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Determination of Double Bond Positions in Isomeric Eicosadienoic Acids from Sea-urchin Lipid

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Masayuki KōCHI*

The double bond positions in the isomeric eicosadienoates from the lipid of a sea urchin species, *Anthocidaris crassispina*, were determined by means of mass spectrometry, periodate-permanganate oxidation, alkaline isomerization, GLC analysis on capillary column and hydrazine partial reduction followed by periodate-permanganate oxidation.

It was found that two isomers containing non-methylene interrupted double bonds exist in sea-urchin lipid. One of the isomers yielded mainly nonanoic and suberic acids on oxidative cleavage, and gave no characteristic absorption of conjugated dienes in the UV region after alkaline isomerization. These results indicate that the isomer is a hitherto unknown acid, i.e., 3,11-eicosadienoic acid. The other isomer was characterized as 5,11-eicosadienoic acid which is rarely demonstrated in marine organisms. It produced nonanoic, glutaric and adipic acids on direct oxidative cleavage and nonanoic, pentadecanoic, glutaric and undecanedioic acids on oxidative cleavage of its partial reduction products.

The eicosadienoic acids generally known to be present in natural products are the 8,11- and 11,14-eicosadienoic acids,^{1,2} and the occurrence of a few eicosadienoic acids with the double bonds at positions different from these have also been confirmed.³⁻⁸ We have already reported⁹⁻¹¹ that the sea-urchin lipid contains 11,14-eicosadienoic acid and also large quantities of other eicosadienoic acids (isomeric 20:2 acids) thought to have double bonds at unique positions. In the previous paper¹¹ these isomeric

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20:2 acids were isolated and purified from the gonads of the sea-urchin (*Anthocidaris crassispina*), and these fatty acids were confirmed as straight chain eicosadienoic acids.

Thus, in order to establish the structures of these isomeric 20:2 acids, the double bond positions were determined. The results will be reported here.

EXPERIMENTAL

Sample Materials As reported previously,¹¹ the methyl esters of the fatty acids obtained from the gonad lipids of the sea-urchin *Anthocidaris crassispina* were separated using seven different techniques; the fractions used here were M-6 (97.8% pure as eicosadienoic acid), M-7 (98.4% pure), and M-7' (93.7% pure), the latter fraction was prepared from new sample material using the same procedure as the preparation of fraction M-7.

Mass Spectral Analysis A Japan Electronic Optics Laboratory Company Limited mass spectrometer model JMS-01 SG, with ionizing voltage of 75 eV and sample temperatures of 32°C, was used for the measurements.

GLC Analysis (1) Using a packed column (10% DEGS, 3 m), the analytical conditions were the same as previously reported.¹⁰ However, the low monocarboxylic and dicarboxylic acids were analyzed at column temperatures of 120°C and 160°C, respectively. The standards used were the C₆-C₁₀ saturated monocarboxylic acids and the C₂-C₁₁ saturated dicarboxylic acids. (2) The analytical conditions for using the capillary column are shown in Table 1.

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Oxidative Degradations Following the method of Rudloff,¹² oxidative degradations were carried out at the double bond positions on about 3 mg of each of the fractions M-6 and M-7 using sodium periodate - potassium per-

manganate in tertiary butanol. The mono- and di-carboxylic acids obtained were then extracted with ethyl ether. An aliquot of the extract was distilled under vacuum to remove the ethyl by distillation; the residue was methylated to the methyl ester which was then analyzed by GLC. The remaining extract was separated into mono- and di-carboxylic acid fractions and then treated as above. The esters obtained were analyzed by GLC to identify the fatty acids.

Determination of the Amount of Conjugated Unsaturated Fatty Acids

About 3mg of a methyl ester sample was weighed and made to 25ml by adding *n*-hexane. Following the standard method,¹³ absorbances in the 210-346 mμ wavelength region were measured on a portion of the solution, and then the amount of conjugated unsaturated fatty acids was calculated.

Determination of the Amount of Non-conjugated Unsaturated Fatty Acids

Following the standard method,¹³ about 3mg of a methyl ester sample was weighed and then isomerization was accomplished by heating in a 6.6% solution of potassium hydroxide - ethylene glycol. The reaction solution was diluted with methanol and the absorbances in the 210-346 mμ wavelength region were measured; similar to the above, the amount of conjugated unsaturated fatty acids was calculated. The difference between the amounts of conjugated unsaturated fatty acids before and after alkaline isomerization was taken as the amount of nonconjugated unsaturated fatty acids. As a control, methyl linoleate (special grade, Wako Pure Chemicals Company) was used and examined under the same conditions.

Partial Reduction Following the method of Aylward,¹⁴ a hydrazine-methanol solution was added to about 10mg of fraction M-7', and then allowed to react at 50°C for 4 hours to give partial reduction of the double bonds.

Silver Nitrate Impregnated TLC Similar to the previously reported

method,⁹ a 300 μ thick silica gel G plate (20 X 20 cm) was prepared and activated by heating at 110^oC for 120 minutes. After applying the methyl ester sample as a band, it was developed in methanol - chloroform (0.5: 99.5,v/v). After air drying, a 1% iodine - methanol solution was sprayed on both edges of the plate to determine the band position.

RESULTS

Mass Spectral Analysis The mass spectrum of fraction M-7 is shown in Figure 1. The molecular ion m/e 322 for the methyl ester of the isomeric 20:2 acid agreed with the molecular weight of the fatty acid methyl ester. Although the methyl esters of high fatty acids generally show strong parent peaks,¹⁵⁻¹⁹ the isomeric 20:2 acid one was weak. This indicates that the molecular ion of the isomeric 20:2 acid fragments easily. Peaks characteristic for methyl esters at m/e 31, 74 and methoxy-carbonyl ion fragments at m/e 59, 87, 101, 115 and 129 were observed. The base peak at m/e 41 is a hydrocarbon fragment and other numerous hydrocarbon fragments C₁ - C₁₂ were also observed, but specific fragments which related to the double bond positions were not observed.

GLC Analysis of the Oxidative Degradation Products Oxidative degradations of fractions M-6 and M-7 gave the mono- and di-carboxylic acid compositions as shown in Table 2. Almost all of the mono-carboxylic acids produced from both fractions was nonanoic acid. Therefore, for this 20:2 acid, it is clear that one of the double bonds is between carbon atoms 11 and 12. On the other hand, the di-carboxylic acids produced were glutaric, adipic and suberic acids. However, the relative amounts of these acids were somewhat different in fractions M-6 and M-7; in the former fraction (M-6) the amount of suberic acid was larger, and in the latter fraction the amounts of adipic and glutaric acids were larger.

Oxidative degradation of a fatty acid possessing a divinyl methane type arrangement of double bonds should produce malonic acid from degradation of the carbon chain between the double bonds, but only a trace amount was detected; this may be due to further decomposition in the oxidative degradation process,²⁰ or there may be no divinyl methane type double bonds present.

The above oxidative degradation products suggest the structures for the 20:2 acid as shown in Figure 2. Using a packed column as previously reported,¹¹ GLC analysis showed a single peak for this isomeric 20:2 acid, but from the oxidative degradation products it is reasonable to consider it to be a mixture of 2 - 4 types of 20:2 acids, which differ in the double bond positions; also the ratios of these types are different between fractions M-6 and M-7. Therefore, of the possibilities shown in Figure 2, it appears that the main component of fraction M-6 is either 3,11- or 8,11-20:2 acid, and that of fraction M-7 is either 5,11- or 6,11-20:2 acid.

The oxidative degradation of 5,11- or 6,11-20:2 acid should theoretically produce equal amounts of glutaric acid and adipic acid. However, fractions M-6 and M-7 both gave more adipic than glutaric acid. This may be due to adipic acid being formed from the oxidative degradation intermediates malonic acid semialdehyde or malonaldehyde, which are obtained from the carbon chain on the carboxylic acid side of the 3,11-20:2 acid, and from the carbon chain between the double bonds of the 8,11-20:2 acid, respectively; alternately, a compound with the same retention time as the dimethyl ester of adipic acid may be formed during GLC analysis. These possibilities were investigated by carrying out oxidative degradations under the same conditions on glutaric acid and linoleic acid. However, such compounds were not produced. Therefore, the differences in the amounts of glutaric and adipic

acids obtained were probably due to the highly water-soluble nature of glutaric acid, which makes quantitative recovery difficult; this has also been observed by Korn.²¹

Ultraviolet Absorption Spectral Analysis Before and After Alkaline

Isomerization In order to clarify the structure of the main component in fraction M-6, ultraviolet absorption spectral analysis was carried out before and after isomerization. Figure 3 shows the absorption curves obtained. Fraction M-6 showed almost no absorptions in this wavelength region before the isomerization, but after isomerization a conjugated diene absorption was observed at a wavelength of 233 m μ . However, this absorption was very weak compared to the absorption obtained after isomerization of methyl linoleate, the control compound examined under the same conditions.

The amounts of conjugated unsaturated fatty acids, before and after isomerization, were calculated from the absorbances. The results obtained are shown in Table 3. Under these conditions, the methyl linoleate control completely isomerized to a conjugated dienoic acid, but most of the 20:2 acid in fraction M-6 was not isomerized. From the above results, it is clear that the main isomeric 20:2 acid component of fraction M-6 is either a divinyl ethane (ethylene interrupted) type, or a fatty acid with the double bond positions further apart.²² Therefore, since previous data indicated either the 3,11- or 8,11-20:2 acid for fraction M-6, this clearly shows that the main fatty acid is the 3,11-20:2 acid.

Further, from the data in Table 3, the amounts of fatty acids containing the divinyl methane (methylene interrupted) type double bond arrangement were calculated, and were compared to the amounts from GLC analysis as shown in Table 4. For methyl linoleate the alkaline isomerization and GLC results were in fair agreement, but for fraction M-6 some differences

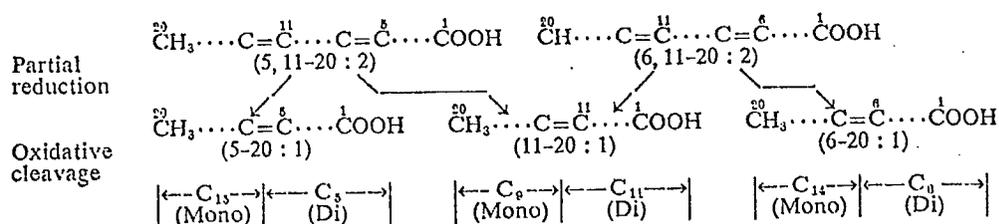
were observed in the amounts of divinylmethane type dienoic acids and of other fatty acids. These differences indicate that the fraction contains, besides the 3,11-20:2 acid, a small amount of the 8,11-20:2 acid, which was not detected by GLC analysis due to overlap with the large 3,11-20:2 acid peak, and thus determined as the 3,11-20:2 acid. This is also observed in the methyl linoleate case where some differences are detected when the two analyses are studied in detail.

GLC Analysis Using a Capillary Column The oxidative degradation results clearly showed that there were 2 - 4 types of fatty acids present in both fractions M-6 and M-7. Thus, investigations into the number of 20:2 acids in each fraction were carried out by GLC analysis using a capillary column. The gas chromatograms obtained are shown in Figure 4. The number of 20:2 acid peaks detected were 4 for fraction M-6, and 2 for fraction M-7. The peak D component of fraction M-6 was identified as the 11,14-20:2 acid because it had the same retention time as a standard sample. Also, because the relative retention time of peak C is 2.43 and its separation factor from the 11,14-20:2 acid peak is 1.07,²³ this component (C) was identified as the 8,11-20:2 acid.

Although GLC analysis using a packed column showed the isomeric 20:2 acid as a single peak,¹¹ GLC analysis using a capillary column gave, for both fractions M-6 and M-7, a separation into 2 peaks, peak A (relative retention time 2.29) and peak B (relative retention time 2.33). Thus, p.726 the isomeric 20:2 acids in both fractions are 3,11-20:2 acid and either the 5,11- or the 6,11-12:2 fatty acid. By comparing the ratio of peaks A and B obtained from the capillary column chromatogram to the ratio of the 3,11- and 5,11- (or 6,11-)20:2 acid obtained by oxidative degradation of the above fractions M-6 and M-7, it is clear that the peak A component is either the

5,11- or 6,11-20:2 acid and the peak B component is the 3,11-20:2 acid.

Oxidative Degradation of the Partially Reduced Product Whether the composition of peak A is the 5,11- or the 6,11-20:2 acid was determined. If the partially reduced compounds are oxidatively degraded, the 5,11- and 6,11-20:2 acids would produce the fatty acids as shown in the following equations. Therefore, by investigating the composition of the mono- and di-carboxylic acids produced, the structure of the isomeric 20:2 acid can be determined.



The gas chromatogram of fraction M-7' (after partial reduction with hydrazine) is shown in Figure 5. On this chromatogram, two 20:1 acid peaks, B and C, were detected. Here, C is the 11,20:1 acid peak, and B can be either the 5- or 6-20:1 acid peak because of its shorter retention time.

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Next, the partially reduced compounds were separated by silver nitrate impregnated TLC, and the isolated monoenoic acid fraction was oxidatively degraded by the addition of a sodium periodate - potassium permanganate solution. The composition of the mono- and di-carboxylic acids obtained are shown in Table 5. The mono-carboxylic acids nonanoic and pentadecylic, and the di-carboxylic acids glutaric and undecanedioic were produced in large quantities. Thus, because large amounts of pentadecylic and glutaric acids were obtained, it appears that the composition of peak B is the 5-20:1 acid. Therefore, the structure of the isomeric 20:2 acid was established

as the 5,11-20:2 acid. Although myristic and adipic acids were also detected among the degradation products, the amounts of these fatty acids were small compared to pentadecylic and glutaric acids; moreover, a difference was observed in the mole percentages of the fatty acids (myristic and adipic). Therefore, these fatty acids can be considered as by-products²⁴⁾ of the oxidative degradation process.

DISCUSSION

Sea-urchin lipid contains two types of 20:2 acids which possess unique double bond positions, and were characterized structurally as the 3,11- and 5,11-20:2 acids.

It is known that many unsaturated fatty acids found in natural products belong to the oleic acid, linolic acid or linolenic acid groups, and a common structural feature is that they all possess the divinyl methane type arrangement of double bonds.^{1,25-27} However, besides these fatty acids, other fatty acids exist which contain unique structures.^{2,28} For the 20:2 acids, although the 8,11- and 11,14-20:2 acids are widely distributed in natural products,^{1,2} the 14,17-20:2 acid, which belongs to the ω₃ series, is rarely found.³ Of the 20:2 acids which contain unique structural features, the 5,11-,³⁻⁷ 5,13-,^{6,7} 7,10-,⁸ and 10,13-20:2⁸ acids are known to exist; and others, the 6,11-⁶ and 8,13-20:2 acids,⁶ have been assumed to be present.

Sea-urchin lipid contains the 3,11-20:2 acid which has a double bond at the third carbon atom; moreover, this fatty acid has a very unique structure with the 2 double bonds in a hexamethylene interrupted arrangement. This result is the first known example of this fatty acid occurring in a natural product. The known fatty acids which are found in natural sub-

stances and have a double bond at the third carbon atom are the following: 3-16:1,²⁹⁻³¹ 3-18:1,³⁰⁻³² 3,6-16:2,⁸ 3,9-18:2,²⁹ 3,9,12-18:3,^{29-31,33} and 3,9,12,15-18:4³⁴ acids. Also, fatty acids which contain hexamethylene interrupted arrangements have been reported: 5,13-20:2,^{6,7} 5,13-22:2,^{32,35,36} and 7,15-22:2⁷ acids. However, a fatty acid with a structural characteristic similar to the 3,11-20:2 acid has not been observed previously in natural substances.

The 5,11-20:2 acid has been found in the seeds and leaves of the ginkgo tree,^{3,4} and in the livers of rats,^{5,6} and recently it has also been found in persimmon.⁷ Besides this acid, a dienoic acid with the double bonds at the 5,11- positions is the 18:2 acid,^{3,4,37,38} and is the main fatty acid component of the slime mold.³⁸ Besides these fatty acids there are many other fatty acids which possess the tetramethylene interrupted arrangement,^{3-5,7,29-31,33,34,39-41} but, except in some plant seed oils,^{29,31,33,34} their contents are small. The sea-urchin is noteworthy as a marine organism because of its rather large content of the 5,11-20:2 acid. p.728

In general, many of the fatty acids containing the unique double bond arrangement are found in plant seed oils^{3,4,29-36,39,40,42} (plant kingdom), but are rarely found in animal lipids.⁵⁻⁸ The presence of the 3,11- and 5,11-20:2 acids in sea-urchin lipids is interesting from the comparative biology aspect.

SUMMARY

Using the isomeric 20:2 acid fractions M-6 and M-7, whose preparations are described in a previous report, and a freshly prepared fraction M-7', the structures of the isomeric 20:2 acids present in sea-urchin lipid were determined. That is, the double bond positions of the isomeric 20:2 acids were determined by mass spectral analysis, oxidative cleavage with sodium periodate - potassium permanganate, ultraviolet absorption

spectral analysis before and after alkaline isomerization, GLC analysis with a capillary column, and oxidative cleavage of compounds which were partially reduced with hydrazine.

As a result, it was established that the main 20:2 acids were 2 stereoisomers with unique double bond arrangements; their structures were characterized as the 3,11-20:2 acid and the 5,11-20:2 acid.

The 3,11-20:2 acid obtained here is the first known occurrence of this fatty acid in natural substances. Also, although the 5,11-20:2 acid is known to exist in other natural substances, the sea-urchin is a rare example which contains large quantities of this fatty acid.

Table 1. Conditions for gas-liquid chromatography using capillary column

Apparatus:	SHIMADZU Gas Chromatograph Model GC-5A
Column:	45 m x 0.05 cm, stainless steel
Solid support:	Column wall.
Stationary phase:	Butanediol succinate polyester (BDS)
Temperatures:	Column, 168°C; injection and detector, 280°C
Carrier gas:	Nitrogen at 2.2 ml/min
Detector:	SHIMADZU Hydrogen Flame Ionization Detector Model FID-5
Sensitivity:	1000 MΩ
Range:	0.08 V
Theoretical plates (n):	Approximately 23,000
Analysis time:	Approximately 23 min to methyl palmitate

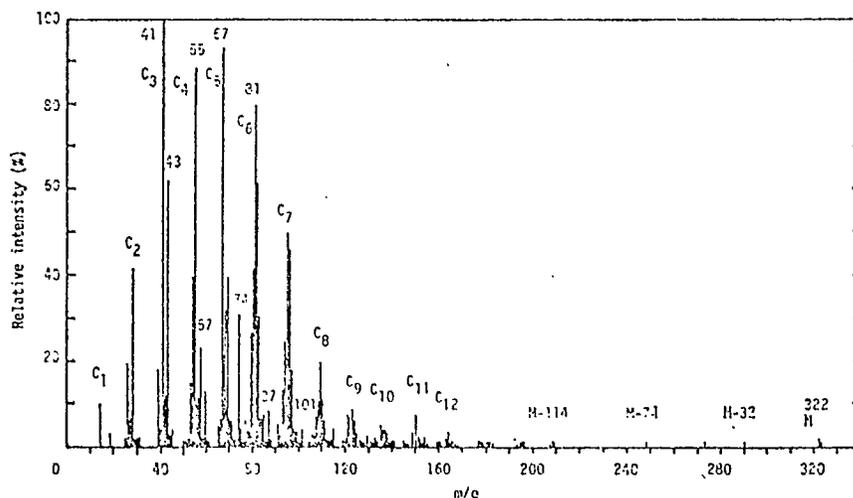


Fig. 1. Mass spectrum of isomeric eicosadienoate fraction, M-7, obtained from lipid of sea-urchin gonad by chromatographic procedures.

Table 2. Composition of oxidative cleavage products of isomeric eicosadienoate fractions, M-6 and M-7, estimated as methyl esters of mono- and dicarboxylic acids (mole %)

Fatty acid	Fraction	
	M-6	M-7
Monocarboxylic		
C ₈	Trace	—
C ₉	Trace	Trace
C ₉	100.0	100.0
Dicarboxylic		
C ₃	Trace	—
C ₄	Trace	—
C ₅	22.3	34.6
C ₆	27.7	50.0
C ₇	Trace	Trace
C ₈	50.0	15.4
C ₉	Trace	—

M-6 and M-7 were fractionated from lipid of sea-urchin gonad by chromatographic procedures

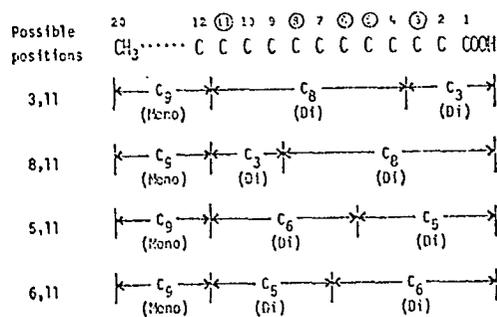


Fig. 2. Possible positions of double bonds in isomeric eicosadienoic acid deduced from C₇-monocarboxylic acid, and C₅-, C₆- and C₈-dicarboxylic acids obtained by periodate-permanganate oxidation.

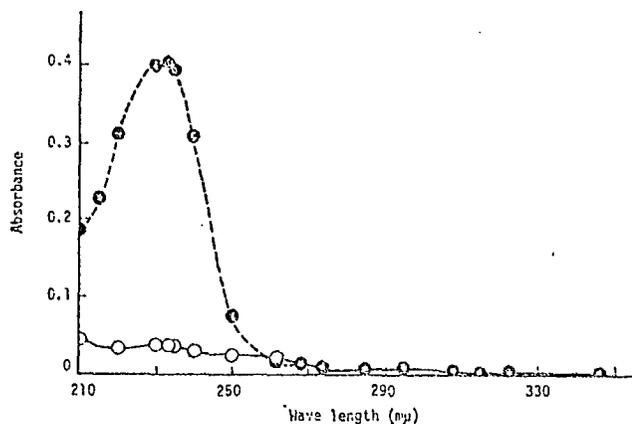


Fig. 3. Absorption spectra of isomeric eicosadienoate fraction, M-6, before and after alkaline isomerization.

Solid line, before alkaline isomerization (0.1030 g/l).

Dotted line, after alkaline isomerization (0.0600 g/l).

Table 3. Proportion of conjugated acids in isomeric eicosadienoate fraction, M-6 and control specimen, commercially available linoleate before and after alkaline isomerization

Fatty acid	Fraction M-6		Methyl linoleate	
	Before isomerization	After isomerization	Before isomerization	After isomerization
Conjugated dienes	0.30%	6.02%	0.20%	94.72%
" trienes	0	0.02	0.02	0.38
" tetraenes	0	0	0	0
" pentaenes	0	0	0	0
Others	99.70	93.96* ¹	99.78	4.90* ²

*¹ Almost exclusively isomeric eicosadienoate

*² Almost exclusively oleate

Table 4. Composition of isomeric eicosadienoate fraction, M-6 and control specimen, commercially available linoleate, calculated from the data shown in Table 3, compared with their composition by GLC analysis

Fatty acid	Fraction M-6		Methyl linoleate	
	Alkaline isomerization	GLC	Alkaline isomerization	GLC
Conjugated dienes	0.30%	0.3%	0.20%	—
" trienes	0	—	0.02	—
Methylene interrupted				
" dienes	5.72	1.5	94.52* ³	94.1
" trienes	0.02	—	0.36* ⁴	0.2
Others	93.96* ¹	98.2	4.90* ²	5.7

*¹ and *², the same as those showed in Table 3; *³, linoleate; *⁴, linolenate

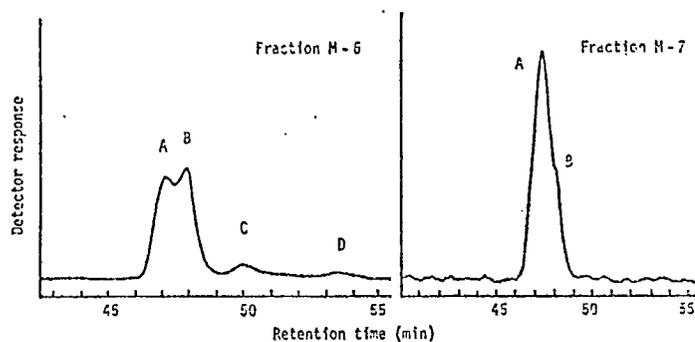


Fig. 4. Gas chromatograms of isomeric eicosadienoate fractions, M-6 and M-7 (capillary column, BDS, 45 m).

Peaks: A, 5, 11- or 6, 11-eicosadienoate; B, 3, 11-eicosadienoate; C, 8, 11-eicosadienoate; D, 11, 14-eicosadienoate.

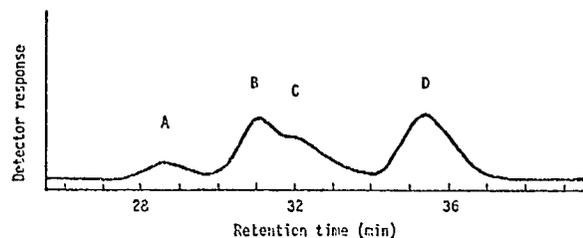


Fig. 5. Gas chromatogram of hydrazine reduced isomeric eicosadienoate fraction, M-7' (packed column, DEGS, 3 m).

Peaks: A, arachidate; B, assumable other eicosenoate; C, 11-eicosenoate; D, isomeric eicosadienoate.

Table 5. Composition of oxidative cleavage products of monoenoic acids formed by hydrazine partial reduction of isomeric eicosadienoate fraction, M-7', estimated as methyl esters of mono- and dicarboxylic acids

Monocarboxylic acid		Dicarboxylic acid	
Component	mole %	Component	mole %
C ₅	0.8	C ₄	1.1
C ₉	37.2	C ₅	35.4
C ₁₀	0.5	C ₆	3.6
C ₁₂	0.6	C ₇	3.4
C ₁₃	1.9	C ₈	1.6
C ₁₄	8.4	C ₉	7.1
C ₁₅	36.4	C ₁₀	11.8
C ₁₆	14.2	C ₁₁	36.0
		C ₁₂	Trace

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