Mass Spectrometry of Perdeuterated Molecules of Biological Origin. Fatty Acid Esters from *Scenedesmus obliquus**

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ABSTRACT: To define the role of mass spectrometry for dealing with structural problems of highly deuterated natural products, the gas chromatographic and mass spectral properties of perdeuterated fatty acid esters from *Scenedesmus obliquus* which had been grown in D_2O have been studied. Of the 20 esters which were characterized, deuterium distribution was essentially random, and ranged from 96.9 to 98.6% of maximum possible incorporation. Structures of unsaturated esters were determined after conversion into trimethylsilyloxy or *O*-isopropylidene derivatives. The previously established ability to distinguish cis and trans isomers of monoenes using the latter derivative was not changed by perdeuteration. Deuterated molecules showed markedly shorter retention times than their protium counterparts. Using combination gas chromatography–mass spectrometry and multiple-ion detection, the $C_{21}HD_{41}O_2$ and $C_{21}D_{42}O_2$ forms of deuterated methyl 3,7,11,15-tetramethylhexadecanoate were resolved on a 9-ft packed column and shown to differ in retention time by 2 sec. Using the latter compound as a model the effects of scanning the mass spectrum on the side of the unresolved chromatographic peak were studied, and were found to have little effect on the accurate determination of deuterium content. In the high-resolution mass spectra of perdeuterated esters the most important doublet is CD_2 *vs.* O, which differ by 33 millimass units, and can be resolved at $R < 10{,}000$. Principal modification required for conventional computer programs used for high-resolution mass spectrometry was partial deletion of the high fractional mass test for doubly charged ions, due to the high positive mass defect of ions containing large numbers of deuterium atoms.

The successful cultivation of algae in D_2O (Crespi *et al.*, 1959; Chorney *et al.*, 1960) has resulted in recent years in the availability of a variety of highly deuterated natural products. These compounds are uniquely well suited for studies of biosynthetic pathways, intermediary metabolism, and enzyme structure and mechanism (for instance: Thompson, 1963; Saur *et al.*, 1968; Rokop *et al.*, 1969). As the scope of biological experiments involving highly deuterated substrates increases, greater demands will develop for chemical and physical techniques to deal with structural problems involving such compounds. We have therefore sought to define and develop the role of mass spectrometry in structural problems relating to perdeuterated compounds of biological origin, using the fatty acids from *Scenedesmus obliquus* as a model system. These compounds are a particularly useful example since the mass spectra of their protium analogs have been investigated in detail (Ryhage and Stenhagen, 1963; McCloskey, 1970), allowing a clear assessment of the effects of extensive deuterium substitution on their mass spectra.

Principal uses of mass spectrometry which we shall describe are in the structure determination of perdeuterated compounds, and for the accurate determination of deuterium content and distribution, including the molar percentage of each labeled species. Specific location of residual hydrogen can, in principle, be made, depending on knowledge and occurrence of structurally suitable fragment ions. In the present study we have characterized 20 perdeuterated fatty acids as their methyl-d_3 esters by direct combination gas chromatography–mass spectrometry. A preliminary report on some of the gas chromatographic and mass spectral properties of perdeuterated fatty acid esters has appeared earlier (McCloskey *et al.*, 1967). In addition, Dinh-Nguyên and Stenhagen (1966) have published the mass spectrum of chemically synthesized methyl perdeuteriodocosanoate.

The use of high-resolution mass spectrometry is facilitated by the high positive mass defect of ions containing large numbers of deuterium atoms. Existing computer programs for calculations and handling of high-resolution data can be used with relatively minor changes, *e.g.*, in the algorithm for distinguishing doubly charged ions from singly charged species with high mass defects.

Materials and Methods

Partial Isolation of Perdeuterated Fatty Acid Esters. A crude mixture of fatty acids was obtained from Merck, Sharp & Dohme of Canada, Ltd. The acids had been previously isolated from *Scenedesmus obliquus* grown in 99.8% D_2O (Williams *et al.*, 1966). As part of the original isolation, the lipid fraction had been saponified in CH_3OH–0.5 N NaOH (9:1) for 1–2 hr. Final purification was obtained by the Merck group by esterification, vacuum distillation, and resaponification by refluxing in methanol–NaOH (A. J. Williams, personal communication, Sept 1968).

Mass spectra of components of the crude mixture showed the presence of some methyl esters, which were removed by resaponification of 200 mg of the crude mixture in CH_3OH–0.5 N NaOH (9:1). The free fatty acids were converted into

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the acid chlorides by thionyl chloride, and esterified by methanol-d_4 in pyridine.

A portion of the mixture of perdeuterated esters was fractionated by preparative thin-layer chromatography using silica gel impregnated with 5% $AgNO_3$, and petroleum ether (30–60°)–diethyl ether (4:1) as developing agent. Six bands were obtained (numbered in increasing order from the origin), and the principal components were identified by their mass spectra: band 1, 7.4 mg, 18:3; 2, 4.2 mg, 18:3; 3, 81.2 mg, 16:2, 18:2; 4, 6.0 mg, 16:1, 18:1, 18:2; 5, 16.7 mg, 16:1, 18:1; 6, 26.4 mg, 16:0, 18:0. Each fraction was further separated by preparative gas–liquid chromatography (6 ft. × 4 mm glass column, 15% EGS liquid phase), allowing complete isolation of the major components, and preparation of subfractions containing minor components for analysis by gas chromatography–mass spectrometry.

Preparation of O-Isopropylidene and Trimethylsilyloxy Derivatives of Unsaturated Esters. Stereospecific oxidation of unsaturated esters to the corresponding diols or tetraols was carried out by OsO_4 using the procedure of Wolff *et al.* (1966), with the exception that the osmate ester was reduced by a suspension of Na_2SO_3 in dioxane rather than in methanol to avoid partial transesterification of the methyl-d_3 esters. Further condensation with acetone produced the *O*-isopropylidene derivative (McCloskey and McClelland, 1965). Alternatively, solution of the diol or tetraol in pyridine followed by reaction with bis(trimethylsilyl)acetamide–trimethylchlorosilane (100:1) yielded either the di- (Capella and Zorzut, 1968) or tetratrimethylsilyl derivative (A. J. Polito, J. Naworal, and C. C. Sweeley, private communication, July 1969[1]). In the case of both types of derivatives, samples were introduced into the mass spectrometer by direct injection of the reaction mixture into the gas chromatograph coupled to the mass spectrometer.

Calculation of Deuterium Content. Total percentage of deuteration and the per cent molar distribution of each isotopically labeled molecular species was calculated from molecular ion abundances, or from suitable fragment ions in certain cases. A computer program (DCALC)[2] was written and used for routine calculation of these values, following essentially the same procedure used for manual calculations. Data (input variables) required for the calculation are: (a) number of replaceable hydrogens in the molecule, (b) number of isotope peaks measured, excluding the ^{13}C isotope peak of the fully deuterated molecular ion, (c) number of carbon atoms in the ion, (d) mass of the fully deuterated molecule, whether or not actually present in the mass spectrum, and (e) masses and relative intensities of the deuterium-containing isotope species of the ion chosen for measurement. No provisions are made for the presence of two atoms of ^{13}C in any given ion or of ^{17}O or ^{18}O, since their low abundance has little effect on the final values obtained. This method of calculation does not assume a statistically random distribution of isotopic species, unlike more common methods of calculation (Beynon, 1960).

Steps in the calculation procedure are outlined as follows,

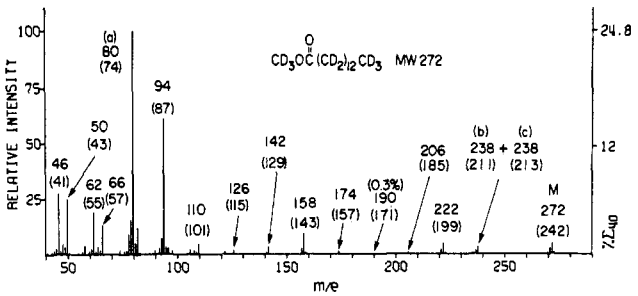


FIGURE 1: Mass spectrum of methyl tetradecanoate-d_{30} (I). Numbers in parentheses refer to mass values of the corresponding ions from methyl tetradecanoate.

using data from the molecular ion of methyl tetradecanoate-d_{30} (Figure 1) as an example. Input data as listed above, are: (a) 30 replaceable hydrogens, (b) 3 peaks measured, (c) 15 carbon atoms, (d) 272, and (e) *m/e* 270, 0.70%; *m/e* 271, 2.60%; *m/e* 272, 4.70%.

(1) Relative abundance values are corrected for contributions from ^{13}C isotope peaks associated with the ion of next lower mass (see Biemann, 1962). For a C_{15} ion the correction will be 15 × 1.09 (natural abundance of ^{13}C) = 16.4% of the abundance of the lower mass ion. Corrected values in the present example then become: *m/e* 270, 0.7% (*i.e.*, no correction); *m/e* 271, 2.49%; *m/e* 272, 4.29%.

(2) Using corrected abundance values, each isotopic species is expressed as a molar percentage of the total (100%). When calculated by computer, isotopic compositions are assigned to each value and are listed: 9.4% H_2D_{28}, 33.2% HD_{29}, 57.4% D_{30}.

(3) The percentage of H relative to the total number of hydrogen isotopes in each ion is calculated: 6.67% in $C_{15}H_2D_{28}$, 3.33% in $C_{15}HD_{29}$, and 0% in $C_{15}D_{30}$.

(4) The percentage of H in each ion relative to the total hydrogen isotopes in the molecule is obtained by multiplying the above values by the appropriate mole fraction of each isotopic species: 6.67 × 0.094 = 0.62% H from $C_{15}H_2D_{28}$; 3.33 × 0.332 = 1.11% H from $C_{15}HD_{29}$; 0 × 0.574 = 0% H from $C_{15}D_{30}$.

(5) The sum of hydrogen contents, 1.73%, gives the total extent of deuteration, 98.3%.

Mass Spectrometry. Low-resolution mass spectra were recorded on an LKB 9000 gas chromatograph–mass spectrometer, using either 1% SE-30 (0.25 in. × 9 ft) or 1% OV-17 (0.25 in. × 6 ft) stationary phases; ion source and carrier gas separators, 250°; ionizing electron energy 70 eV. Spectra were recorded in 4–6 sec on the apex of the gas chromatographic peak.

High-resolution mass spectra of methyl hexadecanoate-d_{34} (III) and methyl 3,7,11,15-tetramethylhexadecanoate-d_{42}(IX) were obtained on a CEC 21-110B instrument, using photographic recording, and exact mass measurement of the entire mass spectra. The samples were introduced by an unheated direct inlet probe into the ion source maintained at 90° (compound III) or 210° (IX). The ionizing electron energy was 70 eV.

Results and Discussion

Gas–liquid chromatography, which normally plays a major role in the characterization of fatty acid esters, must

[1] We are indebted to Professor Sweeley for making available to us the details of his procedure and mass spectrum of the tetra(trimethylsilyloxy) derivative of methyl linoleate.

[2] A copy of this program, written in FORTRAN IV, is available on request from the authors.

TABLE I: Perdeuterated Fatty Acids Isolated as Methyl-d_3 Esters from *Scenedesmus obliquus*.

Compound	Approximate % of Total Fatty Acid Content	Per Cent of Maximum Deuteration[a]	Distribution of Deuterium Label (Mole Per Cent)	Mass (Relative Abundance)[b] of Diagnostically Important Ions[c]
Methyl tetradecanoate-d_{30} (I)	0.4	98.3 (97.8)	9.4% H_2D_{28}; 33.2% HD_{29}; 57.4% D_{30}	Figure 1
Methyl pentadecanoate-d_{32} (II)	0.2	98.2	3.5% H_3D_{29}; 9.1% H_2D_{30}; 29.8% HD_{31}; 57.6% D_{32}	80a(100), 94(63), 158-(7.5), 238(3.3), 254-b,c(3.0), 288M(9.0)
Methyl hexadecanoate-d_{34} (III)	18.0	98.6	12.0% H_2D_{32}; 25.4% HD_{33}; 62.6% D_{34}	80a(100), 94(78), 158(12.0), 254(4.0), 270b,c(2.4), 304M(5.6)
Methyl heptadecanoate-d_{36} (IV)	0.2	98.0 (97.7)	4.8% H_3D_{33}; 13.5% H_2D_{34}; 34.8% HD_{35}; 46.9% D_{36}	80a(100), 94(63.8), 158(8.1), 270(3.2), 286b,c(2.3), 320M(8.9)
Methyl octadecanoate-d_{38} (V)	0.2	98.4	13.2% H_2D_{36}; 35.6% HD_{37}; 51.2% D_{38}	80a(100), 94(67), 158(11), 286(4.0), 302b,c(2.4), 336M(8.4)
Methyl 12-methyltetradecanoate-d_{32} (VI)	0.2	98.1	2.3% H_3D_{29}; 9.6% H_2D_{30}; 33.5% HD_{31}; 54.6% D_{32}	Figure 3
Methyl 5,9-dimethylpentadecanoate-d_{36} (VII)	0.2	95.6 (96.0)	6.0% H_4D_{32}; 18.7% H_3D_{33}; 27.5% H_2D_{34}; 24.0% HD_{35}; 23.8% D_{36}	Figure 4
Methyl 14-methylhexadecanoate-d_{36} (VIII)	0.2	98.1	3.1% H_3D_{33}; 11.9% H_2D_{34}; 34.2% HD_{35}; 50.7% D_{36}	80a(100), 94(66), 158-(9.1), 230h(0.9), 250g(0.6), 254f(0.8), 256(0.5), 258(0.5), 286b,c,e(2.1), 302d-(0.3), 320M(11)
Methyl 3,7,11,15-tetramethylhexadecanoate-d_{42} (IX)	0.2	96.9 (95.4)	3.8% H_4D_{38}; 10.4% H_3D_{39}; 25.3% H_2D_{40}; 34.4% HD_{41}; 26.1% D_{42}	Figure 5
Methyl tetradecenoate-d_{28} (X)	0.2	98.1	13.1% H_2D_{26}; 27.8% HD_{27}; 59.1% D_{28}	80a(77), 94(40), 140k-(7.7), 188j(13), 232i-(8.3), 234b(4.3), 268M(3.4)
Methyl tetradecenoate-d_{28} (branched) (XI)	0.2	97.9	2.1% H_3D_{25}; 9.7% H_2D_{26}; 32.9% HD_{27}; 55.3% D_{28}	46(100), 80a(22), 94-(16), 188j(5.1), 232i-(5.0), 234b(5.1), 268M(17)
Methyl pentadecenoate-d_{30} (XII)	0.2	98.1	10.8% H_2D_{28}; 34.8% HD_{29}; 54.4% D_{30}	46(100), 80a(62), 94-(45), 204j(6.6), 248i-(9.0), 250b(4.8), 284M(2.8)
Methyl pentadecenoate-d_{30} (branched) (XIII)	0.2	97.9	2.3% H_3D_{27}; 11.2% H_2D_{28}; 33.6% HD_{29}; 52.9% D_{30}	46(100), 80a(27), 94-(20), 204j(2.6), 248i-(4.5), 250b(5.6), 284M(15.2)
Methyl *cis*-9-hexadecenoate-d_{32} (XIV)	4.5	98.2	9.9% H_2D_{30}; 39.1% HD_{31}; 51.0% D_{32}	80a(77), 94(66), 172k-(6.7), 220j(9.9), 264i-(19), 266b(8.4), 300M(4.7)
Methyl heptadecenoate-d_{34} (XV)	0.2	98.3	12.4% H_2D_{32}; 34.8% HD_{33}; 52.8% D_{34}	80a(74), 94(67), 188k-(4.3), 236j(6.2), 280i-(14), 282b(5.6), 316M(2.4)

TABLE I (*Continued*)

Compound	Approximate % of Total Fatty Acid Content	Per Cent of Maximum Deuteration[a]	Distribution of Deuterium Label (Mole Per Cent)	Mass (Relative Abundance)[b] of Diagnostically Important Ions[c]
Methyl heptadecenoate-d_{34} (branched) (XVI)	0.2	98.2	13.0% H_2D_{32}; 33.9% HD_{33}; 53.1% D_{34}	80a(51), 94(48), 188k-(2.8), 236j(3.8), 280i-(9.5), 282b(3.4), 316M(2.3)
Methyl *cis*-9-octadecenoate-d_{36} (XVII)	13.0	98.3	12.6% H_2D_{34}; 36.4% HD_{35}; 51.0% D_{36}	Figure 7
Methyl hexadecadienoate-d_{30} (XVIII)	0.2	98.1	10.0% H_2D_{28}; 36.4% HD_{29}; 53.6% D_{30}	74(100), 80a(10), 90-(62), 106(39), 216j-(2.5), 260i(2.6), 262b-(2.5), 296M(6.0)
Methyl 9,12-octadecadienoate-d_{34} (XIX)	58.0	98.1	2.1% H_3D_{31}; 10.9% H_2D_{32}; 35.6% HD_{33}; 51.4% D_{34}	Figure 10
Methyl octadecatrienoate-d_{32} (XX)	0.2	98.2	11.4% H_2D_{30}; 34.7% HD_{31}; 53.9% D_{32}	Figure 12

[a] Values in parentheses refer to the calculated deuterium content of *m/e* 80, without correction for unresolved hydrocarbon ions. [b] Relative abundance values represent perdeuterated ion species, and do not include H-containing peaks. [c] Letters refer to ion types designated in the text.

be used with caution if the molecule is extensively deuterated since retention times shorten with increasing deuteration. Thus methyl palmitate-d_{34} precedes and completely separates from its protium analog on short-packed columns (McCloskey *et al.*, 1967). Retention time characteristics of a number of perdeuterated esters isolated in the present study are compared with protium esters in Figure 2. The usual linear relationship between logarithm of retention time and number of carbon atoms is obtained, indicating that a self-consistent set of retention data for a homologous series in the labeled compounds can be established if a sufficient number of known points are available. The nearly parallel relation between the protium and deuterium curves in Figure 2 implies that the retention times of members of a homologous series of labeled esters can be predicted by measurement of the retention time difference between any protium–deuterium pair, *i.e.*, by establishment of a single point on the lower curve. However, similar data obtained from a less polar liquid phase (5 ft, 2% OV-1, 170°) show a definite convergence of the two lines, which when extrapolated cross at approximately C_{10}. These data indicate that the degree of analogy in gas chromatographic behavior that can be drawn between protium and deuterium forms of a molecule depends strongly on the nature of the liquid phase employed. As an integral part of the mass spectrometer, the gas chromatograph serves to provide valuable information concerning the identity of homologs that is highly complimentary to data produced from the mass spectra, although far less useful for the unambiguous determination of structure. In the present study primary reliance has therefore been placed upon mass spectrometry for structural characterization, and on gas chromatography (using the total ion current chromatogram) for separation of components and identification of suspected homologs and branched chain components.

Measurement of Deuterium Content. Essentially three types of information can generally be provided by the mass spectrum of an isotopically labeled molecule: (i) the total isotopic

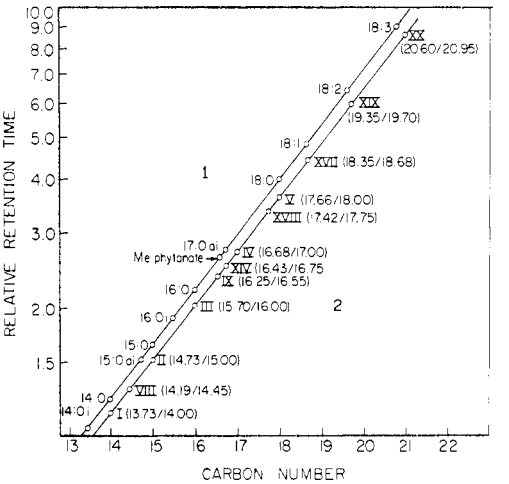


FIGURE 2: Comparison of gas chromatographic behavior of normal (line 1) and perdeuterated fatty acid esters (line 2). Glass column 6 ft × 4 mm; 15% EGS on Diatoport S, 80–100 mesh; temperature, 170°, 40 cm³ of N_2/min. Roman numerals refer to compounds listed in Table I. Values in parentheses give the carbon numbers of the perdeuterated ester on the protium ester scale, followed by the deuterium ester scale. Carbon numbers are equal to the number of carbon atoms in the parent acid, and were determined relative to methyl stearate as internal standard.

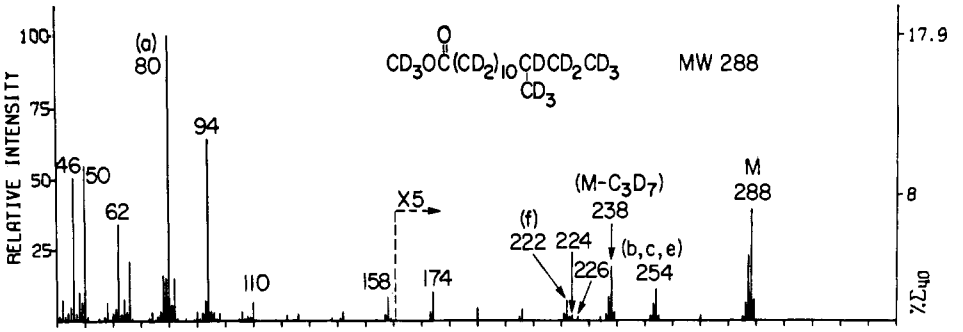


FIGURE 3: Mass spectrum of methyl 12-methyltetradecanoate-d_{32} (VI).

content of the molecule, usually expressed as a percentage of the maximum possible content; (ii) the molar percentage of each labeled species; (iii) the distribution or exact location of the labels. As the number of deuterium atoms in an ion increases, the ^{1}H-isotope peaks M − 1 and M − 2 represent a smaller fraction of the total hydrogen content of the ion. Measurement of the ratio M:M − 1:M − 2..., therefore, becomes less sensitive and more accurate in the calculation of isotopic content. For example, a change of 2% in the ratio M:M − 1 (~1:1) in methyl butyrate-d_{10} represents 0.05% change in molecular deuteration, while in the case of methyl tetradecanoate-d_{30} the same change in ratio represents a difference in deuterium content of approximately 0.02%.

A potential source of error in the measurement of deuterium content arises from partial fractionation of labeled species on the gas chromatographic column. Thus if the mass spectrum of perdeuterated methyl tetradecanoate were recorded on the late side of the eluting gas chromatographic peak the ratio of molecular ion abundances, HD_{29}/D_{30}, would be an invalid measure of the extent of deuteration because of slight enrichment of HD_{29} species. As will be evident from later discussions, errors introduced in this way are negligible if mass spectra are recorded on or near the top of the gas chromatographic peak. However if the exact shape of the peak is not evident because of chromatographic overlap from other peaks, slight error may be introduced, probably no more than 0.2% of D content for compounds and conditions used in the present study.

The extent of deuteration and distribution of labeled molecular species for the deuterated esters identified in the present study are listed in Table I.

Mass Spectra of Perdeuterated Fatty Acid Esters. As a consequence of essentially complete deuteration, two important factors influence correct mass identification and artifact recognition in the mass spectrum. First, since the nominal mass of deuterium is 2, *the mass of any perdeuterated ion must be an even number.* This rule pertains to all singly and doubly charged ions containing an odd or even number of electrons, and any number of even-mass atoms (carbon, deuterium, oxygen, nitrogen, or sulfur). The only peaks in the spectrum which occur at odd *m/e* values represent ions containing H, ^{13}C, or other minor isotopes, and must be suitably reduced in relative intensity in accordance with the usual statistical rules which govern their abundances (Beynon, 1960). In addition, recognition of artifacts which do not contain deuterium is facilitated by the characteristic pattern of lower isotope peaks due to small amounts of protium (*e.g.*, *m/e* 222, 272 in Figure 1). Assuming an approximately uniform distribution of the label in the molecule, the isotopic pattern associated with any given ion will closely resemble that of other ions in the same mass region of the spectrum. This fact has been useful in distinguishing ions arising from gas chromatographic column bleed and other impurities which might normally lead to misinterpretation of the spectrum, particularly of a trace component recorded at high sensitivity.

The mass spectrum of an isotopically labeled molecule can in general be predicted from that of the unlabeled molecule, provided the structural origins or elemental compositions of fragment ions are known in sufficient detail. In a perdeuterated molecule, the shift in mass (*i.e.*, mass difference) from a nonlabeled to a labeled ion is simply equivalent to the number of hydrogen isotopes in the ion. Thus, as indicated in Figure 1, the molecular ion of methyl tetradecanoate ($C_{15}H_{30}O_2$) shifts from *m/e* 242 to 272 in the labeled compound reflecting the presence of 30 deuterium atoms. As discussed in the following section, mass spectra obtained directly from partially isolated mixtures of perdeuterated esters have resulted in the characterization of 20 perdeuterated esters.

Normal Chain Esters. The mass spectrum of perdeuterated methyl tetradecanoate (I, Figure 1) is representative of the five saturated normal chain esters which were identified (Table I), and exhibits many ions which occur in the spectra of other long chain esters. The fully labeled molecular ion occurs in *m/e* 272. Although the presence of lower isotope peaks of the molecular ion at *m/e* 271 and 270 may lead to the impression that the molecule is not highly labeled, these protium-containing species (HD_{29}, H_2D_{28}, respectively) actually represent a high percentage of deuterium content, as shown in Table I. Relative enhancement of the molecular ion resulting essentially from the greater strength of C–D *vs.* C–H bonds is characteristic in the mass spectra of perdeuterated compounds. An increase of 36% in the abundance of the molecular ion of methyl palmitate-d_{34} relative to the principal oxygen-containing fragment ions has been previously reported (McCloskey *et al.*, 1967). The general appearance of the remainder of the spectrum is similar to that of the unlabeled compound (Ryhage and Stenhagen, 1959) except for appropriate mass shifts, as indicated in Figure 1. Ions of the upper mass region in Figure 1 are of lower relative abundance than those shown by Ryhage and Stenhagen (1959). These differences are due to the different geometries of the instruments

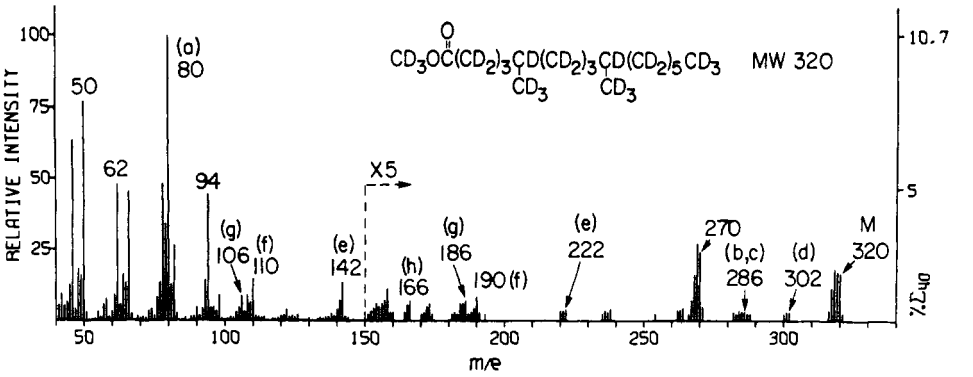
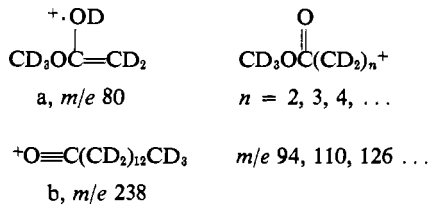


FIGURE 4: Mass spectrum of methyl 5,9-dimethylpentadecanoate-d_{36} (VII).

employed (180 *vs.* 60° deflection) and are not the result of isotopic substitution.

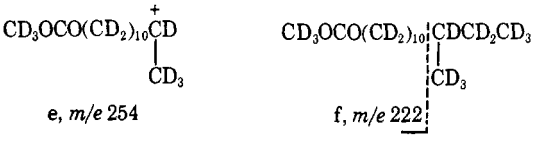
The characteristic *m/e* 74 resulting from hydrogen rearrangement from C-4 to the carbonyl oxygen is observed at *m/e* 80 (ion a). Members of the prominent series $CD_3OCO(CD_2)_n^+$ are likewise observed at mass values calculated from the number of hydrogen isotopes in the ion. The acylium ion arising from loss of a methoxyl-d_3 radical occurs at the same nominal mass (b, *m/e* 238) as the highly rearranged M-C_2D_5



(c) involving elimination of C-2 and -3 (Spiteller *et al.*, 1966). The two ion species are readily distinguished at higher resolving power, as discussed in a later section. Low mass hydrocarbon ions, normally of little diagnostic value, occur at *m/e* 46 ($C_3D_5^+$), 50 ($C_3D_7^+$), 62 ($C_4D_7^+$) and 66 ($C_4D_9^+$).

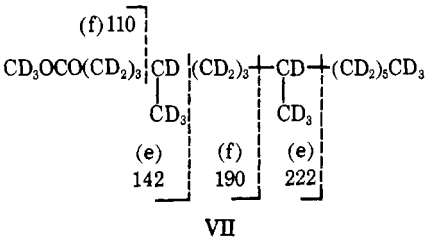
Branched Chain Esters. Detection of methyl branching in a long-chain ester can usually be made by the occurrence of a small but highly significant peak at M-15 (Ryhage and Stenhagen, 1960a), presumably due to loss of a methyl radical from the branch point. In the case of perdeuterated esters this diagnostic ion (d) shifts to M-18 (CD_3), and has served as the principal indicator of branching for compounds VI–IX (Table I). Additional confirmation of chain branching is gained by the shorter gas chromatographic retention times of VI–IX compared with the normal chain esters of the same molecular weight.

Effects of monomethyl alkyl branching are represented by the spectrum of methyl 12-methyltetradecanoate-d_{32}, Figure 3. The position of a methyl branch is normally determined in two ways. The inherently greater stability of a secondary carbonium ion results in preferential formation of ions of type e. However the presence of such ions cannot be regarded as unambiguous evidence, since they are isomeric with members of the $CD_3OCO(CD_2)_n^+$ series. Evidence for structures of type e often rests largely on their higher intensity relative to that in normal chain esters (Ryhage and Stenhagen, 1960a). However, in the present case further contribution to



m/e 254 arises from the acylium ion b, as well as loss of C_2D_5 (c) associated with C-2 and -3 as in the case of compound I. More diagnostic evidence for the location of the branch is given by ion f, which although isomeric with other members of the $CD_3OCO(CD_2)_n^+$ series, also occurs with one and two rearranged deuterium atoms (Ryhage and Stenhagen, 1960a; McCloskey and Law, 1967). The distinctive pattern of three peaks thus produced in the mass spectrum of VI (*m/e* 222, 224, 226) indicates the position of branching to be C-12, the anteiso structure. This interpretation was confirmed by the mass spectrum of a reference sample of the protium form of VI, which gave a virtually identical pattern of ion abundances, appropriately shifted in mass.

A somewhat more complex appearance is observed in the upper mass range of VII, Figure 4. A relatively high protium content (Table I) produces unusually high lower isotope peaks for ions in the upper mass range, although the interpretation of the mass spectrum is not materially affected. The presence of *m/e* 80 and members of the $CD_3OCO(CD_2)_n^+$ series through $n = 3$ (*m/e* 110) shows no modification of structure through C-4. As seen by comparison with Figures 1 and 3 mass 142 shows unusual enhancement, indicating the probability of methyl branching at C-5. The required absence of the $n = 4$ member (*m/e* 126) cannot be confirmed



VII

due to the presence of numerous low peaks in that mass region. In the mass spectra of multiply branched esters (Ryhage and Stenhagen, 1960b) the rearranged ions corresponding to *m/e* 224 and 226 in Figure 3 are usually too low for diagnostic purposes; similar conclusions pertain to the

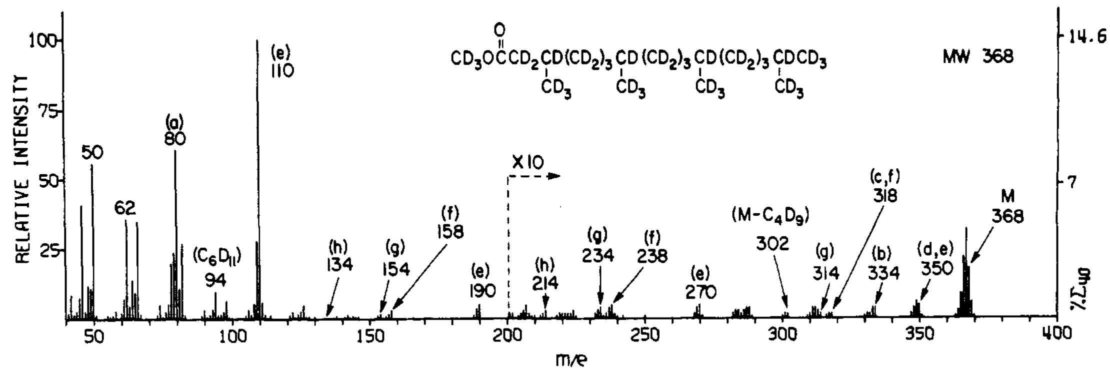
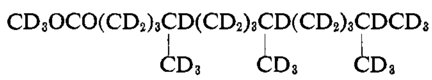


FIGURE 5: Mass spectrum of methyl 3,7,11,15-tetramethylhexadecanoate-d_{42} (IX).

multiply branched perdeuterated esters included in the present study. A small peak (g) at *m/e* 106 corresponds to loss of CD_3OD from *m/e* 142, a process which occurs from secondary (branched) ions but not from other members of the $CD_3OCO(CD_2)_n^+$ series. The complete absence of *m/e* 206 is highly indicative of substitution at C-9, which would be expected to block its formation as a simple cleavage process. Although there is no apparent enhancement of the preferred cleavage leading to e (*m/e* 222), its CD_3OD elimination product g (*m/e* 186) is present, further supporting the structure of VII as methyl 5, 9-dimethylpentadecanoate-d_{36}. Insufficient data are available to determine whether isobranching may be present, a possibility raised by the presence in the same fraction of methyl phytanate (IX, discussed in the following section). The tribranched structure VIIa might be expected



VIIa

to yield a very small peak at *m/e* 246, M − (CD_3 + CD_3OD + D_2O), characteristic of a terminal isopropyl group (Ryhage and Stenhagen, 1960a,b). Although a small peak is present

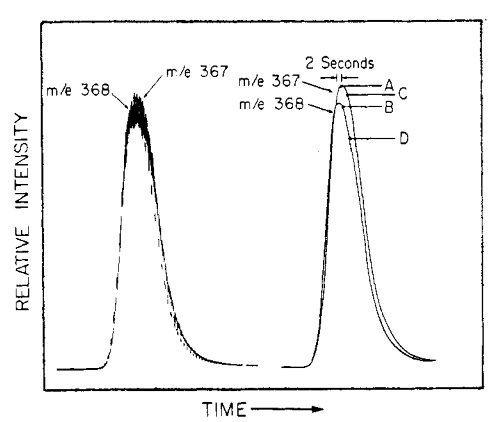
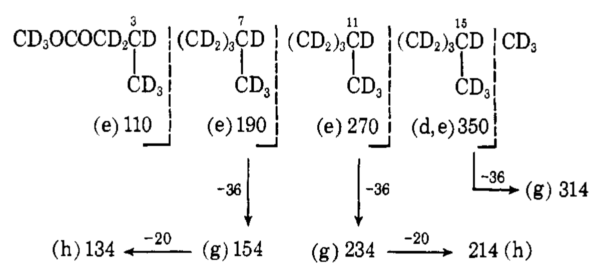


FIGURE 6: Gas chromatographic elution profiles of the perdeuterated ($C_{21}D_{42}O_2$) and monoprotium ($C_{21}HD_{41}O_2$) forms of compound IX, recorded by rapid successive monitoring of *m/e* 368 and 367, respectively. A–B and C–D represent hypothetical points of recording mass spectra on the top and side of the elution profile. Glass column 9 ft × 4 mm, 1% SE-30, 30 cm³ of He/min, temperature 180°; retention time approximately 4.5 min.

(<0.1% relative intensity) above background, lack of total separation of VII from other minor gas chromatographic peaks renders objective judgment impossible.

One of the most interesting minor components examined was compound IX, determined by its mass spectrum (Figure 5) to be the methyl-d_3 ester of a perdeuterated, saturated C_{20} acid. Its chromatographic elution prior to the normal chain C_{17} acid ester IV, as shown in Figure 2, and a peak at *m/e* 350 (ion d) provided a strong indication of extensive branching. Methylation at C-3 was evident from the shift of *m/e* 94 (see Figure 1) largely to *m/e* 110, the remainder being attributed to the $n = 6$ member of the monotonically decreasing $[C_nD_{2n-1}]^+$ series (*m/e* 46, 62, 78...). All members of the $CD_3OCO(CD_2)_n^+$ series through $n = 16$ (*m/e* 318) are present except $n = 7$ (*m/e* 174) and 12 (*m/e* 254), suggesting additional branching on C-7 and -11. These assignments are corroborated by peaks corresponding to the sequential elimination of CD_3OD (g) and D_2O (h) from the secondary branched ions *m/e* 190 and 270



The remaining question as to whether a fourth methyl branch existed was answered with less certainty, as is usually the case with iso- or anteisobranching (Ryhage and Stenhagen, 1960a). The possibility of isobranching is raised by the presence of a small (~0.1%) characteristic peak (Ryhage and Stenhagen, 1960a,b) at *m/e* 294 [M − (CD_3 + CD_3OD + D_2O)], which exhibits a lower isotope peak of the expected intensity. Elimination of CD_3OD from the branched ion *m/e* 350 (d) as represented above probably occurs, but may also be generated from any other of the three probable M − CD_3 ions. The general interpretation of the spectrum as indicated in Figure 5 is supported by the complete high-resolution mass spectrum of IX, including the exact molecular mass (found: 368.5836; calculated for $C_{21}D_{42}O_2$; 368.5821). The structure thus formulated on the basis of its mass spec-

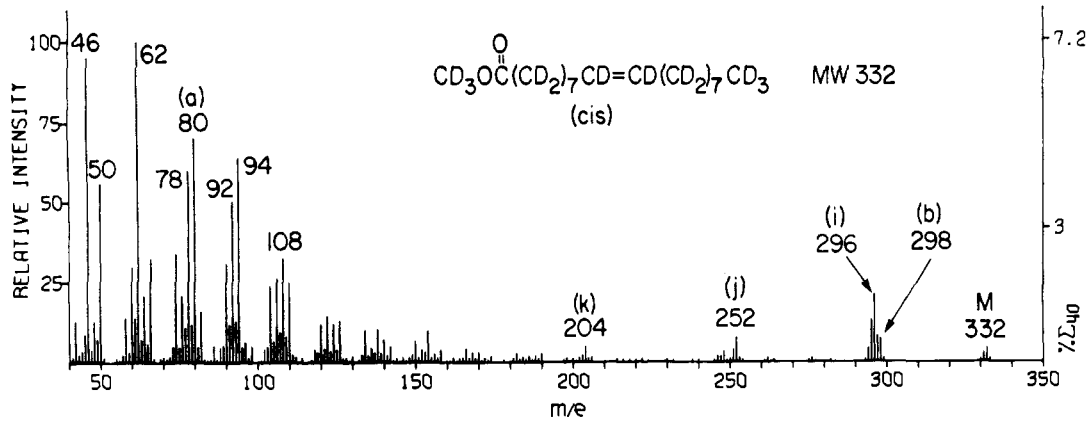


FIGURE 7: Mass spectrum of methyl *cis*-9-octadecenoate-d_{36} (XVII).

trum is methyl 3,7,11,15-tetramethylhexadecanoate-d_{42} (perdeuteriomethyl phytanate). Comparison of the mass spectrum of IX with that of methyl phytanate (Sen Gupta and Peters, 1966) showed the expected similarity of fragmentation pattern, and the existence of the protium forms of the corresponding principal diagnostic ions discussed above.

Examination of Table I shows that the percentage maximum deuterium incorporation values are slightly lower in the branched chain compounds VII and IX. During initial stages of interpretation of their spectra, which exhibited relatively large lower isotope peaks in the upper mass range (*e.g.*, Figures 4, 5), the necessity arose of confirming their identities as protium-containing forms of the molecular ion. This conclusion was verified by rapid, successive monitoring of *m/e* 367 and 368 during elution of a sample of IX (Figure 5) from the gas chromatograph into the mass spectrometer, a technique first reported by Sweeley and coworkers (1966). As seen in Figure 6, slight chromatographic separation of the two elution profiles is observed, indicating that the two ions are associated with different molecules, and are not related by electron impact-induced fragmentation. The perdeuterated component ($C_{21}D_{42}O_2$) elutes approximately 2 sec earlier than its monoprotium counterpart ($C_{21}HD_{41}O_2$), in accordance with the known behavior of deuterium-containing molecules (McCloskey *et al.*, 1967, and references therein; Waller *et al.*, 1969). The effective chromatographic resolution of these two molecules, which differ only by the presence of one hydrogen isotope in a total of 42, offers an excellent example of the use of the multiple ion detector in dealing with separation problems of this type. Similar results were obtained from measurements of the perdeuterio- and monoprotium molecular ions of VII.

Examination of the mass spectra of all compounds obtained from *Scenedesmus obliquus* revealed essentially random distribution of residual protium within each molecule. No fragment ions were found to contain protium in amounts significantly greater than in the molecule as a whole, although it is evident from Table I that some variation exists in the molecular distribution of isotopic species. Ions of type a from compounds I, IV, VII (*m/e* 80), and IX (*m/e* 110) were measured for deuterium content to determine whether slight incorporation of protium might have occurred during saponification. Although a decrease in the accuracy of measurement is expected due to interference from other ion species at *m/e* 78 ($C_5D_9^+$) and 80 ($C_5D_{10}^+$), the protium contents in each case were not significantly different from that of the corresponding molecular ion.

The availability of experimentally obtained elution profiles shown in Figure 6 provides a model from which the effects of chromatographic separation upon calculated deuterium content can be estimated. Since deuterium content values are obtained from ratios of isotope peaks, it is necessary that the relative concentrations of various isotopic species present in the ion source of the mass spectrometer at the time the spectrum is recorded be truly representative of the mixture before chromatography. As shown in Figure 6, errors in measurement of the true ratio of *m/e* 367 to 368 are introduced when the spectrum is recorded on the edge of the profile (C–D) rather than near the apex (A–B). Based on the composition $C_{21}D_{42}O_2$, and neglecting additional lower isotope peaks, total deuterium content was calculated from data corresponding to measurements made at the two positions on the elution profile. The resulting values are: A–B, 98.60%, and C–D, 98.52%. The closeness of these values indicate that for molecules containing a large number of deuterium atoms, gas chromatographic fractionation when using packed columns for sample introduction will not lead to significant errors in measurement of deuterium content. As a corollary, it is evident that in such cases, differences in deuterium content can be measured with high accuracy due to the relative insensitivity of ion abundance ratios to changes in total deuterium content. Likewise, the effect diminishes in molecules containing fewer numbers of deuterium atoms.

Unsaturated Esters and Their Derivatives. Determination of chain length and degree of unsaturation in long-chain esters by mass spectrometry is made primarily through the molecular ion and a characteristic fragmentation pattern in the upper mass region (Hallgren *et al.*, 1959). As shown in Table I, 2 major (XIV, XVII) and 3 minor (X, XII, XV) perdeuterated normal chain monoenes were characterized by their mass spectra. Interpretation of the mass spectra of this series can be represented by methyl oleate-d_{36} (XVII), Figure 7, which represented approximately 13% of the total fatty acid composition of *Scenedesmus obliquus*.

The molecular ion of XVII occurs at *m/e* 332, 4 mass units (D_2) lower than for the corresponding saturated ester V (Table I). Loss of a methoxyl-d_3 radical (ion b) or elimination of CD_3OD (i) is observed as in spectra of the protium counter-

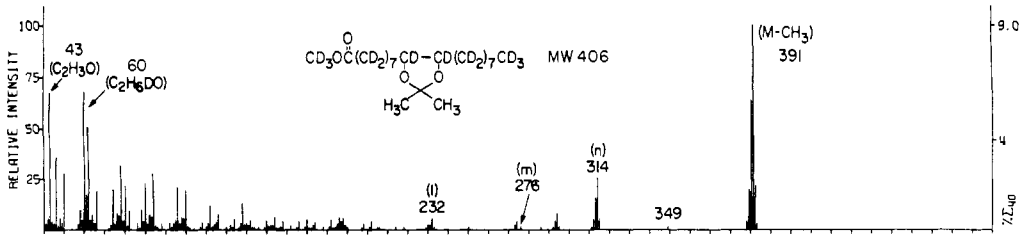


FIGURE 8: Mass spectrum of the *O*-isopropylidene derivative (XVIIb) of methyl *cis*-9-octadecenoate-d_{36}.

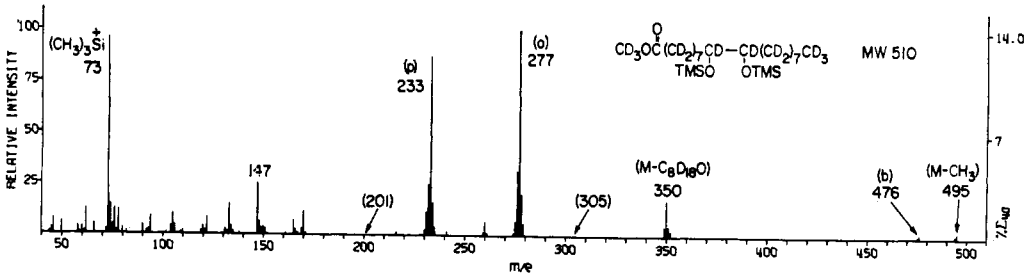
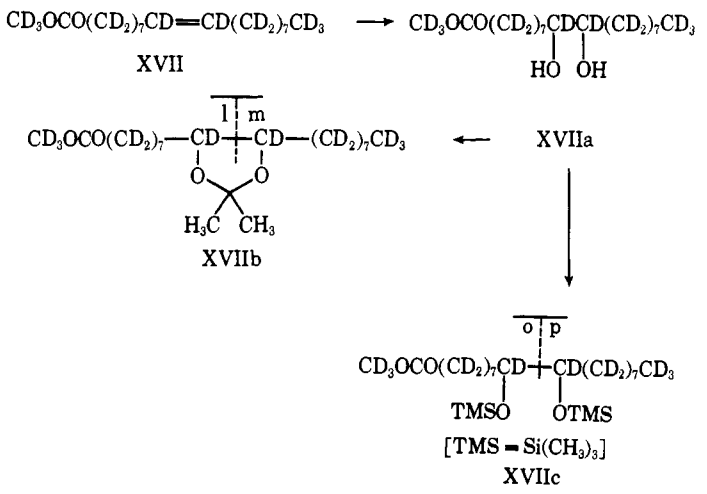


FIGURE 9: Mass spectrum of the ditrimethylsilyloxy derivative (XVIIc) of methyl *cis*-9-octadecenoate-d_{36}.

parts (Hallgren *et al.*, 1959), confirming the identity of *m/e* 332 as the molecular ion of a monoenoic ester. Further indication of unsaturation is provided by ion j (*m/e* 252), which corresponds to the characteristic elimination of $C_3H_6O_2$ from the ester moiety of unsaturated methyl esters by an as yet undetermined mechanism. Of similar importance is ion k, *m/e* 204, arising from elimination of $C_6D_{12}O_2$, in analogy to the behavior of methyl oleate and methyl linoleate. The lower mass range of Figure 7 is highly populated with hydrocarbon ions, principally of the unsaturated series C_nD_{2n-1} (*e.g.*; *m/e* 46, 62, 78). The prominence of these ions apparently results from fragmentation induced by the double bond, and thus competes effectively with the ubiquitous ester ion a (*m/e* 80). Although the preceding interpretation clearly identifies XVII as the ester of a C-18 monoene, mass spectra of the perdeuterated esters resemble their protium counterparts in that double-bond position and geometry cannot be determined directly. These features can be readily ascertained however by conversion into suitable derivatives.



Of the numerous methods which have been proposed (see McCloskey, 1970), the most generally satisfactory procedures involve stereospecific oxidation of the olefinic bond to a vicinal diol (XVII → XVIIa), followed by condensation with acetone, (XVIIa → XVIIb; McCloskey and McClelland, 1965), or conversion into the ditrimethylsilyl ether, XVIIc (Capella and Zorzut, 1968; Argoudelis and Perkins, 1968). In the isopropylidene derivative XVIIb the position of the original double bond can be determined from cleavages β to the ring oxygens, affording ions of type l and m. Geometry of the double bond is retained in the 1,3-dioxolane ring system of the derivative (cis → erythro or trans → threo). Isomers can then be distinguished by the relative abundance of the ion (n) resulting from loss of a dioxolane ring methyl radical, followed by sequential elimination of ketene and methanol (McCloskey and McClelland, 1965). However, since ions l and m are of low abundance, detection of smaller unresolved amounts of other positional isomers is difficult. The trimethylsilyloxy derivative XVIIc is therefore useful since the highly stabilized ions o and p resulting from cleavage of the bond between the two substituted carbons are of high abundance (Capella and Zorzut, 1968; Argoudelius and Perkins, 1968).

The C_{16} and C_{18} monoenes from *Scenedesmus obliquus* were converted into *O*-isopropylidene and trimethylsilyloxy derivatives and subjected to gas chromatography–mass spectrometry. Mass spectra of the derivatives of the principal C_{18} component are shown in Figures 8 and 9, and serve to demonstrate the approach used. Following the previously established interpretations presented for protium derivatives of this type (McCloskey and McClelland, 1965), the masses of ions l and m (232 and 276, respectively) establish the position of the ring and therefore of the double bond as C-9,10.

In the case of protium derivatives, the cis configuration leads to a greater abundance of ion n compared with trans. However, in extrapolating the use of such relationships to

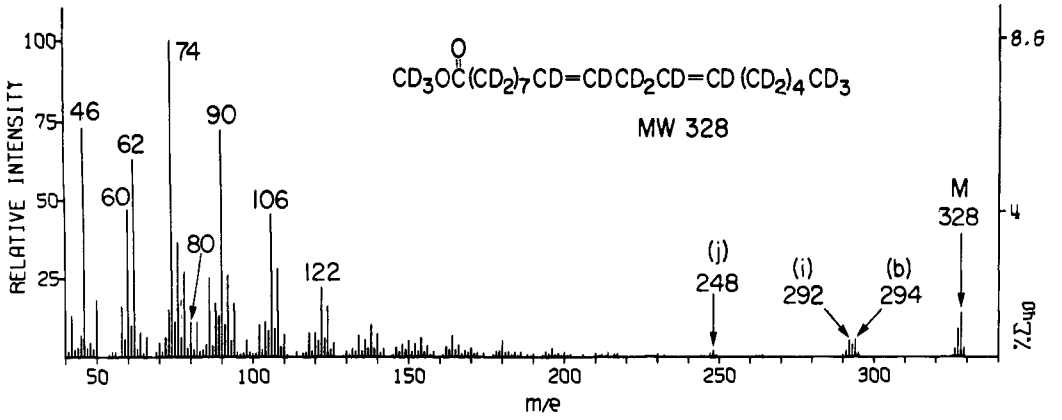


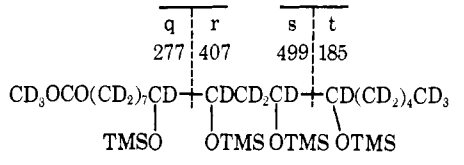
FIGURE 10: Mass spectrum of methyl 9,12-octadecadienoate-d_{34} (XIX).

the deuterated analogs, the effects of isotopic substitution on the abundance of ion n must be considered. Accordingly, the C_{18}-monoene XVII was isomerized by heating with selenium at 200° to a mixture of cis and trans isomers, the *O*-isopropylidene derivatives of which were introduced into the gas chromatograph–mass spectrometer. Following the usual gas chromatographic behavior of *erythro*- and *threo*-*O*-isopropylidene derivatives (Horning and Tanaka, unpublished results, 1964; McCloskey and McClelland, 1965; Wolff *et al.*, 1966), the erythro (cis) isomer showed the shorter retention time, and was identical with that of the principal C_{18} derivative from *Scenedesmus obliquus*. The abundance of ion n, as with the protium analogs, was greater for erythro (3.8% Σ, 0.23 the abundance of M − CH_3, and 3.6 times its daughter ion *m/e* 294), than threo (2.3% Σ, 0.11 of M − CH_3, 2.5 times the abundance of *m/e* 294). The relative intensity of n in the spectrum of XVIIb was 0.25 of M − CH_3 (Figure 8), establishing the structure of the principal C_{18} component XVII as methyl *cis*-octadecenoate-d_{36}.

The mass spectrum of the trimethylsilyloxy derivative XVIIc is shown in Figure 9. The intense peaks o (*m/e* 277) and p (233) clearly mark the position of the original double bond as C-9, 10. Mass values of other major ions in the spectrum are in accordance with the detailed interpretation presented by Capella and Zorzut (1968). Minor ions at *m/e* 305 and 201 correspond to ions o and p for a small amount of a Δ^{11} derivative of unknown configuration.

Based on considerations similar to the foregoing interpretations, XIV was determined to be methyl *cis*-9-hexadecenoate-d_{32}. In addition, mass spectral and gas chromatographic data of derivatives showed trace amounts of methyl *cis*-7-hexadecenoate-d_{32}, methyl *trans*-9-octadecenoate-d_{36}, and methyl 11-octadecenoate as previously mentioned. As shown in Table I, three trace components (XI, XIII, XVI) were identified as being both branched and unsaturated. Their mass spectra were similar to their normal chain unsaturated isomers, reflecting the low competitive influence of alkyl branching compared with unsaturation in the direction of fragmentation processes. Indication of branching was obtained from gas chromatographic retention data, which showed earlier retention times for XI, XIII, and XVI than for their normal chain isomers of the same molecular weight. In addition, from data in Table I it can be seen that competitive influence of alkyl branching produces uniformly lower abundances of the ester-containing ions *m/e* 80 and 94 in the branched esters XI, XIII and XVI, compared with their normal chain isomers (X, XII, XV).

Three multiply unsaturated esters (XVIII, XIX, XX) were isolated from the fatty acids of *Scenedesmus obliquus*, of which perdeuterated methyl 9,12-octadecadienoate (XIX) was the principal constituent. Its mass spectrum, shown in Figure 10, is similar in many respects to that of methyl oleate-d_{36}, and follows the general pattern and interpretation offered by Hallgren *et al.* (1959). The molecular ion appears at *m/e* 328, 4 mass units (D_2) lower than methyl oleate-d_{36}. The acylium ion b from loss of the OCD_3 radical occurs at *m/e* 294, and *m/e* 292 (i) two mass units lower arises from elimination of CD_3OD. Expulsion of $C_3D_6O_2$ (ion j) from the molecular ion follows the analogous characteristic behavior of methyl linoleate. Similar to methyl oleate, the lower mass region contains predominantly hydrocarbon ions. Confirmation of the points of unsaturation as $\Delta^{9,12}$ was made by conversion of the ester to the tetratrimethylsilyloxy derivative (A. J. Polito, J. Naworal, and C. C. Sweeley, private communication, July 1968) XIXa, after oxidation to the corresponding tetraol. The mass spectrum of XIXa, shown in Figure 11, reveals the positions of substitution to be C-9, 10 and 12, 13. Stabilization of the positive charge offered by ether oxygens results in simple cleavage products q and t, while ions r and s which contain three trimethylsilyloxy



functions are also represented through elimination of $(CH_3)_3SiOD$: *m/e* 316 and 408, respectively. The peak at *m/e* 407 in Figure 11 is predominantly the lower isotope peak of *m/e* 408. The molecular ion is not observed, as is common with polysilylether derivatives of long chain compounds (Eglinton *et al.*, 1968; A, J. Polito, J. Naworal, and C. C. Sweeley, private communication, July 1968); however, information afforded by the molecular weight was obtained from the molecular ion of the underivatized compound XIX. The remainder of the spectrum in Figure 11 follows the interpre-

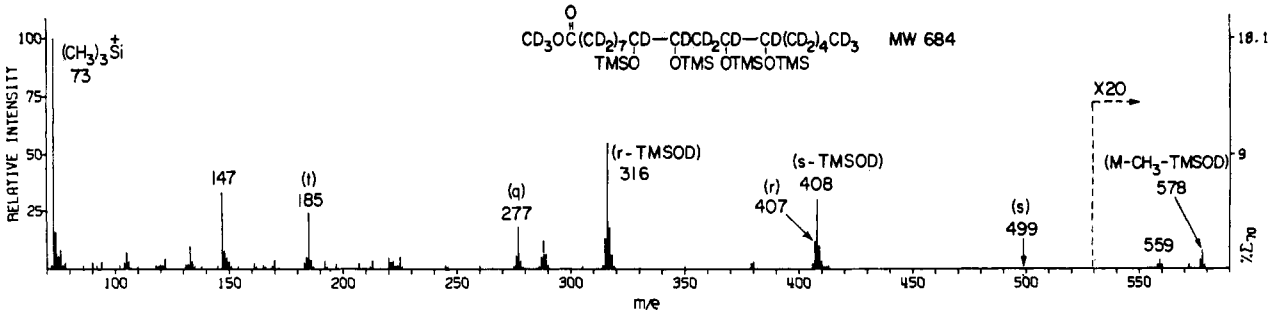


FIGURE 11: Mass spectrum of the tetratrimethylsilyloxy derivative (XIXa) of methyl 9,12-octadecadienoate-d_{34}.

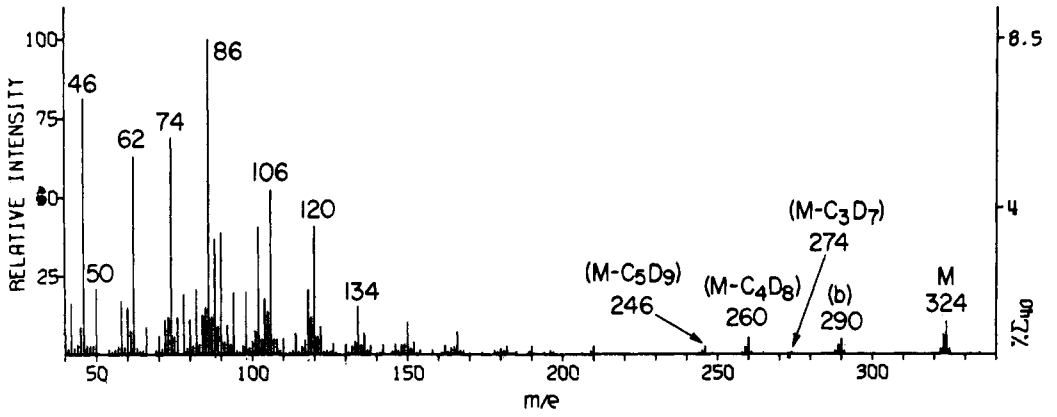


FIGURE 12: Mass spectrum of methyl octadecatrienoate-d_{32} (XX).

tation of the tetrasilyloxy derivative of methyl linoleate (McCloskey, 1970), with appropriate shifts in mass.

The most highly unsaturated ester isolated was the triene XX, the mass spectrum of which is shown in Figure 12. The molecular ion of *m/e* 324 identifies XX as the ester of an 18-carbon acid triene. Losses of OCD_3 (b, *m/e* 290), C_3D_7 (*m/e* 274), C_4D_8 (*m/e* 260), and C_5D_9 (*m/e* 246) are analogous to the behavior of methyl linolenate (Hallgren *et al.*, 1959). Abundant hydrocarbon ions of the lower mass region generally follow the patterns exhibited by other members of the unsaturated series, X–XIX, except for the high intensity of *m/e* 86 ($C_6D_7^+$) which is a characteristic ion type for trienes (Hallgren *et al.*, 1959; Holman and Rahm, 1966). No trace was found of methyl 4,7,10,13-hexadecatetraenoate, the acid of which was reported as isolated from *Scenedesmus obliquus* by Klenk and Knipprath (1959). Its absence in the present case cannot be regarded as significant however, since no effort was made to reproduce their conditions for growth or isolation. The importance of growing conditions on polyunsaturated fatty acid content has been shown for several phytoflagellates and algae by Hulanicka *et al.* (1964).

TABLE II: Selected Mass Doublets Encountered in the High-Resolution Mass Spectra of Highly Deuterated Molecules.

Doublet[a]	Mass Difference (Millimass Units)
H_2–D	1.5
CD–^{13}CH	2.9
CD–N	11.0
C_3–D_2O_2	18.0
ND–O	22.2
CD_2–O	33.3
OD_4–C_2	51.3
D_6–C	84.6

[a] Higher mass member listed first.

High-Resolution Mass Spectrometry of Perdeuterated Compounds. Determination of the elemental composition of a molecule by accurate measurement of its mass is a highly useful aspect of mass spectrometry, particularly when dealing with compounds of unknown structure. In the case of a perdeuterated molecule the basic principles of the technique are unchanged, although the identities of the more commonly encountered doublets, shown in Table II, are different. If residual protium is present in an ion, difficulty will be encountered in distinguishing between H_2 and D, whose mass difference is only 0.0015 mass unit. If the peak in question is a singlet, highly accurate mass measurement may afford a means of selecting the correct composition (Burlingame, 1968) although such distinction is usually of limited importance in the interpretation of a spectrum. Likewise, lower isotope peaks due to protium (*e.g.*, *m/e* 366, 367 from IX, Figure 5) will result in the presence of the doublet CD *vs.* ^{13}CH, the resolution of which is generally not practical.

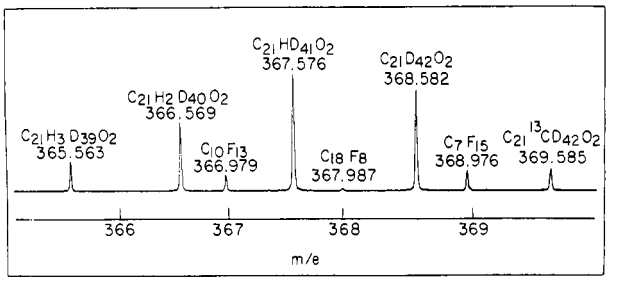


FIGURE 13. Recorded mass spectrum of the molecular ion region of methyl 3,7,11,15-tetramethylhexadecanoate-d_{42} (IX), with fluorocarbon mass standard, at resolution 11,000.

Since deuterium has approximately twice the mass defect of protium (0.014 *vs.* 0.008) ions containing large numbers of deuterium atoms will exhibit unusually high fractional mass values. As an extreme example, Katz and coworkers (Aitzetmüller *et al.*, 1969) have measured the molecular mass of perdeuterated loroxanthin, a xanthophyll pigment of composition $C_{40}D_{56}O$, to be 640.7749. As shown by the recorded partial spectrum in Figure 13, the appearance of the spectrum from this effect may be somewhat confusing, necessitating care in the identification of masses when using fluorocarbons or other conventional mass standards.

Modification of the computer program used in this laboratory for production of complete high-resolution mass spectra required only two minor changes for work with perdeuterated molecules. The mass of the hydrogen isotope was changed from 1.00782 to 2.01410. Since, as shown in Figure 13, fractional mass values approach 0.4–0.5 for ions in the upper mass range, these ions would normally be considered as doubly charged, and therefore listed separately from the element map. Therefore for each high-resolution mass spectrum a nominal mass value is given to the computer with other parameter data, above which the test for doubly charged species (by high fractional mass) does not operate. This value is usually set to slightly over half the molecular weight of the compound involved, and so does not interfere with identification of true doubly charged ions.

The high-resolution mass spectrum of methyl hexadecanoate-d_{34} (III) in the element map format (Biemann *et al.*, 1964) is shown in Figure 14, and reveals many characteristics inherent in the high-resolution spectra of perdeuterated compounds. Ions are listed in increasing mass from top to bottom and increasing oxygen content from left to right. Each entry is read as a carbon/hydrogen value, followed by the error in millimass units between found and theoretical mass values for the composition listed, and a logarithmic indication (*) of abundance. The molecular ion is therefore listed in the lower right corner of the map as $C_{17}D_{34}O_2$, with an error of less than 1 millimass unit. Since the computer was not instructed to consider protium, ions of odd mass do not appear. ^{13}C and H isotope peaks are therefore listed separately. Lower even-mass isotope peaks due to protium cannot be distinguished from the corresponding composition containing deuterium (first doublet of Table II). The entry at *m/e* 302 is therefore listed $C_{17}D_{33}O_2$, although the true composition is $C_{17}H_2D_{32}O_2$. The principal doublet encountered in the map is CD_2–O, which differ by 33 millimass units, and is represented by members of the $CD_3OCO(CD_2)_n^+$ and C_nD_{2n-1}

m/e	CD	CDO	CDO2
50	3/ 7 0*******C	2/ 5 0***	
52	4/ 2 1**		
54	4/ 3 1**		
56	4/ 4 1**		
58	4/ 5 0****	3/ 3-0****	
60	4/ 6-0****	3/ 4-0**	
62	4/ 7-0******	3/ 5-1***	2/ 3-1****
64	4/ 8-1*****	3/ 6-1***	2/ 4-1**
66	4/ 9-1******	3/ 7-1****	2/ 5-1*
70	5/ 5 0**		
72	5/ 6-0**		
74	5/ 7 0****	4/ 5-0**	
76	5/ 8 0***	4/ 6 0**	
78	5/ 9 0*****	4/ 7-0***	3/ 5 1***
80	5/10-0****	4/ 8-0**	3/ 6-1********(a)
82	5/11-0****	4/ 9-0***	3/ 7 0*****
86	6/ 7 0**		
88	6/ 8-0*		
90	6/ 9-0***	5/ 7-0***	
92	6/10 0***	5/ 8 0***	4/ 6 1**
94	6/11 0****	5/ 9-0**	4/ 7 0*******
96	6/12 0***		4/ 8-0***
98	6/13 0***	5/11 0**	
102	7/ 9-0*		
104	7/10-0*	6/ 8-0*	
106	7/11-0***	6/ 9 0***	
108	7/12-1**	6/10-1***	5/ 8-0*
110	7/13-0***	6/11-1*	5/ 9-0****
112	7/14-1**		5/10-1*
114	7/15-1*	6/13-1*	
118	8/11-0*		
120		7/10-0*	
122	8/13 0**	7/11-0**	
124	8/14 0*	7/12-0**	6/10 0*
126	8/15-0**		6/11-0***
128	8/16-0*		6/12 0**
134	9/13 0*		
138	9/15-0*	8/13-1**	
140			7/12 0*
142	9/17-1*		7/13-0****
144			7/14-1**
150	10/15-0*		
154		9/15-0*	
156			8/14 0*
158			8/15-1****
174			9/17-2**
190			10/19-0**
206			11/21-0**
222			12/23-0**
224	14/28-0*		
238			13/25-2*
252			14/26 0*
254			14/27 0***
268		16/30-0**	
270		16/31-0***(b)	15/29-2*(c)
302			17/33 1***
304			17/34-0*****M
	CD	CDO	CDO2

FIGURE 14: High-resolution mass spectrum in the element map format of methyl hexadecanoate-d_{34} (III).

series, occurring at *m/e* 94, 110, 126, and 142. This doublet, which is resolved at resolution less than 10,000, replaces CH_4 *vs.* O (Δ = 0.036 mass unit) as most common in fatty acid ester spectra.

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Occurrence of 2-Aminoethylphosphonic Acid in Human Brain*

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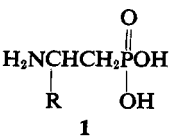
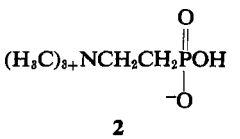
ABSTRACT: The occurrence of 2-aminoethylphosphonic acid (AEP) in acid hydrolysates of nonpolar lipid (hexane soluble) and proteinaceous residue (hexane–methanol insoluble) fractions of human brain tissue has been unambiguously established. This was accomplished using a new, highly sensitive technique for analysis of AEP in biological materials. This method involves acylation and esterification of hydrolysates of tissue extracts, detection and isolation by gas–liquid chromatography, and unambiguous characterization by mass spectrometry.

The presence of 2-aminoethylphosphonic acid (**1**, R = H) in hydrolysates of human brain has been established using a new, highly sensitive and unambiguous technique. The occurrence in human brain of AEP[1] which possesses the biologically unusual carbon–phosphorus bond, suggests that it and perhaps other, closely related, naturally occurring phosphonic acids (Kittredge and Roberts, 1969; Quin, 1967), may have important, but as yet undiscovered, functions in human biochemistry.

Following its initial isolation from ciliated protozoa of sheep rumen (Horiguchi and Kandatsu, 1959), AEP and four closely related compounds have been isolated from a variety of natural sources—principally marine invertebrates and microorganisms (Kittredge and Roberts, 1969; Quin, 1967). Surprisingly, no systematic study of the occurrence of aminoethylphosphonic acids in higher animals has been made, although the presence of AEP in goat liver (Kandatsu and Horiguchi, 1965) and bovine brain (Shimizu *et al.*, 1965) and of trimethylaminoethylphosphonate (**2**) in human aorta (Bishop, 1968) has been reported.



1



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[1] Abbreviation used is: AEP, 2-aminoethylphosphonic acid.