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LC/MS Analysis of Cyclo Fatty Acid-containing Triacylglycerols in Cottonseed Oil

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Abstract: We measured the constituent triacylglycerol of cotton oil, the only edible oil containing a cyclo fatty acid. Due to the difficulty of obtaining a standard for triacylglycerols containing cyclo fatty acids, cotton oil is analyzed using cyclo triacylglycerol in kapok oil as a reference. Analytical methods used liquid chromatography fast atom bombardment mass spectrometry (LC / FAB-MS) using a matrix of 3% m-NBA containing 3 mM NaCl, and orthogonal transfer method for APCI / TOF-MS. FAB mass spectral properties of triacylglycerols containing cyclo fatty acids can be observed with $[M + Na]^+$, $[M-1]^+$ molecular ions, and [M- (RCOO)] + fragments. In the orthogonal APCI / TOF-MS spectrum, only the $[M-1]^+$ molecular ion appears, so that the presence of triacylglycerol containing CPFA becomes apparent. On the other hand, chain triacylglycerol can be observed as $[M + 1]^+$ molecular ions and $[M- (RCOO)]^+$ fragments. Based on these observations, triacylglycerols containing ten types of cyclo fatty acids were successfully successfully detected in crude cottonseed oil. In addition, the APCI/TOF-MS technique successfully detected dilinoleoyl-marvalyl-glycerol and palmtoyl-linoleoyl-marvalyl glycerol in the purified cottonseed oil.

Keywords: LC/FAB-MS, Cyclo Fatty Acid, Kapok Oil, Cottonseed Oil

1. Introduction

Prior to the 2000s, numerous studies into cyclo fatty acid-containing triacylglycerols (or cyclopropane fatty acids, CPFAs) were published. However, recent publications are sparse, and LC data relating to these compounds have yet to be reported. Examples of typical oils that contain CPFAs such as malvalic acid, sterculic acid, and dihydrostearic acid, are kapok oil and cottonseed oil [1–5]. Oil cake containing CPFAs tends to be avoided as a feed for livestock [6] because it causes infertility. Thus, the use of kapok oil, which contains >15% CPFAs, is limited to industrial products. In recent years, with improvements in the purification methods available for kapok oil, CPFA-free kapok oil has become available for purchase in Indonesia, although it has yet to be employed in food-based applications.

In contrast, cotton seeds contain approximately 0.16% of cyclo fatty acids, where cottonseed meal contains 0.03% and crude cottonseed oil contains 0.16%. Indeed, the only edible lipid of CPFA-containing triacylglycerols is cottonseed oil. In Japan and the United States, cottonseed oil has been used as a raw material for high-quality mayonnaise since prior to World War II. Crude cottonseed oil contains ~1% CPFAs, and this is reduced to <1 ppm upon refining; this level of CPFAs cannot be detected by gas chromatography (GC). From crude cottonseed oil, cotton salad oil can be produced through gum removal, deacidification, decolorization, wintering, and deodorization. It should be noted that the established standard for detecting residual CPFAs in cotton salad oil for quality control in mayonnaise and salad dressing manufacturing facilities is the Halfen test [7].

Previously, Horn et al. [8] performed an analysis of CPFA-containing triacylglycerols. More specifically, they introduced the harvested oil into a mass spectrometer using a micromanipulator directly from cotton root and leaf tissue, and reported that it contained up to 44% CPFAs. They analyzed it in the neutral loss mode of triple-quaddle pole MS. Namely, the profiles of the precursor triacylglycerol of the fatty acids of malvaric acid/linoleic acid (a), steracrylic acid (b), and dihydrosteracrylic acid (c) are shown.

During the analysis of triacylglycerols containing cyclo

fatty acids, the lipid is saponified and then methyl esterified. In addition, it is common practice to use silver nitrate in methanol to prepare ether and ketone derivatives for GC analysis. In contrast, during liquid chromatography (LC) analysis, the cyclo triacylglycerol is saponified and then UV-detected as a phenacyl ester derivative [6]. As such, the analysis of cyclo fatty acids is laborious, and a method of directly analyzing cyclo fatty acid-containing triacylglycerols is desired. In this context, Bland et al. [9] analyzed the triacylglycerols of cottonseed oil using LC and GC techniques. Although 17 components were separated by GC analysis, no details were provided regarding the cyclo triacylglycerols. However, we previously reported a method for the analysis of triacylglycerols by liquid chromatography-fast atom bombardment-mass spectrometry (LC/FAB-MS) [10]. Thus, we herein analyze the cyclo triacylglycerols present in cottonseed oil using our previously described LC/FAB-MS method.

2. Experimental

2.1. Samples and Reagents

Kapok and cotton seeds were used as samples of CPFAs, and refined kapok oil and cotton salad oil were used as refined oil standards. Thus, the kapok and cotton seeds (5–6 seeds) were ground in a mortar and extracted three times with acetone (1 mL). The extract was then filtered and concentrated to 1 mL under a flow of nitrogen gas to obtain the crude kapok and cottonseed oils. The eluents used for HPLC analysis were methanol (HPLC grade), acetonitrile (HPLC grade), acetone (HPLC grade), and sodium chloride (special grade reagent), and these were obtained from Kanto Chemical Co., Ltd. (Tokyo, Japan). *m*-Nitrobenzyl alcohol (*m*-NBA) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Brownlee Spheri 5 ODS (5 μm, 250 mm, 4.6 mm I.D; Perkin Elmer, Waltham, MA, USA) was used as a separation column for the CPFA-containing triacylglycerols, and a Develosil C30-UG–5 (5 μm, 250 mm × 4.6 mm I.D) column manufactured by Nomura Chemical Co., Ltd. (Seto, Japan). The following elution conditions were also used:

Elution Condition 1: flow rate, 0.7 mL; ODS column; solution A, 70% acetonitrile; solution B, 30% dichloromethane.

Elution Condition 2: flow rate, 0.8 mL; C30 column; eluent, 1% ethanol in acetone/methanol (6:4).

Elution Condition 3: flow rate, 0.8 mL; C30 column; solvent A, methanol; solvent B, acetone; gradient procedure, 60% B for 10 min, gradually increase to 90% B over 60 min, maintain at 90% B for 20 min.

Elution condition 4: flow rate, 0.8 mL; C30 column; solvent A, methanol; solvent B, acetone; gradient procedure, 60% B for 10 min, gradually increase to 90% B over 50 min, maintain at 90% B for 30 min.

The following abbreviations are employed for the constituent fatty acids of the triacylglycerol

Table 1. *Fatty acid abbreviations and molecular weights.*

Fatty Acid	Abbreviation	M.W
Myristic Acid	M	228
Palmitic Acid	P	256
Heptadecadienoic acid	Hdi	266
Stearic Acid	S	284
Oleic Acid	O	282
Linoleic Acid	L	280
Lnolenic Acid	Ln	278
Malvalic Acid	Ma	280
Sterculic Acid	Sc	294
Dihydrosterculic Acid	Dsc	296

2.2. Instrumentation

The mass spectrometer employed herein was equipped with a Frit-FAB interface manufactured by JEOL Ltd. A SX-102 and JMS700 (JEOL, Tokyo, Japan) double-focusing mass spectrometer was employed along with:, HP1090 (Hewlett-Packard GmbH, Waldbronn, Germany) and HP1100 pumps (Agilent Technologies, Santa Clara, CA, USA), a Waters Model 590 pump (Milford, MA, USA) for feeding the matrix. In the m-NBA matrix, a 3 mM NaCl solution containing 3% m-NBA in methanol was mixed post-column at a rate of 0.2 mL/min. Its introduction into the FAB target was set at 2-8 μL/min using a pneumatic splitter. For time-of-flight MS (TOF/MS), a JMS-T100LP (JEOL) instrument was used. Protonation occurred through atmospheric-pressure chemical ionization (APCI- orthogonal type) by the addition of 1% ethanol to the eluent.

3. Result and Discussion

3.1. Determination of the Chain Triacylglycerols and Cyclo Triacylglycerols of Kapok Oil Using the C18 Column

Since it was difficult to obtain a standard CPFA-containing triacylglycerol, analysis was performed using crude kapok oil as a reference substance since it contains multiple CPFAs. Using a C18 column for separation, the molecular weights and constituent fatty acids can be determined by the FAB of the triacylglycerol using the NaCl-containing m-NBA matrix. More specifically, the molecular weights of the triacylglycerols containing chain triglycerides and CPFAs can be determined from the $[M+Na]^+$ ions. The key difference between the spectra of these two substances is that $[M-1]^+$ ions are generated for the CPFA-containing triacylglycerols, while $[M+1]^+$ ions are generated for the chain triacylglycerides. In addition, the intensity ratio of the $[M-(RCOO)]^+$ fragment ion indicates that the constituent fatty acids of the cyclo triacylglycerol are proportional to the constituent fatty acids of the chain triacylglyceride. Figure 1 shows the FAB/MS chromatograms of the crude kapok oil and refined kapok oil obtained using an ODS column. As indicated, crude kapok oil has a complex chromatogram of overlapping chain triacylglycerols and cyclo triacylglycerols. In addition, Figure 2 shows the spectrum of trilinolein (LLL), which is a chain triacylglycerol of the same molecular weight as the malvalic acid-containing cyclo triacylglycerol (LLMa).

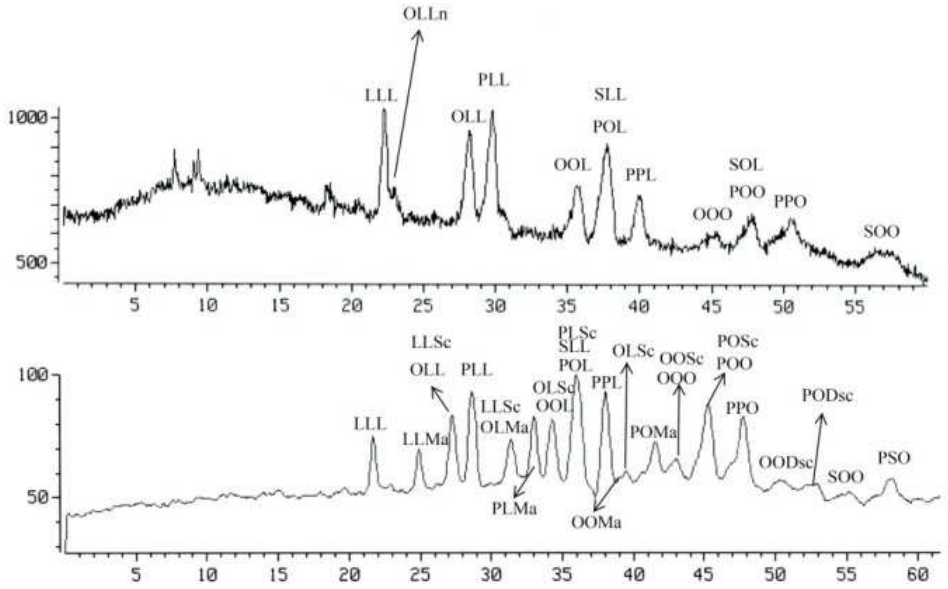

Figure 1. *The FAB/MS total ion chromatograms of crude kapok oil (bottom) and refined kapok oil (top). HPLC elution condition 1 was employed.*

The presence of the $[M-1]^+$ ion at m/z 877 confirms the presence of CPFAs, which have a higher ionic strength than the $[M+1]^+$ ion observed at m/z 879. The formation of $[M-1]^+$ ions is considered to be due to the desorption of H from cyclopropene.

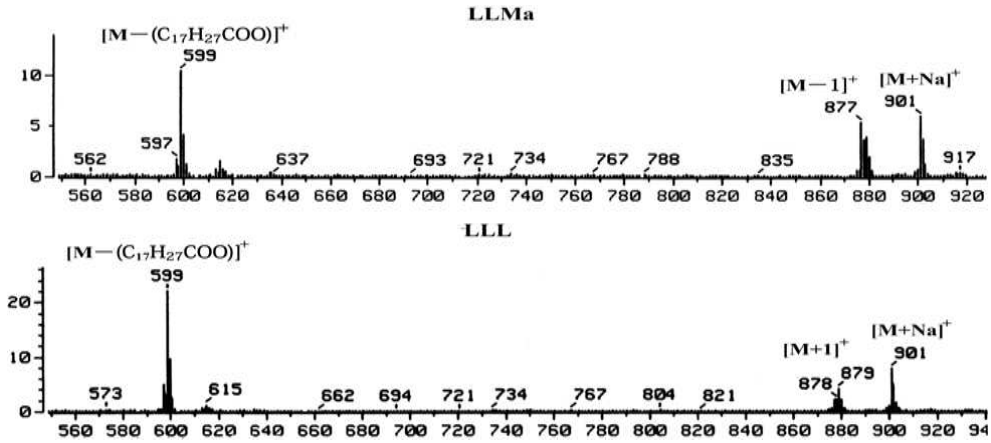

Figure 2. *FAB/MS spectra of the crude kapok oil.*

3.2. APCI/TOF-MS of the CPFAs

Figure 3 shows the spectra of the LLMa and PLMa present in the crude kapok oil. It should be noted that the orthogonal introduction of a CPFA-containing triacylglycerol by APCI/TOF-MS differs from the direct introduction, and fragment ion generation is not observed. In contrast, in the direct introduction APCI method, in the direct introduction APCI method, $[M-(RCOO)]^+$ ions, which are fragment ions of constituent fatty acids, are observed in addition to $[M-1]^+$ and $[M+1]^+$ of molecular ions. Therefore, since the spectrum of the cyclic triacylglycerol is a simple spectrum of molecular ions as show in Figure 3, the existence of the cyclo triacylglycerol can be easily estimated. In addition, the molecular ion strength of CPFA-containing triglyceride $[M-1]^+$ is > 10 times stronger than that of the chain triglyceride, and so is suitable for use in microanalysis.

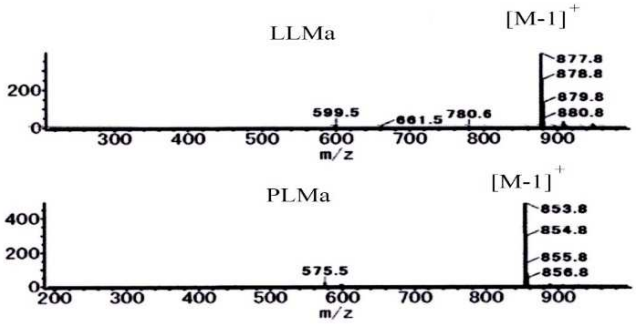

Figure 3. *TOF/MS spectra of the kapok crude oil.*

In addition, the molecular ion strength of CPFA-containing triglyceride $[M-1]^+$ is > 10 times stronger than that of the chain triglyceride, and so is suitable for use in microanalysis.

However, since fragments corresponding to constituent fatty acids are not observed, inexperienced analysts must predict the elution order of cyclo triacylglycerols from the elution behavior of chain triglycerides. Furthermore, it should be noted that formation of the [M−1]+ ion is likely due to the desorption of H from the cyclopropene group, as described above for the FAB method. Figure 4 shows the total ion chromatogram of crude kapok oil with 1% ethanol added to the eluent (C30 column). Since it is difficult to analyze chain triacylglycerols and cyclo triacylglycerols containing more than 15 components (such as in the case of crude kapok oil), the information obtained using the TCN (theoritical carbon number) value is essential for identification. In addition, as shown in Figure 5, LLMa and PLMa contents of ≤1 ppm were detected in cotton salad oil, but their corresponding spectra could not be obtained. These results therefore indicate that the orthogonal APCI/TOF-MS method is effective for trace analysis but not for qualitative analysis.

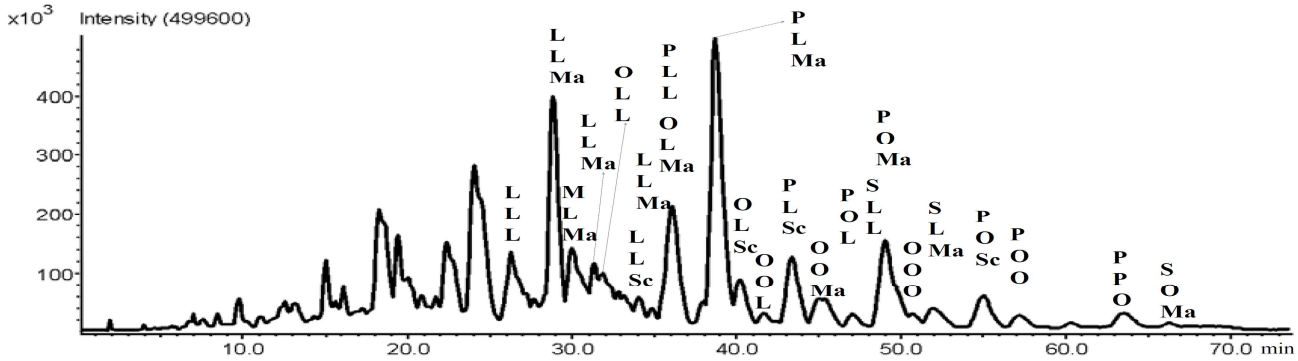

Figure 4. *Total ion chromatogram of the crude kapok oil. HPLC elution condition 2 was employed.*

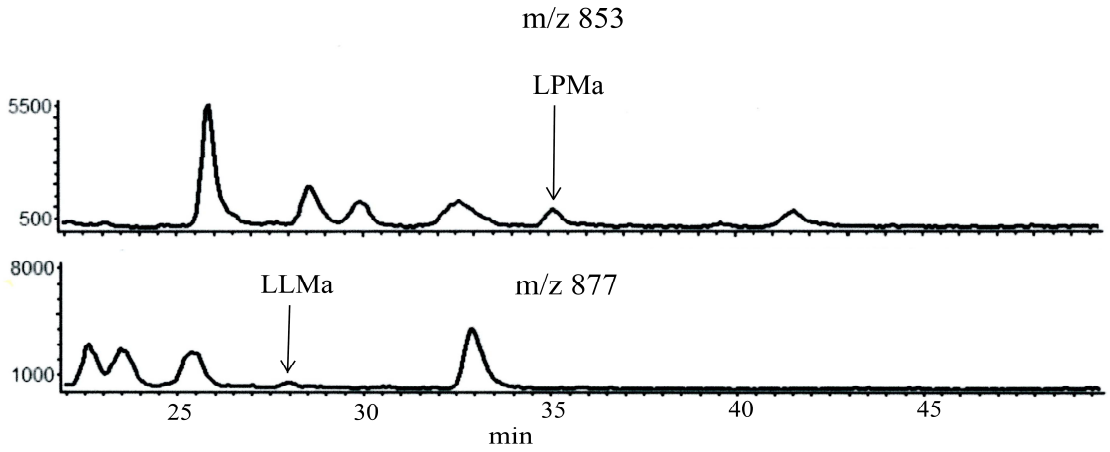

Figure 5. *Mass chromatograms of cotton salad oil.*

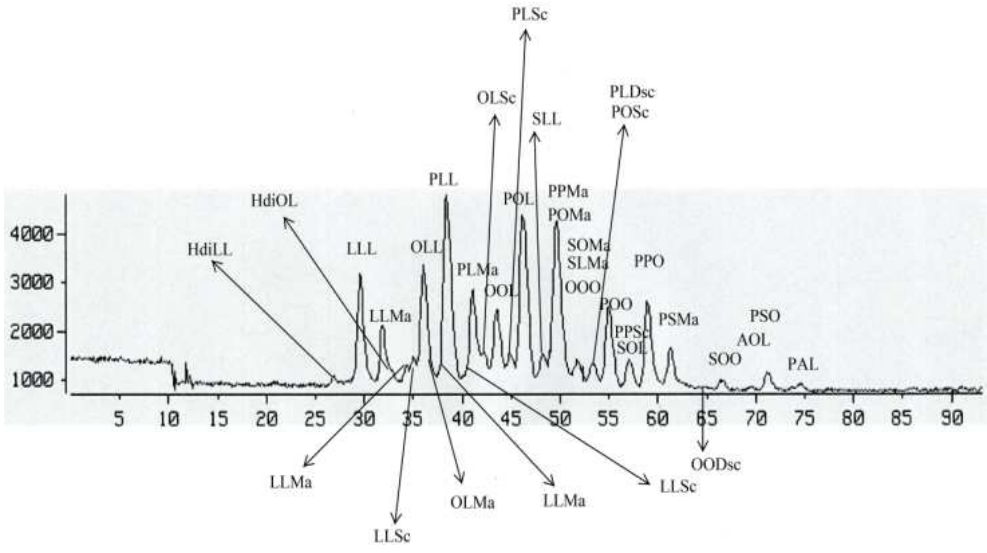

Figure 6. *FAB/MS total ion chromatogram of the kapok crude oil. HPLC elution condition 3 was employed.*

3.3. Analysis of the Cyclo Fatty Acid-containing Triacylglycerols Using a C30 Column

The analysis of kapok oil can be carried out using a C30 column, where chain and cyclo triacylglycerols containing 30 or more components can be identified, with no overlap being observed for 3 or more components. Thus, Figure 6 shows the chromatogram obtained for kapok oil using a C30 column. Compared with the ODS columns, separation was improved, but still insufficient. However, as mentioned above, the LC/FAB method is sufficient for identification of the triacylglycerols. It should be noted here that stereoisomers are formed in the presence of linoleic acid, which contains a conjugated double bond. In addition, trilinolein can be expected to have a stereoisomer, but its presence in vegetable oil has not been confirmed. In kapok oil, the presence of stereoisomers can be predicted from the presence of multiple LLMa and LLSc signals in the total ion chromatogram of Figure 6. It shoud be noted that triacylglycerols containing heptadecadienoic acid having 17 carbon atoms was detected from kapok oil at 26.9 and 32.7 min.

3.4. Analysis of the Cyclo-fatty Acid-containing Triacylglycerols in Cottonseed Oil Using a C30 Column

The analysis of cottonseed crude oil was then carried out using the CPFAs of kapok crude oil as a reference, and triacylglycerols containing 10 types of CPFAs (i.e., LLMa, LLSc, PLMa, OLSc, PLSc, PPMa, PLDsc, PSMa, PODsc, and SLDsc) were detected. Thus, Figure 7 shows the total ion chromatogram of the cottonseed crude oil, while Figure 8 shows the mass spectrum of PLDsc. Since the triacylglycerol containing dihydrostericuric acid also generates [M−1]+ ions, it is thought that H is also desorbed from the cyclopropane group. We also note that APCI/TOF-MS detected triacylglycerols containing both LLMa and PLMa cyclo fatty acids from the refined cottonseed oil, but using the Frit-FAB system, no cyclo triacylglycerols were detected, likely due to the low amount of sample introduced into the mass spectrometer (i.e., <1/200 compared to APCI).

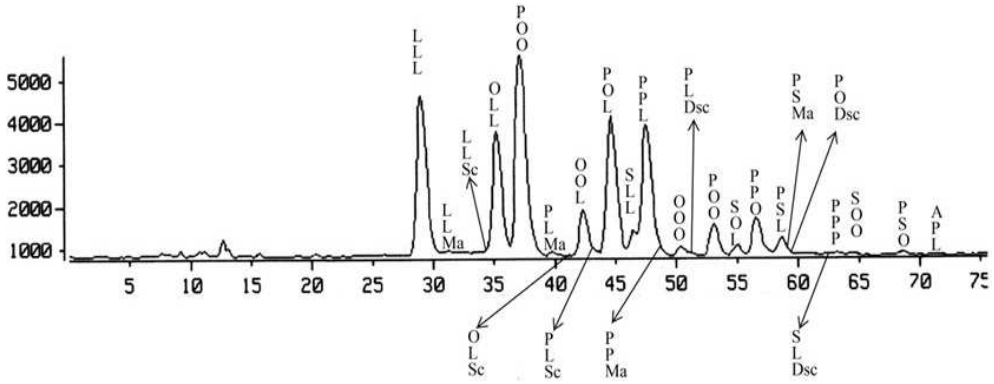

Figure 7. *Total ion chromatogram of the cotton crude oil. HPLC elution condition 4 was employed.*

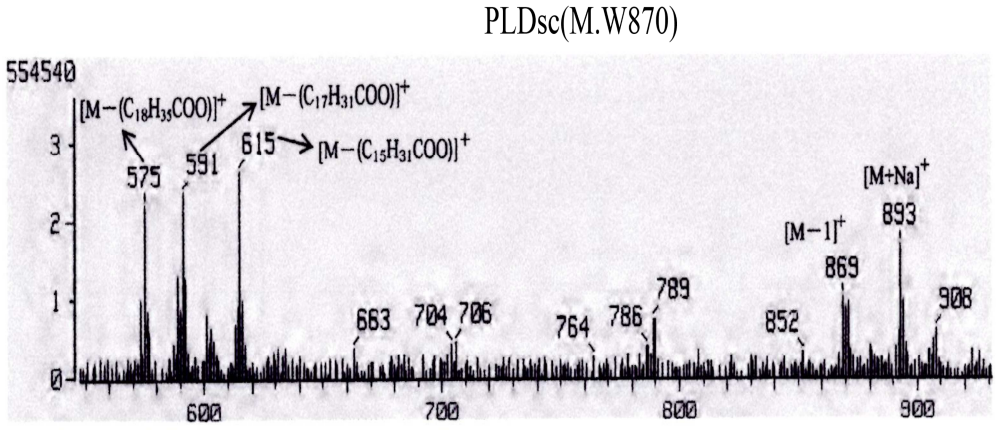

Figure 8. *The mass spectrum of PLDsc.*

4. Conclusion

Analysis of the cyclo fatty acid-containing triacylglycerols present in cottonseed oil was carried out using liquid chromatography-fast atom bombardment-mass spectrometry (LC/FAB-MS). Thus, triacylglycerols containing 10 kinds of cyclo fatty acids were identified using the cyclopropane fatty

acid (CPFA)-containing triacylglycerols of kapok oil as a reference. Although quantification was not achieved, the developed method was suitable for the detection of trace amounts of dilinoleoyl-malvalyl-glycerol and palmtoyl-linoleoyl-malvalyl-glycerol from cottonseed salad oil.

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