Effect of Varietal or Subvarietal Alterations on the High Order Compositeness Status of Fats from the Same Biological Species. The Glyceride Structures of Three *Myristica malabarica* Mace Fats

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Abstract

Mace fats from three morphologically indistinguishable subvarieties of *Myristica malabarica* and from the same geographical area were investigated for high order compositeness (HOC) indices (2,3) by the azelaoglyceride analysis technique (1). The fats had saturated acid contents of 36.5, 43.7, and 49.8% by molecules, and the differences in the component acid compositions were adequate to establish differences in the source of biological origin in all three cases. The subvarieties thus showed uncommonly large alterations in fat metabolism genes for any particular biological species so far reported.

All three fats were of the GS_3 nil type, and the GS_2U values of the HOC indices were +1.9, +2.6, and +1.8 respectively. These differences were within the limits of experimental error, and all three fats showed the same HOC index in spite of large differences in component acids. Subvarietal alterations involving large changes in component acids can hence take place without altering the HOC indices.

This is perhaps possible only if there are separate genes controlling HOC in the fat metabolism genes complex, and these are not necessarily affected in the same manner by factors which produce changes in the genes that control component acid compositions. HOC is thus seen to be a new and independent structural feature of natural fats, which, in the future, has to be considered along with theories of bio-esterification to give complete explanations for triglyceride structure.

Introduction

IN ORDER TO TRACE the biological factors controlling the distribution and degree of HOC (2,3) in natural fats, the seed fats from five different species of the genus *Cassia,* Family Leguminacae, were examined for their HOC indices (4) by methods used earlier in the series (1–3). The fats were from *Cassia occidentalis,* Sm 23.7 (Sm = molar percentage, saturated acids). *Cassia fistula,* Sm 27.1; *Cassia auriculata,* Sm 28.1; *Cassia sophora,* Sm 31.3; and *Cassia tora,* Sm 32.6. All the fats were of the GS_3-nil type, and the GS_2U values for the HOC indices were −1.3, −1.3, +7.9, +3.4, and −1.6, respectively (4). The large variability in the HOC indices in fats from different species of the genus *Cassia* showed that the degree of HOC is not a genus characteristic but may be a species or varietal characteristic.

Examination of seed fats from seven different varieties of peanut showed that, though sometimes there were appreciable differences in component unsaturated compositions, the saturated acid contents varied only between 18 to 20%, and the proportions of GS_2U were within 1% of those required by the Glyceride Type Distribution Rule (GTDR) (1). The fats thus showed no HOC indices (3). This result showed that alterations to produce morphologically different varieties can take place without affecting the fat metabolism genes with respect to HOC indices or proportions of saturated and unsaturated acids or, in some cases, full component acid compositions. However, when the seed fats from brown and white seeded varieties of *Sesamum indicum* were examined, a different relationship was observed (5). The former had Sm 16.0 and HOC index GS_3-nil, GS_2U −1.0 (1) whereas the latter, with Sm 14.0, showed HOC index of GS_3 nil, GS_2U +3.4 (5). Hence the degree of HOC was essentially a varietal characteristic.

It is widely recognized that the same biological varieties collected from different geographical areas frequently show appreciable differences in component acid compositions of seed fats though there may not be any detectable morphological alterations in the plants themselves. The changes involved are sometimes caused by differences in climatic, soil, or other environmental conditions. However, they can be and frequently are caused by changes in genes which are not connected with morphological differentiations. Such subvarietal forms are frequently called "geographic varieties." They will retain their differences in acid compositions even when grown under the same environment. Variations of HOC indices in geographical varieties are of interest in connection with glyceride structure because different specimens of fat of the same biological variety which are obtained from different geographical areas may well be derived from different geographical varieties. Interrelationships between variations in HOC indices and component acids in geographical varieties or subvarieties can be of the same type as in normal varieties, and they may be of two kinds: a) the HOC indices may remain unchanged during changes in component acid compositions and b) HOC indices may increase with an increase in saturated acid contents or may decrease with an increase in saturated acid contents.

In the case of GS_3-nil seed fats from two geographical varieties of *Vateria indica,* with saturated acid contents of 57.1 and 59.3% respectively, the GS_2U values for the HOC indices were +2.2 and +5.4 respectively (6). The HOC indices showed a measurable increase with an increase in saturated acid content, and the significance of this variability in the prediction of the glyceride structure of natural fats from component acid composition and other biological constants has been discussed elsewhere (6).

Variations in HOC indices with changes in the

component acid compositions of varietal or subvarietal seed fats however cannot readily elucidate the genetic factors controlling HOC. On the other hand, if it is found that the HOC indices can remain unchanged during substantial variations in the component acid composition which is produced by varietal or subvarietal differentiations, then this can form immediate and interesting support for the possibility that HOC is an independent structural feature, which is controlled by separate HOC genes in the fat metabolism genes complex. This will be of considerable utility in the further development of the HOC concept.

The screening of normal and geographical varieties derived from different parts of the country showed that considerable differences in component fatty acid compositions (indicated by variations of 10 to 20 units in iodine value was frequently observed although the differences in the proportions of saturated acids present in fats from the same biological species was of a much lower order. Instances where different varieties from the same biological species showed more than 4 to 5% difference in saturated acid contents were rare and were altogether absent in many genera. This was particularly marked in cases where the total saturated acid content was below about 35–40%. However, in some isolated species, saturated acid variations of the order of 4 to 5% or higher were more common. In these instances perhaps the fat metabolism genes are of a more labile or unstable type and are more likely to undergo deep-seated changes under conditions which produce varietal or subvarietal alterations.

During this investigation one of the authors (A.R.S.K.) made an unusually interesting collection of three subvarietal mace fats of the species *Myristica malabarica* from different localities in the Cochin district of South India. There is no appreciable variation in climatic, soil, or other environmental conditions in this small area of 500 square miles near the sea. Hence the differences in the seed fats could not be attributed to differences in the environments. The original trees were not examined for they grew in rather inaccessible reserve forests. They were however reported to be collected from identical trees. The samples of mace were quite similar in shape, color, texture, and aroma to authentic *M. malabarica* mace specimens.

It may be noted that mace derived from different species of *Myristicacae* invariably show characteristic and marked differences in the above respects. Though mace from some species of *Myristica* (for example, *Myristica fragrans*) contains considerable quantities of essential oil, *M. malabarica* mace does not contain any essential oil.

This collection of three mace fats showed uncommonly large variations in component acids for varietal or subvarietal fats from any particular biological species so far indicated in the literature. A detailed report of the glyceride structure of these fats is now given from the point of view of variations in HOC indices which were produced during the changes in component acid composition.

Experimental Section

The mace samples did not show any visible signs of disease or damage as compared with authentic samples. Therefore there is little possibility that any alterations in the fats could have been caused by either of these factors. The air-dried samples were ground in a mortar, and 20-g specimens were distilled in a flask connected to an essential oil trap. No separate layer of essential oil could be observed in the trap after six hours of distillation. Hence the essential oil content of all three mace specimens was negligible.

For removal of oil the ground samples were extracted with petroleum ether, bp 60–80C in a Soxhlet. The crude fat contains an acidic resin, giving a deep red color with alkali. It was refined by dissolving in diethyl ether and washing repeatedly with 1% aqueous potassium hydroxide until the aqueous alkaline extracts remained colorless. The physical and chemical characteristics of the fat required for the calculation of the glyceride structure were determined by previous methods (1,2,3) and are given in Table I. Since it has been observed that the use of different techniques for the determination of component acids leads to substantial differences in composition (7) and since it has only been possible thus far to determine the HOC indices by oxidation techniques, oxidation methods were used for fatty acid analysis wherever possible.

Component Acid Compositions of the Mace Fats in Relation to Their Sources of Biological Origin. The acid compositions of the mace fats were calculated from the saturated acid contents as determined by oxidation procedures (1,2,3) and the characteristics of the mixed fatty acids. The saturated acids contained only palmitic and stearic acids (ester fractionation). The unsaturated acids, which consisted almost entirely of C_{18} acids, contained only mono- and diethenoid acids since the hexabromide tests were negative. The final values calculated are given in No. 15, Table I.

These results show that Mace Fats 1 and 3 have a difference of about 14 units percentage in saturated acid contents. This indicates clearly a difference in

TABLE I

Characteristics of Three *Myristica malabarica* Mace Fats

Details	Mace Fat I	Mace Fat II	Mace Fat III
1. Fixed oil content of dried mace			
crude fat, %	25.0	32.0	33.0
refined as in text, %	16.0	26.0	27.0
2. MP °C	9.4	21.1	24.4
3. IV (Hanus', 30 min)	66.6	57.0	53.0
4. Hehner value	91.7	94.0	95.4
5. Unsaponifiables, %	1.9	0.4	0.8
6. Insoluble acids: on fat, %	89.8	93.6	94.6
MP	42.2	44.4	45.0
IV	62.2	55.0	54.0
7. Unesterifiable acids, %	1.5	2.8	1.6
8. Nonfat lactones in Bertram acids, %	nil	nil	nil
9. Bertram acids from unsaponifiables, esterifiable resin acids, unesterifiable resin acids	nil	nil	nil
10. Bertram acids, fat basis			
a)	31.3	38.2	44.5
b)	.....	38.2	45.0
mean	31.3	38.2	44.8
11. Saturated fatty acids			
on fat, %	31.3	38.2	44.8
Mean MW	269.0	264.0	264.0
MP	56.6	56.6	56.6
12. Mixed fatty acids on fat, %	88.3	90.8	93.0
Triglycerides in fat, %	92.3	95.0	97.2
13. Mean MW of dibasic acids from insoluble azelaoglycerides	200.0	201.0	200.0
14. IV of unsaturated acids (calcd.)	96.3	95.0	104.2
Approx. mean MW of unsatd. acids	282	282	281
15. Acids in mixed fatty acids, %			
a) saturated acids	35.4	42.1	48.2
C_{16} and lower as palmitic	16.4	30.1	34.4
C_{18} and higher as stearic	19.0	12.0	13.8
b) unsaturated acids	64.6	57.9	51.8
monoethenoid (oleic)	60.2	54.9	43.8
diethenoid (linoleic)	4.4	3.0	8.0
proportions of linoleic per 100 parts of unsaturated acids	6.8	5.2	15.5
16. Saturated acids in mixed fatty acids, mol. %	36.5	43.7	49.8
17. Unsaturated acids in mixed fatty acids, mol. %	63.5	56.3	50.2

the source of biological origin for it is well known that fats from the same biological source, grown under the same environmental conditions as in the present instance, do not show any appreciable differences in saturated acid contents or component acid compositions. The possibility of difference in source is further supported by the fact that the relative proportion of linoleic acid in unsaturated acids in Mace Fat 3 is very much higher than in Mace Fat 1 (Table I). Such variations cannot normally occur in fats from the same biological source, grown under the same environments.

There is a difference of about 7 units percentage between the saturated acids contents of Mace Fats 1 and 2 and between Mace Fats 2 and 3. This can normally lead to the suspicion that Mace Fat 2 is simply a mixture of Fats 1 and 3 in about equal proportions. However, in the present instance, this possibility is ruled out by the following considerations.

a) Unesterifiable acids can be determined to an accuracy of 0.1–0.2% by methyl alcohol sulphuric acid esterification (2,3). The proportions of such acids are nearly the same (1.5 and 1.6%) in Fats 1 and 3 but are nearly double this value (2.8%) in Mace Fat 2.

b) The mean molecular weights of saturated acids can be determined to about 1 unit percentage by oxidation methods. Mace Fats 1 and 3 have values of 269 and 264 for this analytical feature. A 50:50 mixture of 1 and 3 should show a value of about 266–67 whereas the value for Mace Fat 2 was 264.

c) Mace Fat 1 contained 6 parts of linoleic acid per 100 parts of unsaturated acids while Fat 3 contained 16 parts. A 50:50 mixture of 1 and 3 should show linoleic acid content of 11 parts per 100 parts of unsaturated acids whereas Mace Fat 2 contained only 5 parts (Table I).

These three observations are adequate to establish that the three specimens represent fats from three distinct geographical varieties of *M. malabarica*.

Glyceride Structure

GS_3 Contents. Crystallization of fats from acetone-methyl alcohol under specified conditions at 25C for 72 hr (8) did not give crystalline precipitate in any case. Hence GS_3 was absent in all three fats. Further it has been shown that there is a quantitative relationship between the GS_3 contents of C_{16}–C_{18} acids in seed fats and the value of the function (MP of total saturated acids, °C—MP fat, °C) (9,8). According to this, when the value of this function is above 23.6, 26.8, 30.0, and 33.2, the GS_3 contents will be lower than 0.4, 0.2, 0.1, and 0.05% respectively. The value of this function for Mace Fats 1,2, and 3 are 49.2, 37.5, and 34.2, respectively (Table I). Hence GS_3 contents are below 0.05% in all cases.

GS_2U Contents. The GS_2U was determined as GS_2A by the azelaoglyceride technique described earlier (1) and included the refinements developed later (2,3). The analysis of the mace fats was comparatively simple since the fats were comparatively "clean" and did not give rise to any Bertram acids from unsaponifiable matter, unesterifiable resin acids, or esterifiable resin acids (determined by appropriate individual estimations). The Bertram acids also did not contain any lactone impurities as ascertained by lead salt crystallization from alcohol (3). The mean molecular weight of the dibasic acids, isolated from the insoluble azelaoglycerides according to procedures already recorded (10,11), were appreciably higher than that required for azelaic acid. It is noteworthy that all these fats showed dibasic acid mean molecular weights of 200–201. The experimental data for the calculation of the glyceride structure as well as the calculation of the results are given in Table II.

TABLE II
The Glyceride Structure of Three *Myristica malabarica* Mace Fats

Details	Mace Fat I	Mace Fat II	Mace Fat III
1. GS_3 by crystallization	nil	nil	nil
2. Fat oxidized, g: a)	1.095	1.578	1.521
b)	.....	2.010	1.759
3. IAG[a] on fat, wt. %: a)	57.8	67.2	74.2
b)	.....	66.2	73.9
4. IV of S from IAG: a)	1.2	2.4	2.4
b)	....	2.1	1.8
5. IAG on fat after correction a)	57.6	66.9	73.8
for above, wt. % b)	.....	65.9	73.6
6. Satd. acids from SAG,[b] wt. %: a)	0.1	0.6	0.8
b)	....	0.6	0.9
7. GSA_2, on fat, corresponding to a)	0.3	1.6	2.0
no. 6 above, wt. %: b)	....	1.6	2.3
8. Apparent azelaoglyceride number: a)	57.9	68.5	75.8
b)	.....	67.5	75.9
Mean	57.9	68.0	75.9
9. Lactone matter and Bertram acids from nonfat sources in azelaoglycerides, %	nil	nil	nil
Azelaoglycerides, wt. %	57.9	68.0	75.9
Azelaoglyceride number (% triglycerides as in Table 1)	62.7	71.6	78.1
10. Saturated acids in azelaoglycerides, %	54.1	56.2	59.0
11. Satd. acids in GS_2A, %	69.3	68.7	68.7
12. Satd. acids in GSA_2, %	38.2	37.4	37.6
13. GS_2A in GS_2A-GSA_2 mixture, %	31.1	43.0	53.4
GSA_2 in GS_2A-GSA_2 mixture, %	31.6	28.6	24.7
14. GS_2U in triglycerides, wt. %	34.4	47.5	59.1
GSU_2 in triglycerides, wt. %	38.5	35.1	30.5
GU_3 in triglycerides, wt. %	27.1	17.4	10.4
15. GS_2U in triglycerides, mol. %	34.9	48.2	59.7
GSU_2 in triglycerides, mol. %	38.5	34.9	30.2
GU_3 in triglycerides, mol. %	26.6	16.9	10.1

[a] IAG—Insoluble azelaoglycerides in the magnesium salt separation.
[b] SAG—Soluble azelaoglycerides in the magnesium salt separation.

Results

A comparison of the glyceride type structures experimentally determined with those calculated according to the GTDR (1,3) is given in Table III. A total variation of 16.3% is present in the saturated acid contents of the three mace fats. A total difference of 25% in GS_2U contents, 8% in GSU_2 contents, and 16.5% in GU_3 contents is observed. However it is seen that the HOC indices show a variation of only 0.8% in the GS_2U values. This is well within the limits of accuracy of $\pm 0.75\%$ of GS_2U attainable by the azelaoglyceride technique hence the HOC indices of all three fats may be considered identical.

The results thus show that large changes can occur in the component acid composition of varietal and subvarietal fats without producing any detectable alterations in the HOC indices. This is normally feasible only if two conditions co-exist: a) the existence

TABLE III
Comparison of the Glyceride Type Structures of Three *Myristica malabarica* Mace Fats with GTDR Values

	Mace Fat I	Mace Fat II	Mace Fat III
1. Sm	36.5	43.7	49.8
2. GS_3 found	nil	nil	nil
3. GS_2U found	34.9	48.2	59.7
4. GS_2U GTDR	33.0	45.6	57.9
5. GS_2U found—GS_2U GTDR	+ 1.9	+ 2.6	+ 1.8
6. GSU_2 found	38.5	34.9	30.2
7. GSU_2 GTDR	43.5	39.9	33.6
8. GSU_2 found—GSU_2 GTDR	− 5.0	− 5.0	− 3.4
9. GU_3 found	26.6	16.9	10.1
10. GU_3 GTDR	23.5	14.5	8.5
11. GU_3 found—GU_3 GTDR	+ 3.1	+ 2.4	+ 1.6
12. HOC index: GS_3-nil, GS_2U	+ 1.9	+ 2.6	+ 1.8

of separate genes to control the HOC status on the one hand and to control the component acid composition on the other, b) comparatively greater resistance of HOC genes toward the factors which usually produce large alterations in the genes controlling the component acid composition. This leads to the probable conclusion that the HOC status of natural fats is a new and independent structural feature which has to be taken into consideration along with theories of esterification to explain triglyceride structure.

Independence of HOC Genes and Influence on HOC Variations in Interspecific Fats. When HOC genes are independent, an increase in HOC indices can take place with an increase in saturated acid content on the one hand and with a decrease in saturated acid content on the other during varietal or subvarietal alterations. Both these possibilities have already been realized though not in geographical varieties in all instances.

In the white variety of *Sesamum indicum* an increase in HOC index occurred along with a decrease in saturated acid content as compared with the brown variety (loc. cit.). In the geographical varieties of *Vateria indica* seed fats an increase in HOC indices took place along with an increase in saturated acid contents as well (loc. cit.). Thus in varietal and subvarietal fats HOC indices can show either increase or decrease with increase in saturated acid contents.

There is at present no method for predicting changes in HOC indices brought about by possible alterations in HOC genes. Calculation of glyceride structure according to biosynthesis theories will, in the future, be principally of use in ascertaining the value of HOC indices and HOC ranges in particular biological sources. The observed range of variation in HOC indices for fats from a specified biological species or variety is termed the HOC range for that particular biological source (5).

A study of the general distribution and magnitude of HOC indices in natural fats from different families along with an attempt to see whether any of the present glyceride structure theories can explain the structure of all natural fats either by themselves or with the help of the HOC concept can possibly lead to further advances in the knowledge of natural fat biosynthesis.

Conclusion

The presence and degree of HOC in natural fats appear to be controlled by independent sets of genes in the fat metabolism genes complex. Hence all kinds of variations in HOC indices of seed fats can take place during varietal and subvarietal alterations in any given biological species.

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