Identification of putative chemical markers in white wine (Chasselas) related to nitrogen deficiencies in vineyards

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Aim: Wine quality is influenced by the nitrogen nutrition of grapevines in the vineyard. A deficiency of this nutrient will affect grape quality, decrease yeast available nitrogen (YAN) and influence alcoholic fermentation. Chasselas wines from nitrogen-deficient grapes (YAN < 140 mg N/L) are systematically more astringent and bitter and less fruity than those from grapes with higher YAN content (Spring et al., 2014). The aim of this study was to identify chemical markers in wine linked to nitrogen deficiencies in the vineyard.

Methods and results: Wine samples produced from grapes growing in nitrogen-deficient vineyards with nitrogen treatment (HN) and without it (LN) were used over four consecutive years (2006–2009). They were all analysed at the same time (2012) with electronic-nose, GC-MS and UHPLC-TOFMS techniques. A metabolomics approach was used for a comprehensive survey of volatile and nonvolatile compounds in order to identify markers related to nitrogen nutrition. Volatile markers with alcohol and ester functions and nitrogen-containing compounds were found and tentatively identified by GC-MS. Additionally, 16 nonvolatile markers were putatively identified by UHPLC-TOFMS, including compounds from diverse chemical classes, namely, amino acids, vitamins, hormones, organic acids, phenolic compounds and polysaccharides.

Conclusion: The nitrogen nutrition of grapevines has a clear but complex effect on the chemical composition of wine. Several markers were tentatively identified and their role in wine composition discussed according to the actual knowledge reported in the literature.

Significance of the study: This study is an important starting point for selecting the most relevant chemical markers in wine, and for determining whether organoleptic problems are related to nitrogen nutrition deficiency in the vineyard and changes in vineyard management are needed.

Keywords: nitrogen deficiency, nitrogen fertilisation, wine metabolomics, chemical markers, white wine, electronic nose, GC-MS, UHPLC-TOFMS
INTRODUCTION

The nitrogen nutrition of vines and particularly berries has an important effect on grape and wine quality (Bell and Henschke, 2005). The management of this nutrient in the vineyard is often challenging and can impact economic, environmental and quality aspects. Excessive nitrogen levels result in vigour rather than grape quality (Wheeler and Pickering, 2003), and they increase the risk of diseases, such as grey rot (Conradie and Saayman, 1989). On the other hand, nitrogen deficiencies in the vineyard alter grape composition (Bell and Henschke, 2005; T. Garde-Cerdán et al., 2015; Perez-Alvarez et al., 2017; Gutiérrez-Gamboa et al., 2020), resulting in fewer flavour precursors (Choné et al., 2006) and a decrease in nitrogen-containing compounds (Schreiner et al., 2014), thereby influencing fermentation rate and yeast metabolism.

Yeast can only use part of the nitrogen present in the berry, commonly referred to as yeast available nitrogen (YAN), which is in the form of primary amino acids and ammonium. A YAN value of 140 mg N/L is generally considered to be the minimum level necessary to ferment a clarified must to dryness (Bell and Henschke, 2005; Jiranek et al., 1995). This value can vary with must sugar content, vine varieties and wine style. Tahum et al. (2019) reported an optimal YAN value of 130 mg N/L for cool-climate Riesling. In the case of red cultivars, which are fermented along with the skin, even lower values have been reported: 100 mg N/L for Pinot Noir (Schreiner et al., 2018) and 60 mg N/L for Merlot (Stockert et al., 2013). This can be explained by the contribution of N contained in the skin, which is about 29 % of the berry’s total YAN (Stines et al., 2000). To prevent stuck or sluggish fermentations, winemakers commonly supplement grape juice with ammonium salt. Supplementation with amino acids or with ammonium will induce different aroma profiles (Garde-Cerdán and Ancín-Azpilicueta, 2008; Miller et al., 2007). The timing of the nitrogen addition also influences the production of the aroma compounds (Seguonot et al., 2018). The complex correlation between wine flavour and YAN values was underlined by Ugliano et al. (2007), who recommended performing YAN analyses before nitrogen supplementation in the cellar. According to our unpublished study of Swiss white wines produced from nitrogen-deficient grape juice (YAN < 140 mg N/L), these practices can decrease, but not eliminate, quality defects such as insufficient aroma quality and pronounced bitterness and astringency.

Torrea and Henschke (2004) studied the impact of must nitrogen content on the sensory properties of Chardonnay wine and reported lower ratings for positive sensory descriptors, such as fruity and floral, for wines made with low-nitrogen juices. The obvious correlation between these sensory descriptors and the concentration of YAN in grapes of different vine varieties indicates the existence of chemical molecules in wine that could be markers for nitrogen deficiencies in vineyards. Previous studies have pointed to certain volatile compounds (i.e., higher alcohols and esters) that are systematically reported in studies on nitrogen nutrition. Webster et al. (1993) reported that significant differences can be found in the volatile profile and sensory analysis of Riesling wines, even after three and five years of storage, depending on the level of N supplementation in the vineyard. Fewer results have been reported for the influence of N nutrition on nonvolatile compounds, and they have often been contradictory with respect to total acidity or total phenols (Bell and Henschke, 2005). Choné et al. (2006) reported that higher N status in vines results in a diminution of total phenolic content in white Sauvignon grapes, just as it does in red varieties. Another study showed that foliar nitrogen treatment of Cabernet-Sauvignon vines decreased the flavanol concentration in the wine (Gutiérrez-Gamboa et al., 2017). However, Paru et al. (2015) reported increasing anthocyanin and flavanol content in red wine samples after foliar treatment with urea, but the flavanols were not affected by the treatment. Unfortunately, no information is available regarding the nitrogen content in the grape musts of the different variants; therefore, it is unclear whether or not the non-treated variant suffered a nitrogen deficiency. Indeed, nitrogen-deficient vineyards react differently to nitrogen supplementation than do vineyards where N is already in sufficient concentration (Moreira et al., 2011). Bell and Henschke (2005) published a review regarding the effect of nitrogen on the chemical composition of wine that demonstrated the complexity of this question. In view of this complexity, a holistic approach (i.e., studying many compounds in wine to identify the chemical markers related to nitrogen nutrition) may be interesting.

Using metabolomics, this study aims to provide an overall survey of ‘all possible’ low-molecular-weight compounds of complex molecular blends, by highlighting significant statistical variations.
across samples and identifying chemical markers. The markers are identified by database matches using experimental data gathered via high-resolution mass spectrometry spectra (HRMS) and MS/MS fragmentation patterns (using either liquid chromatography–mass spectrometry or gas chromatography–electronic impact–MS). Following spectral deconvolution, the data matrix (sample feature area × m/z–RT pairs) is processed by multivariate data analysis methods to highlight major trends and identify biomarkers of interest. This approach has proven to be a valuable tool for the study of complex systems. In the past decade, the number of publications on grape and wine metabolomics has expanded dramatically. In their reviews, Cozzolino (2016) and Pinu (2018) demonstrated the importance of metabolomic approaches in grape and wine research. Such approaches have been used to compare the metabolomic profiles of different grape varieties (Arapitsas et al., 2020), as well as to study the plant metabolites’ responses to abiotic stress, such as water stress (Pinaud et al., 2017), and to investigate oenological techniques, such as sulfite addition (Roullier-Gall et al., 2017) and storage (Gougeon et al., 2019). Moreover, untargeted techniques using MS detection have enabled the identification of new substances in wine (Arapitsas et al., 2016).

Ciampa et al. (2019) recently investigated the effect of nitrogen nutrition on the grape metabolome using the NMR technique and found evidence of significant differences in Nero di Troia grapes for valine, leucine, isoleucine, proline and malic acid. Focusing on the effect of nitrogen nutrition on the grape metabolome, other studies have reported changes in nitrogen-containing compounds (Pérez-Alvarez et al., 2017). The effect of grape juice composition – particularly amino acids and fatty acids – on wine’s yeast metabolism is also a well-studied area (Fairbairn et al., 2017; Pinu et al., 2014; Pinu et al., 2019). However, these studies have often been conducted on model growing media (Rollero et al., 2015) or on grape juice with YAN values higher than 140 mg N/L, and they have often focused on volatile-compound production (Fairbairn et al., 2017; Garde-Cerdan and Ancín-Azpilicueta, 2003). To our knowledge, no study has yet examined the effect of nitrogen deficiency in the vineyard on the composition of wine’s volatile and nonvolatile metabolomes.

The purpose of the present study was to identify the chemical compounds in white wine (Vitis vinifera L. cv. Chasselas) which are related to grape and berry nitrogen nutrition, and to evaluate their relevance as markers for determining whether wine was produced from nitrogen-deficient grapes. Two groups of wine were compared, which were both produced from Chasselas grapes from the same vineyard known to give grapes with low YAN content (<140 mg N/L). Two nitrogen management treatments were applied on the field to produce (a) nitrogen-deficient grapes (LN) with no nitrogen supplementation and (b) grapes with higher N content (YAN > 180 mg N/L) (HN) using foliar nitrogen treatment during the veraison stage. Wines were produced from these two variants of grapes and analysed. Untargeted analyses of volatile and nonvolatile compounds with the putative identification of key metabolites using MS-based platforms were carried out. To identify markers correlated with nitrogen deficiency, the results obtained were combined and analysed using statistical methods.

MATERIALS AND METHODS

1. Grape source

The experiments in the field were conducted in a vineyard at the Agroscope research station in Changins, canton of Vaud, Switzerland, between 2006 and 2009. The vineyard had been planted in 2000 with Itis vinifera L. cv. Chasselas (clone 800) that had been grafted onto rootstock 3309C. The vines were trained to a single Guyot training system (300 x 35 cm). The annual mean precipitation evenly distributed among the seasons was 1009 mm, and the local average temperature during the growing period (April to October) was 14.9°C. The vineyard soil was a compact background moraine with 0–9 mass % total carbonate as CaCO3 and about 1 % of organic matter. The soil was rich in K (4.1 %–4.9 %) and was provided with the usual levels of Mg (4.1 %–4.9 %). The experiments, performed in four replications of 20 plants, were organised in randomised blocks according to the following protocols: low-nitrogen (LN) grapes without treatment or high-nitrogen (HN) grapes with foliar nitrogen treatment during the veraison stage [stage BBCH 83-85] (four applications of 10-unit N/ha from the beginning to the end of August with urea poor in biuret (< 0.55 g/L; 0.05 % p/p) Folur®). The experiment was maintained with no changes made to it for four years.

2. Winemaking and storage

Approximately 150 kg of grapes were vinified per experimental variant (pooling the four agronomical
repetitions) following the standard Agroscope protocol: the grapes were crushed, sulfated (50 ppm SO₂), and cold settled overnight after enzyme addition at 12 °C. Alcoholic fermentation started without nitrogen correction with yeast addition (Bourgoblanc®, 20 g/hL) at 20 °C, and was monitored daily by density measurement. At the end of alcoholic fermentation, the wines were centrifuged and lactic bacteria were added (Viniflora CH35, 1 g/hL) to guarantee the completion of the malolactic fermentation. The wines were then stabilised with 50 ppm SO₂ and stored for one month at 0 °C. Finally, the wines were filtrated with a 0.65-µm filter and bottled. Bottles were stored under controlled conditions (10–12 °C, in a dark room) until the analyses began.

3. Analytical methods

3.1. Must and wine standard chemical analysis

Must samples were collected from the tank before fermentation and were analysed with FTIR (WineScan, Foss) to determine standard parameters (Oe°, pH, total acidity, tartaric acid and malic acid). Formol index was determined with the titration method described by Aerny (Aerny, 1996) and converted to YAN (mg N/L) using a correction factor of 14, which we determined on the basis of a linear correlation between the two parameters using more than 100 samples in 2014 and 2015 (unpublished results).

Wine samples were prepared in 2012 using three bottles of each variant from vintages of 2006–2009. The three bottles were homogenised and divided into three parts: one part for standard analysis, one for sensory analysis and one for metabolomics.

Standard analyses (ethanol, pH, total acidity, glycerol, tartaric acid and malic acid) were performed with FTIR (WineScan, Foss) using in-house calibration.

3.2. Sensory analysis of wine

The sensory analysis was performed by a trained panel of 12 people from Agroscope, each of whom had several years of practice in the sensory analysis of wine. Wines were analysed in the first few months after bottling in each year and a second time immediately before the metabolomics study in 2012. Wines were evaluated using predefined quality attributes on a scale from 1 (bad, absent) to 7 (excellent, high). The results were processed with the FIZZ software (version 2.50) of Biosystems (F 21560, Couternan).

3.3. Electronic nose (SMart Nose®) analyses

A volatile metabolite analysis was performed using a SMart Nose® instrument (LDZ, Marin, Switzerland) equipped with a Combi PAL autosampler (CTC Analytics AC, Zwingen, Switzerland). Samples were prepared in vials containing 0.5 g of NaCl. After an addition of 2 mL of wine, the samples were incubated for 10 min at 60 °C with agitation before the injection of a 2.5-mL sample from the headspace. The needle temperature was 110 °C, and the injector temperature was 160 °C. Scan analyses were performed in the mass range of 10–180 amu with a scan speed of 500 ms/amu. The total acquisition time was 170 s, and the purge time between samples was 60 s. Three replicates were measured for each sample. The analyses occurred in a randomised order.

3.4. Identification of VOCs by GC MS/NPD

Samples were extracted using a divinylbenzene/ carboxen/polydimethylsiloxane (DVB/CAR/PDMS) StableFlex fibre, 2 cm, 50/30 μm (Supelco, Bellefonte, PA, USA). The fibre was conditioned before use according to the supplier’s recommendations (270 °C for 60 min). The analyses were carried out using a 7890B GC coupled with a 5977A mass selective detector (MSD) (Agilent Technology, Santa Clara, CA, USA) and a nitrogen-phosphorus detector (NPD). Injections were performed using an MPS2 autosampler equipped with Maestrol software, V.1.4.8.14/3.5 (Gerstel, Sursee, Switzerland).

The headspace of 2 mL of wine samples was extracted for 180 min at 45 °C with an agitation rate of 500 rpm. The bound volatiles were desorbed for 1 min at 250 °C in the injector, using its splitless mode for 60 s and then opening the split valve (split flow = 35 mL/min). The volatile compounds were separated on a TRB FFAP fused silica capillary column (60 m × 0.32 mm, 0.5 μm film; Teknokroma, Barcelona, Spain) with helium as the carrier gas at a constant flow of 2.0 mL/min (25.298 cm/s).

The oven temperature was programmed as follows: 2 min at 40 °C, then heated to 90 °C at a rate of 6 °C/min, held for 1 min, and then heated to 220 °C at a rate of 12 °C/min, with a final hold time of 8 min.
The settings of the NPD were as follows: 300 °C with flow rates of H2 at 3 mL/min, air at 60 mL/min, and makeup at 10 mL/min and a bead voltage of 1.1 V. The MS settings were as follows: transfer line at 250 °C, source temperature at 230 °C, and analytes monitored in SCAN mode between 30 and 300 m/z without solvent delay.

The detector response signals were integrated using Chemstation Data Analysis software version E.02.00.493 (Agilent Technologies). The NIST/EPA/NIH mass spectral library (NIST11) version 2.0 g (NIST, Gaithersburg, MD, USA) was used for peak identification.

3.5. UHPLC-TOF analysis (sample preparation, analysis conditions)

Lyophilised wine samples (10 mg ± 2 mg) were dissolved in 10 mL water/ethanol (50:50 v/v) solution. LC-HRMS data were acquired using a UHPLC-PDA-TOFMS instrument (LCT Premier, Waters) equipped with an electrospray ionisation (ESI) source. Detection was ensured by a TOF-MS in W-mode. The m/z range was 100–1000 Da with a scan time of 0.25 s, capillary voltage of 2.8 kV, cone voltage of 40 V, desolvation temperature of 250 °C, source temperature of 120 °C and desolvation gas flow of 600 L/h. The MS was calibrated using the sodium formate, and leucine enkephalin was used as the lock mass. The injection volume was 1 µL. Each extract was profiled independently in the ESI positive and negative ionisation modes using two orthogonal separation methods. First, a reverse phase (RP)-based separation was accomplished using an Acquity UPLC BEH C18 column (50 × 1.0 mm i.d., 1.7 µm, Waters) with a linear gradient of 0.1 % formic acid (FA) in water (solvent A) and 0.1 % FA in acetonitrile (Solvent B) from 98 % to 2 % of A in 5 min, followed by 2 min at 2 % A, then returned to the initial conditions for 1 min at a flow rate of 0.3 mL/min. Second, extracts were profiled using hydrophilic interaction chromatography (HLIC) with an Acquity UPLC BEH Amide column (50 × 1.0 mm i.d., 1.7 µm, Waters). The gradient used was as follows: 5 % A for 2 min, then 35 % A for 4 min, followed by 35 % A for 1 min and then back to the initial conditions for 1 min before the next injection. All the extracts were injected randomly with an interposition of blank solvent every six injections.

4. Statistical analysis

The UHPLC-TOF-MS fingerprints were processed with MZmine 2.20 (Pluskal et al., 2010) for mass signal extraction and alignment from 0.2 to 5 min with m/z values ranging from 100 to 1000 m/z. The GridMass 2D (Treviño et al., 2015) peak detection algorithm was used for peak recognition. Each peak list was deisotoped and aligned using the RANSAC alignment method and then gap-filled. Adducts and complex ions were removed from the peak list before being exported to the “.csv” file-format. Before statistical analysis, each data table containing the list of m/z-RT pairs and the corresponding area in each sample was normalised by sum and concatenated. Principal component analyses (PCA) and orthogonal partial least square discriminant analyses (OPLS DA) models were evaluated with the SIMCA P software (version 14, Umetrics, Umeå, Sweden). For each model, a leave-one-subject-out cross-validation was performed to assess the model fit. The validity of the discriminant models was verified using permutation tests (Y scrambling) and CV analysis of variance (ANOVA) (p-value < 0.05).

RESULTS AND DISCUSSION

1. Characterisation of wine samples (standard chemical analyses of must and wine and sensory analyses of wine)

Earlier trials have demonstrated the efficiency of foliar nitrogen treatment in the vineyard regarding the nitrogen content of grape must (Verdenal et al., 2015). In our study, a significant difference was found for each vintage between the YAN values in the grape must of the two variants: 97–127 mg N/L in the LN variants and 182–237 mg N/L in the HN variants (Table 1). The foliar nitrogen treatment increased the YAN in the must by 105–110 mg N/L. Among the other chemical parameters analysed in the grape must, only the concentration of malic acid was influenced by the treatment, increasing by 0.4–0.6 g/L in HN variants. The concentration of malic acid is strongly related to grape maturity, which may explain the strong vintage dependence of this parameter. Despite the fertilisation effect that might be expected, the foliar nitrogen treatment had no significant impact on the yield at a 95 % confidence level. In contrast, the vapour, measured by the weight of pruning wood, increased significantly in two out of the four years with fertilisation, from 585 to 770 g vine in 2007, and from 613 to 703 g/vine in 2008. Even if the same experimental block was used over the four-year period, no cumulative effect was observed in the measured parameters.

The alcoholic fermentation of all musts was completed within 13 days without any problems.
As expected, the LN variants needed three to four more days than the HN variants to arrive at a sugar concentration of less than 1 g/L. This difference could have been dealt with by adding nitrogen salt to the LN must in the cellar; however, this can change the initial composition of YAN, which we wanted to avoid.

The targeted quantitative analysis of the standard chemical parameters of wine was repeated in 2012 for all the samples before the comprehensive metabolomics study (Table 2). A significant difference was only noted for total acidity, which was lower in HN, in agreement with the results of Reynard et al. (2011). The pH, which is commonly negatively correlated to the total acidity, slightly increased in HN. However, the difference between the two variants was not statistically significant at the 95% level. As expected, the concentration of lactic acid, produced in the second fermentation from the malic acid of the grape must, was mostly higher in HN. However, because of the low concentration recorded and the limited number of samples (four samples by variants), the difference was not found to be statistically significant at the 95% level.

A sensory evaluation of the wines was carried out each year after bottling and was repeated in 2012 before the comprehensive metabolomics study to confirm the conservation of the typical differences observed between LN and HN. The 2012 results confirmed positive correlations between the nitrogen content of grapes (LN, HN) and the hedonic descriptor general-impression and sensory descriptors: fruit aroma, body and acidity (Figure 1). Furthermore, bitterness, herbaceous aroma and stress aroma were lower in HN. Stress aroma is a general descriptor used to characterise wines without varietal aromas and expressing notes of hay, naphthalene and wet tissue. The most important differences found by the panel were for stress aroma (1.3 note or 18% on average) and bitterness (1.1 note or 15% on average).

The ANOVA showed a significant difference between LN and HN in terms of sensory quality. However, regarding the vintage, no significant effects were found between the different years. This confirms that despite the inhomogeneity of these samples in terms of age, they are representative of the effect of nitrogen content and can be used to identify chemical markers using comprehensive metabolomic investigation.

2. Analyses of volatile metabolites by electronic nose (Smart Nose®) and GC-MS

2.1. MS fingerprint using an electronic nose

MS-based electronic noses are used for rapid screening, classification and discrimination in food analyses, quality control and authenticity analyses. Electronic noses provide unresolved MS fingerprints of the sample gas phases, which are further submitted to principal component analysis (PCA). The advantage of this method is its speed, and its greatest disadvantage is that the compounds present cannot be identified or quantified. For this reason, GC-MS was used to complete the analyses.

### Table 1. Yield and standard analysis of must samples from different vintages (2006–2009).

| Vintage | Treatment | Yield [kg/m²] | Soluble solids [°Brix] | Total acidity [g/L] | Tartaric acid [g/L] | Malic acid [g/L] | YAN [mg N/L] |
|---------|-----------|---------------|------------------------|---------------------|---------------------|-----------------|--------------|
| 2006    | LN        | 1.0           | 19.2                   | 5.7                 | 5.5                 | 2.3             | 101          |
|         | HN        | 1.1           | 19.4                   | 5.5                 | 5.1                 | 2.8             | 210          |
| 2007    | LN        | 1.3           | 19.3                   | 5.7                 | 5.4                 | 2.2             | 98           |
|         | HN        | 1.5           | 19.0                   | 6.1                 | 5.6                 | 2.8             | 206          |
| 2008    | LN        | 1.4           | 17.6                   | 7.9                 | 5.9                 | 4.0             | 127          |
|         | HN        | 1.5           | 17.2                   | 8.4                 | 6.1                 | 4.6             | 237          |
| 2009    | LN        | 1.1           | 20.7                   | 4.7                 | 5.1                 | 1.4             | 97           |
|         | HN        | 1.2           | 20.7                   | 5.0                 | 5.2                 | 1.8             | 182          |
| Mean ± SD | LN      | 1.2 ± 0.2     | 19.2 ± 1.3             | 6.0 ± 1.4           | 5.5 ± 0.3           | 2.5 ± 1.1 b    | 106 ± 14 b   |
|         | HN        | 1.3 ± 0.2     | 19.2 ± 1.4             | 6.3 ± 1.5           | 5.5 ± 0.5           | 3.0 ± 1.2 a    | 209 ± 23 a   |

The analyses of must were carried out in each of the four years. Treatment indicates the two variants which were compared: LN as the control (without treatment) and HN as the variant with foliar nitrogen treatment during veraison. Total acidity is expressed as tartaric acid. Mean values with different letters in the same column indicate a statistically significant difference at α ≤ 0.05.
TABLE 2. Standard analysis of wine samples from different vintages (2006–2009).

| Vintage | Treatment | Alcohol [v/v%] | pH   | Total acidity [g/L] | Tartaric acid [g/L] | Lactic acid [g/L] | Total polyphenol index |
|---------|-----------|----------------|------|---------------------|---------------------|------------------|-----------------------|
| 2006    | LN        | 11.6           | 3.33 | 3.8                 | 1.5                 | 1.5              | 6.7                   |
|         | HN        | 11.8           | 3.38 | 3.4                 | 1.4                 | 1.8              | 7.0                   |
| 2007    | LN        | 12.1           | 3.52 | 3.7                 | 1.6                 | 1.6              | 4.5                   |
|         | HN        | 12.0           | 3.59 | 3.2                 | 1.5                 | 2.0              | 4.3                   |
| 2008    | LN        | 11.2           | 3.37 | 4.6                 | 1.7                 | 2.5              | 4.1                   |
|         | HN        | 11.2           | 3.36 | 4.4                 | 1.8                 | 2.7              | 4.3                   |
| 2009    | LN        | 12.7           | 3.57 | 3.7                 | 1.4                 | 1.3              | 4.7                   |
|         | HN        | 12.4           | 3.56 | 3.2                 | 1.5                 | 1.3              | 4.7                   |
| Mean ± SD| LN       | 11.9 ± 0.6     | 3.45 ± 0.12 | 4.0 ± 0.4 a | 1.6 ± 0.1 | 1.7 ± 0.5 | 5.0 ± 1.2 |
|         | HN       | 11.9 ± 0.5     | 3.47 ± 0.12 | 3.6 ± 0.6 b | 1.6 ± 0.2 | 2.0 ± 0.6 | 5.1 ± 1.3 |

The analyses of the wines were performed at the same time in 2012. Treatment indicates the two variants that were compared: LN as the control (without treatment) and HN as the variant with foliar nitrogen treatment during veraison. Total acidity is expressed as tartaric acid. Mean values with different letters in the same column indicate a statistically significant difference at α ≤ 0.05.

FIGURE 1. Sensory analysis of wines from different vintages.
Sensory analysis was carried out in 2012 by a trained panel of 12 people. Wines were judged using predefined quality attributes on a scale from 1 (bad, absent) to 7 (excellent, high). For clearer visualisation, this scale was reduced to a 0–5 scale. The dashed line corresponds to HN variants, the solid line to LN variants. Descriptors marked with an asterisk are significantly different between the two variants (α ≤ 0.05).
of volatile metabolites and identify potential markers.

The PCA of the electronic nose results of the wine set, given as intensity by mass in the range of 42–180 m/z, differed between LN and HN (Figure 2). The two principal components (PC1 and PC2) accounted for 67% of the total variance. LN variants are mostly placed in the positive part of the PC2 axis for each vintage. An ANOVA was carried out to identify the variables responsible for this distinction. The results showed that two variables, 56 and 57 m/z, significantly differed between LN and HN. According to the YAN content in the must, two additional variables, 53 and 55 m/z, were found to significantly differ. Even when the different samples were separated on the first axis, no clear classification was observed according to vintage over all the samples. This result suggests that PC1 is not related to the vintage, but to other parameters, which could not be elucidated in this study.

2.2. Putative identification of potential volatile chemical markers by GC-MS

The effect of YAN content on the volatile composition of wine has been extensively studied (Bell and Henschke, 2005; Fairbairn et al., 2017; Garde-Cerdán and Ancín-Azpilicueta, 2008; Ugiano et al., 2007; Gutièrez-Gamboa et al., 2020). Our study focused on the markers that resulted in the differentiation between LN and HN variants on the SmartNose results. For this reason, only the masses 56, 57, 55 or 53 m/z were monitored during the GC-MS analyses of the wine samples. The SIM chromatograms of the wines from the two variants (LN and HN) were compared. Substances that were found at systematically higher or lower concentrations in LN versus HN are listed in Table 3. The identification was performed by matching experimentally obtained MS spectral data and retention index values with those in the literature.

The potential markers found in the GC-MS analyses can be classified into three groups, as noted in Table 3: higher alcohols, esters and nitrogen-containing molecules.

2.2.1. Higher alcohols

Higher alcohols are produced during fermentation by the yeast from either amino acids or sugar (Áyrapá, 1971). Different higher alcohols react differently to the nitrogen nutrition of the
vineyard. As expected, the concentrations of 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol decreased in HN variants. The same trends have been reported in the literature for similar studies (Bell and Henschke, 2005; Tahim and Mansfield, 2019). In contrast, the concentration of 2-methyl-1-propanol increased in the HN variants, as has also been reported by Webster et al. (1993). Higher alcohol content depends on the concentration and composition of available ammonium (YAN). Several studies have demonstrated that the addition of ammonium salts or diverse amino acids to low-nitrogen-containing must results in different higher alcohol profiles (Fairbairn et al., 2017; Garde-Cerdán and Ancín-Azpilicueta, 2008). In a synthetic fermentation medium with YAN ranging from 70 mg N/L to 330 mg N/L, Rollero et al. (2015) found a negative quadratic effect of nitrogen for 2-phenylethanol, 2-methyl-1-butanol and 3-methyl-1-butanol, with a maximum concentration of 150–200 mg N/L, in accordance with results reported by Ayräpää (1971). The addition of ammonium salt to the LN must may lessen the difference in concentration of higher alcohols between the LN and HN variants. However, the difference in the composition of YAN between the variants may lead to differing volatile profiles.

Recent publications have reported that 2-methyl-1-butanol and 3-methylbutanol have suppressing effects on the fruity notes in red wine (Cameleyre et al., 2015; de-la-Fuente-Blanco et al., 2016); this result could account for the differences in fruity notes in our study.

2.2.2. Esters

Generally, esters are found in higher concentrations in wines produced from must containing high levels of YAN (Bell and Henschke, 2005). In our study, ethyl-9-decenoate and the esters of succinic acid, which were identified as potential markers, were present in lower concentrations in the wines from HN variants.

Ethyl-9-decenoate, an unsaturated fatty acid ester, was reported by Novo et al. (2014) as being the only ester in their study whose concentration was influenced by the nitrogen content of the grape must.

The remaining three esters (Table 3) are formed from combinations of succinic acid and alcohols. To our knowledge, only diethyl succinate has been reported in previous wine-fermentation studies. The concentration of the diethyl-succinate decreased with the addition of amino acids to the must before fermentation (Garde-Cerdán and Ancín-Azpilicueta, 2008), indicating that high nitrogen content represses the formation of the succinic ester, which is consistent with the results of our study.

2.2.3. Nitrogen-containing substances

N-(3-methylbutyl)-acetamide was found at higher concentrations in wines from HN variants, benzothiazole was found at lower concentrations. N-(3-methylbutyl)-acetamide concentration is known to increase with the storage time of white wine. This effect may explain why this molecule was not identified as a marker of nitrogen treatment

| RT [min] | SIM mass [m/z] | Substance | Chemical class | RI literature | RI measured | Identification | Molecular formula | Correlation |
|----------|----------------|-----------|----------------|---------------|-------------|---------------|------------------|-------------|
| 11.00    | 56             | 2-methyl-1-propanol | higher alcohol | 1084         | 1088        | MS, RI     | C8H16O     | positive     |
| 14.50    | 56             | 2- and 3-methyl-1-butanol | higher alcohol | 1225        | 1236        | MS, RI     | C6H16O     | positive     |
| 21.40    | 56             | diethyl succinate | esters      | 1697         | 1710        | MS, RI     | C6H12O     | negative     |
| 21.48    | 55             | ethyl 9-decanoate | esters      | 1675         | 1717        | MS, RI     | C7H14O     | negative     |
| 22.55    | 56             | butyl-ethyl succinate | esters    | 1820         | 1814        | MS, RI     | C8H16O     | negative     |
| 23.36    | 56             | N-(3-methylbutyl)-acetamide | nitrogen-containing | 1895        | 1881        | NPD, MS, RI | C7H16NO   | positive     |
| 23.69    | 56             | ethyl 3-methylbutyl succinate | esters | 1892         | 1909        | MS, RI     | C7H16O     | negative     |
| 24.00    | 56             | 2-phenylethanol | higher alcohol | 1944         | 1940        | MS, RI     | C6H10O     | negative     |
| 25.06    | 53             | benzothiazole | nitrogen-containing | 1984        | 2038        | NPD, MS, RI | C8H8N   | negative     |

Correlation indicates the trend of the substance in relationship with HN variants. RT: retention time; SIM: selected-ion monitoring; RI: retention index; NPD: nitrogen phosphorous detector; MS: mass spectrum; Std: chemical standard injected. Columns used in GC analysis: DB-Wax, DB-FFAP (National Institute of Standards and Technology NIST/EPA/NIH mass spectral library NIST17), 'tentatively identified.
in previous studies. In most nutrition studies, wine samples are analysed one year after bottling at the latest. In our study, the bottles were stored for a duration of three to six years. Over this period of time, the concentration of N(3methylbutyl)acetamide can increase by a factor of 5–10 (Pérez-Coello et al., 1999).

Benzothiazole is a sulphur-containing heterocycle molecule that is present in wine, although in concentrations below the perception threshold (Bellavia et al., 2000). Until now, benzothiazole has not been reported in relation to nitrogen supplementation.

All these volatile metabolites in wine may be chemical markers related to the nitrogen nutrition status of the vineyard. However, it is rather unlikely that these molecules are directly responsible for the typical organoleptic defects observed in wines with stress aroma. In this study, we could not identify any molecules that were only present in the LN or in the HN variants. This result supports the hypothesis that the loss of balance between several volatile compounds of wine causes the organoleptic defects observed in wines with stress aroma. The effect of the putative markers on the aromatic profile of the wine could be taken up in future scientific studies.

3. Analyses of nonvolatile metabolites in wine by UHPLC-MS-based metabolomics

The metabolite profiling of nonvolatile molecules of wine was performed by high-performance liquid chromatography–time of flight mass spectrometry (UHPLC-TOFMS). All the profiles were acquired in triplicate on two different columns with different retention modes: reversed-phase in C18 and HILIC columns. These two orthogonal retention modes allowed for better separation and identification of apolar compounds in the C18 column and polar compounds in the HILIC column. The LC-MS data, recorded in positive and negative electrospray ionisation (ESI) modes, were processed as described by Marti et al. (2014). The results, characterised by their m/z ratio, retention time, and area, were analysed by principal component analyses followed by a discriminant analysis approach (OPLS-DA) to obtain a classification model and to highlight potential markers linked to nitrogen nutrition.

The number of detected features varied significantly, depending on the different chromatographic separation and ionisation modes used. Most detected features were polar, as revealed by the analyses in HILIC mode (152 features in ESI- and 262 features in ESI+). This mode detected nearly twice as many features as the C18 column (68 features in ESI- and 182 features in ESI+). Overall, 96 features overlapped between both orthogonal profiling methods. The features were putatively identified based on accurate mass and isotopic distribution scores to calculate the molecular formula (± 6 ppm) and database matching (Arapitsas et al., 2012). The Dictionary of Natural Products (DNP on DVD, CRC press v25.1) and KnapSack databases were used as entry lists to match the compounds based on HRMS data. A list of
compounds was built using 'wine or grape or vitis' as keyword filters. After removal of adducts and other ESI artefacts, the identification procedure revealed 140 putatively annotated features, which corresponds to an annotation of level 3 according to Schymanski et al. (2014). The resulting dataset was used for the multivariate data analysis.

As a preliminary step, PCA was applied as a form of exploratory data analysis to provide an unsupervised overview of the LC–MS fingerprints (Figure 3a). Interestingly, the first principal component (PC1 axis, 27 % of total variance) clustered the nitrogen content, whereas the second component was mainly involved in vintage year separation (PC2 axis, 14 % of total variance). To focus on the most relevant biomarkers related to nitrogen content, a supervised model was set up using orthogonal projection to latent structure discriminant analysis (OPLS-DA, Figure 3b). The model predictive capability was good (R2Y = 0.99, Q2Y = 0.96). Then, an Splot was used to rank the variables according to their contribution to the OPLS-DA model. The upper-right corner encompassed metabolites upregulated in HN, whereas the lower-left corner clustered downregulated compounds (Figure 3c). Overall, 16 biomarkers from several chemical classes (amino acids, vitamins, hormones, organic acids, polysaccharides and phenolic compounds) were found to be highly correlated with nitrogen treatment (Table 4).

### 3.1. Amino acids

Unsurprisingly, amino acids are strongly represented among the potential markers. Their concentration significantly increases in grapes after foliar nitrogen treatments in vineyards (Verdenal et al., 2015), which also impacts their levels in wine despite their partial consumption by yeast during fermentation. Proline, the most important secondary amino acid in grapes, is an interesting candidate. The proline concentrations in grape must and wine are high, because very little proline is taken up by the yeast during fermentation. An increase in concentration is often observed due to the release of proline by yeast (Ough et al., 1991). In the Chasselas grape variety, the proline concentration is typically between 150 and 300 mg/L in the grapes (Verdenal et al., 2015) and between 200 and 700 mg/L in the wine (data not published). Primary amino acids are only found in low concentrations in wine, because these molecules are assimilated by yeast during fermentation. The kinetics of amino acid utilisation by yeast during fermentation was investigated by Jiranek et al. (1995), who defined three groups (A, B and C) according to their uptake speed. Tyrosine and tryptophan belong

#### TABLE 4. Putatively annotated metabolites in wine samples numbered according to the S-plot of Figure 3c.

| Peak | m/z    | RT [min] | Mode          | Molecular formula | Appm | Isotope score | Putative identification     | Ratio HN/LN |
|------|--------|----------|---------------|-------------------|------|---------------|-----------------------------|------------|
| 1    | 149.0090 | 0.85     | HILIC-ESI(-)  | C4H6O6            | -0.9 | 98            | tartaric acid               | 0.11       |
| 2    | 289.0740 | 2.45     | HILIC-ESI(-)  | C15H14O6         | 9.0  | 93            | pentahydroxyflavan         | 0.17       |
| 3    | 447.0878 | 0.80     | HILIC-ESI(-)  | C21H20O11        | 10.0 | 93            | kaempferol-3-glucoside     | 0.20       |
| 4    | 191.0195 | 0.98     | HILIC-ESI(-)  | C6H8O7           | -0.9 | 98            | citric acid                | 0.24       |
| 5    | 341.1076 | 4.11     | HILIC-ESI(-)  | C12H22O11        | -3.7 | 96            | trehalose                   | 0.37       |
| 6    | 193.0343 | 1.45     | HILIC-ESI(-)  | C6H10O7          | -5.5 | 91            | glucuronic acid             | 0.38       |
| 7    | 115.0040 | 0.42     | HILIC-ESI(-)  | C4H4O4           | 3.2  | 98            | fumaric acid                | 0.50       |
| 8    | 133.0142 | 0.42     | HILIC-ESI(-)  | C4H6O5           | 0.1  | 98            | malic acid                  | 0.60       |
| 9    | 117.0184 | 0.28     | HILIC-ESI(-)  | C4H6O4           | -7.4 | 90            | succinic acid               | 0.70       |
| 10   | 170.0821 | 0.30     | HILIC-ESI(+)| C8H11NO3         | 6.0  | 90            | pyridoxine                  | 2.20       |
| 11   | 116.0724 | 1.63     | HILIC-ESI(+)| C5H9NO2          | -0.9 | 98            | proline                     | 2.61       |
| 12   | 205.0960 | 0.75     | C18-ESI(+)| C11H12N2O2        | 1.0  | 98            | tryptophan                  | 3.15       |
| 13   | 265.1424 | 0.25     | C18-ESI(+)| C15H20O4         | 3.9  | 90            | abscisic acid               | 4.72       |
| 14   | 166.0883 | 0.40     | HILIC-ESI(+)| C9H11NO2         | 9.5  | 99            | phenylalanine               | 5.75       |
| 15   | 182.0816 | 2.49     | HILIC-ESI(+)| C9H11NO3         | 6.9  | 99            | tyrosine                    | 6.71       |
| 16   | 175.1202 | 0.22     | C18-ESI(+)| C6H14N4O4         | 7.0  | 97            | arginine                    | 15.21      |
to class C, being the least preferred by yeast. Phenylalanine is classed in the intermediate group B. Similar results were reported by other authors (Pims et al., 2014). These classifications explain the higher concentrations of the three amino acids in the wines from the HN variants. Arginine, which is the most abundant amino acid in the grape must of Chasselas, is rapidly assimilated by yeast (results not published), but it will not be completely assimilated if there is enough nitrogen in the grape must during fermentation, which is often the case for the HN variants. Pims et al. (2014) found a limited consumption of this amino acid during the fermentation of musts of Sauvignon blanc as well.

3.2. Vitamins

Most vitamins produced by plants present amino acids as precursors (Miret and Munné-Bosch, 2014); it can therefore be hypothesised that fertilisation has an influence on vitamin concentration. However, in the literature, only vitamin B1 has been reported to increase with inorganic nitrogen fertilisation in plants (Mozafar, 1993). Other studies have indicated that fertilisation has no significant effect on the vitamin content of plants (Miret and Munné-Bosch, 2014). In our study, the foliar nitrogen treatment increased the concentration of pyridoxine (vitamin B6) in wine. Pyridoxine is present in must and wine in concentrations of 0.2–0.7 µg/L (Ribéreau-Gayon et al., 2004). Wainwright (1970) reported that pyridoxine deficiency can influence sulphur-containing amino acid synthesis in the grapes and increase hydrogen sulphide release during fermentation. In this study, this effect was not observed.

3.3. Hormones

Plants react to nutritional and environmental stress by regulating their hormone concentrations. In this study, the concentration of abscisic acid, a hormone implicated in the control of grape ripening and development, was lower in the LN variants. This study is the first to report this hormone in relation to nitrogen deficiency; until now, it has only been reported as a response of plants and grape berries to water stress (Chaves et al., 2010). Nitrogen fertilisation can provoke water-stress-like situations due to the stimulation of important vegetative growth, high canopy transpiration and rapid soil water depletion in the summer (Scholasch and Rienth, 2019). This effect may explain why we found higher abscisic acid concentrations in the HN variants in our study.

3.4. Organic acids

The results of previous studies on the effect of nitrogen nutrition on concentrations of organic acids are contradictory. In this study, we identified six organic acids by UHPLC-TOFMS as putative markers in the wine: tartaric acid, citric acid, glucuronic acid, fumaric acid, malic acid, and succinic acid. Their concentrations decreased in HN variants. Tartaric acid is the most abundant acid in the grape and is one of the most important acids in the wine. As an important component, tartaric acid was quantified during this study in the grape must (5.5 g/L; Table 1) and in the wine (1.6 g/L; Table 2). In contrast to the results of the UHPLC-TOFMS metabolomic study, the concentrations measured in the wine by the colorimetric method did not differ between the two variants (Table 2). These results can be explained by the differences in the sample preparations. The UHPLC-TOFMS analyses were performed on lyophilised samples, in contrast to the other analyses in which the wine was used directly. During lyophilisation, tartaric acid forms precipitates with calcium and potassium ions, which have poor solubility in a mixture of water and ethanol (50:50 v/v %). This precipitation phenomenon already occurs in the winemaking process and during storage, depending on the temperature and alcohol content of the wine. These uncontrollable parameters can have more influence on the tartaric acid concentrations of wines than can the nitrogen nutrition of a vineyard. This behaviour leads to the exclusion of tartaric acid from the list of markers.

Citric acid, fumaric acid and malic acid are all involved in the citric acid cycle. These acids are produced in varying quantities by the grapes and during fermentation. Fumaric acid was found only in trace amounts in the grapes, and the concentration remained low in the wine, at 1.5–8.3 mg/L; (Ding et al., 1995). In contrast citric acid can reach 1.0 g/L in the wine. A considerable amount of malic acid was present in the grapes, and the production of this acid increased with foliar nitrogen treatment (Table 1). In this study, alcoholic fermentation was followed by malolactic fermentation, in which malic acid is transformed into lactic acid. We observed a positive correlation between the lactic acid concentration in the wine and the N content in the must (Table 2). The concentration of malic acid in these wines, corresponding to the
read due after malolactic fermentation, was lower than 0.3 g/L (LOD of WineScan). In practice, malolactic fermentation is stopped when malic acid can no longer be measured in the wine. We assume that the negative correlation between the malic acid concentration in the wine and the N content of the must was a result of slow fermentation due to lower nitrogen content, the end point was closer to 0.3 g/L of malic acid compared to HN variants, in which the malic acid was quickly and completely consumed.

However, even if these acids show a correlation with N nutrition in the vineyard, these components are not ideal markers for wine, because their concentrations are strongly influenced by the winemaking process, especially by malolactic fermentation. When malolactic fermentation is performed, these acids can be entirely consumed by the bacteria (Davis et al., 1986). On the other hand, citric acid can be added to the wine as an oenological additive to solubilise iron.

Glucuronic acid is a sugar acid derived from glucose. In general, the free form of this acid is quickly metabolised in the plant tissue and remains only in low concentrations in plants. The literature provides minimal information about the amount of this acid in grapes and wine (0–6 mg/L) (de Valme García Moreno et al., 2002). This uronic acid is involved in glucuronidation, which produces more water-soluble products, facilitating their accumulation in vacuoles or their elimination from the cells.

Succinic acid is the main carboxylic acid produced by yeast during alcoholic fermentation. This acid is formed in the citric acid cycle, and during fermentation its concentration increases to 0.5–2.0 g/L. The concentration of succinic acid was lower in HN variants. This difference is reflected in the concentration of succinate esters, which were found to be potential markers (Table 3). In contrast to the effect of nitrogen addition to grape juice during fermentation, the effects of foliar nitrogen applications in vineyards on succinic acid formation has not yet been reported. The addition of nitrogen to grape juice during fermentation is currently practiced to prevent sluggish fermentation, resulting in a decrease in succinic acid concentration if the original YAN content is low (Rollero et al., 2015). However, the addition of certain amino acids (glutamate, asparagine, proline, glutamine, threonine and GABA) increases succinate production (Bach et al., 2009). Although several factors (yeast strains, fermentation conditions, temperature, aeration and must composition) can influence succinic acid concentration during fermentation (Rollero et al., 2015), succinic acid remains an interesting candidate as a marker. Further investigation is necessary to verify whether these factors, due to different oenological practices, mask the effect of nitrogen status on the vineyard.

3.5. Phenolic compounds

Pentahydroxyflavan and kaempferol-3-glucoside were identified as potential markers from the polyphenol family. Their concentrations decreased in HN variants. Pentahydroxyflavans form a class of chemical substances that differ in the positions of hydroxyl groups. In grapes and wine, flavan-3-ols (3,5,7,3′,4′-pentahydroxyflavan), namely catechin and epicatechin, are present in relatively high quantities. In white wine, concentrations of between 1 and 46 mg/L for catechin and of between 0.1 and 60 mg/L for epicatechin can be found, depending on the grape variety and oenological methods. A study showed that foliar nitrogen application in Cabernet-Sauvignon vines has a considerable effect on the flavanol concentration in wine (Gutiérrez-Gamboa et al., 2017). In accordance with our results, the study’s treatments decreased catechin and epicatechin concentration.

Kaempferol-3-glucoside is the main derivative of kaempferol in grapevines. This molecule represents 6%–28% of the total flavonols in white grapes and is the third most abundant after quercetin-3-glucoside and quercetin-3-glucuronide (Castillo-Muñoz et al., 2010). The concentration of kaempferol-3-glucoside is low in grapes, 0.5–4.5 mg/kg for white varieties and 2.5–6.5 mg/kg in red grape skins (Zhang et al., 2015). To our knowledge, no quantitative data have been reported for white wine.

In this study, the total polyphenol index was measured to compare the polyphenol contents of the wines (Table 2). No difference was found between LN and HN in this index in the wine, in contrast to the study of Choné et al. (2006) who observed lower total phenolic content (TP) of the Sauvignon blanc grape must with high nitrogen status of vines. However, in Bell and Henschke’s (2005) review, the results regarding the effects of nitrogen nutrition on TP values are contradictory. Further studies are necessary to understand the reaction of polyphenols to nitrogen deficiency in the vineyard.
3.6. Polysaccharides

Trehalose, a natural disaccharide formed from two α-glucose units, was identified as a putative marker. The concentration of trehalose decreased in HN variants. Trehalose is absent from the grapes, but present in the wine at a concentration of between 150 mg/L and 600 mg/L (Bertrand et al., 1975). This disaccharide is well known as a protective agent produced by the yeast during fermentation under stress conditions, such as nitrogen deficiency (Novo et al., 2005). It has been proposed as a biomarker to detect nitrogen deficiency during alcoholic fermentation in wine yeast (Gutierrez et al., 2013). Yeast produces more intracellular trehalose in cultures with low nitrogen. Even though we did not find data regarding the release of trehalose in the wine, this accumulation may explain the higher content of trehalose in the LN variants.

CONCLUSION

This untargeted differential approach, which included volatile and nonvolatile metabolites, revealed approximately 25 compounds in Chasselas wine that were influenced by nitrogen deficiency in the vineyard. These putative markers represent different chemical groups and have different biological functionalities in grapes or yeast metabolism. Some of these compounds are present in the grapes and indicate changes in grape composition as a function of nitrogen nutrition (amino acids, vitamins) or as reaction to stress (hormone, phenolic compounds). Others, mainly the volatile compounds, are produced during fermentation and reflect the influence of nitrogen nutrition on yeast metabolism (higher alcohols, esters, succinic acid and disaccharide). Finally, some of the molecules are produced during storage, indicating that the vineyard’s nitrogen nutrition can affect the ageing capability of wine.

The concentrations of amino acids, pyridoxine, aspartic acid (ABA), 2-methyl-1-propanol and N(3methylbuthyl)-acetamide were positively correlated with the supplementation of the vineyard’s nitrogen nutrition. In contrast, higher alcohols, such as 2- and 3-methyl-1-butanol and 2-phenylethanol, and succinic esters, ethyl-9-decenolate, benzothiazol, catechin (epicatechin), succinic acids and trehalose decreased.

The relevance of these putative markers should be further verified by quantitative measurements of these compounds in wine samples. Other results (not reported here) showed that the effect of the vineyard’s nitrogen nutrition on the composition of wine is strongly linked to vine variety. The effects of different winemaking practices (e.g., nitrogen addition before fermentation or prolonged skin contact), different yeast strains and different vine varieties should also be studied to determine the limits of the validity of different markers.

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