Effect of supercritical CO_2 plant extract and berry press cakes on stability and consumer acceptance of frozen Baltic herring (*Clupea harengus membras*) mince

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Abstract

A promising way of processing Baltic herring, *Clupea harengus membras*, is turning the fish into boneless mince. However, Baltic herring is prone to lipid oxidation, which possess a challenge for industrial applications. The aim of this work was to study the efficacy of press cakes from Finnish berries and a supercritical CO_2 plant extract to limit lipid oxidation during frozen storage of Baltic herring mince and to determine the impact of these additions on consumer acceptance in a fish product. Peroxide value, formation of volatile oxidation products and loss of polyunsaturated fatty acids showed that the tested natural additives decreased oxidation to a greater or similar extent as conventional antioxidants during 10-month storage. While potential of berry press cakes and plant extracts as "green label antioxidants" was shown, consumer study indicated need for further research to reach both optimal antioxidative efficacy and sensory properties.

Keywords

Baltic herring, fish, lipid oxidation, frozen storage, berry press cake, CO_2 extract

1. Introduction

Oxidation is the major cause of quality deterioration for fatty fish, such as Baltic herring (*Clupea harengus membras*) (BH). BH is a subspecies of Atlantic herring found in the Baltic Sea region. Compared to its Atlantic counterpart, it is smaller in size, usually between 15-20 cm and leaner, but still containing 5-10% of lipids depending on the season (Aro, Tahvonen, Mattila, Nurmi, Sivonen & Kallio, 2000). BH is rich in polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Aro et al. 2000). EPA and DHA are vital for growth and development and important for cardiovascular health. In addition, both fatty acids play a role in balancing immune functions and reducing inflammation. DHA is important for cognitive functions and mental health, and for the eyesight (Hashimoto, Hossain, Al Mamun, Matsuzaki & Arai 2017). In Finland, BH is commercially the most important fish with its share of the total catch varying between 70-90%. In the year 2019, for example, 113 million kilograms of Baltic herring were caught in Finland (Natural Resources Institute Finland, 2020).

Despite its commercial importance and beneficial nutritional aspects, only a small portion of BH caught in Finland is used for food purposes, while the majority is used as feed for fur animals (Natural Resources Institute Finland, 2019). One major factor limiting the food use and processing of BH is its small size and the abundance of small bones. Processing it into fillets is hence difficult, yielding a high percentage of by-products. For BH and other similar small pelagic fishes, a more favorable way of processing is turning it into a consistent and boneless mince using industrial machinery developed for separating the heads, skin, and bones from the fish muscle.

BH is rich in dark muscle and thus abundant in heme pigments, which are considered to be significant endogenous catalysts of lipid oxidation. The Fe^{2+} in heme can autoxidize to Fe^{3+}, releasing superoxide anion radicals, which can be further converted to hydrogen peroxide; the latter in turn promotes the oxidation of lipids (Maqsood, Benjakul, & Kamal-Eldin, 2012). High PUFA and heme contents of BH make it susceptible to oxidation, especially when cellular structures are broken down and the lipids are exposed to hemoglobin and other pro-oxidants present during mincing. Mincing also increases the surface area of fish muscle in contact with oxygen in the air, further promoting oxidation. Lipid oxidation decreases nutritional quality and causes formation of undesirable flavors with fishy and rancid odor, as well as compounds with possible adverse health effects (Rundblad, Holven, Ottestad, Myhrstad & Ulven, 2017). Oxidation poses a challenge for industrial applications, especially because the catch season of BH extends only from autumn to spring, and for the product to be available all-year-round the fish mince has to be stored frozen for several months.

Consumer awareness and demand for "green label" products are continuously increasing. Previously, several studies have shown the efficacy of plant phytochemicals as antioxidants (Määttä-Riihinen, Kähkönen, Törrönen & Heinonen, 2005; Puganen, Kallio, Schaich, Suomela & Yang, 2018) and inhibitors of lipid oxidation in food systems (Püssa, Pällin, Raudsepp, Soidla & Rei, 2008; Tarvainen, Nuora, Quirin, Kallio & Yang, 2015; Tarvainen, Quirin, Kallio & Yang, 2016). For instance, in a study previously carried out by our group (Tarvainen et al., 2015), addition of plant extracts decreased the formation of cholesterol oxidation products in fish patties during cooking and subsequent cold storage. In our previous study using *in vitro* assays, lingonberry (*Vaccinium vitis-idaea* L.), bilberry (*Vaccinium myrtillus* L.), and sea buckthorn (*Hippophaë rhamnoides* L.) have shown strong antioxidative activities (Tian, Puganen, Alakomi, Uusitupa, Saarela & Yang, 2018).

Despite their potential effect in inhibiting oxidation, addition of natural antioxidants may be challenging in regard to sensory acceptance, particularly if they are added at high concentrations. The hypothesis of the study was that natural antioxidants ("green label antioxidants") like berry press residues from lingonberry (L), bilberry (B) and sea buckthorn (SB) can reduce lipid oxidation in Baltic herring fish mince during frozen storage to a similar extent as conventional antioxidants. Therefore, the main objective of this study was to investigate the capability of berry press cakes and a supercritical CO_2 plant extract in suppressing lipid oxidation during frozen storage of BH fish mince in comparison to conventional synthetic antioxidants. Further, the impact of antioxidant additions on consumer acceptance was studied in a traditional fish product prepared from the fish mince.

2. Materials and methods

2.1 Materials

Gutted and be-headed BH, BH fillet with skin, and BH fillet without skin were purchased from a local fish processing company, Martin Kala Oy (Turku, Finland). The fish samples were kept on ice after gutting and filleting, and were produced into minced masses within 24 hours.

"Antimicrobial blend" (AB) extract mixture was purchased from Flavex (Flavex Naturextrakte GmbH, Rehlingen, Germany). AB is a mixture of seven supercritical CO_2 plant extracts, containing 30% sage (*Salvi fruticosa* M.) extract, 20% hop (*Humulus lupulus* L.) extract, 15% licorice root (*Glycyrrhizia uralensis* F.) extract, 15% temulawak (*Curcuma xanthorrhiza* R.) extract, 10% clove bud (*Syzygium aromaticum* L.) extract, 5% oregano (*Origanum vulgare* L.) leaf extract, and 5% ajowan fruit (*Trachyspermum ammi* (L.) Sprague ex Turrill) extract. Lingonberry-bilberry (LB) press cake was purchased from a juice pressing company, Kiantama Oy (Suomussalmi, Finland) under the name lingonberry press cake. However, based on anthocyanin content and composition of the press cake it was concluded to also contain bilberry (see discussion in chapter 3.1). Sea buckthorn (SB) press cake was from Polarforma Oy (Tornio, Finland). *L*-Ascorbic acid, α-tocopherol and ethylenediaminetetraacetic acid calcium disodium salt (EDTA) were bought from Sigma (Sigma-Aldrich Co, St. Louis, Missouri, USA).

Reference compounds of flavonoids, such as myricetin, quercetin, kaempferol, isorhamnetin, delphinidin, delphinidin 3-*O*-glucoside, cyanidin, cyanidin 3-*O*-galactoside, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-arabinoside, petunidin, peonidin, malvidin, and malvidin 3-*O*-glucoside, were purchased from Extrasynthese (Genay, France). Caffeic acid, *p*-coumaric acid, protocatechuic acid, 2,4,6-trihydroxybenzaldehyde, *trans*-cinnamic acid, and *trans*-ferulic acid were purchased from Sigma-Aldrich Co. (St. Louis, U.S.A.). Solvents of LC and/or MS grade, including acetone, acetonitrile, 1,4-dioxane, ethyl acetate, formic acid, heptane, hydrochloric acid, and methanol, were purchased from Honeywell Riedel-de Haën Co. (Seelze, Germany).

2.2 Production and storage of BH fish minces

The BH raw materials were processed into BH fish minces at the Finnish fish processing facility Kolvaan Kala Oy (Säkylä, Finland) using a modified meat bone separator, Baader 600 (Thomeko Oy, Helsinki, Finland), capable of separating skins and bones while mincing the fish muscle.

Three types of BH were used as raw materials for producing the fish mince; gutted and beheaded BH (A), BH fillets with skin (B), and BH fillets without skin (C0). Since other fishes than Baltic herring were commonly processed at the facility, separation of skins and bones during production of mince was not optimized for BH in the used process and therefore full removal of bones and skin was not guaranteed for raw materials A and B. Therefore, the antioxidants were tested in the fish mince from BH fillets without skin (C0), since fully skin- and bone-less is a common commercial standard for fish mince.

EDTA (E385) was used at a level of 0.075 g/kg fresh weight (f.w.) of fish mince (C1). Combination of vitamins E (α-tocopherol) and C (L-ascorbic acid) was added at concentrations of 0.1 and 2.0 g/kg f.w. (C2), respectively. The natural antioxidants and their levels added were AB supercritical CO_2 plant extract (1.0 g/kg) (C3), dried press cakes of LB (30.0 g/kg) (C4), and SB (30.0 g/kg) (C4). The concentration of AB was chosen based on a previous study by Tarvainen et al. (2015). The amount of press cakes added was comparable to what has previously been used for SB in mechanically deboned meat (Püssa et al, 2008). Prior to addition to the fish mince, the dried berry press cakes were milled into smaller particle size powders using a blender (Chef XL titanium, type KVL80, Kenwood Limited, Havant, U.K.) with a grinder attachment by mixing at full speed for approximately 1 minute. Antioxidants were added and thoroughly manually mixed with part of fish mince C0 immediately after the mince was produced. Manual mixing was continued for several minutes to ensure even distribution of additions, which was also confirmed visually in the case of natural additives. BH minces with added (C1-5) and without added antioxidants (A, B and C0) were frozen within 24 hours after production, and stored at -20 °C for 0, 2, 4, 6 or 10 months before analysis.

2.3 Lipid analysis

Total lipid contents of three BH raw materials (A, B and C0) and SB press cake were measured gravimetrically after modified Folch extraction (n=2) (Aro et al., 2000). Lipid contents of the fish minces after antioxidant additions were calculated based on the proportions and, reported or measured lipid contents of the antioxidant additions.

For peroxide value (PV) and fatty acid (FA) analysis, all fish minces (A, B and C0-5) from the first (0 months) and last (10 months) time points were subjected to lipid extraction according to the method described by Lee, Trevino, and Chaiyawat (1996) to minimize lipid oxidation during extraction. Each sample was extracted once and the extract was used for both analyses. PV (n=2) was determined spectrophotometrically at 500 nm using a modified ferric thiocyanate method by Lehtonen, Kemmo, Lampi, and Piironen (2011).

FAs (n=3) were analyzed with gas chromatography (GC) with flame ionization detector (FID) as methyl esters prepared with an acid-catalyzed method according to Christie (2003). C19:0 (1,2-dinonadecanoyl-*sn*-glycero-3-phosphatidylcholine, Larodan, Solna, Sweden) was used as an internal standard. A Shimadzu GC-2030 equipped with an AOC-20i auto injector and an FID (Shimadzu corporation, Kyoto, Japan) was used to analyze methylated fatty acids (FAMEs). The FAMEs were separated with a DB-23 (60 m × 0.25 mm i.d., liquid film 0.25 µm, Agilent Technologies, J.W. Scientific, Santa Clara, CA, U.S.A.) column. The column oven was first held at 130 °C for 1 min, then increased to 170 °C at a rate of 6.5°C/min, followed by increase at a rate of 2.75 °C/min to 205 °C and holding for 18 min, after which temperature was increased 30 °C/min until 230 °C was reached, and this temperature was held for 2 min. Helium was used as a carrier gas. The peaks were identified using external standards, Supelco 37 Component FAME mix (Supelco, St. Louis, MO, U.S.A.), 68D (Nu-Check-Prep, Elysian, MN, U.S.A.), and GLC-490 (Nu-Check-Prep, Elysian, MN, U.S.A.). The FAs were quantified using internal standard and correction factors determined with standard mixtures.

2.4. The content of carotenoids, tocopherols and tocotrienols in sea buckthorn press cake

Total carotenoid content of the SB press cake was determined according to Hornero-Méndez and Mínguez-Mosquera (2001) with a slight modification. Briefly, 0.5 g of ground SB press cake in duplicate was extracted with 35 mL of acetone for 1h, followed by second extraction with 40 mL acetone for 1 h. The samples were covered with foil and mixed constantly during extractions, and before collecting the supernatant the tubes were centrifuged at 5000 × g for 5 minutes. The volume of combined supernatants was made up to a final volume of 100 mL with acetone and filtered using 0.45 µm PTFE filters. Absorbance of the extract was measured at 472 and 508 nm, and the total content of carotenoids (µg/mL) in the extract was calculated as a sum of the "red" (C^R) and "yellow" (C^Y) fractions (Hornero-Méndez et al. 2001).

The tocopherol and tocotrienol content of the SB press cake was determined by normal phase high performance liquid chromatography (NP-HPLC) with fluorescence detection (FLD) according to Schwartz, Ollilainen, Piironen and Lampi (2008). The lipids were extracted in duplicate from the SB press cake by modified Folch extraction and were dissolved in heptane. *D*, *L*-Tocol was added as an internal standard. The analysis was preformed using a HPLC system consisting of Shimadzu Nexera LC-20AD XR pump, SIL-20AC autosampler (set to 4 °C), CTO-20AC prominence column oven (set to 30 °C) and RF-20A prominence fluorescence detector (Shimadzu Corp., Kyoto, Japan) and equipped with a Phenomenex Luna® 3 µm silica column (250 × 4.6 mm) (Phenomenex®, Torrance, CA, U.S.A.). Tocopherols and tocotrienols were separated isocratically using a mobile phase mixture of 3% 1,4-dioxane and 97% heptane (v/v) at flow rate of 2 mL/min. The excitation wavelength was 295 nm and the emission wavelength 325 nm. External standards of all tocopherols (*α*-, *β*-, *γ*- and *δ*-) and tocotrienols (*α*-, *β*-, *γ*- and *δ*-) were used for identification and the internal standard concentration was used for quantification.

2.5 Analysis of phenolic compounds in berry press cakes

The extraction of phenolic compounds in berry press cakes was carried out using two methods, due to the presence of anthocyanins. Anthocyanins were extracted from 3.0 g of powder samples using 15 mL of acidic methanol (methanol/hydrochloric acid, 99/1, v/v), whereas non-anthocyanin compounds were extracted from 5.0 g of press cake powders with 10 mL of ethyl acetate. Both extractions were assisted by ultra-sonication, followed by centrifugation. The detailed procedure was reported previously (Tian et al., 2019).

Qualitative analysis of phenolic compounds was performed on a ultra-high performance liquid chromatography (UPLC) system, equipped with a diode-array detector (DAD), an Apollo II electrospray ion source (ESI), and a quadrupole/time-of-flight tandem mass spectrometer (Q-TOF) (Bruker Corp., Billerica, MA, U.S.A.). The chromatographic separation was conducted at room temperature using a Phenomenex Aeris ™ peptide XB-C18 column (150 × 4.60 mm, 3.6 μm, Phenomenex®, Torrance, CA, U.S.A.). The injection volume was 10 μL, and the total flow rate was set to 1 mL/min. A binary solvent system was applied in the analysis of anthocyanins, including formic acid/water as solvent A (5/95, v/v) and formic acid/acetonitrile as solvent B (5/95, v/v). The LC gradient program was: 0−1 min with 4−6% solvent B, 1−2 min with 6−8% B, 2−14 min with 8−11% B, 14−20 min with 11−16% B, 20−25 min with 16−24% B, 25−28 min with 24−80% B, 28−29 min with 80−20% B, 29−31 min with 20−4% B, and 31−35 min with 4% B (**Supplemental Table 1)**. For other phenolic compounds, the mobile phase consisted of formic acid/water (solvent A, 0.1/99.9, v/v) and formic acid/acetonitrile (solvent B, 0.1/99.9, v/v). The following LC gradient was applied: 0−15 min with 8−10% solvent B, 15−20 min with 10−13% B, 20−25 min with 13−16% B, 25−40 min with 16−22% B, 40−45 min with 22−25% B, 45−50 min with 25−40% B, 50−55 min with 40−60% B, 55−60 min with 60−30% B, 60−63 min with 30−8% B, and 63−65 min with 8% B

(**Supplemental Table 2)**. The peaks in LC chromatograms were recorded in the wavelengths of 520 (for anthocyanins), 360 (flavonols), 320 (hydroxycinnamic acids), and 280 nm (hydroxybenzoic acids and other phenolics).

Approximately 0.4 mL/min of eluent was directed to ESI-Q-TOF system. Mass spectrometric analysis was carried out in both positive and negative ion modes. The capillary voltage was 4500 V for positive ion mode, and 3500 V for negative ion mode, end plate offset was set to 500 V. Nebulizer gas pressure, the flow rate of drying gas, and drying gas temperature was 2.5 bar, 11 L/min, and 280 °C, respectively. Internal calibration was performed using sodium formate in the beginning of each injection. The mass was scanned in the range of 20 to 1500 *m/z*. Mass spectra were processed with Compass Data Analysis software.

A Shimadzu LC-30AD liquid chromatograph system was used in the quantitative analysis of phenolic compounds, connected to a SIL-30AC auto-sampler, a CTO-20AC column oven, and a SPD-M20A photodiode array detector (Shimadzu Corp., Kyoto, Japan). The chromatographic conditions were the same as in the UPLC-DAD-ESI-Q-TOF analysis. The samples were extracted in triplicates, and each extract was analyzed twice. All compounds were quantified with an external standard method as reported previously (Tian et al., 2017). The information regarding quantitative analysis, such as selection of reference standards, and equation of calibration curves, is given in **Supplemental Table 3**.

2.6. Volatile analysis

Volatiles of all fish minces were analyzed after 0, 2, 4, 6 and 10 months of frozen storage, according to the protocol of Damerau, Kamlang-ek, Moisio, Lampi, and Piironen (2014). For the analysis, 3 g ± 0.01 g in quintuplicates of each fish mince was weighed in 20-mL headspace vials and stored at -20 °C until analysis. Volatiles were extracted using headspace solid phase microextraction (HS-SPME) using TriPlus RSH autosampler (Thermo Scientific, Switzerland) equipped with a DVB/CAR/PDMS-fiber (50/30 µm film thickness; Supelco, U.S.A.). Sample holder was cooled at +5 °C. Extraction parameters were as follows: Incubation at 40 °C for 20 min, extraction at 40 °C for 30 min and desorption 6 min at 240 °C. Extracted volatiles were analyzed with TRACE 1310 GC (Thermo Scientific, Switzerland) coupled with a TSQ 8000 evo mass spectrometer detector (Thermo Scientific, Switzerland). The column was Supelco SPB-624 (30 m × 0.25 mm i.d., 1.4 µm film thickness; Supelco, U.S.A.). The GC operation conditions were the following: helium flow 1.4 mL/min; oven temperature 40 °C for 5 min, then increased by 5 °C/min to 200 °C and held at 200 °C for 10 min. MS was operated in EI mode, with a voltage of 70 eV and the scan range was from 50 to 300 amu. Identification of compounds was performed by matching mass spectra with the database NIST MS Search library (version 2.3, National Institute of Standards and Technology, Gaithersburg, Maryland, U.S.A.) and by comparing with retention time and MS spectra of standards.

2.7 Consumer study

Consumer studies were conducted at three different time points to measure the sensory acceptance and liking of the fish minces. The consumer studies focused on perceived differences between fish minces with different antioxidants and was conducted thrice to observe the potential changes in consumer perception due to extended storage. The three time points (TP) studied were: TP1= 0 months, TP2= 2 months, and TP3= 6 months of frozen storage of fish minces. The consumer studies were carried out in a sensory laboratory complying with ISO 8589. Microbial stability was determined to ensure the safety of the fish minces prior to consumer tests (data not shown).

The volunteer participants were recruited from the Aistila Consumer Register of the University of Turku. A total of 158 consumers participated in the tests, 55, 52, and 51 in TP1, TP2, and TP3, respectively. Of TP2 participants, 60% (31/52) took part in TP1. All participants in TP3 (51/51) took part also in TP1 or TP2. Of TP3 participants, 47% (24/51) participated at both TP1 and TP2. The majority of the participants reported to eat fish frequently: 11−21 % reported to use fish 1−3 times/month, 63−72 % 1−2 times/week and 12−13 % “several times/week”, only 0−2 % less frequently than monthly.

The consumer tests included six samples; fish mince B (fillets with skin) without added antioxidants and fish minces C1-C6 supplemented with antioxidant additions. The fish mince B from fillets with skin was chosen as control as it was the most similar raw material to the one traditionally used in evaluated fish dish.

Since the raw fish minces were not edible as such, and the aim was to study the effect of the additions in a typical fish product, fish minces were prepared into fish loaves according to the following recipe: 67% fish mince, 15% egg, 3% toast crumb (without peel), 13% heavy cream and 1% salt. The ingredients of the recipe (without fish) were weighed 8-fold and mixed in a separate container. The uniformly mixed mass was divided into 8 parts and mixed with each fish mince. The batter was baked in aluminum molds (Pirkka Foil Tray 1.5 L, 230 × 110 × 60 mm) in an oven for 35 min at 200 ° C. The samples were prepared and cooked with the same conditions for each test (TP1, TP2 and TP3).

The participants evaluated the samples in a random order. Liking ratings were requested for the following factors: smell, appearance, color, texture, taste, overall appeal. A 9-point hedonic scale was used for evaluating sensory acceptance (1 = dislike extremely to 9 = like extremely). In addition, respondents were asked to provide one-word product descriptions and any other comments they might have. Data were collected with Compusense Cloud version 8.4 (Compusense Inc., Guelph, Ontario, Canada).

2.8 Statistical analysis

Fatty acids and PV values of different fish minces were compared using one-way ANOVA and Tukey's HSD test in SPSS (IBM SPSS Statistics, version 25.0.0.1, IBM, New York, USA). Differences were considered statistically significant if *p*-value was below 0.05. Principal Component Analysis (PCA) using the Unscrambler® X version 10.4.1 (Camo Process AS, Oslo, Norway) was applied to averaged peak area data to determine the correlation of volatiles and samples at different time points. Data were mean-centered and weighed (1/sdev) for PCA using Unscrambler. SPSS was also used for statistical analysis of consumer test data.

3. Results and Discussion

3.1 Characterization of raw materials

The lipid content and composition of the SB press cake was analyzed to take into account the effect of SB lipids on the lipid oxidation of fish mince. The SB press cake contained a significant amount of lipids, 39.5 ± 0.6 mg/100 mg, and the major FAs were 16:0, 16:1(*n*-7) and 18:1(*n*-7), which have previously been reported as the major FAs in the pulp and peel of the SB berry (Yang & Kallio 2001).

Carotenoids in SB press cake have been shown to have radical scavenging activities (Gao, Ohlander, Jeppson, Björk & Trajkovski, 2000). Total amount of carotenoids was 872.5 ±5.3 mg/kg (as is) of press cake. Majority (97%) of total carotenoids belonged to the "yellow fraction", consisting of compounds such as *β*-carotene and *β*-cryptoxanthin. The finding is in line with a previous study by Andersson, Olsson, Johansson, and Rumpunen (2009), where the total content of carotenoids in SB berries from different cultivars, harvest occasions and years was on average 834.8 mg/kg dry weight (d.w.) Based on the estimated total content of carotenoids in the press cake, carotenoid concentration in the fish mince C5 with added SB press cake was 26.2 mg/kg f.w.

The total content of tocopherols and tocotrienols in the SB press cake was 285.6 ±0.6 mg/kg (as is). The most abundant compound was *α*-tocopherol (269.1 ±0.9 mg/kg), followed by *β*-tocopherol (9.2 ±0.1 mg/kg), *γ*-tocotrienol (4.0 ±0.1 mg/kg) and *α*-tocotrienol (3.3 ±0.1 mg/kg). Only traces of *γ*-tocopherol and *δ*-tocopherol were detected. A previous study reported a total content of 316.6 to 1250.9 mg/kg d.w., with a mean value of 561.5 mg/kg d.w., of tocopherols and tocotrienols for fresh SB berries from different subspecies and cultivars (Andersson, Rumpunen, Johansson & Olsson, 2008). The lower content in press cake can be explained by losses through juice pressing and oxidation during processing and storage. Also, the composition of tocopherol and tocotrienol compared to the study by Andersson et al. (2008), indicated losses through oxidation. They identified *α*-, *γ*-, and *δ*-tocopherol, traces of *β*-tocopherol and *α*-, *γ*-, and *δ*-tocotrienol in their studied SB cultivars. The total content of tocopherols and tocotrienols in C5 was 8.6 mg/kg f.w. based on the 3% addition of SB press cake.

Table 1

The lipid contents of prepared BH fish minces are presented in **Table 1**. The lipid content of different types of fish minces (A, B, and C0) varied between 4.2 w-% for BH fillet without skin and 5.2 w-% with skin. BH fillet with skin contained more lipids than gutted BH, which is likely due to the presence of bones and other lean parts in the gutted herring compared to the fillet with skin. The lipid content of BH varies depending on the season being the lowest in the beginning of the summer (Aro et al., 2000). The BH used in this study were caught in the late spring, and the lipid content is in line with the previous findings reported for BH caught this time of the year (Aro et al., 2000). The incorporation of SB press cake increased the lipid content to 5.2 w-%, due to the high lipid content of SB press cake, whereas addition of other antioxidants did not significantly influence the lipid content in the fish minces.

Table 2

The fatty acid compositions of BH fish minces A, B and C0 are presented in **Table 2**. Most FAs differed significantly among samples due to low standard deviations resulting from the sampling method. However, when comparing the averages, the most notable differences were in the contents of 16:1 (*n*-7) (palmitoleic acid), 18:1 (*n*-9) (oleic acid), 18:2 (*n*-6) (linoleic acid) 18:3 (*n*-3) (α-linolenic acid), 20:5 (*n*-3) (EPA) and 22:6 (*n*-3) (DHA). In addition to having the highest lipid content, fish mince B produced from fillet with skin had a lower relative content of EPA and DHA, and higher content of oleic acid compared to A and C0. C4 with LB press cake contained more 18:2 (*n*-6) and 18:3(*n*-3) compared to all other fish minces. In C5 with SB press cake the levels of 16:0 and 16:1(*n*-7) were elevated compared to other minces due to the abundance of these FAs in the SB press cake.

For the CO_2 plant extract AB and the berry press cakes, the content of phenolic compounds was of interest based on their potential antioxidant activity. The main constituents of AB were curcumenes (10 %), humulones and lupulones (10%), eugenol (7%), carnosic acid and carnosol (5%), thymol (3%), carvacrol and thymoquinone (3%), and licoricidin and licorisoflavane A (2%) (Tarvainen et al., 2016). Phenolic compounds in the berry press cakes were identified by comparing the retention times, UV absorption, and mass spectra with reference compounds and previous literatures (Fang, Veitch, Kite, Porter & Simmonds, 2013; Dudonné et al., 2015; Chhonker et al., 2016; Tian et al., 2017). LC chromatograms and quantitative results are given in **Supplemental Figure 1** and **Supplemental Table 4**.

Figure 1 and **Supplemental Table 5** show the concentration of identified compounds in berry press cake samples. The total content of detected phenolic compounds in the LB press cake was 166.9 mg/100 g f.w., of which anthocyanins accounted for over 75% (**Figure 1a**). The major anthocyanins were glycosides of cyanidin (33% of detected phenolics), delphinidin (21%), petunidin (7%), malvidin (5%), and peonidin (4%); which was in contrast to the reports of lingonberry containing cyanidin derivatives only (Ek, Kartimo, Mattila & Tolonen, 2006; Tian et al., 2017). This strongly indicated the presence of bilberry, which is known to contain all of the anthocyanins detected (Tian et al. 2017). Wild bilberry and lingonberry bushes often grow as a mixed population in the forest, and a low amount of bilberry is unavoidable in commercial harvesting of lingonberry. Based on the contents of delphinidins, petunidins, malvidins, and peonidins in the LB press cake and bilberry, and the total anthocyanin contents of bilberry and lingonberry (Tian et al. 2017), the percentage of bilberry in the press cake was estimated to be approximately 20%. This contamination had a significant impact on the anthocyanin composition of the press cake as the total anthocyanin content in bilberry compared to lingonberry is up to 11 times higher (Tian et al. 2017). The concentrations of cyanidin and delphinidin aglycones were 8.0 and 3.8 mg/100 g f.w., respectively; and other three anthocyanidins were detected at trace amounts. As the primary flavonols in LB press cake, the content of quercetin aglycone (12.7 mg/100 g) was four times higher than its glycosides, likely due to enzymatic hydrolysis during juice pressing. Phenolic acids in LB press cakes represented 7% of the total content of detected phenolics, mostly as ferulic acid (4.5 mg/100 g), protocatechuic acid (2.1), cinnamic acid (2.1), *p*-coumaric acid (2.1), and 2,4,6-trihydroxybenzaldehyde (1.0).

Figure 1

As shown in **Figure 1b**, the total content of detected phenolics in SB berry press cake was 42.5 mg/100 g f.w., 85 % of which were flavonols. Isorhamnetins represented for 73% of total content of phenolics detected, including both aglycone (64%) and glycosides (9%). Quercetins, mainly as quercetin aglycone, were present at a concentration of 4.1 mg/100 g of fresh matters. Phenolic acids were the second most abundant group of phenolics in the SB press cake. Two unknown phenolic acid derivatives together accounted for 10% of detected phenolics, followed by acetylbenzoic acid (4%). Berries of SB are rich in proanthocyanidins. Yang and co-workers extracted proanthocyanidins from the berries of SB (wild and cultivated) using a mixture of acetone, water, and acetic acid (80/19.5/0.5, v/v/v). Total content of proanthocyanidins in SB berries of finnish origin ranged from 590 to 1940 mg/100g dry matter (Yang, Laaksonen, Kallio & Yang, 2016). In the present study, proanthocyanidins were not detected in the SB extract, likely due to their low extraction efficiency by ethyl acetate. Moreover, since previous research reported no presence of anthocyanins in SB berries of Finnish origin (Koponen, Happonen, Mattila & Törrönen, 2007; Tian et al., 2017), anthocyanins were not analyzed from the SB press cakes.

3.2 Effects of antioxidants on stability of Baltic herring fish mince during frozen storage

3.2.1 PV and loss of EPA and DHA during storage

During lipid oxidation, PUFAs such as EPA and DHA are degraded and thus their loss is an indicator of oxidation. Relative losses of EPA and DHA in all eight fish minces during 10 months of frozen storage are presented in **Table 3**. Fish minces with berry press cake additions showed the lowest losses of both EPA and DHA, indicating improved preservation of oxidation sensitive PUFAs. A study by Sancho, Lima, Costa, Mariutti, and Bragagnolo (2011) also analyzed decreases in EPA and DHA concentrations during frozen storage of cooked white hake fish balls. Reported losses of EPA and DHA in the control fish ball during the 120 d frozen storage period were 43% and 44%, respectively, but addition of coriander leaves and annatto seeds decreased the losses to 9% for EPA and 7% for DHA. Joaquin, Tolasa, Oliveira, Lee and Lee (2008) observed significant decreases up to 45% in the concentrations of EPA and DHA in minced herring tissue during 4 month of frozen storage. Treatment of the mince with milk protein concentrate (4% and 6%) retained more PUFAs than untreated mince. In our work, the losses of EPA and DHA were smaller for the control (C0) than in both mentioned studies despite the longer 10-month storage period. Better preservation of EPA and DHA in this study is most likely explained by different pre-treatment of fish mince, and due to the different content and composition of lipids and other components in BH compared to white hake and herring. Despite smaller overall losses of these PUFAs, differences between the control fish mince and the fish minces with added berry press cakes were observed. For C4 and C5, the losses of EPA were only 4.1% and 3.8% and of DHA 6.6% and 7.5%, respectively. Hence, the addition of berry press cakes reduced the degradation of these PUFAs by approximately half.

PV was analyzed as an indicator for primary lipid oxidation. The PV data of all fish minces at the beginning and end of the storage test are presented in **Table 3**. PV increased in all samples during the storage period. Interestingly, fish minces C2 and C4 had a higher increase in PV compared to C0. This may be caused by accumulation of hydroperoxides, reducing the degradation of hydroperoxides to secondary oxidation products. In our previous study, proanthocyanidins and flavan-3-ols present in lingonberry contributed strongly to the antioxidative activities of berry extracts (Tian et al., 2018). However, as the PV is based on an indirect measurement of hydroperoxides, other oxidative species in the sample can cause a false positive reaction. In case of C2 vitamin C could have acted as pro-oxidant oxidizing the Fe^{2+} to Fe^{3+} instigating a stronger color formation. The anthocyanins from the LB press cake in C4 were not extracted to the lipid fraction, as no color was observed for the C4 lipid extract and no absorption of lipid extract itself at 500 nm was detected. Nevertheless, it cannot be excluded that the C4 lipid extract contained compounds interfering with the PV measurement, since the method was not optimized or normally used for such a matrix. Furthermore, PV by itself does not always best represent the oxidative state as it only accounts for primary oxidation. Especially during long-term oxidation studies, analysis of secondary oxidation products is needed.

Table 3

3.2.2 Formation of volatile compounds during storage

Volatile compounds at 0, 2, 4, 6 and 10 months were analyzed using HS-SPME-GC-MS to follow the formation secondary volatile lipid oxidation compounds in all fish minces with and without antioxidant additions. Chromatograms of fish mince C0 after 0 and 10 months of frozen storage are presented in **Supplemental Figure 2**. A total of thirty-seven volatiles naturally occurring in fish (**Supplemental Table 6)** were identified. Volatile data was analyzed by PCA and compounds with highest impact on the model were selected for the final PCA model. The PCA model showing the distribution of samples and selected volatiles is presented in **Figure 2**. PC-1, accounting for 80% of the variation between samples (**Figure 2**), separates them based on quantities of oxidation related volatiles. Samples in the lower left corner are associated with 2- and 3-methylbutanal, which are Strecker aldehydes commonly found in fresh BH based on natural degradation reactions occurring in fresh BH (Aro, Tahvonen, Koskinen & Kallio, 2003), whereas samples in the right side of the plot are associated with lipid oxidation related volatiles, such as hexanal, 2(*E*)-hexenal, 4(*Z*)-heptenal, 2-ethylfuran, 2,4-heptadienal and 3,5-octadien-2-one. Differences in oxidation related volatiles between samples are visible already in the 0-month measurement. This is likely due to the fact that there was a 24 h delay between preparation of the fish minces and volatile analysis at 0-month time point, during which time the samples, stored at + 4 °C, were already oxidizing. Differences between volatile profiles of the fish minces were most prominent at 2-month storage time. The highest formation of all lipid-derived volatiles during storage was obtained for 1-penten-3-ol and hexanal in all fish minces. Sampels, Åsli, Vogt and Mørkøre (2010) also found 1-penten-3-ol to be the most abundant volatile in marinated herring fillets. Other volatile oxidation compounds detected in the marinated herring fillets were propanal, butanal, 2- and 3-methylbutanal, hexanal, heptanal, nonanal and 2-penten-1-ol, all of which were also found in the present study (**Supplemental Table 4**). Propanal, 4(*Z*)-heptenal, 2-hexenal and 1-penten-3-ol that are known to correlate with fishy odor (Joaquin et al., 2008) increased during the storage time in all samples. A similar formation of 4(*Z*)-heptenal and 1-penten-3-ol was observed by Aro et al. (2003) for fresh BH during storage at 6 °C. The most abundant volatile compounds in the start of their storage test were 2- and 3-methylbutanal, and hexanal. After 3 days amounts of 4(*Z*)-heptenal, 2-heptanone and octatriene, and after 5 days the amounts of hexanal, heptanal, 1-penten-3-ol and octadienes increased significantly, indicating oxidation of PUFAs. A decreasing trend for 2- and 3-methylbutanal was found by Aro et al. (2003) during storage. In the present study a similar trend was noticed for the first 6 months of storage. Overall, similarities in formation behavior of volatile compounds could be found between the study on refrigerated BH (Aro et al., 2003) and this study on frozen BH mince despite the greatly different temperatures and time frames, which indicates that lowering the temperature mainly slows down oxidation reactions without major alteration of oxidation pathways in BH.

Figure 2

Fish minces C2 to C5 are located on right side of PC-1 only after 10 months of storage (**Figure 2**), indicating low formation of these secondary volatile lipid oxidation products during the first 6 months of storage. The lowest formation was seen in C3 correlating well with low PV at 10 months. However, in comparison to all other additions, the AB extract in C3 contained natural flavor compounds from the plants used to produce the extract, which may have influenced the volatile analysis by competing with formed lipid oxidation compounds during extraction of volatiles, resulting in an underestimation of volatile oxidation products. The variance in PC-2 (**Figure 2**) was mainly due to higher content of lipid oxidation products in comparison to volatiles contributing to the natural flavor of fresh BH after 4 months of storage. Further, there was a slight increase in Strecker aldehydes and 3-hydroxy-2-butanone after 10 months of storage for B, C1, C2, C4 and C5 while these compounds decreased for the first 6 month of storage in all samples.

3.2.3 Evaluation of the oxidative stability during storage

Fish minces produced from different types of BH used in the study, A, B, and C0, showed differences in lipid oxidation during frozen storage. Interestingly, out of the three raw materials, C0, prepared from fillets without skin, showed the highest reduction of EPA and highest increase in PV value during the 10-month storage period. Based on the volatile data, the lipid oxidation in C0 occurred at a similar rate as in B, whereas the extent of oxidation was slightly higher for A.

Out of all the tested antioxidant additions, EDTA had the least effect on lipid oxidation. In a study by Let, Jacobsen, and Meyer (2007), addition of EDTA was shown to efficiently delay the oxidation of fish oil enriched salad, but not of fish oil enriched milk emulsion (Let, Jacobsen, Pham & Meyer, 2005). The lack of effect in the present study may have been related to the fact that the heme iron was mostly still bound to heme, and EDTA was thus not able to chelate it. Alternatively, lipid oxidation catalysis by transition metals may not have been a significant oxidation initiation pathway during frozen storage. Several other processes have been suggested as the initiators of lipid oxidation in fish, including activity of lipoxygenase (German, Chen & Kinsella, 1985).

The combination of α-tocopherol and ascorbic acid has traditionally been used as an antioxidant in food industry, due to the ability of ascorbic acid to regenerate α-tocopherol. Previously, combination of these vitamins was shown to inhibit lipid oxidation of ground meat during illuminated display at + 4 °C (Mitsumoto, Faustman, Cassens, Arnold, Schaefer & Scheller, 1991). Neither vitamin on its own was as efficient as the combination of the two. In the present study, combination of vitamins E and C was not as effective in delaying lipid oxidation as berry press cakes and the plant extract. The BH fish mince C2 with addition of these two vitamins showed however moderately lower formation of lipid oxidation derived volatiles and loss of EPA and DHA compared to the control (C0).

AB consisting of seven plant CO_2 extracts has previously been shown to delay oxidation of triacylglycerols in Atlantic salmon fillets (Tarvainen et al., 2015) and cholesterol oxidation in fish patties made from Atlantic salmon (Tarvainen et al., 2016). In both studies, AB was the most efficient or one of the most efficient out of tested plant extracts. The effect was likely due to the synergetic effects between the extracts of the seven plant species in the AB. In the present study, AB incorporated into BH fish mince decreased oxidation during frozen storage of uncooked mince based on volatile data, FA data and PV. Sage extract, most abundant component of AB, was seen to delay lipid oxidation and formation of fishy odors in hairtail fish balls during cold storage (Guan, Ren, Li & Mao, 2019).

There are few studies reporting the effect of berry press cakes or whole berries on lipid oxidation in fish or meat. Whole berries made into marinades were seen to delay lipid oxidation of preserved herring fillets (Sampels et al., 2010). In a study by Püssa et al. (2008), 1, 2 or 4% of dried SB powder macerated in ethanol was added to mechanically de-boned chicken and turkey. SB enriched meats showed significantly lower TBARS after 6 days of cold storage, with the concentrations of 2% and 4% being the most potent in preventing oxidation. The analysis of polyphenol content also showed that the polyphenols of SB, mainly flavonol glycosides, were relatively stable during the storage test, supporting the potential of SB as an antioxidant in muscle foods.

The effect of different phenolic compounds on protein and lipid oxidation measured in protein-liposome systems have been analyzed by Viljanen, Kivikari, and Heinonen (2004). Studied phenolic compounds included cyanidin, delphinidin and pelargonidin derivatives, two procyanidins, and ellagic acid. Out of the aglycons, pelargonidin was the most effective in reducing formation of hydroperoxides, but cyanidin glycosides were the best inhibitors out of glycosylated compounds (Viljanen et al., 2004). Almost one third of the phenolic compounds in the LB press cake in the present study were cyanidin glycosides, but controversially, the peroxide values were the highest for the fish mince with added LB press cake. In the study by Viljanen et al. (2004), pelargonidin, cyanidin and cyanidin glycosides were most efficient in reducing formation of hexanal as well. The LB press cake in the present study decreased the formation of hexanal by 77% compared to the control BH fish mince, at the 6-month storage point. However, it is impossible to compare pure compounds to whole berries (berry press cakes in this instance) due to the synergistic effects of different compounds, and hence, no conclusions about antioxidative effects in a food system can be made only by the polyphenol content of a specific berry species. Differences in food matrices also add to the complexity of antioxidative and pro-oxidative reactions. In case of SB press cake addition to BH fish mince not only the phenolic compounds but also carotenoids, tocopherols and tocotrienols could have contributed to the antioxidant effects observed. However, the total content of tocopherols and tocotrienols in C5 corresponded to only 0.5% of the total content of these compounds added in C2. Therefore, the tocopherols and tocotrienols in C5 could contribute to synergetic effects, but the actions of these compounds alone may not explain the higher storage stability observed in C5 compared to C2. Tocopherols and tocotrienols in LB press cake were not analyzed in the present study, but due to the lower lipid content of lingonberry and bilberry compared to sea buckthorn, their role in the antioxidative effect of LB is likely even less significant.

3.3 Consumer acceptability of the antioxidant additions in fish loaf

Consumers rated the overall liking as well as liking of odor, color, appearance, texture, and taste of the fish product prepared form the fish mince with and without the berry press cake and plant extract additions. A hedonic scale of 1-9 was used in the rating: 1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4= dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7= like moderately, 8= like very much, 9= like extremely (**Figure 3** and **Supplemental Figure 3**).

Figure 3 shows that the basal fish mince (B), and the fish minces with addition of EDTA (C1) or combination of vitamins E and C (C2) were found to be more pleasant in overall liking than the samples containing AB extract (C3), LB (C4), or SB (C5) press cake. B and C4 were found to be significantly more pleasant at TP3 (after 6 months of frozen storage) compared to the first evaluation at TP1 (0 months) (p <0.05). The samples containing AB extract and berry press cakes received an average rating of 4-5 for overall liking, i.e., between “dislike slightly” or “neither like nor dislike”.

Figure 3

The hedonic ratings for smell, taste, appearance, color and texture are reported in **Supplemental Figure 3**. The addition of plant CO_2 extract or berry press cakes lowered the overall pleasantness ratings of the samples, and the verbal descriptions indicated the liked/unliked characteristics of the samples (data not shown).

Based on the consumer study, orthonasal off-flavours such as rancidity were not perceived from any of the samples even when evaluated at the third time point after the fish minces had been frozen for 6 months. Participants were however not asked to rate the intensity of rancid odor or flavor. The added antioxidants appeared to suppress the fishy taste and apparently also the saltiness. The respondents wanted the taste of the fish to be more distinguishable in products with added plant extract and berry press cakes. As described in section 2.7, 98−100 % of the panelists were frequent fish eaters and thus probably preferred traditional characteristics over the novel combinations of fish with the plant extract or berry press cakes. This preference was seen in the liking profiles of the samples in every TP. As seen in **Figure 3** and **Supplemental Figure 3**, B, C1 and C2, were found to be both more pleasant and tasty than the samples fortified with berry fractions or plant extract (C3, C4 or C5). Their presence in the fish mince reduced the overall appeal of the samples. Fish loaf made from fish mince C4 containing LB press cake was found to be the least pleasant in color and appearance. The pleasantness ratings at TP3 were similar as at TP1 and TP2, although they tended to be slightly higher compared to TP1 and TP2.

4 Conclusions

According to reduction of EPA and DHA and the formation of volatile oxidation products, the BH fish minces with additions of the CO_2 plant extract (C3) and berry press cakes (C4 and C5) showed reduced lipid oxidation compared to fish minces without additions (A, B, and C0). Also, the PV indicated reduced lipid oxidation in the fish mince with the CO_2 plant extract (AB) or SB press cake addition. The present study showed the potential of using plant extracts and berry press cakes to reduce lipid oxidation of BH fish mince during frozen storage. However, further research is needed to optimize the concentrations and addition of the plant extracts and press cakes into fish mince or fish products in order to reduce the lipid oxidation without compromising the sensory properties of the products. Furthermore, consumer information and education may increase the acceptability of products with added plant extracts and berry press cakes as there is a growing demand for natural additives.

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Conflict of interest statement

Authors declare no conflicts of interest.

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