J Am Oil Chem Soc (2016) 93:183–191
DOI 10.1007/s11746-015-2769-2

ORIGINAL PAPER

Ratios of Regioisomers of the Molecular Species of Triacylglycerols in Lesquerella (*Physaria fendleri*) Oil Estimated by Mass Spectrometry

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Received: 14 September 2015 / Revised: 11 November 2015 / Accepted: 24 November 2015 / Published online: 30 December 2015

Abstract The ratios of regioisomers of 72 molecular species of triacylglycerols (TAG) in lesquerella oil were estimated using the electrospray ionization mass spectrometry of the lithium adducts of TAG in the HPLC fractions of lesquerella oil. The ratios of ion signal intensities (or relative abundances) of the fragment ions from the neutral losses of fatty acids (FA) as α-lactones at the *sn*-2 position (MS^3) of the molecular species of TAG were used as the ratios of the regioisomers. The order of the preference of FA incorporation at the *sn*-2 position of the molecular species of TAG in lesquerella was as: normal FA > OH18 (monohydroxy FA with 18 carbon atoms) > diOH18 > OH20 > diOH20, while in castor was as: normal FA > OH18 > OH20 > diOH18 > triOH18. Elongation (from C_{18} to C_{20}) was more effective than hydroxylation in lesquerella to incorporate hydroxy FA at the *sn*-1/3 positions. The block of elongation in lesquerella may be used to increase the content of hydroxy FA, e.g., ricinoleate, at the *sn*-2 position of TAG and to produce triricinolein (or castor oil) for industrial uses. The content of normal FA at the *sn*-2 position was about 95 %, mainly oleate (38 %), linolenate (31 %) and linoleate (23 %). This high normal FA content (95 %) at the *sn*-2 position was a big space for the replacement of ricinoleate to increase the hydroxy FA content in lesquerella oil. The content of hydroxy FA at the *sn*-1/3 positions was 91 % mainly lesquerolic acid (85 %) and the content of normal FA was 6.7 % at the *sn*-1/3 position in lesquerella oil.

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Keywords Lesquerella oil · *Physaria fendleri* · Triacylglycerols · Regioisomers · Quantification · Mass spectrometry

Introduction

Lesquerella (*Physaria fendleri*) oil can be used in industry for similar purposes to those of castor oil [1]. Lesquerolic acid (Ls, $OH^{14}20{:}1^{11}$) is the major fatty acid (FA, about 56 %) in lesquerella oil [2] while ricinoleic acid ($OH^{12}18{:}1^{9}$) is the major FA (about 90 %) in castor oil [3]. For the abbreviations of FA, see the appendix of Table 1.

We have recently identify and quantify ten molecular species of diacylglycerols (DAG), 74 molecular species of triacylglycerols (TAG) and 13 molecular species of tetraacylglycerols in lesquerella oil using HPLC and electrospray ionization mass spectrometry (ESI–MS) [4–7]. The content of ten DAG combined was about 1 %, 74 TAG was about 98 % and 13 tetraacylglycerols was about 1 % [6, 7]. The contents of the molecular species of TAG were: LsLsO (31.3 %), LsLsLn (24.9 %), LsLsL (15.8 %), LsL-OH20:2 (4.3 %), LsO-OH20:2 (2.8 %), and LsLn-OH20:2 (2.5 %) [6]. Minor monohydroxy and dihydroxy FA were identified in lesquerella oil and their structures were different from those of minor FA in castor oil as $OH^{12}18{:}2^{9,14}$ ($OH^{12}18{:}2^{9,13}$ in castor oil) and $diOH^{12,13}18{:}2^{9,14}$ ($diOH^{11,12}18{:}2^{9,13}$ in castor oil) [4]. Since the structures of the minor FA in lesquerella and castor oils were different, the minor FA were likely not the artifacts and oxidation products.

We have estimated the ratios of the regioisomers of 65 molecular species of TAG in castor oil [8–12]. We used the ratios of relative abundances of the fragment ions from the neutral losses of FA as α-lactones at the *sn*-2 position of the

Table 1 Ratios of regioisomers of the molecular species of TAG in HPLC fractions of lesquerella oil

TAG (HPLC Fraction #)	Acyl chain at the *sn*-2	Ratio in %
LsLs-diOH18:1 (18)	Ls : diOH18:1	44 : 56
LsLs-diOH18:2 (19)	Ls : diOH18:2	26 : 74
LsLs-OH18:3 (21, 22)	Ls : OH18:3	18 : 82
Ls-OH20:2-OH18:2 (21, 22)	Ls : OH20:2:OH18:2	8 : 2 : 90
LsR-OH20:2 (23)	Ls : R : OH20:2	1.4 : 95 : 4
LsLs-OH18:2 (23, 24)	Ls : OH18:2	4 : 96
LsLsR (25, 26)	Ls : R	2 : 98
LsLs-OH20:2 (26)	Ls : OH20:2	62 : 38
LsLn-diOH20:0 (28)	Ls : Ln : diOH20:0	1.2 : 99 : 0.4
LsLn-OH18:2 (29)	Ls : Ln : OH18:2	0.4 : 95 : 5
RLn-OH20:2 (29)	R : Ln : OH20:2	5 : 95 : 0.2
Ln-OH20:2-OH20:2 (31)	Ln : OH20:2	100 : 0.3
LsRLn (31)	Ls : R : Ln	1.4 : 6 : 93
LsL-diOH20:0 (31)	Ls : L : diOH20:0	1.0 : 98 : 0.7
LsO-diOH20:1 (31)	Ls : O : diOH20:1	5 : 95 : 0.2
LsO-diOH18:0 (31)	Ls : O : diOH18:0	0.4 : 99 : 0.3
LsLn-OH20:2 (32, 33)	Ls : Ln : OH20:2	0.2 : 99 : 0.5
LsLsLn (34–36)	Ls : Ln	0.9 : 99
LsLsPo (36)	Ls : Po	0 : 100
LsL-OH20:2 (36)	Ls : L : OH20:2	1.4 : 98 : 0.4
LsLsL (37, 38)	Ls : L	0.5 : 99.5
LsO-OH20:2 (38, 39)	Ls : O : OH20:2	0.5 : 91 : 9
LsLsP (40, 41)	Ls : P	4 : 96
LsLsO (40, 41)	Ls : O	0.8 : 99
LsLnLn (43, 44)	Ls : Ln	0.5 : 99
LsO-OH20:0 (44)	Ls : O : OH20:0	1.5 : 98 : 0.7
LsO-23:0 (44, 45)	Ls : O : 23:0	0.6 : 98 : 1
LsLsS (44, 45)	Ls : S	2 : 98
LsS-OH18:3 (45)	Ls : S : OH18:3	1.5 : 74 : 25
LsLLn (46, 47)	Ls : L : Ln	1 : 49 : 50
OLn-OH20:2 (47)	O : Ln : OH20:2	29 : 70 : 1
LsLPo (48)	Ls : L : Po	0.2 : 60 : 40
LsO-16:2 (48)	Ls : O : 16:2	0.9 : 12 : 87
LsLnP (48, 49, 50)	Ls : Ln : P	0.4 : 95 : 5
LsLL (48, 49, 50)	Ls : L	3 : 97
LsOLn (49, 50)	Ls : O : Ln	0.8 : 40 : 59
LsOPo (51)	Ls : O : Po	1.0 : 30 : 69
SLn-OH20:2 (51)	S : Ln : OH20:2	1.5 : 98 : 1.0
OL-OH20:2 (51)	O : L : OH20:2	40 : 60 : 0.3
LsLP (51, 52)	Ls : L : P	0.7 : 89 : 10
LsOL (52, 53)	Ls : O : L	3 : 29 : 68
LsSLn (53, 54)	Ls : S : Ln	0.4 : 1.7 : 98
LsLn-20:1 (53, 54)	Ls : Ln : 20:1	0.2 : 90 : 10
LsOP (55)	Ls : O : P	0.8 : 84 : 15
LsOO (55, 56)	Ls : O	0.3 : 100
LsLS (56, 57)	Ls : L : S	4 : 84 : 12
LsL-20:1 (56, 57)	Ls : L : 20:1	0.2 : 67 : 33
LsLn-20:0 (58)	Ls : Ln : 20:0	0.4 : 63 : 37
LsOS (59, 60)	Ls : O : S	0.4 : 98 : 2
LsO-20:1 (59, 60)	Ls : O : 20:1	1.5 : 95 : 3
LsL-20:0 (60, 61)	Ls : L : 20:0	0.2 : 50 : 50
OLnPo (61)	O : Ln : Po	5 : 14 : 81
LnPPo (61)	Ln : P : Po	30 : 6 : 64
OLLn (62)	O : L : Ln	32 : 44 : 24
LsLn-24:1 (62)	Ls : Ln : 24:1	0.05 : 97 : 3
LLnP (62)	L : Ln : P	56 : 18 : 26
OPoPo (63)	O : Po	9 : 91
LsO-20:0 (63, 64)	Ls : O : 20:0	1 : 69 :30
OPLn (65)	O : P : Ln	30 : 22 : 48
LLP (65)	L : P	58 : 42
OOLn (65, 66)	O : Ln	38 : 62
OLL (65, 66)	O : L	85 : 15
LSLn (65, 66)	L : S : Ln	44 : 33 : 23
OPPo (66, 67)	O : P : Po	33 : 22 : 45
OOPo (67)	O : Po	16 : 84
OOL (68)	O : L	28 : 72
OLP (68)	O : L : P	17 : 66 : 17
OSLn (70)	O : S : Ln	23 : 14 : 63
OOP (70, 71)	O : P	50 : 50
OSPo (70, 71)	O : S : Po	7 : 18 : 75
OLS (72)	O : L : S	23 : 44 : 33
OOS (75)	O : S	58 : 42

R ricinoleic acid (OH18:1), *Ls* lesquerolic acid (OH20:1), *O* oleic acid (18:1), *L* linoleic acid (18:2), *Ln* linolenic acid (18:3), *S* stearic acid (18:0), *P* palmitic acid (P), *Po* palmitoleic acid (16:1)

molecular species of TAG (MS^3) as the ratios of the regioisomers. The fragmentation mechanisms were recently proposed by Grossert *et al*. using ESI–MS and density functional theory computations [13], and were different from those of Hsu and Turk proposed earlier, from the neutral loss of FA as α,β-unsaturated FA at the *sn*-2 position (MS^3) [14, 15]. The recent proposed fragmentation mechanisms included the first neutral loss of free FA at the *sn*-1/3 position from $[TAG + Li]^+$ to form cationized DAG γ-lactone (5-membered lactone ring, MS^2) and then the second neutral loss of FA as α-lactone (3-membered lactone ring) at the *sn*-2 position from the cationized DAG γ-lactone (MS^3) (Fig. 1a) [13].

Regioisomers of ABC type TAG do not differentiate at the *sn*-1 and *sn*-3 positions, thus the stereoisomers of ABC and CBA are the same regioisomers. The ratios of regioisomers of the molecular species of TAG are the ratio of the abundances of FA at the *sn*-2 position. The preferences of the FA at the *sn*-2 position of the molecular species of TAG in castor oil were: normal FA > monoOH-FA > diOH-FA > triOH-FA [8, 10–12]. We have also reported that the

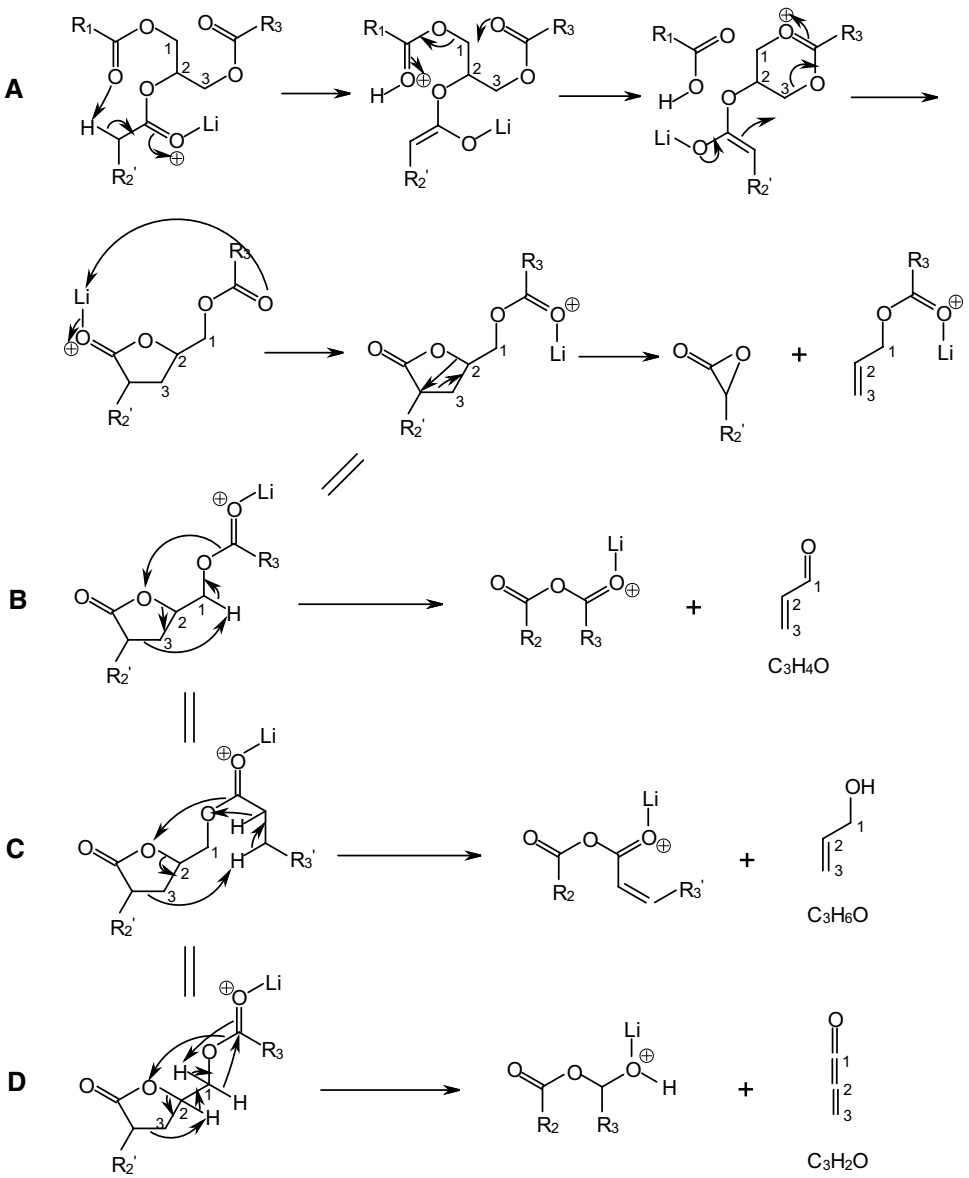

Fig. 1 Proposed MS fragmentation mechanisms. The numbers *1*, *2* and *3* in the figures are the *sn*-1, 2 and 3 positions of the glycerol backbone of triacylglycerols. *Three equal signs* show that the four fragment ions were the same. **a** The first neutral loss of free FA at the *sn*-1/3 position from $[TAG + Li]^+$ to form cationized DAG γ-lactone (5-membered lactone ring, MS^2) and then the second neutral loss of FA as α-lactone from the cationized DAG γ-lactone at the *sn*-2 position (3-membered lactone ring, MS^3). This fragmentation mechanisms were proposed by Grossert *et al*. [13]. Most of the electron pair transfers showing as *arrows* are added here. **b** The neutral loss of the glycerol backbone as C_3H_4O from $[TAG + Li]^+$, see Figs. 4 and 6. **c** The neutral loss of the glycerol backbone as C_3H_6O from $[TAG + Li]^+$, see Figs. 6 and 7. **d** The neutral loss of the glycerol backbone as C_3H_2O from $[TAG + Li]^+$, see Figs. 6 and 7

preference of FA at the *sn*-2 position of the molecular species of TAG containing three normal FA in olive oil as: unsaturated normal FA > saturated normal FA [16]. We report here the estimation of the ratios of the regioisomers of the molecular species of TAG and the proposition that the elongase as the target to increase the content of hydroxy FA in lesquerella oil.

Materials and Methods

Plant Materials

Lesquerella (*Physaria fendleri*) oil was obtained as described recently [4]. Castor (*Ricinus communis* L.) oil used previously was from Sigma (C-7277, Lot 60K0843). The previous analytical methods of castor oil were similar to those of lesquerella oil described here as follows.

HPLC fractionation of the Molecular Species of Triacylglycerols in Lesquerella Oil

HPLC fractionation was carried out on a liquid chromatograph (Waters Associates, Milford, MA, USA), using an absorbance detector (Waters, Model 2487) at 205 nm. Molecular species of TAG were separated using a C_{18} analytical column (Gemini, 250 × 4.6 mm, 5 µm, C18, Phenomenex, Torrance, CA, USA) with a linear gradient from 100 % methanol to 100 % 2-propanol in 40 min, at 1 mL/min flow rate. For fractionation, 1 mg of lesquerella oil in ethanol (50 µl) was chromatographed at 22 °C (room temperature). Fractions were collected every 30 s and

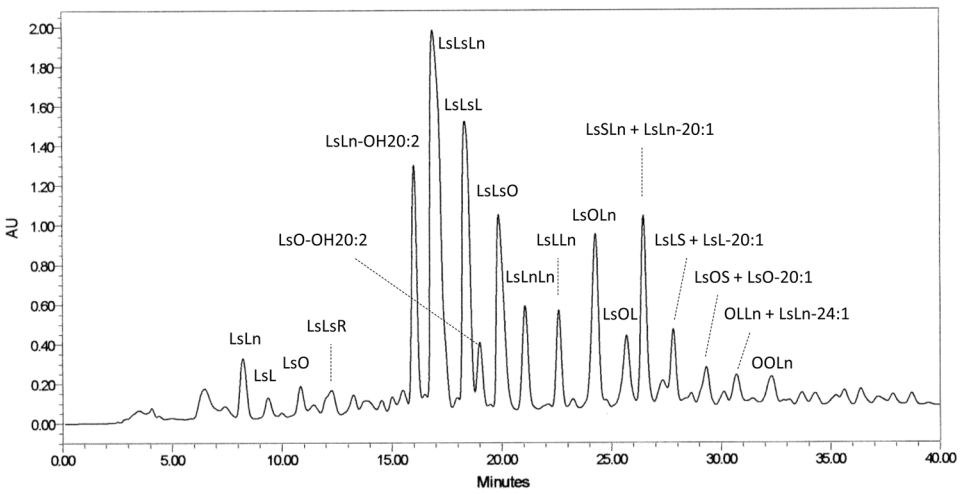

Fig. 2 HPLC chromatogram of lesquerella oil (1 mg) for fractionation (0.5 min/fraction) [4]. For HPLC conditions, see "Materials and Methods". The variation of the retention times of the eight HPLC fractionations (on the same day) pooled together was less than 0.1 min. For retention times of minor HPLC peaks, refer to TAG and HPLC fraction numbers in Table 1

corresponding fractions were pooled from 8 HPLC runs. HPLC fractions were used for MS studies. The final methanol solutions of samples were prepared for direct infusion into the mass spectrometer by combining approximately one-fourth or half of each HPLC fraction with 50 μL of methanol solution of 100 mM lithium acetate and diluting to a total volume of 250 μL.

Electrospray Ionization Mass Spectrometry (ESI–MS)

An LCQ Advantage ion-trap mass spectrometer (MS 2.0) with Xcalibur 2.0 SR2 software (ThermoFisher Scientific, San Jose, CA, USA) was utilized for MS analysis of the various molecular species of TAG. The infusion at a 2.5 μL/min flow rate from a syringe (250 μL) 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: sheath gas (nitrogen) flow rate, 10 arbitrary units (au); aux/sweep gas flow rate, 0 au; spray voltage, 4 kV; capillary temperature, 200 °C; capillary voltage, 5 V; tube lens offset, 15 V. Scan conditions were as follows: isolation width, 1.0 *m/z*; normalized collision energy, 27–42 %; scan ranges 100–2000 *m/z*. Acquire time was usually 3 min.

Results and Discussion

Lesquerella oil was fractionated by C_{18} HPLC at 0.5 min/fraction as the chromatogram shown in Fig. 2 [4]. Samples of the methanol solutions of the individual HPLC fractions and lithium acetate were infused into the MS from the syringe pump for MS studies. The ratios of the ion signal intensities (or relative abundances) of the fragment ions from the neutral losses of FA as α-lactones at the *sn*-2 position (MS^3) were used as the ratios of the regioisomers. Figure 1a is the proposed fragmentation mechanism.

Table 1 lists the ratios (%) of regioisomers of the molecular species of TAG in lesquerella oil. The following is the example for the estimation of the ratio of regioisomers of the molecular species of AAB type TAG following the HPLC fractionation of lesquerella oil. We have used this method to estimate the ratios of regioisomers of the molecular species of AAB type TAG in castor oil [8, 10–12]. Figure 3 is the MS^2 spectrum of the molecular ion $[M + Li]^+$ at *m/z* 931.6 from the HPLC fraction #48 of lesquerella oil (Fig. 2, from 23.5 min to 24 min) containing both LsLL and LsOLn. Four fragment ions from the neutral losses of free FA are shown in Fig. 3 as $[M + Li - L]^+$ at *m/z* 651.4, $[M + Li - O]^+$ at *m/z* 649.4, $[M + Li - Ln]^+$ at *m/z* 653.4 and $[M + Li - Ls]^+$ at *m/z* 605.3. Ls is also shown as $[Ls + Li]^+$ at *m/z* 333.2 in Fig. 3. In the HPLC fraction #48, the content of LsLL was much higher than that of LsOLn, so the relative abundance of $[M + Li - L]^+$ at *m/z* 651.4 is very high compared to those of the fragment ions from the neutral losses of FA from LsOLn (O and Ln). Usually hydroxy FA is shown as $[FA + Li]^+$ and normal FA is shown as $[M + Li - FA]^+$ [17].

Figure 4 is the MS^3 spectrum from the fragment ion $[M + Li - L]^+$ at *m/z* 651.4 in Fig. 3. The precursor ion $[M + Li - L]^+$ at *m/z* 651.4 was proposed as being DAG γ-lactones (Fig. 1a) [13]. Figure 4 shows the fragment ions $[M + Li - L - L + 2]^+$ at *m/z* 373.2 and $[M + Li - L - Ls + 2]^+$ at *m/z* 327.3, the second neutral losses of FA as (L − 2) and (Ls − 2). Both (L − 2) and (Ls − 2) were proposed as being FA α-lactones (Fig. 1a) [13]. The fragment ion $[M + Li - L - Ls + 2]^+$ at *m/z* 327.3 cannot be seen in Fig. 4 because it was so low and buried under the background baseline. This fragment ion can be seen in Fig. 5. Figure 5 is the enlarged section of these two fragment ions in Fig. 4. The ratio of the relative abundances of the fragment ions $[M + Li - L - Ls + 2]^+$ at *m/z* 327.3 and $[M + Li - L - L + 2]^+$ at *m/z* 373.2 in Fig. 5 were used as the ratio of the regioisomers LLsL and LsLL as 3:97

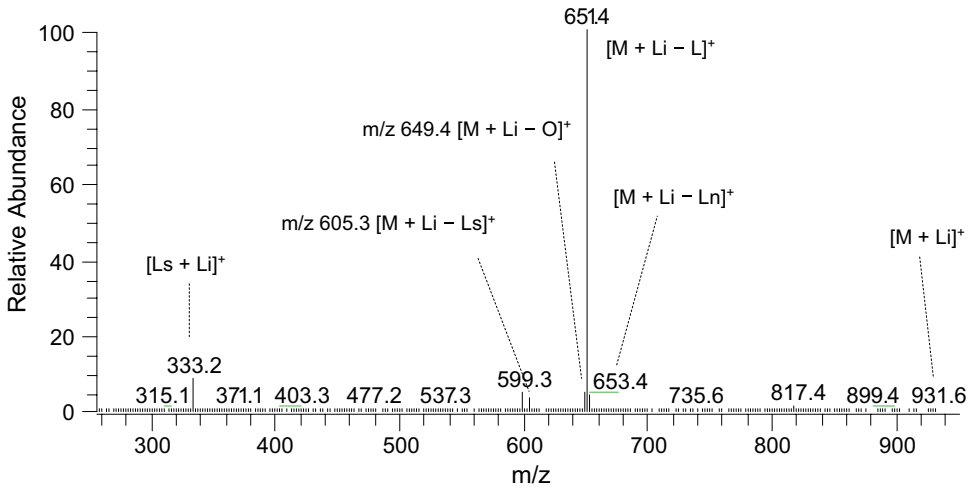

Fig. 3 MS^2 spectrum of the molecular ions $[M + L\ i]^+$ at *m/z* 931.6 from the HPLC fraction #48 of lesquerella oil containing both LsLL and LsOLn

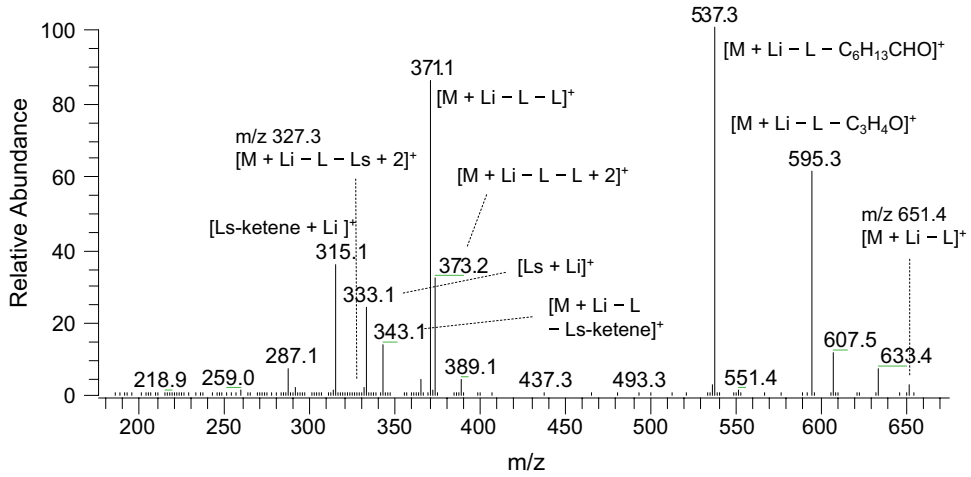

Fig. 4 MS^3 spectrum from the fragment ion $[LsLL + Li - L]^+$ at *m/z* 651.4 in Fig. 3 from the HPLC fraction #48 of lesquerella oil

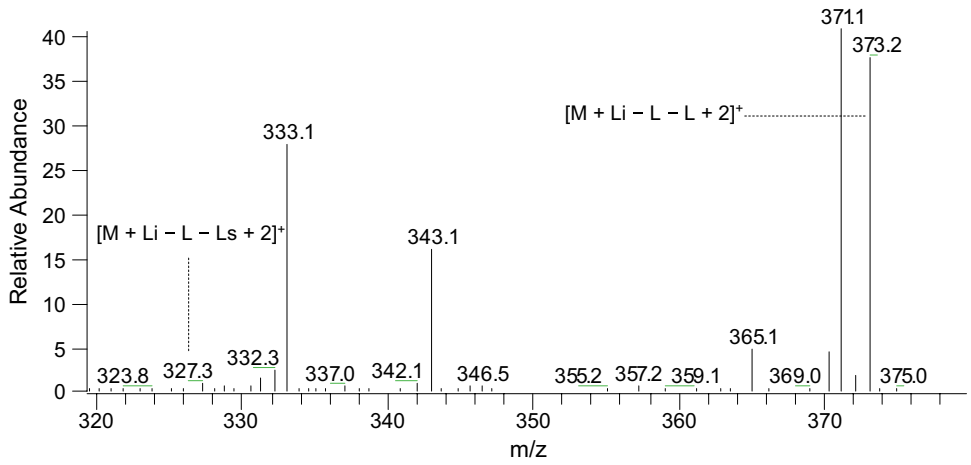

Fig. 5 The partial spectrum of Fig. 4 including the fragment ions of $[LsLL + Li - L - Ls + 2]^+$ at *m/z* 327.3 and $[LsLL + Li - L - L + 2\ ]^+$ at *m/z* 373.2

(Table 1). We used the original MS spectra of Xcalibur to estimate the ratios, not the PowerPoint or PDF files as Fig. 4.

The prominent fragment ion $[M + Li - L - C_3H_4O]^+$ at *m/z* 595.3 in Fig. 4 was from the neutral loss of the glycerol backbone to form FA anhydride (Fig. 1b). We have earlier proposed the fragmentation mechanism of this fragment ion [8] using the MS^2 precursor ion, DAG containing a 1,3-diazolane five-membered ring, proposed by Hsu

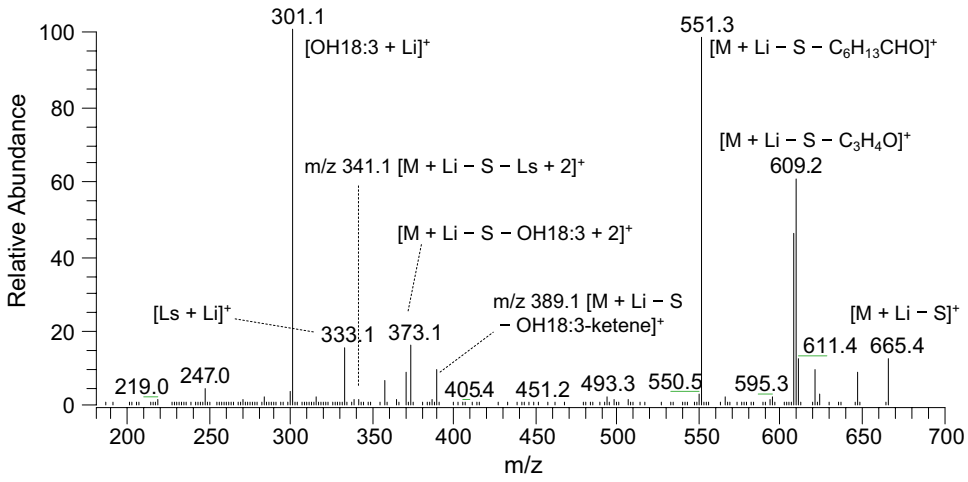

Fig. 6 MS^3 spectrum from the fragment ion [LsS-OH18:3 + Li − S]$^+$ at *m/z* 665.4. This precursor ion was from the MS^2 spectrum (not given here) of the molecular ion [LsS-OH18:3 + Li]$^+$ at *m/z* 949.6 from the HPLC fraction #45 of lesquerella oil

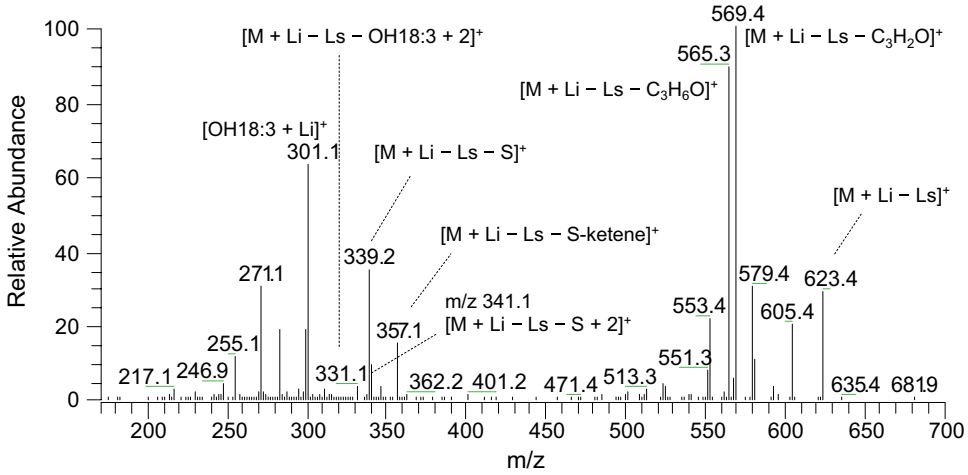

Fig. 7 MS^3 spectrum from the fragment ion [LsS-OH18:3 + Li − Ls]$^+$ at *m/z* 623.4. This precursor ion was from the MS^2 spectrum (not given here) of the molecular ion [LsS-OH18:3 + Li]$^+$ at *m/z* 949.6 from the HPLC fraction #45 of lesquerella oil

and Turk [14]. We proposed here again the fragmentation mechanism (Fig. 1b) using the newly proposed precursor ion of DAG γ-lactones [13]. The prominent fragment ion [M + Li − L − $C_6H_{13}CHO$]$^+$ at *m/z* 537.3 in Fig. 4 was formed from the cleavage between C-13 and C-14 of the lesquerolic acid chain next to hydroxyl group at C-14. The fragmentation mechanism of the cleavage was proposed as the Fig. 5 of the reference #4. All of the fragment ions described in Fig. 4 were the common fragment ions in the MS^3 spectra of the TAG lithium adducts [8–12, 18].

The following is the example for the estimation of the ratio of regioisomers of the molecular species of ABC type TAG following the HPLC fractionation of lesquerella oil. We have used this method to estimate the ratios of regioisomers of the molecular species of ABC type TAG in castor oil [10–12]. There was only one molecular species of TAG lithium adduct, [LsS-O H18:3 + Li]$^+$ at *m/z* 949.6, from the HPLC fraction #45 (Fig. 2, from 22 to 22.5 min) [4]. The MS^2 spectrum (not given here) of the molecular ion, [LsS-OH18:3 + Li]$^+$ at *m/z* 949.6, showed the prominent fragment ions of [M + Li − Ls]$^+$ at *m/z* 623.4 (relative abundance 77 %), [M + Li – S]$^+$ at *m/z* 665.4 (100 %) and [M + Li − OH18:3]$^+$ at *m/z* 655.5 (62 %). Figure 6 is the MS^3 spectrum from the precursor fragment ion [LsS-OH18:3 + Li − S]$^+$ at *m/z* 665.4. Figure 6 shows the fragment ions [M + Li − S − OH18:3 + 2]$^+$ at *m/z* 373.1 and [M + Li − S − Ls + 2]$^+$ at *m/z* 341.1. From the relative abundances of these two fragment ions, the ratio of the two regioisomers, Ls-OH18:3-S and SLs-OH18:3, was estimated at 94:6. Figure 7 is the MS^3 spectrum from the fragment ion [LsL-OH18:3 + Li − Ls]$^+$ at *m/z* 623.4 from the HPLC fraction #45. Figure 7 shows the fragment ions [M + Li − Ls − S + 2]$^+$ at *m/z* 341.1 and [M + Li − Ls – OH18:3 +2]$^+$ at *m/z* 331.1. From the relative abundances of these two fragment ions, the ratio of the two regioisomers, LsS-OH18:3 and Ls-OH18:3-S was estimated at 75 : 25. From these two ratios of regioisomers, 94 : 6 (Ls-OH18:3-S : SLs-OH18:3) and 75 : 25 (LsS-OH18:3 : Ls-OH18:3-S), the ratio of the three regioisomers was estimated at 1.5 : 74 : 25 (SLs-OH18:3 : LsS-OH18:3 : Ls-OH18:3-S) as shown in Table 1. We assumed the same response of hydroxy FA and normal FA on the second neutral loss of

FA as α-lactone at the *sn*-2 position (Fig. 1a). There is no regiospecific standard of TAG containing both hydroxy FA and normal FA available to confirm this assumption. The variations of the ratios of regioisomers from within-day and inter-day analysis were minor when the ratios were compared among the two or three HPLC fractions sequentially (Table 1) of the same molecular species of TAG.

Figure 6, the MS^3 spectrum from the precursor fragment ion $[LsS\text{-}OH18{:}3 + Li - S]^+$ at *m/z* 665.4, shows three prominent fragment ions from the losses of the glycerol backbone to form acid anhydride, $[M + Li - S - C_3H_6O]^+$ at *m/z* 607.2, $[M + Li - S - C_3H_4O]^+$ at *m/z* 609.2 and $[M + Li - S - C_3H_2O]^+$ at *m/z* 611.4. Figure 4 shows only one prominent fragment ion from the loss of the glycerol backbone, $[M + Li - S - C_3H_4O]^+$ at *m/z* 595.3 (Fig. 1b). Figure 7, the MS^3 spectrum from the fragment ion $[LsS\text{-}OH18{:}3 + Li - Ls]^+$ at *m/z* 623.4, shows two prominent fragment ions from the losses of the glycerol backbone, $[M + Li - S - C_3H_6O]^+$ at *m/z* 565.3 and $[M + Li - S - C_3H_2O]^+$ at *m/z* 569.4. The fragmentation mechanisms of the two fragment ions, $[M + Li - S - C_3H_6O]^+$ and $[M + Li - S - C_3H_2O]^+$ were proposed as Fig. 1c, d. In this study, we have obtained many MS^3 spectra and only Figs. 6 and 7 (except LsLs-OH18:3) show these two fragment ions from the loss of C_3H_6O and C_3H_2O. The precursor ions of Figs. 6 and 7 were diacylglycerol lithium adducts, Ls-OH18:3 and S-OH18:3, individually, and the common FA of these two precursor ions was OH18:3. This is the only FA with three double bonds detected in lesquerella oil (Table 1). The structure of OH18:3 was proposed as $OH^{12}18{:}3^{9,14,16}$ [4]. We did not detect OH18:3 in castor oil and the two fragment ions from the losses of C_3H_6O and C_3H_2O were not detected from castor oil. The MS^3 spectrum (not shown here) of precursor ion $[M + Li - Ls]^+$ at *m/z* 665.4 from LsLs-OH18:3 containing OH18:3 in the HPLC fraction #22 (Fig. 2, from 10.5 to 11 min) showed the prominent fragment ion $[M + Li - Ls - C_3H_4O]^+$ at *m/z* 609.3 and the minor fragment ion $[M + Li - Ls - C_3H_6O]^+$ at *m/z* 607.3, and the fragment ion $[M + Li - Ls - C_3H_2O]^+$ at *m/z* 611.3 was not detected. This HPLC fraction contained about equal amounts of LsLs-OH18:3 and Ls-OH20:2-OH18:2 [6] with the same mass as $[M + Li]^+$ at *m/z* 991.7.

Table 1 gives the ratios of the regioisomers of 72 molecular species of TAG in lesquerella oil. The order of the preference of FA incorporation at the *sn*-2 position of the molecular species of TAG in lesquerella was as: normal FA > OH18 > diOH18 > OH20 > diOH20. The part of this order including elongation was OH18 > diOH18 > OH20. Table 1 shows two TAG, LsR-OH20:2 and LsLsR, containing R (OH18) and Ls (OH20) and their ratios (R : Ls) were 95 : 1.4 and 98 : 2 at the *sn*-2 position individually. Table 1 also shows two TAG, LsLs-diOH18:1 and LsLs-diOH18:2, containing diOH18 and Ls (OH20) and their ratios (diOH18 : Ls) were 56 : 44 and 74 : 26 at the *sn*-2 position individually. The ratios of diOH18 : Ls were much closer than the ratios of R : Ls. Therefore the order was OH18 > diOH18 > OH20. Table 1 also shows three examples that OH20 (Ls) > diOH20 as well as many examples that the normal FA is predominate at the *sn*-2 position.

We have reported the ratios of the regioisomers of the molecular species of TAG in castor oil [8, 10–12]. We have also reported the identification of lesquerolic acid in castor oil by GC–MS [19] and LC–MS [8]. The order of the preference of FA incorporation at the *sn*-2 position of the molecular species of TAG in castor oil was as: normal FA > OH18 > OH20 > diOH18 > triOH18. The part of this order including the elongation, OH18 > OH20 > diOH18, was not reported earlier. Table 1 of reference #12 showed two TAG, RLLs and RSLs, containing R (OH18) and Ls (OH20) and their ratios (R : Ls) were 4 : 2 and 10 : 5 at the *sn*-2 position individually. The same table also showed five TAG containing R (OH18) and diOH18 and their ratios were between 5 : 1 and 20 : 1. Therefore OH20 was in the order between the two as OH18 > OH20 > diOH18. The two orders of lesquerella and castor were similar but different. The orders of the preference of FA incorporation at the *sn*-1/3 positions of the molecular species of TAG would be reversed of the order of *sn*-2.

Elongation (from C_{18} to C_{20}) and degree of hydroxylation affect the ratio of the regioisomers of the molecular species of TAG while desaturation seems not as important. Elongation seems more effective than hydroxylation to incorporate hydroxy FA into TAG at the *sn*-1/3 positions in lesquerella as the order of OH20 > diOH18 > OH18. The contents of TAG containing diOH18 and diOH20 were very low [6]. The block of elongation (from C_{18} to C_{20}) may be used to increase the content of hydroxy FA, e.g., ricinoleate, at the *sn*-2 position of TAG in lesquerella. The results will be similar to the contents of various FA at the *sn*-1 and *sn*-1/3 positions in castor oil (Table 2) when the elongase activity was low in castor. Lesquerella is desired to produce ricinoleate because castor, the commercial source of ricinoleate, contains toxic substances such as ricin. The content of normal FA at the *sn*-2 position was 95 % in lesquerella oil (Table 2 and the following) and there is plenty room for ricinoleate to occupy at the *sn*-2 position replacing normal FA. The block of elongation may produce triricinolein (or castor oil) in lesquerella for industrial uses.

We have recently reported the contents (%) of all of these 74 molecular species of TAG in lesquerella oil [6]. From these contents and the ratios of regioisomers (Table 1) of the molecular species of TAG, the contents of individual FA at the *sn*-2 and *sn*-1/3 positions were estimated as shown in Table 2. In more detail, the estimation methods are given as follows. For the individual FA content at the *sn*-2

Table 2 The contents (%) of various fatty acids at the *sn*-1 and *sn*-1/3 positions of triacylglycerols in lesquerella oil and castor oil

FA	*sn*-2	*sn*-1/3
Lesquerella oil		
diOH18:0	0.0002	0.040
diOH18:1	0.017	0.013
diOH18:2	0.022	0.008
diOH20:0	0.0007	0.070
diOH20:1	0.0001	0.025
R, OH18:1	1.08	0.084
OH18:2	0.30	0.028
OH18:3	0.12	0.029
OH20:0	0.0002	0.015
Ls, OH20:1	1.19	85.47
OH20:2	0.34	5.08
S	0.28	1.48
O	37.59	1.74
L	22.90	0.78
Ln	31.30	0.85
P	1.11	0.80
Po	0.85	0.091
20:0	0.23	0.18
20:1	0.25	0.72
23:0	0.0013	0.064
24:1	0.0018	0.029
Total	97.59	97.59
Castor oil		
R	72.77	86.56
Ls	0.21	0.46
S	1.05	0.05
O	6.74	0.68
L	6.37	0.41
Ln	0.14	0.6
P	1.22	0.35
Total	88.50	89.11

position of TAG in lesquerella oil, the formula was used as: contents (%) of molecular species of TAG [6] multiplying the % of each regioisomer of the molecular species of TAG (Table 1), then divided by 100. Since there were 74 molecular species of TAG [6] and three different FA at the *sn*-2 position of ABC type TAG, there were more than 200 products. The products from the same FA were combined and the total of each FA at the *sn*-2 position are shown in Table 2. For the FA contents at the *sn*-1/3 positions, the % of ratios of the regioisomers of TAG at the *sn*-1/3 position were obtained from those at the *sn*-2 position (Table 1). For example, Table 1 shows that the ratio of the regioisomers of Ls-OH20:2-OH18:2 (ABC type) as 8 : 2 : 90 (Ls : OH20:2 : OH18:2 at *sn*-2). The ratio of these FA at *sn*-1/3 of this TAG was estimated at 92 : 98 : 10 by subtracting each number from 100 (total 200). For the AAB type TAG, LsLs-diOH18:1, the ratio of (Ls : diOH18:1) was 44 : 56 at the *sn*-2 position (Table 1) and the ratio of those at *sn*-1/3 was (100 – the ratio %) × 2 and was 112 : 88 (total 200). For the AAA type TAG, 200 was used for the *sn*-1/3 positions. The contents of individual FA at the *sn*-1/3 positions was estimated as that of *sn*-2, except divided by 200 instead of 100. AAA type TAG, LsLsLs and OOO, reported recently [4, 6] are not in Table 1 because there is no regioisomers of AAA type TAG. For the estimation of the contents (%) of various FA in lesquerella oil, the formula is as follows: (content of individual FA at *sn*-2 + content of the FA at *sn*-1/3 × 2) / 3.

The contents of FA at the *sn*-2 position (Table 2) were in the order as: O (37.59 %) > Ln (31.30 %) > L (22.90 %) > Ls (1.19 %). Also the order was as: normal FA (94.51 %) > OH20 (1.53 %) > OH18 (1.5 %) > diOH18 (0.039 %) > diOH20 (0.0008 %). The FA at the *sn*-2 position was predominately normal FA (94.5 %), mainly O, Ln and L. The contents of FA at the *sn*-1/3 positions were in the order as: Ls (85.47 %) > OH20:2 (5.08 %) > O (1.74 %), also in the order as: OH20 (90.57 %) > normal FA (6.73 %) > OH18 (0.14 %) > diOH20 (0.095 %) > diOH18 (0.061 %). The FA at the *sn*-1/3 positions was predominately OH20 (90.6 %), mainly Ls. The content of normal FA at the *sn*-1/3 positions was 6.7 %. Table 2 agreed with earlier report that the vast majority of lesquerella oil's hydroxy FA was at the *sn*-1/3 positions of TAG [20]. Table 2 also agreed with earlier report that the vast majority of OH18 FA in lesquerella oil was at the *sn*-2 position [21]. This is the first report to estimate the contents of individual FA at the *sn*-2 and *sn*-1/3 positions of TAG using mass spectrometry as far as we are aware of. The total FA at the *sn*-2 and *sn*-1/3 positions of TAG combined in lesquerella oil were both as 97.59 % (Table 2), the rest were diacylglycerols (about 1 %) and tetraacylglycerols (about 1 %).

Table 2 also shows the contents of various FA at the *sn*-2 and *sn*-1/3 positions of TAG in castor oil. The contents were estimated from the contents of the molecular species of TAG [19] and the ratios of regioisomers of the molecular species of TAG [8, 12] in castor oil. Fifteen molecular species of TAG containing ricinoleate and normal FA were quantified in 2003 [19] and 65 molecular species of TAG (including TAG containing polyhydroxy FA) in castor oil were later identified [11, 22–24]. Both *sn*-2 and *sn*-1/3 positions of TAG in castor oil were predominated by ricinoleate as 72.8 % (*sn*-2) and 86.6 % (*sn*-1/3) individually. The content of normal FA at *sn*-2 (15.5 %) was higher than that at *sn*-1/3 (2.1 %). The total FA at the *sn*-2 and *sn*-1/3 positions of TAG in castor oil were both about 90 % (Table 2), the rest (about 10 %) were the many minor molecular species of TAG, DAG and tetraacylglycerols which were not quantified [19]. Among the normal FA at the *sn*-2 position of TAG in castor oil, the contents of O and L were high but not Ln, while in

lesquerella oil, O, L, Ln were all high. Table 2 shows that Ls is not predominate at the *sn*-1/3 positions of TAG in castor oil, while Ls is predominate at the *sn*-1/3 positions of TAG in lesquerella oil. This agrees with the preference of FA of the molecular species of TAG at the *sn*-1/3 positions as OH20 > diOH18 > OH18 (lesquerella) and OH20 > OH18 (castor), lesquerella was more effective than castor.

From the FA contents at the *sn*-2 and *sn*-1/3 positions (Table 2), the FA contents in lesquerella oil were estimated as follows comparing to the FA contents in parenthesis recently reported using GC [2]: Ls, 57.4 % (56.5 %); O, 13.7 % (13.8 %); Ln, 11.0 % (11.3 %); L, 8.15 % (7.0 %), OH20:2, 3.5 % (3.3 %); S, 1.08 % (1.9 %); P, 0.90 % (1.1 %); R, 0.42 % (0.6 %). These estimated results were from the contents (%) of the 74 molecular species of TAG [6] and the ratios of the regioisomers of 72 molecular species of TAG (Table 1), and were agreed with the FA contents of lesquerella oil estimated by GC [2]. LsLsLs and OOO have no regioisomers. This indicated that our quantification methods of the molecular species of TAG in lesquerella oil including HPLC with evaporative light scattering detector and ESI–MS comparing the ion signal intensities (or the relative abundance) of the molecular species of TAG in a HPLC peak were accurate. The accuracy included that the ratios of ion signal intensities (or the relative abundances) of the molecular species of TAG (MS^1) were the same as the ratios of the contents of the molecular species of TAG in an HPLC peak. Also the ratios of the total ion signal intensities (or the relative abundances) from the prominent ions combined (MS^2) from molecular species of TAG with the same mass were the same as the ratios of the contents of the molecular species of TAG with the same mass in an HPLC peak. The ion signal intensities of the molecular species of TAG with similar polarity (within the same HPLC peak) were the same (or almost the same).

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