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Identification of free disaccharides and other glycosides in wine

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ABSTRACT

Free soluble carbohydrates of different wine samples were analyzed by GC–MS as their trimethylsilyloximes using a methylsilicone column. Besides α,α-trehalose, several β-glucosylglucoses such as cellobiose, sophorose, laminaribiose and gentiobiose were the main disaccharides identified. With the exception of gentiobiose, these disaccharides are now reported for the first time in wine. Lactose (4-O-β-D-galactopyranosyl-D-glucose), previously described in this product, was also tentatively identified. Several free glycosides: β-ethyl-glucoside and seven glyceryl-glycosides (including glucosides and galactosides) were also identified for the first time in wine. On the contrary, disaccharides in grape juice were mainly constituted of fructose derivatives, including sucrose, and no glycosides were detected. Although the total amount of disaccharides was different in white wines (<50 mg/L) from those in rosé and red wines (80–130 mg/L), the chromatographic profile was noticeably similar in all wine samples. The method here reported allows the identification of several carbohydrates which have not been previously detected in wines and could contribute to increase the understanding of enzymatic activity during winemaking.

1. Introduction

Free carbohydrates in wine are mainly constituted of monosaccharides, sugar alcohols, sugar acids and disaccharides. Several of them are natural constituents of musts, while others are formed as a result of fermentation. Wine monosaccharides have been studied in detail [1–4], the presence of glucose, galactose, fructose, mannose, arabinose, rhamnose, ribose and xylose have been reported.

Wine sugar alcohols consist of linear polyalcohols and cyclitols. Erythritol, threitol, ribitol, arabitol, xylitol, sorbitol, mannitol, and traces of galactitol have been reported in different wines and sherries [1,5,6]. Regarding cyclitols, *myo*-inositol, *scyllo*-inositol [5] and *chiro*-inositol [7] have been reported in different wines, whereas the presence of quercitol (1,3,4/2,5-cyclohexanepentol) has been only found in wines aged in contact with oak wood (barrels or chips) but not in wines aged in bottles [7].

Sugar acids have special relevance in those wines affected by the action of "noble rot" (*Botrytis cinerea*) [8,9] that attacks grapes in humid climate conditions, causing the production of higher sugar and acid contents. Gluconic [5] and galacturonic acids [8] are those sugar acids found in greater amounts in these wines.

α,α-Trehalose has been reported to be the main disaccharide in wine [10] formed as a result of the metabolic activity of yeasts. Its level has been reported within 0–611 mg/L in wines [10] and within 0–53 mg/L in sherries [11]. In *Saccharomyces cerevisiae*, under normal growth conditions, trehalose accumulates after cells enter the stationary phase and also acts as a protectant that contributes to survival during stress conditions [12]. Small amounts of other disaccharides have been reported: sucrose within 20–120 mg/L [1]; other disaccharides (isomaltose, lactose and turanose) within 5 and 50 mg/L, and possible traces of melibiose and gentiobiose [10]. However, there are currently still a number of minor carbohydrates in wines without a conclusive identification.

Although analytical methods used for the characterization of wine carbohydrates have been mainly based on LC [2,3], GC–MS with capillary columns have been also utilized, since it affords the high resolution necessary to analyze such a complex mixture. Trimethylsilyl ethers (TMS) [10,13] and TMS oximes (TMSO) [1] have been used as derivatives for GC analysis.

In the present work, a method based on GC–MS of TMSO has allowed the identification of the main disaccharides of wine and other simple glycosides; their formation process is also discussed.

2. Materials and methods

2.1. Standards

Cellobiose (4-O-β-D-glucopyranosyl-D-glucose), isomaltose (6-O-α-D-glucopyranosyl-D-glucose), gentiobiose (6-O-β-D-glucopyranosyl-D-glucose), lactose (4-O-β-D-galactopyranosyl-D-glucose), laminaribiose (3-O-β-D-glucopyranosyl-D-glucose), β-phenyl-D-glucoside and sucrose (α-D-glucopyranosyl-β-D-

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Table 1
Description of the wine and must samples.

Samples	Type	Grape variety	Origin
Red wines	Young	Tempranillo	Ribera de Duero cellar, Spain
	Young	Tempranillo	Ribera de Duero cellar, Spain
	Young	Tempranillo	Ribera de Duero cellar, Spain
	Young	Cabernet-Sauvignon	Rioja cellar, Spain
	Young	Cabernet-Sauvignon	Rioja cellar, Spain
	Oak aged	Tempranillo + Cabernet-Sauvignon + Merlot	Peñafiel, Valladolid, Spain Bottled
	Oak aged/dry "Amarone"	Corvina + Rondinella + Molinara	Valpolicella, Verona, Italy Bottled
White wines	Young	Verdejo	Rueda cellar, Spain
	Young	Verdejo	Rueda cellar, Spain
	Young	Albariño	Rias Baixas cellar, Spain
	Young/medium-sweet	Airén	Tomelloso (La Mancha), Spain; bottled
Rosé wines	Young	Garnacha	Navarra cellar, Spain
	Young	Garnacha	Navarra cellar, Spain
Musts	Fresh	Verdejo	Rueda cellar, Spain
	Pasteurised	Muscat	Bottled grape juice
	Fresh	Muscat	Obtained in the laboratory

fructose) were obtained from Sigma Chemical Co. (St. Louis, US). Melibiose (6-O-α-D-galactopyranosyl-D-glucose), α,α-trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) and turanose (3-O-α-D-glucopyranosyl-D-fructose) were purchased from Fluka (Buchs, Ch). Sophorose (2-O-β-D-glucopyranosyl-D-glucose) was acquired from Sarsynthèse (Merignac, France).

Retention data and mass spectra for those compounds whose standards were not commercially available were obtained from different sources: α-glucosides (glyceryl-glucosides and ethyl-glucoside) from sake (Kuromatsu-hakushika, Tatsuuma-honke brewing Co., Ltd., Japan) as reported by [14] as provenient from the action of α-glucosidase from *Aspergillus oryzae* on glucose and glycerol, β-glyceryl-glucosides from leaves of *Lilium* spp. [15], α-glyceryl-galactosides from alga nori sheets (Blue dragon, G Costa & Co. Ltd., UK) [16] and β-glyceryl-galactosides from sugar mixtures obtained by transglycosidation with β-galactosidase [17]. This last product was kindly gifted by Dr. Montilla (CSIC, Spain). Extracts were centrifuged for 15 min at 5000 × *g* and immediately refrigerated until analysis.

2.2. Samples

Different types of industrially manufactured still wines including red, white and rosé were provided by cellars from different Spanish wine making areas. These wines were manufactured in 10,000 L stainless-steel tanks according to traditional practices without any storage or ageing in oak barrels. Samples were taken after clarification and stabilization procedures and before bottling. For comparison, three bottled still wines were purchased in different markets: a Spanish medium-sweet white, a Spanish red aged in oak cask and an Italian dry red from partially desiccated grapes (Amarone) also aged in oak. Three different samples of must were examined: one from the tank of a cellar, a commercial grape juice bottled and pasteurised, and the juice of 100 g fresh white grapes, pressed, filtered and centrifuged in the laboratory. Table 1 summarizes the description of these samples.

Wine and must samples were collected, centrifuged for 15 min at 5000 × *g* and immediately refrigerated until analysis. Each analytical assay was performed at least in duplicate.

2.3. Analysis of free carbohydrates

2.3.1. Derivatization

0.5 mL of sample or 1 mL standard (1 mg mL^{-1} in methanol:water 30:70, v/v) was mixed with 0.125 mL or 1 mL of a 70% ethanolic solution of β-phenyl-D-glucoside (1 mg/mL), which was used as internal standard. After drying the samples under vacuum, 350 μL of 2.5% hydroxylamine chloride in pyridine were added and heated at 75 °C for 30 min. Silylation reaction was carried out with 350 μL of hexamethyldisilazane (HMDS) and 35 μL of trifluoroacetic acid (TFA) at 45 °C for 30 min [18]. Derivatized samples were centrifuged and 1 μL of supernatant was injected into the injection port of the gas chromatograph. While non-reducing carbohydrates gave only one chromatographic peak, reducing carbohydrates presented two isomers corresponding to *syn* and *anti*, or *E* and *Z* isomers.

2.3.2. GC–MS analysis

GC–MS analysis was carried out using a Hewlett-Packard 7890A gas chromatograph coupled to a 5975C quadrupole mass detector operating in electronic impact (EI) mode at 70 eV (both from Agilent, Palo Alto, CA, USA). Analyses were carried out in split mode (1:40) on a 30 m × 0.25 mm i.d. × 0.25 μm film thickness TRB-1 column (Teknokroma, Barcelona, Spain). The oven was heated at 200 °C for 15 min, then programmed to 270 °C at a heating rate of 15 °C min^{-1}, then programmed to 290 °C at 1 °C min^{-1} and held for 30 min. Injector temperature was 300 °C and the transfer line was thermostatised at 280 °C. Helium at ∼1 mL min^{-1} was used as carrier gas. Acquisition was done using a HPChem Station software (Hewlett-Packard, Palo Alto, CA, USA).

Linear retention indices (I^T) were calculated from the retention times of TMSO disaccharides and suitable *n*-alkanes.

Wine disaccharides were identified by comparison of their retention times and experimental spectra with those of standards run in the laboratory under identical operation conditions.

2.3.3. GC-FID analyses

Samples were also injected in a HP 7890A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) with a flame ionization detector (FID) for quantitative purposes using nitrogen as carrier gas and the same column and temperature program described above. Carbohydrate quantitative data were obtained from FID peak areas using the response factor (RF) relative to β-phenyl-D-glucoside (internal standard) calculated over the expected range. In those cases where no standards were available, RF were estimated as 1. Detection (LOD) and quantification limits (LOQ) were 0.84 μg L^{-1} and 2.8 μg L^{-1}, respectively. Reproducibility was evaluated by the quintuplicate analysis of a red wine sample being the relative standard deviation lower than 10% for the target compounds (disaccharides and glyceryl-glycosides).

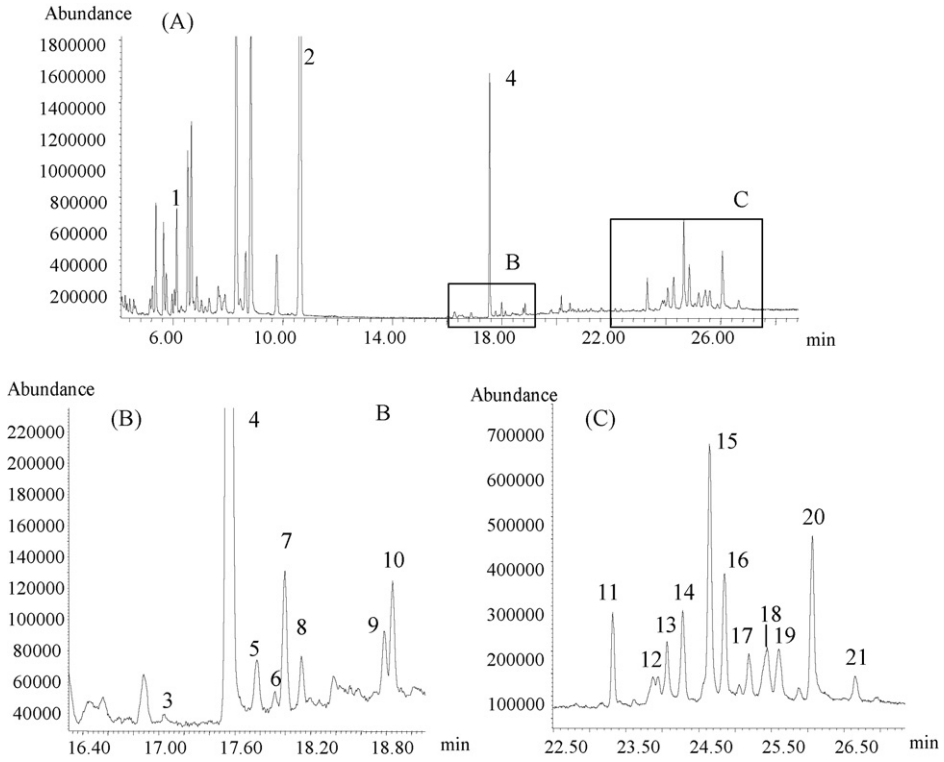

Fig. 1. GC profile of TMSO carbohydrates from a red wine. (1) β-Ethyl-glucoside, (2) *myo*-inositol, (3) β-glyceryl-galactoside 1, (4) β-phenyl-glucoside (internal standard), (5) α-glyceryl-glucoside 1, (6) α-glyceryl-glucoside 2, (7) unknown glyceryl-glycoside, (8) β-glyceryl-galactoside 2, (9) β-glyceryl-glucoside 1, (10) β-glyceryl-glucoside 2, (11) α,α-trehalose, (12) fructose disaccharides + epicatechin, (13) lactose *E*, (14) lactose *Z* + cellobiose *E* + catechin, (15) cellobiose *Z* + laminaribiose *E*, (16) laminaribiose *Z*, (18) unknown disaccharides, (19) sophorose + unknown disaccharides, (20) gentiobiose *E*, and (21) gentiobiose *Z*.

3. Results

3.1. Disaccharides

The chromatographic profile obtained by GC–MS of the free carbohydrates (as TMSO) of a red wine sample is shown in Fig. 1. Monosaccharides, linear polyalcohols, cyclitols, amino acids, carboxylic acids and sugar acids which elute before *myo*-inositol have been already described [19] and they will not be commented upon in this study.

Several peaks eluting between 23 and 27 min were identified as disaccharides (see Fig. 1C). It is noteworthy that the disaccharide profile was very similar in all examined samples. This profile showed an unexpected complexity as a large number of disaccharide peaks was detected. Other wine components such as catechin and epicatechin (peaks 12 and 14, respectively) were also eluted in this zone, although their presence does not interfere in the identification of disaccharides. This resulted in a high and complex background signal which made difficult the identification of minor compounds. Nevertheless, the main disaccharides could be easily identified, due to the coincidence of both I^T and mass spectra with those of standards. Sucrose was not detected in any sample; its absence was clearly established since it eluted in a very clean region of chromatogram (t_R = 22.03 min).

The main disaccharides found in all wine samples were α,α-trehalose, cellobiose, sophorose, laminaribiose, and gentiobiose. Peak 13 displayed the same retention index (I^T = 2846) as lactose (isomer *E*) and a very similar mass spectrum (although not identical); probably the difference was due to the high and variable background that the chromatogram presented in this zone (as described above); thus, lactose identification was not completely confirmed. Bertrand et al. [10] also described tentatively the presence of lactose. Other disaccharides, some of them containing fructose, were characterized by their I^T (2839 and 2842) and mass spectra. Isomaltose, turanose and melibiose tentatively described in wines [10] have not been found in these samples.

To determine the origin of the mentioned disaccharides, a must sample obtained in the laboratory by pressing fresh white grapes was analyzed. Although we have not found previous data about disaccharides in musts, excepting for minute amounts of sucrose, it was worth to check if some disaccharides could be present. The chromatographic profile was different from those of wine: several small peaks corresponding to disaccharides appeared within 21.7 and 26 min (data not shown). They were characterized by their MS patterns as fructose derivatives (especially by fragments at *m*/*z* 217 and 437); among them, only sucrose could be positively identified. A similar profile was found in a commercial bottled grape juice, as well as in a must sample coming from a cellar.

3.2. Other glycosides

A peak appearing at 6.1 min (I^T = 1970) was tentatively assigned as ethyl-glucoside from its mass spectrum (ions at *m*/*z* 147, 204 and 361, Fig. 2a). Since sake has been reported to contain α-ethyl-glucoside [20], a sample of this alcoholic drink was analyzed in the same conditions as wine (Fig. 3). Two peaks with ratio 110:1 appeared with I^T 1912 and 1969 respectively: the former was α-ethyl-glucoside and was absent in wines, whereas the second minor peak displayed the same linear retention index and mass spectrum as peak 1 (Fig. 1A) from wine. It was tentatively assigned as β-ethyl-glucoside.

Up to seven peaks with I^T within 2336 and 2446 and with very similar mass spectra were detected in wines. These spectra were characterized by *m*/*z* fragments at 361 and 337 (see Fig. 2b); the former coming from a glycosylated sugar ring (ion *m*/*z* 451-TMSiOH) and the second including a glycosidic carbon

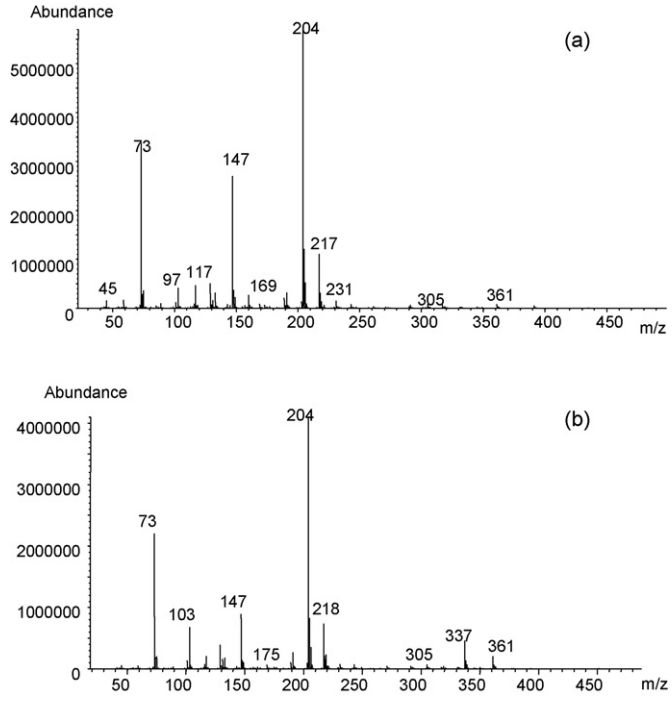

Fig. 2. TMS mass spectrum at 70 eV of (a) ethyl-glucoside and (b) glyceryl-glycoside.

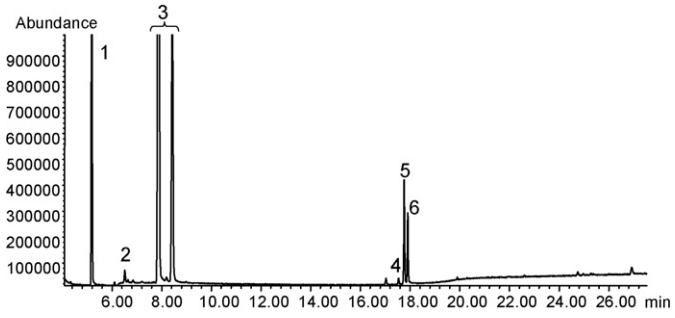

Fig. 3. GC profile of TMSO carbohydrates from a sake sample. (1) α-Ethyl-glucoside, (2) β-ethyl-glucoside, (3) glucose, (4) β-phenyl-glucoside (internal standard), (5) α-glyceryl-glucoside 1, and (6) α-glyceryl-glucoside 2.

and a glycerol molecule (ion *m/z* 191-TMSi + $C_9H_{23}O_2Si_2$). Therefore these peaks were assigned as glyceryl-glycosides.

Different glycosides (α- or β-glyceryl-glucosides or glyceryl-galactosides) were used as standards. Mass spectra of all these compounds were almost identical, thus they must be distinguished by their retention times (Table 2). Peaks found in wine were assigned as α- and β-glyceryl-glucosides and β-glyceryl-galactosides (see Fig. 1B). The main peak appearing in wine with this spectrum (peak 7) was also characterized as glyceryl-glycoside by its mass spectrum but its I^T (2385) was different from the other mentioned above and could not be identified. To the best of our knowledge, this is the first time that all these glycosides are reported in wine.

Table 2
Linear retention indices (I^T) of ethyl- and glyceryl-glycosides found in the analyzed samples.

Sample	Compounds	I^T
Sake	α-Ethyl-glucoside	1912
	β-Ethyl-glucoside	1969
	α-Glyceryl-glucosides	2374, 3281
Lillium	β-Glyceryl-glucosides	2440, 2446
Algae	α-Glyceryl-galactosides	2322, 2365
Lactose solution	β-Glyceryl-galactosides	2336, 2392
Wine	β-Ethyl-glucoside	1969
	α-Glyceryl-glucosides	2374, 3281
	Unknown glyceryl-glycoside	2385
	β-Glyceryl-galactosides	2336, 2392
	β-Glyceryl-glucosides	2440, 2446

Three bottled wines from different origins, as described in Section 2.2 were also analyzed. Chromatographic profiles from these samples including all disaccharide and glycosides were very similar to those described above. Sucrose was not detected in any sample, even in a medium-sweet white sample. The Italian sample (which was elaborated from partially dried grapes) displayed a higher quantity of disaccharides, as well as the red Spanish wine aged in oak. The quercitol peak appearing in these two samples overlapped with the ethyl-glucoside peak. The presence of disaccharides coming from hemicellulose and polyphenol hydrolysis (as rhamnosides and apiosides) was not detected. Such compounds probably were difficult to identify in the background, where they probably overlap with other wine components.

3.3. Quantitative analysis

After identification, 10 samples of cellar wines described in 2.2 were analyzed by GC-FID, using β-phenyl-glucoside as internal standard. Results are summarized in Table 3. Glyceryl-glycosides have been given as the sum of the seven described peaks (only traces were found for the β-glyceryl-galactoside with I^T 2336 in most of the cases). About disaccharides, due to the coelution problems described above, some of them have been given as sums: (fructose disaccharides + epicatechin), (cellobiose + laminaribiose + an unidentified disaccharide + catechin) and a third group of unidentified disaccharides, at least one of them containing fructose.

In general, white wines had less disaccharides (<50 mg/L) than that in rosé and red wines (80–130 mg/L). α,α-Trehalose was very

Table 3
Content (mg/L) of disaccharides and glycosides in young red (5) rosé (2) and white (3) wine samples.

mg/L	Red wines		Rosé wines		White wines	
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
Ethyl-glucoside	5.0	8.7	7.6	9.7	5.6	7.4
Glyceryl-glycosides	7.7	9.1	5.5	7.6	4.0	7.4
α,α-Trehalose	3.4	10.2	18.1	26.1	tr[a]	0.6
Fructose disaccharides + Epicatechin	7.2	9.3	7.7	9.6	3.0	6.3
Lactose	5.2	7.2	4.0	5.3	1.2	3.0
Cellobiose + Laminaribiose + Catechin + Unknown	24.1	31.9	28.3	34.1	5.9	11.6
Sophorose	8.3	12.6	13.6	17.0	4.5	8.5
Gentiobiose	13.8	18.4	14.2	16.1	3.8	7.1
Unknown disaccharides	9.1	15.2	14.6	17.9	5.8	13.2

[a] tr: traces.

low in whites and the highest levels appeared in rosé wines, whereas gentiobiose was the main disaccharide found in red wines.

4. Discussion

The presence of gentiobiose in wines, tentatively reported by Bertrand et al. [10], is confirmed in this work, whereas cellobiose, sophorose and laminaribiose are reported by first time in wine. Moreover, α,α-trehalose previously detected [10] in wines has been also detected here. Apart from this last disaccharide, which is a well-known metabolite of yeasts, the majority of these disaccharides and also ethyl- and glyceryl-glycosides presented a β-glycosidic linkage: sophorose, laminaribiose, cellobiose and gentiobiose are β-glucosyl-glucoses substituted in 2, 3, 4 and 6 position respectively. This seems to indicate that they have been formed from glucose by transglycosidation action of a β-glucosidase. β-Ethyl-glucoside and β-glyceryl-glucosides would be formed similarly from glucose and ethanol or glucose and glycerol respectively (both alcohols are major components of wine). Similarly, several β-galactosyl derivatives (lactose and glyceryl-galactosides) could be due to the action of a β-galactosidase.

The differences described in Section 3.1 among disaccharides from must (fructose containing disaccharides) and those of wine (mainly glucose containing disaccharides) suggest that the formation of the last may be attributed to enzymatic activities acting in winemaking instead of grape contribution, probably from wine microorganisms, mainly yeasts and lactic acid bacteria involved in alcoholic and malolactic fermentation, respectively.

Similarity of GC profiles obtained from very different wines (see Table 1) may indicate that these disaccharides are not depending on grape variety. On the contrary, wine contains several sources of glycosidases: a grape endoglycosidase capable of hydrolysing monoterpene disaccharide glycosides [21] may explain a high diversity in the disaccharide structures identified in wines, since this enzyme operates by cleaving the aglycone moiety by a single step mechanism. However, this enzymatic activity during winemaking still needs to be clarified. Additionally, wine microorganisms include yeasts (*S. cerevisiae* and non-*Saccharomyces* species) and lactic acid bacteria which possess β-glucosidases [22] suggesting the formation of disaccharides during fermentation.

The possibility of some transglycosidation by acid reversion could also be considered, but this reaction is not specific and should give both β and α glycosides from glucose and fructose; in addition, this reaction is difficult in solutions as diluted as wine is.

Further work could be done in future to develop an accurate GC method to quantitatively determine disaccharide composition of a high number of both white and red wines.

5. Conclusions

Several disaccharides (cellobiose, sophorose, laminaribiose) and glyceryl-glycosides, as well as ethyl-glucoside have been identified in wines for the first time. Presence of other disaccharides previously detected by other authors such as gentiobiose, α,α-trehalose and lactose has also been confirmed. Most of these compounds are β-linked carbohydrates which seem to indicate that they have been formed by transglycosidation action of a β-glucosidase from glucose or a β-galactosidase from galactose. On the contrary, disaccharides in grape juice were mainly constituted by fructose derivatives and no glycosides were detected.

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