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Short communication

SPE-HPTLC of procyanidins from the barks of different species and clones of *Salix*

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ABSTRACT

A SPE-HPTLC method was developed for the qualitative and quantitative analysis of procyanidin B_1 in willow barks. The chromatography was performed on HPTLC silica gel layer with the mobile phase chloroform–ethanol–formic acid (50:40:6 v/v/v), in the Automatic Developing Chamber—ADC 2. The methanol extracts from willow barks were purified by SPE method on RP-18 silica gel columns with methanol–water (7:93 v/v) as the eluent. The presence of procyanidin B_1 was revealed in the majority of investigated willow barks. The content of procyanidin B_1 varied from 0.26 mg/g in the extract of *Salix purpurea* clone 1067–2.24 mg/g in the extract of *Salix alba* clone 1100. The method was validated for linearity, precision, LOD, LOQ and repeatability.

1. Introduction

The willow bark (*Salicis cortex*) is a herbal remedy used in the treatment of rheumatism, pain and fever [1,2]. According to the monograph in the 5th European Pharmacopoeia [3], the quality control of the drug is based on the analysis of salicin and its esters. However, it was suggested [2,4,5] that pharmacological activity of willow bark can depend on the presence of other compounds as well [2,4,5].

Proanthocyanidins are oligomers or polymers of flavan-3-ols [6]. These compounds are integral part of human diet because of their common distribution in the plant kingdom [6]. Rich sources of the proanthocyanidins are fruits (apple, grape, cranberry), chocolate, red wine, and herbal remedies—the inflorescences of hawthorn (*Crataegi inflorescentia*) and the maritime pine bark (*Pini maritimae cortex*) [6]. Proanthocyanidins have different pharmacological activities, including antioxidative, antihypertensive and anti-inflammatory activities [6]. It was confirmed that the anti-inflammatory activity of procyanidin dimers—B_1 and B_2, may be partly due to the inhibition of transcription nuclear factor-kappa B (NF-κB) [7].

The chemical composition of proanthocyanidins in willow barks is poorly recognized [8–13]. Until now, the presence of some dimeric proanthocyanidins (procyanidin B_3, B_6, B_7) besides the trimeric derivatives (procyanidin C_2 and C_4) were revealed in *S. caprea* [8–10], *S. sieboldiana* [10] and *S. pet-susu* [11]. Nahrstedt et al. [13] isolated procyanidin B_1 and B_3 besides catechin, epicatechin, gallocatechin and two flavan-3-ol trimers from the bark of *Salix purpurea*.

The most popular method used in the analysis of proanthocyanidins in plant matrices is *reversed phase high-performance liquid chromatography* (RP-HPLC) [14–17]. *Thin-layer chromatography* (TLC) was also used for the separation of procyanidins [18–20]. Vovk et al. [18] optimized the separation of procyanidin B_1 and B_2 on cellulose plates. Procyanidin B_2 and procyanidin C_1 were analyzed by TLC in the rhubarb roots (*Rhei radix*) [19], whereas procyanidin B_2 was resolved from procyanidins C_1 and D_1 in the extract from the leaves and flowers of hawthorn (*Crataegi flos cum folio*) [20].

The aim of our research was to develop and optimize TLC method for separation and quantification of procyanidin B_1 and its isomer—procyanidin B_2 in the bark of 16 species and 6 clones of the genus *Salix*. TLC was chosen for this study because this method is suitable for the analysis of a large number of samples within a relatively short period of time [21].

2. Experimental

2.1. Plant material

The barks of 16 species and 6 clones of the genus *Salix* were collected in March 2004. The barks of *S. acutifolia*, *S. daphnoides*, *S. eriocephala*, *S. fragilis*, *S. purpurea* and *S. triandra* originated from natural habitat (Poznań, Poland). The barks of *S. alba* clone 1100, *S.* × *americana* clone 1036, *S. daphnoides* clone 1095, *S. purpurea* clone 1067, *S. purpurea* clone 1132 and *S. triandra* clone 1044 were

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obtained from willow collection of the University of Warmia and Mazury, Olsztyn (Poland). The barks of *S. lapponum*, *S. melanostachys*, *S. nakamurana* var. *yezoalpina*, *S. rosmarinifolia* and *S. silesiaca* were collected from the Botanical Garden of the University of Warsaw (Poland). The barks of *S. herbacea*, *S. matsudana* 'Totuosa', *S. sachalinensis* 'Sekka', *S.* × *sepulcralis* and *S. viminalis* were obtained from the Medicinal Plants Garden of the Medical University of Gdańsk (Poland).

2.2. Sample preparation

2.2.1. Extraction

Dried and pulverized bark (1 g) was exhaustively extracted with methanol ($T = 60\,^{\circ}C$). From the obtained extract the solvent was evaporated under reduced pressure and the residue was dissolved in methanol (5 ml).

2.2.2. Sample purification—SPE

Methanol extracts (200 μl) were purified on SPE glass columns (4 mm × 140 mm) filled up with Lichroprep™ RP-18 (40–63 μm, 150 mg) (Merck, Darmstadt, Germany) and eluted with 6 ml of solvent (methanol–water 7:93 v/v). Eluates were lyophilized (Lyovac GT 2, Finn-Aqua Santasalo-Sohlberg, Germany) and dissolved before analysis in 300 μl of methanol.

2.3. Chemicals and reagents

Chloroform, methanol, and phosphoric acid were analytical grade from POCH (Gliwice, Poland). Formic acid of analytical grade was obtained from Merck (Darmstadt, Germany). Water of chromatography gradient grade was from J.T. Baker (Deventer, Holland). Ethanol was from Polmos (Starogard Gd., Poland). Vanillin of analytical grade was from Loba Feinchemie (Fischamend, Austria).

Standard of (+)-catechin was obtained from Fluka (Buchs, Switzerland). Procyanidin B_1 and procyanidin B_2 originated from Sigma (St. Louis, MO, USA) (Fig. 1).

Standard compounds (1 mg) were dissolved in methanol (1 ml).

2.4. Thin-layer chromatography

2.4.1. Conditions of chromatographic separation

TLC experiments were carried out on 5 cm × 10 cm silica gel 60 high-performance thin-layer chromatographic (HPTLC) plates (Merck, Darmstadt, Germany) with the mixture of chloroform–ethanol–formic acid (50:40:6 v/v/v) as a mobile phase. For quantitative analysis 10 cm × 10 cm silica gel 60 HPTLC plates were used.

Standard solutions and samples were applied to the plates by means of a HPTLC-Applicator AS-30 (Desaga, Nümbrecht, Germany)—the band length was 5 mm, the application volume was 2–6 μl. Plates were developed at room temperature and 50% humidity in an Automatic Developing Chamber—ADC 2 (Camag, Muttenz, Switzerland), previously equilibrated with mobile phase vapour (both chamber saturation time and layer preconditioning time were 5 min). The migration distance was 25 mm.

Spraying was performed with vanillin/H_3PO_4 reagent [18] (10 ml of H_3PO_4 was added to a solution of 1 g vanillin in 70 ml ethanol), by use of a Sprayer SG 1 and a Thermoplate S ($T = 75\,^{\circ}C$, $t = 3$ min) (Desaga, Nümbrecht, Germany).

Densitograms were obtained with a CD-60 densitometer (Desaga, Nümbrecht, Germany) under visible light at $\lambda = 500$ nm after spraying with vanillin/H_3PO_4 reagent. For qualitative analysis linear scans were obtained with the slit dimensions of 0.02 mm × 2 mm and for quantitation the chromatograms were scanned with a meander 7 mm wide with slit dimensions 0.4 mm × 0.4 mm.

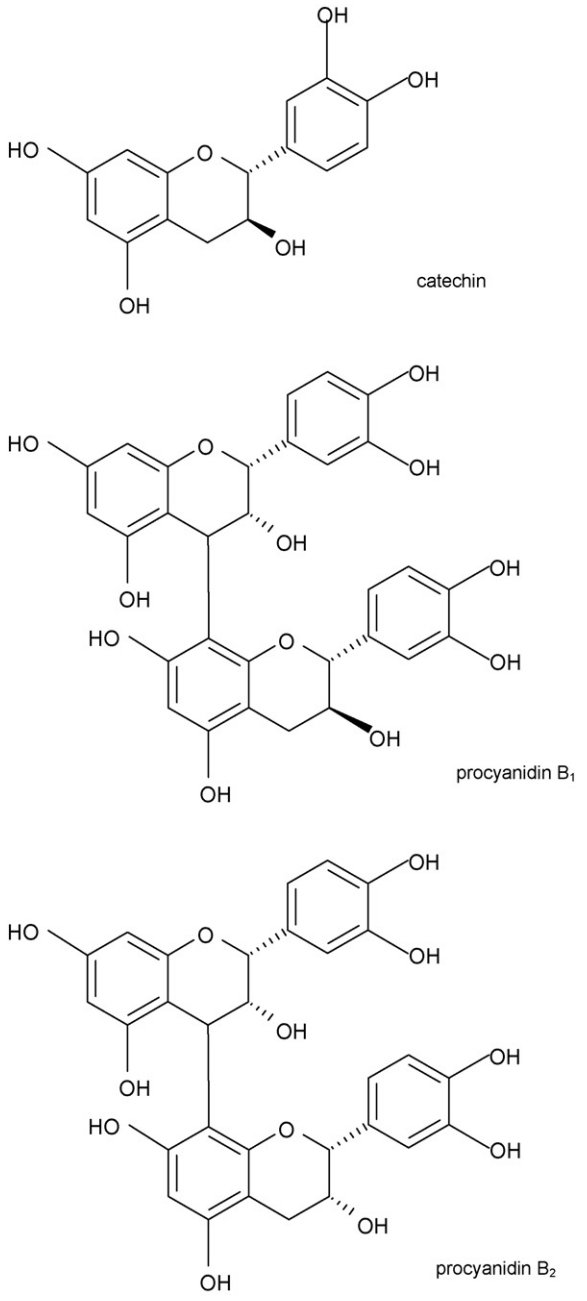

Fig. 1. The structures of analyzed compounds.

2.4.2. Validation

The method was validated for linearity, limits of detection (LOD) and quantitation (LOQ), precision, repeatability and accuracy, which were estimated according to the literature data [22].

Standard solutions of procyanidin B_1, containing concentrations in the range 0.10–0.50 mg ml^{-1}, were prepared for quantitative analysis. Calibration curves ($y = ax + b$, correlation coefficient $r = 0.9992$, slope $a = 0.66 \pm 0.21$; intercept $b = 6.64 \pm 4.56$) were plotted as the value of peak area against the amount of standard.

The limits of detection (LOD) and quantitation (LOQ) were calculated as signal to noise ratio, S/N >3 and 10, respectively.

Instrument precision, expressed as coefficient of variation (%CV), was checked by repeated scanning ($n = 7$) of the spot of procyanidin B_1 (700 ng spot^{-1}) after spraying at $\lambda = 500$ nm.

The repeatability of the method, expressed as %CV, was measured by analysis of seven spots of procyanidin B_1 standard solution (each 700 ng spot^{-1}) after spraying at $\lambda = 500$ nm.

The variability of the method was expressed as intra-day precision [%CV], which was studied by analysis of seven spots of procyanidin B_1 standard solution (each 700 ng spot^{-1}) per plate on three different HPTLC plates.

The accuracy of the SPE method was confirmed by recovery studies for procyanidin B_1. The sample containing respectively 300, 450 and 600 ng of compound in 50 μl of solvent was put onto SPE column and analyzed according to the elaborated SPE procedure. The estimated recovery rate for procyanidin B_1 was 89.9 ± 2.1%.

3. Results and discussion

The optimization of TLC separation of the standard mixture consisting of catechin, procyanidin B_1 and its isomer procyanidin B_2 (Fig. 1) was performed on different types of adsorbents, namely: cellulose, polyamide, modified silica gels (RP-18W, LiChrospher RP-18W) and silica gels chemically modified by cyanopropyl and hydroxyl groups (CN and DIOL). Catechin was included as a standard to the mixture of flavan-3-ol derivatives due to the fact of its occurrence in willow bark [23].

The visualization of flavan-3-ol spots was carried out by spraying with vanillin/H_3PO_4 reagent [18] and heating the plate at 75 °C for 3 min. In contrary to another tested reagents as the solution of vanillin in concentrated HCl [20] or 3% solution of $FeCl_3$ in methanol [19], this spraying reagent gave fast and specific, rose-coloured spots of flavan-3-ols (Fig. 2a).

The best resolution was achieved on the HPTLC silica gel plates using the mixture of chloroform–ethanol–formic acid (50:40:6 v/v/v), as a mobile phase (Fig. 2a). The obtained resolution factors (R_S) for procyanidin B_1/catechin and procyanidin B_2/catechin were 1.7 and 0.9, respectively (Fig. 2a). The correct separation of two compounds, especially for quantitative analysis purposes, should posses factor R_S not less than 0.8 [21]. The development of plates was performed in the Automatic Developing Chamber ADC 2 (Camag). The ADC 2 chamber enables to control the humidity. Humidity is very important parameter of chromatographic process, particularly if silica gel is used as stationary phase. This type of adsorbent has uncovered silanol groups, whose activity is blocked by water in the air [24]. The optimal separation was reached at humidity 50%. The usage of vertical or horizontal chambers, instead of ADC 2 chamber resulted in co-elution of all compounds on the front of mobile phase. This report presents the separation of two isomers—procyanidin B_1 and procyanidin B_2 on silica gel layer for the first time.

The presence of procyanidin B_1 was confirmed in 19 among 22 examined willows. It was revealed through the comparison of the retardation factor values—hR_F ($R_F \times 100$) of standards and rose-coloured spots, which occurred on the chromatograms of *Salicis cortex* extracts. However, the presence of procyanidin B_1 was not found in the bark of *S.* × *americana* clone 1036, *S. rosmarinifolia* and *S. silesiaca*. On the other hand, the performed TLC analysis of the extracts confirmed the absence of procyanidin B_2 in willow bark [13].

Moreover, parallel with procyanidin B_1 ($hR_F = 66$) and catechin ($hR_F = 93$) two further compounds; A ($hR_F = 46$) and B ($hR_F = 80$) were recognized as flavan-3-ol derivatives (rose-coloured spots/vanillin reagent) (Fig. 2a). On the basis of the literature data [18–20] it was stated that the spot observed below

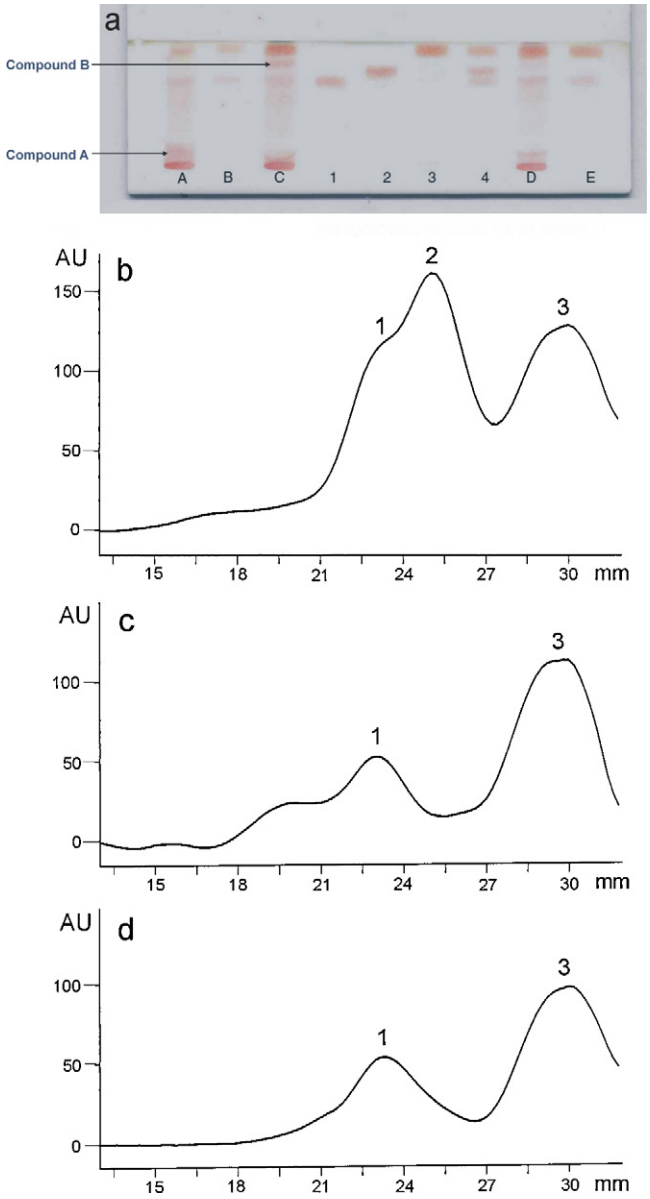

Fig. 2. HPTLC chromatogram of analyzed willow barks (A–E) and standards (1–4), densitogram of the standard mixture (b), densitogram obtained from crude methanol extracts of *S. alba* clone 1100 bark (c) and densitogram obtained from the SPE purified methanol extracts of *S. alba* clone 1100 bark (d): A – methanol extract of *Salix purpurea* bark, B – SPE purified methanol extract of *Salix purpurea* bark, C – methanol extract of *S. fragilis* bark, D – methanol extract of *S. alba* clone 1100 bark, E – SPE purified methanol extract of *S. alba* clone 1100 bark, 1 – procyanidin B_1, 2 – procyanidin B_2, 3 – catechin, 4 – standard mixture 1, 2, 3. Adsorbent: HPTLC Si 60, mobile phase: chloroform–ethanol–formic acid (50:40:6 v/v/v), vanillin/H_3PO_4 reagent [18].

procyanidin B_1 (compound A) could be the trimer of flavan-3-ols—probably procyanidin C [18–20], whereas the spot present above procyanidin B_1 (compound B) could be procyanidin B_3 [13] (Fig. 2a).

On TLC chromatograms of the analyzed methanol extracts several spots of unidentified substances were observed (Fig. 2c). These compounds complicated quantification of procyanidin B_1. For these reasons purification of methanol extracts from willow bark by SPE was carried out as described in Section 2.2.2 (Fig. 2d).

The elaborated method was validated for linearity, detection and quantitative limits, precision and repeatability. The LOD for procyanidin B_1 was found to be 50 ng and LOQ was 170 ng. Instrumental

precision was 1.26% CV (coefficient of variation), repeatability 7.85% CV, and intra-day precision 8.73% CV.

The quantitative analysis of procyanidin B_1 was realized only for selected willows, containing more than 0.2 mg/g of this compound, namely: *S. eriocephala*, *S. fragilis*, *S. purpurea*, *S. matsudana* 'Totuosa', *S. sachalinensis* 'Sekka', *S. alba* clone 1100, *S. purpurea* clones 1067 and 1132.

The determined content of procyanidin B_1 in analyzed willow barks varied in the range 0.26–2.24 mg/g of dry plant material. The highest procyanidin B_1 concentration was found in the bark of *S. alba* clone 1100 (2.24 mg/g), whereas the lowest was in *S. purpurea* clone 1067 (0.26 mg/g). The concentration of procyanidin B_1 was also relatively high in the barks of the following willows: *S. eriocephala*, *S. fragilis* and *S. matsudana* 'Tortuosa' (0.79 mg/g, 0.57 mg/g and 0.42 mg/g, respectively).

The results of qualitative and quantitative analysis showed significant differences in the content of procyanidin B_1 within the population of willows. It is supposed that the reported anti-inflammatory activity of proanthocyanidins [6,7] is the basis of the therapeutic effects of especially the white willow bark (*S. alba*) with the highest concentration of flavan-3-ol derivatives.

4. Conclusion

A SPE-HPTLC method of procyanidin B_1 determination in willow bark was elaborated. This is the first report on separation of two isomers—procyanidin B_1 and procyanidin B_2 on silica gel layer. The described SPE-HPTLC method can be useful for determinations of procyanidin B_1 and B_2 in other plant matrices.

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