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Review Article

Glycosidically Bound Volatile Aroma Compounds in Grapes and Wine: A Review

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Abstract: Volatile aroma compounds in plants are typically found both as "free" and "bound" to a sugar moiety. When bound, these compounds are not odor active; however, upon hydrolysis of the glycoside, these compounds may then be volatilized. In grapes and wine, a large proportion of volatile aroma compounds are found in the bound form. A review of glycosides in grapes and in wine is presented with a focus on identified glycoside structures, their biosynthesis, their potential roles in the plant, and methods for their analysis. Studies of these compounds and their concentration changes during the winemaking process are discussed.

Key words: glycosides, enzyme hydrolysis, acid hydrolysis, grapes, wine, aroma

Introduction

The first volatile aroma glycosides were identified in rose in 1969 (Francis and Allcock 1969). While their presence was suggested in 1974 (Cordonnier and Bayonove 1974), it was not

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until 1982 that these aroma precursors were first identified in grape (Williams et al. 1982a). The first evaluations on the sensory properties of glycosides in grapes and wines were done by Noble and coworkers. They demonstrated that although glycosides may be perceived as bitter in model solutions, they do not occur in high enough concentrations to contribute to bitterness in wine (Noble et al. 1987, 1988). Further, they observed that glycosides in terpenic grapes and wines were "an important reserve of potential wine flavor" (Nobel et al. 1987). In addition to these studies, Noble and coworkers observed that ~90% of the monoterpenes were found in the glycosylated, or "bound," form in Muscat of Alexandria grapes (Park et al. 1991).

This early foundational work demonstrated the necessity of glycoside analysis to study grape aroma composition. Since the discovery of volatile aroma glycosides in grape, researchers have studied ways to exploit this potential flavor reserve as a means to improve wine aroma. In addition, analysis of volatile aroma glycosides has become increasingly important to the wine industry with the discovery of smoke-taint glycosides (Hayasaka et al. 2010b). In these cases, it was observed that large fires burning in close proximity to vineyards during the grapegrowing season produced smoke-tainted grapes and wine. In addition, concentrations of off-aromas appeared to increase during fermentation, leading to a hypothesis that smoke-taint glycosides were being hydrolyzed during fermentation, "freeing" the off-aromas and resulting in smoke-tainted wines (Kennison et al. 2008). This hypothesis was later confirmed (Hayasaka et al. 2010b).

The scope of this review is not intended to be exhaustive, but to inform the reader on progress made in the field of volatile aroma glycosides in grape and wine research and to draw attention to areas where further studies may prove useful. Many past reviews are recommended

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if further detail is required. Winterhalter and Skouroumounis have a comprehensive review of volatile aroma glycosides in a variety of plants (Winterhalter and Skouroumounis 1997). More specific reviews on glycoside formation (Bowles et al. 2006, Jones and Vogt 2001, Vogt and Jones 2000), enzyme hydrolysis (Sarry and Günata 2004), and enzymatic hydrolysis effects on winemaking (Günata et al. 1993) are recommended as well. The current review will present structures of identified glycosides, information on how glycosides are formed and theories on their roles in plants, analytical methods for their characterization and quantitation, and an overview of the role of winemaking processes on glycoside concentration and hydrolysis. The information presented will be primarily focused on glycosides in grapes and wine; however, when few or no studies have been done on grape, information on other plants will be given.

Structures of Glycosides

Aglycones. Glycosides are comprised of an aglycone that is linked to one or more sugar moieties: that is, the glycone. In grapes as with other plants, straight chain alcohols, volatile terpenoids, shikimic acid metabolites, and norisoprenoids (Winterhalter and Skouroumounis 1997) have all been identified as aglycones of volatile aroma glycosides. Examples of these from the different classes are shown (Figure 1). These different classes of aglycones, however, do not behave the same way upon hydrolysis of their corresponding glycosides. Typically, monoterpene glycosides will produce a volatile aroma compound directly upon hydrolysis (Figure 2A). In contrast, norisoprenoid glycosides may produce odorless products after hydrolysis which require further chemistry (e.g., acid catalyzed rearrangements) to produce the volatile aroma compound (Sefton et al. 2011, Winterhalter and Skouroumounis 1997). This is well demonstrated by a

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proposed hydrolysis scheme of the norisoprenoid β-damascenone (Kinoshita et al. 2010) (Figure 2B).

Glycones. To date, all glycosides of aroma compounds have been shown to include a direct linkage of the aroma compound to a β-D-glucose moiety (Winterhalter and Skouroumounis 1997). This is in contrast to other metabolites that may be linked to other sugars, such as the case of quercetin, which, in addition to a glucose, may also be glycosylated to glucuronic acid (Castillo-Muñoz et al. 2007) (Figure 3). Volatile aroma monosaccharide glycosides, or glucosides, are often found esterified to a malonyl group (Sarry and Günata 2004). The addition of other sugars to the glucose moiety will form disaccharide, trisaccharide, and higher order saccharide glycosides. In grapes, rhamnose and arabinose (Williams et al. 1982a) along with apiose (Voirin et al. 1990) have been identified as terminal sugars in disaccharide glycosides. Figure 4 shows the structures of identified glycones in grape, together with their names, and common names, when available. An additional β-D-glucose has been identified as the terminal sugar moiety of volatile aroma disaccharide glycosides in other plants (Winterhalter and Skouroumounis 1997). In addition, although identified in other plants, such as tomato (Tikunov et al. 2010) and apple (Herderich et al. 1992), to date, no higher order saccharides beyond disaccharides have been identified as glycosylated to volatile aroma compounds in grape.

In summary, because volatile aroma compounds are often not directly produced from hydrolysis of glycosidic precursors, as is the case with norisoprenoids, it can be difficult to link volatile aroma compounds to specific precursors. While many volatile aroma glycosides have been identified in grape, many identified in other plants have not been found in grape. It is not clear if these compounds are not present in grapes, if they exist in low levels that are difficult to

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95 detect, or if limitations in our current analytical methods have prevented their identification thus
96 far. In order to improve our understanding of glycosidic hydrolysis mechanisms and improve our
97 ability to quantify them, further studies on glycoside structures are needed.

98 Formation and Roles of Glycosides in Grapes

99 Several theories exist as to why plants glycosylate flavor compounds (Jones and Vogt 2001,
100 Stahl-Biskup et al. 1993). In recent years there have been an increasing number of studies on
101 biosynthesis of glycosides with regard to the enzymes that catalyze their formation and the
102 cellular locations of these enzymes. However, many of these studies have been done on model
103 plant systems and not on grapes. Those studies done on grapes have generally focused on the
104 enzymes involved in formation of glycosylated compounds not related to aroma, such as
105 flavonoid and anthocyanin glycosides (Ono et al. 2010). Knowledge of why and how plants
106 glycosylate volatile aroma compounds may allow us to manipulate their formation through
107 alterations in production practices (light exposure, water stress, etc.), thereby altering the aroma
108 profile of the plant and/or fruit.

109 Biosynthesis of Glycosides in Plants and Location in Grape Berries

110 Our understanding of the formation of glycosides is largely based on studies done in model plant
111 systems (i.e., *Arabidopsis thaliana*). These studies indicate that glycosides are produced by
112 glycosyltransferase enzymes, which add an activated sugar moiety to the aglycone (Figure 5)
113 (Bowles et al. 2006, Vogt and Jones 2000). UDP-glucose, -rhamnose, -galactose, -xylose, and
114 glucuronic acid have all been identified as activated sugars (Bowles et al. 2006, Jones and Vogt
115 2001). A variety of functional groups present on an aglycone have been shown to be acceptors
116 for these activated sugars, including -COOH, -NH2, -SH, -OH, C-C, among others. Based on the

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117 solubility of glycosyltransferases and their lack of targeting information, many researchers have
118 assumed the glycosyltransferases are located in the cytosol of the plant cell (Bowles et al. 2006,
119 Jones and Vogt 2001). Primary protein sequences of glycosyltransferases support this theory;
120 however, it may also be possible that they are associated with the cytosolic side of membrane
121 compartments (Bowles et al. 2006) or as part of multienzyme complexes (Burbulis and Winkel-
122 Shirley 1999). In addition, the presence of a luteolin tri-glycosyltransferase was shown in the
123 vacuole of *Secale cereale* (Anhalt and Weissenböck 1992) and the association of a
124 *Chrysoplenium americanum* glycosyltransferase with the endoplasmic reticulum has been
125 proposed (Ibrahim 1992). To our knowledge, there have been no studies published on subcellular
126 locations of glycosyltransferases in grape.

127 Over 240 putative glycosyltransferases have been identified in *Vitis vinifera* based on
128 screening of the grape genome (Jaillon et al. 2007). However, the number of these putative genes
129 that are truly glycosyltransferase genes and the number actually expressed are yet to be
130 determined. In addition, very little is known about substrate specificity of glycosyltransferases in
131 plants, let alone in grape (Jones and Vogt 2001). Most studies to date have focused on the
132 biosynthesis of flavonol glycosides. Ono et al. (2010) characterized two grape flavonol
133 glycosyltransferases, VvGT5 and VvGT6. Enzymatic activity was assessed by the percentage of
134 the aglycone that was able to be glycosylated. VvGT5 had no activity on 14 tested polyphenolics
135 and showed varying activity toward quercetin (100%), kaempferol (51.5%), and isorhamnetin
136 (5.6%) (Ono et al. 2010). VvGT5 also was specific to the activated sugar UDP-glucuronic acid
137 as no activity was seen with the other activated sugars tested. VvGT6 was similarly specific to
138 flavonols, and while it showed activity to UDP-glucose and UDP-galactose, it was inactive

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139 toward other activated sugars. Similar substrate specificity is seen in glycosyltransferases in
140 other plants (Vogt and Jones 2000). If glycosyltransferases are specific to a narrow group of
141 compounds, much of the data may not be applicable to volatile aroma glycosyltransferases.
142 These results stress the need for studies on the biosynthesis of volatile aroma glycosides
143 specifically.

144 **Glycosides as a Flavor Reserve and as Detoxificants**

145 Plant cell vacuoles function as metabolite reserves and are integral to detoxification (Marty
146 1999). Glycosides have been identified in the vacuole of the cell, which may corroborate these
147 theories (Ferreres et al. 2011, Martinoia et al. 2000, Zhao et al. 2011). Grape berries lack
148 structures capable of storing small lipophilic molecules (Lund and Bohlmann 2006), unlike the
149 trichomes of mint, for example. Correspondingly, grapes appear to have higher concentrations of
150 glycosides relative to the volatile aroma counterparts (Koundouras et al. 2009, Park et al. 1991).
151 Typically, many aglycones have low solubility in aqueous solutions. For example, linalool has a
152 predicted log octanol water coefficient of 3.38, but when glycosylated it has a predicted log K_{ow}
153 of 2.33 (estimates using KOWWIN in EPISuite,
154 www.epa.gov/opptintr/exposure/pubs/episuite.htm). The addition of a sugar moiety greatly
155 increases the solubility of the compounds, preventing diffusion across cellular membranes, and
156 thus provides a convenient storage form (Bowles and Lim 2010).

157 Most active aroma compounds are lipophilic; however, high localized concentrations of
158 lipophilic molecules can be toxic to a plant, by disrupting cellular membranes, for example
159 (Sikkema et al. 1995). In one study, cells from different types of plants were exposed to high
160 levels of volatile aroma compounds, (i.e., menthol). Plant cells that glycosylated greater than

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40% of the compounds, such as pear, for example, were able to continue growing while the others, including grape, could not (Berger and Drawert 1988). Additional studies have shown concentrations of 1.5 mg/g fresh weight of hydrocarbon monoterpenes (i.e., α-pinene) and monoterpenols (i.e., nerol) are enough to induce cell death during initial growing stages of plant cells in vitro (Figueiredo et al. 1996). These results support the hypothesis that plants may glycosylate volatile flavor compounds as a detoxification strategy (Hösel 1981). Further, glycosylation appears to stabilize nucleophilic aglycones, preventing their reactivity with other cellular structures by electron transfer reactions (Jones and Vogt 2001). It has been proposed that improved aqueous solubility through glycosylation of small lipophilic compounds might also prevent their diffusion into the tonoplast, inhibiting their ability to move outside of the vacuole (Wink and Roberts 1998). These findings suggest that movement of glycosides throughout the cell is limited or at least highly regulated.

The Translocation Theory

During photosynthesis plants take up carbon and produce sugars. This process predominately occurs in the leaves. The sugars are then transported from these "sources" to various "sink" organs of the plant, such as roots and reproductive organs. In grapes, the berries begin to accumulate sugar at veraison. As such, it may be possible that flavor compounds may accumulate in such a way as well. Translocation of these lipophilic aroma compounds would likely be in the glycosidic form. Once a compound is glycosylated, it can be moved throughout the cell via membrane transport systems that recognize the sugar moieties (Bowles et al. 2006). The apoplast is one such transport pathway in the plant cell. Glycosides have been identified in the apoplast of the plant cell along with their corresponding glycosidases (Dietz et al. 2000,

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Samuels et al. 2002) which supports the feasibility of glycosides as a translocation form of volatile aroma compounds. Further, a study in peppermint, *Mentha piperita* L., showed that (-)-menthone produced in leaves was first converted to (+)-neomenthol, glycosylated, and finally translocated and accumulated in the rhizomes of the plant (Croteau and Martinkus 1979).

In contrast, several studies indicate that glycoside translocation may not be a means of flavor precursor accumulation in grape berries. To test the theory of glycoside translocation, Gholami and colleagues studied two grape varieties, a terpenic variety, Muscat of Alexandria, and a non-terpenic variety, Syrah (Gholami et al. 1995). Muscat inflorescence clusters were grafted to Syrah vines and vice versa. The data showed that the Muscat berries grown on Syrah vines contained similar levels of terpenes to Muscat berries grown on Muscat vines, indicating that little glycoside transport occurred. The effect was similar with Syrah. Syrah berries grown on Muscat vines did not show an increase in terpene content relative to the control. A recent study of smoke-taint glycosides obtained similar results (Hayasaka et al. 2010a). Labeled guaiacol was fed to grape berries for 1 to 2 days and concentrations of the labeled glycoside in the various plant tissues were analyzed after 35 days. Grapes that were fed labeled guaiacol produced labeled glycoside but nearby leaves failed to show any labeled glycoside. Similarly, leaves that were fed labeled guaiacol produced the labeled glycoside but proximal berries did not accumulate the glycoside. These findings indicate that glycoside translocation may not be a means of flavor precursor accumulation in grape berries. However, the study by Croteau and Martinkus (1979) might indicate that studies done thus far may not have accounted for possible conversions of the parent compound prior to glycosylation and translocation. Further, in many cases, it is possible that the actual aglycone has not been identified due to subsequent

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reactions/rearrangements that occur following hydrolysis. This has been observed, for example with β-damascenone, where multiple glycoside precursors have been reported (Sefton et al. 2011), although none include direct conjugation to β-damascenone. Therefore, as further discussed in the following section, current analytical methods do not allow us to fully predict aglycone composition, and a less targeted approach will be necessary to account for different aglycone structures. In addition, our knowledge of potential glycone composition and substitution is hindered by our relatively limited knowledge of intact glycoside structures.

In summary, when found in high concentrations, volatile aroma compounds can be toxic to plant cells. While studies have been conducted on glycosyltransferases, few have been done in grape, and among those, none have been done on the enzymes that catalyze glycosylation of volatile aroma compounds. Studies in other plant systems have shown glycosyltransferase enzymes have high specificity, thus suggesting the need for studies specific to flavor compounds. In addition, a greater knowledge of where these enzymes are located within the plant cell may provide further insight into the feasibility of other possible roles of glycosides in plants, such as translocators of volatile aroma compounds.

Analytical Techniques to Measure Glycosides

As previously stated, volatile aroma glycosides are found in higher levels than their unbound counterparts. As such, their analysis is crucial to studies on aroma profiles in grapes and wine. In order to correctly infer how fermentation, climate, viticultural practices, and other variables affect grape and wine volatile aroma profiles, these glycoside analytical methods must be reliable. Many methods exist, some of which analyze portions of the glycoside (i.e., the

aglycone), while others analyze the intact glycosidic precursor. An understanding of the principles of these methods will provide insight into potential method biases.

Preparatory techniques. Typically, analysis of grape and wine glycosides begins with a preparative chromatographic technique to isolate and concentrate the glycosidic fraction. The most frequently used method is solid-phase extraction (SPE), which can be done relatively quickly and is fairly inexpensive. For wine and grape analysis, the sorbent is typically reverse phase, often C-18 (Williams et al. 1982b) or Amberilite XAD-2 (Günata et al. 1985). Samples are loaded (after homogenization and either filtration or centrifugation to remove solids, in the case of grapes) and the column washed with water, which removes the highly polar compounds, such as salts and free sugars. Subsequently, elution of glycosides and unbound volatile aroma compounds is performed sequentially, using organic solvents with differing polarities. SPE can be done on an analytical scale or a preparative scale. Günata et al. (1985) indicated that XAD-2 is better able to retain free monoterpene alcohols than C-18 phases, allowing for better recovery of the volatile aroma fraction. However, the XAD-2 phase may be unable to separate glucose from the glycosides completely, which may hinder further analyses (Williams et al. 1995). Additionally, different C-18 phases may show differences in selectivity (Hampel et al. 2014). Research objectives should guide the choice of phase.

Subsequent to SPE, further separation may be desired depending on the research objectives. Countercurrent chromatography, size exclusion chromatography, or preparative HPLC are often used to further separate the glycosidic fraction (Winterhalter and Skouroumounis 1997). Iterations of countercurrent chromatography have been used to purify norisoprenoid glycosides in Riesling leaves, which were then structurally elucidated using NMR

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248 (Skouroumounis and Winterhalter 1994). The authors note that countercurrent chromatography
249 is associated with fewer artifacts and has better recoveries than solid sorbent techniques such as
250 preparative HPLC and size exclusion chromatography.

251 **Analysis of released aglycones.** *Enzyme hydrolysis.* After obtaining the glycoside fraction
252 from the grape or wine sample, the fraction may be first hydrolyzed and the released aglycones
253 may be analyzed, typically using gas chromatography (GC). Glycosidic hydrolysis is done either
254 enzymatically or with the use of acid. Enzymatic hydrolysis is highly dependent on the choice of
255 enzyme. Monosaccharide glycosides in grape may be hydrolyzed by endo- or exo-glucosidases.
256 However, disaccharide glycosides will only be hydrolyzed in a stepwise approach using two or
257 more enzymes or by the use of an endo-glycosidase (Figure 6). Many commercial enzyme
258 preparations have exo-glycosidase activities. Endo-glycosidase enzymes are able to hydrolyze
259 the glycosidic linkage to the aglycone, regardless of the number of sugar moieties: that is, they
260 will have activity on monosaccharide and disaccharide glycosides, unlike the exo-glycosidases.
261 As all grape and wine glycosides identified to date have a glucose moiety directly attached to the
262 aglycone, the use of an endo-glucosidase should theoretically hydrolyze the glycosides in
263 entirety. An endo-glucosidase was isolated and purified from *Aspergillus niger* grown on a
264 monoterpene glycoside-containing medium (Shoseyov et al. 1988). While the enzyme was
265 thermally stable and had optimum activity at pH 3.4, consistent with typical grape and wine pH
266 conditions, enzyme activity decreased with ethanol percentages greater than 9%. Additionally,
267 activity was highly inhibited by glucose. Inhibition of glycosidases by ethanol and glucose is a
268 problem for many enzyme preparations as well and many have pH optima outside a relevant
269 range for grapes and wine (Aryan et al. 1987, Günata et al. 1990). Further, while studies have

indicated that fewer rearrangements of the released aglycone may occur during enzymatic hydrolysis (Cordonnier and Bayonove 1974, Günata et al. 1985, Mateo and Jiménez 2000), artifacts may still be produced via enzymic oxidation (Hampel et al. 2014, Winterhalter and Skouroumounis 1997). These findings indicate that enzyme hydrolysis will likely be unable to free a large number of aglycones and may potentially lead to identification of artifactual aglycone structures.

Acid hydrolysis. Acidic hydrolysis is a widely used technique to liberate aglycones from glycosides. As with enzymatic hydrolysis, subsequent to hydrolysis, aglycones are usually analyzed by GC. Acid hydrolysis is less cost prohibitive and can be done more quickly compared to enzymatic hydrolysis. Acid and enzyme hydrolysis procedures were compared in a study (Loscos et al. 2009), and while the enzyme hydrolysis occurred over 16 hours, the acid hydrolysis was completed in one hour. Seven different grape varieties, both red and white, were tested, and while the enzymatic hydrolysis appeared to release a higher concentration of terpenes (3 to 10x) for five of the seven cultivars, released norisoprenoid concentrations were greater by a factor of 10 for the acid hydrolysis procedure for all cultivars. Furthermore, it has been theorized that acid hydrolysis in the grape berry is the most likely route for *in planta* liberation of monoterpenes from their corresponding glycosides rather than endogenous enzymatic hydrolysis (Williams et al. 1982c), as endogenous grape glycosidases are highly inhibited by glucose (Aryan et al. 1987, Günata et al. 1990). In Williams et al. (1982c), monoterpene glucosides were hydrolyzed both with a glucosidase and also by acid. Acid hydrolysis yielded a wider variety of compounds than did enzymatic hydrolysis. In addition, an isolated glycoside fraction from

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grapes hydrolyzed with acid appeared to more closely resemble a grape berry volatile profile than the enzymatically hydrolyzed sample.

While acid hydrolysis may be more reflective of grape and wine aroma than enzymatic hydrolysis, it is precisely this difference that hinders identification of the intact glycosidic precursor. For example, at pH 3.0, linalyl, geranyl, and neryl glucosides all produced linalool and α-terpineol as major products (Williams et al. 1982c). While this hydrolysis may be more indicative of what happens during the winemaking process, analysis of the hydrolysis products will not yield credible information on the structure of the glycosidic precursor. Increasingly lower pH is associated with more rearrangements of the aglycones, producing more artifacts (Croteau 1987, Hampel et al. 2014, Sefton et al. 1989, Williams et al. 1982c). Both enzymatic and acid hydrolysis techniques have drawbacks and benefits, and ultimately it is up to the researcher to decide which technique may be more congruent with their specific research goals.

Analysis of glycones. *The glycosyl-glucose method.* Most current glycoside analytical methods are qualitative, focusing on the identity of the aglycone. Because few glycosidic structures are known and few standards are available, traditional methods to quantify glycosides are difficult. Based on the knowledge that glycosidic structures all contain one glucose moiety, researchers created a method to quantify glycosides based on released glucose (Williams et al. 1995). An estimation of glycoside concentration is made by isolating the glycosidic fraction, hydrolyzing it with acid, and using an enzyme assay to quantify released glucose. A control is used to account for free glucose present in the initial sample. However, in addition to volatile aroma compounds, other compounds, such as phenolics, are glycosylated. In the case of red grapes, this interference is of particular note, as there are high amounts of glycosylated anthocyanins present. In order to

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achieve a glycosyl-glucose (G-G) concentration that is more reflective of the glycosylated volatile aroma compounds, several studies have explored adaptations of the original method. Iland et al. (1996) took a subsample of the grape homogenate, acidified it, and used a spectrophotometric assay (Somers and Evans 1977) to obtain the anthocyanin concentration. The quantification of anthocyanins was done using malvidin 3-glucoside for the external calibration curve. This calculated anthocyanin concentration was subtracted from the "total G-G" (TGG) concentration to give the "red-free G-G" concentration. The authors point out, however, that while this approach is effective in grapes, it is not recommended for wine because the pigments found in wine are found in polymeric form, whereas they are monomeric in grapes. A G-G value that is more representative of just the volatile aroma glycoside concentration in wine must account for these other sources of glycosylated interferences. To improve upon the G-G method further by accounting for phenolic glycosides beyond anthocyanins, other researchers used the Folin–Ciocalteu reagent to quantify the phenolic glycoside content in gallic acid equivalents, which when subtracted from the TGG, yielded the "phenol-free G-G" (PFGG) (Zoecklein et al. 2000). Despite the improvements, some phenolic glycosides were still present in the PFGG. These interferences, while greatly diminished, will hinder accurate quantification, which may artificially inflate the observed concentration of flavor related G-G. However, the G-G method and related techniques remain valuable tools for quantification of volatile aroma glycosides as they are relatively quick and inexpensive, requiring no instrumentation beyond a spectrophotometer.

Analysis of intact glycosides. *Derivitization techniques.* If structural knowledge of the glycosidic precursor is desired, then a technique that analyzes the glycoside in its intact form is

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335 necessary. Glycosides may be derivatized to increase their volatility, enabling them to be
336 analyzed by GC. Typically, this is done by acetylation, methylation, or silylation (Winterhalter
337 and Skouroumounis 1997). While derivatization has been shown to be an effective tool for
338 structural elucidation of glycosides, it is considered a "dirty" technique. For example, Wells
339 notes that residual trimethylsilyl (TMS) derivatizing agent from previous runs will derivatize
340 compounds as they are separated on the GC column (Wells 1999). Consequently, more routine
341 instrument maintenance and cleaning is necessary. In addition, TMS may react with certain
342 functional groups to produce silylation artifacts (Little 1999), leading to possible erroneous
343 identifications. For all methods, an empirical determination of derivatization efficiency is needed
344 to determine the necessary conditions to complete the derivatization (Pierce 1968). This
345 efficiency is substrate dependent and, as many glycosides are not available commercially as
346 standards, empirical determination is often neglected, which may lead to incomplete
347 derivatization.

348 *HPLC-techniques.* In lieu of derivatization and subsequent GC analysis, liquid chromatography-
349 mass spectrometry (HPLC-MS) can be used. This approach has gained increasing popularity as
350 the technology has improved. HPLC-MS techniques are generally soft-ionization techniques, as
351 opposed to electron ionization GC-MS, and thus do not produce unique compound fragmentation
352 spectra. In order to glean more structural information, HPLC coupled to tandem MS (MS/MS)
353 approaches may be used. Pseudo-molecular ions are filtered by the first MS and then fragmented
354 in a collision cell by a gas with a variable charge applied. The resulting fragments are then
355 detected by the subsequent MS. Analysis of these so-called product-ion scans enables insight
356 into the structure of the molecule. Guaiacol glycosides were tentatively identified in grapes using

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this approach (Hayasaka et al. 2010a). Figure 7 illustrates how analysis of the product ion spectrum can be used for tentative identification by assigning structures to fragments. However, it should be noted that more definitive identifications with these techniques are more difficult. For example, there may be isobars or isomers with similar MS/MS spectra so the actual identification is tentative. Beyond this, very few volatile aroma glycoside standards are commercially available, and as such they must be synthesized in order to identify compounds using a standard, as was done for the guaiacol glucoside (Hayasaka et al. 2010b). In addition, while more compound specific MS/MS fragmentation data is being included in MS spectral databases, limited MS/MS data is available for volatile aroma glycosides.

To summarize, the choice of a method for analysis of aroma glycosides should be based on the experimental objectives of the researcher. Ideally, research into precursors of volatile aroma compounds should be done using a method that analyzes the intact glycoside. While new technology may improve our ability to analyze these compounds, with the use of HPLC-MS/MS for example, these new methods may be hindered by limited availability of authentic standards and limited MS/MS database entries.

Glycoside Studies in Wine

Glycosides are a major source of untapped flavor in wine. While the glycosides often have a bitter taste, they do not contribute to bitterness in wine (Noble et al. 1987, 1988). However, upon hydrolysis, the released volatile aroma compounds may affect the aroma profile of a wine, as previously suggested (Wilson et al. 1986, 1984, Günata et al. 1985) and shown (Francis et al. 1999). Despite being relatively thermodynamically unstable in the acidic environment of a wine, most glycosides remain intact throughout the winemaking process and, to

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379 a lesser extent, in the bottle (Zoecklein et al. 1999). Many studies have looked for ways to
380 exploit this flavor reserve during the winemaking process. Studies have been conducted on the
381 effect of *Saccharomyces* strain, other yeasts, malolactic fermentation, skin contact, temperature,
382 and additions of exogenous glycosides as a way to increase glycosidic hydrolysis. In general,
383 however, a fundamental lack of understanding is hindering our ability to use flavor compound
384 glycosidic precursors to optimize flavor attributes. In addition, the results of these studies are
385 conflicting which prevent us from drawing definitive conclusions. The cause of these conflicting
386 results stems from a variety of sources. Glycosidic hydrolysis of a single precursor may produce
387 more than one volatile aroma compound whether by enzymatic or acid hydrolysis in wine. In
388 addition, some glycosidic precursors will produce odorless products, such as polyols. This
389 underlies the necessity of a global approach for the analysis of glycosides. Both the intact
390 glycosides and the free volatile compounds should be monitored in order to draw conclusions
391 about fermentation effects. Beyond that, sensory studies are required to determine if the
392 procedures to increase glycoside hydrolysis make a significant difference on aroma profiles to
393 the final wine.

394 **Enzymatic additions.** Much of the initial work on enhancing hydrolysis of glycosides during
395 the winemaking process began with studying the addition of enzyme preparations to musts and
396 wines. Endogenous grape enzyme preparations were found to be largely ineffective due to
397 inhibition by sugar and ethanol and by low activity at wine pH (Aryan et al. 1987, Günata et al.
398 1990). Subsequent studies have looked at the effect on exogenous enzyme preparations. Some of
399 these preparations appear to be more effective than others, with the most successful having
400 arabinosidase, apiosidase, and rhamnosidase activity in addition to glucosidase activity

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(Cabaroglu et al. 2003) using a stepwise approach to hydrolyze the disaccharide glycosides (Günata et al. 1993). However, as pointed out by Sarry and Günata (2004), there are two major issues to consider with the use of enzyme preparations. The first is formation of off-aromas caused by cinnamate esterase activity of the enzyme preparation. This, in concert with decarboxylation activity from *S. cerevisiae* during fermentation, can produce off-aromas due to production of volatile phenols (Chatonnet et al. 1992, Dugelay et al. 1992). The other potential issue is loss of color from the hydrolysis of anthocyanins to anthocyanidins (Le Traon-Masson and Pellerin 1998).

Skin contact and temperature. One study compared the effects of skin contact on free volatile compounds, glycosides, and enzymatically released volatile compounds (Palomo et al. 2006). The authors proposed the idea of fermentations done with skin contact and subsequent enzymatic hydrolysis as a way of producing wines with higher concentrations of volatile aroma compounds. Three fermentation treatments done in duplicate on Muscat blanc were carried out at 18°C with either no skin contact, 15 hours of skin contact, or 23 hours of skin contact. Released compounds were measured following enzymatic hydrolysis with a commercial enzyme preparation, AR2000, which has activity for all reported glycosides (Baek and Cadwallader 1999). Free and released volatiles were measured by GC-MS. A descriptive analysis of the wines was done according to a previous method (Noble et al. 1984). Wines with skin contact showed higher concentrations of glycosides and free compounds in addition to more perceived body as determined by sensory analysis. The authors suggested that the addition of glycosidases together with skin contact during winemaking may be a possible way to increase the concentration of free volatiles in wine. However, in this experiment, it is highly likely that the higher concentration of volatile aroma

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423 compounds in the wines with skin contact was due not to the increased concentration and
424 subsequently hydrolyzed glycosides but rather to the extraction of more free compounds from
425 the skins of the grapes. In addition, glycosides were first extracted and subsequently
426 enzymatically hydrolyzed. Because the enzymatic hydrolysis was done in a buffered solution
427 rather than the wine matrix, it is likely that these results would be different if the enzyme was
428 added directly to the wine for the purposes of commercial winemaking. Finally, while phenolic
429 off-aromas were not found in appreciable levels, as noted above, enzyme preparations have been
430 shown to produce high levels of these compounds when in the presence of the wine matrix. It is
431 likely these would have been present if the enzymes had been added to the wine directly.
432 Additional studies are necessary to assess whether skin contact in addition to enzymatic
433 hydrolysis during the winemaking process will truly effect glycoside concentration and
434 subsequent hydrolysis and ultimately result in a change in aroma of the resulting wine.

435 Other researchers assessed the effect of prefermentation soak temperature on Cabernet
436 Sauvignon glycoside concentration (McMahon et al. 1999). A cold soak at 10°C for three days
437 was shown to increase glycoside content. An even greater enhancement was seen with an
438 ambient soak at 20°C for three days. It was also shown that glycoside concentration was higher
439 when the must was pressed before fermenting to dryness. However, there was no analysis of the
440 free volatile compounds. As such, the authors could not conclude if these changes in glycoside
441 contact affected the aroma profile of the resulting wines.

442 **Strains of *Saccharomyces cerevisiae*.** Studies on the effect of *Saccharomyces cerevisiae* strain
443 on glycoside hydrolysis are conflicting. In one study, several strains of *S. cerevisiae* strains were
444 compared for their glycosidase activity (Zoecklein et al. 1997). Strains showed varying ability to

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hydrolyze glycosides. However, these changes were comparatively small among strains and the authors point out that these differences were likely to have little or no sensory impact because the concentrations of the released compounds were below reported thresholds in wine. Importantly, the authors noted the resulting differences in glycoside levels were difficult to correlate with levels of released compounds, since acid-catalyzed rearrangements were indicated as suggested elsewhere (Croteau 1987). Further, correlation of glycoside levels and released aglycones was complicated due to the possibility of absorption and/or metabolism of released compounds by yeast, as also suggested elsewhere (Di Stefano et al. 1992).

One study examined the fate of Chardonnay glycosides in both a model matrix and fermenting wine (Chassagne et al. 2005). Glycosyl-glucose was tracked throughout fermentation and the glycone moieties themselves were identified and quantified. The authors were able to show that the *S. cerevisiae* strains tested showed varying glycosidase activity ranging from 18 to 57% hydrolysis. Glycosides were also extracted from yeast cells, and it was shown that sorption of glycosides to yeast cells was not a significant effect, contrary to the theory proposed by Di Stefano et al. (1992). However, Chassagne et al. (2005) did not include an analysis of the released volatiles, so the fate of the hydrolyzed glycosides is not clear. In addition, there was no sensory component to the study. Therefore, whether or not the hydrolyzed glycosides contributed significantly to a change in aroma could not be determined.

Use of other yeasts. *Saccharomyces* is the preferred wine yeast due to its ethanol tolerance and because it produces few off-aromas (Swiegers et al. 2005). However, early in the winemaking process other yeasts may be present and may influence the aroma of finished wine (Swiegers et al. 2005). Glycosidase activities were compared in several strains of *S. cerevisiae*, several other

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wine yeasts, and a non-native wine yeast, *Candida molischiana* (Fernandez-Gonzalez et al. 2003). The study used a model system of yeast cultures coinoculated with glycosides isolated from Muscato bianco to compare glycoside content during fermentation and the subsequently released flavor compounds. Among the yeasts tested, *Hanseniaspora uvarum* and *C. molischiana* were best able to hydrolyze the glycosides. While *C. molischiana* produced more compounds and in higher concentrations than *H. uvarum*, it also produced higher levels of 4-vinylguaiacol and 4-vinylphenol, which are associated with phenolic off-aromas. Further studies to investigate whether these results are reproduced in wine are warranted.

Malolactic bacteria. In addition to effect of yeast strain on glycoside hydrolysis, many studies have been done to assess the possible ability of malolactic bacteria, in particular *Oenococcus oeni*, to hydrolyze volatile aroma glycosides (Barbagallo et al. 2004, Boido et al. 2002, D'Incecco et al. 2004, Grimaldi et al. 2005). Researchers compared a control fermentation without inoculation to fermentations with *O. oeni* and found varying decreases in glycoside concentration and varying increases in released compounds depending on the strain of *O. oeni* added (Ugliano et al. 2003). However, the work was done using model wine. Indeed, while other studies corroborate these findings, much of the data indicates that although malolactic bacteria show glycosidase activity, they do not significantly contribute to glycoside hydrolysis during wine fermentations, and therefore they are unlikely to contribute significantly to wine aroma via released aroma compounds (Boido et al. 2002).

Although a variety of fermentation parameters have been studied with regard to glycoside content and hydrolysis, our understanding of the reaction mechanisms is incomplete. Model systems may help to elucidate some of the basic mechanisms of glycoside hydrolysis during

winemaking, but studies in wine are necessary to understand how much these factors contribute to a change in the aroma composition and sensory profiles of wine. The literature is conflicting with regard to the influence of many of these factors on glycoside hydrolysis, likely because of inherent differences in glycoside content and concentration among cultivars, differing grape maturities at harvest, and potentially the effect of climate on production of glycosides by the grape berry, among others. In addition, the various analytical approaches for measuring glycosides may yield different results. The acidic environment of wine, the potential presence of several yeasts, malolactic fermentations, and changes in fermentation conditions will also all have some effect on glycoside content and concentration. It is possible these factors have additive or subtractive effects on glycosidic hydrolysis, but these interactive effects have not been well studied. Furthermore, all of these effects may be unimportant overall if there is no appreciable sensory impact on the wine. Many previous studies have either not included a sensory component or have shown no sensory impact on the resulting wine despite an increase in glycoside hydrolysis. In some cases, the resulting wines had off-aromas. It is likely that this approach of trying to hydrolyze more glycosides during fermentation will only be helpful for some varieties that accumulate high enough concentrations of glycosides.

Conclusion

Our understanding of volatile aroma glycosides has greatly improved since the identification of the first glycosides in 1969. Since then, studies have elucidated more structures and this has given rise to more studies on glycoside formation and the roles of glycosides in the plant. New methods to analyze these compounds have been created and they in turn have improved our ability to study the effects of various winemaking conditions on glycoside content and

hydrolysis. Despite this progress in the field, much is still not known. Many structures have still yet to be identified. Studies on glycosyltransferases specific to volatile aroma compounds and specifically in *Vitis vinifera* are needed to improve our understanding on the formation of these potential aroma precursors in grape. In addition, improved analytical techniques are needed to correlate glycoside content and hydrolysis to sensory differences in winemaking studies. Although glycosides represent a large source of potential flavor, we remain largely unable to tap into this flavor reserve to improve grape and wine flavor.

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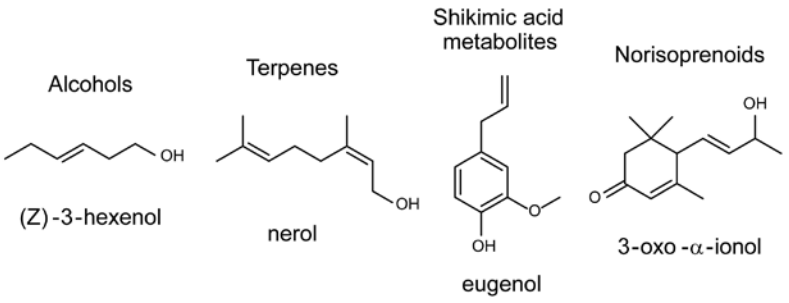

Figure 1 Classes of compounds glycosylated and examples.

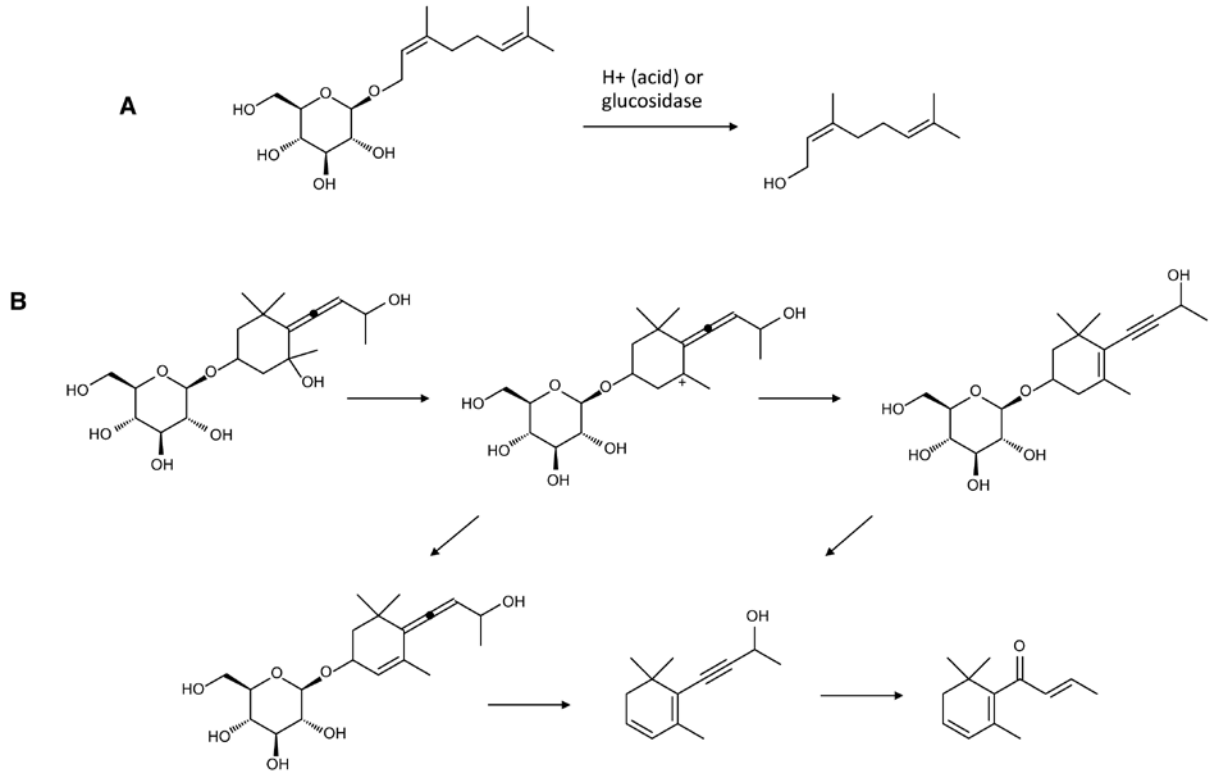

Figure adapted from Kinoshita et al. 2010.

Figure 2 (**A**) Hydrolysis scheme of neryl-β-D-glucopyranoside to nerol. (**B**) Proposed formation of β-damascenone from a glycosidic precursor.

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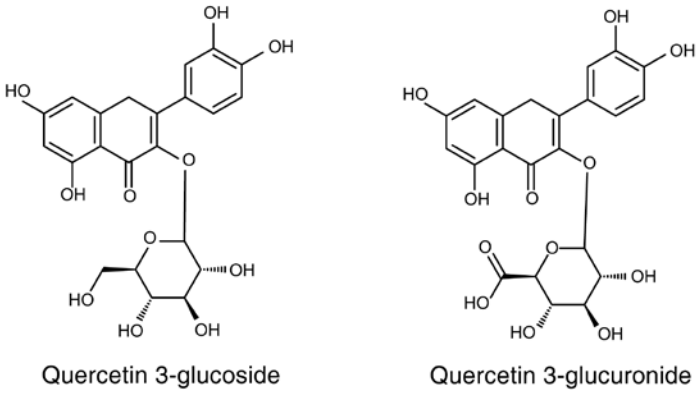

Figure 3 Quercetin glycosylated to glucose and glucuronic acid.

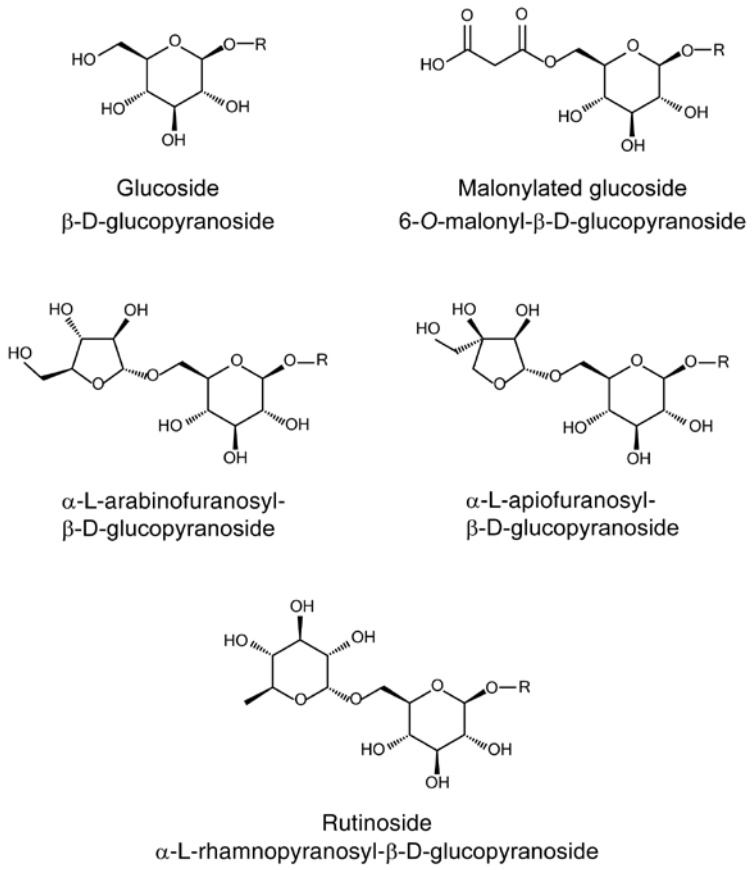

Figure 4 Identified glycones in grape.

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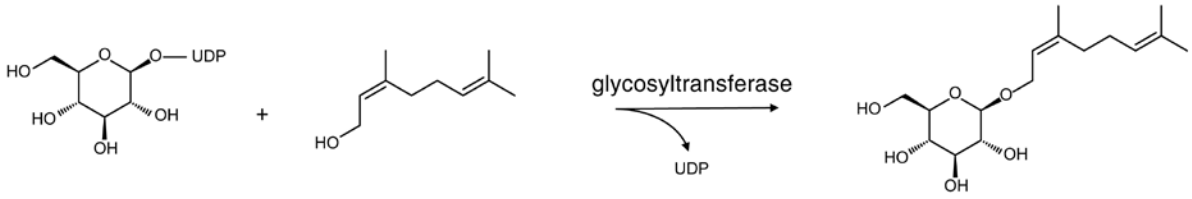

Figure 5 Synthesis of neryl-β-D-glucopyranoside from UDP-glucose and nerol.

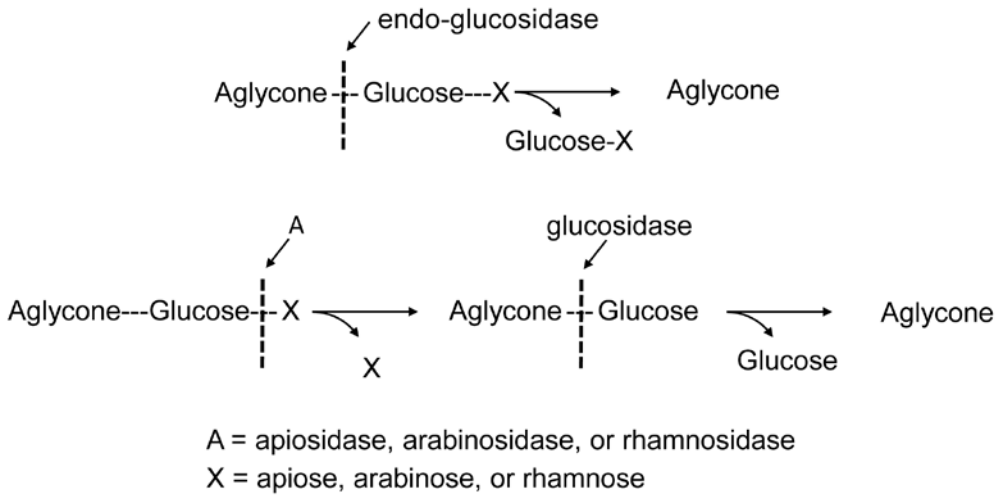

Figure 6 Hydrolysis of disaccharide glycosides.

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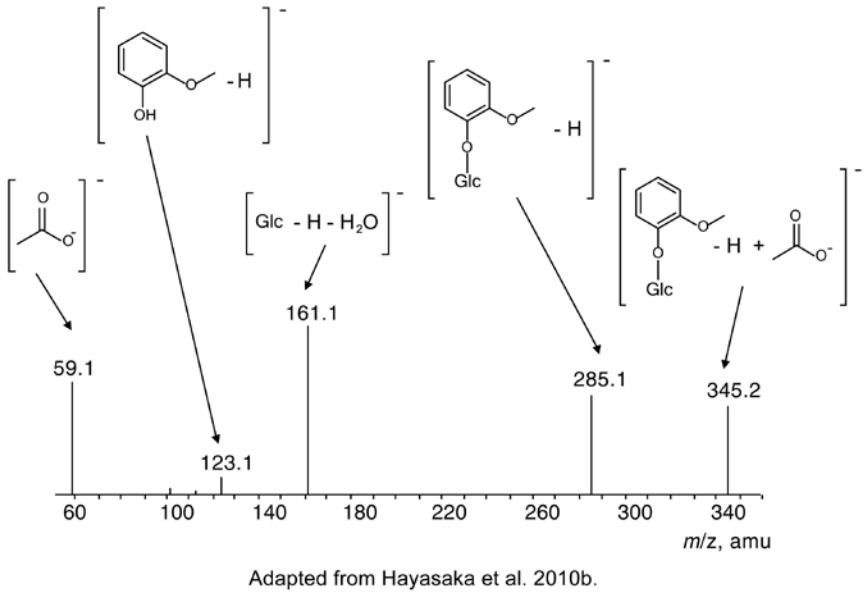

Figure 7 Guaiacol-β-D-glucoside product ion scan. Adapted from Hayasaka et al. (2010b), with permission.