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Fractionation of Orange Peel Phenols in Ultrafiltered Molasses and Mass Balance Studies of Their Antioxidant Levels

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Orange peel molasses, a byproduct of juice production, contains high concentrations of phenols, including numerous flavanone and flavone glycosides, polymethoxylated flavones, hydroxycinnamates, and other miscellaneous phenolic glycosides and amines. Extensive fractionation of these phenols was achieved by adsorption, ion exchange, and size exclusion chromatography. Size exclusion chromatography effectively separated the different classes of flavonoids in ultrafiltered molasses, including the polymethoxylated flavones, flavanone-*O*-trisaccharides, flavanone- and flavone-*O*-disaccharides, and, finally, flavone-*C*-glycosides. Mass spectral analysis of the early-eluting flavonoid fractions off the size exclusion column revealed a broad collection of minor-occurring flavone glycosides, which included, in part, glycosides of limocitrin, limocitrol, and chrysoeriol. Most hydroxycinnamates in the molasses were recovered by ion exchange chromatography, which also facilitated the recovery of fractions containing many other miscellaneous phenols. Total antioxidant levels and total phenolic contents were measured for the separate categories of phenols in the molasses. Inhibition of the superoxide anion reduction of nitroblue tetrazolium showed that a significant amount of the total antioxidant activity in orange peel molasses was attributable to minor-occurring flavones. The miscellaneous phenolic-containing fractions, in which a large portion of the total phenolic content in molasses occurred, also constituted a major portion of the total antioxidants in ultrafiltered molasses.

KEYWORDS: Flavonoids; hydroxycinnamates; flavone glycosides; antioxidants; citrus byproducts; peel

INTRODUCTION

Citrus flavonoids have been extensively studied for their biological actions (*1*), and as early as 1936 Szent-Györgyi and Ruszynák reported the capillary protective effects of certain citrus flavonoids in conjunction with ascorbic acid (*2*). These early findings have been widely confirmed, and most pharmacological actions of citrus flavonoids are now thought to be linked to their inhibition of enzymes involved in inflammation. Phosphodiesterases that regulate cytokine production by activated human monocytes are inhibited by the lipophilic polymethoxylated flavones (*3*), whereas the hydroxylated flavanone and flavone glycosides generally inhibit components of inflammation responsive to antioxidants (*4*−*7*). Studies have shown close correlations between the antioxidant actions of flavonoids and the inhibition of lipoxygenase (*8*, *9*), an important target of anti-inflammation therapeutics.

The importance of antioxidation in the biological actions of citrus flavonoids makes this a critical activity to evaluate. Currently, there is interest in the recovery of citrus phenols as functional food ingredients with targeted pharmacological endpoints. Juice-processing byproducts are promising sources of these compounds because of the high concentrations at which many of these phenols occur (*10*, *11*). In this study we show that composite collections of minor phenolic constituents in ultrafiltered molasses, which share common structural and chromatographic properties, occur at levels comparable to the main flavonoids, and although these latter compounds have been studied in peel byproducts, there has been little definitive evaluation of the total antioxidant activities of these groups of compounds and of the other groups of minor-occurring compounds in orange peel molasses. This study provides a mass balance of the phenolic content of the main classes of phenols in orange peel ultrafiltered molasses as well as a mass balance of their antioxidant levels. These analyses show that much of the total phenolic content and antioxidant activity of citrus byproducts can be attributed to the numerous minor phenolic compounds in the peel.

MATERIALS AND METHODS

Sample Preparation. Concentrated early/mid season (February) orange peel molasses was obtained from a local citrus-processing plant in Florida. The molasses was diluted 1:4 with water, allowed to settle overnight at 4 °C, and centrifuged at 10000g_{max} for 60 min to remove the bulk of undissolved solids. The partially clarified molasses was filtered through a grade 161 glass fiber filter (Scientific Specialities

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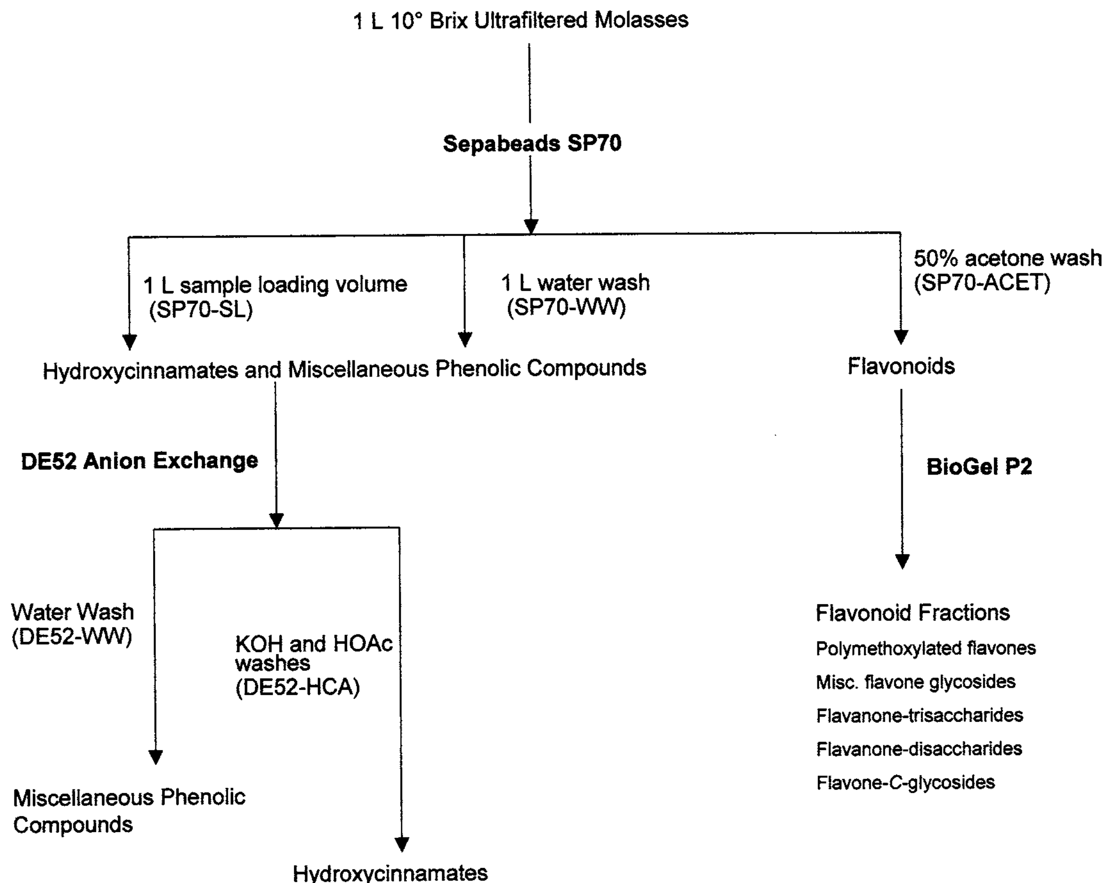

Figure 1. Fractionation scheme including SP70, DE52, and P2 column chromatography.

Group, Mt. Holly Springs, PA). Clarified molasses was ultrafiltered through a Romicon model HF4 hollow fiber cartridge ultrafiltration system (Romicon, Inc., Woburn, MA) with a 10000 Da molecular weight cutoff. The ultrafiltered molasses was adjusted to 10 °Brix with deionized water and to pH 3.0 with phosphoric acid.

Flavonoid Fractionation. Ultrafiltered molasses (1 L) was passed through a Sepabeads SP70 column (1.5 cm × 29 cm) (Supelco, Bellefonte, PA) equilibrated with water. The flow rate was 3 mL/min to ensure optimal bonding to the resin (*12*). The sample-loaded column was subsequently washed with 1 L of water. The initial 1 L sample loading fraction (SP70-SL) and subsequent water wash fraction (SP70-WW) were separately rotary evaporated to ~100 mL (**Figure 1**). The compounds retained on the column were eluted with 50% acetone (500 mL). The 50% acetone eluant (SP70-ACET) was rotary evaporated to a minimal volume and adjusted to 15% ethanol. Half of this fraction was applied to a Bio-Gel P2 column (5 cm × 82 cm) (Bio-Rad, Hercules, CA) equilibrated and run with 15% ethanol. Column fractions (20 mL) were collected, and every 10th fraction was analyzed by HPLC.

The SP70-SL and SP70-WW fractions were run through a DE52 cellulose (Whatman, Clifton, NJ) anion exchange column (2.5 cm × 25 cm), and the hydroxycinnamates were recovered as described previously (*11*). The sample loading and 500 mL water wash fractions obtained off the DE52 column prior to the elution of the hydroxycinnamates were combined to form DE52-WW.

HPLC Methods. Measurements of citrus flavonoids were made with high-pressure liquid chromatography (HPLC). The polymethoxylated flavones were analyzed with an Alltech Alltima C8 5 μm analytical column (4.6 mm × 100 mm). Elution conditions included a two-solvent gradient composed initially of 10 mM phosphoric acid/acetonitrile (90:10, v/v) and increased in a linear gradient to 60:40 (v/v) over 15 min. A final composition of 10 mM phosphoric acid/acetonitrile (55:45, v/v) was achieved by a subsequent linear gradient over 35 min using a flow rate of 0.75 mL min^{-1}. Flavonoid glycosides were analyzed using the same column, using 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) 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}. Instrumentation included an Agilent 1100 quaternary pump with a 1050 photodiode array detector and a 1100 chromatography workstation. The hydroxycinnamates were analyzed with HPLC methods described previously (*11*).

Flavonoid Hydrolysis. Samples of P2 column fractions were initially extracted three times with equal volumes of chloroform to remove flavonoid aglycons, primarily the polymethoxylated flavones, originally in the sample. The remaining glycosidic flavonoids in the aqueous phase were hydrolyzed by refluxing for 1 h in 1 M hydrochloric acid. The solutions were cooled and extracted with chloroform to recover the hydrolyzed flavonoid aglycons. The chloroform extracts were vacuum-dried and analyzed by HPLC-MS. Authentic standards of limocitrin, limocitrol, and chrysoeriol were corun with the hydrolyzed samples to determine overlap between the sample peaks and the standards.

Liquid Chromatography−Electrospray Ionization−Mass Spectral Analysis. Mass spectral (MS) analysis of the flavonoids was carried out with a Waters ZQ single-quadrupole mass spectrometer equipped with a Waters 2695 HPLC pump and a Waters 996 photodiode array (PDA) detector (Waters Corp., Milford, MA). PDA detection was monitored between 230 and 600 nm. Data handling was done with MassLynx software ver. 3.5 (Micromass, Division of Waters Corp., Beverly MA). The flavonoids were analyzed with the MS detector using an Alltech Alltima C8 5 μm analytical column (4.6 mm i.d. × 100 mm). Postcolumn split to PDA and mass ZQ detector was 10:1. MS parameters were as follows: ionization mode, ES+; capillary voltage, 3.0 kV; extractor voltage, 5 V; source temperature, 100 °C; desolvation temperature, 225 °C; desolvation N_2 flow, 465 L h^{-1}; cone N_2 flow, 70 L h^{-1}; scan range, 150−900 amu; scan rate, 1 scan/s; cone voltages, 20, 40, and 60 eV.

Measurement of Total Phenols. Measurements of total phenols were done with the Folin−Ciocalteu assay as described by Sellappan et al. (*13*) with modifications. Dried samples (~30 mg) were accurately weighed and dissolved in 5 mL of dimethyl sulfoxide (DMSO). Aliquots (10−40 μL) of dissolved samples were added to 0.375 μL of sodium carbonate solution (100 g/500 mL of H_2O) and shaken for 2 min. Folin−

Table 1. Flavonoid Composition of Phenolic Fractions (**Figure 1**) Obtained from SP70 Sepabeads Chromatography of Ultrafiltered Molasses[a]

column fraction	SIN	QHME	NOB	TMS	HMF	TAN	ISR	HSP	NR	HSPTS	NR4′G	6,8DCGAP	CON
SP70-ACET	26.9 ± 0.9	5.2 ± 0.4	31.2 ± 2.3	6.8 ± 1.1	11.5 ± 1.2	2.7 ± 1.0	57.5 ± 0.9	437 ± 4.0	245 ± 8.1	209 ± 6.9	172 ± 25.6	30.1 ± 14.3	7.2 ± 0.9
SP70-WW	0	0	0	0	0	0	0	16.5 ± 3.2	20.9 ± 3.3	28.9 ± 3.2	40.6 ± 6.1	14.9 ± 1.1	6.2 ± 2.2
SP70-SL	0	0	0	0	0	0	0	5.3 ± 1.8	7.7 ± 2.1	18.4 ± 5.7	33.7 ± 10.1	14.0 ± 6.5	2.3

[a] Abbreviations: SIN, sinensetin; QHME, quercetagetin hexamethyl ether; NOB, nobiletin; TMS, tetramethylscutellarein; HMF, heptamethoxyflavone; TAN, tangeretin; ISR, isosakuranetin rutinoside; HSP, hesperidin; NR, narirutin; HSPTS, hesperetin trisaccharide; NR4′G, narirutin 4′-glucoside; 6,8DCGAP, 6,8-di-*C*-glucosylapigenin; CON, coniferin. Values are milligrams and are averages of analyses of triplicate replicates of 1 L of ultrafiltered molasses fractionation.

Ciocalteu phenol reagent (0.125 mL of 2.0 N: Sigma F-9252) was added, followed by the addition of 1.0 mL of deionized water. Each tube was brought up to a total of 100 μL of DMSO. The reactions were shaken in the dark for 2 h. Absorbance readings were taken at 725 nm. Calibration curves were made with gallic acid (0.241 μg/μL) and narirutin (2.13 μg/μL). From absorbance readings the micrograms of gallic acid equivalents and micrograms of narirutin equivalents per microgram of sample were calculated. The results are averages of three replicates in the sample series.

Measurement of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity. Measurement of DPPH radical scavenging activity was performed as described by Yamaguchi et al. (*14*) with minor modifications. DPPH (1 mL of 1 mM in 90% ethanol) was combined with 10−40 μL of sample. The reactions were shaken at room temperature under dark conditions and were run to completion for 3 h. The reduction of absorbance at 517 nm was monitored as the DPPH radical scavenging activity. Standard curves were run with gallic acid, and reduction levels of the samples were converted to equivalents of gallic acid (eqs 1 and 2). Reactions run with samples dissolved in DMSO were accompanied with gallic acid standard reactions also run with the same amounts of DMSO.

$$[(A_{\text{initial}} - A_{3\text{h}})/\text{mg of sample}] \times [\text{mg of GA}/(A_{\text{initial}} - A_{3\text{h}})] = \text{DPPH reduction of gallic acid relative to sample (mg of GA/mg of sample)} \quad (1)$$

$$(\text{mg of GA/mg of sample}) \times \text{total mg of sample} = \text{total DPPH reducing equivalents (mg of GA)} \quad (2)$$

Measurement of Superoxide Anion Scavenging Activity. Superoxide radical formation was monitored by the nonenzymatic reduction of nitroblue tetrazolium (NBT). Assays were performed as described by Robak and Gryglewsi (*15*) with minor modifications. NBT (0.50 mL of 156 μM in 0.1 M potassium phosphate, pH 6.8) was combined with NADH (0.50 mL of 468 μM in deionized water) and 1−20 μL of diluted phenolic fraction. The reaction was started by adding 20 μL of phenazine methosulfate (PMS) (240 μM). The PMS stock solution was prepared and kept at ice temperature. New stock solutions were made daily. The reactions were monitored with a Shimadzu UV-2401-PC spectrophotometer for 2.0 min. Concentrations (IC_{50}) of samples that inhibited 50% of the NBT reduction were measured. All IC_{50} values were corrected to a standard change in total absorbance of 1.0 at 560 nm for the difference in the final absorbance of the reaction without phenolic sample and with sufficient phenolic sample to achieve complete NBT reduction inhibition. A unit of inhibitory activity was the inhibition of 50% NBT reduction, corresponding to the inhibition at a sample concentration at the IC_{50} value. Specific activities were determined as 1 unit/IC_{50}, and total inhibition activities were calculated as 1 unit/$IC_{50(\text{mg of sample})}$ × total dry weight of sample (mg).

RESULTS

Chromatographic Fractionation of Citrus Phenolics in Ultrafiltered Molasses. *SP70 and DE52 Anion Exchange Chromatography.* Fractionation of the phenols in orange peel molasses was achieved by adsorption, anion exchange, and size exclusion chromatography (**Figure 1**). Initial fractionation involved chromatography on SP70 Sepabeads resin, where the initial-eluting sample loading (SP70-SL) and water wash (SP70-WW) fractions from this column primarily contained hydroxycinnamates and numerous other miscellaneous phenolic compounds (discussed below). In contrast, the acetone/water (1:1, v/v) eluted material (SP70-ACET) contained the bulk of the flavonoids in ultrafiltered molasses, particularly the lipophilic polymethoxylated flavones (84.3 mg total) (**Table 1**). Nobiletin, heptamethoxyflavone, and sinensetin comprised 83% of the total polymethoxylated flavone content. The flavonoid glycosides were also largely recovered in this fraction (1.212 g total), particularly hesperidin and narirutin, whereas the more polar flavonoids, that is, narirutin 4′-glucoside, hesperetin trisaccharide, 6,8-di-*C*-glucosylapigenin, and the phenolic glycoside, coniferin, also occurred in increasing amounts in the SP70-WW and SP70-SL fractions. Further fractionation of the phenols in SP70-SL and SP70-WW was achieved by ion exchange chromatography (**Figure 1**). Major portions of the hydroxycinnamates in ultrafiltered molasses bind to DE52 anion exchange resin (*11*), and in the present fractionation scheme, these compounds were subsequently eluted in the DE52-HCA fraction by sequential washes of the DE52 resin with 0.04 M KOH and 10% acetic acid. In contrast, numerous other UV-absorbing compounds, including coniferin and phlorin, failed to bind to the DE52 anion exchange resin and were detected in the DE52-WW fraction. **Table 2** shows that 84.8 mg of hydrolyzable ferulic acid and 28.9 mg of *p*-coumaric acid occurred in DE52-HCA, whereas DE52-WW contained far lower amounts. SP70-ACET contained 64.0 mg of ferulic acid, or ∼39% of the total hydrolyzable ferulic acid in ultrafiltered molasses.

Table 2. Levels of Hydrolyzable Hydroxycinnamic Acids in SP70 and DE52 Column Fractions[a]

sample	*p*-coumaric acid (mg)	ferulic acid (mg)
SP0-ACET	9.5 ± 4.0	64.0 ± 23.4
DE52-HCA	28.9 ± 2.1	84.8 ± 7.4
DE52-WW	9.8 ± 2.1	15.5 ± 2.9

[a] Column fractions are defined in **Figure 1**. Values are averages of triplicate replicates of the SP70 and DE52 chromatographic fractionations of 1 L of 10° Brix ultrafiltered molasses.

P2 Size Exclusion Chromatography. Flavonoids in SP70-ACET were further fractionated by P2 size exclusion chromatography (**Figure 2**). The first material off the P2 column (fraction A) showed an absence of flavonoids, whereas some of the individual peaks in the HPLC chromatogram (**Figure 3A**) of this material exhibited UV spectra similar to those of hydroxycinnamates. Little else is known of the chemical composition of this initial column fraction, although further studies showed this fraction to possess antioxidant activity (discussed below). The next P2 column fractions (B and C) contained polymethoxylated flavones and compounds tentatively identified as flavone glycosides on the basis of their characteristic flavone UV spectra. These compounds occurred in the HPLC chromatograms as the broad collections of compounds eluting prior to the polymethoxylated flavones (**Figure 3B**,**C**),

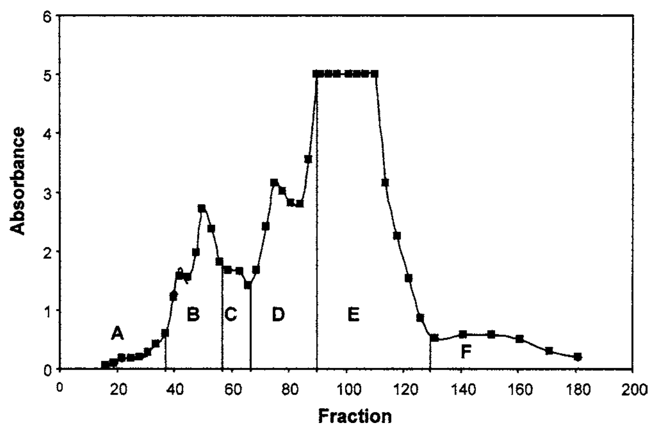

Figure 2. Bio-Gel P2 column fractionation of flavonoids in SP70-ACET. Acetone-eluted flavonoids were dissolved in 50 mL of 15% ethanol and applied to a P2 column (5 cm × 80 cm), which was run at 1 mL min^{-1} with 15% ethanol. Groups of pooled fractions included (A) initial-eluting compounds (16–38); B, PMFs and methoxylated flavone glycosides (39–56); C, PMFs and miscellaneous flavones (57–69); D, flavanone trisaccharides (70–92); E, flavanone disaccharides (93–130); and F, miscellaneous late-eluting flavones (131–180).

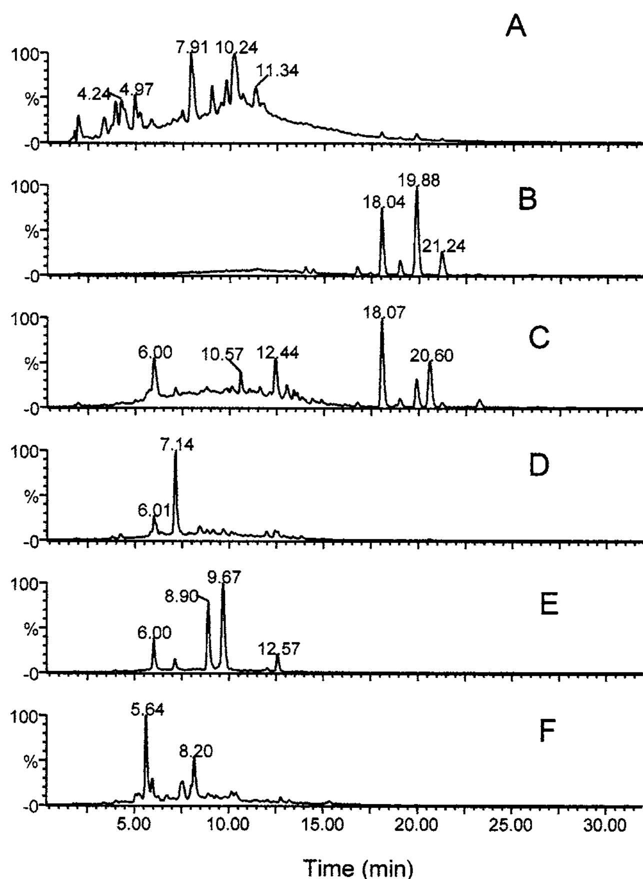

Figure 3. HPLC chromatograms (330 nm) of pooled fractions from P2 size exclusion chromatography. Fractions A–F correspond to the same fractions listed in **Figure 2**. In fractions B and C the polymethoxylated flavones eluted between 18.0 and 24 min. In fraction D the flavanone trisaccharides, narirutin 4′-glucoside and hesperetin trisaccharide, eluted at 6.01 and 7.14 min, respectively. In fraction E the flavanone disaccharides narirutin, hesperidin, and isosakuranetin rutinoside eluted at 8.9, 9.67, and 12.57 min, respectively. The main compound in fraction F, 6,8-di-*C*-glucosylapigenin, eluted at 5.64 min.

and due to the flavone-like UV spectra of these compounds, they were distinguishable from the compounds in the initial fraction A. The flavanone trisaccharides, mainly hesperetin trisaccharide and narirutin 4′-glucoside, eluted in the subsequent fraction D, followed by the flavanone disaccharides, hesperidin and narirutin (fraction E). The HPLC chromatograms of these fractions are illustrated in panels D and E of **Figure 3**, respectively. The final P2 column fraction (F) contained 6,8-di-*C*-glucosylapigenin as the main constituent **(Figure 3F)**, along with numerous other minor-occurring compounds, many of which exhibited hydroxycinnamate-like UV spectra.

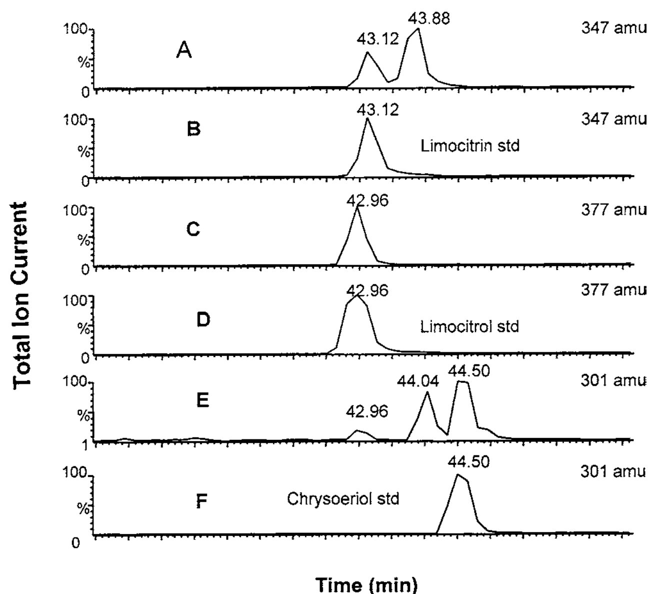

Figure 4. HPLC-MS of aglycons detected in acid-hydrolyzed SP70-ACET and their standard compounds. Total ion current chromatograms B, D, and F are of limocitrin, limocitrol, and chrysoeriol standards at 347, 377, and 301 amu, respectively. Total ion current chromatograms A, C, and E are measured with the hydrolyzed aglycon products at 347, 377, and 301 amu, respectively.

Initial characterizations of the minor constituents in fractions Band C provided evidence that these fractions contain, in part, flavone glycosides and, in particular, glycosides of partially methoxylated flavones. The flavone components of these compounds were analyzed by their chromatographic properties relative to purified standards and by the compounds' UV and MS properties. The P2 column fractions B and C were initially extracted with chloroform to remove flavone aglycons originally present in these samples. Analyses of these initial extracts showed no evidence of flavone aglycons other than the polymethoxylated flavones. The extracted fractions were then acid hydrolyzed and re-extracted with chloroform to recover newly released flavone aglycons. The remaining unhydrolyzed glycosides and other polar compounds remained in the aqueous phase. HPLC-MS analysis of the hydrolysis products showed new flavone aglycons with $[M + H]^+$ ions at 301, 347, and 377 atomic mass units (amu), indicating the possible presence of the partially methoxylated flavones chrysoeriol (5,7,4′-trihydroxy-3′-methoxyflavone), limocitrin (3,5,7,4′-tetrahydroxy-8,3′-dimethoxyflavone), and limocitrol (3,5,7,4′-tetrahydroxy-6,8,3′-trimethoxyflavone), respectively. Analysis of the mass spectral total ion currents at these molecular weights showed that these newly formed compounds, chromatographed by C8 reversed-phase HPLC (**Figure 4A**,**C**,**E**), exhibited exact peak overlaps with authentic standards (**Figure 4B**,**D**,**F**). The UV spectra and MS of the newly formed compounds also matched those of the standards. Additionally, mass spectral analysis of the original nonhydrolyzed column fractions showed the presence of compounds with prominent fragment ions at 301, 347,

Table 3. Phenolic Content and Total DPPH Radical Scavenging Capacity of Fractions from SP70 and DE–52 Column Chromatography of Orange Peel Ultrafiltered Molasses[a]

sample	phenolic content (mg of gallic acid equiv)	DPPH scavenging capacity (mg of gallic acid equiv)
SP70-SL	1308 ± 124	117 ± 3
SP70-WW	303 ± 48	36 ± 6
SP70-ACET	1193 ± 146	131 ± 18
DE52-HCA	414 ± 64	18 ± 2
DE52-WW	1289 ± 132	129 ± 25

[a] Values are averages of three series of chromatographic runs. Folin–Ciocalteu assays were run in triplicate.

and 377 amu, with molecular ions $[M + H]^+$ of these compounds attributable to glucosides or (glucose + rhamnose) species of these putative flavones (data not shown).

Mass Balance of the Phenolic Contents and Antioxidant Activities of the Fractions Recovered from Ultrafiltered Molasses. The phenolic contents (measured as milligrams of gallic acid equivalents) of SP70-SL, SP70-WW, and SP70-ACET, along with the DE52-HCA and DE52-WW column fractions, are listed in **Table 3**. Slightly more than half (57%) of the total phenolic content in molasses occurred in SP70-SL and SP70-WW, whereas only 43% occurred in SP70-ACET. When the SP70-SL and SP70-WW fractions were combined and run through the DE52 anion exchange column, the bulk of the phenolic content (1289 mg) occurred in DE52-WW rather than in DE52-HCA (414 mg); the latter represented only 15% of the total phenolic content of ultrafiltered molasses.

Total DPPH radical scavenging capacities of the ultrafiltered molasses phenolic fractions were also measured as milligrams of gallic acid equivalents (**Table 3**). The DPPH radical scavenging capacities of the SP70 and DE52 column fractions closely paralleled their total phenolic contents. DE52-HCA contained 18 mg of gallic acid equiv of DPPH radical scavenging capacity, whereas DE52-WW contained 129 mg of gallic acid equiv. These results show that the total DPPH radical scavenging capacities of the DE52 column fractions and, thus, of the combined SP70-SL and SP70-WW fractions were not mainly attributable to the hydroxycinnamates, but rather to other compounds in these fractions. The SP70-ACET and DE52-HCA fractions had a total of only 49% of the overall DPPH radical scavenging capacity in the ultrafiltered molasses.

The dry weights, total phenolic contents, and DPPH radical scavenging activities of the flavonoid fractions obtained from P2 size exclusion chromatography of SP70-ACET are listed in **Table 4**. The DPPH radical scavenging activities of the different column fractions were determined relative to gallic acid; thus, they are listed as micrograms of gallic acid per microgram of sample. The low values for this ratio in **Table 4** reflect the stronger radical scavenging activity of gallic acid compared to the P2 column fractions. From these ratios and the total dry weights of the column fractions, total DPPH radical scavenging capacities (milligrams of gallic acid equivalents) of the separate column fractions were determined. The initial-eluting P2 column fraction A exhibited the lowest total DPPH radical scavenging activity (3.37 mg), whereas the combined polymethoxylated flavone and miscellaneous flavone fractions B and C had total DPPH reducing equivalents (20.50 mg of gallic acid) similar to the flavanone-rich trisaccharide (D) (13.80 mg) and disaccharide (E) (21.83 mg of gallic acid) fractions. The total phenolic contents, expressed as milligrams of gallic acid equivalents, of the P2 column fractions are also listed in **Table 4**. The Folin–Ciocalteu assay gave micrograms of dry weight (DW) of sample per micrograms of gallic acid ratios between 0.27 and 0.49 for the P2 column fractions, whereas when narirutin was used as the reference compound, these values ranged from 0.64 to 1.13 (data not shown).

The antioxidant properties of the phenolic fractions recovered from orange peel ultrafiltered molasses were further evaluated by their inhibition of nonenzymatic reduction of NBT by superoxide. Activity levels were calculated in terms of inhibitor concentrations (IC_{50}) at which 50% inhibition of NBT reduction occurred. The initial-eluting fraction A from the P2 size exclusion column exhibited the lowest IC_{50} value (highest inhibitory activity) relative to the other P2 column fractions (**Table 5**). IC_{50} values for these other fractions ranged between 42 and 86 ppm. The total units of inhibitory activity were highest for the flavanone fractions D and E, although the total inhibitory activities for the other P2 column fractions were all similar in magnitude. There were 36854 total inhibitory units contained in the P2 column fractions, representing the SP70-ACET fraction. DE52-WW contained 31199 units of inhibition of superoxide anion reduction of NBT, whereas DE52-HCA contained 15452 units. These relative amounts are in agreement with the earlier DPPH scavenging activities for these column fractions.

DISCUSSION

Significant emphasis has been placed on the development of value-added products from agricultural waste, including the byproducts of citrus processing, which constitute approximately half of the entire processed crop. Research on the processing waste of a number of crops has focused on the production of value-added food ingredients with targeted biological effects on human health and nutrition. Accordingly, one possible means of creating additional economic value for citrus byproducts is the recovery and use of the abundant antioxidants in these materials as functional food ingredients. The antioxidant activities of citrus juice and allied byproducts have been previously studied, including analyses of citrus oils (*16*), seeds and peel (*17*), and juice (*18*). Dried orange peel contains up to 5%

Table 4. Dry Weights and Phenolic Content of Fractions Recovered from 1 L of 10°Brix Orange Peel Ultrafiltered Molasses[a]

fraction	DPPH reducing equivalents (μg of GA/μg of sample)	dry wt (mg)	total DPPH reducing (mg of GA equiv)	μg of GA/μg of sample	total phenolic content (mg of GA equiv)
A: initial-eluting fraction	0.0150 ± 0.001	225 ± 51	3.37	0.278 ± 0.023	62
B–C: polymethoxylated flavones and miscellaneous flavones	0.0225 ± 0.002	911 ± 30	20.5	0.315 ± 0.040	286
D: flavanone trisaccharides	0.0205 ± 0.004	671 ± 90	13.8	0.435 ± 0.067	291
E: flavanone disaccharides	0.0211 ± 0.0017	1035	21.83	0.494 ± 0.042	511

[a] Total phenolic contents, expressed as mg of GA equivalents, were calculated as (sample dry weight × μg of GA/μg of sample) = mg of GA equivalents. Values are averages of analyses of two replicate P2 chromatographic runs.

Table 5. IC_{50} Values of Inhibition by Flavonoid Fractions Obtained from P2 Column Chromatography of Nonenzymzatic NBT Reduction

fraction	IC_{50} (ppm)	total inhibition units
A: initial-eluting material	20.5	7058
B: PMFs and miscellaneous flavones	42.6	3350
C: PMFs and miscellaneous phenols	42.0	5629
D: flavanone trisaccharides	58.8	9734
E: flavanone disaccharides	86.4	6486
F: end run, miscellaneous flavones and hydroxycinnamates	40.3	4597

hesperidin and other flavanone glycosides (*10*), and these polyphenols have been previously considered to be major antioxidants in the peel. Yet, a study of the contribution of peel phenols, including the major flavanone glycosides, to the total antioxidant activities in peel showed no clear relationship between flavanone content and antioxidant activity (*17*). Flavanone glycosides typically accounted for only minor portions of the total antioxidants.

The study of phenols in citrus fruit and processing byproducts has typically focused on the abundant hydroxycinnamates and flavonoids, the latter consisting of flavanone and flavone glycosides and the polymethoxylated flavones (*10*, *11*). Although the flavonoids in citrus have been extensively studied (*19*), there are still numerous minor-occurring compounds that remain uncharacterized. In our present study, the chromatographic separations of the flavonoids in ultrafiltered molasses on P2 size exclusion resin provided excellent recoveries of several newly detected groups of minor-occurring compounds, which were shown by HPLC-PDA and HPLC-MS to contain flavone glycosides and, in certain cases, hydroxycinnamates. HPLC-MS analyses of a number of compounds in these groups confirmed their glycoside structures by the occurrence of molecular mass ions consistent with the presence of either glucose or glucose + rhamnose moieties of flavones with $[M + H]^+$ ions of 301, 347, and 377 amu. These latter fragment ions were subsequently shown to be due to chrysoeriol, limocitrin, and limocitrol. The presence of these compounds was confirmed by the overlaps of authentic samples of these flavones with aglycons obtained after sample hydrolysis. Exact matches were also observed with the UV and mass spectra of the compounds in the hydrolyzed samples and the authentic standards. Flavone glycosides of chrysoeriol, limocitrin, and limocitrol have been reported earlier in several *Citrus* species (*20*−*22*), but have not been reported in *C. sinensis*.

As shown in **Table 4**, the sets of compounds containing these minor-occurring flavones have nearly the same combined phenolic content and DPPH radical scavenging activity as the main flavanone glycosides, narirutin and hesperidin. This suggests that these compounds collectively play important roles in the antioxidant activities of citrus byproducts. In addition to antioxidation, other beneficial biological activities have been observed with similar flavone glycosides (*7*, *23*−*26*), and it is anticipated that these sets of compounds in ultrafiltered molasses may also exhibit similar properties and, thus, attract attention as new nutraceuticals or specialty food ingredients.

Hydroxycinnamates and the numerous other miscellaneous phenols also constitute important sets of phenols in the ultrafiltered molasses. Hydroxycinnamates in citrus largely occur as hydrolyzable conjugates (*27*), where a small number of polar feruloyl and courmaroyl *O*-glucuronides and neutral sugar esters have thus far been identified (*28*, *29*). The fractionation of the phenols in orange peel molasses confirms the polar nature of many of the hydroxycinnamates, when these compounds failed to bind to the hydrophobic SP70 resin, and, thus, occurred in the sample loading and water wash fractions.

The chromatographic separations of the polar hydroxycinnamates in DE52-HCA led to the further recovery of a fraction containing numerous other miscellaneous polar phenols, containing, in part, coniferin and phlorin. Although this fraction remains largely uncharacterized, the mass balance measurements showed that this fraction contained the largest portions of phenols (57%) and antioxidants (51% total DPPH radical scavenging activity) in ultrafiltered molasses. The hydroxycinnamates contributed only minor portions to the total phenolic content and antioxidant activity for the phenols in molasses (**Table 3**). The results in **Table 3** also illustrate the close correlations in this study between the total phenolic content and total DPPH reduction by the fractionated citrus phenols.

Similar distributions of total antioxidant activities for the different fractions of phenols in ultrafiltered molasses were observed for the assays measuring the inhibition of superoxide anion reduction of NBT, in which the miscellaneous phenol fraction (DE52-WW) contained nearly half of the total NBT inhibition activity. For this assay, the original flavonoid fraction, SP70-ACET, contained only 44% of the total antioxidant activity levels. High inhibitory activity levels were observed for the P2 column fractions, including the earliest-eluting P2 column fraction A, and for the broad collections of flavone glycosides in fractions B and C.

In conclusion, the results of this study provide new insights into the chemical composition and relative abundance of many of the structurally diverse phenols in orange peel and orange peel ultrafiltered molasses. These fractionated phenols are likely to act in foods as important radical scavengers, protecting against damaging biological oxidative stress. The phenolic content and antioxidant mass balances indicate that the preponderance of these protective phenols occur as miscellaneous polar compounds, the compositions of which are still largely unknown. These studies extend to complex sets of minor-occurring flavones fractionated by P2 size exclusion chromatography. Initial characterizations of these minor-occurring flavonoids provide evidence of partially methoxylated flavones, intermediate in structure and putative biological activities between the polar hydroxylated flavone glycosides and the lipophilic polymethxylated flavones. The abundance of these compounds, as well as of the other fractionated phenols, presents opportunities for the use of orange peel and peel extracts as future nutraceuticals and health-related ingredients for the food industry.

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