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Aldehyde formation in frozen mackerel (*Scomber scombrus*) in the presence and absence of instant green tea

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Abstract

The effect of frozen storage on lipid peroxidation in Atlantic mackerel (*Scomber scombrus*) stored for up to 26 weeks at −10 or −80 °C (control), with and without green tea antioxidants, was investigated. Hydroperoxides (PV) and aldehydes (TBARS) were measured by HPLC and LC–MS and hexanal by GC. There was an increase in peroxide value which was associated with an increase in aldehydes, followed by hexanal increase with storage time and at a higher temperature of −10 °C compared with samples stored at −80 °C. Although TBARS is a common assay used to follow malondialdehyde formation, other aldehyde products can also react with thiobarbituric acid to give the red chromogen. Analysis of aldehyde–TBA adducts by LC–MS confirmed the presence of malondialdehyde and, in particular, we report the production of gluteraldehyde for the first time in stored frozen fish. Green tea (at 250 ppm) substantially slowed down the oxidation process, whereas at 500 ppm it was less effective.

Keywords: Frozen fish; Lipid peroxidation; Malondialdehyde; Gluteraldehyde; Hexanal; Green tea

1. Introduction

Atlantic mackerel contains 2–4% lipids which comprise high levels of polyunsaturated acids that are prone to oxidation, resulting in off-flavours, changes in colour and texture and loss of nutrients (Ackman, 1989; Ericson & Hung, 1997; Harris & Tall, 1994; Kolakowska, 2002; Saeed & Howell, 2002). Primary lipid peroxidation products include hydroperoxides that are unstable and decompose to generate various secondary products, such as aldehydes, that can contribute to food rancidity. Aldehydes are cytotoxic compounds due to their reactivity with nucleophiles and sulphydryl groups of protein and nucleophilic acids or related amino acids. Consequently, in the body, they induce cell injury and death and result in several diseases, such as atherosclerosis and cancer (Esterbauer, 1982; Hoberman & San George, 1988). In addition, cytotoxicity increases with increasing chain length of the aldehydes (Esterbauer, Zollner, & Schaur, 1990). Unlike free radicals, aldehydes can easily diffuse from the production site and spread the damage further (Esterbauer, 1982; Esterbauer et al., 1990).

Aldehydes can cross-link with different compounds, such as proteins, in muscle, and consequently increase muscle hardness and toughness. The reaction of aldehydes, particularly those with di-functional aldehydic groups, such as gluteraldehyde (GLA) and malondialdehyde (MDA), with amino groups in proteins or DNA, can result in structural damage and change in their functionality (Addis, 1986; Gerrard & Brown, 2002; Nair, Cooper, Vietti, & Turner, 1986). In addition, cross-linking with proteins can result in aggregation and protein insolubility. According to sensory assessment, an increase in hexanal levels correlates with the enhancement of rancid odour and off-flavours. Secondary and final oxidation products are a reliable indicator of flavour deterioration in fish

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products (Shahidi, 1998). Therefore, lipid oxidation products, namely MDA, GLA and hexanal, were investigated to follow the development of oxidative rancidity in Atlantic mackerel.

In this study, instant green tea was tested as a natural antioxidant to prevent or retard the development of oxidative rancidity in fish, since it has been reported that synthetic antioxidants may cause toxicity (Barlow, 1990; Kaur & Kappor, 2001; Prior & Cao, 2000). Tea catechins possess strong scavenging capacity for free radicals and are reported to have significantly higher scavenging activities than have vitamin E and ascorbic acid; they also possess metal-chelating capacity (Jo, Son, Son, & Byun, 2003; Nanjo et al., 1996; Tang, Kerry, Sheehan, & Buckley, 2002; Unno, Sugimoto, & Kakuda, 2000). However, the role of whole instant tea as an antioxidant in fatty fish has not been assessed. Many methods have been used to follow the extent of lipid oxidation, including peroxide value (PV) and thiobarbituric acid-reactive substances (TBARS), gas chromatography (GC) and liquid chromatography–mass spectrometry (LC–MS). TBA can react with aldehydes to produce specific pigments. In the present study, the TBARS method by LC–MS was used, not only to identify MDA but also GLA. TBA can react with products of lipid peroxidation, such as hydroperoxides and conjugated aldehydes, to produce substances that absorb at 535 nm, similar to the product of MDA with TBA. GC was used to determine hexanal (Dahle, Hill, & Holman, 1962; Draper & Hadley, 1990; Kakuda, Stanley, & van de Voort, 1981).

2. Materials and methods

2.1. Materials

Atlantic mackerel (*Scomber scombrus*) was supplied by M & J Seafood, Farnham, UK. Instant green tea was provided by Tokyo Nikken Foods Co. Ltd. Japan. 3,3,3-Tetraethoxypropane (TEP), thiobarbituric acid (TBA) and glutaraldehyde standard, sodium thiosulphate, starch, potassium iodide, glacial acetic acid and chloroform were purchased from Sigma–Aldrich Company Ltd., Poole, England. All reagents used were of analytical grade.

2.2. Methods

2.2.1. Sample preparation

Eighty matched deboned fillets (about 6 kg) of Atlantic mackerel were used. In order to facilitate mixing of green tea, the fillets were minced and divided into groups. One group was stored at −80 °C, a second group was frozen at −10 °C without antioxidant as a control, and the third and fourth groups were mixed with 250 or 500 ppm green tea, respectively, and stored at −10 °C. Tests were conducted at time zero, and subsequently at 4, 8, 16 and 26 weeks of storage, to investigate lipid oxidation during frozen storage.

2.2.2. Lipid extraction from Atlantic mackerel

Lipid was extracted from fresh minced mackerel by the Bligh and Dyer method, as modified by Saeed, Fawthrop, and Howell (1999). Minced fish was homogenised with methanol and chloroform (2:1) for 4 min. The fish homogenate was centrifuged for 10 min at 3000*g*. The lower chloroform phase, containing the lipid, was filtered and chloroform was evaporated. The oil extracted was stored under nitrogen at −80 °C for further analysis.

2.2.3. Peroxide value

Air was expelled from a conical flask by using nitrogen gas. Fish oil (0.5 g) was weighed into the flask, followed by adding 10 ml chloroform and 15 ml acetic acid. After that, 1 ml of fresh saturated potassium iodide was added. The flask was sealed and swirled gently to mix the solution for 1 min and then placed in a dark cupboard for 1 min. Subsequently, about 1 ml of starch solution was added. The coloured solution was titrated with 0.002 N sodium thiosulfate solution. A control sample without the fish oil was also analysed. Peroxide value was calculated as follows:

$$\mathrm{PV} = \frac{(\text{PV titre} - \text{PV blank}) \times N \times 1000}{\text{Weight of fat used}},$$

where PV titre is the ml of sodium thiosulphate solution used in sample, PV blank, ml of sodium thiosulphate used in blank, and N is the concentration of thiosulphate solution.

2.2.4. Thiobarbituric acid-reactive substances (TBARS)

2.2.4.1. General. Thiobarbituric acid (TBA) reacts with TBA-reactive substances (TBARS), such as MDA, under acidic conditions to form a chromagen which can be measured by HPLC (Young & Trimble, 1991) and LC–MS at 532 nm.

2.2.4.2. Sample preparation. Fish muscle (1 g) was homogenised with 2.5 ml distilled water, followed by the addition of 100 μl of BHA to prevent further oxidation and 500 μl of 25% HCl and 500 μl 1% TBA. The mixture was vortexed for 20 s and incubated in a water bath at 90 °C for 1 h and cooled to 20 °C. Methanol (HPLC grade) (1 ml) and 400 μl of NaOH (1 M) were added to 400 μl of sample. The mixture was centrifuged at 13,000*g* for 10 min, and transferred to a vial for analysis. An aliquot (50 μl) was injected into the HPLC (Spectra system AS 3000) for analysis. The red complex separation was achieved on a 150 × 3 mm column containing Luna 5 μm phenylhexyl C18 packing (Phenomenex, Macclesfield, UK) and the sample was eluted with phosphate buffer (pH 6.5) solution. The flow rate was 1 ml/min. The peak assignment and quantification were performed by using standards which were prepared from 1,1,3,3-tetraethoxypropane.

2.2.4.3. Liquid chromatography–mass spectrometry (LC–MS). LC–MS equipment (ThermoFinnigan, San Jose, CA) consisted of a Surveyor MS pump, an autosampler with a

20 μl loop, and a PDA/UV detector. The UV detector was set to record at 530, 280 and 450 nm. Mass spectra were recorded in the negative ion mode for MDA and positive ion for GLA in the range m/z 240 to m/z 600. Zoom scan mode operation, by an interfaced LCQ Deca XP plus mass spectrometer fitted with an ESI source (ThermoFinnigan), was used to measure the collision-induced MS–MS spectrum of the adduct form. The software for the control of the equipment and acquisition and treatment of data was Xcalibar version 1.3. Compound separation was achieved on a 150 × 3 mm column containing Luna 5 μm phenylhexyl packing (Phenonemex, Macclesfield, UK). The chromatographic conditions were: flow rate 0.3 ml/min, sample injection volume 50 μl and mobile phases (A) 2% acetonitrile, 0.5% acetic acid, pH 2.68 and (B) 99.5% acetonitrile, 0.5% acetic acid. A line gradient was used and the run time was 35 min. The spray needle was set to 3.5 kV in the negative ion mode. The inlet capillary temperature was maintained at 350 °C, gas flow rate 80 arbitrary units and auxiliary gas flow 10 (arbitrary units). Mass detection was performed in the negative mode, for m/z between 200 and 500. In order to detect the TBA:GLA product, more sensitive target MS^n experiments were used to seek compounds with a particular molecular ion that might otherwise have been overlooked, e.g. m/z 353 (for TBA:GLA).

2.2.5. Hexanal determination

2.2.5.1. Method. The hexanal method was kindly supplied by Masterfood plc (Masterfoods, Central Nutrition and Microbiological Laboratories, UK).

2.2.5.2. Standards preparation. Stock hexanal (0.1 g) was weighed into a 100 ml volumetric flask with cyclohexane. Stock isobutyl acetate (0.1 g) was weighed into a 100 ml volumetric flask with cyclohexane. Internal standard, 2 ml of stock isobutyl acetate (IBA), was pipetted into 100 ml volumetric flask and was made up to 100 ml with cyclohexane. Working standard: 2 ml of both stock solutions hexanal and isobutyl acetate were pipetted into a 100 ml volumetric flask and made up to volume by adding cyclohexane.

2.2.5.3. Sample preparation. The sample (10 g) in triplicate was weighed and saturated sodium chloride solution (150 ml) and an internal standard, isobutyl acetate (2 ml) were added. The mixture was refluxed on a heating mantle for 15 min and left to cool for 15 min. The cyclohexane layer was injected into the gas chromatograph. The chromatographic conditions were: run time 17.60 min and injection volume 4 μl. The retention time, for isobutyl acetate, was approximately 6.2 min and, for hexanal, 7 min. The concentration of hexanal was calculated as follows:

$$\frac{\text{sample ratio}}{\text{average standard}} \times \text{hexanal standard concentration} \times \frac{\text{internal standard amount}}{\text{weight of sample}}$$

Average standard ratio = average ratio of calibration working standard run and Internal standard amount = 2 ml.

2.3. Statistical analysis

The results are expressed as means ± SD. Significant differences values were assessed with the unpaired Student's t-test. The significance level chosen for the statistical analysis was $p < 0.05$.

3. Results and discussion

3.1. General

In the present work the influence of time (up to 26 weeks) and temperature (−10 °C and −80 °C) on lipid deterioration, in the presence and absence of green tea antioxidant (250 and 500 ppm), produced during the frozen storage of fatty fish, in particular mackerel fish, was significant. The results of all experiments indicated an increase in lipid oxidation products with storage time; the highest value was observed in the sample stored at −10 °C without green tea.

3.2. Peroxide value (PV)

The PV value rose sharply until 16 weeks and then fell dramatically in all samples (Fig. 1). A faster increase in PV values was obtained at −10 °C without green tea, in contrast to a slow increase at −80 °C (Fig. 1), confirming previous results (Saeed & Howell, 2002). PV values were significantly higher in samples stored at −10 °C without green tea than in antioxidant-treated samples, particularly at 250 ppm green tea concentration ($p < 0.05$) (Fig. 1). In contrast, samples mixed with 500 ppm of green tea at −10 °C had higher PV values than samples treated with 250 ppm, which indicated that a low tea concentration was more effective in controlling

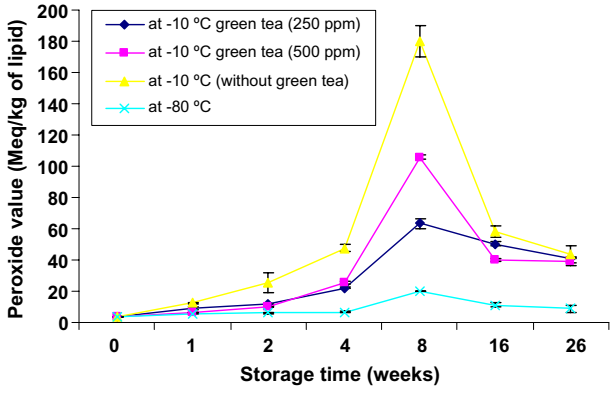


Fig. 1. Peroxide values obtained for mackerel stored at −80 °C and −10 °C in the presence or absence of green tea (250 or 500 ppm) for up to 26 weeks. Data represent the means ± SD of three experiments.

oxidation; it has been reported that a higher concentration of green tea may act as a pro-oxidant (Honglian & Etsuo, 2001). A decrease in the level of primary oxidation products is related to hydroperoxide degradation, producing secondary lipid peroxidation products (Undeland, 2001).

3.3. Hexanal conten

Fig. 2 shows a GC chromatogram of hexanal extracted from fish muscle stored at −10 °C for 8 weeks. Hexanal levels rose significantly after 16 weeks in samples stored at −10 °C without antioxidant compared with samples stored at −10 °C with 250 ppm green tea ($p < 0.01$) (Fig. 3). There was a major increase ($p < 0.001$) in samples stored at −10 °C compared with those stored at −80 °C. These results confirmed the trend for PV and TBARS results and the increasing concentration on storage showed that hexanal is one of the final products of lipid peroxidation (Sanches-Silva, Rodrýguez-Bernaldo de Quirós, López-Hernández, & Paseiro-Losada, 2004).

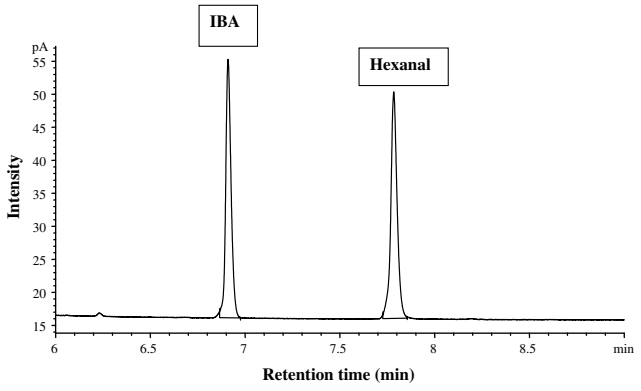


Fig. 2. Gas chromatography (GC) profile of hexanal extracted from mackerel fish stored at −10 °C for up to 4 weeks.

3.4. Thiobarbutric acid-reactive substances (TBARS)

3.4.1. General

Fig. 4 shows a typical TBARS HPLC chromatogram obtained from fish muscle. Overall, there was an increase in the level of TBARS during frozen storage, particularly in samples stored at −10 °C compared with −80 °C ($p < 0.01$) or −10 °C in the presence of green tea (250 ppm) ($p < 0.05$) (Fig. 5). After 10 weeks, there was a decrease, probably due to thiobarbituric acid (TBA)-reactive substances cross-linking with proteins (Saeed et al., 1999) (Fig. 5). As mackerel is a fatty fish, the values obtained over the whole storage period were relatively high and similar to previous studies (Aubourg, Sotelo, & Perez-Martin, 1982; Kurade & Baranowski, 1987; Saeed & Howell, 2002).

3.4.2. LC–MS of aldehyde–TBA adducts

TBARS is a common assay used to follow lipid oxidation in foodstuffs (Ladikos & Lougovois, 1999), based on spectrophotometric determination of malondialdehyde. However, other aldehyde products can react with thiobarbutric acid and thus the TBARS method can also be used

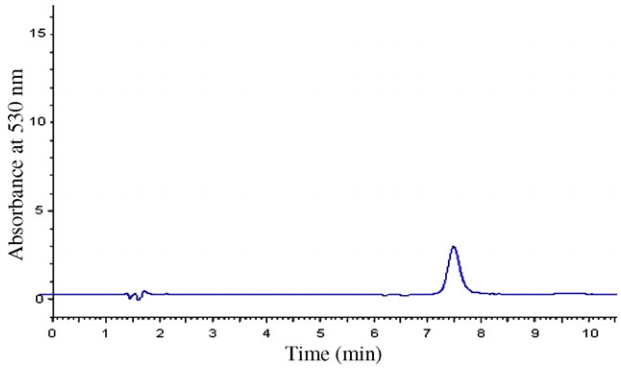


Fig. 4. HPLC Chromatogram of TBARS extracted from fresh fish.

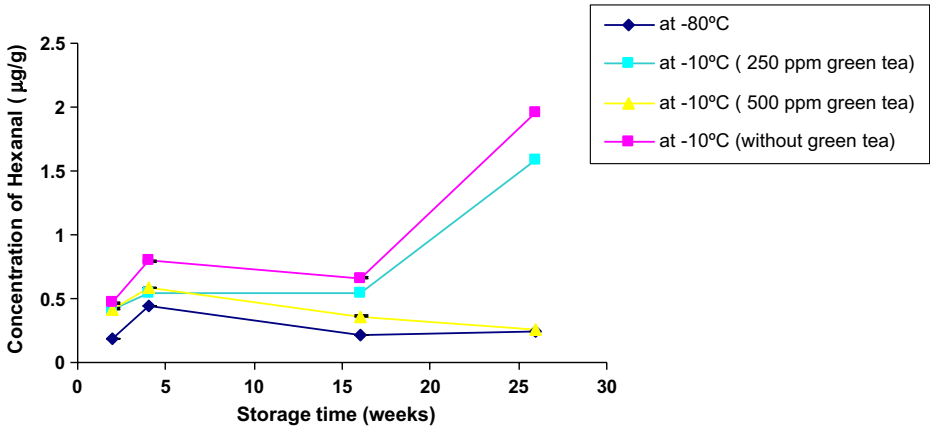


Fig. 3. Hexanal concentration in lipids extracted from mackerel stored at −80 °C and −10 °C with 0, 250 or 500 ppm of green tea for up to 26 weeks. Data represent the mean ± SD of three experiments.

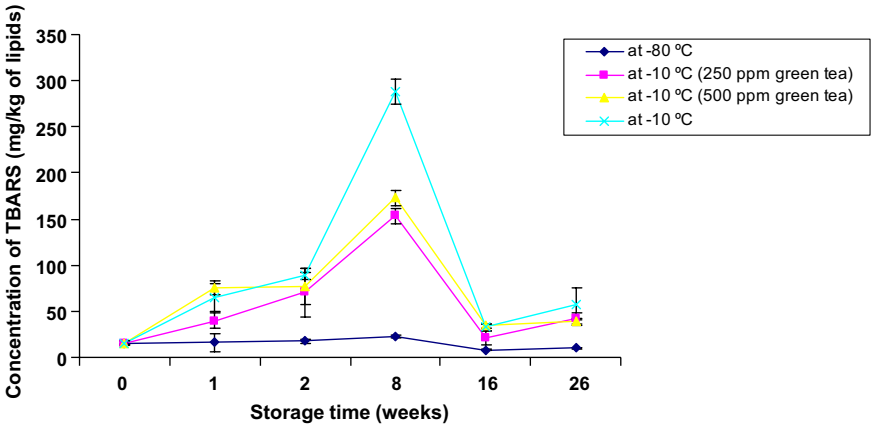
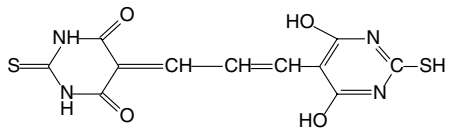


Fig. 5. Thiobarbituric acid test values obtained for mackerel stored for up to 26 weeks at −80 °C and at −10 °C with 0, 250 or 500 ppm of green tea. Data represent the mean ± SD of three experiments.

to assess other aldehydes formed during lipid oxidation (Vyncke, 1975). The red chromogen was analysed by LC–MS in full scan mode for the identification of aldehyde–TBA adducts. Aldehyde–TBA adducts were identified by comparing the retention time (t_r) with the standard and confirmed by LC–MS. A full scan chromatogram at 200–500 m/z for the parent condensation adduct for MDA–TBA is shown in Fig. 6. Absorbance maxima for the parent MDA:TBA 1:2 complex were measured at 525–530 nm; the results agreed with previous studies (Guillen-Sans & Guzman-Chozas, 1998; Janero, 1990; Jardine, Antolovich, Prenzler, & Robards, 2002). Identification was based on the negative parent ion at m/z 323 (MS) (Fig. 7a) and subsequent fragmentation pattern, consisting of a loss of ~102 mu from the parent ion to leave a base peak fragment m/z ~121 in MS^2 (Fig. 7b) and a loss of ~144 mu that gave a base peak fragment ~179 in MS^3 (Fig. 7c). Possible fragmentation products are shown in Fig. 8.

The chemical structure of GLA with TBA is illustrated in Fig. 9 and its possible fragmentations in Fig. 10. Analysis by MS showed that the parent ion $[M-H]^+$ was within +1 mu of the theoretical value for the fish sample and the standard. The GLA:TBA complex fragmented to give product ions of m/z ~335 (MS^2) (Fig. 11a) and ~194 (MS^3) (Fig. 11b).

In this study we have shown the formation of glutaraldehyde in frozen fish for the first time and confirmed its



$C_{11}H_8N_4O_4S_2$
324.33
323.998698

Fig. 6. Chemical structure for MDA:TBA (1:2) adduct.

presence and that of malondialdehyde by LC–MS. Recently, the presence of aldehydes in foods, including meat, has attracted attention because of their effect on food quality and safety. Aldehydes including saturated aldehydes, such as hexanal, formaldehyde and propanal, extracted from the headspace by GC analysis of fish tissue, have been reported (da Cunha Veloso, da Silva, Santos, & Andrade, 2001). In addition, an unsaturated aldehyde like malonaldehyde (Saeed & Howell, 2002), extracted from fish muscle, is a well known lipid peroxidation product, while other unsaturated aldehydes, which can be formed from the breakdown of hydroperoxides, like gluteraldehyde, need more investigation. Since fatty fish like mackerel have a high content of polyunsaturated fatty acids, lipid peroxidation products, particularly aldehydes, contribute to rancidity and food deterioration (Tsaknis, Lalas, & Evmorfopoulos, 1999), including protein aggregation and toughening of fish during frozen storage (Saeed & Howell, 2002) and DNA damage. Protein–aldehyde cross-linking results from the interaction of aldehydes with the ε-amino group in lysine, sulfhydryl group of cysteine and the imidazole group of histidine (Hoberman & San George, 1988), leading to changes in the primary structure of proteins that induce changes in the secondary and tertiary structures (Meng, Chan, Rousseau, & Li-Chan, 2005). Changes in protein structure cause changes in protein functionality and may ultimately cause extensive cell damage and death (Chen, Yang, Jiao, & Zhao, 2002).

In this study, instant whole green tea at low concentration (250 ppm) was an effective antioxidant. This result supports previous findings that natural phenolic compounds are effective in preventing rancidity of many lipid systems, in particular fish oils (Medina, Satue-Gracia, German, & Frankel, 1999; Ramanathan, 1992) and minced muscle (Fagbenro & Jauncey, 1994; Ikawa, 1998), and also act as anticarcinogenic substances. The biological and chemical properties of tea are related to the polyphenols content, called catechins, including (+)-catechin (C),

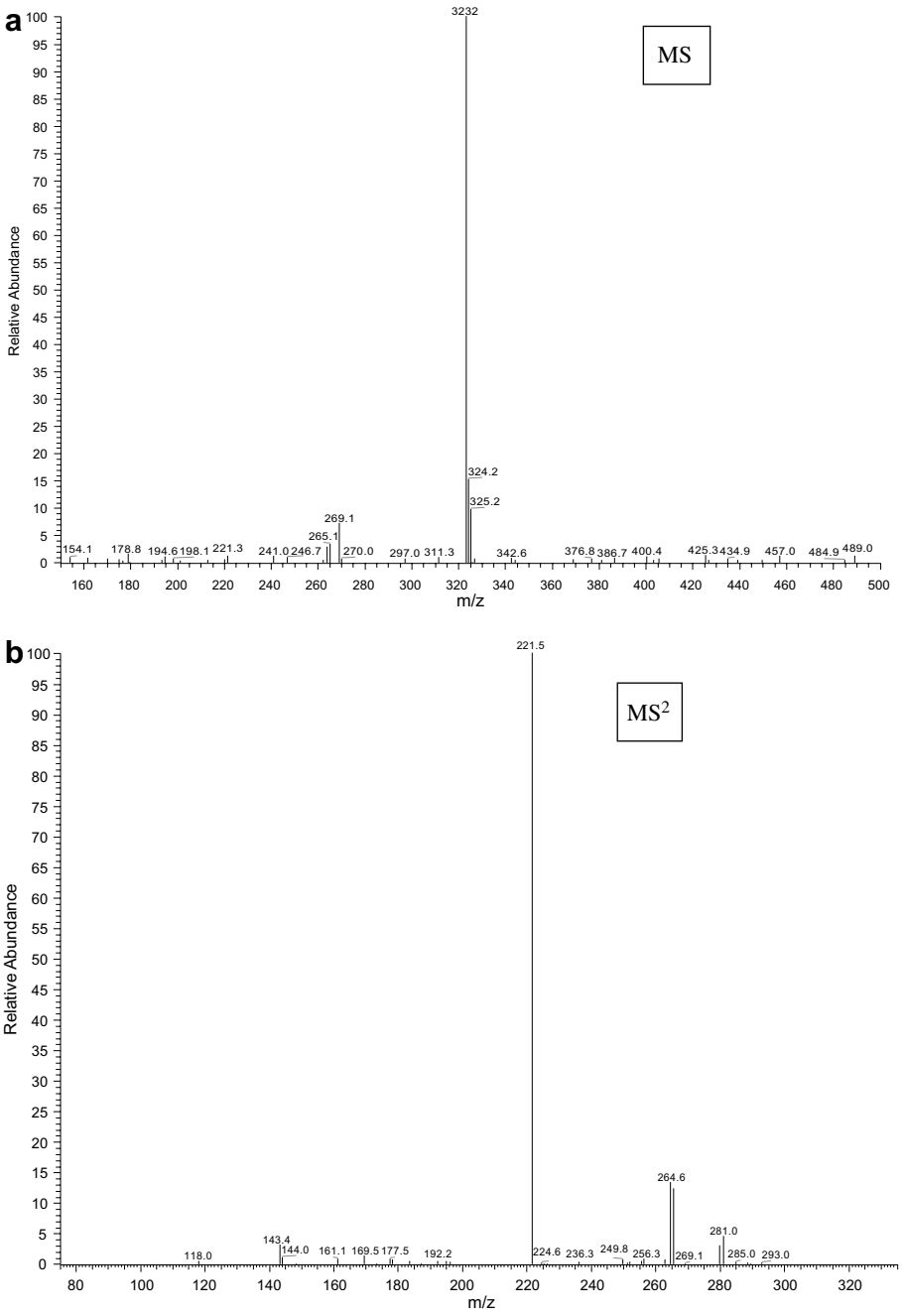


Fig. 7. Mass spectra of TBA:MDA obtained from lipid extracted from frozen mackerel stored at −10 °C for up to 8 weeks. LC–MS conditions are as described in the experimental section. (a) MS–MS spectrum of m/z 323, (b) MS^2 spectrum of 221 m/z and (c) MS^3 spectrum of 178 m/z.

(−)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epicatechin gallate (EGC), (−)-epigallocatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG) (Ninomiya, Unten, & Kim, 1997) (Fig. 12); the structure comprises OH groups which scavenge free radicals generated during lipid peroxidation and chelate metal ions. Compared with alpha tocopherol, catechins are reported to have a substantial antioxidants effect when they are mixed with cooked meat and fish products (Tang, Kerry, Sheehan, Buckley, & Morrisey, 2001a; Tang, Kerry, Sheehan, Buckley, & Morrissey, 2001b; Tang et al., 2002) due to protection or regeneration of alpha tocopherol which already exists in the meat or in the fish muscles. Moreover, green tea extracts were found to be good antioxidants, more effective than rosemary, in canola oil, pork lard and chicken fat heated to 100 °C (Chen et al., 1998) and had a higher antioxidant activity than those of vitamin C and E. However, as our results show, the high level instant green tea of 500 ppm was not as effective as was 250 ppm for reducing peroxidation of unsaturated fatty acids.

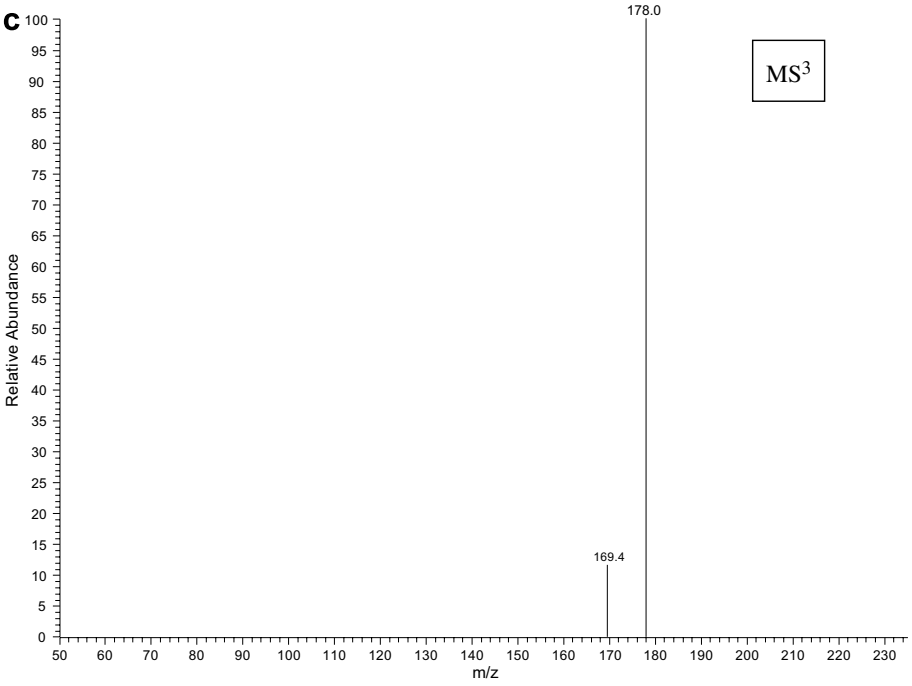


Fig. 7 (*continued*)

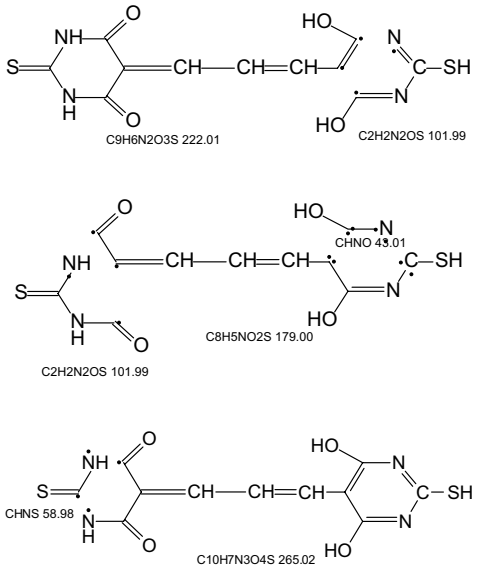


Fig. 8. Possible fragmentation of MDA:TBA adducts during MS^n.

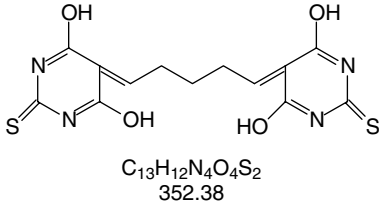


Fig. 9. Chemical structure for TBA:GLA adduct.

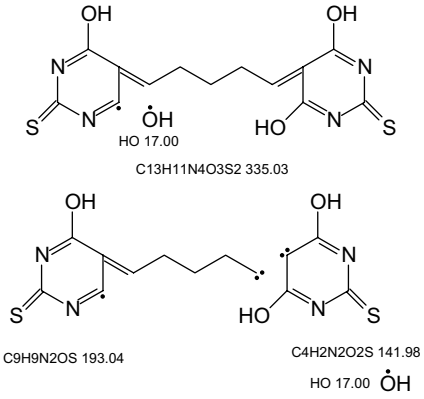


Fig. 10. Possible fragments obtained from parent peak (353 *m*/*z*) for GLA:TBA adducts formed during collision-activated dissociation.

In many instances, for example in our previous work (Sarkardei & Howell, in press), we have found that very high levels of rosemary act as a prooxidant due to production of excessive levels of antioxidant free radicals (Halliwell & Chirico, 1993). In this study, it is possible that 250 ppm of green tea was sufficient to scavenge the free radicals until 16 weeks, whereas 500 ppm was excessive After, 16 weeks of storage, 500 ppm produced lower levels of lipid oxidation products, particularly hexanal, which is the final product. After 26 weeks, inhibition of lipid oxidation was minimal, as all the antioxidants were probably used up.

In addition, it has been pointed out that a high concentration of tea induce apoptosis in many type of cells (Chen et al., 2002; Razat & John, 2005; Zho, He, Cheng, & Xin,

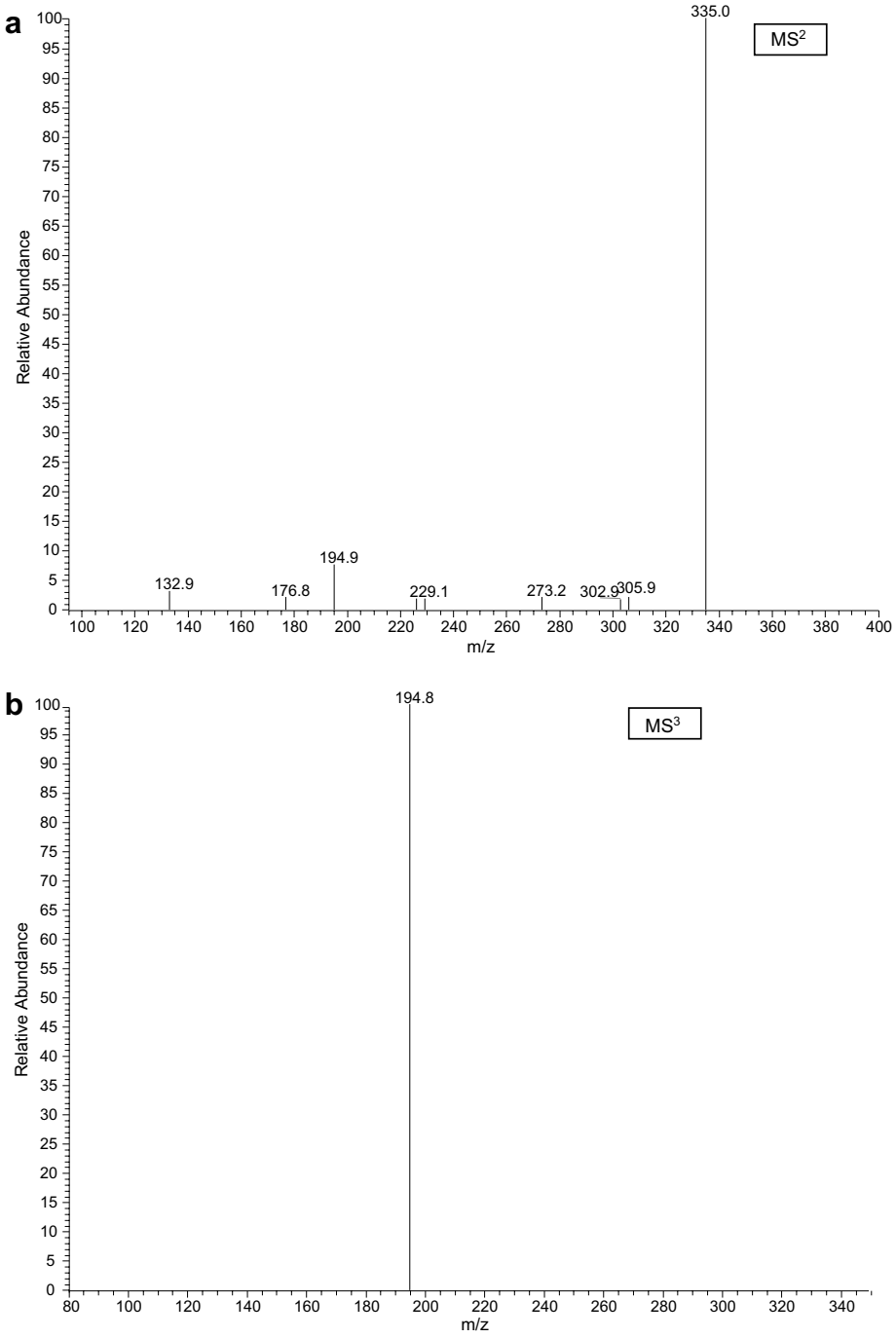


Fig. 11. Mass spectra of TBA:GLA obtained from lipid extracted from frozen mackerel stored at −10 °C for up to 16 weeks. (a) MS^2 spectrum of 335 *m*/*z* and (b) MS^3 spectrum of 194 *m*/*z*.

1989). The effect of antioxidants depends on many factors, such as mixing ability, activity in different lipid systems, and stability at the time of processing (Giese, 1996; Houlihan & Ho, 1985). We have shown that whole instant green tea, used at the right concentration, may delay or inhibit lipid oxidation and can be used as a natural antioxidant in raw minced fatty fish tissue to enhance preservation. Further studies on ascertaining optimum levels of green tea, including polyphenol fractions, for food preservation and their effect on safety using cultured human cells, are underway.

4. Conclusion

Lipid peroxidation products, particularly aldehydes, contribute to rancidity and food deterioration, toughening of fish during frozen storage, as well as protein and DNA damage via cross-linking. In this study we have shown

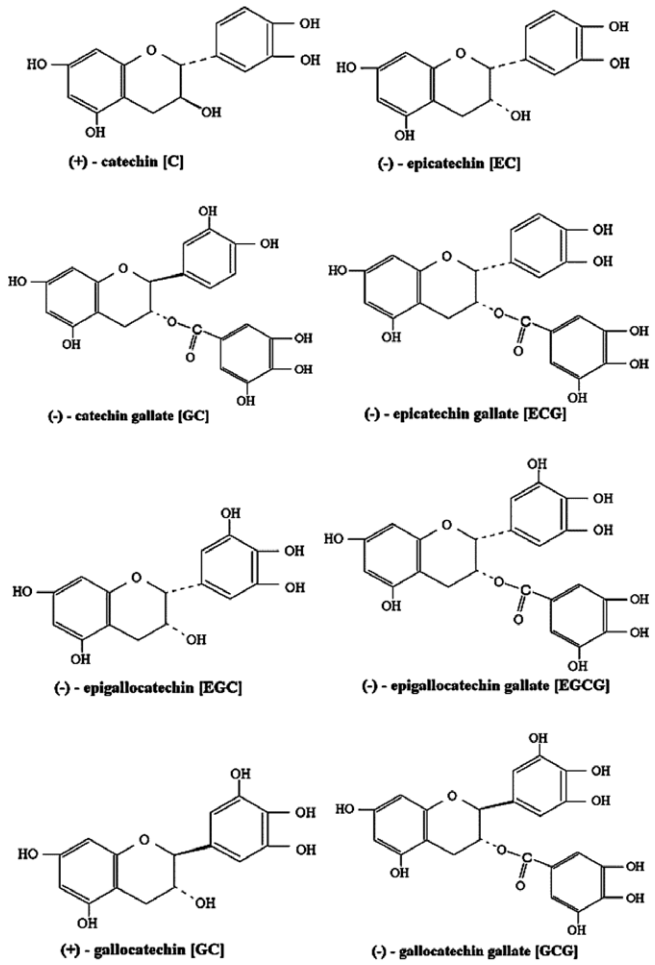


Fig. 12. Chemical structures of tea cathechins.

the formation of glutaraldehyde in frozen fish for the first time and confirmed its presence and that of malondialdehyde by LC–MS. Green tea, used at the right concentration may delay or inhibit lipid oxidation and can be used as a natural antioxidant in both food preservation and medical applications. However, studies on optimum levels of green tea require further investigation.

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