Phenols in Citrus Peel Byproducts. Concentrations of Hydroxycinnamates and Polymethoxylated Flavones in Citrus Peel Molasses

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In addition to the main flavanone glycosides (i.e., hesperidin and naringin) in citrus peel, polymethoxylated flavones and numerous hydroxycinnamates also occur and are major phenolic constituents of the molasses byproduct generated from fruit processing. Although a small number of the hydroxycinnamates in citrus occur as amides, most occur as esters and are susceptible to alkaline hydrolysis. This susceptibility to alkaline hydrolysis was used in measuring the concentrations of hydroxycinnamates in citrus peel molasses. The highest concentrations of hydroxycinnamates occurred in molasses of orange [*C. sinensis* (L.) Osbeck] and tangerine (*C. reticulata* Blanco.) compared to grapefruit (*C. paradisi* Macf.) and lemon [*C. limon* (L.) Burm.]. Concentrations of two phenolic glucosides, phlorin (phloroglucinol-*β*-*O*-glucoside) and coniferin (coniferyl alcohol-4-*β*-*O*-glucoside), were also measured. Measurements of the polymethoxylated flavones in molasses from several tangerine and orange varieties showed that these compounds occurred in the highest amounts in Dancy tangerine, whereas samples from two other tangerine molasses contained significantly lower levels, similar to those in the molasses samples from late- and early/mid-season oranges.

Keywords: *Orange; grapefruit; lemon; tangerine; Rutaceae; flavonoid; hydroxycinnamic acid*

INTRODUCTION

Orange processing in the United States produces ∼700000 tons of peel byproduct solids annually (*1*). An intermediate step in peel processing is the production of molasses, during which the peel is limed and pressed and the resulting press liquor is evaporated to at least 35.5 °Brix. As a result, high concentrations of many of the secondary metabolites in citrus occur in the peel molasses (*2*, *3*). Among these compounds are flavonoids and limonoids, described to have numerous potential beneficial biological actions in humans (*4*−*7*). Although the main phenolic constituents of citrus peel are flavanone and flavone glycosides (Figure 1), there are also numerous hydroxycinnamates, coumarins, psoralens, and polymethoxylated flavones (*8*−*13*).

Although the biological actions of a number of common citrus flavonoids (e.g., hesperidin, diosmin, naringin, and tangeretin) have been extensively studied in vitro and in vivo, much less attention has been given to the potential biological activities of the hydroxycinnamates and of the numerous other minor flavonoids in citrus. However, hydroxycinnamates in other food crops have been widely reported to exhibit potentially beneficial actions, including their roles as antioxidants (*14*−*17*), anticancer agents (*18*−*20*), and antimicrobial agents (*21*−*23*).

The polymethoxylated flavones in citrus have also been shown to exhibit anticancer effects against human cancer cell lines as well as anti-inflammatory and cardioprotective actions (*4*, *5*). These beneficial actions suggest new value-added uses for these compounds as nutraceuticals and specialty ingredients, and studies of the occurrence of the polymethoxylated flavones in byproducts generated from citrus fruit processing are currently of considerable interest to the citrus industry. Previously, we reported the concentrations of the main flavanone glycosides in orange peel molasses (*2*). In the current study the levels of additional phenolic constituents in citrus peel and peel molasses are measured, with emphasis on the polymethoxylated flavones and hydroxycinnamates. Measurements of the hydroxycinnamates were based on the total levels of hydroxycinnamic acids occurring in the peel and molasses following alkaline hydrolysis.

MATERIALS AND METHODS

Sample Preparation. Samples of Valencia and early to mid-season orange molasses were obtained in January and May 2000, respectively, from citrus processing plants in central Florida. Lemon peel molasses was obtained from a processing plant in California in 1998 and stored frozen. Preparation of orange peel molasses for ultrafiltration involved dilution of 4 L of 65 °Brix molasses with 4 L of water followed by centrifugation at $10000g_{max}$ for 30 min to remove undissolved solids. The partially clarified molasses was filtered through grade 161 glass fiber filters (Scientific Specialities 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.

Dancy, Clementine, and Sunburst tangerine and Marsh grapefruit molasses were prepared in the laboratory according to the method reported by Grohmann (*24*).

Sample Purification. The recovery of hydroxycinnamates in orange peel molasses and peel extracts was achieved by the binding of these compounds to water-equilibrated DE-52 cellulose resin (Whatman, Hillsboro, OR) packed in a glass

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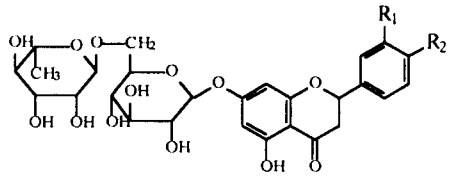
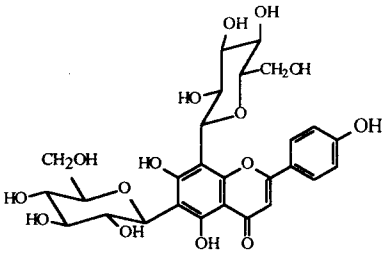
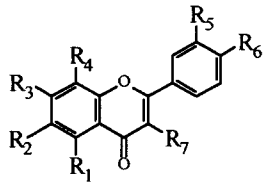




	R_1	R_2
Hesperidin	R_1 (OH)	R_2 (OCH_3)
Narirutin	R_1 (H)	R_2 (OH)
Narirutin-4'-glucoside	R_1 (H)	R_2 (glucose)

6,8-di-*C*-glucosylapigenin



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Sinensetin	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3	H
Tetramethylscutellarein	OCH_3	OCH_3	OCH_3	H	H	OCH_3	H
Isosinensetin	OCH_3	H	OCH_3	OCH_3	OCH_3	OCH_3	H
Nobiletin	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	H
Tangeretin	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3	H
Heptamethoxyflavone	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3

Figure 1. Structures of the main flavonoids in orange peel molasses.

column (12 cm × 2.5 cm i.d.). Fifty milliliters of 7 °Brix ultrafiltered orange peel molasses was loaded onto the column, which was subsequently washed with 300 mL of deionized water. The initial water wash contained the majority of the free sugars and flavonoids. The washed DE-52 column resin was then placed in a Büchner funnel and rapidly washed with 100 mL of 0.4 M KOH, 100 mL of 10% acetic acid, and 300 mL of deionized water, sequentially. HPLC analysis of the resulting hydroxycinnamate fraction (described below) confirmed that negligible changes occurred in the hydroxycinnamates during this procedure. The hydroxycinnamates were recovered from the resulting high-salt solution by passing the hydroxycinnamate fraction through water-equilibrated SP-70 resin (Supelco, Bellefonte, PA) packed in a glass column (25 cm × 2 cm i.d.). The hydroxycinnamates were eluted with 200 mL of methanol.

A similar technique was used to remove the hydroxycinnamates interfering with the HPLC analyses of coniferin and feruloylputrescine. Prior to HPLC analysis of coniferin and feruloylputrescine in citrus molasses, hydroxycinnamates were removed by passing 2 mL aliquots of 10 °Brix ultrafiltered molasses through an NH_2 Sep-Pak (Waters Corp., Milford, MA) cartridge prewashed with dilute acetic acid and deionized water, sequentially. The coniferin and feruloylputrescine quantitatively eluted in the water washes and were subsequently analyzed by HPLC, whereas the hydroxycinnamates remained bound to the cartridge.

Solvent extractions of whole dried orange peel were prepared according to a modified technique of Kanes et al. (*8*). Dried powdered peel (300 mg) was shaken in 5 mL of dimethyl sulfoxide/methanol (1:1, v/v) overnight at room temperature.

Ester Hydrolysis. Hydroxycinnamic acid esters in 20 °Brix molasses were hydrolyzed by the addition of equal volumes of 4 N KOH to the molasses. The hydroxycinnamate esters in dried, powdered peel or peel tissues were hydrolyzed by the addition of 5 mL of 2 N KOH to 300 mg of peel. Hydrolyses of the esters in fresh peel were performed by homogenization of 10 g of fresh peel in 100 mL of vacuum-degassed 2.5 N KOH. All hydrolyses were run under vacuum at room temperature for 30 min. The reactions were stopped by neutralization with concentrated HCl.

Fruit Tissue Preparation. Fifteen fruits of Rhode Red Valencia, Dulce Riberia, and Hamlin sweet orange were collected at the Florida Citrus Arboretum (Florida Department of Agriculture, Winter Haven, FL). The thin outer flavedo and thicker inner albedo portions of the peels were hand-removed by razor blade. The outer membranes of the juice sacs were collected. Tissues of each fruit were kept separately and analyzed individually. Tissue samples were dried at 70 °C and milled to a fine powder. The remaining juice sacs were homogenized and centrifuged to obtain clear juice serum and pelleted serum residue.

Chromatographic Methods. Measurements of citrus phenols were made with high-pressure liquid chromatography (HPLC) using a Perkin-Elmer 250 binary LC pump with a Hewlett-Packard system 1050 photodiode array detector and a 1050 chromatography workstation. The hydroxycinnamates were analyzed with an Alltima (Alltech Associates, Inc., Deerfield, IL) C18 5 μm analytical column (10 cm × 4.6 mm i.d.), using a two-solvent gradient, composed initially of 10 mM phosphoric acid/methanol (90:10, v/v) and increased in a linear gradient to (78:22, v/v) over 15 min. A final composition of 90% methanol was achieved by a subsequent linear gradient over 35 min using a flow rate of 0.75 mL min^{-1}. The polymethoxylated flavones were analyzed with an Alltech Alltima C8 5 μm analytical column (10 cm × 4.6 mm i.d.). Elution conditions included a two-solvent gradient, composed initially of 10 mM phosphoric acid/acetonitrile (90:10, v/v) and increased with linear gradients identical to that described for the C18 column.

Phlorin concentrations (monitored at 270 nm) were determined with a Dynamax-60A phenyl column (25 cm × 4.6 mm i.d.) (Rainin Instrument Co., Woburn, MA). Elution was accomplished with a two-solvent gradient, composed initially of 10 mM phosphoric acid/methanol (97:3, v/v) and then increased with a linear gradient over 15 min to 83:17 (v/v) using a flow rate of 0.75 mL min^{-1}. Analyses of coniferin (270 nm) and feruloylputrescine (330 nm) were run with an Alltech Alltima C8 5 μm analytical column (10 cm × 4.6 mm i.d.) using the elution conditions described for the Alltima C18 column.

Concentrations of hydroxycinnamic acids and flavonoids were calculated by comparing integrated peak areas of the individual compounds to that of peak area per microgram values of the corresponding standards. Peak area per micro-

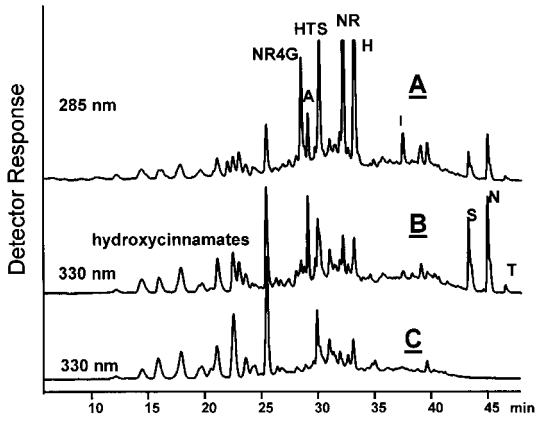


Figure 2. Reversed phase HPLC chromatograms of phenolic constituents of Valencia ultrafiltered orange peel molasses (A and B) and purified molasses hydroxycinnamate fraction (C). Known flavonoid markers are NR4G (narirutin-4′-glucoside), A (6,8-di-*C*-glucosylapigenin), HTS (hesperetin trisaccharide), NR (narirutin), H (hesperidin), I (isosakuranetin rutinoside), S (sinensetin), N (nobiletin), and T (tangeretin).

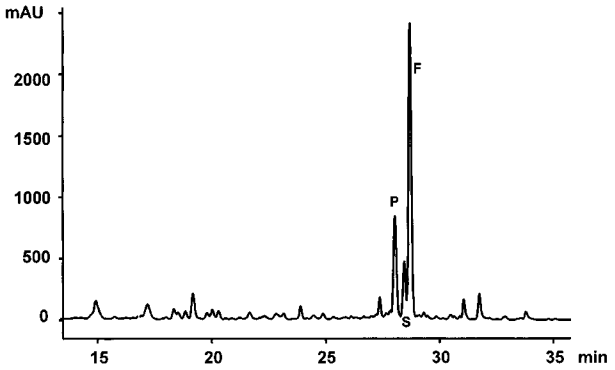


Figure 3. Reversed phase HPLC chromatogram of alkaline hydrolyzed ultrafiltered Valencia orange molasses recorded at 330 nm. Peaks: (F) ferulic acid; (S) sinapic acid; (P) *p*-coumaric acid.

gram conversion factors were determined by running standard curves at 330 nm for flavones and hydroxycinnamic acids or at 285 for flavanone glycosides. In each case, detector responses were linear with increasing amounts of injected compound. Compound assignments were made by comparison of the UV spectra of the individual peaks to the standard compounds and by cochromatography with spiked aliquots of the standard compounds. Flavonoid standards were obtained from authentic flavonoid samples isolated by Horowitz and Gentili (*13*, *25*) and Tatum and Berry (*26*, *27*).

RESULTS AND DISCUSSION

Hydroxycinnamates. Distinct groupings of the hydroxycinnamates and flavonoids are evident in C18 reversed-phase HPLC chromatograms of ultrafiltered orange peel molasses (Figure 2A,B). Polar hydroxycinnamates eluted initially, followed sequentially by flavanone and flavone glycosides and the polymethoxylated flavones. A purified hydroxycinnamate fraction was obtained from the ultrafiltered molasses through the tight binding of the hydroxycinnamates to different anion-exchange resins. The hydroxycinnamates were subsequently recovered from the anion-exchange resins by sequential elution with 0.4 M KOH and dilute acetic acid. Final recovery of the hydroxycinnamates into methanol was achieved by adsorption of the hydroxycinnamates to SP70 resin, followed by methanol elution. The recovery of the hydroxycinnamates on either NH_2-silica or diethylaminoethane (DEAE) anion-exchange resins enhanced the detection of numerous early-eluting (11−26 min) as well as later-eluting (29−34 min) hydroxycinnamate compounds (Figure 2C). Almost all of the peaks in the chromatogram of the purified hydroxycinnamate fraction exhibited characteristic hydroxycinnamate-like UV spectra, indicating that the hydroxycinnamates were efficiently separated from the other major phenolic components in molasses. Also separated from the hydroxycinnamates were the large amounts of free sugars in the molasses samples (*24*), which coeluted with the flavonoids in the initial water washes (data not shown).

Almost all of the molasses hydroxycinnamates, with the main exception of feruloylputrescine, hydrolyzed under alkaline conditions and yielded almost exclusively free ferulic, *p*-coumaric, and sinapic acids (Figure 3).

Table 1. Concentrations of Hydrolyzable Hydroxycinnamic Acids in 20 °Brix Ultrafiltered Molasses from Six Commercial Citrus Cultivars after Alkaline Hydrolysis (Analyses Done in Triplicate)

	total concentration (μg mL^{-1})		
cultivar	ferulic	*p*-coumaric	sinapic
Valencia orange (late-season)	702 ± 15	284 ± 20	156 ± 20
early/mid-season orange[a]	527 ± 11	168 ± 9	71 ± 2
Dancy tangerine	1146 ± 34	270 ± 9	55 ± 2
Sunburst tangerine	1266 ± 19	197 ± 5	trace
Marsh grapefruit	236 ± 7	37 ± 1	64 ± 3
lemon	94 ± 1	337 ± 2	63 ± 4

[a] Mainly Hamlin sweet orange.

Table 2. Concentrations of the Main Flavonoids and Total Hydrolyzable Hydroxycinnamic Acids in 20 °Brix Ultrafiltered Valencia Orange Peel Molasses (Analyses Done in Triplicate)

compound	μg mL^{-1} molasses
p-coumaric acid (hydrolyzed)	337 ± 50
ferulic acid (hydrolyzed)	703 ± 54
sinapic acid (hydrolyzed)	249 ± 6
narirutin-4′-*O*-glucoside	464 ± 18
6,8-di-*C*-glucosylapigenin	184 ± 16
hesperetin-tri-*O*-glycoside	278 ± 18
narirutin (naringenin-7-*O*-rutinoside)	628 ± 8
hesperetin-7-glucoside	1614 ± 8
hesperidin	1672 ± 28

Table 3. Hydrolyzable Hydroxycinnamic Acid Content (Parts per Million) of the Fruit Tissues of Samples from Three Orange Varieties (Analyses Done in Triplicate)

variety	ferulic	*p*-coumaric	sinapic
	Flavedo		
Rhode Red Valencia	3103 ± 39	1360 ± 20	270 ± 40
Dulce Riberia	2451 ± 89	1427 ± 158	ND/trace
Hamlin	2480 ± 65	392 ± 18	499 ± 14
	Albedo		
Rhode Red Valencia	402 ± 9	trace	400 ± 16
Dulce Riberia	309 ± 7	trace	254 ± 15
Hamlin	394 ± 8	trace	295 ± 20
	Membrane		
Rhode Red Valencia	353 ± 2	trace	132 ± 10
Dulce Riberia	299 ± 16	trace	159 ± 3
Hamlin	295 ± 23	84 ± 12	207 ± 17
	Residue		
Rhode Red Valencia	218 ± 8	trace	trace
Dulce Riberia	242 ± 17	trace	trace
Hamlin	220 ± 12	trace	trace
	Juice Serum		
Rhode Red Valencia	61 ± 1	7 ± 1	7 ± 1
Dulce Riberia	49 ± 1	9 ± 1	9 ± 1
Hamlin	45 ± 2	9 ± 1	7 ± 1

Table 4. Concentrations of Phlorin, Coniferin, and Feruloylputrescine in 10 °Brix Ultrafiltered Molasses (Analyses Done in Triplicate)

sample	μg mL^{-1} molasses		
	phlorin	coniferin	feruloylputrescine
Valencia orange	894 ± 7	32 ± 6	81 ± 4
early/mid-season orange	651 ± 27	21 ± 1	46 ± 1
Marsh grapefruit	1796 ± 25	45 ± 1	250 ± 3
lemon	703 ± 20	75 ± 5	ND[a]

[a] None detected.

Only trace levels of caffeic acid were detected. This is consistent with the hydroxycinnamates in citrus occurring primarily as alkaline hydrolyzable esters (*9*, *10*).

Due to the large number of structures in which these hydroxycinnamates occur in the orange (Figure 2C), and the fact that almost all of these compounds occur as hydrolyzable esters, measurements of the hydroxycinnamate concentrations in peel molasses were based on the levels of total hydroxycinnamic acids released by alkaline hydrolysis. As shown in Table 1, the total levels of ferulic, *p*-coumaric, and sinapic acids in alkaline-hydrolyzed 20 °Brix Valencia molasses were 702 ± 15, 284 ± 20, and 156 ± 20 μg mL^{-1}, respectively. Moderately lower concentrations of the hydrolyzed hydroxycinnamic acids occurred in the early/mid-season molasses. Following alkaline hydrolysis, the molasses samples prepared from two varieties of tangerine contained higher levels of hydroxycinnamic acids than in either orange molasses. The main increases in the hydroxycinnamic acids in hydrolyzed tangerine peel molasses occurred in the levels of ferulic acid. Levels of hydrolyzable *p*-coumaric acid remained comparable to those in the orange molasses. Wide variations in the levels of sinapic acid occurred in hydrolyzed molasses prepared from the orange and tangerine varieties. Far lower levels of hydroxycinnamic acids were observed in the samples of alkaline-hydrolyzed molasses from the lemon and grapefruit (Table 1). Unlike the other citrus varieties, the main hydroxycinnamic acid resulting after alkaline hydrolysis of lemon molasses was *p*-coumaric acid.

A comparison of the concentrations of the hydroxycinnamic acids released after alkaline hydrolysis to the concentrations of the main flavonoids in Valencia ultrafiltered molasses (Table 2) shows that hydroxycinnamates are major phenolic constituents in molasses. In contrast, very low levels of free, nonconjugated ferulic acid (20 ± 3 μg mL^{-1}), *p*-coumaric acid (17 ± 1 μg mL^{-1}), and sinapic acid (30 ± 3 μg mL^{-1}) were detected in unhydrolyzed peel molasses, consistent with the trace levels of free, nonconjugated hydroxycinnamic acid previously observed in fresh peel (*9*).

Distributions of the hydrolyzable hydroxycinnamates were measured in the different portions of three varieties of sweet orange (Rhode Red Valencia, Dulce Riberia, and Hamlin) (Table 3). Hydroxycinnamates had been previously shown to occur in the highest concentrations in the flavedo of Shamouti sweet orange (*9*). The levels of hydrolyzed hydroxycinnamic acids in the sample of flavedo of the Rhode Red Valencia peel (Table 3) were found to be similar to those in the Shamouti peel. In contrast, notable differences occurred in the levels of sinapic and *p*-coumaric acids in the hydrolyzed flavedo of Hamlin and Dulce Riberia sweet orange; that is, negligible sinapic acid was observed after hydrolysis of Dulce Riberia flavedo, whereas much higher amounts occurred in the flavedo of the other two samples. Additionally, the hydrolyzed flavedo from Hamlin orange fruit contained much lower levels of *p*-coumaric acid than in the samples from the two other varieties. Interestingly, hydrolysis of the albedo and juice sac membranes produced nearly equal levels of ferulic and sinapic acids in each of the samples from the different orange varieties, whereas only trace levels of *p*-coumaric acid were detected. This is similar to observations made previously for the albedo of Shamouti sweet orange (*9*). Hydrolyzed juice serum residue contained ferulic acid but only trace levels of *p*-coumaric acid and sinapic acid. Samples of juice sera from three different varieties of orange contained very low levels of the hydroxycinnamates. Ferulic acid occurred at levels nearly 9-fold higher than *p*-coumaric and sinapic acids in the hydrolyzed serum. This contrasts sharply with the relative proportions of these compounds in the other hydrolyzed portions of the fruit, suggesting that there are marked qualitative differences in the hydroxycinnamate profiles in the serum and the other tissues of the orange.

Phenolic Glucosides. Two phenolic glucosides, phlorin and coniferin, have been reported in orange peel (*25*, *26*). High concentrations of phlorin were observed in the ultrafiltered orange, grapefruit, and lemon peel molasses samples examined in this study (Table 4). Also in

Table 5. Concentrations of Polymethoxylated Flavones in 20 °Brix Ultrafiltered Molasses of Orange, Tangerine, Grapefruit, and Lemon (Analyses Done in Triplicate)

compound	μg mL^{-1}						
	Dancy tangerine	Sunburst tangerine	Clementine tangerine	late-season[a] orange	early-season[b] orange	Marsh grapefruit	lemon
isosinensetin (5,7,8,3′,4′-pentamethoxyflavone)	142 ± 1	11 ± 1	5 ± 1	18 ± 4	ND	ND	ND
sinensetin (5,6,7,3′,4′-pentamethoxyflavone)	127 ± 2	14 ± 1	15 ± 1	99 ± 2	56 ± 1	12 ± 1	ND
hexa-*O*-methylquercetagetin (3,5,6,7,3′,4′-hexamethoxyflavone)	ND	ND	ND	22 ± 1	12 ± 1	ND	ND
nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone)	711 ± 3	46 ± 2	22 ± 1	95 ± 2	88 ± 1	33 ± 1	ND
3,5,6,7,8,3′,4′- heptamethoxyflavone	26 ± 1	13 ± 1	40 ± 2	33 ± 1	22 ± 1	13 ± 1	ND
tetramethylscutellarein (5,6,7,4′-tetramethoxyflavone)	16 ± 1	3 ± 1	4 ± 1	31 ± 1	10 ± 1	6 ± 1	ND
tangeretin (5,6,7,8,4′-pentamethoxyflavone)	90 ± 1	4 ± 1	2 ± 1	12 ± 1	14 ± 1	7 ± 1	ND
5-desmethylnobiletin (5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone)	22 ± 1	ND	ND	trace	trace	ND	ND

[a] Valencia orange. [b] Mainly Hamlin orange.

Table 4 are the concentrations of coniferin and feruloylputrescine. Phlorin and feruloylputrescine occurred in the highest concentrations in the grapefruit peel molasses sample. Feruloylputrescine was not detected in the sample of lemon molasses. These findings are consistent with the relative concentrations for these compounds previously measured in citrus peel (*28*).

Polymethoxylated Flavones. Concentrations of the polymethoxylated flavones in the orange, tangerine, lemon, and grapefruit 20 °Brix molasses samples are shown in Table 5. The polymethoxylated flavones occurred in the highest concentrations in molasses prepared from the tangerine and orange varieties. The molasses prepared from Dancy tangerine contained nearly 10-fold higher amounts of the polymethoxylated flavones than in the molasses prepared from the other two tangerine or orange varieties. Far lower concentrations of polymethoxylated flavones occurred in the grapefruit molasses. No polymethoxylated flavones were detected in the lemon molasses sample, consistent with similar analyses of juice samples (*29*).

Despite the low concentrations of the polymethoxylated flavones in orange peel, the molasses produced during peel processing represents a potential source for these flavonoids. From 1000 kg of 65 °Brix Valencia orange molasses, ~1.3 kg of total polymethoxylated flavones is recoverable, that is, 0.057 kg of isosinensetin, 0.32 kg of sinensetin, 0.07 kg of hexa-*O*-methylquercetagetin, 0.31 kg of nobiletin, 0.11 kg of heptamethoxyflavone, 0.10 kg of tetramethylscutellarein, and 0.04 kg of tangeretin. On the basis of the values in Table 2, 3.7 kg of total hydrolyzable hydroxycinnamic acids is recoverable from 1000 kg of 65 °Brix Valencia orange molasses. These are comparable to the 2.04 and 5.4 kg of narirutin and hesperidin, respectively, in the same volumes of ultrafiltered molasses. These values suggest that during the processing of molasses into value-added coproducts, large recoveries of the polymethoxylated flavones as well as the hydroxycinnamates are achievable.

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