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Racemization at C-2 of naringin in pummelo (*Citrus grandis*) with increasing maturity determined by chiral high-performance liquid chromatography

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ABSTRACT

The relative content of (2*S*)- and (2*R*)-naringin in the albedo of pummelo during maturation in the entire season was determined by normal-phase HPLC using Chiralpak IB, a polysaccharide-derived chiral stationary phase, and *n*-hexane/ethanol doped with 0.5% TFA as mobile phase. A sigmoid curve was obtained showing variation from 95.3% of (2*S*)-naringin in very immature fruits to 53% in mature fruit samples (2.3 and 14.4 cm diameter, respectively). A comparison was made with previous results obtained for grapefruit and sour orange and a tentative explanation of the bitter taste of sour orange is proposed. The Chiralpak IB is much more efficient with respect to the Chiralcel OD used for the other two *Citrus* species and separation and resolution factors of 1.73 and 9.2, respectively, were achieved. Authentic samples of naringin and neohesperidin were also separated into their C-2 diastereomers with Chiralpak IB and isolation of the pure diastereomers of naringin was accomplished.

1. Introduction

Flavanone glycosides occur in large amount in *Citrus* species and play a major role in pharmacological and nutritional effects [1,2]. Formulations of some *Citrus* species are used in Japanese and Chinese Pharmacopoeia as crude drugs [3,4]. In particular, naringin is the main constituent of the albedo (inner peel) of *Citrus grandis* Osbeck (1.6–4.4 mg/g) and in some cultivars very minor amounts of neohesperidin are also present [5]. Naringin is a chemopreventive agent toward mutagenesis of heterocyclic amines mediated by a cytochrome P 450 [6] and, among nine compounds, shows the strongest inhibitory activity of Vascular Endothelial Growth Factor Secretion (VEGF) from breast cancer cells at less than 0.1 μmol/L [7]. Naringenin, the aglycone of naringin formed in the intestinal epithelia by enzymatic hydrolysis, also inhibits cytochrome P 450 mediated oxidation of some drugs [8]. Nevertheless, the relevance of stereochemistry at the C-2 stereogenic center was not considered in these studies as well as in other ones, although it is well known that interactions of an enzymatic system leading to a functionalization of a substrate are stereospecific [9].

We have reported the direct separation of (2*R*)- and (2*S*)-naringin from the albedo of grapefruit (*Citrus paradisi*) and sour orange (*Citrus aurantium*) using a simple sample preparation and isocratic normal-phase HPLC with a cellulose tris-3,5-dimethylphenylcarbamate chiral selector coated on silica gel (Chiralcel OD-H) [10,11]. This method allowed to determine the relationship between the stereochemistry at C-2 and the increasing maturity of the fruits. In this article we report the separation of the C-2 diastereomers of naringin from the albedo of pummelo (*C. grandis*) which is chemotaxonomically related to grapefruit and sour orange. This was accomplished using a Chiralpak IB column, the same chiral selector of Chiralcel OD but chemically bonded to the silica gel support. Separation and resolution factors are strongly improved and isolation of (2*R*)- and (2*S*)-naringin is done. Also we compared the relative amount of these diastereomers in grapefruit, sour orange and pummelo during maturation and a tentative explanation of the bitter taste of sour orange is proposed.

2. Experimental

2.1. Sample preparation

Fruits of *C. grandis* at various stages of maturation were obtained from the same tree during the entire season (June–December 2007) and stored 2 or 3 days before proceeding with the analysis of the albedo. Samples were collected in an experimental field at Palazzelli, Lentini, Syracuse and at the Botanical Garden of the University of Catania, downtown Catania. Albedo (40 g, fresh weight) was cut into small pieces. These were homogenized for 5 min in a Waring blender in 200 mL of 50% methanol in water and 10 g of Celite was added during the homogenization. The homogenate was filtered immediately under vacuum through a Büchner funnel lined with Whatman no. 91 filter paper. The use of Celite was crucial to avoid the formation of a cloudy methanol–water solution when

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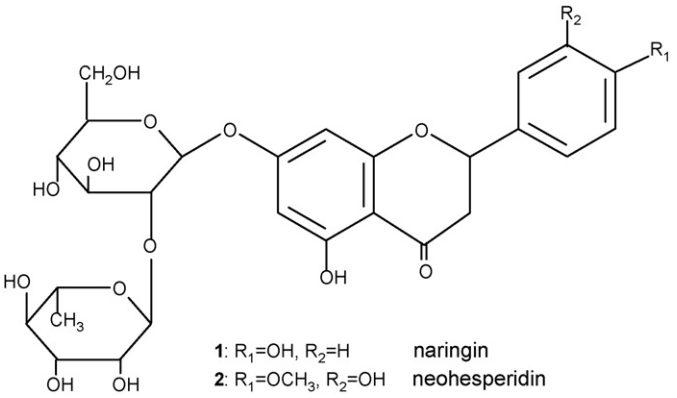

Fig. 1. Structures of naringin and neohesperidin.

working with albedo from mature fruits. A 10 mL aliquot of the solution was then added to 40 mL of 96% ethanol and dried over excess anhydrous sodium sulfate (three times) and then filtered through a non-sterile 0.2 μm PTFE syringe filter for HPLC analysis.

2.2. Chemicals and solvents

Naringin (naringenin-7-*O*-neohesperidoside) was purchased from Fluka (Buchs, Switzerland). Neohesperidin (hesperitin-7-*O*-neohesperidoside) was purchased from Extrasynthèse (Lyon, France). HPLC-grade *n*-hexane and absolute ethanol were obtained from Carlo Erba (Milano, Italy). HPLC-grade *tert*-butyl methyl ether was obtained from Fluka.

2.3. HPLC apparatus and conditions

The HPLC apparatus consisted of a Jasco pump PU 980 with Rheodyne 20 or 100 μL sample loops, a low pressure mixer LG-1580-02 and a line degasser 1550-54, a Uvidec 100-III UV spectrophotometric detector operating at 292 nm (all from Jasco). Chromatograms were acquired and processed using a computer-based Jasco Borwin 2 software. Circular dichroism (CD) spectra were recorded on a Jasco 810 spectropolarimeter (Tokyo, Japan), using 1 mm cell.

Chromatographic separations were performed on a Chiralpak IB column, 250 mm × 4.6 mm i.d., whose chiral selector is cellulose tris-3,5-dimethylphenylcarbamate chemically immobilized onto 5 μm silica gel, supplied by Chiral Technologies (Illkirch, France). A column in-line filter with 0.5 μm stainless steel frit of 3 mm diameter from Rheodyne was used to protect the HPLC column. Disposable PTFE filters of 0.2 μm pore size were used for filtration of sample solutions. Dead time (t_0) was measured by injection of tri-*tert*-butylbenzene as a non-retained marker [12]. HPLC chromatographic parameters such as α and R_s were calculated according to the usual procedure [13].

3. Results and discussion

3.1. Separation of the C-2 diastereomers of authentic flavanone glycosides

At an early stage of the method development we studied authentic naringin and neohesperidin, whose structures are shown in Fig. 1. We chose the Chiralpak IB column for the separation of the C-2 diastereomers (epimers) of these compounds since this chiral stationary phase (CSP) affords the use of solvents as mobile phase different from the usual hexane/alcohol mixtures [14]. Also, we needed a better resolution of the naringin epimers from the neohesperidin epimers with respect to the one experienced in the sour orange study.

Table 1
Chiral HPLC resolution of naringin (**1**) and neohesperidin (**2**) on Chiralpak IB.

Compd.	Mobile phase[a]	t_1	t_2	k'_1[b]	α	R_s
1	A	22.2	36.7[c]	8.23	1.73	9.2
1	B[d]	8.8	13.0[c]	2.65	1.65	7.6
1	C	4.8	6.1[c]	0.97	1.56	4.4
2	A	26.0	35.0[c]	9.84	1.38	4.1
2	B	12.4	16.3[c,e]	4.17	1.38	3.5
2	C	5.6	6.7[c]	1.34	1.34	2.5

[a] A = *n*-hexane/ethanol doped with 0.5% of TFA 70:30; B = *n*-hexane/ethanol doped with 0.5% of TFA 60:40; C = *n*-hexane/ethanol doped with 0.5% of TFA/*tert*-butyl methyl ether 20:30:50. Flow rate at 1.2 mL/min in all cases; t_0 = 2.4 min; λ = 292 nm.
[b] Retention factor of the first eluted peak.
[c] 2*S*-diastereomer.
[d] Experimental conditions used for semipreparative isolation.
[e] Peak area 79.4% with respect to 20.6% of t_1 peak.

Table 1 shows the chromatographic results for the separation of (2*R*/2*S*)-naringin (**1**) and (2*R*/2*S*)-neohesperidin (**2**) using as CSP the Chiralpak IB and three solvent mixtures (A–C) as mobile phases. In general, the separation factor (α) and resolution factor (R_s) are much better than those previously obtained using a Chiralcel OD column for both compounds [11]. They reach good values for compound **1** (1.73 and 9.2, respectively) and for compound **2** (1.38 and 4.1, respectively), using as mobile phase *n*-hexane/ethanol doped with 0.5% TFA, 70:30. A slight decrease in the separation and resolution factor values occurs using a more polar mixture of hexane/alcohol as mobile phase B. The use of the more "exotic" mobile phase C (hexane/alcohol/*tert*-butyl methyl ether) drastically reduces the elution time of the C-2 diastereomers but separation and resolution factors are also reduced.

Validation of the configuration assignment as (2*R*)- and (2*S*)-naringin from the first and second major peaks, respectively, as shown in Table 1 was obtained from the chiroptical behaviour of the C-2 diastereomers. In fact, isolation of the single epimers of naringin was performed using the conditions in line 2 of Table 1 by 100 μL repeated injections of a 4 mg/mL solution of **1** in *n*-hexane/ethanol (1:1). Collection of the eluates corresponding to the two major chromatographic peaks, centrifugation at 5000 × *g* for 10 min and rotoevaporation of the supernatants furnished about 2 mg of the single epimers. The CD spectra of isolated materials were measured in ethanol. A strong negative band centered at 292 nm and a weak positive band centered at 330 nm were observed for the epimer with higher elution time. This is indicative of (2*S*)-configuration by knowledge of the Cotton effects in the CD spectra of flavanone glycosides [10,15]. Analogously, the CD spectrum of commercial neohesperidin (**2**) shows a pronounced negative band centered at 288 nm related to the (2*S*)-configuration and this agrees with the larger second peak as reported in Table 1. Indeed, the relative amount of (2*R*)- and (2*S*)-neohesperidin are 20.6 and 79.4, respectively, from the relative areas. The predominance of one epimer with respect to the other one in this commercial sample of **2** is undoubtedly due to the extraction of it from a *Citrus* species.

3.2. Separation of naringin C-2 diastereomers in albedo extract of pummelo

We have recently studied the variation of the relative content of (2*R*)- and (2*S*)-naringin in the albedo of grapefruit [10] and sour orange [11] during maturation. This ratio affects the quality and taste of marmalades and juices and, from a biosynthetic point of view, it is interesting to observe the variation of the epimeric ratio during ripening of the fruit. Since pummelo (*C. grandis* Osbeck) is strictly chemotaxonomically related to sour orange and grapefruit

Table 2
Average composition of 2*S*/(2*S* + 2*R*)-naringin in pummelo albedo as a function of seasonal growth.

Sample	Collection site[a]	Pummelo diameter (cm)	Season day	2*S*/(2*S* + 2*R*) (%)
1	A	2.3	1	95.0
2	A	2.4	5	94.7
3	B	6.0	26	84.6
4	A	7.3	40	77.2
5	A	8.2	47	76.2
6	B	10.8	112	55.0
7	A	12.2	118	55.0
8	A	12.0	145	54.6
9	A	13.3	186	55.8
10	B	14.4	193	54.4

[a] A = Palazzelli (Syracuse) and B = Botanical Garden (Catania).

and it is considered their direct ancestor [16], we wanted to investigate the variation of the relative concentrations of naringin epimers during maturation of this fruit.

Table 2 summarizes the results for all of the samples. They were collected from the same tree over the months from two different collection sites. For very small fruits (samples 1 and 2) the albedo of 8 or 9 samples was used; for very mature fruits (samples 9 and 10) a portion of the albedo of a single fruit was sufficient for extraction, as discussed in Section 2.1. The HPLC parameters for the determination of (2*R*)- and (2*S*)-naringin were chosen according to the results shown in line 2 of Table 1. The relative percentage of (2*S*)-naringin is calculated from the integrated areas of the peaks. It is clear that this percentage undergoes a marked change with increasing maturity of the pummelo. In very small fruits, the naringin is almost all (2*S*) and remains very abundant during the very early part of the season (1–5 days of collection). As the fruit matures, more

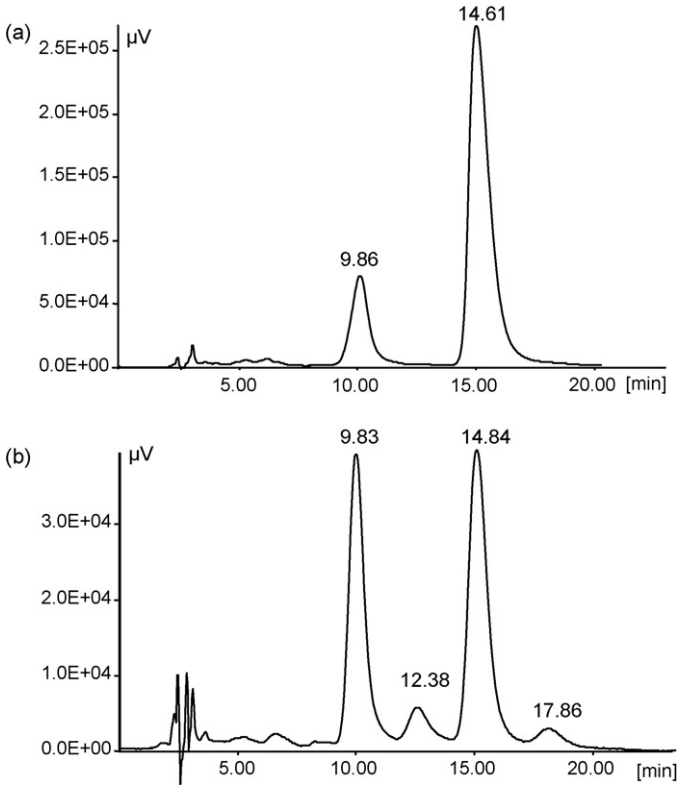

Fig. 2. HPLC separation of the C-2 diastereomers of naringin from albedo of pummelo as a function of fruit maturity (diameter): (a) 6 cm and (b) 12 cm, on Chiralpak IB. Mobile phase *n*-hexane/ethanol doped with 0.5% of TFA, 60:40, at a flow rate of 1.2 mL/min.

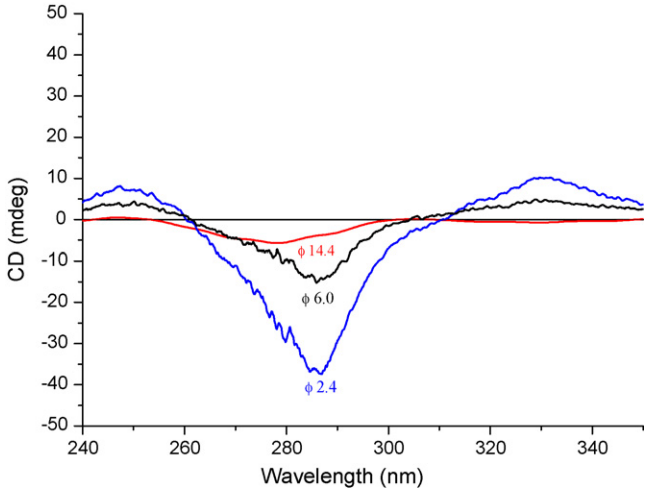

Fig. 3. CD spectra (ethanol) of extracts of the albedo of pummelo as function of the fruit diameter (ϕ).

(2*R*)-diastereomer appears until the diastereomeric percentage at ripeness is 54% in (2*S*).

As an example, Fig. 2 shows the separation of the epimers of naringin in albedo extracts of immature (a) and mature fruits (b) immediately injected after extraction in the Chiralpak IB HPLC system. The identification of the peaks is based on the results obtained

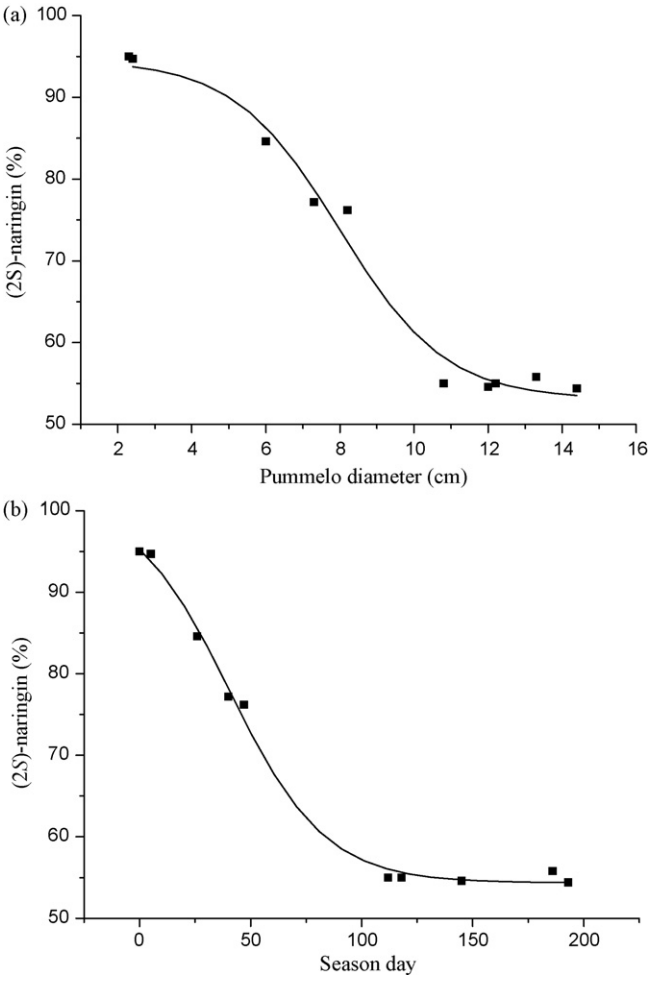

Fig. 4. Variation of (2*S*)-naringin as percentage of both diastereomers in the albedo of pummelo as a function of fruit diameter (a) and seasonal collection day (b).

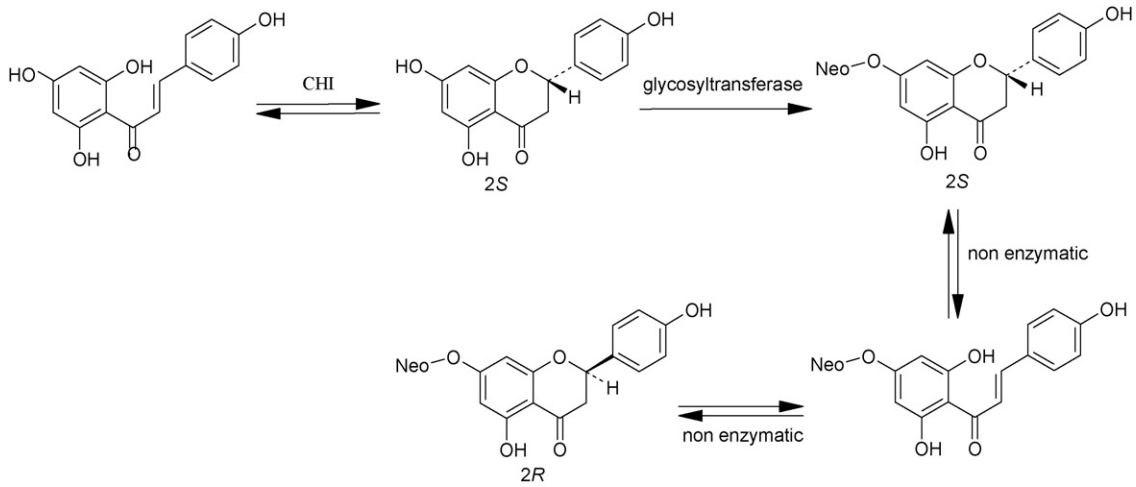

Fig. 5. Biosynthesis of naringin and its non-enzymatic epimerization.

for the authentic compound **1** as discussed in the previous section. In mature sample (b) two additional peaks eluting at 12.4 and 17.9 min are present and remain unidentified. Indeed, coinjection with authentic neohesperidin rules out the identification as neohesperidin. In any case, this compound is predominantly found as (2*S*)-diastereomer in other *Citrus* species and in commercial samples [3,11,17].

Fig. 3 shows the qualitative CD spectra of the extracts of the albedo of pummelo as a function of the fruit diameter. The strong negative band associated to the Cotton effect of (2*S*)-naringin markedly decreases during maturation and, at ripeness, the band is centered at slightly lower λ. This fact agrees with the presence of an additional compound in the extract as shown in Fig. 2(b) contributing to the CD band. For this reason, these data cannot be used for quantitation of the relative content of naringin epimers. In addition, the quality of the albedo used for extraction is different from very small fruits to large ones, being very spongy and wet in the latter case and this feature prevents a correct estimation of the concentration. Thus, the epimers is much better quantified using HPLC separation of them.

3.3. Analysis of C-2 diastereomers of naringin in pummelo during maturation

From the data in Table 2, the variation of the ratio (2*S*)/(2*R* + 2*S*) naringin as a function of the pummelo diameter was obtained using Microcal Origin software and Fig. 4(a) shows the relationship. The sigmoid curve is very characteristic and implies an initial strong enzymatic activity building and maintaining a level of 95.3 ± 2.2% of (2*S*)-naringin and then a rapid decrease to reach a final 53.0 ± 2.1% to full size of the fruit. Also from the data in Table 2, the change in the ratio (2*S*)/(2*R* + 2*S*) naringin was related to the seasonal collection day and this was done for fruits collected in both sites A and B. Again a sigmoid curve was obtained, as shown in Fig. 4(b).

These results are well explained considering the biosynthesis of naringenin, as depicted in Fig. 5 [18,19]. Flavanones are formed by cyclization of chalcones mediated by the enzyme chalcone isomerase (CHI). A Michael nucleophilic attack of the 2′-OH group on the α,β-unsaturated ketone leads to (2*S*)-flavanone. Additional glycosidation steps, catalyzed by glycosyltransferases, form the flavanone glycosides. In particular, the (2*S*)-naringin, biosynthesized in sterospecific way and stored in the fruit vesicles, during maturation undergoes a non-enzymatic racemization at C-2 (epimerization) via ring opening, leading to an equilibrium mixture of both C-2 diastereomers. Recently, it has been observed the location of (2*S*)-naringenin in the CHI crystal structure: although a commercial mixture of (2*S*)- and (2*R*)-naringenin was used for cocrystallization, only the (2*S*)-enantiomer bound in the active site of CHI, an enzyme "possessing a structurally unique fold" [20].

Also, non-enzymatic racemization at C-2 was demonstrated by capillary electrophoresis for some flavanone glycosides and flavanones at high pH and temperature [21].

The sigmoid curves in Fig. 4 are similar to those obtained for grapefruit and sour orange [10,11]. For comparison, the 2*S* percentages at ripeness are 57.3 and 69.7, respectively. The much higher value found for sour orange with respect to the values obtained for grapefruit and pummelo can be related to a higher acidity and more persistent enzymatic activity. Also, the bitter taste of this fruit can be due to the higher content of the (2*S*)-epimer. Inconclusive results from a panel of 10 judges were obtained when studying the taste of individual diastereomers of naringin [22] and a dedicated study to a taste/stereochemistry relationship using statistically representative system is desirable.

4. Conclusions

The normal-phase HPLC analysis for the separation of the C-2 diastereomers of naringin and neohesperidin using Chiralpak IB as CSP is simple and gives very good separation and resolution factors. This procedure was applied to the isolation of pure C-2 diastereomers of naringin and can be scaled up to afford sizeable amounts of single diastereomers of this intriguing nutraceutical for specific taste and pharmacological assays. Moreover, this procedure allowed to determine accurately the relative amount of both C-2 diastereomers of naringin in albedo of pummelo during maturation. At ripeness, the equilibrium mixture of both diastereomers is similar to the one obtained for grapefruit but is different from the value obtained for sour orange. The open question is if the larger amount of (2*S*)-naringin in mature sour orange is responsible of its bitter taste.

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