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RAPID COMMUNICATION

Flavanone glycosides in Brazilian orange juice

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Authentic samples of oranges, frozen concentrated orange juice and pulp-wash, and retail samples of freshly squeezed orange juice and frozen concentrated orange juice have been collected in Brazil and analysed for the flavanone glycosides (FG) narirutin and hesperidin by reversed phase HPLC with UV detection at 280 nm. The juice from hand-squeezed fruit gave narirutin and hesperidin concentrations of 16–142 mg l^{-1} and 104–537 mg l^{-1}, respectively. The ratio of hesperidin to narirutin showed varietal difference with Pera having the highest ratio (mean 8.4) and Baia the lowest (3.6). Frozen concentrated orange juice contained higher quantities of FG with narirutin ranging from 62 to 84 mg l^{-1} and hesperidin from 531 to 690 mg l^{-1} (after dilution to 12°Brix). In frozen concentrated orange juice pulp-wash, the narirutin level ranged from 155 to 239 mg l^{-1} and hesperidin from 1089 to 1200 mg l^{-1}. The analysis of 23 samples of freshly squeezed juice from the Brazilian market place showed that the FG content of most samples (9.1 to 94.8 and 105.8 to 586.6 mg l^{-1}, respectively, for narirutin and hesperidin) was similar to those found for authentic ones, indicating that these orange juices were not adulterated. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

The fruit juice industry is one of the biggest agricultural businesses in the world, with a trade exceeding 10 billion (US) dollars (Patel, 1994). Brazil is the largest orange producer and is responsible for 80% of the international market for frozen concentrated orange juice (Robardis & Antolovich, 1994). Most Brazilian orange juice is exported as concentrate but the Brazilian retail market is largely based on freshly squeezed orange juice (FSOJ) sold in aseptic packaging and on fresh oranges which are available at relatively cheap prices in local markets. The Brazilian orange juice industry also recovers juice from the orange pulp by an aqueous extraction procedure. The material recovered, called pulp-wash, is widely used in drink manufacture. As might be expected, FSOJ commands higher prices than frozen concentrated orange juice (FCOJ) and pulp-wash. Therefore there is always the possibility of pulp-wash and/or FCOJ being substituted for FSOJ. Marketing this product as 100% pure orange juice, obtained from freshly squeezed oranges without the addition of sugar, water, acids or preservatives, represents a fraud against the consumer and an economic loss for the honest processor.

At present there are few data available for flavanoid glycosides (FG) in Brazilian orange juice. Mouly *et al.* (1994) have analysed FG in several samples of orange juice but only three were from Brazil. Other published data (Rouseff *et al.*, 1987) relate to varieties which are not produced in significant quantities in Brazil. The varieties Pera and Natal account for 71% of the oranges produced in Brazil (Steger, 1990). Data on the FG content of the Pera variety are available for oranges grown in Florida (Rouseff *et al.*, 1987). However, to our knowledge there are no data available on the levels of FG in the Natal variety or Brazilian Pera. In addition, data from the commercial samples produced in Brazil such as commercial frozen concentrated orange juice and pulp-wash and from retail markets have not been previously reported.

Flavanoid Glycosides have been used to detect admixtures of citrus juices such as adulteration of orange juice by grapefruit (Rouseff, 1988) or viceversa. Mouly *et al.* (1994) used factorial discriminant analysis

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to differentiate varieties of *Citrus sinensis* according to the amount of FG and through the presence of three unknown peaks. Kirksey *et al.* (1995) have used the ratio narirutin/hesperidin to detect the addition of pulp-wash in orange juice, a practice prohibited in several countries.

The current study provides FG data from authentic samples of the most important varieties of oranges produced and processed in Brazil. It has been used to assess the authenticity of orange juice samples from the Brazilian market place.

MATERIALS AND METHODS

Standards

Naringin was obtained from Sigma, hesperidin from Aldrich and narirutin from Apin Chemicals (Oxon, UK). Narirutin and naringin were dissolved in the mobile phase (water:acetonitrile:tetrahydrofuran:acetic acid, 80:16:3:1, v/v/v/v). Hesperidin was dissolved in dimethylformamide:water (2:1, v/v). Standards were prepared weekly by appropriate dilutions with chromatographic mobile phase to give concentrations ranging from 5.0 to 85.0 mg l^{-1} for hesperidin, 2.5 to 20.0 mg l^{-1} for narirutin, 4.0 to 20.0 mg l^{-1} for naringin.

Recovery studies

Reagent blanks were analysed for the presence of possible interferents and no extraneous peaks were observed. In order to ensure that the FG were correctly identified and quantified, samples were spiked with different concentrations of narirutin (26.4 to 76.8 mg l^{-1}), hesperidin (149.0 to 403.5 mg l^{-1}) and naringin (20.2 to 83.2 mg l^{-1}).

Quality control

An In-House Reference Material (IHRM) was prepared from a sample of well-mixed orange juice (1 litre) divided into portions of 12 ml and stored in a domestic freezer until required. Initially 10 samples of the IHRM were analysed and the average and standard deviation (SD) were obtained for hesperidin and narirutin so as to establish the repeatability of the method. Subsequently, the IHRM was analysed with each batch to ascertain that the method was under control. To accept the batch, the value obtained for the IHRM had to be between the average ± 2 SD, otherwise the batch was rejected.

Samples

Authentic samples of oranges from different varieties as well as commercial concentrated orange juice and pulp-wash were collected from processing plants in the State of São Paulo (Brazil). Retail samples (frozen concentrated orange juice and freshly squeezed orange juice) were purchased from supermarkets in the metropolitan area of Campinas (State of São Paulo, Brazil), during the years of 1995/1996.

Sample preparation

The citrus fruits were hand-squeezed and the juices filtered through a stainless steel sieve (1.25 mm). Frozen concentrated orange juice and frozen concentrated pulp-wash were diluted to 12 °Brix with Millipore water. Retail freshly squeezed orange juices were sieved before use. All of the samples were stored frozen.

Sample analysis

The analyses were conducted according to Mouly *et al.* (1994), as follows.

The juice samples (5 ml) were mixed with dimethylformamide (DMF) (10 ml) and ammonium oxalate (0.05 M, 10 ml) and placed in a steam bath at 90°C for 10 min. The samples were then cooled and the volume made up to 50 ml with Millipore water. The solutions were centrifuged at 10°C until clear. The clarified juice was then filtered through Acrodisc filters (0.45 μm nylon, Gelman Sciences), and placed in a 2 ml ambered flask for liquid chromatography analysis.

High performance liquid chromatography (HPLC)

A C18 Nucleosil 5 μm (250×4.6 mm) column was used with a guard-column (Alltima C18 5 μm, 7.5×4.6 mm) at room temperature. Injections of 20 μl were made using a solvent of water:acetonitrile:tetrahydrofuran:acetic acid (80:16:3:1, v/v/v/v) and a flow of 1 ml min^{-1}. FG were detected using UV at 280 nm.

The FG were identified in the samples by comparing the retention time with that of standards and quantified by comparing the integrated peak areas (Spectra Physics Integrator) with that of an external standard. Peak identity was confirmed by using a Spectra Focus Scanning Detector (Spectra Physics). This equipment takes spectra from three points at different times across the HPLC peak and compares these spectra. If these spectra are identical, the peak is considered pure; i.e. no interferents are present. A peak purity index is calculated automatically where 100% is perfectly pure. Typically in this work, peak purity indices of 97–100% were achieved.

RESULTS AND DISCUSSION

Quality control

The repeatability of the method has been determined using data from the IHRM. Twenty six measurements gave means of 54.4 mg l^{-1} (SD = 2.2), CV = 4.0% and

Table 1. Recovery of flavanone glycosides added to orange juice

Flavanone glycosides	n	Amount added ($mg\,l^{-1}$)	Amount found ($mg\,l^{-1}$)	Recovery (%)
Narirutin	16	26.4–76.8	27.2–88.8	97.1–122 $\bar{X}$ = 112.1 (SD = 9.1)
Hesperidin	16	149–404	149–476	96.0–118 $\bar{X}$ = 106.5 (SD = 6.6)
Naringin	14	20.2–162	20.5–177	98.8–110 $\bar{X}$ = 103.0 (SD = 8.3)

n = number of spiked samples.

$263\,mg\,l^{-1}$ (SD = 6.7), CV = 2.6% for narirutin and hesperidin, respectively. Table 1 shows the recoveries obtained for spiked samples. Means of 112% (SD = 9.1), 107% (SD = 6.6), 103% (SD = 8.3) were established for narirutin, hesperidin and naringin, respectively. No correction for recovery was therefore necessary. These recoveries are in accordance with those cited by Mouly *et al.* 1994. Reagent blanks demonstrated that no background interferents were present.

Authentic samples

Freshly squeezed orange juice

The concentration of FG found for authentic samples of hand squeezed juice are shown in Table 2. The results proved variable. Narirutin and hesperidin concentrations ranged from 16.1 to $142\,mg\,l^{-1}$ and 104 to $537\,mg\,l^{-1}$, respectively. The ratio hesperidin/narirutin varied from 3.2 to 11.3. As anticipated from previous literature, neither naringin nor neohesperidin were detected in any of the samples analysed (Galensa & Herrmann, 1980; Greiner & Wallrauch, 1984; Galensa *et al.*, 1986; Rouseff *et al.*, 1987; Mouly *et al.*, 1994; Ooghe *et al.*, 1994). The concentrations of the FG were similar to those cited by several authors for narirutin (18 to $120.7\,mg\,l^{-1}$) and hesperidin (122 to $379\,mg\,l^{-1}$) (Rouseff *et al.*, 1987; Mouly *et al.*, 1994; Ooghe *et al.*, 1994). Previous work has emphasised the differences which may occur due to variety and also due to different extractor pressures (Fisher, 1978; Rouseff *et al.*, 1987; Kirksey *et al.*, 1995).

The following average ratios of hesperidin/narirutin were obtained for the varieties Pera, Natal, Valência, Hamlin, Baía and Lima: 8.4; 5.5; 5.3; 4.2.; 3.6; 6.4, respectively. The Pera variety presented the highest ratio among the samples analysed and proved sufficiently different from the rest of the samples to suggest that this represented a genuine varietal difference. The lowest hesperidin/narirutin ratio was found for the Baía variety. Ooghe *et al.* (1994) also reported low ratios from Baía although no information on the geographic origin of the samples was provided.

Rouseff *et al.* (1987) also provided information on the concentrations of FG in different orange varieties. Pera, Baía, Hamlin and Valência contained 41, 31, 27, $27\,mg\,l^{-1}$ of narirutin and 208, 135, 122, $151\,mg\,l^{-1}$ of hesperidin, respectively. These values are in good agreement with our own. No literature data are available for the Natal variety.

Frozen concentrated orange juice (FCOJ) and frozen concentrated orange pulp-wash (FCOPW)

Table 3 shows the results for FG in FCOJ. Narirutin and hesperidin concentrations were found to be much higher than those in hand-pressed orange juice. The mechanical pressure exerted by industrial plant is

Table 2. Concentrations of flavanone glycosides in authentic samples of hand-squeezed orange juice from Brazil

Variety		Narirutin ($mg\,l^{-1}$)	Hesperidin ($mg\,l^{-1}$)	Ratio (Hesperidin/ Narirutin)
Pera	1	52.9	269	5.1
	2	19.7	212	10.8
	3	31.1	228	7.3
	4	30.8	253	8.2
	5	16.1	182	11.3
	6	20.0	133	6.6
	7	30.9	350	11.3
	8	62.4	399	6.4
	9	39.8	304	7.6
	10	26.4	228	8.6
Natal	1	43.7	295	6.7
	2	35.1	189	5.4
	3	24.0	104	4.3
	4	38.4	149	3.9
	5	33.5	243	7.3
Valência	1	79.7	291	3.7
	2	35.4	194	5.5
	3	40.4	219	5.4
	4	42.9	321	7.5
Hamlin	1	69.5	321	4.6
	2	72.9	342	4.7
	3	71.9	253	3.5
	4	142	537	3.8
Baía	1	68.8	265	3.9
	2	135	427	3.2
Lima	1	21.7	111	5.1
	2	29.4	223	7.6
		range: 16.1–79.6 $\bar{X}$ = 48.7 SD = 31.7	range: 104–537 $\bar{X}$ = 260.8 SD = 97.9	range: 3.2–11.3 $\bar{X}$ = 6.3 SD = 2.3

Table 3. Concentrations of flavanone glycosides in frozen concentrated orange juice (FCOJ) from Brazil, diluted to 12° Brix

	Variety	Narirutin ($mg\,l^{-1}$)	Hesperidin ($mg\,l^{-1}$)	Ratio (Hesperidin/Narirutin)
FCOJ	Hamlin	97.8	646	6.6
	Pera	62.4	591	9.5
	Pera	72.4	687	9.5
	Pera	71.2	613	8.6
	Pera	83.1	659	7.9
	Pera	79.8	690	8.6
	Pera	75.3	656	8.7
	*	84.0	531	6.3
		range: 62.4–84.0	range: 531.0–689.9	range: 6.3–9.5
		$\bar{X}$ = 78.3	$\bar{X}$ = 634	$\bar{X}$ = 8.2
		SD = 10.6	SD = 53.6	SD = 1.2

* Mixture of several varieties not identified by the producer.

greater than that which can be achieved manually and this leads to greater extraction of FG from the wall membrane and albedo (Fisher, 1978; Kirksey *et al.*, 1995). The amount of FG in FCOJ ranged from 62.4 to 84.0 ($mg\,l^{-1}$) and from 531 to 690 ($mg\,l^{-1}$) for narirutin and hesperidin, respectively. The ratios of hesperidin/narirutin (6.3 to 9.5) were within the upper range found for hand pressed orange juice, which was 3.2 to 11.3. The average ratio for hand-pressed orange juice was 6.4 (SD 2.3) but for FCOJ it was 8.2 (SD = 1.2). The lower standard deviation found for the industrial product may be because this material is produced from a large amount of oranges of different varieties and origins. The composition of the final juice will therefore reflect the average composition. Similarly, the use of industrial extractors provides a consistent juicing pressure which will prevent variability occurring from this factor.

In FCOPW (Table 4) the amount of FG found were much higher than those detected in FCOJ and in hand-squeezed orange juice. The narirutin ranged from 155 to 239 $mg\,l^{-1}$ and hesperidin from 1089 to 1200 $mg\,l^{-1}$. However the ratios were basically the same (5.0 to 7.1). Similar results were recorded by Greiner and Wallrauch (1984) for hesperidin but they did not report results for narirutin. They found that hesperidin concentrations of six samples of pulp-wash ranged from 624 to 1031 $mg\,l^{-1}$ with an average of 904 $mg\,l^{-1}$. They also determined hesperidin in FCOJ finding an average of 388 $mg\,l^{-1}$ (range 211 to 642 $mg\,l^{-1}$).

It has been postulated that the ratio hesperidin/narirutin in pulp-wash differs from that in orange juice, and this ratio could be used as a reference value to detect the addition of pulp-wash in orange juice (Kirksey *et al.*, 1995). According to these authors, a ratio less than 2 should be considered suspect. Ooghe *et al.* (1994) stated that if the ratio hesperidin/narirutin is low, ranging from 2.52 to 3.93, the orange juice may contain pulp-wash. However, for Brazilian orange pulp-wash this method cannot be applied because the ratio between hesperidin and naringin in orange juice and pulp-wash is similar. Nevertheless, the absolute concentrations of FG present are much higher in pulp-wash than in orange juice (1089 to 1200 and 104 to 537 $mg\,l^{-1}$; 155 to 239 and 16.1 to 79.6 $mg\,l^{-1}$, respectively, for hesperidin and narirutin). These relatively high levels of FG detected in pulp-wash could provide an indicator of addition of pulp-wash to freshly squeezed orange juice. Where the levels of narirutin and hesperidin are found in higher concentrations than for authentic samples, there is an indication that either pulp-wash, FCOJ or a mixture of both has been added to the freshly squeezed orange juice.

Retail samples

Retail frozen concentrated orange juice (RFCOJ)

Table 5 shows the results for FG in RFCOJ. The samples analysed showed a similar range of FG concentration (57.3 to 64.7 $mg\,l^{-1}$ and 439 to 520 $mg\,l^{-1}$ for narirutin and hesperidin, respectively) when compared to authentic FCOJ. Similarly, the ratio hesperidin/narirutin (7.5 to 9.1) was similar to those from authentic

Table 4. Concentrations of flavanone glycosides in frozen concentrated orange pulp-wash (FCOPW) from Brazil, diluted to 12 °Brix

		Narirutin ($mg\,l^{-1}$)	Hesperidin ($mg\,l^{-1}$)	Ratio (Hesperidin/Narirutin)
FCOPW	1	239	1193	5.0
	2	180	1200	6.7
	3	175	1089	6.2
	4	155	1093	7.1
		range: 154.8–239.1	range: 1089.3–1200	range: 5.0–7.1
		$\bar{X}$ = 187.4	$\bar{X}$ = 1143.9	$\bar{X}$ = 6.3
		SD = 36.2	SD = 60.9	SD = 0.9

Table 5. Concentrations of flavanone glycosides in retail frozen concentrated orange juice (RFCOJ), from Brazil, diluted to 12 °Brix

		Narirutin ($mg\,l^{-1}$)	Hesperidin ($mg\,l^{-1}$)	Ratio (Hesperidin/ Narirutin)
RFCOJ	1	58.6	439	7.5
	2	64.7	511	7.9
	3	57.3	520	9.1

Table 6. Concentrations of flavanone glycosides in Brazilian retail freshly squeezed orange juice (FSOJ)

Retail FSOJ	Narirutin ($mg\,l^{-1}$)	Hesperidin ($mg\,l^{-1}$)	Ratio (Hesperidin/ Narirutin)
n = 24	range:9.1–94.8	range: 106–587	range: 2.6–14.6
	$\bar{X} = 35.0$	$\bar{X} = 266$	$\bar{X} = 8.3$
	SD = 17.6	SD = 137	SD = 3.0

n = number of analysed samples.

hand-squeezed orange juice and commercial FCOJ. These figures suggest that these samples have not been adulterated with pulp-wash in amounts that could be detected by this method.

Freshly squeezed orange juice (FSOJ)

Table 6 shows a summary of the results obtained for FG in retail FSOJ. Twenty three of the 24 samples analysed fell within the range established for authentic samples (narirutin 16.1 to 142 $mg\,l^{-1}$ and hesperidin 104 to 537 $mg\,l^{-1}$). Similarly, the ratio hesperidin/narirutin found for 21 FSOJ fell within the range of authentic samples (3.5 to 11.3) while three others were just outside (11.4, 11.7, 11.9). One sample proved interesting. Although the ratio was within the authentic range, the concentration of both narirutin and hesperidin were both higher than the authentic range (94.8 and 586.6 $mg\,l^{-1}$, respectively). These data suggest that this sample may contain either FCOJ or FCOPW or both. Other samples of the same brand were also analysed and the FG were within the authentic range. However, all samples of this brand, and another brand showed the presence of an unknown peak eluting after hesperidin and a further investigation of these samples was conducted.

Identification of an unknown peak in two brands

The Scanning UV Spectra of this unknown peak showed a maximum absorption at approximately 260 nm which is completely different from those observed for the FG standards and authentic samples. It has been suggested by Robards & Antolovich (1995) that some preservatives such as sorbate, methyl and propylparabens could be added to orange juice. Several preservatives were therefore examined by LC and sorbic acid matched the retention time. This preservative is used as a mold and yeast inhibitor (The Merck Index, 1989).

Sorbic acid was therefore chromatographed under the same conditions as used for FG analysis. The retention time matched that of the unknown peak and the scanning UV spectra also showed good agreement. To support this identification, sorbic acid and the sample were run on on a second column (Hicarbosphere ODS, 3 μm, 150×4.6 mm) and the peak showed the same retention time for standard and sample. Finally the sample and a standard of sorbic acid was analysed by LC-APcI-MS (VG Platform I). Both standard and sample provided a spectra which was predominantly a single ion mass 111.05 corresponding to the deprotonated molecular ion ($[M\text{-}H]^-$) confirming the identity as sorbic acid. The amount of sorbic acid detected in the samples ranged from 111 to 130 $mg\,l^{-1}$.

Although the Brazilian legislation (Act number 55871/65) allows the addition of 1000 $mg\,l^{-1}$ of sorbic acid to fruit juices this preservative must be declared on the label. In both case neither of the two brands declared the addition of sorbic acid. The juices have been sold as 100% pure orange juice obtained from fresh oranges.

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