Journal of Chromatography, 442 (1988) 175–186
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 412

COMPARATIVE HIGH-PERFORMANCE LIQUID AND GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF PHENOLIC GLUCOSIDES IN SALICACEAE SPECIES

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(Received February 11th, 1988)

SUMMARY

The phenolic glucosides of seven willow species with different glucoside patterns were extracted, purified and analysed by gas–liquid (GLC) and high-performance liquid chromatography (HPLC). Two sample preparation methods were used. It was shown that the HPLC and GLC methods give comparable qualitative and quantitative results for the phenolic glucoside contents of the tested willows. Consequently, both methods can be used for species-specific screening of the glucoside patterns of Salicaceae species.

INTRODUCTION

In recent years, the phenolic glucosides of Salicaceae species have been the subject of increasing interest in plant–animal interaction ecology[1,2], in identifying willow species[3–5] and in the pharmaceutical use of willow bark and poplar leaves for phytotherapy[6,7].

In the 1960s, the phenolic glucosides of several willow species were thoroughly investigated by Thieme (*e.g.*, refs. 3 and 8) and Pearl and Darling (*e.g.*, ref. 9). Thieme isolated most of the willow glucosides currently known and used them in the quantitative analysis of central European Salicaeae species. Glucosides were fractionated by Thieme on a polyamide column, separated by paper chromatography and quantified by spectrophotometry[10]. In spite of the different analytical procedures used, his results[3,11,12] are comparable to those obtained in later investigations with using gas-liquid (GLC) and high-performance liquid chromatography (HPLC)[5,13,14].

The development of instrumental column chromatography during the last decade has made it possible to continue Thieme's work using more efficient methods. Julkunen-Tiitto[15] developed a GLC method in which silylated phenolic glucosides

0021-9673/88/$03.50

can be detected after extraction with 80% acetone followed by ethyl acetate liquid–liquid extraction and purification on a small polyamide column. Egloff[4] used reversed-phase gradient HPLC to separate the lower phenolic glucosides and flavonoids after extraction with methanol and sample purification on C_{18} cartridges. This method has been optimized for the investigation of *Salix daphnoides* and *S. purpurea* species (both rich in phenolic glucosides and flavonoids), and for the general screening of salicylates in willow and poplar species with the HPLC–UV–VIS coupling technique[7,14].

The number of willow species is very high, with more than 50 species in Europe, but only a small number of them have so far been screened for phenolic glucosides. The detailed analyses of the glucoside patterns of different willow species requires considerable analytical work. Moreover, the methods used should be comparable to each other. In this study, the phenolic glucosides of bark, whole twigs and leaves of seven willow species native to Finland were extracted and analysed by HPLC and GLC methods and the results were compared. The species used in this work have previously been only partly investigated for all phenolic glucosides.

EXPERIMENTAL

Materials

The bark (*S. phylicifolia* L., *S. myrsinifolia* Salisb., *S. pentandra* L.) and budleaf (*S. pentandra* L.) samples used were obtained from a winter dormant individual (female) in January 1987. The whole twig and leaf samples (*S. lanata* L., *S. hastata* L., *S. myrsinites* L., *S. rosmarinifolia* L.) were collected in August 1986, also from one individual (female). The current year growth twigs (without buds) were used for the bark and twig samples only. Composite twig and bark samples were clipped into small pieces and immediately oven-dried at 48°C for 8 h. The leaves were dried intact. After drying, the samples were milled to dust and stored in glass containers in a desiccator at −20°C.

Equipment and chromatographic conditions

A Waters Assoc. HPLC system consisting of two M-6000 solvent delivery systems, a Waters system controller (Model 720), a WISP-710B automatic sample injector and a Hewlett-Packard 1040A high-speed spectrophotometer equipped with DPU software were used. The separations were carried out on a 100 × 4 mm I.D. Knauer cartridge filled with Spherisorb ODS II (3 μm) particles as the stationary phase. The gradient used is described in Table I.

A Hewlett-Packard Model 5890 capillary gas chromatograph equipped with a flame ionization detector and Model 7673A auto-injector were used. An OV-1 fused-silica capillary column (25 m × 0.32 mm I.D.) with a phase layer of 0.25 μm was used throughout. The column temperature was programmed from 190 to 295°C at 8°C/min. The detector and injector temperatures were 300 and 230°C, respectively. Helium was used as the carrier gas and the splitting ratio was 1:14. All solvents were of analytical-reagent or HPLC grade.

Sample preparation

Method I. A 50–200-mg amount of dried material was extracted in a Soxhlet

TABLE I

CONDITIONS USED IN HPLC GRADIENT ELUTION

Flow-rate, 1 ml/min. Solvent A: 1.8% tetrahydrofuran + 0.5% orthophosphoric acid. Solvent B: 100% methanol.

Time (min)	*Solvent A*	*Solvent B*
Initial	100	0
5	100	0
10	85	15
20	70	30
30	65	35
40	50	50
45	50	50
Rinsing	0	100
Equilibration	100	0

extractor with 80% aqueous acetone, followed by liquid–liquid extraction with ethyl acetate. The concentrated extract was purified on a polyamide column. The freeze-dried extract was derivatized by trimethylsilylimidazole in pyridine[15].

Method II for HPLC. A 200–500-mg amount of dried material was extracted in a clipping homogenisator once with 25 ml and then with 40 ml of methanol. The residue was washed with 20 ml of methanol and the solvent was then evaporated using a vacuum evaporator. The temperature of the water-bath was not more than 40°C. The sample was resolved in 9 ml of methanol–water (7:2) and 1 ml of internal standard solution was added. A 3-ml volume of the extract was purified on bond Elut C_{18}-octadecyl (500 mg) solid-phase extraction columns. The injection volume was 10 or 5 μl.

Method II for GLC. A 20–150-mg amount of dried material was extracted twice with 25 ml of methanol as above and the residue was washed with 15 ml of methanol. The dried extract was resolved in 10 or 15 ml of methanol. A 5-ml volume of the extract was purified as above. A 200–500-μl volume of the extract was evaporated under nitrogen and derivatized as above.

Quantification

HPLC results were calculated using glucoside references and resorcinol as an internal standard. The standard mixture for HPLC contained 7.56 mg of salicin (puriss. grade, Carl Roth, Karlsruhe, F.R.G.), 5.98 mg of salicortin, 6.14 mg of tremulacin, 0.73 mg of picein, 0.59 mg of vimalin and 0.60 mg of triandrin (isolated and purified at ETH, Zürich) and 1 ml of internal standard solution (4.51 mg/ml resorcinol) in 10 ml of methanol. The injection volume was 10 μl. Fragilin, 2′-O-acetylsalicortin and salireposide were identified from the UV spectra and the elution order in previous work[4,14]. The content of salireposide was corrected using coefficients of 0.54 (at 220 nm) and 0.55 (at 270 nm) relative to salicin[14]. Owing to their instability in the reference solution and identical UV spectra, fragilin and 2′-O-acetylsalicortin were calculated after salicin and salicortin, respectively.

In GLC analysis, each component was identified by comparing its chromato-

TABLE II

PHENOLIC GLUCOSIDE CONTENT OF THE CURRENT GROWTH BARK OF SALICACEAE SPECIES

The results are means of three to five subsample analyses (% on a dry weight basis, S.E. in parentheses). GLC I and GLC II indicate methods and analysis by capillary GLC and HPLC indicates the method and analysis by HPLC.

Species	*Method*	*Salicin*	*Fragilin*	*Salicortin*	*Picein*	*Vimalin*	*Triandrin*	*Acetylsalicortin*	*Salireposide*
S. phylicifolia	GLC I	0.253 (0.015)	0	0.698 (0.058)	0.275 (0.023)	1.14 (0.02)	2.23 (0.09)	0	0.394 (0.079)
	GLC II	0.268 (0.062)	0	1.521* (0.040)	0.231 (0.019)	1.03 (0.04)	1.98 (0.07)	0	*
	HPLC	0.209 (0.006)	0	*	0.253 (0.012)	1.29 (0.03)	2.19 (0.06)	0	*
S. myrsinifolia	GLC I	0.320 (0.013)	0.379 (0.018)	1.87 (0.09)	0.432 (0.007)	0	0.374 (0.020)	0.585 (0.089)	0
	GLC II	0.322 (0.006)	0.336 (0.003)	2.38 (0.01)	0.451 (0.007)	0	0.338 (0.007)	0.889 (0.040)	0
	HPLC	0.293 (0.003)	0	1.71 (0.02)	0.449 (0.003)	0	0.338 (0.010)	*	0
S. pentandra	GLC I	0.082 (0.012)	2.59 (0.05)	0.124 (0.015)	0	0	0	5.59 (0.52)	0
	GLC II	0.069 (0.003)	1.88 (0.09)	0.104 (0.011)	0	0	0	6.12 (0.17)	0
	HPLC**	Trace	1.74	Trace	0	0	0	6.12	0

* Interference with other component.
** Results for one subsample analysis.

graphic retention time with that of the authentic component and most of the detected compounds were confirmed by GLC–mass spectrometry (at the Department of Chemistry, University of Joensuu). The standard compounds used were, in elution order, salicin, fragilin, picein, salidroside, vimalin, triandrin, tremuloidin, salicortin/salireposide, 2′-O-acetylsalicortin and tremulacin. The analysed compounds were quantified using salicin as a reference component and the results were corrected using the response factor and percentage recovery of each component. 2′-O-Acetylsalicortin was calculated after the response factor of salicortin.

RESULTS AND DISCUSSION

The results of the glucoside analysis of willow bark, twigs and leaves are shown in Tables II, III and IV, respectively. There are distinct qualitative and quantitative differences in the glucoside patterns between species. Generally, it is possible to distinguish most willow species on the basis of their phenolic glucoside content of both bark (twigs) and leaves.

The main glucoside in *S. rosmarinifolia* twigs and leaves is salicortin, which is accompanied by a relatively large amount of salireposide in twigs and tremulacin in leaves. The concentration of salicylates in this southern, low-growth form species shows a considerable resemblance to that in the northern, low-growth form *S. myrsinites*. The main difference in glucoside pattern between these species is seen in the existence of picein in the bark of *S. myrsinites*. Moreover, the twigs of northern *S. lanata* and *S. hastata* are rich in salicylates, especially salicortin, whereas their leaves contain only a trace amount of glucosides.

S. pentandra, S. myrsinifolia and *S. phylicifolia* are widespread willow species in Finland. The glucoside composition of *S. pentandra* is characteristic because of a large amount of 2′-O-acetylsalicortin. Earlier, only the bark of *S. fragilis* was quantitatively shown to contain 2′-O-acetylsalicortin[4]. Thieme[12] had detected an unknown compound with an R_F value of 0.74 in both of these species. On the basis of our analysis, this unknown compound is probably 2′-O-acetylsalicortin, which was first isolated by Egloff[4] in the bark of *S. pentandra* and *S. fragilis*. The qualitative composition of glucosides in *S. pentandra* budleaves is similar to that of mature leaves. However, the total amount is more than ten times higher in budleaves[1]. The relative amounts of the main glucosides in budleaves and in bark are equal. Instead of a large amount of salicylates, the winter-dormant bark of *S. phylicifolia* contains an appreciable amount of triandrin and vimalin. *S. myrsinifolia* bark contains most of the glucosides investigated in this study.

Our results indicate that capillary GLC and HPLC are equally effective in the identification of most phenolic glucosides. Differences will be observed in the identification of minor glucosides, fragilin and tremuloidin. If fragilin is determined in small amounts by GLC it will not be found at all by HPLC owing to the detection limit of the method (see below). Moreover, there is always the possibility that small amounts of tremulacin may have decomposed to tremuloidin and 2′-O-acetylsalicortin to fragilin during injection in GLC (*e.g.*, it may have been catalysed by unknown matrix substances in the extract). The same trend was not observed in GLC standard runs with pure tremulacin and 2′-O-acetylsalicortin. On the other hand, the physiological state of actively growing plants is always changing, so that precursors, inter-

TABLE III

PHENOLIC GLUCOSIDE CONTENT OF THE CURRENT GROWTH TWIGS OF SALICACEAE SPECIES

The results are means of up to ten subsample analyses (% a dry weight basis, S.E. in parentheses).

Species	*Method*	*Salicin*	*Salicortin*	*Picein*	*Triandrin*	*Tremuloidin*	*Tremulacin*	*Salireposide*
S. myrsinites	GLC I	1.02 (0.08)	7.60 (0.65)	0.172 (0.013)	0	0	0	0
	GLC II	1.05 (0.20)	6.59 (1.79)	0.183 (0.055)	0	0	0	0
	HPLC**	1.23	7.48	0.185	0	0	0	0
S. lanata	GLC I	0.405 (0.063)	6.65 (0.62)	0.505 (0.050)	0.053 (0.005)	0	0	0
	GLC II**	0.300	8.15	0.515	0.060	0	0	0
	HPLC	0.439 (0.11)	7.18 (0.013)	0.502 (0.002)	0	0	0	0
S. hastata	GLC I	0.775 (0.091)	7.53 (0.97)	0.028 (0.005)	0	0	0	0
	GLC II	0.577 (0.039)	7.52 (0.16)	0.026 (0.006)	0	0	0	0
	HPLC	0.557 (0.028)	7.42 (0.06)	0.019 (0.001)	0	0	0	0
S. rosmarinifolia	GLC II	0.471 (0.009)	6.14 (0.17)	0	0	Trace	0.120 (0.016)	*
	HPLC	0.636 (0.021)	6.25 (0.01)	0	0	0	0.175 (0.006)	1.14 (0.03)

* Interference with other component.
** Results for one subsample analysis.

TABLE IV

PHENOLIC GLUCOSIDE CONTENT OF THE LEAVES OF SALICACEAE SPECIES

The results are the mean values of up to five subsample analysis (% on a dry weight basis, S.E. in parentheses).

Species	*Method*	*Salicin*	*Fragilin*	*Salicortin*	*Acetylsalicortin*	*Tremulacin*	*Tremuloidin*	*Picein*
S. rosmarinifolia	GLC I	1.17 (0.14)	0.073 (0.009)	5.85 (0.44)	0	1.85 (0.19)	0.308 (0.037)	0
	GLC II	1.34 (0.11)	0.084 (0.009)	6.76 (0.68)	0	1.87 (0.03)	0.280 (0.023)	0
	HPLC	1.42 (0.01)	0	7.13 (0.20)	0	2.89 (0.05)	*	0
S. myrsinites	GLC I	0.793 (0.080)	0	9.70 (0.70)	0	2.05 (0.17)	0.127 (0.015)	0
	GLC II	0.856 (0.001)	0	9.81 (0.14)	0	2.53 (0.09)	0.113 (0.007)	0
S. pentandra	GLC II	0.078 (0.007)	1.81 (0.24)	0.064 (0.008)	9.24 (0.84)	0	0	0
	HPLC**	Trace	1.61	Trace	11.14	0	0	0
S. lanata	GLC I	0.022 (0.001)	0	0.148 (0.005)	0	0	0	0.019 (0.001)
	HPLC	Trace	0	0	0	0	0	0.015 (0.001)
S. hastata	GLC I	0.009 (0.001)	0	Trace	0	0	0	0
	GLC II	0.013 (0.001)	Trace	Trace	0	0	0	0
	HPLC	0	0	0	0	0	0	0

* Identified in a peak group (see Fig. 2).

** Results for one subsample analysis.

mediates or decomposed products even of glucosides induced by normal plant metabolism may occur. However, the existence of tremuloidin as a genuine glucoside has been argued over for several years[8] and the question has still not been definitively answered. In our study, a relatively large amount of tremuloidin was detected by GLC in the leaves of *S. rosmarinifolia* (Fig. 1, Table IV). In HPLC tremulodin was also qualitatively identified in a peak group (Fig. 2).

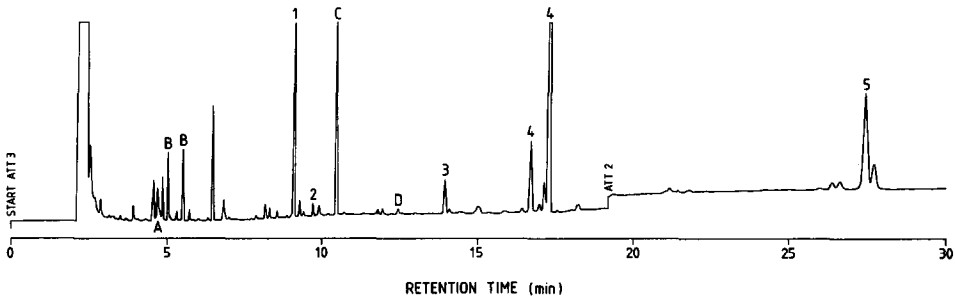

Fig. 1. GLC trace of TMS derivatives of phenolic glucosides in the leaves of *S. rosmarinifolia* (method II). Peaks: 1 = salicin; 2 = fragilin; 3 = tremuloidin; 4 = salicortin; 5 = tremulacin; A = fructose; B = glucose; C = sucrose; D = (+)-catechin.

The quantitative results of the GLC and HPLC analyses of phenolic glucosides show a good correlation with each other. All pairs of GLC (method I) and HPLC (method II) analyses of twig and bark samples and of *S. rosmarinifolia* leaf samples yielded r^2 higher than 0.999 ($n = 3$–5, $P < 0.001$). This is very useful in comparative studies of the inter-species variation in willow glucosides. Moreover, the quantitative differences found between the HPLC and GLC results are far smaller than the observed intra-specific variation in phenolic glucoside content[6,13,14].

In HPLC, the on-line identification of peaks from their UV spectra, using high speed UV–VIS detection systems equipped with a diode-array system (Fig. 2), is very helpful for the correction of the peak identification of such complex mixtures as plant extracts. Peak identification is very effective in dealing with willow glucosides, owing to their different UV spectra[16]. It is especially useful in the identification of tremulacin, because in several species (*e.g., S. myrsinifolia* leaves) co-eluting substances have been found. UV peak identification is also needed in order to distinquish salireposide from the flavonoid naringenin-7-glucoside (occurring in *S. daphnoides*). The mobile phase on its own is not selective enough to separate these glucosides.

The quantification by HPLC was optimized by two-wavelength detection. The phenolic glucosides have an absorption maximum in the lower UV region between 210 and 230 nm and a second maximum between 260 and 280 nm. For all other glucosides, except tremulacin, 220 and 270 nm were chosen for quantification. As tremulacin has a low absorption at 270 nm but a very broad band at 210–230 nm, 230 nm was chosen as the second wavelength. Two-wavelength detection will yield validated results if the quantification on both channels is identical.

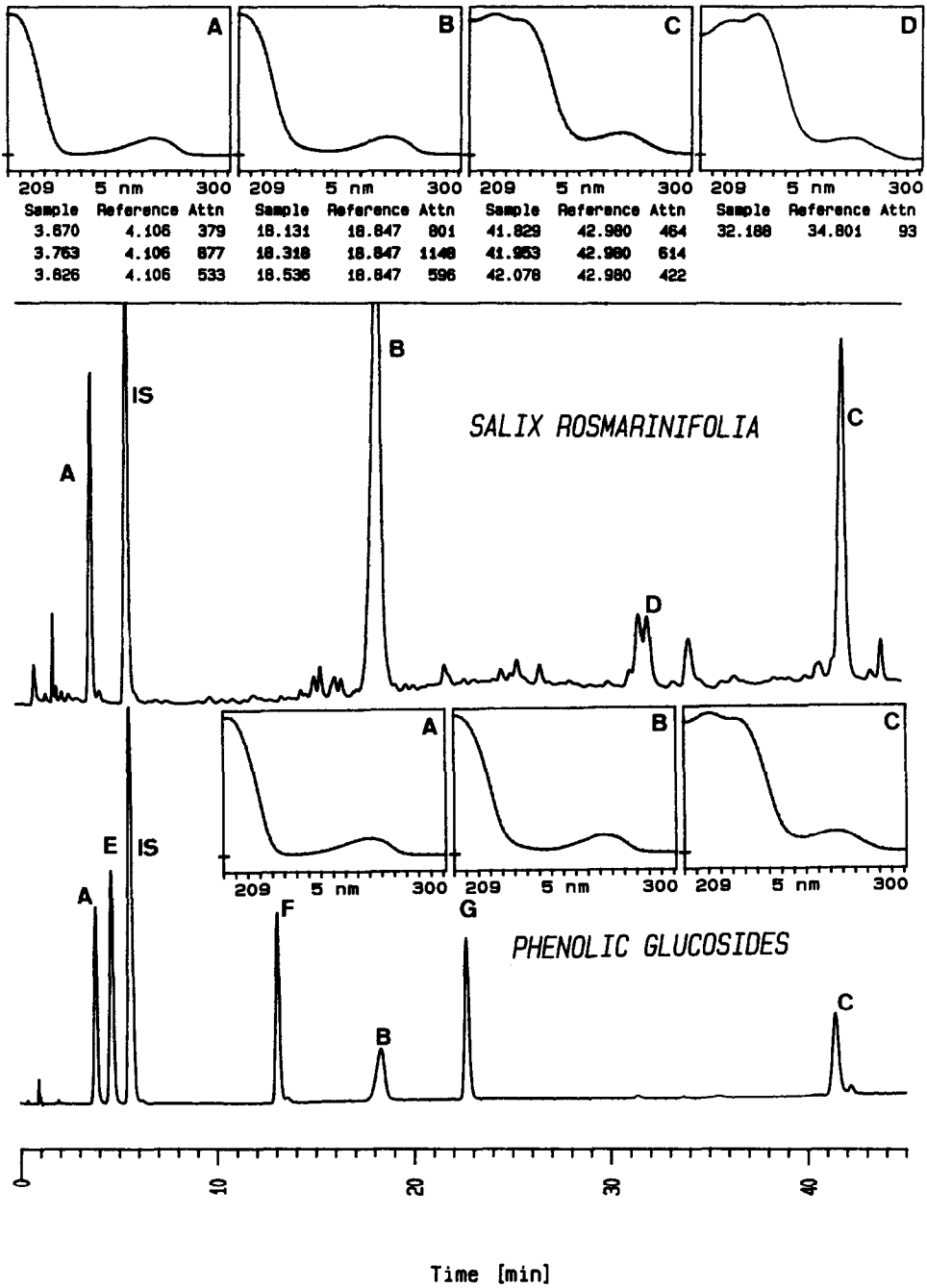

Fig. 2. HPLC traces of *S. rosmarinifolia* leaves (above) and the most important phenolic glucosides (below) of *Salix* species with peak identification of salicylates from UV spectra. A = Salicin; B = salicortin; C = tremulacin; D = tremuloidin (the peak overlap with a flavonoid); IS = internal standard; E = picein; F = triandrin; G = vimalin. Detection: glucosides, 270 nm/200 mAU; *S. rosmarinifolia*, 220 nm/700 mAU.

Without a very selective sample preparation, the detection of trace amounts of phenolic glucosides by HPLC is limited, because the molar absorptivity of some glucosides is not very high. In particular, this puts limitations on the UV detection of salicylates at 270 nm. A higher sensitivity is reached with detection at 220 or 213 nm. However, in a few instances under these conditions, an inconvenient mass transfer of unidentified compounds on the reversed phase resulted in a considerable baseline shift (*e.g., S. caprea* bark). The identification limit using UV spectra was observed to be 1 mg/g (dry weight) for salicylates. However, salicortin eluted as a broad peak, which increased its identification limit to 5 mg/g (dry weight).

The detection limits of phenolic glucosides using GLC was shown to be far lower than those using HPLC. The resolution of glucosides by high-sensitivity capillary GLC is also very high. The analysis of phenolic glucosides by GLC needs sample derivatization to increase their volatility and decrease their polarity. Tri-sil Z silylation reagent was shown to be the most powerful derivatization reagent for these glucosides. Moreover, the technique for injection of the derivatized sample is critical, especially if the boiling points of the components are different. During slow manual injection, syringe needle fractionation will occur. The fast injection technique used in this study mostly eliminates sample discrimination during injection.

The response factors of different glucosides are given in Table V. The response factor of one of the main salicylates, salicortin, was very close to that of salicin, whereas tremulacin, one of the predominant glucosides in some willow leaves, was found to be difficult to analyse by GLC. Its response factor was only 1.8 (Table V) and it eluted late. The molecular weight of tremulacin is 528, so its volatility even after derivatization is not high enough and consequently some more may be lost in relation to salicin during split injection.

High-sensitivity capillary GLC generally requires a high purity of glucoside extracts. The crude extract (*e.g.*, with methanol or 80% acetone) contains many primary and secondary metabolites (*e.g.*, pigments, flavonoids, polymeric phenolics, tannins), the existence of which in silylated samples will cause contamination problems in the injector and in the split system. Extra peaks, unreproducible retention times and baseline drifts will indicate the gradual concentration and decomposition of these impurities in the hot injector. All this may render the glucoside analysis difficult, with overlapping impurities, the need for daily cleaning of the injector and reduced column efficiency. This is why method I was developed, with precautions

TABLE V

RELATIVE RESPONSE FACTORS (SALICIN = 1.000) OF THE FLAME IONIZATION DETECTOR

Glucoside	*Response factor*	*Glucoside*	*Response factor*
Salicin	1.000	Salidroside	1.070
Fragilin	1.000	Triandrin	1.034
Salicortin	1.093	Vimalin	1.134
2'-O-Acetylsalicortin	1.093	Salireposide	1.045
Tremulacin	1.786	Tremuloidin	1.000
Picein	1.000		

against changes in the glucoside composition during sample preparation. As preliminary methodological studies indicated no decomposition during instrumentally long extraction procedures, it was accepted for routine glucoside analysis[15]. By this method the aqueous eluate from the second purification step on the polyamide column yielded most of the glucosides and the 50% ethanol eluate contained salireposide and tremulacin.

Method II was developed for HPLC. Methanol is a suitable solvent for the extraction of phenolic glucosides found in willow species. Flavonoids, which may also be useful in the identification of different willow species, are soluble in methanol and the glucosidic types are eluted and detected by HPLC. A large number of unidentified flavonoids with UV spectra comparable to that of luteolin-7-O-glucoside (identified in *S. purpurea*[17]) were detected in the leaves of *S. hastata*. All the other species analysed in this study were low in flavonoids. Minor peaks with similar UV spectra to that of luteolin-7-O-glucoside were detected only in the leaves of *S. rosmarinifolia* and *S. lanata* and in the bark of *S. pentandra*. A disadvantage of methanolic extraction is its poor selectivity, so in some instances interference with analytes and unknown compounds (Tables II and III) occurred.

As with HPLC, method II for GLC allowed the analyis of glucosides in a single run. However, salireposide, which is present in a few species, overlapped with salicortin and the higher level of contaminants in some samples disturbed the routine GLC analysis. Moreover, the high recoveries of all the glucosides by GLC were consistent with the recoveries obtained by Egloff[4] for HPLC sample preparation.

This comparative study has shown, that GLC and HPLC methods are appropriate for the analysis of phenolic glucosides. GLC and HPLC analyses carried out in two laboratories yielded comparable qualitative and quantitative results for willow glucosides.

ACKNOWLEDGEMENTS

The authors are greatly indebted to Prof. Dr. H. Thieme (Karl Marx University, Leipzig, G.D.R.) for providing several authentic reference components of phenolic glucosides. They also thank Miss. Sinikka Sorsa for technical assistance with the routine glucoside analyses.

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