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Antioxidant properties of ferulic acid-based lipophenols in oil-in-water (O/W) emulsions / Cantele, C., Martina, K., Potenziani, G., Rossi, A.M., Cardenia, V., Bertolino, M.. - In: LEBENSMITTEL-WISSENSCHAFT + TECHNOLOGIE. - ISSN 0023-6438. - 189:(2023). [10.1016/j.lwt.2023.115505]

Availability:

This version is available at: 11696/85882 since: 2025-02-26T15:52:46Z

Publisher:

Elsevier

Published

DOI:10.1016/j.lwt.2023.115505

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Antioxidant properties of ferulic acid-based lipophenols in oil-in-water (O/W) emulsions

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ARTICLE INFO

Keywords:

Lipophilization
Alkyl ferulates
Antioxidants
Oil-in-water emulsions
Lipid oxidation

ABSTRACT

Butyl ferulate (BF) and octyl ferulate (OF) were investigated to enhance oxidative stability of 1.0% vegetable oil-in-water (O/W) emulsions at pH 3.5 and 7.0 at different concentrations (5, 10, 15, 20, and 25 mg/L of emulsion). Particle size distribution, droplet surface charge, hydroperoxides (PV), hexanal and nonanal were monitored for 14 days at 35 °C. Higher concentrations of BF and OF generated smaller particles at pH 7.0 but not at pH 3.5. Control showed the highest negative surface charge (-58.38 ± 0.31 mV, pH 7.0) compared to BF and OF (-46.71 ± 0.06 mV at levels >0.10 mg/L). Both alkyl ferulates counteracted lipid oxidation, especially at pH 3.5, where PV decreased up to 96% compared to the control and delayed hexanal and nonanal formation. A low content of lipophenols allowed to achieve a tremendous shelf-life extension, demonstrating that BF and OF could be of great interest for food application.

1. Introduction

Since fats are predominant ingredients in foods, lipid oxidation is one of the chief chemical reactions that deteriorates food quality, impacting flavor (development of rancid volatiles, like pentanal, hexanal, nonanal and decanal), nutritional value (loss of health-promoting lipids, such as omega-3 fatty acids), protein functionality (co-oxidation), and biological tissues (production of toxic compounds, such as hydroperoxides, aldehydes, and ketones) (Bayram & Decker, 2023; Frankel, 2014). High degree of unsaturation results in a decisive increase in the rate of oxidation, with the result that mono- and polyunsaturated fatty acids are the most unstable, yet also being the most sought after by consumers. Oil-in-water (O/W) emulsions, which make up the majority of foods, are a favorable system for rapid oxidation reactions, as the contact area between lipids and water containing prooxidants (transition metals, enzymes, and photosensitizers) is very large, facilitating their interaction (Li et al., 2020; Li et al., 2023; McClements & Decker, 2018). One

solution to delay lipid oxidation in emulsion systems and increase the shelf-life of health-promoting lipid-containing foods is to use antioxidant compounds (Bravo-Díaz, 2022; Jiang & Charcosset, 2022; Li et al., 2020; Liu et al., 2022; Zhang, Fan, Liu, & Li, 2023).

In addition, the hydrophilicity of most antioxidants brings an important limitation to their use in lipid-containing foods. According to the polar paradox, less polar antioxidants are most effective in more polar systems such as O/W emulsions (Laguerre et al., 2009) and in such multiphase systems the partitioning of antioxidants at the interface region – where oxidation reactions take place – is crucial and can be achieved with amphiphilic antioxidants, such as lipophenols (McClements & Decker, 2018). Lipophenols consist of a phenolic component bound to hydrophobic molecules like alkyl chains, fatty acids, and phytosterols. The lipophilization should enable their positioning at the oil-water interface, with the lipid part in oil droplets and the phenolic part facing the water. Many studies demonstrated the *in vitro* antioxidant capacity of certain lipophenols, involving different phenolic structures

Abbreviations: O/W, oil-in-water; BF, butyl ferulate; OF, octyl ferulate; PV, peroxide value; DPPH, 2,2'-diphenyl-1-picrylhydrazyl; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; GC/FID, gas chromatography/flame ionization detector; HS-SPME-GC/MS, headspace-solid phase microextraction-gas chromatography/mass spectrometry; DLS, dynamic light scattering; DVB/CAR/PDMS, divinylbenzene/carboxen/polydimethylsiloxane.

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<https://doi.org/10.1016/j.lwt.2023.115505>

Received 20 July 2023; Received in revised form 30 October 2023; Accepted 4 November 2023

Available online 9 November 2023

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conjugated to alkyl chains of different lengths (Liu & Yan, 2019; Sørensen et al., 2014; Torres de Pinedo, Peñalver, & Morales, 2007; Xu, Zhang, et al., 2022). Some lipophenols have also been tested in model systems (Elder, Coupland, & Elias, 2021; Lu et al., 2021; Panya et al., 2012; Szydłowska-Czerniak, Rabciej, Kyselka, Dragoun, & Filip, 2018; Sørensen, Nielsen, Yang, Xu, & Jacobsen, 2012), in food products (Elder, Coupland, Hopfer, & Elias, 2022; Marzocchi & Caboni, 2018; Sørensen et al., 2012), and in biological systems (Alemán-Jiménez et al., 2021; Moine et al., 2021; Totani, Tateishi, Takimoto, Shinohara, & Sasaki, 2012; Xu, Li, et al., 2022). Moreover, esters of gallic acid with different alkyl chains are already employed as antioxidant additives in food products (E310-propyl gallate; E311-octyl gallate; E312-dodecyl gallate) (Figueroa-Espinoza & Villeneuve, 2005). However, each antioxidant behaves differently depending on its molecular structure and the surrounding medium (Villeneuve et al., 2021). Therefore, only the study of the lipophenols in both model and real food systems can make their use by food companies realistic and feasible. The so-called *cut-off* theory (Laguerre et al., 2009) rationalizes the non-linear behavior of the synthesized lipophilic phenolic esters in terms of antioxidant activity as the existence of a critical chain length (CCL) for which maximum activity can be achieved. The CCL depends not only on the type of phenolic compound but also on the system in which it acts (Laguerre, Bily, Roller, & Birtić, 2017). For example, the CCL for alkyl caffeates was found to be 8 in O/W emulsions and in fish oil-enriched mayonnaise, while 2 and 12 in milk enriched with fish oil and in O/W microemulsions, respectively (Laguerre et al., 2017). Gallic acid performed best in O/W emulsions when esterified with a 3 carbon atoms alkyl chain, while in liposomes when it has 10 carbon atoms (Laguerre et al., 2017). Regarding ferulate alkyl esters, the CCL in liposomes and in O/W microemulsions was found to be 7 and 8 carbon atoms, respectively (Laguerre et al., 2017), while methyl and butyl ferulates were the two most effective lipophenols in fish oil enriched milk (Sørensen, Lyneborg, Villeneuve, & Jacobsen, 2015). In addition, ferulic acid represents one of the most abundant phenolic acids largely widespread in plant tissues and it is well known for its strong ability to scavenge free radicals due to the presence of substituent in *para* position and a carboxylic group with an adjacent unsaturated C–C double bond (Shahidi et al., 2022).

It is also reported that the potency of the antioxidant activity of phenolics or other antioxidants can change in the presence of a neutral or an acidic environment (Bayram & Decker, 2023; Huang, Frankel, Schwarz, & German, 1996; Li et al., 2020a; Tian et al., 2022), due to different factors, such as the variation of the redox potential based on the pH of the surrounding system, the possible different positioning of molecules in the system, or even the different rate of degradation of molecules (Bayram & Decker, 2023; Bayram, Laze, & Decker, 2023; Jovanovic, Steenken, Hara, & Simic, 1996). To the best of our knowledge, there is a lack of information regarding the ability of ferulate alkyl esters to counteract lipid oxidation as related to different pH conditions in O/W emulsions stored for a prolonged period with no oxidation inducers.

In the present study, therefore, we hypothesized that, based on what reported in the literature, the lipophilization of ferulic acid with a short (C4) and medium (C8) alkyl chain could be a successful strategy to enhance the oxidative resistance toward lipid oxidation during the storage of food emulsions. Two different ferulate alkyl esters (butyl ferulate, BF, and octyl ferulate, OF) were tested at different concentrations in O/W emulsions stored at 35 °C for 14 days. In addition, the effect of pH (3.5 and 7.0) on their activity was also investigated.

2. Materials and methods

2.1. Chemicals

Propan-2-ol, *n*-hexane, iso-octane, ethanol absolute anhydrous, 1-butanol, and hydrochloridric acid (37%) were purchased from Carlo Erba (Milan, Italy), while methanol from VWR (Milan, Italy). Ferulic

acid, butanol, Dowex 50 X8, polyethylene, ethyl acetate, pyridine, acetic anhydride, toluene, thionyl dichloride, chloroform, 1-octanol, piperidine, Tween 20 (polyethylene glycol sorbitan monolaurate), ferrous sulfate heptahydrate, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), ferrozine, silicic acid (100–200 mesh, 75–150 µm, acid washed), activated charcoal, cumene hydroperoxide, barium chloride dihydrate, butylated hydroxytoluene (BHT), ammonium thiocyanate, and sodium phosphate monobasic dihydrate were provided by Merk (Darmstadt, Germany). Ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA) and sodium phosphate dibasic anhydrous were supplied by Fluka (Milan, Italy). Ultrapure water was prepared in a Milli-Q filter system (Millipore, Milan, Italy).

2.2. Synthesis of butyl and octyl ferulates (1 and 5)

Both alkyl ferulates were synthesized in the Department of Drug Science and Technology of the University of Turin.

2.2.1. Butyl ferulate (1)

One hundred mg of ferulic acid (0.5 mmol) were dissolved in 4 mL of butanol and 150 mg of Dowex 50 X8 (previously activated) was added. The reaction was left under reflux overnight. The solution was filtered and dried under vacuum. Then it was purified by column chromatography in petrolether:ethyl acetate (9:1, v/v) to obtain the desired product as a transparent oil in 86% yield.

2.2.2. Octyl ferulate (5)

Ferulic acid (202 mg, 1 mmol) was dissolved in anhydrous pyridine (3 mL). Acetic anhydride (0.5 mL) was added, and the mixture was stirred at room temperature overnight. The solution was evaporated after addition of toluene and dried to obtain a white solid (*p*-acetoxy-ferulic acid 2) that was converted without any further purification.

4-*O*-Acetyl ferulic acid (1 mmol) was dissolved in toluene (5 mL) and 0.35 mL of thionyl dichloride were added. The mixture was heated to reflux for 30 min then the solution was evaporated to obtain a white solid (4-*O*-acetoxy-feruloyl chloride 3) that was used without further purification.

To a solution of 4-*O*-acetoxy-feruloyl chloride 3 (1 mmol) in 5 mL of chloroform, 1-octanol was added, and the reaction was left under shaking at room temperature. Water was added and organic phase extracted twice. The desired 4-*O*-acetoxy-octyl ferulate (4) was isolated by column chromatography to obtain 243 mg of desired product (70% yield over three steps).

A solution of compound 4 was dissolved in 95% ethanol, piperidine was added (5 mL). The reaction was monitored by TLC analysis (ethyl acetate:methanol, 9:1; v/v) and when conversion was complete it was partially dried under vacuum. The crude was neutralized with aqueous acetic acid and extracted twice with water and ethyl acetate (1:1; v/v) to obtain pure desired product in quantitative yield.

The quality control of butyl and octyl ferulates was carried out through thin layer chromatography (TLC), nuclear magnetic resonance (NMR; Figures S1–S4 of Supplementary Material) and gas chromatography coupled with mass spectrometry (GC/MS) (see details in the Supplementary Material).

2.3. Evaluation of the antioxidant properties of butyl (BF) and octyl ferulates (OF)

The antioxidant properties of BF and OF were assessed through *in vitro* assays in terms of radical scavenging activity (DPPH*) and iron chelating activity. For both assays four different concentrations of BF and OF were prepared in ethanol (50, 100, 200, and 400 µmol/L, final concentrations) according to Sørensen et al. (2014) and tested in triplicate (*n* = 3).

2.3.1. DPPH[•] radical scavenging assay

Radical scavenging activity was evaluated according to Sørensen et al. (2014) with slight modifications. Briefly, 100 μ L of each concentration of the ferulate alkyl esters were added in a 96-well microplate and immediately added with 100 μ L of a DPPH[•] ethanolic solution (0.1 mmol/L). After 30 min of incubation in darkness at 23 °C, the absorbance was read at 517 nm in a BioTek Synergy HT spectrophotometric multi-detection 96-well microplate reader (BioTek Instruments, Milan, Italy) against a negative control in which the sample was replaced by ethanol. BHT was used as positive control at the same concentrations of BF and OF. Results were expressed as percentage of inhibition (%I) according to the following equation:

$$\%I = \left(\frac{Abs_{blk} - Abs_{sample}}{Abs_{blk}} \right) \times 100 \quad [1]$$

where Abs_{blk} is the absorbance of the negative control and Abs_{sample} is the absorbance of the tested sample at 517 nm after 30 min.

2.3.2. Iron chelating assay

The ability to chelate ferrous ions was determined according to (Yen & Chung, 1999), modified as detailed in (Cantele et al., 2020). EDTA (same concentrations) served as the positive control. Results were expressed as percentage inhibition (%I).

2.4. Stripping of the oil

Polar compounds were removed from 30 g of oil (a mixture of soybean and sunflower oils) according to (Boon et al., 2008) in a chromatographic column (diameter 3 cm; length 35 mm) packed with silicic and activated charcoal. While stripping, collected triacylglycerols were held in an ice bath and protected from light. Then the solvent was removed with a rotary evaporator (Rotavapor, R-210, Buchi, Flawil, Switzerland). Quality control of stripping step was conducted using gas chromatography with flame ionization detection (GC/FID) (Cardenia et al., 2018) (details in Fig. S9 of Supplementary Material) and peroxide value determination (Shantha & Decker, 1994).

2.5. Preparation of the oil-in-water (O/W) emulsions and Experimental conditions

Oil-in-water (O/W) emulsions were prepared as described by Cardenia, Waraho, Rodríguez-Estrada, McClements, and Decker (2011), with stripped oil (1.0% of the total weight of the emulsion; w/w), Tween 20 as non-ionic emulsifier (0.1% of the total weight of the emulsion; w/w), and 10 mmol/L phosphate buffer solution (pH 7.0 and 3.5). After removing the solvent, BF and OF were dissolved into the stripped oil at different concentrations (5, 10, 15, 20, 25 mg/L of emulsion) by stirring. Tween 20 and buffer solution were then added, mixed with an IKA T25 digital Ultra-Turrax® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 2 min at maximum speed (20,000 rpm) to obtain a coarse emulsion, and then homogenized by ultrasounds sonication for 3 min at 100 W to obtain a fine emulsion. During the sonication process, emulsions were held in an ice bath, to maintain the temperature ≤ 30 °C. Control samples (without lipophenols) were prepared for each experiment. Subsequently, 1 mL of each emulsion was transferred into 20 mL headspace vials secured with aluminum caps with PTFE/silicone septa and stored in darkness at 35 °C for 14 days. Three independent experiments ($n = 3$) were carried out for each sample and each analysis.

2.6. Evaluation of the physical and oxidative stability of the emulsions

2.6.1. Particle size distribution and zeta-potential measurement

The emulsions were diluted in the corresponding 10 mmol/L buffer solution (pH 7.0 and 3.5) used for their preparation at a ratio of 1:50 (v/v), and then particle size distribution and droplet surface charge (zeta-

potential) were measured through dynamic light scattering (DLS) with a Zetasizer Pro (Malvern Instruments, Worcestershire, UK). The measurements were repeated three times for each independent experiment.

2.6.2. Primary oxidation products

Lipid hydroperoxides were measured in the emulsions as primary oxidation products as reported by Cardenia et al. (2011). Briefly, aliquots of emulsions were dispersed in iso-octane:propan-2-ol and the supernatant was then dissolved in methanol:butanol (2:1; v/v) with 15 μ L of ammonium thiocyanate and 15 μ L of FeCl₂. Absorbance was read at 510 nm and quantification was performed based on a cumene hydroperoxide standard calibration curve in the range of 10–300 μ mol/L ($y = 2.6872x + 0.0135$; $R^2 = 0.9973$). Results were expressed as millimoles of hydroperoxides per kilograms of oil (mmol/kg oil).

2.6.3. Secondary oxidation products

Headspace solid-phase microextraction gas chromatography coupled with mass spectrometry (HS-SPME-GC/MS) (QP-2010 Plus GC/MS, Shimadzu, Kyoto, Japan) was used to determine hexanal and nonanal content as markers of the secondary oxidation reactions. Vials were heated at 40 °C for 10 min, and a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (df 50/30 μ m; 1 cm; Supelco, Bellefonte, PA, USA) coated fiber was exposed in the headspace for 5 min at the same temperature. The fiber was then desorbed in the inlet of the GC at 260 °C in split mode (1:50) and using helium as carrier gas (34.8 m/s linear velocity). The analytes were resolved in a RTX-5 fused silica capillary column (20 m $\times$ 0.10 mm, film thickness 0.10 μ m; Restek, Bellefonte, PA) with the following oven temperature program: from 40 °C (held for 1 min) to 100 °C with a constant rate of 5 °C/min; then to 240 °C at 30 °C/min and held for 1 min. Transfer line, and ion source temperature were set at 230 °C, and 200 °C, respectively. Ions were acquired in scan mode in a 33–350 m/z range with a scan velocity of 1666 amu/s. Hexanal and nonanal were identified through the mass spectra contained in the NIST08s library and by injection of the pure standards under the same analytical conditions. The quantification was achieved by external standard method (hexanal, 0.212–750 mmol/kg oil, $y = 43.98x + 5100$; nonanal, 0.146–350 mmol/kg oil, $y = 49.67x + 3215$). Results were expressed as μ mol/kg oil.

2.7. Statistical analysis

The results of all data were reported as means and standard deviation of three independent experiments ($n = 3$). Statistical differences were ascertained by analyzing the results with one-way ANOVA combined with Tukey's post hoc test using IBM SPSS statistical software (version 27; IBM, Chicago, IL, USA). Normality and homoscedasticity were checked before performing the ANOVA test with, respectively, Shapiro-Wilk and Levene's tests with the same software. Differences with a p -value < 0.05 were considered significant. Lag phases of hydroperoxides, hexanal, and nonanal formation were determined as the first data point significantly different (p -value < 0.05) from day 0 using one-tailed Dunnett's post hoc test.

3. Results

3.1. Synthesis and characterization of butyl and octyl ferulates

The synthesis of butyl and octyl esters was attempted by esterification of ferulic acid and, because of the different reactivity, availability, and physical state of butanol and octanol, two different synthetic routes were pursued (Fig. 1).

Butyl ester **1** was obtained by direct acid catalyzed Fischer esterification in presence of solid supported sulfonic acid, while a four steps synthetic scheme was followed for the synthesis of octyl ester **5**. A mild procedure of esterification with butanol in presence of Dowex H⁺ cation-exchange resin was exploited to efficiently obtain butyl ferulate in

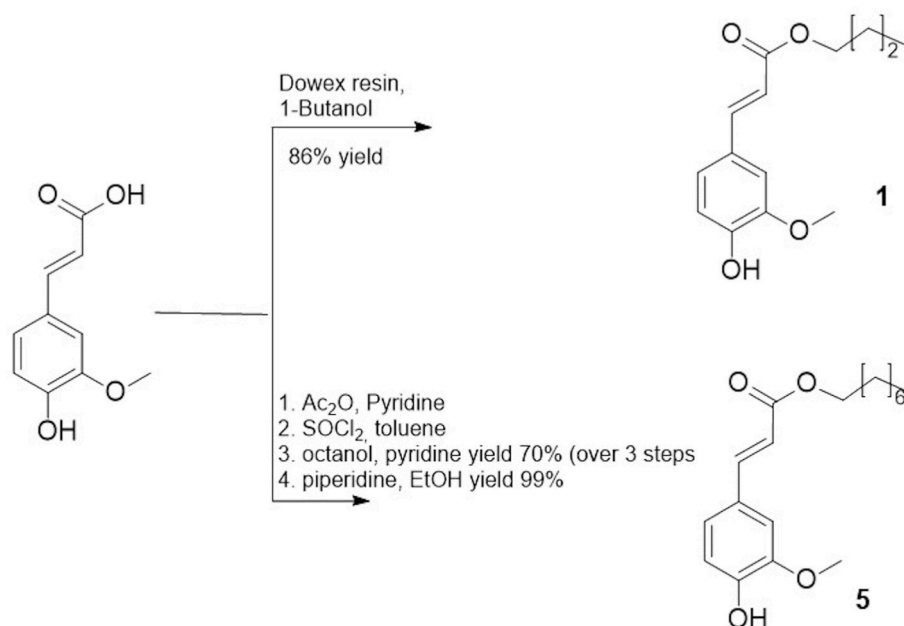


Fig. 1. Schematic representation of ferulate esters 1 and 5 syntheses.

excellent yield (Turhanen, Leppänen, & Vepsäläinen, 2019). When synthesis of octyl ester was approached the previous scheme resulted inefficient and the protection of the phenolic moiety was performed to obtain the ferulic acyl chloride that was further reacted with octanol and deprotected with piperidine in ethanol with an overall yield of 70% (Panda et al., 2012). The purity of synthesized products was fully characterized by TLC, GC/MS and NMR and were delivered to further study only when purity was higher than 99%.

3.2. Antioxidant properties

Both BF and OF did not display iron chelating activity, while anti-radical activity was observed (Fig. 2).

For each tested compound the scavenging activity was affected by the concentration applied. BHT showed significant increase in its activity only at 400 µmol/L ($p < 0.001$), while lipophilized compounds

showed earlier effects at 100 µmol/L ($p < 0.001$) compared to BHT. At 200 µmol/L BF and OF already neutralized 50% of DPPH, while at 400 µmol/L BHT barely reached 35% inhibition. BF displayed slightly superior scavenging efficiency compared to OF. Interestingly, as the concentration increased the gap between the two narrowed; the OF showed a lower activity of 30%, 20%, 15%, and finally 10% at 50 µmol/L, 100 µmol/L, 200 µmol/L, and 400 µmol/L, respectively.

3.3. Effects of BF and of on the physical stability of the O/W emulsions

Fig. 3 shows the particle size distribution of the emulsions with BF and OF at pH 7.0 and 3.5 over 14 days of storage.

In general, particle size ranged 194 – 242 nm at the beginning of the experiments. BF and OF had similar effects on emulsion droplet size within each environmental condition. At pH 3.5, BF- and OF-enriched emulsions showed no significant differences compared to the control at any concentration on any tested day ($p > 0.05$) (Fig. 3b and 3d). Contrarily, at pH 7 the presence of lipophenols led to a decrease in particle size on the last day (Fig. 3a and 3c). However, while the addition of more than 5 mg/L of BF did not lead to a progressive decrease in droplets dimension (Fig. 3a), in OF particle size decreased in relation to its concentration. In fact, particle size was 272.35 ± 3.18 nm in the control, while with 25 mg/L OF resulted in 203.40 ± 6.22 nm (Fig. 3c). However, all emulsions, including controls, remained physically stable without significant changes in particle size over time in all four experiments ($p > 0.05$).

The droplet surface charge under both acidic and neutral conditions is reported in Fig. 4.

Emulsions with BF and OF were characterized by negative values of zeta-potential at both pH values since day 0. At pH 7.0 (Fig. 4a and c), the negative charge drastically increased as the days progressed ($p < 0.001$). The control displayed significantly higher zeta-potential compared to BF and OF emulsions ($p < 0.001$), as their presence reduced the negative charge as related to their concentration. In fact, the control ranged from -10.22 ± 2.31 mV (day 0) to -54.41 ± 1.02 mV (day 14), while BF and OF at 25 mg/L reached -35.48 ± 2.14 mV and -40.95 ± 0.06 mV, respectively, on the last day. At pH 3.5 (Fig. 4b and d) a similar behavior was found, even if the values resulted less negative. At day 14 the control reached a value of -5.85 ± 0.71 mV, whereas the addition of BF reduced the negative charge (ranged from -1.53 ± 0.01

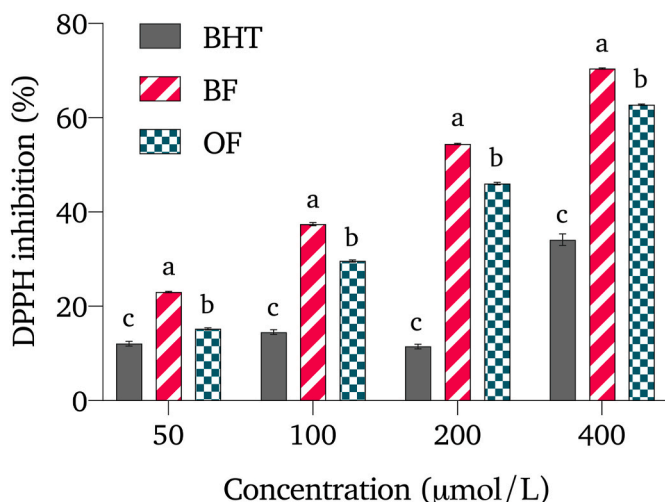


Fig. 2. Results of DPPH radical scavenging activity of different concentrations (50, 100, 200, and 400 µmol/L) of BF and OF and of positive control (BHT). Each bar represents the mean $\pm$ standard deviation of three independent replicates ($n = 3$). Different letters within the same concentration indicate means significantly different at $p < 0.05$.

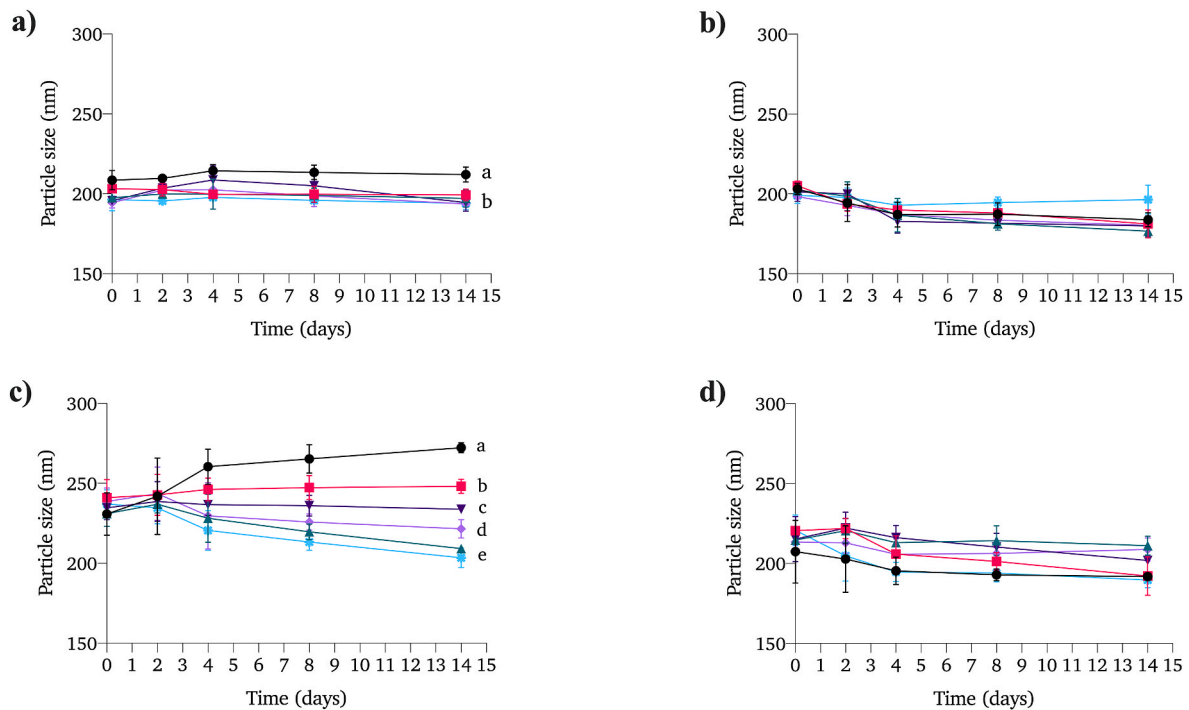


Fig. 3. Particle size distribution (nm) of 1% O/W emulsions without (control) (●) and with butyl ferulate (BF) and octyl ferulate (OF) at 5 mg/L (■), 10 mg/L (▲), 15 mg/L (▼), 20 mg/L (◆), and 25 mg/L (*) throughout the 14 days of storage. **a)** BF at pH 7.0; **b)** BF at pH 3.5; **c)** OF at pH 7.0; **d)** OF at pH 3.5. Each data point represents the mean $\pm$ standard deviation of three independent replicates ($n = 3$). Some error bars lie within the data points. Different letters indicate means significantly different within the day at $p < 0.05$.

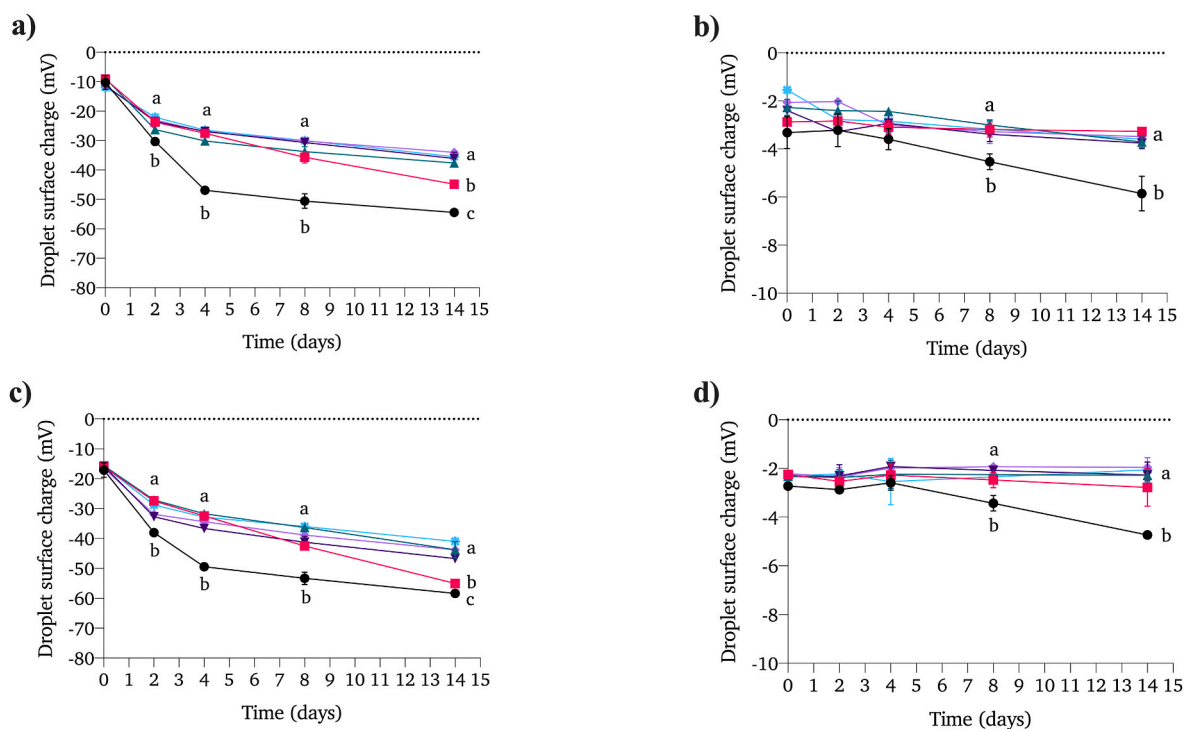


Fig. 4. Droplet surface charge (mV) of 1% O/W emulsions without (control) (●) and with butyl ferulate (BF) and octyl ferulate (OF) at 5 mg/L (■), 10 mg/L (▲), 15 mg/L (▼), 20 mg/L (◆), and 25 mg/L (*) throughout the 14 days of storage. **a)** BF at pH 7.0; **b)** BF at pH 3.5; **c)** OF at pH 7.0; **d)** OF at pH 3.5. Each data point represents the mean $\pm$ standard deviation of three independent replicates ($n = 3$). Some error bars lie within the data points. Different letters indicate means significantly different within the day at $p < 0.05$.

mV to -3.58 ± 0.15 mV). On the other hand, the OF restrained the drop and a steady trend was observed for all concentrations tested ($p > 0.05$).

3.4. Effects of BF and of on the oxidative stability of the O/W emulsions

Increasing the concentrations of lipophenols the lipid hydroperoxides content in emulsions significantly ($p < 0.001$) decreased (Fig. 5). The controls at both pH values showed a significant increase in hydroperoxides already after 4.5h. In contrast, the lag phase was drastically increased when BF and OF were added and, again, this antioxidant activity was related with their concentration. In fact, with the lowest concentration (5 mg/L) of both BF and OF a 5-fold increase in the lag phase of the emulsions at pH 7 was achieved, and 10 days of lag phase were reached with BF at 10 mg/L and 15 mg/L and with OF at 10 mg/L, reaching 14 days with the remaining samples (Fig. 5a and c). In terms of the maximum concentration observed, the acidic environment favored better oxidative stability than the neutral one, and this concerned all the samples, including the controls, with values up to 282.10 ± 17.30 mmol/kg oil and 500.00 ± 47.29 mmol/kg oil at pH 7.0 in BF- and OF-experiments (Fig. 5a and c), respectively, and 178.89 ± 23.34 mmol/kg oil and 246.68 ± 25.85 mmol/kg oil at pH 3.5 (Fig. 5b and d). In general, despite the more successful results obtained under acidic conditions, considerably lower levels of hydroperoxides accumulation were found in all the samples added with both lipophenols with respect to the controls (more than 100 times lower).

Similar results were found also for hexanal and nonanal (Figs. 6 and 7). The control showed their presence at both pH values from the very beginning of the experiment, and their content rose steeply after only 1 day. On the contrary, when lipophenols were added to the emulsions, the formation of the two aldehydes was significantly delayed in relation to the concentration of the two antioxidants, even to the point of being impeded at the highest concentrations of BF and OF. At pH 7.0, the addition of both BF and OF at 5 mg/L was already able to slow down the decomposition of the hydroperoxides, with the hexanal concentration rising steeply only from day 4 and day 10 for OF and BF, respectively

(Fig. 6a and c). Nonanal behaved similarly, but with even longer delays in its formation. The greatest inhibition was attained by the two highest concentrations of BF and OF (20 and 25 mg/L), where hexanal and nonanal were found to be extremely low and, in some cases, not present at all for the entire experiment (Fig. 7a and c). The acidic environment again promoted greater oxidative stability in all the samples including the controls (Fig. 6b and d, Fig. 7b and d), where less than half of the values observed in the experiments conducted at pH 7.0 were found. When lipophenols were added to the emulsions, at pH 3.5 only the 5 mg/L concentrations displayed a slight increase in hexanal and nonanal contents, but with values that were still markedly lower than in the neutral environment.

4. Discussion

The aim of this work was to investigate whether the use of two alkyl ferulates could be a worthwhile solution to enhance the oxidative stability of oil-in-water (O/W) emulsions, exploiting the amphipathic nature of butyl ferulate (BF) and octyl ferulate (OF) that would allow their partitioning at the oil-water interface. Moreover, being surface active, they could also affect the chemical and physical properties of the interfacial region, altering the electrostatic repulsion forces between the droplets if they possess a charge, and lowering their interfacial tension, thus impacting the physical stability of the emulsion among other things (Homma, Suzuki, Cui, McClements, & Decker, 2015; McClements & Decker, 2018).

Ultrasonics-assisted sonication successfully resulted in emulsions with a sufficiently small droplet size. Small particles in emulsions are of great scientific interest because significantly more kinetically stable over time, and the bioactive molecules contained in the system are more soluble and with better controlled/prolonged release, digestibility, and functionality in the upper gastrointestinal tract (Amiri-Rigi, Kesavan Pillai, & Naushad Emmambux, 2023). During the experiment, no physical destabilization was observed in any emulsion at both pH evaluated, meaning that all the samples were stable against coalescence,

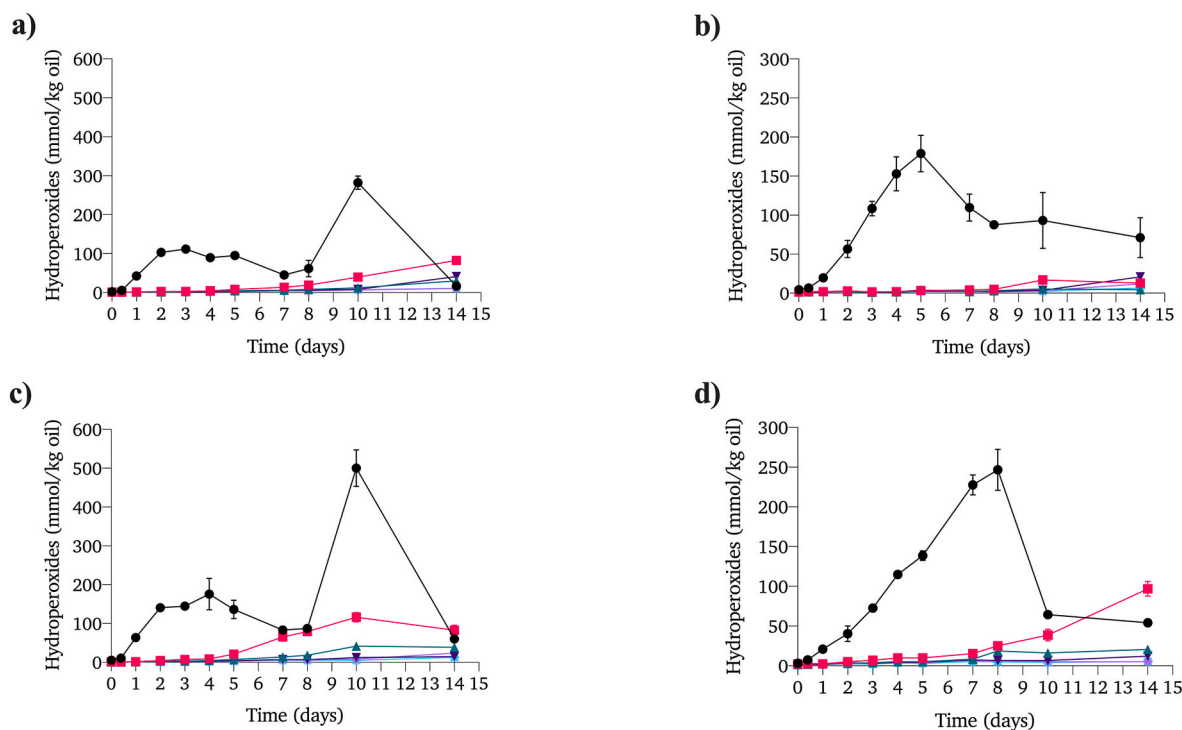


Fig. 5. Hydroperoxides content (mmol/kg oil) of 1% O/W emulsions without (control) (●) and with butyl ferulate (BF) and octyl ferulate (OF) at 5 mg/L (■), 10 mg/L (▲), 15 mg/L (▼), 20 mg/L (◆), and 25 mg/L (☆) throughout the 14 days of storage. a) BF at pH 7.0; b) BF at pH 3.5; c) OF at pH 7.0; d) OF at pH 3.5. Each data point represents the mean $\pm$ standard deviation of three independent replicates ($n = 3$). Some error bars lie within the data points.

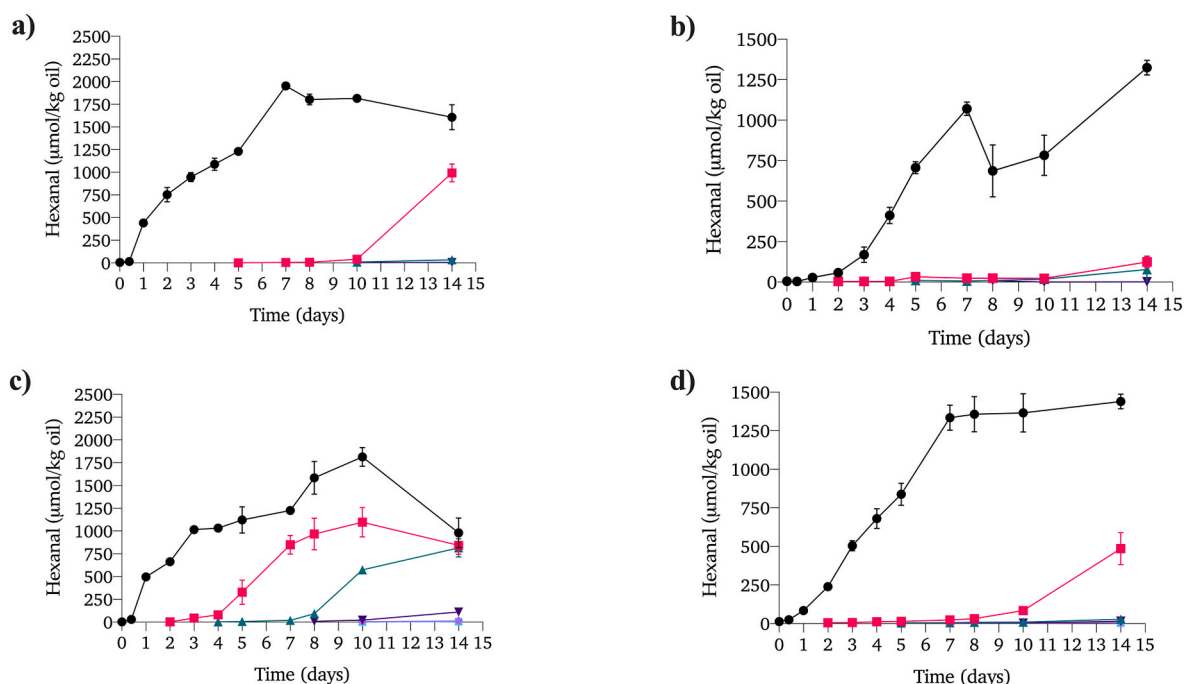


Fig. 6. Hexanal content ($\mu\text{mol/kg oil}$) of 1% O/W emulsions without (control) (●) and with butyl ferulate (BF) and octyl ferulate (OF) at 5 mg/L (■), 10 mg/L (▲), 15 mg/L (▼), 20 mg/L (◆), and 25 mg/L (☆) throughout the 14 days of storage. **a)** BF at pH 7.0; **b)** BF at pH 3.5; **c)** OF at pH 7.0; **d)** OF at pH 3.5. Each data point represents the mean $\pm$ standard deviation of three independent replicates ($n = 3$). Some error bars lie within the data points.

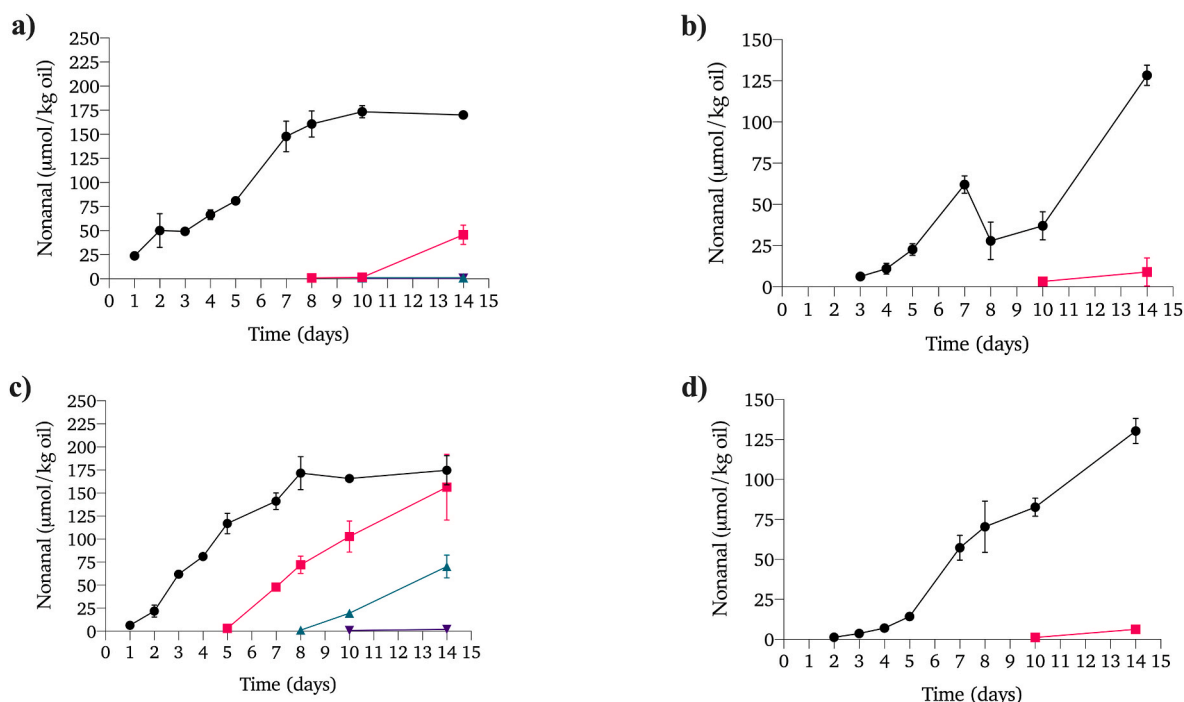


Fig. 7. Nonanal content ($\mu\text{mol/kg oil}$) of 1% O/W emulsions without (control) (●) and with butyl ferulate (BF) and octyl ferulate (OF) at 5 mg/L (■), 10 mg/L (▲), 15 mg/L (▼), 20 mg/L (◆), and 25 mg/L (☆) throughout the 14 days of storage. **a)** BF at pH 7.0; **b)** BF at pH 3.5; **c)** OF at pH 7.0; **d)** OF at pH 3.5. Each data point represents the mean $\pm$ standard deviation of three independent replicates ($n = 3$). Some error bars lie within the data points.

flocculation, or creaming. However, it is interesting to observe how differences were never noted within each day by increasing the concentration of both lipophenols, except for the last day of both BF and OF experiments under neutral conditions, where larger particles were observed as the percentage of lipophenols decreased. McClements and Decker (2018) explained how certain amphiphilic antioxidants with an

appreciable amount of nonpolar groups, that allow direct adsorption at the oil–water interface due to the hydrophobic effect, can act as co-emulsifiers, partially replacing the original emulsifier and thus improving the physical stability of emulsions. However, a precondition for this to occur is that the antioxidant is present in the emulsion in a sufficient concentration relative to the emulsifier. In the present study, it

is plausible that the lipophenols located themselves in the interfacial region and filled the available gaps between the polysorbate molecules or placed immediately behind them, helping to lower, if only slightly, the interfacial tension of the droplets and thus to maintain greater stability over time when their concentration is > 0.5 % relative to the emulsifier. Indeed, in the absence of the lipophenols an increasing trend in size over time was quite clear and became statistically different from the other samples at day 14 in both BF- and OF- experiments. In addition, Sørensen, Nielsen, Yang, Xu, and Jacobsen (2012) reported that Tween 20 favors the solubility of some phenolic compounds in the lipid phase of emulsions, contributing to determine their different partitioning into the system. On the other hand, at pH 3.5 no significant differences between samples and no increasing trends were ever noted, and therefore it is assumed that both lipophenols did not affect the interfacial tension of the droplets under acidic conditions. Further studies are necessary to understand why lipophenols affect surface tension depending on pH.

On the other hand, small particle sizes can worsen the oxidative stability by increasing the surface area of the oil droplets exposed to prooxidants-containing water (Jacobsen et al., 2000). Additionally, oxidized lipid species such as hydroperoxides, once are formed, being more polar due to the presence of the oxygen molecule, tend to migrate at the interface, where will encounter a prooxidant agent, causing it to decompose (Laguerre et al., 2017). This decay will produce highly reactive peroxy and alkoxy radicals, which will then spread the oxidation reactions by snatching an electron from a nearby unsaturated lipid. In other words, lipid oxidation in O/W emulsions is not only a matter of chemical reactions, but also of the physical arrangement of the molecules in the overall system and of the speed at which they move (Laguerre et al., 2017). In our study, however, smaller droplets size showed greater oxidative stability. In fact, regarding the emulsions at pH 7.0, when added with both BF and OF, even if they showed smaller particle size compared to the control, they simultaneously exhibited an unequivocally greater oxidative stability. This could be explained by the ability of these two antioxidants to properly partition at the site of oxidation reactions, which likely occurred at both pH values. Regarding the concentrations, 5 mg/L of BF and OF was already able to increase the oxidative stability of the system, especially at low pH value. In fact, the elongation of the hydroperoxides lag phase was remarkable for both lipophenols in all four experiments compared with the controls, and this would represent an advantage for the food industry, as a very small amount of these antioxidants would suffice to greatly improve the shelf life of emulsion-based food products. Since secondary oxidation products are formed from primary products, it is not surprising to observe a similar behavior in terms of oxidative stability for hexanal and nonanal, the formation of which coincided with the lag phase of the hydroperoxides. In other words, in the control and in all emulsions at pH 7.0, an increased presence of both the aldehydes was observed, reflecting the higher propagation of the hydroperoxides. Contrarily, where lower levels of hydroperoxides were found, lower levels of aldehydes were also detected. Our findings are in line with the actual literature, which shows that other lipophilized phenolic compounds also performed satisfactorily in terms of antioxidant activity in emulsified systems. Methyl and butyl ferulate were found to be highly efficient in fish oil enriched milk; on the other hand, octyl ferulate acted as prooxidant in the same system (Sørensen et al., 2015). This highlights how different systems lead to obtaining different results, thus requiring the evaluation of antioxidants under different conditions. Lipophilization of dihydrocaffeic acid led to excellent results in fish oil-in-water emulsions (Sørensen, Nielsen, et al., 2012). Panya et al. (2012) found that C4 and C8 rosmarinic acid alkyl esters were the most effective in inhibiting the formation of hydroperoxides and hexanal. Finally, lipophilized gallic acid and hydroxytyrosol were able to significantly improve the oxidative stability of olive oil-in-water emulsions (Almeida et al., 2016; Losada Barreiro, Bravo-Díaz, Paiva-Martins, & Romsted, 2013). However, BF and OF proved to be more potent than other lipophilized antioxidants reported in literature and compared to the antioxidants normally used by the industries. In

fact, the hydroperoxide values found in emulsions with BF and OF were lower than in O/W emulsions prepared with erythorbyl fatty acid esters, derivatives of lipophilized ascorbic acid (Kim, Yu, Yang, Choi, & Chang, 2023). Huang et al. (1996) found that emulsions added with 1.16 mmol/L α -tocopherol reached in 4 days a hydroperoxides content of ~100 mmol/kg oil at pH 3, and ~150 mmol/kg oil at pH 7. Similar results were also found with 1.16 mmol/L Trolox-added emulsions, with ~30 mmol/kg oil at pH 3 and > 150 mmol/kg oil at pH 7. In the present study, BF and OF at 5 mg/L never exceeded 5 mmol/kg oil and 10 mmol/kg oil, respectively, at both pH values until day 4. Again, hydroperoxides found in the present work were also lower compared to O/W emulsions added with similar concentrations of α - and δ -tocopherol and BHT stored at 37 °C (Chaiyasit, McClements, & Decker, 2005). The strength of BF and OF, though, lies in their exceptional antioxidant efficacy in this type of system. In fact, the concentrations used in this study were in the range of 0.02 mmol/L – 0.1 mmol/L (on the total emulsion volume) and, considering that the typical concentration of an antioxidant is 0.2 mmol/L (McClements & Decker, 2000), BF and OF could be of great interest to the food industry. Indeed, since 20 mg/L of BF and OF (corresponding to 0.08 mmol/L) already drastically prevented the formation of hydroperoxides and volatile compounds, they would enable industries to tremendously reduce the amount of antioxidant to be added to food.

The *in vitro* antioxidant assays revealed that the great oxidative stability given by the presence of BF and OF is due to their ability to neutralize free radicals rather than their ability to chelate transition metals. This is actually not surprising, as it is well known that the ability to chelate iron is given by the presence of a catechol or galloyl group able to form iron-phenolic complexes (Sørensen et al., 2014), which are not present in the molecular structure of ferulic acid. In contrast, it has a hydroxyl group capable of yielding an electron to a free radical. It is therefore plausible that, by positioning themselves at or close to the interface, BF and OF acted as scavengers of the newly formed radicals and prevented the formation of hydroperoxides. BF proved to be slightly more effective than OF in scavenging the DPPH $\cdot$. Sørensen et al. (2014) noted that C4-esterified ferulic acid had the same ability to neutralize DPPH $\cdot$ as C8-esterified ferulic acid, but a slightly higher reducing power. Similar results were found with quercetin, which displayed the highest antiradical activity when esterified with short-chain fatty acids (Oh, Ambigaipalan, & Shahidi, 2019), while in other studies different outcomes were achieved, reporting equal or lower activity than octyl ferulate (Oh & Shahidi, 2017; Viskupicova, Danihelova, Ondrejovic, Liptaj, & Sturdik, 2010). More in-depth analyses are needed to understand why BF is more powerful than OF in this essay. We hypothesize that it is a matter of a greater spatial impediment of OF compared to BF that leads to the lower effectiveness against the radical. In fact, side chains on the aromatic ring of a phenolic compound slow down the reaction with DPPH due to steric hindrance that makes phenol access to the DPPH radical site difficult (Schaich, Tian, & Xie, 2015).

The high antioxidant efficiency of both esters was confirmed by the changes in the droplet surface charge over time. As oxidation proceeded, compounds generated by lipid degradation with pK_a below the operating pH reached the interface, making the surface more negative. Therefore, in the control, where numerous oxidation products were produced, the zeta-potential became drastically more negative as the storage progressed. In contrast, lipophenols led to a steadier surface charge trend over time, probably due to their capability of hampering the oxidation reactions. Additionally, despite being non-ionic, Tween 20 still exhibited a negative zeta-potential, and this can probably be explained by the presence of free fatty acid impurities in Tween 20 itself (Waraho, Cardenia, Rodriguez-Estrada, McClements, & Decker, 2009). The higher negative charge of the droplets at pH 7.0 compared to those at pH 3.5 explains the lower oxidative stability under neutral conditions. Low pH values, despite making iron more soluble in water, promote protonation of the particles, thus repelling the transition metals with the same charge (Li et al., 2023; McClements & Decker, 2000). Conversely, a

strong negative charge attracts cations, which can then trigger oxidation reactions. Thus, once again BF and OF prove to be of particular interest to the food industry, since the charge of the droplets partly controls the rate of lipid oxidation in O/W emulsions, as has been widely demonstrated (McClements & Decker, 2000). In this study, pH played a role in the antioxidant performances of the two alkylferulates. It has already been observed that acidic conditions somewhat slow down oxidation reactions, reducing the formation of hydroperoxides and related volatile compounds. For instance, α -tocopherol, myricetin, and taxifolin have been reported to be more effective in O/W emulsions at pH 3.0 and 4.0 than at pH 7.0 (Bayram et al., 2023; Huang et al., 1996). Besides the issue of the droplet charge, another explanation could be related to antioxidants' redox potential, which varies according to the pH of the system (Jovanovic et al., 1996). The antiradical activity is consequently reliant on the pH of the surrounding environment since the capacity to quench free radicals by transferring an electron or a hydrogen atom depends on the redox potential of the molecule. Furthermore, two other hypotheses include the different partitioning of BF and OF in the system and degradation rate. When pH is lowered, antioxidants can move in the system getting closer to the oxidation sites, with a consequent slower degradation (Huang et al., 1996). Other studies are needed to investigate these hypotheses.

5. Conclusions

Physically and oxidatively stable O/W emulsions can be accomplished by using ferulic acid lipophilized with 4 and 8 carbon atoms alkyl chains in both neutral and acidic conditions. Oxidative stability was promoted more at pH 3.5 than at pH 7.0 when the lipophenols were added to the emulsions, significantly limiting the formation of hydroperoxides and their decomposition into hexanal and nonanal. Although the antioxidant capacity was related to the concentration of BF and OF, even their lowest levels led to a significant increase in the emulsions' resistance to oxidation. Their antioxidant activity proved to be due to their ability to scavenge free radicals, while they were unable to chelate ferrous ions. These results suggest that BF and OF could be used, even in small concentrations, as efficient antioxidants in the formulation of emulsion-based foods to successfully preserve bioactive lipids such as polyunsaturated fatty acids and thereby decreasing the presence and intake of harmful compounds originating from oxidation reactions. However, further research is needed to investigate their behavior in real food systems, which are more complex matrices that could influence their antioxidant activity.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Carolina Cantele: Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Katia Martina:** Validation, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Giulia Potenziani:** Formal analysis, Investigation. **Andrea Mario Rossi:** Writing – review & editing. **Vladimiro Cardenia:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration. **Marta Bertolino:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2023.115505>.

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