Determination of malondialdehyde in traditional fish products by HPLC

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The oxidation state of traditional fish products was measured by determining the malondialdehyde (MDA) level by HPLC and the results were compared to those given by a spectrophotometric method. The procedure involves oxidation of the products by incubation at 40 °C for 3 d. Samples were steam distilled in a Kjeldahl distillation apparatus and the MDA was determined in the aqueous distillates by HPLC, using a μ-Bondapak C18 column, with mixed mobile phase of 1% acetic acid–acetonitrile (85 + 15; v/v). A total time of 2 min was necessary to assay each distillate and only MDA was detected. MDA can be determined at a level of 1.5×10^{-8} mol l^{-1}. The highest rate of oxidation of the samples, as shown by the changes in the TBA test and MDA concentration determined by HPLC, was observed in smoked fish and the lowest in dried–salted fish.

Introduction

Fish is an important commodity in the diet of most people in Mediterranean region.

There is a broad range of fish products in the Greek market, all having characteristics different from those of the fish from which they are prepared. The main reason for processing fish today is to produce a pleasantly flavoured alternative form fish; normally the texture is firmer than that of the raw material. However the products can also have a longer shelf life than that of the fish from which they are made, due to the preservative action of salt and smoke.[1]

Lipid oxidation is one of the major causes of food spoilage, particularly in fat containing foods like traditional fish products. It leads to the development of various off-odours generally called rancid and discoloration, which render these foods unacceptable or reduce their self-life. In addition, oxidative reactions can decrease the nutritional quality of food, and certain oxidation products are potentially toxic.[2]

Malondialdehyde (MDA), is usually one of the well-known secondary products, has been measured by the TBA method. The TBA test involves the reaction of 2-thiobarbituric acid (TBA) with MDA in edible oils to produce a chromogen which can then be determined spectrophotometrically at 532–535 nm. The major problem of the method is a lack of specificity. The TBA reacts with products of lipid peroxidation such as hydroperoxides and conjugated aldehydes to generate substances which absorb at 535 nm, similar to the adduct of MDA and TBA.[3–6] Thus, the analysis of fatty foods by the spectrophotometric procedure is subject to misinterpretation.

Most of the MDA present in fatty foods exists bound to other food constituents and very little of it exists in the free form. Thus acid must be added to the food to be analysed in order to liberate the MDA.[7]

An HPLC method has been developed for determining total MDA in vegetable oils, after conversion of the MDA released from its precursor, to the dansyl–pyrazole derivative.[8]

In this HPLC method,[7] the quantitation of malondialdehyde (MDA) in aqueous distillates from freeze-dried chicken meat was determined using a mixed mobile phase of 1% acetic acid and acetonitrile (15 + 85; v/v), with a UV detector, at a level of 1.0×10^{-6} mol l^{-1}. In another HPLC method,[9] the determination of MDA in vegetable oils was obtained using a mobile phase of 1% acetic acid and acetonitrile (85 + 15; v/v) with a UV detector to determine MDA at a level of 1.0×10^{-9} mol l^{-1}.

The scope of the present work was to determine a suitable method for the measurement of the degree of rancidity in traditional fish products.

Experimental

Reference compounds and solvents

TBA reagent 1,1,3,3-tetramethoxypropane (TMP) was purchased from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) and acetic acid (reagent grade) were purchased from Merck Ltd. (Darmstand, Germany).

Dried–salted fish (*Gadidae* spp., *Octopus vulgaris* and *Scomber scombrus*), salted fish (*Engaulis encrasicolous, Clupea pilchardus, Sarda sarda* and *Gadidae* spp. roe), smoked fish (*Clupea harengus*) and marinated fish (*Clupea harengus, Octopus vulgaris* and a mixture of *Boops boops, Trachurus trachurus, Meana meana* and *Spicara vulgaris* called Savori) were obtained from the central fish market of Athens (Greece).

Instrumentation

Chromatographic determinations were performed on a Millipore-Waters (Milford, MA, USA) liquid chromatograph equipped with 600E pump and a Waters 486 tunable absorbance UV detector. A computer integrator running a Waters Baseline 815 software was employed to record retention times and chromatograms and to evaluate peak areas. The method used was adapted from Tsaknis *et al*.[9] A reverse-phase column Waters μ-Bondapak C18, 3.9 × 300 mm, particle size 10 μm was used at ambient temperature. Chromatograms were monitored at 254 nm and with a sensitivity of 0.01 absorbance units full scale (AUFS); the mobile phase was 1% acetic acid–acetonitrile (85 + 15; v/v); the flow-rate was 2.5 ml min^{-1}; the pressure was 1300–1500 psi; the injection volume was 20 μl; the retention time for MDA was 1.44 min.

Preparation of standards

The standards were prepared according to the procedure described by Tsaknis *et al.*[9]

MDA standards. TMP (10 μl) was accurately diluted to 10 ml with 0.1 mol l^{-1} HCl in a screw-capped test tube and immersed into a boiling water bath for five minutes, then quickly cooled with tap water (solution X). A working stock solution of MDA was prepared by pipetting 1.0 ml of the hydrolysed acetal (solution X) into a 100 ml volumetric flask and diluting to volume with water. The working stock solution was 6.07×10^{-5} mol l^{-1} acetal or 4.37 μg ml^{-1} malondialdehyde. A 1 + 9 dilution of the working stock solution was made before preparing the actual standard curves. These standards were also used for the TBA method.

TBA solution. A 0.02 mol l^{-1} solution of 2-thiobarbituric acid in 90% glacial acetic acid was prepared.

Preparation of samples

The skin and bones were removed from the fish products. Approximately 50 g of muscle from each sample were milled with a Vorwerk Thermomix 3300 (Vorwerk, Paris, France) at a speed of 12 and placed into a glass Petri dish. Samples were oxidised in an oven (Memmert TW40U, Hamburg, Germany) and held at a constant temperature of 40 °C for 3 d.

Quantification

A portion (5 g) of oxidised (3rd day) or 5 g of non-oxidised (1st day) sample was accurately weighed and slurried in a beaker with 80 ml of water and the pH value was adjusted to 1.5–1.8 with 2 mol l^{-1} HCl. The flasks were connected to a standard micro-Kjeldahl unit and distilled. The distillation was conducted as quickly as possible using the maximum heater setting and terminated when 50 ml of distillate was collected in a 50 ml volumetric flask (this usually took about 15 min). A portion (5 ml) of the distillate was used for the TBA test and 20 μl for HPLC analysis.

Standard calibration graphs were prepared for MDA by plotting peak area measurements at 254 nm *versus* concentration. The recovery of 98.16% (see Table 1) was used to calculate the results.

TBA test

This was performed according to the method of Kakuda *et al.*[7] A 5 ml sample was mixed in a screw capped tube with 5 ml TBA reagent. The tubes were heated into a boiling water bath for 30 min, cooled with tap water and the absorbance was measured at 532 nm with a Hitachi 3210 Spectrophotometer (Hitachi Ltd., Tokyo, Japan).

Table 1 Recovery values for MDA measured by HPLC

	MDA standards/mg		
No.	Without distillation	After distillation	Recovery (%)
1	1.000	0.97	97
2	2.000	1.98	99
3	3.000	2.94	98
4	4.000	3.95	98.7
5	5.000	4.89	97.8
6	6.000	5.93	98.8
7	7.000	6.84	97.7
8	8.000	7.88	98.5
9	9.000	8.81	97.9
		Average	98.16

Results and discussion

Method development

The method of Malondialdehyde determination as reported by Kakuda *et al.*[7] proved unsuccessful when applied to the determination of free MDA in traditional fish products because with the suggested mobile phase (1% acetic acid–acetonitrile 15 + 85 v/v) no peaks were detected. When the percentage of the water phase (1% acetic acid) was reduced below 80% a considerably increased retention time and a flat MDA peak was observed, while with an increase above 89% the recovery of MDA appeared to be lower. After many trials the most suitable mobile phase was found to be 1% acetic acid–acetonitrile at a ratio of 85 + 15 (v/v). The detection limit of the present method is 1.5×10^{-8} mol l^{-1}, while the method suggested by Kakuda *et al.*[7] could only detect 1×10^{-6} mol l^{-1}. This former method was subsequently adopted for all HPLC work. Typical chromatograms for the MDA standard (1.8 mol $l^{-1} \times 10^{-10}$) and *Clupea harengus* oxidised sample are shown in Fig. 1.

Stability of the MDA standards

Nine series of MDA standards were prepared in triplicate and immediately assayed by HPLC. After analysis, the standards were stored at 5 °C for 8 d and then were reanalysed by HPLC. Table 2 shows the concentration of the standard solutions and their corresponding peak areas on the 1st and the 8th day.

Recovery test

A working stock solution (100 mg MDA 100 ml^{-1} H_2O) was prepared using the same method as described previously. Nine standard solutions were prepared using 1 to 9 ml of the working stock solution to give final concentrations of 1 to 9 mg 100 ml^{-1} respectively. These solutions were distilled following the same procedure as in the sample preparation. The distillates were subsequently assayed by HPLC.

The recoveries (Table 1) are within a range of 97 to 99% (mean 98.16%). This represents a significant improvement on the 70% recovery of MDA when determined by the TBA test,[10] 73.2% for the distillation procedure,[7] or 86.7, 78.9 and 88.3% for the single extraction, distillation and heating reflux procedures, respectively,[11] and demonstrates a further benefit of this method.

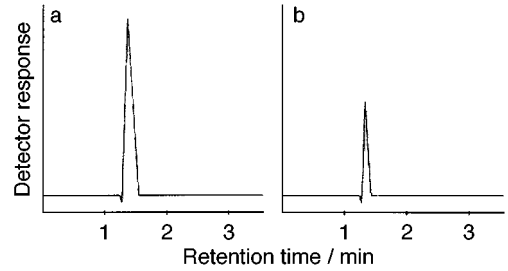

Fig. 1 Chromatograms showing (a) an oxidised fish sample and (b) an MDA standard (1.8 mol $l^{-1} \times 10^{-10}$).

Table 2 Stability of MDA standards during storage at 5 °C for 8 d[a]

Concentration/ mol $l^{-1} \times 10^{-10}$	Peak area 1st Day[b]	8th day[b]	Loss (%)
0.3	545 ± 0.13	525 ± 0.09	3.67
0.9	1 677 ± 0.44	1 629 ± 0.17	2.86
1.8	3 430 ± 0.28	3 398 ± 0.26	0.93
2.7	5 538 ± 0.43	5 509 ± 0.19	0.52
3.31	6 598 ± 0.22	6 502 ± 0.28	1.45
3.92	7 963 ± 0.32	7 856 ± 0.34	1.34
4.52	9 014 ± 0.45	8 866 ± 0.12	1.64
5.16	9 871 ± 0.17	9 815 ± 0.57	0.57
5.72	10 903 ± 0.61	10 817 ± 0.46	0.79

[a] Statistical analysis of the data reported in Table 2 reveals that there was no difference between the MDA peak areas for day 1 and day 8 (Students' *t* test) at the 5% level of significance**.** Thus the MDA standards are stable during 8 d storage at 5 °C . [b]Average ± standard deviation (n = 3).

Table 3 HPLC results of the MDA concentration (mol^{-1}) of oxidised and non-oxidised samples

Sample	Non-oxidised	Oxidised
Dried-salted fish—		
Gadidae spp.	0.39	3.44
Octopus vulgaris	0.12	2.21
Scomber scombrus	0.69	3.23
Salted fish—		
Engaulis encrasicolous	1.01	4.06
Clupea pilchardus	1.03	4.16
Sarda sarda	0.39	3.57
Gadidae spp. roe	0.73	4.02
Smoked fish—		
Clupea harengus	2.84	8.50
Marinated fish—		
Clupea harengus	2.43	8.01
Octopus vulgaris	1.69	5.10
Savori (mixture of *Boops boops, Trachurus trachurus, Meana meana* and *Spicara vulgaris)*	2.12	6.69

Comparison of MDA levels in traditional fish products by the HPLC and TBA test method

Samples of non-oxidised and oxidised traditional fish products were prepared as described previously. The TBA test and HPLC method tested these samples for MDA content. The HPLC results are shown in Table 3. An explanation for the different levels of MDA in the above products is that only peroxides which possessed unsaturation β- or τ- to the peroxy radical are capable of undergoing cyclisation with the ultimate formation of MDA. Such peroxides could only be produced from fatty acids containing three or more double bonds.[4] These fatty acids are common in fish as seen in fatty acid determinations from other workers.[12–15]

The highest rate of oxidation of the samples, as shown by the changes in the TBA test and MDA concentration determined by HPLC (see Table 3 and Figs. 2 and 3), was observed in smoked fish and the lower in dried–salted fish.

A correlation of the results from the HPLC and TBA test for non-oxidised and oxidised fish samples is given in Figs. 2 and 3, respectively.

The sensitivity of the TBA test was lower than the HPLC method: *i.e.* 7.8×10^{-6} mol l^{-1} MDA and 0.5×10^{-8} mol l^{-1} MDA, respectively. As expected, the TBA values for MDA were found to be higher than those given by the HPLC method. An explanation for this is that the TBA reagent can react with a variety of compounds present in oxidised lipids other than MDA. MDA is only one of many compounds associated with rancidity.[16]

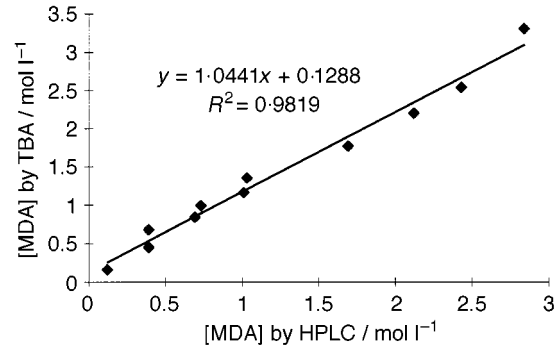

Fig. 2 Correlation of HPLC concentrations (mol l^{-1} MDA) *vs*. TBA test concentrations (mol l^{-1} MDA) of fresh samples.

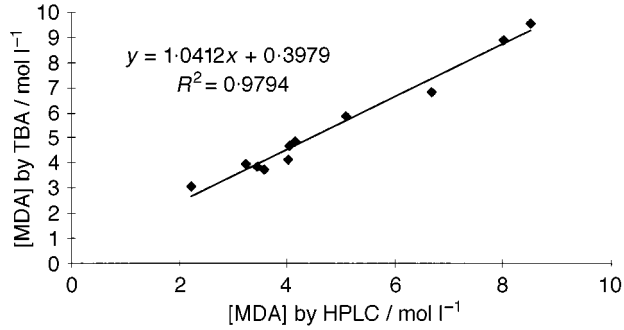

Fig. 3 Correlation of HPLC concentrations (mol l^{-1} MDA) *vs*. TBA test concentrations (mol l^{-1} MDA) of oxidised samples.

The HPLC method was faster since only a total of 2 min per injection was required for the analysis of traditional fish products. This method also is more accurate and specific because the results do not depend on the formation of a coloured complex. Interpretation depends on the fact that oxidation is a complex procedure producing a variety of compounds, all of which may affect rancidity assessment.

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