Flavanones in *Citrus fruit*: Structure–antioxidant activity relationships

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Abstract

Epidemiological surveys have shown an inverse relationship between the intake of fruit and the incidence of coronary heart disease and some type of cancer. Data found in the literature regarding the flavonoids in general while this study focuses on flavanones, a subclass of flavonoids which occurs in *Citrus fruit*. The aim of this work is to elucidate the antioxidant or pro-oxidant behaviours of some common flavanones and to determine their activity–structure relationships as antioxidant using the crocin bleaching inhibition assay. The compounds studied were regarding both the aglycon form and the glycoside form. Data evidence that the substitution of the 7th OH group of the flavanones by a neohesperidoside influences the relationship between structure and antioxidant activity. In fact, the 3′,4′-catechol structure and the O-methylation, in the aglycone forms, do not result significant. On the other hands, in the glycosylate forms, the 3′,4′-catechol structure noticeably increases the antioxidant power and the O-methylation decreases the antioxidant activity. The influence of the O-glycosylation with a rutinose molecule is neglectable.

Keywords: Flavonoids; Flavanones; *Citrus fruit*; Structure–activity relationship; Crocin bleaching method

1. Introduction

The human diet contains important micronutrients, such as vitamin C, vitamin E, carotenoids and flavonoids, essential for maintenance of human health. In consequence of, the dietary route is a primary means of modulating endogenous antioxidant protection. Evidence in vitro (Sies & Stanl, 1995) suggests that many of these compounds can directly react with oxidants and free radicals showing an important role in modulating oxidative stress. Multiple dietary sources of these compounds are present, in virtually, all plant material. Three types of flavonoids occur in *Citrus fruit*: flavanones, flavones and flavonols (Calabrò et al., 2004). Epidemiological studies on dietary Citrus flavonoids have been associated with reduced risk of coronary heart disease placing a new perspective on these components; they have been suggested as one of the possible cancer-preventing agents, too (Calabrò et al., 2004; Hertog, Feskeens, Holmann, Katan, & Kromhout, 1993; Stavric, 1993). The interest in these classes of compounds is due to their pharmacological activity as radical scavengers (Cotelle et al., 1996). Structurally the flavonoids have phenolic groups. These phenolic groups serve as a source of a readily available "H" atoms such that the subsequent radicals produced can be delocalized over the flavonoids structure. The chemical nature of the flavonoids depends on structural class,

degree of hydroxylation, other substitutions and conjugations, and degree of polymerization (Calabrò et al., 2004).

The aim of the present study was to elucidate the antioxidant and pro-oxidant behaviours of some common flavanones and to determine their activity–structure relationships as antioxidant or prooxidant by using the crocin bleaching inhibition assay (Tubaro, Micossi, & Urini, 1996). The flavanones in the aglycone form (molecules not attached to sugar moieties), studied here, include *Naringenin* (4′,5,7-trihydroxyflavanone), *Hesperitin* (3′,5,7-trihydroxy-4′methoxyflavanone), *Isosakuratenin* (3,5-dihydroxy-4′-methoxyflavanone), *Heridictyol* (5,7,3′,4′ tetrahydroxyflavanone), while in the glycoside form (molecules with sugar moieties) are *Hesperidin* (Hesperitin-7-rutinoside), *Narirutin* (Naringenin-7-rutinoside), *Neohesperidin* (Hesperitin-7-neohesperidoside) *Neoeriocitrin* (Heridictyol-7-neohesperidoside), *Naringin* (Naringenin-7-neohesperidoside). The selection of these compounds was based on chemical structure characteristics, availability and prevalence in *Citrus fruit* (Fig. 1).

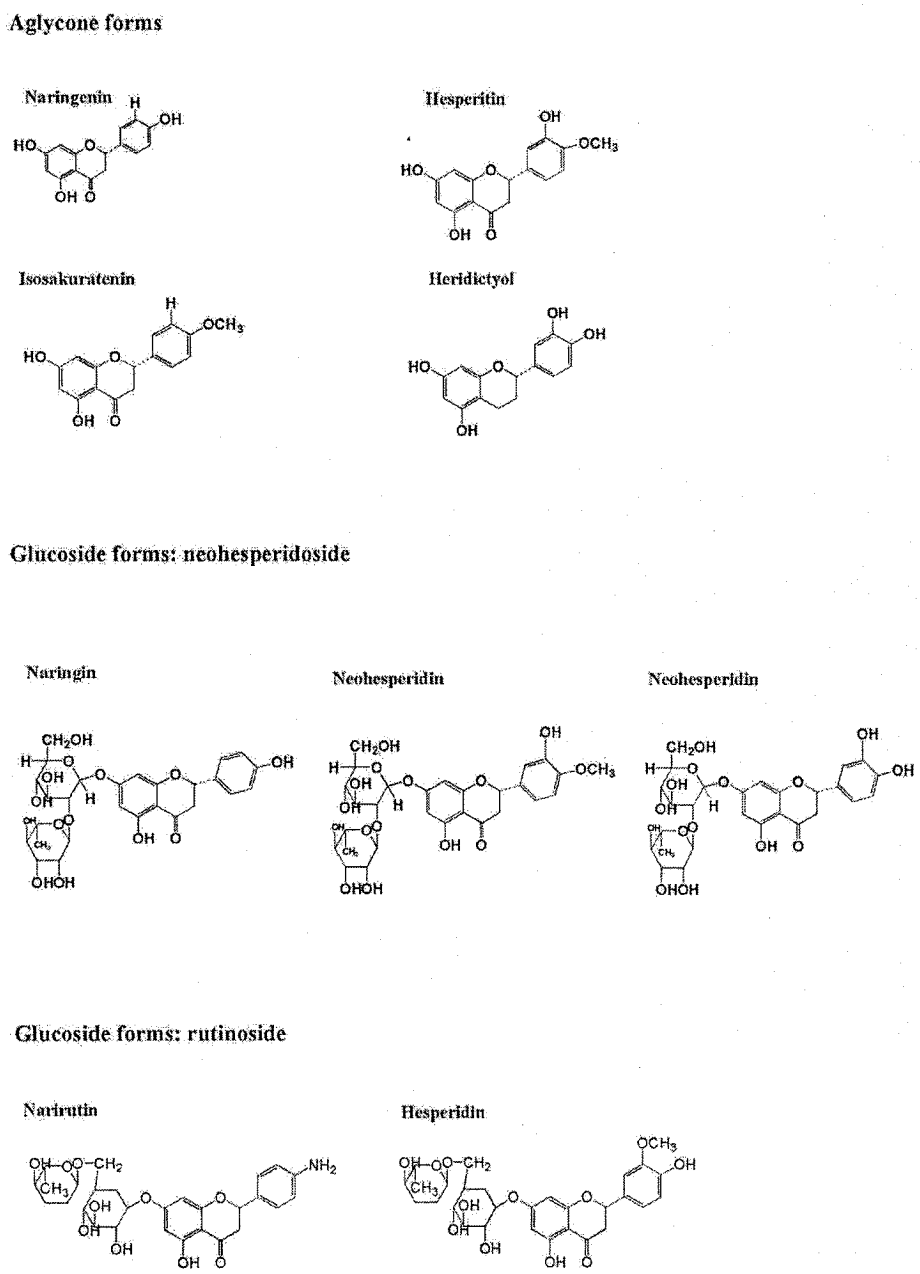

Fig. 1. Structure of flavanones in the aglycone and glycoside forms.

2. Materials and methods

The crocin bleaching method was used to determine the antioxidant capacity of the glycosylated flavanones: Naringin, Neohesperidin, Neoeriocitrin, Hesperidin, Narirutin and the related aglycones such as Naringenin, Hesperitin, Heridictyol and Isosakuratenin. All the standards were purchased from Extrasynthese, Genay, France and they were used at 0.01 mM in hydrophilic (water) environments. This method is based on the oxidation (bleaching) of crocin by peroxyl radicals produced from ABAP [2,2′-azo-bis(2-amidinopropane)] (Waco Chemicals) (Finotti & Di Majo, 2003; Hertog et al., 1993). Crocin was isolated from saffron purchased from Sigma Chemical Co. (St. Louis, Mo, USA) by methanol (Merck) extraction, after that repeated extraction with diethyl ether (Merk, Darmstadt, Germany) to remove possible traces of lipids and other contaminants. The concentration of crocin was calculated using the absorption coefficient ($\varepsilon = 1.33 \times 10^5$ mol^{-1} cm^{-1} at 433 nm).

2.1. The competition kinetics test

This method is based on the crocin bleaching as a result of its oxidation by a source of radicals, ABAP. Carbon-centered radicals, generated by thermal decomposition of ABAP, add molecular oxygen yielding, in a diffusion controlled reaction, peroxy radicals (Barclay et al., 1984; Pryor et al., 1993). These radicals bleach the crocin, and the rate is followed as specific decrease of absorbance at 443 nm (Tubaro et al., 1996). The bleaching rate of crocin by peroxyl radicals in the absence (V_0) and in presence (V_a) of antioxidants were recorded for 10 min. The value of K_a/K_c, calculated from the slope of the linear regression of the plot of [A]/[C] vs. V_0/V_a, indicates the relative capacity of the compound under analysis to interact with peroxyl radicals. The bleaching rate of crocin became linear approximately 2 min after the addition of the diazocompound and recorded for 10 min. According to the competition kinetics (Bors, Michel, & Saran, 1984), the crocin bleaching by a peroxyl radical (V_0) decreases in presence of an antioxidant competing for the peroxy radicals and the new bleaching rate is V_a. The reaction followed the competitive reaction equation:

$$V_0/V_a = 1 + K_a/K_c * [A]/[C],$$

where K_a represents the rate constant for the reaction between antioxidant and peroxyl radicals; K_c is the rate constant for the reaction between crocin and peroxyl radicals; [A] is the concentration of antioxidant and [C] is concentration of crocin; V_0 and V_a represent the rates of the reaction of crocin (V_0) or of antioxidant (V_a) with peroxyl radical. Reactions were carried out at 40 °C. Blank without crocin were run to rule out spectral interferences between the compound and crocin.

The antioxidant capacity of different compounds was measured by the same procedure comparing each kinetic analysis with the kinetic crocin bleach containing only ABAP.

2.2. Statistical analysis

The analysis on the same compound was made three replications and results were expressed as weighted mean and for each value were calculated the standard deviation. Statistical analysis was carried out using SPSS software by SPSS inc. ILUSA. The ANOVA test used to determine the statistical differences. The criterion for statistical significance was $P \leqslant 0.005$. Data presented in tables show the calculated means and the different letters indicate significant differences at $P \leqslant 0.005$ Duncan's test.

3. Results and discussion

The antioxidant activity of flavanones in vitro depends upon the arrangement of functional groups about the nuclear structure: nine different flavanones were measured for antioxidant properties in this study. All compounds were members of the family of flavanones. Special attention was paid to the number position, O-glycosilation and O-methylation of free hydroxyl groups on these compounds. Other similar previously published studies evaluated fewer compounds which were members of the different family of flavonoids and did not focus on the same subgroup of flavonoids (Arena, Fallico, & Maccarone, 2001; Rice-Evans, Miller, & Paganga, 1997).

3.1. Influence of the hydroxyl groups

The spatial arrangement of substituents influences the antioxidant activity more than the flavan backbone. The configuration and the total number of hydroxyl groups influence several mechanisms of the antioxidant activity. This is particularly evident in flavanones with a substitution of the Neohesperidoside group in the 7th position. This study puts in evidence that, in the aglycone form, the 3′,4′-di-hydroxy substitution does not influence so much the antioxidant activity as shown in Table 1. In fact, the difference between the antioxidant power of the *Heridictyol*, which has four hydroxyl groups substitutions with 3′,4′-di-OH-structure, and that of the *Naringenin*, which has three OH substitution on the structure but no 3′,4′-di-OH-structure, does not result significant.

On the other hands, in the flavanones glycosylated with a neohesperidose of the 7th OH group, the 3′,4′-catechol structure the antioxidant power is noticeably increased.

Table 1
Influence of the C_7 neohesperidoside conjugation about effects of the hydroxyl groups and O-methylation on the antioxidant power

Compounds	C_5	C_7	$C_{3'}$	$C_{4'}$	K_a/K_c
Aglycone forms					
Naringenin[bc]	OH	OH	H	OH	2.73 ± 0.45
Heridictyol[abc]	OH	OH	OH	OH	2.90 ± 0.39
Hesperitin[ab]	OH	OH	OH	OCH_3	3.13 ± 0.77
Glycoside forms					
Naringin[bc]	OH	Ramnosil α-1,2 glucose	H	OH	2.41 ± 0.33
Neoeriocitrin[a]	OH	Ramnosil α-1,2 glucose	OH	OH	3.73 ± 0.34
Neohesperidin[c]	OH	Ramnosil α-1,2 glucose	OH	OCH_3	2.14 ± 0.30
F Anova	4.47*				

(Different letters indicate significant differences at $P \leqslant 0.005$ Duncan's test).

Table 2
Comparison between methoxylate flavanones with corresponding hydroxylate forms

Compounds	C_5	C_7	C'_3	C'_4	K_a/K_c
Threehydroxylate forms					
Naringenin[a]	OH	OH	H	OH	2.73 ± 0.45
Isosakuratenin[a]	OH	OH	H	OCH_3	3.52 ± 0.41
Tetrahydroxylate forms					
Heridictyol[a]	OH	OH	OH	OH	2.90 ± 0.39
Hesperitin[a]	OH	OH	OH	OCH_3	3.13 ± 0.77

(Different letters indicate significant differences at $P \leqslant 0.005$ Duncan's test).

In fact, Table 1 shows that *Neoeriocitrin*, the neohesperidoside form of the Heridictyol has a greater antioxidant activity, expressed as K_a/K_c, in comparison to the *Naringin* (Naringenin neohesperidoside compound). The importance of the catechol system in the flavanones glycosylated is confirmed by the antioxidant behaviour of *Neohesperidin*, hydroxylate on the 3′ and methoxylate on the 4′ positions but no 3′,4′-di-hydroxy substitution. *Neohesperidin* has proved to have an antioxidant activity ($K_a/K_c = 2.14$) lower than the *Neoeriocitrin* ($K_a/K_c = 3.73$). This could be caused by the methoxyl group or by the lack of the 3′,4′-di-OH-structure.

A possible explanation of the above mentioned could be that neohesperidose sugar causes the flavanone molecule to get a spatial configuration such as to stabilize the so formed radical. Oxidation of a flavonoid occurs on the B-ring when the catechol is present yielding a fairly stable *ortho*-semiquinone radical through facilitating electron delocalization.

Furthermore, a 3′4′-catechol structure in the B-ring strongly enhances the lipid peroxidation inhibition (Dugas et al., 2000; Mora, Paya, Rios, & Alcaraz, 1990). Flavanones glycosylated with neohesperidose lacking catechol system form relatively unstable radicals and are weak scavengers.

3.2. Influence of the O-methylation

The difference in antioxidant capacity between the polyhydroxylated and the polymethoxilated flavanones is due to the differences either in hydrophobicity and in molecular planarity. The influence of the O-methylation in the aglyconic flavanones is neglectable. On the other hands, in the flavanones, replaced with a neohesperidoside molecule in the 7th position, a methoxylation in the 4th position noticeably decreases the antioxidant power. Table 2 shows that the comparison between the antioxidant capacity of the hydroxylate aglyconic forms (*Naringenin and Heridictyol*) and that of the methoxylate aglyconic forms (*Isosakuratenin and Hesperitin*) did not produce considerable results under these experimental conditions. In the glycosylated analogous forms, as shown in Table 1, the substitution of the hydroxyl group in the 4th position with methoxyl one, apparently decreased the antioxidant activity.

In fact, the *Neohesperidin*, glycosylate on the 7th and methoxylate on the 4th positions, has proved to have an antioxidant power ($K_a/K_c = 2.14$) lower than the *Neoeriocitrin* ($K_a/K_c = 3.73$), analogous form to Neohesperidin but no methoxylate. It could be argued that, in the glycosylated flavanones, the blockage of the C'_4 hydroxyl group with methylation inhibits the anti-oxidation power, which could be caused by the introduction of the electron-donating group or by the lack of the 3′,4′-di-OH-structure. Presence of electrondonating or withdrawing groups at the aromatic system and glycosylation with neohesperidoside in the 7th position strongly influence the redox potential of phenols. The presence of electron-donating groups makes the aromatic system rich in electrons. This confers a higher degree of instability to the flavanone phenoxyl radicals. Furthermore, this study puts in evidence that, the introduction of the neohesperidose in the 7th position gives significant properties to flavanones. The comparison between the antioxidant power of the *Hesperitin* (aglyconic and methoxylate form) and that of the *Neohesperidin* (glycosylated and methoxylated form) puts in evidence the effect of the conjugation on the antioxidant properties. *Hesperitin* has proved to have an antioxidant activity higher than the *Neohesperidin*; this demonstrates that the introduction of the neohesperidose influences the antioxidant power. This could be caused by the lack of hydroxyl group in 7th position, but the comparison between Naringenin and Naringin rule out it. The antioxidant action of *Naringin*, flavanone glycosylated with neohesperidose lacking hydroxyl group, has not revealed a significant difference in the antioxidant power than *Naringenin*, analogous but not glycosylated. Therefore, it could be hypothesized that the sugar molecule in the 7th position is able to interact with the methoxyl group in the 4th position and reduce the antioxidant power.

3.3. Influence of the O-glycosylation

In this work, as shown in Table 3, the antiradical activity of the aglycon molecule (*Hesperetin and Naringenin*) vs. the corresponding neohesperidoside

Table 3
Comparison between aglycon with corresponding neohesperidoside and rutinoside forms

Compounds	C_5	C_7	C_3'	C_4'	K_a/K_c
Aglycone forms					
Hesperitin[a]	OH	OH	OH	OCH_3	3.13 ± 0.77
Naringenin[ab]	OH	OH	H	OH	2.73 ± 0.45
Neohesperidoside forms					
Neohesperidin[b]	OH	Ramnosil α-1,2 glucose	OH	OCH_3	2.14 ± 0.30
Naringin[ab]	OH	Ramnosil α-1,2 glucose	OH		2.41 ± 0.30
Rutinoside forms					
Hesperidin[ab]	OH	Ramnosil α-1,6 glucose	OH	OCH_3	2.81 ± 0.07
Narirutin[ab]	OH	Ramnosil α-1,6 glucose	H	OH	2.46 ± 0.22

(Different letters indicate significant differences at $P \leqslant 0.005$ Duncan's test).

(*Neohesperidin and Naringin*) and rutinoside (*Hesperidin and Narirutin*) forms were compared. Flavanones glycosylate have shown, except for Neohesperidin, an antioxidant power comparable to that expressed by free flavanones. The difference between the antioxidant power of the Hesperetin, free flavanone, and that of the Neohesperidin, flavanone glycosylate with neohesperidose (ramnosil α-1,2 glucose), is really considerable. In fact, only *Hesperitin* has shown to have an antioxidant activity ($K_a/K_c = 3.13$) higher than the *Neohesperidin* ($K_a/K_c = 2.14$). In this case, O-glycosylation at hydroxyl position influences radical-scavenging activities and this could be caused by the steric effect which perturbs the planarity and the ability to delocalize electrons (van Acker et al., 1996). On the other hand, the antioxidant activity of the *Hesperidin* ($K_a/K_c = 2.81$), flavanone glycosylate with rutinose (ramnosil α-1,6 glucose) is actually neglectable. Therefore, it could be hypothesized that the kind of sugar in the 7th position (neohesperidose or rutinose) and the position of methoxyl group (3'th or 4'th position) influence the ability to delocalize electrons. A possible explanation of the above mentioned could be that rutinose sugar causes the flavanone molecule to get a planar configuration such to stabilize the so formed radical.

4. Conclusions

The scavenging properties of antioxidant compounds are often associated with their ability to form stable radicals. The data got from this study show the different antioxidant power contribution that each of the flavanonic compounds can bring to food depending on their chemical structure. The differences in antioxidant potential caused by the different substitution groups is probably a consequence of the altered spin-distribution and stabilization of the flavonoid phenoxyl radical (Mayouf & Lemmetyinen, 1993). These results show a clear relationship between the planar character of flavanone compounds and antioxidant power. The substitution of the 7th OH group of the flavanone compounds by a neohesperidosyl group influences the relationship between structure and antioxidant activity. The results from this work demonstrate that: (a) The 3',4'-di-hydroxy substitution, in the aglycone form, does not influence so much the antioxidant activity (comparison between *Heridictyol* and *Naringenin*); On the other hand, in the flavanones glycosylated with a neohesperidose of the 7th OH group, the 3',4'-catechol structure noticeably increased the antioxidant power (as it can be see in *Neoeriocitrin* comparison vs. *Naringenin*). Oxidation of a flavonoid occurs on the B-ring when the catechol is present yielding a fairly stable *ortho*-semiquinone radical through facilitating electron delocalization. Flavanones glycosylated with neohesperidose lacking catechol system form relatively unstable radicals and are weak scavengers. (b) The influence of the O-methylation in the aglyconic flavanones is neglectable. On the other hands, in the flavanones, replaced with a neohesperidoside molecule in the 7th position, a methoxylation in the 4th position noticeably decreases the antioxidant power (comparison between *Neohesperidin* and *Neoeriocitrin*). The presence of electron-donating groups makes the aromatic system rich in electrons. This confers a higher degree of instability to the flavanone phenoxyl radicals. Therefore, it could be hypothesized that the sugar molecule in the 7th position is able to interact with the methoxyl group in the 4th position and reduce the antioxidant power.

(c) Flavanones glycosylate have shown, except for Neohesperidin, an antioxidant power comparable to that expressed by free flavanones. *Hesperitin* has shown to have an antioxidant activity higher than the *Neohesperidin*. O-glycosylation at hydroxyl position influences radical-scavenging activities and this could be caused by the steric effect which perturbs the planarity and the ability to delocalize electrons (van Acker et al., 1996). (d) The antioxidant activity flavanone glycosylate with rutinose (as it can be see in *Hesperidin* comparison vs. *Hesperitin*) is actually neglectable. Therefore, it could be hypothesized that the kind of sugar in the 7th position (neohesperidose or rutinose) and the position of methoxyl group (3'th or 4'th position) influence the ability to delocalize electrons.

This piece of information should be useful for identifying foods rich in such protective components for the development of safe food products and additives with appropriate antioxidant properties. Antioxidants represent a group of substances very important in diet but we cannot ignore that these compounds, when in high concentrations or in particular environmental conditions, can act like pro-oxidants inducing radicals reaction.

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