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QUANTITATION OF POLYMETHOXYLATED FLAVONES IN ORANGE JUICE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A quantitative high-performance liquid chromatographic (HPLC) procedure for the determination of the five major polymethoxylated flavones (PMFs) in orange juice has been developed. It employs a unique ternary solvent system with coupled UV–fluorescence detection. The dual detectors were employed to determine the presence of interfering substances and served as a cross check on quantitation. Stop flow UV and fluorescence scanning was used to identify peaks and determine the presence of impurities. Although all five citrus PMFs fluoresce, some HPLC fluorescence peaks were too small to be of much practical use. All five citrus PMFs could be quantitated satisfactorily with the fixed wavelength UV (313 nm) detector.

The HPLC procedure has been used to evaluate each step in the preparation. The optimum extracting solvent was selected and one time consuming step was eliminated, as it was found to be unnecessary. HPLC values for nobiletin and sinensetin are in good agreement with the thin-layer chromatographic (TLC) values in the literature. HPLC values for the other three flavones were considerably lower than those reported in the literature. The HPLC procedure is considerably faster than the TLC procedure with equal or superior precision and accuracy.

INTRODUCTION

While flavones are widely distributed in the plant kingdom, various citrus species are notable for their relatively large concentrations of highly substituted polymethoxylated flavones (PMFs). The highest concentrations of PMFs are found in citrus peel with much lower amounts found in the juice[1]. Certain citrus species contain characteristic PMF concentration patterns. Thus, relative concentrations of PMFs can be used to detect qualitatively the presence of one species in the juice of another[2]. Methoxylated flavones were originally suspected to contribute to occasional citrus juice bitterness[3,4], but were later reported to exist below taste threshold levels in orange juice[1].

Methoxylated flavones also produce important physiological responses in the higher animals. Kupchan *et al.*[5] reported tetramethylscutellarein to be a cytotoxic agent toward different strains of carcinoma cells. Other citrus PMFs have been re-

ported by Robbins[6] in *in vitro* experiments to regulate erythrocyte aggregation and concentration in human blood. He suggested possible dietary control of the high blood viscosity syndrome using foods such as citrus as a source of PMFs.

Separation and quantitation of these compounds has until now been done via the TLC–spectrophotometric procedure developed by Swift[3] and later modified by Veldhuis *et al.*[1]. The method is extremely lengthy, involving several manipulative steps which are possible sources of inaccuracy. Therefore, the goal of this study was to develop a more rapid method to accurately determine PMF levels in orange juice. These values could then be used to establish dietary PMF ingestion levels from orange juice for clinical studies.

EXPERIMENTAL

Reagents and standards

All chromatographic and extraction solvents were high purity UV grade purchased from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). Mallinckrodt (St. Louis, Mo., U.S.A.) Nanograde benzene was used for the benzene extractions. All water used in the mobile phase was deionized, distilled and filtered with a 0.22-μm Millipore (Bedford, Mass., U.S.A.) filter before use.

PMF standards were prepared by Lyle J. Swift, formerly of the U.S. Fruit and Vegetable Products Laboratory, Winter Haven, Fla., U.S.A. and were supplied by R. C. Robbins of the Department of Food Science and Human Nutrition at the University of Florida, Gainesville, Fla., U.S.A. Chromatographic analysis indicated that the standards could be used without further purification. Standard PMF solutions were prepared by adding the appropriate amount of solid flavone to a small volumetric flask and diluting to volume with absolute ethanol. The flasks were sonicated to facilitate dissolution.

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A pump and U6K injector were used as the solvent-sample delivery system in isocratic experiments, a Perkin-Elmer (Norwalk, Conn., U.S.A.) Series 3 pump with programmable gradient was used in all gradient studies. A Tracor (Austin, Texas, U.S.A.) Model 970A variable wavelength UV detector equipped with the wavelength scanning accessory was used to determine the spectra of trapped peaks in stop flow experiments. Two 8-μl cells were used as reference and sample cell, respectively. A Waters Assoc. Model 440 UV detector with a 313-nm filter kit was used as the general UV absorbance monitor. Both the general fluorescence monitoring and stop flow emission and excitation spectrum experiments were done with a Perkin-Elmer Model 204A fluorescence detector equipped with a square 20-μl flow cell. Individual PMF concentrations were determined from their integrated peak areas using the external standard method. A Spectra-Physics (Santa Clara, Calif., U.S.A.) Model 4000 integrator-printer plotter was used .

Chromatographic conditions

The PMFs were separated isocratically in the reversed-phase mode using a DuPont Zorbax C_8 column, 25 cm $\times$ 4.6 mm I.D. Isocratic mobile phase composi-

tion consisted of tetrahydrofuran (THF)–acetonitrile–water (22:6:72). Mobile phase solvents were degassed with vacuum and sonication before use. The flow-rate was 1.5 ml/min with a column head pressure of approximately 2200 p.s.i. The column was operated at ambient temperature, usually 22–25°.

Gradient mobile phase composition consisted of THF–water (22:78) for the weak solvent and THF–acetonitrile–water (22:40:38) for the stronger solvent. The concentration of THF was held constant throughout the solvent program to minimize baseline shifts. Initially, 15% of the strong solvent was mixed with the weak solvent. Thus, the mobile phase consisted of THF–acetonitrile–water (22:6:72). This composition was maintained for 10 min. Then the concentration of the strong solvent was increased linearly to 50% in 1 min. This composition (THF–acetonitrile–water, 22:20:58) was held constant for 7 min. Finally, the system was purged at 100% strong solvent for 5 min.

Each flavone peak was trapped in the detector cell as it reached its maximum peak height. Its UV absorption or fluorescence spectrum was obtained by scanning the wavelength region 390–200 nm and 220–550 nm, respectively. Then the flow was resumed.

Resolution of incompletely resolved chromatographic peaks was estimated using the method of Snyder[7].

Sample preparation

Single strength or reconstituted orange concentrate juices, to which Celite was added, were used and filtered with suction through Whatman No. 1 filter paper. Five grams of sodium hydroxide were rapidly dissolved in each 100 ml of juice that was to receive the alkaline treatment and allowed to stand for 30 min. All juices were extracted three times with 25 ml of benzene. The benzene extracts were combined and evaporated to dryness using a rotary evaporator with aspirator vacuum at 40°. Each residue was redissolved in 2.00 ml of absolute ethanol, filtered through a 1.2-μm filter and stored (refrigerated) in a septum sealed vial until injection.

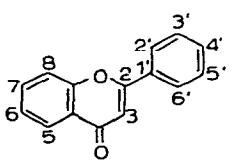
Identification of peaks

Chromatographic peaks were identified in several ways. Retention times of unknown peaks were compared with retention times of authentic compounds. Sample extracts were fortified with small amounts of standard material one at a time and re-chromatographed to see if the peak of interest increased in height. Spiked peaks were also checked for peak symmetry to determine if more than a single component might be present. Stop flow UV and fluorescence scans were obtained for each of the five major PMFs and compared to standards.

RESULTS AND DISCUSSION

Chromatographic separation

All of the PMFs have the basic flavone structure shown below. Since they



Sinensetin	5,6,7,3',4'-pentamethoxyflavone
Nobiletin	5,6,7,8,3',4'-hexamethoxyflavone
Heptamethoxyflavone	3,5,6,7,8,3',4'-heptamethoxyflavone
Tetramethylscutellarein	5,6,7,4'-tetramethoxyflavone
Tangeretin	5,6,7,8,4'-pentamethoxyflavone

differ only in the position and number of methoxy groups, differences in polarity and solubility are subtle. Owing to the aromatic nature of these compounds, a C_{18} column was used for the initial separation. A C_8 column was later found to give slightly better separation and was used from that point on.

The choice of mobile phase constituents and proportions is critical to the effective separation of these flavones. Water–methanol or water–acetonitrile in various proportions could not adequately separate nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) from 3,5,6,7,8,3′,4′-heptamethoxyflavone. Resolution between these two compounds was never greater than 0.5 using 10-μm column packing material. If 5-μm column packing material was used, the resolution increased to approximately 0.8. Water–THF was the only solvent system which adequately resolved these two compounds. Resolution with water–THF (75:25) is approximately 1.3. It was found

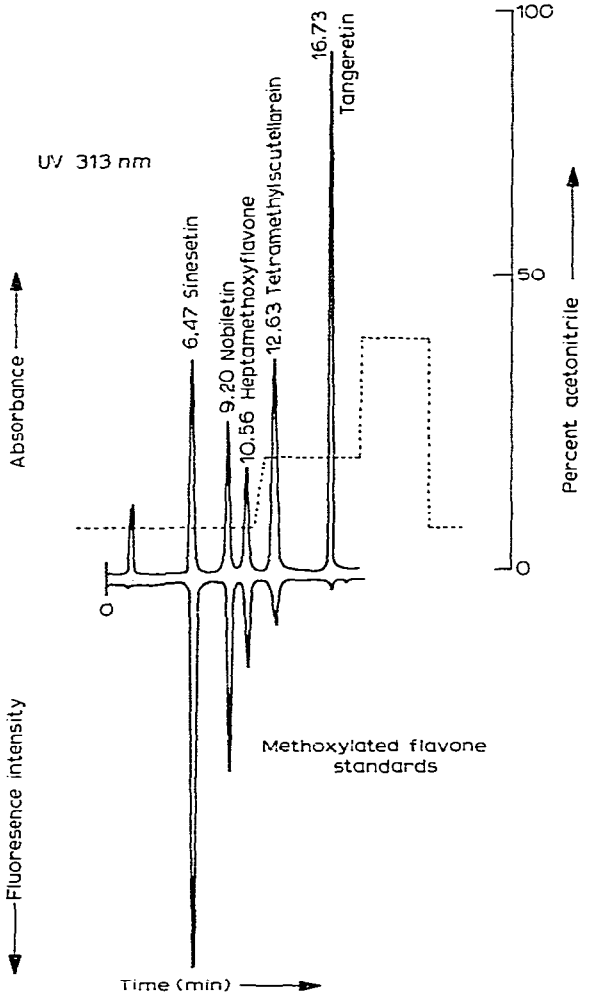


Fig. 1. Liquid chromatograms from a 5-μl injection of 100 ppm each of the five major polymethoxylated flavones in citrus. Upper chromatogram: UV absorbance (313 nm) at 0.1 a.u.f.s. Lower chromatogram: fluorescence determined with Fluoromonitor (ex. 360 nm, em. >415 nm) at 30×. Chromatographic conditions see text.

that by adding a small amount of acetonitrile and decreasing the THF concentration, the peaks became narrower and heptamethoxyflavone shifted further away from nobiletin, thus further increasing the resolution. The optimum ternary solvent mixture of water–THF–acetonitrile was 72:22:6, which placed heptamethoxyflavone equally distant between nobiletin and tetramethyscutellarein. This solvent system was satisfactory for all juice samples tested except those that had been treated with NaOH. The sodium hydroxide-treated samples required a solvent gradient to elute compounds formed as a result of the alkali treatment in a reasonable length of time. An example of the gradient separation is illustrated in Fig. 1 for the five standard PMFs. Since the concentration of THF remains constant, the solvent program is illustrated in terms of percent acetonitrile only. It is superimposed on the UV chromatogram in Fig. 1.

Detector response

It should be noted from Fig. 1 that UV absorbance at 313 nm more effectively detects all five major citrus PMFs than fluorescence. While the fluorescence detector is extremely sensitive to sinensetin, it is much less sensitive to the other flavones. The spectral characteristics of these compounds are given in Table I.

TABLE I

PMF SPECTRAL CHARACTERISTICS

Compound	*Fluorescence maxima (nm)**		*UV absorbance maxima (nm)*		
	Excitation	*Emission*	*Found**	*Literature***	*Log ε***
Sinensetin	346 (47)***	447 (71)	328	329	4.43
Nobiletin	352 (47)	462 (82)	331	333	4.45
Heptamethoxyflavone	330 (66)	466 (70)	342	341	4.38
Tetramethylscutellarein	331 (47)	492 (98)	319	318	4.58
Tangeretin	335 (53)	415 (71)	320	323	4.49

* THF–acetonitrile–water (22:6:72).

** Data from ref. 4; solvent: absolute ethanol.

*** Peak width at half height in parentheses.

Swift[4] reported the UV maxima for these compounds in absolute ethanol. To determine the authenticity of the standards used in these experiments, the individual standards were dissolved in absolute ethanol and their UV maxima were determined. There was excellent agreement with the values reported by Swift[4]. Even values obtained in the ternary solvent mobile phase using the stop flow technique differed not more than 3 nm from Swift's values. Since the extinction coefficients do not vary greatly, the relative sensitivities of these compounds can be explained by the proximity of their UV maxima to that of the 313-nm source.

As a means of qualitative identification, fluorescence excitation and emission maxima were determined for the five standards using the stop flow method. To further characterize these flavones their emission and excitation peak widths at half heights were also determined. Miller and Faulkner[8] have shown that the combination of such spectral features will allow excellent qualitative identification of compounds from liquid chromatography effluents.

Fluorescence excitation maxima for all five citrus PMFs are within 22 nm of

one another while emission maxima occur over a 77 nm range. Using a filter fluorometer with a narrow pass excitation filter (360 nm maximum and 55 nm band pass width) and a sharp cut-off emission filter (415 nm) it should be possible to detect all of the five flavones with approximately equal sensitivity. However, judging from their fluorescence peak heights in Fig. 1, it can be seen that some PMFs appear to have stronger fluorescence than others. To determine if this uneven response was an instrumental artifact, a variable wavelength fluorescence detector was used in place of the filter fluorometer. Excitation and emission wavelengths were set to optimize fluorescence for the smallest chromatographic peak, tangeretin. However, relative peak heights did not change. Thus, tangeretin's small fluorescence peak is not due to fluorescence undetected because of filter choice.

It has been reported[9,10] that the presence of oxygen will reduce fluorescence intensity. To determine if oxygen was causing the reduced fluorescence of the other four flavones, a chromatogram of the five standards was obtained in the usual manner. Helium was bubbled through the solvent saturating it and the chromatogram repeated. There was no change in peak heights. Thus, oxygen is not the cause of the reduced fluorescence intensities of the four methoxylated flavones.

Several factors could be responsible for these widely varying fluorescence intensities. Apparently there are sizable differences in the quantum efficiencies between these flavones. In addition, it was observed during the stop flow scans that some fluorescence peaks increased in size when they were trapped in the cell. Tangeretin was tripled in size within 10 min. This suggests that tangeretin exhibits some delayed fluorescence possibly due to phosphorescence, which is unusual for a solution at room temperature. Therefore, in the case of tangeretin, the small fluorescence peak size is due to delayed fluorescence and limited detector residence time under constant flow conditions. Tangeretin may also have a lower quantum efficiency than sinensetin.

Therefore, the fluorescence detector is not well suited as a general detector for these PMFs. While it is extremely sensitive to sinensetin, it is extremely insensitive to tangeretin under constant flow conditions.

Sample preparation

As a means of sample clean up, Swift[4] added NaOH until a 5% solution was achieved and allowed the solution to stand for 2 h before extraction. Veldhuis *et al.*[1] modified the procedure by shortening the alkaline reaction time to 30 min.

To determine the effectiveness of the NaOH treatment one portion of an orange juice (OJ) sample was treated with NaOH in the manner described by Veldhuis *et al.*[1] before extraction, while the other was immediately extracted. The resulting chromatograms are illustrated in Figs. 2 and 3. Both UV absorbance (313 nm) and fluorescence were monitored.

The purpose of treating the sample with NaOH was to obtain an extract with fewer interfering compounds. However, in comparing the UV chromatograms of the NaOH treated sample with that of the untreated sample, it becomes apparent that while a few peaks have been eliminated, several new peaks are observed. Specifically, two peaks occur near the sinensetin peak (t_R = 6.5 min). Usually one of these compounds will coalesce with sinensetin (see Fig. 3) causing a positive error in the integration of this peak. There are also two large peaks (t_R = 20.85 and 22.51 min) that elute after tangeretin, which are not present in the untreated juice. Apart from ex-

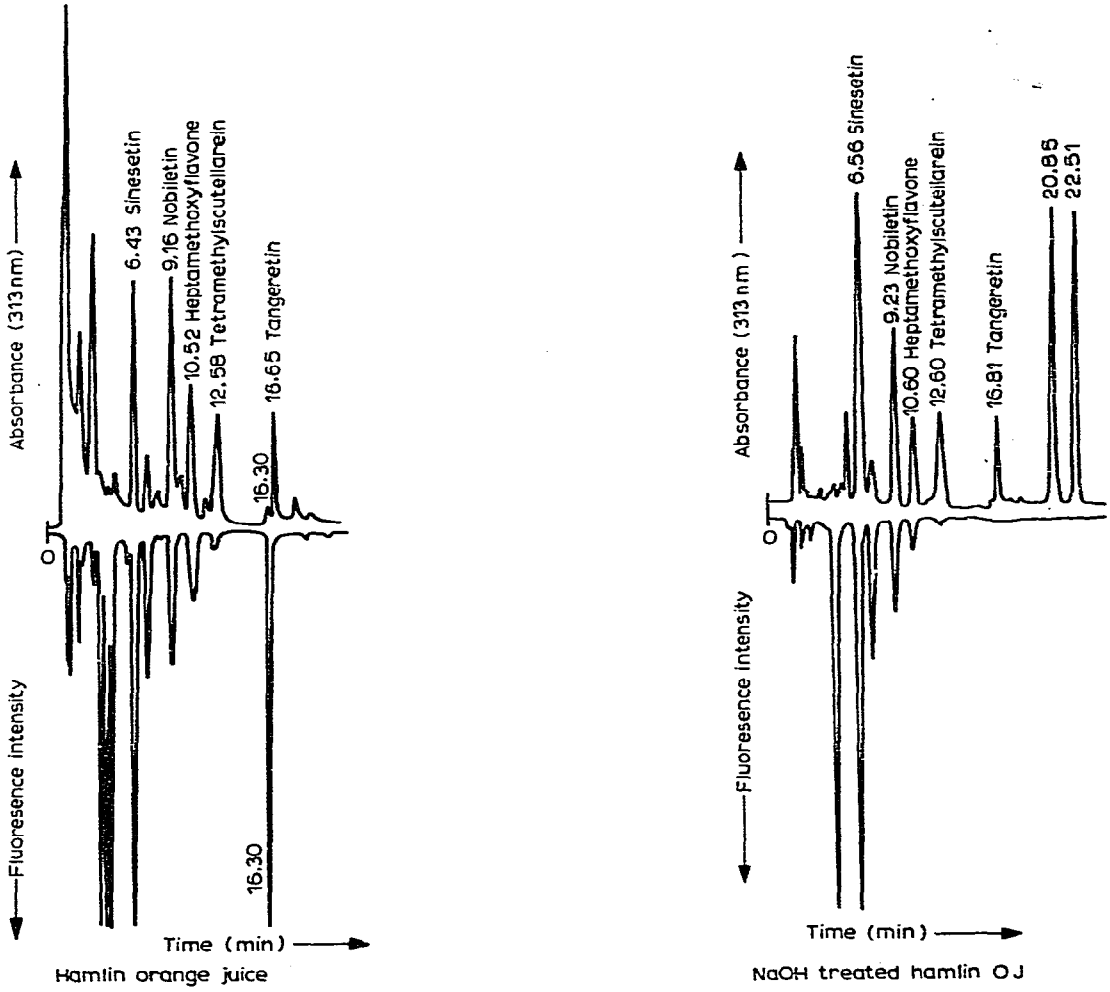


Fig. 2. Liquid chromatograms from a 20-μl injection of an orange juice extract. Chromatographic conditions and detectors as in Fig. 1.

Fig. 3. Liquid chromatograms from a 20-μl injection of an extract from an orange juice that has been treated with NaOH for 30 min before extraction. Chromatographic conditions, and detectors as in Fig. 1.

tending analysis time, these peaks present no problem in the gradient mode. However, if these samples were analyzed isocratically, these peaks would appear at much longer retention times. Usually they appear in subsequent chromatograms as low broad peaks that often interfere with the peaks of interest. On the other hand, the UV chromatogram of the juice sample that was not treated with NaOH contains two small potentially interfering peaks (one preceding tetramethylscutellarein and the other just following nobiletin) that are absent or reduced in the NaOH-treated sample. However, these potentially interfering peaks account for less than 10% of the height of the peaks of interest so any error caused by the inclusion of these peaks with the peaks of interest would be minimal.

There are some interesting qualitative differences between the NaOH-treated and untreated juice chromatograms obtained with a fluorescence detector. The NaOH-treated sample gave a much simpler chromatogram. Each of the five major citrus PMF peaks were well separated with good peak shape. The fluorescence peak at 7.2 min is probably due to the same substance Swift[4] observed as a blue-white band between nobiletin and sinensetin in his TLC studies. Judging from the trends in retention time and methoxylated substitution pattern observed in this study this compound is probably 3,5,6,7,3′,4′-hexamethoxyflavone reported by Tatum and Berry[11] in citrus peel. However, no fluorescence characteristics have been found in the literature and no authentic sample was available to test the validity of this tentative identification.

In the chromatogram of the untreated juice sample there were several major extraneous peaks that preceded the sinensetin peak and a very intense unidentified peak which occurred at 16.3 min (tangeretin occurs at 16.7 min). As the retention time of this latter peak was so close to that of tangeretin both fluorescence maxima were determined (340 nm and 447 nm) and compared to that of tangeretin. They are sufficiently different to indicate that the peak is due to something other than tangeretin. Judging from the lack of UV absorbance at the same retention time as the large fluorescence peak, this compound is apparently a relatively weak UV absorber and does not interfere with the UV peak of tangeretin (313 nm).

Peak shape studies suggest that there is an additional compound underneath the heptamethoxyflavone peak. Stop flow spectral scans gave falsely high values for heptamethoxyflavone. The tetramethylscutellarein peak (12.6 min) is also somewhat distorted but its intensity is so low it was not possible to determine from peak shape studies or stop flow spectral scans whether another compound is definitely present.

To determine the effects of NaOH treatment on individual and total PMF content, portions from the same lot of fresh squeezed Hamlin OJ were given two different NaOH treatments while a third portion received no treatment. The NaOH-treated samples differed only in the length of alkaline reaction time before extraction. Table II contains the concentrations of individual PMF's as determined from UV and fluorescence detectors. Integration of both fluorescence and UV chromatographic peaks should give complementary values.

Of the five major citrus PMFs, tangeretin was found in the lowest concentra-

TABLE II

EFFECT OF NaOH TREATMENT ON METHOXYLATED FLAVONES IN ORANGE JUICE

Juice concentration given in ppm; All samples were extracted 3 times with benzene.

Treatment	*Sinensetin*		*Nobiletin*		*Heptamethoxy-flavone*		*Tetramethyl-scutellarein*		*Tangeretin*		*Total*	
	UV	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*
None	0.86	0.92	1.18	1.12	0.68	1.10	0.36	0.40	0.07	—	3.15	3.54**
NaOH (30 min)	1.04	0.84	0.90	1.00	0.66	0.74	0.30	0.32	0.06	—	2.96**	2.90
NaOH (60 min)	1.08	0.72	0.78	0.88	0.64	0.70	0.28	0.30	0.06	—	2.84**	2.60

* Contains falsely high value from heptamethoxyflavone.

** Contains falsely high value from sinesetin.

tion in OJ. There was no significant difference between NaOH-treated and untreated samples. Since the fluorescence detector was too insensitive to determine tangeretin, no comparison could be made with the UV values. There was substantial agreement between UV and fluorescence values for tetramethylscutellarein. The fluorescence values for this flavone were slightly higher in the untreated juice, possibly due to a fluorescent impurity. However, the UV values also show a slight decrease in tetramethylscutellarein concentration due to NaOH treatment and further decrease with increased alkaline reaction time. However, these differences are so slight that it is questionable whether they are significant.

Heptamethoxyflavone values suggest that this PMF is not affected by NaOH treatment. While the fluorescence value of the untreated juice is substantially higher than the alkaline treated samples, the corresponding UV values are very similar. The discrepancy between the UV and fluorescence values can be resolved as this peak was found to contain a fluorescent impurity.

Fluorescence sinensetin values decrease when the juice sample is treated with NaOH and continues to decrease as the alkaline reaction time is increased. Thus, it appears that sinensetin is degraded by NaOH. Sinensetin UV values are actually higher in the NaOH-treated samples than the untreated samples. This is probably due to an unresolved impurity which is formed as a result of the alkaline treatment and has been discussed earlier. Thus, the UV values do not reflect true sinensetin content.

The major PMF found in OJ is nobiletin. It also appears to be degraded by NaOH as evidenced by the drop in its concentration when treated with NaOH. Its concentration continues to drop the longer it remains in alkaline solution before extraction. Since both UV and fluorescence values are in excellent agreement, the possibility of this trend being due to some artifact is remote.

It can be seen from Table II that the total PMF content decreases when OJ samples are treated with alkali and that the decrease is proportional to the length of alkaline reaction time before extraction. While the UV total PMF concentration of the 1 h treated sample is only 10% lower than that of the untreated sample, it should be remembered that the alkaline-treated samples also contain an interfering substance in the sinensetin peak giving sinensetin and total PMF values which are falsely high. Comparing fluorescence values it appears that 27% of the PMFs from the untreated juice are lost when the sample is allowed to react with alkali for 1 h. This again is not correct as the total fluorescence values contain falsely high heptamethoxyflavone values. A reasonable estimation of the magnitude of the effect of NaOH treatment may be obtained by comparing the values of total UV PMF content of the untreated sample with total fluorescence PMF content of the alkali treated samples. Thus, there is approximately a 9% loss of total PMF content with the 30-min NaOH treatment and an 18% loss for the 1-h treatment.

Hydroxylated flavones are known to react under alkaline conditions. Seshadri[12] has used color changes in alkaline buffers to determine the number and positions of hydroxyl groups in flavones. Therefore, it should not be surprising that the methoxylated flavones should also react in alkali, but at a much slower rate.

Extraction efficiencies

Since Veldhuis *et al.*[1] used benzene to extract PMFs from OJ while Ting *et al.*[2] used chloroform, these and other solvents were evaluated for their ability to extract

PMFs from juice. All samples were taken from the same lot of fresh Hamlin OJ. Table III illustrates the results of this study. It can be seen that while chloroform is more convenient to work with and does not present the safety hazard benzene does, it is only about half as effective in extracting PMFs from OJ. When juice is treated with NaOH the chloroform extract exhibits the same problems as previously discussed for the NaOH-treated benzene extraction. Since there are no qualitative advantages in terms of types or numbers of compounds co-extracted with the flavones and in light of the severe quantitative short-comings ,chloroform is not the solvent of choice when total citrus PMF extraction is attempted.

TABLE III

SOLVENT EXTRACTION EFFICIENCIES

Solvent	*Sinensetin*		*Nobiletin*		*Heptamethoxy-flavone*		*Tetramethyl-scutellarein*		*Tangeretin*		*Total*	
	UV	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*
Benzene	0.64	0.66	1.04	1.01	0.56	1.66	0.26	0.62	0.08	—	2.58	3.95
Chloroform	0.51	0.34	0.61	0.53	0.42	0.86	0.15	0.32	0.04	—	1.64	2.05
MIBK	0.55	0.54	1.10	0.92	0.74	1.40	0.29	0.44	0.06	—	2.74	3.30
Light petroleum	0.08	0.12	0.34	0.39	0.38	0.54	0.12	0.20	0.04	—	0.96	1.25

In terms of total PMFs found, methyl isobutyl ketone (MIBK) is more effective than chloroform and only slightly less effective than benzene. It is interesting to note that MIBK appears to be even more effective than benzene (as evidenced by UV concentration values) in extracting heptamethoxyflavone. However, it also extracts a very large amount of relatively polar compounds which greatly complicate the early portion of the chromatogram and possibly interfere with sinensetin. Total PMFs extracted with light petroleum were very low, indicating that this solvent is also less desirable than others as an extracting solvent. Toluene and hexane were also evaluated. As might be expected, toluene gave almost the same PMF values as benzene, and hexane gave values very similar to those of light petroleum.

Recovery studies

Base level concentrations of the five citrus PMFs were determined on a single lot of single strength Hamlin OJ. Known amounts of each of the five PMFs were added at two levels to a different portion of the same lot of OJ and analyzed. The results are shown in Table IV. Excellent recoveries were obtained for all of the PMFs, except heptamethoxyflavone which was inexplicably (and repeatably) low. Swift[4] reported excellent recoveries for all five citrus PMFs. However, his values were based upon amounts of standards recovered from silica gel plates only. No recovery test from known amounts of standards added to a sample were reported. No other recovery studies have been found in the literature.

Precision

The overall precision of this method is illustrated in Table IV. To determine

TABLE IV

FLAVONE RECOVERY STUDY

UV detection at 313 nm.

	Mean amount found (ng) *	*Amount added (ng)*		*Mean amount recovered*		*Recovered (%)*	
Sinensetin	344 (50)**	200	300	236	310	118	103
Nobiletin	474 (24)	300	450	284	422	95	94
Heptamethoxyflavone	210 (20)	100	150	62	104	62	69
Tetramethylscutellarein	100 (4)	100	150	92	132	92	88
Tangeretin	82 (8)	76	150	78	170	103	113

* 10-μl injection of juice extract.

** Standard deviation in parentheses, $n = 10$.

base level concentrations of citrus PMFs, five samples of the same juice were extracted and analyzed in duplicate. It can be seen from the standard deviation values that the overall precision is excellent for a natural product analysis at the ppm level. As might be expected, sinensetin has the lowest level of precision as evidenced by its 15% relative standard deviation (RSD). Heptamethoxyflavone has a relatively high RSD (9.5%) because it is relatively small and close to nobiletin. Since this study was performed isocratically, the tangeretin peak came out as a low broad peak. Thus, the precision for this peak (RSD = 9.8%) could probably be improved by optimizing integration parameters for a peak of this shape. Nobiletin and tetramethylscutellarein had RSDs of 5.1 and 4.0%, respectively.

Detection limits and linearity of response

UV detection limits for the fixed-wavelength (313 nm) detector range from 2 ng for tetramethylscutellarein and sinensetin to 8 ng for heptamethoxyflavone. Detection limits were considerably higher with the variable wavelength UV detector. (Detection limits were defined as that amount that would give a peak height of twice the peak to peak noise level.)

Fluorescence detection limits were not established because of the extremely unequal response to the PMFs in this study.

Linearity ranges between PMF concentrations and peak areas were established for the fixed wavelength UV detector. The response of all five flavones was found to be linear between 50 ng and 1.0×10^4 ng. All sample injections were well within this range. A linear least squares fit of this data indicated that sinensetin had the best correlation coefficient ($r = 0.999$) while tetramethylscutellarein had the lowest ($r = 0.991$).

PMF values in orange juice

HPLC flavone values for OJ are considerably lower than the corresponding TLC values reported by Veldhuis *et al.*[1]. A comparison between HPLC flavone values and those reported by Veldhuis *et al.* is shown in Table V. The HPLC values are from a typical frozen concentrated orange juice (FCOJ). All juices were reconstituted to 12° Brix. Total HPLC PMFs were only about half that found in the TLC procedure. Of the five flavones, only nobiletin values were comparable. Sinensetin values were

slightly lower while the other three flavones were considerably lower. Since the TLC procedure employed a NaOH treatment in sample preparation (which appears to reduce the concentrations of some PMFs, see Table II), it is surprising to find PMF values higher than those of the HPLC procedure which omits this step. Seasonal or varietal differences can not account for the magnitude of difference between these two sets of data.

TABLE V

COMPARISON OF PMF VALUES IN FCOJ RECONSTITUTED TO 12° BRIX

Method	*PMF concentration (ppm)*					
	Sinensetin	*Nobiletin*	*Heptamethoxyflavone*	*Tetramethylscutellarein*	*Tangeretin*	*Total*
HPLC	0.73	1.00	0.57	0.27	0.13	2.7
TLC*						
Average	0.90	1.31	0.81	0.51	0.45	4.24
Range	0.7–2.05	0.8–1.95	0.4–1.6	0.25–1.05	0.2–1.05	2.25–6.20

* Data from ref. 1; 37 samples taken over a 16-year period.

If the lower HPLC flavone values were due to incomplete extractions, recovery values would have to range between 25–80% to account for the differences observed. Since the data in Table IV indicate that recoveries are almost complete (with the exception of heptamethoxyflavone), other possibilities must be considered.

In Fig. 3, it can be seen that a UV-absorbing (and apparently non-fluorescing) impurity is found along with sinensetin in juice samples that have been treated with NaOH. This impurity is also reflected in sinensetin UV concentration levels (Table II) that are higher than either the corresponding fluorescence or non-treated juice sinensetin values. These falsely high UV concentration levels are in good agreement with those of Veldhuis *et al.*[1]. Thus, it is possible that the TLC–UV spectrophotometric procedure also incorporated this same impurity.

Tangeretin has the greatest discrepancy between concentrations determined by HPLC and TLC. TLC concentration levels are almost four times higher than those obtained by HPLC. A possible explanation can be seen from Fig. 3. It should be noted that two strong UV-absorbing (and non-fluorescing) peaks are formed as a result of the NaOH treatment and elute after tangeretin (at 20.85 and 22.51 min, respectively). Since the TLC procedure was done in the normal phase mode, these peaks would elute close to the solvent front and before tangeretin. (Long-wavelength UV irradiation was used to locate the weakly fluorescing tangeretin band on the streaked TLC plates.) Therefore, it is possible that some of this non-fluorescing material might have been scraped off with tangeretin. Thus, when the scrapings were redissolved, these compounds would cause an abnormally high UV absorbance which would then be calculated as tangeretin.

There is, however, good qualitative agreement between the two methods. In both methods nobiletin and sinensetin are the major methoxylated flavones found in orange juice accounting for approximately 60% of the total PMF content. Both methods find tangeretin to be the flavone in lowest concentration.

CONCLUSION

The HPLC procedure for the analysis of PMFs in OJ offers several advantages over the existing TLC–spectrophotometric method. Overall analysis time is less than half that required for the TLC method. Sample preparation time has been reduced by elimination of the NaOH treatment (which has been shown to be unnecessary). The actual analysis of the juice extract is much simpler and thus less subject to inaccuracies due to successive sample manipulations. Precision is excellent for a natural product analysis at the ppm level.

The use of combined UV and fluorescence detectors was invaluable in determining the presence of impurities and served as a cross check on quantitation. Using UV and fluorescence stop flow spectral data along with retention times, it was possible to identify unequivocally the five major methoxylated flavones from other peaks with similar retention times.

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