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Note

High-performance liquid chromatographic analysis of secoiridoid and flavone glycosides in closely related *Gentiana* species

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Species of the genus *Gentiana* (Gentianaceae) are characterized by the presence of bitter glycosides of the secoiridoid type. Recently, we reported on analytical high-performance liquid chromatography (HPLC) and preparative-scale separation of these glycosides by medium-pressure liquid chromatography[1]. Polyphenolics, such as flavonoids and xanthones, are also considered to be important chemotaxonomic markers in the family Gentianaceae. Thus, a phytochemical investigation of *Gentiana* species should include at least these two classes of compounds.

The use of a photodiode array detector in HPLC represents an important advance in peak identification. However, the UV spectra of flavonoids or xanthones of related structures are often very similar. In such cases, the characterization of separated polyphenolics can be improved when reagents inducing a shift of the absorption maxima are added to the eluate by means of a post-column derivatization system[2]. In the present paper, we show the advantages of reversed-phase HPLC with photodiode array detection and post-column derivatization for the chemotaxonomical investigation of gentians. Two closely related Himalayan species of the section Frigida, *Gentiana farreri* and *G. sino-ornata* were studied and compared with their hybrid, *Gentiana "Macaulayi"*. The three species are among the most beautiful gentians and are often cultivated in botanical gardens[3].

EXPERIMENTAL

Plant material and extracts

Gentiana farreri BALF., *G. sino-ornata* BALF. and *G. "Macaulayi"* (= *G. sino-ornata* × *G. farreri*) were culvitated at the Alpine Botanical Garden, Champex VS, Switzerland, by E. Anchisi. The dried plant material (leaves and stems) was extracted with solvents of increasing polarities: light petroleum (b.p. 80–95°C), chloroform and methanol. The secoiridoid and the flavonoid fractions for HPLC analyses were obtained by column chromatography of the crude methanolic extract on Polyamide SC 6 (Macherey Nagel, Düren, F.R.G.) with 20 and 90% aqueous methanol, respectively.

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HPLC

HPLC was carried out on a column packed with Hypersil RP-8, 5 μm (10 cm × 4.6 mm I.D., Hewlett-Packard), with methanol–water as mobile phase. To avoid tailing of phenolic compounds, phosphoric acid was added to the solvents (0.3 ml/l). The mobile phase was delivered by a SP 8700/SP 8750 pump (Spectra Physics, San José, CA, U.S.A.). The chromatograms and the UV–VIS spectra were recorded with a photodiode array detector HP 1040A, coupled with an HP-85 personal computer (Hewlet-Packard). Details of the post-column derivatization are given in ref. 2.

Isolation

The methanolic extract of *Gentiana "Macaulayi"* (4 g) was separated by column chromatography on Polyamide SC 6 (particle size ⩽ 70 μm; 46 cm × 25 mm I.D.) with aqueous methanol. The fractions eluted with 40 and 60% methanol were

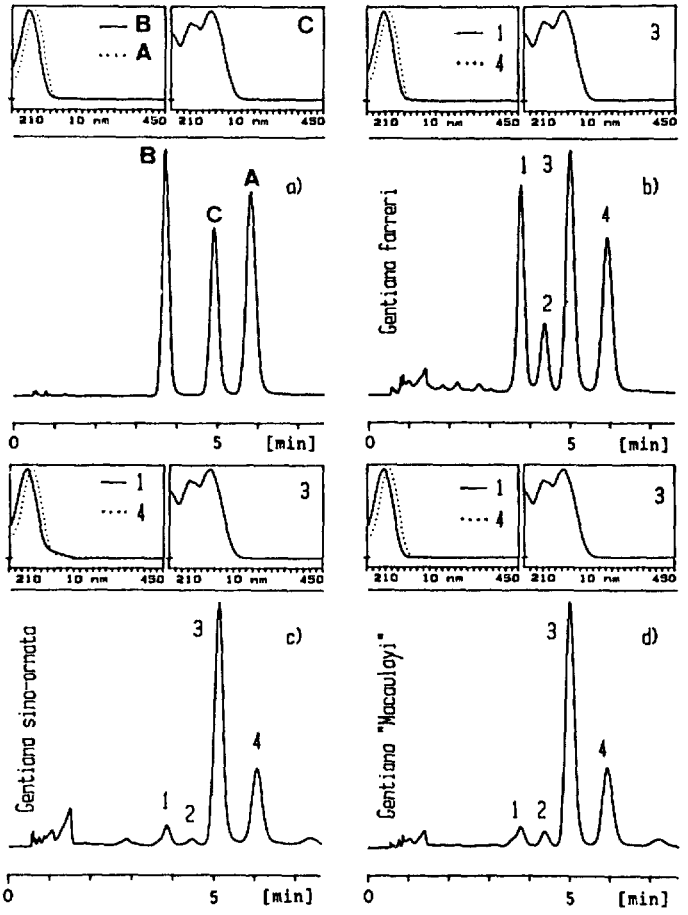


Fig. 1. RP-HPLC separation with photodiode array detection of secoiridoid glycosides: authentic samples (a) and from gentians (b–d). Column: Hypersil RP-8, 5 μm, 10 cm × 4.6 mm I.D. Eluent: 20% aqueous methanol; flow-rate 1.5 ml/min. Detection: 240 nm, UV spectra from 210 to 450 nm.

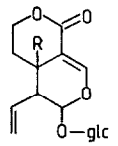
purified over Sephadex LH-20 (Pharmacia) (methanol as eluent) and yielded 20 mg of flavone E and 36 mg of flavone F, respectively.

RESULTS AND DISCUSSION

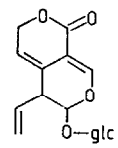
Fig. 1 shows the RP-HPLC separations with photodiode array detection (LC–UV) of secoiridoids from *Gentiana farreri*, *G. sino-ornata* and from their hybrid, *G. "Macaulayi"*. The glycosides 1–4 were separated in less than 10 min and compared with authentic samples (retention times, UV spectra), analysed under the same conditions. The constituents 1 (swertiamarin, B), 3 (gentiopicrin, C) and 4 (sweroside, A) are present in all three species, but at different concentrations. The chromatograms of *G. sino-ornata* and *G. "Macaulayi"* are similar, but clearly different from that of *G. farreri*. Strongly bitter secoiridoids, such as esters of hydroxybenzoic or diphenylcarbonic acids, were not detected. Peak 2 corresponds to a glycoside which has not yet been identified.

The flavonoid fractions of the above mentioned gentians were separated on RP-8, using a methanol–water gradient. The resulting chromatograms and the spectra recorded on-line are represented in Fig. 2. Seven flavonoids could be detected, showing three types of spectra. The glycosides 1–3 have classical flavone spectra with two well distinguished absorption maxima near 270 nm (band II) and 330 nm (band I). The spectra of compounds 4 and 5 show a split band II of lower intensity than that of band I. The spectra of the glycosides 6 and 7 are characterized by the band I at 320 nm and by an additional shoulder at 290 nm. These spectra are similar to those observed with C-glycosylflavones esterified with cinnamic acids[4,5].

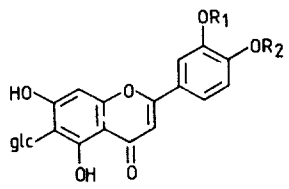
The comparison of the chromatograms indicates a similar distribution of flavonoids for *G. farreri* and *G. "Macaulayi"*. *G. sino-ornata* presents a different flavonoid pattern which is characterized by compound 7 and by the absence of glycoside



A sweroside R = H
B swertiamarin R = OH



C gentiopicrin



D isoorientin $R_1 = R_2 = H$
E isoorientin -4'-O-glucoside $R_1 = H$, $R_2 = glc$
F isoscoparin $R_1 = CH_3$, $R_2 = H$

5. The HPLC separations were repeated and shift reagents added to the eluent as previously described[2]. Fig. 3 shows the UV spectra of compound 4 from *G. farreri*, *G. sino-ornata* and *G. "Macaulayi"*. Similar shifts of the absorption can be observed after addition of the shift reagents (weak base, strong base, aluminium chloride). Glycoside 4 was identified as isoorientin (D) by comparison with an authentic sample[2]. The main constituents (compounds 1 and 5) were isolated from the methanolic extract of the hybrid *G. "Macaulayi"* by column chromatography on polyamide (methanol–water) and Sephadex LH-20 (methanol). Flavones 1 and 5 were identified by comparison with authentic samples and published data (UV, ^{1}H, ^{13}C NMR, hydrolyses) as isoorientin-4'-O-glucoside (E) and isoscoparin (F), respectively[7–10]. The scarce amount of plant material available did not allow the isolation of the constituents from *G. farreri* and *G. sino-ornata*. The identification of flavone 1 in the two parent gentians and of 5 in *G. farreri* was possible by comparison of their retention times and their UV spectra with those obtained from *G. "Macaulayi"*.

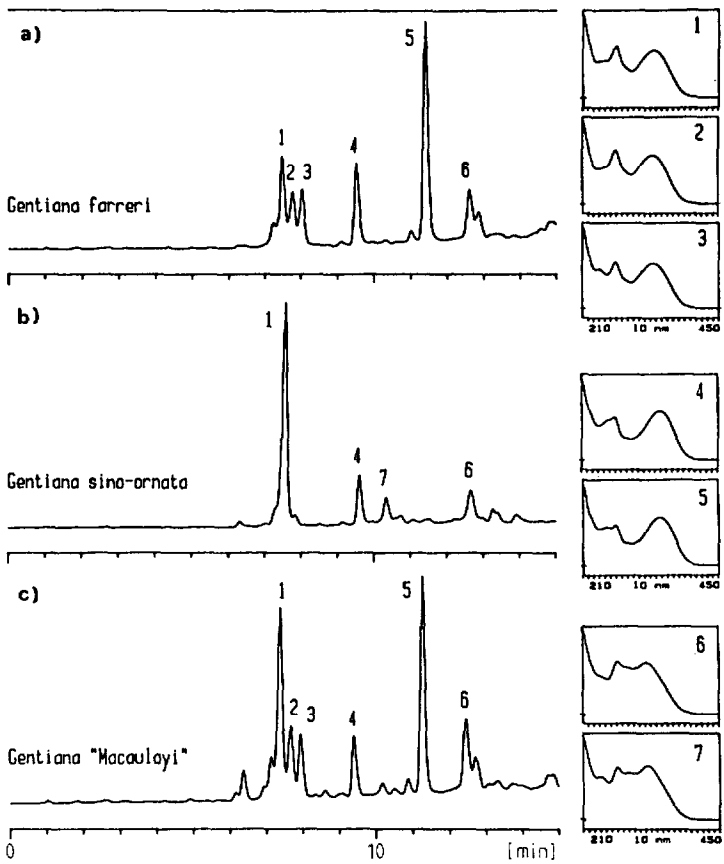


Fig. 2. RP-HPLC separation with photodiode array detection of flavone glycosides from gentians. Column as in Fig. 1. Eluent: (acidified) aqueous methanol, 20 to 50% in 15 min; flow-rate 1.5 ml/min. Detection: 254 nm, UV spectra from 210 to 450 nm.

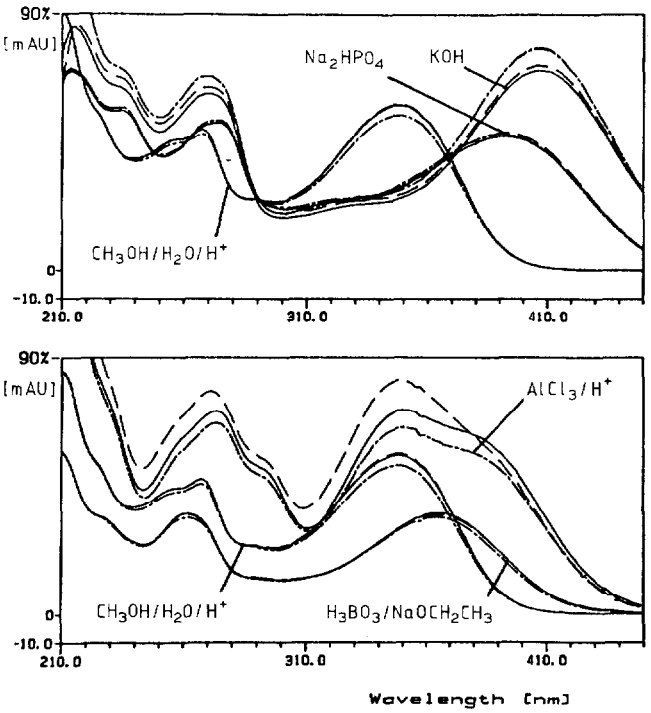


Fig. 3. On-line UV spectra of flavone 4 (isoorientin), in pure eluent and with shift reagents added by a post-column derivatization system. ——, *Gentiana farreri*; ---, *G. sino-ornata* ·–·, *G. "Macaulayi"*.

CONCLUSION

Gentians of the section Frigida are characterized by the C-glycosylflavones isoorientin (D) and the corresponding 4'-O-glucoside[11,12]. This has been shown in the present work. Isoscoparin is a less common glycoside and typical of species from the section Crossopetalum[13]. We have now detected this flavone for the first time in gentians of the section Frigida. The absence of xanthones is another characteristic of this section and has been confirmed in this study. The investigation of the hybrid has shown that its flavonoid pattern is similar to that of *G. farreri*, whereas its secoiridoid content resembles more that of *G. sino-ornata*.

High-performance liquid chromatography, coupled with a photodiode array detector, is a rapid method and of great interest for chemotaxonomical studies. The study of *Gentiana farreri*, *G. sino-ornata* and of their hybrid, *G. "Macaulayi"*, has shown that important conclusions can be drawn, even from small amounts of plant material. The peak identification and the characterization of separated polyphenolics can be improved when shift reagents are added to the eluent through a post-column derivatization system.

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