Purification of Arachidonic Acid from Fungal Single-Cell Oil *via* Al_2O_3-Supported $CuSO_4$ Column Chromatography

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ABSTRACT: A new method has been proven successful to obtain high-purity arachidonic acid (AA), an important human nutrient, from fungal single-cell oil *via* Al_2O_3-supported $CuSO_4$ column chromatography; the stationary phase is stable and readily reusable. In the first step, the mixed FA extracted from saponified fungal single-cell oil, containing 47.3% AA, were purified *via* urea inclusion to afford a fraction of PUFA containing 74.9% AA with an 85.9% yield. The enriched AA fraction was subsequently passed through an Al_2O_3-supported $CuSO_4$ column with a hexane/acetone eluent to provide AA with 90.8% purity at a 46.5% yield. The total yield for the two-step process was 39.9%.

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KEY WORDS: Aluminum oxide, arachidonic acid, column chromatography, cupric sulfate, polyunsaturated fatty acids, purification, saponification, single-cell oil, urea inclusion.

Arachidonic acid (all-*Z*-5,8,11,14-eicosatetraenoic acid), a PUFA that is an essential FA in human nutrition and a direct precursor for a number of biologically active eicosenoids such as prostaglandins, leukotrienes, thromboxanes, and other related metabolites (1), has been shown to have several beneficial effects, including prevention of coronary heart diseases, hypertriglyceridemia and blood platelet aggregation; and the reduction of blood cholesterol, thus reducing the risk of arteriosclerosis (2,3), inflammation, and cancer. In addition, AA is necessary for visual acuity and cognitive development of infants (4).

AA mainly occurs in various animal fats and in lipids or oils produced through fermentation by fungi, such as *Mortierella alpina* and *M. sect. schmuckeri* (5,6), and by microalgae, such as *Porphyridium cruentum* (7). Some commercial products rich in AA have been the main source of high-purity AA in recent years. For example, AA from fungal single-cell oil (ARASCO) (Martek Biosciences Corp., Columbia, MD) contains 38–44% AA; SUN-TGA (Suntory Ltd., Tokyo, Japan), 22–37%; and oil of AA (Wuhan Fuxing Biotechnology Pharmaceutical Corp., Ltd., Wuhan, China), a single-cell oil, ≥42%. However, these oils are not suitable for pharmaceutical applications, which require highly purified AA.

Most of the recent methods to obtain PUFA from oils have been based on the physicochemical properties of FA, such as solubility; polarity; the number, position, and geometric configuration of the double bonds; and chain length. But each method has its advantages and disadvantages. For examples, a lipase-catalyzed reaction and low-temperature crystallization are efficient methods to enrich PUFA (8–11). Vali *et al.* (12) used low-temperature crystallization, selective esterification, and solvent extraction to increase the AA content in single-cell fungal oil from 38.8 to 95.3% with a total yield of *ca.* 71%; however, the low temperature of crystallization (−40°C) is not readily adaptable to industrial-scale production. Urea inclusion (13) is a simple and efficient way to separate PUFA from saturated and monounsaturated FA, but by itself is not sufficient to obtain high-purity target PUFA. Furthermore, to obtain high-purity PUFA, column chromatography methods, especially Ag^+-silica gel column chromatography, have been used widely. Scholfield (14) used a silver nitrate-impregnated silica column and Özcimder and Hammers (15) used silver nitrate-HPLC to separate FAME. Guil-Guerrero *et al.* (7) combined urea inclusion and Ag^+-silica gel column chromatography to obtain high-purity (~97%) AA and EPA in 39.5 and 50.8% yields, respectively, from the red microalga *P. cruentum.* The resolving power of Ag^+-impregnated silica gel is attributed to the reversible charge-transfer complexation of Ag^+ with carbon–carbon double bonds (7,15). However, according to many references and our experiments, silver nitrate on silica gel has notable disadvantages under commonly encountered laboratory conditions: It decomposes under light; it is caustic; the Ag^+ ion is easily reduced to Ag by PUFA (16); the silver nitrate is not easily recycled and reused; and silver nitrate is expensive. These disadvantages make silver nitrate unfavorable for commercial production of high-purity AA.

Because of the electronic structural comparability of Cu^{2+} - and Ag^+, Cu^{2+} may also form reversible charge-transfer complexes with double bonds. But Cu^{2+}, especially $CuSO_4$, is more stable than Ag^+ ($AgNO_3$) under normal laboratory conditions, it is easy to recycle, and it is much cheaper. If $AgNO_3$ were replaced by $CuSO_4$ as stationary phase in column chromatography, the manufacturing costs of high-purity AA products should be much lower, and the method would be more suitable for industrial production. In this paper, we report the first purification process of AA from fungal single-cell oil *via* Al_2O_3-supported $CuSO_4$ column chromatography.

MATERIALS AND METHODS

Materials. Fungal single-cell oil containing ≥42% AA was a product of Wuhan Fuxing Biotechnology Phamaceutical Corp. (Wuhan, China). Standard FA were purchased from Sigma-Aldrich Chemical Co. (Guangzhou, China). All solvents and

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reagents used in these experiments were analytical grade and were obtained from commercial sources.

Extraction of FA from fungal single-cell oil. Fungal single-cell oil (35.3 g) was added to a solution of KOH (15.1 g) in 99.5% (vol/vol) ethanol (400 mL). The mixture was refluxed for 50 min with constant agitation in a nitrogen atmosphere. The reaction mixture was cooled to 5°C for 1 h and was then filtered to remove the oil residue. The residue was washed with 100 mL ethanol (at 5°C). The combined filtrate was concentrated (rotary evaporation) to about 150 mL. Distilled water (450 mL) was added to the concentrated filtrate, and the unsaponifiable matter was separated by extraction with hexane (2 × 80 mL). The aqueous/EtOH phase containing saponified matter was acidified to pH 2 with 1:1 HCl/H_2O (vol/vol), and the FA were recovered by extraction with hexane (3 × 150 mL). The combined extracts were washed with distilled water to pH 7, dried over anhydrous sodium sulfate, and the FA (20.0 g), which were obtained by removing hexane at 45°C, contained 47.3% AA.

Fractionation with urea inclusion. PUFA were isolated by urea inclusion from the FA above according to literature procedure (13,17,18). The solvent was 99.5% (vol/vol) ethanol; the FA/urea/ethanol ratio was 1:2:8 (by wt); the crystallization temperature was −15°C. After filtration, condensation, washing with water, and extraction with hexane, PUFA (12.0 g) were obtained by removing hexane at 45°C (rotary evaporator), of this 12.0 g, 74.9% was AA.

Esterification of PUFA. PUFA (12.0 g) were added to an absolute methanol solution containing 13% BF_3 (48.0 g), and the mixture was refluxed for 10 min with constant agitation in a nitrogen atmosphere. The mixture was cooled to room temperature, and 150 mL of saturated NaCl solution was added. The PUFA methyl esters were extracted with hexane (3 × 100 mL) and dried over anhydrous sodium sulfate. The PUFA methyl esters (12.3 g) were obtained by removing hexane at 40°C (rotary evaporator).

Preparation of Al_2O_3-supported $CuSO_4$ column. Al_2O_3 (33.3 g) (chromatography; 44–178 μm) was suspended in a saturated aqueous solution of $CuSO_4 \cdot 5H_2O$ (29.9 g), which corresponded to 22.7% of dry Cu(II) on Al_2O_3. The mixture was dried and activated for 16 h at 150°C in vacuum drying oven (0.67 kPa), then this material was cooled to room temperature under the same pressure and was kept in a desiccator.

The column (a 2 × 30 cm glass column) (designated as column-1) was packed with 40 g Al_2O_3-supported $CuSO_4$, and the height of the stationary phase was about 16 cm.

Fractionation of PUFA methyl esters. A hexane solution of PUFA methyl esters (2 mL, containing 200–500 mg PUFA methyl esters) was applied onto column-1 to study the effect of sample load on separation. Various binary mixed solvents were used as eluents to select elution mode. The flow rate was controlled at 3 mL/min; twenty 30-mL eluate fractions were collected.

Recycling of Al_2O_3-supported $CuSO_4$. Acetone (100 mL) was added to column-1, which had been used as described in the preceding paragraph, to wash off the residue of PUFA esters. The resulting solid mixture was dried to a free-flowing powder in open air, and then this material was activated again under the same conditions (16 h at 150°C; 0.67 kPa) and prepared for use in a new column (designated as column-2). Column-2 was recycled again with the same method to prepare column-3.

GC analysis. The FA and PUFA compositions were analyzed with Agilent Technologies 6980N gas chromatograph equipped with an FID (Palo Alto, CA). The column used was an HP-5 (30 m × 0.32 mm; Hewlett-Packard, Wilmington, DE). The oven time–temperature profile was as follows: 120°C (4 min), 8°C per min to 240°C, 240°C (10 min). The temperatures of the injector and detector were set at 250 and 260°C, respectively. One microliter of sample was injected at a split ratio of 1:10. The internal standard was pentadecanoic acid methyl ester (15:0 methyl ester). The peaks of these esters were identified by comparison with standard FA processed under the same chromatographic conditions. Each sample was analyzed in triplicate.

RESULTS AND DISCUSSION

The purification of AA from fungal single-cell oil was accomplished by the following process: oil saponification and FA extraction, fractionation of FA with urea inclusion to enrich PUFA, PUFA methylation, and elution through Al_2O_3-supported $CuSO_4$-column chromatography to obtain highly purified AA. In the present work, fractionation of FA with urea inclusion was carried out under the conditions described in the Materials and Methods section, and 74.9% AA content was achieved with 85.9% yield. Table 1 shows the FA composition of saponified single-cell oil at various stages of purification. The saturated and monounsaturated FA for which analyses were made were removed by the urea inclusion process, and the content of diunsaturated FA was reduced. The method was proven reliable and effective for the concentration of PUFA (17).

Selection of eluents for gradient elution. The PUFA methyl ester contents following elution by various pure and binary mixed solvents through the Al_2O_3-supported $CuSO_4$ column are shown in Table 2. The nonpolar solvent hexane had less separation selectivity than hexane/acetone binary solvents. On the other hand, in the range of 0.05–0.2% acetone in hexane, the stronger the polarity of the binary mixed solvents was, the higher the purity of the AA. However, when the polarity of the mixed solvents (>0.2% acetone in hexane) is too strong, poor separation selectivity and lower purity result. Combining the better purity and recovery of AA, the gradient elution was chosen as follows: (i) 50 mL 0.05% acetone in hexane; (ii) 100 mL 0.1% acetone in hexane; (iii) 100 mL 0.2% acetone in hexane (vol/vol). AA content and recovery rate are given in Table 1.

Effect of sample load on separation. The PUFA methyl esters were separated by column-1 using the gradient elution described in the preceding paragraph (flow rate: 3 mL/min). The recoveries of the products containing AA were more than 45%. The effect of sample load on separation is shown in Figure 1,

TABLE 1
FA Composition (wt%)[a] and AA Content at Different Stages of Enrichment of AA from Single-Cell Oil

FA	Saponification	Urea inclusion	Chromatography column-1
14:0	0.36 ± 0.05		
16:0	8.64 ± 0.15		
18:0	5.48 ± 0.13		
18:1	5.64 ± 0.14		
18:2	2.79 ± 0.13	4.24 ± 0.05	
18:3	3.06 ± 0.12	6.67 ± 0.12	3.06 ± 0.02
20:1	0.98 ± 0.01		
20:2	1.25 ± 0.08		
20:3	5.59 ± 0.09	8.81 ± 0.13	2.62 ± 0.01
20:4	47.3 ± 0.3	74.9 ± 0.3[b]	90.7 ± 0.5[c]
21:4	3.24 ± 0.01	2.61 ± 0.03	3.58 ± 0.01
22:0	4.22 ± 0.01		
24:0	10.9 ± 0.01		

[a]Each sample analyzed in triplicate. Data presented as mean ± SD.
[b]The recovery rate was 85.9%.
[c]The recovery rate was 46.5%. AA, arachidonic acid.

TABLE 2
PUFA Ester Composition (wt%) and AA Content at Al_2O_3-Supported $CuSO_4$ Column[a] Chromatography Stage with Various Solvents

	Applied eluents: % acetone in hexane[b]					
PUFA	0.0%	0.05%	0.1%	0.2%	1%	5%
18:2	9.30 ± 0.14	12.9 ± 0.21	2.08 ± 0.01		4.15 ± 0.03	4.14 ± 0.05
18:3	8.07 ± 0.12	17.1 ± 0.17	8.20 ± 0.06	3.00 ± 0.02	10.0 ± 0.11	6.57 ± 0.12
20:3	9.76 ± 0.14	11.8 ± 0.06	8.06 ± 0.05	2.52 ± 0.01	7.04 ± 0.03	8.71 ± 0.13
20:4	72.5 ± 0.2 (91.3 ± 0.2)[c]	50.7 ± 0.2 (87.3 ± 0.3)[c]	78.1 ± 0.2 (77.6 ± 0.2)[c]	90.6 ± 0.5 (45.6 ± 0.2)[c]	76.1 ± 0.3 (78.2 ± 0.3)[c]	74.8 ± 0.3 (92.7 ± 0.3)[c]
21:4			1.88 ± 0.02	3.28 ± 0.01	1.58 ± 0.01	2.51 ± 0.03

[a]Column-1 (40 g Al_2O_3-supported $CuSO_4$ and 2 mL hexane solution of 358 mg PUFA methyl ester). The flow-rate was controlled at 3 mL/min.
[b]Each sample analyzed in triplicate. Data presented as mean ± SD.
[c]AA recovery. For abbreviation see Table 1.

which demonstrates that the purity of AA changed with increasing sample load. The purities of the product were high when the sample loads were less than 1.2 g. However, the purities of AA decreased significantly once the load exceeded 1.2 g. So, the loading amount should be limited to less than 1.2 g/100 g Al_2O_3-supported $CuSO_4$.

Recycling of Al_2O_3-supported $CuSO_4$. Table 3 shows the separation efficiency of the recycled Al_2O_3-supported $CuSO_4$ as stationary phase under the same eluting conditions with the same loading amounts. The purities and recoveries of AA products slightly decreased when column-2 and column-3 were used. However, the change in separation efficiency was so small that the stationary phase could be recycled at least three times in order to reduce the cost.

The Cu^{2+} ion, similar to Ag^+ ion, can form complexes with the double bonds of the PUFA methyl esters. The retention of PUFA methyl esters on Al_2O_3-supported $CuSO_4$ should be controlled by their degree of unsaturation. The influence of the concentration of acetone in hexane on the retention of the PUFA methyl esters may be due to competitive complex formation with Cu^{2+} sites. We compared the results obtained by using two different stationary phases: Al_2O_3-supported $CuSO_4$ and silica gel-supported $AgNO_3$. Table 4 shows that the Al_2O_3-

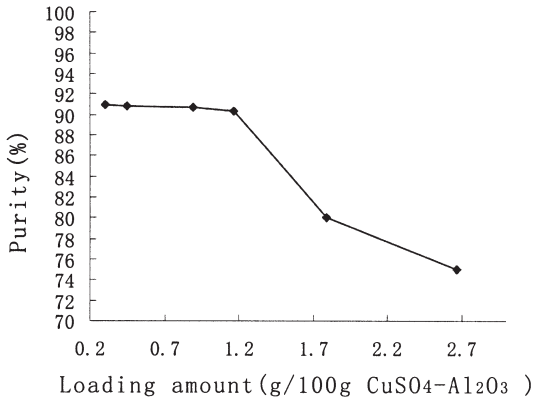

FIG. 1. The purity of arachidonic acid as a function of sample load on column.

TABLE 3
The Separation Efficiency of Initial and Recycled Al_2O_3-Supported $CuSO_4$ Column Chromatography[a]

Column[b]	AA purity[c]	AA recovery[c]
Column-1	90.7 ± 0.5	46.5 ± 0.2
Column-2	90.4 ± 0.5	45.5 ± 0.2
Column-3	89.7 ± 0.5	43.9 ± 0.2

[a]The elution follows this sequence: (i) hexane with 0.05% acetone; (ii) hexane with 0.1% acetone; (iii) hexane with 0.2% acetone (vol/vol). The flow rate was 3 mL per min.
[b]Each column had 40 g Al_2O_3-supported $CuSO_4$, and 358 mg PUFA methyl esters in 2 mL hexane solution was loaded.
[c]Each sample analyzed in triplicate. Data presented as mean ± SD.

TABLE 4
Comparison of Separation Efficiency of Silica Gel-Supported $AgNO_3$ and Al_2O_3-Supported $CuSO_4$ Column Chromatography

Column	AA purity[a]	AA recovery[a]
$CuSO_4$-Al_2O_3[b]	90.7 ± 0.5	46.5 ± 0.2
Ag+-silica gel[c]	91.0 ± 0.4	55.5 ± 0.2

[a]Each sample analyzed in triplicate. Data presented as mean ± SD.
[b]Column-1 (see Table 3).
[c]Silica gel-supported $AgNO_3$ column was prepared according to Guil-Guerrero et al. (7): 100 g silica gel (60–200 μm) and silver nitrate (10 g) in 95% (vol/vol) ethanol (250 mL) was mixed for 10 min. Ethanol was evaporated and the silver-impregnated silica gel was activated by overnight heating at 120°C. The silica gel-supported $AgNO_3$ (15 g) thus activated was packed into a glass column. PUFA esters (358 mg) were loaded onto the column and eluted with solvents in the following sequence: (i) hexane with 0.5% acetone; (ii) hexane with 1% acetone; (iii) hexane with 5% acetone; (iv) hexane with 10% acetone (vol/vol).

supported $CuSO_4$ column chromatography provided a slightly lower recovery rate of AA. Even so, the stability, recyclability, low cost, and easy availability of $CuSO_4$ make Al_2O_3-supported $CuSO_4$ column chromatography a valuable method for the purification of PUFA. With further development, Al_2O_3-supported $CuSO_4$ column chromatography may be a competitive commercial method for the fractionation of PUFA esters.

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