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A test-tube vinification method for high-throughput characterisation of the oenological and aromatic potential of white wines

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ABSTRACT

The quality of wine largely depends on aroma perception, but the genetic basis explaining the variations of aroma compound concentrations in wines is still fragmentary. To unravel links between genetic variations and aroma compound variations in hundreds of genotypes, we developed a small-scale, high-throughput test-tube vinification (TTV) method capable of producing white wines that reveal the genetic potential at the scale of a single vine stock. We evaluated this method on commercial grapevine varieties (Riesling, Gewurztraminer, Chardonnay, Chasselas, Floreal, Muscat à petits grains blancs) and genotypes resulting from a bi-parental cross, covering a wide aromatic palette. The wines produced were described by usual oenological parameters and GC-MS profiling of volatile compounds. We compared the wines obtained with the TTV method to commercial wines and to wines obtained from larger fermentation volumes (5–10 L). Our results show that the TTV method is suitable to produce white wines on a very small scale, i.e., less than 100 mL and that these small-scale wines faithfully reflect the aromatic potential of the different varieties, as would larger volume methods. The proposed method is a high-throughput approach to assess the oenological potential of hundreds of grapevine genotypes from grape material harvested on a single vine. This wine-focused direct phenotyping method will pave the way for a better understanding of the genetic determinism of wine aromas, especially for molecules that are not directly present in grapes, such as volatile thiols and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN).

KEYWORDS: Small-scale winemaking, wine, volatile compound, aromatic potential, genetic variability, GC-MS

INTRODUCTION

The aroma of grapes is one of the major traits considered in the selection of grapevine varieties through centuries (Lacombe *et al.*, 2012) and is still a crucial determinant of wine quality. A wide variety of compounds contribute to the aromatic profile of grapes; the main classes include mono- and sesquiterpenes, norisoprenoids, methoxypyrazines, furan derivatives, and products of the lipoxygenase and phenylpropanoid pathways (Ebeler and Thorngate, 2009; Robinson *et al.*, 2014). Although variations in aromatic potential between grape varieties have long been documented, the genetic determinants of grape aromas are still relatively poorly characterised (Lin *et al.*, 2019). In recent years, several studies have aimed at deciphering the genetic determinism of the biosynthesis of grape aromas. The combination of quantitative trait locus (QTL) mapping with candidate gene characterisation has led to the identification of genes involved in the biosynthesis and metabolism of grape volatile compounds such as monoterpenes (Doligez *et al.*, 2006; Battilana *et al.*, 2009; Duchêne *et al.*, 2009; Ilc *et al.*, 2016; Lin *et al.*, 2020), sesquiterpenes (Bosman *et al.*, 2023) or methoxypyrazines (Guillaumie *et al.*, 2013; Dunlevy *et al.*, 2013). However, knowing the grape composition is not sufficient to accurately predict the quality and the characteristics of the wine.

Indeed, yeast-derived aroma compounds produced during alcoholic fermentation include alcohols, esters, aldehydes, and volatile fatty acids, which considerably influence the organoleptic properties of wines. Alcohols and esters quantitatively constitute the majority of the volatile components in wines (Cordente *et al.*, 2012). Alcohols can impart a strong, pungent smell and taste when present in high concentrations but result in a fruity character at low concentrations. Up to thirty odorant esters were quantified in wines (Antalick *et al.*, 2014), and among them, the fatty acid ethyl esters and the acetate esters are known to contribute to the fruity notes of wines (Ebeler and Thorngate, 2009). The formation of these compounds is influenced by the yeast strain used for fermentation (Lambrechts and Pretorius, 2000). Thus, in the last decade, studies have been used with several wine yeasts to dissect the genetic determinism of metabolic traits such as acetic acid production (Marullo *et al.*, 2006), adaptation to sulfite (Zimmer *et al.*, 2014) or fermentation rates (Ambroset *et al.*, 2011). Similarly, QTL mapping was also used for the detection of genomic regions influencing the production of volatile compounds by yeast during wine alcoholic fermentation (Steyer *et al.*, 2012).

Although many studies have investigated the genetic bases of grape aroma biosynthesis (Lin *et al.*, 2019) and despite the increasing knowledge about wine composition, the genetic bases of wine aroma compound diversity are still relatively unexplored. To date, studies have been undertaken to identify grapevine genomic regions influencing the production of grape aroma compounds, but not directly wine aroma compounds. This is explained by the difficulty of performing simultaneously the large numbers of vinifications required to

achieve sensitive QTL mapping using large populations in segregation (Eder *et al.*, 2018). To meet this challenge, high-throughput vinification methods that can be used to produce wine from single vines in segregating progenies are required. High-throughput fermentation methods have been developed for the characterisation of yeast strains for producing wine (Liccioli *et al.*, 2011) or beer (Zhao *et al.*, 2022), albeit with different purposes.

In this work, we developed a small-scale and high-throughput vinification method in test tubes to assess white grapevine varieties' oenological and aromatic potential. This method was validated using ten varieties covering a broad aromatic palette and compared to vinifications in larger volumes: carboy vinification (CV) (5 – 10 L) and commercial wines.

MATERIALS AND METHODS

1. Plant material

Grape material used in this study originated from a selection of grapevine varieties and genotypes (Table 1), including the traditional grapevine *Vitis vinifera* varieties Riesling, Gewurztraminer, Chardonnay, Chasselas, Muscat à petits grains blancs, one commercial hybrid variety (Floreal) and genotypes from a biparental cross between Riesling (Ri) and Gewurztraminer (Gw) (Ri × Gw) cultivated in the INRAE vineyard in Bergheim (48.215750 N, 7.347083 E) (Duchêne *et al.*, 2012). Four genotypes from the progeny were selected to produce small-scale wines based on the contrasting aromatic character of the grapes, that was evaluated by tasting the berries in the vineyard at maturity to classify them as neutral, aromatic Muscat-like, aromatic Gewurztraminer-like and aromatic Riesling-like. 0238E was aromatic Gewurztraminer-like, 0074E and 4095G were aromatic Muscat-like and 0211E was aromatic Riesling-type.

2. Test-tube vinification (TTV)

Grapes (1 to 2.5 kg) were hand-harvested from at least 3 plants per genotype on October 2nd, 2019. Berries were destemmed and pressed using an Automatic Sieve C80® (ROBOT COUPE SNC, Vincennes, France). The juice was automatically separated from the waste constituted by skins, seeds, and stalks. Fifteen mL of juice were aliquoted to determine the initial grape-must composition (pH, sugar concentration, total acidity, and assimilable nitrogen). After extraction, the juice was protected by the addition of SO₂ (60 mg/L) and centrifuged (1427 g for 10 minutes at 16 °C). The pectolytic enzyme (Enzylia HCL 1 mL/hL from AEB-France, Sigolsheim, France) was added to improve the settling of the grape must. After 24 h of decantation at 10 °C and racking, the musts were complemented when necessary i) with rectified concentrated must (Oenofrance, Eguisheim, France) to reach at least 200 g/L of sugars, ii) with diammonium phosphate (Azoferm from AEB-France, Sigolsheim, France) to reach at least 200 mg/L assimilable N to prevent sluggish fermentations (Conde *et al.*, 2007). 85 mL of musts were then transferred to 100 mL glass tubes (3 tubes for Muscat, 5 tubes for the other genotypes) and were fermented after active dry yeast addition at 0.2 g/L.

The yeast used was the commercial strain *Saccharomyces cerevisiae* var. *bayanus* (Levulia® GC from AEB-France, Sigolsheim, France) and was prepared by rehydrating the yeast at 10 times its weight in water at 25 °C for 30 min. The five tubes were used as replicates for each wine except for Muscat (3 replicates). The tubes were plugged with a rubber stopper fitted with a flexible bubbler tube whose end was immersed in a tube of water to prevent any oxidation of the wine during fermentation (Supplementary Figure 1). Alcoholic fermentation was performed in a temperature-controlled environment at 22 °C. The end of the alcoholic fermentation was visually estimated, i.e., when dead yeasts had sunk to the bottom of the tubes, and the release of CO₂ had stopped. Fermentations were not weight-monitored due to the technical challenges associated with managing this process, which might be amenable to hundreds of genotypes. After fermentation, the wines were decanted after cooling to 10 °C. They were then stabilised with 80 mg/L of sulfur dioxide (potassium metabisulfite 15 % solution from AEB-France, Sigolsheim, France) and stored in a climatic chamber at 10 °C for 2 months before analysis.

The implementation of 50 TTVs required an area of 0.3 m². Two people managed the pre-fermentation operations (destemming, crushing, extraction of juices, settling, yeasting), while the follow-up of the vinifications was carried out by a single person.

3. Carboy vinification (CV)

Grapes (10 to 16 kg) of Riesling, Gewurztraminer, Chardonnay, and Floreal were harvested between September 19th and October 9th, 2019 (Table 1). The whole grapes were destemmed and pressed, and then the free-run juice was allowed to settle at 4 °C for one day after the addition of SO₂ (60 mg/L). The juices were then fermented in 5 or 10 L

carboys at 22 °C after yeast inoculation with the same yeast strain (0.2 g/L) as described above. All vinifications were protected from oxidation by CO₂ inerting. The area required to set up the CV was 8 m². Fermentations were considered to be complete when the density of wine approached 995 g/L. After fermentation, the wines were racked. The clear wines were stabilised with 80 mg/L of sulfur dioxide (potassium metabisulfite 15 % solution from AEB-France or Sulfivin K150® from IOC, Epernay, France) and stored in the INRAE wine cellar at 15 °C in bottles until analysis. Three bottles were used as replicates for each wine and analysed in March 2021.

4. Commercial wine samples

Nine commercial wines (3 Riesling, 2 Gewurztraminer, and 4 Chardonnay) from vintage 2019, different geographical origins, and different winemakers were selected (Table 1). One bottle of each wine was purchased from winemakers or local wine stores and analysed in triplicate. Aromatic quality, typicality, and absence of defects in the wines were assessed by a panel composed of three females and two males belonging to the laboratory staff. All of them had extensive wine-tasting experience.

5. Analyses of grapes and wines

The pH was measured with a 340i pH-meter (WTW, Weilheim, Germany), and titratable acidity (TA) of the must was measured with a TitroLine® titrator (SI Analytics, Mainz, Germany) and expressed as g/L of sulfuric acid (H₂SO₄). A PAL-1 portable electronic refractometer (ATAGO, Tokyo, Japan) was used to determine the total soluble solids (TSS) of the berry juice and, consequently, the sugar content (g/L). The potential alcohol content of the berries (PAC, % v/v) is calculated with a coefficient of 16.83 g/L of sugar required

TABLE 1. Overview of the wines produced in 2019 according to three methods.

Variety or Genotype	Test-tube vinification (TTV) (85 mL)	Carboy vinification (CV) (5–10 L)	Large volume vinification LV (> 100 L)
Chardonnay	F*-Colmar Huben	F-Colmar Huben	F-Chablis F-Limoux F-Fontenay F-Saint Frichoux
Gewurztraminer	F-Bergheim	F-Wintzenheim Rotenberg	F-St Hippolyte F-Amerschwyr
Riesling	F-Bergheim	F-Bollenberg	F-Ribeauvillé F-Eguisheim G-Durbach
Floreal	F-Colmar Huben	F-Colmar Huben	
Chasselas	F-Colmar Huben		
Muscat à petits grains blancs	F-Colmar Huben		
0074E	F-Bergheim		
0211E	F-Bergheim		
0238E	F-Bergheim		
4095G	F-Bergheim		

*F: France; G: Germany.

to produce 1 % volume of alcohol (Ribéreau-Gayon *et al.*, 2006). Glucose and fructose content (g/L), ammonium nitrogen ($[\text{NH}_4^+]$, mg/L), and α -amino nitrogen ($[\alpha\text{-NH}_2]$, mg/L) were determined using a UV-visible Maxi-analyser (Oenolab diagnostics, Hendaye, France). Yeast assimilable nitrogen (YAN) content was calculated as the sum of $[\text{NH}_4^+]$ and $[\alpha\text{-NH}_2]$. The alcohol content (% v/v) was determined by near-infrared spectrometry using an Alcoquick 4000 analyser (Oenolab Diagnostics, Hendaye, France).

6. Analysis of volatile compounds by headspace-solid phase micro-extraction and gas chromatography-mass spectrometry

6.1. Extraction and analysis

Volatile compounds were extracted from wines by headspace solid phase micro-extraction (HS-SPME). Three mL of wine were transferred into a 20 mL SPME vial containing 3 mL of NaCl 5M, 100 μL of Na_2SO_3 at 10 g/L, and 10 μL of 3-octanol at 10 mg/L (internal standard). The mixture was homogenised with a vortex shaker for 30 s, and then the volatiles were sampled by HS-SPME with a DVB/CAR/PDMS fibre (divinylbenzene/Carboxen/polydimethylsiloxane, 50/30 μm , StableFlex, Supelco). Incubation and extraction were performed at 40 °C for 20 and 50 min, respectively, under continuous agitation (250 rpm). After extraction, the fibre was desorbed in the injector at 250 °C for 5 min. The incubation, extraction, and desorption of volatile compounds were performed automatically by an MPS2-XL autosampler (Gerstel GmbH & Co.KG, Germany). The gas chromatography analyses were carried out in a 6890N gas chromatograph (Agilent, CA, USA) coupled to a 5975 single quadrupole mass spectrometer (Agilent, CA, USA). The injections were performed in splitless mode for 1 min, and the volatile compounds were analysed using a DBWax 30 m \times 0.32 mm \times 0.5 μm column (Agilent J&W, CA, USA) with helium as the carrier gas at a constant flow of 1.5 mL/min. The oven temperature conditions were 45 °C for 1 min, increasing by 20 °C/min to 82 °C held for 1 min, then 2.7 °C/min to 235 °C, held for 16 min. The mass detector was operated in electronic ionisation mode (70 eV). The source and transfer line temperatures were 230 °C and 270 °C, respectively. The detection was performed in scan mode in the range between m/z 29 and 400. Chromatograms and spectra were recorded using the Agilent ChemStation software (B.07.06). A pooled quality control (QC) sample was prepared by mixing same-volume aliquots of all wine samples (3 mL of each). The extraction and analysis sequence of all samples was randomised to avoid systematic bias. A blank (empty vial) and a QC sample were analysed at the beginning and the end of each sequence and between each series of 10 samples to assess sample stability during analysis as well as inter/intra-day variability. Injection of a liquid mixture of n-alkane standards (C_7 - C_{35}) was also run to calculate linear retention indices (LRI) (van Den Dool and Kratz, 1963).

6.2. Data processing

Raw data acquired with the Agilent ChemStation software (B.07.06) were converted using the Agilent MassHunter GC/

MS Translator software (B.07.04) and processed using the Agilent MassHunter Unknown Analysis software (B.08.00).

First, deconvolution was performed using data from QC samples as a representative sample in which all volatile metabolites were expected to be present. A library search with the NIST14 database was carried out to identify the detected compounds. Each proposed identification was manually checked and confirmed by comparison with mass spectra and retention time of standards. When standards were not available, identification was confirmed by LRI published in the literature. Unidentified metabolites were coded as MxTy, where x is the m/z and y is the retention time in seconds. Compounds originating from the SPME fibre or bleeding of the column (SiO_2 derivatives) and contaminants were removed. Finally, 108 volatile compounds were identified in the QC samples representative of all the wines analysed (Supplementary Table 1).

Second, the list of the compounds found in the QC samples was then exported in .CEF (Compound Exchange Format) and a targeted analysis method was built using the Agilent MassHunter Quantitative Analysis software (B 08.00). The ions monitored for the target compounds are presented in Supplementary Table 1. All integrations obtained by the targeted quantitative analysis method were manually inspected before validation. Peak areas were normalised by dividing the area of each peak by the area of the internal standard 3-octanol, and then this ratio was multiplied by the mean area of the internal standard in all samples. As absolute concentrations were not available, normalised peak area (NPA) will be used throughout the text.

7. Analysis of varietal thiols

4-Methyl-4-sulfanylpentan-2-one (4MSP), 3-sulfanylhexan-1-ol (3SH), and 3-sulfanylhexyl acetate (3SHA) were quantified in wines by gas chromatography-tandem mass spectrometry (GC-MS/MS) adapted from Thibon *et al.* (2015). Briefly, 20 mL of wine sample were spiked with 50 μL of 6-sulfanylhexanol (6SH, 30 $\mu\text{mol/L}$, EtOH), 4-methoxy-2-methyl-2-butanethiol (MMBT, 30 $\mu\text{mol/L}$, EtOH), and ethyl maltol (EM, 100 $\mu\text{g/L}$, EtOH) as internal standards. The wines were percolated through an SPE column (HR-X, 500 mg 6 mL, Macherey Nagel, France) previously conditioned with 6 mL of methanol and 2 mL of water. After the adsorption step, the SPE columns were rinsed twice with 2 mL of water/ethanol (90/10, v/v), and the compounds were eluted with 3 mL of pentane/dichloromethane (50/50; v/v), followed by 3 mL of dichloromethane/methanol (95/5; v/v). Organic phases obtained were blended, dried over anhydrous sodium sulfate, and concentrated to 150 μL under a nitrogen stream.

8. Statistical analysis

Statistical analyses and data visualisation were carried out using different packages of the R software version 4.1.0 (R Core Team, 2021). Boxplots were obtained through the ggplot2 package (Wickham, 2016) and used to highlight the variability of the volatile compounds. Principal component analysis (PCA) was conducted using the factoextra package

(version 1.0.7) (Kassambara and Mundt, 2020). Partial least square-discriminant analysis (PLS-DA) was performed using the mixOmics package (version 6.6.2) (Rohart *et al.*, 2017), and its performance was assessed through 5-fold cross-validation (CV) repeated 10 times. To estimate variable contribution, variable importance on projection (VIP) scores were calculated, and variables with VIP scores exceeding 1.00 were considered as the most relevant variables. Clustered heatmaps were constructed using the pheatmap package (version 1.0.12) with the complete linkage method based on Euclidean distance. Differential analyses among the different grape varieties were performed using Tukey's honest significant difference method followed by false discovery rate (FDR) correction using the Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995). Metabolites of interest were considered significantly different when the false discovery rate was below 5 % (FDR < 0.05).

RESULTS

1. Oenological parameters of grape and wine

Before the fermentation process, the grape juices intended for TTV were analysed to determine the oenological parameters (Supplementary Table 2A). Among the grapevine *Vitis vinifera* varieties, Gewurztraminer had the highest sugar content (245.9 g/L) and, consequently, the highest PAC (14.6 % v/v). Riesling had the lowest pH (3.11) and high TA (5.5 g/L H₂SO₄). Floreal had the lowest PAC (11.71 % v/v), and Chardonnay had the lowest YAN (128 mg/L). Among the genotypes from the Ri × Gw progeny, two of them were close to the parental Gw (0074E and 0211E) with high sugar content (236.45 and 243.50 g/L, respectively), whereas the two others (0238E and 4095G) were close to the parental Ri with low pH (3.06 and 3.11, respectively).

For the wines obtained by TTV, fermentations were completed between 15 and 25 days (Supplementary Table 2A). The oenological parameters are shown in Table 2. The results showed that almost all sugar was fermented into alcohol, yielding dry wines, as the maximum residual sugar concentration was 4.4 g/L. Riesling had the lowest pH (2.82), whereas Gewurztraminer had the highest (3.65). The alcohol content of the wines obtained was between 11.0 and 15.1 % v/v for Chasselas and genotype 0211E, respectively.

The oenological parameters for the wines obtained by CV are shown in Table 2. Riesling had the lowest pH (3.08). Chardonnay and Floreal were the driest wines, with residual sugar contents of 3.07 and 0.73 g/L, respectively. In contrast, Gewurztraminer (8.30 g/L) and Riesling (12.10 g/L) are classified as medium dry and medium sweet wines, respectively, according to the Regulation of the European Community no 753/2002 (Commission Regulation, 2002).

2. Volatile compounds profiling of wines obtained by test-tube vinification

A total of 108 volatile compounds were detected by HS-SPME-GC-MS in wines obtained by TTV. The main classes included esters (E) with 33 detected compounds (30.6 % of the total number of detected compounds), monoterpenes (MT) with 27 detected compounds (25 %) and alcohols (AL) with 13 detected compounds (12.0 %). Eight benzenoids (BZ, 7.4 %), 8 volatile fatty acids (FA, 7.4 %), 7 sesquiterpenes (ST, 6.5 %), 4 norisoprenoids (C13, 3.7 %), 2 aldehydes and ketones (CO, 1.9 %) and 2 volatile phenols (VP, 1.9 %) were also detected. Some molecules from the lactones and sulfur-containing volatiles family were also detected (Figure 1A).

TABLE 2. Oenological parameters of the wines obtained by test-tube vinification (TTV) and carboy vinification (CV). Data are given by the mean ± standard deviation (n = 5 for TTV except for Muscat n = 3 and n = 3 for CV).

Variety or Genotype	TTV (85 mL)			CV (5–10 L)		
	pH	Glucose + Fructose (g/L)	Alcohol (% v/v)	pH	Glucose + Fructose (g/L)	Alcohol (% v/v)
Chardonnay	3.30 ^{d*} ± 0.02	1.44 ^{bcd} ± 1.52	13.8 ^{ab} ± 0.25	3.23 ^b ± 0.01	3.07 ^c ± 0.29	14.2 ^a ± 0.13
Floreal	3.53 ^b ± 0.02	0.44 ^{cd} ± 0.31	12.6 ^{ab} ± 0.88	3.38 ^a ± 0.01	0.73 ^d ± 0.21	12.4 ^c ± 0.06
Gewurztraminer	3.65 ^a ± 0.04	0.94 ^{cd} ± 0.68	15.0 ^a ± 1.27	3.18 ^c ± 0.01	8.30 ^b ± 0.17	13.6 ^b ± 0.03
Riesling	2.82 ^a ± 0.02	4.42 ^a ± 2.28	13.0 ^{ab} ± 0.85	3.08 ^d ± 0.01	12.10 ^a ± 0.00	14.0 ^a ± 0.07
Chasselas	3.39 ^c ± 0.02	0.30 ^d ± 0.00	11.0 ^b ± 1.67			
Muscat à petits grains blancs	3.03 ^e ± 0.01	1.43 ^{bcd} ± 1.04	14.2 ^{ab} ± 0.86			
0074E	3.31 ^d ± 0.02	3.26 ^{abc} ± 1.41	15.0 ^a ± 1.22			
0211E	3.05 ^e ± 0.02	4.02 ^{ab} ± 2.4	15.1 ^a ± 0.72			
0238E	2.92 ^f ± 0.02	1.04 ^{cd} ± 1.26	13.2 ^{ab} ± 2.87			
4095G	2.90 ^f ± 0.02	0.78 ^{cd} ± 0.69	15.1 ^a ± 0.47			

*Different letters indicate significant differences at p ≤ 0.05, according to Tukey's honest significant difference test.

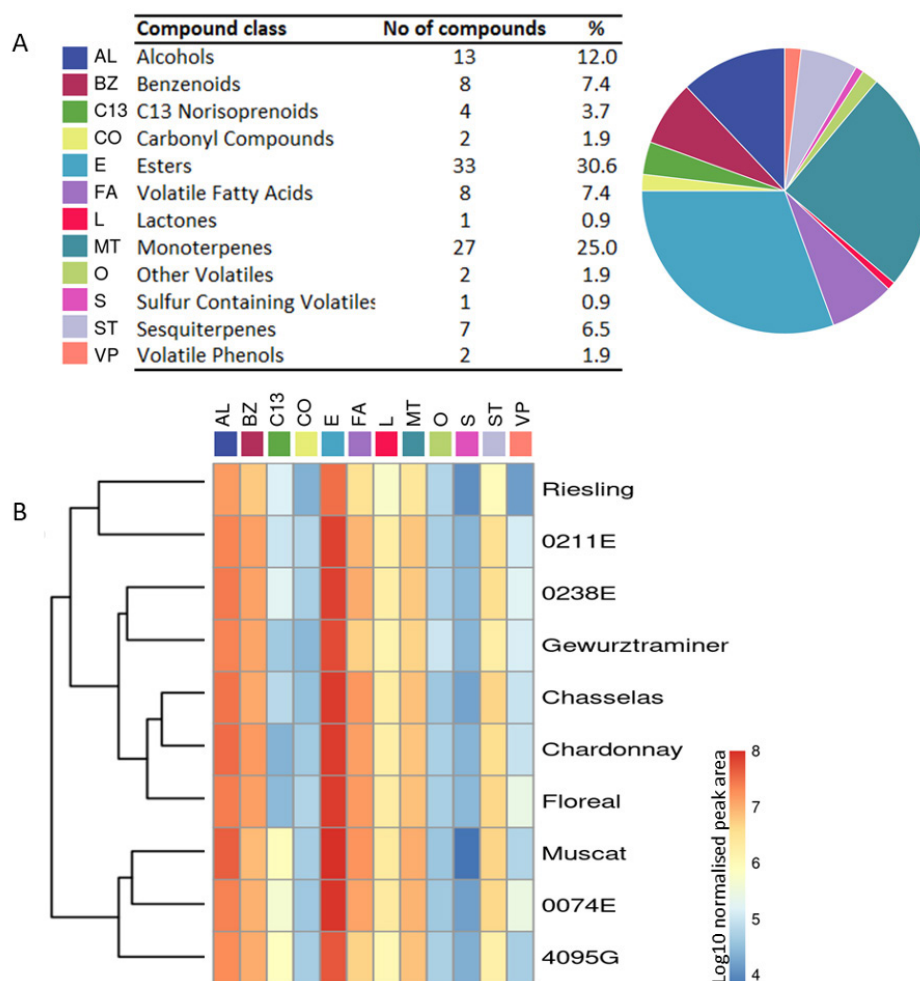


FIGURE 1. HS-SPME-GC-MS volatile profiling of 2019 wines obtained by test-tube vinification from grapes of Riesling, Gewurztraminer, Chardonnay, Chasselas, Floreal and Muscat à petits grains blancs varieties and genotypes (0074E, 0211E, 0238E, 4095G) from the cross between Riesling and Gewurztraminer.

Volatile compounds are grouped as alcohols (AL), benzenoids (BZ), norisoprenoids (C13), carbonyls compounds (CO), esters (E), volatile fatty acids (FA), lactones (L), monoterpenes (MT), sulfur-containing volatiles (S), sesquiterpenes (ST), volatile phenols (VP) and other volatiles (O). (A) The number of volatile compounds by class is indicated and expressed as a percentage of the total number of detected compounds. (B) Log₁₀ normalised peak areas for each class of compounds are given by shades of red, yellow or blue colours according to the scale bar. Hierarchical clustering highlights similarity among varieties or genotypes.

A heatmap depicting the total areas for each class of compounds provides a visual representation of the distribution across the different varieties or genotypes (Figure 1B). Of the chemical groups found in the volatile fraction of the wine samples, esters were present in the highest abundance, followed by alcohols, acids and benzenoids, whereas varietal compounds from the monoterpenes, norisoprenoids and sesquiterpenes families were present in lower abundance.

However, this overview per class of compounds should be considered with caution as it may not precisely depict the behaviour of individual compounds. This is notably the case of TDN, whose NPA was the highest in Riesling and 0238E (Figure 3 and Supplementary Figure 2), despite the apparent lower overall C₁₃ richness in these two wines (Figure 1B). In this case, the discrepancy can be attributed to the unknown compound M69T878, whose NPA was significantly greater

in Muscat, 4095G, and 0074E compared to the other wines (Supplementary Figure 2 and Supplementary Table 3).

A heatmap presenting the individual volatile compounds highlights high variation between varieties and genotypes (Supplementary Figure 2). The compounds with the largest NPA were isoamyl alcohol, isoamyl acetate, 2-phenylethanol, C₆, C₈, C₁₀ ethyl esters, and C₆ and C₈ alcohols and acids (Supplementary Figure 2). All these compounds are volatile metabolites produced by yeast during fermentation, supporting the fact that the TTV method produced actual wines. For example, isoamyl alcohol was found in high abundance in all varieties except for Riesling and 0074E (Supplementary Figure 4). Esters such as 2-phenylethyl acetate had the highest NPA in Chardonnay (Supplementary Figure 4). In addition, Chasselas had the highest NPA for octanoic, decanoic, and dodecanoic acids. We also detected variations among all the wines for the production

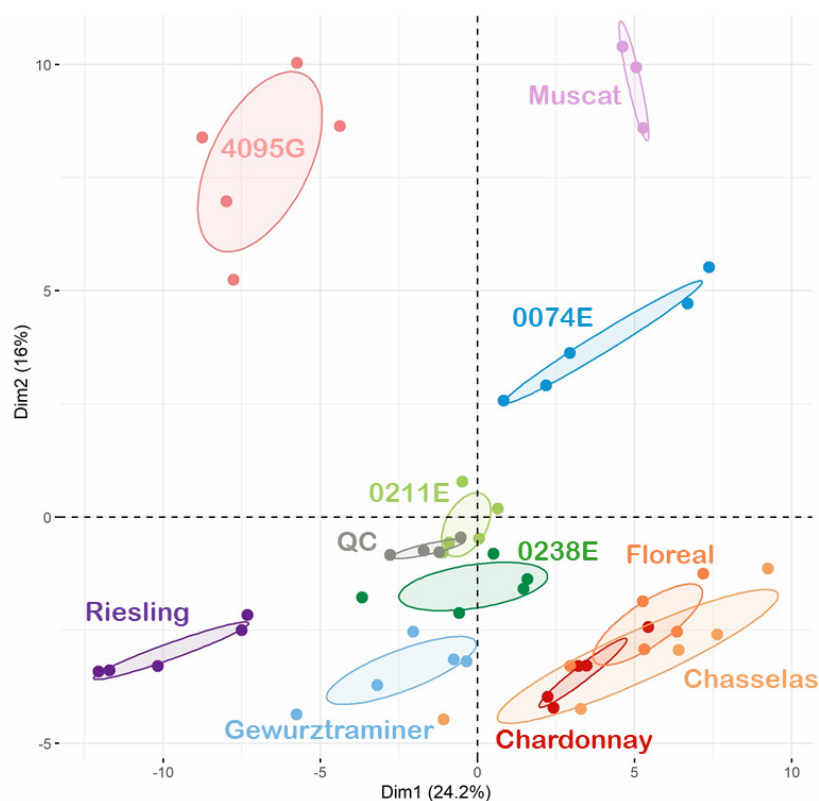


FIGURE 2. Principal component analysis (PCA) on the whole data set (including volatile compounds, oenological parameters, and varietal thiols) of 2019 wines obtained by test-tube vinification with the Riesling, Gewurztraminer, Chardonnay, Chasselas, Floreal and Muscat à petits grains blancs varieties and with the genotypes (0074E, 0211E, 0238E, 4095G) from the cross between Riesling and Gewurztraminer.

of alcohols, such as 1-pentanol, 1-hexanol, 2-ethyl-1-hexanol, and 2-methyl-1-propanol. Varietal compounds, such as monoterpenes or norisoprenoids, were present in much lower quantities. Muscat and genotypes 0074E and 4095G exhibited the highest abundance of monoterpenols such as linalool, α -terpineol, *trans*-2-pinanol or hotrienol. Their derivatives, such as linalyl acetate, nerol oxide, neryl acetate or terpenyl acetate, were also predominant in these three varieties. Gewurztraminer was the variety with the highest NPA for citronellol and terpendiol. Monoterpenes such as α -terpinene, β -myrcene, *trans*- β -ocimene, and limonene were detected with high NPA in Muscat and 4095G.

The hierarchical clustering of wines according to the profile of the volatile compounds detected (Figure 1B and Supplementary Figure 2) showed that the wines obtained from TTV were grouped in four clusters. The first cluster grouped intensely-flavoured Muscat-like varieties (Muscat, 0074E and, to a lesser extent, 4095G) at the opposite side of the cluster, which was composed of Riesling and 0211E. Gewurztraminer and 0238E formed the third cluster, while Floreal was classified close to the cluster of non-aromatic grape varieties (Chardonnay and Chasselas).

A principal component analysis (PCA) on the whole data set, including volatile compounds, oenological parameters, and varietal thiols of wines obtained by TTV, was performed to visualise the data set and to determine whether the TTV

method allows differentiation between grapevine varieties (Figure 2). QC samples formed a well-defined compact cluster near the centre of the plot, showing the high repeatability of the method. In the following data analysis, the QC samples were removed from the data set. The first two dimensions, explaining 24.2 % and 16 % of the variance, respectively, allowed a good differentiation of the wines. Muscat and 0074E appeared in the upper right part, while the 4095G genotype stood alone in the upper left part. Dimension 1 opposed Riesling on the left to non-aromatic grape varieties such as Chasselas and Chardonnay on the right of the graph. Dimension 2 tends to separate the aromatic Muscat-like varieties at the top from the other aromatic varieties (such as Gewurztraminer and 0238E). Supplementary Figure 3 shows the contribution of the variables to the principal components.

The differential analysis with TTV data identified 67 volatile compounds whose NPA was significantly different between grape varieties or genotypes (Supplementary Table 3). Among them, there were 20 monoterpenes, 18 esters, 7 alcohols, 5 benzenoids, 3 C_{13} -norisoprenoids, 3 sesquiterpenes, 2 volatile phenols and 3 varietal thiols. Figure 3 shows an arbitrary selection of molecules representing the main varietal types in the dataset. The other molecules for which there are statistical differences are shown in Supplementary Figure 4. 4095G and Muscat had the highest NPA of 14 monoterpenes, while varieties such as 0238E, Chardonnay, or Chasselas

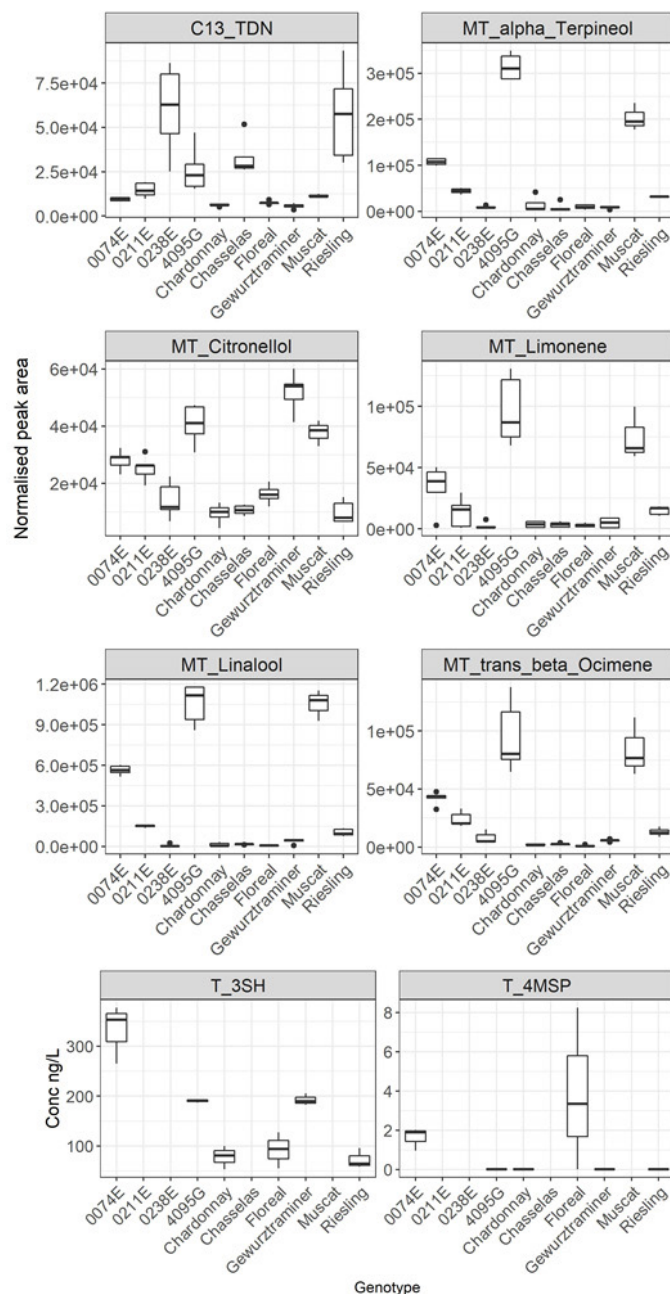


FIGURE 3. Variations of some significant volatile compounds of 2019 wines obtained by test-tube vinification with the Riesling, Gewurztraminer, Chardonnay, Chasselas, Floreal, and Muscat à petits grains blancs varieties and with the genotypes (0074E, 0211E, 0238E, 4095G) from a cross between Riesling and Gewurztraminer.

The results of pairwise comparisons are described in Supplementary Table 3. C13: norisoprenoids, MT: monoterpenes, T: varietal thiols.

had low NPA, if any, of terpenes. When compared to 0238E, we observed NPA up to 33-fold higher for α -terpineol in 4095G and up to 143-fold higher for linalool in Muscat (Supplementary Table 3). While 4095G and Muscat had high linalool and linalyl acetate NPA, we observed low NPA for *cis*-linalool oxide in comparison with the other varieties, except for Riesling. Similarly, limonene or *trans*- β -ocimene were present at high levels in Muscat and 4095G. For example, we observed a 25- and 65-fold difference between Muscat and Floreal for limonene and *trans*- β -ocimene, respectively. Citronellol showed the highest NPA in Gewurztraminer and

hotrienol in 0074E (up to 5 and 50 times when compared to Chardonnay, respectively). In addition, we noticed high NPA for TDN in Riesling and 0238E in comparison with other wines. Sesquiterpenols (nerolidol, *cis,cis*-farnesol and dihydrofarnesol) were not detected or were present with very low NPA in 4095G, Gewurztraminer, and Riesling, while Chasselas was the variety with the highest NPA of these C₁₅ compounds. Concerning the varietal thiols, 3SH was detected with greater abundance in Gewurztraminer, 4095G and especially in 0074E, while 4MSP was only detected in Floreal and 0074E.

3. Comparison of wines obtained by test-tube vinification and carboy vinification

First, PCA based on the whole data set for CV (volatile compounds, varietal thiols, and oenological parameters) was performed to ensure that the method allowed differentiation between grape varieties (Supplementary Figure 5). The first three dimensions, explaining 82.6 % of the variance (35.1 % for axis 1, 28.3 % for axis 2, and 19.2 % for axis 3), allowed a good differentiation of grape varieties. To differentiate the wines of Riesling, Gewurztraminer, Floreal, and Chardonnay obtained from both TTV and CV methods according to the volatile profile, we performed an unsupervised PCA using the significant volatile compounds previously identified in the differential analysis with the TTV method (ANOVA $p < 0.05$). We observed clear discrimination between Gewurztraminer and Riesling wines, while Chardonnay and Floreal wines did not exhibit complete separation (Figure 4A). In parallel, a supervised Partial Least Squares Discriminant Analysis (PLS-DA), a classification technique widely used for discrimination in metabolomics studies (Gronski *et al.*, 2015; Mendez *et al.*, 2020), was performed to identify the most discriminative compounds among varieties, regardless of the vinification method (Supplementary Figure 6). The PLS-DA identified 29 discriminative metabolites with VIP scores greater than 1.00 (Supplementary Table 4). 4MSP appeared to be the most contributory metabolite to the separation between Floreal and the other samples, as also illustrated in Figure 3 and Figure 4B.

4. Discrimination of wines obtained by different vinification methods according to the grape varieties

We next compared the profiling of the volatile compounds of wines obtained with the TTV method to those obtained by small-scale vinification and traditional white winemaking. For this, wines from three commercial grapevine varieties (Riesling, Gewurztraminer, Chardonnay) were selected. Prior to volatile profiling, the wines were briefly tasted by an expert panel, and the results confirmed that they were typical of the original grape variety and free from defects (data not shown). To visualise if the samples could be classified according to the grape variety regardless of the vinification method, an unsupervised PCA was performed on the volatile compounds datasets from wines produced using three different vinification methods (TTV, CV, and traditional winemaking). The first three dimensions accounted for 55.1 % of the variance (26.1 % for axis 1, 16.7 % for axis 2, and 12.3 % for axis 3). As shown in Figure 5, samples were clearly separated into three classes that represented the three grape varieties (*i.e.* Riesling, Gewurztraminer and Chardonnay).

DISCUSSION

Although the last decades have seen the rise of numerous studies deciphering the genetic determinism of grape aroma compounds, the knowledge is still fragmentary, especially for molecules that are not directly present in the musts, such as TDN or volatile thiols. Experimental wines are currently produced in carboys from 5 to 10 L but are not suitable for

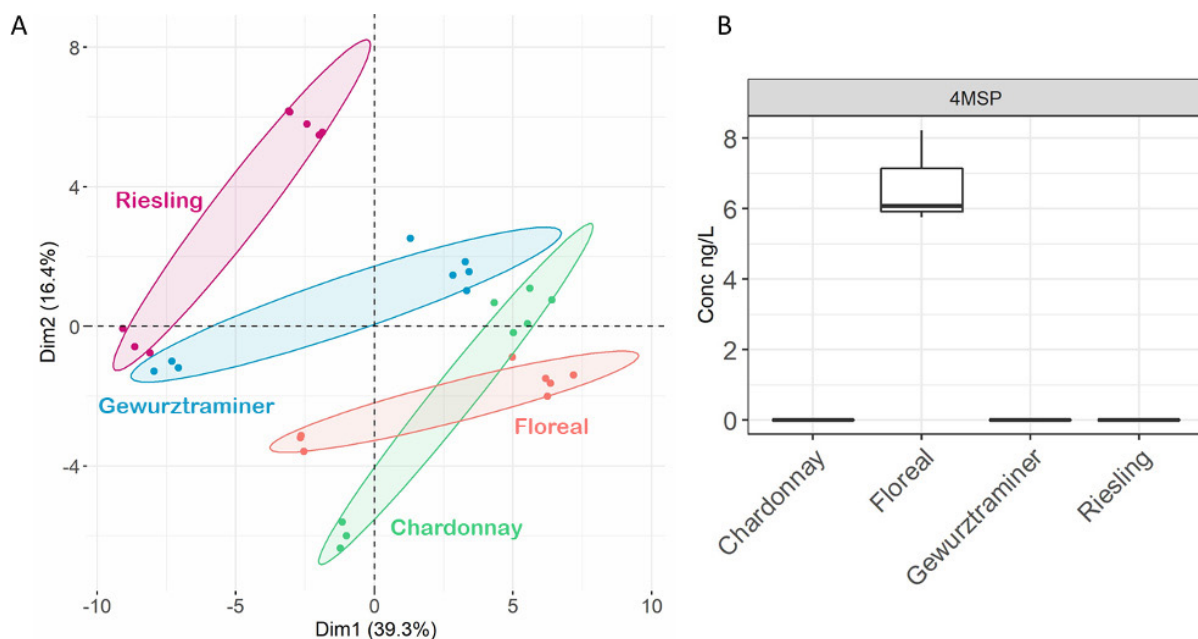


FIGURE 4. Discrimination of wines obtained by test-tube vinification (TTV) and carboy vinification (CV) with Gewurztraminer, Riesling, Chardonnay and Floreal varieties.

(A) Principal component analysis (PCA) of differential volatile compounds from 2019 wines obtained by TTV and CV. (B) The concentration of 4MSP in wines obtained by CV.

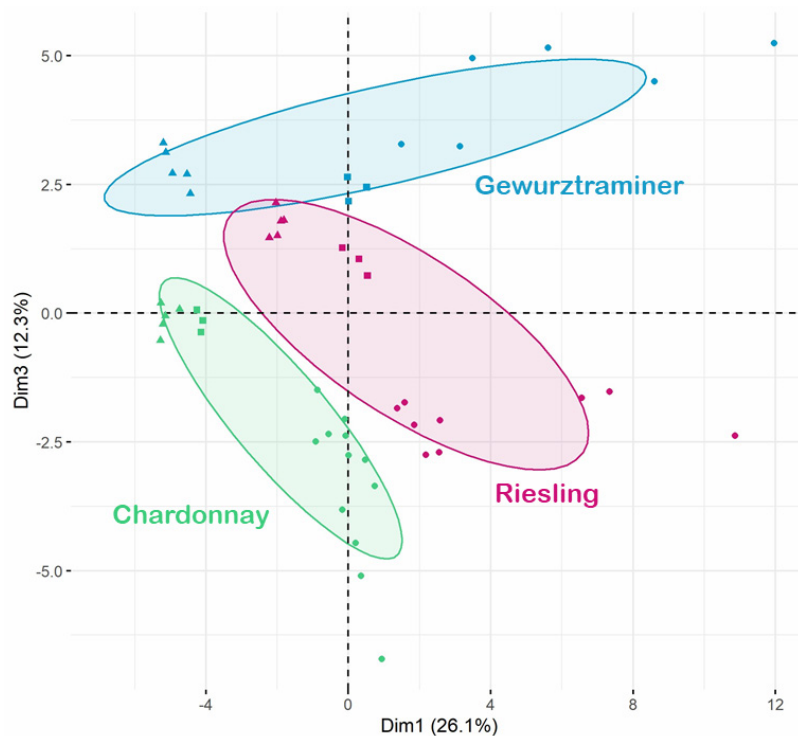


FIGURE 5. Principal component analysis (PCA) on the volatile compounds datasets from 2019 wines produced using three different vinification methods (triangles for test-tube vinification, squares for carboy vinification, and dots for traditional winemaking) with Riesling, Gewurztraminer and Chardonnay varieties.

genetic studies involving hundreds of genotypes. Hence, there is a need to develop a simple, standardised method on a very small scale.

In this work, we developed a specific protocol to produce white wines from less than 100 mL of must. TTVs were conducted from berries harvested at maturity and originated from commercial varieties (Riesling, Gewurztraminer, Chardonnay, Chasselas, Floreal, Muscat à petits grains blancs) and genotypes from a $Ri \times Gw$ progeny, representing a wide diversity of aromatic potential. Our first intention was to align closely with the local vinification practices. This is why we used a yeast strain that is empirically recognized locally for preserving the original aromatic characteristics of the grape. This yeast strain is commonly employed in the production of wines at INRAE Colmar, ensuring a faithful representation of the regional winemaking tradition.

The choice was also made to normalise sugar concentration and assimilable N to ensure that fermentation proceeds steadily to completion (Conde *et al.*, 2007). Moreover, it has been shown that YAN levels can influence aroma production during fermentation (Garde-Cerdán and Ancín-Azpilicueta, 2008; Hernández-Orte *et al.*, 2005; Hernández-Orte *et al.*, 2006).

If we had not adjusted the observed differences in N content in the musts among the different grape varieties (Supplementary Table 2A), we would have obtained aromatic profiles that would have confused the effects of genotype

with those of nitrogen availability. The influence of genetics on must composition, in particular acidity and YAN, and its subsequent impact on aromatic profiles remains a topic that warrants independent consideration.

Small-scale vinification procedures have been used by Keyzers and Boss (2010) and Boss *et al.* (2018), albeit without emphasis on high-throughput characteristics and comparison with traditional winemaking. The main objective of this work was to develop a TTV method that can produce wines that meet the standards of oenological criteria and is suitable to reveal genetic differences in aromatic profiles. To this end, the quality of the wines produced was assessed according to two main criteria: the oenological parameters and the wine aromas through the profiling of volatile compounds. In addition, the wines obtained with the TTV method were compared to those obtained by laboratory-scale and traditional white wine vinification methods.

All the fermentations conducted with the TTV method almost reached dryness within 25 days, and the resulting wines exhibited oenological parameters (pH, residual sugar, and alcohol content) consistent with the expectations (Table 2 and Supplementary Table 2A). All the wines analysed complied with the usual oenological standards. These results prove that the method we developed is adapted, from an oenological point of view, to the vinification of white wines in very small volumes.

Numerous studies have been conducted over the last decades to better understand the origin of grape and wine aroma (Guth, 1997; Ferreira *et al.*, 2000; Francis and Newton, 2005; Ebeler and Thorngate, 2009). Given the importance of the aroma on the quality of the wine, we analysed the volatile compounds of the wine by GC-MS. Among the volatile compounds detected, fermentation-derived aroma compounds, including volatile fatty acids, esters, and higher alcohols, were predominant and detected NPA were greater than those of varietal compounds. In agreement with previous works (Bakker and Clarke, 2012), esters were the most represented class regarding the number of detected compounds. They play an important role in wine aromas as they contribute to fruity notes. For example, isoamyl acetate, with its banana-like aroma, predominates in many wines, with concentrations up to 10 mg/L (Ebeler and Thorngate, 2009). Chardonnay TTV wines were rich in esters when compared to other compound families, especially isoamyl acetate, isoamyl decanoate, or 2-phenylethyl acetate. Isoamyl acetate and 2-phenylethyl acetate have been related to the ‘peach’ aroma of Chardonnay (Lee and Noble, 2003), and Siebert *et al.* (2018) recently pointed out a combination of esters involved in the ‘stone fruit’ aroma in Chardonnay wines. Other fermentation esters produced by yeast during alcoholic fermentation include ethyl acetates of fatty acids, mainly ethyl hexanoate, ethyl octanoate, and ethyl decanoate (Ribéreau-Gayon *et al.*, 2006). With their pleasant odours of fruits such as pineapple, apricot, grape, or apple, they contribute to the aromatic profile of young white wines (Robinson *et al.*, 2014). We found notable levels of these medium-chain fatty acid ethyl esters, which is again in agreement with the literature. Numerous alcohols, another group of compounds formed by the yeast during fermentation either directly from sugars or amino acid catabolism (Cordente *et al.*, 2012), were also detected. According to Ribéreau-Gayon (2006), the main higher fermentation alcohols (*i.e.* components with more than two carbon atoms) are 2-methyl-1-propanol and amyl alcohols (a mixture of 2-methyl-1-butanol and 3-methyl-1-butanol), which is consistent with our data. They contribute to a wine’s aromatic complexity when present at low concentrations. C₆-alcohols, such as 1-hexanol and *cis*-3-hexen-1-ol, are common alcohols already present in grapes with a characteristic “green” aroma reminiscent of leaves and fresh-cut grass (Ilc *et al.*, 2016). We found significant differences in the relative abundance of these compounds across varieties, with Riesling wines having the highest NPA (Supplementary Figure 4). Other predominant fermentation-derived compounds are aliphatic acids (Styger *et al.*, 2011). We detected aliphatic acids derived from fatty acids (C₆ to C₁₂) in all wines and, in particular, in Chasselas. Although these acids are generally unpleasant, with descriptors ranging from sweaty and cheesy to rancid, they can be transformed into more pleasant-smelling compounds, such as esters and lactones, during ageing (Bakker and Clarke, 2012; Robinson *et al.*, 2014).

Nevertheless, wine aromas do not only depend on the most abundant compounds but are also largely influenced by compounds present at low concentrations

(Francis and Newton, 2005). Some of these compounds originating from grapes exhibit very strong odours, contributing to the varietal aroma of wines. It is well established that compounds of the terpene family, such as monoterpenoids (C₁₀ compounds), sesquiterpenoids (C₁₅ compounds), and C₁₃-norisoprenoids are of great importance for wine aroma. In our study, we identified 38 compounds from the terpene family (27 monoterpenoids, seven sesquiterpenoids, and four C₁₃-norisoprenoids). As expected, intensely-flavoured Muscat-like varieties (Muscat, 0074E and 4095G) revealed high NPA of monoterpenols, such as linalool, α -terpineol or hotrienol, that convey the floral, fruity, and citrus characters of these wines (Ribéreau-Gayon *et al.*, 2006). Similarly, non-Muscat aromatic varieties such as Gewurztraminer had high levels of terpenols, specifically the highest citronellol NPA of all varieties studied. As recently reported (Zhang *et al.*, 2017, Yue *et al.*, 2020), we also noticed significant NPA of monoterpenes (*i.e.*, limonene, α -terpinene, β -myrcene or *trans*- β -ocimene) only in varieties able to synthesise high levels of terpenols such as Muscat, 4095G or 0074E.

Riesling wines had the highest NPA of TDN. This C₁₃-norisoprenoid compound is known to exhibit a petroleum-like scent or ‘wet stone’ odour in young Riesling wines, which greatly participates in the aromatic typicality of this variety (Winterhalter and Schreier, 1994; Schüttler *et al.*, 2015). Our NPA values are consistent with the published literature, which reports TDN concentrations in young Riesling wines up to five times higher than those of other cultivars (Sacks *et al.*, 2012; Black *et al.*, 2015). Interestingly, the 0238E genotype appeared to inherit its TDN profile from its Riesling parent, as 0238E wines contained as much TDN as Riesling wines.

As Sefton *et al.* (2011), we found that β -damascenone was not typical of a particular grape variety, as it did not belong to the discriminating compounds. It is interesting to note that β -damascenone has also been found to act as an aroma enhancer for ethyl esters associated with berry fruit aroma (Pineau *et al.*, 2007), indirectly impacting the overall wine aroma.

Volatile thiols are known to be important to the varietal characteristics of certain wines. Among these compounds, 4MSP, 3SH, and 3SHA have been identified as key molecules of young wines, imparting pleasant fruity aromas such as blackcurrant bud, passion fruit, and grapefruit. These molecules are undoubtedly associated with the typical aroma of Sauvignon Blanc wines (Darriet *et al.*, 1995; Tominaga *et al.*, 1998) but have also been identified in a wide range of varieties such as Gewurztraminer (Tominaga *et al.*, 2000) or Riesling (Schüttler *et al.*, 2015). In accordance with Dournes *et al.* (2020), our results revealed high concentrations of 4MSP in Floreal wines (Figure 3), driving the differentiation of this variety from the others.

Our results demonstrate that it is possible to produce white wines on a very small scale, *i.e.*, less than 100 mL, from a much smaller amount of grape material than the experimental vinifications described so far. In addition, the laboratory-

scale vinification devices that are already available require not only sufficient availability of the raw material but also a substantial surface area to place the fermenters, as well as a large workforce to manage the whole winemaking process when screening grapevine population for volatile composition. The proposed TTV method allows a single person to manage hundreds of vinifications on a laboratory bench simultaneously.

By comparing the volatile profile determined in our study, we observed distinct patterns of volatile compounds between varieties. Indeed, a clear classification according to the varieties was achieved by the vinification method, supporting the ability of TTV wines to reflect the aromatic potential of the grape variety accurately.

Nevertheless, commercial wines are commonly characterised by high variability. In our study, the selected commercial wines originated from different wine regions, winemakers, or even countries, and they have used uncontrolled plant material (clone, rootstock) and undergone different viticultural practices and winemaking processes. Despite these multiple sources of variability, our results show that the varietal effect is predominant over the other factors such as viticultural practices, environmental factors, or winemaking processes. Therefore, the TTV method described here can produce wines that reflect the grape variety without any bias compared to experimental vinification and traditional white winemaking. However, due to its small scale, the high-throughput TTV technique has a few limitations compared to larger-scale vinification. In particular, if the TTV is well suited to analytical approaches, the very small wine volumes obtained do not allow thorough sensory analyses. Although it is possible to perform an olfactory evaluation of wine aroma with a panel of judges, the small wine volume is not sufficient to perform a full wine-tasting protocol that includes an evaluation of the mouthfeel of the wines.

CONCLUSION

In this work, we developed a high-throughput, small-scale vinification method to produce white wines from single vine plants. After ensuring that the wines produced by TTV were compliant with oenological criteria, profiling of wine volatile compounds revealed aromatic profiles consistent with those expected for different grape varieties. In addition, a comparison of the profile of the volatile compounds of TTV wines to those obtained by small-scale vinification and traditional white winemaking highlighted a differentiation of the wines according to the grape varieties, regardless of the vinification method.

This high-throughput, small-scale vinification method might be amenable to producing hundreds of white wines, making it possible to assess the oenological potential of hundreds of grapevine genotypes using the harvest of a single vine. This method provides access to the quantification of molecules, such as volatile thiols and TDN, that are not present before fermentation. This direct phenotyping method centred on wine will find major applications in the detection

of QTL of molecules of interest directly in wines and opens up new perspectives not only for a better understanding of the genetic determinism of wine aromas but also for improving the selection of new grape varieties with desirable properties.

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DATA AVAILABILITY

Raw data have been deposited to the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gkad1045, PMID:37971328) with the identifier MTBLS9537.

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