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Determination of Aldehydic Lipid Peroxidation Products with Dabsylhydrazine by High-Performance Liquid Chromatography

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A high-performance liquid chromatographic method has been developed to measure adlehyde products of lipid peroxidation including *n*-alkanals, *trans*-2-alkenals, 4-hydroxy-2-nonenal, and malonaldehyde. The chromophoric reagent dabsylhydrazine reacts with various aldehydes produced from two different lipid peroxidation model systems: Fe^{2+}/H_2O_2 and Fe^{3+}/vitamin C oxidation of arachidonic and linoleic acids, respectively. The formed products were carefully characterized by IR, MS, and NMR spectral analysis and proved to be hydrazone rather than 2-pyrazoline or pyrrole derivatives. EI-MS of all the derivatized straight-chain aldehydes showed a characteristic (M − 28) fragment in the spectra with high consistency. Physicochemical properties including melting points are also described. The detection limits in the range of 11 pmol (5 ng) have been reached by using an NP Octadecyl C_{18} reversed-phase column. 4-Hydroxy-2-nonenal and hexanal were found to be the most abundant aldehydes in the lipid peroxidation systems described. The dose–response curve showed great linearity and exhibited good reproducibility of this procedure for quantitative estimation.

Most polyunsaturated fatty acids (PUFAs) will be degraded to aldehydes or other products when attacked by free radicals through a chain cleavage reaction leading to aldehydes.[1] Among these different kinds of aldehydes, *n*-alkanals, related α,β-unsaturated aldehydes including *trans*-2-alkenals, 4-hydroxy-2-alkenals, and 4-hydroxy-2-nonenal, and malonaldehyde are most widely encountered and studied because of their profound biological effects.[2-5] Recently, with the elucidation of signal transduction pathways, the effects of 4-hydroxy-2-alkenals on phospholipases C and D were also investigated.[6,7] It was postulated that these reactive aldehydes might act as "second toxic messengers".[3] Various methods for qualitative and quantitative analysis of these aldehydes were developed.[8,9] Recently reported methods included liquid chromatography/thermospray mass spectrometry (LC/MS) following derivatization with (pentafluorobenzyl) (PFB)hydroxylamine hydrochloride[10]) and a modified method of HPLC analysis of the fluorescent 1,3-cyclohexanedione (CHD) aldehyde derivatives.[11] Nevertheless, the most widely used technique is that employing 2,4-dinitrophenylhydrazine as derivatizing agent followed by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) analysis. In order to increase the sensitivity of the analysis, we are investigating an alternative method by using the most convenient and commonly used equipment in the laboratory to obtain a whole picture of lipid peroxidation aldehyde products.

Since dabsyl chloride was first synthesized in this laboratory in 1975,[12] this chromophoric reagent has been demonstrated to be powerful in the microdetermination of amino acids,[13] aliphatic amines,[14] and polyamines.[15] In 1987, dabsylhydrazine was also synthesized from the reaction of dabsyl chloride with hydrazine hydrate and successfully applied to quantitative estimation of monosaccharides by HPLC.[16] Because of its high sensitivity and deeper color (orange to red) than that of 2,4-dinitrophenylhydrazine (yellow) as compared on TLC plates, dabyslhydrazine was evaluated for its merit in the determination of aliphatic aldehydes of lipid peroxidation products.

The most commonly used method to determine aldehyde or ketone compounds was by taking advantage of the amino (NH_2) group forming a stable Schiff base (C=N) with the carbonyl (C=O) functional group. 4-Hydroxy-2-nonenal (HNE) was found to be one of the major products of microsomal lipid peroxidation after derivatizing with 2,4-dinitrophenylhydrazine to form the corresponding hydrazone.[17] Later, acrolein, crotonaldehyde, and HNE were characterized as 2-pyrazoline derivatives by reacting with methylhydrazine.[18,19] In 1993, HNE was found to form

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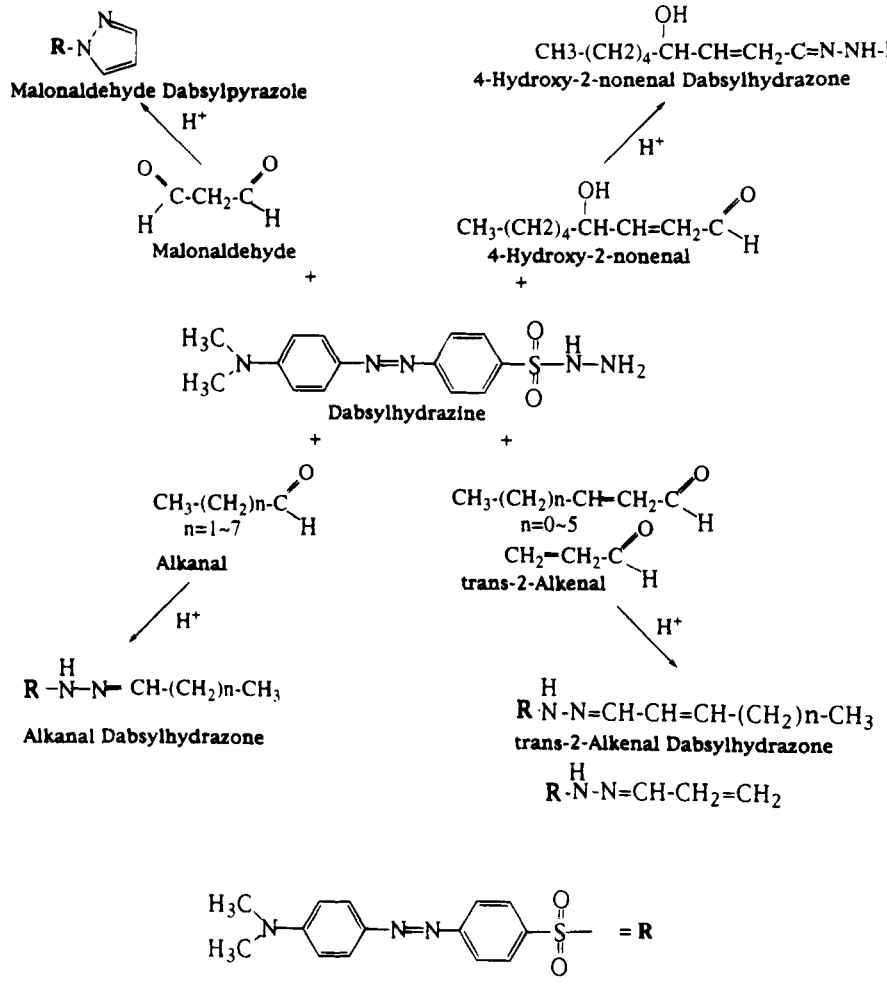

Figure 1. Formation of alkanal, *trans*-2-alkenal, and 4-hydroxy-2-nonenal dabsylhydrazones and malonaldehyde dabsylpyrazole.

another pyrrole derivative when reacted with a primary amine.[20] Because both aldehyde 2-pyrazolines and aldehyde hydrazones had the same molecular weights we first examined the structures of these aldehydes derivatized with dabsylhydrazine by MS, IR, NMR, distortionless enhancement by polarization transfer (DEPT), and two-dimensional proton–carbon heteronuclear-correlated spectroscopy (HETEROCOSY) analysis. Finally, we described a new procedure for the separation and quantitative estimation of these aldehyde derivatives by HPLC.

EXPERIMENTAL SECTION

Instrumentation. IR spectra were measured in Perkin-Elmer 1760 FT-IR spectrophotometer using a pressed pellet consisting of 1 mg of sample and 30–40 mg of KBr. NMR spectra were taken in a NMR spectrophotometer (JEOL, EX-400, Japan Electronic, ltd., Tokyo). Mass spectra (EI-MS) were studied in a Finnigan 4510 quadrupole mass spectrometer (TSQ-46C), FAB and high-resolution mass (HRMS) were measured in a GC/LC/MS spectrometer (JEOL, SX-102A). Electronic spectra were recorded on a Hitachi U-3210 double-beam spectrophotometer. The melting points of these new compounds were measured in a melting point apparatus (Ishii Co., Tokyo, Japan) and are uncorrected.

HPLC separations were performed on a Waters Associates instrument with a two-pump Model 510 solvent delivery system, a Model U6K manual injector, a Model 410 absorbance monitor at 436 nm, an automated gradient controller, and a 745B data module. The HPLC was performed on a Nacalai Tesque Cosmosil $5C_{18}$ (8 mm i.d. × 250 mm) packed column, J. T. Baker NP Octadecyl C_{18} (4.6 mm i.d. × 250 mm), 5 μm, 120 Å, Bakerbond HPLC column, and Waters Associate Nova-Pack C_{18} Guard-Pack Inserts. A linear gradient elution program (curve 6) [solvent A, water; solvent B, acetonitrile (40–80%, 60 min, 5 min hold)] and an isocratic elution program [solvent A, water; solvent B, acetonitrile (90%, 10 min or 60%, 30 min)] were commonly used. A flow rate of 1 mL and an absorbance unit at full scale (AUFS) of 0.061 were normally employed.

Chemicals. Octyl aldehyde, *trans*-2-nonenal, *trans*-2-octenal, *trans*-2-heptenal, *trans*-2-hexenal, and *trans*-2-pentenal were purchased from Aldrich Chemical Co. (Milwaukee, WI). 4-Hydroxy-2-nonenal was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Malonanldehyde bis(dimethylacetal) 1,1,3,3-tetramethoxypropane was purchased from Tokyo Chemical Industry (Tokyo, Japan). Crotonaldehyde, acrolein, nonanal, heptanal, hexanal, pentanal, butyraldehyde, propionaldehyde, hydrazine hydrate, tetrahydrofuran, ferric chloride, ferrous chloride, ascorbic acid, hydrogen peroxide, and dabsyl chloride were purchased from E. Merck Co. (Darmstadt, Germany). 2,4-Dinitrophenylhydrazine, arachidonic acid (90%), and linoleic acid (99%) were purchased from Sigmal Chemical Co. (St. Louis, MO). Acetonitrile (HPLC grade) was obtained from Mallinckrodt Chemicals (St. Louis, MO). All aqueous solutions were made with

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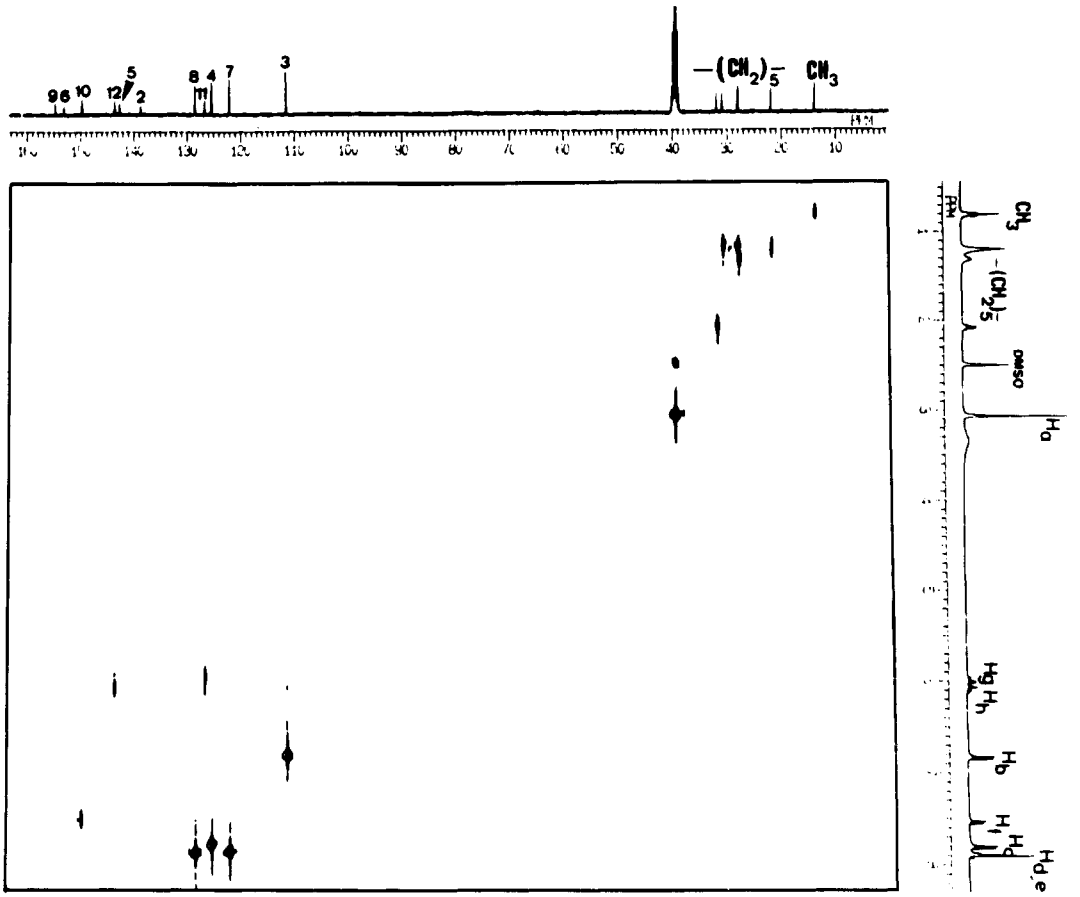

Figure 2. Two-dimensional proton–carbon HETEROCOSY spectrum of DH-NE, in DMSO-d_6 at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Peak number of C and alphabetic assignments of H are as described in Table 1.

water purified by a Milli-Q system (Millipore). All other solvents and reagents were of the analytical grade.

Synthesis of *n*-Alkanal and *trans*-2-Alkenal Dabsylhydrazone (Figure 1). Dabsylhydrazine was synthesized by reacting dabsyl chloride with hydrazine hydrate,[16] and the ^{1}H NMR, IR, and MS spectra were in agreement with previously published data. Dabsylhydrazine (50 mg) was dissolved in 75 mL of dichloromethane containing 2.6–6% acetic acid and mixed well with 0.16 mmol of alkanal. An excess of *n*-alkanal (*trans*-2-alkenal) is recommended because of difficult removal of dabsylhydrazine. The resulted mixture was allowed to stand at room temperature for 30 min. Completion of the hydrazone formation can be easily visualized by TLC analysis [solvent system, *n*-hexane/ethyl acetate [50:50 (v/v)]] with dabsylhydrazine as standard. The reaction mixture was then partitioned with 25 mL of 10% aqueous $NaHCO_3$ two times. The dichloromethane layer was separated with a separatory funnel. The aqueous solution was combined and extracted with 10 mL of dichloromethane twice, and the extract was added to the original dichloromethane fraction. The pooled dichloromethane solution was concentrated to dryness on a rotary evaporator (<37 °C) to obtain the orange or red alkanal (*trans*-2-alkenal) hydrazone products.

Synthesis of 4-Hydroxy-2-nonenal Dabsylhydrazone (Figure 1). Dabsylhydrazine (1 mg) was dissolved in 1.5 mL of dichloromethane containing 1 mg of 4-hydroxy-2-nonenal and 0.06% acetic acid. The mixture was vortex mixed thoroughly and allowed to stand at ambient temperature for 30 min. The formation of the hydrazone product was obtained by repetitive chromatographic purification by HPLC [Nacalai $5C_{18}$ (8 × 250 mm) packed column; isocratic elution program [solvent A, water; solvent B; acetonitrile (60%, 30 min)], the ratio of the retention time of the 4-hydroxy-2-nonenal hydrazone to dabsylhydrazine is 2.1].

Synthesis of Malonaldehyde Dabsylpyrazole (Figure 1). Malonaldehyde bis(dimethylacetal) (40 mg) was added to 75 mL of dichloromethane containing 0.4 N HCl and the solution refluxed for 1 h at 50 °C. Dabsylhydrazine (50 mg) was dissolved in the reaction mixture with constant stirring. After 30 min standing at room temperature, the resulted mixture was worked up by the same procedure as the *n*-alkanal dabsylhydrazones and gave red malonaldehyde dabsylpyrazole product.

Oxidation of Fatty Acids in Both Fe^{2+}/H_2O_2 and Fe^{3+}/Vitamin C Lipid Peroxidation Model Systems and Extraction of Aliphatic Aldehydes. The conditions of the lipid peroxidation model system were modified according to refs 19 and 21. Arachidonic acid (1.5 mg/mL) or linoleic acid (1.5 mg/mL) was dissolved in an aqueous solution (1 mL) containing 0.05 M Tris-HCl (pH 7.4), 40 mM $FeCl_2$, and 40 mM H_2O_2 (Fe^{2+}/H_2O_2 system) or in an aqueous solution (1 mL) containing 0.05 M Tris-HCl (pH 7.4), 20 μM $FeCl_3$, and 500 μM vitamin C (Fe^{3+}/vitamin C system). After incubation at 37 °C for 4 h, these solutions were extracted with 0.5 mL of dichloromethane twice using vortex mixing. The combined dichloromethane extracts were transferred to scintillation vials and evaporated to dryness by rotatory evaporator.

HPLC Analysis of *n*-Alkanal, *trans*-2-Alkenal, and 4-Hydroxy-2-nonenal Dabsylhydrazones and Malonaldehyde Dab-

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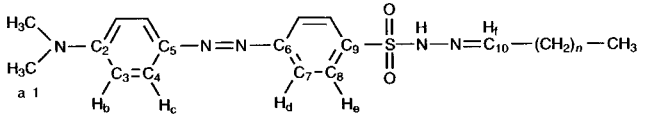
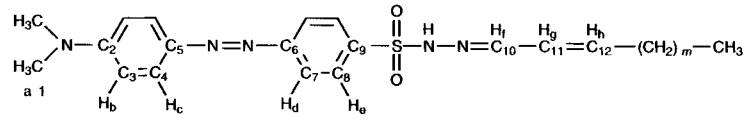
Table 1. ^{1}H NMR Chemical Shifts (in ppm) and Coupling Constants (in Hz) and ^{13}C NMR Chemical Shifts (in ppm) of the Alkanal and *trans*-2-Alkenal Dabsylhydrazones



compd	^{1}H NMR; ^{13}C NMR											
	H_a; C_1	H_b; C_2	H_c; C_3	H_d; C_4	H_e; C_5	H_f; C_6	$(CH_2)_n$; C_7	$(CH_2)_n$; C_8	$(CH_2)_n$; C_9	$(CH_2)_n$; C_{10}	$(CH_2)_n$; $(CH_2)_n$	CH_3; CH_3
						Alkanal						
propionaldehyde (n = 1)	3.08s, (6H); 39.5	6.85d, J = 9.27, (2H); 139.4	7.82, J = 9.28, (2H); 111.6	7.89s, (4H), 125.4	7.89s, (4H); 142.6	7.26t, J = 4.89, (1H); 153.1	122.0	128.4	154.6	2,09m, (2H); 152.3	25.2	0.90t, J = 7.33, (3H); 10.5
butyraldehyde (n = 2)	3.08s, (6H); 39.5	6.85d, J = 9.28, (2H); 139.2	7.82d, J = 9.28, (2H); 111.6	7.90s, (4H); 125.4	7.90s, (4H); 142.6	7.23t, (1H); 153.1	122.0	128.5	2.05m, (2H); 154.6	151.6	1.35m, (2H); 19.1, 33.6	0.74t, J = 7.33, (3H); 13.3
pentanal (n = 3)	3.08s, (6H); 39.5	6.85d, J = 9.28, (2H); 139.5	7.82d, J = 9.28, (2H); 111.6	7.89s, (4H); 125.4	7.89s, (4H); 142.6	7.22t, J = 5.38, (1H); 153.1	121.9	128.4	2.07m, (2H); 154.6	1.30p, J = 7.45, (2H); 151.3	1.11m, (2H); 21.3, 27.8, 31.3	0.75t, J = 7.32, (3H); 13.5
hexanal (n = 4)	3.08s, (6H); 39.5	6.85d, J = 9.27, (2H); 138.6	7.90d, J = 4.39, (2H); 111.6	7.98, (4H); 125.3	7.98s, (4H); 142.6	7.17t, J = 5.37, (1H); 153.0	121.8	2.04m, (2H); 128.2	1.28−1.35p, J = 7.71, (2H); 154.1	1.15−1.20p, J = 7.33, (2H); 149.1	1.06−1.12m, (2H); 21.9, 25.7, 30.6, 31.7	0.75t, J = 7.08, (3H); 13.9
heptanal (n = 5)	3.09s, (6H); 39.5	6.85d, J = 9.28, (2H); 138.7	7.82d, J = 9.28, (2H); 111.6	7.90s, (4H); 125.3	7.90s, (4H); 142.6	7.24t, J = 5.37, (1H); 153.1	122.0	128.5	2.07m, (2H); 154.7	152.2	1.10−1.30m, (8H); 21.9, 25.5, 27.8, 30.9, 31.5	0.74t, (3H); 13.9
octanal (n = 6)	3.08s, (6H); 39.5	6.84d, J = 8.79, (2H); 138.6	7.80d, J = 8.79, (2H); 111.6	7.90s, (4H); 125.4	7.90s, (4H); 142.6	7.24t, (1H); 153.1	122.0	128.5	2.07m, (2H); 154.7	152.3	1.08−1.32m, (10H); 22.0, 25.5, 28.0, 28.3, 31.2, 31.5	0.76t, J = 6.84, (3H); 13.8
nonanal (n = 7)	3.09s, (6H); 39.5	6.85d, J = 9.28, (2H); 138.6	7.82d, J = 8.79, (2H); 111.6	7.90s, (4H); 125.4	7.90s, (4H); 142.6	7.23t, J = 5.38, (1H); 153.1	122.0	128.5	2.07m, (2H); 154.7	152.3	1.01−1.32m, (12H); 22.0, 25.5, 28.1, 28.6, 28.7, 31.2, 31.5	0.76t, J = 7.08, (3H); 13.9



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compd	^{1}H NMR; ^{13}C NMR													
	H_a; C_1	H_b; C_2	H_c; C_3	H_d; C_4	H_e; C_5	H_f; C_6	H_g; C_7	H_h; C_8	$(CH_2)_m$; C_9	$(CH_2)_m$; C_{10}	$(CH_2)_m$; C_{11}	$(CH_2)_m$; C_{12}	$(CH_2)_m$; $(CH_2)_m$	CH_3; CH_3
							trans-2-Alkenal							
acrolein	3.08s, (6H); 39.5	6.85d, J = 9.28, (2H); 138.7	7.82d, J = 8.79, (2H); 111.6	7.91s, (4H); 125.4	7.91s, (4H); 142.6	7.59d, J = 9.77, (1H); 153.2	6.32dt, J = 15.99, 10.63, 1H; 122.1	5.60dd, J = 13.89, 10.74, 2H; 128.4	154.9	149.3	133.4	125.3		
crotonaldehyde (m = 0)	3.08s, (6H); 39.5	6.85d, J = 8.79, (2H); 138.7	7.82d, J = 9.28, (2H); 111.6	7.91s, (4H); 125.4	7.91s, (4H); 142.6	7.55d, J = 9.28, (1H); 153.1	6.04.–6.10m, (2H); 122.1	128.4	154.8	149.7	128.0	138.5		1.77d, J = 6.53, (3H); 18.1
trans-2-pentenal (m = 1)	3.09s, (6H); 39.5	6.85d, J = 9.28, (2H); 138.7	7.85d, J = 9.28, (2H); 111.6	7.91s, (4H); 125.4	7.91s, (4H); 142.6	7.56d, J = 9.27, (1H); 153.2	6.01m, (1H); 122.1	6.14m, (1H); 128.5	154.8	149.8	2.11m, (2H); 125.7	144.9	25.1	0.94t, J = 7.33, (3H); 12.5
trans-2-hexenal (m = 2)	3.09s, (6H); 39.5	6.85d, J = 9.27, (2H); 138.7	7.82d, J = 9.28, (2H); 111.6	7.91s, (4H); 125.5	7.91s, (4H); 142.6	7.55d, J = 8.79, (1H); 153.2	6.01m, (1H); 122.1	6.09m, (1H); 128.5	154.8	149.8	2.08m, J = 7.33, (2H); 126.8	143.4	1.36d, J = 7.82, (2H); 21.2, 34.1	0.83t, J = 7.57, (3H); 13.5
trans-2-heptenal (m = 3)	3.09s, (6H); 39.5	6.85d	7.82d, J = 9.28, (2H); 111.6	7.91s, (4H); 125.4	7.91s, (4H); 142.6	7.55d, J = 8.79, (1H); 153.7	6.00m, (1H); 122.1	6.08m, (1H); 128.5	154.8	2.10m, (2H); 149.8	126.7	1.26p, J = 7.33, (2H); 143.6	1.31m, J = 7.33, (2H); 21.6, 30.1, ,31.7	0.83t, J = 7.33, (3H); 13.7
trans-2-octenal (m = 4)	3.08s, (6H); 39.5	6.85d, J = 9.28, (2H); 138.7	7.82d, J = 9.27, (2H); 111.6	7.91s, (4H); 125.4	7.91s, (4H); 142.6	7.54d, J = 9.28, (1H); 153.2	6.00dd, J = 15.63, 9.28, 1H; 122.1	6.10dt, J = 15.63, 8.79, 1H, 128.5	154.8	149.8	2.09m, (2H); 126.7	143.6	1.20–1.35m, (6H); 21.8, 27.6, 30.7, 32.0	0.82t, J = 7.08, (3H); 13.8
trans-2-nonenal (m = 5)	3.08s, (6H); 39.5	6.85d, J = 9.28, (2H); 138.6	7.82d, J = 9.27, (2H); 111.6	7.90s, (4H); 125.4	7.90s, (4H); 142.6	7.54d, J = 9.28, (1H); 153.1	6.00dd, J = 15.63, 8.79, 1H; 122.1	6.08dt, J = 14.39, 8.54, 1H; 128.5	154.8	149.6	2.09m, (2H); 126.7	143.4	1.21–1.33m, (8H); 22.0, 28.0, 28.2, 31.0, 32.0	0.82t, J = 6.83, (3H); 13.9

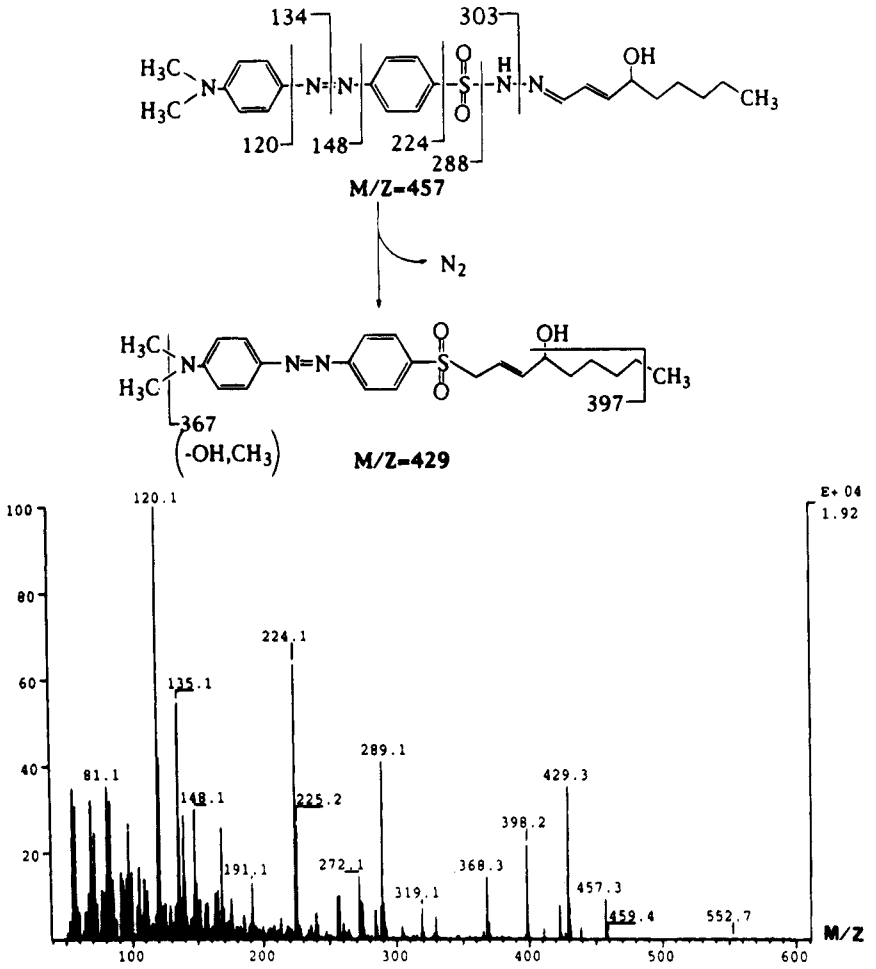

Figure 3. Electron impact mass spectrum of 4-hydroxy-2-nonenal dabsylhydrazone. Ionization energy was 70 eV.

sylpyrazole. Standard stock solutions were prepared by adding 1 mg of an individual sample prepared as described above to 4 mL of acetonitrile. Aliquots of 50 μL from each sample (0.25 μg/μL) were added to a vial that could be sealed with a Teflon cap and then diluted to 1 mL to make a final concentration of 12.5 ng/μL. Duplicate samples were prepared similarly except with 1% acetic acid in the reaction mixtures. These solutions were used directly for HPLC analysis. A linear gradient elution program was employed for the separation of *n*-alkanals including nonanal, octylaldehyde, heptanal, hexanal, pentanal, butyraldehyde, and propionaldehyde; *trans*-2-alkenal including *trans*-2-nonenal, *trans*-2-octenal, *trans*-2-heptenal, *trans*-2-hexenal, *trans*-2-pentenal, crotonaldehyde, and acrolein; 4-hydroxy-2-nonenal; and malonaldehyde. The details of the chromatographic conditions are described in the Instrumentation section.

Recovery Studies of *n*-Alkanal, *trans*-2-Alkenal, and 4-Hydroxy-2-nonenal Dabsylhydrazonse and Malonaldehyde Dabsylpyrazole. Recovery efficiencies of dabsylhydrazone derivatives were measured by using an aqueous solution (1 mL) containing 0.05 M Tris-HCl (pH 7.4), standard *n*-alkanals, and *trans*-2-alkenals (10 μg/mL, each). The aqueous solution was partitioned with 0.5 mL of dichloromethane two times with vigorously vortexing. The pooled dichloromethane solution was evaporated to dryness and redissolved in acetonitrile (1 mL) with 1% acetic acid. Dabsylhydrazine (1 mg) was dissolved in this acetonitrile solution and allowed to stand at room temperature overnight. The final solution was used for HPLC analysis directly.

HPLC Analysis for the Determination of Alkanal, *trans*-2-Alkenal, 4-Hydroxy-2-nonenal, and Malonaldehyde in the Lipid Peroxidation System. A dry extract of aliphatic aldehyde was obtained as described above and redissolved in acetonitrile (1 mL) containing dabsylhydrazine (0.25 mg) and 1% acetic acid. This acetonitrile solution was allowed to stand at room temperature overnight and then used for HPLC analysis.

RESULTS AND DISCUSSION

Characterization of Alkanal, *trans*-2-Alkenal, and 4-Hydroxy-2-nonenal Dabsylhydrazones. Alkanal, *trans*-2-alkenal, and 4-hydroxy-2-nonenal dabsylhydrazones were synthesized as described in the Experimental Section. The names, yields (%), and melting points (°C), respectively, of these 14 dabsylhydrazone derivatives are as follows: propionaldehyde, 55, 167–168; butyraldehyde, 58, 164–166; pentanal, 67, 162–164; hexanal, 72, 178 dec; heptanal, 76, 140–142; octylaldehyde, 67, 112–113; nonanal, 61, 117–118; acrolein, 54, 154–155; crotonaldehyde, 68, 183–184; *trans*-2-pentenal, 70, 166–168; *trans*-2-hexenal, 50, 171–172; *trans*-2-heptenal, 85, 169–170; *trans*-2-octenal, 62, 156–157; and *trans*-2-nonenal, 73, 169–170. These new compounds were fully identified by their UV–visible, IR, NMR (Figure 2), and mass spectra (Figure 3). The physicochemical data of malonaldehyde dabsylpyrazole agreed with our laboratory's previous work.[22]

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Assignments of functional groups are based on previous literature.[23,24]

trans-2-Nonenal dabsylhydrazone show a typical azo dye peak at 443 nm (orange color observed), which is shifted to 503 nm (pink-red color observed) in acidic conditions accompanied by a large increase in absorbance at the same concentration (0.005 mg/mL). This characteristic can be widely used in the enhancement of the sensitivity of aldehyde detection in HPLC and TLC analysis. The molar absorptivities and maximal absorption peaks of *trans*-2-nonenal dabsylhydrazone (0.005 mg/mL) in neutral, acidic, and alkaline conditions are listed below ($\epsilon \times 10^{-4}$; nm): acetonitrile (2.74; 443), 0.1 N HCl in aetonitrile (5.25; 503), 0.1 N NaOH in acetonitrile (3.38; 427), (4.83, 283), (5.82; 243), (9.28; 213).

The IR spectra (KBr pellet, cm^{-1}) of alkanal dabsylhydrazones are shown below ($n = 12-14$): N—H stretch (3203 ± 9); aliphatic C—H stretch (2959 ± 7, 2863 ± 9, 2927 ± 4); C=N stretch (1601 ± 1); N=N stretch (1521 ± 1, 1422 ± 1); aliphatic C—H bend (1368 ± 3, 1446 ± 1); SO_2 stretch, asymmetric (1314 ± 1), symmetric (1169 ± 2, 1141 ± 1);[14] aromatic C—H bend, weak overtone, or combination bands (1650–2000, 846 ± 2, 822 ± 2, 748 ± 1, 691 ± 2). *trans*-2-Alkenal dabsylhydrazones also show the following: C=C stretch (1647 ± 2, $n = 7$); aliphatic C—H out-of-plane band (983 ± 2, $n = 6$) in addition to the bands shown as alkanal dabsylhydrazones.

The ^1H NMR data are given as chemical shifts (in ppm, δ) downfield from tetramethylsilane (TMS), multiplicity, coupling constants (in Hz), and number of protons. The ^{13}C NMR data are given relative to TMS in chemical shift (in ppm, δ), and the carbon assignments of *trans*-2-nonenal were confirmed by two-dimensional proton–carbon HETEROCOSY (Figure 2) and DEPT experiments. The ^1H NMR and ^{13}C NMR data of alkanal and *trans*-2-alkenal dabsylhydrazones are shown in Table 1. The ^1H NMR (400.13 MHz, $CDCl_3$) spectral data of 4-hydroxy-2-nonenal dabsylhydrazone are listed below: δ 0.86 (t, 3H, CH_3), 1.09–1.40 (m, 8H, $(CH_2)_4$), 3.11 (s, 6H, $(CH_3)_2N$), 4.20 (m, 1H, CHOH), 6.01 (dd, J = 15.80, 5.91 Hz, 1H, CH=CHCHOH), 6.31 (ddd, J = 15.78, 9.16, 0.95 Hz, 1H, CH=CHCHOH), 6.74 (d, J = 9.16 Hz, 2H, aromatic hydrogen ortho to dimethylamino group), 7.39 (d, J = 9.16 Hz, 1H, N=CH), 7.87 (d, J = 1.96 Hz, 2H, aromatic hydrogen meta to dimethylamino group), 7.88 (d, J = 2.04 Hz, 2H, aromatic hydrogen meta to sulfonyl group), 7.99 (d, J = 8.68 Hz, aromatic hydrogen ortho to sulfonyl group).

The chemical shifts of H_h and C_{12} of the α,β-unsaturated functional group of *trans*-2-alkenal dabsylhydrazones ($m = 0-5$) are further downfield than H_g and C_{11} because electron delocalization results in a lower electron density at the H_h and C_{12} position except for acrolein dabsylhydrazone. The question is Whether the 2-pyrazoline or hydrazone structure of the products formed from the dabsylhydrazine with *trans*-2-alkenals and 4-hydroxy-2-nonenal can be easily solved by the DEPT because the numbers of downward CH_2 peaks of the 2-pyrazoline product will be one more than the hydrazone products.

The EI mass spectral data (m/z, % relative intensity) of alkanal, *trans*-2-alkanal, and 4-hydroxy-2-nonenal dabsylhydrazones are presented in Table 2, and the characteristic EI mass spectrum of 4-hydroxy-2-nonenal dabsylhydrazone is shown in Figure 3. EI mass spectra were normally recorded at an ionization energy of 20 eV, if not especially indicated. The contribution of the ^{34}S isotope (4.4%) to the M + 2 and fragment +2 peaks confirms these sulfur-containing compounds. The relative intensity of the molecular ion of these derivatized aliphatic aldehydes was greatly enhanced (above 38% in alkanal; above 23% in *trans*-2-alkenal dabsylhydrazones) by comparing with direct mass spectrometric analysis without derivatization (below 3% from pentanal and other large alkanals).[25] Low-resolution FAB (positive) of *trans*-2-nonenal dabsylhydrazone also show high-resolution intensity, 441 (78, M^+), 442 (100, $[M + 1]^+$), which indicates that dabsylhydrazine might

Table 2. Mass Spectral Data of Alkanal, *trans*-2-Alkenal, and 4-Hydroxy-2-nonenal Dabsylhydrazones

compound (M)	mass spectra m/z (% relative intensity)
alkanal	
propionaldehyde (359)	359 (38, M^+), 331 (10, $[M - N_2]$), 257 (2), 224 (24), 148 (25), 120 (100)
butyraldehyde (373)	373 (100, M^+), 345 (7, $[M - N_2]^+$), 304 (4), 289 (8), 272 (4), 224 (20), 148 (18), 120 (11)
pentanal (387)	387 (100, M^+), 359 (13, $[M - N_2]^+$), 304 (10), 289 (10), 272 (6), 224 (22), 148 (25), 120 (63)
hexanal (401)	401 (100, M^+), 373 (31, $[M - N_2]^+$), 316 (12), 289 (14), 272 (33), 224 (30), 148 (27), 120 (79)
heptanal (415)	415 (58, M^+), 387 (5, $[M - N_2]^+$), 304 (12), 272 (6), 224 (30), 148 (43), 135 (37), 120 (100)
octanal (429)	429 (40, M^+), 414 (2, $[M - CH_3]^+$), 401 (6, $[M - N_2]^+$), 304 (27), 289 (10), 224 (23), 148 (42), 135 (40), 120 (100)
nonanal[a] (443)	443 (46, M^+), 428 (3, $[M - CH_3]^+$), 415 (6, $[M - N_2]^+$), 304 (7), 289 (16), 272 (15), 257 (16), 224 (14), 148 (47), 135 (51), 120 (100)
trans-2-alkenal	
acrolein[a] (357)	357 (54, M^+), 329 (100, $[M - N_2]^+$), 272 (31), 257 (4), 224 (54) 148 (27), 120 (58)
crotonaldehyde (371)	371 (100, M^+), 343 (33, $[M - N_2]^+$), 289 (8), 272 (5), 240 (5), 224 (39), 148 (14), 120 (30)
trans-2-pentenal (385)	385 (33, M^+), 357 (7, $[M - N_2]^+$), 289 (13), 272 (7), 257 (6), 240 (7), 224 (31), 148 (30), 135 (12), 120 (100)
trans-2-hexenal (399)	399 (74, M^+), 371 (10, $[M - N_2]^+$), 289 (19), 272 (8), 224 (44), 148 (37), 135 (8), 120 (100)
trans-2-heptenal (413)	413 (23, M^+), 385 (37, $[M - N_2]^+$), 289 (24), 272 (11), 257 (11), 224 (27), 148 (36), 135 (25), 120 (100)
trans-2-octenal (427)	427 (32, M^+), 399 (100, $[M - N_2]^+$), 289 (49), 272 (20), 257 (17), 224 (35), 148 (49), 135 (39), 120 (100)
trans-2-nonenal[a] (441)	441 (49, M^+), 413 (38, $[M - N_2]^+$), 289 (36), 224 (24), 148 (38), 135 (67), 120 (100)
4-hydroxy-2-nonenal (457)	459 (1, $[M + 2]^+$), 458 (2, $[M + 1]^+$), 457 (9, M^+), 429 (35, $[M - N_2]^+$), 398.2 (22), 368.3 (15), 289 (41), 257 (6), 224 (64), 148 (30), 135 (54), 120 (100)

[a] High-resolution mass data are also shown in the text. EI condition, 70 eV.

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be a good tool to detect structural changes in proteins that are modified by a α,β-unsaturated aldehyde.[26,27]

Apart from the typical aldehyde dabsylhydrazone ions, all spectral data are characterized by key fragments of *m/z* (M − 28) due to loss of N_2. High-resolution EI mass mesaurement of the composition and the error (ppm/mmu) of the ion of (M − 28) appearing in the spectra of nonanal, acrolein, and *trans*-2-nonenal dabsylhydrazones are listed below (*m/z*, composition, ppm/mmu): nonanal (415.2283, $C_{23}H_{33}O_2N_3S$, −2.5/−1.0); acrolein (329.1168, $C_{17}H_{19}O_2N_3S$, −9.0/−3.0); *trans*-2-nonenal (413.2133, $C_{23}H_{31}O_2N_3S$, −0.8/−0.4). It seems that the lost N_2 is derived from the $SO_2NHN{=}C$ group rather than the $N{=}N$ group between aromatic rings because the mass spectrum of dabsylhydrazine did not show the (M − 28) ion.[16] Besides, the relative intensities of the (M − 28) ion of *trans*-2-alkenal dabsylhydrazone is higher than alkanal dabsylhydrazone, which implies that α,β-unsaturated functional group might be helpful in the enhancement of N_2 loss, but formation of the 2-pyrazoline intermediate ion seems impossible. The detailed rearrangement mechanism of losing N_2 still remains to be investigated.

On the basis of these spectrometric features, the structures of these aldehyde hydrazone products are identified as alkanal, *trans*-2-alkenal, or 4-hydroxy-2-nonenal 4′-[[4-(dimethylamino)-phenyl]azobenzene]sulfonylhydrazone as depicted in Figure 1.

Reaction of Dabsylhydrazine with Alkanals, *trans*-2-Alkenals, and 4-Hydroxy-2-nonenal. Alkanals, *trans*-2-alkenals, and 4-hydroxy-2-nonenal are readily reacted with dabsylhydrazine, and the formation of dabsylhydrazone products of individual aldehyde or their mixtures was accelerated and completed with 2.6−6% acetic acid catalysis at room temperature. The yields (%) of alkanal and *trans*-2-alkenal dabsylhydrazones described above are about 50−85%, which is due to great loss during the microscale operation (50 mg of dabsylhydrazine) and can be elevated to at least 90% if 1 g of dabsylhydrazine is used as starting material. We suggest that 50 mg be used as starting material for preparation of reference hydrazones.

The 2-pyrazoline products formed by derivatizing α,β-unsaturated carbonyl compounds with methylhydrazine did not appear in the products of dabsylhydrazine derivatives. This reaction appears to involve subsequent addition of NH across the C=C and is generally regarded as having a high degree of steric hindrance. We also do not expect these hydrazone products will be closed to become 2-pyrazoline ring products under more vigorous conditions (HOAc/HBr, 2 h of reflux) without the required anti form because of geometrical prohibition.[28] Besides, the pyrrole product seems also not been made in this case although dabsylhydrazine also contains a primary amine functional group.

Sensitivity and Linearity. The sensitivity of *trans*-2-nonenal dabsylhydrazone standard was measured by dissolving 1 mg of this compound in 1 mL of acetonitrile and then diluting to 0.5 ng/μL with acetonitrile. A 10 μL aliquot of the diluted sample (0.5 ng/μL) was injected and gave a peak height response within the linear range of the dose−response curve and a signal-to-noise ratio of 4.0. Therefore, the minimal practical detection limit for *trans*-2-nonenal or other alkanals, *trans*-2-alkenals, and 4-hydroxy-2-nonenal is about 5 ng (11.3 picomol). This picomole detection limit would be elevated by adding 1% acetic acid in the detection samples and changing the absorbance monitor to 503 nm simultaneously. Otherwise, employment of an electrochemical detector might also greatly increase the detection limit of this method to femtomoles.[29] Application of this method directly on the TLC plates by using a densitometric technique is also still being developed.

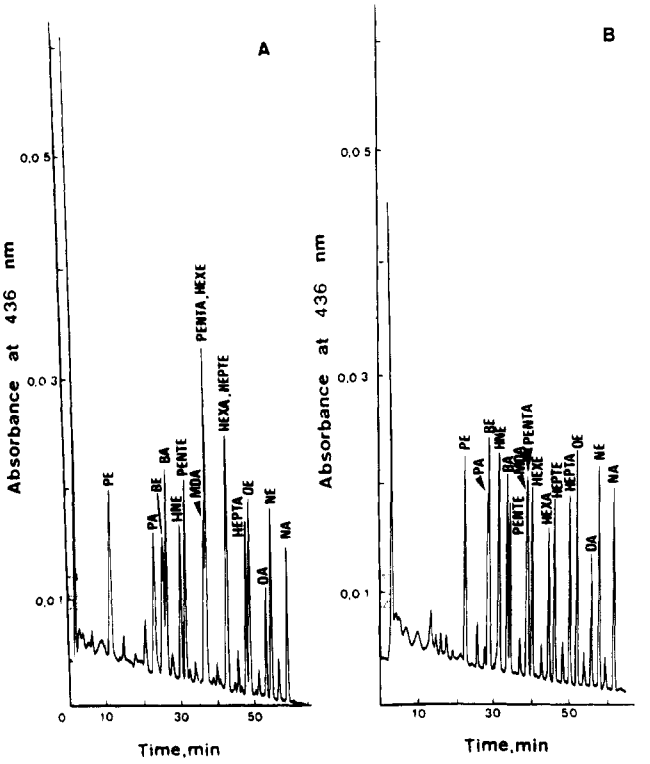

Figure 4. HPLC separation of alkanal, *trans*-2-alkenal, 4-hydroxy-2-nonenal dabsylhydrazones and malonaldehyde dabsylpyrazole. Chromatographic conditions: J. T. Baker NP Octadecyl C_{18} column; mobile phase, linear elution program, solvent A, water; solvent B, acetonitrile (40−80%, 60 min, 5 min hold); flow rate 1.0 mL/min; AUFS 0.061; (A) 24 μL of individual aldehyde dabsylhydrazones and DH-MDA (12.5 ng/μL, each) in acetonitrile. (B) 24 μL of individual aldehyde dabsylhydrazones and DH-MDA (12.5 ng/μL, each) in acetonitrile containing 1% acetic acid. Abbreviations: DH, dabsylhydrazone; PE, acrolein (2-propenal); PA, propionaldehyde; BE, crotonaldehyde (2-butenal); BA, butyraldehyde; HNE, 4-hydroxy-2-nonenal; PENTE, *trans*-2-pentenal; MDA, malonaldehyde; PENTA, pentanal; HEXE, *trans*-2-hexenal; HEXA, hexanal; HEPTE, *trans*-2-heptenal; HEPTA, heptanal; OE, *trans*-2-octenal; OA, octanal; NE, *trans*-2-nonenal; NA, nonanal.

Dose−response curves for alkanal, *trans*-1-alkenal, 4-hydroxy-2-nonenal dabsylhydrazones and malonaldehyde dabsylpyrazole were measured and plotted (data not shown). Good proportionality exists between amounts (ng) of the individual aldehydes and peak area of the HPLC chromatogram. The correlation coefficients (*r*) of these regression lines lie between 0.999 and 1 except for the dabsylhydrazones of butyraldehyde (0.997), hexanal (0.997), and heptanal (0.998). Excellent fit and linearity indicate that this method is suitable for quantitative determination.

HPLC Separation of Standard Alkanal, *trans*-2-Alkenal, and 4-Hydroxy-2-nonenal Dabsylhydrazones and Malonaldehyde Dabsylpyrazole. Standard stock solutions (12.5 ng/μL,

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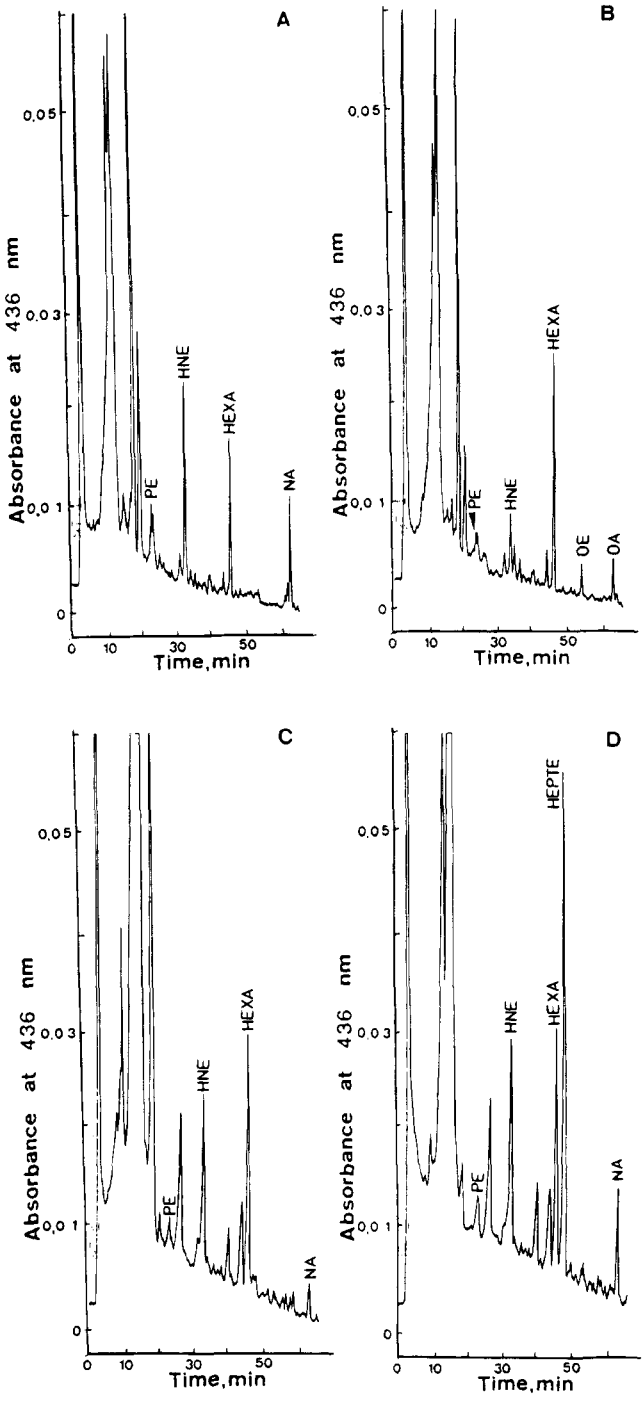

Figure 5. High-performance liquid chromatographic profiles of alkanal, *trans*-2-alkenal, and 4-hydroxy-2-nonenal dabsylhydrazones and malonaldehyde dabsylpyrazole formed in the lipid peroxidation systems. The chromatographic condition and abbreviations are as described in Figure 4: (A) oxidation of linoleic acid with Fe^{2+}/H_2O_2; (B) oxidation of arachidonic acid with Fe^{2+}/H_2O_2; (C) oxidation of linoleic acid with Fe^{3+}/vitamin C; (D) oxidation of arachidonic acid with Fe^{3+}/vitamin C.

each) of alkanal, *trans*-2-alkenal, and 4-hydroxy-2-nonenal dabsylhydrazones and malonaldehyde dabsylpyrazole were prepared by the procedure described in the Experimental Section. The C_{18} reverse column (J. T. Baker C_{18}) with a linear gradient water–acetonitrile mobile phase [solvent A, water; solvent B, acetonitrile (40–80%, 60 min, 5 min hold)] gave sharp peaks for the neutral derivatized aldehydes (Figure 4A). Eleven of 16 derivatized aldehydes had good resolution except for peak area integration

Table 3. Amount of *n*-Alkanal, *trans*-2-Alkenal, 4-Hydroxy-2-nonenal, and Malonaldehyde Produced from Arachidonic Acid and Linoleic Acid in Both Fe^{2+}/H_2O_2 and Fe^{3+}/Vitamin C Lipid Peroxidation Model Systems

	linoleic acid (nmol/mg of acid)		arachidonic acid (nmol/mg of acid)	
compound	Fe^{2+}/H_2O_2	Fe^{3+}/vitamin C	Fe^{2+}/H_2O_2	Fe^{3+}/vitamin C
alkanal				
propionaldehyde	4.74	nd	3.26	nd
butyraldehyde	25.94	nd	43.47	nd
pentanal	4.36	6.22	6.19	11.41
hexanal	90.38	115.84	138.11	123.37
heptanal	6.76	4.27	4.13	1.58
octylaldehyde	trace	3.13	nd	3.87
nonanal	28.47	5.92	11.80	18.18
trans-2-alkenal				
acrolein	58.69	21.85	11.91	40.11
crotonaldehyde	nd	4.32	nd	nd
trans-2-pentenal	4.96	1.68	9.02	2.29
trans-2-hexenal	nd	trace	trace	5.43
trans-2-heptenal	7.23	10.87	trace	580.17
trans-2-octenal	16.51	5.29	43.10	19.57
trans-2-nonenal	trace	12.46	nd	22.70
4-hydroxy-2-nonenal[b]	20.77	14.48	6.91	20.62
malonaldehyde[b]	4.75	6.78	6.75	12.44

[a] nd, not detected. [b] Not corrected by recovery efficiency.

of pentanal/*trans*-2-hexenal and hexanal/*trans*-2-heptenal dabsylhydrazones. Better resolution was obtained by adding 1% acetic acid in this standard stock solution under the same chromatographic conditions (Figure 4B), which resolved 14 peaks except for merging of malonaldehyde/pentanal dabsylhydrazones. As a matter of fact, this merged peak still can be differentiated into two peaks by a data module to get quantitative estimation of 16 all derivatized aldehydes.

Apart from the quantitative estimation, qualitative information about the aldehyde analyzed can be obtained via the retention time. *trans*-2-Alkenal dabsylhydrazones were always eluted before alkanal dabsylhydrazones (comparing the same carbon numbers), which means the polarity of the former is greater than the later, also corresponding to the speculation from the melting points described above. Besides, the retention time of 4-hydroxy-2-nonenal dabsylhydrazone (DH-HNE) in neutral medium is longer than butyraldehyde dabsylhydrazone (DH-BA) but shorter than it in acidic medium, which should be carefully examined during HPLC analysis.

Recovery of Alkanals and *trans*-2-Alkenals. Recovery efficiencies of alkanals and *trans*-2-alkenals added to the Tris-HCl buffer and then extracted with dichloromethane and derivatized with dabsylhydrazine were determined. Recoveries were calculated by inputting each chromatographic area into the individual regression lines of alkanal and *trans*-2-alkenal dabsylhydrazones obtained from the dose–response curves. The average recoveries of alkanals (51%) are higher than that of *trans*-2-alkenals (33%), indicating that lower polarity alkanals (except for butyraldehyde and pentanal) are much more easily extracted by dichloromethane. Besides, both kinds of aldehydes exhibited a biphasic trend, with medium values for the shorter chain aldehydes (C_3 and C_4) and the highest recoveries with the C_6 chain length, followed by a reduction for the less polar aldehydes.

Determination of Aldehyde Contents in Lipid Peroxidation Model Systems. Dry extracts of aldehydes from Fe^{2+}/H_2O_2 and Fe^{3+}/vitamin C model systems were prepared and derivatized with dabsylhydrazine as described in the Experimental Section. Four representative profiles of arachidonic acid and linoleic acid with two kinds of lipid peroxidation model systems, respectively, were given in Figure 5. Each peak was characterized on the basis of its retention time compared to the HPLC profile of standard aldehyde derivatives shown in Figure 4B. The amounts of aldehydes produced from lipid peroxidation of these two polyunsaturated acids (PUFAs) were listed in Table 3 and were corrected by recovery efficiencies except for 4-hydroxy-2-nonenal dabsylhydrazone and malonaldehyde dabsylpyrazole. The amounts of 4-hydroxy-2-nonenal and hexanal (above 90.38 nmol/mg of acid) are relatively high in both peroxidation systems of the two PUFAs, corresponding to the previous data.[1] Besides, nonanal (28.47 nmol/mg of acid) exhibits the highest amount in the Fe^{2+}/H_2O_2 oxidation system of linoleic acid (Figure 5A). Panels B and D of Figure 5 also show that *trans*-2-heptenal appeared at an extremely high level after 4 h of Fe^{3+}/vitamin C oxidation of arachidonic acid but at only a trace level in Fe^{2+}/H_2O_2 oxidation of arachidonic acid. It is worth noting that several unknown peaks with high absorbance appearing in the HPLC chromatogram (Figure 5) still remain to be identified. We believe that the present method is a straightforward procedure without tedious purification steps for aldehyde determination and can be futher extensively applied to the biological samples avoiding most visible and UV absorbance impurities that preexisted in the samples.

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