Natural Deuterium Distribution in Long-Chain Fatty Acids Is Nonstatistical: A Site-Specific Study by Quantitative ^{2}H NMR Spectroscopy

Isabelle Billault,*[a] Sébastien Guiet,[a] Françoise Mabon,[b] and Richard Robins[a]

Quantitative ^{2}H NMR spectroscopy has been used to study the site-specific natural occurrence of ^{2}H in common unsaturated fatty acids. A marked nonstatistical isotopic distribution of ^{2}H is observed in both methyl oleate and methyl linoleate. By chemical modification, the internal isotopic ^{2}H signatures of these products have been partially accessed. Notably, it can be seen that (1) the sites of desaturation show a strong impoverishment at only one ethylenic position of each desaturation; (2) the level of impoverishment reflects the source of the hydrogen atoms present; and (3) a gradient of ^{2}H content occurs along the chain. These effects can in part be related to the mechanisms proposed for the enzymes responsible for the various steps of biosynthesis of unsaturated fatty acids in plants.

KEYWORDS:

dehydrogenation · deuterium · fatty acids · isotope effects · NMR spectroscopy

Introduction

Triacylglycerides are major carbon storage compounds in the seeds of many plant species and can constitute up to 60% dry weight of the seed. The principal fatty acids present in most seed oils have a chain length of 16 or 18 carbon atoms and possess between zero and three *Z*-configured double bonds. Such long-chain fatty acids are synthesised from acetyl-CoA by the fatty acid synthase (FAS) complex.[1, 2] During chain elongation under the action of FAS, C_2 units are repeatedly added to the growing chain, ultimately leading to the formation of palmitoyl-ACP (C16:0) and stearoyl-ACP (C18:0) (ACP = acyl carrier protein). Hydrogen atoms are derived either from the methyl group of acetate or from NAD(P)H/H^+. For those positions in which the carbon comes from the carboxy group of acetate (uneven sites), hydrogen is introduced by the actions of the NADPH-dependent β-ketoacyl-ACP reductase and the NAD(P)H-utilising enoyl-ACP reductase. The even-numbered carbon atoms of the fatty acid contain one hydrogen atom derived from acetate and one from the NAD(P)H-utilising enoyl-ACP reductase. The products of FAS are saturated fatty acids, which may be modified by desaturase enzymes. In plants, the initial oxidation of stearoyl-ACP is catalysed by a soluble di-iron enzyme,[3] which catalyses the elimination of two vicinal *pro-R* hydrogen atoms[4] to form oleoyl-ACP (C18:1,Δ9). Further desaturation to linoleic (C18:2,Δ9,12) and linolenic (C18:3,Δ9,12,15) acids occurs on the endoplasmic reticulum by the action of insoluble desaturases.[5]

Due to their differing physico-chemical properties, the isomers of hydrogen—^{1}H and ^{2}H (D)—have different reaction velocities and binding constants.[6] The result of this is that during enzyme-catalysed synthesis a kinetic isotope effect (k_H/k_D) is observed. This leads to a nonstatistical distribution of isotopes in the products. With the normal primary effect ($k_H/k_D > 1$), that is, scission/formation of a C−H bond, enrichment of the residual substrate (and impoverishment of the product) in the heavy isotope tends to occur. The secondary effect, that is, the influence of adjacent hydrogen atoms, can lead to enrichment or impoverishment in the product, depending on the enzymatic mechanism.[7, 8] The combination of these effects is that a different D/H ratio can potentially be established at each hydrogen site in a molecule. This leads to a nonstatistical pattern in which some sites are impoverished and others enriched relative to the mean. Thus, the molecules exist as a population of monodeuterated isotopomers, the isotopic fractionation. This site-specific natural isotopic fractionation in deuterium is most readily measured by NMR spectroscopy, the so-called SNIF-NMR technique[9, 10] (SNIF = site-specific natural isotope fractionation). The derived data may be used to obtain information on the biosynthetic origin of the molecule(s) under investigation.

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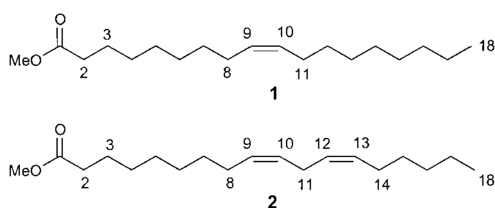
Deuterium SNIF-NMR measurements performed on fatty acid clusters have shown that variation in the $(D/H)_i$ ratios along the chains could be directly related to the environment experienced during the growth of the organism.[8] However, the degree of site-specific resolution is not sufficient to understand directly the underlying mechanistic causes of this nonstatistical distribution. Nevertheless, independent of environment or biological origin, a consistent observation is that a significant factor in the nonstatistical distribution of ^2H is an impoverishment at the site(s) of desaturation.[11–14] Such depletion could indicate a kinetic isotope effect during the action of the desaturase enzyme(s). As a result of the coincidence of the ethylenic hydrogens, only mean measurements of the $(D/H)_{9,10}$ (oleic acid) and the $(D/H)_{9,10,12,13}$ (linoleic acid) ratios can be made. To overcome this problem, a method has been developed for the chemical modification of the methyl esters of oleic acid **1** and linoleic acid **2** which allows the $(D/H)_i$ ratios to be measured in greater detail. Methyl esters are an advantageous starting material, as they show both good solubility and NMR spectroscopic resolution and can easily be obtained by transesterification of triacylglycerols.



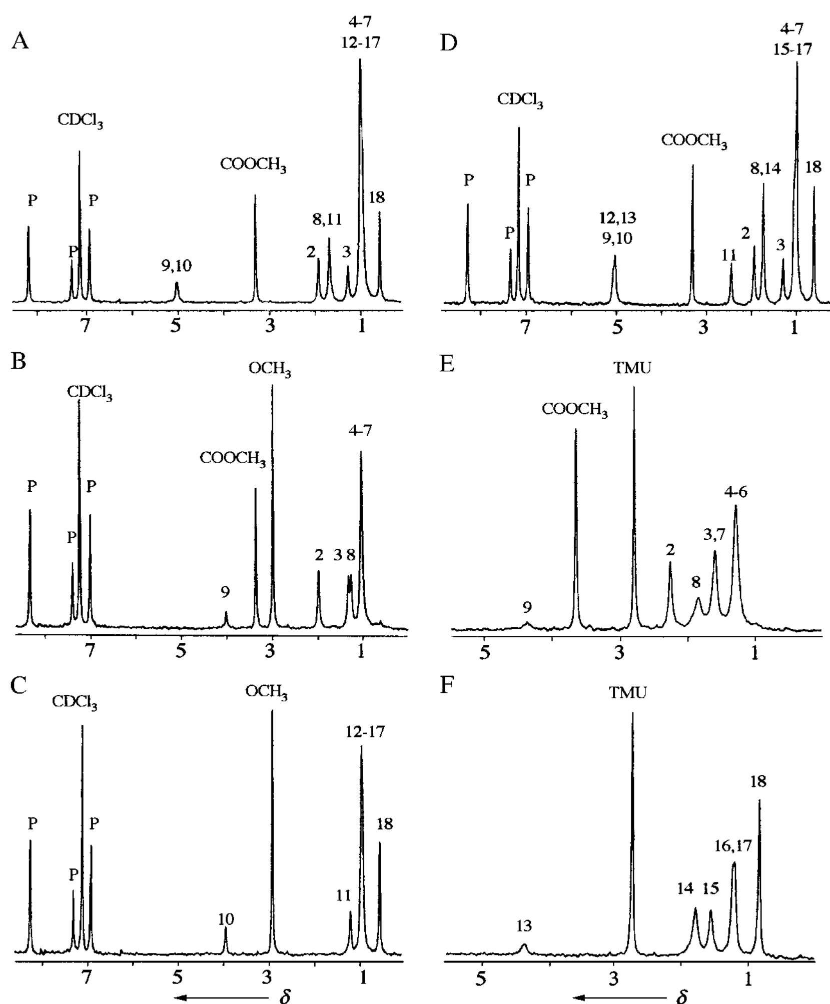


Figure 1. *^2H NMR spectra (61.4 MHz) of methyl oleate (**1**) (A); derivatives **6** (B) and **7** (C) (sites of **7** numbered following **1**); methyl linoleate (**2**) (D); derivatives **13** (E) and **15** (F) (sites of **15** numbered following **2**). P = pyridine, TMU = 1,1,3,3-tetramethylurea (internal references).*

A conventional approach to determining kinetic isotope effects exploits competition between enriched and nonenriched substrates. Using substrates specifically enriched in ^2H, strong kinetic isotope effects have been reported at C9 for the insoluble Δ^9-desaturase (yeast)[15] and at C12 for the Δ^{12}-desaturase (*Arabidopsis thaliana*).[16] Such studies have exploited genes overexpressed in *Saccharomyces cerevisiae*.

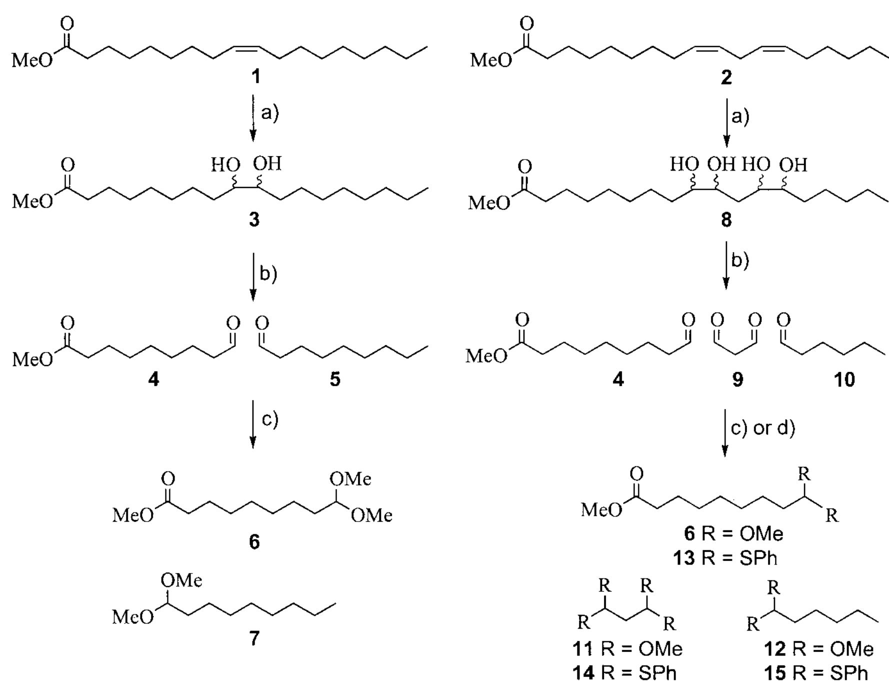
An analysis of the ^2H SNIF-NMR spectra of the derivatives **6** + **7**, and **13** + **15**—derived, respectively, from **1** and **2**—allows the direct observation of the exact position of the kinetic isotope effects during the action of both the Δ^9-desaturase and the Δ^{12}-desaturase at natural abundance (Figure 1). The positional effects are found to differ from those reported previously. In addition, the alternating pattern of the hydrogen sources during the elongation steps of FAS and a slight fractionation during chain elongation can be seen. To the best of our knowledge, no such study of the kinetic isotope effects at natural abundance during the biosynthesis of unsaturated fatty acids has previously been reported.

Results

Chemical modifications

Chemical modification of methyl oleate (**1**) was performed in three steps (Scheme 1). Treatment of **1** with a catalytic amount of OsO_4 and NMO (1.4 equiv) gave the intermediate 9,10-dihydroxy ester **3**.[17] Further oxidation of **3** was carried out in the presence of sodium periodate in MeOH to provide aldehydes **4** + **5**.[18, 19] After filtration, aldehydes **4** + **5** in MeOH were directly submitted to acidic conditions ($TsOH \cdot H_2O$) to yield the dimethylacetals **6** + **7**, which could be easily recovered in pure form following flash chromatography. Overall yields were 70% for both **6** and **7**.

A similar strategy was used to modify methyl linoleate (**2**), which possesses two double bonds (Scheme 1). *Z*-Hydroxylation of **2** in the presence of a catalytic amount of OsO_4 and NMO (2.6 equiv) provided the 9,10,12,13-tetrahydroxy ester **8** which, when treated with sodium periodate in MeOH, gave aldehydes **4** + **9** + **10**. When this mixture was submitted to acidic conditions in methanol, corresponding dimethylacetals **6** + **11** + **12**



Scheme 1. Chemical modification of methyl oleate (1) and methyl linoleate (2). a) OsO_4, NMO, $CHCl_3/H_2O$ (100:1, v/v); b) $NaIO_4$, MeOH; c) TsOH, molecular sieves (3 Å), MeOH; d) PhSH, TsOH, toluene. NMO = 4-methylmorpholine-N-oxide, Ts = toluene-4-sulfonyl.

could be detected by gas chromatography. Unfortunately, **11** + **12** proved too volatile to be isolated in satisfactory yields. Therefore, the strategy was modified by treating aldehydes **4** + **9** + **10** in toluene with thiophenol and TsOH · H_2O in a Dean–Stark apparatus to yield dithioacetals **13** + **15**.[20] During this protection step, **14** could not be isolated. Dithioacetals **13** + **15** were easily separated by flash chromatography and both were obtained in 69% yield.

In the course of such chemical modifications, a risk exists of introducing isotopic fractionation due to kinetic and thermodynamic isotopic effects intrinsic to the manipulations. While these are difficult to avoid completely, they can be minimised by the judicious choice of conditions, notably those which give high yields and avoid changes of state. Thus, all steps have been optimised to improve overall yield and strict protocols have been established. Except for the formation and separation of **13** + **15**, complete conversion of starting material at each step was checked by gas chromatography. Experiments were performed several times to examine the reproducibility of the chemical modification and separation. Furthermore, conducting the modification protocol (Scheme 1) from methyl oleate (**1**) in the presence of 5% MeOD confirmed that there was no significant exchange between the solvent and the fatty acid chain (data not shown). A number of internal checks were performed, confirming that no significant fractionation due to the manipulations occurred (see below).

^{2}H NMR measurements

The surface below the ^{2}H NMR signal is directly proportional to the number of monodeuterated isotopomers present. The site-specific isotopic ratio $(D/H)_i$ is defined in Equation (1) and expressed in parts per million (ppm):[10]

$$\left(\frac{D}{H}\right)_i = \frac{D_i}{H_i} = \frac{N_{D,i}}{P_i N_H} \qquad (1)$$

where $N_{D,i}$ is the number of monodeuterated isotopomers of type i, N_H is the number of fully protonated molecules, and P_i is the number of equivalent hydrogen atoms at site i. Figure 1 shows the ^{2}H NMR spectra obtained from methyl oleate (**1**), methyl linoleate (**2**) and their corresponding derivatives **6** + **7** and **13** + **15**. Details of the calculation of $(D/H)_i$ values from these spectra are given in the Experimental Section.

Independent of those factors in the chemical modification and purification discussed above, the accuracy of the estimation of $(D/H)_i$ is dependent on a number of NMR-related parameters. Standard deviation is a function of the signal-to-noise ratio, line shape, homogeneity in the NMR tube and spectrometer stability during the acquisition. In the present study, in which the signal-to-noise ratio varied from < 10 (C9 and C13 for **13** and **15**, respectively) to about 200, most $(D/H)_i$ values were determined with an accuracy of < 5% deviation.

The site-specific nonstatistical natural isotope distribution of ^{2}H in methyl oleate (**1**) and its derivatives is reported in Table 1. Equivalent data for methyl linoleate (**2**) are reported in Table 2. Values for $(D/H)_i$ for **6** + **7** (Table 1) and **13** + **15** (Table 2) are the means from two independent chemical modifications and NMR acquisitions on the same batch of methyl ester.

A direct comparison of the (D/H) values determined at sites C2, C3, C18 and COOMe for starting materials **1** or **2** and their respective products **6** + **7** and **13** + **15** shows that no significant fractionation due to the chemical modifications and purification occurred. Examining those sites that are resolved in the derivative spectra, but which are unresolved in the spectra of **1** or **2** supports this conclusion. Thus, the mean value of 104.4 ppm calculated from $(D/H)_9$ and $(D/H)_{10}$ of **6** and **7**, respectively, compares favourably with the $(D/H)_{9,10}$ = 105.6 ppm measured for **1**. Similarly, calculation from $(D/H)_8$ and $(D/H)_{14}$ of **13** and **15**, respectively, gives a mean value of 137.1 ppm, which is not significantly different from $(D/H)_{8,14}$ = 140.7 ppm measured directly from methyl linoleate (**2**).

Since, on this basis, no fractionation could be detected during the chemical modification and purification, further $(D/H)_i$ ratios of methyl oleate (**1**) and methyl linoleate (**2**) were obtained by calculation (Tables 1 and 2). With $(D/H)_3$ = 131.0 ppm from methyl oleate (**1**), calculation of $(D/H)_8$ gave 147.0 ppm. Since $(D/H)_{11}$ could be directly obtained from **7**, another calculation gave $(D/H)_8$ = 150.4 ppm. The validity of this estimate is confirmed by using the $(D/H)_8$ value to calculate $(D/H)_3$ = 127.6 ppm,

Table 1. $(D/H)_i$ values (in ppm) of methyl oleate (**1**) and derivatives **6** and **7**.[a]

Compd	Carbon atom number 2	3	4–7	8	9	10	11	12–17	18	COOMe	total
1	147.5 (2.6)	131.0 (2.1)	130.8[b] (2.0)	131.3[c] (2.4)	105.6[d] (4.5)	105.6[d] (4.5)	131.3[c] (2.4)	130.8[b] (2.1)	123.4 (2.7)	132.6	129.6
6	150.7 (3.3)	139.0[e] (2.7)	140.6 (1.7)	139.0[e] (2.7)	86.4 (3.4)						
7[f]						122.3 (4.0)	112.2 (2.7)	128.2 (2.2)	129.8 (3.0)	131.8 (1.5)	131.0
deduced value	147	131[g]	141	147[g]	86	122	112	128	123	133	131

[a] Values given are the means of two separate determinations, each analysed in triplicate. For the definition of $(D/H)_i$, the isotopic ratio at carbon atom *i* of **1**, see Equation (2). The standard deviations are given in brackets. The deduced values are those obtained either by direct measurement on **1** + **6** + **7** or by calculation. [b] Sites 4–7 and 12–17 all resonate at the same frequency. [c] Sites 8 and 11 resonate at the same frequency. [d] Sites 9 and 10 resonate at the same frequency. [e] Sites 3 and 8 resonate at the same frequency. [f] To maintain continuity, the sites in **7** are numbered following their position in **1**. [g] Calculated $(D/H)_i$ values.

Table 2. $(D/H)_i$ values (in ppm) of methyl linoleate (**2**) and derivatives **13** and **15**.[a]

Compd	Carbon atom number 2	3	4–6	7	8	9	10	11	12	13	14	15	16–17	18	COOMe	total
2	141.3 (2.5)	126.5 (4.4)	119.9[b] (1.7)	119.9[b] (1.7)	140.7[c] (2.0)	99.8[d] (2.0)	99.8[d] (2.0)	98.6[d] (2.1)	99.8[d] (2.0)	99.8[d] (2.0)	140.7[c] (2.0)	119.9[b] (1.7)	119.9[b] (1.7)	119.0 (1.9)	132.9 (2.1)	120.3
13	143.9 (2.8)	112.8[e] (0.8)	134.4 (1.1)	112.8[e] (0.8)	136.4 (4.0)	65.7 (6.9)										
15[f]										62.8 (2.1)	137.8 (4.8)	98.7 (2.0)	109.1 (2.0)	116.7 (0.5)	129.0 (0.5)	118.5
deduced value	141	126	134	99[g]	136	66	135[g]	99	135[f]	63	138	99	109	119	133	118

[a] Values given are the means of two separate determinations, each analysed in duplicate. For the definition of $(D/H)_i$, the isotopic ratio at carbon atom *i* of **2**, see Equation (2). The standard deviations are given in brackets. The deduced values are those obtained either by direct measurement on **2** + **13** + **15** or by calculation. [b] Sites 4–7 and 15–17 all resonate at the same frequency. [c] Sites 8 and 14 resonate at the same frequency. [d] Sites 9, 10, 12 and 13 resonate at the same frequency. [e] Sites 3 and 8 resonate at the same frequency. [f] To maintain continuity, sites of **15** are numbered following their position in **2**. [g] Calculated $(D/H)_i$ values.

which is comparable with the $(D/H)_3 = 131.0$ ppm measured directly from **1**.

Similarly, with $(D/H)_3 = 126.5$ ppm from methyl linoleate (**2**), calculation of $(D/H)_7$ provided 99.1 ppm. A mean $(D/H)_{10,12} = 135.4$ ppm could be calculated from $(D/H)_9$ and $(D/H)_{13}$ obtained from **13** and **15**, respectively.

Discussion

The samples of methyl oleate (**1**) and methyl linoleate (**2**) used were not of the same botanical origin—*Helianthus annus* (sunflower) and *Carthamus tinctorius* (safflower), respectively. Therefore, absolute $(D/H)_i$ values cannot be compared directly between the fatty acids studied here. Nevertheless, internal comparisons are possible, within both data sets, for methyl oleate (**1**) and methyl linoleate (**2**). Three isotopic effects that can be related to the biosynthesis of fatty acids can clearly be observed in both fatty acids.

Firstly, is is apparent that the hydrogen atoms stably incorporated into the fatty acid during the early steps of the biosynthesis of the chain have a lower mean (D/H) value than those added later in biosynthesis (Figure 2). Thus, mean $(D/H)_i$ values from sites in derivatives **6** and **13** (representing the

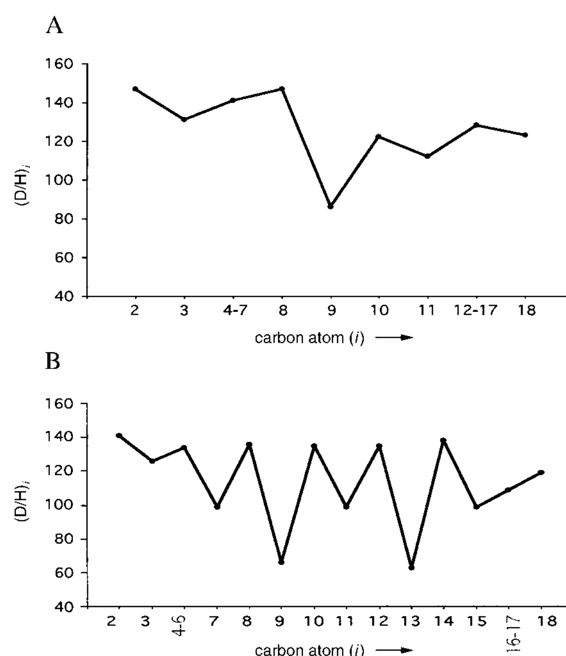


Figure 2. Alternating pattern of $(D/H)_i$ values for methyl oleate (**1**) (A) and methyl linoleate (**2**) (B).

acetate units added later in the chain) are greater by 12 – 15 ppm than those from sites of **7** and **15**, respectively (Table 3). This indicates that during the chain elongation process there is a net overall small positive kinetic isotope effect, whereby those molecules bearing light isotopes are favoured during biosynthesis. A similar trend has previously been reported for the incorporation of heavily enriched acetate into fatty acids in both yeast and several algae.[4, 21–24] The present study, however, is the first time that such a phenomenon has been observed at natural abundance.

Table 3. *Mean $(D/H)_i$ values for clusters of carbon positions from derivatives **6** and **7** and **13** and **15**.*

Fatty acid	Derivative	i	$(D/H)_i$ [ppm]
Methyl oleate (**1**)	**6**	[2 – 9]	138
	7	[10 – 18]	126
Methyl linoleate (**2**)	**13**	[2 – 9]	125
	15	[13 – 18]	110

The second deduction that can be made is that even methylenic sites, derived from the C2 position of acetate, possess $(D/H)_i$ values greater than those at equivalent odd-numbered (C1-derived) sites. While a tendency can be seen in **1** (Figure 2 A), an alternating pattern is particularly clear in **2** (Figure 2 B). This fluctuation is of the order of 15 to 25 ppm. During biosynthesis of fatty acids by the FAS complex, NAD(P)H/H^+ is used as reductant by the two acyl-ACP reductases. Two hydrogen atoms are thus incorporated from NAD(P)H/H^+ for odd-numbered methylene positions and one from each of malonyl-CoA and NAD(P)H/H^+ for even-numbered methylene groups.[1, 2] Due to equilibration, the (D/H) ratio of the hydrogen atoms transferred by NAD(P)H/H^+-utilising reductases will essentially reflect the (D/H) ratio of the water of the environment in which the plants were cultivated, plus the kinetic isotope effect of these reactions.

In contrast, the situation at the even-numbered positions is more complex. Here, one hydrogen atom is derived from the NAD(P)H/H^+-dependent enoyl reductase and one from acetyl-CoA via malonyl-CoA.[1, 2] The (D/H) ratio of the acetate-derived hydrogen atom can be influenced by two factors. On the one hand, $k_H/k_D = 1.15$ has been calculated for acetyl-CoA carboxylase which will favour the retention of ^2H at C2 of malonyl-CoA.[21] This will lead to enrichment at these positions relative to the starting acetate. The fact that the C18 position—which is derived solely from acetate—has values in both **1** and **2** (123 and 119 ppm, respectively) lower than those of the resolved methylene groups is compatible with this positive kinetic isotope effect for malonyl-CoA synthesis. In addition, the possibility exists of an exchange between malonyl-CoA and cell water, which could modify the concentration of ^2H derived from acetate. This post-malonate exchange, proposed by Sedgwick et al.[22] to explain a loss of ^3H label, appears, however, to be rather variable and organism-dependent.[4, 23, 24] The data presented here indicate that, during biosynthesis in oil seeds, minimal or negligible exchange has occurred, since extensive exchange would have eliminated the observed alternating pattern.

The third and most marked observation relates to the effect on ^2H distribution of the action of the Δ^9- and Δ^{12}-desaturases. The isotopic fractionation at the ethylenic positions of methyl oleate (**1**) and methyl linoleate (**2**) can be obtained from their respective derivatives **6** + **7** and **13** + **15** (Tables 1 and 2). For both **1** and **2**, odd-numbered sites of double bonds (C9 for **1** and C9 and C13 for **2**) are strongly depleted compared to the statistical ^2H distribution. Allowing for the alternating pattern already discussed, the ^2H intensity at C9 of **1** and C9 and C13 of **2** can be estimated from the neighbouring uneven-numbered positions. Values of 120, 105 and 100 ppm, respectively, are obtained. Thus, these sites show depletions of about 34, 40 and 37 ppm, respectively, in excess of the methylenic positions. These impoverishments could result either from secondary kinetic isotope effects in the course of desaturation or from a very marked difference in the initial values of the $(D/H)_i$ ratio at the *pro-R* and *pro-S* positions of the substrate. The latter explanation can be dismissed on the basis that both hydrogen atoms at the C9 and C13 positions originate from reductase activity in FAS and that the isotopic incorporations are very similar, irrespective of whether the hydrogen is introduced in a *re* or *si* reaction.[4] Thus, it can be concluded that these depletions represent secondary kinetic isotope effects due to desaturation. From the estimated values of these sites without desaturation (see above and Figure 2), approximate values for the k_H/k_D values at C9 in **1** and C9 and C13 in **2** of 1.4, 1.6 and 1.6, respectively, can be estimated.

Even-numbered sites of double bonds (C10 for **1** and C10 and C12 for **2**), in contrast, show no such isotopic depletion. If it is assumed that the stereochemistry of fatty acid biosynthesis in higher plants is the same as reported for several algae,[4] then the C10 and C12 positions prior to desaturation will contain a *pro-S* hydrogen atom derived from acetate and a *pro-R* hydrogen atom derived from NADPH/H^+. Desaturation universally appears to abstract the pro-*R* hydrogen atoms;[4, 25, 26] thus, the remaining hydrogen at the C10 and C12 positions is that derived from acetate. In the absence of any secondary kinetic isotope effect at these positions in the desaturase, these positions should therefore reflect those of the neighbouring methylene groups, as is found to be the case.

Desaturation by the insoluble Δ^9- and Δ^{12}-desaturases of *S. cereviseae* and *A. thaliana* respectively, have both previously been shown to involve a strong kinetic isotope effect[27] at the C9 and C12 positions, but not at the C10 and C13 positions.[15, 16] The data presented here show that, when the isotopic values at natural abundance are analysed, a strong secondary kinetic isotope effect is observed for the soluble Δ^9-desaturase in both sunflower and safflower. The extent of the secondary kinetic isotope effect has not previously been determined for this reaction. The impoverishment of both **1** and **2** at site C9 is in accord with the mechanism proposed in which H abstraction at C9 is the slow initiating step of desaturation.[15, 16] In linoleate **2**, the Δ^{12}-desaturation in safflower shows impoverishment at position C13, rather than at position C12. The extent of impoverishment is similar to that obtained for the Δ^9-desatur-

ase[15] and is therefore compatible with a comparable mechanism. However, in previous studies a strong total kinetic isotope effect at position C12 for the *A. thaliana* Δ^{12}-desaturase was reported.[16, 27] As previously discussed, the impoverishment seen at the C9 and C13 positions is not simply due to the alternating values in the methylene backbone, and the value at C12 fits closely with these values. This discrepancy requires further investigation.

Conclusions

Deuterium SNIF-NMR spectroscopy, combined with chemical modification of methyl oleate (**1**) and methyl linoleate (**2**), has proved to be a powerful tool for the analysis of ^{2}H distribution at natural abundance in fatty acids. Data pertaining both to the biosynthesis of the backbone long-chain fatty acid and to subsequent metabolism can potentially be obtained. In order to probe further these phenomena, fatty acids from the same biological source are being isolated. Of particular interest are the substrate – product relationship during desaturation, regio- and stereospecific effects during desaturation, and natural derivatives, notably oxygenated fatty acids (e.g. vernolic acid and ricinoleic acid).

Experimental Section

Materials: Methyl oleate (**1**) (from *Helianthus annus*, sunflower; lot 69H5228) and methyl linoleate (**2**) (from *Carthamus tinctorius*, safflower; lot 65H0257) were purchased from Sigma – Aldrich.

^{2}H NMR spectroscopic measurements: The samples were previously characterised by their ^{1}H and ^{13}C NMR spectra. The ^{2}H NMR spectra were recorded with a Bruker DPX 400 spectrometer operating at 61.4 MHz and fitted with a ^{19}F field-frequency-locking device. The T_1 values were measured by using an inversion recovery sequence[28] with twelve inversion time values ranging from 5 ms to 6 s and calculated using the T_1 calculation software provided. T_1 values of references pyridine and 1,1,3,3-tetramethylurea (TMU) are in both cases longer than those of fatty acids and their derivatives. The T_1 values of references were therefore chosen to determine the repetition time ($>5T_1$). Acquisition parameters: number of scans, 10000 – 21500 (depending on the sample); sweep width, 1197 Hz; acquisition time, 5.7 s; pulse (90°), 11.5 µs; $T=310$ K and broad-band decoupling. Sample preparation: lipid derivatives (0.4 – 0.7 g), solvent $CHCl_3/CCl_4$ (1:4, 3.3 g), the external reference pyridine (0.2 g) or TMU (0.07 g) and the field-frequency locking material C_6F_6 (60 µL) was mixed, then filtered and introduced into a 10-mm NMR tube. Three spectra were recorded for each sample except for **13** and **15** (two spectra) and $(D/H)_i$ values were calculated from all measurements after exponential multiplication associated with a line broadening of 1 Hz. The quantitative calculation of the monodeuterated isotopomers was performed by using a curve-fitting algorithm based on a complex least squares treatment of the ^{2}H NMR signal.[29]

$(D/H)_i$ ratios of samples were calculated from Equation (2) using an internal reference of either pyridine or TMU. The isotopic ratio of TMU, $(D/H)_{TMU}$, was precisely calibrated on the V.SMOW scale[10] and $(D/H)_{PYR}$ for pyridine was calibrated relative to TMU.

$$\left(\frac{D}{H}\right)_i = \frac{P_{ref}\, m_{ref}\, M_S\, S_i}{P_i\, m_S\, M_{ref}\, S_{ref}} \left(\frac{D}{H}\right)_{ref} \quad (2)$$

where P_i and P_{ref} are the stoichiometric numbers of hydrogen atoms in site i and in the reference, S_i and S_{ref} are the surface areas of the signal, M_S, m_S and M_{ref}, m_{ref} are, respectively, the molecular weight and mass of the sample and the reference used.

Chemical modification: General methods: ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded with a Bruker DRX 500 spectrometer. Chemical shifts are given relative to Me_4Si in $CDCl_3$. Flash chromatography was performed with Silica Gel 60 (40 – 60 µm, Merck). Petroleum ether (b.p. 45 – 60 °C) was distilled before use. Reaction was followed by gas chromatography on an HP-5 capillary column (30m × 0.32 mm, film thickness 0.52 µm); carrier gas, He, 1.2 mL min^{-1}; split injection 1:40; FID temp., 280 °C; thermal gradient, 100 °C (1 min)/10 °C min^{-1}/240 °C (1 min)/10 °C min^{-1}/280 °C (1 min)/ 10 °C min^{-1}/298 °C (2 min).

Methyl-9,9′-bis(methoxy)nonanoate (6) and 1,1′-bis(methoxy)nonane (7): A solution of OsO_4 in *t*BuOH (2.5 wt%, 0.46 mL) was slowly added to a solution of methyl oleate (**1**) (1.0 g, 3.4 mmol) and NMO (0.52 g, 4.8 mmol) in $CHCl_3$ (20 mL) and H_2O (0.2 mL) at 4 °C. The mixture was stirred and kept at 4 °C for 1 h, then allowed to warm to room temperature. After 24 h the mixture was co-evaporated with toluene (3 × 30 mL). The intermediate diol **3** was taken up in MeOH (75 mL), then treated with sodium periodate (0.8 g, 3.7 mmol) and stirred at room temperature for 12 h. After filtration, the solution of aldehydes **4** and **5** in MeOH was treated with $TsOH \cdot H_2O$ (0.76 g, 3.99 mmol) and heated at 40 °C for 2 h. The solution was cooled at room temperature and molecular sieves (3 Å, 9.0 g) were added. After 14 h, the mixture was filtered, the MeOH removed by evaporation, and the residue taken up in Et_2O. The organic phase was washed with aqueous $NaHCO_3$ (100 mL, 1 wt%), dried ($MgSO_4$), filtered and concentrated. Flash chromatography of the residue (petroleum ether (b.p. 45 – 60 C°)/Et_2O, 95:05, then 90:10) gave **6** (0.56 g, 71% yield) and **7** (0.44 g, 70% yield). **6**: ^{1}H NMR ($CDCl_3$): $\delta=$ 4.29 (t, $J=5.5$ Hz, H9), 3.61 (s, 3H, MeOCO), 3.25 (s, 6H, 2OMe), 2.24 (t, $J=7.4$ Hz, 2H, H2, H2′), 1.54 (m, 4H, H3, H3′, H8, H8′), 1.27 (m, 8H, CH_2); ^{13}C NMR ($CDCl_3$): $\delta=174.3$, 104.6, 52.6, 51.4, 34.1, 32.5, 29.3, 29.2, 29.1, 25.5, 24.9.—**7**: ^{1}H NMR ($CDCl_3$): $\delta=4.30$ (t, $J=5.7$ Hz, 1H, H1), 3.25 (s, 6H, 2OMe), 1.58 (m, 2H, H2, H2′), 1.33 – 1.15 (m, 12H, CH_2), 0.83 (t, $J=6.7$ Hz, 3H, Me); ^{13}C NMR ($CDCl_3$): $\delta=104.7$, 52.6, 32.6, 31.9, 29.6, 29.5, 29.3, 24.7, 22.7, 14.1.

Methyl-9,9′-bis(phenylthio)nonanoate (13) and 1,1′-bis(phenylthio)hexane (15): A solution of OsO_4 in *t*BuOH (2.5 wt%, 0.5 mL) was slowly added to a solution of methyl linoleate (**2**) (1.0 g, 3.4 mmol) and NMO (1.05 g, 8.84 mmol) in $CHCl_3$ (50 mL) and H_2O (0.5 mL). The mixture was stirred and kept at room temperature for 28 h. After co-evaporation with toluene (3 × 30 mL), the intermediate **8** was taken up in MeOH (70 mL) then treated with sodium periodate (1.6 g, 7.5 mmol) for 12 h. After filtration, toluene (70 mL) was added. The organic phase was washed with sodium thiosulfate solution (150 mL, 5% (w/v)) and the aqueous phase extracted with toluene (4 × 20 mL). Organic phases were combined, dried ($MgSO_4$) and filtered.

PhSH (3.5 mL, 34 mmol) and $TsOH \cdot H_2O$ (0.08 g, 0.4 mmol) were added to the solution of aldehydes **4**, **9** and **10** in toluene. The mixture was heated under reflux in a Dean – Stark apparatus until no more water separated (ca. 9 h). The mixture was cooled, diluted with Et_2O (80 mL), washed with aq NaOH solution (2 M, 200 mL), then sat. NaCl solution, and dried ($MgSO_4$). Flash chromatography of the residue (petroleum ether (45 – 60 C°)/Et_2O, 100:1 then 100:3) gave **13** (0.90 g, 69%) and **15** (0.70 g, 69%). **13**: ^{1}H NMR ($CDCl_3$): $\delta=7.40$ (m, 4H, Ph), 7.27 – 7.18 (m, 6H, Ph), 4.33 (t, $J=6.5$ Hz, 1H, H9), 3.61 (s, 3H, MeOCO), 2.23 (t, $J=7.5$ Hz, 2H, H2, H2′), 1.79 (m, 2H, H8, H8′), 1.54 (m, 4H, H3, H3′, H7, H7′), 1.28 – 1.17 (m, 6H, CH_2); ^{13}C NMR ($CDCl_3$): $\delta=$

174.3, 134.4, 132.7, 128.9, 127.7, 58.5, 51.5, 35.8, 34.1, 29.0, 28.9, 27.0, 24.9.—**15**: ^{1}H NMR ($CDCl_3$): $\delta = 7.41$ (m, 4H, Ph), 7.28–7.19 (m, 6H, Ph), 4.35 (t, $J = 6.5$ Hz, 1H, H1), 1.80 (m, 2H, H2, H2′), 1.56 (m, 2H, H3, H3′), 1.29–1.16 (m, 4H, CH_2), 0.83 (t, $J = 7.0$ Hz, 3H, Me); ^{13}C NMR($CDCl_3$): $\delta = 134.5$, 132.8, 128.9, 127.7, 58.5, 35.9, 31.3, 26.8, 22.5, 14.1.

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[1] J. Ohlrogge, J. Browse, *Plant Cell* **1995**, *7*, 957–970.
[2] J.L. Harwood in *Plant Biochemistry* (Eds.: P.M. Dey, J.B. Harbourne), Academic Press, San Diego, **1997**, pp. 237–272.
[3] B.G. Fox, J. Shanklin, A. Jingyan, T.M. Loeher, J. Sanders-Loehr, *Biochemistry* **1994**, *33*, 12776–12786.
[4] A.G. McInnes, J.A. Walker, J.L.C. Wright, *Tetrahedron* **1983**, *39*, 3515–3522.
[5] E.B. Cahoon, Y. Lindquist, G. Schneider, J. Shanklin, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 4872–4877.
[6] E. Wada, T. Ando, K. Kumazawa in *Stable Isotopes in the Biosphere* (Eds.: E. Wada, T. Yoneyama, M. Minagawa, T. Ando, B.D. Fry), Kyoto University Press, Kyoto (Japan), **1995**, pp. 7–14.
[7] H.-L. Schmidt, H. Kexel, M. Butzenlechner, S. Schwarz, G. Gleixner, S. Thimet, R.A. Werner, M. Gensler in *Stable Isotopes in the Biosphere* (Eds.: E. Wada, T. Yoneyama, M. Minagawa, T. Ando, B.D. Fry), Kyoto University Press, Kyoto (Japan), **1995**, pp. 17–35.
[8] G.J. Martin in *Stable Isotopes in the Biosphere* (Eds.: E. Wada, T. Yoneyama, M. Minagawa, T. Ando, B.D. Fry), Kyoto University Press, Kyoto (Japan), **1995**, pp. 36–58.
[9] G.J. Martin, M.L. Martin, *Tetrahedron Lett.* **1981**, *22*, 3525–3528.
[10] M.L. Martin, G.J. Martin in *NMR Basic Principles and Progress, Vol. 23* (Eds.: P. Diehl, E. Fluck, H. Günther, R. Kosfeld, J. Seelig), Springer, Berlin, **1990**, pp. 1–61.
[11] A. Royer, N. Naulet, F. Mabon, M. Lees, G.J. Martin, *J. Am. Oil Chem. Soc.* **1999**, *76*, 365–373.
[12] M. Aursand, F. Mabon, G.J. Martin, *Magn. Reson. Chem.* **1997**, *35*, S91–S100.
[13] B. Quémerais, F. Mabon, N. Naulet, G.J. Martin, *Plant Cell Environ.* **1995**, *18*, 989–998.
[14] A. Lai, M. Casu, G. Saba, F.P. Coriongiu, M.A. Dessi, *Magn. Reson. Chem.* **1995**, *33*, 163–166.
[15] P.H. Buist, B. Behrousian, *J. Am. Chem. Soc.* **1996**, *118*, 6295–6296.
[16] P.H. Buist, B. Behrousian, *J. Am. Chem. Soc.* **1998**, *120*, 871–876.
[17] V. Van Rheenen, R.C. Kelly, D.Y. Cha, *Tetrahedron Lett.* **1976**, *23*, 1973–1976.
[18] A.S. Perlin in *Oxidation* (Ed.: R.L. Augustine), Marcel Dekker, New York, **1969**, pp. 189–211.
[19] C.A. Burton in *Oxidation in Organic Chemistry* (Ed.: K.B. Wiberg), Academic Press, New York, **1965**, pp. 367–407.
[20] D.J. Ager, *J. Chem. Soc. Perkin Trans. 1* **1983**, 1131–1136.
[21] B. Sedgwick, J.W. Cornforth, *Eur. J. Biochem.* **1977**, *75*, 465–479.
[22] B. Sedgwick, J.W. Cornforth, S.J. French, R.T. Gray, E. Kelstrup, P. Willadsen, *Eur. J. Biochem.* **1977**, *75*, 481–495.
[23] K. Saito, A Kawaguchi, Y. Seyama, T. Yamakawa, S. Okuda, *J. Biochem.* **1981**, *90*, 1697–1704.
[24] K. Saito, A. Kawaguchi, S. Nozoe, Y. Seyama, S. Okuda, *Biochem. Biophys. Res. Commun.* **1982**, *108*, 995–1001.
[25] B. Behrousian, P.H. Buist, J. Shanklin in *Abstr. Pap. 14th International Symposium on Plant Lipids* (Cardiff, UK) **2000**, A26.
[26] G.J. Schroepfer, K. Bloch, *J. Biol. Chem.* **1965**, *240*, 54–63.
[27] It is important to note that in refs. [15] and [16] enriched $^{2}H_2$ substrates were used to examine the desaturase activities. Thus, the values obtained reflect the combined primary and secondary kinetic isotope effects.
[28] R. Freeman, H.D.W. Hill, *J. Chem. Phys.* **1963**, *54*, 3140–3143.
[29] Y.L. Martin, *J. Magn. Reson. Ser. A* **1994**, *111*, 1–10.

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