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Clean recovery of antioxidant flavonoids from citrus peel: Optimizing an aqueous ultrasound-assisted extraction method

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ABSTRACT

One of the main reasons for unsuccessful recovery of flavonoids from citrus by-products is the absence of effective extraction procedures. In this work, flavonoid fractions were obtained from citrus peels (lime, orange and tangerine) growing in South American cultivars using an optimized aqueous ultrasound-assisted extraction method with high yield (40.25 ± 12.09 mg of flavonoid fraction/g peel). Total phenolic content in flavonoid fractions obtained from different sources was 74.80 ± 1.90, 66.36 ± 0.75 and 58.68 ± 4.01 mg GAE (gallic acid equivalents)/g, for lime, orange and tangerine, respectively.

The composition of flavonoid fractions was established by using HPLC/MS. Orange peel contained hesperidin, neohesperidin, diosmin, nobiletin and tangeritin, being the most complex source. Tangerine peel was the simplest source and contained only hesperidin and neohesperidin. Using the thiobarbituric acid-reactive substances (TBARS) assay it was demonstrated that all flavonoid fractions were able to inhibit cupper (Cu^{2+}) or peroxynitrite ($ONOO^-$) induced human low density lipoprotein (LDL) oxidation. Differences in the antioxidant activity of individual components from flavonoid fractions were also observed.

1. Introduction

Due to their high flavonoid content, citrus peels could be exploited by both pharmaceutical and food industries. In spite of this, the compounds present in citrus peel are usually processed as by-products or wasted, resulting in environmental pollution. One of the main reasons for this is the absence of effective extraction procedures to obtain the flavonoids from the citrus peels (Ma et al., 2008). Additionally, instrumental methods for rapid and solid structural characterisation and analysis must be developed. HPLC coupled with mass spectrometry (HPLC–MS), has been a selective technique to analyze citrus flavonoids in orange extracts (De Lourdes Mata Bilbao, Andres-Lacueva, Jauregui, & Lamuela-Raventos, 2007). Mass spectrometry with electrospray ionization is a soft technique that mainly forms protonated $[M+H]^+$ or adduct ions; in most cases, no fragmented ions are observed. However, collision-induced dissociation (CID) and tandem mass spectrometric techniques have shown to be useful in obtaining important structural information for the characterisation of underivatized flavonoid aglycones and glycosides. The low-energy CID MS/MS spectra of various flavonoid aglycones and glycosides were described in LC–ESI/MS (Huck, Huber, Ongania, & Bonn, 2000).

Hesperidin is the most abundant flavanone glycoside found in citrus peel. It is known that hesperidin improves the vascular integrity, decreases capillary permeability and is given as a supplement to patients with blood vessel fragility (Valensi et al., 1996). Hesperidin also shows anti-inflammatory and immunomodulatory effects (Yeh et al., 2007). Furthermore, in association with naringin, the hesperidin might reduce cholesterol levels (Lee et al., 1999). It is also reported that hesperidin has the ability to inhibit copper-induced low density lipoprotein (LDL) oxidation (Cirico & Omaye, 2006). Numerous studies have shown that micronutrients provided by diets including various flavonoids can protect LDL from oxidative modification (de Whalley, Rankin, Hoult, Jessup, & Leake, 1990). The key role played by oxidized LDL in the initial and advanced stages of the atherosclerotic lesions has been well established (Witztum & Steinberg, 1991). Modified LDL is not recognized by the LDL-receptors apo (B/E), but instead, is taken up in a non-regulated manner through scavenger receptors present in the surface of monocytes and macrophages. This process leads to the accumulation of esterified and unesterified cholesterol in the macrophages and, consequently, to the formation of foam cells, the hallmark of the atherosclerotic lesion (Esterbauer, Gebicki,

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Puhl, & Jurgens, 1992). Oxidant species such as nitric oxide (·NO) and superoxide anion ($O_2^{\cdot-}$) are produced in the vascular endothelium, and can generate the highly oxidant specie peroxynitrite ($ONOO^-$). In the presence of $ONOO^-$, LDL undergoes oxidation on both lipidic and proteic portions, increasing lipid oxidation products and nitrotyrosine, a fingerprint for peroxynitrite oxidation formed by tyrosine residues nitration (Shishehbor et al., 2003).

Citrus flavonoid composition was subject of HPLC studies in Japan (Kawaii et al., 2001), USA (Berhow, Kanes, and Vandercook, 1996) and Spain (Rio et al., 1997). Surprisingly, there are very few studies about citrus peel flavonoid composition considering species and local cultivars of South America, despite Brazil is the main citrus producer in the world and with Argentina generate about 23% of global orange production. In this work, a clean, environmental friendly and efficient methodology which allows extraction of flavonoid from citrus peels using Colombian cultivars was developed. Operational conditions for HPLC–ESI–MS analysis of flavonoids were optimized. Antioxidant activity of flavonoid fractions, hesperidin and its aglycone hesperetin, was assessed measuring the capacity to inhibit low density lipoprotein oxidation both copper and peroxynitrite-induced. Furthermore, individual compounds from flavonoid fractions were evaluated regarding antioxidant activity on liver microsomes in order to establish structure–activity relations.

2. Materials and methods

2.1. Chemicals

Methanol HPLC grade and formic acid were obtained from Merck (Darmstadt, Germany). Hesperidin, Folin–Ciocalteau reagent, thiobarbituric acid, were reactive grade and purchased from Sigma (St. Louis, MO, USA). Reference standards of flavonoids were purchased from CrhomaDex Inc. (Irvine, USA). Calcium hydroxide USP grade was from HRA Uniquimica (Medellín, Colombia), other commercial grade reagents were obtained from local distributors.

2.2. Extraction experimental design and sample preparation

The citrus peels were obtained from three species: tahiti lime (*Citrus latifolia*), sweet orange (*Citrus sinensis*) and oneco tangerine (*Citrus reticulata*). The ripeness degree and post-harvest conditions were according to those set in the Colombian Technical Norms (ICONTEC) (CTN-4085, CTN-4086 and CTN-4087) for commercial citrus fruits. The citrus peel composed of flavedo and albedo, were removed from pulp, processed in fresh, or dried at 40 °C until constant weight according to experimental design, powered in a conventional food mixer and sifted through 0.2 mm mesh. For dry weight calculation, the humidity percentage in citrus peel was calculated (74.97% ± 0.080).

For extraction of hesperidin and other related citrus flavonoids such as naringin, we took advantage of their very low solubility in water (<20 ppm) (El-Nawawi, 1995). The extraction was ultrasound-assisted and was carried out in ultrasonic cleaning bath (Elma-LC60H, Singen, Germany), operating at 60 kHz, as reported recently (Ma et al., 2008).

In order to optimize the extraction process a factorial design (2^2) was used to identify the effect of two active factors and its levels on yield percentage and total phenolic compounds content. Tangerine peel was used as model. It was evaluated the effect of water content in the citrus peel material (0% and 75%) and the extraction time (30 and 90 min); the citrus peel/water ratio (g/mL) was fixed in 1/10, and $Ca(OH)_2$ was used as basifying agent, after evaluation of other agents (data not shown).

For measurement, precipitates were obtained by centrifugation after acidification with chlorhydric acid until pH 7 and then collected for yield estimation and an amount of product was dissolved in dimethyl sulphoxide:methanol (2:1 v/v), filtrated through 0.45 μm nylon membrane and used for HPLC analysis and total phenolic content through Folin–Ciocalteau method, the results are expressed as mg gallic acid equivalents per gram (GAE/g). The hesperidin isolation was accomplished by successive acid/base precipitations from flavonoids fraction. The hesperetin was obtained by atmospheric pressure acid-catalyzed hydrolysis of hesperidin (Grohmann, Manthey, & Cameron, 2000) and used as model to establish differences between glycoside and aglycone. Structural identification of glycoside and aglycone was made by HPLC/MS comparing fragmentation profile with reference standards.

2.3. HPLC–DAD ESI–MS analysis

The HPLC analysis were performed using an Agilent 1200 Series LC/MSD-Q (Santa Clara, CA, USA) with a photodiode array detector set at 290 and 360 nm (monitoring wavelength). A Hypersil BDS (C8) (Alltech, Nicholasville, KY, USA), 250 × 4.6 mm, 5 μm column was used at 25 °C. The mobile phase consisted of water with 0.1% formic acid (eluent A) and acenonitrile (eluent B), used in isocratic program with 75% A and 25% B. The flow-rate was 0.75 mL/min. The ESI-MS spectra were acquired in positive mode. The ESI source conditions were optimized in Selected Ion-Monitoring (SIM) mode, using the automated Flow-Injection Analysis (FIA). The molecules of the protonated adduct $[M+Na]^+$ (m/z 633) was selected as the target ion for hesperidin. The parameters and ranges used are listed as follows: drying N_2 (10–12 L/min at 350 °C); nebulizing N_2 (52–60 psi); capilar voltage (2–5 kV). In order to obtain a Collision Induced Dissociation (CID) it was used the fragmentor voltage (70–190 V).

Both, the highest abundance of target ion and fragmentation were obtained with the conditions: drying N_2; 13 L/min, nebulizing N_2; 60 psi, capilar voltage; 4.8 kV, fragmentor; 100 V.

2.4. Antioxidant activity

LDL was isolated through discontinuous density gradient centrifugation procedure (Londoño, Guerrero, Aristizabal, and Arango, 2006) using a Beckman XL-100 ultracentrifuge. The concentration of protein was determined by the Bradford's method using a commercial kit (Fluka Chemika AG, Buchs, Swizerland). Peroxynitrite, used as oxidant agent, was synthesised by the quenched-flow method using vacuum at the end of a cooled T junctions-flasks system (De Lima, Morfim, Teixeira, & Creczynski-Pasa, 2004). Peroxynitrite concentration (2.68 mM) was guaranteed into LDL solution (500 μg/mL) by successive additions; the reaction mixture was maintained at 37 °C during 3 h. The samples were tested at 10 μg/mL. For copper-induced oxidation, LDL (300 μg/mL) was incubated with $CuSO_4$ (100 μM) during 12 h at 37 °C. The samples were tested at 1000, 500, 100, and 10 μg/mL.

The liver microsomes were obtained by differential centrifugation with calcium aggregation (De Lima et al., 2004). The fractions obtained were frozen at −70 °C. The protein concentration was determined by the Bradford's method.

The extent of lipid peroxidation was determined by thiobarbituric acid-reactive substances (TBARS) method (Bird & Draper, 1984). In all experiments controls were used by adding all reagents except lipids or compounds. The amount of TBARS was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and results are shown as μM of malondialdehyde (MDA) equivalents/mg protein.

2.5. Statistical analysis

Data are presented as means ± SEM. The statistic significance of differences among groups was evaluated by one-way ANOVA using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). The differences between the means were assessed using Newman–Keuls multiple comparisons post-test and significance was identified with a value of $p < 0.05$. For extraction analysis, a screening design was made using StatGraphics Plus 5.1.

3. Results and discussion

3.1. Extraction

Extraction of bioactive compounds from vegetable materials with a solvent is a classical operation applied in many industrial processes, particularly the pharmaceutical industry. It is obvious that medical interest in plants derived drugs has led to an increased need for ideal extraction methods, which could obtain the maximum of the bioactive constituents in a shortest processing time with a low cost. Ultrasonic-assisted extraction has been proven to significantly decrease extraction time and increase extraction yields in many vegetable materials (Vinatoru, 2001).

Recently, it was reported the hesperidin extraction from *C. reticulata* peel (Ma et al., 2008). The optimum ultrasonic conditions were determined as: frequency of 60 kHz, extraction time of 60 min, and temperature of 40 °C and methanol as solvent. In the present work as illustrated in Fig. 1 the highest yield was obtained with dry material and 30 min of extraction, however at 30 min with wet material the lowest yield was obtained. It is possible to explain this behavior because the dry material has more porosity and the solvent diffusion rate could be higher.

With wet material it was seen that the yield increased with the rise of time, however this increment is not selective to phenolic compounds as can be seen in Fig. 2. The total phenolic content does not change significantly ($p > 0.05$) over the time.

In general, the yields and total phenolic content from dry material were higher than those in wet material ($p < 0.01$) and the time of extraction had no influence on the phenolic content. Hence we set the extraction parameters as: ultrasound-assisted extraction, frequency of 60 kHz, extraction time of 30 min, and temperature of 40 °C, citrus peel/water ratio (g/mL) 1/10, $Ca(OH)_2$ as basifying agent and water as solvent. With this parameter yield of 40.25 ± 12.09 mg/g was obtained and the total phenolic content was 19.595 ± 2.114 mg GAE/g of peel dry matter.

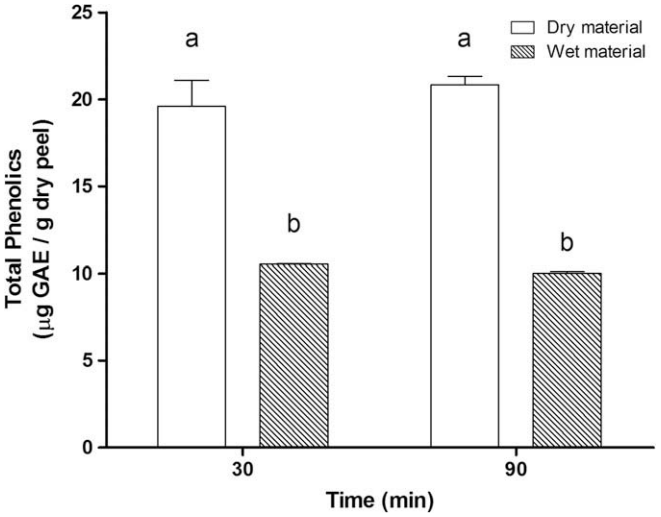


Fig. 2. Comparison of total phenolics content in both dry and wet material submitted to ultrasound-assisted extraction during two time lapses. Data are shown as means ± SEM. Means with the same letter are not significantly different at the $p = 0.05$ level. Means with different letter are significantly different at the $p < 0.01$ level.

Extraction of flavonoid fraction was allowed on lime, orange and tangerine peels and the total phenolic content was measured through Folin–Ciocalteau method, showing that lime peel has the higher content of phenolic compounds followed by orange peel and finally tangerine peel, as can be seen in Fig. 3.

3.2. HPLC/DAD–MS analysis

In this work, we applied a LC/ESI/MS with CID-in source approach in order to elucidate and characterize the flavonoid glycosides structure, achieving structural information on (i) the carbohydrate sequence, (ii) the aglycone moiety and (iii) the linkage in the diglycosidic.

For chemical characterisation, the carbohydrate and aglycone ion nomenclature was adopted from Domon and Costello (13) (Table 1). The flavanones hesperidin and neohesperidin showed characteristic ions: 633 $[M+Na]^+$, loss of rhamnose 465 $[Y_1+H]^+$, formation of characteristic ion 449 (Z_1 or Y^*) by loss of an internal dehydrated glucose residue and a mechanism involving a mobile proton from the aglycone to the terminal rhamnose (Cuyckens,

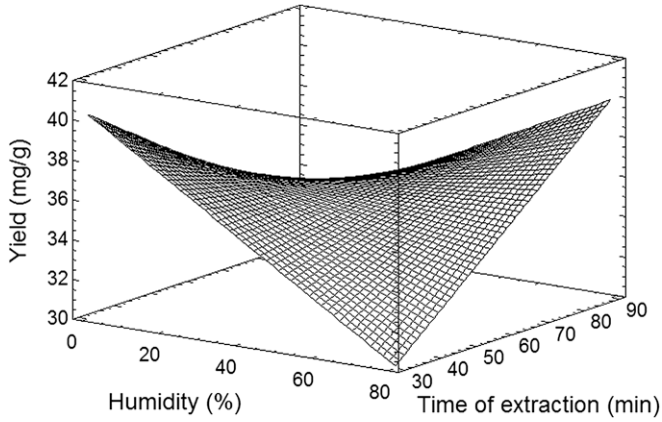


Fig. 1. Optimization of extraction process. Tangerine peel was extracted with experimental conditions as material and methods. The effect of two factors (humidity of peels and time of extraction) was evaluated.

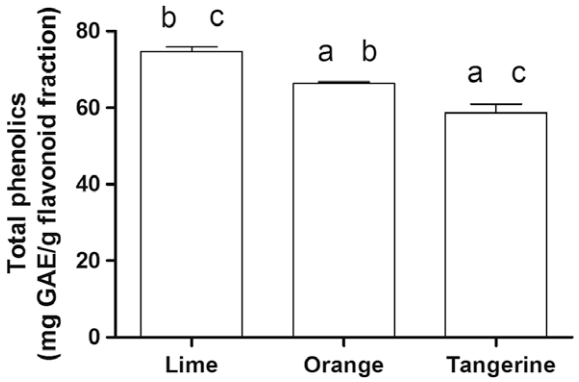
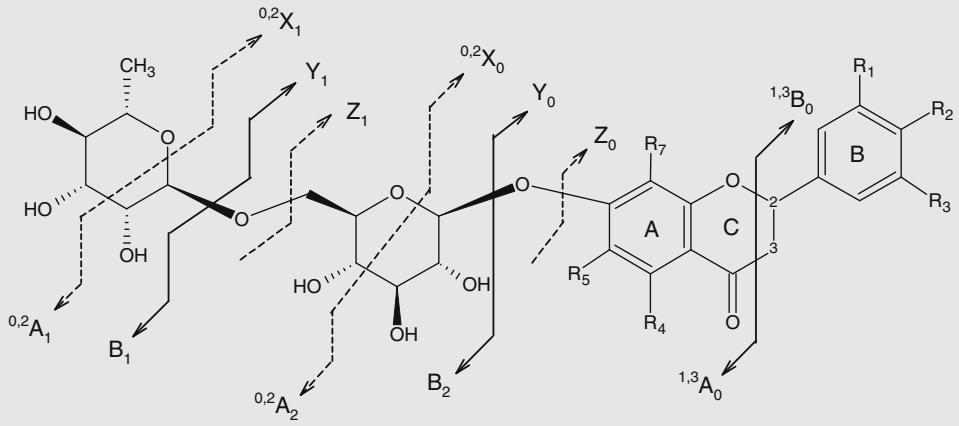


Fig. 3. Flavonoid extraction was allowed on lime, orange and tangerine peels using the optimized method. Total phenolics content was measured. Data are shown as mean ± SEM. Samples are statistically different, bars with the same letter represents the pair comparison: (a) $p < 0.05$, (b) $p < 0.01$ and (c) $p < 0.001$.

Table 1
Chemical structures with ion nomenclature followed for flavonoid glycosides. Chromatographic and spectral data of flavonoids obtained from different sources.



Compound	R1	R2	R3	R4	R5	R6	R7	[M+H]+ (*m/z*)	UV λ_{max} (nm)	Sources			
										C	O	T	L
Flavanones (single C_2–C_3)													
(**1**) Hesperidin	OH	OCH_3	H	OH	H	O-glu-rham (1 → 6)	H	611	285, 330	X	X	X	X
(**2**) Neohesperidin	OH	OCH_3	H	OH	H	O-glu-rham (1 → 2)	H	611	280, 336	X	X	X	X
Flavones (double C_2–C_3)													
(**3**) Diosmin	OH	OCH_3	H	OH	H	O-glu-rham (1 → 6)	H	609	253, 350	X	X		X
(**4**) Isorhofolin	H	OH	H	OH	H	O-glu-rham (1 → 6)	H	579	267, 338	X			
(**5**) Nobiletin	H	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	403	250, 272, 334		X		
(**6**) Tangeritin	H	H	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	373	270, 325		X		
(**7**) Hesperetin	OH	OCH_3	H	OH	H	OH	H	303	284, 330				

C, commercial flavonoid fraction; O, orange peel; T, tangerine peel; L, lime peel.

Ma, Pocsfalvi, & Claeys, 2000), protonated aglycone hesperetin 303 $[Y_0+H]^+$, aglycone Retro-Diels–Alder (RDA) fragmentation by both 1–4 and 0–3 bond scission to give 153 $[^{0,3}A+H]^+$ and 177 $[^{1,4}B-H_2+H]^+$ ions, respectively. The linkage in the diglycosidic moiety (1 → 6 or 1 → 2) was determinated from Y_0/Y_1 ion ratio and $Y_0 - Z_1$ relationship, according with literature (Cuyckens et al., 2000). Flavones diosmin and isorhoifolin were identified by its Y_0/Y_1 ions (301/463 and 271/433, respectively) (Cuyckens et al., 2000). The polymethoxylated flavones showed MS and UV profiles similar to those reported previously (Weber et al., 2006), with ions 425 $[M+Na]^+$, 403 $[M+H]^+$, 373 $[M-OCH_3]^+$ for nobiletin and 395 $[M+Na]^+$, 373 $[M+H]^+$, 343 $[M-OCH_3]^+$, 211 $[M-5OCH_3]^+$ for tangeritin.

Table 1 shows chromatographic and spectral (MS and UV) information of compounds which were identified based on their UV spectra, molecular and fragment ions. The presence of these compounds was differentially observed in the sources. Orange peel represents the most diverse source due to presence of polymethoxylated flavones nobiletin and tangerintin. Tangerine peel is a most simple source containing only hesperidin and its isomer neohesperidin, for this reason it might be a feasible material for hesperidin isolation. On the other hand, the commercial flavonoid fraction (sold as hesperidin 80%) differs of studied citrus peel for isorhoifolin only.

A typical chromatographic profile of orange peel is shown in Fig. 4. Since the peak resolution is acceptable ($\alpha > 1.5$) it is possible to propose this method as a rapid identification tool for flavonoids in quality control of citrus by-products. The ions 633/303, 631/301, 601/271, 425/373, 395/343 might be useful for quantitative analysis of hesperidin, neohesperidin, diosmin, isorhoifolin, nobiletin, tangeritin, respectively; using single ion monitoring approach (HPLC/ESI/SIM/MS).

3.3. Inhibition of peroxynitrite-induced oxLDL

Hesperidin is the most abundant flavanone glycoside found in citrus. Citrus peels also contain important flavonoids, such as narigin, narirutin, diosmin and eryocitrin (Schieber, Stintzing, & Carle, 2001). The bioactivities of citrus polymethoxylated flavones, such as nobiletin and sinensetin have been also reported. For instance, nobiletin showed immunomodulatory and antiatherogenic activity (Kurowska & Manthey, 2004).

In studies with copper-induced LDL oxidation, hesperidin had predominantly antioxidant effects when evaluated at 9.3 μM either alone or mixed with quercetin, ferulic acid and catechin (Cirico & Omaye, 2006). In contrast, single compounds quercetin and ferulic acid at 20 μM were prooxidant and only the mixture showed antioxidant effect. In our study, no prooxidant effect was seen when hesperidin was assessed at concentrations higher than 10 μg/mL (16.3 μM). Nevertheless, concentrations higher than 100 μg/mL of flavonoid fraction or 163 μM of hesperidin were not the most effective, showing a stationary effect (Fig. 5). On the other hand, the flavonoid fraction at 10 μg/mL was more active than hesperidin at the same concentration ($p < 0.001$), showing a synergist effect of the components of the fraction at low concentration.

When we compared composition and activity, it can be seen that changes in individual composition does not affect the antioxidant activity of flavonoid fractions. In fact, all the flavonoid fractions obtained from different sources and the commercial fraction showed the same activity ($p > 0.05$) in spite of the exclusive presence of isorhoifolin in commercial fraction and nobiletin and tangeritin in orange peels (Fig. 6). In effect, total phenolic content was not a key feature for antioxidant activity and it is probably that the differences between samples are not significant as to produce a change in antioxidant activity.

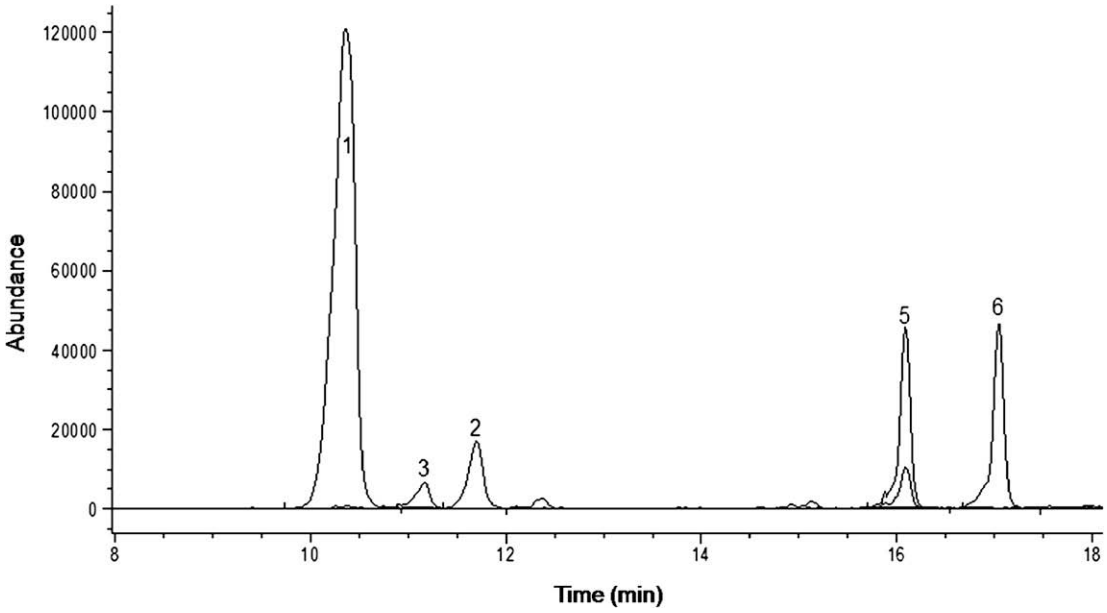


Fig. 4. The typical HPLC profile of flavonoid fraction from orange peel. HPLC–EIC–MS chromatogram of $[M+Na]^+$ ions: hesperidin (1) and neohesperidin (2) (633), diosmin (3) (631), nobiletin (5) (425), tangeritin (6) (395).

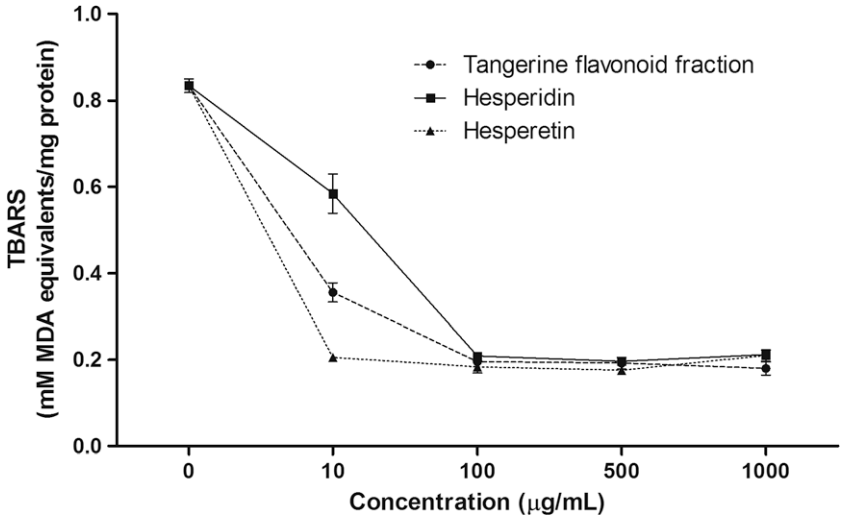


Fig. 5. Concentration-dependent inhibition of the copper-induced LDL oxidation by tangerine flavonoid fraction, hesperidin and hesperetin. Data are shown as means ± SEM.

In order to study the antioxidant activity of individual components from flavonoid fraction, we evaluate the compounds found in the more complex source (orange) on liver microsomes lipoperoxidation.

It was seen that hesperetin (aglycone), nobiletin and tangeritin (polymethoxyflavons) are more active that diosmin, hesperidin and neohesperidin (glycosides) (Fig. 7), showing an interesting structure–activity relationship as already reported for flavonoids, which describe that antioxidant activity decrease with glycosilation and enhance with (1) hydroxyl groups in ring B, (2) C2–C3 double bond in conjugation with a 4-oxo function, and (3) the ability of the flavonoids to interact with the lipid bilayers (lipophylicity) (Rice-Evans, Miller, & Paganga, 1996).

According to our results, both aglycone (hesperetin) and glycoside (hesperidin) showed similar antioxidant properties when evaluated in the peroxynitrite-oxidized LDL model at the same concentration (10 μg/mL) (Fig. 6). Nevertheless, when a concentration–response experiment was performed (Fig. 5), hesperidin was less active than hesperetin when evaluated at 10 μg/mL, suggesting a role of sugar moiety. In general, it was reported that aglycones are more potent antioxidants than their corresponding glycosides (Williamson, Plumb, & Garcia-Conesa, 1999). For example, genistein is inferior to its aglycone genistein to attenuate peroxynitrite-induced oxidation of LDL (Lai & Yen, 2002). Aside from occupying free OH groups necessary for hydrogen abstraction and radical scavenging, any sugar substituent is capable of (i) diminishing coplanarity of the B-ring relative to the rest of the flavonoid, and/or (ii) changing hydrophilicity and altering access to lipid peroxyl and alkoxyl radicals during propagation of lipoperoxidation (Kelly, Anthony, & Dennis, 2002). Though glycosides are usually weaker antioxidants than aglycones, bioavailability is sometimes enhanced by a glucose (Hollman et al., 1999). In light of the evidence that glycosidic bonds are often cleaved at the gut level (Kobayashi, Tanabe, Sugiyama, & Konishi, 2008), the influence of sugar moieties on antioxidant properties is of questionable significance in

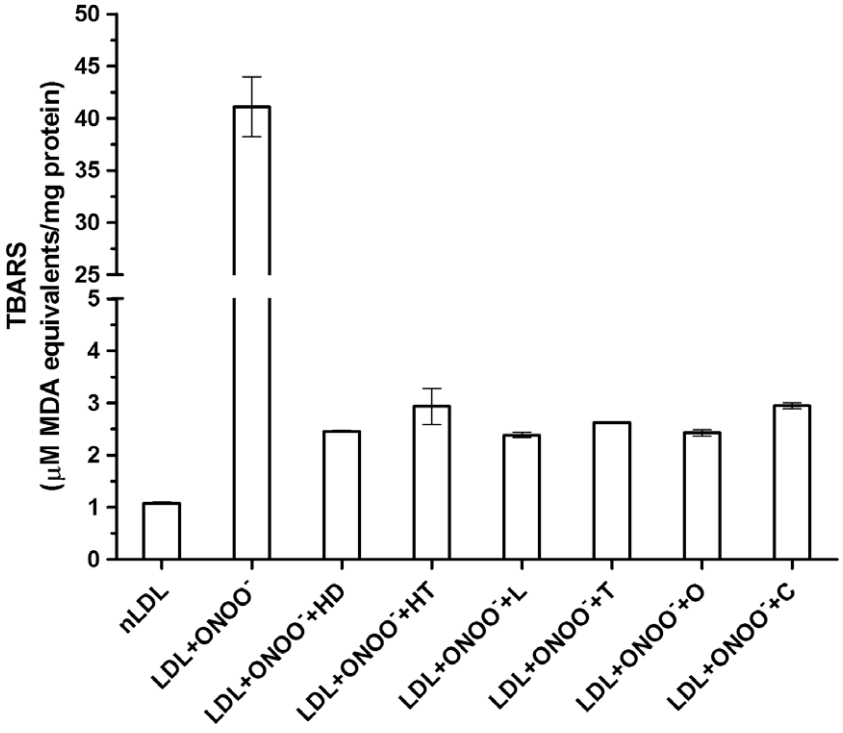


Fig. 6. Effect of flavonoid fractions, hesperidin and hesperetin on peroxynitrite-induced LDL oxidation. HD, hesperidin; HT, hesperetin; L, flavonoid fraction from lime; T, flavonoid fraction from tangerine; O, flavonoid fraction from orange; C, commercial hesperidin. Data are shown as means ± SEM.

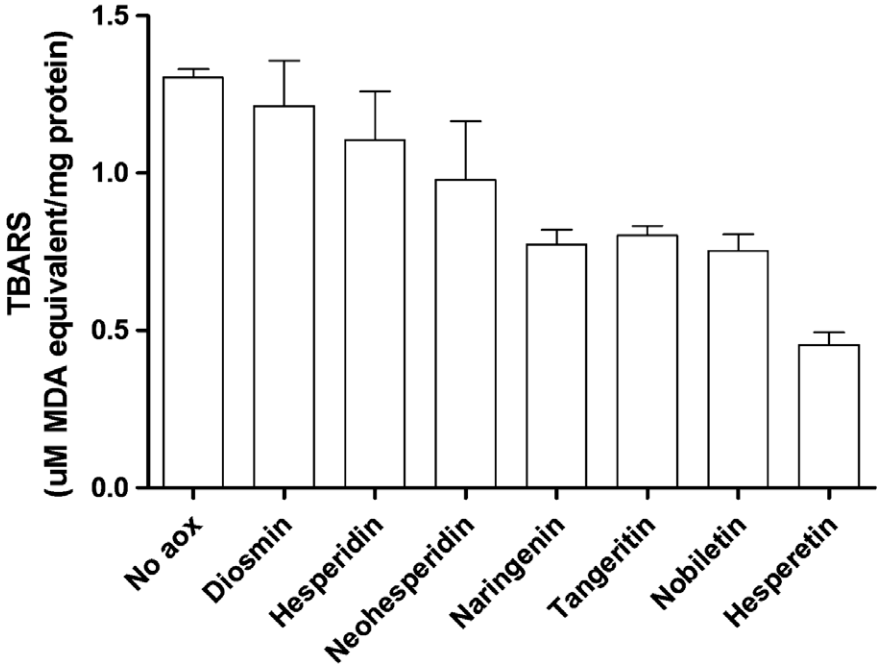


Fig. 7. Effect of individual components from flavonoid fractions on liver microsomes lipoperoxidation (no Aox: control without antioxidant). Data are shown as mean ± SEM.

humans, since removal of the glycosidic substituent by enteric enzymes or bacteria likely increase the *in vivo* activity of dietary flavonoids like hesperidin.

Acknowledgments

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