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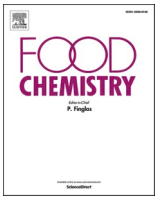

Antioxidant peptides from alternative sources reduce lipid oxidation in 5% fish oil-in-water emulsions (pH 4) and fish oil-enriched mayonnaise

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ABSTRACT

Bioinformatics tools were used to predict radical scavenging and metal chelating activities of peptides derived from abundant potato, seaweed, microbial, and spinach proteins. The antioxidant activity was evaluated in 5% oil-in-water emulsions (pH4) and best-performing peptides were tested in mayonnaise and compared with EDTA. Emulsion physical stability was intact. The peptide DDDNLVLPEVYDQD showed the highest protection against oxidation in both emulsions by retarding the formation of oxidation products and depletion of tocopherols during storage, but it was less efficient than EDTA when evaluated in mayonnaise. In low-fat emulsions, formation of hydroperoxides was reduced 4-folds after 5 days compared to control. The concentration effect of the peptide was confirmed in mayonnaise at the EDTA equimolar concentration. The second-best performing peptides were NNKWVPCLEFETEHGFVYREHH in emulsion and AGDWLIGDR in mayonnaise. In general, the peptide efficacy was higher in low-fat emulsions. Results demonstrated that peptide negative net charge was important for chelating activity.

1. Introduction

The use of antioxidant peptides as sustainable ingredients in food industry has gained interest in the last decade as a potential alternative to replace the synthetic antioxidants. Ethylenediaminetetraacetic acid (EDTA) is a widely used synthetic antioxidant highly efficient in preventing primary and secondary oxidation in low pH oil-in-water emulsions such as mayonnaises due to its chelating activity (Ghorbani Gorji et al., 2016; Jacobsen et al., 2001a). However, even though synthetic antioxidants are permitted in food industry, there is a concern about their use because toxic/carcinogenic effects have been described when they are used in high concentrations (Ghorbani Gorji et al., 2016). Due to this, natural antioxidant peptides may be preferred even if synthetic antioxidants are more economical. In food matrices, some bioactive peptides can slow down or inhibit lipid oxidation processes thereby contributing to the chemical stability of the system (McClements & Decker, 2018). Proteins and peptides exhibit their antioxidant activity by different mechanisms such as inactivating reactive oxygen species, scavenging free radicals, chelating prooxidant transition metals, reducing hydroperoxides or modifying physical properties of food

Abbreviations: Asp, aspartic acid; BST, Backscattering and Transmission; CHE, chelator antioxidant activity; Cys, cysteine; E, emulsion sample; EDTA, ethylenediaminetetraacetic acid; FRAP, ferric reducing antioxidant power; GC-FID, Gas Chromatography with Flame Ionization Detection; GC–MS, Gas Chromatography-Mass Spectrometry; Glu, glutamic acid; HPLC-FLD, High-Performance Liquid Chromatography with Fluorescence Detection; Lys, lysine; M, mayonnaise sample; ORAC, oxygen radical absorbance capacity; P, peptides obtained from potato, particularly patatin; Phe, phenylalanine; pI, isoelectric point; PMC, predicted metal chelator; PRS, predicted radical scavenger; PV, peroxide value; R, peptides obtained from spinach; S, peptides obtained from seaweed, *E. denticulatum*; SCA, scavenger antioxidant activity; SDS, Sodium Dodecyl Sulfate; Trp, tryptophan; TSI, turbiscan stability index; TW20, Tween 20; Tyr, tyrosine; U, peptides obtained from microbial organism, *Methylococcus capsulatus*.

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systems in a way that separate reactive species (Elias et al., 2008; Tkaczewska, 2020). Antioxidant peptides are not active when embedded in the parent proteins but can be released from them by different processes, especially by enzymatic hydrolysis (Gregersen et al., 2021; Hajfathalian et al., 2017). They are usually up to 30 amino acids, and their amino acid composition, structure, charge, and hydrophobicity influence their functional and bioactive properties including their antioxidant activity (García-Moreno et al., 2020a; Görgüç et al., 2020; Yesiltas et al., 2022). These peptides can be derived from different alternative sources such as plants, marine and microbial (Chandra et al., 2020; Hajfathalian et al., 2017; Nadeeshani et al., 2021). Also, they can be obtained from protein-rich side-streams in food industrial processes.

For instance, potato peptides have previously been demonstrated to have antioxidant properties by scavenging free radicals and/or as metal chelators in oil-in-water emulsions (García-Moreno et al., 2021; García-Moreno et al., 2020b; Yesiltas et al., 2022). Another plant-based protein, the ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO), is the most abundant protein on earth and is considered an attractive and sustainable source for producing enzymatic hydrolysates exhibiting antioxidant activity (Udenigwe et al., 2017). Moreover, some protein hydrolysates from seaweed species were previously reported to prevent oxidative deterioration: the kelp hydrolysates of *Ecklonia cava* in fish oil-in-water emulsions at 60 °C for 12 days (Heo et al., 2003); a synthesized peptide derived from *Palmaria palmata* (SDITRPGGNM) showed the highest oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) compared to other peptide sequences tested from this protein source (Harnedy et al., 2017). In addition, peptides obtained from protein-rich biomass of methane metabolizing bacteria (*Methylococcus capsulatus and Ralstonia* sp.) have been reported as good emulsifiers with antioxidant activity in model low-fat oil-in-water emulsions (Yesiltas et al., 2022).

Olsen et al. (2020) showed that bioinformatics tools can be developed for predicting antioxidant activity of peptides using machine learning algorithms. Using this bioinformatics-assisted approach, several alternative protein sources were investigated in our previous work to identify specific proteins of high abundance (Yesiltas et al., 2022). Following *in silico* analysis and prediction of embedded antioxidant peptides, 35 peptides were assayed for *in vitro* antioxidant activity. The 11 best performing peptides were further validated in a model emulsion system (5% fish oil-in-water emulsions) at pH 7. Simple oil-in-water emulsions are useful and suitable models to test antioxidant peptides. However, as few studies have been carried out in real model food products, there is a lack of understanding of the peptide's mechanism to enhance physical and oxidative stability and the possible interactions between those peptides and the food matrix. Thus, the present study provides new insight into the use of these peptides not only in low-fat emulsions as test models but also in a real food product, mayonnaise. Mayonnaise, as a high-fat emulsion-based food product, is prone to lose its oxidative stability due to its high content of iron and usually also a high content of polyunsaturated oil and is therefore a good model food to evaluate antioxidant peptides (Yesiltas et al., 2021). The structure of the interfacial region of mayonnaise plays a critical role in the lipid oxidation process as well as the type of oil used (Ghorbani Gorji et al., 2016). Furthermore, it has been reported that the low pH values due to vinegar and lemon juice in mayonnaise leads to prooxidative effect by the release of iron from the egg yolk that is used as emulsifier (Ghorbani Gorji et al., 2016; Jacobsen et al., 1999a; Jacobsen et al., 2001b). Therefore, increased iron concentrations coming from the oil–water interface to the aqueous phase increase the possibility of interactions with already existing lipid hydroperoxides located at the emulsion droplet surface (Jacobsen et al., 1999a; Jacobsen et al., 2001b).

Here, we investigate the antioxidant activity of the same set of peptides as in our previous work (Yesiltas et al., 2022), in low-fat emulsions at pH 4 and mayonnaise. This was done to investigate if amino acid composition, charge, and structural characteristics (size, secondary structure and amphiphilicity), as well as the specific antioxidant mechanisms (e.g., radical scavenging or metal chelating), and concentration might affect the final physical and oxidative stabilities of emulsion systems at pH 4. This investigation consisted of three parts, i) the study of antioxidant activity of the 11 synthetic peptides in 5% fish oil-in-water emulsions at pH 4, ii) evaluation of the best performing peptides from study i) in a mayonnaise storage experiment for the determination of changes in physical and oxidative stability, and iii) the evaluation of the effect of peptide concentration based on the best performing peptide in mayonnaise.

2. Materials and methods

2.1. Materials

Selected antioxidant peptides (purity > 70% by HPLC (High-Performance Liquid Chromatography)) were synthetically produced by Synpeptide Co., Ltd. (Shanghai, China). Refined and deodorized fish oil (cod liver oil) was provided by Vesteraalens A/S (Sortland, Norway) and stored in brown bottles at −40 °C until use. The fatty acid composition of the fish oil was determined using fatty acid methyl ester (FAME) analysis by GC-FID (Gas Chromatography with Flame Ionization Detection) in a previous study and reported as the following: myristic acid, C14:0 (4.0), Palmitic acid, C16:0 (9.2), Palmitoleic acid, C16:1n-7 (8.3), Stearic acid, C18:0 (2.2), Oleic acid, C18:1n-9 (15.8), Vaccenic acid, C18:1n-7 (4.1), Linoleic acid, C18:2n-6 (2.5), Linolenic acid, C18:3n-3 (0.2), Eicosenoic acid, C20:1n-9 (11.4), Eicosapentaenoic acid, C20:5n-3 (8.8), Cetoleic acid, C22:1n-11 (5.4), and Docosahexaenoic acid, C22:6n-3 (11.4) (Yesiltas et al., 2021). Tocopherol contents and peroxide value (PV) of the fish oil were analyzed and alpha-tocopherol, beta-tocopherol, gamma-tocopherol, and delta-tocopherol were 156.3 ± 0.05 μg/g oil, 2.8 ± 0.22 μg/g oil, 107.4 ± 0.59 μg/g oil, 39.7 ± 0.78 μg/g oil, respectively by HPLC-FLD (High-Performance Liquid Chromatography with Fluorescence Detection) analysis and PV was 0.24 ± 0.05 meq O_2/kg oil. Refined and deodorized rapeseed oil was provided by AAK Sweden AB (Malmö, Sweden), stored at −40 °C until use, and its fatty acid composition (determined by FAME analyses) was previously reported as follows: C16:0 (4.5), C18:1n-9 (60), C18:1n-7 (2.5), C18:2n-6 (19), C18:3n-6 (9.4), C20:1n-9 (1.5) (Yesiltas et al., 2021). The tocopherols and PV of the rapeseed oil were alpha-, 226.8 ± 3.26 μg/g oil; beta-, 58.2 ± 0.72 μg/g oil; gamma-, 299.7 ± 4.35 μg/g oil; delta-tocopherol, 6.6 ± 0.27 μg/g oil and PV, 0.76 ± 0.05 meq O_2/kg oil. EDTA disodium salt hydrate, purity ≥ 99% was purchased from Sigma-Aldrich (Søborg, Denmark) and used as a positive control in mayonnaise experiment due to its well-known antioxidant activity. *N*-heptane HPLC grade was purchased from VWR Chemicals (Søborg, Denmark). Volatile standards were purchased from Sigma-Aldrich (Søborg, Denmark). Bottles of 50 mg from Calbiochem (Søborg, Denmark) of purity ≥ 95% were used for each tocopherol standard (alpha-, beta-, gamma-, and delta-tocopherol). All the other chemicals and solvents used were of analytical grade.

2.2. Methods

2.2.1. Emulsion and mayonnaise production

First, a storage experiment of 5% fish oil-in-water emulsions at pH 4 was carried out as described in the next section (2.2.1.1). The antioxidant activity of the selected peptides were only reported in low-fat emulsions at pH 7 (Yesiltas et al., 2022). As the food system we would like to test the activity of peptides is mayonnaise, we tested the antioxidant activity of the peptides in a low-fat emulsion at pH 4. Second, based on the results of the low-fat emulsion study, a selection of the four most efficient peptides shown as M3, M4, M5 and M6 in Table 1 was included in the mayonnaise storage experiment (production of fish oil-enriched mayonnaises and experiment parameters are shown in section 2.2.1.2). The information regarding peptide codes, amino acid sequence, length, isoelectric point (pI), and predicted radical scavenging

Table 1
Peptide codes and their amino acid sequence, length, isoelectric point (pI), predicted radical scavenger (PRS) scores, and predicted metal chelator (PMC) scores.

Peptides	Amino acid sequence	Length	pI*	Net charge at pH 7*	Net charge at pH 4*	PRS score	PMC score	Emulsion codes**	Mayonnaise codes***
111-P-SCA	KWGPLRW	7	11.39	2	2	0.46	0.22	E2	–
113-P-SCA	VPFYFEHGPHI	11	6.05	−0.8	1.8	0.64	0.22	E3	–
123-S-SCA	DFPVR	5	6.68	0	0.2	0.38	0.26	E4	–
124-S-SCA	AGDWLIGDR	9	3.71	−1	−0.1	0.43	0.20	E5	M3
125-U-SCA	HWYD	4	4.87	−0.9	0.2	0.59	0.23	E6	–
128-U-SCA	MLWQYKPK	8	10.21	2	2	0.54	0.20	E7	M4
132-R-SCA	NNKWVPCLEFETEHGFVYREHH	22	5.79	−1.8	3.7	0.50	0.23	E8	M5
133-R-SCA	YWTMWK	6	9.49	1	1	0.53	0.20	E9	–
135-P-CHE	HCPSH	5	7.23	0.1	2	0.51	0.30	E10	–
139-P-CHE	YKLLHCPSHLQCKN	14	8.74	2.1	4	0.47	0.26	E11	–
144-P-CHE	DDDNLVLPEVYDQD	14	0.52	−6	−3	0.45	0.30	E12	M6

Abbreviations in the peptide codes: P, patatin-derived peptides obtained from potato; S, lectin-derived peptides obtained from seaweed; U, peptides derived from obtained from microbial organism; R, RuBisCO-derived peptides obtained from spinach; SCA, scavenger antioxidant activity; CHE, chelator antioxidant activity.
* pI and net charge at pH7 and pH4 were calculated using peptide property calculator (Innovagen AB, Lund, Sweden).
** E1 is not included as it is the control emulsion without any antioxidant peptide.
*** M1 and M2 are not showed as they are the control mayonnaises prepared without any antioxidant peptide and with EDTA, respectively.

and chelating activity scores are listed in Table 1, in accordance with previous reports (Yesiltas et al., 2022).

2.2.1.1. Production of 5% fish oil-in-water emulsions. Emulsions were produced as described previously (Yesiltas et al., 2022). Briefly, 5% fish oil-in-water emulsions (220 g) stabilized with 1 wt% of Tween 20 (TW20) and added 0.05 wt% antioxidant peptides were produced by dissolving peptides in 10 mM sodium acetate-10 mM imidazole buffer at pH 4 and shaken at 100 rpm in water bath at 50 °C for 2 h and continued to be shaken overnight at room temperature (20 °C) in darkness to assure that the peptides were dissolved completely. Next day, TW20 was added in the peptide solution and the final solution was adjusted to pH 4 by adding 1 M HCl. One emulsion was prepared with only TW20 as a control. Preliminary homogenization was performed using Ultra Turrax (Ystral, Ballrechten-Dottingen, Germany) for 3 min at 16000 rpm while fish oil was added into the aqueous phase within the first minute of mixing. Secondary homogenization was performed by running the primary emulsion through a Microfluidizer (M110L Microfluidics, Newton, MA, USA) equipped with a ceramic interaction chamber (CIXC, F20Y, internal dimension 75 μm) at 9 kpsi pressure for three passes. Sodium azide (0.05%) and 50 μM of $FeSO_4$ was added into the final emulsion and stirred with a spoon. The final pH of the emulsion was measured, ranging from 3.98 to 4.04. Emulsions were stored for 8 days at room temperature (20 °C) in darkness. Samples were collected for physical characterization and oxidative stability analyses during storage.

2.2.1.2. Production of fish oil-enriched mayonnaises. Based on the results obtained from low-fat emulsion storage experiment, four peptides were selected with the following codes: 124-S-SCA, 128-U-SCA, 132-R-SCA and 144-P-CHE, shown in Table 1, to test their antioxidant activity in the mayonnaise during storage. Peptides (75 mg/kg) were dissolved in distilled water by shaking in a water bath at 50 °C during 2 h, leaving them overnight under stirring at 100 rpm at room temperature (20 °C) in darkness. Different concentrations of peptides were studied in mayonnaise, 75, 165, 330, and 660 mg/kg, with 75 mg/kg being the concentration used for EDTA in industry (Jacobsen et al., 2001a). A concentration of 165 mg/kg was included for the peptide to test the equimolar concentration of EDTA. Following 330 and 660 mg/kg were included to test the double and quadruple concentrations of equimolar concentration of EDTA, respectively.

Mayonnaises enriched with fish oil were produced using Stephan mixer (UMC 5, Hameln, Germany) under vacuum at 1200 rpm according to (Meyer & Jacobsen, 1996). The mayonnaises were produced based on the listed ingredients: 64% rapeseed oil, 16% fish oil, 9.2% distilled water, 4% estragon vinegar (7%), 4% egg yolk, 3% salt, 1.2% lemon juice, 1% sugar, 0.3% salt, 0.2% Grindsted FF DC stabilizer and 0.1% potassium sorbate. Once produced, mayonnaises were kept in 100 mL closed brown bottles containing approximately 75 g each to get a similar headspace and stored up to 28 days at room temperature (20 °C) in darkness. Samples were taken at the following sampling points on days 0, 3, 7, 14, 21 and 28 and subjected to physical and oxidative stability analyses. The final pH of the mayonnaises ranged from 3.92 to 3.97.

2.2.2. Physical stability of emulsions and mayonnaises

Low-fat emulsions and mayonnaises were characterized by Turbiscan analysis, measurement of their droplet size distribution and zeta potential.

2.2.2.1. Turbiscan analysis. Physical stability of the low-fat emulsions was measured with Turbiscan Tower (Formulaction, Toulouse, France) at the first day of the production and the last day of the storage for 10 min each. A volume of 10 mL of emulsions were transferred into special vials, which were then placed into the instrument to be scanned at the length of the vials measuring backscattering (BS) and transmission (T). Obtained results were used to investigate if the sample experienced creaming, sedimentation, or flocculation. In addition, instrument reports a Turbiscan stability index (TSI), which is calculated based on T and BS values with the following equation,

$$TSI\,(t) = \frac{1}{N_h} \sum_{t_i=1}^{tmax} \sum_{z_i=z_{min}}^{z_{max}} |BST\,(t_i,\, z_i) - BST(\,t_{i-1}, z_i)\,| \quad (1)$$

where t_{max} is the measurement point at time t, when the TSI is calculated, z_{min} and z_{max} are the lower and higher selected limits,

respectively, N(h)= (z_{max}-z_{min})/Δh is the number of height position for the scan and BST (Backscattering and Transmission) is the signal that is considered (BS when T < 0.2%, otherwise T is taken). Measurements were run as a single determination for checking if the stability of the emulsions were acceptable.

2.2.2.2. Droplet size distribution. The droplet size distribution was measured by laser diffraction technique using Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) after 1 and 8 days of storage for low-fat emulsions and after 14 and 28 days of storage for mayonnaises. Before analysis, mayonnaise samples were diluted in SDS (Sodium Dodecyl Sulfate) buffer (10 mM NaH_2PO_4, 5 Mm SDS, pH 7) in the ratio of 1:9 (sample:buffer) and placed in ultrasonic bath at 25 °C for 20 min (twice) as previously described (Yesiltas et al., 2021). Droplet size analysis was carried out in low-fat emulsion and mayonnaise samples by diluting in recirculating water set at 3000 rpm until an obscuration of approximately 12–15% was reached (Jacobsen et al., 1999b). Results of droplet size distribution were carried out in triplicate, and they were given as the surface weighted (D[3,2]) and volume weighted (D[4,3]) mean diameters.

2.2.2.3. Zeta potential. The surface charge of oil droplets was determined on day 1 for low-fat emulsions using Zetasizer Nano ZS (Malvern Instruments, Ltd., Worcestershire, UK). Prior to analysis, emulsions were diluted (0.032 g of emulsion in 40 g of distilled water) and mixed (vortex). Then, samples were placed in a DTS-1070 disposable folded capillary cell (Malvern Instruments, Ltd., UK). Measurements were carried out in triplicate at 25 °C using a zeta potential range of (−) 100 to (+) 50 mV and samples were analyzed with 100 runs.

2.2.3. Oxidative stability of emulsions and mayonnaises

Oxidative stability analyses such as the measurement of PV, tocopherols, and secondary volatile oxidation products were carried out on the samples collected on days 0, 1, 2, 5, and 8 for low-fat emulsions and on days 0, 3, 7, 14, 21, and 28 for mayonnaises during the storage experiments.

2.2.3.1. Extraction of lipids. Frozen low-fat emulsions (−40 °C) were thawed and lipids from the emulsions were extracted using chloroform/methanol (1:1, v/v) according to a method described by Bligh and Dyer (1959) with a modification to reduce the amount of solvent used (30 mL chloroform and 30 mL methanol). The procedure for mayonnaises was different. The frozen mayonnaises (−40 °C) were thawed, and the oil phase was separated by centrifugation (Jacobsen et al., 1999a).

2.2.3.2. Peroxide value. Peroxide value (PV) was measured on the obtained lipid extracts (section 2.2.3.1) based on the ferro-thiocyanate method using a spectrophotometer at 500 nm (Shimadzu UV-1280, Holm&Halby, Brøndby, Denmark) as was described by Shantha and Decker (1994). Measurements were carried out in duplicate, and results were expressed in meq O_2/kg of oil.

2.2.3.3. Tocopherols. Tocopherol contents on the lipid extracts (section 2.2.3.1) were measured by normal phase HPLC (AOCS Official Method 8-89, 1998). The HPLC system was a 1100 series from Agilent Technologies with the following characteristics: mobile phase of heptane/2-propanol (100/0.4, v/v), isocratic pump with a flow rate of 1.0 mL/min, injection volume 20 μL, Waters Spherisorb 3 μm Silica column (4.6 mm I.D. × 150 mm), Waters Spherisorb 5 μm Silica guard column (4.6 mm I.D. × 10 mm) and fluorescence detection performed at 290 nm (excitation wavelength) and 330 nm (emission wavelength). Approximately 0.04 g of each sample was weighed and dissolved in 1 mL of heptane to be injected in the HPLC-FLD. Measurements were conducted in duplicate and results were expressed in μg of each tocopherol/g of oil.

2.2.3.4. Secondary oxidation volatile products. Volatile compounds were measured using dynamic headspace combined with GC–MS (Gas Chromatography-Mass Spectrometry). For extraction, 4 g of sample were weighed in pear-shaped glass tubes and heated in a water bath at 60 °C for 30 min with a nitrogen flow of 150 mL/min. Volatile compounds were collected on Tenax GR packed tubes by dynamic headspace. Additionally, in mayonnaise samples volatile acids were removed by using s-shaped tubes filled with KOH which were included before the Tenax GR tubes according to Hartvigsen et al. (2000). The volatile products trapped on the Tenax GR tubes were desorbed by using an ATD-400 automatic thermal desorbed connected to a GC for the separation of the volatile compounds. The GC system was an Agilent 6890 (Palo Alto, CA, USA) with the following characteristics: DB 1701 fused silica capillary column (0.25 mm I.D. × 30 m, 1 μm film thickness; J&W Scientific, Folsom, CA, USA). The oven conditions used for 5% fish oil-in-water emulsions were described previously by Yesiltas et al. (2022). Briefly, the oven program had an initial temperature of 45 °C for 5 min, which was increased 1.5 °C/min until reaching 55 °C, and increased 2.5 °C/min until reaching 90 °C, and finally increased 12 °C/min until 220 °C and kept for 4 min. The oven conditions for mayonnaise were as follows: the initial temperature was 35℃ and kept for 3 min, then the temperature was increased by 3 °C/min to 120 °C and then by 7 °C/min to 160 °C. Finally, the temperature was increased by 15 °C/min to 200 °C and kept for 4 min. GC was equipped with an Agilent HP 5973 mass spectrometer to analyze the volatile compounds (Network Mass Selective Detector, Agilent Technologies, 70 eV; mass to charge ratio scan between 30 and 250) and they were identified by MS-Library searches (Wiley 138 K, John Wiley and Sons, Hewlett Packard). The quantification was carried out for the following volatile compounds: 2-ethyl-furan, 1-penten-3-one, 1-penten-3-ol, pentanal, 1-pentanol, hexanal, 2,3-pentandione, *(E)*-2-hexenal, heptanal, *(E)*-2-heptenal, *(Z)*-4-heptenal, octanal, *(E,E)*-2,4-heptadienal and *(E,E)*-2,4-decadienal in fish oil-in-water emulsions and 2-ethyl-furan, 1-penten-3-one, 1-penten-3-ol, pentanal, *(E)*-2-pentenal, 1-pentanol, hexanal, 2-penten-1-ol, *(E)*-2-hexenal, heptanal, *(E)*-2-heptenal, *(E,E)*-2,4-heptadienal and *(E,Z)*-2,6-nonadienal in mayonnaise samples. The calibration curve was prepared from a stock standard solution containing these volatile compounds. Seven different concentrations of diluted stock solution were added to fish oil-in-water emulsion (produced the same as E1) for low-fat emulsion analysis and added to mayonnaise produced with only rapeseed oil for the analysis of mayonnaise samples. Analysis was performed in triplicate and the results were expressed in ng/g of sample.

2.2.3.5. Percentages of inhibition. The antioxidant activity of the different peptides was assessed by the percentages of inhibition. They were calculated based on PV and three volatile compounds (2-ethyl-furan, hexanal and *(E,E)*-2,4-heptadienal) development to compare the emulsion systems for the storage experiments. The following formula was used for the calculation:

$$\%\ inhibition = \left(\frac{(Control\ sample - Antioxidant\ sample)}{Control\ sample}\right) x\,100$$

2.2.4. Statistical analysis

Data analysis was performed with IBM SPSS Statistics (v 23, IBM, Armonk, NY, USA). Mean and standard deviation values were considered as descriptive statistical parameters for emulsions and mayonnaise results. Data were subjected to one-way analysis of variance (ANOVA) and significant differences between means were assessed by Tukeýs post-hoc test at a 95% confidence level (differences were considered significant at $p \leq 0.05$).

3. Results and discussion

We previously reported the identification, abundance and potential release of antioxidant peptides derived from sustainable sources by

using bioinformatics and proteomics, which could allow the design of a more targeted production process (Yesiltas et al., 2022). The antioxidant activity of these peptides has already been confirmed *in vitro* and in low-fat emulsion model system at pH 7 (Yesiltas et al., 2022). This work investigates the influence of pH on the antioxidant activity of these previously identified peptides when added to 5% fish oil-in-water emulsions at pH 4. Moreover, the best performing 4 peptides in low-fat emulsions were further tested in mayonnaise.

3.1. Physical and oxidative stabilities of 5% fish oil-in-water emulsions

3.1.1. Physical stability of emulsions

Low-fat emulsions showed good physical stability in general as indicated by the low values obtained (<1) for Turbiscan stability index (TSI) (see Fig. S1 in the Supplementary material). However, TSI values increased to 3 or even above 3 at day 8 of storage for E5 (124-S-SCA), E6 (125-U-SCA) and E11 (139-P-CHE), which indicates instability. In the case of E5 (124-S-SCA) and E6 (125-U-SCA), the value of pH (4) is close to their pI (3.71 and 4.87 respectively) which generates net charges close to zero in both (Table 1). This can explain a reduction in the solubility of the peptides making them precipitate and thus, an insufficient surface charge density. In turn, it can result in a loss of electrostatic stability which would imply coalescence of the emulsion droplets and emulsion destabilization (Ma & Chatterton, 2021). On the other side, regarding E11 (139-P-CHE), the pH value was below its pI (8.74) which led to a positive net charge (4) (see Table 1). However, it seemed that the surface charges of droplets were not sufficient to prevent flocculation by electrostatic repulsion. As previously reviewed by Ma and Chatterton (2021) some possible reasons could be the different adsorbed configurations of peptides as well as the low peptides' concentration that led to an insufficient peptide' coverage of droplets and thus, a loss of electrostatic stabilization. Droplet size and zeta potential results of low-fat emulsions during storage are reported in Table S1 (Supplementary material). Results for D[3,2] were in the range of 0.131 and 0.136 μm on day 1 and similar values were observed on day 8. D[4,3] values were also very similar during storage and ranged between 0.226 and 0.260 μm for all emulsions. Considering zeta potential, low absolute values (<30 mV) were observed in all cases. This is due to the non-ionic nature of TW20 leading to low electrostatic repulsion between oil droplets. Despite the low zeta potential values, good physical stability of low-fat emulsions was observed due to the strong emulsifying ability of TW20 (1 wt% of the emulsion) as a surfactant, known to rapidly move to the oil–water interface during homogenization and stabilize the oil droplets (Zhao et al., 2021). Furthermore, as droplet size and zeta potential remained very similar during the storage experiment, these results did not support the increased TSI values previously mentioned for E5 (124-S-SCA), E6 (125-U-SCA) and E11 (139-P-CHE). In our previous study, similar results were obtained over an 8-day period for 5% fish oil-in-water emulsions at pH 7 stabilized with TW20 and also containing peptides as antioxidants, where D[3,2] ranged between 121 and 132 nm, D[4,3] between 188 and 229 μm, and zeta potential between (−) 7.5 and (−) 4.7 mV (Yesiltas et al., 2022).

3.1.2. Oxidative stability of emulsions

3.1.2.1. Peroxide value. PV results of low-fat emulsions are presented in Fig. 1A and the statistical analysis is showed in Table S2 (Supplementary material). Due to increasing temperature, exposure to oxygen and high shear during emulsion production, lipid oxidation reactions may take place during emulsion production (Berton-Carabin et al., 2014). Indeed, we observed considerably high PV of emulsions at day 0 ranging from 14.6 ± 0.62 to 54.5 ± 7.23 meq O_2/kg oil, which was significantly higher than PV of fresh fish oil (PV < 0.1 meq O_2/kg oil). Moreover, the initial concentration of PV significantly differed between low-fat emulsions at day 0. Thus, PV of E12 (144-P-CHE) was the lowest value while E10 (135-P-CHE) and E11 (139-P-CHE) presented a significantly higher PV compared to rest of the emulsions and remained the highest also on day 1 (Fig. 1A and Table S2, Supplementary material). During storage, the significant increase in PV was observed from day 0 to 1 for only E1 (control) and E8 (132-R-SCA), from day 1 to 2 for all emulsions except for E12 (144-P-CHE), which increased from day 0 to 2, and finally from day 2 to 5 for all emulsions. At day 2, when the significant increase in PV took place for most emulsions, the difference between some samples was also significant. The E10 was significantly higher than the other emulsions while the most stable three emulsions were in the following order: E5 (124-S-SCA) > E8 (132-R-SCA) > E12 (144-P-CHE), latter is the most oxidatively stable (Table S2, Supplementary material).

These results differ from what was observed in the previously mentioned study, which contained the same set of antioxidant peptides added to 5% fish oil-in-water emulsions stabilized with TW20 at pH 7 and stored for 8 days, where PV already significantly increased from day 0 for most of the emulsions instead of day 1 (Yesiltas et al., 2022). In general, at pH 7 the control emulsion (E1) showed a significant, almost linear, PV increase throughout the storage. E9 (133-R-SCA) exhibited promising antioxidant activity in low-fat emulsion at pH 7 with a lag phase until day 5 (Yesiltas et al., 2022), while in the present study the

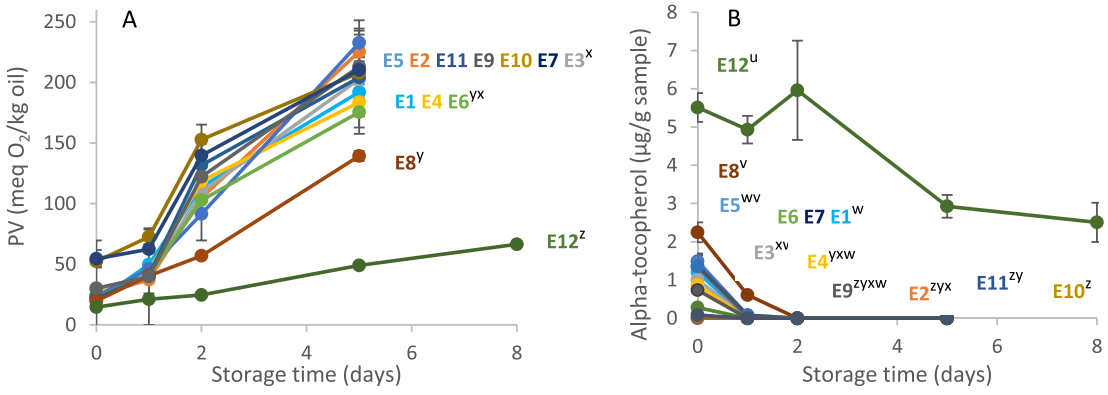

Fig. 1. Formation of A) primary oxidation products (PV), and the consumption of B) alpha-tocopherol in 5% fish oil-in-water emulsion at pH 4 during storage. Each value in the figure was presented as the mean ± standard deviation (n = 2). Emulsion codes: E1, without any antioxidant peptide; E2, containing 111-P-SCA; E3, containing 113-P-SCA; E4, containing 123-S-SCA; E5, containing 124-S-SCA; E6, containing 125-U-SCA; E7, containing 128-U-SCA; E8, containing 132-R-SCA; E9, containing 133-R-SCA; E10, containing 135-P-CHE; E11, containing 139-P-CHE; E12, containing 144-P-CHE. Abbreviations: P, patatin-derived peptides obtained from potato; S, lectin-derived peptides obtained from seaweed; U, peptides derived from obtained from microbial organism; R, RuBisCO-derived peptides obtained from spinach; SCA, scavenger antioxidant activity; CHE, chelator antioxidant activity. $^{z\text{-}u}$ Letters indicate significant differences between samples at A) day 5, and B) day 0 (ANOVA, Tukey's post-hoc test, $p \leq 0.05$).

same peptide provided one of the highest PV on day 2 in low-fat emulsion at pH 4.

3.1.2.2. Tocopherol content. The oxidative stability of emulsions was also measured by analyzing the consumption of the four tocopherol homologues (alpha, beta, gamma, and delta tocopherol) present in the fish oil used during storage (Fig. 1B and Table S3 and Fig. S2, Supplementary material). Emulsions showed similar trends in the content of the different tocopherol homologues, with alpha and gamma tocopherol being the ones present in the highest concentration. At day 0, low content of tocopherols was found in all emulsions for all types of tocopherols (Table S3, Supplementary material). It was attributed not only to the low oil amount used in the emulsion production but also tocopherols might be consumed during the emulsification process while PV increased. In most emulsions the alpha tocopherol almost entirely consumed on day 1, except for E8 and E12 which had the highest contents from the beginning and during storage. The E8 (132-R-SCA) had a reduction from day 1 to 2 while E12 (144-P-CHE) showed a significant decrease from day 2 to 5 (Fig. 1B and Table S3, Supplementary material). Gamma tocopherol significantly decreased ($p \leq 0.05$) in all samples at day 1, except for E12; and was almost fully consumed at day 2, except for E8 and E12 (Table S3, Supplementary material).

It is noteworthy that both alpha and gamma tocopherols significantly increased in concentration from day 1 to 2 for E12 (144-P-CHE) compared to the rest of emulsions (Table S3, Supplementary material). A possible explanation could be a regeneration of oxidized tocopherols as previously reported due to other antioxidants (peptides in this case) (Elias et al., 2008; Yesiltas et al., 2018). Furthermore, these results were in line with PV, as the highest tocopherol contents indicated the better antioxidant activity of the peptides controlling the formation of lipid oxidation products during storage. Regarding beta and delta tocopherol contents, E12 was again the richest of these tocopherols during storage (Table S3, Supplementary material).

In general, the highest consumption of all tocopherols was observed for E10 (135-P-CHE) and E11 (139-P-CHE), while the best performing peptides in preventing tocopherol consumption were E12 (144-P-CHE) and E8 (132-R-SCA). These results partly agree with the previous study regarding low-fat emulsions at pH 7 (Yesiltas et al., 2022), where peptides in E11 (139-P-CHE) and E12 (144-P-CHE) were reported as the best in preventing the consumption of tocopherols.

3.1.2.3. Secondary oxidation volatile compounds. Fig. 2 shows the content of 2-ethylfuran and *(E,E)*-2,4-heptadienal derived from n-3 and hexanal derived from n-6 polyunsaturated fatty acids. Although 14 volatiles compounds were identified and quantified (Table S4 and Fig. S3, Supplementary material), these 3 selected volatiles had higher concentrations and were representative of the rest during storage. In general, significantly increasing concentrations were found after a lag phase, which differed based on the peptide used as well as the volatile compound formed. For 2-ethylfuran (Fig. 2A), the lag phase was 1 day for E2 (111-P-SCA) and E5 (124-S-SCA), and 2 days for E1 (control), E3 (113-P-SCA), E4 (123-S-SCA) and E8 (132-R-SCA), however, no lag phase was found for the rest of emulsions. A slightly faster increase showed the development of *(E,E)*-2,4-heptadienal, compared to 2-ethylfuran, which was significant on day 1 for the E1, E2, E3, E6, E11, on day 2 for the E4, E5, E7, E8, E9, E10 and on day 8 for E12 (Fig. 2B). Finally, for hexanal (Fig. 2C), the significant increase occurred mainly on day 2 except for E4, E5, E6 and E8 (day 5) and E12 (day 8).

The increase in the secondary oxidation products was attributed to decomposition of lipid hydroperoxides, which mainly occurred on days 1 and 2. Considering the significantly low content of these three volatile compounds found in E12 (144-P-CHE) followed by E8 (132-R-SCA) during the entire storage period showed that these two peptides had the highest antioxidant activity, in line with the PV and tocopherols results (sections 3.1.2.1 and 3.1.2.2).

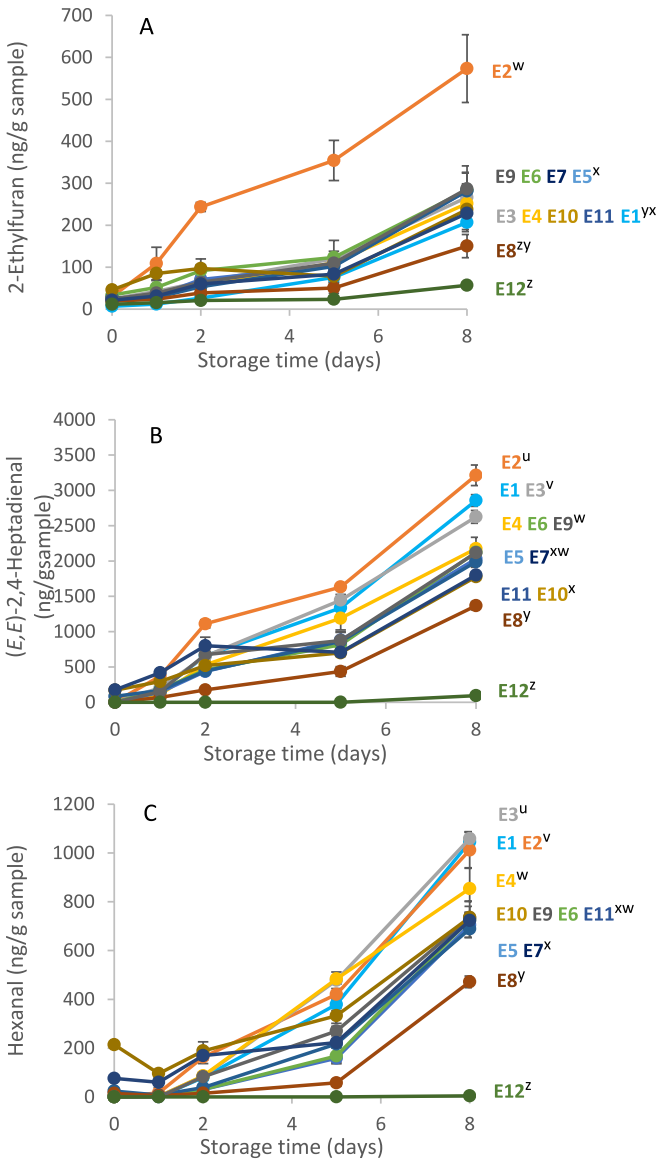

Fig. 2. Formation of some secondary oxidation products in 5% fish oil-in-water emulsion at pH 4 along the storage: A) 2-ethylfuran, B) *(E,E)*-2,4-hepatadienal and C) hexanal. Each value in the figure was presented as the mean ± standard deviation (n = 3). Emulsion codes: E1, without any antioxidant peptide; E2, containing 111-P-SCA; E3, containing 113-P-SCA; E4, containing 123-S-SCA; E5, containing 124-S-SCA; E6, containing 125-U-SCA; E7, containing 128-U-SCA; E8, containing 132-R-SCA; E9, containing 133-R-SCA; E10, containing 135-P-CHE; E11, containing 139-P-CHE; E12, containing 144-P-CHE. Abbreviations: P, patatin-derived peptides obtained from potato; S, lectin-derived peptides obtained from seaweed; U, peptides derived from obtained from microbial organism; R, RuBisCO-derived peptides obtained from spinach; SCA, scavenger antioxidant activity; CHE, chelator antioxidant activity. $^{z\text{-}u}$ Letters indicate significant differences between samples at day 8 (ANOVA, Tukey's post-hoc test, $p \leq 0.05$).

According to these results, the best performing peptides in low-fat emulsions at pH 4 were 144-P-CHE (E12) and 132-R-SCA (E8) because of the reduction in the formation of primary and secondary lipid oxidation products as well as the prevention of tocopherol depletion during storage. The antioxidant activity of 144-P-CHE (E12) is mainly attributed to its high metal ion chelating activity (PMC score of 0.30). This promising activity could be related to its richness in aspartic acid (Asp) and the presence of glutamic acid (Glu) residues on its structure (Elias et al., 2008). Likewise, peptide 132-R-SCA (E8) also presented

promising antioxidant activity, which could be due to specific amino acids such as phenylalanine (Phe) and cysteine (Cys) residues on its structure. Indeed, the presence of multiple Cys residues have been related to increase metal cations chelation (Gregersen et al., 2016). This would explain that 139-P-CHE (E11) peptide showed high chelation ability because the two Cys residues on its sequence. Peptide 144-P-CHE (E12) also performed well in preventing lipid oxidation and tocopherol consumption in low-fat emulsions at pH 7 (Yesiltas et al., 2022). The different pH could also alter the secondary structure of peptides being one of the factors leading to changes in their antioxidant activity. In fact, Yuan et al. (2018) reported an increased antioxidant activity when secondary structure changed from α-helix to β-strand in yogurt peptides.

As previously reviewed, pH and pI influenced the ability of peptides to chelate metals since the electrostatic charge of peptides is dependent on both (Elias et al., 2008). In the case of 144-P-CHE peptide, it has a negative net charge in both emulsions at pH 4 and pH 7 (as both pH values are above its pI at 0.52, see Table 1). Therefore, electrostatic attraction might take place between the negatively charged peptides and the cationic prooxidant transition metal ions favoring the peptide chelating activity and thus inhibiting lipid oxidation reactions. However, while 132-R-SCA (E8) at pH 4 was the second-best peptide inhibiting lipid oxidation, 133-R-SCA (E9), as a shorter peptide, showed a good potential as antioxidant at pH 7. It can be noted that both peptides (132-R-SCA and 133-R-SCA) came from RuBisCO and shared common amino acids in their sequence; tyrosine (Tyr), tryptophan (Trp), and lysine (Lys) which might be responsible for the scavenging activity (Elias et al., 2008). Also, they differ in pI and 132-R-SCA was more positively charged at pH 4 compared to 133-R-SCA (as the pH was below the pI for 132-R-SCA) while 132-R-SCA at pH 7 showed a negative net charge which did not explain the more pronounced oxidation levels for E9 at pH 4. However, this was observed for E11 (139-P-CHE) in comparison to emulsions at pH 7 (Yesiltas et al., 2022), where the increased positive net charge of the peptide at pH 4 led to lower chelating activity. Regarding the previous case, besides net charge, the differences between antioxidant activity at pH 4 and 7 could be also due to the differences in solubility of iron at pH 7 and 4. 133-R-SCA has the same positive net charge at both pH values (Table 1), and thus the change in the antioxidant activity is presumably due to the solubility of the iron which increased its accessibility at the oil–water interface of the emulsion at pH 4 more than at pH 7 conditions. On the other hand, at day 5, E6 containing 125-U-SCA in emulsions at pH 7 (Yesiltas et al., 2022) showed the highest PV, while it had the third lowest PV among the emulsions at pH 4 in the present study; however, the PV was much lower (50 meq O_2/kg oil) than what is obtained in this study (175 meq O_2/kg oil). The peptide 125-U-SCA is negatively charged at pH 7 and slightly positively charged at pH 4; therefore, the antioxidant activity could be positively influenced by lower iron solubility at pH 7 (despite its negative net charge). Overall, the remarkably high lipid oxidation rates observed for most emulsions in this study, compared to the previous study by Yesiltas et al. (2022), might be explained by this higher solubility of iron at low pH and the reduced chelation by generally shifted charges to more positive values, which decrease the affinity of peptides to bind metal ions (Berton-Carabin et al., 2014).

Further, the peptide location in low-fat emulsions at pH 7 was previously investigated by Yesiltas et al. (2022) and no interaction between peptides and TW20 at the oil–water interface was reported for the same 11 peptides studied in this study. As the peptides were predominantly located in the aqueous phase of the oil-in-water emulsions at pH 7, peptides could mainly exhibit metal chelating activity in the water phase where the metal ions are present, while they were not acting as radical scavengers as lipid radicals can be found at the interface or in the oil phase. Overall, since all peptides assessed were mainly located in the aqueous phase (Yesiltas et al. 2022) and all emulsions were subjected to the same production process (same droplet size distribution) and were stabilized with the same surfactant type and concentration (similar interface thickness and charge), the differences observed in the oxidative stability of emulsions were mainly due to the different antioxidant activity of peptides. Thus, according to the oxidative results obtained for the low-fat emulsions study at pH 4 and the previous results reported at pH 7 for the same peptides (Yesiltas et al., 2022), peptides 144-P-CHE, 132-R-SCA, 124-S-SCA and 128-U-SCA were selected to investigate how their use may impact the physical and oxidative stability of a real food emulsion system with low pH like mayonnaise.

3.2. Physical and oxidative stabilities of fish oil-enriched mayonnaises

3.2.1. Physical stability of mayonnaises

Although some significant differences were observed in droplet size during storage, the magnitude of these differences were very small for both D[3,2] and D[4,3]. Thus, they were not considered of any importance with respect to their effect on oxidative stability (Table S5, Supplementary material). Results for D[3,2] were in the range of 3.94 and 4.09 μm on day 14 with similar droplet size values on day 28 (3.99 and 4.39 μm) and D[4,3] values ranged between 5.01 and 5.47 μm on day 14 and varied between 4.95 and 5.71 μm on day 28. Thus, mayonnaise samples with such droplet sizes can be expected to be physically stable emulsions as also reported in a previous study (Jacobsen et al., 2000). Overall, the low-fat emulsions and mayonnaises presented differences regarding their droplet size. Mayonnaise samples had bigger oil droplets than low-fat emulsions, as previously reported in other studies (Alemán et al., 2015; Sørensen et al., 2012; Yesiltas et al., 2021). This was expected because of the emulsification method used for preparation of the emulsions (Stephan mixer vs. microfluidizer) as well as the use of different emulsifiers (egg yolk vs. TW20) and oil contents (80 vs. 5%).

3.2.2. Oxidative stability of mayonnaises

3.2.2.1. Peroxide value. The control mayonnaise (M1) showed a significant increase in PV from day 7 reaching a final PV of 20.9 ± 0.53 meq 0_2/kg of oil on day 28 (Fig. 3A and Table S6, Supplementary material).The evolution of PV during storage of the emulsions containing peptides significantly differed (Fig. 3A). M3 (124-S-SCA), M5 (132-R-SCA) and M6 (144-P-CHE) presented a lag phase of 7 days with a significant increase in PV at day 14 and onwards (Table S6, Supplementary material). However, the 128-U-SCA peptide added to M4 resulted in a shorter lag phase (3 days) compared to the previous peptides. Despite that M2 showed the shortest lag phase by increasing PV significantly at day 3, the PV on days 14, 21 and 28 of storage period was significantly lower for this mayonnaise containing EDTA compared to the rest of the mayonnaises (Fig. 3A and Table S6, Supplementary material).

Among emulsions containing peptides, the lowest formation of hydroperoxides at the end of storage (day 28) was for M6 (13.8 ± 0.18 meq 0_2/kg of oil), which was stabilized with the peptide 144-P-CHE. This might be related to its antioxidant properties (chelating activity) due to some amino acid residues present in its structure (such as Asp and Glu) which provide a very low isoelectric point (pI = 0.52) and further a high negative net charge due to pH (García-Moreno et al., 2020a). In contrast, M5 containing the peptide 132-R-SCA presented, in general, the highest PV compared to the other mayonnaises on days 14, 21 and 28 (5.6 ± 0.01, 14.4 ± 0.40 and 22.0 ± 0.22 meq O_2/kg of oil, respectively) with no significant differences to control on days 21 and 28 (Fig. 3A and Table S6, Supplementary material). On the other hand, M3 containing peptide 124-S-SCA showed intermediate antioxidant activity at the end of the storage (17.5 ± 0.37 meq O_2/kg of oil) (Fig. 3A and Table S5, Supplementary material).

3.2.2.2. Tocopherol content. Alpha and gamma tocopherols were found in highest amounts in mayonnaise due to their higher content in fish and rapeseed oils. A significant consumption of these two tocopherol homologues was observed from 7 to 14 days in most mayonnaises except for M2 for alpha and M2 and M5 for gamma tocopherols (Table S7,

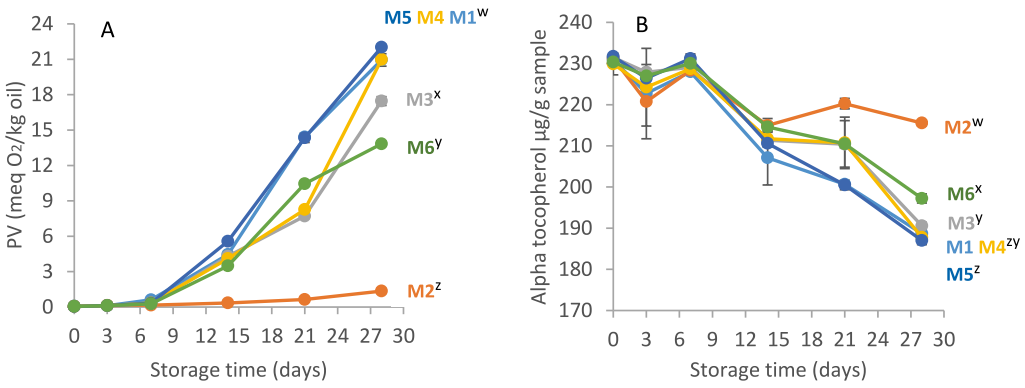

Fig. 3. Mayonnaise concentration of A) primary oxidation products (PV) and B) alpha-tocopherol throughout the storage. Each value in the figure was presented as the mean ± standard deviation (n = 2). Mayonnaise codes: M1, without any antioxidant peptide; M2, containing EDTA; M3, containing 124-S-SCA; M4, containing 128-U-SCA; M5, containing 132-R-SCA; M6, containing 144-P-CHE. Abbreviations in peptide codes: P, patatin-derived peptides obtained from potato; S, lectin-derived peptides obtained from seaweed; U, peptides derived from obtained from microbial organism; R, RuBisCO-derived peptides obtained from spinach; SCA, scavenger antioxidant activity; CHE, chelator antioxidant activity; EDTA, ethylenediaminetetraacetic acid. $^{z\text{-}w}$ Letters indicate significant differences between samples at day 28 (ANOVA, Tukey's post-hoc test, $p \leq 0.05$).

Supplementary material). This indicated that these tocopherols were consumed due to their action as a natural chain breaking antioxidant, which was in line with the subsequent increase in the formation of primary oxidation products. In contrast, there were no significant decreases in beta and delta tocopherols during storage for most mayonnaises (Table S7, Supplementary material).

Both alpha and gamma tocopherol significantly decreased in M2 compared to the rest of the mayonnaises with a reduction of 6.79% and 6.82%, respectively, from day 0 to day 28. It was followed by M6 (144-P-CHE), where the reduction was 14.37% and 8.63%, respectively. However, higher reductions of alpha and gamma tocopherols were observed for the rest of the mayonnaises during the storage: M1 (control), 18.28% and 9.98%; M3, 17.67% and 9.88%; M4, 18.31% and 9.57% and M5, 19.29% and 10.88%, respectively. This might indicate that the main part of the antioxidant activity was due to the chelating properties of EDTA, and 144-P-CHE used in these mayonnaises, which agreed with the lowest PV found in these samples (Fig. 3A).

3.2.2.3. Secondary oxidation volatile compounds. In mayonnaises, 13 volatiles were identified and quantified (Table S8 and Fig. S5, Supplementary material). The development of 2-ethylfuran, *(E,E)*-2,4-heptadienal and hexanal during 28 days of storage is shown in Fig. 4 as they had a representative trend for the other volatile compounds. The increase in concentration of these volatiles was also reported in other mayonnaise storage experiments (Alemán et al., 2015; Yesiltas et al., 2021). For 2-ethylfuran and *(E,E)*-2,4-heptadienal, a similar lag phase was found for all mayonnaises containing peptides (7 days), until the significant increase in concentration occurred on day 14 (Fig. 4A,B and Table S8, Supplementary material). The exception was M6 (144-P-CHE), for which the lag phase was of 14 days with the significant increase occurring at day 21. However, a shorter lag phase was observed for hexanal with an increment in concentration from day 3 for M3-M5 and from day 7 for M6 (Fig. 4C and Table S8, Supplementary material).

Overall, PV and volatile compounds showed a similar trend in mayonnaise samples with increasing contents from day 14, which was also in accordance with the reduction of tocopherols at large. In summary, after M2 (EDTA), M6 (144-P-CHE) had the highest oxidative stability with the lowest PV and content of volatile compounds. However, the rest of the mayonnaises were not clearly different from the control at the end of the storage, except for M3 (124-S-SCA) in terms of primary oxidation and tocopherol consumption which followed M6.

3.3. Concentration effect of one selected peptide in mayonnaise

The higher molar concentration of an antioxidant may be advantageous for smaller peptides when all the peptides were added in the same mass concentration. Therefore, a range of concentrations of 144-P-CHE were tested including the equimolar concentration of EDTA when added in mayonnaise to determine the concentration effect on antioxidant activity of the peptide. The concentrations assayed were: 75 mg/kg (commonly used mass concentration for EDTA), 165 mg/kg (half of the EDTA equimolar concentration of the peptide), 330 mg/kg (EDTA

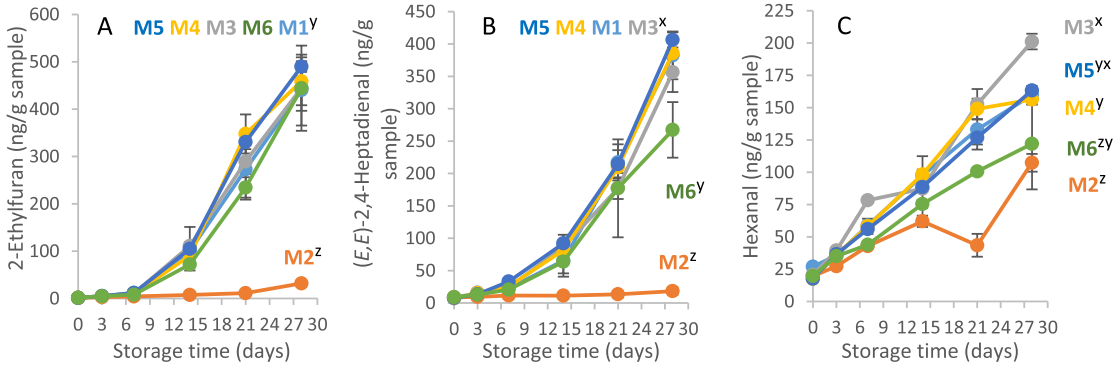

Fig. 4. Mayonnaise concentration of secondary oxidation products during storage A) 2-ethylfuran, B) *(E,E)*-2,4-heptadienal and C) hexanal. Each value in the figure was presented as the mean ± standard deviation (n = 3). Mayonnaise codes: M1, without any antioxidant peptide; M2, containing EDTA; M3, containing 124-S-SCA; M4, containing 128-U-SCA; M5, containing 132-R-SCA; M6, containing 144-P-CHE. Abbreviations: P, patatin-derived peptides obtained from potato; S, lectin-derived peptides obtained from seaweed; U, peptides derived from obtained from microbial organism; R, RuBisCO-derived peptides obtained from spinach; SCA, scavenger antioxidant activity; CHE, chelator antioxidant activity; EDTA, ethylenediaminetetraacetic acid. $^{z\text{-}x}$ Letters indicate significant differences between samples at day 28 (ANOVA, Tukey's post-hoc test, $p \leq 0.05$).

equimolar concentration of the peptide), and 660 mg/kg (twice the EDTA equimolar concentration of the peptide). Additionally, a control mayonnaise without any added antioxidant (MP1) and a mayonnaise prepared with EDTA at 75 mg/kg (MP6) were investigated. The physical stability results showed that the droplet size of the mayonnaises was not affected by the use of different concentrations of peptides within the tested range (data not shown).

Formation of hydroperoxides (PV) after 28 days of mayonnaise storage was significantly higher with the peptide concentrations of 660, 165 and 75 (MP6, MP4 and MP3) followed by the control (MP1) (Fig. 5A). However, the concentration of 330 (MP5) had significantly lower PV at the end of storage. The content of alpha-tocopherol on day 28 showed a significantly higher decrease in mayonnaise produced with 660, 165 and 75 concentrations while lower consumption was observed in the MP5 (330) and control (MP1) (Fig. 5B). The secondary oxidation products showed lower increase over storage for 2-ethylfuran in MP5 (330) compared to the rest of mayonnaises, whereas only MP4 had significantly higher values of hexanal and *(E,E)*-2,4-heptadienal when compared with all mayonnaises. EDTA was the most efficient antioxidant for PV and all volatiles compounds throughout the storage time (Fig. 5A and 5C-E).

These results indicated that even if the concentration influences the oxidative stability of the mayonnaises, it is not the only factor. Overall, the 144-P-CHE peptide exhibited a concentration dependent antioxidant effect in mayonnaises, with an optimum concentration at 330 mg/kg. However, 144-P-CHE was not as efficient antioxidant as EDTA in mayonnaise. This might be explained by higher binding constants towards metal ions, notably iron, in EDTA than 144-P-CHE. In fact, the major iron-carrier in egg yolk is the protein phosvitin (phosvitin-Fe^{3+}). When pH decrease, it causes the breakdown of egg yolk structure releasing the Fe^{3+} from the oil–water interface into the aqueous phase (Jacobsen et al., 1999a). The EDTA's strong affinity towards Fe^{3+} leads to forming strong complexes by its binding constant of $1.3 \times 1025\ M^{-1}$ in mayonnaise (Jacobsen et al., 2008) which is assumed to be a value several orders of magnitude higher than that of 144-P-CHE. Likewise, pH could be another factor influencing the metal binding ability of different antioxidants. For instance, EDTA is specifically sensitive to pH values around 4, which increases its chelating activity (Jacobsen et al., 2008). An influence of the ratio between EDTA: iron on the antioxidant activity of EDTA in mayonnaise has been also previously reported (Halliwell & Gutteridge, 1989). Overall, further studies are needed to identify the binding constants of the antioxidant peptides, to better understand their lower antioxidant capacity compared to EDTA. The binding constants of peptides should be investigated using Surface Plasmon resonance and switchSense (Canabady-Rochelle et al., 2018; El Hajj et al., 2021).

3.4. Comparison of the results obtained from low-fat emulsions and mayonnaises

To compare the peptides' antioxidant effect in the different emulsion systems (mayonnaise and low-fat emulsion at pH 4) and the emulsions at pH 7 investigated in the previous study (Yesiltas et al., 2022), % inhibition was calculated. The % inhibition was based on PV, 2-ethylfuran, (*E,E*)-2,4-heptadienal and hexanal development during storage. According to these results (Table S9, Supplementary material) the % inhibition confirmed that 144-P-CHE peptide was the most efficient antioxidant in inhibiting the formation of hydroperoxides and the 3 mentioned volatiles when added to mayonnaises and emulsions at pH 4. However, it is noteworthy that better antioxidant properties were obtained in low-fat emulsion at pH 4 than in mayonnaise for this peptide. Considering PV, 74% inhibition at day 5 was found for emulsions (pH 4) while the maximum inhibition for mayonnaises was 56% at day 7 (Table S9, Supplementary material). Similar to PV, the % inhibition in emulsions at pH 4, for hexanal and (E,E)-2,4-heptadienal reached the highest value (100%) during all the storage experiment, while 2-ethylfuran reached a maximum of 73% at day 8. These results were much higher than mayonnaises where 2-ethylfuran and hexanal showed a maximum of 32% or 25% at day 7, and (E,E)-2,4-heptadienal of 30% at day 28 (Table S9, Supplementary material). This better antioxidant activity of peptides in low-fat emulsions compared to mayonnaises can be explained due to the food matrix effect. As previously reviewed, there are many molecules in foods that can react with peptides leading to a modification of their structure and thus affecting their activity (Kamdem & Tsopmo, 2019; Tkaczewska, 2020). The chemical reactivity of peptides within the food matrix can include interactions with free radicals, lipid oxidation products or carbohydrates (sugars) (Kamdem & Tsopmo, 2019). For instance, reactive species such as hydroxyl radicals can be generated in foods and can react and modify peptides availability and activity; peptides containing lysine, tryptophan, cysteine, and methionine are particularly susceptible to the free radicals' attack (Kamdem & Tsopmo, 2019). Also, possible reactions of secondary lipid oxidation products (aldehydes) with bioactive peptides have been reviewed, as well as reactions between amine groups from peptides and reducing sugars to form complex products (Maillard reaction) (Kamdem & Tsopmo, 2019). All these interactions may occur in different food matrices such as mayonnaises, compromising/reducing the peptides activity. On the other hand, the 144-P-CHE peptide did not show the best antioxidant activity at pH 7 compared to the other peptides, which could be explained by the effect of net charge at different pH values.

Further, low-fat emulsions presented the highest PV and volatile compound contents compared to mayonnaises. This might be explained by them having smaller droplet size, thereby increasing their total surface area, in turn favoring the contact between substrates and prooxidants. In fact, the droplet size effect has been previously reported in both types of emulsion (high- and low-fat content) (Azuma et al., 2009; Jacobsen et al., 2000). Other authors reported no significant effect of droplet size; however, they assumed the main factor to be related to oil-phase volume fraction because when it increases, the aqueous phase decreases proportionally thereby decreasing the amount of water-soluble prooxidants such as metal ions (Berton-Carabin et al., 2014). Furthermore, other factors affecting lipid oxidation could be viscosity and added Fe^{2+}. The Fe^{2+} of emulsions at pH 4 corresponded to a final concentration of 2.8 mg/kg whereas the concentration of iron in mayonnaise is calculated as 1 mg/kg based on the iron content reported in egg yolk and mayonnaise formulation. Also, the more viscous the system is, such as mayonnaises, the more difficult for the metals and radicals to initiate and propagate lipid oxidation reactions because of lower diffusivity. Thus, despite the similar pH in mayonnaises and low-fat emulsions (pH 4), the different iron amounts, viscosity, oil-phase volume or tocopherol contents could be some of the reasons for the lower oxidation rates found in mayonnaises regardless the shorter storage for emulsions.

4. Conclusions

Bioinformatically-predicted antioxidant peptides from natural alternative sources (potato, seaweed, microbial and spinach particularly from RuBisCO) showed different antioxidant efficacy in low-fat model emulsions (pH 4) and a complex high-fat food emulsion (i.e., mayonnaise). The difference in peptides' performance in mayonnaise and low-fat emulsions denoted the importance of peptides' concentration, composition, and structure (e.g., pI and charge) on the emulsion stabilization. It is remarkable how peptides' net charge significantly affects the ability of peptides to chelate metals. These results clearly show that anionic peptides, located in the aqueous phase of emulsions improve the oxidative stability of emulsions at pH 4 and mayonnaises. Further, the present results demonstrated that it is not possible to extrapolate the antioxidant peptide potential from one matrix to another mainly due to the complexity of real food systems and because all the factors involved in the peptides' behavior when considering both systems. Overall, the 144-P-CHE peptide (DDDNLVLPEVYDQD) derived from potato patatin

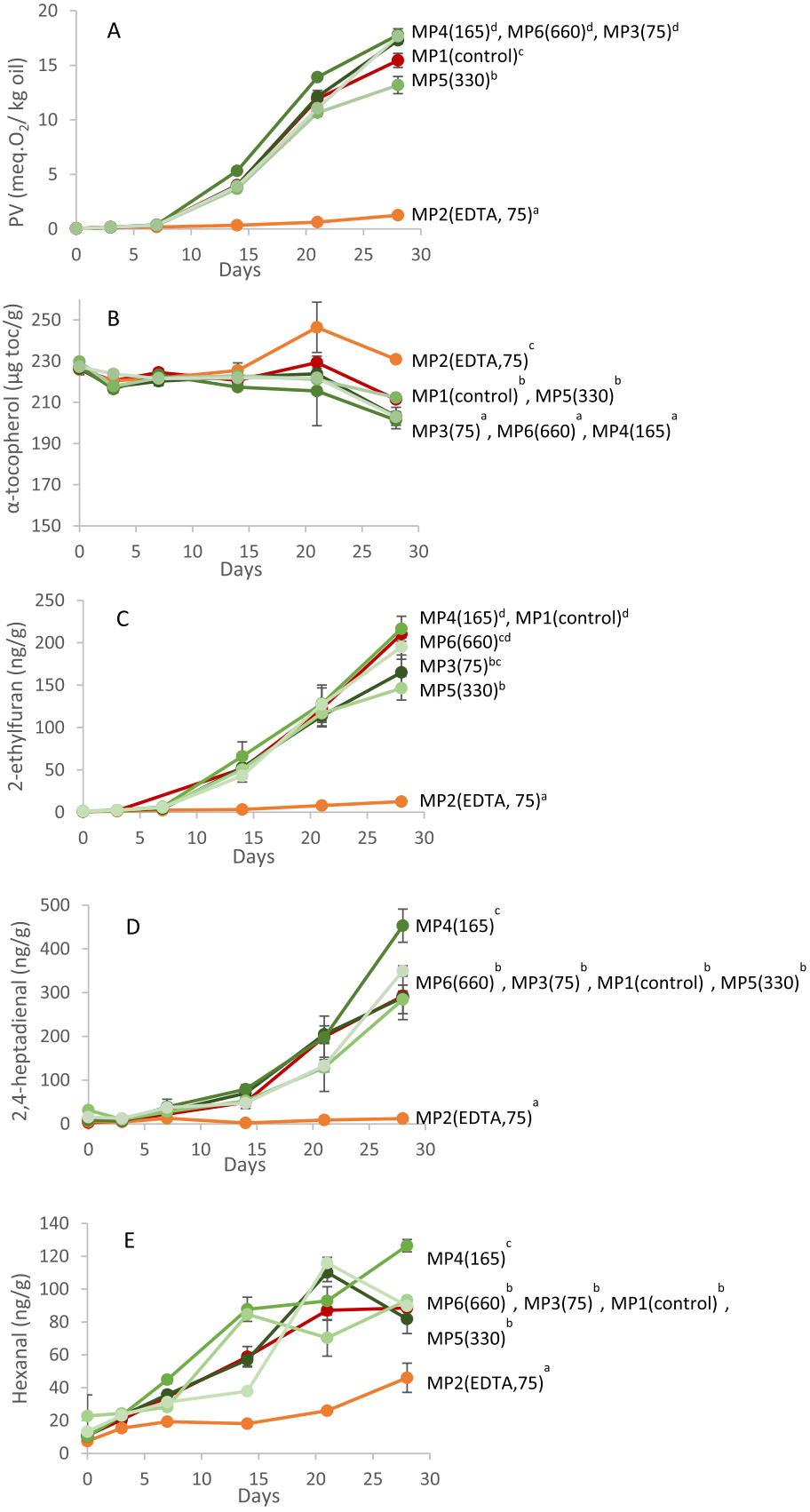

Fig. 5. Development of A) primary oxidation products (PV), B) alpha-tocopherol content, C) 2-ethylfuran, D) *(E,E)*-2,4-heptadienal and E) hexanal in mayonnaises containing different concentrations of 144-P-CHE during storage. The codes have the peptide content in mg/kg in parenthesis. Statistical differences at 28 days were shown on the codes using the letters a-d ($p \leq 0.05$).

was the best performing peptide compared to the rest and significantly inhibit lipid oxidation reactions in both types of emulsion systems. This indicates a superior chelating activity of peptides with the very low isoelectric points (<4), which are negatively charged at pH 4, as is the case for 144-P-CHE. However, the second-best antioxidant peptide was different for the two emulsion systems: 132-R-SCA (NNKWVPCLEFETEHGFVYREHH), spinach RuBisCO-derived peptide, in low-fat emulsions and 124-S-SCA (AGDWLIGDR), a seaweed-derived peptide, in mayonnaises. Finally, these different observations indicated that several characteristics in the final emulsion systems affected the peptides' antioxidant properties such as pH, charge, type of emulsifier, iron binding capacity or oil content. The alteration of the sensory attributes of food due to bitterness of antioxidant peptides can be considered as one of the biggest drawbacks/challenges in relation to food applications. Therefore, future work should be carried out in order to assess sensory properties and allergenicity not only of the antioxidant peptides but also of the protein hydrolysates from which they come from in order to get a complete evaluation of their safety.

CRediT authorship contribution statement

Elisa Varona: Formal analysis, Visualization, Writing – original draft. **Pedro J. García-Moreno:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Simon Gregersen:** Conceptualization, Writing – review & editing. **Tobias H. Olsen:** Conceptualization, Formal analysis, Investigation. **Paolo Marcatili:** Conceptualization, Methodology, Formal analysis, Investigation. **Francesc Guardiola:** Writing – review & editing. **Michael T. Overgaard:** Conceptualization, Funding acquisition. **Egon B. Hansen:** Conceptualization, Funding acquisition, Project administration. **Charlotte Jacobsen:** Conceptualization, Methodology, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Betül Yesiltas:** Conceptualization, Methodology, Formal analysis, Visualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136498.

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