Variability of Degree of High Order Compositeness in Fats From the Same Biological Species: Fats From Geographic Varieties of *Pongamia glabra*, *Nerium thevetifolium* and *Moringa pterygosperma*

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Abstract

Seed fats from four geographic varieties of *P. glabra* (Sm 20.2, 27.1, 25.2 and 24.7), three of *N. thevetifolium* (Sm 33.5, 36.3 and 33.1) and two of *M. pterygosperma* (Sm 23.2 and 20.6) showed high order compositeness (HOC) indices of GS_3nil, GS_2U +5.2, −3.1, +5.2 and +9.0, GS_3nil, GS_2U +0.9, +7.0 and +2.0 and GS_3nil, GS_2U +2.0 and +7.3 respectively. Since geographic varieties are morphologically indistinguishable no fixed relationship can any longer be expected between the proportions of acids and glycerides in different specimens of fat from the same biological variety. Prediction of glyceride structures of different specimens of fat from the same variety can hence be made only to the extent of HOC ranges just as in the case of fats from different varieties or species.

Fats from geographic varieties belonging to different biological species show different kinds of relationships between the component saturated and unsaturated acids. No quantitative or even qualitative correlation exists between the HOC indices and the proportions of saturated acids in fats from geographical varieties belonging to the same biological source.

Introduction

SIGNIFICANT DIFFERENCES were observed in the degrees of high order compositeness (HOC indices) (1) of seed fats from five closely related species in genus *Cassia*, family Leguminaceae (2) and also in brown and white seeded varieties of Sesamum indicum (3). Different specimens of fat from the same biological variety, but collected from different geographic regions (geographic varieties), showed two types of relationships in this respect: in one case, namely three *Myristica malabarica* mace fats, the HOC indices remained unchanged in spite of very large variations in saturated acid contents (4) while in another case, namely *Vateria indica* seed fats (5), significant differences in HOC indices were observed for different specimens showing only small differences in saturated acid contents. The morphologically distinguishable varieties or sub-varieties constitute the basic units in biological differentiation, and hence variability of HOC indices in fats from geographic varieties will lead to the result that there is no fixed relationship between the proportions of acids and glycerides even in different specimens of fat from the same biological source and hence that prediction of glyceride structure is possible only to the extent of HOC ranges (3,5) even in such cases. To gather further data on the issue, we have been collecting seeds of biological species from different geographic regions which have not so far evolved any morphologically distinguishable varieties. The collections were made from Delhi representing North Indian types and Kerala State representing South Indian types. The two areas are separated by a distance of 2,000 miles and by many geographical barriers. The North Indian types grow in a colder climate than the South Indian and the composition of the fats may help to throw further light on the possible influence of climatic conditions on mutations in fat metabolism genes involved in the development of different geographic varieties. The glyceride structures of seed fats from four geographic varieties of *P. glabra*, three of *N. thevetifolium* and two of *M. pterygosperma* are reported.

Experimental Procedures

All fat specimens examined were extracted in the laboratory by the cold percolation method (room temperature) which dissolves minimal proportions of non-glyceridic materials (6). GS_3 contents were determined as earlier reported (7,8,1): no GS_3 was present in any of the fats. Determinations of GS_2U were conducted by basically the same methods as earlier reported (8,1), but improvements have been worked out both in the oxidation procedure as well as in the azelaoglyceride separation.

Improved Procedure for Oxidation of Fats

One to two grams of fat (smaller quantities are adequate when saturated acid contents are high) are oxidized by the acetic acid acetone permanganate procedure (8) keeping the concentration of fat in acetone at 0.5% or less. Ten grams of powdered permanganate per gram fat is used in all cases. All the permanganate is added in one lot as also all the acetic acid required (8) and oxidation is complete when the mixture has been refluxed with occasional shaking for 5 hr or till the permanganate is decolorized. Under these conditions the double bonds are broken down completely and no correction is necessary for unoxidized fat. Incomplete oxidation may result if the fat is oxidized in more concentrated solutions. Reaction products are worked up as earlier (8) but the use of powdered oxalic acid and 1:1 sulfuric acid is more convenient than sodium bi-sulfite and sulfuric acid for dissolution of manganese dioxide.

Improved Procedure for Azelaoglyceride Separation

When azelaoglyceride precipitation is conducted at 25C for 30 min (8), the magnesium salts of GS_2A may not separate quantitatively in the IAG fraction

in case of fats containing less than about 30% saturated acids. This incompleteness of precipitation is caused by intersolubility effects exerted by soluble magnesium soaps of GA_3 and of lower monobasic acids produced during oxidation. In order to remove error from this source the following alterations in procedure have been adopted. The isolated ether soluble oxidation products of the fat are heated in a current of air on a water bath till most of the volatile acids are removed (odor). The lower acid free residue is dissolved in very dilute ammonia, if necessary with the aid of minimal heating, and after cooling to room temperature is precipitated with 15 ml of 15% ammonium chloride and 35 ml of 15% magnesium sulfate solutions. A solution volume of 100 to 150 ml/g fat oxidized is ordinarily used at point of precipitation, but higher volume ratios are allowable. Lower volume ratios may produce incomplete precipitation due to intersolubility effects. The precipitated mixture is cooled at 10–15 C for 2 hr, filtered using a cooled funnel and washed free of mother liquor using water cooled to 10 C. The IAG and SAG fractions are subsequently isolated as usual (8).

Recovery of Last Traces of GS_2A From SAG Fractions

When the saturated acids in the SAG fraction amount to more than 1.5% to 2.0% of the rat, there is possibility of a little GS_2A being still retained in the SAG. When this occurs the retained GS_2A is recovered as follows. The SAG, from 2 g fat, is washed in ethereal solution three times, with 20 ml of 4% solution of freshly prepared sodium bicarbonate solution each time. This removes the GA_3 almost completely. The solvent is removed from the bicarbonate insoluble fraction which is again submitted to the azelaoglyceride separation using the same volumes of solutions and other conditions as before. Any insoluble precipitate obtained is added to the main IAG fraction for further analysis. The filtrates in the second separation are added to the bicarbonate washings for retrieving the total SAG as usual (8).

Further Analysis of IAG and SAG Fractions

The IAG fractions are weighed to constant weight and would contain all the GS_3 and GS_2A along with some GSA_2 and also various non-glyceride materials. This is hydrolyzed with alcoholic potash and separated into fractions giving water soluble and water insoluble magnesium salts by two magnesium salt precipitations, as reported before (8). The material recovered from the insoluble magnesium salts is termed the Bertram acids. The Bertram acids will contain all the higher saturated fatty acids present in the IAG, high molecular weight saturated acids from non-glyceridic sources, if any, and also various types of lactonic materials and unoxidized unsaponifiables. The SAG fraction is not weighed to constant weight but is directly hydrolyzed and the Bertram acids isolated (8).

Recovery of Dibasic Acids and Non-glyceridic Materials Giving Soluble Magnesium Salts From IAG Fractions.

The combined filtrates from the Bertram acid separations of the IAG fractions are acidified, concentrated till magnesium sulfate starts crystallizing and extracted six times with diethyl ether (9,10). Nearly quantitative recovery of dibasic acids is effected by this. The dibasic acid fractions may contain the following non-glyceridic impurities: High molecular weight non-fatty acids giving water soluble magnesium salts; lactonic material giving soluble magnesium salts in presence of excess ammonia; and unoxidized unsaponifiables dissolving colloidally in hot soap solutions. All these impurities are of the water-insoluble type. They may be qualitatively tested by heating the dibasic acid fractions with a large amount of water. When present they can be quantitatively isolated by heating repeatedly with water and filtering through moist filter paper. The water-insoluble fractions are recovered by being dissolved with diethyl ether. The purified dibasic acid fraction is examined for equivalent weight necessary for azelaoglyceride calculation. The weight of water-insoluble fractions present is to be deducted from the IAG for these calculations. In some fats nonglyceridic materials of the above type have amounted to 2% to 3% on fat basis. As a matter of fact some of the earlier results by the azelaoglyceride technique (8) are in some error due to the nonrecognition of the presence in the IAG of various types of non-glyceridic impurities which can interfere with the azelaoglyceride calculations. The Bertram acid filtrates from the SAG are usually discarded (8).

Analysis of Bertram Acids From IAG and SAG Fractions

These are separated into three fractions by lead salt crystallization from alcohol. (i) Fraction giving lead salts insoluble in boiling alcohol after a minimum of two precipitations. Obviously this fraction would consist entirely of non-fat acids. (ii) Fraction giving lead salts insoluble in cold alcohol. This would represent the higher saturated acids present in the triglycerides. (iii) Fraction giving lead salts soluble in cold alcohol. This would contain all the lactonic material giving magnesium salts insoluble in water along with unoxidizable unsaponifiables which are not soluble in soap solutions, and some amount of acids of the same type as separate in fraction (ii). An approximate idea of the proportions of total non-glyceridic material in this fraction is obtained from its acid value by calculating it as a mixture of acids of the same mean mol. wt. as in (ii) along with materials of no acid value (lactones, unsaponifiables, etc.). During lead salt crystallizations the acidic fractions from soluble lead salts may undergo a little esterification and the correct acid values are obtained after hydrolysis with alcoholic potash. The proportions of non-glyceridic materials in the Bertram acids from the IAG are to be subtracted from the weight of the Bertram acids as well as from that of the IAG for the azelaoglyceride calculations. When Bertram acids from SAG amount to less than 1% to 2% of fat, lead salt analysis of the same may be omitted.

Separate Estimation of Total Bertram Acids From Non-Glyceride Sources

Non-glyceridic materials in many oils and fats, namely unsaponifiables, esterifiable resin acids, non-esterifiable resin acids sometimes produce various proportions of Bertram acids on oxidation which separate entirely with the IAG during azelaoglyceride separation (11). Small proportions of these present in the IAG can produce large errors in the calculated values of GS_2A and GSA_2. These acids are generally of the type giving lead salts soluble in hot alcohol and their proportions cannot be obtained from any of the determinations earlier described. The total Bertram acids produced from all

TABLE I

Characteristics of Seed Fats From Four Geographic Varieties of *Pongamia glabra*, Three of *Nerium thevetifolium* and Two of *Moringa pterygosperma*

	P. glabra				*N. thevetifolium*			*M. pterygosperma*	
	I (NI)[a]	II (SI)[b]	III (SI)	IV (SI)	I (SI)	II (SI)	III (NI)	I (NI)	II (SI)
Iodine val. per cent[c]	79.0	76.0	75.0	76.0	68.0	63.0	90.0	63.0	65.0
Hehner val. per cent	95.3	95.7	95.8	95.8	95.9	93.7	94.9	95.4	95.6
Non-glyceridic material, consol. per cent	4.9	5.7	5.8	5.8	5.2	5.4	5.7	1.8	1.8
Fatty acids per cent	90.4	90.0	90.0	90.0	90.7	88.3	89.2	93.6	93.8
Triglycerides per cent	94.5	94.0	94.0	94.0	94.8	92.1	93.2	97.8	98.0
Bertram acids per cent	19.5	24.8	24.2	24.0	30.1	31.7	29.2	23.8	21.0
S, per cent	18.9	24.8	24.2	24.0	29.2	30.8	28.3	23.4	20.6
S, Mean Mol. wt.	298.0	289.0	308.0	312.0	265.0	265.0	265.0	312.0	306.0
U, Mean Mol. wt. ca.	282.0	282.0	282.0	282.0	282.0	282.0	282.0	282.0	282.0
S, per cent mol.[d]	20.2	27.1	25.2	24.7	33.5	36.3	33.1	23.2	20.6
U, per cent mol.	79.8	72.9	74.8	75.3	66.5	63.7	66.9	76.8	79.4
Dibasic acids from IAG, Mean Mol. wt.	192.0	192.0	194.0	192.0	186.0	184.0	186.0	184.0	184.0

[a] NI = North Indian origin.
[b] SI = South Indian origin.
[c] per cent = percentage on refined fat, wt. basis.
[d] per cent mol. = percentage molecules in mixed fatty acids or triglycerides.

the non-glyceridic sources present in a fat is now estimated in the following way. The mixed acids are esterified without removal of unsaponifiable matter and the isolated reaction products (inclusive of all the unesterified acids) are oxidized by the acetic acid acetone permanganate oxidation procedure as described earlier. The products of oxidation are worked up with diethyl ether and separated into acidic and neutral fractions with the help of dilute ammonia; the separation is repeated a second time for the acidic fraction. The purified acidic fraction is hydrolyzed with alcoholic potash and then submitted to Bertram separation twice as usual (8). Material recovered from insoluble magnesium soaps represent the Bertram acids from total non-glyceride sources. The proportions of these have to be subtracted from the IAG and also from the proper fraction of the Bertram acids from the IAG, namely fraction giving hot alcohol soluble lead slats, for calculating the proportions of GS_2A.

In addition to the data discussed above, a knowledge of the Hehner value, unsaponifiables and unesterifiable acids are required for calculating the glyceride structure. These characteristics are determined by standard methods.

Procedure for correcting the experimental results for the proportions of various nonglyceridic impurities discussed above is as follows (all weights are based on 100 parts of refined fat). 1) weight $GS_2A + GSA_2$ in IAG equals weight IAG − weight GS_3 − weight Bertram acids giving hot alcohol insoluble lead salts in IAG (A) − weight lactones and unsaponifiables in Bertram acids from IAG (B) − weight water-insoluble material in dibasic acid fraction from IAG (C) − weight Bertram acids from total non-glyceridic sources (D). 2) weight saturated acids in $GS_2A + GSA_2$ mixture equals weight Bertram acids from IAG − A − B − D − weight saturated acids in GS_3. 3) Mixed fatty acids in fat = Hehner value per cent − unsaponifiables per cent − A − B − C − D. 4) Total Saturated acids in fat equals Bertram acids from IAG and SAG − A − B − D.

The necessary chemical characteristics of the fat specimens now analyzed are given in Table I and the glyceride structure data in Table II.

Results and Discussion

Seed fats from four geographic varieties of *P. glabra* were examined. Fat I was of North Indian origin while II, III and IV were from different locations in South India. The iodine values of the fats did not show much variation in spite of varia-

TABLE II

Glyceride Structures of Seed Fats From Four Geographic Varieties of *Pongamia glabra*, Three of *Nerium thevetifolium* and Two of *Moringa pterygosperma*

	P. glabra				*N. thevetifolium*			*M. pterygosperma*	
	I (NI)[a]	II (SI)[b]	III (SI)	IV (SI)	I (SI)	II (SI)	III (NI)	I (NI)	II (SI)
GS_2A- GSA_2, per cent[c]	35.2	50.5	43.2	40.6	55.4	53.1	52.8	43.6	36.1
S, per cent in $GS_2A + GSA_2$	53.7	48.9	56.0	59.1	52.6	58.0	53.6	53.7	57.1
S, per cent in GS_2A, theory	72.1	71.5	72.8	73.1	70.3	70.3	70.3	74.0	73.6
S, per cent in GSA_2, theory	41.4	40.6	42.2	42.5	39.3	39.3	39.3	43.5	43.0
GS_2A, per cent (mean of 2–4 determinations)	14.2	13.6	19.5	22.2	23.8	32.2	24.3	14.6	16.6
Glyceride types, per cent									
GS_2U	15.7	15.1	21.6	24.5	26.8	36.2	27.4	16.3	18.6
GSU_2	26.3	46.2	29.4	22.9	40.6	26.8	36.6	36.9	24.9
GU_3	52.5	32.7	43.0	46.6	27.4	29.1	29.2	44.6	54.5
Glyceride types, per cent mol.[d]									
GS_2U	16.2	15.9	22.2	25.0	28.9	40.0	30.0	16.0	18.3
GSU_2	27.7	49.1	31.1	24.2	42.9	29.1	39.3	37.3	25.2
GU_3	56.1	35.0	46.8	50.8	28.2	30.9	30.7	46.7	56.5
Glyceride types, per cent mol. GTDR.									
GS_2U	11.0	19	17	16	28	33	28	14	11
GSU_2	39.0	43	43	42	45	44	44	41	39
GU_3	58.0	38	40	42	27	23	22	45	50
HOC index, GS_3 nil, GS_2U	+5.2	−3.1	+5.2	+9.0	+0.9	+7.0	+2.0	+2.0	+7.3

[a] NI = North Indian origin.
[b] SI = South Indian origin.
[c] Per cent = Percentage on refined fat, weight basis.
[d] Per cent = Percentage molecules in mixed fatty acids or triglycerides.

tions in saturated acid content of from 19% to 25%. All the fats contained unusual proportions of various non-glyceridic materials. They also showed appreciable variation in the mean mol. wt. of the total saturated acids indicating changes in the chain-length composition of the latter. Appreciable variations can hence occur in the component saturated acid compositions of geographic varieties. All three South Indian varieties showed Sm above 24 while the North Indian variety showed Sm below 20. The dibasic acids recovered from the IAG showed eq. wt. 96–97 in all cases in spite of the variations in the mean mol. wt. of the saturated acids.

The variations in the HOC indices were instructive. Fats I and III showed the same index of GS_3 nil, GS_2U + 5.2; they are however derived from areas about 2,000 miles apart and show a difference in Sm of about five units which is about the maximum difference usually met with in such variations. Large alterations can thus take place in the fat metabolism genes without necessarily producing differences in the factors which determine the magnitude of the HOC indices. This example provides further support for the concept of independent HOC genes put forward from the results of examining three *Myristica malabarica* mace fats (4). Fat II is unusual in showing a negative HOC index of GS_3 nil, GS_2U − 3.1. Fat IV, also from South India, shows the highest HOC index in the series, namely, GS_3 nil, GS_2U + 9.0. The total variation in the HOC index amounts to 12 GS_2U units in a saturated acid content of 25% molecules. In the case of fat with the highest HOC index, saturated acids in the GS_2U in the HOC index correspond to about 25% of the total saturated acids. *P. glabra* forms the first instance where mutations in fat metabolism genes have been found to occur with changes in the HOC genes on the one hand (Fats I, II and IV) and without changes in HOC genes on the other (Fats I and III).

N. thevetifolium fats I and II were from different localities in South India while III was of North Indian origin. The fats showed large variations in iodine value, from 63 to 90; the variations in saturated acid contents were however small, within three units per cent. This is in notable contrast to the behavior of *P. glabra* seed fats wherein appreciable variations in saturated acid contents occurred without any significant changes in the iodine values of the oils. There is no quantitative or even qualitative correlation between variations in iodine value and saturated acid contents in fats from geographic varieties of the same biological species. *N. thevetifolium* seed fats differed from *P. glabra* seed fats in another important respect: the saturated acids from all three specimens of the former showed the same mean mol. wt. Large changes in component unsaturated compositions can take place without any detectable alterations in the total or relative proportions of component saturated acids. All three fats contained dibasic acids of eq. wt. 92–93 in the IAG. *N. thevetifolium* fats I and II showed small HOC indices of GS_3 nil, GS_2U + 1.0 and +2.5 respectively while fat II showed HOC index of GS_3 nil, GS_2U + 7.0, the highest in the series. The variations in this species are thus limited to 6 GS_2U units against 12 units observed with *P. glabra* seed fats.

M. pterygosperma fat I was of North Indian origin while fat II was South Indian. There was little difference in iodine value between the two, and both showed presence of minimal proportions of non-glyceridic matter. In the case of *P. glabra* and *N. thevetifolium* the South Indian specimens showed higher saturated acid contents, but this was reversed with *M. pterygosperma* where the North Indian specimen showed Sm 3 units per cent higher than the South Indian. Development of higher saturated acid contents in geographic varieties in warmer regions is hence not obligatory though it is a common phenomenon. Although the saturated acids showed substantially different mean chain lengths, as in the case of *P. glabra,* the equivalent weight of the dibasic acids in the IAG remained constant at 91–92.

The two specimens of *M. pterygosperma* fats showed different HOC indices. The North Indian with Sm 23.8 had HOC index of Gs_3 nil, GS_2U + 2.0 whereas the South Indian specimen with Sm 20 showed HOC index of Gs_3 nil, GS_2U + 7.3. The S in the GS_2U present in the higher HOC index corresponds to 25% of the total saturated acids in the fat; this is the highest ratio observed with *P. glabra* seed fats as well. HOC indices can thus increase, decrease or remain unchanged with increase in saturated acid contents in different geographic varieties from particular biological species.

The proportions of GS_2U and GU_3 in most of the fats now examined are higher than those possible according to any of the glyceride structure theories so far recorded (12–16) and they show a varying degree of deviation from GTDR as represented by the differing HOC indices (1). Obviously there can be no conceivable fixed arithmetical relationship between the proportions of different glyceride types and of saturated acids in any instance in the different geographic varieties from the same biological species now studied. All attempts at accurate prediction of glyceride structure (12–16) were based on the assumed constancy of some such relationships in the same biological species, or in wider groups, and such attempts are no longer dependable. The only prediction now possible is an approximate one making use of the HOC ranges, namely observed range of variability of HOC indices for particular biological species or varieties (3,5). The HOC ranges of *P. glabra, N. thevetifolium* and *M. pterygosperma* are GS_3 nil, GS_2U−3.1 to +9.3, GS_3 nil, GS_2U + 0.9 to +7.3 and GS_3 nil, GS_2U + 2.0 to +7.3 respectively.

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