Development of a Fluorescence Sensor to Monitor Lipid Oxidation. 1. Fluorescence Spectra of Chitosan Powder and Polyamide Powder after Exposure to Volatile Lipid Oxidation Products

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The fluorescence spectra of chitosan and polyamide powder after exposure to lipid oxidation volatiles were obtained by solid-sample fluorescence spectrophotometry. Chitosan powder fluoresced after exposure to the volatile oxidation products from oxidizing methyl arachidonate and methyl linolenate. Chitosan powder also fluoresced after exposure to pure malonaldehyde vapor. Chitosan fluorescence after exposure to volatile malonaldehyde had an excitation maximum at 395 nm and an emission maximum at 460 nm. These wavelength maxima were similar to the fluorescence wavelength maxima of chitosan after exposure to the lipid oxidation volatiles. Chitosan did not fluoresce after exposure to hexanal and 2,4-decadienal at concentrations produced during methyl linoleate oxidation and appeared to be selective to malonaldehyde for fluorescence formation. Chitosan fluorescence spectra are compared to the fluorescence spectra of polyamide powder after exposure to lipid oxidation volatiles and after exposure to volatile malonaldehyde.

INTRODUCTION

Fluorescence spectrophotometry, which has been used to study lipid oxidation, is characterized by two distinct spectra: excitation and emission. Emission occurs at a lower frequency (or longer wavelength) than the incident light (excitation) because the energy of the emitted radiation differs from that absorbed by an amount equal to the vibrational energy lost to the surroundings (Campbell and Dwek, 1984).

Solution fluorescence spectrophotometry has been used as a method to measure the extent of peroxidation in rat liver subcellular organelles (Chio et al., 1969; Itoh et al., 1988), rat heart sarcosomes (Dillard and Tappel, 1971), rat retinol components (Eldred and Katz, 1989), red blood cells (Goldstein and McDonagh, 1976), T cell and B cell membranes (Hendricks and Heidrick, 1988), and human brain tissue (Taubold et al., 1975). The fluorescence of these tissues has been attributed to the formation of fluorescent age-related (lipofuscin) pigments due to the reaction of lipid oxidation products with primary amino group containing compounds (Kikugawa and Beppu, 1987).

Examination of the literature on the fluorophores formed in solution from the reaction of oxidizing fatty acids or lipid oxidation breakdown products (aldehydes, malonaldehyde, hydroperoxides) with primary amino group containing compounds (proteins, amino acids, DNA, poly(L-lysine), primary amines) reveals two distinct groups of fluorophores based on fluorescence wavelength maxima. One fluorophore, with an excitation maximum between 320 and 366 nm and an emission maximum between 410 and 460 nm, is due to the reaction of oxidizing methyl linoleate, methyl linoleate hydroperoxides, and aldehydes (other than malonaldehyde) with a variety of amino compounds (Kikugawa et al., 1984a, 1985; Leake and Karel, 1985; Beppu et al., 1986; Iio and Yoden, 1987). The second fluorophore, with an excitation maximum between 370 and 405 nm and an emission maximum between 450 and 470 nm, is due to the reaction of malonaldehyde with amino compounds (Chio and Tappel, 1969a,b; Reiss et al., 1972; Fujimoto et al., 1984; Kikugawa et al., 1984b, 1985; Beppu et al., 1986).

Porter et al. (1983) used solid-sample fluorescence spectrophotometry and polyamide-coated thin-layer chromatography plates to study lipid oxidation and browning reactions in food systems. They also discussed the structural characteristics of polyamide. The typical polyamide, poly(ϵ-caprolactam), is formed by heating aqueous solutions of ϵ-caprolactam in a sealed tube at 223 °C. The product, nylon 6 or Perlon, has a highly ordered crystalline structure. The primary amino groups of polyamide are the end groups of the polyamide chain.

Another polymer with primary amino groups is chitosan. Chitosan is widely available from chitin, which is a waste product of the seafood-processing industry with an estimated 1.2×10^5 metric tons annually accessible on a worldwide basis (Knorr, 1991). The occurrence and availability, processing, structures, and properties of chitin and chitosan have been the subject of two international conferences (Muzzarelli and Pariser, 1978; Hirano and Tokura, 1982) and various fundamental works (Muzzarelli, 1977; Muzzarelli et al., 1986). The use of chitinous polymers in food processing and food biotechnology has been reviewed by Knorr (1984, 1986, 1991). Chitosan, a copolymer of glucosamine and *N*-acetylglucosamine units, is produced by deacetylating chitin in hot, concentrated sodium hydroxide. The primary amino groups of chitosan are on the repeating units of the polymer.

This study was undertaken to determine the feasibility of using solid-sample fluorescence spectrophotometry as a method to measure lipid oxidation in food systems. The basis of the method is the stable fluorophore formed from the reaction of volatile aldehydes (which are generated during lipid oxidation) with the primary amino group of chitosan or polyamide. The fluorescence spectra of both chitosan and polyamide after exposure to lipid oxidation

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volatiles are discussed. The potential use of chitosan and polyamide as the basis for a fluorescence sensor is also discussed.

EXPERIMENTAL PROCEDURES

Materials. Chitin was purchased from Fluka Chemical Corp., Ronkonkoma, NY. 1,1,3,3-Tetramethoxypropane (TMP), hexanal, and 2,4-decadienal were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Polyamide powder was scraped from flexible polyamide thin-layer chromatography plates (Polygram polyamide 6 UV_{254}), which were purchased from Brinkman Instruments, Inc., Westbury, NY. Methyl arachidonate, methyl linolenate, and methyl linoleate, which had a purity of greater than 99%, were purchased from Nu Chek Prep Inc., Elysian, MN.

Chitosan powder was prepared by deacetylating chitin powder. Chitin flakes were ground to a particle size of less than 150 μm in an analytical mill. Approximately 4 g of the chitin powder was treated for 2 h with 300 mL of a boiling 50% sodium hydroxide solution in a 500-mL round-bottom flask. Nitrogen gas was bubbled through the solution to minimize degradation of the polymer. After 2 h, the intermediate product was cooled slightly, filtered through Schleicher & Schuell analytical paper No. 595, and washed with distilled water. The powder was returned to the flask and treated with fresh sodium hydroxide for another 2 h to continue the deacetylation. After the final 2-h treatment, the chitosan powder was filtered, washed with distilled water, washed with acetone, and then allowed to dry at room temperature. The chitosan prepared according to this method was 85–90% deacetylated as determined by the titration method described by Broussignac (1968).

Malonaldehyde was prepared by acid hydrolysis of the acetal 1,1,3,3-tetramethoxypropane (TMP), according to the method of Kurechi et al. (1980). Ten millimoles (1.65 g) of TMP and 0.90 mL of 1.0 N HCl were mixed in a 10-mL volumetric flask. After the addition of distilled water to 10 mL, the flask was stoppered and incubated in a 37 °C water bath for 1 h to produce a 1 M malonaldehyde solution.

Experimental Apparatus. In separate experiments, chitosan was exposed to lipid oxidation volatiles from oxidizing fatty acid methyl esters and to pure aldehyde vapor in a 50-mL Erlenmeyer flask. Approximately 100 mg of chitosan powder was spread evenly on the bottom of the flask. A strip of filter paper, which was attached to the stopper of the flask, served as a support for (1) 50 μL of a fatty acid methyl ester (methyl arachidonate, methyl linolenate, or methyl linoleate), (2) 50 μL of hexanal, (3) 50 μL of 2,4-decadienal, or (4) 3 μL of a 1 M malonaldehyde solution. After the fatty acid methyl ester or the aldehyde solution was placed on the strip of filter paper, the flask was stoppered and placed in an oven at 85 °C. After exposure to the volatiles from the oxidizing fatty acid methyl esters or after exposure to the aldehyde vapors, the chitosan was removed from the flask and the fluorescence spectra of the exposed powder were scanned by solid-sample fluorescence spectrophotometry.

In three other experiments, polyamide powder (100 mg) was exposed to 50 μL of oxidizing methyl arachidonate, 50 μL of oxidizing methyl linolenate, and 1 μL of a 1 M malonaldehyde solution at 85 °C in the same experimental apparatus.

Solid-Sample Fluorescence Spectrophotometry. The fluorescence spectra of chitosan and polyamide after exposure to lipid oxidation volatiles and after exposure to aldehyde vapor were obtained by solid-sample fluorescence spectrophotometry in a Perkin-Elmer LS-5 fluorescence spectrophotometer equipped with a solid-sample attachment and a 3600 data station. All fluorescence spectra were scanned with the excitation and emission slit widths set at the smallest setting, 3 nm.

For the fluorescence experiments with chitosan powder, the spectra were scanned relative to a quinine sulfate fluorescence standard and a chitosan control, which was heated at the experimental temperature but was not exposed to lipid oxidation volatiles or aldehyde vapor. For the fluorescence experiments with polyamide powder, the spectra were also scanned relative to a quinine sulfate fluorescence standard and a polyamide control, which was heated at the experimental temperature but was not exposed to lipid oxidation volatiles or aldehyde vapor. The fluorescence intensities, given in arbitrary units, of the exposed samples were measured relative to the intensities of the controls. To prepare the quinine sulfate fluorescence standard, 300 μL of a quinine sulfate solution (100 μg/mL in 0.1 N HCl) was dispersed on 60 mg of Celite filter aid powder and allowed to dry. The fluorescence intensity of this standard was 35 (arbitrary units) at the excitation wavelength maximum (360 nm) and the emission wavelength maximum (450 nm). (The wavelength maxima are denoted by the following notation: EX_{max}/EM_{max} = 360/450 nm.) The chitosan control sample had a fluorescence intensity of 10 at EX_{max}/EM_{max} = 395/460 nm. The polyamide control sample had a fluorescence intensity of 15 at EX_{max}/EM_{max} = 360/425 nm and 395/460 nm.

The excitation spectra of the chitosan powder samples after exposure to fatty acid methyl ester oxidation volatiles and after exposure to volatile malonaldehyde were scanned from 260 to 420 nm with the emission wavelength fixed at 463 nm. The emission spectra were scanned from 420 to 630 nm with the excitation wavelength fixed at 395 nm. The excitation spectra of the chitosan powder samples that had been exposed to hexanal vapor were scanned from 260 to 380 nm with the emission wavelength fixed at 430 nm. The emission spectra were scanned from 380 to 630 nm with the excitation wavelength fixed at 360 nm.

For fluorescence studies with polyamide powder and volatile malonaldehyde, the excitation spectra were scanned from 260 to 420 nm with the emission spectra fixed at 460 nm, and the emission spectra were scanned from 420 to 630 nm with the excitation wavelength fixed at 395 nm. The excitation spectra of polyamide powder after exposure to methyl linoleate oxidation volatiles were scanned from 260 to 410 nm with the emission wavelength fixed at 425 nm, and the emission spectra were scanned from 410 to 630 nm with the excitation wavelength fixed at 360 nm.

The excitation spectra of polyamide powder after exposure to methyl arachidonate oxidation volatiles at initial oxidation times (30 min or less at 85 °C) were scanned from 260 to 410 nm with the emission wavelength fixed at 445 nm. The emission spectra were scanned from 410 to 630 nm with the excitation wavelength fixed at 365 nm. As oxidation proceeded, the wavelength maxima shifted to higher wavelengths and were scanned accordingly. At intermediate oxidation times (45 min), the excitation spectra were scanned from 260 to 420 nm with the emission wavelength fixed at 460 nm and the emission spectra were scanned from 420 to 630 nm with the excitation wavelength fixed at 390 nm. At longer oxidation times (60 min or longer), the excitation spectra were scanned from 260 to 420 nm with the emission wavelength fixed at 470 nm and the emission spectra were scanned from 420 to 630 nm with the excitation wavelength fixed at 395 nm.

RESULTS AND DISCUSSION

Chitosan Fluorescence Spectra. Chitosan powder fluoresced after exposure to volatile oxidation products from oxidizing polyunsaturated fatty acid methyl esters. Figure 1 shows the excitation spectra (A) and the emission spectra (B) of chitosan fluorescence after exposure to oxidizing methyl arachidonate (MA), oxidizing methyl linolenate (MLN), and oxidizing methyl linoleate (ML). Chitosan fluorescence after exposure to the volatiles from all three oxidizing unsaturated fatty acid methyl esters had an excitation maximum between 395 and 397 nm and an emission maximum at 463 nm. The fluorescence spectra of chitosan after exposure to the volatiles from the oxidizing fatty acid methyl esters were very broad. Leake and Karel (1985) noted that the solution fluorescence spectrum of lysozyme after exposure to oxidizing lipid was quite broad. They hypothesized that the observed fluorescence spectrum of lysozyme after exposure to oxidizing linoleate could be the sum of several spectra of different fluorescent structures formed from the reaction of various aldehydes with lysozyme since several classes of aldehydes (alkanals, alkenals, alkadienals) are produced during linoleate oxidation.

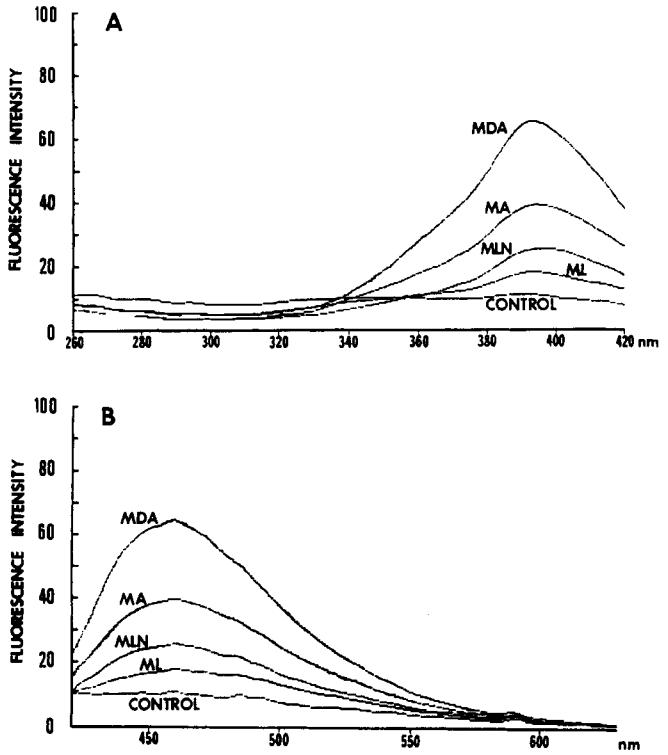

Figure 1. Fluorescence excitation (A) and emission (B) spectra of chitosan powder after exposure to 50 μL of oxidizing methyl arachidonate (MA) at 85 °C for 75 min, 50 μL of oxidizing methyl linolenate (MLN) at 85 °C for 75 min, 50 μL of oxidizing methyl linoleate (ML) at 85 °C for 240 min, and 3 μL of a 1 M malonaldehyde (MDA) solution at 85 °C for 75 min. The spectra were scanned as described under Solid-Sample Fluorescence Spectrophotometry of Experimental Procedures. The fluorescence intensity is given in arbitrary units.

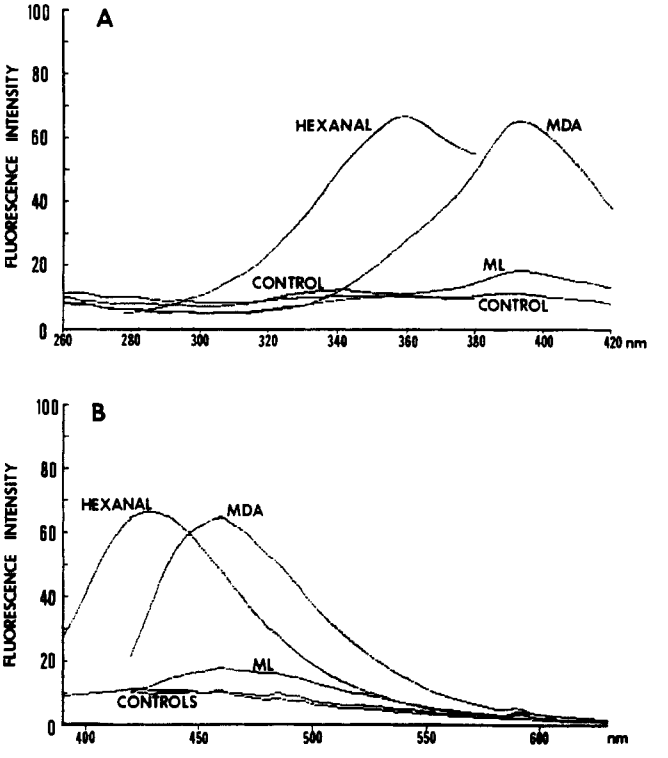

Figure 2. Fluorescence excitation (A) and emission (B) spectra of chitosan powder after exposure to 50 μL of oxidizing methyl linoleate (ML) at 85 °C for 240 min, 50 μL of hexanal at 85 °C for 90 min, and 3 μL of a 1 M malonaldehyde (MDA) solution at 85 °C for 75 min. The spectra were scanned as described under Solid-Sample Fluorescence Spectrophotometry of Experimental Procedures. Fluorescence intensity is given in arbitrary units.

The fluorescence spectra of chitosan powder after exposure to volatile malonaldehyde (MDA) are also shown in Figure 1. The wavelength maximum of chitosan fluorescence after exposure to malonaldehyde vapor was similar to the wavelength maximum of chitosan after exposure to the volatiles from the oxidizing unsaturated fatty acid methyl esters with an excitation maximum at 395 nm and an emission maximum at 460 nm. Malonaldehyde, a dialdehyde, is a product of lipid oxidation and is formed during the oxidation of unsaturated fatty acids which have three or more double bonds, such as linolenic acid and arachidonic acid (Dahle et al., 1962; Pryor et al., 1976).

The reaction of malonaldehyde with a variety of primary amino group containing compounds has been studied extensively by solution fluorescence spectrophotometry. Generally, the reaction of malonaldehyde with DNA (Fujimoto et al., 1984), amino acids (Shimasaki et al., 1988), proteins (Beppu et al., 1986), and primary amines (Kikugawa and Ido, 1984) in solution produced fluorescence with an excitation maximum between 370 and 405 nm and an emission maximum between 450 and 470 nm. These fluorescence wavelength maxima are similar to the fluorescence wavelength maxima of chitosan powder after exposure to oxidizing arachidonate, oxidizing linolenate, oxidizing linoleate, and volatile malonaldehyde.

Hexanal and 2,4-decadienal are the two major aldehyde breakdown products formed during linoleic acid oxidation (Gaddis et al., 1961; Grosch, 1987). Chitosan fluoresced only slightly after exposure to methyl linoleate oxidation volatiles, even after 4 h of oxidation at 85 °C (Figures 1 and 2). This fluorescence had an excitation maximum at 395 nm and an emission maximum at 463 nm. These maxima are similar to the wavelength maxima of chitosan fluorescence after exposure to volatile malonaldehyde and after exposure to methyl arachidonate and methyl linolenate oxidation volatiles.

Chitosan did not fluoresce after exposure to concentrated 2,4-decadienal vapor at 85 °C for 90 min. Chitosan fluorescence had an excitation maximum at 360 nm and an emission maximum at 430 nm after exposure to concentrated hexanal vapor (Figure 2). Chitosan, however, did not fluoresce at EX_{max}/EM_{max} = 360/430 nm after exposure to hexanal at lower concentrations produced during linoleate oxidation (Figure 2). The fluorescence of chitosan at EX_{max}/EM_{max} = 395/463 nm after exposure to oxidizing methyl linoleate could be due to malonaldehyde formed from the oxidation of alkadienals which are produced during linoleate oxidation. Lillard and Day (1964) found that significant quantitities of malonaldehyde were not observed in methyl linoleate until the late stages of oxidation, and they hypothesized that the dienals formed through degradation of primary linoleate hydroperoxides may in turn oxidize to give malonaldehyde.

Polyamide Fluorescence Spectra. Polyamide powder also fluoresced after exposure to lipid oxidation volatiles. Figure 3 shows the excitation spectra (A) and the emission spectra (B) of polyamide powder after exposure to oxidizing methyl arachidonate (MA), oxidizing methyl linoleate (ML), and volatile malonaldehyde (MDA). Polyamide powder fluorescence after exposure to methyl linoleate oxidation volatiles had an excitation maximum between 360 and 365 nm with a broad shoulder at 380 nm and an emission maximum between 425 and 435 nm. These wavelength maxima are similar to the fluorescence wavelength maxima of chitosan powder fluorescence after exposure to concentrated hexanal vapor. The fluorescence

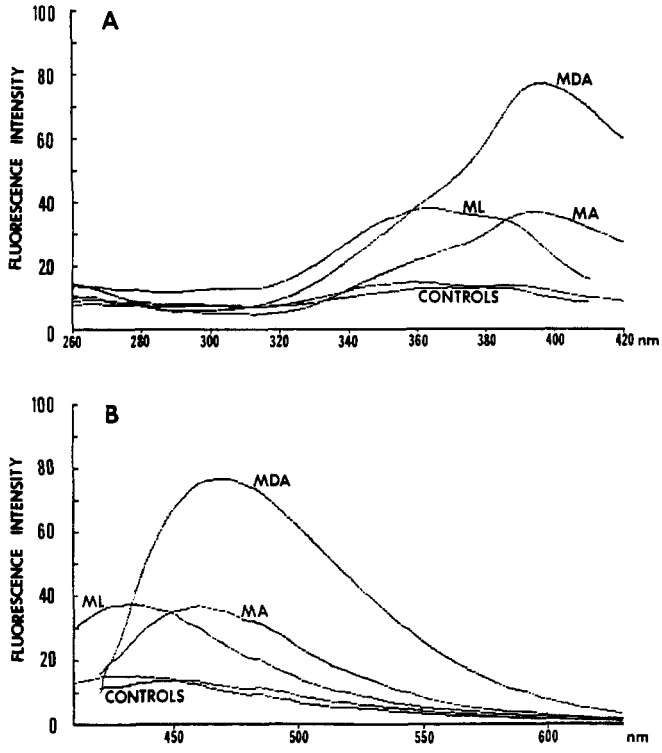

Figure 3. Fluorescence excitation (A) and emission (B) spectra of polyamide powder after exposure to 50 μL of oxidizing methyl arachidonate (MA) at 85 °C for 90 min, 50 μL of oxidizing methyl linoleate (ML) at 85 °C for 90 min, and 1 μL of a 1 M malonaldehyde (MDA) solution at 85 °C for 60 min. The spectra were scanned as described under Solid-Sample Fluorescence Spectrophotometry of Experimental Procedures. Fluorescence intensity is given in arbitrary units.

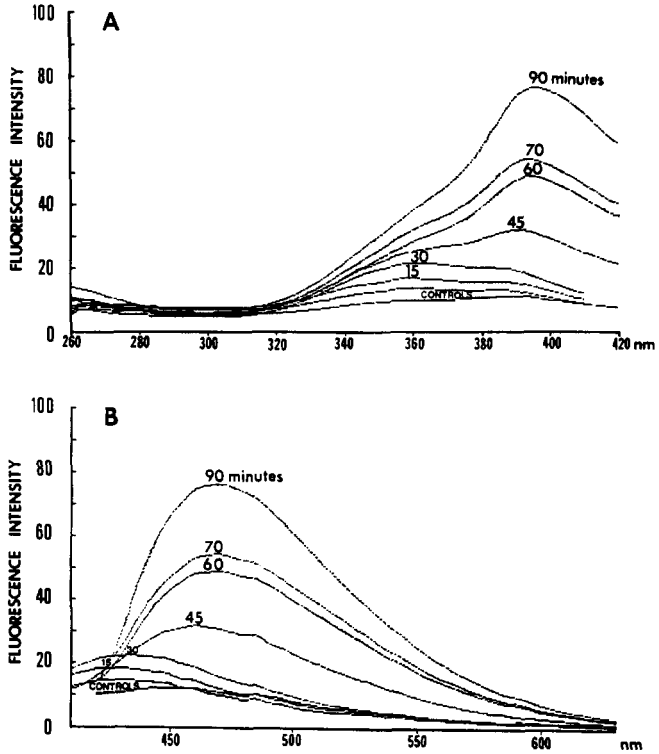

Figure 4. Fluorescence excitation (A) and emission (B) spectra of polyamide powder after exposure to 50 μL of oxidizing methyl arachidonate at 85 °C as a function of oxidation time. The spectra were scanned as described under Solid-Sample Fluorescence Spectrophotometry of Experimental Procedures. Fluorescence intensity is given in arbitrary units.

spectra of polyamide powder after exposure to linoleate oxidation volatiles could be the composite spectra of several aldehydes that are formed during linoleate oxidation.

For solution fluorescence studies, similar wavelength maxima (EX_{max} = 330–360 nm and EM_{max} = 410–460 nm) have been observed in the following reaction sytems: (1) linoleate, 2,4-hexadienal, and crotonaldehyde with serum albumin (Fletcher and Tappel, 1971); (2) linoleic acid hydroperoxides with amino acids (Shimasaki et al., 1982); (3) linoleate with lysozyme (Leake and Karel, 1985); (4) linoleate hydroperoxides, heptanal, and 2,4-decadienal with human erythrocyte ghosts (Beppu et al., 1986); (5) oxidized methyl linoleate with amylamine (Iio et al., 1987); and (6) alkanals, 2-alkenals, and 2,4-alkadienals with methylamine (Kikugawa and Sawamura, 1987).

Porter et al. (1983) used solid-sample fluorescence and polyamide-coated thin-layer chromatography plastic plates to measure the volatile carbonyls arising from oxidizing linoleic acid, oxidizing soy lecithin, oxidizing crushed potato chips, and oxidizing freeze-dried carrots. In all cases, the fluorescence of the polyamide plates had an excitation maximum at 356 nm and an emission maximum at 422 nm. These fluorescence wavelength maxima are similar to the wavelength maxima of polyamide fluorescence after exposure to methyl linoleate oxidation volatiles (Figure 3).

Polyamide powder fluorescence after exposure to volatile malonaldehyde had an excitation maximum at 395 nm and an emission maximum at 460 nm (Figure 3). These wavelength maxima are identical to the wavelength maxima of chitosan fluorescence after exposure to volatile malonaldehyde and after exposure to oxidizing arachidonate and oxidizing linolenate. Polyamide powder fluorescence spectra after exposure to oxidizing arachidonate had three distinct wavelength maxima depending on the extent of oxidation (Figure 4). At initial oxidation times (30 min or less at 85 °C), polyamide fluorescence had an excitation maximum at 365 nm and an emission maximum at 445 nm. At intermediate oxidation times (45 min), the fluorescence had an excitation maximum at 390 nm and an emission maximum at 460 nm. At longer oxidation times (60 min or greater), the fluorescence had an excitation maximum at 395 nm and an emission maximum at 470 nm. This shift in wavelength maxima could reflect the formation of two maxima (365/445 and 395/470 nm) and their intermediate composite. A variety of carbonyl products are formed during arachidonate oxidation (Grosch, 1987). The formation of fluorescence at EX_{max}/EM_{max} = 365/445 nm could reflect the reaction of saturated or unsaturated aldehydes with polyamide powder, and the fluorescence at EX_{max}/EM_{max} = 395/470 nm could result from the reaction of volatile malonaldehyde with the primary amino end groups of polyamide.

A study (Weist, 1991) has shown that chitosan fluorescence formation is proportional to the generation of volatile malonaldehyde during the oxidation of polyunsaturated fatty acid methyl esters. If fluorescence is proportional to oxidation product (malonaldehyde or any aldehyde responsible for fluorescence) formation and if the generation of the oxidation product (malonaldehyde) is the rate-limiting step in the overall fluorescence formation reaction, then chitosan (or polyamide) could be used as the basis for a fluorescence sensor to monitor the generation of volatile aldehydes during lipid oxidation in food and biological systems. Polyamide, which fluoresced after exposure to linoleate oxidation volatiles, could be used to monitor oxidation in many food systems since linoleate is prevalent in many foods. Chitosan, which appeared to be selective to malonaldehyde for fluorescence formation, could be used to monitor the generation of

volatile malonaldehyde during lipid oxidation in food and biological systems.

ABBREVIATIONS USED

MDA, malonaldehyde; MA, methyl arachidonate, MLN, methyl linolenate; ML, methyl linoleate; EX_{max}, excitation wavelength maximum; EM_{max}, emission wavelength maximum; TMP, 1,1,3,3-tetramethoxypropane.

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Registry No. Chitosan, 9012-76-4; nylon 6, 25038-54-4; malonaldehyde, 542-78-9; hexanal, 66-25-1; 2,4-decadienal, 2363-88-4.