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The Compositional Characterisation and Antioxidant Activity of Fresh Juices from Sicilian Sweet Orange (*Citrus sinensis* L. Osbeck) Varieties

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Epidemiological evidence has suggested that consumption of fruit and vegetables reduces the risk of both cancer and cardiovascular diseases, potentially through the biological actions of components such as vitamin C, vitamin E, flavonoids and carotenoids. *Citrus* species are extremely rich sources in vitamin C and flavanones, a class of compounds which belongs to the flavonoids family. A comparison of the phenolic compositions, the ascorbic acid contents and the antioxidant activities of fresh Sicilian orange juices from pigmented (*Moro*, *Tarocco* and *Sanguinello*) and non-pigmented (*Ovale*, *Valencia* and *Navel*) varieties of orange (*Citrus sinensis* L. Osbeck), was undertaken. The simultaneous characterisation and quantification of the major flavanone, anthocyanin and hydroxycinnamate components were attained by HPLC with diode array detection. Differences between varieties in terms of the flavanone glycoside content, particularly hesperidin, were observed, with the *Tarocco* juices reporting the highest content. Furthermore, cyanidin-3-glucoside and cyanidin-3-(6″-malonyl)-glucoside were predominant in all the pigmented varieties, but their concentration was higher in the juices of the *Moro* variety. Quantitatively, the major antioxidant component of all juices was ascorbic acid and its concentration was significantly correlated ($r = 0.74$, $P < 0.001$) with the total antioxidant activity of the juices, determined *in vitro* using the ABTS radical cation decolorization assay. Similarly, hydroxycinnamates ($r = 0.73$, $P < 0.01$) and anthocyanins ($r = 0.98$, $P < 0.001$) content showed a good correlation with the determined antioxidant capacity. Therefore orange juices, particularly those rich in anthocyanins, may represent a significant dietary source of flavonoids.

Keywords: Flavanone glycosides; Anthocyanidin glycosides; Hydroxycinnamic acids; Ascorbic acid; *Citrus sinensis* varieties; Fresh juices

INTRODUCTION

Epidemiological evidence has suggested that consumption of fruit and vegetables reduces the risk of both cancer and cardiovascular diseases, potentially through the biological actions of components such as vitamin C, vitamin E, flavonoids and carotenoids.[1–3] *Citrus* species are extremely rich sources in vitamin C and flavanones, a class of compounds which belongs to the flavonoids family. Flavanones are present in fruit and vegetables as glycosides, usually rutinosides (6-*O*-α-L-rhamnosyl-D-glucosides) and neohesperidosides (2-*O*-α-L-rhamnosyl-D-glucosides) attached at position 7.[4] Lemon, lime, mandarin and sweet orange contain essentially rutinosides, mainly of hesperetin, while grapefruit and bitter orange contain neohesperidosides, mainly of naringenin. Some varieties of sweet orange are also characterised by a high content of anthocyanins, a class of flavonoids widely used as natural colorants for food products. These compounds are present both in the juices and the tissues of fruit; thus, *citrus* fruit and their derived products represent a major source of flavanones and other flavonoids and may contribute significantly to the total dietary flavonoid intake in humans.[5]

Citrus flavonoids have shown various biological activities. For example, it has been reported that dietary orange and grapefruit juices

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are able to lower LDL cholesterol in rabbits.[6] Cholesterol-lowering effects by *citrus* flavonoids have also been demonstrated in cultured liver cells (HepG2) and they were associated with a reduced synthesis of hepatic cholesterol esters through regulation of apolipoprotein B metabolism.[7] Others have shown a 22% tumor incidence reduction in male rats fed orange juice after induction of colon cancer by azoxymethane.[8] Furthermore, we have recently shown that hesperetin glucuronides, potential *in vivo* metabolites of the citrus flavonoid hesperidin, are able to protect human skin fibroblasts from UVA-induced oxidative stress.[9]

Thus, in addition to ascorbic acid, *citrus* fruits and their constituents are proposed to be an important dietary source of biologically active compounds such as flavanones and their consumption may exert health beneficial effects. However, the number and diversity of components in sweet orange requires complex analytical techniques for their separation and identification. In addition, preliminary sample preparation, which entails the risk of degradation and loss of certain components, is normally required. Various HPLC methods have been described for the analysis of individual classes of components so that, in order to characterise the phenolic profile of an orange juice, separate analysis have been required for the flavanone glycosides, the hydroxycinamic acids and the anthocyanins.[10–13] Furthermore, while the antioxidant activity of selected phenolic components have been already investigated, little data on the qualitative and quantitative profile of the specific classes of flavonoid compounds in orange juice and their relation with antioxidant potential of this beverage are available.

In this paper, a rapid method for the simultaneous HPLC characterisation and quantification of the hydroxycinnamate, flavanone and anthocyanin profile of fresh orange juices, which does not involve a complex sample preparation, is described. Furthermore, the ascorbic acid content of the juices is reported and the relation between the flavanone glycosides, anthocyanin pigments, hydroxycinnamic acids and ascorbic acid levels and their antioxidant potential *in vitro* is investigated.

MATERIALS AND METHODS

Orange Juice Samples and Sample Preparation

Fresh juices of three non-pigmented, *Ovale*, *Valencia* and *Navel*, and three pigmented, *Sanguinello*, *Tarocco* and *Moro*, orange varieties cultivated in Sicily (Italy) were obtained and stored at −20°C for HPLC analysis or at −80°C for total ascorbic acid determination. Juices were thawed, centrifuged at 3000 rpm to remove solids and the supernatant was clarified by filtration through Sterile Millex® filters, 29 mm diameter, 4 cm^2 filtration area, 0.22 μm pore size (Millipore Corporation, Bedford, USA). The filtered juices were then used for HPLC analysis, total ascorbic acid (ascorbic acid and dehydroascorbic acid) and total antioxidant activity determination.

HPLC Analysis

The HPLC analysis of each juice was performed a minimum of three times on Hewlett-Packard 1100 system (Palo Alto, CA, USA) consisting of an autosampler with Peltier temperature controller, a quaternary pump with degasser and a photodiode array detector. The column was a Nova-Pak C_{18} (Waters Corp., Milford, USA), 4.6 × 250 mm with a 4 μm particle size and it was maintained at 30°C during analysis. Solvents were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK). Samples were injected by means of an autosampler with a 100 μl fixed loop and the volume of injection was 50 μl. Flow rate was 0.5 ml/min and the mobile phase was constituted by solvent A, 20% methanol/water with 0.1% hydrochloric acid, and solvent B, acetonitrile. The solvents were mixed using a linear gradient held at 95% solvent A for 10 min and then decreased to 50% solvent A at 50 min, returned to 95% solvent A at 55 min and held at these conditions for a further 5 min. Samples of filtered juice were opportunely diluted in solvent A and added up with an internal standard (10 mg/l chrysin, final concentration) prior to injection. The identification and quantification of the peaks were carried out from the retention times and photodiode array detection between 200 and 600 nm, in comparison with authentic standards HPLC grade (Extrasynthese, Genay, France). Specifically, flavanone glycosides and hydroxycinnamic acids were identified from chromatograms recorded at 320 nm, by comparing retention time and spectral characteristics with that of commercially available pure standards run with the same HPLC conditions. Similarly, cyanidin-3-glucoside was identified from chromatograms recorded at 520 nm, in comparison with the retention time and UV–Vis. spectrum of a pure standard. However, cyanidin-3-(6″malonyl)-glucoside was a putative assignment, based on a report that has identified this anthocyanin as the other major anthocyanin component present in red orange varieties, and other peaks were identified as anthocyanin components on the basis of their spectral characteristics and were quantified as cyanidin-3-glucoside.

Total Ascorbic Acid

Total ascorbic acid was measured by a fluorimetric method.[14] The measurement of the fluorescence

intensity of each juice samples was carried out a minimum of three times on a Shimadzu RF-1501 fluorimeter (Shimadzu Europe—UK Branch, Milton Keynes, UK), excitation wavelength 348 nm, emission wavelength 423 nm. The concentration of ascorbic acid was then obtained relative to a response curve obtained by using ascorbic acid standard (Sigma–Aldrich Ltd., Poole, UK).

Total Antioxidant Activity (TAA)

The total antioxidant of the juices was measured a minimum of three times by using the TEAC method[15] based on the oxidation of the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) by potassium persulphate to form a radical ($ABTS^{\bullet+}$) and the direct scavenging of the pre-formed radical. ABTS and potassium persulphate were purchased from Sigma–Aldrich (Poole, Dorset, UK). ABTS was dissolved in 5 mM PBS, pH 7.4, to a 7 mM concentration and reacted with a solution of potassium persulphate (2.45 mM final concentration in water). The mixture was allowed to stand in the dark, at room temperature for 12–16 h before use, to assure the complete formation of the stable $ABTS^{\bullet+}$. The radical cation solution was then opportunely diluted with PBS and mixed with aliquots of filtered juice (2.5–40 μl) to a final volume of 1 ml. Absorbance readings were taken at 30°C, exactly at 1 min and up to 4 min after the mixing, on a Hewlett-Packard spectrophotometer, model HP 8453, equipped with a peltier temperature control (Cheadle Heath, Stockport Cheshire, UK). The TAA was calculated from the percentage of reduction of the absorbance at 734 nm, indicating the ability of the compound to scavenge $ABTS^{\bullet+}$, as a function of the volume of juice, compared to that induced by a Trolox® standard solution (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Aldrich Chemical Co., Gillingham, Dorset, UK), under the same experimental conditions.

Statistical Analysis

Results were expressed as mean ± SD. Correlations were obtained by using Pearson's correlation coefficient and a $P \geq 0.05$ was considered significant.

RESULTS

The HPLC method with diode array detection here reported allowed the rapid and simultaneous determination of the major classes of phenolic components in orange juices, flavanone glycosides, hydroxycinnamic acids and anthocyanidin glycosides (the latter only present in the pigmented varieties).

Figures 1 and 2 show the HPLC chromatograms at 320 λ_{max}, displaying the separation of flavanone glycosides and hydroxycinnamic acids, of the juices of the non-pigmented and pigmented varieties respectively. The chromatograms at 520 λ_{max}, showing the separation of anthocyanin glycosides in the pigmented varieties only, are presented in Fig. 3. The identification and quantification of the peaks are given in Tables I and II, for the non-pigmented and pigmented varieties, respectively.

The hesperidin content of the pigmented varieties (range: 174–218 mg/l) was about 2–3 fold higher than that of the non-pigmented (range: 52–122 mg/l). Similarly, narirutin levels were approximately 2–3 fold higher in the pigmented oranges (range: 14–18 mg/l) than the non-pigmented varieties (range: 5–10 mg/l). As shown in Tables I and II, the hydroxycinnamic acids levels were also relatively higher in the pigmented oranges.

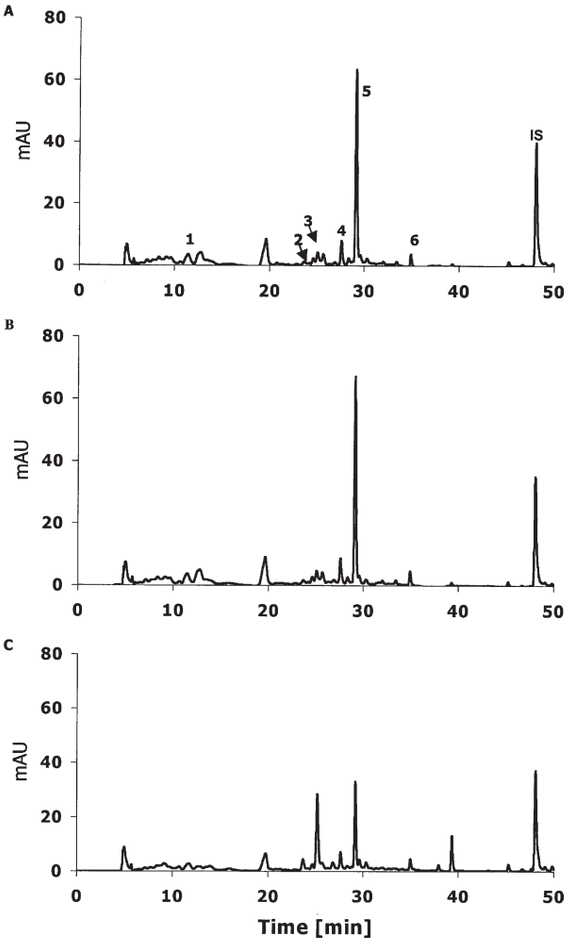

FIGURE 1 HPLC-PDA chromatograms at 320 nm of the juices obtained from non-pigmented orange varieties: (A) *Ovale*, (B) *Navel*, (C) *Valencia*. See Table I for peak identification, IS = chrysin.

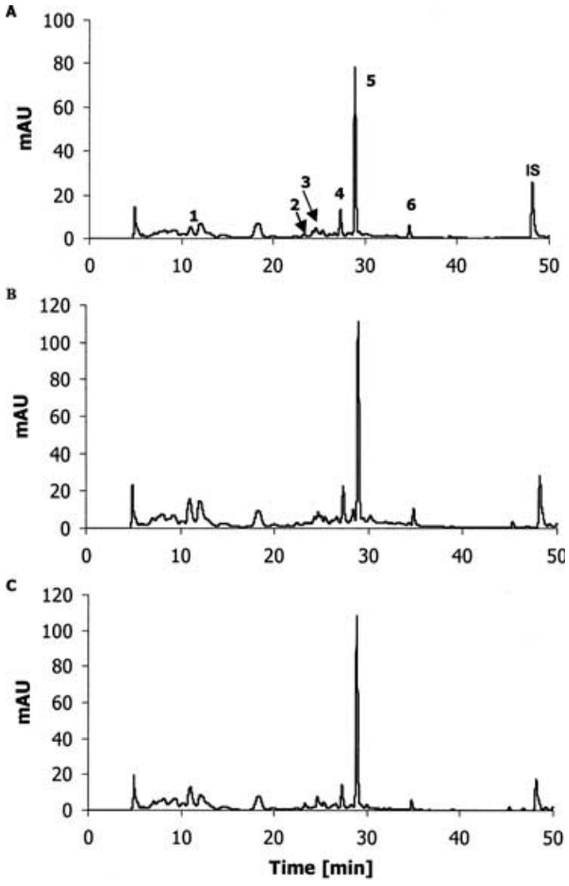

FIGURE 2 HPLC-PDA chromatograms at 320 nm of the juices obtained from pigmented orange varieties: (A) *Sanguinello*, (B) *Moro*, (C) *Tarocco*. See Table II for peak identification, IS = chrysin.

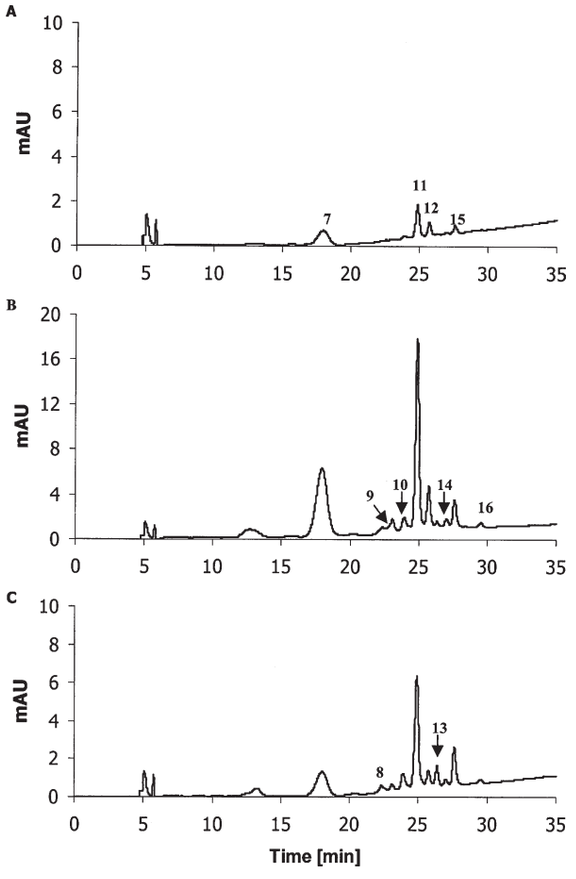

FIGURE 3 HPLC-PDA chromatograms at 520 nm of the juices obtained from the pigmented orange varieties: (A) *Sanguinello*, (B) *Moro*, (C) *Tarocco*. See Table II for peak identification.

Although the levels of the major anthocyanin pigments varied considerably between varieties, cyanidin-3-glucoside and cyanidin-3-(6″-malonyl)-glucoside (the identity of the latter pigment as reported by Maccarone *et al.*[16]), were predominant in all the varieties. However, in the juices of the *Moro* variety, which showed the highest content of anthocyanin pigments, the relative amounts of cyanidin-3-glucoside and cyanidin-3-(6″-malonyl)-glucoside were similar (46 and 54 mg/l, respectively), whereas in the *Tarocco* variety cyanidin-3-(6″-malonyl)-glucoside was the major component (25 mg/l). Furthermore, uncharacterised anthocyanidin conjugates, peaks 9, 14 and 16, were detected only in juices of the *Moro* variety, peak 13 was found only in juices of the *Tarocco* variety and peak 8 and 10 were not detected in the *Sanguinello* variety (Fig. 3).

The ascorbic acid levels of the juices under investigation are reported in Table III. The juices from pigmented oranges exhibited the highest

TABLE I Hydroxycinnamic acids (HCA) and flavanone glycosides (FG) content, as determined by HPLC-PDA in orange juices obtained from three non-pigmented varieties*

Compound class	Compound name	Peak No†	Orange variety		
			Navel	*Valencia*	*Ovale*
HCA	Chlorogenic acid	1	1.94 ± 0.12	1.84 ± 0.03	2.32 ± 0.59
	p-Coumaric acid	2	0.16 ± 0.04	0.15 ± 0.04	0.47 ± 0.30
	Ferulic+Sinapic acid	3	0.76 ± 0.01	1.11 ± 0.03	1.31 ± 0.61
FG	Narirutin	4	5.96 ± 0.21	4.57 ± 0.96	10.17 ± 4.49
	Hesperidin	5	100.75 ± 10.35	52.05 ± 13.22	121.73 ± 27.57
	Didymin	6	2.80 ± 0.01	1.86 ± 0.38	4.80 ± 2.20

* Amounts are in mg/l juice, mean ± SD of a minimum of three determinations for each variety of juice. † See chromatograms at 320 nm, Fig. 1.

TABLE II Flavanone glycosides (FG), hydroxycinnamic acids (HCA) and anthocyanidin glycosides (AG) content as determined by HPLC-PDA in orange juices obtained from three pigmented varieties*,†

Compound class	Compound name	Peak No‡	Orange variety		
			Sanguinello	*Moro*	*Tarocco*
HCA	Chlorogenic acid	1¶	1.40 ± 0.26	4.80 ± 4.15	5.45 ± 5.49
	p-Coumaric acid	2¶	1.40 ± 0.26	1.42 ± 1.91	1.61 ± 1.31
	Ferulic+Sinapic acid	3¶	5.91 ± 1.11	4.57 ± 3.76	3.68 ± 1.55
FG	Narirutin	4¶	17.22 ± 3.24	18.25 ± 2.79	14.17 ± 2.25
	Hesperidin	5¶	189.20 ± 35.59	174.28 ± 13.13	217.77 ± 48.19
	Didymin	6¶	6.60 ± 1.24	6.80 ± 1.05	5.54 ± 0.38
AG	Cyanidin-3-glucoside	7‡	5.18 ± 3.99	46.30 ± 19.88	10.33 ± 11.63
	Anthocyanin conjugate	8‡	nd	1.21 ± 0.35	1.57 ± 0.38
	Anthocyanin conjugate	9‡	nd	2.83 ± 0.28	nd
	Anthocyanin conjugate	10‡	nd	3.37 ± 0.14	3.04 ± 0.61
	Cyanidin-3-(6″-malonyl)-glucoside§	11‡	7.33 ± 1.55	53.98 ± 1.06	25.08 ± 6.36
	Anthocyanin conjugate	12‡	2.36 ± 0.57	9.23 ± 0.11	2.50 ± 0.68
	Anthocyanin conjugate	13‡	nd	nd	3.04 ± 0.72
	Anthocyanin conjugate	14‡	nd	1.37 ± 0.15	nd
	Anthocyanin conjugate	15‡	1.58 ± 0.59	6.72 ± 0.51	7.53 ± 1.69
	Anthocyanin conjugate	16‡	nd	1.19 ± 0.12	nd

*Amounts are mg/l juice, mean ± SD of a minimum of three determinations for each variety of juice. nd = Not detected. ‡See chromatograms at 520 nm, Fig. 3. ¶See chromatograms at 320 nm, Fig. 2. §Identification from Ref. [15].

ascorbic acid content, with no significant difference between varieties, and the *Navel* non-pigmented juices reported the lowest amount.

The total antioxidant activity (TAA) of all juices is also reported in Table III. The TAA of *Ovale* juices (5.32 ± 0.32 mM) was higher than that of *Valencia* and *Navel* juices (4.79 ± 0.08 and 3.49 ± 0.13 mM, respectively) and the TAA of the highly pigmented *Moro* was the highest of all juices. The pigmented *Sanguinello* juices showed a TAA, 4.37 ± 0.34 mM, lower than the other red varieties but higher than the non-pigmented *Navel* juices.

An excellent correlation (Table IV) was observed between the TAA of the juices and their ascorbic acid concentration ($r = 0.74$, $P < 0.001$). Also, TAA and hydroxycinnamic acids levels were well-correlated ($r = 0.73$, $P < 0.01$) while a correlation between the measured antioxidant activity and flavanone glycosides content was not appreciable. However, the total anthocyanidin glycosides content was highly correlated with the total antioxidant activity ($r = 0.98$, $P < 0.001$) of the pigmented oranges varieties.

DISCUSSION

The HPLC method here provided a good simultaneous separation of three distinct classes of phenolic compounds, flavanone glycosides, anthocyanin glycosides and hydroxycinnamic acids. Therefore, this method could be extremely useful, due to its simplicity and rapidity, for the characterisation of the phenolic profile of other beverages, food or plant material extracts.

The results showed that fresh orange juices have high antioxidant capacity, as measured by the improved TEAC assay, which is mainly due to their high ascorbic acid content and in part to the phenolic constituents, particularly anthocyanins for the pigmented varieties *Moro* and *Tarocco* for which the relative ratio anthocyanins/flavanone glycosides was 1:1.5 and 1:4, respectively. The flavanone glycosides, although the major phenolic components in the orange juices, contributed minimally to the antioxidant potential. This is not after all surprising: it has, in fact, been demonstrated that during

TABLE III The ascorbic acid content and TAA values of juices obtained from three non-pigmented varieties of oranges*

Orange variety	Ascorbic acid (mM)	TAA (mM)
Ovale	3.01 ± 0.07	5.32 ± 0.32
Navel	2.62 ± 0.30	4.79 ± 0.08
Valencia	2.21 ± 0.17	3.49 ± 0.13
Sanguinello	3.27 ± 0.27	4.74 ± 0.04
Moro	3.32 ± 0.25	5.83 ± 0.16
Tarocco	3.11 ± 0.07	5.05 ± 0.23

*Mean ± SD of a minimum of three determinations.

TABLE IV Pearson's correlation coefficients and relative *P* values between the TAA and the ascorbic acid, flavanone glycoside, hydroxycinnamic acid and anthocyanin glycoside content of juices obtained from pigmented and non-pigmented varieties of oranges

Components	Correlation coefficient (*r*)	*P**
Total ascorbic acid	0.74	< 0.001
Flavanone glycosides	0.44	ns
Anthocyanin glycosides	0.98	< 0.001
Hydroxycinnamic acids	0.73	< 0.01

*ns = Not significant.

refrigerated storage a significant fraction of the soluble flavanones precipitate and integrate in the cloudy fraction of the juice.[17]

Our observations are generally consistent with a previous report in which juices from four of the six described varieties were analysed and their antioxidant action was ascribed to the phenolic content.[18] In particular, concentrations of anthocyanins and hydroxycinnamic acids were highly correlated with TAA values, estimated using the ferrylmyoglobin/ABTS method, while ascorbic acid seemed to play a minor role. However, other reports have shown that the main contribution to the TAA of *citrus* juices is provided by ascorbic acid.[19,20]

The results of this comparison of the antioxidant potential of juices from different varieties of oranges, suggest that fresh orange juices, particularly those of the pigmented varieties, may represent a more significant source of antioxidant components in the diet. However, it is still unclear whether they can exert those antioxidant effects *in vivo*, depending on their bioavailability. A study from Ameer *et al*.[21] has provided the first evidence that flavonoids derived from citrus, namely the flavanone glycosides naringin and hesperidin are absorbed after oral administration of a 500 mg dose of the pure compounds or grapefruit and orange juice or the whole fruit. Recent studies have provided evidence for the presence of the glucuronidated hesperetin and naringenin in the circulation.[21,22] In this context, our observations that hesperetin glucuronides can exert protective effects on human skin fibroblasts in a UVA-induced oxidative stress models have important implications.[9]

Data on bioavailability of pure anthocyanins are also very limited but they have led to the conclusion that in general anthocyanins, cyanidin in particular, are much more resistant to metabolism by the gut microflora than other flavonoids.[23] Recently, Tsuda *et al*.[24] have described the absorption of cyanidin-3-glucoside and its rapid entry in the circulation as glucoside in rats orally administered the anthocyanin by stomach intubation. Furthermore, Miyazawa *et al*.[25] have demonstrated the direct intestinal absorption, in the glycoside form, of cyanidin-3-glucoside and cyanidin-3,5-diglucoside into rats and humans after oral supplementation. The observations of this study would suggest that cyanidin-3-glucoside and other anthocyanins can potentially act as antioxidants *in vivo*.

Several studies have investigated bioavailability of commercial extracts rich in anthocyanins. Lietti *et al*.[26] have showed accumulation in the skin, liver, kidney and heart of rats, following intraperitoneal and intravenous administration of a commercial *Vaccinium myrtilus* preparation. In addition, Morazzoni *et al*.[27] reported that plasma concentration of anthocyanins reached rapidly peak level and that the anthocyanins were absorbed intact, following administration of a single oral dose of a commercial preparation of *Vaccinium myrtillus* in rats.

Although there is a need to expand the knowledge on the absorption, metabolism and bioavailability of flavonoids, particularly anthocyanins, there is accumulating evidence that metabolites and conjugates of flavonoids represent the bioactive form *in vivo*. In fact, there have been some reports that glucuronide metabolites of flavonoids act as antioxidants in biological fluids, following the ingestion of dietary flavonoids[28–30] and that they are able to exert other biological effects, probably independent from their antioxidant activity.[31–33] Therefore, consumption of orange juices, particularly those rich in anthocyanins, which represent a significant dietary source of potentially bio-active flavonoids, might have important beneficial health implications.

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