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High-speed counter-current chromatography of apple procyanidins

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Abstract

Apple procyanidins were separated by high-speed counter-current chromatography using a type-J multilayer coil planet centrifuge. Several two-phase solvent systems with a wide range of hydrophobicities from a non-polar hexane system to polar *n*-butanol systems were evaluated their performance in terms of the partition coefficient and the retention of the phase. The best separation of procyanidins B and C was achieved with a two-phase solvent system composed of *n*-butanol–methyl *tert*.-butyl ether–acetonitrile–0.1% trifluoroacetic acid (2:4:3:8) using the lower phase as a mobile at a flow-rate of 1.0 ml/min. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Catechin and epicatechin are flavonoids that are widely distributed in plant-derived foods such as wine, green tea, chocolate and various fruits. Polyphenols in apples contain dihydrocalcons, phenolic acids and others up to 50% of the total mass while the rest consists of monomers, dimers, trimers and oligomers of catechin and epicatechin which are called apple procyanidins (apple condensed tannins; ACTs). Recently, dimers, trimers and oligomers of the procyanidins have attracted attention in the fields of pharmacology and food chemistry because of their beneficial effect on the circulatory system [1] as well as being efficient free radical scavengers [2,3]. Physiological activities, such as hair-growth promotion [4], anti-allergy [5], antibiotic [6] and inhibitory activity against enzymes and receptors [7–10] were also reported. These properties of procyanidins depend on the degree of polymerization of catechin and/or epicatechin. In order to designate the physiological activities according to the different degree of polymerization, it is necessary to establish an efficient, reliable separation method.

Analytical-scale separations of procyanidins have been reported using normal-phase [11], reversed-phase [12] and size-exclusion [13] liquid chromatography. However, the preparative separation of the procyanidins has not been reported, and in addition these chromatographic methods tend to cause irreversible adsorption of analytes onto the column packing materials.

Since counter-current chromatography (CCC) is a liquid partition chromatographic method free of solid

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support matrices, the method eliminates various complications arising from the use of a solid support [14–17]. Among all the existing CCC instruments, the high-speed CCC is the most advanced form in terms of partition efficiency and separation time [18,19], and it has been used for separation and purification of a wide variety of natural products since the last decade. The recent model of high-speed CCC, that facilitates the stationary phase retention for polar solvent systems [20], is particularly useful for separation and purification of hydrophilic apple procyanidins. Several reports have been published for the separation of condensed tannins extracted from sorghum grain [21] and for the purification of oligomeric hydrolyzable tannins extracted from *Heterocentron roseum* [22].

In the present study, high-speed CCC was applied to the separation of monomers, dimers, trimers and oligomers of catechin and/or epicatechin from ACTs using a set of two-phase solvent systems.

2. Experimental

2.1. Apparatus

Fig. 1 shows a photograph of the type-J high-speed CCC centrifuge (Hitachi Tokyo Electronics, Tokyo, Japan). The apparatus holds a multilayer coil separation column and a counter-weight symmetrically at a distance of 10 cm from the central axis of the centrifuge. The small separation column was fabricated by winding a single piece of 1.0 mm I.D. and ca. 44 m long PTFE (polytetrafluoroethylene) tubing (Tokyo Rikakikai, Tokyo, Japan) directly onto the holder hub making five coiled layers between a pair of flanges (β=0.5–0.6). The large column was similarly made by winding 21 m×2.0 mm I.D. tubing making four coiled layers (β=0.5–0.62). The total capacity of small and large columns is about 35 ml and 72 ml, respectively. The speed of the apparatus was regulated at 1000 rpm with a speed

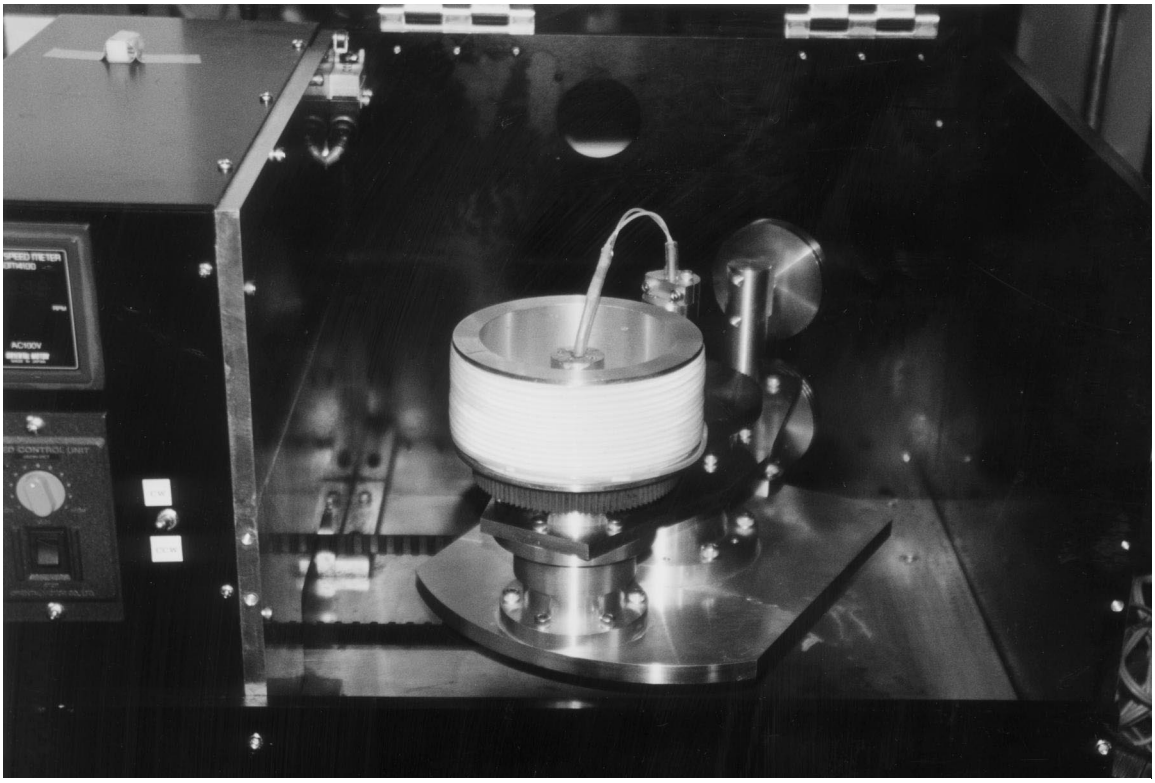

Fig. 1. Photograph of the type J multilayer coil planet centrifuge.

controller. The coil rotates around its axis as it simultaneously revolves around a central axis, producing an efficient mixing of the two phases while retaining a sufficient amount of the stationary phase in the column.

2.2. Reagents

Hexane, ethyl acetate, methanol, acetonitrile, methyl *tert*.-butyl ether (MTBE) and trifluoroacetic acid (TFA) were all glass-distilled chromatographic grade (Kanto, Tokyo, Japan). Catechin, epicatechin were obtained from Sigma (St. Louis, MO, USA). Procyanidin B1, B2 and C1 were gifts from Nikka Whisky Distilling Co. (Chiba, Japan). Other chemicals were of reagent grade.

2.3. Preparation of apple procyanidins (apple condensed tannins: ACTs)

The preparation of ACTs obtained from apple juice by column liquid chromatography using several packing materials has been described in detail elsewhere [13,23]. Purified ACTs were the mixture of monomeric flavan-3-ols and procyanidin oligomers from dimers to pentadecamers [23].

2.4. Measurement of partition coefficient

The following five solvent systems were selected based on the K_D values of catechin, epicatechin, procyanidin B1, B2, C1 and ACTs: (i) hexane–ethyl acetate–methanol–water (1:1:1:1), (ii) ethyl acetate–acetic acid–water (4:1:4), (iii) *n*-butanol–water (1:1), (iv) *n*-butanol–acetic acid–water (4:1:5) and (v) *n*-butanol–MTBE–acetonitrile–0.1% TFA (2:4:3:8).

Each solvent mixture was throughly equilibrated in a test tube and the two phases separated.

Using these solvent systems, the K_D values were determined as follows: about 1.5 ml of each phase was delivered into a test tube to which about 10 mg of the sample was added. The contents were thoroughly mixed and then allowed to settle at room temperature. After two clear layers were formed (the tube was centrifuged if necessary), an aliquot (usually 1 ml) of each phase was pipetted and diluted with 0.5 ml of methanol to determine the absorbance at 280 nm using a Shimadzu UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan). K_D was expressed as the solute concentration in the upper phase divided by that in the lower phase.

2.5. Measurement of stationary phase retention

Experiments were performed according to the standard procedure described elsewhere [24]. The 35-ml volume coil was used for the measurement of the stationary phase retention. In each measurement, the coil was first entirely filled with the stationary phase. Then, the apparatus was rotated at 1000 rpm while the mobile phase was pumped into the column at a flow-rate of 1.0 ml/min. The effluent from the outlet of the column was collected in a 50-ml graduated cylinder to measure the volume of the stationary phase eluted from the column as well as the total elution volume of the mobile phase. The elution was continued for 35–40 min until the total elution volume exceeded the column capacity of 35 ml when the retention of stationary phase was stabilized. Then the centrifuge was stopped and the column contents emptied into a graduated cylinder by connecting the inlet of the column to a pressured nitrogen line. The column was then washed with several milliliters of methanol and finally flushed with several milliliters of the stationary phase which was to be used for the next experiment.

2.6. CCC separation of ACTs

In each separation, a 72-ml capacity multilayer coiled column of the type-J high-speed CCC centrifuge was first entirely filled with the stationary phase and a sample solution containing 50 mg of ACTs was injected into the column using an EYELA type SV-6010 sample injector (Tokyo Rikakikai). Then, the apparatus was rotated at 1000 rpm while the mobile was pumped into the column by the EYELA LP 1100 pump at 1.0 ml/min flow-rate. The effluent from the outlet was continuously monitored at 280 nm, the maximum wave length of ACTs and procyanidin, with an EYELA UV-9000 absorbance monitor (Tokyo Rikakikai) and fractionated using an LKB 2112 Redirac fraction collector (LKB Instruments, Bromma/Stockholm, Sweden). An aliquot of each fraction was diluted with methanol and the

absorbance was measured at 280 nm with a Shimadzu UV-1200 spectrophotometer.

2.7. Analysis of CCC fractions

Aliquots of CCC fractions corresponding to various portions of the major peaks were lyophilized and analyzed by reversed-phase high-performance liquid chromatography (HPLC). The analysis was performed using an EYELA HPLC system and an Inertsil ODS-3 column (150 mm×4.6 mm I.D., 5 μm particle size) (GL Science, Tokyo, Japan). Each lyophilized powder was dissolved in the mobile phase composed of 0.05% TFA–acetonitrile (91:9, v/v) at a flow-rate of 1.0 ml/min. The standard procyanidins B1, B2 and C1 were collected from ACTs by normal-phase HPLC [12]. The components in the fractions were confirmed from their retention times.

3. Results and discussion

3.1. Composition of ACTs and their polarities

Purified ACTs are a mixture of monomeric catechins and procyanidin oligomers from dimers to pentadecamers [23]. The chemical structures of monomers, dimers and trimer in ACTs are shown in Fig. 2. Monomers (catechin and epicatechin), dimers (procyanidin B1 and B2) and trimers (mainly procyanidin C1) are contained in the ACTs at about 15%, 10% and 7%, respectively. Compared with the monomers, dimers, trimers, and oligomers are more hydrophilic, and the polarity of procyanidinns gradually increases with a degree of polymerization (data not shown). Consequently, the partition coefficient of the monomers may be very different from those of their oligomers in some solvent systems.

3.2. Measurement of partition coefficient of catechin, epicatechin and ACTs

In the CCC technique, successful separation requires a proper choice of two-phase solvent systems. In order to achieve efficient separation of catechin and epicatechin from ACTs, the partition coefficient (K_D) values of these compounds were determined in a series of solvent systems in a wide range of hydrophobicities from a non-polar hexane system to several polar *n*-butanol systems. The K_D values of catechin, epicatechin and ACTs were determined spectrophotometrically according to a standard test tube procedure as described in the Experimental section.

The K_D values of catechin, epicatechin and ACTs

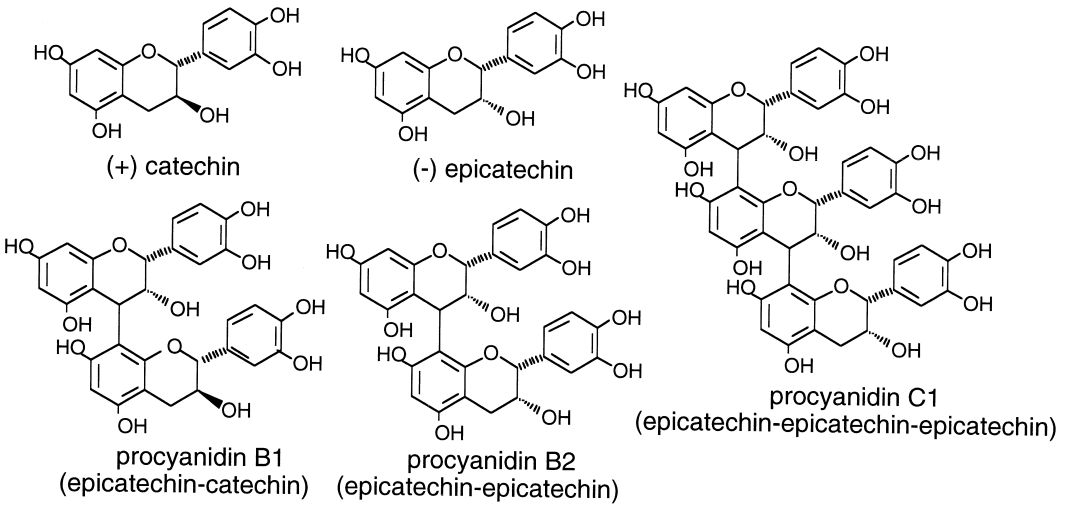

Fig. 2. Structures of catechin, epicatechin, procyanidins B1, B2 and C1.

composed of monomers, dimers, trimers and oligomers are listed in Table 1. The chromatographic process in CCC is based on the partition of a solute between the two liquids that are used as the mobile and stationary phase, respectively. The K_D value is therefore the most important parameter in CCC. This value of around 1 are desirable in CCC because a solute with $K_D=1$ elutes once the equivalent of one coil volume of the mobile phase has passed through the coiled column. The K_D values of catechin, epicatechin and ACTs in two solvent systems containing hexane and/or ethyl acetate are substantially smaller than 1.0, and more importantly the K_D values of ACTs are very similar to those of the monomers. In the hexane–ethyl acetate–methanol–water solvent system, the α value of epicatechin/ACTs is 1.25 and α values ranging from 1.1 to 1.19 of monomers/ACTs are observed in the ethyl acetate–acetic acid–water solvent system. It was indicated that the separation of dimers and trimers from their monomers is not feasible. On the other hand, in the hydrophilic solvent systems containing *n*-butanol, the K_D values of ACTs are much smaller than those of catechin and epicatechin ($\alpha=1.7–5.4$) suggesting that monomers such as catechin and epicatechin are more hydrophobic than their oligomers present in ACTs and it seems likely that procyanidins B1, B2 and C1, i.e., dimers and trimers, may be separable from the monomers by CCC. Therefore, we tested these four polar solvent systems for the stationary phase retention in the high-speed CCC column.

3.3. Measurement of stationary phase retention in multilayer coil separation column

CCC is a support-free liquid–liquid partition chromatographic technique where the liquid stationary phase is retained in the column by the aid of a centrifugal force field. Therefore, in contrast with other chromatographic methods, the amount of the stationary phase retained in the column varies widely according to the applied experimental conditions. Since the resolution of solute peaks in CCC largely depends upon the volume of the stationary phase

Table 1
Partition coefficient of catechin, epicatechin and apply procyanidins (ACTs)

Solvent systems	Catechin	Epicatechin	ACT
hexane:ethyl acetate:methanol:water (1:1:1:1)	0.01	0.05	0.04
ethyl acetate:acetic acid:water (4:1:4)	0.50	0.46	0.55
n-butanol:water (1:1)	5.93	4.05	1.1
n-butanol:acetic acid:water (4:1:5)	2.73	1.71	1.00
n-butanol:MTBE:acetonitrile:0.1% TFA (2:4:3:8)	4.18	4.74	2.33

Table 2
Stationary phase retention into small separation column

Solvent systems	Stationary phase retention (%)	
	Upper phase	Lower phase
n-butanol:water (1:1)	7.0	50.0
n-butanol:acetic acid:water (4:1:5)	7.0	0.0
n-butanol:MTBE:acetonitrile:0.1% TFA (2:4:3:8)	85.7	82.1

retained in the column, it is extremely important to maximize the phase retention.

Generally speaking, the retention of the stationary phase in the column is determined by the β values (distance between the coil and its axis divided by the revolution radius) [25] and the physical properties of the solvent system [26]. The β values of the coiled column used in the present studies range from 0.5 to 0.75 which are suitable for most of the two-phase solvent system [25]. Although effects of the physical parameters of the two-phase solvent system on the retention of the stationary phase is extremely complex, a settling time of two solvent phases in a test tube represents their composite effect on the retention of the stationary phase [26]. All solvent systems employed in the present studies showed the settling times less than 20 s which promises a satisfactory retention of the stationary phase if the aqueous phase (heavier phase) is eluted through the column in the head to tail direction or the organic phase (lighter phase) in the opposite direction.

Table 2 shows percentage retention of the stationary phase for three pairs of hydrophilic two-phase solvent systems using both upper and lower phases as the mobile phase. Each measurement was performed at 1.0 ml flow-rate at 1000 rpm. The best retention of 85.7% for the upper phase and 82.1% for the lower phase were obtained from the solvent system composed of *n*-butanol–MTBE–acetonitrile–0.1% TFA (2:4:3:8). The results indicate that the CCC separations of procyanidin oligomers in ACTs may be feasible with this solvent system using either phase as the stationary phase.

3.4. CCC separation of ACTs

Fig. 3 shows a chromatogram obtained from ACTs using the solvent system composed of *n*-butanol–MTBE–acetonitrile–0.1% TFA (2:4:3:8) by dual-mode CCC. After filling the small multilayer coil (35-ml capacity) with the lower aqueous stationary phase, 1 ml of the sample solution containing 51 mg

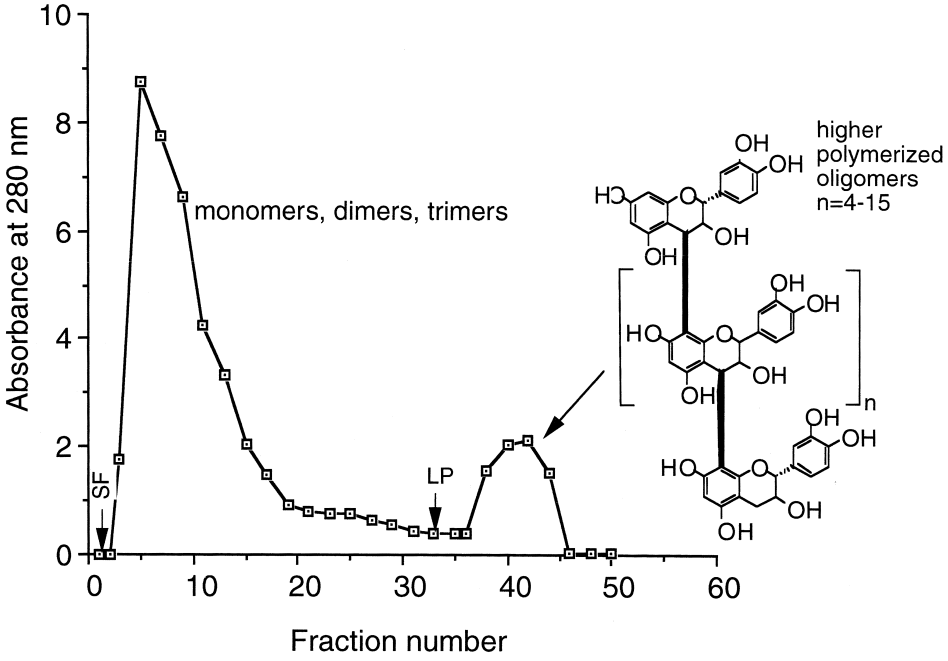

Fig. 3. Counter-current chromatogram of apple condensed tannin obtained by the type J multilayer coil planet centrifuge. Experimental conditions: column is a 1.0 mm I.D. PTFE multilayer coil, 35 ml capacity; sample is the solution consisting of 50 mg ACTs in 1 ml each upper and lower phase; solvent system is *n*-butanol–MTBE–acetonitrile–0.1% TFA (2:4:3:8); stationary phase is the lower aqueous phase; mobile phase is the upper organic phase; flow-rate=1.0 ml/min; revolution=1000 rpm; SF=solvent front; LP=lower phase eluted in the reversed direction.

of ACTs was injected into the column. The separation was performed by pumping the upper organic phase at a flow-rate of 1.0 ml/min at 1000 rpm. The effluent was monitored at 280 nm and fractions were collected at 3 ml per tube. The solvent front marked SF emerged at the third tube. After the elution of the first peak containing a mixture of monomer, dimer and trimer of procyanidin, the lower phase was pumped into the column in the reversed direction to facilitate rapid elution of the higher polymerized oligomers. The retention of the stationary phase was estimated at 82% of the total column capacity. As show in the previous paper, the ACTs used as the CCC sample was the mixture of monomers, dimers, trimers and oligomers from tetramers to pentadecamers of procyanidin [23]. The first peak corresponds to a mixture of monomers, dimers and trimers of procyanidin and the second peak should correspond to the oligomer.

In order to improve the peak resolution, the separation was performed with a larger-capacity (72-ml) multilayer coil. Fig. 4 shows CCC separation of ACTs using the same solvent system but with the upper phase as the stationary phase. In this separation five main peaks were resolved. Based on the HPLC analysis, peak fractions were each identified as procyanidin oligomers, unknown trimers, procyanidin B1, procyanidin B2, C1 mixed fraction and monomers in the order of elution. This elution order of procyanidins is well correlated with the degree of polymerization. The original ACTs sample and its CCC fractions were analyzed by reversed-phase HPLC as shown in Fig. 5. CCC fractions 20 and 21 corresponding to the second peak contained unknown procyanidin trimer (Fig. 5B). CCC fractions 36 and 37 corresponding to the third peak contained only procyanidin B1 (Fig. 5C). Fractions 50 and 51 contained a large amount of procyanidin

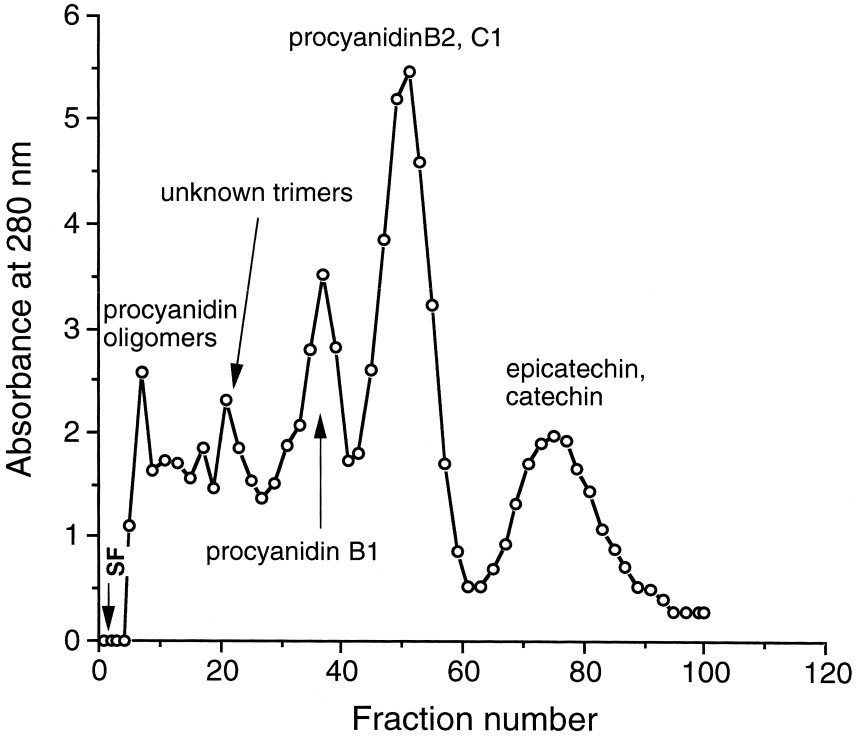

Fig. 4. Separation of procyanidins by the type J CPC. Experimental conditions: column is a 2.0 mm I.D. PTFE multilayer coil, 72 ml capacity; sample is the solution consisting of 100 mg ACTs in 1 ml each upper and lower phase; solvent system is *n*-butanol–MTBE–acetonitrile–0.1% TFA (2:4:3:8); stationary phase is the upper organic phase; mobile phase is the lower aqueous phase; flow-rate=1.0 ml/min; revolution=1000 rpm; SF=solvent front.

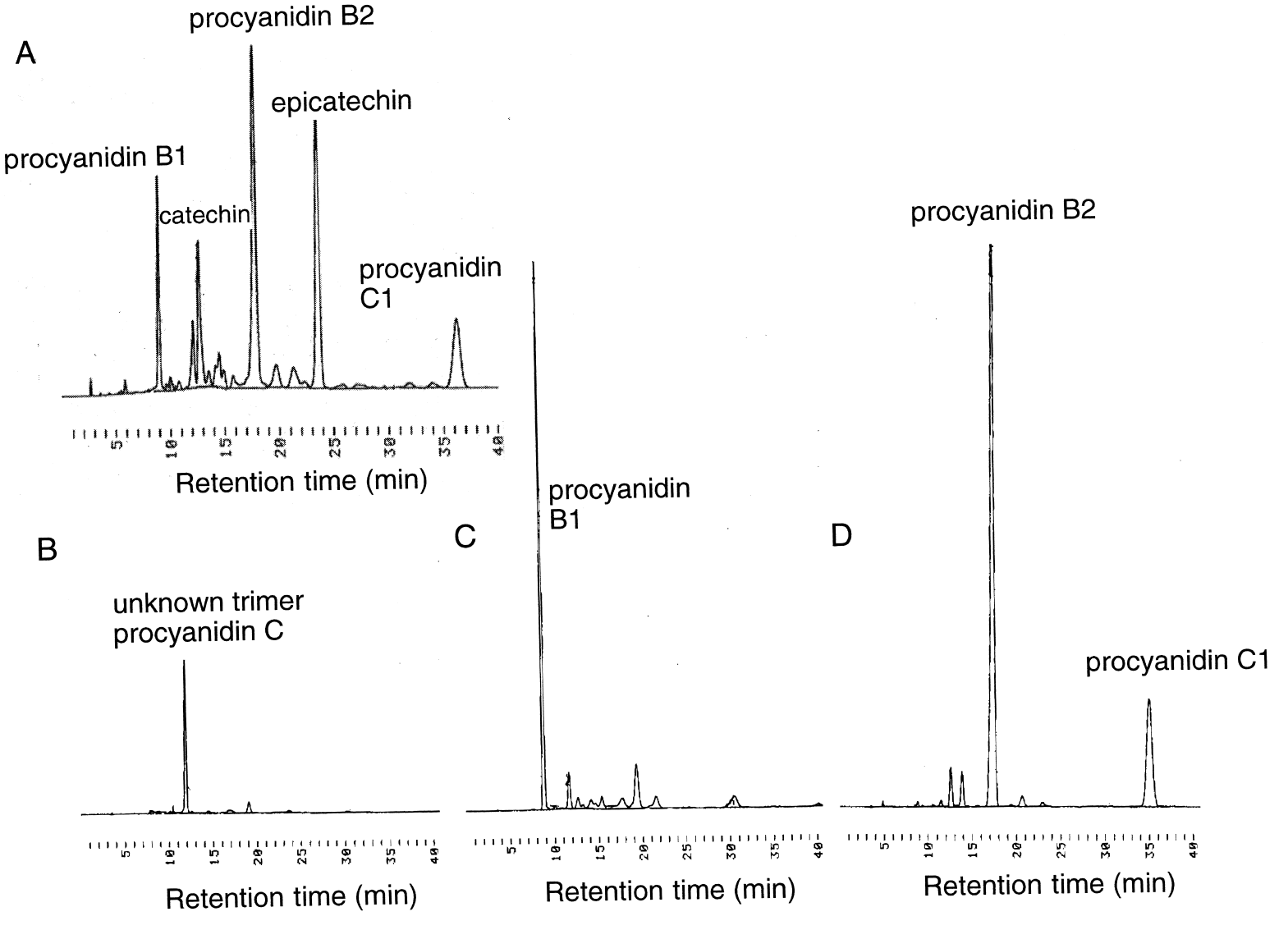

Fig. 5. Reversed-phase HPLC profiles of ACTs (A), fractions 20–21 (B), 36–37 (C) and 50–51 (D). The HPLC conditions are shown in the Experimental section.

B2 mixed with a small amount of procyanidin C1 (Fig. 5D).

The overall results of the above studies indicate that the high-speed CCC technique can be used to purify monomers, procyanidins B1 and B2 from ACTs using a polar *n*-butanol solvent systems. We are planning to determine the structure of the unknown trimer and study its physiological activities. Also the one-step separation of procyanidin dimers and trimers from crude apple polyphenols may be carried out by pH-zone-refining CCC without prepurification by liquid chromatography.

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