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Distribution of Furanocoumarins in Grapefruit Juice Fractions

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The reported effects of grapefruit (*Citrus paradisi* Macf.) juice on oral bioavailability of certain prescription drugs have led to the discovery of the inhibition by compounds in grapefruit of cytochrome P450 3A4 (CYP3A4) in the intestinal wall and liver. Recent evidence indicates that furanocoumarins related to bergamottin [5-[(3′,7′-dimethyl-2′,6′-octadienyl)oxy]psoralen] are primarily responsible for the grapefruit effect, yet the exact mechanisms and roles that specific compounds play in this effect are still uncertain. In the current experiments freshly extracted grapefruit juice was separated into four fractions, consisting of raw finished juice (~5% fine pulp), centrifugal retentate (~35% fine pulp), centrifuged supernatant (<1% pulp), and coarse finisher pulp. The relative concentrations of furanocoumarins in each of these grapefruit juice fractions were measured by HPLC-MS. These measurements showed that the centrifugal retentate had the highest furanocoumarin content, containing 892 ppm of bergamottin, 628 ppm of 6′,7′-dihydroxybergamottin, 116 ppm of 6′,7′-epoxybergamottin, 105 ppm of 7-geranyloxycoumarin, and ~467 ppm of furanocoumarin dimers. These high furanocoumarin concentrations make this fraction a useful starting material for preparative-scale isolations of these compounds. MS analysis of this furanocoumarin-enriched fraction provided evidence of additional furanocoumarins in grapefruit juice that remain to be fully characterized and evaluated for their roles in the grapefruit−drug interactions.

KEYWORDS: Furanocoumarins; psoralens; grapefruit juice; grapefruit pulp

INTRODUCTION

Early observations by Bailey et al. (*1*−*3*) provided indications that compounds in grapefruit juice influence the oral bioavailability of certain prescription drugs. Initially, these influences were believed to be due to naringin, a flavonoid in grapefruit juice, but studies have now shown that a series of furanocoumarins are the primary responsible agents (*3*−*7*) through their inhibition of intestinal CYP3A4. Other studies have shown that compounds in grapefruit juice may also mediate drug efflux, possibly via the P-glycoprotein transporter (*8*, *9*). Although it is speculated that the furanocoumarins are possibly involved in this inhibition, direct evidence of the inhibition of the P-glycoprotein transporter by the furanocoumarins is not firmly established. The majority of the active furanocoumarins appear to be analogues of bergamottin [5-[(3′,7′-dimethyl-2′,6′-octadienyl)oxy]psoralen], 6′,7′-dihydroxybergamottin, and 6′,7′-epoxybergamottin (**Figure 1**), including a number of dimers of these compounds. The potencies of these latter dimers as CYP3A4 inhibitors appear to far surpass that of the monomeric species (*4*). A number of the dimers have been structurally identified (*10*−*14*), but many other furanocoumarins in grapefruit juice that also appear to be either dimers or other types of complex conjugates remain uncharacterized. Examinations of the HPLC-MS total-ion-current (TIC) chromatograms at molecular masses of the dimers thus far reported in grapefruit juice (i.e., 708 and 726 amu) provide evidence of a number of additional compounds at these molecular masses that may possibly be involved in the grapefruit−drug interaction phenomenon.

A major limiting factor to the study of the roles of specific compounds in the grapefruit−drug interaction phenomenon is the exceedingly low levels at which many of the active furanocoumarins occur. Unlike the flavonoids in grapefruit, most of the active grapefruit furanocoumarins are not concentrated in the peel and peel oil, but are primarily located in the juice (*10*, *14*). The objectives of this study were to separate grapefruit juice into easily obtained fractions enriched in serum (centrifugal supernatant), juice cloud (centrifugal retentate), and pulp. The original grapefruit juice served as the control sample. Measurements of the relative levels of these compounds in each of the four specific fractions of grapefruit juice point to the preferential binding of the furanocoumarins to the particulate juice cloud and, thus, identify this material as the most practical fraction to be used in preparative-scale isolations of these bioactive compounds.

MATERIALS AND METHODS

Sample Preparation. Juice from cv. Marsh white grapefruit was prepared with an FMC in-line extractor, and the juice was finished with a screw finisher set at normal settings. The finished juice, containing ~5% pulp (v/v), was centrifuged with a Westfalia continuous

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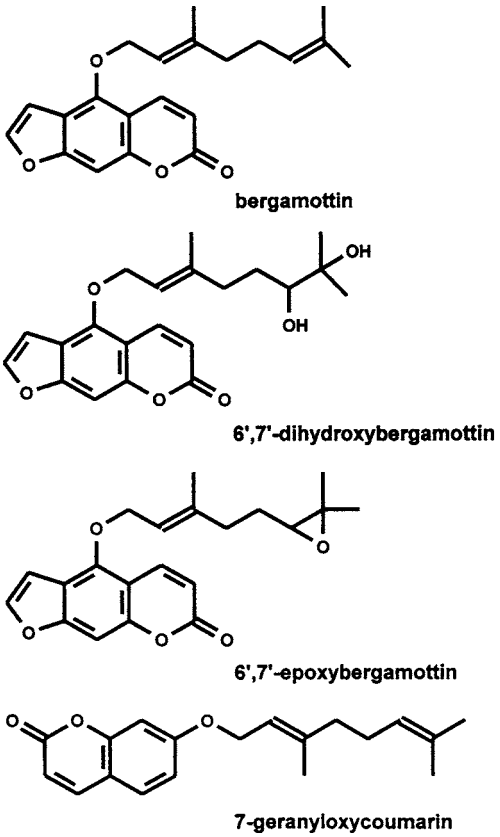

Figure 1. Structures of bergamotttin, 6′,7′-dihydroxybergamottin, 6′,7′-epoxybergamottin, and 7-geranyloxycoumarin.

flow centrifuge with the retentate adjusted to contain ~35% (v/v) pulp content and the supernatant effluent with <1% (v/v) of pulp. Finisher pulp, raw finished juice, centrifugal retentate, and supernatant were analyzed for furanocoumarin content. Centifugal retentate comprised nearly 5% of the unprocessed juice.

Furanocoumarin Extraction. The furanocoumarins were quantitatively extracted into ethyl acetate using a previously reported method of Widmer and Haun (*15*) with slight modifications. Twenty grams each of unprocessed juice, retentate, supernatant, and finisher pulp were extracted successively three times with 20 mL of ethyl acetate. Recoveries of >99% were achieved for the compounds listed in **Table 1**. Extracts from each sample were combined, and the ethyl acetate was evaporated to dryness with a Savant rotary vacuum concentrator; each residue was redissolved in 2 mL of tetrahydrofuran. Due to the lability of these compounds, temperatures >40 °C were avoided during sample drying.

Chromatographic Analysis. Previously published HPLC-PDA and HPLC-API-MS methods of analyses of furanocoumarins in grapefruit were used with modifications (*15*−*18*). Each extract (20 μL) was analyzed with a Waters Alliance high-pressure liquid chromatograph (HPLC), equipped with a Waters 996 photodiode array (PDA) detector and a Waters/Micromass ZQ single-quadrupole mass spectrometer. Separation was accomplished on a 150 × 3.9 mm i.d. Nova-Pak C-18 column (Waters, Milford, MA), with a multistep linear water/acetonitrile/0.05% formic acid gradient at a flow rate of 0.75 mL/min. The gradient of water/acetonitrile/0.05% formic acid started at 85:10:5, increased to 55:40:5 in 15 min and to 25:70:5 in 25 min, followed by another linear segment to 0:95:5 in 12 min, and was held there isocratically for 13 min. Because most of the grapefruit furanocoumarins showed a characteristic UV peak near 310 nm, the chromatograms were recorded at this wavelength. 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). Postcolumn split to the PDA and mass ZQ detector was 10:1. MS parameters were as follows: ionization mode, ES+; capillary voltage, 3.0 kV; extractor voltage, 5V; source temperature, 100 °C; desolvation temperature, 225 °C; desolvation N_2 flow, 465 L/h; cone N_2 flow, 70 L/h; scan range, *m*/*z* 150−900; scan rate, 1 scan/s; cone voltages, 20, 40, and 60 eV. Single ion monitoring (SIM)-MS analysis was conducted with the same instrument parameters, with the exception of monitoring at specific mass ions (*m*/*z* 709 and 727), rather than conducting full scans between *m*/*z* 150 and 900. SIM-MS increased by 100−200-fold the sensitivity of detection of most of the grapefruit furanocoumarins, particularly the trace-occurring furanocoumarin dimers at *m*/*z* 709 and 727. For bergamottin the lower limits of detection by SIM-MS were measured in the range of 12 pg (signal/noise 22), whereas ~20 ng was required to provide UV spectra by HPLC-PDA analysis. The lower limit of detection of the bergamottin peak by HPLC-UV-PDA was 1.2 ng (signal/noise 7).

Component Identification. The chromatographic peaks were characterized by a combination of UV (PDA) and ESI-MS analysis. The UV traces of the HPLC chromatograms were compared with the TICs at *m*/*z* 339, 355, 373, 709, and 727, corresponding to $[M + H]^+$ ions of bergamottin, 6′,7′-epoxybergamottin [5-[(3′,7′-dimethyl-6′-epoxy-2′-octenyl)oxy]psoralen] and 6′,7′-dihydroxybergamottin [5-[(3′,7′-dimethyl-6′,7′-dihydroxy-2′-octenyl)oxy]psoralen] and of previously identified dimers (*10*−*14*). The $[M + H]^+$ and $[M + Na]^+$ peaks, if present, obtained at 20 V cone volts, were used to determine the molecular masses of most compounds. Fragmentation data at higher cone voltages often provided additional structural information. Collision-induced fragmentation of bergamottin analogues, including many of the unknown compounds, nearly uniformly produced a prominent 203 amu fragment corresponding to bergaptol (5-hydroxypsoralen). Authentic samples of bergamottin, 6′,7′-dihydroxybergamottin, 6′,7′-epoxybergamottin, 7-geranyloxycoumarin, and bergaptol were obtained from a previous study (*19*).

Table 1. Concentrations of Furanocoumarins/Coumarins (Parts per Million) in Grapefruit Juice Fractions[a]

retention time (min)	*A*/μg	raw juice	retentate	supernatant	pulp	compound	MS fragment ions (*m*/*z*)
16.8	UK[b]	20.7	35.9	21.8	79.8	FC620	203/337/355/373/621
18.7	41972	89.0	628.1	57.1	139.1	6′,7′-dihydroxybergamottin	203/337/355/373
24.4	UK	2.0	57.5	0.0	6.7	FC574	203/355/373/389/557/575
25.5	UK	5.7	57.8	0.7	6.7	FC418	203/215/337/355/377/419
26.7	UK	0.8	14.8	0.1	2.0	FC648	203/259/631/649
28.4	UK	5.8	82.0	0.6	9.5	FC608	203/237/353/407/609
29.3	36065	16.2	116.4	1.7	9.4	6′,7′-epoxybergamottin	203/355/377
38.5	52542	6.0	105.6	1.0	8.7	7-geranyloxycoumarin	163/299
39.4	UK	3.3	238.1	0.0	30.2	FC726[c]	203/337/355/373/727
41.1	48476	45.0	892.7	4.5	89.1	bergamottin	203/339
45.9	UK	2.1	50.7	0.0	4.9	FC708	203/337/355/507/709
47.3	UK	0.0	32.1	0.0	2.0	FC744	203/355/357/709
51.1	UK	0.6	53.0	0.0	7.5	FC708	203/337/355/373/534/709
53.3	UK	3.1	94.3	0.0	11.8	FC692	203/339/355/693

[a] The main fragment ions detected at 20 and 40 V for identified furanocoumarins and unknown compounds are listed. Zero values in the table indicate levels below the detection limit. [b] Unknown compound concentrations, estimated by using the integrated peak area per microgram value for 6′,7′-dihydroxybergamottin. [c] Fragmentation data similar to that of the 726 amu compounds analyzed previously (*15*).

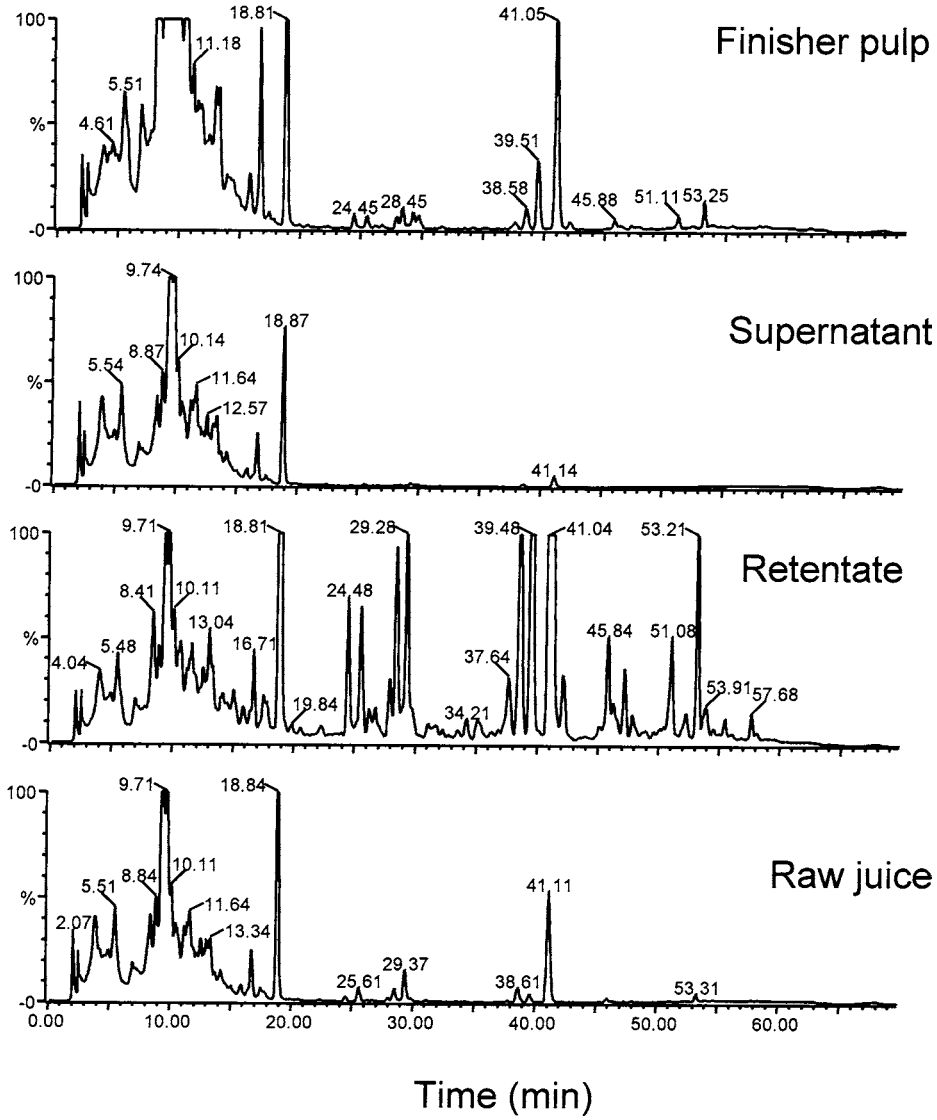

Figure 2. Chromatograms of grapefruit juice fractions. To show relative distribution of the components, all chromatograms are normalized to the bergamottin peak (41.05 min) appearing in the finisher pulp trace. The bulk of peaks between 5 and 15 min are mainly attributable to flavanone glycosides.

RESULTS AND DISCUSSION

Freshly extracted grapefruit juice was processed via centrifugation into four fractions differing in pulp and juice cloud content. The centrifugal retentate represented the fraction with the highest content of particulate juice cloud material, whereas the supernatant contained the least. The finisher pulp fraction contained mainly larger cell wall fragments. The furanocoumarins in these four grapefruit juice fractions (including the original unprocessed juice) were quantitatively extracted into ethyl acetate and analyzed by HPLC-MS. The reversed-phase HPLC chromatograms of the extracts of these fractions (**Figure 2**) were dominated by peaks attributable to flavonoids, hydroxycinnamates, furanocoumarins, and related compounds. The flavonoids in grapefruit, primarily naringin, neohesperidin, and poncirin, are polar flavanone neohesperidosides (*20, 21*) that occurred with the hydroxycinnamates in the early (5−15 min) portions of the chromatograms. These early-eluting compounds were not quantitatively extracted into ethyl acetate and were not part of the distribution analysis. In contrast, the majority of the furanocoumarins in grapefruit showed elution times substantially later than the those of the polar flavonoids and hydroxycinnamates, and with the distinctive UV spectra with sharp absorption wavelength maxima near 310 nm, these latter compounds were easily detected by PDA analysis.

Levels of the main furanocoumarins in the four grapefruit juice centrifugal fractions are listed in **Table 1**. The furanocoumarins occurred in the highest concentrations in the centrifugal retentate, containing 892 ppm (μg/g of wet weight) of bergamottin, 628 ppm of 6′,7′-dihydroxybergamottin, 116 ppm of 6′,7′-epoxybergamottin, 105 ppm of 7-geranyloxycoumarin (**Figure 1**), and ∼467 ppm of furanocoumarin dimers. Far lower levels of these furanocoumarins occurred in the pulp fraction and unprocessed juice. The retentate supernatant is depleted of these compounds, although it is rich in other coumarin and psoralen monomers, a number of which have been identified previously (*19, 22, 23*). With the exception of the calculations made for the furanocoumarin dimers, the concentrations of the known compounds were calculated on the basis of the peak area conversion factors measured with purified standards. Using a method similar to that of Ohta et al. (*14*), levels of the unknown compounds, including the dimers, were calculated on the basis of the peak area conversion factor measured for 6′,7′-dihydroxybergamottin at 310 nm. Actual concentrations of the unidentified furanocoumarins in **Table 1** will deviate from these calculated amounts depending on the structural disparities

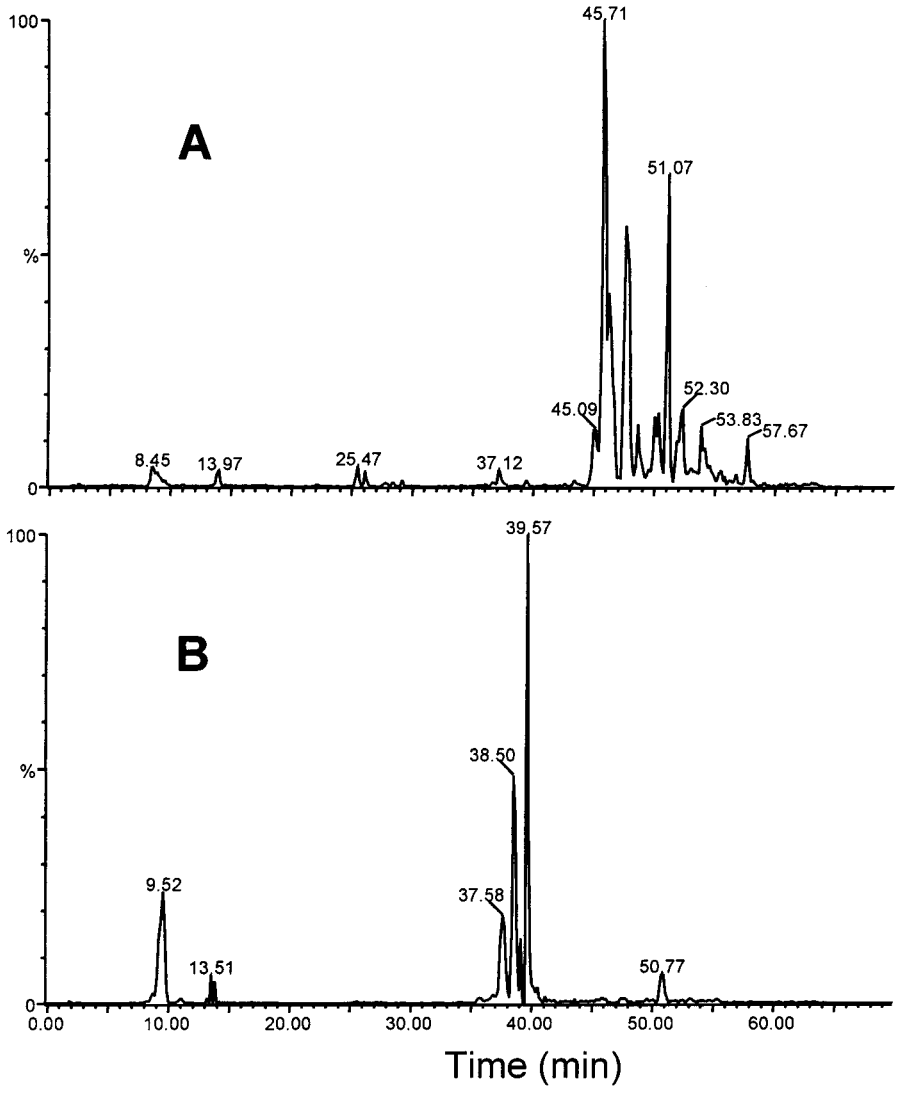

Figure 3. TIC chromatograms at *m*/*z* 709 (**A**) and 727 (**B**) of the ethyl acetate extract of grapefruit juice centrifugal retentate. MS detection was achieved with ESI and cone voltage at 20 V. Separation conditions are identical to those in **Figure 1**.

between these unknowns and 6′,7′-dihydroxybergamottin. Because many of these compounds showed MS fragmentation consistent with structures containing 6′,7′-dihydroxybergamottin subunit(s) (i.e., the occurrence of the *m*/*z* 203, 337, and 355 ions) (**Table 1**), these measurements provide reasonable estimates of the furanocoumarin contents of these compounds relative to 6′,7′-dihydroxybergamottin. The identification and/or partial characterizations of the compounds in **Table 1** are based on the UV and MS properties of the compounds. Identifications of bergamottin, 6′,7′-dihydroxybergamottin, 7-geranyloxycoumarin, and 6′,7′-epoxybergamottin were based on the identical chromatographic and UV and MS spectroscopic properties of these compounds relative to purified standards. The remaining unknowns in **Table 1** were described by their molecular masses, collision-induced fragmentation, and UV spectra. Five of these compounds showed molecular masses of 708 and 726 consistent with the occurrence of dimers (*12*). Further detection of furanocoumarin dimers in the four fractions was achieved by analysis of the TIC chromatograms and subsequent SIM-MS analysis at these two molecular masses. Evidence of 10 compounds with a molecular mass of 708 and of three compounds with a molecular mass of 726 was shown by the TIC chromatograms at *m*/*z* 709 and 726 ($[M + H]^+$) (**Figure 3**). SIM analyses at *m*/*z* 709 and 727 for the $[M + H]^+$ ions confirmed these findings (data not shown). The presence of these 709 and 727 amu $[M + H]^+$ compounds is consistent with the furanocoumarin dimers reported by Fukuda and coworkers (*10*−*14*). The latter group characterized compounds termed paradisins A−C, including two 727 amu $[M + H]^+$ dimers of 6′,7′-dihydroxybergamottin, linked tail-to-tail and head-to tail, and a 709 amu $[M + H]^+$ dimer of two subunits of 6′,7′-epoxybergamottin linked tail-to-tail. Although Harris (*16*) has similarly reported the occurrence in grapefruit juice of a series of 709 and 727 amu $[M + H]^+$ head-to-tail furanocoumarin dimers, termed "orthospiroesters", no chemical characterizations were reported. On the basis of the distinctive UV spectra of the putative orthospiroesters, Widmer and Haun (*15*) have recently reported the detection of six such compounds by HPLC-PDA.

Of further interest were the 692 amu furanocoumarin eluting at 53.3 min and the 574 amu furanocoumarin eluting at 24.4 min. The MS of the 692 amu compound exhibited collision-induced fragmentation consistent with a mixed heterodimer between 6′,7′-epoxybergamottin [355 $[M + H]^{+1}$; 339 ($- H_2O$); 203 amu] and bergamottin (neutral mass loss of 338 amu) (**Figure 4A**), whereas the MS of the 574 amu furanocoumarin exhibited collision-induced fragmentation consistent with a mixed conjugate between 6′,7′-dihydroxybergamottin [373 $[M + H]^{+1}$; 355 ($- H_2O$); 337 ($- 2H_2O$); 203 amu] and bergaptol (neutral mass loss of 202 amu) (**Figure 4B**). It is

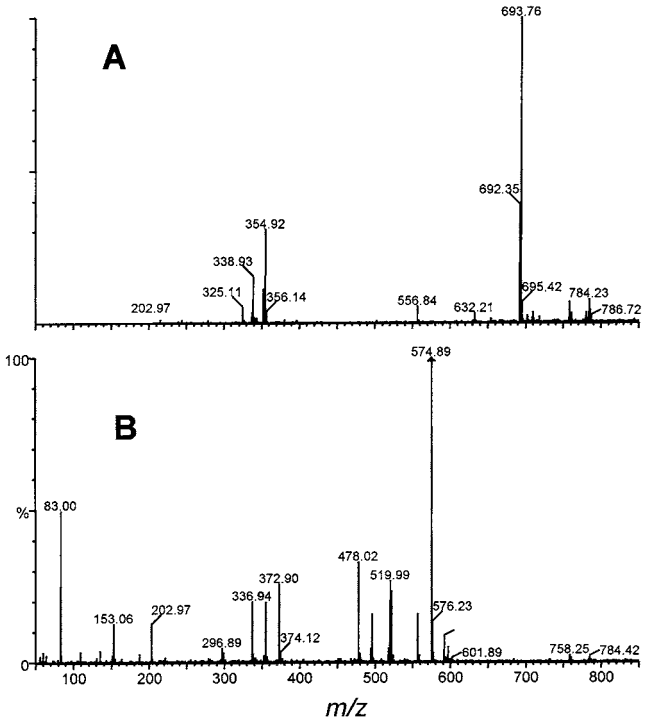

Figure 4. ESI-MS of furanocoumarins eluting at 53.3 min with a molecular mass of 692 amu (**A**) and at 24.4 min with a molecular mass of 574 amu (**B**). The fragment at *m*/*z* 355 in (**A**) suggests the detection of 6′,7′-epoxybergamottin, whereas the mass difference between the molecular ion $[M + H]^+$ at 693 and the fragment at *m*/*z* 355 suggests the loss of bergamottin at *m*/*z* 338. The fragment at *m*/*z* 373 in (**B**) suggests the detection of 6′,7′-dihydroxybergamottin, whereas the mass difference between the molecular ion $[M + H]^+$ at 575 amu suggests the loss of bergaptol at *m*/*z* 203.

important to note that, in contrast to these compounds, all previously reported grapefruit furanocoumarin dimers have been limited thus far to compounds composed of epoxybergamottin and 6′,7′-dihydroxybergamottin, variously linked via the terpene side chains (tail-to-tail) or via the terpene chain to the aromatic ring (head-to-tail). The MS data in **Figure 4** are consistent with other mixed heterodimers and thus widen the consideration of other possible structures involved in the grapefruit−drug interactions.

Finally, the findings in **Table 1** point to an additional property of the grapefruit furanocoumarins, particularly the dimers. Although the relatively high concentrations of these compounds in the pulp fractions have already been reported (*10*, *14*, *16*), our findings point to the significant differences in the occurrences of these compounds in the pulp and juice cloud material. Importantly, the finisher pulp fraction contains far less of the later-eluting furanocoumarins, relative to the retentate; this indicates that these compounds are mainly associated with the small particulate juice cloud material. The nature of these associations is presumed to be hydrophobic, due to the extreme lipophilic properties of these compounds, but the specificity exhibited toward the cloud material over the pulp remains poorly understood. Earlier studies have shown that grapefruit juice cloud contains a much higher abundance of organelles, lipid bodies, and cellular membranes, relative to the pulp (*24*, *25*), and it is likely that the lipophilic furanocoumarins selectively bind to these lipid-rich constituents of the juice cloud. This may show a propensity of the furanocoumarins to bind and interact with biological membranes in intact tissue. These lipophilic interactions may play important roles in contributing to the biological actions of these compounds.

Table 2. Calculated Amounts of the Main Grapefruit Furanocoumarins in Centrifugal Retentate Obtained from 10 kg of Unprocessed Grapefruit Juice[a]

compound	total (mg)
bergamottin	446
7-geranyloxycoumarin	52
6′,7′-epoxybergamottin	18
6′,7′-dihydroxybergamottin	314
furanocoumarin dimers	233

[a] Recovered retentate volume is ∼5% of original grapefruit volume (i.e., 500 g). Amounts of the identified compounds are calculated from ppm values listed in **Table 1**. Amounts of furanocoumarin dimers are estimated as described in **Table 1**.

Regardless of the nature of these interactions, the distributions of the furanocoumarins in the four grapefruit fractions show that initial fractionation of grapefruit juice to obtain the centrifugal retentate is an important improvement to the efficiency of preparative-scale isolations of these compounds over liquid/liquid extractions of grapefruit juice. **Table 2** lists the calculated amounts of a number of these main grapefruit furanocoumarins, based on the concentrations in retentate (**Table 1**) from 10 L of grapefruit juice (∼5% retentate). The use of such centrifugal retentates has been successfully applied in our laboratory to isolate sets of a number of the trace-occurring furanocoumarin dimers for further evaluations of their roles in inhibiting liver microsomal CYP3A4 and the P-glycoprotein transporter.

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