Printed in the Republic of Korea
ANALYTICAL SCIENCE
& TECHNOLOGY
Vol. 30 No. 2, 102-111, 2017
https://doi.org/10.5806/AST.2017.30.2.102

# Identification of triacylglycerols in coix seed extract by preparative thin layer chromatography and liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry

**Hee-Jung Sim[1], Seul gi Lee[1], Na-Hyun Park[1], Youna Kim[1], Hyun-Woo Cho[2] and Jongki Hong[1,★]**
[1]*College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea*
[2]*Department of Chemistry, Kyonggi University, Suwon 16227, Korea*

(Received February 3, 2017; Revised February 12, 2017; Accepted February 12, 2017)

**Abstact:** Here we reported a methodology for identification of triacylglycerols (TAGs) and diacylglycerols (DAGs) in coix seed by preparative thin layer chromatography (prep-TLC) and non-aqueous reversed-phase liquid chromatography (NARP LC)-atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (MS/MS). Lipid components were extracted from coix seed by reflux extraction using *n*-hexane for 3 hr. TAGs and DAGs in coix seed extract were effectively purified and isolated from matrix interferences by prep-TLC and then analyzed by LC-APCI-MS and MS/MS for identification. TAGs were effectively identified taking into consideration of their LC retention behavior, APCI-MS spectra patterns, and MS/MS spectra of $[DAG]^+$ ions. In MS/MS spectra of TAGs, diacylglycerol-like fragment $[DAG]^+$ ions were useful to identify TAGs with isobaric fragment ions. Based on an established method, 27 TAGs and 8 DAGs were identified in coix seed extract. Among them, 15 TAGs and 8 DAGs were for the first time observed in coix seed. Interestingly, some of TAGs isolated by prep-TLC were partly converted into DAGs through probably photolysis process during storing in room temperature. Thus, degradation phenomenon of TAGs should be considered in the quality evaluation and nutritional property of coix seed. LC-APCI-MS/MS combined with prep-TLC will be practical method for precise TAG and DAG analysis of other herbal plants.

**Key words:** triacylglycerol, diacylglycerol, coix seed, Prep-TLC, LC-APCI-MS/MS

## 1. Introduction

*Coix lachryma-jobi L.* var. *ma-yuen Stapf*, commonly called adlay, is a crude drug in the family of Gramineae and has been long used as a crop due to rich source of triacylglycerols (TAGs).[1,2] Diacylglycerols (DAGs) in coix seed are also present in minor amounts but have significant nutritional and metabolic importance. Coix seed has been used in Asian countries for the treatment of warts, rheumatism, female endocrine system and neuralgia from ancient times.[3,4] The structural elucidation of TAGs and DAGs in coix seed is important with regard to nutritional functions, quality control, lipid metabolism,

---

★ Corresponding author
Phone : +82-(0)2-961-9255 Fax : +82-(0)2-961-0357
E-mail : jhong@khu.ac.kr

and authenticity establishment.

The variety of TAG species is huge due to a high number of fatty acids present in plants. These TAGs are characterized by the total carbon number (CN), the number, position and configuration (cis/trans) of double bond (DB) in fatty acid (FA) acyl chains and the stereospecific position of FAs on the glycerol skeleton (*sn*-1, 2 or 3). The knowledge of stereospecific positions of individual FA is highly important, because FAs at *sn*-1 and *sn*-3 of the TAG are hydrolyzed during digestion and absorption of food while FAs at the *sn*-2 position remain intact.[5,6] This results in the different bioavailability of FAs depending on their *sn*-position.[7,8] Thus, identification of TAGs and DAGs in coix seed is required for nutritional and pharmaceutical understanding of coix seed widely used as food and medicine. There are, however, a few reports on determination and identification of TAGs and DAGs in coix seed.[9,10]

Several analytical methods including high performance liquid chromatography (HPLC)[11,12] and gas chromatography (GC)[13,14] have been used for analysis of TAGs. Specially, non-aqueous reverse-phase HPLC (NARP-HPLC) has been widely used in the TAGs separation considering the insolubility of these compounds in aqueous phases.[15-17] However, the severe problems in the analysis of TAGs are the lack of chromophore and the limitation of authentic standards. Reference standards are required for quantification and identification of TAGs in quality control of herbal medicine but the limited standards are commercially available. Recently, LC-based atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) have been widely used for structural elucidation of TAGs and DAGs because of easy coupling to non-aqueous mobile phase systems and high ionization efficiency for non-polar species.[18-20] Also, the stereospecific analysis using APCI mass spectra enables the determination of fatty acids in *sn*-2 position (TAG regioisomers) using the relative intensity of the diacylglycerol-like $[DAG]^+$ fragment ions.[20,21]

The aim of this study was to propose a methodology for comprehensive analysis of TAGs and DAGs in coix seed by LC-APCI-MS and MS/MS. This study included 1) TLC purification of TAGs and DAGs in coix seed extract, 2) identification of TAGs and DAGs based on APCI-MS spectral patterns and MS/MS fragmentation characteristics, and 3) observation of TAG degradation under room temperature. Some of mass fragment ions were shown to be diagnostic ions for the elucidation of regiospecific TAG isomers. A total of 35 lipid components including 27 TAGs and 8 DAGs were identified in coix seed extract by LC-APCI-MS method. Among them, 15 TAGs and 8 DAGs were for the first time observed in coix seed. This established methodology could play important role for nutritional and medicinal evaluation of coix seed and understanding TAG metabolism in plant.

## 2. Materials and Methods

### 2.1. Materials and reagents

Acetonitrile, dichloromethane, hexane, diethyl ether and acetic acid were purchased from J.T Baker (Phillipsburg, NJ, USA). All reagents and organic solvents were of HPLC analytical grade. Three authentic standards, 1,2,3-trilinoleylglycerol (18:2/18:2/18:2, LLL), 1-palmitoyl-2-oleoyl-3-linoleoylglycerol (16:0/18:1/18:2, POL), and 1,2,3-tripalmitoylglycerol (15:0/15:0/15:0, PPP) were purchased from Sigma Aldrich (St. Louis, MO, USA). In addition, 1,2,3-trioleylglycerol (OOO) was isolated by previously reported method.[18] TLC silica gel plate was purchased from Merck (Darmstadt, Germany).

### 2.2. Sample preparation

Dried coix seed were pulverized and one gram of powder was placed into 30 mL of hexane. The sample mixture was extracted for 30 min by a reflux extraction method. After the extract had been centrifuged at 3000 rpm for 10 min, the hexane extract was filtered through a 0.2 μm membrane filter (GHP membrane filters, Woongki Science, Seoul, Korea). The hexane extract was analyzed by prep-TLC and LC-APCI-MS.

### 2.3. Prep-TLC preparation

The 1 mL of hexane extract was spotted onto a

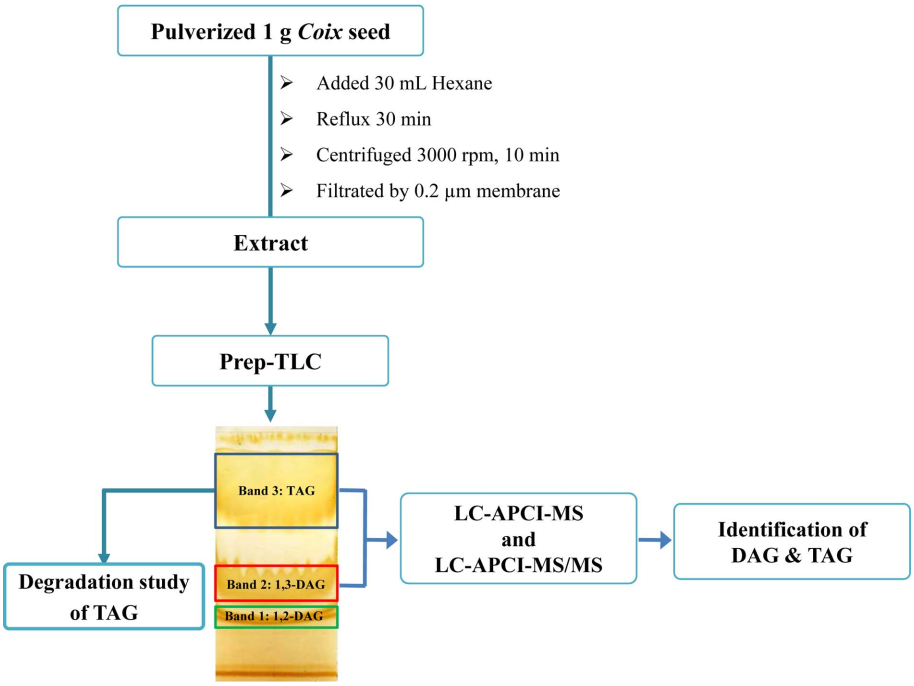

*Fig.* 1. Overall procedure for identification of DAGs and TAGs in coix seed.

TLC plate coated with silica gel. Another spotted TLC plates were also prepared with the same method. The TLC plates were simultaneously developed in a solvent system containing hexane/diethylether/acetic acid (80:20:2, v/v/v).[14] After development and drying, three bands were identified by reacting DAGs and TAGs with iodine. The bands of TAGs and DAGs on non-iodine treated TLC plate were scrapped out and dissolved in 1 mL of developing solvent using ultrasonic bath (15 min). These extracts were centrifuged at 3,000 rpm for 10 min and the supernatants were collected and filtrated through a 0.2 μm membrane filter. Ten microliters of the resulting solution was injected into the LC-MS/MS system. Overall procedure for analysis of TAGs and DAGs in coix seed is shown in *Fig.* 1.

### 2.4. LC-APCI-MS/MS conditions

All APCI-MS experiments were performed using an API 3200 instrument (MDS Sciex, Concord, ON, Canada) equipped with an APCI source. Chromatographic separation of the coix seed extracts in an HPLC system (Agilent 1200 series) was performed using the same analytical column as our previous study.[15] Mobile phase A (acetonitrile) and B (dichloromethane) was used and the linear gradient elution was as follows: 0-90 min, 25 %-40 % B, and then return to the initial condition for 10 min. The flow rate was set at 0.68 mL/min. In positive-ion APCI mode experiments, mass spectrometric conditions were as follows: curtain gas, 10 psi; temperature, 400 °C; nebulizing gas, 40 psi; heating gas, 0 psi; nebulizer current 3; the mass scan range was m/z 100-1000 in the full-mass scan. A syringe pump was used for direct analysis of the three reference compounds at a flow rate of 10 μL/min. The APCI-MS/MS experimental conditions were as follows: declustering potential (DP) 55 V and collision energy (CE) 43 V; OOO, DP 55 V and CE 45 V; POL, DP 55 V and CE 35 V. The $[M+H]^+$ and $[DAG]^+$ ions were selected as precursor ions in the positive-ion APCI mode.

## 3. Results and Discussion

### 3.1. prep-TLC for separation of TAGs and DAGs in coix seed

To effectively identify the TAGs and DAGs, they should be completely separated from complex matrix interferences of coix seed. Also, because there are several types of TAGs and DAGs with different or similar skeletons in the coix seed extracts, their precise analysis is not an easy task. In this study, prep-TLC using hexane/diethylether/acetic acid (80:20:2, v/v/v) was applied for the elimination of matrix interference and isolation of TAGs from DAGs. As shown in *Fig*. 1, lipid components in coix seed were clearly separated three main bands which are corresponding to 1,2-DAGs (band 1), 1,3-DAGs (band 2), and TAGs (band 3). Three bands on prep-TLC plate were separately extracted as described above (section 2.4) and then extracts were analyzed by LC-APCI-MS and MS/MS. By using prep-TLC, the regioisomer bands of 1,2-DAG and 1,3-DAG could be well separated due to their different polarities. Also, the isolation of DAGs and TAGs from coix extract using prep-TLC was useful to investigate the TAG degradation.

To identify DAGs and TAGs in coix seed extract, individual bands 1, 2, and 3 in prep-TLC plate were analyzed by LC-APCI-MS in positive ion mode. Total ion chromatograms (TICs) of *Fig*. 2(A) and (B) showed characteristic fingerprinting of 1,3-DAGs and 1,2-DAGs in coix seed extract, respectively, though weakly visualizing by iodine. Eight 1,2-DAGs were clearly detected in band 1, whileas any significant DAG peaks did not observe 1,3-DAGs in band 2 of prep-TLC. A total of 27 TAG peaks were observed in band 3, as shown in *Fig*. 2(C). Individual DAGs and TAGs could be clearly characterized without any interference and overlapped peaks by APCI-MS.

### 3.2. Identification of TAGs and DAGs composition in coix seed extract by APCI-MS and MS/MS

#### 3.2.1. Identification of TAGs by APCI-MS

To identify the TAG components of band 3 by LC-APCI-MS, three approaches were applies as follow: 1) considering HPLC retention behavior combined with equivalent carbon numbers (ECN), 2) sorting MS/MS spectral patterns according to structural characteristics such as AAA, ABA, AAB, and ABC types, and 3) referring to previously reported MS and MS/MS spectral data of TAGs observed in related plants and oils.

At first, the retention order of TAGs in RP-HPLC system is followed ECN, which is defined as

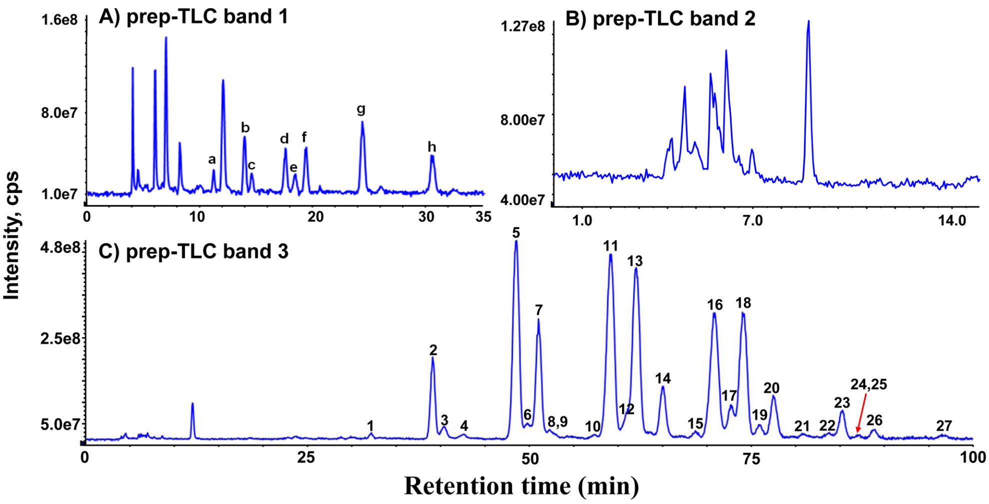

*Fig*. 2. TICs of (A) band 1, (B) band 3, and (C) band 3 extracts of coix seed separated by prep-TLC. Peak identities are the same as *Tables* 1-3.

ECN=CN-2DB (CN: number of carbon atoms, DB: number of double bonds).[17,18] For example, LLL (ECN=42), LLO (ECN=44), POL (ECN=46) and OOO (ECN=48) were eluted in this order on RP-HPLC system. The retention behavior of the TAGs with identical ECN is mainly influenced by the unsaturation degree and acyl chain length. For TAGs with the same ECN, their retention increase as DB number in acyl chains decrease. In this study, the TAGs with ECN=46 such as GLL (Rt=57.35), LOO (59.08), SLL (60.86), OLP (62.01) and PLP (65.06) were well resolved under optimized HPLC conditions [*Fig.* 2(C)]. Thus, the LC elution order of TAGs combined with ECN could be good supplemental data for identification of TAGs by LC-APCI-MS.

Second, TAGs were identified from APCI-MS and MS/MS spectra based on protonated molecules $[M+H]^+$ used for the molecular weight assignment, $[DAG]^+$ fragment ions for distinguishing the TAGs regioisomers, and acylium ions $[RCO]^+$ for the identification of individual fatty acids of TAG. Also, APCI-MS spectra of TAGs provided important information on unsaturation degree because the intensity of the $[M+H]^+$ ion depends on the number of sites of unsaturation in the molecule.[18] The APCI-MS spectra of the TAGs with more unsaturation exhibited more intense $[M+H]^+$ peaks but relatively weak intense $[DAG]^+$ peaks. In other words, those of TAG with less unsaturation produced less intense $[M+H]^+$ peaks but more intense $[DAG]^+$ peaks. Thus, the $[M+H]^+/\sum[DAG]^+$ ratios of TAGs could provide information on unsaturation degree of fatty acids substituted at glycerol backbone. *Tables* 1 and 2 summarizes retention times combined with ECN, $[M+H]^+/\sum[DAG]^+$ ratio, $[AA]^+/[AB]^+$ ratios, and DAGs fragment ions for TAGs extracted from coix seed. For example, the $[M+H]^+/\sum[DAG]^+$ ratio of LLL (18:2/18:2/18:2) was higher than that of OOO (18:1/18:1/18:1) as shown in *Table* 1. The $[M+H]^+/\sum[DAG]^+$ ratio of AAB/ABA type TAGs were higher than 0.21 when DB is ≥ 4 and those of ABC type TAGs except for unseparated TAGs which have ambiguous ratio due to the presence of common $[DAG]^+$ ion were higher than 0.05 when DB is ≥ 3.

In addition, four types of TAGs in band 3 could be readily discriminated by their different APCI-MS spectra patterns, as shown in *Fig.* 3. For typical example, the MS spectrum of OOO with AAA-type exhibited $[M+H]^+$ ion and one $[DAG]^+$ ion [*Fig.*

*Table* 1. AAA and AAB/ABA types of triacylglycerols identified in coix seed extract by LC-APCI-MS

| | Types of TAG | Peaks | Rt | ECN[a] | CN[b] | DB[c] | $[M+H]^+$ | $[M+H]^+/\sum[DAG]^+$ | $[AA]^+/[AB]^+$ | DAG fragment ions (*m/z*) |
|---|---|---|---|---|---|---|---|---|---|---|
| AAA | LLL (18:2/18:2/18:2) | 2 | 39.08 | 42 | 54 | 6 | 879 | 0.66 | | LL *m/z* 599 |
| | OOO (18:1/18:1/18:1) | 16 | 70.73 | 48 | 54 | 3 | 885 | 0.12 | | OO *m/z* 603 |
| AAB | LnLL (18:2/18:2/18:3) | 1 | 32.11 | 40 | 54 | 7 | 877 | 0.87 | $[LL]^+/[LnL]^+$=45:69 | LL *m/z* 599, LnL *m/z* 597 |
| | OLL (18:1/18:2/18:2) | 5 | 48.52 | 44 | 54 | 5 | 881 | 0.39 | $[LL]^+/[LO]^+$=94:100 | LL *m/z* 599, LO *m/z* 601 |
| | PLL (16:0/18:2/18:2) | 7 | 51.00 | 44 | 52 | 4 | 855 | 0.34 | $[LL]^+/[PL]^+$=86:100 | LL *m/z* 599, PL *m/z* 575 |
| | GLL (20:1/18:2/18:2) | 10 | 57.35 | 46 | 56 | 5 | 909 | 0.37 | $[LL]^+/[GL]^+$=61:100 | LL *m/z* 599, GL *m/z* 629 |
| | LOO (18:2/18:1/18:1) | 11 | 59.08 | 46 | 54 | 4 | 883 | 0.21 | $[OO]^+/[LO]^+$ =71:100 | OO *m/z* 603, LO *m/z* 601 |
| | SLL (18:0/18:2/18:2) | 12 | 60.86 | 46 | 54 | 4 | 883 | 0.31 | $[LL]^+/[SL]^+$=87:100 | LL *m/z* 599, SL *m/z* 603 |
| | POO (16:0/18:1/18:1) | 18 | 74.00 | 48 | 52 | 2 | 859 | 0.04 | $[OO]^+/[PO]^+$=70:100 | PO *m/z* 577, OO *m/z* 603 |
| | GOO (20:1/18:1/18:1) | 21 | 80.68 | 50 | 56 | 3 | 913 | 0.04 | $[OO]^+/[GO]^+$=53:100 | GO *m/z* 631, OO *m/z* 603 |
| | SOO (18:0/18:1/18:1) | 23 | 85.25 | 50 | 54 | 2 | 887 | 0.01 | $[OO]^+/[SO]^+$=54:100 | SO *m/z* 605, OO *m/z* 603 |
| | SSL (18:0/18:0/18:2) | 24 | 86.70 | 50 | 54 | 2 | 887 | 0.007[d] | $[SS]^+/[SL]^+$=100:66 [d] | SS *m/z* 607 SL *m/z* 603 |
| | AOO (20:0/18:1/18:1) | 27 | 96.47 | 52 | 56 | 2 | 915 | 0.002 | $[OO]^+/[AO]^+$=60:100 | AO *m/z* 633, OO *m/z* 603 |
| ABA | OLnO (18:1/18:3/18:1) | 6 | 49.74 | 44 | 54 | 5 | 881 | 0.23 | $[OO]^+/[OLn]^+$=38:100 | OLn *m/z* 599, OO *m/z* 603 |
| | PLP (16:0/16:0/18:2) | 14 | 65.06 | 46 | 50 | 2 | 831 | 0.01 | $[PP]^+/[PL]^+$=46:100 | PL *m/z* 575, PP *m/z* 551 |
| | POP (16:0/18:1/16:0) | 20 | 77.45 | 48 | 50 | 1 | 833 | 0.008 | $[PP]^+/[PO]^+$=38:100 | PO *m/z* 577, PP *m/z* 551 |

[a]ECN=equivalent carbon numbers, [b]CN=carbon numbers, [c]DB=number of double bonds, [d]The ratio is ambiguous because of common $[DAG]^+$ ion.

*Table* 2. ABC types of triacylglycerols identified in coix seed extract by LC-APCI-MS

| | Types of TAG | Peaks | Rt | ECN[a] | CN[b] | DB[c] | $[M+H]^+$ | $[M+H]^+/\sum[DAG]^+$ | $[AC]^+/[BC]^+/[AB]^+$ | Fragment (*m/z*) |
|---|---|---|---|---|---|---|---|---|---|---|
| ABC | LLnO (18:2/18:3/18:1) | 3 | 40.32 | 42 | 54 | 6 | 879 | 0.46 | $[LO]^+/[LnO]^+/[LLn]^+$= 69:73:75 | LO *m/z* 601, LnO *m/z* 599, LLn *m/z* 597 |
| | LnLP (18:3/18:2/16:0) | 4 | 42.53 | 42 | 52 | 5 | 853 | 0.54 | $[LnP]^+/[LP]^+/[LnL]^+$= 50:62:73 | LnP *m/z* 573, LP *m/z* 575, LnL *m/z* 597 |
| | OLnP (18:1/18:3/16:0) | 8 | 52.24 | 44 | 52 | 4 | 855 | 0.19 [d] | $[OP]^+/[LnP]^+/[OLn]^+$= 70:79:100 [d] | OP *m/z* 577, LnP *m/z* 573, OLn *m/z* 599 |
| | PLPo (16:0/18:2/16:1) | 9 | 52.24 | 44 | 50 | 3 | 829 | 0.02 [d] | $[PPo]^+/[LPo]^+/[PL]^+$= 2:100:42 [d] | PL *m/z* 575, LPo *m/z* 573, PPo *m/z* 549 |
| | OLP (18:1/18:2/16:0) | 13 | 62.01 | 46 | 52 | 3 | 857 | 0.1 | $[OP]^+/[LP]^+/[OL]^+$= 93:94:100 | OP *m/z* 577, LP *m/z* 575, OL *m/z* 601 |
| | OLG (18:1/18:2/20:1) | 15 | 68.68 | 48 | 56 | 4 | 911 | 0.12 | $[OG]^+/[LG]^+/[OL]^+$= 76:83:100 | OG *m/z* 631, LG *m/z* 629, OL *m/z* 601 |
| | LOS (18:2/18:1/18:0) | 17 | 72.67 | 48 | 54 | 3 | 885 | 0.07 | $[LS]^+/[OS]^+/[LO]^+$= 62:69:100 | LS *m/z* 603, OS *m/z* 605, LO *m/z* 601 |
| | SLP (18:0/18:2/16:0) | 19 | 75.95 | 48 | 52 | 2 | 879 | 0.005 | $[SP]^+/[LP]^+/[SL]^+$= 53:80:100 | SP *m/z* 579, LP *m/z* 575, SL *m/z* 603 |
| | LOA (18:2/18:1/20:0) | 22 | 83.65 | 50 | 56 | 3 | 913 | 0.05 | $[LA]^+/[OA]^+/[LO]^+$= 63:73:100 | LA *m/z* 631, OA *m/z* 633, LO *m/z* 601 |
| | ALP (20:0/18:2/16:0) | 25 | 86.70 | 50 | 54 | 2 | 887 | 0.003 [d] | $[AP]^+/[LP]^+/[AL]^+$= 74:89:100 [d] | AL *m/z* 631, LP *m/z* 575, AP *m/z* 607 |
| | SOP (18:0/18:1/16:0) | 26 | 88.90 | 50 | 52 | 1 | 861 | 0.004 | $[SP]^+/[OP]^+/[SO]^+$= 56:99:100 | SP *m/z* 579, OP *m/z* 577, SO *m/z* 605 |

[a]ECN=equivalent carbon numbers, [b]CN=carbon numbers, [c]DB=number of double bonds, [d]The ratio is ambiguous because of common $[DAG]^+$ ion.

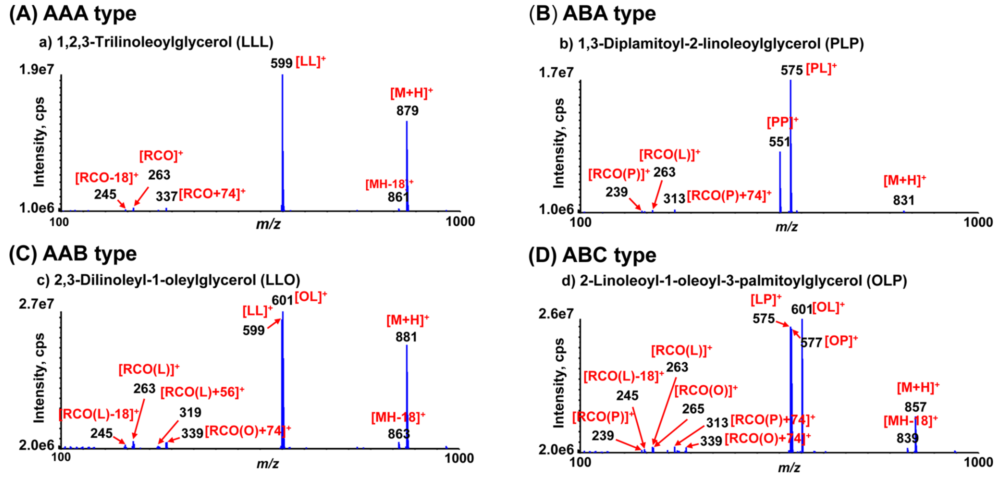

*Fig.* 3. Typical APCI-MS spectra of four types of TAGs observed in coix seed.

3(A)], those of LnLL and OLL with ABA/AAB-types showed $[M+H]^+$ ion and two $[DAG]^+$ fragment ions [*Fig*. 3(B, C)], and that of LLnO with ABC-type exhibited three $[DAG]^+$ fragment ions [*Fig*. 3(D)]. The discrimination of AAB-type or ABA-type could be performed based on $[DAG_{AA}]^+/[DAG_{AB}]^+$ ratio. $[DAG_{AA}]^+/[DAG_{AB}]^+$ ratio for ABA type is substantially below the statistically expected value of 0.5 whereas that of AAB type is greater than 0.5.[20] Equally, FAs at *sn*-2 for ABC-type could be identified from the lowest abundance $[DAG]^+$ ion. It can be explained that the lowest abundant peak $[DAG]^+$ might be energetically unfavorable in the loss of a FA from the *sn*-2 position.[21] For identification of FAs at *sn*-2 position, contributions of A+2 and A+3 isotopes should be subtracted from $[DAG]^+$ fragment ions with m/z values higher by two units, e.g., A+2 isotopic peak from $[LP]^+$ fragment ion at m/z 575 contributes to the peak of $[OP]^+$ at m/z 577. As a result, 27 TAGs were classified to AAA, AAB, ABA, ABC types as shown in *Tables* 1 and 2.

There are two analytical applications of the $[DAG_{AA}]^+/[DAG_{AB}]^+$ ratios in identification of TAGs. First, the $[DAG_{AA}]^+/[DAG_{AB}]^+$ ratios could be used to distinguish the TAG regioisomers when AAB- and ABA-types were completely separated. Second, the TAG content ratio of AAB- and ABA-type in coix seed could be calculated based on linear equation observed using $[DAG_{AA}]^+/[DAG_{AB}]^+$ ratios. In this study, the amount of AAB-type TAGs in coix seed was apparently greater than ABA-type TAGs from the $[DAG_{AA}]^+/[DAG_{AB}]^+$ ratios.

### 3.2.2. Identification of TAGs by APCI-MS/MS

For further identification of four types of TAGs in band 3, the CID MS/MS technique was applied for abundant $[DAG]^+$ ions appeared in APCI-MS spectra. The MS/MS spectra of $[DAG]^+$ ions showed several characteristic ions including $[RCO+74]^+$, $[RCO+54]^+$, $[RCO]^+$ and $[RCO-18]^+$ which enable to identify individual fatty acids substituted in glycerol backbone.

Especially, the MS/MS spectra of $[DAG]^+$ ions were helpful to identify the co-eluted TAGs in TIC. For example, the MS spectrum of peak 8 (52.24 min) exhibits the $[M+H]^+$ ions at m/z 855 and 829, and the $[DAG]^+$ ions at m/z 549, 573, 575, 577, and 599, as shown in *Fig.* 4(A). Observation of several $[DAG]^+$ ions reflected the presence of two TAGs at Rt 52.24 min. Based on the mass values of $[M+H]^+$ ions and $[DAG]^+$ ions, these $[DAG]^+$ ions were tentatively assigned as m/z 549 $[PPo]^+$, 573 $[PLn]^+$, 575 $[PL]^+$, 577 $[PO]^+$, and 599 $[OLn]^+$ in the MS spectrum. Thus, the peak eluted at Rt 52.24 min could be apparently identified as the mixture of OLnP and PLPo. Also, the MS/MS spectra of $[DAG]^+$ ions at m/z 577 $[PO]^+$ and 599 $[OLn]^+$

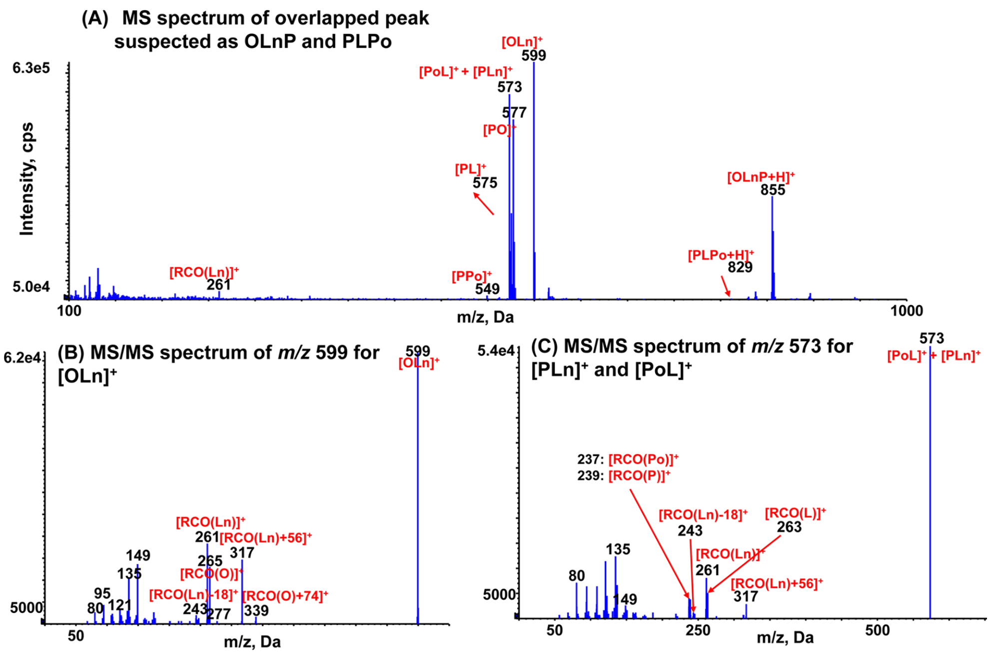

*Fig.* 4. (A) LC-APCI-MS spectrum of overlapped peak 8 containing OLnP and PLPo, (B) MS/MS spectrum of m/z 599 for $[OLn]^+$ ion, and (C) MS/MS spectrum of m/z 573 for $[PLn]^+$ and $[PoL]^+$ ion.

showed the acylium ions at m/z 265 [RCO(O)]$^+$, 239 [RCO(P)]$^+$ and m/z 265 [RCO(O)]$^+$, 261 [RCO(Ln)]$^+$ [*Fig*. 4(B)], respectively. The MS/MS spectrum of at m/z 573 [PLn]$^+$, however, showed the acylium ions not only at 239 [RCO(P)]$^+$, 261 [RCO(Ln)]$^+$ but also at m/z 237 [RCO(Po)]$^+$, 263 [RCO(L)]$^+$ as shown in *Fig*. 4(C). This results indicate that the [DAG]$^+$ ion at m/z 573 could be assigned as [PLn]$^+$ and [LPo]$^+$. The overlapped peak no. 8 were identified as OLnP and POLn on the basis of observation of [M+H]$^+$ ions and MS/MS spectra of [DAG]$^+$ ions. Similarly, other TAGs in coix seed could be clearly assigned.

In summary, identification of TAGs in coix seed was successfully performed based on the prep-TLC separation, the retention behavior of TAGs on RP-LC, the [DAG$_{AA}$]$^+$/[DAG$_{AB}$]$^+$ ratios in their MS spectra, and several acylium ions in MS/MS spectra of [DAG]$^+$ ions. Two TAGs with AAA-type, 11 TAGs with AAB-type, 3 TAGs with ABA-type, and 11 TAGs with ABC-type from coix seed were identified and tentatively characterized (*Tables* 1 and 2). Among them, 15 TAGs were for the first time identified in coix seed.

### 3.2.3. Identification of DAGs by APCI-MS

DAGs comprise approximately 5 % of neutral lipids in coix seed,[22] however, very little is known of DAGs in coix seed. To identify DAGs in coix seed, band 1 of prep-TLC in coix seed extract was analyzed by LC-APCI-MS (*Fig*. 2(A) and 5). A total of 8 DAGs were observed in band 1 of prep-TLC for coix seed extract. Identification of DAGs by LC-APCI-MS was performed on the basis of the presence of [M+H]$^+$, [M+H-18]$^+$, and other characteristic acylium ions such as [RCO]$^+$ and [RCO+74]$^+$. The retention of DAGs on RP-LC was also increased with ECN ascending order like TAGs. For example, LL (ECN=28), LO (ECN=30), OO (ECN=32), SP (ECN=34) and SS (ECN=36) were eluted in this order, as shown in *Fig*. 2(A). The intensity of the [M+H]$^+$ ion depends on the degree of unsaturation in the molecule, as shown in *Table* 3. The APCI-MS spectra of DAGs (LL, LO, PL, and OO) which have more than two DBs showed strong intensities for both [M+H]$^+$ and [M+H-18]$^+$ ions. On the other hands, the [M+ H]$^+$ ions of PO (DB=1), PP (DB=0), PS (DB=0) and SS (DB=0) did not observe in their MS spectra, yet [M+H-18]$^+$ ions observed as strong intensities. All of 8 DAGs extracted from coix seed were tentatively characterized on the basis of retention behavior on RP-LC and MS spectral information. To the best of our knowledge, the structural characterization of DAGs was for the first time reported in coix seed. Also, this method could be very useful to investigate biosynthesis and plant metabolism of TAGs.

## 3.3. Degradation of TAGs

In this study, the degradation of TAG extract obtained by prep-TLC was observed after standing for ten days at room temperature. After TAG extract was standing at room temperature for ten days, the resulting solution was analyzed by APCI-MS. As shown in *Fig*. 6(A), some of TAG peaks in band 3 might be partly converted into DAGs. In this study, the proposed degradation pathways are depicted in *Fig*. 6(B). Among observed DAG peaks, major three

*Table* 3. Diacylglycerols identified in coix seed extract by LC-APCI-MS

| Peaks | DAG | Rt | ECN$^a$ | CN$^b$ | DB$^c$ | [M+H-18]$^+$ or [M+H]$^+$ | Other ions |
|---|---|---|---|---|---|---|---|
| a | LL (18:2/18:2) | 11.32 | 28 | 36 | 4 | 599, 617 | 337, 263[RCO(L)]$^+$ |
| b | LO (18:2/18:1) | 13.82 | 30 | 36 | 3 | 601, 619 | 339, 337, 265[RCO(O)]$^+$, 263[RCO(L)]$^+$ |
| c | PL (16:0/18:2) | 14.48 | 30 | 34 | 2 | 575, 593 | 337, 313, 263[RCO(L)]$^+$ |
| d | OO (18:1/18:1) | 17.53 | 32 | 36 | 2 | 603, 621 | 339, 265[RCO(O)]$^+$ |
| e | PO (16:0/18:1) | 18.38 | 32 | 34 | 1 | 577 | 339, 313, 265[RCO(O)]$^+$, 239[RCO(P)]$^+$ |
| f | PP (16:0/16:0) | 19.38 | 32 | 32 | 0 | 551 | 313, 239[RCO(P)]$^+$ |
| g | SP (18:0/16:0) | 24.54 | 34 | 34 | 0 | 579 | 341, 313, 267[RCO(S)]$^+$, 239[RCO(P)]$^+$ |
| h | SS (18:0/18:0) | 30.94 | 36 | 36 | 0 | 607 | 341, 285, 267[RCO(S)]$^+$ |

$^a$ECN=equivalent carbon numbers, $^b$CN=carbon numbers, $^c$DB=number of double bonds

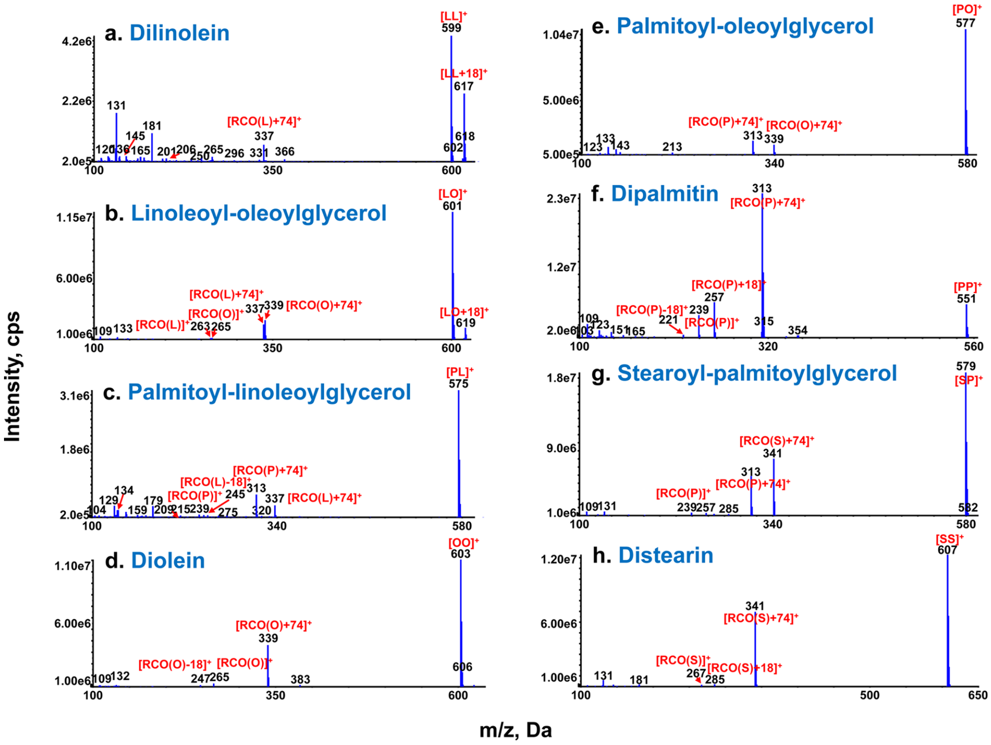

*Fig.* 5. LC-APCI-MS spectra of 8 DAGs observed in coix seed.

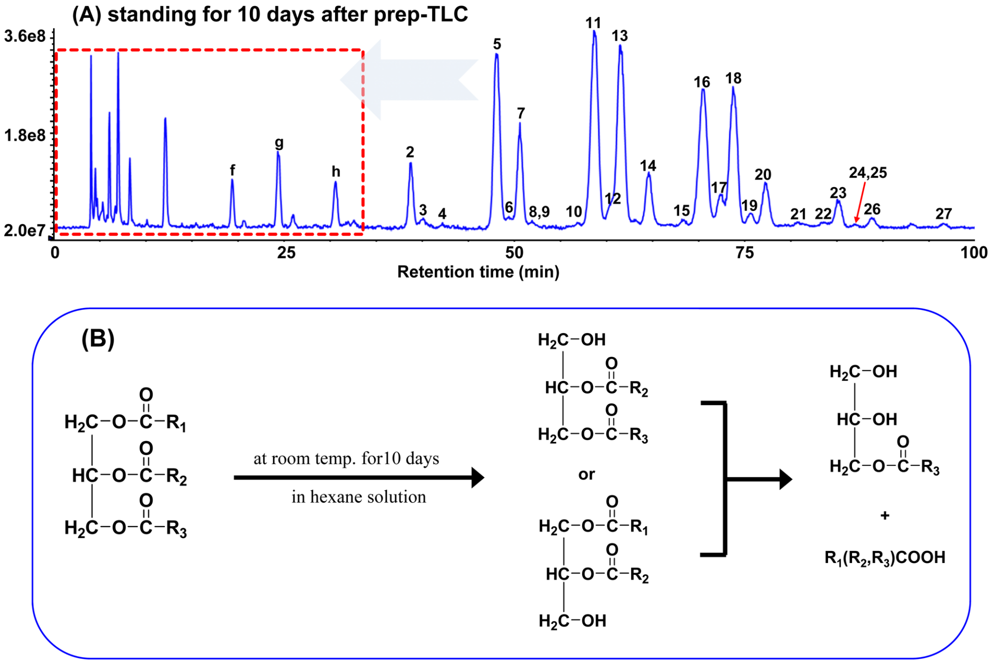

*Fig.* 6. (A) TICs of band 3 extract after standing for 10 days at room temperature and (B) degradation pathways of TAGs. Peak identities are the same as *Tables* 1-3.

peaks (f-h) were tentatively identified as PP, PS and SS on the basis of their APCI-MS spectra. Interestingly, these major components are the saturated DAGs with palmitic and stearic acid moiety. No unsaturated DAGs were observed. In future, the degradation phenomenon of TAG should be reflected in quality evaluation and nutritional property of coix seed.

## 4. Conclusions

A new analytical method based on LC-APCI-MS combined with prep-TLC was proposed for identification of TAGs and DAGs in coix seed. The feasibility of prep-TLC has been demonstrated for the rapid isolation of TAG and DGA from coix seed extract under developing solvent system. Individual bands were analyzed by LC-MS/MS and TAGs and DAGs were successfully characterized by LC retention behavior, MS spectral patterns and MS/MS spectral patterns. The MS/MS spectra of $[DAG]^+$ ions were useful to identify TAGs with isobaric fragment ions and even low amount. Among 27 TAGs and 8 DAGs identified by LC-APCI-MS/MS, 15 TAGs and 8 DAGs were observed for the first time in coix seed. In future, the developed prep-TLC system can be useful tool for isolation of lipids from plants and other oils. Also, the established method can be used for chemical profiling and quality control of TAGs and DAGs in coix seed and other related plant.

## Acknowledgements

This study was financially supported by the Ministry of Food and Drug Safety of Korea (2011, 0012671) and the National Research Foundation of Korea (No. 2017R1A2B4005616).

## References

1. C. C. Kuo, , W. Chiang, G. P. Liu, Y. L. Chien, J. Y. Chang, C. K. Lee, J. M. Lo, S. L. Huang, M. C. Shih, and Y. H. Kuo, *J. Agric. Food Chem.*, **50**, 5850-5855 (2002).
2. D. W. Huang, Y. H. Kuo, F. Y. Lin, Y. L. Lin, and W. Chiang, *J. Agric. Food Chem.*, **57**, 2259-2266 (2009).
3. Tanimura, *Chem. Pharm. Bull.*, **9**, 47-53 (1961).
4. S. O. Kim, S. J. Yun, B. Jung, E. H. Lee, D. H. Hahm, I. Shim, and H. J. Lee, *Life Sci.*, **75**, 1391-1404 (2004).
5. F. Yu, J. Gao, Y. Zeng, and C. X. Liu, *J. Ethnopharmacol.*, **119**, 252-258 (2008).
6. M. J. Jiménez, L. Esteban, A. Robles, E. Hita, P. A. González, M. M. Muñío, and E. Molina, *Process Biochem.*, **45**, 407-414 (2010).
7. M. C. Michalski, C. Genot, C. Gayet, C. Lopez, F. Fine, F. Joffre, J. L. Vendeuvre, J. Bouvier, J. M. Chardigny, and K. Raynal-Ljutovac, Prog. *Lipid Res.*, **52**, 354-373 (2013).
8. S. D. Stamatov and J. Stawinski, *Org. Biomol. Chem.*, **5**, 3387-3800 (2007).
9. Z. M. Xiang, M. ZHU, B. L. Chen, and Y. Chen, *Chin. J. Chinese materia medica*, **30**, 1436-1438 (2005).
10. Fan Zhu, *Trends Food Sci. Tech.*, **61**, 160-175 (2017).
11. M. H. Lee, H. K. Park, and I. W. Kim, *Anal. Sci. Tech.*, **19**, 189-193 (2006).
12. M. Lisa, M. Holcapek, T. Rezanka, and N. Kabatova, *J. Chromatogr. A.,* **1146**, 67-77 (2007).
13. C. Ruiz-Samblás, L. Cuadros-Rodríguez, A. González-Casado, F. P. Rodríguez García, D. L. Mata-Espinosa, P. and J. M. Bosque-Sendra, *Anal. Bioanal. Chem.*, **399**, 2093-2103 (2011).
14. E. D. Dodds, M. R. McCoy, L. D. Rea, and J. M. Kennish, *Lipids* **40**, 419-428 (2005).
15. H. J. Sim, J. H. Kim, S. K. Lee, E. H. Kim, Y. H. Jin, E. K. Seo, and J. Hong, *Bull. Korean Chem. Soc.*, **36,** 1707-1709 (2015).
16. M. Fasciotto and A. D. Pereira Netto, *Talanta*, **81**, 1116-1125 (2010).
17. Q. Zhou, B. Gao, X. Zhang, Y. Xu, H. Shi, and L. Yu, *Food Chem.*, **143**, 199-204 (2014).
18. A. Jakab, K. Héberger, and E. Forgács, *J. Chromatogr. A*, **976**, 255-263 (2002).
19. A. Acheampong, N. Leveque, A. Tchapla, and S. Herson, *J. Chromatogr. A*, **1218**, 5087-5100 (2011).
20. M. Lísa and M. Holcãpek, *Anal. Chem.*, **85**, 1852-1859 (2013).
21. T. Rezanka, L. Nedbalová, and K. Sigler, *J. Chromatogr. A*, **1467**, 261-269 (2016).
22. H. C. Lu, P. L. Jiang, Light R. C. Hsu, C. L. Chyan, and Jason T. C. Tzen, *Biosci. Biotechnol. Biochem.*, **74**, 1841-1847 (2010).