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The nature of cyclic fatty acids formed in heated vegetable oils

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SUMMARY

The nature of cyclic fatty acids formed in heated vegetable oils

The nature of the complex mixtures of monocyclic fatty acids formed in heated sunflower oils and linseed oil are summarized. The analytical approach involved fractionation of total cyclic fatty acids by silver ion high-performance liquid chromatography and characterization by gas chromatography - mass spectrometry of the picolinyl esters and/or dimethyloxazoline derivatives and Fourier transform infrared spectroscopy of the methyl esters. The cyclic fatty acids derived from oleate were saturated, those from linoleate were monoenes and those from linolenate were dienes. They contained five- and six-membered rings and differed according to the positions and configurations of the double bonds and rings.

Possible mechanisms for their formation via allylic radical intermediates are discussed.

KEY-WORDS: Cyclic fatty acids- Heated linseed oil- Heated sunflower oil.

1. INTRODUCTION

Numerous monomeric cyclic fatty acids are formed in heated vegetable oils. Their occurrence, structures and biological effects have been reviewed (Sebedio and Grandgirard, 1989). They are absorbed extremely well in the intestines of rats and they have been found to be potentially toxic. When they were fed to pregnant rats there was an increase in mortality of the pups during the first days of life. It was suggested that cyclic fatty acids from linolenic acid were more toxic than those from linoleate, which indicates that the cyclic fatty acids formed in sunflower oils may be relatively harmless, although this needs to be confirmed. Toxicity experiments were carried out, at best, using a total cyclic monomer fraction but the specific cyclic compounds involved in toxicity have yet to be determined. Cyclic monomers have been detected at low but varying levels (0.01-0.7%) in commercial frying oils but the levels required to produce a physiological effect are unknown.

Over the past 30 or so years, information has accumulated on the structure of these fatty acids (Sebedio and Grandgirard, 1989). They either have five- or six-membered rings and those derived from linoleate in sunflower oil are monoenoic whereas those from linolenate in linseed oil are dienoic. The mixtures have been simplified by hydrogenation to remove the double bonds, and the skeletal structures were determined by gas chromatography-mass spectrometry (GC-MS) of the methyl esters. The configurations of the rings have also been assigned. The degree of unsaturation and geometry of the double bonds of many of the native fatty acids were determined on the basis of GC-MS and GC-Fourier Transform Infrared Spectroscopy (FTIR), respectively, of the methyl esters. The complete characterization of all components, particularly with respect to the positions of the double bonds, had not been undertaken and this was the objective of our studies.

The approach was to convert the heated oils to fatty acid methyl esters and isolate a total cyclic fatty acid methyl ester fraction by a combination of urea adduction, column chromatography and preparative high-performance liquid chromatography (HPLC) of the non-adducted fraction. The cyclic fatty acids were then fractionated according to degree of unsaturation, configuration of the double bonds, and size and configuration of the ring by silver ion HPLC as their phenacyl esters. The fractions, containing a limited number of fatty acids, were then amenable to characterization by GC-MS as their picolinyl esters and dimethyloxazoline (DMOX) derivatives. These derivatives have been used with great success in elucidating the structures of fatty acids, particularly in determining the position of double bonds and the position and nature of other structural features (Dobson and Christie, 1996). The assignments of structures were greatly aided by performing GC-MS of the DMOX derivatives of hydrogenated and deuterated as well as native fatty acid fractions.

2. MATERIALS AND METHODS

2.1. Isolation of cyclic fatty acids

A normal sunflower oil and a linseed oil were heated under nitrogen at 275°C in the laboratory. Normal and high-oleate sunflower oils were used in small-scale frying trials with french fries as detailed elsewhere (Dobson *et al.*, 1996a). The temperature varied between 140 and 200°C during the trials. The oils were converted into fatty acid methyl esters and then separated by column chromatography on silicic acid. The less polar fractions were submitted to urea fractionation; the adducts contained the usual fatty acids, while the non-adducted fraction contained a mixture of the cyclic fatty acids and either linoleic or linolenic acid, depending on the type of oil. The non-adducted fraction was further purified by preparative reversed-phase HPLC on a C_{18} column in the recycle mode in order to reduce the amount of linoleic/linolenic acid.

2.2. Derivatization procedures

Esterified fatty acids were hydrolysed with ethanolic potassium hydroxide as described previously (Christie *et al.*, 1993). Methyl ester derivatives were prepared by sodium methoxide-catalysed transesterification (Christie, 1989) and phenacyl derivatives were prepared as described elsewhere (Wood and Lee, 1983). Fatty acid methyl esters were hydrogenated with hydrogen and platinum oxide catalyst (Christie, 1989) and were deuterated with deuterium gas and Wilkinson's catalyst (Dickens *et al.*, 1982). Picolinyl ester derivatives were prepared by the method of Balazy and Nies (1989) and DMOX derivatives according to Fay and Richli (1991).

2.3. Silver ion high-performance liquid chromatography

A Spectra-Physics Model 8700 solvent delivery system was used (Spectra-Physics Ltd, St. Albans, UK.), together with a Cunow model DDL 21 detector (Severn Analytical Ltd, Shefford, UK). A stream-splitter (approx. 10:1) was inserted between the column and the detector to enable collection of fractions. A column (4.6 x 250 mm) of Nucleosil™ 5SA (HPLC Technology Ltd, Macclesfield, UK) was converted to the silver ion form as described elsewhere (Christie, 1987).

For micro-preparative purposes, 0.5 to 1 mg of phenacyl esters were applied to the column in 10 µL of dichloroethane. The column was eluted with a gradient of dichloromethane-dichloroethane (1:1, by volume) (Solvent A) and dichloromethane-dichloroethane-acetonitrile (49:49:2) (Solvent B). For cyclic monoenes from sunflower oils, solvent A was maintained for 5 minutes, after which there was a linear gradient from 100% A to 75% A-25% B over 60 minutes. For cyclic dienes from linseed oil, the same gradient was started immediately and run over 50 minutes, and there was an additional final gradient to 100% B over a further 5 minutes. The flow-rate was 1 mL/min. A standard solution of methyl hexadecanoate was added to each fraction as an internal standard for subsequent quantification by GC, following transmethylation.

2.4. Analytical gas chromatography

Methyl esters of fatty acids were analysed either on a Carlo Erba Model 4130 (Erba Science, Swindon, UK) or a Hewlett Packard Model 5890 Series II (Hewlett Packard Ltd., Wokingham, UK) capillary gas chromatograph, fitted with split/splitless injection, and equipped with a capillary column (0.25 mm i.d. x 25 m in length, 0.2 µm film thickness) of fused silica coated with Carbowax 20M™ or CP-Wax 52CB™ (Chrompack UK Ltd, London) for routine analyses. Hydrogen was the carrier gas. After holding the temperature at 170°C for 3 min, the column was temperature-programmed at 2°C/min to 210°C, then was held at this point for a further 10 minutes. Capillary columns of CP-Sil 84™ (0.22 mm x 25 m x 0.22 µm; Chrompack UK Ltd.), BPX70™ (0.22 mm x 50 m x 0.25 µm; SGE(UK) Ltd., Milton Keynes) and OV-275 (0.22 mm x 25 m x 0.2 µm; Chrompack UK Ltd.) were also used. Details of conditions are given in the appropriate publications (Christie *et al.*, 1993; Dobson *et al.*, 1995; Dobson *et al.*, 1996b).

2.5. Gas chromatography-mass spectrometry

Fatty acids were subjected to GC-MS in the form of the picolinyl ester and DMOX derivatives. Either a Hewlett Packard Model 5890 gas chromatograph attached to a Model 5970 Mass Selective Detector or a Carlo Erba Mega Series gas chromatograph connected to a Kratos 8/90 double-focusing magnetic sector instrument (Kratos Analytical, Manchester, UK.) were used. For the former instrument, a fused-silica capillary column (0.25 mm x 25 m x 0.25 µm), coated with a cross-linked (5% phenylmethyl) silicone (CP-Sil 8CB™, Chrompack UK Ltd) was used and picolinyl esters were injected onto the column at 70°C (held for 0.5 min), and temperature-programmed to 220°C at 60°C/min, when this was held for 40 min. For the latter instrument, an equivalent column (DB-5MS™, 0.25 mm x 30 m x 0.25 µm; J & W Scientific) to the former was used. Picolinyl esters were injected on to the column at 80°C (held for 3 min), temperature-programmed to 200°C at 30°C/min, then to 280°C at 2°C/min and finally to 320°C at 10°C/min. DMOX derivatives were injected at 80°C (held for 3 min), temperature-programmed to 160°C at

20°C/min then to 325°C at 4°C/min. DMOX derivatives were also analysed on a BPX70™ (0.22 mm x 50 m, 0.25 µm) capillary column. The temperature was held for 80°C for 3 min, temperature-programmed to 160°C at 20°C/min and then to 260°C at 2°/min. The column outlet was connected directly into the ion source of the mass spectrometer operated at an ionization energy of 70 eV.

2.6. Gas chromatography-Fourier transform infrared spectroscopy

The gas-phase infrared spectra were obtained on a Bruker IFS 85 Fourier transform spectrometer, connected via a gold-coated light-pipe (0.8 mm x 20 cm) to a Carlo Erba 5160 gas chromatograph fitted with an on-column injector, a DBwax fused silica capillary column (0.32 mm x 30 m, 0.5 µm, J & W. Scientific) and a flame ionisation detector. The carrier gas (helium) flow rate was 2 mL/min (velocity 35 cm/sec). The oven temperature was programmed from 35 to 80°C at 30°C/min, to 150°C at 10°C/min and then to 220°C at 5°C/min.

Nitrogen make-up gas was introduced into the transfer line to reduce peak broadening within the light-pipe (maintained at 220°C), resulting in a total gas flow rate of 5 mL/min. The spectral resolution was 8 cm^{-1}, and time resolution was reduced from 12 collected interferograms to 4 effective ones by adding 3 interferograms in real time. A narrow band (4800-600 cm^{-1}) mercury-cadmium-telluride (MCT) detector was used.

Bands around 970 cm^{-1} and 660 cm^{-1} corresponded to *a trans* double bond in a straight chain and a *cis* double bond in a cyclohexenyl ring, respectively. A band around 710 cm^{-1} was indicative of a *cis* double bond in a straight chain and/or a cyclopentenyl ring.

3. RESULTS AND DISCUSSION

3.1. Chemical characterization of cyclic fatty acids

The phenacyl esters of the cyclic fatty acids, derived from linoleate in laboratory-heated sunflower oil, were separated by silver ion HPLC into several peaks and eight fractions were collected (Christie *et al.*, 1993) (Figure 1). Analysis of the fractions as the methyl esters by GC revealed many components. Each fatty acid was characterized by GC-MS of the picolinyl esters of not only the native fractions but also of the hydrogenated and deuterated components. The type and position of the ring and position of double bonds in the straight chain, but not in the ring, were determined. In all but the first silver ion HPLC fraction the fatty acids were monocyclic and monoenoic in nature.

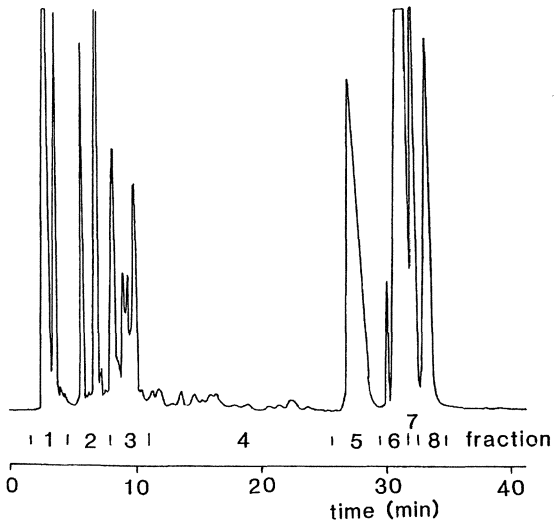

Figure 1
Silver ion HPLC separation of phenacyl esters of cyclic fatty acids derived from linoleic acid in heated sunflower oil. Elution conditions as in Materials and Methods.

The mass spectrum of the picolinyl ester of a monoenoic fatty acid with a cyclohexyl ring between C-5 and C-10 of the original chain and a double bond at C-12 is shown as an example (Figure 2A). The molecular ion is at *m/z* 371 and a gap of 82 amu between *m/z* 178 and *m/z* 260 allows the size and position of the ring to be assigned. The double bond is located by a gap of 26 amu between *m/z* 274 and *m/z* 300. The mass spectrum of the hydrogenated compound (Figure 2B) also has the ions locating the ring but the molecular ion is 2 amu higher (*m/z* 373), and regular gaps of 14 amu above *m/z* 260 verifies the saturated nature of the straight chain.

The monoenes were derived from linoleic acid and each of the double bonds reacted to about the same degree. About half of the total monoenes were produced by cyclization from one double bond in the direction of the other to form cyclopentene rings from C-8 to C-12 and C-10 to C-14 of the original chain, with loss of the double bonds at C-12 and C-9, respectively (Figure 3A). Cyclization occurred with about equal probability in both directions to give only one of the two possible ring configurational isomers in each case. Although the positions of the double bonds in the rings could not be determined from the mass spectra, in some cases (Formulae I and II in Figure 4) they appeared to remain at the original C-9 or C-12 positions. This seems reasonable because from the mass spectra of derivatized cyclohexenyl fatty acids from linolenate, without doubt the double bond in the ring was always at C-12 (see below). In other cases, the double bond appeared to migrate to a substituted ring carbon (for example Formulae III and IV). This was suggested by the formation of two ring geometrical isomers upon hydrogenation of a single fatty acid. Perhaps surprisingly, cyclohexenyl fatty acids were not detected.

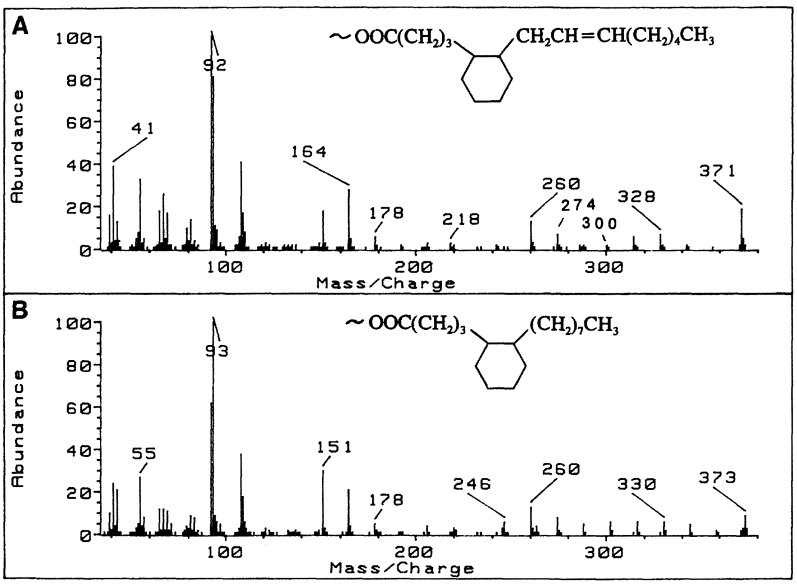

Figure 2
Mass spectra of picolinyl ester derivatives of **A.** Native and **B.** Hydrogenated cyclopentyl monoenoic fatty acid (Formula VII in Figure 4).

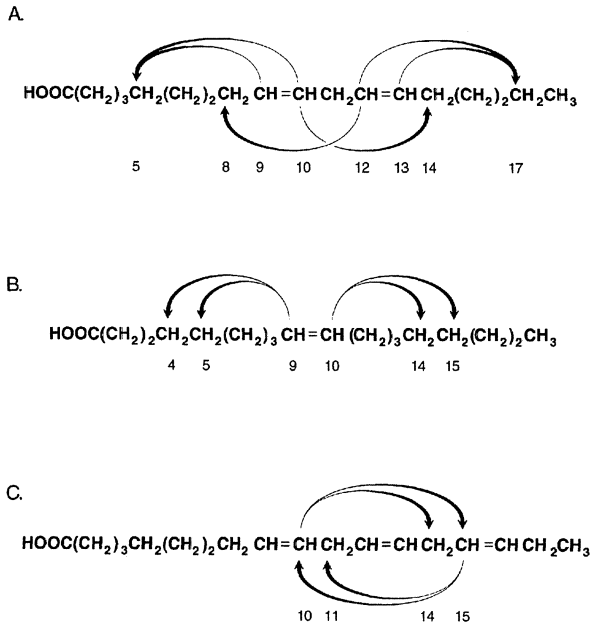

Figure 3
Carbon atoms involved in cyclization of **A.** Linoleic acid **B.** Oleic acid and **C.** Linolenic acid

Cyclization also occurred from a double bond towards the ends of the molecule to form fatty acids with either a cyclopentane ring between C-5 and C-9 (Formula V) or C-13 and C-17 (Formula VI) or a cyclohexane ring between C-5 and C-10 (Formula VII), C-12 and C-17 (Formula VIII) or C-13 to C-18 (Formula IX) together with a *cis* double bond (determined by GC-FTIR) in the straight chain at either the original C-9 or C-12 positions. Cyclization again occurred with approximately equal probability in each direction (Figure 3A), but this time most ring configurational isomers were represented. Only in the cyclohexane fatty acids were isomers with *trans* double bonds detected. They eluted in an earlier HPLC fraction than the corresponding *cis* fatty acids and were formed presumably by stereomutation at high temperature; they appeared to exist in a restricted range of ring configurations.

The saturated cyclic fatty acids (earliest eluting silver ion HPLC fraction) were examined from normal and high-oleate sunflower oils which had been used in a small-scale frying trial (Dobson *et al.*, unpublished). Many peaks were revealed by GC (Figure 5), all of which were saturated because the profile remained unchanged after hydrogenation. They were examined as their DMOX derivatives by GC-MS and could be divided into two distinct groups; the later eluting peaks were bicyclic, but the mass spectra were difficult to interpret, whereas the earlier eluting peaks were monocyclic. The bicyclic fatty acids were more abundant (as a proportion of the total cyclic fatty acids) in the normal sunflower oil whereas the monocyclic fatty acids were greater in the high-oleate sunflower oil. They were presumably derived from linoleate and oleate, respectively.

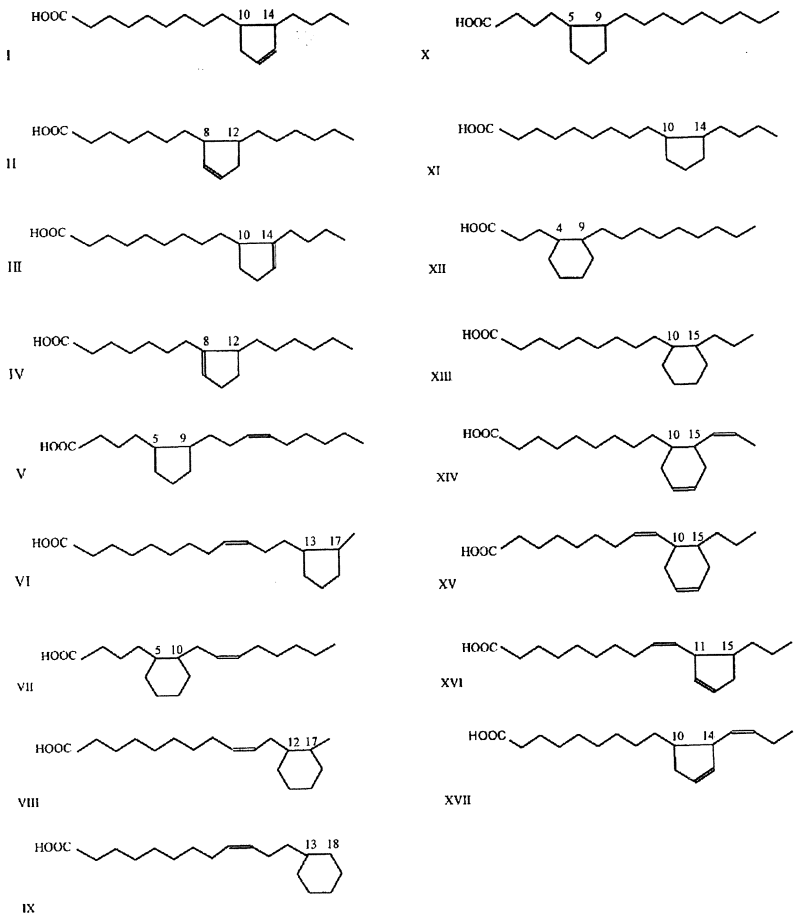

Figure 4
Structural formulae of cyclic fatty acids derived from linoleic acid (**I-IX**) and oleic acid (**X-XIII**) in heated sunflower oil
and linolenic acid (**XIV-XVII**) in heated linseed oil.

Eight monocyclic saturated fatty acids, comprised of four basic structures, were characterized. There were cyclopentyl fatty acids with rings from C-5 to C-9 (Formula X) and C-10 to C-14 (Formula XI), and cyclohexyl fatty acids with rings from C-4 to C-9 (Formula XII) and C-10 to C-15 (Formula XIII). The cyclopentyl fatty acids were about twice as abundant as the cyclohexyl fatty acids. Cyclization occurred in both directions and was favoured slightly more towards the terminal methyl end of the molecule than towards the carboxyl group (Figure 3B). Each structure was represented by two components corresponding to *cis* and *trans* ring isomers.

Sixteen cyclic dienoic fatty acids formed from linolenic acid were characterized from heated linseed oil (Dobson *et al.*, 1995). There were approximately equal amounts of cyclohexenyl and cyclopentenyl fatty acids comprising four basic structures. There were fatty acids with cyclohexenyl rings formed from C-10 to C-15 with the double bond shifted to either the C-16 (Formula XIV) or C-8 (Formula XV) positions, and fatty acids with cyclopentenyl rings formed from C-11 to C-15 (Formula XVI) and C-10 to C-14 (Formula XVII) with a double bond at either the original C-9 or C-15 positions, respectively. The presence of an ion corresponding to a retro Diels-Alder fragment in the mass spectra of derivatized cyclohexenyl fatty acids unequivocably proved that the double bonds in the rings remained at the C-12 position. It seems likely that the double bonds in the rings of the cyclopentenyl fatty acids were also at C-12 but this could not be confirmed by mass spectrometry.

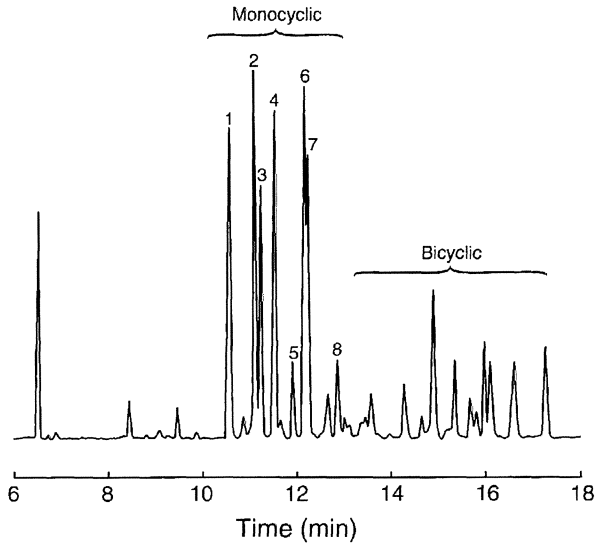

Figure 5
Partial gas chromatographic trace of saturated cyclic fatty acid methyl esters derived from normal sunflower oil. Conditions as in Materials and Methods. Peaks 1 and 4, and 2 and 6 contain a cyclopentyl ring from C-5 to C-9 (Formula X in Figure 4), and C-10 to C-14 (Formula XI), respectively, of the original chain in oleic acid. Peaks 3 and 5, and 7 and 8 contain a cyclohexyl ring from C-4 to C-9 (Formula XII), and C-10 to C-15 (Formula XIII), respectively.

Each of the four basic structures was represented by all four possible geometrical isomers arising from variations in configuration of the ring and of the double bond in the straight chain. In contrast, not all configurational isomers were present in the products from linoleic acid. For example, *trans* double bonds were only in the straight chains of six- and not five-membered ring fatty acids. Cyclization involved only the double bonds at C-9 and C-15 and, again in contrast to the situation for linoleic acid, did not involve the double bond at C-12 and was always directed towards the other double bond and not towards the ends of the molecule (Figure 3C).

Another group of workers have studied similar dienoic compounds from heated linseed oil (Mossoba *et al.*, 1994)(Mossoba *et al.*, 1995) and there was agreement with our study for ten of the structures. Their approach was to analyse the total mixture as DMOX derivatives by GC-MS. The advantage of an approach that analyses simplified fractions produced by silver ion HPLC was evident because an additional three components, which by GC overlapped with other peaks in the total mixture, were revealed in our study. Also, we believe that three of the fatty acids in the other study were misidentified because the mass spectra were, understandably, wrongly interpreted. In this context, we have found the use of deuterated fatty acids to be of great value.

The gas chromatographic properties, including equivalent chain lengths, of the cyclic dienes were examined on capillary columns of different polarities (Dobson *et al.*, 1996b). Identification and quantification of all fatty acid methyl esters was achieved only by using two different columns (CPWax-52CB and BPX-70 or CPSil 84). The method was used to determine the cyclic fatty acid compositions in the livers of rat mothers and pups following feeding of the pregnant females with a total cyclic diene mixture (unpublished results). The relative proportions of the individual cyclic dienoic fatty acids were different to those in the oil, indicating that there had been selective incorporation.

3.2. Possible mechanisms for formation of cyclic fatty acids

We have proposed that cyclic fatty acids are formed in heated vegetable oils via radical intermediates (Christie *et al.*, 1993; Dobson *et al.*, 1995). The simplest case is that of saturated monocyclic fatty acids from oleate which may produce four allylic radicals at C-8, C-9, C-10 and C-11. These may undergo 1,6- (Figure 6A) and 1,5-hydrogen (Figure 6B) shifts followed by cyclization between the radical and an olefinic carbon to form cyclopentyl and cyclohexyl products, respectively (Table I). Each radical gives rise to one observed product resulting in two cyclopentyl (Formulae X and XI) and two cyclohexyl fatty acids (Formulae XII and XIII) in total (not including ring geometrical isomers). However, there are eight possible products and it is unclear why four of them were not observed and why for some radicals a 1,5-hydrogen shift was favoured whereas for others a 1,6-hydrogen shift was favoured.

Table I
Expected monocyclic saturated products from heated oleate via radical intermediates

Double bond position	Radical position	Ring position* 1,5-hydrogen shift	Ring position* 1,6-hydrogen shift
9	8	4-9	(9-13)
9	11	10-15	(6-10)
8	10	(9-14)	5-9
10	9	(5-10)	10-14

* Positions labelled according to original positions in oleic acid. Bracketed ring positions were not observed.

In linoleate, allylic radicals may be formed from C-8 to C-14. The 9,12- pentadiene unit will give the C-11 radical most easily, but the more stable radicals at C-9 and C-13 will be favoured in this five-carbon delocalized system. In addition, the double bonds can be involved individually to produce radicals at C-8 and C-14 which may be delocalized to C-10 and C-12, respectively. With

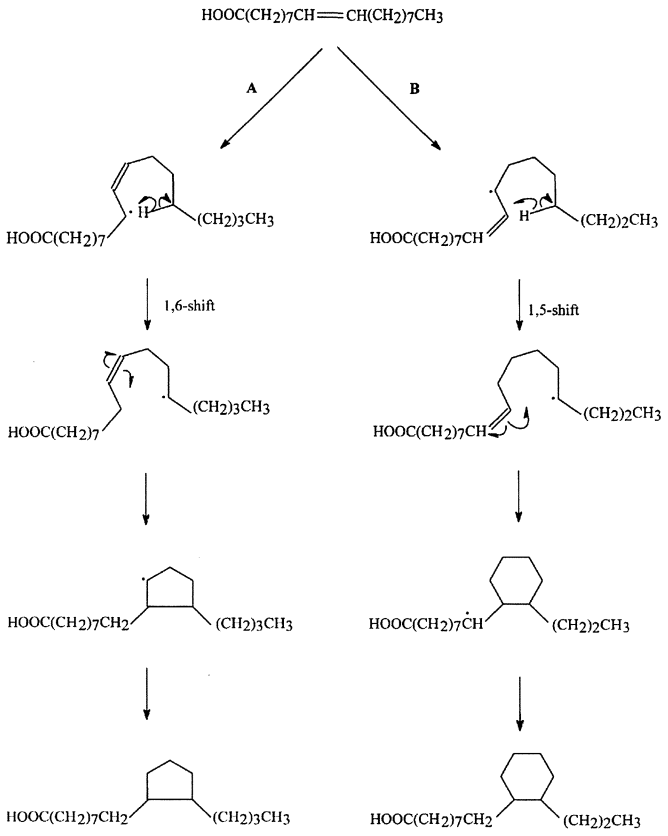

Figure 6
Possible mechanisms for the formation of
A. 9-(2'-Butylcyclopentyl)nonanoic acid (Formula XI in Figure 3) and **B.** 9-(2'-Propylcyclohexyl)nonanoic acid (Formula XIII)

Table II
Expected monocyclic monoenoic products from heated linoleate via radical intermediates

Double bond positions	Radical position	Ring (double bond) position*		
		1,5-hydrogen shift	1,6-hydrogen shift	No hydrogen shift
9,12	8	[4-9(12)]		8-12(9) [8-13(9)]
9,12	11	[7-12(9)] [10-15(12)]	[6-10 (12)] [12-16(9)]	
8,12	10	[9-14(12)]	5-9(12)	
10,12	9	5-10(12)	10-14(12)	[9-13(10)]
9,12	14	13-18(9)		[9-14(12)]10-14(12)
9,11	13	12-17(9)	8-12(9)	[9-13(11)]
9,13	12	[8-13(9)]	13-17(9)	

* Positions in cyclic products labelled according to original positions in linoleic acid.
Bracketed ring positions were not observed.

respect to the arrangement within the radical/diene unit, the radicals at C-9 and C-13 are similar, as are those at C-8 and C-14, and C-10 and C-12.

Cyclization between a radical and an olefinic carbon may occur with or without a preceding 1,5 or 1,6-hydrogen shift to give monocyclic monoenoic fatty acids. In total, there are sixteen possible products (not including double bond and ring geometrical isomers), some of which may be derived by two different routes (Table II). Seven (Formulae I, II, V-IX) of the nine observed products can thus be accounted for and the remaining two (Formulae III and IV) may be formed by thermal rearrangement of the ring double bond of two of these products (Formulae I and II).

Nine of the possible products were not observed. On the basis of the similarity of the C-8 and C-14 radicals, one might have expected to have detected a cyclohexyl fatty acid with a ring from C-4 to C-9 and a double bond at C-12 (Table II); the radicals within each of the other pairs of similar radicals (C-9 and C-13, and C-10 and C-12) gave analogous observed products to one another. Considering that the radical at C-11 is relatively unstable, it is perhaps not surprising that the four possible products from this radical were not observed. Presumably the remaining four products [8-13(9), 9-13(10), 9-13(11) and 9-14(12)] were not detected because competing mechanisms (cyclization with 1,5-, 1,6- or no hydrogen shifts) favoured the formation of other products. Indeed all radicals (apart from the C-11 radical) produced at least one product.

Linolenate can produce radicals from C-8 to C-17 resulting in twenty possible products. Only four (Formulae XIV-XVII) were observed, all of which could be predicted from allylic radical mechanisms involving the double bonds at C-9 and C-15. Products involving loss of the C-12 double bond were not detected. Also, products involving cyclization with olefinic carbons of the C-9 double bond and radicals nearer the carboxyl group (i.e. C-4 to C-9 and C-5 to C-9 rings with double bonds at C-12 and C-15) were not detected. Again it would appear that the stability of the various radicals and the outcome of competing mechanisms, in the presence of double bonds which are not necessarily involved directly in the cyclization process, are important factors influencing the types of products formed.

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