Phenylpropanoid-substituted Procyanidins and Tentatively Identified Procyanidin Glycosides from Hawthorn (*Crataegus* spp.)

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Key words
- *Crataegus* spp.
- Rosaceae
- procyanidins
- cinchonain II b
- procyanidin glycosides

Abstract
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The rational use of hawthorn leafs and flowers from *Crataegus* spp. for declining cardiac performance is mainly due to flavon-C-glycosides and oligomeric procyanidins (OPC). From OPC-enriched extracts from different batches, a dimeric phenylpropanoid-substituted procyanidin (cinchonain II b, **1**) was isolated and characterized by MS, CD, and NMR. Also the presence of higher oligomeric cinchonains (degree of polymerization 3 to 8) in hawthorn extracts was shown by a specific ultrahigh-pressure liquid chromatography-ESI-qTOF-MS method. Interestingly, strong evidence for the occurrence of oligomeric procyanidin hexosides was found by ultrahigh-pressure liquid chromatography-ESI-qTOF-MS analysis which additionally revealed the presence of peaks indicative of dimeric procyanidin hexosides by their exact mass, which were clearly distinguishable from the cinchonain II type peaks.

Abbreviations
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DP: degree of polymerization
OPC: oligomeric procyanidin
UHPLC: ultrahigh-pressure liquid chromatography

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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Introduction
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Hawthorn leaves and flowers consist of the dried flowering branches of *Crataegus monogyna* Jacq. (Lindm.), *C. laevigata* (Poiret) DC. (Rosaceae), and, more rarely, *C. pentagyna* Waldst. & Kit. ex Willd., *C. nigra* Waldst. & Kit., and *C. azarolus* L. Pharmaceutical preparations of hawthorn are considered as a rational based phytomedicine against declining cardiac performance corresponding to the functional classification class I and II, as defined by the New York Heart Association (NYHA) [1]. Also a recent meta-analysis indicates a significant benefit in symptom control and physiologic outcome from hawthorn extract as an adjunctive treatment of chronic heart failure [2]. The main constituents are flavonoids (up to 2%), such as vitexin, vitexin-2″-rhamnoside, hyperoside, rutin, approximately 3% of oligo- and polymeric B-type procyanidins [3], triterpenes, phenolic acids, amines, xanthines, and polysaccharides [4]. From the functional point of view, flavone-C-glycosides and OPCs are considered to be the main active compounds (ESCOP 2003). For a recent summary of the phytochemistry and problems concerning the analysis of procyanidins from hawthorn, see [3] and references cited there. Despite the fact that OPCs are claimed to be part of the active ingredients, data published on the cardiac activity of hawthorn did not use pure OPCs but multicompound extracts, standardized on procyanidin content [5–7]. On the other side, the B-type OPCs from hawthorn are a complex heterologous series with DP 2 to 13 and a polymeric procyanidin fraction. Therefore, an efficient methodology for the purification of oligomeric procyanidins with defined DP has been established in order to obtain sufficient amounts of test compounds for detailed structure-activity studies [8]. During the large scale purification of defined OPCs, a further homologous series of oligomeric cinchonains has been identified from the OPC fraction, a substance class described in this report for the first time for hawthorn leaves and flowers.

Materials and Methods
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General analytical techniques

NMR spectra of the peracetylated derivatives were recorded in CDCl$_3$ (δ 7.26 and 77.00 ppm) on a Varian Unity plus 600, a Varian INOVA 500, or a Varian m400 spectrometer. CD spectra were measured with a Jasco J-815 CD spectrometer in MeOH. Optical rotations were measured with a Perkin-Elmer 341 digital polarimeter in MeOH. Analytical TLC was carried out on silica gel plates (0.2 mm; Merck) using ethyl acetate/water/formic acid (90 : 5 : 5) as the eluent. Compounds were visualized as red colored spots by spraying with vanillin-HCl reagent. Peracetylation of compounds was performed in pyridine/acetic acid anhydride (1 : 1) at room temperature for 24 h in the dark [9]. Analytical HPLC equipment was supplied from Waters: controller 600 unit, PDA 996, scanning fluorescence detector 474, autosampler 717 plus, in-line degasser AF, Millenium32 software.

Plant material

Two batches of the herbal drug material *Crataegi folium cum flore* (batches no. 52467097 and 09363903), conforming to the specifications of Ph. Eur. 6, were obtained from Caesar & Loretz. Reference samples (voucher samples IPBP-240 and 249) are stored at the Institute of Pharmaceutical Biology and Phytochemistry. Additionally fresh plant material (leaves and flowers) from *C. monogyna* L. (identified by A. H.) was collected on May 28th 2011 at the Medicinal Plant Botanical Garden of the University of Münster, Germany. The material was dried at room temperature; a reference sample (voucher sample IPBP-251) is stored at the Institute of Pharmaceutical Biology and Phytochemistry.

Preparation of fraction E4 and subfraction c
(see also Supporting Information, Fig. 1S)

Isolation and characterization of OPC clusters was performed as previously described [8]. The herbal material from batch 52467097 (5.15 kg) was extracted with acetone/water (7 : 3 v/v) and successively concentrated *in vacuo*, filtered to remove precipitated chlorophyll, further concentrated and defatted with petroleum benzine. Successive extraction of the aqueous phase with EtOAc yielded an EtOAc-fraction (126 g, corresponding to 2.6%) and a water fraction (859 g, corresponding to 16.7%, related to the starting material). The EtOAc fraction was resuspended in ethanol and centrifuged. The resulting supernatant, corresponding to 85 g of dry mass, was subjected to Sephadex® LH-20 chromatography [675 × 55 mm, eluents: ethanol 96% 13 L, methanol 9 L, acetone/water (7 : 3) 3.9 L, flow 1.5–2 mL/min, fraction size 15 mL]. All fractions eluted were investigated by TLC, and fractions with comparable composition were combined. The subfraction E4 (elution from 5.8 to 8.4 L, yield 1.83 g) contained high amounts of a dimeric procyanidin cluster and was subjected to preparative HPLC for the isolation of cinchonains.

The dried water fraction was divided into a MeOH-soluble extract and a MeOH-insoluble residue. An aliquot of the MeOH-soluble extract (1.30 g) was further fractionated by MPLC [RP18, 18–32 µm, 100 Å, 36 × 500 mm, Best Technik, flow 8 mL/min, step gradient MeOH 10% (25 min) → MeOH 30% (1 h) → MeOH 50% (1 h) → MeOH (1 h)] to yield the procyanidin-containing subfraction c (elution from 0.75 to 1.25 L) (0.16 g). Subfraction c was further subjected to preparative HPLC on a diol stationary phase (Uptisphere®120 Å, bonding OH, 6 µm, 21.2 × 250 mm; Interchim) using a binary gradient of acetonitrile (A) and methanol/water (95 : 5) (B) for the isolation of procyanidin clusters DP3 to

13. Gradient: 0 min: A 100% → 30 min: A 60% → 40 min: A 60% → 45 min A 65% → 50 min: A 0% → 55 min: A 0% → 60 min: A 100%; flow rate 10 mL/min.

Isolation of cinchonain IIb

Isolation of cinchonains was performed from subfraction E4 by preparative HPLC on a diol stationary phase (Uptisphere®120 Å, bonding OH, 6 µm, 21.2 × 250 mm; Interchim) using a binary gradient with acetonitrile (A) and MeOH/water (95 : 5) (B) ($t_{0\,min}$ 100% A, $t_{35\,min}$ 70% A, $t_{39\,min}$ 100% B, $t_{44\,min}$ 100% B 5 min isocratic). Besides a major peak of dimeric OPCs, an unknown compound eluting at t_R = 26.7 min was collected to yield fraction E4-3 (13.9 mg from 302 mg of fraction E4) (**Fig. 2S**, Supporting Information). By UHPLC-ESI-TOF-MS analysis, this fraction was shown to contain a substance with the monoisotopic mass of a dimeric cinchonain (m/z 739, [M – H]$^-$), but still was contaminated with substantial amounts of a dimeric OPC (m/z 577 [M – H]$^-$) (**Fig. 4S**, Supporting Information).

Further preparative purification of this fraction (13 mg) was performed on a RP18 stationary phase (Hypersil® ODS, 5 µm, 250 × 16 mm), eluted with a binary gradient of MeOH (A) and 0.1% TFA (B), $t_{0\,min}$ 5% A, $t_{15\,min}$ 30% A, $t_{25\,min}$ 40% A, $t_{30\,min}$ 100% A; flow 10 mL/min (**Fig. 3S**, Supporting Information). Five peaks were obtained under these conditions from E4-3, of which the third eluting peak at t_R = 19.3 min was isolated (E4-3/3, yield 6.5 mg) and identified by MS/MS (positive mode) as a dimeric cinchonain (m/z 741 [M + H]$^+$).

ESI-q-TOF analysis of subfraction c and fragmentation analysis of cinchonain IIb (m/z 739)

A solution of 0.1 mg/mL subfraction c in isopropanol 50% was directly infused into an Apollo ESI source of a Bruker Daltonics micrOTOF QII (Bruker Daltonics). Direct infusion was rationalized for getting higher signal intensity for fragmentation analysis. Settings: flow rate 3 µL/min in the negative mode; mass range m/z 50–1000; nebulizer gas nitrogen, 0.4 bar; dry gas nitrogen, 4.0 L/min, 180 °C; capillary voltage 4500 eV; end plate offset − 500 eV; transfer time 100 µs; prepulse storage 5.0 µs; ion energy − 3 eV; low mass m/z 320. Collision cell RF settings were combined to each single spectrum of 5000 summations as follows: 2500 summations with 300 Vpp + 2500 summations with 130 Vpp. Collision energy for m/z 739 was varied between − 5 eV and − 80 eV in order to detect a wide range of fragments. Instrument calibration and internal data set calibration was performed using a 10 mM sodium formiat solution in 50% isopropanol that was directly infused into the ESI source by a syringe pump at 3 µL/min.

UHPLC-ESI-qTOF-MS of procyanidin clusters and cinchonains

Test solutions: 0.1 mg/mL subfraction c, prepared from batch 52467097, was dissolved in isopropanol 50%. Preparation of test solutions from batch 09363903 and from *C. monogyna* leaves and flowers was performed as follows: 3 g of herbal drug was pulverized in liquid nitrogen and sieved over a 0.800-mm sieve (DIN 4188). 0.50 g of the pulverized material was extracted with 10 mL of acetone : water (7 : 3 v/v) by a rotor-stator system (Ultra-Turrax; ICA) for 6 × 1 min; the suspension was filtered over a glass filter (Duran®; Schott). The organic part of the solvent from the combined extracts was removed using a rotary evaporator, and the resulting aqueous suspension transferred to a 20.0-mL flask, which was filled up with water.
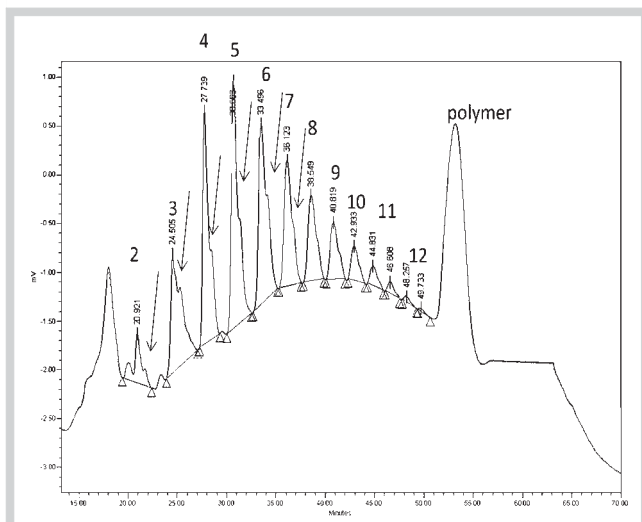
Fig. 1 Representative HPLC chromatogram of OPC-enriched subfraction c, containing clusters of DP 2 to DP 12 and polymeric procyanidins; arrows indicate oligomeric cinchonains at the peak tails of procyanidin peaks, numbers give degree of polymerization (DP). Gradient: acetonitrile (**A**), MeOH:water (95:5) (**B**) gradient ($t_{0\,min}$ 100% A, $t_{20\,min}$ 70% A, $t_{45\,min}$ 50% A, $t_{50\,min}$ 100% B 5 min. isocratic).

Fig. 2 Structure of cinchonain IIb (**1**) isolated from hawthorn leaves and flowers and the respective cinchonain IIb peracetate (**1a**) used for structure elucidation.

Chromatographic separation was performed on a Dionex 3000 RS liquid chromatography system (Thermo Fisher Scientific, Inc.) on an Uptisphere 120 Å, bonding OH, 6 μm, 250 × 4.6 mm column (Interchim) with a binary gradient modified according to [10] [A: acetonitrile/acetic acid (98:2), B: methanol/water/acetic acid (95:3:2)] at 40 °C and 0.8 mL/min: 0–38 min 100% A → 60% A, 38–45 min 60% A, 45–50 min 60% A → 0% A, 50–60 min 0% A, 60–65 min. Reequilibration time: 5 min. The injection volume was 10 μL. Eluted compounds were detected using a Bruker Daltonics micrOTOF-QII time-of-flight mass spectrometer equipped with an Apollo electrospray ionization source in the negative mode at 2 Hz over a mass range of m/z 50–3000 using the following settings: nebulizer gas nitrogen, 5 bar; dry gas nitrogen, 9 L/min, 180 °C; capillary voltage 2600 V; end plate offset − 500 V; transfer time 140 μs; prepulse storage time 15 μs; ion energy − 5.0 eV; low mass m/z 300. Collision cell RF settings were combined to each single spectrum of 2500 summations as follows: 1250 summations with 300 Vpp + 1250 summations with 600 Vpp. Internal data set calibration (HPC mode) was performed for each analysis using the mass spectrum of a 10-mM sodium acetate solution in 50% isopropanol that was infused during LC reequilibration using a divert valve equipped with a 20-μL sample loop.

Supporting information

The extraction scheme of hawthorn leaves and flowers, the fragment list of cinchonain IIb, HPLC chromatograms of fraction E4 and E4-3, and spectra for cinchonain IIb are available as Supporting Information.

Results and Discussion
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Recently, an HPLC method on the diol stationary phase for the effective preparative isolation of oligomeric procyanidin clusters (mixtures 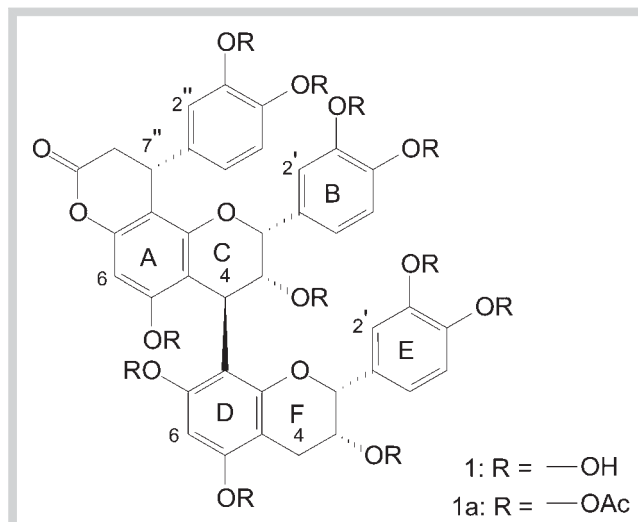of procyanidins with identical molecular weight but different stereochemistry and linkage types) with defined DP from hawthorn leafs and flowers has been described [8]. During the large scale OPC isolation from the procyanidin-enriched subfraction E4 (see Materials and Methods section), procyanidin clusters with DP 3 to 13 were isolated in good yields as distinct peaks (see Fig. 1 in [8]), but additionally it was observed that in cases where the preparative system was overloaded with high substance amounts, minor peaks were detected typically in the peak tails of procyanidins or in some cases within the valleys between the main peaks (**○ Fig. 1**). For structure elucidation of compounds from these peaks, the following experiments were performed on a Sephadex LH20 fraction (E4) from the ethylacetate partition of an acetone/water extract (extraction scheme, see **Fig. 1S**, Supporting Information), focusing on the unknown compound eluting directly after the dimeric procyanidin cluster DP 2: by preparative HPLC on the diol stationary phase, this peak was isolated as fraction E4-3. By rechromatography of E4-3 on RP18, a pure fraction E4-3/3 (yield 6.5 mg) was isolated and characterized as a phenylpropanoid-substituted procyanidin from the cinchonain-type **1** (**○ Fig. 2**).

The structure of **1**, including the stereochemistry, was elucidated on the basis of its HR-MS data (**○ Fig. 3**; **Table 1S**, Supporting Information, additionally lists typical MS fragments of cinchonain IIb after CID) and spectroscopic data (NMR, CD; for respective spectra, see Supporting Information **Figs. 5S** and **6S**) of its peracetate **1a** in comparison to recently published data [11]. The use of cinchonain peracetates is rationalized by an improved interpretation of NMR spectra by suppression of rotamer formation (9, 29, 30). All measurements were in perfect congruence with the respective published data sets (10).

The identification of naturally occurring flavan-3-ols with a phenylpropanoid substitution of the cinchonain-type started 30 years ago by Nonaka and Nishioka and their coworkers [12–14]. The structures at that time were determined on the basis of spectroscopy followed by controlled degradation with identification of the resulting compounds. Comparison of the CD spectra of similar cinchonain analogues later has led to revision of the stereochemistry of cinchonains Ia-Id, IIa, and IIb as well as for the
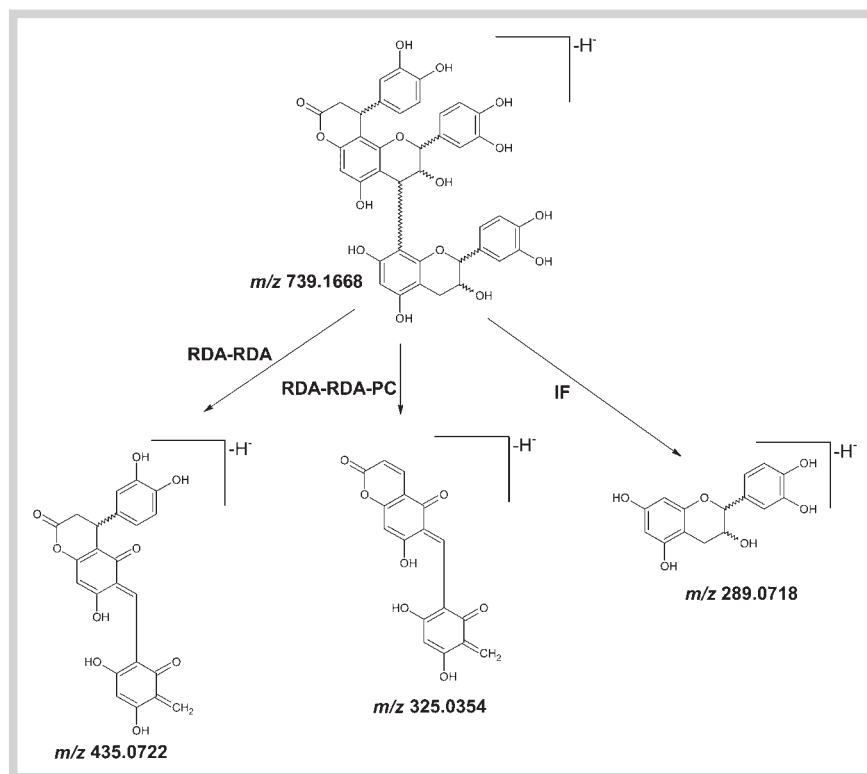
Fig. 3 Diagnostically important fragments of cinchonain dimer and their theoretical *m/z* values. The fragments are indicative for the linkage of the C_9 moiety to the upper flavanol unit (*m/z* 289) and to an A-Ring (*m/z* 435) as well as the presence of a dihydroxylated phenyl substructure within the C_9 moiety (*m/z* 325) (RDA: retro-Diels-Alder reaction, PC: phenyl cleavage, IF: interflavan fission).

kandelins A1, A2 and B1, B2 [15]. The current generally accepted structure of cinchonain II b is shown in ⦿ **Fig. 2**. Our present study is the first report on cinchonains for the genus *Crataegus*. The occurrence of cinchonain-type flavan-3-ols within the Rosaceae family is strictly limited and, to our knowledge, until now only described for the genera *Rhaphiolepis* [16] and *Eriobotrya* [17–19].

Based on our findings of the dimeric phenylpropanoid-substituted procyanidin, further investigations were performed to elucidate if, additionally, a homologous series of cinchonains is detectable in the herbal material. For that, the procyanidin-enriched subfraction c (see extraction scheme, **Fig. 1S**, Supporting Information) was investigated by ESI-qTOF-MS. A series of weak MS-signals (series A) were detected that showed an offset of 162 u relative to the more prominent series of dimeric (*M* = 578 u) to hexameric (*M* = 1730 u) procyanidins (series B). Double charged members [M – 2H]$^{2-}$ of both series became increasingly dominant as their molecular weight exceeded 1000 u and were used for the identification of larger molecules. The exact *m/z* values for series A are indicative for di- to hexameric procyanidins conjugated to a $C_9H_6O_3$ moiety, as typically described for cinchonains [13]. The MS/MS signals of the most abundant ion *m/z* 739 from series A match the data published for cinchonain II isomers [20] and can predominantly be attributed to the fragmentation reactions of interflavan fission (IF), phenyl cleavage (PC), heteronuclear ring fission (HRF), and retro-Diels-Alder reaction (RDA) also known from proanthocyanidins [21,22]. Fragments of specific diagnostic value are (i) *m/z* 435 which results from repeated RDA and indicates linkage of the C_9 moiety to the A-ring of one flavanol subunit, (ii) *m/z* 289 which results from IF when the C_9 moiety is linked to the upper flavanol subunit (the complementary fragment *m/z* 449 could also result from repeated HRF when the C_9 moiety is linked to the lower flavanol unit), and (iii) *m/z* 325 which can be explained by PC of *m/z*

435, the neutral loss of 110 u being indicative of the presence of a dihydroxylated phenyl ring within the C_9 moiety (⦿ **Fig. 3**). Further fragmentation data and an MS/MS spectrum are given in Supporting Information, **Table 1S** and **Fig. 4S**, respectively. Based on the detection behavior of directly infused cinchonains, an UHPLC ESI-qTOF-MS analysis with optimized MS settings on a diol-modified silica column allowed the chromatographic separation and detection of the members of both series A (oligomeric cinchonains) from the monomer up to heptamers (⦿ **Fig. 4 A**) and series B (oligomeric procyanidins) by narrow base peak chromatograms (⦿ **Fig. 4 B**).

Detection of a homologous series of cinchonains was reproduced by investigation of two other samples, one being a commercial herbal drug of *Crataegus* leaves and flowers, meeting the specifications of European Pharmacopoeia. Further on, a collection of leaves and flowers from *C. monogyna* was investigated. Therefore, extracts prepared by acetone : water (7 : 3) were tested by ESI-qTOF-MS, which revealed unambiguously the existence of cinchonains DP 2 to 7.

However, procyanidin hexosides show the same nominal but not exact masses and apparently – as demonstrated for the dimeric procyanidin hexoside – a similar fragmentation pattern which is comparable to the members of series A described here in our investigations [23]. Hence, high mass accuracy is required to distinguish them from the nominally isobaric cinchonains. The mass spectrometry instrumentation used in the present study allows the unambiguous discrimination of nominally isobaric cinchonains and procyanidin hexosides up to the level of hexamers (*M* = 1892 u). For detailed comparison of the measured *m/z* signals with the theoretical data of cinchonains and procyanidin hexosides, see ⦿ **Table 1**. This investigation and exact differentiation seems necessary because procyanidin hexosides have also been tentatively reported to occur in the genus *Crataegus* based on LC-MS analysis with low mass accuracy [24–26]; as no further

Table 1 Comparison of the measured *m/z* signals with the theoretical data of cinchonains and the nominally isobaric procyanidin hexosides. The instrument's mass accuracy of ± 5 ppm allows a clear discrimination up to DP 6 [DP degree of polymerization, refers to the number of (epi)catechin building blocks].

| DP	Theoretical data of cinchonains			Measured	Theoretical data of procyanidin hexosides		
	Mol. form.	Theor. *m/z*	Err [ppm]	*m/z*	Err [ppm]	Theor. *m/z*	Mol. form.
2	$C_{39}H_{31}O_{15}$ [M – H]⁻	739.1668	4.2	739.1699	– 24.5	739.1880	$C_{36}H_{35}O_{17}$ [M – H]⁻
3	$C_{54}H_{43}O_{21}$ [M – H]⁻	1027.2302	0.8	1027.2310	– 19.9	1027.2514	$C_{51}H_{47}O_{23}$ [M – H]⁻
4	$C_{69}H_{55}O_{27}$ [M – H]⁻	1315.2936	0.1	1315.2937	– 16.0	1315.3148	$C_{66}H_{59}O_{29}$ [M – H]⁻
5	$C_{84}H_{67}O_{33}$ [M – H]⁻	1603.3570	3.0	1603.3618	– 10.2	1603.3782	$C_{81}H_{71}O_{35}$ [M – H]⁻
6	$C_{99}H_{78}O_{39}$ [M – 2H]²⁻	945.2066	0.5	945.2070	– 10.5	945.2169	$C_{96}H_{82}O_{41}$ [M – 2H]²⁻
7	$C_{114}H_{90}O_{45}$ [M – 2H]²⁻	1089.2383	– 3.2	1089.2348	– 12.7	1089.2348	$C_{111}H_{94}O_{47}$ [M – 2H]²⁻
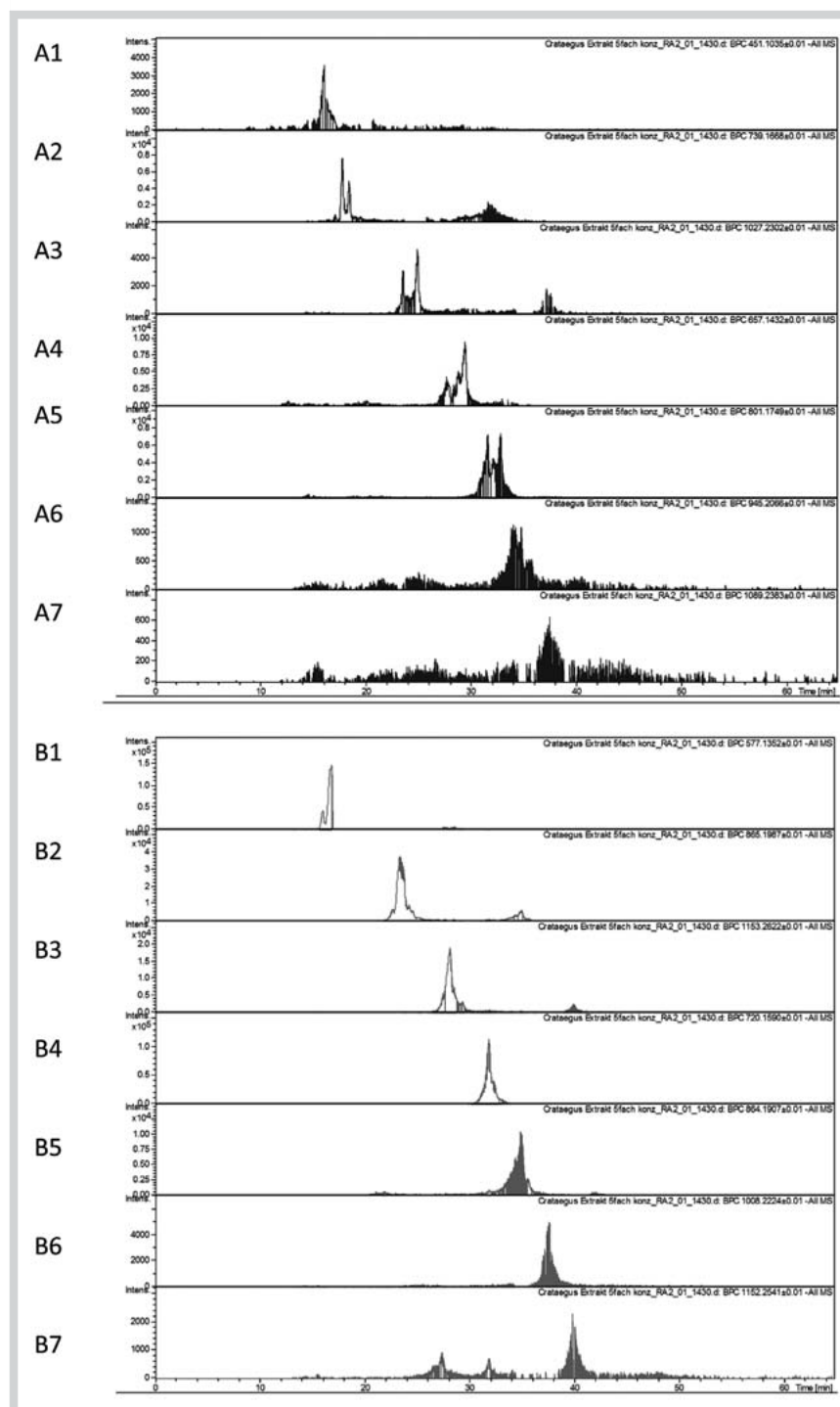
Fig. 4 Extracted base peak chromatograms for identification of monomeric to heptameric cinchonains (**A**) and dimeric to octameric procyanidins (**B**) after UHPLC ESI-qTOF-MS on the diol stationary phase of subfraction c. Base peak chromatograms used for analysis of oligomeric cinchonains in series A: A_1 *m/z* 451.1035 [M – H], A_2 *m/z* 739.1668 [M – H], A_3 *m/z* 1027.2302 [M – H], A_4 *m/z* 657.1432 [M – 2H], A_5 *m/z* 801.1749 [M – 2H], A_6 *m/z* 945.2066 [M – 2H], A_7 *m/z* 1089.2833 [M – 2H]. Base peak chromatograms for analysis of oligomeric procyanidins in series B: B_1 *m/z* 577.1352 [M – H], B_2 *m/z* 865.1987 [M – H], B_3 *m/z* 1153.2622 [M – H], B_4 *m/z* 720.1590 [M – 2H], B_5 *m/z* 864.1907 [M – 2H], A_6 *m/z* 1008.2224 [M – 2H], A_7 *m/z* 11 152.2541 [M – H].
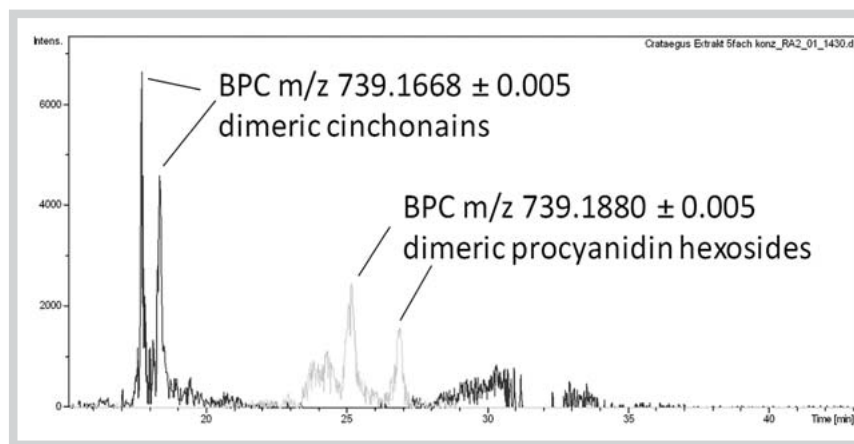
Fig. 5 Overlay of base peak chromatograms from hawthorn extract (subfraction c) for dimeric cinchonains and dimeric procyanidin hexosides.

supportive data were presented in these studies, we suggest that the respective analytical signals might in part be attributed to the nominally isobaric cinchonains. The presence of procyanidin glycosides in the Rosaceae family has been convincingly demonstrated by Porter et al., who observed indicative chemical shifts in a ^{13}C-NMR study of procyanidins isolated from *Cydonia oblonga* which yielded glucose upon acid hydrolysis [27].

The evaluation of UHPLC-ESI-qTOF-MS chromatograms and spectra obtained in our studies with OPC-enriched subfraction c, also containing the homologous series of cinchonains, additionally revealed the presence of a pair of peaks indicative for dimeric procyanidin hexosides by their exact mass (*m/z* 739.1880). These peaks were clearly separated from the two cinchonain II-type peaks (*m/z* 739.1668) (◉ **Fig. 5**). Unfortunately, a further MS/MS fragmentation of these peaks for detailed structural analysis was not possible due to low peak intensity. From these data, we can additionally assume the presence of a dimeric procyanidin glycoside with one hexose as a sugar moiety. Because two peaks are identified with the same mass, the existence of isomer pairs of the cinchonains and the tentative procyanidin hexosides can be expected. O-Glycosylation of the procyanidin backbone can be hypothesized to occur mainly at the hydroxyl groups at positions 7, 3, 5, 3′, or 4′ as shown for synthetic catechin glucosides [28]. It seems interesting that certain flavan-3-ol glycosides show different intestinal stability and absorption characteristics compared to the unglycosylated compounds: e.g., catechin-3′-O-glucoside and catechin-7-O-glucoside are apparently better absorbed in an intestinal rat perfusion assay [28]. From these findings, further studies on pharmacokinetic and pharmacodynamic properties of oligomeric procyanidin glycosides and also for the group of cinchonains may be worth to be performed.

Acknowledgements
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Conflict of Interest
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The authors state that they have no conflict of interest.

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