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Technical Brief

Evaluation of Glycosyl-Glucose Analytical Methods for Various Glycosides

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The accuracy of two methods for determination of glycosyl-glucose (GG) in grapes and wine has been tested using several glycoside standards containing alkyl, benzenoid, and phenolic aglycones. The total GG method using C-18 sorbent material was found to exhibit poor recoveries of benzenoid glycosides. The recoveries for the phenol-free GG method were satisfactory for alkyl and benzenoid glycosides. In addition, an adaption of the phenol-free GG method to 96-well microplate format has been demonstrated.

Key words: Glycosides, glycosyl-glucose, GG

Many potential aroma compounds present in grapes are in the form of nonvolatile, flavorless glycosides. These conjugates have attracted much interest as precursors of aroma and flavor compounds in wine [1,12,14]. A variety of methods have been used to analyze glycoside content of grapes and wines. Williams et al. isolated glycosides on C-18 sorbent and analyzed the resulting material by hydrolysis, followed by gas chromatography (GC) and GC/mass spectrometry (GC/MS) [10,11]. Gunata et al. extracted glycosides with XAD®-2 sorbent prior to hydrolysis and GC [3]. Individual glycosides have also been isolated by countercurrent chromatography and analyzed by hydrolysis and GC/MS [15], nuclear magnetic resonance (NMR) and MS [13], and high-performance liquid chromatography (HPLC) followed by fast atom bombardment (FAB)-MS [5].

A simple method for the determination of the total glycoside pool in a sample by quantitation of glycosyl-glucose (GG) was developed by Williams et al. [9]. The GG method is based on the one-to-one molar ratio of glucose to glycosides found in grapes. The method involves isolation of glycosides by C-18 solid-phase extraction (SPE), followed by hydrolysis and enzymatic determination of the released glucose. The method was further optimized by Iland et al. [4]. Because the glycoside fraction in black grapes is dominated by glycosylated anthocyanins, Iland added a spectrophotometric measurement of anthocyanins, which is subtracted from the GG value to obtain an estimate of "red-free" GG. Zoecklein et al. [16] also modified the GG method by performing the glycoside extraction at pH 13, so that the phenolic glycosides would be ionized and not retained by the sorbent. This phenol-free GG (PFGG) measurement is intended to give a more focused estimate of the pool of aroma and flavor precursors. The GG methods have been employed by Zoecklein et al. to examine the effects of viticultural practices on grape quality [18,19] and to determine the effects of processing techniques on wine glycoside levels [7,17].

Accurate determination of the concentration of glycosidically-bound aroma and flavor precursors depends on obtaining a quantitative recovery of many different glycosides. The aroma and flavor compounds found in grapes include several compound classes, with varying properties, including terpenes, aliphatic alcohols, benzene derivatives, and C_{13}-norisoprenoids. Although the total GG (TGG) method was thoroughly tested with n-octyl-glucoside as a surrogate [9], it has not been tested with other glycosides. Since Mateo et al. [6] have demonstrated that glycosides can be fractionated on C-18 stationary phase, it cannot be taken for granted that n-octyl-glucoside is an adequate surrogate for all glycoside residues. Juice from nonterpene dominated grape varieties has been found to contain considerable fractions of glycosides of nonaliphatic metabolites [2,8]. For that reason, this work examined the recovery of benzenoid and phenolic glycosides by the TGG and PFGG methods.

Solid-phase extraction products have recently become available in 96-well microplate format. This format allows many samples to be processed simultaneously, with minimal solvent usage, and can be coupled with microplate enzymatic determination of glucose to streamline the analysis. This paper also describes the modification of the PFGG method to microplate format.

Materials and Methods

Reagents and standards. Reagents were obtained from Fisher Scientific (Pittsburgh, PA). Arbutin (hydroquinone-ß-D-glucoside), salicin (2-[hydroxymethyl]phenyl-ß-D-glucoside), rhapontin (3,3',5-trihydroxy-4'-methoxystilbene-3-O-ß-D-

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glucoside), phloridzin (phloretin-2'-ß-D-glucoside), (+)-rutin hydrate (quercitin-3-(6-deoxy-α-L-mannopyranosyl)-ß-D-glucopyranoside), n-octyl-ß-D-glucoside, phenyl-ß-D-glucoside, and genistin (4',5,7-trihydroxyisoflavone-7-glucoside) were all obtained from Sigma-Aldrich (St. Louis, MO). Hexokinase/glucose-6-phosphatase enzymatic test kits for glucose were obtained from Boehringer Mannheim (Indianapolis, IN). Standard solutions were prepared in distilled water with 2 g/L potassium bitartrate (see Table 1 for concentrations).

Standard column GG assays. GG determinations were carried out according to the method described by Iland et al. [4], as modified by Zoecklein et al. [16]. Grape juice samples were clarified, and wine samples diluted to less than 7% alcohol (v/v).

For total GG, aliquots of juice or wine were adjusted to pH 2.25 using concentrated hydrochloric acid. Samples (2 mL) were loaded onto 500-mg trifunctional C-18 SPE (solid-phase extraction) cartridges (Waters, Milford, MA) and washed three times with 10 mL of distilled water. The glycosides were then eluted with 1.5 mL ethanol followed by 3.5 mL distilled water, and then hydrolyzed with 2.0 mL 3 N sulfuric acid at 100°C for 1 hr. Samples were cooled and passed through the regenerated (10 mL methanol followed by 10 mL distilled water) C-18 columns, then 1 mL was neutralized with 1 mL 3 N sodium hydroxide and 0.5 mL 1 mM triethanolamine buffer (pH 7.6). Glucose was determined enzymatically in the neutralized samples using a microplate reader (Labsystems Multiskan MCC/340, Helsinki, Finland).

For phenol-free GG, aliquots of juice or wine were adjusted to pH 13 using 10 N NaOH, loaded onto 200 mg Oasis® HLB conditioned (4.0 mL methanol followed by 4.0 mL distilled water) cartridges (Waters), and washed with distilled water. The glycosides were then eluted with ethanol, hydrolyzed, and analyzed as described above, except that the hydrolysates were not passed back through the columns.

Microplate GG assays. Phenol-free GG assays were carried out using 30-mg Oasis® HLB extraction plates (Waters). Wine samples were diluted 1:2 with distilled water. Juice and diluted wine samples were adjusted to approximately pH 13 by adding 0.15 mL 10 N sodium hydroxide to 1.0 mL of sample. The extraction plates were conditioned by passing 0.5 mL methanol and 0.5 mL distilled water through each well. Aliquots of 0.5 mL of sample were loaded, and the sorbent washed three times with 0.5 mL water. The rinse water was pulled through the sorbent with vacuum, and a clean collection plate was fitted under the extraction plate. The samples were eluted from the sorbent by 0.15 mL of ethanol, followed by 0.35 mL of water. The elution solvent was pulled through the sorbent with vacuum. The samples were transferred to conical reaction vials containing 1 mL 4.5 N sulfuric acid. The vials were capped and placed in a heating block at 100°C for one hour. After cooling, 0.2 mL of each sample was neutralized with 0.2 mL 3 N sodium hydroxide and 0.1 mL triethanolamine buffer, and the glucose was determined by enzymatic assay.

Table 1 Single component analyte recoveries for the total glycosyl-glucose (TGG) method.

Compound	Concn (µM)	Recovery		n
		Average	SD	
n-Octyl-glucoside	323	92%	14%	3
Arbutin	363	0	—	3
Phloridzin	208	95%	10%	3
Phenyl-glucoside + salicin	400	16%	18%	3
Rhapontin	240	85%	4%	4
Rutin	50	69%	25%	4
Phenyl-glucoside	99	0	—	7
Salicin	100	0	—	3

Results and Discussion

A desire to improve quality control of our GG measurements led us to prepare a solution of phenolic glycosides (rhapontin, phloridzin, and arbutin) and nonphenolic benzenoid glycosides (phenyl-glucoside and salicin) as a check sample. The total glycoside concentration was 1091 µM, 200 µM of which was nonphenolic. Analysis by the conventional methods [9,16] gave recoveries of 35% for TGG and 133% for PFGG. The low recovery for TGG caused considerable concern. Although none of the standards are compounds known to exist in grapes, they were chosen to mimic, as closely as possible, the benzenoid and phenolic glycosides found in grapes. Mateo et al. [6] showed that some benzyl glycosides are more polar and, therefore, less strongly retained than aliphatic glucosides on C-18 sorbent, which may explain the poor recoveries in the TGG determination.

Analysis of individual standards indicated that n-octyl-glucoside and polyphenolic glycosides exhibit high recoveries, while benzenoid glycosides are essentially not recovered by the C-18 GG method (Table 1). The 200-mg Oasis® HLB columns were tested as a replacement for C-18, with mixed results. Aromatic residues were well retained, but the recovery of n-octyl-glucoside was erratic.

The poor recovery of some glycoside compounds leads us to question the utility of TGG values as indicators of potential aroma and flavor in nonterpene wines. When the overwhelming contribution of anthocyanins to the total glycoside concentration in red wine and juice (approximately 75% for Pinot noir and Shiraz berries [4]) is considered as well, the correlation between TGG and aroma and flavor becomes even more tenuous.

The phenol-free GG method, on the other hand, exhibits good recoveries for n-octyl-glucoside and the nonphenolic aromatic glycosides. The recoveries of three glycoside standards using the microplate PFGG method are shown in Table 2. Williams et al. [9] determined that some polymeric sorbents (XAD®-2 and XAD®-16) retain free glucose and are therefore unsuitable for GG determination. We have tested the Oasis® HLB by challenging it with 100 g/L glucose solutions, with no detectable glucose retention. We have also analyzed nonhydrolyzed fractions collected from Chardonnay and Cabernet Sauvignon juice with the Oasis® HLB columns and found no detectable free glucose, indicating that glucose retention is not a problem on that sorbent.

Table 2 Analyte recoveries for the microplate phenol-free glycosyl-glucose (PFGG) method.

Compound	Concn (μM)	Recovery		n
		Average	SD	
n-Octyl-glucoside	97	83%	3%	4
Phenyl-glucoside	99	105%	5%	4
Salicin	100	90%	4%	4

The microplate method was found to increase sample throughput, compared to the standard column format, by allowing a single analyst to process up to 96 samples simultaneously. Sample and solvent flow through the 30-mg columns frequently occurs at appropriate speeds without application of vacuum, and water or ethanol can be applied with an eight channel multipipettor. The microplate PFGG method was compared to the conventional PFGG method for the analysis of 23 Cabernet Sauvignon wine samples, and the measured PFGG concentrations for the two methods (ranging from 44 to 99 μM) did not differ significantly at $p = 0.05$.

Conclusions

The use of C-18 sorbents in the TGG method was found to introduce potential error due to nonretention of aromatic glucosides. This is a particular problem for low-terpene grape varieties. Retention of aromatic compounds was not found to be a problem with the PFGG method, indicating that this method may be better suited to determination of the pool of potential aroma and flavor compounds in low-terpene varieties. Due to the lack of available standards, questions remain about the recovery of glycoside classes, which are neither aliphatic nor aromatic (for example, C_{13}-norisoprenoids).

Comparison of 96-well microplate and standard column format versions of the PFGG method indicates that they produce comparable results. The microplate version was found to be advantageous in terms of increased sample throughput and reduced analyst effort.

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