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Protein Sequestration of Lipophilic Furanocoumarins in Grapefruit Juice

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Supporting Information

ABSTRACT: The sequestration of grapefruit furanocoumarins by foods was investigated by characterizing the binding between these compounds and foods with contrasting protein, fat, and carbohydrate compositions. Individual grapefruit furanocoumarins exhibited contrasting affinities to foods, where the lipophilic bergamottin and several structurally related dimers bound to foods more tightly than the more polar 6′,7′-dihydroxybergamottin. From the investigation of different classes of macromolecules in foods, water-soluble proteins were found to be the major constituents responsible for furanocoumarin sequestration. Studies using bovine serum albumin as a model protein demonstrated the dissociation of grapefruit furanocoumarins from the insoluble juice cloud particles and the subsequent formation of water-soluble bovine serum albumin−furanocoumarin complexes. Fluorescence binding assays further demonstrated the binding of bergamottin and 6′,7′-dihydroxybergamottin to bovine serum albumin. These results demonstrate that proteins can be sequestration agents of these important dietary furanocoumarins.

KEYWORDS: *Grapefruit, bergamottin, tryptophan fluorescence, protein binding, bovine serum albumin*

INTRODUCTION

Grapefruit (*Citrus paradisi* Macf.) is widely consumed and has a number of purported beneficial effects against chronic diseases.[1] Inhibition of oral carcinogenesis and reduction in cardiovascular mortality and coronary heart disease are a few examples of oxidative stress-related diseases potentially inhibited by grapefruit polyphenols.[2−4] However, despite these protective effects, consumption of grapefruit and grapefruit juice (GFJ) in the U.S. has decreased by nearly 50% in the past decade,[5] primarily because of concerns regarding the widely publicized grapefruit−drug interactions.[6,7] In these interactions, phenolic furanocoumarins (FCs) in GFJ, such as 6′,7′-dihydroxybergamottin (DHB) and bergamottin (BM), irreversibly inhibit the human intestinal cytochrome P450 3A4 isozyme (CYP3A4) involved in the metabolism of certain widely prescribed medications.[8] This inhibition involves an irreversible mechanism-based covalent attachment of the GFJ FCs to the active site of the CYP3A4 protein.[9−11]

In the course of our previous studies, it was shown that a number of fungi passively sequester GFJ FCs and that this process could be used to lower the concentrations of FCs in GFJ.[12,13] This sequestration was more pronounced with the more lipophilic GFJ compounds, particularly BM and modified dimers (Figure 1). It became of interest, therefore, whether other common components of human diets could also sequester lipophilic GFJ FCs and, thus, possibly influence the distribution and bound states of these compounds. In our present study the abilities of different foods to sequester the FCs in GFJ were analyzed, as were the respective roles of different classes of food macromolecules. Results of this study showed that the lipophilic GFJ FCs are effectively sequestered by soluble food proteins away from the juice cloud particles to which these compounds are normally bound.

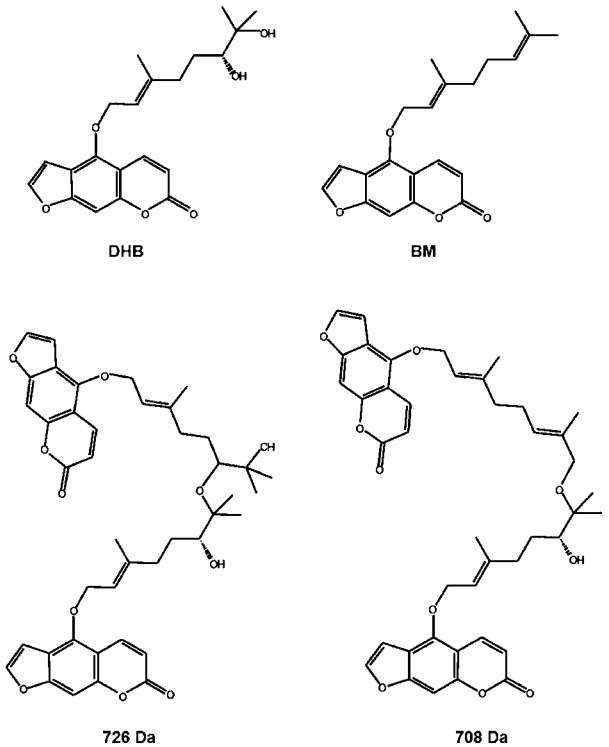

Figure 1. Structures of BM, DHB, and the main 708 and 726 Da dimers in GFJ.

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MATERIALS AND METHODS

Reagents and Standards. Dimethylsufoxide (DMSO) was purchased from Fisher Chemical (Fair Lawn, DE). Bovine serum albumin (BSA) (product number A7030) was purchased from Sigma-Aldrich (St. Louis, MO). The Bradford protein assay was run with a BSA protein standard ampule from Thermo Scientific. BM, 6′,7′-epoxybergamottin, and DHB were isolated as reported previously.[14] Bergapten (5-methoxypsoralen) (product number 27,572-7) was purchased from Aldrich Chemical Co.

Preparation of Foods. The foods analyzed in this study were purchased from a local grocery store. Two samples of ground beef (containing either 10 or 30% fat) were cooked in a microwave for 4 min, while salmon was thinly sliced (5 mm) and cooked in a microwave for 3 min. The grease and water from the microwaved foods were drained. The cooked ground beef and salmon, along with chopped fresh mushrooms (*Agaricus bisporus* L.), wheat bread, and frozen precooked corn were dried under vacuum at 40 °C for 48 h. These dried foods along with untreated long grain rice were ground separately to fine powders with a coffee grinder. GFJ was prepared by mixing commercial GFJ concentrate (355 mL) with 1.5 L of water and adjusted to pH 5.0 with a 2 M KOH solution to minimize acid hydrolysis of the FCs.[15]

HPLC Analysis of GFJ FCs. The separation and detection of the GFJ FCs were achieved using a Waters Alliance 2695 HPLC system coupled with a Waters 996 photodiode array detector and a Waters ZQ mass spectrometer as described previously.[12,13]

Interactions of GFJ FCs with Foods. GFJ (50 mL) was mixed with 1.0 and 2.0 g of dried powdered food samples and shaken at 300 rpm for 8 h at 25 °C. The mixtures were vacuum-filtered through a Whatman No. 1 filter, and the remaining residues were washed with 200 mL of water to completely remove any unbound FCs. The FCs in the filtered GFJ or those remaining bound to the food residues were extracted and analyzed by high-performance liquid chromatography (HPLC). FCs were not detected as normal components in any of the untreated foods used in this study (data not shown).

Fractionation of Protein, Carbohydrate, and Lipid Components of Corn and Salmon. Lipids were removed from cooked corn and salmon according to Folch et al.[16] (Figure 2A). The powdered food samples (7−10 g) were homogenized in 200 mL of chloroform/methanol (2:1) for 1 min and shaken for 1 h, and the slurry was vacuum-filtered through a Whatman No. 1 filter. The remaining residue was extracted 3 more times with additional aliquots of the solvent mixture and filtered, followed by two extractions with 100 mL of hexane/isopropanol (3:2). The presence of lipids in the final filtrates was examined using 250 μm silica gel HLF thin-layer chromatography (TLC) Uniplates (Analtech, Newark, DE) developed with chloroform/methanol/water (60:30:5) for polar lipids and hexane/diethyl ether/acetic acid (80:20:1.5) for nonpolar lipids.[17] The removal of the extractable lipids from the corn and salmon residues (residue 1 in Figure 2A) was confirmed by the absence of the two classes of lipids on TLC plates spotted with 4 × 10 μL of 20-fold concentrated final filtrates from the above solvent extractions.

Soluble carbohydrates and proteins were removed from the defatted corn powder by acidic and basic water extractions as published previously,[18−20] with modifications. The lipid-free residue 1 (Figure 2A) of corn (15 g) was extracted by Polytron (Kinematica) homogenization (1 min at room temperature) in either 200 mL of water adjusted to pH 4.0 with 1 M HCl or in 200 mL of water adjusted to pH 10.0 with 1 M NaOH and then centrifuged at 10000*g* for 10 min. The resulting insoluble pellets were re-extracted with new aliquots of water solutions until proteins and carbohydrates were no longer detected in the final supernatants by colorimetric protein and carbohydrate assays.[21,22] To ensure the removal of any further remaining soluble carbohydrates and proteins from corn residues 2A and 2B (remaining after pH 4 and 10 washes, respectively), additional extractions were performed at pH 2.0 or 12.0 at 80 °C, producing the filtered residues 3A or 3B, respectively. No water-soluble proteins or carbohydrates were detected in these final acid and base treatments. Residues 2A and 2B and 3A and 3B were neutralized, collected by filtration, and vacuum-dried to produce fine powders.

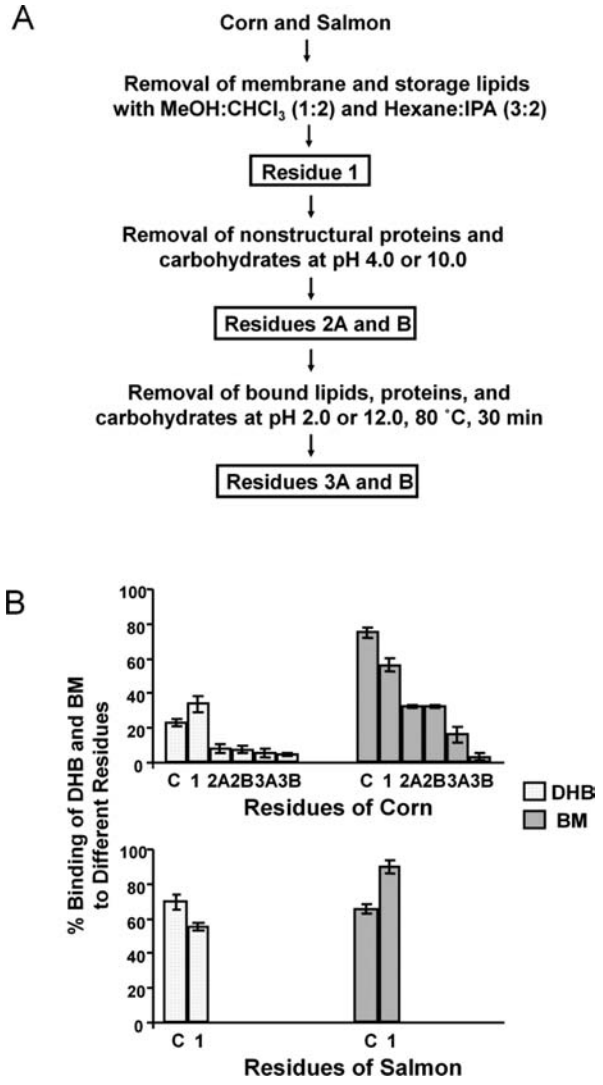

Figure 2. (A) Fractionation strategy to investigate separate components (residues 1−3) of corn and salmon that sequester FCs in GFJ and (B) their binding (%) of DHB and BM in GFJ. Bars represent standard errors of means.

A total of 2 g of each residue was shaken with 50 mL of GFJ at 200 rpm at room temperature for 8 h. Concentrations of the DHB and BM remaining in the GFJ or bound to the various residues were analyzed by HPLC. Because of the high protein content of cooked, dried salmon (60−70%),[23] only the defatted residue 1 of salmon was tested for GFJ FC sequestration.

Preparation of Salmon Proteins and Analysis of GFJ FC Sequestration. A total of 5 g of defatted salmon powder (residue 1) was homogenized with 50 mL of 0.025 M potassium phosphate buffer (pH 7.25), and the homogenate was stirred for 2 h at 4 °C. The resulting slurry was centrifuged at 10000*g* for 15 min, and the supernatant was decanted and saved as a crude protein extract. This extract was collected 3 times by rehomogenizing, stirring, and centrifuging the remaining pellet residues with additional 50 mL aliquots of the phosphate buffer. The protein of the combined extracts was precipitated at 0 °C with ammonium sulfate (80% saturation), and the precipitate was dissolved in 20 mL of 0.025 M potassium phosphate buffer (pH 7.25) and dialyzed through Spectra/Por membrane [molecular weight cut-off (MWCO) = 12 000−14 000 kDa, Spectrum Laboratories, Inc., Rancho Dominguez, CA] against the same buffer. The protein concentrate (15 mL of 10 mg of protein mL^{-1}) was added to GFJ concentrate (15 mL), and the resulting sample was shaken for 18 h at 4 °C. GFJ concentrate (15 mL) with no added salmon protein was shaken with the same volume of

Table 1. Recovery (%) of DHB, BM, and the Minor-Occurring 726 and 708 Da Dimers from Different Sources of Foods after Mixture with GFJ[a]

	dry weight of foods used (g)							
	1.0				2.0			
	DHB	BM	726 Da	708 Da	DHB	BM	726 Da	708 Da
beef (10% fat)	39.5 a	76.2 a	76.6 a	87.8 a	57.7 a	75.9 b	84.7 b	95.1 a
beef (30% fat)	36.1 a	71.7 a	70.8 a	81.4 a	41.6 a	78.7 b	86.6 b	97.8 a
salmon	43.5 a	77.1 a	79.8 a	80.5 a	53.9 a	93.5 a	100.0 a	100.0 a
mushroom	12.0 b	74.4 a	81.4 a	87.0 a	35.3 b	80.6 b	87.4 b	93.7 a
bread	10.5 b	63.8 a	68.1 a	76.5 a	31.1 b	73.4 b	79.8 b	93.8 a
corn	15.7 b	65.8 a	76.6 a	77.6 a	21.3 b	70.4 b	93.8 b	95.5 a
rice	0.0 c	59.4 a	73.5 a	80.0 a	4.1 c	73.8 b	72.4 b	96.6 a
control[b]	0.0 c	0.0 b	0.0 b	0.0 b	0.0 c	0.0 c	0.0 c	0.0 b

[a]The percent recovery of DHB and BM from foods was calculated on the basis of concentrations of FCs in control, untreated GFJ samples. Letters indicate a significant difference between means of triplicates within columns by LSD (p = 0.05). [b]DHB and BM were not detected in dried foods used in this study and set to 0.0%.

0.025 M phosphate buffer and subjected to the same preparation procedures. The buffer-treated GFJ sample as well as the salmon protein-treated GFJ sample were centrifuged at 10000g for 20 min at 4 °C, and the resulting supernatants were passed through 0.45 μm polytetrafluoroethylene (PTFE) membrane filters (SUN-SRi, Rockwood, TN). The filtered GFJ samples (2.0 mL) were applied to a gel filtration column (2.5 × 33.0 cm) prepared with Sephadex G-100 resin (Sigma). Fractions (1.2 mL fraction^{-1}) were collected after the void volume (~25 mL), determined in a previous run with Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) at a flow rate of 0.3 mL min^{-1}. Proteins in the eluted fractions were analyzed by the Bradford protein assay.[21] Fractions containing proteins were combined as a protein fraction, while later-eluting fractions not containing proteins were combined as a non-protein fraction. The GFJ FCs in both fractions were analyzed by HPLC.

Interactions of GFJ FCs with Carbohydrates. GFJ (50 mL) was mixed with 2 g of cellulose (Sigma), pectin (Sigma), and isolated cell wall powder of *A. bisporus* for 4 h and filtered through Whatman No. 1 filters. Mycelial tissue of *A. bisporus* was obtained from cultures as described in Myung et al.,[13] and cell wall material of the mycelia was isolated as described by Novaes-Ledieu and Garcia-Mendoza.[24] The resulting filtrates were subjected to HPLC analysis.

Interactions of GFJ FCs with BSA. GFJ (30 mL) was shaken with or without 1.8 g of BSA (Sigma) for 4 h. The shaken samples were centrifuged at 10000g for 20 min, and the supernatant was collected (fraction F1 in Figure 4). The supernatant was passed through a 0.45 μm filter to produce F2 (Figure 4), and the resulting filtrate was additionally passed through an Anotop 0.20 μm membrane filter (Alltech, Deerfield, IL) to produce F3 (Figure 4). The FCs in these fractions were analyzed by HPLC. A total of 4 mL of F3 were also dialyzed against four 1 L changes of water over 24 h.

Fluorescence Measurements of GFJ FC Protein Binding. Fluorescence spectra were recorded with a Perkin-Elmer LS-55 spectrofluorometer. Fluorescence binding assays were run according to Soares et al.,[25] with modifications. Quenching the intrinsic tryptophan fluorescence of the model protein BSA was measured with 282 nm excitation (slit width of 2.5 nm) and 348 nm emission (slit width of 10 nm). Binding assays were measured with BSA (0.16 μM) in 0.10 M potassium phosphate buffer at pH 6.8. Spectra of the tryptophan fluorescence of BSA were scanned between 290 and 450 nm with 282 nm excitation. Aliquots (5, 10, and 15 μL) of stock solutions (2.50, 1.25, 0.83, 0.50, 0.25, and 0.12 mM) of FC standards in DMSO were added to 3.0 mL portions of the protein solutions. Contributions of these added volumes of DMSO to the fluorescence quenching were measured and were negligible. GFJ FCs exhibit high intrinsic fluorescence with emission wavelength maxima typically above 500 nm[26] but also exhibit low fluorescence intensity at 348 nm at the higher concentrations (>10 μL) used in the binding assays. These low levels of fluorescence at 348 nm were measured separately in the buffer blanks (minus BSA) and subtracted from the 348 nm fluorescence emission measurements with BSA.

Data Analyses. All experiments were performed with at least three replicates, and analysis of variance and Fisher's least significant difference (LSD) at p = 0.05 were applied to determine significant differences among the means of controls and treatments using STATISTICA 7.0 (Statsoft Co., Tulsa, OK).

RESULTS

Sequestration of GFJ FCs by Foods. The sequestration of DHB, BM, and the minor-occurring 708 and 726 Da dimers in 50 mL aliquots of GFJ by 1.0 and 2.0 g portions of different foods is shown in Table 1. Sequestration of the more polar DHB exhibited distinct differences between cooked beef and salmon versus mushroom, bread, and corn. For the cooked meats, sequestration of DHB ranged from 36.1 to 43.5%, whereas DHB sequestration by the remaining foods ranged from only 10.5 to 15.7%. There was a similar difference among these groups of foods in the sequestration by 2.0 g amounts. Negligible sequestration of DHB occurred by either 1.0 or 2.0 g of rice. In contrast, much higher amounts of sequestration occurred for the more lipophilic BM and 708 and 726 Da dimers in 50 mL of GFJ by both 1.0 and 2.0 g of the dried powdered foods. There were modest increases in the sequestration of these compounds by 2.0 g versus 1.0 g amounts. Unlike the negligible interactions between DHB and rice, 1.0 and 2.0 g of rice appeared to sequester large portions of the BM and 708 and 726 Da dimers. For 1.0 g of rice, substantially more of the 726 and 708 Da dimers were bound (73.5 and 80.0%, respectively) than BM (59.4%).

Interactions between Food Macromolecules and GFJ FCs. The binding of GFJ FCs to food lipids was examined by comparing FC sequestration by whole foods versus defatted food residues. Total lipids of corn and salmon (Figure 2A) were removed, and the resulting defatted powders (residue 1) were evaluated for their sequestration of DHB and BM in 50 mL of GFJ. As shown in Figure 2B, sequestration of 32.4% of the DHB in 50 mL of GFJ occurred with 2.0 g of defatted corn residue 1 in contrast to 23.1% with the original non-defatted corn powder and 56.6% of the DHB was sequestered by 2.0 g of defatted salmon powder compared to 70.7% sequestration by the original salmon powder. Opposite trends were observed with BM, where 55.7% of the BM in 50 mL of GFJ was sequestered by 2.0 g of defatted corn residue 1 in contrast to 74.7% with 2.0 g of the original corn powder. More binding of BM occurred with

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defatted salmon (90.3%) in contrast to the original dried powdered salmon (64.9%) (Figure 2B).

The potential binding of GFJ FCs by complex food carbohydrates was evaluated by similar studies with purified cellulose and pectin and lyophilized cell wall powder isolated from *A. bisporus*, with the latter composed largely of chitin and chitosan.[24] Mixing 50 mL portions of GFJ with 2.0 g amounts of pectin, cellulose, and *A. bisporun* cell walls at room temperature for 2 h yielded no sequestration of the GFJ FC to these complex food carbohydrates (data not shown).

The binding of DHB and BM to the defatted corn residue 1 was subsequently compared to defatted corn residues from which the soluble proteins and carbohydrates were removed. This was accomplished by extractions of corn residue 1 with water at pH 4.0 or 10.0 to yield dried powdered residues 2A and 2B, respectively (Figure 2A). The resulting DHB binding to 2A and 2B was reduced by 67 and 70%, respectively, compared to the original residue 1, and the binding of BM to these same residues decreased by 57%. Further extractions at pH 2.0 or 12.0 at 80 °C had only small additional effects on the binding of DHB, but final total decreases of 79 and 96%, respectively, occurred in the binding of BM to these residues (3A and 3B) compared to the original residue 1 (Figure 2B).

Involvement of proteins in sequestration of GFJ FCs was further examined by several additional techniques, including gel filtration chromatography, selective pore-size membrane filtration, and dialysis. The first studies involved the use of gel filtration chromatography of putative complexes formed between the GFJ FCs and soluble proteins obtained from cooked salmon. As shown in Figure 3A, soluble salmon proteins eluted in early fractions (1−13) off the gel filtration column after the initial void volume. In a separate chromatographic run, the complete amounts of FCs in 0.45 μm PTFE-filtered GFJ were recovered in later-eluting fractions (40−55) with no detection of proteins (Figure 3B). In sharp contrast, when GFJ premixed with salmon proteins was applied to the gel filtration column, BM as well as the highly lipophilic 708 and 726 Da dimers coeluted with the early eluting salmon protein fraction and were absent in the later-eluting non-protein fractions (Figure 3C). Unlike these lipophilic FCs, only 7% of the DHB eluted with the main salmon protein fractions (Figure 3C).

Interaction of BSA with GFJ FCs. Further studies of the interactions between GFJ FCs and soluble proteins were made by analyzing the fractionation and direct binding of these compounds to BSA. Results in Figure 4A show that centrifugation of untreated GFJ (fraction F0) removed large portions of the GFJ FCs, except DHB in fraction F1, and additional amounts of FCs were also sequentially removed with 0.45 and 0.20 μm filters in fractions F2 and F3, respectively. In fraction F3, only approximately 10% of the BM and the 726 Da dimer remained in solution and complete removal occurred for the 708 Da dimer. In contrast, 33% of the more freely soluble hydrophilic DHB remained in solution after 0.20 μm filtration. However, with the addition of BSA to GFJ, nearly all of DHB, BM, and the 708 and 726 Da dimers were recovered through the entire fractionation (F0−F3) (Figure 4B), suggesting that these compounds remained soluble through association with BSA. Consistent with these findings were the observations that following dialysis of the −BSA GFJ F3, the DHB and the lipophilic FCs occurred largely in the dialysate (Table 2). However, following dialysis of the +BSA GFJ F3, the lipophilic BM and the 708 and 726 Da dimers were largely present (77, 88, and 90%, respectively) in the retentate (Table 2) and only trace

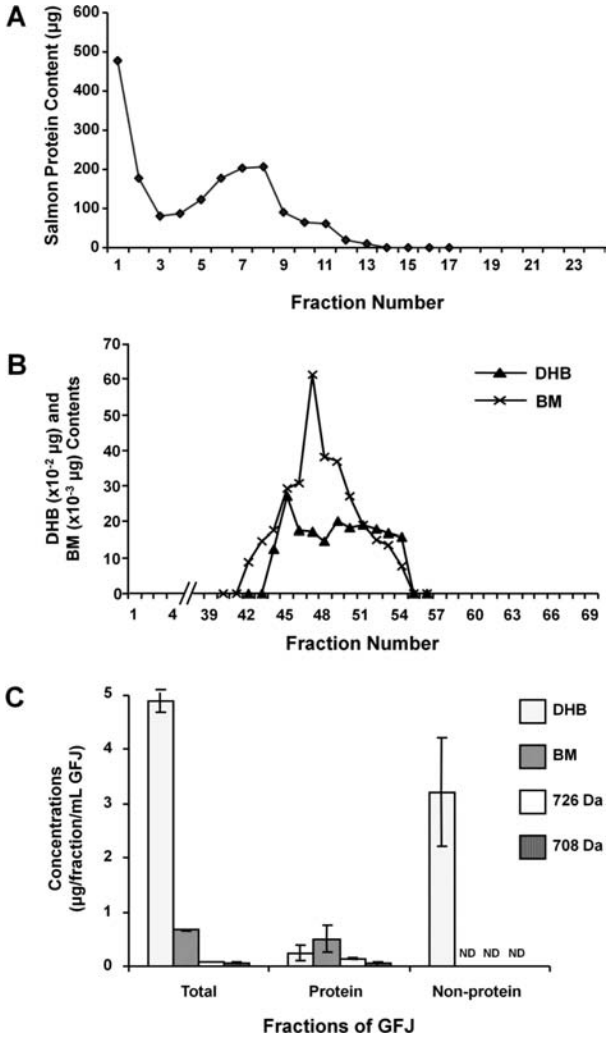

Figure 3. (A) Fractionations of total salmon proteins after gel filtration chromatography, (B) fractionations of DHB and BM in GFJ after gel filtration chromatography, and (C) contents of DHB, BM, and 726 and 708 Da dimers in protein or non-protein fractions after gel filtration of salmon protein-treated GFJ. Fractions containing proteins were collected and combined as the protein fraction, while fractions not containing proteins were also collected and combined as the non-protein fraction, followed by HPLC analysis. Bars represent standard errors of means. ND = not detected.

levels occurred in the dialysate, with the exception of DHB, of which 31% occurred in the dialysate. Corroborating findings were also made with gel filtration chromatography of the BSA-treated GFJ F3 (see Figure S1 of the Supporting Information).

In addition to the filtration studies discussed above, binding of GFJ FCs to proteins was further demonstrated by the quenching by DHB and BM of the intrinsic tryptophan fluorescence of BSA associated with ligand binding.[27,28] Figure 5 shows the quenching of the tryptophan fluorescence spectrum of BSA at pH 6.8 by BM and DHB, respectively. Plots of the binding curves ((F_c/F) versus [FC]) show a biphasic binding curve for BM at low concentrations (Figure 6). Fluorescence quenching curves with BSA at pH 6.8 by two other GFJ FCs, 6′,7′-epoxybergamottin and bergapten (5-methoxypsoralen), were nearly identical to DHB (data not shown).

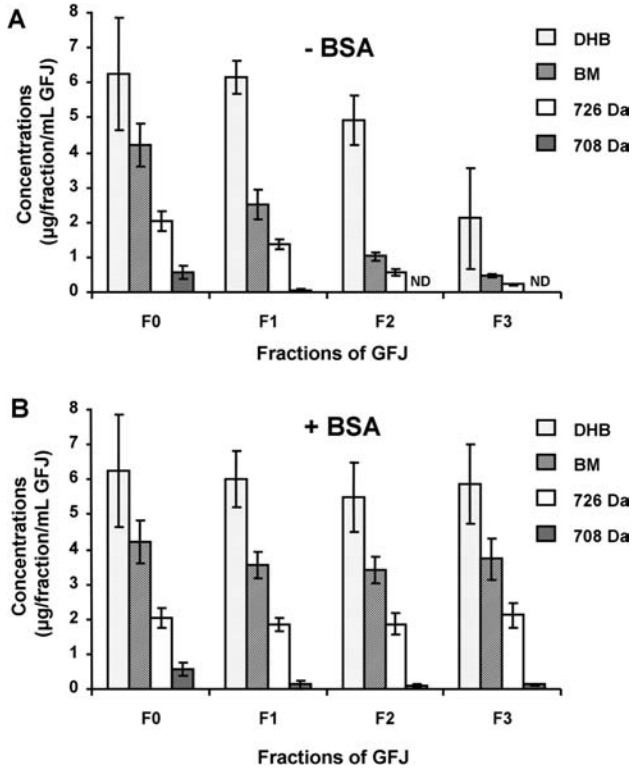

Figure 4. Concentrations of DHB, BM, and 726 and 708 Da dimers in (A) BSA-untreated (−BSA) or (B) BSA-treated (+BSA) GFJ before (F0) or after fractionations (F1−F3). GFJ samples were fractionated: F0, GFJ prior to fractionation; F1, GFJ supernatant after centrifugation at 10000*g* for 20 min; F2, 0.45 μm filtered GFJ after centrifugation; and F3, 0.2 μm filtered GFJ after 0.45 μm filtration. Bars represent standard errors of means. ND = not detected.

Table 2. Distribution of GFJ FCs Following Dialysis of ±BSA-Treated GFJ Fraction F3[a]

	total	retentate	dialysate
		+BSA GFJ Fraction F3	
DHB	23.5 ± 4.5	6.5 ± 3.4 (28)	14.2 ± 3.3 (31)
BM	14.9 ± 2.4	11.5 ± 1.7 (77)	0.5 ± 0.4 (3)
706 Da	8.5 ± 1.5	7.5 ± 1.8 (88)	0.3 ± 0.3 (3)
728 Da	2.1 ± 0.4	1.9 ± 0.7 (90)	0.5 ± 0.4
		−BSA GFJ Fraction F3	
DHB	8.4 ± 5.7	1.2 ± 0.9 (14)	12.5 ± 7.7 (100)
BM	1.9 ± 0.2	0.2 ± 0.1 (12)	0.9 ± 0.6 (48)
706 Da	0.9 ± 0.1	0.1 ± 0.0 (11)	0.5 ± 0.3 (57)
728 Da	ND[b]	ND	ND

[a]Values (μg of FC/4 mL of GFJ) are means ± standard deviations of assays run in triplicate. Values in parentheses are amounts (percent) relative to total amounts. [b]ND = not detected.

DISCUSSION

The sequestration of dietary phenolic compounds and the associated effects of protein−polyphenolic complex formation impact a number of important factors ultimately influencing digestion[29,30] and the oral bioavailabilities, pharmacokinetics, perception, and blood transport of these compounds.[31−36] These effects are likely to similarly impact the bioavailabilities of important citrus juice components, including the GFJ FCs. Supporting this was the observation of food and protein sequestration of the FCs in GFJ shown in Table 1, where 1.0 g portions of selected dried foods sequestered major portions of

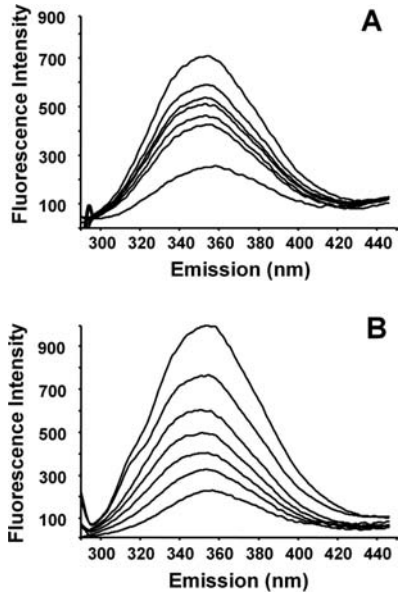

Figure 5. BSA (0.16 μM) tryptophan fluorescence quenching by (A) BM and (B) DHB with 282 nm excitation. BM concentrations ranged from 0, 0.103, 0.206, 0.257, 0.412, 1.03, 2.06, to 20.6 μM for spectra (top to bottom). DHB concentrations ranged from 0, 0.90, 1.80, 3.1, 5.9, 9.2, to 18.0 μM for spectra (top to bottom).

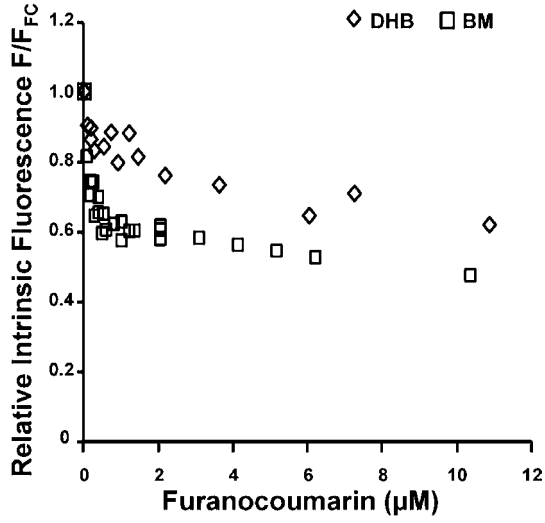

Figure 6. BSA (0.16 μM) in 0.10 M potassium phosphate buffer (pH 6.8) fluorescence quenching curve by (◇) DHB and (□) BM.

the lipophilic BM and the 708 and 726 Da modified dimers of BM from 50 mL of GFJ and nearly complete sequestration of the dimers occurred with 2.0 g portions of these foods. As reported for other polyphenolic−protein complex formations,[33,37,38] the lipophilic properties of the GFJ FCs played influential roles in this sequestration, as evidenced in this present study by the much higher sequestration of BM and the more lipophilic 708 and 726 Da dimers compared to DHB. Also shown in Table 1 were the contrasting levels of sequestration of DHB by the cooked meats compared to mushrooms, bread, corn, and rice, while these differences were not observed with BM and the other lipophilic compounds. A further difference between DHB and the lipophilic FCs was the lack of sequestration of DHB by rice, whereas rice sequestered large portions of the BM and the 708 and 729 Da dimers. The levels of sequestration of these latter

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compounds by rice were similar in magnitude to the levels of sequestration by cooked salmon. Considering the sharply contrasting compositions of these two foods,[23,39,40] it is reasonable to hypothesize that food sequestrations of GFJ FCs may likely involve a number of different mechanisms influenced by food composition as well as by the chemical structures of the GFJ compounds.

The role of food composition on GFJ FC sequestration was investigated next by measuring the binding by different classes of food macromolecules, including lipids, carbohydrates, and proteins. To test the possible involvement of food lipids, dried powdered corn and salmon were extracted with organic solvents to remove the lipids from these food materials. The removal of lipids from cooked salmon powder resulted in a 20% loss of DHB binding but a 39% increase in BM binding. This increase in the binding of BM by the lipid-depleted salmon meat rules out lipids as a major food component in the sequestration of the GFJ FCs, while it suggests a major role for the salmon meat proteins. The sharp losses in the GFJ FC sequestration for the extensively acid- and base-washed corn residues 2A and 2B and 3A and 3B were also supportive of the potential roles of soluble proteins in the sequestration of these GFJ compounds.

This involvement of soluble proteins in the GFJ FC sequestrations was further supported by the observations of putative protein−FC complexes by gel filtration chromatography. Pertinent to these observations is the fact that large portions of the lipophilic FCs in GFJ are associated with insoluble GFJ cloud particles,[41,42] where the major components of citrus juice cloud are insoluble proteins complexed with low-molecular-weight compounds.[41] As shown in Figure 3, combining GFJ with salmon proteins appeared to effectively solubilize BM and the 708 and 726 Da dimers away from the juice cloud particles. This solubilization of the particulate-associated FCs was also observed with dialysis and gel filtrations of +BSA-treated GFJ. Notable were the apparent sharp differences in protein−FC complex formation between BM and DHB with the salmon proteins and BSA, and in a manner similar to the differences in sequestration shown in Table 1, the polar DHB exhibited far less affinity for protein binding. This was further demonstrated by the differences in the BSA fluorescence quenching curves of DHB and BM in Figure 6, where BSA fluorescence quenching occurred at far lower BM concentrations compared to DHB.

The efficacy of food proteins as carriers for dietary flavonoids has been recently analyzed,[37] and elements of this study corroborate central findings made in our present study with the GFJ FCs. A variety of food proteins, including milk and egg proteins and fish gelatin hydrolysates were compared as carriers for flavan-3-ols. Results showed fish gelatin to be one of the best protein-flavonoid carriers, an observation consistent with our findings with the sequestration and binding of the lipophilic BM and its related dimers by cooked salmon and salmon proteins. Most efficient carriers of the flavonoids were flexible open structures, as present in random-coil proteins.[37] This is similar to the earlier finding by Haslam,[38] where open, random-coil types of conformations, particularly in the proline-rich caseins and gelatin, are particularly efficient binders of phenolic ligands. The enhanced involvement of random-coil, open protein structures as flavonoid carriers may have significance in mediating binding of phenolics in the gastrointestinal (GI) tract, where protein unfolding and denaturation occur because of the pepsin−HCl (pH 2) and pancreatin digestions with bile salts.[43] To better understand the degrees to which food sequestration and protein binding influence the bioavailability of the GFJ FCs, these conditions of the GI tract need to be considered further.

Finally, the binding of structurally diverse polyphenols to a number of plasma proteins, including human serum albumin, bovine γ-globulin, and hemoglobin, has been studied,[31−34,44] and the binding of the GFJ FCs to BSA observed in this present study suggests that similar binding of these compounds may also occur with structurally related human serum albumin. The sharply contrasting affinities of DHB and BM to BSA observed in this study suggest that significant differences may occur in the plasma protein transport and bound concentrations of these compounds in blood and, therefore, influence the pharmacokinetics and bioavailabilities of these compounds.[35] Importantly, multiple binding sites occur with human serum albumin, and binding to this carrier protein is influenced by allosteric effects induced by endogenous ligands,[35,36,45,46] much of which is influenced by the diet. Studies of these potential dietary influences on the serum protein binding of the GFJ FCs and on the resultant bioavailabilities of these compounds are important because of actions of these FCs in the grapefruit−drug interactions in humans.

■ ASSOCIATED CONTENT

Supporting Information

(A) Gel filtration chromatogram of the co-elution of BSA and BM and the separate elution of DHB in +BSA GFJ F3 and (B and C) total, retentate, and dialysate contents of grapefruit FCs in (B) +BSA GFJ F3 and (C) −BSA GFJ F3 after dialysis (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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