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USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE SEPARATION OF FLAVONOL GLYCOSIDES AND FLAVONOL SULPHATES

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SUMMARY

Forty flavonol glycosides have been separated by reversed-phase high-performance liquid chromatography (HPLC) on a C_8 column by gradient elution with methanol–acetic acid–water mixtures. The results indicate that relative retention times are a useful adjunct for purposes of characterisation. Retention times are inversely correlated with increasing glycosylation, although the position of glycosylation in the flavonol moiety has a significant effect on mobility. The value of HPLC as an additional criterion for characterisation is illustrated by the identification of the quercetin arabinoside of *Foeniculum vulgare* as guaijaverin. Flavonol sulphates tend to overlap with glycosides under the above conditions but they can be separated by ion-pairing with tetrabutylammonium phosphate. HPLC of a crude extract of *Oenanthe crocata* leaves provided quantitative measurements of the relative amounts of sulphated and unsulphated flavonoids.

INTRODUCTION

Reverse-phase high-performance liquid chromatography (HPLC) using gradient elution with aqueous–methanolic–organic acid solvent-systems is now well established in flavonoid research[1] but its main application has been in quantitative analysis, *e.g.* refs. 2 and 3. Its value for resolving mixtures of closely related structures has not been explored to the same extent, although many different flavonoid glycosides have been separated on a C_{18} column[4]. Its effectiveness in relationship to paper chromatographic and TLC procedures has also not been widely studied, although it has been applied successfully to resolve mixtures of glycosides not readily separated by other procedures[5].

We have now tested a range of some forty flavonol glycosides in an HPLC system, in order to further explore the relationship between structure and retention time and to compare retention time with R_F values. We have also used HPLC as an additional criterion in the identification of a quercetin 3-arabinoside of uncertain configuration. Additionally, we have shown that flavonol sulphates can be resolved from neutral glycosides by HPLC using ion-pairing with tetrabutylammonium phosphate.
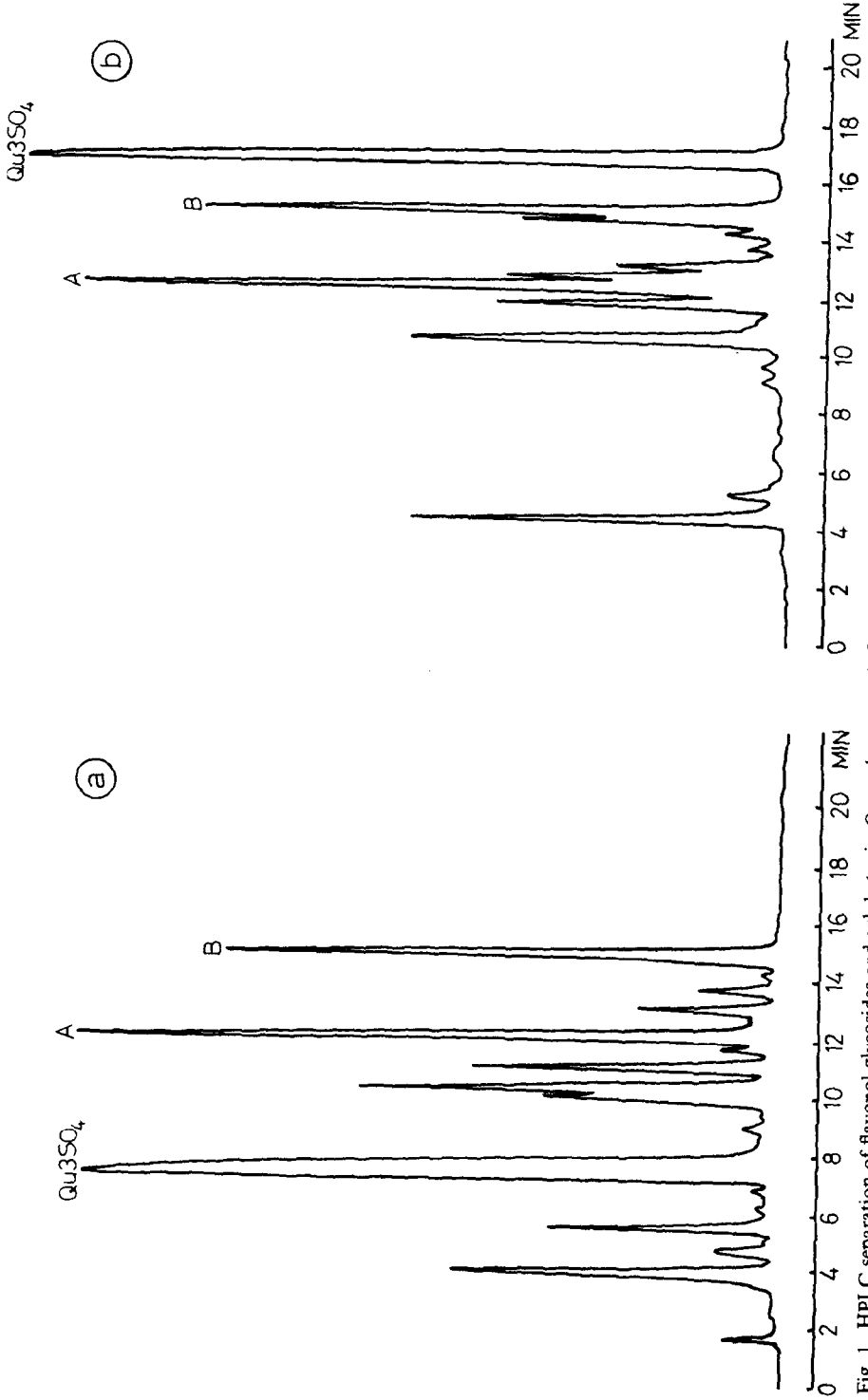
Fig. 1. HPLC separation of flavonol glycosides and sulphates in *Oenanthe crocata* before (a) and after (b) ion-pairing. Key: A = kaempferol 3-rutinoside, B = isorhamnetin 3-rutinoside, Qu3SO$_4$ = quercetin 3-sulphate.

EXPERIMENTAL

Flavonoid samples

        Practically all the flavonol glycosides and sulphates were available from the collection in this laboratory. Guaijaverin was kindly supplied by Professor H. Geiger.

Apparatus

        An Applied Chromatography Systems liquid chromatograph and solvent programme were used, with UV-absorbance detection at 365 nm.

Separation of flavonol glycosides

        A Partisil 5 CCS/C_8 (Whatman) column 250 × 5 mm I.D. was used, the solvents being A, water; and B, methanol–acetic acid–water (90:5:5). The initial solvent composition was 25% B in A and gradient elution was linear with a 2% increase of B in A per min for 20 min and flow-rate of 1.7 ml/min. All compounds were run in duplicate or triplicate, between 20 and 70 μl of approximately 0.1% solutions of the various glycosides were injected, singly and in mixtures (as indicated in Tables I–III). Quercetin 3-glucoside and kaempferol 3-glucoside were used as internal-reference compounds for the calculation of relative retention times.

Separation of flavonol sulphates

        Columns used were an analytical (250 × 4 mm I.D.) and a semi-preparative (250 × 8 mm I.D.) packed with Spherisorb S5 ODS-2 (Hichrom, Reading, U.K.). Solvents used were as follows: A, water; B, methanol–water–acetic acid (90:5:5); C, 0.01 *M* aq. tetrabutylammonium phosphate; D, water buffered to pH 2.2 with water–acetic acid–formic acid (156:3:1); and E, 0.01 *M* tetrabutylammonium phosphate in solvent D. For ion-pairing systems the column was equilibrated by passing 50 ml (analytical column) of solvent through the column at initial solvent composition (usually 15% B). Purified flavonol sulphates were analysed using solvents A and B. The flow-rate was set at 1.7 ml/min and initial solvent composition was 15% B in A. Gradient elution was linear, increasing by 2% B per min. This system was used to obtain retention times of sulphated flavonoids under normal reversed-phase conditions. For analytical ion-pairing, solvent C was substituted for solvent A, all other conditions remained unchanged (Table 5).

        When analysing crude extracts of *Oenanthe crocata* leaf, it was found that slight alterations in the retention times of other compounds as well as sulphated compounds occurred with the addition of ion-pair reagent. Results were improved by buffering the aqueous mobile phase to pH 2.2 (as in solvents D and E). This enabled comparisons to be made between chromatograms produced with and without ion-pairing conditions (Fig. 1).

        Flow-rate and gradient elution conditions were kept as for pure samples (see above).

        Crude extracts were prepared by extracting fresh leaf material with 70% ethanol, evaporating the extract to dryness at 40°C, then taking up the residue in 30% methanol. This was then filtered through a Millipore 0.45-μm sieve before injection onto the column.

        Semi-preparative HPLC can be carried out under reversed-phase or ion-pairing

conditions. A mixture of quercetin and isorhamnetin 3-sulphates was purified using the 250 × 8 mm I.D. column and solvents A and B. The flow-rate was set at 1.8 ml/min, initial solvent composition was 15% B in A with linear gradient at 2% B per min.

A crude extract of *Oenanthe crocata* (prepared as for analytical HPLC) was separated by ion-pairing, using the same column and solvents B and C. Initial solvent composition was 30% B in C, gradient elution was linear at 1% B per min, flow-rate 4.5 ml/min. Before sample injection the column was equilibrated with *ca.* 100 ml of solvent at initial composition.

RESULTS AND DISCUSSION

Retention times of flavonol glycosides

The results of determining retention times of twenty kaempferol glycosides, eleven quercetin glycosides and seven myricetin derivatives are shown in Tables I–III. We have found that a C_8 (octasilyl) column, namely Partisil CCS/C_8, gives better resolution of flavonol glycosides than does a C_{18} (octadecylsilyl) column, such as μBondapak C_{18} and LiChrosorb RP-18, the most common types of packing used previously. Gradient elution with increasing proportions of methanol–acetic acid–

TABLE I

HPLC RETENTION TIMES AND R_F VALUES OF KAEMPFEROL GLYCOSIDES

Glycoside	t_R (min)*	t_R K3G (min)**	R_F (× 100) in***	
			BAW	Water
3,7-Diglucoside	7.62	0.54	28	57
3-Triglucoside-7-rhamnoside	7.77	0.56	35	78
3-Rutinoside-7-glucoside	8.22	0.58	15	47
3-(2^G-Glucosyl) rutinoside	9.65	0.69	40	54
3-Sophoroside-7-rhamnoside	9.81	0.70	40	71
3-Glucoside-7,4'-dirhamnoside	10.82	0.77	52	53
3-Glucoside-7-rhamnoside	11.58	0.82	40	30
7-Glucoside	12.85	0.90	54	02
3-Robinobioside-7-rhamnoside	13.98	1.00	40	54
3-Glucoside	14.13	1.00	70	13
3,7-Dirhamnoside	14.37	1.00	56	41
3-Rhamnoside	16.69	1.18	78	28
7-Rhamnoside	19.05	1.35	75	02
3-Gentiotrioside	10.86	0.76	31	33
3-Sophoroside	11.08	0.77	45	29
3-Gentiobioside	11.86	0.83	43	27
3-Neohesperidoside	12.25	0.85	53	55
3-Xylosylglucoside	12.57	0.88	55	29
3-Glucuronide	14.16	1.00	53	67
3-Arabinopyranoside	15.83	1.10	84	17

   * Mean of three measurements.
   ** Retention time relative to kaempferol 3-glucoside.
   *** Published R_F data in *n*-butanol–acetic acid–water (4:1:5) (BAW) and in water on Whatman No. 1 paper.

water (90:5:5) in water was used in all cases. Retention times in the C_8 system range from 7.62 min for kaempferol 3,7-diglucoside to 19.05 min for kaempferol 7-rhamnoside (Table I). These compare with retention times of between 10.05 min for kaempferol 3-sophoroside-7-glucoside to 20.09 min for kaempferol 7-rhamnoside on a LiChrosorb RP-18 column, eluted with 5% aqueous formic acid and methanol gradients[4]. The difference in retention time, for example, between the 3-neohesperidoside and 3-rutinoside of quercetin in the C_{18} system is 0.69 min, while in our C_8 system this is increased to 1.40 min (Table II).

TABLE II

HPLC RETENTION TIMES AND R_F VALUES OF QUERCETIN GLYCOSIDES

Glycoside	t_R (min)	t_R Q3G (min)*	R_F (× 100) in	
			BAW	Water
5-Glucoside	9.73	0.84	22	02
3-Glucoside	11.64	1.00	58	08
3-Arabinopyranoside	13.22	1.10	70	07
4'-Glucoside	13.52	1.12	48	01
3-Rhamnoside	13.65	1.17	72	19
3-Sophoroside	9.65	0.77	45	31
3-Neohesperidoside	10.74	0.85	50	43
3-Rutinoside	12.12	0.96	45	23
3-Glucoside	12.58	1.00	58	08
3-(2^G-Glucosyl)rutinoside	9.67	0.79	37	19
3-Gentiobioside	8.31	0.68	36	46
3-Glucoside	12.20	1.00	58	08
3-Gentiobioside	8.18	0.69	37	19
3,4'-Diglucoside	8.98	0.74	35	29
3-Glucoside	12.20	1.00	58	08

  * Retention time relative to quercetin 3-glucoside.

TABLE III

HPLC RETENTION TIMES AND R_F VALUES OF MYRICETIN, LARYCITRIN AND SYRINGETIN GLYCOSIDES

Flavonol glycoside*	t_R (min)	t_R S3GAL** (min)	R_F (× 100) in	
			BAW	Water
Myricetin 3-galactoside	9.38	0.69	37	09
Myricetin 3-rhamnoside	11.06	0.81	60	15
Larycitrin 3-galactoside	11.64	0.85	43	09
Syringetin 3-galactoside	13.65	1.00	51	08
Larycitrin 3-rhamnoside	13.92	1.02	65	06
Syringetin 3-arabinoside	15.56	1.13	64	24
Syringetin 3-rhamnoside	16.32	1.20	83	41

  * Larycitrin = myricetin 3'-methyl ether, syringetin = myricetin 3',5'-dimethyl ether.
 ** Retention time relative to syringetin 3-galactoside.

Absolute retention times are useful in indicating the order in which related glycosides separate when injected as a mixture, but they are rather variable, possibly due to temperature variation on the column. On the other hand, relative retention times give a better comparison between runs (see Table II) and are less subject to change. Use of an internal standard is advisable when screening plant extracts for their flavonol glycosides and the readily available 3-glucosides or 3-rhamnosides are particularly suitable for this purpose, because they tend to elute late in this system.

The main structural feature affecting the mobilities of flavonol glycosides on a C_8 column is the extent of glycosylation. It is clear, for example, both in the kaempferol and quercetin series (Tables I and II), increasing glycosylation lowers retention time so that triglycosides elute off before diglycosides and diglycosides before monoglycosides. As observed before on C_{18} columns[4], glucosides are eluted ahead of arabinosides which come off ahead of rhamnosides (cf. Table II). Again, a diglycoside with only glucose moieties will elute sooner than one containing one or two rhamnose units. For example, in the kaempferol series, the 3,7-diglucoside, the 3-glucoside-7-rhamnoside and the 3,7-dirhamnoside elute at 7.62, 11.58 and 14.37 min, respectively.

The effect of position of glycosylation on retention time is more complex. Generally, a glucose at the 7-position appears to cause a glycoside to be eluted earlier than it otherwise would be expected; compare kaempferol 3,7-diglucoside which elutes off before any of the 3-triglycosides studied (Table I). However, 7-rhamnosides, appear to be held on a C_8 column for a much longer time than related 7-glucosides. However, in the case of kaempferol 3-sophoroside-7-rhamnoside, the extra sugar in the 7-position gives it a lower t_R than kaempferol 3-sophoroside. The effect of glycosylation at other positions in the flavonol nucleus is even more unpredictable. Quercetin 5-glucoside has a t_R of 9.73 min, the 3-glucoside of 11.64 min and the 4′-glucoside at 13.52 min (Table II).

The effect of methylation in the flavonol moiety of flavonol glycosides expectedly increases retention on the C_8 column. This can be seen in the data of Table III, with the monoglycosides of myricetin and its 3′-methyl ether and 3′, 5′-dimethyl ether. Thus, myricetin 3-galactoside elutes at 9.38 min, larycitrin 3-galactoside at 11.64 min and syringetin 3-galactoside at 17.65 min. The corresponding 3-rhamnosides elute at 11.06, 13.92 and 16.32 min, respectively.

Comparison of HPLC retention-times with R_F values on paper in a typical butanolic and an aqueous system (Tables I–III) shows that there is little direct relationship between the behaviour of a given glycoside in the different systems. Relative retention time on a C_8 or C_{18} column would therefore seem to be a useful independent criterion for establishing the novelty of a particular flavonol glycoside. Similarly, HPLC provides an additional means of comparison in the co-chromatography of an isolated glycoside with a standard.

In terms of resolving power, paper chromatography (PC) —or thin-layer chromatography (TLC) on microcrystalline cellulose— and HPLC would seem to be comparable. Closely related pairs of glycoside, e.g., the 3-arabinofuranosides and 3-arabinopyranosides, are separable both on paper and on HPLC. Correspondingly, pairs more or less inseparable on paper, e.g. the 3-glucosides and 3-galactosides, are unresolvable on HPLC. This has been our experience (unpublished data) on C_8 columns and is also true on a C_{18} column[4]. The two procedures should be used in a complementary manner, since occasionally mixtures of glycosides running together on PC will be resolved by HPLC[6] and vice versa.

In this laboratory, we have found HPLC to be invaluable for checking the homogeneity of flavonol glycosides separated on paper or TLC. HPLC is particularly successful at revealing the presence of unsuspected glycosides as minor components in plant extracts containing mixtures of partly methylated flavonol glycosides, *e.g.* of the various myricetin derivatives listed in Table III.

Identification of foeniculin by HPLC

Foeniculin was isolated from leaves of fennel, *Foeniculum vulgare*[7,8] and was subsequent listed[9] as a quercetin 3-arabinoside, apparently different from other known arabinosides, namely avicularin (the 3-α-L-arabinofuranoside), guaijaverin (the 3-α-L-arabinopyranoside) and polystachoside (the 3-β-L-arabinoside). More recently, foeniculin was isolated from plants in the Epacridaceae and shown, on the basis of enzymic data and R_F values, to be different from avicularin and polystachoside[10]. Direct comparison with guaijaverin seemed to be in order and our results (Table IV) confirm that foeniculin is indeed identical to the 3-α-L-arabinopyranoside.

Standard chromatographic comparison in nine solvents on three different supports and electrophoretic comparison show no separation between an authentic sample of guaijaverin and foeniculin. Additionally, HPLC was carried out and a mixture of the two samples gave a simple sharp peak eluting at 16.93 min. Under the same conditions, avicularin clearly separated at 17.35 min. These two isomers also separate

TABLE V

CHROMATOGRAPHIC COMPARISON OF FOENICULIN AND GUAIJAVERIN

Chromatographic system	R_F (× 100)	
	Foeniculin	*Guaijaverin*
Paper		
n-Butanol–acetic acid–water (4:1:5)	65	65 (73)*
n-Butanol–ethanol–water (4:1:2:2)	46	46
Water	05	05 (08)
15% aq. Acetic acid	25	25 (31)
Phenol–water (4:1)	56	56 (54)
Chloroform–acetic acid–water (2:1:1)	13	13
Silica gel TLC		
Ammonium hydroxide–acetic acid–water (2:1:1)	63	65
Polyamide TLC		
Methanol–acetic acid–water (19:1:1)	27	27
Ethanol–water (3:2)	25	25
Electrophoresis		
Borate buffer pH 10	1.23**	1.23**
3 h 40 V/cm		
HPLC		
C$_8$ column, methanol–acetic acid–water	16.93 min***	16.93 min***
(90:5:5) and water		

   * Data for avicularin (quercetin 3-α-L-arabinofuranoside).
  ** Mobility relative to quercetin 3-glucoside.
 *** Time of elution.

in a C_{18} column and 16.16 and 16.55 min, respectively[4]. This example shows that HPLC usefully complements other chromatographic techniques for the characterisation of those flavonol glycosides in which the sugar moieties exist in different configurations or are differently linked to the aglycone.

HPLC of flavonol sulphates

Sulphated flavonols, which have been reported in over twenty angiosperm families, usually occur in conjunction with flavonol glycosides and their analysis requires separation from such compounds[11]. While paper electrophoresis in an acid buffer is a useful technique for such separations[11] other methods of separation are desirable, especially for quantitative determination. We have now found that these conjugates are readily separated by HPLC on a C_{18} column by gradient elution with methanol–acetic acid–water and water mixtures (Table V). The 3-sulphates of kaempferol, quercetin and isorhamnetin are well separated from each other and from related flavonol glycosides. Some overlap in retention times is, however, possible if highly-glycosylated flavonols or C-glycosyl flavones were also present in an extract (cf. Tables I and II).

TABLE V

HPLC RETENTION TIMES OF FLAVONOL SULPHATES WITH AND WITHOUT ION PAIRING

Flavonol	Without ion-pairing		With ion-pairing*	
	t_R (min)	t_R Qu3R** (min)	t_R (min)	t_R Q3R (min)
Quercetin 3-sulphate	6.0	0.37	18.2	1.11
Kaempferol 3-sulphate	7.2	0.45	19.3	1.21
Isorhamnetin 3-sulphate	8.4	0.51	19.7	1.23
Quercetin 3'-sulphate	9.2	0.57	22.5	1.37
Rutin	14.2	0.88	14.2	0.88
Quercetin 3-rhamnoside (quercitrin)	16.2	1.00	16.2	1.00

　* In the presence of 0.01 M tetrabutylammonium phosphate.
　** Retention time relative to quercetin 3-rhamnoside.

This difficulty, however, is readily resolved by application of an ion pair reagent[12,13]. Tetrabutylammonium phosphate proved to be suitable and flavonol sulphates were then eluted from the column after, instead of before, the quercitrin marker (Table V). In the course of these experiments, it was found that control of pH is advisable in ion-pairing separations and the water in the eluting solvent mixture was replaced by acetic acid buffer pH 2.2.

HPLC was then applied to an Oenanthe crocata leaf extract, which contains a mixture of kaempferol 3-rutinoside and isorhamnetin 3-rutinoside, together with quercetin and isorhamnetin 3-sulphates[14]. The results of HPLC separation before and after ion-pairing are illustrated in Fig. 1. It is clear that ion-pairing (Fig. 1b) produces sharper peaks for the sulphated compounds and hence improves the accuracy of the quantitative analysis. Semi-preparative separation of flavonol sulphates, either from a crude plant extract of Oenanthe or using a partly purified flavonol sulphate fraction, was successful on a larger diameter column using the same

solvent system. If ion-pairing is used in the semi-preparative separation, the reagent is readily removed by final purification of the sulphate using paper electrophoresis.

This appears to be the first report of the HPLC of sulphated flavonols. HPLC with and without ion-pairing provides an excellent procedure for specifically detecting these charged flavonols in plant extracts, which contain normal flavonol glycosides as well. We believe that this technique has certain advantages over other available methods and will be generally useful in the separation and quantitative analysis of these naturally occurring plant conjugates.

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