ENRICHMENT OF α-(ALPHA) LINOLENIC ACID OF BASIL SEED OIL, *Ocinum Basillium* L. BY FRACTIONAL CRYSTALIZATION AND CRYSTALIZATION IN UREA INCLUSION COMPLEXES

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ABSTRACT

Enrichment of α-(alpha) linolenic acid (ALA) of basil seed oil, Ocinum basilicum L. can be done by fractional crystalization and crystalization of fatty acid in urea inclusion complexes (UIC) methods. In this research, the ALA of fatty acid of basil seed oil was fractionated by fractional crystallization in methanol solution at -3, -13 and -25 °C and by crystallization in urea solution (ratio 1:2) at 4, 2, -6 and -8 °C. The ALA percentages were analyzed by GC and GC/MS. The results showed that percentage of ALA obtained from fractional crystallization at -25 °C increase from 65.16 to 91.40, and acquired from UIC is 98.8 at 2 °C

Keywords: *ALA, fractional crystallization, urea inclusion complexes and basil seed oil (Ocinum basilicum L.)*

INTRODUCTION

Generally, fatty acid of plant seed oils is classified as saturated and unsaturated fatty acid. Unsaturated fatty acids content, such as oleic, linoleic, includes arachidonic, eicosapentaenoic acids always higher than saturated fatty acid, except in coconut oil. In soybean oil, unsaturated fatty acid attained >90% [1], sown variety of *Safflower* oil >94% [2]. Among some unsaturated fatty acid, conjugated linolenic acid in tung seed oil, catalpa seed oil, pomegranate seed oil, bitter gourd oil, marigold seed oil and karela seed oil, are present in large quantities and can account for 31–80% [3-4]. Meanwhile, the variety of basil seed oil contains of ALA range from 43.8–64.8% [5].

Isolation and purification methods used to obtain fractions rich in unsaturated fatty acid from plant seed oil are commonly based on differences in the polarity and/or spatial configuration of fatty acids present in the extract. These differences are mostly associated with the number of double bonds in the carbon chain and hence, fatty acids can be separated according to their degree of unsaturated [6]. The carbon chain will be determined to hydrophob phase**,** whereas unsaturated determined by molecule geometrics of fatty acid, with the result every fatty acid has temperature transition or melting point in different temperature. Thus a simple fatty acid separation can be done by controlling crystallization temperature (crystallization method).

ALA conjugated can be isolated from oil seed in methanol solution of pomegranate and tung seed oil by fractional crystallization at 0-4 °C for 8 h [3]. They also conducted crystallization of flaxseed oil in methanol solution, at t 4 and -18 °C. The process was carried out twice until the ALA mixture reached 70% and 80% purity. The enrichment of γ-linolenic acid (GLA) were employed from *Borago officinalis* and *Echium fastuosum* seed oil and free fatty acids (FFAs) [7]. Different solution of seed oils and FFAs from these two oils at 10, 20 and 40% (w/w) were crystallized at 4, -24, and -70 °C, respectively using hexane, acetone, diethyl ether, isobutanol and ethanol as solvent. Best results for *B. officionalis* and *E. fastuosum* FFAs in hexane, reaching highest concentration of GLA 58.8% and 39.9% respectively.

Conventional crystallization method have restrictiveness to produce appropriate fatty acid purity, so recently researcher used urea inclusion complex method, UIC and combined with chromatography method (TLC, column or HPLC) which impregnating with silver-ion [6]. Application of UIC method suggested simplicity, ease of scaling, effective, efficient and ecological friendliness [8].

The selectivity urea to form inclusion complexes with long chain organic compounds based on factors:

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(a) carbon chain length, (b) presence of unsaturated in molecule, (c) degree of unsaturated. The formation of these complexes was found to be use a useful technique for separation of mixture of saturated and unsaturated organic compounds, e.g. fractionation of mixture of FFAs [1], whereas the distribution of urea between the crystalline phase and the solvent was not significantly affected by FFAs composition of oils nor the overall ratio of FFAs to urea [8].

UIC methode was used to purify docosahexanoic acid (DHA) from the marine heterotropic microalga *Crypthecodinium conii* CCMP 316 [9]. The highest of DHA fraction (99.2%) produced from urea to fatty acid ratio (w/w) (3.5) at crystallization temperature of 4 and 8 °C. Wu et al. [10] were optimizing conditions UIC for separation and purification linoleic acid from sunflower oils. They were obtained optimal condition at urea to fatty acid ratio (w/w) (0.94), 95% ethanol to urea (v/w) (5.00), a crystallization temperature of 18 °C for 5 h with the result 87.8% purity and 83.4% recovery. Urea solution in methanol 35% can be used to partial separated and removed of FFAs of sterols monoasilgliserol and other components from tocotrienols [11]. UIC from 30 organic acids and test their activity by *in vitro* as antibacterial and antifungal was synthesized [12]. The UIC of capric acid, pamoac acid and 3-hydoxybenzoic acid found to be most active ones. The combination of IUC-chromatography impregnated by silver-ion was used to separate GLA methyl ester with the result purity >84% [13], whereas the twice application of UIC to enrichment GLA contents in lipid extract *Mucor zychae* MTCC 5420 has successfully performed [14]. GLA percentage increased from 8.7% to 63.5% (first UIC) and a 92.7% (second UIC).

The potentially of ALA as a basic material of insect sex pheromones, such as *Manduca sexta* L. (Tobacco leaf eaters) and *Mulberry Pyralid, Glyphodes* pyloalis Walker (murbey leaf eaters), which requires a high level of purity, so it is interesting to study the fractional crystallization and UIC methods for ALA enrichment in *O. basilicum* L. seed oil.

EXPERIMENTAL SECTION

Materials

Chemical that used in this research from E merck includes: n-hexane, methanol, urea, KOH, H_2SO_4, HCl, $NaHCO_3$, NaCl, and MgSO4 anhydrous. N_2 gas from PT Tirta Austenite Tbk. and dry ice product of PT. Petrokimia, sample of basil seeds from Materia Medica, Batu Malang.

Instrumentation

Soxhlet Extractor, rotary evaporator, freezer box, magnetic stirrer, Buchner funnel, reflux reactor, gas chromatography (GC) instrument: HP 5890 Series II, column (HP 5, L 30 m), column temperature [150 °C (2 min) -300 °C (2 min), rate 10 °C/min], injector and detector temperature at 300 °C, FID detector for ALA analysis from crystallization method and column (HP 1, L 10 m), column temperature [100 °C (5 min) -250 °C (5 min), rate 10 °C/min], injector temperature and detector (250 °C), FID detector for ALA analysis from crystallization method IUC, gas chromatography-spectrometry mass (GC-MS) instrument: Shimadzu GCMS-QP 2010S, column (Rtx-5 MS, L. 30 m, ID 0.25 mm), He carrier gas and El ion sources.

Procedure

Isolation of basil seeds oil

Isolation of basil seeds oil was conducted in Soxhlet extractor, wherein 100 g blended basil seeds put in Soxhlet extractor and than extracted with 300 mL n-hexane for 9 times circulation. The solvent were removed from the extract using rotary evaporator and completely removal under gentle stream of N_2 gas. The oil was storage then to dark bottle and keeps it at refrigerator.

Hydrolysis of basil seeds oil

50 g of basil seeds oil, put into round flask, and add 100 mL methanol. A volume 100 mL of 11% KOH solution added and stirs up at temperature 60 °C until clear solution (90 min approach). The result of reaction move on separation funnel, added 250 mL of distilled water and 62.5 mL of n-hexane, and then shakes it. Take water phase and added 1 M H_2SO_4 until pH 1, and organic phase separated. The organic phase containing FFAs was washed 3 times with the same volume of distilled water and dried by added $MgSO_4$ anhydrous.

Isolation ALA from basil seeds oil

Fractional crystallization. 52.4 g FFAs from basil seed oil was dissolved into 185 mL methanol warmed at 50 °C in a flask. After flushing with nitrogen, the flask was placed into around dry ice in Styrofoam box (17 x 17 x 15 cm) at 3, -13 and -25 °C respectively. Set of temperature used dry ice packed with newspaper. Crystals filtered by Buchner funnel which packed with Styrofoam, filled dry ice wrapped up with newspaper. The temperature of Buchner funnel keep at 3 °C, through removed or added a portion of dry ice. The crystals removed and filtrate continued at -13 and -25 °C to obtain linolenic acid. The crystallization

process was repeated 3 times until ALA fraction reached highly percentage.

Urea inclusion complexes (UIC). 25 g of FFAs from basil seed oil mixed with a hot solution of urea in methanol (50 g urea/200 mL methanol) at round flask. After cooled until room temperature followed by streaming of N_2 gas in round flask and than closed. The round flask put on freezer box at 6 °C for overnight. Formed crystal filtered with Buchner funnel. Filtrate stored and crystal washed 3 times with 25 mL cold n-hexane. This liquid combined with filtrate, and then put on separation funnel, added 150 mL distilled water and 10 mL of 6 M HCl. Then it extracted two times with 80 mL n-hexane. The same process was freeze for mixture FFAs in methanol at 2, -6, and -10 °C.

Analysis of ALA. 1 g of ALA put on round flask, added 0.5 mL methanol and 0.1 mL sulfuric acid. The mixture was refluxed for 1 h at 80 °C. The reflux liquid removed to separation funnel. The organic phase neutralized with saturated $NaHCO_3$ until pH 7 and than washed 3 times with 25 mL distillated water. Following salting out process with added saturated NaCl several drop until the mixture clearly separated. The organic phase separated and than dried by added $MgSO_4$ anhydrous. The same procedure used to esterification for FFAs resulted from hydrolysis and trans-esterification basil seed oil as product comparation. Furthermore, ALA methyl esters were analyzed with GC and GC-MS.

RESULT AND DISCUSSION

The composition of basil seeds oil

Isolation of basil seed oil *O.basilicum* L by extraction method using n-hexane solvent for 9 times circulation, with the result rendemen basil oil average 15.8% to dry seeds. The refractive index (t = 25 °C) and specific gravity of oil 1.470 and 0.858 respectively. Percentage of hydrolysis of seed oil to FFAs achieved 92.03%.

Analysis result with GC (Fig. 1a) compilated with total ionic chromatogram (TIC) and mass spectra from each peak**.** The composition fatty acid of basil seed oil can be showed in Table 1.

Fractional and UIC crystallization of ALA

Fractional crystallization method for acquiring ALA with higher percentage based on differential frozen point between saturated and unsaturated fatty acid component of basil seed oil. Saturated fatty acid as lauric, palmitic and stearic had carbon chain which well-arranged, so when the temperature set at 3 °C, most of the components will be frozen. On the contrary, oleic and linoleic acid which have one and two carbon unsaturated.

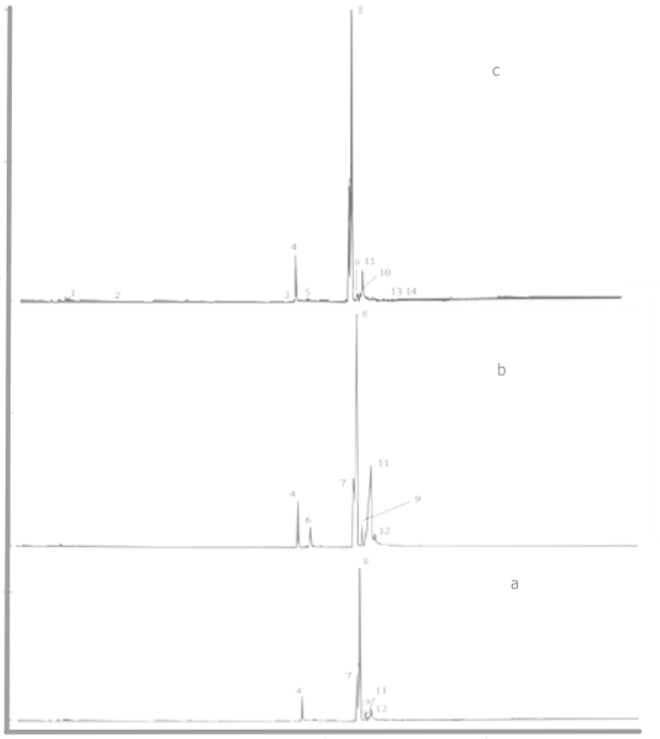

Fig 1. Chromatogram GC of methyl ALA esters
(a) basil seed oil
(b) crystallization at -13 °C
(c) crystallization at -25 °C

Table 1. The composition fatty acid of basil seeds oil

Tipe of Fatty Acid	Percent
Palmitic	4.87
Stearic	3.67
Lauric	1.21
Oleic	6.89
Linoleic	18.18
α-Linolenic (ALA)	65.16

The unsaturated carbon skeleton formed fold, so their molecule uncoordinated and the result were obtained is liquid phase. Furthermore, when the enviroment was set at -13 °C, component of oleic and linoleic mostly frozen and remained filtrate contain of fully ALA, it caused unsaturated fatty acid-carbon skeleton higher than another components. Furthermore, when temperature FFAs solution set at -25 °C found that all remain sampel frozen. This step can not find filtrate. It showed that FFAs contained another component which has unsaturated-carbon skeleton much more than ALA.

The increasing of percentage of ALA obtained from fractional crystallization of basil seed oil was found by comparing with the chromatogram of GC (Fig. 1b and 1c to Fig. 1a). ALA content in crystal obtained at freezing temperature -25 °C reach 91.4% or percentage of ALA increased 26.24%. But ALA reserved temperature, critical temperature at -18 °C [3],

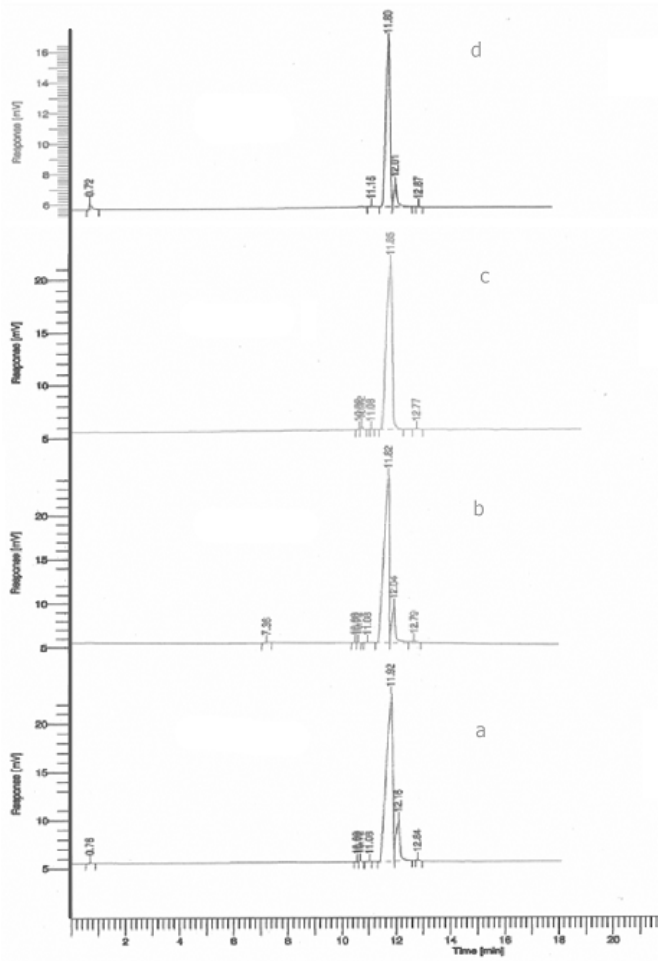

Fig 2. Chromatogram GC of methyl ALA esters of UIC method at a. -8 °C, b. -6 °C ,c. 2 °C and d. 4 °C

when cooled at -13 °C acquiring lower percentage (45.37%) compared with ALA of basil seed oil (65.10%). It means ALA molecules tend to long distance and weak molecule interaction, so tend to show liquid form or as filtrate, just the opposite under critical temperature, ALA molecules tend to close distance, so tend to interaction each other and latest freezing or as crystalline. It was found ALA component at crystallization above critical temperature or saturated and unsaturated fatty acid at crystallization under critical temperature. This showed between saturated and unsaturated fatty acid always interact each other or molecule of fatty acid trap each other when reserved process.

ALA isolation by UIC crystallization method, this research based on the differential ability of saturated and unsaturated fatty acid to interact with urea molecule. On urea solution, fatty acids formed IUC caused (1) the carboxyl group of fatty acid can provide a site for hydrogen bonding and (2) the crystal structure of organic hydrogencarboxylate can incorporate a highly ordered. Infinite layer of hydrogencarboxylate anion linked together by relatively short O-H…H interactions and this structure is able to organize the corresponding cation in an accentric layered or one dimensional frame work. The fatty acid with unsaturated carbon-chain skeleton which well-arranged may not influence cohesively of complex structure which formed between urea and fatty acid. So the fatty acid of this group tend to free, not formed complex and will be as filtrate. The ALA which have highest degree of unsaturated carbon mostly form in filtrate.

Based on chromatogram GC (Fig. 2), the relative percentage of ALA in filtrate at separation using IUC method, found that all variation of temperature crystallization acquiring average above 85% and percentage of highest ALA 98.80%, for crystallization at 2 °C. The result showed that on urea solution, saturated fatty acid molecules have strong interaction and easy to fill channel formed by urea molecule in solution. On the contrary, unsaturated fatty acids have carbon chain convoluted at geometric skeleton were difficult to fill channel, so it tend to form filtrate. The UIC method of urea-fatty acid complex can be done at higher temperature with the result raised higher average relatively percentage and rendemen of ALA. UIC method is more efficient and effective than fractional crystallization method.

CONCLUSION

Fractional crystallization and IUC crystallization methods can be used to increase the ALA percentage of basil seed oil. The percentage of ALA resulted from fractional crystallization at -25 °C increased from 65.16 to 91.40%, and acquired from UIC (ratio urea to fatty acid 1:2) is 98.8% at 2 °C. UIC ALA crystallization method is technically more practical and efficient than fractional crystallization method.

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