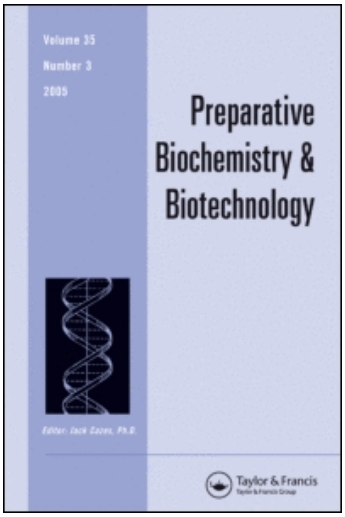

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Separation and Preparative Purification of Arachidonic Acid from Microbial Lipids by Urea Inclusion Reaction and HPLC

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Abstract: Arachidonic acid (AA) was separated and purified from microbial lipids by the combined method of urea inclusion reaction and reversed-phase high performance liquid chromatography. At first, AA was concentrated from free fatty acids made from microbial lipids by a urea inclusion reaction. The optimum conditions were as follows: methanol was the suitable solvent, the ratio of free fatty acids to urea to methanol was 1:2:8 (wt/wt), and the temperature of the urea inclusion reaction was -10°C. The AA content was increased from 38% to 79%, and then AA was purified on a C_{18} preparative column (300 mm × 30 mm I.D., $d_p = 15$ μm), using methanol-water (95:5, v/v) as the mobile phase, at a flow rate of 5 mL/min. The purity of AA after two steps purification reached 99%. This result indicates that the combined method of the urea inclusion reaction and reversed-phase high performance liquid chromatography is a promising technique for purification of AA.

Keywords: Arachidonic acid, Polyunsaturated fatty acids, Urea inclusion reaction, Reversed-phase high performance liquid chromatography, Separation, Purification

INTRODUCTION

Arachidonic acid (5,8,11,14-eicosatetraenoic acid, AA - Figure 1), belonging to polyunsaturated fatty acids (PUFAs) of the n-6 family, is an essential dietary component for the human body and an important precursor of many eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes.[1–3]

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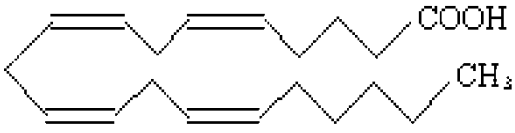

Figure 1. Chemical structure of arachidonic acid.

AA has various physiological functions and plays an important role in adjusting the cardiovascular system and infant nutrition.[4–6]

In recent years, the isolation and purification of PUFAs has taken the attention of many researchers, due to their broad physiology activity. At present, there are two developing aspects in the study of AA products. One is microbial lipids by fermentation with fungi, and the other is highly purified AA biological preparation. The former product is applied as a nutrition enhancer and functional healthcare food, while the latter are normally used as bio-pharmacy and chemical materials. In the last decade, AA fermented by microbes has been focused in many countries, such as America, Japan, canada,[7–9] and especially in China, where the production of AA in 50-ton fermentors was performed.[10]

Over the last 20 years, several methods have been developed to separate and purify PUFAs, such as crystallizing at low-temperature,[11] the urea inclusion reaction (UIR),[12–14] supercritical CO_2 extraction,[15,16] silver resin chromatography,[17,18] the lipase catalyzed reaction,[19] and reversed-phase high performance liquid chromatography (RP-HPLC).[20,21] However, there have been fewer reports on efficient methods for the preparative purification of AA, since it was very difficult to separate all the components because their properties were close to each other and any improvement in the separation of some compounds will result in decreased separation capacity for the remaining compounds. In the present study, we report a combined method of UIR and reversed-phase HPLC to separate AA from microbial lipids.

EXPERIMENTAL

Materials

The microbial lipids (AA: 38.3%, GC) were offered from Wuhan Alking Bioengineering Co. Ltd (Wuhan, Hubei, China). HPLC reagents were of chromatographic grade and purchased from Burdick & Jackson (Honeywell, Muskegon, MI, USA). Other organic solvents were analytical grade and were purchased from Shanghai Chemical Factory, Shanghai, China.

Apparatus

Reversed-Phase HPLC

The chromatographic system (Waters LC series 4000, Milford, MA, USA) consisted of a quaternary pump, a 10 mL injection loop, a Waters 410 differential refractometric detector, and a Waters Chem-Station for data analysis (Millennium 32, 3.20 version). A Rheodyne Model 3725 injector (Cotati, CA, USA) was used to inject preparative samples. The separation was performed with a μ-Bondpak^{TM} (Waters, Milford, MA, USA) C_{18} column (300 mm × 30 mm I.D., $d_p = 15$ μm). A Shen Sheng R-201 rotary evaporator (Shanghai, China) was used to concentrate the liquid samples. The mobile phase consisted of methanol-water (95:5, v/v) and the flow-rate was 5 mL/min. All chromatographic separations were carried out at room temperature. The injection volume of the samples was 2 mL.

GC-MS

The GC-MS system consisted of a gas chromatograph (Perkin-Elmer Auto System XL GC, Norwalk, CT, USA), a mass selective detector (Perkin-Elmer Turbo Mass) and a Perkin-Elmer Chem-Station for data analysis. The running conditions were as follows: Separation was performed on a silex capillary column of SE-54 (30 m × 0.25 mm, 0.25 μm) made by SULPECO (Bellefonte, PA, USA). The oven temperature was initially set at 80°C, and was then raised to 250°C at 5°C/min and maintained for 10 min. The injector and detector temperatures were set at 250°C and 280°C, respectively. Helium was used as the carrier gas at a constant flow-rate of 0.8 mL min^{-1} after an initial head pressure of 100 kPa, with the split ratio at 30:1.

The capillary column was directly interfaced to a PE Turbo mass-selective detector, which was operated in the electron impact (EI) mode. The energy of the electron beam was 70 eV. The ion source temperature was 180°C. Full-scan acquisition mode was used for detection. The mass scanning range is from 45 to 350 (m/z). Samples of 1.0 μl were injected manually.

Separation Procedures

Preparation of Free Fatty Acids

The mixture of 50 g microbial lipids and 300 mL of 0.5 mol/L NaOH-ethanol in a rocked flask was put in a waterbath at 80°C for 1 h to recirculate and render the lipids saponified. Afterwards, appropriate volumes of water were injected into the rocked flask to make the soap dissolve completely, and then the solution was acidified with 1.0 mol/L HCl to pH 2-3. The solution was further extracted with ether-petroleum ether (1:1, v/v) and washed with

distilled water to a neutral pH. Finally, free fatty acids (FFA) were obtained by low-pressure rotary evaporation at 60°C. The iodine value of the FFA was measured by Wijs-GB/T 5009.37–1996.[22] The content of AA in the free fatty acids was measured with a gas chromatograph.

Preparation of Fatty Acid Methyl Esters (FAME)[23]

Concentration of AA by UIR

After the urea was dissolved thoroughly, the preparative FFA or FAME was added to the solution (the compounding ratios are shown in Table 1). The solution was mixed adequately and recirculated in 70°C water for 1 h. Then, the sample was put into a low temperature installation for overnight. The next day, the sample was taken out and quickly filtered with a Buchner funnel with a vacuum pump. Afterwards, the same volume of water was added to the filtered liquid phase of the sample. The solution was then acidified to pH 2–3 with 1.0 mol/L HCl (this step was eliminated for the FAME). The aqueous layer of the solution was extracted with ether-petroleum ether (1:1, v/v), two or three times. Then, the organic layers were combined, and washed with distilled water to neutral pH. Finally, PUFAs or esters in the liquid phase were obtained by rotary evaporation at 60°C. At the same time, the solid phase of the sample was dissolved by the addition of the same amounts of water, and adjusted pH 2–3 with 1.0 mol/L HCl (this step was eliminated in the FAME). FFA or FAME was obtained from the solid part through the same procedure as described above. The PUFAs or esters obtained from the two phases were weighed, respectively. The iodine values of free fatty acids and methyl esters were measured by Wijs.[22] The content of AA in FFA and FAME were measured with GC.

Separation of AA by HPLC

After being trans-methylated, the FFA obtained by the urea inclusion reaction was dissolved in appropriate volumes of absolute methanol (0.2–0.3 g/mL). The solution was filtered through a 0.2 μm membrane filter. Afterwards, the solution was purified by HPLC on a μ-Bondpak™ C_{18} preparative

Table 1. The ratio of FFA or FAME, urea and solvent

Sample number	Free fatty acids or esters	Urea	Solvent
1	1(FFA)	2	10
2	1(FFA)	2	8
3	1(FFA)	3	8
4	1(FFA)	2	6
5	1(FAME)	2	8
6	1(FAME)	3	8

column (300 mm × 30 mm I.D., $d_p = 15$ μm), using methanol-water (95:5, v/v) as the mobile phase, at a flow rate of 5 mL/min and detected by a 410 Waters differential refractometer. The injection volumes of samples were 2 mL. The products were collected in stages every 2 min, and the effluent liquid was mixed for every 3 cuvettes. The samples were obtained by low-pressure rotary evaporation at 60°C.

Analyses and Identification of Concentrated Products by GC-MS

All condensates were analyzed and identified using gas chromatography-mass spectrometry. The peaks of the total ion current chromatogram were scanned and the corresponding mass spectrogram was obtained. The fatty acids species can be identified from the fragments in the ion spectra by matching the compounds in the Wiley standard spectrogram library. The AA contents were calculated by a peak area normalization method.

RESULTS AND DISCUSSION

Effects of Urea/Acids (esters) Ratio and the Reactants Included by Urea on UIR

According to the data of Table 1, the effects of the urea inclusion reaction were studied. The common reaction conditions were: Temperature: −10°C, Solvent: methanol. The results are shown in Figure 2.

Amongst all of the groups tried (as listed in Table 1), it can be seen that Group 2 (FFA: urea: methanol = 1:2:8(wt/wt)) was the most suitable for concentration and recovery of AA in six groups, which is different from previous reports on DHA isolation by the urea inclusion reaction.[13,14] The content of AA in the liquid phase increased, while the total recovery percentage of AA decreased. The results also indicate that the FFA reacted with urea more easily than FAME. Moreover, the solvent simply played the role of dissolving urea in this reaction. So, the quantity of solvent has little effect on the content of AA.

Effects of Temperature on UIR

We investigated the effects of temperature on the urea inclusion reaction; the ratio of FFA:urea,:methanol was 1:2:8.

It is shown, in Figure 3, that the effects of temperature on the urea inclusion reaction were significant. It can be seen that the content of AA in the liquid phase increased, evidently, with the decrease of temperature when the temperature was above −10°C. However, the increase of AA

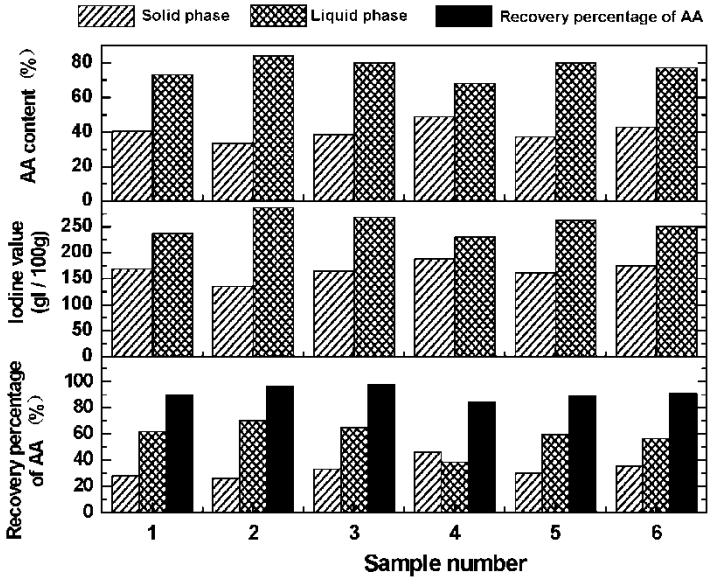

Figure 2. Effects of urea/acids (esters) and the reactants included by urea on the urea inclusion reaction; Temperature: −10°C; Solvent: methanol.

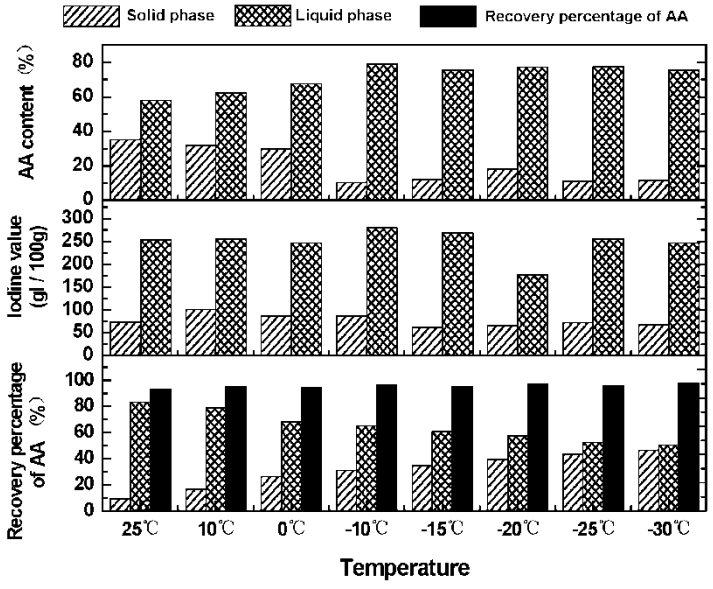

Figure 3. Effects of temperature on the urea inclusion reaction; FFA: urea: methanol = 1:2:8(wt/wt).

Table 2. Effects of solvents on UIR; FFA: urea: alcohol = 1:2:8 (wt/wt); Temperature: −10°C

	Iodine value (gI/100 g)		AA content (%)		AA recovery percentage (%)		
Solvent	SP[a]	LP[b]	SP	LP	SP	LP	SP + LP
Methanol	99.87	251.89	15.83	67.98	19.5	74.7	94.2
Ethanol	88.80	252.95	25.56	64.19	28.3	67.5	95.8
Isopropyl alcohol	91.32	236.15	28.43	59.78	24.1	60.8	84.9

[a]SP: Solid phase; [b]LP: Liquid phase.

content was not so obvious when the temperature arrived below −10°C. Furthermore, in view of the lower temperature, the energy consumption and equipment investment would be very costly. So, the optimum urea inclusion temperature was selected at −10°C.

Effects of Solvents on UIR

The effects of solvents on the urea inclusion reaction are reported in Table 2.

Under the same conditions, methanol was more suitable for the concentration of AA than ethanol and isopropanol. In addition, urea was more difficult to dissolve in ethanol than in methanol; the reaction temperature would exceed 80°C, whereas the reaction temperature of methanol with urea is 70°C. Thus, not only free fatty acids were prone to decomposition or oxidation, but also energy consumption and cost would increase. Hence, methanol was superior to ethanol and isopropyl alcohol.

Effects of Secondary Urea Inclusion Reaction

The products of the liquid phase at −15°C from the first extraction mentioned above were processed with urea, using the proposed procedure once again. The reaction temperature was −10°C. The results are shown in Table 3.

Table 3. The results of secondary UIR; FFA: urea: solvent = 1:2:8 (wt/wt); Temperature: −10°C

Temperature (°C)	Iodine value (gI/100 g)	AA content (%)	AA recovery percentage (%)
−10	231.85	77.32	69.5
−20	283.11	82.10	65.6
−30	282.70	83.71	68.4

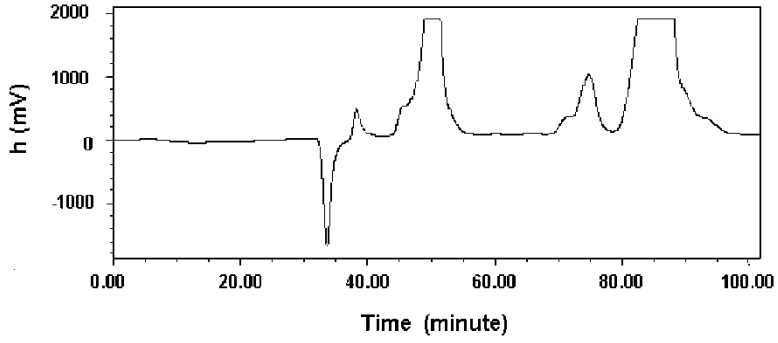

Figure 4. HPLC Chromatogram of AA methyl ester, with substantial overloading, on a C_{18} preparative column, using methanol-water (95:5, v/v) as the mobile phase at a flow rate of 5 mL/min. The injection volume of samples was 2 mL.

The content of AA in the products of the liquid phase was found to increase slightly (from 77% to 83%) when the sample was enriched twice. There may be saturation for urea to include free fatty acids. Therefore, it is of little significative for the secondary urea inclusion reaction.

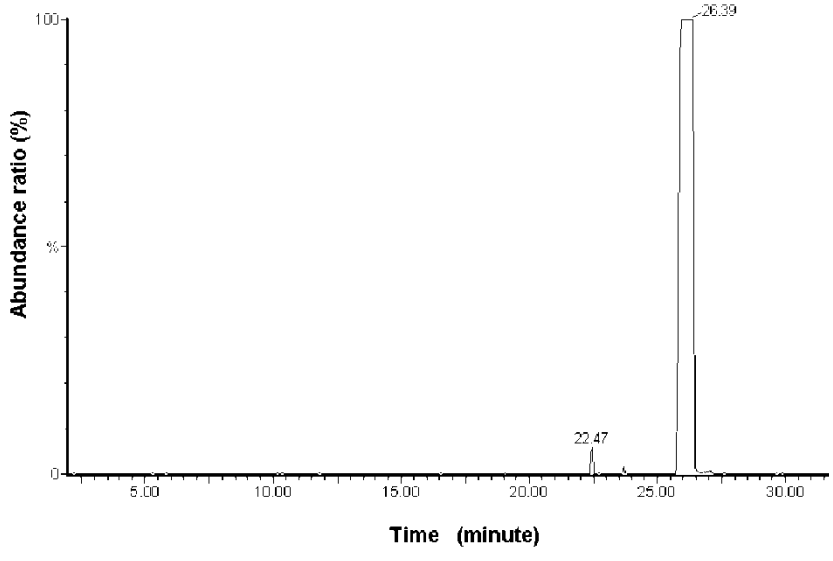

Figure 5. GC-MS total ion current chromatogram of sample from preparative reverse-phase HPLC; performed on SE-54; temperature programme: 80°C–250°C (10 min). The energy of the electron beam was 70 eV. The mass scanning range is from 45 to 350 (m/z); 1.0 μL samples were injected manually.

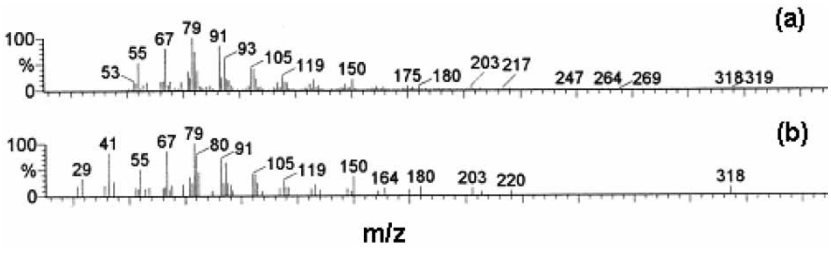

Figure 6. The mass spectrum of AA methyl ester; a) mass spectrum of sample; b) standard mass spectrum of AA methyl ester in Wiley Library.

Preparative Separation of AA by Reversed-Phase HPLC

The samples were separated and purified by preparative reversed-phase HPLC, with substantial overloading; the injection volumes of samples were 2 mL. The HPLC chromatogram is shown in Figure 4.

Identification of AA by GC-MS

According to Figure 4, we collected the effluent liquid every 2 min, from 40 min to 100 min. After being concentrated by rotary evaporation at 60°C, the samples were analyzed and identified by GC-MS. The total ion current chromatogram of the sample in 83–88 min is given in Figure 5.

The mass spectrum of the HPLC chromatographic peak with the retention time of 26.39 min was compared with that of the AA methyl ester standard mass spectrum (as shown in Figure 6). It can be seen that they were very similar with the same base peak at m/z 79, and other major peaks at m/z 55, 91, 105, and 150. So, from the information given by the GC-MS, it can be confirmed that the main HPLC chromatographic peak was the AA methyl ester. The AA content was 99.5%, calculated by the area normalization method.

CONCLUSION

In this paper, a simple separation and preparative purification method for AA from microbial lipids is presented. At first, AA was concentrated by a urea inclusion reaction from free fatty acids made from microbial lipids. Then, the samples were separated and purified by preparative reversed-phase HPLC, with substantial overloading. Finally, milligram quantities of AA at higher than 99% purity were obtained. The experimental results show that the content of AA increased as the iodine value of free fatty acids increased. But, the recovery percentage of AA decreased while the content

of AA increased. In summary, the proposed method, which combined the use of the urea inclusion reaction and reversed-phase HPLC, can provide highly efficient preparative purification of AA from microbial lipids.

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