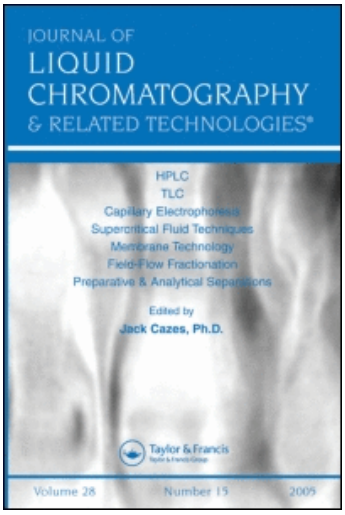

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PREPARATIVE SEPARATION OF UNSATURATED FATTY ACIDS ESTERS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY (CPC)

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ABSTRACT

A novel method for separation of fatty acid ethyl esters by Centrifugal Partition Chromatography is reported in this paper. Data are first presented for laboratory-scale separations of fatty acid esters derived from cereal oil, primarily oleic, linoleic and linolenic acids, and from fish oil, containing, primarily, icosapentenoic and docosapentenoic acids. The method does not employ column packing resins; long-chain unsaturated fatty acids which are generally susceptible to oxidation on catalytic surfaces are readily purified by CPC. Scale-up design for industrial applications is discussed.

INTRODUCTION

Long chain unsaturated fatty acids and their esters have been cited recently(1) as therapeutic agents for prevention of adult diseases -

cerebral apoplexy, arteriosclerosis etc. Production of these compounds in a highly purified state, for use as food additives and pharmaceuticals, is one of the critical biotechnology problems in the fats and oils industry. Conventional separation methods, such as distillation under conditions of high vacuum and temperature, and column liquid chromatography are not yet suitable in terms of both the technological and economical aspects.

We have developed a novel separation method, utilizing Centrifugal Partition Chromatography (CPC)[2], and have applied the technique to the separation of fatty acid esters, particularly to the long-chain unsaturated fatty acid esters which are susceptible to rapid oxidation during preparative-scale separation processes.[3]

The CPC method does not employ column packing resins. Instead, two-phase solvent mixtures are used as separation media; one phase serves as the stationary phase and the other as the mobile phase. The stationary phase is contained in the partition channels of a series of partition cartridges contained in the rotor of a flow-through centrifuge; the mobile phase passes through the stationary phase forming tiny droplets. The stationary phase is retained in the cartridges, even at high mobile phase flow rates, by virtue of the centrifugal force that is developed by the centrifuge rotor. A change of flow direction makes upper or lower phase available as stationary phase; these correspond to "normal ascending" and "reversed descending" chromatography, respectively.

We have applied the CPC method to the purification of unsaturated fatty acid esters (oleic, linoleic, linolenic, arachidonic, icosapentenoic and docosahexenoic acids) from fish, cereal and microbial sources.

Recovery was checked gravimetrically and the purity of each fraction was assayed by gas chromatography.

Since the CPC method is based on two-phase liquid-liquid partition chromatography, in the complete absence of a solid stationary phase support, and the separation is rapid, it is ideally suited for separation of labile compounds which would ordinarily be decomposed or lost upon passage through and in contact with conventional column packing materials[4], [5]. Moreover, quantitative recovery of the compounds is readily realized with excellent cost performance. Scale-up design for industrial applications is shown and feasibility studies are discussed.

EXPERIMENTAL

Materials

Ethyl esters of oleic, linoleic, linolenic acid were prepared by direct ethanolysis of cereal oils. Icosapentenoic (EPA) and docosahexenoic (DHA) acid esters from fish oils were obtained from Gasukuro Kogyo, Ltd., Japan. Compositions of the fatty acid ester in the starting materials subjected to separation are shown in Table 1.

Purity Assay and Identification

Fatty acid ethyl esters were identified and assayed by gas chromatography (Shimadzu GC-9A, equipped with data processing unit C-R3A, and FID detector). A packed column, containing Advance-DS coated Chromasorb W (5%), 3.1 mm x 2.1 m, was used.

TABLE 1

Composition of Starting Materials of the Fatty Acid Ethyl Esters Subjected to CPC Separation.

Material	Ethyl Esters	Composition (*)
Cereal Oil	Oleic	18.2%
	Linoleic	14.8%
	Linolenic	59.3%
	Others	7.7%
Fish Oil	Icosapentenoic	57.5%
	Docosahexenoic	10.7%
	Others	31.8%

(*) Calculated from gas chromatographic peak areas.

Centrifugal Partition Chromatography

Laboratory scale separations were performed with a Centrifugal Partition Chromatograph, CPC Model LLN (Sanki Engineering Limited, Nagaokakyo, Kyoto, Japan). A flow sheet of the system is given in Fig. 1. Partition micro-cells are contained in rectangular cartridges; one cartridge contains 400 micro cells with a total net volume of 21.3 ml. Twelve cartridges were connected in series around the rotor of the centrifuge. Thus, in the present work, 4800 partition micro-cells, with total net volume 256 ml, were used for the laboratory-scale separations.

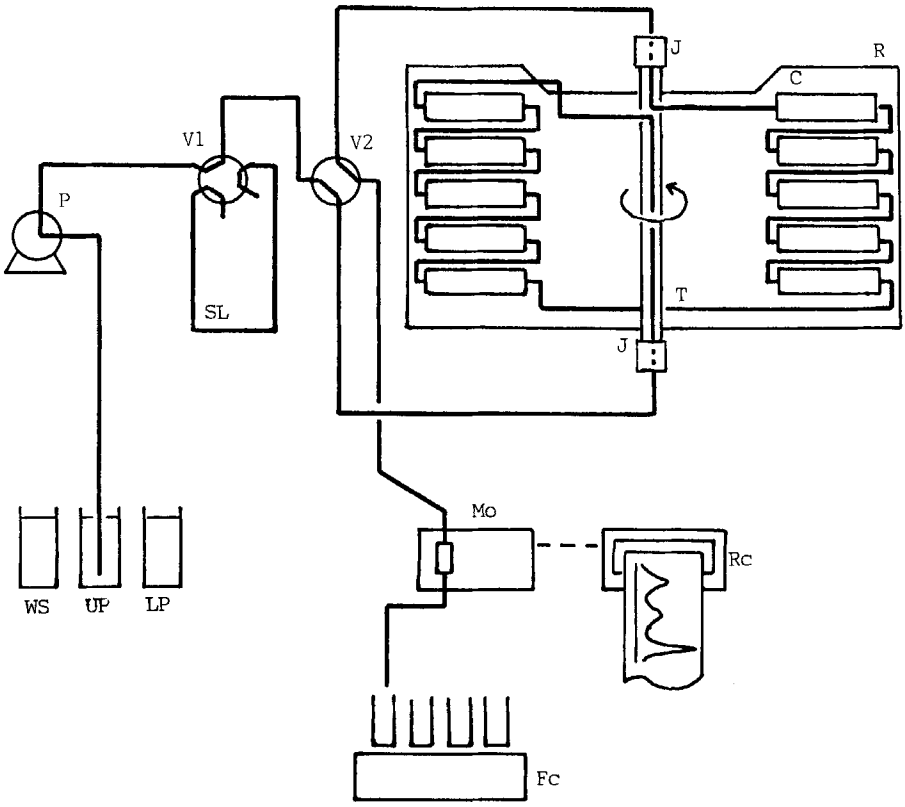


FIGURE 1: System flow sheet, Centrifugal Partition Chromatograph (CPC^{TM}).

WS: Wash Solvent
UP: Upper Phase Solvent
LP: Lower Phase Solvent
P: Constant Flow Pump
V1: 6-Way Valve (Sample Injector)
R: Rotor
V2: 4-Way Valve (Elution Mode)
T: Connecting Tubes
J: Rotary Seal Joint
Rc: Recorder
C: Partition Micro Cells
Mo: Flow Cell Monitor
Fc: Fraction Collector

A pilot plant system, CPC Model MF-007 (Sanki Engineering Limited, Nagaokakyo, Kyoto, Japan) was used for scale-up tests, based on the data obtained with the laboratory unit. The stacked-disk type rotor of the main centrifugal unit, 300 mm (diameter) x 200 mm (height) x 2, contains 1,200 partition cells; total net volume is 6,800 ml. As much as 500 grams of crude materials have been processed, within a few hours, with this pilot plant system.

Eluates from the CPC system were collected in fractions of equal volume, and the fatty acid esters in each fraction were identified by gas chromatography. Chromatograms for the CPC separation were obtained by plotting the peak heights of the fatty acid esters for the fractions, determined by gas chromatography, versus their elution volumes.

Solvents

Two-phase partition solvent systems used for separation of the fatty acid esters were prepared by the combination of hydrocarbons (n-hexane or n-heptane) and water-miscible solvents (acetonitrile, methyl or ethyl alcohol) either with or without water. All the solvents used were a high purity reagent grade.

ELUTION MODE IN THE CPC METHOD

In the CPC method, elution mode may be switched from the normal ascending mode to the reversed descending mode (and vice versa) during operation, within a single run, to accommodate a broad range of sample component polarities and/or hydrophobicities.

When the upper layer (the less polar phase) is used as mobile phase and the lower layer (the more polar phase) is used as the stationary phase (during the first elution), the elution mode is "normal ascending" and the sample components are eluted in order of their hydrophobicities. The more polar components remain, primarily, in the lower stationary phase, but they are gradually developed and separate from each other during the first normal phase elution. The remaining components in the stationary phase may then be individually recovered by changing the solvent flow direction and the elution mode from the normal ascending to the reversed descending (during the second elution). These operational modes are illustrated in Fig. 2.

Selection of the elution mode for the first and second stage elutions depends on the nature of the required separation and the scale of separation to be performed.

RESULTS AND DISCUSSIONS

Partition coefficients for the compounds distributed between the selected two phase-solvent systems can be easily calculated from the CPC retention volumes[6]. The values for fatty acid ethyl esters in three different two-phase solvent systems (n-hexane/acetonitrile, n-hexane/methyl alcohol/water and n-hexane/ethyl alcohol/water) are shown in Fig. 3 - 5. The partition coefficients of the desired compounds and those of the other esters were taken into account when selecting the two-phase solvent systems and elution modes for separation of fatty acid ethyl esters.

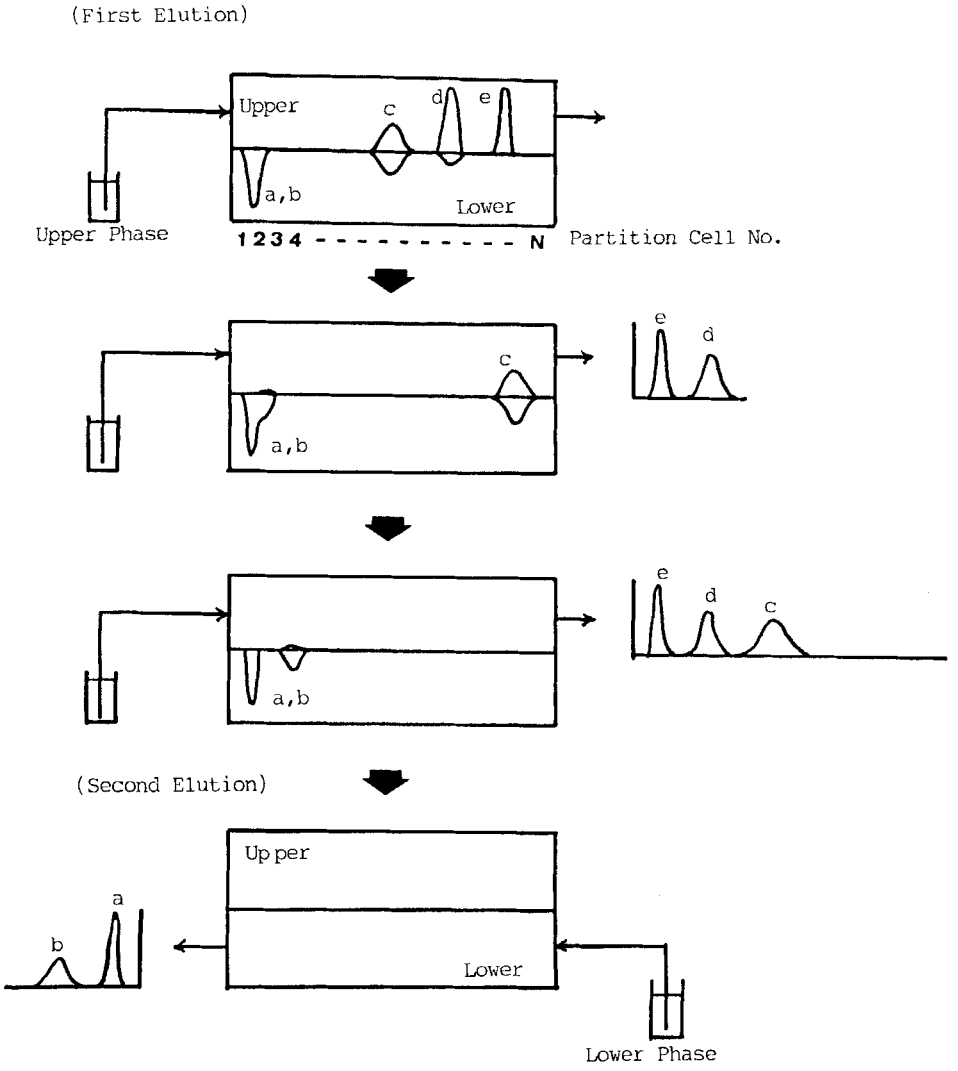


FIGURE 2: Illustration of elution modes in Centrifugal Partition Chromatography (CPC™).

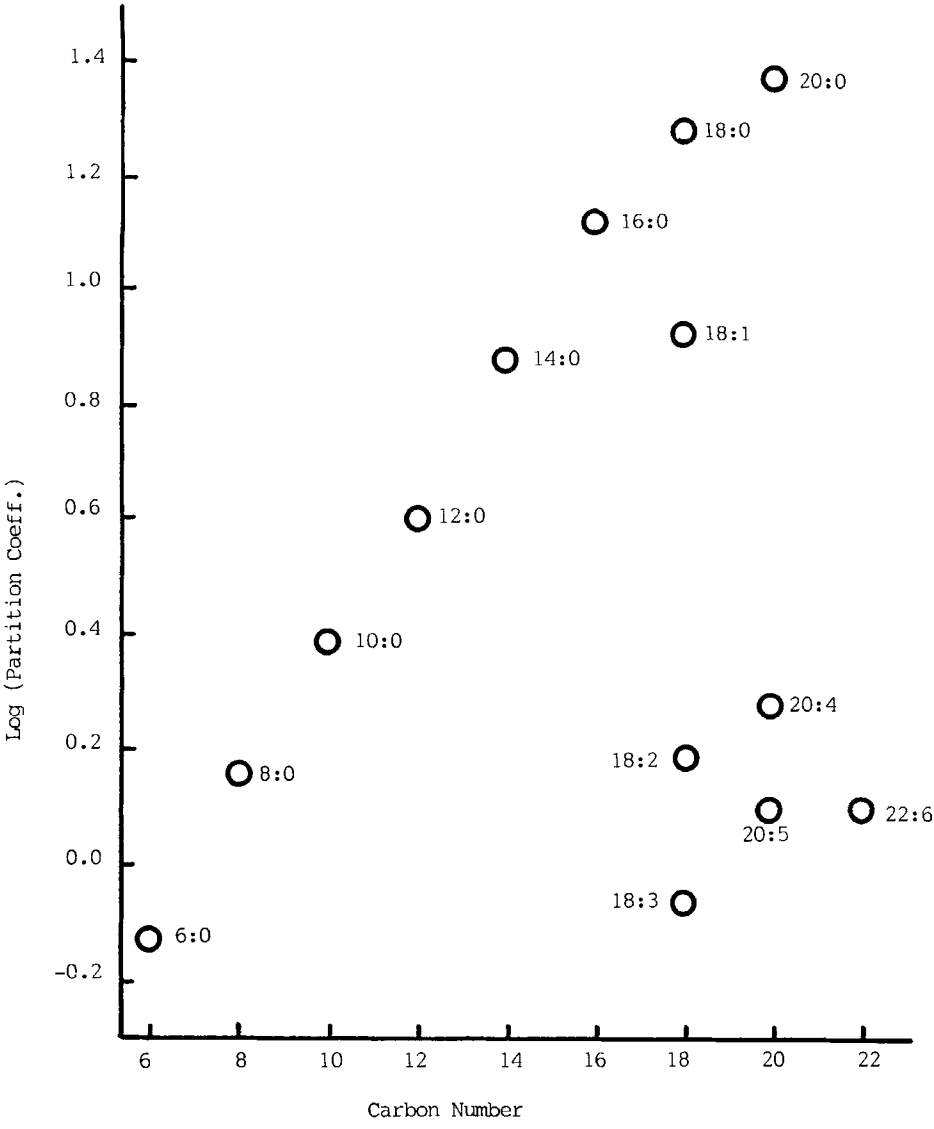


FIGURE 3: Partition coefficients of fatty acid ethyl esters in n-Hexane/Acetonitrile (1 / 1) two-phase solvent system.

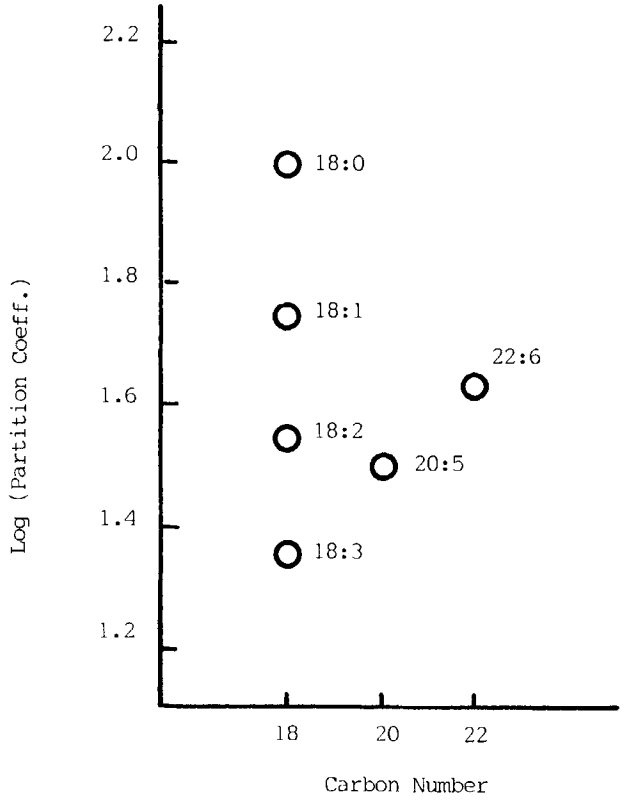


FIGURE 4: Partition coefficients of fatty acid ethyl esters in n-Hexane/Methyl Alcohol/Water (1 / 0.9 / 0.1) two-phase solvent system.

The two-phase solvent system, n-hexane/acetonitrile (1:1), is well suited for the separation of the C_{18} series of fatty acid esters because the partition coefficients of $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ esters are distributed over a wide range in this two-phase system. Fatty acid ethyl esters from cereal oil sources were subjected to separation on a laboratory scale (Fig. 6). Linolenic ($C_{18:3}$) and linoleic ($C_{18:2}$) acid esters were separated during the first (normal ascending) elution and

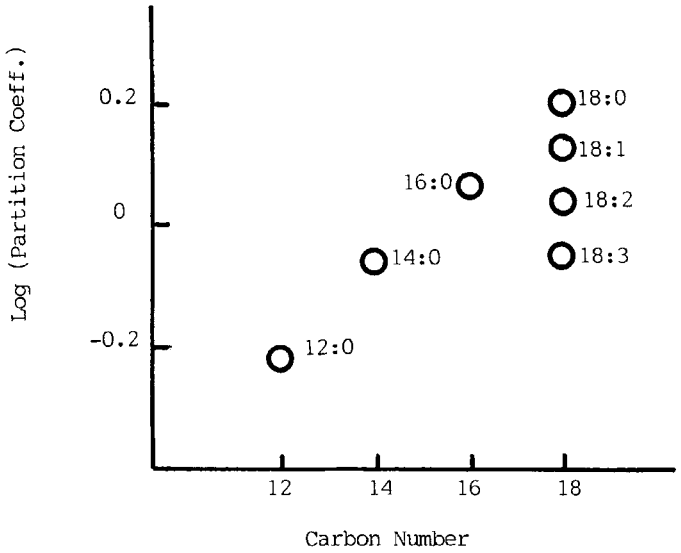


FIGURE 5: Partition coefficients of fatty acid ethyl esters in n-Hexane/Ethyl Alcohol/Water (1 / 0.9 / 0.1) two-phase solvent system.

oleic ($C_{18:1}$) acid ethyl ester was recovered by switching the elution mode; however, this fraction partly overlaps with the palmitic acid ester.

In a scaled-up separation of the same material, with the CPC MF-007 system all the compounds were fractionated through the second reversed descending mode elution after the first normal phase elution. A flow sheet of the separation process, together with purity and yield of each fatty acid ester, are shown in Fig. 7.

For the separation of icosapentenoic ($C_{20:5}$, EPA) and docosahexenoic ($C_{22:6}$, DHA) acid esters from fish oils, the n-hexane/acetonitrile two-

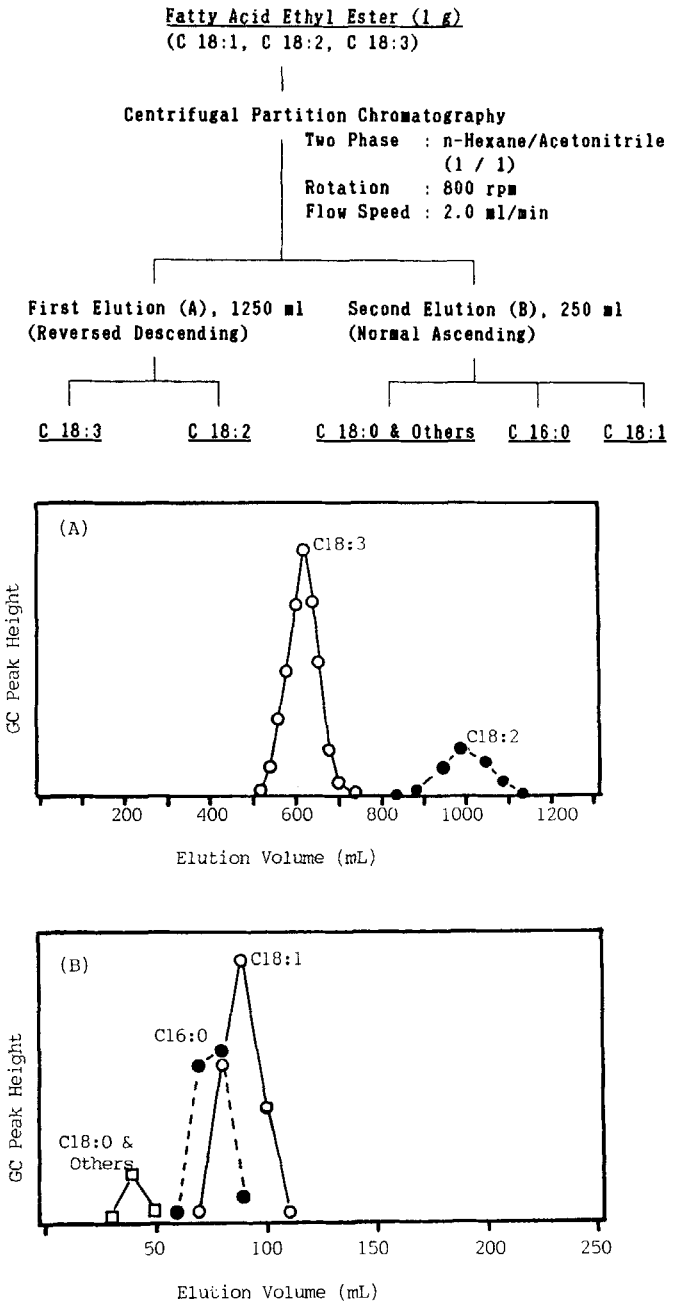
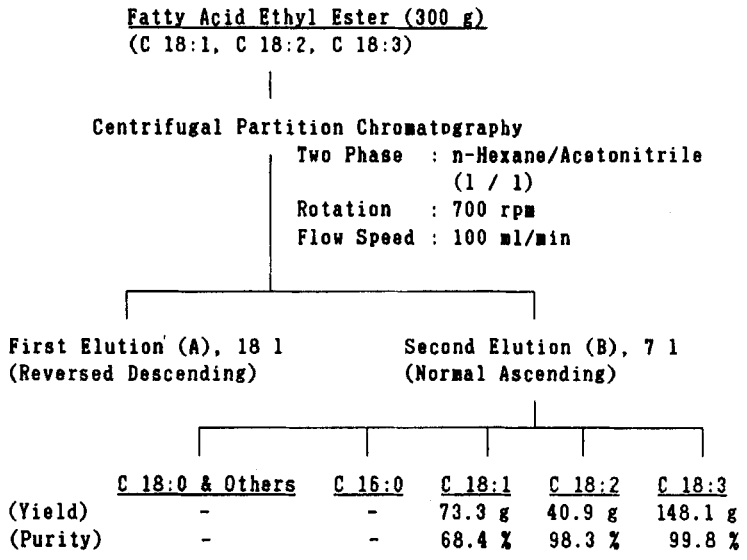


FIGURE 6: Laboratory-scale separation of linolenic, linoleic and oleic acid ethyl esters by Centrifugal Partition Chromatography.



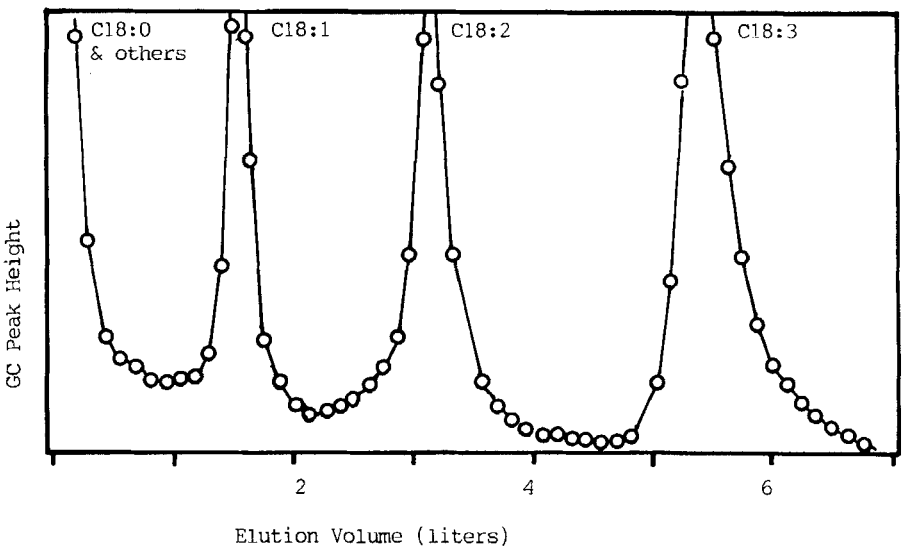


FIGURE 7: Semi-pilot-scale separation of linolenic, linoleic and oleic acid ethyl esters by Centrifugal Partition Chromatography.

phase solvent system is not adequate because the partition coefficients between these two phases for EPA and DHA esters are quite similar. When n-hexane/methanol/water (1 / 0.9 /0.1) two-phase solvent is used, these compounds tend to distribute preferentially in the upper phase, but their partition coefficients differ from each other to some extent. Separation of EPA and DHA ethyl esters was achieved during the second normal ascending mode elution, following development of the compound, within the partition channels, during the first, reversed descending mode elution (Fig. 8).

When the optimum two phase solvent system and elution mode were adopted, the CPC method proved to be practical for the preparative separation of long-chain unsaturated fatty acids with high purity and excellent yield.

SCALE UP DESIGN OF CPC SYSTEM

Scale-up design of the system was examined for industrial applications of the CPC method. The centrifugal component of the industrial system is composed of circular disk-type (instead of the cartridge type) partition cells and the rotor is constructed by stacking several of the partition cell disks (Fig. 9). Productivities of these industrial CPC systems were estimated from experimental data obtained with the MF-007 system. These values are given in Table 2. The system flow sheet for an industrial CPC plant is shown in Fig. 10.

The running cost of the separation process depends, primarily, on the products/solvent consumption ratio and the amount of the solvents that can

Fatty Acid Ethyl Ester (1 g)
(C 20:5, C 22:6)

Centrifugal Partition Chromatography
Two Phase : n-Hexane/Methyl Alcohol /Water (1/ 0.9/ 0.1)
Rotation : 600 rpm
Flow Speed : 3.3 ml/min

First Elution (A), 3800 ml (Reversed Descending)

Second Elution (B), 250 ml (Normal Ascending)

		Fr. I	Fr. II
(Yield)		328 mg	433 mg
(Purity)	C 20:5	21.0 %	95.8 %
	C 22:6	36.8 %	0.8 %

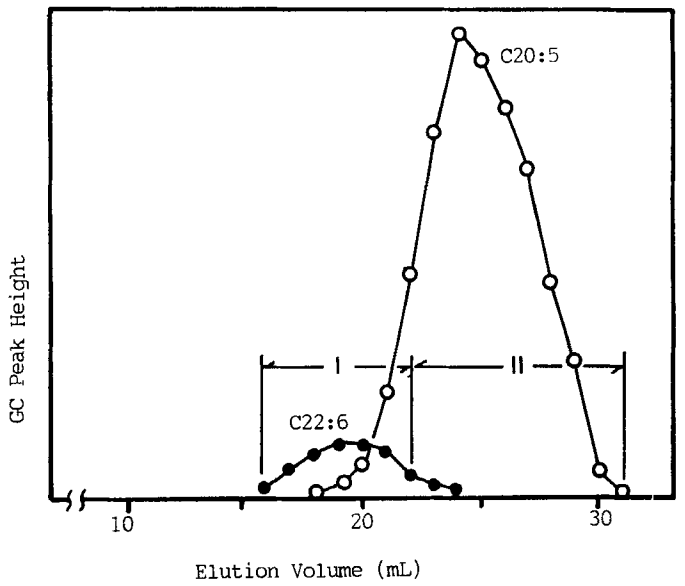


FIGURE 8: Laboratory-scale separation of eicosapentenoic and docosahexenoic acid ethyl esters by Centrifugal Partition Chromatography.

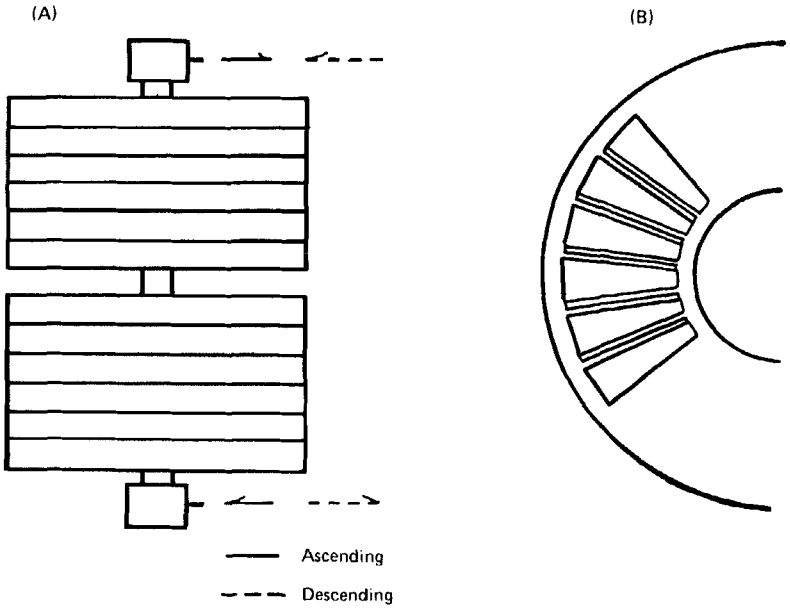


FIGURE 9: Centrifuge Main Frame of the industrial CPC system.
(A) Rotor construction. (B) Circular disk-type partition cells.

TABLE 2

Industrial-Scale CPC System Specifications.

Model	Rotor Size (mm)	Net Vol. (Liters)	Productivity (g/hr)*	Note
MF-003	300(d) X 200(h)	3.5	15 - 50	Semi-Pilot
MF-007	300(d) X 200(h) X 2	6.8	30 - 100	Pilot
MF-025	600(d) X 300(h)	23.8	100 - 350	Commercial
MF-050	600(d) X 300(h) X 2	47.6	200 - 700	Commercial
MF-100	900(d) X 300(h) X 2	106.9	500 - 1500	Commercial

* Ability for crude material processing in the case of fatty acid ester separation using n-hexane / acetonitrile (1 / 1) two-phase solvent system.

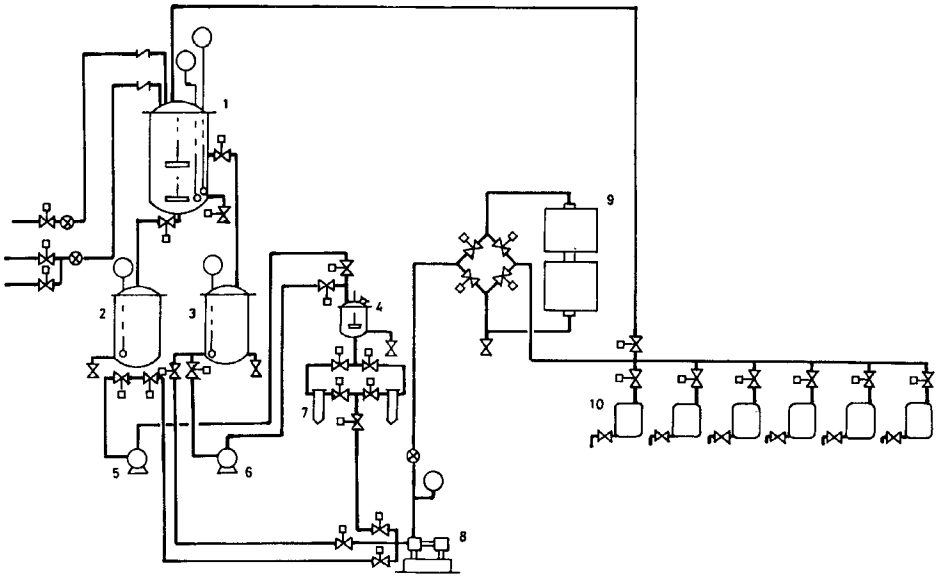


FIGURE 10: CPC industrial system flow sheet.

(1) Mixing & separation tank for two-phase solvent system

(2) Upper phase solvent tank

(3) Lower phase solvent tank

(4) Sample solution tank

(5) Upper phase solvent metering pump

(6) Lower phase solvent metering pump

(7) Filter

(8) Main pump (high pressure, constant flow pump)

(9) Main frame (Flow-through centrifuge)

(10) Fraction collector

be recovered and re-used. The ratio is expected to be larger, probably by an order of magnitude, than when the same separation is performed by column liquid chromatography or preparative HPLC. Two-phase solvents used for CPC are recovered by distillation as azeotropic mixtures and recycled after separating them into two phases. More than 95% recovery of each solvent (except water) is often readily realized. The energy cost for the solvent recovery is estimated to be less than a few dollars per Kg of the products. Manpower costs are dramatically decreased through direct microcomputer control of the entire process.

Industrial operation of the CPC system for the separation of fatty acid esters, particularly EPA and DHA esters, is now in progress. Detailed engineering data will be reported in the near future.

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