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Fourier Transform Infrared Spectroscopic Analysis of the Polymethoxylated Flavone Content of Orange Oil Residues

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Preliminary studies have shown that orange peel polymethoxylated flavones (PMFs) exhibit beneficial biological properties in animals. These properties have increased the demands for these compounds as candidate nutraceuticals and specialty food ingredients. Orange oil residues are a likely commercial source of the PMFs, and a rapid, solvent-free method for the analysis of the PMFs in orange oil residues has been developed based on Fourier transform infrared (FTIR) spectroscopy. The intensities of the FTIR vibrations of the phenyl ring ν(C=C) stretch at 1515 cm^{-1} of the PMFs can be used, relative to the intensity of the carbonyl stretch at 1733 cm^{-1} of the non-PMF orange oil residue components, to measure PMF content. Excellent correlations for the ratios of the intensities of these vibrations and the total PMF content were observed irrespective of the source, viscosity, and presence of particulate material. The detection limit by this method is approximately 0.1% PMF.

KEYWORDS: Flavonoids; methoxylated flavones; FTIR; nutraceuticals; nonvolatile oil residues; citrus byproducts; value-added products

INTRODUCTION

Orange peel contains diverse flavonoids, including numerous flavanone and flavone *O*- and *C*-glycosides, and the polymethoxylated flavones (PMFs) (*1*, *2*). The latter compounds occur mainly as highly methoxylated flavone aglycones. Biotesting of these compounds has shown that the PMFs exhibit anticancer and antiinflammation actions (*3*−*5*), as well as triglyceride and low-density lipoprotein cholesterol-lowering properties (*6*). These biological actions have created interest by the food and nutraceutical industries for the use of these compounds as specialty ingredients with the above, targeted pharmacological end points.

Because of the lipophilic nature of these compounds and the fact that these compounds originally occur in the oil glands of orange peel, high concentrations of PMFs are recovered in orange oil during fruit processing. Subsequent vacuum distillations of orange oil run during the recovery of volatile fractions (*7*) produce large amounts of nonvolatile oil residues, a portion of which are the PMFs. Analysis of the PMFs in these residues is usually performed by reversed phase high-performance liquid chromatography (HPLC) (*8*, *9*), which requires the use of purified standards. In contrast, Fourier transform infrared (FTIR) spectroscopy is shown in this study to provide a rapid, solvent-free analysis of the total PMF content in orange oil residues. This analytical technique does not require the availability of purified PMF standards and significantly shortens the analysis time for these compounds in the oil residues.

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MATERIALS AND METHODS

FTIR Spectroscopy. Orange oil residues were obtained from four local companies. No further processing of the samples was done prior to FTIR measurements with a PerkinElmer Spectrum One FTIR spectrometer. Measurements were recorded at room temperature and were summations of eight scans. Samples were applied as thin films on PTFE infrared cards (International Crystal Laboratories, Garfield, NJ).

HPLC Chromatography. Three hundred milligram portions of the orange oil residues were added to 10 mL of acetone and shaken at room temperature for 2 h. The PMF contents of the samples were analyzed by HPLC using an Agilent 1100 quaternary pump and chromatography workstation and a 1050 Photodiode Array detector and autosampler (Agilent, Wilmington, DE). PMFs were chromatographed with a Zorbax Eclipse XDB-C8 (4.6 mm × 150 mm) 5 μm column. Elution conditions included a three-solvent gradient composed initially of 2% formic acid/water/acetonitrile (5:85:10, v/v/v) and increased in linear gradients to 5:60:35 (v/v/v; 30 min), then to 5:55:40 (v/v/v; 10 min), and finally to 5:45:50 (v/v/v; 10 min) at a flow rate of 0.75 mL min^{-1}. PMFs were identified by their distinctive UV spectra (*9*). Quantitations were made using peak area conversion factors determined with purified standards.

PMF Fractionation. An orange oil residue (30 g) containing 4% PMF was rapidly stirred overnight at room temperature in 350 mL of 95% ethanol. The suspension was allowed to settle, and the ethanol was decanted, and the remaining residue was briefly shaken with 50 mL of 95% ethanol and allowed to settle. The additional 95% ethanol wash was decanted and added to the original 95% ethanol extract. The remaining residue was re-extracted with 95% ethanol, and the combined ethanol extracts were dried by rotary evaporation (8 g). The ethanol insoluble material (22 g) was extensively dried by rotary evaporation and was used as the PMF-free residue. HPLC analysis of the ethanol insoluble material showed a negligible PMF content (<0.1%).

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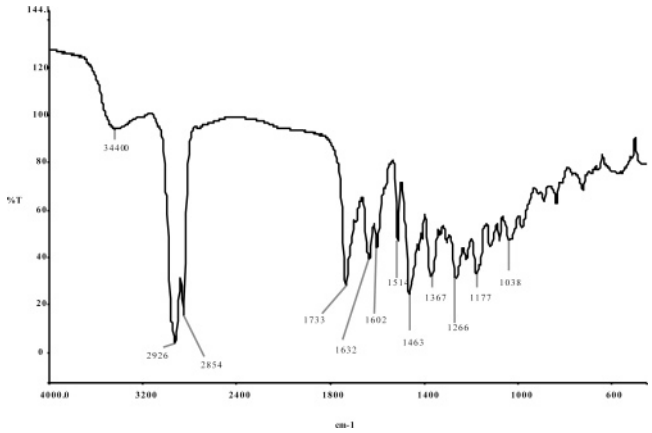

Figure 1. FTIR spectrum of an orange oil residue (4% PMF). The spectrum is a summation of eight scans taken with the residue thinly applied to a PTFE IR card.

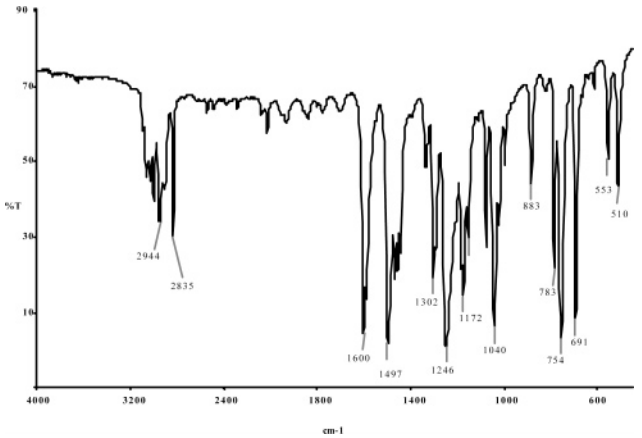

Figure 2. FTIR spectrum of anisole. The spectrum is a summation of eight scans with pure anisole thinly applied to a PTFE IR card.

The PMF-containing ethanol extract (300 mg) was dissolved in 95% ethanol and chromatographed on LH-20 resin (Whatman, Clifton, NJ) in a glass column (5.0 cm × 25 cm) with 95% ethanol. Fractions (15 mL) were collected, and every third fraction was analyzed for PMF content by UV spectroscopy or HPLC. The fractions containing the six main PMFs were pooled and dried by rotary evaporation. Analysis by HPLC showed that the PMF content of this material was 78%. This PMF-enriched material was used to measure the FTIR of the collective PMFs of the original orange oil residue.

RESULTS AND DISCUSSION

FTIR of Orange Oil Residues. The major nonvolatile constituents of orange oil include PMFs, conjugated phytosterols (*10*, *11*), fatty acids (*12*), waxes (*13*), and carotenoids (*14*). PMFs in orange oil consist of six main compounds [sinensetin (5,6,7,3′,4′-pentamethoxyflavone), quercetagetin hexamethyl ether (3,5,6,7,3′,4′-hexamethoxyflavone), nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), tetramethylscutellarein (5,6,7,4′-tetramethoxyflavone), 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin (5,6,7,8,4′-pentamethoxyflavone)], and the concentrations of these compounds in orange oil have been previously reported (*15*). A collection of other minor methoxylated flavones also occurs (*1*), but this collection usually comprises less than 3% of the total PMF content. In this study, the PMF contents of orange oil residues are reported as the combined levels of the six main compounds.

Although detailed chemical characterizations of orange oil residues have thus far received little attention, the properties of these materials are consistent with their lipid composition. This lipid composition is reflected in the FTIR spectra of orange oil residues (**Figure 1**), where similarities occur between this spectrum and the IR spectra of long chain hydrocarbons and fatty acids (data not shown). The vibrations between 2800 and 3000 cm^{-1} are dominated by the methylene ν(C−H) asymmetric stretch at 2926 cm^{-1}. This is consistent with the presence of long chain fatty acids, waxes, carotenoids, and phytosterols (*16*). Likewise, the vibration at 1733 cm^{-1} reflects the presence of the carbonyls of phytosterols and fatty acids.

FTIR of PMFs. Yet, a number of the vibrations above 1500 cm^{-1} in the FTIR spectra of oil residues are unaccounted for by long chain fatty acids and hydrocarbons, but these features can be attributed to functional groups of the PMFs. FTIR spectral analyses were made of the six main PMFs in orange oil, and the vibrational energies are summarized in **Table 1**. A few of the key vibrations of flavones, including a number of the PMFs, have been studied previously (*17*−*20*), although the complete infrared (IR) spectra of the PMFs have not been fully characterized. Applicable to the study of the IR spectra of the PMFs are the assigned phenyl ring ν(C═C) and mixed alkyl/aryl methyl ether ν(C−O−C) vibrations of the much simpler molecule, anisole (phenylmethyl ether) (*21*) (**Figure 2**). A comparison of the wavenumbers (cm^{-1}) of the functional groups for anisole and the PMF, sinensetin, is shown in **Table 1**. For anisole, the asymmetric methoxy ν(C−H) stretch occurs at 2835 cm^{-1}, and this vibration is similarly seen in the FTIR spectrum of sinensetin, at 2838 cm^{-1} (**Figure 3**). The ν(C−O−C) asymmetric stretches for the mixed phenyl/methyl ether linkages of anisole occur at 1246 and 1040 cm^{-1} (**Figure 2**). For sinensetin, there are a number of vibrations in this region, and this complexity is likely due to the multiple methoxyl substituents in sinensetin. The intense ν(C═C) vibrations of the phenyl ring of anisole occur at 1600, 1586, and 1497 cm^{-1} (**Figure 2**). These vibrations in the IR spectra of sinensetin are not as readily assigned, although there are multiple vibrations in this region (**Figure 3**), including a sharp vibration at 1515 cm^{-1}. This vibration can be reasonably assigned to the ν(C═C) stretch of the flavone phenyl rings.

Not observed in the anisole FTIR spectrum is the carbonyl stretch of the PMFs, which occur between 1612 and 1652 cm^{-1}

Table 1. Infrared Vibrations of Anisole and Orange Oil Residue PMFs

compound[a]	ν(C–H) methoxy	ν(C═O)	ν(C═C) phenyl	ν(C–O–C)	ν(C–H) in plane
anisole	2835		1600, 1586, 1497, 1467, 1454, 1441	1246, 1040	1172, 1152, 1077, 1020, 994
SIN	2838	1634	1599, 1515, 1486, 1418	1264, 1019	1144, 1117, 1049, 989, 957
NOB	2829	1622	1590, 1516, 1408	1210, 1150	1107, 1076, 1040, 1012, 972
TMS	2835	1633	1600, 1511, 1484, 1456, 1421	1258, 1145, 1021	1179, 1115, 1096, 1042, 981, 948
HMF	2829	1645	1588, 1515, 1463, 1407	1215, 1147	1105, 1081, 1050, 1020, 1004, 980, 962
TAN	2848	1652	1606, 1586, 1512, 1424, 1406	1217, 1155	1104, 1073, 1030, 1014, 1003, 966
QHME	2838	1612	1601, 1514, 1421	1256, 1221, 1147	1121, 1091, 1071, 1009, 992

[a] Abbreviations: SIN, sinensetin; NOB, nobiletin; TMS, tetramethylscutellarein; HMF, heptamethoxyflavone; TAN, tangeretin; QHME, quercetagetin hexamethyl ether.

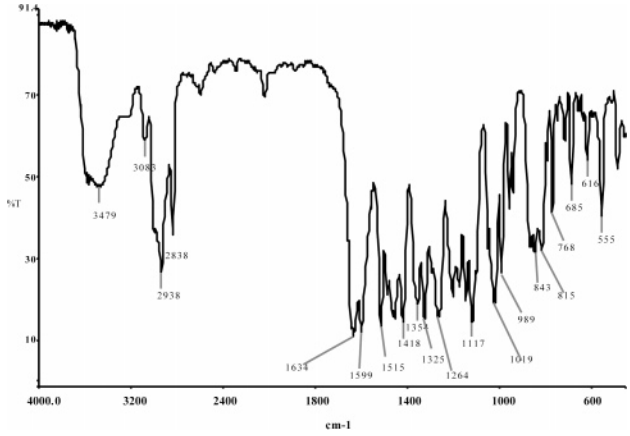

Figure 3. FTIR spectrum of sinensetin. The purified sinensetin standard was dried as a dilute acetone solution on a PTFE IR card.

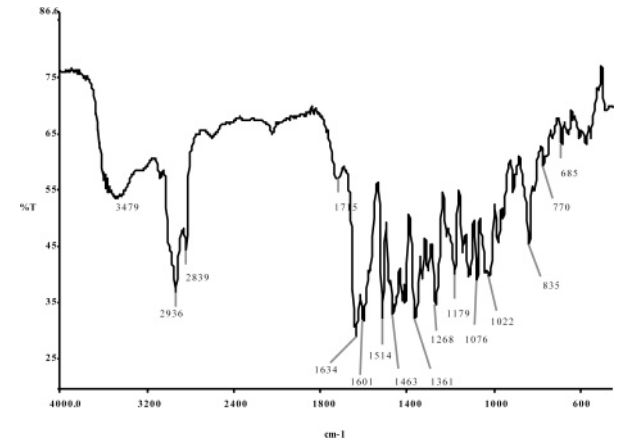

Figure 4. FTIR spectum of the collective PMFs isolated by 95% ethanol extraction and LH20 column chromatography. The spectrum is a summation of eight scans taken with the residue thinly applied to a PTFE IR card.

(**Table 1**). These ν(C═O) vibrations of the PMFs occur at significantly lower energies than for most sterols and polyunsaturated fatty acids, where the carbonyl stretch typically occurs between 1752 and 1710 cm^{-1}. The lower vibrational energies of the flavone carbonyl stretches are the result of decreased bond strengths of the carbonyl bonds in the PMFs due to resonance structures of these compounds (*17*−*20*). Importantly, this shift in the vibrational energies of the PMF carbonyl stretches allows these vibrations in the PMFs to be clearly distinguishable from those of the other non-PMF constituents.

FTIR of PMF and Non-PMF-Enriched Oil Residue Fractions. An oil residue fraction containing the bulk of the PMFs, with only trace non-PMF residue content, was obtained from a series of 95% ethanol extractions and LH20 column chromatography. Analysis by HPLC of the PMF fraction recovered from LH20 chromatography in 95% ethanol showed a PMF content of 76% and a PMF profile nearly identical to the original residue (data not shown). The FTIR spectrum of this PMF-enriched fraction is shown in **Figure 4**. The near absence of other non-PMF constituents is evident by the low intensity of the ν(C═O) vibration at 1724 cm^{-1}. Excellent agreement occurs between this spectrum and that for purified sinensetin (**Figure 3**).

To characterize and assign other vibrations in the FTIR of orange oil residues to the PMFs, the FTIR spectum of the collective PMFs (**Figure 4**) was compared to the FTIR spectrum

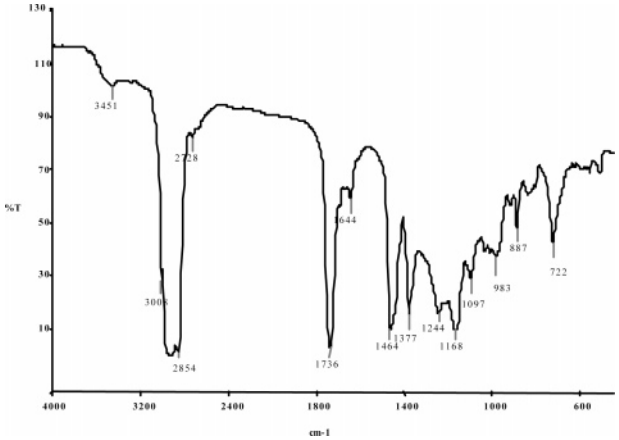

Figure 5. FTIR spectum of the non-PMF residue recovered after 95% ethanol extraction. The spectrum is a summation of eight scans taken with the residue thinly applied to a PTFE IR card.

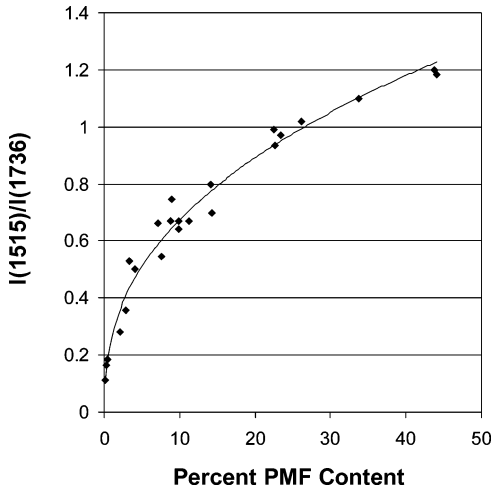

Figure 6. Correlation between the ratios ($I_{1515\ cm^{-1}}/I_{1723\ cm^{-1}}$) and PMF concentrations. The solid line represents the equation $y(I_{1515\ cm^{-1}}/I_{1723\ cm^{-1}}) = x(\text{percent PMF content})^{0.4048}$. The R^2 value for data fit was 0.9788.

of an ethanol-extracted PMF-free residue fraction. This latter material was prepared by the removal of the PMFs from orange oil residues by extensive extraction with 95% ethanol. The remaining ethanol insoluble oil residue was shown by HPLC analysis to contain only a trace PMF content (data not shown). The FTIR spectrum of the PMF-free residue (**Figure 5**) showed the absence of the phenyl ring ν(C═C) vibration at 1514/1515 cm^{-1} and only a very weak peak near the PMF ν(C═O) vibration at 1644 cm^{-1}.

Of significance is the fact that there are no vibrations in the non-PMF residue (**Figure 5**) overlapping with the PMF phenyl ring vibration at 1515 cm^{-1}, nor are there vibrations associated with the PMFs that overlap with the non-PMF carbonyl stretch at 1736 cm^{-1}. This allows the use of the intensity of the phenyl ring vibration at 1515 cm^{-1} as an indicator of the relative PMF content in the orange oil residue. To correct for variations in the thickness of the residue applied to the sample cards, the non-PMF carbonyl stretch at 1736 cm^{-1} was used as an internal standard, and measurements were taken as the ratios of the intensities of the vibrations at 1515 and 1723 cm^{-1}, measured relative to a baseline defined from approximately 2700 to 1800 cm^{-1}. These ratios were measured for a series of oil residue samples from different commercial sources, consisting of distinct oil stocks and consistencies. Samples with low PMF content

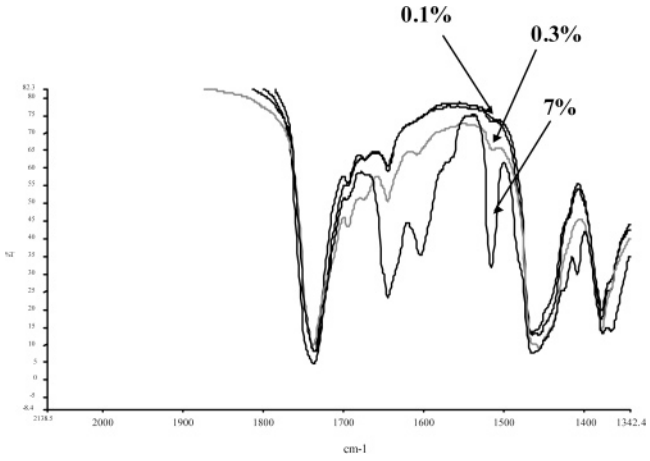

Figure 7. FTIR spectra of phenyl ring ν(C═C) vibration at 1515 cm^{-1} of PMF-free residue (unmarked) and residues containing 0.1, 0.3, and 7% PMF.

tended to be thin oils, while high PMF residues were often thick and contained particulate material. For all samples, the PMF contents were also measured by HPLC. The correlation between the ratios ($I_{1515\ cm^{-1}}/I_{1723\ cm^{-1}}$) and PMF concentrations is shown in **Figure 6**. The most sensitive portion of the correlation curve to PMF concentration occurs at low PMF concentrations. **Figure 7** shows that the lowest level of PMF detectable with this technique is approximately 0.1%.

Although this analysis does not provide information on the levels of individual PMFs, it provides a rapid, solvent-free measurement of the total PMF content in orange oil residues. The 95% ethanol extraction of the PMFs from the bulk of the oil residue provides a rapid and easy enrichment and recovery of the PMF from the oil residues. LH20 size exclusion chromatography provides a means of isolating high percentage PMF material, consisting of relative proportions of the individual PMFs similar to those in the original oil residues.

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