viburnitol has replaced pinitol as the major cyclitol, and the microsymbiont involved in nitrogen fixation in both Russian olive and alder is an actinomycete in the genus *Frankia* (Silvester, 1977).

It is possible this difference in cyclitol composition may reflect a basic difference in the metabolic pathways involved in symbiotic nitrogen fixation where *Rhizobium* is the microsymbiont (legumes) as compared to nonlegumes where the microsymbiont *Frankia* sp. is functioning. Evidence for the validity of any such hypothesis will have to await the examination of a larger number of legume and nonlegume species.

ACKNOWLEDGMENT

Registry No. Glucose, 50-99-7; fructose, 57-48-7; sucrose, 57-50-1; *myo*-inositol, 87-89-8; *chiro*-inositol, 643-12-9; trehalose, 99-20-7; sinitol, 10284-63-6; (−)-viburnitol, 488-76-6.

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Isolation and Identification of a Branched Quercetin Triglycoside from *Ribes rubrum* (Saxifragaceae)

Fred Siewek, Rudolf Galensa, and Karl Herrmann*

Quercetin 3-O-(2″-O-α-L-rhamnopyranosyl-6″-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside was isolated from leaves of red current (*Ribes rubrum*) by means of preparative HPLC. Almost complete identification of the glycoside was carried out with a 2-mg sample after benzoylation of the partially hydrolyzed triglycoside and separation of the benzoates by normal-phase HPLC. Total identification by spectroscopic methods such as UV, IR, ^{1}H NMR, ^{13}C NMR, and FAB-MS verified the results obtained after hydrolysis and determined the branched structure of the saccharide.

In the past the isolation of flavonol glycosides was carried out by column chromatographic and paper chromatographic methods (Mabry et al., 1970; Harborne and Mabry, 1982). Disadvantages of these methods are the loss of time, an often inadequate separation, and therefore an insufficient purity of the isolated glycoside. Preparative high-performance liquid chromatography (HPLC) is a time-saving and more efficient method and is also qualified for the isolation of sensitive substances. The preparative column efficiency is comparable with that of an analytical column, filled with the same stationary phase. Hence, each analytical separation can be transferred to preparative dimension. Only the flow rate must be adapted.

In the past the identification was performed after isolation by means of the UV spectra in methanol and after addition of shift reagents (Markham, 1982). Acid and enzymatic hydrolyses were attached (Mabry et al., 1970;

Markham, 1982). The gas chromatograph is used more and more in analyses of the structural members of flavonols (Harborne and Mabry, 1982).

The determination of sugars by HPLC is difficult, because the indication sensitivity is often insufficient when the available quantity is too low. The benzoylation of sugars (Galensa, 1984) and its liquid chromatographic separation are a suitable method. Each derivated hydroxyl group leads to a considerable increase in sensitivity (about a 1000-fold for each hydroxyl group; e.g., sorbitol, with its six hydroxyl groups, gains a 6000-fold increase in indication sensitivity). Also, flavonol glycosides are simultaneously determinable in small amounts (rutin, e.g., has ten hydroxyl groups). The nonpolar derivates may be separated isocratically on normal and reverse phase in the case of sugars and flavonol aglycons. For the separation of mono-, di- and triglycosides on normal phase, a mobile-phase system was developed, which allows the determination of sugars, aglycons, and the above-mentioned glycosides in a period of 20 min (see Materials and Methods). Separation of glycosides on nonpolar reverse phase was possible

Institut für Lebensmittelchemie der Universität Hannover, D-3000 Hannover 91, F.R.G.

using a gradient elution system.

In fruit juice analyses highly glycosidated flavonols may be used as proof for adulteration, so there is an interest in an easy method of isolation and a method for a safe and reliable identification. Henning (1982) detected fruit juice adulteration of strawberry concentrate with elderberry juice by determination of flavonol glycosides.

MATERIALS AND METHODS

Analytical HPLC of Flavonol Glycosides. The chromatography was performed with an HPLC system consisting of an pump (type LC-XPD), an gradient programmer (type LC-XPD), and an UV detector (type LC-UV) (all Philips-Pye Unicam, Kassel, F.R.G.). A Rheodyne 7125 injection valve (Berkeley, California) with a 10-μL sample loop and a 3390-A reporting integrator (Hewlett-Packard, Frankfurt, F.R.G.) were used. The analyses were performed with a 250 × 4.6 mm i.d. stainless steel column of Ultrasphere ODS, 5 μm. Gradient elution was carried out with solvent A (1% acetic acid) and solvent B (acetonitrile), from 5% B in A to 30% B in A in 45 min with a flow rate of 0.8 mL/min and with detection at 360 nm.

Analytical HPLC of Benzoates. The chromatographic system is the same as described under Analytical HPLC of Flavonol Glycosides. A 300 × 3 mm i.d. glass column of LiChrosorb Si 60, 5 μm (Merck, Darmstadt, F.R.G.) was used. Isocratic elution was carried out with a mixture of isooctane/diethyl ether/acetonitrile (150 / 90 / 30) at a flow rate of 1 mL/min and with detection at 231 nm.

Preparative HPLC of Flavonol Glycosides. For preparative experiments, a Pye Unicam HPLC instrument equipped with a preparative pump head (0.028–27.972 mL/min), an UV detector (see Analytical HPLC of Flavonol Glycosides) with a flow cell, preparative, and a photocell filter was used. Injections were performed by using a Rheodyne 7125 injection valve with a 2-mL sample loop. The chromatographic flow was 14 mL/min. After a first rough fractionation by means of a solvent containing 16% acetonitrile in 1% acetic acid, another two runs using a solvent of 14% acetonitrile in 1% acetic acid were necessary to obtain a complete separation of the glycoside. Each fraction was freeze-dried.

Preparation of Sample. Leaves (150 g) from "Fays Fruchtbare" were extracted 3 times with 500 mL of 70% methanol each time. By means of evaporation the extract was reduced to a small volume and filtered. The blank solution was ready for separation on a polyamide column (Grisebach and Barz, 1969). The elution of nonphenolic compounds with 1000 mL of doubly distilled water was followed by elution of phenolic compounds with 2000 mL of methanol (column 250 × 40 mm). The methanol fraction was evaporated and filled up to a volume of 50 mL with 70% methanol.

Hydrolysis/Benzoylation. One to two milligrams of the isolated glycoside and 1 mg of technical enzyme (EL 1-77, Röhm Darmstadt, F.R.G.) were dissolved in 2 mL of doubly distilled water and left in a water bath at 36 °C for 70 min. During this time only a partial hydrolysis was observed. The technical enzyme shows lack of selectivity, which is necessary for complete hydrolysis of unknown substances. We found glycosidase (α-rhamnase, β-glucosidase) and esterase activities. This is only a small spectrum of all activities of the enzyme preparation, which are not given by the producer. For complete hydrolysis the incubation time was extended to a period of 24 h while the experimental conditions were the same. After addition of 10 mL of ethanol to the hydrolyzed samples, they were evaporated to dryness under vacuum. The benzoylation
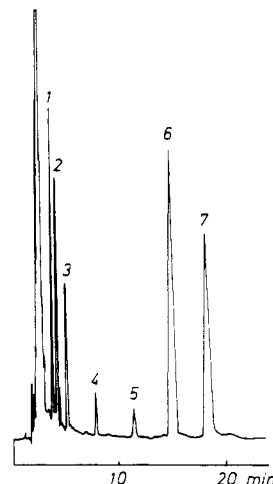
Figure 1. HPLC separation of the benzoates after partial hydrolysis of the isolated triglycoside: 1, α-rhamnose; 2, β-rhamnose; 3, α- and β-glucose, not separated; 4, quercetin; 5, quercetin 3-O-β-D-glucoside; 6, quercetin 3-O-β-D-rutinoside; 7, quercetin 3-O-dirhamnosylglucoside.

Table I. Ultraviolet–Visible Absorption Spectra of Quercetin 3-O-β-D-Dirhamnosylglucoside

	maximum, nm
MeOH	254, 265 sh, 312 sh, 354
NaOMe	271, 327, 400
AlCl$_3$	274, 298 sh, 423
AlCl$_3$/HCl	270, 299, 368 sh, 402
NaOAc	272, 323, 382
NaOAc/H$_3$BO$_3$	261, 303 sh, 373

process is described in detail by Galensa (1984).

RESULTS AND DISCUSSION

After preparative separation an amount of 130 mg of glycoside was obtained. A purity of 98% was determined by analytical HPLC on reverse phase using a water–acetonitrile gradient. The isolation from leaves was preferred to the isolation from fruits because of the higher concentrations of flavonol glycosides in leaves. Another advantage is the absence of anthocyanins in leaves, which could interfere with the separation process because of similar retention times to the ones of flavonol glycosides.

After partial hydrolysis, derivatization, and separation of the benzoates, the following intermediates were determined (Figure 1). After total hydrolysis the ratio of quercetin/glucose/rhamnose was found to be 1/1/2. By means of two hydrolyses the structure of the unknown triglycoside was obtained to the stage of quercetin 3-O-β-D-rutinoside, and also, the third bound sugar molecule could be determined.
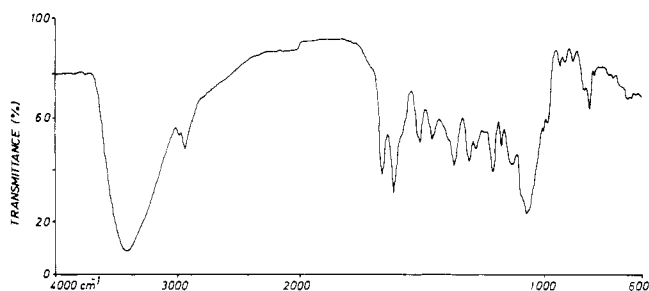
For the determination of the aglycon and the glycosidic linkage position also the UV spectra were recorded, first in methanol and then after addition of the usual shift reagents. Spectral data are given in Table I. They are typical for quercetin glycosides only substituted at position 3.

For further characterization the glycoside was measured by means of the KBr technique in the infrared part of the spectrum (Figure 2). The spectrum shows absorptions that are typical for the analyzed glycoside. In the case of flavonol glycosides the aglycon is the most absorbing part and therefore the IR spectrum only gives information about it and its substitution pattern. Quercetin 3-O-glycosides, e.g., all show similar spectra.

The question arose whether the glycoside has a linear or a branched structure. This was the only question that

Table II. Carbon Resonances in the ^{13}C NMR Spectra of Quercetin, Rutinose, and Neohesperidose Bound in Glycosides in Comparison with the Data Obtained from the Isolated Triglycoside in Me$_2$SO-d_6

		C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
quercetin	Harborne and Mabry (1982)	146.9	135.8	175.9	160.8	98.3	164.0	93.5	156.2	103.1
	found	156.6	132.7	177.2	161.2	98.9	164.8	93.6	156.4	103.7

		C-1'	C-2'	C-3'	C-4'	C-5'	C-6'
quercetin	Harborne and Mabry (1982)	122.1	115.2	145.1	147.7	115.7	120.1
	found	121.6	115.2	144.9	148.4	116.1	121.2

		C-1"	C-2"	C-3"	C-4"	C-5"	C-6"	C-1	C-2	C-3	C-4	C-5	C-6
rutinose	Harborne and Mabry (1982)	101.5	74.2	76.1	70.8	76.8	67.1	100.7	70.4	70.4	72.2	68.2	17.5
neohesperidose	Harborne and Mabry (1982)	98.4	76.9	77.1	70.1	77.3	60.9	100.5	70.5	70.8	72.2	68.3	20.9
	found	98.7	77.3	77.2	70.6	75.7	67.1	100.8	70.6	70.6	71.9	68.3	17.7
								100.6	70.4	70.4	71.9	68.3	17.3

Figure 2. IR spectrum of quercetin 3-*O*-dirhamnosylglucoside.

Figure 3. Quercetin 3-*O*-(2″-*O*-α-L-rhamnopyranosyl-6″-*O*-α-D-rhamnopyranosyl)-β-D-glucopyranoside. Negative FAB-MS.

could not be answered after the evaluation of the hydrolyses. Therefore, the ^{13}C NMR spectrum of the glycoside was recorded (Bruker Cryospec WM 400) in Me$_2$SO-d_6 as the solvent. The spectral data are shown in Table II.

For the involved carbon atoms the formation of a glycosidic linkage causes a downfield shift in the ^{13}C NMR spectrum (Harborne and Mabry, 1982). This shift was obtained for glucose at C-1″, C-2″, and C-6″ and for both rhamnoses only at carbon C-1‴/ⁱᵛ. The resonance lines caused by rhamnose had a double intensity in comparison to those observed for glucose or quercetin. Both rhamnoses were combined only with one glycosidic linkage from C-1‴/ⁱᵛ to the C-2″ and C-6″ of the glucose unit. The glucose itself was linked to the C-3 of the aglycon by C-1″.

Also, the Fast atom bombardment mass spectrum (FAB-MS) was recorded (Kratos MS 50 S with Kratos FAB source) by using glycerol as the matrix (Figure 3). The interpretation of the mass spectrum was carried out in full accordance with the other analytical results. The mass m/z 755 corresponds to the molecular weight less one proton (M – H$^+$). In the case of linear as well as branched saccharides, the cleavage of one rhamnose molecule leads to an anion with the mass m/z 609. Cleavage of the second rhamnose would be possible only in case of the linear structure. Quercetin 3-glucoside anion (m/z 461) would occur. This mass did not appear because the cleavage of a second molecule from an already charged diglycoside would generate a second negative charge. This would be very improbable. Therefore, the absence of the quercetin 3-glucoside anion mass proves the branched structure of the saccharide and supports the NMR results.

The isolated quercetin triglycoside is a suitable indicator for the detection of adulteration of black currant juice with red currant juice. It was not detectable in 14 species of black currants but was found in eleven species of red currants (3–20 mg/kg). Only in three red species was it not indicated. On the market only juice mixtures of several species are available, and therefore the glycoside was found in all analyzed trade products of red currant juice and concentrate. Four of twenty-two black currant juices of trade showed detectable amounts of the glycoside, and it
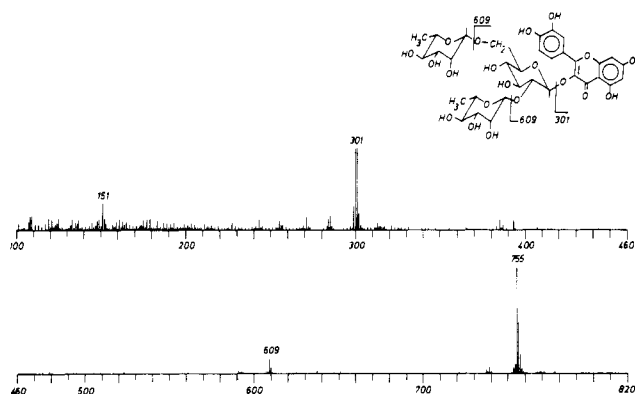
may be taken for granted the four samples were adulterated by red currant juice.

The glycoside was already isolated and identified from leaves of *Cerbera manghas* (Apocyanaceae), a wild plant growing at the Pacific Ocean coast, by Sakushima et al. (1980), by Vancraenenbroeck et al. (1978) from hop (*Humulus lupulus*), and by Buttery and Buzzell (1978) from leaves of soya bean (*Glycine max*).

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