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Non-aqueous reversed-phase high-performance liquid chromatography of synthetic triacylglycerols and diacylglycerols

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Abstract

Reversed-phase high-performance liquid chromatography (HPLC) methods were developed for the separation of molecular species of 45 synthetic triacylglycerols and diacylglycerols. These methods used linear gradients of methanol–isopropanol and UV detection at 205 nm as well as radioactive flow detection, which we add for metabolic studies. The elution orders of triacylglycerols and diacylglycerols depend on the polarity of their fatty acid constituents with elution time increasing as polarity decreases. The elution orders of triacylglycerols depending on their fatty acid constituents were as follows: ricinoleic acid<linolenic acid<palmitoleic acid<myristic acid<palmitelaidic acid<linoleic acid<linolelaidic acid<oleic acid<palmitic acid<elaidic acid<petroselinic acid<petroselaidic acid<stearic acid, while the elution orders of diacylglycerols were: palmitic acid<oleic acid<stearic acid. For both classes of glycerides, elution corresponded closely with chain length, degree of unsaturation and presence of polar groups and they were similar to the elution orders of fatty acids on an aqueous C_{18} HPLC which we reported recently. Other structural features also affect elution order, as triacylglycerol containing a *cis*-fatty acid elutes slightly earlier than its isomer containing a *trans*-fatty acid. Additionally, higher polarity in the *sn*-2 position causes earlier elution; Diacylglycerol with a hydroxy group at *sn*-2 position of the glycerol backbone and triacylglycerol with a polar group on the fatty acid chain at *sn*-2 position elute slightly earlier than their respective *sn*-1(3) isomers.

Keywords: Triacylglycerols; Diacylglycerols

1. Introduction

Triacylglycerols in living systems are complex mixtures and their analysis is tedious and usually requires HPLC separation. The HPLC separation of molecular species of triacylglycerols has been reported for almost 20 years [1–13], however, there have been few reports of HPLC separation for diacylglycerols [6]. Non-aqueous C_{18} reversed-phase HPLC has been extensively used for the separation of complex triacylglycerol mixtures in milk fat [1–5], soybean oil [6–8], oils of marine, algal and microbial origin [9], avocado oil [10] and synthetic mixtures [6,11,12]. Silver-ion HPLC for the separation of molecular species of triacylglycerols has also been reported [13]. In these studies, mass spectrometry [1–3,6,7,13] and gas chromatography [4,5,9] were used to identify the fatty acid constituents of triacylglycerols. Stereospecific analysis of triacylglycerols cannot be accomplished by these techniques. However, some stereospecific analysis of triacylglycerols has been accomplished by lipase

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hydrolysis followed by chiral HPLC of the diacylglycerol dinitrophenyl urethane derivatives [9].

Almost all of the C_{18} HPLC for the separation of molecular species of triacylglycerols reported [1–12] used non-aqueous eluents, though the eluent compositions varied. The eluents used were toxic in all cases [1–12] and absorbed strongly in the UV region in most cases. We report here a new HPLC method, using the less hazardous eluent methanol–isopropanol to separate molecular species of triacylglycerols and diacylglycerols. This solvent system allows the detection by UV at 205 nm and does not interfere with detection by flow-through liquid scintillation counting which we use for metabolic studies. Some elution characteristics of triacylglycerols and diacylglycerols are given here.

2. Experimental

HPLC was carried out on a liquid chromatograph consisting of a chromatography manager (Millennium V 2.15, Waters Associates, Milford, MA, USA) operated by a computer (PC 586), an in-line degasser (Waters), a pump (Waters, 600 controller), an injector (7125, Rheodyne, Cotati, CA, USA) and a photodiode array detector (Waters 996) detecting at 205 nm. A C_{18} column (25 cm×0.46 cm, 5 μm, Ultrasphere C_{18}, Beckman, Fullerton, CA, USA) was used. The eluent was a linear gradient starting at 100% methanol to 100% isopropanol in 40 min at a flow-rate of 1 ml/min (system 1 of Table 1). A gradient starting at 100% methanol to 50% methanol (in isopropanol), was also used for the separation of diacylglycerols and ricinoleate-containing triacylglycerols to increase the retention times and improve the separation (system 2 of Table 1). When necessary an evaporative light scattering detector (ELSD, MKIII, Varex, Rockville, MD, USA) was used to detect saturated compounds. The drift tube temperature of the ELSD was set at 75°C. Nitrogen gas flow of the nebulizer was set at about 1 l/min and N_2 gas pressure was about 22 p.s.i. (1 p.s.i.=6894.76 Pa). The N_2 pressure on the regulator of the N_2 tank was set at about 80 p.s.i. The triacylglycerol and diacylglycerol standards and castor oil were purchased from Sigma (St. Louis, MO, USA). The diacylglycerol and unsaturated triacylglycerol standards and castor oil were dissolved in isopropanol at a concentration of 1 mg/ml. Saturated triacylglycerol standards were dissolved in methylene chloride because they do not dissolve well in methanol, isopropanol or hexane. About 5 μg of standards were injected on the HPLC column. The back pressure of these HPLC runs ranged from about 550 p.s.i. initially to 2300 p.s.i. by the end of the runs (System 1).

The relative retention times of the five compounds indicated by a superscript d in Table 1 are from a single HPLC run of the mixture. The relative retention times of all other forty compounds are based on normalization to the one of these five compounds that elutes most closely and resolved. For example, triolein has a retention time of 32.83 min as shown in Table 1, with a different C_{18} column, retention time is 32.06 min and that of trielaidin is 32.64 min. The relative retention time of trielaidin with the original column in Table 1 is obtained by direct proportion and is 33.43 min. This relative retention time corrects for different HPLC runs, long-term column usage, different columns, etc. and the reproducibility is better than 0.5%. The relative retention times of two closely eluting compounds in Table 1 were obtained by sequential HPLC runs using the same column. The elution orders for two closely eluting compounds, such as the *sn*-1(3) and *sn*-2 isomers of triacylglycerols containing a fatty acid with a single double bond, were confirmed by the repetitive HPLC analysis.

The castor microsomal incubation of 1-palmitoyl-2-[1-^{14}C]-oleoyl-*sn*-glycero-3-phosphocholine, the putative substrate of oleoyl-12-hydroxylase, is the same as we recently described [14]. The triacylglycerol fraction was from a silica HPLC [15] of intact lipid extract from the incubation. The radioactivity flow detector (Flow-one, Model CR; Packard, Downers Grove, IL, USA) included a flow cell of 0.5 ml, with delay time of 30 s compared to the photodiode array detector. The flow-rate of liquid scintillation fluid (Ready Safe, Beckman) was 3 ml/min.

3. Results and discussion

Since we are investigating the biosynthesis of

triacylglycerols in castor bean and other oil seeds, a suitable HPLC system was required for the separation of molecular species of triacylglycerols and diacylglycerols. In the HPLC solvent systems we report here, only methanol and isopropanol were used in linear gradients. Both a UV detector at 205 nm and a radioactivity flow detector can be used simultaneously for the identification of radioactive metabolites. In previous reports an ELSD [1–3,6,7,9,10,13] and a refractive index detector [4,5,8,11,12] were used. The UV detector at 205 nm is used to detect unsaturated compounds and is not sensitive to saturated compounds. Detection at 205 nm cannot be used when the eluent contains methylene chloride [1,6,7,13] or acetone [4,5,8,10,12,13]. Eluents used in some of the previous HPLC systems [2,3,9] could also have used UV detection. Any solvent causing significant quenching in liquid scintillation counting must also be avoided as an eluent component for radioactivity flow detection. The eluents used in the present HPLC systems, methanol–isopropanol, have the least quenching effect on liquid scintillation counting compared to the eluents used in previous HPLC systems [1–13] which contain quenching solvents such as acetone, acetic acid, methylene chloride, acetonitrile and/or propionitrile. In contrast to the eluents previously used, the eluents used in this study, methanol–isopropanol, are less toxic and less hazardous environmentally. Methanol is less toxic than acetonitrile, the commonly used eluent component [1–13], according to the exposure limits shown in the Material Safety Data Sheets. Isopropanol (rubbing alcohol) is less toxic than any other eluent component used [1–13]. Isopropanol has been used previously [2,3,9] as an eluent component while methanol has not.

The relative retention times of 45 synthetic diacylglycerols and triacylglycerols are shown in Table 1. The retention times of 35 synthetic compounds listed in Table 1 have not previously been reported [6,11–13]. The diacylglycerols and triacylglycerols in Table 1 are arranged in the order of elution (decreasing polarity). These HPLC systems used one C_{18} column and good separation efficiency (theoretical plate number illustrated by peak width and retention time) was obtained as shown in Fig. 1. Some previous reports used two [6,7] or three columns [11] connected in series and longer running times up to 100 min [7] were needed. We have previously used two C_{18} columns in series to improve the separation of fatty acids from that achieved with a single column [16].

The elution orders of triacylglycerols and diacylglycerols in reversed-phase HPLC systems depend on their polarities with elution time increasing as polarity decreases. Diacylglycerols eluted earlier than triacylglycerols, as shown in Table 1, because diacylglycerols contain two fatty acids and one free hydroxy group on the glycerol backbone and are thus more polar than triacylglycerols. The elution orders of 1,3-dipalmitin≤1,3-diolein<1,3-distearin; 1,2-dipalmitin≤1,2-diolein<1,2-distearin as shown in Table 1 correspond to the elution order of palmitic acid<oleic acid<stearic acid that we have previously observed for fatty acids [16]. 1-Palmitoyl-3-stearoyl-*rac*-glycerol eluted between 1,3-dipalmitin and 1,3-distearin as expected.

The elution order (polarity order) of triacylglycerols containing three identical fatty acids is shown in Table 1, and the order is similar to that of their fatty acid constituents as we recently reported [16]. The triacylglycerol and diacylglycerol relative retention times directly depend on their fatty acid constituents. The exceptions to the elution orders of a few closely-eluting triacylglycerols and fatty acids may be because the eluent (methanol–water) used for fatty acids [16] was different from that used for triacylglycerols. The general phenomena that affect retention times of fatty acids, as we previously reported [16], are: the presence of polar substituents, e.g. hydroxy, which increase polarity; the presence of a double bond, triple bond and cyclopropane ring, which also increase polarity; geometry/position of double bond, triple bond, cyclopropane ring and polar group; and chain length which decrease polarity with increasing chain length. These phenomena of fatty acids can also be found in triacylglycerols and diacylglycerols as shown in Table 1, for example: the presence of a hydroxy group or double bond on the fatty acid increases the polarity of the triacylglycerol and diacylglycerol; the increase of the number of double bonds on the fatty acid increases the polarity of triacylglycerol and diacylglycerol; the increase of chain length of fatty acid decreases the polarity of triacylglycerol and diacylglycerol.

The elution order of mixed triacylglycerols in this

Table 1
Relative retention times of diacylglycerols and triacylglycerols in C_{18} HPLC[a]

Diacylglycerols and triacylglycerols	Relative retention times (min)	
	System 1[b]	System 2[c]
Triricinolein (from castor oil)	7.63	7.68
Diricinoleoyl-linolenoyl-glycerol (from castor oil)	11.74	12.53
Diricinoleoyl-linoleoyl-glycerol (from castor oil)	12.93	14.44
Diricinoleoyl-oleoyl-glycerol (from castor oil)	14.52	16.83
1,3-Dipalmitin	16.17	18.48
1,3-Diolein	16.19	18.67
1,2-Dioleoyl-*sn*-glycerol[d]	16.32	18.95
1,2-Dipalmitoyl-*sn*-glycerol	16.36	18.97
1-Palmitoyl-3-stearoyl-*rac*-glycerol[e]	18.45	22.63
1,3-Distearin	21.10	27.13
1,2-Distearoyl-*rac*-glycerol	21.35	27.62
Trilinolenin[d]	23.28	
Tripalmitolein	26.69	
Trimyristin	26.80	
Tripalmitelaiden	27.43	
Trilinolein[d]	27.92	
Trilinolelaidin	28.41	
1,2-Dimyristoyl-3-oleoyl-*rac*-glycerol	28.85	
1,2-Dimyristoyl-3-palmitoyl-*rac*-glycerol	29.06	
1,2-Dilinoleoyl-3-oleoyl-*rac*-glycerol	29.41	
1,2-Dilinoleoyl-3-palmitoyl-*rac*-glycerol	29.50	
1-Myristoyl-2-oleoyl-3-palmitoyl-*rac*-glycerol	31.02	
1,2-Dioleoyl-3-linoleoyl-*rac*-glycerol	31.27	
1,2-Dipalmitoyl-3-myristoyl-*rac*-glycerol	31.34	
1-Palmitoyl-2-oleoyl-3-linoleoyl-*rac*-glycerol	31.35	
1,3-Dipalmitoyl-2-linoleoyl-glycerol	31.43	
1-Palmitoyl-2-elaidoyl-3-linoleoyl-*rac*-glycerol	31.56	
Triolein[d]	32.83	
1,2-Dioleoyl-3-palmitoyl-*rac*-glycerol	32.93	
1,3-Dioleoyl-2-palmitoyl-glycerol	32.96	
1,3-Dipalmitoyl-2-oleoyl-glycerol	33.12	
1,2-Dipalmitoyl-3-oleoyl-*rac*-glycerol	33.16	
Tripalmitin	33.27	
1-Linoleoyl-2-oleoyl-3-stearoyl-*rac*-glycerol	33.32	
Trielaidin	33.43	
Tripetroselinin	33.66	
Tripetroselaidin	34.08	
1,2-Dioleoyl-3-stearoyl-*rac*-glycerol	34.73	
1,3-Dioleoyl-2-stearoyl-glycerol	34.76	
1-Palmitoyl-2-oleoyl-3-stearoyl-*rac*-glycerol	34.91	
1,2-Distearoyl-3-myristoyl-*rac*-glycerol	35.20	
1,3-Distearoyl-2-oleoyl-glycerol	36.64	
1,2-Distearoyl-3-oleoyl-*rac*-glycerol	36.72	
1,2-Distearoyl-3-palmitoyl-*rac*-glycerol	37.07	
Tristerin[d]	38.86	

[a] For HPLC conditions, see Section 2.
[b] Linear gradient from 100% methanol to 100% isopropanol in 40 min.
[c] Linear gradient from 100% methanol to 50% methanol in isopropanol in 40 min.
[d] These triacylglycerols are used for normalization of retention times to get relative retention times for system 1.
[e] This triacylglycerol is for system 2.

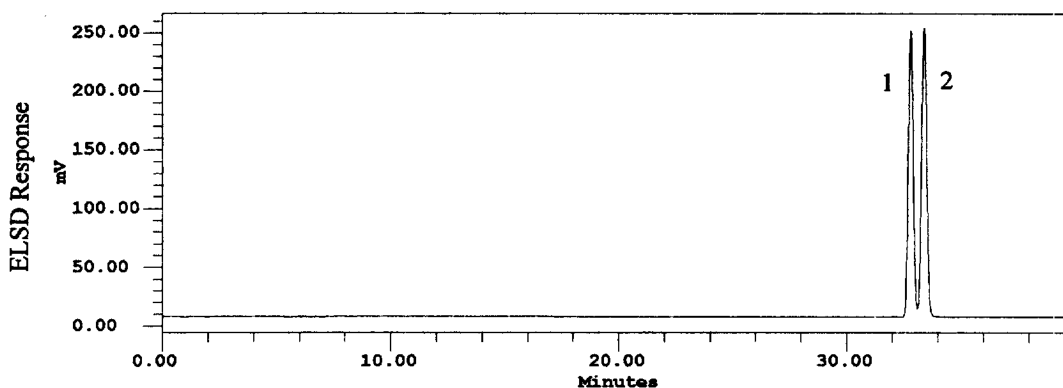

Fig. 1. HPLC separation of triacylglycerols with different double bond configurations, (1) triolein, retention time 32.82 min; and (2) trielaidin, 3.40 min. C_{18} column was used with a linear gradient of 100% methanol to 100% isopropanol in 40 min at a flow-rate of 1 ml/min. For other HPLC conditions see Section 2.

non-aqueous reversed-phase HPLC also directly depends on their constituent fatty acids. The order is as follow: ricinoleic acid [D(+)-12-hydroxy-*cis*-9-octadecenoic acid]<linolenic acid<palmitoleic acid<myristic acid (14:0)<palmitelaidic acid (16:1; *trans*-9)<linoleic acid<linolelaidic acid (18:2; *trans,trans*-9,12)<oleic acid<palmitic acid<elaidic acid (18:1; *trans*-9)<petroselinic acid (18:1; *cis*-6)< petroselaidic acid (18:1; *trans*-6)<stearic acid. We can find many examples by comparing the elution orders of two triacylglycerols containing only one different fatty acid: trimyristin<1,2-dimyristoyl-3-oleoyl-*rac*-glycerol<1,2-dimyristoyl-3-palmitoyl-*rac*-glycerol < 1-myristoyl-2-oleoyl-3-palmitoyl-*rac*-glycerol<1,2-dipalmitoyl-3-myristoyl-*rac*-glycerol. There are many other examples found in Table 1 and we have found no exception to the rules. This order of constituent fatty acids of triacylglycerols is not exactly the same as that of diacylglycerols given here and is also slightly different from the order of triacylglycerols in another non-aqueous reversed-phase HPLC system [6].

The HPLC separations reported here used the synthetic triacylglycerols and diacylglycerols with exactly known structures, while previous reports were the HPLC of triacylglycerols and diacylglycerols with unknown *sn*-stereospecific positions, unknown positions of double bonds and/or unknown configurations of double bonds. However, some comparison of elution orders with previous reports can be made. The elution orders reported here are not exactly the same as those of the previous reports, not only the pairs with the same partition numbers (carbon number minus twice the number of double bonds) but also the pairs with different partition numbers [12]. Some methods have been developed to calculate the relative retention times of triacylglycerols such as partition number, equivalent carbon number, theoretical carbon number and matrix model [17]. However, minor factors affecting the relative retention times such as *sn*-stereospecific positions, positions of double bonds and/or configurations of double bonds were not taken into account.

The effect of double bond configuration/position on the retention of triacylglycerols was not shown previously in non-aqueous reversed-phase HPLC systems [1–12]. However, some triacylglycerols with *trans*-configuration of the double bond on the fatty acid and with a C-6 double bond are listed in Table 1. A triacylglycerol containing a *cis*-fatty acid eluted slightly earlier than its isomer containing a *trans*-fatty acid. An example of the separation of *cis*- and *trans*-configurations is shown in Fig. 1 with almost baseline separation. Some other examples of configurations can also be found in Table 1. The *trans*-fatty acids are present in plants and bacteria as minor fatty acids, but are a concern for nutrition and health. Triacylglycerol with a C-9 double bond (oleic acid or elaidic acid) elutes slightly earlier than its isomer with C-6 double bond (petroselinic acid or petroselaidic acid) such as: triolein<tripetroselinin; trielaidin<tripetroselaidin. This corresponds to our

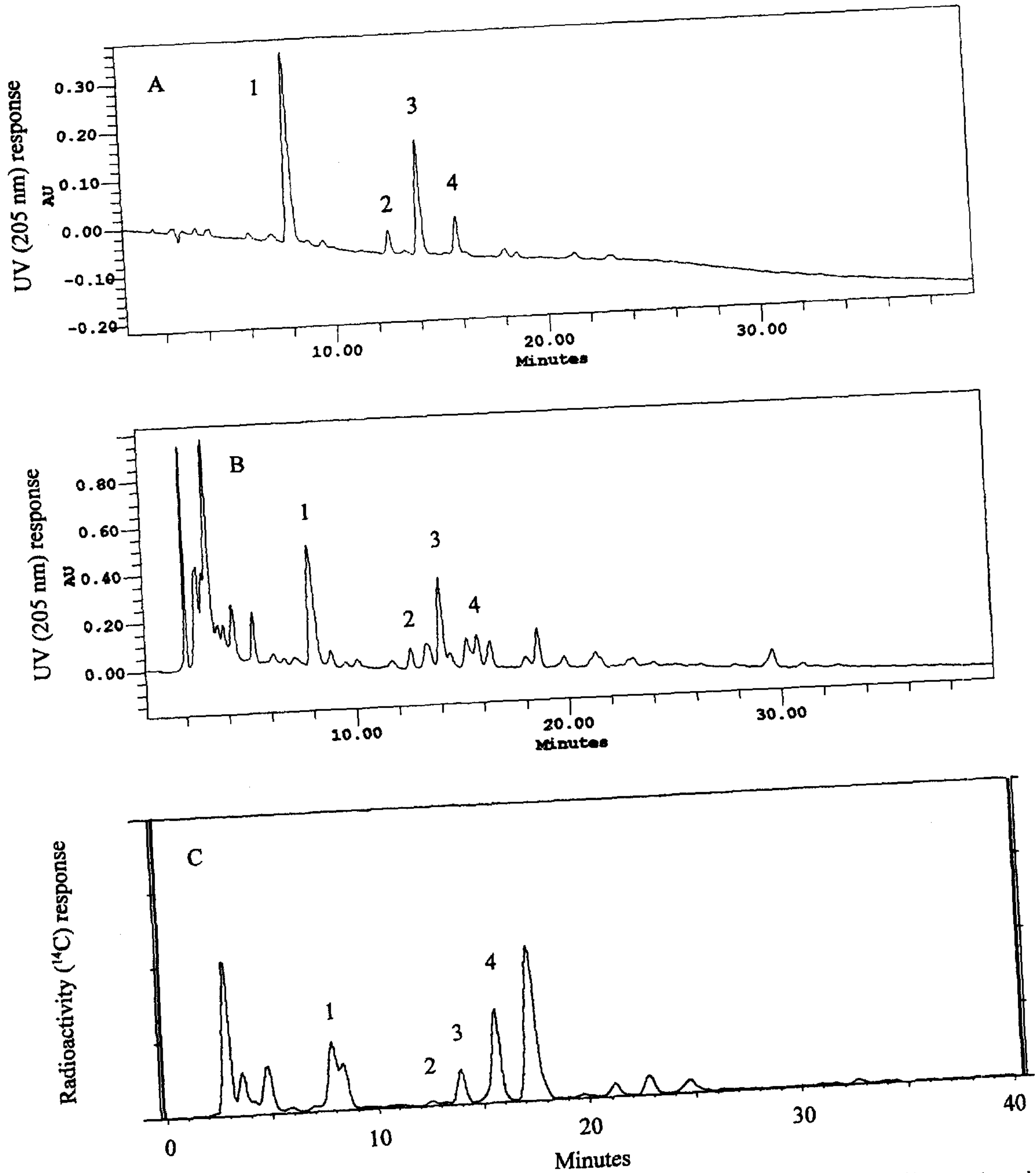

Fig. 2. HPLC identification of triacylglycerols from the castor microsomal incubation of 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine. The HPLC conditions are the same as those of Fig. 1. (1) triricinolein, (2) diricinoleoyl-linolenoyl-glycerol, (3) diricinoleoyl-linoleoyl-glycerol and (4) diricinoleoyl-oleoyl-glycerol identified are indicated in the chromatograms. (A) HPLC of castor oil (80 μg) with the retention times of triacylglycerols: (1) 7.94 min (2) 12.62 min (3) 14.05 min (4) 15.79 min. (B) HPLC of triacylglycerol fraction of the lipid extract from the incubation which was co-chromatographed with castor oil (80 μg). The retention times are: (1) 7.95 min (2) 12.61 min (3) 14 min (4) 15.75 min. (C) Radiochromatogram of the same HPLC run as (B). The retention times obtained from radioactive flow detector were: (1) 7.9 min (2) 12.6 min (3) 14.0 min (4) 15.7 min.

previous observation of the elution order of fatty acids that a *cis*-double bond (or a *trans*-double bond) at C-10 of the methyl ester of octadecenoic acid decreased the retention time most, with slightly increased retention times as the double bond shifted toward either end of the chain [16].

The effect of *sn*-stereospecific positions of fatty acids on the relative retention time is very small and has not been previously reported. 1,3-Diacylglycerol elutes slightly earlier than its 1,2-isomer as shown in Table 1: 1,3-dipalmitin>1,2-dipalmitoyl-*sn*-glycerol; 1,3-diolein>1,2-dioleoyl-*sn*-glycerol; 1,3-distearin> 1,2-distearoyl-*rac*-glycerol. It seems that a hydroxy group at *sn*-2 position of the glycerol backbone is slightly more polar than the hydroxy groups at *sn*-1 and *sn*-3. Triacylglycerol with the fatty acid containing a double bond (slightly polar) at *sn*-2 position elutes slightly earlier than its isomer with the fatty acid containing a double bond at *sn*-1(3) position, for example: 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol< 1,3-dioleoyl-2-palmitoyl-glycerol; 1,3-dipalmitoyl-2-oleoyl-glycerol < 1,2-dipalmitoyl-3-oleoyl-*rac*-glycerol; 1,2-dioleoyl-3-stearoyl-*rac*-glycerol<1,3-dioleoyl-2 -stearoyl-glycerol;1,3 -distearoyl-2-oleoyl-glycerol<1,2-distearoyl-3-oleoyl-*sn*-glycerol. This seems limited to the triacylglycerols without the fatty acid which is more polar than oleic acid. It appears that diacylglycerol with a hydroxy group at *sn*-2 position of the glycerol backbone and triacylglycerol with a polar group on the fatty acid chain at *sn*-2 position elute slightly earlier than their respective *sn*-1(3) isomers. The constituent fatty acids at *sn*-1 and 3 positions contribute equally to the polarity of 1,3-diacylglycerols and the racemates cannot be separated.

We are currently using this HPLC method (system 1) in metabolic studies. The radioactive triacylglycerols incorporated from the incubation of 1-palmitoyl-2-[1-^{14}C]-oleoyl-*sn*-glycero-3-phosphocholine, the putative substrate of oleoyl-12-hydroxylase, in castor microsomes were identified by HPLC as shown in Fig. 2. The radioactive metabolites, triricinolein, diricinoleoyl-linolenoyl-glycerol, diricinoleoyl-linoleoyl-glycerol and diricinoleoyl-oleoyl-glycerol, were identified by HPLC with simultaneous detections of mass by UV at 205 nm and radioactivity. Triricinolein, diricinoleoyl-linolenoyl-glycerol, diricinoleoyl-linoleoyl-glycerol, and diricinoleoyl-oleoyl-glycerol in castor oil were used as standards. The identification was by co-chromatography of radioactive metabolites and standards, and the identical retention times of radioactive metabolites and standards were obtained through radioactivity and UV detections. The labelling of metabolites was also quantified as shown in Fig. 2C. Some radioactive metabolites shown in the radiochromatogram (Fig. 2C) have not been identified as yet, but this HPLC is of great help in elucidating the process of triacylglycerol production in castor.

We have developed these HPLC systems which have advantages over the HPLC systems previously reported [1–13] for metabolic studies of triacylglycerols and diacylglycerols. The elution characteristics given here can be helpful in identification of triacylglycerols and diacylglycerols while the standards are not available. We have previously given the elution characteristics of fatty acids [16] and gibberellins, a group of plant hormones [18], and have used those elution characteristics to aid in the identification of unknown compounds.

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