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Radical-scavenging proanthocyanidins from sea buckthorn seed

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Abstract

Water–acetone (3:7) extract of sea buckthorn seeds was separated by Sephadex LH-20 column chromatography into nine fractions. Three of the fractions exhibited strong radical-scavenging activities by DPPH· analysis and contained flavan-3-ols. Four monomeric flavan-3-ols, catechin (1), epicatechin (2), gallocatechin (3) and epigallocatechin (4), along with two dimeric procyanidins, catechin(4α-8)catechin (5) and catechin(4α-8)epicatechin (6), were isolated. The dimeric proanthocyanidins were investigated by HPLC, in combination with electrospray ionization mass spectrometric detection. The more abundant polymeric proanthocyanidins were fractionated and their chemical constitutions studied by acid-catalysed degradation in the presence of toluene-α-thiol. The results showed the polymers to be highly heterogeneous, with catechin, epicatechin, gallocatechin and epigallocatechin all being constituent components of both the extension as well as the terminating units. The mean degree of polymerization (mDP) was 12.2. The proportion of prodelphinidins was 81.2%.

Keywords: *Hippophae rhamnoides*; Sea buckthorn; Proanthocyanidins; Antioxidant activity

1. Introduction

Sea buckthorn (*Hippophae rhamnoides*) is a fascinating plant growing widely in various regions of Asia, Europe, and North America (Rousi, 1971). It was used as a medicinal plant in Tibet as early as 900 AD (Lu, 1992). It has had many diverse uses, from controlling soil erosion to being a source of horse fodder, nutritious foods, drugs and skin-care products. In recent years, sea buckthorn has been recognized as a versatile nutraceutical crop with great economic potential and environmental value in China, which has led to development of over 2×10^6 ha of sea buckthorn orchards.

Currently, the use of some naturally occurring antioxidant molecules in foods, as well as preventive and therapeutic medicine, is gaining popularity. Although high contents of natural antioxidants, including ascorbic acid, tocopherols, carotenoids and polyphenols, have been detected in sea buckthorn berries (Fu, Yang, & Yang, 1993; Gao, Ohlander, Jeppsson, Björk, & Trajkovski, 2000; Heinonen, Meyer, & Frankel, 1998; Rösch, Bergmann, Knorr, & Kroh, 2003; Yao & Tigerstedt, 1992) and its leaves (Chumbalov, Mukhamed'yarova, & Polyakov, 1976; Mukhamed'yarova & Chumbalov, 1977), few studies have investigated the antioxidant compounds in the seeds. The purpose of the present work was to isolate and identify the antioxidant compounds from sea buckthorn seeds.

2. Materials and methods

2.1. General

(+)-Catechin, (−)-epicatechin, (+)-gallocatechin and (−)-epigallocatechin were obtained from Sigma (St. Louis, MO, USA). Kieselgel 60 F_{254} HPTLC (10 × 10 cm, 0.25 mm) was obtained from Merck (Darmstadt, Germany). Sephadex LH-20 gel was obtained from Pharmacia (Sweden). ODS-AQ(C18) was obtained from YMC (Japan).

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2.2. Extraction

Sea buckthorn seeds were obtained from berries of *Hippophae rhamnoides* L. subsp. Sinensis Rousi harvested from Taiyan in Sanxi Province. One hundred and fifty gram of sample were ground in a Wiley mill to pass through 20 mesh (840 μm), then extracted with 3×800 ml of H_2O–Me_2CO (3:7; 2 h with continuous stirring). Extracts were pooled and rotary evaporated under vacuum at 35 °C to remove Me_2CO. The resulting extract (500 ml) was washed with 3×500 ml of hexane to remove lipid-soluble substances, then rotary-evaporated to remove the residual hexane.

2.3. Chromatographic separation of monomeric flavan-3-ol and proanthocyanidins

The aqueous fraction was applied to a 60×450 mm Sephadex LH20 column equilibrated with H_2O. The column was eluted with H_2O–EtOH, the EtOH content being increased from 0% to 70% in increments of 10%, and then H_2O–Me_2CO (3:7) to obtain nine fractions (A–I). Fractions G, H were further treated chromatographically, alternating between ODS-AQ and Sephadex LH-20. Fractions were collected and monitored by thin-layer chromatography (TLC). Fractions of the same composition were combined and, if necessary, re-chromatographed until homogeneous samples were obtained. Fraction I was further fractionated by Sephadex LH 20 column chromatography. The column was washed sequentially using the solvent systems and volumes described in Table 1 to obtain I1–I8 (fractions). These fractions were later rotary-evaporated, freeze-dried and stored at −15 °C.

2.4. Thiolysis of the proanthocyanidins

A method based on that of Guyot, Marnet, and Drilleau (2001) was adopted to perform the thiolysis. Briefly, 50 μl of solution of each fraction (2 mg/ml in MeOH) was mixed together with 50 μl of MeOH acidified with concentrated HCl (3.3%, v/v) and 100 μl of benzyl mercaptan (5% v/v in MeOH). After sealing, the mixture was shaken and heated at 40 °C for 30 min. The resulting solution was analyzed by reversed-phase HPLC–ESI MS.

Table 1
Solvent systems used to fractionate sea buckthorn seed proanthocyanidins and yields of fractions

Solvent system[a] (v/v)	Volume (ml)	Yield (%)	Fraction
90% EtOH (v/v)	1000	6.4	I-1
10% acetone, 80% EtOH	1200	25.4	I-2
20% acetone, 65% EtOH	1000	30.3	I-3
30% acetone, 50% EtOH	1000	19.3	I-4
40% acetone, 35% EtOH	1000	6.2	I-5
50% acetone, 15% EtOH	1100	8.8	I-6
60% acetone	1500	2.0	I-7

[a] Solvents consisted of water and 0.2% HOAc (v/v).

For the quantitation of flavan-3-ol monomers and benzyl mercaptan adducts, dihydroquercetin was used as a quantitative standard. The response factors of the other products relative to dihydroquercetin were used for their estimation, as indicated by Meagher, Lane, Sivakumaran, Tavendale, and Fraser (2004). The response factors were 0.26 and 0.07, respectively, for terminal PC and PD flavan-3-ol units. For the extender flavan-3-ol thiol adducts, response factors of 0.25 and 0.06, respectively, were obtained for PC and PD benzylthioethers. To calculate the apparent mean degree of polymerization (mDP), the sum of all subunits (flavan-3-ol monomer and benzyl mercaptan adducts, in moles) was divided by the sum of all flavan-3-ol monomers (in moles).

2.5. Reversed-phase HPLC–ESI MS analyses of oligomeric proanthocyanidins, thiolysis media and hydrolysis products of polymers

A Waters 2690 chromatography system equipped with a diode array detector (Water 996 DAD) and a quadruple mass spectrometer (Waters Platform ZMD 4000) with an electrospray interface was used for analyses of oligomeric proanthocyanidins, thiolysis media and hydrolysis products of polymers. Separation was performed on a Purospher STAR RP18 (250×4.6 mm i.d., 5 μm) column at room temperature. For the thiolysis media, the elution conditions were as follows: solvent A, MeOH/HCOOH/H_2O (15:1:84, v/v/v); solvent B, MeOH/HCOOH/H_2O (85:1:14, v/v/v). Linear gradients: solvent B 5–100% in 30 min. For hydrolysis products, solvent A was replaced with 5% (v/v) formic acid in H_2O and the linear gradient was 15–55% B in 45 min. For oligomeric proanthocyanidins, solvent A was replaced with 0.1% HCOOH in H_2O; solvent B was replaced with 80% CH_3CN in H_2O. Linear gradients were: solvent B 5–50% in 20 min, 50–100% in 40 min. The solvent gradient described above was applied at a flow rate of 1 ml/min. The detection wavelength of the diode array detector was set at 280 nm for flavan-3-ol as well as their benzylthioethers and oligomeric proanthocyanidins and 520 nm for anthocyanidins. Components in the thiolysis media were identified with the mass spectrometer in negative mode, using a setting of 50% for compound stability, and 25% for ion trap drive. Positive mode ESI MS was employed to identify anthocyanidins in hydrolysis products with 50% for compound stability, and 80% for ion trap drive level with target mass set at 300 m/z.

2.6. TLC analysis

TLC analysis of chromatographic fractions was performed on Kieselgel 60 F_{254} HPTLC 10×10 cm, 0.25 mm (Merck) which was developed with toluene:Me_2CO:HOAc (3:3:1), according to the method earlier reported (Sun, Leandro, Ricardo da Slilva, & Spranger, 1998). The plates were visualized by spraying with 1% vanillin solution in

20% H_2SO_4 in MeOH (v/v) which revealed the flavans and proanthocyanidins as orange to reddish spots.

2.7. BuOH–HCl hydrolysis

This reaction was performed with a small sample of proancyanidins (1 mg) in a sealed vial containing 5% HCl in *tert*-BuOH (2 ml) and the mixture heated at 95 °C in a boiling water bath for 1 h. The anthocyanins formed were chromatographed by reverse-phase HPLC coupled with electrospray ionization mass spectrometry.

2.8. Free radical-scavenging activity on DPPH

This experiment was carried out according to the Brand-Williams, Cuvelier, and Berset (1995) method. Different concentrations of the sample solution (0.1 ml) were added to 3.9 ml of a 6×10^{-5} M MeOH DPPH· solution, followed by determining the decrease in absorbance at 516 nm at different times until the reaction reached a plateau. The resulting calibration curve for DPPH· between 0.5×10^{-5} and 6×10^{-5} mol/l, was used to calculate the DPPH· concentration ($C_{DPPH\cdot}$) in the reaction medium with the following formula:

$$Abs_{516} = 11251 \times (C_{DPPH\cdot})$$

The reaction kinetics were plotted for each antioxidant concentration tested. These graphs were used to determine the percentage of DPPH· remaining at the steady state and subsequently the values were transferred onto another graph, showing the percentage of residual steady state DPPH·. As a function of the sample concentration, antiradical activity was defined as the amount of antioxidant required to lower the initial DPPH· concentration by 50% (EC_{50}).

2.9. Identification

Purified procyanidin compounds were identified by ^1H NMR ^{13}C NMR on a Bruker Advance 500 instrument. Samples (20–30 mg) were dissolved in 0.5 ml deuterated methanol and chemical shifts were referenced to the ^1H ^{13}C chemical shifts of the deuterated solvent. Compounds were also identified by electrospray mass spectrometry (ESMS), using a negative ion probe with sample dissolved in acetonitrile–water (1:1 v/v).

3. Results and discussion

3.1. Identification of monomeric flavan-3-ol and oligomeric proanthocyanidins

The crude extract of sea buckthorn seed (SCT) on Sephadex LH 20 column chromatography yielded nine fractions, as shown in Fig. 1. The $DPPH^-$ radical-scavenging activity of the fractions is shown in Fig. 2. The activity of fraction C and G–I was higher than that of the crude extract, and the activity of fractions B and D were almost the same as that of the crude extract. However, fraction A, which was the major constituent in the water–acetone (3:7) extract from sea buckthorn seeds, exhibited the lowest activity.

Examination of fraction G on silica TLC revealed a highly complex mixture which, on repeated chromatography, alternating between Sephadex LH-20 and ODS-AQ, afforded (1) catechin (2) epicatechin (3) and gallocatechin (4) epigallocatechin. The presence of all the these flavanols, which were regarded as representative of the terminal units of proanthocyanidin oligomers and polymers, provides the first indication of a highly heterogeneous mixture of the proanthocyanidins which were likely to be present in the extract.

The spots in TLC chromatograms of fraction H and I (Fig. 3), upon with spraying vanillin–H_2SO_4 reagent, suggested that oligomeric and polymeric proanthocyanidins were contained in these fractions, respectively (Sun et al., 1998). Acid hydrolysis of fractions H and I, followed by HPLC analysis with UV–visible diode array detection, showed that both cyanidin and delphindin were formed, an indication that both procyanidins and prodelphinidins were present in these fractions.

Characterization of the oligomeric proanthocyanidins with limited numbers of flavan-3-ol units ($DP < 3$) in fraction H was achieved by HPLC–UV analysis, combined with ESI mass spectrometry. The latter technique is currently a powerful detection tool for on-line identification of plant constituents in crude extracts. Recently, application of this technique for the analysis of proanthocyanidin oligomers and polymers extracted from chocolate, grape and wine allowed the determination of the nature of polymeric proanthocyanidin units, their degree of polymerization, type of interflavanoid bonds and presence of gallate derivatives (Fulcrand, Remy, Souquet, Cheynier, & Moutounet, 1999; Monagas, Gómez-Cordovés, Bartolomé, Laureano, & Ricardo da Slilva, 2003; Wollgast, Pallaroni, Agazzi, & Anklam, 2001).

The DAD-UV spectra of all marked peaks in the chromatogram of fraction H (Fig. 4) were catechin-like with an absorbance maximum at 277–280 nm. The molecular masses of the separated compounds indicated that fraction H contained dimeric proanthocyanidins (Table 2, Fig. 5). The signals at m/z 593 = $[M-H]^-$ (compounds 3–6) indicate that the corresponding compounds are mixed dimers consisting of one (epi)gallocatechin (gc) and one (epi)catechin (c) unit in contrast to m/z 577 = $[M-H]^-$ (two c units, compounds 7–9, dimeric procyanidin) or m/z 609 = $[M-H]^-$ (two gc units, compound 1–2, dimeric prodelphinidin).

In order to distinguish the flavanol units in proanthocyanidins, Porter (1988) developed a nomenclature depending on the positions of the interflavanoid bonds at ring A and C: a T-unit (top) has only one interflavanoid linkage at C_4, whilst the M-units (middle) have an additional linkage at C_6 or C_8, and the B-unit (base) has one interflavanoid bond at C_6 or C_8 (Fig. 6).

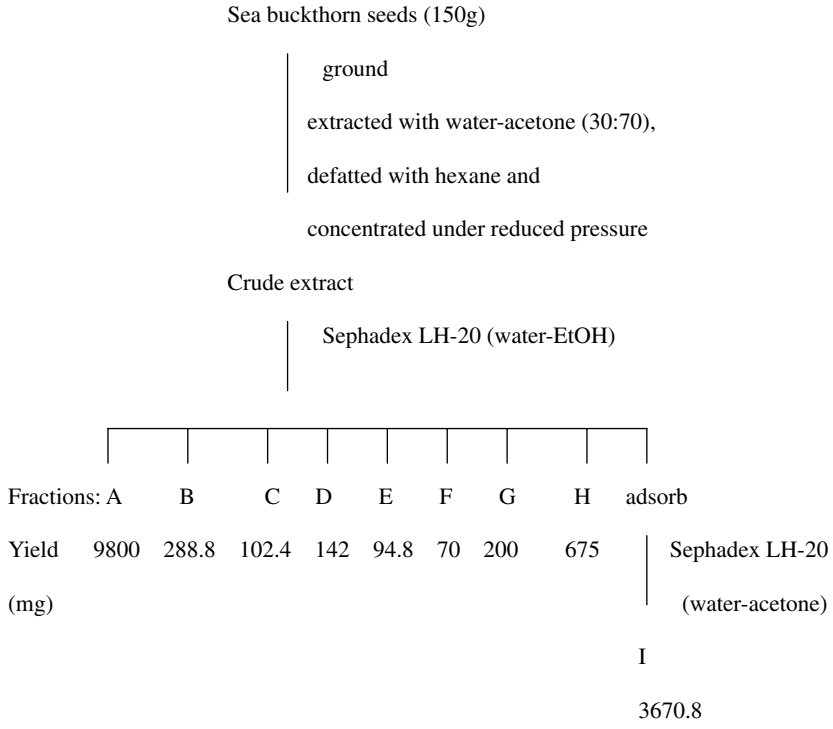

Fig. 1. Scheme of preparation of antioxidant compounds from sea buckthorn seeds and yields of fractions separated by Sephadex LH-20 column chromatography.

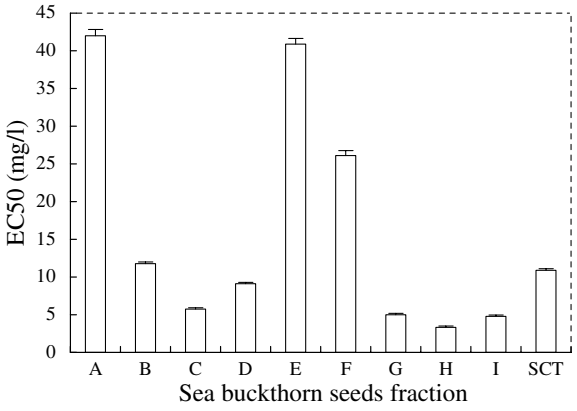

Fig. 2. Concentration of the antioxidant necessary of reduce, by 50% the oxidation (EC50) as determined by DPPH·. Measurement was carried out in triplicate. Means and standard deviation are indicated.

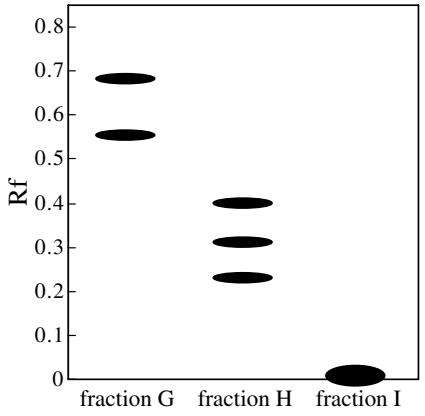

Fig. 3. TLC chromatogram of fractions G, H and I.

As expected by the suggested general scheme for proanthocyanidins (Friedrich, Elberhardt, & Galensa, 2000), the loss of a phloroglucinol unit ($C_6H_6O_3$), as well as losses due to retro-Diels–Alder (RDA) fission and interflavanoid cleavage, are the predominant fragmentation pathways of proanthocyanidins. Retro-Diels–Alder fission of the heterocyclic ring for c–c and gc–gc resulted in the ions *m/z* 425 and *m/z* 441, respectively. The ions corresponding to their subsequent water elimination were detected at *m/z* 407 and *m/z* 423 (Fig. 5a and d; Table 2). Interflavanic bond cleavage of c–c and gc–gc, through the quinone-methine mechanism, resulted in ion fragments detected at *m/z* 287, *m/z* 303 ($[M_T-3H]^-$, methylenic quinone) and *m/z* 289, *m/z* 305 ($[M_B-H]^-$, flavan-3-ol monomer) (Fig. 5a and d), respectively, the latter ion being more abundant than the former, which is in agreement with the observations of other authors (Monagas et al., 2003). For c–gc and gc–c, retro-Diels–Alder fission resulted in the ion fragments detected at *m/z* 441, *m/z* 425, respectively, and the ions corresponding to their subsequent water elimination being detected at *m/z* 423 and *m/z* 407 (Fig. 5b and c), since this type of fragmentation is considered to occur in the top unit of the proanthocyanidin molecule. Interflavanic fragmentation resulted in the ion fragments detected at *m/z* 305, *m/z* 289 ($[M_B-H]^-$, flavan-3-ol monomer) for c–gc and gc–c, respectively, whereas the ion fragments at

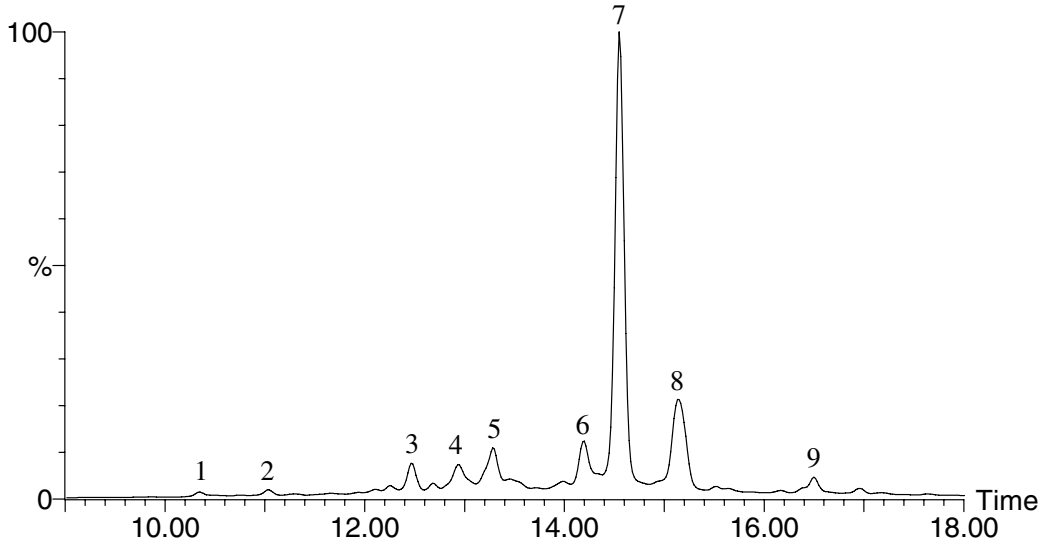

Fig. 4. HPLC chromatogram of fraction H of sea buckthorn seed obtained by Sephadex LH-20 column chromatography at 280 nm. Peak numbers are referenced to Table 2.

Table 2
Determination of dimeric proanthocyanidins in fraction H: pseudomolecular and main fragment ions: c catechin, gc gallocatechin, RAD retro-Diels–Alder

Peak no.	t_R (min)	$[M-H]^-$	RAD product	$[M-C_6H_6O_3-H]$	Interflavan bond cleavage product		Sequence
					$[M_B-H]$	$[M_T-3H]$	
1	10.3	609	441	483	305	303	gc–gc
2	11.1	609	441	483	305	303	gc–gc
3	12.4	593	425	467	289	nd	gc–c
4	13.0	593	441	nd	305	nd	c–gc
5	13.3	593	441	nd	305	nd	c–gc
6	14.2	593	441	nd	305	nd	c–gc
7	14.5	577	425	451	289	287	Procyanidin B3
8	15.2	577	425	451	289	287	Procyanidin B4
9	16.5	577	425	451	289	287	c–c

nd, not detected.

m/*z* 287 and *m*/*z* 303 corresponding to $[M_T-3H]^-$ by this cleavage were not detected (Fig. 5b and c). Ions detected at *m*/*z* 451 for (c–c), at *m*/*z* 467 (for gc–c), and at *m*/*z* 483 (for gc–gc) resulted from the loss of a fragment equivalent to a phloroglucinol unit ($C_6H_6O_3$) which has been interpreted as an inversion reaction involved in the biosynthesis of proanthocyanidins (Friedrich et al., 2000). The main fragmentation pathways of dimers are shown in Fig. 7.

The structure of dimeric proanthocyanidins was elucidated by the detection of the pseudomolecular ion and the ion resulting from its RAD fission in the upper T-units without preparative isolation of the compounds. Additionally, the detection of the lower B-units as $[M_B-H]^-$ and the upper units as $[M_T-3H]^-$ from the interflavanoid cleavage can be used to support the results. The mass spectrometric data of these compounds 4–7 showed that they are two different types of mixed dimers (Table 2). These results showed that sea buckthorn seed proanthocyanidins, besides procyanidins, consist of mixed proanthocyanidins in addition to pure prodelphinidins. Among the proanthocyanidin dimers were catechin(4α-8)catechin, and catechin(4α-8)epicatechin, both of which were identified from their ^{13}C NMR data.

3.2. Thiolysis of fractionated polymeric proanthocyanidins on Sephadex LH-20

In contrast to simple oligomers, polymeric proanthocyanidis (DP > 5) are very difficult to resolve by HPLC techniques since the number of possible isomers increases with degree of polymerization. Therefore, only fractions containing mixtures of polymers can be isolated, using purification techniques such as normal-phase HPLC (Yanagida et al., 2000), C18 Sep-Pak cartridge (Sun et al., 1998) and absorption chromatography on Fractogel TSK HW-40 (Yanagida, Kanda, Shoji, Ohnishi-Kameyama, & Nagata, 1999). Chromatography on Sephadex LH-20, with elution

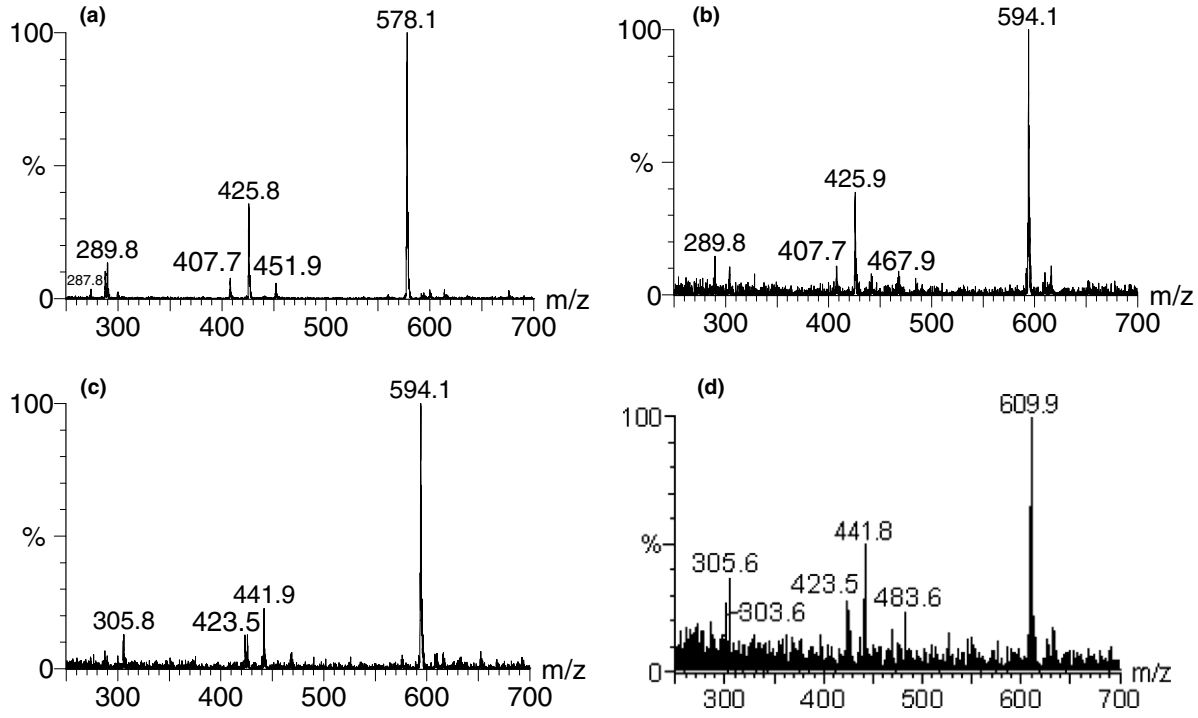

Fig. 5. Mass fragmentation pattern of proanthocyanidins under ESI-MS in negative mode: (a) c–c, (b) gc–c, (c) c–gc, (d) gc–gc. c catechin, gc gallocatechin.

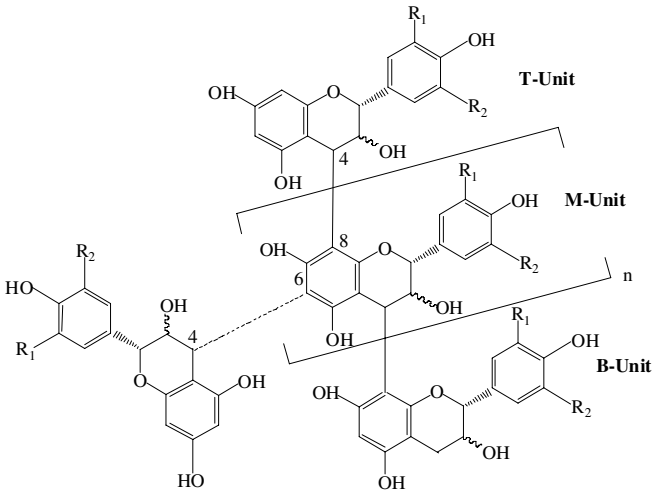

Fig. 6. Basic structure of proanthocyanidins. R_1, R_2 = H, propelargonidins; R_1 = H, R_2 = OH, procyanidins; R_1, R_2 = OH, prodelphinidins.

by aqueous alcohol or aqueous acetone, has commonly been applied to separate native-form proanthocyanidins from plant extracts according to the degree of polymerization (Gabetta et al., 2000; Kennedy & Tayor, 2003; Meagher et al., 2004). In an effort to determine the major constituents of bioactive fraction I, fraction I was further fractionated by Sephadex LH 20 column chromatography which yielded seven fractions (Table 1).

The gross structure of a proanthocyanidin polymer is characterized by the nature of its constitutive extension and terminal flavan-3-ol units and its degree of polymerization (DP), i.e., average number of units in the polymers. Among the methods available to investigate the structures of proanthocyanidin polymers, acid-catalysed degradation in the presence of toluene-α-thiol is of particular interest as it distinguishes between extension units and terminal units. The average composition of proanthocyanidin polymers in the fractions was determined using acid-catalysed degradation in the presence of toluene-α-thiol, followed by reverse-phase HPLC analysis with UV–visible detection. In thiolysis reactions, all the extension subunits of proanthocyanidins are attacked by benzyl mercaptan to form the corresponding benzylthioether. Only the terminal unit is released as the free flavan-3-ol. The components in the thiolysis media were separated by HPLC (Fig. 8) and their structures studied using ESI-MS (Fig. 9).

A typical HPLC chromatogram of the thiolysis products is shown in Fig. 8a. Sea buckthorn seed tannin gave ten main peaks. Four early eluting compounds (t_R 5.8 min, $[M-H]^-$ m/z; 305; t_R 8.4 min, $[M-H]^-$ m/z; 305; t_R 8.9 min, $[M-H]^-$ m/z; 289; t_R 11.6 min, $[M-H]^-$ m/z; 289) were identified, respectively, as gallocatechin (peak 1), epigallocatechin (peak 2), catechin (peak 3), and epicatecin (peak 4) by comparison of their t_R, UV–visible spectra and mass spectra with those of standards (Fig. 9a and b).

Two compounds (peaks 8 and 9) eluting at 20.3 and 21.4 min, showing $[M-H]^-$ at m/z 411 were identified as catechin benzylthioether by comparing their t_R, UV–visible spectra and mass spectra with the thiolysis products of cat-

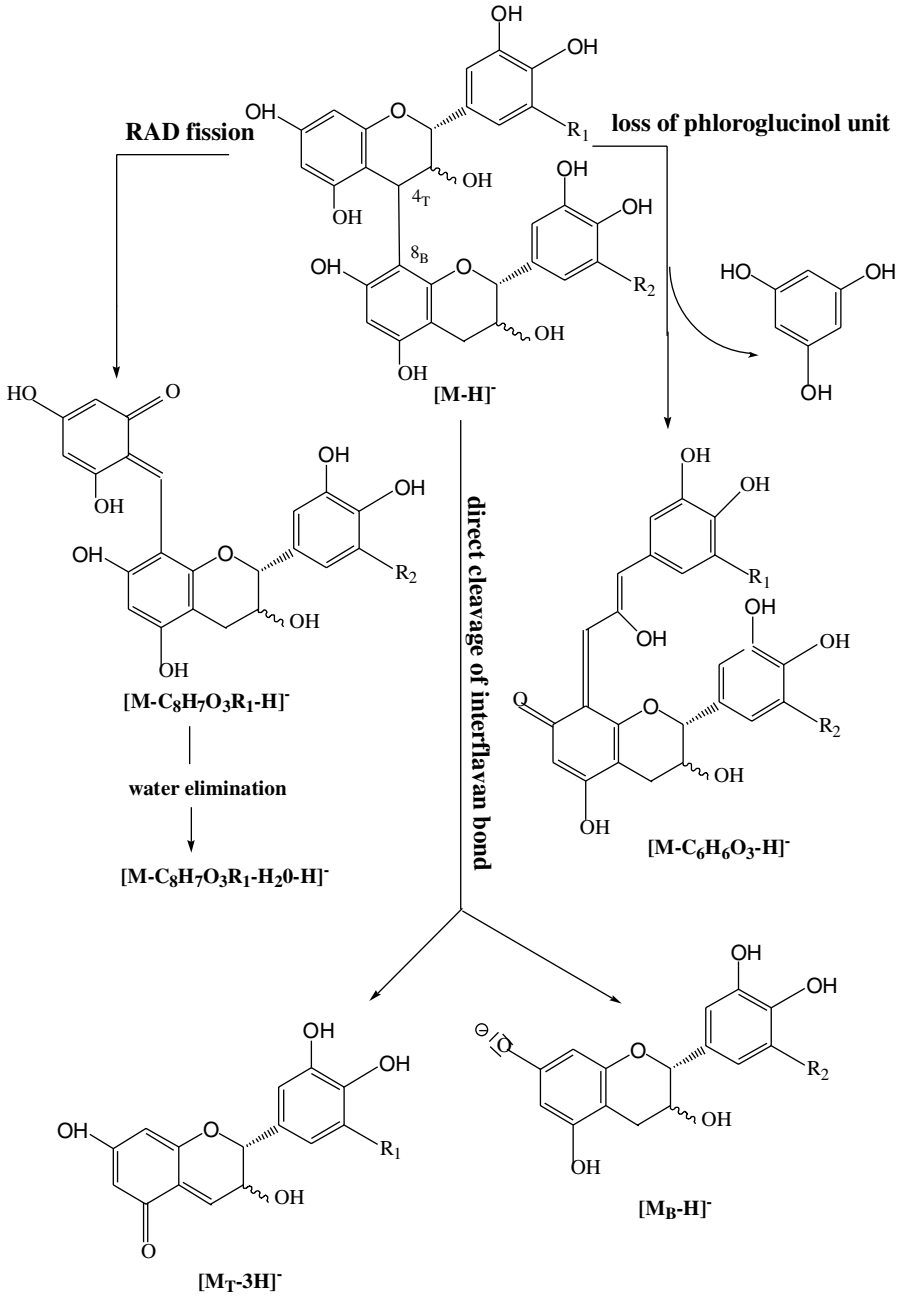

Fig. 7. Main fragmentation pathways of dimeric proanthocyanidin by negative ESI.

echin(4α-8)catechin (Figs. 8 and 9c). The mass spectra of compound 10 (t_R 23.3 min, $[M-H]^-$ m/z; 411) was very similar to that of catechin benzylthioether, which suggests that the latter compound corresponds to benzylthioepicatechin. Three compounds, eluting at 16.5, 18.0 and 18.8 min, showed $[M-H]^-$ at m/z 427, with characteristic fragment ions $[(M-Ph-CH_2-S)-H]$ at m/z 303, suggesting that they were stereoisomeric (epi)gallocatechin benzylthioethers (peak 5, 6, 7), although the actual conformations on C-2, 3, 4 remain to be established (Figs. 8a and 9d).

The structural composition and characteristic data obtained by thiolysis degradation of each of the proanthocyanidin fractions are presented in Table 3. Catechin, epicatechin, gallocatechin and epigallocatechin were found in each fraction, both as extension and terminal units, with gallocatechin being particularly abundant in terminal units. Prodelphinidin units predominated in the extended chains and were the major components of all tannin fractions. For total extract (fraction I), gallocatechin represented 56.1% of the terminal units, whereas 81.9% of the extension units consisted of prodelphinidin units. The total extract showed mDP of 12.2, with 81.2% of prodelphinidins. The average degree of polymerization (mDP) calculated from thioacidolysis data increased from 4.5 in fraction I-1 to 31.6 in fraction I-6 (slightly decreased to 28.5 in the last one), as expected from its exclusion from Sephadex

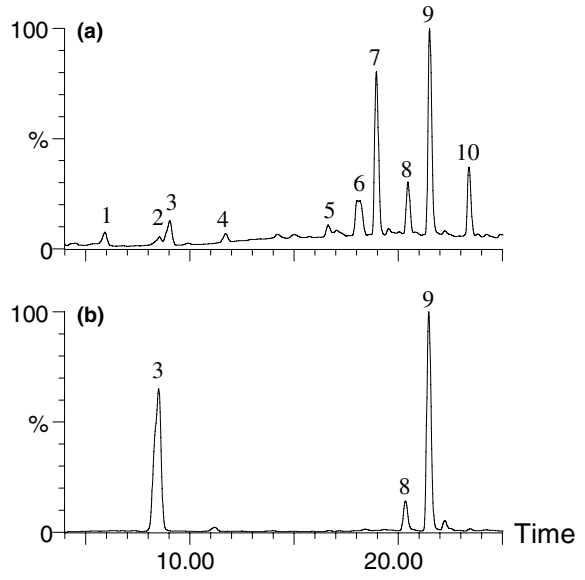

Fig. 8. HPLC chromatogram of thiolysis media of sea buckthorn proanthocyanidins (fraction I-5) detected at 280 nm. (a) Fraction I; (b) procyanidin B3. Peaks 1–10 were identified with the aid of ESI-MS, and their mass fragmentation patterns are shown in Fig. 9.

LH20. The proportion of prodelphinidins increased significantly from 51.4% (fraction I-1) to 84.6% (fraction I-3), then remained at 87% from fraction I-4 to fraction I-7.

The proportion of fraction I-1–I-7 was evaluated as weight relative frequency in the percentage of fraction I. The proanthocyanidin with mDP 9.1, 13.2 and 17.0 represented the three major clusters. The distribution not only was centred on the mean but also was unimodal, as shown in Table 1. As a consequence, the estimated mDP (12.2) and proportion of prodelphinidins (81.2%) of the total extract (fraction I) represented the major class in the proanthocyanidin sample studied.

4. Conclusion

Three antioxidant fractions separated by Sephadex LH-20 column chromatography were found to contain monomeric, oligomeric and polymeric flavan-3-ol, respectively. Sea buckthorn proanthocyanidins proved to be highly heterogeneous. Four monomeric flavan-3-ols and two dimeric procyanidins were isolated and identified. The structures of more dimeric proanthocyanidins were elucidated by HPLC

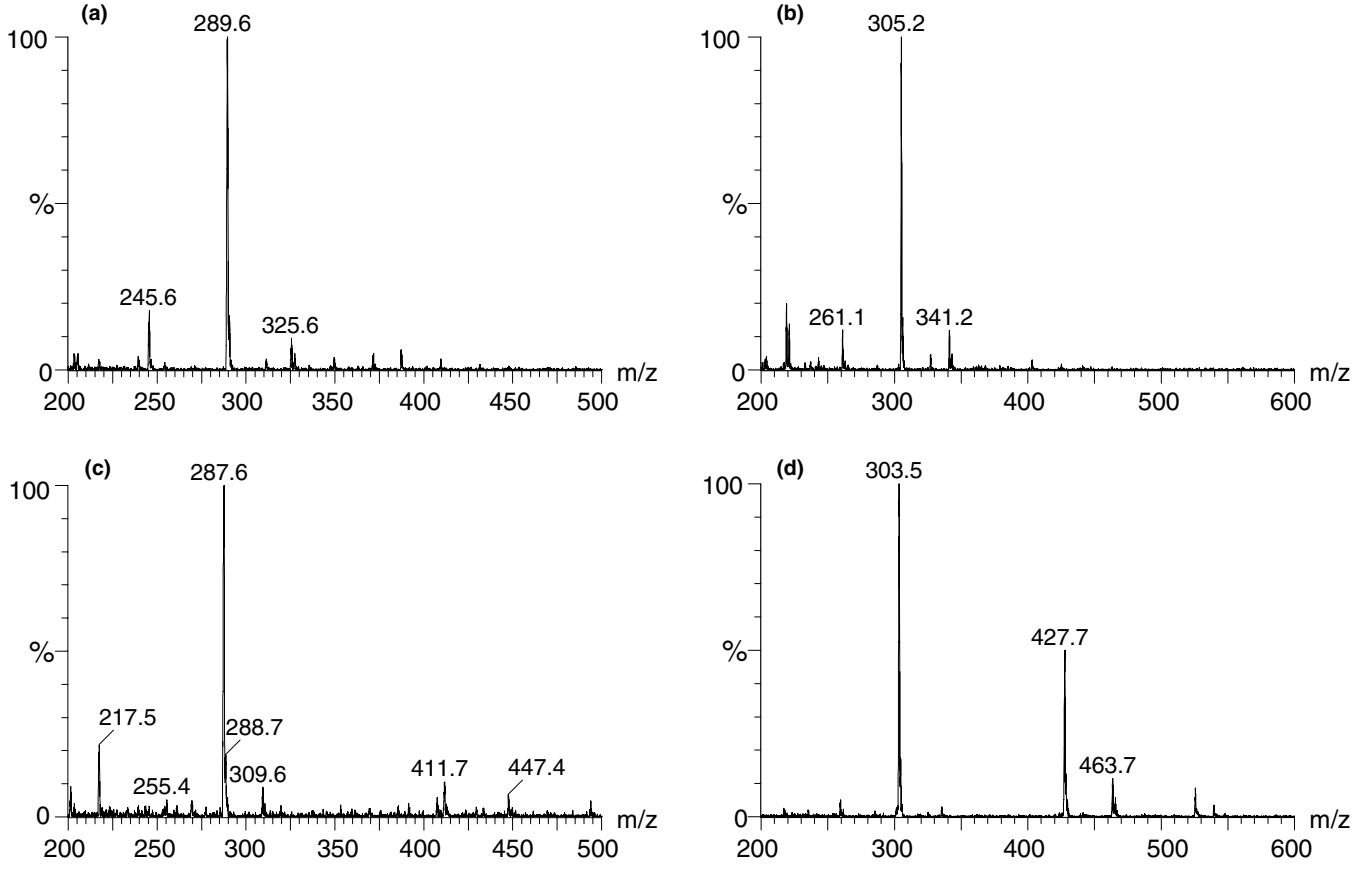

Fig. 9. Mass fragmentation pattern of components in the thiolysis media of sea buckthorn proanthocyanidins: (a) (epi)catechin; (b) (epi)gallocatechin; (c) (epi)catechin benzylthioether; (d) (epi)gallocatechin benzylthioether.

Table 3
Structural composition and characteristics of sea buckthorn seed proanthocyanidin fractions determined by HPLC following thioacidolysis degradation[a]

Fraction	Terminal units				Extension units			mDP	Prodelphinidins (%)
	GC	EGC	Cat	EC	GC + EGC	Cat	EC		
I-1	8.2	3.3	8.1	2.7	39.9	27.3	10.6	4.5	51.4
I-2	5.7	1.6	2.7	1.1	61.9	19.7	7.3	9.1	69.2
I-3	4.2	1.8	1.1	0.5	78.6	12.1	1.8	13.2	84.6
I-4	3.8	1.2	0.7	0.3	82.5	10.6	1.1	17.0	87.4
I-5	2.1	0.9	0.6	0.2	85.5	9.9	0.9	26.8	88.5
I-6	1.8	0.6	0.5	0.2	85.2	10.6	1.0	31.6	87.7
I-7	2.1	0.7	0.5	0.2	85.1	10.5	0.9	28.5	87.9
Total extract	4.6	1.3	1.6	0.7	75.2	13.7	2.9	12.2	81.2

[a] GC, EGC, Cat, EC are the abbreviations for gallocatechin, epigallocatechin, catechin and epicatechin units. All amounts represent relative concentrations (in mol).

in combination with electrospray ionization mass spectrometric detection. The result showed that sea buckthorn seed dimeric proanthocyanidins, besides procyanidins, consist of mixed proanthocyanidins, in addition to pure prodelphinidins. Polymers are predominantly of prodelphinidin-type, with (epi)gallocatechin (75.2%) as the main extender unit. In addition, gallocatechin (4.6%) was the prevalent terminal unit. Plant proanthocyanidins, known as functional food factors, have recently attracted increasing attention, due to the rapidly growing volume of evidence associating these compounds with a wide range of potential health benefits (Santos-Buelga & Scalbert, 2000). Sea buckthorn seed proanthocyanidin may have potential as a natural antioxidant.

Appendix

(1) Catechin
ESMS gave an $[M-H]^-$ at m/z: 289. ^{1}H NMR (500 MHz, methanol-d_4): δ 6.84 (1H, d, $J = 1.5$ Hz, H-2′), 6.77 (1H, d, $J = 8.2$ Hz, H-6′), 6.73 (1H, dd, $J = 1.5$, 8.2 Hz, H-5′), 5.93 (1H, d, $J = 2.1$ Hz, H-6), 5.86 (1H, d, $J = 2.1$ Hz, H-8), 4.57 (1H, d, $J = 7.4$ Hz, H-2), 3.78 (1H, m, H-3), 2.87 (1H, dd, $J = 5.4$, 16 Hz, H-4α), 2.54 (1H, dd, $J = 8.1$, 16 Hz, H-4β). ^{13}C NMR (125 MHz, methanol-d_4): δ 158.3, 158.0, 157.4, 146.7 (2×), 132.7, 120.5, 116.6, 115.8, 101.4, 96.8, 96.0, 83.3, 69.3, 28.9.

(2) Epicatechin
ESMS gave an $[M-H]^-$ at m/z: 289. ^{1}H NMR (500 MHz, methanol-d_4): δ 6.97 (1H, d, $J = 1.7$ Hz, H-2′), 6.8 (1H, dd, $J = 1.7$, 8.2 Hz, H-6′), 6.76 (1H, d, $J = 8.2$, Hz, H-5′), 5.94 (1H, d, $J = 1.3$ Hz, H-6), 5.92 (1H, d, $J = 1.3$ Hz, H-8), 4.81 (1H, s, H-2), 4.17 (1H, t, $J = 2.9$ Hz, H-3), 2.87 (1H, dd, $J = 4.6$, 6.4 Hz, H-4α), 2.75 (1H, dd, $J = 4.6$, 6.4 Hz, H-4β). ^{13}C NMR (125 MHz, methanol-d_4): δ 158.5, 158.2, 157.9, 146.4, 146.3, 132.8, 119.9, 116.4, 115.8, 100.6, 96.9, 96.4, 80.4, 67.9, 29.7.

(3) Gallocatechin
ESMS gave an $[M-H]^-$ at m/z: 305. ^{1}H NMR (500 MHz, methanol-d_4): δ 6.39 (2H, s, H-2′, 6′), 5.72 (1H, d, $J = 2.3$ Hz, H-6), 5.86 (1H, d, $J = 2.3$ Hz, H-8), 4.53 (1H, d, $J = 7.1$ Hz, H-2), 3.87 (1H, m, H-3), 2.82 (1H, dd, $J = 5.3$, 16 Hz, H-4α), 2.52 (1H, dd, $J = 7.8$, 16 Hz, H- 4β). ^{13}C NMR (125 MHz, methanol-d_4): δ 158.1 (C-7), 157.9 (C-5), 157.1 (C-8α), 147 (C-3′, 5′), 134.3 (C-4′), 131.9 (C-1′), 107.5 (C-2′,6′), 101.0 (C-4α), 96.6 (C-6), 95.8 (C-8), 83.1 (C-2), 69.0 (C-3), 28.3 (C-4).

(4) Epigallocatechin
ESMS gave $[M-H]^-$ at m/z: 305. ^{1}H NMR (500 MHz, methanol-d_4): δ 6.52 (2H, s, H-2′, 5′), 5.96 (1H, d, $J = 1.8$ Hz, H-6), 5.94 (1H, d, $J = 1.8$ Hz, H-8), 4.74 (1H, s, H-2), 4.16 (1H, s, H-3), 2.87 (1H, dd, $J = 4.5$, 16.8 Hz, H-4α), 2.76 (1H, dd, $J = 2.7$, 16.8 Hz, H-4β). ^{13}C NMR (125 MHz, methanol-d_4): δ 158.3 (C-7), 157.9 (C-8α), 157.6 (C-5), 147.0 (C-6′), 134.0 (C-4′), 132.0 (C-3′), 107.5 (C-1′,2′), 100.6(C-5′), 96.9 (C-6, 4α), 96.4 (C-8), 80.2 (C-2), 67.9 (C-3), 29.5 (C-4).

(5) Catechin(4α → 8)catechin
ESMS gave an $[M-H]^-$ at m/z: 577. ^{13}C NMR (125 MHz, methanol-d_4): δ 29.1, 38.9, 69.2, 73.9, 82.8, 84.4, 96.4, 97.2, 97.6, 102.6, 107.5, 108.5, 115.5, 115.8, 116.5, 116.7, 120.2, 120.9, 132.2, 132.9, 145.8, 145.9, 146.1, 146.3, 146.4, 155.2, 156.0, 156.2, 157.4, 158.9

(6) Catechin(4α → 8)epicatechin
ESMS gave an $[M-H]^-$ at m/z: 577. ^{13}C NMR (125 MHz, methanol-d_4): δ 30.3, 39.4, 67.9, 74.3, 80.4, 84.3, 97.8, 98.2, 99.9, 107.2 (2×), 107.7, 108.3, 109.1, 116.5, 116.9, 121.7 (2×), 132.2, 132.7, 146.6, 146.8, 146.9, 147.2, 155.4, 156.1, 156.8, 157.8, 157.9, 159.2.

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