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A novel approach to discriminate the volatilome of *Vitis vinifera* berries by Selected Ion Flow Tube Mass Spectrometry analysis and chemometrics.

Thomas Baerenzung dit Baron[a,b], Olivier Yobrégat[c], Alban Jacques[a], Valérie Simon[b], Olivier Geffroy[a,*]

[a]*PPGV – Physiologie, Pathologie et Génétique Végétale, Toulouse INP-Purpan, University of Toulouse, 31076 Toulouse, France.*

[b]*LCA – Laboratoire de Chimie Agro-Industrielle, UMR 1010 INRAe/Toulouse INP-ENSIACET, University of Toulouse, 31030 Toulouse, France.*

[c]*IFV – Institut Français de la Vigne et du Vin pôle Sud-Ouest, 81310 Peyrole, France.*

Corresponding author. E-mail address: olivier.geffroy@purpan.fr (O. Geffroy).

Abstract

This study investigated a quick way to access the composition in volatile compounds of 23 grape varieties through a scan of their volatilome by SIFT-MS coupled with simple chemometrics approaches such as analysis of variance (ANOVA), principal component analysis (PCA) and hierarchical ascendant classification (HAC).

Grape varieties were distinguishable based on their SIFT-MS volatilome scan with O_2^+, H_3O^+ and NO^+ as reagent ions, and on the combination of these three scans. For its ability to ionize most compounds, to efficiently fragment them to generate ions with distinct m/z ratio, and to enhance the differentiation of compounds of similar masses, O_2^+ reagent ion should be preferentially considered. The use of one single ion rather than three enables to limit the time of analysis and the number of variables to be treated. The technique allowed the distinction of high and low aroma compounds producers as confirmed by headspace solid-phase microextraction followed by gas chromatography-mass spectrometry (HS-SPME/GC-MS) analyses.

SIFT-MS is a quick and interesting tool with potential application in various fields of viticulture such as phenotyping of grape varieties or non-targeted studies on the impact of environmental factors or viticultural practices on grape aroma composition.

1. Introduction

Wine aroma compounds come from multiple origins. They can emerge from the plant or the fruit itself, alcoholic and malolactic fermentation, or aging. When these volatiles originate from the grape, they are known as varietal aroma compounds. These molecules are responsible for wine typicity and often differ in occurrence and concentration between grapevine varieties and terroirs (Souza Gonzaga et al., 2021).

Grapevine varietal aroma compounds have diversified chemical structures and belong to several structural groups, such as terpenes, methoxypyrazines, polyfunctional thiols, phenylpropanoids, C13-norisoprenoids, C6 compounds or glycosylated aroma precursors (Robinson et al., 2014). They are responsible for a large diversity of aromas notably floral, fruity and spicy notes for free and hydrolyzed bound monoterpenols (Rapp & Mandery, 1986), C13-norisoprenoids (Winterhalter & Rouseff, 2001) and sesquiterpenes (Wood et al., 2008) respectively; bell pepper for methoxypyrazines (Allen et al., 1991); citrus for polyfunctional thiols (Darriet et al., 1995; Tominaga et al., 1996); spicy and smoky for volatile phenols (Hayasaka et al., 2010; Ilc et al., 2016); or fresh leaves for C6 compounds (Previtali et al., 2021). These compounds are considered as some of the most important molecules driving wine quality and appreciation (Zhu et al., 2016) which means that studying these molecules is crucial for the wine industry.

Concentrations of these compounds range from µg/L to ng/L or less, which often implies that the sample preparation steps to concentrate the analytes are as important as the analysis itself. Diverse extraction techniques are primarily considered, such as liquid-liquid extraction (LLE), solid phase extraction (SPE), solid phase microextraction (SPME), and stir bar sorptive extraction (SBSE) (Marín-San Román et al., 2020). Conventional LLE and SPE techniques, although still widely used, have many drawbacks. Such methods are time-consuming and require the use of organic solvents, that are often

harmful to the environment and to operators. For these reasons, SPME and SBSE techniques were developed to provide numerous advantages over the previous ones (solvent free extraction, extraction and concentration in a single step). Whatever the extraction technique, it is generally associated with an analysis by gas chromatography (GC) using different types of detectors: mass spectrometry (MS) (Geffroy et al., 2015), flame ionization detector (FID) (Gammacurta et al., 2017) or pulsed flame photometric detector (PFPD) (Gürbüz et al., 2013).

Considering analytical techniques, Gas Chromatography – Mass Spectrometry (GC-MS) is the preferred combination for volatiles analysis. However, it suffers from the same issues as the preparation techniques. Such analysis is usually long. GC-MS runs last between 45 minutes to 2 hours and might require some complex method development (Chin & Marriott, 2015; Sadgrove & Jones, 2015). More recently in parallel to GC based methods, the use of Chemical Ionization coupled with Mass Spectrometry (CIMS) has been reported in literature. Real-time - high sensitivity analyses are some of the advantages common to CIMS techniques, such as Proton Transfer Reaction Mass Spectrometry (PTR-MS) (Lubes & Goodarzi, 2017) and Selected Ion Flow Tube Mass Spectrometry (SIFT-MS), which was first commercially available in 2008 (Spanel & Smith, 2011). Their development concerns diverse fields such as medical diagnostics (Catala et al., 2020), air pollution control processes (Vitola Pasetto et al., 2020), air quality studies (Ghislain et al., 2020) or volatiles monitoring in the food industry (Sumonsiri & Barringer, 2013). Among these technologies, SIFT-MS is particularly promising as it enables to limit samples preparation and analyze gas composition within a few minutes.

SIFT-MS allows the study of both organic (Debono et al., 2018; Thevenet et al., 2021) and inorganic molecules (Vitola Pasetto et al., 2019a, 2019b). The device can directly analyze the sample headspace and measure relative abundances of each recorded mass in scan or Selected Ion Monitoring (SIM) mode. This soft ionization technology uses multiple reagent ions generated using microwaved water plasma to ionize the sample headspace compounds in a flow tube. Recent SIFT-MS devices can generate 8 different reagent ions: 3 cations (H_3O^+, NO^+, $O_2^{+\cdot}$) and 5 anions (NO_3^-, NO_2^-, O^-, O_2^-, OH^-),

thus expanding the analysis capacity of the machine. Yet, in biological sciences, cations are preferentially used as they ionize most organic compounds (Hera et al., 2017). Most of the previous research work using this equipment focused on SIM mode capabilities of the SIFT-MS, monitoring only a small fraction of the matrix compounds (Ghislain et al., 2019; Scotter et al., 2005; Smith & Španěl, 2011). Recently some studies investigated the whole metabolic profiles of olive and argan oils, using cations and scan mode. These analyses were proven to be efficient as means to associate oils and their geographical origin, with consistency and limited analysis times (Kharbach et al., 2018; Ozcan-Sinir, 2020).

As this analytical technique has never been used on grapevine, the aim of this research was to investigate the ability of SIFT-MS coupled with chemometrics to quickly discriminate grapevine varieties on the basis of their volatile fingerprint.

2. Material and methods

2.1. Reagent and materials

Guaiacol, β-ionone and β-caryophyllene (ACS reagent, 98 %) were obtained from ExtraSynthese (Lyon, France). Sodium chloride (NaCl, ACS reagent, 99 %) and polydimethylsiloxane/divinylbenzene/carboxen (PDMS/DVB/CAR, 50/30 μm) SPME fibers were provided by Sigma Aldrich (Saint-Louis, Missouri, USA). SPME vials, septa and caps were purchased from Perkin Elmer (Waltham, Massachusetts, USA). *n*-alkanes (C9 – C17) mix standard for retention index determination was obtained from the standards of pure compounds (99 %, Merck, Darmstadt, Germany). Ultra-pure water from a Milli-Q system (Millipore, Burlington, Massachusetts, USA) was used throughout. Hydrogen used as the GC carrier gas was produced by a COSMOSMF.H2 (CJ Lab, La Frette, France).

2.2. Grapevine samples

2.2.1 Sampling

In 2020, 23 grape varieties from a germplasm collection located in the southwest of France were sampled at 3 different dates according to their theoretical timing of veraison: early, middle and late (Parker et al., 2013) (*Table* 1)**.** This approach was preferred to an approach based on technological maturity and sugar concentration, as the content in varietal aroma compounds is well known to be impacted by the lapse of time since veraison, and notably the environmental conditions over this period (Van Leeuwen et al., 2020). Three grape varieties, namely, Négrette, Duras and Tardif which were not classified by Parker et al. (2013) and for which veraison occurs at a similar period than Merlot, Syrah and Tannat respectively, were included in the experimental design at the corresponding sampling dates. Samples from early, middle, and late cultivars were harvested on 26 August, 17 September, and 28 September respectively. To investigate the impact of maturity on the volatile composition, the variety that exhibited during the first sampling date the lowest maturity level, as reflected by its sugar concentration, was also sampled during the two following dates. The studied vintage was characterized by warm and dry conditions of climate. The timing of sample collection corresponded to commercial harvests for the local varieties grown on neighboring blocks from the same wine estate (*i.e.*, Sauvignon, Fer) which took place about 40 days after veraison. The vineyard representative of the area (longitude: 43°50'24.9"N, Latitude: 1°50'57.8"E), with a 2.20 m x 1 m spacing between rows and vines, was planted in 2004. It was grafted on Gravesac rootstock and trained with vertical shoot positioning on a cordon pruning system. The sanitary status of all the accessions was regularly monitored, and the plant material was free of grapevine virus diseases, especially fanleaf and leafroll types 1, 2 and, 3. For each grape variety, the sampling area was composed of 6 continuous vines. This vineyard was known to be homogenous, and the sampling area did not exceed 800 m^2. This indicates that observed differences in grape volatile composition within this experimental site for a similar phenological stage are likely to be due to cultivars rather than to soil heterogeneity. For each grape variety, three samples consisting of 100 berries from both sides of the row and several parts of the bunch (50 on each side of the row), were collected.

2.2.2   Maturity parameters analysis

For each 100-berry sample, 50 g of grapes were used for physico-chemical analysis (sugar concentration, titratable acidity, pH, weight of a single berry) and 50 g for SIFT-MS analysis. The rest was frozen at -20 °C eventually for further HS-SPME/GC-MS analysis.

Before the start of the analysis, the number of berries was counted in 50 g samples to calculate berry weight. Samples were then gently crushed, the extracted juice was transferred into 1.5 mL tubes, centrifugated for 1 min at 10000 rpm and the supernatant was used for the measurements. Sugar concentration (°Brix) was measured with a MA885 Wine Refractometer (Milwaukee, Wisconsin, USA), pH was determined using a PHM 210 MeterLab pH meter (Radiometer, Copenhagen, Denmark). Titratable acidity (TA) expressed as g/L of tartaric acid was measured according to the method of the Organisation Internationale de la Vigne et du Vin (OIV, 2009) using a 1 M NaOH solution.

2.3. Selected Ion Flow Tube – Mass Spectrometry (SIFT-MS) analysis

2.3.1. Sample preparation

On sampling day, 50.0 ± 0.1 g of grapes were gently crushed and transferred in a 1 L Schott bottle (Verres Vagner, Toulouse, France) sealed with a Teflon secured screw cap, an experimental set-up inspired by Spanel *et al.* (2002). The bottle was kept for 6 hours at room temperature and then transferred in a temperature-controlled water bath for 40 min at 40 °C to reach headspace equilibrium. A blank experiment with an empty bottle was systematically performed with the same bottle used for the final analysis.

2.3.2. SIFT-MS analysis

The Selected Ion Flow Tube Mass Spectrometer used in this study (SIFT-MS, model Voice 200 Ultra, Syft Technologies, Christchurch, NZ) was set to full scan mode (from 15 to 250 m/z) using positive ions (H_3O^+, NO^+, $O_2^{+\cdot}$). Each precursor ion was selected in a sequenced way by a first quadrupole mass filter and was then injected to the flow tube with a N_2 flow as a carrier gas (Alphagaz, Air Liquide, 99,9999 %, Paris, France). Nitrogen flowrate was set at 2.0 TorrL/s. The headspace of the sample was introduced by a calibrated capillary at a sampling flowrate of 0.3 TorrL/s. The analytes reacted with

the selected precursor in the flow tube kept at 119 °C and 0.06 kPa and generated product ions with specific mass-to-charge ratios (*m/z*) that were quantified by a second quadrupole mass spectrometer. The volatile headspace compounds were directly extracted from the bottles to the SIFT-MS flow tube system. Calibration procedure was made before each instrument start to ensure linearity, range, accuracy, and precision by injecting a mix of certified gas standards (Air Liquide America Specialty Gases LLC, Plumsteadville PA, USA). LabSyft 1.6.2. software (Syft Technologies) was used for data acquisition and analysis. For each triplicate sample per variety and for each precursor ion, four full mass scans were recorded with the three last ones used for calculation.

Preliminary experiments were conducted to assess that berry quantity and equilibration time were not saturating the SIFT-MS analysis potential. Indeed, to ensure linearity, reagent ions need to be in excess to still be present and analyzed by the detector after ionizing matrix compounds (data not shown). Instrumental repeatability was estimated at 9 %, 8 % and 7 % for H_3O^+, NO^+ and O_2^+ respectively, from 10 grape samples by averaging the RSDs obtained for each *m/z* value recorded for the three full mass scans. Nine homologous batches of grapes stored prior to analysis at 4°C were used for reproducibility tests. Over 3 consecutive days, 3 batches were analyzed per day and reproducibility was estimated as described above at 11 %, 9 % and 10 % for H_3O^+, NO^+ and O_2^+ respectively

2.4. HS-SPME/GC-MS analysis

2.4.1.   HS-SPME conditions

To optimize sampling, an approach based on stratified sampling was implemented (Parsons, 2017). The segmentation proposed by SIFT-MS analyses and chemometric treatment was used as the strata (§2.5). Four cultivars belonging to distinct homogenous groups and that shall reflect the diversity in volatile compounds composition within our panel were selected to perform HS-SPME/GC-MS analysis. For the selected cultivars, frozen grapes were subsampled in three 4 g—groups (corresponding to the 3 replicates sampled for the SIFT-MS analysis), directly blended, and put in 20 mL screw cap vials. Samples were prepared and analyzed based on the method published by Perestrelo et al., (2011),

which can be summarized as follows: 2 g of NaCl and 5 mL milliQ water were added to the vial, the mix was then vigorously shaken. Finally, after adding a magnetic bar, the vials were shut with aluminium/PTFE screw septa and stirring speed was set at 800 rpm. Vials were then placed in thermostated baths and heated for one hour at 60 °C (± 3 °C) to reach equilibrium.

The PDMS/DVB/CAR fiber was inserted into the vial headspace and left for 1 hour to extract compounds. The fiber was then manually inserted into the injection port of the GC for desorption at 270 °C for 1 min then 20 °C /min to 250 °C (held for the whole run).

2.4.2. GC-MS analysis

GC-MS analyses were performed on a Clarus 680 gas chromatograph coupled with a Clarus 600 SQ Mass Spectrometer (Perkin Elmer, Waltham, Massachusetts, USA). Separation was conducted on a DB-5ms capillary column (Agilent, Santa Clara, CA, USA) of 30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness. The carrier gas was hydrogen at a constant flow rate of 1.0 mL/min. The GC-MS oven was set as follows: 40 °C for 5 min then 2 °C /min to 220 °C finally 40 °C /min to 280 °C (hold 1 min). The injector was kept at 270 °C. Injection was in splitless mode with a deactivated glass liner with an internal diameter of 1.0 mm. The split valve, at 50:1, was opened after 60 s.

The MS fragmentation was performed by electron impact ionization mode (70 eV). The transfer line, source and quadrupole temperatures were 250 °C, 230 °C, and 150 °C, respectively. MS acquisition was carried out in full scan mode (40-300 m/z). Identification of the volatile organic compounds was done by comparing the mass spectra of the samples with the data system of the National Institute of Standards and Technology (NIST 2017, Gaithersburg, Maryland, USA). The confirmation of molecule identity was performed by comparing the retention indices of compounds, determined after injection of a series of n-alkanes (C9–C17) under the analytical conditions previously described, with literature data.

The GC-MS method repeatability was estimated by analyzing 5 aqueous solutions (V = 10 mL) spiked with 500 μL of 20 μg/L guaiacol, β-ionone and β-caryophyllene. The analysis was conducted using the

same protocol as in the grape study. The results gave relative standard deviations (RSDs) lower than 3 % for the 3 standards.

All data treatments were conducted using Xlstat 3.1. software (Addinsoft, Paris).

Primarily, physico-chemical analyses were treated with one-way analysis of variance (ANOVA), considering the sampling date as a factor. For each ion, masses with a *m/z* ratio below 100 and abundance below noise were removed from the data set to eliminate most of fragmentation ions and reduce noise. Indeed, fragments below 100 of *m/z* ratio which are similar between terpenes (Amadei & Ross, 2011; Dhooghe et al., 2008) or other compounds class (Diskin et al., 2002) could also match with the molecular ion of smaller compounds. Such choices also enable to take into consideration most of the grape aroma compounds. Indeed, to our knowledge and besides small aldehydes that are generally not specific to some cultivars such as acetaldehyde, C6 compounds are the smallest molecules contributing to grape varietal aroma. Within this family, hexanal which has the lowest molecular weight is likely to generate through SIFT-MS ionization at least one molecular ion with 100 *m/z*.

Then, data were treated through a one-way ANOVA to determine masses that enables to discriminate grapevine varieties. Finally for each reagent ion and for each variety, discriminating masses were averaged, analyzed by Principal Component Analysis (PCA), and Hierarchical Clustering Analysis (HCA) was applied on the main PCA components explaining 99% of variance to create varieties similarity groups.

The sum of chromatographic peak areas obtained by GC-MS by family of compounds were also analyzed by ANOVA.

3. Results and discussion

3.1. Physico-chemical parameters analysis

Physico-chemical parameters measured on the studied grape varieties are shown in supplementary fig. 1. The cultivar that exhibited the lowest sugar concentration with 22.0 ± 0.2 °Brix during the 1st sampling date was Sémillon. Consequently, this variety was also sampled on the two following sampling date to investigate the sampling time effect.

Brix, TA levels, pH, and berry weights were not significantly impacted by the sampling date at $P < 0.05$ (Table 2) which tends to indicate that no bias was introduced by the date of sampling. On average, a lower maturity level, as reflected by a lower sugar concentration and a higher acidity, would have been expected for the cultivars harvested during the 3rd date. Although not statistically significant, such trend can be observed for Carignan, Grenache and Mourvèdre, some Mediterranean varieties not planted in the area of study that have a high thermal need. The fact that these requirements have been somehow satisfied under our experimental conditions might be the consequence of the very warm and dry weather conditions of the studied vintage.

Large differences in sugar concentration, TA, pH, and berry weight can be observed between varieties harvested during the same sampling date. Despite an expected similar timing of veraison, such differences could be explained by variations in leaf area to fruit ratio, a proxy well correlated to fruit composition and wine quality (Kliewer & Dokoozlian, 2005). As the cordon pruning system used on the vineyard is known to promote regularity in leaf hedge development (Jackson, 2001), we can assume that differences in grape yields are mainly involved. This yield effect through its berry weight component can be clearly observed for Merlot, that exhibits a relatively high sugar concentration and small berry size, and Cinsaut that is characterized by a low sugar concentration and a large berry size. Besides berry weight, it is also worth mentioning that the cordon pruning system might have enhanced differences in number of clusters per vine, and therefore in crop load between cultivars, by only maintaining basal buds whose fertility strongly differ according to genotypes (Rosner & Cook, 1983).

3.2. Grapevine varieties discrimination by SIFT-MS

An example of SIFT-MS spectrum obtained for H_3O^+, NO^+ and O_2^+ is represented on Fig. 1 (Carignan data). Similar profiles were obtained with other varieties (data not shown). For the three reagent ions, the pattern of the signal is quite comparable. Several groups of masses were observed around 105, 119, 135, 149, 161, 177 and 208 *m/z.* These masses are commonly associated to terpenoids fragmentation by SIFT-MS (Amadei & Ross, 2011; Dhooghe et al., 2008) but could be the result of numerous reactions including other compounds classes and smaller molecules. Molecular clusters and adducts are also bound to happen with this technology. H_3O^+ can react with H_2O, forming $H_3O^+.H_2O$ with up to 3 water molecules and NO^+ is known to attach to compounds creating molecule.NO^+ adducts (Diskin et al., 2002; Smith et al., 2003; Sovová et al., 2011; Wang, 2006).

The preliminary ANOVA treatment showed that among the 150 *m/z* ratio between 100 and 250 with an abundance above the background noise, 84 enabled to significantly discriminate the grape varieties with O_2^+, 62 with NO^+ and 81 with H_3O^+.

PCA plots for each reagent ion and for all reagent ions pooled together showed that most of the varieties were aligned along one of the axes and that the Carignan had a different production than the other varieties (Fig. 2). Eigen values in two principal components (PC1 and PC2) were 56.94 %, 56.61 %, 69.82 % and 61.05 % for H_3O^+, NO^+, O_2^+ and all ions combined respectively. As the segmentation appears to be mainly drawn by the Carignan sample, data were also treated after removing data from this cultivar. Such removal helped to understand the varieties layout on the PCA plot but did not change the output of HCA (results not shown).

For the 3 reagent ions and the 3 reagent ions pooled together, the composition of the clusters only slightly differed (Fig. 3). The O_2^+ and all ions combined HCAs were composed of a first cluster, made of Carignan (colored in black and afterwards referred as Carignan cluster), a second cluster made of Semillon 1, 2 and 3, Chenin, Tardif, Cabernet Franc, Fer, Mourvèdre, Tannat, Syrah, Cabernet Sauvignon and Grenache (colored in red and afterwards referred as Mourvèdre cluster), a third cluster made of Muscat à petits grains blancs, Gewurztraminer, Colombard, Malbec, Merlot, Gamay, Pinot

noir, Chardonnay and Sauvignon (colored in green and afterwards referred as Merlot cluster) and then a last cluster made of Cinsaut, Duras and Négrette (colored in purple and afterwards referred as Duras cluster).

For H_3O^+, Sémillon 3, Fer, Tardif and Chenin moved from the Mourvèdre cluster to the Duras cluster and Cinsaut, from the Duras cluster to the Merlot cluster. Besides these changes, the Mourvèdre and Carignan clusters remained unaltered. For NO^+, Tardif, Chenin and Sémillon 3 moved to the Duras cluster while Fer and Sémillon 1 moved to the Merlot cluster. As for the Carignan cluster, it remained unaltered. Large changes in berry volatile compounds are known to occur during maturation and notably over the last days prior to harvest (Geffroy et al., 2014; Robinson et al., 2014). This may explain why the three Sémillon samples do not always belong to the same cluster. However, the segmentation proposed with NO^+ is particularly inconsistent as Sémillon samples are scattered across 3 different groups. The one obtained with O_2^+ and all the ions combined support that the cultivar has a larger impact than the maturity on grape volatilome.

The varieties grouping never seems to be made according to berry skin color as several examples of white or black varieties belonging to the same group can be observed (i.e., Sémillon and Mourvèdre, or Colombard and Merlot). Even if varieties harvested during the 3 sampling dates can be found in the different clusters, it can be pointed out that the Merlot cluster is mainly composed of varieties with an early timing of veraison. As the SIFT-MS measurements have shown a good reproducibility, this grouping is more likely to be due to a real similarity in volatile composition rather than particular operating conditions the day of analysis.

Differences in varietal aroma composition between cultivars have been little studied simultaneously, enabling accurate comparison through an untargeted headspace approach like ours. Most of these studies have been focusing on wines, or on grapes using a limited number of *Vitis vinifera* genotypes or only local cultivars (Pinu, 2018). When such works are carried out on wines, the comparison

between white and red grape cultivars becomes impossible due to the great differences in winemaking techniques and notably the prefermentative removal of skins for white wines.

However, some white varieties that are close to each other on the HAC plots are well known to exhibit similar aromatic features. This is the case for Sauvignon and Colombard, and particularly for Muscat à petits grains blancs and Gewurztraminer that are known to produce great quantities of 3-mercaptohexanol and monoterpenols, respectively (Roland et al., 2012; Song et al., 2018). This closeness is somehow surprising for the varietal thiol producing cultivars as these aroma compounds are found in grapes under a non-volatile precursor form which is *a priori* not detectable through SIFT-MS measurements (Roland et al., 2012).

Our results also showed that in most cases, varieties connected by family ties are close by in the different cluster's layout. This can be observed for Pinot noir, Chardonnay and Gamay that all belongs to the Merlot cluster and are known to have a parent/offspring relationship (Bowers et al., 1999). This is also the case for Colombard, Sauvignon and Gewurztraminer that are all related to Savagnin, and for Colombard, Chardonnay and Gamay that are Gouais offsprings (Lacombe et al., 2013). Cabernet Franc and Cabernet Sauvignon, or Malbec and Merlot duos are other examples of siblings and parent/offspring connections showing a similar volatile composition (Lacombe et al., 2013). These links would need more research to understand the underlying cause of correlation and causality. However, the variety lineage hypothesis cannot be validated for some varieties such as Sauvignon and Cabernet Sauvignon, Cabernet Franc and Merlot, or Chenin and Colombard that are positioned far away in the HCAs despite their parentage relationship. Due to the huge heterozygosity within the *Vitis vinifera* species, crossings can generate offsprings that greatly differ between them and from their parents, and notably in grape aroma composition (Velasco et al., 2007).

3.3. Identification of the most relevant reagent ion for grape varieties discrimination

The differences in spectrum shape between the three reagent ions are clearly visible in Fig. 4 and Supplementary Fig 1. H_3O^+ and O_2^+ had comparable spectra, while NO^+ gave lower abundances in general, leading to some undetected masses being absent from the spectrum.

These differences could be explained through the abilities of these ions to ionize compounds. NO^+ tends to only produce molecular ions with little to no fragmentation and is also known to produce adducts which leads to smaller compounds being moved into a higher mass range. H_3O^+ is prone to protonation and has a middle fragmentation effect, while O_2^+ is supposed to induce the most fragmentation (Amadei & Ross, 2011; Dhooghe et al., 2008; Koss et al., 2016).

By creating more fragmentation, we can assume that O_2^+ may promote the differentiation of compounds with the same molecular weight such as nerol, geraniol, or α-terpineol found in grapes and imparting key aromatic characteristics (Robinson et al., 2014). This property is likely to be particularly valuable for discriminating *Vitis vinifera* berries whose diversity in aroma compounds is usually lower than wines whose production process involves fermentations and aging.

The facts that i) O_2^+ enabled the strongest discrimination between varieties as reflected by the 84 *m/z* between 100 and 250 significantly impacted by this factor during the preliminary data treatment, ii) the highest eigen values for PC1 and PC2 were observed for the plot built with ions generated using O_2^+, iii) H_3O^+ and NO^+ gave different cluster combinations in comparison with O_2^+ and all ions combined, and iv) the three Sémillon samples were part of the same cluster for O_2^+, tends to strengthen the superiority of this latter reagent ion. In comparison with the previous studies using scan mode in which the segmentation was made using H_3O^+, NO^+, O_2^+ (Kharbach et al., 2018; Ozcan-Sinir, 2020), this finding is not negligeable as the use of the single ion O_2^+ enables to reduce the time of analysis and the number of variables which facilitates the data treatment.

It must also be mentioned that if O_2^+ may have induced more ions with a low molecular weight, the noise generated by such small ions may have been limited by only selecting m/z ratio above 100.

3.4. Differences in spectrum profile between clusters for O_2^+

The spectrum with average abundances for each *m/z* ratio per group enabled to better understand the differences in volatile composition between the four clusters of varieties obtained with O_2^+ (Fig. 5). Differences were mainly explained through different production levels of the same compounds. In most cases, the Carignan cluster exhibited the highest signal intensity while abundances for the Merlot and Mourvèdre clusters were lower. For the Duras cluster, abundances were generally intermediate. The aroma composition of Carignan grapes or wines has been the subject of little comparative research with other cultivars. One of the most consistent studies was conducted on wines vinified through several winemaking techniques made from Spanish Grenache and Carignan grapes from two different sites (Geffroy et al. 2015). Among the grape-derived aroma compounds with the highest concentration in control wines (in absolute value expressed as µg/L), Carignan was characterized by a higher content in benzaldehyde, 4-vinylguaiacol and β-damascenone. Although benzaldehyde and 4-vinylguaiacol are known to be released by yeast enzyme activity from a glycosylated precursor, they can also be found under a free form in grapes (Ugliano & Moio, 2008). Despite the ability of O_2^+ to generate fragmentation ions, it can be assumed that some masses represented on the spectrum could possibly be affiliated with molecular ions. If this would be the case for benzaldehyde, vinylguaiacol and β-damascenone, a higher signal would be expected for the Carignan cluster for 106, 150, 190 m/z and for 177 which is produced through soft ionization of β-damascenone (Unpublished data, Baerenzung dit Baron, 2021). This can be confirmed on the spectrum, as abundances of these four ions are greater for the Carignan cluster than for the Mourvèdre cluster that included the Grenache variety.

The weak signals observed at 152 and 166 m/z could be related to the molecular ions of 2-isopropyl-3-methoxypyrazine (IPMP) and 2-Isobutyl-3-methoxypyrazine (IBMP). For both ions, abundances are greater for the Carignan cluster. Although the presence of these compounds has not been formally identified in this cultivar, they are known to be rather ubiquitous, notably IBMP (Koch et al., 2010). The fact that higher concentrations are found in the least ripe Carignan sample is not completely incoherent as the content of these molecules is known to decrease over maturation (Kotseridis et al., 1999). However, if this hypothesis cannot be entirely excluded, it is unlikely as these compounds are

usually found at a trace level in grapes (ng/kg) which might be under the limit of detection of 100 ppt to 1 ppb in a gas phase for this equipment (Lehnert et al., 2019).

Also found at a trace level, sesquiterpenes are another family of semi-volatile compound discovered more than a decade ago in Syrah grapes (Wood et al., 2008). Even if no signal can be observed at 218 m/z which would correspond to the molecular ion of rotundone, significant levels of abundance can be found for the Carignan cluster for 208 and 209 m/z. Such masses could be related to other sesquiterpenoids identified in grapes (Li et al., 2020).

It must also be pointed that some m/z ratio signals are particularly specific of some clusters. This is the case for 154 and 183 that are only found in the Merlot cluster. The 154 m/z signal could be related to the molecular ion of some monoterpenols such as nerol, geraniol, or α-terpineol. This hypothesis is particularly conceivable as some varieties from the Merlot cluster like Gewurztraminer or Muscat à petits grains blancs contain remarkable levels of these compounds (Song et al., 2018).

Among the masses not yet described, those around 136 *m/z* may have been produced by the ionization of monoterpenes or monoterpenols such as β-cyclocitral or geraniol. Indeed, when reacting with O_2^+, β-cyclocitral is known to produce 137/123/121 *m/z* ions while geraniol produces 136/123 *m/z* ions (Syft Technologies Limited, 2015). In addition to β-damascenone, masses around 177 and 190 could also be linked to trans-β-ionone or other sesquiterpenoids, while masses between 117-121 and 104-109 could be linked to fragments of bigger compounds, or smaller compounds like phenols, alkanes, or aldehydes (Amadei & Ross, 2011; Dhooghe et al., 2008; Diskin et al., 2002; Sovová et al., 2011; Syft Technologies Limited, 2015).

3.5. *HS-SPME/GC-MS analysis*

To unveil the biochemical compounds analyzed by SIFT-MS, Carignan, Mourvèdre, Merlot and Duras samples which i) belong to four different clusters for the different reagent ions and their combination, and ii) occupy a central position on the HAC plot for each of the 4 clusters built with O_2^+, were selected for GC-MS analysis.

In total, 20 different volatile compounds were identified in the grape samples (Table 3). Given the higher number of masses detected through SIFT-MS with a significant abundance for a single variety (Fig 1), and notably those above 150 m/z that are less likely to be related to fragmentation ions, it can be hypothesized that SIFT-MS has a greater sensitivity to detect volatiles than the selected HS-SPME/GC-MS method.

The chromatographic analysis sensitivity could be improved by using other sampling techniques such as SBSE or dynamic adsorbent sampling like Tenax TA ® (Elmore et al., 1997; Petronilho et al., 2014). On the other hand, two-dimensional chromatography (GCxGC) would mean higher peak capacity and resolution (Petronilho et al., 2014; Takase et al., 2015). High resolution mass spectrometry working in MS or MS/MS mode makes the characterization of volatile compounds more feasible by answering many questions, regarding the analytical characterization of both their structure, composition, and concentration in a very sensitive manner (Alvarez-Rivera et al., 2019).

Five main types of compounds were identified, sorted by their metabolic pathway of origin and/or class: aldehydes, alcohols, phenols, monoterpenoids, and C13- norisoprenoids (Table 3). In most cases, large differences were observed between the four studied varieties which tends to confirm the relevancy of the clustering performed using the SIFT-MS data.

Also, Carignan was proven to produce more C13-norisoprenoids and small alcohols or aldehydes (Fig 5), consistent with literature (Geffroy et al., 2015) and the previous hypothesizes formulated (§3.2). Of all the varieties, it was the one for which we detected the most different compounds and in greater quantities for most of them (Table 3). Duras produced as many monoterpenes as the Carignan; Mourvèdre had a balanced production while Merlot produced more phenols than the other groups (Fig 5).

The fact that Merlot who belongs to the same cluster than Gewurztraminer and Muscat à petits grains blancs doesn't exhibit the highest monoterpenes content deserves further content. This result is particularly linked to the choice of the cultivars made to represent each cluster for GC-MS

measurements, and to the chemometric pipeline used. Indeed, the multivariate data treatment enabled to discriminate varieties on the basis of similarity of their whole volatile fingerprint and did not focus on a few selected masses that might differ between varieties from a same cluster. Adding weight to specific ions associated with key impact compounds would be a way to turn the model into a choice matrix, helping breeders into variety selection. Our chemometric treatment combining PCA and HAC has proved effective in the past and in our study to create groups of similarity. However, other authors who implemented an untargeted approach like ours with SIFT-MS decided to implement either partial least-squares discriminant analysis (PLS-DA) or soft independent modeling of class analogy (SIMCA) (Kharbach et al., 2018; Ozcan-Sinir, 2020). The first method which might provide misleading results if the validation step is not properly performed, is particularly adapted to the analysis of large, highly complex data like those obtained in metabolomics studies (Gromski et al., 2015). While Kharbach et al. (2018) have chosen to base their discrimination on the combination of O_2^+, H_3O^+ and NO^+, we studied in most cases the segmentation proposed by each reagent ion separately which greatly contributes to simplify the data and limit the number of variables making PLS-DA less relevant. The second one which requires a training data set with known characteristics and class membership, and do not always lead to classification of samples into non-overlapping classes was less adapted to our experimental design and notably the stratified sampling approach (Vanden Branden & Hubert, 2005).

The method we developed could be particularly valuable for the phenotyping of new varieties. The breeding of resistant varieties to fungal disease such as downy mildew (*Plasmopara viticola*) or powdery mildew (*Erysiphe necator*) is very dynamic nowadays due to the global demand for reducing the use of pesticides. In many winegrowing regions, initiatives are taking place to cross local varieties with certified resistant genotypes. The method we developed could be implemented to select ideotypes that presents similar aroma characteristics than the local parent (Le Cunff, L et al., 2021). This potential use seems even more relevant as our method appears to enable grouping according to genetic similarity.

However, one must bear in mind that most of the SIFT-MS discrimination seems to be made on monoterpenols, C13-norisoprenoids, phenols, and small alcohols or aldehydes production. Other key compounds driving wine quality such as non-volatile varietal thiols or glycosidic precursors released during the winemaking process, alkyl-methoxypyrazine or rotundone might not be detectable and included in the clustering criterion. Accessing the full aroma potential of grapes through SIFT-MS would involve additional preparation steps. Such preparation might include the use of ethanol or heating to enhance aroma extraction from the skin (Alegre et al., 2020; Geffroy et al., 2015) and the implementation of acid or enzymatic hydrolysis to release bound compounds (Dziadas & Jeleń, 2016).

It cannot be excluded that the SIFT-MS discrimination would have been based on other compounds during vintages with distinct (and notably cooler) conditions of climate. The 2020 vintage was characterized by hot temperatures and little summer rainfall, some features that are known to enhance the accumulation of phenolic compounds, monoterpenols and C13-norisoprenoids (Duley, 2021; Robinson et al., 2014).

4. Conclusion

This work which is novel in various aspects provided several interesting findings. Grapevine varieties can be easily discriminated based on their volatile fingerprint using SIFT-MS and chemometrics. The composition of the homogeneous clusters of grape varieties only slightly differed according to reagent ions. However, O_2^+ appears to be the best suited notably due to its ability to generate more fragmentation ions and to promote the differentiation of similar weight compounds. Additionally, O_2^+ reagent ion generated the most discriminating ions and enabled the grouping of three Sémillon samples harvested at distinct sampling dates. The use of one single ion rather than three is particularly interesting as it allows to reduce the time of analysis and the number of variables facilitating the data treatment. Differences between clusters produced with O_2^+ were mainly explained through different production levels of the same compounds with, in most cases, the Carignan cluster exhibiting the

highest signal intensity, the Merlot and Mourvèdre clusters the lowest, while abundances were generally intermediate for the Duras cluster.

These findings were confirmed by HS-SPME-GC-MS analysis. The discriminating molecules were proved to belong to 4 families of volatile compounds: small alcohols or aldehydes, phenols, monoterpenols, and C13-norisoprenoids.

Our results show that SIFT-MS is a suitable technique which could find various applications in the wine industry. It could help nurserymen and winegrowers in the phenotyping of new varieties, particularly those resistant to fungal diseases whose development is expanding worldwide. It could also be useful for researchers to study the impact of winegrowing techniques on berry volatile composition using an untargeted approach. The method developed has the advantage of requiring a small amount of grapes, not needing a complex preparation step, and providing a quick response. However, it did not enable to get access to the full potential of the grapes notably aroma found under a bound form. More research is necessary to optimize our model and enhance the release of these latter compounds.

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.

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Table 1

Grape varieties investigated during the study with their related theoretical timing of veraison according to Parker *et al*. (2013), and berry skin color.

Variety	Timing of veraison	Berry skin color
Cabernet Franc	Middle	Red
Cabernet Sauvignon	Middle	Red
Carignan	Late	Red
Chardonnay	Early	White
Chenin	Late	White
Cinsault	Middle	Red
Colombard	Middle	White
Duras	Middle[a]	Red
Fer	Middle	Red
Gamay	Early	Red
Gewurztraminer	Early	Pink
Grenache	Late	Red
Malbec	Middle	Red
Merlot	Middle	Red
Mourvèdre	Late	Red
Muscat à petit grains blancs	Early	White
Négrette	Middle[a]	Red
Pinot Noir	Early	Red
Sauvignon	Early	White
Sémillon	Early	White
Syrah	Middle	Red
Tannat	Late	Red
Tardif	Late[a]	Red

[a]timing of veraison not mentionned by Parker et al (2013).

1 **Table 2**

2 Means, standard deviations, and significance of the ANOVA for the physico-chemical parameters measured on the grape samples. The three Sémillon samples

3 are included in the data.

Grape samples	Sugar concentration (°Brix)			Titrable Acidity (g/L of tartaric acid)			pH			Berry weight (g)		
1st sampling date (n = 21)	23.6	±	0.7	5.05	±	0.59	3.52	±	0.10	1.72	±	0.21
2nd sampling date (n = 33)	23.2	±	0.6	6.10	±	0.52	3.59	±	0.09	1.80	±	0.18
3rd sampling date (n = 21)	22.8	±	0.6	4.56	±	0.52	3.53	±	0.09	1.72	±	0.18
P-value	*0.702*			*0.128*			*0.836*			*0.946*		

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6   **Table 3**

7   GC peak area of volatile compounds identified in scan mode in Carignan, Duras, Merlot, and Mourvèdre grapes samples using HS-SPME/GC-MS methodology.

8   Means of three biological replicates per variety.

RT (min)	RI exp	RI lit	Compound	Mass (g/mol)	Chemical groups	Peak areas (x10^{6})			
						Carignan	Duras	Merlot	Mourvèdre
17.79	903	902[a]	Heptanal	114.2	Aldehyde	3.6	1.6	1.8	2.3
21.40	959	947[a]	2-heptenal	112.2	Aldehyde	nd	nd	nd	2.9
21.52	961	960[a]	Benzaldehyde	106.1	Aldehyde	2.2	nd	nd	nd
23.88	997	991[a]	3-octanol	130.2	Alcohol	nd	nd	0.6	nd
24.31	1004	998[a]	Octanal	128.2	Aldehyde	2.2	0.6	0.7	0.9
24.77	1011	1012[b]	2,4-heptadienal	110.2	Aldehyde	1.2	nd	nd	nd
26.25	1033	1031[a]	Benzyl alcohol	108.1	Alcohol	nd	0.8	nd	nd
26.83	1042	1043[c]	Benzeneacetaldehyde	120.2	Aldehyde	nd	0.6	nd	nd
30.6	1100	1098[a]	Linalool	154.3	Monoterpene	0.6	2.3	nd	1.2
30.79	1102	-	Unidentified monoterpene	-	Monoterpene	2.2	1.2	nd	0.2
30.91	1104	1100[a]	Nonanal	142.2	Aldehyde	2.4	1.2	1.4	3.2
36.29	1189	1191[a]	Methyl salicylate	152.2	Phenol	nd	0.1	nd	nd
36.55	1193	1199[a]	γ-terpineol	154.3	Monoterpene	0.8	0.7	nd	1.1
37.98	1216	1219[a]	β-cyclocitral	152.2	Monoterpene	0.7	nd	nd	0.3
40.04	1249	1252[a]	Geraniol	154.3	Monoterpene	nd	1.3	nd	nd
46.24	1352	1364[a]	β-damascenone (-Z)	190.3	C13-norisoprenoid	nd	nd	nd	0.2
47.56	1375	1384[a]	β-damascenone (-E)	190.3	C13-norisoprenoid	13.4	3.0	3.9	5.7
48.48	1391	1393[d]	Amylphenol	164.2	Phenol	41.9	38.8	49.4	31.3
51.58	1445	1455[e]	Trans-Geranylacetone	194.3	C13-norisoprenoid	1.5	nd	nd	nd
53.16	1473	1488[a]	Trans-β-ionone	192.3	C13-norisoprenoid	0.4	nd	nd	nd

9   [a](Adams, 2007), [b](Varlet et al., 2006), [c](Cho et al., 2007), [d](Mjøs et al., 2006), [e](Adams et al., 2005). *nd*: not detected.

10 **Figures**
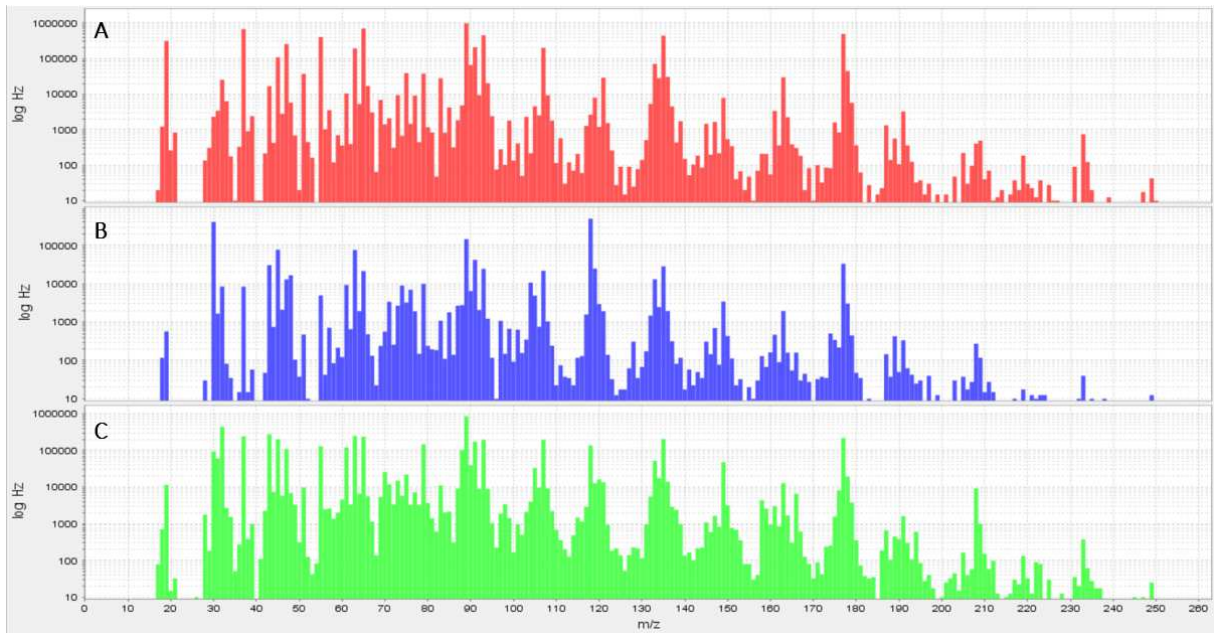
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12 **Fig 1**. Example of SIFT-MS grapevine fingerprint obtained using (A) H_3O^+, (B) NO^+ and, (C) O_2^+. as reagent
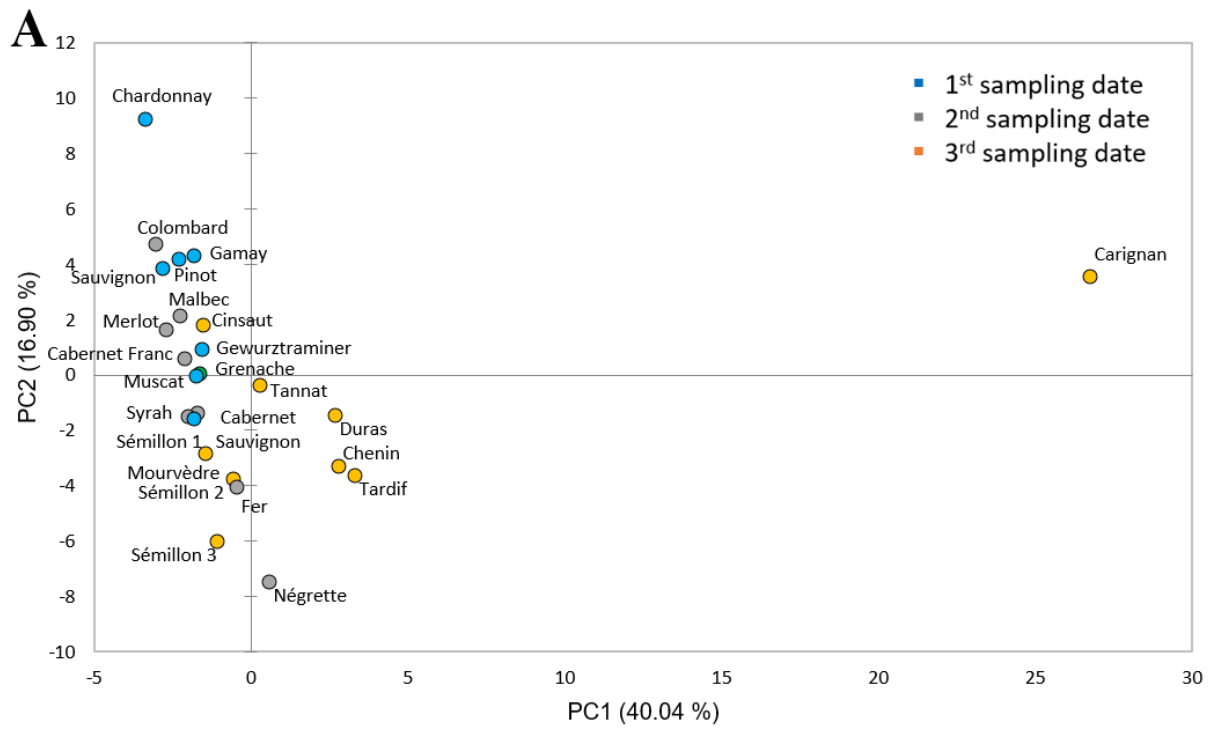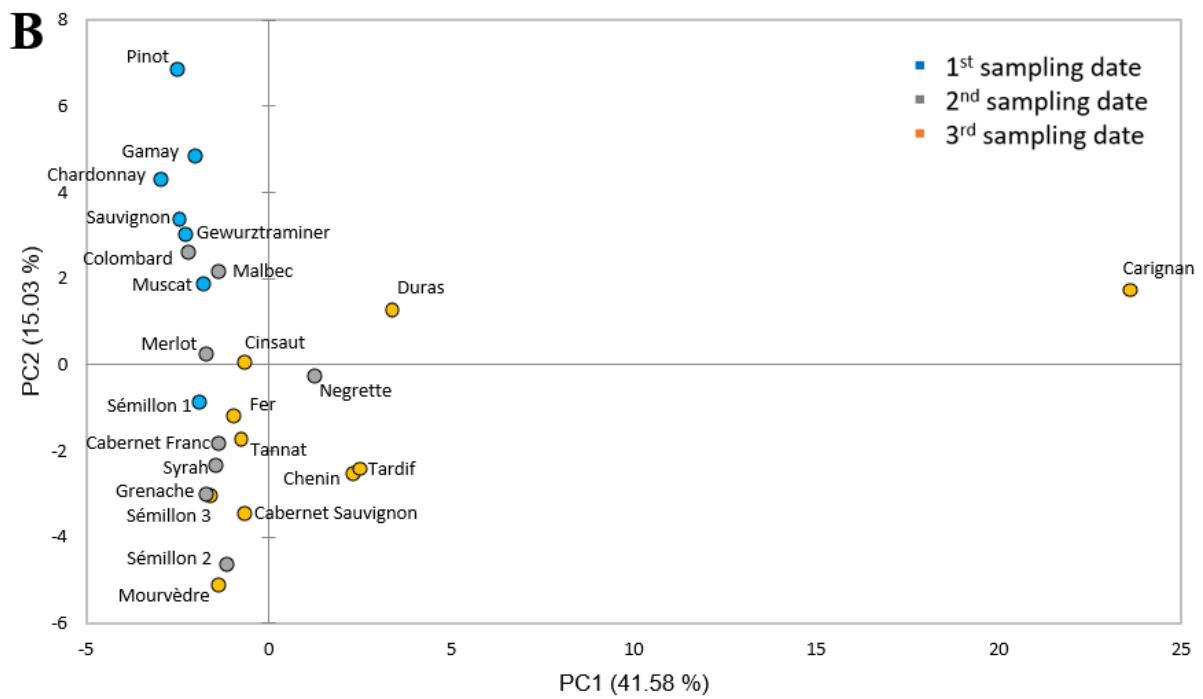
13 ion – Carignan data.

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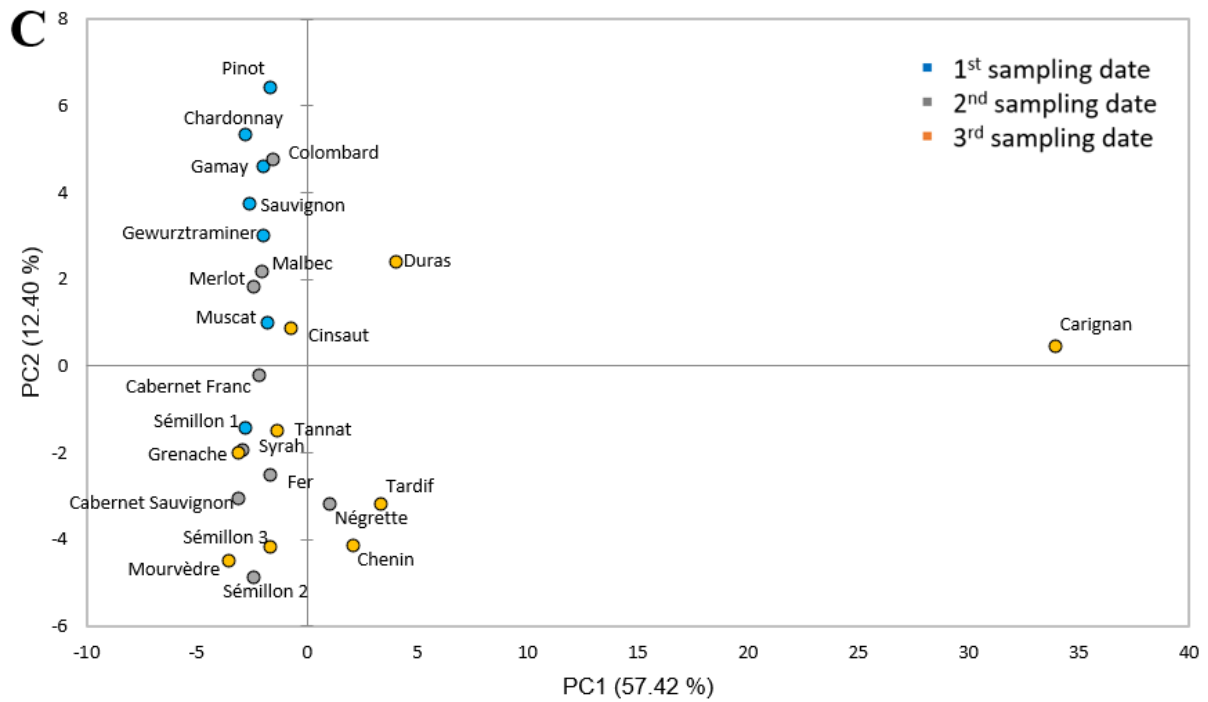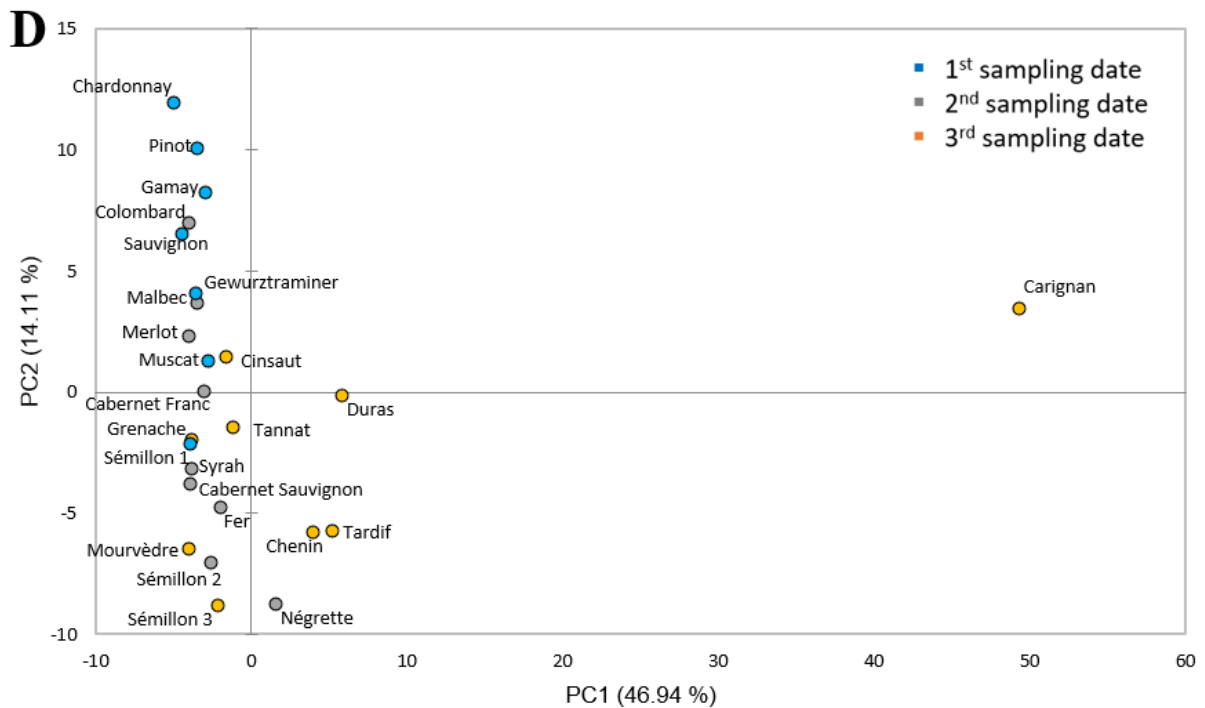
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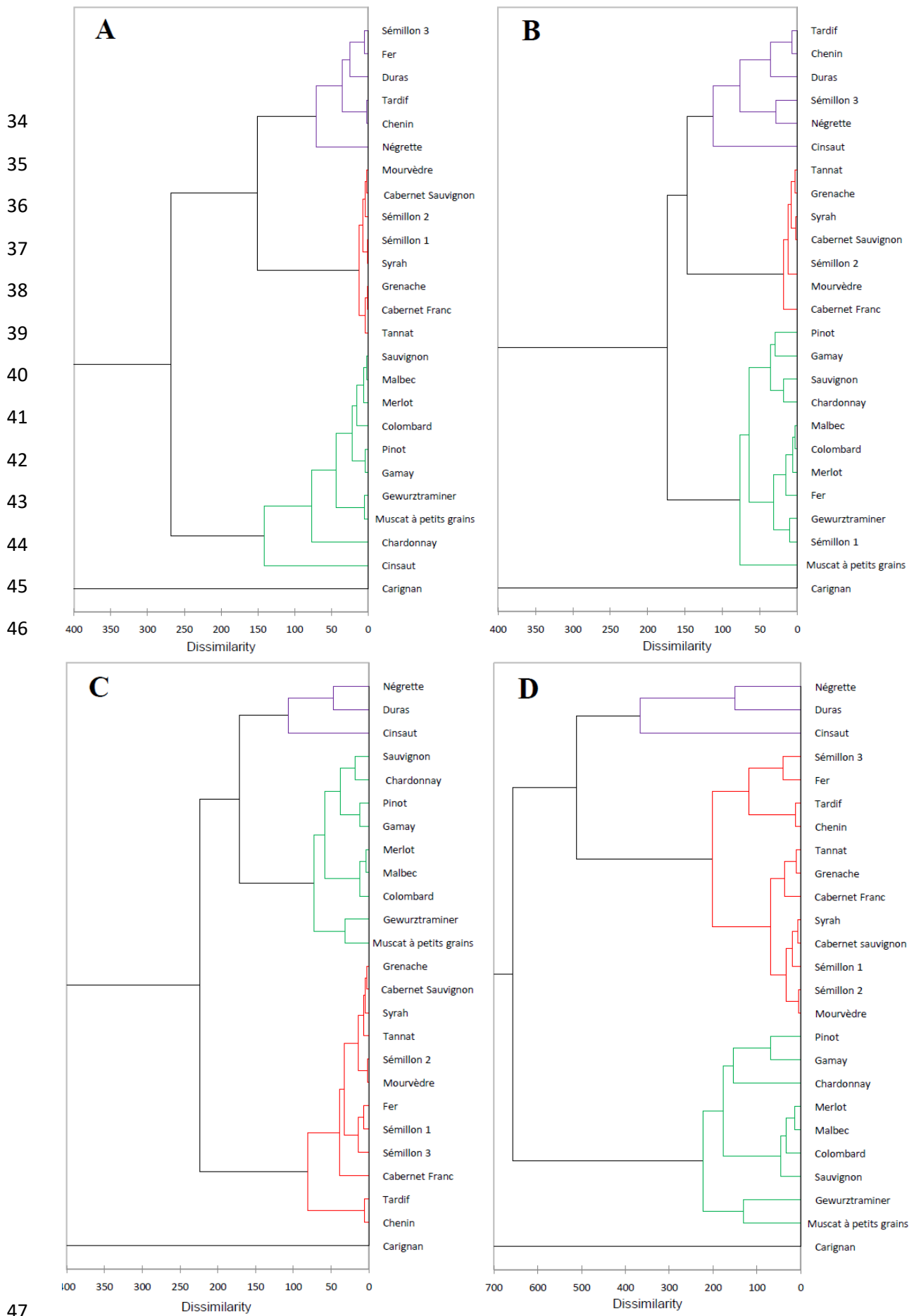

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Fig 2. Factor scores for a principal component analysis (PCA) performed on the SIFT-MS data for the studied grape varieties considering (A) only the H_3O^+ reagent ion, (B) only the NO^+ reagent ion, (C) only the O_2^+ reagent ion, and (D) all the reagent ions. Sémillon 1, 2, and 3 represent respectively the Sémillon sampled on the 1st, 2nd, and 3rd sampling date.
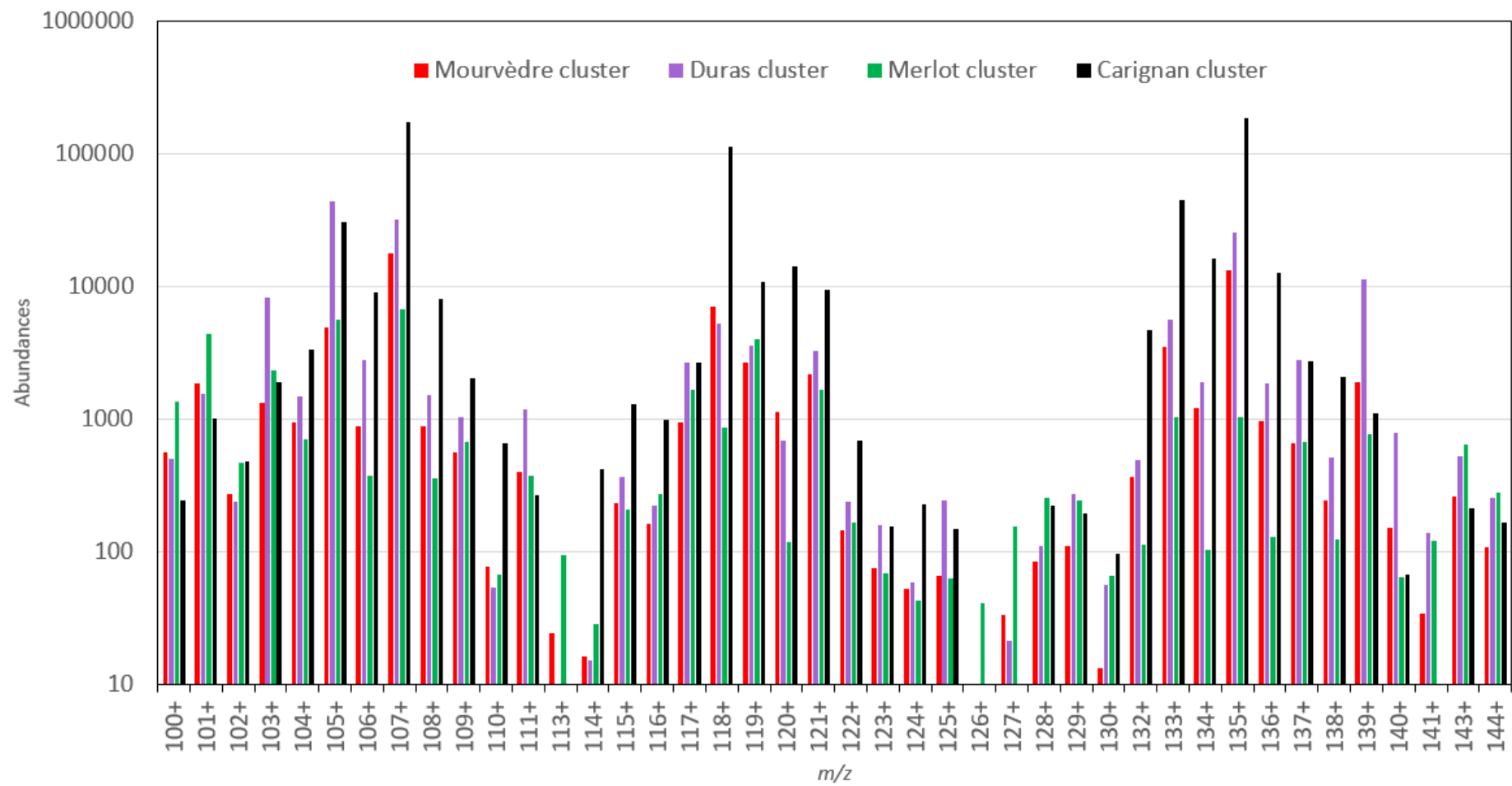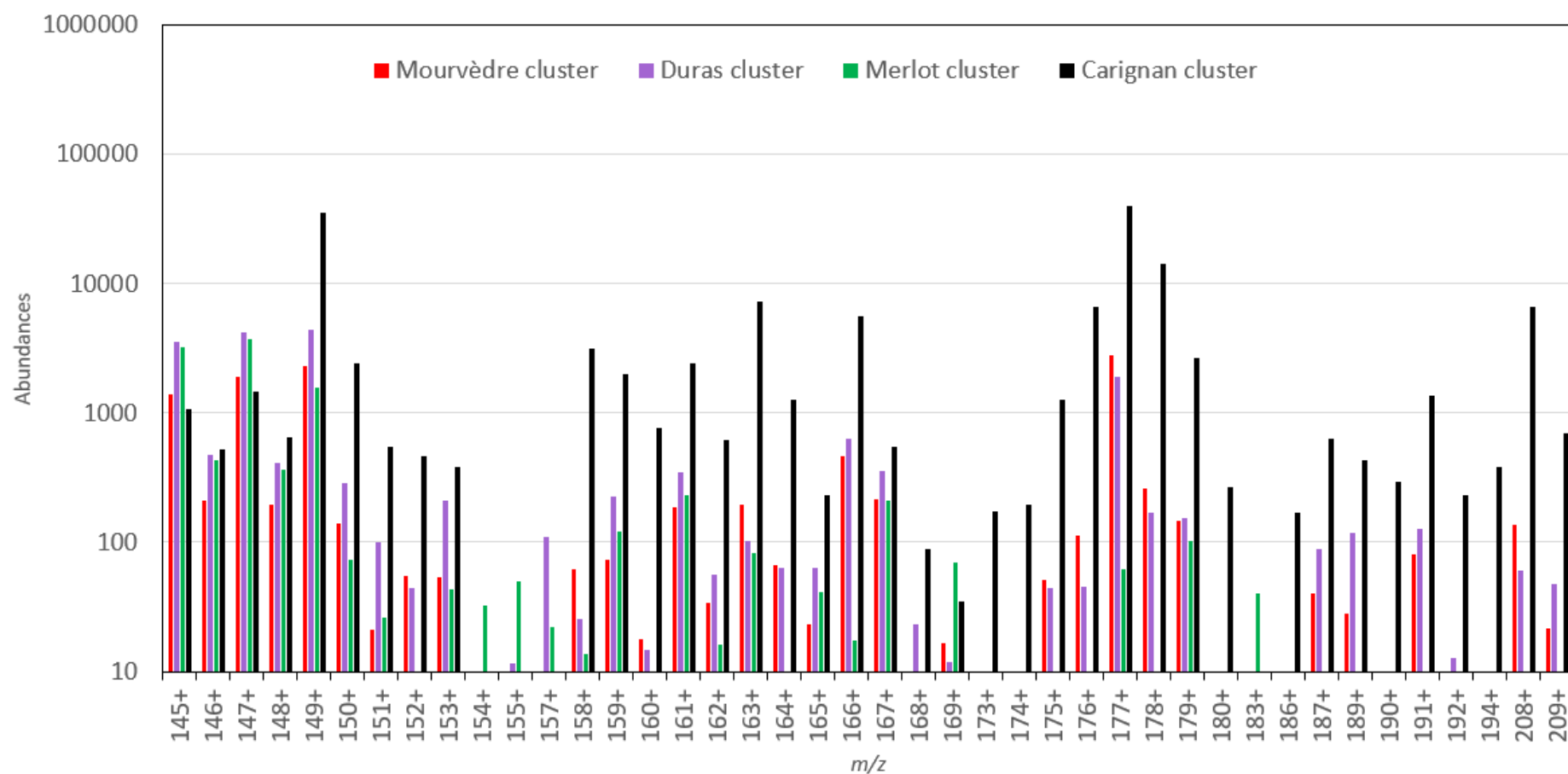
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Fig 3. Hierarchical Cluster Analysis (HCA) generated using principal component analysis (PCA) coordinates for (A) H_3O^+ reagent ion, (B) NO^+ reagent ion, (C) O_2^+ reagent ion, and (D) all reagent ions.

A

B

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Fig 4. Mean SIFT-MS spectrum obtained with O_2^+ reagent ion for all the grapevine varieties combined in the Carignan cluster (n = 3), Duras cluster (n = 9),
Mourvèdre cluster (n = 36), and Merlot cluster (n = 27). Masses from 100 to 144 *m/z* (A) and from 145 to 209 *m/z* (B). Only the masses that enabled to
discriminate the varieties after the ANOVA data pretreatment are shown.

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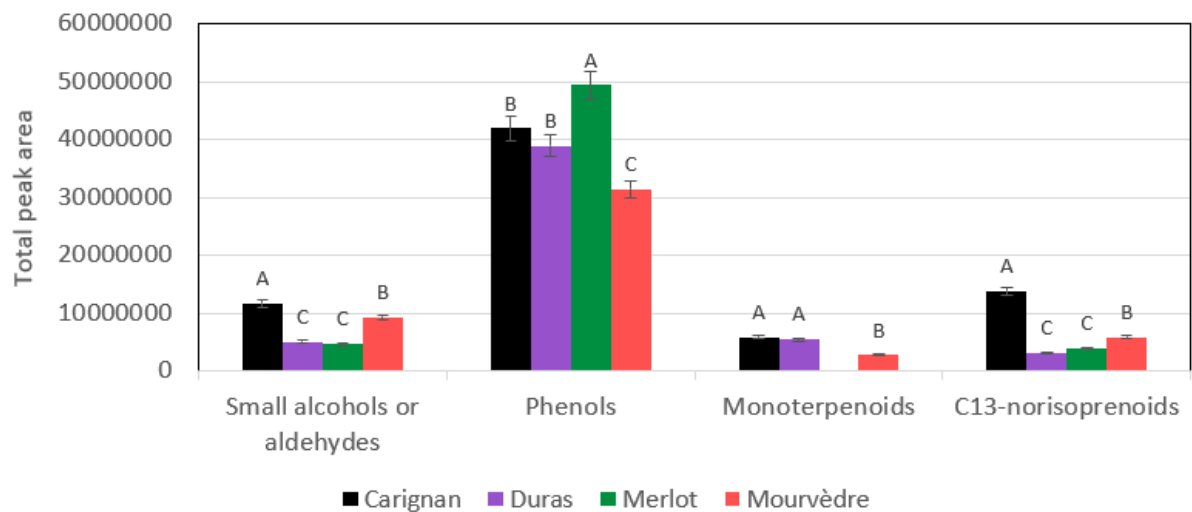
Fig 5. Total GC peak areas obtained for the 4 main classes of compounds identified by HS-SPME/GC-MS (small alcohols or aldehydes, phenols, monoterpenoids and C13-norisoprenoids) for the 4 representative varieties studied. Means of three replicates per variety. Error bars represent standard deviation of the means. Different letters indicate significant differences between varieties at *P < 0.05.*

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Graphical abstract