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Biochemical Analysis and in Vivo Hypoglycemic Activity of a Grape Polyphenol−Soybean Flour Complex

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ABSTRACT: Defatted soybean flour (DSF) can efficiently sorb, concentrate, and stabilize polyphenols, but not sugars, from Concord grape juice, to yield grape polyphenol-enriched DSF. Sorption of grape polyphenols to DSF particles was dependent on the ratio of DSF and grape juice concentrate used, but not time of mixing or pH. Depending on ratios of starting materials, 1 g of grape polyphenol-enriched DSF contained 1.6−10.4 mg of anthocyanins, 7.5−93.1 mg of proanthocyanidins, and 20.5−144.5 mg of total polyphenols. LC-MS analysis of grape juice samples before and after addition and removal of DSF and eluate from grape polyphenol-enriched DSF confirmed that a broad range of grape compounds were sorbed to the DSF matrix. Finally, grape polyphenol-enriched DSF was able to significantly lower blood glucose levels in hyperglycemic C57BL/6J mice. The data indicate that grape polyphenol-enriched DSF can provide a high-protein, low-sugar ingredient for delivery of concentrated grape polyphenolics.

KEYWORDS: *Concord grape juice, defatted soy flour, anthocyanins, proanthocyanidins, polyphenols, hypoglycemic, diabetes*

INTRODUCTION

Grapes are among the most popular fruits in the world and are consumed raw and dried (raisins) or as a juice. Multiple scientific studies and traditional beliefs have linked fresh grapes, grape extracts, wine, and individual phenolic compounds from grapes with beneficial health effects. For example, Concord grape (*Vitis labrusca*) juice diminished symptoms of insulin resistance, metabolic syndrome, and cognitive decline in animals and people.[1−3] The major classes of polyphenols contained in Concord grape juice are anthocyanins, which make up 46% of the total polyphenols, followed by tartarate esters of hydroxycinnamic acids (29%) and proanthocyanidins (10%). Monomeric flavan-3-ols, flavonols, hydroxybenzoic acids, and free hydroxycinnamic acids comprise 15% of the phenolic content, whereas stilbenes, such as resveratrol, account for <1%.[4] The beneficial polyphenolic compounds in grapes coexist with considerable amounts of sugars (15%) and associated calories (288 kJ or 69 kcal/100 g). The glycemic index for grapes is 46 compared to 38 for apples and 25 for grapefruit.[5,6]

Polyphenols have a natural binding affinity for proteins, and insoluble protein−polyphenol complexes are formed most efficiently at pH values near the isoelectric point of the interacting proteins.[7] The interaction of polyphenols with bovine serum albumin (BSA) protein was found to be mediated by either hydrogen bonding in the case of polar polyphenols or hydrophobic interactions for nonpolar polyphenols.[8] Furthermore, in stoichiometry studies in which polyphenol was in excess over protein, precipitates were found to be composed of a fixed polyphenol to protein ratio, which may be a function of the size of the polyphenol molecule and the surface area of the protein.[8] Proline-rich proteins and proteins of >20 kDa appear to have the highest binding affinity for polyphenols due to their open conformation and greater surface area for interaction.[9]

Leveraging the binding affinity between polyphenols and proteins, we recently reported that defatted soybean flour (DSF) can sorb, concentrate, and stabilize polyphenol compounds from berry juices, while excluding highly polar sugars.[10] In this study we have applied the polyphenol sorption process to Concord grape juice. Sorption efficiency of grape polyphenols to the DSF matrix as a function of DSF concentration, the pH of the juice−DSF mixture, and incubation time was investigated. The major classes of grape polyphenols sorbed to the DSF matrix were determined by LC-MS analysis. As virtually all classes of grape polyphenols have been shown to have antidiabetic/antiobesity activity,[11] we also tested grape polyphenol-enriched DSF in an acute hyperglycemic mouse model to verify antidiabetic activity.

MATERIALS AND METHODS

Comparative Sorption of Grape Polyphenols. The polyphenol sorption capacity of full-fat soybean flour (FSF; Hodgson Mill Inc., Effingham, IL, USA), defatted soybean flour (DSF; Hodgson Mill

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Inc.), and soy protein isolate (SPI; Supro40, Solae, St. Louis, MO, USA) were compared. Concord grape juice concentrate (68 °Brix; Fruitsmart, Grandview, WA, USA) was diluted 4 times with water, and 5 g/L of FSF, DSF, or SPI was mixed with 10 mL of this grape juice. The effect of pH on sorption capacity was examined separately by adjusting the pH of the 3 times diluted grape juice after addition of 30 g/L of DSF to pH 2, 3, 5, 6, or 7. Time-dependence experiments were performed using DSF (100 g/L) mixed with 5 times diluted grape concentrate for 5, 15, or 30 min on a magnetic stir plate. In all cases triplicate samples were prepared for each condition tested. Juice−flour mixtures were centrifuged for 10 min at 3200*g* (Eppendorf, 5810R), and supernatants were filtered prior to quantification of grape anthocyanins, proanthocyanidins, or total polyphenols.

Quantification of Anthocyanins, Proanthocyanidins, Total Polyphenols, and Total Carbohydrates. The pH differential method[12] was used to measure total monomeric anthocyanins in grape juice, and absorbance was measured at 520 and 700 nm with a UV−vis spectrophotometer (Shimadzu UV-2450 or Synergy HT Multi-Detection Microplate Reader, BioTek). Concentration of monomeric anthocyanins (mg/L) was calculated as cyanidin 3-*O*-glucoside equivalents. The 4-dimethylaminocinnamaldehyde (DMAC) assay[13] was used to quantify total proanthocyanidins. Sample absorbance was determined at 640 nm against a proanthocyanidin B2 (ChromaDex, Irvine, CA, USA) standard curve. Total polyphenols were quantified according to the Folin−Ciocalteu method,[14] and samples were read at 726 nm against a gallic acid (Sigma) standard curve. The levels of anthocyanins, proanthocyanidins, or total polyphenols bound to the DSF were calculated by subtracting their concentration in the DSF-treated juice supernatants from that measured in untreated juice samples and dividing by grams of DSF added. Total carbohydrates in grape juice samples were quantified by using the phenol−sulfuric acid method,[15] which measures hydrolyzed neutral sugars in oligosaccharides, proteoglycans, glycoproteins, and glycolipids. Juice samples were read against a standard curve made from a 1:1 solution of fructose and glucose.

Biochemical Composition. DSF (100 g/L) was mixed with triplicate 20 mL volumes of 3 times diluted grape juice. Grape juice and DSF were separated by centrifugation. The original juice sample and the three juice supernatants were subjected to C18 SPE column (J. T. Baker, Phillipsburg, NJ, USA) purification to remove sugars prior to LC-MS analysis. Briefly, the same volume (400 μL) of each juice sample was applied to individual C18 SPE columns, and each column was washed with 3 bed volumes (15 mL total) of acidified water (1% acetic acid). Each sample was eluted from the C18 SPE column with 4 volumes of methanol containing 1% acetic acid, and then each 20 mL sample was concentrated to a final volume of 1 mL by rotary evaporation (Büchi, Flawil, Switzerland) prior to LC-MS. The three samples of grape polyphenol-enriched DSF solids were subjected to six rounds of elution with 20 mL volumes of acidified methanol (methanol/water/acetic acid, 75:20:5), and eluates were concentrated to 20 mL prior to LC-MS analysis to confirm the presence of compounds in DSF matrix. Samples were separated and analyzed with UPLC-MS (Dionex Ultimate 3000 RSLC UPLC system) and a triple-quadrupole Varian 1200 L (Varian Inc., Palo Alto, CA, USA) mass spectrometer equipped with electrospray ionization (ESI) interface using a Phenomenex RP C8 reverse-phase column (size 150 mm × 2 mm, 3 μm particle size). LC-MS conditions have been previously described in Roopchand et al.[10] The integrated mass peak area for each compound was quantified for the original grape juice sample and in the three juice supernatant samples, which were then averaged. For each compound, the difference in mass peak area between original juice sample and averaged juice supernatants was calculated and used to estimate the percentage of individual compounds sorbed to the DSF matrix (i.e., removed from the original juice). Quantification of cyanidin 3-*O*-glucoside in the samples was performed against a standard curve of cyanidin 3-*O*-glucoside standard (Polyphenols Laboratories, Sandnes, Norway).

To quantify the sorption of resveratrol, DSF (30 g/L) was added to 50 mL of store-bought organic Concord grape juice (Santa Cruz Organic, Orville, OH, USA) and mixed at room temperature for 5 min. The juice was separated from the flour by centrifugation and filtered through a 0.22 μm syringe filter. Grape juice samples before and after addition and removal of DSF were prepared according to a previously published method[16] prior to LC-MS quantification of resveratrol. Resveratrol-spiked (20 μg) and nonspiked apple juice samples were subjected to the same extraction and quantification procedures to calculate recovery. Resveratrol was quantified against resveratrol standard (Sigma). Data are the average of two independent experiments that yielded similar results.

Hypoglycemic Effect of Grape Polyphenol-Enriched DSF. Grape juice was diluted 2 times, and polyphenols were sorbed to 100 g/L DSF. The amount of anthocyanins bound to DSF was estimated as described above. As a control, DSF was also mixed with water acidified with citric acid to match the pH of the DSF−grape juice mixture (pH 3.5). The juice and water-treated DSF mixtures were centrifuged, and pellets were freeze-dried and powdered. Grape polyphenol-enriched DSF and the water-treated control DSF were formulated in 75% Labrasol (Gattefossé Corp., Paramus, NJ, USA) for in vivo experiments. The protocol was approved by the Rutgers University Institutional Care and Use Committee and followed federal and state laws. Five-week-old male C57BL/6J mice (10−20 g) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and fed a regular diet ad libitum (Purina, 5015) during their 1 week acclimatization period. At 6 weeks, mice were placed on a very high fat diet (VHFD; 60% kcal fat; Research Diets D12492) for 12 weeks to induce obesity and hyperglycemia. Body weights were measured weekly. Mice were randomly divided into experimental groups (n = 5 mice per group), fasted for 4 h, and then gavaged with indicated doses of grape polyphenol-enriched DSF or DSF control (300 mg/kg) formulated in a 75% Labrasol−water solution (vehicle). Blood glucose readings were taken using a glucometer (AlphaTRAK 32004-02, Abbott Animal Health); animals were fasted during the testing period. Metformin (300 mg/kg; water solution) was administered as positive control.

Statistics. Statistics were performed with Statistica v. 10 (StatSoft). One-way ANOVA was used to determine significance among three or more groups followed by the indicated posthoc test. Paired t tests were performed within groups (before vs after treatment).

RESULTS AND DISCUSSION

Sorption of Grape Polyphenols to Different Soybean Products. The capacities of FSF, DSF, and SPI (5 g/L) to sorb grape polyphenols from 4 times diluted Concord grape juice concentrate were compared. The efficiency of sorption was correlated with the amount of protein in the matrix, and SPI sorbed the highest levels of grape anthocyanins, proanthocyanidins, and total polyphenols, followed by DSF and FSF (Table 1). The anthocyanin level in Concord grapes has been estimated at 1.2 mg/g of fresh weight;[17] thus, a serving of grapes (46 g or a half cup)[18] delivers 55 mg of anthocyanins. Therefore, 3.9, 5.3, and 8.2 g of grape polyphenol-enriched SPI, DSF, and FSF matrix, respectively, can concentrate and deliver

Table 1. Sorption of Polyphenols from Concord Grape Juice to Different Soy Products[a]

matrix	% protein[b]	ACNs[c] (mg/g)	PACs[d] (mg/g)	TP[e] (mg/g)
FSF	33	6.7 ± 1.4 a	45.6 ± 9.9 a	74.4 ± 5.1 a
DSF	47	10.4 ± 0.5 a	62.6 ± 7.2 a	144.5 ± 14.8 b
SPI	82	14.3 ± 2.3 b	93.1 ± 7.7 b	280.3 ± 39.4 c

[a]Flour concentrations were 5 g/L in 4 times diluted grape juice Different letters indicate significant differences (ANOVA, Tukey post hoc, $p < 0.05$). [b]Based on product nutrition label. [c]Calculated as cyanidin 3-*O*-glucoside equivalents. [d]Calculated as proanthocyanidin B2 equivalents. [e]Calculated as gallic acid equivalents.

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the level of anthocyanins present in one serving of Concord grapes. DSF was used as the matrix in remaining analyses.

DSF Sorbs and Concentrates Grape Polyphenols, But Not Sugars. The relationship between DSF concentration and sorption of major classes of grape polyphenols was further examined by adding increasing amounts of DSF (10, 30, or 100 g/L) to a 4 times dilution of grape juice concentrate. As the concentration of DSF added to the juice increased, the levels of anthocyanins, proanthocyanidins, and total polyphenols sorbed to the matrix decreased (Figure 1A). To determine whether the matrix also sorbed sugars from the grape juice, the concentration of total carbohydrates was measured in the original grape juice and in the grape juice supernatants after the addition and removal of increasing amounts of DSF.[15] There were no significant differences in sugar concentration, indicating that sugars were not sorbed or concentrated by the DSF matrix, but rather remain in the juice (Figure 1B).

pH and Time Dependence of Polyphenols Sorption to DSF. The grape juice concentrate had a pH of 3.5, which is near the isoelectric point of soybean proteins. To determine whether sorption efficiency could be improved at lower or higher pH, aliquots of 3 times diluted grape juice were mixed with 30 g/L of DSF, and HCl or NaOH was used to adjust the pH to 2, 3, 3.5, 5, or 7. The amount of total polyphenols and anthocyanins sorbed to the DSF was estimated as described under Materials and Methods. As shown in Figure 2A, the amount of polyphenols or anthocyanins sorbed to the DSF matrix remained optimal at pH 3.5, but decreased at lower or higher pH values. Anthocyanins are unstable in neutral/basic environments; therefore, sorption to DSF could not be accurately measured at pH 7. Increasing the incubation time of DSF and 5 times diluted grape juice from 5 to 30 min did not increase the level of grape anthocyanins, proanthocyanidins, or total polyphenols sorbed to DSF (Figure 2B).

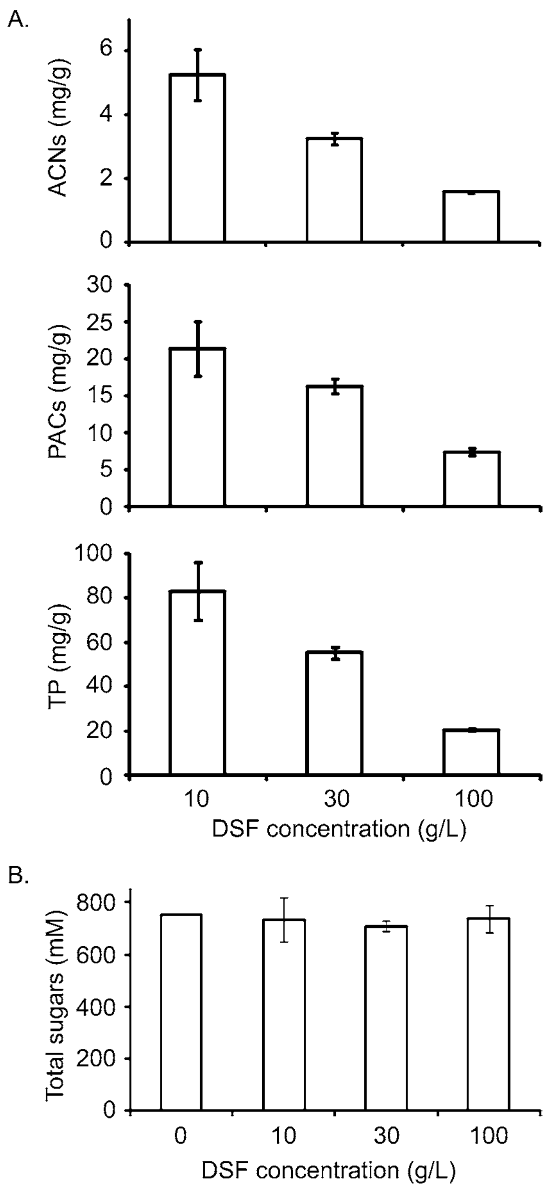

Figure 1. Sorption of grape polyphenols to defatted soybean flour (DSF): (A) amounts of grape anthocyanins (ACNs; cyanidin 3-*O*-glucoside equivalents), proanthocyanidins (PACs; proanthocyanidin B2 equivalents), and total polyphenols (TP; gallic acid equivalents) sorbed to increasing amounts (g/L) of DSF from 4 times diluted grape juice concentrate; (B) concentration of total carbohydrates present in 4 times diluted grape juice concentrate before (0 g/L DSF) and after addition and removal of 10, 30, and 100 g/L of DSF.

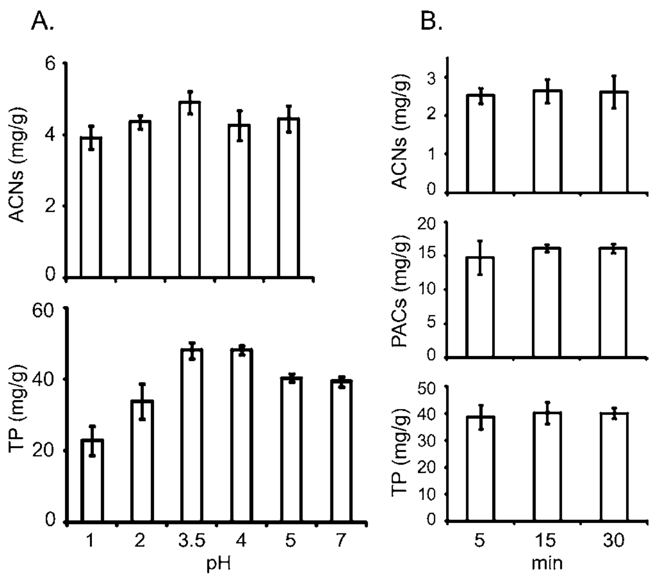

Figure 2. pH and time dependence of grape polyphenol sorption to DSF: (A) amounts of grape anthocyanins (ACNs) and total polyphenols (TP) sorbed to DSF after adjustment of DSF–juice mixture to indicated pH values; (B) concentration of grape ACNs, PACs, and TPs sorbed to DSF after mixing of DSF (100 g/L) and 5 times diluted grape concentrate for 5, 15, or 30 min.

Biochemical Analysis of Grape Polyphenol-Enriched DSF. We investigated which polyphenols contained in Concord grape juice could be sorbed to the DSF matrix and the relative effectiveness of their sorption. DSF (100 g/L) was mixed with 3 times diluted grape juice concentrate in triplicate, and solids were separated from the juice. Grape juice samples taken before and after the addition and removal of 100 g/L DSF were purified by C18 SPE cartridges to remove sugars prior to LC-MS analysis. The grape polyphenol-enriched DSF solids were eluted with acidified methanol as described, and eluates were also subjected to LC-MS analysis to confirm the presence of compounds in the DSF matrix. Table 2 summarizes the masses, major mass fragments, and retention times corresponding to the grape compounds sorbed to the DSF matrix (i.e., removed

Table 2. Concord Grape and DSF Compounds Eluted from Grape Polyphenol-Enriched DSF Matrix

compound	peak mass area ($\times 10^8$)	% sorbed to DSF	MS (m/z) ESI $[M]^+$	MS (m/z) ESI $[M + H]^+$	MS (m/z) ESI $[M - H]^+$	fragments (m/z)	t_R (min)
Concord Grape							
cyanidin 3-*O*-glucoside	110.6	67	449			287	7.8
delphinidin 3-*O*-glucoside	109.4	69	465				6.9
malvidin 3-*O*-glucoside	34.26	58	493				8.7
peonidin 3-*O*-glucoside	27.61	65	463				8.6
petunidin 3-*O*-glucoside	46.6	63	479				7.9
cyanidin 3-*O*-(6″-*O*-acetyl)glucoside	18.7	67	491			287	9.7
delphinidin 3-*O*-(6″-*O*-acetyl)glucoside	39.27	76	507			303	9.0
petunidin 3-*O*-(6″-*O*-acetyl)glucoside	17.57	68	521			317	9.8
malvidin 3-*O*-(6″-*O*-acetyl)glucoside	10.06	59	535			331	10.4
cyanidin 3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	58.32	90	595			287	11.5
peonidin 3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	9.754	81	609				12.2
delphinidin 3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	120.2	92	611				10.8
malvidin 3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	20.01	73	639				12.2
peonidin 3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside	5.965	31	771				11.5
delphinidin 3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside	21.48	55	773			303	10.1
petunidin 3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside	9.395	33	787			317	10.8
malvidin 3-*O*-(6″-*p*-coumaroyl)-5-*O*-diglucoside	15.88	31	801			301	11.5
quercetin	13.74	94			301		16.0
quercetin 3-*O*-glucoside and -galactoside	9.837	62			463		12.0
quercetin 3-*O*-glucoronide	55.24	83			477		12.8
myricetin *O*-glycoside	10.01	55			479		11.1
myricetin *O*-glucoronide	7.2	82			493		11.9
coumaric acid	33.73	67			163		12.5
caffeic acid	27.89	66			179		10.5
ferulic acid	44.58	91			193		2.2
coutaric acid	107.9	69			295		10.9
caftaric acid	179.2	69			311		9.1
fertaric acid	45.69	74			325		11.4
(epi)C-(epi)C dimer	4.128	49			577		9.7
catechin	3.204	43			289		9.3
(epi-)catechin	11.51	47			289		10.3
epicatechin 3-*O*-gallate	3.737	90			441		12.4
DSF							
daidzin				417		255	12.85
genistin				433		271	15.67
acetyldaidzin				459		255	17.66
acetylgenistin				475		271	20.69
malonyldaidzin				503		255	15.89
malonylgenistin				519		271	18.38
malonylglycitin				533		285	16.04

from the juice) and confirmed in eluates of the grape polyphenol-enriched DSF. DSF compounds were also detected in the eluates. The peak mass areas for compounds detected in the untreated grape juice are shown and were used to estimate the percentage of each compound sorbed by the DSF matrix as described under Materials and Methods. DSF sorbed 58−76% of the individual anthocyanins present as monoglucosides, either with or without acetyl moieties. DSF also sorbed 73−92% of anthocyanin monoglucosides containing coumaroyl moieties and 31−55% of anthocyanin diglucosides containing coumaroyl moieties. In parallel, the levels of cyanidin 3-*O*-glucoside in grape juices before and after the addition and removal of DSF were quantified by LC-MS using cyanidin 3-*O*-glucoside standard. The concentration of cyanidin 3-*O*-glucoside in the untreated grape juice sample was estimated to be 465 mg/L, and the DSF matrix sorbed 67% of this amount; therefore, the concentration of cyanidin 3-*O*-glucoside in the flour was estimated to be 3.1 mg/g. Assuming that cyanidin 3-*O*-glucoside comprises 10% of the total anthocyanin content of Concord grape juice, as recently reported,[4] the total anthocyanins concentration in the untreated juice can be estimated to be 4.65 g/L. This anthocyanin concentration estimated by LC-MS is about 20 times higher than the concentration calculated using the pH differential method (207 mg/L); therefore, the colorimetric method may significantly underestimate anthocyanin concentrations. In addition to anthocyanins, DSF sorbed 66−91% of hydroxycinnamic acids, 49% of dimeric proanthocyanidins, 43−90% of individual catechins, and 55−94% of flavonols (Table 2).

The ability of DSF to sorb resveratrol from grape juice was determined after the addition and removal of DSF (30 g/L) to a 50 mL sample of Concord grape juice. The untreated juice and juice supernatant samples were prepared as described under Materials and Methods and then analyzed by LC-MS. Resveratrol-spiked (20 μg) apple juice was processed in identical fashion, and recovery was estimated to be 68%. The initial concentration of resveratrol in grape juice was 1.86 μg/mL, and after the addition and removal of DSF from the grape juice, the resveratrol level was below the limit of detection (<10 ng), indicating that DSF was able to sorb most of the 93 μg of the resveratrol initially present in the juice.

The efficiency of grape polyphenol sorption to the DSF matrix is a function of binding affinity between individual compounds and the DSF matrix, the relative concentrations of the various compounds in the juice, and the availability of binding sites in the matrix. The contributions of each of these factors are compound-specific, and an accurate quantification of the binding efficiency for each polyphenol would require individual standards. Assuming equal affinity of sorption, compounds present in the grape juice at high concentration (e.g., cyanidin 3-*O*-glucoside) have a lower percentage, but higher amount, of compound sorbed than compounds present in low amounts (e.g., resveratrol).

Hypoglycemic Activity of Grape Polyphenol-Enriched DSF. Anthocyanin-enriched extracts from blueberries[19] as well as blueberry polyphenol-enriched DSF[10] have a hypoglycemic effect in diabetic C57BL/6J mice. To verify whether grape polyphenol-enriched DSF would have a similar hypoglycemic effect, we compared single-dose administration of vehicle (VEH; 75% Labrasol), grape polyphenol-enriched DSF (G-DSF), and DSF in C57BL/6J mice. Metformin (MET) was administered as a positive control. Before treatment (t = 0 h), all groups had similar levels of blood glucose (ANOVA, p = 0.14); however, significant differences between groups were observed 6 h post-treatment (ANOVA, $p = 7.2 \times 10^{-4}$). The 300 and 600 mg/kg doses of grape polyphenol-enriched DSF significantly reduced blood glucose levels in mice compared to VEH or DSF alone (Figure 3). The amount of anthocyanins in the grape polyphenol-enriched DSF was 2.5 mg/g; therefore, the 300 and 600 mg/kg doses of grape polyphenol-enriched DSF delivered 0.75 and 1.5 mg/kg of anthocyanins, respectively. The data indicate that grape polyphenol-enriched DSF preserves the antidiabetic activities of grape anthocyanins and other polyphenols. The blood glucose lowering effect was also an indirect demonstration that grape polyphenols sorbed to DSF are bioavailable. These data suggest that grape polyphenol-enriched DSF may provide a novel ingredient for the creation of nutritious, low-sugar, and high-protein food ingredients useful for the dietary management of diabetes or metabolic syndrome.

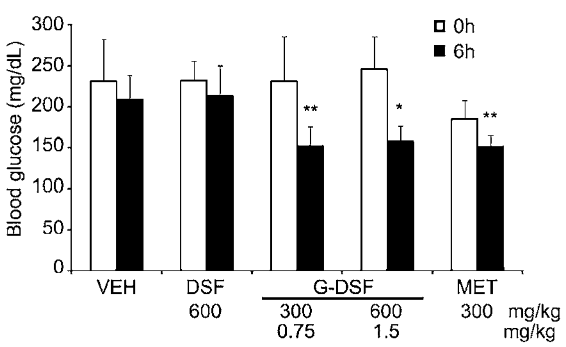

Figure 3. Hypoglycemic activity of grape polyphenol-enriched DSF. Blood glucose levels of C57BL/6J mice were taken before and 6 h after treatment with 75% Labrasol (VEH), DSF, grape polyphenol-enriched DSF (G-DSF), or metformin (MET). The second row of numbers represents the amount of anthocyanins (ACNs) delivered in the indicated dose of grape polyphenol-enriched DSF. Each bar represents the mean ± SD (n = 5) of data. *, p = 0.05; **, p = 0.01 (Dunnett's test relative to DSF group). Significance was confirmed within groups using two-tailed, paired t tests.

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Notes

The authors declare the following competing financial interest(s): D.E.R., M.A.L., B.F., and I.R. have equity in Nutrasorb LLC, which has interest in developing polyphenol sorption technology.

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ABBREVIATIONS USED

ACN, anthocyanins; PAC, proanthocyanidins; TP, total polyphenols; DSF, defatted soybean flour; FSF, full-fat soybean flour; SPI, soy protein isolate.

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