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ULTRAVIOLET–VISIBLE SPECTROSCOPY OF PHENOLIC COMPOUNDS IN PLANT EXTRACTS USING POST-COLUMN DERIVATIZATION

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SUMMARY

High-performance liquid chromatography coupled with ultraviolet–visible spectroscopy, using a photodiode array detector, was applied to the investigation of plant extracts for polyphenolic compounds. Simultaneous detection at different wavelengths and measurement of the UV spectrum of each separated compound during the elution allow an easy and rapid identification of flavonoids and xanthones. However, as some of these compounds have closely related structures, a characterization by their UV spectra is insufficient. Derivatization with reagents inducing a shift of the UV absorption maxima is required and furnishes additional structural information. In the present work, shift reagents were added to the eluate using a post-column derivatization system. The reagents were adapted in order to be compatible with the solvent system used for the chromatographic separation. The technique was applied to the analysis of crude extracts of various *Gentiana* species.

INTRODUCTION

The recent availability of a photodiode array detector for high-performance liquid chromatography (HPLC) enables a marked improvement in the possibilities of peak identification[1]. It provides a simultaneous record of chromatograms obtained at different wavelengths, and the measurement of the UV spectrum of each eluted compound make it a method of choice for the analysis of complex mixtures. This technique is of special interest for the screening of plant extracts for biologicallyactive compounds and for chemotaxonomical studies.

The present work describes some applications of the method to crude methanolic extracts of species of the genus *Gentiana* (Gentianaceae) in order to detect the presence of flavonoid and xanthone glycosides. Compounds of these two classes can easily be distinguished by their characteristic UV spectra[2]. An identification of the peaks of a chromatogram is possible by comparing their retention times and UV spectra with those of authentic samples. Using the storage facilities of a data bank,

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an extensive range of investigations is possible. However, for compounds having closely related structures, this technique might not be sufficient for an unambiguous identification.

In order to complete the characterization of phenolic compounds, reagents inducing a shift of the absorption maxima can be used. The application of such reagents to flavonoids and xanthones has been extensively described[3,4]. A weak base (sodium acetate) deprotonates only the more acidic phenolic groups, while a strong base (sodium methanolate) reacts with all phenolic groups, except those forming a hydrogen bond with the keto function of the flavonoid or xanthone skeleton. Aluminium chloride in neutral solution forms complexes with *ortho*-dihydroxyl groups and/or with keto functions having a hydroxyl group in position α or peri. The former complexes are unstable when HCl is added. *ortho*-Dihydroxyl groups also form a chelate complex with boric acid. For flavonoids and xanthones, all these reactions are in general carried out in methanol. They provide useful information about the type of flavonoid or xanthone as well as the oxidation pattern and the location of free hydroxyl groups. In the present work, reagents were added to the eluate using a post-column derivatization system. Therefore, the UV spectra showed characteristic shifts of the absorption maxima. As the separations were achieved under acidic aqueous conditions, the shift reagents cited above required some adaptations.

EXPERIMENTAL

Apparatus

The solvent delivery system comprised two M-6000 pumps, a M-720 gradient controller and a U6K injector (Waters). The photodiode array detector HP-1040A (Hewlett-Packard) coupled with an HP-85 personal computer (Hewlett-Packard) was used for recording chromatograms and UV–Vis spectra. For post-column derivatization, an Eldex Model A-30-5-2 (Eldex Labs., Menlo Park, CA, U.S.A.) consisting of a pump and a reaction coil was employed. Shift reagents were added to the eluent at a flow-rate of 0.3 ml/min.

HPLC conditions

Separations were performed on a LiChrosorb RP-8 column, 7 μm (25 cm × 4.6 mm I.D.) (Knauer), equipped with a 4-cm precolumn, or on a Hypersyl RP-8 column, 5 μm (10 cm × 4.6 mm I.D.) (Hewlett-Packard). Gradients of methanol–water were used. To avoid the tailing of phenolic compounds, sulphuric acid was added to the solvents in order to adjust the pH to about 3.5 (0.3 ml of concentrated acid per litre of solvent).

Shift reagents

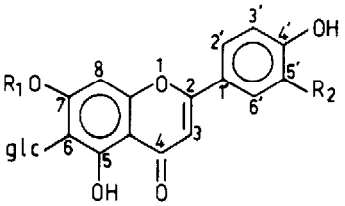
For comparison purposes, the classical shift reagents were prepared according to standard procedures[3]. The reagents used in the post-column derivatization system were as follows: weak base, sodium monohydrogenphosphate, 0.5 *M* solution in water; strong base, potassium hydroxide, 0.3 *M* solution in water; aluminium chloride, 0.3 *M* solution in water (with this reagent, the reaction coil was heated to 60°C); boric acid, a methanol–water (1:1) solution containing boric acid (0.7 *M*) and sodium acetate (0.1 *M*).

Samples

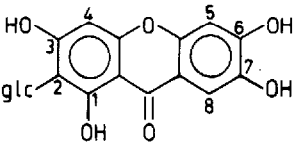
The dried plant material was extracted with solvents of increasing polarities: light petroleum (b.p. 80–95°C), chloroform and methanol. The following plants of the Gentianaceae family were used in the present investigation: *Gentiana verna,* collected in Switzerland (aerial parts); *Gentiana lactea,* collected in Chile (aerial parts) and *Gentiana ramosa,* collected in Switzerland (leaves and roots). Solutions to be analyzed were prepared by dissolving 5 mg of a methanolic extract in 1 ml of methanol. The injected volumes were 10–20 μl. A fraction, obtained from the crude methanolic extract of *Gentiana lactea* after purification by column chromatography on polyamide S C 6 (Macherey, Nagel & Co.) with 70% aqueous methanol, was also subjected to HPLC analysis. All the authentic samples were obtained in the course of previous studies.

RESULTS

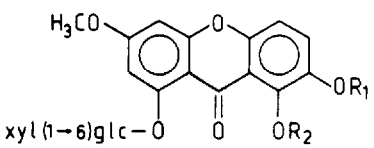
Reversed-phase chromatography on octadecylsilyl bonded columns with methanol–water containing acetic acid, phosphoric acid or formic acid has been successfully employed in many separations of polar flavonoid aglycones and flavonoid glycosides[5–8]. The same packing material used with different methanol–water mixtures as the solvent seems to be an ideal system for the separation of naturally occurring xanthone glycosides[9]. As extracts of *Gentiana* species usually contain flavonoid and xanthone glycosides of different polarities and oxidation patterns, a linear gradient elution was developed. The best results were obtained with methanol–water under acidic conditions (pH about 3.5) in order to avoid peak tailing. Thus, the classical shift reagents were adapted in order to be compatible with these conditions. Sodium monohydrogenphosphate and potassium hydroxide were used as the weak and strong



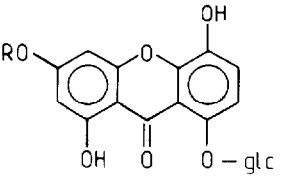
A $R_1 = H$, $R_2 = OH$ isoorientin
B $R_1 = R_2 = H$ isovitexin
C $R_1 = CH_3$, $R_2 = H$ swertisin



D Mangiferin



E $R_1 = R_2 = H$ swertianin-1-O-primeveroside
F $R_1 = H$, $R_2 = CH_3$ gentiacaulein-1-O-primeveroside
G $R_1 = R_2 = CH_3$ decussatin-1-O-primeveroside



H $R = CH_3$ bellidifolin-8-O-glucoside
I $R = H$ desmethylbellidifolin-8-O-glucoside

bases, respectively, instead of sodium acetate and sodium methanolate. Aluminium chloride formed a complex only involving the keto function. Furthermore, this reaction did not go to completion when the eluate flowed through the cell. The reaction rate was increased by heating the reaction coil to 60°C, The presence of *ortho*-dihydroxyl groups was evidenced with boric acid–sodium acetate.

The UV spectra of the flavone-C-glycoside isoorientin (A) recorded in the HPLC solvent with and without shift reagents are shown in Fig. 1. The absorption maxima and the observed shifts are listed in Table I and are compared with the values obtained using the classical reagents. It appears that all the values are similar. Only a small difference in the shape of band I was noted when aluminium chloride was used. This example clearly demonstrates that the modified shift reagents can be employed for post-column derivatization in the analysis of plant phenolics.

Fig. 2 shows the separation of a crude extract of *Gentiana verna*. Peaks 1–5 could be identified by comparison of their retention times and UV spectra with those of authentic samples obtained from a previous investigation, as mangiferin (D), isoorientin (A), swertianin-1-O-primeveroside (E), gentiacaulein-1-O-primeveroside (F) and decussatin-1-O-primeveroside (G), respectively. In order to ascertain the struc-

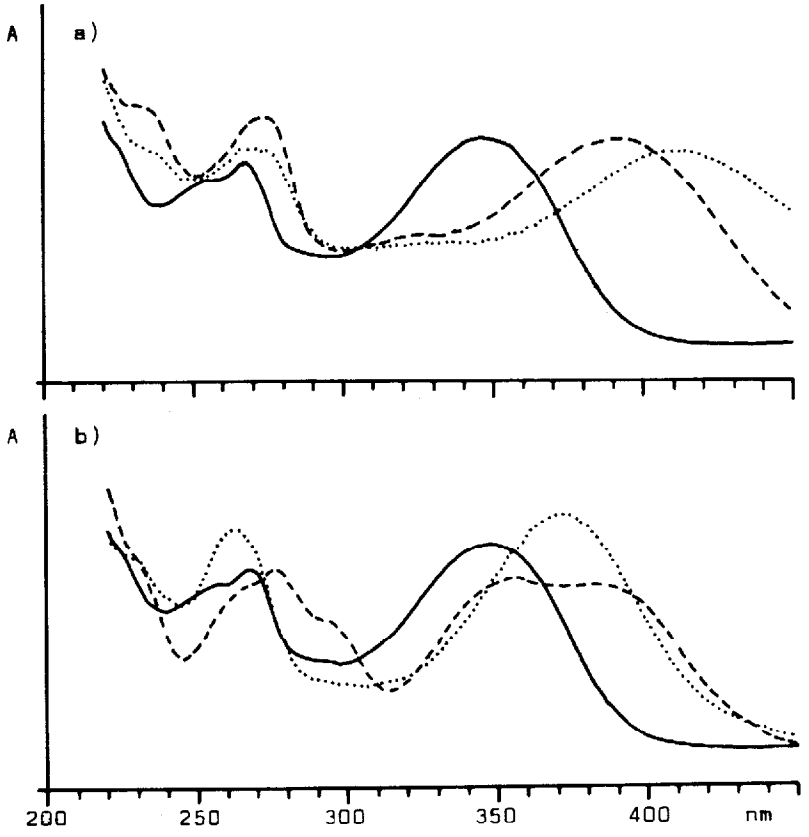


Fig. 1. UV spectra of isoorientin (A). a, In pure eluent (——), Na_2HPO_4 (– – –) and KOH (. . . . .); b, in pure eluent (——), $AlCl_3$ (– – –) and H_3BO_3/NaO_2CCH_3 (. . . . .).

TABLE I

ABSORPTION MAXIMA OF ISOORIENTIN (A) INDUCED BY SHIFT REAGENTS COMPATIBLE WITH HPLC AND BY CLASSICAL REAGENTS

	LC conditions			*Classical shift reagents*[3]		
	Reagent	λ_{max} *(nm)*	*(shift)*	*Reagent*	λ_{max} *(nm)*	*(shift)*
Pure solvent	Methanol–water	267		Methanol	271	
	pH 3.5	350			349	
Weak base	Na_2HPO_4	272	(+ 5)	CH_3COONa	276	(+ 5)
	0.5 *M*	390	(+40)		393	(+44)
Strong base	KOH	267	(+ 0)	CH_3ONa	267	(− 4)
	0.3 *M*	413	(+63)		406	(+57)
Aluminium	$AlCl_3$	275	(+ 8)	$AlCl_3$–HCl	279	(+ 8)
chloride	0.3 *M*	358	(+ 8)		361	(+12)
		384	(+34)		384	(+35)
Boric acid	H_3BO_3/CH_3COONa	262	(− 5)	H_3BO_3/CH_3COONa	265	(− 6)
	0.7 *M*/0.1 *M*	373	(+23)		377	(+26)

tures, the separation was repeated and a shift reagent added to the eluate by means of the post-column derivatization kit. The UV data of the identified flavonoid and xanthone glycosides are given in Table II. It is noteworthy that the UV spectra of 4 (= F) and 5 (= G), typical of 1,3,7,8-substituted xanthones, are very similar, but differ greatly when a strong base is added as illustrated in Fig. 3a. The absorption maxima of decussatin-1-O-primeveroside (G), devoid of any free hydroxyl groups, are not shifted, whereas for gentiacaulein-1-O-primeveroside (F), bathochromic shifts, due to the presence of a hydroxyl group at position 7, are observed.

The authentic samples A (isoorientin), C (swertisin), D (mangiferin), H (bel-

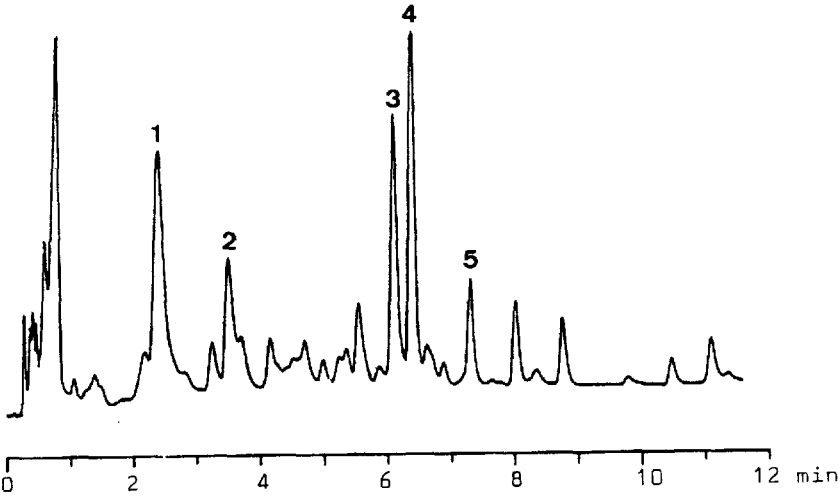


Fig. 2. HPLC separation of a crude methanolic extract of *Gentiana verna*. Column: Hypersyl RP-8. Eluent: methanol–water, gradient from 20% to 80% methanol in 15 min. Flow-rate: 2 ml/min. Detection: 240 nm.

TABLE II

ABSORPTION MAXIMA (nm) OF FLAVONOID AND XANTHONE GLYCOSIDES FROM *GENTIANA VERNA* (SEE CHROMATOGRAM IN FIG. 2)

sh = Shoulder.

Peak	*Compound*	*Pure solvent*	*Weak base*	*Strong base*	*Aluminium chloride*	*Boric acid*
1	Mangiferin (D)	238	239	238	232	232
		256	267	275	265	261
		315	300	350	345	332
		363	387	404	410	372
2	Isoorientin (A)	267	272	267	275	262
		350	390	413	358/384	373
3	Swertianin-1-O-	238	238	243	243	243
	primeveroside (E)	266	266	282	278	278
		313	313	310 sh	335	307
		385	385	≈430	440	392
4	Gentiacaulein-1-O-	240	240	247	240	240
	primeveroside (F)	252	252	272	252	252
		304	304	300 sh	304	304
		362	362	405	362	362
5	Decussatin-1-O-	242	242	242	242	242
	primeveroside (G)	250	250	250	250	250
		302	302	302	302	302
		360	360	360	360	360

lidifolin-8-O-glucoside) and I (desmethylbellidifolin-8-O-glucoside) could easily be separated within 17 min as shown in Fig. 4a. The separation of the crude extract of *Gentiana lactea* was achieved under the same conditions (see Fig. 4b). The values of the absorption maxima for each separated compound are given in Table III. Retention times and UV spectra of the substances corresponding to peaks 1–3 and 5, when compared with the data from the authentic samples, allow the identification of mangiferin (D), isoorientin (A), desmethylbellidifolin-8-O-glucoside (I) and bellidifolin-8-O-glucoside (H) in the crude extract. The glycosides H and I have similar UV spectra, but they can be distinguished from each other by addition of a base which produces an important bathochromic shift in the spectrum of I as shown in Fig. 3b. The occurrence of 1,3,5,8-substituted xanthones in *Gentiana lactea* is of great chemotaxonomical interest since this species belongs to the sub-genus *Gentianella* which seems to be characterized by this type of xanthones[10].

In order to detect minor constituents in the extract of *Gentiana lactea,* the major glycoside I (corresponding to peak 3 in Fig. 4a) was removed by polyamide column chromatography with methanol–water. In the purified fraction a previously undetected compound appears in peak 3′ (Fig. 4c). The UV spectrum of this constituent is typical of a 1,3,7,8-substituted xanthone (see Table III). The structure of this xanthone has not yet been determined. Peak 4 is also of interest as the UV spectrum of the compound corresponding to this peak is very similar to that of swertisin (C). The retention times of peak 4 and swertisin are also similar. However, when a weak base was added to the eluate, a bathochromic shift of band II could be observed, whereas this band is not shifted in swertisin (Fig. 5). Without the use of

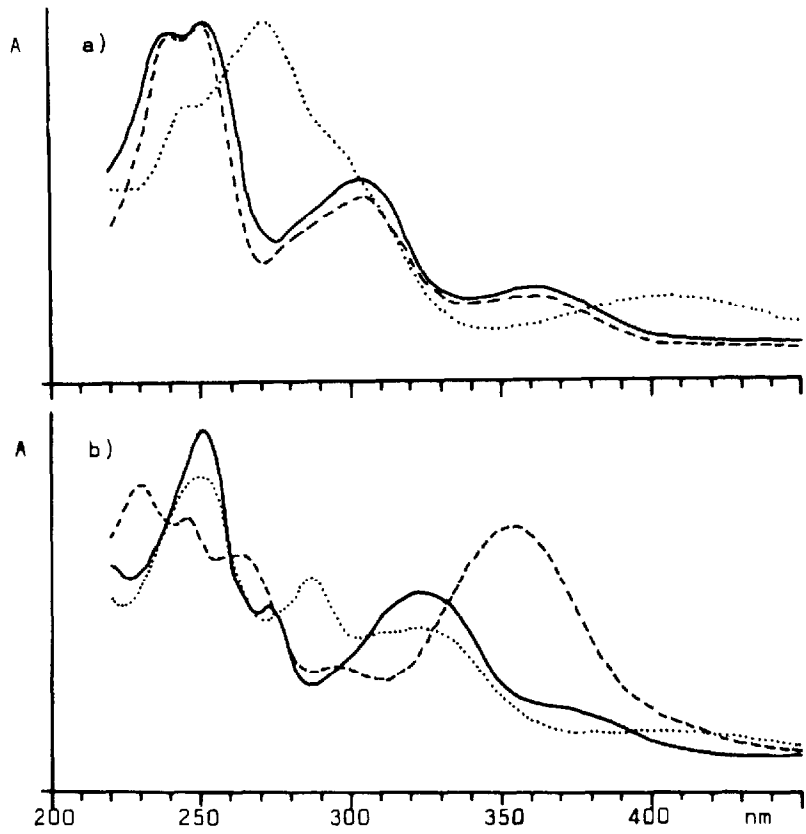


Fig. 3. UV spectra of xanthone glycosides. a, 1,3,7,8-substitution pattern: gentiacaulein-1-O-primeveroside (F) in pure eluent (——) and KOH (. . . . .); decussatin-1-O-primeveroside (G) in pure eluent and KOH (- - - -). b, 1,3,5,8-substitution pattern: bellidifolin-8-O-glucoside (H) and desmethylbellidifolin-8-O-glucoside (I) in pure eluent (——); with addition of Na_2HPO_4, compound H (. . . . .) and I (- - - -).

shift reagents, a wrong conclusion would have been drawn since the unknown flavonoid glycoside 4 possesses a free hydroxyl group at position 7. It was finally identified as isovitexin (B)[11].

The previously investigated *Gentiana ramosa* was subjected to HPLC–UV analysis. Fig. 6a shows the separation of the methanolic leaf extract. The main peaks 2 and 3 could easily be identified as desmethylbellidifolin-8-O-glucoside (I) and bellidifolin-8-O-glucoside (H), respectively by comparison with the retention times and the UV spectra of authentic samples. The minor peaks 1 and 3 correspond to the flavone-C-glycosides isoorientin (A) and swertisin (C), respectively whereas the other constituents were not identified. A root extract of this gentian was also analysed (Fig. 6b). The major xanthone glycosides I and H corresponding to peaks 2 and 4 occur in the underground organs of *Gentiana ramosa*. However, no flavonoid could be detected as all the other peaks corresponded to unidentified xanthones or to non-phenolic constituents.

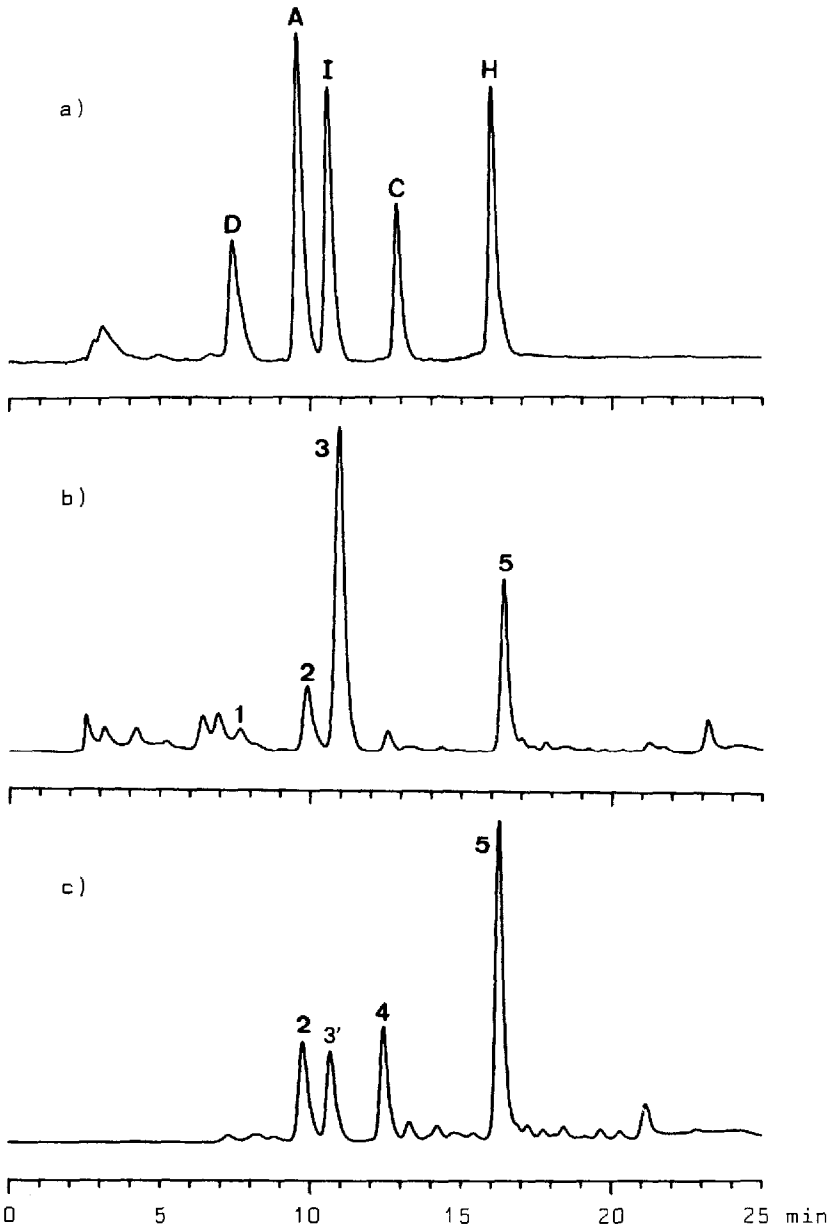


Fig. 4. HPLC separations of authentic samples and extracts of *Gentiana lactea*: a, mangiferin (D), isoorientin (A), desmethylbellidifolin-8-O-glucoside (I), swertisin (C) and bellidifolin-8-O-glucoside (H); b, crude methanolic extract; c, purified fraction. Column: LiChrosorb RP-8. Eluent: methanol–water, gradient from 30% to 80% methanol in 25 min. Flow-rate: 1.5 ml/min. Detection: 254 nm.

CONCLUSIONS

HPLC coupled with a photodiode array detector is a suitable method for the screening of polyphenolic compounds in plant extracts or other complex mixtures of

TABLE III

ABSORPTION MAXIMA (nm) OF FLAVONOID AND XANTHONE GLYCOSIDES FROM *GENTIANA LACTEA*

For chromatograms, see Fig. 4; values for mangiferin (D) (peak 1) and isoorientin (A) (peak 2) are identical with those listed in Table II. sh = shoulder.

Peak	*Compound*	*Pure solvent*	*Weak base*	*Strong base*	*Aluminium chloride*	*Boric acid*
3	Desmethylbellidifolin-8-O-glucoside (I)	250	230	230	264	
		272	246	258	277 sh	Not measured
		325	262	294	323 sh	
		375	355	356	353	
3′	Unidentified xanthone	238	235	245	232	238
		261	269	275	273	261
		312	345	345	323	312
		375	387 sh	416	408	375
4	Isovitexin (B)	268	276	277	278/305	270
		336	384	398	339/379	336
5	Bellidifolin-8-O-glucoside (H)	250	251	257	266	250
		274	287	280	278	274
		326	325	349	356	326
		373	≈415	398	–	373

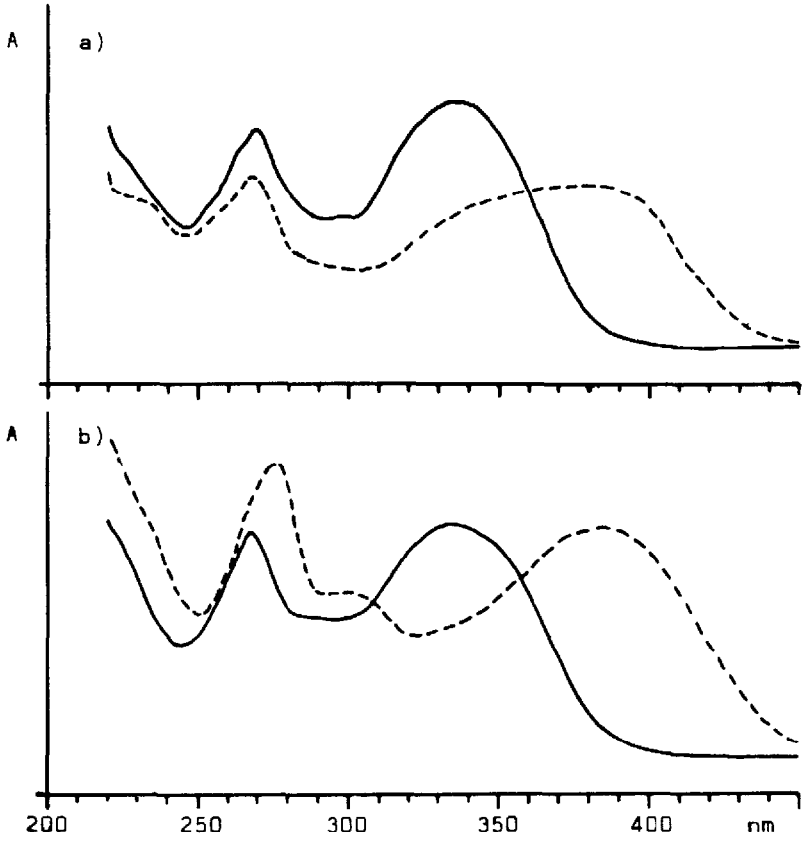


Fig. 5. UV spectra of (a) swertisin (C) and (b) the flavonoid identified as isovitexin (B) in *G. lactea* (peak 4 in Fig. 4c) in pure eluent (——) and Na_2HPO_4 (– – –).

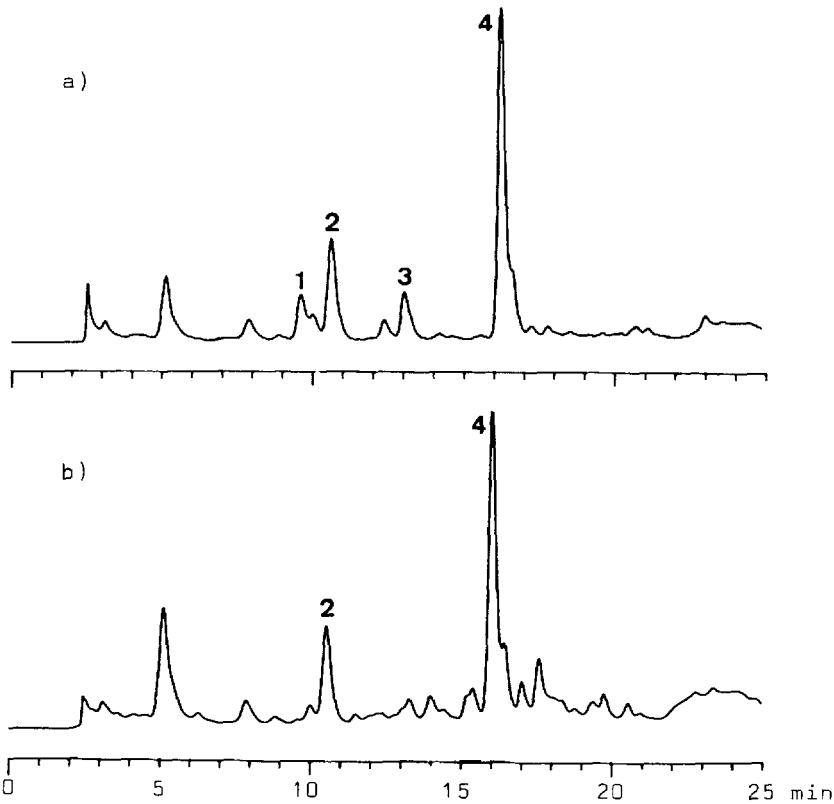


Fig. 6. HPLC separations of crude methanolic extracts of *Gentiana ramosa*: leaves (a) and roots (b). Conditions as in Fig. 4.

biological origin. The possibility of comparing retention times and the UV spectra of compounds with those of authentic samples stored in a data bank allows a fast and easy identification. As exemplified by the analysis of the extract of *Gentiana lactea*, wrong conclusions can be drawn when an identification is limited to the retention time and UV spectrum. When the UV spectra are similar, the addition to the eluate of specific reagents producing characteristic shifts of the absorption maxima can be used as a means of distinguishing compounds. This simple method using a post-column derivatization device greatly improves the reliability of peak identification. All shift reagents employed in the present work gave identical results to those obtained with the classical shift reagents. On-line HPLC-UV–Vis spectroscopy appears to be a very powerful method for chemotaxonomical studies. The small amount of sample needed (50–100 μg of crude plant extract) allows the analysis of very rare and small species as well as single plant parts of herbarium vauchers. Phenolic constituents such as flavonoids or xanthones which have closely related structures and similar chromatographic behaviour can easily be distinguished from each other in the course of the elution. The addition of shift reagents to the eluate furnishes additional structural information such as the oxidation pattern and the position of free phenolic hydroxyl groups. However, the method has some limitations, especially

if no authentic samples are available for comparison. In such a case it should be combined with liquid chromatography–mass spectrometry (LC–MS)[12]. In general, LC–MS would be an ideal complementary technique for this kind of phytochemical investigation.

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