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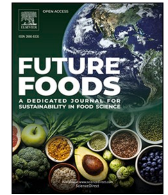

Mechanistic insights to the strong antioxidative capacity of lingonberry press cake during recovery of fish protein ingredients

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ABSTRACT

Lingonberry press cake (LPC) has been shown to limit lipid oxidation in fish filleting co-products during pH-shift processing. To explore the underlying mechanism, this study subjected LPC to pH-shift processing (native pH → pH 12 → pH 5), and analyzed the resultant fractions for phenolic content and antioxidant capacity. It was observed that LPC experienced a 23.73 % reduction in total phenolic content (TPC) when the initial homogenate was adjusted to pH 12; however, no significant further losses were noted during centrifugation or subsequent adjustment to pH 5. Both LPC and the soluble fraction at pH 5 ("S2") demonstrated effective inhibition of hemoglobin (Hb)-mediated lipid oxidation in washed cod mince (WCM) model system. Additionally, the insoluble fraction at pH 5 ("P2") exhibited the strongest binding to WCM. Proanthocyanidin A1 and cyanidin 3-O-galactoside were identified as the most effective antioxidants in LPC. Overall, this study affirms LPC's value as an effective natural antioxidant ingredient in muscle foods and proposes an innovative strategy for valorizing multiple food side streams together to support sustainable development.

1. Introduction

Lingonberry (*Vaccinium myrtillus*) is a small berry native to the boreal forests of the Northern Hemisphere. It is seldom consumed in its raw form due to its notably tart flavor and is predominantly utilized in the production of various forest fruit derivatives, such as jams, preserves, juices, and syrups. In Sweden, approximately 8000 tons of wild lingonberries are harvested each year (Zhang et al., 2024). When juicing lingonberries, a lingonberry press cake (LPC) is generated as a rest raw material, which is usually discarded and can easily cause environmental pollution. However, LPC exhibits significant potential for valorization due to its substantial polyphenol content. It is noteworthy that the freezing and pressing process employed during juice production does not deplete the polyphenol concentrations; instead, it may enhance further extraction of these compounds by opening up structures (Witczak et al., 2021). It has earlier been described how berry-derived antioxidant extracts can be combined with e.g. meat (Lorenzo et al., 2018) or fish (Sampels et al., 2010) to prevent lipid oxidation; something which call for valorization of LPC.

Another source of food rest raw materials, currently underutilized for food production, are fish filleting side streams. In herring filleting, rest raw materials constitute approximately 60 % of the total fish weight, including heads, backbones, viscera, and tails (Wu et al., 2022b). Currently, the most common uses of such biomasses are for low-value bulk products, such as fodder meal, fodder oil or silage (Sajib et al., 2022, 2021). However, as a viable option, the pH-shift method is an effective tool for extracting functional protein from fish co-products and converting them into food. This technology is based on protein solubilization in water induced by changing the pH to extreme acid or alkaline pHs, thereby allowing the separation of proteins from lipids and insoluble materials such as scales and bones, followed by precipitation of the proteins at their isoelectric point (pI). However, it has been seen that when applying this method on fish co-products rich in hemoglobin (Hb), Hb can become activated as a pro-oxidant, leading to lipid oxidation (Wu et al., 2021a).

Previous research has shown that lipid oxidation in Hb-rich fish co-products can be effectively mitigated by combining them with antioxidant-rich plant food residues during pH-shift processing, recently referred to as cross-processing (Zhang et al., 2023a, 2023b). This strategy mitigates lipid oxidation in a clean-label manner and facilitates the upcycling of residual materials from the plant-based food industry while reducing the costs associated with antioxidants. Furthermore, it

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establishes a foundational framework for exploring the impact of pH for the antioxidant capacity of polyphenols. When herring co-products were pH-shift processed with 30 % LPC (dw/dw), the storage period of the resulting protein isolates was significantly extended from 3 days without LPC to 21 days (Zhang et al., 2023a). It was later shown that even down to an LPC-level of 2.5 %, fish protein isolates were protected from oxidation during the pH-shift processing itself (Zhang et al., 2024). However, so far, it is not known which antioxidants that are co-extracted from LPC during the pH-shift processing, and whether soluble or insoluble antioxidants of LPC play the largest role in protecting the fish lipids from oxidation. This knowledge would aid both the refining and expansion of the cross-processing concept to combat lipid oxidation during the production of protein ingredients from fish or other sources rich in heme-proteins. Therefore, the aims of this study were to: (i) investigate how pH-shift processing affects the total phenolic content (TPC) and in vitro antioxidant capacity of LPC; (ii) explore how different LPC fractions derived during pH-shift processing affect Hb-mediated lipid oxidation in a fish matrix; and (iii) elucidate which antioxidant components of the LPC-fractions are the most associated with reductions in lipid oxidation.

2. Material and methods

2.1. Chemicals and reagents

Hydrochloric acid, sodium chloride, trichloroacetic acid (TCA), thiobarbituric acid (TBA), tris aminomethane (Tris), chloroform, iron (III) chloride hexahydrate, trifluoroacetic acid, gallic acid, sodium acetate, acetic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), iron(II) sulfate heptahydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium dihydrogen phosphate, disodium hydrogen phosphate and streptomycin sulfate were purchased from Sigma Chemical A/S (St. Louis, MO, USA). Sodium hydroxide, acetone and methanol were obtained from Honeywell Riedel-de Haën AG (Germany) while Folin-Ciocalteu reagent and sodium carbonate were from Merck & Co. lnc. All chemicals were analytical grade or higher purity.

2.2. Preparation of lingonberry press cake (LPC) fractions derived from pH-shift processing

LPC used in this study was provided by *Grangärde AB* in Sweden in November 2019 and stored at −80 °C until utilized. LPC consists of peels, leftover flesh, seeds, and stems. LPC fractions were prepared to investigate their antioxidant mechanisms during the pH-shift processing of herring co-products, as noted by Zhang et al. (2022). This process, referred to as “cross-processing,” initially involved adding LPC to herring co-products at a 30 % (dw/dw) level. To simulate the cross-processing methodology previously used (Zhang et al. (2022), 31.1 g of LPC was here combined with 786.3 g of cold water at 4 °C . Then the mixture was homogenized (L5M-A, Silverson, UK) at 8000 rpm for 90 s. The mixture (initial pH 3.05) was then adjusted to pH 12.0 by 2.0 M NaOH, and the homogenate (H1) was incubated for 15 min before centrifugation at 8500 × *g* for 20 min at 4 °C. The supernatant and pellet were collected as S1 and P1, respectively. The S1 was then adjusted to pH 5.0 by using 2.0 M HCl, and this acidified homogenate (H2) was centrifuged at 8500 × *g* for 20 min at 4 °C. The supernatant and pellet were collected as S2 and P2 respectively. The whole process was carried out on ice, and samples from all steps of the process were freeze-dried before being stored at −20 °C. Fig. 1 shows the LPC fractions under investigation in this study.

2.3. Characterization of LPC and LPC-fractions as antioxidants

2.3.1. Total phenolic content (TPC)

The method was performed as described by Zhang et al. (2022) with some modifications. A 0.1 g of freeze-dried sample was mixed with a 5 mL extraction solution (70 % methanol containing 1 % trifluoroacetic acid) and vortex for 15 s. After sonication and incubation in a water bath (60 °C, 100 rpm) for 30 min, the samples were centrifuged at 5000 × *g* for 5 min at 4 °C and the supernatant was collected. The pellets were

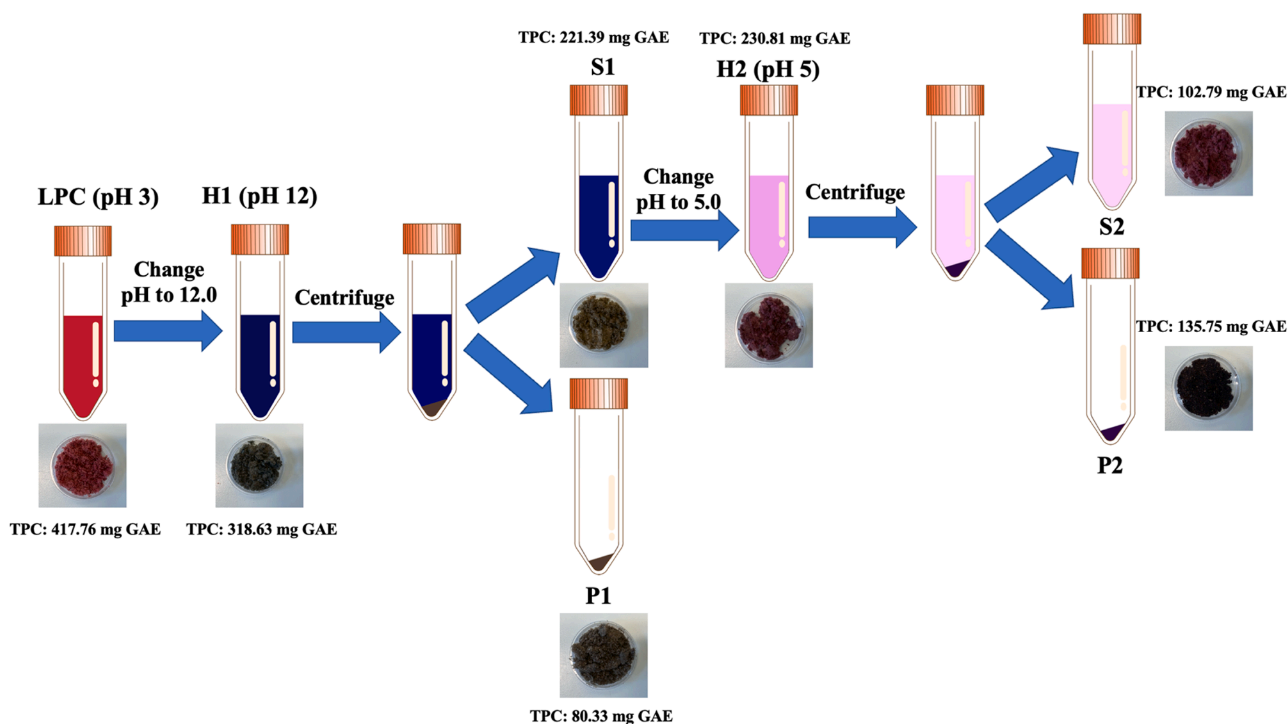

Fig. 1. Flow chart over the alkaline pH-shift processing of lingonberry press cake (LPC) and the abbreviations used for the different fractions. H1 represents the LPC-in-water homogenate at pH 12.0, S1 represents supernatant at pH 12.0, P1 represents pellet at pH 12.0, H2 represents S1 after adjusting its pH to pH 5.0, S2 represents the supernatant at pH 5.0 and P2 represents pellet at pH 5.0.

re-dissolved in 5 mL extraction solution and vortexed, sonicated and centrifuged as above. The supernatant was collected and combined with that obtained after the first centrifugation cycle. The extracts (500 μL) were mixed with 250 μL aqueous sodium carbonate (Na_2CO_3) solution at 13.5 % (w/v) and 250 μL of Folin-Ciocalteu aqueous reagent solution (1:5, v/v), followed by incubation for 10 min and centrifugation at 15, 000 × *g* for 5 min. The absorbance of the supernatant was measured at 765 nm by using a spectrophotometer (Cary 60 UV–vis, Agilent technologies, USA). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g DW).

2.3.2. Analysis of key phenolic compounds

The key antioxidative components of LPC and its fractions were identified as conducted by Bunea et al. (2013). Three mL of acidified methanol (0.3 % HCl v/v) were added to 0.2 g of freeze-dried LPC fractions, followed by sonication for 15 min. The sample was centrifuged at 2000 rpm for 10 min and the supernatant collected. The extraction was repeated and supernatants combined prior to their storage at −20 °C. Before analysis, the combined supernatants were centrifuged at 5500 rpm for 20 min and filtered through a 0.45 μm filter before analysis with LCMS.

Quantitation of phenolics was achieved by Liquid Chromatography Mass Spectrometry/Mass Spectrometry (LC–MS/MS) using a 6500+ QTRAP triple-quadrupole mass spectrometer (AB Sciex, 11432 Stockholm, Sweden) which was equipped with an ESI Turbo Spray Ion Drive source and operated in the positive-ion mode. Chromatographic separations were performed on Waters BEH Premier C18 column (5 μm, 2.5 cm × 4.6 mm). LC–MS grade water (100 % solvent A) and MeOH (100 % solvent B) both containing 0,1 % formic acid were the mobile phases for gradient elution. The column flow rate was 0,8 mL/min and the column temperature was 50 °C, the autosampler was kept at 12 °C. LC starting conditions at 2 % B, 2 min 10 % B, 2,5 min 25 % B, 4 min 45 % B 4,5 min 100 % B held for 30 s, 4,5–6 min 2 % B, total runtime 6 min. The MRM transitions were optimized for the analytes one by one by direct infusion containing 25 mM of each phenolic molecule. The Q1/Q3 pairs were used in the MRM scan mode to optimize the collision energies for each analyte, and the two most sensitive pairs per analyte were used for the subsequent analyses. The retention time window for the scheduled MRM was 1 min for each analyte. The two MRM transitions per analyte, the Q1/Q3 pair that showed the higher sensitivity was selected as the MRM transition for quantitation. The other transition acted as a qualifier for the purpose of verification of the identity of the compound. The standards (including catechin, epicatechin, procyanidin B1, procyanidin B2, procyanidin A1, procyanidin A2, procyanidin C1, cyanidin-3-galactoside) were dissolved in aqueous methanol (50 % v/v) to prepare a five-point external standard calibration curves for all analysis. The concentration of anthocyanins in the samples was determined using regression analysis ($r^2 > 0.99$).

2.3.3. Ferric-reducing antioxidative power (FRAP)

The FRAP method described by Malaver et al. (2015) was used. The FRAP reagent was mixed with acetate buffer (300 mM, pH 3.6), 10 mM TPTZ in 40 mM HCI, and 20 mM $FeCl_3$ in a proportion of 10:1:1 (v/v/v). The 50 μL sample was then mixed with 1.5 mL FRAP reagent in microtubes. The absorbance was recorded at 593 nm immediately after incubation at 37 °C for 15 min. A standard calibration curve in the range from 0 μM to 2 μM of ferrous sulfate ($FeSO_4$•$7H_2O$) was plotted. Results were expressed as Fe^{2+} equivalents (mmol)/mL of samples.

2.3.4. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH method was performed as described by Lee et al. (2006). The DPPH solution in methanol (6×10^{-5} M) was prepared fresh, and 3 mL of this solution was mixed with 100 μL of sample (LPC, S1, P1, S2 and P2). The samples were then incubated for 20 min, where after the decrease in absorbance at 515 nm was recorded (A_E). To prepare blanks (B), methanol was added to the freshly prepared DPPH solution instead of the sample and its absorbance was recorded as A_B. Radical scavenging activity was calculated using the following formula:

$$\% \text{ Inhibition} = [(A_B - A_E)/A_B] \times 100$$

2.4. Antioxidant assessment of LPC and its fractions in a fish-based oxidation system

2.4.1. Preparation of trout hemolysate

Rainbow trout *(Oncorhynchus mykiss)* was obtained from *Antens laxodling AB*, Alingsås, Sweden. The bleeding were performed according to Ghirmai et al. (2020). The bleeding of trout was approved by the regional animal ethics committee in Gothenburg, permit number 167-2013. Trout hemolysates were then prepared as described by Wu et al. (2021c). Four volumes of ice-cold saline (1.7 % NaCl in 1 mM Tris, pH 8) were added to the heparinized trout blood. The sample was centrifuged at 700 × *g* at 4 °C for 10 min and the supernatant was removed. Then, 10 vol of saline were added to the pellet and the sample was mixed manually into a homogenous solution. The sample was then centrifuged at 700 × *g* at 4 °C for 10 min where after the supernatant was removed. The last step was repeated 2 times. Cells of the pellet were lysed in 3 volumes of 1 mM Tris (pH 8) for 1 h on ice. One-tenth volume of 1 M NaCl was finally added to the cells before centrifugation at 28,000 × *g* for 15 min at 4 °C. The supernatant, i.e., hemolysate, was collected and stored at −80 °C.

2.4.2. Preparation washed cod muscle (WCM)

Fresh cod *(Gadus morhua)* was caught in Skagerrak on 13th September 2021. WCM was prepared from cod fillets as described by Wu et al. (2022d). Briefly, minced fish muscle was washed in 3 volumes of cold distilled water and stirred manually for 2 min before settling on ice for 15 min. The slurry was then filtered through a sieve to dewater. Then, the mince was washed twice in 3 volumes of cold 50 mM phosphate buffer at pH 6.6 according to above. In the last wash, the mince was homogenized with a polytron (Ultra Turrax, IKA Werks, Intermed Labassco) and the slurry was finally centrifuged at 15,000 × *g* at 4 °C for 25 min. The pellet was collected as WCM, which was put as a thin layer into a zip-lock plastic bag and frozen at −80 °C. The entire washing process was performed on ice.

2.4.3. Separation of LPC and S2 samples

Freeze-dried LPC and S2 samples underwent separation treatment by using the method described by Lee et al. (2006) with a few modifications. Ten mL of methanol were added to 0.5 g of samples; the mixture was sonicated for 10 min and then centrifuged at 3000 × *g* for 10 min at 4 °C. The supernatant was loaded on a Sephadex LH-20 (15 g) column (ID = 1.5 cm, length = 20 cm) from which seven fractions were obtained by the use of the following solvents at a flow rate of 2.5 mL/min 100 %: Milli-Q water; 50 % aqueous ethanol (v/v); 100 % ethanol; ethanol: methanol (1:1, v:v); 100 % methanol; 80 % aqueous acetone (v:v). These fractions were evaporated under nitrogen and then redissolved in 50 % aqueous ethanol (v/v) and stored at −80 °C.

2.4.4. Preparation of oxidation system

The oxidation system was prepared as described by Lei et al. (2022). The thawed WCM was adjusted to pH 7 in a beaker by using 1.0 mol/L NaOH solution, and if needed 1.0 mol/L HCl. The original moisture content of the WCM was 86.7 ± 2.2 %, and Milli-Q water was added to reach 90 %. Streptomycin sulfate stock solution (2 % w/v) was added to give a final concentration of 200 ppm. LPC and LPC-fractions derived in the pH-shift process (S1, P1, S2 and P2) were then stirred in manually to the WCM at an equal DW-basis or TPC-basis. Trout hemolysate was thereafter added to a final concentration 25 μM Hb/kg of WCM and the sample (25 g in total) was transferred to the bottom of a 250 mL E-flask wrapped in aluminum foil. All steps were performed on ice and the final samples were stored on an ice bed in cooler bags. Sub-samples were

taken from E-flasks daily. The whole ice storage trial was conducted twice.

2.4.5. Partitioning of LPC fractions into cod muscle membranes

The method was performed as described by Lee et al. (2006) with a few modifications. 0.01 g of dried powdered LPC fraction (S1, P1, S2 or P2) was premixed with 1 mL Milli-Q water in a 100 mL beaker. Thereafter 18 g WCM was added into the beaker and mixed with a glass rod for 30 s. To monitor how much of the LPC fractions that partitioned into the WCM membranes, the LPC fraction-fortified WCM was washed with Milli-Q water (1:3, w:w) followed by centrifugation at 8500 g. After this, the WCM was washed twice with sodium phosphate buffer (pH 6.6) (1:3, w:w) with centrifugation in between. After centrifugation (25 min, 15, 000 × g, 4 °C) and removal of the supernatant from the last washing step, trout hemolysate (0.345 μM Hb) was added to the resulting WCM pellet. A control sample of WCM was also prepared using the same three washing steps, but without pre-mixing with the LPC fractions. All samples obtained were subjected to ice storage as described in Section 2.4.4.

2.4.6. Redness loss measurement

Redness (a^*) loss during storage of WCM-samples was measured according to Lei et al. (2022) using a Minolta CR-400 Chroma Meter (Minolta Camera Co., Osaka, Japan). A white calibration plate was used to calibrate the instrument and the colorimeter probe was pressed against the top of the petri dish in which samples were stored in a layer having the same thickness as in the E-flasks (Section 2.4.4).

2.4.7. Analysis of lipid oxidation products

Lipid oxidation products (hydroperoxides and carbonyls) were analyzed as described by Wu et al. (2021). One gram of muscle sample was mixed with 10 mL chloroform: methanol (2:1) and homogenized for 30 s by a T 25 high-speed disperser (IKA, Staufen, Germany). The upper phase (water–methanol) was used to analyze thiobarbituric acid reactive substances (TBARS) while the bottom phase (chloroform) was used to analyze peroxide value (PV). Results were expressed in μM peroxide or μM TBARS/kg muscle.

2.5. Statistical analysis

All trials were conducted at least twice ($n \geq 2$). One-way analysis of variance (ANOVA) was conducted using SPSS Statistics version 19.0 (IBM, New York, NY, USA) to identify significant differences. Differences with a significance level (p) below 0.05 were considered significant.

3. Results

3.1. TPC of LPC-fractions obtained throughout the pH-shift processing

TPC levels (mg GAE/g DW) of each LPC fraction obtained in the pH-shift process are shown in Fig. 1. Prior to pH-shift processing, the absolute TPC of the crude LPC was measured to be 417.8 mg GAE, on DW basis, at its native pH of 3.05. This initial TPC was considered as 100 % to which other fractions were related. As the crude LPC was homogenized in water, and its pH was increased to 12 (H1), the absolute TPC dropped from 417.8 mg GAE to 318.6 mg GAE. Thus, a TPC reduction of 23.7 % occurred during homogenization in water and the first pH adjustment. After subsequent centrifugation, the absolute TPC of S1 and P1 were 221.4 mg GAE and 80.3 mg GAE, respectively. After S1 was adjusted to pH 5 to obtain H2, the absolute TPC went from 221.4 to 230.8 mg GAE, thus increased slightly by 4.25 %. The final centrifugation step which delivered S2 and P2, provided102.8 and 135.8 mg GAE, respectively, in these phases.

3.2. Antioxidative capacity of LPC in vitro and in the WCM oxidation system

As shown in Table 1, FRAP levels of the different LPC-fractions derived in pH-shift processing decreased as follows: P2 > crude LPC > S2 > S1 > P1 while DPPH percentages decreased in the following order: S2 > S1 > P1 > crude LPC > P2. As the two methods showed different results, a real muscle matrix was used to verify the antioxidative capacities of the crude LPC fractions. Fig. 2 illustrates the PV, TBARS values, and redness loss when the LPC-fractions were added to Hb-fortified WCM at the same DW basis. In presence of 25 μmol Hb/kg WCM, the PV increased rapidly in control samples, reaching a maximum of 671.34 μM lipid hydroperoxide (LHP)/kg WCM at day 2 (Fig. 2A). As shown in Fig. 2B, the most effective antioxidants were the crude LPC and S2, which were able to suppress lipid oxidation by up to 4 days. TBARS results followed a similar trend, and the extension of the oxidation lag phases were as follows: crude LPC > S2 > S1 > P2 > P1 > C, ($p < 0.05$). The redness loss results (Fig. 2C) confirmed this trend, with the crude LPC and S2 being the most effective antioxidants. In addition to adding the LPC-pH-shift fractions on the same DW-basis, these fractions were also added to WCM on basis of the same TPC. These results are shown in Fig. 3. Also here, the crude LPC and S2 were the most capable of delaying redness loss and preventing PV and TBARS formation, followed by S1, P1 and then P2. Thus, the order between P1 and P2 was different from when LPC-fractions were added on a weight basis.

Table 1
Ferric-reducing antioxidative power and 1,1-Diphenyl-2-picrylhydrazyl of LPC and its different pH-shift fractions. Data show mean values ± SD ($n = 3$).

Fractions	FRAP (Fe^{2+} mmol/g DW)	DPPH (%)
LPC	3346.96 ± 256.74^b	38.27 ± 3.32^d
S1	2514.29 ± 248.85^c	88.86 ± 5.48^a
P1	2074.29 ± 183.48^c	58.90 ± 0.68^b
S2	3242.32 ± 245.97^b	92.31 ± 0.09^a
P2	4539.46 ± 364.87^a	16.76 ± 3.05^e

3.3. Effect of LPC-fraction carrier solvent for lipid oxidation in WCM

Although P2 showed the highest TPC and FRAP levels (Tables 1 and S2), its antioxidative capacity in Hb-spiked WCM was the lowest (Figs. 2 and 3), which may be related to the carrier solvents used in the different trials. In order to compare the effect of the different carrier solvents used in the in vitro antioxidant tests vs. in WCM (50 % ethanol in TPC/FRAP, and Milli-Q water in the WCM oxidation system), P2 was dissolved in each of the two solvents prior to adding it to WCM for assessing lipid oxidation products and redness loss. Fig. 4 shows that the PV of WCM when fortified with P2 dissolved in Milli-Q remained low up to 2 days, with a slight increase at day 3, and then a rapid increase at day 4, reaching a peak of 584.55 μM LHP/kg. However, P2 dissolved in 50 % ethanol provided a low PV during the first 5 days of storage, after which it increased rapidly. TBARS showed a similar trend, with a PV-lag phase of 3 days with Milli-Q and 5 days with 50 % ethanol. Redness loss of WCM with P2 dissolved in Milli-Q dropped from 15.16 to 4.01 at day 4, whereas the redness loss with 50 % ethanol dropped from 16.38 to 4.08 at day 7. These results showed that P2 had a higher solubility in 50 % ethanol and thus could provide a stronger antioxidative effect.

3.4. Binding ability of the different LPC-fractions to WCM

Fig. 5 shows PV (A), TBARS (B) and redness loss (C) after one wash of the fortified in a WCM-system with 0.01 g DW of each LPC-fraction. The PV of the control (i.e., without LPC or LPC fraction) and P1 quickly reached the maximum on day 2, whereas WCM with crude LPC, S2, S1 and P2 reached the maximum PV at day 4. TBARS results followed the same trend. With respect to redness loss, the control sample declined rapidly on day 1, followed by WCM fortified with P1. Although the P2 sample initially had a lower redness than the control due to black particles in P2 affecting the redness measurement, the oxidation peak was consistent with that of LPC, S1, and S2 from the second day of storage.

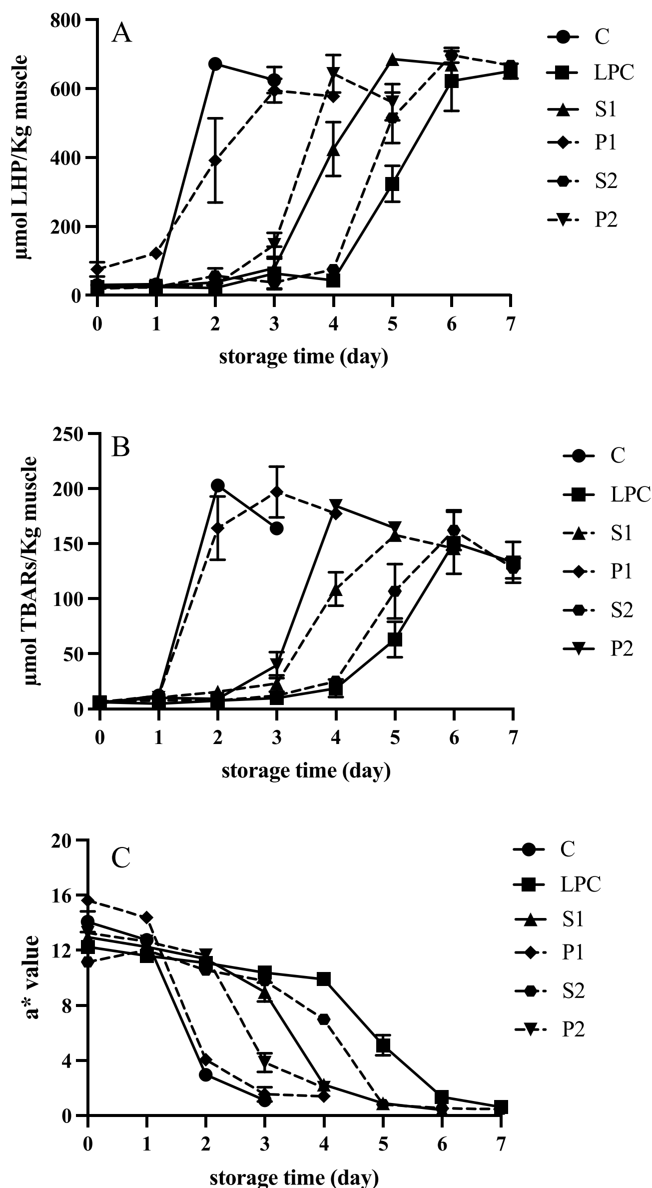

Fig. 2. PV (A), TBARS (B) and Redness loss (C) during ice storage when the same dry weight (DW) (=0.01 g) of LPC and its fractions were added to WCM (pH 7) with 25 µM Hb. C represents WCM with 25 µM Hb/kg, added as hemolysate (oxidation system), LPC represents oxidation system with crude lingonberry press cake, S1 represents oxidation system with the LPC supernatant obtained at pH 12.0, P1 represents oxidation system with the LPC-pellet obtained at pH 12.0, S2 represents oxidation system with the LPC-supernatant obtained at pH 5.0 and P2 represents oxidation system with the LPC-pellet obtained at pH 5.0. Data are shown as mean values ± SD (n = 2).

When comparing WCM samples which had been fortified with the same DW of LPC pH-shift fractions prior to ice storage without (Fig. 2) or with (Fig. 5) a pre-wash, the latter showed faster increase in PV and TBARS as well as faster redness loss than the unwashed sample. The washed control sample reached maximum PV at day 1 (508.26 µM LHP/kg) and the unwashed one at day 2 (671.39 µM LHP/kg). For WCM with S2 and crude LPC, lag phases were reduced from 4 to 2 days after washing, and the PV as well as TBARS peak values decreased by 41.16 % and 17.09 %, respectively. Further, WCM with S1 reached the oxidation peak on day 4 after washing, instead of at day 5 for the unwashed sample. The maximum PV's were also reduced by 75.3 % after washing. For the WCM sample with P1, the oxidation peak was not shifted after washing, however, the maximum PV was significantly higher than that

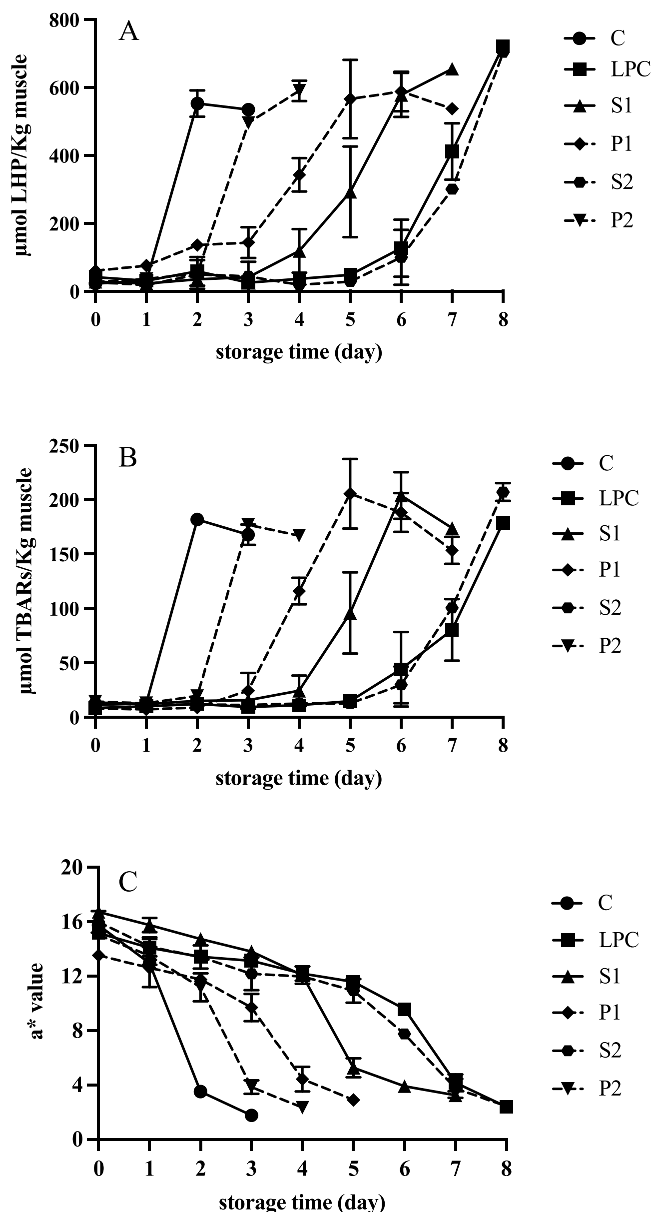

Fig. 3. PV (A), TBARS (B) and Redness loss (C) during ice storage when the same total phenolic content (TPC) (=1.78 mg GAE) of the LPC and its fractions were added to WCM (pH 7) with 25 µM Hb. Data are shown as mean values ± SD, replicates per treatment were n = 2. For abbreviations, see Fig 2.

for the unwashed corresponding sample ($p < 0.05$). For WCM with P2, the lag phase was reached after 2 days instead of 4 days after washing. TBARS and redness loss data showed the same trends as those of PV.

3.5. Antioxidative components in LPC and S2 fractions

Compared to the other fractions, crude LPC and S2 showed the strongest antioxidative ability; thus, the components preventing lipid oxidation in these samples were further investigated (Fig. 6 and Table S1). Fig. 6A and B show TBARS after adding crude LPC and S2, respectively, into WCM, when dissolved in different solvents. LPC and the S2 fraction in 50 % aqueous ethanol were the most effective inhibitors of TBARS formation providing 4 and 3 days lag phase, respectively. All other solvents, including the control gave similar lag-phases, 1 day.

Table S1 shows that proanthocyanidin A1 and cyanidin 3-O-galactoside (Cy3gal) were enriched in the 50 % aqueous ethanol extracts of LPC

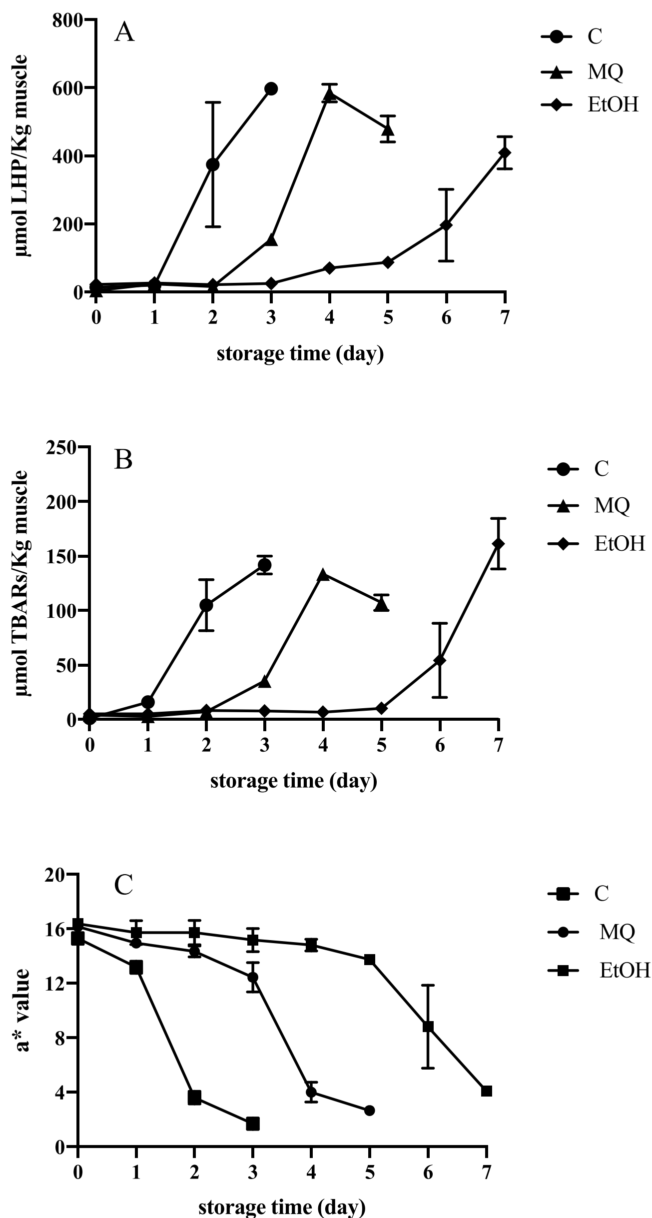

Fig. 4. PV (A), TBARS (B) and Redness loss (C) during ice storage when WCM was fortified with P2 dissolved in Milli-Q or in 50 % ethanol (providing 2 % P2-in-ethanol extract w/w in the oxidation system). Data are shown as mean values ± SD. Replicates per treatment were n = 2. C, MQ and EtOH represent the control sample and samples fortified with P2 in Milli-Q water and ethanol, respectively.

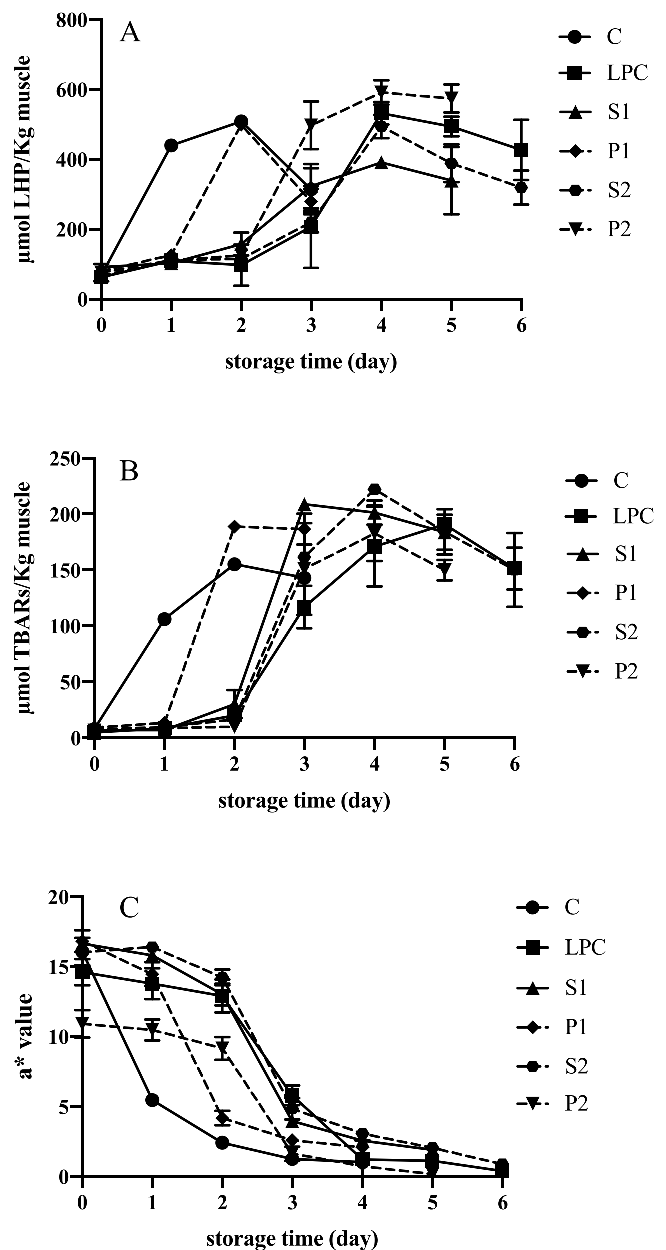

Fig. 5. PV (A), TBARS (B) and Redness loss (C) during ice storage of WCM (pH 7, 25 μM Hb, 90 % moisture). The WCM was washed one time after it was fortified with the same dry weight (=0.01 g) of LPC or its fractions. The final LPC concentration was 0.26 mg /g wet WCM. Data are shown as mean values ± SD. Replicates per treatment were n = 2.

and S2, with proanthocyanidin A1 showing highest concentrations. Cy3gal levels in the Milli-Q and 50 % ethanol extracts of LPC were 3.19 μg/mL and 2.39 μg/mL, respectively, with differences being significant ($p < 0.05$). In contrast, the Cy3gal concentration in the 50 % aqueous ethanol extract of S2 (1.53 μg/mL) was slightly higher than in the S2 Milli-Q extract (1.20 μg/mL).

To investigate whether proanthocyanidin A1 and Cy3gal are the key antioxidants of LPC and S2, the same mole of these molecules as was found in the 50 % aqueous ethanol extracts of LPC and S2, were added to the WCM in pure form; TBARS results are shown in Fig. 6C. When added individually, proanthocyanidin or Cy3gal did not delay oxidation relative to the control with only Hb, whereas when both compounds were added together, the oxidation lag phase was extended by 1 day, irrespective of the level added.

4. Discussion

4.1. Stability of phenolic compounds during pH-shift processing of LPC

The stability of phenolic compounds in LPC can be highly influenced by the surrounding pH, as evidenced by the documented changes in TPC throughout the pH-shift process (Fig. 1). The transition from pH 3.05 to 12 resulted in a substantial decrease in TPC (23.73 %), which may be attributed to the irreversible destruction of phenolic structures in alkaline environments (Xu et al., 2015). At pH 3.05, the phenolic compounds of LPC are protonated, enhancing their stability. However, as pH increases to 12, deprotonation takes place, rendering the hydroxyl groups on the phenolic compounds more susceptible to nucleophilic attack by hydroxide ions, causing degradation (Wu et al., 2022a). The main

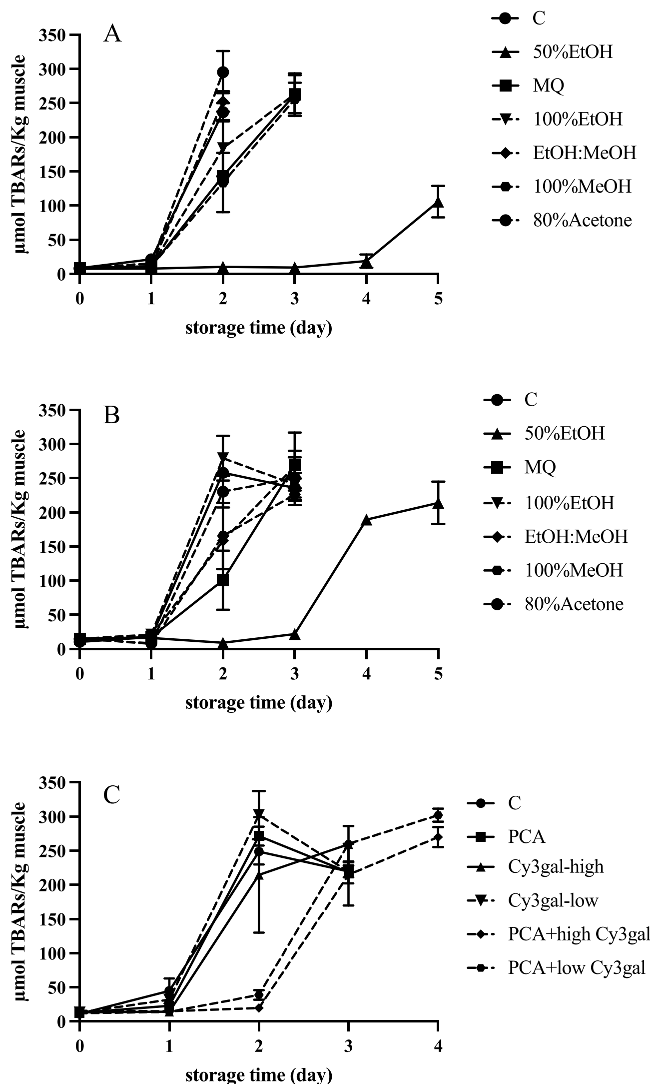

Fig. 6. TBARS of WCM fortified with LPC (A), the separated S2 fraction (B) and anthocyanin standards added at the same level as was found in LPC and S2 (C). The pH of WCM was 7, the moisture content 90 % and it contained 25 µM Hb. Replicates per treatment were $n = 2$. Data points show mean values ± SD. C, EtOH, MeOH, PCA and Cy3gal are the short of control, ethanol, methanol proanthocyanidin and cyanidin 3-O-galactoside.

phenolic compounds in LPC are proanthocyanidins, which accounts for 71 % of the TPC (Kylli et al., 2011). Dimeric proanthocyanidins linked by A- or B-type interflavanyl bonds are unstable under extremely alkaline conditions, and the oxidation rate of proanthocyanidins is directly correlated to an increase in pH (Xu et al., 2015). Besides proanthocyanidins, anthocyanins are the second largest contributor to the TPC in lingonberry, which are rapidly hydrolyzed into ionized chalcone at a pH of 12 (Friedman and Jürgens, 2000). Therefore, when the pH is adjusted to acidic values, the lost phenols cannot be recovered.

4.2. *Effects of TPC, binding capacity, and solvent on the ability of LPC and LPC fractions to delay to Hb-mediated lipid oxidation of WCM*

To investigate the antioxidative capacity of crude LPC and different LPC fractions obtained during the pH-shift process, an oxidation model system comprising WCM and trout Hb as the pro-oxidant was employed. Previous research e.g. on trout and mackerel fillets has revealed that blood is the main driver of lipid oxidation, and that Hb contributes to over 90 % of its pro-oxidative capacity (Cai et al., 2013; Richards and Hultin, 2002; Richards and Li, 2004). The contribution of TPC to the antioxidant capacity of LPC and the different LPC pH-shift fractions was investigated by adding the latter at the same TPC level to Hb-spiked WCM. This revealed that there was no positive correlation between TPC the antioxidant capacity of LPC/LPC-fractions in the used fish model system (Fig. 3). The antioxidant activity of phenolic compounds is strongly dependent on their chemical structure, such as the number of benzene rings and the presence of substituents as hydroxyl- and methyl groups, as these structural features determine their reducing ability and their stability as radicals.

Interestingly, the antioxidant abilities of LPC and its fractions in the WCM model system also contradicted those ascertained via the DPPH assay (Table 1). The ranking order of antioxidative capacities as determined by this assay was S2 > S1 > P1 > LPC > P2 while that measured in the WCM system on a same DW-basis was LPC > S2 > S1 > P2 > P1 (Fig. 2). This observation was consistent with the study of Lee et al. (2006), which revealed that the ability of different cranberry fractions to scavenge DPPH free radicals did not correspond to their relative abilities to inhibit lipid oxidation in WCM (Lee et al., 2006). These findings suggest that phenolics might employ other mechanisms besides free radical scavenging to inhibit lipid oxidation and/or that DPPH radicals are not representative for radicals being present in an oxidizing fish muscle system. For example, the ferrylhemoglobin (ferrylHb)-scavenging activity was proposed as an additional mechanism of alleviating the pro-oxidative effect of Hb by flavonoids. In this sense, flavonoids retard hydrogen-peroxide-mediated oxidation of oxyHb to metHb, in which ferrylHb is formed as an intermediate (Gebicka and Banasiak, 2009). Furthermore, it has been found that the binding of phenolics to Hb reduced the dissociation of heme from metHb (Wu et al., 2022a, 2022c). Earlier findings have revealed that this is the most critical step in Hb-mediated lipid oxidation (Richards et al., 2009). As further discussed below, the ability of polyphenols to precipitate proteins (Jongberg et al., 2014; Lund, 2021) also imply that Hb-precipitation could be induced as yet another way of mitigating Hb-mediated oxidation.

A washing experiment was also performed to investigate the binding of LPC and its fractions to WCM, with a specific focus on the membranes which are considered the primary substrate for Hb-mediated lipid oxidation (Richards et al., 2009). As shown in Fig. 5, P2 presented the highest binding capacity among the tested fractions as the oxidation lag phase was not affected by the washing. The phenolic hydroxyl groups in polyphenols can inhibit lipid oxidation by reducing the formation of metHb, thereby lowering the oxidative activity of Hb (Wu et al., 2022a). In addition, proanthocyanidins can also be used as reducing and chelating agents to maintain Hb in a ferrous state, and proanthocyanidins as reducing agents have a higher inhibitory effect on Hb mediated lipid oxidation than proanthocyanidins as chelating agents (Maestre et al., 2009).

Moreover, polyphenols can not only inhibit Hb mediated lipid oxidation, but also inhibit lipid oxidation by binding to phospholipid membranes and myofibrillar proteins. Most of the derivatives of polyphenolics are theoretically predicted to insert relatively deep in the membrane i.e., become embedded in between lipid chains, therefore being prone to scavenge radicals from both the initiation and propagation stages of lipid oxidation (Ossman et al., 2016). Furthermore, alterations in membrane fluidity as a result of polyphenol-partitioning into cell membranes may constitute yet another inhibitory mechanism of muscle systems, which could determine the rate of heme-mediated lipid oxidation (Kathirvel and Richards, 2009). Moreover, phenolic compounds could scavenge free radicals and form a chelate with transition metal catalysts to limit lipid oxidation (Zamora and Hidalgo, 2016). The present study did not rule out that polyphenols also bound to the myofibrillar proteins as the reactivity of polyphenols to proteins is known to be high with both covalent and non-covalent bonds being formed (Jongberg et al., 2014; Lund, 2021). Such binding may hamper protein oxidation, but at the same time yields protein-phenol complexes

that promote the loss of amino acid side chains in protein cross-linking reactions (Guo et al., 2021). Likely, binding to myofibrillar proteins moves the polyphenols away from the active site of oxidative attack, and thus their antioxidative capacity would be reduced. This could explain why the LPC fraction binding the hardest was not the most antioxidative.

The choice of solvent to dissolve LPC or the P2 fraction also influenced the anti-oxidative capacity (Figs. 2 and 4). This could contribute to the discrepancy between results from the in vitro assays and the WCM-Hb system in terms of antioxidant capacity order. While ethanol was employed in the former, Milli-Q water was used in the latter. As shown in Fig. 4, when P2 was dissolved in 50 % aqueous ethanol, its antioxidant capacity exhibited a considerable enhancement compared to when dissolved in Milli-Q water. This can be ascribed to the higher polarity of water compared to aqueous ethanol, thus, better solubilizing non-polar/hydrophobic groups and thereby creasing the antioxidation capacity (Khoo et al., 2017; Qi et al., 2023). A previous study found that using aqueous ethanol led to higher extraction of TPC and monomeric anthocyanins from 'Violet Nori' rice compared to using water alone; maximum TPC was achieved at a 50 % ethanol level (Catena et al., 2020). In our study, P2 was likely generated through cross-linking between anthocyanins and biomolecules such as proteins or carbohydrates of the LPC (Li et al., 2014). However, ethanol has been observed to interfere with such bindings by disrupting intramolecular hydrogen bonds among the side chains of the proteins involved. Introduction of ethanol may thus dissociate anthocyanin-protein complexes and render the solubilized anthocyanins accessible for interaction with other molecules, something which ultimately could enhance the antioxidant capacity of P2 (Shao et al., 2012).

4.3. Antioxidative components in LPC and its fractions

It was observed that both LPC and S2 contained the highest levels of proanthocyanidin A1 and Cy3gal, particularly when eluted with 50 % ethanol solution from the sephadex column (Table S1). Proanthocyanidin A1 and Cy3gal were therefore further studied with respect to their changes during the pH-cycle used in the pH-shift process for a better understanding of their roles in lipid oxidation (Fig. 6C). The exposure of proanthocyanidins and Cy3gal to an extremely alkaline environment (pH 12) rendered them unstable and susceptible to degradation, which aligns with earlier observations (Xu et al., 2015). Stanley et al. (2015) observed that alkaline processing of cocoa powder resulted in a reduction of 23–66 % in the levels of epicatechin and proanthocyanidins. Additionally, this treatment significantly altered the color, shifting from red to blue, and led to a 20 % decrease in TPC. In contrast, Lu et al. (2011) reported that the stability of these compounds was relatively unaffected when the pH was adjusted from 12 to 5. Both Cy3Gal and Proanthocyanidin A1 isolated from cranberries, peanut skins and persimmons, have proven strong antioxidant capacities. Yan et al. (2002) reported that Cy3Gal exhibited higher free radical scavenging capacity and overall antioxidant activity compared to other anthocyanins extracted from cranberry fruit. This finding was corroborated by Jordheim et al. (2007). Dong et al. (2013) compared the free radical scavenging capacity of B-dimers and A-dimers of proanthocyanidin isolated from peanut skins and persimmons, with the corresponding subunits in both aqueous and lipid systems. The results demonstrated that A-dimers exhibited comparable or even higher antioxidant capacity than B-dimers in the lipid-containing systems, supporting the effective antioxidative capacity of Procyanidin A as observed in WCM of our study. Consequently, both Cy3Gal and Procyanidin A were considered the strongest antioxidants in LPC, with procyanidin A1 showing particularly effective antioxidant properties in muscle.

5. Conclusions

This study aimed to understand the mechanisms underlying the strong antioxidative properties of lingonberry press cake (LPC) observed during protein extraction from fish co-products using the pH-shift method. When subjected to pH-shift processing (native pH → pH 12 → pH 5), LPC demonstrated a reduction in total polyphenol content (TPC) under alkaline conditions (pH 12) and maintained stable TPC levels during subsequent adjustments to pH 5. Notably, LPC and its fraction obtained from the precipitation step at pH 5 (i.e., the second supernatant, "S2") effectively mitigated hemoglobin (Hb)-mediated lipid oxidation in a washed cod muscle (WCM) model system. This finding contrasted with the results from in vitro antioxidant assays (FRAP, DPPH), highlighting the limitations of these assays and suggesting other mechanisms beyond radical scavenging or low molecular weight iron (LMW-Fe) reduction. Proanthocyanidin A1 and Cy3Gal were identified as the strongest antioxidants of LPC. Overall, this study paves the way for further investigations of berry-derived antioxidants and their potential applications in muscle foods. Moreover, it proposes a strategy for valorizing antioxidant-rich co-products from juice production which will reduce environmental impacts of this value chain, at the same time as economic benefits are increased.

Ethical statement

The research presented does not involve any animal or human study.

CRediT authorship contribution statement

Xueqing Lei: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Haizhou Wu:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Ling Liu:** Writing – review & editing, Supervision, Project administration. **Jingnan Zhang:** Writing – review & editing, Resources, Methodology. **Ingrid Undeland:** Writing – review & editing, Supervision, Project administration.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2024.100484.

Data availability

Data will be made available on request.

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