Molecular Species of Triglycerides in *Camellia japonica* Seed Oil

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冬栢種實油의 트리글리세리드의 分子種에 관한 硏究

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要 約

冬栢種實油에서 컬럼크로마토그래피로 脂質 成分을 相互 分離하였다. 여기서 얻은 트리글리세리드의 一部를 취하여 알칼리로 加水分解하거나 또는 pancreatic lipase로 加水分解하여, 트리글리세리드의 構成 脂肪酸 또는 1,3-位置와 2-位置에 結合한 脂肪酸 組成을 調査하였다. 나머지 트리글리세리드는 16% $AgNO_3$-TLC로 二重結合數별로 나누었으며, 이렇게 나누어진 分劃을 다시 HPLC로 PN별로 再分劃 하였다. 여기서 얻어진 劃分들 중 PN이 같은 것은 모두 모아서 알칼리 加水分解로 PN의 總脂肪酸 組成을, pancreatic lipase로 1,3-位置에 結合한 脂肪酸의 組成을 調査하였다. 여기서 얻어진 結果로 부터 構成 트리글리세리드 分子種을 算出하였더니 다음과 같은 結果를 얻었다.

1) 種實油는 透明한 液體로 그 含量이 73.5%였으며, 그 중 트리글리세리드가 94.8%, 極性 脂質이 2.0%, 炭化 水素가 1.8%였다.

2) $AgNO_3$-TLC로 트리글리세리드를 分劃하였더니 5개의 劃分을 얻었으며, 大部分의 트리글리세리드가 二重 結合數가 3~5개인 band 2와 band 3에 80% 이상 存在하였다.

3) $AgNO_3$-TLC에서 얻은 각 劃分은 모두가 HPLC상에서 PN 46, 48 및 50으로 나누어졌으며 全體 分劃에서 PN 48이 78.13%로 제일 많았으며, 다음으로 PN 50이 12.04%였으며, PN 46이 9.83%였다.

4) 全體 트리글리세리드 分子種 중에서 0.1 mol % 이상을 차지하고 있는 分子種이 43種이었으며, OOO와 POO가 각각 39.8 mol %와 25.8 mol %로 제일 많았으며, 그 다음으로 OPO가 5.5 mol %, OOL가 4.8 mol %, POS가 3.9 mol %, SOO가 3.5 mol %, POL이 3.0 mol %였다. 또 冬栢種實油에 存在하는 트리글리세리드의 分子種의 組成은 1,3-random, 2-random 分布說에 따라 構成되어 있었다.

I. Introduction

As triglycerides(TG_s) are the main component of naturally occurring lipids and their structure elucidation is of great importance to food and confectionary fat manufacturers, a great deal of effort has been applied to their analysis.[1~6] At present, separ-

ation of TGs according to their chain length or carbon numbers by gas liquid chromatography (GLC) has become a routine procedure.[7~12] However, the GLC method gives insufficient information to provide a complete triglyceride composition, even when combined with the total fatty acid composition or distribution.

For more detailed information about TG composition, separation methods other than the carbon number distribution are necessary. Argentation chromatography[13~15] and enzymatic splitting thchniques[16~18] provide such methods and actually have been used by many workers[19~21] in combination with GLC.

Recently, the application of high performance liquid chromatography(HPLC) to the analysis of TGs has been extensively studied. Plattner and others[22] separated the TGs of some seed oils both by chain length and degree of unsaturation using a HPLC equipped with a μ-Bondapak C_{18} column with an acetonitrile-acetone solvent. They observed linear relationships between chain length and log of retention volume for saturated TGs and between the number of double bonds and log of retention volume for TGs of the same chain length. Wada[23] fractionated the TGs from soy bean oil into six peaks on a μ-Bondapak C_{18} column of HPLC with methanol-chloroform(9 : 1, v/v).

Camellia japonica L. belongs to the *Theaceae* family and is grown in the southern coastal areas of Korea. *Camellia japonica* seed oil(CSO) has been used in cosmetics and as a substitute for olive oil in the Far East, and has attracted the attention of food scientists and nutritionists as an antiblood-clotting foodstuff these days.[24] Clinical studies[25] on mice have indicated that CSO can reduce the risk of diseases linked to meat rich in a special fatty acid. Included among these are high serum cholesterol, blood clotting and diabetes. The TG structure may play an important role in the physical properties, stability and nutritional value of lipids. However, limited information about CSO is available to the best of our knowledge.

This paper describes the composition of molecular species to TGs from CSO by HPLC in combination with $AgNO_3$-TLC, pancreatic lipase splitting technique and GLC.

Ⅱ. Material and Experimental

Seeds of *Camellia japonica* were collected in the area of Goh Je County, Kyueng-Nam Province, Korea, in October of 1989. Total lipids were extracted from the husked and smashed seeds with chloroform-methanol(2 : 1, v/v).[26] The triglyceride (TG) fraction of the seed oil[27] was isolated by passing it through a silica gel column with a mixture of hexane-diethyl ether(97 : 3, v/v) as the eluting solvent. The TG fraction was evaporated to dryness in a stream of nitrogen, a small portion of which was hydrolyzed by 10% KOH-ethanol[28] and the fatty acids thus obtained were methylated by 14% BF_3-methanol according to the method of Metcalfe.[29] A known amount of methyl eicosanoate ($C_{20:0}$) was added to the fatty acid methyl esters (FAME) to be analyzed.

Some portions of the remaining TGs were enzymatically hydrolyzed as follows[17~30] : To 1mg of the TGs in a 5m*l* screwcap vial were added 0.5m*l* of 0.2M tris-HCl buffer(pH 8.0) solution, 0.5m*l* of 2.2% $CaCl_2$ solution and 0.5m*l* of 0.1N bile salts solution. Contents of the vial were homogenized and hydrolyzed by 0.2mg of pancreatic lipase(Sigma, St. Louis, USA) in a water bath at 40℃ for 20minutes. At the end of the reaction time, the contents were acidified with 1m*l* of 6N HCL solution and the reaction was halted by addition of 1m*l* ethyl alcohol. The hydrolyzates were transferred quantitatively to a small separatory funnel and thoroughly extracted with diethyl ether. The ether extract was washed with distilled water several times until the washings were neutral to Toyo test paper

(Toyo Roshi Co., Tokyo, Japan), dried over sodium sulfate, filtered and the solvent removed. The hydrolytic products were redissolved in a small quantity of diethyl ether, streaked on preparative TLC plates, and they were developed in the solvent system of n-hexane-diethyl ether(80 : 20, v/v). Silica gel powder was scraped from the bands corresponded to 2-monoglycerid(2-MG) and free fatty acid fraction. It was transferred to a small stoppered-Erlenmyer flask and extracted with chloroform-methanol(95 : 5, v/v) three times. The combined extracts of 2-MG fraction were evaporated to dryness, directly transmethylated with 14% BF_3-methanol and analyzed by GLC. The free fatty acid fraction was methylated by the method described above after solvent removal.

The FAME mixture[31] was then analyzed on a stainless steel column (3m × 3cm i. d.) packed with 15% DEGS on Chromosorb W in a Shimadzu Model 6A gas liquid chromatograph(GLC) equipped with a flame ioniozation detector(FID). Nitrogen was used as the carrier gas(flow rate, 40 m*l*/min.), and the column and detector temperature were 190℃ and 250℃, respectively. The peak area percentage in all the runs was calculated on a Shimadzu Chromatopac C-EIB.

All the rest of the fractionated triglyceride was separated on $AgNO_3$-TLC plates prepared as follows[32~35], glass plates(20 × 20cm) were spread with silica gel 60 GF 254-H_2O slurry impregnated with 16% $AgNO_3$ and dried in a dark place. The TLC plates were then activated by heating in a light-proof oven at 120℃ for 1 hour before use. A diethyl ether solution of the TG_s of CSO was streaked on three or four $AgNO_3$-TLC plates, which were developed in a mixture of chloroform-ethanol(99 : 1, v/v) and then sprayed with 0.02% 2', 7'-dichloro- fluorescein. The bands on TLC were detected by UV-light and then scraped. The combined silica gel was extracted five times with chloroform-methanol (19 : 1, v/v).

The chloroform-methanol extract of each TG band separated on $AgNO_3$-TLC was evaporated to dryness, redissolved in acetonitrile-acetone-methanol-chloroform(3 : 3 : 3 : 1, by volume), and fractionated on a Waters HPLC(Model 6000) instrument equipped with a differential refractometric detector (Model R401, Waters Associates Inc., Milford, Massachusetts, U.S.A.) and stainless steel tubing(30.0cm × 3.9mm i. d.) packed with μ-Bondapak C_{18}(Waters Associates Inc.) by using a mixture of acetonitrile-acetone-methanol-chloroform(3 : 3 : 3 : 1, by volume) at a flow rate of 0.8m*l*/min.[39] The tubing was kept at 30℃ by water circulation. The partition numbers (PN_s) of TG components on HPLC were established by comparison with those of standard TG_s, such as 1, 2-dimyristoyl-3-lauroyl-rac-glycerol(PN=40), 1, 2-dimyristoyl-3-oleoyl-racglycerol(PN=44), 1, 2-dipalmitoyl-3-myristoyl-racglycerol(PN=46), 1, 3-dipalmitoyl-2-oleoyl-racglycerol(PN=48), triolein(PN=48), 1, 3-dioleoyl-2-stearoyl-rac-glycerol(PN=50) and 1, 3-distearoyl-2-oleoyl-rac-glycerol (PN=52).

For simplicity and accuracy of analysis, all the fractions having the same PN in the TLC bands were combined together. The collected fraction for each PN was divided equally into two portions. One portion has hydrolyzed by alkali[28] and the resulting fatty acids were methylated with 14% BF_3-methanol[29] and analyzed by GLC along with the internal standard, methyl eicosanoate.[31,35] The remaining portion was enzymatically hydrolyzed by pancreatic lipase.[17,30] The fatty acids released from the position 1 and 3 of L-glycerol of TG were separated from 2-monoglyceride on TLC, and also methylated to be analyzed by GLC. The fatty acid composition of the 2-monoglyceride was calculated from the equation[36], mol % of fatty acid at position 2=3 × [mol % of fatty acid of TG]−2 × [mol % of fatty acid at position 1 and 3].

A portion of the TG components collected from HPLC effluent was subjected to GLC analysis. The

work was carried out on a Shimadzu GC-8A equipped with a FID and glass tubing(0.5m × 3mm i.d.) containing 2% Dexil on Chromosorb W(60~80 mesh, Nishio, Tokyo, Japan). The column temperature was programmed from 270℃ to 330℃ at the rate of 2℃/min., the injection port & detector was kept at 330℃, and the flow rate of carrier gas(N_2) was 60ml/min.

Table 1. Fatty acid composition —overall, at 1,3-position and 2—position-of total triglycerides in *Camellia japonica* seed oil

Fatty acid	Overall	1,3-Position (wt %)	2-Position
$C_{16:0}$	11.79(12.76)[a]	14.03(15.17)	6.65(7.24)
$C_{16:1}$	0.46(0.50)	0.25(0.27)	0.85(0.93)
$C_{18:0}$	3.58(3.51)	5.74(5.62)	1.31(1.29)
$C_{18:1}$	76.10(75.16)	74.03(73.04)	78.08(77.54)
$C_{18:2}$	7.17(7.13)	5.53(5.49)	11.02(11.02)
$C_{18:3}$	0.38(0.38)	0.21(0.21)	0.78(0.78)
$C_{20:1}$	0.52(0.47)	0.22(0.20)	1.32(1.20)

a) Mol %

Table 2. Percentage of each PN triglyceride fraction by HPLC

Band No.	Partition No. (PN)	Weight %	Weight % to total
1	46	6.39	0.19
	48	59.57	1.78
	50	34.04	1.02
2	46	3.10	0.72
	48	80.57	18.78
	50	16.33	3.81
3	46	8.90	5.21
	48	79.22	46.39
	50	11.88	6.96
4	46	21.34	2.61
	48	77.13	9.45
	50	1.53	0.19
5	46	38.10	1.10
	48	60.00	1.73
	50	1.90	0.06

Ⅲ. Results and Discussion

The oil level of the seed is 73.5%. The oil is a clear fluid which crystallizes in a refrigerator(at 4 ~5℃) and consists mainly of TG(94.8%) with minor components such as polar lipid(2.0%), hydrocarbon (1.8%) and sterol ester (1.4%). The fatty acid composition of the TG_s fractionated from CSO used in this study is shown in Table 1. COS is characterized by oleic acid(76.05%) palmitic acid(11.77%), linoleic acid(7.25%) and stearic acid(3.75%), and minor components such as palmitoleic acid, eicosamonoenoic acid and linolenic acid are also present.

The first step in the analysis was fractionation of TG_s according to their total double bond numbers on $AgNO_3$-TLC. The developing solvent of chloroform-ethyl alcohol separated the TG_s into five bands. The TG_s were mainly concentrated in band 3 (58.56%), followed by band 2(23.3%) and band 4 (12.25%) (Table 2).

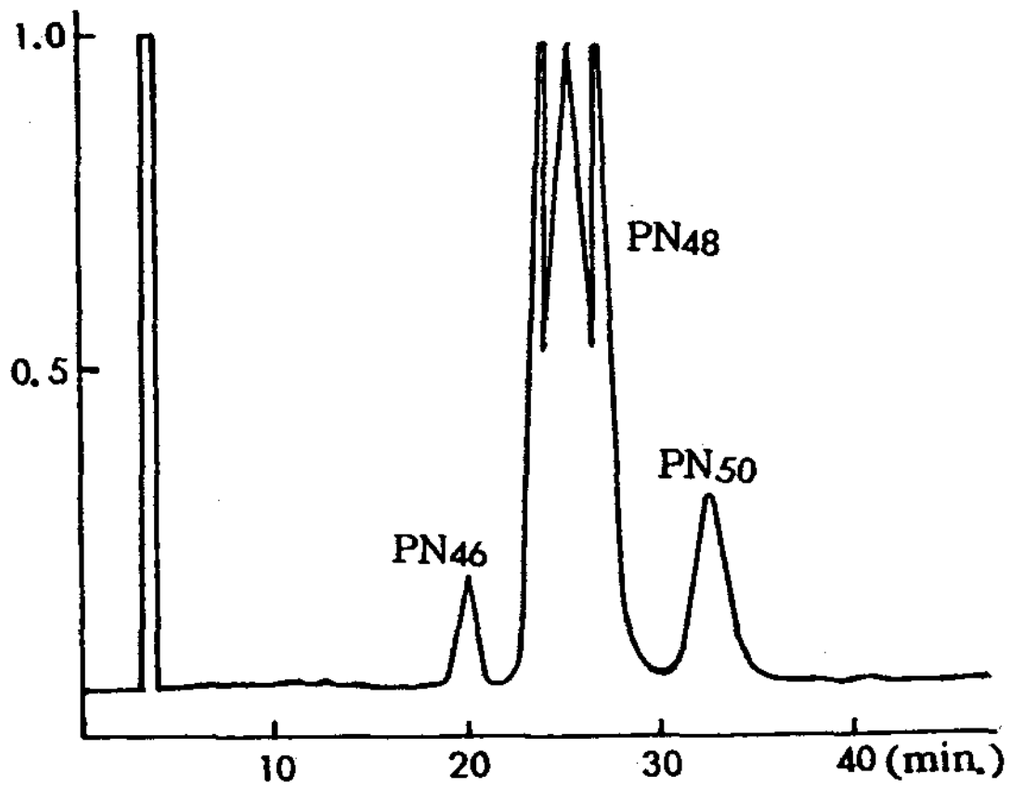

Fig. 1. HPLC of triglycerides from band 2 of *Camellia japonica* seed oil separated on $AgNO_3$-TLC.

Each PN was confirmed by co-running the standards ; 1,3-dipalmitoyl-2-myristoyl-rac-glycerol(PN =46), triolein(PN=48) and 1,3-dioleoyl-2-stearoyl-rac-glycerol(PN=50). The operational conditions are described in material and experimental.

The TG_s recovered from each band on $AgNO_3$-TLC was fractionated by HPLC. TG_s on reversed phase HPLC separated mainly according to their partition numbers(PN). PN can be empirically expressed as, PN=TG-2DB, where TG and DB denote the numbers of total carbon and double bonds in the acyl chains of a TG, respectively. As seen in Fig. 1, three peaks corresponding to PN 46, 48 and 50 appeared on all the HPLC chromatograms of $AgNO_3$-TLC bands and each PN fraction was collected. Table 2 reveals the weight percentage of each separated fraction on HPLC of TG_s bands, followed by PN 50 and PN 46. A small quantity of each PN fraction was qualitatively analyzed by GLC for more detailed information about TG composition. Three components of TC 50, 52 and 54 could be observed in the PN 46 with two components of TC 52 and 54 in both PN 48 and 50.

The fatty acid compositions-overall, at 1,3-position and 2-position-of triglycerides present in all the PN_s are listed in Table 3. As the TG_s of PN 46, 48 and 50 fractions are characterized by six, seven and five fatty acids respectively, they could be theoretically expected to comprise at least 56, 84 and 35 TG species without taking the isomers into consideration[37].

By combining the HPLC information with the fatty acid composition and TC components of each PN fraction, its TG composition was computed and the results are given in Table 4. The molar percentages of TG species were calculated according to the methods of Vander Wal[38] and Jurriens.[19]

As expected from the high level of unsaturated fatty acids sin CSO, most of TG species all occupy unsaturated fatty acids at the 2-position of their glycerol backbones. The results indicate the presence of 43 TG species of which levels exceed 0.1 mol % in CSO. The OOO and POO are the most predominant TG_s(39.8ml%, 25.8mol%) and other TG_s of importance are OPO(5.5mol %), OOL (4.8 mol %), POS(3.9mol %), SOO(3.5 mol %) and POL(3.0mol %). Caglioti[40] reported the triglycerides in olive oil, of which fatty acid composition was very similar to that of CSO, were predominantly composed of OOO(43.5%), POO(18.4%), OOL(6.8%), POL(5.9%) and SOO(5.1%). This result is in considerable agreement with our results. The composition of the TG molecular species in CSO is very similar to that obtained from the combinations of TG_s based on 1,3-random, 2-

Table 3. Fatty acid composition —overall, at 1,3-and 2-position —of each PN triglyceride

PN[a]	46			48			50		
Wt %	9.83			78.13			12.04		
FA[a]	Overall	1,3-position	2-position[b]	Overall	1,3-position	2-position[b]	Overall	1,3-position	2-position[b]
				(mol %)					
$C_{16:0}$	31.09	42.63	8.01	13.16	16.23	7.02	7.54	9.69	3.24
$C_{16:1}$	0.58	0.21	1.32	0.53	0.34	0.91	–	–	–
$C_{18:0}$	5.32	7.61	0.74	2.17	3.09	0.33	11.79	14.76	5.85
$C_{18:1}$	53.87	42.63	76.35	74.95	74.56	75.73	70.97	68.81	75.29
$C_{18:2}$	8.39	6.80	11.57	6.98	4.45	12.04	8.07	6.22	11.77
$C_{18:3}$	0.75	0.12	2.01	1.03	0.57	1.95	–	–	–
$C_{20:1}$	–	–	–	1.18	0.76	2.02	1.63	0.52	3.85

a) FA : Fatty acid PN : Partition number

b) Calculated from the equation : mol%, FA at 2-position=3 × [mol% overall FA] −2 × [mol% FA at 1,3-position]

Table 4. Molecular species of triglycerides in *Camellia japonica* seed oil

PN[a]	TC[b]	Species	Mole (%)	PN	TC	Species	Mole(%)
46	50	PoPO	0.05[c](tr.)[d]	48	52	PoOS	0.12(0.1)
		PPoP	0.40(tr.)[e]			SPoO	0.27(0.2)
		POPo	0.05(tr.)			PoSO	0.08(0.1)
		PLP	4.50(0.5)			PPoE	0.27(0.2)
		PPL	3.40(0.3)			PEPo	0.12(0.1)
		PoPoS	0.05(tr.)			PoPE	0.12(0.1)
		POSPo	0.05(tr.)	54		PLE	0.27(0.2)
52		SPLn	0.06(tr.)			LPE	0.27(0.2)
		PSLn	0.06(tr.)			PEL	1.78(1.6)
		PLnS	0.91(0.1)			SOL	0.61(0.5)
		PLO	0.52(tr.)			SLO	0.50(0.4)
		OPL	2.94(0.3)			OOO	45.43(39.8)
		POL	29.90(3.0)			PoOE	0.12(0.1)
		SPoL	0.37(tr.)			OPoE	0.47(0.4)
		PoSL	0.05(tr.)			PoEO	0.12(0.1)
		PoLS	0.06(tr.)			SLnS	1.95(1.7)
		PoOO	0.06(tr.)			OSL	0.08(0.1)
		OPoO	0.06(tr.)			SSLn	0.08(0.1)
	54	SOLn	0.06(tr.)	50	52	PPE	0.27(0.1)
		OSLn	0.06(tr.)			PEP	1.65(0.5)
		SLnO	1.10(0.1)			POS	12.55(3.9)
		LSL	0.52(tr.)			SPO	2.70(0.8)
		SLL	1.24(0.1)			PSO	0.55(0.2)
		OOL	48.28(4.8)		54	SSL	0.11(tr.)
		OLO	5.25(0.5)			SLS	1.53(0.5)
48	52	PLS	0.40(0.4)			SOO	11.09(3.5)
		SPL	0.40(0.4)			OSO	5.19(1.6)
		PSL	0.09(0.1)			POE	0.50(0.2)
		POO	29.45(25.8)			OPE	0.27(0.1)
		OPO	6.32(5.5)			PEO	2.20(0.7)

a) PN : Partition number
b) TC : Total acyl carbon number
PN : TC − 2 × DB (double bond number)
c) Mol % in each PN TG fraction
d) Mol % to total TGs
e) Tr. : trace (below 0.1 mol %)

random theory of distribution[41] as shown in Table 5. Vander Wal[42] indicated that the triglyceride structure of olive oil apparently conformed more closely to the 1,3-random, 2-random theory of distribution than to any other.

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Table 5. Triglyceride composition of *Camellia japonica* seed oil calcuated on the basis of 1,3-random, 2-random theory

TG species	mol %	TG species	Mol %
SSS[a]	0.37	LLS	0.13
SSU	1.30	LSL	0.03
USS	1.30	SLeLe	tr.
SUS	3.44	LeLeS	tr.
SSL	0.10	LeSLe	tr.
LSS	0.10	UUU	43.05
SLS	0.48	UUL	3.22
SSLe	tr[b]	LUU	3.22
LeSS	tr.	ULU	6.00
SLeS	0.03	UULe	0.12
SUU	12.18	LeUU	0.12
UUS	12.18	ULeU	0.42
USU	4.61	ULL	0.44
SUL	0.91	LLU	0.44
LUS	0.91	LUL	0.24
SLU	1.68	ULLe	0.02
ULS	1.68	LeLU	0.02
USL	0.34	ULeL	0.03
LSU	0.34	LLeU	0.03
SULe	0.03	LULe	0.01
LeUS	0.03	LeUL	0.01
SLeU	0.12	LLL	0.03
ULeS	0.12	LLLe	tr.
USLe	0.01	LeLL	tr.
LeSU	0.01	LLeL	tr.
SLLe	tr.	ULeLe	tr.
LeLS	tr.	LeLeU	tr.
SLeL	0.01	LeULe	tr.
LLeS	0.01	LLeLe	tr.
LSLe	tr.	LeLeL	tr.
LeSL	tr.	LeLLe	tr.
SLL	0.13	LeLeLe	tr.

a) S : Saturated fatty acids ($C_{16:0} + C_{18:0}$)
U : Monounsaturated acids ($C_{16:1} + C_{18:1}$)
L : Linoleic acid
Le : Linolenic acid
b) Below 0.01 mol %

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