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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FLAVONOIDS IN BARLEY AND HOPS

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SUMMARY

Gradient elution from a reversed-phase high-performance liquid chromatography column was used to separate and quantitate the flavonol glycosides and simple flavanol oligomers extracted from hops and barley. Whereas flavonol monoglycosides, diglycosides and triglycosides, and flavanol monomers, dimers and trimers were readily separated, polymeric flavanols were not resolved. A column temperature of 30°C was found optimal for the separation of flavonol aglycones by isocratic elution.

INTRODUCTION

Flavonoids are very widely distributed in the plant world and a great diversity of chemical structures has been encountered[1]. Most of these polyphenols contain catechol and phloroglucinol units within their structures, which confer on them an important range of chemical reactivities. Notably, flavonoids are very susceptible to oxidation and may also undergo both intramolecular and intermolecular hydrogen bonding, and most show a capacity to chelate metals[2]. Unfortunately, the reactivity of flavonoid materials present in barley and hops can cause problems at several stages in the brewing process[3]. Most important of the problems known to be associated with flavonoids is the formation of unacceptable hazes and undesirable flavours in beer[4,5]. For these reasons the measurement of flavonoids in brewing materials and in beers is a prominent feature of judicious quality control. Until recently, the standard methods for measuring polyphenols have suffered from shortcomings in specificity, speed or precision[6]. The advent of high-performance liquid chromatographic (HPLC) techniques has greatly enhanced the measurement of a variety of simple phenolic constituents[7–10], including flavonoids from a number of plant sources[11–16], but the applications in brewing science have been relatively few[17–21]. In this study, HPLC on reversed-phase columns was used to measure the contents of specific components in grains of barley (*Hordeum vulgare*) and in flower cones of hops (*Humulus lupulus*). The flavonoids of interest were flavonol glycosides, *e.g.* rutin (Fig. 1) and simple flavanol oligomers, *e.g.* procyanidin B-3 (Fig. 2). The results obtained indicated that reversed-phase HPLC has wide applicability for the measurement of flavonoids in the raw materials of brewing.

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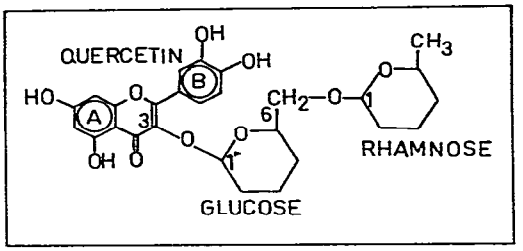

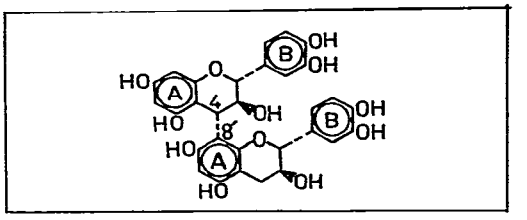

Fig. 1. Structure of rutin as an example of a flavonol glycoside. Other flavonol glycosides may differ in the number of B-ring hydroxyl groups in the flavonol aglycone (*e.g.* myricetin has three, quercetin two, kaempferol one, and galangin none) or in the glycosidic units.

Fig. 2. Structure of procyanidin B-3, a dimer of (+)-catechin as an example of a simple flavanol oligomer. Other oligomers may be derived from combination of (−)-epicatechin (two B-ring hydroxyl groups) or (+)-gallocatechin (three B-ring hydroxyl groups).

EXPERIMENTAL

Chemicals and equipment

Kaempferol, (−)-epicatechin, and diphenyl boric acid 2-aminoethyl ester were obtained from Sigma (Poole, Great Britain). Galangin and pelargonidin chloride were obtained from Fluorochem (Glossop, Great Britain). Rutin and (±)-catechin were obtained from Koch-Light Labs. (Colnbrook, Great Britain). Delphinidin chloride was obtained from Apin (Cardiff, Great Britain). Quercetin, quercitrin, cyanidin chloride and myricetin were obtained from ICN (Plainview, NY, U.S.A.). Apart from HPLC-grade solvents, all other chemicals were obtained from BDH (Poole, Great Britain).

HPLC was performed on a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph equipped with two Model 6000 solvent delivery systems, a Model 660 solvent programmer, a Model U6K injector, a Model 440 absorbance detector and a Model 730 data module. The pre-packed reversed-phase column (30 cm × 3.9 mm I.D.) contained μBondapak C_{18} (Waters Assoc.) and was eluted with degassed and filtered mixtures of 2.5% (v/v) analytical grade acetic acid (BDH) in water containing organic modifiers[22]. For different purposes, the organic modifier used (HPLC grade) was either methanol (Rathburn, Walkerburn, Great Britain), acetonitrile (Rathburn) or tetrahydrofuran (Fisons, Loughborough, Great Britain), and was included either at a fixed concentration (isocratic elution) or automatically increased in concentration (gradient elution) during chromatographic development. A mobile phase flow-rate of 2.0 ml/min was used throughout this study, and a standard column temperature of 30°C was maintained by a glass water-jacket connected to a circulating system. Detection was carried out at either 254 nm, 280 nm, 365 nm or 546 nm as required, with a detector sensitivity of 0.1 a.u.f.s. Samples (5–50 μl) were injected through a Microliter 810 syringe (Hamilton, Reno, NV, U.S.A.).

Extraction of flavonoids from plant material

Extracts were prepared from ground hops or barley grains in acetone–water (75:25, v/v) as described previously[23]. The extracts so prepared were either used directly for HPLC analysis or fractionated further when necessary. As a first frac-

tionating step, an acetone–water extract was saturated with sodium chloride and then allowed to separate into two liquid phases. The upper liquid phase was then mixed with an equal volume of hexane, and phase separation was allowed for a second time. The lower liquid phase obtained by the second separation contained the bulk of the simple flavanols and flavonol glycosides, and was termed the flavonoid extract. The procedure not only accomplished a four-fold concentration of the simple flavonoids present in the acetone–water extract but also removed unwanted phenolic acids, depsides and polymeric flavanols.

Preparative chromatography of flavonoids

Flavonoid extracts from hops (100 g) or barley (500 g) were evaporated to dryness under vacuum at 30°C and then dissolved in ethanol (50 ml). The ethanol solutions were then applied to columns (100 cm × 2.5 cm I.D.) of Sephadex LH-20[24] (Pharmacia, Uppsala, Sweden) and eluted for 5 days with ethanol (50 ml/h). The distribution of flavonols and flavanols in the fractions collected (15 ml) was determined respectively by absorption measurements at 350 nm and reactivity with dimethylaminocinnamaldehyde[23]. Pooled fractions were then concentrated and chromatographed on columns (30 cm × 1.5 cm I.D.) of Sephadex G-25 Superfine (Pharmacia) eluted with increasing concentration of methanol in water[25]. Whereas this procedure was sufficient to purify most flavonoids, certain flavonol glycosides required final purification by preparative paper chromatography[26]. Identities[24,25] were assigned to the recovered flavonoids from the results of two-dimensional cellulose thin-layer chromatography (TLC), and examination of the products formed on hydrolysis with acid, alkali or enzymes.

Simultaneous extraction and hydrolysis of hop flavonoids

Each sample (10 g) of finely ground hops was refluxed in 150 ml of methanol–4 *N* HCl (75:25, v/v) for 2 h. After cooling, the hop debris was removed by filtration and washed twice with methanol (75 ml). Filtrate and washings were then combined and made up to 400 ml with water. The combined filtrate was then shaken with hexane, (100 ml) and, after the separated hexane phase had been discarded, the red filtrate was adjusted to 500 ml with methanol. A sample (10 ml) of the filtrate was centrifuged (15,000 *g* for 30 min) clear and examined by HPLC for flavonol aglycones and anthocyanidins[27] (flavylium ions).

Thin-layer chromatography

Two-dimensional chromatography on thin layers (20 cm × 20 cm) of cellulose (Eastman-Kodak, Rochester, NY, U.S.A.) was used for the identification of flavonoids and their products. Chromatograms were developed in the first dimension with isoamyl alcohol–acetic acid–water (2:1:1, v/v/v) and in the second dimension with acetic acid–water (6:94, v/v). For detection of flavonoids the three different spray reagents used contained either ferric chloride–potassium ferricyanide[26], diphenylboric acid 2-aminoethyl ester[25], or 4-dimethylaminocinnamaldehyde[23].

Raw materials

The samples of the hops used were from the varieties; Brewer's Gold, Bullion, Challenger, Comet, Fuggles, Hallertau, Northern Brewer, Pride of Ringwood, Talis-

man and Wye Target. The barley varieties examined were; Ackermann 253, *ant*-13, Aramir, Ark Royal, Arkil, Athos, Emma and Roland.

Procyanidins

Samples of procyanidins B-1, B-2, B-3 and B-4 were kindly given by Dr. E. Haslam of the University of Sheffield, Great Britain. Procyanidin B-3 was also synthesised by the method of Eastmond[28].

RESULTS AND DISCUSSION

Flavonol glycosides in hops

Samples (5–25 μl) of the unfractionated acetone–water (75:25, v/v) extracts of hops were injected directly on to the HPLC column. Owing to the complex composition of these extracts, gradient elution was required to achieve adequate resolution of the constituent flavonol glycosides. Three organic modifiers were tested in conjunction with acetic acid–water (2.5:97.5, v/v) as eluents, and were ranked according to the separations obtained. Accordingly, in order of decreasing separating efficacy the solvents were graded (i) tetrahydrofuran (THF), (ii) acetonitrile, (iii) methanol.

An example of the separation of flavonol glycosides obtained from Challenger hops, which was representative of the ten varieties of hops that were examined, is shown in Fig. 3. Typically the chromatographic profile of hop flavonols consisted of four peaks and numerous incompletely resolved minor peaks. The identities of the major flavonol components were assigned on the results of co-chromatography with standards, obtained by isolation from hops[25] when not available commercially. Sixteen substances that displayed the properties of flavonol glycosides were isolated from hop extracts by chromatography on either Sephadex LH-20 or Sephadex G-25. Whereas complete chemical characterisation of only six of these substances was accomplished, tentative designations were assigned to the remainder. Quantitatively the predominant glycosides present in hops were monoglycosides and diglycosides of both kaempferol and quercetin. The most numerous group of glycosides present consisted of members that displayed the chromatographic mobilities of triglycosides on cellulose TLC and on dextran gels. A separation of selected glycosides of quercetin is shown in Fig. 4. With unfractionated hop extracts, the presence of many minor components slightly obscured the HPLC separation of the four major components, which were the β-3-rutinosides and β-3-glucosides of quercetin and kaempferol. Nonetheless, any large differences seen between the chromatographic profiles of extracts obtained from different varieties of hops were invariably in their relative contents of these four glycosides. A comparison of the HPLC profiles of extracts prepared under standard conditions provided, therefore, estimates of variability in the contents of flavonol glycosides in hops. For greater precision in the measurement of differences in the contents of flavonols in different hop varieties, a less complex separation was required.

Hydrolysis of flavonol glycosides

To simplify analysis, flavonols were simultaneously extracted from ground hops and hydrolysed in methanolic HCl to release their aglycones. Although the

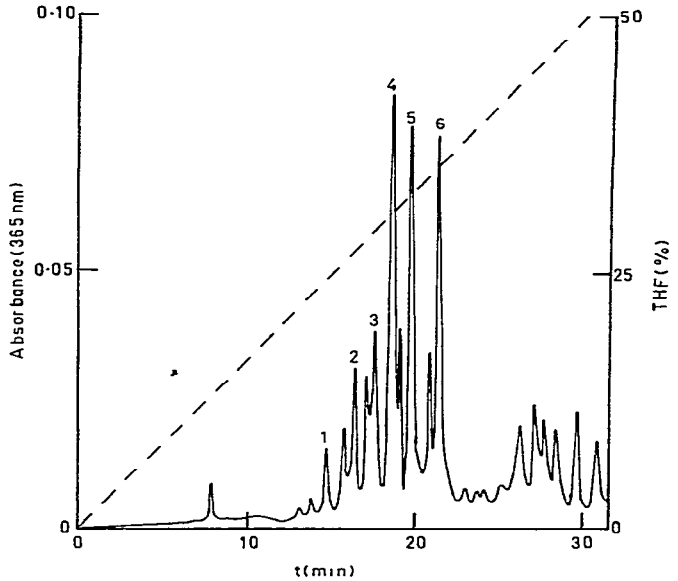

Fig. 3. Separation of flavonol glycosides from hops by gradient elution on μBondapak C_{18}. Peaks: 1 = quercetin triglycoside; 2 = quercetin neohesperidoside; 3 = quercetin rutinoside; 4 = quercetin glucoside; 5 = kaempferol rutinoside; 6 = kaempferol glucoside. The broken line is the percentage of THF in the mobile phase.

hydrolysis rates of isolated flavonol 3-glycosides is rapid[1], a reaction time of 2 h was required to ensure maximum recoveries of aglycones from the ground hop material, suggesting that rate of extraction determined the overall yields. Prolonged (4 h) or

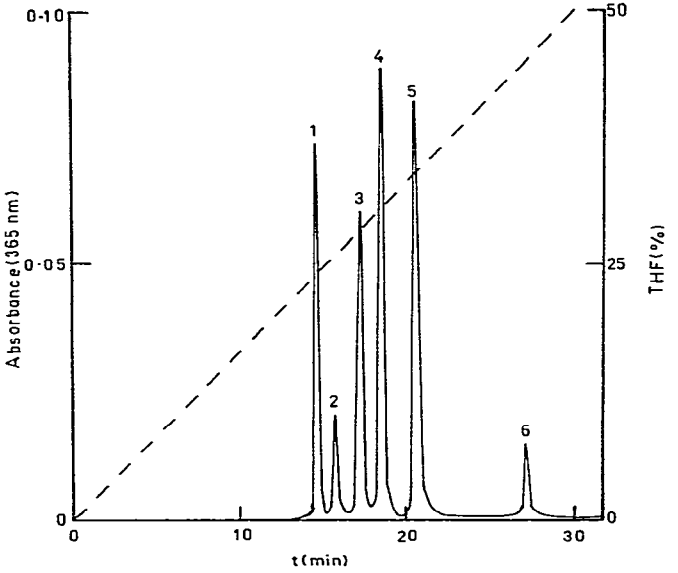

Fig. 4. Separation of quercetin glycosides by gradient elution on μBondapak C_{18}. Peaks: 1 = quercetin triglycoside; 2 = quercetin neohesperidoside; 3 = quercetin rutinoside; 4 = quercetin glucoside; 5 = quercetin rhamnoside; 6 = quercetin. The broken line is the percentage of THF in the mobile phase.

repeated (2 h) extraction cycles increased the overall yields by no more than 5% total and were considered unnecessary. Precautions to minimise autoxidation[29] were judged effective, since rutin yielded 98% of the theoretical yield of quercetin, and neither myricetin, quercetin nor kaempferol were denaturated significantly, when these were included as controls in experiment. When samples of hops were hydrolysed in triplicate, the coefficients of variation (C.V.) for measurements of kaempferol and quercetin were never greather than 2.5%

The amounts of aglycones recovered from hydrolysed hop cones were measured by HPLC using isocratic elution, which facilitated rapid repetitive analysis. Hydrolysates from hops contained kaempferol and quercetin as the predominant flavonol aglycones and traces of another substance similar in chromatographic behaviour to myricetin. Accordingly, the chromatographic system was calibrated with these three aglycones for measurements of peak areas.

Separation and quantitation of flavonol aglycones

Gradient elution was not required for satisfactory resolution of the three flavonol aglycones and, using a solvent containing equal volumes of methanol and acetic acid–water (5:95, v/v), the analysis was completed in 15 min with a column temperature of 30°C. With the appropriate integration parameters, peak area measurements of aglycone standards were reproducible (C.V. = 2.5%, n = 10) and linearly related to sample loadings in the range 0–1 μg. Since it has been claimed[14] that elevated temperatures improve the separation of flavonols on reversed-phase columns, the effect of changes in the range 10–60°C on the separation of mixtures of four aglycones was investigated. Components of this mixture were well separated and eluted in the order myricetin: quercetin: kaempferol: galangin.

Van 't Hoff plots of retention data for the four flavonol aglycones showed the classical dependence of log k' and $1/T$ (where k' = capacity ratio and T = temperature)[30]. Linear regression analysis of the data gave values for the slopes and intercepts of the four straight lines obtained. From the slopes the enthalpies of transition (stationary to mobile phase) for each aglycone were calculated to be: 6.54, 6.42, 6.31 and 6.20 kcal/mol, for myricetin (myr), quercetin (que), kaempferol (kae) and galangin (gal), respectively, and increased slightly with increasing retention. This result signifies that temperature variations had only a small effect on column selectivity. Selectivity, however, depends both on the enthalpies and entropies of transition of solutes[30]. Calculation of the standard entropies of transition from intercept values of −4.278, −4.014, −3.710, and −3.296 for myricetin, quercetin, kaempferol and galangin was not possible since the phase ratio of the column[30] was not known. Nevertheless, the differences in the intercept values obtained from the Van 't Hoff plots (*cf.* ref. 31) suggested a marked dependence of column selectivity on the entropies of transfer for these solutes, which may differ in the contiguity of their orientations on the stationary surface[30].

Although temperature variations had little effect on column selectivity an optimum temperature of 30°C for separation was found. Peak skewness[30] progressively increased as the temperature of the column was decreased below 30°C, presumably owing to non-linearity of the absorption isotherm at low temperatures. Moreover, maximum values for the resolution of neighbouring pairs of aglycones (myr/que = 4.26, que/kae = 4.76, kae/gal = 8.5) in the chromatograms and measurements of

plate numbers (*ca.* 6000 plates/m) were obtained for separations performed at 30 °C. These changes in column efficiency may be explained by the effects of temperature on solvent viscosity and swelling of the stationary phase[30].

The contents of flavonols, measured as aglycones, did not differ greatly in samples of ten varieties of hops harvested in 1980. Contents of kaempferol ranged from 0.82 to 1.63 mg/g and averaged 1.20 mg/g, while contents of quercetin ranged from 0.32 to 1.44 mg/g and averaged 0.92 mg/g. The contents of the four major flavonol glycosides and of their parent aglycones found in three varieties of hops are given in Table I.

TABLE I

CONTENTS OF MAJOR FLAVONOL GLYCOSIDES AND TOTAL FLAVONOL AGLYCONES IN THREE VARIETIES OF HOPS

Hop variety	*Flavonol glycosides (mg/g)*				*Total flavonol* aglycones (mg/g)*	
	Kaempferol (kae)		*Quercetin (que)*			
	Glucoside	*Rutinoside*	*Glucoside*	*Rutinoside*	*Kae*	*Que*
Bullion	0.62	0.68	0.08	0.13	0.82	0.52
Challenger	0.44	0.60	0.37	0.26	0.89	0.85
Talisman	0.31	1.16	1.04	0.91	0.87	1.32

* Measured after hydrolysis of total glycosides.

Flavanols in barley

The acetone–water extract of barley contained many UV-absorbing substances, which interfered grossly with the chromatography of simple flavanols. The problem of separation was ameliorated greatly by prefractionation of the acetone–water extract so that the simple flavanols were freed largely from contaminating phenolic acids and polymeric tannins. Preparative-scale chromatography of flavanol extracts on dextran gels yielded catechin, three flavanol dimers, four flavanol trimers and polymeric material. The HPLC separation of a mixture of the isolated propelargonidin, procyanidin B-3 and prodelphinidin B-3 dimers is shown in Fig. 5. The superior selectivity of reversed-phase HPLC permitted the resolution of two minor trimeric components, which were not well separated from the two major trimeric[32] components of barley by chromatography on dextran gels. Moreover, HPLC of the hydrolysis products[27] of the trimers revealed that two of the trimeric flavanols yielded either cyanidin or delphinidin exclusively. In contrast, the other two trimers yielded mixtures of cyanidin and delphinidin, although one or other of these anthocyanidins clearly predominated. These findings are entirely consistent with proposals made recently for the structures of flavanol trimers in barley[33].

All the simple flavanol components were identified by their retention times in HPLC profiles of flavanol extracts of barley, even though some were present in relatively small amounts. The failure of the prefractionating procedure to eliminate polymeric material completely from the flavanol extract, however, diminished the resolution of the smaller molecules. When recovered flavanol polymers were chromatographed on μBondapak C_{18} they were eluted as a broad band that extended through-

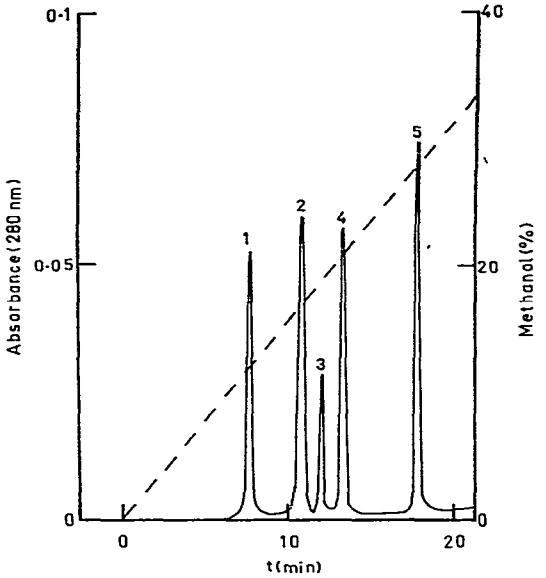

Fig. 5. Separation of flavanol dimers and monomers by gradient elution on μBondapak C_{18}. Peaks: 1 = prodelphinidin B-3; 2 = procyanidin B-3; 3 = propelargonidin; 4 = (+)-catechin; 5 = (−)-epicatechin. The broken line is the percentage of methanol in the mobile phase.

out the chromatogram. Despite this interference, the major flavanols were sufficiently resolved (Fig. 6) to permit their recognition and measurement. Extracts obtained from seven varieties of malting barley all contained measurable amounts of catechin, prodelphinidin B-3 and procyanidin B-3 (Table II). In contrast, none of these was detected in the mutant barley *ant*-13, which is blocked in the biosynthesis of flavanols[34].

Flavanols in hops

Unlike barley, hops contained a preponderance of polymeric material that was not separated sufficiently from the simple flavanols in the prefractionating stage to permit satisfactory HPLC of the flavonoid extract. To separate the simple and polymeric flavanols, the flavonoid extract of hops was fractionated on a column of Sephadex LH-20 eluted with ethanol. During elution with 1–1.5 l of ethanol, both catechin and epicatechin were eluted in a single peak. Flavanol dimers were recovered from a peak that was eluted during the passage of 2.75–3.25 l of ethanol. Four dimeric procyanidins were present, though in different amounts, and these were separated preparatively by chromatography on Sephadex G-25. The identities of the isolated dimers were assigned on the basis of thin-layer and high-performance liquid cochromatography with authentic samples. The four procyanidin dimers were resolved well by HPLC when mixtures containing approximately equal amounts of each were chromatographed (Fig. 7). In Challenger hops the relative amounts present of the dimers B-1, B-2, B-3 and B-4 were 3:0.5:10:5. Although all these dimers are procyanidins, both delphinidin and cyanidin were detected by HPLC in the hydrolysates of whole hops. Most strikingly the ratio of cyanidin to delphinidin measured depended on the hop variety and varied from 1.2 (Northern Brewer) to 6.2 (Talisman)

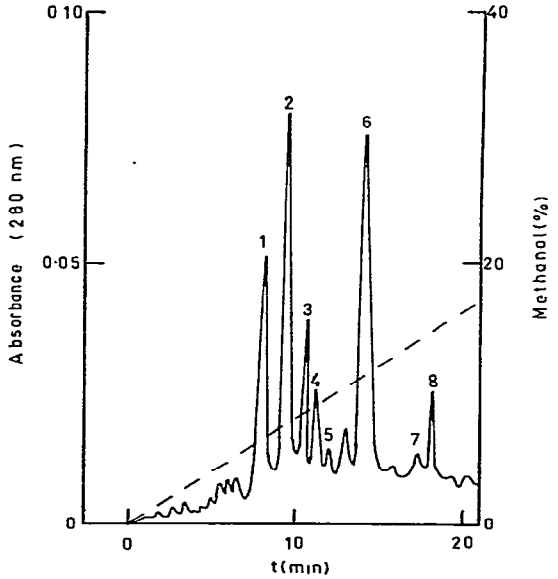

Fig. 6. Separation of flavanol trimers, dimers and monomer from barley by gradient elution on μBondapak C_{18}. Peaks: 1, 3, 4, 7 = trimers; 2 = prodelphinidin B-3; 5 = oxidised flavanol; 6 = procyanidin B-3; 8 = catechin. The broken line is the percentage of methanol in the mobile phase.

and averaged 2.5. The source of the delphinidin of hops is possibly the polymeric flavanol fraction. Five flavanols, tentatively identified as trimers, were resolved by HPLC and measured but collectively amounted to only 7.0 mg per 100 g of hops. The B-series procyanidin dimers amounted to 17.0 mg per 100 g of hops, whereas the contents of (−)-epicatechin and (+)-catechin were 19.4 mg and 26 mg, respectively, per 100 g of Challenger hops.

CONCLUSIONS

HPLC in the reversed-phase mode was suitable for the analysis of flavonol glycosides and simple flavanols in barley and hops. Doubtless, the presence in plant extracts of polymeric flavanols, which were not resolved by the column used, complicated otherwise facile separations. Even so, complex mixtures of flavanol oligo-

TABLE II

CONTENTS OF FLAVANOL MONOMER (CATECHIN) AND FLAVANOL DIMERS IN SEVEN VARIETIES OF MALTING BARLEY

Flavanol	*Content (mg/kg)*	
	Range	*Mean*
Catechin	30–95	51
Procyanidin B-3	65–350	191
Prodelphinidin B-3	108–450	230

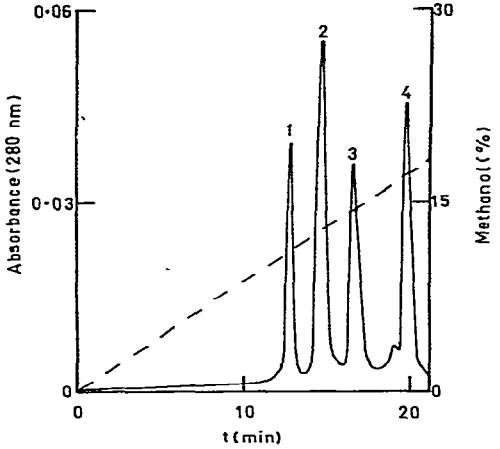

Fig. 7. Separation of procyanidin dimers present in hops by gradient elution on μBondapak C_{18}. Peaks: 1 = procyanidin B-3; 2 = procyanidin B-1; 3 = procyanidin B-4; 4 = procyanidin B-2. The broken line is the percentage of methanol in the mobile phase.

mers and flavonol glycosides were separable using gradient elution. The methods described can be used for measuring specific flavonoids in brewing materials as a useful supplement to existing quality controls.

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