Characterization of Orange Juice (*Citrus sinensis*) by Polymethoxylated Flavones

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As a complement to the flavanone glycoside pattern, the pattern of polymethoxylated flavones (PMFs) may be very efficient in detecting orange juice (*Citrus sinensis*) falsifications. The method developed is based on gradient HPLC followed by photodiode array detection after extraction of the PMFs in benzene. Separation is done with a 15 cm Waters Novapak RP18 column and a mobile phase gradient, which increases the acetonitrile level in the presence of tetrahydrofuran. *C. sinensis* authenticity criteria have been proposed for the seven main PMFs present in orange juice based on their average relative peak areas and the corresponding standard deviations ($\bar{x} \pm \sigma$) at 340 mn. The addition of small quantities of tangerine and Murcott juice may be established by significant changes in the PMF pattern.

Keywords: *Adulteration; orange juice; polymethoxylated flavones*

INTRODUCTION

Orange juice products may be adulterated very easily by adding water or other (cheaper) juices or components, such as acids and sugars, in optimal proportions. Therefore, fruit juice control has to be based on relevant parameters that, on the basis of their number and diversity, give an optimal guarantee for an honest product.

In the past many conventional parameters have been determined, which, however, could be adapted easily by unfair producers or manufacturers (Attaway et al., 1988; Ooghe and De Waele, 1982; Ooghe, 1990). Due to their lowered cost, even L-amino acids have been added during the past years (Ooghe et al., 1994). New specific techniques have to be looked for to discourage potential falsifiers.

Recently, liquid chromatography techniques have been developed to determine the levels of natural plant pigments, such as flavonoids as flavanone glycosides and polymethoxylated flavones (Kirksey et al., 1988; Ooghe et al., 1994; Rouseff and Ting, 1979; Schnüll, 1990; Sendra et al., 1988; Veldhuis et al., 1970; Wade, 1992).

As discussed for the flavanone glycosides (Ooghe et al., 1994), the aim of this work is fourfold: optimization of an HPLC method for polymethoxylated flavones to be used as a routine procedure for the analysis of orange juice; proposition of some analytical standards for the polymethoxylated flavones to guarantee the authenticity of *Citrus sinensis*; determination of the influence of fruit juice technology on the flavonoids; determination to what extent addition of some other citrus juices may be detected.

POLYMETHOXYLATED FLAVONES

The generic term "polymethoxylated flavones" (PMFs) represents a chemical family of flavones with a number of methoxyl groups equal to or greater than 4 (CEN, 1991). They are almost exclusively found in citrus in a very specific and characteristic distribution for each fruit (Tatum et al., 1978). They are present in the leaves, as well as in all fruit parts: peel, flavedo, albedo, membranes, juice. The peel is a much richer source of PMFs than the juice (Sendra et al., 1988).

A survey and the chemical structures of the most important PMFs in citrus are given in Figure 1.

It has been found that PMFs have pharmacodynamic properties. So tetra-*O*-methylscutellarein should be an active cytotoxic agent toward different strains of carcinoma cells (Kupchan et al., 1965). Sinensetin and nobiletin should be effective in decreasing erythrocyte aggregation and sedimentation in human blood (Robbins, 1976). Several of these flavones do possess antimicrobial and antiviral activities (Huet, 1982).

Finally, Gaydou et al. (1987) mentioned the possible use of the PMF determination for chemotaxonomic studies of *Citrus* species.

EXPERIMENTAL PROCEDURES

A. Description of the Samples. Five different groups of citrus juice products have been considered.

Group 1 comprised 100 authentic orange juice samples freshly squeezed by Solasun, Melle (B), on an industrial basis; these "SOL..." samples, characterized by their origin, variety, and/or squeezing date, have been reduced to 22 samples by preparing blended samples from orange juices from the same origin/variety and pressing period.

Group 2 consisted of 32 authentic orange juice samples obtained from the Schutzgemeinschaft der Fruchtsaftindustrie (SGF), Zornheim (D), and coded "SGF.../0" or "SGF.../1".

Group 3 was made up of 20 samples from SGF, either obtained by a different extraction technology or blended with other citrus juices ("SGF.../2 to SGF.../7").

Group 4 comprised 17 authentic citrus juices or concentrates not belonging to the *C. sinensis* variety (code name ending on the name of the juice).

Group 5 consisted of 16 self-prepared "falsified" orange juices containing 10, 20, or 30% (m/m) of another citrus juice, coded "SIN + x%...".

The juices of groups 1 and 2 may be considered authentic orange juices. All juice samples were kept at −18 °C in a deep freezer until analysis.

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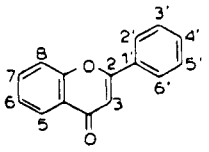

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Heptamethoxyfl.	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$
Nobiletin	-	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$
Quercetogetin	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	-	$-OCH_3$	$-OCH_3$
Scutellareïn	-	$-OCH_3$	$-OCH_3$	$-OCH_3$	-	-	$-OCH_3$
Sinensetin	-	$-OCH_3$	$-OCH_3$	$-OCH_3$	-	$-OCH_3$	$-OCH_3$
Tangeretin	-	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	-	$-OCH_3$

Figure 1. Names and chemical structures of some important PMFs in *C. sinensis*.

B. Sample Preparation. The Brix value (expressed as grams of sucrose/100 g of juice) of all juices has been determined at 20 °C by means of an Abbe-Zeiss refractometer. Concentrates have been diluted either to the original juice strength, if known, or to 11.2° Brix, i.e., the value generally recognized as the minimum Brix value for industrially prepared orange juices.

The sample preparation of the juices and diluted concentrates for the polymethoxylated flavones is based on that of Rouseff and Ting (1979). As a result of its better sensitivity, better reproducibility, simplicity, rapidity, and lower cost, this method is preferred above the method proposed by Sendra et al. (1988).

A 40 mL sample is pipetted into an especially developed centrifuge tube of 50 mL with a stopper and a narrow bottleneck of about 8 mm i.d. After addition of 5 mL of benzene, the contents are mixed strongly by hand and centrifuged (Christ centrifuge, type UJ 15) at 3500 rpm for 5 min. Water is added to raise the separation surface benzene/juice just into the bottleneck. The upper benzene layer is pipetted off very carefully into a stoppered tube of 20 mL. A further 3 mL of benzene is added to the once extracted juice in the centrifuge tube, and shaking and centrifugation is repeated as described above. The upper benzene layer is pipetted again into the glass tube, and the mixed extracts are dried under a nitrogen stream in a water bath at 40 °C. The residue is dissolved in 1 mL of methanol and, after mixing on a vortex mixer, filtered through a small 0.45 μm membrane filter (Gelman nylon acrodisc) using a syringe.

C. Chromatography. The chromatographic HPLC gradient system described by Rouseff and Ting (1979) has been adapted to obtain an optimal separation using a 15 cm Waters RP18 column (Novapak 4 μm; 3.9 × 150 mm). For a full description of the gradient HPLC system equipped with a PDA detector, please refer to our previous paper (Ooghe et al., 1994).

1. Preparation of the Standard Solution. At the time of our experiments only two PMFs were commercially available: sinensetin (Extrasynthèse 1156) and tangeretin (Roth 5111). A stock solution of both components was prepared separately containing 0.5 mg of each component in 1.0 mL of HPLC methanol (Janssen 26.828.56).

The standard solution is obtained by mixing together 50 μL of tangeretin stock solution, 150 μL of sinensetin stock solution, and 800 μL of HPLC methanol. This standard solution is used only to identify unknown peaks by their relative retention times.

2. Working Conditions: column, Waters Novapak 4 μm (3.9 × 150 mm); column temperature, 35 °C; eluent flow rate, 0.8 mL/min; injection volume, 20 μL; HPLC gradient, see Table 1 ; wavelength range PDA, 210–400 nm; chromatogram wavelength, 340 nm; analysis time, 30 min; global time, 45 min.

3. Chromatography. Twenty microliters of standard solution is injected into the HPLC system. Sinensetin elutes after about 8 min and tangeretin after about 16 min. Also, 20 μL of each sample is injected. Identification of sinensetin and tangeretin is based on retention times and spectra. The other PMFs are identified on the basis of their elution order and spectra obtained from the literature (CEN, 1991; Kirksey et al., 1988; Sendra et al., 1988). If tangeretin is not present in the juice, 20 μL of tangeretin stock solution is added to 380 μL of the unknown sample and another 20 μL is injected.

Table 1. Waters HPLC Gradient Program for the Determination of Polymethoxylated Flavones (Flow Rate 0.8 mL/min)

time (min)	% tetrahydrofuran	% acetonitrile	% water	curve
0	16	0	84	*
30	16	42	42	6
33	16	0	84	3
45	16	0	84	3

As a result of the availability of only two PMF standards, the real concentrations and the extraction efficiencies of all PMFs could not be calculated. Nevertheless, it could be established that this extraction procedure was reproducible, yielding relative PMF values with a coefficient of variation (N = 5) better than 4% for the seven main peaks considered.

Using the software package Maxima 820, version 3.3 (Waters Chromatography Workstation), the relative area of all peaks present in a relative concentration above 1% is calculated at 340 nm. At this wavelength a maximum of information is obtained.

RESULTS AND DISCUSSION

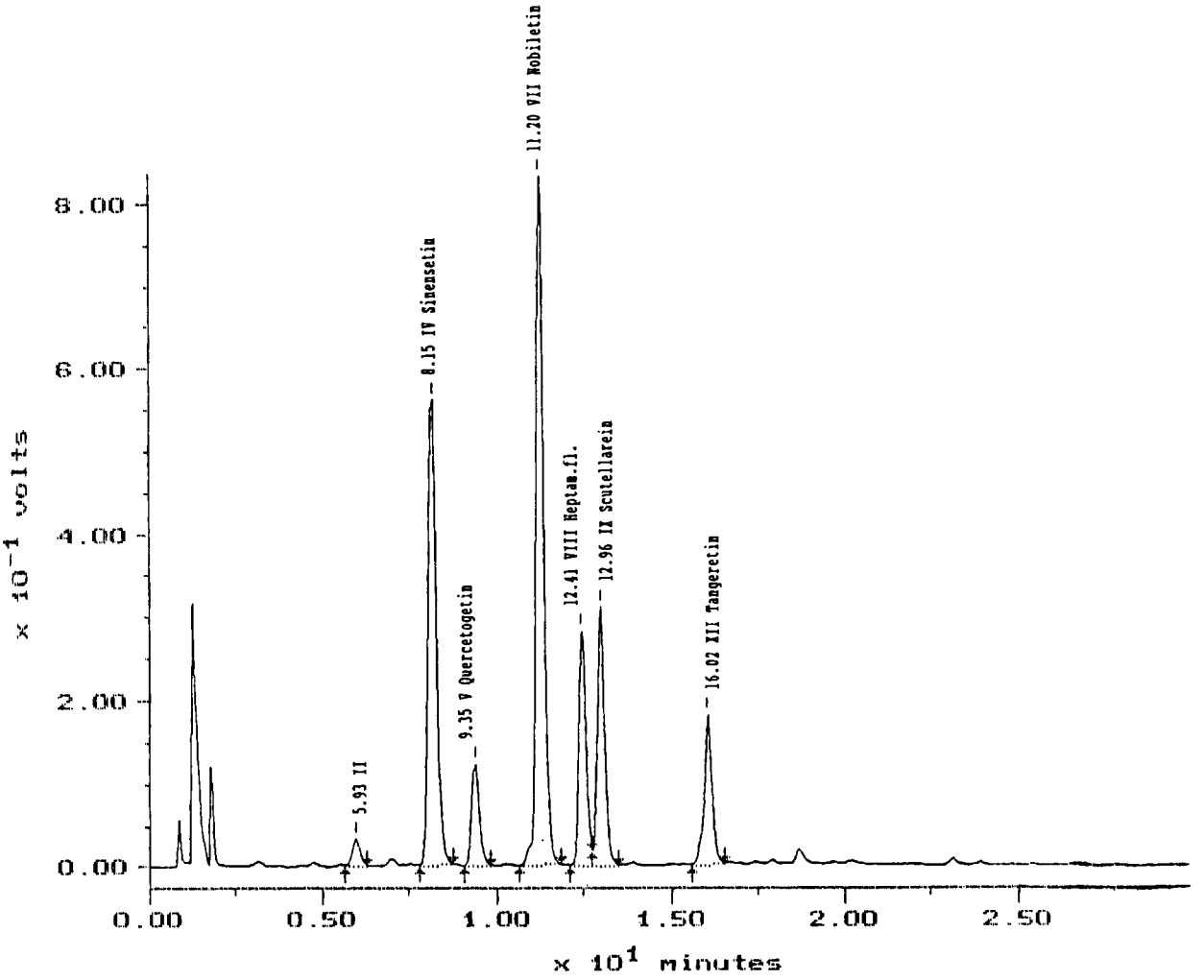
As in the flavanone glycoside paper (Ooghe et al., 1994), a report is obtained using the software package Maxima containing the chromatogram at 340 mm (Figure 2). It seems, after analysis of all authentic orange juices (groups 1 and 2), that seven peaks are systematically present with a relative peak area of at least 1% of the total peak areas: an unknown peak (X), sinensetin (SIN), quercetagetin (QUE), nobiletin (NOB), heptamethoxyflavone (HEP), scutellarein (SCU), and tangeretin (TAN).

Because it can be assumed from a probit diagram that the results obtained may be considered to be normally distributed, the average values and standard deviations of these seven PMFs have been calculated (Table 2). Outliers first have been eliminated using a statistical F test (7 degrees of freedom in the numerator, 47 degrees of freedom in the denominator, 99.5 and 99.0% probability). A procedure of internal testing was started and repeated until no further outliers occurred. In both ways 3 of the 54 samples have been eliminated.

These three samples are a Valencia orange juice from Morocco showing a relatively high heptamethoxyflavone and a relatively low scutellarein content, a Pera juice from Brazil with relatively high sinensetin and tangeretin values and a relatively low scutellarein value, and a blood orange juice from Tunesia, which not only has relatively low sinensetin and relatively high nobiletin values but also contains a number of additional peaks after tangeretin accounting for more than 30% of the total peak area (Figure 3).

Considering now the 7 average values $\bar{x}$ and standard deviations σ of the 51 remaining orange juice samples as a standard, it is possible to compare any other sample by means of an F test to that standard and to calculate the percent probability that the sample is falsified or not. The larger the F value obtained, the higher the probability that the juice is not an authentic orange juice.

The PMF results obtained from the other samples—either obtained by a different technology or blended with other citrus juices (group 3) or authentic citrus



DETECTOR: 340.0 nm

PK#	Component Name	Retention Time (minutes)	Relative Time	Peak Area	Area Percent
1	II	5.926	0.370	490371	1.39
2	IV Sinensetin	8.148	0.509	9099121	25.79
3	V Quercetogetin	9.352	0.584	1874225	5.31
4	VII Nobiletin	11.204	0.699	12538035	35.53
5	VIII Heptam.fl.	12.407	0.775	4054305	11.49
6	IX Scutellarein	12.963	0.809	4426359	12.54
7	XII Tangeretin	16.018	1.000	2802159	7.94
TOTAL				35284576	

Figure 2. Chromatogram at 340 nm of the PMFs present in *C. sinensis* and corresponding Maxima report.

juices not belonging to the *C. sinensis* variety (group 4) or the self-prepared falsified orange juices (group 5)—are summarized in Tables 3 and 4. In these tables some more acidic citrus juices such as lemon, lime, grapefruit, bergamot, and pommerans juices were not considered, because the PMFs present show a completely different pattern. These samples contain not only some of the seven PMFs considered in this study but also a lot of quantitatively important, yet unidentified, PMF peaks, mostly after tangeretin.

A comparison of the authentic orange juices to their corresponding concentrates shows that there is good agreement between the results, except for tangeretin. Using the paired t test of Student (5 degrees of freedom),

Table 2. Average Relative Peak Areas (Ratios) and Standard Deviations of PMFs at 340 nm in Authentic Orange Juice ($N = 54$) after Elimination of Three Outliers by an F Test

PMF	$\bar{x} \pm \sigma$	PMF ratio	$\bar{r} \pm \sigma$
X	1.345 ± 0.179	SIN/QUE	4.496 ± 0.707
SIN	26.779 ± 1.969	SIN/HEP	2.201 ± 0.423
QUE	6.104 ± 1.056	SIN/TAN	4.169 ± 0.942
NOB	34.576 ± 1.751	NOB/SIN	1.299 ± 0.127
HEP	12.555 ± 2.232	NOB/HEP	2.845 ± 0.550
SCU	11.962 ± 1.533	NOB/SCU	2.935 ± 0.380
TAN	6.678 ± 1.215	NOB/TAN	5.323 ± 0.867
		HEP/SCU	1.082 ± 0.324
		HEP/TAN	1.955 ± 0.539
		TAN/QUE	1.152 ± 0.384

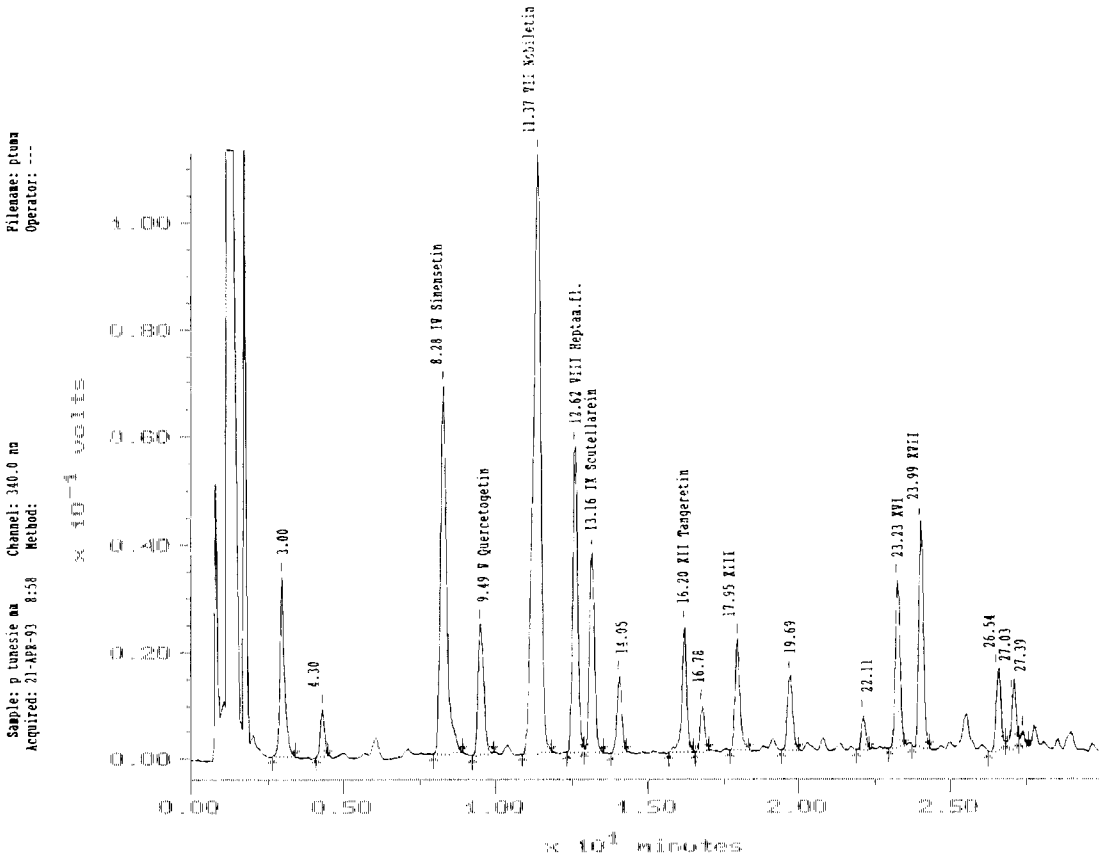


Figure 3. PMF chromatogram at 340 nm of blood orange juice.

Table 3. Relative PMF Peak Areas at 340 nm in Orange Juice Samples Obtained by a Different Technology (Further Extraction) or Blended with Other Citrus Juices, Pulp Wash, or Cells (Group 3)

no.	code	X	SIN	QUE	NOB	HEP	SCU	TAN
55	SGFBR20/4	1.53	28.46	7.31	33.76	13.26	11.00	4.69
56	SGFBR20/6	1.90	30.07	6.83	33.37	11.77	11.10	4.97
57	SGFBR20/7	1.77	28.25	6.76	32.73	12.26	12.34	5.90
58	SGFBR21/5	1.88	28.25	6.72	33.50	12.64	11.02	6.00
59	SGFBR24/3	1.49	26.18	6.40	32.94	12.79	12.89	7.31
60	SGFBR26/1	1.57	23.49	5.43	37.20	12.05	9.90	10.37
61	SGFBR28/1	1.61	23.16	5.41	37.61	11.64	9.91	10.67
62	SGFBR64/5	1.25	27.13	6.82	35.81	13.42	11.03	4.55
63	SGFBR69/3	1.42	27.38	5.07	40.94	10.77	9.57	4.05
64	SGFBR69/4	1.46	28.10	5.30	38.68	11.09	10.14	5.24
65	SGFBR69/5	1.51	27.85	5.12	39.14	11.19	10.04	5.15
66	SGFIT55/4	1.51	27.92	7.43	29.07	12.16	14.00	7.93
67	SGFIT59/5	1.81	29.40	9.51	29.15	14.46	10.35	5.25
68	SGFIT60/4	1.43	28.22	5.14	35.44	9.02	13.78	6.97
69	SGFIT62/4	2.15	30.77	10.03	28.08	12.45	11.43	5.10
70	SGFIT32/4	1.46	30.87	5.23	35.40	7.92	12.44	6.69
71	SGFIT33/3	1.65	32.44	5.69	34.96	8.41	11.23	5.61
72	SGFIT33/4	1.59	30.69	6.19	32.60	9.40	12.63	6.91
73	SGFIT39/4	1.75	28.59	7.03	29.24	10.83	14.37	8.19
74	CITPW	1.20	26.73	5.80	34.46	11.36	12.85	7.60

no significant differences ($\alpha < 0.01$) are calculated for X, SIN, QUE, NOB, HEP, and SCU. For tangeretin, however, a t value of 3.033 is obtained, indicating a significant ($\alpha < 0.05$) relative enhancement of tangeretin during concentration.

As may be remarked from Table 5, almost all samples containing pulp wash or cells do not show elevated F values, indicating that they are acceptable as authentic orange juices. On the other hand, the addition of Lima and/or tangerine juice (Table 6) clearly seems to be detectable due to the very high F values (falsification or blending probability about 100%).

Table 4. Relative PMF Peak Areas at 340 nm of Some Citrus Juices Not Belonging to the *C. sinensis* Variety (Group 4) and Self-Prepared Falsified Juices (Group 5)

no.	code	X	SIN	QUE	NOB	HEP	SCU	TAN
75	CITMUR1	1.47	7.09	0.60	60.77	4.85	2.77	22.47
76	CITMUR2	1.52	7.22	0.49	60.36	4.92	2.71	22.78
77	CITTAN1	0.58	3.20	0.41	44.81	14.68	2.90	33.42
78	CITTAN2	0.63	3.46	0.53	43.83	13.81	3.40	34.34
79	CITBAIA	1.10	23.64	5.12	35.25	11.95	13.46	9.47
80	CITLIMA1	0.84	25.54	4.02	38.05	10.40	11.68	9.47
89	SGFMUR3	0.80	3.88	0.20	57.06	4.55	1.79	31.72
90	SGFTAN3	0.94	4.49	0.71	47.48	15.11	3.09	28.18
91	SGFLIMA2	1.29	22.25	3.73	40.79	9.10	9.03	13.82
92	SIN	1.39	25.79	5.31	35.53	11.49	12.54	7.94
93	SIN + 10% TAN	1.34	23.14	4.70	36.28	11.90	11.45	11.18
94	SIN + 20% TAN	1.23	20.85	4.28	37.32	11.93	10.66	13.73
95	SIN + 30% TAN	1.15	18.48	3.69	38.12	12.33	9.58	16.64
96	SIN + 10% MUR	1.37	23.95	4.81	37.64	10.86	11.80	9.58
97	SIN + 20% MUR	1.38	22.60	4.47	39.79	10.31	11.02	10.42
98	SIN + 30% MUR	1.45	20.83	4.02	41.75	9.83	9.96	12.15

The influence of technology, using a second or third extraction of the oranges, is not clear: the higher F values probably may be attributed to the use of "polycitrus" juices, containing hybrids.

From Table 6 it also may be clear that it is not a problem at all to differentiate Murcott and tangerine juice from *C. sinensis* juice as a result of the enormously high F values. Otherwise, it may be confirmed that Baia belongs to the sweet oranges, which is less evident however for Lima.

Considering finally the self-prepared orange juices falsified with 10–30% Murcott or tangerine juice (group 5), it seems in both cases that the addition of 10% of each juice clearly may be established on the basis of strongly enhanced F values, giving rise to a blending probability from about 40% (without addition) to 100%

Table 5. *F* Value and Falsification Probability of Orange Juices Containing Pulp Wash or Cells, Based on Seven PMF Values

		F test	
no.	code	*F* value	% probability
55	SGFBR20/4	0.528	21.6
56	SGFBR20/6	1.658	84.6
57	SGFBR20/7	1.208	68.0
58	SGFBR21/5	3.024	98.6
59	SGFBR24/3	0.634	29.8
62	SGFBR64/5	1.816	88.3
63	SGFBR69/3	3.910	99.6
64	SGFBR69/4	1.648	84.4
65	SGFBR69/5	2.248	94.4
73	SGFIT39/4	4.084	99.8
74	CITPW	0.335	8.5

Table 6. *F* Value and Falsification Probability of Some Citrus Juices and Self-Prepared Falsified Orange Juices, Based on Seven PMF Values

		F test	
no.	code	*F* value	% probability
75	CITMUR1	437.500	100.0
76	CITMUR2	442.400	100.0
77	CITTAN1	444.700	100.0
78	CITTAN2	464.700	100.0
79	CITBAIA	1.299	72.3
80	CITLIMA1	2.546	96.7
89	SGFMUR3	585.500	100.0
90	SGFTAN3	363.500	100.0
91	SGFLIMA2	42.100	100.0
92	SIN	0.838	45.3
93	SIN + 10% TAN	11.766	100.0
94	SIN + 20% TAN	28.983	100.0
95	SIN + 30% TAN	58.116	100.0
96	SIN + 10% MUR	7.186	100.0
97	SIN + 20% MUR	16.977	100.0
98	SIN + 30% MUR	39.874	100.0

(after addition of 10% or more). Addition of both citrus juices causes a relative decrease of sinensetin and a relative increase of tangeretin, so that the sinensetin/tangeretin ratio is an important indicator to trace such blendings.

Other relevant average PMF ratios $\bar{r}$, also summarized in Table 2, also may be important to obtain a quick indication about a possible falsification. If three or more of these ratios are not within the limits defined by $(\bar{r} \pm \sigma)$, an *F* test is necessary to confirm a possible blending with another juice.

CONCLUSION

A fast and simple routine procedure has been developed or adapted from the literature. After a short sample preparation, gradient HPLC is used followed by photodiode array detection. An RP-18 Novapak column (3.9 × 150 mm) is used, and a gradient is made by partly replacing water by acetonitrile in the presence of tetrahydrofuran. The PDA detector is necessary to confirm the identification of the polymethoxylated flavones, on the basis of (relative) retention times, by their spectra.

Second, average relative peak areas and standard deviations at 340 nm of the seven main PMFs have been proposed to guarantee the authenticity of *C. sinensis*. In this way a PMF standard $(\bar{x} \pm \sigma)$ for orange juice is established to compare an unknown juice by a statistical *F* test. This results in an *F* value corresponding to a percent probability that the unknown sample is falsified and does not belong to the *C. sinensis* variety.

In a third part, we have established a possible influence of technology on the PMFs and the influence of the addition of other citrus juices. Technology in general seems not to have a clear influence on the relative PMF pattern. The addition of tangerine (*Citrus reticulata*) and Murcott juice (a tangor or hybrid of orange and tangerine), however, may be established by significant changes in the PMF pattern.

Finally, we could establish that the determinations of the flavanone glycosides and the polymethoxylated flavones are complementary techniques and have by preference to be used together to optimally detect falsifications of *C. sinensis* juice.

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