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Short Communication

TLC-densitometric determination of 2,3-*cis*-procyanidin monomer and oligomers from Hawthorn (*Crataegus laevigata* and *C. monogyna*)*

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Introduction

The structure elucidation of procyanidins isolated from the flowers and the leaves of hawthorn, *Crataegus laevigata* (Poiret) DC. (syn. *C. oxyacantha* auct. non L.) and *C. monogyna* Jacq. (syn. *C. oxyacantha* L. p.p.) [1] has been achieved over the past 20 years (−)-epicatechin; [4,8]-2,3-*cis*-3,4-*trans*:2′,3′-*cis*-procyanidin (B_2), [4,6]-2,3,-*cis*-3,4-*trans*:2′,3′-*cis*-procyanidin (B_5), [4,8:4,8]-2,3-*cis*-3,4-*trans*:2′,3′-*cis*-3′,4′-*trans*:2″,3″-*cis*-procyanidin (C_1) and [4,8:4.8]-2,3-*cis*-3,4-*trans*:2′,3′-*cis*-3′,4′-*trans*:2″,3″-*cis*-3″,4″-*trans*: 2‴,3‴-*cis*-procyanidin were definitely identified [2]. In addition, (+)-catechin, procyanidins B_1, B_4, B_5 (dimers) and D_1 (tetramer) were also detected in leaves and fruits of *C. monogyna* [3].

Previous toxicological and pharmacological experiments have shown that procyanidins are important contributors to the pharmacological properties of hawthorn; moreover the improvement of the coronary blood flow, the increase of heart contractility and the decrease of arterial blood pressure are closely related to the stage of procyanidin polymerization [4–8].

Colorimetric determinations of procyanidins as previously described [9] do not measure the relative proportions of each monomer or oligomer and lack specificity. A study on the separate determination of the main biologically active procyanidins found in the drug was therefore undertaken.

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Experimental

Procyanidins (standards) isolation

Powder (100 g) of *C. laevigata* and *C. monogyna* (leaves and flowers) was extracted with acetone–water (7:3, v/v) (800 ml). After acetone evaporation under reduced pressure, the aqueous suspension was extracted with toluene–petroleum ether (1:1, v/v) (3 × 200 ml), then with ethyl acetate–2-butanol (1:1, v/v) (3 × 100 ml). These last fractions were evaporated to give a green-brown residue (7 g) which was further purified by HSCCC. This last step was performed using an horizontal flow-through planet centrifuge [10, 11] with an Ito Multilayer coil [12] (P.C. Inc., Potomac, MD, USA) equipped with a 2.6 mm i.d. column (length: 66 m; capacity: 350 ml); the solvent system was prepared by equilibrating, in a separating funnel, a mixture of water–ethyl acetate–2-butanol (600:558:42, v/v/v), upper phase (stationary phase) and lower phase (mobile phase) pumped into the column at 4.5 ml min^{-1}. The rotational speed was 800 rpm and the samples (500 mg) were successively injected after partition in the solvent system (10 ml). The pertinent fractions were detected by TLC analysis, pooled and further purified by preparative TLC on silica gel (mobile phase:ethyl acetate–formic acid–acetic acid–water 75:3:2:20, v/v/v/v, upper phase) to provide four major fractions of procyanidins (approximately 60 mg each) eluted from the adsorbent with methanol. Before evaporation, the methanol solutions were quickly filtered through a small column of Sephadex LH-20 (Pharmacia LKB Uppsala, Sweden). The evaporation residues (white powders) were stored under nitrogen at −20°.

Mass spectrometry

FAB–MS spectra were recorded on an AEI MS 902 mass spectrometer. The samples were dissolved in a thioglycerol matrix and placed on a steel target prior to bombardment with Ar atoms of energy 7–8 kV.

Extraction procedure for the procyanidins determination

Approximately 6.0 g (accurately weighed) of ground dried plant, introduced in a percolator (capacity: 60 ml), was allowed to macerate, for 2 h, in 10 ml of acetone–water containing 1% acetic acid (7:3, v/v), then percolated with the same solvent mixture so as to obtain 50 ml within 2 h. The solution was quantitatively transferred into a 250 ml flask and concentrated under reduced pressure at room temperature to completely remove the acetone. The resulting aqueous suspension was filtered on a small cotton plug. For hawthorn dried extracts, the sample (1.5 g) was dispersed (ultrasonic bath) in 15 ml of 1% acetic acid in water and filtered under the same conditions. The filtrate (colloidal solution) was directly transferred onto a chromatographic column (i.d. 20 mm) filled with Sephadex LH-20 in water. The flask and the filter were rinsed with 3 × 12 ml water and the filtrates successively transferred onto the column. The aqueous eluate (approximately 50 ml) was discarded and the column was further eluted with methanol–acetone (1:1) (flow-rate: 0.5 ml min^{-1}). The first fraction of 5 ml was discarded, then the eluate was collected in a volumetric flask so as to obtain 20 ml (sample solution) or a volume adjusted according to the procyanidins concentrations in the sample.

TLC-densitometry

Adsorbent: HPTLC pre-coated plates of silica gel 60 F_{254} (20 × 10 cm) were obtained from Merck (Darmstadt, FRG); they were not activated. Applications: 10 μl of the

solutions of (−)-epicatechin (supplied by Fluka, Buchs, Switzerland) dissolved in methanol–acetone–water 1:1:1, v/v/v (6 to 70 mg 50 ml^{-1}) and 10 μl of the sample solutions were applied 15 mm from the lower edge of the plates with an automatic apparatus Linomat IV (obtained from Camag Muttenz, Switzerland) in narrow bands (9 mm width, rate of delivery: 6 s μl^{-1}, total number of bands: 12) which were dried under a current of hot air before development. Mobile phase: ethyl acetate–formic acid–acetic acid–water 75:3:2:20, v/v/v/v (upper phase). This mobile phase was allowed to travel a distance of 100 mm when the plate was developed in an unsaturated tank at room temperature. Detection: after drying at 120°C for 5 min, a 1% vanillin solution in hydrochloric acid (8 ml/plate) was sprayed on the warm plate. After 10 min, the chromatograms were covered with a glass plate to prevent the release of hydrochloric acid inside the densitometer. Densitometry: the bands were measured with a dual-wavelength TLC scanner CS-930 from Shimadzu (Kyoto, Japan). The following settings were used: reflection mode, Vis detection at 515 nm, linear scanning, beam size 0.05 × 5 mm, peak minimum width 100, delt "Y" 0.10 mm. Calculations: each determination corresponded to the mean value calculated from the integration results of 12 chromatograms [four different (−)-epicatechin concentrations repeated twice for the calibration curve and four bands of the sample solution].

Results and Discussion

Isolation of procyanidin standards

The isolation of the major procyanidins of hawthorn was achieved using firstly an original and convenient HSCCC (high-speed counter-current chromatography) method followed by preparative TLC on silica gel. Evident advantages of HSCCC for the isolation on a preparative scale of proanthocyanidins were the reduced pre-clean-up of the plant extract, the high resolution within a short time (2 h) in the dark and the suppression of irreversible binding on adsorbents and of degradation on solid supports. The identification of four fractions was achieved by FAB–MS [13]. Fraction I corresponded to (−)-epicatechin as confirmed by co-chromatography (TLC) with an authentic sample. Fractions II, III and IV were respectively identified as procyanidins B_2 [FAB–MS: 579 (MH^+)], procyanidin C_1 [FAB–MS: 867 (MH^+)] and D_1 [FAB–MS: 1155 (MH^+)]. In addition, low amounts of another dimer (II′, procyanidin B_5 ?) and another trimer (III′) peak, (Fig. 1) were also detected; fraction IV could correspond to a mixture of several isomeric tetramers.

Extraction procedure for the determination of procyandins

The first step included an acetone–water extraction of the powder. Clean-up by partition into various solvents was not efficient for the quantitative extraction of the procyanidins. A simple acetone evaporation of the initial extract led to the precipitation of interfering substances (lipids, pigments . . .) so that the purification on Sephadex was feasible without any other clean-up procedure. A careful check of the content of each solution or precipitate led to the conclusion that no procyanidin oligomer (mono-, di-, tri- and tetramers) loss occurred during the complete extraction procedure.

TLC-densitometric determination

The improvements of the TLC conditions for the separation of procyanidins was achieved using procyanidin standards; they were found more efficient than those

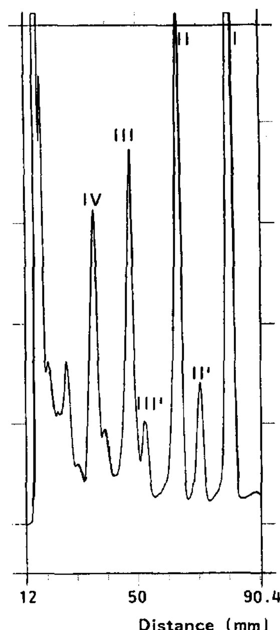

Figure 1
Scanning profile of a TLC chromatogram of a purified extract of *C. laevigata* leaves and flowers. Adsorbent: silica gel 60. Mobile phase: ethyl acetate–formic acid–acetic acid–water (75:3:2:20, % v/v/v/v, upper phase). Detection: 1% vanillin in hydrochloric acid (absorption) = 515 nm. I = (−)-epicatechin; II′ = procyanidin B_5 (?); II = procyanidin B_2; III = procyanidin C1; III′ = trimer; IV = procyanidin D_1 (+ other isomeric tetramers?).

described earlier for hawthorn [14] and more recently for other drugs [15]. However, UV–vis spectrometry of the chromatograms allowed the detection of interferences at Rf values corresponding to the procyanidins oligomers and specially at the tetramer position. Therefore, the use of a specific reagent was essential, and 1% vanillin–phosphoric acid, 3% aqueous Fe(III)chloride, 5% phosphomolybdic acid, Folin–Ciocalteau, and 1% vanillin–hydrochloric acid reagent were tested. This last reagent was found to be the most specific. Furthermore, the colours developed with each procyanidin oligomer was comparable to that of (−)-epicatechin at the same concentration (superimposable calibration curves). Therefore, (−)-epicatechin was suitable for the determination of all oligomers. Concentrations of 1.2 to 14 μg of (−)-epicatechin per 10 μl spotted afforded a linear calibration graph with an r value (correlation coefficient) typically greater than 0.995. The results of the quantitative analysis of one sample (six determinations) showed that the variation coefficient was lower than 6%. The direct densitometry of the chromatograms was more rapid, more accurate and reproducible than the chromatographic method involving the extraction of procyanidins from the adsorbent and a further colorimetric determination [14].

Determination of procyanidins

Results obtained with samples of *C. laevigata* (one sample of flowers, one sample of flowers and leaves and one sample of a spray-dried hydro-ethanolic extract) are given in Table 1 and a typical scanning profile is presented in Fig. 1. It is evident that the concentrations of procyanidins are lower in the flowers than in the leaves. Among several hawthorn extracts obtained from both *C. laevigata* and *C. monogyna* and corresponding either to the Pharmacopœial monographs (P.V. V, D.A.B. 8, Ph. Helv. VI, Ph. Fr. IX) either to commercially available samples, only one extract (Table 1) shows acceptable concentrations of procyanidins when compared with the powder;

Table 1
Concentrations of procyanidins in *C. laevigata*: comparison between two samples of different composition and a commercially available extract (% calculated with reference to the dried drug or extract)

Procyanidins	Flowers	Leaves and flowers	Extract
(−)-Epicatechin	0.58%	0.85%	1.18%
Procyanidin B_5 (7)	0.09%	0.18%	traces
Procyanidin B_2	0.87%	1.09%	1.78%
Procyanidin C_1 + another trimer	0.79%	1.02%	0.43%
Procyanidin D_1 (+ other tetramers?)	traces	0.74%	traces
Total	2.33%	3.88%	3.39%

however the tetramer fraction was not detectable and the advantage of the extraction was finally not evident.

It can be concluded that the proposed method could be conveniently applied to the standardization of hawthorn pharmaceutical forms. Current studies on a more appropriate extraction procedure are now in progress.

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