Similarly to the polarized light images in Fig. 5, the microstructure of PS-CLW consists of smaller crystals compared to PS-BEW.

3.4 Network rigidity and hardness



Fig. 7 Complex modulus as function of the shear strain obtained from the amplitude sweep.

Hybrid fat blends are good to improve the structuring functionality of gelators that are used to formulate oleogels. This functionality in terms of the rigidity and the shear sensitivity was investigated by performing an amplitude sweep. The results are visualized in Fig. 7 and summarized in Table 4. Fig. 7 clearly illustrates the improved rigidity of combining PS with waxes. The 6BEW and 1.5CLW oleogels had the lowest rigidity while the rigidity of 6CRW was similar to the 30PS reference. Remarkably, adding PS to the weak 6BEW and 1.5CLW resulted in a big increase in rigidity. Ramírez-Gómez et al. (2016) analyzed the effect of combining CLW with fully hydrogenated soybean oil in which the same trend was observed [23]. The increase in rigidity was less pronounced when comparing 6CRW with PS-CRW. This can be linked to their microstructure where it was hypothesized that both PS-BEW and PS-CLW showed co-crystal formation between the PS and the wax while PS-CRW had clear wax center surrounded by PS. Additionally, the reduced crystal size of PS might enhance the formation of the network compared to 30PS. Similar findings occurred for the yield

stress and the flow point, where PS-CLW and PS-BEW showed a bigger increase compared to PS-CRW. Even though the previous results indicated big changes between 6BEW and PS-BEW, the phase shift angle remained similar around 6°, which is characteristic for solid-like viscoelastic products. Regarding the texture analysis, the structure of the oleogels was too weak to achieve the trigger force 0.03 N. As a result, the actual hardness of the oleogels could not be detected. When comparing the hybrid fat blends, the highest hardness was found for PS-BEW.

Table 4 Physicochemical properties of the oleogels (6BEW, 1.5CLW, 6CRW), the reference (30PS)and the hybrid fat blends (PS-BEW, PS-CLW, PS-CRW). Letters a-f indicate significant differencesbetween the samples (p<0.05).</td>

| | a | | | | |
|--------|--------------------------------------|------------------------------|------------------------------|----------------------------|------------------------------|
| | Complex modulus | Phase shift angle | Yield stress | Flow point | Hardness |
| | in LVR (Pa) | in LVR (°) | (Pa) | (Pa) | (N) |
| 6BEW | 9.4 E+03 ± 1.9 E+03 a | 5.6 ± 0.2 ^{a,c,d,e} | 6.7 ± 1.4 ª | $33 \pm 6^{a,c}$ | < 0.03 |
| 1.5CLW | 2.8 E+03 ± 1.2 E+01 ^a | 4.2 ± 0.2 ^{a,b} | 2.1 ± 0.5 ° | 8 ± 1 ° | < 0.03 |
| 6CRW | 2.3 E+04 ± 4.4 E+03 ^{a,b} | $8.0 \pm 0.4^{b,f}$ | 7.3 ± 2.6 ^a | 7 ± 1 ° | < 0.03 |
| 30PS | 3.1 E+03 ± 4.6 E+03 ^b | $8.9 \pm 0.4^{a,b,f}$ | 10.7 ± 3.5 ^a | 31 ± 2ª | 0.09 ± 0.01 ^a |
| PS-BEW | 6.2 E+05 ± 3.2 E+05 ^{a,b,c} | 6.4 ± 1.1 ^d | 237.6 ± 154.5 ^{a,b} | $409 \pm 76^{a,b,c}$ | 1.68 ± 0.27 ^b |
| PS-CLW | 1.5 E+05 ± 2.7 E+04 ^{a,b,c} | 10.1 ± 0.3 ° | 55.3 ± 11.6 ^{a,b} | 229 ± 50 ^{a,b,c} | 0.42 ± 0.02 ^c |
| PS-CRW | 1.1 E+05 ± 6.8 E+03 ° | 11.6 ± 0.9 ^{c,f} | 27.9 ± 1.3 ^b | 109 ± 3 ^b | 0.66 ± 0.03 ^d |

4 Conclusion

The structuring functionality of oleogels can be improved by combining waxes with palm stearin into hybrid fat blends. During crystallization of PS-CRW, the CRW crystallizes first, acting as a seeding template for PS. This resulted in the formation of fat crystals containing a center that was rich in CRW, surrounded by PS. For PS-BEW and PS-CLW, co-crystallization was hypothesized. The size of the PS crystals in the three hybrid fat blends did not change on the nanoscale but seemed to be smaller on the microscale compared to the reference 30PS, which resulted in an increased rigidity and sensitivity to shear for the hybrid fat blends. This increase was also observed when comparing the hybrid fat blends to the oleogels.

Author contributions

Conceptualization: Filip van Bockstaele; *Methodology*: Kato Rondou, Ivana A. Penagos, Fien De Witte; *Formal analysis and investigation*: Kato Rondou, Marie Romanus, Ellen Verwee, Ivana A. Penagos; *Writing - original draft preparation*: Kato Rondou; *Writing - review and editing*: Ellen Verwee, Ivana A. Penagos, Fien De Witte, Andre G. Skirtach, Filip Van Bockstaele; *Funding acquisition*: Koen Dewettinck, Filip Van Bockstaele; *Resources*: Andre G. Skirtach, Koen Dewettinck, Filip Van Bockstaele; *Supervision*: Koen Dewettinck, Filip Van Bockstaele.

ORCID: Kato Rondou: 0000-0002-7066-2902, Ellen Verwee: 0009-0007-7878-8650, Ivana A. Penagos: 0000-0003-3214-3025, Fien De Witte: 0000-0002-2216-4028, Koen Dewettinck: 0000-0002-5984-932X, Andre G. Skirtach: 0000-0002-4468-7620, Filip Van Bockstaele: 0000-0001-6878-249X

Funding

This work was supported by BOF-UGent (Bijzonder Onderzoeksfonds UGent): [BOF/STA/202009/049], the FWO (Fonds Wetenschappelijk Onderzoek): [Hercules Grant AUGE/17/29], [DUBBLE – ESRF TRAVEL], [1SA5321N], [1128923N], [1002620N] and Vandemoortele Lipids NV.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Data availability statement

The dataset used in this manuscript is available in Zenodo at https://doi.org/10.5281/zenodo.13120416.

5 References

- [1] Kolattukudy, P.E., Chemistry and biochemistry of natural waxes. Amsterdam: Elsevier Scientific Pub. Co. 1976.
- [2] Patel, A.R., Babaahmadi, M., Lesaffer, A., and Dewettinck, K., Rheological Profiling of Organogels Prepared at Critical Gelling Concentrations of Natural Waxes in a Triacylglycerol Solvent. Journal of Agricultural and Food Chemistry, 2015. 63(19): p. 4862-4869.
- [3] Doan, C., Tavernier, I., Sintang, M.D., Danthine, S., Walle, D., Rimaux, T., and Dewettinck, K., *Crystallization and Gelation Behavior of Low- and High Melting Waxes in Rice Bran Oil: a Case-Study on Berry Wax and Sunflower Wax*. Food Biophysics, 2017. **12**.
- [4] Lim, J., Hwang, H.-S., and Lee, S., *Oil-structuring characterization of natural waxes in canola oil oleogels: rheological, thermal, and oxidative properties.* Applied Biological Chemistry, 2017. **60**(1): p. 17-22.
- [5] European Union. (2008). Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. https://eurlex.europa.eu/eli/reg/2008/1333/oj
- [6] Commission Regulation (EU) No 1147/2012 of 4 December 2012 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council as regards the use of beeswax (E 901), carnauba wax (E 903), shellac (E 904) and microcrystalline wax (E 905) on certain fruits Text with EEA relevance. https://eur-lex.europa.eu/legalcontent/EN/TXT/?uri=CELEX%3A32012R1147
- [7] Barroso, N.G., Okuro, P.K., Ribeiro, A.P.B., and Cunha, R.L., *Tailoring Properties of Mixed-Component Oleogels: Wax and Monoglyceride Interactions Towards Flaxseed Oil Structuring.* Gels, 2020. **6**(1).
- [8] da Silva, T.L.T., Arellano, D.B., and Martini, S., *Interactions between candelilla wax and saturated triacylglycerols in oleogels*. Food Research International, 2019. **121**: p. 900-909.
- [9] da Silva, T.L.T., Arellano, D.B., and Martini, S., *Physical Properties of Candelilla Wax, Monoacylglycerols, and Fully Hydrogenated Oil Oleogels.* Journal of the American Oil Chemists' Society, 2018. **95**(7): p. 797-811.
- [10] Yap, P.H., de Man, J.M., and de Man, L., *Polymorphism of palm oil and palm oil products*. Journal of the American Oil Chemists' Society, 1989. **66**(5): p. 693-697.
- [11] De Witte, F., Penagos, I.A., Rondou, K., Moens, K., Lewille, B., Tzompa-Sosa, D.A., Van de Walle, D., Van Bockstaele, F., Skirtach, A.G., and Dewettinck, K., *Insights in the Structural Hierarchy of Statically Crystallized Palm Oil*. Crystals, 2024. **14**(2): p. 142.
- [12] Penagos, I.A., Rondou, K., Dewettinck, K., and Van Bockstaele, F., *Exploring the impact of waxes and emulsifiers on the crystallization of palm stearin: a time-resolved synchrotron X-ray scattering study*, in AOCS Annual Meeting & Expo. 2023: Denver, Colorado, USA.
- [13] Andersen, M.E., Andersen, U., Wiking, L., Rasmussen, J.T., Corredig, M., and Gregersen, S.B., The exploration of milk fat crystallization in milk fat globules by confocal Raman microscopy. Food Structure, 2024. 40: p. 100372.
- [14] Neofytos, D.D., Gregersen, S.B., Andersen, U., and Corredig, M., In situ single-droplet analysis of emulsified fat using confocal Raman microscopy: insights into crystal network formation within spatial resolution. Soft Matter, 2024.
- [15] Dalen, G., Velzen, E.J.J., Heussen, P., Sovago, M., Malssen, K., and Duynhoven, J., Raman hyperspectral imaging and analysis of fat spreads. Journal of Raman Spectroscopy, 2017. 48: p. 1075-1084.

- [16] Doan, C.D., To, C.M., De Vrieze, M., Lynen, F., Danthine, S., Brown, A., Dewettinck, K., and Patel, A.R., *Chemical profiling of the major components in natural waxes to elucidate their role in liquid oil structuring*. Food Chemistry, 2017. **214**: p. 717-725.
- [17] Acevedo, N.C., Marangoni A.G., Characterization of the nanoscale in triacylglycerol crystal networks. Crystal Growth & Design, 2010. 10(8): p. 3327–33. https://doi.org/10.1021/cg100468e
- [18] Langford, J.I., Wilson, A.J.C., Scherrer after sixty years: a survey and some new results in the determination of crystallite size. Journal of Applied Crystallography, 1978. **11**(2): p. 102–13. https://doi.org/10.1107/S0021889878012844
- [19] Rondou, K., De Witte, F., Rimaux, T., Dewinter, W., Dewettinck, K., Verwaeren, J., and Van Bockstaele, F., *Multiscale analysis of monoglyceride oleogels during storage*. Journal of the American Oil Chemists' Society, 2022. **99**(11): p. 1019-1031.
- [20] Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9, 671–675 (2012)
- [21] Patel, A. R., Babaahmadi, M., Lesaffer, A., Dewettinck, K., Rheological Profiling of Organogels Prepared at Critical Gelling Concentrations of Natural Waxes in a Triacylglycerol Solvent. Journal of Agricultural and Food Chemistry, 2015. 63(19): p. 4862-4869. https://doi.org/10.1021/acs.jafc.5b01548
- [22] Toro-Vazquez, J.F., Alonzo-Macias, M., Dibildox-Alvarado, E., and Charó-Alonso, M.A., *The Effect of Tripalmitin Crystallization on the Thermomechanical Properties of Candelilla Wax Organogels*. Food Biophysics, 2009. **4**(3): p. 199-212.
- [23] Ramírez-Gómez, N.O., Acevedo, N.C., Toro-Vázquez, J.F., Ornelas-Paz, J.J., Dibildox-Alvarado, E., and Pérez-Martínez, J.D., *Phase behavior, structure and rheology of candelilla wax/fully hydrogenated soybean oil mixtures with and without vegetable oil.* Food Research International, 2016. **89**: p. 828-837.
- [24] Kellens, M., Meeussen, W., and Reynaers, H., *Crystallization and phase transition studies of tripalmitin*. Chemistry and Physics of Lipids, 1990. **55**(2): p. 163-178.
- [25] Dassanayake, L.S.K., Kodali, D.R., Ueno, S., and Sato, K., *Physical Properties of Rice Bran Wax in Bulk and Organogels*. Journal of the American Oil Chemists' Society, 2009. 86(12): p. 1163.
- [26] Watanabe, K., Okawa, S., Miyakawa, O., Nakano, S., and Shiokawa, N., Mechanism of anisotropic dimensional changes in wax patterns prepared by the softened wax technique. II. Determination of paraffin crystal size. Dent Mater J, 1982. 1(1): p. 55-66.
- [27] Koch, K. and Ensikat, H.-J., The hydrophobic coatings of plant surfaces: Epicuticular wax crystals and their morphologies, crystallinity and molecular self-assembly. Micron, 2008. 39(7): p. 759-772.
- [28] Liu, C., Zheng, Z., Meng, Z., Chai, X., Cao, C., and Liu, Y., *Beeswax and carnauba wax modulate the crystallization behavior of palm kernel stearin.* LWT, 2019. **115**: p. 108446.
- [29] den Adel, R., van Malssen, K., van Duynhoven, J., Mykhaylyk, O.O., and Voda, A., Fat Crystallite Thickness Distribution Based on SAXD Peak Shape Analysis. European Journal of Lipid Science and Technology, 2018. 120(9): p. 1800222.
- [30] Ribeiro, A.P., Masuchi, M.H., Miyasaki, E.K., Domingues, M.A., Stroppa, V.L., de Oliveira, G.M., and Kieckbusch, T.G., *Crystallization modifiers in lipid systems*. J Food Sci Technol, 2015. **52**(7): p. 3925-46.
- [31] Schie, I.W., Rüger, J., Mondol, A.S., Ramoji, A., Neugebauer, U., Krafft, C., and Popp, J., High-Throughput Screening Raman Spectroscopy Platform for Label-Free Cellomics. Analytical Chemistry, 2018. **90**(3): p. 2023-2030.

- [32] Yashchenok, A., Masic, A., Gorin, D., Shim, B.S., Kotov, N.A., Fratzl, P., Möhwald, H., and Skirtach, A., Nanoengineered Colloidal Probes for Raman-based Detection of Biomolecules inside Living Cells. Small, 2013. **9**(3): p. 351-356.
- [33] Okuro, P.K., Tavernier, I., Bin Sintang, M.D., Skirtach, A.G., Vicente, A.A., Dewettinck, K., and Cunha, R.L., Synergistic interactions between lecithin and fruit wax in oleogel formation. Food & Function, 2018. **9**(3): p. 1755-1767.