

Measurement of Malondialdehyde as a Biomarker of Lipid Oxidation in Fish

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How to cite this paper: Rizzo, M. (2024) Measurement of Malondialdehyde as a Biomarker of Lipid Oxidation in Fish. *American Journal of Analytical Chemistry*, 15, 303-332.

<https://doi.org/10.4236/ajac.2024.159020>

Received: August 26, 2024

Accepted: September 26, 2024

Published: September 29, 2024

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Abstract

Oxidative reactions are key part of the stress response in marine organisms, which are exposed to a wide variety of environmental stressors. Lipid peroxidation (LPO) occurs in response to oxidant attack, giving rise to unsaturated aldehydes as malondialdehyde (MDA), the main biomarker for LPO assessment. Levels of MDA are measured in biological samples of different fish matrices to determine the oxidative effect of physical or chemical agents, particularly represented by metals. The most used assay is the spectrophotometric determination of thiobarbituric acid (TBA) as MDA-TBA₂ adduct. Selectivity is enhanced by separating the adduct by chromatographic methods such as high-performance liquid chromatography or by using alternative derivatization reagents. Because, in biological organism, MDA is found free or protein bound, the two forms should be measured, and the extraction procedures are a crucial step in the processing of the biological sample. This review focused on MDA determination procedures used to assess the effect of experimental and environmental stress induced in fish.

Keywords

Malondialdehyde, Thiobarbituric Acid, Fish, Metals

1. Introduction

In marine species, oxidative reactions are important components of the stress response, because these organisms are exposed to a wide variety of anthropogenic and environmental stressors, as physical or chemical agents. Moreover, factors such as temperature, salinity, water pH, mineral composition have a significant impact on stressor intensity [1].

Fish are particularly vulnerable to water pollution, with various levels of susceptibility to environmental toxicants, able to activate endogenous reactive oxygen

species (ROS). A continuous exposure to stressors can lead to ROS-mediated oxidative damage [2].

Metals, especially heavy metals, are important contaminants of aquatic environments worldwide and are well-known inducers of oxidative stress. Assessment of oxidative damage and antioxidant defenses in fish can reflect metal contamination of the aquatic environment [3] [4]. Therefore, fish can be used as bioindicators of metals in the environment by studying the induction of oxidative stress. [5].

The assessment of toxic effects under stressful environmental conditions needs an integrated approach with the appreciation of the balance between prooxidant manifestations and antioxidant (enzymatic and non-enzymatic) defenses in biological systems [6] [7].

Polyunsaturated fatty acid (PUFA) present in phospholipids and glycolipids are exceptionally susceptible to non-enzymatic lipid peroxidation (LPO), due to multiple unsaturated bonds present in their structure [8] [9].

Non-enzymatic antioxidants such as malondialdehyde (MDA) and glutathione, and enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST) have often been implicated as effective biomarkers of stress-induced physiological alterations in fish [10].

However, MDA, as non-enzymatic oxidative stress markers, is reported in literature strictly correlated to changed environmental conditions [11] and is still the main and most studied product of the peroxidation of PUFA [12].

A reliable determination of MDA concentration in biological samples, especially for lipid-rich samples like fish, is a formidable challenge from both an analytical and toxicological perspectives. Indeed, MDA determination requires the chemical conversion to derivatives with improved physicochemical properties for separation and detection because MDA cannot be determined by spectrophotometry as free dialdehyde. Therefore, to be quantified, MDA needs to change its chemical properties by proceeding with a derivatization process which takes advantage of the electrophilic character of the MDA molecule, forming adducts that have been used for its measurement.

Aim of this review is to analyze the literature dealing with the determination of MDA derived from LPO induced in fish by physical and chemical agents or environmental pollutants such as metals, by using different derivatization agents coupled with various separation procedures and detection methods.

2. MDA as Biomarkers of LPO

MDA is an oxidation product originating from PUFA with more than two double bonds. The oxidation rate of fatty acids increases with the number of double bonds, while saturated and monounsaturated ones are poorly oxidizable [13] [14].

Chemically, is a small and reactive organic molecule with two aldehyde groups at the carbon 1 and 3 position, and because of its pH-dependent tautomeric

chemical property, it exists in different forms in aqueous solution. At higher pH, MDA displays lower chemical reactivity, whereas at lower pH it appears in equilibrium between its protonated enol aldehyde and the dialdehyde form [15].

The reactivity of the two aldehyde groups towards nucleophiles enables MDA to form adducts, resulting in biomolecular damage [16].

3. MDA Determination by Thiobarbituric Acid Assay

In the field of environmental science, researchers use the thiobarbituric acid (TBA) assay to assess MDA and to measure the impact of pollutants on fish. This assay may determine how contaminants like heavy metals contribute to oxidative stress in marine and freshwater ecosystems.

Depending on the research focus, samples can be derived from various tissues and prepared to extract lipids, which are the primary targets in the assay that consists in a condensation of MDA with two molecules of TBA, forming an MDA-TBA₂ adduct. Unfortunately, TBA is not a selective reagent and may react with other similar substances that are formed during the LPO process, expressed as thiobarbituric acid reactive substances (TBARS). Moreover, adducts may derive from the oxidation obtained by the elevated temperatures required by the method, and heating may damage the biological samples and produces an overestimation of LPO. The kinetic reaction producing MDA-TBARS adduct strongly depends on the temperature, pH and TBA concentration. The adducts are pink pigments and their color intensity is commonly measured using a spectrophotometer, typically at a wavelength of 532 nm and weighted as MDA equivalents. The intensity of the color correlates with the amount of LPO in the sample [17].

The methods to determine MDA are usually validated according to international guidelines for standardized methods to measure linearity, recovery, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). The biological samples solutions are read against the reagent blank and compared with pooled samples spiked with MDA standard prepared from 1,1,3,3 tetraethoxypropane (TEP) [18]-[20].

In 1944, a “substance arbitrarily called B”, able to condense in acid solution with TBA producing a pink pigment, has been described [21]. Few years later, a complex formed by reaction of TBA and PUFA oxidation product has been characterized [22]. In 1951, MDA has been identified as the compound responsible of a red color observed in food fat rancidity and in the biological oxidation of PUFA, when treated with TBA reagent [23].

For the first time, in 1958, a mechanism of reaction involving one molecule of MDA with two molecules of TBA has been proposed to form a colored product with a maximum absorbance (λ_{abs}) at 532 nm [24]. Moreover, [25] studied the TBA reactivity by using mercuric chloride and sulfhydryl reagents to measure LPO in mouse liver microsomes.

Reference [26] reported the mechanism of reaction of different alk-2-enal derivatives with TBA, under different aerobic conditions. Three colored pigments

absorbing at different λ_{abs} (yellow 455 nm, orange 495 nm, and red 532 nm) have been obtained by monitoring the pH, temperature, time and oxygen. They described how the initial reaction phase produced a colorless 1:1 adduct, which was later converted into the yellow and finally in red pigment, under aerobic conditions. High performance liquid chromatography (HPLC) was used to separate the adducts, but the red pigment formed from the aldehydes could not be distinguished from the red 1:2 MDA-TBA adduct by absorption spectrum and HPLC separation.

Investigating the LPO products, *i.e.* MDA, 4-hydroxynonenal (4-HNE) and the F₂-isoprostane 15(*S*)-8-*iso*-prostaglandin, [27] reported a comparison study concluding that MDA measurement is comparable to the other LPO products, but it requires special precautions at the pre-analysis stage, beside the spectrophotometric, chromatographic or mass spectrometric assays used.

In biological materials, MDA may occur in a free and covalent bound forms with proteins, nucleic acids, and other substances. The required pH to obtain the maximum yield of total (*i.e.* free and bound) MDA varied from one biological material to another, depending on the nature of the derivatives present [28]-[30].

By studying the TBA reactivity towards aldehydes species, the dependence on pH was highlighted, reaching a maximum responsiveness around 3, and the strength of the acid used could affect the reactivity of the nucleophilic reagent. In addition, because of the presence of ferric ion, the assay could be affected by the presence of a chelating agent as ethylene diamine tetra-acetic acid (EDTA) [31].

Since the TBA test was first introduced, it has been widely used for years in lipid oxidation study, and, for many decades, it was primarily applied to determine rancidity in food. To select a suitable method for the measurement of the degree of rancidity in traditionally wide range products of fish markets, [32] compared LPO in fresh fish muscle samples and in fish samples, oxidized by incubating at 40°C for 3 days. Samples were steam distilled in a Kjeldahl distillation apparatus and the MDA was determined in the aqueous distillates by HPLC, as previously described [33].

Between the several approaches that have been developed to assess MDA content in biological matrices, commercial kits have been chosen for MDA detection, to facilitate the standardization of the analytical method, along the time [34] [35].

Up to date, despite its limited analytical specificity, the TBARS are still widely used to determine LPO in different biological samples as human serum, low density lipoprotein, and HepG2 cell lysate, reaching a LOD of 1.1 μM [36].

3.1. Sample Preparation

Three ways to prepare samples were reported by [37], to enhance the accuracy, *i.e.* an automatic trituration of the sample, a manual trituration in mortar with pestle, and a combination of the two methods.

A critical step in TBA assay is the sample preparation procedure, which involves heating samples under acid condition, and it could be considered as a stress

condition itself. Several studies reported a wide range of different condition of derivatization reaction. Samples were heated to 100°C for up to 1 h [38], or to 90°C for a minimum time of 30 min [39].

Because decomposition of lipid hydroperoxides, an overestimation of results may occur. Then, it has been proposed to add a chain-breaking antioxidant, as a butylated hydroxytoluene (BHT) to stabilize samples and prevent further LPO. Some Authors [38]-[40] observed a reduced amount of MDA-TBA₂ adduct, while [41] noticed that BHT included in the sample preparation procedure did not influence the amount of detected product.

The acid used in the sample preparation step may affect the detected MDA level, because the treatment with acid could have a hydrolytic action on MDA protein-bound fraction, allowing a detection of total MDA. The time necessary for an alkaline hydrolysis has been defined, and no significant differences have been reported in the range between 30 to 60 min, using sodium hydroxide (NaOH) by varying the concentration from 3M to 6M [40]. Comparable results for free and bound MDA in human plasma were obtained by using a similar alkalization/acidification procedure and by analyzing MDA as MDA-TBA₂ adduct by HPLC [42].

The TBARS measurement has been developed and tested in comparison with a commercially available 10-cm chromatographic column kit to gain in stability, precision and recovery, by using HPLC. Eight different acids for the hydrolysis of the samples and a series of phosphate buffer-acetonitrile-methanol mixtures were considered to improve the TBA adducts peak. Analysis of variance was used to assess relations between the proposed HPLC method and the kit assay [43].

3.2. Sample Extraction

A reliable measurement of MDA, particularly in lipid-rich samples, is challenging for every analytical technique, and thus, any special precaution at the pre-analysis stage is useful, especially the extraction procedures.

The method is usually carried out by direct extraction, which is applicable to raw fish involving maceration of the sample in solution of extraction agent. To enhance the selectivity towards TBARS in biological sample with high lipid contents, n-butanol was successfully used by [38]-[40].

A well-drawn study reported a comparison between a spectrophotometric and a chromatographic assay for MDA determination. They used a direct injection of samples into an HPLC system; however, this requires an additional precipitating agent such as methanol and subsequent centrifugation [44].

A derivatization reaction by using TBA in phosphoric acid has been proposed, heating for 60 min in a boiling water bath. After cooling in an ice bath, butanol was added as extracting agent. The absorbance of the organic layer was measured at 520 and 535 nm, enhancing the selectivity of the method [45].

Hydroperoxide derivative may interfere with MDA determination by reacting with TBA and producing overestimation. To improve selectivity toward MDA, the optimum pH was found to be 3.5 using acetate buffer. After heating at 95°C

for 60 min, the red pigment produced was extracted with *n*-butanol-pyridine mixture and estimated by the absorbance at 532 nm [46].

By using the “Bligh & Dyer” [47] liquid-liquid extraction (LLE) with trichloromethane (TCM), [48] proposed a sample pretreatment to enhance selectivity toward MDA, adding methanol (MeOH) to ameliorate the extraction. All the lipids and hydroperoxide were found in the apolar layer, while MDA was found in the polar layer, represented by a methanolic-water mixture.

Because interferences from cellular components may represent a critical point for a validation of the analytical method, an extraction of MDA-TBA₂ adduct by using methyl-tert-butyl ether (MTBE) has been proposed, demonstrating an acceptable linearity and precision in cellular oxidative stress models [49].

In the last century, the distillation has been considered as a comparable method, because the colored adduct MDA-TBA₂ is considerably affected by the acid. In fact, in 1964, a modification of the distillation process has been successful in the extraction of the adduct without acid-heat treatment [50]. Moreover, a spectrophotometric measure at 530 nm showed that heating without acid accelerated the condensation of TBA with MDA without affecting the absorbance.

Reference [51] used a TBA assay to measure oxidative rancidity due to the urea in different fish species, by comparing a direct extraction using trichloroacetic acid (TCA) and distillation. After a forced oxidation period, TBA value increased to a greater extent with the direct extraction procedure than with the distillation method, showing the lower recovery using the distillation technique.

A comparison between acid extraction and distillation methods has been used to evaluate the effectiveness of both extraction TBA methods for monitoring LPO in food with high fat contents (>10%). The TBA values were highly correlated for both the extraction and distillation methods. Perchloric acid (PCA) was preferred because this extraction method was faster, easier to perform, and accurate as the distillation method for monitoring lipid oxidation in meat [52].

A determination of MDA-TBA₂ adduct was performed in muscle of smoked and dried-salted fish, after aqueous extraction and distillation in a Kjeldahl apparatus. The separation performed on reversed phase HPLC was fast (2 min) and selective for MDA, because any other interference substance was detected [32].

3.3. Detection Techniques

The analytical methods to determine MDA using TBA assay, even if they have been published in the past century, are still used today by many authors, albeit with small variations, by using spectrophotometric determination, or chromatographic separation.

An HPLC method was proposed to be faster and less susceptible to side effects for the quantitation of MDA-TBA₂ adduct in aqueous distillates [53]. Afterwards, a more sophisticated assay has been developed using a Fourier-transform infrared spectroscopy and high-field ¹H and ¹³C nuclear magnetic resonance. This study was able to describe the complete structure of the red crystalline MDA-TBA₂ adduct

[54].

The use of liquid chromatography (LC), coupled with mass spectrometry (MS) and MS-MS detection techniques, has been proposed to study the structures of the pink MDA-TBA₂ adduct, as well as a common unstable yellow interference compound, which absorbs at 455 nm, indicating that TBA should be purified from the presence of impurities before use [55].

A sensitive and selective determination of free and total MDA may be reached by using the ultra-high performance liquid chromatography (UHPLC) coupled to a quadrupole time-of-flight high-resolution MS. This detector solves the types of interference present in the methods and can be used with any type of sample [56].

Recently, a method using HPLC coupled with photodiode array (PDA) was fully validated in terms of linearity, selectivity, accuracy, precision, stability, and robustness, demonstrating to overcome the limitations of the spectrophotometric method in terms of specificity and sensitivity [37].

4. MDA Determination by 2,4-Dinitrophenylhydrazine

One of the most widely used derivatization agent for aldehydes and ketones is 2,4-dinitrophenylhydrazine (DNPH). The hydrazine group is a strong nucleophile, and this helps the reaction that takes place at room temperature and in a slightly acidic environment with MDA, giving 1-(2,4-dinitrophenyl)pyrazole as confirmed by MS [57] and the corresponding hydrazone derivative (HDZ) as reported by [58]. MDA has been measured in human plasma after protein precipitation by PCA. After centrifugation, DNPH solution was then added, mixed and incubated for 10 min at room temperature. Hexane has been used to extract and separate HDZ from aqueous phase. By using HPLC coupled with ultraviolet (UV) detection, they measured a total MDA reaching a LOD of 0.3 nmol/ml.

To improve recovery, [59] suggested a longer incubation time (60 min) to have a complete alkaline hydrolysis of bounded MDA, reaching 88.5%. Moreover, the quantification of MDA by HPLC was improved by using an internal standard as methyl-MDA.

A more specific assay was needed to determine both MDA and formaldehyde as products of LPO in brain, and to monitor an experimental oxidative stress. DNPH was used to separate HDZ by gas chromatography (GC) and quantitate by MS with single ion monitoring [60].

To increase sensitivity, the analyte extraction represents a critical point in sample preparation procedures, then a choice of a solid phase extraction (SPE) led a LOD of 3 ng/ml when 1-(2,4-dinitrophenyl)pyrazole was measured by HPLC [61].

A high selectivity for the total MDA determination was reached in homogenate rat brain by measuring HDZ, by HPLC with PDA detector. The extraction by SPE was chosen to enhance specificity and avoid the risk of overestimation, reaching a LOD of 5 ng/ml. Chromatographic measurement of DNPH concentration, ensured the completeness of MDA determination in biological samples [62].

Reference [63] proposed a validated assay of MDA in cell cultures without

previous purification of HDZ, with a recovery ranged from 96.27% to 99.91%. Moreover, HDZ was determined in rat serum and liver homogenates, reaching a LOD of 0.1 nmol/ml [64]. DNPH was added to the acidified sample, followed by vigorous shaking. The resulting solution was incubated at room temperature and protected from light for 30 min, forming HDZ, easily quantifiable using a HPLC with UV detector.

The use of DNPH agent coupled with UHPLC-MS produced a more accurate and specific assay to determine free and total MDA in blood fish, obtained from *Scolopsis bilineatus* caudal vein. MDA was extracted using acidic precipitation and alkaline hydrolysis followed by acidic precipitation, respectively. HDZ quantification was achieved by internal standardization using deuterated MDA [56].

5. MDA Determination by Other Agents

Other derivatization agents have been chosen in fish matrices, especially when they are utilized as food for human consuming. Due to their high reactivity, hydrazines are one of the most popular reagents used for the quantitation of carbonyls.

The phenyl-hydrazine (PH) has been proposed to eliminate interferences in the formation of MDA-TBA₂ adduct [65]. A sensitive analytical method, by using GC-MS, has been developed to detect total MDA under mild conditions (25°C, pH 4.0, 30 min). Before the derivatization step, an alkaline hydrolysis was performed to obtain free MDA [65].

Halogen containing hydrazine, such as pentafluorophenylhydrazine (PFPH), allows for enhanced sensitivity using GC-MS with electron-capture negative ionization. This derivatization reagent has allowed to reduce the inherent deviations from the presence of nitrite in food matrices [66].

A very high recovery of MDA from spiked matrices (96%) was reached by using 2,4,6-trichlorophenylhydrazine as derivatization agent. A *n*-hexane was chosen for the extraction procedure, and the separation of the cyclic derivatization product was obtained by capillary GC coupled with electron-capture detection (ECD) [67].

An interesting derivatization method used pentafluorobenzyl bromide (PFBeBr), exploiting the MDA nucleophilicity for its derivatization in aqueous acetone with this nucleophilic derivatization reagent. The derivative product was best suited for the quantitative GC-MS analysis of native, non-conjugated MDA in human biological samples [68].

Reference [69] proposed diaminonaphthalene (DAN) as a highly specific derivatization agent to determine free and total MDA. The same reagent was chosen by [70] for the determination of free MDA contained in fish tissue, detecting a naphthodiazepinium ion at 311 nm, by using HPLC-UV.

The N-methyl-2-phenylindole is a derivatization reagent that reacts with MDA under acidic conditions to give a carbocyanide product. A commercial kit based on N-methyl-2-phenylindole was compared to the traditional TBA assay to

measure their reactivity towards aldehydes, by testing a selection of the more appropriate acidic medium. Both commercial kit and TBA assay, tested in acetate buffer with EDTA, determined similar MDA concentration [71].

Research on mechanistic aspects of the colorimetric assay has been described by [72], seeking a selective assay for MDA in the presence of 4-HNE. It has been shown that the nature of the acid plays a crucial role in the oxidative fragmentation of intermediates into chromophores.

6. Methods to Determine MDA in Fish Tissues

Many studies reported MDA determination as a measure of oxidative damage, providing insights into environmental stressors affecting both wild and farmed fish species.

These measurements are essential for understanding the health and environmental impacts on aquatic life, particularly, in resident fish species that have been proposed as sentinel species to assess the possible effect of anthropogenic activities and for monitoring transitional environment pollution [73]-[75].

The liver as well as the gills, are the biological matrices preferred for the determination of MDA because their content in PUFA. Both organs are usually the main target for toxic effects of pollutants in fish [76].

MDA determination in fish muscle is a common procedure used to assess oxidative stress and LPO and it should be noted that MDA levels depend on the fish species, as observed by [77] in a white sucker, a species of freshwater cypriniform fish, relatively tolerant of turbid and polluted waters. Moreover, [78] reported that different fish species such as Iberian chub, common carp, goldfish, rainbow trout and Andalusian barbel, have different liver MDA levels. The highest value of MDA in liver tissue was 3 times the lower value.

Table 1 summarizes the different analytical approaches for the determination of MDA in fish tissues used in four selected studies, reporting: species and origin; sample preparation, MDA derivatization, extraction and detection; LOD and LOQ; recovery range; fish tissue; MDA level; reference.

Reference [44] compared three different methods (A, B and C) for MDA determination in fresh (sardines and farmed sea bream) and frozen (South Africa hake) fish muscle samples chosen for their different fat content, kept in a refrigerated chamber and analyzed periodically over a period of 19 days, to study a rancidity development. Method A measured the MDA-TBA₂ adduct at 530 nm with a spectrophotometer, according to [51]. Method B used an HPLC separation of MDA-TBA₂ adduct that was detected through a spectrofluorometer, according to the method described by [43]. Method C used a different derivatization reagent forming MDA-DNPH adduct, determined by the method described by [64]. Results were expressed as MDA measured in fish muscle of sardine (*Sardina pilchardus*), farmed sea bream (*Sparus aurata*), and South African hake (*Merluccius capensis*). All methods were fully validated, reporting a lower recovery level (under 71%) for method A, and the best recovery, accuracy and specificity for method C, by using

HPLC-UV for MDA-DNPH determination. HPLC determination of MDA-DNPH adduct by was greater than MDA-TBA₂ adduct. A fluorometric detector (FD) had better efficiency than the traditional spectrophotometric measures, reaching the best LOD and LOD.

Table 1. Methods for MDA determination in fish tissues.

Species and origin	Sample preparation MDA derivativization, extraction, and detection	LOD° μmol/kg	LOQ\$	Recovery range %	Fish tissue	MDA level	Ref.
<i>Sardina pilchardus</i> wild fish from local market	Method A: - derivativization with TBA, 100°C, 60', cooling at room temperature - MDA-TBA ₂ extraction by TCA - spectrophotometry λ _{abs} , 530 nm ® [51]	0.16° 0.23\$		66 - 71	Muscle from fresh fish	50 - 675 μmol/kg C _{max} on the 15 th days of the chilled storage at 2°C ± 1°C	
<i>Spaurus aurata</i> farmed sea fish from local market	Method B: - deproteinization with TCA and methanol (1:3) - derivativization with TBA, 97°C, 60', cooling at -20°C for 20' - MDA-TBA ₂ extraction by MeOH - filtration on PTFE, 0.2 mm - HPLC-FD, λ _{ex} 525 nm, λ _{em} 560 nm ® [43]	0.10° 0.17\$		100 - 108	Muscle from fresh fish	5 - 20 μmol/kg C _{max} on the 4 th days of the chilled storage at 2°C ± 1°C	[44]
<i>Merluccius capensis</i> frozen fish from local market	Method C: - derivativization with DNPH at room temperature for 10' - MDA-DNPH direct injection - HPLC-UV, 310 nm ® [64]	0.20° 0.26\$		90 - 112	Muscle from frozen fish	5 - 30 μmol/kg C _{max} on the 7 th days of the chilled storage at 2°C ± 1°C	
<i>Salmo salar</i>	- homogenized tissue in 1 mL of 1% sulfuric acid in PBS - alkaline hydrolysis with 6M NaOH - incubation in a water bath at 60°C for 30' - centrifugation at 2800 rpm 10' - derivativization with DNPH in the dark at room temperature 60' - MDA-DNPH direct injection - HPLC-PDA, 310 nm ® [44] [64]	0.195° 0.39\$		92.4 - 104.2	Liver, muscle stored at -80°C until analysis	26.6 - 45.7 μmol/kg liver 2.6 - 4.64 μmol/kg muscle	[79]
<i>Caprinus carpio</i>	- homogenized tissue added with EDTA solution - MDA-TBA ₂ - HPLC-FD ® original study	0.015\$		91.2 - 107.6	Liver stored at -80°C until analysis	0.02 ± 0.004 μmol/g liver	[40]
<i>Rastrelliger kanagurta</i> from local market	- homogenized tissue added with TCM:MeOH (2:1, v/v +BHT) - flushed with N ₂ gas - added with EDTA - derivativization with TBA, 100°C for 30' - MDA-TBA ₂ - spectrophotometry λ _{abs} , 532 nm	0.1° 1.31\$		85.95 - 86.98	muscle stored at 4°C for six days	2.67 nmol/g on 1 st storage day 11.80 nmol/g on 5 th storage day	[80]

Legend of symbol: ®references of original analytical protocols cited by Authors; °LOD limit of detection; \$LOQ limit of quantitation.

Reference [79] reported the determination of MDA present in salmon liver using HPLC, through the derivatization with DNPH using a variable time of reaction from 30 to 60 min, to improve the sensitivity of the method. Moreover, to determine the total MDA, alkaline hydrolysis was chosen. The LOQ of MDA was 0.39 $\mu\text{mol/L}$, which was comparable to the values previously reported [44] [64]. The recovery of the spiked MDA liver samples was in the range of 92.4% to 104.2%. This study reported a higher MDA concentration in salmon liver (from 26.6 to 45.7 $\mu\text{mol/kg}$) as compared to muscle fresh tissue (from 2.6 to 4.64 $\mu\text{mol/kg}$).

An interesting comparative study on MDA determination in various biological samples (human plasma, fish liver tissue and cells in culture) has been reported. Liver tissue of common carp (*Cyprinus carpio*) was used to determine MDA-TBA₂ adduct by HPLC coupled with FD. Samples were frozen in liquid nitrogen and, following addition of ice-cold potassium phosphate buffer (PBS) and EDTA as metal chelating agent, were homogenized. After centrifugation, supernatants were collected and stored (at -80°C) until analysis. Before assessing MDA, tissue supernatants were diluted 10 times with distilled water. In supernatant of 10% carp liver homogenate, MDA level varied from 0.015 to 0.027 $\mu\text{mol/g}$ tissue [41].

TBARS assay was used to assess the freshness quality of Indian mackerel market fish, evaluating the quality changes every 48 hours, up to 6 days storage [80]. This study focused on the validation of the analytical method by following the report published by the National Institute of Nutrition and Seafood (NIFES) [81].

In muscle of *Rastrelliger kanagurta*, the TBARS concentration varied from 2.67 nmol/g (on the 1st day of storage) to 14.33 nmol/g (on the 6th day of storage) where the threshold value of 13.89 nmol/g is generally acceptable for rancidity testing. The assay showed a high correlation coefficient (0.983) with storage time, indicating a strong relationship between MDA content and freshness [82].

7. Effects of Experimental Oxidative Stress Determined by Physical and Chemical Agents in Fish

To evaluate the impact of pollutants and environmental stressors on fish health, MDA has been determined as LPO biomarker in both freshwater and marine wild fish, and in species that have been artificially stressed to observe the oxidative stress induced by such exposure.

Table 2 summarizes the methods used to determine MDA in fish experimentally stressed by physical and chemical agents, described by four selected studies, reporting: species and geographic origin; MDA derivatization and detection; artificial stressor agent; fish tissue; MDA level determined in control fish; MDA level determined in stressed fish; references.

7.1. Salinity

Salinity measures the amount of dissolved salts in water, usually expressed in parts per thousand (ppt), and represents one of the most important abiotic regulators

for maintaining physiological homeostasis in aquatic ecosystems. Hyper-saline habitats (salinity > 35 ppt) are among the toughest water environments [83].

Table 2. Methods for MDA determination in fish experimentally stressed by physical and chemical agents.

Species and geographic origin	MDA derivatization and detection	Artificial stressor agent	Fish tissue	MDA level determined in control fish	MDA level determined in stressed fish	Ref.
<i>Notopterus chitala</i> Sundarban estuary West Bengal	- MDA-TBA ₂ - spectrophotometry $\lambda_{\text{abs}} = 532 \text{ nm}$ ® [29]	salinity exposures 0 - 3 - 6 - 9 - 12 ppt	Liver Gills	6 - 7 nmol/mg protein	+23.88-80.59% $C_{\text{max}} = 12 \text{ ppt}$ $p < 0.05$ +25.35-90.14% $C_{\text{max}} = 12 \text{ ppt}$ $p < 0.05$	[84]
<i>Clarias Gariepinus</i> Kafrelsheikh city Egypt	- MDA-TBA ₂ - spectrophotometry $\lambda_{\text{abs}} = 532 \text{ nm}$ ® [45]	salinity exposures 0 - 4 - 8 - 12 ppt salinity exposures 0 - 4 - 8 - 12 ppt heat stress 32°C for 72 h	Liver Gills Liver Gills	NR	140 - 160 nmol/g liver $p < 0.05$ 190 - 230 nmol/g gills $p < 0.05$ 155 - 175 nmol/g $p < 0.05$ 210 - 260 nmol/g $p < 0.05$	[87]
<i>Oreochromis niloticus</i> Kafrelsheikh area Egypt	- MDA-TBA ₂ - spectrophotometry $\lambda_{\text{abs}} = 532 \text{ nm}$ ® [45]	salinity exposures 0 - 15 ppt ammonia exposure 5 mg/L continuous: daily intermittent: every 3 days	Liver Gills	10 - 20 nmol/g	40 - 60 nmol/g $C_{\text{max}} = 15 \text{ ppt} +$ continuous ammonia	[91]
<i>Mugil cephalus</i> Oman sea Oman	- assay kit - MDA-TBA ₂ - spectrophotometry $\lambda_{\text{abs}} = 532 \text{ nm}$ ® [25]	salinity exposure 35 mg/L for 30', 60', 120' metal exposure: NiCl ₂ 5 mcg/L = T ₁ 10 mcg/L = T ₂ 15 mcg/L = T ₃	Gills	0.12 - 0.15 nmol/mg	T ₂ = 0.42 nmol/mg T ₃ = 0.46 nmol/mg $C_{\text{max}} = 30 \text{ min}$ $p < 0.01$	[94]

Legend of symbol: ® references of original analytical protocols cited by Authors; NR, not reported.

An antioxidant defense system impairment may be determined in fish by salinity fluctuations, as shown by recent studies reporting as a hyperosmotic environment induces acute oxidative stress in freshwater fish species [84]. MDA was measured as MDA-TBA₂ adduct, as previously described [29], and showed a significant increase in gills and liver, at five different salinity exposures (0 - 3 - 6 - 9 - 12 ppt), in response to an acute stress in adult female *Notopterus chitala*. This freshwater species has a physiological activity in water where salinity range varies between 0.01 and 3 ppt. A considerable MDA increase (90%) was measured in gills of fish exposed to the highest water salinity level. In fact, gills represent the most important osmoregulatory organ in fish, ensuring efficient maintenance of internal salt and water balance [85].

7.2. Salinity and Heat

Climates change and the increased temperatures have caused a high evaporation rate, especially during the summertime [86]. Some fish species have high capacity to adapt to diverse environmental conditions, as African catfish (*Clarias gariepinus*), a popular commercial fish. Nevertheless, the impact of water salinities determined stress on the health fish status, as reported by [87]. The oxidative status in gills and liver was determined by measuring TBARS at 532 nm, as previously described [45]. MDA resulted markedly increased in all groups, reaching the highest concentration at 12 ppt of exposure saline. Moreover, after heat stress, all fish showed higher MDA levels than before heat stress, especially in liver.

Similar results were reported studying the effect of enhanced salinity and concurrent water temperature on developing enzymatic and non-enzymatic activities in juvenile sablefish (*Anoplopoma fimbria*) produced by ROS [88].

7.3. Salinity and Ammonia

In water, there is an equilibrium between ionized (NH_4^+) and gaseous (NH_3) ammonia, affected by pH and salinity. Due to the seawater buffering system, ammonia and pH are positively correlated, while an increase of salinity reduces the NH_3 formation [89].

Nile tilapia (*Oreochromis niloticus*), a freshwater fish of the Cichlid family, is a fish of growing interest for aquaculture due to its successful growth in brackish water conditions [90].

Reference [91] determined the combined effects of water salinity and total ammonia (TAN). Fish were exposed to a salinity water gradually raising (1 ppt daily) from 0 to 15 ppt, and then exposed to continuous (daily) or intermittent (every 3 days) TAN at 5 mg/L. MDA was determined by using the TBA-based method, as previously reported [45].

The highest levels of oxidative stress were found in gills and liver homogenates of Nile tilapia reared at the highest salinity and exposed to continuous ammonia, and MDA increased to the same extent in both organs. Interestingly, growing Nile tilapia in brackish water with a salinity range of 10 - 12 ppt has been achieved with no negative impact on the stress resistance of the fish by a dietary regimen [92].

7.4. Salinity and Metals

The physicochemical properties of water play an important role in metal solubility [93].

The toxic effects of the heavy metal, as nickel dichloride (NiCl_2), were investigated in the gray mullet (*Mugil cephalus*), a bottom-related fish species. LPO was evaluated by measuring MDA in gills as MDA-TBA₂ adduct with an assay kit, as previously described [25]. MDA levels significantly increased in response to increasing nickel dichloride concentration ($p < 0.01$), showing the highest value after 30' and returning to the same levels as the control group post salinity challenge. Fish could recover from toxic effects over time when contamination was removed [94].

8. Environmental Metal Pollutants in Fish

Various metal ions, metals, metalloids and metalorganics are involved in oxidative stress in fish [95]-[98]. Then, MDA level is commonly reported to assess the extent of LPO and oxidative damage induced by metals. Interestingly, both increase and decrease in enzyme activity and enhanced and reduced level of non-enzymatic components have been described in fish, after metal exposure [7].

The fish organ most susceptible to high MDA levels is typically the liver. This is because the liver is the main site of lipid metabolism and is, therefore, subject to intense oxidative stress, especially under conditions of exposure to environmental contaminants and toxins. The liver plays a crucial role in detoxification and, as a result, tends to accumulate LPO products such as MDA [99]. Besides liver, kidney, muscle and brain are organs that accumulate MDA, making them susceptible to oxidative stress in fish [100]-[102]. Moreover, gills are particularly susceptible to MDA accumulation because directly exposed to the aquatic environment and thus to the pollutants contained in water [103].

Metal ions dissolved in ambient water are absorbed through gills and other permeable body surfaces, and metals bound to solid particles are ingested and absorbed through the intestinal epithelium [104]-[106]. Because these two major routes of metals exposure, metal accumulation in fish living in polluted waters shows a variable number of different metals in different organs, depending on the ionization and redox state of the chemical elements [107].

Table 3 summarizes the methods for determining MDA in environmentally contaminated fish described in seven selected studies, reporting: species and geographic origin; MDA derivatization and detection; contaminant; fish tissue; MDA level; references.

Table 3. Methods for MDA determination in environmentally contaminated fish.

Species and geographic origin	MDA derivatization and detection	Contaminant	Fish tissue	MDA level	Ref.
<i>Clarias garepinus</i> Ogun River Nigeria	- MDA-TBA ₂ - λ _{abs} = 532 nm ® [109]	Metals (Zn, Cu, Cd, As, Pb)	Liver	40 nM/mg protein 177% p < 0.01	[108]
			Gills	60 nM/mg protein 168% p < 0.001	
			Kidneys	38 nM/mg protein 102% p < 0.01	
			Heart	21 nM/mg protein 71% p < 0.05	
			Muscle	1.47 ± 0.39 nmol/mg protein	
<i>Cyprinus carpio L.</i> , Bafra fish Lake Turkey	- MDA-TBA ₂ - λ _{abs} = 532 nm ® [28]	Metals (Cd, Co, Cu, Ni, Fe, Se, Zn, Mn, As)	Liver	0.58 ± 0.08 nmol/mg protein	[110]
			Gonads	0.79 ± 0.22 nmol/mg protein	
			Gills	2.49 ± 0.02 nmol/mg protein	

Continued

<i>Salmo trutta</i>	- MDA-TBA ₂	Arsenic	Kidneys	*↑ + 30%	[113]
S1 Presa River	- λ _{abs} = 532 nm		Liver	*↑ + 53%	
RS Bravona River Corse, France	® [46]				
<i>Clarias gariepinus</i>		Metals (Cd, Cr, Cu, Fe, Mn)	Liver	C _{max} in Spring-season	[114]
Orontes River	- MDA-TBA ₂			S1 = 17 mmol/g tissue	
S1	- λ _{abs} = 532 nm			S2 = 43 mmol/g tissue	
S2	® [28]		RS = 15 mmol/g tissue		
RS			C _{max} in Autumn-season		
Turkey			Muscle	S1 = 8 mmol/g tissue	
<i>Oncorhynchus mykiss</i>		Heavy metal	Ovaries	S1 = 0.52 μmol/g	[115]
Jhelum River	- MDA-TBA ₂			S2 = 0.77 μmol/g	
S1 Verinag hatchery	- λ _{abs} = 532 nm			p < 0.05	
S2 Panzath hatchery Kashmir	® [116]				
<i>Solea solea</i>		Metals metalloids (As, Cd, Cr, Cu, Pd, Hg, Mn, Ni, Se, V, Zn)	Liver	15.5 ± 12.9 nmol/g	[117]
<i>Mullus barbatus</i>	- MDA-DNPH			19.6 ± 27.9 nmol/g	
<i>Sardina pilchardus</i>	- HPLC-PDA			103 ± 17.1 nmol/g	
<i>Scomber scombrus</i>	- λ _{abs} = 310 nm			16.5 ± 12.6 nmol/g	
Ionic Sea Italy	® [62]				
<i>Esox Lucius</i>	- MDA-(N-methyl-2phenylindole)	MeHg	Liver	#r ² = 0.81	[118]
St. Maurice River	- λ _{abs} = 586 nm			p < 0.001	
Québec, Canada	- read in a 96-microplate kit				

Legend of symbols: ®references of original analytical protocols cited by Authors; *MDA concentration increase in comparison to the reference site; #correlation between MDA and MeHg liver concentration. S1 = Site 1; S2 = Site 2; RS = Reference Site.

8.1. Metals

Metals and metalloids can accumulate in various fish tissues, especially when they are raised in contaminated water. The measurement of MDA levels in fish tissues provided insights into the oxidative stress induced by metal exposure.

Metal pressure in farmed fish under captive conditions is a main concern, as it can lead to oxidative stress. Fish farming in contaminated water bodies or using feeds with trace metals leads to the accumulation of these metals in fish tissues, adversely affecting their health and growth.

Because heavy metal accumulation may lead to oxidative damage in aquatic animals, [108] correlated metal levels with indicators of oxidative stress, such as MDA. They performed a comparative MDA evaluation in kidney, liver, gills, and heart of African catfish (*Clarias gariepinus*) found in a river near an industrial site and fish collected from fish farms to be used as reference sites. They described in detail all the procedures used to minimize oxidative stress in the pre-treatment samples, *i.e.*, fish transportation (fish were kept alive for at least 24 h), organ removal (in ice-cold and buffer solution), and homogenization (in buffer pH 7.4). After centrifugation (10,000 g for 20 min, at 0 °C - 4 °C), the supernatant was stored at -20 °C until analysis. LPO was determined by measuring the TBARS as

previously described [109]. MDA was quantitated as nmol/mg of protein, in fish living in a polluted site compared to those collected from fish farming and resulted elevated in all fish organs, more significantly in gills.

Reference [110] discussed the importance of studying aquatic ecosystem, considered as an indicator of health in both animal and human. The levels of selected metals (Cd, Cu, Co, Ni, Fe, Se, Zn, Mn, As), the antioxidant enzymatic activity and MDA concentration were determined in the muscle, liver, gills, gonads, and kidney of *Cyprinus carpio L.*, a freshwater fish. Part of supernatant was taken for protein determination using the Bradford method [111]. The MDA was detected through the TBA assay, at 532 nm [28]. The results showed high LPO in all tissues, especially in gills, suggesting that fish act as a bio-indicator of environmental contamination by heavy metals.

Arsenic can be absorbed through the gills and can induce oxidative stress and disrupt antioxidant defenses, as reported in *in vivo* and *in vitro* experimental models [112]. Environmental condition on bioaccumulation of arsenic, producing oxidative stress, was assessed in wild trout (*Salmo trutta*) living in a river suffering of past mining contamination [113]. Arsenic was measured in freshwater fish tissues and in water. MDA was measured as MDA-TBA₂ adduct, as previously reported [46] and significant differences were observed in all organs except gonads. The results showed a 53% and 30% increase in MDA in the liver and kidney of wild trout compared to fish caught at the reference site (RS), although the arsenic organotropism was kidney > liver > gills > fins > gonads > muscle.

African catfish (*Clarias gariepinus*) derived from two differently contaminated sites (S1 and S2) of Orontes River polluted by metals, were used to measure LPO by comparing the MDA concentration determined in fish collected in S1 and S2 with fish caught at the RS. The adduct MDA-TBA₂ was determined in the muscle and liver homogenates, as previously described [28]. MDA levels measured at the polluted sites were higher than those at the reference site during the Spring season in the liver and during the Fall season in the muscle. Both liver and muscle MDA levels were significantly lower in winter at all sites. Correlation analysis showed a positive relationship between MDA and metal content (Cd, Cr, Cu) in water [114].

To determine the severity of pollution effects in two different hatcheries (S1 and S2) contaminated with different levels of heavy metals, oxidative stress was assessed in rainbow trout ovaries [115]. MDA content was evaluated by measuring the MDA-TBA₂ adduct using a spectrophotometric assay at 532 nm, as previously described [116]. MDA levels were lower in fish collected from S1 compared to fish collected from S2, which was strongly correlated with the severity of heavy metal stress ($p < 0.05$) and showed increased LPO.

Reference [117] reported the measurement of metals, metalloids and LPO in four different fish species. MDA was determined as MDA-DNPH derivative purified by SPE from liver homogenates using HPLC-PDA, as previously described [62]. In all fish species, MDA levels were significantly correlated with specific elements and especially with the total contaminant load along the trophic levels, *i.e.*

from benthic (*Solea solea*) to demersal (*Mullus barbatus*) and pelagic (*Sardina pilchardus* and *Scomber scombrus*) compartments.

8.2. Metalorganic Derivatives

Oxidative stress is a suggested effect of methylmercury (MeHg) bioaccumulation in fish. A recent study examined the relationship among MeHg and LPO levels, by MDA determination in fish liver of a northern pike from St. Maurice River (Québec, Canada). An original analytical method, by using a microplate kit and N-methyl-2phenylindole as derivatizing agent for MDA has been described, at pH acid, in a 45°C water bath for 1 h. A standard curve was assessed using the MDA stock solution provided with the kit. The digestate was centrifuged and the absorbance of a 150 µL part of the supernatant was read in a 96-well microplate, at 586 nm. During sample pretreatment, BHT (5 mM) was used to prevent oxidation, then liver samples were homogenized and centrifuged. MDA concentration founded in *Esox lucius* liver was normalized to the protein content [111] and best correlated with MeHg concentration.

The multiple linear models were used, and a strong correlation was found between the predicted and measured MDA levels in fish liver, indicating that methylmercury concentration increased LPO [118].

9. Discussion

Pollution is an issue of great concern because of the effects that pollutants can have on habitats, organisms, and ecosystems, as also on human quality of life and public health, especially when it comes to risk assessment for fish consumption [119]. Bioindicators, which include biological processes, species, or communities, represent a valuable tool to assess environmental properties and how they change over time.

Fish are considered excellent bioindicators for the evaluation of environmental quality of the aquatic systems [120] and sediments [121]. Trace metals are typical classes of environmental pollutants with prooxidant effects, which can both depress the antioxidants capacity to remove oxyradicals or enhance the intracellular formation of ROS [122].

Activation of oxidases and inhibition of scavenger systems can lead to oxidative damage. When the concentration of radical species is such as to saturate the self-regulating systems present in living organisms, a state of oxidative stress is encountered, which is therefore not limited to carrying out the physiological functions mentioned above, but also irreversibly damages the cells [123].

A specific biomarker for oxidative stress caused by metals does not exist and therefore a complex approach should be taken. Since LPO is one of the first mechanisms of cell damage by xenobiotics, the levels of non-enzymatic markers of oxidative stress, such as MDA, are reported in the literature to be strictly correlated with altered environmental conditions [124].

In a toxicity study of oxidation products, hydroperoxide was found to be much

more toxic than secondary products such as MDA. However, while peroxy radicals have no significant mobility, MDA diffuses from the site of production and can cause oxidative damage [125] [126].

MDA levels are measured in biological samples of various edible species to determine the oxidative effect of physical or chemical insults. When MDA levels are high, proteins and nucleic acids are irreparably altered. However, if MDA scavenging and redox signal regulation are functioning properly, MDA elevation may represent acclimation processes rather than damage, as MDA may play a beneficial role by activating regulatory genes involved in fish defense and reproduction. Thus, it has been suggested that MDA may function as a protective mechanism rather than an indicator of damage. For example, in observations of salt-stressed fish, MDA increased transiently and decreased rapidly when the stress was removed [87] [88].

Therefore, the role of this compound as either a damaging or protective agent depends on its production, scavenging, and signal modulation, and thus relies on the enzymatic activity of aldehyde dehydrogenase. Its expression is induced to control the level of aldehyde compounds (such as MDA) by oxidizing them to their corresponding carboxylic acids, thereby restoring low cellular levels. This mechanism can then serve as a signal rather than causing damage to the cell, as has also been described in plants [127]. MDA may have a physiological and protective function as a signalling molecule stimulating gene expression and cell survival, but also, may have a cytotoxic role inhibiting gene expression, promoting cell death and multifactorial health disorder [128] [129].

In almost all reviewed studies, LPO was considered as a complex process resulting from free radical reactions in biological membranes. When lipid hydroperoxides cleave double bonds of unsaturated fatty acids and destroy membrane lipids, elevated levels of MDA result. Over time, many studies have used MDA as an indicator of LPO resulting from oxidative stress in fish related to environmental pollutants, and its formation is clearly implicated in the symptoms of environmentally stressed fish.

The growing conditions of farmed fish may determine metal composition of fish tissues as well as the response that these fish exhibit to metal toxicity [130]. The presence of MDA is commonly detected in farmed fish organs to determine site contamination and metal exposure under captive conditions [131].

This review has highlighted that the study of LPO effects in fish, by measuring MDA concentrations in various tissues and organs of experimentally or naturally metal-intoxicated fish, is mainly carried out using the TBA assay. This can be considered an acceptable choice, despite the limitations of this assay already discussed. Very often, MDA-TBA₂ determination has been combined with the quantification of enzymatic biomarkers of LPO to study the antioxidant system (SOD, CAT, GPx, GST) and the alteration of oxidative damage parameters [109] [110] [113] [114] [116].

Since by its first application in biological samples, in 1944 [21], this assay has

been proposed by Authors, up to date, by attending to the critical issues brought about by the acidity of the reaction environment, the heating used to promote the derivatization reaction, and the extraction procedures to purify the adduct.

The determination of MDA in lipid-rich matrices may require analytical methods with higher specificity than the spectrophotometric assay, in fact, the TBARS assay led to an overestimation of LPO in biological samples compared to other derivatization reagents determined by HPLC or LC coupled with different type of detection techniques, *i.e.* PDA or MS [132].

Very rarely, the reported data referred to method validation, as recommended by international guidelines. The use of commercial tests can then offer standardized procedures and reagents to ensure consistency and reproducibility between different laboratories. The commercial kits are fully validated and designed to be more specific, reducing the likelihood of interference from other substances in the sample. Nevertheless, these kits are generally cost effective, making them suitable for regular monitoring without significantly impacting the budget. The reactivity of the TBA assay towards aldehydes has been compared to commercial tests based on the TBA assay [43] or other derivatization agents [71], demonstrating their simplicity and speed, making them accessible for routine analysis in various settings, including laboratories and quality control environments.

To ensure the comparability of results and the validity of MDA as a measure of LPO in experimental and toxicological studies, scientific reports should include detailed information on the analytical and pre-analytical conditions used, assuming that samples have been properly handled and stored, since LPO can occur in matrices rich in PUFA during storage and processing activities. Fish lipids differ from mammalian lipids because they contain up to 40% of PUFA with high susceptibility to degradation, such as oxidation, which has been shown to determine the formation of off-flavor components, reduced quality during different storage conditions, loss of nutritional value and even formation of anti-nutritional molecules [133].

10. Conclusions

This review aims to demonstrate the different methods to assess LPO by determining MDA in biological samples of fish as a biomarker of oxidative stress, evoked or naturally occurring.

Under environmental stress or developmental signals, MDA levels increase, and this may result in either defense signaling if MDA accumulates transiently or trigger cell death if there is a sustained accumulation of MDA in the cells.

Many factors (*e.g.* stimulus and conditions of peroxidation) modulate the formation of MDA from lipids. Only certain lipid peroxidation products generate MDA (always at low yields), and MDA is neither the sole end-product of lipid peroxide formation and degradation nor a substance generated exclusively by LPO.

The most used chemical derivatization reagent for the detection of MDA and

other yet unnamed TBARS in fish matrices is TBA. To date, no consensus has been reached on the procedure for sample preparation and derivatization temperature and time.

Although the MDA content is overestimated due to the lack of correction, a spectrophotometric method has been widely used. When using HPLC, specificity problems may persist because other adducts formed may have the same retention time and may be indistinguishable from the MDA-TBA₂ adduct. Detection techniques must then be selected to detect interfering compounds.

Despite the undoubted advantages of a room temperature reaction, derivatization with DNPH or other reagents is rarely used for the determination of LPO in fish.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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