Journal of Chromatography, 508 (1990) 236–240
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 310

Note

High-performance liquid chromatographic determination of five widespread flavonoid aglycones

A. HASLER and O. STICHER*
Department of Pharmacy, Swiss Federal Institute of Technology (ETH) Zurich, CH-8092 Zürich (Switzerland)
and
B. MEIER
Zeller AG, CH-8590 Romanshorn (Switzerland)
(First received October 30th, 1989; revised manuscript received January 26th, 1990)

Flavonoids are widespread compounds of the flora and there is special interest in medicinal plants. They normally occur as glycosides but it is difficult to determine all glycosides[1] in a crude plant extract because most reference compounds are not available commercially. Isorhamnetin, kaempferol, luteolin, myricetin and/or quercetin have been recognized as the major aglycones of medicinal plants such as *Arnica* sp., *Betula* sp., *Calendula officinalis*, *Epilobium* sp., *Equisetum arvense*, *Tussilago farfara*, *Filipendula ulmaria*, *Ginkgo biloba*, *Juglans regia*, *Primula* sp., *Prunus* sp., *Sambucus nigra*, *Solidago virgaurea*, *Tilia* sp. and *Verbascum* sp.

The hydrolysis of the glycosides and their spectrophotometric detection as an aluminium chloride chelate complex is the current method for determining the amount of flavonoids in a plant extract[2,3]. This method, described in several Pharmacopoeias, is not very specific and permits only a limited statement regarding the total flavonoids in plants. A detailed description of the qualitative and quantitative composition of the aglycones is not possible. With using reversed-phase high-performance liquid chromatography (RP-HPLC) and diode-array detection[4], a simple and rapid method was developed and validated for determining the above aglycones.

The conditions of the hydrolysis were optimized and tested with rutin. For the hydrolysis of rutin hydrochloric acid and trifluoroacetic acid[5] were tested in various concentrations together with the solvents acetone and methanol. Complete hydrolysis of rutin could only be achieved within 30 min by using methanol and hydrochloric acid.

EXPERIMENTAL

Standards and solvents

Isorhamnetin, kaempferol, luteolin, myricetin and quercetin dihydrate (Rotichrom HPLC) were obtained from Roth (Basle, Switzerland) and morin (Fluka

0021-9673/90/$03.50

standard for microscopy) and rutin (purum) were purchased from Fluka (Buchs, Switzerland). All the organic solvents used were of HPLC grade (Romil Chemicals, Shepshed, U.K.). Trifluoroacetic acid (purum), hydrochloric acid and orthophosphoric acid (analytical-reagent grade) were obtained from Fluka. Pure water was delivered by a NANOpure Cartridge System (Skan, Basel-Allschwil, Switzerland). Bond Elut C_{18} (3 ml) disposable extraction columns (Analytichem International, Harbor City, CA, U.S.A.) were used for sample clean-up.

Columns

Knauer (Berlin, F.R.G.) pre-packed column cartridges (100 × 4 mm I.D.) filled with the following different C_{18} materials were examined: Hypersil ODS, 3 and 5 μm (Shandon, Runcorn, U.K.); LiChrospher RP-18, 10 μm (Merck, Darmstadt, F.R.G.); LiChrosorb RP-18, 5 μm (Merck); Nucleosil 100-C_{18}, 3 and 5 μm (Macherey, Nagel & Co., Düren, F.R.G.); Partisil ODS-3, 5 μm (Whatman, Maidstone, U.K.); and Spherisorb S 5 ODS II, 5 μm, and S 3 ODS II, 3 μm (Phase Separations, Queensferry, U.K.).

Apparatus

HPLC analyses were performed using a Hewlett-Packard instrument (79994A Analytical Workstation, 1090 LC, 1040 DAD). The mobile phase consisted of solvent A (methanol) and solvent B (0.5% orthophosphoric acid) with the following gradient (% A): 0 min, 38%; 12 min, 48.2%; 12.01 min (washing), 100%; 17 min, 100%; and 17.01 min (equilibration), 38%. The flow-rates were 1 ml/min (3 μm), 2 ml/min (5 μm) or 3 ml/min (10 μm), the column temperature 25.0°C, injection volume 10 μl and detection was effected at 370 nm (flavonols) or 349 nm (luteolin).

Sample preparation

A 4-g amount of pulverized plant or plant extract material was refluxed with 70 ml of methanol and 10 ml of 25% hydrochloric acid for 30 min. After cooling, the solution was filtered through a G3 glass filter covered with an LS 14 paper filter. The supernatant was washed with 100 ml of methanol. The solution was evaporated under vacuum to about 80 ml and then diluted to 100 ml with methanol in a volumetric flask. A 5-ml volume of this solution was filtered through Bond Elut C_{18} which was equilibrated with methanol. The cartridge was washed with 4 ml of methanol and the solution was diluted to 10 ml with methanol in a volumetric flask. A 10-μl volume of this solution was injected into the HPLC system.

RESULTS AND DISCUSSION

The method was applied to *Ginkgo* and *Betula* analysis. The identification of the peaks after hydrolysis (*Betula*, myricetin and quercetin; *Ginkgo*, quercetin, kaempferol and isorhamnetin) was made with an automated library search system from Hewlett-Packard (Operating software, Rev. 5.03, 1988). This application includes the peak purity (comparison of the spectra upslope, apex, downslope and correlation between the peak at different wavelengths) and compares the spectra of the peaks in the chromatogram with the spectra of the reference compounds stored in the library. This comparison also considers the retention time (time window ±6%) of the peaks in

the chromatogram and in the library. Myricetin and quercetin in *Betula* and quercetin and kaempferol in *Ginkgo* were identified with a peak purity match index of >998 and a library match index of >994 (maximum 1000).

The linearity of the determination of all flavonoid aglycones and of the internal standard (morin) was verified by regression analysis (seven-point measurement, $r^2 \geqslant 0.994$). The determination was carried out with six samples (Table I). Each sample was injected three times by HPLC or measured by UV–VIS sopectrometry.

It is well known and was confirmed by our experimental work that the flavonoids require very well elaborated chromatographic procedures[7]. Different mobile and stationary phases were evaluated. The examination of the mobile phases was restricted to solvents currently used in RP-HPLC such as acetonitrile, 1,4-dioxane, ethanol, methanol, 2-propanol and tetrahydrofuran. A good separation of the five aglycones in less than 12 min was obtained with a methanol–water gradient. In addition, the influence of the various solvents on the UV spectra was studied, but no significant differences were observed.

The well known tailing problem with flavonoids, which depends on the mobile and especially on the stationary phase, disturbs the automatic integration. Therefore, different C_{18} materials were tested. Further, the influence of the addition of 0.5% of orthophosphoric acid to the solvent was examined. This modifier markedly reduced the tailing. With the requirements of high peak symmetry ($1 \leqslant T_f < 1.2$, at 5% peak height)[8], t_R (last peak) < 12 min, and $R_s > 1.5$, a successful and reproducible separation could be obtained only with Hypersil ODS, 5 μm (Table II and Figs. 1–3). The differences between the tested materials were very significant (Table III).

Comparing the chromatographic parameters of the different stationary phases, it can be concluded that reversed-phase materials have to be evaluated for the analysis of flavonoids.

Morin, a flavonol derivative that is commercially available and found so far only in four Moraceae sp. and one Anacardiaceae sp.[9], is proposed as an internal standard. However, quantitative analyses were established with external standards.

TABLE I

DETERMINATION OF THE FLAVONOLS IN *BETULA* AND *GINKGO*

Component	*Betulae folium, Dixa Lot No. 237*		*Ginkgo extractum, Flachsmann Lot No. 85375: HPLC*
	HPLC	*UV/VIS*[a]	
Myricetin (%)	0.34		–
Quercetin (%)	0.61		0.39
Kaempferol (%)	–		0.51
Isorhamnetin (%)	–		0.10
Calculated as total amount of:			
Aglycones (%)	0.95		1.00
Hyperoside (%)	1.47	1.57	–
Rutin (%)	–	–	2.12

[a] Determination according to ref. 6.

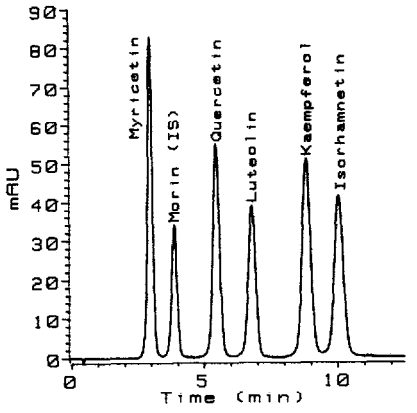

Fig. 1. Chromatogram of the five aglycones separated on Hypersil ODS, 5 μm. Amounts (μg per 10 μl) of the different aglycones injected: myricetin, 0.889; morin, 0.903; quercetin, 0.865; luteolin, 0.856; kaempferol, 0.999; isohamnetin, 0.863.

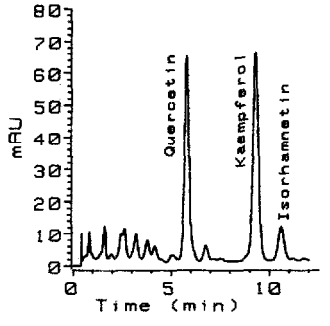

Fig. 2. Chromatogram of *Ginkgo* extractum after hydrolysis.

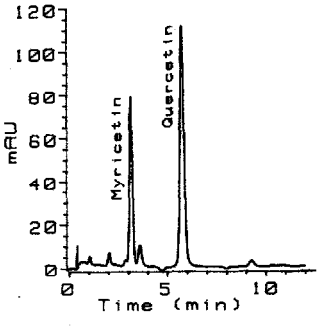

Fig. 3. Chromatogram of *Betulae folium* after hydrolysis.

TABLE II

CHARACTERIZATION OF THE CHROMATOGRAPHIC PARAMETERS OBTAINED WITH HYPERSIL ODS, 5 μm

T_f = Tailing factor according to ref. 8; t_R = retention time.

t_R (min)						T_f
Myricetin	*Morin*	*Quercetin*	*Luteolin*	*Kaempferol*	*Isorhamnetin*	
2.984	3.868	5.464	6.803	8.832	10.056	1.06

TABLE III

CHARACTERIZATION OF THE TESTED COLUMNS

R_s (5/6) = resolution factor between kaempferol and isorhamnetin; T_f = tailing factor according to ref. 8; t_R = retention time.

Stationary phase	t_R (min)[a]						R_s (5/6)	T_f
	1	*2*	*3*	*4*	*5*	*6*		
Hypersil, 3 μm	6.137	7.89	10.244	12.062	14.955	16.273	2.341	2.14
Hypersil, 5 μm	2.984	3.868	5.464	6.803	8.832	10.056	1.966	1.06
LiChrosorb, 5 μm	4.329	5.346	7.527	9.056	11.558	12.668	1.449	1.43
LiChrosorb, 10 μm	3.741	4.856	6.843	8.243	10.843	12.036	0.976	2.86
Nucleosil, 3 μm	8.639	10.762	13.785	15.925	19.276	20.608	2.79	1.82
Nucleosil, 5 μm	6.848	8.524	11.163	13.252	16.048	17.392	2.526	1.56
Partisil, 5 μm	5.278	6.87	8.876	10.843	13.328	14.445	0.698	5.83
Spherisorb, 3 μm	7.83	9.729	12.733	14.665	18.096	19.524	1.142	3.47
Spherisorb, 5 μm	3.41	4.529	6.257	7.613	10.188	11.42	1.135	5.14

[a] 1 = Myricetin; 2 = morin; 3 = quercetin; 4 = luteolin; 5 = kaempferol; 6 = isorhamnetin.

ACKNOWLEDGEMENT

Financial support from Zeller (Romanshorn, Switzerland) is gratefully acknowledged.

REFERENCES

1 K. Dallenbach-Tölke, Sz. Nyiredy, B. Meier and O. Sticher, *Planta Med.*, 53 (1987) 189.
2 H. Glasl and U. Becker, *Dtsch. Apoth.-Ztg.*, 124 (1984) 2147.
3 T. J. Mabry, K. R. Markham and M. B. Thomas, *The Systematic Identification of Flavonoids*, Springer, Heidelberg, New York, 1970, pp. 51–61.
4 B. Meier and O. Sticher, *Pharm. Ind.*, 48 (1986) 87.
5 M. Morrison, *Phytochemistry*, 27 (1988) 1097.
6 *Pharmacopoea Helvetica*, Editio Septima, Eidgenössische Drucksachen- und Materialzentrale, Bern, 1987.
7 D. J. Daigle and E. J. Conkerton, *J. Liq. Chromatogr.*, 11 (1988) 309.
8 M. T. Gilbert, *High Performance Liquid Chromatography*, Wright, Bristol, 1987, p. 11.
9 W. Karrer, *Konstitution und Vorkommen der Organischen Pflanzenstoffe*, Ergänzungsband 2, Birkhäuser, Basle, Boston, Stuttgart, 1981, p. 349.