

Applications of Chromic Acid-Celite Columns to Lipid
Analysis. Location of Double Bond Position in
Submicro- and Microgram Amounts of
Methyl Octadecenoates

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A procedure for locating the double bond position in a series of methyl octadecenoates is detailed. Submicrogram or microgram amounts of substrate dissolved in CS₂ are brought in contact with a very small column of chromic acid on Celite, and the oxidation products (carboxylic acids) are eluted, converted to methyl esters, and resolved by gas-liquid chromatography. Beside the acids resulting from scission of the double bond, acids containing one less carbon atom arise from oxidation of the allylic carbons on both sides of the double bond so that pairs of peaks appear on the chromatogram. All positions from $\Delta 3$ to $\Delta 17$ were located successfully. The $\Delta 2$ position failed to oxidize.

This paper describes a procedure which utilizes a very small column of aqueous chromium trioxide on a Celite support to oxidatively cleave carbon-carbon double bonds brought briefly in contact with it. The scission products are then eluted and readily identified by gas-liquid chromatography (glc). The method has been thoroughly tested by applying it to submicrogram and microgram amounts of a series of methyl octadecenoates with *cis* double bonds in each position, as well as to a number of the *trans* isomers.

Chromic acid oxidation of carbon-carbon double bonds has been extensively studied (1) but outside of one report (2) little use of this reaction as a tool in lipid analysis has been made. When the double bond in a methyl octadecenoate is attacked on the CrO₃-Celite column, the molecule is cleaved, giving a monocarboxylic acid and a monomethyl ester of a dicarboxylic acid. In addition, the allylic carbon atoms on both sides of the double bond are attacked, giving a monocarboxylic acid and a monomethyl ester of a dicarboxylic acid, each containing one less carbon atom than the double bond scission products. Thus, each pure methyl ester would theoretically yield four fragments with the exception of the $\Delta 2$ and $\Delta 17$ isomers where only three are possible. The allylic oxidation products are always produced in lesser amounts than are the double bond oxidation

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products under the experimental conditions employed. The appearance and identification of the pair of monocarboxylic acids and the pair of half-acid esters, where applicable, pinpoint the site of unsaturation.

The monocarboxylic acids produced in the oxidation (with the exception of C_1-C_3) can be eluted from the column with CS_2 . However, the half-acid esters generated on the column show varying elution patterns with CS_2 ; those with 10 or more carbon atoms in the chain elute completely; whereas those below C_8 do not elute at all. The C_9 and C_8 are partially eluted with CS_2 . Half-acid esters that do not elute with CS_2 can, with the exception of C_3 and C_2 , be eluted with isopropyl ether. The differences in the ability to elute with CS_2 is fortuitous as it eliminates all possibility of confusion due to overlap of the pairs of cleavage products during glc.

Although the pair of monocarboxylic acids alone suffice to locate the double bond, identification of the pair of monomethyl esters of the dicarboxylic acids not only substantiate the position but are also essential, as it turns out in this study, for locating the double bond when it occupies the 15, 16, and 17 positions.

REAGENTS AND EQUIPMENT

Analytical Grade Celite² (also called Analytical Filter Aid) and Celite 545 are products of the Johns-Manville Corp., Lompoc, Calif. They are available from various supply houses; carbon disulfide (Baker Chem. Co., Phillipsburg, N.J.) and isopropyl ether (99+%, Aldrich Chem. Co., Milwaukee, Wis.) were used as received; chromium trioxide was a product of the Fisher Scientific Co., Silver Spring, Md.; methyl esters of monocarboxylic acids were purchased from Nu-Chek-Prep., Inc., Elysian, Minn.; dimethyl esters of the dicarboxylic acids were obtained from the Aldrich Chemical Co. (Milwaukee, Wis.) and the Eastman Kodak Chem. Co. (Rochester, N. Y.), or they were prepared from commercially available dicarboxylic acids.

Melting point capillaries open at both ends, 100 mm long, 1.2–1.4-mm i.d. \times 1.6–1.8-mm o.d. (Kimble No. 34,500) were cut approximately in half and used for columns; a commercially available pressure device was found very convenient throughout this study. It consisted of a rubber squeeze-type bulb with a netted reservoir and a connecting tube fitted with a rubber capillary holder such as that supplied with Microcaps (available through various supply houses). A capillary holder can also be made by puncturing the top of a rubber medicine dropper holder.

² Reference to brand or firm names does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

EXPERIMENTAL

Preparation of chromic acid on Celite. Chromium trioxide (1.0 g) was dissolved in distilled water (1 ml) in a 4-in. mortar and Analytical Grade Celite (4.0 g) was added.³ The contents were ground with a pestle until homogeneously yellow. The powder was kept in a screw-cap vial at room temperature during the working day and at -18°C when not in use. Under these conditions, it retained its ability to cleave double bonds for at least 6 months.

Preparation of oxidizing column. A cut capillary was dabbed into the CrO_3 -Celite powder until approximately 1.8–2.0 cm was retained. The powder was pushed into the capillary with the straight portion of an ordinary paper clip or other suitable tamper and held there while the powder was packed into a compact column approximately 1 cm in length with another tamping device. The capillary was cut approximately 1 cm below (arbitrarily called exit end) and 2–3 cm above the column.

Oxidation of substrate. From 0.5 to 5 μg of substrate dissolved in up to 6 μl of CS_2 was applied to the column with a 10- μl syringe by placing the syringe needle tip lightly on the surface of the bed and expelling the solution slowly. After 15 min the column was eluted with 15–20 μl of CS_2 using the pressure device. The first 6–7 μl (about 6–7 mm) of effluent emerging was removed with a clean syringe.

Formation of methyl esters. The contents of the syringe was slowly applied to the surface of a compact column of Celite 545, approximately 1.5 cm long, contained in a cut capillary (3). The acids were then converted to methyl esters using the 2-min micro procedure previously described (3), except that the diethyl ether in the Diazald solution was replaced by an equal volume of 2-(2-ethoxyethoxy)ethanol. The methyl esters were eluted from the column with CS_2 . The first 9–10 μl of effluent were removed and analyzed by glc as described below.

Elution with isopropyl ether. If the analysis of the CS_2 effluent does not furnish sufficient information to establish the site of unsaturation, the oxidizing column is treated as follows: The exit end of the capillary is rinsed by filling it with CS_2 from a syringe and withdrawing and discarding the solution. This ensures that no residual acids from the CS_2 effluent remain on the glass which might subsequently be mistaken for the dicarboxylic acid monomethyl esters. The oxidation column is then eluted with five 10- μl aliquots of isopropyl ether using the pressure device. The effluent (slightly colored) is withdrawn after each aliquot and applied to a compact column of Celite 545, approximately 3 cm long, contained in a cut capillary. The ether is evaporated almost instantly by pulling a very slight vacuum on

³ It is very important that no other Celite be substituted. Several other grades of Celite were tried but were found to give incomplete or no oxidation of the double bonds.

TABLE 1
 PRODUCTS IDENTIFIED FROM THE OXIDATION OF METHYL OCTADECENOATES
 ON A CHROMIC ACID-CELITE COLUMN

Double bond position	Double bond scission products identified		Allylic oxidation products identified	
	MA ^a	HA ^b	MA ^a	HA ^b
<i>cis</i> isomers				
3	C15	—	C14(39-44) ^c (42) ^d	—
4	14	C4	13(27-39) (28)	—
5	13	5	12(35-37) (36)	C4(30-34) (32) ^e
6	12	6	11(35-42) (38)	5(31-37) (33) ^e
7	11	7	10(48-59) (56)	6(55-58) (56) ^e
8	10	8	9(34-40) (36)	7(9-15) (13) (37-43) (40) ^e
9	9	9	8(37-45) (42)	8(25-27) (26)
10	8	10	7(28-34) (31)	9(38-40) (39)
11	7	11	6(37-40) (39)	10(41-47) (43)
12	6	12	5(34-39) (37)	11(44-51) (48)
13	5	13	4(22-27) (24)	12(45-54) (50)
14	4	14	—	13(53-61) (58)
15	—	15	—	14(51-53) (52)
16	—	16	—	15(20-23) (21)
17	—	17	—	16(45-92) (66)
<i>trans</i> isomers				
5	C13	C5	C12(53-57) (55)	C4(36-48) (43) ^e
6	12	6	11(44-48) (47)	5(37-51) (44) ^e
7	11	7	10(59-64) (62)	6(42-52) (47) ^e
9	9	9	8(31-42) (36)	8(30-34) (32)
11	7	11	6(37-40) (39)	10(41-47) (43)
12	6	12	5(37-43) (39)	11(44-50) (47)

^a Monocarboxylic acids.

^b Half-acid esters.

^c Range of allylic oxidation as percentage of double bond scission products.

^d Average allylic oxidation as percentage of double bond scission products.

^e In isopropyl ether extract.

the end of the capillary opposite the end of application. After the ether evaporates from the final aliquot, the capillary is inserted through the septum (3) with the colored end up and exposed to diazomethane vapor (3). After 2 min, the column is eluted with CS₂, and the first 9-10 μl of effluent are analyzed by glc.

Gas-liquid chromatography. The conditions for glc were: instrument,

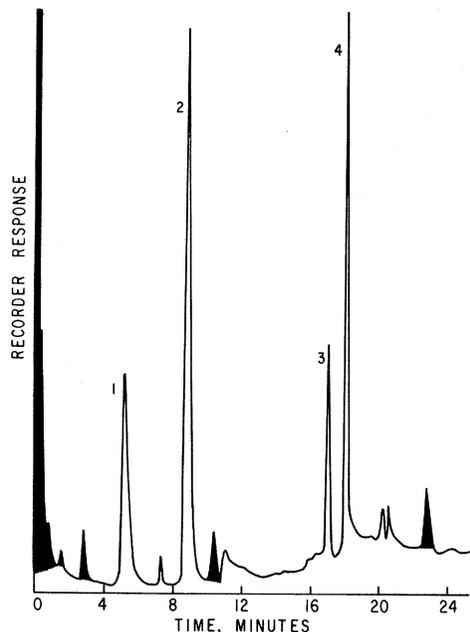


FIG. 1. Gas-liquid chromatogram of methyl esters of fragments formed from oxidation of 2.5 μg of methyl-*cis*-octadec-9-enoate on a CrO_3 -Celite column eluted with CS_2 . For conditions, see text. Identification of peaks: (1) methyl octanoate, (2) methyl nonanoate, (3) dimethyl octanedioate, and (4) dimethyl nonanedioate. Shaded areas are present in control and/or solvent blank.

Hewlett-Packard 5750 A; column, 8 ft \times $\frac{1}{8}$ in. silanized stainless-steel packed with 7.5% ethylene glycol adipate and 2% H_3PO_4 on 90-100 mesh Anakrom ABS; detector, FID; helium flow rate, 30 ml/min; injection port temperature, 250°C; detector temperature 250°C, column temperature 50 to 195°C programmed at 10°C/min following a hold of 4 min at 50°C; the range was set at 10 and attenuation usually at $\times 2$.

Retention times of authentic methyl esters were used as evidence that the expected fragments had been formed. Mass spectra were obtained on the peaks to verify identification. The LKB-9000 gas chromatograph-mass spectrometer was used. It was operated at an ionizing energy of 70 eV, accelerating voltage of 3.5 kV, and an ion source temperature of 290°C. Helium was the carrier gas and was supplied at a head pressure of 40 psi.

Peak areas were obtained by integration using an Infrotronics CRS-1 chromatographic readout system.

RESULTS AND DISCUSSION

Table 1 lists the compounds subjected to the oxidation procedure and the fragments identified. The extent of allylic oxidation products expressed

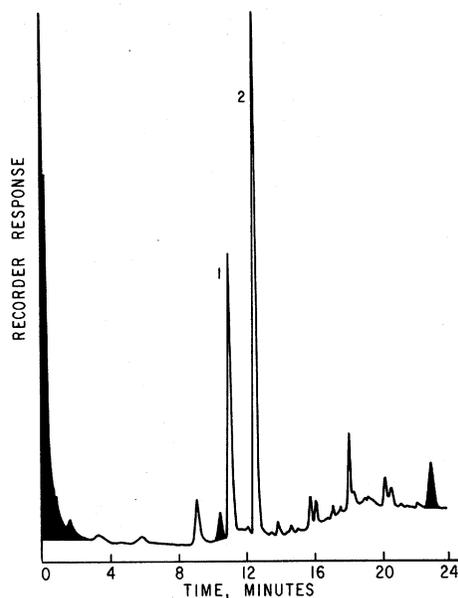


FIG. 2. Gas-liquid chromatogram of methyl esters of fragments from oxidation of 3 μg of methyl-*trans*-octadec-7-enoate on a CrO_3 -Celite column eluted with CS_2 . For conditions, see text. Identification of peaks: (1) methyl decanoate and (2) methyl undecanoate. Shaded areas are present in control and/or solvent blank.

as percentage of the double bond oxidation products is given in parentheses. The figures in the first set represent the range found when the procedure was repeated four or more times and, in the second set, their average. As these figures appear to be characteristic for each position, they can serve as a guide in selecting the pair of peaks which have arisen from oxidation of the double bond and the allylic carbon atoms in the event that peaks due to impurities appear on the chromatogram. A control gas chromatographic analysis should always be run on the preparation containing the unsaturated compound to be oxidized, and a blank should be carried through the entire procedure on the solvents if any doubt exists as to the true position of the double bond.

All substrates gave the expected monocarboxylic acid except those with double bonds at positions 15, 16, and 17. In these, the expected products (propionic, acetic, and formic acids, respectively) could not be isolated from the oxidation column. Failure to recover these acids may not have been due solely to an unfavorable partition ratio because propionic acid carried through the procedure was partially recovered. More polar solvents (CH_2Cl_2 , isopropyl ether) also failed to recover propionic acid resulting from double bond oxidation or allylic oxidation.

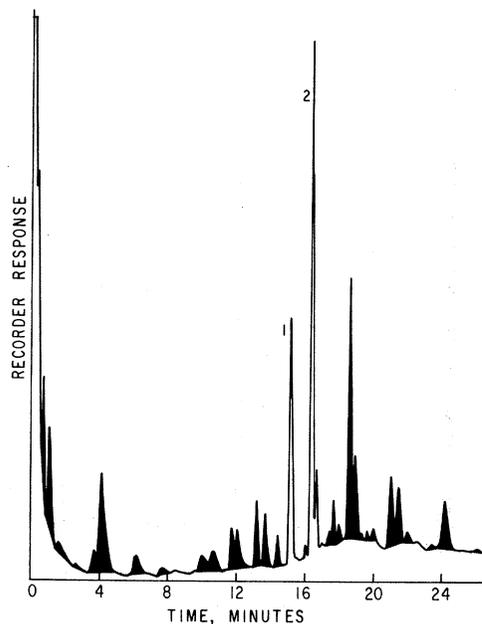


FIG. 3. Gas-liquid chromatogram of methyl esters of fragments from oxidation of 3 μg of methyl-*trans*-octadec-7-enoate on a CrO_3 -Celite column eluted with isopropyl ether following CS_2 elution (see Fig. 2). For conditions, see text. Identification of peaks: (1) dimethyl hexanedioate and (2) dimethyl heptanedioate. Shaded areas are present in control and/or solvent blank.

All substrates also gave the expected dicarboxylic acid monomethyl ester with the exception of the $\Delta 3$ isomer. The expected product from the $\Delta 3$ isomer and the $\Delta 4$ allylic oxidation product, monomethyl malonate, was not found in the isopropyl ether extract. Solvents more polar than isopropyl ether were tried as eluants but were found to elute too much chromic acid or were unsatisfactory in other ways. These included diethyl ether, nitromethane, ethyl acetate, and 2-heptanone.

Figure 1 is a reproduction of the chromatogram obtained from the oxidation of 2.5 μg of methyl-*cis*-octadec-9-enoate. All four fragments are essentially completely eluted with CS_2 , making elution of the oxidation column with isopropyl ether unnecessary. Actually, all four fragments will appear on the CS_2 chromatogram only when positions 9 through 13 are oxidized.

Figures 2 and 3 show the results of oxidizing 3 μg of methyl-*trans*-octadec-7-enoate. The CS_2 eluate (Fig. 2) contains only the expected monocarboxylic acid methyl esters and no dicarboxylic acid dimethyl esters. The latter appear in the isopropyl ether extract (Fig. 3).

Although other solvents (CH_2Cl_2 , petroleum ether, benzene, and CCl_4) were successfully tried in the oxidation step, they were not given serious consideration as a substitute for CS_2 because of this solvent's superior

gas chromatographic qualities for flame ionization detection, especially in the analysis of substrates giving rise to the shorter-chain acids. One may, of course, run the reaction and elute the column with isopropyl ether, thereby obtaining the monocarboxylic acids and the monomethyl esters of the dicarboxylic acids together. However, even though this would save one elution step, a methylation step, and a gas chromatographic run, it is not recommended because of the possibility of losing the shorter-chain acids by volatilization from the capillary while evaporating the isopropyl ether and also because of the possibility of overlap of the peaks during glc. Overlap would occur under the conditions used in this study with methyl laurate and dimethyl glutarate, double bond scission, and allylic oxidation products, respectively, from the $\Delta 6$ isomer. Oxidation products from the $\Delta 5$ isomer would also be very close on the chromatogram so that some doubt would exist as to the true position of the double bond.

The *cis* $\Delta 2$ and the *cis-trans* $\Delta 2$ mixture did not oxidize at all, even when reaction time was extended to 1 hr. This is probably due to the electron-withdrawing ability of the ester carbonyl in conjugation with the double bond. To test this, methyl-*cis*-octadec-2-enoate was reduced to the corresponding alcohol and the hydroxyl group was acetylated. The resulting acetate, upon oxidation in the standard manner, gave the expected product (C_{16} monocarboxylic acid) and the allylic oxidation product (C_{15} monocarboxylic acid (20% of scission product)). One would thus expect double bonds conjugated with an oxo or an oxidizable hydroxyl group to be resistant to oxidation under the specified conditions.

It was noted that there were differences in the time needed for complete oxidation of the double bond. Positions 3 through 11 were completely oxidized within 5 min. Positions 12 through 16 were completely oxidized within 15 min. Position 17, the terminal position, was oxidized to the extent of 85–90% within 15 min but was still not completely cleaved in 1 hr. An incubation time of 15 min was therefore considered suitable.

The percentage of allylic oxidation product generated in the oxidation of the $\Delta 17$ isomer was quite variable (Table 1) and it was observed that, if incubation periods shorter than 15 min were used, the allylic oxidation product would exceed the scission product, and in some cases (5 min incubation) it exceeded 200%.

Although the extent of allylic oxidation of the other isomers was less variable and also of a lesser magnitude, it was still a formidable amount. Allylic oxidation by chromic acid appears to be a relatively minor reaction with straight chain alkenes when carried out in classical solvent systems (4), and Hallgren and Larsson (2) reported that the allylic oxidation product from the chromic acid oxidation of a docosenyl glyceryl ether was only about 17% of the double bond oxidation product. Double bond location using a permanganate–periodate system resulted in only about 1% allylic oxidation (5).

The method was applied successfully to the location of both double

bonds in methyl linoleate and methyl linelaidate. Both compounds (3–4 μg) gave the expected scission and allylic oxidation products, i.e., hexanoic and pentanoic acids from the $\Delta 12$ bond and monomethyl azelate and monomethyl suberate from the $\Delta 9$ bond.

Oxidation of 3 μg of methyl linolenate resulted in the location of the bond at $\Delta 9$ only. As expected from the results obtained with the monoenoic methyl esters, the propionic acid produced from scission of the $\Delta 15$ position could not be isolated from the column.

Several olefins were also investigated. The double bond in from 1 to 3 μg of 9-octadecene, 1-hexadecene, and 2-, 3-, and 4-decene yielded the expected monocarboxylic acid scission and allylic oxidation products in ratios similar to those obtained with the identical position in the methyl octadecenoate.

The most popular methods for locating double bonds are ozonolysis and permanganate–periodate oxidation, although several other approaches are available (6–9). The author is not aware of any method which has been applied directly to submicrogram amounts of substrate, although several would seem to have the potential to do so (10–13). The need for sensitive procedures that can be readily utilized in any laboratory has become increasingly apparent as improved techniques for isolating trace constituents from natural products become available. The method described in this report fulfills these aspects, and, although the study has been limited to a few classes, it should be possible to extend it to other classes either as described or with appropriate modifications.

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