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Analytical Methods

Measurement of malondialdehyde in fish: A comparison study between HPLC methods and the traditional spectrophotometric test

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ABSTRACT

Alternative methods to the traditional spectrophotometric determination of the malondialdehyde-thiobarbituric acid (MDA-TBA) complex (method A) and to the overestimation of MDA levels in the TBA reaction have been develop for the evaluation of lipid oxidation in fish. In this study, two HPLC separation methods of the MDA-TBA (method B) and MDA-dinitrophenylhydrazine (MDA-DNPH) adduct (method C) were investigated and compared to the traditional spectrophotometric TBA test (method A) in samples of chilled fish (hake, sea bream and sardine). Detection limits were 0.16, 0.10 and 0.20 μM MDA and quantification limits were 0.23, 0.17 and 0.26 μM MDA, for methods A, B and C, respectively. Recovery of method B ranged between 100% and 108% and of method C between 90% and 112%. Method A presented low recovery levels (under 71%). Overall method performance followed the order HPLC method MDA-DNPH > HPLC method MDA-TBA > traditional spectrophotometric TBA test. Though showing a better accuracy and specificity, method C had, however, some disadvantages, a relatively high limit of detection (0.20 μM MDA) and a lower reproducibility at lower MDA contents in standards and samples. Nevertheless, these are not critical drawbacks for an application in routine fish analysis, given the high MDA concentrations in oxidised fish. The application of the modified HPLC methods in fish samples with different levels of MDA, showed that these methods are useful for the samples with low amounts of oxidation products, such as chilled hake as well as in samples with high levels of oxidation, like 15 days chilled stored sardine.

1. Introduction

Lipid oxidation in food is associated with the development of rancidity and oxidative deterioration. Fish, on account of its high content of polyunsaturated fatty acids (PUFA), is highly susceptible to lipid oxidation during manipulation, processing, and cooking. As a consequence of oxidative spoilage, lipid hydroperoxides are formed, which, in turn, are unstable and decompose to aldehydes, ketones, alcohols, acids or hydrocarbons (St. Angelo, 1996). These so-called secondary oxidation products can change food quality, namely, colour, texture, flavour and odour (Fernández, Pérez-Álvarez, & Fernández-López, 1997). One of the most important products of oxidation is malondialdehyde (MDA), which is thought to be a carcinogenic initiator and mutagen. MDA has often been used as marker of oxidative damage in biological samples (Kinter, 1995) and foods (St. Angelo, 1996). The most widely used method for determination of MDA is the spectrophotometric determination of the pink fluorescent MDA-thiobarbituric acid (MDA-TBA) complex produced after reaction with 2-thiobarbituric acid (TBA) at low pH and high temperature (Bergamo, Fedele, Balestrieri, Abrescia, & Ferrara, 1998). This simple technique is used in fish analysis, whenever an assessment of lipid oxidation is required (Panpipat & Yongsawatdigul, 2008). Reaction occurs by attack of the monoenolic form of MDA on the active methylene groups of TBA. Visible and ultraviolet spectrophotometry of the pigment confirms its primary maximum at 532–535 nm and a secondary one at 245–305 nm (Sinnhuber, Yu, & Yu, 1958). The intensity of colour is a measure of MDA concentration (Tarladgis, Pearson, & Dugan, 1964) and has been correlated with rancidity (Zipser, Kwon, & Watts, 1964). Reaction kinetics depends on the concentration of TBA solution, temperature and pH (Fernández et al., 1997).

Several variations of MDA-TBA method exist, namely, a method for fish lipids was described by Ke and Woyewoda (1979) and for fish by Vyncke (1970). Furthermore, various procedures are generally performed in food: direct heating of the sample with TBA (Sinnhuber & Yu, 1958), sample distillation (Ke, Cervantes, & Robles-Martinez, 1984), lipid extraction with organic solvents (Pikul, Leszczynski, & Kummerow, 1989) or acid extraction of MDA (Squires, 1990), followed by acid reaction with TBA. In spite of these improving modifications, the traditional spectrophotometric TBA test has been criticised for its lack of sensitivity (Squires, 1990) and its high inaccuracy, since TBA reacts not only with MDA but also with many other compounds (for instance, carbohydrates, amino acids, pyridines, pigments, etc.) (Guillen-Sans & Guzman-Chozas, 1998), interfering in the TBA assay and resulting in

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considerable overestimation, as well, variability in the results (Mateos, Lecumberri, Ramos, Goya, & Bravo, 2005). The traditional spectrophotometric TBA test has also shown problems when used in frozen Gadidae fish species, like saithe, haddock, cod and others. During such storage, these species can produce an important amount of formaldehyde which in turn was shown to interfere in the traditional spectrophotometric TBA test (Aubourg, 1999; Careche & Tejada, 1988). Furthermore, the high temperatures (95–100 °C), extended incubation times (up to 150 min) (Sakai, Habiro, & Kawahara, 1999; Volpi & Tarugi, 1998) and strong acidic conditions (pH 1.5–3.5) commonly required for the reaction of MDA with TBA may cause an artifactual peroxidation of sample constituents even in the presence of added antioxidants. For this reason and in order to eliminate interferences in the formation of the MDA-TBA red pigment, more sensitive and advanced methods of analysis of biological matrices by capillary gas chromatography with electron-capture and mass spectrometry and liquid chromatography–mass spectrometry were developed (Cighetti, Debiasi, Paroni, & Allevi, 1999; Jardine, Antolovich, Prenzler, & Robards, 2002; Stalikas & Konidari, 2001). Also more specific high-performance liquid chromatographic (HPLC) approaches using reversed-phase chromatography (Draper & Hadley, 1990; Squires, 1990) with UV/VIS absorption (Cesa, 2004) or with fluorimetric detection (De las Heras, Schoch, Gibis, & Fischer, 2003) have been used. Derivatisation of MDA with 2,4-dinitrophenylhidrazine (DNPH) and conversion into pyrazole and hydrazone derivatives, has been also found to allow a specific estimation of MDA, particularly, if combined with HPLC separation (Mateos et al., 2005). Absorption at 310 nm is used to calculate MDA concentration. This method has been used to determine MDA levels in biological samples, such as rat and human plasma (Pilz, Meineke, & Gleiter, 2000) or rat urine (Ekström, Garberg, Egestad, & Högerg, 1988). However, it has not been applied yet to fish samples.

Though the mentioned disadvantages, conventional spectrophotometric MDA-TBA methods are preferred because of their simplicity. Therefore, development of a simple, sensitive and specific MDA-TBA method has remained a challenge. The objective of this study was to develop an improved sensitive and specific HPLC method for MDA determination in fish with different fat contents and different degrees of rancidity, using TBA or DNPH as derivatising reagents and compare these two methods with the traditional spectrophotometric technique (TBA test).

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and reagents used were analytical grade of the highest purity. Potassium dihydrogenphosphate (KH_2PO_4), potassium hydroxide (KOH), hydrochloric acid fuming 37% (HCl), glacial acetic acid (CH_3COOH), trichloroacetic acid (TCA, CCl_3COOH), perchloric acid (PCA, $HCLO_4$), ethylenediaminetetracetic acid (EDTA, $C_{10}H_{16}N_2O_8$) and propyl gallate ($C_{10}H_{12}O_5$) were purchased from Merck (Darmstadt, Germany). 2-thiobarbituric acid (2-TBA, $C_4H_4N_2O_2S$) and 1,1,3,3-tetraethoxypropane (TEP, $C_{11}H_{24}O_4$) were from Sigma (St. Louis, MO, USA). 2,4-dinitrophenylhydrazine (DNPH, $C_6H_6N_4O_4$) was from Fluka (Deisenhofen, Germany). HPLC grade organic solvents were used: methanol (CH_3OH) and acetonitrile (CH_3CN) were from Merck (Darmstadt, Germany). Aqueous solutions were prepared with Milli-Q purified water.

2.2. Materials

Sardine (*Sardina pilchardus*) and farmed sea bream (*Sparus aurata*) were bought fresh from a local supermarket. South African hake (*Merluccius capensis*) was purchased frozen from a local fish processor and thawed overnight in a refrigerated chamber. For the study of rancidity development all fish species were kept in a refrigerated chamber (2 ± 1 °C) and analysed periodically over a period of 19 days.

2.3. Sample preparation

For MDA extraction, a portion (15 g) of minced fish was weighed in a 50 ml centrifuge tube and diluted with 30 ml of 7.5% TCA solution (7.5% (p/v) TCA, 0.1% (p/v) EDTA, 0.1% (p/v) propyl gallate). Mixture was then homogenised with a Polytron PT3000 blender for 1 min at 5000 rpm and filtered through filter paper (Whatman #1). Filtrate was centrifuged for 10 min at 6000 rpm. Supernatant (extract) was used in both TBA and DNPH methods.

2.4. Traditional spectrophotometric TBA determination of MDA-TBA (TBA Test)

This MDA determination (method A) was performed according to Vyncke's methodology (Vyncke, 1970). TEP (1,1,3,3-tetraethoxypropane) was used as the MDA standard, without hydrolysis prior to the TBA reaction. A standard curve was made from TEP diluted in 7.5% TCA solution, at concentrations 2.0, 4.0, 6.0, 8.0 and 10.0 μM. 5 ml sample supernatant, standard or blank was transferred into a screw-capped tube, 5 ml of 20 mM TBA solution was added, mixture was vigorously agitated in a vortex and placed in a boiling water bath for 60 min. After cooling, MDA-TBA complex was measured at 530 nm using an UNICAM Helyos spectrophotometer. Results were expressed as micromoles MDA present in 1 kg of muscle.

2.5. HPLC determination of MDA-TBA

HPLC separation of MDA-TBA adduct (method B) was performed according to the method described by Seljeskog, Hervig, and Mansoor (2006), with modifications in the sample deproteinisation procedure (see sample preparation, above). TEP was used as the MDA standard, without hydrolysis prior to the TBA reaction. A standard curve was made from TEP diluted in 7.5% TCA solution, at concentrations of 0.6, 1.3, 2.5, 5.0, 10.0 μM. 0.5 ml sample supernatant, standard or blank was transferred into a screw-capped tube, 1.5 ml of 40 mM TBA solution was added, mixture was vigorously agitated in a vortex and placed in a hot water bath at 97 °C for 60 min. After cooling in a freezer at −20 °C for 20 min, 3 ml methanol was added and mixed in a vortex. The resulting solution was filtered through a 0.2 μm PTFE membrane (Acrodisc® CR 25 mm Syringe Filter, PALL Life Sciences) into autosampler vials. HPLC analysis was performed using an Agilent 1100 Series system, equipped with pump, degasser, autosampler, spectrofluorimetric detector and system controller with a PC control program. Separation of MDA-TBA was done using a Phenomenex Gemini C18 column (5 μm, 150 × 4.6 mm), operated isocratically with a HPLC mobile phase pumped at 1.0 ml/min and consisting of 50 mM KH_2PO_4 buffer solution, methanol and acetonitrile in the proportion 72:17:11 (v/v). In this method, injection volume was 10 μl, sample run took 8 min and retention time for MDA-TBA was near 5.5 min. Spectrofluorimetric detector wavelengths were set at 525 nm (excitation) and 560 nm (emission). Results were expressed as micromoles MDA present in 1 kg of muscle.

2.6. HPLC determination of MDA-DNPH

MDA-DNPH adduct detection (method C) was based on the method described by Mateos et al. (2005), modified in order to ob-