Effect of different oleogelators on lipolysis and curcuminoid bioaccessibility upon in vitro digestion of sunflower oil oleogels

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Abstract: Sunflower oil enriched with curcuminoid compounds (CUs) was gelled by adding 5% (w/w) saturated monoglycerides (MG), rice bran waxes (RW) or a mixture of β-sitosterol and γ-oryzanol (PS). The resulting oleogels differed for rheological properties and firmness due to the difference in gel network structure. PS oleogel was the firmest sample followed by RW and MG ones. Upon in vitro digestion, fatty acid release as a function of digestion time was greatly affected by oleogel structure: the extent of lipolysis decreased as oleogel strength increased (PS<RW<MG). On the other hand, the nature of the oleogelator affected CUs bioaccessibility, which was lower in oleogels containing crystalline particles (MG and RW). These findings appear interesting in the attempt to develop oleogels able to control lipid digestion as well as to deliver bioactive molecules in food systems.

Dear Editor,

I would like to submit the revised manuscript entitled *"Effect of different oleogelators on lipolysis and curcuminoid bioaccessibility upon in vitro digestion of sunflower oil oleogels"* by Sonia Calligaris, Marilisa Alongi, Paolo Lucci and Monica Anese for consideration for publication in *Food Chemistry*.

We thank the Reviewers for supplying their useful observations that, in our opinion, allowed us to further improve the quality and the clarity of the manuscript. We hope that in this form it will be suitable for publication in Food Chemistry.

Best regards

Marilisa Alongi

Ms. Ref. No.: FOODCHEM-D-19-03433R1

Title: Effect of oleogelator type on lipolysis kinetics and curcuminoids bioaccessibility upon in vitro digestion of sunflower oil-based oleogels

Answers to Reviewers' comments

(Please find grouped text pertaining to Reviewer 1, 2, 3 or 4, and to the Editor; Reviewer text is in normal, and Answer text is italics, for each numbered item)

Reviewer #1:

This version of the manuscript has been improved significantly and it is thus more acceptable for publication in Food Chemistry. Most of my comments have been addressed, except for the fact tha there is no rhyme of reason for the concentrations of the systems chosen. We have no idea if other concentrations have the same effect. This is not going to change with any number of revisions here. The study is also only an in vitro study. These are early days in this research, so this may be OK. The manuscript is well written, clear, and the science is sound. The scope of the work, more limited. It is OK to consider publication as is.

The authors thank the reviewer for his/her appreciation of the general idea of the work. Indeed, further research considering also ex vivo and in vivo models is required to shed some light on this topic which is still in its infancy.

Reviewer #2:

The authors did a good work on improving the manuscript, which results from excluding the samples of EC. Despite this good work, there are still some major points that should be clarified before considering the manuscript for publication.

Line 228-230. The amount of ingredient or additive depends on the material used and the food where is used. Therefore, should not be assumed that this concentration was selected because can be used in all food applications. To do so the authors should indicate the food product and explain in detail the amount allowed by EU regulations or FDA of each gelator, as example. Is rice bran wax approved to use in foods in EU and USA as texturizer?

We agree with the reviewer that our consideration was too general. Moreover, being early days in this research, the reference to the final food application is premature. Only after the comprehension of the effect of structure on the digestive fate of oleogels, it would be possible to speculate applications in both the food and pharmaceutical fields. We modified the text accordingly (lines 220-222).

How did the authors check the size above the 6 um "However, to the aim of the present work, the determination of particles up to 5.5 <mu>m was exhaustive. In fact, the determination was applied to digested oleogel and oil samples in which, as reported also in the literature, the biggest family expected present an average diameter below this value". The way that this was verified should be included in the manuscript. This should be taken in account for size and Zeta potential.

In this work, we determined the particle size and zeta potential only on the micellar phase obtained after the intestinal digestion phase. This micellar phase was obtained after intense centrifugation (30,000 g x 70 min) of the sample as reported in line 156 and as suggested by the literature (Ahmed et al., 2012). These centrifugation conditions were selected after preliminary trials based on the conditions applied in the literature, being capable to remove big particles from the sample while maintaining in dispersion micelles. Only in this way, the sample was suitable for BAC assessment.

If we understand well the issue raised by the reviewer, he/she is suggesting studying the destructurization of the sample during digestion, considering not only the mixed micellar phase, that was the topic of the present work but also the digested sample before centrifugation as well as the sediment recovered upon digestion and centrifugation. This is a really interesting topic that should be properly addressed with appropriate experiments in further studies.

To improve the clarity of our manuscript, we better explicated in the M&M and R&D sections which samples were considered in this study (line 160).

The justification for the difference between the BAC and lipolysis is still not clear. The degradation can one of the justifications but also the extraction procedure and the gel structure. Both MG and Wax are organized in strong crystalline lamellar structures and BS in tubular structures. Can this have any influence on the CU BAC?

The reduced BAC in MG and RW containing systems is for sure tricky and not easy to explain. Besides oxidation, it cannot be excluded a role of the matrix in entrapping CUs, as suggested by the reviewer. Being no literature on this aspect, we can only speculate the reasons for such results and more research is needed on this topic. More comments were added in the text (lines 353-362).

Another of the doubts is how the authors considered the max lipolysis, once by the Figure is not clear that the maximum was reached at the same time for all the samples.

We understand the issue of the reviewer. In the experiments, we considered the FFA_{max} as the maximum value reached the plateau. However, we agree with the fact that it should be better to compare samples at a defined length of time. For this reason, we repeated some experiments to confirm results and compare data at 30 min of lipolysis, improving in this way also the quality of Figure 2.

Reviewer #3:

The authors have made considerable revision to address the comments raised by all reviewers. The paper in its current state, nevertheless, requires some corrections, because after eliminating some of the data and revising the text some key issues have remained:

The authors have deleted the kinetics analysis from the text, but still refer to the effect of oil structuring on lipolysis kinetics (title, highlights, discussion (line 322), and conclusions). More proper reference would be to the total free fatty acids release.

Suggested changes were made.

Lines 235-237: the results are not similar (your statistical analysis showed differences).

The text was modified accordingly (lines 230-231).

In general, the authors did not suggest an explanation why one system showed higher G' compare to the other. Please do ... this is necessary.
Lines 250-251: why would the crystal morphology lead to different gel firmness? Justify.
Why was the PS sample firmer than the other two? Explain/justify.

The last three reviewer considerations were carefully considered and this part of the manuscript was deeply revised (lines 234-241 and 254-258). We hope that in this version the effect of network structure on system properties is clearer than in the previous one.

What is the source of the different surface electrical charge showed in the zeta potential analysis? Can you suggest a mechanism?

Based on literature data we can only speculate an effect of surface-active species present in RW and PS. We modified the text accordingly (lines 308-312).

Lines 323-325: Ashkar et al. (2019) did not show that the final strength of the oleogel is the most critical factor affecting lipolysis, they concluded that different structuring agents and gelation mechanisms demonstrate different susceptibility to digestive lipolysis. Fix.

The text was modified as indicated (lines 325-326).

Lines 348-350: how does the involvement in gelation/crystallization relate to exposure to oxidation? Explain in detail.

The authors should consider interactions between the MG or RW with the CU affecting the results seen in the bioaccessibility.

Unfortunately, to our knowledge in the literature, there is no clear evidence on the effect of lipid physical state on bioactive molecule bioaccessibility. We added some comments in the text on this aspect (lines 348-350 and 353-362).

The sample size (not thickness) is missing in the firmness method section. Add.

Details were added to the text (line 112).

The manuscript should be edited by an English editor.

The manuscript was submitted to English revision.

Reviewer #4:

Please revise the manuscript in accordance with the comments below.

1- It must be pointed out in the text, how much time was necessary to structure the <gamma>-oryzanol and <beta>-sitosterol oleogel.

Details were added to the text (lines 104-105).

2- The authors must provide the specifications of the compression probe used in the firmness measurements in the materials and methods section.

Details were added to the text (line 113).

Editor's Comments:

The original four referees have now commented on your revised manuscript. All indicate that great strides have been made in revising the R0. One accepted the paper as is. Unfortunately, three others still have major issues for you to address. Please consider the comments of all reviewers very carefully and either adopt or rebut each and every point made. In your rebuttal please indicate the line number in the revised manuscript corresponding to each change that has been made and possibly use yellow highlighting or color in the text to indicate the edits.

More and more these days, manuscripts are being rejected at the R1 and R2 stages for poor English. Please don't let this happen to you! Due to dwindling resources, typesetters can no longer edit the English, so there's a new category, "Reject Due to Poor Language (regardless of the scientific quality)", in the Decision Box of this EES system. At least one of the reviewers notes English problems in your submission. I strongly suggest that the paper be scrutinized very carefully by a native speaker of the language with a background in food chemistry, or pay for a reputable service to do this, before the R2 is submitted via the EES portal.

Highlights

Sunflower oil was enriched with curcuminoids

Curcuminoid-enriched oil was gelled with different gelators

Obtained oleogels differed for rheological properties and firmness

Lypolysis kinetic during *in vitro* digestion was affected by gel structure

The type of gelator, instead of gel strength, affected curcuminoid bioaccessibility

Effect of different oleogelators on lipolysis and curcuminoid bioaccessibility upon *in vitro* digestion of sunflower oil oleogels

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Abstract

Sunflower oil enriched with curcuminoid compounds (CUs) was gelled by adding 5% (w/w) saturated monoglycerides (MG), rice bran waxes (RW) or a mixture of *β*-sitosterol and *γ*-oryzanol (PS). The resulting oleogels differed for rheological properties and firmness due to the difference in gel network structure. PS oleogel was the firmest sample followed by RW and MG ones. Upon *in vitro* digestion, fatty acid release as a function of digestion time was greatly affected by oleogel structure: the extent of lipolysis decreased as oleogel strength increased (PS<RW<MG). On the other hand, the nature of the oleogelator affected CUs bioaccessibility, which was lower in oleogels containing crystalline particles (MG and RW). These findings appear interesting in the attempt to develop oleogels able to control lipid digestion as well as to deliver bioactive molecules in food systems.

Keywords

Oleogel; Curcuminoids; Lipolysis; Bioaccessibility; Structure

1. Introduction

Oil gelation is a relatively novel strategy in which liquid oils are converted into semi-solid materials (Co & Marangoni, 2012; Patel & Dewettinck, 2016). Different oil gelators have been proposed in the literature, including low-molecular weight compounds (e.g. monoglycerides, waxes, sorbitan tristearate, 12-hydroxystearic acid, phytosterols, fatty alcohols, and fatty acids) and high-molecular weight polymers, such as ethylcellulose and chitin (Co & Marangoni, 2012; Nikiforidis & Scholten, 2015; Patel & Dewettinck, 2016; Rogers, Wright, & Marangoni, 2009; Singh, Auzanneau, & Rogers, 2017). These compounds directly gel into liquid oil by forming a self-assembly network according to different mechanisms. Most low-molecular weight gelators (e.g. monoglycerides, waxes, fatty acids, and fatty alcohols) are able to organize themselves into crystalline networks that entrap and retain oil (Co & Marangoni, 2012). The final oleogel structure strictly depends on crystal shape, number, and size, as well as on physical molecular interactions among building blocks. Differently, mixtures of phytosterols-sterol esters promote oil gelation through the formation of a supramolecular network of hollow double-walled tubules (Bot & Agterof, 2006; Calligaris, Mirolo, Da Pieve, Arrighetti, & Nicoli, 2014; Sawalha *et al*., 2015). Finally, ethylcellulose is able to form a network in oil *via* hydrogen bonding between polymer strands (Davidovich-Pinhas, 2016), whereas chitin develops a particle filled network by polymer aggregation (Nikiforidis & Scholten, 2015).

The interest in oleogels has increased dramatically in the last decade due to their potential application as replacers of common hard stock fat (i.e. saturated and trans fatty acids) in different food products (Patel & Dewettinck, 2016; Singh *et al*., 2017; Wang, Gravelle, Blake, & Marangoni, 2016). More recently, oleogels have also been proposed as efficient tools to modulate lipid digestion and deliver nutrients and bioactive molecules (O'Sullivan, Davidovich-Pinhas, Wright, Barbut, & Marangoni, 2017; Ashkar, Laufer, Rosen-Kligvasser, Lesmes, & Davidovich-Pinhas, 2019; Tan, Peh, Marangoni, & Henry, 2017a; Tan, Peh, Siow, Marangoni, & Henry, 2017b; Tan, Peh, Lau, Marangoni, & Henry, 2017c). On this topic, O'Sullivan *et al.* (2017), studying the

digestion of an ethylcellulose-based oleogel enriched with *β*-carotene, noted that oil structuring reduced lipolysis, probably by hindering lipase activity. Consequently, the residence time of *β*-carotene in the digestive tract increased leading to an evenly arrayed absorption over time with no spikes and drops in plasma concentration (O'Sullivan *et al.*, 2017). According to Yu, Shi, Liu, and Huang (2012), monostearin might increase the stability of curcuminoids loaded into oleogels, by preventing their recrystallization or precipitation (Yu *et al.*, 2012). However, these authors did not observe differences between the oleogel and the liquid oil in terms of curcuminoid bioaccessibility. The latter is expressed as the fraction of molecules enclosed in the micelles that can be potentially absorbed through the intestinal epithelium, thus becoming available for physiological functions (Ferruzzi, 2010).

Besides these studies, recently Ashkar *et al.* (2019) confirmed that oleogelation of canola oil with ethylcellulose, a *β*-sitosterol+*γ*-oryzanol mixture, or mono- and di-glycerides reduced the extent of oil lipolysis during *in vitro* digestion. As reported by these authors, such effect can be modulated by selecting the gelator type and concentration. Interestingly, also some *in vivo* studies highlighted the significant impact of lipid physical state on post-prandial plasma triglycerides, glycemia, and appetite when comparing the co-ingestion of a carbohydrate-rich meal with EC-oleogel instead of liquid oil (Tan *et al.*, 2017a, 2017b, 2017c).

Based on this evidence and with the final aim of designing food with tailor-made functionalities, it appears fundamental to improve the knowledge on the fate of oleogels and loaded bioactive molecules upon digestion. Therefore, the aim of the present study was to investigate the effect of gelator type and derived oleogel structure on oil lipolysis and on the bioaccessibility of curcuminoids (CUs) during *in vitro* digestion. Curcuminoids were chosen as model lipophilic bioactive molecules due to their weel-known health-promoting capacity (Aggarwal, Kumar, & Bharti, 2003; Aziz *et al.*, 2013; Su, Wang, & Chi, 2017; Zheng *et al.*, 2014). CUs are extracted from turmeric and include three major compounds: bisdemethoxycurcumin, demethoxycurcumin and curcumin (Shishu & Maheshwari, 2010). Although the latter is the most abundant, representing

nearly 80% of total CUs, the health-promoting capacity of the mixture of the three CUs in turmeric extract is higher than that generated by the sole curcumin (Chakravarty, Chatterjee, Yasmin, & Mazumder, 2009; Račková *et al.*, 2009). In this study, sunflower-oil and oleogels containing 5% (w/w) of gelator (saturated monoglycerides, rice-bran waxes, and *β*-sitosterol+*γ*-oryzanol mixture) were enriched with CUs and characterized for mechanical and rheological properties as well as for the CUs stability during storage. Afterward, CUs-enriched oil and oleogels were *in vitro* digested to assess lipolysis and CUs bioaccessibility.

2. Materials and methods

2.1. Materials

High oleic sunflower oil (fatty acid profile reported in Table S1) was kindly provided by Olitalia srl (Forlì, Italy) and turmeric extract (NNCL2065, ext. dry conc. std 20:1) was purchased from Network Nutrition – IMCD spa (Milan, Italy). Bisdemethoxycurcumin (BDMC), demethoxycurcumin (DMC) and curcumin (C) analytical standards, α-amylase from Bacillus sp., porcine pepsin, porcine lipase, porcine bile extract, amyloglucosidase from Aspergillus niger, HCl, NaOH, $CaCl_2(H_2O)_2$, Na_2CO_3, $NaHCO_3$, NaCl, KCl, KH_2PO_4, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, and $MgSO_4$ were purchased from Sigma Aldrich (Milan, Italy). Myverol™ saturated monoglycerides (fatty acid composition: 1.4% C14:0, 59.8% C16:0, 38.8% C18:0; melting point 68.05 ± 0.5 °C) were purchased from Kerry Bioscience (Bristol, UK); β-sitosterol (75.5% β-sitosterol, 12.0% β-sitostanol, 8.4% campesterol, 3.0% other) and γ-oryzanol (99% purity) were purchased from Nutraceutica srl (Monterenzio, Italy); rice wax was purchased from Kahl GmbH & Co. KG (Reinbek, Germany). All solvents were purchased from Sigma–Aldrich (Milan, Italy). Acetonitrile and 2-isopropanol were of HPLC grade. Deionized water (System advantage A10®, Millipore S.A.S, Molsheim, France) was used for all the analyses.

2.2. Methods

2.1.1. Oil enrichment

The turmeric extract was added to sunflower oil (5 mg/g, w/w) and the mixture was stirred for 2 h at 80 °C in the dark under nitrogen atmosphere, to avoid CUs and oil oxidation. The mixture was cooled to room temperature and filtered (Chromafil PET-20/25, 0.20 µm, 25 mm Düren, Germany) to remove insoluble particles.

2.1.2. Oleogel preparation

Oleogels were prepared by mixing CUs-enriched sunflower oil with 5% (w/w) of saturated monoglycerides (MG) (Da Pieve, Calligaris, Co, Nicoli, & Marangoni, 2010), rice waxes (RW) (Doan, Van De Walle, Dewettinck, & Patel, 2015), or a mixture of *β*-sitosterol and *γ*-oryzanol (PS) (2:3 w/w) (Calligaris *et al.*, 2014). The mixtures were heated under stirring in dark conditions for 30 min at 80 °C for MG and RW, and for 45 min at 90 °C for PS, until melting was reached. MG and BW samples were quiescently cooled to 20 °C and stored at this temperature. PS oloege was cooled to 4 °C and kept at this temperature for 12 hours before being stored at 20 °C. All samples were then analysed after 2 days of storage at 20 °C.

2.1.3. Oleogel storage

Aliquots of 5 g of CUs-enriched oil and oleogels were placed into 10 mL vials and stored at 20 °C under dark for increasing time. Samples were collected after 60 and 100 days and analyzed for CUs content.

2.1.4. Firmness

Oleogel firmness was determined using a texture analyzer (TA.XT Plus, Stable Micro Systems Ltd, Godalming, UK) equipped with a 5 kg load cell. Forty grams of 25-mm-thick sample were compressed with a 35-mm-diameter compression platen at a crosshead speed of 1.5 mm/s

(Giacintucci *et al.*, 2018) and firmness was expressed as the maximum force (N) applied to the samples.

2.1.5. Rheological measurement

Rheological properties of oleogels were determined with a Haake Rheostress 6000 (Thermo Scientific, Rheostress, Haake, Germany). Aliquots of about 5 g of sample were transferred on a 40-mm parallel-plate geometry system thermostated at 20 °C and the measuring gap was set at 2 mm. Samples were equilibrated for 5 min before testing to allow relaxation. Stress sweep measurement in the range of 0.1 to 1000 Pa was carried out at 1 Hz frequency to determine the linear viscoelastic region. Frequency sweep was carried out by applying a fixed stress value chosen in the linear viscoelastic region with a frequency scan of 0.1 to 10 Hz. Data were acquired and managed by applying the software Haake Rheowin v.4.60.0001 (Thermo Fisher Scientific). The critical stress was computed as the stress leading to a 10% G′ decrease during the stress sweep. G′ and G″ were compared at 1 Hz. The tangent of the phase angle (Tanδ) was computed as the ratio between the two moduli (G″/G′) during the frequency sweep.

2.1.6. Macroscopic appearance

Gel images were acquired by using an image acquisition cabinet (Immagini and Computer, Bareggio, Italy) equipped with a digital camera (EOS 550D, Canon, Milan, Italy). The digital camera was placed on an adjustable stand positioned 40 cm in front of a black cardboard base where the sample was placed. Light was provided by four 23 W frosted photographic floodlights, in a position allowing minimum shadow and glare. Other camera settings were: shutter time 1/250 s, F-Number F/2,8 and focal length 60 mm. Images were saved in jpeg format resulting in pictures of 5184 × 3456 pixels, 72 × 72 dpi.

2.1.7. Polarized light microscopy

Polarized light microscopy was carried out by using a Leica DM 2000 optical microscope under polarized light conditions (Leica Microsystems, Heerbrugg, Switzerland). A small portion of gel was placed on a glass slide, covered with a cover slide and observed at 20 °C. Images were taken at 200× magnification using a Leica EC3 digital camera and elaborated by the Leica Suite Las EZ software (Leica Microsystems, Heerbrugg, Switzerland).

2.1.8. In vitro *digestion*

In vitro digestion was carried out according to the protocol proposed by Minekus *et al*. (2014). Briefly, the simulated salivary (SSF), gastric (SGF) and intestinal (SIF) fluids were prepared and stored at 4 °C. The fluids were preheated to 37 °C just before *in vitro* digestion. The oral phase was started by adding to 0.25 g sample (oil or oleogel), 6 μL of $CaCl_2(H_2O)_2$ (0.3 M), 194 μL of water and 800 μL of a 6.4 mg/mL α-amylase solution, prepared in SSF and providing 75 U/mL activity in the final mixture. The sample was maintained at 37 °C under stirring for 2 min. At the end of the oral phase, the pH was adjusted to 3.0 with 40 μL HCl (1 M). Subsequently, 140 μL water and 1.82 mL of a 0.31 mg/mL pepsin solution, prepared in SGF and providing 2,000 U/mL activity in the final mixture, were added to start the gastric phase. The mix was stirred at 37 °C for up to 2 h. At the end of the gastric phase, the pH was adjusted to 7.0 with 30 μL NaOH (1 M). The intestinal phase was initiated by adding 8 μL $CaCl_2(H_2O)_2$ (0.3 M), 262 μL of water, 3.2 mL of 22.15 mg/mL lipase solution, prepared in SIF and providing 100 U/mL activity in the final mixture, and 0.5 mL of 160 mM bile extract prepared in SIF. The mix was stirred at 37 °C for up to 2 h. At the end of the intestinal phase, samples were centrifuged at 30,000 *g* for 70 min at 4 °C (Beckman Avanti tm J-25, Beckman Instruments Inc., Palo Alto, CA, USA) and the supernatant, i.e. the mixed micellar phase, was collected.

2.1.9. *Particle size and zeta potential of digested samples*

The particle size distribution of the mixed micellar phase of digested oil and oleogels was measured by dynamic laser light scattering (Zetasizer NanoZS, Malvern Instruments, Worcestershire, UK). Samples were diluted 1:100 (v/v) with deionized water and placed in a cell where the laser light, set at 173 ° angle, was scattered by the particles. Particle size was reported as volume-weighed mean diameter in nm. The ζ-potential was also measured by placing the diluted sample in a capillary cell equipped with two electrodes to assess particle electrophoretic mobility.

2.1.10. *Free fatty acid release*

The amount of free fatty acids (FFA) released from the sample during the intestinal phase of *in vitro* digestion was measured by using a titration method (Ahmed, Li, McClements, & Xiao, 2012). Immediately after the addition of lipase, the pH of the digestion mixture was monitored and maintained at 7.00 by adding 0.25 M NaOH. The volume of NaOH added to the sample was recorded and used to calculate the percentage of FFA released during lipolysis (Equations 1 and 2):

$$V_t = 2 \times \left[\frac{m_{oil}}{MW_{oil}} \frac{1000}{C_{NaOH}}\right] \quad \text{Equation 1}$$

$$\text{FFA (\%)} = \frac{V_e}{V_t} \times 100 \quad \text{Equation 2}$$

where V_e was the experimental volume of NaOH used for the titration, V_t was the theoretical volume required to titrate the fatty acids released by complete hydrolysis of triglycerides in the reaction vessel, assuming 2 FFA are produced for each triacylglycerol molecule (L) (Li, Hu, Du, Xiao, & McClements, 2011), m_{oil} was the mass of oil in the reaction vessel (g), MW_{oil} was the average molecular weight of sunflower oil (g mol^{-1}) and C_{NaOH} was the concentration of the sodium hydroxide (mol L^{-1}). The maximum value of free fatty acids released during digestion was determined after the plateau was reached, i.e. after 30 min from the beginning of intestinal phase. This value was considered as an indication of the maximum lipolysis and was reported as FFA_{max}.

2.1.11. Curcuminoid quantification and bioaccessibility computation

CUs-enriched oil and oleogels were diluted in isopropanol (100 μg/mL) and transferred to glass vials for UHPLC analysis, while the mixed micellar phase recovered after *in vitro* digestion, was submitted to extraction. Briefly, 2 mL of water was added to the digested samples followed by 2 mL of isopropanol. The sample was vigorously hand- shaken for 1 min and vortexed for 15 s. A mixture of salts ($MgSO_4$/NaCl 2.0:1.5, w/w) was then added and shaking was repeated under the same conditions. The resulting mixture was centrifuged at 5000 *g* for 15 min and the supernatant was recovered for UHPLC analysis.

To validate the extraction procedure for CUs analysis in digested samples, accuracy was evaluated by means of recovery experiments, analyzing digested samples fortified with three different amounts of CUs (5, 10 and 20 μg for each CUs), whereas precision, expressed as the repeatability of the method, was determined in terms of relative standard deviation (RSD) from recovery experiments at each fortification level. In all cases, the average recovery ranged from 92 to 100% with repeatability (CV%) lower than 2%, revealing the suitability of the procedure for the quantitative extraction of CUs from digested samples.

Different chromatographic conditions were employed to obtain a baseline separation of BDMC, DMC, and C. Fig. S1 shows the chromatogram of CUs in enriched oil samples obtained by applying the optimized chromatographic conditions. The UHPLC system used was a Shimadzu Prominence LC-20A coupled with a RF-20A fluorescence detector (Shimadzu, Milan, Italy). The column was an Agilent Poroshell C18 column (150 mm x 4.6 mm x 2.7 µm) thermostated at 30 °C. The mobile phase was a mixture of 0.2% aqueous phosphoric acid water and acetonitrile (85:15, v/v) at 0.45 mL/min flow rate. The excitation wavelength (λex) and emission wavelength (λem) were set a 430 and 524 nm, respectively. Standard stock solutions of BDMC (0.25 mg/mL), DMC (0.25 mg/mL) and C (0.5 mg/mL) were prepared in acetonitrile and intermediate working CUs solutions were prepared weekly from the stock standard solution by appropriate dilution with acetonitrile and

stored in the dark at 4 °C. Calibration curves were obtained for each curcuminoid (0.1 - 500 ng on column) and presented $R^2 > 0.998$ in all cases.

CUs bioaccessibility was calculated as the percentage ratio between the concentration of compound incorporated in the micelles after *in vitro* digestion and its concentration in the undigested sample.

2.1.12. Statistical analysis

Results are averages of three measurements carried out on two replicated experiments and are reported as means ± standard deviation. Analysis of variance (ANOVA) was performed using R (version 3.2.3, The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to check the homogeneity of variance and the Tukey test was used to test for differences between means ($p < 0.05$).

3. Results and discussion

3.1. Oleogel physical and chemical properties

Oleogels containing sunflower oil enriched with CUs were prepared by using 5% (w/w) of saturated monoglycerides (MG), rice waxes (RW) and a mixture of *β*-sitosterol and *γ*-oryzanol (PS). This concentration was selected being effective in gelling vegetable oils as previously reported by different authors (Da Pieve *et al.*, 2010; Calligaris *et al.*, 2014; Doan *et al.*, 2015).

Table 1 shows firmness, rheological parameters (critical stress, G′, G″ and Tan*δ*), and micro- and macroscopic images of oleogels prepared by using MG, RW, and PS as oil gelators. The original stress sweep and frequency sweep curves are reported in Fig. S2. From the macroscopic images it can be noted that all samples were self-standing materials, even if with different structures. In particular, MG and RW oleogels were opaque sytems with cream-like structure, whereas the PS oleogel appeared as a transparent solid. From a rheological point of view, the gel behavior was confirmed for all samples, being the storage modulus (G′) higher than the loss modulus (G″) and Tan$\delta < 1$ (Zetzl *et al.*, 2014). Considering G′ and G″ at 1 Hz, the MG-based sample showed the lowest values, followed by PS and RW. On the other hand, PS demonstrated the highest critical

234 stress value, which represents the beginning of the non-linear region and accounts for the structure
235 breakdown required to onset flowing (Doan *et al.*, 2015).
236 As well known, G′ and G″ are indexes of the elastic and viscous behavior of the matrix,
237 respectively; whereas the critical stress is an indicator of the stress needed to break the gel network.
238 Thus, from the rheological parameters it can be concluded that, in spite of the reduced elastic
239 behavior, PS presented the highest stress resistance. This is also consistent with firmness values that
240 followed the order PS>RW>MG (Table 1).
241 These results are in agreement with those reported by Fayaz, Calligaris & Nicoli (2019). These
242 authors compared the ability of different molecules to gel sunflower oil, and attributed the different
243 gel properties to the peculiarities of the network structure formed by each oil gelator. As well
244 reported by Sawalha *et al.* (2013, 2015), *β*-sitosterol+*γ*-oryzanol self-assemble into a network by
245 alignment of cross-linked tubules into helical ribbons strongly stabilized by hydrogen bonds. The
246 dimension of these tubules is around 10 nm, which is smaller than the wavelength of visible light
247 accounting for system transparency (Bot & Agterof, 2006; Bot, Den Adel, & Roijers, 2008; Bot *et*
248 *al.*, 2011). or this reason, tubules are not detectable by using polarised light microscopy and they
249 can be imaged by SEM solely (Sawalha *et al.*, 2013). The peculiar network arrangement of *β*-
250 sitosterol+*γ*-oryzanol conferred to the system a higher stress resistance in comparison to the
251 crystalline networks formed by MG and RW. It should be remembered that the ability of these
252 molecules to gel into oil is associated to their self-assembly into crystals (Da Pieve *et al.*, 2010,
253 Doan *et al.*, 2015). As can be observed in the polarized light microscopy images (Table 1), the
254 morphology of crystals (bright areas) was different in MG and RW-based oleogels: small needle-
255 like crystals were formed in MG containing system, whereas large dendritic crystals were observed
256 in RW oleogel, in well agreement with the literature (Da Pieve *et al.*, 2010; Doan *et al.*, 2015;
257 Fayaz *et al.*, 2019). Both MG and RW crystals further aggregated mainly by hydrogen bonds, van
258 der Waals and hydrophobic forces to finally develop into three-dimensional gel network. As

demonstrated by Fayaz *et al*. (2019), the physical molecular interactions among crystal aggregates are expected to be stronger in rice wax-containing sample than in MG-based oleogel.

3.2. Curcuminoid stability

After the characterization of oleogels, further research was carried out to investigate the impact of oleogel preparation conditions on CUs content. As already mentioned, oleogels were prepared by heating oil at temperatures higher than the melting temperatures of the gelators. Being CUs sensitive to heat, their degradation could occur during oleogel preparation. The concentration of BDMC, DMC, and C in turmeric extract, enriched oil and oleogels was thus determined by UHPLC. BDMC, DMC, and C concentration in turmeric extract accounted for 10,142 ± 10 μg/g, 53,277 ± 79 μg/g and 147,761 ± 249 μg/g, respectively. As expected, C was the most abundant compound, followed by DMC and BDMC (Yu & Huang, 2012). Based on CUs concentration in the turmeric extract and in freshly prepared oil (Table 2), it can be observed that these compounds were completely solubilized during oil enrichment. BDMC, DMC and C concentration in oleogels did not differ significantly from those found in freshly prepared oil and ranged between 39 and 41; 264 and 274; 1,049 and 1,112 $\mu g/g_{oil}$, respectively. These results suggest that the preparation methodology applied to produce oleogels did not induce CUs degradation. It can be noted that BDMC, DMC, and C accounted for nearly 2%, 14% and 84% of CUs, respectively, in oil and oleogels.

Samples were further analyzed for CUs content during storage at 20 °C. As shown in Table 2, CUs concentration did not change up to 60 days of storage, whereas a significant decrease was recorded at 100 days of storage in all samples. In any case, the measured differences did not allow highlighting a clear effect of oleogel structure on CUs stability.

3.3. Physical properties, lipolysis and bioaccessibility of in vitro digested oleogels

The aim of the second part of the research was to study the effect of oil gelator type, and thus oleogel structure, on the digestive fate of oil and CUs. The particle size distribution of the micellar

phases obtained after *in vitro* digestion of oil and oleogels was firstly investigated (Fig. 1a). A multimodal particle size distribution was observed for all samples, revealing the presence of small and large particles. The smallest particle family detected in oil, PS and RW showed an average diameter ranging from 90 to 190 nm, attributable to the presence of mixed micelles formed upon digestion (Salvia-Trujillo *et al*., 2017). By observing particle size distributions, it is evident that RW and PS produced broader distributions than that observed in oil. This result suggests the formation of micelles with unhomogeneous size probably due to the fact that gel structure interfers with the digestion process (O'Sullivan *et al*., 2017). In particular, the gel network might represent a physical barrier to the access of lipase to its substrate or could directly interfere with lipid digestive components (McClements, Decker, & Park, 2009), resulting in a less efficient micelle formation. In this regard, it is important to highlight that micellarization plays a pivotal role in determining the absorption of loaded lipophilic bioactive compounds, but no correlation has been demonstrated between size and bioaccessibility (Salvia-Trujillo *et al*., 2017).

Differently, the major particle family in MG presented an average diameter of 28 nm. Such difference could be attributed to the ability of MG to act as surfactants during *in vitro* digestion, thus promoting the formation of smaller micelles (Reis *et al*., 2008).

Beside the presence of particles smaller than 200 nm, as shown in Fig. 1a, all analyzed samples also presented a particle family with an average diameter of 4,800-5,500 nm. This family is attributable to the presence of undigested lipid dropletssurrounded by anionic species, such as free fatty acids and bile salts (Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013; Singh, Ye, & Horne, 2009; Zou *et al.*, 2016),and resulting in complex colloidal structures with negative charge (Zhang *et al.*, 2016).

The negative charge of particles in the micellar phase was confirmed by ζ-potential measurements (Fig. 1b). As well known, this parameter provides information about the surface electrical charge of particles and depends on the adsorbed species at the oil-water interfaces (Salvia-Trujillo *et al*., 2017). All digested oleogels presented negatively charged particles, with a ζ-potential ranging from

-50 to -62 mV. Unstructured oil and MG-containing oleogel were characterized by the most negatively charged micelles upon digestion, whereas RW- and PS-containing samples presented a higher ζ-potential, confirming the ability of different structuring agents to affect not only micelle size but also their surface charge (Fig. 1). To explain these results it can be speculated that some surface active species contained in RW and PS might play a role in modifying the total surface charge of the micelles. Moreover, some authors observed a relationship between the electrical charge upon digestion and the lipolysis degree (Qian, Decker, Xiao, & McClements, 2012). Lipid digestion was thus monitored by measuring free fatty acid (FFA) release during the intestinal phase of *in vitro* digestion (Fig. 2). In can be observed that the FFA release increased during digestion for all analyzed samples and reached a *plateau* within 30 min of intestinal digestion. This behavior is in agreement with literature data (McClements & Li, 2010; O'Sullivan, Barbut, & Marangoni, 2016). However, the extent of lipid digestion was significantly affected by oil structure. The unstructured oil presented the highest value of lipid digestion (FFA_{max}= 54%) in comparison with oleogels. This value agreed with literature findings, reporting a maximum lipolysis for unstructured sunflower oil around 57% (Ye *et al.*, 2019). Among oleogels, MG and RW presented similar lipolysis extents (nearly 45%), whereas PS-based oleogel showed the lowest lipolysis upon *in vitro* digestion, being 20% lower than that of oil.

These results suggest that the presence of differently structured supramolecular networks in oil affected the free fatty acid release, probably by hindering lipase access to triacylglycerol digestion sites. Thus, different oil gelators and gelation mechanisms seem to critically affect lipolysis, in agreement with results reported byAshkar *et al.* (2019) with reference to canola oil-based oleogels containing MG and PS. It should be noted that no universal conclusion on the relation between the gel strength and lipolysis can be gathered, as both gel strength (Yang *et al.*, 2018; Sawalha *et al.*, 2015) and lipolysis extent (Ye *et al.*, 2019) may be differently affected by fat composition. Still, it can be inferred that it may be possible to modulate fat digestibility and availability by selecting a proper oleogel structure. Several authors observed an interdependence between lipolysis efficiency

and micelle characteristics (Qian *et al.*, 2012; Salvia-Trujillo *et al.*, 2017). In fact, besides resulting in the highest lipolysis extent (Fig. 2), MG-containing oleogel also presented the smallest and most negatively charged mixed micelles (Fig. 1), comparable with those observed in the unstructured oil. Since these differences could affect the bioaccessibility of loaded lipophilic bioactive compounds, to get an insight into the effect of different structuring agents on this feature, CUs concentration and bioaccessibility in the *in vitro* digested oil and oleogels were assessed (Table 3). As well known, bioaccessibility measures the percent transfer of the bioactive molecules from the lipid phase to the aqueous one, in which CUs are incorporated into mixed micelles (Ferruzzi, 2010). Only CUs enclosed in the micellas are then available to be absorbed by the intestinal epithelium cells. A significant decrease ($p < 0.05$) in the concentration of CUs was observed for both oil and oleogels in the micellar phase after *in vitro* digestion, as compared to undigested samples (Table 2). Regarding the susceptibility of different CUs upon digestion, curcumin presented the lowest BAC, being more susceptible to oxidation as compared to BDMC and DMC (Gordon Luis, Ashley, Osheroff, & Schneider, 2015), in agreement with the results reported by Yu *et al.* (2011, 2012). In addition, the gelator significantly affected CUs concentration and bioaccessibility in digested samples, in contrast with the results found for the undigested oleogels (Tables 2 and 3). The bioaccessibility of CUs included into PS oleogel was comparable to that of oil ($p > 0.05$), suggesting that the tubular network did not compromise the release of CUs into digestive fluids impairing their bioaccessibility. On the other hand, oleogels structured through crystalline networks (MG- and RW-based oleogels) presented the lowest CUs content and bioaccessibility upon *in vitro* digestion. It can be speculated an effect of the presence of crystalline particles on CUs BAC. In particular, CUs could be involved in the gelation/crystallization process, being more exposed to oxygen and/or other oxidants present in the digestive mixture. To our knowledge, in the literature there are no indications on this aspect. However, some evidences show an effect of the lipid crystalline network structure on β-carotene oxidative degradation. On this regards, some authors (Calligaris, Valoppi, Barba, Anese, & Nicoli, 2018; Martins, Cerqueira, Cunha, & Vicente, 2017)

demonstrated the critical role of biomolecule location inside the lipid crystal network. Even if more research is needed on this aspect, it can be inferred that also during digestion the presence of fat crystals could impact not only the chemical stability of bioactive molecules, but also their release into the digestive fluids and thus their bioaccessibility.

4. Conclusions

Results reported in the present study confirm that oleogelation could be a profitable strategy to modulate lipid digestion while delivering bioactive molecules. Oleogel structure seems to affect the lipolysis extent and the bioaccessibility of loaded lipophilic bioactive compounds. Regarding lipid digestion, gel strength resulted critical in affecting the rate and extent of lipolysis. On the contrary, the choice of the gelling agent had an impact on CUs bioaccessibility.

Based on this knowledge, it can be suggested that oleogel development would not only offer the food industry a plastic fat replacer but could represent a strategy to modulate lipid digestion and deliver health benefits. Matching the reduction of fat uptake and the improved bioaccessibility of bioactive molecules might provide the consumers with functional foods potentially able to tackle the risk of obesity and cardiovascular diseases, currently representing major issues for public health.

Abbreviations

BDMC = bisdemethoxycurcumin; C = curcumin; CUs = curcuminoids; DMC = demethoxycurcumin; MG = monoglycerides; PS = mixture of β-sitosterol and γ-oryzanol; RW = rice wax.

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Conflict of interest

There are no conflicts of interest to declare.

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Captions for figures

Fig. 1. Particle size distribution (a) and ζ-potential (b) of oil and oleogels obtained by using 5% (w/w) of monoglycerides (MG), rice wax (RW) and phytosterols (PS), after *in vitro* digestion. Different letters (a-c) mean significant differences ($p < 0.05$) among samples.

Fig. 2. Concentration of free fatty acids (FFA) during *in vitro* digestion of oil and oleogels containing 5% (w/w) of monoglycerides (MG), rice wax (RW) or phytosterols (PS). Different letters (a-c) mean significant differences ($p < 0.05$) among samples.

Fig. S1. UHPLC trace of CUs enriched oil. 1: bisdemethoxycurcumin; 2: demethoxycurcumin; 3: curcumin.

Fig. S2. Stress and frequency sweep curves of monoglyceride- (MG), rice wax- (RW) and phytosterol- (PS) based oleogels.

*Declaration of Interest Statement

DECLARATION OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author.

Udine, 15th May 2019

Signed by all authors as follows:

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Monica Anese

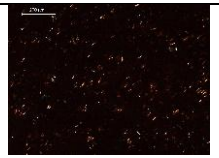
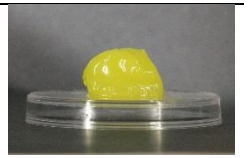
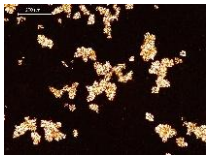
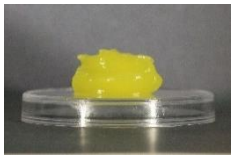
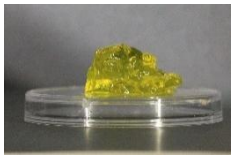
Author contribution

Sonia Calligaris: Conceptualization, Writing - Review & Editing, Supervision; Marilisa Alongi: Investigation, Formal analysis, Writing - Original Draft; Paolo Lucci: Methodology, Validation, Writing - Review & Editing; Monica Anese: Writing - Review & Editing, Supervision.

Table 1

Table 1

Firmness, G', G'', Tanδ, critical stress, and microscopic and macroscopic images of oleogels obtained by using 5% (w/w) of monoglycerides (MG), rice wax (RW) and phytosterols (PS).

Gelling agent	Firmness (N)	G' (Pa)	G" (Pa)	Tanδ	Critical stress (Pa)	Microscopic appearance	Macroscopic appearance
MG	0.91 ± 0.08 [c]	25090 ± 4044 [b]	3075 ± 513 [b]	0.15 ± 0.01 [b]	6.6 ± 0.1 [b]		
RW	3.49 ± 0.24 [b]	37413 ± 2591 [a]	4384 ± 539 [ab]	0.15 ± 0.01 [b]	16.7 ± 3.1 [b]		
PS	5.65 ± 0.42 [a]	29410 ± 4646 [ab]	6026 ± 808 [a]	0.21 ± 0.01 [a]	128.5 ± 28.6 [a]	n.a.	

Different letters (a-c) mean significant differences ($p < 0.05$) of structural properties among oleogels.
n.a. Images not acquired.

Table 2

Table 2

Bisdemethoxycurcumin (BDMC), demethoxycurcumin (DMC) and curcumin (C) contents in oil and oleogels obtained by using 5% (w/w) of monoglycerides (MG), rice wax (RW) and phytosterols (PS), during storage at 20 °C under dark.

	BDMC ($\mu g/g_{oil}$)			DMC ($\mu g/g_{oil}$)			C ($\mu g/g_{oil}$)		
Sample	0 days	60 days	100 days	0 days	60 days	100 days	0 days	60 days	100 days
Oil	$41.2 \pm 1.8^{A,a}$	$38.5 \pm 0.2^{A,a}$	$31.6 \pm 0.1^{B,a}$	$274 \pm 12^{A,a}$	$251 \pm 1^{A,a}$	$193 \pm 3^{B,b}$	$1112 \pm 48^{A,a}$	$974 \pm 5^{B,a}$	$820 \pm 5^{C,b}$
MG	$39 \pm 1.5^{A,a}$	$38.9 \pm 1.8^{A,a}$	$32.1 \pm 0.3^{A,a}$	$268 \pm 10^{A,a}$	$250 \pm 12^{A,a}$	$192 \pm 2^{A,b}$	$1049 \pm 38^{A,a}$	$974 \pm 49^{A,a}$	$812 \pm 6^{B,bc}$
RW	$41.0 \pm 2.8^{A,a}$	$37.3 \pm 0.3^{A,a}$	$29.8 \pm 0.2^{B,b}$	$272 \pm 15^{A,a}$	$246 \pm 1^{A,a}$	$200 \pm 2^{B,ab}$	$1104 \pm 57^{A,a}$	$968 \pm 2^{A,a}$	$794 \pm 3^{B,c}$
PS	$39.7 \pm 2.3^{A,a}$	$38.2 \pm 0.3^{A,a}$	$31.3 \pm 0.2^{B,a}$	$265 \pm 15^{A,a}$	$247 \pm 2^{A,a}$	$207 \pm 1^{B,a}$	$1079 \pm 58^{A,a}$	$1007 \pm 8^{A,a}$	$853 \pm 3^{B,a}$

Different capital letters (A-C) mean significant differences of curcuminoid concentration during storage. Different lowercase letters (a-c) mean significant differences ($p < 0.05$) of curcuminoid concentration between oil and oleogels.

Table 3

Table 3

Bisdemethoxycurcumin (BDMC), demethoxycurcumin (DMC) and curcumin (C) content and bioaccessibility (BAC) in oil and oleogels obtained by using 5% (w/w) of monoglycerides (MG), rice wax (RW) and phytosterols (PS) after *in vitro* digestion.

Sample	BDMC		DMC		C	
	Concentration ($\mu g/g_{oil}$)	BAC (%)	Concentration ($\mu g/g_{oil}$)	BAC (%)	Concentration ($\mu g/g_{oil}$)	BAC (%)
Oil	22.9 ± 3.8 ^{a}	55.6 ± 9.1 ^{a}	141.9 ± 22.8 ^{a}	51.7 ± 8.3 ^{a}	520.4 ± 85.9 ^{a}	50.4 ± 7.7 ^{a}
MG	13.1 ± 0.5 ^{c}	33.6 ± 1.4 ^{b}	87.3 ± 5.5 ^{b}	32.5 ± 2.1 ^{c}	333.9 ± 31.5 ^{b}	31.8 ± 3.0 ^{b}
RW	13.7 ± 3.4 ^{bc}	33.3 ± 8.4 ^{b}	94.0 ± 22.6 ^{b}	34.5 ± 8.3 ^{bc}	376.5 ± 93.0 ^{ab}	29.2 ± 8.4 ^{b}
PS	20.5 ± 2.0 ^{ab}	51.4 ± 5.0 ^{a}	130.5 ± 11.1 ^{ab}	49.1 ± 4.2 ^{ab}	489.3 ± 39.7 ^{ab}	45.2 ± 3.7 ^{a}

Different letters (a-c) mean significant differences ($p < 0.05$) of curcuminoid concentration or bioaccessibility among samples.

Figure 1

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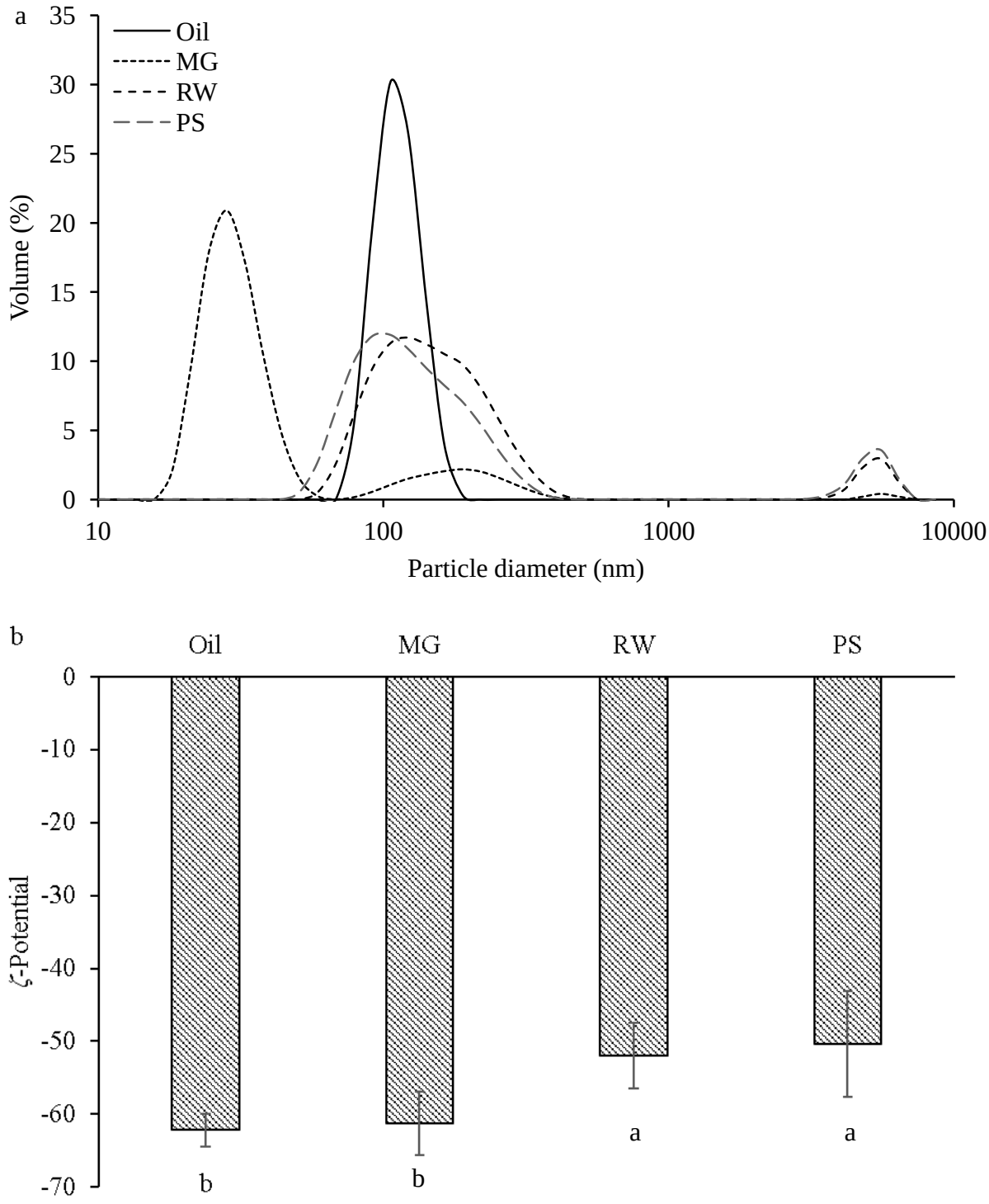

Fig. 1

Figure 2

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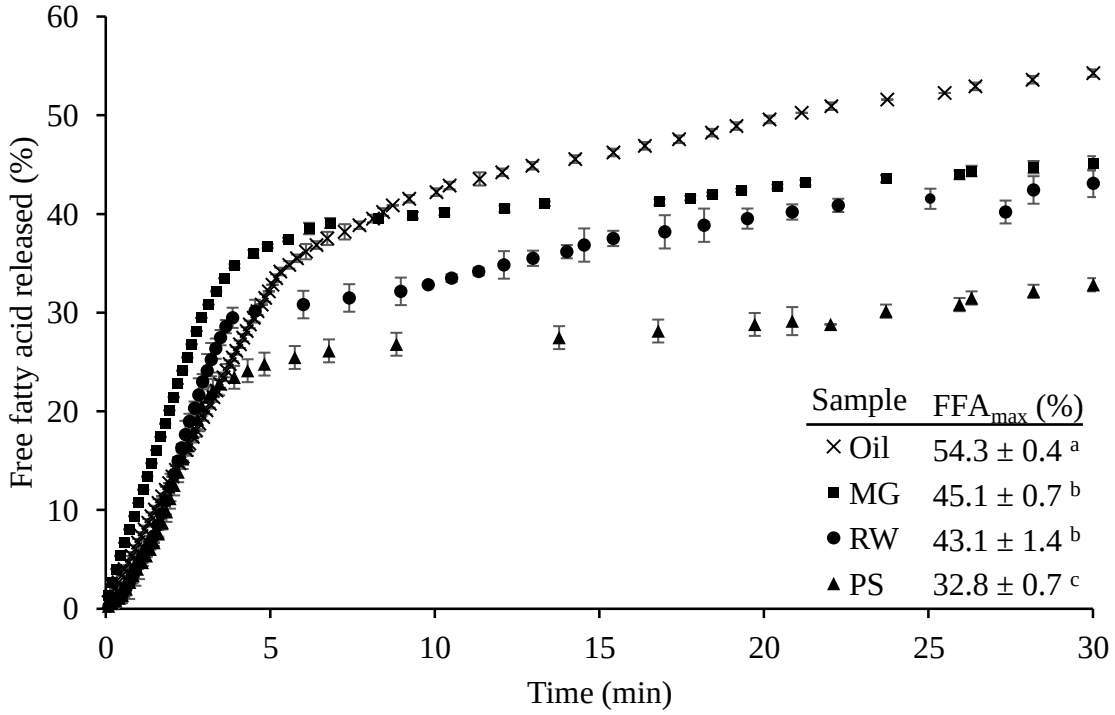

Fig. 2.

Table S1

Total fatty acid profile of high-oleic sunflower oil

Fatty acid	Relative abundance (%)
C16:0	5.1 ± 1.0
C16:1 $^{\Delta 9c}$	0.2 ± 0.0
C18:0	3.0 ± 0.1
C18:1$^{\Delta 9c}$	81.6 ± 1.5
C18:2 $^{\Delta 9c,12c}$	8.3 ± 0.8
C18:3 $^{\Delta 9c,12c,15c}$	0.2 ± 0.0
C20:0	0,3 ± 0.0
C20:1 $^{\Delta 11}$	0.3 ± 0.1
C22:0	1.1 ± 0.2

Legend for fatty acids – m:n Δx, m=number of carbon atoms, n= number of double bonds, x= position of double bonds.

Supplementary Material_Fig. S2

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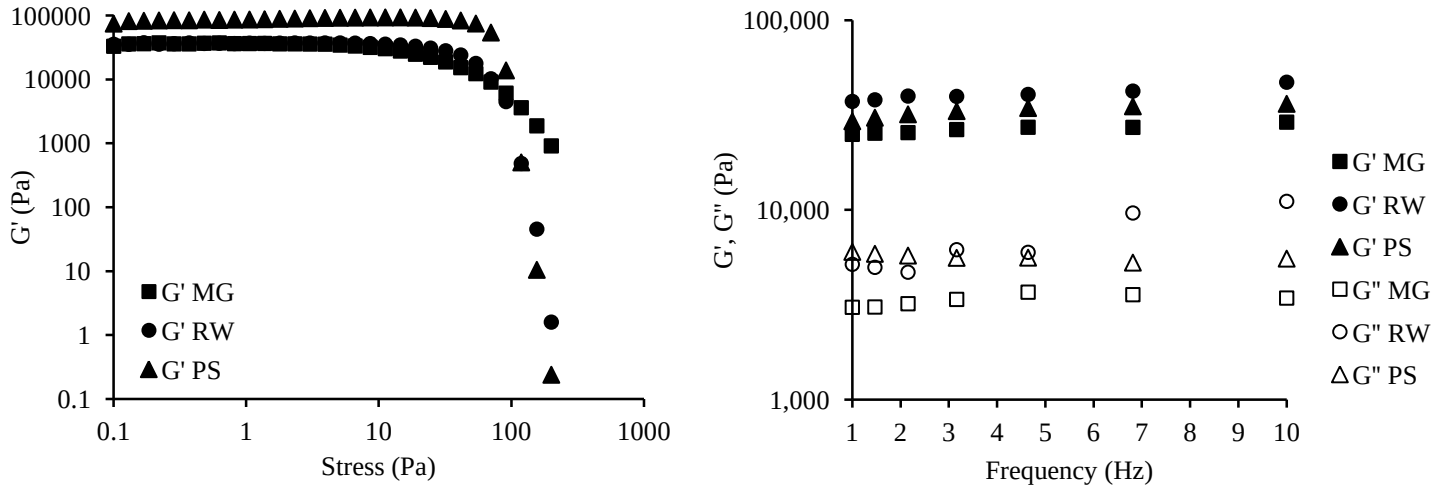

Fig. S2