Technical

Derivative UV Spectra of Lipid Conjugated Dienes

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Zeroth and second order derivative UV spectra of some lipids and model substances having a conjugated system of double bonds were recorded.

The second derivative spectra showed fine spectral structures with at least three negative peaks. The wavelengths of their minima are different for *trans, trans* and *cis,trans* geometric isomers, which allowed determination of each individual isomer in a mixture without any separation.

The derivative spectra of 2,4-hexadienes, some acetyl esters of unsaturated fatty alcohols and autoxidized linoleic acid, soybean oil and soybean lecithin are discussed.

The autoxidation of polyunsaturated fatty acids is accompanied by an increase in UV absorbance with a maximum at about 234 nm, which is characteristic of conjugated dienic systems (1). This increase is proportional to the uptake of oxygen and to the concentration of hydroperoxides formed in the early stages of reaction (2). Therefore, measurement of absorbance at that wavelength is useful as a method to determine the degree of lipid oxidation.

It has been suggested that little information dealing with these methods can be found in the literature (3), and that development of UV chromophores cannot be taken as a measure of the degree of oxidation unless the composition of the fatty acid mixture is known (4). Nevertheless, the change in absorbance at about 234 nm for a given substance can be used as a relative measure of oxidation (3), and absorbance or $E_1^{1\%}{}_{cm}$ values at the same wavelength have been used as an indication of crude degummed soy oil quality (5), to determine deleterious effects of bleaching on soy oil (6), as a measure of conjugated dienes hydroperoxides in peanut products (7) and soy oil (8), to follow deterioration of cottonseed and peanut frying oils (9), etc. Besides, the peroxide value has been correlated with the differential area between the derivative spectra of oils before and after percolation through alumina (10), and with the increase in absorbance at 234 nm in the oxidation of peanut butter (7). The rate of increase in absorbance at 234 nm is used in the assay of lipoxygenase (11).

In spite of the mentioned applications, there is a lack of a more general and more widely accepted procedure based on UV absorbance to determine lipid oxidation. This can be due, among other reasons, to the fact that the band at 234 nm is usually just a shoulder over the higher band at about 215 nm associated to the carboxyl group in carboxylic acids and esters, and this overlapping makes it difficult to obtain comparable and reproducible results when samples different in source and nature are evaluated.

In searching for a method to measure lipid oxidation based on the UV spectra, which could avoid interference due to chromophores other than conjugated dienes, and as the first step in that study, the derivative spectra of some lipids and model substances were recorded and are reported herein. An unexpected result of this work has been the unveiling of the fine spectral structure of conjugated dienes, which allowed determining individual geometric isomers in a mixture by derivative UV spectrophotometry without any separation.

Derivative spectra. In derivative spectrometry the first or higher order derivative of absorbance with respect to wavelength is recorded versus wavelength. For a simple absorbance band the shape of the first derivative spectrum shows a maximum and a minimum, going through zero between extremes at the wavelength of maximum absorbance. The most characteristic feature of the second derivative spectra (SDS) is a strong minimum at the wavelength of maximum absorbance.

Two important advantages of derivative spectrometry are its use in quantitative analysis when the peak of the analyte is obscured by a large overlapping band due to an interference or by turbidity, and the resolution effect. The UV-visible spectra usually contain several overlapping bands which often can be resolved by differentiation. The enhancement of resolution increases with the derivative order (12,13), and its magnitude depends on the band shapes (14) as well as on the relative band widths and heights (15).

Methods to obtain derivative spectra include optico-mechanical, electronic and mathematical means. Although most spectra shown in this paper were obtained with a spectrophotometer capable of giving first and second derivatives directly, when absorbance data from the literature was used, the method of Savitzky and Golay (16) was used. In this method the digital data of absorbance is processed; the derivative at any wavelength is calculated by taking a number of points at each side of that wavelength, multiplying absorbance values by appropriate coefficients and adding them together. This addition gives, at the chosen wavelength, the derivative of a least squares best polynomial that is fitted to the original data. Although the use of just five points (cubic fit) or seven points (quartic fit) along wavelength are generally satisfactory with regard to noise level and peak broadening, seven points for a cubic fit were necessary in the case mentioned further on.

EXPERIMENTAL

Pheromones and 2,4-hexadienes were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). Linoleic acid (99%) and soybean lipoxygenase were purchased from Sigma Chemical Co. (St. Louis, Missouri). Spectroscopic grade n-hexane, analytical grade absolute ethyl alcohol or pH9 borate buffer were used as solvents.

Isomeric 2,4-hexadienes were analyzed by GLC with a 25 m long, 0.2 mm I.D. high performance Hewlett-Packard fused silica capillary column, crosslinked 5% phenyl methyl silicone, film thickness 0.33 micron. The gas chromatograph used was a Hewlett-Packard HP5840A equipped with flame ionization detector (FID). Flow rates of hydrogen and air were 30 and 240 ml/min, respectively. Nitrogen inlet pressure was 0.5 kg/cm^2, and auxiliary make-up flow rate was 30 ml/min. Temperatures of injector, oven (isothermic) and detector were 300, 60 and 200 C, respectively.

Absorbance and derivative UV spectra were measured in 10 mm pathlength quartz cells with a Hewlett-Packard 8450A UV-vis spectrophotometer and were recorded with a Hewlett-Packard 7225B graphics plotter.

Autoxidation of linoleic acid with lipoxygenase (final concentration about 200 U/ml) was carried out under the conditions described by Axelrod et al. (17) for the assay of the enzyme in pH9 0.2M sodium borate buffer. The reaction also was carried out in the same conditions but without lipoxygenase present.

Autoxidation of refined soybean oil and native soybean lecithin took place by air at room temperature in the dark with no solvent.

RESULTS

Spectra of hexadienes. The simplest substances having a conjugated system of double bonds with terminal methyl groups, as they occur in lipids, are 2,4-hexadienes. The spectra of two isomers, *cis*-2,*trans*-4-hexadiene (I) and *trans*-2,*trans*-4-hexadiene (II), were recorded. The absorbance bands have maxima at about 226 nm (*trans,trans*) and 229 nm (*cis,trans*); they are similar in shape, having a shoulder at each side of the maximum. This feature is enhanced in the SDS which show for each isomer three major minima at about the same wavelengths as absorbance maximum and shoulders (Fig. 1).

The SDS of mixtures of both substances have the same form as those of the components; the strong overlapping does not allow any resolution, and wavelengths of the minima have values in between. Nevertheless, a procedure was devised to determine the concentration of each isomer in a mixture from its spectrophotometric data.

When the SDS of a single isomer is recorded at different concentrations, it is observed that zero crossing points take place at fixed wavelengths (Fig. 2). This is a general characteristic of SDS, related to the fact that inflection points on the absorbance band for a given substance always occur at the same wavelength, regardless of absorbance values.

Two of these zero crossing points were selected, at 228.3 nm for *trans*-2,*trans*-4 hexadiene and at 240.2 nm for the *cis,trans* isomer. Thus, when the SDS of a mixture of both substances is measured, the derivative value at 228.3 nm is proportional only to the *cis,trans* isomer concentration and the derivative value at 240.2 nm is proportional only to the *trans,trans* isomer concentration. By means of calibration curves the

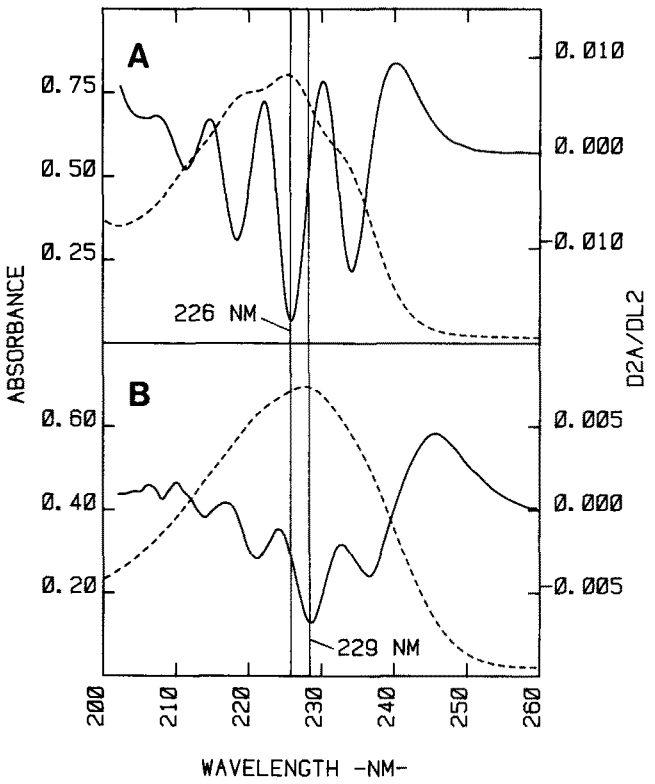

FIG. 1. Spectra in absorbance (- - -) and second derivative (——) of *trans*-2,*trans*-4-hexadiene (A) and *cis*-2,*trans*-4-hexadiene (B) in n-hexane.

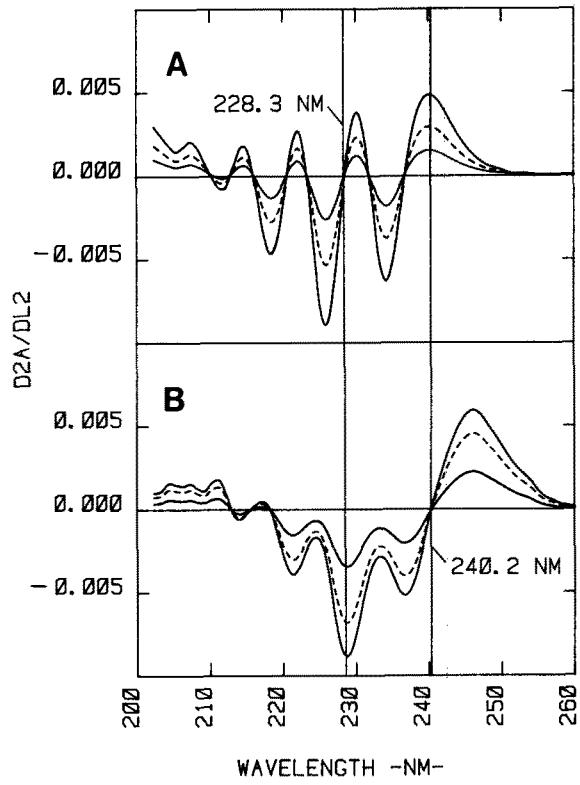

FIG. 2. Second derivative spectra of *trans*-2,*trans*-4-hexadiene (A) and *cis*-2,*trans*-4-hexadiene (B) at different concentrations showing zero crossing points. Solvent n-hexane.

concentration of the individual components can be determined.

A series of laboratory prepared mixtures of *trans, trans* and *cis,trans* 2,4-hexadienes were analyzed by this method with good recoveries (Table 1). The calibration curves

$$[trans,trans]\ (\mu mol/l) = -0.23 + 3148\ (d^2A/d\lambda^2)_{240\cdot2}$$

and

$$[cis,trans]\ (\mu mol/l) = -0.47 - 4591\ (d^2A/d\lambda^2)_{228\cdot3}$$

(correlation coefficients = 0.9999) were utilized.

No calibration curves were necessary to determine the ratio of geometric isomers concentrations in a mixture, but a constant factor giving reason of the difference in derivative values at the wavelengths of zero crossings for equal concentrations of both isomers had to be applied. For 2,4-hexadienes that ratio can be determined by

$$R = [cis,trans]/[trans,trans] = -1.474\ (d^2A/d\lambda^2)_{228\cdot3}/(d^2A/d\lambda^2)_{240\cdot2}$$

Ratios found in this way in several mixtures resulted in good agreement with known values (Table 1).

Spectra of fatty alcohols and esters. Some pheromones having the structure of fatty alcohols or their acetyl esters with a conjugated system of double bonds were studied. The spectra in absorbance and in second derivative of *trans*-8,*trans*-10-dodecadien-1-ol (III), *trans*-8,*trans*-10-dodecadien-1-yl acetate (IV), *trans*-7,*cis*,-9-dodecadien-1-yl acetate (V) and *cis*-9, *trans*-11-tetradecadien-1-yl acetate (VI) were recorded.

Similar spectra resulted either for both *trans,trans* or both *cis,trans* substances, but each pair can be clearly differentiated from the other by their SDS (Fig. 3). The absorbance bands have maxima at approximately 228 nm (*trans,trans*) and 233 nm (*cis,trans*); they are similar in shape with a shoulder at each side and, like the hexadienes, this feature is enhanced by the derivative process which gives SDS with three major minima at about the same wavelengths as absorbance maxima and shoulders.

However, in the SDS of mixtures of *trans,trans* and *cis,trans* pheromones, unlike the hexadienes, the fine spectral structure is almost lost. The larger bathochromic shift of the *cis,trans* isomer (about 5 nm) allows the overlapping of positive lobes of one component with negative ones of the other, yielding a disguised spectrum. Nevertheless, because appropriate zero crossing points can be selected (Fig. 4), the procedure described to determine the concentration of each isomer in the mixture is still useful.

Furthermore, it was possible to "recover" or "isolate" the spectrum of each component from the spectrum of the mixture. For this purpose two series of normalizing factors had to be calculated from the spectrum of each pure isomer. For substances IV and V these factors are

$$f_\lambda^{IV} = (d^2A/d\lambda^2)_\lambda^{IV}/(d^2A/d\lambda^2)_{244\cdot7}^{IV}$$

and

$$f_\lambda^{V} = (d^2A/d\lambda^2)_\lambda^{V}/(d^2A/d\lambda^2)_{234\cdot4}^{V}$$

where λ subscripts represent wavelengths along the interval of interest, taken for instance at each nm.

Once two values, $(d^2A/d\lambda^2)_{244\cdot7}^{M}$ and $(d^2A/d\lambda^2)_{234\cdot4}^{M}$, have been measured on a mixture of IV and V, the digitalized spectrum of each substance can be calculated by

$$(d^2A/d\lambda^2)_\lambda^{IV} = f_\lambda^{IV} \cdot (d^2A/d\lambda^2)_{244\cdot7}^{M}$$

and

$$(d^2A/d\lambda^2)_\lambda^{V} = f_\lambda^{V} \cdot (d^2A/d\lambda^2)_{234\cdot4}^{M}$$

TABLE 1

Analysis of Mixtures of Geometrical Isomers of 2,4-Hexadiene by Derivative Spectrophotometry

	Concentration (μmol/l)					
	Known[a]		Found		Ratio [c,t]/[t,t]	
Sample	c,t	t,t	c,t	t,t	Known[a]	Found[b]
c,t	31.3	0.2	31.2	0.2		
A	28.3	3.1	28.9	3.0	9.13	9.07
B	22.0	9.2	21.6	9.3	2.39	2.33
C	15.75	15.4	16.1	15.7	1.02	1.05
D	9.45	21.6	8.9	22.1	0.44	0.42
E	3.15	27.7	3.2	28.4	0.11	0.13
t,t	0.5	30.3	1.4	30.9		

[a]GLC.
[b]Without calibration curves.

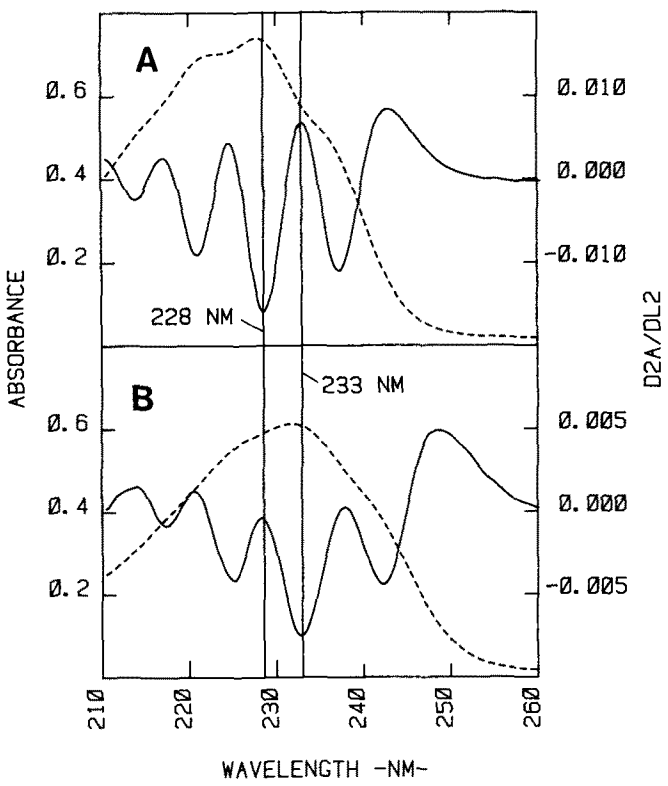

FIG. 3. Spectra in absorbance (- - -) and second derivative (——) of *trans*-8,*trans*-10-dodecadien-1-yl acetate (A) and *trans*-7, *cis*-9-dodecadien-1-yl acetate (B) in n-hexane.

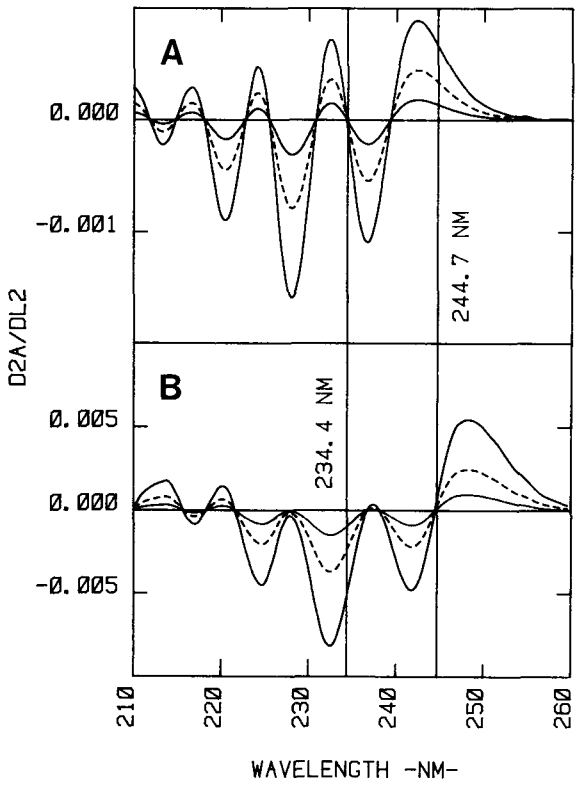

FIG. 4. Second derivative spectra of *trans*-8,*trans*-10-dodecadien-1-yl acetate (A) and *trans*-7,*cis*-9-dodecadien-1-yl acetate (B) at different concentrations showing zero crossing points. Solvent n-hexane.

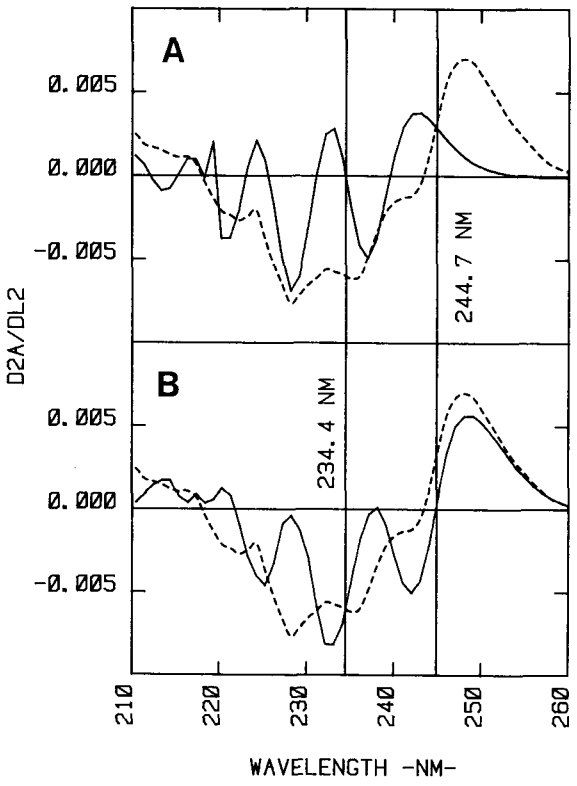

FIG. 5. Second derivative spectrum of a mixture of *trans*-8-, *trans*-10-dodecadien-1-yl acetate and *trans*-7,*cis*-9-dodecadien-1-yl acetate (- - -) and the spectrum of each substance (——) "recovered" from the mixture. Solvent n-hexane.

and plotted as in Figure 5.

Spectra of autoxidized linoleic acid. The autoxidation of linoleic acid produces four isomeric hydroperoxy acids, 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic (VII.A); 13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoic (VIII.A); 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic (IX.A), and 9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoic (X.A) acids (Fig. 6). These acids or their methyl esters (VII.B–X.B) have been separated by HPLC either directly (18-20) or as the hydroxy acid derivatives obtained by reduction of the hydroperoxy groups (18,20,21).

The spectrophotometric data available (1,18) about substances related to linoleic acid but having a conjugated system of double bonds shows that neither the wavelengths of the maxima nor the molar absorptivities are significantly affected either by the presence or absence of a hydroxy or hydroperoxy group or, if present, by its position along the carbon chain. Thus, λmax and εmax values of substances VII.B–X.B (18) are approximately equal in the pair *cis,trans* and in the pair *trans,trans*. Hence the four isomers can give only two different spectra by which geometrical isomers, but not positional isomers, can be differentiated.

The absorbance spectra of substances VII.B and VIII.B separated by HPLC are seen (18) as broad bands very similar in shape; they are shifted 3 nm apart, a shift analogous to that observed in *cis,trans* and *trans,trans* isomers of conjugated fatty acids (22-24) and in 2,4-hexadienes. Each spectrum presents at least two shoulders, one at each side of the maximum.

Since pure geometrical isomers of conjugated diene substances derived from linoleic acid were not available, the absorbance spectra of VII.B and VIII.B published by Chan and Levett were used to obtain their SDS by the following procedure. The small figure was enlarged photographically, the abscissa was marked each nm,

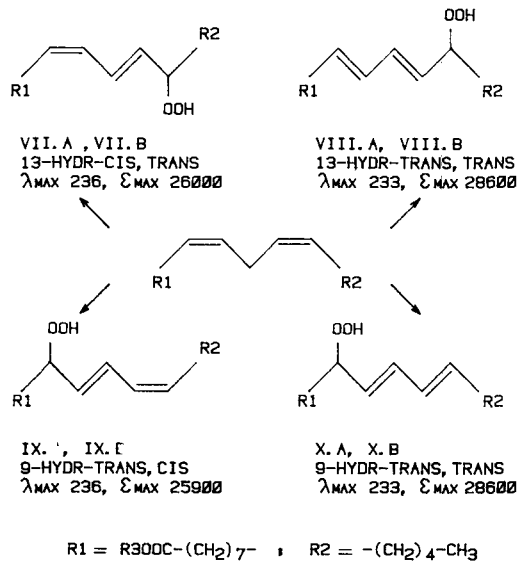

FIG. 6. Isomeric hydroperoxy acids (VII.A–X.A; R3 = H) formed in linoleic acid autoxidation and their methyl esters (VII.B–X.B; R3 = CH_3). λmax and εmax values are indicated for the esters.

absorbance values were measured (in millimeters) from the bases to the curves, and finally the digital data was processed by the method of Savitzky and Golay using seven points for a cubic fit to avoid excessive noise.

All features of the SDS so obtained (Fig. 7) were similar to that of 2,4-hexadienes and pheromones studied with regard to shape and relative positions of minima and zero crossing points. Thus, for example, the minima of the *cis,trans* isomer are bathochromically shifted with respect to those of the *trans,trans* one.

A SDS similar to that of substance VII.B was obtained experimentally. Lipoxygenase catalyzes the peroxidation of methylene interrupted polyunsaturated fatty acids giving only or preponderantly *cis,trans* products (25), yet *cis,trans/trans,trans* ratios depend on the substrate concentration, temperature, presence of cosubstrates, etc. The SDS of linoleic acid autoxidized in the presence of lipoxygenase at optimum conditions (isoenzyme 1) (17) is shown in Figure 8 (A). In spite of differences in solvents and methods by which they were obtained, the similarity of this spectrum with that of substance VII.B (Fig. 7 [B]) is remarkable, with minima and zero crossings occurring at the same wavelengths.

Since no conditions are known leading only to *trans,trans* isomers, linoleic acid was oxidized in the same medium as before but with no lipoxygenase present. A mixture of geometric isomers resulted and, as expected, minima and zero crossings underwent an hypsochromic shift as shown in Figure 8 (B).

When the SDS of lipoxygenase-oxidized linoleic acid (normalized by multiplying it by an adequate factor to compensate the difference in concentration) was subtracted from that of linoleic acid autoxidized with no lipoxygenase present, the result was an SDS with the same features of shape, maxima and minima as that obtained from the absorbance spectrum of substance VIII.B (*trans,trans*) published by Chan and Levett (18).

Spectra of autoxidized soybean oil and soybean lecithin. Conjugated diene compounds of soybean oil have been studied by UV spectrophotometry and by HPLC (8). Among the chromatographic peaks, four were identified by their UV absorbance as due to conjugated dienes.

The spectra in absorbance and second derivative autoxidized soybean oil and autoxidized soybean lecithin are shown in Figure 9. Their minima have values (234-235 nm) between those of *cis,trans* and *trans,trans* hydroperoxides derived from linoleic acid.

In spite of the highly complex composition of these products, their SDS in the wavelength range heretofore considered (220-260 nm) cannot be differentiated from those of much simpler compounds; the neat shape with three negative peaks is retained. This can be explained by considering the properties of the spectra as well as the chemical nature of the products. Although UV SDS is capable of distinguishing between dienic geometric isomers, it is not, or it is very little, influenced either by the length of the carbon chain or by the position of the double bonds along the same or by the occurrence of hydroperoxy or related groups near the double bonds. The products contain no substances with chromophores which could interfere with conjugated dienes, except

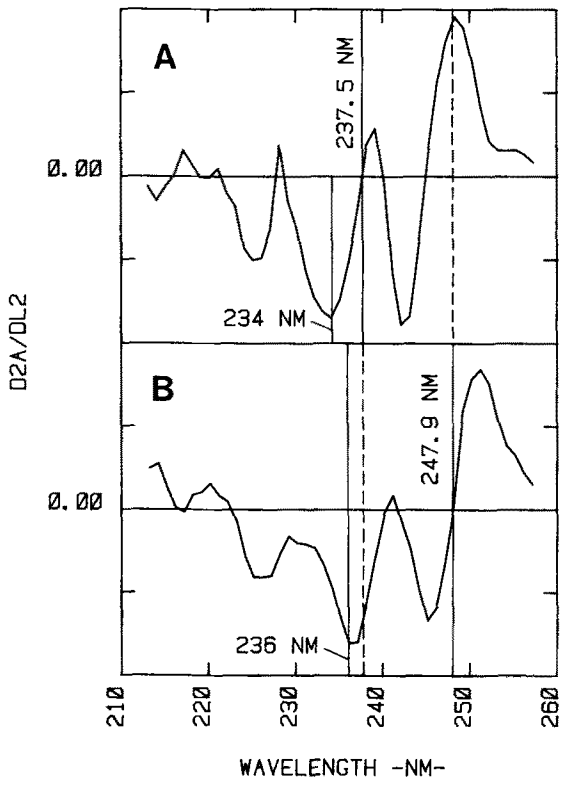

FIG. 7. Second derivative spectra of methyl 13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoate (A) and methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate (B) calculated from literature data.

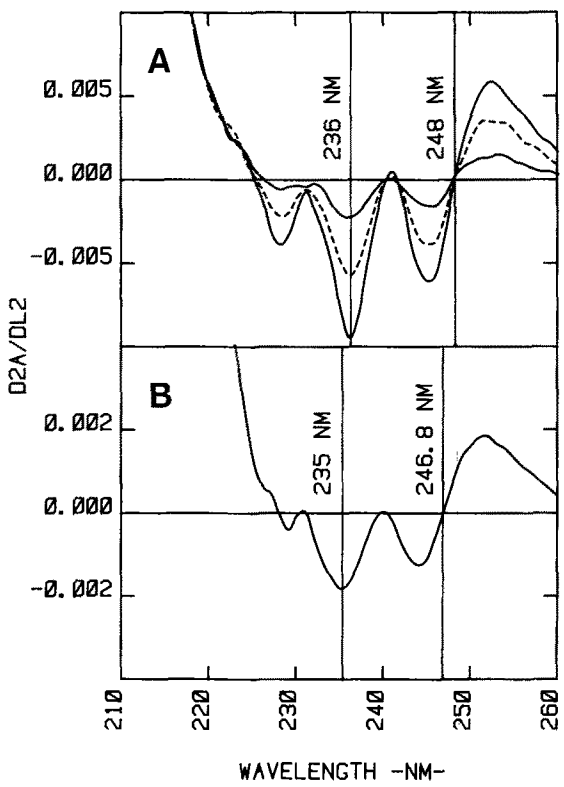

FIG. 8. Second derivative spectra of autoxidized linoleic acid with (A) and without (B) lipoxygenase. Solvent pH 9 borate buffer.

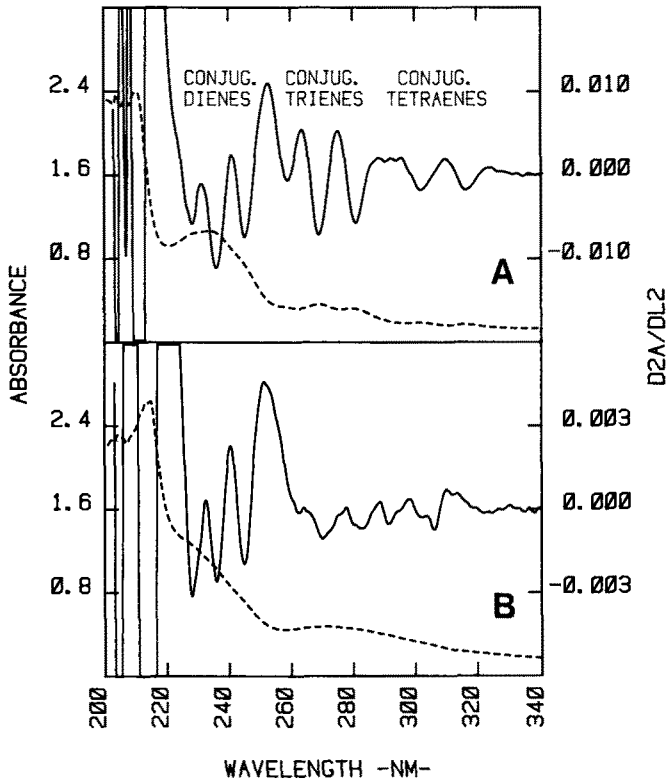

FIG. 9. Spectra in absorbance (- - -) and second derivative (——) of autoxidized soybean oil (A) and autoxidized soybean lecithin (B). Solvent n-hexane.

perhaps a slight overlapping with the smaller bands of conjugated trienes at 250-260 nm.

DISCUSSION

The knowledge on SDS of conjugated dienes and their properties opens a series of possibilities in the field of lipid analysis. Since under certain conditions the derivative spectra are not affected by an absorbance background, correlations between derivative values and oxidative status of fats and oils should be expected to be better than those mentioned in the intoduction.

Analysis of mixtures of isomers derived from the autoxidation of lipids has been performed by infrared spectrophotometry (26,27), but this technique, besides other drawbacks, is not reliable in determining minor amounts of isomers in the mixture.

Lipid conjugated dienes have been separated by adsorption HPLC, while reversed phase HPLC separates the mixture only according to the geometric isomerism of the double bonds and not according to the position of the hydroperoxy group (18).

It is shown in this paper that derivative spectrophotometry offers the possibility of developing very simple non-separative methods for the analysis of mixtures of conjugated diene geometric isomers with advantages over other methods in rapidity and cost. Besides, the ratio of geometric isomer concentrations, useful in the study of lipid autoxidation mechanism, can be determined easily by derivative spectrophotometry.

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REFERENCES

1. Bolland, J.L., and H.P. Koch, *J. Chem. Soc.* 445 (1985).
2. Farmer, E.H., and D.A. Sutton, *Ibid.* 119 (1943).
3. Gray, J.I., *J. Amer. Oil Chem. Soc. 55*:539 (1978).
4. Holman, R.T., and G.O. Burr, *J. Am. Chem. Soc. 68*:562 (1946).
5. Ong, V.T.L., *Fette Seifen Anstrichm. 82*:169 (1980).
6. Van Den Bosch, E., *J. Amer. Oil Chem. Soc. 50*:421 (1973).
7. St. Angelo, A.J., R.L. Ory and L.E. Brown, *Ibid. 52*:34 (1975).
8. Brown, H.G., and H.E. Snyder, *Ibid. 59*:280 (1982).
9. DuPlessis, L.M., P. Van Twisk, P.J. Van Niekerk and M. Steyn, *Ibid. 58*:575 (1981).
10. Defrancesco, F., C. Defrancesco and A. Boccardi, *Riv. Ital. Sostanze Grasse 57*:85 (1980).
11. MacGee, J., in *Methods of Enzymatic Analysis*, edited by U. Bergmeyer, Academic Press, 1965, p. 411.
12. Cahill, J.E., *Am. Lab. 11*:79 (1979).
13. Talsky, G., L. Mayring and H. Kreuzer, *Angew. Chem. 90*:840 (1978).
14. Smith, R.C., *Rev. Sci. Instr. 34*:296 (1963).
15. Giese, A.T., and C.S. French, *Appl. Spectrosc. 9*:78 (1955).
16. Savitzky, A., and M.J.E. Golay, *Anal. Chem. 36*:1627 (1964).
17. Axelrod, B., T.M. Cheesbrough and S. Laakso, *Methods Enzymol. 71*:441 (1981).
18. Chan, H.W.S., and G. Levett, *Lipids 12*:99 (1977).
19. Chan, H.W.S., and F.A.A. Prescott, *Biochim. Biophys. Acta 380*:141 (1975).
20. Koskas, J.P., J. Cillard and P. Cillard, *J. Chromatogr. 258*:280 (1983).
21. Patee, W.E., and J.A. Singleton, *J. Agric. Food Chem. 27*:216 (1979).
22. Gunstone, F.D., and W.C. Russell, *J. Chem. Soc.* 3782 (1955).
23. Ahlers, N.H.E., R.A. Bret and N.G. McTaggart, *J. Appl. Chem. 3*:433 (1953).
24. Jackson, J.E., R.F. Paschke, W. Tolberg, H.M. Boyd and D.H. Wheeler, *J. Amer. Oil Chem. Soc. 29*:229 (1952).
25. Porter, N.A., B.A. Weber, H. Weenen and J.A. Khan, *J. Am. Chem. Soc. 102*:5597 (1980).
26. Privett, O.S., W.O. Lundberg, N.A. Khan, W.E. Tolberg and D.H. Wheeler, *J. Amer. Oil Chem. Soc. 30*:61 (1953).
27. Sephton, H.H., and D.A.Sutton, *Ibid. 33*:263 (1956).

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