Sunflower wax recovered from oil tank settlings: revaluation of a waste from the oilseed industry

Running title: Sunflower waxes recovered from oil tank settlings.

Authors: Cintia Elizabeth Redondas, Erica Raquel Baümler, Amalia Antonia Carelli

Institutional affiliations: Planta Piloto de Ingeniería Química (PLAPIQUI, UNS-CONICET), Departamento de Ingeniería Química, Universidad Nacional del Sur (UNS)-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), 8000 Bahía Blanca, Argentina

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Abstract:

BACKGROUND: Sunflower oil industries produce a large amount of waste that nowadays are not commercially exploited, as it is the case of oil tank settlings. In this sense, the recovery of a by-product of high added value, such as sunflower waxes, would increase the commercial value of these wastes. In this original research paper, a method that allows the recovery and purification of this by-product was developed. The waxes were characterized and its potential use as organogelator agent was investigated.

RESULTS: The waste sample was composed of 45.1% of oily material, being 16.9% of this oily fraction, impure waxes. The purification was performed through two different methods, obtaining three waxes with different degrees of purity. All the waxes were composed of wax esters in the range of 40-60 carbon atoms, exhibiting traces of carotenes, as well as free fatty acids and free fatty alcohols. In addition, the presence of phospholipids was observed in two of them. The third wax presented a higher total content of wax esters, physicochemical characteristics (color and thermal behavior) similar to those of commercial sunflower waxes, and was the most efficient organogelator agent, requiring only a small amount of wax (1.5%) to structure high oleic sunflower oil.

CONCLUSION: It was verified that sunflower waxes could be recovered from oil tank settlings. Additionally, a purification method that allowed the obtention of sunflower

waxes with similar physicochemical properties to those of commercial waxes was developed. The purified waxes were capable of structure high oleic sunflower oil.

Keywords: Sunflower waxes, oil tank settling, differential scanning calorimetry, color, composition.

Introduction:

Sunflower (*Helianthus annuus* L.) is grown in many regions of the world, from Argentina to Canada and from Central Africa to the Russian Federation. In 2016, Argentina was located in the third place of worldwide producers of sunflower seeds (3.0 million tons), preceded by Ukraine (13.6 million tons), and the Russian Federation (11.0 million tons)¹. Sunflower waxes (SW) are located mainly in the hull of the seed (80-83% of the total wax content), being the wax content in concentrations up to 3 - 4% depending of the genotype of the seeds, the geographic origin and the maturity stage of the plant from which the waxes were obtained, amongst other reasons^{2,3}. SW are being considered as an alternative for rice bran, carnauba, and candelilla wax and have multiple potential uses, including the production of edible films to act as coatings to extend the shelf-life of food products^{4,5} and the ability to structure vegetable oils to form oleogels and emulsions⁶⁻⁸. Due to the purity of its chemical composition, SW has its own functional characteristics, showing stronger gelling capacity, improving oil binding and lubricating capacity at lower concentrations than other vegetable waxes^{6,9,10}. In fact, unlike other vegetable waxes that can include hydrocarbons, alcohols, ketones and aldehydes in their composition, recovered SW consisted almost entirely of long-chained esters (C40 – C60), derived from fatty acids (C14 – C34) and saturated fatty alcohols (C18 – C34)^{11,12}. During the oil extraction process, SW are extracted along with the oil, and are later removed in the winterization process. However, throughout the storage of the crude oil, SW tend to crystallize and set down

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together with other minor components constituting an oily sediment named “oil tank settlings”, which makes this waste a potential source of SW. Nowadays, commercial SW are not recovered from the wastes of the storage tanks (tank settlings). Instead, SW are recovered from the filter cakes from the winterization process, and the oil tank settlings are being used for to animal feed with low commercial value. Several studies were developed using commercial waxes^{7,9,13–16} but scarce are the studies applying recovered waxes from sunflower oil processing^{5,17}. Additionally, Argentina produces 1.1 million metric tons of sunflower oil, and a large proportion of this oil is exported as crude oil (ranking 3rd worldwide)^{18,19}, generating this activity a large amount of oil tank settlings that could be commercially exploited. Therefore, it is very important to develop methods that allows the recovery and purification of this by-product with high commercial value from the oil tank settlings.

The aim of this work was to recover, purify and characterize sunflower waxes from oil tank settlings of crude sunflower oil. The waxes will be characterized through their purity, color, distribution of wax esters, thermal behavior and their potential use as organogelator agents.

Materials and Methods

Materials

A waste sample from oil tank settlings of unrefined sunflower oil was kindly provided by a local industry. This material consisted of a mud conformed mainly by oil,

phospholipids, waxes and solid particles. Commercial SW (CSW) were supplied by Koster Keunen Inc. (Watertown, CT, USA). CSW are a light yellow, granular solid obtained from the winterization of sunflower oil²⁰. For the elaboration of oleogels, high-oleic sunflower oil (HOSO) was purchased in a local supermarket.

Wax recovery and purification

Sunflower waxes (SW) were recovered through a modification of the procedure proposed by Martini et al.¹⁷. Briefly, approximately 300 g of tank settlings were mixed with hexane (analytical reagent grade, boiling point 62-70 °C, Dorwil) at a ratio of 2:1 hexanes: oil tank settlings (wt/wt), and heated in a water bath at 55 °C for 30 min, continuously stirring. After, the insoluble particles were left to decant and the supernatant, composed by hexane, oil, phospholipids and waxes, was collected. The supernatant was stored in a freezer, at approximately -18 °C, for 18 h, allowing the crystallization of the wax esters. The crystals were separated from the liquid phase using a centrifuge at 1318 g for 20 min. This procedure was performed several times and the recovered portions were mixed before the purification process.

Two different methods were developed for the purification of SW. In the first one, known from now on as method A, the mixture of SW and remaining impurities was subjected to successive washing with cold hexane until no color was visually observed in the liquid phase, eliminating the remaining oil and other hexane soluble compounds.

Between each wash, the mixture was centrifuged at 1318 g for 20 min. To eliminate any polar impurities remnants the samples were subsequently washed using 50 mL acetone at room temperature, followed by centrifugation at 1318 g for 30 min. This procedure was repeated three times. It was observed inside the centrifuge bottle, that the waxes separated in two fractions: a light brown portion (SWLA) located on the top of the bottle, and a dark brown portion (SWDA) placed on the bottom. As this fact suggested a different degree of purity between fractions, they were collected separately conforming two different samples.

In the second purification procedure (method B), the acetone washings from method A were replaced by ethanol washings at room temperature. In order to eliminate any remaining colorants, the impure waxes were filtered through a bed of diatomaceous earth (4g, Celite® Hyflo), using hexane at room temperature as solvent. The eluate was collected, and the solvent removed. The recovered waxes were named SWB.

Performance of waxes obtained in oleogels elaboration

To evaluate the performance of the obtained waxes in the elaboration of oleogels the critical gelling concentration (C^*) was determined. C^* is defined as the minimum concentration of SW necessary to achieve gelation of liquid oil after 24 h of cooling at room temperature inside a test tube⁶. Oleogels were prepared dissolving a determined amount of wax material (from 10 to 100 g wax material/kg of oleogel, with increments

of 5 g wax material/kg of oleogel) in HOSO, previously heated to 80 °C in a water bath. The molten oil - wax mixture was maintained at 80 °C for 5 min, with continuous stirring. Approximately 20 g of the molten oleogels were deposited in test tubes of 20 mm of diameter and 150 mm and then cooled to 25 °C for 24 h inside a refrigerator with controlled temperature. Afterwards, the test tubes were inverted. C* was determined as the concentration in which no observable flow was detected inside the test tubes after inversion. This procedure was performed in duplicate. The oleogels were named as follows: OSWLA (SWLA as gelling agent), OSWDA (SWDA as gelling agent), OSWB (SWB as gelling agent) and OCSW (CSW as gelling agent).

Color measurement

The color of SWLA, SWDA, SWB and CSW, oleogels and HOSO was determined using a Hunter Lab Ultra Scan XE colorimeter with Universal Software (Hunter Associates Laboratory Inc., Reston, VA, USA) with Universal Software (Hunter Associates Laboratory Inc., Reston, VA, USA). The CIE – Lab parameters L* (lightness), a* (positive: redness; negative: greenness) and b* (positive: yellowness; negative: blueness), were measured in triplicate. In addition, the color saturation, known as chroma value (C), was calculated according to the following equation²¹:

$$C = \sqrt{a^{*2} + b^{*2}}$$

Wax Characterization

Wax esters analysis

Wax esters determination was performed according to the method proposed by Carelli et al.²². In this method, a column chromatography on a double phase of silver nitrate-impregnated silica gel followed by gas chromatography (GC-FID) with on-column injection is used. The solvents used (ethyl ether, n-hexane and n-heptane) were of chromatographic grade (J.T. Baker Inc., Phillipsburg, NJ, USA). Lauric acid arachidyl ester ($C_{32}H_{64}O_2$, Sigma Chemical Co., St. Louis, MO) was used as an internal standard for the quantitative analysis, and wax standards from C32 to C44 of 99% of purity (Sigma Chemical Co., St. Louis, MO) were used for the qualitative analysis.

Approximately 3 mg of waxes (weighted accurately), 1 mL of internal standard and two drops of Sudan I dye (1% in n-hexane, used to control the completion of the wax elution) were dissolved in n-hexane and loaded into a glass column (length = 400 mm; i.d. = 15 mm) that had silver nitrate impregnated silica gel (3 g) placed at the bottom and silica gel 2% hydrated (12 g) placed on the top. The waxes were eluted with n-hexane/ethyl ether (99:1% v/v) at a flow rate of 3 mL/min.

The first portion (approximately 20 mL) of the eluted solvent was separated and collected to determine the presence, or absence, of hydrocarbons. The second portion (wax fraction) was collected in a different flask. The elution was completed when the dye reached a mark made on the glass column, 2 cm above the interphase between

the 2% hydrated silica gel and the silver nitrate impregnated silica gel. The eluted fractions were evaporated to dryness using a stream of nitrogen at room temperature and diluted with 1 mL of n-heptane for chromatographic analysis. As previously mentioned, the extracts obtained were analyzed by GC-FID (Clarus 580, Perkin Elmer, MA, USA) with an on-column injector, a HP-5 fused-silica column (5% diphenyl and 95% dimethylpolysiloxane, length = 15 m, i.d. = 0.32 mm, film thickness = 0.25 μ m, Hewlett-Packard, Palo Alto, CA, USA) and hydrogen as carrier gas, with a flow rate of 3 mL/min. The oven temperature programming was as follows: initial temperature 80 °C hold for 1 min, increase at 20 °C/min to 240 °C, increase at 5 °C/min to 325 °C and hold for 6 min, increase at 20 °C/min to 340 °C and hold for 27 min. The FID detector was programmed at 350 °C and the injector was programmed from 80 to 320 °C at 20 °C/min. The injection volume was 2 μ L. Two injections were performed for two independent analyses.

Thin-layer Chromatography

In order to determine the qualitatively composition of the remaining impurities (e.g. free fatty acids, free fatty alcohols, phospholipids and carotenes), the recovered waxes were separated by TLC. SWLA, SWDA and SWB were spotted on a 20 x 20 cm glass TLC plate precoated with silica gel ("Baker" Si250, 250 μ m, J.T. Baker Inc., Phillipsburg, NJ, USA). Diethyl ether and n-hexane were of chromatographic grade (U.V.E., Grand Bourg, Bs. As., Argentina), while acetic acid (Anedra – Research AG S.A., Tigre, Bs. As.,

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Argentina) was analytical grade. The elution solvent was hexane/diethyl ether/acetic acid (85:15:2).

L- α -phosphatidylcholine ($\geq 99\%$ of purity) was acquired from Sigma Chemical Co. (St. Louis, MO, USA). Arachidyl alcohol, 1-heneicosanol and 1-docosanol and a mixture containing 1-decene, 1-undecene, 1-dodecene, 1-tridecene and 1-tetradecene (kit 91, mixture 24c, Analytical Standard) were purchased from PolyScience Corporation (Chemical Division). Oleic acid (90% of purity) was provided by Aldrich Chemical Co. (Milwaukee, WI, USA). The content of a β -carotene soft gel capsule (Nutra manufacturing Inc., Greenville, SC, USA) was emptied into a tube, and 5 mL of n-hexane was added and stirred until complete dissolution. This solution was used as a β -carotene standard for the TLC. Oleic acid methyl ester ($C_{18}H_{34}O_2$, 99% purity, Sigma Chemical Co.) was used as a wax standard with the purpose of corroborate if there was co-elution of some compound with the waxes. Approximately, 50 mg of each wax sample was dissolved in 1.5 mL of n-hexane. Standards and the wax solution (6 μ L of each one, divided into 2 drops) were applied onto the TLC plates. The bands were visualized by placing the TLC plates onto a chamber under a saturated I_2 atmosphere for 2 min. The retention factor (R_f) is the relative distance travelled by a particular compound with respect to the solvent front. This parameter is used to identify qualitatively different groups of compounds. After the development and visualization of the spots, it was calculated according to the following equation:

$$R_f = \frac{\text{Distance between the center of the spot and the spotting line}}{\text{Distance between the solvent front and the spotting line}}$$

Thermal Behavior

The thermal behavior, melting and crystallization profiles of SWLA, SWDA, SWB and CSW were studied through differential scanning calorimetry (DSC) with a Discovery DSC apparatus (TA Instruments, New Castle, DE, USA). Samples of approximately 8 mg of wax were placed in an aluminum pan, that were then hermetically closed with an aluminum lid and placed in the DSC chamber. In order to completely melt and eliminate the crystalline history of the waxes, the samples were allowed to equilibrate at 125 °C for 30 min. After this the samples were cooled to 25 °C at a rate of 5 °C/min, and this temperature was maintained for 60 min to allow the complete crystallization of the waxes. This cooling cycle was followed by a heating cycle from 25 to 125 °C at a rate of 5 °C/min. The analysis was performed in triplicate.

The melting temperature (T_m), melting enthalpy (ΔH_m), onset temperature (T_{on}), crystallization temperature (T_c) and crystallization enthalpy (ΔH_c) were determined from the thermograms using the software TA Instruments' Universal Analysis. The melting entropy (ΔS_m) of the purified waxes was calculated according to the formula:

$$\Delta S_m = \frac{\Delta H_m}{T_m}$$

Oleogel characterization

Polarized microscopy

The microstructure of the oleogels was visualized by polarized light microscopy (PLM) with a Carl Zeiss optical microscope (Phomi III Pol). Samples were prepared by depositing a drop of the molten oleogels onto a glass microscope slide previously heated and pressed gently with a coverslip of the same material. The slides were stored at 25 °C in a temperature control refrigerator for 24 hs before visualization. Imaging was conducted at ambient temperature using 160x objective lens.

Texture analysis

The firmness, determined as the maximum force required to penetrate the oleogels, was determined using the texture profile analysis (TPA) test with the Texture Analyzer TA Plus (Lloyd Instruments, England). Approximately, 14 g of the molten mixtures of HOSO - wax were poured into cylindrical containers (i.d. = 33 mm, height = 20 mm) that were quickly placed in a refrigerator with temperature control, set at 25 °C, for 24 h to allow the complete crystallization of the waxes. The samples were penetrated to half their height (10 mm), using a cylindrical probe with a round head (diameter = 11.8 mm, length = 57.2 mm) at a speed of 1 mm/s, according to the procedure described in Palla et al.²³. The analysis was performed in triplicate.

Rheological analysis

The viscoelastic properties of the oleogels were determined using a Paar Physica rheometer (model MCR 301, Anton Paar GmbH, Austria) with a Peltier temperature control system. The geometry used was parallel plate (d. = 50 mm, PP50, Anton Paar) with a gap of 1000 μm . The samples were prepared following the same procedure as the samples for the textural analysis. A round slice of approximately 3 mm of height of the oleogels was used for each independent analysis. The analysis was performed in triplicate.

In order to determine the linear viscoelastic region, an oscillatory stress sweep test was performed at a fixed frequency of 10 rad/s and a strain range from 0.01 to 100%. The storage (G') and loss (G'') moduli were determined at 25 $^{\circ}\text{C}$, using an oscillatory frequency sweep test from 0.01 to 100 rad/s, with a fixed strain of 0.05%. The data was acquired using the software Rheoplus/32 V3.40 and was later analyzed with the software OriginPro 9.1.0 (OriginLab Corporation, USA).

Statistical analysis

The results were analyzed through a two-way ANOVA and LSD Fisher test ($\alpha = 0.05$) with the statistical analysis software InfoStat (Version 2018, Grupo InfoStat, FCA, National University of Cordoba, Argentina)²⁴.

Results and Discussion

The waste sample of the oil tank settling was composed of $54.9 \pm 3.8\%$ of hexane insoluble material and $45.1 \pm 5.6\%$ of oily material; being 16.9% of this oily fraction impure waxes.

Performance of waxes obtained in oleogels elaboration

In order to determine the potential use of the recovered waxes as organogelants agents, SWLA, SWDA and SWB were used in the production of oleogels with HOSO. The C^* values for each wax are shown in **Table 1**. When considering the concentration of the wax material, it was found that amongst the recovered waxes, SWB were the most efficient gelling agent, with only a small amount of wax material needed to completely gel HOSO. This value was slightly higher than that obtained using CSW and those reported by Patel¹² ($C^* = 5$ g wax material/kg of oleogel) and Blake et al.⁹ ($C^* = 10$ g wax material/kg of oleogel) for CSW. Oleogels developed with SWLA and SWDA required higher concentrations to generate gels that did not flow when the test tubes were inverted (**Table 1**). These differences will be further analyzed when comparing the wax esters profile of the waxes.

Wax Characterization

Color measurement

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Regarding their color, significant differences ($p < 0.05$) were found in L^* , a^* and b^* for SWLA, SWDA and SWB (**Table 2**). All of the samples presented medium to high L^* values, indicating their brightness, and positive values of a^* and b^* , showing a tendency toward red and yellow. SWB presented the highest L^* values, being the brightest and less pigmented (smallest number of red and yellow components).

Chalapud et al.¹¹ analysed SW recovered from filter cakes coming from conventional and high-oleic sunflower-oil winterization, that were purified by successive washings with cold n-hexane, also found high L^* values (88.41 and 85.49 for conventional and high-oleic hybrids, respectively) similar to those of SWB. Through colorimetry it was possible to verify that SWDA were the darkest wax due to the lack of luminosity and not to the pigmentation since it possessed a smaller amount of red and yellow components than SWLA, represented by a smaller C value (**Table 2**). This may be due to the fact that during crystallization the waxes retain a great number of colored impurities, originating crystals with differences in their densities, which after centrifugation generates the color separation of the two fractions of waxes. In general, the a^* and b^* values were not far from those reported by Chalapud et al.¹¹ ($a^* = 8.48$ and 8.61 ; $b^* = 7.61$ and 9.49 for conventional and high-oleic hybrid, respectively), except for SWB whose b^* value was appreciably lower, indicating the elimination of substances with yellow hue when the purification method B is applied. CSW were slightly darker ($L^* = 85.80 \pm 0.23$) and presented a smaller number of red component

($a^* = 6.51 \pm 0.02$) and a higher number of yellow components ($b^* = 3.88 \pm 0.09$) than SWB.

The oleogels presented medium to low values of L^* and positive values of b^* , indicating their tendency towards yellow (**Table 2**). OSWB were the brightest (highest L^* values) and less pigmented oleogels (smallest C values). OSWLA and OSWDA showed a tendency towards red (positive a^* values), while OSWB and OCSW showed a tendency towards green (negative a^* values). Specifically, the a^* values of OSWLA and OSWDA were related to the color of the waxes used as the gelling agent, while the negative a^* values of OSWB and OCSW were related to the greenish color of HOSO (**Table 2**) and not to the color of the waxes.

No significant differences were found in L^* for OSWLA and OSWDA (**Table 2**). When comparing these oleogels, it was observed that OSWLA was slightly darker than OSWDA due to a greater amount of pigments (higher C value) and not a difference in their luminosity. This is the same behavior observed in SWLA and SWDA (**Table 2**). However, there were significant differences between these two oleogels and the ones developed using SWB and CSW.

Wax ester analysis

Representative chromatograms of the wax esters distribution of SWLA, SWDA, SWB and CSW are shown in **Fig. 1**, while the results of the quantitative analysis are presented in **Table 3**. The analysis of the first fraction from the column chromatography revealed absence of hydrocarbons in all the wax samples. SWLA, SWDA, SWB and CSW were composed of wax esters in the range of 40 - 60 carbon atoms. In SWLA, SWDA and SWB the higher percentages of wax esters corresponded to C46, C48, C44, C50, followed by C52 and C54, representing 82.8% of the total wax esters in SWLA, while for SWDA and SWB it was 79.8% and 85.5%, respectively. In addition, it was observed the presence of small amounts of wax esters with odd numbers of carbons (SWLA= 10.8%, SWDA= 10.3% and SWB= 9.1%). CSW presented a very similar wax esters distribution to that of SWB, with the higher percentages of wax esters corresponding to C46 (135.11 ± 22.41 g wax esters/kg wax material), C44 (114.26 ± 18.94 g wax esters/kg wax material), C48 (89.79 ± 14.88 g wax esters/kg wax material), C50 (50.76 ± 8.41 g wax esters/kg wax material) and C52 (39.22 ± 6.50 g wax esters/kg wax material). All the wax esters profiles were close to that reported for waxes from winterization tanks of conventional sunflower oil recovered by Chalapud et al.¹¹. SWB presented an intermediate percentage of wax esters $\geq$ C46 (86.07%) with respect to SWLA (83.2%) and SWDA (88.73%). Differences between sunflower wax profiles can arise from the hybrid seed processed, the waste used to recover the waxes, and their purification treatment, among other factors.

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The quantitative analysis of the wax esters also showed that SWLA contained 24.8% of aliphatic esters while SWDA had 28.3% of said esters, indicating that they still had an appreciable number of other components. In contrast, SWB had a considerable higher amount of wax esters (76.6%), with 91.4% esters of even carbon number. In spite of this, it was evident the presence of other compounds, representing approximately 23.4% of the total weight.

The differences between the C^* values of the samples could be explained with total of wax esters in each sample: SWB and CSW presented a total wax content almost three times greater than SWDA, while the latter had a wax content slightly higher than SWLA (**Table 3**). As previously mentioned, all the wax samples were composed of wax esters, but also contained, to a greater or lesser extent, remaining impurities. In **Table 1** the C^* values, expressed as a function of the concentration of the wax esters, are shown. SWLA and SWDA required a similar concentration of wax esters (2.46 and 2.54%, respectively) to completely structure HOSO. These C^* values were higher than those required when using SWB and CSW as gelling agents (1.14 and 0.79%, respectively). Hwang et al.¹⁰ and Doan et al.¹³ have stated that impurities within the waxes altered their gelling properties, and, as we have been discussing, SWLA and SWDA were waxes that presented a high content of impurities, while SWB and CSW contained a smaller amount of impurities.

Thin-layer Chromatography

The sample studied in this work came directly from the storage tanks of crude oil, which is the first step of the purification process. In this stage, the particulate material is left to decant, along with other chemical components that sunflower oil possesses. Considering that sunflower oil is composed of triacylglycerols, phospholipids and pigments, such as carotenoids³, a TLC was performed in order to determine which of these compounds remained in the waxes after the purification process (**Table 4**). The R_f value of the wax standard was 0.58. After the development of the TLC plate, a dark orange-brown spot correspondent to phospholipids ($R_f = 0$) was observed in SWLA and SWDA. A lighter spot was observed in SWB, indicating that this sample contained only traces of phospholipids. SWDA possessed traces of triacylglycerols, while SWLA and SWB presented traces of triacylglycerols and monoacylglycerols, corroborating the existence of sunflower oil within all of them. Traces of carotenes were observed in SWLA and SWDA, but surprisingly also in SWB indicating that these compounds are not removed by filtration through diatomaceous earth. Finally, SWLA, SWDA and SWB presented traces of free fatty alcohols and free fatty acids.

Thermal Behavior

The thermal behavior of SWLA, SWDA and SWB and their comparison with CSW is presented in **Fig. 2**. As several authors have stated, SW are chemically homogeneous waxes, composed almost entirely of esters (97-100%) from C39 to C60^{9,11,12,25}. Their

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thermal transition is characterized by a single peak correspondent to the melting or the crystallization of the mentioned esters. This fact is observed in the heating thermograms but not in the cooling thermograms of all the samples (**Fig. 2**) where the cooling transition of the wax esters (T_c) was not characterized by a single peak. Instead, T_c were composed of two overlapping peaks in SWDA ($T_c = 68.68\text{ }^{\circ}\text{C}$ and $T_c = 73.02\text{ }^{\circ}\text{C}$, **Fig. 2d**) and CSW ($T_c = 71.06\text{ }^{\circ}\text{C}$ and $T_c = 74.43\text{ }^{\circ}\text{C}$, **Fig. 2h**), three overlapping peaks in SWB ($T_c = 71.36\text{ }^{\circ}\text{C}$, $T_c = 71.88\text{ }^{\circ}\text{C}$ and $T_c = 72.70\text{ }^{\circ}\text{C}$, **Fig. 2f**) and four overlapping peaks in SWLA ($T_c = 68.88\text{ }^{\circ}\text{C}$, $T_c = 71.24\text{ }^{\circ}\text{C}$, $T_c = 72.71\text{ }^{\circ}\text{C}$ and $T_c = 73.83\text{ }^{\circ}\text{C}$, **Fig. 2b**). Since all of the samples presented a single peak, corresponding to the T_m of the wax esters (peak I, **Fig. 2a, 2c, 2e** and **2g**), it could be assumed that the separation of the peak I in T_c may be due to differences in the crystallization of the wax esters that composed each sample (**Table 3**). Notably, in CSW one of these peaks was a very narrow peak. This type of peak could indicate that a portion of the wax esters crystallized in a perfectly crystalline manner²⁶. For the quantitative analysis, the overlapping peaks were integrated together. In addition, SWLA and SWB presented a second peak (II) at lower T_c (57.19 and 61.85 $^{\circ}\text{C}$, respectively), indicating the presence of an impurity that crystallized along with the waxes.

SWB and CSW exhibited only one peak corresponding to T_m of the sunflower wax esters (peak I, **Fig. 2e** and **2g**, respectively). However, peak I in CSW was broader than that of SWB. The presence of other peaks in the melting thermograms of SWLA and

SWDA (**Fig. 2a** and **2c**, respectively) was a clear indicator of the presence of minor components that act as impurities, affecting the crystallization and morphology of the wax crystals. In both thermograms the endothermic peak (I) corresponded to T_m of the sunflower wax esters, while a second endothermic peak (II), at lower temperatures ($T_m = 66.46\text{ }^{\circ}\text{C}$ and $T_m = 68.58\text{ }^{\circ}\text{C}$, respectively) and a third endothermic peak (III), at higher temperatures ($T_m = 84.44$ and $T_m = 85.46\text{ }^{\circ}\text{C}$, respectively) was an indicative of the presence of other components.

In **Table 5** the statistical analysis of the melting and crystallization parameters of SWLA, SWDA, SWB and CSW is shown. With respect to T_m of peak I, the statistical analysis did not reveal significant differences ($p > 0.05$) between SWLA and CSW, but there were significant differences ($p < 0.05$) between these samples with respect to SWDA and SWB, being the latter the one with the smallest T_m value. To explain these results two things must be taken into account: that T_m increases with the increase in the length of the chain of wax esters, and that SWDA was the sample that had the highest proportion of crystallizable waxes between C50 and C60 (**Table 3**). Despite these differences, T_m of all of the samples were in accordance with values obtained by other authors for recovered SW ($T_m = 77.34\text{ }^{\circ}\text{C}$ ²⁷; $T_m = 76.70$ and $80.53\text{ }^{\circ}\text{C}$ ¹¹; $T_m = 70 - 80\text{ }^{\circ}\text{C}$ ²⁸).

There were significant differences for ΔH_m of SWLA, SWDA, SWB and CSW ($p < 0.05$), being CSW the SW that presented the highest value (**Table 5**). When comparing the ΔH_m values of the recovered waxes, it was observed that there was a marked increase in the enthalpy of SWB, followed by SWDA and finally, SWLA. This could be related to the fact that SWB contained a total wax content much higher than SWLA and SWDA (**Table 3**), resulting in an increase in the amount of energy required to completely melt Blake et al.⁹ reported a ΔH_m value for commercial SW slightly higher than that of SWB (195 ± 29.8 J/g), while Chalapud et al.¹¹ reported higher ΔH_m values for recovered SW (202.77 and 204.35 J/g). Additionally, ΔH_m was expressed as a function of the wax esters (**Table 5**). As with T_m , ΔH_m augments as the length of the chain of the wax esters increases, since more energy is required to overcome the intermolecular forces between them. SWLA was the sample with the highest proportion of higher molecular weight odd number wax esters ($>C_{50}$, 27.88%), followed by SWLA (20.41%), CSW (19.20%), and finally SWB (17.89%).

Even though the T_c values of SWLA, SWDA, SWB and CSW were lower than expected, they were still comparable with T_c values obtained by Martini and Añon (74.3 – 74.8 °C)²⁸. In addition, significant differences ($p < 0.05$) were observed in ΔH_c , being CSW the sample that released the greatest amount of energy during the crystallization stage.

Blake et al.⁹ stated that ΔS_m can be used as a measure of the crystallinity of the waxes. The chemical homogeneity of SW allows the formation of homogeneous crystals in size and shape, that interact strongly with each other, generating a very stable crystalline network, as indicated by a higher ΔS_m . However, when the sample has other minor compounds (chemical heterogeneity), mixed crystals are formed. These crystals arrange in a disorganized manner causing a decrease in ΔS_m . SWB presented the highest ΔS_m value (**Table 5**) of all the recovered waxes, and, therefore, the highest crystallinity. This was expected since SWB was the sample with the highest total wax content thus, the lowest amount of remaining impurities (**Table 3**). However, this value was lower than that obtained for CSW (**Table 5**), indicating that SWB were not as crystalline as CSW. A similar value for ΔS_m was reported by Blake et al.⁹ for commercial waxes [557 mJ/(g.K)]. SWLA presented the lowest ΔS_m value, indicating the lowest crystalline order. As previously mentioned, this was due to the fact that SWLA had the lowest total wax content, thus, the highest percentage of impurities.

Oleogel Characterization

Polarized light microscopy

The microstructure of OSWLA, OSWDA, OSWB and OCSW was visualized through polarized light microscopy (**Fig. 3**). As previously reported by other authors^{6,7,14,29}, SW crystals aggregate and form a three-dimensional structure capable of retaining the liquid oily phase (in this case, HOSO) within its pores. Specifically, Blake et al.⁹ reported

that when visualized under polarized light, SW crystals (anisotropic material presenting birefringence) appeared as bright structures with a fibrous, needle-like morphology in canola oil, which was observed as black since it is optically isotropic. Yilmaz and Ogutcu⁷ reported the same morphology of the SW crystals in olive oil, however, they measure the crystal length using XRD.

The differences between C* for OSWLA, OSWDA, OSWB and OCSW could be explained with the morphology of the SW crystals. OSWB (**Fig. 3c**) and OCSW (**Fig. 3d**) presented a network composed of fibrous, needle-like crystals, with the length ranging from 26 – 52 μm and 21 – 45 μm , respectively. These micrographs were similar to those reported by Blake et al.⁹ and Yilmaz and Ogutcu⁷. However, the crystals in OSWB and OCSW were slightly longer than those measured by Blake et al.⁹ (15 and 25 μm). As previously discussed, SWB and CSW possessed a considerable amount of wax esters in their composition. These wax esters are the responsible for the needle-like morphology due to the interaction within parallel laminar planes, resulting in high lateral crystal growth rates³⁰. This needle-like morphology is the most desirable shape to gel a large volume of oil, due to their ability to arrange into a crystalline network capable of immobilize a large amount of oil^{30,31}. Blake and Marangoni³² observed the morphology of de-oiled oleogels of SW in peanut oil using cryo-SEM and reported that SW crystals formed platelet-like crystals and not fibrillar crystals as observed with polarized light microscopy. The authors suggested that the needle-like crystals were

the edges of platelets, while Hwang et al.³³ suggested that the small platelets could fold over themselves, observing the bent part as a needle-like crystal under polarized light.

Neither in OSWLA (**Fig. 3a**) nor OSWDA (**Fig. 3b**) the fibrillar morphology was observed. Instead, SW appeared to be finely dispersed grain-like crystals. In addition, both oleogels presented darker, yellowish - brown stains, as well as granules of the waxes that did not solubilize in HOSO. Dassanayake et al.³⁰ and Toro-Vazquez et al.³¹ stated that impurities alter the crystallization process and thus, the morphology of the SW, resulting in smaller crystals structures and weaker packing arrangements. As previously discussed, SWLA and SWDA had a considerable amount of impurities, so this could explain the lack of fibrous crystals within OSWLA and OSWDA network.

Texture analysis

No significant differences ($p < 0.05$) were found between the firmness of OSWB (0.10 ± 0.02 N) and OCSW (0.13 ± 0.04 N). However, there were significant differences ($p > 0.05$) between these two oleogels and OSWDA (0.26 ± 0.08 N), being the latter the gel that showed the maximum value of firmness. In order to understand these results two things must be taken into consideration: the amount of SW in each of the wax materials and the microstructure of the crystal network in each oleogel. As previously discussed, SWDA required a larger amount of wax esters to completely gelled HOSO,

than SWB and CSW (**Table 1**). This resulted in higher concentration of wax esters in OSWDA. Regarding the microstructure of the SW crystals, Hwang et al.¹⁰ suggested that a network composed of a large amount of small crystals increase the firmness of the oleogels, since this network could resist to a greater extent the deformations generated during the penetration test.

Finally, the firmness of OSWLA could not be determined with the Texture analyzer. When examining the remains of these oleogels after the penetration, it was observed that the center showed a more viscous appearance than the exterior of the oleogels. This was not evidenced during the determination of C^* , because possibly the gelled outside of OSWLA hold the non-gelled interior.

Rheological analysis

Patel et al.³⁴ defined a "gel" as a substance that exhibits more elastic behavior than viscous behavior, represented by a greater storage modulus (G') than loss modulus (G''). Furthermore, this behavior must continue under the application of low shear for a time period in the order of seconds, creating a plateau known as the linear viscoelastic region.

The G' and G'' values of the oleogels developed in this work are represented in **Fig. 4a**. OSWDA, OSWB and OCSW fulfilled the definition of "gel" ($G' > G''$). At low ω OSWLA

was found to be slightly more elastic than viscous ($G' > G''$). With an increase in ω , both G' and G'' increased. However, at $\omega = 8.92$ rad/s a crossover between G' and G'' , indicating that at medium to high frequencies the system behaved more like a viscous sol than an elastic gel. OSWDA were the oleogels that showed the lowest frequency dependence, as given by somewhat linear curves, while OSWLA showed the most frequency dependence, as given by the non-linear curves in **Fig. 4a**. In addition, OSWLA presented the lowest G' values, indicating that these samples were not as elastic as the others oleogels. The elastic behavior of an oleogel is attributed to the arrangements of the crystalline network, and as previously discussed, SWLA did not form the expected fibrillar crystals, instead, they appeared to be finely dispersed crystals (**Fig. 3a**) with less entanglement between the crystals. As shown in **Fig. 3c**, SWB acquired the fibrillar morphology necessary to generate gels with loose entanglements, and hence, high elasticity. This crystal morphology was not observed in OSWDA. OSWB and OSWDA showed similar G' along the frequency sweep, being their G' values lower than those of OCSW.

The structure of the oleogels was evaluated analyzing the damping factor ($\tan \delta$)³⁵. This rheological parameter describes the ratio between the elastic and viscous components (G' and G'' , respectively) of the oleogels under shear at a constant strain ($\tan \delta = G''/G'$)³⁶. For a gel to be considered a "strong gel" the damping factor must be ≤ 0.1 , indicating the preponderance of the elastic portion of the sample. In general, as

the structure becomes increasingly weak, the viscous portion of the oleogels becomes more significant, thus, the damping factor increases its value, up to the point when $\tan \delta \geq 1$. These are the gels that are considered “weak gels”³⁴.

None of the samples fulfilled the definition of “strong gel”, since they all presented values of $\tan \delta$ higher than 0.1 (**Fig 4b**). Despite this, OSWDA were the oleogels that presented the smallest values of $\tan \delta$ at low angular frequency (ω), and therefore, the strongest structure, being these values slightly higher than 0.1. OSWLA were the oleogels that presented the highest values of $\tan \delta$ (> 0.9). As previously mentioned, these values closer to the unity at low ω were a clear indicative that these samples possessed a weak structure, and that the viscous portion of the viscoelastic behavior of the oleogels was the predominant. This could explain why the firmness of these gels could not be determined: SWLA were able to structure HOSO, but the generated gels presented such a weak structure, that they could not tolerate the minimum force required by the texture analyzer. Finally, OSWB were the oleogels that presented a damping factor similar to CSW at low ω (**Fig 4b**).

Conclusion

It was verified that sunflower waxes could be recovered from oil tank settlings, and that the purification method B allowed the obtention of sunflower waxes with similar physicochemical properties to those of commercial and recovered sunflower waxes. In

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addition, it was observed that SWB had a lower amount of remaining impurities than SWLA and SWDA, and therefore, had a higher total content of waxes. Also, SWB were the most efficient structuring agent, developing oleogels that showed similar color, microstructure, firmness and rheological properties as the gels developed with commercial SW. This recovery and purification process also have great relevance, because it gives the oil industry the possibility to reprocess a residue that is currently eliminated or used as animal feed. The next step of our research will be the development of oleogels with different properties, which could be utilized in various industries, such as the food industry.

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Figure Legends

Figure 1. Chromatogram of recovered sunflower waxes. (a) SWLA, (b) SWDA, (c) SWB and (d) CSW.

Figure 2. Thermal behavior of: SWLA (a) melting, (b) crystallization; SWDA (c) melting, (d) crystallization; SWB (e) melting, (f) crystallization; CSW (g) melting, (h) crystallization.

Figure 3. Polarized light microscopy images of a) OSWLA, b) OSWDA, c) OSWB and d) OCSW at their critical gelling concentration.

Figure 4. Frequency sweeps for oleogels developed at their correspondent critical gelling concentration. a) G' and G'' are shown as filled and open symbols, respectively.

Table 1. Critical gelling concentration (C*) of SWLA, SWDA, SWB and CSW.

Wax	C* (g wax material/Kg of oleogel)	
	Wax material	Wax esters
SWLA	100	24.7
SWDA	90	25.4
SWB	15	11.4
CSW	10	7.9

Table 2. Colour parameters of the recovered waxes, HOSO and oleogels.

	L*	a*	b*	C
Wax material				
SWLA	65.43 ± 0.19 ^b	10.79 ± 0.02 ^c	11.46 ± 0.04 ^c	15.74 ± 0.04 ^c
SWDA	56.11 ± 5.03 ^a	9.61 ± 0.45 ^b	8.18 ± 1.14 ^b	12.62 ± 1.08 ^b
SWB	87.74 ± 0.06 ^c	9.00 ± 0.03 ^a	1.76 ± 0.21 ^a	9.17 ± 0.01 ^a
Oil				
HOSO	90.57 ± 0.02	-2.25 ± 0.01	5.96 ± 0.02	6.37 ± 0.02
Oleogels				
OSWLA	29.03 ± 1.46 ^a	8.66 ± 0.14 ^d	17.89 ± 0.48 ^d	19.88 ± 0.42 ^d
OSWDA	29.06 ± 1.20 ^a	7.03 ± 0.13 ^c	16.60 ± 0.75 ^c	18.08 ± 3.17 ^c
OSWB	39.13 ± 0.29 ^c	-2.07 ± 0.09 ^b	4.32 ± 1.63 ^a	4.80 ± 1.50 ^a
OCSW	37.97 ± 0.33 ^b	-2.35 ± 0.30 ^a	5.63 ± 0.78 ^b	6.10 ± 0.83 ^b

Mean values ± confidence intervals are shown, n = 3. Values in the same column followed by different letters are significantly different (p<0.05) by the Fisher test.

Table 3: Composition of the wax esters in SWLA, SWDA and SWB (g wax esters/Kg wax material).

	SWLA	SWDA	SWB
C40	0.05 ± 0.04 ^a	Tr. ^a	Tr. ^a
C41	Tr. ^a	Tr. ^a	Tr. ^a
C42	3.17 ± 0.70 ^c	1.66 ± 0.07 ^b	2.61 ± 0.57 ^a
C43	0.84 ± 0.13 ^b	0.59 ± 0.02 ^a	1.54 ± 0.42 ^a
C44	33.04 ± 3.38 ^c	25.74 ± 0.80 ^a	87.98 ± 16.96 ^b
C45	4.42 ± 0.38 ^b	3.88 ± 0.11 ^a	14.22 ± 2.28 ^b
C46	60.70 ± 2.51 ^b	58.82 ± 1.46 ^a	213.08 ± 28.97 ^c
C47	4.79 ± 0.18 ^b	5.09 ± 0.16 ^a	17.32 ± 2.11 ^c
C48	46.70 ± 1.26 ^a	52.84 ± 1.12 ^a	165.54 ± 21.70 ^b
C49	3.49 ± 0.14 ^a	4.23 ± 0.16 ^a	11.49 ± 1.84 ^a
C50	28.60 ± 2.02 ^a	35.98 ± 2.58 ^a	91.20 ± 15.13 ^a
C51	2.69 ± 0.24 ^a	3.62 ± 0.30 ^b	7.74 ± 1.63 ^a
C52	21.45 ± 3.20 ^a	30.66 ± 2.27 ^b	62.08 ± 12.61 ^a
C53	2.07 ± 0.36 ^a	3.18 ± 0.33 ^b	5.54 ± 1.48 ^a
C54	13.77 ± 3.20 ^b	21.74 ± 2.34 ^c	35.13 ± 6.41 ^a
C55	2.59 ± 0.72 ^a	3.69 ± 0.32 ^a	5.66 ± 5.12 ^a
C56	7.75 ± 1.79 ^a	13.25 ± 1.20 ^b	20.61 ± 4.10 ^a
C57	1.76 ± 1.03 ^{a, b}	2.66 ± 0.33 ^b	3.35 ± 2.58 ^a
C58	4.85 ± 1.33 ^b	8.44 ± 0.91 ^c	11.89 ± 2.63 ^a
C59	1.38 ± 0.81 ^b	2.03 ± 0.18 ^b	2.48 ± 0.94 ^c
C60	2.77 ± 0.98 ^b	4.90 ± 0.61 ^c	6.18 ± 3.00 ^a

Total content	246.92 ± 24.44^a	283.02 ± 15.33^a	759.57 ± 130.86^b
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Mean values $\pm$ confidence intervals are shown (n=4). Values for the same component in the same row followed by different letters are significantly different ($p < 0.05$) by the Fisher test. Tr. traces ($< 0.1\%$).

Table 4: R_f values of standards and recovered waxes.

	PC	FA	MAG	FFA	TAG	Carotene
Standard	0.00	0.06	0.12	0.25	0.42	0.81
SWLA	0.00	0.05	0.09	0.25	0.42	0.72
SWDA	0.00	0.05	-	0.25	0.41	0.77
SWB	0.00	0.08	0.13	0.20	0.41	0.88

PC: L – α – phosphatidylcholine, FA: fatty alcohols, MAG: monoacylglycerols, FFA: free fatty acids, TAG: triacylglycerols.

Table 5: Melting and crystallization parameters of SWLA, SWDA, SWB and CSW.

	SWLA	SWDA	SWB	CSW
T_m (°C)	76.76 ± 0.31^b	78.65 ± 0.48^c	75.13 ± 1.05^a	76.86 ± 0.54^b
ΔH_m (J/g wax material)	73.45 ± 4.41^a	128.11 ± 5.26^b	167.71 ± 9.05^c	213.98 ± 16.79^d
ΔH_m (J/g wax esters)	297.46 ± 17.86^a	452.65 ± 18.58^b	220.80 ± 11.91^c	277.90 ± 21.80^d
$T_{on\ m}$ (°C)	72.42 ± 0.06^c	72.48 ± 0.60^c	69.02 ± 0.46^a	70.40 ± 0.19^b
ΔS_m [mJ/(g.K)]	218.86 ± 39.67^a	364.14 ± 14.80^b	481.19 ± 26.15^c	611.34 ± 48.11^d
T_c (°C)	$70.67 \pm 3.18^{a,b}$	69.39 ± 1.53^a	72.12 ± 0.56^c	$70.96 \pm 0.57^{b,c}$
ΔH_c (J/g wax material)	75.52 ± 4.89^a	146.81 ± 5.45^b	164.35 ± 8.94^c	217.13 ± 10.13^d
$T_{on\ c}$ (°C)	74.71 ± 0.39^c	73.41 ± 0.29^a	74.01 ± 1.02^b	74.43 ± 0.09^c

Mean values $\pm$ confidence intervals are shown (n=3). Values in the same row followed by different letters are significantly different ($p < 0.05$) by the Fisher test.