Quantification of Glycosides in Grapes, Juices, and Wines through a Determination of Glycosyl Glucose†,‡

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An analytical assay for glycosylated secondary metabolites of grapes, juices and wines, through quantification of glycosyl glucose (G-G), has been optimized and validated. The glycosides are isolated on C_{18} reversed-phase silica gel and hydrolyzed with sulfuric acid at 100 °C; the released glucose is determined enzymatically. The accuracy and precision of the assay, determined from the gradients and coefficients of determination of linear regression curves for the recovery of a standard glycoside from wine, juice or homogenized fruit extract ranged, respectively, from 0.957 to 1.021 and from 0.991 to 0.997. Some examples of application of the assay are presented—the decrease of G-G in wines with aging, the range of G-G in Shiraz juices from different regions, and the development of G-G in ripening fruit.

Keywords: *Glycosides; quantification; Vitis vinifera grapes; glycosyl glucose*

INTRODUCTION

The role of glycosylated secondary metabolites as flavor precursors in fruits is attracting attention. This in turn has stimulated interest in the development of methods for the analysis of these constituents and has highlighted the need for techniques to quantify glycosides (Williams, 1993; Williams and Allen, 1995). For example, in viticultural research there is a requirement for simple and rapid analytical methods to determine the concentration of glycosylated forms of these compounds in grape berries. Existing methods for quantifying terpene glycosides in grapes and wines are laborious and time-consuming and require sophisticated instrumentation (Voirin et al., 1992a,b). An earlier distillation method for the estimation of potentially volatile (including glycosidically bound) monoterpenes, although requiring only simple apparatus, is restricted in application to floral grape varieties (Dimitriadis and Williams, 1984).

Typically, the aglycons of plant glycosides are structurally complex and highly diverse. An obvious focus, therefore, in developing a quantitative assay for glycosides is the sugar moiety. This means of quantification through the carbohydrate part of the molecule is aided by the availability of many simple, specific, and highly sensitive methods for measuring the concentration of sugars. Such an approach has been used in determining the concentration of a sweet diterpene glycoside, stevioside, in *Stevia rebaudiana* leaf extracts (Mizukami et al., 1982).

A common feature of the glycosylated secondary metabolites present in grapes, and in many other plant tissues, is that the compounds are glucosides in which the glucose moiety may or may not be further substituted. Hydrolysis of the glycosides, therefore, yields an equimolar proportion of aglycons and of D-glucose; the latter is termed the glycosyl glucose (G-G). On the basis of this reasoning, a determination of the G-G concentration will permit an estimation of the total concentration of glycosylated secondary metabolites present in the fruit. We described a prototype method (Abbott et al., 1993) involving the following steps: (a) isolation of a glycosidic fraction from juice by selective retention of the glycosides on a C_{18} reversed-phase (C_{18} RP) adsorbent; (b) hydrolysis of this glycosidic fraction to liberate the glucose; and (c) measurement of the concentration of the released glucose using a hexokinase/glucose-6-phosphate dehydrogenase (HK/G-6-PDH) enzymic determination. We now describe the further development, refinement, and analytical validation of this assay. The efficacy of this method for the determination of total glycosylated secondary metabolites in grapes, juice, and wine is illustrated.

MATERIALS AND METHODS

Materials. Sep-Pak Plus C_{18} RP solid phase extraction cartridges, with an internal volume of 0.7 mL and containing 360 mg of adsorbent, were obtained from Millipore Aust. Pty Ltd. They were used with a 20 mL reservoir and a Sep-Pak vacuum manifold obtained from the same supplier. All solvents were redistilled, and water was treated with a Milli-RO/Milli-Q system (Millipore Aust.) before use. Glucose concentration of hydrolysates was determined with a HK/G-6-PDH spectrophotometric assay kit (Boehringer Mannheim Australia Pty Ltd, catalog no. 716 251), following the instructions contained in the kit and scaled-down for use in a 96-well microtiter plate and a microplate reader (UVmax, Molecular Devices Corp., Palo Alto, CA). XAD-2, XAD-16 resins, and polyvinylpolypyrrolidone (PVPP) adsorbent were obtained from Sigma Chemicals (Sigma-Aldrich Pty Ltd, Aust.), and XAD-2 was also assessed as a commercially packed cartridge (Spe-ed Amberlite XAD-2, Applied Separations). Acid hydrolyses were carried out in an aluminum block heating module (Pierce Chemical Co.). Novoferm 12 (Novo Ferment AG, Switzerland) was used for enzymic hydrolyses.

Standard glycosides used were *n*-octyl glucoside (*n*-OG) and rutin (Sigma Chemicals), apiin (Carl Roth, GmbH & Co.,

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Karlsruhe, Germany), naringin (Apin Chemicals Ltd, Oxfordshire, U.K.), and phloridzin (Calbiochem).

Commercial red wines used were a 1990 Coonawarra Shiraz and a 1992 Victorian Cabernet Sauvignon/Cabernet Franc/Merlot blend. The white wines were made from Semillon grapes from the 1993, 1992, 1991, 1989, 1988, 1987, 1986, and 1983 vintages, grown in the same area in the Hunter Valley (NSW) and vinified by the same winery. Shiraz fruit was sourced from the Barossa Valley (SA), from either a commercial vineyard or a viticultural field trial plot of the SA Department of Agriculture. Muscat of Alexandria fruit was harvested from the University of Adelaide research vineyards. These samples were homogenized with an Ultra-Turrax T25 high-speed homogenizer with an F25N dispersing head (Janke & Kunkel GmbH & Co., Germany). Shiraz juices were prepared from 1992 commercially harvested fruit samples from viticultural areas of South Australia (SA), New South Wales (NSW), and Victoria. A 1990 Semillon juice from Padthaway (SA) was also used. Juices were expressed by light pressing of whole grape bunches in a water bag press; samples were stored at −20 °C until analyzed.

G-G Assay. Juices were analyzed directly, and no sample preparation was required for wines with an alcohol strength less than 15%; wines with an alcohol strength greater than 15% should be diluted for analysis. Homogenized fruit extracts were prepared from 50-berry samples. These were homogenized for 45 s, a known weight (*ca.* 1 g) was treated with ethanol (10 mL, 50%), and the solution was shaken at 5 min intervals for 1 h and then centrifuged at 3500 rpm for 5−7 min; an aliquot of the supernatant (5 mL) was mixed with water (20 mL) and taken directly for, or held frozen (−20 °C) prior to, analysis.

Step 1, Isolation of Glycosides. The C_{18} RP cartridges were pretreated with methanol (*ca.* 10 mL) followed by water (*ca.* 10 mL). An appropriate volume of sample, *e.g.* homogenized grape extract (20 mL), wine (5 mL), or juice (10 mL), was loaded onto the cartridge at room temperature at a flow rate of *ca.* 2−3 mL/min. The cartridge was then washed with water (3 × 15 mL), and the glycosides were eluted with ethanol (1.5 mL), followed by water (*ca.* 3 mL); the glycoside eluate was adjusted to 5.0 mL with water.

Step 2, Hydrolysis. To aliquots (0.5 mL) of the above glycoside eluate was added H_2SO_4 (1.0 mL, 2.25 M) to give solutions for hydrolysis that contained 1.5 M H_2SO_4 and 10% v/v ethanol. A control solution was similarly prepared for each glycoside eluate, with water (1.0 mL) added in place of the H_2SO_4 solution, for determination of the free (nonglycosidic) glucose concentration of the eluate. A reagent blank was made with ethanol (0.5 mL, 30%) in place of the glycoside eluate and acidified as above. Five calibration solutions containing glucose (*e.g.* 100, 200, 300, 400, and 500 μM or other appropriate concentrations) in 1.5 M H_2SO_4 and 10% v/v ethanol were made up daily. The sample, blank, and calibration solutions, in screw-capped test tubes, were heated at 100 ± 2 °C for 1 h; the controls were held at room temperature. After heating, the samples were cooled to room temperature. For all experiments, duplicate aliquots of glycoside solutions were taken for hydrolysis and further analysis.

Step 3, Analysis of D-*Glucose.* The D-glucose concentration of each of the sample, blank, control, and calibration solutions from step 2 above was analyzed using a HK/G-6-PDH enzyme assay kit. Aliquots of the solutions after hydrolysis (80 μL) were transferred to the wells of a microtiter plate, and 2 M NaOH solution (120 μL) was added. For the control solutions, water was substituted for the NaOH solution. Solution 1 (100 μL) of the assay kit (triethanolamine buffer, pH 7.6, with NADP and ATP) was added to each well. The plate was shaken in the plate reader and the absorbance read at 340 nm. Solution 2 (diluted 1 to 12.5) (25 μL) from the assay kit (HK/G-6-PDH enzyme mixture) was added to each well, the plate again shaken, and after 20 min, the absorbance at 340 nm read. The absorbance difference was used to determine the concentration of glucose from a standard curve prepared from the calibration solutions. The G-G (micromoles) in solution loaded onto a C_{18} RP is given by

$$[(\Delta A_s - \Delta A_b) - (\Delta A_c - \Delta A_b)]mV(DF)$$

where ΔA_s is the absorbance difference for sample, ΔA_b is the absorbance difference for blank, ΔA_c is the absorbance difference for control, m is the gradient of the calibration curve, V is the volume of sample in liters, and DF is the dilution factor.

The accuracy and precision of the procedure was determined by analysis of matrix samples (see Figure 1) prepared as follows: For experiments a−c, nine solutions (20 mL) containing 0, 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6, and 7.2 μmol of added *n*-OG were made from aliquots of the matrix (45 mL) by adding the appropriate volume of aqueous 10 mM *n*-OG solution and adjusting to 50 mL with water. The solutions were analyzed in duplicate. For experiment d, a similar procedure was followed with solutions (10 mL) containing 0, 1.0, 2.5, 5.0, 7.5, 10.0, and 12.5 μmol of added *n*-OG, prepared by taking 10 mL of the juice sample, adding the appropriate volume of 7.85 mM *n*-OG aqueous solution, and adjusting the volume to 25 mL with water.

Investigations of the Solid Phase Extraction Step. XAD-16 resin (1 g) or XAD-2 resin cartridges were conditioned with methanol (1 mL) and water (10 × 1 mL). In A, in Table 2, after juice was loaded (1 mL onto XAD-16 or 5 mL onto XAD-2), the resins were washed with water (3 × 10 mL) and eluted with methanol (4 mL); the residue, after solvent evaporation, was taken up in water (5 mL) for determination of the glucose concentration. In the second series of experiments on XAD, *i.e.* B in Table 2, juice (0.5 mL) was loaded onto the resins which were then washed by suspension in water (1 mL) for 15 min, drained, and washed by passage of water (3 × 10 mL); this procedure was repeated three times. The XAD-2 was then suspended in water (1 mL), and an aliquot (0.2 mL) was taken for glucose determination; the XAD-16 was eluted with methanol (4 mL), which was evaporated, and the glucose concentration determined as above. To evaluate PVPP, juice (10 mL) was passed in sequence through a combination of PVPP (1 g) and C_{18} RP cartridges. The experiments were made with the PVPP adsorbent first or second in line, following which the PVPP was suspended in 1 M H_2SO_4 (10 mL) and heated at 100 °C for 1 h. An aliquot of this solution was analyzed using the HK/G-6-PDH assay.

To determine the influence of sample ethanol concentration on the efficiency of the C_{18} RP isolation step (see Figure 3), a series of solutions was prepared from an 80% dilution of a red wine (originally containing 13.3% ethanol) with an appropriate volume of ethanol or water, to give solutions containing 10, 11, 12, 13, 14, 15, 16, 18, and 20% v/v ethanol; samples (1 mL) were then taken for G-G assay.

RESULTS

Determination of the Accuracy, Precision, and Detection Limit of the Assay. Extracts of a black or white grape homogenate and a white wine were used as test matrices. *n*-OG was added to portions of each matrix so that a series of samples was available with an increasing concentration of the added glycoside. Aliquots from each sample were then analyzed for G-G by the assay procedure. The results of these experiments are given by curves a−c in Figure 1. The coefficients of determination and gradients of the linear regression equations were all close to 1. The actual gradients of the linear regression equations, ranging from 0.957 to 1.021, and the coefficients of determination, ranging from 0.997 to 0.991, demonstrate, respectively, the high accuracy and precision of the assay. A similar experiment made on a red grape juice gave a linear relationship (gradient 0.973, $r^2 = 0.996$) between the G-G determined and the amount of *n*-OG added to the juice, extending up to 17.5 μmol of G-G (see curve d in Figure 1).

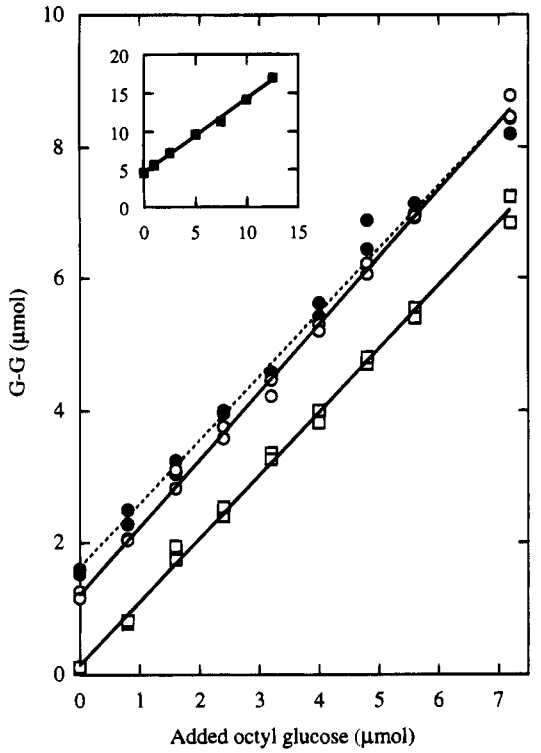

Figure 1. G-G determined on a series of sample matrices to which additions of *n*-OG were made ranging from 0 to 7.2 μmol, in duplicate sample matrices a–c, and from 0 to 12.5 μmol in sample matrix d, singly. The sample matrices were as follows: (a, □) an extract of a Muscat of Alexandria grape homogenate; (b, ○) an extract of homogenized Shiraz grapes; (c, ● a Semillon wine; and (d, ■) a Shiraz grape juice (see inset). The gradients and coefficients of determination of linear regressions were (a) 0.957 and 0.997, (b) 1.021 and 0.997, (c) 0.964 and 0.991, and (d) 0.973 and 0.996.

Table 1. G-G for Eight Replicate Analyses Made on Two Different Samples Using the Assay Procedure

sample	G-G (μmol) ($n = 8$)	SD	rel SD (%)
homogenized Shiraz extract	0.58	0.04	7
Shiraz juice	3.48	0.07	2

To further define the precision, eight replicate analyses of a homogenized fruit extract and a juice were made, both from Shiraz grapes. The mean values, standard deviations, and relative standard deviations for these experiments are given in Table 1.

The limit of detection for G-G in a sample taken for analysis was calculated (Miller and Miller, 1988) as 3 times the standard deviation of control determinations ($n = 16$) at 0.10 μmol. A quantification limit of 0.35 μmol was assigned as 10 times this standard deviation.

Evaluation of Adsorbents Other than C_{18} RP. Experiments were undertaken to explore the possible use of XAD-2 (Gunata et al., 1985), XAD-16, and PVPP. The polystyrene resins were examined in two different forms, *i.e.* as laboratory-packed adsorbent beds and, in the case of XAD-2 in experiment A (see Table 2), in prepacked cartridge form. The XAD-16 has a surface area and capacity greater than that of XAD-2. Both of these adsorbents had the disadvantage of retaining free glucose in addition to the adsorbed glycosides. This was demonstrated from control experiments in which, after loading a red juice sample and washing the adsorbent, the glucose concentration of the eluate was determined without acid hydrolysis (see Table 2). The data show that, even with extensive washing of the adsorbent beds, both the XAD-2 and XAD-16 retained free glucose. Repeatedly resuspending the adsorbents in the wash water, a highly laborious and inefficient procedure, did improve removal of the interfering glucose from XAD-16 but was still inadequate for XAD-2.

Table 2. Glucose Retained on Three Solid Phase Adsorbents after Passage of Grape Juice followed by Water Washing

washing procedure	glucose (μmol) retained on XAD-16	XAD-2	C_{18} RP
A. standard washing	19.5 ($n = 1$)	1.4 ($n = 3$)	0.03 ($n = 2$)
B. washing the resuspended bed	0.04 ($n = 2$)	0.4 ($n = 3$)	nd[a]

[a] nd, not determined.

Table 3. Glycosides Isolated from a Red Juice (10 mL) by Different Combinations of Solid Phase Adsorbents, As Determined by G-G Assay

adsorbent combination	G-G retained by the first adsorbent (μmol)	G-G retained by the second adsorbent (μmol)
C_{18} followed by PVPP	12.5	0.55
PVPP followed by C_{18}	6.65	4.03
C_{18} followed by C_{18}	12.5	1.81

A second study involved sequential loading of a 10 mL volume of a high G-G juice onto C_{18} RP and PVPP and determination of the G-G retained by each adsorbent. For comparative purposes, an experiment was also made with two C_{18} RP cartridges in sequence. Loading of the juice onto the C_{18} RP and PVPP adsorbents in both forward and reverse order was investigated. It should be noted that the G-G retained on PVPP was determined by direct hydrolysis of the solid adsorbent because adsorbed compounds could not be eluted from this material. Data for the experiments on PVPP are given in Table 3. The experiments demonstrated that there was no benefit in using a combination of adsorbents. The small amount of G-G (*ca.* 10%) found in the second of the two combined C_{18} RP cartridges showed that the first cartridge was overloaded as a consequence of the high G-G content of the juice used for this experiment (see below).

Variables Associated with Sample Loading of the C_{18} RP Cartridge. *Capacity of C_{18} RP Cartridges for Juice Glycosides.* The capacity limitations of C_{18} RP in retaining glycosylated constituents from juice were investigated. Figure 2 shows results of an experiment in which the G-G retained on a series of C_{18} RP cartridges was determined after each cartridge had been loaded with a different volume of a white grape juice. There was a linear relationship ($r^2 = 0.994$) between the G-G determined and the volume of juice loaded up to 30 mL (corresponding to 5.8 μmol of G-G), beyond which the G-G values declined.

Sample Volume. Individual C_{18} RP cartridges were each loaded with a different volume of a proportionally diluted red wine sample. The cartridges were then eluted, and the G-G retained on each was compared. Data for this experiment, given in Table 4, show that dilution did not significantly affect the capacity of the cartridge to isolate glycosides and that the cartridges could retain G-G from a large volume of dilute sample.

Sample Ethanol Concentration. A series of red wine samples was prepared with ethanol concentrations ranging from 10 to 20%. Individual C_{18} RP cartridges were each loaded with an equal volume of wine from the series, and the G-G retained on each was compared. The results (Figure 3) show a decrease in the efficiency

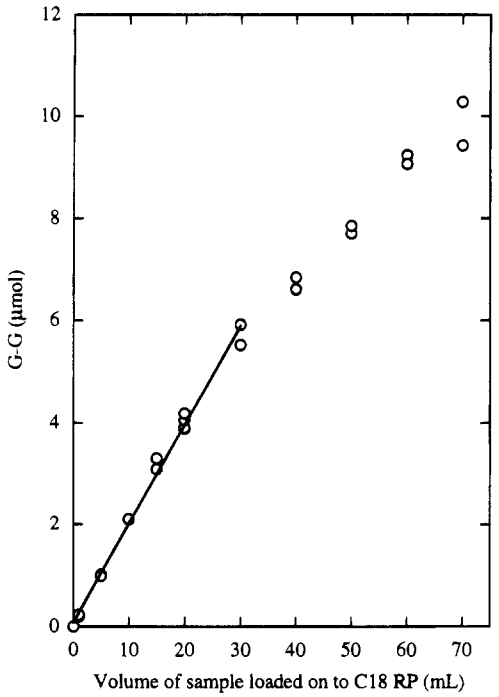

Figure 2. G-G determined on different volumes of a Semillon grape juice. Individual C_{18} RP cartridges (replicated) were loaded with different volumes of the juice. The linear regression curve shown was constructed for the samples from 0 to 30 mL.

Table 4. Effect of Sample Volume on G-G Determination

vol (mL) of sample loaded onto the C_{18} RP[a] (dilution)	mean G-G (μmol) (n = 2)	SD
1 (undiluted)	2.10	0.07
10 (10% dilution)	2.01	0.01
100 (1% dilution)	2.07	0.05

[a] The same red wine was used with different volumes of proportionally diluted sample loaded onto the C_{18} RP.

of glycoside retention when the wine ethanol concentration exceeded 15%.

Volume of Ethanol Necessary To Elute the C_{18} RP Cartridge. In the developmental stage of the assay (Abbott et al., 1993), the solvent eluate from the C_{18} RP was evaporated to dryness before the hydrolysis reaction. To avoid this evaporation step, an investigation was made to find the minimum volume of ethanol necessary to elute the glycosides. This was determined from the results of a series of recovery experiments similar to those described under Determination of the Accuracy, Precision, and Detection Limit of the Assay above. The variable in these experiments was the volume of ethanol used to elute the C_{18} RP cartridges (*i.e.* 1.0, 1.2, and 1.5 mL). On the basis of the recoveries obtained (*i.e.* 92.2, 94.8, and 102.2%, respectively), which were calculated from the gradients of the recovery curves, the volume chosen for use was 1.5 mL.

Experiments on Hydrolysis of Glycosides. *Acid versus Enzyme Hydrolysis.* Differing periods of glycoside hydrolysis, using either glycosidase enzyme catalysis as previously employed (Abbott et al., 1993) or acid catalysis, were applied in determining the G-G of a red juice. The experiment was also conducted after the concentration of *n*-OG in the juice sample was adjusted to 200 μM. Results from these experiments are given in Figure 4. It is evident that glycosidase hydrolysis of the juice glycosides was initially slower than that

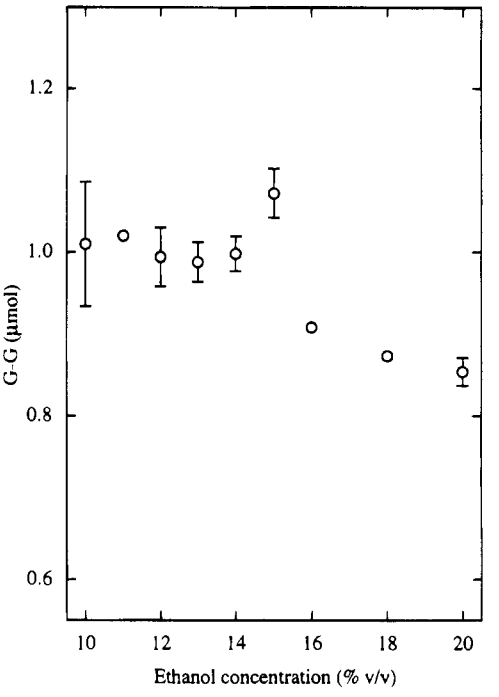

Figure 3. G-G determined on samples prepared from a red wine adjusted to give a range of ethanol concentrations. Means (○) and standard deviation bars are shown, with n = 2, except for the 10% ethanol value, where n = 6. Error bars are not shown where SD < 0.01.

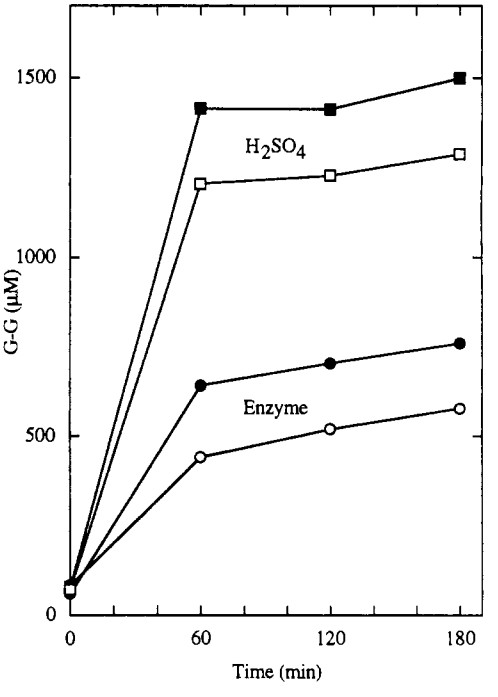

Figure 4. G-G determined (and expressed as concentration in μM) on the glycosides isolated from a Shiraz grape juice, before (unshaded symbols) and after (shaded symbols) addition of *n*-OG to give a concentration of 200 μM for that substrate, and hydrolyzed for different periods with (○) Novoferm 12 at pH 5.0 and 55 °C or with (□) 1.0 M H_2SO_4 at 100 °C.

effected by acid. The enzyme preparation gave satisfactory release of glucose from the added *n*-OG substrate, yet it was incapable of total release of glucose from the endogenous fruit glycosides in an acceptable hydrolysis time.

Hydrolysis Kinetics of Standard Glycosides. The extent of liberation of glucose from a range of standard glycosides was observed over different periods of hy-

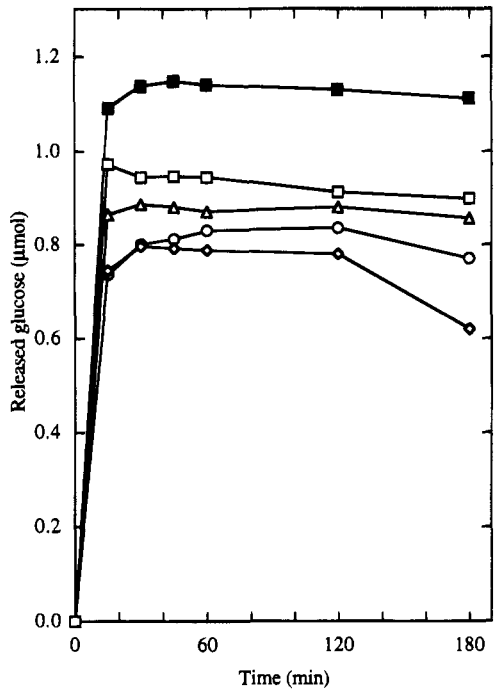

Figure 5. Determination of the rate of glucose release from five reference glycosides hydrolyzed in aqueous solution with 1.5 M H_2SO_4 at 100 °C for periods up to 180 min. The particular reference glycoside, the amount in the solution taken for hydrolysis, and the glucose released, expressed as percent recovery at 60 min, were as follows: (■) naringin, 1.28 μmol, 89.1%; (□) phloridzin, 1.01 μmol, 93.5%; (△) rutin, 1.00 μmol, 87.2%; (○) *n*-OG, 0.80 μmol, 103%; and (◇) apiin, 0.91 μmol, 86.4%.

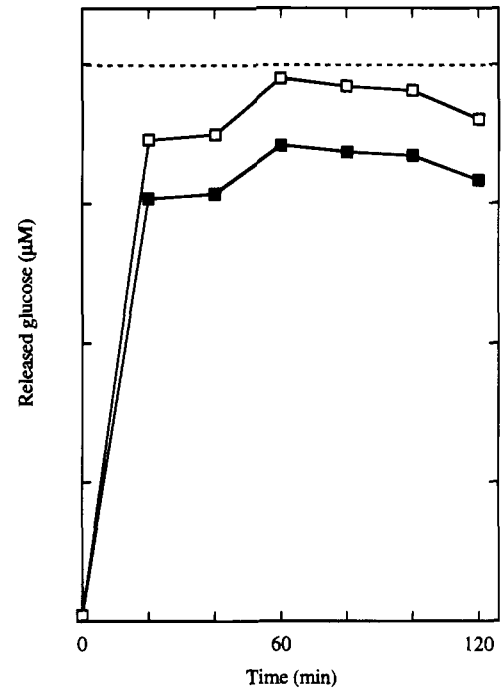

Figure 6. Glucose released from a standard solution of *n*-OG (200 μM) hydrolyzed in 10% aqueous ethanol solution with 1.5 M H_2SO_4 at 100 °C over 120 min, determined using the HK/G-6-PDH kit with glucose calibration solutions either (■) untreated with acid and ethanol or (□) heated with 1.5 M H_2SO_4 and 10% ethanol, as in the G-G assay procedure.

drolysis. The results (Figure 5) show that, although complete hydrolysis of the four phenolic glycosides (apiin, naringin, phloridzin, and rutin) was apparently achieved in 30 min, *n*-OG required 60 min. Importantly, all of the glucose release curves in Figure 5 plateaued between 60 and 120 min, demonstrating that the acid conditions caused no degradation of the released glucose in this period. The recovery of glucose from these reference compounds, which were at commercial purity only, ranged from 86 to 103%.

Influence of Ethanol on Acid Hydrolysis. The G-G values of several different juices were compared after hydrolyses were made in 10 or 20% ethanol solution. The results (data not shown) confirmed that acid hydrolysis in the presence of 20% ethanol gave G-G values consistently lower than those obtained in 10% ethanol. Following this observation, an experiment was undertaken to minimize the ethanol interference without the necessity of removing the organic solvent prior to the hydrolysis reaction. The results (Figure 6) show the determination of glucose released from a standard *n*-OG solution by acid hydrolysis in the presence of 10% ethanol. These experiments were made with the glucose calibration standards for the HK/G-6-PDH determination prepared with and without acid and ethanol. The difference between the two curves shown in Figure 6 indirectly quantifies the magnitude of the ethanol interference and demonstrates that the interference can be compensated for with an appropriate calibration procedure using acid- and ethanol-treated calibrants.

Examples of Application of the G-G Assay. The G-G values of eight Semillon wines of different ages were determined. These white wines were vinified in one winery using fruit from the same area and harvested over 10 years; the wines had been held in cellar storage from the year of vintage. The data given in Figure 7 show an inverse correlation between the measured G-G and the age of the wines. In another study, the G-G values of 158 Shiraz juices were measured and the data plotted as a frequency histogram (Figure 8). The wide range of values indicates the variability in concentration of glycosylated secondary metabolites present in these samples. Figure 9 shows the changes in berry G-G with Shiraz grape ripening as indicated by juice soluble solids in degrees Brix.

DISCUSSION

The isolation of glycosides from wines and juices by selective retention of the compounds on a solid phase adsorbent is a commonly used technique (Gunata et al., 1985; Williams et al., 1982). By washing the adsorbent with water following the adsorption step, free sugars and other polar constituents can be removed while the less polar glycosides are retained. Elution with an organic solvent then gives the glycosidic fraction. Reversed-phase silica gel has been found to be a particularly suitable adsorbent for this purpose (Williams, 1993). The commercial availability of this adsorbent in uniform, prepacked cartridges was an advantage in development of the isolation step in the assay.

Having obtained a glycosidic isolate, logical successive steps of hydrolyzing the glycosides, and quantifying the D-glucose released (Abbott et al., 1993), have been refined into a simple protocol enabling determination of the concentration of glycosides in the original sample.

The accuracy and precision of the assay were evaluated by a series of experiments in which the recovery of a standard glycoside from a juice, wine, or homogenized grape extract was determined. For incremental addition experiments of this type, the gradients and coefficients of determination of the linear regression curves are ideally one (Miller and Miller, 1988). The

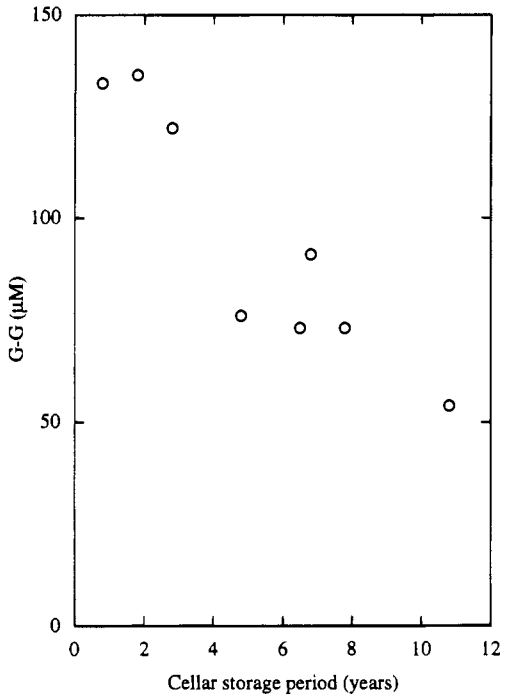

Figure 7. Effect of years of cellar storage on concentration of G-G (μM) of Semillon wines of different age. The wines were made from fruit harvested from successive vintages. The cellar storage period was calculated from the date of vinification to the date of analysis.

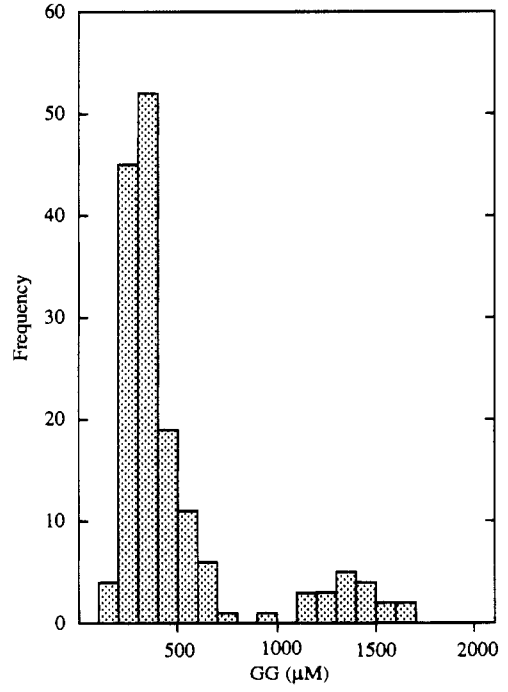

Figure 8. Histogram based on the juice G-G concentration of 158 samples of Shiraz grape juices from different vineyards during the 1992 vintage.

results of these experiments (Figure 1) validated the assay protocol for each sample matrix.

Further variables associated with the solid phase isolation of the glycosidic fraction were investigated. The C_{18} RP was found to be capable of efficiently retaining glycosides from a large volume of dilute sample. The volume of a juice sample applied to the C_{18} RP cartridges and its G-G concentration are, however, important factors in isolating the glycosides quantitatively. Comparison of the data given in Figures 1 and 2 demonstrates that while a 10 mL volume of a particular juice containing 5–17.5 μmol of glycosides could be reliably assayed, a 40 mL volume of a second juice, although containing less than 7 μmol of G-G, gave deviant results. Furthermore, the data in Table 3 show that a 10 mL volume of a third juice overloaded a single cartridge, albeit with greater than 12.5 μmol of G-G. The degree of partitioning of different glycosides into the reversed phase and the presence of adsorbable but nonglucosylated constituents that reduce the capacity of the C_{18} RP may partly account for these observations. In practice, 10 mL of undiluted juice was taken for analysis. If, however, a juice gives a G-G of greater than 5 μmol, a second analysis on a smaller volume is recommended. For the analysis of less concentrated samples, *i.e.* homogenized grape extracts, the problem of obtaining values below the 0.35 μmol quantification limit of the assay was overcome by taking a 20 mL or greater sample volume.

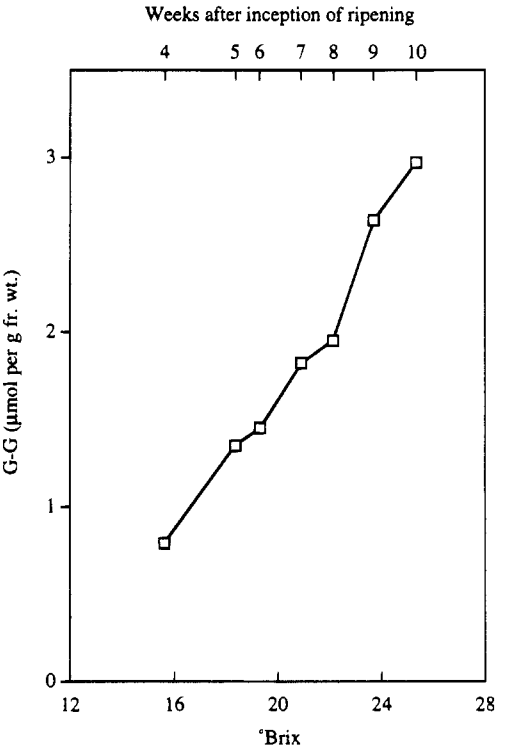

Figure 9. G-G values of Shiraz berry samples harvested at different stages of ripeness. Data are mean values from 50-berry samples from each of three vines.

The C_{18} RP cartridges were found to be suitable for all liquid matrices tested: glycosides were retained with high efficiency from homogenized grape extracts with an ethanol concentration of 10% and from wines containing 15% alcohol. Experience showed that this efficiency was not compromised by sample loading techniques, and samples could be applied to the cartridges over a reasonable range of flow rates and at temperatures near to or just below ambient. However, cold samples at temperatures <5 °C gave low G-G values, a factor to be recognized when juices taken from cold storage are analyzed. In comparison with the C_{18} RP cartridges, the difficulty of removing free glucose from the glycosides isolated by XAD resins, and the need for extensive washing of the latter adsorbents, precluded their use in the assay. There was also no benefit in using the phenol-selective adsorbent, PVPP, in the isolation step.

In the developmental stage of the assay, glycosidase enzyme-catalyzed hydrolysis of the isolated glycosides had been employed (Abbott et al., 1993); however, concern about the ability of the enzyme preparations to act on all grape glycosides within a reasonable time was justified by the data in Figure 4. Acid-catalyzed hydrolysis was found to be a more efficient technique for the release of glucose from fruit glycoside isolates (Figure 4) or from a range of standard glycosides (Figure 5) and was thus adopted. Early experiments with 1.0 M H_2SO_4 had indicated complete hydrolysis of *n*-OG in 60 min, although small uncontrolled variations in hydrolysis temperature could give low recoveries at this acid strength. Accordingly, 1.5 M H_2SO_4 was employed in the assay procedure. The reported stability of glucose to H_2SO_4 (Selvendran et al., 1979) under conditions similar to, and indeed more vigorous than, those used in the assay was confirmed by the recovery experiments in Figure 5.

In principle, hydrolysis of the glycosides was possible without elution of the compounds from the C_{18} RP, but experiments demonstrated that in situ hydrolysis was inefficient and, under all conditions tested, gave lower G-G values (data not shown). Accordingly, solvent elution of the cartridges prior to hydrolysis was essential. Choice of a solvent for this purpose was limited to one that would elute the C_{18} RP in a minimum volume and offer no, or minimal, interference to the assay protocol, thus eliminating the need for a time-consuming solvent-removal step. Although ethanol had the necessary properties, its presence imposed a variable in the acid hydrolysis reaction, and it was necessary to dilute the ethanol in the glycoside eluate prior to acid hydrolysis to minimize the interference. The residual interference, possibly resulting from ethyl glycoside formation under the conditions of acid catalysis, was compensated for by applying the same conditions of acid hydrolysis to the glucose standards used in calibrating the HK/G-6-PDH determination in the third step of the assay (see Figure 6). A further refinement would be the use of *n*-OG, rather than glucose, as the calibrant for the assay.

The commercial HK/G-6-PDH enzymic assay kit used was reliable and suffered no interference from any of the substrates analyzed. Importantly, acid degradation products of the aglycons offered no interference, as evidenced by the recoveries in Figure 1. The activity of the kit enzymes was checked, as recommended by the suppliers, by additions of standard glucose solutions to individual sample determinations. The analytical throughput was increased by making use of a microtiter plate system for this third step. This also decreased the volume of reagents and enzyme solutions used, lowering the total analysis cost.

Extensive application of this assay will be needed to establish its value. The ability to readily and simply quantify changes in grape and wine composition, as exemplified by the data in Figures 7–9, indicates the potential of this assay to provide objective, analytical information to viticulturists and oenologists.

CONCLUSION

An assay protocol has been developed for the quantitative analysis of glycosylated secondary metabolites of grapes, juices, and wines. The assay, made through a determination of glycosyl glucose concentration, has been optimized to provide

- quantitative isolation of the glycosides through retention on, and elution from, a C_{18} RP cartridge;
- complete hydrolysis of the isolated glycosides, with minimal degradation of released glucose, by use of acid catalysis;
- analysis of glucose, free from interference by the aglycons or their decomposition products.

The assay is rapid, accurate, precise, and specific for the glucose released from the glycosides. The simplicity of the protocol means that it is amenable to automation, an aspect that is currently being evaluated. Optimization of methods for the preparation of fruit samples for assay is the subject of a further paper.

We believe that this assay will prove to be a valuable tool for viticulturists investigating the influences of vine growing practices on grape composition. In being able to quantify glycosylated secondary metabolites, including the potential flavor compounds of grapes, the assay holds promise of an objective measure of grape composition pertinent to wine quality. In postharvest applications, the G-G assay could be used to measure the effects of fermentation and aging on glycosylated secondary metabolites, which are of importance to the sensory character of wines.

This assay could find wider application in the assessment of horticultural products in which glycosidically bound volatile compounds are important to flavor. It should also be of value to research in the field of plant-derived glycosides generally. The possibility of using quantitative analysis of glycosides for the quality control of particular plant extracts (Sticher, 1993), or to determine the glycoside status of different plant tissues (Tazaki et al., 1993), emphasizes the broader needs for such a technique.

ABBREVIATIONS USED

HK/G-6-PDH, hexokinase and glucose-6-phosphate dehydrogenase enzyme system for the determination of glucose; G-G, glycosyl glucose; *n*-OG, *n*-octyl β-D-glucopyranoside; C_{18} RP, C_{18} reversed-phase silica gel; PVPP, polyvinylpolypyrrolidone.

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