Measuring primary lipid oxidation in food products enriched with colored microalgae

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Abstract

Microalgae are a valuable alternative source of n-3 LC-PUFA and have already proven their potential in different food products. However, enrichment of food products with n-3 LC-PUFA implies an increased sensitivity to lipid oxidation. Numerous analytical techniques have already been developed to determine and follow lipid oxidation. Photoautotrophic microalgae often contain, besides n-3 LC-PUFA, other compounds like carotenoids and chlorophylls. These colored compounds may interfere with the standard analytical techniques. This study contributes to optimize a simple and low-cost method to measure the degree of primary lipid oxidation in food products enriched with photoautotrophic microalgae. The standard iodometric titration and three spectrophotometric methods (FOX, IDF and CD & CT) were investigated. The FOX method was selected as the preferable method, although interference due to the presence of metal ions could occur. This could partially be solved by a supplemental step with TPP addition. However, as this additional step did not change the trend in oxidative values during storage, it was suggested to normalize the values of the FOX method to week zero to investigate the trend of oxidation during storage of products enriched with photoautotrophic microalgae.

Keywords

photoautotrophic microalgae, colored food product, peroxide value, lipids, hydroperoxide determination

Abbreviations

CD & CT	conjugated dienes & conjugated trienes
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FOX method	ferrous oxidation xylenol orange method
IDF method	ferric thiocyanate based international dairy federation method
n-3 LC-PUFA	omega-3 long chain poly unsaturated fatty acids
TPP	triphenylphosphine

1. Introduction

Microalgae, rich in omega-3 long chain poly-unsaturated fatty acids (n-3 LC-PUFA), could provide a sustainable, vegetarian alternative for fish and fish oil (Ryckebosch et al., 2012a). Microalgae are unicellular organisms and are the primary producers of n-3 LC-PUFA, which are mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Morales-Sánchez et al., 2015; Rawat et al., 2013). Microalgae have already proven their potential in different food products (Gheysen et al., 2018b).

Unsaturated lipids, in particular those containing n-3 LC-PUFA, are highly susceptible to lipid oxidation due their chemical structure, which includes a large number of double bounds. Lipid oxidation leads to an undesired and rancid flavor, with a low threshold value (Frankel, 2005). Furthermore, the nutritional value related to n-3 LC-PUFA is reduced (Ryckebosch et al., 2013) and toxic compounds, linked to the development of inflammatory diseases, cancer and atherosclerosis, can be formed (Vieira et al., 2017).

Different forms of lipid oxidation can occur: autoxidation, photo-oxidation and enzymatic oxidation. In each of these processes, hydroperoxides are however formed as primary oxidation products. These products are subsequently transformed into a broad range of secondary oxidation products (Frankel, 2005; Schaich et al., 2013).

Numerous analytical methods, covering different pathways of the complex oxidation process, are available and can be classified in five groups based on what they measure: the absorption of oxygen, the loss of initial substrate, the formation of free radicals, primary oxidation products and secondary oxidation products. The most commonly used analytical methods belong to the latter two groups. They can be divided in more simple and low-cost methods, such as titration or spectrometry, and more complex methods, such as chromatography, fourier-transform infrared spectrometry and nuclear magnetic resonance spectrometry (Antolovich et al., 2002; Barriuso et al., 2013; Shahidi and Zhong, 2005).

This study contributes to create a simple and low-cost method to measure the degree of primary lipid oxidation in products enriched with microalgae. Microalgae often contain carotenoids and chlorophylls (Ryckebosch et al., 2012a) and these colored compounds may interfere with the standard analytical techniques, warranting this study. By extension, this method can be used for other food products rich in carotenoids and chlorophylls, without the presence of microalgae.

The iodometric assay, a titration method, is the standard method for the determination of the amount of hydroperoxides expressed as the peroxide value. This method is based on the reduction of lipid hydroperoxides with potassium iodide in an acidic solution with the formation of iodine. The formed iodine is then titrated with a standardized solution of sodium thiosulfate. Starch is often used to indicate the endpoint of the titration. The simplicity is the biggest asset of this method, which is however time-consuming and labor-intensive. Moreover, it requires a large amount of sample and creates a significant amount of chemical waste (Barriuso et al., 2013; Frankel, 2005; Shahidi and Zhong, 2005).

In addition to the iodometric assay, several spectrometric assays have been developed to measure the peroxide value. The ferrous oxidation xylenol orange (FOX) and the ferric thiocyanate based International Dairy Federation (IDF) method are based on the same principle. Hydroperoxides initiate the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) in an acidic medium. Ferric ions form a blue-purple colored complex with xylenol orange, in case of the FOX method, or a red colored complex with thiocyanate, in case of the IDF method. The intensity of the color formation is measured spectrophotometrically at 560 and 500 nm respectively (Bou et al., 2008; Nielsen et al., 2003). Apart from hydroperoxides, conjugated dienes (CD) and trienes (CT) are also formed during the primary oxidation and they can be detected by their absorption peak at 234 and 268 nm respectively. A good correlation between the amount of conjugated dienes and the peroxide value has been found (Farhoosh and Moosavi, 2009; Marmesat et al., 2009).

The amount of sample needed for the spectrophotometric methods is lower compared to titration methods. As the detection is less arbitrary, the reproducibility is higher compared to the iodometric titration. Interference of compounds absorbing at the wavelength of measuring or compounds also forming a complex with xylenol orange or thiocyanate might occur (Frankel, 2005; Shahidi and Zhong, 2005).

To develop a simple and low-cost method to measure the primary lipid oxidation of food products enriched with photoautotrophic microalgae, the amount of primary oxidation products of an aqueous suspension enriched with photoautotrophic microalgal biomass was measured by the iodometric titration, FOX, IDF and CD & CT assays. Each of these methods was applied to the lipids which were first extracted from the sample. During this extraction undesired extra oxidation might occur, a possibility that was also investigated in this study as well as oxidation during the measurement itself. Moreover, interference from ferrous and ferric ions was also investigated.

2. Materials and Methods

2.1. Sample preparation

2.1.1. Microalgal biomass

A photoautotrophic brown microalga, *Isochrysis* sp., was selected to screen four simple and low-cost methods to measure the primary lipid oxidation in enriched suspensions. It was obtained as freeze dried biomass from Proviron (Hemiksem, Belgium).

Commercial, and thus refined, fish oil, which was used to obtain more insight in the impact of ferrous and ferric ion addition, was obtained from Inve (Dendermonde, Belgium). The heterotrophic microalga *Schizochytrium* sp., which is more prone to oxidation (Gheysen et al., 2018a), was selected to obtain more insight in the possible occurrence of oxidation of enriched suspensions during lipid extraction and during measuring the amount of hydroperoxides. The freeze dried biomass was obtained from Mara Renewables Corporation (Dartmouth, Canada).

2.1.2. Preparation of enriched suspensions

The aqueous suspension enriched with microalgal biomass was prepared, treated, freeze dried and stored as described in Gheysen et al. (2018a).

2.2. Analyses

2.2.1. Lipid extraction

The lipids were extracted from the freeze dried suspension according to Ryckebosch et al. (2012b) with the modification described in Gheysen et al. (2018a). Briefly, to 100 mg freeze dried suspension 4 mL methanol, 2 mL chloroform and 0.4 mL water were added. After vortexing the sample, 2 mL chloroform and 2 mL water were additionally added, followed by vortexing and centrifugation. The upper phase was removed and the lower organic layer was kept in a clean tube. The remaining pellet was re-extracted with 4 mL chloroform/methanol (1/1), vortexed and centrifuged. The extract was pooled with the first extract. The previously described steps were repeated a second time whereby the solvent

phases were pooled with those of the first extraction. The extract was filtered through a filter paper (Whatmann n°1, Sigma Aldrich, Bornem, Belgium) with a sodium sulphate layer and the filter was washed with chloroform/methanol (1/1). The solvents were removed by rotary evaporation.

For the **lipid extraction with argon flushing** the previously described method was adapted by deoxygenation of the solvents (chloroform, methanol, water and chloroform/methanol (1/1)) by flushing with argon during 30 min prior to use in the extraction.

For the **lipid extraction with butylated hydroxytoluene (BHT) addition,** 4 mM of the synthetic antioxidant BHT was added to the extraction solvents (chloroform, methanol and chloroform/methanol (1/1)), prior to use in the extraction.

2.2.2. Iodometric titration

The iodometric titration was performed according to AOCS method Cd 8-53 (AOCS, 2003).

2.2.3. FOX method

The determination of the primary oxidation products using the FOX method, also named the FOX-2 method was performed according to Wrolstad et al. (2005) with some slight modifications. In brief, 10 mg extracted lipids, obtained according to section **2.2.1**, were dissolved in chloroform/methanol 7/3 (9.9 mL) and this solution was diluted 1/10 (0.99 mL dissolved extract + 8.91 mL chloroform/methanol 7/3). The absorbance of the diluted sample (A_{DS}) was measured at 560 nm. This is necessary to remove the background noise of the sample (especially for food products enriched with colored microalgae) and is often not implemented in the current methodologies. Subsequently, xylenol orange (Sigma-Aldrich, Bornem, Belgium) (50 µL; 10 mM) and Fe^{2+} chloride solution (50 µl; 18 mM, acidified with HCl (1 µL; 10 M)) were added. The absorbance of the sample (A_S) was measured at 560 nm after exactly 5 min of incubation at room temperature. A blank of the Fe^{2+} chloride solution (A_B) was measured to determine the freshness of the Fe^{2+} solution and thus to prevent the use of a Fe^{2+} solution whereby Fe^{2+} is already oxidized to Fe^{3+}. A Fe^{3+} chloride standard solution (10 µg/mL) was prepared to set up

the calibration curve of Fe^{3+} (zero to 3.1 µg Fe^{3+}/mL) versus absorbance (zero to 0.9). Samples with an absorbance outside this range were diluted additionally. The PV value was calculated by following equation:

$$PV = \frac{[(A_S - A_{DS} - A_B) * m_i]}{W * 55.84 * 2}$$

where A_S is the absorbance of the sample, A_{DS} is the absorbance of the diluted sample to measure the background noise, A_B is the absorbance of the blank, m_i is the inverse of the slope of the calibration curve, W is the mass of the sample (g), 55.84 is the atomic mass of Fe^{3+}. In the numerator of the equation, the residual absorbance (which is thus the absorbance corrected for the absorbance of background noise and blank) is multiplied by the inverse of the slope resulting in the corresponding amount of Fe^{3+} (mass). This value is divided by the molecular mass of iron (55.84 g/mole) and the sample mass, resulting in a value expressed as mmole Fe^{3+}/kg lipids. Based on the chemical reaction of the method, each mole lipid hydroperoxide can transform two mole Fe^{2+} into two mole Fe^{3+}. Therefore, the value is divided by two (factor 2 in the equation). This results in the final peroxide value, expressed as meq hydroperoxides/kg lipids.

2.2.4. Adapted FOX methods

a. FOX method with triphenylphosphine (TPP) addition

The adaptation of the FOX method with TPP was performed according to Nourooz-Zadeh et al. (1994) with some slight adaptations. Instead of adding 8.91 ml chloroform/methanol (7/3) to 0.99 mL of the dissolved sample as described in section **2.2.3**, 7.91 mL chloroform/methanol (7/3) was added together with 1 mL, 10 mM TPP. The mixture was kept at room temperature for 15 min, to allow the hydroperoxides to react with TPP. This step was followed by adding xylenol orange and Fe^{2+} chloride solution as described earlier in section **2.2.3**.

Based on the equation below, the corrected peroxide value can be calculated:

$$PV_{\text{without TPP}} - PV_{\text{with TPP}} = PV_{\text{corrected for interference}}$$

With $PV_{\text{without TPP}}$, the peroxide value obtained via the standard FOX method (section 2.2.3) and $PV_{\text{with TPP}}$, the peroxide value obtained via the TPP adapted FOX method (section 2.2.4.a).

b. FOX method with argon flushing

The FOX method (section **2.2.3**) was adapted by deoxygenation of the solvent (chloroform/methanol (7/3)) by flushing with argon during 30 min prior to use.

c. FOX method with BHT addition

BHT (4 mM) was added to the chloroform/methanol (7/3) mixture which was used in the method described in section **2.2.3**.

2.2.5. IDF assay

The IDF assay was performed according to Shantha and Decker (1994). The method is comparable with the FOX method described in section **2.2.3** except for some slight differences. Ammonium thiocyanate solution was used instead of xylenol orange. To prepare this solution 30 g ammonium thiocyanate (Carl Roth, Karlsruhe, Germany) was weighed and water was added to a volume of 100 mL. Furthermore the complex formation was measured at a wavelength of 500 nm.

2.2.6. CD & CT assay

The CD & CT assay was performed according to the IUPAC method described by Abuzaytoun and Shahidi (2006). The extracted lipids (10 mg, section **2.2.1**) were dissolved in iso-octane (10 mL) and 3 mL of this solution was brought into a quartz cuvet. The absorbance of the CD & CT was measured spectrophotometrically at 234 and 268 nm respectively. A blank of iso-octane (3 mL) was used as reference in the spectrophotometer. The amount of CD & CT was calculated based on the following equation:

$$\text{CD or CT} = (A * F)/(W*a)$$

Where A represents the absorbance at 234 or 268 nm, F is the dilution factor, W is the mass of the sample (kg) and a is the slope of the calibration curve. Calibration curves were based on conjugated linoleic acid and conjugated linolenic acid (Larodan, Solna, Sweden), for conjugated dienes and trienes respectively.

2.2.7. Determination of secondary oxidation

Volatile secondary oxidation products were analyzed with head space solid phase micro-extraction gas chromatography coupled with mass spectrometry (HS-SPME GC-MS) according to Giri et al. (2010) with the modifications described by Gheysen et al. (2018a).

2.2.8. Determination of metals

The determination of metals was based on the method of Ashoka et al. (2009). Briefly, extracted lipids (10 mg) were ashed overnight in a muffle oven at 550 °C. The ash was subsequently dissolved in 10 mL 50% nitric acid. The metal concentration was measured with inductively coupled plasma optical emission spectrometry (ICP-OES) (Varian, PTY LTD, Australia). For each of the screened metals an external calibration curve was set up. The amount of metals was expressed as µg/mg extracted lipids.

2.3. Statistical analysis

Results were statistically evaluated by a one way ANOVA (analysis of variance). The differences were determined by a *post hoc* Tukey test. All statistical tests were performed with $\alpha = 0.05$ (JMP Pro 12.1, SAS Institute Inc.).

3. Results and discussion

3.1. Comparison of four low-cost assays

The formation of primary lipid oxidation products in an aqueous suspension enriched with a photoautotrophic microalga was followed during 12 weeks of storage of 37°C. Determination of peroxide value based on the standard iodometric titration failed as the color change was not visible due to color of the suspension. Therefore, no results of this assay could be shown. No further adaptations to this method were investigated as the visual determination of the endpoint is always difficult in colored samples and a large sample size is required. **Figure 1** shows the amount of primary oxidation products formed over time as measured by three different spectrophotometric assays. A comparable trend could be observed for the FOX and IDF method (**Figure 1a**), which is logic as the measurement of hydroperoxides is in both techniques based on the same principle. An increase over time was observed which implies the initiation of lipid oxidation during storage. On the other hand, no remarkable changes in the conjugated compounds were observed in the CD & CT measurements (**Figure 1b**). A high value was observed at week zero, after which the amount of conjugated compounds remained the same. This may indicate that during storage no further lipid oxidation occurred or that the peak maximum of the primary oxidation was located between two measuring points. However, both hypothesis were clearly aberrant from the conclusion based on the FOX and IDF method. To decide which of both observed trends was more likely, secondary lipid oxidation products were measured by HS-SPME GC-MS (**Figure 1c**). The volatile compounds showed an increase over storage time. These results confirmed the trend measured by the FOX and IDF method. The CD & CT method did not seem suitable for colored samples. Possibly, other conjugated compounds, like carotenoids and phenols, in the lipid extract of aqueous suspensions enriched with photoautotrophic microalgae interfere with this method and possibly explain the high value of CD & CT at week zero (Orefice et al., 2015).

No significant differences between the values obtained via the FOX and IDF method could be observed at none of the measured time points, which was expected as both techniques are based on a comparable mechanism. This also implies that compounds interfering with the reaction mechanism have the same impact on both analytical techniques. Measuring the background noise of the sample (A_{DS}) and using it to correct the value, was an important improvement of the methodology especially for food products enriched with colored microalgae. The value of this background noise was however different for both techniques as the selected wavelength of measurement was different. The signal-to-noise ratio, calculated as the signal of the sample over the signal of the background, was respectively ¼ and 3 for the IDF and FOX method at week zero. This difference can be explained by the higher absorption of other compounds, for example carotenoids, at a wavelength of 500 nm (IDF method) compared to 560 nm (FOX method). Although the results were corrected for the background noise, the lower signal-to-noise ratio may lead to an increase of the detection limit and a lower reproducibility. A higher relative standard deviation (although not significant, F-test, $\alpha = 0.05$) was indeed observed for the IDF (with an average of 23.3%) compared to the FOX method (with an average of 9.3%). Therefore, the FOX method was selected as the method of choice for colored suspensions enriched with photoautotrophic microalgae.

3.2. Insight in the interference of the FOX method

In **Figure 1a**, it could be observed that even after the correction for the background noise, the peroxide value at week zero was already 20 meq hydroperoxides/kg lipids. This can imply that the oxidation in the sample or during extraction already started or that other compounds interfere with the method as has been reported by Bou et al. (2008), who suggested pigments, ascorbic acid, free iron and proteins as the main interfering compounds. Although pigments have often been suggested as interference of the FOX method (Hornero-Méndez et al., 2001; Ryckebosch et al., 2013), they were not a causative factor for the interference in this study as the peroxide value was already corrected for this background noise. The FOX method was performed on a lipid extract of the enriched suspension (section **2.2.3**),

and as such it can be assumed that the more water-soluble compounds, ascorbic acid and proteins, were removed by the counter extraction with water. The presence of metal ions in the lipid extract was further investigated in section **3.2.1**.

3.2.1. Presence of metal ions

The incorporation of microalgal biomass can imply the addition of metal ions like iron, magnesium, calcium, zinc, etc. originating from their cultivation medium. Despite that the metal ions from the cultivation medium can be co-harvested with the biomass, it is not necessarily so that these ions interfere with the FOX method as prior to this assay a lipid extraction was performed. Therefore, ICP-OES was performed on the lipid extract of the suspensions enriched with *Isochrysis*. The extracted lipids contained amounts of calcium (15.31 ± 0.03 µg/mg lipid), iron (0.084 ± 0.004 µg/mg lipid), magnesium (9.75 ± 0.03 µg/mg lipid), manganese (0.83 ± 0.03 µg/mg lipid) and zinc (0.80 ± 0.02 µg/mg lipid).

To obtain more insight in the impact of iron on the FOX method, commercial, and thus refined and free from endogenous amounts of metals, fish oil was used. **Figure 2** shows the impact of Fe^{3+} and Fe^{2+} addition to a fresh fish oil sample. Addition of low amounts of Fe^{3+} (1.7 and 3.4 µg/mg fish oil respectively) resulted in a significant increase in peroxide value, which was linear with the added amount. In contrast, addition of similar amounts of Fe^{2+} (0.9 and 1.8 µg/mg fish oil respectively) had no significant impact on the peroxide value, although a slight increase could be visually observed. These results can be explained by the higher reaction rate of xylenol orange with Fe^{3+} compared to Fe^{2+} (Gay et al., 1999). Bou et al. (2008) suggested free iron as interfering metal, because an iron-xylenol orange complex is formed in the FOX method. This complex formation is however not selective for iron as xylenol orange also forms complexes with a large number of other metal ions depending on the pH and the ion charge density (Brajter and Olbrych-Śleszyńska, 1983; Belleza and Villaraza, 2014). Amongst others Zn^{2+}, Tb^{3+}, Gd^{3+}, Cu^{2+}, Ni^{2+}, Co^{2+}, etc. have been reported as good complex formers with xylenol orange while Ca^{2+}, Mn^{2+}, etc. react weakly with xylenol orange (Belleza and Villaraza, 2014; Bou

et al., 2008; Colston and Robinson, 1997; Mizuguchi and Yotsuyanagi, 2001). The presence of the metal ions observed in the lipid extract can thus possibly (partially) explain the elevated peroxide value on week zero.

3.2.2. Reduction of interference

As seen in section **3.2.1** the presence of metal ions can interfere with the FOX method and can thus overestimate the amount of hydroperoxides. Ideally, this interference should be removed. The addition of triphenylphosphine (TPP) to the FOX method can help to eliminate the interference. TPP forms a complex with the hydroperoxides present in the sample to form TPPO (Gotoh et al., 2011; Wang et al., 2016). By adding an excess of TPP, none of the hydroperoxides can oxidize Fe^{2+} to Fe^{3+}, and thus lead to an increase in the complex formation of xylenol. In the presence of TPP, all the remaining absorption is thus caused by the presence of interfering compounds like metals, etc. (Nourooz-Zadeh et al., 1994; Södergren et al., 1998).

Figure 3 shows the impact of the addition of a supplemental step with TPP during the FOX method. The adaptation of the FOX method with TPP indeed reduced the amount of hydroperoxides measured in the sample. This suggests that the hypothesis from section **3.2.1** that amongst others the presence of metal ions resulted in an elevated peroxide value and thus an overestimation of the peroxide value. Analogous results were observed by using the modified FOX method with TPP on plant based materials (spinach, avocado, potato, etc.) (DeLong et al., 2002).

It was however observed that both methods, with and without TPP, showed almost the same trend. Therefore it could be concluded that the interference only had an impact on the absolute value, but not on the trend observed during storage. Besides it was seen that the peroxide value was not reduced to zero by the adaptation of the method with TPP. This indicated that possibly the extracted oil was already oxidized, possibly during the extraction of lipids or during the FOX method itself. This was investigated in section **3.3**.

3.3. Oxidation during analysis

As suggested in the previous section the oil could possibly be oxidized during extraction or during measuring the peroxide value. Therefore an aqueous suspension enriched with the heterotrophic microalga *Schizochytrium* was used. This enriched suspension is more sensitive to lipid oxidation compared to the suspensions used in section **3.1** and **3.2** (Gheysen et al., 2018a), and therefore more suitable to study the impact of adaptations to the extraction method and analysis on lipid oxidation. After all, if in a sample prone to oxidation, no oxidation occur during extraction or measuring the peroxide value this will also not be the case in a sample less prone to oxidation.

3.3.1. Argon flushing

Figure 4 shows the impact of argon flushing during lipid extraction and during the FOX method on the peroxide value. Argon flushing replaces dissolved oxygen from the solvents and extracted lipids. By removing oxygen, the lipid oxidation process is delayed (Schaich, 2016). No significant differences in peroxide value between samples with and without argon flushing were observed. This indicated that no oxidation occurred during the extraction of the lipids, or during the FOX method and thus that the preventive action of flushing with argon has no additional value. To the best of our knowledge, no results about the impact of argon flushing on the lipid extraction and on the FOX method have been described earlier.

3.3.2. BHT addition

BHT, a synthetic antioxidant, can inhibit lipid oxidation as a chain breaking antioxidant (Jacobsen et al., 2013). **Figure 5a** shows the impact of BHT addition during lipid extraction and during the FOX method on the peroxide value. It can be observed that addition of BHT during lipid extraction reduced the peroxide value significantly, while no impact of BHT addition during the FOX method was observed. More detailed analysis of the lipid extraction however showed that BHT addition during extraction also significantly increased the total lipid content (**Figure 5b**). The total extracted lipids increased from 72%

to 102%, which is above the theoretical maximum lipid content of 100%, and much higher than the expected value for the freeze dried suspension. This increased lipid content could be explained by a co-extraction of BHT with the lipids, implying that not all compounds in the lipid extract were indeed lipids. This was proven by a blank extraction in the presence of BHT, in which more than 95% of the BHT was co-extracted and was does counted as 'lipids' (results not shown). This means that the amount of lipids used for the FOX method was underestimated, which explains the lower peroxide value obtained by BHT addition during extraction. A recalculation of the peroxide value based on the real amount of lipids in the extract (the obtained peroxide value divided by the following ratio: $\frac{Lipid\ content\ via\ extraction\ without\ BHT}{Lipid\ content\ via\ extraction\ with\ BHT}$) resulted in a value of 425 ± 10 meq hydroperoxides/kg lipids. From this, it could thus be concluded that the addition of BHT had no significant effect during lipid extraction. These results indicated that no oxidation occurred during the extraction of the lipids and thus that no further preventive actions were needed. No significant effect of BHT addition, and thus no oxidation, during the FOX method could be observed either, which was in line with the results of Södergren et al. (1998). The hypothesis, that the extracted oil was oxidized during the extraction of lipids or during the FOX method itself, suggested in section **3.2** could thus be rejected.

4. Conclusions

Different methods were screened for their potential in the determination of primary oxidation products in suspensions enriched with photoautotrophic microalgae. The standard iodometric titration was not a valuable technique as the color change was not visible in the colored sample and therefore this assay could not be used. The spectrophotometric methods, FOX, IDF and CD & CT, could be used for the determination of lipid hydroperoxides (FOX and IDF) or conjugated compounds (CD & CT). In contrast to the results obtained by the FOX and IDF method, the trend obtained with the CD & CT assay was not consistent with the results of the secondary oxidation. Therefore the CD & CT method was not selected for further optimization. Possibly the high interference of chromophore compounds with conjugated bounds has resulted in the aberrant trend. Only limited differences could be observed between the FOX and the IDF method as both methods are based on the same principle. Measuring the background noise of the sample to correct the obtained value improved the methodology by excluding interference of the color of the sample. This background noise was much higher for the IDF compared to the FOX method resulting in a higher (although not significant) relative standard deviation of the former. Based on this observation, the FOX method was selected as the preferred method for colored suspensions.

Despite the FOX method was selected, interference by the presence of metal ions occurred. This could be partially solved by the addition of a supplemental step whereby TPP was added to eliminate the interference. The trend observed in the standard peroxide value and peroxide value corrected with TPP showed almost the same trend. Therefore it could be concluded that the interference only had an impact on the absolute value, but not on the trend observed during storage. Furthermore in this study it was observed that enriched suspensions sensitive to lipid oxidation did not oxidize during lipid extraction nor during the FOX method and thus this could also not explain the higher than zero values at the first time point.

Therefore it is suggested, based on previous observations, to measure the lipid hydroperoxides in suspensions enriched with colored photoautotrophic microalgae with the FOX method after which a normalization on week zero can be implemented to investigate the oxidative stability during storage. This allows comparing the trend of different samples, irrespective of the interference. Further research should be executed to identify and eliminate all potential non-hydroperoxide absorbances and how to eliminate them.

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Conflict of interest

The authors have declared no conflict of interest.

Ethical approvel

No conflicts, informed consent, human or animal rights applicable.

Informed consent

Not applicable

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Figure captions

Figure 1: The amount of hydroperoxides (a) or conjugated compounds (b) of an aqueous suspension enriched with photoautorophic microalgae (Isochrysis) measured by the FOX, IDF (a) or CD & CT (b) method and the amount of secondary oxidation compounds (c).

Figure 2: The impact of Fe^{3+} and Fe^{2+} addition in a fresh fish oil sample on the peroxide value measured by the FOX method, expressed as meq hydroperoxides/kg lipids. Statistical differences are indicated with a different letter (α=0.05).

Figure 3: The impact of TPP addition on the peroxide value of an aqueous suspension enriched with photoautotrophic microalgae (Isochyrsis) measured by the FOX method, expressed as meq hydroperoxides/kg lipids.

Figure 4: Impact of argon flushing during extraction and FOX method on the peroxide value of an aqueous suspension enriched with heterotrophic microalgae (Schizochytrium), expressed as meq hydroperoxides/kg lipids. No statistical differences were observed (α=0.05).

Figure 5: Impact of BHT addition during extraction and FOX method on the peroxide value of an aqueous suspension enriched with heterotrophic microalgae (Schizochytrium), expressed as meq hydroperoxides/kg lipids (a). Impact of BHT addition during extraction on the total lipid content of an aqueous suspension enriched with heterotrophic microalgae (Schizochytrium), expressed as % (b). Statistical differences are indicated with a different letter (α=0.05).