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Ratios of Regioisomers of Triacylglycerols Containing Dihydroxy Fatty Acids in Castor Oil by Mass Spectrometry

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Abstract The triacylglycerols (TAG) containing dihydroxy fatty acids have been recently identified by mass spectrometry in castor oil. These new dihydroxy fatty acids were proposed as 11,12-dihydroxy-9-octadecenoic acid (diOH18:1), 11,12-dihydroxy-9,13-octadecadienoic acid (diOH18:2) and 11,12-dihydroxyoctadecanoic acid (diOH18:0). The ratios of regioisomers of the TAG were estimated by fragment ions from the loss of fatty acids at the *sn*-2 position as α,β-unsaturated fatty acids by electro spray ionization-mass spectrometry of the lithium adducts (MS^3). The content of regioisomeric diOH18:1-OH18:1-diOH18:1 (ABA, with two different fatty acids) was about 92% in the total of stereoisomeric diOH18:1-OH18:1-diOH18:1, OH18:1-diOH18:1-diOH18:1 and diOH18:1-diOH18:1-OH 18:1 combined. The approximate contents of other regioisomers were as follows: diOH18:1-OH18:1-OH18:1 (92%), diOH18:1-diOH18:0-diOH18:1 (91%), diOH18:2-OH18:1-OH18:1 (80%) and diOH18:0-OH18:1-OH18:1 (96%). The ratios of regioisomers of TAG (ABC) containing three different fatty acids were estimated as about 7:1:2 (OH18:1:diOH18:1:diOH18:2) and about 7:2:1 (OH18:1:diOH18:0:diOH18:1). Ricinoleate (OH18:1) was predominately at the *sn*-2 position of TAG (both AAB and ABC) containing dihydroxy fatty acids and ricinoleate. Dihydroxy fatty acids were mainly at the *sn*-1,3 positions of TAG containing dihydroxy fatty acids and ricinoleate in castor oil. The ratios of the three regioisomers of TAG (ABC) containing three different fatty acids by mass spectrometry are first reported here.

Keywords Dihydroxy fatty acids · Triacylglycerols · Regioisomer · *sn*-2 position · Mass spectrometry · Castor oil

Introduction

Ricinoleate (12-hydroxyoleic acid, OH18:1), a monohydroxy fatty acid, has many industrial uses such as the manufacture of aviation lubricant, plastic, paint and cosmetics. Ricinoleate occurs as acylglycerols (AG) in castor oil, and about 70% of castor oil is triricinolein (triricinoleoylglycerol) [1]. Castor oil is the only commercial source of ricinoleate. We have previously identified and quantified 14 molecular species of triacylglycerols (TAG) containing ricinoleate in castor oil using high-performance liquid chromatography (HPLC) and mass spectrometry (MS) methods [1, 2]. We have also recently identified 12 molecular species of AG containing dihydroxy fatty acids in castor oil [3]. These dihydroxy fatty acids were new fatty acids and were proposed as 11,12-dihydroxy-9-octadecenoic acid (11,12-dihydroxyoleic acid, diOH18:1), 11,12-dihydroxy-9,13-octadecadienoic acid (diOH18:2) and 11,12-dihydroxyoctadecanoic acid (11,12-dihydroxystearic acid, diOH18:0) [3]. Dihydroxy fatty acids were reported in microbial culture converted from oleic acid [4] and ricinoleic acid [5].

The presence of a hydroxyl group on fatty acid drastically changes the physical properties of the oil, e.g., viscosity, pour point, melting point, heat of fusion, solubility, crystal structure, and polymorphism [6]. Because of the physical and chemical changes from the normal fatty acids,

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many industrial uses of ricinoleate have been found. The physical and chemical properties of dihydroxy fatty acids and AG containing dihydroxy fatty acids are different from those of ricinoleate and normal fatty acids. The dihydroxy fatty acids and the AG containing dihydroxy fatty acids can be used in industry similarly to those of ricinoleate with different physical properties. Dihydroxy fatty acids have not been used in industry to date as there has been no practical source but, in the future they may be isolated from castor oil. However, the total AG containing dihydroxy fatty acids was about 2.5% of castor oil and the individual molecular species of AG containing dihydroxy fatty acids were at the levels of about 0.5% of castor oil or less [3].

The regioisomers of the molecular species of naturally occurring triacylglycerols (AAB) containing three normal fatty acids (non-hydroxylated fatty acids) have been quantified by MS based on the premise that the loss of the acyl group from the *sn*-1 or *sn*-3 position is energetically favored over the loss from the *sn*-2 position using linear calibration curves. The ratios of the regioisomers could be determined from the linear calibration curves of the ratios of $[AA]^+$ and $[AB]^+$ derived from various concentrations of regiospecific ABA and AAB standards [7, 8].

We have identified and quantified the regiospecific TAG in castor oil [9, 10] and olive oil [11] using the fragment ions of mass spectrometry of lithium adducts from the loss of the fatty acid at the *sn*-2 position as α,β-unsaturated fatty acid [12]. The ratios of regioisomers of TAG (AAB, with two different fatty acids, and ABC, with three different fatty acids) containing dihydroxy fatty acids by mass spectrometry are presented here. Due to lack of standards, we could not use the calibration curves to quantify the regioisomers. We assumed the linear calibration curves using the current method for quantifications as our earlier reports [9, 10]. Regiospecific structures of AG affect the physical property of AG for industrial uses [6]. The regiospecific and stereospecific identification and quantification can help us to understand the biosynthesis of AG for the development of transgenic oil seed plants to produce dihydroxy fatty acids.

Experimental Procedures

Materials

Lithium acetate was obtained from Sigma (St. Louis, MO, USA). HPLC and GC grade methanol and 2-propanol (Burdick & Jackson) for LC-MS were purchased from VWR International (West Chester, PA, USA). High purity nitrogen for LC-MS was acquired from Praxair (Oakland, CA, USA). Research grade (99.999%) helium (Praxair) was used as a collision gas.

HPLC Fractionation of the Molecular Species of AG in Castor Oil

The fractionation of the molecular species of AG in castor oil was as previously reported [1]. Chromatographic fractionation was performed using a Waters HPLC (Waters Associate, Milford, MA, USA) and a C_{18} analytical column (Gemini, 250 mm, 4.6 mm, 5 μm, C18, Phenomenex, Torrance, CA, USA). One mg of castor oil was chromatographed at 22 °C (room temperature) with a linear gradient from 100% methanol to 100% 2-propanol in 40 min, at a 1 mL min^{-1} flow rate, and detected at 205 nm. One-half minute fractions were collected and analogous fractions were pooled from 15 HPLC runs. Fractions eluted before triricinolein (fraction #20, retention time 9.5–10.0 min) were used for MS studies. The final sample solutions prepared for direct infusion into the MS were the mixtures of 200 μL of methanol solution containing about one-fourth of each fraction and 50 μL of methanol solution of lithium acetate (100 mM).

ESI-MS^3 of Triacylglycerols

An LCQ Advantage quadrupole ion-trap mass spectrometer with Xcalibur 1.3 software (ThermoFinnigan, San Jose, CA, USA) was utilized for MS analysis of the various molecular species of AG. The infusion at a 2.5 μL/min flow rate from a syringe pump produced stable singly charged lithiated parent ions which were subsequently fragmented for MS^2 and MS^3 analysis. ESI source conditions were as follows: 50 arbitrary units (au) nitrogen sheath gas flow rate, 4.5 kV spray voltage, 250 °C ion-transfer capillary temperature, 1.5 *m/z* isolation width, 160–1,000 *m/z* mass range, 5 min acquisition time, 38 V capillary voltage and normalized collision energy ranging 35–39% for $MS^2$and MS^3 fragmentations, varying between molecular species of AG and fatty acids.

Results and Discussion

Castor oil was fractionated (0.5 min/fraction) by reversed-phase C_{18} HPLC and the HPLC chromatogram was published earlier [1]. The fractions eluted before triricinolein (retention time 9.5 min) were used for MS studies. Figure 1 shows the TAG containing dihydroxyl fatty acids identified in the HPLC fractions 11–19 (retention time 5.0–9.5 min) from castor oil [3]. Diacylglycerols were in the fractions 9–12 (retention time 4.0–6.0 min).

MS^1 of the lithium adducts of Fraction 12 (retention time 5.5–6.0 min, the spectrum not shown here) showed the ion at *m/z* 971.6 (lithium adduct of TAG, diOH18:1-OH18:1-diOH18:1, ABA, with two identical fatty acids,

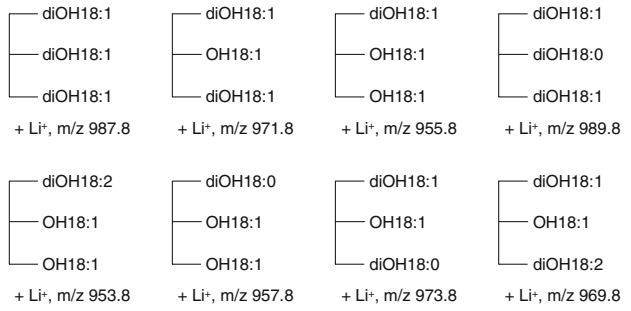

Fig. 1 The structures of the most abundant regioisomers of the triacylglycerols containing dihydroxy fatty acids in castor oil. The calculated *m/z* of the lithium adducts of triacylglycerols are also shown

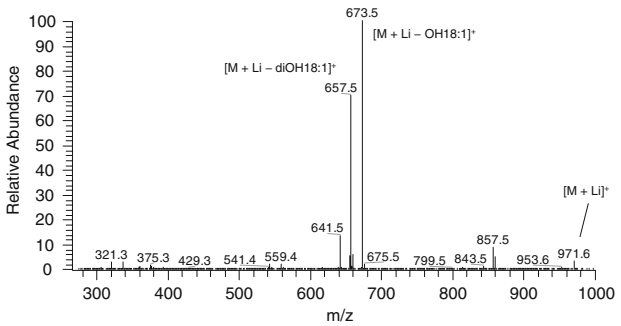

Fig. 2 Ion trap mass spectrum of ESI-MS^2 of diOH18:1-OH18:1-diOH18:1 $[M + Li]^+$ at *m/z* 971.6 in HPLC fraction 12 of castor oil (collision energy 36%). *diOH18:1* is dihydroxyoleate, *OH18:1* is ricinoleate

Fig. 1) and the ion at *m/z* 987.6 (lithium adduct of TAG, diOH18:1-diOH18:1-diOH18:1, AAA, with three identical fatty acids, Fig. 1). The ion at *m/z* 971.6 was the most prominent ion. Figure 2 is the MS^2 spectrum of diOH18:1-OH18:1-diOH18:1 $[M + Li]^+$ at *m/z* 971.6. The two prominent fragment ions were from the losses of ricinoleate OH18:1, $[M + Li - OH18:1]^+$ at *m/z* 673.5, and diOH18:1, $[M + Li - diOH18:1]^+$ at *m/z* 657.5.

Figure 3 is the MS^3 spectrum of $[M + Li - diOH18:1]^+$ at *m/z* 657.5 from Fig. 2. The fragment ions from the loss of fatty acid specific at the *sn*-2 position as α,β-unsaturated fatty acid were $[M + Li - diOH18:1 - OH18:1 + 2]^+$ at *m/z* 361.2 and $[M + Li - diOH18:1 - diOH18:1 + 2]^+$ at *m/z* 345.2. Both (OH18:1 − 2) and (diOH18:1 − 2) were α,β-unsaturated fatty acids. The abundances of these two fragment ions *m/z* 361.2 and *m/z* 345.2 represented the relative contents of fatty acids at the *sn*-2 position [7]. The content of regiospecific (or stereospecific) diOH18:1-OH18:1-diOH18:1 was estimated as about 89% in the total of the three stereospecific diOH18:1-OH18:1-diOH18:1, diOH18:1-diOH18:1-OH18:1 and OH18:1-diOH18:1-diOH18:1 combined. The approximate contents of the same TAG in other HPLC fractions were

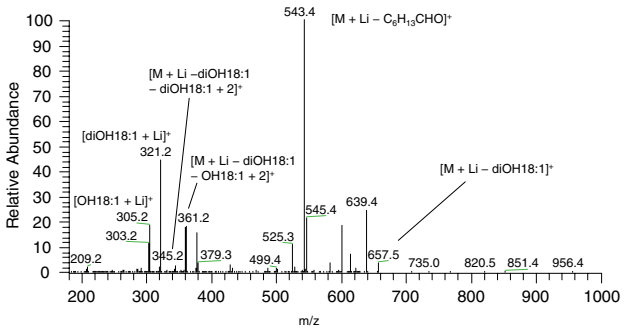

Fig. 3 Ion trap mass spectrum of ESI-MS^3 of diOH18:1-OH18:1-diOH18:1. This was from $[M + Li - diOH18:1]^+$ at *m/z* 657.5 of Fig. 2 (collision energy 38%). For abbreviations see Fig. 2. $C_6H_{13}CHO$ is an aldehyde

94% (fraction 13, 6.0–6.5 min), 92% (fraction 14, 6.5–7.0 min), 94% (fraction 15, 7.0–7.5 min), 89% (fraction 16, 7.5–8.0 min), 89% (fraction 17, 8.0–8.5 min), and 94% (fraction 18, 8.5–9.0 min). The approximate contents were similar and at the average of 92%. The resolution of TAG containing dihydroxy fatty acids was not good for this HPLC system. The base ion at *m/z* 543.4 (Fig. 3) was from the loss of $C_6H_{13}CHO$. The loss of $C_6H_{13}CHO$ (or shown earlier as $C_7H_{14}O$) has been previously reported [9, 10, 13] from the cleavage between C-11 and C-12 of the ricinoleoyl chain on TAG containing ricinoleate.

The contents of the other regioisomeric AAB were estimated as described as that of diOH18:1-OH18:1-diOH18:1. The approximate content of regioisomer diOH18:1-OH18:1-OH18:1 (ABB), $[M + Li]^+$ at *m/z* 955.7, was estimated as follows: 95% (fraction 13, 6.0–6.5 min), 95% (fraction 14, 6.5–7.0 min), 95% (fraction 15, 7.0–7.5 min), 88% (fraction 16, 7.5–8.0 min), 86% (fraction 17, 8.0–8.5 min) and 92% (fraction 18, 8.5–9.0 min), at the average of 92%. The content of regioisomer diOH18:1-diOH18:0-diOH18:1 (ABA), $[M + Li]^+$ at *m/z* 989.7, was about 91% (fraction 13, 6–6.5 min). The content of regioisomer diOH18:2-OH18:1-OH18:1 (ABB), $[M + Li]^+$ at *m/z* 953.7, was estimated as about 80% (fraction 15, 7.0–7.5 min). The content of regioisomer diOH18:0-OH18:1-OH18:1 (ABB), $[M + Li]^+$ at *m/z* 957.8, was estimated as about 97% (fraction 15, 7.0–7.5 min) and about 94% (Fraction 16, 7.5–8.0 min), at the average of 96%.

The ratios of the three regioisomers of TAG (ABC) also can be estimated by the abundances of the fragment ions (MS^3) from the loss of the fatty acids at the *sn*-2 position as α,β-unsaturated fatty acids [7]. For diOH18:1-OH18:1-diOH18:2 (ABC, see Fig. 1) in fraction 15 (7–7.5 min), the MS^2 spectrum (not shown here) of $[M + Li]^+$ at *m/z* 969.5 showed the fragment ions from the losses of the three fatty acids as follows: $[M + Li - diOH18:1]^+$ at *m/z* 655.5, $[M + Li - diOH18:2]^+$ at *m/z* 657.5 and [M + Li −

$OH18:1]^+$ at *m/z* 671.4. The MS^3 spectrum (not shown here) of $[M + Li - diOH18:1]^+$ at *m/z* 655.5 showed the fragment ions from the losses of fatty acids at the *sn*-2 position as α,β-unsaturated fatty acids, $[M + Li - diOH18:1 - OH18:1 + 2]^+$ at *m/z* 359.2 and $[M + Li - diOH18:1 - diOH18:2 + 2]^+$ at *m/z* 345.2. The ratio of the abundances of these two fragment ions was 75:25. So the ratio of the contents of OH18:1 (ricinoleate) and diOH18:2 at the *sn*-2 position was about 75:25. The MS^3 spectrum (not shown here) of $[M + Li - diOH18:2]^+$ at *m/z* 657.5 showed the fragment ions from the losses of fatty acids at the *sn*-2 position as α,β-unsaturated fatty acids, $[M + Li - diOH18:2 - OH18:1 + 2]^+$ at *m/z* 361.2 and $[M + Li - diOH18:2 - diOH18:1 + 2]^+$ at *m/z* 345.2. The ratio of the abundances of these two fragment ions was 93:7. So the ratio of the contents of OH18:1 (ricinoleate) and diOH18:1 at the *sn*-2 position was about 93:7. The MS^3 spectrum (not shown here) of $[M + Li - OH18:1]^+$ at *m/z* 671.4 showed the fragment ions from the losses of fatty acids at the *sn*-2 position as α,β-unsaturated fatty acids, $[M + Li - OH18:1 - diOH18:1 + 2]^+$ at *m/z* 359.2 and $[M + Li - OH18:1 - diOH18:2 + 2]^+$ at *m/z* 361.2. The ratio of the abundances of these two fragment ions was 42:58. So the ratio of the contents of diOH18:1 and diOH18:2 at the *sn*-2 position was about 42:58. We have obtained three ratios from three MS^3 spectra of diOH18:1-OH18:1-diOH18:2 (ABC).

Using two of the three ratios obtained (MS^3) for each estimation, the ratio of three fatty acids at the *sn*-2 position of diOH18:1-OH18:1-diOH18:2 (ABC) could be estimated three times from the fragment ions from the losses of different fatty acids (MS^2). From the two ratios, 93:7 (OH18:1:diOH18:1 at the *sn*-2 position) and 75:25 (OH18:1:diOH18:2 at the *sn*-2 position), the ratio of these three fatty acids at the *sn*-2 position were estimated as about 71:5:24 (OH18:1:diOH18:1:diOH18:2). From the two ratios, 93:7 (OH18:1:diOH18:1 at the *sn*-2 position) and 42:58 (diOH18:1:diOH18:2 at the *sn*-2 position), the ratio of these three fatty acids at the *sn*-2 position were estimated as about 85:6:9 (OH18:1:diOH18:1:diOH18:2). From the two ratios, 75:25 (OH18:1:diOH18:2 at the *sn*-2 position) and 42:58 (diOH18:1:diOH18:2 at the *sn*-2 position), the ratio of these three fatty acids at the *sn*-2 position were estimated as about 64:15:21 (OH18:1:diOH18:1:diOH18:2). Three ratios of three fatty acid contents (OH18:1:diOH18:1:diOH18:2) at the *sn*-2 position (regioisomers) were obtained as 71:5:24, 85:6:9 and 64:15:21. The variation might be due to the different abundances of the fragment ions produced from the losses of fatty acids as α,β-unsaturated fatty acids at the *sn*-2 position (MS^3) from different fatty acids at both precursor ions and fragment ions, especially the difference between ricinoleate and dihydroxy fatty acids. All of the three ratios showed that ricinoleate was predominately at the *sn*-2 position of diOH18:1-OH18:1-diOH18:2 (ABC) in castor oil. The average of these three ratios was about 7:1:2 (OH18:1:diOH18:1:diOH18:2) and the variation was about 15%. The ratios of the three regioisomers of TAG (ABC) by mass spectrometry were first reported here.

For diOH18:O-OH18:1-diOH18:1 (ABC, see Fig. 1) in fraction 13 (6–6.5 min), the MS^2 spectrum (not shown here) of $[M + Li]^+$ at *m/z* 973.5 showed the fragment ions from the losses of the three fatty acids as follows: $[M + Li - diOH18:0]^+$ at *m/z* 657.4, $[M + Li - diOH18:1]^+$ at *m/z* 659.4 and $[M + Li - OH18:1]^+$ at *m/z* 675.4. The MS^3 spectrum (not shown here) of $[M + Li - diOH18:0]^+$ at *m/z* 657.5 showed the fragment ions from the losses of fatty acids at the *sn*-2 position as α,β-unsaturated fatty acids, $[M + Li - diOH18:0 - OH18:1 + 2]^+$ at *m/z* 361.2 and $[M + Li - diOH18:0 - diOH18:1 + 2]^+$ at *m/z* 345.3. The ratio of the abundances of these two fragment ions was 88:12. So the ratio of contents of OH18:1 (ricinoleate) and diOH18:1 at the *sn*-2 position was about 88:12. The MS^3 spectrum (not shown here) of $[M + Li - diOH18:1]^+$ at *m/z* 659.4 showed the fragment ions from the losses of fatty acids at the *sn*-2 position as α,β-unsaturated fatty acids, $[M + Li - diOH18:1 - OH18:1 + 2]^+$ at *m/z* 363.2 and $[M + Li - diOH18:1 - diOH18:0 + 2]^+$ at *m/z* 345.2. The ratio of the abundances of these two fragment ions was 81:19. So the ratio of the contents of OH18:1 (ricinoleate) and diOH18:0 at the *sn*-2 position was about 81:19. The abundances of the fragment ions, $[M + Li - OH18:1 - diOH18:0 + 2]^+$ at *m/z* 361.4 and $[M + Li - OH18:1 - diOH18:1 + 2]^+$ at *m/z* 363.4 from the MS^3 spectrum (not shown here) of $[M + Li - OH18:1]^+$ at *m/z* 675.4 were very low and were not used for the estimation. From the two ratios, 81:19 (OH18:1:diOH18:0 at the *sn*-2 position) and 88:12 (OH18:1:diOH18:1 at the *sn*-2 position), the ratios of these three fatty acids at the *sn*-2 position of diOH18:O-OH18:1-diOH18:1 (three regioisomers) was estimated as about 73:17:10 (OH18:1:diOH18:0:-diOH18:1) or about 7:2:1 if considering the 15% variation.

Ricinoleate was predominately at the *sn*-2 position of TAG (both AAB and ABC) containing dihydroxy fatty acids and the dihydroxy fatty acids were predominately at the *sn*-1,3 positions in castor oil. The hydroxylation of ricinoleate to form dihydroxy fatty acids occurred in vivo mainly at the *sn*-1,3 positions from triricinolein or the dihydroxy fatty acids were mainly incorporated at the *sn*-1,3 positions. The identification of fatty acids at the three stereospecific positions of TAG containing dihydroxy fatty acids in seed oil can help to elucidate the biosynthetic pathway. The AG containing dihydroxy fatty acids in castor oil can be isolated for industrial uses. The total content is low in castor oil (about 2.5%) [3], however, the

content can be increased in the transgenic plant for future uses. The estimation of the ratios of regioisomers of TAG can help to understand the physical properties of the oil [6] and it will help in the industrial uses.

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