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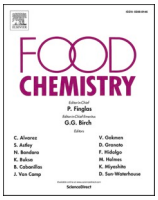

Alkyl chain length modulates antioxidant activity of gallic acid esters in spray-dried emulsions

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ABSTRACT

Lipid oxidation is a well-recognized issue in dried food emulsions, such as infant milk formula. Antioxidants can be used to mitigate this issue; however, their efficiency in such complex systems is far from understood. In this study, antioxidant polarity is varied through the alkyl chain length of gallic acid esters (0–16 carbon atoms) incorporated to O/W emulsions that are subsequently spray-dried. During processing and subsequent storage of the samples, antioxidants with more than eight carbon atoms are effective. Both for encapsulated fat and surface free fat, we observe a slight cut-off effect, meaning that beyond eight alkyl groups, a more nonpolar antioxidant is slightly less effective. Depending on the antioxidant polarity, lipid oxidation is faster either in the encapsulated or in the surface free fat. The insights obtained contribute to understanding lipid oxidation in low moisture food emulsions, and thus lead to effective antioxidant strategies.

1. Introduction

Many food products such as mayonnaise, salad dressing and infant milk formula consist of several immiscible phases, with one phase dispersed in the other as droplets which are stabilized by an emulsifier (e.g., proteins). When such oil-in-water (O/W) emulsions contain polyunsaturated fatty acids, lipid oxidation can readily occur, which has a negative impact on the sensorial and nutritional quality of the products (Schaich, 2005). Lipid oxidation has become a renewed challenge in the current context of enrichment of targeted food products with the long-chain polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), because of their positive impact on human health (Ganesan, Brothersen, & McMahon, 2014). The World Health Organization recently even set regulations for minimum amounts of EPA and DHA in infant formula products. It is now well-known that EPA and DHA improve immune responses, cognition functions and visual acuity, when sufficiently consumed in early life (Joint, 2010; Lien, Richard, & Hoffman, 2018).

In bulk oils and wet O/W emulsions, lipid oxidation pathways have been studied extensively, which has led to insights in the activity of various antioxidants (Shahidi & Zhong, 2010). In bulk oils, polar antioxidants are generally more effective in delaying lipid oxidation compared to relatively nonpolar ones, as described in pioneering work more than 40 years ago (Porter, Black, & Drolet, 1989). It was argued that if lipid oxidation is initiated at the air-oil interface, more polar antioxidants with interfacial activity would be more effective because of their accumulation at this site (Frankel, Huang, Kanner, & German, 1994). Later, the effect of antioxidant polarity in bulk oils was assessed by systematically incrementing the length of the alkyl chain grafted on phenolic acids, forming so-called 'phenolipids', and it was found that also extrinsic factors such as traces of water influence lipid oxidation greatly (Laguerre et al., 2015, Laguerre et al., 2011). The theory that the air-oil interface is the main site of oxidation was later argued to be unlikely, and it was shown that association colloids in bulk oils can act as pro-oxidants (Homma, Suzuki, Cui, McClements, & Decker, 2015). Thus, antioxidant effectiveness in bulk oils seems to be dependent on both intrinsic factors (such as antioxidant hydrophobicity) and extrinsic factors (Laguerre et al., 2015; Phonsatta et al., 2017).

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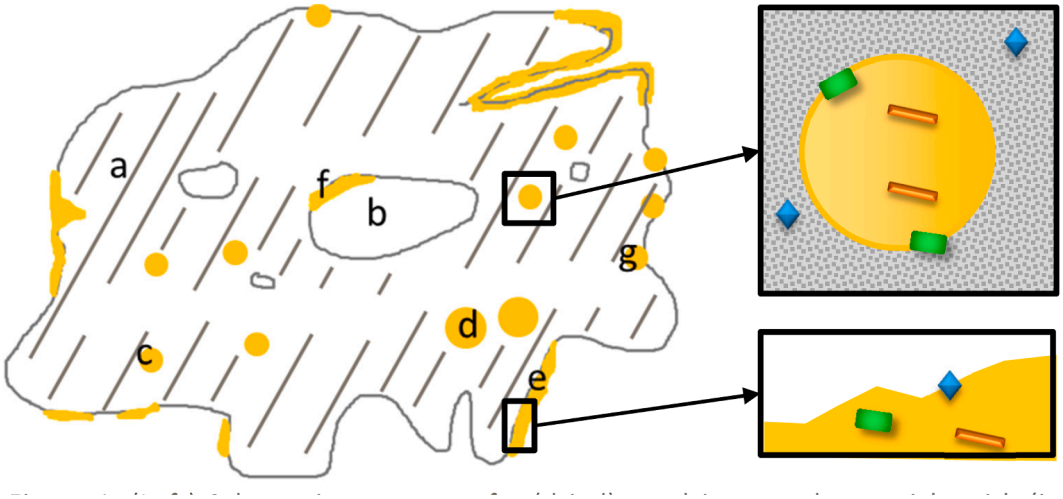

Fig. 1. (Left) Schematic structure of a (dried) emulsion powder particle with (in yellow) fat, and (grey stripes) matrix material, generally present in a glassy state. The particle contains multiple compartments: matrix of sugars and proteins (a), vacuole (b), encapsulated fat (c), coalesced encapsulated fat (d), surface free fat (e), inner free fat (f) and surface globular fat (g). (Right) The blue diamonds, green rectangles and orange rods depict the possible locations of relatively polar, amphiphilic and nonpolar antioxidants, in either encapsulated (top panel) or surface fat (bottom panel), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Opposite to bulk oils, in wet O/W emulsions, the most nonpolar antioxidants were initially reported to be the most effective, which was termed as 'the polar paradox' (Porter et al., 1989). In such wet emulsions, the antioxidant polarity was also systematically varied, and the antioxidants with an intermediate alkyl chain length were the most effective. This gave further nuance to the polar paradox, and is known as the 'cut-off effect' (Laguerre et al., 2015, Laguerre et al., 2009). The most likely explanation for this effect is the tendency of amphiphilic molecules to accumulate near the oil–water interface, which is postulated to be the site of lipid oxidation initiation in an emulsion (Laguerre et al., 2015; Laguerre, Bily, Roller, & Birtić, 2017; Phonsatta et al., 2017). However, this effect seems to be dependent on extrinsic factors as well; it was found that antioxidant effectiveness in emulsions also depends on the phase to which it was added (oil or water phase), on the emulsifier, and on the polarity of the oxidation initiator (da Silveira et al., 2021; Phonsatta et al., 2017; Stöckmann, Schwarz, & Huynh-Ba, 2000).

Infant milk formulas are generally sold as powders, which have to be reconstituted by dispersion in water before consumption. In these powders, oil is either entrapped as droplets in a dry matrix, or present as free fat on the surface (Fig. 1) (Vignolles, Jeantet, Lopez, & Schuck, 2007). This structural difference from wet O/W emulsions is expected to affect lipid oxidation, but insights in the oxidation behavior in low moisture food emulsions are still lacking (Barden & Decker, 2016; Velasco, Dobarganes, & Márquez-Ruiz, 2003). Some studies interpret the data of a simultaneous degradation of antioxidants and formation of lipid oxidation products in encapsulated fat as an evidence for a different lipid oxidation status between the droplets (Morales, Marmesat, Ruiz-Méndez, Márquez-Ruiz, & Velasco, 2015; Velasco, Marmesat, Dobarganes, & Márquez-Ruiz, 2006). In contrast, the oxidation of surface free fat is reported to start with the degradation of antioxidants, during which the formation of lipid oxidation products is suppressed, followed by a rapid increase in lipid oxidation products, which is comparable to the situation in bulk oils (Morales et al., 2015; Velasco et al., 2006). In dried emulsions, it is expected that the oil-solid matrix interface, which surrounds the encapsulated fat droplets, has an effect on lipid oxidation that largely differs from the role of the oil–water interface in wet emulsions. Velasco and coworkers recently tested different gallic acid alkyl esters in such products, and found that the most nonpolar one that they tested (lauryl gallate) was the most effective at delaying lipid oxidation in both the free and encapsulated fat (Velasco, Holgado, Dobarganes, & Márquez-Ruiz, 2009). If oxidation of surface free fat would proceed in a similar manner as in bulk fat, it may be expected that polar antioxidants would be the most effective, but that was not the case (Velasco et al., 2009).

To assess whether an optimal alkyl chain length also applies in dried systems, a next step would be to use a broader range of phenolipids with increasing alkyl chain length, up to nonpolar ones. We assume that phenolipids with a medium chain length are especially of great interest for application in dried emulsions given the combined protective effects found in both wet emulsions, and in bulk oil. Furthermore, these antioxidants may be instrumental in preventing lipid oxidation in liquid emulsions (the starting material for the powders), and preventing early oxidation events, which has been shown to be detrimental to the subsequent oxidative stability of the dry product (Sánchez, Cuvelier, & Turchiuli, 2016).

In the present study, we aimed to unravel the effect of phenolipid chain length on lipid oxidation in the free and encapsulated fat fractions of dried emulsions. For this, the polarity of the antioxidant gallic acid was systematically varied by grafting alkyl chains of increasing length (gallic acid [G0], propyl [G3], octyl [G8], lauryl [G12] and hexadecyl gallate [G16]), and these gallates were incorporated in O/W emulsions that were subsequently spray-dried. The obtained powders were incubated at 40 °C under relative humidity of 50%, and lipid oxidation was measured over time with nuclear magnetic resonance (^{1}H NMR), which allowed for measuring a range of oxidation markers simultaneously (Merkx, Hong, Ermacora, & Van Duynhoven, 2018). We compared initial formation of lipid oxidation products using a curve fit procedure, which allowed us to chart differences on a quantitative, objective basis.

2. Material and methods

2.1. Materials

Whey protein isolate (WPI) with purity 97.0–98.4% (BiPro®, Davisco, Switzerland), and sodium caseinate (SC) with purity 97% (Excellion™, Sodium Caseinate S, Friesland Campina, the Netherlands) were used as dairy protein sources. Maltodextrin with a dextrose equivalent of 21 was kindly provided by Nutricia, Danone, (Utrecht, Netherlands). Sunflower oil was obtained from a local supermarket, mixed with fish oil (3 wt% of total oil) (MEG-3, DSM Nutritional Products, Canada), and stripped with alumina powder (MP EcoChromet

ALUMINA N, Activity: Super I, Biomedicals) to remove impurities and endogenous antioxidants, in particular tocopherols (Berton, Genot, & Ropers, 2011). *n*-Hexane and 2-propanol were obtained from Actu-All Chemicals (Oss, the Netherlands). Deuterated chloroform and dimethylsulfoxide ($CDCl_3$ and DMSO-d_6) were purchased from Euriso-top (Saint-Aubin, France). Ultrapure water (18.2 MΩ) was used for all experiments, and prepared using a Milli-Q system (Millipore Corporation, Billerica, MA, USA).

2.2. Synthesis of gallic acid esters

The synthesis of gallic acid alkyl esters was performed according to an established procedure (Durand et al., 2019). In brief, gallic acid (850.6 mg, 5 mmol) and its corresponding alcohol (15 mmol) were dissolved in dry *p*-dioxane (10 mL) in a 50-mL round bottom flask. Next, concentrated sulfuric acid (5 mmol, 273 μL) was added, the mixture was refluxed for 8 h and the reaction's progress was monitored by thin layer chromatography. The solvent was removed under vacuum and the alkyl gallates were separated through column chromatography (20% ethyl acetate in methylene chloride). The purity was measured with ^{1}H and ^{13}C NMR, using a Bruker AVIII-HD-500 at 500 MHz and 126 MHz, respectively, in DMSO-d_6 (Appendix A). For all gallates the purity was >99%.

2.3. Preparation of the aqueous phase

The day before the emulsions were made, all glassware was cleaned with detergent and rinsed with ultrapure water to ensure that it was free of possible contaminants that might influence oxidation. First, WPI (1.22 wt% of final emulsion) was dissolved in ultrapure water for 30 min at room temperature by gentle stirring, next sodium caseinate (4.87 wt %) was added, and the mixture was stirred for 2 h at 50 °C. Finally, maltodextrin DE 21 (27.9 wt%) was added and the solution was stirred for an additional 30 min.

2.4. Preparation of the emulsions

Emulsions containing the different antioxidants (gallic acid [G0], propyl [G3], octyl [G8], lauryl [G12] and hexadecyl gallate [G16]) were prepared (total mass of each emulsion was 246 g). The antioxidants were first dissolved in methanol in such concentrations that adding 100 μL methanolic solution to the oil leaded to a concentration of 600 μmol of antioxidant / kg oil (equivalent to 100 mg/kg for gallic acid). Methanol was next evaporated by placing the mixture under a flow of nitrogen. Stripped oil (14.8 wt% sunflower oil and 0.46 wt% fish oil), with or without antioxidant was added to the previously prepared aqueous phase to form the emulsion. First, a coarse emulsion was made by high-speed stirring at 11,000 rpm for 1 min with a rotor–stator homogenizer (Ultra-turrax IKA T18 basic, Germany). The coarse emulsion was then passed two times through a lab scale colloid mill with gap width of 0.32 mm (IKA Magic Lab, Staufen, Germany), operated for 1.5 min at 26,000 rpm.

2.5. Spray drying of the emulsions

The emulsions were spray-dried using a Büchi B-290 laboratory spray dryer (Büchi Labortechnik AG, Flawil Switzerland). The inlet air temperature was set to 180 °C and the flow rate was between 35 and 45% to obtain an outlet air temperature of 100 °C. The aspirator was set to 90%. The amount of powder obtained was about 20 g with an average particle size of 46 ± 8 μm and an average moisture content of 3.0 ± 0.4 wt%. The powder yield was ~16% of the initial dry matter, which was relatively high compared to other values reported in literature for high-oil emulsions using lab-scale spray dryers (Langrish, Marquez, & Kota, 2006).

2.6. Sample incubation

Aliquots of powder (1.5 g) were distributed in 50-mL plastic cups without lids, which were incubated in a climate chamber (Memmert, Büchenbach, Germany) at 40 °C and 50% relative humidity in the dark. At regular time intervals, samples from two aliquots were taken for further measurements.

2.7. Free and encapsulated fat extraction

Surface free fat and encapsulated fat were extracted using the methods described by Kim et al. and Sánchez et al., with small adjustments (Kim, Chen, & Pearce, 2005; Sánchez et al., 2016). In brief, 1.5 g powder was washed three times with 6 mL hexane. For each washing step, the hexane and powder mixture were rotated vertically for 10 min at 20 rpm. Prior to filtration, the powder was allowed to sediment to the bottom of the tube (~5 min), and next the hexane phase was filtered two times (No. 4, Whatman, Maidstone, Kent, UK). Hexane was then evaporated under a stream of nitrogen at 25 °C until constant weight and the remaining oil was frozen at −80 °C and stored for 48 h to three weeks before further measurements were performed.

To extract the encapsulated fat, the powder (collected after extraction of surface free fat) was reconstituted by adding 1 mL of ultrapure water to 0.25 g of powder at 50 °C and by vortexing two times 1 min. The extraction was then performed by adding 8 mL hexane–isopropanol (3:1 v/v) to 1.5 mL reconstituted emulsion. The mixture was centrifuged at 5000x*g* for 20 min and the upper layer, containing the hexane and extracted lipids, was carefully separated from the bottom layer. The hexane then was evaporated in the same way as for the surface free fat.

2.8. Measurements

2.8.1. Oil droplet size

The oil droplet size in the emulsion was measured by static light scattering (Malvern Mastersizer 3000, Malvern Instruments Ltd., Malvern, Worcestershire, UK). The refractive index was 1.465 for the dispersed phase (mix of stripped sunflower and fish oil) and 1.33 for the dispersant (water). The absorption index was 0.01. The average droplet size (D[3,2]) of the emulsions was 0.9 ± 0.04 μm (Fig. A1).

2.8.2. Powder particle size

The powder particle size was measured with light microscopy imaging (Malvern Morphology 4, Malvern instruments Ltd., Malvern, Worcestershire, UK). The powder was spread over the microscope glass slide by the automatic powder dispenser with an applied air pressure of 3 bar.

2.8.3. Lipid oxidation by NMR

Hydroperoxides (primary oxidation products), aldehydes (secondary oxidation products), gallates (antioxidants) and triacylglycerols (as a reference for the total amount of oil) were simultaneously quantified by ^{1}H NMR using an Advance III 600 MHz spectrometer equipped with a 5 mm cryo-probe at 295 K, following the method described by (Merkx et al., 2018). In brief, 550 μL 5:1 $CDCl_3$/DMSO-d_6 was added to a total of 30–60 μL extracted oil (as described in 2.4) and transferred to 5-mm NMR tubes (Bruker, Billerica, MA, USA). From the recorded single pulse experiment, the glycerol backbone peaks at δ 4.4 pm were used for the quantification of the amount of triacylglycerols, and the gallate peak at 7.1 for the quantification of the amounts of gallates. From the band selective pulse, the region between δ 13.0 and 8.0 ppm was selectively excited for the quantification of the lipid oxidation products. The hydroperoxide signals resonate between δ 11.3 and 10.6 ppm and the aldehydes between δ 9.8 and 9.4 ppm. The calculations, including a factor that accounts for intensity loss during the selective pulse, were described in (Merkx et al., 2018). The data was processed with the Bruker TopSpin 4.0.6 software.

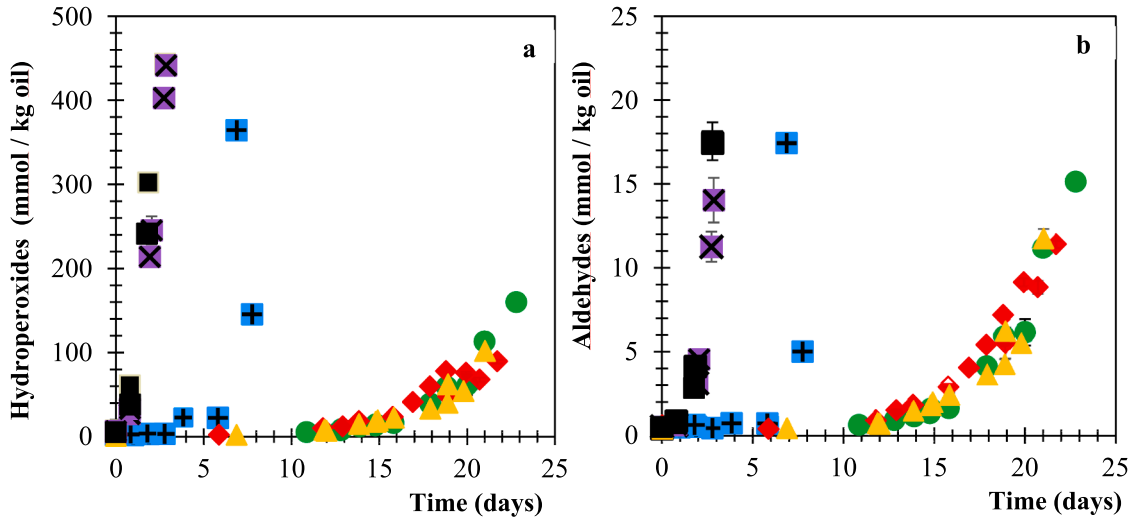

Fig. 2. Formation of hydroperoxides (a) and aldehydes (b) in the encapsulated fat of the spray-dried emulsions over incubation. Symbols correspond to the systems containing gallates with different alkyl chain length: gallic acid (G0) (X), propyl gallate (G3) (+), octyl gallate (G8) (●), dodecyl gallate (G12) (▲) and hexadecyl gallate (G16) (◆); and to the blank (no antioxidant) (■). Error bars denote standard deviations of two measurements on the same sample. The outcomes of the independent replicates are shown as separate points.

2.8.4. Sorption isotherm

The sorption isotherms of one sample containing G8 and one sample containing G12 were determined with a dynamic vapor sorption elevated temperature analyzer (Surface Measurement System, London, UK) at a temperature of 40 °C. For the measurement, 10 ± 1 mg of sample was used. The relative humidity was varied between 0 and 90 % with steps of 10% and the sample weight was equilibrated at each step. Equilibration was considered completed when the change in mass was<0.001 mg/min. The temperature, humidity and mass were recorded every min.

2.9. Experimental design and data fitting procedure

Two spray-dried emulsions were prepared independently for each gallate used. Per time point, two aliquots of powder were taken, the free and encapsulated fat fractions were extracted, and further measurements were performed. We considered various standard equations to fit the experimental hydroperoxide concentration data, and found that an exponential equation (equation (1)) was best suited based on the Akaike criterion that encompasses agreement of fit, and number of parameters used. Furthermore, we systematically checked the distribution of the residuals.

$$y = y_0 e^{kt} \quad (1)$$

In this equation y is the hydroperoxide concentration (mmol/kg oil), y_0 the hydroperoxide concentration just after spray drying (mmol/kg oil), k is indicative for hydroperoxide formation rate (day^{-1}) and t the time (days). Please note that k should not be interpreted as an actual reaction rate, since it does not capture the full complexity of the cascaded reactions involved in lipid oxidation. Yet, it is useful to compare our samples in a quantitative, objective manner.

Both y_0 and k were used as fitting parameters, and the residual sum of squares was minimized based on relative differences with the measured data. In this way, all data points were equally weighed in the determination of the parameters that otherwise would be completely dominated by the highest values measured. The estimated y_0 value was compared with the measured initial hydroperoxide concentration. The 95% confidence intervals for k and y_0 were calculated using the Student's *T*-distribution formula in Excel (Microsoft Office 2016) with a sample size of 2 (two emulsions were produced and incubated independently).

3. Results and discussion

Regarding the general properties of the powder that we prepared, it had an average moisture content of 3.0 ± 0.4 wt% after spray drying; the sorption isotherms (moisture content against water activity) at 40 °C are shown in Fig. A2, and were identical for both antioxidants used, as expected. The powder had an average surface free fat content of 2.3 ± 0.3 wt% (compared to the total mass of powder, this represented around 7.6 wt% of the total lipids), and an average particle size of 46 ± 8 μm.

3.1. Oxidation in encapsulated fat

Lipid oxidation was measured with ^{1}H NMR spectroscopy, which allows for measuring a range of hydroperoxides and a range of aldehydes simultaneously; more information about this method can be found in the work of Merkx and coworkers (Merkx et al., 2018). The amounts of hydroperoxides and aldehydes formed over time in the encapsulated fat are shown in Fig. 2a and b, respectively. High levels of oxidation products were formed after a few days for the blank system that did not contain any antioxidant. The amounts of hydroperoxides and aldehydes were only slightly lower when gallic acid was present, and the antioxidant effectiveness increased with the alkyl chain length. The gallates with a medium to long alkyl chain (G8, G12 and G16) were all able to delay the formation of high amounts of lipid oxidation products by around fifteen days.

Although we added all gallates to the oil phase before making the emulsion and spray drying it, they can be expected to rapidly partition between the dispersed phase, the interface and the continuous phase. The actual partitioning coefficients (log [p_w^o]) between a vegetable oil and citric acid buffer are negative for gallic acid (~−1) and increases linearly with the gallates alkyl chain lengths up to G4 (Freiría-Gándara, Losada-Barreiro, Paiva-Martins, & Bravo-Díaz, 2018). This indicates that especially G0 would preferably locate in the polar water phase, and thus likely end up to a large extent in the glassy matrix after spray drying, where it has a very limited mobility making it ineffective. Furthermore, it has been reported that gallic acid (G0) can form non-covalent bonds with both caseinate and whey proteins (at pH 6.0 and 7.0 respectively), which could cause it to be even less effective (Cao & Xiong, 2017; Zhan et al., 2020). Based on the results of caffeic acid esters in fish oil-enriched milk discussed above (Alemán et al., 2015), one could expect G3 to be relatively effective compared to G8-G16, but this

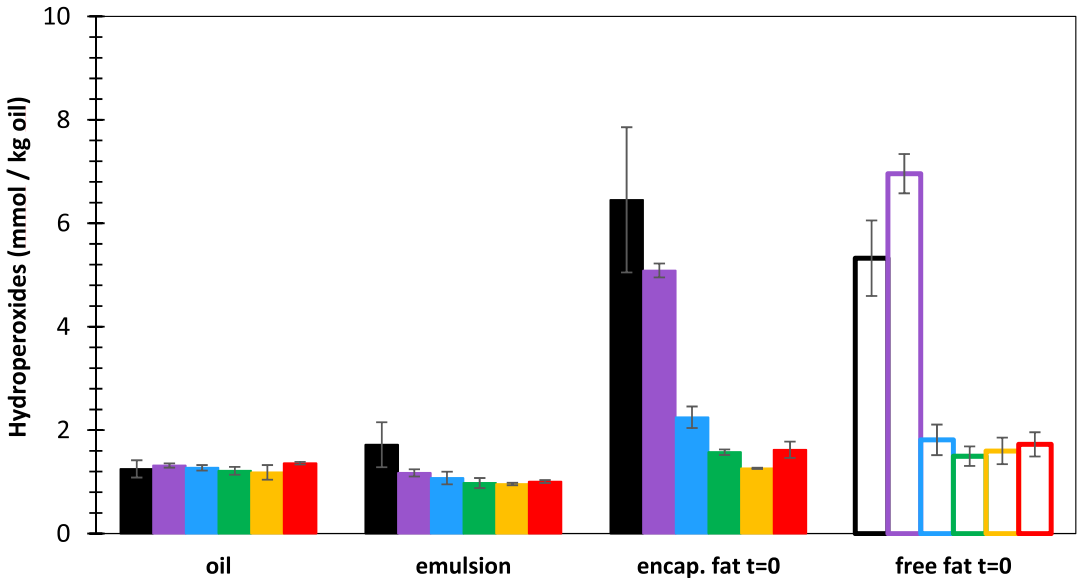

Fig. 3. Formation of hydroperoxides during processing. The bars with different colours correspond to systems with no antioxidant (blank) or gallates with various alkyl chain lengths. From left to right: Blank (black), G0 (purple), G3 (blue), G8 (green), G12 (yellow) and G16 (red). Error bars denote standard deviations of two independent experiments that are both measured twice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was clearly not the case (Fig. 2). G3 has been reported to partition mainly to the oil–water interface in wet (surfactant-based) emulsions (Losada Barreiro et al., 2013). Part of this interface may become glassy, therewith reducing the accessibility of G3 to the oxidizing lipids, or we can also not exclude that during drying the partitioning of G3 may be shifted to the surrounding glassy matrix. Additionally, based on the results of Freiría-Gándara and coworkers, the gallates with alkyl chain lengths $\geq$ G8 were expected to end up in the encapsulated oil at higher concentrations compared to G0 and G3, with little differences between G8, G12 and G16. We think that this can explain, for a large part, the reported results in effectiveness to prevent lipid oxidation.

In the work of Velasco and coworkers on freeze-dried emulsions, it was concluded that their most hydrophobic gallate (G12), was the most effective in the encapsulated fat (Velasco et al., 2009), which is not completely in line with our results because we do not see a distinct difference between G8, G12 and G16 (Fig. 2). This is actually more in line with another study about roasted peanuts (which can be regarded as a low-moisture system with dispersed lipids), in which hydrophobic antioxidants (in this case, G8 and G12) were also found to be the most effective (Phonsatta et al., 2017). The suggested rational was that polar antioxidants were not readily present in the oil phase, which made them less effective than more nonpolar ones. In wet O/W emulsions, gallates with an intermediate polarity (~G3) were generally the most effective (Losada Barreiro, Bravo-Díaz, Paiva-Martins, & Romsted, 2013; Stöckmann et al., 2000), although this has been shown to be dependent on the type of emulsifier or oxidation initiator as well (da Silveira et al., 2021; Phonsatta et al., 2017; Stöckmann et al., 2000). Also for fish oil-enriched milk, which is a wet emulsion system that can be compared with our model dairy emulsion, the caffeic acid esters with intermediate alkyl chain lengths (G1-G4) were the most effective (Alemán et al., 2015). Since the interface is often regarded as the actual site where lipid oxidation occurs in a wet emulsion (Berton-Carabin, Ropers, & Genot, 2014; Laguerre, Tenon, Bily, & Birtić, 2020), this suggests that the antioxidant has to be close to this site to be effective. Romsted and Bravo-Diaz have developed a pseudophase kinetic model to determine the partitioning of chain-breaking antioxidants in surfactant-stabilized emulsions (Romsted & Bravo-Díaz, 2013; Romsted & Zhang, 2002). With this method, they showed that the effectiveness of gallates with an intermediate polarity (~G3) could be linked to their partitioning in high concentrations at the interface (Losada Barreiro et al., 2013). Yet, it is questionable to which extent this pseudophase kinetic model can be used for our system for two reasons: (1) we worked with a protein-stabilized emulsion consisting of different protein types, which has not been covered using with the pseudophase model, and (2) the matrix surrounding the droplets is in the glassy state which can influence diffusivity greatly compared to the situation for which the pseudophase model was derived (fully liquid surfactant-stabilized emulsions). Therefore, in a spray-dried emulsion, it is still unknown whether the oil-matrix interface is also the location where lipid oxidation is initiated.

Another aspect that we considered, is that during spray drying a fraction of the gallates might be chemically degraded. Therefore, we measured the concentrations of gallates by NMR and found no marked differences in the concentrations of the relatively nonpolar gallates (G8, G12 and G16) (G0 cannot be measured), compared to the amounts in the emulsion or in the oil (Fig. A3). A recent study on the thermal stability of gallic acid showed that this phenolic compound is hardly degraded in aqueous solution when subjected to a temperature of 100 °C for half an hour (Volf, Ignat, Neamtu, & Popa, 2014). All this suggests that in our systems, the gallates were hardly affected by the sample preparation procedure, if at all.

We did find differences in the initial amounts of lipid oxidation products in the different powders, and that is why we have traced their formation throughout the production process. The concentration of hydroperoxides in the oil (prior to emulsification), in the wet emulsions and directly after spray drying in the encapsulated fat is shown in Fig. 3. None of the antioxidants totally prevented an onset of lipid oxidation during processing, which is in line with the study of Sánchez and co-workers (Sánchez et al., 2016). A slight increase in hydroperoxides can be observed for the blank during emulsification, which is in line with studies on homogenization of milk protein-stabilized emulsions (Anna F Horn, Barouh, Nielsen, Baron, & Jacobsen, 2013; Horn, Nielsen, Jensen, Horsewell, & Jacobsen, 2012). The subsequent spray drying had a more pronounced effect in that respect, probably due to the high temperatures (~100 °C) used in the process (Baik et al., 2004). When comparing the effectiveness of the gallates during processing and storage, we generally observed that the antioxidants that are the most effective during storage were also the most effective during the processing (Fig. 3). One exception was propyl gallate (G3), which was relatively effective during

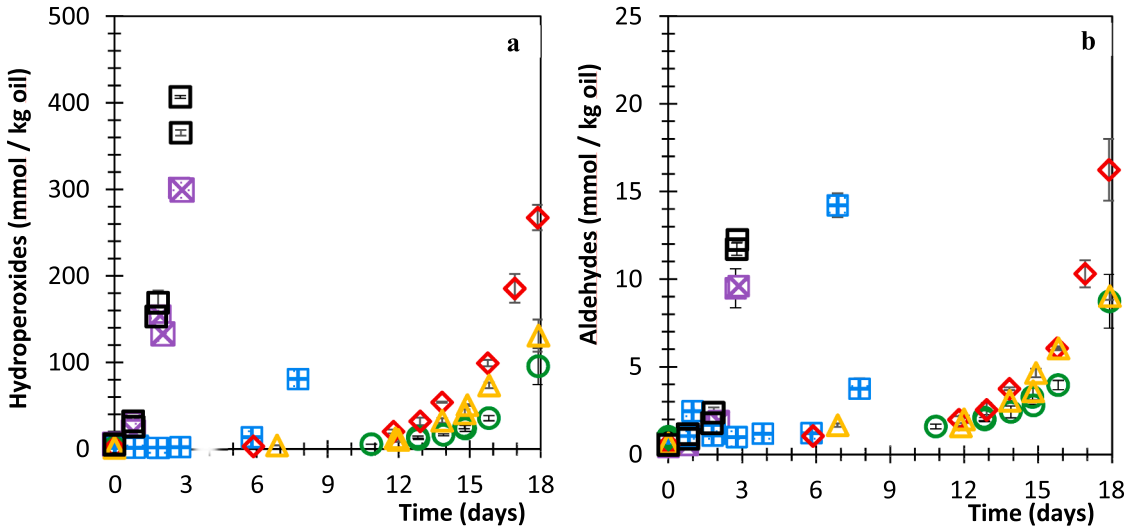

Fig. 4. Formation of hydroperoxides (a) and aldehydes (b) in the free fat of the powders over time. Symbols correspond to the systems containing gallates with a varied alkyl chain length: gallic acid (G0) (╳), propyl gallate (G3) (+), octyl gallate (G8) (●), dodecyl gallate (G12) (▲) and hexadecyl gallate (G16) (◆); and to the blank (no antioxidant) (■). Error bars denote standard deviations of two measurements on the same sample. The outcomes of the independent replicates are shown as separate points. For clarity, the data until 18 days are shown. The graphs with the data until 25 days can be found in Figs. A4 and A5.

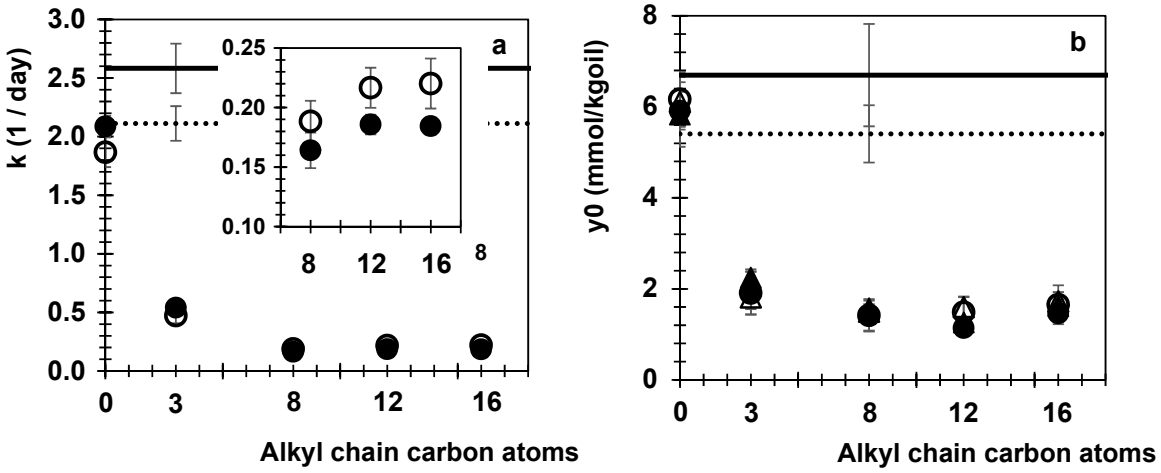

Fig. 5. The fitting parameters k (indicative for hydroperoxide formation (d^{-1})) (a) and y_0 (concentration of hydroperoxides in the dried emulsion at $t = 0$) (b) against the alkyl chain length of the gallates for free fat (open symbols) and encapsulated fat (closed symbols). The dashed and solid lines denote the values for the free and encapsulated fat in emulsions without antioxidant, respectively, and the triangles (Δ) in (b) denote the experimentally measured values. Error bars represent 95% confidence intervals, some being within the marker points. The insert in panel (a) is a magnification of the right part of the graph with k for G8 to G16.

processing, but not that much during storage. This can be related to previous studies showing that in wet emulsions, moderately amphiphilic gallates such as G3 can be effective, which is often related to their partitioning at the interface (Losada Barreiro et al., 2013; Stöckmann et al., 2000). Yet, this is not the case in the powders, probably due to its partitioning as described above. Clearly, positive effects obtained in wet emulsions (prior to spray drying) are not always indicative for effects occurring during storage of the powders, which we already concluded when we compared our results to fish oil-enriched milk (Alemán et al., 2015). Although the amounts of lipid oxidation products that are formed prior to drying are very limited, it is thought that they can still play a significant role in the development of oxidation later on (Sánchez et al., 2016), which may also explain variations between outcomes reported in literature (Drusch, Serfert, Scampicchio, Schmidt-Hansberg, & Schwarz, 2007).

To wrap up, phenolipids have to be relatively nonpolar to optimally protect the encapsulated fat in dried emulsions, compared to wet emulsions. For wet emulsions, there are three possible explanations for the fact that more nonpolar antioxidants are less effective: (1) reduced mobility of the antioxidant molecule at longer alkyl chain length, (2) self-aggregation of the antioxidant, therewith lowering mobility, and (3) localization of the antioxidant away from the interface (Laguerre et al., 2015; Phonsatta et al., 2017). Since increasing the alkyl chain length of the gallates from G8 to G12 to G16 did not greatly influence oxidation in the encapsulated fat, these effects do not play major roles in the encapsulated fat of dried emulsions. However, especially explanation (1) and (2) could (together with the similar p_w^o *for G8-G16* (Freiría-Gándara et al., 2018)) explain why our most hydrophobic antioxidant (G16) is not the most effective antioxidant in the encapsulated fat. In addition, it has been reported previously that the complexity of real food

emulsions (such as milk and mayonnaise) can contribute to a less obvious effect of the alkyl chain length of antioxidants on their effectiveness compared to model O/W emulsions (Alemán et al., 2015; Laguerre et al., 2009).

3.2. Oxidation in surface free fat

In a similar manner as done for the encapsulated fat, we now discuss how lipid oxidation proceeds in surface free fat. In the next section, we use a fitting procedure to compare both fat fractions, and the respective effects that antioxidants (at 600 μmol/kg oil) have on the overall course of hydroperoxide formation. For surface free fat, we observed similarities with the trends observed for the encapsulated fat: compared to the blank without antioxidant, gallic acid slightly decreased the concentration of lipid oxidation products, but the G8, G12 and G16 gallates delayed lipid oxidation much more effectively during processing and subsequent storage (Fig. 3, Fig. 4). For surface free fat, the formation of hydroperoxides seemed to be a function of the alkyl chain length amongst the three longest carbon chain antioxidants tested. In fact, G8 seemed to be more effective than G12, which in turn was more effective than G16, and this was visible in the formation of both primary and secondary lipid oxidation products (whether these differences are statistically significant is further discussed in section 3.3). In wet emulsions, this is known as the cut-off effect (Laguerre et al., 2009). This cut-off effect was not found for dried emulsion by Velasco and coworkers, who concluded that hydrophobic gallates are more effective for the free fat fraction, compared to more hydrophilic antioxidants (Velasco et al., 2009). Yet, a possible cut-off effect for dried emulsions could probably not be studied because only G0, G3 and G12 gallates were used. From a structural point of view, it has been proposed that free fat has similarities with bulk oil (i.e., a limited air-oil interface, and a potentially important role of dispersed colloidal structures based on polar lipids) (Laguerre et al., 2015; Morales et al., 2015; Velasco et al., 2006). Regarding the pathways of lipid oxidation in bulk oil, the so-called polar paradox was formulated, which implies that the most hydrophilic antioxidants are the most effective (Porter et al., 1989; Velasco et al., 2006), as exemplified for a homologous series of gallates (Phonsatta et al., 2017). For our dried emulsions, this relation was clearly not found: the antioxidant has to be hydrophobic enough to partition into the oil phase of the wet emulsion (which is probably not the case for G0 and G3 as described above). When increasing the alkyl chain length further, the antioxidants seem to become less effective, although the actual differences are small (Fig. 4) (also see section 3.3). Such a slightly better performance of the G8 antioxidant, compared to G16, might be due to a higher affinity for the interface, which can be an asset when pro-oxidant colloidal structures are involved (Frankel et al., 1994; Laguerre et al., 2015). This tends to validate the hypothesis for a substantial involvement of dispersed colloidal structures in the surface free fat. Given the fact that stripped oil was used, it is likely that such structures could be formed as early as the homogenization process.

3.3. Initial hydroperoxide formation

The dry powders were stored under conditions where the oxygen concentration was constant and thus non-limiting. Here, we only considered the initial stages of oxidation (when typically <10% of the oxidizable bonds have reacted, which corresponds to hydroperoxide concentrations below 50 mmol / kg oil (Fig. A6)), so we can assume that the concentration of oxidizable bonds did not largely change compared to the initial concentration.

The data were fitted by nonlinear regression using Eq. (1), and k and y_0 were both used as fitting parameters (for motivation of the choices made, please consult the materials and methods section). In general, the accuracy of the fit that we found is high for all curves tested, and also the residuals were distributed evenly, which is a requirement for any fitting equation (the outcomes of the fit are shown together with the data points <50 mmol/kg oil in Fig. A6, and the residual plot is shown in Fig. A7). In doing so, we compare the different gallates directly based on the effect they have on the course of the initial hydroperoxide formation in the dried emulsions. The values found for y_0 were compared with the actual measured values at $t = 0$ (triangles, Fig. 5b), and found to be in very good agreement. The values for k and y0 are plotted against the alkyl chain length of the gallates in Fig. 5a and b, respectively.

When considering both k and y_0, we can confirm that in both fractions the relatively nonpolar antioxidants (G8, G12 and G16) were by far more effective at delaying lipid oxidation, both initially (y_0) and during incubation (k) than their shorter counterparts, of which G3 was still more effective compared to gallic acid. The data, which have proved to be very reproducible (as revealed by the low standard deviations), also allow us for establishing comparisons in the performance of antioxidants, even when differences are very small.

When using no antioxidant or a relatively polar antioxidant, lipid oxidation was faster in the encapsulated fat than in the surface free fat, but this trend switched when increasing the alkyl chain length (Fig. 5 and Figs. A4 & A5). An obvious difference between the free and encapsulated fat that may explain this crossover is the nature of the interface to which it is exposed, either air, or the solid matrix. This may imply that the differences in antioxidant effectiveness are related to the affinity of the antioxidant for the interface. Especially for the surface free fat, the antioxidant effectiveness seemed to be reduced beyond G8 (Fig. 4a). This effect seemed less marked for the encapsulated fat, and this led to this switchover (Fig. 5a) around G8. Since the 95% confidence intervals overlap slightly for G8, G12 and G16 (error bars in Fig. 5a, insert), the significance of this effect is subject to discussion.

When we did not use any antioxidant, we observe that encapsulated fat oxidized faster than free fat, although the differences were rather small. In literature, it has been reported frequently that surface free fat oxidizes faster (Morales et al., 2015; Velasco et al., 2006), which is mostly ascribed to a better oxygen availability for this fraction (Velasco et al., 2003, Velasco et al., 2009). On the other hand, it is well known that oxygen is able to diffuse through glassy matrices, although the diffusion rate is rather low and affected by the matrix components present (Drusch et al., 2009). For example, low molecular weight carbohydrates (i.e., high dextrose equivalent) tend to decrease the oxygen diffusivity (Drusch et al., 2007), which was relevant for our model system, containing maltodextrin (dextrose equivalent of 21). On the other hand, our model system contained relatively high amounts of proteins, which has shown to increase the free volume of the matrix material and therewith the accessibility of oxygen to oil (S Drusch et al., 2009). Recently, Linke et al. showed that the so-called internal oxygen (oxygen in oil and powder particle) only has a minor influence on oxidation of spray-dried emulsions (Linke, Linke, & Kohlus, 2020). They concluded that the transfer rate of oxygen through the matrix determines oxidation of encapsulated fat, and that this transfer rate can be relatively high compared to the reaction rate. Since we found hardly any difference between free and encapsulated fat, we expect that oxygen mass transfer limitations in the matrix did not apply in the systems that we studied.

4. Conclusion

In this work, we varied the alkyl chain length of gallic acid alkyl esters and measured lipid oxidation in the surface free fat and in the encapsulated fat of a spray-dried emulsion. For both fat fractions, we have shown that the alkyl chain length has to be relatively long (so, the antioxidant molecule should not be too polar) in order to be effective at delaying lipid oxidation. Gallates with an alkyl chain length of ≥G8 are expected to mainly partition into the lipid phase, where they can actively counteract lipid oxidation. The oil-matrix interface probably does not play such an essential role at controlling lipid oxidation in powder compared to wet O/W emulsions. In fact, in the latter case, the oil–water interface is a critical locus, which results in amphiphilic

antioxidants usually being more effective than hydrophobic ones. In the present work, a slight cut-off effect seems to be observed especially for the surface free fat, even though the corresponding differences in antioxidant effectiveness $\geq$G8 are very small.

The lipid oxidation events that take place during processing can have a major influence on the subsequent lipid oxidation during storage, and it is therefore important to include this in the evaluation of antioxidants. The relatively nonpolar antioxidants are the most effective in preventing lipid oxidation during processing, which makes us conclude that differences in initial amounts are an important parameter to consider. A limitation here may be the detection/quantification thresholds of the available analytical methods, as relevant concentrations may be rather low.

According to our findings, the patterns of the lipid oxidation reaction in the free fat and in the encapsulated fat fractions are always very close. Yet, we highlight a moderate but consistent switchover effect. Encapsulated fat oxidized faster than surface free fat when using no antioxidant or the polar gallates, whereas the opposite trend was highlighted when using relatively nonpolar gallates. This switchover is probably caused by the most nonpolar gallates used that are less effective in the surface free fat, whereas they are still relatively effective in the encapsulated fat.

All the insights obtained through this work do not just improve our basic understanding of lipid oxidation in low moisture food emulsions, but also paves the way for more effective antioxidant strategies in related food products.

CRediT authorship contribution statement

Sten ten Klooster: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Pierre Villeneuve:** Writing – review & editing, Investigation, Methodology. **Claire Bourlieu-Lacanal:** Writing – review & editing, Investigation, Methodology. **Erwann Durand:** Writing – review & editing, Investigation, Methodology. **Karin Schroën:** Conceptualization, Investigation, Methodology, Writing – review & editing, Supervision. **Claire Berton-Carabin:** Conceptualization, Investigation, Methodology, Writing – review & editing, Supervision, Funding acquisition.

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.

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Appendix A ^{1}H and ^{13}C NMR settings

NMR data were acquired using a Bruker AVIII-HD-500 at 500 MHz and 126 MHz, respectively, in DMSO-d_6.

Propyl gallate, C3GA: 1H NMR (500 MHz, DMSO-d_6) δ 9.26 (s, 2H), 8.93 (s, 1H), 6.95 (s, 2H), 4.17 (t, J = 6.5 Hz, 2H), 1.70 – 1.57 (m, 2H), 0.93 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, DMSO-d_6) δ 166.31, 146.00, 138.79, 120.02, 108.90, 64.13, 30.81, 14.36.

Octyl gallate, C8GA: 1H NMR (500 MHz, DMSO-d_6) δ 9.25 (s, 2H), 8.93 (s, 1H), 6.95 (s, 2H), 4.15 (t, J = 6.5 Hz, 2H), 1.70 – 1.60 (m, 2H), 1.44 – 1.16 (m, 10*H*), 0.86 (t, J = 6.9 Hz, 3H). 13C NMR (126 MHz, DMSO) δ 166.30, 146.00, 138.79, 120.02, 108.90, 64.41, 40.49, 40.33, 40.16, 39.99, 39.83, 39.66, 39.49, 31.69, 29.12, 29.10, 28.75, 26.01, 22.54, 14.42.

Dodecyl gallate, C12GA: 1H NMR (500 MHz, DMSO-d_6) δ 9.26 (s, 2H), 8.94 (s, 1H), 6.94 (s, 2H), 4.15 (t, J = 6.5 Hz, 2H), 1.69 – 1.60 (m, 2H), 1.40 – 1.34 (m, 2H), 1.32 – 1.22 (m, 16H), 0.85 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, DMSO-d_6) δ 166.30, 145.99, 138.79, 119.99, 108.88, 64.41, 31.77, 29.51, 29.49, 29.47, 29.45, 29.19, 29.15, 28.74, 25.99, 22.57, 14.44.

Hexadecyl gallate, C16GA: 1H NMR (500 MHz, DMSO-d_6) δ 9.24 (s, 2H), 8.92 (s, 1H), 6.94 (s, 2H), 4.15 (t, J = 6.5 Hz, 2H), 1.68 – 1.60 (m, 2H), 1.42 – 1.22 (m, 26H), 0.88 – 0.83 (m, 3H). 13C NMR (126 MHz, DMSO-d_6) δ 166.30, 146.00, 138.79, 120.00, 108.90, 64.41, 40.48, 40.40, 40.31, 40.15, 39.98, 39.81, 39.65, 39.48, 31.76, 29.50, 29.48, 29.45, 29.18, 29.16, 28.75, 26.00, 22.57, 14.43.

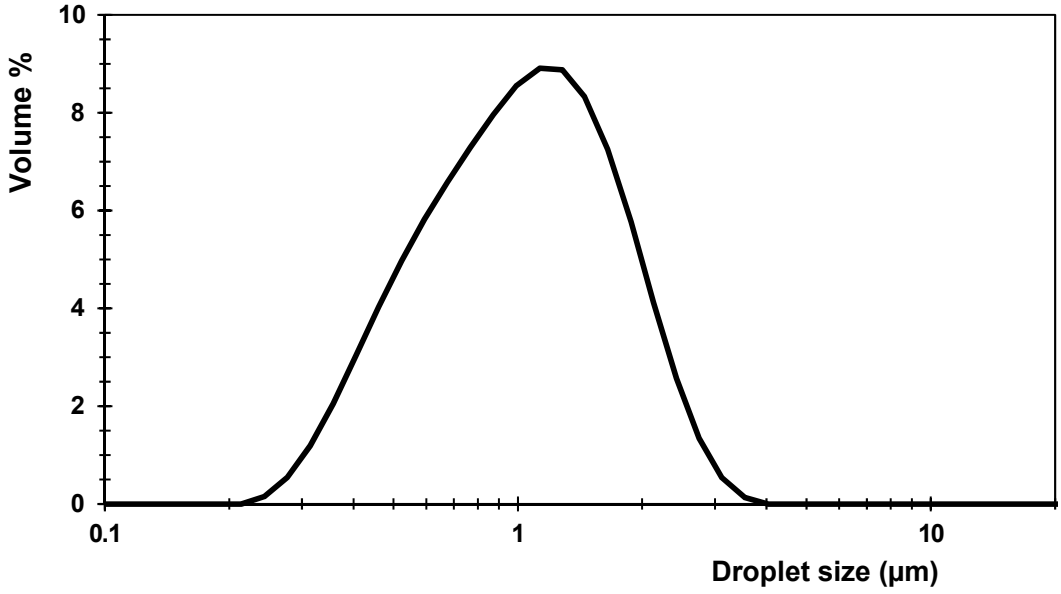

Fig. A1. Droplet size distribution (volume-based frequency [%] as a function of particle size [μm]) of the emulsion.

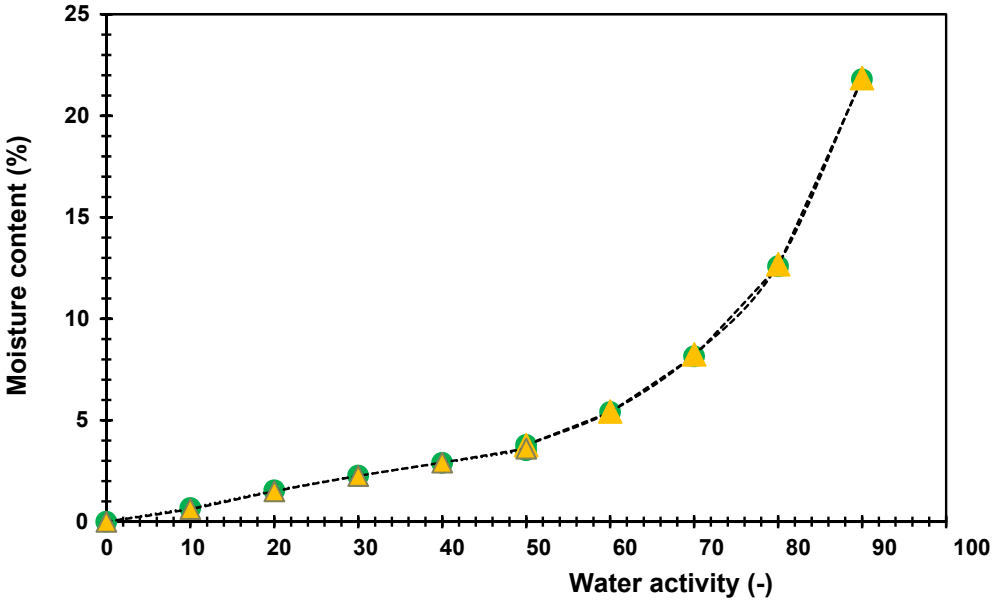

Fig. A2. Sorption isotherms of spray dried samples containing G8 (●) and G12 (▲) at 40 °C.

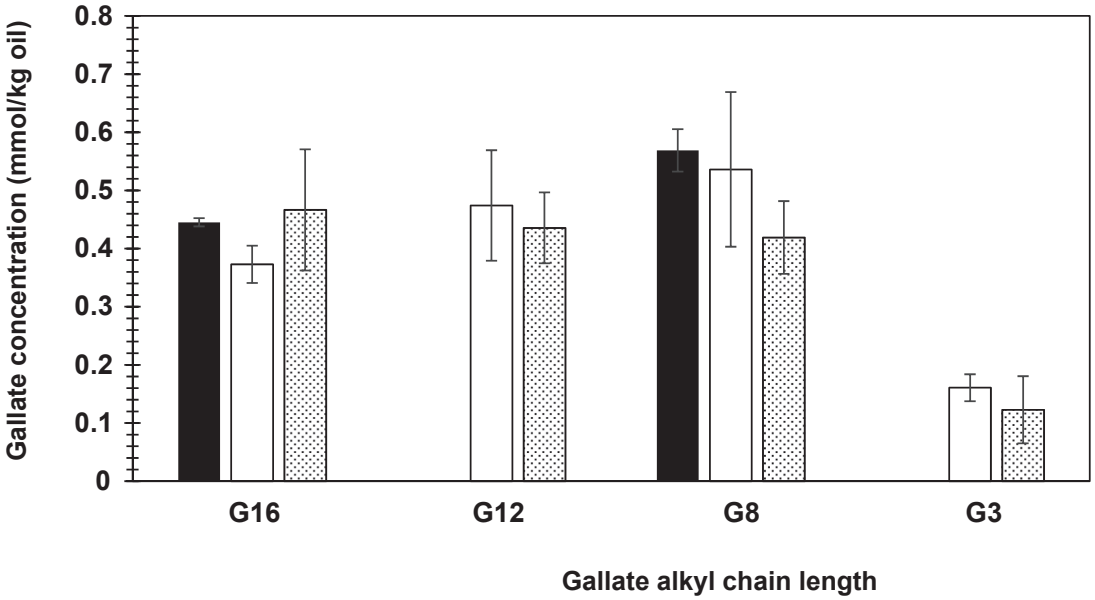

Fig. A3. The concentrations of gallates as measured by NMR in the oil (filled bar), emulsion (empty bar) and encapsulated fat (dotted bar). The error bars denote standard deviation of two independent experiments that are both measured twice.

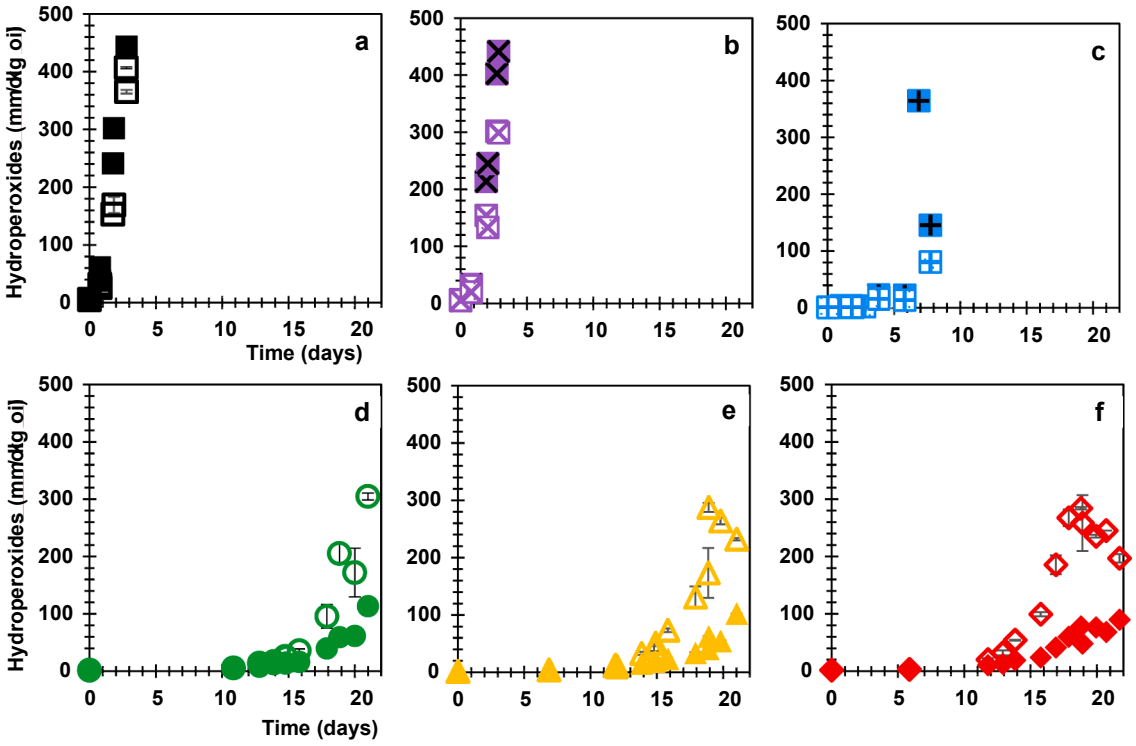

Fig. A4. Formation of hydroperoxides over time for the blank (a, ■), G0 (b, ╳), G3 (c, +), G8 (d, ●), G12 (e, ▲) and G16 (f, ◆). Open symbols denote free fat, closed symbols encapsulated fat. Error bars denote standard deviations of two measurements on the same sample. The outcomes of the independent replicates are shown as separate points.

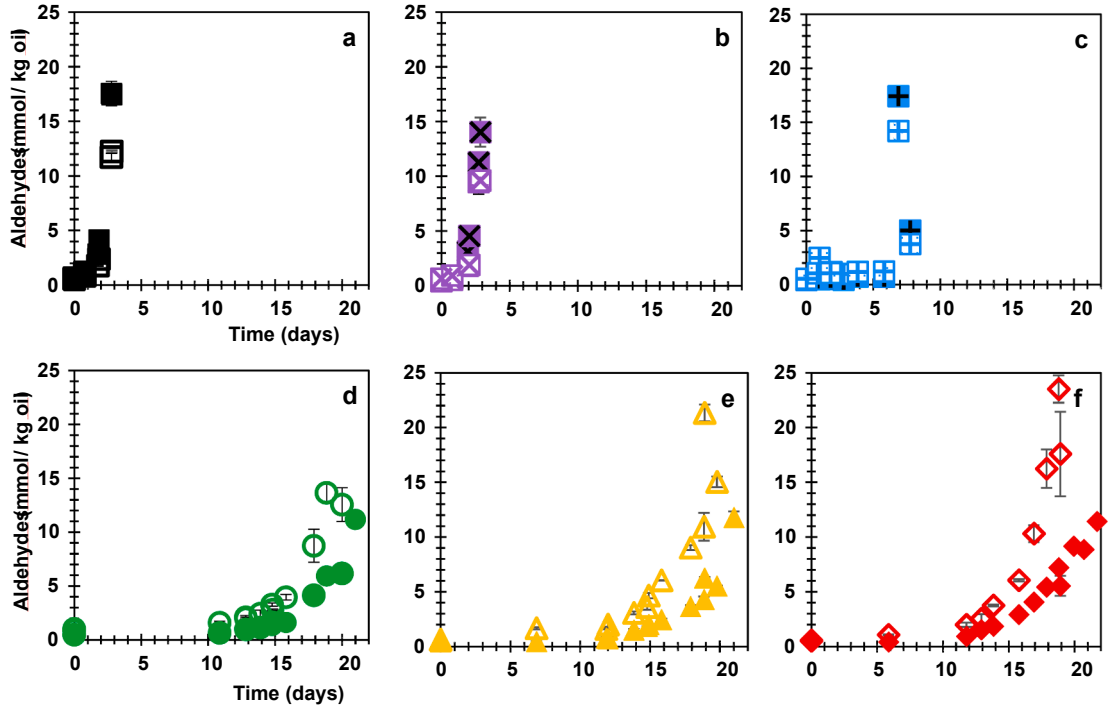

Fig. A5. Formation of aldehydes over time for the blank (a, ■), G0 (b, ╳), G3 (c, +), G8 (d, ●), G12 (e, ▲) and G16 (f, ◆). Open symbols denote free fat, closed symbols encapsulated fat. Error bars denote standard deviations of two measurements on the same sample. The outcomes of the independent replicates are shown as separate points.

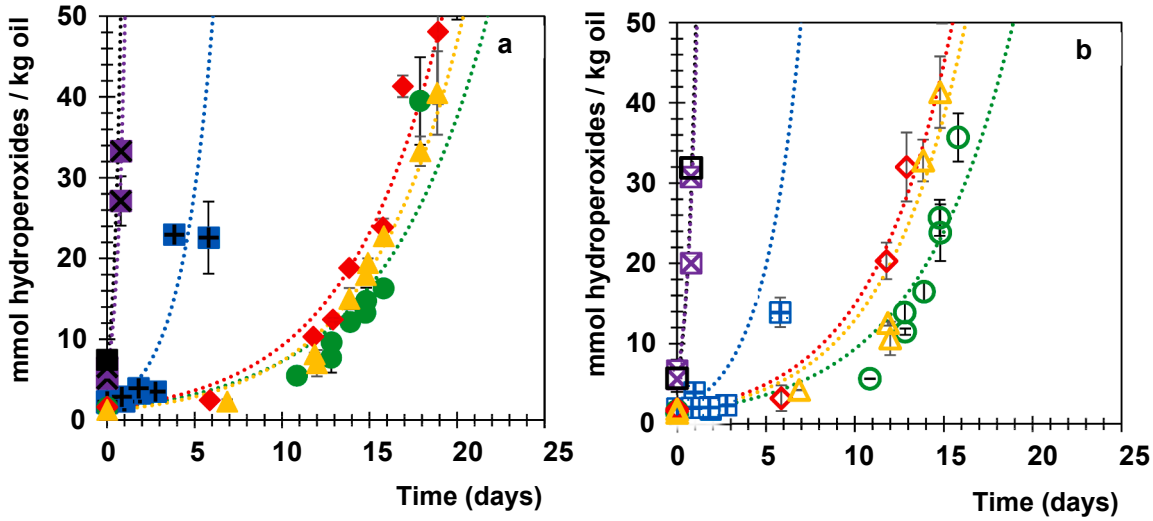

Fig. A6. Formation of hydroperoxides in the encapsulated fat (a) and in the free fat (b) of the powders over time, showing values until 50 mmol / kg oil. Symbols correspond to the systems containing gallates with different alkyl chain length: gallic acid (G0) (X), propyl gallate (G3) (+), octyl gallate (G8) (●), dodecyl gallate (G12) (▲) and hexadecyl gallate (G16) (◆); and to the blank (no antioxidant) (■). The dotted lines correspond to the outcome of fitting the data of the two repeats simultaneously to Equation (1). Error bars denote standard deviations of two measurements on the same sample. The outcomes of the independent replicates are shown as separate points.

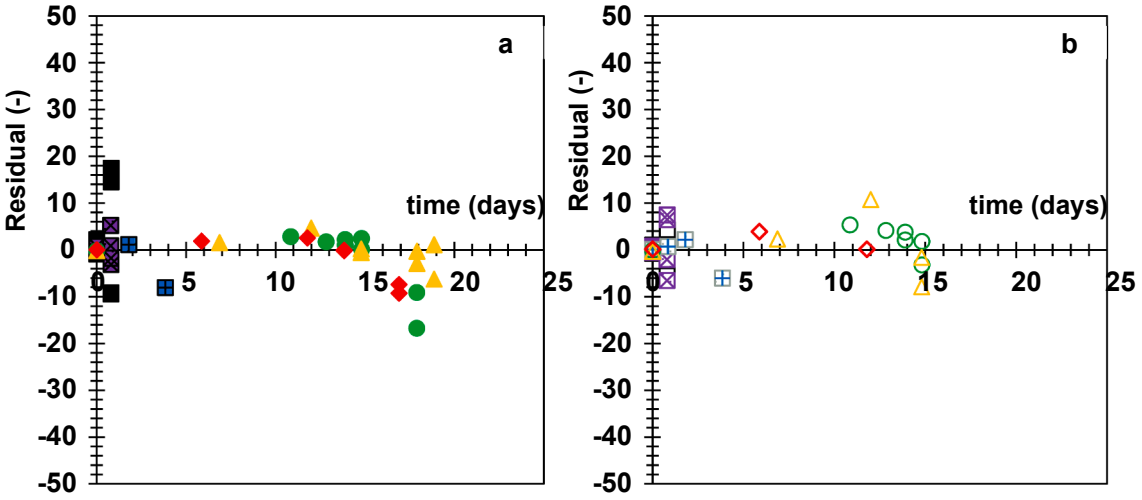

Fig. A7. Residual plots obtained through fitting the two repeats simultaneously to Equation (1) for the free fat (a) and the embedded fat (b) of the powders. Symbols correspond to the systems containing gallates with different alkyl chain length: gallic acid (G0) (X), propyl gallate (G3) (+), octyl gallate (G8) (●), dodecyl gallate (G12) (▲) and hexadecyl gallate (G16) (◆); and to the blank (no antioxidant) (■).

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