*Journal of Chromatography*, 537 (1991) 453–459
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 754

# Note

## Isocratic column liquid chromatographic separation of a complex mixture of epicuticular flavonoid aglycones and intracellular flavonol glycosides from *Cistus laurifolius* L.

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(First received November 28th, 1989; revised manuscript received August 7th, 1990)

Reversed-phase high-performance liquid chromatography (RP-HPLC) is the method of choice for the separation flavonoid compounds [1]. HPLC protocols have been developed for the identification of methylated flavonoid aglycones [2], but the separation of different isomeric 3-O-methylflavonol derivatives is still difficult [3]. Thin-layer chromatography (TLC) on polyamide is able in some instances to separate closely related 3-O-methylflavonol or flavone isomers [4]. The very complex mixture of epicuticular flavonoids from *Cistus laurifolius* [5–7] is mainly composed of closely related isomeric quercetin mono- and dimethyl ethers. To study this material we developed a simple isocratic RP-HPLC system that allows a good resolution of these isomeric methyl ethers. In contrast to most other eluents, which utilize methanol or acetonitrile, the isocratic separation takes advantage of the dipole–dipole interactions of tetrahydrofuran with the flavonoids. The eluent system developed for analytical RP-HPLC also gave good results in preparative isolation even of the minor flavonol aglycones by reversed-phase low-pressure liquid chromatography (RP-LPLC).

## EXPERIMENTAL

### *Methylated flavonoid aglycones (Fig. 1)*

Leaves of *C. laurifolius* were washed with chloroform to extract the epicuticular resin–wax mixture from their surface. Waxes were separated from the resin by dilution of the mixture in hot methanol (50–60°C) (1 g of extract in 50 ml of methanol) and cooling for 12 h in a freezer (−20°C). This resulted in the precipitation of the waxes, leaving about 95% of the crude resin in the supernatant.

Terpenoids were separated from the flavonoid aglycones by column chromatography (CC) on a Sephadex LH-20 (Pharmacia, Freiburg, F.R.G.) column (100 cm × 3.0 cm I.D.) with methanol as eluent. Terpenoids were eluted first and identified by TLC on silica gel 60 (Merck, Darmstadt, F.R.G.) [solvent, toluene–ethyl acetate

0021-9673/91/$03.50 

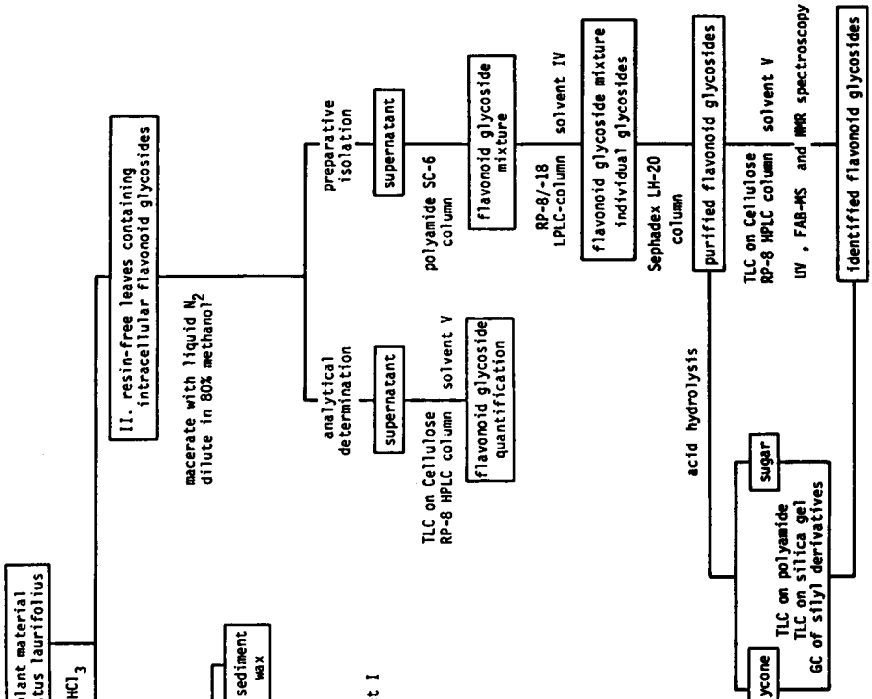


Fig. 1. Extraction, isolation and identification of epicuticular and intracellular flavonoids of *C. laurifolius*.

(9:1)], sprayed with concentrated sulphuric acid and heated at 100°C for 10 min. Then the coumarins (scopoletin and ayapin) were eluted, followed by flavonoid bands. Coumarins and flavonoids were detected at 350 nm with a hand UV lamp. For analytical HPLC of single leaf extract, the removal of terpenoids was not necessary.

For preparative separations, the flavonoid fraction were taken up in 3 ml of solvent I [water–methanol–acetonitrile–tetrahydrofuran (65:10:5:25)] and injected directly onto an RP-18 (alternatively RP-8) LPLC column (40 cm × 3 cm I.D.), particle size 40–60 μm, prepared in the laborators [8] according to a method described by Glatz [9]. A Duramat D-80 pump with pulsation suppression (Chemie und Filter Verfahrenstechnik, Heidelberg, F.R.G.) was used. Flavonoids were eluted by a stepwise gradient of water (containing 0.5% trifluoroacetic acid)–methanol–acetonitrile–tetrahydrofuran from 75:5:5:18 to 70:8:5:18 to 65:10:5:20 (solvents IIa, b and c) at a flow-rate of 8 ml/min and a pressure of 3–4 bar. The column was equilibrated before use with 200 ml of solvent IIa. Fractions were collected and monitored by detection with a hand UV lamp at 350 nm. If necessary, the separation was repeated to enrich individual flavonoids.

Further purification of individual flavonoids was achieved by chromatography on a Sephadex LH-20 column (100 cm × 2 cm I.D.) with methanol as eluent. Purified flavonoids were identified by comparision with authentic samples [5–7] according to their relative retention times in HPLC, co-chromatography by TLC using polyamide DC-6 and DC-11 [mobile phase, toluene–methyl ethyl ketone–methanol (65:25:15 or 8:1:1)] and microcrystalline cellulose [mobile phase, water–acetic acid (6:4)] (Macherey-Nagel, Düren, F.R.G.) and UV spectrometry. The 5-O-methylflavonols were also characterized by mass and $^1$H NMR spectrometry [7].

For analytical HPLC analysis, the resin samples, after precipitation of the waxes, were dried under nitrogen, weighed and dissolved in 1 ml of methanol per 10 mg of resin. Aliquots of 50 μl were dried under nitrogen and diluted again in about 200–300 μl of solvent I. Samples of 30 μl were then injected onto a Hypersil MOS-RP-8 HPLC column (125 mm × 4.6 mm I.D.) (particle size 5 μm) (Chromatographie Service, Eschweiler, F.R.G.). A Spectra-Physics (Santa Barbara, CA, U.S.A.) HPLC system was used with a Rheodyne 20-μl loop and a Shimadzu C-R6A UV detector (Shimadzu Europa, Duisburg, F.R.G.). Isocratic elution was performed with solvent system III: water (containing 0.5% phosphoric acid)–methanol–acetonitrile–tetrahydrofuran (68:6:6:20) at a flow-rate of 1.2 ml/min. Preparatively isolated flavonoid mixtures were dried under nitrogen and an aliquot was diluted in solvent I and injected onto the HPLC column.

External standards were prepared from crystalline samples of quercetin-3-O-methyl ether (que-3-OMe), que-3,3′-diOMe, que-3,7-diOMe, que-5,3′-diOMe, que-7,3′-diOMe and the coumarin scopoletin. Quercetin, que-3,7,3′,4′-tetraOMe, kampferol (kae), kae-3,7,4′-triOMe, apigenin (ap) and ap-4′-OMe were purchased from Roth (Karlsruhe, F.R.G.).

*Flavonol glycosides (Fig. 1)*

The chloroform-washed leaves were macerated in liquid nitrogen and extracted twice in 80% methanol (300 ml per 100 g of leaves) for glycoside analysis. The combined supernatants were carefully evaporated and fractionated by CC on 200 g of polyamide SC-6 (Macherey, Nagel & Co.) in a 40 cm × 7.5 cm I.D. column with

consecutive elution with 2 l of water, 8 l of water–methanol (1:1) and 3 l of methanol as eluents. Fractions were dried, taken up in 3 ml of solvent system I and applied to the equilibrated column with stepwise gradient elution with water (containing 0.5% trifluoracetic acid)–methanol–acetonitrile–tetrahydrofuran from 85:4:4:7 to 82:5:5:8 to 80:6:6:9 (solvents IVa, b and c). Individual bands were recovered after UV detection at 350 nm with a hand UV lamp.

Sugars were identified by hydrolysis of 1 mg of flavonoid glycoside with 2 ml of 2 *M* trifluoracetic acid for 30 min, evaporation of the acid and extraction of the aglycone with diethyl ether. Aglycones were detected by TLC on polyamide DC-6 with toluene–methanol–methyl ethyl ketone–acetylacetone (3:3:2:1). The water soluble sugars were identified by TLC [10] on silica gel plates (Merck). About 0.5–1 mg of the dried sugar was silylated with 20 $\mu$l of N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) (Macherey, Nagel & Co.) in 50 $\mu$l of pyridine for 1 h at room temperature and subsequently analysed by gas chromatography on a 10-m OV-1 column [11] using a Hewlett-Packard Model 5710 gas chromatograph with a flame ionization detector and a Hewlett-Packard Model 3380 integrator (Hewlett-Packard, Boeblingen, F.R.G.) with temperature programming from 110°C (2 min isothermal) to 240°C at 6°C/min and a carrier gas (nitrogen) flow-rate of 2 ml/min. Individual sugar standards were obtained from Roth or Merck.

For analytical HPLC studies of flavonoids, one leaf (500 mg fresh weight) was macerated in liquid nitrogen taken up in 5 ml of 80% methanol in a glass vial and stored for 12 h at 4°C. A 100-$\mu$l volume of the supernatant was evaporated to dryness under nitrogen and the residue was dissolved in solvent I, injected directly on to a Hypersil MOS-RP-8 (5 $\mu$m) column and eluted with water (containing 0.5% phosphoric acid)–methanol–acetonitrile–tetrahydrofuran (81:6.5:5.5:7) (solvent system V) at a flow-rate of 1.2 ml/min (Fig. 1).

In some instances the flavonoid pattern was checked by TLC on microcrystalline cellulose [mobile phase, water–acetic acid (8:2)].

Crystalline standards (kae-3-rhamnosidoglucoside or que-3-rhamnosidoglucoside) were available from Roth or obtained by preparative RP-LPLC (myr-3-galactoside, myr-3-rhamnoside, que-3-galactoside). Kae-3-rham-glc was added as an external standard to the macerated leaves to determine the total recovery of flavonoid glycosides from the leaves (recovery = 90–100% of the flavonoids). After every preparative run the RP-LPLC column was cleaned by elution in the opposite direction with 200 ml of water (containing 2% phosphoric acid)–acetonitrile–tetrahydrofuran (10:45:45) under the prevailing optimum elution conditions.

## RESULTS AND DISCUSSION

The resin of *C. laurifolius* consists of 31 phenolic aglycones (29 different flavonoids and two coumarins). Some of these aglycones were present only in negligible amounts and did not appear in the HPLC profile of the aglycone mixture (Fig. 2). The isocratic HPLC system was able to separate the 5-O-methyl derivatives well from the rest of the flavonol aglycones. This result is not especially dependent on the use of tetrahydrofuran in the solvent. Short retention times for 5-O-methyl flavonoids have been reported with other columns and eluent systems [12,13]. 5-Hydroxy groups form strong intramolecular hydrogen bonds with the 4-keto group of the heterocyclic ring,

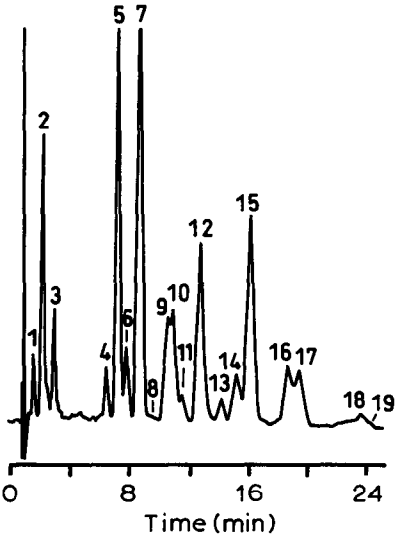


Fig. 2. HPLC of the crude resin of *C. laurifolius* after precipitation of the waxes. Hypersil MOS-RP-8, 5 μm; column, 125 mm × 4.6 mm I.D.; solvent system, III; flow-rate, 1.2 ml/min; UV detection at 350 nm. Peaks: 1 = scopoletin (coumarin); 2 = que-3,5,3′-triOMe (traces of the coumarin ayapin); 3 = que-5,3′-diOMe; 4 = luteolin; 5 = que-3-OMe (traces of lut-3′-OMe); 6 = que-3,4′-diOMe; 7 = que-3,3′-diOMe (traces of quercetin and apigenin); 8 = naringenin; 9 = que-3′-OMe; 10 = kae-3-OMe; 11 = lut-7-OMe; 12 = que-3,7-diOMe; 13 = que-7-OMe (traces of que-3,7,3′,4′-tetraOMe); 14 = que-3,7,3′-triOMe (traces of ap-4′-OMe); 15 = ap-7-OMe; 16 = que-7,3′-diOMe; 17 = kae-3,7-diOMe; 18 = ap-7,4′-diOMe; 19 = kae-7-OMe.

reducing its polarity and increasing the interaction with the hydrocarbon chains of the column matrix. If the protons are replaced with methyl groups, the polarity of the 4-keto group is enhanced, which leads to shorter retention times for the 5-O-methyl flavonoids. The use of tetrahydrofuran in the solvent is important for the separation of isomeric 3-O-methylflavonols. Previously, que-3,4′-diOMe could be separated from its 3,3′-isomer only by TLC on polyamide-11 [4]. Now it is possible to separate these closely related isomers by RP-HPLC. The separation and determination of the 3,7- and 3,4′-diOMe and the 3,7,3′- and 3,7,4′-triOMe flavonol isomers was completely successful. This system can also be applied to the separation of flavone isomers, as shown for ap-7-OMe and ap-4′-OMe.

As the complex flavonoid pattern of *C. laurifolius* results in only slightly different retention times for individual flavonoids, even the solvent composition of the sample before injection is very crucial. Dissolving the sample in methanol prevents the separation of the two 5-O-methyl derivatives and leads to co-elution of luteolin (lut), que-3-OMe and que-3,4′-diOMe and que-7-OMe, que-3,7,3′-triOMe and ap-7-OMe, respectively.

The conditions for analytical HPLC were directly transferred to the preparative RP-LPLC for the isolation and identification of several flavonoid aglycones after pre-fractionation of the pure resin on a Sephadex LH-20 column. As shown in the one-step purification of the 5-O-methyl derivatives from the other flavonoids [7], this

method leads to the isolation of que-3,4′-diOMe, a minor resin compound. A 20-fold concentration was achieved by chromatographing the Sephadex fraction twice on an RP-18 LPLC column using solvent system I. After a final Sephadex step the mixture contained 71% of que-3,4′-diOMe, 24% of que-3,3′-OMe and 5% of que-3-OMe, compared with 3% of que-3,4′-diOMe in the crude flavonoid mixture. The concentrated 4′-O-methyl isomer was identified by UV spectrometry and HPLC and TLC

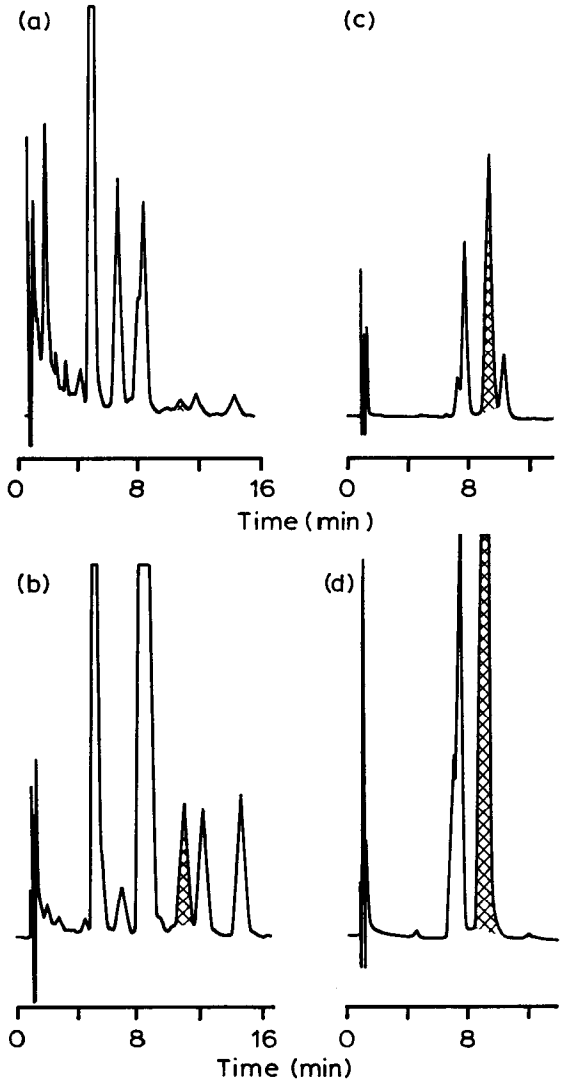


Fig. 3. Stepwise purification and preparative isolation of quercetin-3-O-β-arabinoside (shaded). Hypersil MOS RP-8, 5 μm; column, 125 mm × 4.6 mm I.D.; solvent system, V; flow-rate 1.2 ml/min; UV detection at 350 nm. (a) Crude intracellular flavonoid extract; (b) after polyamide SC-6 column, eluent 50% methanol; (c) after one RP-18 LPLC passage, solvent system V; (d) after purification by column chromatography on Sephadex LH-20, eluent methanol.

co-chromatography with an original standard. The following components were purified to 95% que-3-OMe, 97% apigenin and 92% kae-3-OMe by injecting the flavonoid mixture twice onto the LPLC column with subsequent purification by CC on Sephadex LH-20. In the same manner it was possible to identify some minor flavonoids from the complex resin mixture not detected previously [6], *e.g.*, luteolin, lut-7-OMe and que-7-OMe.

In addition to the aglycone isolations, the purification of intracellular flavonol glycosides was also substantially improved by using he RP-LPLC technique by modifying the HPLC solvent. The purification of minor components including que-3-arabinoside (que-3-ara) on an RP-18 LPLC column is illustrated in Fig. 3. After elution of the crude extract from a polyamide SC-6 column, the fraction that contained the main amount of que-3-ara was subsequently chromatographed using the RP-18 LPLC system (solvent V). The desired component was concentrated 10-fold in one step. After purification by Sephadex LH-20, the substance was suitable for submission to $^1$H NMR analysis. In the same way, other glycosides were purified with the aid of RP-LPLC and crystallized from methanol–water solutions.

When closely related or isomeric flavonoids are present, the application of RP-HPLC or RP-LPLC mainly based on tetrahydrofuran as eluent may be the method of choice for preparative isolation and identification. Even complex mixtures of flavonoids are well separated with the LPLC technique with only slight modifications of the solvent system used for HPLC. The choice of RP-8 instead of RP-18 did not influence the separation significantly.

ACKNOWLEDGEMENTS

We thank V. Wray (Braunschweig, F.R.G.) for NMR analysis of myr-3-gal, myr-3-ara, myr-3-rham-glc and que-3-ara-glc, E. Wollenweber (Darmstadt, F.R.G.) for samples of que-7-OMe, que-3,4′-diOMe and of que-3,7,4′-triOMe, G. Weissenboeck (Köln, F.R.G.) for a sample of que-3-glc, D. Strack (Braunschweig, F.R.G.) for a sample of kae-3-glc and B. Ellis (Vancouver, Canada) for his help with the preparation of the manuscript.

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