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Physico-chemical Characteristics of Nanovesicle−Carbohydrate Complexes in Grape Juice Concentrate

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It is generally assumed that polyphenols, such as anthocyanins in fruit juice, exist in a free soluble state and are readily available for absorption in the gastro-intestinal tract. In the present study, we have investigated the interaction of polyphenols with soluble carbohydrate polymers, such as pectin and lipid nanovesicles, that are generated during homogenization of the fruit tissue during juice extraction. A commercially available grape juice concentrate contained nearly 25% of polyphenol fraction bound to macromolecules that were nondialyzable. Treatment of dialyzed juice with cellulase, pectinase, and β-galactosidase did not cause the release of bound polyphenols; however, treatment with triton X-100 caused an increased release of bound polyphenols. The dialyzate contained relatively more -3-O glucosides and -3-O-acetoyl glucosides in comparison to the bound fraction which was enriched in -3-O-coumaroyl glucosides, suggesting qualitative differences in the bound and the free anthocyanin composition. Electron microscopic analysis of the juice fractions revealed the presence of electron-dense nanovesicle−fiber complexes ranging from 10 to 200 nm in diameter. Such complexes were absent in the dialyzate fraction. Cellulase treatment did not change the morphology of the complexes; however, treatment with pectinase and β-galactosidase disrupted the complexes, releasing vesicular structures, suggestive of the pectin nature of the fibrous matrix. The dialyzed and the dialyzate fractions also showed differences in their ^{1}H NMR and fluorescence spectral characteristics. The dialyzed fraction containing polyphenol−pectin complexes showed no superoxide scavenging capacity, reduced hydroxyl radical scavenging activity, and high 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, indicating potential changes in functionality because of the complex formation.

KEYWORDS: Polyphenols; grape juice; carbohydrates; glucosides; anthocyanin

INTRODUCTION

Macromolecular organization of food involves several types of chemical interactions between food monomers and polymers manifesting into cells and tissues. Food microstructure is the organization of elements within a food and their interactions. Knowledge about the microstructure of foods is vital to control the properties of foods, because there is a causal connection between structure and functionality (*1*). The microstructural elements in plant-derived foods include cell walls, starch granules, proteins, water and oil droplets, fat crystals, gas bubbles, and so forth. The nutrients found in the natural cellular compartments or within the assemblies produced during processing need to be released prior to or during digestion for their efficient absorption in the intestine. Nutrient absorption depends not only on the complete disruption of the cellular structure but also on the presence of and interaction with other food components. In addition, the physical state of the food matrix plays a key role in the release, mass transfer, accessibility, and biochemical stability of many food components (*2*). The food microstructure is an important factor in the release and bioavailability of several nutrients and has enormous consequences in assessing the nutraceutical properties of foods used in the prevention and therapy of some chronic degenerative diseases (*3*).

Polyphenols are the most widely occurring phytochemicals with a considerable physiological and morphological importance in plants, and these secondary metabolites are thus an abundant source of nutraceuticals in our diet. They have been proposed and shown to play several roles in the prevention of degenerative diseases by acting as antiallergic, antimicrobial, anti-inflammatory, antiatherogenic, antioxidant, cardio-protective, and vasodilatory agents. Grapes are rich sources of phenolic compounds such as flavan-3-ols (catechins), anthocyanins (in red grapes), and flavonols (*4*). Anthocyanins are water-soluble

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pigments responsible for all the color differences between grapes and the resulting wines. They have also been associated with the health benefits derived from consuming high levels of fruits, vegetables, and wine. However, the bioavailability of anthocyanins is estimated to be very low, and their metabolism is still not fully understood. Because a major part of the polyphenols ingested (75–99%) is not found in urine, they may have been either not absorbed through the intestinal barrier, absorbed and excreted in the bile, or metabolized by colonic microflora (*3*). McDougall et al. reported that polyphenols are transiently bound to food matrices during digestion and that this protects the more labile anthocyanins from degradation (*5*).

In most plants, anthocyanins are found uniformly dissolved in the vacuolar solution of epidermal cells. However, in certain species, they are found localized in discrete regions of the cell vacuole (*6*). Such pigmented bodies, which are membrane-bound organelles that provide an intense coloration in the vacuoles of mature plant cells, have been described by Pecket et al. as anthocyanoplasts (*7*). Later, these globular inclusions have been reported as protein matrices (*8, 9*) without a membrane boundary or an internal structure (*10, 11*). These insoluble protein matrices, called anthocyanin vacuolar inclusions (AVIs), are thought to sequester anthocyanins, thereby increasing their stability and reducing the inhibition of certain vacuolar enzymes (*6*). Protoplasts of anthocyanin-containing grapevine cells (*Vitis vinifera* cv. Gamay) (*12*) contained anthocyanin-containing vesicles (anthocyanoplasts), whereas no anthocyanoplasts were found in isolated vacuoles, and it was concluded that anthocyanoplasts are mainly located in the cytoplasm. Similar studies using a grapevine cell suspension culture implied the vacuolar localization of numerous small pigmented bodies (1–3 μm in diameter), which presumably coalesced inside the vacuole to form a single large body up to 15 μm in diameter. These bodies freely moved around the majority of the cell by Brownian motion on unfixed samples, being retarded only by the inner face of a visible membrane. Furthermore, when released into the buffer, they remained intact for a short time with no apparent delimiting membrane. This evidence indicated their nature as macromolecular complexes of vacuolar localization (*13*).

A new insight into the structure and formation of AVIs in flower petals was obtained by using light and electron microscopy (EM) (*14*). Investigations on AVIs in the epidermal cells of different regions of lisianthus flower petals revealed three different forms of AVIs: vesicle-like, rod-like, and irregular shaped. Electron microscope examinations showed no membrane encompassing the AVI; instead, the AVI itself consisted of membranous and thread-like structures throughout. It was also found that anthocyanins accumulated as vesicle-like bodies in the cytoplasm and were contained in the prevacuolar compartments (PVCs). These vesicle-like bodies were transported into the central vacuole by merging of the PVCs and the central vacuole in the epidermal cells. The anthocyanin-containing vesicle-like bodies were subsequently ruptured to form threads in the vacuole. During homogenization of grapes for juice processing, these structures may be further modified and influence the microstructure of the juice, potentially affecting its properties. The objective of this study was to analyze the physical and chemical characteristics of nanovesicle− carbohydrate complexes of the polyphenols found in grape juice.

MATERIALS AND METHODS

Analyses of concentrated grape (Concord) juice were carried out to study the physico-chemical properties of polyphenol nanovesicles. A commercial preparation of Welch's grape concentrate made from Concord grapes was purchased from a local grocery store. All chemicals were purchased from Sigma Chemical Co.

Sample Preparation: Dialysis. The grape juice concentrate was thawed at 4 °C, made into aliquots, and stored at −20 °C. Dialysis was conducted overnight whenever dialyzed juice was required for the following day. About 10 mL of the thawed juice was filled in a Spectra Por molecular porous membrane tubing (molecular weight cutoff of 6–8 kDa). The dialysis was carried out in 2 L of 10 mM Tris hydrochloride (pH = 7.5) or, in some cases, against distilled water, at 4 °C. The dialyzed juice was collected and used fresh for the analysis.

Estimation of Total Polyphenol Content. The total polyphenol content was estimated by using the Folin−Ciocalteau method (*15*). To each 1 mL sample, 5 mL of distilled water was added along with 0.5 mL of Folin−Ciocalteau reagent (2 N). The samples were then vortexed and allowed to incubate for 5 min. Next, 1 mL of 5% (w/v) sodium carbonate solution was added to each sample. The samples were then vortexed and incubated in the dark for 1 h. After incubation, the samples were mixed well, and the absorbance was measured at λ_{max} = 725 nm by using a spectrophotometer (Beckman DU 800). A standard curve was generated by using catechin with concentrations ranging from 25 to 200 μg/mL. The grape polyphenol concentrations are expressed as weight catechin equivalent.

Effect of Sucrose on Total Polyphenols. A 10 mL sample of dialyzed juice was mixed with 10 mL of 5% or 10% sucrose solution, and the mixture was incubated at room temperature for 30 min. It was then centrifuged at 50 000 rpm (105000*g*) in a Beckman L-60 ultracentrifuge using a 60Ti rotor for 1 h at 4 °C. The samples were examined for the presence of a pellet and analyzed for the total polyphenol content. The control samples were mixed with 10 mL of 10 mM Tris buffer, pH = 7.5, instead of the sucrose solutions and subjected to the same centrifugation regime.

Effect of Triton X-100 on Total Polyphenols. Triton X-100, a nonionic detergent, was used to examine the possible inclusion of polyphenols in lipid-containing vesicles. A 10 mL sample of the dialyzed juice was mixed with 10 mL of 0.01, 0.02, or 0.04% (v/v) of Triton X-100 (final concentrations of 0.005, 0.01, and 0.02% v/v), and the mixture was incubated at room temperature for 30 min. It was then centrifuged at 50 000 rpm (105000*g*) in a Beckman ultracentrifuge using a 60Ti rotor for 1 h at 4 °C. The supernatant was collected and subjected to dialysis for 12 h before analysis for the total phenol content. The pellets did not contain any polyphenols and were discarded. For the control samples, 10 mL of the dialyzed juice was mixed with 10 mM Tris buffer, pH = 7.5, and was subjected to the same centrifugation and dialysis regime.

Effect of Digestive Enzymes on Total Polyphenols. The effect of digestive enzymes, such as trypsin and lipase, on the dialyzed grape juice was studied at varying pH. Dialyzed juice under the following conditions was used for the analysis: (a) pH adjusted to 2 by using HCl, (b) treated with trypsin at 1 U/mL, (c) treated with trypsin at 1 U/mL and pH adjusted to 2 by using HCl, (d) treated with lipase at 1 U/mL, and (e) treated with lipase at 1 U/mL and pH adjusted to 8 by using NaOH. Each treated sample was incubated for 4 h at 25 °C, and the total polyphenols were measured.

Transmission Electron Microscopy (TEM) Analysis. TEM was used to determine the microstructure of the polyphenols in the dialyzed juice (control) and in the samples subjected to the various treatments. A 10 mL sample of the dialyzed juice was treated with enzymes at 1 U/mL and incubated for 6 h at their optimum temperature (cellulase at 37 °C, pectinase at 25 °C, and β-galactosidase at 30 °C). These were then dialyzed overnight by using a SpectraPor molecular porous membrane tubing (molecular weight cutoff of 6–8 kDa) in 2 L of 10 mM Tris HCl (pH = 7.5) at 4 °C. The dialyzed juice was collected the following day and used for EM. A fomvar-coated nickel grid was floated on a droplet of the preparation with the coated side facing the solution for 1 min. The grid was removed, blotted dry, and stained with 1% uranyl acetate for 30 s. The excess stain was removed, and the grid was examined by using a transmission electron microscope (Leo 912 B).

Antioxidant Assay. The ability of the grape juice concentrate, the dialyzed juice fraction, and the dialyzate to scavenge superoxide, hydroxyl, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was evaluated by standard procedures as explained below. The results were

statistically analyzed by using a Graphpad software (*16*).

Superoxide Anion Radical Scavenging Assay. The superoxide anion radical ($O_2^{\bullet -}$) scavenging capacity of grape polyphenols was determined as described by Boveris (*17*). The $O_2^{\bullet -}$-dependent oxidation of epinephrine to adrenochrome was followed spectrophotometrically at $\lambda_{max} = 480$ nm. The control reaction mixture consisted of 1175 μl of 20 mM Tris buffer (pH = 7.5), 600 μl of 1 mM xanthine, 25 μL of xanthine oxidase (EC1.1.3.22, made up in Tris buffer at 1:4 ratio), and 200 μl of 10 mM epinephrine. The grape juice concentrate, the dialyzed fraction, and the dialyzate were added to obtain concentrations of 2.5, 5, 10, and 20 μg of polyphenols in the reaction mixture (2 mL). The mixture was vigorously shaken, and the initial and final (after 30 min) absorbances were measured. The radical scavenging capacities of the grape juice concentrate and its dialyzed fractions were expressed as percent quenching relative to the control (taken as 100% without grape fractions).

Hydroxyl Radical Scavenging Assay. This assay was based on the method described by Halliwell et al. (*18*). The diluted grape juice concentrate, the dialyzed fraction, and the dialyzate were added to obtain concentrations of 2.5, 5, 10, and 20 μg of polyphenols in the reaction mixture (2500 μl). This was added to 690 μl of 2.5 mM 2-deoxyribose (made up in 10 mM phosphate buffer, pH = 7.4) and 100 μl of 1 mM ferric chloride (made up in 1.04 mM ethylenediaminetetraacetic acid). The samples were kept in a water bath at 37 °C, and the reaction was started by adding 100 μl of 1 mM ascorbic acid and 10 μl of freshly prepared 0.1 M H_2O_2. After 20 min, the reaction was stopped by adding 1 mL of 2.8% cold trichloroacetic acid and 0.5 mL of 0.5% thiobarbituric acid. The samples were then boiled for 8 min and cooled, and the absorbance was measured at $\lambda_{max} = 532$ nm. The hydroxyl radical scavenging capacity of the grape juice polyphenols was expressed as percent quenching relative to the control (taken as 100% without grape fractions).

DPPH Radical Scavenging Assay. The ability of polyphenols present in the grape juice concentrate, the dialyzed fraction, and the dialyzate to scavenge free radicals (total antioxidant activity) was also measured by using DPPH (*19*). A 0.2 mL aliquot of each phenolic fraction (to give a final total phenol content of 2.5, 5, or 10 μg) was mixed with 0.8 mL of 0.1 mM ethanolic DPPH. The solution was vortexed well and incubated at room temperature for 30 min. The samples were then clarified by centrifugation at 15 000*g* for 40 s, and the absorbance was measured at $\lambda_{max} = 517$ nm. The control sample was 0.2 mL of 95% ethanol. The antioxidant activity was expressed as percent DPPH scavenging, calculated as [(control absorbance − quenched extract absorbance)/control absorbance] × 100.

Mass Spectral Analyses. Anthocyanins were isolated by hydrophobic interaction chromatography by using a C_{18} Sep-pak column (1 mL) and elution with 1 mL of 100% methanol. Chromatographic analyses of anthocyanins (100 μg) isolated from the grape juice concentrate, the dialyzed juice fraction, and the dialyzate were performed on an Agilent 1100 series LC-MSD instrument in a positive ion mode. An Xterra RP18 column (3.9 × 150 mm) was used for the separation. The elution was performed by using mobile phase A (water:methanol:formic acid, 45:45:10 v/v) and mobile phase B (water: formic acid, 90:10 v/v). The flow rate was 0.8 mL/min, and the compounds eluted were detected at 520 nm. The gradient used was as follows: 0–15 min, 70% B; 15–35 min, 20% B; 35–45 min, 100% A; and 45–65 min, 70% B. Electrospray ionization (ESI) was performed with an API-ES mass spectrometer. Nitrogen was used as the nebulizing and drying gas. The ESI conditions were as follows: nitrogen pressure, 60 psig; drying gas, 12 L/min at 350 °C; ion spray voltage, 4000 V; and fragmentor voltage, 80V. A sample injection volume of 50 μl (100 μg) was used for all the samples.

NMR Analysis. The NMR spectral scans of (a) the dialyzed grape juice concentrate, (b) the dialyzed juice treated with pectinase at 1 U/mL, (c) the dialyzate, and (d) the malvidin-3-O-galactoside chloride (standard) were performed with a Bruker 600 MHz FT-NMR spectrophotometer in a 5 mm NMR tube. A quantity of 8 mL of each sample (except the standard) was concentrated by using conditioned AMICON Ultra-4 centrifugal filter devices (10 000 nominal molecular weight limit) to 500 μL in a swinging bucket centrifuge at 4000*g* at 4 °C. The samples were then washed twice with 2.5 mL of D_2O. This procedure was followed by centrifugal concentration to a final volume of 500 μL. Finally, the samples were washed with D_2O (99.9% purity) and concentrated to a final volume of 700 μl. A quantity of 1 mg of malvidin-3-O-galactoside chloride was dissolved in 700 μl of D_2O (99.9% purity) and used as a standard. ^{1}H NMR spectra of the samples were recorded at 300 K after accumulating 100 scans with an acquisition time of 2.28 s and a spectral width of 7183.90 Hz. Solvent suppression of the residual water signal was conducted for all scans (broad singlet at δ_1H = 4.8).

Table 1. Total Polyphenol Content in Grape Juice Preparations (Mean ± Standard Error from Eight Independent Estimations)[a]

sample	total polyphenols (μg/mL)
grape juice concentrate	627 ± 85 b,c
dialyzed fraction of grape juice concentrate	143 ± 13 a,c
dialyzate of grape juice concentrate	445 ± 30 a,b

[a] a denotes the statistical significance at $p < 0.05$ from grape juice concentrate, b denotes the significance from dialyzed fraction of grape juice concentrate, and c denotes the significance from dialyzate of grape juice concentrate.

Fluorescence Spectral Analysis. Fluorescence spectra of the polyphenols isolated from the grape juice concentrate, the dialyzed fraction, and the dialyzate were generated by using a Varian (Cary Eclipse) fluorescence spectrophotometer. The scanning was performed in the synchronous mode from 260 to 700 nm at a PMT voltage of 800 V, with a δ wavelength of 20 nm, and with an excitation and emission slit of 10 nm each. Samples of 2 mL, each containing 100 μg/mL of polyphenols, were measured for fluorescence in quartz cuvettes.

Statistical Analysis. Statistical analyses were conducted by using GraphPad Prism software, version 4. Results having two means were compared by using a Student's t test. Results with multiple means were compared by using a one-way analysis of variance, which was followed by a Tukey's test to evaluate the level of significance. Significantly different means ($p < 0.05$) are denoted by different letters.

RESULTS

Polyphenol Content of Grape Juice Concentrate, Dialyzed Fraction, and Dialyzate. The polyphenol contents of the grape juice concentrate, the dialyzed fraction, and the dialyzate are shown in **Table 1**. The original concentrated grape juice contained about 627 μg/mL of polyphenols. After dialysis by using a 6–8 kDa molecular weight cutoff dialysis membrane, 445 μg/mL or about 70% of the polyphenols was obtained in the dialyzate solution (significant at $p < 0.05$). Nearly 143 μg/mL or about 22% was recovered in the dialyzed juice. Thus, a statistically significant ($p < 0.05$) amount of polyphenols was retained in nondialyzable complexes.

Physico-chemical Properties of the Dialyzed Juice Fraction. The juice fraction obtained by dialysis of grape juice concentrate was subjected to various treatments to analyze the physico-chemical properties of carbohydrate−polyphenol complexes.

(a) Effect of Sucrose. The sedimentability of carbohydrate−polyphenol complexes was tested by using sucrose solutions at 2.5 and 5% final concentrations (**Table 2**). It was found that the addition of sucrose resulted in darker solutions; however, no sediments were observed after 1 h of centrifugation at 105000*g*, during which time insoluble carbohydrate polymers and membrane vesicles normally sediment. The total polyphenols decreased (significant at $p < 0.05$) by more than 50% (64 and 67 μg/mL) for both treatments in comparison to the control (143 μg/mL). This may be due to the polymerization of the polyphenols.

(b) Effect of Triton X-100. It was hypothesized that polyphenol micro-aggregates are vesicular in nature, and treatment with

Table 2. Effect of Sucrose, Triton X-100, and Digestive Enzymes on the Dialyzed Fraction of Grape Juice Concentrate (Mean ± Standard Error from Three Independent Estimations)[a]

treatment	concentration levels of treatments	total polyphenol content (mg/L)
sucrose	control	143 ± 13 b,c
	2.5%	64 ± 1 3 a
	5.0%	67 ± 15 a
Triton X-100	control	143 ± 13 b,c,d
	0.005%	115 ± 32 b,c
	0.01%	94 ± 25 b,c
	0.02%	71 ± 25 a,b,c
digestive enzymes	control	143 ± 13 f
	pH = 2.0	129 ± 6
	trypsin	136 ± 11
	trypsin/pH = 2.0	136 ± 13
	pancreatic lipase	128 ± 10
	pancreatic lipase/pH = 8.0	111 ± 10 a

[a] Statistically significant means are denoted by different letters.

a detergent such as Triton X-100 would rupture the lipid vesicular membrane surrounding the polyphenol-containing vesicle. This would result in more polyphenols being released into the surrounding medium. After centrifugation of the incubated mixture, small pellets were observed. The supernatant was subjected to further dialysis for 12 h, after which the total polyphenols of the supernatant dropped by 32% (115 μg/mL), 44% (94 μg/mL), and 59% (71 μg/mL), for juice treated with 0.005, 0.01, and 0.02% Triton X-100, respectively. The polyphenols released by increased leakiness of any polyphenol-containing vesicles would have been lost during the second dialysis, resulting in a decreased polyphenol content in the supernatant (significant from the control at a concentration of 0.02%, $p < 0.05$, **Table 2**).

(c) Effect of Digestive Enzymes. Digestive enzymes such as trypsin and lipase were used to study the stability of the polyphenol micro-aggregates in the dialyzed fraction of grape juice concentrate. The pH was varied to simulate conditions that are normally encountered in the stomach. It was found that the total polyphenol content was unaffected at pH = 2.0 or with trypsin (alone or at pH = 2.0). With the lipase treatment at pH = 8.0, the total polyphenol content dropped by nearly 24% in comparison to the control (significant at $p < 0.05$), whereas the total polyphenol content was unaffected for the dialyzed juice treated with lipase alone (**Table 2**).

Ultrastructural Analysis of Juice Fractions. The retention of a significant amount of polyphenols inside the dialysis bag after extensive dialysis suggested that these may be complexed to materials of large molecular weight. To obtain a better structural understanding of such entities, electron microscopic analyses of the juice preparations were conducted. A brief staining with uranyl acetate provides positive staining to electron-dense materials attached to the fomvar-coated grids. A representative morphology of the macromolecular complexes from the dialyzed juice is shown in **Figure 1**. Dark staining areas varying in diameter from 10 to 100 nm were detectable (arrows). Many of the aggregates also revealed the presence of minute vesicular structures. Carbohydrate polymers by themselves do not stain with uranyl acetate; therefore, the visible structures must contain components such as polyphenols that would react with uranyl acetate. The treatment with Triton X-100 (**Table 2**) did not fully release the polyphenols, suggesting that a large proportion of the polyphenols are bound potentially to carbohydrate polymers. A representative field of dialyzate subjected to the same analysis did not reveal any such

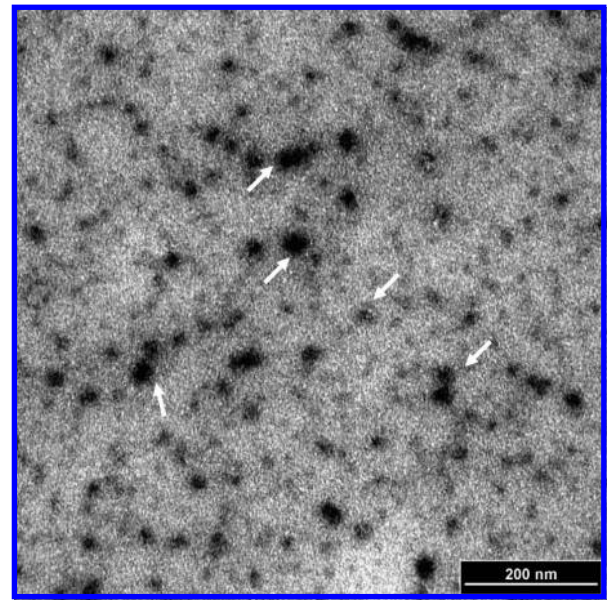

Figure 1. Transmission electron micrograph of pectin−polyphenol−lipid nanovesicle complexes in dialyzed grape juice concentrate as revealed by positive staining with uranyl acetate. The complexes are marked by white arrows. The micrograph is representative of several independent experiments.

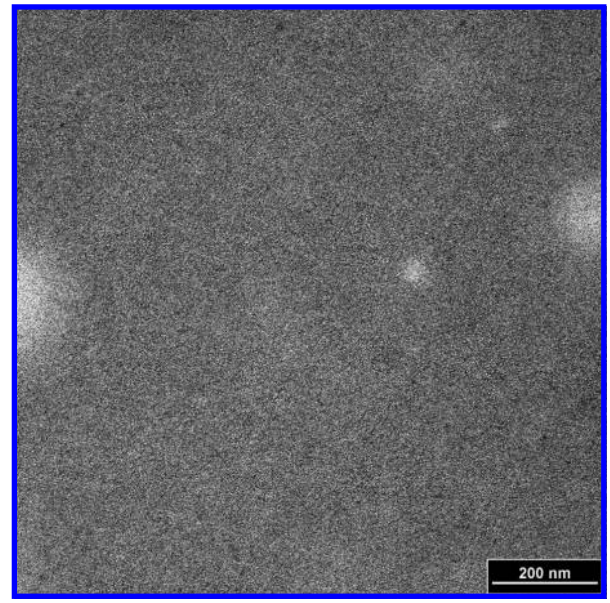

Figure 2. Transmission electron micrograph of a fomvar-coated grid exposed to the dialyzate fraction and stained by exactly identical procedures as explained in **Figure 1**. There were no visible complexes as the ones observed in **Figure 1** in any area of the field. The figure is representative of several independent observations.

structures (**Figure 2**), indicating the true nature of such macromolecular complexes in the dialyzed juice.

To determine the nature of potential carbohydrate moieties involved in the formation of macromolecular complexes, the dialyzed juice was subjected to a treatment with carbohydrate-degrading enzymes. Since grapes are primarily acidic and rarely contain starch after ripening, amylases that catabolize starch were not used. In these experiments, the dialyzed juice was treated with cellulase or enzymes that degrade pectin, such as pectinase and β-galactosidase, and the juice was examined after a second dialysis conducted to remove any digested materials. The electron micrograph of the cellulose-treated dialyzed juice

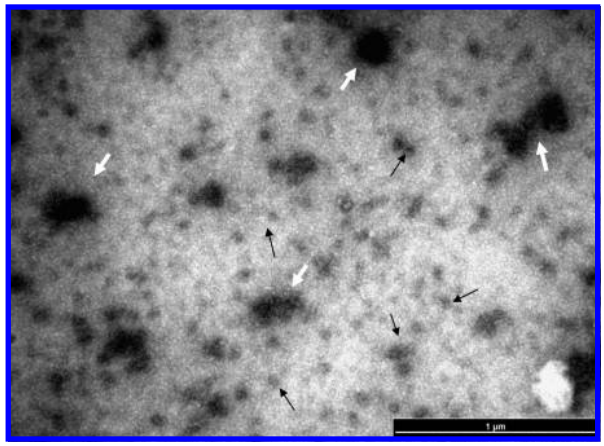

Figure 3. Transmission electron micrograph of macromolecular complexes and vesicles in dialyzed grape juice concentrate as revealed by positive staining with uranyl acetate. The dialyzed juice was treated with cellulase and subjected to further dialysis prior to EM. The undigested complexes are marked by white arrows. Regions showing vesicular aggregates are shown by black arrows. The micrograph is representative of several independent experiments.

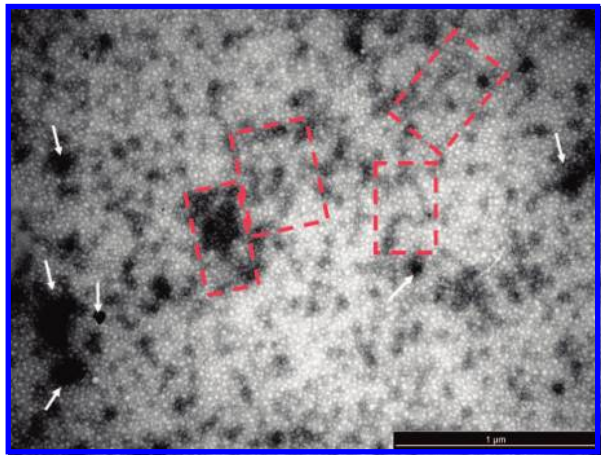

Figure 4. Transmission electron micrograph of macromolecular complexes and vesicles in dialyzed juice subjected to treatment with pectinase and further subjected to dialysis before EM. The grid was stained with uranyl acetate. The undigested complexes are marked by white arrows. Regions showing vesicular aggregates are shown by rectangular boxes. The micrograph is representative of several independent experiments.

is shown in **Figure 3**. In general, the complexes remained similar to those in untreated juice (**Figure 3**, white arrows) with lightly stained vesicular structures (**Figure 3**, black arrows). However, the treatments with pectinase (**Figure 4**) or β-galactosidase resulted in the disruption of the macromolecular complexes into lighter staining vesicular aggregates (**Figure 4**, boxed region; **Figure 5**, white arrows).

Polyphenol Composition of Juice Fractions. To evaluate potential differences in the polyphenol composition of the grape juice concentrate, the dialyzed juice, and the dialyzate, the polyphenols were isolated from these fractions by hydrophobic interaction chromatography, and equal amounts were subjected to HPLC-MS. The results are shown in **Table 3**. There were major differences in the polyphenol compositions of the three samples. The dialyzed fraction contained -3-O-diglucosides of cyanidin and delphinidin that were barely detectable in the original juice and dialyzate. -3-O-glucosides of delphinidin, cyanidin, and malvidin were present in the juice and the dialyzate, but they were at barely detectable levels in the

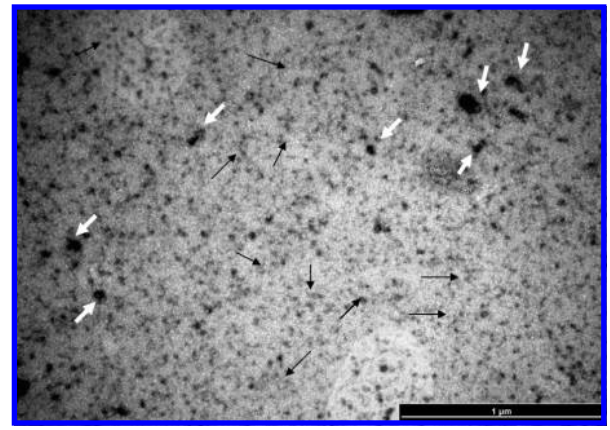

Figure 5. Transmission electron micrograph of macromolecular complexes and vesicles in dialyzed juice subjected to treatment with β-galactosidase and further subjected to dialysis before EM. The grid was stained with uranyl acetate. The undigested complexes are marked by white arrows. Regions showing vesicular aggregates are shown by thin black arrows. The micrograph is representative of several independent experiments.

dialyzed juice. The dialyzate showed significantly higher levels of cyanidin-3-O-glucoside ($p < 0.05$). Malonyl glucoside of malvidin was at detectable levels only in the dialyzed juice. The -3-O-acetoyl glucosides of delphinidin, petunidin, and malvidin were nearly evenly distributed in the polyphenol fraction of the concentrated juice and dialyzate but at a lower level (~50%) in the polyphenols of the dialyzed fraction (significantly different at $p < 0.05$). The polyphenols from the dialyzed fraction also showed the presence of feruloyl and coumaroyl glucosides of cyanidin. The polyphenols from the concentrated juice contained a significantly higher proportion of coumaroyl glucosides of delphinidin and petunidin than those of the dialyzed fraction. Interestingly, the polyphenols from the dialyzed fraction also contained nearly double the amount of coumaroyl glucoside of malvidin in comparison to the polyphenols of the juice concentrate or the dialyzate (significantly different at $p < 0.05$). The macromolecular complexes perhaps may provide better conditions of interactions for relatively more-hydrophobic polyphenols.

^{1}H NMR Analyses of Grape Juice. Proton NMR analyses of various juice fractions were conducted to understand the chemical nature of the macromolecular components. The NMR spectrum of pure malvidin-3-O-galactoside chloride showed resonances characteristic of the A, B, and C ring protons and the glucose moiety (**Figure 6A**). The spectrum shows well-resolved resonances from the aromatic and aliphatic sugar protons, spanning from 6 to 8.4 ppm and from 3.5 to 4 ppm, respectively. There are two anomeric duplets at 4 and 5.15 ppm. The 3′ and 5′ positions of malvidin are methylated, and the signals from the two -O-CH_3 groups appear at 3.75 ppm as sharp singlets. The signals from the 2′ and 6′ protons appear at 6.95 and 7.25 ppm as duplets (designated as 2). The signal from the proton at position 4 of the B ring is potentially the most deshielded and was projected to appear at 8.8 ppm as a duplet (peak designated as 1). The signals from the protons at C6 and C8 of the A ring are projected to appear at 6.25 and 6.35 ppm (group designated as 3). In general, the spectrum of malvidin-3-O-galactoside chloride is very similar to that of other anthocyanins but with fewer aromatic protons.

The ^{1}H NMR spectrum of the dialyzed grape juice was acquired under the exact same conditions as those employed for authentic malvidin-3- O-galactoside (**Figure 6B**). An interesting aspect of this spectrum is the near disappearance of

Table 3. LCMS Profile of the Polyphenols Present in Grape Juice Concentrate, Dialyzed Fraction, and Dialyzate (Mean ± Standard Error from Three Independent Estimations) [a]

anthocyanins identified by LCMS	anthocyanin (% wt basis)		
	grape juice concentrate	dialyzed fraction	dialyzate
cyanidin-3-O-diglucoside	BDL	0.30 ± 0.01	BDL
delphinidin-3-O-diglucoside	BDL	5.72 ± 0.1	BDL
delphinidin-3-O-glucoside	9.83 ± 1.8	BDL	10.57 ± 0.7
cyanidin-3-O-glucoside	6.02 ± 2.0 c	BDL	9.19 ± 0.2 a
petunidin-3-O-glucoside	traces	BDL	traces
malvidin-3-O-glucoside	3.42 ± 1.8	4.43 ± 0.01	5.24 ± 0.8
malvidin-3-O-malonyl glucoside	BDL	2.52 ± 0.2	BDL
delphinidin-3-O-acetoyl glucoside	3.29 ± 0.9 c	2.80 ± 0.1 c	5.94 ± 0.5 a,b
petunidin-3-O-acetoyl glucoside	6.73 ± 2.4 b	3.27 ± 0.1 a,c	8.42 ± 1.9 b
malvidin-3-O-acetoyl glucoside	9.58 ± 1.4 b	3.65 ± 0.01 a,c	7.31 ± 2.1 b
cyanidin-3-O-feruloyl diglucoside	BDL	2.47 ± 0.9	BDL
cyanidin-3-(6′-coumaroyl) glucoside	BDL	3.09 ± 0.02	BDL
delphinidin-3-(6′-coumaroyl) glucoside	12.43 ± 0.3 c	BDL	8.32 ± 2.3 a
petunidin-3-(6′-coumaroyl) glucoside	10.28 ± 2.2 b,c	2.50 ± 0.01 a	4.06 ± 0.4 a
malvidin-3-(6′-coumaroyl) glucoside	38.41 ± 8.2 b	69.34 ± 3.6 a,c	40.96 ± 8.0 b

[a] BDL, below detection limit; The letters a, b, and c denote statistical significance at $p < 0.05$ for grape juice concentrate, dialyzed fraction of grape juice concentrate, and dialyzate, respectively. Single values were not subjected to statistical comparison.

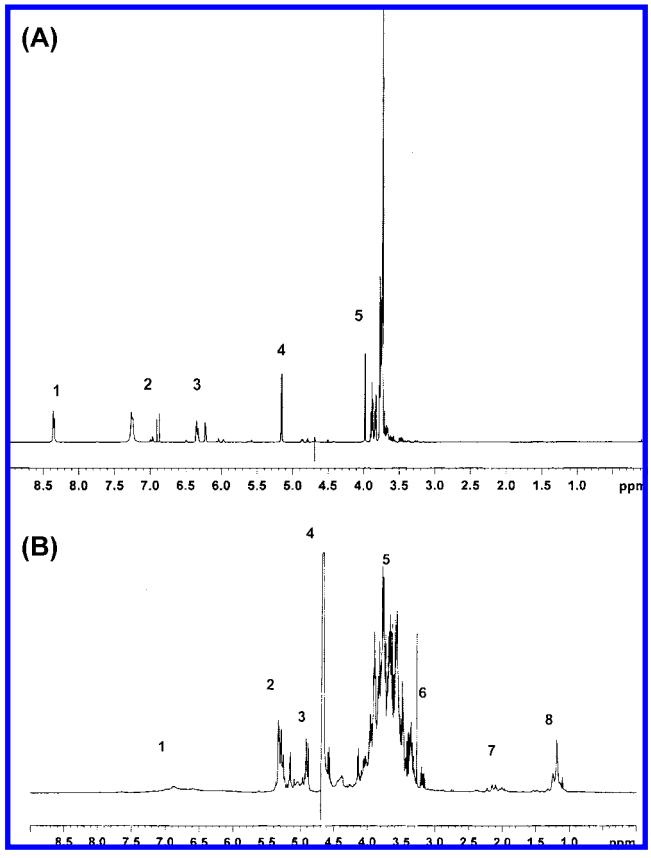

Figure 6. (**A**) ^{1}H NMR spectrum of malvidin-3-O-galactoside chloride recorded in D_2O. Peaks at 1, 2, and 3 correspond to protons in the aromatic region. Peak 4 is unidentified. The signals from galactose occur between 3.5 and 5 ppm. The sharp singlet at 3.7 ppm corresponds to the -O-methyl resonance from the B ring. (**B**) ^{1}H NMR spectrum of dialyzed juice recorded in D_2O. Broad peaks at 1 may correspond to aromatic protons. Peak 4 is the suppressed water signal. Peaks 2, 3, and 5 originate from carbohydrate protons. Peak 6 may originate from an -O-methyl group which is distinct from the sharp singlet at 3.7 ppm. Peak 8 corresponds to lipid methylene protons.

the protons in the aromatic region, potentially because of molecular interactions and broadening. Such a region is designated by 1 in the spectrum. Because of the complex nature of carbohydrates, the carbohydrate region of the signals appears to range from 3.5 to 5.5 ppm with multiple peaks and a broad multiplet (peaks designated by 2, 3, 5, and 6). Peak 4 is a remnant of the suppressed water protons. Additional signals are observed at 1.2 ppm, potentially indicating the presence of methylene and methyl protons arising from lipid vesicles (peaks designated as 8). The signals arising at 2.2 ppm (designated as 7) are unknown.

Pectinase treatment of the dialyzed juice resulted in a significant reduction of the carbohydrate resonances (**Figure 7A**) appearing between 3 and 5.2 ppm (peaks designated as 1, 3, and 4). Peak 2 is a suppressed water proton signal at 4.8 ppm. The signals which appears upstream of 4 ppm in the carbohydrate signals of dialyzed juice (**Figure 6B**, peaks 2 and 3) appear to have been considerably reduced, indicating that they are polygalacturonic-acid-specific protons. Also, the sharp singlets among the carbohydrate signals may arise from -O-CH_3 groups of anthocyanins and pectin. The signals from the lipid methylene protons (group designated as 6) appearing at 1.2 ppm appear to have increased after pectinase treatment. The resonance signals from the unknown components (group designated as 5) also show an increase in intensity after pectinase treatment.

The ^{1}H NMR spectrum of the concentrated polyphenols in the dialyzate (**Figure 7B**) shows a complex group of signals (designated as 1–6). Interestingly, the signals from the aromatic protons appear to be missing or masked. The carbohydrate region between 3 and 4 ppm is much simplified. The triplet at 1 ppm (peak 6) is suggestive of a $-CH_2-CH_3$ group. The signals from the anthocyanins appear to be totally different from those characteristic of pure anthocyanins, potentially indicating complex formations or structural alterations.

Fluorescence Spectrum of Juice Fractions. The fluorescence spectra of the polyphenols isolated from the concentrated juice, the dialyzed juice, and the dialyzate (**Figures 8A**, **B**, and **C**) also show differences. The fluorescence spectrum of the polyphenols from the juice concentrate has two well-defined emission maxima at 383 nm from the B ring and at 635 nm from the A and C rings, with a shoulder at 570 nm (**Figure 8A**). In the polyphenols of the dialyzed juice, these peaks are shifted to 370 and 641 nm with shoulders at 425, 550, and 700 nm (**Figure 8B**). The peak intensities in all peaks are reduced by more than 50%. The fluorescence spectrum of the polyphenols from the dialyzate has a shape similar to that of the

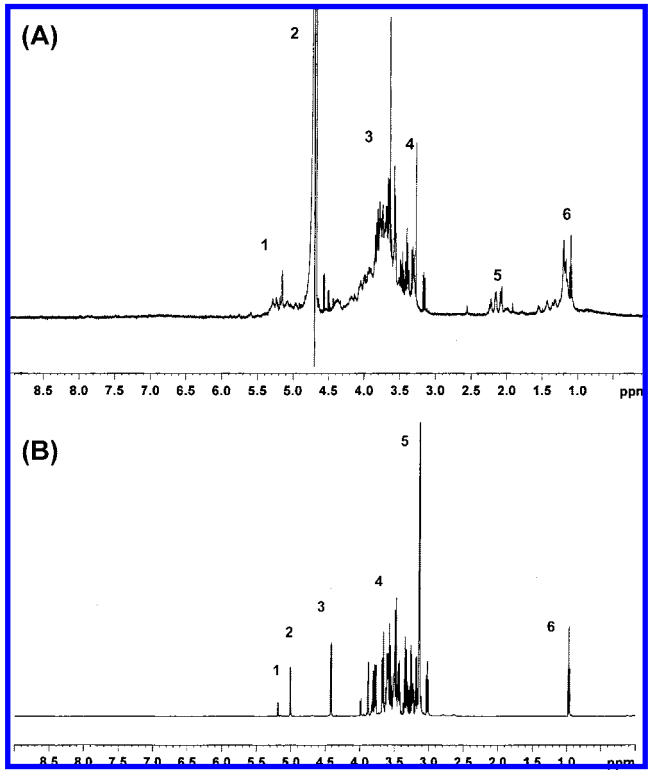

Figure 7. (**A**) ^{1}H NMR spectrum of pectinase-treated dialyzed juice recorded in D_2O. Broad peaks at 1 appear to have been reduced in intensity after pectinase treatment. Peak 2 originates from water. The carbohydrate proton signals (3–4.5 ppm) appears to be reduced in intensity. The sharp singlets in this group may originate from -O-methyl protons. Peaks at 5 are unidentified. Peaks at 6 may originate from lipid methylene (broad) and methyl protons (CH_3-CH_2, triplet). (**B**) ^{1}H NMR spectrum of the dialyzate containing polyphenols recorded in D_2O. The aromatic proton signals are missing. Peaks 1–4 may originate from various sugar moieties of polyphenols. Peak 5 at 3.2 ppm may originate from -O-methyl groups. Peak 6 corresponds to a methyl group with an adjacent CH_2.

polyphenols from the concentrate; however, the signal intensity at 633 nm is considerably reduced, suggesting potential changes in the A and C rings structures (**Figure 8C**).

Antioxidant Activity. To evaluate the functional properties of the various juice fractions, the ability of the polyphenols in concentrated grape juice, the dialyzed juice fraction, and the dialyzate to scavenge superoxide, hydroxyl, and DPPH radicals was assessed (**Figure 9A**, **B**, and **C**). The polyphenol concentrations were varied in typical assay systems from 2.5, 5, 10, and 20 μg per assay volume.

The superoxide radical scavenging potential of the polyphenols present in the grape juice concentrate, the dialyzed fraction, and the dialyzate is shown in **Figure 9A**. The dialyzed fraction showed no scavenging potential at any of the polyphenol concentrations tested. The concentrate and the dialyzate showed a dose-dependent increase in superoxide scavenging ability that reached 100% at about a 20 μg level. The polyphenols from the concentrate showed a lower scavenging potential than the polyphenols from the dialyzate at 5 μg (32% vs 67%) and 10 μg (70% vs 89%) polyphenol concentrations (significantly different at $p < 0.05$).

The hydroxyl radical scavenging capacity of the polyphenols present in the grape juice concentrate, the dialyzed fraction, and the dialyzate is shown in **Figure 9B**. All three samples showed an increasing scavenging potential up to around 5 μg, after which point the rate curve plateaued (dialyzed juice and

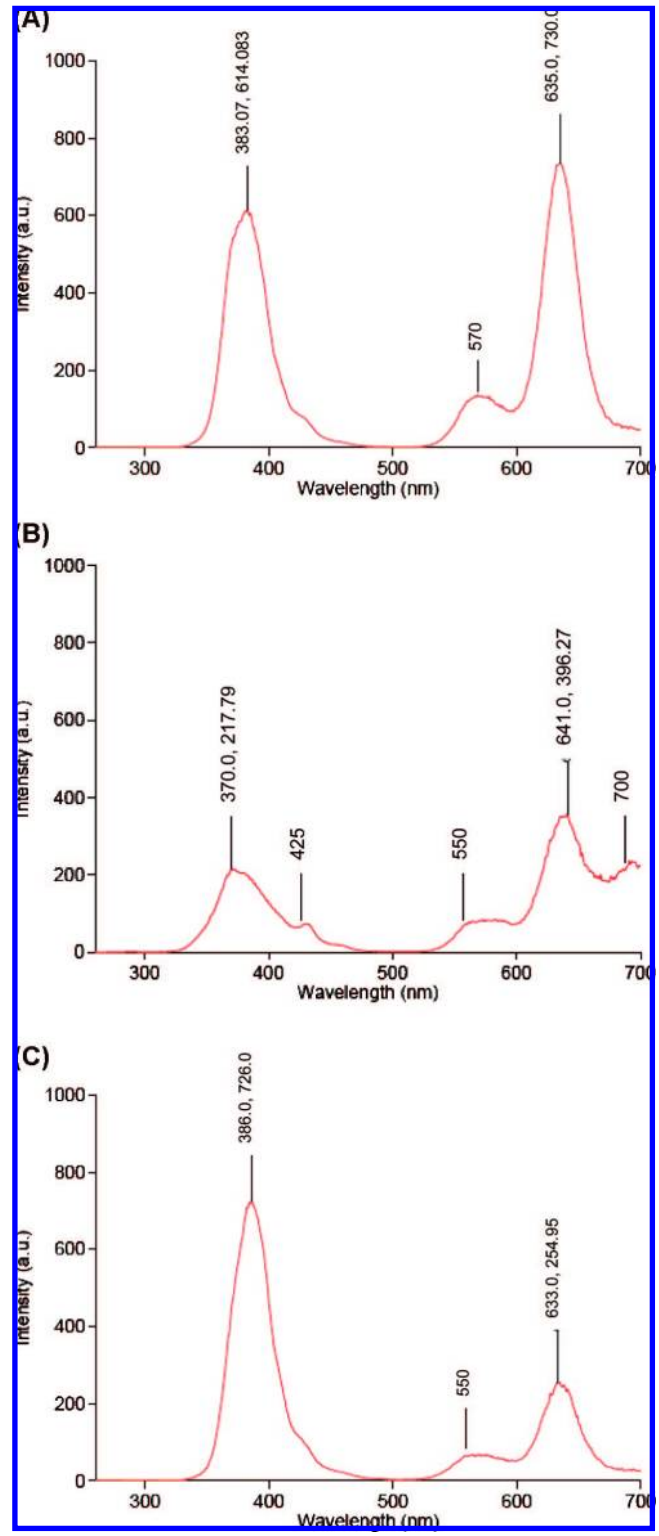

Figure 8. (**A**) Fluorescence spectrum of polyphenols isolated from grape juice concentrate. The solution contained 100 μg/mL of polyphenols. (**B**) Fluorescence spectrum of polyphenols isolated from dialyzed grape juice concentrate. The solution contained 100 μg/mL of polyphenols. (**C**) Fluorescence spectrum of polyphenols isolated from the dialyzate fraction of grape juice. The solution contained 100 μg/mL of polyphenols.

dialyzate) or showed a slight decline (concentrate). The concentrate reached a maximum scavenging potential of 63% at the lowest polyphenol concentration (5 μg), after which point it declined to 33% at 20 μg. Similarly, the dialyzate reached a maximum scavenging potential of 67% at a polyphenol concentration of 10 μg. The dialyzed juice showed a relatively lower

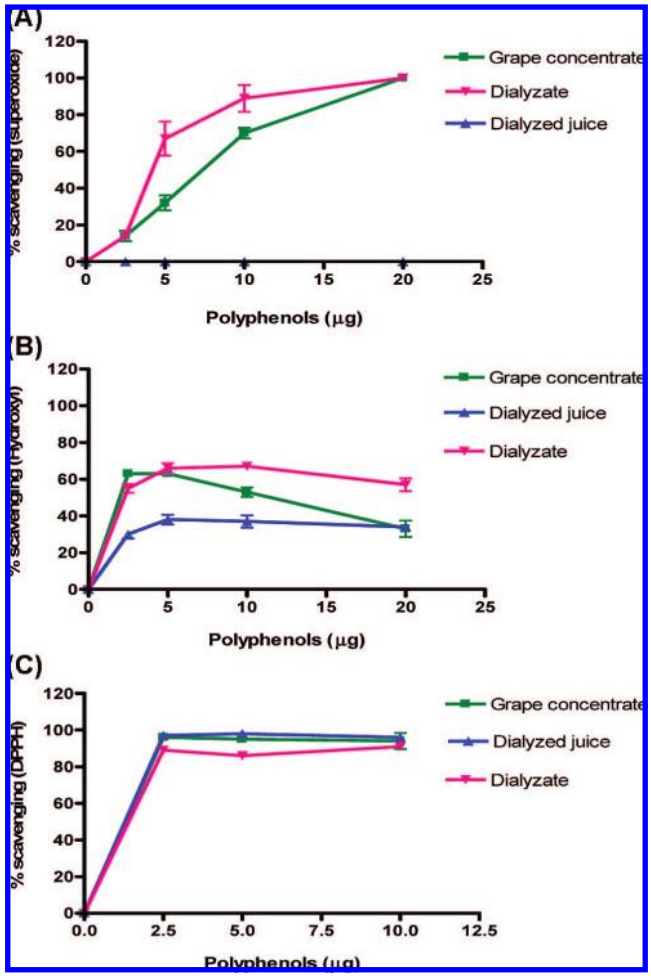

Figure 9. (**A**) Superoxide scavenging efficiency of the polyphenols from grape juice concentrate, dialyzed juice, and dialyzate. (**B**) Hydroxyl radical scavenging efficiency of polyphenols isolated from grape juice concentrate, dialyzed juice, and dialyzate. (**C**) DPPH radical scavenging efficiency of polyphenols isolated from grape juice concentrate, dialyzed juice, and dialyzate. The results are mean ± standard error from three independent analyses (statistically significant at $p < 0.05$ at 5 and 10 μg between dialyzate and the concentrate for A and at 2.5, 5, and 10 μg between the dialyzed juice and the concentrate as well as the dialyzate).

hydroxyl radical scavenging potential with a maximum of 38% at a 5 μg polyphenol concentration (significantly different at $p < 0.05$ for 2.5, 5, and 10 μg of polyphenols as compared to that from the juice concentrate and the dialyzate, and at 20 μg from the dialyzate).

The DPPH radical scavenging capacity of the polyphenols present in the grape juice concentrate, the dialyzed fraction, and the dialyzate is shown in **Figure 9C**. All three samples showed similar scavenging potentials with a maximum of about 96–98% for the polyphenols from the juice concentrate and dialyzate, whereas the dialyzed juice fraction showed a slightly lower scavenging potential of about 91% at 10 μg of polyphenols. The DPPH scavenging capacity of all three samples plateaued after 5 μg of polyphenols.

DISCUSSION

Fruits have a heterogeneous assembly of macromolecular structures, such as cell wall components comprising cellulose and pectin, various membranes that provide compartmentalization, and simple chemical components that provide several qualities such as color, taste, flavor, and so forth. The ripening process involves the controlled deterioration of the structural components. Cellulose and pectin are broken down into smaller units to form soluble fiber components. Starch or organic acids are converted into sugars. The membrane phospholipids are catabolized into neutral lipids such as diacylglycerols and free fatty acids. The accumulation of degradation products in the membrane results in the microvesiculation of the membrane. Thus, a ripe fruit provides a variety of components that impart nutritional quality in either the fresh or the processed form (*20–23*).

Polyphenols are nutritionally important components that provide health beneficial properties to fruits and their processed products (*24*). Fruit juice is an important medium for obtaining polyphenols, soluble and insoluble fibers, vitamins, and various other components. Fruit juices can be either whole fruit blend, such as tomato and citrus, or clarified juices, such as grape and various berries. The method of juice preparation may affect the composition of the juice. For instance, hot break processing inhibits pectinolytic enzymes, resulting in the formation of large molecular-weight pectic polymers that give a thick consistency to tomato juice and sauce (*21, 22*). Commercially available grape juice concentrate is processed through several steps that involve homogenization, filtration, pasteurization, and concentration by heat or other methods. Exposure to such physical parameters can alter the composition and intermolecular interactions, because the compartmentalization of the cells is disrupted, and several cell components (cell wall, membrane, polyphenols, flavor, organic acids, etc.) are free to interact with each other. Such interactions may alter the functionality of various ingredients from that observed for the free molecules. Essentially, juice concentrates may show properties that are quite different than those observed for the individual constituents. The complexity of the juice composition potentially suggests the existence of interactions between the various types of molecules. Such interactions may affect the nature of transit, stability, and bioavailability of key components such as polyphenols in the gastro-intestinal system. The bioavailability of anthocyanins is in the range of 0.61–1.82% (*25*), which is not very high when compared to the amount present in the juice or in fruits. This suggests that a good portion of polyphenols may escape absorption in the small intestine but are carried over to the large intestine, where they may exert an unknown physiological function or get metabolized and absorbed into the body. It is generally assumed that the food matrix in fruit juices may not exert an influence on the availability of polyphenols. But fruit juice processing may change the molecular interactions between various components, thus altering the structure, bioavailabilty, and physiological properties. Recent studies have suggested the existence of potential interactions between polyphenols and polysaccharides in wine (*26*). The present study explores the fine structure of commercial grape juice to understand potential interactions between polyphenols, carbohydrates, and lipids.

Our study confirms the existence of polyphenols that are bound to or confined in lipid vesicles. Nearly 25% of the total polyphenols is present in such forms. Ultrastructural studies show the presence of electron-dense complexes in the nondialyzable fraction of grape juice concentrate. A similar analysis of the dialyzate does not show the existence of any such complexes, confirming the true nature of the macromolecular complexes in dialyzed juice. Neutral carbohydrates by themselves do not interact with electron-dense atoms such as those of uranium; potentially, large molecular-mass pectins with free galacturonic acids may interact with such atoms. If this were the case, staining would have been nonspecific and general

throughout the specimen because of the soluble pectic components rather than confined to discrete areas as observed in our study. The macromolecular entities also contain vesicular structures that may have originated from the anthocyanin vacuolar inclusions or from membrane microvesiculation during homogenization, that would have trapped polyphenols within. Thus, the structures observed in our study may arise from a complex formation between pectic polysaccharides and vesicles containing polyphenols. Polyphenols conjugated with sugars may also interact with the pectic chains through their carbohydrate moieties. The pectic nature of the macromolecular complexes is revealed by the disruption of their structure after being treated with pectinolytic enzymes such as polygalacturonase and β-galactosidase, releasing nanovesicles. Treatment with cellulase did not affect the integrity of the complexes as revealed by the ultrastructural analysis. Despite the disruption in the macromolecular assembly, polyphenols were not released from the enzyme-treated juice during extended dialysis (data not shown), indicating that the polyphenols are still complexed within the lipid vesicles and possibly small pectic components. The Triton X-100 treatment of the dialyzed juice caused over 50% of the polyphenols to be released from the complexes, strongly suggesting that a large portion of the polyphenols are confined within the lipid vesicles. Also, these structures were resistant to treatment with enzymes normally present in human digestive systems, indicating their stable structure. Only the alkaline lipase treatment caused a minor release of polyphenols, again showing their lipid association. However, its effectiveness is also potentially low because fruit membrane lipids are not rich in triacylglycerols, its preferred substrate.

There were interesting differences in the polyphenol composition of the dialyzed juice and the dialyzate, suggesting preferential interactions between the macromolecular complexes and polyphenols. The simple -3-O-glucosides of delphinidin and cyanidin are not retained in the dialyzed fraction but are present in the dialyzate. However, the diglucosides of these anthocyanidins were preferentially retained in the dialyzed fraction. Perhaps, the diglucoside moiety may provide a better interaction with the pectic components in the macromolecular complexes. If only the vesicular portion of the complexes retained polyphenols, a qualitative change in the polyphenol composition would not occur as the vesicles would trap the polyphenols in equal proportion during homogenization. This again supports the existence of pectin−polyphenol interactions, even after treatment with pectinolytic enzymes. Acetoyl glucosides are present in all the fractions but at a slightly lower level in the dialyzed fraction. The largest proportion of malvidin-3-coumaroyl glucoside (69%) was retained in the dialyzed fraction, suggesting a preferential interaction of the relatively more hydrophobic anthocyanins in the macromolecular complexes. This may affect the bioavailability of such anthocyanins because they will pass through the stomach and small intestine without undergoing digestion and absorption. In cell cultures, anthocyanins containing malvidin 3-O-coumaroyl glucosides are more effective against breast cancer cells, whereas in xenografts of MCF-7 in an athymic mouse, anthocyanins containing malvidin-3-O-glucosides are more effective (*27*)(also Hakimuddin et al., unpublished). Such differences may stem from molecular interactions of anthocyanins with macromolecules that affect their bioavailability.

Spectroscopic analyses of the juice preparations showed unique compositional features. The original juice concentrate, dialyzed juice, pectinase-treated dialyzed juice, and concentrated dialyzate were extensively washed with D_2O and finally brought to the same polyphenol content. Authentic malvidin-3-galactoside chloride was also used to obtain comparisons. The spectra were recorded in D_2O rather than deuterated dimethyl sulfoxide or any other solvent usually used to obtain ^1H NMR spectra of polyphenols (*28*) because of the large molecular-weight complexes present in the juice preparations that may become insoluble in organic solvents. Despite this, the signals from authentic malvidin-3-O-galactoside chloride in D_2O were comparable to those published in the literature (*28, 29*). In these studies, the proton at C4 was the most deshielded, and the signal appeared at 9.07 ppm rather than at 8.4 ppm as in our analysis. The signal from the protons at C6 and C8 appeared at 7.1 ppm, both appearing as doublets, whereas these signals were observed at 6.9 ppm in our analysis. The protons at 2′ and 6′ (on the B ring) are equivalent and appeared as a singlet at 8.03 ppm (7.25 ppm in our analysis). The O-methyl protons at 3′ and 5′ appeared as a singlet at 4.05 ppm, which is close to the value of 3.75 ppm observed in our study. Thus, the signals from authentic malvidin-3-O-galactoside resembled those reported in the literature. An interesting observation in our study was that the resonances from the aromatic region of polyphenols were suppressed in the dialyzate preparation and not at all visible when present as a complex in the juice concentrate and the dialyzed juice. The signals from C4, C6, C2′, and C6′ were barely visible and appeared to have undergone a broadening, suggesting hindered motion, energy transfer, and increased spin–lattice relaxation times. However, the signals from the glucose moiety were visible in the carbohydrate region between 3 and 5 ppm. The polyphenol mixture in the juice preparations contains acetoyl and coumaroyl moieties, the signals from which may also appear in the spectrum. The carbohydrate signals were prominent in the dialyzed juice, whereas they were reduced considerably after the pectinase treatment. The spectrum also showed a broad signal potentially originating from lipid methylene protons, indicating the presence of lipid vesicles in the juice (compare this resonance with that from dialyzate, **Figure 7A**,**B** where a distinct triplet from a methyl group is visible).

The fluorescence spectra of the polyphenols isolated from the juice concentrate, the dialyzed juice, and the dialyzate showed interesting differences. By using a synchronized method, the solution was excited at a specific wavelength, the fluorescence was measured at 20 nm over the excitation wavelength, and the process was repeated from 260 to 700 nm. Even though the polyphenol concentration in each sample was the same, the fluorescence output from the polyphenols from the dialyzed sample was quite low. The fluorescence emission peaks for the polyphenols from the juice concentrate and the dialyzate were nearly identical, with similar maximal values. The dialyzate polyphenol preparation showed a slight shift in the emission peak from 386 to 370 nm that may be due to structural changes such as disruption in conjugation in the flavonoid ring. Thus, it appears that during processing, some polyphenols may undergo structural changes that, in turn, may affect their function.

Polyphenols have a very strong antioxidant activity that has been attributed to their structure (*30*). The most efficient antioxidant activity is observed when the structure shows unsaturation between carbons 2 and 3 (C_2-C_3) of the C ring and in the presence of a free hydroxyl group at position 3 (3−OH) of the C ring. Antioxidant activity has been measured by using several techniques such as DPPH, FRAP, ORAC, and so forth (*31*). In the present study, we evaluated the superoxide, hydroxyl, and DPPH radical scavenging capacity of the polyphenols isolated from the original juice concentrate, the

dialyzed juice, and the dialyzate to monitor if potential differences existed in the antioxidant activity because of complex formations. It was interesting to note that the superoxide scavenging property of the polyphenols from the dialyzed juice was undetectable, whereas dose-dependent increases in activities were observed in the polyphenols from the juice concentrate and the dialyzate. A lower magnitude of scavenging was observed for hydroxyl radicals by polyphenols from the dialyzed fraction. All the fractions showed a similar scavenging potential toward DPPH radicals. This, again, may suggest structural modifications in at least some proportion of the polyphenols that may alter their ability to scavenge the free radicals. This may be due to complex formations involving the key hydroxyl groups, making them less amenable to the exchange of protons. Thus, conventional processing may reduce some of the key functionalities of polyphenols. Improvements in processing technology may have to be adopted to help preserve the nutritional quality of fruit juice.

As a comparison to juice concentrate, we also have examined juice derived from fresh grapes, cherries, and mangos (prepared in the laboratory by homogenization in an aqueous medium) for the existence of vesicle−carbohydrate complexes by EM. In all these cases, we were able to observe structures similar to those observed for the grape juice concentrate (Jacob and Paliyath, unpublished). Thus, such structures appear to be a common feature of fruit juice preparations irrespective of the method used for the preparation of the juice or the concentrate. As discussed earlier, the homogenization, extraction, and concentration steps would have facilitated the association of different cellular macromolecules into well-defined structures that may affect the food properties and functionality of ingredients associated with such structures.

In a recent study (*26*), it was reported that nearly 35% of the polyphenols in red wine is bound with soluble dietary fiber and is not accessible for analysis. It was also proposed that such polyphenol−soluble fiber complexes may pass through the intestine without the polyphenols getting absorbed and reach the colon. Colonic fermentation may further release these polyphenols and provide an antioxidant environment in the colon. The present study supports this finding. Despite the loss in some functional aspects, the polyphenol−carbohydrate−lipid complexes may play some hitherto unidentified functional role in human diet.

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