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Fresh and Commercially Pasteurized Orange Juice: An Analysis of the Metabolism of Flavonoid Compounds

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Orange juice is a rich source of flavonoids, mainly the flavanones hesperidin and narirutin, associated with health benefits in humans. The objective of this study was to analyze the uptake of flavonoids in humans after the consumption of two types of orange juice, fresh squeezed (fresh juice, FJ) and commercially extracted and pasteurized (processed juice, PJ). Preliminary measurements showed that the main flavanones in PJ were approximately three-fold higher than in FJ. This study involved healthy volunteers including 12 men and 12 women, aged 27 ± 6, with a BMI of 24 ± 3 kg/m². Volunteers drank 11.5 mL/kg body weight of fresh orange juice, and after an interval of 30 days they drank the same quantity of pasteurized orange juice. Urine was collected from each volunteer during 24 hours following juice consumption. Urine metabolites were recovered by solid phase extraction, and measured by HPLC–ESI–MS. Analyses of the urine samples showed high concentrations of glucuronic acid and sulfate conjugates of hesperetin and naringenin. The data indicate that the concentrations of the flavanone metabolites following consumption of PJ were approximately three times higher than for FJ, thus matching the relative doses of these compounds in the juices provided to the volunteers.

Orange juice is a dietary source of citrus flavonoids such as hesperidin (hesperetin-7-rutinoside) and narirutin (naringenin-7-rutinoside), which have been linked to health benefits in several epidemiologiocal studies (Kimmons et al., 2009; O'Neil et al., 2009, 2011). Evidence shows that the intake of these compounds is associated with a reduction of chronic disorders such as cancer, hypercholesterolemy, inflammation, and heart disease (Asgary and Keshvari, 2013; Devaraj et al., 2011; Ghorbani et al., 2012).

Among certain consumers, there is a belief that fresh orange juice may exert higher levels of health benefits than what is obtained from commercially produced, pasteurized orange juice. Although debatable, it is a fact that orange juice processing methods influence the concentrations of many of the juice components. Fresh juice was previously shown to contain higher levels of the orange fruit polymethoxylated flavones compared to commercially extracted and pasteurized juice (Bai et al., 2012). On the other hand, flavonoid glycosides, alkaloids, and limonoids were present at higher concentrations in commercially extracted and pasteurized juice (Bai et al., 2012). The objective of this study was to determine if there were differences in the uptake of flavonoids in humans after the consumption of fresh and commercially processed juices.

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Materials and Methods

Subjects. Participants included 24 volunteers, 12 men and 12 women, aged 27 ± 6, healthy, with BMIs of 24 ± 3 kg/m². All volunteers were nonsmoking and did not use medication, hormones, or dietary supplements. The study was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences at São Paulo State University (UNESP), Araraquara, Brazil (CAAE 00558712.5.0000.5426).

Study design. Each subject was submitted to two treatments. They drank 11.5 mL/kg body weight of fresh squeezed orange juice and the same quantity of commercially extracted and pasteurized orange juice. There was a month interval between the two treatments and collection of the samples. This protocol was conducted at the Laboratory of Nutrition, Faculty of Pharmaceutical Sciences, UNESP.

Orange juices. Two standard boxes of fresh oranges (cultivar Pera Rio) and 20 L of commercially extracted and pasteurized orange juice (PJ), made from the same batch of fruit, were provided by Fisher Group (Matao, Brazil). The processed orange juice was stored in 1-L bottles at −20 °C for 4 weeks. The fresh juice, prepared with a commercial fresh juicer (FJ), was produced with the same fruit on the morning before starting the procedure for each subject.

Urine samples. Urine was collected from each volunteer during 24 h after ingestion of juices. The total volume collected was homogenized and 200-mL samples were passed through a C18 Sep Pac. The metabolites were eluted with methanol and dried under vacuum. Measurements of citrus metabolites were moni-

tored by high-performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC–ESI–MS).

Statistical analysis. Results from each experimental group were pooled and presented as mean ± standard deviation. Statistical differences of the urinary excretion of flavonoids were tested comparing FJ and PJ orange juice data sets, using the software Sigma Stat 3.0.

Results and Discussion

Table 1 shows the anthropometric, hemodynamic, and biochemical characteristics of the participants of this study. The data showed that all measurements were within normal standards, which are compatible with healthy subjects. Analyses of the flavonoid metabolites in the urine samples obtained from the participants after the consumption of both types of juice showed high concentrations of glucuronic acid and sulfate conjugates of hesperetin and naringenin (Table 2). The chromatographic peaks of flavanones 557 amu, 381 amu and 477 amu, 447 amu, identified in the urine samples, are shown in Figures 1 and 2, respectively.

Table 1. Anthropometric, hemodynamic, and biochemical characteristics of the volunteers participating in the study.

Parameters[z]	Men (12)	Women (12)
Anthropometric		
BMI (%)	24.5 ± 3[y]	23.6 ± 3
Body fat (%)	23.4 ± 5	30 ± 3
Waist circumference (cm)	85.5 ± 10	73.7 ± 7
Blood pressure		
Systolic (mmHg)	122 ± 10	112 ± 11
Diastolic (mmHg)	67 ± 8	72 ± 10
Biochemical		
Glucose (mg/dL)	84.8 ± 7	81 ± 4
Insulin (mg/dL)	8.3 ± 2	10.8 ± 7
Total cholesterol (mg/dL)	185.6 ± 30	166.8 ± 22
HDL-cholesterol (mg/dL)	45.7 ± 8	63.2 ± 9
White blood cell (µg/dL)	6.6 ± 1	6.7 ± 1

[z]All parameters were within normal references.
[y]Values are expressed as mean ± standard deviation.

The flavanone metabolites were detected during the 24-h period following ingestion. Vallejo et al. (2010) reported that no further excretion of flavonoid metabolites following orange juice ingestion occurs after 24 h. We observed that during the 24-h urine collection, the excretion of metabolites after ingestion of PJ was greater than after ingestion of the FJ, and these differences were highly significant (Table 2).

Flavanones and their metabolites exhibit distinctive ultraviolet spectra with wavelength maxima between 280 and 286 nm. Peaks that exhibited such spectra, as well as exhibiting mass ions anticipated for flavanone conjugates were assigned as metabolites. Mass spectra of these peaks frequently exhibited neutral mass losses of 176 atomic mass units which were consistent with glucuronic acid conjugates. Neutral losses of 80 atomic mass units were also common, and were attributed to sulfate conjugates. Verification of these assignments awaits compound isolations and further spectroscopic analyses.

Peaks A–C tentatively assigned to sulfate conjugates of hesperetin glucoronides appeared at 10.2, 10.9, and 11.1 min, respectively. Metabolite A occurred in the urine of participants following the consumption of PJ, but not in the urine of participants consuming FJ. Metabolites B and C exhibited levels 11 and 2.4 times higher in the urine of participants consuming PJ compared to the levels in the urine of participants consuming FJ. Metabolite D with a retention time of 14.8 min is tentatively assigned to a sulfate conjugate of hesperitin, and the difference in the amount of this metabolite was 2.5 times greater after ingestion of PJ than after ingestion of FJ. Peaks E (rt 9.6) , F (rt 10.7), G (rt12.5) and H (rt 12.8), tentatively assigned to different naringin glucuronides, were present, respectively, at 1.7, 1.7, 2.3, and 2.7 times higher after ingestion of PJ compared to the levels following the ingestion of FJ. HPLC–ESI–MS analysis at m/z 477 for the peak with a retention time of 11.6 min revealed that this metabolite was not found in the urine 24 h after ingestion of FJ, but was found in the urine after intake of PJ. Similarly, for the tentatively assigned peaks for hesperetin glucuronides at 13.2 min (J), 13.6 min (K), and 15.7 min (L) the urine levels following the consumption of PJ were 2.29, 2.48, and 1.9 times higher, respectively, than following consumption of FJ. These values are consistent with the initial measurements showing that the flavonoid concentrations in the PJ were approximately three-fold higher than in the FJ.

Table 2. Metabolite urinary excretion after consumption of commercially extracted and pasteurized (PJ) and fresh squeezed orange juice (FJ).

				Metabolite excretion (µg/ 200 mL urine)		
Flavonone metabolite	Molecular ion	Retention time (min)	Peak	PJ	FP	*P*
Hesperetin glucoronide sulfate	557	10.2	A	1.75 ± 0.8[z,y]	---	---
		10.9	B	40.16 ± 13.5	3.59 ± 0.8	<0.001
		11.1	C	40.64 ± 6.5	17.30 ± 13.7	<0.001
Hesperetin sulfate	381	14.8	D	153.9 ± 28.6	61.6 ± 11.2	<0.001
Naringenin glucuronide	447	9.6	E	3.98 ± 0.46	2.3 ± 0.26	<0.001
		10.7	F	16.75 ± 2.12	9.66 ± 0.9	<0.001
		12.5	G	23.19 ± 3.9	9.95 ± 3.6	<0.001
		12.8	H	25.06 ± 4.0	9.26 ± 3.0	<0.001
Hesperetin glucuronide	477	11.6	I	3.38 ± 1.12	---	---
		13.2	J	31.44 ± 4.41	13.72 ± 5.1	<0.001
		13.6	K	107.66 ± 14.43	43.46 ± 12.9	<0.001
		15.7	L	3.68 ± 0.8	1.93 ± 0.34	<0.001

[z]Significantly different from between PJ and FJ ($P < 0.001$).
[y]Values are expressed as mean ± standard deviation.

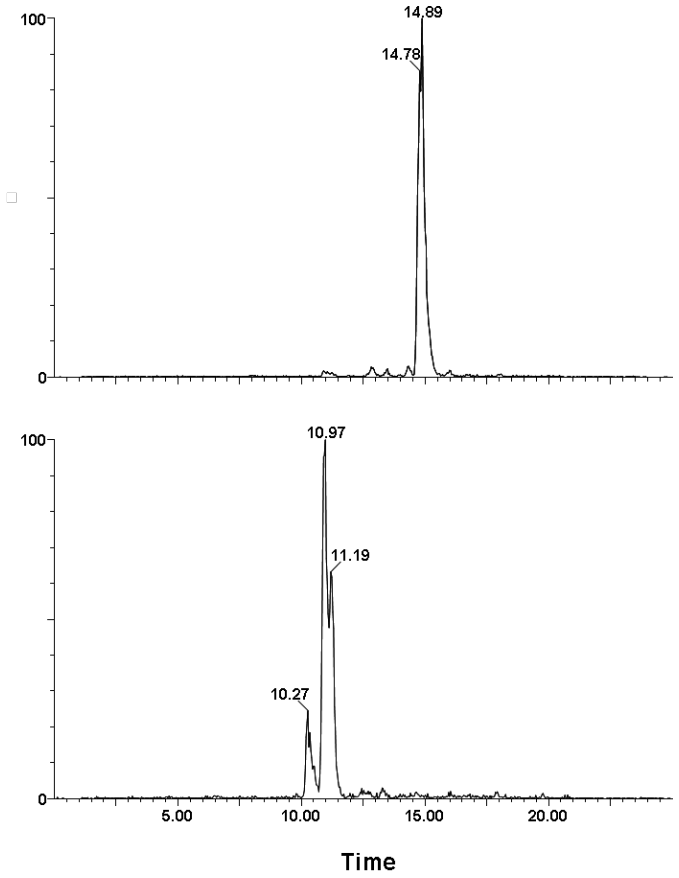

Fig. 1. Representative chromatograms of urine metabolites detected. Hesperitin sulfate 381 m/z (**top**) and hesperitin glucuronide sulfate 557 m/z (**bottom**).

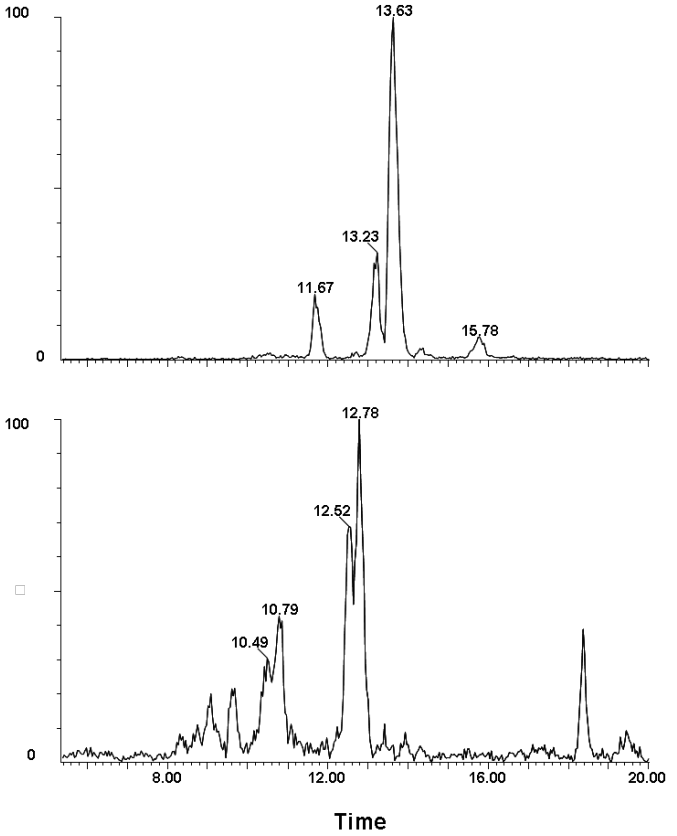

Fig. 2. Representative chromatograms of urine metabolites detected. Hesperitin glucuronide 477 m/z (**top**) and naringenin glucuronide 447 m/z (**bottom**).

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