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SEPARATION AND IDENTIFICATION OF FATTY ACID IN TRIACYLGLYCEROL ISOLATED FROM CALOPHYLLUMINOPHYLLUM OIL

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ABSTRACT

Nyamplung (Calophylluminophyllum) is one of the mangrove plants. Its seed has a significantly high non-edible oil content of 70.4%. Therefore, most researches were focused on the conversion of this oil into biodiesel. In this work, the proximate composition of C. inophyllum seed, and cis-trans fatty acids of triacylglycerols (TAG) in its oil were investigated. The identification method of double bond position in the corresponding cis and trans isomer was also presented. The position of double bond in fatty acid was determined by characteristic ions. Cis and trans configuration in double bond positions was identified by relative intensity of ions. TAG was obtained in high purity (99%) via batchwise multistage liquid extraction. Fatty acids of TAG identified include palmitic acid (C16:0, 11.37%), palmitoleic acid (C16:1c, 2.65%), margaric acid (C17:0, 0.68%), stearic acid (C18:0, 16.21%), elaidic acid (C18:1t, 0.41%), oleic acid (C18:1c, 32.49%), linoleaidic acid (C18:2t, 0.62%), linoleic acid (C18:2c, 22.99%), linolenic acid (C18:3c, 2.23%), arachidic (C20:0, 6.97%), gondoic acid (C20:1c, 1.74%), and behenic acid (C22:0, 1.64%). It was shown that the proposed method can easily distinguish the double bond position and the cis-trans configuration in oleic, linoleic and linolenic acid methyl esters.

Keywords: *C. inophyllum*, edible oils, fatty acids, liquid extraction, *triacylglycerols.*

INTRODUCTION

Indonesia has many tropical flora that can be used as healthy food and traditional treatment that benefits human health. One of the plants is the *Calophyllum* genus of *Clusiaceae* family. The *Calophyllum* genus is a tropical plant that consists of 180-200 different species that are famous for rich in a number of bioactive compounds [1]. One species of the *Calophyllum* genus is *Calopylluminophyllum Lin* (*C. inophyllum*). Indonesia has many species of *C. inophyllum* that spread over Sumatera, Java, Nusa Tenggara, Sulawesi and Bali [2].

Some parts of *C. inophyllum* plant have been proven to be beneficial to human health including sap for treating wound, bark for serving as antiseptic and disinfectant, root for treating wounds and coronary heart disease, and leaves for treating disease inflammation of eyes. *C. inophyllum* seed contains 40-73% crude oil [3]. Since *C. inophyllum* oil is considered as nonedible, most researchers focused on the conversion of this oil into biodiesel. However, Lee and Gibot[4] reported that this seed oil can be used as edible oil after refinement and detoxification.

According to Aparamarta *et al.* [5], crude *C. inophyllum* oil is dominated by 78.30% triacylglycerol (TAG), 5.35% diacylglycerol, and 8.51% free fatty acid (FFA). TAG is the largest content of oil in the *C. inophyllum* which is an ester with glycerol and three fatty acids. Crude *C. inophyllum* oil was dominated by C18:1 followed by C18:2, C18:0 and C16:0 [6-9]. Some fatty acids have benefit for human health. One of them is oleic acid (C18:1c). The other name for systematic name is cis-9-octadecenoic [3].This fatty acid has many benefits, such as protecting from free radicals and other oxidative stressors [10], reducing blood pressure while increasing good HDL cholesterol in women [11], and prevent cancer [12]. The isomer of oleic acid is vaccenic (11-Octadecenoic) and elaidic acids (9-octadecenoic) [13]. These fatty acids have trans configuration which may be harmful for human health, such as causing cardiovascular disease, cancer, obesity, and diabetes [14]. Some researchers reported C18:1 as oleic acid [3,6,15]. Aparamarta *et al* [16]reported that 5 fatty acids include C18:1 were identified in crude *C. Inophyllum* by GC-MS analysis. This work only uses qualitative analysis and identification of fatty acid based on the double bond position.The information about *cis-trans* isomers of fatty acids has never been reported.

Then, the objective of this work was to determine the proximate composition of *C. inophyllum* seed and to characterize the composition of double bond position and*cis-trans* fatty acids of the TAG in the seed oil by a simple and accurate method with qualitative and quantitative analyses. The fragmentation of fatty acid methyl ester in TAG was also presented.

MATERIALS AND METHODS

Materials

Crude *C. inophyllum* oil was obtained from Koperasi Jarak Tani Lestari (Central Java, Indonesia). Silica gel was purchased from Merck (New York, USA). Standard fatty acids, tripalmitin and triolein were purchased from Sigma Chemical Corporation (St. Louis, MO). Thin-layer chromatography (TLC) aluminium plates

(20 cm x 20 cm x 250 µm) were obtained from Merck (Darmstadt, Germany). All reagents and solvents were obtained from commercial sources.

Determination of composition of seed

Protein, fiber and ash contents of *C. inophyllum* seed were determined following the methods of AOAC [17]. The lipid content of *C. inophyllum* seed was determined as described by P. Shiu *et al.* [18]. The content of moisture was measured using a halogen moisture analyzer as defined by Gunawan *et al.* [19]. The content of mineral was measured using an inductively coupled plasma optical emission spectrometry (ICP-EOS) as defined by Gunawan *et al*. [20].

Separation of TAG from crude *C. inophyllum* oil

The separation of TAG from crude *C. inophyllum* oil was described by Aparamarta *et al.* [5]. Polar (methanol) and non polar (petroleum ether) solvents extraction were used in this study. The process of extraction was repeated 8 times. The petroleum ether fraction was defined as the non polar lipids fraction (NPLF). The methanol extract was collected and concentrated to give the polar lipids fraction (PLF). Both fractions were then analyzed using TLC and GC-MS.

Thin layer chromatography analysis

TLC was employed to qualitatively analyze the sample as defined by Gunawan *et al*. [19]. TLC paper that has been stained by the sample was immersed in a mobile phase of hexane: ethyl acetate: acetic acid at 90: 10: 1 (v/v/v).

Gas chromatography analysis (GC)

A Shimadzu GC-17A equipped with a split–split injector and a flame ionization detector (FID) was employed for analyzing TAG. Separations were carried out on a DB-5HT (15 m x 0.32 mm i.d.; Agilent Technologies, Palo Alto, CA) to quantify TAG, DAG, MAG and FFA. The temperatures of injector and detector were both fix at 370 °C. The temperature of column was initiated at 80 °C, increased to 365 °C at 15 °C/min, and maintained at 365 °C for 8 min. The nitrogen as the carrier gas with a linear velocity of 30 cm/s at 80 °C and split ratio 1:50 was used in this work. The sample was mixed in 1 mL ethyl-acetate, and 1 µL of this solution was taken and injected into the HT-GC instrument.

The second column was useda Rtx-2330 (30 m x 0.25 mm i.d., Supelco, Bellefonte, PA). This column was used to identify *cis* and *trans* fatty acid in TAG. The temperatures of detector and injector were set at 250 °C, the temperature of column was set at 180 °C and then increased to 245 °C at 1 °C/min and held for 10 min. Capillary head pressure, vent velocity and purge velocity were 150 kg/cm^2, 2–3 mL/min and 100 mL/min, respectively.

Gas chromatography mass Spectrofotometer analysis (GC-MS)

Identification of compounds in the sample was performed as defined by Gunawan *et al*. [19]. Separation of the compounds were carried out on an HP-5MS (5% -phenyl)-methylsiloxane non-polar column (30 m × 0.32 mm i.d. × 0.25 µm film thickness, Hewlett-Packard, Avondale, PA, USA) as brief described by Gunawan *et al.* [20].

RESULTS

The proximate and mineral composition of *C. inophyllum* seed is shown in Table-1and Table-2.

Table-1. Proximate composition of *C. inophyllum* seeds.[a],.

Constituents	Composition (wt.%)	
	This Work	Atabani and Cesar (2013)
Protein	3.37±0.54	NA[a]
Lipids	70.38±0.32	40-73
Fiber	12.86±0.24	NA
Ash	0.71±0.03	NA
Nitrogen	1.99±0.98	NA
Free Extract		

[a] Not Available

Table-2. Mineral contents of *C. inophyllum* seeds[a].

Minerals	Content, ppm
K	35.21±1.05
Ca	17.64±2.32
Fe	10.56±2.45
Mn	3.27±0.24
Cu	15.19±0.78
Zn	ND[b]
Mg	3.40±2.94
Na	3.97±1.65
P	3.33±2.13
Si	0.75±0.66
Al	0.16±0.15
S	ND

[a] Obtained from three independent experiments.
[b] Not detected.

It was found that dried *C. inophyllum* seed has high crude lipid content (70.38%). The lipid content of dried *C. inophyllum* seed reported in this workagrees with the previous work. Atabani and Cesar[3]reported that lipids content of *C. inophyllum* seed was 40-73%. The variability of oil content was attributed to *C. Inophyllum* cultivar, harvesting age, and diversity of agronomic factors. Among the minerals, potassium has the highest

and aluminum has the lowest contents. No report is available regarding the *C. inophyllum* seed proximate and mineral compositions.

After multistage stirred batch-wise solvent-solvent extraction, it was separated by 2 layers, namely NPLF and PLF. For PLF, the color is not changes. This result is not the same of NPLF. The color was change from dark green to bright yellow. The color change of NPLF may be caused by its increased TAG content. Aparamarta *et al.* [5] described that the content of TG in NPLF was increased from 78.3 to 98.53 wt%. However, the PLF color remained the same as crude oil. Aparamarta *et al* [20] reported that xanthone was detected in the crude oil which can be used for medical purposes such as anti HIV virus [21]. Xanthone probably is why *C. inophyllum* oil is toxic. In this work, TAG compound was successful concentrated in the NPLF with the potential of being used as edible oil.

It is a proved correlation between consumption of food that contains low-density lipoprotein (LDL) and *trans* fatty acids. High LDL level is associated with risk of cardio vascular disease. It causes death and becomes a trending focus in some parts of the world [22]. More consumption of *trans* fatty acids make a higher risk of disease like coronary heart disease (CHD), while *cis* isomers can decrease this disease [23]. Then, it is essential to know the composition of fatty acids and determine *trans* fatty acids content in *C. inophyllum* oil.

In this study, fatty acid in TAG's were developed into fatty acid methyl ester (FAME) to improve volatility of sample and symmetry of peak for providing more acurate data in gas chromatography. Identification of fatty acid composition was performed by GC as can be seen on Table-3.

Table-3. The composition of fatty acids.

Lipids	**Composition (wt.%)**		
	This work	**Atabani, 2013**	**Sahoo *et al*, 2007**
Saturated			
C16:0	11.37±0.96	14.7	12.01
C17:0	0.68±0.24	ND	ND
C18:0	16.21±1.49	ND	12.95
C20:0	6.97±0.29	0.8	ND
C22:0	1.64±0.60	ND	ND
Unsaturated Monoenes			
C16:1c	2.65±0.09	0.3	ND
C18:1t	0.41±0.06	ND	ND
C18:1	32.90±0.07	46.1	34.09
C18:1c	32.49±1.68	13.2	ND
Dienes			
C18:2	23.61±0.25	24.7	38.26
C18:2c	22.99±0.35	ND	ND
C18:2t	0.62±0.12	ND	ND
Trienes			
C18:3	2.23±0.09	0.2	0.3
C18:3c	2.23±0.09	ND	ND
C18:3t	ND	ND	ND

The result of Table-3 was agreed with previous report that palmitic acid (Cl6:0), stearic acid (Cl8:0), oleic acid (Cl8:lc), linoleic acid (Cl8:2c), and linolenic acid (Cl8:3c) were detected in the TAGs by GC-MS[16]. Crane *et al* [6] was detected palmitic acid, linolenic acid, linoleic acid, oleic acid, and stearic acid in the TAGs by high performance liquid chromatography (HPLC). However, no report was available regarding the double bond position in the corresponding *cis* and *trans* isomer.

To clarify the effectiveness of the gas chromatography in analyzing *cis* and *trans* isomers, mass spectrometry (MS) analysis is introduced. The *cis* and *trans* configuration of double bond in unsaturated fatty acid can be determined from the base peak of mass spectrometric characteristic ions of the corresponding

FAME as can be seen in Table-4. The difference between of GC and GC-MS result is the exits of *trans* configuration. The GC-MS has not the presence of trans configuration. It was GC-MS can be a failure to differentiate between *cis* and *trans* isomers causing misidentification of FAME [22].

The *cis* and *trans* configuration of double bond in unsaturated fatty acid can be determined from the base peak of mass spectrometric characteristic ions of the corresponding FAME. The ion at *m/z* 74 was the base ion peak of the C_6-C_{26} saturated FAME. It was due to the Mclafferty rearrangement and α-cleavage [24]. The ion at *m/z* 55, 67, and 79 were the base ion peak of the C_6-C_{26}polyunsaturated, biunsaturated and monounsaturated FAME's, respectively [25]. Moreover, Hejazi *et al* [26] reported that the configuration of central double bond at *m/z* 79 was *cis* and at *m/z* 95 was *trans*. The result of the fatty acid fraction analysis of triacylglycerols is summarized in Table-4.

Table-4. Gas chromatographic retention time and mass spectrometric characteristic ions of fatty acid methyl esters.

Peak no. (Retention time)	**Name** (Molecular weight)	**Characteristic ions *m/z*** (relative intensity)
1. (RT=9.88 min)	9-Hexadecenoic acid (Z) methyl ester, Palmitoleic acid methyl ester (MW= 268); C16:1	M^+ 268(10.81%), 236(40.54%), 208(21.62%), 194(32.43%), 166(8.11%), 152(32.43%), 137(18.92%), 123(27.03%), 110(37.84%), 96(67.57%), 83(64.86%), 74(83.78%), 69(78.38%), 55(100%)
2. (RT=10.01 min)	Hexadecanoic acid methyl ester, Palmitic acid methyl ester (MW= 270); C16:0	M^+ 270(22.86%), 239(14.29%), 227(28.57%), 213(2.86%), 199(8.57%), 185(11.43%), 171(8.57%), 157(2.86%), 143(28.57%), 129(11.43%), 115(2.86%), 101(11.43%), 87(77.14%), 83(8.57%), 74(100%), 69(11.43%), 55(17.14%)
3. (RT=10.66 min)	Heptadecanoic acid methyl ester, Margaric acid methyl ester (MW= 284); C17:0	M^+ 284(18.75%), 267(3.13%), 253(12.50%), 241(18.75%), 227(3.13%), 213(3.13%), 207(25.00%), 199(12.5%), 185(9.38%), 171(3.13%), 157(3.13%), 143(28.13%), 129(9.38%), 115(3.13%), 101(6.25%), 87(78.13%), 74(100%), 69(19.43%), 55(21.88%)
4. (RT=11.11 min)	9,12,15-Octadecatrienoic acid (Z,Z,Z) methyl ester, Linolenic acid methyl ester (MW= 292); C18:3c	M^+ 292(7.89%), 262(5.26%), 261(3.95%), 232(3.24%), 236(6.58%), 191(3.53%), 163(3.42%), 148(26.32%), 134(5.24%), 121(23.68%), 108(42.11%), 95(65.79%), 79(100%), 67(52.63%), 55(39.47%)
5. (RT=11.13 min)	9,12-Octadecadienoic acid (Z,Z) methyl ester, Linoleic acid methyl ester (MW= 294); C18:2c	M^+ 294(27.50%), 263(22.50%), 262(3.14%), 235(2.50%), 220(7.50%), 195(2.50%), 178(7.50%), 164(12.50%), 150(20.00%), 136(5.00%), 123(20.00%), 109(40.00%), 95(80.00%), 81(95%), 67(100%), 55(52.50%)
6. (RT=11.17 min)	9-Octadecenoic acid (Z) methyl ester, Oleic acid methyl ester (MW= 296); C18:1c	M^+ 296(17.50%), 265(4%), 264(85.00%), 237(10.00%), 222(42.50%), 199(2.50%), 180(30.00%), 166(12.50%), 152(15.00%), 138(20.03%), 125(27.5%), 111(37.50%), 97(70.00%), 83(72.50%), 69(77.50%), 55(100%)
7. (RT=11.31 min)	Octadecanoic acid methyl ester, Stearic acid methyl ester (MW= 298); C18:0	M^+ 298(27.78%), 267(13.89%), 255(27.78%), 227(2.78%), 213(2.78%), 199(16.67%), 185(5.56%), 171(2.78%), 157(2.78%), 143(30.56%), 129(8.33%), 115(2.78%), 101(5.56%), 97(8.33%), 87(8.33%), 74(100%), 55(19.12%)
8. (RT=12.65 min)	11-Eicosenoic acid (Z) methyl ester, Gondoic acid methyl ester (MW= 324); C20:1c	M^+ 324(5.26%), 292(34.21%), 281(47.37%), 263(5.26%), 250(13.16%), 207(57.89%), 191(13.16%), 179(7.89%), 165(7.89%), 141(13.16%), 123(15.79%), 111(18.42%), 97(42.11%), 87(44.74%), 74(42.11%), 69(50.00%), 55(100%)
9. (RT=12.85 min)	Eicosanoic acid methyl ester, Arachidic acid methyl ester (MW= 326); C20:0	M^+ 326(29.73%), 295(2.70%), 283(21.62%), 269(2.70%), 255(2.70%), 241(5.41%), 227(8.11%), 213(2.71%), 207(10.81%), 199(10.81%), 185(8.11%), 171(2.71%), 163(1.35%), 157(2.70%), 143(27.03%), 129(10.81%), 115(2.70%), 97(10.81%), 87(10.81%), 74(100%), 69(18.92%), 55(25.42%)
10. (RT=15.12 min)	Docosanoic acid methyl ester, Behenic acid methyl ester (MW= 354): C22:0	M^+ 354(33.33%), 323((7.14%), 311(23.81%), 297(2.38%), 283(40.48%), 269(6%), 255(11.90%), 241(2.38%), 213(2.38%), 207(85.71%), 199(11.90%), 185(5.13%), 171(2.38%), 143(30.95%), 129(9.52%), 115(7.14%), 101(8.11%), 97(14.29%), 87(78.57%), 74(100%), 55(28.57%)

Typical GC-MS fragmentation of saturated fatty acid methyl esters (margaric ester) is shown in Figure-1. Peaks 2, 3, 7, 9 and 10 were identified as unsaturated fatty acid (palmitic, margaric, stearic, arachidic, and behenic acid methyl esters, respectively). Their GC-MS fragmentation were confirmed by M^+, $(M^+ - C_2H_5)$, $(M^+ - C_3H_7)$, $(M^+ - C_4H_9)$, $(M^+ - C_5H_{11})$, $(M^+ - C_6H_{13})$, $(M^+ - C_7H_{15})$, $(M^+ - C_8H_{17})$, $(M^+ - C_9H_{19})$, $(M^+ - C_{10}H_{21})$, $(M^+ - C_{11}H_{23})$ and *m/z* 79, which was the base peak ion of the C_6–C_{26} saturated FAME.

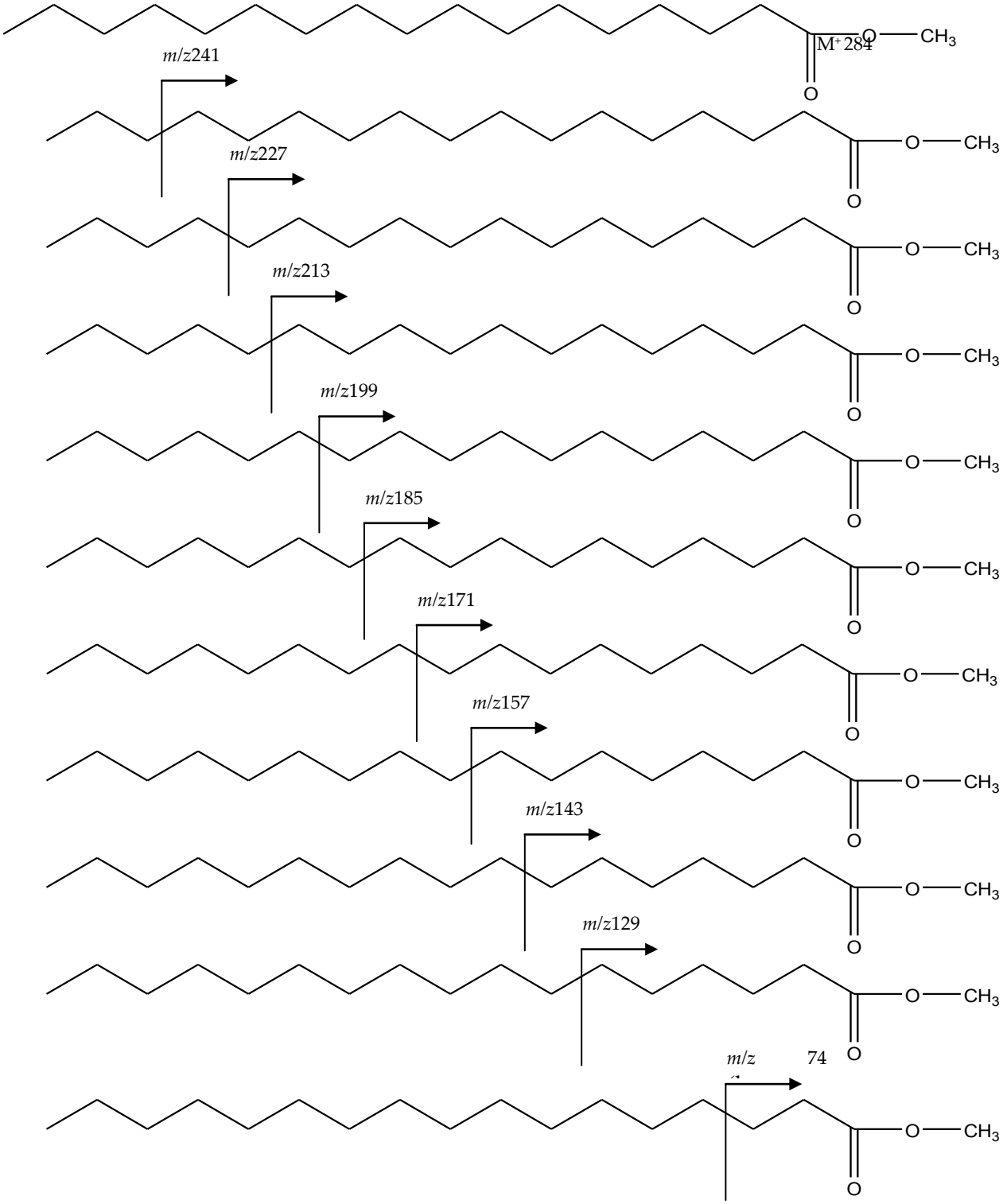

Figure-1. Cleavages leading to major fragment ions in margaric acid methyl esters (C17:0).

The fragmentation of GC–MS in peak 4 (RT=11.11 min) was indicated a molecular ion (M^+) at 292 (relative intensity: 7.89%). Its fragmentation mechanism is defined as follows: after the i-induced cleavage and γ-

hydrogen atom transfer, *m/z* 260 ($M^+ - CH_3OH$) was produced; *m/z* 261 ($M^+ - OCH_3$) was the result of the *α*-cleavage of carbonyl after the *α*-cleavage of carbonyl; ion $C_6H_7^+$, *m/z* 79, was the result of double-bond transfer and the cleavage of *α*. Other ions also were identified at *m/z* 55, 67, 95, 109, 121, 134, 148,162, 191, 232, 236, 261 and 292 (Figure-2). The characteristic ions of polyunsaturated fatty acids ester were *m/z* 67, $[M-31]^+$, and molecular ion[25]. A peak at *m/z* 108 (ω ion) was characteristic for polyunsaturated fatty acid methyl ester with n-3 terminal group [26]. From this m/z, it can be identified that double bonds in Δ12 and Δ15. Moreover, polyunsaturated fatty acid methyl ester with double bonds in Δ9, 12 position was identified at 236 (α ion) [27].Hejazi *et al*[26] reported that a mass spectrum with a base peak at m/z 79 means that the central of double-bond (Δ12) is *cis* configuration; on the other hand, a base peak at m/z 95 indicated that the central of double-bond is *trans* configuration. In this study, the significant reductions of ions were proposed for the determination of double bond position in the corresponding *cis* and *trans* isomer. The other double bond positions in Δ9 and Δ15 were *cis* configuration based on relative intensity of *m/z* 236 and 108 are higher than 2% and 40%, respectively. Our proposed method based on data that was provided by Hejazi *et al.* [26]. It was said that Δ9 and Δ15 were *cis* configuration based on relative intensity of *m/z* 236 and 108 are 6.7% and 60%, respectively. This result agrees with another literature that the summation relative intensity of *m/z* 95 and 67 are below than 150%. This proposed method based on previous worked by Mjos and Pettersen[27]. It was reported that the cis configuration at Δ15 was obtained based on relative intensity of *m/z* 95 and 67 are 58% and 58%. The double bond positions Δ9, Δ12 and Δ15 were cis configuration based on the base peak at *m/z* 79 and characteristic ions at *m/z* 261 and 292 [25].

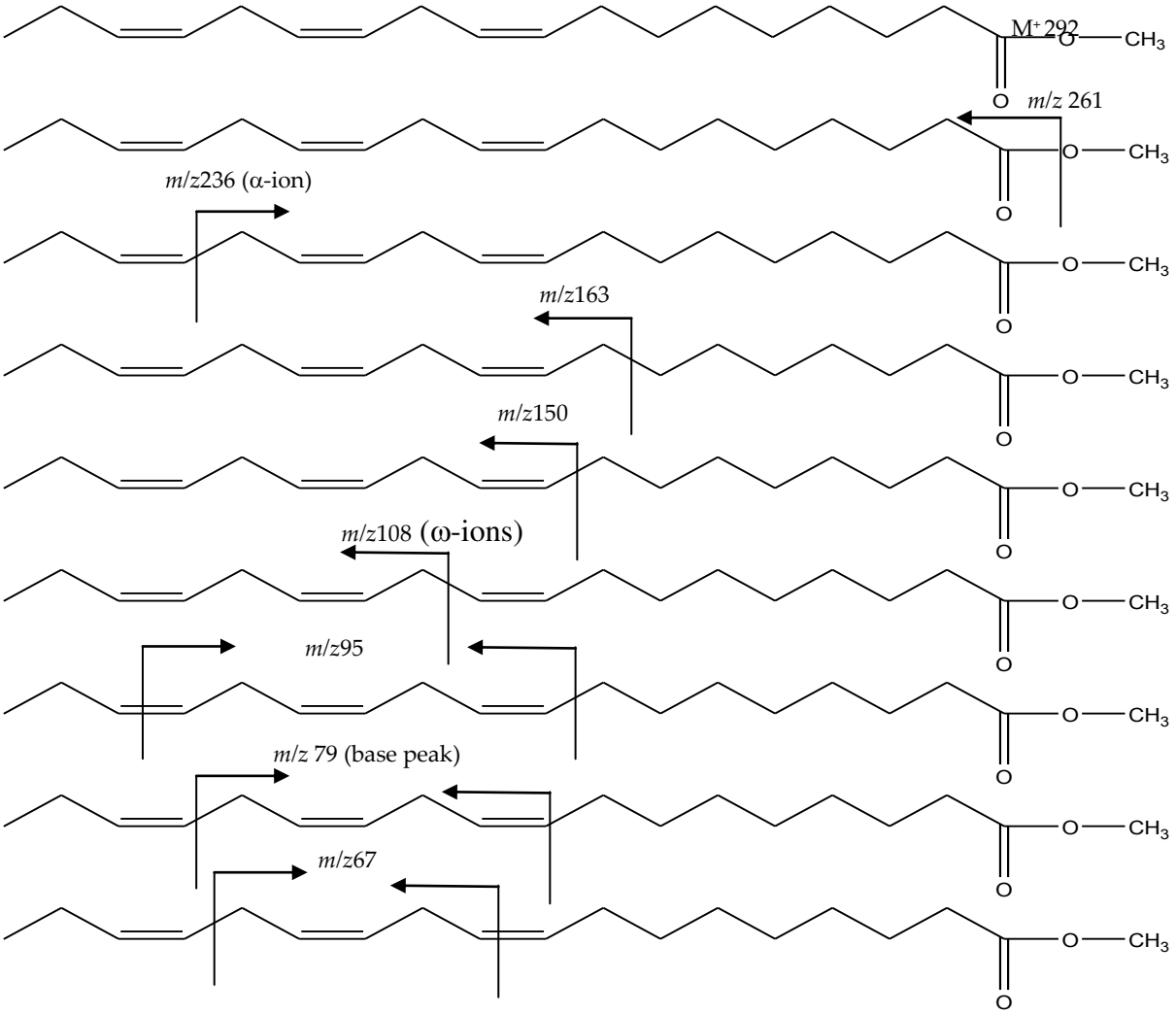

Figure-2. Cleavages leading to major fragment ions in linolenic acid methyl esters (C18:3c).

As the result, peak 4 was recognized as linolenic acid methyl ester (9,12,15-octadecatrienoic acid (Z,Z,Z) methyl ester).

For peak 5 with RT= 11.13 min (linoleic acid methyl ester), the mass spectrum is shown in Table-4. Its fragmentation mechanism is defined by the molecular ion

(M^+) at *m/z* 294 and the γ-hydrogen atom transfer and i-induced cleavage ($M^+ - CH_3OH$) at *m/z* 262. Moreover, the peak at *m/z* 263 ($M^+ - OCH_3$) was the result of the *α*-cleavage of carbonyl. The base peak at *m/z* 67, ($C_5H_7^+$), was due to the cleavage of *α*and the result of double bond transfer. Peaks also were observed at *m/z* 55, 81, 95, 110, 123, 136, 150, 164, 178, 195, 220, and 235 (Figure-3). The determination of double bond positions in Δ9 and Δ12 was identified at *m/z* 235 (α-ion) [27]. The other double positions in Δ9, andΔ12 were identified at m/z 150 (ω-ion) [26]. The double bond positions in Δ9 was *cis* isomer because relative intensity at m/z 235 is higher than 2%. This proposed method was based on data provided by Hejazi *et al.* [26]. It was reported that relative intensity of m/z 235 was 5.3%. Qin *et al* [25] was reported that the double bond positions Δ9, and Δ12 were cis configuration based on the base peak at *m/z* 67 and characteristic ions at *m/z* 263 and 294. It was confirmed that this peak is 9, 12 - Octadecadienoic acid methyl ester (linoleic acid methyl ester).

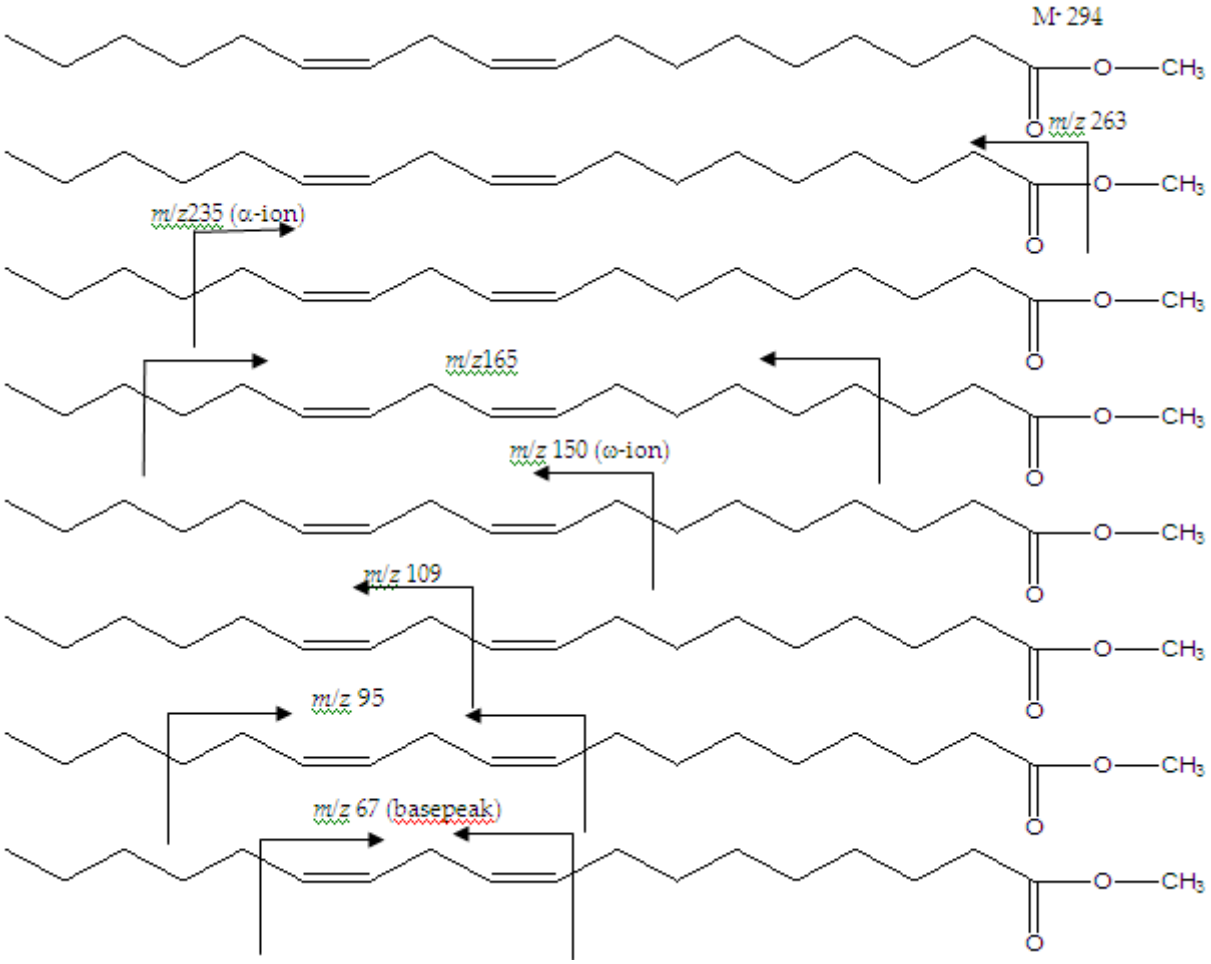

Figure-3. Cleavages leading to major fragment ions in linoleic acid methyl esters (C18:2c).

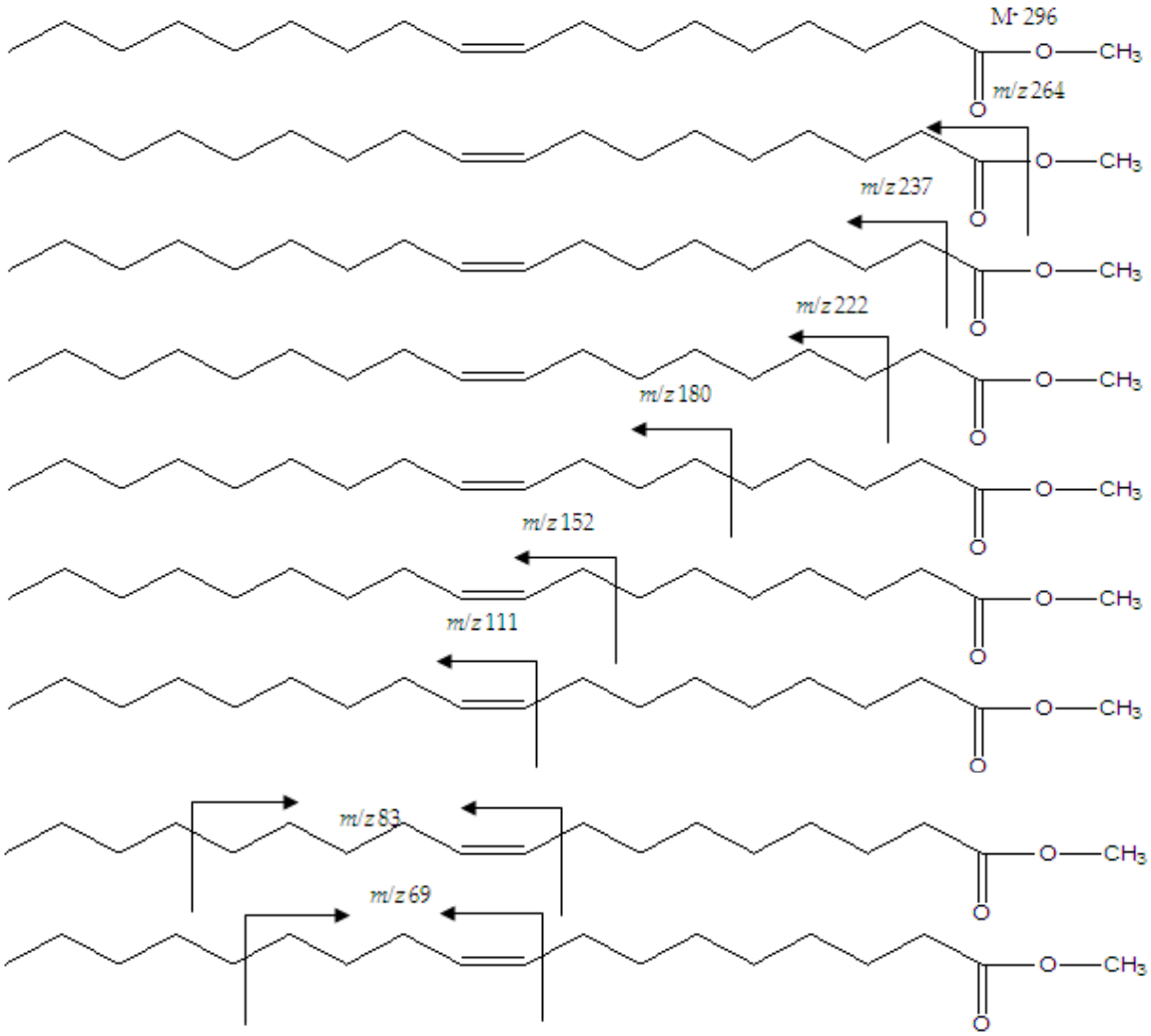

Figure-4. Cleavages leading to major fragment ions in oleic acid methyl esters (C18:1c).

Figure-4 shows the typical GC-MS fragmentation of monounsaturated (oleic) fatty acid methyl esters. Its fragmentation mechanism was identified by the molecular ion (M^+). The ion at *m/z* 264 (M^+-OCH_3) was the result of the α-cleavage of carbonyl. After the i-induced cleavage and γ-hydrogen atom transfer, ion at $M^+ - CH_3OH$ was identified. The ion at *m/z* 55 was the base peak ion of C_6–C_{26}monosaturated FAME [25, 28]. The determination of double bond positions in Δ9 was identified at *m/z* 111. Based on relative intensity higher than 2%, it can be identified as *cis* configuration. This proposed method agrees with the other literature. Qin *et al*. [25] said that the double bond positions Δ9 was cis configuration based on the base peak at *m/z* 55 and characteristic ions at *m/z* 264 and 296. Therefore, peaks 1, 6, and 8 were identified as palmitoleic, oleic, and gondoic acids methyl esters, respectively. These results agree with previous report that 9- oleic acid (C18:1 (9)) and 11- cis- vaccinic acid (C18:1 (11)) were detected by Gas Chromatography in the Sauromatum appendix [30].

CONCLUSIONS

Twelve fatty acids were identified in the TAGs. *Trans* fatty acids of TAGs were detected in this study with amount lower than 1%. The present work clearly demonstrated that *C. inophyllum* oil is potentially became healthy oil because it contains very little *trans*-isomers

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