

Short Communication

Thin-layer chromatographic analysis of proanthocyanidins from *Ribes nigrum* leaves*

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Introduction

Blackcurrant leaves are traditionally used in Europe for the treatment of rheumatic disease. We have observed that the anti-inflammatory activity (in carrageenan-induced rat paw-oedema) is mainly due to proanthocyanidins and particularly to prodelphinidins [1]. Prodelphinidins are less frequently encountered in the plant kingdom than procyanidins. However, in the case of *Ribes nigrum*, the proanthocyanidin fraction contains chiefly prodelphinidins instead of procyanidins, as is also evident from the structures of the main oligomeric compounds, which are formed solely from (epi)gallocatechin units [2].

In preceding papers [1, 2] the occurrence in *R. nigrum* of five prodelphinidins has been described: gallocatechin (GC) **3**, epigallocatechin (EGC) **4**, gallocatechin-(4 α ->8)-gallocatechin **5**, gallocatechin-(4 α ->8)-epigallocatechin **6** and the new trimer: gallocatechin-(4 α ->8)-gallocatechin-(4 α ->8)-gallocatechin **10**.

In continuing studies on the constituents of blackcurrant leaves, a further five, known, compounds have been isolated: catechin (C) **1**, epicatechin (EC) **2**, gallocatechin-(4 α ->8)-catechin **7**, gallocatechin-(4 α ->6)-gallocatechin **8** and catechin-(4 α ->8)-gallocatechin-(4 α ->8)-gallocatechin **9**. This report describes the identification and TLC fingerprint of these 10 proanthocyanidins. The simultaneous

examination of their R_f values and colorations with the vanillin-hydrochloric acid reagent gives structural and stereochemical information which is of use in the quality control of medicinal products based on blackcurrant leaves.

Experimental

Extraction and isolation of the proanthocyanidins

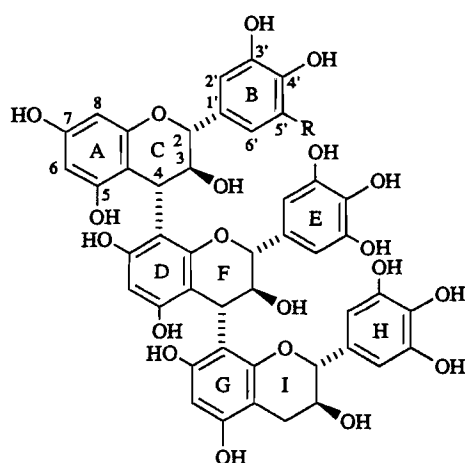
The extraction and purification of the proanthocyanidins followed the procedure recently described [2]. The crude extract was fractionated using a medium-pressure Superformance column filled with reversed-phase RP 8 and the elution was carried out with water-acetone (9:1, v/v). The purification of proanthocyanidins was effected on Sephadex LH 20, using as solvent system ethanol with increasing concentrations of methanol. The elution from the Sephadex followed the order: **1**, **2**, **3**, **4**, **7**, **5** and **6** (eluted simultaneously), **8**, **9** and **10**.

Structure determination of the proanthocyanidins

¹³C NMR spectra were measured at 400 MHz in acetone-d₆-water (1:1, v/v); chemical shifts are given in δ (ppm) relative to TMS. MS were recorded with FAB in the positive mode; the samples were dissolved in a glycerol matrix. IR spectra (KBr discs) were recorded

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9 C-(4 α ->8)-GC-(4 α ->8)-GC R=H
 10 GC-(4 α ->8)-GC-(4 α ->8)-GC R=OH

min) at room temperature, using the upper phase of ethyl acetate–water–formic acid–acetic acid (70:20:3:2, v/v/v/v) as mobile phase [6]. Following development, the plates were dried and sprayed with a 1% vanillin solution in methanol–hydrochloric acid (8:2, v/v). The colours of the bands were observed 5 min and 24 h after spraying. The R_f values were as follows: 1 (0.89), 2 (0.875), 3 (0.80), 4 (0.78), 5 (0.41), 6 (0.42), 7 (0.51), 8 (0.6), 9 (0.21) and 10 (0.15).

Results and Discussion

Up to now, 10 proanthocyanidins have been isolated from *R. nigrum*. They can be subdivided into three groups, according to the increasing degree of polymerization: four monomers 1, 2, 3 and 4 (1 and 2 are minor components); four dimers, comprising two major prodelphinidins, 5 and 6, and two minor compounds, 7 and 8, discussed below; and two trimers, including the new prodelphinidin 10 and the mixed proanthocyanidin 9.

Determination of the structure of the dimers 7 and 8 and of the trimer 9

Compound 7 was the first substance to be eluted after the monomers. The $[M + 1]^+$ peak m/z 595 suggested a dimeric structure with mixed B-ring flavan units. This was confirmed by treatment with 1.4 M hydrochloric acid, which generated cyanidin and delphinidin. Degradation by treatment with 0.1 M hydrochloric acid yielded catechin from the lower or terminal unit. Examination of the ^{13}C NMR

spectrum of 7 showed signals in the aromatic region at 114.8–116 ppm, corresponding to procyanidins and at 108 ppm corresponding to C-2' and C-6' of prodelphinidins.

The chemical shifts of C-2 and C-3 indicated that the configuration is 2*R*, 3*S*, 4*S* in the two units [3, 7]. These data indicated structure 7, the same as the GC-(4 α ->8)-C previously isolated from *Salix caprea* [8], *Quercus dentata* [4], *Thuja occidentalis* [9], and recently from *Cistus incanus* [5]. The IR and ^{13}C NMR spectral data were identical with those previously reported [4].

Compound 8 was eluted on Sephadex LH 20 after 5 and 6. It has the same molecular weight (610) as the known major dimers 5 and 6 [2]. Treatment with ethanolic 0.1 M hydrochloric acid liberated gallic acid. The IR spectrum indicated two pyrogallol rings: two distinct bands at about 1520 and 1535 cm^{-1} and a single band near 730 cm^{-1} , thus different from the spectra of procyanidin models [10]. Examination of the aromatic region of the ^{13}C NMR spectrum revealed signals at 108 ppm, corresponding to C-2' and C-6' of two pyrogallol rings (B and E), and at 146 ppm, corresponding to C-5' and C-3' of the same rings. The chemical shifts of C-2 and C-3 indicated the configuration 2*R*, 3*S*, 4*S*. Accordingly, compound 8 is gallic acid-(4 α ->6)-gallic acid, previously isolated from *Cistus incanus* [5].

The mass spectral data and the results of the hydrolysis experiments for the trimer 9 indicated a mixed B-ring oxidation pattern and suggested possible identity with a proanthocyanidin recently found in *Croton lechleri* [3]. Moreover, the ^{13}C NMR spectral data were superimposable. Hence, the compound is probably C-(4 α ->8)-GC-(4 α ->8)-GC 9.

Thin-layer chromatography

Examination of the plates 5 min after spraying with vanillin–hydrochloric acid reagent showed that flavan-3-ols (monomers and oligomers) afforded a red colour, which allows the proanthocyanidins and flavonoids present in the leaves of *R. nigrum* to be distinguished; however, the next day, re-examination of the plate allowed some of the stereoisomers to be differentiated. Prodelphinidins comprising gallic acid unit(s) only (3, 5, 8, 10) changed to violet at room temperature, while those containing epigallocatechin unit(s) (4, 6) became brown.

Evaluation of the R_f values revealed a characteristic TLC fingerprint for the *R. nigrum* proanthocyanidins. Four points may be noted:

(1) R_f values decrease with increasing degree of polymerization (R_f 0.75–0.9 for monomers; 0.4–0.6 for dimers; and 0.15–0.3 for trimers). This order is the same on silica gel, with dibutyl ether–diethyl ether–isobutanol–acetic acid (5:2:2:1, v/v/v/v) [11]; nevertheless, this solvent system does not provide a good separation between the dimers and trimers. On cellulose, the R_f values do not depend on the average molecular weight.

(2) Procyanidins are eluted before prodelphinidins of equivalent constitution.

(3) Dimers with a (4 α ->8) inter-flavan linkage are eluted after their (4 α ->6) isomers; this elution order is the reverse of that described earlier for Sephadex LH 20.

(4) R_f values of C-3 diastereoisomers are too close to allow quantitative densitometric determination of each constituent. The HPTLC method, however, can be used for the quantitative determination of some of the major oligomeric prodelphinidins. These observations corroborate and complete the previous studies on the chromatographic behaviour of flavan-3-ols [12].

In summary, the main oligomeric prodelphinidins of *R. nigrum* can be identified by HPTLC; this method could also be utilized as a proanthocyanidin screen for determining their

occurrence in other plants and for quantification of the major dimers. We are currently investigating the development of an appropriate densitometric method.

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