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Polymethoxylated flavones in Brazilian orange juice

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Sinensetin has been quantified in authentic samples of Brazilian orange juice. In addition, six further polymethoxylated flavones (PMFs) have been determined in terms of their relative amounts. The PMFs were extracted into toluene and analysed using reversed phase HPLC with detection at 340 nm. Peak identification was based on the UV-visible spectra and the elution order described in the literature. Hand-squeezed orange juices contained a mean of 0.10 (SD 0.04) mg^{-1} sinensetin with the highest concentrations found in Pera and Natal varieties. Commercial samples of frozen concentrated orange juice (FCOJ), frozen concentrated pulp-wash (FCOPW), retail FCOJ and retail freshly squeezed orange juice (FSOJ) typically contained at least ten times more sinensetin than those found for samples squeezed by hand. The PMFs peak area ratios for these sample classes were examined further using canonical discriminant analysis. This procedure could distinguish the hand-squeezed juices of Pera and Hamlin varieties from those of Natal and Valência. Similarly, hand-squeezed juices could be readily distinguished from the commercial samples of FCOJ, FCOPW, retail FCOJ and retail FSOJ. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Authentic orange juices are produced exclusively from the fleshy part of the orange, with no pulp-wash, sugar, preservatives, or other ingredients added (Vogels *et al.*, 1996). Brazil is the most important producer of orange juice in the world and is responsible for 80% of the international market in frozen concentrated orange juice (FCOJ) (Robards and Antolovich, 1994). FCOJ is not widely consumed in Brazil, due to the availability of fresh oranges at affordable prices. However, the retail market for freshly squeezed orange juice (FSOJ) has grown considerably in the last few years with many brands now available. The prices of FCOJ and pulp-wash are lower than FSOJ which makes them potentially applicable to the fraudulent extension of FSOJ. Similarly, other fruit juices may be potential adulterants when the season or market price offers the possibility of economic advantage.

Polymethoxylated flavones (PMFs) have been used in the past to identify and characterise several kinds of juices. Thin layer chromatography was the preferred technique in the early seventies. Veldhuis *et al.* (1970) analysed five PMFs in FCOJ produced from 1952 to 1968. The main objective was to evaluate the taste threshold of these compounds in commercial FCOJ. Ting *et al.* (1979) used the technique of high performance liquid chromatography (HPLC) to analyse PMFs in tangerine and orange juices, and it was concluded that this method could be used to detect the presence of one juice in the other. Similarly, Gaydou *et al.* (1987) differentiated orange and mandarin peel oil using PMFs. Ooghe *et al.* (1994) measured the composition of PMFs in several varieties of orange juice and used the relative peak area and the correspondent standard deviation to detect juice adulteration.

Data from PMFs in Brazilian orange juice are scarce. Heimhuber *et al.* (1988) analysed one sample of pulp-wash from Brazil. They indicated that adulteration of orange juice with peel or pulp-wash could be detected from the amount and distribution of PMFs. Details of the country of origin of the samples analysed by Ooghe *et al.* (1994) are not described although it is likely that

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some came from Brazil. Similarly, little information is available concerning orange juice in the retail market place.

The objective of this work was to determine the relative amounts of PMFs (as peak area ratios) for the most important varieties of oranges available in Brazil. In addition, we have analysed commercial FCOJ, pulp-wash and samples from the Brazilian market place for comparative purposes.

MATERIALS AND METHODS

Standard

Sinensetin (Apin Chemicals, Oxon, UK) (5 mg) was dissolved in 50 ml of methanol. Chromatographic standards (prepared weekly) were obtained by diluting appropriate amounts of the concentrated solution. The concentrations for sinensetin ranged from 0.2 to 4.0 mg l^{-1}.

Quality control

An in-house reference material (IHRM) was analysed throughout the period of study to ascertain that the method was in control and to determine the reproducibility of the method. The IHRM was prepared from a sample of orange juice (1 l) which was well mixed and divided in vials containing 12 ml each and these were frozen at −20°C until analysis. Initially, eight analyses of the IHRM were performed and the average and standard deviation (SD) were obtained for the ratio of the six PMFs analysed (total of 10 results). A sample of IHRM was included with each batch of 12 samples. To accept the batch, the value obtained for the IHRM should be between the average ±2SD, otherwise the analysis was rejected.

Samples

Authentic samples of oranges from different varieties and commercial concentrated orange and pulp-wash were obtained from producers 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 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. FSOJ was sieved before using. All of the samples were stored at −20°C.

Sample analysis

The analyses of the samples were conducted following the principles of the method described by Ooghe *et al.* (1994) with modifications as described below.

Samples of orange juice (5 ml) were transferred to a 25 ml centrifuge tube. Five ml of toluene were added and the solution mixed in a Vortex at high speed for 10 s, following centrifugation at 3000 rpm for 10 min at 10°C. The upper phase was pipetted off and the extraction step was repeated twice. The organic fractions were pooled and evaporated under a nitrogen stream at approximately 50°C. The residue was dissolved in 1 ml methanol and injected into the chromatographic system.

Samples of concentrated juice were diluted to 12° Brix and then treated as for single strength juice. The concentration of sinensetin was recorded after dilution to permit comparison between concentrated and single strength juices.

High performance liquid chromatography (HPLC)

The HPLC consisted of a Waters 625 LC System, autosampler Gilson 231 XL and a Spectra Focus UV-Vis detector (Spectra Physics). A 20 μl loop was used for injection. Solvents were HPLC grade. The mobile phase was a ternary mixture of water:acetonitrile: tetrahydrofuran (53:43:4, v/v/v). The column was a C18 Nucleosil 5 μm (250×4.6 mm i.d., Alltech) with a guard-column Alltima C18 5 μm (7.5×4.6 mm i.d., Alltech). The column was kept at room temperature (±22°C) and the flow rate was 0.7 ml min^{-1}. The wavelength was adjusted to 340 nm. The peak areas were determined using a Spectra Physics integrator. Peak identities were confirmed by a Spectra Focus Scanning Detector (Spectra Physics), under the same conditions as described above.

Peak identification was based on the elution order cited by the literature (Ting *et al.*, 1979; Rouseff and Ting, 1979; Ooghe *et al.*, 1994), and by comparing the UV spectra of PMFs with those in the literature (Sendra *et al.*, 1988).

RESULTS AND DISCUSSION

Sample preparation and high performance liquid chromatography

The procedure used in this work showed some improvements in relation to those published previously (Ting *et al.*, 1979; Veldhuis *et al.*, 1970; Rouseff and Ting, 1979; Ooghe *et al.*, 1994). PMFs have been extracted from orange juice using benzene (Veldhuis *et al.*, 1970; Rouseff and Ting, 1979; Ooghe *et al.*, 1994) and chloroform (Ting *et al.*, 1979). However, it is desirable to use extraction solvents of low toxicity where possible. In the present study, toluene was used because

it is less toxic than benzene and chloroform (The Sigma Aldrich Library of Chemical Safety Data, 1985). Secondly, the chromatographic separation was conducted using an isocratic system that separated all PMFs (Fig. 1), which is much simpler than using a gradient system.

Peak purity and identification

The peak identities were based on the elution order cited by several authors (Ting *et al.*, 1979; Rouseff and Ting, 1979; Ooghe *et al.*, 1994). A scanning UV detector (Spectra Physics) was used to characterise and identify the PMFs (Sendra *et al.*, 1988). The order of elution was: peak I: sinensetin, II: quercetogetin, III: nobiletin, IV: heptamethoxyflavone, V: scutellarein, VI: tangeretin. When samples were analysed, five UV spectra were taken across each peak. The good agreement of these spectra indicated that no UV absorbing species were coeluting with the species of interest.

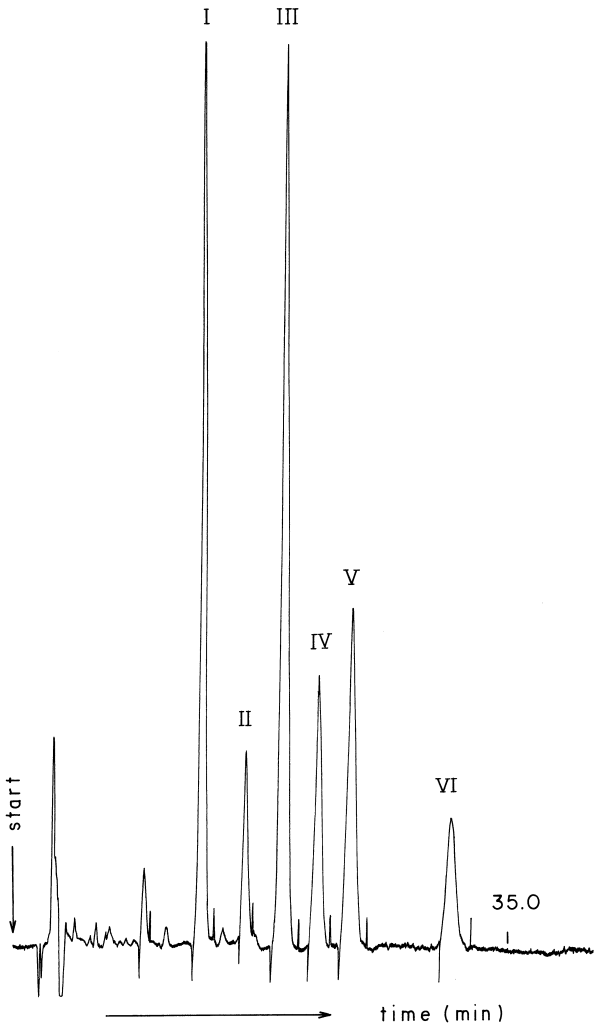

Fig. 1. HPLC of PMFs from an orange juice extract. Chromatographic conditions: Column C18 Nucleosil 5 μm (250×4.6 mm i.d., Alltech), pre-column C18 μm (7.5×4.6 mm i.d., Alltech), mobile phase water:acetonitrile:tetrahydrofuran (53:43:3), flow 0.7 ml min^{-1}, UV 340 nm. Peak I sinensetin, II quercetogetin, III nobiletin, IV heptamethoxyflavone, V scutellarein, VI tangeretin.

Quantitation

Sinensetin showed a linear response within the range studied: 0.2 to 4.0 mg l^{-1} ($r = 0.999$ or better). Samples were quantified against this external standard. Unfortunately the absence of available standards for other PMFs prevented their quantitative measurement. Ooghe *et al.* (1994) overcame this problem by describing the PMF content of samples using relative peak areas and we have adopted the same practice in order to permit a comparison of data.

Quality control

Table 1 provides data on the precision of the analytical method from 18 determinations of an IHRM measured throughout the period of study. The SD ranged from 0.02 to 0.14 for the ratios measured and was considered satisfactory. This range no doubt reflects the differences in concentration of the individual compounds. No background interferents were observed from reagent blanks.

Authentic, commercial and retail samples of orange juice

Table 2 provides data from hand-squeezed oranges. The levels of sinensetin ranged from a mean of 0.10 (SD 0.04) mg^{-1}. Similar quantities were found by Sendra *et al.* (1988) who reported a mean value of 0.09 mg l^{-1}. It is apparent from the data that sinensetin concentrations differ with plant variety. Pera, Natal, Baía and Lima varieties gave almost double the concentration of sinensetin when compared with Valência and Hamlin. Varietal differences in peak ratios were also observed. For example, the peak ratio VI/II for variety Pera was lower than that of Natal, Valência, Hamlin and Baía varieties.

Table 3 indicates that FCOJ, after dilution to 12° Brix, contained considerably more sinensetin than was found in the hand-squeezed juices with concentrations ranging from 1.27 to 2.36 mg l^{-1}. This effect is undoubtedly due to the increased extraction pressure which commercial equipment is able to exert. Several authors have emphasised the influence of extraction pressure on juice composition including the hesperidin content, pectin content and UV polyphenolic absorption (Petrus and Dougherty, 1973; Gherardi *et al.*, 1980; Cohen *et al.*, 1984; Kirksey *et al.*, 1995). Kanes *et al.*, 1993 have indicated that PMFs are found in high concentration in the peel and in low concentration in the juice.

The concentrations of sinensetin given in Table 3 are in agreement with previous reports for FCOJ. Veldhuis *et al.* (1970) found levels varying from 0.50 to 2.05 mg l^{-1} of sinensetin using thin layer chromatography. Sendra *et al.* (1988) determined sinensetin

Table 1. Relative peak areas of PMFs and level of sinensetin ($mg\,l^{-1}$) for the IHRM

	n	I/II	I/IV	I/VI	III/I	III/IV	III/V	III/VI	IV/V	IV/VI	VI/II	Sinensetin
$\bar{x}$	18	3.69	2.91	3.60	1.39	4.08	1.99	4.95	0.49	1.22	1.03	1.96
SD		0.13	0.11	0.14	0.02	0.09	0.02	0.04	0.02	0.04	0.02	0.07

n = number of samples analysed.
peak I: sinensetin, II: quercetogetin, III: nobiletin, IV: heptamethoxyflavone, V: scutellarein, VI: tangeretin.

Table 2. Relative peak area of PMFs and sinensetin levels ($mg\,l^{-1}$) in authentic hand-squeezed samples of orange juice (see Table 1 for peak identification)

		I/II	I/IV	I/VI	III/I	III/IV	III/V	III/VI	IV/V	IV/VI	VI/II	Sinensetin
Pera $n=6$	$\bar{x}$	2.07	4.06	6.20	1.49	6.00	1.46	9.34	0.24	1.53	0.34	0.12
	SD	0.27	0.59	1.25	0.26	1.15	0.48	3.22	0.04	0.20	0.06	0.04
Natal $n=4$	$\bar{x}$	2.71	2.02	2.24	2.13	4.20	1.65	4.67	0.41	1.11	1.32	0.12
	SD	0.50	0.64	0.73	0.20	0.86	0.43	1.01	0.15	0.07	0.51	0.06
Valência $n=3$	$\bar{x}$	2.54	2.54	3.36	1.68	4.25	1.20	5.70	0.28	1.36	0.81	0.07
	SD	0.82	0.30	0.68	0.09	0.28	0.18	1.49	0.03	0.45	0.36	0.02
Hamlin $n=3$	$\bar{x}$	4.62	1.85	2.46	2.80	5.12	1.59	6.63	0.32	1.33	1.85	0.06
	SD	1.99	0.19	0.64	0.66	0.83	0.59	0.42	0.14	0.28	0.47	0.03
Baía $n=1$		4.19	3.09	5.10	1.25	3.86	1.65	6.37	0.43	1.65	0.82	0.11
Lima $n=1$		1.90	3.79	5.26	1.11	4.20	1.06	5.82	0.25	1.39	0.36	0.10

Table 3. Relative peak area of PMFs and sinensetin levels ($mg\,l^{-1}$) in authentic samples of frozen concentrated orange juice (FCOJ) and in authentic samples of frozen concentrated pulp-wash (FCOPW), both diluted to 12° Brix (see Table 1 for peak identification)

		I/II	I/IV	I/VI	III/I	III/IV	III/V	III/VI	IV/V	IV/VI	VI/II	Sinensetin
FCOJ $n=3$	$\bar{x}$	4.69	2.59	3.39	1.58	4.06	2.67	5.31	0.66	1.32	1.40	1.84
	SD	0.36	0.36	0.40	0.13	0.39	0.19	0.21	0.06	0.15	0.26	0.55
FCOPW $n=2$	$\bar{x}$	4.47	3.21	5.07	1.51	4.79	2.78	7.61	0.58	1.60	0.88	1.30
	SD	0.03	0.58	0.34	0.17	0.31	0.02	0.37	0.03	0.18	0.05	0.19

concentrations in composite samples of FCOJ. A mean value of $0.8\,mg\,l^{-1}$ was obtained for samples from two factories having a 'low' level of PMFs and a mean value of $2.9\,mg\,l^{-1}$ from those having a 'high' level of PMFs. According to the authors, this difference was due to the type of extractor which the factories employed to produce the juice. The peak ratios for FCOJ showed fair agreement when compared to authentic samples of hand-squeezed orange. However, peak area ratio I/II was lower for Pera, Natal and Valência hand-squeezed juices and ratio III/V was consistently lower for all hand-squeezed juices.

PMFs in FCOPW (Table 3) proved very similar to FCOJ with, for instance, no significant observable differences in sinensetin concentrations. However, some differences in relative amounts of the PMFs are apparent from the calculated ratios, with I/VI and III/VI, for example, higher in the two samples of pulp-wash analysed. Ooghe *et al.* (1994) found that samples of orange juice containing pulp-wash could not be distinguished from authentic samples on the basis of PMF data.

Retail FCOJ (Table 4) showed similar amounts of sinensetin and peak ratios when compared to authentic FCOJ (i.e. that collected directly from factories, Table 3). Some differences can be observed in peak ratios I/II and VI/II. Veldhuis *et al.* (1970) found similar values of sinensetin in retail (0.80 to $1.45\,mg\,l^{-1}$) and commercial samples of FCOJ (0.50 to $2.05\,mg\,l^{-1}$).

Retail freshly squeezed orange juice (RFSOJ) (Table 5) gave concentrations of sinensetin between those found in hand-squeezed orange juices and FCOJ. Only one sample showed a concentration which reached the levels found in the FCOJ samples. Admixture of a product such as FCOJ or pulp-wash high in PMFs might be one reason for this occurrence. However, it is not possible to rule out other reasons including use of fruit variety (e.g. Pera) having a higher PMF content or the use of extraction equipment having a high operating

Table 4. Relative peak area of PMFs and sinensetin levels ($mg\,l^{-1}$) in retail samples of frozen concentrated orange juice (RFCOJ), diluted to 12° Brix (see Table 1 for peak identification)

		I/II	I/IV	I/VI	III/I	III/IV	III/V	III/VI	IV/V	IV/VI	VI/II	Sinensetin
RFCOJ $n=3$	$\bar{x}$	3.89	2.99	4.12	1.38	4.11	2.19	5.67	0.53	1.38	0.95	2.03
	SD	0.11	0.17	0.32	0.07	0.05	0.08	0.21	0.02	0.03	0.05	0.24

Table 5. Relative peak area of PMFs and sinensetin levels (mg l^{-1}) in retail samples of freshly squeezed orange (see Table 1 for peak identification)

FSOJ $n=14$		I/II	I/IV	I/VI	III/I	III/IV	III/V	III/VI	IV/V	IV/VI	VI/II	Sinensetin
	$\bar{x}$	3.75	3.06	4.81	1.31	3.99	2.30	6.29	0.56	1.58	0.79	1.02
	SD	0.49	0.36	0.60	0.10	0.39	0.20	0.70	0.09	0.18	0.15	0.46

Table 6. Summary for peak ratio in authentic (hand-squeezed) and in authentic samples of orange juice from literature (see Table 1 for peak identification)

		I/II	I/IV	I/VI	III/I	III/IV	III/V	III/VI	IV/V	IV/VI	VI/II	Sinensetin
Authentic samples ($n=18$)	$\bar{x}$	2.82	2.91	4.11	1.85	4.94	1.47	6.88	0.31	1.37	0.92	0.10
	SD	1.25	1.05	1.93	0.60	1.15	0.42	2.67	0.11	0.28	0.65	0.04
Ooghe *et al.* (1994)	$\bar{x}$	4.50	2.20	4.17	1.30	2.85	2.94	5.32	1.08	1.96	1.15	n.a.
	SD	0.71	0.42	0.94	0.13	0.55	0.38	0.97	0.32	0.54	0.38	n.a.

* = mg l^{-1}.
n.a. = not available.

pressure. Ooghe *et al.* (1994) found relatively high levels of sinensetin and tangeretin, and a relatively low scutellarein value in Pera oranges from Brazil. They therefore excluded this sample from their statistical evaluation.

The majority of the peak ratios found in RFSOJ were similar to the ones found for hand-squeezed samples. Some differences were observed for peak ratio III/V.

From the results obtained for FSOJ and FCOJ it can be observed that industrial processing has some influence on the peak ratios I/II and III/V (i.e. sinensetin/quercetogetin; nobiletin/scutellarein) when compared to hand-squeezed orange juice. Ooghe *et al.* (1994) compared authentic orange juices with their corresponding concentrates. No differences in most of the PMFs were observed except for tangeretin which increased after the concentration process.

From Table 6, it can be seen that authentic hand-squeezed samples showed a much higher SD than the retail samples of FSOJ. This reflects the fact that industrial processing involves the mixing of large quantities of juice and hence observed values will tend towards the mean. This effect is also apparent from the data presented by Ooghe *et al.* (1994) in which composite samples were analysed and resulted in data with a much smaller standard deviation. A similar observation was made by Martin *et al.* (1996) on ^{2}H SNIF/NMR and ^{13}C SIRA/MS data from samples of industrial-processed and hand-squeezed orange juice. Typically, standard deviations were 50% higher in the latter.

An appreciation of the tabulated data is difficult to achieve by visual inspection. Canonical discriminant analysis (CDA) was therefore investigated in order to classify the sample groups. As can be seen from Fig. 2 (varieties Lima and Baía were excluded due the low number of samples), the method separated varieties Pera and Hamlin from the remaining varieties but was not able to distinguish between Natal and Valência. Commercial FCOJ, FCOPW and retail samples of

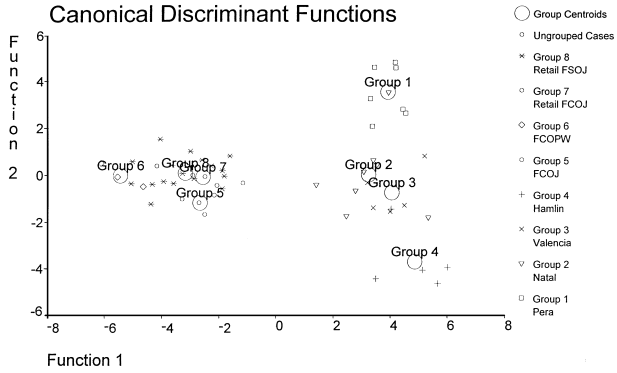

Fig. 2. Canonical discriminant analysis of several kinds of orange juice using PMF data (FCOJ: frozen concentrated orange juice; FCOPW: frozen concentrated orange pulp-wash; FSOJ: freshly squeezed orange juice).

freshly squeezed orange juice were distinct from hand-squeezed orange juice. This no doubt reflects the greater concentration of PMFs in these samples.

Heimhuber *et al.* (1988) discussed the possibility of using the variability in the amount and distribution of PMFs in juice, pulp-wash and peel to detect adulteration. They observed visual differences in the PMF chromatograms of hand-pressed orange juice, juice diluted commercially from concentrate, and pulp-wash; no quantitative data were given. Our data do not lead to the same conclusions. Although the amount of PMFs vary for each kind of juice analysed, insufficient differences were observed to detect the addition of pulp-wash to orange juice. As Ooghe *et al.* (1994) suggest, the method is best applied to detecting the addition of non-*Citrus sinensis* juices such as tangerine to orange juice.

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