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INFLUENCE OF ORGANIC MODIFIERS ON THE ISOCRATIC REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FLAVONOL GLYCOSIDES FROM BREWER'S HOPS

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SUMMARY

The elution order in reversed-phase high-performance liquid chromatography of certain flavonol glycosides found in hops was influenced by the concentration of tetrahydrofuran (THF) used under isocratic conditions. Whereas acceptable resolution of all compounds of interest was obtained with the appropriate water–THF mixtures, solvent mixtures containing methanol instead of THF were much less satisfactory. The solvent strength of acetic acid, when included in eluent mixtures was found to be greater than that of THF, which was in turn greater than that of methanol.

INTRODUCTION

Flavonoids were included as examples of important naturally occurring compounds in one of the earliest applications[1] of modern high-performance liquid chromatography (HPLC). Since then reversed-phase[2] has supplanted adsorption as the preferred mode of chromatographic separation, especially so with respect to flavonoids. Frequently, methanol has been used as an organic modifier for the isocratic elution of flavonol glycosides[3–5] but the use of gradients of methanol[6,7], acetonitrile[8], and tetrahydrofuran (THF)[9,10] has been reported also. In one of the more comprehensive chromatographic studies[6] conducted on flavonoids, mixtures of up to 40 substances were separated using a combination of isocratic and gradient elution with methanol as the organic modifier. This method, however, failed to separate some flavonol rutinosides from their corresponding glucosides. Other instances of the failure of methanol-containing mobile phases to effect this required separation adequately on reversed-phase columns have been recorded[4,7]. Rutinosides and glucosides are perhaps the most common molecular conjugates of the ubiquitous flavonoids[11], so an HPLC method proffering general applicability in flavonol analysis should be capable at least of resolving these species.

In an earlier communication[9] on the gradient elution chromatography of crude extracts of flavonols from brewer's hops we claimed that THF afforded a better separation than methanol when used as the organic modifier. From the study[12] of the

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effects of different organic modifiers on the retention of relatively simple organic solutes containing various functional groups it was concluded that significant selectivity differences existed between several water–organic modifier mobile phases. Notably, phenols were significantly retarded when eluted from reversed-phase columns with THF compared with methanol[12], despite the generally higher eluotropic strength of the former solvent[13].

In routine analysis isocratic elution rather than gradient elution of solute mixtures is the preferred mode[14] of chromatography. For some separations, gradient elution may be germane only to a preliminary "scouting" of solvent compositions leading to optimisation of isocratic conditions. In this respect general recommendations for the selection of solvent compositions suitable for isocratic elution from the results of gradient elution have been made already[14,15]. From the results of our previous studies[9] in which different organic modifiers were used in the gradient elution of hop flavonols, volume fraction, φ, of 0.15–0.30 for THF and 0.30–0.40 for methanol were calculated[15] to be suitable for isocratic elution. Our first attempts to separate mixtures of known flavonol glycosides by isocratic means using solvent mixtures containing acetic acid ($\varphi = 0.025$) and either THF ($\varphi = 0.2$) or methanol ($\varphi = 0.4$) failed, however, to separate adequately all components. Consequently a study was made of the effects of variations in modifier concentration on the retention of each of those flavonoids of interest.

We presently report that polar group selectivity differences enhanced the separation of some flavonoids in THF-containing mobile phases relative to mixtures containing methanol. Moreover, the inclusion of acetic acid in the mobile phase used for isocratic elution enabled an adjustment of retention times to be made without disturbing relative retentions.

EXPERIMENTAL

HPLC was performed on a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph using a reversed-phase column as described previously[9]. Samples of flavonoids were obtained either from commercial sources or by isolation[16] from brewer's hops (*Humulus lupulus*). The retention times of flavonols and their glycosides were measured under standardised isocratic conditions in which the volume fraction (φ) of the organic modifier under test (THF, methanol) or acetic acid was varied in different runs. Measurements were made as customary[17] to calculate the chromatographic parameters: capacity ratio, k', selectivity factor, α, resolution, R_S, and plate number, N.

Retention times for each solute were measured in duplicate at each of at least five different concentrations of organic modifier. From these measurements values of k' were calculated for each solute when eluted at each volume fraction (φ) of modifier. The orders of elution as indicated by the retention times were then confirmed using appropriate mixtures of solutes. Solvent mixtures were chosen so that the behaviour of each solute was examined over a practically useful range (1–10) of k' values. Accordingly it was valid to apply the linear relationship[14,15]

$$\ln k' = \ln k_0 - S\varphi \qquad (1)$$

to describe approximately isocratic retention behaviour, where φ is the volume fraction of the given organic modifier and S is a coefficient related to the solvent strength of that modifier when used pure as the mobile phase. Values obtained for ln k_0 refer to the elution of solute in acetic acid–water (2.5:97.5, v/v) but may be of little practical or theoretical significance[14].

RESULTS AND DISCUSSION

THF versus methanol as organic modifier

Acceptable resolution of all seven flavonoids was obtained using most of the THF-containing mixtures but less success with methanol-containing mixtures was evident. Linear regression analysis of the plots ln k' *versus* φ using the results given in Tables I and II gave values for ln k_0 (intercept) and S (slope) and yielded correlation

TABLE I

CAPACITY RATIOS (k') AND VALUES OF S AND k_0 FOR FLAVONOIDS ELUTED WITH ACIDIFIED SOLVENTS CONTAINING DIFFERENT VOLUME FRACTIONS (φ) OF TETRAHYDROFURAN

Column, μBondapak C_{18} (30 cm × 3.9 mm I.D.); solvents contained different volume fractions of THF made up in aqueous acetic acid (φ = 0.025); flow-rate, 2 ml/min; temperature, 30°C.

Common name	k'					S	k_0
	$\varphi = 0.15$	$\varphi = 0.20$	$\varphi = 0.225$	$\varphi = 0.25$	$\varphi = 0.30$		
Quercetin 3-rutinoside	8.83	3.12	2.12	1.42	0.77	16.22	88
Quercetin 3-glucoside	11.28	4.79	3.32	2.27	1.27	14.59	93
Kaempferol 3-rutinoside	13.16	5.01	2.98	1.92	1.00	17.40	164
Kaempferol 3-glucoside	15.60	5.80	4.25	2.87	1.48	15.57	146
Quercetin 3-rhamnoside	21.55	7.88	5.45	3.80	1.93	15.91	211
Myricetin	–	–	–	15.97	6.22	18.99	1863
Quercetin	–	–	–	25.33	9.50	18.50	2441

TABLE II

CAPACITY RATIOS (k') AND VALUES OF S AND k_0 FOR FLAVONOIDS ELUTED WITH ACIDIFIED SOLVENTS CONTAINING DIFFERENT VOLUME FRACTIONS (φ) OF METHANOL

Column, μBondapak C_{18} (30 cm × 3.9 mm I.D.); solvents contained different volume fractions of methanol made up in aqueous acetic acid (φ = 0.025); flow-rate, 2 ml/min; temperature, 30°C.

Common name	k'					S	k_0
	$\varphi = 0.35$	$\varphi = 0.40$	$\varphi = 0.45$	$\varphi = 0.50$	$\varphi = 0.60$		
Quercetin 3-rutinoside	6.57	4.41	1.59	1.08	0.50	10.65	250
Quercetin 3-glucoside	6.33	4.45	1.63	1.13	0.51	10.30	219
Kaempferol 3-rutinoside	11.56	7.67	2.63	1.70	0.73	11.44	602
Kaempferol 3-glucoside	10.88	7.40	2.59	1.75	1.03	9.87	308
Quercetin 3-rhamnoside	10.32	6.98	2.49	1.67	0.73	10.96	455
Myricetin	–	6.28	2.41	1.65	1.38	10.01	276
Quercetin	–	12.92	4.72	3.18	0.76	10.58	706

coefficients of 0.9918–0.9960 (THF) and 0.9632–0.9878 (methanol). The straight-line plots obtained for both solvents were then appraised with a view to devising optimal isocratic conditions.

At all concentrations of THF in the mobile phase the aglycones quercetin and myricetin were well separated from each other and from the flavonol glycosides (Table I). Moreover the quercetin rhamnoside (quercitrin) was always eluted after the other glycosides but before the aglycones. The order of elution of the rutinosides and glucosides depended on the concentration of THF used. Quercetin glucoside preceded kaempferol rutinoside with volume fractions below 0.2 but this order was reversed at higher concentrations (Table I). This observation reconciles reports of different orders for flavonol glycosides eluted by THF using different gradient programmes. Using a gradient of THF increasing from 25% to 50% (v/v) Asen[10] reported the order of elution to be quercetin 3-rutinoside (QR), kaempferol 3-rutinoside (KR), quercetin 3-glucoside (QG) and kaempferol 3-glucoside (KG). In contrast the flavonol glycosides were eluted in the order QR, QG, KR and KG by a gradient increased from 0% to 50% (v/v) THF[9].

Solvent mixtures containing methanol achieved less separation of the flavonoids than was required. Notably the rutinoside and the glucoside of quercetin were not eluted with adequate resolution at any concentration of methanol (Table II). Although QG was eluted slightly ahead of QR, in agreement with others[4,6,7], this order was reversed at higher concentrations. A similar reversal of order occurred with the glucoside and rutinoside of kaempferol which were poorly resolved also. Separation was complicated further by the similar retentions of myricetin, quercetin rhamnoside, and kaempferol rutinoside. Except at the higher concentrations of methanol used myricetin preceded these two glycosides, confirming previous reports[4,6,7]. This study concentrated on the effects of organic modifiers on the retention of a few flavonoid solutes but some comments concerning possible correlations between retention and structure are appropriate.

Quite clearly the glycosylation of an OH group produced a much greater hydrophilic interaction[6] in THF-containing solvent than in methanol-containing solvent[3]. Moreover, the shielding effect[6] on other hydrophilic substituents caused by the introduction of different sugars seemed less in aqueous THF than in aqueous methanol, and accordingly the different glycosides were much more readily separated in the former solvent.

Acetic acid and solvent strength, S

The coefficient S in eqn. 1 is a measure of the solvent strength of the organic modifier[15], but this is not a constant characteristic for a given solvent. Nevertheless, comparisons between values of S in Table I and Table II indicate that THF is the stronger eluent for flavonoids in the reversed-phase system used. This is in general agreement with other findings but the S values given herein are *ca.* 1.5 times greater than corresponding values quoted for other solutes[15]. It has been noted elsewhere[18], however, that polymeric flavonoids displayed S values higher than those of simple molecules and that these values increased with molecular weight. It should be considered also that the S values given herein are for mixtures of THF- or methanol-containing acetic acid.

Following earlier reports[3,10] of successful separation of phenolic compounds

by reversed-phase chromatography it has become a widespread practice to include small amounts (0.1–5%)[3,7] of acetic acid in the solvent mixtures used for this purpose. Ostensibly acetic acid has been included for sound reasons, such as the suppression of ionisable acidic groups[3] when present, or to decrease the tailing of peaks[19] or for unspecified improvements in separation[7]. Surprisingly, the influence of acetic acid on the retention of solutes is an aspect of eluent composition which has been relatively neglected. In this study the effect of variations of acetic acid content (φ = 0–0.075) in aqueous THF (φ = 0.25) on the retention of flavonoid solutes was measured. For the solutes examined no effect of acetic acid on peak tailing was obvious but acetic acid had a pronounced influence on retention. In Fig. 1 values of ln k' for five flavonol glycosides are plotted against φ for acetic acid. Straight lines were drawn following regression analysis of the data which yielded correlation coefficients from 0.9897 to 0.9997. The similarities in slope and calculated S values indicated that separation was almost independent of acetic acid content. Calculations of selectivity factors (α) for pairs of solutes indicated a maximum change of 12% and a mean change of *ca.* 8% over the range of acetic acid concentrations studied. As indicated by the values of S obtained (Fig. 1) acetic acid is a stronger solvent than either THF or methanol in the system studied. It is conceivable that the powerful influence of acetic acid as an organic modifier might be overlooked either when devising elution programmes or when comparing retention data obtained by different workers with different solvents.

Crude extracts of hops

Owing to the pronounced dependence of elution order on both the quantitative and qualitative composition of the eluent used, other studies [13,15,20] provided some-

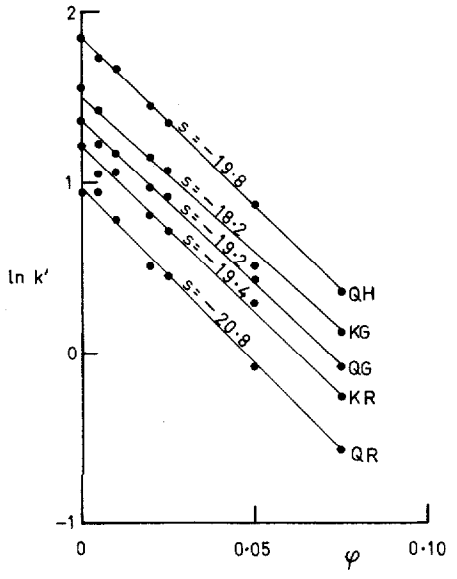

Fig. 1. Variation of ln k' of flavonol glycosides *versus* φ for acetic acid in aqueous tetrahydrofuran (φ = 0.25). The flavonol glycosides were: QH = quercetin 3-rhamnoside; KG = kaempferol 3-glucoside; QG = quercetin 3-glucoside; KR = kaempferol 3-rutinoside; QR = quercetin 3-rutinoside. Slopes, s, obtained and lines fitted by regression analysis of data points.

what fallible guidance to optimum conditions for isocratic elution of hop flavonols. Our previous studies[9] showed that four glycosides were predominant in the flavonoid constitution of hops, and the results in Tables I and II indicated that isocratic conditions for their separation were limited to a few possible options. To test the accuracy of these predictions a range of solvents were tested which were expected to vary considerably in separating efficiency.

Samples (10 μl) of a crude extract containing flavonol glycosides obtained from hops[9] were chromatographed using isocratic elution with solvents containing three different concentrations of THF made up with and without acetic acid (φ = 0.025). At a THF volume fraction of 0.2 glycosides QG and KR co-chromatographed, and at a volume fraction of 0.25 baseline resolution of the four major hop glycosides was not achieved. In contrast all four glycosides were well resolved (resolution QR/QG = 2.11, QG/KR = 1.52, KR/KG = 1.73) by the 25-cm column (N = 6400 plates/m) when the volume fractions of THF and acetic acid were 0.15 and 0.025, respectively. The flavonols of interest were eluted with retention times between 13 min and 23 min. When the corresponding solvent without acetic acid was used the relevant retention times were increased to between 22 min and 40 min, although separation was effective. Inclusion of acetic acid in the solvent mixture therefore permitted separations to be performed in an acceptable time which could not otherwise be achieved by variations in THF content alone.

As expected from the observed behaviour of individual flavonoids (Table II) and simple mixtures, methanol-containing solvents failed to separate adequately the flavonol glycosides in crude extracts of hops. Elution with a solvent containing methanol (φ = 0.35) and acetic acid (φ = 0.025) separated hop flavonols into two main peaks, the first of which to emerge contained quercetin rutinoside and glucoside while the second contained corresponding glycosides of kaempferol.

From this study it appears that some general conclusions drawn on the behaviour of relatively small solutes in reversed-phase chromatography[13–15,20] may not be directly applicable at present to more complex molecules, such as flavonol glycosides. Notably, the effects of changes in solvent composition on solute retention were imperfectly predictable from theoretical models based on other solutes[13,14]. Reversals in the elution order on changing composition have been noted before[3] and were attributed to the relative solubilities of solutes in solvent mixtures of different organic contents. While the use of acidified THF rather than acidified methanol was demanded by the special requirements of this study it seems possible that extension of this work to other solutes might reveal more features of the relationship between retention and structure.

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