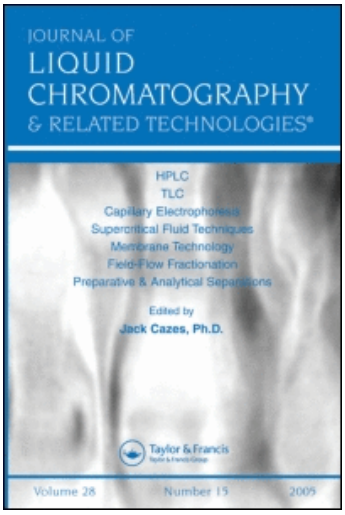

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Identification and Quantification of the Molecular Species of Acylglycerols in Castor Oil by HPLC Using ELSD

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ABSTRACT

Sixteen molecular species of acylglycerols (AG) in castor oil have been identified and quantified. Evaporative light scattering detection (ELSD) responses of different amounts of the standards of molecular species of AG were nearly linear and similar. In general, the addition of a double bond and a hydroxyl group on the acyl chain and the shortening of the acyl chain decreased the ELSD response of AG slightly. The quantification of molecular species of AG was based on the percentage peak area in the HPLC chromatogram. Triricinolein (RRR) constituted about 71% of castor oil. The contents of the molecular species of diricinoleoyltriacylglycerol (RR-TAG) were, in total, about 18% and were in the order of RRO (8.8%), RRL (6.6%), RRS (1.1%), RR-lesqueroleate (0.67%), RRP (0.47%) and

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RRLn (0.15%). The level of R-TAG was about 0.3%. The level of TAG containing no ricinoleate was less than 0.1%. Diricinoleoylglycerol, a diacylglycerol, was also identified and quantified (0.14%) in castor oil.

Key Words: Identification; Quantification; Molecular species; Triacylglycerol; Caster oil; HPLC; ELSD.

INTRODUCTION

Ricinoleate (R, a hydroxy fatty acid) has many industrial uses, such as the manufacture of aviation lubricant, plastics, paints, coatings, and cosmetics. Fatty acids (FA) occur as acylglycerols (AG) in castor oil, and 90% of its FA is ricinoleate.[1] Castor oil is the only commercial source of ricinoleate. However, castor bean contains the toxin, ricin, and potent allergens, which makes it hazardous to grow, harvest, and process. It would be desirable, instead, to produce ricinoleate from a transgenic oil seed lacking these toxic components. In order to develop a transgenic plant that produces seed oil containing a high level of ricinoleate as much as 90%, as in castor oil, it is important to know the biosynthetic pathway of castor oil and to identify the key enzymatic steps that drive ricinoleate into triacylglycerols (TAG) in castor bean. The biosynthetic pathway of castor oil was established and the key enzymatic steps were identified recently.[2,3] Identification and quantification of the molecular species of AG in castor oil, the end products of the biosynthesis, will help to understand the control mechanism to produce ricinoleate containing AG. It will also help the industrial uses of castor oil.

Four molecular species of TAG have been identified and quantified in castor oil, as triricinolein (RRR, 68.2%), diricinoleoyl–dihydroxystearoyl–glycerol (4.9%), diricinoleoyl–oleoyl–glycerol (RRO, 7.5%), and diricinoleoyl–linoleoyl–glycerol (RRL, 8.3%) by countercurrent distribution.[1] However, the minor molecular species of AG could not be detected by this method. Some molecular species of AG in castor oil have been identified by MS/MS.[4] The separation and detection of molecular species of AG by C_{18} HPLC,[5] using absorbance detection (205 nm) are both much better than the countercurrent distribution method. We have identified four molecular species of TAG on a C_{18} HPLC chromatogram of castor oil as RRR, RRO, RRL, and RRLn (diricinoleoyl–linolenoyl–glycerol) by GC analysis of FA components of HPLC peaks.[2] Identification of the minor HPLC peaks by this method was unsuccessful. We have recently identified 61 molecular species of AG incorporated from six radiolabeled FA individually in microsomal incubations.[6] They are potentially present in castor oil, because these FA are endogenous. The HPLC peaks of castor oil have also been designated by

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the retention times (RT) of the molecular species of AG.[6] However, the identification of the HPLC peaks needs to be confirmed by GC of the FA components. The quantitative results are difficult to obtain by absorbance detection. The evaporative light scattering detector (ELSD) is a universal mass detector and it has been used to quantify the molecular species of TAG in human milk.[7] We report, here, the use of C_{18} HPLC and ELSD to quantify the molecular species of AG in castor oil.

EXPERIMENTAL

HPLC was carried out on a liquid chromatograph (Waters Associates, Milford, MA), using an absorbance detector (Waters 2487) at 205 nm or an ELSD (MK III, Alltech Associates, Deerfield, IL) at a flow-rate of 1 mL/min. The drift tube temperature of the ELSD was set at 80°C. The nitrogen gas flow of the nebulizer of the ELSD was set at 1.0 L per min. The nitrogen pressure on the regulator of the nitrogen tank was set at about 65 p.s.i. Molecular species of AG in castor oil were separated as we previously reported,[5] using a C_{18} column (25 × 0.46 cm, 5 μm, Ultrasphere C_{18}, Beckman Instruments Inc., Fullerton, CA) with a linear gradient starting with 100% methanol to 100% 2-propanol in 40 min. For the separation and detection of lipid classes in castor oil, castor oil was separated according to Singleton and Stikeleather[8] on a silica column (25 × 0.46 cm, 5 μm, Luna, silica (2), Phenomenex, Torrance, CA) with a linear gradient starting with 2-propanol/hexane (4 : 3, v/v) to 2-propanol/hexane/water (4 : 3 : 0.75, v/v) in 20 min, then held isocratically for 20 min. For the separation and detection of free FA in castor oil, castor oil was separated on a C_{18} column with a linear gradient, starting with 85% methanol in water (containing 0.5% of acetic acid as ion suppressor) to 100% methanol (containing 0.5% of acetic acid) in 40 min.[9] The castor oil used was from Sigma (St. Louis, MO, C-7277, Lot 43H0790). The AG standards were obtained from Sigma or Nu-Chek (Elysian, MN). Triricinolein was fraction collected from castor oil on C_{18} HPLC. 1,2-Diricinoleoyl-*rac*-glycerol (1,2-RR) was obtained by the lipase hydrolysis of RRR.

Fractions (0.5 mL each) were collected from the HPLC of castor oil. The fractions containing different molecular species of AG [Fig. 1(A)] were hydrolyzed and methylated (methanolic HCl) to form fatty acid methyl esters (FAME) for GC analysis. Methanolic HCl (4 M) was prepared from acetyl chloride and anhydrous methanol (Instant methanolic HCl kit, Alltech Associates, Deerfield, IL). Half milliliter of methanolic HCl was added to the HPLC fractions and the mixture was kept at room temperature overnight. The mixture was extracted twice with 0.5 mL of hexane. The extract was dried and then dissolved in 50 μL of hexane, 2 μL was used for GC analysis.

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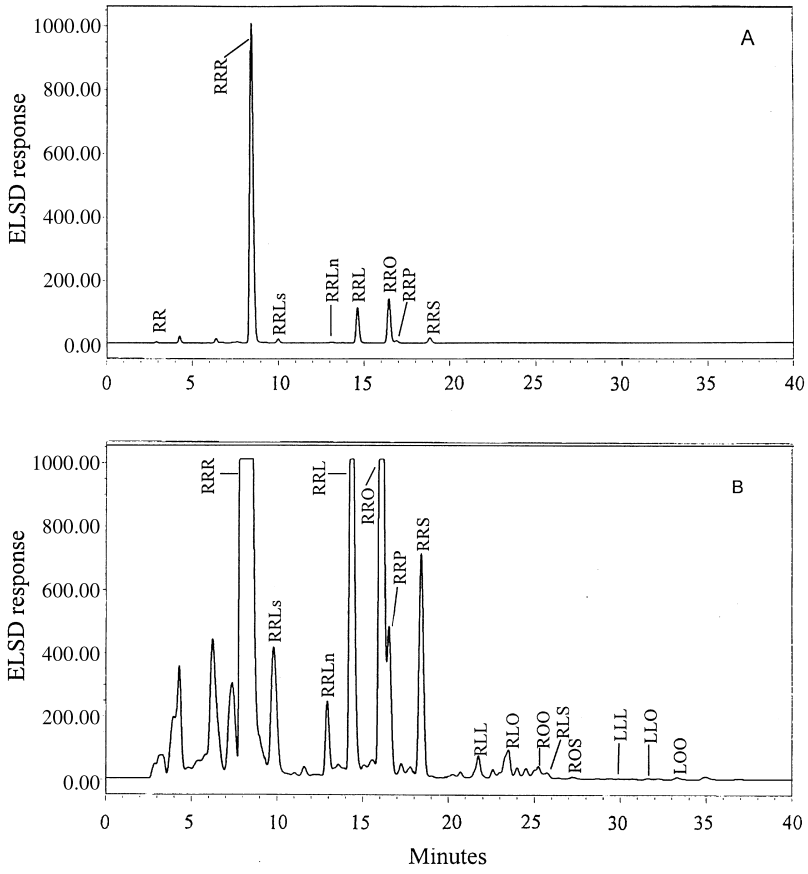

Figure 1. HPLC chromatograms of castor oil detected by ELSD. (A) 0.032 mg of castor oil in 4 μL ethanol. (B) 0.79 mg of castor oil in 100 μL ethanol. For HPLC conditions, see experimental. For abbreviation of AG, see Table 1.

RESULTS AND DISCUSSION

Castor oil is a mixture of many molecular species of AG. The lipid classes, other than AG in castor oil (0.5 mg in ethanol), could neither be detected on silica HPLC for the separation of lipid classes[8] nor on C_{18} HPLC for the separation of free FA by UV detector at 205 nm.[9] Figure 1 shows the C_{18} HPLC chromatograms separating the molecular species of AG in castor oil using ELSD. Figure 1(A) shows the separation of major molecular species of AG. All of the peaks are within the figure range on the y-axis, but many minor

AG are below the limit of detection. Using a much higher amount of castor oil, the chromatogram of Fig. 1(B) shows several minor molecular species of AG in addition, but the HPLC peaks of major molecular species of AG such as RRR, RRL, and RRO, are out of the range and cannot be quantified.

The identified molecular species of AG in castor oil are shown in Fig. 1 and Table 1. We have recently designated the HPLC peaks (UV at 205 nm) of castor oil by the RT of the molecular species of AG incorporated from six ^{14}C-labeled FA in microsomal incubations.[6] The HPLC peaks of the molecular species of AG shown in Fig. 1 have been confirmed by GC of FA methyl esters of the HPLC fraction collected (0.5 min) corresponding to the peaks. The peak at 10.0 min [Fig. 1(A)] was identified as RRLs (RR-lesqueroleate) by GC–MS of its FAME, as well as its TMS derivatives, by comparing with the MS of the major component in lesquerolic seeds, lesquerolic acid. It was formerly reported as 20 : 1 OH by MS/MS.[4]

The ELSD responses to different molecular species of TAG (Fig. 2) and DAG (Fig. 3) are almost linear, except for tristearin (SSS) of Fig. 2. We do not know why the response to SSS was unusually low at low amount. In general, the addition of a double bond and a hydroxyl group on the FA and shortening the acyl chain length decreased the ELSD response of AG, e.g., OOO > LLL > LnLnLn, SS > OO > LL, OOO > RRR, 1,2-SS > 1,2-PP. The ELSD response of the 1,3-isomers was less than that of 1,2-isomers, e.g. 1,2-OO > 1,3-OO. The differences of the ELSD responses to various molecular

Table 1. Molecular species of acylglycerols (AG) identified and their contents (%) in castor oil.[a]

Acylglycerols[b]	%	Acylglycerols	%
1,2-RR	0.14	RLL	0.08
RRR	70.92	RLO	0.13
RRLs	0.67	ROO	0.05
RRLn	0.15	RLS	0.02
RRL	6.58	ROS	0.01
RRO	8.80	LLL	0.002
RRP	0.47	LLO	0.005
RRS	1.08	LOO	0.01

[a]The unknowns in Fig. 1(A) are at RT 4.3 min about 0.90%; at 6.4 min, 0.70%. One might be diricinoleoyl–dihydroxy-stearoyl–glycerol.
[b]Abbreviation: R, ricinoleic acid; Ls, lesquerolic acid; Ln, linolenic acid; L, linoleic acid; O, oleic acid; P, palmitic acid; S, stearic acid.

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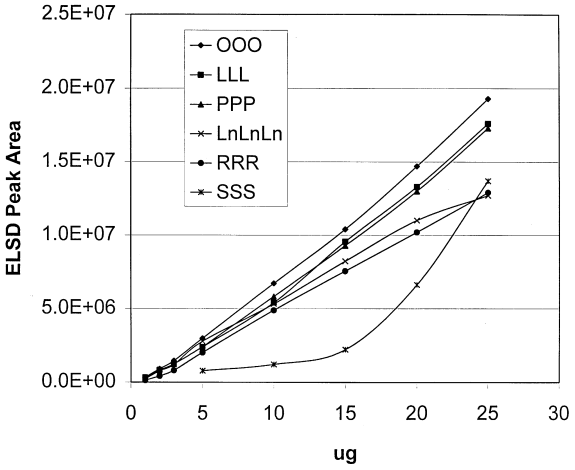

Figure 2. ELSD responses (integration units) of the molecular species of triacylglycerols. Data shown are the averages of duplicate HPLC runs. For HPLC conditions, see experimental. For abbreviation of AG, see Table 1.

species of AG were small (Figs. 2 and 3), as reported earlier.[7] Percentage peak area in chromatograms of ELSD response has been used to quantify the molecular species of TAG in human milk.[7] Since about 90% of FA in castor oil is ricinoleate, and most of the molecular species of AG contain ricinoleate, we used the same method to quantify the molecular species of AG in castor oil.

The contents of the molecular species of AG in castor oil are shown in Table 1. These were obtained from the peak areas of both chromatograms in Fig. 1, because Fig. 1(A) shows no peaks of minor AG and the three major AG in Fig. 1(B) are off scale. Earlier quantitative results of RRR (68.2%), diricinoleoyl–dihydroxystearoyl–glycerol (4.9%), RRO (7.5%), and RRL (8.3%) by countercurrent distribution[1] are similar to the results in Table 1. However, diricinoleoyl–dihydroxystearoyl–glycerol is not given in Table 1. According to elution characteristics of TAG,[5] it would elute earlier than RRR and might correspond to the peaks of RT at 4.3 min or 6.4 min [Fig. 1(A)]. However, its content was not as high as 4.9%. The exact structure of dihydroxystearate is unknown and it was not detected earlier by MS/MS.[4] Table 1 also includes a diacylglycerol, diricinoleoylglycerol (RR). Diacylglycerol content in olive oil have previously been determined by ^{13}C-NMR.[10]

In conclusion, we have identified and quantified sixteen molecular species of AG in castor oil (Table 1). Triricinolein made up to about 71% of castor oil. The levels of the molecular species of RR-TAG were in the order of ricinolea-

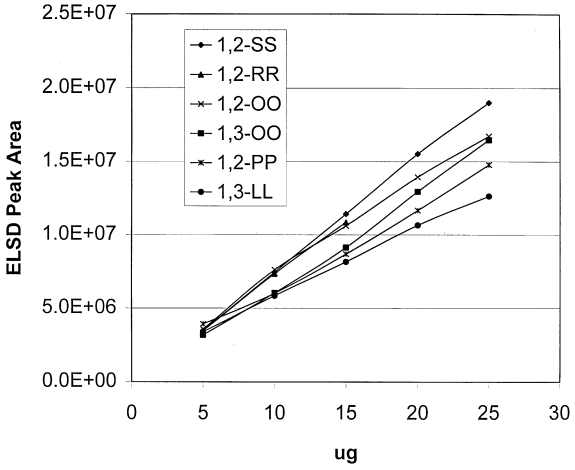

Figure 3. ELSD responses (integration units) of the molecular species of diacylglycerols. 1,2-RR was off scale at 20 and 25 μg. Data shown are the averages of duplicate HPLC runs. For HPLC conditions, see experimental. For abbreviation of AG, see Table 1.

te > oleate > linoleate > stearate > lesqueroleate > palmitate > linolenate and made up to about 18% in total. The level of R-TAG was about 0.3%. The level of TAG containing no ricinoleate was less than 0.1%. Diricinoleoylglycerol in castor oil was identified and quantified (0.14%).

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