Journal of Chromatography, 540 (1991) 469–474
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 922

Short Communication

Separation of flavonoid glucosides from their galactosidic analogues by thin-layer chromatography

JAROMIR BUDZIANOWSKI
Chair and Department of Pharmaceutical Botany, K. Marcinkowski Medical Academy, Wieniawskiego 1, 61-712 Poznan (Poland)

(First received July 16th, 1990; revised manuscript received October 16th, 1990)

ABSTRACT

Particular problems in flavonoid analysis such as the separation of mixtures of glucosyl and galactosyl analogues appears to be solvable by means of thin-layer chromatography in classical flat chambers. Each of the nine pairs of such analogues tested, including flavonol 3-O-glycosides (three pairs), flavonol 3-O-diglycosides (two pairs), mono-C-glycosylflavones (one pair), di-C-glycosylflavones (one pair) and C,O-glycosylflavones (two pairs) could be resolved into individual components in at least one of the nine chromatographic systems described. The method is recommended for homogeneity control of C-glycosylflavones and any flavonoid which releases both glucose and galactose on hydrolysis.

INTRODUCTION

A great variety of chromatographic system can be employed for the analysis and isolation of plant flavonoids, which may form complex mixtures [1–3]. Particular problems exist when epimeric glycosides, such as glucosides and the corresponding galactosides, occur together in the sample to be analysed. For instance, it has been concluded, that C-glucosyl and C-galactosyl pairs are generally non-resolvable unless either high-performance liquid chromatography or silica gel thin-layer chromatography (TLC) of permethylated compounds is used [4]. Fortunately, it sometimes appeared to be possible to identify the structures of the individual compounds of glucosyl–galactosyl analogue mixtures without their separation from each other [5,6]. Our recent investigations of flavonoids in four *Solidago* L. (Golden Rod) taxa indicated the presence of at least five pairs of such analogues which were isolated (only one of them could be resolved chromatographically into individual components) and their structures deduced on the basis of hydrolytic and NMR spectral data [6]. Our most recent success in separating by traditional chromatography (TLC and column chromatography) two C,O-glycosylflavones, namely 7-O-glucoside and 7-O-galac-

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V.

toside of 6-C-[glucosyl-(1–2)-glucosyl]apigenin [7], encouraged us to undertake further studies.

This paper describes simple TLC methods for the separation of some flavonoid O- (or C-) glucosides from their O- (or C-) galactosidic analogues.

EXPERIMENTAL

Compounds

The compounds studied are listed in Table I. Compounds 1a, 2b (in a mixture with 2a), 3a, 4a, 4b and 5b (in a mixture with 5a) were from *Solidago canadensis* L. var. "scabra" [6], 1b was obtained from Roth (Karlsruhe, Germany), 3b (in a mixture with 3a) from *Solidago virgaurea* L. [6], 5a from *Tulipa gesneriana* L. cv. "Paradae" [8], 6b and 7b from *Polygonatum multiflorum* All. [9], 7a from *Solidago graminifolia* (L.) Salisb. [10], 8a and 8b from *Stellaria media* (L.) Vill. [7] and 2a from 5a, 6a from 8a–8b, 9a from 8a and 9b from 8b, all by acidic hydrolysis [7].

TLC

Plastic- (0.20 mm thick) or glass-backed (0.25 mm thick) precoated plates of silica gel 60 (non-activated) and cellulose (0.1 mm thick), 10 × 10 cm or 10 × 20 cm, from Merck (Darmstadt, Germany) were used. Polyamide plates (sizes as above) were hand made by spreading a slurry composed of 10 g of polyamide 6D (Riedel-de Haën, Seelze-Hannover, Germany), 1 g of cellulose MN 300 (Macherey, Nagel & Co., Düren, Germany) and 60 ml of ethanol (96%) followed by air drying; the layer thickness was *ca.* 0.2 mm.

The following solvent systems were prepared from analytical-reagent grade chemicals and doubly distilled water:

(A) ethyl acetate–formic acid–water (50:3:10, v/v/v; upper phase) (this work);
(B) ethyl acetate–formic acid–water (50:4:10, v/v/v; upper phase) (this work);
(C) ethyl acetate–methanol–water (20:3:3, v/v/v; upper phase) (this work);
(D) chloroform–methanol–water (40:10:1, v/v/v) [11];
(E) water-saturated butanone [12];
(F) butanone–methanol–water (8:1:1, v/v/v) [7];
(G) ethyl acetate–acetic acid–formic acid–water (100:11:11:27, v/v/v/v) [13];
(H) water-saturated phenol [14];
(J) chloroform–methanol–butanone–acetylacetone (9:4:2:1, v/v/v/v) [6].

Methanolic solutions of flavonoids —separate for each individual analogue (with the exceptions of 2b, 3b and 5b) and each pair of analogues— were employed. They had the following concentrations: 0.5 mg/ml for individual compounds 1a, 1b, 2a, 3a, 4a, 4b, 5a, 6a, 6b, 7a, 7b, 8a, 8b, 9a and 9b and 1.0 mg/ml for pairs of analogues (natural from plants, 2a–2b, 3a–3b and 5a–5b, or prepared by mixing of individual analogues in a 1:1 ratio, remaining compounds).

Solutions were applied to the plates manually as streaks *ca.* 2 mm × 1 cm (*ca.* 3 µl or 5 µl, *i.e.*, *ca.* 1.5–5 µg of flavonoid material) 0.9 cm from the lower edge of the plate. For the determination of the sequence of analogues, the general order of application was glucoside, galactoside, pair. The application zone was then thoroughly dried with a hair dryer operating in the "no heating" mode. A 15-ml volume of the mobile phase was placed in a classical flat-bottomed chamber (internal dimensions

23 × 6 × 22 cm; Camag, Muttenz, Switzerland) without saturation pads. After 5 min the plates were inserted and developed for a distance of 9.0 or 18.0 cm. Air conditions were temperature 23 ± 2°C and relative humidity 55 ± 5%.

The developed plates were air dried for 1 h before detection or repeated development. For detection the chromatograms were viewed under UV light at 365 nm before (all compounds brown) and after spraying with a 0.1% ethanolic solution of β-aminoethanol diphenylboric acid ester (Roth), followed by drying with a warm stream of air (quercetin derivatives, orange fluorescence; all other compounds, yellow-green fluorescence).

For purposes of hR_F calculation, the approximate centre of the maximum fluorescence of each spot was taken, immediately after detection with the reagent. The hR_F values reported in Table I were determined only from the plates (in the case of silica gel only the plastic-backed form) run to a distance of 9 cm and are the average values of three experiments.

Compound 5a was used as a reference on each chromatogram as an indicator of distortions of the chromatographic process.

RESULTS AND DISCUSSION

The choice of solvent systems followed from an earlier observation that two epimeric C,O-glycosylflavones from *Stellaria media* (8a and 8b; see Table I) could be separated in eluents based on butanone [7], and that quercetin 3-rhamnoglucoside (4a) could be separated from its 3-rhamnogalactoside (4b) in an eluent based on chloroform [6]. As butanone and chloroform belong to group VI and VIII, respectively, according to Snyder's classification of solvents [15], one could conclude, that solvents suitable for separation of glucoside–galactoside analogues should be sought within these two groups. Hence, solvents such as ethyl acetate, acetone (group VI), chloroform and phenol (by analogy with *m*-cresol) (group VIII) were tested. Their strength was increased by adding a polar modifier such as water, as recommended for polar substances [16]. To bring more water into the organic phase, an alcohol (methanol) or acid (acetic or formic) was used. Formic acid was particularly useful for suppressing the tailing effect of quercetin derivatives on silica gel layers.

Some eluents containing these solvents, reported in the literature, such as chloroform–methanol–water (40:10:1) [11], ethyl acetate–methanol–water (50:3:10) [17], ethyl acetate–acetic acid–formic acid–water (100:11:11:27) [13] and water-saturated phenol [14], appeared useful, whereas others such as ethyl acetate–butanone–formic acid–water (5:3:1:1) [1], ethyl acetate–formic acid–water (6:1:1) [18] or (10:2:3) [19] and chloroform–methanol–water (6:4:1) [20] or (7:3:0.5) [1] gave none or poor resolution, perceptible only when individual analogues were chromatographed side by side, but not when they were run together. Eluent A above [ethyl acetate–formic acid–water (50:3:10)] was derived from the published composition ethyl acetate–methanol–water (50:3:10) [17] by replacing methanol with formic acid.

All the experiments indicated that sufficient selectivity may depend on the proportions of the components of the mobile phase and the type of stationary phase used, as is already well known [16,21].

Finally, nine chromatographic systems were chosen for the analysis of nine pairs of analogues in an experimental manner similar to that described by Van Beek *et al.*

TABLE I

CHROMATOGRAPHIC DATA FOR THE SEPARABLE PAIRS OF GLUCOSYL–GALACTOSYL ANALOGUES

Quercetin = 3,5,7,3′,4′-pentahydroxyflavone; kaempferol = 3,5,7,4′-tetrahydroxyflavone; isorhamnetin = 3,5,7,4′-tetrahydroxy-3′-methoxyflavone; apigenin = 5,7,4′-trihydroxyflavone.

No.	Compound[a]	hR_F								
		Silica gel						Avicel		Polyamide
		A	B	C	D	F	G	E	H	J
1a	Quercetin 3-O-glucoside	35	43	—[b]	—	—	—	—	—	—
1b	Quercetin 3-O-galactoside	30	37	—	—	—	—	—	—	—
2a	Kaempferol 3-O-glucoside	44	50	47	47	53	76	—	—	—
2b	Kaempferol 3-O-galactoside	36	43	43	39	49	73	—	—	—
3a	Isorhamnetin 3-O-glucoside	—	45	—	50	—	—	—	—	—
3b	Isorhamnetin 3-O-galactoside	—	42	—	47	—	—	—	—	—
4a	Quercetin 3-O-[rhamnosyl-(1–6)-glucoside] (rutin)	—	—	—	—	—	—	—	—	23
4b	Quercetin 3-O-[rhamnosyl-(1–6)-galactoside]	—	—	—	—	—	—	—	—	33
5a	Kaempferol 3-O-[rhamnosyl-(1–6)-glucoside]	—	38[c]	30	—	—	—	—	64	—
5b	Kaempferol 3-O-[rhamnosyl-(1–6)-galactoside]	—	33[c]	28	—	—	—	—	74	—
6a	8-C-Glucosylapigenin (vitexin)	39	48	43	—	—	—	—	61	30
6b	8-C-Galactosylapigenin	43	52	45	—	—	—	—	76	39
7a	6-C-Glucosyl-8-C-arabinosylapigenin (schaftoside)	—	—	—	—	64	71	—	74	—
7b	6-C-Galactosyl-8-C-arabinosylapigenin (isocorymboside)	—	—	—	—	62	67	—	83	—
8a	6-C-[Glucosyl-(1–2)-glucosyl]apigenin 7-O-glucoside	—	—	—	—	41	54	14[c]	—	—
8b	6-C-[Glucosyl-(1–2)-glucosyl]apigenin 7-O-galactoside	—	—	—	—	33	49	10[c]	—	—
9a	6-C-Glucosylapigenin 7-O-glucoside (saponarin)	—	—	—	—	63	72	28[c]	—	—
9b	6-C-Glucosylapigenin 7-O-galactoside (neosaponarin)	—	—	—	—	55	67	24[c]	—	—

[a] Glucosyl and galactosyl as β-pyranosyl, rhamnosyl and arabinosyl as α-pyranosyl.
[b] — = Data not included because analogues were not separated.
[c] After two developments.

[22]. The data obtained including structures, adsorbents, solvent systems used and hR_F values, are given in Table I. For clarity of presentation, hR_F values reported are only when noticeable separations occurred when the analogues were run together to a distance of 9 cm. In some instances the differences in hR_F values were very small (*e.g.*, 0.02 for 5a and 5b in solvent C), but the resolution was reliable owing to the extreme sharpness and compactness of the bands. As can be seen, none of the ten systems used was universal. Thus, quercetin, kaempferol and isorhamnetin 3-O-monoglycosides (compounds 1a–1b, 2a–2b and 3a–3b) and also kaempferol 3-O-rutinoside (5a) and its 3-O-robinobioside (5b) were separable on silica gel with system B, whereas for isorhamnetin derivatives 3a and 3b, solvent D [11] was the best; in solvent B formally the same ΔhR_F value was obtained but the bands were broader. Similarly, 5a and 5b were better separated with system C. In turn, system F [7] was excellent for C- or C,O-glycosylflavones containing two (compounds 7a–7b and 9a–9b) or three (compounds 8a–8b) simple sugars in the molecule. Similar to solvent F was solvent G, recommended for the analysis of flavonoids in medicinal plants [13]. On cellulose (Avicel) layers, solvent E [12] was effective for C,O-glycosylflavones 8a–8b and 9a–9b [7], and was particularly convenient for preparative column chromatography [7]. Solvent H, recommended by Harborne and Williams [14] for difficult to separate compounds, was good for the separation of two kaempferol biosides (5a–5b) and C-glycosylapigenins (6a–6b and 7a–7b). However, compounds analogous to kaempferol derivatives 5a–5b, quercetin 3-O-rutinoside (4a) and its 3-O-robinobioside (4b) could be separated only on polyamide 6D with solvent J [6].

With all systems except H and J and for 8-C-glycosylapigenins (6a–6b); the glucosyl (O- or C-) derivatives had higher hR_F values than the corresponding galactosyl derivatives.

Moreover, it was also found that biphasic solvent systems are much better than monophasic ones {*e.g.*, acetone–butanone–formic acid (10:9:1), reported as being capable of separating quercetin 3-O-glucoside from its 3-O-galactoside [23]} in that they produced sharper resolution and narrower bands. The resolutions can be improved by running the chromatograms to a longer distance and/or by repeated development (two or three times). Increasing the solvent strength by enrichment with polar components was less effective.

Although it cannot be claimed that at least one of the solvent systems used in this work will be suitable for the resolution of other flavonoid pairs (already known or to be isolated in future), they do seem at least to be valuable for the compounds listed in Table I. Quercetin, kaempferol and isorhamnetin 3-O-glycosides are very common in the plant kingdom [14], as are C-glycosylapigenins among the C-glycosylflavones [4].

The method described should be applied when an apparently homogeneous glycoside would release a glucose together with a galactose on hydrolysis. As a first try, system B is recommended. For C-glycosylflavones, systems E, F and H should be tested.

The effectiveness of the method was confirmed by its application to the flavonoids isolated from the four *Solidago* taxa [6], where all pairs of glucosyl–galactosyl analogues (1a–1b to 5a–5b) were clearly separated into individual components.

REFERENCES

1 K. R. Markham, in J. B. Harborne, T. J. Mabry and H. Mabry (Editors), *The Flavonoids*, Chapman & Hall, London, 1975, Ch. 1, pp. 1–44.
2 K. Hostettmann and M. Hostettmann, in J. B. Harbone and T. J. Mabry (Editors), *The Flavonoids; Advances in Research*, Chapman & Hall, London, 1982, Ch. 1, pp. 1–16.
3 J. B. Harborne (Editor), *The Flavonoids; Advances in Research Since 1980*, Chapman & Hall, London, 1988.
4 J. Chopin and G. Dellamonica, in J. B. Harborne (Editor), *The Flavonoids; Avances in Research Since 1980*, Chapman & Hall, London, 1988, p. 91.
5 F. Imperato, *Phytochemistry*, 24 (1985) 2136.
6 J. Budzianowski, L. Skrzypczak and M. Wesołowska, *Sci. Pharm.*, 58 (1990) 15.
7 J. Budzianowski and G. Pakulski, *Planta Med.*, (1990) in press.
8 J. Budzianowski and L. Skrzypczak, *Pol. J. Chem.*, 53 (1979) 1489.
9 J. Chopin, G. Dellamonica, E. Besson, L. Skrzypczakowa, J. Budzianowski and T. J. Mabry, *Phytochemistry*, 16 (1977) 1999.
10 J. Budzianowski, *Sci. Pharm.*, 60 (1990) in press.
11 K. Nakano, M. Takatani, T. Tomamitsu and T. Nohara, *Phytochemistry*, 22 (1983) 2831.
12 K. W. Nicholls, B. A. Bohm, *J. Nat. Prod.*, 45 (1982) 453.
13 H. Wagner, S. Bladt and E. M. Zgainski, *Plant Drug Analysis*, Springer, Berlin, Heidelberg, New York, 1983, p. 164.
14 J. B. Harborne and C. A. Williams, in J. B. Harborne, T. J. Mabry and H. Mabry (Editors), *The Flavonoids*, Chapman & Hall, London, 1975, Ch. 8, pp. 379, 413 and 418.
15 L. R. Snyder, *J. Chromatogr. Sci.*, 16 (1978) 223.
16 K. Dallenbach-Toelke, S. Nyiredy, B. Meier and O. Sticher, *J. Chromatogr.*, 365 (1986) 63.
17 W. Olechnowicz-Stepień, *Diss. Pharm. Pharmacol.*, 19 (1967) 91.
18 T. Brasseur and L. Angenot, *Phytochemistry*, 27 (1988) 1487.
19 H. Wagner, M. A. Iyengar, E. Michahelles and W. Herz, *Phytochemistry*, 10 (1971) 2547.
20 Y. N. Shukla and R. S. Thakur, *Phytochemistry*, 29 (1990) 239.
21 S. Nyiredy, C. A. J. Erdelmeier, B. Meier and O. Sticher, *Planta Med.*, 51 (1985) 241.
22 T. A. Van Beek, R. Verpoorte and A. Baerheim-Svendsen, *J. Chromatogr.*, 298 (1984) 289.
23 T. Brasseur and L. Angenot, *J. Chromatogr.*, 351 (1986) 351.