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A new fluorimetric method for simultaneous determination of lipid and protein hydroperoxides in muscle foods with the use of diphenyl-1-pyrenylphosphine (DPPP)

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ABSTRACT

A new fluorimetric assay for simultaneous determination of lipid and protein hydroperoxides in muscle foods by the use of diphenyl-1-pyrenylphosphine (DPPP) was proposed in the study. Non-fluorescent DPPP that reacts with hydroperoxides stoichiometrically to yield fluorescent DPPP oxide, was used as a fluorescent dye to determine lipid and protein peroxidation in fish samples during frozen storage. The novel sensitive DPPP-assay developed for analysis under fluorescence microscopy conditions was based on this reaction. A good linear correlation between the amount of lipid hydroperoxides measured by the developed DPPP-assay and the peroxide value measured by iodometric titration was observed. Also, increase in fluorescence intensity from the reaction of DPPP with protein hydroperoxides in fish samples showed linear relation to the amount of cumene hydroxide added over a wide concentration range. The method was successfully applied on fish samples and can be used for fast assessment of primary oxidation stress in muscle foods.

1. Introduction

Unsaturated lipids are susceptible to oxidation reactions when exposed to different external factors including heat and light, as well as pro-oxidants such as enzymes, metals and metalloproteins (Frankel, 1998). The formation of primary lipid oxidation products, particularly lipid hydroperoxides is produced during peroxidation chain reactions by free radicals and enzymes such as lipoxygenase. These lipid hydroperoxides are odourless and flavourless but play a role of oxidative mediators due to their instability leading to further free radical and oxidation reactions generating a high variety of non-volatile and volatile compounds (Frankel, 1998). Besides generation of off-flavours, their breakdown in food products may result in a loss of nutrients leading to a decreased quality and nutritional value (Diplock et al., 1998; McClements & Decker, 2017; Shahidi & Zhong, 2010). Generally, the formation of secondary lipid oxidation products from the breakdown of primary oxidation products is the main reason for rancidity, generation of off-flavours and nutritional losses in foods (Frankel, 1998; Shahidi & Zhong, 2010). Therefore, a prompt determination of lipid hydroperoxides is needed to take actions for retarding the progression of lipid oxidation in a product.

Until recently, proteins were not considered biologically significant targets for reactive oxygen species, but newer investigations have shown that proteins form hydroperoxides as well, thus contributing to carbonylation and formation of cross-linkages (Gebicki & Gebicki, 1999; Bou, Chen, Guardiola, Codony, & Decker, 2010). Similar to lipid oxidation mechanism, particular reactive oxygen species (ROS) such as $^{\bullet}OH$, $O_2^{\bullet-}$ and $ROO^{\bullet}$ along with metal cations (iron, copper) can catalyze the abstraction of a hydrogen from a susceptible amino acid residue, resulting in the formation of a protein radical (Xiong, 2000). Sulfur-containing amino acids such as cysteine and methionine are the most susceptible to oxidation among other muscle proteins (Park & Xiong, 2007). The abstraction of a hydrogen atom gives birth to a protein carbon-centered radical ($P^{\bullet}$) which is further transformed into a peroxyl radical ($POO^{\bullet}$) in the present of oxygen, and an alkyl peroxide (POOH) through abstraction of a hydrogen atom from a neighbour molecule. Further chemical transformations involving $HO_2^{\bullet}$ yield alcoxyl radical ($PO^{\bullet}$) and its hydroxyl derivative (POH). As a result of the oxidative damage of muscle proteins, formation of oxidised derivatives and conversion of one amino acid residue to a different one, take place (Xiong, 2000). In addition, the oxidation of the side chain of certain amino acids (lysine, proline, arginine and threonine) results in formation of carbonyl residues deamination reactions, as well as fragmentation of the peptine backbone and the reaction with reducing sugars (Schiff base mechanism) or by coupling with non-protein carbonyl compounds such as malondialdehyde (MDA) (Park & Xiong, 2007). This

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further leads to a number of problems in the food industry, such as aggregation and loss of solubility of proteins (Bou et al., 2010; Cropotova, Mozuraityte, Standal, & Rustad, 2019). These highly oxidised proteins decrease the nutritional and sensory quality of muscle foods, such as meat or fish (Cropotova et al., 2019), leading to decreased shelf life of the product. Analysis of protein carbonyls is a widely applied method for assessment of later stages of protein oxidation reactions arising from primary oxidative stress (Cropotova & Rustad, 2019). However, besides determination of protein carbonyls, measuring protein hydroperoxides along with lipid hydroperoxides in a product can become a useful marker of oxidative stress at propagation stages of oxidation reactions.

A number of methods have been proposed for the determination of lipid and protein hydroperoxides in biological samples using the ferrous oxidation-xylenol orange method (Bou, Codony, Tres, Decker, & Guardiola, 2008; Du & Gebicki, 2004; Gay & Gebicki, 2003), flow injection analysis (Akasaka, Takamura, Ohrui, Meguro, & Hashimoto, 1996; Akasaka & Ohrui, 2000), nuclear magnetic resonance spectroscopy (Frankel, Neff, & Weisleder, 1990), iodometric hydroperoxide assay (Jessup, Dean, & Gebicki, 1994) and other chemical methods of analysis (Nakamura and Maeda, 1991; Nielsen, Timm-Heinrich, & Jacobsen, 2003). However, all the reported methods lack a simple and sensitive procedure for simultaneous and at the same time robust extraction and analysis of both lipid- and protein hydroperoxides. All existing methods require a separate extraction and/or purification step which makes the analysis more sophisticated and time-consuming.

The present paper displays a new rapid and sensitive method for simultaneous determination of lipid and protein hydroperoxides in muscle foods, in particular fish products based on a direct fluorescent labelling with diphenyl-1-pyrenylphospine (DPPP). The main benefit of the developed method is that the fluorescent signal of DPPP is neither affected by pigments absorbing at 560 nm nor iron or chelators present in muscle foods (Bou et al., 2010). Moreover, simultaneous determination of lipid and protein hydroperoxides can be directly performed in chloroform and water/methanol phases obtained after the extraction step by the Bligh and Dyer (1959) method, and does not require additional procedures for lipid and protein isolation and their further dilution in organic solvents such as it was done in previous studies with DPPP (Bou et al., 2010). In addition, small amounts of sample can be used for analysis. Thus, the proposed method can be applied for determination of lipid hydroperoxides in lean fish or meat, as well as protein hydrolysates that require big amounts of sample to be used for extraction of lipids.

A modification of the DPPP-fluorimetric assay by the use of fluorescence microscopy was proposed in the study as a rapid, non-invasive and cost-efficient method aiming to not only quantify lipid and protein hydroperoxides in muscle foods, but also visualize them by fluorescence imaging. The protocol was compared with the DPPP-assay for determination of lipo- and water-soluble hydroperoxides validated under microplate format conditions. The use of the developed fluorescence microscopy DPPP-assay can become a good alternative for scientific laboratories and facilities lacking multi-detection microplate readers equipped with fluorescence detectors.

2. Materials and methods

2.1. Reagents and materials

Methanol (purity), chloroform (purity), cumene hydroperoxide (80% purity), and 2,6-di-tertbutyl- 4-methylphenol (BHT) were acquired from Sigma Aldrich Co. (Germany). Dipheny-1-pyrenylphosphine (DPPP) was purchased from Thermo Fisher (USA). Bi-distilled water was obtained using a Milli-Q Integral System (Millipore Co., Billerica, MA, USA).

Fillets of Atlantic herring (*Clupea harengus*), Atlantic mackerel (*Scomber scombrus*), Rainbow Trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) and Atlantic cod *(Gadus morhua)* were used as raw material in the study as experimental samples for optimization and validation of the fluorimetric DPPP-assay. Atlantic cod was used just for the recovery of the DPPP-assay for determination of protein hydroperoxides. The fish fillets were purchased from a local retailer (Ravnkloa, Trondheim, Norway), minced using a benchtop blender (Bosch, Germany) and placed in polyethylene bags (portions of 500g fish mince per bag, oxygen permeability 25 cm^3/m^2) for frozen storage at −20 °C for 1, 3 and 6 months before analyses.

Before determination of hydroperoxides, the fish fillets were defrosted in a cold room at 4 ± 1 °C overnight. Analyses were performed in three replicates before and after freezing, and the average value with standard deviation were calculated.

2.2. Chemical and physical assays

2.2.1. Extraction of lipids

The total amount of lipids was extracted from the fish minces by the Bligh and Dyer (1959) method, which uses a binary mixture of chloroform and methanol diluted with distilled water as an extraction medium (Bligh & Dyer, 1959). The extraction was performed in duplicate. Extracted lipids in chloroform phase and oxidation products in water/methanol phase were stored at −80 °C prior to analyses.

2.2.2. Determination of peroxide value by conventional iodometric titration method

Peroxide value (PV) was measured by using the iodometric titration method described in AOCS official methods (Cd 8b-90) (AOCS, 2003). The end point of titration was assessed potentiometrically with an automatic titrator (TitroLine 7800, Xylem Analytics, Mainz, Germany) coupled with a platinum electrode (Pt 62). The analysis was performed in four replicates and the results were expressed in (meq $O_2 \cdot kg^{-1}$ lipid as a mean value ± SD.

2.2.3. Fluorometric method based on diphenyl-1-pyrenylphospine (DPPP)

After the last centrifugation step of the Bligh and Dyer (1959) lipid extraction method, three layers are formed in a centrifuge tube: an upper (water-methanol phase), a middle thin layer (disk) of precipitated proteins, and a bottom – chloroform layer. The resulting bottom (chloroform phase) layer was carefully collected (avoiding grabbing of the middle disk of protein fraction in its periphery with the tip of a pipette) and used for determination of lipid (liposoluble) hydroperoxides (LOOH). The upper layer (water-methanol phase) was collected and used for determination of water-soluble TBARS as secondary lipid oxidation products (data not shown). Finally, the middle thin layer of precipitated proteins was collected for determination of protein hydroperoxides (PrOOH). For determination of LOOH, the lipid amount was determined in the collected chloroform layer by Bligh and Dyer (1959) to express lipid hydroperoxides per kg oil. For determination of PrOOH, the resulting protein fraction (protein disk pellets) was first washed with 20 ml 10% TCA in an Eppendorf polypropylene graduated tubes (50 ml; Fisher Scientific, UK) and further re-pelleted by centrifugation by using SORVALL RC-5B PLUS Superspeed Centrifuge (Thermo Scientific, Waltham, Massachusetts, US). The collected protein pellets were further dissolved the in 20 ml 0.1M NaOH, followed by neutralization with 1/10 vol 1M HCl and centrifugation, as described by Grintzaliset al. (2013). The PrOOH concentration in the resulting supernatant can be expressed per protein (in μM) after determination of protein content, or wet weight of fish. In the present study, total amount of protein hydroperoxides was expressed per wet weight of fish. For quantification of PrOOH by DPPP-assay, the protein solution was mixed with methanol with the ratio 1:10.

The fluorescence assay proposed in the paper is a modification of the methods by Bou et al. (2010) and Santas, Guzmán, Guardiola, Rafecas, and Bou (2014) based on the quantitative reaction of phosphine moiety of the DPPP with various hydroperoxides (lipo- and

hydrosoluble) producing a high intensity fluorescent DPPP-oxide which can be fluorometrically detected at a wavelength of emission/excitation 360 ± 50/380 ± 50 nm (Akasaka, Sasaki, Ohrui, & Meguro, 1992; Bou et al., 2010). 100 μL chloroform and methanol extracts were transferred to Eppendorf polypropylene graduated microtubes (1.5 ml; Fisher Scientific, UK) placed on a 96-tube sample rack. The rack was kept on ice, and 100 μL of 150 μM dipheny-1-pyrenylphosphine (DPPP) dissolved in chloroform containing 3 mM 2,6-di-tertbutyl-4-methylphenol (BHT) was added in each of the microtubes. The microtubes were capped tightly with flat polypropylene lids to prevent solvent evaporation during the experiment and vortexed for 30 s. The 96-tube sample rack with the samples was incubated in a water bath at 60 °C for 120 min under constant agitation, and then placed on ice for 10 min in a cold room in the dark at 4 °C to stop the reaction. Further, the samples were vortexed for 30 s and subsequently transferred to a 96-microwell plate (200 μL each well) for fluorescence scanning. The measurements were performed by reading the sample fluorescence at λ_{ex} = 360/10 and λ_{em} = 415/10 nm with a Spark multimode microplate reader (Tecan, Austria).

For determination of lipo- and hydrosoluble peroxides in the samples, two standard calibration curves were prepared using cumene hydroperoxide (CumOOH) dissolved in chloroform and methanol, respectively. CumOOH was selected for calibration due to its good solubility both in chloroform and methanol. Hydrogen peroxide (HP) is soluble in water, alcohols and ethers, but not soluble in apolar solvents such as chloroform. The results obtained from the calibration curve expressed as mmol CumOOH equivalents per kg of sample (oil or fish wet weight) were transformed in meq $O_2 \cdot kg^{-1}$ sample by multiplying the former by a factor of 2 (Shantha & Decker, 1994).

For fluorescence imaging, the samples and CumOOH-standards were placed on glass microscope slides with a Pasteur pipette after incubation with DPPP, covered by cover slips and directly visualized on a Zeiss Axio Imager Upright microscope equipped with Zeiss Plan-Apochromat 10 × objective and AxioCam ERc5s with a filter set 49 DAPI (EX 365/50, FT 395, EM 445/70). The acquired micrographs were processed using a classical image-processing protocol of the ImageJ software, and the total fluorescence intensity was measured, as described in Cropotova, Mozuraityte, Standal, and Rustad (2018).

Lipid and protein hydroperoxide contents were determined from standard curves prepared on the basis of CumOOH-standards, and were compared with the results obtained by the DPPP-microplate assay.

2.3. Statistical analysis

All experiments were conducted in triplicate, and data were reported as the mean ± standard deviation. Simple correlations among the studied parameters were evaluated by the Pearson coefficient by using SigmaPlot software, version 14 (Systat 183 Software Inc. USA). When appropriate, the developed fluorometric assay was compared with the conventional methods of analysis of peroxide value using a paired Student's *t*-test. The criterion for significance was set at $P < 0.05$.

Linear regression analysis was performed to validate the method performance by comparing the lipid hydroperoxide content expressed as peroxide value determined by the standard iodometric titration method with the lipid hydroperoxide content measured by the optimized DPPP-assay.

For validation of the DPPP-assay for determination of protein hydroperoxides, a regression analysis of fish protein extracts prepared with addition of different amounts of CumOOH starting from 0.0 to 2.5 nmols in 100 μL of methanol media, was carried out. In the method validation, a fresh cod mince specially selected with the initially lowest content of protein hydroperoxides (< 1 meq $O_2 \cdot kg^{-1}$ fish), was used. This helped to identify the recoveries of the amounts of CumOOH standard added to protein extract in methanol obtained from cod mince, as well as limits of detection. It has been decided that if the recoveries at the used levels of addition of CumOOH to the protein extract are high enough (< 85%), the proposed DPPP-assay possesses sufficient accuracy and can be used in the analysis of protein hydroperoxides (AOAC, 1993; Bou et al., 2010).

3. Results and discussion

The following experimental parameters were considered for method optimization in the study: reaction time and method sensitivity and precision. The reaction time varies over a wide range depending on the incubation temperature and the type of solvent and hydroperoxides (lipo- or water-soluble) present (Bou et al., 2010; Santas et al., 2014), and thus was checked for both methanol and chloroform media. The method sensitivity and precision depend on the linearity region of the calibration curves and the relative standard deviation (RSD) of the fluorescence intensity signal arisen from DPPP-standard reacted with lipid and protein hydroperoxides within this linearity range. Therefore, the linearity region of the calibration curves for determination of lipid and protein hydroperoxides in chloroform and methanol media, respectively, was determined along with RSD of the fluorescence signal.

3.1. Reaction time

The reaction time was tested for DPPP in each of the solvents separately. It was revealed that in both 100% methanol and 100% chloroform media, the reaction with DPPP reached a plateau after 90 min of incubation at 60 °C (Fig. 1). The signal was stable from 90 to 180 min for DPPP in both methanol and chloroform solvents. A number of methods for hydroperoxides determination with the use of DPPP-dye suggest performing the reaction for 60 min at 60 °C (Akasaka et al., 1992; Bou et al., 2010). However, the reaction time may vary depending on the type of solvent and hydroperoxides (lipo- or water-soluble) to be determined (Bou et al., 2010; Santas et al., 2014). Therefore, based on previous studies, it has been decided to perform the measurements after longer incubation times to ensure the completion of the reaction (Bou et al., 2010; Santas et al., 2014). Thus, a reaction duration of 120 min was selected as optimal for the optimized DPPP-method for determination of both lipid and protein hydroperoxides.

3.2. Linearity of the calibration curves and method sensitivity

The linearity of the method was checked through preparation of calibration curves made for determination of lipid (lipo-soluble) and protein (water-soluble) hydroperoxides in different media with the use of different amounts of the standard (CumOOH). The experimental trials revealed, that for lipo-soluble hydroperoxides, the reaction was linear (R = 0.998) from 0.0 to 70.0 meq $O_2 \cdot L^{-1}$ by using CumOOH standard in chloroform media. A high correlation coefficient (R = 0.996) was also obtained for the linear segment of the calibration curve ranging from 0.0 to 50.0 meq $O_2 \cdot L^{-1}$ for determination of water-soluble hydroperoxides in methanol media. The detection limits for both lipo- and water-soluble hydroperoxides are in close agreement with the previously reported DPPP-based assays developed for edible oils and food products (Akasaka et al., 1992; Bou et al., 2010).

3.3. Precision of the method

For the calibration curve made for determination of lipo-soluble hydroperoxides, the relative standard deviation (RSD) of the fluorescence intensity signal arisen from CumOOH standard was within the linearity range of the method, ranging from 1.2% to 4.6% (n = 6). While for the DPPP-assay, the reproducibility of calibration curve used for the measurement of water-soluble hydroperoxides, ranged from 2.2% to 5.7%. Thus, according to AOAC recommendations, the precision of the DPPP-method proposed for simultaneous determination of lipid and protein hydroperoxides can be considered satisfactory (AOAC,

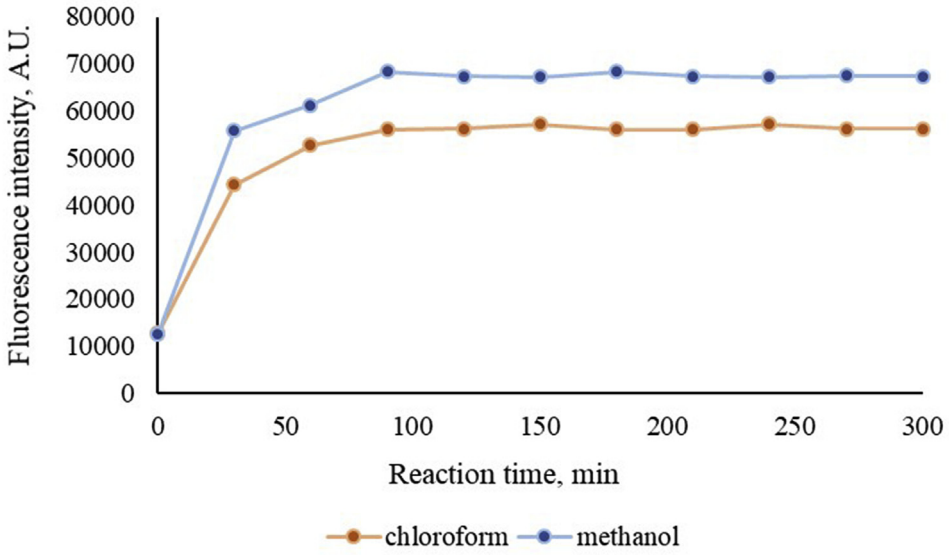

Figure 1. Kinetic reaction of DPPP with cumene hydroperoxide after incubation at 60 °C in chloroform and methanol media (example is shown for 2.0 nmol cumene hydroperoxide in 100 μL solvent).

Table 1
Lipid and protein hydroperoxides determined in fish samples by conventional iodometric method (lipid hydroperoxides) and modified fluorimetric assay based on DPPP-probe.

Sample type	Lipid hydroperoxide content (meq $O_2 \cdot kg^{-1}$ lipid)			Protein hydroperoxide content (meq $O_2 \cdot g^{-1}$ fish)	
	determined by iodometric titration	determined by DPPP-assay in microplate format	determined by DPPP-assay in microplate format	determined by DPPP-assay in microplate format	determined by DPPP-assay in microplate format
1-month storage					
Atlantic herring	3.18 ± 0.51	2.96 ± 0.12	2.86 ± 0.14	3.47 ± 0.21	3.52 ± 0.16
Atlantic mackerel	5.52 ± 0.23	5.03 ± 0.11	5.11 ± 0.25	7.18 ± 1.24	7.03 ± 0.92
Rainbow Trout	0.61 ± 0.09	0.74 ± 0.08	0.58 ± 0.18	1.23 ± 0.44	1.09 ± 0.61
Atlantic salmon	0.87 ± 0.14	0.76 ± 0.05	0.93 ± 0.23	1.12 ± 0.71	1.24 ± 0.22
3-month storage					
Atlantic herring	5.89 ± 1.07	5.11 ± 1.02	5.24 ± 1.04	22.13 ± 3.25	22.35 ± 2.92
Atlantic mackerel	8.31 ± 2.42	7.75 ± 1.71	7.92 ± 0.95	31.72 ± 4.21	31.43 ± 3.11
Rainbow Trout	1.23 ± 0.74	1.04 ± 0.51	1.58 ± 0.58	12.46 ± 2.84	11.98 ± 1.83
Atlantic salmon	1.16 ± 0.21	1.34 ± 0.32	1.23 ± 0.53	15.63 ± 3.17	15.12 ± 2.74
6-month storage					
Atlantic herring	12.28 ± 2.61	11.79 ± 1.02	11.97 ± 1.64	38.52 ± 5.51	39.01 ± 3.92
Atlantic mackerel	11.62 ± 1.35	10.85 ± 0.91	11.22 ± 0.95	92.17 ± 4.04	90.83 ± 3.51
Rainbow Trout	6.84 ± 2.04	6.32 ± 1.11	6.38 ± 1.08	24.37 ± 7.82	25.04 ± 6.01
Atlantic salmon	4.33 ± 0.81	3.94 ± 0.72	4.11 ± 0.53	36.11 ± 3.36	36.94 ± 3.96

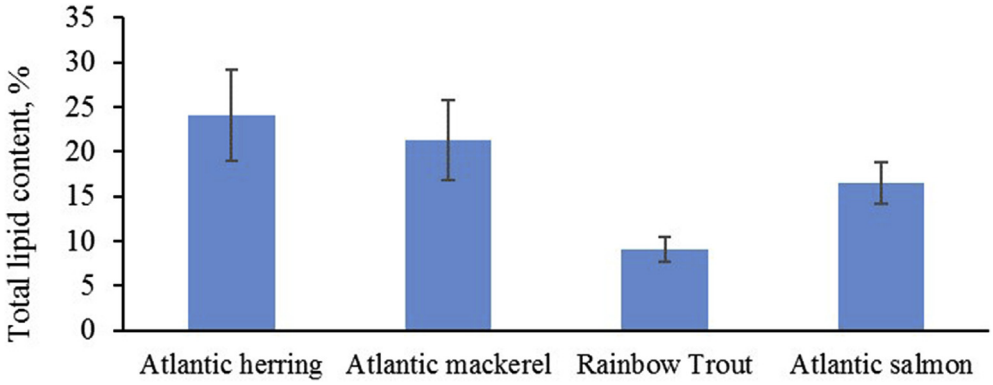

Fig. 2. Total lipid content in experimental fish samples.

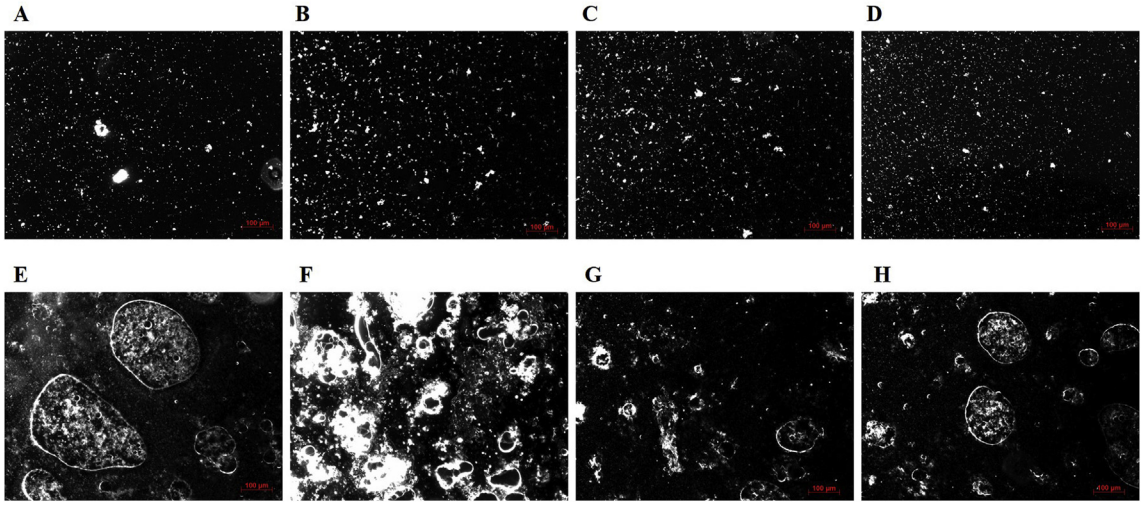

Fig. 3. Fluorescence visualization of lipid (A–D) and protein (E–H) hydroperoxides in chloroform and methanol extracts of fish samples after 6-month frozen storage: A, E – Atlantic herring; B, F – Atlantic mackerel; C,G – Rainbow Trout; D, H – Atlantic salmon. Scale bar length is 100 μm.

1993).

3.4. Method recovery and validation

For lipid hydroperoxides measured in a microplate format, the obtained results displayed a good correlation ($R = 0.998$, $p < 0.05$) between lipid hydroperoxide content measured by the proposed DPPP-assay and peroxide value determined by standard iodometric titration (Table 1).

According to the obtained results (Table 1), herring and mackerel samples were characterized with significantly ($p < 0.05$) higher lipid peroxide values compared to salmon and trout during the frozen storage. This tendency can be explained by a significantly higher lipid content in herring and mackerel compared to salmon and trout (Fig. 2), and respectively higher amounts of polyunsaturated fatty acids prone to oxidation (Frankel, 1998; Shahidi & Zhong, 2010).

Mincing and subsequent frozen storage of the fish minces resulted in the muscle cell damage leading to release of pro-oxidants accelerating oxidation, as well as lipid oxidation products from the rupture of adipocytes (Standal et al., 2018). Taking into account that Atlantic herring and mackerel are richest in dark muscle containing haem-proteins, their mincing and freezing may also result in increased lipid peroxidation due to denaturation of myoglobin and also release of free iron (Tokur & Korkmaz, 2007).

The recoveries determined by increasing water-soluble hydroperoxide content through addition of an appropriate amount of CumOOH to protein extracts obtained from cod mince were 94–97% (data not shown), suggesting high recovery of the method according to AOAC recommendations (AOAC, 1993). This proved that CumOOH-standard can reliably be used to construct a calibration curve for determination of water-soluble hydroperoxides in protein extracts. The amount of water-soluble hydroperoxides determined in protein extracts of fish minces displayed the same trend as for lipo-soluble hydroperoxides. Thus, herring and mackerel accounted for significantly higher protein hydroperoxide content compared to salmon and trout (Table 1).

As mentioned above, mincing and freezing caused rupture of fish cell membranes, leading to liberation of lipid oxidation products and pro-oxidants that further interacted with proteins, resulting in protein peroxidation. Generally, free radicals not only attack lipid membranes of cells causing lipid peroxidation, but also target membrane proteins, leading to lipid-protein, lipid-lipid and protein-protein oxidation and cross-linking (Du & Gebicki, 2004; Griffiths, 2000). Primary lipid and protein oxidation reactions yield lipo- and water-soluble hydroperoxides, respectively, further resulting in a variety of secondary low-molecular-weight compounds (Frankel, 1998). The secondary lipid and protein oxidation products lead to a decrease in sensory and nutritional quality of muscle foods such as fish or meat. Therefore, it is important to measure protein hydroperoxides along with lipid hydroperoxides in a product to assess lipid and protein oxidation at a propagation step to see the tendency towards further oxidation reactions.

To check the repeatability of results and validate the developed fluorometric assay for fluorescence microscopy format, the DPPP-treated lipid and protein extracts of fish minces and CumOOH-standards were transferred from a 96-well microplate on glass microscope slides, covered by cover slips and directly visualized on a Zeiss Axio Imager Upright microscope as described in *Materials and Methods*. Lipid and protein hydroperoxide contents were determined from the standard curves constructed based on mean fluorescence intensity coming from CumOOH-standards, and the obtained results were compared with the DPPP-microplate assay. The Pearson's correlation coefficients between the DPPP methods performed in microplate format and fluorescence microscopy format for determination of lipid ($R = 0.998$, $p < 0.05$) and protein ($R = 0.999$, $p < 0.05$) hydroperoxides, proved that the modified DPPP-assay for fluorescence microscopy format has a high degree of accuracy. The mean values of lipo- and water-soluble hydroperoxides for each of the fish sample obtained by the DPPP-assay performed in fluorescence microscopy format are shown in Table 1. It is clearly seen that lipid hydroperoxide values determined by the DPPP-assay conducted in fluorescence microscopy format closely agree with those obtained by the standard iodometric titration, suggesting the high precision of the method.

The microscopic visualization of lipid and protein hydroperoxides is displayed in Fig. 3.

The lipid and protein hydroperoxides are fluorescent on the dark background of the displayed micrographs. It is clearly seen that the amount and fluorescence intensity of the both lipo- and water-soluble hydroperoxides displayed in the fluorescence micrographs (Fig. 3) are gradually increasing during frozen storage. This phenomenon can be explained by the negative effect of frozen storage on lipid and protein oxidation in the analyzed fish minces due to a greater membrane damage over time (Standal et al., 2018). Thus, freezing favors the rupture of the cell membranes liberating various pro-oxidants that further come in contact with lipids and proteins generating lipo- and water-soluble hydroperoxides.

4. Conclusion

The present study has shown that a novel fluorimetric assay based

on stoichiometric reaction of DPPP with lipid and protein hydroperoxides yielding fluorescent DPPP oxide, is a reliable, fast and sensitive method able to detect, visualize and quantify both lipo- and water-soluble hydroperoxides in muscle foods such as fish samples. It has been revealed that the developed fluorimetric assay and the standard iodometric titration method displayed similar results for lipid peroxides in different fish samples analyzed during frozen storage, indicating high repeatability and validation of experimental data.

The main benefits of the assay are: rapidness, high accuracy and reproducibility, and simplicity as it can be performed both in a microplate reader or fluorescence microscopy format, depending on equipment availability and requirements for relative or absolute quantification. Moreover, the modified DPPP-assay requires low sample, standard and solvent amounts, which can be extremely useful in analysis of lipid and protein hydroperoxides in lean fish or meat, as well as protein hydrolysates.

CRediT authorship contribution statement

Janna Cropotova: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Turid Rustad:** Project administration, Resources, Writing - review & editing.

Declaration of competing interest

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

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