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Racemization at C-2 of Naringin in Sour Oranges with Increasing Maturity Determined by Chiral High-Performance Liquid Chromatography

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Naringin is the major flavanone-7-*O*-glycoside of sour orange, and it is mainly responsible for the bitter taste of the fruit. The relative content of (2*S*)- and (2*R*)-naringin in the albedo of sour oranges during maturation in the entire season was determined by normal phase HPLC using Chiralcel OD-H as chiral stationary phase and *n*-hexane/ethanol doped with 0.5% of TFA as mobile phase. HPLC traces were complicated by the presence of (2*S*)-neohesperidin, and a software-guided analysis was developed to assess the relative amount of the C-2 diastereomers of naringin. A sigmoid curve was obtained showing variation from 94% of (2*S*)-naringin in very immature fruits to 69.7% in mature fruit samples. Spontaneous epimerization of (2*S*)-naringin in the ethanolic solutions of the albedo was noted after prolonged keeping for less acid samples. The separation of the C-2 diastereomers of other flavanoid-7-*O*-glycosides present in *Citrus* (neoeriocitrin, neohesperidin, and eriocitrin) was also obtained by chiral HPLC and afforded the identification, including stereochemistry, of the eluting peaks in the HPLC traces of the albedo extracts.

KEYWORDS: Naringin; diastereomer separation; chiral stationary phases; *Citrus* fruit; maturation of sour orange; racemization

INTRODUCTION

Flavanone glycosides are found in high concentrations in *Citrus*, and their distribution in various tissues for several taxa has been widely studied (*1*). The albedo of sour orange (*Citrus aurantium*) contains large amounts of naringin, neohesperidin, and neoeriocitrin (39, 24, and 22% respectively) relative to other parts of the fruit, and these percentages are very similar to those of the juice (*1*, *2*). These compounds as well as other flavanone glycosides play a major role in nutritional and pharmacological effects. In particular, naringin is the main constituent responsible for the bitter taste (*3*) of juice and marmalade, and it is chemopreventive toward mutagenesis of heterocyclic amines mediated by a cytochrome P-450 (*4*). Naringenin, the aglycone of naringin, formed in the intestinal epithelia by enzymatic hydrolysis, also inhibits cytochrome P-450 mediated oxidation of some drugs (*5*). However, the relevance of the stereochemistry of the C-2 stereogenic center has not been considered. A pioneering study, based on circular dichroism spectra, reported the variation of the C-2 stereochemistry of naringin with increasing maturity of grapefruit (*Citrus paradisi*) (*6*). More recently, this was confirmed by direct separation of (2*R*)- and (2*S*)-naringin in albedo of grapefruits collected at various maturation steps, using chiral HPLC in normal phase mode, and a sigmoid curve for the decrease of (2*S*)-naringin as a function of grapefruit diameter was obtained (*7*).

The purpose of the present study was to investigate if a similar behavior occurs in the chemotaxonomically related sour orange. The method development by chiral HPLC was, however, complicated by the presence in the extract of sour orange albedo of high concentrations of (2*S*)-neohesperidin, which is absent in the albedo of grapefruit. This gave a complex HPLC profile, and a software-guided analysis of the peak areas was necessary to establish the relative amounts of the C-2 diastereomers of naringin. Also, in the HPLC ethanolic solution of some samples a spontaneous racemization at C-2 was observed during standing at room temperature. At an early stage of the method development, to assess the identity and the C-2 stereochemistry of HPLC peaks, we also studied authentic naringin and other flavanone glycosides, and incorrect stereochemical characterization of the commercial samples was noted.

MATERIALS AND METHODS

Chemicals and Reagents. Naringin (naringenin-7-*O*-neohesperidoside) was purchased from Fluka (Buchs, Switzerland). Neohesperidin (hesperitin-7-*O*-neohesperidoside), neoeriocitrin (eriodictyol-7-*O*-neohesperidoside), eriocitrin (eriodictiol-7-*O*-rutinoside), and narirutin (naringenin-7-*O*-rutinoside) were purchased from Extrasynthèse (Lyon, France). HPLC-grade *n*-hexane and absolute ethanol were obtained from C. Erba (Milano, Italy). All other chemicals were of analytical grade.

Plant Materials. Fruits of *C. aurantium* at various stages of maturation were obtained from the same tree for each sample during the entire season, June 2005−March 2006, and stored at 2 °C for 1 or 2 days before proceeding with the analysis of the albedo. Santa Marina

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and Cassibile genotypes were collected in an experimental field at Palazzelli, Lentini, Syracuse. Cassibile samples were also collected at the Botanical Garden of the University of Catania and downtown Catania.

Sample Preparation. Albedo (40 g) was cut into very small pieces. These pieces were homogenized for 5 min in a Waring blender covered with a Teflon-lined lid at room temperature in 150 mL of 50% methanol in water, and 10 g of Celite was added during the homogenization. The homogenate was filtered twice immediately under vacuum through a Büchner funnel lined with Whatman no. 91 filter paper. Addition of Celite prevented formation of a cloudy methanol−water solution, particularly 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 Whatman no. 91 fluted filter paper. The alcoholic solution obtained was filtered through a nonsterile 0.2 μm PTFE syringe filter for HPLC analysis.

Chromatographic Conditions. The HPLC systems consisted of a Varian 5060 liquid chromatograph with a Knauer injector with a 20 μL sample loop, a Jasco Uvidec 1000-III UV spectrophotometric detector operating at 292 nm, and a Hewlett-Packard 3396 integrator. The column was a 250 mm × 4.6 mm i.d. Chiralcel OD-H (cellulose tris-3,5-dimethylphenylcarbamate) coated on 5 μm silica gel (Chiral Technologies Europe, Illkirch, France). In system 1, the mobile phase consisted of *n*-hexane/ethanol doped with 0.5% of trifluoroacetic acid (TFA), (60:40) at 1.2 mL/min. In system 2, the mobile phase consisted of *n*-hexane/ethanol doped with 0.5% of TFA (75:25) at 1.2 mL/min. The column dead time (t_0) was measured by injection of tri-*tert*-butylbenzene as a nonretained sample. The capacity factor [$k' = (t - t_0)/t_0$], separation factor ($\alpha = k_2'/k_1'$), and resolution factor [$R_s = 2(t_2 - t_1)/(w_1 + w_2)$] were calculated for the standard flavanone glycosides according to the usual procedure (*8*). Duplicate analyses were performed at ambient temperature, both for albedo samples and for standard compounds.

Analysis of Chromatographic Peaks. The HPLC traces of the extracts of sour orange albedo often showed a shoulder on the major peak that was not marked and integrated by the available integrator. The peaks of a chromatogram were therefore digitized with WinDig for Windows 98 (*9*) to get scanned curves with numerical values. These digitized data were entered in commercial software (PeakFit v. 4 from Jandel) to obtain accurate peak areas, determined by integrating the peak equations in the model. Autofit Peaks I residuals for chromatographic Gaussian areas fitting process was selected among several features. Then, iteration was adopted to obtain the best *F* statistic (agreement factor) and r^2 coefficient of determination. The ranges of *F* and r^2 values are between 5000 and 85000 and between 0.995−0.999, respectively, by analysis of all chromatograms.

RESULTS AND DISCUSSION

Flavanone glycosides are formed by cyclization of chalcone glycosides mediated by chalcone-flavanone isomerase (CFI) (*10*). A Michael nucleophilic attack from the OH group in the C-2′ position of ring A to the α,*β*-unsaturated ketone leads to (2*S*)-flavanone (*11*). When, during maturation, the CFI enzyme is inhibited or degraded, (2*S*)-naringin, the flavanoid glycoside present in immature grapefruit, undergoes nonenzymatic racemization at the C-2 position via ring opening, leading to nearly equal amounts of (2*S*)- and (2*R*)-naringin, as shown in **Figure 1** (*6*). We confirmed this pioneering study and obtained for the first time a sigmoid curve of the relative percentage of (2*S*)-naringin in the albedo of grapefruit as a function of the maturation (*7*). It is known that sour orange and grapefruit are species chemotaxonomically related (*12*). Thus, the formation of the naringin diastereomers probably occurs in a similar way.

Separation of Authentic Flavanone Glycoside C-2 Diastereomers. The structures of the various flavanone glycosides investigated are shown in **Figure 2**. These five compounds contain three aglycones having a flavanone skeleton associated

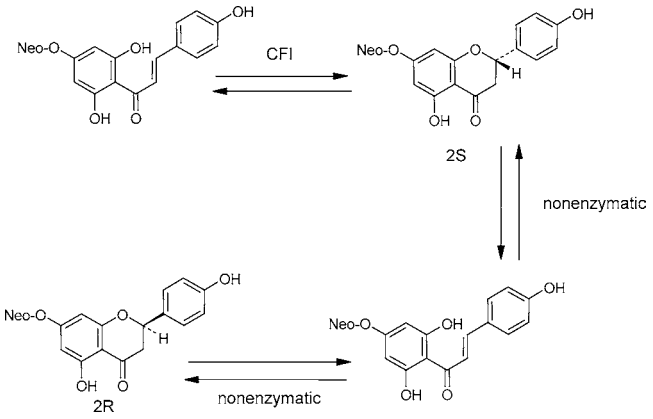

Figure 1. Naringin biosynthesis by stereospecific cyclization of chalcone glycoside and subsequent nonenzymatic cyclization via ring opening.

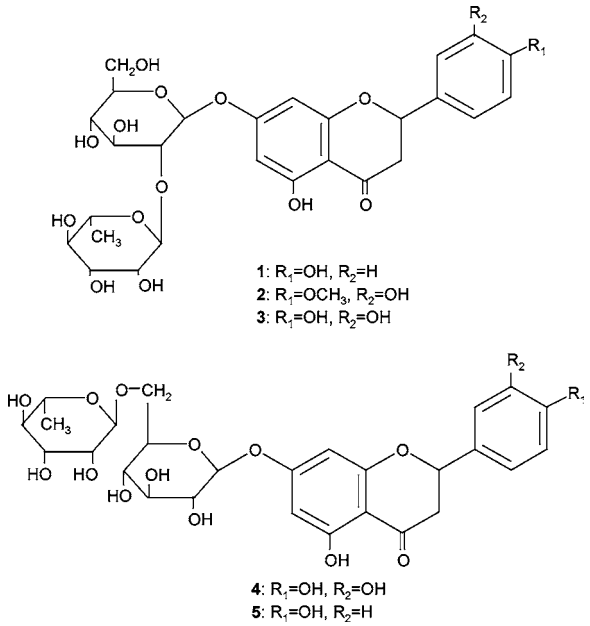

Figure 2. Structures of the studied flavanone glycosides: **1**, naringin; **2**, neohesperidin; **3**, neoeriocitrin; **4**, eriocitrin; **5** narirutin.

Table 1. Enantioselective HPLC Resolution of Flavanone Glycosides on Chiralcel OD-H

compd	A^a (%)	t_1	t_2	$K'_1{}^b$	α	R_s
1	25	21.7	37.6[c]	8.23	1.82	2.13
2	25	22.9	32.4[c]	8.76	1.46	1.33
3	40	15.4	21.1[c]	5.53	1.44	1.04
4	25	12.1	15.5[c]	4.14	1.35	0.82
5	25	8.7		2.68	1.00	

[a] Percentage of ethanol doped with 0.5% of TFA in *n*-hexane at a flow rate of 1.2 mL/min; $t_0 = 2.35$ min; 292 nm. [b] Capacity factor of the first eluted peak. [c] 2*S* diastereomer.

with a neohesperidosyl moiety [naringin (**1**), neoeriocitrin (**2**), and neohesperidin (**3**)] and two aglycones associated with a rutinosyl moiety [eriocitrin (**4**) and narirutin (**5**)]. To optimize the conditions for obtaining the separation of the C-2 diastereomers (epimers) present in the extracts of sour orange albedo, to assess their identity, and to study their C-2 stereochemistry, we separated compounds **1**−**5** into their epimers by chiral HPLC in a normal phase mode.

Table 1 shows the chromatographic results for the separation of (2*R*/2*S*)-flavanone glycosides using as chiral stationary phase the Chiralcel OD-H. Epimer selectivity values (α), ranged from

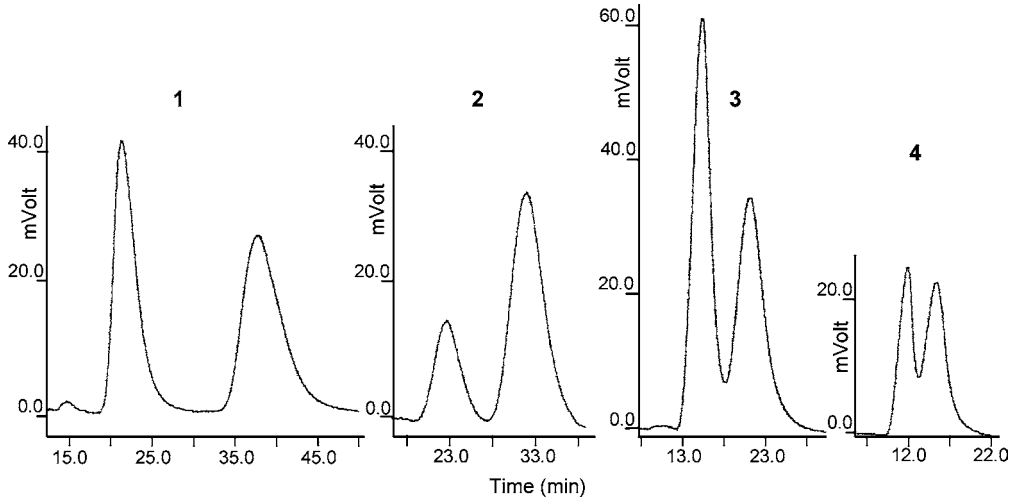

Figure 3. Typical HPLC separation of C-2 diastereomers of compounds **1**–**4**. Conditions were as in **Table 1**.

1.82 for naringin to 1.35 for eriocitrin. Only the epimers of narirutin (**5**) were not separated ($\alpha = 1$). Analogously, the resolution factor (R_s) ranged from 2.1 for naringin to 0.8 for eriocitrin. The values of α and R_s obtained for naringin were much better than those obtained using another polysaccharide-derived chiral stationary phase (Chiralpak AD) and a very similar mobile phase (1.51 and 0.7, respectively) (*7*). The difference in the chiral recognition ability may be due to the different volumes of the helical groove of the cellulose derivative (OD-H) and the amylose derivative (AD) because it is well-known that amylose-derived phases possess a wider and more compact helix (*13*). Narirutin (**5**) was not resolved into its epimers using the composition of the mobile phase reported in **Table 1**, but because this flavanone glycoside is not present in the solution of albedo extracts, no further attempts were made to separate them. Reasonable elution times for the epimers of neoeriocitrin (**3**) can be achieved only by using a more polar mobile phase (40% doped ethanol in *n*-hexane). Much higher elution times for very broad peaks (>1 h) were obtained when using a less polar phase. The strong interaction of neoeriocitrin (**3**) with the chiral stationary phase, resulting in a very high k_1' and the need for a more polar mobile phase for reasonable elution, is due to the presence in the structure of compound **3** of two phenolic groups, which act as hydrogen donor and hydrogen acceptor groups for hydrogen-bonding interaction with the carbamate groups of the phase. This behavior was important for proper method development in the analysis of the solution of albedo extracts. A different interaction of the neohesperidoside group in compounds **1**−**3** with the chiral stationary phase may explain the higher capacity factors (k_1') in comparison with those of compounds **4** and **5**, possessing instead a rutinoside group. Typical separation of the epimers of compounds **1**−**4** is shown in **Figure 3**.

From integration of the peaks, all of the commercial compounds are richer in the 2*S* epimer, particularly neohesperidin (**2**), which shows a diastereomeric purity (dp) $2S/(2S + 2R) =$ 74.3%. Similarly, the dp of naringin (**1**) was 52.3%. However, for the commercial sample (Fluka) the optical rotatory power of (2*S*)-(−)-naringin is indicated as that reported in the literature (*14*), although the stereochemistry at C-2 is not depicted. In contrast, the Extrasynthèse commercial neohesperidin is reported with 2*S* stereochemistry, whereas compounds **3**−**5** are reported in the same catalogue without indication of their C-2 stereochemistry. The predominance of one C-2 diastereomer with respect to another one is undoubtedly due to the extraction of these compounds from *Citrus* species. However, in our opinion, the stereochemistry at C-2 of commercial samples, when indicated, must result from chiroptical measurements. Indeed, chirality in compounds used for enzymatic assays and pharmacological testings must be taken into account due to its pharmacological and biological implications (*15*). The absolute configuration at C-2 of the first and second peaks of compounds **1**−**4**, as reported in **Table 1**, has been assigned by knowledge of the Cotton effects in the circular dichroism (CD) spectra of flavanone glycosides and their aglycones, because the carbohydrate moiety is CD-inactive (*14*, *16*).

Moreover, the assignment of the absolute configuration of the eluting peaks is not automatic in the absence of information about the CD spectra or if authentic samples of C-2 diastereomers are not available. Indeed, the elution order in the HPLC experiments can be different when using reverse phase mode or even a different chiral stationary phase in normal phase mode, as shown for example in the case of naringin and narirutin (*7*). The usefulness of the direct separation of C-2 diastereomers of flavanone glycosides by chiral HPLC using a normal phase isocratic elution should be stressed for its ruggedness and simplicity. Other direct separations of these compounds in chiral HPLC were in fact accomplished by reverse phase using a β-cyclodextrin-bonded phase (*17*) and normal phase gradient elution using a cellulose triacetate diol (*18*).

Separation of Flavanone Glycoside C-2 Diastereomers in Albedo Extracts of Sour Oranges. Flavanone 7-neohesperidosides, compounds **1**−**3**, are intensely bitter and are mainly responsible for the characteristic taste of sour orange and grapefruit. In contrast, flavanone 7-rutinosides, such as compounds **4** and **5**, are tasteless (*19*). A panel of 10 judges tried to study the taste of individual diastereomers of naringin, but inconclusive results were obtained (*6*).

The greatest use of sour oranges as food is in the form of marmalade, particularly in England and Scotland, and the albedo of the fruits is present in it. The adulteration of marmalade and juice with sweet orange (*Citrus sinensis*) affects the quality and the taste of the product (*20*−*22*) and is due to the larger production and lower cost of the sweet orange with respect to the sour orange. The latter is in fact used in Italy mainly as rootstock for sweet oranges.

In this investigation we studied the relative contents of (2*S*)- and (2*R*)-naringin in the albedo of the sour orange during maturation because this ratio can affect the quality and the taste of commercial products. Also, from a biosynthetic point of view, it is interesting to observe the variation of the epimeric ratio

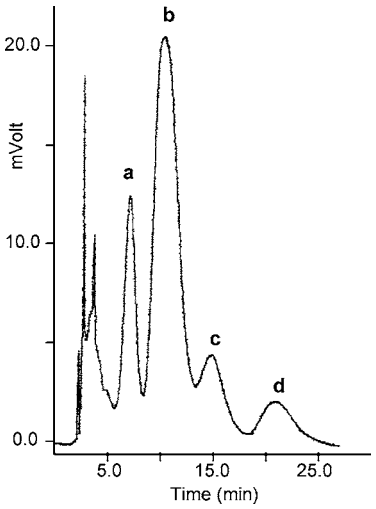

Figure 4. Partial separation of epimers of compounds **1**–**3** in an extract of sour orange albedo. Peaks: a, (2*R*)-naringin; b, (2*S*)-naringin and (2*S*)-neohesperidin; c, (2*R*)-neoeriocitrin; d, (2*S*)-neoeriocitrin.

during ripening of the fruit. A similar study was recently performed by us on the grapefruit (*7*).

The albedo of sour orange is dominated by naringin (**1**), neohesperidin (**2**), and neoeriocitrin (**3**), whereas that of the grapefruit mainly consists of naringin and narirutin (**5**) (*1*, *2*, *23*). **Figure 4** shows the partial separation of the epimers of compounds **1**−**3** in an albedo extract, immediately injected onto the Chiralcel OD-H HPLC system. The identification of the peaks is based on the results obtained for the authentic compounds, but the mobile phase used (40% doped ethanol in *n*-hexane, system 1) was too polar for this purpose. Indeed, although the epimers of neoeriocitrin were well separated (15.1 and 21.2 min, respectively), a single peak grouped (2*S*)-naringin and (2*S*)-neohesperidin together. A fourth peak eluting at 7.3 min was attributed to (2*R*)-naringin. Thus, a better optimization of the method was necessary, and this was achieved using a less polar mobile phase (25% doped ethanol in *n*-hexane, system 2) in isocratic mode until 40 min of elution time and then washing with the more polar mobile phase to clean the HPLC column from the later eluting peaks of neoeriocitrin at more than 60 min with very broad shape. In this way, separation of (2*R*)-naringin, (2*S*)-naringin, and (2*S*)-neohesperidin was achieved, and (2*R*)-neohesperidin was absent in the albedo extracts.

Typical HPLC separation of these compounds from the albedo of sour oranges as a function of maturity of the fruit (diameter ∅) is reported in **Figure 5** for two samples [Santa Marina (**A**) and Cassibile (**B**) collected at Palazzelli, respectively], using conditions of system 2. The absolute configuration is indicated on the peaks, and it is derived by comparison with the study of the authentic samples. The elution order was (2*R*)-naringin, (2*S*)-neohesperidin, and then (2*S*)-naringin. Other chiral HPLC conditions such as gradient or flow rate of the mobile phase were tested, but the best conditions remained those reported. The last peak appears often as a shoulder on the major peak of (2*S*)-neohesperidin, depending on the degree of maturation, and it is not marked by the integrator. Thus, the assessment of the area, and therefore of the relative amount of (2*S*)-naringin, was solved by a software program. First, using the WinDig program, a manual curve digitalization was made from scanned HPLC

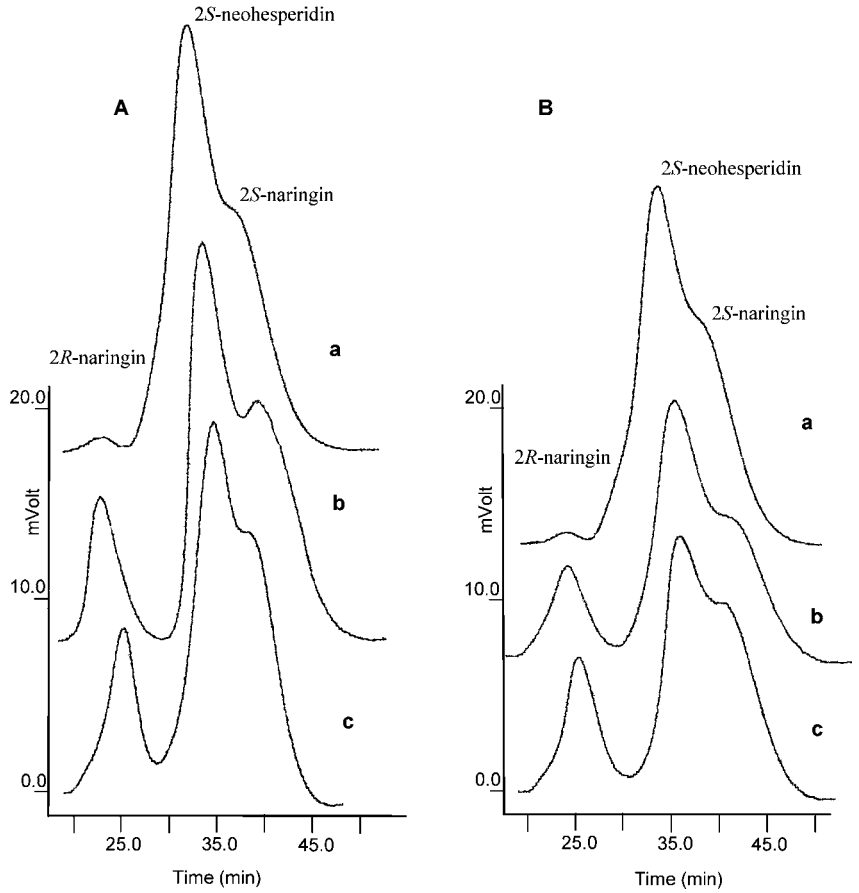

Figure 5. Typical HPLC separation of the C-2 diastereomers of compounds **1** and **2** from albedo of sour orange as a function of fruit maturity (diameter ∅): (**A**) samples of Santa Marina from Palazzelli (**a**, 2.4 cm; **b**, 6.4 cm; **c**, 8.6 cm); (**B**) samples of Cassibile from Palazzelli (**a**, 2.5 cm; **b**, 6.0 cm; **c**, 9.5 cm).

Table 2. Average Composition of (2*S*)/(2*S* + 2*R*)-Naringin in Sour Orange Albedo as a Function of Seasonal Growth

sample	genotype (collection site)[a]	sour orange diameter (cm)	date (dd/mm/yy)	2*S*/(2*S* + 2*R*) %
1	A	2.5	22/6/05	97.8
2	A	3.6	22/7/05	88.2
3	A	5.0	2/9/05	75.4
4	A	6.0	4/10/05	70.1
5	A	6.6	16/11/05	70.3
6	A	6.7	16/12/05	70.0
7	A	6.8	12/1/06	71.2
8	A	7.6	16/2/06	68.5
9	A	8.3	20/3/06	70.4
10	A	9.5	3/5/06	66.0
11	B	2.4	22/6/05	98.5
12	B	3.9	22/7/05	91.7
13	B	5.3	2/9/05	81.4
14	B	6.4	4/10/05	75.7
15	B	8.0	16/11/05	72.5
16	B	6.7	16/12/05	71.9
17	B	7.2	12/1/06	73.4
18	B	7.0	16/2/06	74.8
19	B	8.0	20/3/06	70.1
20	B	8.6	3/5/06	69.0
21	C	1.5	5/7/05	90.0
22	C	2.9	5/7/05	84.2
23	D	2.5	4/7/05	91.9
24	D	4.8	27/7/05	76.1
25	D	5.6	8/9/05	72.6
26	D	6.5	5/10/05	68.5
27	D	8.0	29/11/05	68.1
28	D	7.0	16/12/05	69.0

[a] A, Cassibile (Palazzelli); B, Santa Marina (Palazzelli); C, Cassibile (Botanical Garden, Catania); D, Cassibile (Beato Angelico Square, Catania).

chromatograms. The digitized data were then used as input for PeakFit software, which calculates the area of the peaks, including the shoulder peak, by using a statistically fitting peak function to the submitted data set. The accuracy of the area determination was checked by the *F*, r^2, and residuals values. The agreement between the experimental and calculated peaks was good and, as examples, values of *F* and r^2 were 13647 and 0.997, respectively, for peaks in **Figure 5Aa**, and 40391 and 0.999 for peaks in **Figure 5Ab**.

Analysis of C-2 Diastereomers of Naringin in Sour Orange during Maturation. Using the method developed, the quantization of the individual diastereomers of naringin, based on the calculated areas (*A*) being % (2*S*) = $A_{2S}/A_{2S} + A_{2R}$, was done on a large collection of sour oranges at different ripening stages. The season from June 2005 to May 2006 was followed, collecting fruits once a month, extracting the albedo within 48 h from collection, and immediately injecting 10 μL of the obtained ethanolic solution in the chiral HPLC system.

Table 2 summarizes the results for all of the samples. Four groups of fruits, from different collection sites or genotypes, were examined. They were collected from the same tree over the months to avoid intragroup variability. For very small fruits, the albedo of eight or nine samples was used; for very large fruits the albedo of two samples was sufficient. Thus, the reported diameter for a sample is an average value. Obviously there is not a relationship between diameter and collection day among all of the samples, as shown, for example, between samples 1 and 23, samples 18 and 28, and samples 5 and 26. Nevertheless, it is clear that the ratio 2*S*/(2*S* + 2*R*) undergoes a marked change with increasing maturity of the sour orange. In very small fruits (diameter = 1.5−2.5 cm), such as samples 1, 11, and 23, the naringin is practically all 2*S*, and it is very abundant during the early part of the season. As the fruit

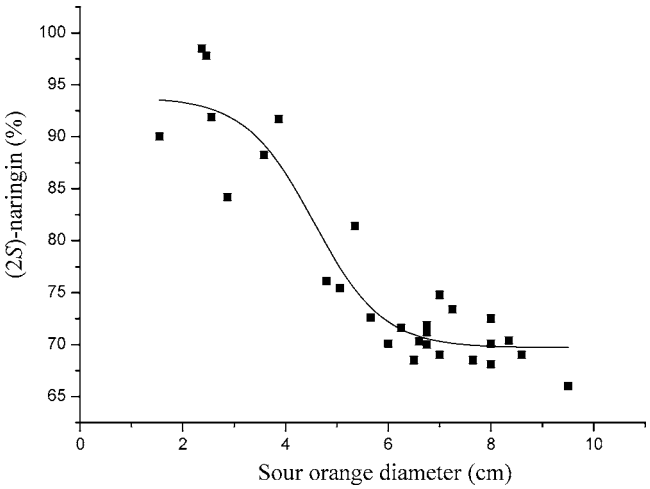

Figure 6. Variation of (2*S*)-naringin as percentage of both diastereomers in the albedo as a function of the sour orange diameter. Data from all samples are given in **Table 2**.

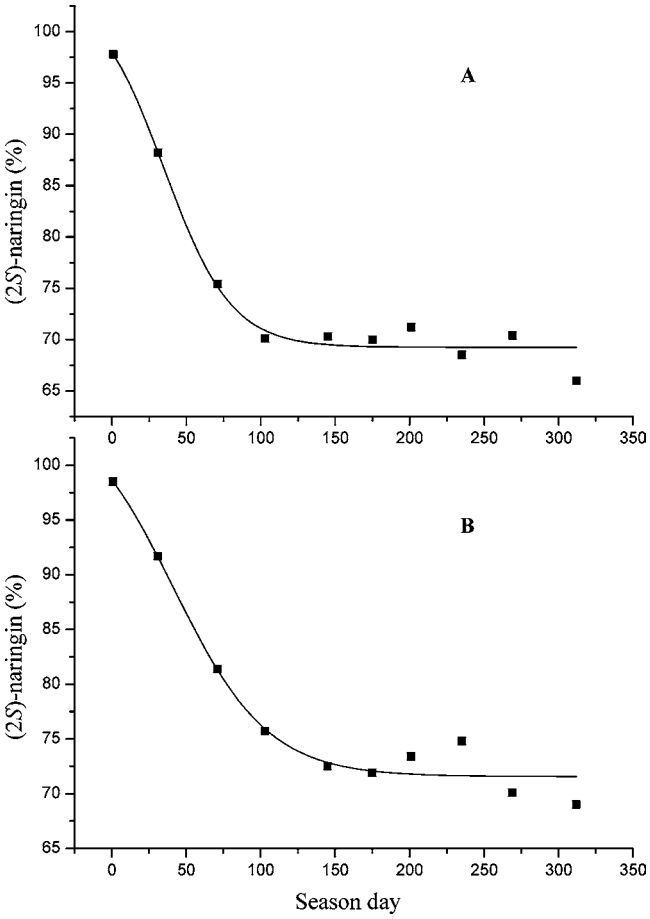

Figure 7. Variation of (2*S*)-naringin as percentage of both diastereomers in the albedo of (**A**) Cassibile (Palazzelli) and (**B**) Santa Marina (Palazzelli) sour oranges as a function of seasonal collection day: (**A**) data from samples 1–10; (**B**) data from samples 11–20 in **Table 2**. Day 1 was June 22, 2005.

matures, more (2*R*) diastereomer appears until the diastereomeric percentage at ripeness is 66−70% (2*S*). From the data in **Table 2**, the variation of the ratio 2*S*/(2*S* + 2*R*) as a function of the sour orange diameter was obtained using Microcal Origin Software, and **Figure 6** shows the relationship. The sigmoid curve obtained is very characteristic and implies an initial strong enzymatic activity building and maintaining a level of 93.8 ±

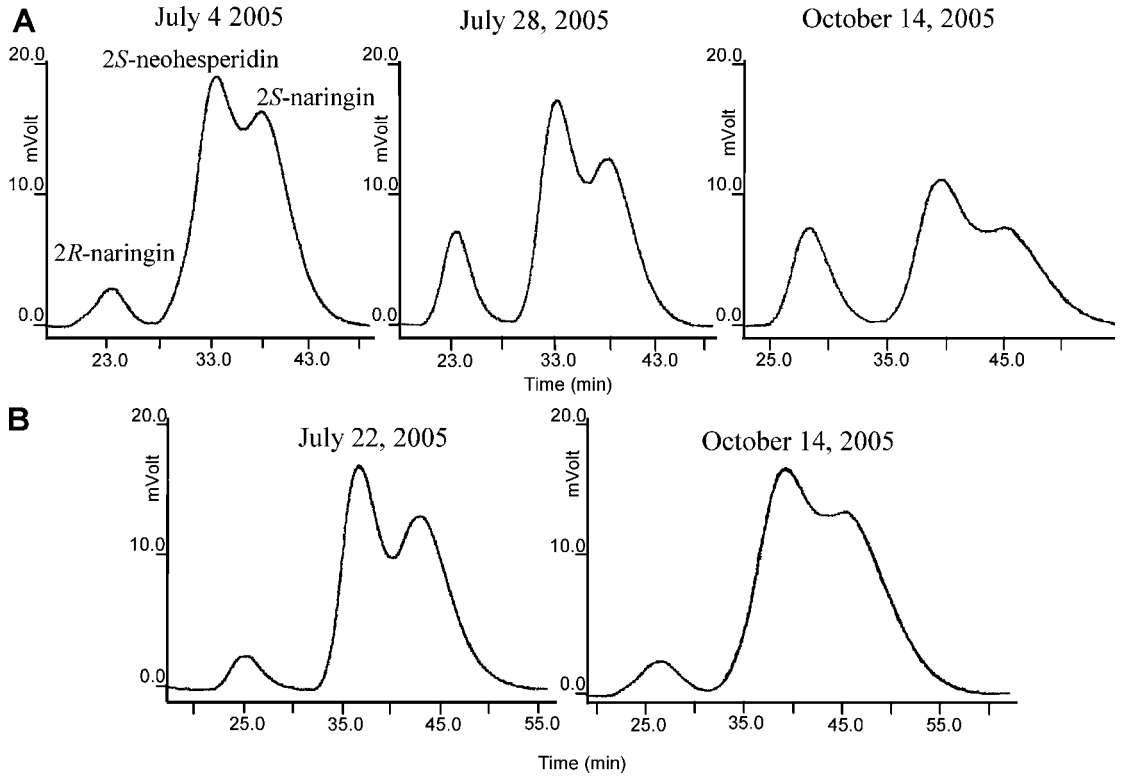

Figure 8. Change in the chromatographic profile of ethanolic HPLC solutions of sample 23 (**A**) and sample 12 (**B**) with standing days and related variation of (2*S*)/(2*R*)-naringin ratio.

2.4% of (2*S*)-naringin and then a rapid decrease to reach a final 69.7 ± 1.1%, from 6.7 cm to full size of the fruit. The χ^2 and r^2 parameters of the Boltzmann curve, taking into account the large number of points, are satisfactory (11.5 and 0.892, respectively). By comparison, the 2*S* percentage at ripeness for grapefruit was 57.3% (*7*) and, in our opinion, this is due to a lower acidity of mature grapefruits. Also, from the data in **Table 2**, the change in 2*S*/(2*S* + 2*R*) naringin ratio was related to the seasonal collection day, and this was possible of course for fruits of the same group and collected on the same tree. **Figure 7A** shows the results for the Cassibile sour orange collected at Palazzelli. It is again a sigmoid curve with good χ^2 and r^2 values, (2.98 and 0.980, respectively), and it is clear that after 100 season days the ratio 2*S*/(2*S* + 2*R*)-naringin remains constant at 69.2 ± 0.7%. A similar sigmoid curve was obtained for fruits of the Santa Marina genotype, as shown in **Figure 7B**, although in this case the final ratio is slightly higher (71.6 ± 0.9) and is reached at about 140 season days. Also, these data confirm the initial enzymatic formation of only the 2*S* diastereomer and then a progressive nonenzymatic epimerization (racemization at C-2) up to an equilibrium between both diastereomers.

The similarity of the results obtained for albedo of the grapefruit and sour orange is consistent with the strict chemotaxonomic relationship between these two *Citrus* species. Indeed, both are hybrids derived directly from pummelo (*Citrus grandis*), which is considered to be their ancestor (*12*). Collection of similar data for pummelo should be interesting. The chiral HPLC profile of the sour orange albedo extract was more complex with respect to that of grapefruit albedo due to the presence of the large peak of (2*S*)-neohesperidin. However, the treatment of the HPLC data with the PeakFit software afforded an accurate analysis of the diastereomeric ratio in naringin. This analysis can be useful in the manufacture of marmalade or functional foods for the selection of sour oranges at a convenient maturity. Also, a relationship between absolute configuration and bitterness is possible, because stereochemistry is important in determining the three-dimensional structure of the receptor protein, which is supposed to bind the sweet compounds (*24*). It has to be noted that neohesperidin remains as the 2*S* diastereomer during maturation of the sour oranges, as shown in **Figure 5** and other unreported data. This is consistent with the results of the Galensa group for sour orange juice and marmalade extract (*17*, *25*).

Epimerization of (2*S*)-Naringin in the HPLC Solutions of Albedo of Sour Orange. During the HPLC analysis as the fruit increased in size and maturity, we always used freshly prepared solutions for chromatographic injections. When these ethanolic solutions were kept at room temperature for months and injected again in the HPLC system, some of these showed a marked change in the chromatographic profile.

Figure 8A shows this behavior for sample 23 reported in **Table 2**, using conditions of system 2. A clear increase in the content of (2*R*)-naringin with respect to the 2*S* diastereomer can be noted after 24 days and much more after 100 days from the first injection. Remarkably, almost no variation was observed for sample 12, as shown in **Figure 8B**. Thus, racemization at C-2 occurs in some samples, and this can be related to the total water-soluble organic acids content (expressed as grams per 100 mL of citric acid) in the corresponding juices. Indeed, acidity values for juice of samples 23 and 12 were 4.0 and 7.0, respectively, at the collection day. The nonenzymatic equilibrium shown in **Figure 1** can explain this result. Previous work on the pH profile of the naringin chalcone−flavanone equilibrium under basic conditions has been reported (*26*). Also, (2*S*)-naringin was reported to racemize easily in aqueous methanolic solution at 70 °C (*17*). Recently, the 2*R*/2*S* diastereomerization barriers were determined by capillary electrophoresis at various basic pH values (*27*) and by a combination of achiral and chiral reverse phase HPLC method in water/methanol 90:10 at 60 °C (*28*). The epimerization via the chalcone was, however, stated

as not applicable, and it was reported that further investigations on this point are in progress (*27*). The spontaneous racemization at C-2 of naringin in ethanolic solutions of albedo extracts observed in this study may be useful in processing products from sour oranges.

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Supporting Information Available: Figure showing the Gaussian line shapes obtained for the HPLC chromatograms shown in **Figure 5A**. This material is available free of charge via the Internet at http://pubs.acs.org.

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