Determination of Phlorin as Peel Marker in Orange (*Citrus sinensis*) Fruits and Juices

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Phlorin (3,5-dihydroxyphenyl β-D-glucopyranoside), an orange peel marker, has been isolated from orange (*Citrus sinensis*) fruits using C-18 reversed-phase column chromatography and the structural formula confirmed by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry. Aqueous extracts of orange peel were followed during more than 2 days at two temperatures, showing increasing content of phlorin in water following time. The phlorin content was determined in various parts of two orange varieties, Navel Late from Spain and Valencia from Brazil. The range of phlorin was only 10−14 mg·L^{-1} in juice but was in a higher concentration in albedo (800−1000 ppm).

Keywords: *Authenticity; peel marker; phlorin; 3,5-dihydroxyphenyl β-D-glucopyranoside (R.N. 28217-60-9); sweet orange; juice quality; HPLC; NMR; MS*

INTRODUCTION

Fruit juice industry has become one of the world's most important agricultural businesses where citrus juices dominate. The trade of commercial citrus juice production, based on concentrates, and consumption are fastly increasing, and among the main productive countries, Brazil represents 80% of frozen orange juice concentrate (FOJC) with around 1.3×10^6 metric tons/year. European community demand is estimated at more than 0.8×10^6 metric tons/year. Commercial citrus juice is dominated by sweet orange (*Citrus sinensis*) juice. The increased demand of orange juice has enhanced the adulteration opportunities which may be from simple dilutions with water and addition of sugar to very sophisticated methods difficult to detect. Some reviews have been published to assess orange juice authenticity (Robards and Antolovich, 1995; Simpkins and Harrison, 1995; Widmer et al., 1992). Among the methods reviewed, some are based on spectroscopy-like proton nuclear magnetic resonance (NMR) (Vogels et al., 1996), near-infrared (Twomey et al., 1995), and study of the stable isotope composition (Yunianta et al., 1995). Other methods are based on chromatographic data determination using high-performance liquid chromatographic (HPLC) profiles of flavanone glycosides (Manthey and Grohmann, 1996; Mouly et al., 1994), methoxylated flavones (Ooghe et al., 1994), and phenolic compounds (Fernandez de Simon et al., 1992). Among such phenolic compounds, 3,5-dihydroxyphenyl β-D-glucopyranoside, also known as phlorin, is a naturally occurring phenol widespread in various parts of plants and particularly in fruits. It was first synthesized by alkaline hydrolysis of phloridzin by Cremer and Sueffert in 1912. Jensen et al. (1973) obtained phlorin in crystalline form by silica gel chromatography of the glucoside fraction of leaves and twigs from *Cornus capitata* and *Cornus kousa*. Phlorin has also been reported in *Cistus laurifolius* (De Pascual Teresa et al., 1986), in *Pseudotsuga menziesii* inner bark (Douglas fir) (Foo and Karchesy, 1989), in shoot laticifer exudate of *Cannabis sativa* (marihuana) (Hammond and Mahlberg, 1988, 1994), in fresh fruits of *Picrasma quassioides* (Yoshikawa et al., 1995), and in fresh bulbs of *Urginea sanguinea* (Majinda et al., 1997). Michael et al. (1995) reported a novel extraction procedure of phlorin in crystalline form from *Viscum rotundifolium*.

Horowitz and Gentili (1961) isolated phloroglucinol β-D-glucoside from grapefruit (*C. paradisi*) and oranges (*C. sinensis*), but no spectra were given. Although the occurrence of phlorin has been previously proposed in orange juice (Johnson et al., 1995; Hammond et al., 1996) the precise spectroscopic data and extraction conditions of pure standard from orange fruits have not been clearly presented. In this paper, we present the isolation of pure phlorin from orange peel of two main cultivars, Valencia and Navel Late, used for industrial orange juice and FOJC productions. The determination of this compound in the various parts, peel, flavedo, albedo, and juice, is presented.

MATERIALS AND METHODS

Materials. Ten kilograms of orange (*C. sinensis*) varieties (Navel Late from Spain and Valencia from Brazil) was purchased on a local market of Marseilles in February−March 1998.

Isolation of 3,5-Dihydroxyphenyl β-D-Glucopyranoside. 3,5-Dihydroxyphenyl β-D-glucopyranoside was isolated using column chromatography (CC) from aqueous albedo extract (aAe) of Navel Late oranges; 2 kg of this orange variety was hand-squeezed, and the crushed peels were put in water (1 L) and heated at 50 °C during 48 h. After filtration of the mixture, the aqueous part was concentrated under vacuum at 40 °C giving a brown oily residue; 2 mL of concentrated aAe was submitted to CC over 12 g of reversed-phase 100 C-18 (0.015−0.035 mm; Fluka), using a 30-cm column (15-mm i.d.).

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Elution was carried out with 100 mL of pure water yielding 15 mg of pure compound (tubes 40−50, 2 mL/tube) with a retention time of 10.3 min using high-performance liquid chromatography (HPLC) (vide infra for HPLC conditions). The compound was obtained as a white crystalline powder, having a melting point of 229−230 °C in agreement with literature (Michael et al., 1995; Cremer and Sueffert, 1912).

High-Performance Liquid Chromatography Determinations. A Beckman system Gold liquid chromatograph equipped with a diode array detector (512 diodes) was used with a Supelcosil LC-ABZ analytical column (Supelco; 250 × 4.6 mm, 5 μm). The elution solvent was a 25 mM potassium dihydrogen phosphate (Sigma) solution adjusted at pH 2 with 85% *o*-phosphoric acid (Carlo Erba). A flow rate of 1 mL·min^{-1} at 25 °C was used, UV detector was at 214 nm, and the diode array scanned from 190 to 310 nm. Samples were filtered on 0.45-μm micropore (Sartorius) and injected into the column with a 10-μL loop. Ascorbic acid (vitamin C; Fluka), phlorin (isolated, vide supra), and phloroglucinol (1,3,5-trihydroxybenzene; Fluka) have a retention time of 4.1, 10.1, and 14.6 min, respectively. Phlorin content was expressed for juices at 11.2 °Brix according to the Association of the Industry of Juices and Nectars from Fruits and Vegetables of the European Economic Community recommendations (council directive 93/77/EEC).

Nuclear Magnetic Resonance Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-400 spectrometer in CD_3OD solutions. Tetramethylsilane (TMS) was used as internal standard in both measurements. ^{13}C resonance multiplicities were established via the acquisition of DEPT spectra (Doddrell et al., 1982).

HPLC−Mass Spectrometry (HPLC−MS). A quadripolar LCQ Finnigan mass spectrometer equipped with electrospray ionization (negative mode −4.2 kV, nitrogen, capillary transfer temperature 200 °C) was used. The injected solution was of 10 ng/μL in methanol−water (50−50, v/v). In analyses, MS/MS selection−excitation of parent ion was at *qz* 0.83/0.25, helium as collision gas.

Phlorin Distribution in Various Fruit Parts. The total soluble solid content as °Brix was measured with a RFM-91 refractometer (Bellingham and Stanley Ltd., England) for juices and macerations. Fruits (2 kg) were hand-squeezed, and the juice was immediately filtered through a sieve (1.25 mm; Prolabo, France) and analyzed by HPLC. The flavedo was carefully separated from albedo with a scalpel. An aliquot part of peels was hand-separated with a vegetable peeler and was composed of 1-mm external part of the peel (flavedo plus some amount of albedo). The white part residue of peels was composed of only albedo. Albedo parts (150 g), 1-mm external parts (200−300 g), flavedo parts (8−9 g), and peels (350−450 g) were extracted with water (300, 300, 20, and 600 mL, respectively) at 40 °C for 2 days. The aqueous parts were separated from raw material by centrifugation, and the supernatant was filtered on 0.45-μm micropore (Sartorius). For each variety, an aliquot part of fruits (1 kg) was hand-squeezed and the juice filtered through a sieve (1.25 mm; Prolabo, France) and analyzed by HPLC. The peels (400 g) were cut in small pieces and extracted with water (600−700 mL) at 25 and 50 °C. At regular intervals of time, 10 mL of aqueous solution was separated from raw material by centrifugation and the supernatant filtered on 0.45-μm micropore before HPLC analyses for phlorin and phloroglucinol contents.

RESULTS AND DISCUSSION

Identification of 3,5-Dihydroxyphenyl β-D-Glucopyranoside. Since industrial orange juice could be adulterated with peel extract (peel pressed or washed with water), the knowledge of compounds present in all parts of orange fruit is important. In Figure 1 are shown the chromatographic profiles of an orange juice and of an aqueous peel extract. These profiles differ by the intensities of three main peaks. One of them has been identified as vitamin C (peak 1). The main unidentified peak (peak 3) is present in greater amount in aqueous peel extract and is very small in juice, which offers the opportunity to use it as a natural marker for sweet orange juice quality control.

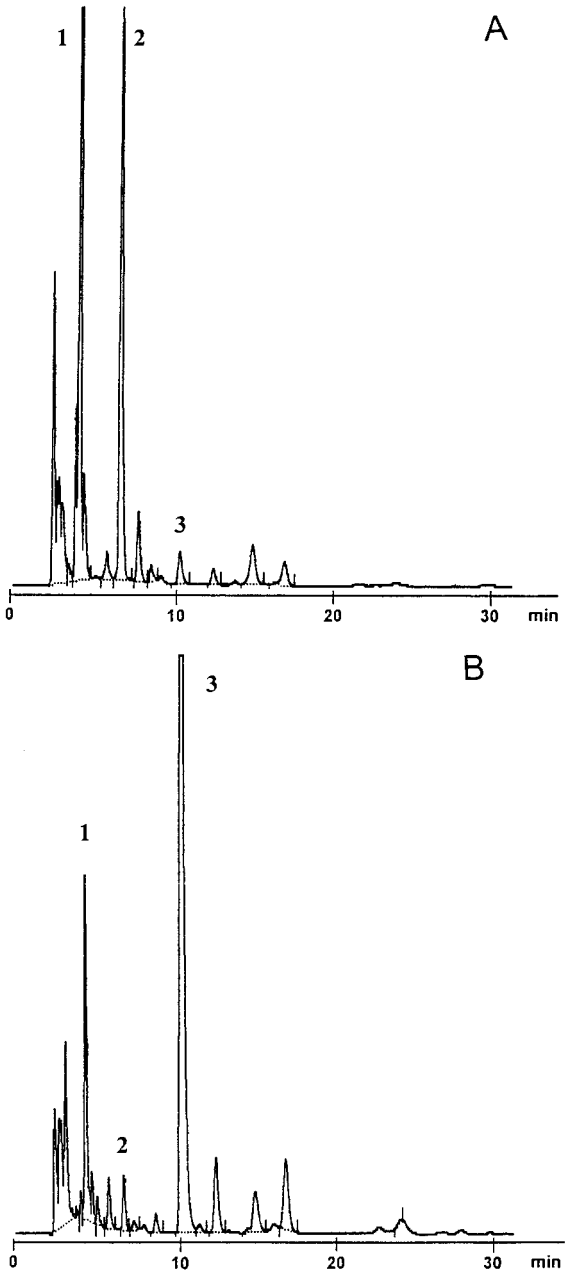


Figure 1. Chromatographic profiles of juice (A) and of aqueous peel extract (B) of sweet oranges, variety Valencia. For chromatographic conditions, see Materials and Methods. Peak identification: 1, vitamin C; 2, unknown; 3, phlorin.

Fractionation of aqueous albedo extract using reversed-phase column chromatography led to the isolation of a pure compound by HPLC as shown in Figure 2. The structure of this compound was determined by NMR analysis (Table 1). The ^{1}H NMR signals showed three aromatic protons at 6.07 ppm (2 H) and 5.96 ppm (1 H). An anomeric proton appeared as a doublet at 4.70 ppm (J = 7 Hz) indicative of a sugar β-configuration. All other ^{1}H and ^{13}C NMR data were in agreement with a phloroglucinol ester associated with a β-D-glucopyranoside moiety (Faure et al., 1987; Mouly et al., 1997). Therefore we established that this compound was 3,5-dihydroxyphenyl β-D-glucopyranoside, known as phlo-

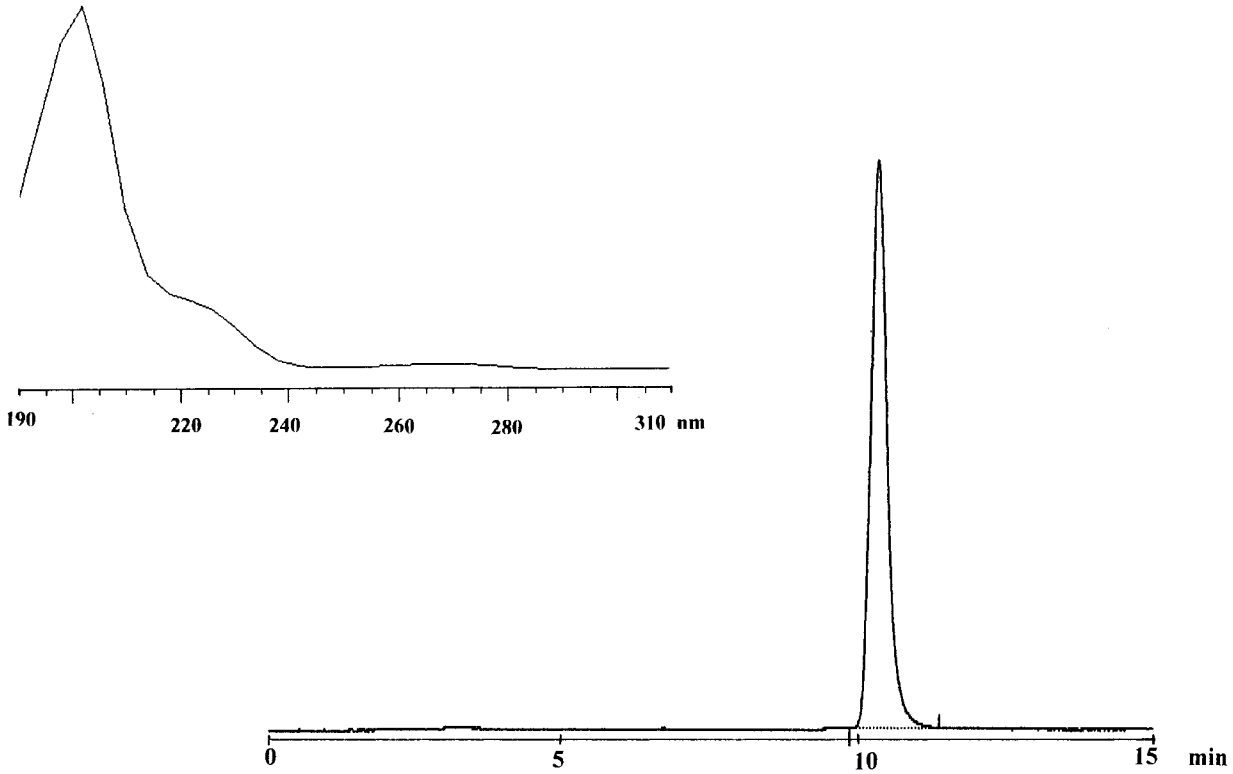
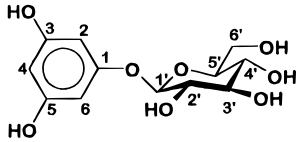


Figure 2. Liquid chromatography profile and UV spectrum from 190 to 310 nm of isolated 3,5-dihydroxyphenyl β-D-glucopyranoside.

Table 1. ^{1}H and ^{13}C NMR Chemical Shifts of 3,5-Dihydroxyphenyl β-D-Glucopyranoside



$\delta^{13}C^a$	group[b]	assignment	δ^1H^a
160.89	C	C-1	
160.15	C	C-3, C-5	
102.09	CH	C-1′	4.70 (d)
97.98	CH	C-4	5.96
96.68	CH	C-2, C-6	6.07
78.11	CH	C-3′	
78.03	CH	C-5′	
74.84	CH	C-2′	3.55−3.50
71.30	CH	C-4′	
62.48	CH_2	C-6′	3.68 (dd) and 3.85 (dd)

[a] In ppm relative to TMS. [b] Determined from DEPT analyses.

rin. The structure was confirmed by negative mode mass spectrometry. The MS/MS on the $[M - H]^-$ peak of this compound gave five characteristic ions in accordance with the structure proposed in Table 1. They were *m/z* $[M - H]^-$ 287 (100%), [phloroglucinol − H]$^-$ 125 (99), [β-D-glucose − H]$^-$ 179 (1), [ion 179 − H_2O]$^-$ 161 (5), and [ion 179 − HCHO]$^-$ 149 (19). The occurrence of phlorin has been previously proposed in orange juice (Johnson et al., 1995; Hammond et al., 1996) without precise spectroscopic data and isolation conditions of pure standard from orange fruits.

Distribution of 3,5-Dihydroxyphenyl β-D-Glucopyranoside in Various Fruit Parts. The isolation and identification of phlorin permitted us to quantify its level in sweet orange peels and juices. The distribution of 3,5-dihydroxyphenyl β-D-glucopyranoside in the different parts of fruits, flavedo, 1-mm external part of peel, albedo, peel, and juice, in Valencia and Navel Late oranges is presented in Table 2. The results showed that the peels contained the major quantity of phlorin compared with the juices. A careful separation of flavedo from albedo shows that phlorin is mainly located in albedo. This result was previously observed by Johnson et al. (1995) and Cancalon (1995). Albedo contains 800−1100 ppm of phlorin (Figure 3). Navel Late oranges contained a higher phlorin level than Valencia peels (1 and 0.8 g·kg^{-1}, respectively). The phlorin content in juice in the two varieties is about 11−14 mg·L^{-1}. Although the phlorin content was found to be 3−5 ppm by Johnson et al. (1995), Hammond et al. (1996) observed that this content depended on origin and varied from 5 to 65 ppm. Our results, 12 ppm for Valencia from Brazil and 14 ppm for Navel Late from Spain, are in agreement with those of the Hammond group which say that hand-pressed juices have levels below 40 ppm. Therefore a high level of phlorin in orange juices should indicate an adulteration with peels.

Table 2. Distribution of 3,5-Dihydroxyphenyl β-D-Glucopyranoside in Various Orange Fruit Part Extracts from Different Origins and Varieties

	Valencia (Brazil)		Navel Late (Spain)	
part of fruit	ratio in fruit (%)	phlorin content[e] (ppm)	ratio in fruit (%)	phlorin content[e] (ppm)
flavedo	4	nd[g]	3	nd
external part of peel[a]	16	59	14	90
albedo[b]	30	833	21	1120
peel[c]	46	803	35	1000
juice[d,f]	54	12	65	14

[a] 1-mm external part of the peel (flavedo plus some amount of albedo). [b] White part of the peel. [c] Flavedo and albedo obtained after juice extraction. [d] Filtered fresh juice. [e] See Materials and Methods for maceration at 40 °C. [f] Expressed at 11.2 °Brix. [g] Not detected.

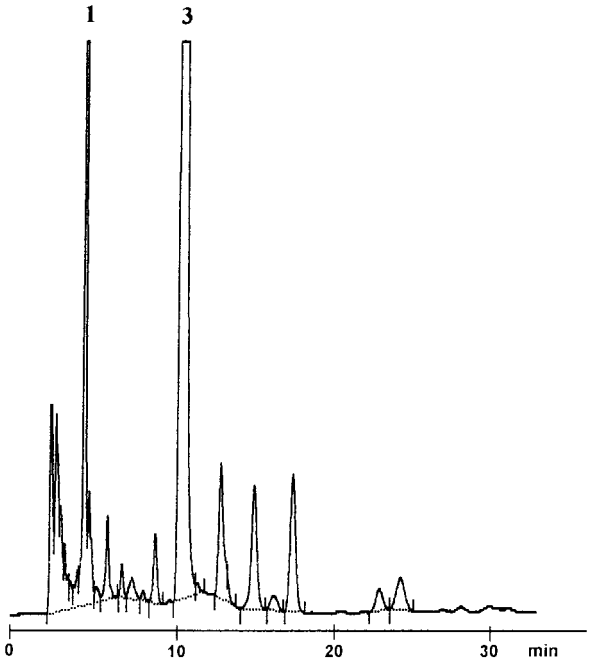


Figure 3. Chromatographic profile of aqueous albedo extract of oranges, variety Navel Late. For chromatographic conditions, see Materials and Methods. Peak identification: 1, vitamin C; 3, phlorin.

Table 3. 3,5-Dihydroxyphenyl β-D-Glucopyranoside Content in Maceration of Peel of Valencia Oranges

	temperature (°C)		
	25	50	
maceration (h)[a]	phlorin content[d] (ppm)	phloroglucinol occurrence[b]	phlorin content[d] (ppm)
5	232	nd[c]	366
10	316	nd[c]	547
24	417	nd[c]	703
30	475	nd[c]	735
35	503	nd[c]	770
48	594	traces	834
53	633	traces	845

[a] See Materials and Methods for maceration. [b] Phloroglucinol determined by HPLC at 214 nm; not detected at 25 °C. [c] Not detected. [d] HPLC at 214 nm.

Table 4. 3,5-Dihydroxyphenyl β-D-Glucopyranoside Content in Maceration of Peel of Navel Late Oranges

	temperature (°C)			
	25		50	
maceration (h)[a]	phloroglucinol occurrence[b]	phlorin content[d] (ppm)	phloroglucinol occurrence[b]	phlorin content[d] (ppm)
5	nd[c]	213	nd[c]	489
10	nd[c]	306	nd[c]	721
24	nd[c]	470	nd[c]	863
30	nd[c]	566	nd[c]	918
35	traces	582	traces	931
48	traces	668	traces	1018
53	<1%	677	traces	1027

[a] See Materials and Methods for maceration. [b] Phloroglucinol determined by HPLC at 214 nm. [c] Not detected. [d] HPLC at 214 nm.

Content of 3,5-Dihydroxyphenyl β-D-Glucopyranoside in Peel Extracts. The quantity of 3,5-dihydroxyphenyl β-D-glucopyranoside extracted with water from Valencia and Navel Late peel oranges has been examined during 2 days at two temperatures. These results, expressed as phlorin content in peels, are given in Tables 3 and 4. Since phlorin hydrolysis during these experiments may be considered, 1,3,5-trihydroxybenzene or phloroglucinol has been determined. Phloroglucinol was detected in only a few samples at a very low level. Phlorin content increased with maceration time. Maxima were observed around 50 h in our conditions. As shown in Tables 3 and 4, the water extraction is more efficient at 50 °C (approximately 1.5 times). As for fruit parts distribution, Navel Late peels are richer in phlorin than Valencia ones.

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