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Effects often overlooked in lipid oxidation in oil-in-water emulsions: Agitation conditions and headspace-to-emulsion ratio

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Abstract

The effects of the agitation conditions and headspace-to-emulsion volume ratio on lipid oxidation in emulsions can be considerable, but have not been systematically investigated yet. In the current paper, lipid oxidation was monitored in model oil-in-water (O/W) emulsions at pH 4.0 and 25°C in the presence of 200 μM iron sulfate. The formation of primary (conjugated dienes and hydroperoxides) and secondary (p-anisidine value and TBARS) oxidation products confirmed that using rotating or shaking devices doubled the rate of oxidation product formation compared to a non-agitated system, as a result of enhanced oxygen transfer. Furthermore, we found that a higher headspace-to-emulsion volume ratio at least doubled the rate of lipid oxidation due to a higher amount of oxygen available per mass of oil, which is in agreement with the kinetics of the reaction. This indicates that the variation in literature data on lipid oxidation in emulsions can be attributed to differences in mixing conditions and volume ratios. These factors are crucial and should be reported systematically along with the agitation conditions, and sampling method. This will enable a better comparison of literature information.

KEYWORDS
agitation, headspace, iron sulfate, lipid oxidation, O/W emulsion

INTRODUCTION

Lipid oxidation is an important challenge to address for food industries as it is one of the main causes of compromised shelf-life in food products containing polyunsaturated fatty acids (PUFAs). In fact, this reaction between unsaturated lipids and oxygen has a detrimental influence on the sensory and nutritional quality of the food products. Many lipid-based foods are oil-in-water (O/W) emulsions (e.g., salad dressing, mayonnaise, milk, infant formula, creams, dairy analogues, etc.), which are dispersions of oil droplets in an aqueous phase stabilized by emulsifiers. Lipid oxidation in emulsions is in general faster and more complex compared to bulk oil. One significant factor is the increased surface area in these systems (Berton-Carabin et al., 2014), which promotes exposure of unsaturated lipids to more oxygen and water-soluble pro-oxidant compounds (free radicals, metals; Laguerre et al., 2017; McClements & Decker, 2000), and thus accelerates the oxidation process. Furthermore, the emulsifiers used in such systems can impact lipid oxidation in various ways (Berton-Carabin et al., 2014). Some emulsifiers inhibit lipid oxidation by scavenging free radicals (Ries et al., 2010), whereas others promote oxidation by generating free radicals or facilitating lipid hydroperoxide breakdown (Cui & Decker, 2015). In general, the interfacial layer plays a crucial role in lipid

oxidation (Berton-Carabin et al., 2014; McClements & Decker, 2000; Waraho et al., 2011), and this has been related to its composition, thickness, and electrostatic charge (Berton-Carabin et al., 2014).

The pH and ionic strength of the aqueous phase are additional factors influencing lipid oxidation in emulsion systems (Genot et al., 2003; McClements & Decker, 2000; Villeneuve et al., 2021). The pH can influence the reactivity and activity of pro-oxidant and antioxidants, by altering the ionization state of functional groups (Villiere et al., 2005). Ionic strength has been connected to repelling or binding charged pro-oxidant metal ions (Mei et al., 1998). Furthermore, the pH may modulate the charge of both the pro-oxidants and of certain lipid molecules. This can induce attractive or repulsive interactions between pro-oxidants and lipid molecules (Petursson et al., 2004), thus affecting lipid oxidation. A high oxygen concentration (Marcuse & Fredriksson, 1968), temperature (Bolland & Ten Have, 1947), and light exposure (Bolland & Ten Have, 1947) were also reported to increase lipid oxidation in O/W emulsions, and are thus often used for accelerated shelf-life tests (Waraho et al., 2011).

Less investigated are factors related to mass transfer such as stirring/agitation (Coupland & McClements, 1996), and the ratio of reactants, more specifically that of oxidizable lipids to oxygen (Marcuse & Fredriksson, 1968). Agitation of the emulsions may lead to a different course of the oxidation reaction (when diffusion is a limiting factor), and the same holds for the ratio of reactants. This can in turn affect oxidation rates due to for example, changes in the total amount of surface area if coalescence occurs (Genot et al., 2003), and changes in the local reactant concentrations due to for example, creaming. In literature, this has also been suggested to lead to hindered oxygen diffusion, although we think that this is questionable for oxygen given its high diffusivity compared to the diffusivity of other components (Schroën & Berton-Carabin, 2022).

It is good to keep in mind that agitation not only influences mass transfer, but may also influence the physical stability of emulsions (flocculation and subsequent coalescence; Genot et al., 2003). According to Yesiltas et al. (2019), creaming can enhance oxidation, which may be a reflection of the changed reactant ratio that has also been linked to differences in lipid oxidation levels in the large creamed droplets and in small droplets that remain in the bulk aqueous phase (Genot et al., 2003). Additionally, pro-oxidants and oxidation products may be exchanged more readily between droplets within a creamed emulsion (Laguerre et al., 2017).

Genot et al. (2003) stressed the importance of agitation conditions, as did others (Marcuse & Fredriksson, 1968), but this is only limitedly picked up in research. Given the fact that so many other factors have been considered in explaining the course of an oxidation reaction, it is rather surprising that this is not given more attention in lipid oxidation studies (Berton-Carabin et al., 2014). The lack of control over the oxygen levels in the incubation containers/tubes (either through the headspace-to-emulsion ratio, or through gas leakage) can cause variability in results. Containers/tubes that are not hermetically sealed or containers/tubes that are repeatedly opened during the incubation period are used, and this could alter the oxygen concentration and ratio in the tube at each sampling time point, affecting the outcomes and interpretations of the experiments. As far as we know, there has been no systematic study yet that links these factors to the oxidative stability of emulsions. Therefore, the aim of our study is to address this gap. We compared lipid oxidation in O/W emulsions incubated on a rotative agitator, or with a shaker, with immobile emulsions, and varied the headspace percentage between 12% and 88% (vol/vol).

We expected that agitation and increased headspace ratio (56% and 88%) would speed up lipid oxidation in O/W emulsions. Primary (i.e., hydroperoxides and conjugated dienes [CD]) and secondary (i.e., p-anisidine and thiobarbituric acid-reactive substances [TBARS]) oxidation markers were measured as function of time, and physical properties (i.e., droplet size distribution, zeta-potential) and more general features (acid value and pH) were monitored.

MATERIALS AND METHODS

Materials

Rapeseed oil purchased from a local supermarket was stripped from surface-active impurities and tocopherols by means of alumina (Berton et al., 2011). Unless stated otherwise, all chemicals, including Tween 20, ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), and alumina powder, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water, purified by a Millipore Milli-Q system (Darmstadt, Germany) was used throughout the study.

Preparation and physical characterization of emulsions

Emulsion preparation

Tween 20 was added to ultrapure water to form a 1 wt % solution, which was gently stirred overnight at room temperature. A coarse emulsion was prepared by homogenizing 10% (wt/wt) stripped rapeseed oil with 90% (wt/wt) of the Tween 20 solution using a high-speed rotor-stator homogenizer (UltraTurrax T25 Basic Disperser with 25 mm diameter blade, Janke & Kunkel, IKA, Staufen, Germany) at 7000 rpm for 2 min. Subsequently, the coarse emulsion was passed through a

high-pressure homogenizer (M-110Y Microfluidizer, Microfluidics, USA) equipped with a F12Y chamber at 800 bar, for three cycles. To limit temperature rise during the emulsification process, the cooling jacket of the homogenizer was filled with iced water. Emulsion preparation was performed twice to obtain independent duplicates. The final iron concentration in all emulsions was adjusted to 200 μM by adding ferrous sulfate powder to the emulsions and dissolving it by vigorous mixing.

Particle size distribution

The emulsion droplet size was determined by static light scattering (Malvern Mastersizer 3000, Malvern Instruments Ltd., Malvern, Worcestershire, UK). The obscuration was between 8% and 12%; the refractive index (RI) of dispersant was set to 1.33 for water, and to 1.46 for the dispersed rapeseed oil.

Zeta potential

The surface charge of the emulsion droplets was assessed through the measurement of the electrophoretic mobility with a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instrument Ltd., UK). Samples were 100-fold diluted with ultrapure water prior to measurement. The same refractive indices as mentioned earlier for static light scattering were used for the continuous and dispersed phases. The results were expressed in mV.

Particle size distribution and zeta potential measurements were performed immediately after emulsification, and at the end of the incubation period.

Assessment of the chemical changes in incubated emulsions

Incubation conditions

Emulsions were distributed in 50-mL tubes. Five different systems were studied to investigate the effect of agitation conditions and headspace-to-emulsion ratio on the oxidative stability of emulsions (Figure 1). To study the effect of agitation conditions, aliquots of 50 mL of emulsion (12% [vol/vol] headspace) were incubated in the dark either on a rotative device (Stuart rotator, SB3, UK) at 5 rpm, on a shaker (Grant instruments, Platform rocker, PMR-30, UK) at a frequency of five cycles per minute, or under immobile conditions (Figure 1a). In addition, to study the effect of the headspace-to emulsion ratio, emulsion aliquots leading to 56% or 88% (vol/vol) headspace in the tubes were incubated on the rotative device.

All emulsions were incubated at 25°C for 8 days, and aliquots of 2.1 mL were taken right after emulsion production (day 0). The emulsions used to investigate the effect of agitation were analyzed every day, those used for headspace-to-emulsion ratio every 2 days. The 12% (vol/vol) headspace emulsion tube was opened daily, that is, same tube for all time points, whereas others (56% and 88% [vol/vol]) were opened once at a time for sampling, that is, one tube per time point (Figure 1b).

Measurements of lipid oxidation markers

Primary lipid oxidation products

The procedure for quantification of CD was adapted from Lethuaut et al. (2002). Briefly, 0.25 mL emulsion aliquots were diluted 100 times in *n*-propanol, and the mixture was centrifuged at 1200*g* for 4 min. The absorbance of the supernatant was recorded between 200 and 310 nm using a UV/VIS spectrophotometer (DU 720 Beckman Coulter, Brea, CA, USA) and the value at 233 nm was used for calculations. Results were expressed in mmol conjugated dienes per kg of oil (mmol CD kg^{-1} oil); using 27,000 M^{-1} cm^{-1} as the molar extinction coefficient of CD at 233 nm.

The hydroperoxide concentration was measured following the methodology of Shantha and Decker (1994). Briefly, emulsions were weighed in tubes and lipids were extracted by mixing with a *n*-hexane:*n*-propanol mixture (3:1 vol/vol). Next, the hexane phase containing the extracted lipids was mixed with methanol:1-buthanol (2:1 vol/vol) and the assay reagent. After 20 min at room temperature, absorbance was determined at 510 nm against a blank containing all reagents except the sample using a spectrophotometer (DU 720 Beckman Coulter, Brea, CA, USA). The hydroperoxide concentration was expressed as mmol per kg of oil and determined using a calibration curve (Figure S1) prepared with a commercial cumene hydroperoxide standard solution.

Secondary lipid oxidation products

To determine the p-anisidine value (pAV), a measure of total aldehydes, lipids were first extracted using 1.5 mL *n*-hexane:*n*-propanol mixture (3:1 vol/vol) as described above. The initial absorbance of the top hexane phase (A_b) was measured at 350 nm, using pure hexane as a blank. One milliliter of this top hexane phase was mixed with 0.2 mL p-anisidine solution (2.5 M in acetic acid). After 10 min, the absorbance at 350 nm (A_s) was measured, using hexane similarly mixed with the p-anisidine solution as a blank. The pAV was calculated as shown in Equation (1).

$$\mathrm{pAV} = (1.2A_s - A_b)/m, \quad (1)$$

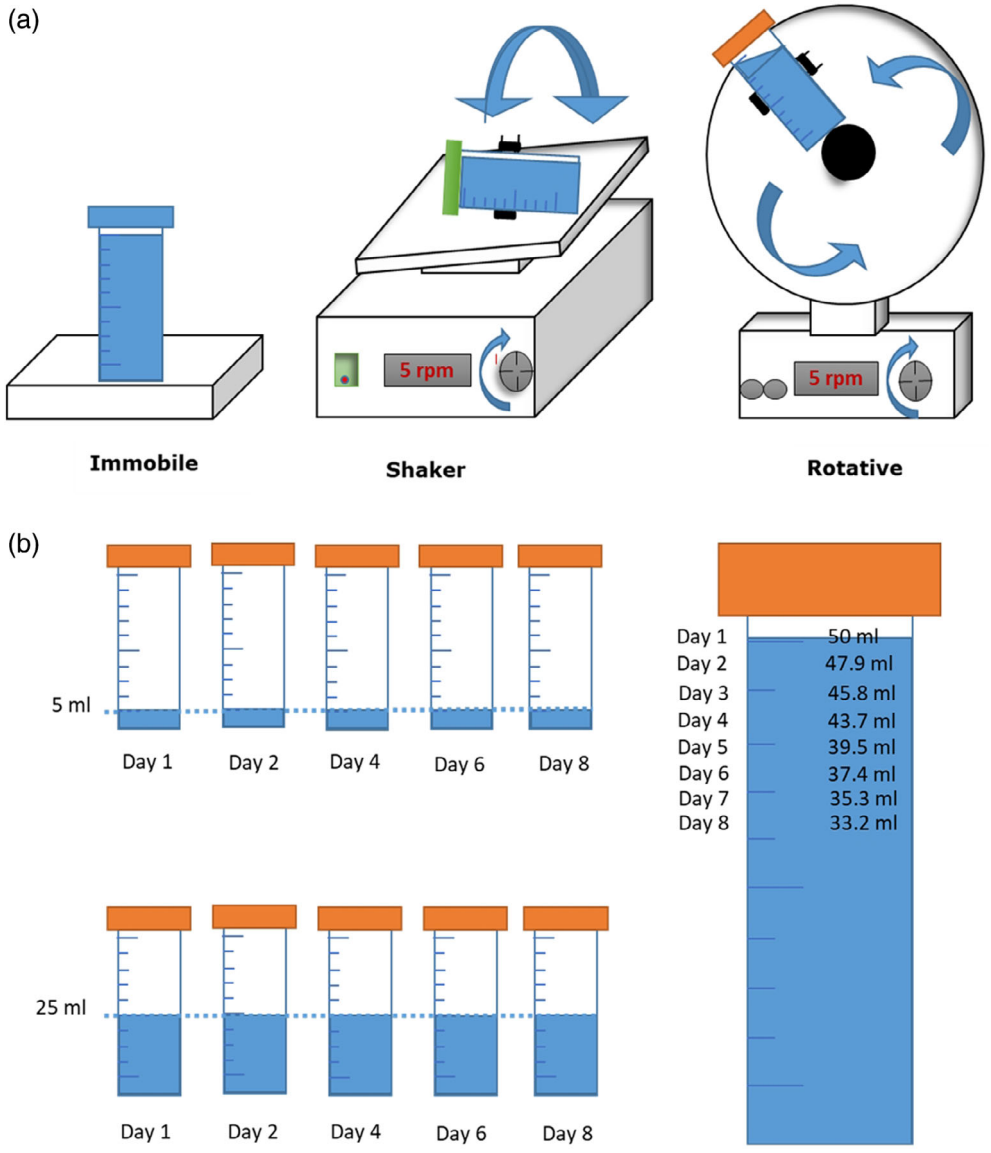

FIGURE 1 Experimental approach: (a) agitation conditions, all vessels contain 88% (vol/vol) emulsion, and 12% (vol/vol) headspace (i.e., air at t0); (b) various headspace ratios of rotated emulsions.

where *m* is the mass (g) of oil per mL hexane. The pAV was expressed in arbitrary units (AU).

Thiobarbituric acid-reactive substances (TBARS) were determined according to an adapted method of McDonald and Hultin (1987). TBA solution was prepared by mixing trichloroacetic acid-thiobarbituric acid (TCA-TBA) solution (15 g TCA, 0.375 g TBA, 1.76 mL of 12 N HCl and 82.9 mL of water) and butylated hydroxytoluene (2% [wt/wt] in ethanol) in a 100:3 ratio. One milliliter emulsion was combined with 2 mL of TBA solution in test tubes and placed in a hot water bath (75°C) for 30 min. The tubes were cooled to room temperature for 10 min and then centrifuged at 4000*g* for 15 min. The absorbance was measured at 532 nm. TBARS concentrations were determined using a standard curve of 1,1,3,3-tetraethoxypropane and expressed as mmol/mg lipids.

Acid value and pH in emulsions

The acid base titration technique described in the AOAC Official Method 969.17 (AOAC, 2005) was used to determine free fatty acids in the starting oil, and in the lipids extracted from the emulsions (at day 0 and 8). The pH was measured once per day in emulsions incubated using different agitation conditions and every two days in emulsions with different headspace-to-emulsion ratio.

Experimental design and statistical analysis

Oxidation experiments were carried out in independent duplicates (i.e., two emulsions prepared and incubated

independently, for all conditions tested), and each measurement was conducted at least twice. Results ($n = 4$) were averaged and statistically analyzed with two-way ANOVA test (time × agitation/headspace ratio) followed by post hoc Tukey's test using SPSS software (version 18, PASW Statistics, US). For the physical stability tests, one-way ANOVA was conducted using six individual results from two independent repetitions and least significant differences were calculated applying Tukey's post hoc test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Physical characterization and lipid hydrolysis in incubated emulsions

The average droplet size, zeta potential, pH and acid value of Tween 20-stabilized emulsions, freshly prepared and after 8 days of incubation are presented in Table 1; the droplet size distributions can be found in Figure S2. For all emulsions, droplet size distributions were monomodal with an average volume mean diameter ($d_{4,3}$) of ~0.16 μm, irrespective of the agitation time and conditions, and no creaming was observed over the timescale of the experiment.

Right after iron addition, the starting pH of all emulsions was around 4.0 and dropped to 3.5 within the first day for all emulsions (Table 1, and Figure S3a). After this first decrease, the pH remained constant during the incubation for most emulsions. An exception was the emulsion incubated with the highest headspace-to-emulsion ratio (88% [vol/vol] headspace) under rotative agitation, for which the pH decreased significantly more to reach 3.2 on day 2 and finally 3.1 on day 8. It is well-documented that metal ions form metal aquo complexes in water that are acidic owing to the ionization of protons from the water ligands, which explains the low pH at the start, right after iron addition to emulsions (Elias, 1992).

The zeta potential of all emulsions was strongly negative (Figure S3b), as reported by others for emulsions stabilized by non-ionic surfactants such as polysorbates (Berton et al., 2011; Cengiz et al., 2019). Stripped rapeseed oil and fresh emulsions containing iron had a low amount of free fatty acids (acid value), 0.03 mg/g oil and 0.08 mg/g oil, respectively (Table 1). After 8 days of incubation at 25°C, the FFA content of all emulsions increased, with some differences depending on agitation method and headspace-to-emulsion ratio. This indicates that triglycerides are limitedly hydrolysed into FFAs (Cengiz et al., 2019). The emulsion incubated in immobile conditions had the lowest FFA content of 0.51 mg/g oil at the end of the incubation period, whereas both rotated and shaken emulsions had similar values around 0.90 mg/g oil, and emulsions with larger headspace (88% [vol/vol] headspace) had the highest FFA content (1.21 mg/g oil), compared to the 12% and 56% (vol/vol) headspace emulsions (~0.97 mg/g oil). Herman and Groves (1993) explored the correlation between FFAs and pH and did detect phospholipid and triacylglycerol hydrolysis in emulsions, alongside with a drop in pH. Interestingly, they still concluded that the measured drop in pH could not be entirely explained by the extent of FFA formation; other possible reasons were discussed, including some oxidative degradation of the lipids in these emulsions.

Another possible explanation for the formation of free fatty acids, and drop in pH is the degradation of the polysorbate (Tween 20) by hydrolysis of the fatty acid ester bond, or oxidation of the polyethylene oxide (POE) chains leading to generation of short chain acids such as formic acid, and various other components such as aldehydes, acids, and ketones (Kerwin, 2008; Kishore et al., 2011), and even trace amounts of fatty acids (Tomlinson et al., 2015). In general, these FFAs have low solubility in water, yet it may be enhanced by the presence of Tween 20 (Cengiz et al., 2019). Formation of weak acids may occur through degradation of hydroperoxides, yet this is most probably not the cause for the pH drop, since the formation of hydroperoxides

TABLE 1 Average particle size, zeta potential, pH and acid value in stripped rapeseed oil, and in emulsions freshly prepared or after 8 days of incubation under various agitation conditions and volume of headspace at 40°C.

Sample name	Particle size (μm)	Zeta potential (mV)	pH	Acid value (mg KOH/g oil)
Stripped oil	-	-	-	0.03 ± 0.01^{a}
Fresh emulsion (iron added)	0.15 ± 0.00^{ab}	-31.9 ± 1.82^{b}	3.92^{d}	0.08 ± 0.00^{b}
12% (vol/vol) headspace—immobile	0.17 ± 0.00^{c}	-31.2 ± 2.19^{b}	3.53^{c}	0.51 ± 0.04^{d}
12% (vol/vol) headspace—Shaker	0.15 ± 0.02^{b}	-31.5 ± 0.48^{b}	3.47^{b}	0.85 ± 0.15^{c}
12% (vol/vol) headspace—Rotary	0.14 ± 0.00^{a}	-35.5 ± 1.10^{a}	3.53^{c}	0.99 ± 0.01^{c}
56% (vol/vol) headspace—Rotary	0.17 ± 0.01^{c}	-	3.44^{b}	0.97 ± 0.10^{c}
88% (vol/vol) headspace—Rotary	0.17 ± 0.01^{c}	-	3.06^{a}	1.21 ± 0.02^{e}

Note: Values are the average ± standard deviation of six measurements on two independently prepared batches. Values with different letters in column are significantly different according to Tukey's *b* test ($p < 0.05$).

become prominent later in the incubation period, whereas pH drops mostly within the first two days of incubation. This effect might still have contributed to the difference in pH drop between emulsions with 12% headspace that oxidize very readily due to the high oxygen concentration, and the other two that have similar amounts of oxygen, and show similar but lower pH drop.

To wrap up, we expect the initial drop in pH to be mostly caused by the formation of metal aquo complexes, particularly when present in their deprotonated state at the interface. Additionally, the generation of FFA formation from triacylglycerol and/or Tween 20 hydrolysis may contribute to further decrease of pH over time (Figure S3a; Chen et al., 2011). In future research, it would be advisable to also monitor other lipid oxidation products, such as volatile compound formation.

Effect of agitation conditions on lipid oxidation

The impact of agitation on the formation of primary and secondary lipid markers is reported in Figure 2. Both agitation methods significantly increased lipid oxidation compared to emulsions that were incubated under immobile conditions ($p < 0.05$). After one day of incubation, the immobile emulsion was already significantly lower in CD (12 mmol/kg oil) than the emulsions incubated using rotative or shaker conditions (16–18 mmol CD/kg oil). This difference between the oxidative fate of the immobile emulsion and the agitated ones became more marked over incubation time, with final concentrations of 36 mmol/kg oil and ~80 mmol/kg oil reached for the immobile and agitated emulsions, respectively.

For hydroperoxides, the differences between agitated and non-agitated emulsions were similar to those found for CDs. The differences became significant on day 2 with values of ~30 and 13 mmol hydroperoxides/kg oil for agitated and immobile emulsions, respectively. The differences developed then further over time, as was also found for CDs, reaching 2.4–2.9-fold higher hydroperoxide concentrations (>60 mmol/kg oil) in agitated emulsions compared to the immobile ones (25 mmol/kg oil) after 8 days of incubation.

The markers for the formation of secondary oxidation products, pAV and TBARS, followed the same trends as the primary oxidation products. Significant differences were first observed after 3 days between immobile and agitated emulsions, with pAV of 10 and ~15 (AU), and TBARS concentrations of 0.14 and ~0.45 mmol/kg oil, respectively. After 8 days of incubation, rotated and shaken emulsions peaked to ~32 (AU) and ~1 mmol/kg oil, whereas the concentrations remained low for the immobile emulsions showing

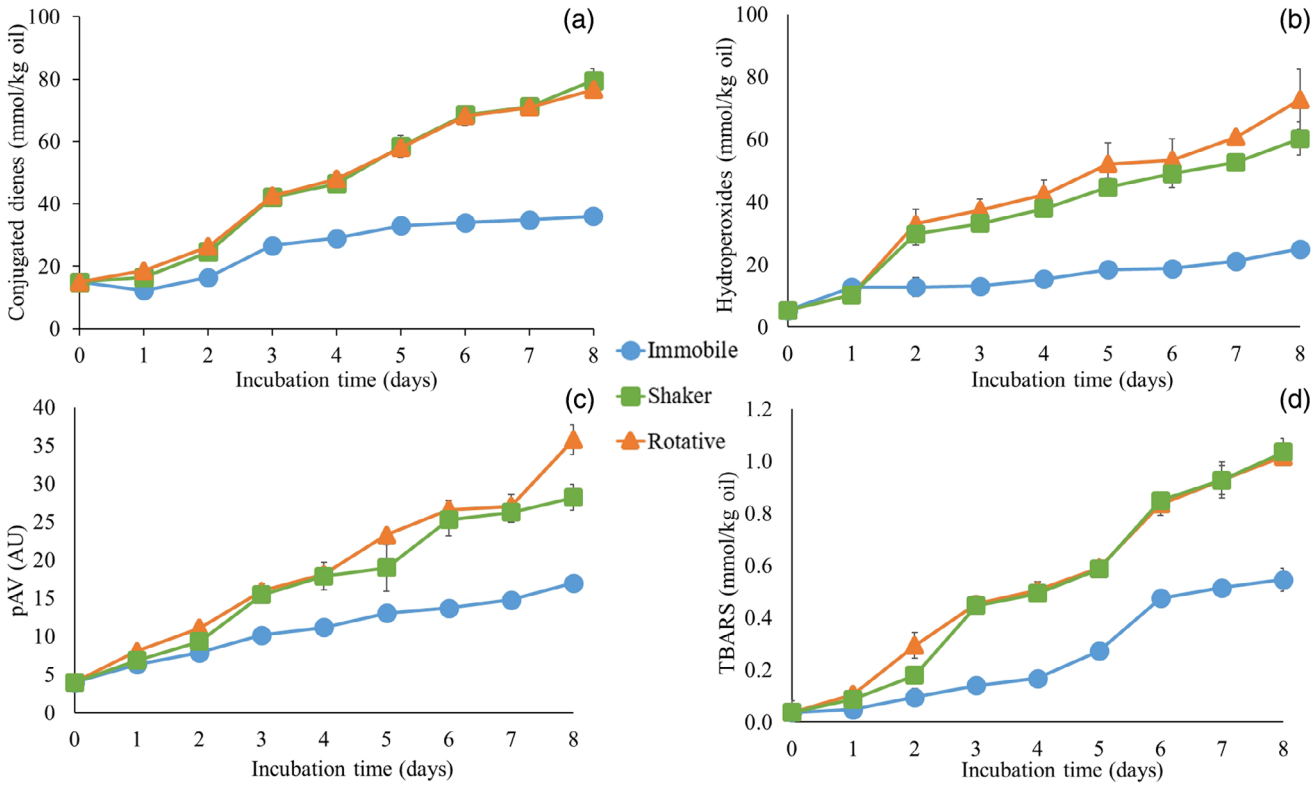

FIGURE 2 Formation of primary (a and b) and secondary (c and d) lipid oxidation products in O/W emulsions stabilized with Tween 20 and incubated with 12% (vol/vol) headspace at 25°C under immobile (●), shaking (■), and rotative (▲) conditions. Error bars represent standard deviations ($n = 4$).

pAV and TBARS of 17 (AU) and ~0.5 mmol/kg oil, respectively.

Genot et al. also reported that development of oxidation products was slow under non-agitated conditions (Genot et al., 2003). The increased oxidation under agitated conditions was related to enhanced mass transfer of for example, oxygen, free radical molecules, ferrous ions, and also oil droplets. We expect that the effects are mostly caused by the larger diffusion distance for oxygen in a system that is standing still. Diffusion is influenced by the viscosity of the medium, and this may be significant for certain formulations where the amount of oxygen is limited and droplet sizes exceed 100 μm (Schroën & Berton-Carabin, 2022). In the present work, the emulsions had a low viscosity and small particle size, and under the conditions used this leads to ideally mixed emulsions with no oxygen diffusion limitation expected to play a role.

In literature, plenty of reasons have been proposed to explain the differences in the course of lipid oxidation between various emulsion formulations. Clearly, oil and emulsifier type/concentration, pro-oxidant/anti-oxidant species and temperature attracted most attention whereas agitation did not. This does not do justice to the effect that this may have, especially when comparing data obtained under different conditions. Our results clearly indicate that agitation speeds up oxidation, and accurate reporting of incubation conditions is essential for fair comparison of data in literature, and reproducibility of work between labs.

Effect of headspace-to-emulsion ratio on lipid oxidation

To investigate the effect of headspace-to-emulsion volume ratio (vol/vol) on the oxidative stability of emulsions containing 200 μM iron, emulsions incubated in closed tubes with 12%, 56%, or 88% (vol/vol) headspace were incubated at 25°C (Figure 3). There was no significant difference in CD concentrations between emulsions incubated with 12% or 56% (vol/vol) headspace, with these concentrations remaining below 76 mmol/kg oil during the full incubation period; however, it is good to point out that the sampling conditions were different (Figure 1). Conversely, the emulsion incubated with 88% (vol/vol) headspace topped at 175 mmol CD/kg oil within one day, to reach 131 mmol CD/kg oil at the end of incubation. The same trend was observed for the hydroperoxide concentrations. Their concentrations in emulsions incubated with 12% or 56% (vol/vol) headspace remained under 73 mmol/kg oil, whereas in the emulsion incubated with 88% (vol/vol) headspace, it reached 261 mmol/kg oil within 2 days before slowly decreasing to 172 mmol/kg oil at the end of the incubation.

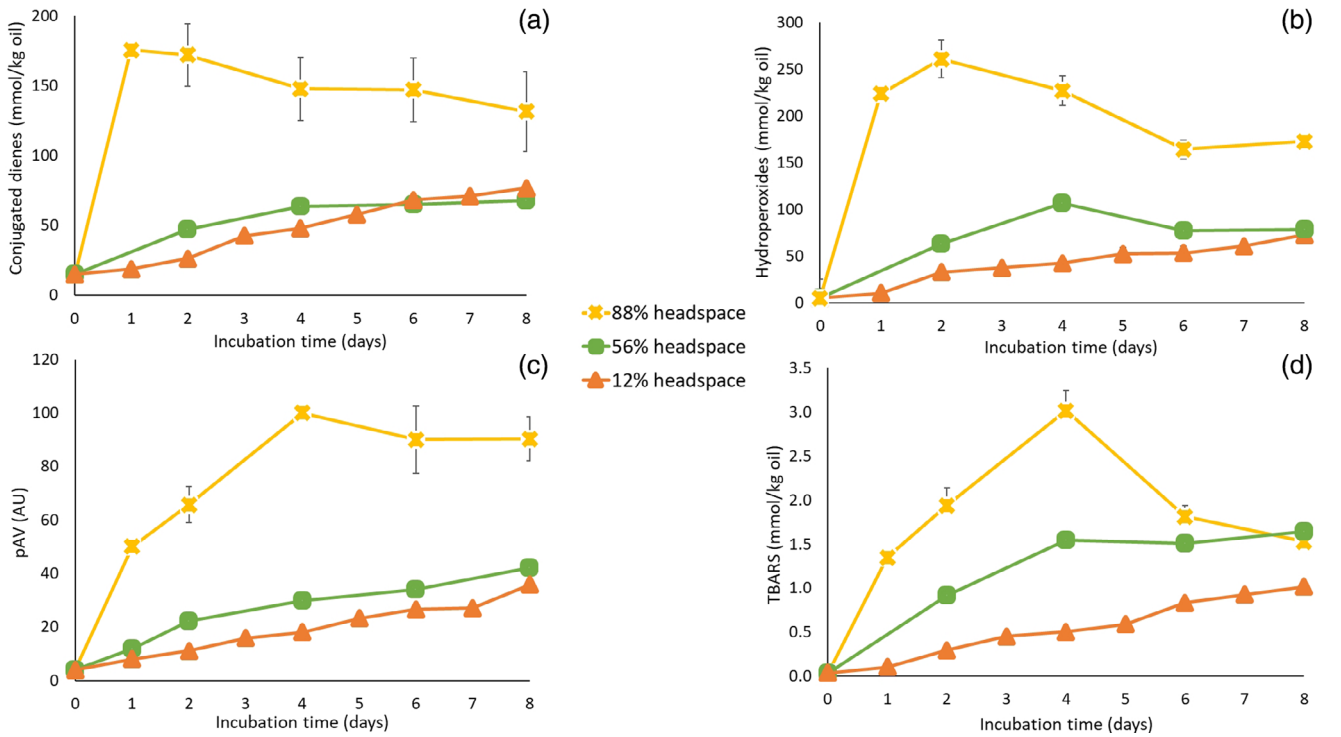

FIGURE 3 Formation of primary (a and b) and secondary (c and d) lipid oxidation products in O/W emulsions incubated at 25°C under rotary conditions with 88% (**x**), 56% (■) or 12% (▲) headspace (vol/vol). Error bars represent standard deviations ($n = 4$).

The impact of the headspace-to-emulsion ratio was also observed in secondary oxidation products, and similar effects were noted as for primary oxidation products, albeit later in time. pAV and TBARS increased during the incubation to reach final concentrations of 36 (AU) and 1.02 mmol/kg oil, in emulsions incubated with 12% (vol/vol) headspace, and 42 (AU) and 1.6 mmol/kg oil, in emulsions incubated with 56% (vol/vol) headspace, respectively. In the emulsion incubated with 88% (vol/vol) headspace, these markers increased to a maximum of 100 (AU) and 3 mmol/kg oil, respectively, after 4 days of incubation, after which values decreased to ~90 (AU) and 1.5 mmol/kg oil at the end of the incubation. This decrease in TBARS concentration and pAV is related to further decomposition of aldehydes into alcohols and acids (Bérces, 1972), which we did not measure as such, but may also have contributed to the pH drop in this emulsion (Table 1).

It is good to stress that different sampling methods were used for the three emulsions, which requires further analysis before a meaningful comparison can be made. The experiments with 56% or 88% (vol/vol) headspace were carried out by taking out a full sample at each time point, whereas for the emulsion incubated with 12% (vol/vol) headspace, the same tube was repeatedly sampled and incubated further. This implies that for the emulsions incubated with 56% or 88% (vol/vol) headspace, the amount of oxygen available for the reaction is determined by the initial headspace volume only (Schroën & Berton-Carabin, 2022), and the rate and extent of the reaction by the ratio of available oxygen relative to oxidizable lipids. In the emulsions with 88% headspace the oxygen-to-oxidizable lipid ratio is approximately eight times higher than for the 56% headspace emulsion. Accordingly, the initial increment of the oxidation products increased. At day 1, for CD, 30 versus 180 mmol CD/kg oil were found for 56% and 88% headspace, respectively. The corresponding hydroperoxide concentrations were 35 versus 230 mmol hydroperoxides/kg oil, respectively. For the emulsion incubated with 12% headspace, we calculated the amount of oxygen available for the reaction over time by considering the amount of oxidation products formed, and the increment in amount of oxygen caused by opening and sampling a given tube multiple times. The actual values are given in the supporting information (Table 2). The amount of oxygen available per kg of oil at the end of incubation was slightly higher than for the emulsion incubated with 56% (vol/vol) headspace, which explains the similarity in the levels of oxidation products found at the end of incubation. This clearly illustrates the importance of not only the incubation conditions and headspace-to-emulsion ratio, but also of the sampling method used. Furthermore, if one is aware of these effects, one can compensate for them and still reach a fair comparison between experiments, and even between research groups.

TABLE 2 Oxygen concentration per kg oil over time.

Sample name	Time (day)	mmol oxygen/kg oil
12% (vol/vol) headspace	0	5.09
	1	7.43
	2	9.72
	3	12.78
	4	15.93
	5	20.05
	6	24.53
	7	30.00
	8	37.38
56% (vol/vol) headspace	0–8	33.10
88% (vol/vol) headspace	0–8	205.61

Marcuse and Fredriksson (1968) studied the effect of oxygen pressure on lipid oxidation in linoleic acid-in-water emulsions stabilized by Tween 20 under shaking (Warburg apparatus at 80 strokes/min and 3 cm amplitude). Lipid oxidation was limited at low oxygen concentration (0.5 and 1% [vol/vol]) because of limited availability of oxygen; it is not expected that any diffusion effects play a role in the work given the agitation used (please check Schroën & Berton-Carabin, 2022 for analysis of relative importance of reaction and mass transfer based on dimensionless numbers). At higher oxygen concentrations (2%–21% [vol/vol]), the reaction was much faster, and also then diffusion was not expected to be limiting.

In literature, also the volume fraction of oil has been considered as a factor that can modulate lipid oxidation. For example, when the oil volume fraction was increased in the range of 6%–44% in safflower oil emulsion containing glycerol, sugars, or sugar alcohols (Sims et al., 1979) observed less lipid oxidation in emulsions (measured by oxygen uptake). We hypothesized that this may be interpreted as an effect of a difference in amount of oxygen per mole of oxidizable lipid (see previous section). In conjunction with that, creaming as elucidated by Genot et al. (2003) may also have consequences for the oxidative stability of emulsions that are standing still, even leading to spatial heterogeneity in case diffusion limitations start playing a role.

CONCLUSION

Our study highlights the importance of the agitation conditions and headspace-to-emulsion ratio on the rate and extent of lipid oxidation in oil-in-water emulsions. Both agitation and increased headspace volume significantly increased the oxidation in a stripped rapeseed oil-in-water emulsion stabilized with Tween 20 and that contained 200 μM ferrous sulfate as oxidation initiator. These results stress the importance of reporting the exact mixing conditions and volume ratios chosen in lipid oxidation

studies, to increase the reproducibility of results and allow comparison among various data from literature.

AUTHOR CONTRIBUTIONS

All authors contributed significantly to the research. Alime Cengiz conducted the experimental work and wrote the final manuscript. Claire Berton-Carabin and Karin Schroën were involved in project- and experimental design, and revision of the final manuscript. Marie Hennebelle contributed to the discussion of the results and to the revision of the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

ETHICS STATEMENT

No human or animal subjects were used in this research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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