LWT - Food Science and Technology 215 (2025) 117314

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

The potential of enzymatic hydrolysis with HS-SPME-GC×GC-ToFMS for the study of volatile signature of grapes

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ARTICLE INFO

Keywords:
Aroma composition
HS-SPME-GC×GC-ToFMS
AR2000 enzyme
Clones

ABSTRACT

This works focuses on developing an optimized methodology for analyzing free and bound volatile compounds in grapes using enzymatic hydrolysis with AR2000 enzyme and HS-SPME-GC × GC-ToFMS. To achieve this purpose, a previous optimization step of enzymatic hydrolysis was implemented and optimal conditions for enzymatic hydrolysis were established using 50 mg of AR2000 enzyme at 35 °C for 24 h, while the best extraction conditions were found to be 40 min at 60 °C. Applying this methodology to clones of Moreto grapes from harvest of 2022 and 2023, it was possible to identify and semi-quantify sixty volatile compounds, with more compounds identifiable in 2022 than in 2023. The results also showed the influence of crop year and genetic intravariability.

1. Introduction

In 1981, Cordonnier and Bayonove proposed a comprehensive classification system of grape aromas based on their origin, categorizing them into varietal, pre-fermentative, fermentative, and post-fermentative types (Cordonnier & Bayonove, 1981). Varietal aromas, specific to each grape variety, are pivotal indicators in assessing the potential for wine production, wine quality and sensory complexity (Ribereau-Gayon et al., 2006). Terpenes are the most extensively studied varietal compounds in *Vitis vinifera* grapes, contributing predominantly to floral and fruity aromas (González-Barreiro et al., 2015). C_{13}-norisoprenoids, derived from grape carotenoids, are also crucial for varietal character and are typically found in glycosylated form (Yuan & Qian, 2016). Additional aromatic compounds like aldehydes, alcohols, ketones, and lactones play significant roles in the distinctive flavor profiles of grape varieties (Gutiérrez-Gamboa et al., 2020).

It was in the 1980s that Williams and colleagues identified terpene glycosides compounds in grapes (Dimitriadis & Williams, 1984; Gunata et al., 1985; Williams et al., 1982). According to literature, the glycosylated forms are more common than the free forms, due to the bonding of aromatic compounds with polar molecules such as amino acids or sugars, thereby stabilizing them within grape skins and pulp (Cabrita et al., 2006; Ferreira & Lopez, 2019). Releasing the volatile aglycone is crucial and can be achieved via acid or enzymatic hydrolysis (Liu et al., 2017).

According to the literature, there is no consensus on the most suitable method for breaking the bond between sugar and aglycone and the choice between acid and enzymatic hydrolysis methods still remains a topic of investigation (Hampel et al., 2014; Loscos et al., 2009). Both hydrolysis processes, acid and enzymatic, have advantages and disadvantages, so the most advisable is to choose one of these approaches, according to the type of compounds to be identified (Chen & Quek, 2023; Dziadas & Jeleń, 2016).

In these studies a sample preparation step is mandatory. Solid phase microextraction (SPME), in which the analytes are volatilized and then adsorbed onto a fiber, has emerged as a promising alternative to conventional techniques (Canuti et al., 2009; Costa Freitas et al., 2012; Souza-Silva et al., 2015). SPME is a fast, simple and inexpensive technique that requires small sample volumes, combines extraction and concentration, does not require the use of solvents, requires little sample handling and can be used on liquid, solid or gaseous samples (Fernandes et al., 2015; Lancioni et al., 2022; Marín-San Román et al., 2020; Nolvachai et al., 2023).

In the study of grapes, SPE is the mostly common method for

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https://doi.org/10.1016/j.lwt.2024.117314
Received 10 October 2024; Received in revised form 20 December 2024; Accepted 30 December 2024
Available online 31 December 2024

analyzing free and bound volatile compounds since it is possible to isolate these fractions. However, due to some disadvantages arising mainly for the use of organic solvents in the extraction processes, innovative methods are emerging aiming to overcome some of those limitations (Panighel & Flamini, 2015). The integration of enzymatic hydrolysis with HS-SPME-GC × GC-ToFMS has been implemented, enabling the comprehensive profiling of both free and bound volatile compounds in grapes. In fact, this approach offers unparalleled analytical depth, enabling detailed characterization of grape aroma profiles while minimizing environmental impact.

In Portugal, a strategic and large-scale approach has been implemented for conserving intra-varietal diversity. This involves preserving representative samples of genotypes from autochthonous Portuguese grapevine varieties (Gonçalves & Martins, 2022). Specifically, Moreto clones were selected because it is an indigenous Portuguese variety, which allows for random selection. In fact, there is significant intra-varietal diversity in quantitative characteristics within each variety. This includes traits of great oenological importance, such as yield, soluble solids content, acidity, and volatile and phenolic composition (Alonso et al., 2004).

Commonly, the aglycones are analyzed by gas chromatography. The use of advanced chromatographic techniques such as comprehensive two-dimensional gas chromatography (GC × GC) further enhances resolution and compound identification in complex matrices, like grape extracts (Könen & Wüst, 2019).

Thus, this work aims to develop a methodology for analyzing varietal volatile compounds, free and bound volatile compounds, in grapes using an enzymatic hydrolysis with AR2000 enzyme and HS-SPME-GC × GC-ToFMS. This is of utmost importance since it is well known that monoterpenes and norisoprenoids are present mainly as non-volatile glycosides in neutral varieties. This methodology developed in this study will be applied to Moreto clones, enabling the assessment of intra-varietal diversity based on their volatile composition. The use of clones is not only a tool for improving the resilience of viticulture, but also for preserving the quality and identity of grape varieties in the face of climate change. Continued development and research into adapted clones are crucial to ensuring the sustainability and competitiveness of the wine sector. The analytical methodology developed, which allows to establish the potential varietal volatile profile of grapes with only one injection, can be further used to contribute to the knowledge of grapes with enological interest. This will give valuable information to winemakers to increase varietal aroma of wines, according to the potential of each grape variety.

2. Materials and methods

2.1. Samples

Five clones of Moreto grapes (M 1, M 2, M 3, M 4, and M 5) from two different crop years were studied. Grapes were harvested in 2022 and 2023 from the PORVID (Portuguese Association for Vine Diversity) vineyard in Pegões. All plants were grown under identical soil, climate, and cultivation conditions. Oenological parameters such as potential alcohol degree, total acidity and pH were measured according to OIV (International Organisation of Vine and Wine) (OIV, 2018).

2.2. Reagents and standards

Sodium chloride was purchased from Honeywell (Seelze, Germany), sodium hidrogenophosfate from Scharlab, (Barcelona, Spain), citric acid monohydrate from Panreac Applichem (Darmstadt, Germany) and AR2000 from Creative Enzymes (Shirley, USA). Ultra pure water was obtained from Millipore (Elix, Interface, Amadora, Portugal). Standards used were commercial hydrocarbon mixture C_8–C_{20} from Supelco (Bellefonte, PA, USA) and mixture of terpenes called MegaMix #1 (Restek, Bellefonte, PA).

2.3. HS-SPME methodology

A carboxen/divinylbenzene/polydimethylsiloxane fiber (CAR/DVB/PDMS), 1 cm, 50/30 μm film thickness, supplied from Supelco, (Bellefonte, PA, USA) were used for HS-SPME extractions. Fiber blanks were run periodically, that is, a blank was carried out before the injection of the first sample of grapes and the remaining blanks were carried out every 3 injections, to ensure the absence of contaminants and/or carryover. HS-SPME extraction was performed according to a previously developed procedure by Fonseca et al., 2024, with some modifications (Fonseca et al., 2024). In a 20.0 mL SPME flask sealed with a Teflon-coated rubber septum/magnetic screw cap, 4 g of grapes previously crushed with the Ultra Turrax T25 basic (IKA Labortechnik, Germany) were weighed, then 2 g of sodium chloride (NaCl) was added, then 50 mg of AR2000 and finally 2 mL of citrate-phosphate buffer solution (pH 5). The salt was added to inhibit yeast activity, preventing the start of alcoholic fermentation. Additionally, as noted in the literature, NaCl increases the ionic strength of the samples, reducing the solubility of the compounds and altering their partition coefficient, thereby enhancing the extraction of analytes (Fonseca et al., 2024; Perestrelo et al., 2011). The flask was then incubated at 35 °C for 24 h. After, the vial was equilibrated for 5 min at 60 °C and then extracted for 40 min at the same temperature. The thermal desorption of the analytes was carried out by exposing the fiber in the GC injection port at 260 °C for 3 min in splitless mode. Fig. 1 summarizes the implemented methodology, comprising the steps of enzymatic hydrolysis, HS-SPME and chromatographic analysis (GC×GC-ToFMS). All measurements were made with three replicates.

2.3.1. Optimization of the use of AR2000 enzyme

To evaluate the efficiency of the AR2000 enzyme action on the release of glycosylated compounds, several parameters were tested namely the amount of enzyme (0, 50 and 100 mg of AR 2000), the enzyme reaction time (0, 24 and 48 h) and the enzyme operating temperature (30, 35 and 40 °C). The parameters indicated were selected based on literature and preliminary studies that allowed for the selection of the best conditions for enzyme activity (Dziadas & Jeleń, 2016; Hjelmeland & Ebeler, 2015; Loscos et al., 2009). In this experiment we tested all combinations possible for the three parameters under study, namely amount of enzyme, reaction time and temperature, and each test was carried out in triplicate.

2.3.2. Optimization of extraction time and temperature

The extraction time and temperature were also optimized, due to their crucial role on the volatility and solubility of the target compounds under study in this work. To optimize the extraction time and temperature, the fiber (CAR/DVB/PDMS) was exposed to different conditions: time of extraction (20, 40 and 60 min) and the extraction temperature (40, 50 and 60 °C) were also considered. The extraction times and temperatures were tested according to the literature (Fonseca et al., 2024; Perestrelo et al., 2011). The parameters were varied individually to test all combinations possible, and each test was conducted in triplicate.

2.4. GC × GC-ToFMS analysis

The analyses were performed on a GC×GC-ToFMS system consisting of an Agilent 8890 GC System (Shanghai, China) with a BenchTOF-Select detector (Markes International, Bridgend, UK). An automatic sampler injector was used (CTC Analysis autosampler PAL-System SepSolve Analytical, Zwingen, Switzerland) and the data were acquired and analyzed with ChromSpace of Markes International, Bridgend, UK. Chromatographic separation was achieved with INSIGHT™ flow modulator (SepSolve Analytical, Waterloo, Canada), equipped with a loop with 50 μL, a BPX5 column (20 m length × 0.18 mm i. d. and 0.18 μm film thickness, from SGE GC column, Trajan, Australia) as first-

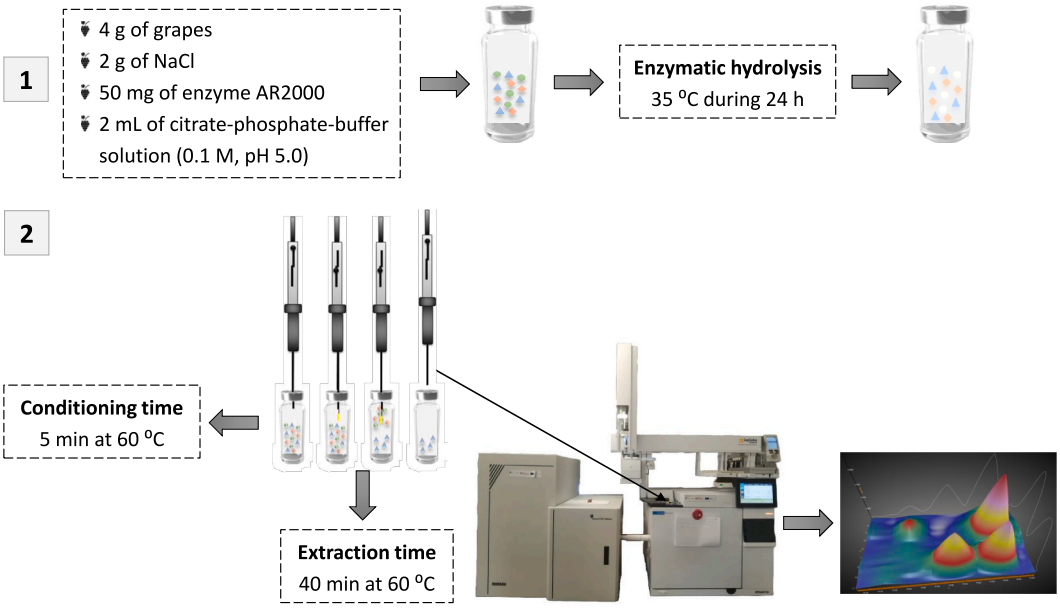

Fig. 1. Enzymatic hydrolysis combined with HS-SPME-GC × GC-ToFMS methodology.

dimension (1D) and a BPX50 column (5 m length × 0.25 mm i. d. and 0.1 μm film thickness, from SepSolve Analytical, Australia) as second-dimension (2D). The modulation period (PM) used was 5 s and flush time (FT) was 200 ms. The oven temperature program began at 40 °C hold for 3 min, raised at 3 °C/min up to 150 °C, then 4 °C/min up to 200 °C, then 10 °C/min up to 260 °C and hold for 5 min. Helium was used as carrier gas with a flow of 0.5 mL/min in the first column and 20 mL/min in the second column. The MS transfer line and source temperatures were set at 270 °C. To determine the characteristic mass fragments, electron ionization (EI) at 70 eV mass spectra of the analytes were recorded at full scan, from 30 to 400 m/z and data acquisition frequency of 50 Hz. The linear retention index values were calculated through analysis of the commercial hydrocarbon mixture C_8–C_{20} (Supelco, Bellefonte, PA, USA), using the same chromatographic conditions. A mixture of terpenes called MegaMix #1 (Restek, Bellefonte, PA) was injected to help identify terpenes. The volatile compounds were first identified by matching mass spectra with the spectra of reference compounds in the NIST mass spectral library (NIST MS Search Program Version 2020), also taking into consideration structure and molecular weight, and by comparing the calculated LRIs with those described in the literature. The relative amount of each compound was calculated as the percent ratio of the respective peak area relative to the total peak area and expressed as percentage.

2.5. Statistical analysis

NCSS 11 Statistical Software (2016) (LLC. Kaysville, Utah, USA) was used to carry out one-way analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) to assess the influence of harvest "year", "clone", or the interaction "year × clone" in Moreto varietal volatile composition. Differences were assessed at a probability level of 0.05, 0.01 and 0.001 using Tukey-Kramer test. OriginPro 2023b SR1 software (OriginLab, Northampton, MA, USA) was used to produce polar heatmaps.

3. Results and discussion

3.1. Oenological parameters of samples

Table 1 shows the oenological parameters of different grapes samples of Moreto (M 1, M 2, M 3, M 4, and M 5). For all the samples, the potential alcohol degree is different between years, especially for samples M 1, M 2 and M 4, where the potential alcohol degree is much higher in 2022, while for samples M 3 and M 5 the potential alcohol degree is only slightly higher, 0.13 and 0.33 (% v/v) respectively. Values of total acidity and pH are within normal values for this variety. This observation highlights the variability in grape composition from year to year, which can significantly affect the winemaking process and the quality of the final product. The higher potential alcohol degree in 2022 for these samples suggests better ripening conditions, which, may be related to climatic conditions.

3.2. Optimization of HS-SPME-GC × GC-ToFMS methodology

3.2.1. Optimization of the AR2000 enzyme

The selection of the most suitable conditions for the occurrence of the enzymatic hydrolysis is a mandatory step (Hampel et al., 2014). According to Fig. 2 (a, b, and c), the results differ notably between not

Table 1
Chemical characterization of different Moreto grape samples.

	Year	Potential alcohol degree (% v/v)	pH	Total acidity (g/L) [a]
M 1	2022	18.13	4.02	2.75
	2023	17.63	4.01	3.65
M 2	2022	21.37	4.05	3.25
	2023	20.83	4.08	3.45
M 3	2022	18.77	3.99	3.25
	2023	18.90	4.07	2.55
M 4	2022	18.37	4.01	3.45
	2023	17.93	4.01	4.10
M 5	2022	19.20	4.07	3.10
	2023	19.53	4.09	3.40

[a] Tartaric acid.

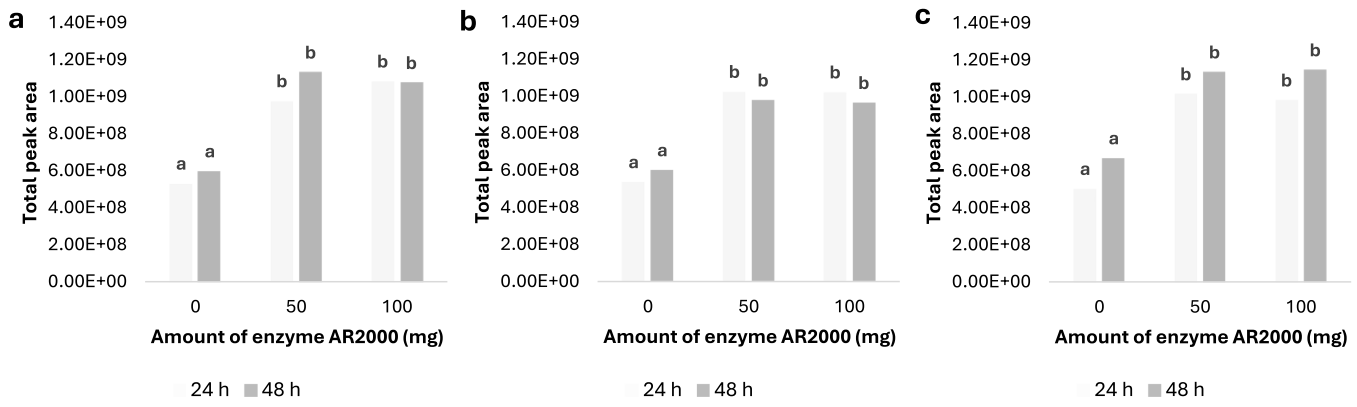

Fig. 2. Optimization of amount of enzyme AR2000 at 30 °C (a), 35 °C (b) and 40 °C (c) for 24 and 48 h. Different letters above the columns means significantly differences ($p \leq 0.05$).

using the enzyme (0 mg) and using the enzyme (50 and 100 mg), with the total areas increasing twofold, these results shows that the enzyme was able to release glycosylated compounds. Furthermore, the enzyme facilitated the identification of two additional compounds, eugenol and α-ionone, that were not detected in assays without the enzyme presence (Hampel et al., 2014; Loscos et al., 2009).

Fig. 2a shows that using 50 mg of AR2000 for 48 h, at 30 °C resulted in a higher total area. Results depicted in Fig. 2b also indicate that using 50 mg of enzyme for 24 h, at 35 °C is the most promising condition, yielding a higher total area with low standard deviation compared to the same conditions shown in Fig. 2a and c. Notably, the results for 100 mg of AR2000 for 24 h were similar to those for 50 mg, at 35 °C, but the latter resulted in a slightly higher total area. According to Fig. 2c, the total area obtained with 50 mg of enzyme over 48 h, at 40 °C, was very similar to that with 100 mg of AR2000 over the same 48 h.

In light of these results, the Tukey-Kramer multiple-comparison test revealed no significant differences. For this reason, we chose to use 50 mg of AR2000 enzyme at 35 °C for 24 h, as the areas obtained for 50 and 100 mg of the enzyme, either at 24 or 48 h, at 40 °C, showed no significant differences. Since the number of compounds identified was similar for both conditions, factors such as the shorter time and smaller amount of enzyme, proved to the essential on the methodology implementation, being chosen in this work.

3.2.2. *Optimization of extraction time and temperature*

Fig. 3 (a, b, and c) presents the results of the optimization of the extraction time (20, 40, and 60 min) and temperature (40, 50, and 60 °C). These conditions were chosen based on previous works (Fonseca et al., 2024; Perestrelo et al., 2011). Generally, the total area of the peaks increases with longer extraction times and higher temperatures. Fig. 3a and b demonstrate that extracting for 60 min yields a significantly higher total peak area compared to the 40 min extraction time. Fig. 3c indicates that extracting for 40 min at 60 °C results in a much higher total area than extracting for 60 min at 50 °C (as shown in Fig. 3b), establishing 60 °C as the optimal extraction temperature, to perform the assays. Additionally, Fig. 3c shows also that the total areas obtained with 40 and 60 min of extraction at 60 °C are similar.

Based on these results, the Tukey-Kramer multiple-comparison test revealed no significant differences between 40 and 60 min at 60 °C. Therefore, the best extraction condition is 60 °C for 40 min, as it achieves comparable total areas to the 60 min extraction but in a shorter time.

3.3. *Application of HS-SPME-GC × GC-ToFMS methodology for analysis of free and bound volatile compounds*

After optimizing the sample preparation steps and selecting the best conditions, the developed methodology was applied to analyze both free and bound volatile compounds. The advantage of this methodology is that it allows to identify end semi-quantify the free and bound volatile compounds (released by enzymatic hydrolysis) in only one injection. The contour plots for the M 1, M 2, M 3, M 4, and M 5 grape samples are

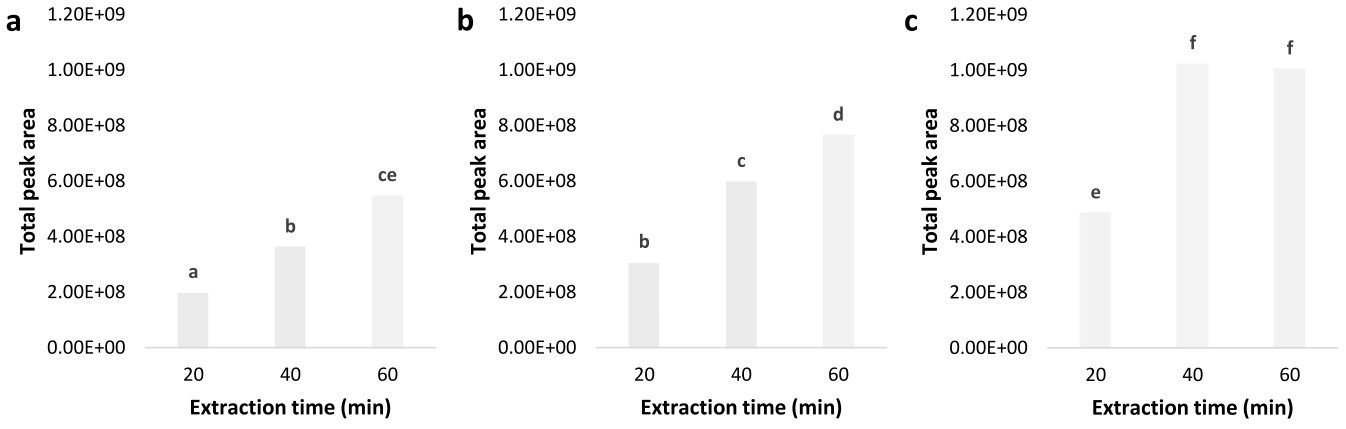

Fig. 3. Optimization of extraction time and temperature 40 °C (a), 50 °C (b) and 60 °C (c). Different letters above the columns means significantly differences ($p \leq 0.05$).

shown in Figs. S1, S2, S3, S4, and S5, respectively.

A total of sixty free and bound volatile compounds were identified and quantified in the different Moreto grape clones, of which fifty-four were varietal volatiles. Specifically, the analysis identified three C_6 compounds, three benzenoids, twenty-four monoterpenes, twenty-four sesquiterpenes, and six C_{13}-norisoprenoids (Table 2). Although fifty-four varietal volatile compounds were identified, the C_6 compounds exhibited the highest relative areas, making them the most prominent ones (Table S1). The results of the ANOVA presented in Table 2 evaluate the effects of the year on different families of compounds. For C_6 compounds and benzenoids all compounds exhibit statistically significant variations based on the year, clone, and the interaction year × clone. However, for the monoterpenes, nine compounds were not statistically significant for the year. Regarding the clone, only *p*-cymene, *p*-cymenene, and menthol were not statistically significant. For the year × clone interaction, *p*-cymene, menthol, and D-verbenone were not statistically significant. Most sesquiterpenes were statistically significant for both year and clone. However, six sesquiterpenes were not statistically significant for the year × clone interaction. All C_{13}-norisoprenoids were statistically significant for the year. However, three C_{13}-norisoprenoids (vitispirane, β-damascenone, and α-ionone) were not statistically significant for the clone. Regarding the year × clone interaction, only α-ionone was not statistically significant.

Table S1 presents the free and bound volatile compounds identified in Moreto grape samples, along with the corresponding mean and standard deviation of relative areas for the years under study. In 2022, cedrene and α-ionone were not detected in the M 1 clone. In clone M 2, cis-thujopsene, β-copaene, and α-ionone were absent. In clone M 4, δ-selinene and α-ionone were not identified. Conversely, in clones M 3 and M 5, all compounds listed in Table S1 were identified in 2022. In 2023, only clone M 5 contained all the compounds. In M 1 clone, cis-thujopsene, β-copaene, and α-ionone were not detected. In clone M 2 α-gurjunene, *cis*-thujopsene, α-amorphene, α-cadinene and α-ionone were not found. In clone M 3, four compounds were absent in 2023: β-copaene, δ-selinene, α-muurolene, and α-ionone. Finally, in clone M 4, α-amorphene, δ-selinene, and α-ionone were not found. These year-to-year variations suggest the influence of climatic conditions. Increased temperatures affect the grapevine cycle, impacting grape ripening and the synthesis of compounds, which may explain the observed differences. It was observed that in 2022 a greater number of volatile compounds were identified than in 2023. This can be explained by the potential alcohol degree, as it was higher in 2022, as previously mentioned (Cabrita et al., 2006; Díaz-Fernández et al., 2022; Fonseca et al., 2024; Petronilho et al., 2014; Van Leeuwen et al., 2020).

In recent years, many studies have examined the volatile composition of wine using HS-SPME-GC × GC-ToFMS (Aith Barbará et al., 2020; Carlin et al., 2016; Könen & Wüst, 2020; Lukić et al., 2020, 2022; Weldegergis et al., 2011; Welke et al., 2013). However, studies on the volatile composition of grapes using this methodology are almost unexplored. Könen & Wüst, 2019 developed a method using HS-SPME-GC × GC-ToFMS to analyze the biosynthetic pathways of sesquiterpene hydrocarbons in grapes of the Lemberger variety (*Vitis vinifera* subsp. *Vinifera*, clone 1 Gm, exocarp). This method revealed the presence of hundreds of components with twenty-five identified as sesquiterpene hydrocarbons (Könen & Wüst, 2019; Fonseca et al., 2024 developed a methodology for analyzing free volatile compounds in grapes using HS-SPME-GC × GC-ToFMS enabling the identification of fifty-two free volatile compounds (Fonseca et al., 2024).

However, as far as we are aware, no studies have been found in the literature in which enzymatic hydrolysis is carried out before HS-SPME-GC × GC-ToFMS. This step is crucial as it allows bound precursors to be released, thereby increasing the number of compounds identified contributing for a more deeply knowledge of grape composition. The importance of enzymatic hydrolysis is evident when comparing this study's results with those in the literature (Fonseca et al., 2024; Könen & Wüst, 2019).

Fig. 4 displays polar heatmaps with a dendrogram for the clones under study, revealing intra-varietal differences among Moreto grapes due to the high genetic complexity that each grapevine variety exhibits. Notably, there is a clear hierarchical grouping between clones M 2 and M 4, and between clones M 3 and M 5.

The polar heatmap is divided into five clusters: Cluster 1 (red) includes hexanal, 2 E-hexenal, and hexanol; Cluster 2 (blue) contains another set of compounds; Cluster 3 (green) encompasses the most compounds; Cluster 4 (lilac) includes another distinct group of compounds; and Cluster 5 (yellow) contains only a single compound, α-ionone. Cluster 1 comprises compounds present in the largest quantities, while Cluster 5 includes compounds present in the smallest quantities. Essentially, the polar heatmap groups compounds into clusters based on their areas, with increasing cluster numbers corresponding to smaller compound areas.

4. Conclusions

This study enables to implement a methodology for analyzing free and bound volatile compounds in grapes, comprising two sequentially steps-firstly an enzymatic hydrolysis with AR2000 enzyme followed by HS-SPME-GC × GC-ToFMS, showing remarkable improvements on the number of glycosylated compounds. Indeed, the optimal conditions for enzymatic hydrolysis were determined to be 50 mg of AR2000 enzyme at 35 °C for 24 h. This process significantly increased the total area of volatile compounds, demonstrating the enzyme's effectiveness in releasing glycosylated compounds and identifying additional compounds such as eugenol and α-ionone. The extraction conditions were also optimized, showing that the most suitable conditions are 40 min at 60 °C. This approach provided similar total areas to those obtained with 60 min of extraction, but in a shorter time, making it more efficient.

Applying the optimized methodology to clones of Moreto grape samples from the 2022 and 2023 harvests, a total of sixty volatile compounds were identified, with fifty-four being varietal volatile compounds. The study revealed significant year-to-year variations in the volatile composition, likely influenced by climatic conditions affecting grape ripening and compound synthesis. For instance, some compounds such as cedrene and α-ionone were absent in certain samples depending on the year, probably indicating the impact of environmental factors.

This analytical methodology will allow a comprehensive study on the potential varietal volatile composition of grapes, in a simpler and faster way. A previous step of enzymatic hydrolysis followed by SPME makes possible to analyze both free and glycosidic bound volatile compounds in only one chromatographic run.

The developed methodology can therefore be further used to establish the varietal volatile signature of grapes from different varieties and to understand the impact of agronomic practice or environmental factors on the volatile composition of grapes.

CRediT authorship contribution statement

Daniela Fonseca: Writing – original draft, Methodology, Investigation, Formal analysis. **Nuno Martins:** Supervision, Software, Resources. **Raquel Garcia:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Maria João Cabrita:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Funding

This research was funded by "Vine&Wine - Driving Sustainable Growth Through Smart Innovation" project n. o C644866286-011 (subproject - BioGrapeSustain), "Mobilizing Agendas for Business Innovation" under the Recovery and Resilience Program. This work is financed by National Funds through FCT – Foundation for Science and Technology under the Project UIDP/05183/2020.

Table 2
The results of ANOVA and MANOVA for the free and bound volatile compounds found in clones of Moreto grapes.

Compounds	[1]DRT (min)[a]	[2]DRt (min)[b]	LRI^{c}_{Lit}	LRI^{d}_{Calc}	ID	ANOVA and MANOVA		
						Year	Clone	Year × Clone
C_6 compounds								
Hexanal	11.64	3.00	801	–	MS, LRI	***	***	***
(2 E)-Hexenal	14.31	3.29	846	–	MS, LRI	***	***	***
Hexanol	14.88	3.05	863	–	MS, LRI	***	**	***
Benzenoids								
Benzaldehyde	20.30	3.90	952	–	MS, LRI	***	***	**
Benzenemethanol	24.04	3.92	1026	1030	MS, LRI	**	**	***
Benzene ethanol	28.13	3.93	1107	1119	MS, LRI	***	***	***
Monoterpenes								
β-Myrcene	21.00	2.82	988	–	S, MS, LRI	***	***	***
α-Terpinene	22.57	2.90	1014	998	S, MS, LRI	***	***	***
p-Cymene	23.08	3.05	1022	1009	S, MS, LRI	NS	NS	NS
Limonene	23.26	2.92	1024	1013	S, MS, LRI	*	***	***
Eucalyptol	23.52	2.91	1026	1018	S, MS, LRI	NS	**	**
cis-β-Ocimene	24.05	2.86	1032	1030	S, MS, LRI	NS	***	***
α-Terpinolene	26.23	2.99	1086	1078	S, MS, LRI	***	***	***
p-Cymenene	26.73	3.22	1089	1089	S, MS, LRI	***	NS	***
Linalool	26.94	3.00	1095	1093	S, MS, LRI	NS	***	***
Hotrienol	27.16	3.20	1101	1098	MS	***	***	***
α-Thujone	27.94	3.44	1101	1115	MS, LRI	***	**	***
Allocimene	28.33	2.94	1128	1124	MS, LRI	*	***	***
Nerol Oxide	29.65	3.18	1154	1153	MS, LRI	***	***	***
cis-Pyran linalool oxide	30.74	3.30	1170	1176	MS, LRI	***	***	***
trans-Pyran linalool oxide	31.03	3.27	1173	1183	MS, LRI	NS	***	***
Borneol isomer	31.08	3.20	1165	1184	S, MS, LRI	NS	***	***
Menthol	31.16	3.06	1167	1186	S, MS, LRI	NS	NS	NS
4-Terpineol	31.33	3.16	1174	1189	S, MS, LRI	NS	***	***
α-Terpineol	32.12	3.23	1186	1207	S, MS, LRI	NS	***	***
Myrtenol	32.25	3.30	1194	1209	MS, LRI	*	***	***
D-Verbenone	33.00	3.64	1204	1226	MS, LRI	NS	***	NS
Nerol	33.25	3.18	1227	1231	S, MS, LRI	***	*	***
Z-Citral	34.06	3.33	1235	1249	MS, LRI	***	**	***
Geraniol	34.46	3.20	1249	1258	S, MS, LRI	***	***	***
Sesquiterpenes								
δ-Elemene	38.32	2.80	1335	1342	MS, LRI	***	***	***
α-Cubebene	38.87	2.80	1345	1354	MS, LRI	***	***	***
α-Ylangene	40.01	2.84	1373	1379	MS, LRI	***	NS	***
α-Copaene	40.30	2.82	1374	1386	MS, LRI	**	**	NS
α-Gurjunene	41.69	2.84	1409	1418	MS, LRI	***	***	***
Cedrene	42.25	2.80	1410	1432	MS, LRI	***	***	***
β-Caryophyllene	42.30	2.89	1417	1434	S, MS, LRI	***	***	***
cis-Thujopsene	42.48	2.83	1429	1438	MS, LRI	***	NS	***
β-Copaene	42.58	2.78	1430	1441	MS, LRI	**	***	***
α-Guaiene	42.69	2.83	1437	1443	MS, LRI	***	***	***
Aromadendrene	43.43	2.87	1439	1462	MS, LRI	**	**	NS
α-Humulene	43.77	2.89	1452	1470	S, MS, LRI	***	***	NS
Alloaromadendrene	44.43	2.88	1458	1487	MS, LRI	NS	***	NS
α-Amorphene	44.54	2.88	1483	1490	MS, LRI	***	***	***
δ-Selinene	44.84	2.89	1492	1497	MS, LRI	***	**	NS
Valencene	45.14	2.87	1496	1505	S, MS, LRI	***	***	***
α-Muurolene	45.47	2.88	1500	1515	MS, LRI	***	***	***
δ-Cadinene	45.93	2.89	1522	1528	MS, LRI	***	***	***
cis-Calamenene	46.18	2.97	1528	1535	MS, LRI	***	***	***
α-Cadinene	46.68	2.87	1537	1549	MS, LRI	**	***	**
α-Calacorene	46.97	3.10	1544	1558	MS, LRI	*	***	*
α-Corocalene	49.50	3.16	1622	1633	MS, LRI	***	***	*
Cubenol	49.91	2.92	1645	1646	MS, LRI	NS	NS	NS
Cadalene	51.39	3.23	1675	1693	MS, LRI	***	***	***
C_{13} - Norisoprenoids								
β-Cyclocitral	33.33	3.43	1217	1233	S, MS, LRI	***	***	***
Vitispirane	36.06	3.11	1286	1293	MS, LRI	***	NS	***
TDN	39.66	3.37	1354	1372	MS	***	***	***
β-Damascenone	40.50	3.39	1383	1390	S, MS, LRI	**	NS	***
α-Ionone	42.33	3.13	1428	1434	MS, LRI	***	NS	NS
β-Ionone	44.57	3.18	1487	1490	S, MS, LRI	***	***	***

[a] [1]DRt(min): first dimension retention time; [b] [2]DRt(min): second dimension retention time; [c] LRI_{calc}: The linear retention index values were calculated through analysis of the commercial hydrocarbon mixture C_8–C_{20}; [d] LRI_{lit}: The linear retention index values from the literature for a 5 % phenyl polysilphenylene-siloxane column; ID - type of identification: S - comparison with retention time and mass spectrum of pure standards (mixture of terpenes called MegaMix #1 (Restek, Bellefont, PA) and commercial hydrocarbon mixture C_8–C_{20} (Supelco, Bellefont, PA, USA) and with NIST mass spectra electronic library; LRI - comparison with linear retention index from literature; MS - comparison with mass spectra from the NIST library or the literature. Statistically significant at $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$, respectively. NS: Not significant.

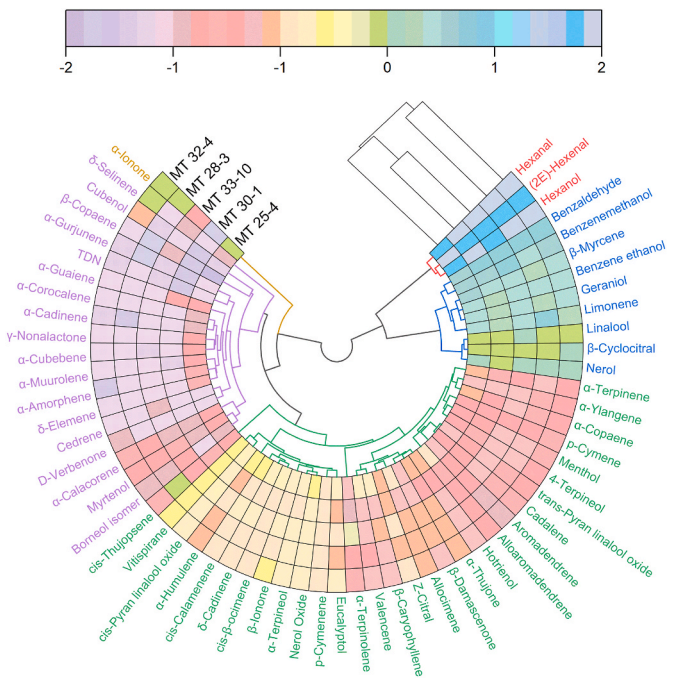

Fig. 4. Polar heatmaps with dendrogram of free and bound volatile compounds identified in clones of Moreto grapes: M 1, M 2, M 3, M 4, M 5.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Daniela Fonseca reports financial support was provided by European Commission, through "Vine&Wine - Driving Sustainable Growth Through Smart Innovation" project n. o C644866286-011 "Mobilizing Agendas for Business Innovation" under the Recovery and Resilience Program. - If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to acknowledge MED https://doi.org/10.54499/UIDB/05183/2020 and CHANGE https://doi.org/10.54499/LA/P/0121/2020.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.117314.

Data availability

Data will be made available on request.

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