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Chemical Characterization of Red Wine Grape (*Vitis vinifera* and *Vitis* Interspecific Hybrids) and Pomace Phenolic Extracts and Their Biological Activity against *Streptococcus mutans*

JOANNE THIMOTHE,[†,‡] ILLEME A. BONSI,[‡] OLGA I. PADILLA-ZAKOUR,[‡] AND HYUN KOO*[,†]

Eastman Department of Dentistry and Center for Oral Biology, University of Rochester Medical Center, Rochester, New York 14620, and Department of Food Science & Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, New York 14456

Grapes are rich sources of potentially bioactive polyphenols. However, the phenolic content is variable depending on grape variety, and may be modified during vinification. In this study, we examined the chemical composition and biological activity of phenolic extracts prepared from several red wine grape varieties and their fermented byproduct of winemaking (pomace) on some of the virulence properties of *Streptococcus mutans* a well-known dental pathogen. Grape phenolic extracts were obtained from *Vitis vinifera* varieties Cabernet Franc and Pinot Noir and *Vitis* interspecific hybrid varieties Baco Noir and Noiret. The anthocyanins and flavan-3-ols content were highly variable depending on grape variety and type of extract (whole fruit vs fermented pomace). Nevertheless, all grape phenolic extracts remarkably inhibited glucosyltransferases B and C (70–85% inhibition) at concentrations as low as 62.5 μg/mL ($P < 0.01$). Furthermore, the glycolytic pH-drop by *S. mutans* cells was inhibited by the grape extracts without affecting the bacterial viability; an effect that can be attributed to partial inhibition of F-ATPase activity (30–65% inhibition at 125 μg/mL; $P < 0.01$). The biological activity of fermented pomace was either as effective as or significantly better than whole fruit grape extracts. The results showed that grape phenolic extracts, especially from pomace, are highly effective against specific virulence traits of *S. mutans* despite major differences in their phenolic content.

KEYWORDS: Grapes; polyphenols; flavan-3-ols; anthocyanins; *S. mutans*; glucosyltransferases; glycolysis

INTRODUCTION

Grapes are one of the world's largest and economically important fruit crops, which are mostly (>80%) used in winemaking (*1, 2*). Wine grapes are rich sources of flavonols, anthocyanins, proanthocyanidins, catechins, and other phenolic compounds (*3*). However, the phenolic content and composition in grapes are variable depending on cultivar, environmental factors, vintage, and extraction/fermentation conditions (*4*). In general, red grape varieties have higher phenolic content, especially anthocyanins, than their white counterparts (*5*). The distribution of phenolics also varies depending on which part of the grape is used for chemical extraction; for example, grape seeds have higher concentrations of catechins and procyanidins than skins and pulp (*6*). Furthermore, grape pomace (a waste byproduct comprised mostly of skins and seeds) obtained by pressing fermented solids during wine production is particularly rich in polyphenols (*3*). The presence of flavonoids in wine and grape products has been associated with their biological activities, including antimicrobial properties (*6, 7*). Nevertheless, the potential use of grape and its byproducts as sources for novel therapeutics to prevent oral disease has received scant attention.

Streptococcus mutans is regarded as a primary microbial agent in the pathogenesis of dental caries, although additional acidogenic microorganisms may be involved (*8*). This bacterium has at least two specific virulence traits that are involved in the formation of cariogenic biofilms on the tooth surface: (1) synthesis of extracellular polysaccharides (mostly glucans) through glucosyltransferases (GTFs) and (2) ability to (i) produce and (ii) tolerate acids (*9, 10*). The GTFs secreted by *S. mutans* synthesize complex glucans from dietary sucrose which are critical for bacterial accumulation on the tooth surface and contribute to the bulk and structural integrity of the biofilms (*11*). Furthermore, the acid production by *S. mutans* results in low pH values in the plaque matrix which contribute to demineralization of tooth enamel and selection of acid-tolerant

* To whom correspondence should be addressed. Tel: 585-273-4216. Fax: 585-273-1237. E-mail hyun_koo@urmc.rochester.edu.
[†] University of Rochester Medical Center.
[‡] New York State Agricultural Experiment Station.

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organisms, such as mutans streptococci. *S. mutans* has developed mechanisms to alleviate the influences of acidification by increasing proton-translocating F-ATPase activity in response to low pH (*10, 12*). F-ATPase transports protons (H^+) out of cells in association with ATP hydrolysis to maintain intracellular pH more alkaline than the extracellular environment pH (*12*). By aiming to disrupt the ability of *S. mutans* to utilize sucrose to form glucans and acids on tooth surface, therapeutic approaches to reducing the formation of cariogenic biofilms could be precise and selective.

Several studies have shown that extracts from wine (*39*) and other fruits, including apple, cranberry, and cocoa, and their phenolic compounds exhibit antibacterial activities against oral pathogens, including *S. mutans* (*13–16*). Recently, we have also shown that flavonol- and proanthocyanidin-rich fractions of cranberry exhibited inhibitory effects against glucan synthesis, acid production, and F-ATPase activity without displaying biocidal activity (*14*).

Considering that grape- and wine-derived byproducts are largely available and harbor a myriad of substances, some of which we have previously shown to display biological activity against *S. mutans*, the aim of this study was to examine comprehensively whether (1) the chemical composition of grape phenolic extracts is variable depending on variety and type of extract (whole fruit vs grape pomace); (2) the grape extracts exhibit biological activity against *S. mutans*; and (3) the biological activity varies depending on grape variety and type of extract. This study is the first step toward identifying whether grape, including its waste product, could be a potential source for extraction of novel cariostatic agents.

MATERIALS AND METHODS

Grape Extracts. Powdered red grape polyphenolic extracts were prepared from whole fruit (WF) and pomace (skins and seeds) after fermentation on skins (FP). *Vitis vinifera* grapes (Pinot Noir and Cabernet Franc) and *Vitis* interspecific hybrids (Baco Noir and Noiret, a recently released wine variety selected for its deep color resulting from a cross between NY65.0467.08 and Steuben) were used. Grapes were obtained from the 2005 harvest season from wineries and orchards across the Finger Lakes region of New York: Pinot Noir grapes from Hosmer Winery (Ovid, NY), Cabernet Franc grapes from Cornell Orchards (Lansing, NY), Baco Noir grapes from Pleasant Valley Winery (Hammondsport, NY), and Noiret grapes from Swedish Hill Winery (Romulus, NY). For pomace preparation, grapes were washed, destemmed, and crushed in a stemmer/crusher and then dispensed into 5-gal fermentation buckets. One millilter of 10% (w/v) solution of potassium meta bisulfite crystal per liter of grape must was added and allowed to stand for 1 h. Diammonium phosphate (DAP), Fermaid, and yeast (*Saccharomyces cerevisiae* - DV10) were added at concentrations of 1, 0.2, and 0.3 g/L, respectively, to each bucket. Buckets were stored at 18 °C for 9–11 days. Must was pressed when the sugar level was below 0.5%. Pomace was bagged and stored at −5 °C, protected from light. Both grape and pomace samples were freeze-dried using a Virtis SR50C freeze drier (Virtis Co., Gardiner, NY).

Polyphenols were extracted from freeze-dried whole grapes and pomace samples as previously described (*17*) with some modifications. Briefly, 200 mL of methanol/ethanol/water (50/25/25%, v/v) was added to 20 g of freeze-dried sample, and the mixture was sonicated in a Branson 2200 sonicator (Fisher Scientific, Agawam, MA) in ice for 20 min and then centrifuged using a Sorvall Instruments RC5C Centrifuge (Dupont Co., Wilmington, DE) at 10000 rpm for 20 min. Sonication and centrifugation steps were repeated. Solvents were evaporated using a Buchi RE 121 Rotovapor (Brinkmann Instruments, Inc., Westbury, NY) at 35 °C and reduced pressure until an aqueous polyphenolic extract was obtained. The aqueous extract was passed through preconditioned C18 Sep Pak cartridges. The cartridges were then washed with 0.01 N aqueous HCl to remove acids, sugars, and other water-soluble compounds and dried with a current of nitrogen. The polyphenols were eluted with absolute methanol. The methanol was evaporated as described above to dryness. Extracts were redissolved in distilled water and lyophilized to obtain powdered extracts.

Determination of Total Phenolic, Anthocyanin, and Flavan-3-ol Content. The total phenolic content in the extracts was measured using the Folin–Ciocalteu (FC) method (*18*). The absorbance of the samples was read after 90 min at 750 nm. Total phenols were expressed in grams of gallic acid equivalents (GAE)/100 g of extract.

The total monomeric anthocyanin content was determined using the pH differential method (*19*) with some modifications. Absorbance was measured at 520 and 700 nm. The total monomeric anthocyanin content was calculated using an absorptivity coefficient and molecular weight of 28000 L/cm/mol and 529 g/mol, respectively, and expressed in grams of malvidin-3-glucoside equivalents/100 g of extract (*20*).

The total flavan-3-ol content was estimated using a modified vanillin assay (*21*). Absorbance was read at 500 nm using a Barnstead Turner SP830 Spectrophotometer (Barnstead International, Dubuque, IA). Absorbance values were corrected for anthocyanin interference by carrying out the reaction as described above in the absence of vanillin (*22*). Total flavan-3-ols were quantified using a standard curve prepared from a catechin standard obtained from Sigma Chemical Co. (St. Louis, MO). Results were expressed as grams of catechin equivalents/100 g of extract.

HPLC Analysis of Grape Extracts. High performance liquid chromatography (HPLC) analysis was conducted using modified methods of Kim and Padilla-Zakour (*23*). The main polyphenolic extracts were analyzed using a reversed-phase HPLC system (Hewlett-Packard model 1100; Palo Alto, CA). A C18 reversed-phase Symmetry Analytical column (5-m × 250-mm × 4.6-mm) with a Symmetry Sentry guard column was used. Powdered extracts were resolubilized in methanol, appropriately diluted, and hydrolyzed with 2 M HCl by heating for 1 h at 90 °C to facilitate individual compound identification against available standards. The linear solvent gradient of binary mobile phases were solvent A, 0.1% phosphoric acid (H_3PO_4) in HPLC-grade water, and solvent B 0.1% H_3PO_4 in HPLC-grade acetonitrile, and these were applied (for 55 min), as follows: 92% A/8% B at 0 min, 89% A/11% B at 4 min, 65% A/35% B at 25 min, 40% A/60% B at 30 min, 40% A/60% B at 40 min, 65% A/35% B at 45 min, 89% A/11% B at 50 min, and 92% A/8% B at 55 min. Flow rate was set to 1 mL/min. The detector was set to 280, 320, 370, and 520 nm. Individual phenolic compounds were identified by comparison of UV–vis spectra and retention times, as well as spiking samples with standards, and were quantified on the basis of generated calibration curves for each compound. Standards tested included gallic acid, caffeic acid, ferulic acid, *p*-coumaric acid, sinapic acid, shikimic acid, chlorogenic acid, catechin, epicatechin, procyanidin B1, procyanidin B2, resveratrol, myricetin, quercetin, kaempferol, isorhamnetin, naringin, delphinidin, cyanidin, petunidin, peonidin, and malvidin. Samples for chemical analyses were analyzed in duplicate.

Bacterial Strains. The bacterial strains used for the production of GTFs were as follows: *Streptococcus anginosus* KSB8 (kindly provided by Howard K. Kuramitsu, State University of New York, Buffalo), which harbors the *gtfB* gene (for GTF B production), and *S. mutans* WHB 410 (*24*), where *gtfB*, *gtfD*, and *ftf* genes were deleted (for GTF C production). The *S. mutans* UA159, a proven virulent cariogenic pathogen and the strain selected for genomic sequencing (*25*), was used for F-ATPase and glycolytic pH-drop studies and antimicrobial assays. The cultures were stored at −80 °C in tryptic soy broth containing 20% glycerol.

GTF Assays. The GTF B and C enzymes (EC 2.4.1.5) were prepared from culture supernatants and purified to near-homogeneity by hydroxyapatite column chromatography as described previously (*24, 26*). GTF B or C was mixed with a 2-fold dilution series of grape phenolic extracts (concentration ranging from 15.6 to 125 μg/mL) or vehicle control (10% ethanol, v/v) and incubated with [^{14}C]glucose-sucrose substrate (0.2 μCi/mL; 200 mM sucrose, 40 μM dextran 9000, and 2% sodium azide in a buffer consisting of 50 mM KCl, 1 mM KPO_4, 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$, pH 6.5) to a final concentration of 100 mM sucrose (*27*). GTF activity was measured by the incorporation of [^{14}C]glucose from labeled sucrose (NEN Research Products, Boston, MA) into

Table 1. Total Phenolic, Anthocyanin, and Flavan-3-ol Contents of Freeze-Dried Grape Phenolic Extracts[a]

sample	total phenolic content (g of GAE eq/100 g of extract)	total anthocyanin content (g of mal eq/100 g of extract)	flavan-3-ol content (g of cat. eq/100 g of extract)
V. vinifera			
Pinot Noir WF	56.0 ± 6.4	4.67 ± 0.04	56.7 ± 0.0
Pinot Noir FP	61.8 ± 0.8	0.59 ± 0.01	48.4 ± 1.3
Cab. Franc WF	42.5 ± 1.1	9.78 ± 0.14	28.0 ± 0.5
Cab. Franc FP	46.9 ± 4.1	6.72 ± 0.06	24.2 ± 1.0
Vitis interspecific hybrids			
Baco Noir WF	34.4 ± 3.1	13.46 ± 0.32	12.3 ± 0.7
Baco Noir FP	51.5 ± 0.4	3.99 ± 0.01	30.0 ± 0.2
Noiret WF	37.1 ± 5.0	22.70 ± 0.20	19.6 ± 0.8
Noiret FP	41.1 ± 1.1	16.54 ± 0.26	18.0 ± 0.1

[a] Mean ± standard deviation of duplicate samples. Abbreviations: WF, whole fruit; FP, fermented pomace; Cab., cabernet; GAE, gallic acid equivalents; mal eq, malvidin-3-glucoside equivalents; cat. eq, catechin equivalents.

glucans (*27*). The GTF enzyme added to each sample for all assays was equivalent to the amount required to incorporate 1–1.5 μmol of glucose over the 4 h reaction (1.0–1.5 U). All of the assays were conducted in triplicate from at least two separate experiments. Although grape extracts contain some weak phenolic acids, it did not influence the pH of the reaction mixture; the pH values were between 6.8 and 6.9, which are within optimum pH for the activity of GTF enzymes (pH 6.5 and 7.0) (*34*).

F-ATPase and Glycolytic pH Drop Assays. F-ATPase assay was performed using permeabilized cells of *S. mutans* UA 159 by subjecting the cells to 10% toluene (v/v) followed by two cycles of freezing and thawing as described elsewhere (*28*). F-ATPase was measured in terms of the release of phosphate in the following reaction mixture: 75 mmol of Tris-maleate buffer (pH 7.0) containing 5 mM ATP, 10 mmol of $MgCl_2$, permeabilized cells, and the grape phenolic extracts (concentration ranging from 15.6 to 125 μg/mL) or vehicle control (10% ethanol, v/v). The released phosphate (over the 10-min reaction time) was determined by the method of Bencini et al. (*29*).

The effects of the grape phenolic extracts on glycolysis were measured by standard pH drop with dense cell suspensions (*28*). Cells of *S. mutans* UA159 were collected at the stationary phase by centrifugation, washed with cold salt solution (50 mM KCl plus 1 mM $MgCl_2$, pH 7.0), and resuspended in salt solution containing the grape phenolic extracts (concentration ranging from 62.5 to 500 μg/mL) or vehicle control (10% ethanol, v/v). Glucose was added in the mixture to give a final concentration of 1% (w/v). The decrease in pH, as a result of glycolytic activity of the bacterial cells, was assessed by means of a glass electrode (Futura Refillable Combination pH electrode, 5 mm diameter, Beckman Instruments, Inc., Fullerton, CA) over a period of 75 min (*28*). All of the assays were conducted in triplicate from at least two separate experiments.

Antimicrobial Assays. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each of the test compound were determined in accordance to CLSI (formerly NCCLS) guidelines (NCCLS, 2000) as described in Koo et al. (*27*). The broth microdilution and macrodilution methods (in tryptic soy broth) were used for the antibacterial tests. The starting inoculum was 1×10^6 colony forming units/mL (at log phase), and the concentration of test compounds ranged from 62.5 to 500 μg/mL (2-fold dilution).

Statistical Analyses. Analysis of covariance (ANCOVA) was used to assess differences in percent enzyme activity between agents at each concentration. The models included categorical variables for agent, concentration, and their interaction. Overall tests were F-tests, and individual comparisons used *t* tests. ANCOVA was also used to assess differences in mean pH levels between agents at each time point. The models included categorical variables for agent, time, and their interaction. Overall tests were F-tests, and individual comparisons used *t* tests. All statistical analyses were performed using SAS software (Version 9.1; SAS Institute Inc., Cary, NC). *P* values reported are two-sided, with a level of significance set at $\alpha = 0.01$.

RESULTS AND DISCUSSION

The wine grapes (Cabernet Franc, Pinot Noir, Baco Noir, and Noiret) analyzed in this study were selected from our initial screening from nine different varieties of *Vitis vinifera*, *V. labrusca*, and *Vitis* interspecific hybrids based on (i) total phenolic content, (ii) biological activity (anti-GTF effect), and (iii) availability (Bonsi et al., 2007 Institute of Food Technologists Annual Meeting, Chicago, IL, Abstract 146-05); the selected grape varieties represent important cultivars for red winemaking, particularly in New York and the northeastern United States. We chose red wine grapes due to their higher phenolic content compared to table grapes (*30*) and because of the availability of pomace as an inexpensive source of extractable material. The polyphenols were extracted from grapes (and its byproduct) by chromatographic methods, which removed most of naturally occurring sugars and acids (which could interfere with our biological assays). The phenolic composition, mainly anthocyanin content, was variable depending on grape variety and on wine processing. Nevertheless, all of the grape phenolic extracts of varieties studied showed significant biological activities against some of the virulence traits of *S. mutans* involved in extracellular polysaccharide synthesis and acidogenicity. It is noteworthy that the inhibitory effects of pomace extracts were either as effective as or significantly better than the fruit extracts.

Chemical Composition of Whole Fruits and Pomace Phenolic Extracts. Table 1 shows the total content of the predominant phenolic compounds by variety for both whole fruit and pomace extracts. The total content of the phenolic compounds was variable depending on variety and wine processing, as reported previously by others (*3, 4, 31, 32*). From the fruit extracts, Pinot Noir had the highest concentration of total phenolics and flavan-3-ols and the lowest anthocyanin content, while Noiret had the highest anthocyanin content. Even though pomace represents a waste material from the wine fermentation process, the extracts produced from pomace for all the varieties studied had either comparable or slightly higher total phenolic content than the whole fruit extracts; total phenols in the pomace was between 41.1 and 61.8 g of GAE/100 g of extract which was similar to that found in *V. vinifera* var. Bangalore blue pomace (*33*). Furthermore, pomace extracts had similar concentrations of flavan-3-ol than the whole fruit, except for Baco Noir, which showed twice the concentration found in the fruit extract. In contrast, the anthocyanins content in all of the pomace extracts was lower than that in whole fruit extracts.

In order to further characterize the composition of the grape extracts, we conducted HPLC analysis of the whole fruit and pomace phenolic extracts after hydrolysis, thus allowing the

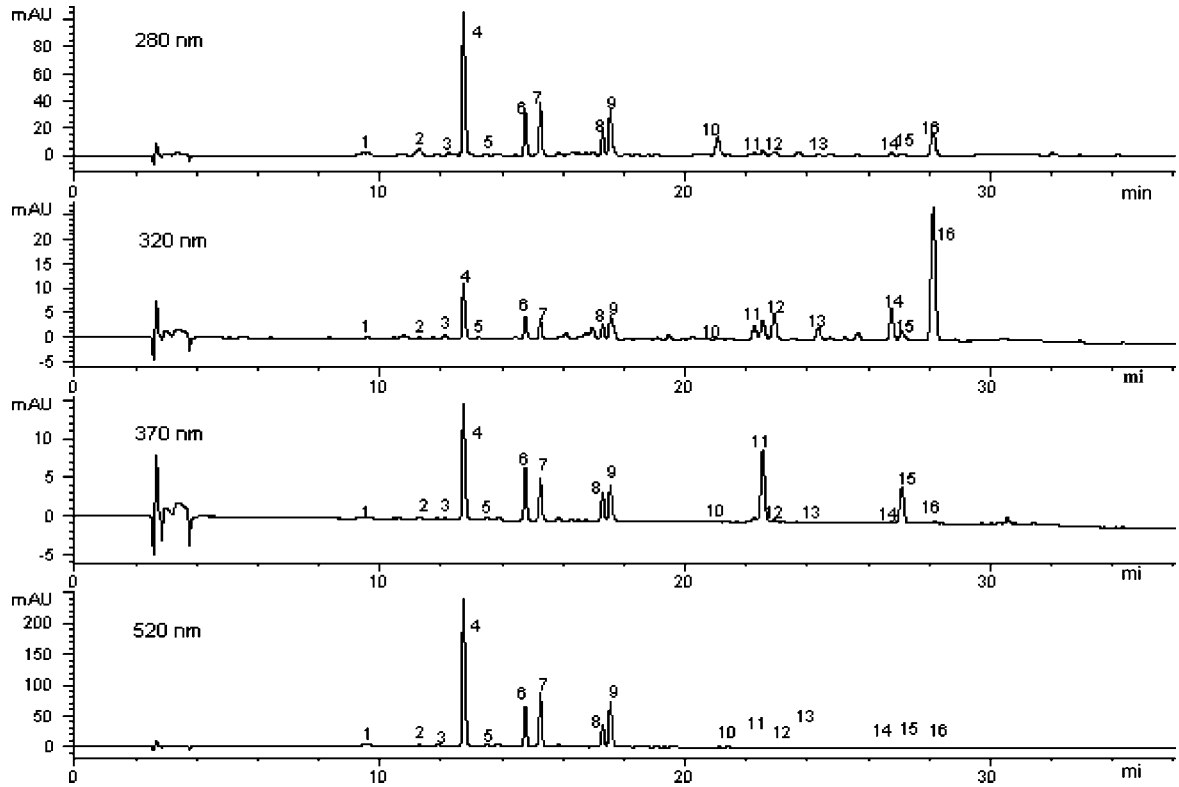

Figure 1. HPLC chromatogram for Noiret whole fruit polyphenolic extract at 280, 320, 370, and 520 nm (different scale size) (mAU = milli absorbance units): 1, procyanidin B1; 2, catechin; 3, procyanidin B2; 4, delphinidin; 5, epicatechin; 6, cyanidin; 7, petunidin; 8, peonidin; 9, malvidin; 10, unknown; 11, myricetin; 12, unknown; 13, resveratrol; 14, unknown; 15, quercetin; 16, unknown.

Table 2. Major Phenolic Compounds Present in Freeze-Dried Grape Phenolic Extracts (g/100 g of Extract) Quantified by HPLC Analysis[a]

	phenolic acids	flavan-3-ols				stilbenes
sample	gallic acid	catechin	epicatechin	procyanidin B1	procyanidin B2	resveratrol
V. vinifera						
Pinot Noir WF	0.104 ± 0.002	9.69 ± 0.12	0.777 ± 0.014	1.23 ± 0.10	1.00 ± 0.01	0.029 ± 0.001
Pinot Noir FP	0.075 ± 0.004	8.10 ± 0.12	0.629 ± 0.004	1.09 ± 0.03	N/D	0.025 ± 0.001
Cab. Franc WF	0.077 ± 0.003	8.69 ± 0.04	0.555 ± 0.031	N/D	N/D	0.028 ± 0.001
Cab. Franc FP	0.100 ± 0.014	9.73 ± 0.01	0.616 ± 0.011	0.68 ± 0.02	N/D	0.022 ± 0.001
Vitis interspecific hybrids						
Baco Noir WF	0.035 ± 0.011	6.34 ± 0.09	0.451 ± 0.004	N/D	0.70 ± 0.06	N/D
Baco Noir FP	0.120 ± 0.011	13.09 ± 0.09	0.966 ± 0.012	N/D	N/D	N/D
Noiret WF	N/D	3.35 ± 0.19	0.831 ± 0.019	4.31 ± 0.17	1.51 ± 0.02	0.058 ± 0.003
Noiret FP	N/D	5.31 ± 0.10	1.021 ± 0.020	5.12 ± 0.08	N/D	0.044 ± 0.001

[a] Mean ± standard deviation of duplicate samples. Abbreviations: WF, whole fruit; FP, fermented pomace; N/D, non-detectable. Gallic acid was the only detectable phenolic acid in the grape extracts.

quantification of the major moieties, for the four varieties studied. **Figure 1** shows a typical chromatographic separation obtained with our chromatographic method. **Tables 2** and **3** presents the concentration of individual phenolic compounds in each of the extracts. The grape extracts showed major differences in the concentration of the various anthocyanins consistent with total phenolic content data presented in **Table 1**. In general, hybrids displayed the highest concentration of delphinidin and petunidin. For example, the concentration of delphinidin and petunidin in Noiret extracts was up to 30 times higher than other grape extracts. Some differences in the composition and concentration of flavonols (e.g., kaempferol) and flavan-3-ols (e.g., catechins and procyanidin B1) were also detected among the extracts; Noiret showed particularly higher concentration of procyanidin B1 and myricetin compared to other extracts. Clearly, the content of anthocyanins and flavan-3-ol (especially monomers) was highly variable depending on (i) grape variety and (ii) type of extract (whole fruit vs fermented pomace).

Biological Activities of Grape Phenolic Extracts against *S. mutans*. *Effects on GTF Activity*. The effects of phenolic extracts on the activity of GTF B are shown in **Figure 2**. All of the extracts significantly reduced the activity of GTF B at all concentrations tested compared to vehicle control ($P < 0.01$) in a dose-dependent manner. The effects of phenolic extracts from *V. vinifera* varieties on GTF activity was similar irrespective of whether the extracts were obtained from whole fruit (WF) or fermented pomace (FP) ($P > 0.01$); the Cabernet Franc extracts were more effective GTF inhibitors than Pinot noir extracts only at concentration of 15.625 μg/mL ($P < 0.01$). In contrast, FP extracts of Baco Noir and Noiret inhibited the glucan synthesis more effectively than the WF extracts ($P < 0.01$). Individual comparisons revealed that extract from Baco Noir FP was the most effective inhibitor among the hybrid extracts. Similar inhibitory profile was observed for GTF C (data not shown).

Glucosyltransferases are specific and proven virulence traits of *S. mutans* associated with (i) the pathogenesis of dental caries

Table 3. Major Phenolic Compounds Present in Freeze-Dried Grape Phenolic Extracts (g/100 g of Extract) Quantified by HPLC Analysis[a]

	flavonols			anthocyanidins				
sample	myricetin	quercetin	kaempferol	delphinidin	cyanidin	petunidin	peonidin	malvidin
V. vinifera								
Pinot Noir WF	N/D	0.232 ± 0.005	0.229 ± 0.001	1.09 ± 0.01	5.56 ± 0.05	0.63 ± 0.01	2.79 ± 0.01	4.70 ± 0.01
Pinot Noir FP	N/D	0.128 ± 0.004	0.165 ± 0.002	N/D	3.96 ± 0.02	N/D	0.43 ± 0.01	1.18 ± 0.01
Cab. Franc WF	0.137 ± 0.002	0.479 ± 0.002	0.141 ± 0.001	3.80 ± 0.04	4.34 ± 0.02	2.23 ± 0.06	2.86 ± 0.01	7.90 ± 0.01
Cab. Franc FP	0.054 ± 0.003	0.375 ± 0.011	0.155 ± 0.006	2.50 ± 0.05	4.43 ± 0.04	1.56 ± 0.01	1.94 ± 0.01	5.80 ± 0.02
Vitis interspecific hybrids								
Baco Noir WF	0.112 ± 0.003	0.347 ± 0.017	0.028 ± 0.010	6.47 ± 0.07	3.56 ± 0.01	4.39 ± 0.03	1.90 ± 0.06	7.42 ± 0.01
Baco Noir FP	0.007 ± 0.001	0.260 ± 0.004	0.055 ± 0.001	2.38 ± 0.01	3.27 ± 0.03	1.50 ± 0.01	0.92 ± 0.01	3.26 ± 0.01
Noiret WF	0.307 ± 0.005	0.157 ± 0.005	N/D	31.08 ± 0.27	7.53 ± 0.16	10.23 ± 0.06	4.30 ± 0.17	6.99 ± 0.26
Noiret FP	0.363 ± 0.007	0.289 ± 0.001	N/D	20.23 ± 0.67	6.20 ± 0.09	8.03 ± 0.01	3.11 ± 0.20	6.30 ± 0.03

[a] Mean ± standard deviation of duplicate samples. Abbreviations: WF, whole fruit; FP, fermented pomace; N/D, non-detectable.

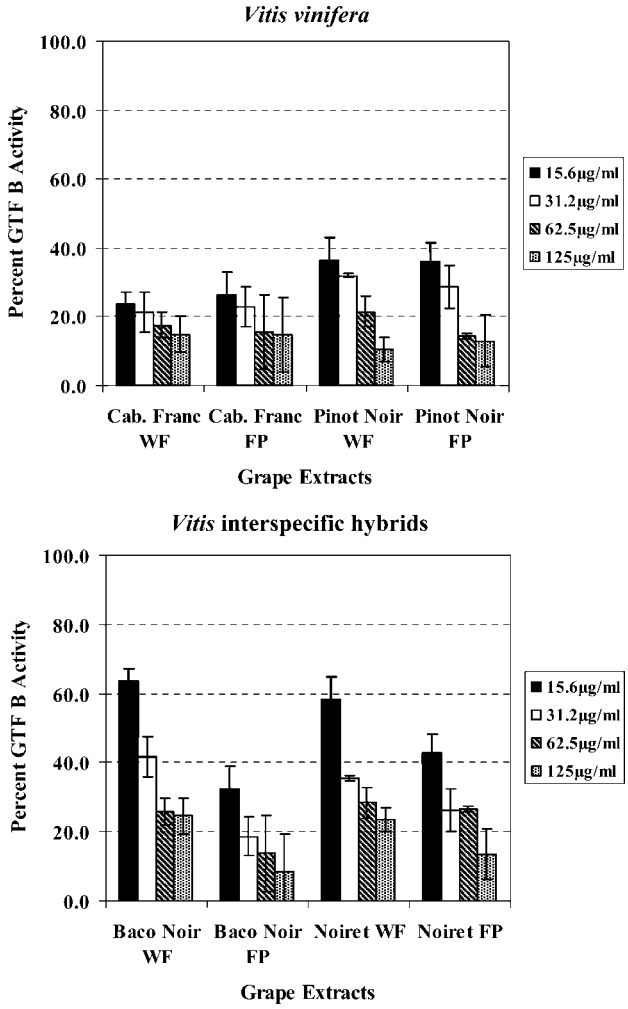

Figure 2. Influence of selected *V. vinifera* and *Vitis* interspecific hybrids grapes extracts on the activity of GTF B in solution. The final concentration of ethanol in each assay is 10% (v/v). The percent of enzyme activity is calculated against a sample with no extract as 100% GTF B activity. All of the grape extracts ($n = 9$) significantly reduced the activity of GTF B at all concentrations tested compared to vehicle control ($P < 0.01$).

and (ii) bulk and structural integrity of dental biofilm (plaque) (*9*). The glucans synthesized by these enzymes promote the binding and accumulation of *S. mutans* and other cariogenic bacteria on the tooth surface and contribute to the formation of the matrix of biofilms (*9, 11, 35*). Therefore, one of the strategies to control biofilm formation and dental caries is to inhibit the activity of GTFs; GTF B (which synthesizes a polymer of mostly insoluble α1,3-linked glucan) and GTF C (which synthesizes a mixture of insoluble α1,3-linked glucan and soluble α1,6-linked glucan) were used in our assays because these enzymes have been shown to be essential for the expression of virulence by *S. mutans* in causing dental caries *in vivo* (*35*). In general, the phenolic extracts from pomace were highly effective GTF inhibitors (>60% inhibition) even at concentrations as low as 15.6 μg/mL; this level of inhibition has not been observed previously in our laboratory (*15, 24*) and would certainly disrupt the formation of cariogenic biofilms based on our *in vitro* and *in vivo* studies (*27, 35*).

The putative bioactive compound(s) of grape phenolic extracts (especially pomace) that are modulating the GTF inhibition are unknown, and we are currently pursuing this issue. Nevertheless, the presence of various flavonoids, such as low molecular weight flavonols, may be associated with enzyme inhibition. We have shown that flavonols, such as quercetin, myricetin, and kaempferol (which were identified in the grape extracts) are effective GTF inhibitors (*14*); the inhibitory effect could be associated with the presence of an unsaturated double bond between C-2 and C-3, which may provide a site for nucleophilic addition by side chains of amino acids in GTFs (*14*). However, the concentration of each of the flavonols present in the grape extracts is 10–100 times lower than that required for the enzyme inhibition (the concentration of the compounds to inhibit the enzymatic activity by 50% was between 25 and 100 μg/mL). On the other hand, flavan-3-ol monomers (catechin and epicatechin) and anthocyanins, which lack a double bond between C-2 and C-3, as well as procyanidin B1 and procyanidin B2 (dimers), exhibited either negligible or modest inhibitory activities even at concentrations as high as 1 mg/mL (*13, 15*). This observation could, in part, explain why the anti-GTF activity of the various grape extracts was not affected despite major qualitative and quantitative differences in anthocyanin and flavan-3-ol content. Clearly, there are other compounds, such as high molecular weight proanthocyanidins (oligomers of flavan-3-ols), that may be involved in the GTF inhibition by the grape phenolic extracts. Further chemical analysis and research with individual compounds is needed to elucidate the specificity and mechanistic details of GTF inhibition by the grape extracts.

Effects on S. mutans Acidogenicity. The effects of grape phenolic extracts from WF and FP on aciduric and acidogenic properties of *S. mutans* were examined by glycolytic pH-drop and F-ATPase activity assays. The *S. mutans* can survive and carry out glycolysis at low pH which can lead to the demineralization of the adjacent dental enamel leading to formation of carious lesions (*36*). *S. mutans* cells rapidly degrade glucose and lower the pH value of the suspension until they can no

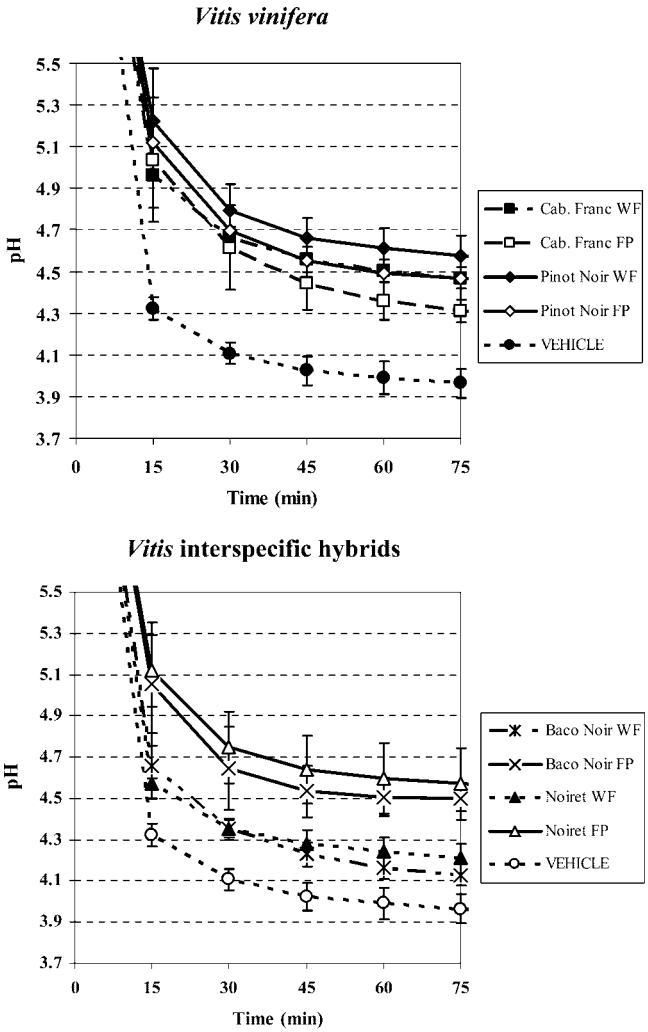

Figure 3. Influence of selected *V. vinifera* and *Vitis* interspecific hybrids grapes extracts on glycolytic pH drop of *S. mutans* UA 159 cells. Planktonic cells in presence of excess glucose and of grape extracts at 500 μg/mL. Values (SD, $n = 6$) from all grape extracts and from vehicle control are significantly different at time points $t_{15\ min}$, $t_{30\ min}$, $t_{45\ min}$, and $t_{60\ min}$ ($P < 0.01$).

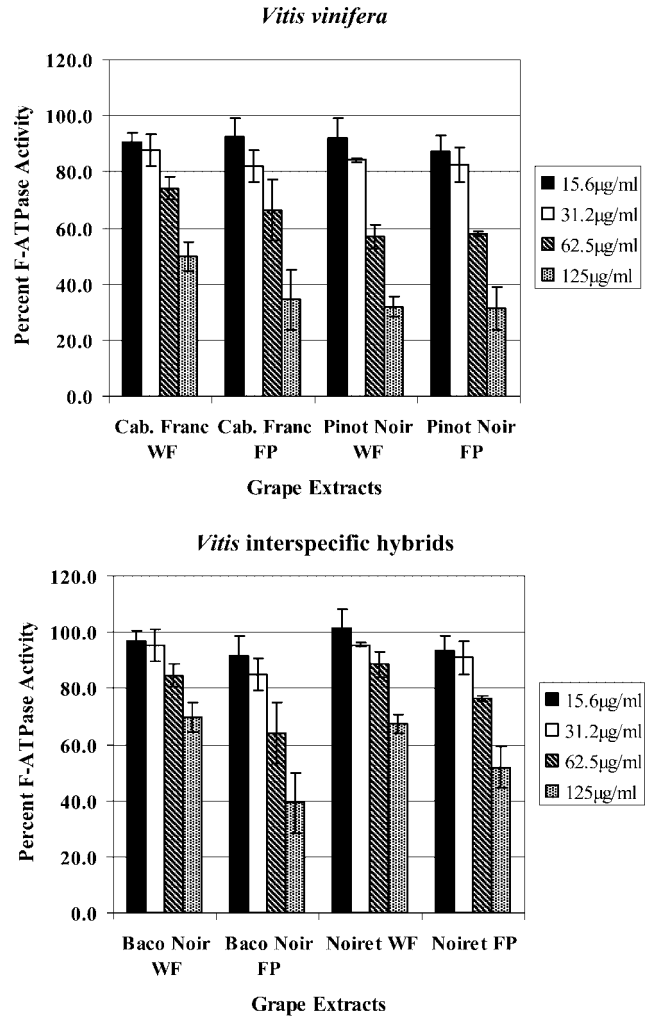

Figure 4. Effects of selected *V. vinifera* and *Vitis* interspecific hybrids grapes extracts on F-ATPase activity. The final concentration of ethanol in each assay is 10% (v/v). The percent of enzyme activity is calculated against a sample with no extract as 100% F-ATPase activity. All grape extracts ($n = 9$) significantly reduced the activity of F-ATPase at 125 μg/mL compared to vehicle control ($P < 0.01$).

longer maintain a cytoplasmatic pH compatible with activity of glycolytic enzymes. Acid sensitization can be rapidly seen in glycolytic pH-drop experiment in which cells are given excess glucose. Thus, the rate of pH drop reflects acidogenic capacities of the cells, while final pH values of the suspensions reflect acid tolerance. The results of pH-drop experiments in suspensions of *S. mutans* UA 159 with excess glucose in the presence of grape extracts (or vehicle control) are presented in **Figure 3**. All grape extracts significantly disrupted acid production of *S. mutans* cells at 500 μg/mL ($P < 0.01$). The presence of grape extracts sensitized the cells to acidification to the point that the final pH values ($t_{75\ min}$) were significantly higher (0.25–0.7 units) than those in the presence of vehicle control ($P < 0.01$), except Baco Noir WF and Noiret WF ($P > 0.01$), indicating that acid tolerance of *S. mutans* was affected; the effects may be related to F-ATPase inhibition since the extracts were devoid of any biocidal activity (as determined by plating aliquots of cell suspension at each time point, and counting the colony forming units/mL). The proton translocating F-ATPase protects *S. mutans* against environmental acid stress by regulating pH homeostasis, which is critical for the optimum function of glycolysis (*13*). Enolase and other enzymes of the glycolytic pathway and the sugar transport system are sensitive to cytoplasmic acidification (*28*). As shown in **Figure 4**, the F-ATPase activity of *S. mutans* UA159 was partially inhibited (23–69%) by the grape extracts at 62.5 μg/mL (except Noiret extracts) and 125 μg/mL ($P < 0.01$). All of the *V. vinifera* grapes extracts (Cab. Franc WF, Cab. Franc FP, Pinot Noir WF, Pinot Noir FP) and Baco Noir FP and Noiret FP inhibited at least 48% of the enzyme activity at 125 μg/mL; these same extracts also showed higher final pH values in the glycolytic pH drop experiments as shown in **Figure 3**. Overall, the F-ATPase sensitivities to grape extracts agree well with the pH drop data in **Figure 3**, and shows that fermented pomace of all grape varieties tested is biologically active against *S. mutans* acidogenicity. Flavonoids have been shown to inhibit various forms of ATPase, including *S. mutans* membrane-associated F-ATPase activity (*14, 37*). Among them, quercetin, myricetin, and their glycosides moderately inhibited the activity of F-ATPases (15–35% inhibition) but only at high concentrations (150–300 μg/mL) (*37*). Anthocyanins (e.g., cyanidin and peonidin) and low-molecular weight flavan-3-ols (e.g., epicatechin) were devoid of any inhibitory effects against F-ATPase (*14, 37*). As observed for GTF activity assays, the biological effects on acidogenicity of *S. mutans* may involve other unidentified compounds, and were not affected by the

differences in the anthocyanins and flavan-3-ols content of the various grape phenolic extracts.

Interestingly, none of the extracts showed effects on the growth of *S. mutans* at concentrations tested in this study (up to 500 μg/mL), although other studies have reported selected antimicrobial activity of grape extracts against *Listeria monocytogenes* (*39*) and of wine extracts against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Streptococcus pyogenes*, and oral streptococci (*7, 39*). However, the antimicrobial activity of wine extracts against oral streptococci was mainly attributed to the presence of organic acids rather than the polyphenols (*39*), which could explain the lack of microbiocidal activity of the grape phenolic extracts tested in this study.

Overall, grape phenolic extracts exhibit biological activity against *S. mutans* by disrupting essential virulence traits (especially GTF activity) of this ubiquitous oral pathogen involved in formation and acidogenicity of dental biofilms without affecting bacterial viability; grape extracts may harbor specific compounds that may be useful for pathogenic biofilm control. Despite major qualitative and quantitative differences in the content of anthocyanins and flavan-3-ols (especially monomers) among different pomace and fruit extracts, it did not influence their biological activities against *S. mutans*. Additional chemical analyses, including characterization of the high molecular weight proanthocyanidins, are needed to identify the putative bioactive compound(s) in the grape extracts. Furthermore, pomace extracts were as effective as or better than the fruit extracts in reducing the activity of GTF and *S. mutans* acidogenicity irrespective of the variety of the grape tested in this study. Clearly, fermented pomace is a promising and feasible (low cost and largely available) source for extraction and isolation of compounds for prevention of oral diseases, such as dental caries.

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