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ARTICLE

High-yield methods for purification of α-linolenic acid from *Perilla frutescens* var. *japonica* oil

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Abstract This study was conducted to investigate the purification methods for high-yield of α-linolenic acid (ALA) from *Perilla frutescens* var. *japonica* oil (PO). PO was treated with 2 g of urea, cooled for 24 h, and 81.75 % of ALA was obtained after gas chromatography–flame ionization detector analysis. Urea complexation at low temperature (refrigeration) is a prospective method for the extraction of unsaturated fatty acids in high yields. Our results suggest that urea treatment with cooling at 10 °C could be used for purification of ALA from PO. It will be enhanced for the development of nutritional, medical, and cosmetic value.

Keywords GC-FID · Linolenic acid · *Perilla frutescens* var. *japonica* · Urea

Introduction

Perilla frutescens is a member of the mint family (Lamiaceae) and an annual herbaceous plant. It is widely cultivated for use as sushi garnish, food supplements as well as in salad, pickles, and oil in China, Japan, and Korea (Nitta et al. 2003). The native origins of this plant are the terrains of India and China (Turchini et al. 2010) and in Korea, this plant is commonly known as "Dlggae." Its leaves and stems are green, with no wrinkles, and it is taller than other *Perilla* species, with a unique flavor (Waldeck et al. 2010). Previous phytochemical investigations of *P. frutescens* involved the isolation of phenolic compounds, including caffeic acid–3-*O*-glucoside, rosmarinic acid–3-*O*-glucoside, rosmarinic acid, luteolin, and apigenin (Ha et al. 2013). *P. frutescens* is known to have anti-allergic, anti-inflammatory, and anti-oxidant activities. It has also been utilized as a therapeutic food for its anti-obesity properties and has shown favorable appropriate activities against human culture cells, such as HL-60, MCF-7, and Hep-G2 (Makino et al. 2003; Akihisa et al. 2006; Kim and Kim 2009; Hong et al. 2010; Ueda et al. 2002).

The oil of *P. frutescens* is consumed principally in India and East Asia and is used for cooking and in traditional therapy (Kurowska et al. 2003); it is also used as a traditional treatment modality for lung disease in China. This oil is rich in polyunsaturated fatty acid; however, the leaves are a poor source of oil with a yield of only 0.2 %. The seed oil contains omega-3 fatty acids, α-linolenic acid (ALA) and omega-6 fatty acids, γ-linolenic acid. In comparison to other plant oils, *P. frutescens* oil contains high levels of linolenic acid, oleic acid, and linoleic acid (Asif 2011). ALA, an essential fatty acid, has a long-chain structure and is a precursor of eicosapentaenoic acid and docosahexaenoic acid (Sinclair et al. 2002). ALA is abundant in vegetable oils, such as soy, canola, and walnut oils. In previous studies, ALA was used as a nutraceutical and pharmaceutical supplement (Kim et al. 2014) and found to have positive effects by having anti-inflammatory (Ren and Chung 2007), anti-thrombotic (Yang et al. 2014), anti-tumor (Numata 1995), improving the bone health

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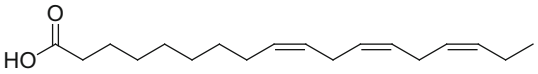

Fig. 1 The structure of ALA

(Kim and Ilich 2011), and preventing brain damage and stroke (Nguemeni et al. 2013). *P. frutescens* contains approximately 60 % ALA (Turchini et al. 2010).

This study was conducted to investigate high-yield methods for the purification of ALA (Fig. 1) from *P. frutescens* var. *japonica* oil (PO) by gas chromatography–flame ionization detector (GC-FID).

Materials and methods

Plant materials

Perilla frutescens var. *japonica* (PF) was supported from the National Institute of Crop Science, Rural Development Administration, Miryang, Republic of Korea (2014). Voucher specimens of PF were deposited at the Herbarium of Department of Integrative Plant Science, Chung-Ang University, Korea.

Instruments and reagents

A medium pressure liquid chromatography (MPLC) was performed using a Biotage Isolera-one using a Biotage snap cartridge (KP-Sil; 100 g, Biotage, Sweden). Samples were analyzed using Agilent 7890A GC (Agilent, USA) equipped with a FID and DB-Wax column (Agilent, 30 mm × 0.25 mm × 0.25 μm). Reagents and solvents, including potassium hydroxide (KOH) and urea, were purchased from Junsei Chemicals Co., Ltd. (Japan). Hydrochloric acid (HCl) was purchased from Samchun Pure Chemicals Co., Ltd. (Korea). Sodium hydroxide (NaOH) and iodomethane were purchased from DaeJung Chemicals and Metals Co., Ltd. (Korea). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemicals Industries, Ltd. (Japan). PO and PF were kept in a FRS-300RWE refrigerator (Dongbu Daewoo Electronics, Korea) and WUF-500 deep freezer (Daihan Scientific Co., Ltd., Korea).

Preparation of PO and solvent fractions

The PF seeds were roasted at 170 °C for 10 min using an electric roasting machine (Dong Bang Machinery, Korea), and these roasted PF seeds were compressed into at 600 °C with an oil press machine, DB-500 (Dong Bang Machinery, Korea), and the collected PO was used. PO was separated by MPLC using *n*-hexane (isocratic: 100, v/v) which was to be used as the *n*-hexane layer. The PO was then separated by MPLC using $CHCl_3$ (isocratic: 100, v/v) and CH_2Cl_2 (isocratic: 100, v/v) were to be used as $CHCl_3$ and CH_2Cl_2 layers, respectively, after removing of *n*-hexane fraction. The collected *n*-hexane, $CHCl_3$ and CH_2Cl_2 layers were concentrated and the final residue was used for this experiment.

Saponification and urea complexation treatment

PO and solvent layers (each 20 mL) were dissolved using MeOH (10 mL) mixed with KOH (2 g) and heated at 65–70 °C for 1 h until homogenous solution. The mixture solution (2 g) was dissolved using MeOH (10 mL) and subjected to urea treatment (2 g). The treated mixture was cooled at 10 °C in a refrigerator for 24 h and then, the treated mixture annexations were crystallized. The crystallized mixture annexations were removed by filtration, and the filtrate was acidified with HCl (pH 2–3). To the separated fatty acids, *n*-hexane (10 mL) and ethyl ether (5 mL) were added and stirred. After removing the residue by filtration, the filtrate was dried using anhydrous sodium sulfate and evaporated with nitrogen gas.

Urea complexation of PO (PU) with varying amounts of urea

Each solvent layer (*n*-hexane, $CHCl_3$, and CH_2Cl_2) was treated with different amounts of urea (no treatment, 0.5, 1, 2, 4, and 8 g). The solvent fractions (*n*-hexane, $CHCl_3$, and

Table 1 GC conditions for ALA analysis

Instrument	GC model Agilent 7890A (Agilent, CA, USA), detector FID (280 °C, H_2 35 mL/min, air 350 mL/min, He 35 mL/min)
Column	DB-Wax (Agilent, 30 mm × 0.25 mm × 0.25 μm)
Injector temperature	250 °C
Column temperature	50 °C (1 min) → 200 °C (5 min) → 230 °C (20 min)
Split ratio	1:20
Separator temperature	250 °C
Ion source temperature	200 °C

Table 2 ALA content from PO based on the different amounts of urea used for treatment

Sample	Urea in *n*-hexane layer (g)						Urea in $CHCl_3$ layer (g)						Urea in CH_2Cl_2 layer (g)					
	0 (PU-1)	0.5 (PU-2)	1 (PU-3)	2 (PU-4)	4 (PU-5)	8 (PU-6)	0 (PU-7)	0.5 (PU-8)	1 (PU-9)	2 (PU-10)	4 (PU-11)	8 (PU-12)	0 (PU-13)	0.5 (PU-14)	1 (PU-15)	2 (PU-16)	4 (PU-17)	8 (PU-18)
ALA (%)	31.98	57.50	37.26	61.35	2.06	65.28	58.26	68.07	68.26	80.50	30.68	45.42	60.89	–	65.37	81.75	25.60	38.59

CH_2Cl_2) without any urea treatment were named PU-1, -7, and -13, respectively, and those treated with varying amounts of urea were named as follows: 0.5 g urea (PU-2, -8, and -14, respectively), 1 g urea (PU-3, -9, and -15, respectively), 2 g urea (PU-4, -10, and -16, respectively), 4 g urea (PU-5, -11, and -17, respectively), and 8 g urea (PU-6, -12, and -18, respectively).

Urea complexation of PO at different temperatures and time points (PT)

The samples of the CH_2Cl_2 layer treated with 2 g of urea were stored in a deep freezer, refrigerator, and room temperature for different periods of time (12, 24, 48, and 72 h).

Methylation for GC-FID

Iodomethane (0.1 mL) and ground NaOH (20 mg) were added to 0.5 mL DMSO with sample and then sonicated for 30 min at room temperature. Distilled water and CH_2Cl_2 were added and the non-polar phase was collected. Collected CH_2Cl_2 layer was evaporated under nitrogen gas (Ciucanu and Kerek 1984; Asres and Perreault 1997) and then the fatty acid methyl ester (FAME) was synthesized.

GC conditions

One microliter (spilt ratio 20) of FAME was injected via an Agilent 7890A autosampler (Agilent, USA) at 250 °C into the DB-Wax column (Agilent, 30 mm × 0.25 mm × 0.25 μm) in an Agilent 7890A (Agilent, USA) equipped with a FID. The column temperature was set at 50 °C for 1 min. The analysis was initiated at 200 °C and increased at a rate of 25 °C/min for 5 min. The column was set and then again started at 230 °C and increased at a rate of 3 °C/min for 20 min. Injector and ion source temperature were set at 250 and 200 °C, respectively. The detailed conditions were shown in Table 1.

Results and discussion

This study was conducted to investigate the purification methods used to obtain high levels of ALA from PO by urea treatment. Urea treatment of saturated fatty acids formed urea inclusion complexes by reducing the saturation degree of fatty acids (Hayes et al. 1998; Lee et al. 2014). Urea complexation efficiently reduced the saturation degree of fatty acids, yielding unsaturated fatty acids (Swern and Parker 1952; Swern 1955). This is a common method for the separation of a mixture of fatty acids, ALA and linoleic acid (Lee et al. 2011). This method was used

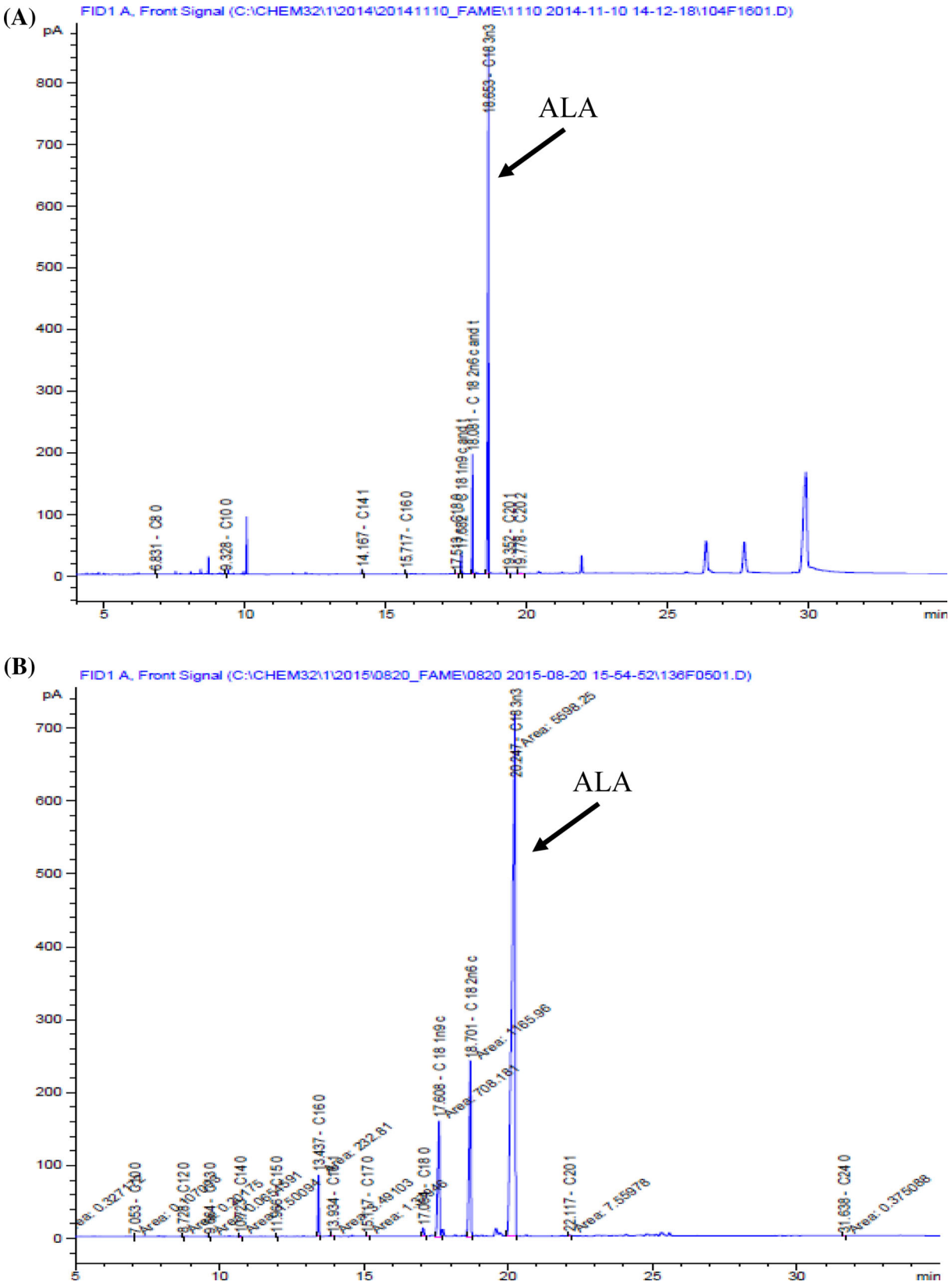

Fig. 2 GC chromatograms of ALA in PU-16 (**A**) and PT-6 (**B**)

Table 3 ALA content in PO at different temperatures and time periods of extraction

Sample	Time in the deep freezer (h)				Time in the refrigerator (h)				Time at room temperature (h)			
	12 (PT-1)	24 (PT-2)	48 (PT-3)	72 (PT-4)	12 (PT-5)	24 (PT-6)	48 (PT-7)	72 (PT-8)	12 (PT-9)	24 (PT-10)	48 (PT-11)	72 (PT-12)
ALA (%)	60.71	44.02	27.22	69.71	51.46	72.03	65.11	53.37	52.70	52.56	53.34	–

for other materials such as fungal lipids (Arjuna 2013), liver oil from dog fish (Stefanov et al. 1997), tuna (Suriani et al. 2014), pine nut oil (No et al. 2015), and soybean oil (Hasnisa and Jumat 2014). The amount of omega-3 fatty acids in tuna increased 2.70-fold with urea treatment (Suriani et al. 2014).

We investigated the optimum amount of urea treatment for each solvent layer (*n*-hexane, $CHCl_3$, and CH_2Cl_2). The amounts of ALA varied based on the amounts of urea used for treatment (no treatment, 0.5, 1, 2, 4, and 8 g; Table 2). Of the treated solvent fractions, PU-16 (PO eluted with CH_2Cl_2 using 2 g urea) had the highest amount of ALA (81.75 %). Figure 2 shows GC chromatograms of ALA in PU-4, -10, and -16. Generally, treatment with 2 g urea (PU-4, -10, and -16) resulted in the highest ALA yield. Treatment with 2 g urea at complexation temperature and time were conducted (Table 3). The amount of ALA in the refrigerated sample (PT-6) was the highest at a complexation time of 24 h (72.03 %). Figure 2 shows GC chromatograms of ALA in PT-6. This result indicates that 2 g urea treatment and 24 h refrigeration are favorable condition to obtain the highest amount of ALA.

To separate unsaturated fatty acids, gradient cooling methods for *Perilla* oils (Gu et al. 2009), lipase-mediated hydrolysis of flax seeds (Rupani et al. 2012), and column chromatography and urea complexation for *Lithospermum erythrorhizon* (Han et al. 2004) were used. Gu et al. (2009) suggested that the method of cooling urea inclusion could be used for purifying linoleic acid. Urea complexation conducted repeatedly with corn, *Perilla*, and linseed oils showed that 89 % of ALA was obtained from *Perilla* (Swern and Parker 1953). The concentration of ALA and linoleic acid focused on alkaline-isomerization reaction time and concentration of NaOH (Lee et al. 2011).

In addition to urea complexation, other methods used for omega-3 fatty acid separation include the distillation method, low temperature crystallization, lipase-catalyzed hydrolysis, and supercritical fluid extraction (Kapoor and Patil 2011). Among them, the distillation method with different boiling points is used for isolation of substances from a fatty acid mixture (Lopes et al. 2009). In low temperature crystallization, solubilization is used to purify fatty acids, esters, and lipids (Brown and Kolb 1955). Urea complexation can not only be used in food industry but also in the field of energy resources such as biodiesel. Urea complexation is used to improve the cold flow and reduce the cold filter plugging point of biodiesel. This method reduced the amount of saturated fatty acids and improved the cold filter plugging point of biodiesel (Lee et al. 2012).

In conclusion, urea complexation at low temperature (refrigeration) is a prospective method for the extraction of unsaturated fatty acids in high yields. Application of urea complexation method for PO eluted with CH_2Cl_2 yields large amount of ALA for use in the field of cosmetics and pharmaceutical food supplements.

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