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Fractionation of lipids and purification of γ-linolenic acid (GLA) from *Spirulina platensis*

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Abstract

The microalgae, *Spirulina platensis*, is an excellent source of γ-linolenic acid (GLA), an essential polyunsaturated fatty acid and a potent nutraceutical. The fatty acid composition of *S. platensis* ARM 740 was determined after transmethylation by gas chromatography (GC). Lipid fractionation was achieved on silica gel column chromatography and preparative TLC. Neutral lipids, glycolipids and phospholipids accounted for 77.0%, 15.6% and 7.4%, respectively, of the total lipid fraction. *S. platensis* ARM 740 was found to contain 94% of the total GLA in the glycolipid fraction. Attempts were made to purify GLA methyl ester by using urea to form inclusion complexes with the saturated and the less unsaturated FAMEs (fatty acid methyl esters), which enhanced the purity of GLA methyl ester to 84%. A further approach to isolate GLA methyl ester with higher purity involved the use of argentated silica gel chromatography. An initial PUFA concentration step frequently adopted by most researchers to increase GLA purity was not necessary in the isolation of GLA from *S. platensis*. A GLA methyl ester with a purity of >96% and a recovery of 66% was obtained. Purity of the isolated GLA methyl ester was confirmed by GC and IR analysis with respect to authentic standard.

Keywords: *Spirulina platensis*; Lipid fractionation; Purification; GLA; Urea complexation; Argentated silica gel chromatography

1. Introduction

The blue-green alga, *Spirulina platensis*, is a potential source of GLA, an essential polyunsaturated fatty acid of excellent economic interest. GLA is a metabolite of linoleic acid (LA) and the first intermediate in the conversion of LA to arachidonic acid (AA) (Gunstone, 1992). Various studies have asserted the pharmaceutical value of GLA, especially in lowering the low density lipoprotein in hypercholesterolemic patients (Ishikawa et al., 1989), alleviation of symptoms of pre-menstrual syndrome (Horrobin, 1983), and atopic eczema (Biagi et al., 1988). Being a precursor of prostaglandin E_1, GLA also exhibits direct and indirect antiviral action (Ziboh, 1989). *In vitro* and *in vivo* studies have shown GLA to selectively kill tumor cells without harming normal cells (Reddy, Prassas, & Das, 1998). Natural sources of GLA contain variable amounts of this acid which rarely exceed 25%. Hence, there has always been a keen interest in producing higher concentrates of GLA.

The methods generally used to obtain PUFA-rich fractions from natural oils are based on differences in the polarity and/or spatial configuration of the fatty acids (FA) present in the extract. These differences, mostly associated with the number of double bonds in the carbon chain, can enable the separation of PUFAs with respect to their degree of unsaturation (Kates, 1986). The common methods of producing PUFA concentrates include winterization, fractional distillation, urea inclusion, high-performance liquid chromatography and argentated silica gel chromatography (Facciotti, Lassner, & Metz, 2000; Guerrero, Madrid, & Belarbi, 2000; Guerrero, Madrid, & Juárez, 2003; Guerrero, Mercado, Maroto, Garcia, & Madrid, 2001; Madrid and Guerrero, 2002; Martínez, Madrid, & Guerrero, 2004; Spurvey and Shahidi, 2000; Yokochi, Usifa, Kamisaka, Nakahara, & Suzuki, 1990). Preparative HPLC methodology is

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expensive and the scalability of HPLC for purifying large quantities of fatty acids is impractical.

Recently, argentated silica gel column chromatography was shown to purify α-linolenic methyl ester from hydrolyzates of perilla oil that also contained linoleic acid and oleic acid methyl esters. Hence, the method was considered suitable for the isolation of GLA methyl ester from *S. platensis*. Also, a major interference from α-linolenic acid (ALA) in the isolation of GLA from seed oils, would not be encountered, due to the absence of α-linolenic acid (ALA) in *S. platensis*. The close similarity in the polarity of GLA and ALA reduces the separation efficiency of the two isomers by chromatographic methods.

In the present study, the composition of lipids with respect to their individual classes was quantified and evaluated for their FAME contents. The purification of GLA from *S. platensis* ARM 740 was attempted by urea complexation and also by argentated silica gel column chromatography.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical reagent grade. GLA methyl ester standard was procured from Sigma Chemical Company (USA) Ltd. The other FAME standards were obtained from SRL (Sisco Research Laboratories) India Ltd.

2.2. Microorganisms

S. ARM-740 was obtained from CFTRI, Mysore, India.

2.3. Medium

The medium used for the cultivation of *S. platensis* was SOT medium containing (g/l) $NaHCO_3$, 16.8; K_2HPO_4, 0.5; $NaNO_3$, 2.5; K_2SO_4, 1; NaCl, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.04; $FeSO_4 \cdot 7H_2O$, 0.01, ethylene diamine tetraacetate, 0.08 and A-5 trace metal solution, 1 ml. The A-5 trace metal solution contained (g/l) H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.22; $Na_2MoO_4 \cdot 2H_2O$, 0.039; $CuSO_4 \cdot 5H_2O$, 0.079 and $Co(NO_3)_2 \cdot 6H_2O$, 0.49 (Hirano et al., 1990).

2.4. Cultivation

Stock cultures of *S. platensis* were maintained and inocula transferred according to Vonshak (Vonshak, 1986). It was cultivated in two carboys, each of 18 l capacity at 28–30 °C and harvested after seven days. Cultures were illuminated with six Philips cool white fluorescent tubes (40 W each) providing 1200 lx. The cells were harvested by filtration through a nylon mesh (33 μm), and the cell paste was lyophilized and stored at −20 °C for further use. The fatty acid methyl esters (FAME) obtained after transmethylation of the dried biomass were analyzed by GC.

2.5. Lyophilization of S. platensis

The cell mass obtained after filtration was lyophilized using a Heto Dry winner. The wet cell mass was frozen overnight at −70 °C and then freeze-dried at −52 °C under vacuum. The dry biomass obtained after freeze-drying was stored in air-tight containers at −20 °C.

2.6. Fractionation of lipids and their analysis

2.6.1. Extraction of lipids using the Bligh and Dyer method (Bligh & Dyer, 1959)

One gram of freeze-dried biomass was treated with a mixture of 100 ml methanol, 50 ml chloroform and 40 ml water, sonicated for 10 min and shaken overnight for maximum extraction of lipids. The mixture was vacuum-filtered using a Buchner funnel through Whatman No. 3 filter paper. The supernatant after filtration was centrifuged at 8000 rpm at 8 °C, treated with 50 ml chloroform and 50 ml water, mixed well and the chloroform layer retrieved after separation in a separating funnel. The emulsion formed was removed with the addition of common salt, which helped in better recovery of the lipids and subsequently GLA. The methanol + water layer (upper layer in the separating funnel) was further treated with about 25 ml chloroform, mixed well and the chloroform layers were combined to obtain total lipids on removal of the solvent. The lipids so obtained were stored under nitrogen atmosphere at −20 °C, until further use.

2.6.2. Lipid fractionation

Lipids from the freeze-dried biomass were extracted with chloroform–methanol–water (2:1:0.8) according to Bligh and Dyer method (Bligh & Dyer, 1959). Separation of the lipids into the individual classes, namely neutral lipids (NL), glycolipids (GL) and phospholipids (PL) was achieved by silica gel column chromatography. Crude lipids obtained from 1 g of freeze-dried biomass were fractionated on a column (2 × 18 cm) of activated silica gel (15 g silica gel 60, 60–120 mesh; Merck). The individual classes were eluted with chloroform (200 ml), acetone (400 ml) and methanol (200 ml) (Gamian et al., 1996). For elution of glycolipids, mixtures of varying proportions of chloroform: acetone to pure acetone were used. The eluates were monitored for various types of lipids on TLC silica gel G plates (Merck).

The mobile phases used for the detection of the lipid classes were: neutral lipids – petroleum ether:diethyl ether:acetic acid (70:30:0.5 v/v); glycolipids – chloroform:methanol: water (75:25:4); phospholipids – chloroform:methanol: water (65:25:4). The spray reagents used were: neutral lipids and phospholipids – 5% (w/v) ethanolic solution of molybdophosphoric acid, followed by heating for 10 min at 180 °C; glycolipids: α-naphthol solution (0.5% w/v in methanol/water, 1:1 v/v), followed by drying the plate and spraying lightly with 95% v/v sulphuric acid and heating at 120 °C for 5 min, after which the glycolipids turned blue. The

absence of glycolipids was confirmed by using the spray reagent for glycolipids for both the NL and PL fractions. The glycolipids were also confirmed by the Molisch test: water + fraction collected from column + conc. sulphuric acid + α-naphthol to give a distinct blue color.

The lipids were quantified gravimetrically prior to fatty acid derivatization and analysis. The fractions, pooled together to constitute the individual lipid classes, were applied to preparative TLC (20 × 20 cm) plates and the corresponding bands were scraped, eluted with the respective solvent, dried with nitrogen gas, and weighed to obtain the amount of each lipid fraction. The analysis was carried out at least five times to confirm the reproducibility of the results.

2.7. Transmethylation of fatty acids in lipids of S. platensis

One gram of freeze-dried biomass were treated with 15 ml of methanol–acetyl chloride mixture (95:5). The mixture was sealed in a Teflon-lined vial under nitrogen atmosphere and heated to 80 °C for 1 h. The vial contents were cooled, diluted with water and extracted with 10 ml of hexane containing 0.01% butylated hydroxyl toluene. The hexane layer was dried over Na_2SO_4 and evaporated to dryness.

2.8. Purification of GLA methyl ester

2.8.1. Fractionation with urea

The FAMEs obtained were dissolved in methanol containing various amounts of urea by heating the mixture to 65 °C, until clear. The urea and urea inclusion complexes were allowed to crystallize at room temperature, and then refrigerated overnight at 0 °C. The mother liquor was separated by vacuum filtration and extracted with 5 ml urea-saturated methanol, 6 ml water and 3 ml concentrated HCl (Cohen & Cohen, 1991). The FAMEs in the methanol layer were extracted into hexane and the urea complexes were rinsed with hexane. The combined hexane extracts were concentrated using nitrogen gas and FAMEs were identified by GC.

2.8.2. Argentated silica gel chromatography

GLA methyl ester was purified by fractionation of FAMEs by argentated silica gel column chromatography (Guerrero, Mercado, Maroto, Garcia, & Madrid, 2001). The fractionation was carried out at least three times to confirm the reproducibility of the results.

The Ag–silica gel was prepared as follows: 10 g of silica gel (100–120 mesh) for column chromatography was slurried in ethanol (20 ml, 10 min) in a round-bottom flask. A solution of silver nitrate (1 g) in 70% ethanol (35 ml) was added. Agitation was continued for a further 10 min. Ethanol was evaporated on a water-bath followed by nitrogen purging. The free-flowing silver-impregnated silica gel was activated by overnight heating at 80 °C. The material was cooled and kept in an amber bottle in a desiccator, until required. The packed height of the glass chromatography column was kept at 3 cm in a 0.5-cm diameter column. The chromatography column was packed as follows: The exit of the chromatography column was plugged with glass wool to retain the solids and the column was first filled with hexane. A flow of 1 ml/min was allowed during the packing. A slurry of 2.3 g of Ag–silica gel in hexane was poured into the column that was half-filled with hexane. The hexane layer was lowered until it was 1 cm above that of the stationary phase. The column was conditioned for 2 h with the outlet flow of hexane going back into the column top.

2.9. Fatty acid analysis

FAMEs were analyzed by GC (Chemito 8510 HR) using a highly polar EGSS-X column procured from Chromatopak,

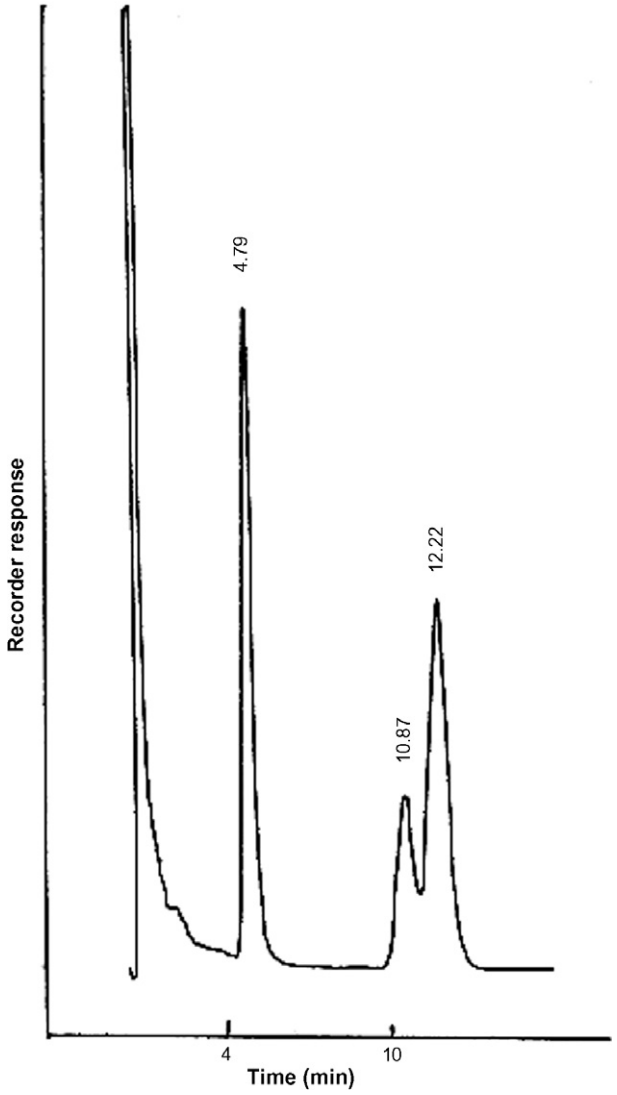

Fig. 1. The two peaks at R_t 10.87 and 12.22 min show the separation of GLA methyl ester from ALA methyl ester by EGSS-X column. ALA methyl ester was not found to be present in *Spirulina platensis* ARM 740. (R_t – 4.79 min – heptadecanoic acid methyl ester (internal standard), R_t – 10.87 min – GLA methyl ester, R_t – 12.22 min – ALA methyl ester).

Mumbai. An injection and detector temperature of 250 °C and a column temperature of 180 °C were used for GC analysis. FAMEs were identified by comparing the retention time of the integrated peak of the individual FAME with those of authentic standard FAMEs. Quantitative determinations were carried out by using heptadecanoic acid methyl ester as the internal standard. A standard curve was plotted for each FAME using the weight and area ratios with respect to the internal standard.

A standard curve of GLA methyl ester was prepared using 0.5–3 mg/ml of *n*-hexane containing 1 mg/ml of the internal standard, heptadecanoic acid methyl ester. Weight ratio was calculated as weight of GLA methyl ester/weight of internal standard. Area ratio was calculated as area of GLA methyl ester/area of internal standard.

3. Results and discussion

In the present study, two carboys, each of 18 l capacity was used for the cultivation of *S. platensis* ARM-740. The cells harvested by filtration were lyophilized and stored in air-tight containers for further analysis.

3.1. Composition of FAMEs

Hundred milligrams of freeze-dried biomass was transmethylated to obtain FAME in the upper hexane layer. Although the hexane layer was found to be free from any adhering biomass or pigments, it was washed with water to remove any traces that may have been present, and passed through a bed of sodium sulphate before analysis. The hexane layer so obtained was concentrated and transferred to a weighed Eppendorf tube and the residual solvent was completely removed by nitrogen purging. The weight of the residue in the Eppendorf tube corresponded to the FAMEs obtained from the biomass. The total FAME content of *S. platensis* ARM 740 was found to be 4.11% on dry weight basis of lyophilized biomass.

FAMEs were analyzed by GC and quantified by extrapolation from the standard curve of each methyl ester. The FAMEs assessed by direct transesterification of the freeze-dried material were found to be comprised of palmitic acid (53.09%), oleic acid (18.37%), GLA (15.8%), linoleic acid (6.57%), myristic acid (4.38%) and stearic acid (1.80%). The GLA content of the lyophilized biomass was found to be 0.64%.

3.2. Resolution of isomers of linolenic acid

To verify the presence of the γ-isomer of linolenic acid in *S. platensis* ARM-740, a mixture of pure standards of γ-linolenic acid methyl ester (ALA) and GLA methyl ester dissolved in hexane was analyzed by GC. Also, pure GLA methyl ester standard without ALA methyl ester was injected under the same conditions. The separation of the peaks of the two isomers (Fig. 1) with different retention times confirmed the efficacy of the EGSS-X column in resolving the two isomers of linolenic acid and hence the presence of only GLA in *S. platensis* ARM-740.

3.3. Composition of lipids

Silica gel column chromatography was used to fractionate lipids into their individual classes based on polarity. Neutral lipids, glycolipids and phospholipids accounted for 77.0%, 15.6% and 7.4% of the total lipid fraction. The neutral lipid fraction was found to contain the non-polar portions such as mono and diacylgycerols and hydrocarbons. The neutral lipid fractions also contained the chlorophyll and carotenoid pigments, which were separated by

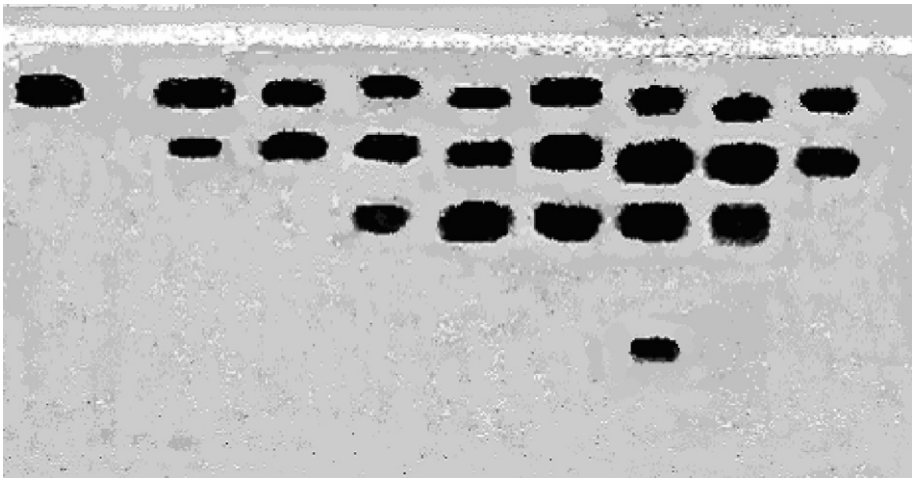

Fig. 2. The bands shown correspond to the glycolipid components stained by α-naphthol acid spray reagent in various column fractions collected by elution with acetone (lane 1: pooled fractions eluted from the column using 50 ml of 75:25 of chloroform:acetone; lanes 2, 3, 4, 5: pooled fractions eluted from the column using 30, 40, 75, 75 ml pure acetone, respectively; lane 6, 7, 8, 9: pooled fractions, each eluted with 50 ml of 80:20 of acetone:methanol).

preparative TLC. The glycolipids stained with the specific stain, α-naphthol, showed blue-purple bands at R_f values of 0.82, 0.7, 0.60, and 0.43 (Fig. 2). Ninety four percent of the GLA was found to be present in the glycolipid fraction. The column fractions corresponding to the individual classes were pooled, concentrated using a flash evaporator, applied to preparative TLC, and analyzed by GC. The total lipid content of *S. platensis* ARM 740 was found to account for 8.7% of the freeze-dried biomass. The fatty acid composition of the fractionated lipids is shown in Table 1.

3.4. Concentration of GLA by urea complexation

Attempts were made to isolate GLA methyl ester by using urea to form inclusion complexes with the saturated and less unsaturated FAMEs, thus isolating the more unsaturated GLA methyl ester. The urea molecules bond together *via* hydrogen bonding, while strong van der Waals attractions exist between the urea molecules and the 'guests', which are held in circular channels of diameter 0.4–0.6 nm. Most urea complexes have a hexagonal crystalline structure compared to the tetragonal structure of pure urea (Harris, 1996; Smith, 1952; Takemoto & Sonoda, 1984).

When mixed fatty acids or esters are crystallized from methanol, the crystallizing urea traps the saturated and less unsaturated acids or esters as clathrates or adducts and leaves the more unsaturated acids or esters in solution. The material recovered from the mother liquor contains GLA at several times the original level depending on the composition of the starting material. In the present work, experimental conditions made use of varying fatty acid/urea ratio (1:3 to 1:100), urea/solvent ratio (1:2, 1:3), and crystallization conditions (RT, 0 °C, tap-water cooling, overnight/immediate crystallization), to increase the purity of the isolated GLA methyl ester. At least 20 trials were undertaken with varying composition of urea: methanol and temperature conditions. In all the trials, GLA methyl ester was found to be always accompanied by linoleic acid methyl ester. A two-step urea adduction to further increase purity of GLA methyl ester was also attempted.

Lower levels of urea for the complexation of FAMEs were found to result in lower purity of GLA methyl ester. When 100 mg of FAME was treated with 300 mg of urea in 2.5 ml of methanol at 65 °C, until clear, the purity of GLA was 66% and the recovery was 34%. Also, instant crystallization of the complexes by tap-water cooling resulted in a

Table 1
Classes of lipids and distribution of fatty acids by these classes (%)[a]

	Neutral lipids	Glycolipids	Phospholipids
C14:0	1.78 ± 0.035	–	–
C16:0	57.5 ± 1.03	42.4 ± 1.06	57.03 ± 0.85
C18:0	1.99 ± 0.055	0.86 ± 0.017	2.79 ± 0.05
C18:1	27.83 ± 0.8	11.97 ± 0.23	13.54 ± 0.39
C18:2	8.8 ± 0.22	2.95 ± 0.05	25.99 ± 0.45
C18:3, γ	1.98 ± 0.05	41.76 ± 0.87	0.64 ± 0.012
GLA distribution	5.4 ± 0.16	94 ± 2.1	0.6 ± 0.018
Fraction distribution	77.9 ± 1.55	15.6 ± 0.28	6.5 ± 0.13

[a] Results are means ± SD of three or more determinations.

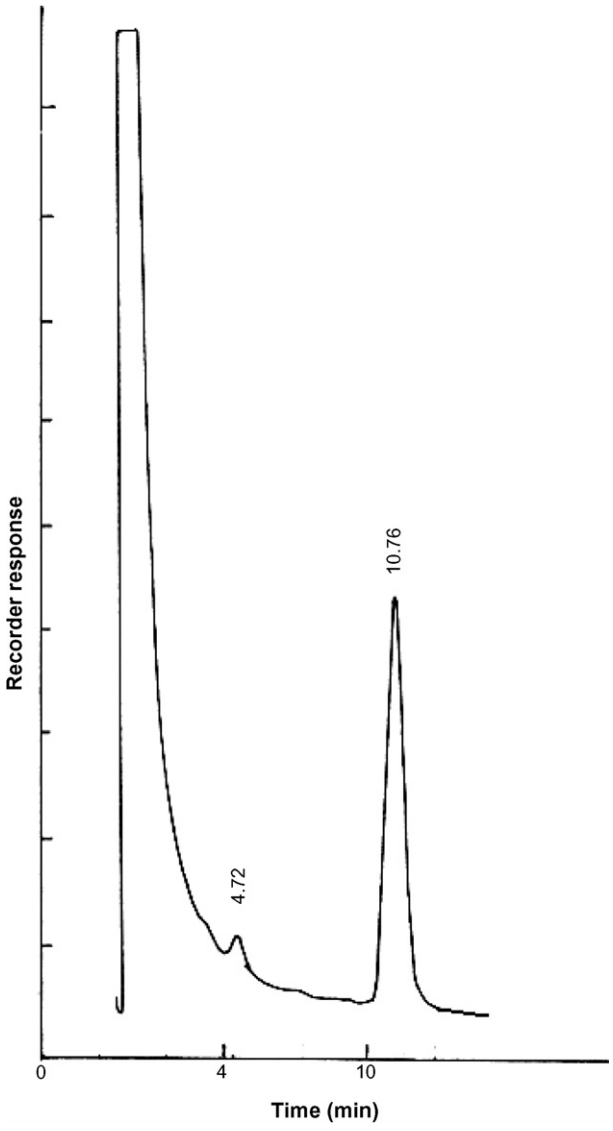

Fig. 3. Chromatogram of purified GLA methyl ester from *Spirulina platensis* ARM-740.

Table 2
Percent individual FAMEs before and after urea complexation[a]

	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3, γ
Before urea complexation	4.38 ± 0.08	53.09 ± 1.16	1.8 ± 0.04	18.37 ± 0.45	6.57 ± 0.12	15.8 ± 0.28
After urea complexation	–	Traces	–	–	15.2 ± 0.37	84 ± 0.91

[a] Results are means ± SD of three or more determinations.

purity of GLA of 56.8% and a recovery of 33%. However, a maximum purity of 84% of GLA methyl ester and a recovery of 64% was obtained when 100 mg of FAME was dissolved in 3 ml methanol containing 1 g urea (by heating the mixture to 65 °C until clear) and the urea inclusion complexes were allowed to crystallize at room temperature followed by overnight refrigeration at 0 °C. Table 2 shows the % fatty acid composition obtained after complexation of the FAME with urea. An additional urea adduction step was found to further reduce the % recovery of GLA. Further attempts were therefore made for the purification of GLA methyl ester by column chromatography.

3.5. Enrichment of GLA by argentated silica gel chromatography

In the silver–silica gel fractionation of fatty acid esters, saturated esters elute before the unsaturated ones. The number, position, and geometric configuration of double bonds determine the elution order of unsaturated fatty esters. The resolving power of Ag–silica gel is attributed to a reversible charge-transfer complexation of Ag^+ with carbon–carbon double bonds (Guerrero et al., 2000). The extent and the strength of complexation control the mobility of a solute, as does the polarity of the mobile phase. The concentration of silver nitrate loaded per unit mass of silica gel influences the surface density of Ag^+ ions on the solid matrix, hence, affecting the retention time of the solute.

For column chromatography, FAME was prepared as follows: 1 g of the freeze-dried biomass of *S. platensis* ARM 746 was transmethylated in air-tight Borosil culture tubes by adding 15 ml of methanol–acetyl chloride mixture and 3 ml hexane and heating at 80 °C for a period of 1 h in a water-bath. The hexane layer was removed using a Finn pipette after proper mixing, transferred to a vial and washed with water to remove cell debris. The hexane extract was passed through a bed of sodium sulphate and concentrated using nitrogen gas to obtain 1 ml hexane containing the FAMEs. 0.5 ml of this solution was diluted to 1 ml with hexane and analyzed by GC to detect the FAME corresponding to 0.5 g of the biomass. 0.5 ml was applied to the argentated silica gel chromatography.

The column was eluted with the following sequence of solvents: hexane (40 ml), hexane with 0.5% acetone

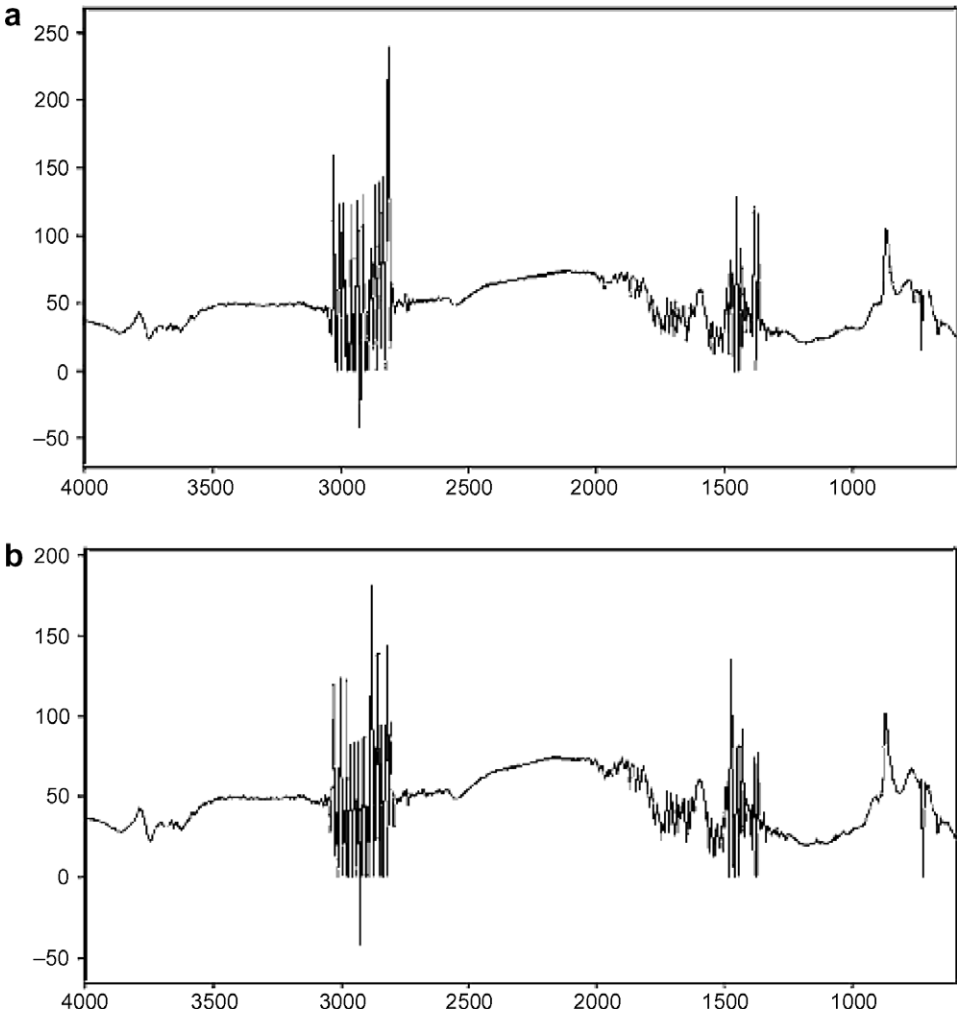

Fig. 4. IR spectra of (a) standard GLA methyl ester and (b) purified GLA methyl ester.

(20 ml), hexane with 0.75% acetone (40 ml), hexane with 1% acetone (20 ml), and hexane with 2% acetone (40 ml) at a flow rate of 1 ml/min. The saturated fatty acid esters were obtained in the hexane fractions. Linoleic acid methyl ester and some amount of palmitic acid methyl ester were found to be present in the fractions eluted with hexane containing 0.75% acetone. The hexane eluates with 1% and 2% acetone were pooled together to obtain GLA methyl ester with over 96% purity (as detected by GC) and a recovery of 66% (Fig. 3). 4.2 mg GLA methyl ester was obtained from 1 g of lyophilized biomass. The presence of GLA methyl ester in the eluates was also confirmed by IR analysis against a standard. The region of the IR spectrum containing most of the complex vibrations (600–1400 cm^{-1}) commonly called the fingerprint region of the spectrum was found to match with that of the standard. Absorption bands were obtained at 715, 1023, 1170, 1245, 1370, 1440, 1760, 2930 cm^{-1} in both the standard and the purified GLA methyl ester (Fig. 4a and b).

4. Conclusions

GLA methyl ester with over 96% purity and a recovery of 66% was obtained using argentated silica gel chromatography. GLA was found to be concentrated mainly in the glycolipid fraction. Successful separation of the lipid fractions was possible using silica gel column chromatography followed by preparative TLC. Optimization of medium composition is suggested to further improve the proportion of glycolipid in the lipid fraction, hence enhancing the yield of GLA in *S. platensis* ARM 740.

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