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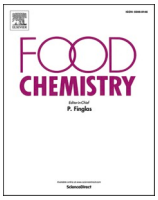

Comparative study of different commercial enzymes on release of glycosylated volatile compounds in white grapes using SPE/GC–MS

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ABSTRACT

Glycosides represent a large source of potential flavor in grape must. Commercial preparations enzymes with glycosidase activity are commonly employed to enhance wine aroma. In this study, we conducted an evaluation of twelve commercial enzymes to assess their effectiveness in releasing volatile compounds from their conjugated forms in a white grape must under laboratory conditions by solid-liquid extraction (SPE) and gas chromatography–mass spectrometry (GC–MS). In this laboratory-level experiment, regardless of the enzymes used, the total concentration of volatile compounds was not statistically affected by the treatments. While the total concentration of volatile compounds remained largely unchanged, four specific volatile groups were significantly affected by the enzyme treatments: acids, alcohols, C_{13}-norisoprenoids, and terpenes. The results also revealed a significant effect of commercial enzymes on individual compounds, which led to a notable increase in the concentration of twenty-one aroma compounds, mainly terpenes. Rapidase Revelation Aroma and Enozym Extra Aroma emerged as the most powerful ones on the must's volatile composition with important ability to release higher concentrations of essential varietal aroma compounds, particularly terpenes and C_{13}-norisoprenoids.

1. Introduction

The wine industry has experienced a substantial growth in the demand for wines with unique and complex aromatic profiles. Aromatic compounds play a pivotal role in the sensory perception and overall quality of wines, by shaping its sensory characteristics (Li et al., 2023).

Currently, more than 1000 volatile compounds have been identified in grapes and wines (Pons et al., 2017; Šikuten et al., 2020), and have been classified into various classes such as acids, alcohols, C6- compounds, C_{13}-norisoprenoids, aldehydes, lactones, terpenes, esters, volatile phenols and many others, each contributing to the overall aroma characteristics of the winem (Dziadas & Jeleń, 2016; Liu et al., 2017). All these compounds are primarily concentrated in the skin and seeds of grapes (Claus & Mojsov, 2018).

Glycoside compounds in grape wines are typically bound to β-D-glucopyranose. In the case of diglycosides, glucose can be bound to other molecules like malonic acid, arabinose, apiofuranose, or rhamnose (Ferreira & Lopez, 2019; Liu et al., 2017; Sarry & Günata, 2004). These odorless compounds, which make up around 90 % of the total precursor concentration, contain aroma and flavor aglycones (Claus & Mojsov, 2018; Dziadas & Jeleń, 2016) and play a crucial role in defining the sensory and varietal attributes of wine (Hjelmeland & Ebeler, 2015) upon the release of free aglycones from glycosides through hydrolysis (Arévalo Villena et al., 2006). The liberation can occur via acid-catalyzed hydrolysis, resulting from the acidic nature of grape must, occurs throughout the winemaking process and participates in the release of bound-aromas (Liu et al., 2017; López et al., 2004) or the activity of endogenous β-glycosidase enzymes (Botelho et al., 2007), that release the associated aromatic compounds in their free form.

However, the acid hydrolysis of non-volatile glycosides is a slow process during winemaking process, and if accelerated through heat, it can negatively impact the quality of the wine (Sefton, 1998). Moreover, the enzymatic hydrolysis reactions performed by glycosidases present in grapes and yeast are less active under fermentative conditions (Günata et al., 1985).

To sort out this limitation, therefore, the use of exogenous glycosidase enzymes has emerged as an innovative and effective strategy to enhance the release of aromatic compounds in grape musts, due to the reactivity of the liberated aglycones in the wine's pH environment (Günata et al., 1988). Also, the use of enzymes can broaden the diversity

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of aromas, providing winemakers with greater versatility in creating wines with unique and distinctive profiles in a scenario of climate change (González Barreiro et al., 2015).

In recent years, there has been a growing interest in utilizing glycosidases to enhance the aromatic profile of white wines (Espejo, 2021). Research studies have demonstrated that the addition of enzyme preparations in white grape varieties leads to an increase in the content of volatile terpene compounds (Rusjan et al., 2009; Rusjan et al., 2012), C_{13}-norisoprenoids (Armada et al., 2010), and ethyl esters (Masino et al., 2008) in wines. However, it is important to note that the impact of enzyme treatment may vary depending on the specific grape variety used. Fia et al. (2016) observed significant alterations in the volatile composition of wine when treating Trebbiano grapes with enzymes. Conversely, Rocha et al. (2005) evaluated the effect of a commercial enzyme preparation (Lallzyme) containing β-glucosidase, pectinase, arabinosidase, and rhamnosidase on white wines made from two Portuguese grape varieties, which yielded different results. The wines produced from the Maria Gomes variety with enzyme treatment exhibited a 9 % increase in total volatile compounds, attributed to elevated levels of monoterpenoids, terpenoids, and aromatic alcohols. However, the composition of wines made from the Bical variety remained unaffected by the enzymatic treatment (Rocha et al., 2005).

Several works report analytical methods for the determination of glycosylated fraction of aroma in grapes. In most of these works, the enzyme Rapidase Revelation Aroma (AR2000) is used to release glycosidically-bound volatiles (Fundira, 2002; Vilanova & Sieiro, 2006; Kang et al., 2012; Ghaste et al., 2015; Wang et al., 1015; Tavernini et al., 2020; Oller-Ruiz et al., 2022). Some comparative studies showed the effect of different commercial enzymes to release glycosylated aroma forms in different cultivars (Fundira, 2002; Armada et al., 2010; Rodríguez-Nogales et al., 2024; Rio Segade et al. 2024) showing different behaviors in basis to the cultivar used.

Despite these findings, there is still limited knowledge regarding the impact of new commercial enzymes on the volatile composition derived from glycosylated compounds in white grape varieties. Understanding and effectively managing the extraction and release of these precursors are essential to know the cultivar aromatic potential.

In this context, the current research explored the effect of twelve different oenological commercial enzymes on the effectiveness of aroma glycoside release by SPE/GC–MS on blended must derived from four aromatic white cultivars (Albariño, Loureira, Treixadura, and Godello) grown in Galicia (NW Spain). In this work a blended of white Galician cultivars were used because they are very aromatic cultivars due to terpenes and C_{13}-norisoprenoids which are responsible for floral and fruity aromas (Falqué et al., 2001; Genisheva & Oliveira, 2009; Losada et al., 2011; Oliveira et al., 2008; Vilanova et al., 2013; Vilanova et al., 2017; Vilanova et al., 2019). Moreover, the blended wines made from white cultivars from Galicia, especially those with Loureira, showed an increase in terpenes and C_{13}-norisoprenoids, improvement the complexity of the wine aroma (Vilanova et al., 2017).

2. Materials and methods

2.1. Grape samples

To evaluate the impact of 12 commercial enzymes on the extraction of glycosidic bound fraction of volatile compounds in grape must by SPE/GC–MS, a blended must from four aromatic white cultivars commonly used in winemaking in Galicia were used. Aromatic white must was composed as a blend of several grape cultivars as follow: Albariño (36 %), Loureira (12 %), Treixadura (12 %) and Godello (40 %). The musts, all from 2022 harvest, were obtained from a commercial winery from AOC Rías Baixas sited in Galicia (Spain). The must chemical parameters were as follow: sugars content (222.3 g/L), pH (3.2), total acidity (5.03 g/L), tartaric acids (2.5 g/L) and malic acid (3 g/L) and yeast assimilated nitrogen (121.0 mg YAN/L).

2.2. Commercial enzymes preparations

To investigate the impact of enzyme extraction on the volatile composition of must, a study was conducted using twelve different commercial enzymes commonly employed in the winemaking process. The commercial enzyme preparation usually consists of blends of different activities, thus the enzymes studied showed in Table 1 were: E1-Rapidase Revelation Aroma (DSM, Montpellier, France); E2-Lallzyme Beta (Lallemand, Aurillac, France); E3 Lallzyme Cuvee Blanc (Lallemand, Aurillac, France); E4-Endozym B-Split (AEB, Barcelona, Spain); E5-Enovin Varietal (Agrovin, Ciudad Real, Spain); E6-Enozym FW (Lamothe-Abiet, Bordeaux, France); E7-Lafazym Arom (Lafford, Bordeaux, France); E8-Rivela (Enartis, San Marino, Italy); E9-Enozym Extra Arome (Agrovin, Ciudad Real, Spain); E10-Trenolin Mash DF (Erbsöh (Geisenheim, Germany); E11-Endozym ICS 10 Arome (AEB, Barcelona, Spain); E12-Trenolin Bouquet (Erbsöh (Geisenheim, Germany).

2.3. Analysis of volatile composition by gas chromatography coupled to mass spectrometry (GC–MS)

2.3.1. Extraction of volatiles from must in free and bound fractions

The extraction of the volatile compounds present in the free and glycosidically-bound fractions of the musts was carried out according to the method described by Oliveira et al. (2008), with some modifications (Vilanova et al., 2019). Aromatic white must, composed by Albariño (36 %), Loureira (12 %), Treixadura (12 %), and Godello (40 %), was centrifuged (5000 rpm / 20 min / 4 °C) and filtered with glass wool. To 75 mL of the centrifuged sample, 3 μg of 4-nonanol was added as an internal standard. A solid phase extraction was performed using SPE columns Chromabond 500 mg (Macherey Nagel). The cartridge was preconditioned with 10 mL dichloromethane, 5 mL methanol, and 10 mL alcohol solution (10 %, *v*/v). The free and glycosidically-bound fractions were successively eluted with 5 mL of azeotropic pentane-dichloromethane solution (2:1, v/v, pH = 5.0) and 7 mL of ethyl acetate, respectively.

Prior to analysis, the free fraction was concentrated to 200 μL by

Table 1
Commercial enzymes applied to bended of Galicia white grape must.

Code	Enzyme	Enzymatic Activity	Commercial	Country
E1	Rapidase Revelation Arome	Pectinase, α- and β-glycosidase	DSM	Montpellier, France
E2	Lallzyme Beta	Pectinases, β-glucosidase, Rhamnosidase, Apiosidase and Arabinofuranosidase	Lallemand	Aurillac, France
E3	Lallzyme Cuvee Blanc	Pectinases and Glycosidase	Lallemand	Aurillac, France
E4	Endozym B-Split	β-glucosidase	AEB	Barcelona, Spain
E5	Enovin Varietal	β-glucosidase	Agrovin	Ciudad Real, Spain
E6	Enozym FW	Glycosidase	Lamothe-Abiet	Bordeaux, France
E7	Lafazym Arom	ß-glucosidase and Pectinase	Laffort	Bordeaux, France
E8	Rivela	Glycosidase, Pectolytic and Hemicellulase	Enartis	San Martino, Italy
E9	Enozym Extra Aroma	β-glycosidase	Agrovin	Ciudad Real, Spain
E10	Trenolin Mash DF	Pectinase and ß-glucosidase	Erbslöh	Geisenheim, Germany
E11	Endozym ICS 10 Arome	β-glucosidase	AEB	Barcelona, Spain
E12	Trenolin Bouquet	ß-glycosidase	Erbslöh	Geisenheim, Germany

evaporating of the solvent using nitrogen. On the other hand, the bound fraction was concentrated to dryness (40 °C, 30 rpm) in a Buchi Multivapor™ (Flawil, Switzerland) and dissolved in 100 μL of 0.1 M citrate-phosphate buffer (pH = 5.0). Fourteen milligrams of each commercial enzyme were added to the glycoside extract, and the mixture was incubated at 40 °C for 18 h. The released aglycons were extracted with azeotrope (pentane-dichloromethane), after the addition of 3 μg of 4-nonanol as internal standard. The organic phase was then concentrated to 200 μL with nitrogen. All extractions were done in triplicate.

2.3.2. Chromatograph analysis

Volatile composition analysis was performed using an Agilent 7890 gas chromatograph (GC) coupled to an Agilent 7000C triple quadrupole mass spectrometer. Samples were injected in split less mode using a volume of 1.5 μL. Chromatographic separation was performed on a DB-WAX ultra-inert (30 m 0.25 mm i.d., 0.25 μm film thickness, Agilent). The injector temperature was 250 °C. The oven temperature was maintained at 60 °C for 2 min, then programmed to rise from 60 °C to 234 °C with a gradient of 3 °C/min and then with a gradient of 5 °C/min to 250 °C and finally programmed 10 min at 250 °C. The carrier gas is helium N60 (Air Liquide), flow at 1 mL/min. The detector is set to electronic impact mode (70 eV), with an acquisition range from 29 *m/z* to 360 *m/z*, and an acquisition rate of 610 ms. Identification was carried out with Mass Hunter Qualitative Analysis software (Agilent) using the NIST library and by comparison with the mass spectra and retention index of chromatographic standards, and data found in the literature. Quantification of volatiles compounds in terms of 4-nonanol was performed with Mass Hunter Quantitative Analysis software (Agilent). The compounds were quantified in terms of 4-nonanol equivalents by comparing the GC retention times Standard compounds 97 % were purchased from Sigma-Aldrich.

We provide information of volatile compounds identified in the Supplementary Material S1.

2.4. Statistical analysis

All data were analysed using the XLSTAT-Pro statistical package (Addinsoft, Paris, France). A one-way ANOVA was used to evaluate the differences among treatments after testing their normality and variance's homoscedasticity. The multiple comparison among enzymes were calculated according to the least significant difference from Tukey's test with a confidence interval of 95 % ($p < 0.05$). The results were presented as the mean of triple measurements. Also, a heat map was generated to assess differences in the statistically significant volatile compounds. Finally, principal component analysis (PCA) was used on chemical groups of wine volatile composition to discriminate among different glycosidic enzymes used.

3. Results and discussion

3.1. Free volatile composition in blended must from white grapes

The identification and quantification of must volatile composition, including both free and glycosidically-bound compounds, are crucial for characterizing the aromatic profile of the grape must. Table 2 shows the total concentration (μg/L) and percentage (%) of free volatile compounds, categorized into several families, such as acids, alcohols, C6-compounds, aldehydes, carbonyl compounds, lactones and terpenes which were detected in the free fraction of the must before any treatments were applied. It is worth noting that C_{13}-norisoprenoids, esters, and volatile phenols were not found in the free fraction of the must before treatment, likely due to their glycosylated state.

Must volatile composition revealed interesting insights into the abundance and diversity of aromatic compounds present in their free forms before any treatments. Among the free families analysed, C6-compounds exhibited the highest concentration, measuring 887.8 μg/L, making up a significant 87.2 % of the total identified volatile compounds quantified. Following closely were aldehydes with 39.8 μg/L (3.9 %), acids with 35.2 μg/L (3.5 %), and alcohols with 29.3 μg/L (2.9 %). Terpenes, although present in lower quantities at 21.1 μg/L, still contributed 2.1 % to the total free volatile concentration. Lactones and Carbonyl compounds were detected at lower levels, with 3.7 μg/L (0.4 %) and 0.7 μg/L (0.1 %), respectively.

Table 2
Concentration (μg/L) of free volatile compounds grouped by chemical groups in the must previous treatments.

Free Volatile Compounds	Mean values μg/L	%
Volatile Acids	35.2	3.5
Alcohols	29.3	2.9
C_6-Compounds	887.8	87.2
Aldehydes	39.8	3.9
C_{13}-norisoprenoids	nd	nd
Esters	nd	nd
Carbonilic Compounds	0.7	0.1
Lactones	3.7	0.4
Terpenes	21.1	2.1
Volatile phenols	nd	nd
Total concentration	**1017.6**	**100**

In total, 16 free volatile compounds were identified in the must. Among these, two acids (hexadecanoic acid, octadecanoic acid), three alcohols (1-butanol, benzyl alcohol, phenylethyl alcohol), five C6-compounds (1-hexanol, trans-3-hexen-1-ol, cis-3-hexen-1-ol, trans-2-hexen-1-ol, cis-2-hexen-1-ol), three aldehydes (benzaldehyde, phenylethanal, 2,5-dimethyl benzaldehyde), one carbonyl compound (acetoin), one lactone (γ-butyrolactone), and one terpene (linalool) were identified.

3.2. Effect of commercial enzymes on glycosylated groups release in grape must

In this laboratory-level experiment, regardless of the enzymes used, the total concentration of volatile compounds released was not statistically affected by the treatments, as shown in Table 3. However, Enozym Extra Aroma, Lallzyme Cuvee Blanc, Endozym β-Split, and Rapidase Revelation Aroma exhibited a promising trend towards producing positive effects on the volatile composition of white blended must. While the total concentration of volatile compounds remained largely unchanged, four specific volatile groups were significantly affected by the enzyme treatments: acids, alcohols, C_{13}-norisoprenoids, and terpenes. Among these groups, acids were the most dominant compounds, showing the highest mean concentration in the must, accounting for 46.4 % of the total volatiles. Alcohols followed next, contributing to 19.4 % of the volatile composition, while C_{13}-norisoprenoids and terpenes represented 2.4 % and 4.7 %, respectively. The remaining families of volatile compounds were unaffected by the enzyme treatments.

When Enozym Extra Aroma was applied to the must extract, a significant increase in total acids, alcohols and C_{13}-norisoprenoids was observed, suggesting a positive impact on these chemical groups' liberation in the must. Terpenes showed the significant higher concentration when Rapidase Revelation Aroma was applied. In contrast, a significant decrease of these chemical groups' concentration was observed when Enozym FW and Lafazym Arom was used. The application of Rapidase Revelation Aroma (AR 2000) to Marula cultivar resulted in a 90 % increase in terpenes (Fundira et al., 2002). Other authors showed the increase of levels of terpene concentration by effect of different enzyme on different cultivars during wine production, such as Emir (Cabaroglu et al., 2003), Maria Gomez (Rocha et al., 2005), Perła Zali and Nachodka (Dziadas & Jeleń, 2011) or Chardonnay, Arneis, Greco and Falanghina (Rio-Segade et al., 2024).

The aroma of white wines plays an important role in defining their overall quality and expressing their unique varietal character (Pérez

Table 3
Volatile composition (μg/L) of blended must from white varieties released with 12 different commercial enzymes.

Volatile Compounds	Concentration (μg/L)												
	Rapidase Revelation Aroma	Lallzyme β	Lallzyme Cuvee Blanc	Endozym β-Split	Enovin Varietal	Enozym Extra Aroma	Trenolin Bouquet	Trenolin Mash DF	Endozym ICS 10 Arome	Enozym FW	Lafazym Arom	Rivela	Sig.
Acids													
Hexanoic acid	17.0	11.4	17.1	18.4	16.2	15.1	13.7	19.0	12.4	15.1	15.1	18.2	ns
2-Hexenoic acid	10.0	7.5	12.4	12.9	10.9	12.7	9.4	11.7	11.0	12.0	12.1	11.9	ns
Octanoic acid	0.6 **bc**	0.60 **c**	1.0 **abc**	1.2 **abc**	1.0 **abc**	1.2 **abc**	1.0 **abc**	1.5 **a**	1.2 **abc**	1.5 **a**	1.3 **ab**	1.3 **ab**	**
Nonanoic acid	0.90 **c**	1.1 **bc**	2.2 **a**	1.9 **ab**	1.8 **abc**	2.0 **ab**	1.8 **abc**	2.6 **a**	2.3 **a**	2.3 **a**	1.9 **ab**	2.5 **a**	***
trans-Geranic acid	3.2	2.3	3.7	3.9	2.5	5.2	3.4	2.9	3.2	2.4	2.3	3.7	ns
Hexadecanoic acid	349.7 **bcd**	421.6 **ab**	395.3 **bc**	370.1 **bcd**	400.7 **abc**	509.4 **a**	301.6 **cd**	374.4 **bcd**	301.1 **cd**	285.1 **cd**	297.8 **cd**	267.4 **d**	***
Octadecanoic acid	38.6	29.7	60.0	54.0	47.1	62.7	34.9	44.0	47.4	47.2	36.8	42.9	ns
Alcohols													
1-Butanol	4.9 **a**	4.3 **ab**	3.2 **abc**	4.5 **ab**	3.2 **abc**	5.2 **a**	4.7 **ab**	2.1 **abc**	3.0 **abc**	0.8 **c**	1.3 **bc**	2.9 **abc**	*
Isoamyl alcohol	4.6 **a**	3.7 **ab**	3.1 **abc**	3.6 **ab**	2.8 **abc**	4.3 **a**	3.7 **ab**	2.7b **abc**	2.7 **abc**	1.0 **c**	1.3 **bc**	3.0 **abc**	*
Furfuryl alcohol	0.8	1.1	4.4	3.7	0.6	5.9	3.3	5.7	0.4	0.4	0.4	0.4	ns
Benzyl alcohol	125.6 **ab**	101.6 **abc**	103.9 **abc**	95.7 **abc**	77.5 **abcd**	139.8 **a**	91.8 **abc**	68.3 **bcd**	68.7 **bcd**	22.4 **d**	38.8 **cd**	55.6 **cd**	***
Phenylethyl alcohol	120.2	84.2	119.0	104.4	88.9	123.2	92.6	96.8	80.3	48.5	64.3	70.5	ns
2-Phenoxyethanol	2.3	1.4	2.3	2.1	1.9	2.5	1.5	1.6	2.0	1.5	1.6	1.5	ns
C6-Compounds													
1-Hexanol	23.1	7.6	16.1	19.4	25.3	10.2	15.9	25.6	5.9	5.1	6.7	26.5	ns
trans-3-Hexen-1-ol	0.5	nd	0.3	0.4	0.5	0.2	nd	nd	nd	nd	nd	0.5	ns
cis-3-Hexen-1-ol	1.9	1.1	1.6	1.6	1.6	1.5	1.5	1.8	1.2	0.6	1.0	1.8	ns
trans-2-Hexen-1-ol	13.2	3.2	9.0	11.9	16.2	4.6	9.9	17.3	2.6	3.2	3.7	15.4	ns
Aldehydes													
Benzaldehyde	5.1 **ab**	5.1 **ab**	5.0 **ab**	4.6 **ab**	4.0 **abc**	6.3 **a**	4.7 **ab**	3.7 **abc**	3.3 **bc**	1.9 **c**	2.8 **bc**	4.2 **abc**	**
Phenylethanal	1.2	1.1	10.5	13.9	1.0	9.5	3.1	1.8	2.2	9.0	3.6	3.3	ns
2,5-Dimethyl benzaldehyde	1.9	1.3	1.4	1.3	0.8	1.0	1.5	1.9	0.5	0.7	0.4	2.3	ns
C_{13}-norisoprenoids													
3-Hydroxy-β-damascone	15.1 **ab**	14.5 **ab**	nd	nd	nd	15.2 **ab**	17.7 **a**	nd	6.4 **bcd**	1.6 **cd**	3.7 **cd**	10.4 **abc**	***
3-Oxo-α-ionol	16.5 **abc**	16.1 **abc**	13.5 **abc**	11.1 **abc**	8.1 **bc**	23.3 **a**	20.3 **ab**	5.6 **c**	6.5 **c**	2.9 **c**	3.9 **c**	12.6 **abc**	**
3-Hydroxy-7,8-dihydro-β-ionol	5.9 **ab**	4.5 **ab**	3.6 **ab**	3.2 **ab**	2.4 **b**	7.5 **a**	4.7 **ab**	3.5 **ab**	4.7 **ab**	2.5 **b**	3.6 **ab**	4.8 **ab**	*
Esteres													
Methyl salicylate	2.8 **a**	1.1 **ab**	1.1 **ab**	0.8 **ab**	1.4 **ab**	1.5 **ab**	1.3 **ab**	0.5 **ab**	0.6 **ab**	0.3 **b**	0.6 **ab**	0.8 **ab**	*
Diethyl malate	0.4	0.3	0.4	0.4	0.2	0.4	0.3	0.4	0.5	0.4	0.3	0.3	ns
Ethyl palmitate	2.7	2.1	15.8	19.5	3.9	14.5	8.7	5.8	0.5	0.9	0.3	nd	ns
Methyl vanillate	11.3	7.4	10.3	11.7	8.4	14.1	9.7	7.4	9.7	6.0	5.0	10.9	ns
Methyl gentisate	62.0	41.9	56.9	55.6	44.1	68.5	49.1	40.9	44.7	39.0	34.9	46.1	ns
Terpenes													
cis-furan linalool oxide	10.5 **a**	3.6 **ab**	3.2 **ab**	6.3 **ab**	2.7 **b**	7.4 **ab**	6.5 **ab**	1.6 **b**	2.7 **b**	2.5 **b**	2.1 **b**	2.0 **b**	**
trans-furan linalool oxide	6.8 **a**	1.9 **bc**	1.4 **bc**	1.3 **bc**	0.8 **c**	1.8 **bc**	3.8 **b**	0.6 **c**	0.9 **c**	1.0 **c**	0.8 **c**	0.6 **c**	***

(*continued on next page*)

Table 3 (*continued*)

Volatile Compounds	Concentration (μg/L)											
	Rapidase Revelation Aroma	Lallzyme β	Lallzyme Cuvee Blanc	Endozym β-Split	Enovin Varietal	Enozym Extra Aroma	Trenolin Bouquet	Trenolin Mash DF	Endozym ICS 10 Arome	Enozym FW	Lafazym Arom	Rivela	Sig.
Linalool	10.5 **a**	4.9 **ab**	5.3 **ab**	4.5 **ab**	3.4 **ab**	4.9 **ab**	6.4 **ab**	2.2 **b**	1.9 **b**	1.8 **b**	1.7 **b**	6.7 **ab**	*
Hotrienol	8.4 **a**	3.2 **b**	5.0 **ab**	4.2 **ab**	3.3 **b**	5.0 **ab**	5.7 **ab**	2.4 **b**	4.1 **ab**	4.3 **ab**	3.9 **ab**	3.7 **ab**	*
α-terpineol	4.7	2.4	3.7	4.5	3.3	3.2	5.5	9.8	7.3	2.1	3.3	2.4	ns
trans-pyran linalool oxide	7.8	4.0	4.5	5.7	5.0	6.5	5.7	4.3	4.9	4.0	4.5	3.7	ns
cis-pyran linalool oxide	2.0 **a**	0.8 **b**	0.9 **b**	1.0 **b**	0.9 **b**	1.3 **ab**	1.2 **ab**	0.9 **b**	0.9 **b**	0.9 **b**	0.9 **b**	0.8 **b**	*
Nerol	0.7 **ab**	0.6 **ab**	0.7 **ab**	0.2 **b**	0.2 **b**	0.9 **a**	0.4 **ab**	0.2 **b**	0.4 **ab**	0.3 **b**	0.4 **ab**	0.6 **ab**	**
Geraniol	2.8 **abc**	3.0 **abc**	3.1 **abc**	1.3 **bc**	1.0 **c**	4.5 **a**	1.6 **bc**	1.3 **bc**	2.3 **abc**	1.7 **bc**	2.8 **abc**	3.9 **ab**	**
HO-Diendiol I	11.9	14.8	12.9	13.4	11.9	16.4	10.1	13.8	8.2	10.0	7.9	13.2	ns
trans-8-Hydroxylinalool	5.1 **a**	4.7 **ab**	6.2 **a**	5.2 **a**	3.4 **ab**	6.8 **a**	3.8 **ab**	4.0 **ab**	4.8 **ab**	0.0 **b**	2.6 **ab**	0.0 **b**	**
Volatile Phenols													
Eugenol	3.7 **a**	2.3 **abc**	3.2 **a**	3.0 **ab**	2.3 **abc**	3.6 **a**	2.6 **abc**	1.9 **abc**	1.6 **abc**	0.7 **c**	0.9 **bc**	1.8 **abc**	**
2-Methoxy-4-vinylphenol	25.1	14.9	82.1	86.7	25.6	77.1	62.6	63.2	47.7	96.4	55.0	66.5	ns
4-Vinylphenol	20.2	11.8	129.8	163.7	34.2	90.5	58.3	92.6	60.0	176.7	85.0	58.6	ns
3,4,5-Trimethoxyphenol	10.7 **ab**	8.6 **ab**	10.6 **ab**	9.5 **ab**	6.7 **ab**	12.6 **a**	6.8 **ab**	4.1 **b**	7.4 **ab**	3.7 **b**	4.6 **b**	8.2 **ab**	**

Signification: *, **, *** and ns indicate a significant difference among enzymes at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively. The different letters indicate significant differences among enzymes for each volatile compound by Tukey's test ($p < 0.05$).

et al., 2022). Among the wide range of aroma compounds found in white wines, terpenes and C_{13}-norisoprenoids stand out as the key contributors to their characteristic aroma (Vilanova & Sieiro, 2006). Despite being present in lower proportions compared to other compounds, terpenes have a profound impact on the sensory experience of white wines because their low threshold.

Principal Component analysis (PCA) was applied to understand the effect of different commercial enzymes activity on release of glycosylated chemical groups responsible of white grape must aroma (Fig. 1). This multivariate analysis helped in interpreting the data and identifying the families of volatile compounds that best discriminate among the enzymes used in the study. The first two principal components (PC1 and PC2) accounted for 75.61 % of the total variance (49.54 % and 26.07 %, respectively). PC1 was mostly correlated with esters, acids, alcohols, terpenes and C_{13}-norisoprenoids, while PC2 was mostly correlated with aldehydes and volatile phenols. The enzymes distribution according the two components show the increase of terpenes and C_{13}-norisoprenoide when Rapidase Revelation of Aroma was applied. Acids and esters increased their concentration when Enozym Extra Aroma was used, however Endozym β-split and Lallyzyme Cuvee Blanc showed a hug influence on Aldehydes release. Finally, Trenolin Bouquet and Lallzyme Beta was correlated with C6-compounds release.

In the context of this study, the conditions employed led to a significant increase in free-volatile compounds, particularly terpenes and C_{13}-norisoprenoids, in the white grape must treated with Rapidase Revelation Aroma. The rise in terpenes and C_{13}-norisoprenoids concentrations contributes to the aromatic complexity and varietal character of the wine. By utilizing Rapidase Revelation Aroma, it can effectively release and amplify these aroma compounds, resulting in white wines with more pronounced floral and fruity notes, thereby suggesting a good candidate to be used for improving white wines quality.

Indeed, it is evident that Enozym Extra Aroma emerged as the enzyme that released the most substantial amounts of volatile compounds compared to the other enzymes used in the study. Notably, Enozym Extra Aroma led to a significant increase in the concentration of acid and ester compounds. Esters are very important in contributing to the fruity and floral aromas in wine, imparting notes of tropical fruits, citrus, pears, apples, and flowers (Iobbi et al., 2023). This enhancement of aromatic complexity and intensity contributes to the overall appeal of the wine. In addition to esters, acids are another important component that significantly influences the sensory experience of white wines. The presence of acids imparts a sensation of freshness, creating a harmonious balance that helps counterbalance any natural sweetness from the fruit or residual sugar in the wine (Chidi et al., 2018).

3.3. Effect of enzyme treatment on individual glycosylated compounds release in grape must

Table 4 exhibited the effects of 12 commercial enzymes on the glycosylated fraction of must volatile composition, showing the individual aroma compounds identified. A total of 43 aroma compounds, belonging to several chemical groups such as acids, alcohols, C6-compounds, aldehydes, C_{13}-norisoprenoids, esters, terpenes, and volatile phenols, were detected and quantified. The results from the one-way ANOVA and Tukey test ($\alpha = 0.05$) revealed that the different commercial enzymes significantly influenced to 21 aroma compounds, accounting for 48.8 % of the total compounds identified. This emphasizes the crucial role these enzymes play in shaping the volatile profile of the mixed white varieties must.

In the acid's family, seven compounds were identified, but only three showed significant differences (octanoic acid, nonanoic acid, and hexadecanoic acid) among the enzymes. Trenolin Mash DF and Enozym FW enzymes recorded the highest values for octanoic acid, but only significantly different to Rapidase Revelation Aroma and Lallzyme β. The same enzymes, along with Lallzyme Cuvee Blanc, Endozym ICS10

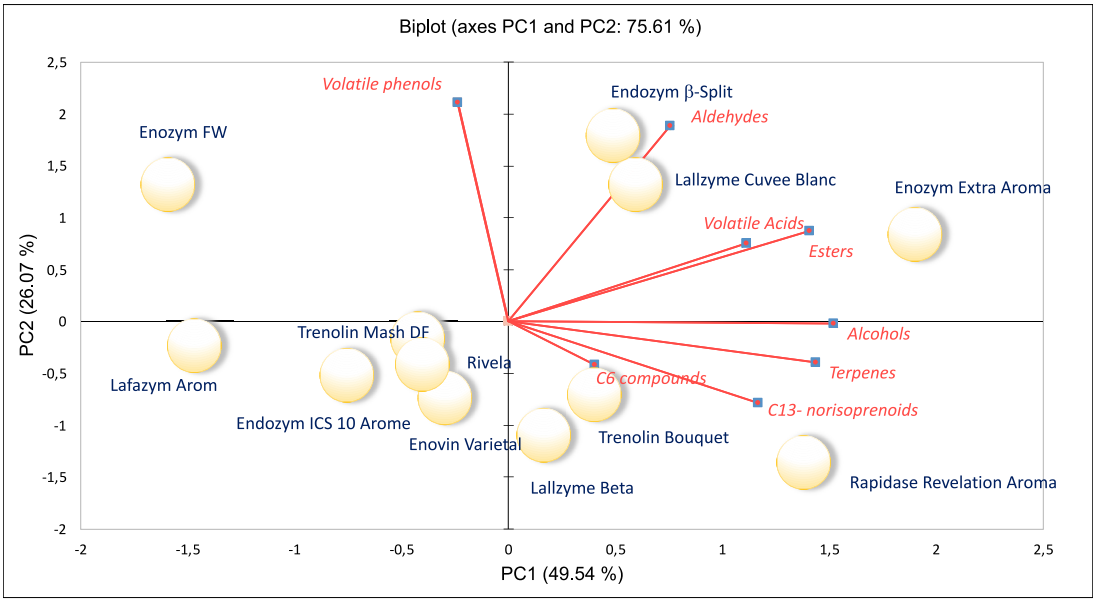

Fig. 1. Principal Component Analysis (PCA) applied on chemical groups of volatile of grape must treated with 12 commercial enzymes.

Arome and Rivela exhibited the highest values for nonanoic acid and also significant different to Rapidase Revelation Aroma and Lallzyme β. Hexadecanoic acid reached the highest value by effect of Enozym Extra Aroma. Conversely, Lallzyme β, Rapidase Revelation Aroma and Rivela demonstrated the lowest concentrations of octanoic acid, nonanoic acid, and hexadecanoic acid, respectively. The other enzymes exhibited intermediate behaviour within this family.

For the alcohols, 1-butanol, isoamyl alcohol, and benzyl alcohol were found to be significantly influenced by the enzymes used. Rapidase Revelation Aroma, along with Enozym Extra Aroma, displayed the highest concentration of 1-butanol and isoamyl alcohol, while Enozym Extra Aroma alone recorded the highest value for benzyl alcohol. Conversely, Enozym FW contributed the lowest values for these three compounds. According to our wok, other authors also reported changes in on the concentration of alcohols by effect of various commercial enzyme preparation on Verdejo wines after fermentation, leading an increase of benzyl alcohol (Rodríguez-Nogales et al., 2024).

Among the three aldehyde compounds identified, only benzaldehyde exhibited statistical significance, with Enozym Extra Aroma showing the highest value and Enozym FW the lowest. The other enzymes displayed intermediate behaviour for this compound.

The C_{13}-norisoprenoids group exhibited significative variations among the identified compounds (3-hydroxy-β-damascone, 3-oxo-α-ionol, and 3-hydroxy-7,8-dihydro-β-ionol) in response to different enzymes used in the must treatment. Trenolin Bouquet and Enozym Extra Aroma enzymes were particularly effective in significantly increasing the concentrations of all three C_{13}-norisoprenoids compounds. However, 3-hydroxy-β-damascone could not be released by Lallzyme Cuvee Blanc, Endozym-β Split, Enovin Varietal and Trenolin Mash DF enzymes. Conversely, Enozym FW and Lafazym Arom resulted in the lowest liberation of C_{13}-norisoprenoids compounds. The remaining enzymes demonstrated moderate effects on this group of compounds. The effect of several commercial enzyme treatment on C_{13}-norisoprenoids showed the evolution of these compounds during alcoholic fermentation depends on the type of enzyme used (Scutarasu et al., 2022).

From ester compounds, Methyl salicylate, showed the highest concentration with the application of Rapidase Revelation Aroma, while Enozym FW showed the opposite trend. The other enzymes displayed an intermediate impact on the liberation of this ester.

Among the terpenes, eleven compounds were identified in the blended white varieties must. The commercial enzymes used had a significant effect, particularly on *cis*-furan linalool oxide, *trans*-furan linalool oxide, linalool, hotrienol, *cis*-pyran linalool oxide, nerol, geraniol, and *trans*-8-hydroxylinalool concentrations. The dominant aromatic glycosides in grapes are terpenes, mostly the glycosides of monoterpenes and C_{13}-norisoprenoids (Parker et al., 2018). Rapidase Revelation Aroma and Enozym Extra Aroma exhibited the highest content of these terpenes, indicating their strong influence on their release. Increase of several terpenes such us α-terpineol, linalool, nerol, geraniol and citroellol by effect of AR 2000 (Rapidase Revelation Aroma) on Marula pulp have been reported by Fundira et al. (2002).

Monoterpenes such us geraniol, linalool and its oxides have low sensory thresholds, therefore their release increase the aroma of wine (Ghaste et al., 2015). In the study conducted by Versini et al. (1994), they reported an important presence of linalool in Albariño musts at a concentration of 20–50 μg/L, while Loureira musts exhibited approximately 5–8 times higher levels of this compound. In the present work, linalool was one of the most abundant terpene compounds in blended musts with high proportion of Albariño (10.5 μg/L) when Rapidase Revelation Aroma was used. This disparity in results can be attributed to the fact that the must use in our experiment was composed of several white varieties and the vintages was different. As a result, when these varieties are combined, the overall aromatic composition of the must can be influenced by the specific proportions of each variety present, potentially leading to variations in linalool concentrations compared to individual musts. In terms of varieties grown for white wines in the NW of the Iberian Peninsula, Galicia and Northern Portugal, Albariño, Loureira, Treixadura and Godello are the typical (Falqué et al., 2001). In the Rías Baixas area, the Albariño variety stands out as the protagonist, known for its aromatic and refreshing wines. In contrast, the Ribeiro area employs a blend of varieties, with Treixadura as the prevailing grape, lending complexity and balance to the wines. In the inland regions of Galicia, such as Ribeira Sacra, Valdeorras, and Monterrei, Godello is the most prevalent (Vilanova et al., 2010). The Loureira and Albariño single wines had the highest concentrations of volatiles. However, the blended white wines, especially those with Loureira, showed increases in terpenes and C_{13}-norisoprenoids (Vilanova & Freire, 2017).

On the other hand, the rest of the enzymes studied showed a more moderate impact on the overall liberation of these terpene compounds when compared to Rapidase Revelation Aroma and Enozym Extra

Table 4
Total concentration (μg/L) and total percentage (%) of chemical groups of volatiles released by treatment with 12 different commercial enzymes.

Volatile groups	Rapidase Revelation Aroma	Lallzyme β	Lallzyme Cuvee Blanc	Endozym β-Split	Enovin Varietal	Enozym Extra Aroma	Trenolin Bouquet	Trenolin Mash DF	Endozym ICS 10 Arome	Enozym FW	Lafazym Arom	Rivela	Sig.
Acids (μg/L)	420.0 **bc**	474.2 **abc**	491.7 **ab**	462.4 **bc**	480.1 **ab**	608.3 **a**	365.8 **bc**	427.2 **bc**	353.1 **bc**	394.5 **bc**	330.7 **c**	375.8 **bc**	***
(%)	43.2	55.2	42.9	40.6	54.7	46.5	41.0	46.3	46.8	46.5	48.3	45.7	
Alcohols (μg/L)	258.3 **ab**	196.3 **abc**	235.9 **ab**	214.0 **abc**	174.9 **abc**	280.8 **a**	197.6 **abc**	177.3 **abc**	157.1 **abc**	74.6 **c**	107.6 **bc**	133.9 **abc**	**
(%)	26.5	22.8	20.6	18.7	19.9	21.4	22.1	19.2	20.8	8.8	15.7	16.3	
C_6 -Compounds (μg/L)	38.7	11.9	27.0	33.3	43.6	16.4	27.3	44.7	9.7	8.9	11.3	44.2	ns
(%)	4.0	1.4	2.4	2.9	5.0	1.3	3.1	4.8	1.3	1.0	1.7	5.4	
Aldehydes (μg/L)	8.2	7.4	17.0	19.9	5.7	16.8	9.3	7.4	6.0	11.5	6.7	9.7	ns
(%)	0.8	0.8	1.4	1.7	0.7	1.3	1.0	0.8	0.8	1.4	1.0	1.2	
C_{13}-norisoprenoids (μg/L)	37.5 **abc**	35.1 **abcd**	17.2 **bcde**	14.2 **cde**	10.6 **cde**	46.0 **a**	42.7 **ab**	9.0 **de**	17.6 **bcde**	7.0 **e**	11.2 **cde**	27.8 **abcde**	***
(%)	3.9	4.1	1.5	1.2	1.2	3.5	4.8	1.0	2.3	0.8	1.6	3.4	
Esters (μg/L)	79.2	52.9	84.5	88.1	57.9	99.0	69.2	54.9	56.0	46.6	41.2	58.0	ns
(%)	8.1	6.2	7.4	7.7	6.6	7.5	7.8	5.9	7.4	5.5	6.0	7.0	
Terpenes (μg/L)	71.2 **a**	43.9 **ab**	47.1 **ab**	47.6 **ab**	35.8 **b**	58.5 **ab**	50.9 **ab**	41.1 **ab**	38.3 **ab**	28.6 **b**	30.9 **b**	37.7 **ab**	**
(%)	7.3	5.1	4.1	4.2	4.1	4.5	5.7	4.5	5.1	3.4	4.5	4.6	
Volatile phenols (μg/L)	59.7	37.7	225.7	262.8	68.8	183.8	130.2	161.8	116.8	277.6	145.5	135.1	ns
(%)	6.2	4.4	19.7	23.0	7.8	14.0	14.5	17.5	15.5	32.6	21.2	16.4	
Total Concentration (μg/L)	**972.8**	**859.4**	**1146.1**	**1142.2**	**877.5**	**1309.5**	**893.0**	**923.4**	**754.7**	**849.3**	**685.1**	**822.2**	ns
(%)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	

Signification: *, **, *** and ns indicate a significant difference among enzymes at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively. The different letters indicate significant differences among enzymes for each volatile compound by Tukey's test ($p < 0.05$).

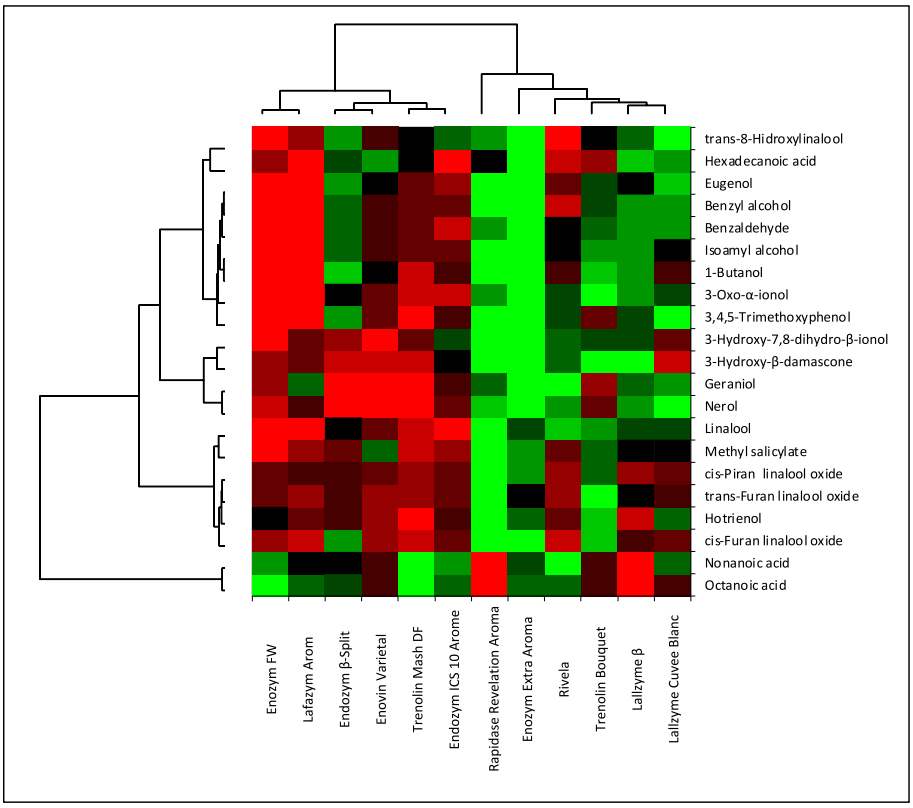

Fig. 2. Heat map representations of the 21 significative volatile compounds according to the commercial enzyme used.

Aroma.

Esters and Terpenes play an important role in the development of fruity and floral aromas. Additionally, C_{13}-norisoprenoids, which are also characterized by floral aromas, are important volatile compounds contributing to the wine aroma due to their low olfactory thresholds (Mateo & Jiménez, 2000). In this sense, Rapidase Revelation Aroma and Enozym Extra Aroma emerge as the top candidates for Galicia winemaking processes, as supported by utilizing these enzymes, winemakers have the opportunity to elevate the terpene content in the must, ultimately resulting in high-quality white wines with enhanced aromatic complexity and pronounced varietal characteristics.

Among the volatile phenols chemical family, the impact of different enzymes was relatively limited, with only two compounds (eugenol and 3,4,5-trimethoxyphenol) being significantly affected. For Eugenol, the most substantial increase in content was observed when Rapidase Revelation Aroma, Lallzym Cuve Blanc, and Enozym Extra Aroma were used. However, only Enozym Extra Aroma displayed the highest release of 3,4,5-trimethoxyphenol. Interestingly, Enozym FW proved to be less effective in breaking the glycosylated link of Eugenol from its precursor, resulting in lower liberation of this compound, as well as 3,4,5-trimethoxyphenol, showing a contrast performance compared to Rapidase Revelation Aroma and Enozym Extra.

The efficiency of the commercial enzymes varied as differences in their ability to release individual compounds when eight commercial preparations were assessed on Verdejo grapes to evaluate their wine aromatic potential (Rodríguez-Nogales et al., 2024).

These findings demonstrate the unique impact of each commercial enzyme on the individual aroma compounds, underscoring the importance of enzyme selection in winemaking to achieve specific aromatic profiles and sensory characteristics in the final product.

Finally, we generated a heatmap based on the commercial glycolytic enzymes used, focusing on the statistically significant twenty-one volatile compounds identified (Fig. 2). The heatmap visually represented the relationships between the enzymes and the concentration levels of these key volatile compounds. By examining the heatmap, we gained valuable insights into how each glycolytic enzyme influenced the production of specific volatile compounds. The clustering patterns in the heatmap provided a clear visualization of enzyme-chemical interactions, highlighting which enzymes were particularly efficient in releasing certain aroma compounds.

The analysis of different metabolite intensities of color led to a clear separation of the 12 enzymes used, revealing two main clusters denoted as A and B (Fig. 2). Cluster A encompassed Enozym FW, Lafazym Arom, Endozym β-Split, Enovien Varietal, and Trenolin Mash DF, exhibiting, on average, lower concentrations of these volatile compounds. In contrast, cluster B consisted of the remaining 7 enzymes, primarily characterized by the presence of terpenes, C_{13}-norisoprenoids, acids, esters, and alcohols. Notably, the enzymes Rapidase Revelation Aroma and Enozym Extra Aroma displayed particularly high abundances of these volatile compounds, outperforming the others and confirming the results obtained from the Principal Component Analysis (PCA). Furthermore, Rapidase Revelation Aroma, is a unique enzymatic formulation of β-D-glucosidase to maximize aromatic potential that has been used in the protocols proposed by several authors (Oliveira et al., 2008; Vilanova et al., 2010; Vilanova et al., 2021).

4. Conclusion

In this study, we conducted an evaluation of 12 commercial enzymes to assess their effectiveness in releasing aromatic compounds from their conjugated forms in a mixed white variety must under laboratory conditions. The results revealed a significant effect of glycolytic enzymes, which led to a notable increase in the concentration of twenty-one aroma compounds. This finding confirms the importance of utilizing glycolytic enzymes in the production of more aromatic wines. However, it is essential to be careful when assuming that all commercial enzymes would exhibit equal effectiveness in hydrolyzing aroma precursors at the specific conditions employed in this research (40 °C and pH of 3.27).

Enzymes can show different glycosidase activities depending on the temperature and pH conditions they are subjected to. Hence, their performance may vary under different experimental setups. Among the commercial enzymes studied in this work, Rapidase Revelation Aroma and Enozym Extra Aroma emerged as the most powerful ones on the must's volatile composition. These enzymes demonstrated an important ability to release higher concentrations of essential varietal aroma compounds, particularly release of terpenes, C_{13}-norisoprenoids, and esters compounds. As a result, they hold significant potential to enhance the aromatic profile of the must, thereby contributing to the overall floral and fruity characteristics of white Galician wines. The findings suggest that these enzymes could play a crucial role in improving the quality of the end product. To improve understanding of these enzymes' mechanisms and optimize aroma extraction during the winemaking process, more in-depth exploration and analysis have to be conducted.

CRediT authorship contribution statement

Liliana Martínez: Writing – review & editing, Validation, Investigation, Formal analysis. **Bianca S. da Costa:** Resources, Investigation, Formal analysis. **Mar Vilanova:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141742.

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