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Institute for Pharmaceutical Biology and Phytochemistry (IPBP)[1], Institute for Organic Chemistry[2], University of Münster, Germany

Preparative isolation of oligomeric procyanidins from Hawthorn (*Crataegus* spp.)

S. Zumdick[1], F. Petereit[1], H. Luftmann[2], A. Hensel[1]

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Prof. Dr. Andreas Hensel, University of Münster, Institute for Pharmaceutical Biology and Phytochemistry (IPBP), Hittorfstraße 56, 48149 Münster, Germany
ahensel@uni-muenster.de

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The oligomeric procyanidins (OPC) from Hawthorn leaves and flowers (Crataegi folium cum flore) are considered to be in part responsible for the cardiotonic clinical activity of the herbal material. Effective methods for rapid isolation of these heterogenous oligomeric clusters with defined molecular weight as reference compounds are not published until now. Therefore the water soluble fraction of an acetone/water (7 + 3) extract of Hawthorn leaves and flowers was fractionated by a combination of MPLC on RP-18 material and preparative HPLC using a diol stationary phase. This procedure resulted in the effective isolation of procyanidins with a distinct degree of polymerization (DP) from dimers DP2 up to tridecamers DP13. Exact mass measurements with negative ESI-TOF/MS were employed to confirm the respective structures of the isolated procyanidins.

Hawthorn leaves and flowers consist of the dried flower bearing branches of *Crataegus monogyna* Jacq. Emend. Lindm., *C. laevigata* (Poiret) and, more rarely, *C. pentagyna* Waldst. et Kit. Ex Willd., *C. nigra* Waldst. et Kit. and *C. azarolus* L. Pharmaceutical preparations of Hawthorn are considered as a rational based phytomedicine for treating declining cardiac performance corresponding to NYHA I and II. Flavonoids, such as flavonol and flavon derivatives, and procyanidins are considered to be the main active compounds, whereas oligomeric procyanidins (OPCs) seem to have the most marked effect (ESCOP 2003). For a recent summary of the phytochemistry and problems concerning the analysis of procyanidins from Hawthorn see Petereit and Nahrstedt (2005), Veit and Wittig (2005) and references cited therein. Because the OPCs are a highly complex heterologues series a big need is seen for isolation of the individual oligomers for quality control but also for more detailed pharmacological and pharmacokinetic studies. Therefore an acetone/water (7 + 3) extract of Crataegi folium cum flore was prepared. After removal of the organic solvent the aqueous phase was extracted with EtOAc leading to proanthocyanidins with a low degree of polymerization (DP2 to about DP4) enriched in this EtOAc phase while the longer chain oligomers are to be found in the aqueous phase beside minor amounts of DP2 to DP4 oligomers (Bicker et al., in press). The aqueous phase was further fractionated by a subsequent combination of MPLC on RP-18 material and pre-

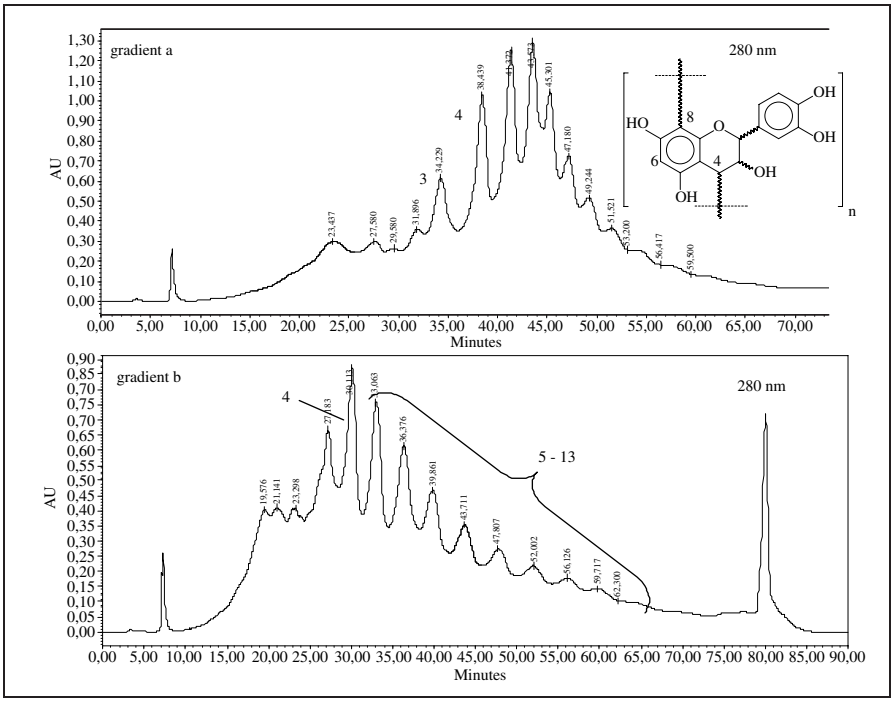

Fig. 1:
Preparative HPLC chromatograms with optimized gradients A and B for separation of OPCs from Hawthorn with DP of 3 and 4 (gradient A) and for OPCs up to tridecamers (gradient B)

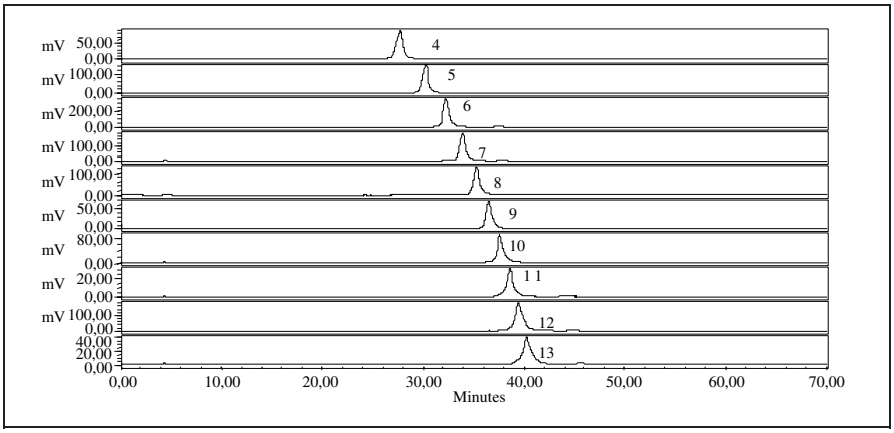

Fig. 2:
Analytical HPLC for purity control of OPCs with defined DP isolated from Hawthorn; numbers indicate degree of polymerization DP

parative HPLC using a diol stationary phase with two different gradient systems. This HPLC method allows the separation of procyanidins according to their degree of polymerization (DP). Trimers and tetramers were isolated with gradient A, whereas tetramers DP4 up to tridecamers DP13 were obtained by gradient B (Fig. 1). Yields, related to the amount of purified extract subjected to preparative HPLC (80 to 365 mg, depending on the chromatographic run), were as follows: gradient A trimers 3.9%, tetramers 9.6%; gradient B tetramers 5.8%, pentamers 6.6%, hexamers 5.9%, heptamers 4.3 %, octamers 3.7%, nonamers 3.1%, decamers 2.4%, undecamer to tridecamer 2.7%. These values correlate not necessarily with the oligomer distribution in the herbal material, because only the aqueous phase from the EtOAc-water partionation was used for the isolation of the oligomers. Additionally higher amounts of DP2 to 4 oligomers are to be expected in the respective EtOAc extract, which on the other side is not suitable for isolation of the long chain oligomers.

Purity of isolated fractions was subsequently controlled by analytical HPLC with a method modified according to Kelm et al. 2006 indicating the presence of highly purified oligomeric clusters (Fig. 2). Exact mass measurements with negative ESI-TOF/MS were employed to confirm the elemental composition of the isolated OPCs: *m/z* (DP3) = 865,1994 (calculated: 865,1974 $[M-H]^-$), *m/z* (DP4) = 576,1298 (576,1262 $[M-2\,H]^{2-}$), *m/z* (DP5) = 720,1601 (720,1579 $[M-2\,H]^{2-}$), *m/z* (DP6) = 864,1936 (864,1907 $[M-2\,H]^{2-}$), *m/z* (DP7) = 1008,2178 (1008,2224 $[M-2\,H]^{2-}$), *m/z* (DP8) = 767,8396 (767,8336 $[M-3\,H]^{3-}$), *m/z* (DP9) = 1296,2874 (1296,2858 $[M-2\,H]^{2-}$). Mass values are given for the monoisotopic peak. The formation of multiply charged ions as the DP increases has already been discussed by Guyot et al. (1997). The decrease in ionization efficiency of procyanidins with increase in DP has previously been mentioned by other authors (Guyot et al. 1997; Gu et al. 2003). We have been able to detect ions up to DP9. The DP of decamer to tridecamer was extrapolated from the measured data.

Summarizing, the described method enables the isolation of highly purified oligomeric procyanidins with a defined DP in relatively good yields. Further investigations will deal with the pharmacological testing of OPCs with a defined DP to have a more rational insight into the claimed therapeutical use for the treatment of heart diseases.

Experimental

1. Plant material

Crataegi folium cum flore (Ch.-B.:52467097) was obtained from Caesar – Loretz, Germany. A reference sample is stored at the Institute of Pharmaceutical Biology and Phytochemistry, Münster.

2. Extraction and purification

Drug material (5 kg) was exhaustively extracted with acetone/water (7 + 3). The combined extracts were evaporated *in vacuo*, filtered to remove precipitated chlorophyll, concentrated and defatted with petroleum. Successive extraction with EtOAc yielded an EtOAc-extract (126 g, 2.5%) and a water extract (859 g, 17.2%). A portion of the water extract (20 g) was divided into a methanol-soluble (9.3 g) and a methanol-unsoluble (8.9 g) part. A part of the methanol-soluble water extract (5.5 g) was applied to MPLC (RP-18, 18–32 μm, 100 Å, 36 × 500 mm, Besta Technik (Germany) flow: 9 mL/min, Waters 501 HPLC pump, fraction collector: Pharmacia LKB Superfrac (Sweden), fraction size: 3 min/test tube, using MeOH 10%, MeOH 50% and MeOH 80% until no compounds eluted anymore. Fractions were analysed by TLC (aluminium sheets, 20 × 20 cm, silica gel 60 F_{254}, Merck (Germany), mobile phase : EtOAc : H_2O : formic acid 90 + 5 + 5 (v/v/v)) with vanillin/HCl and anisaldehyd/sulfuric acid. Subfraction c (457 mg, 0.66% related to the absolute amount of herbal material) was subjected to preparative HPLC.

3. Separation conditions: preparative HPLC

Two Waters 515 HPLC pumps, Waters 2487 dual λ absorbance detector (detection wave length: 280 nm), uniflows degasys DG-2410, waters pump controlle module, software: Millenium^{32}, column: material: Uptisphere®, 120 Å, bonding: OH, 6 μm, 250 × 21 mm, Interchim, France. Mobile phase A: acetonitrile, mobile phase B: methanol/water (95 : 5, v/v). Elution gradient A: 0–35 min 100% A → 40% A, 35–65 min 40% A isocratic, 65–70 min 40% A → 0 % A, 5 min 0% A isocratic, 75–80 min 0 % A → 100% A, 10 min 100% A isocratic. Elution gradient B: 0–15 min 100% A → 60% A, 15–20 min 60% A → 50% A, 20–50 min 50% A → 40% A, 50–68 min 40% A, 68–70 min 40% A → 0% A, 5 min 0% A isocratic, 75–85 min 0% A → 100% A, 10 min 100% A isocratic. Reequilibration time: 10 min, flow rate: 10 ml/min, samples were dissolved in methanol, injection volume: 1 ml.

4. Analytical HPLC

Waters 600 controller, Waters 996 PAD, Waters 474 scanning fluorescence detector, Waters 717 plus autosampler, Waters in-line degasser AF, software: Millenium^{32}, column: material: Uptisphere®, 120 Å, bonding: OH, 6 μm, 250 × 4,6 mm, Interchim, France. Mobile phases A: acetonitrile/acetic acid (98 : 2, v/v), B: methanol/water/acetic acid (95 : 3:2, v/v/v). Elution gradient was modified according Kelm et al. 2006 as follows: 0–35 min 100% A → 40% A, 5 min 40% A isocratic, 40–50 min 40% A → 0% A, 10 min 0% A isocratic, 60–65 min 0% → 100% A. Reequilibration time: 20 min, samples were dissolved in methanol, injection volume was 20 μl, detection: PAD at 280 nm and fluorescence detection (λ_{Ex} 280 nm, λ_{Em} 316 nm).

5. ESI-Tof/MS

ESI-Mass spectras were recorded with Micro Tof (Bruker Datronics, Germany) via loop injection in the negative ion mode. Samples were dissolved in methanol.

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