Technical Brief

Evaluation of the Phenol-Free Glycosyl-Glucose Determination

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Grape glycosides are, in part, an important source of varietal wine aroma and flavor. Aglycones may be aliphatic residues, monoterpenes, sesquiterpenes, norisoprenoids, or phenolic compounds. Studies were undertaken to evaluate and compare methods of isolating non-phenolic or phenol-free glycosides. Phenol - free glycoside fractions were obtained by three isolation methods: the addition of polyvinylpolypyrrolidone (PVPP), isolation at pH 10.0 using C18 solid phase extraction cartridges, or isolation at pH 13.0 using Oasis™ hydrophilic-lipophilic balance (HLB) extraction cartridges. Glycoside separation using the Oasis cartridges reduced the phenol content of the phenol-free glycoside fractions in Chardonnay juice and wines by an average of 49.7% compared to 33.6 % using C18. The differences in the phenol concentration in the phenol-free glycoside fractions with these two methods were greater with red juices and wines. For the Cabernet Sauvignon juice and wines the phenol concentration was reduced by an average 103% and 73% using the Oasis and C18 cartridges, respectively.

KEY WORDS: glycosides, phenol-free glycosides, glycosyl-glucose, GG

Grape-derived secondary metabolites are principal sources of wine aroma, flavor, and color [6]. Aroma and flavor compounds are present as free volatiles and, in part, as sugar-bound nonvolatile precursors, including glycosides [16]. Glycosidically bound compounds can undergo enzyme and/or acid catalyzed hydrolysis during processing and wine aging to yield free volatiles [3,5,16]. The majority of hydrolysis products are odorless or possess only weak aromas or flavors, although a significant number of compounds have not been identified [4,13]. However, the sensory significance of some products of glycoside hydrolysis to varietal wine aroma and flavor has been established, providing justification and rationalization for their quantification and study [13]. A positive correlation between the concentration of grape glycoconjugates and wine quality has been suggested [1].

Traditional measures of grape glycoside hydrolysis products are either time consuming, requiring isolation, hydrolysis, and gas chromatography [15], or are restricted in application to floral, high terpene varieties [2]. Abbott *et al.* [1] and Williams *et al.* [17] proposed a method for the determination of the total pool of glycosidically bound secondary metabolites. Glycoside hydrolysis yields equimolar proportions of D-glucose and the aglycone, the former referred to as glycosyl - glucose (GG). The authors proposed that the determination of GG provides an indirect measure of the total pool of glycosidically bound secondary metabolites, some of which are aroma and flavor precursors.

The determination of total GG measures all grape glycosides including aliphatic residues (C4 - C8 monoterpenes, sesquiterpenes, norisoprenoids, and shikimic acid metabolites. This procedure has been refined to also include a non-colored or red-free glycosyl-glucose measurement, obtained by subtracting the anthocyanin molar equivalent from the total GG value, since the molar relationship between anthocyanin and glucose is 1:1 [8,9]. Although phenolic glycosides are important wine color and structural components, their impact on aroma and flavor is not believed to be substantial. Williams *et al.* [18] suggested an additional determination that would, in part, eliminate phenolic glycosides to produce a phenol-free GG. Zoecklein *et al.* [19] reported the use of polyvinylpolypyrrolidone (PVPP) as a means of removing a significant proportion of phenolic glycosides to produce a phenol-free GG measurement. These analyses have been used to aid in the understanding of the relationships between vineyard management, processing, wine aging and grape glycosides [7,10,11,12,18,20,21,22,23].

This investigation was undertaken to compare the phenol concentration of phenol-free GG fractions obtained using three different techniques. Additionally, the use of a buffer system was evaluated to increase the efficiency of the GG procedure.

Materials and Methods

In study 1, eight replications of Riesling, Chardonnay, Cabernet Sauvignon and Merlot juices and wines were used to evaluate the total and phenol-free glycosyl-glucose (GG) concentrations and the total phenol concentration of the phenol-free GG fractions. The GG procedure used was that of Abbott *et al.* [1] and Williams et al. [17]. Wine samples were diluted to less than 7% alcohol (v/v), and aliquots of clarified juice or wine were adjusted to pH 2.25 for total GG and pH 10 for the phenol-free GG determination using concentrated hydrochloric acid or sodium carbonate (20% w/v), respec-

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Table 1. Mean total phenols, total glycosyl-glucose (TGG μ*M*), phenol-free (pH 10) glycosyl-glucose (PFGG μ*M*), and the phenol concentration of TGG and PFGG fractions.

	Total phenols (mg/L)	TGG (μ*M*)	PFGG (μ*M*)	Tot. phenols TGG fraction (mg/L)	Tot. phenols PFGG fraction (mg/L)
White juice	460	644	490	180	32
White wine	300	250	195	21	4
Red juice	690	1760	850	370	50
Red wine	2500	2980	550	1050	140

tively. Samples were loaded onto 500 mg C18 solid phase extraction cartridges (Waters, Milford, MA), rinsed with distilled water, then the glycosides were eluted with ethanol and hydrolyzed with 1.5 *M* sulfuric acid at 100°C for one hour. After cooling, the hydrolysates were passed through the regenerated extraction cartridges, as described by Iland *et al.* [9], neutralized, and the glycosyl-glucose determined enzymatically (Boehringer Mannheim, Indianapolis, IN). Aliquots for total phenol determination were taken from the original samples and from the total GG and phenol-free GG fractions. Total phenols were determined using the Folin-Ciocalteu reagent which acts predictably with all natural phenols. The analysis was performed by the spectrometric method described by Singleton *et al.* [14]. A parallel experiment (Study 2) was established using polyvinylpolypyrolidone (PVPP). Samples were treated with 10% PVPP for 30 minutes at ambient temperature, filtered and analyzed for total GG, phenol-free GG, and residual phenols as described above.

The phenol -free GG analysis separates those glycosides without phenolic or other functional groups ionizable at pH 10.0 [18]. The separation of phenolic glycosides could be enhanced by increasing the sample pH above 10. However, silica-based C18 solid phase extraction cartridges are limited by the solubility of the silica support at high pH. In order to circumvent this limitation, the use of Oasis™ HLB (hydrophilic, lipophilic balance) (Waters, Milford, MA) 200 mg polymeric reverse - phase solid extraction cartridges was investigated, as suggested by Francis (I. L. Francis, personal communication, 1998). This sorbent contains a patented copolymer reported to provide high and reproducible recoveries particularly when analyte ionization is suppressed. In Study 3, samples of Chardonnay and Cabernet Sauvignon juices and wines (three replicates of each) were analyzed for total and phenol -free GG as described above (pH adjusted to pH 2.25 or 10 and C18 solid phase extraction cartridges) and by loading samples adjusted to pH 13 onto Oasis™ HLB cartridges.

In a fourth study, an evaluation of a modification of the original GG procedure was investigated. The final step of the GG analysis involves neutralizing the acid hydrolyzate with sodium hydroxide and performing the assay for glucose. It can be difficult to adjust the hydrolyzate pH to near neutral with sodium hydroxide without either over or undershooting, as the titration curve for a strong acid and a strong base is very steep near pH 7. Neutralizing the hydrolyzate with a triethanolamine buffer was evaluated using the varieties listed in the first study. The GG analyses for each study were conducted in duplicate

Results and Discussion

Study 1. The mean concentrations of juice and wine phenols, total and phenol-free GG are provided in Table 1. For white juices and wines, the ratio of phenol-free GG to total GG averaged 76% and 77.6%, respectively. A greater difference in this ratio was noted with red juices and red wines. The phenol -free to total GG averaged 48% and 18% for red juices and wines, respectively. Fermentation results in a significant increase in the total versus phenol -free GG concentration in red wines [12,18].

Residual phenols were found in all GG fractions, including phenol-free fractions (Table 1). The phenol concentration in the phenol-free GG fractions of the white juice and white wines ranged from 6.9% to 1.3% of the total phenols present in the original samples. Not surprisingly, a higher residual phenol content was found in the phenol-free GG fractions in reds. The phenol concentration in the phenol -free GG averaged 7.2% and 5.6% of the original red juices and wines, respectively.

Study 2. Juice and wine samples were also treated with PVPP and evaluated for the effect on the concentration of total and phenol-free GG and on the phenol concentration in the GG fractions (Table 2). The ratio of phenol-free GG to the total GG was increased in all samples with the addition of PVPP. As expected, PVPP had a more dramatic effect on the GG concentration of red versus white juices and wines. The total GG fraction concentration was lowered by an average of 55% with the PVPP, the phenol-free GG fraction lowered by an average of 33%.

The PVPP treated juice and wine had lower phenol concentration in the phenol -free GG fractions which were lower than the untreated group (mean value for all treatments 4.9 mg/L GAE *vs.* 56.5 mg/L GAE,

Table 2. Effect of PVPP treatment on mean total glycosyl-glucose (TGG μ*M*) phenol-free (pH 10.0) glycosyl-glucose (PFGG μ*M*) and the phenol concentration of TGG and PFGG fractions.

	TGG (μ*M*)	PFGG (μ*M*)	Total phenols in TGG fraction (mg/L)	Total phenols in PFGG fraction (mg/L)
White juice	472	416	7.8	4.4
White wine	196	163	7.1	3.6
Rid juice	720	550	12.9	5.3
Red wine	430	270	46.2	6.6

Table 3. Comparison of the phenol-free glycosyl-glucose (PFGG) and total phenol concentrations using either C18 Sep Pak (pH 10.0) or Oasis™ cartridge (pH adjustable 13.0).

	Sep Pak		Oasis		Total phenols	
	TGG (μ*M*)	(pH 10.0) PFGG (μ*M*)	(pH 13.0) PFGG (μ*M*)	TGG (mg/L)	Sep Pak pH 10.0 PFGG (mg/L)	Oasis pH 13.0 PFGG (mg/L)
Chardonnay juice	183	122	87	15.8	9.0	7.8
Chardonnay wine	82	54	21	24.8	16.7	12.6
Cabernet Sauvignon juice	1436	341	100	311.4	145.3	9.3
Cabernet Sauvignon wine	1806	303	62	762.4	145.7	11.7

Tables 1 and 2). However, the differences are relatively small compared to the total phenol concentration of the original samples. The phenol concentration of the phenol -free glycosides in samples treated with PVPP represents 0.6% of the phenol concentration in original samples.

Study 3. Phenol-free determination by sample adjustment to pH 10.0 followed by separation using C18 RP was compared to pH adjustment to 13.0 and isolation on Oasis cartridges (Table 3). The Oasis procedure resulted in an average reduction of 67% in the phenol-free GG concentration compared to the C18 treatments. The ratio of phenol-free GG to the total averaged 23.3% and 7.7% for the C18 and Oasis™ treatments, respectively. There were differences in the concentration of phenols found in the two phenol-free GG fractions (Table 3). For the Chardonnay juice and wines, the phenol concentration of the phenol-free glycoside fractions averaged 63% of that found in the total GG fraction using the C18 RP cartridges. With the Oasis cartridges the phenol concentration was reduced to 50.2% of that found in the original total GG samples. The differences in the phenol concentration in the phenol-free GG fractions using these two isolation methods was greater with red juices and wines. Cabernet Sauvignon juices and wines averaged 27% of the phenols found in the total GG fraction using the C18 RP compared to 1.95% found in the Oasis phenol-free fractions.

Study 4. We have found that manually adjusting the pH to ensure that the pH is between 6 and 8 is important for reproducible results from the glucose assay; however, this takes considerable time and effort. Neutralizing 1 mL of hydrolyzate (1.5 *M* sulfuric acid) with 1 mL of 3 *M* sodium hydroxide and 0.5 mL of triethanolamine buffer (1M, pH 7.6) produced samples which were consistently between pH 6 and 8. The buffer solution is similar to that used in the enzymatic assay and corrects for minor fluctuations in acid or base concentrations and volume, resulting in significant savings of time and effort for the analyst. Comparisons of analyses performed with and without the buffer show no difference in assay results (data not shown).

Conclusions

Phenolic glycosides are important wine color and structural components, however, their impact on aroma and flavor is not believed to be substantial. Although the analysis of glycosyl-glucose cannot be considered a direct measure of potential aroma and flavor, the removal of phenol-glycosides may add to its utility. Substantial differences in the phenol concentration of phenol-free glycoside fractions using several isolation methods were demonstrated. The refinement of the analysis of phenol-free glycosyl-glucose may allow for a greater quantification of the influences of vineyard management and wine processing on this group of important grape-derived secondary metabolites.

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