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Argentated silica gel chromatography for separation of γ-linolenic acid from microalgae

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ABSTRACT

A low expense process is developed for recovery of γ-linolenic acid from *Spirulina platensis* biomass. Simultaneous extraction and direct transmethylation of fatty acids from freeze dried biomass and subsequent purification using silver silica gel column chromatography resulted in 95.43% pure γ-linolenic acid. The percent purity of γ-linolenic acid in the fractions varied from 87.18 -95 %. A recovery of 74.05% γ- linolenic acid was achieved using silver silica gel chromatography.

Key words: γ-linolenic acid, silver silica gel column, chromatography, methyl ester.

INTRODUCTION

γ- linolenic acid (GLA) is an important metabolite, which finds wide applications in treating various disorders: rheumatoid arthritis (Soeken 2004), eczema (Wright and Bolton 1989), diabetes, multiple trauma, and premenstrual syndrome (Wantanabe *et al*. 2005). GLA is currently produced from plants (Hirano *et al*.1990) and fungi (Pactrica de Oliveira Carvalho *et al*.1999), Plants require larger area for cultivation, GLA content in plants change with seasonal fluctuations in temperature. On the other hand, fungal oils are contaminated with higher polyunsaturated fatty acids (PUFA) .In addition; the oils are associated with alfa-linolenic acid, an isomer of GLA which makes purification difficult. Thus an alternative and economic source for GLA is *Spirulina platensis*(Cohen *et al*.1993) . Average lipid content found in the alga is 4-7% while the omega-6 fatty acid (GLA) amounts to a maximum of 31% of total fatty acids (Cohen *et al*. 1987). The alga accumulates 1% GLA on dry cell weight basis. Besides, the alpha isomer (γ-linolenic acid) is found absent in the alga. The Current bulk market price of GLA oils mostly from plants is 10-15$ per kilogram. Any new alternative source should need to compete with that price. Many methods are proposed for fatty acid purification, urea fractionation concentrates unsaturated fatty acids. However, it is difficult to remove linoleic acid beyond certain limit fromγ-linolenic acid. This work reports highly pure (95% purity) GLA ester from microalgal biomass using argentated silver silica column chromatography.

MATERIAL AND METHODS

Materials

Column packing

The column employed in the study was a modified 10ml (borosil) pipette (0.5cm diameter); the column exit was blocked with glass wool to retain solids. The tapering end was fitted with a micro tip to facilitate connection between the column and peristaltic pump through solvent resistant tubing, slurry of silver silica gel (600mg) was poured into the column which was half filled with hexane, a slight flow of hexane was allowed while packing the column. About 600mg of silver silica gel is sufficient to give a packed height of 6cm in the column. The column was equilibrated with the solvent system for 2 hour with a flow of 1ml per minute. Hexane was lowered 1cm above the stationary phase while

loading the column, about 30 mg of fatty acid methyl ester dissolved in hexane (2ml) was loaded on to the column. The column was eluted with the following sequence of solvents: 1) hexane with 0% (v/v) acetone; 2) hexane with 1% (v/v) acetone; 3) hexane with 2% (v/v) acetone; 4) hexane with 3% (v/v) acetone; 5) hexane with 4% (v/v) acetone 6) hexane with 5 % (v/v) acetone 7) hexane with 6% (v/v) acetone. The volume of each solvent fraction was kept 5ml however depending on the requirement; fraction containing 4% and 5% acetone in hexane were run twice for eluting unsaturated fatty acids.

Analytical methods

Fatty acid analysis and Quantification

The fatty acid composition was analyzed using a Gas chromatography (GC make- Chemito HR 5820). The fatty acids were identified by correlating the retention time with that of standard fatty acids (Sigma &Co). The column used to identify fatty acids was an EGSS packed column with 10 % w/w chromosorb solid support (Mahajan and Kamat 1995). The column was operated at 180°C in an isothermal mode. The mobile phase was an inert nitrogen gas fixed at a flow rate of 35 ml per min. Injector and detector temperatures were kept at 250°C. The detector of gas chromatography was flame ionizing detector (FID). Fatty acid quantification was done using internal standard method.

Fatty acid extraction and methylation

Microalgal lipids are first extracted and then transesterified (Nagle and Lemke 1990). However, the process consumes time and also expensive. A single step transmethylation was done to obtain fatty acid methyl ester from the algal biomass. About 100mg of the freeze dried biomass was taken in a 5ml borosil glass vial. To the reaction vial, 3ml of methanol -Acetyl chloride (95:5) was added. The vial was closed air tight and the contents were heated to 80°C till 1 hour in the water bath. After cooling to room temperature, the contents were added with 3ml of hexane. The methyl ester was extracted into hexane layer; care was taken to remove moisture if any present in the fatty acid methyl ester with sodium sulphate. The procedure was scaled up for desired amount of fatty acid methyl ester.

Preparation of silver silica gel

Silver silica gel chromatography (Svetlana and Nikolova-Damayanova, B. 2003; Belarbi *et al.*2000) was used for separation of individual fatty acid esters. Silver silica gel was prepared as follows; 20g of silica gel (0.06-0.2mm, 70-230 mesh, mean pore diameter of 60nm) was slurried in 96% (v/v) ethanol (200ml, 10min). Silver nitrate solution (10 g prepared in 70% (v/v)ethanol (approx 35ml) was added drop wise to silica gel .The contents were agitated for 10 min; ethanol was evaporated using rotary evaporator under vacuum at 60^{0}C. The silver silica gel was then kept for activation over night in a hot air oven operating at 120^{0}C.

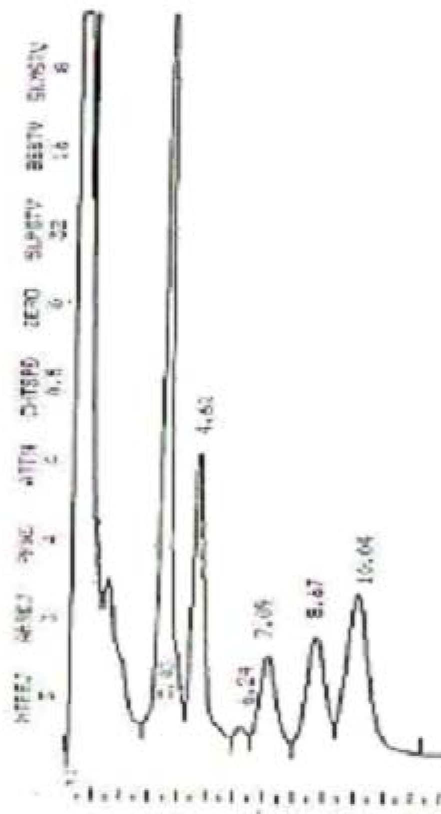

Fig. 1: Fatty acid profile of *spirulina platensis* arm 740 showing various fatty acids and their retention times: palmitic acid (3.43); heptadecanoic acid (internal standard) (4.61);stearic acid (6.24);oleic acid (7.09);linoleic acid ((8.67);gla(10.04)

RESULTS AND DISCUSSION

The Ag-silica gel chromatography works on the principle that the higher unsaturated fatty acids bind to the silver ion attached to the stationary phase while saturated fatty acids elutes with the mobile phase depending on their relative polarity of the fatty acids. Increasing the polarity of the mobile phase sequentially elutes saturated and unsaturated fatty acids from the silver silica gel column. The silver silica gel columns prepared are fairly stable(Ozcimder and Hammers 1980) and can be reused provided that the silver does not get leached during elution.

The fatty acid profile of the alga considered in the present study was shown in Fig.1. As can be seen from the figure, unlike in most fungal oils, where poly unsaturated fatty acids(PUFA) dominate. The present microalgal strain shows only linoleic and ã-linolenic acid as major unsaturated fatty acids. This is an advantage in purification of lower chain unsaturated fatty acid like GLA when PUFA is absent. The sequence of operations in purification

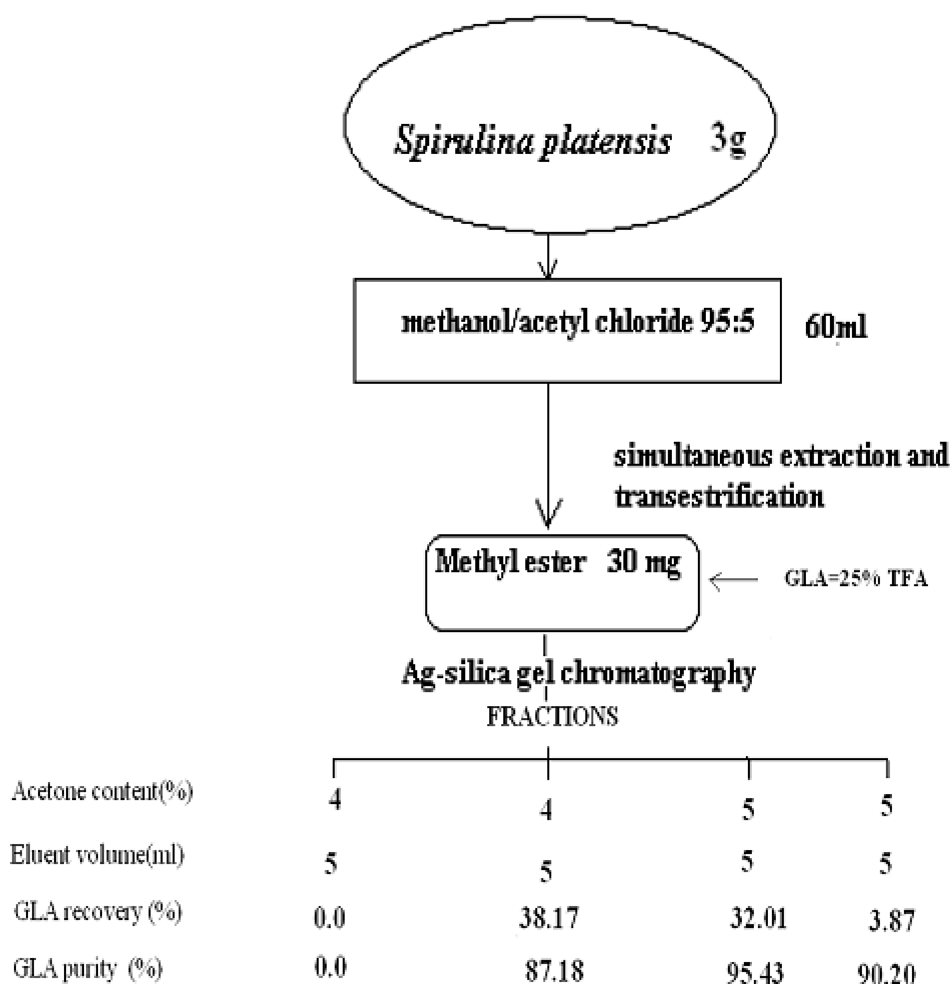

Fig. 2: GLA recovery from *spirulina platensis* freeze dried biomass.
The percent GLA recovery numbers for the various solvent fractions are based on the GLA ester content in total fatty acids.

of GLA from *Spirulina platensis is* summarized in Fig.2. The figure also shows GLA recovery and purity at various stages, the total fatty ester loading with respect to the amount of the stationary phase was about 5% (w/w). The total GLA contained in the various eluent fractions was 74.05% of the GLA that was originally present in the starting total fatty extract.

The fatty acid profiles of various solvent fractions were shown in Table1 Saturated fatty acid esters elute in the third solvent fraction (2% acetone in hexane) because they do not complex with silver ion. Palmitic acid, though a major part of the fatty acid was eluted in the 2% and 3% acetone–hexane fraction, smaller quantities of fatty acid was still observed in 4% mobile phase. Similarly, monounsaturated fatty acid ester (oleic acid) complex relatively less strongly with silver and as a result it is eluted mostly in the solvent fraction only after saturated fatty acid (palmitic acid). Hence, oleic acid was seen eluting after palmitic acid in fraction containing 3% acetone. Only the unsaturated fatty acids bind strongly with silver ion and are eluted depending on the relative degree of unsaturation. Thus linoleic acid is expected to elute initially followed by GLA in fractions following 3% acetone. It can be seen from Table 1 and Table 2 that linoleic acid eluted in fraction containing 3% and 4% acetone, though traces of this fatty acid ester was observed in fractions containing 5% and 6 % acetone. GLA appeared in fractions containing 4% and 5% of the eluted mobile phase.

The percent purity of GLA (Table1) in the fractions obtained varied from 87.18-95.43 %. Data in Table 2 shows the percent fatty acid in the eluted fractions among total fatty acids. GLA was found

Table 1: Data showing elution of principle fatty acids in varying polarity of solvent system and percent purity of GLA in different fractions.

Fraction no.	Conc. of mobile phase	Principle Fatty acids eluted (% acetone in hexane)	% Purity of GLA in each fraction
1	0	- -	
2	1	- -	
3	2	C16:0 -	
4	3	C16:0;C18:1;C18:2	-
5	4	C16:0;C18:1;C18:2	-
6	4	C16:0;C18:2;C18:3	87.18
7	5	C18:2;C18:3	95.43
8	5	C18:2;C18:3	90.20

(-) absent

Table 2: Percent fatty acids eluted in different fractions

Fatty acids	% Fatty acids in different fractions								
	1	2	3	4	5	6	7	8	9
C16:0	-	-	46.4	29.49	9.76	2.34	-	-	
C18:1ω9	-	-	-	37.63	34.85	-	-	-	-
C18:2ω6	-	-	-	36.50	38.84	3.27	1.53	0.42	-
C18:3ω6	-	-	-	-	-	38.17	32.01	3.87	-

(-) absent

highest in fraction 6& 7 with minor impurity of plamitic and linoleic acid respectively. Table 3 shows the recovery percent of all the fatty acids, Palmitic acid has highest recovery(87.99%) followed by linoleic acid (80.56%) , GLA (74.05%) and oleic (72.48%). Thus, purification studies using silver silica gel chromatography resulted in 95.43% pure gamma-linolenic acid with 74.05% recovery.

Table 3: Percent recovery of fatty acids

Fatty acid	% Over all recovery
Palmitic acid (C16:0)	87.99
Oleic acid (C18:1ω9)	72.48
Linoleic acid (C18:2ω6)	80.56
γ-linolenic acid C18:3ω6	74.05

Conclusion

The work shows the practical feasibility of recovering and purification of GLA ester from microalgae using a simple and scaleable process. The process consists of simultaneous extraction and trans-esterification of fatty acids in a single step from the microalgal biomass followed by fractionation on a silver-silica gel chromatography column. The one-step extraction-transesterification is practicable with freeze-dried biomass of *Spirulina platensis*; GLA ester of up to 95% purity can be recovered with yields exceeding 70%. Further, the process can be scaled up and process economics can be studied. Process improvements can be done by replacing the stationary phase (silver-silica gel) with longer-life silver loaded alumino silicate columns. Enhancing the GLA content of alga by selection, gene manipulation, or improved cultivation regimens can further improve the GLA from microalgae. For microalgal GLA to be competitive with either plant or fungal oil derived materials, the price of microalgal biomass (dry basis) should be about $5/kg.

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