Oxidative and physical stability of oil-in-water emulsions prepared with quinoa and amaranth proteins

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Abstract

Interactions of food proteins and lipids under oxidative conditions may lead to alterations in food texture as well as loss of nutritional and sensory quality. Oxidative and physical stability of oil-in-water emulsions stabilized with water-soluble proteins extracted from quinoa (*Chenopodium quinoa*) and amaranth (*Amaranthus caudatus*) were monitored in an oxidation study at 30 °C for 7 days. Alkaline extraction of proteins from the flours followed by acid precipitation and freeze-drying was conducted and purified rapeseed oil was used to prepare emulsions via high-pressure microfluidizer. Protein stabilized emulsions showed lower physical and oxidative stability compared to Tween®20-stabilized emulsions. Lipid oxidation volatile profiles of protein stabilized emulsions indicated advanced oxidation. Comparison with the physically more stable emulsions stored at 6 °C pointed to the role of co-oxidation between proteins and lipids in coalescence of oil droplets and increase in droplet size. Emulsions stabilized with amaranth proteins showed higher resistance to oxidation compared to quinoa protein containing emulsions.

Keywords: protein oxidation; lipid oxidation; emulsion stability; quinoa; amaranth

Introduction

Impacts of chemical reactions of food components are deemed crucial in food systems in terms of safety and acceptability of the product. Among these, oxidative reactions in protein and lipid constituents of foods raise significant issues such as deterioration of sensory attributes, loss of nutritional quality, and undesired textural modifications. Under oxidative conditions, lipid radical species are formed at the initial stage of oxidation which are highly reactive and lead to formation of hydroperoxides. Hydroperoxides in turn are decomposed into secondary oxidation products. Advanced stages of unsaturated fatty acid oxidation lead to release of various volatile compounds that are characterized as off-flavors and odors [1, 2]. Free lipid radicals may induce further reactions in proteins either by hydrogen abstraction or radical addition on susceptible sites creating protein radical species. These species in turn initiate radical transfer to other protein molecules and promoting additional lipid radical formation [3]. Oxidative damage of proteins are manifested as peptide backbone cleavage, amino acid side chain alterations and protein crosslinking. These modifications consequently lead to severe changes in food systems through reduced protein solubility, textural alterations, and loss of functionality and digestibility [4, 5]. Protein-lipid interactions under oxidative conditions also include adduct formations between secondary lipid oxidation products and proteins through reaction of aldehydes with nucleophilic groups on proteins [6-8]. Since some of these volatile carbonyls play a significant role in aroma perception of the food, protein-carbonyl interactions have been the subject of various studies [9-11].

Oil-in-water emulsions are systems where surface active molecules form a layer around the dispersed lipid droplets at the oil-water interface. In protein-stabilized emulsions interfacial layer consists of adsorbed protein molecules that have gone through conformational changes during which hydrophobic interactions are maintained at the lipid interface in order to stabilize the droplets against coalescence [12]. Physical and oxidative stability of emulsions with

proteins depend on several factors such as protein structure, conformation and concentration. Unlike other surfactants proteins form a non-uniform layer around the lipid droplets and - depending on the concentration- may be present in the continuous phase in ample amounts [13]. Oxidation studies on protein-stabilized emulsions have demonstrated several effects of proteins in hindering lipid oxidation such as free radical-scavenging and metal chelating [14-16]. The antioxidant behavior of proteins may depend on abundance of unadsorbed proteins as well as the thickness of adsorbed protein layer around lipid droplets [17, 18]. Studies on physical stability of the emulsions that are affected by protein oxidation agree on the importance of several factors such as pH, thickness of interfacial layer, type of the protein and droplet size [14, 19, 20]. As a result, oxidative modifications of proteins carry significance with respect to both emulsion stability and progress of lipid oxidation.

Quinoa (*Chenopodium quinoa*) and amaranth (*Amaranthus caudatus*) are grains that originate from Andean region. Recent years have seen a rise in demand for diets with sustainable sources of proteins including plant-based foods. Quinoa and amaranth proteins carry a well-balanced amino acid composition with high content of amino acids such as lysine and leucine, thus making them nutritionally of high value.[21] Moreover protein profiles of these grains also suit gluten-free diets for celiac patients and people with gluten sensitivity. These aspects of quinoa and amaranth proteins make them significant subjects for studies that provide an insight into their behavior for the purpose of their utilization in food applications. Furthermore, a recent publication by Jarvis et al. [22] which reveals the successful sequencing of quinoa genome signals that an increase in worldwide quinoa production can be expected, hence making this nutritive grain more accessible for consumption.

In the current study our objectives were to monitor the oxidative stability of oil-in-water emulsions stabilized with water-soluble quinoa and amaranth proteins and investigate the effects of protein-lipid co-oxidation reactions with respect to emulsion stability. Quinoa and

amaranth proteins are less-studied and under-utilized with respect to their incorporation in food applications and we aim to incite further interest in optimizing the use of these nutritionally valuable proteins.

Materials and methods

Materials

Quinoa (*Chenopodium quinoa*) and amaranth (*Amaranthus caudatus*) grains used in the study were of commercial variety and imported from South America by Aduki Ltd. (Finland). Rapeseed oil (Keiju Rypsiöljy, Bunge Finland Ltd., Raisio, Finland) was purchased from a local store. Albumin from bovine serum, linoleic acid (≥99%), Tween®20, 2-propanol (CHROMASOLV®, for HPLC, 99.9%), and heptane (CHROMASOLV®, for HPLC, ≥99%) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Soy milk with vanilla flavor (Alpro C.V.A., Wevelgem, Belgium) to be used as an in-house reference for the gas chromatography – mass spectrometry (GC-MS) method was purchased from a local store. Tocopherol standards (α-, β-, γ-, δ-), aluminum oxide (Al_2O_3, 90 active neutral, activity stage I, for column chromatography, 0.063-0.200 mm, 70-230 mesh ASTM), sodium hydroxide pellets (NaOH), *di*-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), and sodium azide (NaN_3) were acquired from Merck KGaA (Darmstadt, Germany). Water used throughout the study was purified via Milli-Q equipment (Millipore Corp., Bedford, MA, USA).

Flour preparation and protein extraction

Quinoa and amaranth grains were washed under running cold water to remove saponins and then were air-dried at room temperature. Afterwards, grains were milled into fine flour using an ultra-centrifugal mill (Retsch ZM 200, Haan ,Germany) at 10000 rpm with sieve pore size

of 0.5 mm. Total protein contents of the flour samples were measured according to Kjeldahl method with a nitrogen to protein conversion factor of 5.95 [23].

Flours were defatted prior to protein extraction with heptane. Flour samples were mixed with heptane with a ratio of 1:10 (w/v) and left at room temperature with continuous stirring for 12 h. Samples were then centrifuged for 20 min at 14500 *g* and supernatant was discarded. Residual heptane was evaporated in a vacuum oven at room temperature. Protein extraction was performed according to the methods described by Abugoch et al. [24] and Guerreo-Ochoa et al. [25] with minor modifications. Defatted flours were suspended in water (10% w/v) and pH was adjusted to 9.0 using 2 N NaOH. Suspensions were left stirring at room temperature for 2 h and subsequently stored at 4 °C overnight. Afterwards, samples were centrifuged at 9000 *g* for 20 min at 4 °C. Supernatant was filtered through a filter paper (Whatman No. 1), pH was adjusted to 5.0 with 1 N HCl and left at 4 °C for protein precipitation for at least 1 h. Samples were then centrifuged at 9000 *g* for 20 min and supernatant was discarded while the precipitate was suspended in water and mixed using an Ultra-Turrax® T25 homogenizer (IKA®-Werke GmbH & Co. KG, Germany). Solutions were neutralized using 1 N NaOH and freeze-dried to acquire the protein extracts. Samples were taken for protein content measurement before freeze-drying. Protein content of the water-soluble protein extracts were determined spectrophotometrically using a protein assay kit (Bio-Rad *DC*™ Protein Assay, CA, USA). Bovine serum albumin was used to obtain a standard curve.

Emulsion preparation

Rapeseed oil used was stripped of its tocopherols prior to emulsion preparation according to the method described by Lampi et al. [26] with modifications. A glass column (51 cm x 2.9 cm i.d.) was packed with 180 g activated aluminum oxide (kept at 100 °C for 16 h, then at 200 °C for 8 h) and conditioned with heptane. Later, 100 g oil dissolved in 100 mL heptane was eluted

in order to dispose of tocopherols, pro-oxidants and trace metals. Purified oil was then stored in heptane at −20 °C until further use. Normal-phase HPLC connected with a fluorescence detector was used to check the residual tocopherols according to the method described by Schwartz et al. [27]. The results showed no detectable residues of tocopherols.

Heptane portion of the purified oil-in-heptane solution was evaporated under nitrogen flow followed by addition of protein extracts in water and a brief coarse emulsion-making procedure using Ultra-Turrax®. Oil-in-water emulsions were prepared with a final concentration of 5% (w/v) oil and 1% (w/v) protein based on optimization process carried out in preliminary tests which included various emulsion preparation and oxidation storage conditions. Final stable emulsions were obtained by homogenizing the coarse emulsions via a high-pressure M-110Y Microfluidizer® (Microfluidics™, MFIC Corp., MA, USA) at an operating pressure of 600 bar for 10 min of continuous flow process. Next, sodium azide was added into the emulsions with a final concentration of 0.02% (w/v) to prevent possible microbial growth. Oil-in-water emulsions stabilized with Tween®20 instead of proteins were also prepared with the same method as control group. Emulsions were stored in DURAN® glass bottles of 100 mL capacity and dimensions of 56 mm x 105 mm (external diameter x height) and placed at 30 °C in the dark for accelerated oxidation storage with a gentle stirring action by a magnet in order to maintain similar conditions in all emulsion sample groups.

Monitoring oxidation and emulsion stability

Analytical samples were collected from emulsions to monitor the oxidative changes and emulsion stability on days 0, 1, 4, and 7. Analyses were performed in triplicates. Progress of lipid oxidation in the emulsions was monitored via formation of conjugated diene hydroperoxides (CD) and secondary oxidation volatile compounds. CD formation was measured according to a modified method by Lethuaut et al. [19]. An aliquot of the emulsion

151 sample was mixed with 2-propanol and shaken to extract CD into the solvent phase which was
152 followed by centrifugation at 14000 *g* for 20 min. The resulting supernatant was collected and
153 measured at 234 nm using a UV/Vis spectrophotometer (Lambda 25 UV/Vis
154 Spectrophotometer, PerkinElmer, USA). Concentration of CD was expressed as mmol/kg oil
155 (Molar absorptivity, $\varepsilon = 25000\ M^{-1}\ cm^{-1}$). Formation of volatile lipid oxidation products were
156 monitored by detection and identification via headspace solid phase micro extraction gas
157 chromatography – mass spectrometry (SPME-GC-MS) technique. Integrated peak areas of
158 detected and selected volatile compounds were used to compare the progress of oxidation
159 within sampling days. Emulsion aliquots were collected on sampling days in headspace SPME-
160 GC vials (75.5 x 22.5 mm) closed with screw cap. The equipment consisted of an SPME injector
161 (combiPAL, CTC Analytics, USA), a GC (HP 6890 series, Agilent Technologies Inc., DE,
162 USA), and a MS detector (Agilent 5973 Network, Agilent Technologies Inc., DE, USA). The
163 volatiles were extracted using a divinylbenzene/ carboxen/ polydimethylsiloxane
164 (DVB/CAR/PDMS) SPME fiber assembly with 50/30 µm film thickness (Stableflex 23Ga,
165 Supelco, PA, USA). Chromatographic separation of compounds were achieved through a
166 SPB®-624 capillary column with dimensions of 30 m x 0.25 mm i.d. and 1.4 µm film thickness
167 (Supelco, PA, USA). The SPME-GC-MS method employed in the study was developed by
168 Damerau et al. [28] in the analysis of lipid oxidation volatiles from spray-dried emulsions
169 (condition 1). All emulsions samples were prepared using the same composition (i.e. same
170 water and oil content) to minimize the effect of emulsifier matrix difference on the migration
171 of volatiles into the headspace. Furthermore, to ensure the effective release of volatiles an
172 equilibration step was included which involved agitation that mechanically helped the release
173 of volatiles followed by the extraction of volatiles at 40 °C. Equilibration step was carried out
174 at 40 °C for 10 min with an agitator speed of 250 rpm followed by extraction at 40 °C for 30
175 min. Next, fiber was desorbed for 10 min at 250 °C at the GC front inlet in “spitless” mode. GC

was operated with a helium flow of 0.7 mL/min while temperature gradient of the GC oven was set as follows: 40 °C for the first 2 min, then incremental increase at a rate of 5 °C/min until 200 °C followed by an 11 min of fixed temperature of 200 °C. Ionization energy for MS detection was 70 eV and *m/z* scan range was 40-300 amu. Identification of the volatile compounds was based on mass spectral data library Wiley 7N (Wiley Registery™ of Mass Spectral Data, 7th ed., USA) and retention times of these compounds in previously published data [28].

Progress of protein oxidation in emulsions within sampling days was assessed through the changes in tryptophan fluorescence. Sample aliquots were dissolved in phosphate buffer (pH 7.4) and, following necessary dilutions, measured in quartz cuvettes using a fluorometer (LS 55 Luminescence Spectrometer, PerkinElmer Inc., MA, USA). Emission spectra were collected between 300 and 400 nm upon excitation at 283 nm [29].

Changes in droplet size during the course of experiments were monitored via a laser diffraction particle size analyzer (Mastersizer Hydro 3000 SM, Malvern Instruments Ltd., Worcestershire, UK). Rapeseed oil refractive index was set at 1.33 and density at 0.905 g/cm^3. Another set of emulsion samples of all groups were also stored at 6 °C in order to detect differences in emulsions stability. Analytical samples were diluted in water during measurement and data on droplet size distribution, volume mean diameter: D[4,3]; surface mean diameter: D[3,2]; and median droplet size: Dv 50 were collected. Specific surface area (SSA) of droplets was calculated according to the equation [19]:

$$SSA = \frac{6\,\emptyset}{D[3,2]}$$

where Ø is the volumetric oil fraction, D[3,2] the surface mean diameter of the droplets and SSA is expressed in m^2/mL.

Statistical analyses of data were carried out using SPSS software (IBM Corp., v.24.0.0.1). Tukey's HSD test was employed as a post-hoc analysis with ANOVA to mark the significant mean differences at a level of 0.05 (n=3).

Results and discussion

Characterization of emulsion components

Saponins were removed from the grains prior to milling in order to reduce foam formation and thus enable a smoother emulsion-making process via high-pressure homogenization. Although removal of saponins has found to decrease the emulsifying activity, the procedure increases the stability of emulsions produced [30]. Total protein content of quinoa and amaranth flours was measured as 13.02 ± 0.12 and 15.93 ± 0.51 g/100 g flour (dry weight), respectively (moisture content of the same flour samples was measured by Ramos-Diaz et al. [31]). The amount of water-soluble albumin and globulin fractions in quinoa and amaranth protein compositions show variation according to different cultivars. Janssen et al. [32] has tabulated the quinoa protein distributions referring to various sources according to which the content of albumins and globulins among total proteins amount to around 64% in average. Bressani and García [33] reported the albumin and globulin content as 43.4% of total proteins in amaranth (*Amaranthus caudatus)* samples. In the current study, water-soluble proteins extracted from flours were measured as 5.01 ± 0.30 and 5.20 ± 0.45 g/100 g flour (dry weight) in quinoa and amaranth flours, respectively. These values show that around 60% of total water-soluble proteins in quinoa were extracted while this value was around 75% in amaranth. It would be possible to acquire a larger yield under extraction conditions of higher temperature and higher pH [25]. However, these settings were not chosen for this study in order to avoid protein denaturation and undesired oxidation during extraction. Nonetheless, the aim of the protein extraction

process was to obtain enough amount of proteins to be utilized in emulsion-making rather than achieving higher yield.

Fatty acid composition of the purified rapeseed oil used for emulsion-making was analyzed by Lehtonen et al. [34] and found as 4% C16:0, 60% C18:1, 21% C18:2, and 10% C18:3. The composition of fatty acids present in the lipid source for oxidation determines the rate of formation and diversity of lipid oxidation products.

Emulsion droplet size distribution

Utilization of a high-pressure microfluidizer produced emulsions with majority of the droplets sized under 1 µm for both protein-stabilized emulsions. However neither of them displayed as high specific surface area values (SSA) as the Tween®20-stabilized emulsion. Mean SSA values for quinoa, amaranth and Tween®20 group emulsions were 3.69, 2.80, and 6.63 m^2/mL, respectively. Specific surface area elucidates the total surface area or interfacial area per unit volume, hence a higher SSA value points to a smaller droplet size. Droplet size measurements on day 0 showed that emulsions with quinoa proteins had larger interfacial surface area than emulsions with amaranth proteins. This means that quinoa protein-stabilized emulsions provided more area available for oxidative reactions compared to emulsions with amaranth proteins at the beginning of the oxidation experiment. It also indicates that the amount of proteins present in the oil droplet interface were higher in amaranth protein-stabilized emulsions than in quinoa.

Several droplet size parameters monitored over oxidation period at 30 °C are presented for quinoa, amaranth, and Tween®20 emulsions in Table 1. Droplet size distribution of the protein-stabilized emulsions varied noticeably over time during which the volume of large size droplets increased and was numerically reflected in the decrease of SSA values while Tween®20 emulsions remained stable (Fig. 1 a, b, c). Several studies have found that Tween®20 forms a

246 tighter barrier than proteins against coalescence of oil droplets due to its molecular bonds and
247 higher percentage of surface coverage around the oil droplets [17, 35, 36]. This results in
248 increased surface area of oil droplets in Tween®20 emulsions compared to protein-stabilized
249 emulsions.

250 Emulsions containing quinoa proteins displayed earlier formation of larger droplets than
251 amaranth protein-stabilized emulsions which indicates more extensive modification of quinoa
252 proteins and progress of lipid oxidation. On the other hand, changes in the droplet size of
253 emulsions stored at 6 °C were not as remarkable (Fig. 1 d, e, f). At lower temperature, amaranth
254 protein-stabilized emulsion samples were more stable than quinoa protein-stabilized emulsions
255 while emulsions with Tween®20 continued to be physically the most stable. The difference of
256 the droplet size distributions in protein-stabilized emulsion samples between two temperatures
257 could be attributed to the expected greater extent of protein oxidation at higher temperature.

258 **Formation of primary and secondary lipid oxidation products**

259 Progress of lipid oxidation was monitored via measurement of both conjugated diene
260 hydroperoxides (CD) and the release of volatile secondary oxidation compounds. The progress
261 of CD formation followed a similar pattern of growth throughout the oxidation period in all
262 sample groups until day 4. On day 7, CD formation was more advanced in protein-stabilized
263 emulsions than the emulsions with Tween®20 (Fig. 2). This may be an indication that the rate
264 of hydroperoxide decomposition into secondary volatiles increased in Tween®20-stabilized
265 emulsions after day 4, while propagation of lipid radicals interacting with protein radicals
266 continued in protein-stabilized emulsions resulting in ongoing hydroperoxide formation.

267 Secondary oxidation volatiles detected by SPME-GC-MS method was monitored for 7 days.
268 Chromatographic peak areas of compounds detected were integrated in order to be utilized in
269 monitoring the formation of these volatiles and obtain the comparative data rather than

quantitative data. Most abundant 10 compounds detected were charted as chromatographic peak areas versus time in Fig. 3. These compounds were 2-pentylfuran, hexanal, 2-octenal, 2-heptenal, 2,4-heptadienal, 2-pentenal, 3,5-octadien-2-one, 2,4-hexadienal, 5-pentyl-2(5H)-furanone, and nonanal. Even though it may be thought that protein matrix would hinder the release of certain volatiles compared to Tween®20-stabilized emulsions, the volatile profile detected in protein-stabilized emulsions showed higher diversity and abundance. This result combined with the SPME parameters set to maintain effective release of the volatile analytes indicated that the matrix difference between protein- and Tween®20-stabilized emulsions had no effect on the extraction of volatiles. According to the volatile profiles, quinoa protein-stabilized emulsions contained greater amounts of most of these oxidation products compared to amaranth protein-stabilized emulsions. In all emulsions, formation of compounds like 2-pentylfuran, 2-octenal, 2,4-heptadienal, and 5-pentyl-2(5H)-furanone was more pronounced starting from day 4, whereas hexanal, 2-heptenal, 2-pentenal, 3,5-octadien-2-one, 2,4-hexadienal, and nonanal displayed a steadier progress from day 1. Presence of volatiles reported in this study reflected the expected oxidation products originating from oleic, linoleic, and linolenic acid. Hexanal is the main volatile of linoleic acid oxidation and it accumulated over time in all emulsions in abundance. The other dominant volatile 2-pentylfuran is reported to arise as a singlet oxygen oxidation product of linoleic and linolenic acid [37]. Third most abundant volatile detected was 2-octenal, which like 2-pentylfuran and hexanal originates from decomposition of 9-linoleate hydroperoxide [38]. These results point to linoleic acid as the preferred substrate for oxidation in all emulsions. On the other hand, the formation of major oleic acid oxidation product nonanal was consistent in all emulsions. At the end of day 7, aldehydes 2-heptenal, 2,4-heptadienal, and 2-pentenal had formed in higher abundance in Tween®20-stabilized emulsions than in protein-stabilized samples, while the formation of the rest of the reported compounds were more emphasized in protein-stabilized emulsions. Radical

lipid alkyl, alkoxyl and peroxyl species are involved in complex competing mechanisms of side reactions that determine resulting end products of lipid oxidation. [1]. Therefore it is highly probable that presence of protein radicals influence these reactions such as hydrogen abstraction, scission, and recombination which consequently affect the progress of lipid oxidation as well as the formation of secondary lipid oxidation products providing favorable conditions to formation of certain volatiles over others. Moreover, many volatile compounds are known to arise from further oxidation of unsaturated aldehydes [1, 38]. This may be one of the reasons why compounds 2-heptenal, 2,4-heptadienal, and 2-pentenal accumulated in higher amounts in Tween®20-stabilized emulsions as they may have undergone further decomposition in protein-stabilized emulsions in which oxidation was more advanced. Therefore, while assessing the extent of lipid oxidation it is vital to monitor a diversity of compounds to obtain a more comprehensive understanding. Another compound that marked the difference of lipid oxidation between emulsions containing proteins and Tween®20 was 5-pentyl-2(5H)-furanone which formed in significantly higher amounts in the protein-stabilized emulsions. This volatile compound is also generated as a breakdown product of linoleic acid aldehydes [39].

Tryptophan fluorescence and protein oxidation

Oxidation of proteins lead to a significant loss in tryptophan fluorescence in both protein-stabilized emulsions. In emulsions with quinoa proteins the maximum emission wavelength of fluorescence spectra shifted from 345 to 353 nm as oxidation progressed while for emulsions with amaranth protein this shift occurred from 344 to 358 nm (Fig. 4). This so-called red shift in emission wavelengths occur for tryptophan residues as the environment becomes more polar [40]. The increase in polarity is due to the lipid hydroperoxides formed during oxidation which are more polar in nature compared to lipids from which they originate and thus they migrate to the interface where proteins are present [1]. Another factor is that protein modification during oxidation exposes more tryptophanyl residues in the interface to the polar aqueous phase [41].

The changes in fluorescence intensity over oxidation sampling days were presented as mean emission spectra in Fig. 4. In emulsions with quinoa protein, the fluorescence intensity on day 1 was measured as only 54% of the initial day 0 intensity. On day 4 the fluorescence intensity decreased to ~38% of the starting day intensity. Day 7 fluorescence intensity remained also at this level. The unchanging intensity level during the last three days indicates that the majority of the tryptophan degradation took place already during the beginning of the storage at 30 °C in the emulsion with quinoa proteins. On the other hand, fluorescence of amaranth proteins followed a different pattern of oxidation where the degradation of tryptophanyl residues progressed steadily between day 0 and day 4. On day 1 fluorescence was measured as 85.5% of initial intensity which was trailed by a decline to 45.7% of initial fluorescence on day 4. On the final oxidation day of the study, emission intensity was 34.9% of the starting day. The difference in the oxidation rates between quinoa and amaranth proteins may have been caused by several factors. Drzewiecki et al. [42] have reported the composition of secondary structure of soluble quinoa and amaranth proteins according to which amaranth proteins contain higher percentage of α-helices while quinoa proteins cover higher percentage of β-sheets. The propensity for tryptophan residues was found to be higher towards β-sheet conformation than α-helices, especially in exposed fractions of β-strands [43]. Therefore, the secondary structural differences of soluble quinoa and amaranth proteins such as the location and thus availability of tryptophanyl residues for oxidative reactions may have resulted in more rapid oxidation of quinoa proteins. Another factor is the smaller size of the oil droplets stabilized by quinoa proteins compared to amaranth proteins on day 0 (Table 1) which provided a larger surface area for the initiation of oxidation of both proteins and lipids. Co-oxidation of lipids and proteins may take place via several pathways. One of them includes the interactions of secondary lipid oxidation aldehydes with nucleophilic sites on proteins that lead to adduct formations, protein crosslinking, and fluorescent dihydropyridine-like products [8, 44-46]. Another pathway for

protein-lipid co-oxidation involves free radical transfer between these components. Once lipid free radicals are transferred to protein sites prone to hydrogen atom abstraction and thus generating reactive protein radicals, oxidative damage in protein is iterated and follows a parallel pathway to lipid oxidation [1]. This explains higher abundance of conjugated diene hydroperoxides (CD) in protein-stabilized emulsions at the end of day 7 hinting at a continuous formation of primary oxidation products due to the presence of protein and lipid radicals. The extensive degradation of quinoa proteins within one day points to the initiation of protein oxidation through free radical-caused reactions. Most likely the formation of protein radicals took place before that of lipid radicals, especially in emulsions with quinoa proteins, due to high temperature and oxygen uptake as well as promotion of the onset of lipid oxidation hence the higher physical stability of protein-stabilized emulsions kept at 6 °C compared to incubation at 30 °C (Fig. 1 d, e, f). Although there are not many studies on the origins of oxidation onset in multiphase systems where proteins and lipids are in contact, Berton et al. [47] have reported that protein modifications started earlier than lipid oxidation in β-lactoglobulin-, β-casein-, and bovine serum albumin-stabilized oil-in-water emulsions. The relatively earlier start of oxidative reactions in quinoa proteins compared to amaranth proteins also resulted in a more abundant formation of volatile lipid oxidation products. Additionally, advanced oxidative damage to proteins may lead to reduction in physical stability. It has been reported earlier that while moderate oxidation helps increase emulsifying activity of proteins, extensive oxidation weakens protein network at the interfacial layer and leads to lower emulsion stability through aggregation [48-50]. Higher physical stability of emulsions kept at 6 °C support this outcome. Accordingly, rapid protein degradation in quinoa proteins is reflected in lower emulsion stability as the droplet size distribution displays larger size droplets on day 1 compared to emulsions stabilized with amaranth proteins (Fig. 1 a, b). The effects of protein oxidation are visible also in emulsions containing amaranth proteins as the coalescence of oil droplets and

formation of advanced lipid oxidation volatiles trail the pattern of quinoa protein-stabilized emulsions. In agreement with the significant effect of protein degradation towards the oxidative and physical stability of the emulsions, emulsions with Tween®20 maintained better stability throughout the monitoring period at 30 °C.

Conclusion

Our study showed that emulsions stabilized with quinoa and amaranth proteins showed lower oxidative and physical stability compared to those stabilized with Tween®20 stored at 30 °C due to the extensive protein oxidation reflected in the significant decrease in tryptophan fluorescence. Emulsions stabilized with amaranth proteins were oxidatively more stable than those stabilized with quinoa proteins. Earlier stages of reactive species-mediated reactions involve transfer of free radicals in what can be called co-oxidation of proteins and lipids. Profiles of lipids volatiles released also indicated a more advanced oxidation with emulsions containing proteins as compared to emulsions stabilized with Tween®20. According to the droplet size distributions, protein-stabilized emulsions stored at 6 °C maintained their physical stability better than those stored at 30 °C, which was due to lack of extensive protein oxidation. We hypothesize that optimized solutions aimed at hindering oxidation of these Andean grain proteins of high nutritional quality in food emulsion applications would lead to an oxidatively and physically more stable product. Further studies should also focus on improving emulsifying properties of quinoa and amaranth through technological alterations such as deamidation and enzymatic modifications.

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395 **Conflict of Interest**

396 Authors declare that they have no conflict of interest.

397 **Compliance with ethics requirements**

398 This article does not contain any studies with human or animal subjects.

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FIGURE CAPTIONS

Fig. 1 Changes in droplet size distributions of (a) quinoa protein-stabilized emulsions at 30 °C; (b) amaranth protein-stabilized emulsions at 30 °C; (c) Tween®20-stabilized emulsions at 30 °C; (d) quinoa protein-stabilized emulsions at 6 °C; (e) amaranth protein-stabilized emulsions at 6 °C; (f) Tween®20-stabilized emulsions at 6 °C

Fig. 2 Formation of conjugated diene hydroperoxides (CD) in emulsions

Fig. 3 Progress of lipid oxidation in emulsions stabilized with quinoa proteins, amaranth proteins, and Tween®20 in terms of release volatile compounds (a) 2-pentylfuran; (b) hexanal; (c) 2-octenal; (d) 2-heptenal; (e) 2,4-heptadienal; (f) 2-pentenal; (g) 3,5-octadien-2-one; (h) 2,4-hexadienal; (j) 5-pentyl-2(5H)-furanone; (k) nonanal (Significant mean differences between emulsions within each analysis day was denoted with different lowercase letters, at $p < 0.05$)

Fig. 4 Changes in tryptophan fluorescence during protein oxidation in (a) quinoa protein-stabilized emulsions; (b) amaranth protein-stabilized emulsions

552 **TABLES**

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Table 1. Changes in droplet size parameters of emulsions during oxidation at 30 °C.*

Emulsions	Oxidation days	Volume mean diameter, D[4,3] (μm)	Median diameter, Dv 50 (μm)	Specific Surface Area, SSA (m^2/mL)
Quinoa	day 0	7.81 ± 1.89	0.15 ± 0.00	3.69 ± 0.02
	day 1	24.30 ± 6.91	10.28 ± 0.74	0.03 ± 0.00
	day 4	69.37 ± 1.42	33.63 ± 0.45	0.01 ± 0.00
	day 7	45.90 ± 0.85	35.00 ± 0.20	0.01 ± 0.00
Amaranth	day 0	15.27 ± 5.86	0.20 ± 0.01	2.80 ± 0.06
	day 1	8.61 ± 0.03	8.44 ± 0.03	0.04 ± 0.00
	day 4	50.80 ± 3.97	46.83 ± 3.47	0.01 ± 0.00
	day 7	85.53 ± 17.62	34.43 ± 2.34	0.48 ± 0.01
Tween®20	day 0	0.64 ± 0.22	0.06 ± 0.00	6.63 ± 0.23
	day 1	0.37 ± 0.18	0.07 ± 0.00	6.39 ± 0.01
	day 4	0.75 ± 0.16	0.07 ± 0.00	6.37 ± 0.02
	day 7	0.09 ± 0.00	0.07 ± 0.00	6.44 ± 0.00

***** Values denote mean ± standard deviation (n = 3)

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