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Molecular Species of Acylglycerols Incorporating Radiolabeled Fatty Acids from Castor (*Ricinus communis* L.) Microsomal Incubations

JIANN-TSYH LIN,* JENNIFER M. CHEN, LUCY P. LIAO, AND THOMAS A. MCKEON

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710

Sixty-one molecular species of triacylglycerols (TAG) and diacylglycerols produced from castor microsomal incubations incorporating six different ^{14}C-labeled fatty acids have been identified and quantified. The preference for incorporation into TAG was in the order ricinoleate > oleate > linoleate > linolenate > stearate > palmitate. Ricinoleate was the major fatty acid incorporated, whereas stearate, linolenate, and palmitate were incorporated at low levels. Twenty-one molecular species of acylglycerols (HPLC peaks) in castor oil have also been assigned. The levels of TAG in castor oil are RRR (triricinolein) ≫ RR-TAG ≫ R-TAG > no R-TAG. The levels of the molecular species within the groups of RR-TAG, RL-TAG, and LL-TAG individually are ricinoleate > linoleate > oleate > linolenate, stearate, and palmitate. The results of the labeled fatty acid incorporation are consistent with ricinoleate being preferentially driven into TAG and oleate being converted to ricinoleate in castor oil biosynthesis.

KEYWORDS: Molecular species; triacylglycerol; diacylglycerol; castor oil; biosynthesis; HPLC; relative retention times; *Ricinus communis* L.

1. INTRODUCTION

The presence of the hydroxy group on ricinoleate (12-hydroxyoleate) underlies many industrial uses such as the manufacture of lithium grease, plastics, paints, coatings, and cosmetics. Castor oil contains 90% of its fatty acids (FA) as ricinoleate (*1*) and is the only commercial source of ricinoleate. Because castor bean contains the toxin ricin and potent allergens, it is hazardous to grow, harvest, and process. It would be desirable to instead produce ricinoleate from a transgenic oilseed lacking these toxic components. The cDNA for oleoyl-12-hydroxylase, the enzyme catalyzing the hydroxylation of oleate to ricinoleate, has been cloned from castor and expressed in tobacco, resulting in accumulation of low levels of ricinoleate in seed lipid (*2*). Later, the expression of this enzyme in a transgenic *Arabidopsis thaliana* plant was improved but still resulted in low levels of hydroxy FA (*3*) compared to castor oil (20 vs 90% ricinoleate in castor oil). To develop a transgenic plant that produces seed oil containing a high level of ricinoleate, it is important to know the biosynthetic pathway of castor oil and to identify the key enzymatic steps that drive ricinoleate into triacylglycerol (TAG) in castor bean.

We have previously identified 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine (2-oleoyl-PC) as the immediate substrate of oleoyl-12-hydroxylase and oleoyl-12-desaturase in castor microsomes (*4*) and later also shown that 1-acyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (2-oleoyl-2-PE) cannot be used as the immediate substrate for either of these two enzymes (*5*). We have demonstrated the conversion of 2-oleoyl-PC to 2-oleoyl-PE and vice versa (*4*, *5*). In addition to oleoyl-12-hydroxylase, we have also identified phospholipase A_2 and 1,2-diacyl-*sn*-glycerol-acyltransferases in the pathway as the key enzymatic steps that drive ricinoleate into TAG (*4*).

The final step in castor oil biosynthesis can be carried out by acyl-CoA:diacylglycerol acyltransferase (DGAT) and/or phospholipid:diacylglycerol acyltransferase (PDAT) (*6*). We have studied the biosynthetic pathway on the lipid class level instead of molecular species mostly, including phosphatidylcholine (PC), phosphatidylethanolamines (PE), and TAG.

Because the final step in castor oil biosynthesis has been considered to support ricinoleate incorporation, we wanted to evaluate the relative effectiveness for incorporating each of the fatty acids found in castor oil. The identifications of the molecular species of [^{14}C]TAG incorporating [^{14}C]oleate in microsomes of developing sunflower cotyledons were reported (*7*) as LOO, OOO, and LLO (in order of abundance) and of [^{14}C]DAG as LO, LL, and OO. The molecular species of [^{14}C]-TAG incorporating [^{14}C]linoleate (*7*) were LLO, LOO, LLL, and those of [^{14}C]DAG were LO and LL. To further characterize the acylation steps in castor oil biosynthesis, we have compared the incorporation of these six FAs present in castor oil into the molecular species of acylglycerols (AG) using castor microsomal incubations.

* Author to whom correspondence should be addressed [telephone (510) 559-5764; fax (510) 559-5768; e-mail jtlin@pw.usda.gov].

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2. MATERIALS AND METHODS

2.1. Microsomal Incubation. Microsomes from castor (*Ricinus communis* L.) bean were prepared as previously described (*4*, *8*), and microsomal incubations were scaled up to generate suitable amounts of material. The incubation mixture in a total volume of 20 mL included sodium phosphate buffer (0.1 M, pH 6.3), CoA-SH (10 μmol), NADH (10 μmol), ATP (10 μmol), $MgCl_2$ (10 μmol), catalase (20000 units), and a microsomal fraction of endosperm from immature castor bean (300 μL, 2.76 mg of protein). These components were 20 times our previous single incubation (*4*, *5*, *8*) with the addition of CoA-SH. The [^{14}C]FA, ricinoleate (55 Ci/mol, American Radiolabeled Chemicals, Inc., St. Louis, MO), stearate (44 Ci/mol, NEN Life Science Products, Boston, MA), oleate (52 Ci/mol, NEN), linoleate (51 Ci/mol, NEN), linolenate (52 Ci/mol, NEN), and palmitate (56 Ci/mol, NEN) were used individually as incubation substrates. The [^{14}C]FA substrate (5.0 μCi, in 400 μL of ethanol) was added last into a screw-capped bottle containing the incubation mixture, followed by immediate mixing. The mixture was then incubated in a shaking water bath for 60 min at 22 °C. The incubation was stopped by suspension in 75 mL of chloroform/methanol (1:2, v/v), followed by mixing with 12.5 mL of chloroform and 12.5 mL of water. The lower chloroform layer containing the lipid extract was dried and fractionated on a silica HPLC system to separate lipid classes as described below.

2.2. HPLC. HPLC was carried out on a liquid chromatograph (Waters Associates, Milford, MA), using a photodiode array detector (Waters 996) detecting absorbance at 205 nm. Radiolabeled lipids were separated by HPLC and identified by cochromatography with lipid standards by matching the retention times (RT) from the UV detector and flow scintillation analyzer. The TAG and diacylglycerol (DAG) standards were purchased from Sigma (St. Louis, MO) or Nu-Chek Prep, Inc. (Elysian, MN). The flow rate of HPLC eluents was 1 mL/min. The flow rate of liquid scintillation fluid (Ultima Flo M, Packard Instrument Co., Downers Grove, IL) through the flow scintillation analyzer (150TR, Packard Instrument Co.) was 3 mL/min. The flow scintillation analyzer used a 200-μL flow cell.

2.2.1. Separation of Lipid Classes. Lipid classes were separated according to the procedure of Singleton and Stikeleather (*9*) on a silica column [25 × 0.46 cm, 5 μm, Luna, silica (2), Phenomenex, Torrance, CA] with a linear gradient starting at 2-propanol/hexane (4:3, v/v) to 2-propanol/hexane/water (4:3:0.75, v/v) in 20 min and then held isocratically for 20 min. A prepacked silica saturator column (3 × 0.46 cm, 15−25 μm, Phenomenex) was installed between the pump and injector to saturate the mobile phase with silica before it reached the analytical column.

2.2.2. Separation of Molecular Species of TAG and DAG. Molecular species of TAG and DAG were separated as we previously reported (*10*), using a C_{18} column (25 × 0.46 cm, 5 μm, Ultrasphere C18, Beckman Instruments Inc., Fullerton, CA) with a linear gradient starting at 100% methanol to 100% 2-propanol in 40 min.

3. RESULTS AND DISSCUSSION

3.1. Separation of ^{14}C-Labeled Lipid Classes. We have identified ricinoleate (R), stearate (S), oleate (O), linoleate (L), linolenate (Ln), and palmitate (P) in castor microsomal incubations as the constituents of AG, PC, and PE. For the purpose of comparing the incorporation of these FAs into the molecular species of TAG, the end product in the biosynthesis of castor oil, we have used these six [^{14}C]FAs individually as substrates for castor microsomal incubations and have identified and quantified the molecular species of [^{14}C]TAG and [^{14}C]DAG that have incorporated these FAs. The total lipid extracts from the [^{14}C]FA incubations were fractionated by silica HPLC (*9*) to separate the lipid classes. The silica HPLC radiochromatogram separating the lipid classes from the [^{14}C]oleate incubation is shown in **Figure 1**, and the lipid classes are AG, an unknown, PE, PC, and 1-acyl-2-ricinoleoyl-PC (2-ricinoleoyl-PC). The AG fraction included the ^{14}C-labeled TAG, DAG, monoacylglycerols, and FA.

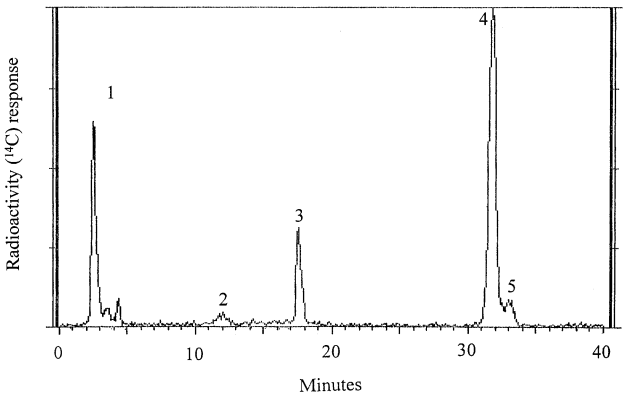

Figure 1. Silica HPLC radiochromatogram for the separation of lipid classes of total lipid extract (2 μL injected from the total 100 μL methanol solution) from the castor microsomal incubation (60 min) with [^{14}C]oleic acid (see Materials and Methods, section 2.2.1): (1) AG (acylglycerols, 2–5 min, including fatty acids); (2) unknown; (3) PE (phosphatidylethanolamines); (4) PC (phosphatidylcholines); (5) ricinoleoyl-PC.

3.2. Separation and Identification of the Molecular Species of [^{14}C]Acylglycerols. The AG fraction was collected and rechromatographed to separate the molecular species of [^{14}C]-AG using C_{18} HPLC (*10*). Cochromatography with standards was used to identify the molecular species of [^{14}C]AG. Because many standards are not commercially available, the assignment of the radiolabeled peaks without standards was inferred from the following: (A) HPLC elution characteristics shown by the molecular species of TAG and DAG (*10*). The elution order of the AG depends on their FA constituents as ricinoleate < linolenate < linoleate < palmitate < oleate < stearate. AG with the same partition numbers elute closely or at the same time. (B) All FA constituents in the AG eluting as a ^{14}C peak were limited to these six endogenous FAs. The AGs containing linolenate, stearate, and palmitate were minor AG compared to those containing ricinoleate, oleate, and linoleate. (C) All six [^{14}C]FAs used can be incorporated into [^{14}C]AG. In the [^{14}C]-oleate incubation, oleate can be metabolized to ricinoleate and linoleate (*2*), so the FA constituents of AG of a ^{14}C peak must contain oleate, ricinoleate, and/or linoleate. FA was unchanged in the other five [^{14}C]FA incubations, so one of the FA constituents of AG of a ^{14}C peak must be the same [^{14}C]FA used for the incubation. (D) The differentiation of *sn*-1(3) and *sn*-2 was based on the relative retention time (RRT) prediction method we had previously developed (*11*) and are, therefore, preliminary.

3.2.1. Molecular Species of [^{14}C]Acylglycerol from [^{14}C]-Ricinoleate Incubation. **Figure 2** depicts the components of the AG fraction (see **Figure 1**) from the [^{14}C]ricinoleate incubation, separated on C_{18} HPLC. The molecular species of AG in castor oil shown in **Figure 2A** are designated for identification purposes and were derived from the combined results of the incubations of the six [^{14}C]FAs described later. **Figure 2B** shows the separation of the molecular species of [^{14}C]AG incorporated from [^{14}C]ricinoleate. The four ^{14}C peaks in **Figure 2B** were identified by matching the retention times (RT) of the four major TAG in castor oil shown in **Figure 2A** and identified previously (*4*), and they are RRR, RRLn, RRL, and RRO. The other ^{14}C peaks were designated by the combined methods described earlier (section 3.2). Because ricinoleate was unchanged in the microsomal incubation, there is no ^{14}C peak after 28 min, where TAG containing no ricinoleate eluted. In this radiochromatogram, TAGs were well separated as groups on the basis of

Table 1. Incorporation of Various Radiolabeled Fatty Acids into Molecular Species of Triacylglycerols and Diacylglycerols in Castor Microsomal Incubations (60 min)

fatty acid[a] (nmol)	radiolabel incorporated in triacylglycerols and diacylglycerols[b] (nmol)
ricinoleate (91)	RR (3.8), RRR (6.7), RRLn (1.9), RRL (12), RRO (9.7), RRS (6.1), RLLn (1.1), RLL (4.1), RLO (3.6), ROO (1.2), RLS (1.0), ROS (0.38)
stearate (114)	RS (0.76), LnS (0.22), RRS + LS (1.8), PS (0.58), SS (0.10), RLnS (0.11), RLS (0.45), ROS (0.35), RSS (0.10), LnSLn (0.12), LSLn (0.14), LSL (0.65), LSO (0.45)
oleate (96)	RR (0.46), RRR + RO (1.8), RRLn (0.16), LL (0.65), RRL (1.3), RRP (2.4), RRO (2.8), OO (2.2), RRS (0.22), RLLn (0.58), RLL (0.74), RLO (1.9), ROO (1.3), RLS(0.10), ROS (0.21), LLLn (0.10), LLL (0.30), LLO (0.92), LOO (0.83), OOO (0.37)
linoleate (98)	RL (2.0), LLn (0.6), LL (3.5), RRL (2.4), LO (1.8), LS (0.48), RLLn (0.57), RLL (2.8), RLO (1.6), RLS (0.39), LLnLn (0.24), LLLn (0.56), LLL (2.2), LLO (1.4), LOO (0.23), LLS (0.25), OLS (0.08)
linolenate (96)	RLn (0.91), LnLn (0.83), LnL (1.8), RRLn (1.7), OLn (1.2), SLn (0.41), RLnLn (0.62), RLLn (1.9), ROLn (1.2), RSLn (0.31), LnLnLn (0.32), LnLLn (0.94), LLLn (1.5), LOLn (1.0), OOLn (0.30), LSLn (0.19)
palmitate (89)	RP (3.2), PLn (0.20), PL (0.78), RRP (0.78), OP (0.40), PP (0.21), PS (0.13), RLnP (0.13), RLP (0.47), RPP (0.16), ROP (0.19), RSP (0.10), LnLP (0.10), LLP (0.45), LOP (0.41), LSP (0.10)

[a] Radioactivity of 5.0 μCi was used for each fatty acid incubation. The amounts (nmol) of fatty acid used are different because the specific radioactivities of fatty acids are different. [b] The molecular species of triacylglycerols and diacylglycerols are shown in **Figures 2** and **3** in HPLC elution order. The fatty acid constituents of acylglycerols are abbreviated as follows: R, ricinoleate; S, stearate; O, oleate; L, linoleate: Ln, linolenate; P, palmitate.

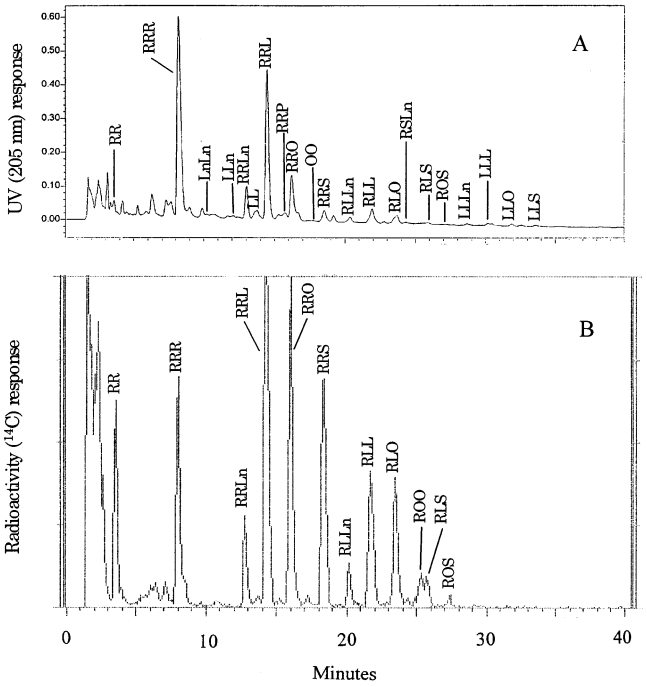

Figure 2. C_{18} HPLC radiochromatogram (B) for the separation of molecular species of acylglycerols (AG) in the AG fraction (see **Figure 1**, 5 μL from 100 μL methanol solution) from the castor microsomal incubation (60 min) of [^{14}C]ricinoleic acid cochromatographed with castor oil (see Materials and Methods, section 2.2.2). The upper UV (205 nm) chromatogram (A) shows the separation of molecular species of AG in castor oil. The molecular species of AG are given as the abbreviations of their fatty acid constituents: R, ricinoleate; S, stearate; O, oleate; L, linoleate; Ln, linolenate; P, palmitate.

differing numbers of ricinoleate, RRR, RR-TAG, R-TAG, and no R-TAG.

3.2.2. Molecular Species of [^{14}C]Acylglycerol from [^{14}C]-Oleate Incubation. **Figure 3** is the radiochromatogram for the AG fraction from the [^{14}C]oleate incubation cochromatographed with castor oil. We have previously shown the conversion of 2-[^{14}C]oleoyl-PC to 2-[^{14}C]ricinoleoyl-PC and 2-[^{14}C]linoleoyl-PC in a castor microsomal incubation (*4*). Because oleate can be further metabolized to ricinoleate and linoleate, there are numerous ^{14}C peaks. The ^{14}C peaks of RRR, RRLn, RRL, and RRO were identified by cochromatography with castor oil. Other

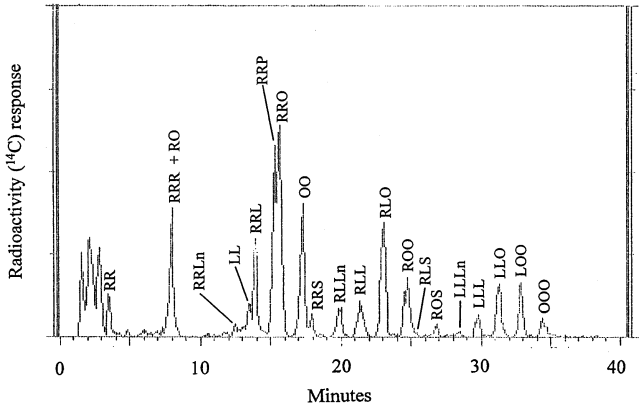

Figure 3. C_{18} HPLC radiochromatogram for the separation of molecular species of acylglycerols (AG) in the AG fraction (see **Figure 1**, 10 μL from 100 μL methanol solution) from the castor microsomal incubations (60 min) of [^{14}C]oleic acid cochromatographed with castor oil (see Materials and Methods, section 2.2.2). For the UV chromatograms, see **Figure 2A**. For the abbreviations of AG, see the caption of **Figure 2**.

^{14}C peaks were identified by cochromatography with the standards 1,2-diolein, trilinolein (LLL), 1,2-linoleoyl-3-oleoyl-*rac*-glycerol (LLO), 1,2-dioleoyl-3-linoleoyl-*rac*-glycerol (OOL), and triolein (OOO). The peak at 28.5 min should be LLLn calculated from the RT of LLL (29.8 min). The standard 1,3-dilinoleoylglycerol (standard 1,2-LL was not available) was also used for cochromatography and was 0.2 min ahead of the ^{14}C peak at 13.5 min. This peak is likely 1,2-dilinoleoyl-*sn*-glycerol, because in our previous paper (*10*), 1,3-dipalmitoylglycerol eluted 0.2 min ahead of 1,2-dipalmitoyl-*sn*-glycerol.

3.3. Levels of Molecular Species of [^{14}C]Acylglycerols. The levels of molecular species of TAG and DAG incorporating the six [^{14}C]FAs in castor microsomal incubations (60 min) are shown in **Table 1**. When the six [^{14}C]FAs incorporated individually into the molecular species of [^{14}C]TAG containing the same RR constituents were compared, the order was RRR (6.7 nmol) > RRO (2.8) > RRL (2.4) > RRLn (1.7) > RRS (+ LS, 1.8) > RRP (0.78). This order may primarily represent the selectivity for the final acylation step to use endogenous RR as substrate. It has been shown previously in castor microsomal incubations that DGAT selectively utilized ricinoleate over oleate containing substrates (*12*, *13*). The level of [^{14}C]RRR was lower than both [^{14}C]RRL and [^{14}C]RRO (**Table 1**) in the castor microsomal incubation with [^{14}C]ricinoleate.

Table 2. Incorporation of Various Radiolabeled Fatty Acids into Triacylglycerols (TAG) and Diacylglycerols (DAG) in Castor Microsomal Incubations (60 min)

fatty acid incubated (nmol)	DAG + TAG (nmol)	DAG (nmol)	TAG (nmol)
ricinoleate (91)	52	3.8	48
stearate (114)	5.8	1.7–3.5	2.4–4.2
oleate (96)	19	3.3–5.1	14–16
oleate (96)[a]	10–12	2.2–4.0	8.3
linoleate (98)	21	8.4	13
linolenate (96)	15	5.2	10
palmitate (89)	7.8	4.9	2.9

[a] Excluding DAG and TAG containing no oleate.

This is in agreement with the results of castor microsomal incubation with [^{14}C]ricinoleate-CoA (*14*). However, in the endosperm incubation with [^{14}C]ricinoleate-CoA, [^{14}C]RRR was the predominant [^{14}C]TAG in the oil body (*14*).

3.4. Levels of [^{14}C]Triacylglycerols and [^{14}C]Diacylglycerols. The levels of incorporation for the six [^{14}C]FA into total TAG and total DAG are shown in **Table 2**. The incorporation into [^{14}C]TAG were in the order ricinoleate > oleate > linoleate > linolenate > stearate > palmitate. These results concur with the previous comparisons of the incorporations of [^{14}C]ricinoleate and [^{14}C]oleate in castor microsomal incubations (*12*, *15*). This order is similar to the relative levels of the molecular species of RR-TAG of castor oil with RRR > RRL > RRO > RRLn, RRS, RRP as shown in **Figure 2A**. Ricinoleate was the major FA incorporated into the molecular species of TAG, whereas linolenate, stearate, and palmitate were minor components.

3.5. Identification and Semiquantification of the Molecular Species in Castor Oil. After the ^{14}C peaks were designated as shown in **Figures 2B** and **3**, AG fractions from the six [^{14}C]-FA incubations cochromatographed with castor oil were used to designate the molecular species of AG in castor oil as shown in **Figure 2A**, which shows the separation of the AG molecular species of castor oil. **Figure 2A** shows the well-separated groups of the molecular species of TAG semiquantitatively as RRR > RR-TAG > R-TAG > no R-TAG. The levels of RR-TAG are RRL > RRO > RRLn, RRS, RRP; those of RL-TAG RLL > RLO > RLLn, RLS; and those of LL-TAG LLL > LLO > LLLn, LLS. These roughly reflected the incorporation of FA into TAG in castor oil and [^{14}C]TAG in **Table 2**. The molecular species of DAG were also designated in **Figure 2A** as RR, LL, OO, LLn, and LnLn. **Figure 2B** shows the RR peak (3.5 min); however, there are many minor peaks shown before RRR in **Figure 2A**, mostly arising from castor microsomal incubation, not strictly from castor oil. Both **Figure 2A** here and the HPLC chromatogram of castor oil reported earlier (Figure 4A in ref *2*) showed the minor peak at 3.5 min. These DAGs in castor oil have not been previously reported. Using the HPLC systems for the separation of lipid classes (*8*) and the separation of FA (*16*), no lipid classes except AG were detected in castor oil (0.5 mg of castor oil in ethanol was injected).

Four TAGs in castor oil have previously been identified and quantified as RRR (68.2%), RRO (7.5%), RRL (8.3%), and diricinoleoyl-dihydroxystearoyl-glycerol (4.9%) using the method of countercurrent distribution (*1*). Eleven TAGs, containing RR (RR-TAG), in castor oil have also been identified earlier by mass spectrometry/mass spectrometry (MS/MS) (*17*). **Figure 2A** shows the designation of 21 AGs in castor oil. However, the RR-TAGs containing dihydroxystearate (*1*), 20:0, 20:1, 20:2, 18:2-OH, and 20:2-OH shown earlier (*17*) are not shown in **Figure 2A**. We have not detected these RR-TAGs in castor oil.

3.6. Conclusion. Sixty-one molecular species of AGs (**Table 1**) incorporating six [^{14}C]FAs individually in castor microsomal incubations were identified and quantified, among them 28 AGs containing ricinoleate. Ricinoleate was the major FA incorporated, whereas stearate, linolenate, and palmitate were incorporated only to a minor extent. Many of these AGs may be present in castor oil in trace amounts. The molecular species of AGs in castor oil are shown in the HPLC chromatogram (**Figure 2A**) semiquantitatively in the order RRR > RRL > RRO > RRLn > RRS > RRP.

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