ELSEVIER

Journal of Chromatography A, 915 (2001) 253–257

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Short communication

Separation of apple procyanidins into different degrees of polymerization by high-speed counter-current chromatography

Yoichi Shibusawa[a,*], Akio Yanagida[a], Mitsuhiro Isozaki[a], Heisaburo Shindo[a], Yoichiro Ito[b]

[a]*Department of Analytical Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*

[b]*Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA*

Received 12 December 2000; received in revised form 15 January 2001; accepted 15 January 2001

Abstract

Apple procyanidins were fractionated by high-speed counter-current chromatography in a one-step operation from apple condensed tannins using a type-J multilayer coil planet centrifuge. The separation of procyanidins was performed with a two-phase solvent system composed of methyl acetate–water (1:1) by eluting the upper phase at a flow-rate of 1.0 ml/min. Each fraction was examined by time-of-flight mass spectrometry. Procyanidins were separated according to their degrees of polymerization.

Keywords: Counter-current chromatography; Apples; Procyanidins; Catechins; Tannins

1. Introduction

Counter-current chromatography (CCC) is a liquid–liquid partition technique that eliminates various complications arising from the use of solid supports [1–4]. Among all existing CCC systems, high-speed CCC is the most advanced form in terms of partition efficiency and separation time [5,6]. This technique has been used for separation and purification of a wide variety of natural products.

Unripe apple contains polyphenols including dihydrocalcons, phenolic acids, and others up to 50% of the total mass of solids while the rest consists of monomers, dimers, trimers and oligomers of catechin and/or epicatechin which are called apple procyanidins. Procyanidins have attracted attention in the fields of pharmacology and food chemistry because of their beneficial pharmacological effects such as promoting hair-growth [7], anti-allergy [8], antibiotic [9] and inhibitory activity against enzymes and receptors [10–13]. In order to describe these pharmaceutical activities according to the different degrees of polymerization, it is necessary to establish an efficient, reliable separation method. Separations of procyanidins have been reported using normal-phase [14,15], reversed-phase [16] and size-exclusion [17] modes of liquid chromatography. However, procyanidins showing polymerization beyond hexamers have not been reported.

In the present study, the high-speed CCC was applied to the separation of monomers, dimers, trimers and oligomers of catechin and/or epicatechin

*Corresponding author.

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00575-1

from apple condensed tannins (ACTs) using hydrophilic two-phase solvent systems. The degree of polymerization of the procyanidin oligomers in the eluted fractions was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis [18].

2. Experimental

2.1. Apparatus

The CCC separation of procyanidin oligomers from ACTs was performed using a type-J coil planet centrifuge (Fig. 1). The apparatus holds a multilayer coiled separation column and a counter-mass symmetrically at a distance of 10 cm from the central axis of the centrifuge. The separation column was fabricated by winding 21 m×2.0 mm I.D. PTFE (polytetrafluoroethylene) tubing (Tokyo Rikakikai, Tokyo, Japan) making four coiled layers (β=0.5–0.62). The total capacity of the column is 72 ml. The speed of the apparatus was regulated at 1000 rpm with a speed controller. The coiled column 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.

2.2. Reagents

Methyl acetate, methanol, acetonitrile, methyl *tert*.-butyl ether (MTBE), and trifluoroacetic acid (TFA) were glass-distilled chromatographic grade (Kanto, Tokyo, Japan). Catechin, epicatechin were obtained from Sigma (St. Louis, MO, USA). ACTs were prepared from unripe apple as described in detail elsewhere [17,18]. Purified ACTs are a mixture of monomeric catechins and/or epicatechin and procyanidin.

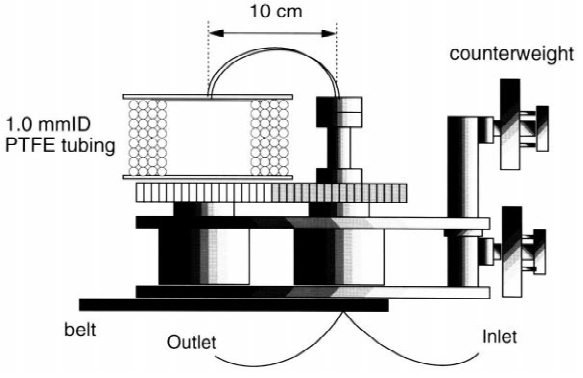

Fig. 1. Schematic drawing of the type-J coil planet centrifuge.

2.3. Procedures

The following four hydrophilic solvent systems were selected based on the partition coefficient values (K_D) of catechin, epicatechin and ACTs: (1) MTBE–acetonitrile–0.1% aqueous TFA (4:1:5); (2) MTBE–acetonitrile–0.1% aqueous TFA (6:3:8); (3) MTBE–acetonitrile–0.1% aqueous TFA (2:2:3) and (4) methyl acetate–water (1:1). Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The K_D values of catechin, epicatechin and ACTs were determined (n>3) spectrophotometrically by the following procedure using the above two-phase solvent systems. About 1.5 ml of each phase was delivered into a test tube to which 1 mg of the each catechin, epicatechin and ACTs was added. The contents were thoroughly mixed and allowed to settle at room temperature. After two clear layers were formed, an aliquot (usually 0.15 ml) of each phase was removed and diluted with 1.35 ml of methanol to determine the absorbance at 280 nm using a Shimadzu UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan). The K_D values were calculated by dividing the absorbance value in the upper phase with that of the lower phase.

In each CCC separation, a 72-ml capacity multilayer coiled column of the type-J high-speed CCC centrifuge was first entirely filled with the stationary lower phase and a sample solution containing 100 mg of ACTs was injected into the column using an EYELA type SV-6000 sample injector (Tokyo Rikakikai). Then the apparatus was rotated at 1000 rpm while the upper mobile phase was pumped into the column by an EYELA LP 1100 pump at a flow-rate of 1.0 ml/min. The effluent from the column was continuously monitored at 280 nm (the I_{max} of the absorption spectra of ACTs) 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. The CCC fractions eluted with the upper organic phase were analyzed by reversed-phase high-performance liquid chromatography (HPLC) described elsewhere [19]. The masses of the fractionated procyanidins showing polymerization beyond hexamers were determined by MALDI-TOF-MS analysis. MALDI-TOF-MS was performed using a Voyager DE RP system (PerSeptive Biosystems, CA, USA) in the linear positive-ion mode. Procyanidin oligomers eluted with the lower phase were ionized by a nitrogen laser (337 nm; 3 ns pulse) and accelerated under 20 kV [18]. 2′4′6′-Trihydroxyacetophenone monohydrate (THAP) was used as matrix compound.

3. Results and discussion

The partition coefficient values (K_D) of catechin, epicatechin and ACTs are listed in Table 1 together with separation factor (α) between catechin and/or epicatechin and ACTs. The K_D value is the most important parameter to determine the peak resolution in CCC. In the four hydrophilic solvent systems tested in this studies, the K_D values of ACTs are much smaller than those of catechin and epicatechin suggesting that monomers are more hydrophobic than their oligomers present in ACTs. In the MTBE–acetonitrile–0.1% aqueous TFA (2:2:3) solvent system, the α value of epicatechin/ACTs is 1.35 indicating that the separation of monomers from their oligomers is not feasible. An α value over 2.0 is usually needed to separate the two solutes each other. Among other three systems, we selected a simple binary system of methyl acetate–water for the separation of procyanidin oligomers from ACTs by CCC.

The capability of the type-J CPC was demonstrated on the separation of apple procyanidins by eluting the 100 mg of the ACTs with the upper phase at a flow-rate of 1.0 ml/min. As shown in Fig. 2, the monomer, dimer and trimer of procyanidins were eluted from the column in increasing order of their degree of polymerization. The CCC fractions eluted with the upper organic phase were assigned by HPLC analysis. After the elution of trimers, the lower aqueous phase was eluted through the column to facilitate a rapid elution of the procyanidins still remaining in the column. The tetramers and pentamers fraction, the pentamers, hexamers and heptamers fraction and higher polymerized oligomer over hexamers fraction were subsequently eluted from the column in this order. The MALDI-TOF-MS spectra of the fractions 46, 52 and 55 are shown in Fig. 3. Interestingly, the elution order is also coincident with the degree of polymerization of catechin and/or epicatechin. The last large peak will contain higher polymerized procyanidin oligomers (over hexamers) and was not assigned by MALDI-TOF-MS under these experimental conditions. The assignment of the oligomers will be investigated further and will be reported in a subsequent paper. The lower stationary phase retained in the column was estimated as 82% of the total column capacity (72 ml) prior to the application of the elution with lower phase. The separation was completed within 3 h.

These results indicate that the present method is capable of fractionating the apple procyanidins from ACTs according to the degree of the polymerization

Table 1
Partition coefficient values of catechin, epicatechin and ACTs

Solvent system	Catechin (*c*)	Epicatechin (*e*)	ACTs (*A*)	Separation factor (α)
MTBE–acetonitrile–0.1% aqueous TFA				
4:1:5	1.56	1.59	0.31	4.97 (*c*/*A*)
6:3:8	1.94	1.55	0.33	4.70 (*e*/*A*)
2:2:3	1.88	1.32	0.98	1.35 (*e*/*A*)
Methyl acetate–water				
1:1	1.65	2.00	0.53	3.11 (*c*/*A*)

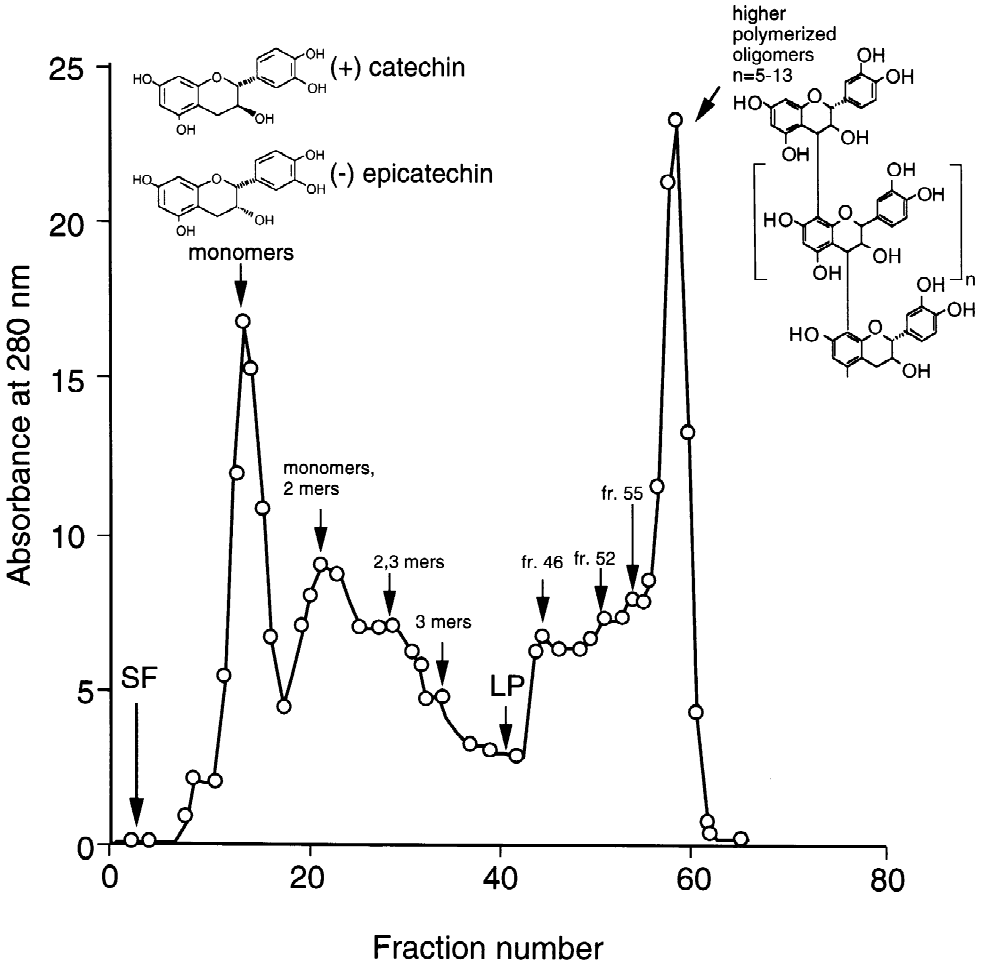

Fig. 2. Separation of procyanidins in the order of their polymerization by the type-J CPC. Experimental conditions: column is a 2.0 mm I.D. PTFE multilayer coil, 72 ml capacity; sample is the solution of 100 mg ACTs in 1 ml each upper and lower phase; solvent system is methyl acetate–water (1:1); stationary 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 column.

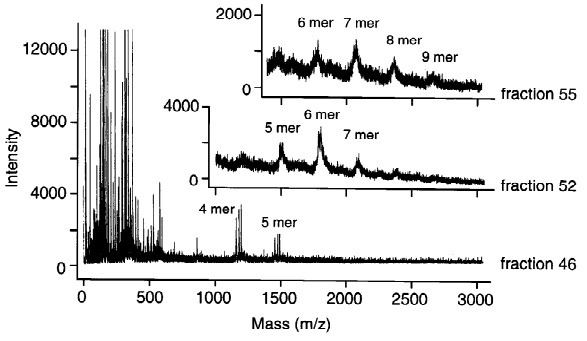

Fig. 3. MALDI-TOF mass spectra of CCC fractions eluted by lower aqueous mobile phase.

of catechin and/or epicatechin. The higher polymerized procyanidins will be separated using modified solvent systems. The experiment is now underway in our laboratory.

Acknowledgements

The authors are indebted to Dr. Henry M. Fales for editing the manuscript with valuable suggestions.

References

[1] Y. Ito, R.L. Bowman, Science 167 (1970) 281.
[2] Y. Ito, R.L. Bowman, J. Chromatogr. Sci. 8 (1970) 315.
[3] T. Tanimura, J.J. Pisano, Y. Ito, R.L. Bowman, Science 169 (1970) 54.
[4] Y. Ito, R.L. Bowman, Anal. Chem. 43 (1971) 69A.
[5] Y. Ito, J. Sandlin, W.G. Bowers, J. Chromatogr. 244 (1982) 247.
[6] Y. Ito, E. Kitazume, J.L. Slemp, J. Chromatogr. 538 (1991) 81.
[7] T. Takahashi, T. Kamiya, A. Hasegawa, Y. Yokoo, J. Invest. Dermatol. 112 (1999) 310.
[8] T. Kanda, H. Akiyama, A. Yanagida, M. Tanabe, Y. Goda, M. Toyoda, Y. Saito, Biosci. Biotechnol. Biochem. 62 (1998) 1284.
[9] A. Scalbert, Phytochemistry 30 (1991) 3875.
[10] A. Yanagida, T. Kanda, M. Tanabe, F. Matsudaira, J.G.O. Cordeiro, J. Agric. Food Chem. 48 (2000) 5666.
[11] S. Guvot, P. Pellerin, J.M. Brillouet, V. Cheynier, Biosci. Biotechnol. Biochem. 60 (1996) 1131.
[12] B.H. Wang, L.Y. Foo, G.M. Polya, Phytochemistry 43 (1996) 359.
[13] M. Zhu, J.D. Phillipson, P.M. Greengrass, N.E. Bowery, Y. Cai, Phytochemistry 44 (1997) 441.
[14] J. Rigaud, M.T. Escribano-Bailton, C. Prieur, J.M. Souquet, V. Cheynier, J. Chromatogr. A 654 (1993) 255.
[15] A. Yanagida, T. Kanda, T. Takahashi, A. Kamimura, T. Hamazono, S. Honda, J. Chromatogr. A 890 (2000) 251.
[16] G.E. Rohy, B. Meier, O. Sticher, J. Chromatogr. A 835 (1999) 59.
[17] A. Yanagida, T. Kanda, T. Shoji, M. Ohnishi-Kameyama, T. Nagata, J. Chromatogr. A 855 (1999) 181.
[18] M. Ohnishi-Kameyama, A. Yanagida, T. Kanda, T. Nagara, Rapid Commum. Mass Spectrom. 11 (1997) 31.
[19] Y. Shibusawa, A. Yanagida, A. Ito, K. Ichihashi, H. Shindo, Y. Ito, J. Chromatogr. A 886 (2000) 65.