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Oxidative stability of protein concentrates from the green seaweed *Ulva* spp.

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ABSTRACT

Protein extracted from *Ulva* spp., a green seaweed, is gaining attention for food applications due to its promising amino acid composition. However, limited information is available on the oxidative status of these extracts upon their production, and on their oxidative stability during storage. This study investigated the oxidative stability of three protein concentrates (PCs) from *Ulva* spp. stored under dark and light conditions for 120 days. PCs were produced in pilot-scale from wild *Ulva* spp. (PC Wild) and from the corresponding pulp (PC Wild pulp). Because these pilot-scale PCs were brown, indicating loss of chlorophyll and oxidation, lab-scale processing of cultivated *Ulva* sp. (PC Cultivated) was carried out for comparison. PC Wild and PC Wild pulp showed increased levels of volatile oxidation products compared to the primary biomass, whereas PC Cultivated showed values similar to its primary biomass, indicating that the lab-scale process was less harsh than the pilot-scale process. Differences in processing or in the balance between pro- and antioxidants in the primary materials could have contributed to these observations. Under light storage conditions, both PC Wild and PC Cultivated showed a greater loss of monounsaturated fatty acids and all PCs had increased levels of oxidation, indicating photooxidation. Lipid hydroperoxide values and volatile compounds reached higher levels on day 0 than on day 120, pointing to a turnover of both primary and secondary oxidation products and likely formation of cross-linked complexes.

1. Introduction

To meet future protein demands, numerous alternative protein sources are currently being investigated [1–3]. In this context, the green seaweed *Ulva* spp. is a promising and underexploited source due to its high protein production potential and bioremediation capacity, with no requirement for land or freshwater resources [4–7]. However, *Ulva* spp. can contain high levels of fiber and ash [8–10], which can impede protein digestibility [11,12]. Protein extraction has been found to improve protein digestibility – both in vitro [13] and in vivo [14], and to increase the content of essential amino acids (EAA), thereby meeting the Food and Agriculture Organization (FAO) recommendations for human consumption [15]. One approach for protein extraction is by mechanical fractionation of *Ulva* spp., producing juice and a pulp fraction. The proteins in the juice can then be precipitated, separated, and dried to

obtain a protein concentrate (PC) [16,17]. The remaining pulp still contains a considerable amount of protein and extracting this with alkaline treatment can increase the total protein yield. However, this process results in a protein concentrate of lower quality [17,18].

The composition of resulting protein concentrates depends strongly on the biomass utilized and the extraction method applied, with protein contents ranging from 10 to 70% of DM [5,13,16,19–22]. In addition, protein concentrates are found to contain other components such as ash, lipids, and fiber [19,21]. Among these constituents, both the proteins and lipids are prone to oxidation [23,24]. The rate and extent, however, depends on the precise composition of the protein concentrate, as well as its extraction and storage conditions [25,26]. To our knowledge, these factors have not yet been investigated for *Ulva* spp. protein concentrates. Oxidation can alter the composition, color, functionality, and sensory properties of protein concentrates, thereby affecting their suitability for

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food applications [26–28], and highlighting the importance to gain knowledge about oxidative development. Lipids are generally more susceptible to oxidation than proteins due to their chemical structure, especially unsaturated fatty acids containing reactive double bonds [29], but radical transfer between lipids and protein occurs [24]. The lipid content in *Ulva* spp. represents a small fraction of 0.5–8% of DM [10,30,31], consisting of storage lipids (e.g. glycerides) and structural lipids (e.g. glycolipids and phospholipids) [32,33]. Glycolipids are observed to dominate in *Ulva* spp. as these are found in the photosynthetic membranes [33], though the overall lipid composition changes with environmental factors such as irradiance, nitrogen availability, and CO₂ supply [32,33]. *Ulva* spp. typically contains high proportions of saturated palmitic acid (C16:0) and polyunsaturated α -linolenic acid (C18:3n3) [10,33–35]. After protein extraction, similar or increased lipid contents have been found in the protein concentrates compared with the original biomass, ranging from 1.7 to 8.6% of DM [17,19,21]. Harrysson et al. [36] investigated the storage stability of dried *Ulva* spp. biomass and found high vulnerability for changes under light conditions, likely due to a high content of chlorophyll that acts as a photosensitizer and thereby initiates lipid oxidation. This resulted in losses of polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA), color changes and the formation of secondary oxidation products e.g. malondialdehyde (MDA). Trigo et al. [13] reported higher levels of volatile oxidation products in protein concentrates produced by pH-shift-based extraction than in the corresponding biomass (both analyzed as ingredients in emulsions). This was hypothesized to be a result of metal-catalyzed, enzymatic and photooxidation during the extraction. However, data is still lacking for other extraction principles and for storage-induced oxidation of *Ulva* spp. protein concentrates.

The aim of this study was to investigate the oxidative stability of protein concentrates derived from *Ulva* spp., after extraction and during storage under dark and light conditions. Protein extraction was carried out both in pilot- and lab-scale, as processing scale was hypothesized to influence the oxidative state. Two protein concentrates were produced in pilot-scale from wild harvested *Ulva* spp.: one from the primary biomass (PC Wild) and one from the pulp (PC Wild pulp) [17], as this can increase the protein yield. A third protein concentrate was produced in lab-scale from cultivated *Ulva* sp. (PC Cultivated). Protein concentrates stored under light conditions were hypothesized to be more susceptible to oxidation, due to the presence of photosensitizers e.g. chlorophyll. Moreover, extraction of protein from wild harvested *Ulva* spp. in pilot-scale was hypothesized to accelerate oxidation due to longer processing time. Oxidation was investigated by analyzing color (i. e., pigment oxidation), fatty acid losses, lipid hydroperoxide values and selected volatile secondary oxidation products such as alcohols and aldehydes (e.g. 2-alkenals, 2,4-alkadienals). These analyses were chosen to evaluate the oxidation that had taken place during processing and storage. Through oxidation, unsaturated fatty acids are expected to convert into primary oxidation products e.g. lipid hydroperoxides and further into secondary oxidation products that cause off-flavor and reduce sensory acceptance.

2. Material and methods

2.1. Chemical and reagents

Milli-Q water was deionized (18.2 M Ω) filtered water (0.22 μ M) from an in-house Milli-Q system, Millipore SAS (Molsheim, France). HCl 37% was obtained from Merck (Darmstadt, Germany) and petroleum ether from Fisher Scientific (Roskilde, Denmark). Methanol and isoctane with HPLC grade, and toluene and acetyl chloride with ACS reagent grade was provided from Sigma-Aldrich Sweden AB (Stockholm, Sweden).

2.2. Protein concentrates

Three different protein concentrates from *Ulva* spp. were used in this study (Table 1). Protein extraction was carried out both in pilot and lab scale to evaluate how processing conditions influence the oxidative state. Due to limited biomass availability at the time of processing, the lab-scale extraction was conducted on cultivated biomass, whereas the pilot-scale extraction used wild *Ulva* spp. PC Wild and PC Wild pulp were provided from a pilot-scale study [17] that processed approximately 3 tons of wild *Ulva* spp. PC Cultivated was produced from approx. 10 kg of cultivated *Ulva* sp. provided by Pure Algae Denmark (Grenaa, Denmark). The cultivated *Ulva* sp. was grown in recirculated cultivation systems and harvested three times in January 2023 to obtain sufficient material. After harvest, biomass was stored at 4 °C and processed in lab-scale the following day of harvest. Protein extraction for PC Cultivated followed the same procedure as for PC Wild: mechanical fractionation (Angel Juicer 8500S, Domotech, Denmark) followed by decantation, 15 min at 1700 x g, 4 °C (SL 40R Centrifuge, Thermo Scientific, USA), acid precipitation (pH 2), separation at 4500 x g, 4 °C for 10 min (SL 40R Centrifuge, Thermo Scientific, USA) and freeze-drying. The three harvests were pooled into one sample.

2.3. Biochemical composition

The protein concentrates provided (PC Wild and PC Wild pulp) were analyzed for biochemical composition by Nissen et al. [17]. The protein concentrate PC Cultivated was analyzed in terms of dry matter (DM), crude protein (CP), ash, and lipids in triplicates. The DM content was measured using a moisture analyzer (HR73 Halogen Moisture Analyzer, Mettler Toledo, USA). Total nitrogen was analyzed by DUMAS combustion (DUMATHERM®, Gerhart Analytical Systems, Königswinter, Germany) using 1.4 mg O₂/mg sample and an O₂ flowrate of 300 mL/min. The CP content was calculated using a nitrogen-to-protein conversion factor of 5.0 for the primary biomass [37] and 5.5 for PC according to previous results [17,18]. Ash content was measured by heating 0.5 g of sample at 550 °C for 5 h using a Muffle Furnace (Nabertherm, Lilienthal, Germany). The lipid content was quantified by digesting 1 g of sample with boiling hydrochloric acid to break the lipoprotein bonds using a Hydrotherm (Gerhardt Analytical systems, Königswinter, Germany). The resulting solution was filtered, dried, and the lipid was subsequently extracted with petroleum ether using a Soxtherm (Gerhardt Analytical systems, Königswinter, Germany). The lipid content was calculated from the initial sample weight and the final weight obtained after drying.

2.4. Storage and sampling

As the protein concentrate from the pilot processing was more granulated than the fine powder from the lab scale processing of cultivated *Ulva* sp., these protein concentrates were grinded to obtain similar surface area. Thus, protein concentrates from wild harvested *Ulva* spp. were grinded to 0.25 mm at 6000 rpm (Ultra Centrifugal Mill, Retsch). Since the powders were different, the smallest grinding mesh available was chosen to ensure equal conditions in terms of surface area. Three g of each protein concentrate was stored at –70 °C until further analysis. These samples were referred to as day 0. Each protein concentrate was allocated into petri dishes (D: 8.5 cm) in portions of approx. 3 g with a height of approx. 0.1 cm for storage. Petri dishes were used to obtain a relatively thin layer of protein concentrates to ensure that oxygen and light (for samples stored under light conditions) would not be limiting factors during storage. Samples were stored under dark and light conditions at 21 °C for 120 days as ambient temperature reflects storage conditions in industry. Storage for 120 days was chosen as it leaves room for oxidation to take place and based on what was possible during the project As the *Ulva* spp. protein concentrates contain chlorophyll, which is an efficient photosensitizer, we wanted to investigate the effect of

Table 1
Overview of the three different protein concentrates (PC).

Name	PC wild	PC wild pulp	PC cultivated
Extraction material	Wild harvested <i>Ulva</i> spp.	<i>Ulva</i> pulp (from the wild harvested <i>Ulva</i> spp.)	Cultivated <i>Ulva</i> sp.
Extraction	Mechanical fractionation and acid precipitation (pH 2)	Homogenization under mildly alkaline conditions (pH 8.5), mechanical fractionation and acid precipitation (pH 2)	Mechanical fractionation and acid precipitation (pH 2)
Decanting before precipitation	–	–	+
Scale	Pilot-scale	Pilot-scale	Lab-scale
Drying of protein concentrates	Freeze-drying	Freeze-drying	Freeze-drying

light exposure as well. The light source was documented to generate wavelengths between 300 and 700 nm with the highest proportion at 550 nm, followed by 425 and 610 nm. Light intensity was 1700–2000 LUX to accelerate light oxidation, and the samples were rotated to ensure similar light exposure. Samples were taken after 7, 30, 60 and 120 days, homogenized, allocated in Eppendorf tubes, and stored at -70°C until further analysis.

2.5. Color analysis

The color of the protein concentrates was determined using a handheld colorimeter (Konica-Minolta, Japan), measuring the color components L^* (black-white), a^* (green-red), and b^* (blue-yellow). The samples were homogenized and placed in a petri-dish ($D = 5.3$ cm) to a height of 0.5 cm before measurement. All samples were analyzed in triplicates. ΔE indicates the total color changes based on the color components L^* , a^* and b^* and was calculated using the following equation:

$$\Delta E = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2}$$

L_2^* , a_2^* and b_2^* denote the values after storage for 7, 30, 60 and 120 days and L_1^* , a_1^* and b_1^* denote the values at day 0.

2.6. Fatty acid composition

Fatty acid composition was analyzed based on the procedure described by Vall-Ilosera et al. [38] with a few modifications. Briefly, 25 mg sample was weighed into a glass tube, and 50 μL methylated C19:0 (1000 $\mu\text{g}/\text{mL}$ in toluene) and 50 μL methylated C21:0 (1000 $\mu\text{g}/\text{mL}$ in toluene) were added as internal standards (Larodan, Sweden), along with 0.9 mL toluene and 1 mL acetyl chloride-MeOH solution. Samples were vortexed for 5 min at 1600 rpm and heated at 70°C for 120 min in a shaking water bath. Samples were cooled in cold water, followed by addition of 0.2 mL Milli-Q water and 5 mL petroleum ether:diethyl ether (4:1). For extraction, the samples were vortexed for 5 min at 1600 rpm, centrifuged at $2500 \times g$ for 3 min, after which the upper phase was transferred to a glass tube and evaporated under nitrogen gas. Samples were re-dissolved in 1 mL isooctane, vortexed (1600 rpm, 5 min) and transferred to gas chromatograph (GC) vials. Samples were analyzed as described by Vall-Ilosera et al. [38] using an Agilent 7890A GC system with an Agilent 5975C triple axis mass spectrometry detector (Santa Clara, CA, USA). Quantification of fatty acids was performed using the GLC-463 fatty acid methyl esters standard mixture (Nu-Check, Prep, Inc., Elysian, MN, USA) and the internal standard C21:0. Fatty acids not included in the standard mixture (C16:1, C16:2, C16:3, C16:4, C18:4 and C20:4) were identified using the NIST08 library search and quantified using the internal standard C19:0.

2.7. Analysis of volatile oxidation products

The samples were analyzed for the dominating volatile oxidation products using solid-phase microextraction (SPME) coupled with a gas chromatography–mass spectrometry (GC–MS), an Agilent GC 7890A

according to Dalsgaard et al. [23] with minor changes. Briefly, 10 mg of sample was transferred to a 10 mL SPME vial together with the internal standard (D-12 hexanal, 100 ng/sample) and 2 mL Milli-Q water. A 75 μm Car/PDMS-coated SPME Fiber (57343-U, North Harrison road, Bellefonte USA) was incubated at 50°C for 30 min in the headspace of each sample while stirring (500 rpm). The fiber was then introduced to the GC inlet at a temperature of 280°C . The volatiles were separated on a HP5-MS column (30 m, 0.25 mm ID, 0.25 μm film thickness; Agilent, Folsom, CA, USA). The initial oven temperature was 35°C and increased to 120°C at a rate of $7^{\circ}\text{C}/\text{min}$, followed by an increase of $35^{\circ}\text{C}/\text{min}$ until 325°C , which was held for 10 min. Helium 6.0 was used as the carrier gas at a constant flow rate of 1.2 mL/min. Detection was performed using an Agilent 5975 with an interface temperature of 280°C , quadrupole at 150°C , and the MS source at 230°C . Fragmentation voltage was 70 eV. The data acquisition was performed in SIM mode over a mass range of 50–250 m/z . Quantification was performed using external standard curves (hexanal, 1-hexanol, heptanal, benzaldehyde, 2-pentylfuran, 2,4-heptadienal, nonanal, 2-nonenal obtained from Merck (Darmstadt, Germany)) at concentrations of 1–1000 ng/mL, using D-12 hexanal (100 ng/sample) as internal standard. These standards were selected based on initial scans, as they represented the dominant oxidation products observed.

2.8. Lipid hydroperoxide value

Folch extraction was performed to extract lipid hydroperoxides from protein concentrates. In brief, the Folch extraction was carried out by mixing ~ 300 mg of seaweed protein concentrates with 2 mL of water and 2 mL of MeOH. Then, 4 mL of chloroform was added, followed by 30 s of mixing. The samples were centrifuged at 3000 rpm for 10 min at 4°C . After centrifugation, the chloroform phase was transferred to a GC-vial and stored in darkness at -21°C . Samples were analyzed for their hydroperoxide concentration using the thiocyanate assay [23]. To prepare the iron (II)/thiocyanate reagent, three solutions were prepared. Solution I was prepared by dissolving 0.4 g of barium chloride dihydrate in 50 mL of MilliQ-water, while 0.5 g of iron (II) sulfate heptahydrate was dissolved in 50 mL of MilliQ-water. The barium chloride solution was slowly added to the iron (II) sulfate heptahydrate solution, followed by the addition of 2 mL of 10 M HCl. The solution was filtered to remove precipitated barium sulfate. Solution II was prepared by dissolving 3 g of ammonium thiocyanate in 10 mL of MilliQ-water. Solution III consisted of a chloroform-methanol ratio (1:1). The iron (II)/thiocyanate reagent was prepared by mixing 250 μL of Solution I with 250 μL of Solution II, followed by dilution to 25 mL with Solution III. To measure the hydroperoxide concentration, 1 mL of sample was mixed with 1 mL of the iron (II)/thiocyanate reagent. After 5 min of incubation at room temperature (21°C), the absorbance was measured at 500 nm, with 700 nm used for background correction, using a UV-3100PC Spectrophotometer (VWR International, Pennsylvania, USA). A sample blank was included to correct for the intrinsic color of the sample. A hydrogen peroxide standard curve (1–60 μM) was prepared and used to calculate the hydroperoxide concentration in the samples.

2.9. Statistical analysis

Results are presented as means \pm SD. Data for the oxidation products were log₁₀-transformed to obtain normal distribution and homogeneity of variance, confirmed by QQ-plot and Shapiro test ($P > 0.05$), and Bartlett test ($P > 0.05$), respectively. One-way analysis of variance (ANOVA) was carried out to test the significance of difference between the different protein concentrates. Differences were regarded as significant at a minimum level at 95% ($P < 0.05$). A Duncan's Multiple Range test was used as a post-hoc test to designate significantly different averages. Statistical analysis was carried out using RStudio. For the principal component analysis (PCA), the dataset was log₂-transformed to stabilize variance and minimize skewness.

3. Results

3.1. Biochemical composition

Three different protein concentrates (PC Wild, PC Wild pulp and PC Cultivated) were produced from three different starting materials (Wild harvested *Ulva* spp., pulp (obtained after the first protein extraction of wild harvested *Ulva* spp.), and cultivated *Ulva* sp., respectively), showing different compositions (Table 2). All three protein concentrates had increased contents of protein and decreased contents of ash compared to their respective primary starting material. Lipids were concentrated in the protein concentrates extracted from primary biomass for both wild (PC Wild) and cultivated *Ulva* sp. (PC Cultivated), whereas extraction from the pulp (PC Wild pulp) resulted in a similar lipid content compared to the starting material. Protein concentrates varied in protein (27.0–40.1% of DM) and lipid content (2.1–11.6% of DM) with the highest content of both observed in PC Cultivated and the lowest content of both observed in PC Wild pulp. The ash content varied from 24.3 to 37.7% of DM with PC Cultivated showing the lowest content, whereas PC Wild and PC Wild pulp had similar contents.

3.2. Color

The color components L*, a* and b* of the three protein concentrates on day 0 and upon storage for 7, 30, 60 and 120 days in dark and light conditions, are shown in Fig. 1. In general, only minor changes were observed for PC Wild and PC Wild pulp, which is consistent with ΔE showing an increase of <1 for PC Wild pulp and around 2 for PC Wild, with slightly larger changes for protein concentrates stored under dark conditions. This was also observed visually, as protein concentrates appeared brown throughout storage (Fig. 2A and B). PC Cultivated increased in lightness (L*) under light storage conditions until day 60, after which it decreased. Under dark storage conditions, lightness increased until day 7, followed by a decline. An increase in a* was observed for PC Cultivated under both dark and light storage conditions, indicating a shift towards less green coloration. For b*, a decrease was observed after 120 days, most pronounced for samples stored under dark conditions, resulting in samples with a less yellow appearance. The total color change for PC Cultivated was highest for the samples stored under light conditions during the first 60 days, after which a decrease in ΔE was observed. By day 120, samples stored in dark conditions showed the largest overall changes (ΔE). Visually, PC Cultivated was greener than

Table 2

Biochemical composition of the starting material (primary biomass/pulp) and the resulting protein concentrates. Protein is listed as crude. All values are based on dry matter. Data for Wild *Ulva* spp. and Wild pulp was provided by Nissen et al. [17]. Data is represented as means \pm SD, $n = 3$.

% of DM	Wild <i>Ulva</i>		Wild pulp		Cultivated <i>Ulva</i>	
	Starting material	Protein concentrate	Starting material	Protein concentrate	Starting material	Protein concentrate
Protein	10.2 \pm 1.2	31.6 \pm 0.6	8.7 \pm 0.7	27.0 \pm 0.3	21.5 \pm 0.1	40.1 \pm 0.1
Lipid	0.7 \pm 0.2	4.6 \pm 0.1	2.2 \pm 0.5	2.1 \pm 0.3	3.3 \pm 0.4	11.6 \pm 0.6
Ash	57.9 \pm 3.5	37.1 \pm 0.5	60.0 \pm 2.6	37.7 \pm 0.1	30.2 \pm 0.3	24.3 \pm 0.2

PC Wild and PC Wild pulp at day 0 and developed a darker color under dark conditions and a lighter color under light conditions (Fig. 2C).

3.3. Fatty acids

The fatty acid content and composition of the protein concentrates (PC Wild, PC Wild pulp and PC Cultivated) were analyzed at day 0 and after 120 days of storage under dark and light conditions (Table 3). In general, C16:0 constituted the highest fraction of fatty acids in all protein concentrates at both day 0 and after 120 days of storage (3.77–29.23 mg/g DM). High levels of the unsaturated fatty acids C16:4, C18:1n7, C18:2n6 and C18:3n3 were also observed for PC Cultivated at day 0 (4.97–6.74 mg/g DM). For PC Wild and PC Wild pulp, SFA represented the largest fraction at day 0, followed by MUFA, whereas PC Cultivated had a notable high PUFA content compared to PC Wild and none in PC Wild pulp. Upon storage, PC Wild showed a high loss of total fatty acids (20.8–21.6%) regardless of light or dark storage (Fig. 3) compared to the PC wild pulp (Fig. 3). This was explained by a higher content of PUFA in PC wild and therefore higher loss of PUFA, followed by MUFA. A greater loss of MUFA (50.3 \pm 0.8%) was observed during storage in light conditions than storage in dark conditions (43.6 \pm 1.3%) for PC Wild. PC Wild pulp showed a minor loss of total fatty acids (0.5–4.5%) after storage for 120 days, primarily due to a loss of MUFA (21.4–26.9%) in both storage conditions. PC Cultivated had the highest content of TFA at day 0 (76.06 mg/g DM) compared to the other protein concentrates, with high contents of PUFA and SFA, followed by MUFA. A total loss of 57.1–58.0% fatty acids was observed for both light and dark conditions after storage for 120 days, due to a significant loss of PUFA (98.1–98.2%) and MUFA (41.2–59.2%). PC Cultivated showed a higher loss of MUFA (59.2 \pm 2.2%) under light conditions compared dark conditions (41.2 \pm 7.1%). Loss of SFA was also observed for PC Cultivated (17.2–21.2%), whereas the loss of SFA in PC Wild and PC Wild pulp was lower (\leq 4.8%).

3.4. Volatile oxidation products

The protein concentrates (PC Wild, PC Wild pulp and PC Cultivated) and their respective crude materials (biomass/pulp) were analyzed for their levels of dominant volatile oxidation products (hexanal, 1-hexanol, heptanal, benzaldehyde, 2-pentylfuran, 2,4-heptadienal, nonanal and 2-nonenal) at day 0 and after 120 days of storage under dark and light (Fig. 4).

PC Wild and PC Wild pulp showed significantly higher levels of oxidation products in the protein concentrates compared to the starting materials at day 0, except for hexanal and benzaldehyde in PC Wild pulp, which were similar or slightly lower. PC Wild pulp showed significantly higher levels of all oxidation products when stored under light conditions than dark conditions. A similar trend was observed for PC Wild, except for 2-pentylfuran, nonanal and 2-nonenal showing no significant differences between storage under light and dark conditions. PC Wild and PC Wild pulp showed higher values for all oxidation products on day 0 compared to samples stored for 120 days. Among the different oxidation products in PC Wild and PC Wild pulp, hexanal was the most abundant (65–537 μ g/10 g DM), with the highest levels seen in PC Wild at day 0.

PC Cultivated showed significantly higher levels of hexanal and 2-

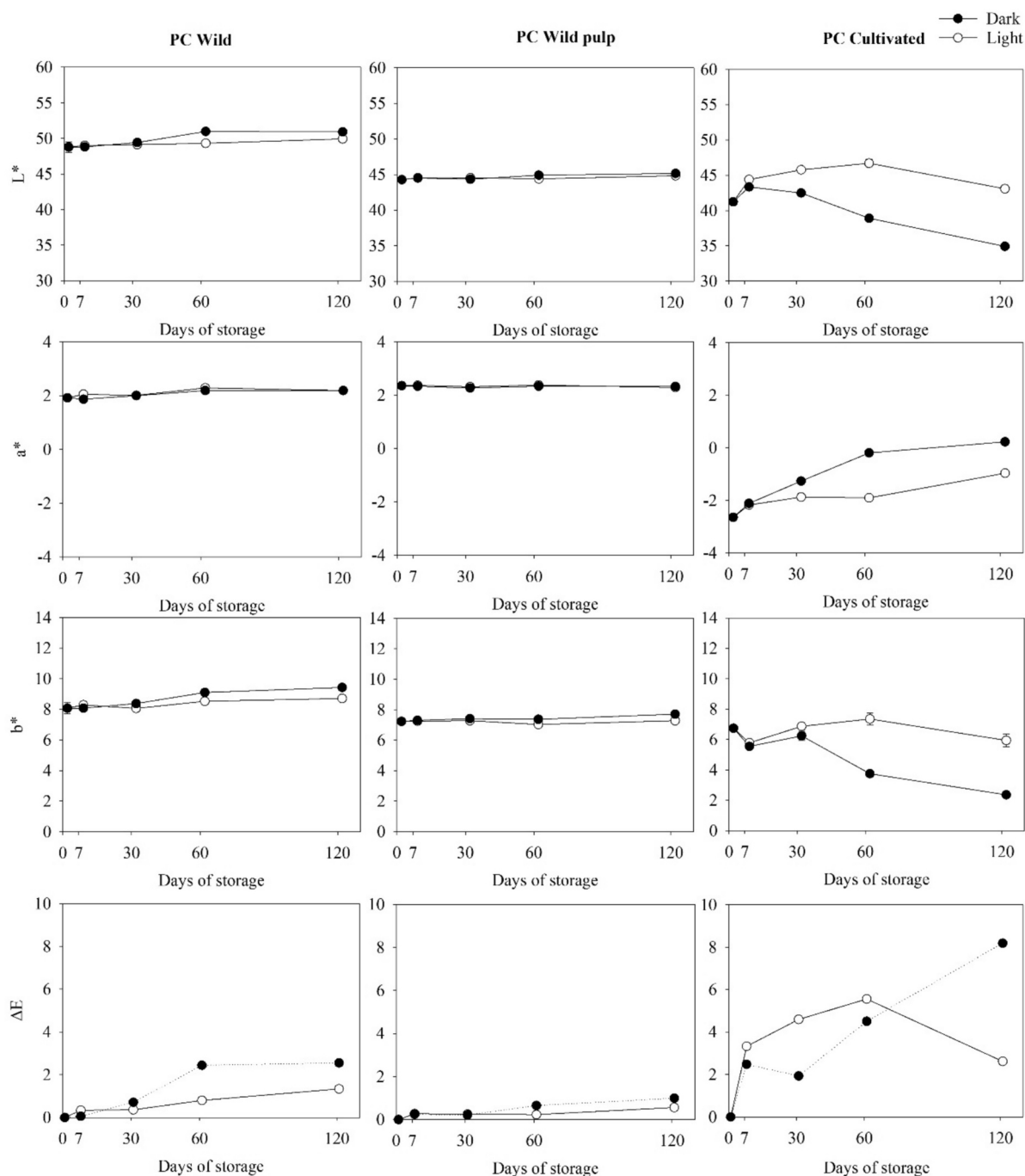


Fig. 1. Color changes (L^* , a^* , b^* and ΔE) of the protein concentrates (PC Wild, PC Wild pulp and PC Cultivated) over storage time from day 0 to 120 when stored under dark and light conditions at 21 °C. ΔE shows the total changes based on the color components L^* (lightness), a^* (green to red) and b^* (blue to yellow). Data is represented as means \pm SD, $n = 3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pentylfuran compared to the primary biomass, while 1-hexanol and benzaldehyde were lower, and the remaining compounds showed no significant differences. Compared to PC Wild samples, PC Cultivated showed lower total oxidation product levels both in the starting material and in the protein concentrate at day 0. During storage, higher levels of volatile oxidation products were observed for the PC Cultivated stored under light conditions compared to dark conditions, except for 2-pentylfuran which showed no significant differences between the two storage conditions. After 120 days, PC Cultivated stored in the dark had oxidation product levels that were similar or lower than day 0, except for heptanal and 2-nonenal, which increased. In contrast, PC Cultivated stored under light conditions resulted in similar or significantly higher

levels of oxidation products when compared to day 0. Lipid hydroperoxide values followed the same pattern as the volatiles (data not shown).

To better assess initial oxidation, lipid hydroperoxide values were analyzed and visualized alongside the other analytes in a PCA-plot (Fig. 5A-B). Lipid hydroperoxides followed the same pattern as the volatiles and contributed to principal component 1, with PC Wild Biomass and PC Wild Pulp at day 0 positioned to the right and PC Cultivated at day 0 to the left, along with protein, lipids, TFA, SFA, MUFA, PUFA, and green color (Fig. 5A). This clearly indicates the higher fatty acid content, including MUFA and PUFA, in the PC cultivated than in the two Wild PC processed in pilot plant facilities (Fig. 5A). The PCA of PC Cultivated alone clearly indicated high content of volatiles and

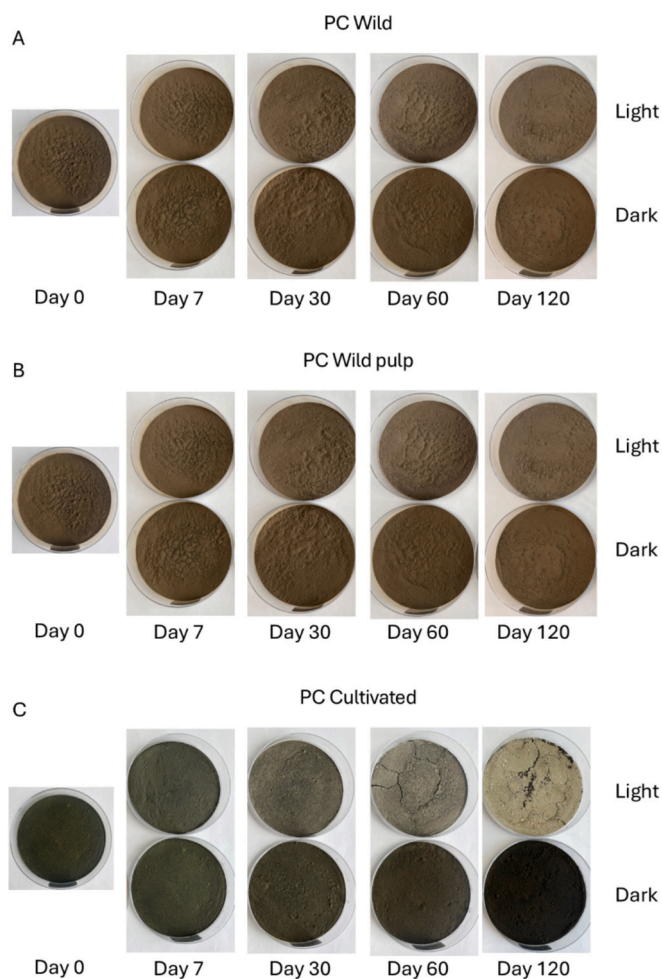


Fig. 2. Color changes of protein concentrates (PC) stored under light and dark conditions for 120 days for A) PC Wild, B) PC Wild pulp and C) PC Cultivated.

lipid hydroperoxides on day 0 (Fig. 5B). However, 1-hexanol, heptanal, nonanal, and 1-nonenal all increased in samples stored in light (PC Cultivated, day 120) compared to day 0, following another pattern than the other volatiles.

4. Discussion

In this study, we compared protein concentrates from wild-harvested *Ulva* spp. obtained after one protein extraction (PC Wild) and a second alkaline (pH 8.5) protein extraction of the resulting pulp (PC Wild pulp). These two protein concentrates have previously shown notable differences in protein content and overall chemical composition, as well as in the amino acid composition and protein digestibility [17]. Therefore, we aimed to assess process-induced oxidative changes and storage stability in relation to changes in the lipid composition as products high in unsaturated fatty acids always will play a role, also in the protein fraction due to potential radical transfer between the lipid and protein [24]. After pilot-scale processing, the protein products appeared very brown, which we have not observed previously in lab-scale processing. Hence, we decided to include lab-scale extracted protein concentrate. However, since wild-harvested *Ulva* spp. was no longer available, cultivated *Ulva* sp. was used instead, resulting in PC cultivated.

4.1. Effect of protein extraction

The cultivated *Ulva* sp. was significantly higher in protein than the wild-harvested *Ulva* spp., and higher than what previously have been

observed for cultivated *Ulva* sp. [39]. It resulted in a higher protein content in PC Cultivated than in the PC Wild biomass. The lipid content was also significantly higher in the cultivated *Ulva* sp. compared to the wild *Ulva* spp., which resulted in a high content of lipids in PC Cultivated compared to the two PC Wilds. From this data, more oxidation might be expected to take place in the PC Cultivated than in the two PC Wild samples both during processing and storage. However, oxidative changes during processing showed otherwise.

Primary (lipid hydroperoxides) and secondary (volatiles) lipid oxidation products are often low in the beginning of storage experiments [23], but in the pilot processed protein concentrates, the lipid hydroperoxide value and the secondary oxidation products were high, already after processing. Even though the lipid content was higher in PC Cultivated, the content of unsaturated FAs was higher after processing compared to the two pilot processed protein concentrates, indicating less damage to the lipid fraction in the PC Cultivated protein samples. More oxidation taken place in the pilot processing was supported by a low percentage of unsaturated fatty acids and a low retention of the green color of the PC Wild and PC Wild pulp compared to the PC Cultivated sample.

The loss of green pigment was most likely caused by a conversion of chlorophyll to other forms. Chlorophyll, containing Mg^{2+} , is green in color, whereas a loss of Mg^{2+} results in a green/brown color (pheophytin). Mg^{2+} can be lost upon exposure to heat or acidic conditions (pH < 6), where the latter could be the case as the protein was precipitated at pH 2. Moreover, enzymatic activity of chlorophyllase or pheophytinase can degrade chlorophyll and pheophytin, respectively, resulting in loss of phytol. Loss of both Mg^{2+} and phytol results in a brown color (pheophorbide) [40], which resembles the color of PC Wild and PC Wild pulp. Other reactions could also have contributed to the color, including enzymatic and non-enzymatic browning. Enzymatic browning results from oxidation of polyphenols to quinones that polymerize into dark pigments [41,42], which, previously, have been detected in protein extraction from *Ulva* spp. [43]. Non-enzymatic browning (Maillard reaction) can be promoted by alkaline conditions or catalyzed by e.g. iron and copper and is elevated at higher temperatures [44]. Therefore, this reaction was more likely for PC Wild pulp where mildly alkaline conditions (pH 8.5) were included in the extraction procedure. Another possible pathway leading to browning is via polymerization of Schiff-bases, which are derived from interactions between e.g., aldehydes and amino acids [45,46]. PC Cultivated was visually green on day 0 (Fig. 2C), indicating a higher content of chlorophyll still complexing Mg^{2+} . In the lab-based extraction setup, enzymatic browning might have been prevented due to shorter processing times, resulting in a faster addition of acid (down to pH 2), which can slow down or prevent the activity of the enzymes responsible [42,47].

All of the protein concentrates contained significant levels of ash (24.3–37.7%), where Nissen et al. [17] documented the presence of high concentrations of transition metals including iron, zinc and copper in PC Wild and PC Wild pulp. Hence, these could act as prooxidants in the lipid oxidation reaction [29].

Volatile oxidation products were detected in the primary materials (biomass/pulp) with the highest concentration of hexanal, followed by benzaldehyde. These compounds may have been generated during cultivation, harvest, sampling, or storage. Upon protein extraction, all the detected secondary oxidation products increased for PC Wild and PC Wild pulp, except for hexanal and benzaldehyde in PC Wild pulp showing similar or reduced content. Trigo et al. [48] observed increased levels of hexanal as well as pentanal and 2-ethylfuran upon protein extraction from *Ulva fenestrata* using a pH shift approach and analysis of the protein concentrates in emulsions with 5 or 10% lipids. Hexanal is a common lipid oxidation product, derived from n-6 PUFA such as linoleic acid (C18:2n6), generated upon transition metal induced oxidation or through enzymatic pathways [29]. Increased levels of hexanal were also observed for protein concentrates from peas compared to the pea flour [49], whereas Tanambell et al. [50] showed lower hexanal content in

Table 3

Fatty acid content (mg/g DM) of the protein concentrates (PC Wild, PC Wild pulp and PC Cultivated) over storage time from day 0 to day 120 under dark and light conditions. Total fatty acids (TFA), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) are shown. Moreover, the loss of TFA is shown along with loss of SFA, MUFA, and PUFA for dark and light conditions, respectively. ND = Not detected/Below limit of quantification. Data is represented as means \pm SD, $n = 2$.

Fatty acids (mg/g DM)	PC Wild			PC Wild pulp			PC Cultivated		
	Start (0)	Dark (120)	Light (120)	Start (0)	Dark (120)	Light (120)	Start (0)	Dark (120)	Light (120)
C14:0	1.10 \pm 0.02	1.00 \pm 0.02	1.04 \pm 0.01	0.24 \pm 0.00	0.25 \pm 0.00	0.27 \pm 0.00	0.74 \pm 0.04	0.52 \pm 0.04	0.63 \pm 0.05
C15:0	0.29 \pm 0.01	0.36 \pm 0.01	0.38 \pm 0.00	0.13 \pm 0.00	0.17 \pm 0.00	0.18 \pm 0.00	0.14 \pm 0.00	0.12 \pm 0.01	0.17 \pm 0.01
C16:0	7.84 \pm 0.14	7.06 \pm 0.36	7.54 \pm 0.08	3.96 \pm 0.07	3.77 \pm 0.04	3.90 \pm 0.02	29.23 \pm 0.30	22.84 \pm 1.88	24.06 \pm 1.21
C16:1n7	1.69 \pm 0.01	0.92 \pm 0.00	0.79 \pm 0.02	ND	ND	ND	2.39 \pm 0.03	1.36 \pm 0.13	0.95 \pm 0.05
C16:1 ^a	0.39 \pm 0.00	0.35 \pm 0.01	0.35 \pm 0.01	0.16 \pm 0.01	0.12 \pm 0.00	0.12 \pm 0.00	2.19 \pm 0.11	1.18 \pm 0.03	1.06 \pm 0.07
C16:2 ^a	ND	ND	ND	ND	ND	ND	0.19 \pm 0.01	ND	ND
C17:0	0.26 \pm 0.00	0.47 \pm 0.01	0.47 \pm 0.00	0.10 \pm 0.00	0.15 \pm 0.00	0.16 \pm 0.00	0.05 \pm 0.00	0.05 \pm 0.00	0.05 \pm 0.00
C17:1n7	0.06 \pm 0.00	0.10 \pm 0.00	0.11 \pm 0.00	0.03 \pm 0.01	0.05 \pm 0.00	0.05 \pm 0.00	ND	ND	ND
C16:3 ^a	ND	ND	ND	ND	ND	ND	1.93 \pm 0.09	ND	ND
C16:4 ^a	ND	ND	ND	ND	ND	ND	6.74 \pm 0.26	ND	0.14 \pm 0.01
C18:0	1.20 \pm 0.01	1.32 \pm 0.01	1.28 \pm 0.03	0.35 \pm 0.00	0.35 \pm 0.0	0.36 \pm 0.00	0.09 \pm 0.01	0.32 \pm 0.25	0.08 \pm 0.00
C18:1n9/12	0.96 \pm 0.01	0.47 \pm 0.02	0.38 \pm 0.00	ND	ND	ND	0.62 \pm 0.01	0.58 \pm 0.27	0.21 \pm 0.00
C18:1n7	1.66 \pm 0.01	0.85 \pm 0.05	0.72 \pm 0.02	0.05 \pm 0.00	0.04 \pm 0.01	0.06 \pm 0.02	5.80 \pm 0.01	3.32 \pm 0.39	2.11 \pm 0.15
C18:2n6	0.25 \pm 0.00	ND	ND	ND	ND	ND	4.97 \pm 0.06	0.41 \pm 0.02	0.25 \pm 0.01
C18:3n6	ND	ND	ND	ND	ND	ND	3.88 \pm 0.08	0.10 \pm 0.01	0.17 \pm 0.01
C18:3n3	0.03 \pm 0.00	ND	ND	ND	ND	ND	4.97 \pm 0.06	0.07 \pm 0.01	0.06 \pm 0.00
C18:4 ^a	ND	ND	ND	ND	ND	ND	3.17 \pm 0.00	ND	ND
C20:0	0.32 \pm 0.00	0.28 \pm 0.02	0.28 \pm 0.00	0.28 \pm 0.00	0.24 \pm 0.00	0.26 \pm 0.00	0.65 \pm 0.01	0.57 \pm 0.03	0.64 \pm 0.04
C20:1	0.90 \pm 0.02	0.49 \pm 0.00	0.46 \pm 0.00	ND	ND	ND	0.18 \pm 0.01	0.14 \pm 0.01	0.24 \pm 0.00
C20:3n6	ND	ND	ND	ND	ND	ND	0.59 \pm 0.01	ND	ND
C20:4 ^a	0.15 \pm 0.01	ND	ND	ND	ND	ND	1.62 \pm 0.02	ND	ND
C20:4n3	ND	ND	ND	ND	ND	ND	0.19 \pm 0.00	ND	ND
C20:5n3	0.36 \pm 0.02	0.05 \pm 0.00	0.06 \pm 0.01	ND	ND	ND	1.55 \pm 0.04	ND	ND
C22:0	0.16 \pm 0.01	0.15 \pm 0.00	0.15 \pm 0.00	0.12 \pm 0.00	0.11 \pm 0.01	0.12 \pm 0.00	1.44 \pm 0.05	1.06 \pm 0.04	1.12 \pm 0.02
C22:4n6	ND	ND	ND	ND	ND	ND	1.07 \pm 0.03	ND	ND
C22:5n3	ND	ND	ND	ND	ND	ND	1.39 \pm 0.05	ND	ND
C22:6n3	0.09 \pm 0.00	ND	ND	ND	ND	ND	0.28 \pm 0.03	ND	ND
C24:1n9	ND	ND	ND	0.16 \pm 0.02	0.09 \pm 0.01	0.09 \pm 0.01	ND	ND	ND
SFA	11.17 \pm 0.18	10.62 \pm 0.37	11.13 \pm 0.12	5.18 \pm 0.07	5.04 \pm 0.05	5.24 \pm 0.02	32.33 \pm 0.19	25.48 \pm 2.25	26.77 \pm 1.32
MUFA	5.65 \pm 0.04	3.19 \pm 0.05	2.81 \pm 0.03	0.41 \pm 0.02	0.30 \pm 0.00	0.33 \pm 0.03	11.18 \pm 0.07	6.58 \pm 0.84	4.57 \pm 0.28
PUFA	0.86 \pm 0.03	0.05 \pm 0.00	0.06 \pm 0.01	ND	ND	ND	32.54 \pm 0.14	0.58 \pm 0.03	0.62 \pm 0.04
TFA	17.69 \pm 0.24	13.86 \pm 0.43	14.01 \pm 0.14	5.59 \pm 0.09	5.34 \pm 0.05	5.57 \pm 0.01	76.06 \pm 0.40	32.63 \pm 3.12	31.95 \pm 1.63
% Loss SFA		4.8 \pm 4.9	0.3 \pm 2.7		2.7 \pm 2.2	-1.2 \pm 1.7		21.2 \pm 6.5	17.2 \pm 3.6
% Loss MUFA		43.6 \pm 1.3	50.3 \pm 0.8		26.9 \pm 4.9	21.4 \pm 2.1		41.2 \pm 7.1	59.2 \pm 2.2
% Loss PUFA		94.4 \pm 0.7	92.5 \pm 0.5		0 \pm 0	0 \pm 0		98.2 \pm 0.1	98.1 \pm 0.1
% Total loss		21.6 \pm 3.5	20.8 \pm 1.9		4.5 \pm 2.5	0.5 \pm 1.5		57.1 \pm 3.9	58.0 \pm 1.9

^a Identified using the NIST08 library search and quantified using internal standard.

acid precipitated pea concentrates than in pea flour and ultrafiltered protein concentrates. n-6 PUFAs are present in *Ulva* spp. biomass [36,38], where a minor decrease in concentration has been observed upon protein extraction using different extraction procedures in lab-scale [19,21]. In this study, PC Cultivated showed the highest content of n-6 PUFA, while it was barely present in PC Wild and none in PC Wild pulp. This is likely due to oxidation of n-6 PUFA upon protein extraction, explaining the high content of hexanal observed in PC Wild and PC Wild pulp on day 0. Disruption of cell membranes facilitated by the protein extraction could promote the exposure of structural lipids to prooxidants, thereby increasing the risk for oxidation [51]. In general, the highest content of MUFA and PUFA was observed in PC Cultivated, whereas PC Wild and PC Wild pulp consisted of 60–90% SFA. Differences in processing conditions could contribute to this, where PC Wild and PC Wild pulp were extracted in pilot-scale. Longer processing times in pilot-scale (4–5 h processing in total) compared to lab-scale (2–3 h processing in total) are likely to facilitate oxidative stress for highly sensitive molecules such as MUFA and PUFA. Duque-Estrada et al. [25] detected a higher content of protein-bound carbonyls as indices for protein oxidation for several commercial soy protein isolates and concentrates compared to similar products generated in lab-scale. This was ascribed to the differences in processing conditions or differences in the composition of the starting material. Also in the present study, the higher degree of oxidation observed for the protein concentrates produced in pilot-scale, supported by higher levels of volatile oxidation products and lower content of unsaturated fatty acids; could be a result

of the different processing conditions or different compositions of the starting material, or both. Regarding raw material differences, potential differences in the balance between endogenous pro- and antioxidants in the biomasses will play a profound role [29,51,52].

4.2. Effect of storage in dark and light conditions

Under light conditions, the color changes were characterized by bleaching, while under dark conditions, the development of dark brown pigments dominated. PC Cultivated, stored under light, had a loss of green color, indicating loss of chlorophyll. Bleaching under light conditions was also observed in a study by Harrysson et al. [36] storing dried *Ulva fenestrata*. Our study used a light source with wavelengths between 300 and 700 nm with the highest proportion at 550, 425 and 610 nm, respectively. Specific chlorophyll derivatives, e.g. chlorophyll *a*, methyl chlorophyllide *a*, zinc and copper pheophytin *a* have absorption maxima close to 425 nm [40], which could have facilitated the bleaching.

To understand the photooxidation, PC Cultivated should be in focus as it still has green color after processing, indicating intact chlorophyll. Although all secondary oxidation products except 2-pentylfuran were higher in samples stored in light than in those stored in dark, the most pronounced increases from day 0 to day 120 were observed for heptanal and 2-nonenal. At the same time loss of both MUFA and PUFA were observed. The loss of PUFA was more pronounced than that of MUFA, likely due to their higher number of double bonds, which makes them

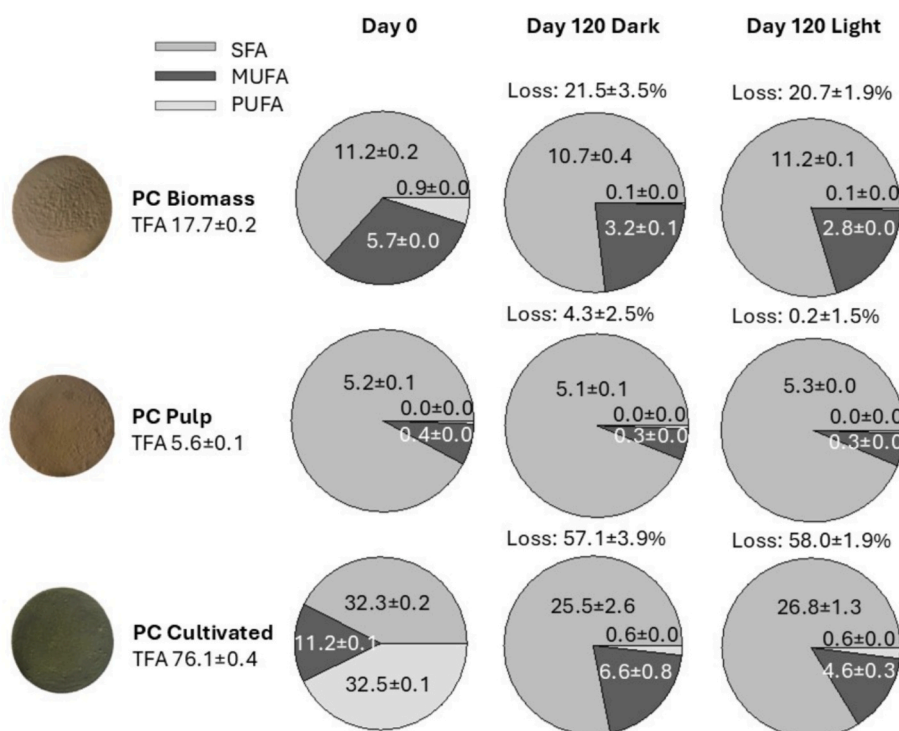


Fig. 3. Total fatty acids (TFA) (mg per g DM) in the protein concentrates: PC Biomass, PC Pulp and PC Cultivated over storage time from day 0 to 120 stored under dark and light conditions. Composition is shown as mg/g DM distributed between saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in the diagrams. Loss of TFA (%) is shown after storage for 120 days in dark and light. The color of the different protein concentrates is included as a picture. Data is represented as mean \pm SD, $n = 2$. PC: Protein concentrate.

more susceptible to oxidation [29]. MUFA was lost to a lesser extent than PUFA, but the loss of PUFA under light and dark conditions was the same, whereas MUFA loss was lower in dark storage. Upon photooxidation of oleic acid with methyl blue as photosensitizer, Lee et al. [53] previously showed increased levels of heptanal and 2-nonenal compared to dark storage, whereas no differences were observed for nonanal. Thus, oxidation of oleic acid might be part of the explanation for the different pattern observed for some of the secondary lipid oxidation products, suggesting chlorophyll and photooxidation being a part of the story. Chlorophyll acts as a type II photosensitizer, generating singlet oxygen [29]. The chlorophyll derivatives would contribute differently to this as they exhibit different absorption maxima [40].

Both n-3 and n-6 fatty acids made up a large fraction of the TFA in the PC Cultivated on day 0, but there was only a minor presence in PC Wild and none in PC Wild pulp. These fatty acids are important for human consumption in a well-balanced ratio to support overall health [29]. They are more susceptible to oxidation, illustrated by the greater loss both with autooxidation in dark and photooxidation in light. In storage they were completely lost for PC Wild, whereas PC Cultivated still had low amounts available. Losses of n-3 and n-6 fatty acids were also observed in other studies storing *Ulva* spp. biomass [36,38]. Harryson et al. [36] also encountered higher oxidation for *Ulva fenestrata* stored under light conditions, in particular increased levels of 4-hydroxy-trans-2-hexenal (HHE), which is an oxidation product deriving from n-3 PUFA [54].

Hexanal is probably the most common secondary oxidation product in literature, and it is an oxidation product derived from n-6 PUFA. However, it can also be a result of degradation of other volatile compounds such as 2,4-decadienal and 2-octenal [29,55]. This could explain why hexanal reached the highest values under light conditions in all three PCs, as no differences in PUFA content were observed under dark and light conditions. Linolenic acid (C18:3n3 or C18:3n6) can oxidize to form 2,4-heptadienal [56], whereas oxidation of oleic acid (C18:1n9) is

suggested to form heptanal [57], and linoleic acid (C18:2n6), besides hexanal can oxidize into heptanal [58], nonanal and 1-hexanol [59]. The diene system of different hydroperoxides can decompose to form furanoids such as 2-pentylfuran [59], which was encountered in minor amounts for all concentrates. However, these compounds can also be formed upon degradation of 2-alkenals such as 2-nonenal in the presence of transition metals such as iron [56,60]. The formation of benzaldehyde can be formed during Strecker degradation of amino acids [60]. This could explain the higher values encountered in cultivated *Ulva* sp. biomass, as well as in its derived PC.

The majority of the secondary oxidation products detected in PC Cultivated were lower after 120 days of storage in darkness compared to day 0. This could be hypothesized to be due to decomposition and/or further reactions with other compounds present, such as proteins. Carbonyl compounds (e.g. aldehydes) can react with free amino groups via Schiff-base formation, resulting in a cross-linked complex that negatively affects the nutritional value of the protein and generate off-flavors as well as yellow-brown pigments [61]. Another suggestion could be aldol condensation (reaction between two carbonyl compounds, such as aldehydes or ketones), leading to a tertiary oxidation product such as 2-butyl-2-octenal, which is an indicator for strong oxidation and severe quality degradation [56]. A greater difference in oxidation product levels was observed for PC Wild and PC Wild pulp stored under dark and light conditions, which both revealed lower levels on day 120 compared to day 0. These concentrates had been subjected to higher formation of volatile oxidation products during their production, thereby facilitating a higher formation of cross-linked oxidation products. However, further investigation is necessary to confirm this.

4.3. Future perspectives

In general, protein concentrates (protein <80%) were expected to encounter a lower storage stability as compared to protein isolates

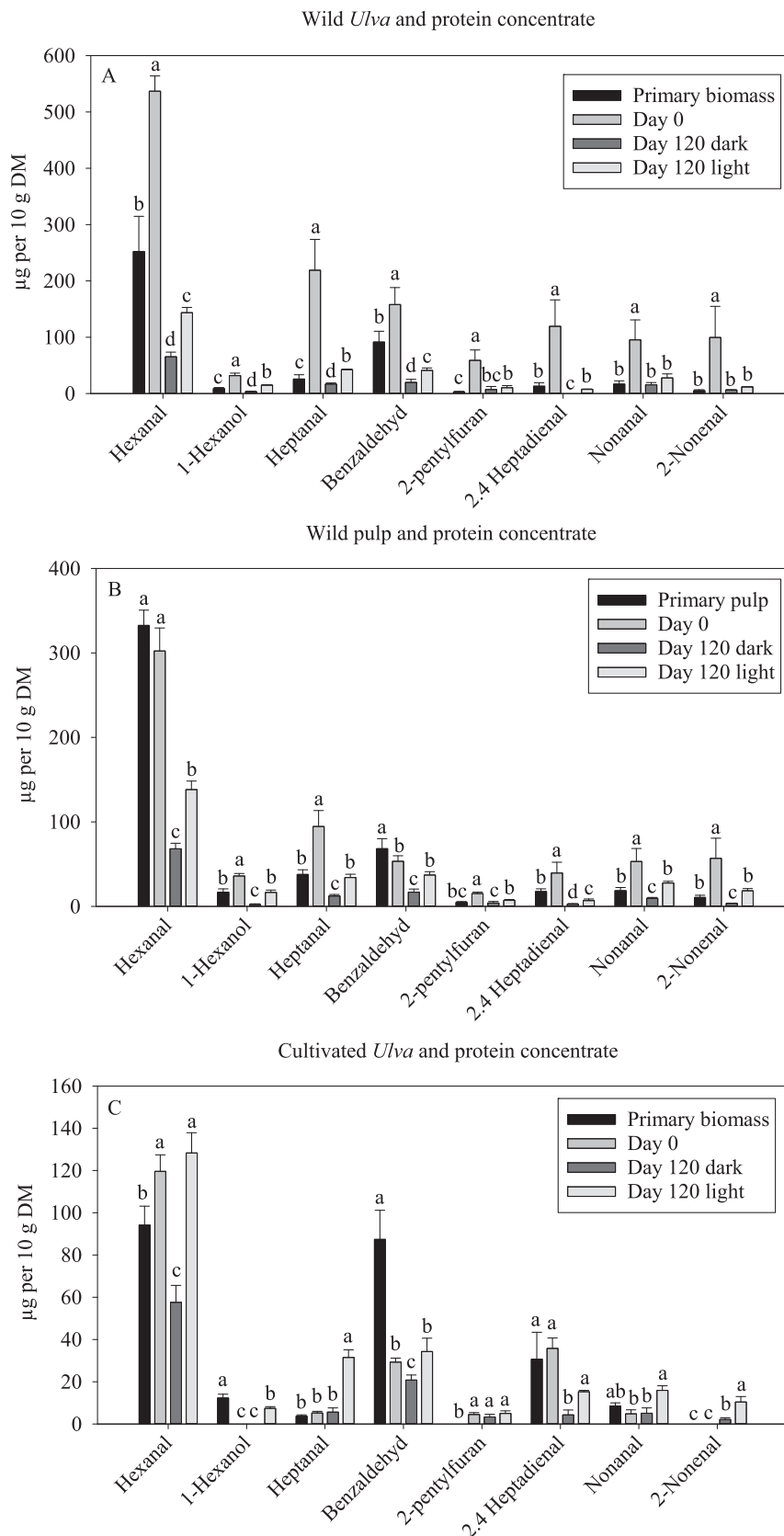


Fig. 4. Concentration of different oxidation products ($\mu\text{g}/10\text{ g DM}$) for PC Wild, PC Wild pulp and PC Cultivated, including extraction material (primary biomass/pulp), protein concentrate on day 0 and protein concentrates stored in dark and light conditions for 120 days. Different oxidations products include hexanal, 1-hexanol, heptanal, benzaldehyde, furan 2-pentyl, 2,4-heptadienal, nonanal, and 2-nonenal. Data is represented as means \pm SD, $n = 3$. Different letters within the same oxidation product indicate significance of difference ($p < 0.05$) compared by one-way ANOVA followed by a Duncan's Multiple Range test.

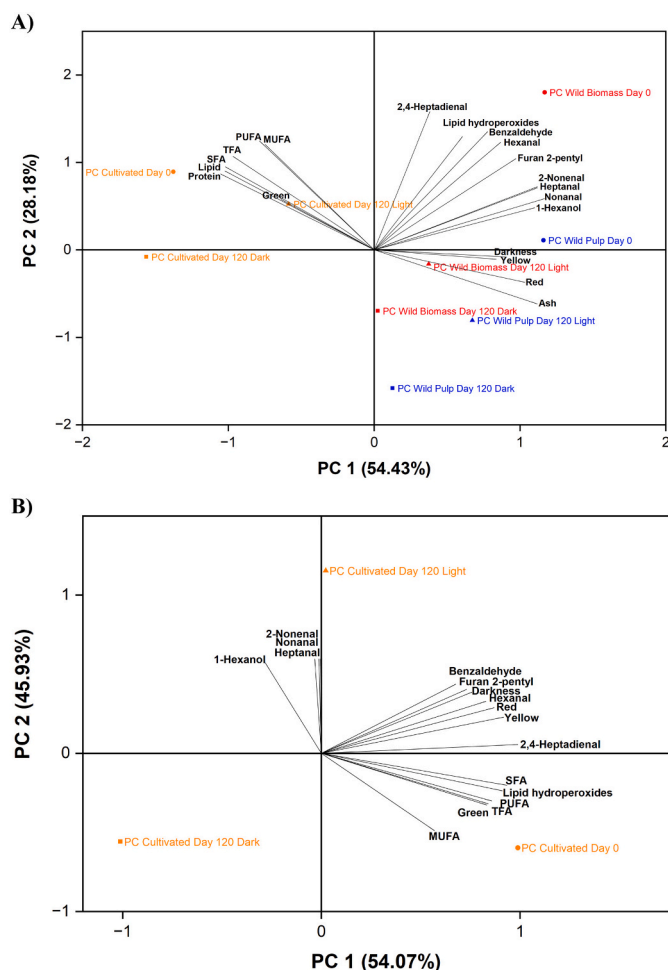


Fig. 5. PCA plot of *Ulva* spp. protein concentrates (PC) from cultivated and wild harvested *Ulva* spp. after processing (Day 0) and after storage for 120 days under light and dark conditions. The wild harvested *Ulva* spp. was extracted twice resulting in PC Wild Biomass after the first extraction and PC Wild Pulp after the second extraction. A) Combined PCA of PC Wild and PC Cultivated. B) PCA of PC Cultivated alone. Data was log₂-transformed for the PCA plot. TFA: Total fatty acids, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, and PUFA: polyunsaturated fatty acids.

(protein >80%) due to a higher content of lipids as well as ash (transition metals) and other pro-oxidants. However, the higher presence of non-proteins may also imply a higher level of endogenous antioxidants, raised nutritional value and sometimes improved protein functionality [62], which is why protein isolates not necessarily is the golden route. Among ways to reduce tentative problems with oxidation could be to vacuum-pack the samples as observed in previous studies [23,36]. This study stored the samples in petri-dishes covered by lids for practical reasons (e.g., to keep an even layer of sample) and to slightly accelerate the oxidation by ensuring access to air and light. Although endogenous antioxidants have been detected in *Ulva* spp., such as ascorbic acid [36], tocopherol [63] and chlorophyll (antioxidative under dark conditions) [64], the extraction of protein induces oxidative stress, which might result in losses of these antioxidants and thereby initiating oxidation. Thus, incorporation of additional antioxidants is often carried out in processed foods [29], which could be a solution for *Ulva* spp. protein concentrates as well.

5. Conclusion

In conclusion, protein concentrates from *Ulva* spp. were highly susceptible to oxidation, with processing conditions and light exposure

strongly influencing quality during storage. Particularly, pilot-scale processing resulted in high oxidation. Color disorientation and oxidation during processing in pilot plant suggested a need for less harsh processing or a need for antioxidant addition during processing. The pronounced oxidation of MUFAs under light and substantial PUFA losses under all conditions highlighted the need for optimized extraction and storage strategies to preserve nutritional and sensory quality. Overall, the results emphasize the importance of controlling oxidation through processing optimization, light protection, and the use of antioxidants or modified packaging to enable stable *Ulva* protein ingredients.

CRedit authorship contribution statement

Signe H. Nissen: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Louise Juul:** Writing – review & editing. **Nikolaj Juel:** Writing – review & editing, Investigation. **Karin Larsson:** Writing – review & editing, Investigation. **Caroline Nebel:** Writing – review & editing, Methodology. **Annette Bruhn:** Writing – review & editing. **Morten Ambye-Jensen:** Writing – review & editing. **Ingrid Undeland:** Writing – review & editing, Supervision. **Trine K. Dalsgaard:** Writing – review & editing, Supervision, Resources, Conceptualization.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Data availability

Data will be made available on request.

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