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C- and *O*-glycosyl flavonoids in Sanguinello and Tarocco blood orange (*Citrus sinensis* (L.) Osbeck) juice: Identification and influence on antioxidant properties and acetylcholinesterase activity

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ABSTRACT

Sanguinello and Tarocco are the blood orange (*Citrus sinensis* (L.) Osbeck) cultivars most diffused worldwide. Reversed phase liquid chromatography coupled with MS-MS analysis showed that these two varieties have a similar chromatographic pattern, characterised by the presence of *C*- and *O*-glycosyl flavonoids. Of the two, Sanguinello was found to be far richer in flavonoids than Tarocco. In the juices, twelve individual components were identified for the first time, namely, four *C*-glycosyl flavones (lucenin-2, vicenin-2, stellarin-2, lucenin-2 4′-methyl ether and scoparin), three flavonol derivatives (quercetin-3-*O*-(2-rhamnosyl)-rutinoside, quercetin-3-*O*-hexoside, quercetin 3-hydroxy-3-methylglutaryl-glycoside), an *O*-triglycosyl flavanone (narirutin 4′-*O*-glucoside) and a flavone *O*-glycosides (chrysoeriol 7-*O*-neoesperidoside). Moreover, the influence of the identified *C*- and *O*-glycosyl flavonoids on the antioxidant and acetylcholinesterase activity of these juices has been evaluated.

1. Introduction

Sanguinello and Tarocco (*Citrus sinensis* (L.) Osbeck) are the most common cultivars of pigmented (blood) sweet orange. These varieties are widely diffused in the Mediterranean basin (mainly Southern Italy and Spain) and in the United States of America (California), and they are consumed worldwide, as fresh or processed products. They are the result of a spontaneous genetic mutation, occurred many centuries ago in native plants from China and repeated, perhaps several times, as oranges spread through the Mediterranean basin. In particular, in Sicily they have been cultivated since the 15th century, becoming a staple of the local citrus industry, so much so that they have received the Protected Geographical Status (IGP).

Differently from the typical blond orange, the flesh and the rind of blood oranges are characterised by the presence of pigmented compounds. The distinctive red flesh colouring is due to the presence of anthocyanins (cyanidin 3-glucoside, cyanidin 3-6″-malonyl glucoside, delphinidin 3-glucoside, peonidin 3-glucoside, cyanidin 3-rutinoside and the 6″-malonyl glucose esters of delphinidin, peonidin and petunidin, Dugo, Mondello, Morabito, & Dugo, 2003; Hillebrand, Schwarz, & Winterhalter, 2004; Maccarone, Rapisarda, Fanella, Arena, & Mondello, 1998). Recent work described the properties of these compounds, pointing out also the presence, in significant amount, of vitamin C, along with hydroxycinnamic acids, hesperidin, narirutin and didymin (Berhow, Tisserat, Kanes, & Vandercook, 1998; Kelebek, Canbas, & Selli, 2008; Rapisarda, Carollo, Fallico, Tomaselli, & Maccarone, 1998; Rapisarda & Intelisano, 1996; Rapisarda et al., 1999) that confer pigmented orange juice superior nutraceutical and biological properties with respect to the blond orange varieties (Buscemi et al., 2012; Grosso et al., 2013; Rapisarda et al., 1999). Very little information is available on the minor phenolics components such as *C*- and *O*-glycosyl flavonoids, as we have recently reported for the Moro blood orange variety (Barreca, Bellocco, Leuzzi, & Gattuso, 2014).

In the present paper we report the identification and quantification of twelve compounds in juice of Sanguinello and Tarocco fruits, carried out simultaneously by means of a single HPLC–DAD–ESI-MS-MS chromatographic course, as well as an in-depth study on the antioxidant and acetylcholinesterase activity of the

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); AChE, acetylcholinesterase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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identified *C*- and *O*-glycosyl flavonoids employing selected fractions obtained by preparative RP-HPLC separation.

2. Materials and methods

2.1. Samples

The fruits of Sanguinello and Tarocco blood orange (*C. sinensis* (L.) Osbeck) were supplied by Vivai Raimondi Spadaro (Messina, Italy). The oblate fruits (average size 5.0–6.2 cm wide and 6.0–7.0 cm high) were harvested in April, about 8 months after the flowering period. The investigation was carried out on 10 samples from the 2012–2013 fruit season.

2.2. Reagents and standard solutions

HPLC-grade acetonitrile and methanol were supplied by Sigma–Aldrich (St. Louis, MO, USA), dimethylformamide (DMF) by Carlo Erba (Milano, Italy). Hesperidin and narirutin were supplied from Extrasynthèse (Genay, France). Vicenin-2, lucenin-2 4′-methyl ether and scoparin were separated from *Citrus limetta* (Barreca, Bellocco, Caristi, Leuzzi, & Gattuso, 2011a), lucenin-2, stellarin-2 and chrysoeriol 7-*O*-neohesperidoside from *Citrus bergamia* (Gattuso et al., 2006), narirutin 4′-*O*-glucoside from *Citrus aurantium* (Barreca, Bellocco, Caristi, Leuzzi, & Gattuso, 2011d) and they were used as standards.

2.3. Sample preparation

The fruits were preliminarily peeled to avoid contamination by flavedo components (Gattuso et al., 2006). Peeled fruits were then hand-squeezed, and the juice sample thus obtained were stored at −20 °C until used for analysis. Prior to analysis, DMF (10.0 mL) was added to the crude juice (10.0 mL) and the mixture was centrifuged for 5 min at 3200 rpm. The supernatant liquid was then filtered through an Iso-Disc P-34, 3 mm diameter PTFE membrane, 0.45 μm pore size (Supelco, Bellefonte, PA, USA).

2.4. LC–MS-MS analysis of flavonoids

LC–MS-MS analyses of juice samples were carried out with a ThermoQuest Model LCQ-Duo equipped with a diode array spectrophotometer and an ion trap mass spectrometer with an electrospray ionisation source (ESI). Separation of each compound was performed on a 250 mm × 4.6 mm i.d., 5 μm Discovery C18 column, supplied by Supelco (Bellefonte, PA), equipped with a 20 mm × 4.0 mm guard column. The column was placed in a column oven set at 30 °C. The injection loop was 20 μL, and the flow-rate was 1.0 mL/min. The mobile phase consisted of a linear gradient of acetonitrile in H_2O as follows: 5–20% (0–15 min), 20–30% (15–20 min), 30–100% (20–35 min), 100% (35–40 min), 100–5% (40–45 min), and 5% (45–55 min). UV spectra were recorded between 200 and 450 nm, and simultaneous detection by diode array was performed at 278 and 325 nm. Operating parameters of the mass spectrometer were set as follows: capillary temperature 250 °C; spray needle voltage set at 4.50 kV; ES capillary voltage +3 and −47 V for positive and negative polarity, respectively; tube lens offset 0 and −25 V for positive and negative polarity, respectively. Nitrogen was used as a sheath gas with a flow of 50 arbitrary units. Mass analysis was carried out in full-scan mode in the 80–900 amu range, both in positive and negative mode. MS-MS spectra were obtained using an applied collision energy of 20–30% of instrument maximum. A source fragmentation of 20 V as a collision energy was used in MS and MS-MS analysis when required. Errors for *m*/*z* ratios for each compound were comprised between 150 and 400 ppm. Each sample was tested three times and gave superimposable chromatograms.

2.5. Identification of compounds

Compounds **1**–**12** were identified by their retention time, UV spectra, MS and MS-MS data and by comparison with standard samples, where available. Full spectroscopic data are reported in Table 1.

2.6. Acid hydrolysis

Juice hydrolysis was carried out by following the procedure described by Hertog, Hollman, and Venema (1992). 6 M HCl (10 mL) in a methanol (25 mL)/water (10 mL) solution was added to 5 mL of each of the juice samples, to a final concentration of 1.2 M HCl in 50% aqueous methanol. Ascorbic acid (50 mg) was added as antioxidant. After refluxing at 90 °C for 20 h under stirring, the solution was allowed to cool to room temperature. The solvents were then evaporated under reduced pressure, and the residue was suspended in 10 mL water/DMF (1:1). The mixture was filtered through an Iso-Disc P-34 membrane and analysed by HPLC.

2.7. Quantitative evaluation of flavonoids content

Quantitative analysis was carried out by mass spectrometry, operating either in positive or negative ion ESI mode with selected reaction monitoring (SRM). The instrumental parameters were optimised for maximal generation of the pseudo molecular ions ($[M-H]^-$ or $[M+H]^+$) and of the characteristic fragment ions – indicated as "neutral loss" – employed for the quantification of each individual compound. Quantification of analytes was obtained by applying a collision energy of 25% of the instrument maximum for flavone *C*-glucosides, 20% for flavone-*O*-glycosides and flavanone-*O*-glycosides, respectively. For lucenin-2, vicenin-2, stellarin-2, lucenin-2 4′-methyl ether and scoparin the precursor ions (*m*/*z* 609, 593, 623, 623 and 461, respectively) and product ions (*m*/*z* 489, 473, 503, 503 and 341, respectively) were in agreement with a neutral loss of 120 amu, corresponding to the characteristic fragmentation of the *C*-linked glucose moiety of the mono- and di-*C*-glucosyl flavonoids. The precursor and product ions of chrysoeriol 7-*O*-neoesperidoside (*m*/*z* 609 and 301), narirutin (*m*/*z* 579 and 271) and hesperidin (*m*/*z* 609 and 301) were in agreement with a neutral loss of 308 amu, corresponding to the characteristic fragment mass of *O*-diglycoside (i.e., a neohesperidose or rutinose disaccharide). Narirutin 4′-*O*-glucoside displayed a neutral loss of 470 amu, typical of *O*-triglycosyl flavonoids. The neutral losses of 306, 146 and 162 amu were used for quercetin 3-hydroxy-3-methylglutaryl-*O*-hexoside, quercetin-3-*O*-(2-*O*-rhamnosyl)-rutinoside and quercetin-3-*O*-glycoside derivative from the precursor *m*/*z* 607, 757 and 463 to product ions at *m*/*z* 301, 611 and 301, respectively. The scan width was set to *m*/*z* 1.0. Standard compounds (0.1–600 mg/L) were analysed twice and used for calibration of the peak areas, by using the Genesis peak detection algorithm integrated in the ThermoQuest software. To confirm the linearity and reproducibility for the quantification of the analytes, the standards were analysed twice, and linear calibration curves were constructed from the averaged peak areas.

This experimental procedure allows to achieve excellent limit of detection (LOD, 0.009–0.025 μg/mL) and limit of quantification (LOQ, 0.027–0.075 μg/mL). LOD was defined as a signal to noise ratio of 3. The LOQ was calculated as three times the LOD. Recovery of analytes was always >98%. Intra- and interday coefficients of variation were below 7% for all analytes.