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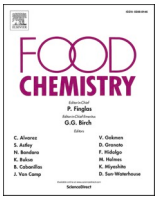

Antioxidant peptides derived from potato, seaweed, microbial and spinach proteins: Oxidative stability of 5% fish oil-in-water emulsions

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ABSTRACT

In this study, we used a combination of quantitative proteomics and bioinformatic prediction for identifying novel antioxidant peptides. Thirty-five peptides from potato, seaweed, microbial, and spinach proteins were investigated. Based on high DPPH radical scavenging activity ($IC_{50} \leq 16$ mg/mL), metal chelation activity, isoelectric point, and high relative abundance in the parent protein sources, 11 peptides were selected. Lipid oxidation retardation was evaluated in 5% fish oil-in-water emulsions stabilized with Tween 20, where emulsion physical stability was unaffected by peptide addition. The secondary structure of selected peptides was similar in the aqueous solution and emulsions, as confirmed by synchrotron radiation circular dichroism spectroscopy. The emulsions containing the selected peptides had lower levels of hydroperoxides and volatile compounds during storage compared to the control (without peptide). This study contributes to elucidating the effect of antioxidant peptides in emulsions and demonstrates the ability of quantitative proteomics and bioinformatics prediction to identify peptides with strong antioxidant properties.

1. Introduction

The increase in the consumption of sustainable food obliges the food industry to develop natural multifunctional ingredients derived from alternative sources such as plant, marine, and microbial-based peptides. In particular, research on natural and sustainable antioxidants to prevent lipid oxidation in food, ultimately affecting nutrition and sensory profiles negatively, is gaining considerable attention (Jacobsen, 2015). For instance, antioxidant peptides embedded in plant proteins (pea, soy, flaxseed, algae, quinoa, and potato) have been reported to exhibit radical scavenging or metal chelating activities (Ashaolu, 2020; Sarmadi & Ismail, 2010), and thus have the potential to replace widely used synthetic antioxidants in the food industry. Moreover, replacing synthetic additives with natural, protein/peptide-based functional ingredients directly improves both safety and nutritional value of the product (Sarmadi & Ismail, 2010). Proteins and peptides may inhibit several lipid oxidation pathways, i.e. inactivating reactive oxygen species, scavenging free radicals, chelating prooxidative transition metals, reducing hydroperoxides, and altering the physical properties of food systems in a way that separates reactive species (Elias et al. 2008).

Although several studies have focused on studying the antioxidant properties of peptides, there is a lack of fundamental understanding of the antioxidant mechanism in model or food systems. Various structural characteristics of peptides (amino acid (AA) sequence and profile, length, pI, etc.) influence their antioxidant activity; however, the structure-activity relationship and molecular understanding of antioxidant peptides has not yet been fully elucidated. Therefore, new and advanced methods (e.g. Quantitative Structure-Activity Relationship (QSAR), Machine Learning) have been applied in this research area. Uno et al. (2020) developed a QSAR model for identifying antioxidant activity of tripeptides using the effective hydrophobicity scale, determined based on the first AA side chain. For example, it was reported that

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peptides containing a cysteine (Cys) residue at any position had higher antioxidant activity than other peptides. While the QSAR study is limited to only very short peptides, other bioinformatic approaches have emerged such as peptide database searching (e.g. BIOPEP-UWM) (Minkiewicz et al., 2019) and machine learning prediction algorithms (e.g. Peptide Ranker (Mooney and Haslam, 2016) and AnOxPePred (Olsen et al., 2020)). Nevertheless, all approaches have limitations. BIOPEP-UWM is limited to only known bioactive peptides within the database. BIOPEP-UWM and Peptide Ranker are both restricted by allowing only peptide sequence inputs. AnOxPePred is capable of processing full protein sequences to predict embedded antioxidant peptides, but accuracy of predictions is limited by the modest size (~1400 peptides in total) of the benchmark data set used to train the model (Olsen et al., 2020). To further evaluate the potential of bioinformatic analysis and prediction in the field of antioxidant peptides and sustainable food ingredients from alternative protein sources, more systematic studies of functional prediction and *in vitro* validation and assessment in food model systems are needed.

Interesting non-animal sources for production of antioxidant peptides are potatoes, seaweed, microbial and spinach proteins. Despite a very modest protein content (1–2% of dry matter), potatoes have also gained attention as a potential plant protein source due to growing magnitude of the starch industry producing towards ~240,000 tons/year of potato protein (García-Moreno et al., 2020a; García-Moreno et al., 2021). Moving from a trial-and-error approach towards more time and cost-efficient methods, García-Moreno et al. (2020b) followed a novel approach combining bioinformatics, testing functionality, and applying bottom-up proteomics to obtain emulsifier peptides from potato side-streams and investigated their *in vitro* antioxidant activity as well. Results revealed that peptides containing the sequence FCLKVGV provided better protection against lipid oxidation in emulsions and showed good *in vitro* 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity.

Seaweed is one of the sources that is increasingly used for food consumption and produced in large amounts. The protein content of seaweed can be up to 50% per dry weight, although it varies according to the season and the species (Pangestuti & Kim, 2015). Heo et al. (2003) studied the potential antioxidant activity of water-soluble enzymatic hydrolysates from a kelp, *Ecklonia cava*, obtained using various proteases and reported the effect of these hydrolysates on controlling lipid oxidation of fish oil emulsions during 12 days of storage at 60 °C. Harnedy et al. (2017) studied the antioxidant activity of synthetic peptides derived from *Palmaria palmata* and reported SDITRPGGQM as an antioxidant peptide based on the high *in vitro* oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) values (152.43 and 21.23 nmol trolox equivalent/μmol peptide, respectively).

During the last decades, there has been increasing attention towards sustainable production of microbial proteins with an excellent nutritional profile as an alternative to animal and plant proteins. Chandra et al. (2020) reviewed the antioxidant activity of bioactive compounds including peptides and proteins from microbial sources. However, there is a need for more in-depth research on antioxidant activity of peptides from microbial sources. Moreover, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), known as the most abundant protein on earth, is also a sustainable source of bioactive peptides (Udenigwe et al., 2017). It has been reported to have a wide range of health promoting effects and applications in food (Di Stefano et al., 2018). For instance, Je et al. (2015) reported that a dipeptide (PC), which was obtained from the large subunit of plant and microalgae RuBisCO, was predicted to be bioactive based on a bioinformatics analysis and results showed *in vitro* reducing potential and inhibitory activity against lipid peroxidation comparable to the activity of glutathione.

In this study, we apply bioinformatics to previously published quantitative proteomics data to identify a range of novel and abundant antioxidant peptides embedded in a representative selection of sustainable protein sources. These include abundant proteins from red seaweed (side-stream of carrageenan production), microbial (single cell protein through fermentation), spinach (a model of green leaves), and potato (side-stream of starch production) to illustrate the broad applicability of the novel methodological approach. Moreover, the approach bypasses the tedious and cost-ineffective trial-and-error approach conventionally used for discovery of novel bioactive peptides. Antioxidant properties of the predicted peptides were validated through *in vitro* tests using synthetic peptides and model emulsion systems. The study consisted of i) testing *in vitro* antioxidant activity of 35 predicted antioxidant peptides, ii) selecting the best performing peptides based on prediction scores, *in vitro* test results, and their abundance in the parent protein sources, iii) determining the changes in secondary structure of the selected peptides in buffer solution, aqueous phase (buffer plus Tween 20 (TW20)), and oil-in-water emulsions, and iv) investigating the physical and oxidative stability of the TW20 stabilized 5% fish oil-in-water emulsions at pH 7 containing 0.05 wt% of selected antioxidant peptides. Applying this novel approach for identifying antioxidant peptides embedded in alternative protein sources also provides new insight and knowledge into designing and developing downstream processes for protein-rich side stream valorization and ultimately, food products with improved oxidative stability.

2. Materials and methods

2.1. Materials

Fish oil (cod liver oil) was provided by Vesteraalen (Norway). The peroxide value (PV) was measured as 0.23 ± 0.00 meq. O_2/kg oil. The fatty acid composition (%, w/w) of the fish oil was as follows: C14:0 (3.7), C16:0 (9.0), C16:1n-7 (9.6), C18:0 (1.9), C18:1n-9 (15.8), C18:1n-7 (4.5), C18:2n-6 (2.4), C18:3n-3 (1.0), C20:1n-9 (13.7), C20:5n-3 (8.4), C22:1n-11 (5.6), and C22:6n-3 (10.7), which was determined using GC analysis of fatty acid methyl esters (FAME) (AOCS official methods Ce 2-66 and 1b-89). The tocopherol content analyzed for the fish oil was as follows: α-tocopherol, 195 ± 3 μg/g oil; β-tocopherol, 5 ± 0 μg/g oil; γ-tocopherol, 116 ± 2 μg/g oil; δ-tocopherol, 43 ± 1 μg/g oil (AOCS Official Method Ce 8–89). Peptides were synthesized by Synpeptide Co., Ltd. (Shanghai, China) to a certified purity of >70%. Physicochemical properties for the 35 peptides, including peptide codes, AA sequence, length, isoelectric point (pI), and net charge at pH 7, are shown in Table 1. Tricaprylin was purchased from Sigma-Aldrich (Brøndby, Denmark). All chemicals and solvents used were of analytical grade.

2.2. Identification of novel peptide antioxidants

Antioxidant peptides were identified using a novel four-stage workflow, with the overall objective of data-driven and sustainable production of natural and functional food ingredients. The workflow capitalizes on recent developments in quantitative proteomics of alternative protein sources, bioinformatic prediction of antioxidant properties in peptides, small scale antioxidant screening assays, and *in silico* peptide sequence analysis (Fig. 1). Small scale screening assays also allowed for preliminary evaluation of peptide solubility. Ultimately, the workflow allows for designing downstream processing by e.g. targeted enzymatic hydrolysis, facilitating release of abundant and verified antioxidant peptides in industrial side- and waste-streams or protein isolates, increasing their value and applicability.

2.2.1. Protein selection

As proof-of-principle, a range of taxonomically diverse sources were included in the study. This was done to ensure a wide distribution of protein classes and to illustrate the broad applicability of the approach. All included protein sources represent an industrially relevant side-stream or isolate, for which quantitative proteomics data are available. Potato (*S. tuberosum*) proteins were selected from industrial protein isolates as a side-stream from starch production (García-Moreno et al.,

Table 1
Predicted antioxidant peptide codes and their amino acid sequence, length, isoelectric point (pI), net charge, and predicted radical scavenger (PRS) and metal chelator (PMC) scores, DPPH half maximal inhibitory concentration (IC_{50}), and emulsion codes.

Peptide No*	Amino acid sequence	Length	pI[0]	Net charge at pH7[0]	PRS score[1]	PMC score[1]	DPPH IC_{50} (mg/mL)[2]	Emulsion code[3]
110-P-SCA	LMQ	3	3.7	0	0.38	0.29	N/A	
111-P-SCA	KWGPLRW	7	11.4	2	0.46	0.22	**5.9 ± 1.6**^{abc}	E2
112-P-SCA	GKELDPR	7	6.9	0	0.35	0.21	23.0 ± 3.3 ^{fg}	
113-P-SCA	VPFYFEHGPHI	11	6.1	−0.8	0.64	0.22	16.3 ± 1.7 ^{def,**}	E3
114-P-SCA	IVDPLSLHIGD	11	3.7	−1.9	0.35	0.26	N/A	
115-P-SCA	PGYHSKTEEEKEKEK	15	5.5	−0.9	0.33	0.17	34.3 ± 3.2 ^{h}	
116-P-SCA	PTNPICINCCSGYKGCNYYSAFG	23	7.7	0.7	0.48	0.17	30.8 ± 0.9^{gh}	
117-P-SCA	ENNRPFAAANEIVPFYFEHGPHIFNS	26	5.2	−1.8	0.52	0.22	38.9 ± 6.6 ^{h,**}	
118-P-SCA	LIYPTGCTTCCTGYKGCYYFGKNGKFVCEG	30	7.9	1.7	0.52	0.12	15.4 ± 5.2^{cdef,**}	
119-P-SCA	FCLKVGV	7	8.6	0.9	0.35	0.19	N/A	
120-S-SCA	RYVWN	5	9.6	1	0.49	0.18	18.7 ± 1.3^{ef}	
121-S-SCA	PEPIPNGAMPQ	11	1.0	−1	0.43	0.29	21.9 ± 2. 9 ^{fg}	
122-S-SCA	QSDSDYSSSGPLGVPDPSDLL	21	0.4	−4	0.51	0.26	37.0 ± 6.6 ^{h}	
123-S-SCA	DFPVR	5	6.7	0	0.38	0.26	**3.9 ± 1.4**^{a}	E4
124-S-SCA	AGDWLIGDR	9	3.7	−1	0.43	0.20	**3.5 ± 0.0**^{a,**}	E5
125-U-SCA	HWYD	4	4.9	−0.9	0.59	0.23	**2.2 ± 0.1**^{a,**}	E6
126-U-SCA	GNTYFWHAFWF	11	7.8	0.1	0.60	0.22	N/A	
127-U-SCA	LGEWINRYFNFWGWTYFPINF	21	6.8	0	0.56	0.17	N/A	
128-U-SCA	MLWQYKPK	8	10.2	2	0.54	0.20	**6.7 ± 2.4**^{abcd,**}	E7
129-U-SCA	FLDSDVYK	8	3.7	−1	0.36	0.22	N/A	
130-R-SCA	VWYA	4	3.5	0	0.55	0.22	**7.0 ± 1.2**^{abcd,**}	
131-R-SCA	EHHNSPGYYDG	11	5.0	−1.8	0.53	0.27	90.8 ± 5.5^{i,**}	
132-R-SCA	NNKWVPCLEFETEHGFVYREHH	22	5.8	−1.8	0.50	0.23	**5.5 ± 2.2**^{ab,**}	E8
133-R-SCA	YWTMWK	6	9.5	1	0.53	0.20	14.1 ± 0.9^{bcdef,**}	E9
134-R-SCA	LEQLFSEHGK	10	5.3	−0.9	0.38	0.25	**2.6 ± 0.5**^{a}	
135-P-CHE	HCPSH	5	7.2	0.1	0.51	0.30		E10
136-P-CHE	MDDAS	5	0.6	−2	0.31	0.23		
137-P-CHE	CEGES	5	0.9	−2.1	0.36	0.23		
138-P-CHE	ALQWMLVI	8	3.7	0	0.44	0.20		
139-P-CHE	YKLLHCPSHLQCKN	14	8.7	2.1	0.47	0.26		E11
140-P-CHE	GMKNVYKLLHCPSHLQCKNIGSN	23	9.7	3.1	0.53	0.25		
141-P-CHE	LGYNLLYCPFSSDDQFCLKVGVVHQN	26	5.0	−1	0.29	0.15		
142-P-CHE	ENPIVLPSTCHDDDNLVLPEVYDQDGHPL	29	3.4	−6.9	0.35	0.26		
143-P-CHE	DFNLVDGDVAAVDPSLLSISV	21	0.4	−4	0.30	0.23		
144-P-CHE	DDDNLVLPEVYDQD	14	0.5	−6	0.45	0.30		E12

* P, S, U, and R denote potato, seaweed, microbial biomass (single cell), spinach (particularly RuBisCO), while SCA and CHE denote scavenger and chelator. The thick line indicates the divide between SCA and CHE peptides.

** These data were published previously in Olsen et al., 2020.

[0] pI and net charge at pH7 were calculated using peptide property calculator (Innovagen AB, Lund, Sweden).

[1] The PRS and PMC scores are values between 0 and 1, with higher scores indicating a higher chance of being either a radical scavenger or metal chelator peptide.

[2] Sodium caseinate had a value of 9.2 ± 2.3^{abcde} mg/mL IC_{50} as the positive control. Peptides with 'N/A' were not analyzed due to gel formation. CHE peptides were not analyzed using the DPPH assay. Statistical differences between samples were shown using letters from a to i ($p < 0.05$).

[3] Emulsion codes were used for emulsion storage study. Control emulsion, E1, did not contain any peptide.

2020b). Microbial proteins were selected from a pilot-scale fermentation of methanotrophic bacteria (*M. capsulatus*) currently used for feed protein production (Yesiltas et al., 2021). Seaweed proteins were selected from a pilot-scale protein isolate of *E. denticulatum* obtained during carrageenan production (Gregersen et al., 2020). Based on quantitative proteomics analysis of the different protein sources in these studies, abundant proteins (selection criteria: molar abundance >1%) determined by relative intensity-based absolute quantification (riBAQ) (Schwanhäusser et al., 2011), were selected for bioinformatic analysis in this study. In spinach (*S. oleracea*), RuBisCO subunits are the most abundant proteins (Yang et al., 2004), and spinach may serve as a well-characterized model for a sustainable, green leaf protein and was therefore also included. The high abundance of RuBisCO subunits was confirmed by quantitative proteomics analysis of a spinach protein extract (unpublished data) but all abundant proteins (riBAQ >1%) were selected. Due to the low protein quality observed in the seaweed protein isolate (i.e. low protein content, extensive smearing in SDS-PAGE analysis, and low number of protein identifications (Gregersen et al., 2020), more stringent requirements were applied to the seaweed proteins (riBAQ >2% and peptide identifications >3 in tryptic analysis). All proteins submitted for prediction are listed in Table S1.

2.2.2. Prediction of antioxidant properties of peptides by bioinformatics

Radical scavenging and metal chelating activity of peptides embedded in the four raw materials were predicted based on the AnOxPePred webserver (https://services.bioinformatics.dtu.dk/service.php?AnOxPePred-1.0) developed using machine learning by Olsen et al. (2020). The algorithm was applied in "exhaustive mode" to reveal all potential antioxidant peptides embedded within high abundance proteins. Thirty-five potentially (high scoring) antioxidant peptides were initially predicted across the four protein sources. Their predicted radical scavenger (PRS) and metal chelator (PMC) scores are reported in Table 1. The 35 peptides were subsequently synthesized to screen for antioxidant activity using small scale assays.

2.2.3. Preliminary screening of in vitro peptide antioxidant activity

2.2.3.1. DPPH radical scavenging activity. The radical scavenging activity of synthesized peptides was determined using the DPPH radical scavenging activity method of Yang et al. (2008) with some modifications. Peptides were dissolved in dimethyl sulfoxide (DMSO) at different concentrations (0.2–50 mM). Equal parts (100 μL) of 0.1 mM ethanolic DPPH solution and a peptide solution were mixed. The mixture was kept in darkness at room temperature for 30 min and the absorbance was measured at 515 nm using a spectrophotometer. The scavenging effect

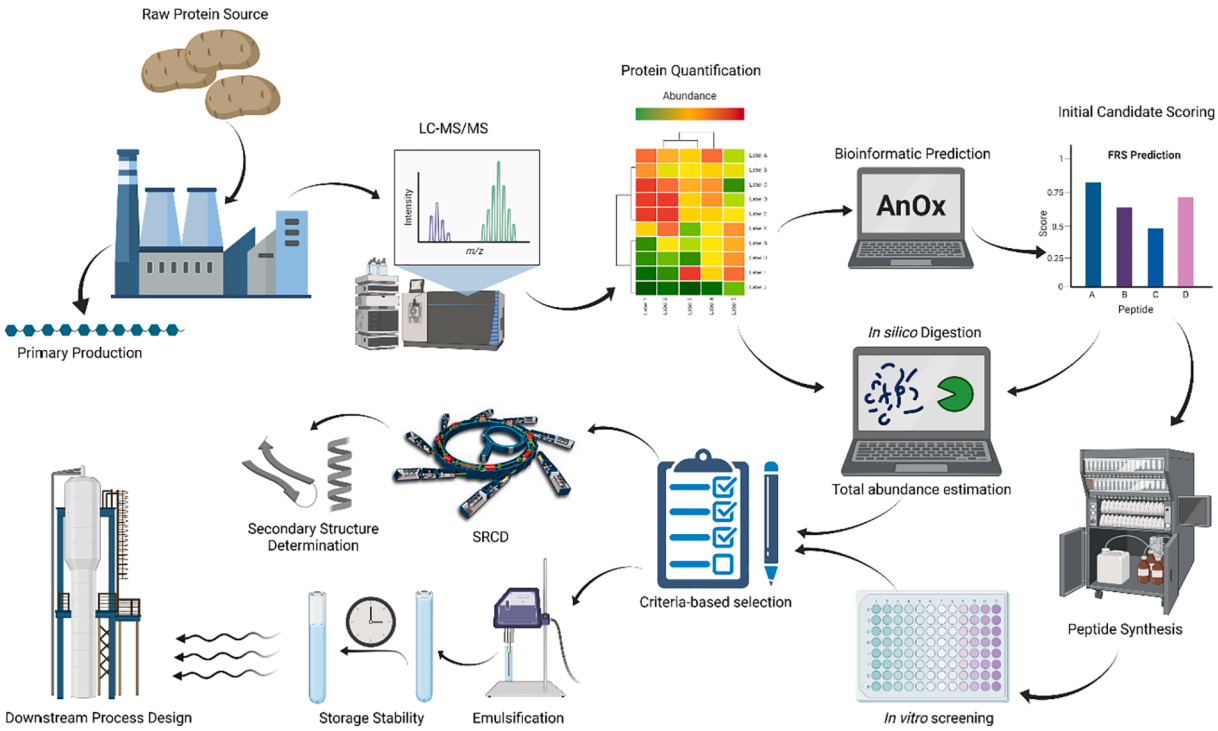

Fig. 1. Schematic illustration of the novel workflow for antioxidant peptide identification presented in this study. From a well-established, industrial production line (here potato starch), a protein-rich side-stream is identified. Following protein identification and quantification by LC-MS/MS, the most abundant proteins are submitted for identification of embedded peptides with predicted antioxidant properties using AnOxPePred (Olsen et al., 2020). Potential antioxidant peptides are synthesized and screened for *in vitro* antioxidant activity. Based on activity, abundance and potential enzymatic release (here by trypsin), a final criteria-based selection is performed. Selected peptides are investigated for changes in secondary structure from solution phase to model emulsion (here using Tween20). Selected peptides are used for up-scaled model emulsion production to investigate physical and oxidative stability during storage, ultimately allowing for the design of a downstream process to release target peptides by enzymatic hydrolysis. Created with BioRender.com.

was calculated as an inhibition percentage following Eq. (1):.

$$DPPH\ inhibition(\%) = \left(1 - \frac{As - A0}{Ab}\right) \times 100 \quad (1)$$

where A_s is the sample absorbance (peptide + DPPH), A_o is the sample blank absorbance (no DPPH), and A_b is the reagent blank absorbance (no peptide). Results were calculated for 50% inhibition concentration (IC50) and presented in mg/mL. Sodium caseinate, a commonly used food ingredient with antioxidant properties, was used as a reference. Butylated Hydroxytoluene (BHT) solution was included as a positive control. All measurements were performed in triplicate.

2.2.3.2. Iron chelating activity. The iron chelating activity of the synthesized peptides was determined according to Farvin et al. (2010), with some modifications. Peptides were dissolved in DMSO at different concentrations (0.2–50 mM). Concisely, 100 μL of antioxidant sample were transferred to the microtiter plate and 110 μL water were added. To start the reaction, 20 μL of 0.5 mM ferrous chloride was added and the plate was shaken. After 3 min, 20 μL of 2.5 mM ferrozine were added and the plate was shaken again. After 10 min in the dark at room temperature, the absorbance was measured at 562 nm. The chelating capacity was calculated in the same way as for DPPH inhibition (Eq. (1)) Ethylenediaminetetraacetic acid (EDTA) was employed as a positive control. All measurements were performed in triplicate.

2.2.4. Tryptic release potential and total peptide abundance

In silico peptide sequence analysis was performed to evaluate the potential release of target peptides by enzymatic hydrolysis with trypsin. Trypsin was selected as model protease due to its high specificity and bulk availability as an industrial enzyme. Thus, such a process is industrially scalable and economically feasible. Tryptic match was ranked from 1 (perfect) to 5 (terrible) based on alignment of the target peptides with products from *in silico* tryptic digest of the parent protein (s) (see supplementary material for further details). Total peptide abundance in the selected sources was based on quantitative proteomics data by means of riBAQ. Within a given quantitative dataset, all identified proteins containing the exact sequence of a predicted peptide (based on multiple sequence alignment (MSA) using CLC Sequence Viewer 8.0 (Qiagen Bioinformatics) were clustered. The sum of riBAQs was then calculated and used as an estimate for maximum relative molar recovery of the peptide (mol target peptide per mol total protein, %) from each protein source. Total abundance and tryptic match scores are summarized in Table S2.

2.2.5. Final peptide selection

Based on *in silico* analysis and *in vitro* screening, a delimitation was performed before further analysis and up-scaled emulsion production. Criteria for selection of free radical scavenger peptides were defined as i) comparable or higher activity than reference (sodium caseinate); ii) solubility under the assay conditions; iii) high total abundance; and iv) good or perfect tryptic match. For final selection, peptides were required to adhere to at least three of the four outlined criteria. The final 11 peptides selected for emulsion production were furthermore subjected to additional *in silico* sequence analysis to reveal peptide uniqueness and/or other potential protein sources. Peptide sequences were analyzed using the "Peptide Search" web-tool (https://www.uniprot.org/peptidesearch/) from UniProtKB (The UniProt Consortium, 2021).

2.3. Secondary structure of the peptides

Total of 2 g of samples were prepared by solubilizing the 11 selected peptides (0.05 wt%, final concentration in emulsion) in 10 mM

phosphate buffer (pH 7), shaken at 100 rpm for 2 h in a water bath at 50 °C and overnight at room temperature to allow complete rehydration. These samples are called peptide solutions in the following. For the preparation of aqueous phase, TW20 (1 wt%, final concentration in emulsion) was added to peptide solutions the next day. Emulsions were prepared with the addition of tricaprylin oil (5 wt%, final concentration in emulsion) into the aqueous phase samples by applying pre-homogenization using a hand-held ultra turrax (POLYTRON® PT1200E, Kinematic Inc., New York, USA) mixing at 18,000 rpm for 30 s, followed by secondary homogenization using a sonicator (Microson XL2000, Misonix, Inc., New York, USA) equipped with a *P*1 probe and run at an amplitude of 75% (maximum amplitude of 180 μm), running 2 passes of 30 s with a break of 1 min between passes (García-Moreno et al., 2021). During sonication the emulsions were surrounded by iced water to minimize the increase in temperature. Control samples, which were used for the baseline correction for the emulsions with peptides, were produced in the same way as described above excluding the parts regarding peptide addition.

Synchrotron radiation circular dichroism (SRCD) measurements were carried out on the AU-CD beamline at the ASTRID2 synchrotron radiation source (ISA, Department of Physics and Astronomy, Aarhus University, Denmark). The optical rotation magnitude and wavelength were checked daily by using camphorsulfonic acid to confirm the operation of the SRCD spectrometer (Miles et al., 2004). A 0.01 cm path length quartz Suprasil cell (Hellma GmbH & Co., Germany) was used for far-UV SRCD measurements at 25 °C. The baseline was corrected using 10 mM phosphate buffer (pH 7) for peptide solutions, buffer and TW20 for aqueous phase, tricaprylin oil-in-water emulsions stabilized with TW20 for emulsions containing antioxidant peptides. The far-UV SRCD spectra were recorded in duplicate from 280 to 170 nm in 1 nm steps, with a dwell time of 2.1 s per point.

The spectra were converted to delta epsilon units using the peptide concentration obtained from absorbance at 205 nm (Anthis & Clore, 2013). The proportions of each secondary structure components were determined using DICHROWEB, a web-based calculation server, which incorporates various methods and a wide range of protein spectral databases (Whitmore & Wallace, 2007). The method used for calculation was CDSSTR with the reference set SMP180 (Abdul-Gader et al., 2011). Due to the nature of the emulsions, in particular due the scattering that occurs from the droplets, the accuracy of peptide concentration determination is lower than for those in solution.

2.4. Emulsion production and storage experiment

A total of 220 g of 5 wt% fish oil-in-water emulsions stabilized with 1 wt% TW20 were produced. Antioxidant peptides (0.05 wt%) were dissolved in 10 mM sodium acetate-10 mM imidazole buffer at pH 7, shaken at 100 rpm in a water bath (50 °C, 2 h) and continued to be shaken at room temperature overnight in darkness, for the peptides to be totally dissolved and rehydrated. The next day, TW20 was added to the peptide solutions and the aqueous phases were adjusted to pH 7 by addition of 1 M NaOH. One emulsion was prepared with only TW20 as a control. Pre-homogenization was performed using ultra turrax (Ystral, Ballrechten-Dottingen, Germany) for 3 min at 16000 rpm. Fish oil was added into the aqueous phase within the first minute of mixing. Secondary homogenization was performed using 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 emulsion obtained after Microfluidizer homogenization and it was stirred with a spoon. The final pH of the emulsion was measured. Emulsions were stored for 8 days at 20 °C in darkness. Samples were collected for physical characterization and oxidative stability analyses during storage.

2.5. Physical stability of emulsions

2.5.1. Emulsion stability

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

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

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, $N(h) = (z_{max}\text{-}z_{min})/\Delta$ h is the number of height position for the scan and BST is the signal that is taken into account (BS when T <0.2%, otherwise T) (Formulaction, 2021). Measurements were run as a single determination for checking if the stability of the emulsions was acceptable.

2.5.2. Zeta potential

The zeta potential was measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., Worcestershire, UK) on day 1. Before the measurement, 0.032 g of each sample was weighed, diluted in distilled water (40 g) and vortexed. DTS-1070 disposable folded capillary cell (Malvern Instruments, Ltd., UK) was used. Measurements from the same sample were carried out in triplicate using a zeta potential range of (-) 100 to (+) 50 mV and samples were analyzed with 100 runs at 25 °C.

2.5.3. Droplet size distribution

The droplet size distribution of the emulsions was measured by laser diffraction using Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) on days 1 and 8. Emulsions were diluted in recirculating water set at 3000 rpm until an obscuration of approximately 12–15% was reached. For particle and dispersant, the refractive indices of sunflower oil (1.469) and water (1.330) were used, respectively. Measurements of droplet size were carried out in triplicate and they were given as the surface weighted (D[3,2]) and volume weighted (D[4,3]) mean diameters.

2.6. Oxidative stability of emulsions

Oxidative stability analyses were carried out on the samples collected on days 0, 1, 2, 5, and 8 during the storage experiment.

2.6.1. Peroxide value

Extraction of lipids was performed based on the method described by Bligh and Dyer (1959) using a reduced amount of chloroform/methanol (1:1, w/w). Two extractions were made from each emulsion sample. PV was determined on lipid extracts using the colorimetric ferric-thiocyanate method at 500 nm according to Shantha and Decker (1994) with a spectrophotometer (Shimadzu UV-1280, Holm&Halby, Brøndby, Denmark). Measurements were performed in duplicate.

2.6.2. Tocopherols

Tocopherols were determined using an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA), equipped with a fluorescence detector. Two g of the lipid extract from the above-mentioned Bligh and Dyer extraction (section 2.2.4.1) was weighed and evaporated under nitrogen and dissolved in 10 mL *n*-heptane. One mL of the heptane solutions were taken into separate vials before injection of an

aliquot on a Spherisorb S5 W column (250x4.6 mm) (Phase Separation Ltd., Deeside, UK). Elution was performed with an isocratic mixture of *n*-heptane/2-propanol (100:0.4, v/v) at a flow of 1 mL/min injection volume of 20 μL in a column (Waters Spherisorb 3 μm Silica, 4.6 mm I.D. ×150 mm), preceded by a guard column (Waters Spherisorb, 5 μm Silica, 4.6 mm I.D. ×10 mm). Detection was done using a fluorescence detector with excitation wavelength set to 290 nm and emission wavelength set at 330 nm according to the AOCS method (1998). Tocopherol standard mix including α, β, γ, and δ-tocopherol standards was used (Calbiochem 613424). Results were calculated using this external quantitative standard. Measurements were performed in duplicate and quantified by authentic standards.

2.6.3. Secondary volatile oxidation compounds – dynamic headspace technique coupled with GC–MS

Approximately 4 g of emulsion was placed in a purge bottle together with 5 mL distilled water and 30 mg of an internal standard (4-methyl-1-pentanol). The purge bottle was subjected to a water bath at 45 °C for 30 min under purging with nitrogen (150 mL/min) and the volatile compounds were trapped in Tenax GR tubes. Afterwards, the tubes were put into an Automatic Thermal Desorber (ATD-350, Perkin Elmer, Norwalk, CN), which was connected to a GC (GC Agilent 6890 N, Palo Alto, CA, USA; Column: DB-1701, 30 m ×0.25 mm ×1.0 μm). The oven program had an initial temperature of 45 °C for 5 min, increasing with 1.5 °C/min until 55 °C, with 2.5 °C/min until 90 °C, and with 12.0 °C/min until 220 °C, where the temperature was kept for 4 min. The individual volatile compounds were separated by MS (MS Agilent 5973, Agilent Technologies, USA; electron ionization mode, 70 eV; mass to charge ratio scan between 30 and 250) and these compounds were identified by MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard). The selected standards were 2-ethyl furan, 1-penten-3-one, pentanal, 1-penten-3-ol, 2,3-pentanedione, 1-pentanol, hexanal, *(E)*-2-hexenal, heptanal, *(E)*-2-heptenal, *(Z)*-4-heptenal, octanal, *(E,E)*-2,4-heptadienal, *(E,E)*-2,4-decadienal and *(E,E)*-3,5-octadiene-2-one. A stock solution of the standards was made in ethanol and diluted into nine levels (0.1–250 μg/mL) for the calibration curve. Each dilution (30 mg) was added into an emulsion produced in the same way as in the control emulsion (E1), which allows the similar release for standards as it was for volatile compounds formed in all emulsions. Analysis was run in triplicate.

2.7. Statistical analysis

Mean value and standard deviations were introduced to Statgraphics 18 (Statistical Graphics Corp., Rockville, MD, USA) for the data analysis. Multiple sample comparison was performed to identify the significant differences between samples at certain sampling days and between sampling days for each sample during storage using Tukey as a post-hoc test at $p < 0.05$ significance level.

3. Results and discussion

3.1. Selection of peptides for emulsion study

Antioxidant peptides were identified using the AnOxPePred webserver, which predicts free radical scavenging and metal chelating properties of embedded peptides, based on the AA sequence of full length proteins. This bioinformatics tool was fed with the sequences of the most abundant proteins in potato, seaweed, microbial samples and spinach RuBisCO subunits (Table S1). The predicted radical scavenging (PRS) and predicted metal chelating (PMC) scores of the peptides studied are shown in Table 1. PRS scores ranged between 0.29 and 0.64, whereas PMC scores ranged from 0.12 to 0.30.

Peptides, which were predicted to have radical scavenging activity, were tested using the *in vitro* DPPH radical scavenging activity assay and results are reported in Table 1. Out of 25 identified radical scavenger peptides, 6 peptides (110-P-SCA, 114-P-SCA, 119-P-SCA, 126-U-SCA, 127-U-SCA, and 129-U-SCA) had very low solubility or resulted in gel formation when dissolved in DMSO. Therefore, these peptides were not further analyzed. Among the analyzed 19 peptides, 8 of them had similar DPPH half maximal inhibitory concentration (IC_{50}) compared to sodium caseinate (9 ± 2 mg/mL), which was used as a positive control ($p < 0.05$). The highest IC_{50} value was 91 ± 5 mg/mL (131-R-SCA) followed by the second highest value at 39 ± 7 mg/mL (122-S-SCA). Cian et al. (2012) reported that IC_{50} values of seaweed protein hydrolysates from *P. columbina*, which were hydrolyzed using trypsin, alcalase, sequentially trypsin + alcalase or alcalase + trypsin, were ranging between 2.7 and 3.0 mg/mL based on the *in vitro* DPPH radical scavenging assay. Rapeseed protein hydrolysate (hydrolyzed with alcalase) had a DPPH radical scavenging IC_{50} value of 0.71 mg/mL (Pan et al., 2011). Peptide IC_{50} values are somewhat higher than previously published complex protein hydrolysates and even though some of the results are comparable, inconsistent conditions and diversity in the methods should be borne in mind (Freitas et al., 2013). Moreover, comparing the peptides synthetically produced and protein hydrolysates may cause differences due to different kinds of impurities as well as synergistic effects of peptides in hydrolysates.

All peptides that were soluble, exhibited radical scavenging activity (with low IC_{50} values) as predicted by bioinformatics; however, there was no direct relationship between PRS and IC_{50} values. Seven (111-P-SCA, 123-S-SCA, 124-S-SCA, 125-U-SCA, 128-U-SCA, 130-R-SCA, and 134-R-SCA) of these 8 peptides with IC_{50} lower than 7 mg/mL had 4–10 AAs, whereas 132-R-SCA had 22 AAs and an IC_{50} value of 5 ± 2 mg/mL. It was expected that shorter peptides would show antioxidant activity, as previous studies also reported that peptides with 2–20 AA residues and with a molecular weight (MW) lower than 3 kDa are considered to have good antioxidant activity (Pan et al., 2020). On the other hand, AA composition, molecular conformation and hydrophobicity has a great impact on the antioxidant activity as well (Yuan et al., 2018). 132-R-SCA, as a longer peptide with 22 AAs (NNKWVPCLEFETEHGFVYREHH) with a MW of 2772 Da, had two His at the C-terminus, which was also the case in another study where the antioxidant peptide (LLPHH) derived from proteolytic digests of a soybean protein was reported to be the most antioxidative peptide among 22 peptides tested (Chen et al., 1998).

Metal chelator peptides were not successfully tested using the ferrozine method due to poor solubility of peptides and poor replicability of results, which might be attributed to the use of DMSO as solvent. Even though DMSO solubilized peptides better compared to water, some peptides did not solubilize completely and were cloudy. An important parameter to be taken into account for the metal chelator peptides was the pI, as it provides information about the electrostatic charge of the peptides. Potential metal ion chelating peptides should preferably be negatively charged as they would attract positively charged metal ions. Thus, peptides with pI lower than 7 might favor chelating activity in emulsions at pH 7. This also highlights the need for refinements in the prediction models, where properties such as solubility and pI should be predicted for a peptide for it to be relevant to study in an aqueous system.

Peptides were also evaluated based on their relative abundance in their parent proteins and their potential to be released by enzymatic hydrolysis using trypsin (see Supplementary material). Applying a range of selection criteria (IC_{50} <16 mg/mL; abundant (>2.5% relative in at least one protein source); high level of tryptic match (Table S2)), eight peptides (111-P-SCA, 113-P-SCA, 123-S-SCA, 124-S-SCA, 125-U-SCA, 128-U-SCA, 132-R-SCA, 133-R-SCA) were selected as strong candidates for targetable radical scavenger peptides. Three metal chelator peptides (135-P-CHE with pI ~7, 139-P-CHE with pI >7 and peptide 144-P-CHE with pI <7) with similar PMS (0.26–0.30, Table 1), were selected for the further studies in emulsions in order to elucidate the effect of charge on chelating activity of peptides.

3.2. Secondary structure of selected antioxidant peptides

Secondary structure of the selected 11 peptides was determined using SRCD in different systems, i.e. peptide solution, aqueous phase, and emulsion (Fig. 2a–k). Despite the challenging conditions employed (high oil and low peptide concentration), it was still possible to get sufficient signal from the peptides due to the high sensitivity of the SRCD instrument having a higher flux of light from synchrotron radiation when compared to a conventional bench top CD instrument. As seen in Fig. 2, secondary structure of the peptides did not change significantly in the studied three different systems meaning that the secondary structure of the peptides in solution were the same as they were in the aqueous phase when mixed with TW20 as well as in emulsions in the presence of TW20 stabilized oil droplets. These findings may suggest that the peptides did not interact with TW20 in the aqueous phase or co-locate with TW20 at the tricaprylin oil-water interface in emulsion. At least not in a manner causing any conformational change within the peptides.

Even though we did not observe conformational changes in this study, β-strand peptides may be promising in terms of increased antioxidant capacity. For instance, Yuan et al. (2018) reported that the changes in secondary structure from α-helix to β-strand increased the antioxidant activity of yoghurt peptides. Yang et al. (2017) studied the structure-activity relationship of synthetic pentapeptides (SHECN and LPFAM) and reported that higher β-sheet content and lower content levels of α-helix were correlated with higher antioxidant activity. Nevertheless, investigating well-defined secondary structure elements for such short peptides in aqueous solution may be futile as they are likely too short to adopt this due to the flexible nature of unrestricted peptide termini in the numeric form unless involved in self-assembly and formation of macromolecular assemblies such as fibrillation or

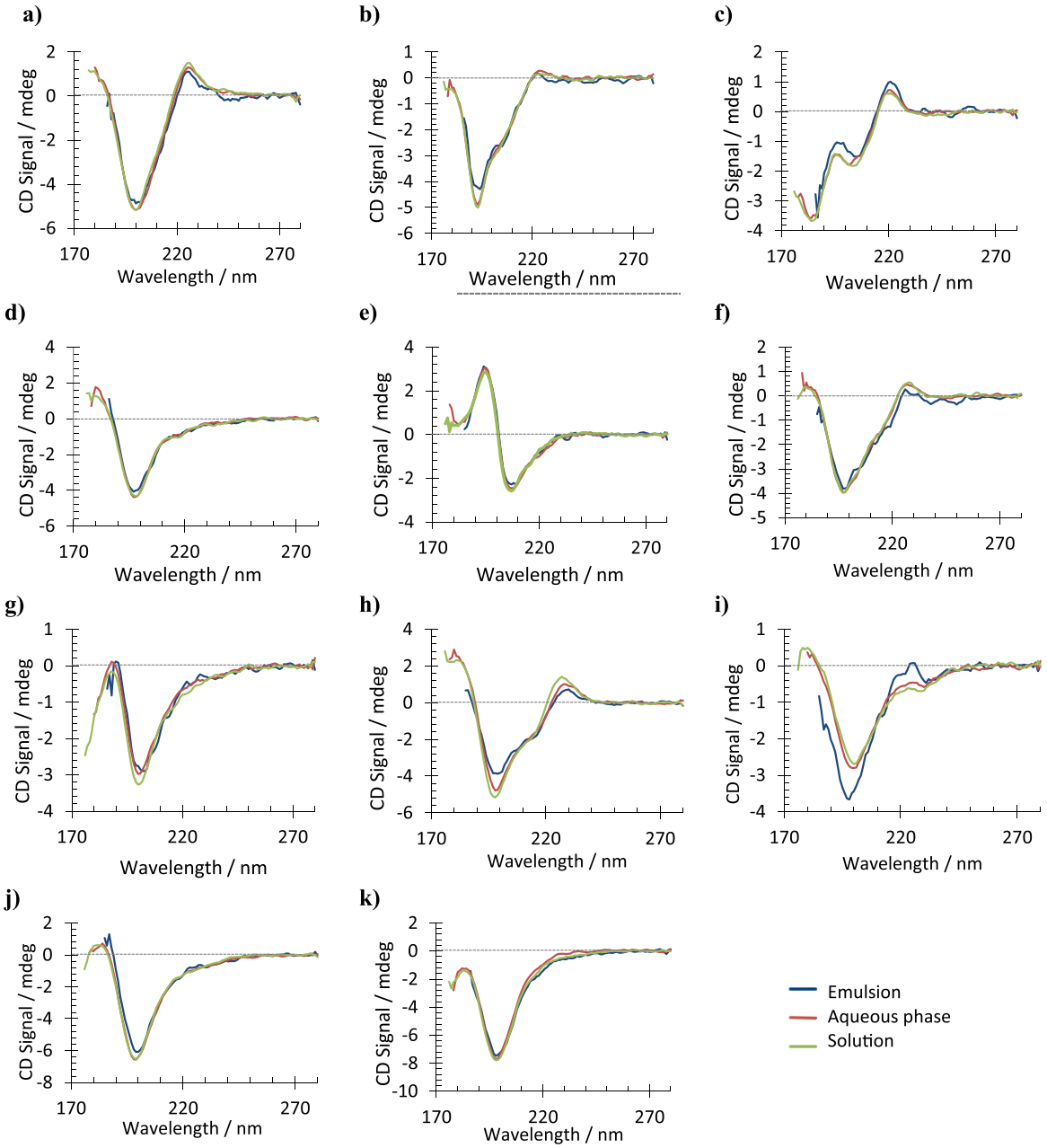

Fig. 2. SRCD spectra of the peptides a) 111-P-SCA, b) 113-P-SCA, c) 123-S-SCA, d) 124-S-SCA, e) 125-U-SCA, f) 128-U-SCA, g) 132-R-SCA, h) 133-R-SCA, i) 135-P-CHE, j) 139-P-CHE, and k) 144-P-CHE in solution (green line), in aqueous phase (red line), and in emulsion (blue line) at pH7. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interaction with other constituents (e.g. the oil/water interface) (Jacob and Unger, 2007; Castelletto et al., 2008). Moreover, generalizing structure-activity relationship based on only two peptides is fundamentally problematic. The data presented here is also a valuable addition in the perspective, which eventually will allow to build more robust and valid models and interpretations.

Fig. 3 shows the secondary structure of the peptides in solution illustrating the α-helix, β-strand, turns and unordered percentages. The secondary structure for the peptides in aqueous phase and in emulsion is not shown since the results were not significantly different compared to peptides in solution. None of the peptides gave signal typical for α-helix structure except 125-U-SCA. However, as the peptide is only 4 AAs long, we find it unlikely to adopt a helical structure. Therefore, this result was attributed to the signal coming from the chiral centers of the AAs. The rest of the peptides displayed predominantly unordered (~50%) or β-strand (~30%) structures.

Even though peptides contained unordered configuration as the major secondary structure (up to ~55% of the total structures), they also adopted β-strand conformation up to ~40%. Previously, García-Moreno et al. (2020b) studied emulsifier peptides, which were predicted to have β-strand structure by bioinformatics, and found that peptides with middle length (13–15 AAs) adopted a proper rearranging at the oil-water interface and reduced the interfacial tension, as well as provided physical stability to 5% fish oil-in-water emulsions when used as the only emulsifier. In another study investigating the emulsifying activity of peptides, two peptides ELQVSARVTLEIEL (14 AAs) and KVKINETVEIKGKFHV (16 AAs), comprising 86% and 81% β-strand structure based on modelling data, respectively, exhibited undisturbed β-strand conformation with alternating hydrophobic and hydrophilic AAs (Yesiltas et al., 2021). Both 139-P-CHE and 144-P-CHE had 14 AAs and complied with the range presented by García-Moreno et al. (2020b). However, there was no change in the secondary structure when the peptides were added in 5% oil-in-water emulsion stabilized with TW20. This could be due to the lower concentration of peptides (0.05 wt%) or the well-established TW20 interfacial layer, which did not interact with these peptides even if they had affinity towards the interface. Moreover, these peptides do not have alternating hydrophobic/hydrophilic character either, which may also explain the reason why they do not appear to go to the oil-water interface and change structure into a more well-defined amphiphilic structure. Except 132-R-SCA (22 AAs), the rest of the peptides tested were short (≤14 AAs), which might also affect their ability to adsorb at the oil-water interface and obtain a more well-defined secondary structure. Cheng et al. (2014) have previously reported that even when in low concentrations, charged peptides have the potential to interact with the adsorbed surfactants at the oil-water interface. Even though TW20 is a non-ionic surfactant, there could be some interactions depending on the amphiphilicity and charged groups of the antioxidant peptides added in the emulsion system as antioxidants. However, SRCD measurements did not show any evidence of interaction between TW20 and the peptides at the concentrations employed. Based on these observations, it seems highly likely that all 11 peptides remain in the aqueous phase and do not interfere with the TW20 interfacial layer or the TW20 micelles present in the aqueous phase.

3.3. Physical stability of the emulsions

3.3.1. Emulsion stability

After emulsions were produced, physical stability of all the emulsions was investigated at day 0. The results of the calculated Turbiscan stability index (TSI) are shown in Fig. S1. TSI values lower than 3 are accepted as a physically stable emulsion (Formulaction, 2021). Results showed that all samples were stable as the TSI values were lower than 1, where no changes in the emulsion stability can be observed by the naked eye at this stage of destabilization. The differences between samples did not indicate any significance, as the obtained values are small. Therefore, all samples were evaluated as in the same range of stability. In addition, creaming was also not observed for any of the samples by the naked eye during storage. This is in agreement with SRCD results, where the addition of peptide did not appear to result in interactions at the oil-water interface and disturbance of the emulsion.

3.3.2. Droplet size distribution

All emulsions (E1-E12, see Table 1) stabilized with 1 wt% TW20 without any antioxidant (E1, control) or containing different antioxidant peptides (0.05 wt%) provided similar D[3,2] and D[4,3] values ranging between 0.121 and 0.132 and 0.188–0.229 μm, respectively (Table S3). The mean droplet sizes were very stable during the 8 days of storage. E1 had larger D[3,2] and D[4,3] compared to E9, E10, E11, and E12, and smaller droplets compared to E4, E5, E7, and E8 ($p < 0.05$). Even though the results showed significant differences, mean droplet sizes varied on a small scale when the structure of the emulsion is considered. This confirms that the addition of peptide has very little or no effect on the physical state of the emulsion. Our results were similar to Cheng et al. (2014), where 10% soybean oil-in-water emulsions were either produced with only 1.11% TW20 or the same amount of TW20 and 2% potato protein hydrolysate, which was reported to have particle sizes measured as 218.1 and 215.3 nm, respectively, using dynamic light scattering. Another study also had similar results where 1 wt% of TW20 was used to stabilize 10% linseed oil and found that the D[4,3] was 0.55

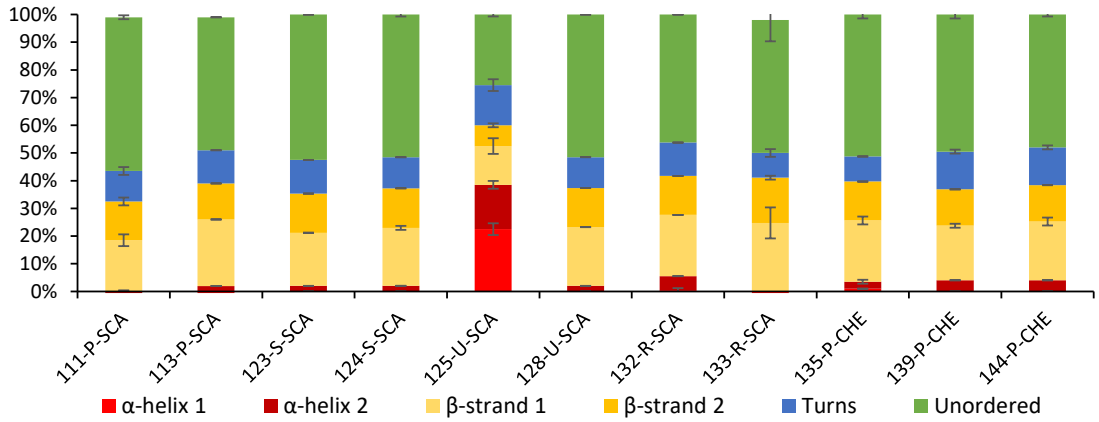

Fig. 3. The secondary structure composition (α-helix, β-strand, turns and unordered in %) of the peptides in solution (0.05 wt% peptides dissolved in 10 mM phosphate buffer at pH7). The results are obtained from deconvolution of the respective SRCD spectra. The numbers 1 and 2 in α-helix and β-strand denote regular and distorted structures, respectively.

μm (Lamothe et al., 2019). This increase in the droplet size is expected as the oil content used in the study was twice as much (10%) in comparison to what we used in our study (5%) while the TW20 amount was 1 wt% in both studies.

3.3.3. Zeta potential

Zeta potential results are presented in Table S3. Results varied from (-) 7.52 ± 1.05 to (-) 4.73 ± 0.15, where significant differences between samples were observed. Results were comparable with a previous study where 1 wt% TW20 was used to stabilize 10% linseed oil at pH 7 and the zeta potential was reported as −10.2 mV (Lamothe et al., 2019). Although TW20 is a non-ionic surfactant, the negative charge of oil droplets might be due to anions derived from the buffer and traces of free fatty acids. Statistical analysis showed that E2, E4, E5, E7, E11, and E12 had significantly lower zeta potential compared to the control, E1 ($p < 0.05$). Although it may have been anticipated that peptides could alter the surface charge of the oil droplets by co-locating at the interface, this was not the case since SRCD results indicate that the peptides locate in the aqueous phase. Thus, the subtle differences observed may be related to residual impurities in the synthetic peptides (e.g. acid and salt). Overall, zeta potential was lower than 30 mV in magnitude, which is considered to be insufficient for providing considerable electrostatic repulsion between oil droplets in emulsions. Therefore, peptides in these emulsions had a minor effect on eliminating destabilization via electrostatic forces.

3.4. Oxidative stability

3.4.1. Peroxide value

Formation of hydroperoxides was followed during the 8-day storage of 5% fish oil-in-water emulsions and the results of the PV are shown in Fig. 4. E1, the control emulsion without any antioxidants, had 14.7 ± 4.7 meq. O_2/kg oil at the beginning of the storage experiment followed by a significant, almost linear, PV increase until the end of the storage, without an observable lag phase. All peptide-containing emulsions in general (with very few exceptions) displayed lower PV throughout the storage experiment. At the last day of storage, all emulsions containing antioxidant peptides showed significantly lower PV compared to the control, which confirms the predicted antioxidant activity of the peptides in 5% fish oil-in-water emulsions. Most of the emulsions (E2, E3, E4, E5, E7, E8, and E11) had significant increases in PV already from day 0 and kept increasing significantly at most of the sampling points, which indicates a lack of metal chelating activity retarding lipid oxidation at the early stages of oxidation (Jacobsen, 2015). E9 containing 133-R-SCA had a lag phase until day 5, which was attributed to its promising antioxidant activity, and after that PV started to increase significantly. E6 containing 125-U-SCA reached its highest PV on day 5 and decreased between day 5 and 8 indicating the start of a more pronounced hydroperoxide decomposition into secondary oxidation products than hydroperoxide formation.

Unexpectedly, E10 containing 135-P-CHE and E12 containing 144-P-CHE started with a higher PV and decreased significantly from day 0 to 2 for E10 and from day 0 to 1 for E12, which then increased significantly for each sampling point until the end of the storage. The high PV already at the production day could be due to the oxidation happening during emulsification or oxidation of peptides during synthesis. However, there is no obvious explanation for the significant decrease and then increase of the PV value during storage for these two samples. High PV (8–37 meq. O_2/kg) at day 0 was also observed in a previous study where emulsifier peptides were used for producing physically and oxidatively stable 5% fish oil-in-water emulsions, which was attributed to lipid oxidation occurring during homogenization (García-Moreno et al., 2021).

3.4.2. Consumption of tocopherols

Tocopherols are antioxidants that are naturally present in fish oil. Therefore, consumption of tocopherols was followed during the 8-day storage of emulsions as an indication of the oxidative stability (Fig. 5). As expected, the results indicated that the consumption trend of the tocopherols were oppositely related to the formation of hydroperoxides as shown in Fig. 4. Tocopherols in the control emulsion (E1) were consumed faster than in the rest of the emulsions with antioxidant peptides. E6 and E10 had the next highest consumption of tocopherols and the strange trend in PV that was noted for the E10, was also observed for tocopherols, first with an increase and then a decrease. E10 contained a metal ion chelator peptide (135-P-CHE), which had a neutral charge at pH 7. Lack of a strong negative charge might have prevented the metal chelating activities of the peptide. On the other hand, most of the peptides decreased or prevented the loss of the tocopherols compared to the E1 during storage. Peptides used in E11 (139-P-CHE) and E12 (144-P-CHE) were the best in preventing tocopherol consumption. The two peptides were predicted to be metal ion chelators, which have positive (2.1) and negative (-6) net charge at pH 7, respectively (Table 1). Tocopherol consumption in E2, E5, E7, E8, and

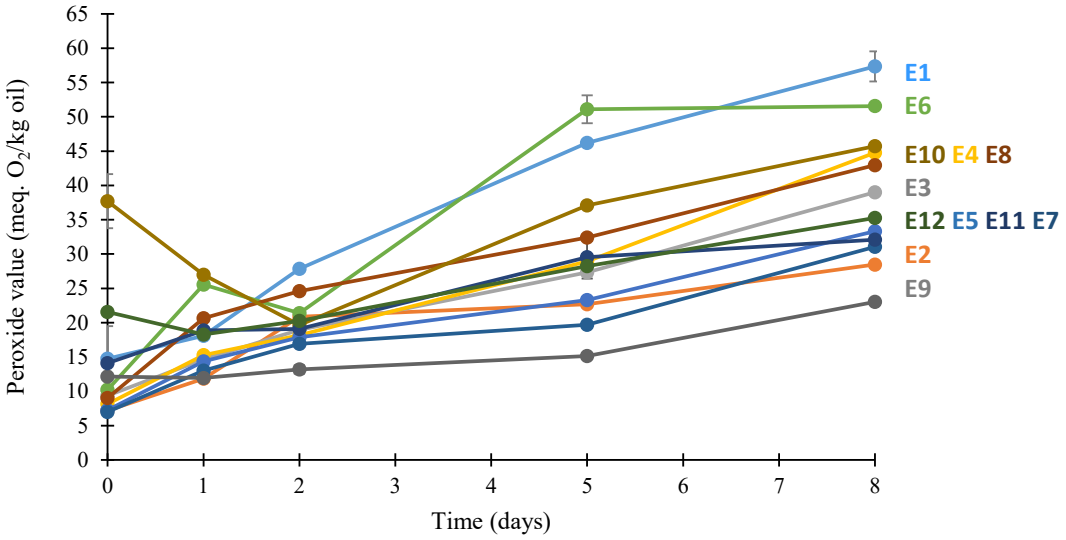

Fig. 4. Peroxide value determined in 5% fish oil-in-water emulsions (pH7) stabilized with Tween 20 and containing antioxidant peptides during 8 days of storage.

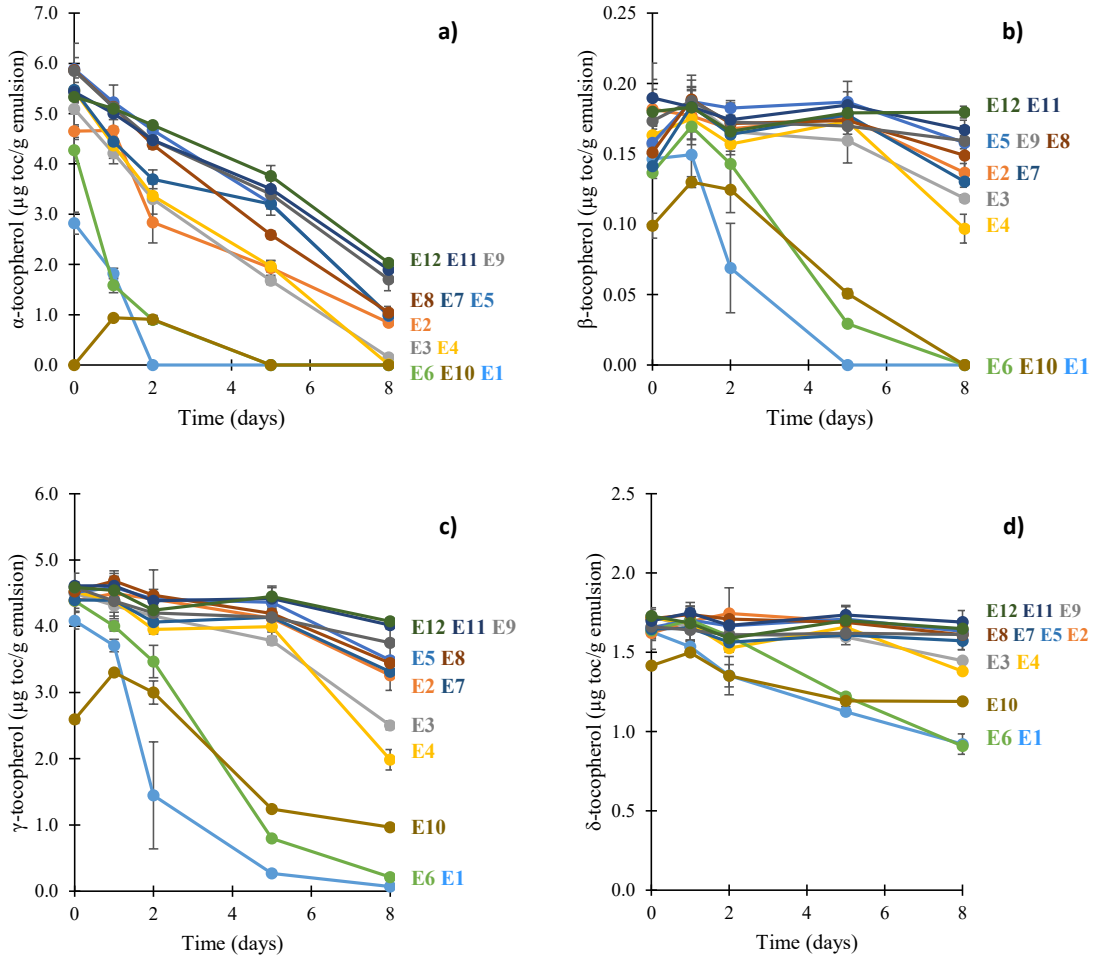

Fig. 5. Consumption of a) α-tocopherol, b) β-tocopherol, c) γ-tocopherol, and d) δ-tocopherol in 5% fish oil-in-water emulsions (pH7) stabilized with Tween 20 and containing antioxidant peptides during 8 days of storage.

E9 containing scavenger peptides (111-P-SCA, 124-S-SCA, 128-U-SCA, 132-R-SCA, and 133-R-SCA, respectively) was also closer to the best performing peptides and there was no direct relationship with the peptide charge or length.

3.4.3. *Development of secondary volatile oxidation compounds*

Four volatile compounds, which were highly abundant and had a representative trend for the other volatile compounds, are presented in Fig. 6. The rest are shown in the supplementary material, Fig. S2. One-penten-3-ol (Fig. 6a), 1-penten-3-one (Fig. 6b), and *(E,E)*-2,4-heptadienal (Fig. 6d) are derived from omega-3 PUFAs and hexanal is derived from omega-6 PUFAs (Fig. 6c) (Genot et al., 2003). Overall, results showed that the control emulsion had the highest formation of secondary oxidation volatile compounds followed by E6 (125-U-SCA) and E10 (135-P-CHE), which is in line with the formation of hydroperoxides and consumption of tocopherols. The highest antioxidant activity of peptides was observed in the following emulsions: E7, E9, E11, and E12, which contained 128-U-SCA, 133-R-SCA, 139-P-CHE, and 144-P-CHE, respectively. Bioinformatic sequence analysis (Table S4) indicates that 128-U-SCA is only found in Methanol dehydrogenase from *M. capsulatus* and both 139-P-CHE and 144-P-CHE are only found in Kunitz Inhibitor (Type-C) from potatoes, thereby making these raw materials potentially unique sources of these antioxidant peptides. In contrast, 133-R-SCA is found in the small subunit of RuBisCO of a wide variety of green plants, thereby potentially making this peptide readily available from many different industrial side streams.

135-P-CHE (HCPSH), which has a His residue on both N- and C-termini in addition to a Cys as radical scavenger AAs (Ashalou, 2020), did not provide the expected oxidative stability for E10. On the other hand, 139-P-CHE (YKLLHCPSHLQCKN, added to E11), which contained the whole sequence of 135-P-CHE, provided better antioxidant activity. This may relate to the presence of Tyr and lysine (Lys), which are not present in 135-P-CHE, as well as an additional Cys. Hydrogen can be easily abstracted from Tyr due to its aromatic ring, which also prevents the peptide from turning into a free radical after the loss of an electron (Elias et al., 2008). Tyr containing peptides were suggested to have antioxidant activity independently from their position in the peptide (Aluko, 2015). Lys binds to and neutralizes negatively charged free radicals due to its positively charged amine groups (Aluko, 2015). Chen and Nawar (1991) reported that the presence of Cys, Trp, and Lys prevented lipid oxidation in milk fat, whereas the control sample lost 50% of the unsaturated fatty acids. Cys containing peptides were also reported to prevent lipid peroxidation with its single electron transfer antioxidant activity mechanism (Ashalou, 2020). Moreover, 139-P-CHE is positively charged at pH 7 (pI = 8.7), which indicates that the metal chelating activity could only be due to some negatively charged regions that still allowed the chelation of metal ions. Alternatively, this peptide had radical scavenging properties, which provided the antioxidant activity observed. This should be further investigated.

Even though all 20 biologically derived AAs can potentially show

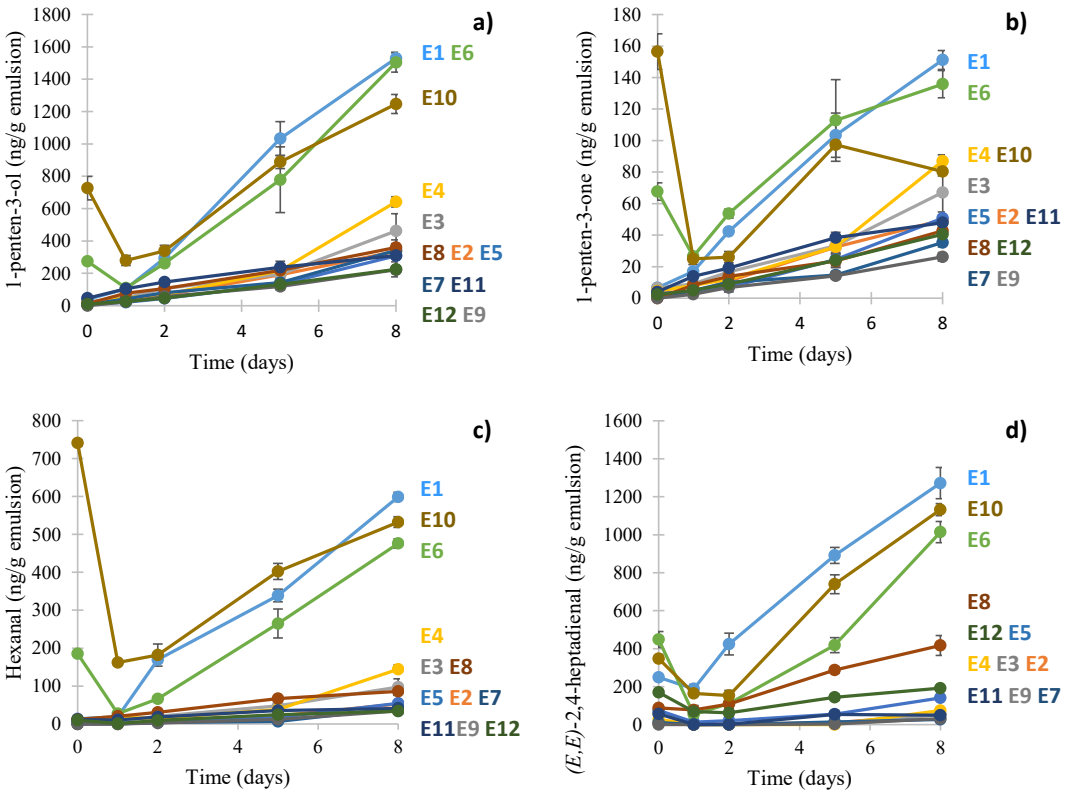

Fig. 6. Development of secondary volatile oxidation compounds a) 1-penten-3-ol, b) 1-penten-3-one, c) hexanal, and d) *(E,E)*-2,4-heptadienal in 5% fish oil-in-water emulsions (pH7) stabilized with Tween 20 and containing antioxidant peptides during 8 days of storage.

antioxidant activity by interacting with free radicals, the ones containing either nucleophilic sulfur-containing side chains (Cys and Met) or aromatic side chains (Trp, Tyr, and Phe) from which hydrogen is easily abstracted, are the most reactive (Elias et al., 2008). 133-R-SCA (YWTMWK), added to E9, provided very good antioxidant activity presumably due to the presence of Tyr, Trp, Met, and Lys. This may indicate that the lipid radicals were scavenged from the aqueous phase as the peptides were shown to be in the aqueous phase. One common AA for five of the peptides was Trp, which was reported to have radical scavenging activity due to its electron-dense aromatic rings (Freitas et al., 2013). Zhao et al. (2021) reported that the peptides preventing lipid oxidation in 10% soybean oil-in-water emulsions highly contained Phe, Tyr, Ala, Lys, arginine (Arg), and aspartic acid (Asp) and added that the latter three AAs could chelate pro-oxidative metal ions (Fe^{2+} and Cu^{2+}) via electrostatic coordination, since their free amino group and free carboxyl group exhibits a pair of free electrons. In addition, the presence of aromatic or hydrophobic AAs were shown to be important contributing factors to the metal chelating ability of the pea seed (*Pisum sativum* L.) enzymatic protein hydrolysate fractions (Pownall et al., 2010).

144-P-CHE (added to E12) also showed good potential in preventing lipid oxidation. 144-P-CHE (DDDNLVLPEVYDQD) is rich in Asp at the both N- and C-termini, which was reported to have metal chelating activity (Elias et al., 2008). Moreover, 0.5 pI and −6 net charge (pH7) for 144-P-CHE kept the carboxylic groups deprotonated and presumably increased its chelating activity for Fe^{2+} ions. 128-U-SCA, 111-P-SCA, and 124-S-SCA added to E7, E2, and E5, respectively, also performed well in decreasing formation of oxidation products and preventing the tocopherols depletion. These peptides varied in length (7 to 9 AAs) and contained several antioxidant AAs such as Tyr, Trp, Met, Asp, Arg, Lys, and Ala exhibiting radical scavenging and/or metal chelating activities as stated above. Alboofetileh et al. (2021) also stated that the lower molecular weight peptides containing hydrophobic and aromatic AAs are reported to exhibit higher antioxidant activity. It should also be borne in mind that antioxidant activity of peptides cannot only be explained by the presence of any single AA. There are other factors that could have an impact such as physical structure of the peptide (e.g. size, length, and conformation), the location of the antioxidant (e.g. in the aqueous phase or at the oil-water interface), and interaction of the antioxidant peptides with other compounds or impurities present (Elias et al., 2008; Zhao et al., 2021; Yesiltas et al., 2021).

The antioxidant peptide location in an emulsion may have an impact on the emulsion oxidative stability. Cheng et al. (2014) studied interfacial peptides adsorbed at the oil-water interface of 10% soybean oil-in-water emulsions (stabilized with TW20) and interpreted the results in two different ways: one as being integrated in the interfacial membrane and the other adsorbing over the inner layer formed by TW20 via weak forces. They concluded that the antioxidant mechanisms of peptides at the emulsion interface were presumably both physical via steric hindrance and electrostatic effects and chemical via radical scavenging activities. However, and according to SRCD results, peptides tested in this study seemed to exhibit their antioxidant activity in the aqueous phase either by chelating metal ions or by scavenging free radicals present in the aqueous phase of the oil-in-water emulsion, which considerably enhanced the oxidative stability of 5% fish oil-in-water emulsions.

4. Conclusions

From the 35 antioxidant peptides predicted by AnOxPePred from various sustainable sources (potato, seaweed, microbial, and spinach), 11 peptides were selected for their assessment in fish oil-in-water

emulsions. Selection criteria included the *in vitro* antioxidant properties, obtainable yields from the protein sources and industrial waste streams, as well the probability of obtaining the peptides by means of tryptic hydrolysis. Several selected peptides such as DDDNLVLPEVYDQD (144-P-CHE from potato), YKLLHCPSHLQCKN (139-P-CHE from potato protein), YWTMWK (133-R-SCA from spinach), and MLWQYKPK (128-U-SCA from microbial biomass) were shown to significantly inhibit lipid oxidation when compared to the control emulsion without added peptide. The length of the best performing peptides varied between 6 and 14 AAs indicating higher antioxidant activity for shorter peptides. Charged peptides (down to −6 or up to 2.1) inhibited oxidation more successfully compared to more neutral peptides (between −0.9 and 0.1) at pH7. The most active peptides did contain a quite high proportion of aromatic and charged residues (e.g. presence of Tyr, Asp, and Lys), in line with previous studies which reported the importance of AA composition. Even though further systematic and molecular studies are needed for elucidating antioxidant mechanisms of the specific AAs in peptides (e.g. emulsions stabilized with other emulsifiers, at lower pH levels or with higher oil content), this study showcases the great potential of using bioinformatics and proteomics for identifying natural and sustainable antioxidant peptides, which are abundant in their parent proteins. Ultimately, the presented methodology allows for targeting specific peptides by e.g. designed enzymatic hydrolysis using a combination of protease specificity and bioinformatic sequence analysis. This approach, which is currently investigated in our labs, is complementary to the traditional trial-and-error approach encountered in functional hydrolysate research and provides an excellent opportunity for a more data-driven process design, adhering to the visions of Industry 4.0. Furthermore, the results provide a valuable addition to the communal dataset of antioxidant peptides, which can serve as a basis for development of improved prediction algorithms.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132699.

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