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Note

High-performance liquid chromatographic determination of the degree of glycosidation of flavonols by use of an ultra-violet diode-array detector*

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Several chromatographic and spectroscopic methods such as thin-layer chromatography (TLC)[1,2], gas chromatography (GC)[3], high-performance liquid chromatography[3], NMR[3] and mass spectrometry (MS)[4] have been applied to the identification of isolated flavonol glycosides. Due to the small amounts and insufficient purity of the material available, the qualitative and quantitative analysis of sugars often proves to be difficult. Thus, a large number of hydrolysis procedures have been proposed, especially for highly glycosidated substances[1,2,5]. An approximate indication of the number of bound sugars can be obtained by applying the Egger test, involving TLC on polyamide[6].

However, if the aglycone (which can easily be determined) is known, the present method gives a more precise estimation of the number of sugar units bound to it. In addition, it allows the qualitative and quantitative determination of all the constituents resulting from the hydrolysis of the glycosides.

EXPERIMENTAL

Enzymatic hydrolysis

The isolated glycoside (1–2 mg) and 1 mg of technical enzyme (EL 1-77; Röhm, Darmstadt, F.R.G.) were dissolved in 2 ml of twice distilled water and left in a water-bath at 36°C for 70 min. During this time only a partial hydrolysis is observed. To complete hydrolysis, the incubation time was extended to 24 h, under the same conditions. Ten millilitres of ethanol were added to the hydrolysed samples, which were then evaporated to dryness under vacuum.

Benzoylation

The benzoylation procedure was that described by Galensa[7].

HPLC

The HPLC system comprised an LC-XPD pump (Pye Unicam, Philips, Kassel,

* Dedicated to Professor Dr. Karl Herrmann on the occasion of his 65th birthday.

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F.R.G.) and a 1040 HP detection system combined with HP 85 and a 82901 M flexible disc drive (Hewlett-Packard, Frankfurt, F.R.G.). A 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 10-μl sample loop and a C-R1A integrator (Shimadzu, Düsseldorf, F.R.G.) were used. The analyses were performed on a 300 × 3 mm I.D. glass column of LiChrosorb Si 60, 5 μm (Merck, Darmstadt, F.R.G.). Isocratic elution was carried out with isooctane–diethyl ether–acetonitrile (150:90:30) at a flow-rate of 1 ml/min, and detection was effected at 230 nm.

RESULTS AND DISCUSSION

Fig. 1 shows the UV spectrum of benzoylated quercetin. There is a main maximum at 233 nm and a secondary one at 295 nm. After benzoylation of all free hydroxyl groups the mesomerism of the flavonol structure becomes limited and the original maximum at 360 nm disappears. If a flavonol glycoside, such as rutin, is benzoylated, there is an increase of the extinction value of the maximum at 233 nm, which is simultaneously shifted to 231 nm. This increased extinction is attributable to the benzoylated hydroxyl groups. The low intensity maximum at 295 nm, almost entirely due to the flavonol chromophore, is shifted to 301 nm without any increase in extinction.

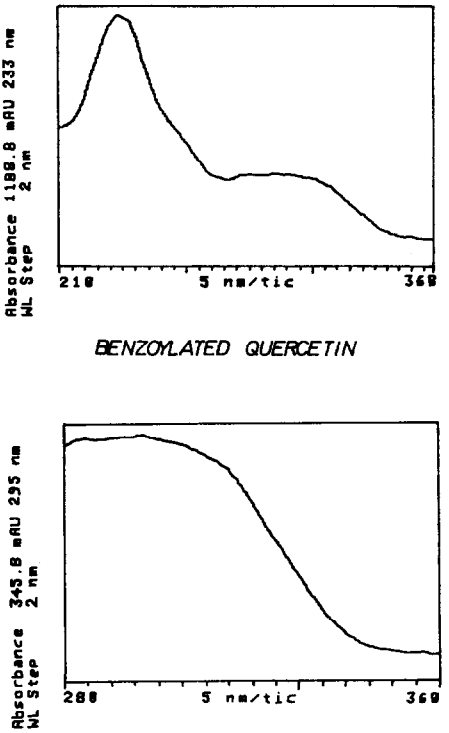

Fig. 1. UV spectra of benzoylated quercetin, taken during a chromatographic analysis. Upper curve shows the whole spectrum (210–360 nm). The lower curve shows part of the whole spectrum (280–360 nm). The absorbance of the second maximum is given and is needed for the calculations of ratios. 5 nm/tic expresses the graduation of the chosen wavelength range.

TABLE I

RELATIONSHIPS BETWEEN DEGREE OF GLYCOSIDATION AND ABSORPTION RATIO

Aglycone or glycoside benzoate	*λ (nm)*		*Number of OH groups*	*Abs. max. 1 / Abs. max. 2*
	max. 1	*max. 2*		
Kaempferol	233	295	4	2.8
Kaempferol-3-O-glucoside	231	301	7	4.9
Kaempferol-3-O-rutinoside	231	301	9	6.0
Kaempferol-7-O-neohesperidoside	231	301	9	5.0
Kaempferol-3-O-rutinoside-7-O-glucoside	231	303	11	6.5
Quercetin	233	295	5	3.4
Quercetin-3-O-glucoside	231	301	8	5.5
Quercetin-4'-O-glucoside	231	309	8	4.5
Quercetin-3-O-galactoside	231	301	8	5.5
Quercetin-3-O-rutinoside	231	301	10	6.5
Quercetin-3-O-dirhamnosylglucoside	231	301	12	7.5
Myricetin-3-O-glucoside	231	301	9	5.9
Myricetin-3-O-rutinoside	231	301	11	7.0

Furthermore, detection sensitivity considerably increases in proportion to the number of benzoylated hydroxyl groups (about a thousand-fold for each group; for instance, sorbitol, with its six hydroxyl groups, gains a six thousand-fold increase in detection sensitivity after benzoylation.

Therefore, it is possible to determine the number of bound sugars by evaluating the absorption ratio of both maxima (231 nm/301 nm), provided that the aglycone is known. As shown in Table I, the number of benzoylated hydroxyl groups can thus

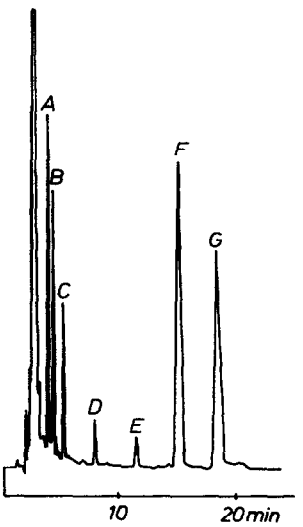

Fig. 2. Separation of benzoylated intermediates after partial enzymatic hydrolysis of a triglycoside.

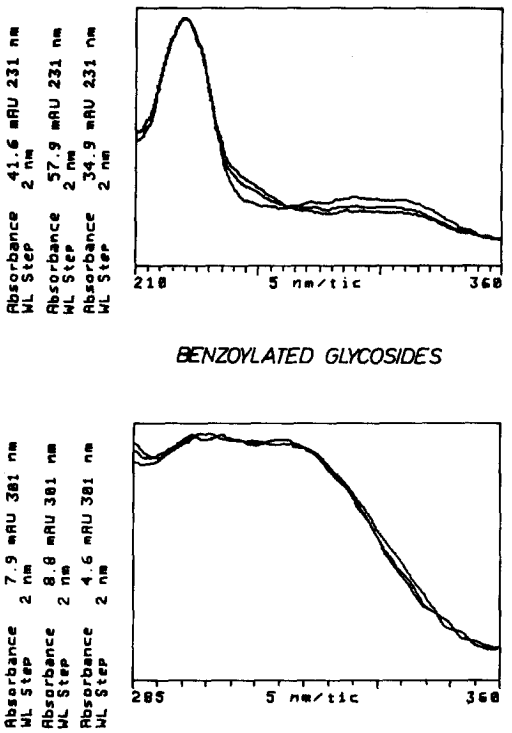

Fig. 3. UV spectra of the three glycosides (E, F, G) normalized and superposed by computer. Maximum wavelength and absorption (mAU) are printed for each spectrum.

be exactly defined, making possible some prediction about the nature of the bound sugars, *e.g.*, the distinction between hexose and methylpentose.

In some cases, knowing the aglycone, the position of the glycosidic linkage can also be determined, as the absorption maximum of the flavonol system is characteristic of the position of its glycosidic linkage (Table I). The direct determination of the degree of glycosidation with the help of a diode-array detector is exemplified by the following analysis of a unknown triglycoside.

After partial enzymatic hydrolysis and benzoylation (see Experimental), the separation by HPLC yielded seven main peaks (Fig. 2). During the analysis, the UV spectra were scanned and printed (superposed as required) after normalization by computer. The evaluation of the seven UV spectra (A–G) demonstrated the presence of three sugars (A–C), quercetin (D), one mono- (E), di- (F) and triglycoside (G). The normalized spectra of the three glycosides (E–G) are given in Fig. 3. α-, β-Rhamnose (A, B), α-, β-glucose (C), quercetin (D), quercetin-3-O-β-D-glucoside (E) and quercetin-3-O-β-D-rutinoside (F) were identified by co-chromatography. Peak G represents unhydrolysed triglycoside. After complete hydrolysis (see Experimental) the glucose/rhamnose ratio was found to be 1:2. By means of ^{13}C, ^{1}H NMR and MS the glycoside was identified as a branched quercetin-3-O-(2″-O-α-L-rhamnopyranosyl-6″-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside, details about the isolation and identification of which will be reported elsewhere. Only the structure of this sac-

charide could not be determined using the present method, as it was necessary to resort to NMR spectroscopy to decide whether its structure was branched or linear.

CONCLUSIONS

Using HPLC and a diode-array detector after benzoylation allows one to determine the degree of glycosidation of flavonols. This determination is more reliable than that obtained using the Egger method[6].

Unequivocal identifications can hardly be provided by comparison only of retention data, these being too easily affected by stationary phase–substance interactions. The present procedure makes use of more reliable, specific extinction ratios for the characterization of flavonols.

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