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Contribution of the Interfacial Layer to the Protection of Emulsified Lipids against Oxidation

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ABSTRACT: The oxidative stability of oil-in-water (O/W) emulsions is highly dependent on the type of emulsifier. The purpose of this work was to investigate the specific role of the adsorbed emulsifiers on lipid oxidation of O/W emulsions. Emulsions of similar droplet size distribution stabilized by minimum amounts of proteins or surfactants were oxidized at 25 °C in the presence of equimolar iron-EDTA complex. The pH and the amount of emulsifier in the aqueous phase were also varied to investigate the role of the droplet charge and the emulsifier in the aqueous phase. Oxygen uptake, conjugated dienes (CD), and volatile compound formation demonstrated that the protein-stabilized interfaces are less efficient at protecting emulsified lipids against oxidation than surfactant-stabilized interfaces. The antioxidant effect of unadsorbed proteins was also confirmed.

KEYWORDS: Lipid oxidation, oil-in-water emulsions, interface, emulsifiers, proteins, surfactants, kinetic model

INTRODUCTION

Oxidation of polyunsaturated fatty acids (PUFA) is a major cause of deterioration of food quality. It is therefore of importance to have a deep understanding of the formulation factors that influence lipid oxidation kinetics in complex food matrixes.

In food products, lipids are often dispersed in an aqueous phase, which affects their oxidative stability.[1−5] It is generally admitted that the interfacial region, which is the contact region between the oil phase and the aqueous phase, represents a critical area in the system. Surface-active compounds (emulsifiers, oxidation products, amphiphilic antioxidants, etc.) that adsorb at the oil−water interface influence lipid oxidation.[2,3,5]

The charge of emulsifiers is currently considered as one of the main factors in the oxidative stability of emulsions because it governs attractive or repulsive forces between metal ions and interfaces. Accordingly, emulsions stabilized with positively charged surfactants were found to be more oxidatively stable than emulsions stabilized with negatively charged surfactants.[6,7] However, the charge effect in protein-stabilized emulsions still remains controversial. Protein-stabilized emulsions were more oxidatively stable at low pHs where proteins are positively charged than at higher pHs in several studies,[7−10] while the reverse effect or no relationships were noticed in other studies.[6,11−13]

As compared to surfactants, proteins have been generally found to better protect the oil phase against oxidation.[12,14−16] This was attributed to both their chelating and free radical scavenging properties as well as their ability to form thick layers at the interface.[5,8,10,17−21] However, considering the amount of emulsifier and the oil droplet surface that has to be covered by the emulsifier in the different studies, it should be concluded that they have been carried out in the presence of a large excess of emulsifier, an important proportion of which is unadsorbed. Indeed, unadsorbed protein or surfactant emulsifiers can affect the oxidative stability of emulsions by interacting with metal ions or by scavenging free-radicals in the aqueous phase.[10,20,22,23] In these experimental conditions, the measured effects of the emulsifiers on lipid oxidation resulted from a combined effect of both adsorbed and unadsorbed emulsifiers. Therefore, one should conclude that the effect of the interfacial layer on lipid oxidation in emulsions has not been clearly elucidated yet.

The aim of this study was to investigate the role of the composition of the interfacial layer on lipid oxidation in emulsions. For this purpose, we used optimized emulsions in which the amounts of emulsifiers were minimized to get physically stable emulsions with concentrations of unadsorbed emulsifiers as low as possible.[24] Chosen emulsifiers included proteins of different structures and interfacial conformations: bovine serum albumin (BSA), β-casein (BCN), and β-lactoglobulin (BLG), and both nonionic and anionic surfactants: Tween 20, Tween 80, and Citrem. The pH was varied for some emulsifiers above and under the isoelectric point of the proteins to investigate the effects of pH and protein charge. Lipid oxidation was measured by methods allowing the characterization of the different stages of the reaction: oxygen (O_2) consumption, formation of conjugated dienes (CD), and formation of selected volatile compounds.

MATERIALS AND METHODS

Materials. Rapeseed oil was purchased in a local supermarket. It was stripped by means of alumina (MP Alumina N-Super I, MP Biomedicals, France) to eliminate impurities and tocopherols.[24] Stripped oil contained less than 2 μg of residual tocopherols per g oil. Medium chain triglycerides (Miglyol) were obtained from International Flavors and Fragrances (Dijon, France). BSA (fraction V, minimum 96% by agarose gel electrophoresis) was obtained from MP Biomedicals (France). BCN (purity ≥98%) was purchased from Lactalis (France). β-Lactoglobulin (BLG) has been purified at the laboratory from Prolacta 90 (Lactalis) by selective precipitation.[25] The absence of contaminants was checked by sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis, gel filtration, and reverse phase high-performance liquid chromatography. Tween 20 (Sigma Ultra grade, average M_w = 1228 g mol^{-1}), Tween 80

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Table 1. Physical Characteristics of the Emulsions Stabilized with Different Emulsifiers[a]

emulsifier	pH	emulsifier initial concentration in aqueous solution (g L^{-1})	emulsifier concentration in aqueous phase of emulsion (g L^{-1}) at t_0	$[d_{3,2}]$ (μm) at t_0	$[d_{3,2}]$ (μm) at t = 48 h	zeta potential (mV) at t_0
BLG	6.7	5.0	1.52 ± 0.09[c]	1.5 ± 0.1	2.1 ± 0.1	−54.5 ± 0.4
BSA	6.7	4.0	1.17 ± 0.11[c]	1.8 ± 0.1	2.0 ± 0.2	−29.8 ± 1.3
BCN	6.7	5.0	0.45 ± 0.12[c]	1.7 ± 0.1	1.7 ± 0.1	−46.5 ± 1.9
Tween 20	6.7	5.0	0.47 ± 0.09[c]	1.4 ± 0.1	1.3 ± 0.1	−9.6 ± 0.8
Tween 80	6.7	5.0	1.68 ± 0.14[c]	1.7 ± 0.1	1.6 ± 0.1	−6.4 ± 0.1
Citrem	6.7	6.0[b]	0.012 ± 0.004[c]	2.2 ± 0.3	2.4 ± 0.1	−96.9 ± 3.3
BLG	3.0	5.0	2.10 ± 0.19	2.2 ± 0.1	2.7 ± 0.2	68.1 ± 1.2
Tween 20	3.0	5.0	0.56 ± 0.10	1.6 ± 0.2	1.6 ± 0.1	−3.8 ± 0.1
BLG	6.7	10.0	6.05 ± 0.10	1.5 ± 0.1	1.7 ± 0.1	nd
BCN	6.7	10.0	3.60 ± 0.10	1.9 ± 0.1	1.9 ± 0.1	nd
Tween 20	6.7	10.0	3.37 ± 0.17	1.6 ± 0.1	1.6 ± 0.1	nd

[a] Data represent the mean and standard deviation of three measurements on three emulsions prepared independently for $[d_{3,2}]$ and emulsifier concentration in the aqueous phase, and the mean and standard deviation of three measurements on one emulsion for the zeta potential. nd: not determined [b] Citrem was incorporated in the oil phase, with a concentration equivalent to 6 g L^{-1} of aqueous solution. [c] Data from Berton et al.[24]

(Sigma Ultra grade, average M_w = 1310 g mol^{-1}), 1,4-piperazinediethanesulfonic acid (PIPES), sodium carbonate (Na_2CO_3), potassium sodium tartrate ($C_4H_4KNaO_6$, $4H_2O$), copper(II) sulfate ($CuSO_4$), Folin−Ciocalteu's phenol reagent, ethylene diamine tetraacetic acid disodium calcium salt (EDTA), iron(II) sulfate ($FeSO_4$), propanal, hexanal, 2-octenal, 1-octen-3-ol, and 2,4-decadienal were purchased from Sigma Aldrich (France). A standard mixture of fatty acid methyl esters (FAME) (Sigma Aldrich, France) was used for the identification of fatty acids. Citric acid ester of fatty acids (Citrem) was obtained from Danisco (Grindsted, Denmark). NaCl and SDS were purchased from Fluka Chemika (France). Monoheptadecanoyl glycerol (purity ≥99.0%, M_w = 344.48 g mol^{-1}) was purchased from Nu-Chek-Prep (Elysian, MN). Sodium hydroxide (NaOH) was purchased from Merck (France). Sulphuric acid, orthophosphoric acid of analytical grade, acetone, methanol, hexane, pentane, and isopropanol of HPLC grade were purchased from Carlo Erba (France). The neutral buffer was composed of PIPES (10 mM) and NaCl (80 mM) and adjusted at pH 6.7. The acidic buffer was composed of orthophosphoric acid (10 mM) and NaCl (80 mM) and adjusted at pH 3.0.

Preparation and Physical Characterization of O/W Emulsions. The day before emulsion preparation, water-soluble emulsifiers (proteins or surfactants) were dispersed in adequate buffer (pH 6.7 or 3.0) and gently stirred overnight at 4 °C to ensure their complete solubilization without foam formation. Citrem, which was not soluble in water, was incorporated in the oil after being dissolved in dichloromethane. The solvent (70 μL g^{-1} oil) was evaporated under nitrogen flow at 35 °C for 1 h. The absence of traces of solvent was checked by solid-phase microextraction (SPME) coupled with gas chromatography (GC) with the parameters detailed below.

O/W emulsions were prepared with 30 g of oil and 70 g of aqueous solution per 100 g of emulsion. The two phases were premixed for 2 min at 15000 rpm using a rotor-stator homogenizer fitted with a 12-mm diameter head (Polytron PT 3000, Kinematica, Littau, Switzerland). The coarse emulsions were then homogenized through a one-stage low-pressure valve homogenizer (A0812W-A-CD, Stansted Fluid Power, Stansted, UK). The size distribution of oil droplets in the emulsions was measured immediately after homogenization with a laser light scattering instrument (Saturn 5200, Micromeritics, Verneuil en Halatte, France). It was daily checked to monitor emulsion stability and reported as volume-surface mean diameter ($[d_{3,2}]$). The emulsifier concentration, pressure, and time of emulsification were adjusted to produce stable emulsions with concentrations of unadsorbed emulsifiers as low as possible but a similar narrow droplet size distribution and average droplet size ($[d_{3,2}]$ comprised between 1.4 and 2.2 μm). Concentrations of emulsifiers in aqueous solutions were 4 g L^{-1} for BSA and 5 g L^{-1} for BCN, BLG, Tween 20, and Tween 80 (Table 1). Concentration of Citrem in oil was 12.9 g L^{-1}, which was equivalent to 6 g L^{-1} of aqueous solution. Nonionic surfactant-stabilized emulsions were homogenized for 5 min at 35 bar, while Citrem- and protein-stabilized emulsions were homogenized for 10 min at 50 bar.

Emulsions with excess emulsifier (BLG, BCN, or Tween 20) in the aqueous phase were prepared according to the following procedure: 30 g of oil and 60 g of aqueous solution were homogenized as previously described. The aqueous solutions contained 5.83 g L^{-1} emulsifier. After homogenization, 10 g of an emulsifier solution (35 g L^{-1}, BLG, BCN, or Tween 20, respectively) were added to the emulsions to obtain a final proportion of 30 g of oil per 100 g of emulsion. The addition of excess of emulsifier *post* emulsification avoided the formation of very small oil droplets during the homogenization stage.

Emulsion droplet charge was evaluated by the measurement of zeta potential (ξ, mV) with a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, UK). Emulsions were diluted 1000 times in salt-free buffers (pH 6.7 or 3.0) before measurements. The Smoluchowski model was applied. The refractive index of rapeseed oil was 1.473 with an absorption coefficient of 0.01. The parameters of the dispersant phase were those of water (viscosity, 0.8872 cP at 25 °C; refractive index, 1.330; and dielectric constant, 78.5).

The concentrations of unadsorbed emulsifiers in the aqueous phase at t_0 and after 48 h of incubation were measured as previously described.[24] Briefly, aliquots of emulsions were centrifuged to separate the aqueous phase from the oil droplets. The aqueous phase was collected and filtered through cellulose acetate 0.45, 0.20, and 0.10 μm-filters (Minisart High-Flow, Sartorius, Germany) to remove the small oil droplets. The amount of unadsorbed proteins was determined according to the method described by Markwell et al.[26] The amount of unadsorbed surfactants was determined by direct transesterification of the fatty acids of the surfactant molecules followed by GC analysis in the presence of monoheptadecanoyl glycerol.

Incubation of O/W Emulsions. The catalyst consisted of an equimolar mixture of $FeSO_4$ and EDTA. Solutions of $FeSO_4$ and EDTA (12 mM) were prepared separately in ultrapure water. Equivalent volumes of each solution were mixed, and the iron-EDTA complex was allowed to form under moderate magnetic stirring for at least 1 h. The mixture was then added to the emulsions to obtain a final concentration of 200 μM of each compound. Aliquots (3 mL) of emulsions were distributed in 20.5-mL headspace vials sealed with

Teflon/silicon septa and aluminum crimp caps. The vials were rotated in the dark at 25 °C and 5 rpm for 48 to 78 h, with a test tube rotator oriented at 30° versus the vertical position.

Measurements of Lipid Oxidation. *Oxygen Uptake.* Oxygen uptake was determined as described by Villière et al.[27] A sample (100 μL) of headspace was injected with a gastight syringe into the injector (split rate 30 mL min^{-1}, injector temperature 50 °C) connected to a fused silica plot column (Molsieve 5A CP7535, length 10 m, internal diameter 0.32 mm, Varian, Les Ulis, France). GC analysis was performed with a HP 5890 series II gas chromatograph (Hewlett-Packard, Böblingen, Germany) coupled to a thermal conductivity detector (TCD) in isothermal mode at 50 °C. TCD temperature was 125 °C. Helium was used as carrier gas with a flow of 2 mL min^{-1}. Peaks corresponding to oxygen were integrated with Borwin software (JMBS Développements, Fontaine, France) and normalized in comparison to the peak area of ambient air. The amount of residual oxygen in the headspace of the vials was calculated from the theoretical proportion of oxygen in air and the volume of headspace in the vials. Results were expressed in mmol of consumed oxygen per kg of oil (mmol O_2 kg^{-1} oil).

Conjugated Dienes. The formation of CD, which are primary oxidation products, was determined according to the method described by Lethuaut et al.[28] Aliquots of emulsions were diluted in isopropanol. The following solutions were centrifuged, and the absorbance of the supernatants was measured at 233 nm with a UV−visible spectrophotometer (Perkin-Elmer Lambda 12, Norwalk, CT, USA). The reference cell contained isopropanol and buffer in the same proportions as in the final dilution of the samples. Results were expressed in mmol of equivalent hydroperoxides per kg of oil (mmol eq HP kg^{-1} oil) with 27000 M^{-1} cm^{-1} as the molar extinction coefficient of CD at 233 nm.

Volatile Compounds. The formation of secondary oxidation products was evaluated by the quantification of two volatile compounds, namely, propanal and hexanal, in the headspace of the samples. These compounds were analyzed by GC paired with a flame ionization detector (FID) after their adsorption on a SPME fiber. The fiber (75 μm Carboxen, polydimethylsiloxane, Supelco, Bellefonte, PA, USA) was exposed in the headspace of the vials for 15 min at 25 °C. The fiber was then transferred into the injector (temperature 250 °C, equipped with a linear of 0.75 mm internal diameter) of a HP 5890 series II gas chromatograph. The purge of the injector was closed for the first 5 min to ensure the complete desorption of the volatile compounds and then opened for the following 15 min with a hydrogen split flow of 30 mL min^{-1}. Volatile compounds were separated on a ZB-624 fused silica capillary column (length, 30 m; internal diameter, 0.32 mm; film thickness, 1.80 μm; J&W Scientific, Chromoptic, Auxerre, France). Hydrogen was used as carrier gas with a flow of 2 mL min^{-1}. The temperature program was as follows: 3 min at 40 °C, heating 10 °C min^{-1} until 210 °C, heating 20 °C min^{-1} until 260 °C, and 4 min at 260 °C (26.5 min total). The FID temperature was set at 250 °C with hydrogen and air flows set to 25 and 250 mL min^{-1}, respectively. The fiber aging was checked weekly by performing an analysis with a standard test tube containing standard volatile compounds dissolved in Miglyol (propanal, pentane, hexanal, 2-octenal, 1-octen-3-ol, and 2,4-decadienal). Volatile compounds were identified by GC (HP 5890 series II gas chromatograph) coupled to mass spectroscopy (MS) (Trace DSQII ThermoFisher Scientific mass spectrometer) in conditions similar to those of the GC-FID analysis, except for helium as carrier gas. Mass spectra were obtained with 70 eV electron impact ionization and continuous scanning from m/z 35 to 350 at a scan speed of 2 scan s^{-1}. They were compared to spectra found in the NIST library. GC-MS identification of volatile compounds was performed on 3 different emulsions. Peaks obtained with GC-FID analysis were integrated with Borwin software. The peak areas were converted into amounts of volatiles compounds (μmol kg^{-1} oil) according to calibration curves. The external calibration was performed with Tween 20-stabilized O/W emulsions prepared with nonstripped rapeseed oil in which known amounts of standard compound solutions had been incorporated. Special attention was given to obtain proportions of the 18 main volatile compounds similar to those observed in oxidized emulsions and to comprise the highest and lowest amounts of volatiles. Calibration emulsions were distributed in vials, equilibrated at 25 °C for at least 30 min. Peak areas were obtained from SPME-GC analysis as previously described. The coefficient of variation (standard deviation divided by average value, %) was calculated for propanal and hexanal with 5 standard test tubes.

Experimental Design and Data Treatment. For each emulsifier or pH, at least two emulsions were prepared independently. Because of the duration of each analysis, oxygen uptake was measured in 2 vials, with three headspace samplings per vial; CD were measured in one vial, with three samplings per vial; volatile compounds were analyzed in one vial, with one sampling per vial. In the particular case of emulsions containing excess emulsifiers in the aqueous phase, only oxygen uptake and the formation of volatile compounds were measured.

The lag phases and rates of oxygen uptake and CD formation were calculated by adjustment by a modified-Gompertz model performed on the whole individual data, according to eq 1. After several tests, this model was found to be well adapted to adjust curves presenting a lag phase, an increasing phase, and a plateau.

$$Y = y_0 + A\exp\left(-\exp\left(1 + \frac{\mu\exp(1) \times (L - t)}{A}\right)\right) \quad (1)$$

where Y is the oxygen uptake (mmol O_2 kg^{-1} oil) or the CD formation (mmol eq HP kg^{-1} oil), y_0 is equal to zero for oxygen uptake or to the initial amount of CD in emulsions (mmol eq HP kg^{-1} oil), t is the time of incubation (h), A is the threshold value of oxygen uptake (mmol O_2 kg^{-1} oil) or CD formation (mmol eq HP kg^{-1} oil), μ is the rate of oxygen uptake (mmol O_2 kg^{-1} oil h^{-1}) or of CD formation (mmol eq HP kg^{-1} oil h^{-1}), and L is the lag period (h).

A nonlinear regression was applied to estimate L and μ with the Marquardt algorithm. A was fixed to 134 mmol O_2 kg^{-1} oil for oxygen uptake or to 80 mmol eq HP kg^{-1} oil for CD formation, as experimentally determined. The initial estimates of L and μ were 1.0 and 1.0, respectively. Asymptote confidence intervals for L and μ values were calculated with a 95% significance level. These calculations were performed with Statgraphics Plus 5.1 software (StatPoint Technologies, Warranton, USA).

Using the estimated values of L and μ, the time ($t_{1/2}$ (h)) corresponding to the incubation time when half the maximum oxygen uptake or CD formation was reached, was calculated from eq 2 as follows:

$$t_{1/2} = L - \frac{\left(\ln\left(-\ln\frac{Y - y_0}{A}\right) - 1\right) \times A}{\mu \times \exp(1)} \quad (2)$$

RESULTS

Physical Characterization of Emulsions. *Particle Size Distribution.* Emulsions stabilized with the different emulsifiers had similar particle size distributions with an average $[d_{3,2}]$ comprised between 1.4 and 2.2 μm at t_0 (Table 1). Although BLG-stabilized emulsions exhibited a slight increase of the average $[d_{3,2}]$, the particle size distributions remained quite constant during incubation. Consequently, differences in oxidation rates for the different emulsions might be attributed to neither the particle size distribution nor the destabilization of the oil droplets.

Surface Charge of Oil Droplets. At pH 6.7, emulsions stabilized with proteins were negatively charged as revealed by zeta potential measurements: from −54.5 mV for the BLG-stabilized emulsion to −29.8 mV for the BSA-stabilized emulsion

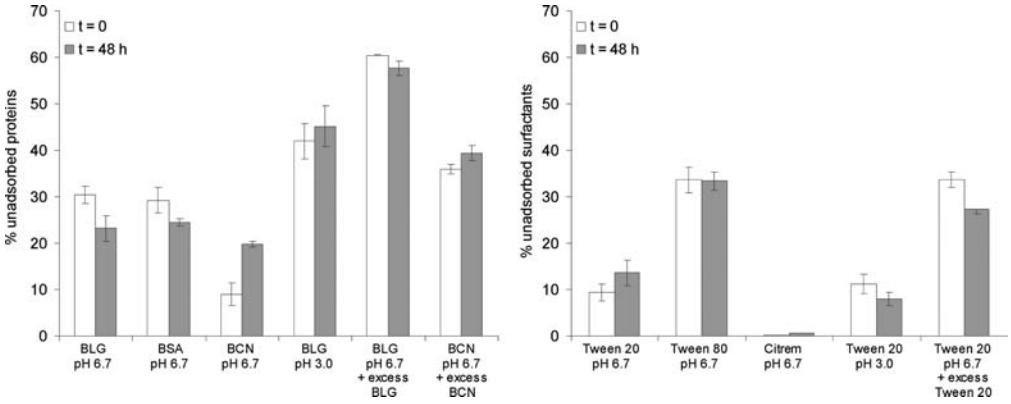

Figure 1. Proportion of unadsorbed emulsifiers in the emulsions, freshly prepared or after 48 h of incubation (25 °C, $FeSO_4$/EDTA; M/M; 1/1). Data represent the means of 2 to 6 measurements for at least 2 emulsions prepared independently. Errors bars correspond to standard deviations.

(Table 1). Emulsions stabilized with nonionic surfactants exhibited a weak net charge, whereas the emulsions stabilized with the anionic surfactant Citrem were the most negatively charged (−96.9 mV). At pH 3.0, the surface charge of the BLG-stabilized emulsion was dramatically reversed (68.1 mV), whereas that of the Tween 20-stabilized emulsion was little modified.

Amount of Unadsorbed Emulsifiers. In the absence of excess emulsifier added after homogenization, emulsions prepared at pH 6.7 contained low amounts of emulsifier remaining in the aqueous phase (0.012 to 1.68 g L^{-1}, Table 1). Emulsions stabilized with BCN, Citrem, and Tween 20 contained less than 20% of the total emulsifier molecules located in the aqueous phase (Figure 1). Emulsions stabilized with BSA, Tween 80, and BLG at pH 6.7 contained about 30% of the total emulsifier molecules in the aqueous phase. This could not be avoided because decreasing the initial concentration of emulsifier resulted in an increase of the average droplet size and a rapid physical destabilization of emulsions. The highest proportion of unadsorbed emulsifiers was found in the emulsion stabilized with BLG at pH 3.0 which contained about 40% (2.1 g L^{-1}) of the total BLG molecules in the aqueous phase. In contrast, emulsions prepared with Tween 20 contained similar amounts of unadsorbed emulsifier, whatever the pH. Distribution of emulsifiers between the interface and the aqueous phase remained constant overall during incubation except in the case of the BCN-stabilized emulsion in which some desorption of the protein probably occurred.[24]

When excess emulsifier (BLG, BCN, or Tween 20) was added after the homogenization of the emulsions prepared at pH 6.7, the amounts of unadsorbed emulsifiers in the aqueous phase were accordingly higher (around 6 g L^{-1}; 3.6 g L^{-1}; or 3.4 g L^{-1}, respectively), as shown in Table 1.

Oxidative Stability of Emulsions at pH 6.7. *Emulsions without Excess Emulsifier.* Oxygen uptake started almost from the beginning of the incubation period for the BLG-stabilized emulsion, whereas a longer lag phase was observed for the other emulsions (Figure 2a). After the lag phase, oxygen consumption increased until a plateau at about 134 mmol O_2 kg^{-1} oil. The plateau corresponds to about 25% of oxygen initially present in the vials and is, at least in part, due to the experimental procedure.[27] In the increasing phase, oxygen uptake was slower in emulsions stabilized with Tween 20, Tween 80, and BCN than in emulsions stabilized with BSA and BLG. No substantial oxygen uptake was observed for the Citrem-stabilized emulsions all along the incubation period. Therefore, a first ranking of the pH 6.7 emulsions according to their oxygen uptake was as follows: BLG < BSA < BCN < Tween 80 ≈ Tween 20 < Citrem.

Amounts of CD in freshly prepared emulsions were lower than 10 mmol eq HP kg^{-1} oil versus 7.8 mmol eq HP kg^{-1} in the stripped oil, which shows that emulsification did not induce substantial oxidation of the oil. Formation of CD enabled us to rank the emulsions in an order roughly similar to that of oxygen uptake, even if the difference between the BSA- and BCN-stabilized emulsions was less obvious (Figure 2b).

Twenty-four volatile compounds were identified during the incubation of emulsions. We chose to focus on the formation of propanal, which is a volatile compound arising from the oxidation of n-3 PUFA, and hexanal, which is a volatile compound arising from the oxidation of n-6 PUFA, both of them being major and current markers of lipid oxidation.[3,29] Formation of volatile compounds showed overall trends similar to those of oxygen uptake and formation of CD (Figure 2c,d). Formation of propanal was enhanced in BLG-stabilized emulsions, while no substantial amount of the compound was detected in Citrem-stabilized emulsions. In between, BCN-, BSA-, Tween 20-, and Tween 80-stabilized emulsions exhibited intermediate trends. As compared to propanal, lower amounts of hexanal were produced in the emulsions whatever the emulsifiers. The formation of hexanal was the fastest in BLG-stabilized emulsions in opposition to Citrem-stabilized emulsions, with BCN-, BSA-, Tween 20-, and Tween 80-stabilized emulsions being intermediate.

To summarize, the combination of the three lipid oxidation measurements (oxygen uptake and formation of CD, hexanal, and propanal) showed concordance among the obtained data. In our experimental conditions, the ranking of the emulsions according to their chemical stability is BLG < BSA ≤ BCN < Tween 80 ≈ Tween 20 < Citrem.

To compare the oxidative stability of the different emulsions with quantitative data, oxygen uptake and CD formation curves were adjusted with a modified-Gompertz model (eq 1). Correlation coefficients (R^2) obtained for the modeling were higher than 90% for all emulsions prepared at pH 6.7 (Table 2), indicating a rather good adjustment with the modified-Gompertz equation.

The shortest lag phases were obtained for the BLG-stabilized emulsion (L = 3.3 h for oxygen uptake and 1.4 h for CD formation). It was the longest for oxygen uptake in the Tween 20-stabilized emulsion (36.3 h) and for CD formation in the

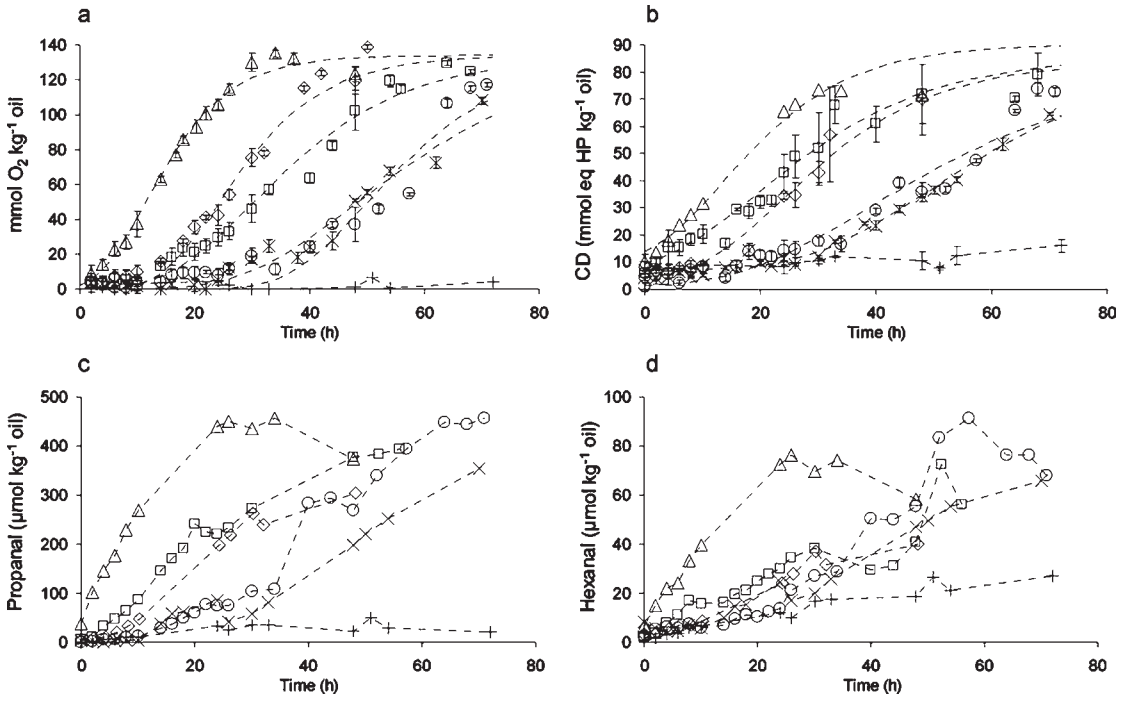

Figure 2. Oxygen uptake (a), formation of CD (b), and volatile compounds (c, propanal; d, hexanal) during the incubation of emulsions stabilized with BLG (△), BSA (◊), BCN (□), Tween 20 (○), Tween 80 (×), or Citrem (+) at pH 6.7, at 25 °C, in the dark, in the presence of $FeSO_4$/EDTA (1/1; M/M; 200 μM). In panels a and b, the dotted curves correspond to the adjusted modified-Gompertz model drawn with the L and μ parameters estimated for each emulsion except for the Citrem-stabilized emulsion. Error bars represent the standard deviations for oxygen uptake and CD measurements. Coefficients of variation were 3.3% and 11.6% for propanal and hexanal formation, respectively (see text).

Table 2. Lag Periods (L_1 and L_2) and Rates of Oxygen Uptake (μ_1) and CD Formation (μ_2) for the Emulsions[a]

	oxygen uptake parameters				CD formation parameters				
emulsion	L_1 (h)	μ_1 (mmol O_2 kg^{-1} oil h^{-1})	R^2 (%)	$t_{1/2\ (1)}$ (h)	L_2 (h)	μ_2 (mmol eq HP kg^{-1} oil h^{-1})	R^2 (%)	$t_{1/2\ (2)}$ (h)	μ_1/μ_2
BLG pH 6.7	3.3 ± 0.4	6.1 ± 0.2	98.3	14.4	1.4 ± 1.5	2.4 ± 0.2	94.2	13.8	2.5
BSA pH 6.7	14.0 ± 0.8	4.8 ± 0.3	97.1	27.9	9.4 ± 2.4	2.0 ± 0.3	93.4	27.3	2.4
BCN pH 6.7	15.0 ± 0.8	3.3 ± 0.1	96.9	35.6	2.5 ± 1.9	1.7 ± 0.2	90.1	22.2	1.9
Tween 20 pH 6.7	36.3 ± 1.8	3.6 ± 0.4	92.0	55.0	16.1 ± 2.7	1.3 ± 0.1	93.0	46.7	2.8
Tween 80 pH 6.7	28.6 ± 1.3	2.5 ± 0.2	95.7	55.5	28.2 ± 1.3	1.4 ± 0.1	97.9	51.8	1.8
BLG pH 3.0	13.4 ± 1.4	3.2 ± 0.2	96.2	34.8	4.7 ± 2.1	1.2 ± 0.1	94.6	27.4	2.7
Tween 20 pH 3.0	68.9 ± 5.2	0.6 ± 0.2	80.1	172.9	49.5 ± 3.3	0.6 ± 0.1	86.8	98.1	1.0
BLG pH 6.7 (excess BLG)	9.9 ± 1.3	3.9 ± 0.3	95.7	27.3	nd	nd	nd	nd	
BCN pH 6.7 (excess BCN)	44.4 ± 1.2	3.9 ± 0.4	94.4	61.8	nd	nd	nd	nd	
Tween 20 pH 6.7 (excess Tween 20)	43.2 ± 1.0	1.9 ± 0.1	97.0	77.9	nd	nd	nd	nd	

[a] Asymptote confidence intervals for L and μ values were calculated with a 95% significance level. Correlation coefficients (R^2) indicate the percentage of dispersion explained by the adjusted model. $t_{1/2\ (1)}$ and $t_{1/2\ (2)}$ are incubation times calculated with the model when half the maximum oxygen uptake or CD formation was reached, respectively. nd: not determined

Tween 80-stabilized emulsion (28.2 h). The highest rates of oxygen uptake and CD formation were obtained for the BLG-stabilized emulsion (6.1 mmol O_2 kg^{-1} oil h^{-1} and 2.4 mmol eq HP kg^{-1} oil h^{-1}, respectively). The lowest rate of oxygen uptake was observed for the Tween 80-stabilized emulsion (2.5 mmol O_2 kg^{-1} oil h^{-1}), whereas the lowest rates of CD formation were obtained for both Tween 20- and Tween 80-stabilized emulsions, with similar values (around 1.3 mmol eq HP kg^{-1} oil h^{-1}). The shortest $t_{1/2}$ values were obtained for the BLG-stabilized emulsion, for which half the maximum of oxygen uptake and CD formation were reached after 14.4 and 13.8 h, respectively. The longest $t_{1/2}$ for oxygen uptake were obtained for both Tween 20- and Tween 80-stabilized emulsions, for which half the maximum oxygen uptake was reached after around 55 h of incubation. The longest $t_{1/2}$ for CD formation was obtained for Tween 80-stabilized emulsion, for which half the maximum CD formation was reached after 51.8 h of incubation. These quantitative parameters confirm the order of oxidative stability

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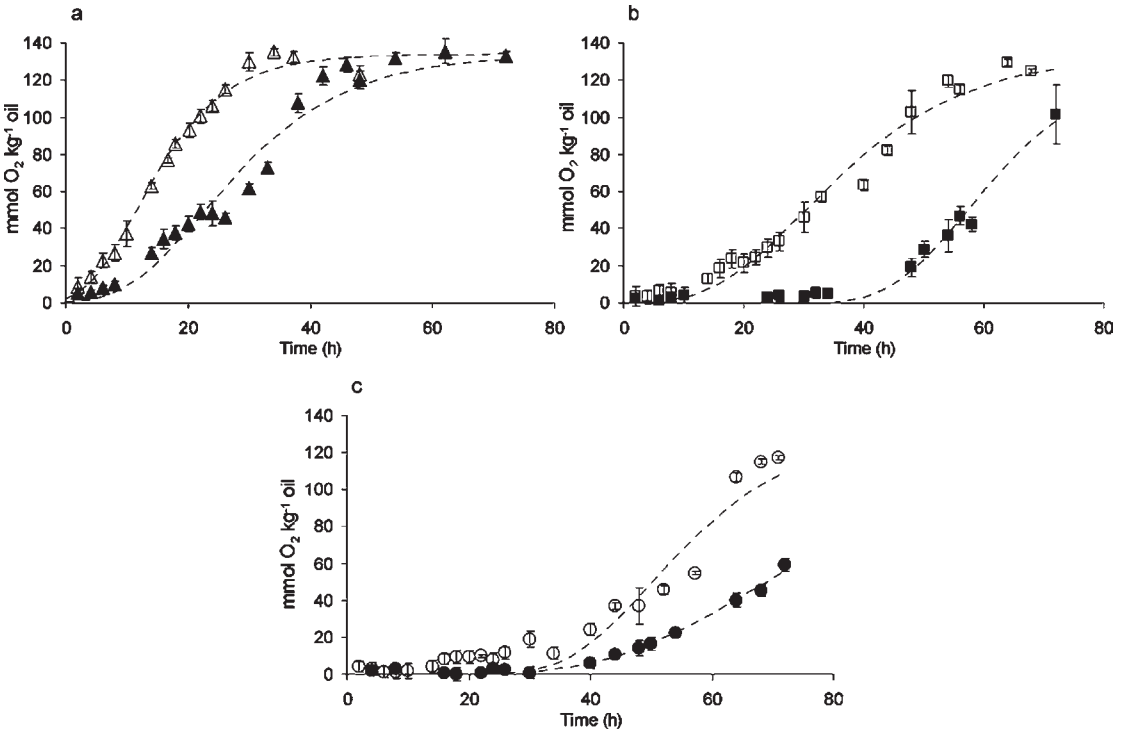

Figure 3. Oxygen uptake during the incubation of emulsions stabilized with BLG at pH 6.7 (a) without (△) or with (▲) excess BLG in the aqueous phase; BCN at pH 6.7 (b) without (□) or with (■) excess BCN in the aqueous phase; or Tween 20 at pH 6.7 (c) without (○) or with (●) excess Tween 20 in the aqueous phase at 25 °C in the dark, in the presence of $FeSO_4$/EDTA (1/1; M/M; 200 μM). Dotted curves correspond to the modified-Gompertz equation with the estimated L and μ parameters. Error bars represent standard deviations.

for the emulsions at pH 6.7 according to the emulsifier, which is definitely as follows: BLG < BSA ≤ BCN < Tween 20 ≈ Tween 80 ≪ Citrem.

Emulsions with Excess Emulsifier in the Aqueous Phase. Most previous studies investigating the effect of the emulsifier on lipid oxidation in O/W emulsions have been carried out in the presence of a large excess of emulsifier in the aqueous phase. To evidence the effect of these unadsorbed emulsifiers, we added excess emulsifier (BLG, BCN, or Tween 20) in the aqueous phase of the emulsions prepared with limited amounts of BLG, BCN, or Tween 20. In the BLG-stabilized emulsion containing excess BLG, the concentration of unadsorbed BLG increased from 1.5 to 6.1 g L^{-1} (Table 1). In these conditions, oxygen uptake was lower than in the BLG-stabilized emulsion without excess BLG (Figure 3a). In the same way, when unadsorbed BCN or Tween 20 were added in the aqueous phase of BCN- or Tween 20-stabilized emulsions, respectively, oxygen uptake was slowed down (Figure 3b,c). A huge increase of the lag phase (L_1 = 44.4 h) was noticed in the presence of excess BCN as compared with the control consisting of BCN-stabilized emulsion with low amounts of unadsorbed BCN (Table 2). Formation of lipid oxidation volatile compounds was also decreased in the presence of the emulsifiers added in the aqueous phase (data not shown). These results demonstrate the antioxidant effect of unadsorbed emulsifiers, unadsorbed BCN being the most effective.

Oxidative Stability of Emulsions at pH 3.0. In order to distinguish between the effect of interface composition and charge, we studied the oxidative stability of emulsions prepared at pH 3.0 when BLG-stabilized droplets carry globally a positive charge, whereas Tween 20-stabilized droplets remain almost noncharged (Table 1). In contrast with the BLG-stabilized emulsion at pH 6.7 (L_1 = 3.3 h), a longer lag phase (L_1 = 13.4 h) was observed in the BLG-stabilized emulsions at pH 3.0 (Figure 4a). Then, oxygen uptake proceeded at a lower rate (3.2 against 6.1 mmol O_2 kg^{-1} oil h^{-1}). In the Tween 20-stabilized emulsion, the lag phase increased from 36.3 to 68.9 h when pH decreased from 6.7 to 3.0. The formation of CD showed roughly similar trends (Figure 4b). Rates of CD formation were slower at pH 3.0 than at pH 6.7 for both emulsifiers (Table 2). In the same way, formation of propanal and hexanal increased less rapidly at pH 3.0 than at pH 6.7 for both emulsifiers (Figure 4c,d)

DISCUSSION

Lipid oxidation was measured by three methods, enabling us to characterize the different stages of the reaction. Results from these three methods corroborate our hypothesis. The oxygen uptake measurement was the most rapid and convenient method, both to acquire and analyze the data. Considering the standard deviations, it was also the most reproducible method and enabled us to better discriminate the different emulsions in regard to their oxidative stability. Beyond the qualitative description of the lipid oxidation curves, the adjustment of the curves with the modified-Gompertz model enabled us to go further in the understanding of the mechanisms. The mathematical model allowed us to estimate the lag phase, L, and the oxygen uptake and CD formation rates, μ. These parameters correspond to the initiation and propagation stages of lipid oxidation, respectively.[30,31] The estimated lag periods (L) were always equal or shorter for CD formation than for oxygen uptake. As CD are formed through the abstraction of a hydrogen atom from a bisallylic position followed by double bond rearrangement and oxygen fixation, this result

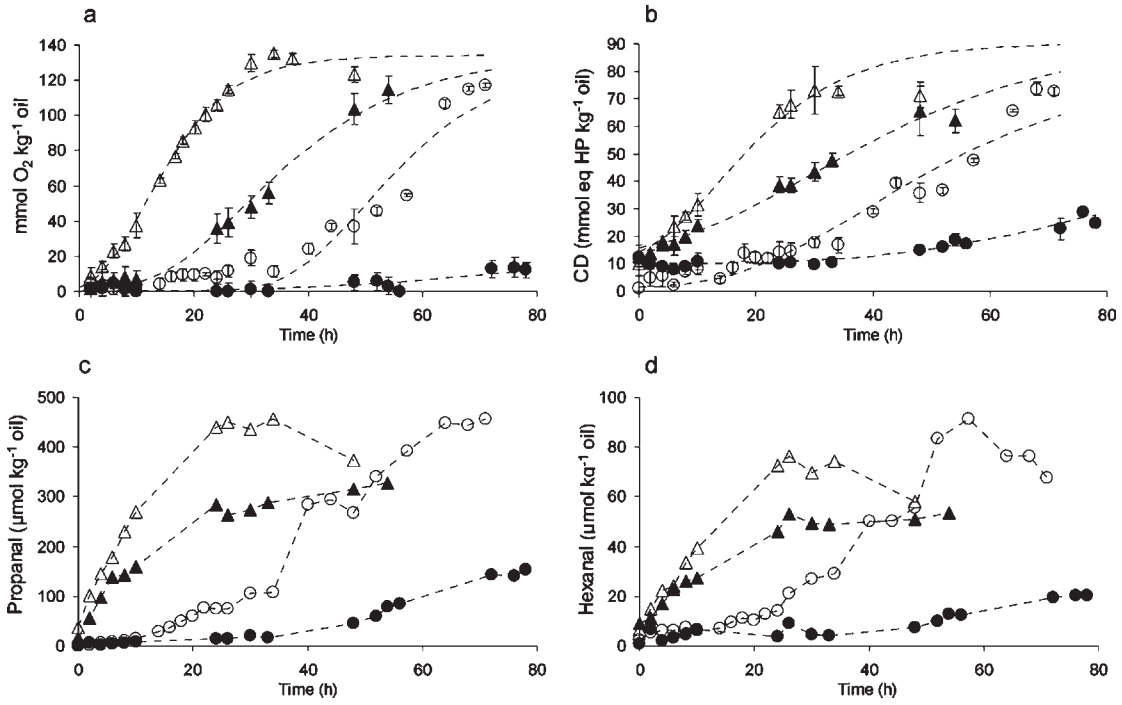

Figure 4. Oxygen uptake (a) and formation of CD (b) and volatile compounds (c, propanal; d, hexanal) during the incubation of emulsions stabilized with BLG at pH 6.7 (△), BLG at pH 3.0 (▲), Tween 20 at pH 6.7 (○), or Tween 20 at pH 3.0 (●), at 25 °C in the dark, in the presence of $FeSO_4$/EDTA (1/1; M/M; 200 μM). In panels a and b, the dotted curves correspond to the modified-Gompertz equation with the estimated L and μ parameters. Error bars represent the standard deviations for oxygen uptake and CD measurements. Coefficients of variation were 3.3% and 11.6% for propanal and hexanal formation, respectively (see text).

probably comes from a higher sensitivity for the spectrophotometric measurement of conjugated dienes than for the headspace-GC measurement of oxygen uptake CD.[32] Oxygen initially dissolved in the aqueous and lipid phases of the emulsion is first and probably rapidly consumed.[33] A decrease of oxygen concentration in the headspace is only detected when a sufficient amount of oxygen has diffused from the gas phase to the liquid phase. The μ_1/μ_2 ratio for the different emulsions, except the Tween 20-stabilized emulsion at pH 3.0, comprised between 1.8 and 2.8. This corroborates the higher maximum oxygen uptake (134 mmol O_2 kg^{-1} oil) as compared to the maximum CD concentration (80 mmol eq HP kg^{-1} oil). This difference can be partly explained by the fact that CD simultaneously form and decompose into secondary oxidation products such as volatile compounds. In fact, for all emulsions, propanal and hexanal were detected at the same time as the formation of CD. In addition, oxygen could be also consumed without CD formation through fixation by monoenic species (namely oleic acid) and by direct reaction of oxygenated free radicals with proteins.[34,35] However, no substantial decrease in the C18:1 content was observed in the oxidized emulsions (data not shown).

In our experimental conditions, namely, the presence of low amounts of unadsorbed emulsifiers and iron/EDTA-catalyzed oxidation, the emulsions stabilized with surfactants exhibited a better oxidative stability than the emulsions stabilized with proteins. As summarized in Figure 5, protein-stabilized emulsions had shorter lag phases and higher rates of oxygen uptake and CD formation than surfactant-stabilized emulsions. This observation differs from previous results that showed a better oxidative stability for emulsions stabilized by proteins.[12,14−16] We assume that the results obtained in these previous studies corresponded to a global effect of the emulsifier, both adsorbed at the oil−water interface and solubilized in a large proportion in the aqueous phase. To test this hypothesis, we added an excess of emulsifier in the aqueous phase of the optimized emulsions. We observed a delay in the oxidation process, especially when unadsorbed proteins were used, confirming previous data.[3,10,20,22,23,36] This also demonstrates that the emulsions designed with low amounts of emulsifiers in the aqueous phase provide information regarding mainly the role of the emulsifier located at the interface on lipid oxidation. When emulsions samples were prepared in such conditions, the proteins adsorbed at the interface do not efficiently protect lipids against oxidation, in comparison to surfactants. We assume that surfactant-stabilized interfaces could protect lipids against oxidation because of their compactness and homogeneity, whereas protein-stabilized interfaces would be more heterogeneous and porous. Surfactants are described in the literature as more surface-active than proteins, forming more compact adsorbed layers.[37−39]

The emulsions stabilized with nonionic surfactants (Tween 20 and Tween 80) exhibited similar lipid oxidation kinetics. These two surfactants only differ by their hydrophobic chains mainly composed of lauric acid or oleic acid, respectively. The size of the hydrophobic chains of the surfactants adsorbed at the interface is therefore not a main factor influencing lipid oxidation in emulsions, as previously suggested.[40]

Our results indicate also that the surface charge of the droplets is not the most crucial factor influencing lipid oxidation in emulsions. First, the positively charged BLG-stabilized emulsion prepared at pH 3.0 remained less oxidatively stable than the surfactant-stabilized one (Figure 4 and Table 2). Second, the order of zeta potential was: Citrem < BLG pH 6.7 < BCN pH

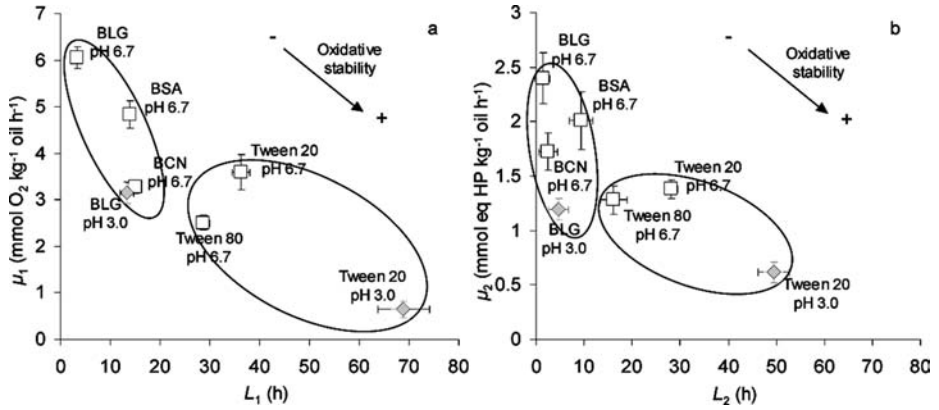

Figure 5. Relationships between the lag periods and (a) the rates of oxygen uptake (mmol O_2 kg^{-1} oil h^{-1}) or (b) the rates of CD formation (mmol eq HP kg^{-1} oil h^{-1}) for the emulsions containing limited concentrations of unadsorbed emulsifiers at pH 6.7 (□) or pH 3.0 (◊). Error bars correspond to the asymptote confidence intervals calculated with a 95% significance level.

6.7 < BSA pH 6.7 < Tween 20 ≈ Tween 80 < BLG pH 3.0 (Table 1), which does not correlate with the order of oxidative stability previously mentioned. In particular, the Citrem-stabilized emulsion had the lowest zeta potential but was the most oxidatively stable emulsion. Thus, the rapid oxidation in the protein-stabilized emulsions at pH 6.7 cannot be attributed only to the negative charge of the droplets. When unadsorbed emulsifiers are in low concentrations, the oxidative stability of emulsions having positively charged interfacial layers and that are stabilized by a protein is not better than the oxidative stability of emulsions stabilized with nonionic surfactants.

BLG and Tween 20-stabilized emulsions were more oxidatively stable at pH 3.0 (phosphoric buffer) than at pH 6.7 (PIPES buffer). These differences can account for several physicochemical factors including pH, droplet charge, unadsorbed emulsifiers, and chemical reactivity of buffers. For Tween 20-stabilized emulsions, L_1 and L_2 increased while μ_1 and μ_2 decreased when decreasing the pH, indicating that both initiation and propagation were slowed down. Because Tween 20 is a nonionic surfactant, the surface charge of the droplets was weakly affected by pH, as demonstrated by zeta potential measurements (Table 1). The amount of unadsorbed Tween 20 did not change substantially between pH 3.0 and pH 6.7 (Figure 1). Thus, in Tween 20-stabilized emulsions, the droplet charge and the amount of unadsorbed emulsifiers were not involved in the observed differences. About the effect of pH on lipid oxidation in emulsions, contradictory results had been obtained. Some authors observed that emulsions were more oxidatively stable at neutral pHs than at acidic pHs.[6,11,12] This was assumed to be due to the higher iron solubility at low pHs. However, others observed the opposite tendency,[7−10] which corresponds to the results we obtained. The lower solubility of metals at neutral pHs than at acidic pHs would result in the precipitation of metal onto the lipid droplets, promoting more efficient lipid oxidation.[7] A possible explanation for these contradictory results is the interference of several mechanisms within the pH effect. Additionally, phosphoric acid and PIPES may intervene in oxidative stability.[41,42] For BLG-stabilized emulsions, L_1 and L_2 increased only slightly when decreasing the pH, whereas μ_1 and μ_2 decreased sharply (Table 2 and Figure 5) showing modifications in the propagation step of the reaction. The same physicochemical factors as those described above may be involved. However, the charge effect, favoring Coulombic interactions between iron ions and the negatively charged interface, cannot be excluded due to the huge increase of zeta potential values from pH 6.7 to pH 3.0 (Table 1). Finally, the amount of unadsorbed BLG was higher at pH 3.0 than at pH 6.7 (Table 1), which could also contribute to decrease the rate of oxidation in the propagation steps and to improve the oxidative stability (Table 2 and Figure 3).

The oxidative stability of emulsions stabilized by proteins at pH 6.7 was in the order BLG < BSA < BCN. The amount of unadsorbed proteins in the aqueous phase that could act as antioxidant increased in the reverse order (Table 1): BCN (0.45 g L^{-1}) < BSA (1.17 g L^{-1}) < BLG (1.52 g L^{-1}), reinforcing the conclusion that oil droplets covered by BLG oxidizes faster than oil droplets covered by BCN. In previous studies performed with excess emulsifiers, casein had already been shown to be more effective in protecting against lipid oxidation than other proteins.[10,19,43] This was usually explained by its ability to chelate metal ions,[10,27,36] to scavenge free radicals,[43] or to form thick interfacial layers.[44] Considering the average diameter ($[d_{3,2}]$) and the amount of excess proteins in the aqueous phase (Table 1), the surface concentrations of each protein were around 1.8 mg m^{-2}, 1.9 mg m^{-2}, and 2.9 mg m^{-2} for BLG-, BSA-, and BCN-stabilized emulsions, respectively. This confirms that BCN formed the most dense interfacial layer surrounding oil droplets and the possible role of the density of the interfacial layer in the oxidation of emulsified lipids.[44] In bovine and caprine casein-stabilized emulsions, the phosphoprotein BCN was recently found by ^{31}P NMR to insert its hydrophobic site compactly into the oil droplets and present its hydrophilic end on the surface. This could participate in the protecting effect against lipid oxidation of BCN compared to BLG and BSA.[45]

This research brings new insights about the effect of the composition of the interfacial layer on lipid oxidation in emulsions. The optimization of emulsion formulation enabled us to demonstrate that protein-stabilized interfaces are less efficient in protecting emulsified lipids against iron/EDTA-catalyzed oxidation than surfactant-stabilized interfaces. The antioxidant effect of unadsorbed proteins was also confirmed. Complementary work is currently being performed in our laboratory to expand our results to various conditions of initiation of oxidation and to characterize the modifications undergone by the interfacial proteins in the oxidizing emulsions.

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ABBREVIATIONS USED

BCN, β-casein; BLG, β-lactoglobulin; BSA, bovine serum albumin; CD, conjugated dienes; EDTA, ethylene diamine tetraacetic acid; FAME, fatty acid methyl ester; FID, flame ionization detector; GC, gas chromatography; HP, hydroperoxides; MS, mass spectroscopy; O/W emulsion, oil-in-water emulsion; PUFA, polyunsaturated fatty acid; SDS, sodium dodecyl sulfate; SPME, solid phase microextraction; TCD, thermal conductivity detector.

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