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High-performance liquid chromatography of diastereomeric flavanone glycosides in *Citrus* on a β-cyclodextrin-bonded stationary phase (Cyclobond I)

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ABSTRACT

The flavavone glycosides prunin, naringin, neohesperidin and narirutin were separated into their diastereomers by high-performance liquid chromatography elution in the reversed-phase mode on a β-cyclodextrin bonded stationary phase (Cyclobond I). Application to the analysis of *Citrus* extracts showed that immature grapefruit fruits contained almost entirely (2*S*)-naringin and (2*S*)-prunin, whereas in grapefruit juice both diastereomers of naringin and narirutin were present (about 60% *S* and 40% *R* isomers). In bitter-orange juice only (2*S*)-neohesperidin was detected and sweet orange juice contained racemic narirutin. Benzoylated flavanone glycosides (naringin, prunin) were also separated on Cylcobond I in the normal-phase mode.

INTRODUCTION

Flavonoids are widely distributed in the plant kingdom [1]. Some flavanone glycosides, however, are unique to *Citrus* [2] and have been considered to be potential taxonomic markers [3]. For example, naringin, the chief bitter constituent in grapefruit (*Citrus paradisi*), is an indicator substance to detect grapefruit juice in sweet orange (*Citrus sinensis*) juice [4–6]. The C-2 position of the flavanone moiety is a chiral centre and (2*R*)- and (2*S*)-flavanone glycosides are diastereomeric by virtue of the carbohydrate present. From chiroptical studies it was concluded that naturally occurring flavanone glycosides have the 2*S* configuration [7]. Gaffield and Lundin [8] also reported that only immature grapefruit contained (2*S*)-naringin whereas both diastereomers were present in the mature fruit. These results were obtained by circular dichroism and NMR spectroscopy.

In recent years, high-performance liquid chromatography (HPLC) has become the technique of choice for the analysis of flavonoids, especially in complex phenolic mixtures of plant extracts [9]. Most of the HPLC separations have been performed on reversed-phase (RP) stationary phases [9]. Although diastereomeric flavanone glycosides differ in their physico-chemical properties, no separation of the isomers by reversed-phase chromatography has been reported. Recently, we reported the resolution of flavanone glycosides on a cellulose triacetate-based chiral stationary phase [10]. Unfortunately, different flavanone diglycosides could not be separated from each other on this stationary phase. *Citrus* extracts often contain more than one flavanone diglycoside (*e.g.*, naringin and narirutin in grapefruit), and therefore their analysis was impossible.

Although benzoylated flavanone glycosides have been resolved into their diastereomers on a silica gel stationary phase by HPLC [5,11], it is advantageous to have an analytical method without a preceding derivatization step.

Cyclodextrin-bonded stationary phases have been used for the separation of enantiomers, diastereomers, structural isomers and other compounds

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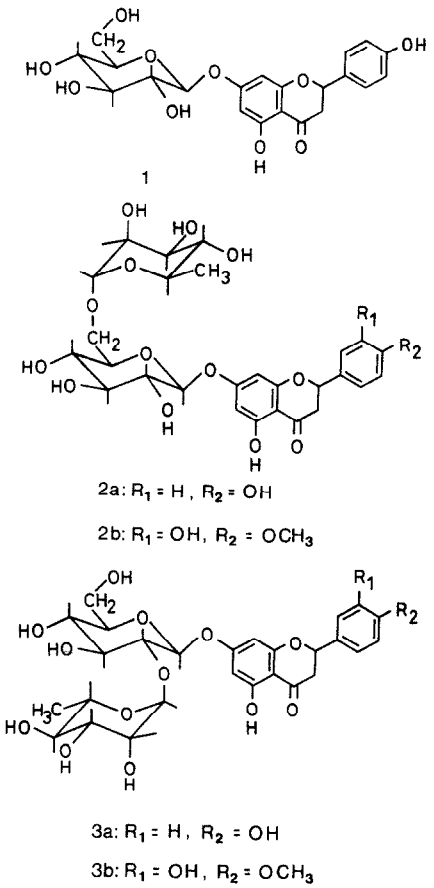

Fig. 1. Structures of flavanone glycosides. 1 = Prunin (naringenin-7-O-glucoside); 2a = narirutin (naringenin-7-O-rutinoside); 2b = hesperidin (hesperetin-7-O-rutinoside); 3a = naringin (naringenin-7-O-neohesperidoside); 3b = neohesperidin (hesperetin-7-O-neohesperidoside).

[12–15]. In this paper we report the direct separation of flavanone glycosides (prunin, naringin, narirutin and neohesperidin, Fig. 1) on a β-cyclodextrin-bonded stationary phase (Cyclobond I) in the reversed-phase mode and its application to *Citrus* extracts. Some benzoylated flavanone glycosides were also resolved in the normal-phase mode on Cyclobond I.

EXPERIMENTAL

Materials

Flavanone glycosides were of HPLC grade and purchased from Roth (Karlsruhe, Germany). The benzoylated flavanone glycosides were prepared as described previously [5,16]. Methanol, acetic acid, isooctane and diethyl ether were of analytical-reagent or HPLC grade and purchased from Baker (Gross-Gerau, Germany). Immature grapefruit (2–3 cm diameter) was a gift from Professor Dr. C. Hoeppe (Kassel, Germany) and bitter-orange juice from Dr. V. Ara (Hannover, Germany). Other samples were from commercial sources.

HPLC

The liquid chromatograph consisted of two Model 114 M pumps (Beckman, Munich, Germany), a high-pressure mixing chamber, a sampling valve (Altex 210; Beckman) equipped with a 20-μl sample loop and a Pye Unicam variable-wavelength UV detector set at 280 nm (Philips, Kassel, Germany). The peaks were recorded with a C-R6A integrator (Shimadzu, Duisburg, Germany). Peak identity was also confirmed with an HP 1040 diode-array detector (Hewlett-Packard, Waldbronn, Germany). The analytical column used was a 250 × 4.6 mm I.D. Cyclobond I (β-cyclodextrin; ASTEC, ICT, Germany). The following gradient was applied: 1 min A [water–methanol–acetic acid (90:10:0.5)]–B (methanol) (95:5), then linear from 5% to 50% B in 25 min. The flow-rate was 1 ml/min.

The configurations of the flavanone glycoside diastereomers were determined by enzymatic hydrolysis of the enriched isomers and determination of the enantiomeric aglycone on a chiral stationary phase [10].

Sample preparation

A 5-ml volume of *Citrus* juice was applied to a polyamide cartridge (Macherey-Nagel, Düren, Germany). After washing with 12 ml of water, the flavonoids were eluted with 6 ml of methanol. Water was added to give a final volume of 10 ml. Immature grapefruit and marmalade were extracted with methanol, the extract was evaporated to dryness and the residue transferred with water to the polyamide cartridge. Washing and elution steps were the same as for the juices.

RESULTS AND DISCUSSION

Fig. 2 shows two chromatograms of a mixture of flavanone glycosides. All four flavanone diglycosides

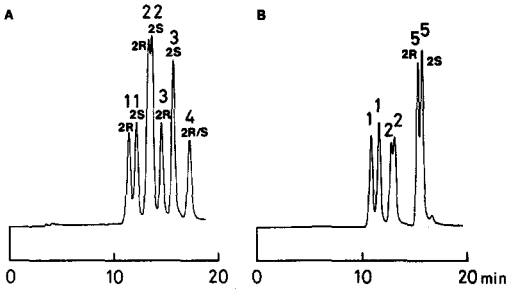

Fig. 2. HPLC of diastereomeric flavanone glycosides on Cyclobond I with gradient elution (see text). Peaks: 1 = naringin; 2 = narirutin; 3 = neohesperidin; 4 = hesperidin; 5 = prunin.

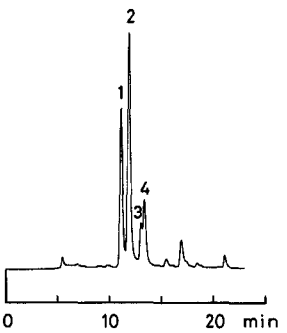

Fig. 4. HPLC of an extract of a commercial grapefruit juice extract. Peaks: 1 = (2*R*)-naringin; 2 = (2*S*)-naringin; 3 = (2*R*)-narirutin; 4 = (2*S*)-narirutin.

(naringin, nariruitin, neohesperidin and hesperidin) are separated from each other (Fig. 2A). The resolution of their diastereomers, however, depends on the sugar moiety. Naringin and neohesperidin (sugar moiety: neohesperidose) are much better separated than the rutinosides. Narirutin is only partially resolved and hesperidin not at all. Naringenin-7-O-glucoside (prunin) is also separated into diastereomers, as shown in Fig. 2B.

A chromatogram of an immature grapefruit fruit (diameter 2–3 cm) extract is shown in Fig. 3. Only about 2% of the naringin has the 2*R* configuration. The same result was reported by Gaffield and Lundin [8], who studied the composition of naringin diastereomers in grapefruit during ripening. They found that as the fruits mature, the amount of (2*S*)-naringin increased up to a final composition of 60% *S* and 40% *R* [8]. These results, obtained by circular dichroism and NMR spectroscopy after the isolation of naringin, could be confirmed by HPLC. Freshly squeezed grapefruit juice (ripe fruit) contained 66% (2*S*)- and 34% (2*R*)-naringin and a grapefruit juice from a commercial source 60% (2*S*)- and 40% (2*R*)-naringin. Fig. 4 illustrates the analysis of a commercial grapefruit juice. The diastereomeric compositions of narirutin and naringin were almost the same.

Prunin (naringenin-7-O-glucoside) has recently been identified in immature grapefruit, but has not been detected in mature grapefruit [17]. Raymond and Maier [18] isolated a chalcone cyclase from grapefruit and it was believed that glycosidation and rhamnosylation of naringin occur before flavanone formation in *Citrus* tissues. However, recently Lewinsohn *et al.* [19] suggested that glycosidation occurs at the stage of the flavanone and the monoglycoside prunin is an intermediate in the biosynthesis of naringin in *Citrus*. The convertion of exogene-

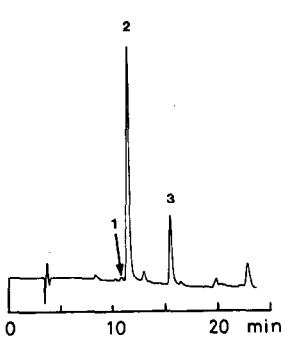

Fig. 3. HPLC of an immature grapefruit extract. Peaks: 1 = (2*R*)-naringin; 2 = (2*S*)-naringin; 3 = (2*S*)-prunin.

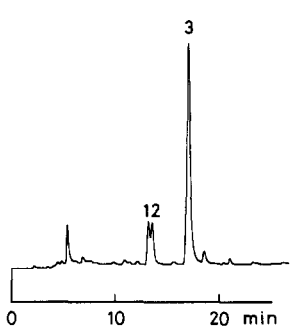

Fig. 5. HPLC of a sweet orange juice extract. Peaks: 1 = (2*R*)-narirutin; 2 = (2*S*)-narirutin; 3 = (2*R*/*S*)-hesperidin.

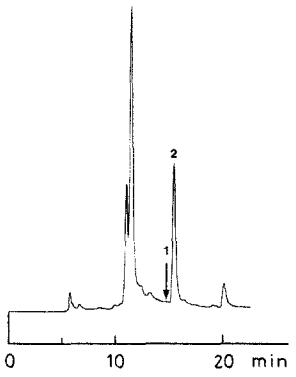

Fig. 6. HPLC of a bitter-orange juice extract. Peaks: 1 = (2*R*)-neohesperidin; 2 = (2*S*)-neohesperidin.

ous naringenin to prunin and further to naringin by grapefruit cell cultures has also been reported [20]. Fig. 3 shows that prunin is present in immature grapefruit almost exclusively as the 2*S* isomer.

Sweet oranges (*Citrus sinensis*) contain only flavanone rutinosides such as narirutin and hesperidin [2]. Fig. 5 shows a chromatogram of an orange juice extract. Narirutin is present with both diastereomers and hesperidin isomers cannot be separated with this method.

Bitter oranges (*Citrus aurantium* subsp. *aurantium*) are used for processing marmelade. In contrast to sweet oranges (*Citrus sinensis*), they contain the flavanone neohesperidosides naringin, neohesperidin and neoeriocitrin [2,21]. The results of the HPLC analysis are shown in Fig. 6. As naringin and neoriocitrin co-elute, their diastereomeric composition cannot be determined; neohesperidin is only detected in the 2*S* configuration. Horrowitz and Jurd [22] reported that the tendency for chalcone formation of flavanone glycosides depends on the substitution pattern of the flavanone. A free 4'-hydroxy group of a flavanone (*e.g.*, naringenin aglycone in naringin and narirutin) favours the reaction. Neohesperidin has a 4'-methoxy group and is therefore more stable than naringin and narirutin. This also has implications for the racemization of flavanone glycosides. The aglycone in naringin was easily racemized by heating (2*S*)-naringin at 70°C in an aqueous methanolic solution. Neohesperidin did not racemize under these conditions, but was racemized in dilute alkaline solution. In a marmalade extract (Fig. 7), most of neohesperidin is still in the 2*S* configuration, despite the heat-processing conditions. It should be mentioned additionally that in commercially available neohesperidin the 2*S* isomer also dominates (Fig. 2).

Cyclodextrin-bonded stationary phases have similar properties to a diol column when used in the normal-phase mode [13,23]. The separation of ben-

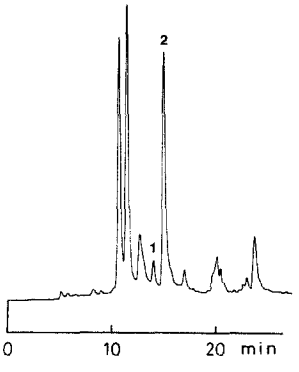

Fig. 7. HPLC of a marmalade extract. Peaks: 1 = (2*R*)-neohesperidin; 2 = (2*S*)-neohesperidin.

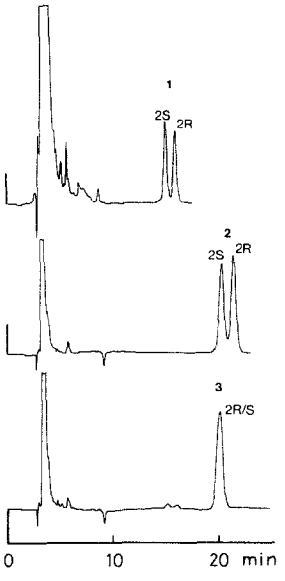

Fig. 8. HPLC of benzoylated flavanone glycosides on Cyclobond I. Mobile phase: isooctane–diethyl ether–methanol (55:40:5 v/v/v) at a flow-rate of 1 ml/min; detection at 231 nm. Peaks: 1 = prunin benzoate; 2 = naringin benzoate; 3 = narirutin benzoate.

zoylated diastereomeric flavanone glycosides has been performed on silica gel [7,11]. Fig. 8 shows that the resolution of benzoylated naringin and prunin can also be achieved on Cyclobond I, while narirutin benzoate is unresolved.

In conclusion, on Cyclobond I the direct HPLC separation of flavanone glycoside diastereomers in the reversed-phase mode and the resolution of their benzoates in the normal-phase mode are possible. Applying gradient elution, the diastereomeric composition of flavanone glycosides in *Citrus* can be determined, with the exception of hesperidin.

With the described HPLC method, the formation of flavanone glycosides can be analysed easily with respect to their C-2 stereochemistry and this may also be of interest in biochemical or enzymatic studies of *Citrus*.

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