Food Research International 173 (2023) 113239

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Oleogelation of extra virgin olive oil by different gelators affects lipid digestion and polyphenol bioaccessibility

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ARTICLE INFO

Keywords:
Extra virgin olive oil
Oleogel
Lipolysis
Polyphenol bioaccessibility
Structure

ABSTRACT

The possibility to steer extra virgin olive oil (EVOO) digestion and polyphenol bioaccessibility through oleogelation was investigated. EVOO was converted into oleogels using lipophilic (monoglycerides, rice wax, sunflower wax, phytosterols) or hydrophilic (whey protein aerogel particles, WP) gelators. *In-vitro* digestion demonstrated that the oleogelator nature influenced both lipid digestion and polyphenol bioaccessibility. WP-based oleogels presented ~100% free fatty acid release compared to ~64% for unstructured EVOO and ~40 to ~55% for lipophilic-based oleogels. This behavior was attributed to the ability of WP to promote micelle formation through oleogel destructuring. Contrarily, the lower lipolysis of EVOO gelled with lipophilic gelators compared to unstructured EVOO suggested that the gelator obstructed lipase accessibility. Tyrosol and hydroxytyrosol bioaccessibility increased for WP oleogels (~27%), while liposoluble-based oleogels reduced it by 7 to 13%. These findings highlight the deep effect of the gelator choice on the digestion fate of EVOO components in the human body.

1. Introduction

Oleogelation is a timely topic increasingly attracting the interest of the scientific community and the food industries. It can be defined as a process able to turn liquid oil into a solid-like material by exploiting the structuring properties of selected molecules, called oleogelators (Patel & Dewettinck, 2016). Two main approaches for oil structuring have been proposed. Direct methods are based on the use of lipophilic oleogelators (single or in combination), which are firstly dispersed in oil, heated above their melting temperature, and then cooled to induce their self-assembly into a three-dimensional network, based on different mechanisms (Marangoni & Garti, 2011). For instance, saturated monoglycerides and waxes form crystalline networks, while phytosterol-phytosterol ester mixtures generate networks made of hollow double-walled tubules (Scharfe et al., 2019). By contrast, ethylcellulose produces a polymeric network based on intermolecular interactions stabilized by hydrogen bonds among the hydroxyl groups (Giacintucci et al., 2018). However, regulatory restrictions limit the applications of these oleogelators. Although monoglycerides can be used as "*quantum satis*", they must be declared as additives on the food label with the number E471. Among waxes, only a few can be used, and their application is limited to the coating of food products (Reg. EU No 1129/2011). Based on hypocholesterolemic capacity, phytosterols are only allowed as functional ingredients in a few products with a daily intake recommended to be lower than 3 g day^{-1} (Reg. EU No 608/2004).

On the other side, indirect oleogelation strategies rely on the formation of an oleogel by removing water from a pre-formed emulsion or upon oil absorption into a porous material obtained by solvent removal from hydrogels or foams (Patel & Dewettinck, 2016), in turn formed by structuring hydrophilic polymers in an aqueous environment. Differently from most lipophilic gelators, the large majority of hydrophilic polymers such as proteins and carbohydrates do not incur into regulatory limitations. In this context, aerogels are novel materials with the interesting capacity of absorbing a significant amount of oil. They are characterized by low density, open porosity, and high surface area, obtained by water removal from hydrogels, using a method that allows the preservation of the solid network structure, typically supercritical carbon dioxide (SCO_2)-assisted drying being the gold standard. Thanks

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https://doi.org/10.1016/j.foodres.2023.113239
Received 23 April 2023; Received in revised form 4 July 2023; Accepted 5 July 2023
Available online 6 July 2023

to these characteristics, aerogels resulted in efficient templates for the preparation of oleogels (Manzocco et al., 2021).

Oleogels have been traditionally studied and developed as a feasible alternative to saturated fats (*e.g.*, animal fats, tropical oils, margarine, and shortenings) due to their good capability to mimic their technological functionalities (Patel & Dewettinck, 2016; Sivakanthan et al., 2022). Most recently, their potential health functionalities have been claimed. In particular, it has been shown that oil structuring could affect lipid digestibility (Ashkar et al., 2019; Calligaris et al., 2020; Marangoni et al., 2007; Marangoni & Garti, 2011; O'Sullivan et al., 2017; Plazzotta et al., 2022) as well as the bioaccessibility of lipophilic compounds contained in the oil (Calligaris et al., 2020; Dent et al., 2022; Luo et al., 2021; Salvia-Trujillo et al., 2017; Zhao et al., 2022). Considering oleogels based on lipophilic oleogelators, it has been shown that the lipolysis extent may be reduced, probably due to the ability of the gelator network to hinder the access of lipolytic enzymes to their substrate (Calligaris et al., 2020; O'Sullivan et al., 2017; Ashkar et al., 2019). At the same time, the bioaccessibility of liposoluble compounds would be modulated by controlling the gastrointestinal behavior of the oleogel through the selection of the proper oleogelator (Calligaris et al., 2020), and the relevant concentration (Dent et al., 2022). For instance, Li et al. (2019) reported that the bioaccessibility of curcumin loaded in an oleogel structured by β-sitosterol and lecithin was higher than the one of curcumin in unstructured oil. Still, this behaviour cannot be extended straightforwardly to all bioactive compounds, as different molecules present very different stability upon processing and further digestion, reasonably resulting in different outcomes depending on the compound under investigation.

On top of this, the digestion of oleogels structured by hydrophilic molecules has received even much lower attention. Recently, Plazzotta et al. (2022) studied the gastrointestinal behavior of oleogels structured by whey proteins aerogel particles. These Authors showed a higher intestinal lipolysis extent of the oleogel in comparison to unstructured oil. It has been hypothesized that the oleogel structure, associated with the presence of proteins could allow faster oleogel destructuring, favoring lipolysis (Plazzotta et al., 2022). Besides this study, to our knowledge, no further research has been published yet on the fate of lipophilic molecules included in oleogels structured by hydrophilic biopolymers during the gastrointestinal transit, and thus their bioaccessibility is still an unexplored topic.

A particularly challenging yet interesting oil to be delivered by using oleogelation is extra virgin olive oil (EVOO), which is an excellent source of lipids, characterized by an optimally balanced fatty acid profile and the presence of health-promoting minor components, such as polyphenols (Martín-Peláez et al., 2017). The most representative and abundant phenols in EVOO are hydroxytyrosol and tyrosol. Both these components act as free radical scavengers in the human body, boosting the endogenous defence systems against oxidative stress (Visioli et al., 2002). The biological functions of EVOO polyphenols are strictly related to their bioaccessibility (BAC), defined as the fraction of the compounds released from the initial matrix in the gastrointestinal lumen and available for intestinal absorption (Salvia-Trujillo et al., 2017). Polyphenols from different sources are known to suffer harsh oxidizing conditions, pH shifts, and enzymatic activity during their transit throughout the gastrointestinal tract (Dinnella et al., 2007; Reboredo-Rodríguez et al., 2021a). As a result, their BAC is generally low upon food digestion (Pripp et al., 2005; Spencer, 2003). As exposed by Reboredo-Rodríguez et al. (2021b), the low pH of the gastric environment reduces the concentration of hydroxytyrosol down to 1.5-fold at the gastric level, while remaining stable in intestinal conditions where the pH is neutral. As a result, the final bioaccessibility at the intestinal level for hydroxytyrosol and tyrosol was found to be relatively low, being 64 and 35%, respectively (Reboredo-Rodríguez et al., 2021b). Besides these data, the possible impact of EVOO structuring on polyphenol bioaccessibility has not been explored so far.

The aim of this study was to address the critical knowledge gap regarding the digestion fate of EVOO-based oleogel systems structured by lipophilic and hydrophilic gelators, by investigating the destructuring behavior, the lipid digestion, and the bioaccessibility of key polyphenols, namely tyrosol and hydroxytyrosol.

To this purpose, oleogels were prepared by using either the direct method based on the gelation capability of saturated monoglycerides, phytosterol-phytosterol ester mixture, sunflower, and rice bran waxes or the indirect method based on whey protein aerogel particles. Oleogels were subjected to digestion simulation by using the INFOGEST standardized *in vitro* protocol. The lipolysis degree and polyphenol bioaccessibility of the oleogels were compared to those of the unstructured EVOO. Results confirmed the possibility to steer the digestion fate of EVOO components by oleogelation.

2. Materials and methods

2.1. Materials

Commercial EVOO was used (fatty acid composition: 7.7% C18:0, 2.3% C18:0, 80.1% C18:1, 8.0% C18:2, 1.9% other; K232: 2.24; K270: 0.06; PV: 7.6 $mEqO_2$ kg^{-1}; viscosity: 77 mPa s). Saturated monoglycerides (Myverol^{TM}, fatty acid composition: 0.24% C12:0, 0.9% C14:0, 60.1% C16:0, 38.4% C18:0, 0.3% C20:0, 0.1% other; melting point 68.1 ± 0.5 °C) were purchased from Kerry Bioscience (Bristol, UK); rice bran wax (Karl Wax GmbH & Co. KG, Reinbek, Germany) was kindly provided by Spica Srl (Sulmona, Italy); sunflower wax was purchased from Kahlwax GmbH & Co. KG (Reinbek, Germany); 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). Whey protein isolate (WP, 94.7% protein content; 74.6% β-lactoglobulin, 23.8% α-lactalbumin, 1.6% bovine serum albumin) was purchased from Davisco Food International Inc. (Le Sueur, MN, USA). Agar technical (Agar No. 3) was purchased from Oxoid Limited (Basingstoke, UK). Nile Red dye, porcine pepsin, porcine lipase, porcine pancreatin (8 × USP), porcine bile extract, HCl, NaOH, $CaCl_2$, Na_2CO_3, NaCl, KCl, KH_2PO_4, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, $MgSO_4$, hydroxytyrosol, tyrosol analytical standards, were purchased from Sigma Aldrich (Milan, Italy).

2.2. Sample preparation

2.2.1. Preparation of oleogels containing lipophilic molecules (direct method)

Oleogels were prepared by using 90% (w/w) EVOO and 10% (w/w) of the following gelators: saturated monoglycerides (MG), rice waxes (RW), sunflower waxes (SW), or a mixture of β-sitosterol and γ-oryzanol (PS) (2:3 w/w). As previously described by Ciuffarin et al. (2023), the mixtures were stirred in dark conditions at 80 °C (MG, RW, and SW) or 90 °C (PS) until the gelator completely melted. Following, MG, RW, and SW mixtures were cooled down to 20 °C, while the PS mixture was kept at 4 °C for 12 h. All samples were then stored in dark conditions at 20 °C and analyzed 48 h after preparation to allow network setting.

2.2.2. Preparation of oleogels containing hydrophilic molecules (indirect method)

Oleogels were prepared as described by Plazzotta et al. (2020). In brief, WP aerogel particles were dispersed into EVOO (0.1 g mL^{-1}), homogenized by a high-speed mixer (13,000 rpm, 3 min, Polytron PT-MR3000, Kinematica AG, Littau, Switzerland), and collected by centrifugation (14,000 ×*g*, 10 min, Beckman, Avanti J-25 centrifuge, Palo Alto, USA). This procedure was repeated twice, obtaining oleogels presenting 80% (w/w) oil content. The samples were stored in dark conditions at 20 °C and analyzed 48 h after preparation.

2.3. Analytical determinations

2.3.1. 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 (g) applied to the samples.

2.3.2. In vitro digestion

In vitro digestion was carried out according to the INFOGEST static protocol (Brodkorb et al. 2019). The simulated salivary (SSF), gastric (SGF), and intestinal (SIF) fluids were preheated at 37 °C just before *in vitro* digestion. Due to the lack of carbohydrates in the digested matrices, amylolytic enzymes were not considered. Four mL SSF, 25 μL 0.3 M $CaCl_2$, and 975 μL water were added to the sample to start the oral phase and the sample was continuously stirred at 37 °C for 2 min. Then, 8 mL SGF, 5 μL 0.3 M $CaCl_2$, and 667 μL of an aqueous pepsin solution providing 2,000 U/mL activity in the final chyme were added to the WP sample, while in the case of MG, RW, SW, PS and control EVOO the enzyme solution was substituted with 667 μL of water. The gastric phase was started by adjusting the pH to 3.0 with 1 M HCl and making up the volume to 20 mL with water. The mix was continuously stirred at 37 °C for 2 h. Following, 8 mL SIF, 4 μL 0.3 M $CaCl_2$, 5 mL of a lipase-pancreatin solution, prepared in SIF and providing 2000 and 100 U/mL activity respectively in the final mixture, and 3 mL of 160 mM bile extract prepared in SIF were added to WP sample. For MG, RW, SW, PS, and control EVOO the lipase-pancreatin solution was substituted with 5 mL of lipase solution at 2000 U/mL activity. The intestinal phase was started by adjusting the pH to 8.00 ± 0.10 with 1 M NaOH and making up the volume to 40 mL with water. The mix was continuously stirred at 37 °C for up to 2 h. A 30 mL aliquot of the mixed micellar phase (*i.e.*, stabilized structures in which lipophilic bioactive components are encapsulated) was recovered after *in vitro* digestion by centrifugation at 30,000 ×*g* for 70 min (Beckman, Avanti J-25 centrifuge, Palo Alto, USA).

2.3.3. Confocal light scanning microscopy (CLSM)

The fluorescent dye Nile Red (0.2% aqueous solution) was used to stain lipophilic molecules. The hanging-drop method was used (Gallier et al., 2012) to analyze samples collected after the gastric and intestinal digestion phases (*i.e.*, digestate). The stained samples were added with an agarose solution (1% w/w) preheated at 50 °C, in a sample:agarose solution ratio of 1:2 (v/v). Two μL of the fluid mix was placed on a microscope coverslip and left to set at room temperature for 1 min. The coverslip with the gelled droplet was then fixed on a concave microscope slide and observed at 100× by confocal laser scanning microscope (Leica TCS SP8 X confocal system, Leica Microsystems, Wetzlar, Germany). Images of confocal micrographs were imported in jpeg format (LasX 3.5.5, Leica Microsystems, Wetzlar, Germany). Image analysis was carried out to assess the oil droplet dimension (Image-Pro Plus 6.3, Media Cybernetics Inc., USA). Images were converted to an 8-bit grey scale and software calibration was applied. Droplet diameter data were provided by the software and further elaborated to obtain the D_{32} using Excel (Microsoft, Redmond, Washington, USA).

2.3.4. Particle size and zeta potential of digested samples

The particle size distribution of the mixed micellar phase of digested oil and oleogels (recovered as described in paragraph 2.3.4) was measured by DLS (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 the cumulative sum of volume-weighed mean diameter expressed in nm. The polydispersity index of distributions (PDI) was used to measure the homogeneity of particle size distribution. The ζ-potential was also measured by placing the diluted sample in a capillary cell equipped with two electrodes to assess particle electrophoretic mobility.

2.3.5. Lipid digestibility

The pH-stat approach was used to determine the extent of lipid digestibility (Mat et al., 2016). $NaHCO_3$ was replaced with NaCl in SSF, SGF, and SIF. According to oleogel composition (80% oil content for WP-based oleogels, 90% for MG, RW, SW, PS-based oleogel), an oleogel amount of 1.25 g for WP oleogel and 1.11 g for MG, RW, SW, PS-based oleogels, corresponding to 1 g oil, was used. The pH of the digestion mixture was monitored immediately after lipase addition (paragraph 2.2.4) and 0.25 M NaOH aliquots were added to maintain a value of 8.0 ± 0.1 (*i.e.*, the optimum of the lipase used in the present study according to its technical specifications) and the volume of NaOH (mL) added to titrate the oleogels was recorded ($V_{oleogel}$). An aliquot of lipid-free WP gelator (0.25 g) corresponding to that contained in the oleogels was digested and the required NaOH volume registered ($V_{aerogel}$) to account for the proteolysis contribution to pH lowering. Finally, the NaOH volume required to titrate unstructured EVOO (1 g) was also recorded (V_{oil}).

The percentage of free fatty acids (FFA) released during lipolysis was calculated according to Eq (1):

$$\text{FFA}\ (\%) = \frac{V_e}{V_t} \times 100 \tag{1}$$

where V_e is the experimental volume, represented by: (i) V_{oil} in the case of EVOO and MG, RW, SW, and PS-based oleogels; (ii) the difference between $V_{oloegel}$ and $V_{aerogel}$ in the case of WP-based oleogel. V_t represents 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 (Li et al., 2011), and was calculated according to Eq. (2):

$$V_t = 2 \times \left[\frac{m_{oil}}{MW_{oil}} \frac{1000}{C_{NaOH}}\right] \tag{2}$$

where m_{oil} is the mass of oil in the reaction vessel (g), MW_{oil} is the average molecular weight of EVOO (879.67 g mol^{-1}) and C_{NaOH} is the concentration of the sodium hydroxide (mol L^{-1}).

2.3.6. Hydroxytyrosol and tyrosol quantification

Hydroxytyrosol and tyrosol were extracted and quantified from 2 g of oil as reported by Ciuffarin et al. (2023) and according to the official COI/T.20/Doc No 29 method in agreement with Reg. UE 432/2012. Acid hydrolysis was applied following the ISO 23942:2022 procedure. UHPLC analysis was performed as previously reported (Lucci et al., 2020).

Total polyphenol content was calculated as the sum of hydroxytyrosol and tyrosol values.

2.3.7. Polyphenol bioaccessibility computation

Phenolic compounds were extracted from the mixed micellar phase following the methodological approach reported by Calligaris et al. (2020). The method was modified to optimize the extraction of the compounds of interest, by modifying the solvent mixture and by adding a d-SPE clean-up step. Briefly, aliquots of 10 mL of micellar phase were placed in a 50 mL falcon tube with 2.5 mL of acetonitrile. The sample was vigorously hand-shaken for 1 min. A 6 g salt mixture ($MgSO_4$/NaCl 2:1, w/w) was then added and shaking was repeated under the same conditions. The resulting mixture was centrifuged at 5,000 ×*g* for 10 min and 1.5 mL of supernatant was carefully taken and placed in a tube with 150 mg $MgSO_4$ and 50 mg PSA for d-SPE clean-up, vortexed, and centrifuged at 10,000 ×*g* for 5 min (Mikro 120, Hettich Italia Srl, Milan, Italy). 1 mL of supernatant was then placed in a vial and dried by a gentle N_2 flow. Acid hydrolysis was applied before UHPLC analysis as

described in Section 2.3.6.

The extraction procedure for polyphenols analysis in digested samples was validated. Accuracy was determined by means of recovery experiments during which digested samples of refined sunflower oil were fortified with three different amounts of polyphenols (25, 100, and 250 μg for tyrosol and hydroxytyrosol, each) and their content was assessed. Precision was determined in terms of relative standard deviation from recovery experiments at each fortification level. In all cases, the average recovery ranged from 75 to 100% with repeatability relative standard deviation lower than 2%, revealing the suitability of the procedure for the quantitative extraction of polyphenols from digested samples.

The BAC of HT and T was calculated as the percentage ratio between the concentration of compound incorporated in the micellar phase recovered from *in vitro* digestion and its concentration in the undigested sample, and was expressed as mg of T or HT per kg_{oil}.

2.3.8. Statistical analysis

Results are reported as mean ± standard deviation and are averages of three measurements carried out on two replicated experiments. Bartlett's test was used to check the homogeneity of variance, the Tukey test and the analysis of variance (ANOVA) were used to test for differences between means ($p < 0.05$), using R (v. 4.0.3, The R Foundation for Statistical Computing, Vienna, Austria). Correlation was measured by the Pearson coefficient.

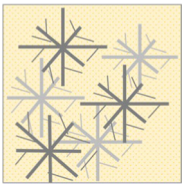
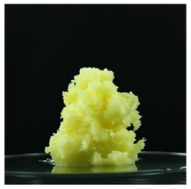
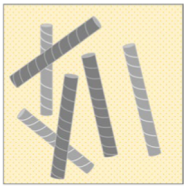
3. Results and discussion

3.1. Oleogel physical properties

The firmness of the oleogels considered in this study is reported in Table 1. All samples have been previously characterized for their structural features elsewhere (Alongi et al., 2022; Ciuffarin et al., 2023; Plazzotta et al., 2022). The 10% concentration of liposoluble gelators was chosen since allowing to obtain self-standing oleogels (Table 1), while the WP-based oleogel contained 80% oil, which is the maximum quantity of oil that can be included in the WP network (Plazzotta et al., 2020).

The oleogels presented firmness values decreasing in the order PS > SW > RW > WP > MG. This behavior can be explained by the different nature of the oleogel network and agrees with the literature (Alongi et al., 2022; Plazzotta et al., 2022; Valoppi et al., 2017). As represented in Table 1, MG, SW, and RW samples were characterized by a three-dimensional network of oleogelator crystals with different morphology. In particular, MG presents crystals with a spherulite conformation characterized by a dendritic shape, while RW and SW present needle-like crystals with different dimensions, smaller for the former and longer for the latter (Ciuffarin et al., 2023; Doan et al., 2017; Valoppi et al., 2017). PS network was formed by hollow double-walled tubules smaller enough to be crossed by the light and accounting for a high firmness but a brittle structure (Scharfe et al., 2019). Finally, in the

Table 1
Structuring elements, network representation, macroscopic appearance and firmness of EVOO-based oleogels obtained by using 10% (w/w) monoglycerides (MG), rice wax (RW), sunflower oil (SW), or phytosterols (PS), and 20% (w/w) whey protein aerogel (WP).

Oleogelator	Structuring elements	Network representation	Macroscopic appearance	Firmness (g)
MG	Crystals			33.6 ± 2.5 [e]
RW	Crystals			115.0 ± 3.0 [c]
SW	Crystals			532.7 ± 23.8 [b]
PS	Hollow tubules			2534.0 ± 71.6 [a]
WP	Porous material			96.7 ± 6.8 [d]

[a–e]: indicate significant differences among oleogels ($p < 0.05$).

case of the WP-based oleogel, the oil was retained by multiple mechanisms, including: (i) oil absorption into the protein particle pores, driven by capillary forces; (ii) oil adsorption onto particle surface; (iii) interactions between oil and hydrophobic protein residues; (iv) oil retainment in the interstices among particles, which create a network based on weak hydrophilic interactions (De Vries et al., 2017). As a result, a semi-solid material with rheological properties analogous to those of laminating fats is obtained, as well demonstrated by Plazzotta et al. (2020, 2022).

3.2. Destructuring behavior under gastrointestinal conditions

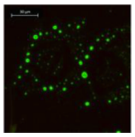
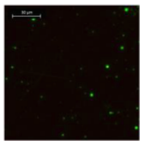
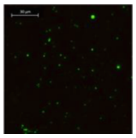
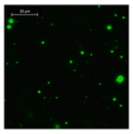
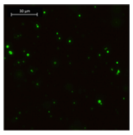
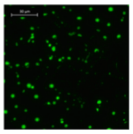
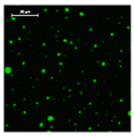
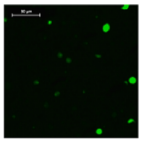
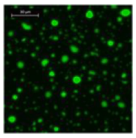
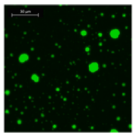
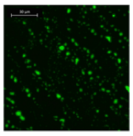
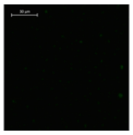
The different oleogel structure (Table 1; Alongi et al., 2022; Ciuffarin et al., 2023) has been previously reported to affect the structural arrangement of samples also during digestion (Plazzotta et al., 2022). The digestive behavior of samples was thus analyzed and compared to that of unstructured EVOO, considered as a reference.

The microstructure generated upon gastric and intestinal phases was observed by CLSM to study the destructuring of oleogels under gastrointestinal conditions. Table 2 reports the confocal micrographs. Upon gastric digestion, unstructured EVOO presented droplets with a D_{32} around 4.5 μm. Analogous small droplets were also observed in the gastric digestate of MG, RW, SW, and PS oleogels (Table 2), which showed droplets with a D_{32} of about 3.4, 2.7, 5.5, and 3.4 μm, respectively. It must be mentioned that after gastric digestion intact oleogel agglomerates, not homogeneously dispersed, were still found in the digestate. This is consistent with the lower number of droplets visually detectable in MG, RW, SW, and PS sample micrographs, as compared to that of the unstructured oil. Residual oleogel structures physically entrapped the oil, thus preventing its release in the form of droplets in the digestive medium. In the case of the WP oleogel, a D_{32} of 4.4 μm was measured after the gastric phase. However, in the CLSM micrograph of this sample, a larger number of droplets was observed. This suggests that a higher amount of oil was released as compared to the other oleogels, resulting in a gastric digestate similar to that of the unstructured oil. This behavior can be explained by the susceptibility of the WP-based aerogel template to gastric digestion. In this regard, the structure of WP-based oleogel, including protein location, has already been thoroughly characterized in our previous work Plazzotta et al. (2022), demonstrating that more than 70% of aerogel proteins were digested during this phase.

The differences among samples became even more evident after the intestinal phase (Table 2). Looking at the CLSM micrograph of EVOO, it can be observed that both the number and the D_{32} (4.2 μm) of particles remained almost unchanged as compared to the gastric phase. Oil was dispersed into the digestive mixture since the beginning of digestion, due to the absence of an oleogel network able to entrap it. Conversely, the number of droplets observed in the CLSM micrograph of MG, RW, SW, and PS samples increased after the intestinal phase of digestion, indicating that oil was physically released from the oleogel network in the digestive mixture. It must be pointed out that, at the end of the intestinal phase, intact oleogel particles could be barely visually detected in the samples. Moreover, it was observed that the D_{32} of MG and RW droplets almost doubled from the gastric to the intestinal phase, accounting for 6.7 and 5.9 μm, respectively, while the D_{32} of SW and PS samples remained almost unchanged (6.3 and 4.3 μm, respectively). These differences could be attributed to the different firmness of the oleogels (Table 1). MG and RW actually presented a lower firmness compared to SW and PS. This may have increased their susceptibility to mechanical disruption during digestion, possibly accelerating oil release in the digestive mixture, and resulting in a more pronounced droplet coalescence (Luo et al., 2021). Moreover, gelator crystals are expected to place themselves at the oil/water interface, contributing to the increase in droplet size (Dong et al., 2020). Nevertheless, in all cases the presence of bile salts, combined with peristalsis simulation, favored oil emulsification, preventing the formation of large droplets. Oil droplets completely disappeared in the WP sample after the intestinal phase, in agreement with previous findings (Plazzotta et al., 2022). Besides allowing the physical release of oil already during the gastric phase, the WP released upon the digestion of the aerogel template would be able to act as surfactants, favoring the emulsification of oil and increasing the exposed surface area (Mat et al., 2020). This, in turn, is expected to increase the susceptibility of oil to lipolysis.

It must be pointed out that CLSM micrographs provide an overview of the appearance of digestate samples on the micro-scale. However, when dealing with the digestibility of lipid matrices, it is crucial to characterize the mixed micellar fraction. Mixed micelles are self-assembled nanoscale-size structures, encapsulating free fatty acids and other lipophilic compounds released during lipid digestion. The micelles below 200 nm can be absorbed through the intestinal epithelium and therefore their role is to carry the encapsulated compounds through the epithelial cells of the small intestine for uptake (Salvia-Trujillo et al., 2017). Since the efficiency of this process is affected by mixed micelle size (Marze et al., 2015), the particle size distribution of the mixed micellar fractions obtained after the intestinal digestion of oleogels and EVOO (control) were analyzed by DLS, and the cumulative distributions are shown in Fig. 1. The 51% of particles in the mixed micellar fraction of unstructured EVOO was smaller than 200 nm, *i.e.*, the threshold for intestinal uptake. A similar profile was observed also in the case of MG, RW, SW, and PS oleogels, in which the ratio of particles below 200 nm was around 50% (Fig. 1). These findings agree with those reported in the literature for sunflower oil gelled by using the same liposoluble gelators here applied (Calligaris et al., 2020; Dong et al., 2020). Conversely, almost 90% of the micelles generated upon WP digestion presented a size below 200 nm, in agreement with the results reported by Plazzotta et al. (2022) on sunflower and flaxseed oil WP-based oleogels, possibly resulting in an increased lipid digestibility. The micellar fraction of the

Table 2
Confocal light scanning microscopy (CLSM) micrograph of the digestate samples obtained upon gastric and intestinal digestion of unstructured EVOO (control) and EVOO-based oleogels obtained by using 10% (w/w) of monoglycerides (MG), rice wax (RW), sunflower oil (SW), or phytosterols (PS), and 20% (w/w) whey protein aerogel (WP).

Sample	EVOO	MG	RW	SW	PS	WP
Gastric Phase						
Intestinal Phase						

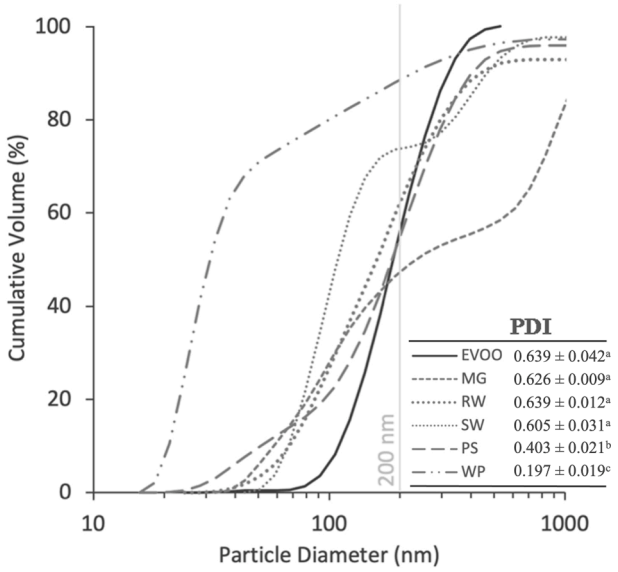


Fig. 1. Cumulative particle size distribution of unstructured EVOO (control) and EVOO-based oleogels obtained by using 10% (w/w) of monoglycerides (MG), rice wax (RW), sunflower oil (SW), or phytosterols (PS), and 20% (w/w) whey protein aerogel (WP).

samples also showed different PDI values (Fig. 1). The PDI of EVOO micellar fraction was the lowest one (around 0.2), indicating that the micelles produced upon its digestion showed a rather homogeneous size. The PDI increased (~0.4) when WP-based oleogel was digested, but the highest values (greater than 0.6) were observed in the micellar fraction deriving from the digestion of liposoluble gelator-based oleogels. This occurred regardless the gelling agent used, indicating that the gelator nature affected the heterogeneity of the micellar phase size. These results can be possibly attributed to the de-structuring behavior of oleogels. It can be hypothesized that the presence of a network made of liposoluble gelators impaired the homogeneous breakage of the oleogel structure and the consequent release of oil. By contrast, in the case of WP oleogels, the oil present in the interparticle spaces was readily released and digested, leading to micelles analogous to those found in the bioaccessible fraction of unstructured EVOO.

Besides particle dimension, also the surface electrical charge of particles at the oil–water interface is well known to play a role in intestinal uptake (McClements, 2004). The ζ-potential of the mixed micellar fractions was thus measured. A negative charge was found in all cases, due to the presence of anionic surfactants absorbed at the oil–water interface, such as bile salts and free fatty acids (Salvia-Trujillo et al., 2013). However, MG, RW, SW, PS, and EVOO presented similar values, ranging from −53 to −56 mV, while the WP ζ-potential was significantly higher (-41 mV). Such a difference can be attributed to the presence of positively charged free aminoacids and peptides derived from protein digestion (Qian et al., 2012).

Based on these results, the destructuring behavior of oleogels seems to be governed in a complex way by both the gel network strength and the oleogelator type. These differences could lead to changes in lipid digestibility and polyphenol bioaccessibility.

3.3. Lipolysis upon in vitro digestion

The lipid digestibility of oleogels and unstructured oil was monitored by measuring the free fatty acid (FFA) released during the intestinal phase of *in vitro* digestion (Fig. 2).

The FFA release during intestinal digestion of all samples followed the typical profile reported in the literature (Li et al., 2011; O'Sullivan et al., 2017), with a steeper increase in the first minutes followed by a

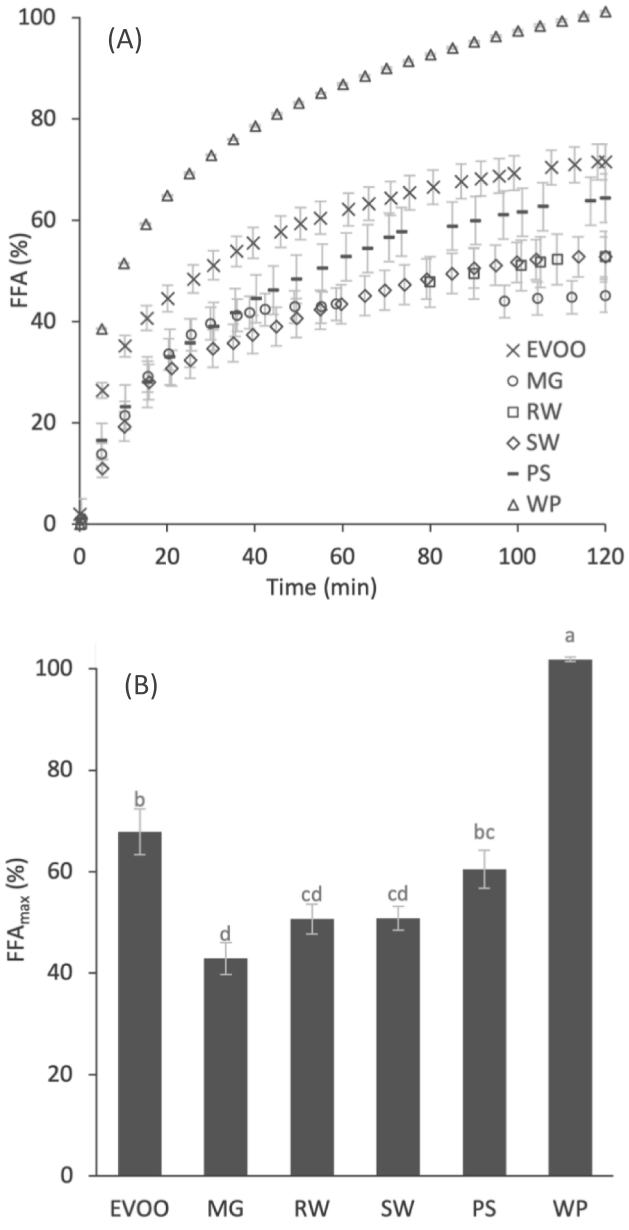


Fig. 2. Free fatty acids (FFA) release kinetics (A) and total free fatty acids (FFA_{max}) released during *in vitro* intestinal digestion (B) of unstructured EVOO (control) and EVOO-based oleogels obtained by using 10% (w/w) of monoglycerides (MG), rice wax (RW), sunflower oil (SW), or phytosterols (PS), and 20% (w/w) whey protein aerogel (WP).

flattening until a *plateau* was approached (Fig. 2A) (Mat et al., 2020; Salvia-Trujillo et al., 2017). However, the rate and extent of lipid digestion were affected by oil structuring. After 2 h intestinal digestion, FFA_{max} accounted for nearly 70% (Fig. 2B) of the unstructured EVOO, in agreement with the literature (Pascoviche et al., 2019). It must be pointed out that the theoretical value of FFA release under gastrointestinal conditions is 66%, due to the selective hydrolysis carried out by lipases preferably in positions 1- and 3- of the triglyceride, thus producing 2 free fatty acids and one monoglyceride (Hofmann & Borgstroem, 1964). However, different lipolysis extents were reported in the literature for oils with different compositions, ranging from 44% for unstructured canola oil (O'Sullivan et al., 2017), to 72% for palm oil (Ye et al., 2019). The remaining undigested oil was actually clearly visible as distinct droplets in the CLSM micrographs of the intestinal digestate (Table 2).

Oleogel structuring with liposoluble gelators led to an overall decrease in FFA_{max} (PS ≥ SW ≈ RW ≥ MG). This behavior can be attributed to the presence of different network structures modulating the lipase accessibility to its active sites. In particular, the use of oleogelators forming crystalline networks reduced lipase activity more than the oleogelator forming fibrillar network (PS).

An opposite effect on lipolysis extent was observed when oil was structured through WP aerogel. The presence of proteins considerably improved the efficiency of lipid digestibility, leading to complete hydrolysis (FFA_{max} ≈ 100%) after the 2-h intestinal digestion, in agreement with previous results on other vegetable oils (Plazzotta et al., 2022). This result was attributed to the ability of WP aerogel to act as an emulsifier in the digestive mix. The finely dispersed oil droplets were stabilized by partially undigested aerogel particles, which placed themselves at the droplet surface, supporting the easy attack by intestinal lipases and complete lipolysis. This evidence agrees with CLSM micrographs showing the complete absence of visible oil droplets in the intestinal digestate (Table 2). WP would actually increase oil susceptibility to gastric enzymes favoring the formation of small oil droplets already in the gastric phase, and leading to their prompt lipolysis during the first stages of the intestinal phase (Plazzotta et al., 2022).

Overall, these results confirm the possibility to steer lipid digestion by oleogelation, thus suggesting the applicability of oleogels not only for their technological advantages but also to accomplish specific nutritional requirements (Qian et al., 2012; Salvia-Trujillo et al., 2017). In this regard, the possibility to tune lipolysis by selecting the proper oleogelator could be exploited to develop different products intended for specific consumer categories. For instance, the use of a lipophilic-based oleogel might be adopted to reduce caloric intake thanks to its ability to restrain lipid digestion, whereas protein-based oleogels can contribute to the efficient deliver of essential fatty acids.

3.4. Polyphenol bioaccessibility

The bioaccessibility of the major phenolic compounds contained in EVOO (*i.e.*, hydroxytyrosol and tyrosol) was further investigated. Unstructured EVOO contained 248.1 ± 4.6 and 93.9 ± 3.3 mg kg^{-1} of hydroxytyrosol and tyrosol, respectively. Since the oleogel preparation process did not affect phenolic concentration (data not shown), these values were used to compute the bioaccessibility in MG, RW, SW, PS, and WP oleogels (Table 3).

Overall, hydroxytyrosol and tyrosol decreased considerably upon digestion in all cases, but significant differences were detected among the samples.

When unstructured EVOO underwent *in vitro* digestion, hydroxytyrosol suffered a dramatic decrease, completely disappearing from digested unstructured EVOO, while tyrosol was halved. These results can be attributed to the different resistance toward the oxidizing conditions in the biological fluids of these two phenolic compounds (Cheng et al., 2002; Pripp et al., 2005). Tyrosol is actually reported to be more stable than hydroxytyrosol, maintaining its antioxidant activity also under critical conditions (Marković et al., 2019).

Our results differ from those reported by Reboredo-Rodríguez et al. (2021b), who observed the presence of HT after intestinal digestion in the micellar phase. These contradictory outputs could be associated with the diversity of both the starting material and the methods applied in the studies. In consideration of the limited literature on this topic, there is a need to reinforce the studies on this topic.

When EVOO was structured by liposoluble gelators, the BAC of hydroxytyrosol and tyrosol changed. The BAC of tyrosol significantly decreased, while that of hydroxytyrosol increased, being detected even after digestion. The increase in hydroxytyrosol concentration in the mixed micellar fraction and thus in its BAC can be associated with a retarded oil digestion (Fig. 2A), leading to partial protection of the bioactive molecule through the shielding carried out by the gelled structure against the gastric environment, followed by a further release in the intestinal fluids. In the case of tyrosol, which is less water soluble than hydroxytyrosol, its BAC decreased due to the entrapment of the molecule into the undigested oleogel (Table 2). Additionally, a possible role of the interactions among phenolic compounds and gelators cannot be excluded as recently demonstrated by Ciuffarin et al. (2023). Given these protection and binding mechanisms, the total phenolic BAC ultimately remained in the same range as observed for unstructured EVOO, accounting for 12.3, 10.0, 12.1, and 7.0% in MG, RW, SW, and PS-based oleogels, respectively. These values corresponded to an overall concentration of bioaccessible phenolic compounds of around 42.4, 34.5, 41.7, and 24.1 mg kg^{-1} in MG, RW, SW, and PS, respectively.

On the contrary, WP allowed a higher retention of hydroxytyrosol and tyrosol than lipophilic gelators, resulting in an improvement of total phenolic BAC (27%, 26.9 mg kg^{-1}). This result can be linked to the presence of emulsifying WP, able to protect hydroxytyrosol from degradative events occurring under gastric conditions. It cannot be excluded also a protective role of whey proteins, especially β-lactoglobulin, as antioxidants during digestion (Liu et al., 2007).

4. Conclusions

The knowledge of the effect of oleogel structure on human digestion behavior appears promising to formulate oleogels with tailored health functionalities, widening their potential application beyond their use as fat-mimetic. This study highlighted how, based on the gelator choice, not only the caloric intake can be modified, but also the BAC of phenols naturally present in EVOO can be steered. In particular, WP-based oleogels presented the highest FFA release (*circa* 100%) and phenolic compound BAC, as a consequence of their peculiar destructuring behavior during digestion. Contrarily, EVOO gelled with lipophilic gelators showed reduced oil lipolysis and BAC, possibly due to the entrapment in the undigested oleogel. The recorded BAC reduction may open the possibility for EVOO phenolic compounds to be carried to the colon, where they can further exert their bioactivity. Indeed, future research should be addressed to understand more in detail the actual fate of the non-bioaccessible fraction of EVOO phenolic compounds.

Table 3
Hydroxytyrosol and tyrosol content and bioaccessibility (BAC) in EVOO (control) and oleogels obtained by using 10% (w/w) of monoglycerides (MG), rice bran waxes (RW), sunflower waxes (SW), or phytosterols (PS), and 20% (w/w) of whey protein aerogels particle (WP) after *in vitro* digestion.

Sample	Hydroxytyrosol		Tyrosol	
	Concentration (mg kg_{oil}^{-1})	BAC (%)	Concentration (mg kg_{oil}^{-1})	BAC (%)
EVOO	n.d.	n.d.	46.3 ± 3.2^{a}	48.1 ± 3.38^{a}
MG	17.2 ± 4.3^{b}	7.0 ± 0.75^{b}	25.1 ± 0.9^{bc}	26.1 ± 0.97^{bc}
RW	13.5 ± 1.2^{c}	5.4 ± 0.49^{c}	21.9 ± 1.7^{c}	22.8 ± 1.77^{c}
SW	13.2 ± 1.6^{c}	5.3 ± 0.66^{c}	30.4 ± 3.1^{b}	31.6 ± 3.25^{b}
PS	2.9 ± 0.2^{d}	1.2 ± 0.08^{d}	24.6 ± 1.7^{c}	25.6 ± 1.81^{c}
WP	42.1 ± 11.1^{a}	17.0 ± 4.50^{a}	50.3 ± 1.8 ^{a}	52.3 ± 1.96^{a}

$^{a–d}$: indicate significant differences in polyphenol concentration in the same column ($p < 0.05$). n.d.: not detectable.

CRediT authorship contribution statement

Francesco Ciuffarin: Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Marilisa Alongi:** Methodology, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Stella Plazzotta:** Methodology, Writing – review & editing. **Paolo Lucci:** Methodology, Validation, Writing – review & editing. **Francesco Paolo Schena:** Conceptualization, Funding acquisition, Writing – review & editing. **Lara Manzocco:** Supervision, Writing – review & editing. **Sonia Calligaris:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This project has been granted by the INNONETWORK PROGRAM of Puglia Region. Title of the project: Processo innovativo per la valorizzazione dell'olio extravergine di oliva monovarietale Coratina come nutraceutico nei processi infiammatori dell'intestino PRIN CORATINA (P8K5PA8). The authors are also grateful to Mr. Giacomo Alberio for contributing to the analyses.

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