An Efficient Method for the Purification of Arachidonic Acid from Fungal Single-Cell Oil (ARASCO)

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ABSTRACT: PUFA, such as arachidonic acid (AA), have several pharmaceutical applications. An efficient method was developed to obtain high-purity arachidonic acid (AA) from ARASCO, a single-cell oil from Martek (Columbia, MD). The method comprises three steps. In the first step, AA was enriched from saponified ARASCO oil by low-temperature solvent crystallization using a polar, aprotic solvent, which gave a FA fraction containing 75.7% AA with 97.3% yield. The second step involved enriching AA content *via* lipase-catalyzed selective esterification of FA with lauryl alcohol. When a mixture of 1 g FA/lauryl alcohol (2:1 mol/mol), 50 mg *Candida rugosa* lipase, and 0.33 g water was incubated at 50°C for 24 h with stirring at 400 rpm, the AA content in the unesterified FA fraction was as much as 89.3%, with *ca.* 90% yield. Finally, a solvent extraction procedure, in which acetonitrile was the extracting solvent, was used to enrich AA from FA fraction dissolved in *n*-hexane. The best results were obtained when 2 g FA was dissolved in 80 mL hexane and extracted twice, each time with 20 mL acetonitrile at –20°C, by allowing 2 h storage. This step gave a FA fraction containing 95.3% AA with 81.2% yield. By using this three-step process the AA content in the saponified single-cell oil (ARASCO) was increased from 38.8 to 95.3% with a total yield of *ca.* 71%.

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KEY WORDS: Arachidonic acid, *Candida rugosa* lipase, crystallization, esterification, homo-γ-linolenic acid, γ-linolenic acid, lipase, single-cell oil, solvent extraction.

Arachidonic acid (all-*cis*-5,8,11,14-eicosatetraenoic acid) is an EFA to human nutrition and is a direct precursor for a number of biologically active eicosenoids, such as prostaglandins, leukotrienes, thromboxanes, and other related metabolites (1). These eicosenoids exhibit regulatory effects on lipoprotein metabolism, blood rheology, vascular tone, leukocyte function, platelet activation, and cell growth. The application of arachidonic acid (AA) to infant diets is particularly important to ensure optimal conditions for developing membrane-rich systems such as brain, nervous, and vascular systems (2,3). In addition, AA is necessary for the visual acuity and cognitive development of infants (4).

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Various processes have been described for production of lipid- or oil-containing AA by fermenting fungi, such as *Mortierella alpina*, *M. sect. schmuckeri* (5,6), and microalgae, such as *Porphyridium cruentum*, AA (7). Some commercial products rich in AA are ARASCO (Martek Biosciences Corporation, Columbia, MD), a single-cell oil with 38–44% AA content, and SUN-TGA (Suntory Limited, Tokyo, Japan), also a single-cell oil, with 22–37% AA content. These oils are encapsulated and marketed as dietary supplements. The pharmaceutical industry requires highly purified products for preclinical and clinical trials (8). The technologies available for purifying PUFA from oils are based on physicochemical properties of FA such as the number, position, and geometric configuration of the double bond; m.p.; polarity; solubility; and chain length.

Ratnayake *et al.* (9) used urea inclusion to purify PUFA by removing most of the saturated and monounsaturated FA. However, this method alone is not sufficient to obtain high-purity target PUFA if the oil contains appreciable amounts of other PUFA. Purification of EPA and AA from *P. cruentum* oil was carried out by combining urea inclusion and silver-silica gel column chromatography (8). In recent years, a number of workers have demonstrated that lipase-catalyzed reactions can be used to enrich PUFA in various oils. Shimada *et al.* (10) increased the AA content from 25 to 50% *via* selective hydrolysis of single-cell oil with *Candida rugosa* lipase. However, the AA content could not be increased to more than 60%, even though the hydrolysis was repeated three times. Enrichment of AA content from 25 to 75% with 71% recovery of its initial content was achieved by repeated selective esterification and urea adduct formation of single-cell oil FA (11). Thus, the processes mentioned above do not yield high-purity (>90%) AA. However, low-temperature solvent crystallization and lipase-catalyzed reaction have been shown to be very effective for enriching PUFA (12–14). In the present study, an attempt was made to obtain high-purity AA with high yield from saponified single-cell oil (ARASCO) FA by using low-temperature solvent crystallization, selective esterification, and solvent extraction.

EXPERIMENTAL PROCEDURES

Materials. Lipase from *C. rugosa* was purchased from Sigma Chemical Co. (St. Louis, MO). Fungal single-cell oil containing

ca. 40% AA was a commercial product of Martek Biosciences Corporation. 1-Dodecanol (lauryl alcohol, 98%) was purchased from Acros Organics (Geel, Belgium). Standard FA were obtained from Sigma Chemical Co. The internal standard pentadecanoic acid (>99%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All solvents and reagents were either HPLC-grade or AR-grade and were obtained from commercial sources.

Preparation of FFA from fungal single-cell oil. Fungal single-cell oil (25 g, pretreated with 200 ppm EDTA) was added to a solution of KOH (5.75 g) in water (11 mL) and 99.5% ethanol (66 mL). The mixture was refluxed at 65°C under nitrogen atmosphere, and the reaction was monitored by analytical TLC (silica gel; eluted with 80:20:0.5, hexane/ethyl acetate/acetic acid, by vol). The saponification was completed in less than 2 h, and the reaction mixture was allowed to cool to room temperature. Distilled water (100 mL) was added to the saponified mixture, and the unsaponifiable matter was separated by extraction with hexane (2 × 50 mL) and discarded. The aqueous phase containing saponifiable matter was acidified to pH 2 with HCl/H_2O = 1:1 (vol/vol). The mixture was transferred to a separatory funnel, and the FA were recovered by extraction with hexane (2 × 100 mL). The extract was washed with water to neutral pH, and the hexane layer was then dried over anhydrous sodium sulfate. The solvent was removed at 40°C to recover FFA, which were then stored at –20°C. The FA obtained with an AA content of 38.8% was designated as FFA-I.

Low-temperature solvent crystallization. FFA-I (4 g) was dissolved in acetonitrile (300 mL) in a stoppered glass vessel. The mixture was stirred with a magnetic stirrer at 50°C under N_2 until all FA were completely dissolved. The solution was allowed to cool at room temperature and then stored in an ultra-low-temperature freezer (Sanyo MDF-192; Gunma, Japan) chamber at –40°C for 48 h. The solid and liquid phases were separated immediately after removal from the cold storage by vacuum filtration using an Ace Büchner funnel (25–50 mm). FFA in the liquid phase were recovered by removing the solvent in a vacuum rotary evaporator at 35°C and were then stored at –20°C. This liquid fraction with 75.7% AA was designated as FFA-II. The yield of AA was defined as the ratio of the weight of recovered AA in the liquid phase to the weight of AA in the saponified single-cell oil.

Selective esterification. The enrichment of AA *via* lipase-catalyzed selective esterification was carried out according to the procedure described by Shimada *et al.* (11) with some modifications. Typically, FFA-II (1.0 g), lauryl alcohol (0.31 g), *C. rugosa* lipase powder 50.0 mg (5% based on weight of the FA substrate), and 0.33 mL water were added to a 7-mL flat-bottomed glass vial sealed with Teflon-lined caps. Simultaneously, selective esterification of FFA-I was carried out under similar reaction conditions except using a FFA/lauryl alcohol molar ratio of 1:1. The reaction mixture was incubated in a water bath for 30 h at 50°C and magnetically stirred at 400 rpm. Aliquots of the reaction mixture (100 μL) were withdrawn at regular times to analyze AA content in the unesterified FA fraction. To this sample, 500 μL *n*-hexane and 100 μL pentadecanoic acid as internal standard were added. The aqueous phase containing unreacted FFA was separated by centrifugation after adding 200 μL 1 N KOH solution. FFA in the aqueous phase were extracted with *n*-hexane after acidification to pH < 2 with aqueous HCl. FA compositions were analyzed by GLC. The unesterified FA fraction, with an AA content of 89.3%, was designated as FFA-III.

Solvent extraction. FFA-III (2 g) was dissolved in *n*-hexane (80 mL) in a conical flask, and the contents were transferred to a separatory funnel. To this solution, 20 mL of acetonitrile was added, and the mixture was shaken vigorously for *ca.* 4 min. The separatory funnel was kept at –20°C for about 2 h to allow maximum separation of a hexane-rich phase and an acetonitrile-rich phase. The lower acetonitrile-rich phase was collected. Extraction was repeated by adding 20 mL of fresh acetonitrile solvent to the hexane-rich phase. After two extractions the acetonitrile-rich fractions were combined and solvent was evaporated under vacuum. This product was designated as FFA-IV and was kept at –20°C.

GLC analysis of FA composition. FFA were converted into their corresponding methyl esters (FAME) by heating with BF_3/methanol. The FFA composition was analyzed by a China Chromatography model 8700F (Taipei, Taiwan) gas–liquid chromatograph equipped with an FID. The column used was SP-2330 (30 m × 0.25 mm i.d.; Supelco, Bellefonte, PA). The temperatures of the injector and detector were set at 250 and 270°C, respectively. The column was held at 160°C for 2 min and then increased to 235°C at a constant rate of 15°C/min, then kept at 235°C for 8 min. One microliter of sample was injected at a split ratio of 1:50. The FAME peaks were identified and calibrated by using authentic standard FA. Pentadecanoic acid ($C_{15:0}$) was used as internal standard.

RESULTS AND DISCUSSION

Enrichment of AA by low-temperature solvent crystallization. Low-temperature solvent crystallization was developed decades ago for separating FA or their esters (15,16). In earlier reports from this laboratory, the concentration of PUFA from borage and linseed oils (12) and the enrichment of EPA and DHA from menhaden oil (17) by low-temperature solvent crystallization were described. In the present investigation, concentration of AA by low-temperature solvent crystallization was designed to achieve a FA fraction enriched in AA and, at the same time, to maintain a high yield of AA. We succeeded in concentrating AA (~76%) with high yield (~97%) by a simple solvent crystallization of FFA-I using acetonitrile as solvent. Table 1 shows the FA composition of saponified single-cell oil (ARASCO) as well as FA compositions after different stages of purification. The results show that all saturated FA, 83% monounsaturated, and 38% diunsaturated FA were removed. The high recovery of AA (97.3%) suggested that AA possesses a high solubility and a low crystallization temperature in acetonitrile. A number of common organic solvents were screened to test their effects

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

FA	Saponification (FFA-I)	Solvent[b] crystallization (FFA-II)	Selective[c] esterification (FFA-III)	Solvent[d] extraction (FFA-IV)
14:0	1.38 ± 0.05			
16:0	13.12 ± 0.10			
16:1	0.63 ± 0.02			
18:0	9.23 ± 0.12			
18:1	16.28 ± 0.20	2.85 ± 0.21		
18:2	6.54 ± 0.13	9.85 ± 0.72	0.53 ± 0.20	
18:3	5.46 ± 0.08	5.44 ± 0.42	4.72 ± 0.43	1.44 ± 0.22
20.0	0.71 ± 0.05	ND[e]		
20:3	4.35 ± 0.08	6.10 ± 0.12	5.34 ± 0.44	3.31 ± 0.31
20:4	38.82 ± 0.34	75.72 ± 0.65 (97.3 ± 0.8)[f]	89.3 ± 0.6 (89.8 ± 2.5)[f]	95.25 ± 1.1 (81.2 ± 2.1)[f] (70.94)[g]
22:0	2.31 ± 0.12	ND		
24:0	1.11 ± 0.06	ND		

[a]Mean absolute deviation of three independent determinations.
[b]Solvent = CH_3CN, solvent/FFA = 75 mL/g, temperature = –40°C, storage time = 48 h.
[c]Reaction conditions: Lipase, *Candida rugosa* = 50.0 mg (5% based on weight of FA), temperature = 50°C, molar ratio of FFA/alcohol = 2:1 (FFA 1.0 g, lauryl alcohol 0.31 g), 0.33 mL water, stirrer speed = 400 rpm, incubation time = 30 h.
[d]Solvent extraction conditions: FFA-III (2 g) was dissolved in 80 mL *n*-hexane and extracted twice with 20 mL acetonitrile after storing at –20°C for 2 h.
[e]ND, not detected.
[f]AA recovery.
[g]Overall recovery.

on the solvent crystallization process. Figure 1 shows the contents and yields of AA in different solvents at different storage temperatures. At a fixed solvent/FFA ratio of 75:1 (vol/wt) and 48 h storage time, crystallization of single-cell FFA either in acetone or in methanol at –80°C resulted in *ca.* 80% AA in the liquid fraction with only moderate yield (*ca.* 65%). On the other hand, crystallizing FA in acetonitrile at –40°C yielded a liquid phase that contained 76% AA with 97% yield. The freezing point of acetonitrile is –45°C; hence, further increases in the AA content by lowering storage temperature are impracticable. Chen and Ju (12) described a two-step crystallization procedure to concentrate PUFA from borage and linseed oil using a mixture of acetone and acetonitrile (70:30 vol/vol) as solvent and –80°C storage temperature. However, attempts to increase AA content by using a mixture of solvents were unsuccessful.

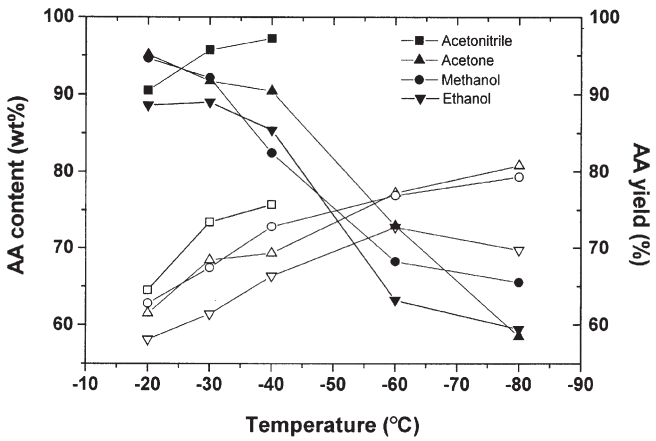

FIG. 1. The effects of storage temperature on the arachidonic acid (AA) content and yield in different solvents. FFA/solvent = 1:75 wt/vol, storage time = 48 h. Closed symbols: AA yield, open symbols: AA content.

Selective esterification. FFA-II contained *ca.* 75% AA (20:4n-6), 6% homo-γ-linolenic acid (20:3n-6), 5.5% γ-linolenic acid (18:3n-6), 10% linoleic acid (18:2n-6), and 3% oleic acid (18:1n-9). Because of the low m.p. and high solubilities of both AA and other PUFA, and the small amount of mono- and diunsaturated FA present in FFA-II, attempts to enrich AA content further by recrystallization of undesired FA using a single solvent were unsuccessful. However, enriching AA content up to 82% with yields less than 60% was achieved by using methanol or a mixture of acetonitrile and acetone at –80°C (data not shown).

Lipase-catalyzed selective esterification of FA is one of the most widely investigated approaches for the enrichment of PUFA (13,14). In the present study, enrichment of AA in both FFA-I and FFA-II by lipase-catalyzed esterification was investigated. Lipase from *C. rugosa* was chosen as the enzyme owing to its FA chain-length selectivity, showing higher activity toward FA with less than 20 carbons (18,19). When the reaction was conducted in a solvent-free system using a FFA/alcohol molar ratio of 1:1 for FFA-I and 2:1 in FFA-II and 5% lipase (based on wt% FFA substrate) at 50°C, the reaction reached equilibrium in 36 h with 52.3% degree of esterification and 24 h with 21% degree of esterification in FFA-I and FFA-II, respectively. At this stage, the AA content in unesterified FFA fraction increased from 38.8 to 64.2% with 93.4% yield and from 75.7 to 89.3% with 89.8% for FFA-I and FFA-II, respectively. A further increase in reaction time had no effect on AA content and yield.

Table 2 shows the time courses of FA composition during the selective esterification of FFA-I. Lipase from *C. rugosa* exhibited substantial selectivity toward lower-chain acids compared to AA. After 36 h, 95% linoleic acid, 84% oleic acid, and 77% palmitic acid were esterified. This lipase shows low selectivity for long-chain saturated FA (behenic acid, lignoceric acid), and their contents remained fairly constant. The contents of myristic acid (14:0) and stearic acid (18:0) changed slightly in the first 8 h of reaction. One of the characteristics of this reaction is the big difference in the selectivity of *C. rugosa* lipase toward palmitic and stearic acids. This lipase exhibits high selectivity for palmitic acid, and 78% of this acid was transformed into ester after 36 h of reaction, whereas for stearic acid the selectivity was practically nil and its content changed only slightly. On the other hand, the PUFA content increased gradually with time. At 36 h, reaction time AA content in the unesterified FFA fraction was 64.4% with 92.2% yield. Owing to the presence of stearic acid and other long-chain saturated FA, the enrichment of AA *via* lipase-catalyzed selective esterification was not very efficient.

TABLE 2
Time Course Variation of FA Composition During Selective Esterification[a] of FFA-I[b]

	FA composition (wt%)											
Time (h)	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:3	20:4	22:0	24:0
0[b]	1.38	13.12	0.63	9.23	16.28	6.54	5.46	0.71	4.35	38.82	2.31	1.11
1	1.14	12.10	0.51	10.40	13.75	4.85	4.30	0.31	5.38	44.37	1.75	0.91
2	0.98	10.57	0.20	10.23	11.61	4.54	4.94	0.25	5.81	47.43	1.56	0.99
4	0.86	9.22	ND	10.70	9.83	3.13	5.17	ND	6.32	51.54	2.12	1.10
8	1.01	6.81	ND	12.04	6.34	1.66	5.52	ND	6.68	55.02	3.19	1.81
16	0.46	4.76	ND	11.02	3.38	0.59	5.91	ND	7.44	61.56	3.11	1.77
24	0.35	3.61	ND	10.53	2.66	0.50	6.52	ND	7.86	63.26 (93.4%)[c]	3.00	1.80
36	ND	2.92	ND	10.33	2.51	0.31	6.11	ND	8.20	64.39 (92.2%)[c]	2.50	1.67
48	ND	3.02	ND	10.22	2.65	0.29	5.94	ND	8.62	65.05	2.37	1.54

[a]Reaction conditions: *Candida rugosa* lipase = 50.0 mg (5% based on weight of FA), temperature = 50°C, FFA/alcohol molar ratio 1:1 (FFA = 1 g, lauryl alcohol = 0.62 g), 0.33 mL of water, stirrer speed = 400 rpm.
[b]Initial FA composition of saponified single-cell oil.
[c]Yield of AA. For abbreviations see Table 1.

When the enrichment of AA by selective esterification was carried out after solvent crystallization, which removed all the saturated and most of the mono- and diunsaturated FA, the result was much better. Table 3 shows the time course of selective esterification of FFA-II obtained after low-temperature solvent crystallization of FFA-I. In the first 8 h of reaction, the content of linoleic and oleic acids decreased rapidly, with a concomitant increase in PUFA content in the unesterified FA fraction. AA content in the unesterified FA fraction kept increasing throughout the reaction, whereas γ-linolenic (18:3n-6), and homo γ-linolenic (20:3n-6) remained fairly constant. At 24 h, AA content reached 89.3% (from an initial content of 75.5%) with 89.9% yield. This AA-rich FA was designated as FFA-III.

Re-esterification. To further increase AA content, the FA fraction (FFA-III) was again subjected to selective esterification. Figure 2 shows time courses of AA content and yield. AA content in the FA fraction increased rapidly, whereas the yield of AA decreased rapidly as the reaction proceeded, especially in the early reaction stage. At a reaction time of 8 h, AA content in the unesterified FA fraction was 94.8% with a corresponding yield of 63.3%. Therefore, AA with a purity of 95% and yields greater than 70% is difficult to achieve by re-esterification.

Solvent extraction. Solvent extraction is a selective separation procedure for isolating and concentrating valuable substances using a mixture of solvents. The procedure depends on the nonuniform distribution of substances between two immiscible or partially miscible liquid phases. Under suitable conditions, a substance of interest can be transferred to one phase while undesired substances are retained in the other. After screening a number of common organic solvents, acetonitrile was selected as the solvent for the extraction and enrichment of AA in FFA-III dissolved in hexane. Acetonitrile and hexane are immiscible, and AA has different solubilities in these two solvents. The ratio of solvents in two phases, number of extractions, and storage temperature are major factors affecting the yield and purity of AA.

TABLE 3
Time Course Variation of FA Composition During Selective Esterification[a,b] of FFA-II[c]

	FA composition (wt%)				
Time (h)	18:1	18:2	18:3	20:3	20:4
0[c]	2.85	9.85	5.44	6.10	75.72
1	2.48	7.81	5.85	5.35	78.48
2	1.63	6.05	6.02	6.01	80.31
4	1.25	3.71	6.11	6.43	82.43
8	0.16	1.18	6.46	6.55	85.65
16	ND	0.92	5.68	6.22	87.18
24	ND	0.53	4.72	5.34	89.30 (89.8%)[d]
36	ND	0.48	4.82	5.17	89.25 (88.5%)[d]

[a]Data are the average of three independent determinations.
[b]Reaction conditions are described in the Experimental Procedures section.
[c]Initial FA composition of FFA-II obtained after low-temperature solvent crystallization.
[d]Yield of AA. For abbreviations see Table 1.

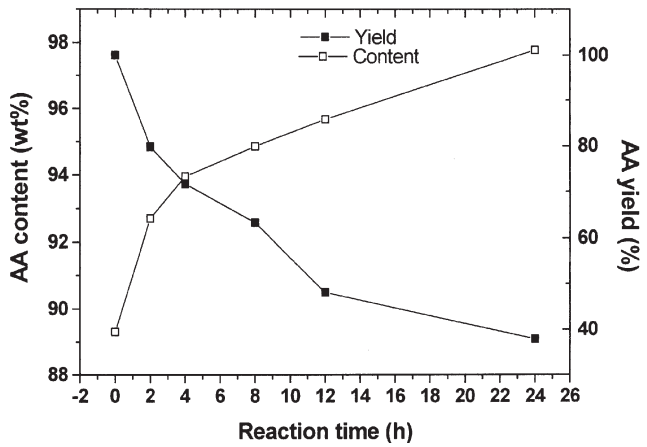

FIG. 2. Time course of AA content and yield during re-esterification of FFA-III. *Candida rugosa* lipase = 50 mg (5% based on FA wt). Molar ratio of FFA to lauryl alcohol = 2:1 (1 g FFA-III, 0.31 g lauryl alcohol). Water = 0.33 mL; temperature = 50°C; stirrer speed = 400 rpm; incubation time = 30 h. For abbreviation see Figure 1.

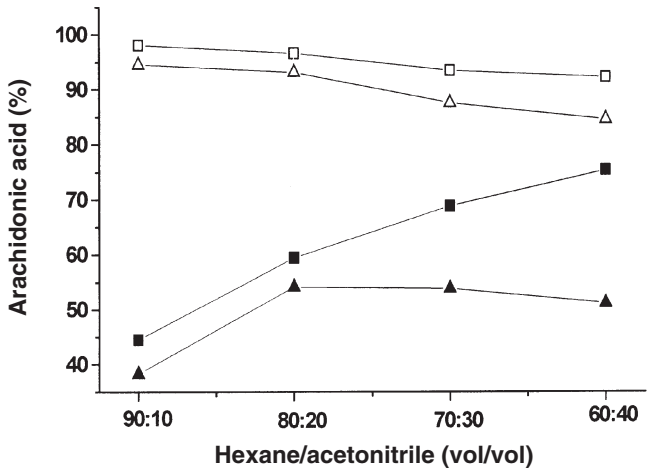

FIG. 3. Effect of solent ratios between hexane and acetonitrile on AA content and yield during solvent extractions. Solvent extraction is described in the Experimental Procedures section. Closed symbols: AA yield; open symbols: AA content. (■,□) first extraction; (▲,△) second extraction. For abbreviation see Figure 1.

Figure 3 shows the AA content and yield obtained in the first and second extractions when different ratios of hexane to acetonitrile were used. Best results were obtained when 2 g of FFA-III was dissolved in 80 mL hexane and extracted twice each with 20-mL acetonitrile at –20°C. About 59.5 and 54.2% of AA were recovered with 96.7 and 93.2% content in the first and second extractions, respectively. The pooled product of two extractions resulted in a FA mixture with 95.3% AA with 81.2% yield, which was designated as FFA-IV.

When extractions were carried out repetitively under similar conditions (2 g of FFA-III dissolved in 80 mL of hexane, stored under –20°C for 2 h and followed by two extractions each with 20 mL acetonitrile), both the content and yield of AA decreased for each extra extraction. About 60% AA with 96.7% purity was extracted in the first extraction. Repetition of solvent extractions yielded 54, 48, and 31% of AA with 93.2, 82.7, and 69.9% content in the second, third, and fourth extraction, respectively. Figure 4 shows AA content and yield as a function of the number of pooled AA-rich acetonitrile fractions in the multiple extractions. As the number of extractions increased, the AA content in the pooled acetonitrile fraction decreased slightly but the corresponding yield increased greatly.

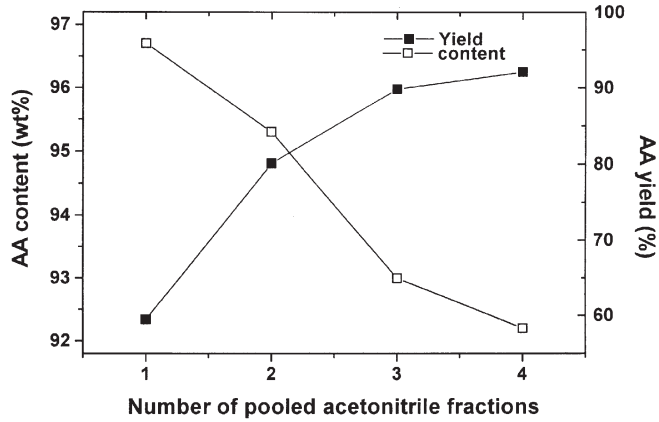

FIG. 4. AA content and yield as a function of the number of pooled acetonitrile fractions obtained in a multiple solvent extraction operation. Solvent extraction is described in the Experimental Procedures section. The acetonitrile fraction from the first extraction gives point 1 in the abscissa. A combined acetonitrile fraction from the first and second extractions gives point 2, etc. For abbreviation see Figure 1.

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