Discernment of Relative Retention Values of Triglyceride α,β Isomers on Reverse Phase High Performance Liquid Chromatography

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Discernment of α,β isomers of triglyceride on reverse phase high performance liquid chromatography (HPLC) was investigated by the use of a multiple regression analysis. The retention effect of triglyceride molecular species was considered to be dependent principally on the addition theorem of chemical potential of both position α and position β (acyl carbon numbers and numbers of double bonds in the acyl group), though it was observed that the retention effect of position α is somewhat larger than that of position β on HPLC when it has the same acyl group combination.

A mathematical model for elution of triglyceride (TG) on reverse phase high performance liquid chromatography (HPLC) has been demonstrated by the authors (1). The multiple regression expression was introduced in order to formulate a concrete form for the prediction of a relative retention time (RRT) of a TG molecular species on HPLC. As it is obvious from the concept of multiple regression analysis, theoretically it is possible to discern the α,β positional isomers of TG. In the previous paper (2), the chromatogram of Park et al. (3) was used for the demonstration of this hypothesis. In this study, the hypothesis was confirmed by analyzing various kinds of TG.

EXPERIMENTAL PROCEDURES

Linseed oil and olive oil were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Cacao butter was supplied by Yunokawa Seiyaku Co., Ltd., Hakodate, Hokkaido, Japan, and palm oil was supplied by Tsukishima Food Industry Co. Ltd., Tokyo, Japan. Walnut oil and peanut oil were obtained from a commercial source. "Ogonori" (*Gracilaria verrucosa*) was

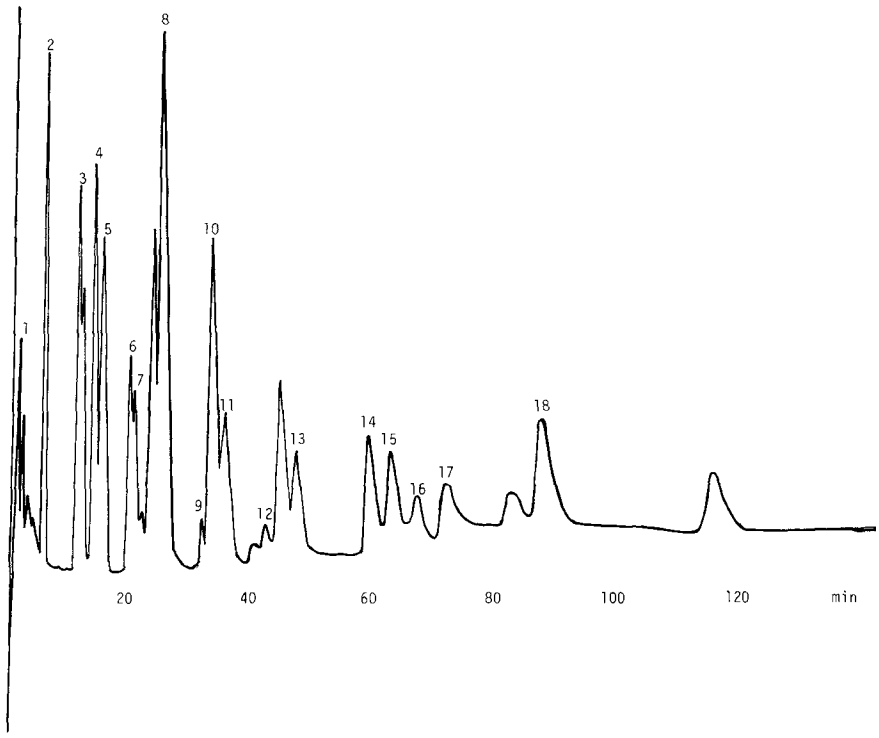

FIG. 1. HPLC chromatogram of triglyceride molecular species mixtures. Conditions: instrument, Hitachi 638-50 liquid chromatograph; column, Chemcosorb I-5C18, 4ϕ × 300 mm (Chemco Ltd., Osaka, Japan); detector, Shodex RI monitor (Showa Denko Ltd., Tokyo); solvent, acetone/acetonitrile (3:2, v/v); flow, 0.5 ml/min; column temp., ambient (20-22 C). The last unnumbered peak lacked the reproducibility of RRT and was therefore rejected. The other unnumbered peaks contained impurities and were therefore rejected. Retention results are shown in Table 1.

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collected at Shinori Coast, Hakodate, Hokkaido, Japan. The total lipid was extracted from "Ogonori" using chloroform/methanol (1:2, v/v), and TG from these sources were purified by preparative thin layer chromatography (TLC) using n-hexane/diethyl ether (4:1, v/v) as developing solvent.

The purified TG was fractionated into major peaks using HPLC. The condition of HPLC is shown in the legend to Figure 1. Peaks on HPLC chromatograms were numbered in sequence of elution. The fatty acid composition and the total acyl carbon number of each collected dominant peak were analyzed by gas chromatography (GC) as previously reported (4). The acyl group combination of each peak was determined by inference of results obtained by GC (4).

Each predominant TG molecular species obtained by HPLC was hydrolyzed with pancreatic lipase (Calbiochem, San Diego, California) as described previously (5) and by Rhizopus lipase (*Rhizopus delemar*, Calbiochem, San Diego, California) according to the method of Iwai et al. (6). The hydrolysate, i.e. 2-monoglyceride (2-MG), was purified by preparative TLC with n-hexane/diethyl ether (1:1, v/v) as developing solvent. The fatty acid residue in 2-MG was determined by GC.

The identified reliable peaks of TG molecular species that had been selected from various sources were combined and reinjected to the HPLC system to obtain the most accurate RRT possible. The eluted material corresponding to peaks appearing on the HPLC chromatogram was converted to methyl ester; TG molecular species were ascertained (monitored) using GC, and the RRTs were calculated. Multiple regression analysis was introduced by considering the RRT as dependent variables and by considering the acyl carbon number and the number of double bonds of a TG molecular species as independent variables.

RESULTS AND DISCUSSION

Figure 1 shows the HPLC chromatogram of the combined mixture of the reliable peaks of TG molecular species.

The RRT of each peak on the chromatogram was calculated by dividing the retention time of each peak by that of triolein (Table 1). By the application of multiple regression analysis to the retention results, the following information was obtained:

- By introducing TG molecular species that consist of acyl groups that have 18 carbons for the multiple regression analysis:

$$100 \times \log(\mathrm{RRT}) = -12.77043 \times \mathrm{D1} - 11.08929 \times \mathrm{D2} - 11.02671 \times \mathrm{D3} + 234.13057 \quad [1]$$

$$\therefore 100 \times \log(\mathrm{RRT}) = -11.89857 \times (\mathrm{D1} + \mathrm{D3}) - 11.08929 \times \mathrm{D2} + 234.13057 \quad [2]$$

was obtained (∵Antipodes such as α,α' isomers can be considered to have the equivalent chemical potential, since α,α' isomers of TG is a symmetry body. Therefore, in this study, the coefficients of D1 and D3 [number of double bonds in position α and α'] were treated as equal). The partial regression coefficient of position α in expression [2] was −11.89857, and that of position β was −11.08929. So, $|-11.89857| > |-11.08929|$ was held. This implies that the retention effect of position α is larger than that of position β.

- By introducing all of the identified TG molecular species shown in Figure 1, the following regression expression was obtained:

$$100 \times \log(\mathrm{RRT}) = 7.44497 \times \mathrm{C1} + 5.09957 \times \mathrm{C2} + 4.75835 \times \mathrm{C3} - 13.69358 \times \mathrm{D1} - 10.45701 \times \mathrm{D2} - 13.53262 \times \mathrm{D3} - 70.45341 \quad [3]$$

$$\therefore 100 \times \log(\mathrm{RRT}) = 6.10166 \times (\mathrm{C1} + \mathrm{C3}) + 5.09957 \times \mathrm{C2} - 13.61310 \times (\mathrm{D1} + \mathrm{D3}) - 10.45701 \times \mathrm{D2} - 70.45341 \quad [4]$$

This regression expression [4] indicates that the retention effect of position α is larger than that of position β on HPLC because the coefficient of C1 + C3 as well as D1 + D3 is somewhat larger than that of C2 and D2, respectively. The multiple correlation coefficient was 0.9950 for [2] and 0.9918 for [4].

This regression expression, i.e. expression [4], may be useful in predicting the RRT of TG molecular species on HPLC. This was ascertained by introducing some α,β positional isomers of TG molecular species, for example, when we have:

16:0 18:1 18:2	(A)	16:0 18:2 18:1	(B)

By using the regression expression [4], (A) will become $100 \times \log(\mathrm{RRT}) = 191.11208$, and (B) will become 100

TABLE 1

Retention Results of Figure 1

No.	M S[a]	100 × log(RRT)	Source
1	Cy Cy Cy	65.822	Standard
2	C C C	103.065	Standard
3	Ln Ln Ln	130.174	Linseed oil
4	La La La	138.365	Standard
5	L Ln Ln	142.257	Linseed oil
6	L L Ln	153.422	Walnut oil
7	O Ln Ln	154.959	Linseed oil
8	L L L	162.937	Walnut oil
9	M M M	173.583	Standard
10	L O L	175.356	Peanut oil
11	P L L	177.969	Walnut oil
12	P A P	185.383	"Ogonori"
13	P L O	190.389	Peanut oil
14	O O O	200.000	Olive oil
15	P O O	202.530	Palm oil
16	P O P	205.295	Cacao butter
17	P P P	208.450	Standard
18	P O S	216.674	Cacao butter

[a]Abbreviations: MS, molecular species; Cy, caprylic acid; C, capric acid; Ln, linolenic acid; La, lauric acid; L, linoleic acid; O, oleic acid; M, myristic acid; P, palmitic acid; A, arachidonic acid; S, stearic acid.

$\times$ log(RRT) = 194.26817. So (A) should, in theory, elute slightly earlier than (B). As the chemical potential of 18:1 is obviously larger than that of 18:2, and, if the retention effect of position α is larger than that of position β, (A) should, in theory, elute earlier than (B). The results obtained indicate that if the two TG molecular species have the same acyl group combination, RRT on HPLC is considered to be affected somewhat more by the acyl group bound in position α than in position β.

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Evaluation of Some Approaches to Liquified Tallow: Stereochemical Consequences of Interesterification

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Several approaches to converting tallow to a liquid state at ambient temperature to facilitate incorporation into poultry feed were examined. Acetone fractionation can be used in a single step to produce a flowable powder and an oil (no additional semi-solid fraction). Partial hydrolyses and interestifications mediated by lipases of *Candida rugosa, Rhizopus delemar* and porcine pancreas did not lead to liquefaction. Interesterification (trioctanoin + glycerol, 1,2-acetonide) in the presence of *C. rugosa* lipase shows a small but significant sterobias indicating that there are stereochemical consequences in such processes that deserve further investigation.

There is a renewed interest in altering the physical properties of tallow so that it might be more satisfactorily used, as an additive for poultry feed (Private communications with D. Gilcrest, president, Fats and Proteins Foundation, Inc., Des Plaines, Illinois). Feed grade animal fat, or yellow grease, is collected from restaurants, as residue from food preparation. Its constitution is therefore both animal and vegetable in origin and is quite variable. It will separate into liquid and semi-solid on standing in much the manner of various grades of tallow. The current process of blending yellow grease to produce a feed would be better served if the feedstock to the mills was entirely liquid; the semi-solid material reportedly hampers the milling process. Chemical fractionation, or alteration, of the yellow grease is one of several alternatives that could accomplish this goal. Because fancy bleached tallow has the highest titer, we felt that any procedure that liquefied the tallow would succeed with tallow-containing mixtures. We report here our results using the several possible chemical approaches to liquefaction with fancy bleached tallow.

MATERIALS

Acetone and acetonitrile were Mallinckrodt Nanograde. Tallow was a gift of Chemol Inc., Greenboro, North Carolina; corn oil (Mazola) and sunflower oil (Wesson) were local purchases; and soy oil was obtained from Central Soya, Decatur, Indiana. Tributyrin (Eastman, Rochester, New York), and tris-heptafluorocamphoratoeuropium, or Eu(hfc)$_3$ (Aldrich, Milwaukee, Wisconsin), were used directly as obtained. Trioctanoin (Eastman, Rochester, New York) was treated with a small amount of octanoyl chloride and pyridine to esterify the dioctanoins present and precipitated several times from cold alcohol. The acetonide of glycerine was synthesized from acetone and glycerol; product bp 51-53 C/0.1 mm. The lipases used were from *Candida rugosa* (Enzyme Development Corp., New York, New York), *Rhizopus delemar* (Seikagaku Kogyo Co. Ltd., Tokyo, Japan), and porcine pancreas (Sigma Chemical Co., St. Louis, Missouri). High performance liquid chromatography was performed with a Perkin Elmer Series 2 pump, a Waters Associates differential refractometer and an ODS (reverse phase) column (4.6 mm $\times$ 25 cm). Gas liquid chromatography of

TABLE 1

Acetone Fractionation of Tallow

T[a]	% Solid	Melting range	T[b]	CP
25	4.4	54-56	>25	23.5
20	8.0	50-53	25	16
15	10.7	49-53	21	13
4	27.1	35-47	19	9

[a]T, T C for crystallization.
[b]Minimum T C at which recovered oil remained liquid for 48 hr.
[c]P, cloud point.