Fractionation of apple procyanidins according to their degree of polymerization by normal-phase high-performance liquid chromatography

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Abstract

A new method was developed for the fractionation of procyanidin oligomers according to their degree of polymerization. Monomeric flavan-3-ols and low molecular mass procyanidins were selectively extracted from the lyophilized powder of apple condensed tannins (ACTs) by methyl acetate extraction. Sequentially, the separation of each oligomer from dimer to pentamer in this extract was carried out by normal-phase high-performance liquid chromatography using a silica-beads packed column. The best separation was achieved with a mobile phase system containing hexane; (1) hexane–methanol–ethyl acetate, (2) hexane–acetone. These sequential treatments can be easily adapted to large-scale fractionation. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Proanthocyanidins, which are called condensed tannins, have a wide distribution in the plant kingdom as secondary metabolites. They are a mixture of oligomers consisting of chains of flavan-3-ol units linked mainly through C4–C8 (or C6) bonds. Edible fruits are one of the richest sources of proan-thocyanidins [1,2], and we consume these compounds every day from the fresh fruits, juices, wines, and the process foods made from fruits. Rather than nutrients, plant proanthocyanidins are known as the functional food factors that possess a variety of physiological activities such as antioxidant [3–6], antimicrobial [7], anti-allergy [8], hair-growth promotion [9,10], anti-caries [11], anti-hypertensive [12] and inhibition against the activities of some physiological enzymes and receptors [13–16]. The greater part of these activities of proanthocyanidins largely depend on their structures and particularly their degree of polymerization. To elucidate the physiological mechanisms of proanthocyanidins and to

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provide these oligomers for a subsequent in vivo study, it is required to establish an effective separation and fractionation method of proanthocyanidin oligomers according to their degree of polymerization.

Until now, many chromatographic separation methods of proanthocyanidins have been developed, but there is no perfect method satisfying the simplicity, separation efficiency and the scale of treatment. Reversed-phase HPLC was the general method for the separation of flavan-3-ol monomers and some small oligomers, especially dimers and trimers [17–21]. However, the elution order of oligomers was not according to their degree of polymerization, and the broad unresolved peaks derived from the higher polymerized oligomers were overlapped on the chromatogram. Chromatography on Sephadex LH-20 or Toyopearl HW-40 column with elution by alcohol–water or acetone–water solvents has commonly been applied to the purification of proanthocyanidins from plant materials [22,23]. In such cases, because the chromatographic mode is adsorption rather than size-exclusion, the separation of proanthocyanidins according to their degree of polymerization is incomplete. Interestingly, the use of acetone–8 M urea as the mobile phase on a HW-40 column resulted in a molecular sieve effect without any interaction based on hydrogen-bonds and hydrophobic adsorptions, and the procyanidin oligomers were eluted in order of their molecular masses [24]. However, this size-exclusion chromatography (SEC) is difficult to carry out on a large scale, and the oligomers larger than trimer were not separated clearly under this SEC condition.

Good separation according to the polymerization degree of proanthocyanidins has been achieved with thin-layer chromatography (TLC) on silica-plate [25,26]. Furthermore, an excellent NP-HPLC method adapted from the TLC method was reported by Rigaud et al. [27]. In their NP-HPLC system, the oligomeric constituents of cacao bean procyanidins were clearly separated from monomer to pentamer on a silica column eluted with dichloromethane–methanol–formic acid–water as the mobile phase. Theoretically this separation method can be applied to large-scale fractionation of proanthocyanidin oligomers, but practically, the use of a chlorinated solvent and strong acid as the HPLC solvent con-

siderably limits the fractionation scale, especially in an industrial process.

The aim of this study was to develop a new NP-HPLC method to separate oligomeric proanthocyanidins according to their degree of polymerization. In this study, apple procyanidins (apple condensed tannins: ACTs) [24,28] were used as the experimental sample. For large-scale fractionation, some usual organic solvents containing hexane were applied as the mobile phase against silica stationary phase. Additionally, to perform the effective fractionation of low molecular mass oligomers from dimers to pentamers by NP-HPLC, the solvent extraction using methyl acetate was tried for the purification process of procyanidin oligomers from ACTs.

2. Experimental

2.1. Materials

Epicatechin (EC) was purchased from Wako Pure Chemicals (Osaka, Japan). Procyanidin B1 (epicatechin-(4β→8)-catechin: PB1) and procyanidin B2 (epicatechin-(4β→8)-epicatechin: PB2) and procyanidin C1 (epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin: PC1) were isolated from apple juice [24].

All other chemicals were of analytical reagent grade. All chromatographic solvents were of HPLC grade.

2.2. Apple procyanidins (apple condensed tannins: ACTs)

The preparation of apple procyanidins (ACTs) obtained from immature apples has been described in detail elsewhere [24,28]. Briefly, the filtered juice was obtained from immature apples by press with the presence of 0.1% potassium pyrosulfite. Next, crude apple polyphenols (CAP) in this juice were prepared by the column chromatography using Sepabeads SP-850 beads (Mitsubishi Kasei, Tokyo, Japan). Finally, ACT fraction was purified from CAP fraction by the Toyopearl HW-40EC (Tosoh, Tokyo, Japan) column chromatography and Sep-Pak C_{18} (Waters Milford, MA, USA) treatment. The organic solvent was

removed from final ACT fraction by evaporation, and the remaining materials was lyophilized as ACT powder.

Purified ACTs were the mixture of monomeric flavan-3-ols (catechin and EC) and procyanidin oligomers ranging in size from dimer (2-mer) to pentadecamer(15-mer) [28]. Other previous reports [29] provided that the proanthocyanidins in apple were composed of only procyanidin-form (i.e., hydroxylation pattern of B-ring; 3′, 4′).

2.3. Reversed-phase HPLC analysis of standard oligomers in experimental samples

The analysis of standard oligomers (catechin, EC, PB1, PB2 and PC1) in the experimental samples and fractions was performed by reversed-phase HPLC. The HPLC condition was described elsewhere [24]. Briefly, the column used was an Inertsil ODS-3 packed column (150 mm×4.6 mm I.D., 5-µm particle size, GL Sciences, Tokyo, Japan). The mobile phase solvents A and B were each a mixture of methanol–0.01 M KH$_2$PO$_4$ (adjusted to pH 2 with phosphoric acid) at volume ratios of (A) 2:8 and (B) 5:5. A 10-µl sample solution was injected and eluted with solvent A for 10 min. Then, linear gradient elution from 0 to 100% solvent B was applied for 40 min. The above chromatography was performed at a flow-rate of 1 ml/min and the absorbance of the eluate was monitored at 280 nm.

2.4. Methyl acetate extraction of procyanidin oligomers from ACTs

Lyophilized powder of 1 g of ACTs was added into 100 ml of methyl acetate. The mixture was stirred for 30 min in room temperature and was filtered. The residue was re-extracted with methyl acetate under the same condition. Then, both filtrates were combined and concentrated by evaporation. Sequentially, this concentrated solution was supplied to normal-phase liquid chromatographic separation. A portion of this concentrated solution was lyophilized as the methyl acetate extract. On the other hand, the final residue of methyl acetate extraction of ACTs was dissolved in distilled water and the solution was lyophilized. Both lyophilized samples

were supplied to size-exclusion chromatographic analysis.

2.5. Size-exclusion chromatography (SEC) of methyl acetate extract of ACTs

The composition of procyanidin oligomers in the methyl acetate extract and/or residue of ACTs was examined by SEC using TSKgel Toyopearl HW-40F (fine grade; Tosoh) column [24]. The mobile phase composed of the mixture of acetone–8 M urea (6: 4 v/v) was used. A 0.5-ml portion of sample solution, prepared by dissolving the lyophilized sample in the mobile phase was loaded on the column (950×12 mm I.D.), and the elution was performed at a flow-rate of 1.0 ml/min with a L-6000 pump (Hitachi, Tokyo, Japan) at room temperature. Three-ml volume fractions were collected using a type SF-2120 fraction collector (Advantec Toyo, Tokyo, Japan), and the phenolic content in the fractions was analyzed by the modified Folin–Ciocalteu method [24]. Finally, the total content of procyanidin oligomers in each SEC fraction was determined by the absorbance at 760 nm using a U-2000 spectrophotometer (Hitachi).

2.6. Nomal-phase high-performance liquid chromatography (NP-HPLC) of methyl acetate extract of ACTs

The separation of procyanidin oligomers in methyl acetate extract was performed by NP-HPLC using a silica-beads packed column. Before NP-HPLC analysis, a portion of lyophilized powder of extract was dissolved in a small amount of methyl acetate as the sample solution. Analytical HPLC separation was carried out on a Hitachi system consisting of a L-6200 intelligent pump, an AS-2000 auto sampler, a L-4200 UV-VIS detector and a D-2500 integrator. The column used was an Inertsil SIL column (150× 4.6 mm I.D., 5-µm particle size) (GL Sciences). The mobile phase composed of hexane–methanol–ethyl acetate (7:3:1) was used. A 20-µl sample solution was injected through the sample port and eluted isocratically at a flow-rate of 1.8 ml/min, and the absorbance of eluate was monitored at 280 nm.

A large-scale HPLC system consisted of a L-6200

intelligent pump (Hitachi) equipped with preparative pump-head unit, a PUS-65 double-planger pump (GL Sciences), a rheodyne 7715 sample injector connected with a 30-ml sample loop, a S-310 UV-VIS detector (Soma Kogaku, Tokyo, Japan) equipped with the flow-cell of 0.1 mm in thickness, and a D-7500 integrator (Hitachi). A L-6200 pump and a PUS-65 pump were arranged in a line with peek tube. A Sokensil s-15/30 (Soken Chemical and Engineering, Tokyo, Japan), which was porous sphere silica-bead (15–30 μm particle size), was used as the material of stationary phase and was packed in the stainless-steel columns (500×50 mm I.D.). The mobile phase solvents A and B were each a mixture of hexane–acetone at volume ratios of (A) 35:65 and (B) 20:80. The lyophilized powder of 12 g of extract was dissolved in 30 ml of methyl acetate as sample solution, and this solution was injected through the sample loop and eluted with solvent A for 80 min. Then, linear gradient elution from 0 to 100% solvent B, was applied for 110 min. Further, the components that remained in the column were then eluted with solvent B for 110 min. The elution was performed at a flow-rate of 57 ml/min and the absorbance of the eluate was monitored at 230 nm. The eluate were fractionated using a type SF-2120 fraction collector (Advantec Toyo). The NP-HPLC fractions by peaks were combined separately, and a little portion of distilled water was added into each combined solution. The organic solvent in each solution was removed by evaporation, and the remaining material was lyophilized. The procyanidin oligomers in these samples were characterized by MS analysis.

2.7. MS analysis of NP-HPLC fractions

The molecular mass profiles of procyanidin oligomers separated by NP-HPLC were determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) MS. The MALDI TOF mass spectra of lyophilized samples were acquired on a REFLEX II (Bruker-Franzen Analytik, Bremen, Germany) instrument and were obtained in the positive-ion mode. The other details of analytical condition of MS were described elsewhere [24,28].

3. Results and discussion

3.1. Selective purification of low molecular mass procyanidin oligomers from ACTs by methyl acetate extraction

Fig. 1 shows the structures of the monomeric and oligomeric constituents in ACTs. About half of constituents in ACTs were composed of monomeric flavan-3-ols (catechin and EC), dimeric procyanidins (PB1 and PB2) and trimeric procyanidins (mainly, PC1), and the other half of those in ACTs are higher polymerized oligomers. Generally, procyanidins are very hydrophilic compounds and their polarities gradually increase with an increase of polymerization degree. In our preliminary experiments, when the ACTs were loaded on the silica column for NP-HPLC, a part of highly polymerized oligomers was irreversibly adsorbed to the silica stationary phase. Therefore, for the elimination of these high polymerized oligomers from ACTs, the solvent extraction was performed. The ACT powder perfectly dissolved in water, acids, alcohols and acetone, and did not
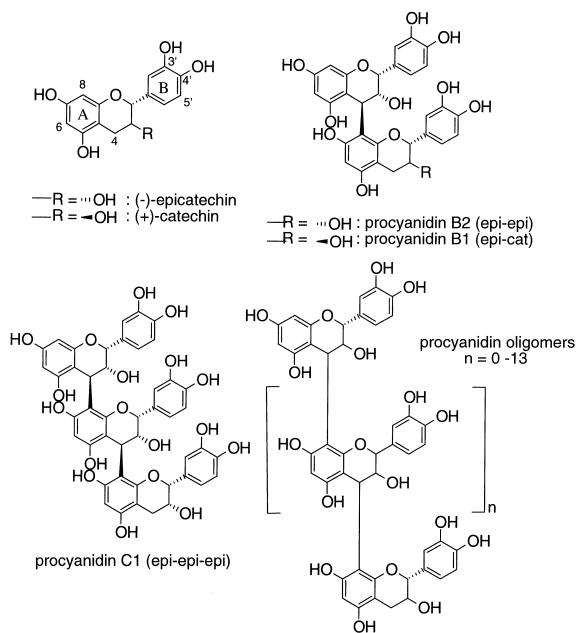

Fig. 1. Structures of monomeric flavan-3-ols and procyanidin oligomers in apples.

dissolve in hexane and chloroform. However, some organic esters such as ethyl acetate and methyl acetate were partially dissolved in the ACT powder. Table 1 shows the yields and recoveries of total solid and procyanidin dimers (PB1, PB2) and trimer (PC1) in each extract of solvent extraction. In comparison with the single extraction data between ethyl acetate and methyl acetate used as the extraction solvent, the recoveries of total solid and the sum of oligomers (PB1+PB2+PC1) in the methyl acetate extract were better than those in the ethyl acetate extract. In particular, the methyl acetate extraction (100 ml, two times) of 1 g of ACT powder gave 583 mg of the extract and 417 mg of the residue, and the yield of the sum of oligomers (PB1+PB2+PC1) in the extract was maintained high (the recovery: 90.8%). This result means that the low molecular mass procyanidins were selectively extracted from ACT powder by the methyl acetate extraction.

Next, the oligomeric composition of methyl acetate extract and/or residue was examined by SEC using TSKgel Toyopearl HW-40F (950×25 mm I.D.). The SEC profile of both the extract and residue of ACTs is shown in Fig. 2. Because the procyanidin oligomers are eluted in order of their molecular weight (i.e. polymerization degree) under this SEC condition [24], the highly polymerized oligomer is eluted faster than the small one from the HW-40F column. Fig. 2 shows that the oligomeric constituents of residue were eluted faster than those of methyl acetate extract, and that the constituents of extract were mainly composed of low molecular mass

oligomers containing monomers, dimers and trimers. Then, both the data shown in Table 1 and Fig. 2 indicate that the methyl acetate selectively extracted the low molecular mass oligomers from ACT powder.

3.2. NP-HPLC separation and fractionation of methyl acetate extract of ACTs using mobile phase containing hexane

Although the silica NP-HPLC technique reported by Rigaud et al. [27] is an excellent separation method of procyanidins according to their degree of polymerization, the composition of the mobile phase (i.e. dichloromethane–methanol–formic acid–water) considerably limits the fractionation scale. Particularly in industrial processes, the use of a large volume of chlorinated solvent could be hazardous and the use of water and strong acid deteriorates the silica stationary phase. We thought that the most suitable NP-HPLC mobile phase for large-scale fractionation of procyanidin oligomers had to be constituted of a few kinds of usual organic solvents having low boiling points and without water and acids.

In our preliminary experiments, when the methanol and ethanol were used as the mobile phase, the procyanidin oligomers in ACTs were not retained on the column. Then, the many solvent mixtures containing hexane were tested as the mobile phase. The methyl acetate extract of ACTs was used as a NP-HPLC sample, because the highly polymerized

Table 1

Yields and recoveries of total solid and dimeric and trimeric procyanidin oligomers in each extract of solvent extraction[a]

Extraction solvent	Extraction times	Total solid in extract		PB1 (mg)[c]	PB2 (mg)[c]	PC1 (mg)[c]	PB1+PB2+PC1	
		Yield (mg)[b]	Recovery (%)				Yield (mg)[c]	Recovery (%)
Methanol	1	1000	100	36.4	136.1	63.0	235.5	100
Ethyl acetate	1	138	13.8	10.9	34.9	12.6	58.4	24.8
Methyl acetate	1	399	39.9	25.9	87.7	31.1	144.7	61.5
Methyl acetate	2	583	58.3	34.6	126.3	53.0	213.9	90.8

[a] Lyophilized power of 1 g of ACT was extracted with 100 ml of each solvent. For the other conditions of the extraction see Experimental.

[b] Yield of total solid in each extract was equal to the weight of lyophilized material of the extract.

[c] Yields of procyanidin dimers (PB1: procyanidin B2) and a trimer (PC1: procyanidin C1) in each extract were determined by reversed-phase HPLC analysis.
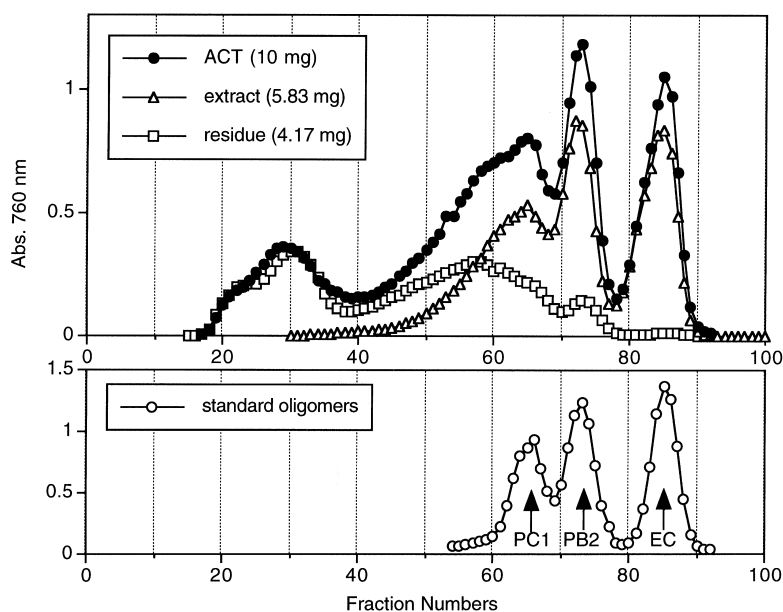
Fig. 2. SEC elution profiles of methyl acetate extract and/or residue of ACTs applied to a Toyopearl HW-40F column and eluted at flow-rate of 1.0 ml/min. Column size: 950×25 mm I.D. Mobile phase: acetone–8 M urea (6:4). Upper chromatograms are the elution pattern of ACTs (10 mg) before extraction, that of methyl acetate extract (5.83 mg) of ACTs and that of the residue (4.17 mg). Lower chromatogram is the elution pattern of the mixture of standard oligomers (EC, PB2 and PC1, 2 mg each) as the molecular weight markers. Each lyophilized sample was dissolved in 0.5 ml of mobile phase solvent, and loaded on the HW-40F column. SEC separation was carried out at room temperature.

oligomers in ACTs do not dissolve in the mobile phase solvent containing hexane.

A first good separation of procyanidin oligomers in this extract was achieved with isocratic elution by hexane–methanol–ethyl acetate (7:3:1) (the data not shown). In this case, the absorbance of eluate was monitored at 280 nm, and the five separated peaks appeared on the chromatogram. However, under these conditions, part of the polymerized oligomers in the extract were not eluted from the column and were retained on the silica stationary phase. It was assumed that this shortcoming was improved by the gradient elution with the increase of mobile phase polarity.

In our further search for the mobile phase solvent containing hexane for NP-HPLC separation, we found that hexane–acetone (approximately, 4:6) separated the oligomeric constituents in methyl acetate extract of ACTs as well as hexane–methanol–ethyl acetate (7:3:1). Generally, acetone is not often used as the HPLC solvent for the separation of

the compounds absorbed by UV rays because acetone also has the nature of an UV absorbent. For this reason, in the mobile phase containing acetone, procyanidins in eluate could not be detected at 280 nm, which is the maximum absorbance wavelength of procyanidins. However, because the relative UV absorbance of procyanidins was stronger than that of acetone at the range of wavelength from 220 to 230 nm, the eluate from the NP-HPLC column could be monitored at this wavelength range using the UV detector attached with the small capacity flow-cell. After the many trials of analytical NP-HPLC separation using this solvent system, we carried out the large-scale fractionation of procyanidin oligomers in methyl acetate extract of ACTs using the large column (500×50 mm I.D.) with gradient elution system. The chromatogram is shown in Fig. 3. Five major peaks appeared on the chromatogram, and the fractions A, B, C, D and E were collected and lyophilized. When the 12 g of extract of ACTs dissolved with 30 ml of methyl acetate were loaded
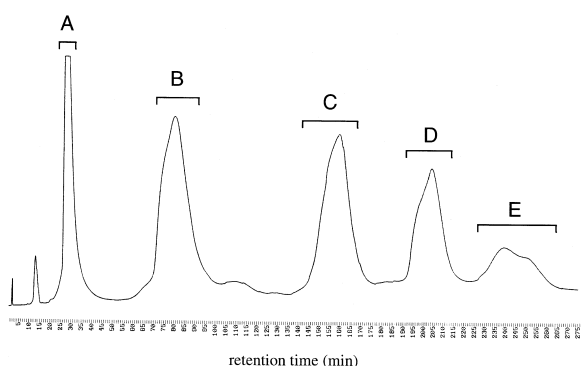
Fig. 3. Preparative NP-HPLC profile of methyl acetate extract of ACT. Column size: 500×50 mm I.D. Solvent composition of mobile phases: hexane–acetone (35:65 and 20:80). The mobile phases were eluted gradually and the absorbance of the eluate was monitored at 230 nm. The fractions obtained are named from A to E. For the details of the preparative conditions, see Experimental.

on the column, 1.69 g, 2.17 g, 2.06 g, 1.67 g and 1.14 g of lyophilized constituents were obtained from fractions A, B, C, D and E, respectively.

The results of reversed-phase HPLC analysis of each fraction is shown in Fig. 4. The constituents in fr. A were only composed of monomers (catechin and EC) and those in fr. B were only dimers (PB1 and PB2). However, those in fr. C, D and E were the complex of many kinds of stereoisomers, and those peaks on each chromatogram were not identified excepting with PC1 in fr. C. Furthermore, the molecular mass distribution of the lyophilized sample obtained from each NP-HPLC fraction was characterized by MALDI-TOF MS analysis. These mass spectra are shown in Fig. 5. The data reveals that the oligomeric procyanidins in the corresponding fr. B, C, D and E are the dimers, trimers, tetramers and pentamers, respectively. These results shown in
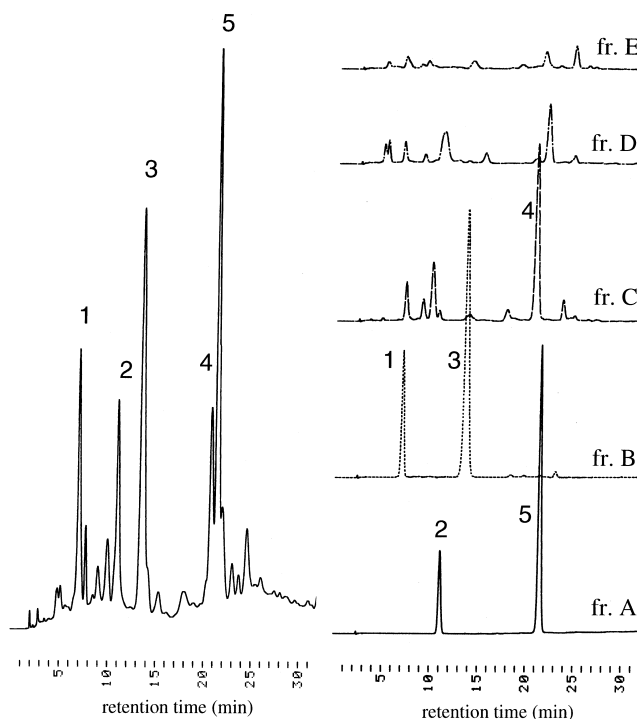

Fig. 4. Reversed-phase HPLC profiles of ACTs and NP-HPLC fractions (fr.) of ACTs. Each lyophilized sample was dissolved in water (1 mg/ml) and analyzed. A left chromatogram is the profile of ACTs. Five right chromatograms are the profiles of NP-HPLC fractions. The numbers of NP-HPLC fractions correspond to those in Fig. 3. The numbers of the identified peaks in each chromatograms are (1) procyanidin B1 (PB1), (2) catechin, (3) procyanidin B2 (PB2), (4) procyanidin C1 (PC1), (5) epicatechin (EC). For the details of the HPLC conditions, see Experimental.
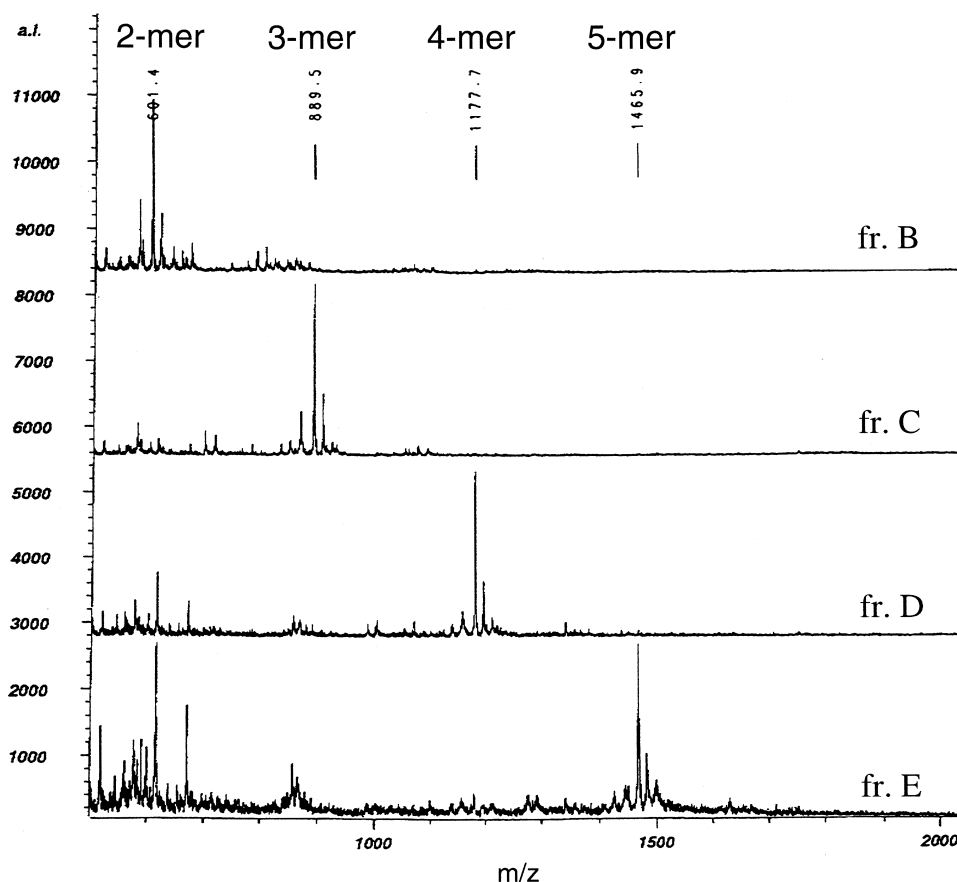
Fig. 5. MALDI-TOF mass spectra of preparative NP-HPLC fractions of methyl acetate extract obtained in the positive-ion mode. The numbers of NP-HPLC fractions correspond to those in Fig. 3. The number shown on each ionic peak ([M+Na]$^+$) indicates the degree of polymerization (number of flavan-3-ol units in each molecular ion).

Fig. 4 and 5 prove that the elution order of the procyanidin oligomers in the methyl acetate extract of ACTs from the silica column coincides with the increasing order of their polymerization degree.

3.3. Applications of this fractionation method and the separated oligomers

Our fractionation method of procyanidin oligomers from ACTs consisted of only the two simple treatments (i.e., methyl acetate extraction and NP-HPLC), and the all organic solvents used in each step did not contain the chlorinated solvent, strong acid or water. In view of the simplicity and solvent recycling, our method can be easily adapted to large-scale fractionation in an industrial process. This

method is not only applicable to apple procyanidins, but also other polymerized polyphenols from other plants (e.g., cacao, grape, persimmon, berries, banana), foods (wine, tea, chocolate) and traditional medicine (cinnamon bark, areca, rhubarb, chinese galls). Furthermore, the large amount of fractionated procyanidin oligomers with different degrees of polymerization may be the important tools of experiments for the elucidation of the physiological mechanism of condensed tannins, and they may also be useful for subsequent in vivo studies.

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