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Natural Deuterium Distribution in Fatty Acids Isolated from Peanut Seed Oil: A Site-Specific Study by Quantitative ^{2}H NMR Spectroscopy

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Quantitative ^{2}H NMR spectroscopy has been used to measure the distribution of deuterium at natural abundance in long-chain fatty acids extracted from the same vegetable oil. Peanut seed oil was selected, due to its suitable oleic and linoleic acid content. The methyl esters of the fatty acids were prepared by transesterification and isolated by modified argentation column chromatography on silica. In order to measure the natural isotopic fractionation of deuterium (D) at the maximum number of positions, the purified methyl oleate and methyl linoleate were chemically cleaved and the $(D/H)_i$ values determined by quantitative ^{2}H NMR spectroscopy. It was thus possible to demonstrate that fractionation in deuterium occurs during the desaturation of oleate to linoleate. Furthermore, the previously observed distribution of deuterium at the sites of desaturation is confirmed, as is the alternating pattern of $(D/H)_i$, which relates to the origin of the pertinent hydrogen atoms. The data obtained are discussed in terms of the kinetic isotopic effects intrinsic to the enzymes—synthetases and desaturases—involved in the biosynthesis of fatty acids.

KEYWORDS:

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

Introduction

During the biosynthesis of natural products, isotopic fractionation can be introduced, due to the kinetic isotope effects (KIEs) associated with the implied reactions.[1, 2] Due to the sensitivity of certain enzymes to the presence of a heavy isotope, the KIEs of some transformations can be high.[3] For hydrogen, for which the mass, volume and vibrational energy differences between the stable isotopes—^{1}H and ^{2}H (D)—are proportionately particularly large, KIEs as high as and even in excess of the maximum theoretical value are encountered.[4] The effect of a series of KIEs specific to each enzyme-catalysed step can accumulate during a biosynthetic pathway, resulting in fractionation of the isotopes between the starting substrate and the final product.[1] As the individual reactions act on different positions in the substrate, this gives rise to a nonstatistical isotope distribution in the final product. The extent to which selection against (or, in rarer cases, for) a heavy isotope has occurred at individual sites in the product therefore reflects the KIEs of the reaction mechanisms involved in its biosynthesis and defines the population of monodeuterated isotopomers.[2]

The synthesis of the long-chain fatty acids of vegetable oils involves a long pathway, susceptible to isotopic fractionation at a number of steps. This synthesis may be divided into three components:[5, 6] the activation of acetyl-CoA as malonyl-CoA by acetyl-CoA carboxylase (ACC), chain initiation from a unit of acetyl-CoA plus a unit of malonyl-CoA and subsequent elongation with malonyl-CoA by the multifunctional and multienzymatic fatty acid synthase (FAS) complex, and desaturation by regio- and stereospecific desaturases. FAS activity leads to the formation of palmitoyl-ACP (C16:0) and stearoyl-ACP (C18:0; ACP = acyl carrier protein), in which the hydrogen atoms are either derived from the methyl group of acetate or are introduced from nicotinamide adenine dinucleotide phosphate reduced form (NADPH) and the aqueous environment (H^+) during the reductase reactions of FAS. At the odd-numbered positions in the fatty acid, in which the carbon comes from the carboxyl group of acetate, hydrogen is introduced entirely from NADPH, whereas the even-numbered carbons have one hydrogen atom derived from acetate and one from the aqueous environment during the enoyl-ACP reductase step.[7] The stearoyl-ACP and, to a lesser extent, palmitoyl-ACP can undergo further modification, the most common of which is desaturation. The removal of two vicinal pro-*R* hydrogen atoms[7] from stearoyl-ACP (C18:0) to form oleoyl-ACP (C18:1,Δ9) is under the control of a soluble Δ^9-desaturase containing two iron centres in the active site.[8] Further desaturation to linoleate (C18:2,Δ9,12) and linolenate (C18:3,Δ9,12,15) involves insoluble desaturases embedded in the membrane of the endoplasmic reticulum.[9]

It has previously been shown that fatty acids extracted from vegetable[10–12] and animal[13] sources show marked nonstatistical

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isotopic distributions for hydrogen. This site-specific natural isotopic fractionation in ^{2}H is most readily examined by quantitative ^{2}H NMR spectroscopy,[14, 15] which allows direct measurement of the natural abundance ^{2}H content in each of the resonances resolved in the spectrum. However, because of the lack of functionality in fatty acids, a considerable degree of overlap occurs in the ^{2}H NMR spectrum and the degree of site-specific resolution is not sufficient for direct examination of the underlying mechanistic causes of the observed ^{2}H distribution.

To overcome this difficulty, methods of chemical modification of fatty acids are being developed to allow improved access to the (D/H)$_i$ ratios. In the first instance, a method for the cleavage of the double bonds of methyl oleate (**3**) and methyl linoleate (**4**) (Scheme 1) has been developed.[16] This procedure involves minimal oxidation, releasing the cleavage products as aldehydes that may then be protected as dimethylacetal (**5**, **6**) or dithiophenylacetal (**7**, **8**) derivatives and separated by chromatography. Methyl esters are advantageous starting materials, as they show both good solubility and good NMR resolution and can easily be obtained by transesterification of triacyl glycerols.[10] Furthermore, they can be separated on the basis of their degree of unsaturation by argentation chromatography.[17, 18]

In this paper, the isotopic distribution of ^{2}H between different fatty acids isolated from the same biological origin is reported. The approach developed has allowed a direct comparison of the nonstatistical distribution of ^{2}H within a single fatty acid population to be studied for the first time.

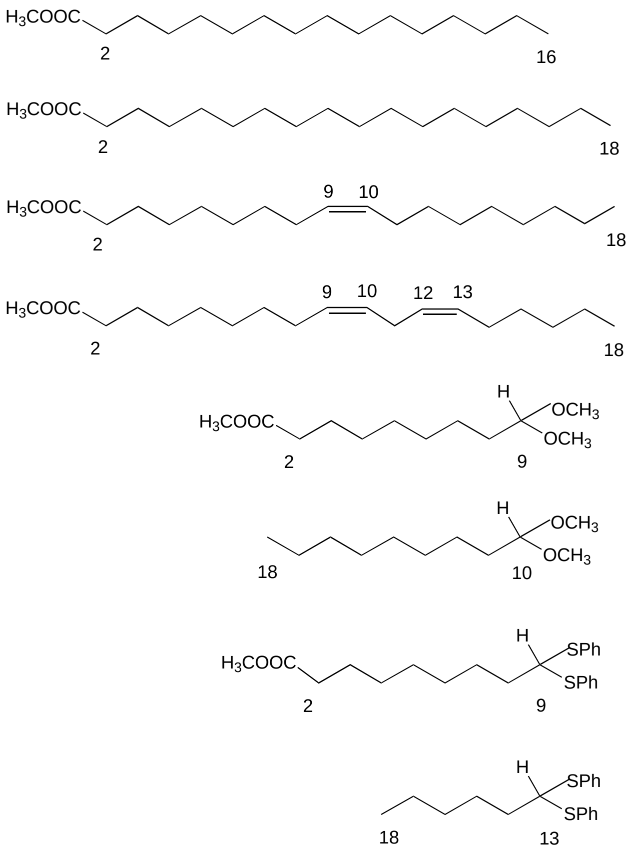
Results

Accessing the ^{2}H isotopic data for the fatty acids of peanut seed oil involved three steps (for details see the Experimental Section): transmethylation of the oil, separation by argentation chromatography and chemical modification. The overall procedure was carried out three times on the same sample of oil.

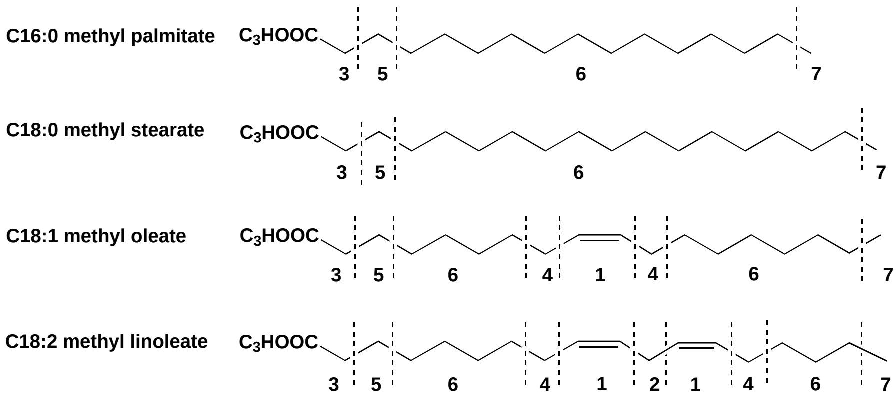
Transesterification

Transesterification gave a mixed fatty acid methyl ester (FAME) preparation composed of methyl palmitate (**1**, $13.3\pm0.4\,\%$), methyl stearate (**2**, $3.3\pm0.4\,\%$), methyl oleate (**3**, $44.5\pm0.7\,\%$) and methyl linoleate (**4**, $38.9\pm0.3\,\%$). For the three principal acids, the experimental error is satisfactory for subsequent analysis. The quantification of the minor product **2** showed poorer reproducibility but, since **1** and **2** were studied together in all subsequent analyses, this variability had negligible impact on the overall values used.

These preparations were submitted to quantitative ^{2}H NMR spectroscopy to determine the overall mean (D/H)$_i$ values[19] for each cluster (Scheme 2) in the spectrum (Table 1). These values were useful for confirming that no isotopic fractionation had been introduced by the workup procedure. The standard errors obtained were satisfactory, being largely <5 ppm. From these data, it can be seen that, as found



*Scheme 1. Structures of the principal fatty acid methyl esters and their derivatives. Note that in compounds **5**–**8** the carbon atoms are numbered according to their positions in the parent fatty acids.*



Scheme 2. Contribution of the methyl esters of the principal fatty acids found in peanut oil to the different clusters found in the ^{2}H NMR spectrum.

Table 1. *(D/H)$_i$ ratios (ppm) measured by ^{2}H NMR spectroscopy for mixed preparations of fatty acid methyl esters obtained from peanut oil.*[a]

Methyl ester of:				Carbon no.				
palmitate **1**	-	-	2	-	3	4–15	16	OCH_3
stearate **2**	-	-	2	-	3	4–17	18	OCH_3
oleate **3**	9, 10	-	2	8, 11	3	4–7, 12–17	18	OCH_3
linoleate **4**	9, 10, 12, 13	11	2	8, 14	3	4–7, 15–17	18	OCH_3
Sample				Cluster[b]				
	1	2	3	4	5	6	7	OCH_3
RMN1	103.1	97.7	140.4	128.0	127.1	130.5	115.8	94.3
	(0.1)	(1.6)	(0.6)	(1.8)	(0.9)	(1.6)	(1.3)	(2.3)
RMN2	92.7	95.5	133.6	119.6	120.9	126.9	146.5[c]	119.0
	(0.9)	(4.9)	(2.2)	(1.1)	(0.8)	(0.7)	(2.3)	(2.2)
RMN3	95.2	97.3	140.1	124.6	125.2	128.8	123.2	129.9
	(3.8)	(7.0)	(5.2)	(3.1)	(3.2)	(3.3)	(3.2)	(3.3)
mean	96.8	97.3	137.8	124.0	124.3	128.7	118.7	–
	(5.2)	(4.3)	(4.2)	(4.2)	(3.0)	(1.9)	(4.3)	–

[a] (D/H)$_i$ values are calculated as described in the Experimental Section. Three acquisitions were made for each sample. For each acquisition, the (D/H)$_i$ values were calculated for each of the three resonances due to the internal reference, pyridine. The standard deviation for each value is given in parentheses. [b] A cluster is defined as a group of unresolved resonances in the ^{2}H NMR spectrum. The contribution to the clusters of each of the four fatty acids studied is given in the top section of the table. For structures, see Scheme 1; for contribution to clusters, see Scheme 2. [c] The (D/H)$_i$ value for cluster 7 of sample RMN2 is based on only two spectral acquisitions as a small impurity was present that made it difficult to determine the area under this peak precisely.

previously for a number of vegetable oils,[10–12] the distribution of ^{2}H is nonstatistical. Notably, *(D/H)$_1$* values of the ethylenic sites—due entirely to positions C9 and C10 in **3** and C9, C10, C12 and C13 in **4**—are relatively impoverished. Similarly, the allylic site (C11) of **4** (*(D/H)$_2$*) is markedly impoverished.

In contrast, the *(D/H)$_3$* value, which represents the methylenic hydrogens at position C2 of all the fatty acids present, appears relatively enriched. When transesterificaton was carried out in enriched methanol (5% MeOD), however, it was confirmed that this position underwent exchange with the reagent,[20] and so these values are not useful for interpretation of biological isotope effects. They can nevertheless still be useful to verify that subsequent steps do not introduce artefacts.

Table 2. *(D/H)$_i$ ratios (ppm) measured by ^{2}H NMR spectroscopy for the mixture of methyl palmitate (**1**) and methyl stearate (**2**) obtained from peanut oil.*[a]

			Cluster[b]		
	3	5	6	7	OCH_3
RMN4	127.6	128.8	120.0	107.7	122.2
	(0.3)	(2.4)	(1.1)	(1.0)	(2.1)

[a] See footnote [a] in Table 1. [b] For the definition of the clusters, see Table 1.

Table 3. *(D/H)$_i$ ratios (ppm) measured by ^{2}H NMR spectroscopy for methyl oleate (**3**) obtained from peanut oil.*[a]

Sample				Carbon no.			
	9, 10	2	8, 11	3	4–7, 12–17	18	OCH_3
RMN5	89.9	136.8	113.7	138.1	126.9	118.9	119.9
	(1.6)	(2.4)	(2.0)	(2.5)	(2.3)	(2.1)	(2.1)
RMN6	93.6	139.1	119.5	124.0	124.4	114.5	132.1
	(5.0)	(1.9)	(0.1)	(3.7)	(1.0)	(0.9)	(1.6)
RMN7	93.8	140.3	120.9	122.9	125.3	112.5	129.8
	(4.2)	(1.3)	(0.4)	(0.8)	(0.5)	(0.6)	(2.2)
mean	92.7	139.0	118.6	123.4	125.4	114.9	127.3
	(4.4)	(2.3)	(3.0)	(2.5)	(1.2)	(2.7)	(4.8)

[a] See footnote [a] in Table 1.

Separation

The fatty acid methyl esters from each transesterification were isolated separately by argentation chromatography.[17] This technique did not separate **1** from **2**, and these were collected and studied as a single fraction. For the three samples, the overall degree of recovery was 93 ± 2%. The purity of each fraction, as determined by GC, was >99%. From the individual fractions, the calculated composition of the starting sample was: **1** + **2**, 16.3 ± 0.4%; **3**, 43.8 ± 0.8%; **4**, 40.0 ± 0.4%. Thus, no compositional discrepancy was introduced in this step.

These preparations were submitted to quantitative ^{2}H NMR spectroscopy. Only one sample (RMN3) was of sufficient size to obtain data for the methyl palmitate (**1**) plus methyl stearate (**2**) fraction (Table 2).

(D/H)$_i$ values were obtained independently on three samples of methyl oleate (**3**) and are presented in Table 3 by carbon number rather than by cluster number.[19] The standard errors range between 1.2 and 4.4 ppm, indicating good reproducibility for the workup procedure. As anticipated, the (D/H)$_{9,10}$ value is the most impoverished and is close to the *(D/H)$_1$* value (Table 1) obtained on the mixed sample.

(D/H)$_i$ values were obtained independently on three samples of methyl linoleate (**4**) corresponding to the same separations as the three samples of **3** and are presented in Table 4 by carbon number rather than by cluster number. The standard errors range between 1.4 and 4.8 ppm, indicating good reproducibility for the workup procedure. As anticipated, the (D/H)$_{9,10,12,13}$ value

Table 4. *(D/H)$_i$ ratios (ppm) measured by ^{2}H NMR spectroscopy on methyl linoleate (**4**) obtained from peanut oil.*[a]

Sample	9, 10 12, 13	11	2	8, 14	3	4–7 15–17	18	OCH$_3$
RMN8	89.0	90.5	140.1	127.4	121.9	121.5	121.3	119.6
	(1.9)	(1.8)	(2.4)	(2.9)	(3.2)	(2.2)	(1.6)	(1.4)
RMN9	88.0	88.5	131.3	124.2	118.6	123.8	119.9	122.7
	(4.2)	(1.7)	(1.1)	(1.1)	(2.7)	(3.0)	(1.4)	(0.7)
RMN10	91.2	95.4	137.6	132.9	120.0	114.6	111.8	129.2
	(1.6)	(2.7)	(1.3)	(0.6)	(2.4)	(1.3)	(0.8)	(1.9)
mean	89.5	91.6	135.9	128.3	120.0	119.8	117.2	123.8
	(3.0)	(3.8)	(4.1)	(4.3)	(2.7)	(4.8)	(1.4)	(5.4)

[a] See footnote [a] in Table 1.

is the most impoverished and is close to the *(D/H)$_1$* value obtained on the mixed sample (Table 1). Similarly, the (D/H)$_{11}$ value is highly impoverished and, when the standard errors in measuring this position are taken into account, does not differ significantly from the *(D/H)$_2$* value obtained on the mixed sample. This indicates that no fractionation had taken place during workup, as the *(D/H)$_2$* value for the mixed sample is due exclusively to the **4** present.

In order to confirm that there had not been any significant overall fractionation due to the workup procedures, the (D/H)$_i$ values obtained from isolated fractions of **1** + **2**, **3** and **4** were used, with the percentage compositional data, to predict values for the initial mixture (Table 5). Overall, these calculated values agree well with those obtained by direct measurement. The small differences for *(D/H)$_1$* and *(D/H)$_2$* may be attributed to the poorer signal-to-noise ratio in the mixture, in which the hydrogens contributing to these clusters are present at a relatively lower molar ratio. The differences seen are not significantly larger than the standard deviations observed. For the value of *(D/H)$_6$*, the difference is larger than the standard error, but still well within the acceptable range of variation.

Table 5. *Comparison of the (D/H)$_i$ ratios (ppm) measured by ^{2}H NMR spectroscopy for the initial mixture of FAMEs and the values calculated for this mixture based on the (D/H)$_i$ ratios determined for the isolated fatty acid methyl esters **1** + **2**, **3** and **4**.*[a]

	Cluster no. 1	2	3	4	5	6	7
calculated (C)	91.6	90.7	135.6	123.1	123.0	122.5	118.9
measured (M)	96.8	97.3	137.8	124.0	124.3	128.7	118.7
	(5.2)	(4.3)	(4.2)	(4.2)	(3.0)	(1.9)	(4.3)
Δ(M – C)	5.2	6.6	2.2	0.9	1.3	6.2	– 0.2

[a] Values given are the mean for the three samples RMN1, RMN2, RMN3. The standard deviations are given in parentheses for the measured values.

Chemical modifications

A partial chemical degradation technique has been developed to reveal isotopic data otherwise masked due to overlapping resonance positions in the ^{2}H NMR spectrum for methyl oleate (**3**) and methyl linoleate (**4**).[16] Essentially, the molecules are cleaved with minimal oxidation at the sites of the double bond(s), the resulting aldehydes are protected and the protected products are isolated by chromatography.

Two samples (see the Experimental Section) of **3** were submitted to partial chemical degradation and the resulting products, methyl 9,9-dimethoxynonanoate (**5**) and 1,1-dimethoxynonane (**6**) were separated by chromatography, giving yields of 71% in each case. These samples were submitted independently to quantitative ^{2}H NMR spectroscopy (Table 6). The overall standard errors are reasonable, ranging from 1.0 to 5.6 ppm. The values of (D/H)$_i$ obtained may be compared to those obtained from the intact molecule of **3**. A direct comparison of the (D/H)$_{18}$ values shows no change, indicating no fractionation due to the procedure in this position. Similarly, the value calculated for the positions C9 + C10 of **3** from the measured values for C9 of **5** and C10 of **6** is 93.2 ppm, which compares favourably with the measured value of 92.7 ppm. Again, the measured (D/H)$_{4-7}$ value of **5** and the (D/H)$_{12-17}$ value of **6** give a calculated value of 125.8 ppm, as against the measured value of 125.4 ppm. Finally, although the displacements of positions C3 and C8 are coincident in the spectrum of **5**, the value for C11 obtained in **6** and for C3 obtained in **3** allows (D/H)$_8$ to be calculated as 133 ppm, and hence (D/H)$_3$ as 123 ppm, the same as measured initially (Table 3, 123.4 ppm). On the basis of these internal checks, it can be concluded that the chemical modification has not introduced aberrant values for (D/H)$_i$ of the sites in **3** thus revealed.

Two samples (see the Experimental Section) of methyl linoleate (**4**) were submitted to partial chemical degradation and the resulting products, methyl 9,9-bis(phenylthio)nona-

Table 6. *(D/H)$_i$ ratios (ppm) measured by ^{2}H NMR spectroscopy for methyl 9,9-dimethoxynonanoate (**5**) and 1,1-dimethoxynonane (**6**).*[a]

	Carbon no.[b] 9	COOCH$_3$	OCH$_3$	2	3, 8	4–7		10	OCH$_3$	11	12–17	18
sample							sample					
5 RMN11	71.5	135.3	122.7	143.5	128.2	129.8	**6** RMN12	116.5	119.0	103.4	124.9	110.8
	(5.6)	(1.0)	(1.2)	(2.8)	(1.4)	(3.0)		(3.3)	(1.4)	(1.3)	(1.0)	(1.6)
5 RMN13	72.3	137.5	120.6	146.6	130.5	130.1	**6** RMN14	113.9	118.0	110.1	121.3	117.7
	(1.4)	(1.6)	(1.6)	(0.3)	(1.7)	(2.0)		(1.8)	(1.5)	(2.1)	(2.9)	(1.2)
mean	71.9	136.4	121.7	145.6	129.4	130.0	mean	115.2	118.5	106.8	123.1	114.3
	(5.6)	(2.1)	(2.5)	(2.8)	(2.8)	(3.2)		(3.5)	(1.5)	(5.2)	(3.6)	(4.8)

[a] See footnote [a] in Table 1. [b] Carbon numbers are given as for **3**: see Scheme 1.

noate (**7**) and 1,1-bis(phenylthio)hexane (**8**), were separated by chromatography, again giving yields of 71% in each case. These samples were also submitted independently to quantitative ^{2}H NMR (Table 7). The overall standard errors are acceptable, ranging from 1.5 to 8.4 ppm for all (D/H)$_i$ values except (D/H)$_9$ and (D/H)$_{13}$. These single carbons gave errors of 12.1 and 7.4 ppm, respectively; these errors were due to the extremely small signal-to-noise ratios for these positions.

The values of (D/H)$_i$ obtained may be compared with those obtained from the intact **4**. In this instance, the (D/H)$_{18}$ value for **8** is significantly lower than that obtained for intact **4**. However, other values are in good agreement. Thus, (D/H)$_2$ shows no change. From the measured (D/H)$_{4–7}$ value for **7** and (D/H)$_{15–17}$ value for **8**, a value of 113 ppm can be calculated for the *(D/H)$_6$* value of **4**, acceptably close to the measured value of 119 ppm. In addition, from the values for C8 (resolved in **7**) and C14 (resolved in **8**), it is possible to calculate a mean of 125.0 ppm, which differs insignificantly from the measured (D/H)$_{8,14}$ value of 128.3 ppm determined in **4**. On the basis of these internal checks, it can be concluded that the chemical modification has not introduced aberrant values for (D/H)$_i$ of the sites in **4** thus revealed.

A large number of sites are measured directly in **7** and **8**. Notably, (D/H)$_{15}$ is seen to be relatively impoverished compared to the mean for methylenic positions. Similarly, both the (D/H)$_8$ and (D/H)$_{14}$ values are obtained and found not to be impoverished. In contrast, the (D/H)$_{11}$ value is markedly impoverished. Further sites may be obtained by calculation. Thus, although in **7** the C3 and C7 resonances are coincident, the value measured directly for C3 in **4** may be used to calculate a value of 94 ppm for (D/H)$_7$. Similarly, the (D/H)$_9$ and (D/H)$_{13}$ values can be determined after cleavage, to allow an estimation of the mean value of (D/H)$_{10,12}$ as 118 ppm by use of the (D/H)$_{9,10,12,13}$ for **4.**

The various measured and calculated values for the fatty acids studied are summarised in Table 8. Where more than one direct measurement has been made, a mean value is given.

Discussion

The procedure described here has, for the first time, given access to a large number of (D/H)$_i$ values for fatty acids from the same biological source. The data can thus be used for studies both from the point of view of comparison of the internal variation in (D/H)$_i$ values within a molecule, as described previously,[16] but also in terms of the intermolecular relationships between the individual fatty acid populations present. The data confirm that the distributions of ^{2}H along the chains of oleate and linoleate are nonstatistical. This can be attributed to three principal causes: the origin of the various hydrogen atoms present, the KIEs of the various enzymes in the FAS complex, and the KIEs of the desaturases acting on stearate and oleate.

The oil studied was extracted from mature seed, in which the biosynthesis of fatty acids had ceased. Thus, the sizes of the populations present represent the level of advancement of the

Table 7. *(D/H)$_i$ ratios (ppm) measured by ^{2}H NMR spectroscopy for methyl 9,9-bis(thiophenyl)nonanoate (**7**) and 1,1-bis(thiophenyl)hexane (**8**).[a]*

	Carbon no.[b]											
	9	OCH$_3$	2	8	3, 7	4–6		13	14	15	16, 17	18
sample							sample					
7 RMN15	60.7	130.1	143.6	120.9	107.8	135.0	**8** RMN16	58.9	127.0	77.6	111.9	110.2
	(11.8)	(1.4)	(2.9)	(5.2)	(3.9)	(1.0)		(6.0)	(3.4)	(1.9)	(1.8)	(0.9)
7 RMN17	58.8	126.5	135.1	127.9	106.5	132.5	**8** RMN18	63.4	125.4	77.7	108.0	108.5
	(10.8)	(1.4)	(0.4)	(6.3)	(2.8)	(1.4)		(3.6)	(0.9)	(0.9)	(0.2)	(0.8)
overall mean	59.8	128.3	139.3	124.4	107.2	133.8	overall mean	61.2	126.2	77.7	110.0	109.4
	(12.1)	(3.0)	(6.7)	(8.4)	(3.9)	(2.2)		(7.4)	(3.4)	(1.9)	(2.9)	(1.5)

[a] See footnote [a] in Table 1. [b] Carbon numbers are given as for **4**: see Scheme 1.

Table 8. *Summary of the values (ppm) measured for (D/H)$_i$ of the fatty acids isolated from peanut oil.*

Methyl ester	Carbon no.													
Palmitate **1**	2	3						4–15						16
Stearate **2**	2	3						4–17						18
Oleate **3**	2	3	4–7		8	9	10	11			12–17			18
Linoleate **4**	2	3	4–6	7	8	9	10	11	12	13	14	15	16–17	18
Initial mixture:														
	137.8	124.3	128.7		124.0	96.8	96.8	97.3	96.8	96.8	124.0		128.7	118.7
Mixture of methyl palmitate **1** and methyl stearate **2:**														
	127.6	128.8						120.0						107.7
Methyl oleate **3:**														
(mean)	143[a]	123[b]	130[c]		133[d]	72[c]	115[e]	107[e]			123[e]			115[a]
Methyl linoleate **4:**														
(mean)	138[f]	120[g]	134[h]	94[d]	124[h]	60[h]	118[d]	92[g]	118[d]	61[i]	126[i]	78[i]	110[i]	113[f]

[a] Mean value of **3** + **5**. [b] Direct value from **3**. [c] Direct value from **5**. [d] Calculated value. [e] Direct value from **6**. [f] Mean value of **4** + **7** or **4** + **8**. [g] Direct value from **4**. [h] Direct value from **7**. [i] Direct value from **8**.

different transformations. In the mixture, only 3% is methyl stearate, indicating that the majority of this fatty acid has been desaturated to oleate during maturation. However, only about 47% of the oleate has been converted into linoleate by Δ^{12}-desaturase. In the case of a normal KIE in the reactions involved in this pathway, a steady enrichment of the residual population of substrate in ^{2}H should occur.[1] The extent of this depends on the state of advancement of the reaction.[21] In the current situation, in which the molecules under consideration are both substrates and products, it is too complicated to calculate KIE values. However, the determined levels of enrichment or impoverishment at different sites show the overall influence of the various isotopic effects that have taken place. Such an analysis is only possible with **3** and **4**, as the resolution of $(D/H)_i$ values in the saturated acid fraction (**1** + **2**) is inadequate.

For positions C3 to C8 of methyl oleate (**3**), a mean $(D/H)_i$ value of 130 ppm can be calculated. Positions C12 – C17 of **3**, however, give a mean $(D/H)_i$ value of only 120 ppm. Similarly, in methyl linoleate (**4**), a mean $(D/H)_i$ value of 120 ppm can be estimated for positions C3 – C8, whereas positions C14 – C17 of **4** give a mean $(D/H)_i$ value of only 106 ppm. These variations are of the same order as observed for methyl oleate and methyl linoleate from *Helianthus annus* (sunflower) and *Carthamus tinctorius* (safflower).[16] The fact that both molecules show variation along the length of the chain can thus be considered a general effect, which may be explained in terms of isotope discrimination during biosynthesis. Essentially, during the repeated elongation of the growing chain through the addition of a unit of acetate in the form of malonyl-CoA,[5, 6] those isotopomers not containing ^{2}H are slightly favoured, due to the less favourable kinetics of reactions involving molecules carrying one or more heavy isotopes.[3, 4] This effect appears to become accentuated as the chain gets progressively longer, resulting in the observed discrepancies between those C_2 units added early in chain elongation and those added later.

Crucially, however, this study also makes the direct comparison of **3** and **4** possible. Discrepancies of 10 – 14 ppm are observed for the $(D/H)_i$ values of the methylene groups located in the 'early' and 'late' portions. This finding clearly indicates that, as the seed matures, there is discrimination against heavy isotopomers during the Δ^{12} desaturation, giving rise to an overall enrichment in the residual pool of oleate. This presumably represents not only KIEs at the level of the Δ^{12}-desaturase but also the transport of oleoyl-ACP to the rough endoplasmic reticulum and its incorporation into phosphatidylcholine.

As observed previously,[16] it is seen that even-numbered methylenic sites, those derived from the C2 position of acetate, display $(D/H)_i$ values greater than those at equivalent odd-numbered (C1-derived) sites. This fluctuation is of the order of 20 ppm and is found throughout the chain. The relatively low values at the odd-numbered sites could be explained by KIEs occurring during the β-ketoacyl-ACP reductase and enoyl-ACP reductase reactions. While no isotopic data are available for these components of the FAS complex, data for soluble aldehyde- and keto-reductases[22–25] and enoyl reductase[26, 27] indicate a significant primary KIE (1.5 – 3), which would give rise to depletion in the hydrides transferred to odd-numbered positions from NADPH.

At the even-numbered positions, however, one of the hydrogen atoms is derived from the aqueous environment and the other from acetyl-CoA through malonyl-CoA. The fact that the values obtained for even-numbered positions are relatively elevated indicates that isotopic discrimination has occurred. Firstly, this could be due to the selectivity of acetyl-CoA carboxylase, which discriminates in favour of ^{2}H retention ($k_H/k_D = 1.15$).[28] In addition, the hydrogen atoms at position C2 of malonyl-CoA possess a certain acidity, resulting in "post-malonate exchange".[29] This phenomenon will favour the more rapid exchanging out of ^{1}H than ^{2}H atoms, further enriching the malonyl-CoA pool in ^{2}H. In view of the 20 ppm difference between the $(D/H)_i$ values observed for even-numbered and odd-numbered sites, it would appear that such an exchange has occurred.

The C18 position is unique in that it possesses three hydrogen atoms derived from acetate and has passed through the intermediacy of malonyl-CoA. The $(D/H)_{18}$ values should therefore reflect the isotopic composition of the initial acetate, allowing for fractionation during chain elongation. In both **3** and **4**, the $(D/H)_{18}$ values are significantly lower than the measured values for the other even-numbered sites; this supports the proposition that these latter sites have undergone isotopic fractionations resulting in enrichment in ^{2}H.

The unsaturated fatty acids of peanut oil show the now well established overall depletion in ^{2}H at the sites of desaturation.[10–13, 16] As shown for oleate from sunflower and linoleate from safflower,[16] the C9 position in both fatty acids is highly impoverished, whereas the C10 is not. In **4**, the C13 of the Δ^{12} desaturation is impoverished, but not the C12. As argued above, the C9 and C13 methylenic groups of the substrate, stearate, are likely to be slightly impoverished due to KIEs associated with the transfer of hydride from NADPH. While these values cannot be determined directly on the stearate, it can be deduced that they fall in the range of 100 – 75 ppm, on the basis of the values determined for the C11 positions of oleate (107 ppm) and linoleate (92 ppm) and the C15 position of linoleate (78 ppm). Thus, the C9 position of oleate is 25 ppm impoverished relative to the C11 position of oleate, while the C13 position of linoleate is between 17 and 31 ppm impoverished relative to the C11 and C15 positions of linoleate. This impoverishment can reasonably be attributed to secondary isotope effects associated with the Δ^9- and Δ^{12}-desaturases.

The measured $(D/H)_{10}$ value for methyl oleate (**3**) and the calculated $(D/H)_{10}$ and $(D/H)_{12}$ values for methyl linoleate (**4**) do not show any apparent impoverishment. Thus, it is evident that, if there is any decrease in the $(D/H)_i$ value at the C10 and C12 positions, it must be very much smaller than that observed for the C9 and C13 positions.

Conclusions

The data presented here indicate that the nonstatistical isotopic distribution previously observed in just one sample each of methyl oleate (**3**; sunflower) and methyl linoleate (**4**; safflower) is a general phenomenon in the fatty acids of plant seed oils. In addition, through the isolation of **3** and **4** from the same botanical origin, it has proved possible to demonstrate that there is a general isotopic fractionation in the transformation of oleate to linoleate. It is now desirable to study the effect of the extent of advancement of the reaction on this phenomenon.

It has previously been observed that a strong KIE at the C9 position occurs during Δ^9 desaturation.[30] The current data indicate that this overall isotope effect contains a significant secondary KIE component. A strong KIE has also been reported at C12 during Δ^{12} desaturation.[31, 32] In this study, in contrast, no secondary component was found for this position. Rather, a secondary isotope effect of similar size to that observed for the C9 position during Δ^9 desaturation was measured at C13, at which negligible isotope effect was reported.[31, 32] This discrepancy indicates that, by observing natural abundance isotopic parameters, it may be possible to obtain isotopic fractionation data not visible by classical enrichment techniques.

Experimental Section

Materials:

The sample of peanut seed oil used was taken from a 2.5-L bottle of pure peanut oil, supplied by Northsea Oils & Grain Industries, Tianjin (China) and purchased in a supermarket in Beijing (China). Reference samples of FAMEs for GC were purchased from Sigma–Aldrich.

^{2}H NMR spectroscopic measurements:

^{2}H NMR spectroscopy was carried out as previously described on a Bruker DPX 400 spectrometer operating at 61.4 MHz and fitted with a ^{19}F field-frequency-locking device.[16]

The (D/H)$_i$ ratios of samples were calculated from Equation (1), where P_i and P_{ref} are the stoichiometric numbers of hydrogens in site i and in the reference, S_i and S_{ref} are the area of the signal, and M_S, m_S and M_{ref}, m_{ref} are the molecular weight and mass of the sample and the reference used, respectively. For the mixture of methyl esters, P_i and M_s take the composition of the mixture into account.[14]

$$\left(\frac{\mathrm{D}}{\mathrm{H}}\right)\mathrm{i} = \frac{P_{ref} \times m_{ref} \times M_s \times S_{si}}{P_i \times m_s \times M_{ref} \times S_{ref}} \times \left(\frac{\mathrm{D}}{\mathrm{H}}\right)\mathrm{ref} \qquad (1)$$

The internal reference (ref) was either pyridine or tetramethylurea (TMU). The isotopic ratio of TMU, (D/H)$_{TMU}$, was calibrated on the V.SMOW scale,[33] and (D/H)$_{pyr}$ for pyridine was calibrated relative to TMU.

The quantitative calculation of the monodeuterated isomers was performed by using a curve-fitting algorithm (INTERLIS; Eurofins Scientific, Nantes).[34]

Transesterification:

A solution of peanut oil (10.0 g) in a solution of sodium hydroxide in methanol (180 mL, 0.5 mol L^{-1}) was heated under reflux for 30 min and a solution of boron trifluoride (50 mL; 25% w/w) was then added. The mixture was further heated for 10 min, after which cyclohexane (50 mL) was added and the mixture was left to cool to ambient temperature. Saturated NaCl solution (10 mL) was added and the aqueous phase was extracted with cyclohexane (3 × 100 mL) and $CHCl_3$ (1 × 25 mL). The combined organic phase was washed with water (100 mL), dried (Na_2SO_4), filtered and concentrated to give 9.8 g of mixed FAMEs. The purity of the mixture was checked by GC (see below) and by ^{1}H NMR (Bruker DRX 500 MHz, $CDCl_3$). The mixture of FAMEs was sufficiently pure to be used for the ^{2}H NMR measurements without further purification. Three transesterification experiments were performed and studied by ^{2}H NMR spectroscopy (RMN1, RMN2, RMN3).

Gas chromatography:

The FAME mixture was analysed by GC on a HP-INNOWAX capillary column (30 m × 0.25 mm, film thickness 0.25 μm). Conditions of analysis: carrier gas, He, 1.2 mL min^{-1}; split injection 1:40; flame-ionisation detector temperature, 250 °C; injector temp, 180 °C; thermal gradient, 180 °C for 1 min, then 180 °C to 202 °C at 10 °C min^{-1}, then 202 °C for 1 min, then 202 °C to 215 °C at 1 °C min^{-1}, then 215 °C for 1 min, then 215 °C to 250 °C at 20 °C min^{-1}, then 250 °C for 1 min.

Relative response coefficients k' were determined for each FAME present in the mixture (C16:0, C18:0, C18:1 and C18:2) relative to methyl myristate (C14:0), according to Equation (2) where S_{ester} and S_{ref} are the areas under the peaks for the respective methyl ester and for methyl myristate, and m_{ester} and m_{ref} are the masses of the respective FAME and methyl myristate present.

$$k' = \left(\frac{S_{ester}}{m_{ester}}\right)\left(\frac{m_{ref}}{S_{ref}}\right) \qquad (2)$$

As all the k' values were equal to 1.00 ± 0.05, the composition of the mixture for the (D/H)$_i$ calculation was determined without correction from the relative peak areas of the chromatogram.

Separation of FAMEs from the mixture:

The separation of FAMEs was performed by modified argentation column chromatography on silica.[17]

Solvents were distilled and dried before use. Cotton and Fontainebleau sand were washed successively with concentrated HCl (15 mL), H_2O (200 mL) and acetone (15 mL) and then oven-dried at 70 °C (4 h).

For a typical separation of 2 g of the FAME mixture, the column was prepared as follows. An aqueous silver nitrate solution (80 mL, 50% w/v) was added with stirring to silica (110 g, Sigma, 70–230 mesh). The silica was then dried in an oven at 120 °C (24 h). The silver-impregnated silica was used as normal silica for flash chromatography and the column was always protected from light. The column (37.5 × 3.0 cm) was filled with the silver-impregnated silica suspended in cyclohexane (100 mL). A pressure of 0.25 bar was applied to the top of the column during preparation of the column and throughout separation.

The solvent gradient used for the separation was as follows: 0.25 L hexane; 2.0 L hexane/AcOEt (99.5:0.5); 1.0 L hexane/AcOEt (99:1); 1.0 L cyclohexane/AcOEt (98:2); 1.0 L cyclohexane/AcOEt (97:3); 1.0 L cyclohexane/AcOEt (96:4); 1.0 L cyclohexane/AcOEt (90:10); 1.0 L (cyclohexane/AcOEt 80:20).

Fractions of 60-mL volume were collected and the presence of FAMEs was checked by GC. After pooling of the fractions containing the same FAME and removal of solvent in vacuo, the purity of each fraction, as determined by GC and ^{1}H NMR, was > 99%.

A typical experiment from 1.95 g of FAME mixture gave 0.3 g of **1** + **2**, 0.8 g of **3** and 0.7 g of **4**. The calculated composition of the starting sample based on the individual fractions was similar to the composition determined by GC.

Four separations on silver columns were carried out and the three fractions obtained from two of these were pooled to give enough material of **1** + **2** to measure by ^{2}H NMR spectroscopy. Thus, one sample (0.7 g) of **1** + **2** (RMN4), three samples (0.8 g, 0.8 g and 1.8 g) of **3** (RMN5, RMN6, RMN7) and three samples (0.7 g, 0.7 g and 1.6 g) of **4** (RMN8, RMN9, RMN10) were obtained and studied by ^{2}H NMR spectroscopy.

Chemical modification:

Chemical modifications were carried out as previously described.[16] As the chemical modifications were performed on 1 g of starting material, two samples of **3** (RMN5, RMN6) and two samples of **4** (RMN8, RMN9) were combined. Hence, two separate chemical modifications have been carried out for each of **3** (on RMN4 and on RMN5 + RMN6) and **4** (on RMN7 and on RMN8 + RMN9). Modification of methyl oleate (**3**) gave derivatives **5** (RMN11, RMN13) and **6** (RMN12, RMN14). Modification of methyl linoleate (**4**) gave derivatives **7** (RMN15, RMN17) and **8** (RMN16, RMN18).

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