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Nitrogen maceration of wine grape: an alternative and sustainable technique to carbonic maceration

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Abstract: Carbonic maceration (CM) consists in placing intact grape bunches into a sealed tank to have a natural or artificially-created carbon dioxide atmosphere. No articles have been published on the comparison between CM and nitrogen maceration (NM). Therefore, the aim has been the use of alternative maceration technique NM in alternative to CM, to create the conditions of anoxia on the Gamay variety obtaining an aromatic wine as well. The results showed a higher concentration of polyphenols and anthocyanins in the macerated wines, especially in NM wine. Concerning VOCs (volatile organic compounds), the CM wine showed the highest content in esters while the total alcohol content was slightly higher in the NM wines. The CM wine had the highest content in volatile medium chain fatty acids (MCFA). E-nose measurements revealed a clear separation among the samples, and the E-nose data, in PCA computation, were generally overlapped by the PCA of VOCs analysis. The NM can be an useful technique to have new style aromatic wine in an environment and economic sustainable way.

Keywords: Wine; Grape Maceration; Carbon Maceration; Nitrogen Maceration; VOCs; E-nose.

1. Introduction

Invented by Michel Flanzy in 1934, carbonic maceration (CM) consists in placing the intact grape clusters into a closed tank with a carbon dioxide-rich atmosphere (Tesniere & Flanzy, 2011). The removal of air in the atmosphere surrounding grapes by totally replacing with carbon dioxide, triggers an anaerobic metabolism (fermentation) without microorganism involvement. It is well known that during grape ripening on vine, ethanol is formed with an expression of ADH (alcohol dehydrogenase) gene (Tesniere et al., 2004), and recently, Xiao et al. (2018) have shown that, as ripening progresses, the O_2 approaches zero in the flesh of grape berry of seeded cultivars. Alcoholic fermentation contributes to maintain cell function under O_2-limiting condition, provided sugars are available. Plants under low-oxygen or no-oxygen availability adapt their metabolism to compensate for the lower ATP production that arises from the limited respiratory activity in mitochondria. The anaerobic catabolism of sucrose is thought to require the activity of sucrose synthase, being this enzymatic reaction more energetically favourable than that of invertase. It is assumed that the importance of sucrose synthase under hypoxia (Zeng et al., 1998) is linked to its role in providing carbon units for fermentation (Guglieminetti et al., 1995), but it has been seen that the sucrose synthase pathway is not the preferential route for sucrose metabolism under hypoxia (Santaniello et al., 2014). The response of fruits and vegetables to very high carbon dioxide concentrations include induction of the glycolytic pathway, fermentation pathways, accumulation of succinate and/or alanine and decreases in pH and ATP levels (Ke et al., 1995). The molecule's primary action appears to be on the kinetics of a reversible reaction within the

tricarboxylic acid (TCA) cycle. Carbon dioxide may influence the glycolytic pathway by affecting the enzymes involved in the pathway or by regulating the fermentative metabolism pathway. The two key enzymes controlling the rate of the glycolytic pathway are ATP: phosphofructokinase (PFK) and pyruvate kinase (PK) (Kays, 1991). Ke et al. (1993) proposed that the increase in PDC and ADH activities observed under high CO_2 atmosphere on different fruits, could be due to an increased rate of synthesis or activation of these enzymes by a decrease in cytoplasmic pH and/or increased substrate concentrations, that is, pyruvate, acetaldehyde and NADH. In TCA cycle, succinate dehydrogenase appears to be the enzyme most significantly inhibited by high CO_2 (with the other TCA cycle enzymes being affected to a greater or lesser extent) and, as consequence, accumulation of succinate and depletion of malate (Ke et al., 1991). The mode of action of CO_2 on respiration could be due to an inhibition of ethylene biosynthesis and/or action, even though in grape berry the role of ethylene in ripening is less important than in climacteric fruit; another hypothesis is the effect on pH change, that is a decrease of cytoplasmatic pH. Thus, the saturation with CO_2 changes significantly the berry cell metabolism physically but also chemically with modification of volatile organic compounds (VOCs) panorama (Zhang et al., 2019). Romieu et al. (1992) found, keeping under 100% CO_2 detached grape berries at 30°C in the dark, that ethanol was near 1.5% (V/V) by the end of anaerobiosis (CO_2 saturation) which varied from 10 to 1 days as indicated by the complete oxidation of succinate; with this ethanol concentration the membrane permeability increases. The maintenance of neutral cytosolic pH is critical for plant survival under anaerobiosis while grape vacuolar pH is very acidic (grape transtonoplastic proton gradient should be about 3 to 4 pH units). The production of ethanol from glycolysis allows to produce 1 mole of ATP that can be used for the transport of two protons into the vacuole (Guern et al., 1989); furthermore, ethanol production from malic acid directly eliminates two protons from the cytosol. In this respect, ethanol formation is a good means for the cell to halt cytosolic acidity. The effect of CO_2 and ethanol formation contributes to the significant increase in aromatic quality due to their higher total content of esters and acetates found in several Spanish wines produced with carbonic maceration (González-Arenzana et al., 2020). Wines produced by white grape kept under carbonic maceration, had higher contents of alcohols and carbonyl compounds, but a lower concentration of C6 alcohols, anyway in this case the CM process is not isolated since some grapes break down when they were placed in the tank, thus permitting the beginning of fermentation by yeasts, therefore, both phenomena occurred simultaneously (Ayestarán et al., 2019). Salinas et al. (1996) found Monastrell grapes kept under CM, had a higher concentration of ethyl esters and fatty acids and a lower alcohol concentration. Finally, high CO_2 gas treatment (95%) leads to anaerobic fermentation in persimmon fruit, thus triggering acetaldehyde metabolism which facilitates polymerization of high soluble tannins (inducing astringency), converting them to insoluble condensed tannins (Min et al., 2014). But if CO_2 has a great effect on berry cell metabolism what happens if oxygen removal is done with nitrogen? Anoxia is known to provoke anaerobic metabolism (fermentation) such as for CM, but nitrogen gas is inert, compared to CO_2, on cell metabolism. Thus, it is expected that anoxia created by nitrogen gas modifies the berry cell metabolism differently from anoxia by CM. In peach fruit kept in anoxia for 72 h, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities were greatly increased (6- and 6.5-fold, respectively) with respect to untreated fruit, followed by an almost 2-fold increase in lactate dehydrogenase (LDH) activity (Lara et al., 2011). Jiménez et al. (2007) tested short nitrogen gas treatment for 6 through 48 h on table and wine grapes, finding a significant increase in *trans*-resveratrol but no VOCs were measured. At our knowledge no paper has been published on the comparison between CM and NM, thus our hypothesis is that the VOCs pattern of wine will change, beyond the other grape and wine characteristics, during the two types of maceration because, as explained above, the two gas act differently in the grape berry cell. Thus, different maceration techniques using different gases (N_2, CO_2 or N_2/AIR), to create the conditions of anoxia, were tested on Gamay grape variety.

2. Materials and Methods

2.1 Raw materials

Bunches of red grape variety (*Vitis vinifera* L.), cv Gamay teinturier, were hand harvested upon Fattoria di Calappiano (Sensi Vigne & Vini, Lamporecchio, Pistoia, Italy) at a sugars content of 213.7 ± 0.7 g/L. After harvest, the grapes were sorted for homogeneous bunch shape and the absence of visual defects or evident diseases, and immediately shipped to the laboratory (DAFE, University of Pisa, Pisa, Italy) to carry out all the analytical determinations (Table 1).

2.2 Laboratory scale maceration and fermentation

After washing and superficial drying, the grape bunches were divided in 12 different sets (2 kg each) to have 3 replications for each type of gas-maceration treatment (100 % N_2 = NM, 100 % CO_2 = CM, N_2/AIR = NOM, AIR). The grape bunches were cut in smaller bunches to permit the insertion inside a 4 L Pyrex glass jar connected with the Department gas pipeline in a continuous flow system, at constant pressure given by a water buffer jar. The gas line of each gas was split into 3 glass jars per gas. The flow speed was adjusted to guarantee, each hour, a complete removal of the jar free volume. This allowed for the complete CO_2 removal in NM and NOM. CO_2 and O_2 concentrations were measured on the outlet pipe by a portable gas analyzer (Helpy, Marvil Eng, BZ, Italy). The NOM treatment was performed to observe the effect of breaking anoxia on the behaviour of berry cell in terms of VOCs production. The NOM gas mixture consisted in keeping the glass jars under nitrogen gas but, every two days, nitrogen was replaced with AIR along for 7 hours; after this time, nitrogen gas was refluxed back into the glass jars. The AIR treatment was performed by flowing the jars at a very low flow rate just to avoid the CO_2 accumulation, with the same system used for maceration. All the tests were run at room temperature (22-25 °C). After 7 days, the jars were opened, and the berries were hand removed from the small bunches, hand squeezed, then the must was obtained by means of a juice extractor (JU3701 Frutelia Centrifuge Moulinex, Ecully, France). Fermentations were carried out in the same glass containers used for gas maceration, thus in three replicates, and the must in each jar was inoculated with 20 g/hL of selected yeasts (Zymaflore Xpure Vin Rouge Laffort 33072 Bordeaux Cedex-France); 30 g/hL of Nutristart (Laffort, 33072 Bordeaux Cedex-France) was added as a fermentation activator. To ensure temperature control during the fermentation process, the jars were kept inside a stainless-steel tank with water circulation at a controlled temperature (22°C). At the end of the alcoholic fermentation period (7 days for all the samples), the wines were taken at higher temperature (25-27°C) for the malolactic fermentation (MLF) which started rapidly and ended in 3 days. Then the wines were removed from the glass jar and filtered (paper filter 0.82 μm), bottled and stored at 10°C for approximately 10 days to perform Wine Scan analysis and 30 days for VOCs one. From each jar, about 1.25 L of wine was obtained, with a yield of about 70%.

2.3 Chemical analysis

The berry juice of harvested grape and from the grapes removed from the maceration jars, were extracted as described above (juice extractor). Most of the juice coming from the macerated samples were fermented as aforementioned, while 20 mL of either of the harvested grape juice or of the macerated berry juices were centrifuged (6869 *g* for 5 min at 22°C). Supernatants were analysed by a calibrated Fourier transform infrared Wine Scan FT 120 (Foss Analytics, Hillerod, Denmark) to determine the following oenological parameters in triplicate: sugars (g/L hexoses), pH, titratable acidity (tartaric acid g/L), volatile acidity (g/L acetic acid), malic acid (g/L), tartaric acid (g/L), citric acid (g/L), total extract (g/L), ashes (g/L), YAN (yeast assimilable nitrogen) (g/L), glycerol (g/L), gluconic acid (g/L) total anthocyanins (mg/L malvidin), and total polyphenols (mg/L gallic acid). For each macerated sample and the control one, three replicated jars were analysed. The accuracy of the Wine Scan analyses was validated by destructive analyses performed with traditional methods during the calibration activity before starting the readings, following the OIV 2021 methods, as previously reported in Bianchi et al. (2021).

2.4 Colour determination

Absorbance at 420, 520 and 620 nm, hue (according to the formula Abs 420 nm/Abs 520) and intensity (according to the formula Abs 420 nm + Abs 520 nm + Abs 620 nm) were calculated following the OIV 2021 methods. The chromatic characteristics were detected by a Benchtop CLM-196 colorimeter (Eoptis-38121 Trento (TN)-ITALY). The colour values were expressed using the native coordinates CIE L * a * b and the cylindrical coordinates, h * and C *, according to the OIV methods (2021). The instrument also measured ΔE* (the colour difference among the samples) and ΔL* (the brightness difference among the samples); in this case the values were calculated using, as the reference colour, the AIR maceration sample.

2.5 VOCs analysis

The analyses were carried out as previously reported (Priser et al., 1997) and modified by De Filippis et al. (2019). Briefly, the VOCs extraction was carried out by liquid-liquid extraction: 100 mL of wine, spiked with 250 μL of an alcoholic solution of 2-

octanol (221 ppm/EtOH) as internal standard, were extracted with 5 mL of CH_2Cl_2 as a solvent. The mix was subjected to magnetic stirring for 1 h at the constant temperature of 21 °C. The emulsion thus obtained was transferred to a separation funnel for 12 h at 4 °C, the organic aromatic extract was finally recovered, dried over Na_2SO_4, and stored at −20 °C until the GC/MS analysis. Each extraction was carried out in triplicate, one each jar.

For High Resolution Gas-Chromatography/Mass Spectrometry (HRGC/MS) analysis, a GC/MS-QP2010 quadrupole mass spectrometer (Shimadzu, Shimadzu corp., Kyoto, Japan) equipped with a DB-WAX UI column (60 m, 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA 95360, USA) was used. The carrier gas was helium (1.3 mL/min) and the used temperature program was the following: 40 °C for 5 min, raised up to 220 °C at a rate of 2 °C/min, and held for 20 min at the maximum temperature. Electron impact mass spectra were recorded with ion source energy of 70 eV, while the temperature was kept at 230 °C. The peak areas were measured using a GC/MS solution program Shimadzu version 2.30 (Shimadzu corp., Kyoto, Japan). Compounds concentrations and identification were computed and performed as previously reported (Piombino et al., 2010). In a few cases, the pure chemical standard was not available, and the identified compounds were labelled as tentative (t).

2.6 E-nose measurements

The employed E-nose consisted of an array of eight quartz microbalances (QMB) coated with modified metallo-porphyrins (5,10,15,20-tetraphenylporphyrin, TPP), whose selectivity depended on the nature of both the central metal and the peripheral substituent of macrocycles (Mn-TPP, Co-p-OCH3 TPP, Sn-TPP, Rh-TPP, Co-p-NO2, Cr-TPP, Co-TPP, Ru-TPP) (Di Natale et al., 2007). The QMB sensors acted as an electromechanical resonator with a natural oscillation frequency ΔF of 20 MHz that change when volatiles interact reversibly with its surface. The signal from the sensors was acquired and formatted through software before processing and analysing it (Santonico et al., 2010).

The protocol for the E-nose detections was adapted from Santonico et al. (2010). Fifty mL of wine sample were first degassed by using partial vacuum using a 500 mL Erlenmeyer flask and a Venturi pump system for 1 min. Afterwards, 5 mL of degassed wine were placed in a 10 mL vial and tempered at 25 ± 2 °C for 30 min. The volatiles accumulated in vial headspace were transferred into the E-nose sensor cells using an air stream for 10 min, which was filtered through a trap filled with anhydrous $CaCl_2$. Pure nitrogen (99.99%) at a constant flow of 100 mL/min was used to clean the sensors for 5 min and the obtained signals were considered as reference for each QMB. The wine sample from each jar was placed into three vials, and three E-nose detections each vial were performed. The average values calculated on those triplicates were used for statistical multivariate computations which were performed as described below.

2.7 Statistical analysis

One-way ANOVA was run (CoStat, Version 6.451, CoHort Software, Pacific Grove, CA, USA) and Tukey's honestly significant difference (HSD) test with $p \leq 0.05$ for multiple comparison, was used.

Multivariate statistics computed on autoscaled E-nose data and on normalized VOC measurements were performed by using Matlab R2013a (MathWorks®, Natick, MA, USA), and PLS Toolbox (Eigenvector Research, Inc., Manson, WA, USA). Namely, a principal component analysis (PCA) and a hierarchical cluster analysis (HCA), performed by the Ward's method and reported as dendrogram (E-Nose) or two-way heat map (VOCs), were computed on E-nose and VOC detections.

3. Results and Discussion

3.1 Chemical characterization

As expected, the first clear evidence is the decrease of sugars and the ethanol increase after 7 days of maceration (Table 1). In comparison to the harvested sample, about 11 % of sugar decrease was measured in the CM vs 17 % in the NM and intermediate value in NOM grape (Table 1). In the AIR maceration, the sugar decrease was similar as the one of the NM but, with a measured alcohol content, significantly lower (0.26 % v/v) vs 0.82, 0.98, and 1.20, respectively in the CM, NOM, and NM.

We found less alcohol content than that reported by Romieu et al. (1992) and also by Pace et al. (2014), but the factors influencing the transformation of sugars in alcohol are several. First of all, the temperature: we worked at 22-25°C while both mentioned authors worked at 28-30°C. This temperature difference accelerates the anaerobic metabolism, as expected, in a hyperbolic way. The treatment length is another factor affecting the response during the maceration: we kept grapes under maceration for 1 week while Romieu et al. (1992) from 1 through 10 days and Pace et al. (2014) used 12 days. The way which CO_2 is produced is another factor. Using the grape respiration to saturate with CO_2 is different by using technical gaseous CO_2; or, again, using a dynamic flow system which always keeps a constant pressure on grape berries is different by using a static system which changes the pressure continuously around the berry. Finally, the ripening stage influences the berry response to maceration as showed by Pace et al. (2014), but also the variety and field growing conditions which modifies the physical and chemical structure of the berry, have an effect on this response. Another important consideration is the way to operate during the maceration period. i.e. Pace et al. (2014), to sample at 3 and 6 days, broke the gas tightness and then closed back the jars, saturating the jar every 12 h with CO_2 technical gas. In contrast, we kept close the jars all the period, and we let the gas flow. Titratable acidity decreased in the macerated samples as expected while it was still in the AIR macerated one (Table 1). The reason of the mentioned decrease is the reduction of malic acid as reported by Romieu et al., (1992) but also the decrease of tartaric acid, probably due to a higher permeability of the membrane (affected by a higher alcohol content) and thus formation of tartaric salts. The decrease of acidity confirms also what observed by Yang et al., (2006) in the CM of Gamay and Baco noir with, who found pH increase, supposing a consume of malic acid to pyruvic acid, but they did not measure them.

Generally speaking, no significant difference in acidity and acid concentration among the different technical gas macerated samples were found while a difference was measured with the AIR macerated one because respiration occurred and not fermentation. Glycerol was detected only in AIR sample at the end of the maceration period as well as gluconic acid (Table 1). These two compounds are usually found in great concentration in grape kept under dehydration to produce Passito wines (Mencarelli & Bellincontro, 2013) together with formation of volatile acidity. Also in this experimental work, volatile acidity was significantly higher in AIR macerated samples, more than twice than the other samples (Table 1). Glycerol and acid acetic formation is a clue of glyceropyruvic fermentation, and likely, indicates an initial step of aerobic fermentation due to a postharvest senescence of the grape berries at room temperature as it has been seen in other fruit (Chervin et al., 1999) and on late harvest grape (Piazzolla et al., 2016). These compounds were expected to be in a greater concentration in the technical gas macerated samples instead of, glycerol was not detected, and acetic acid was significantly lower than in AIR grape, while no difference was observed among the macerated samples. Our hypothesis is that the glyceropyruvic fermentation in technical gas macerated grapes occurs at low rate while alcoholic fermentation goes straight at higher rate (Goold et al., 2017). In line with this hypothesis is the low alcohol amount detected in the AIR sample, by suggesting, together with the highest acetic acid level, a limited and sluggish alcoholic fermentation. Gluconic acid is due to glucose oxidation and usually is in high concentration in wine produce by botrytized grapes (Teissedre & Donèche, 2013) because it is the main metabolite from glucose in yeast-bacteria culture (Pinto et al., 2019). In our case, the grapes were sound, and were washed before treatment, but it is possible that in AIR grapes filamentous fungi (*Penicillium*, and *Aspergillus* spp.), bacteria (*Acetobacter*, and *Gluconobacter* spp.) and wild yeasts (*Saccharomyces* spp.) might grow over the surface or into the grape berry and its microscopic wounds (Pinto et al., 2019), even though we did not observe any surface rotting. Anyway, it is also possible that the high concentration of gluconate is due to the strong oxidation process during the week of the AIR maceration at room temperature, indeed the concentration is low and does not lead to think of a pathogen contamination. Interesting behaviour has been of YAN: the concentration diminished significantly in all the samples but especially in the ones with oxygen (NOM and AIR maceration) (Table 1). A possible explanation of this event it could be, the respiration contributed to the decrease of assimilable nitrogen while, where the fermentation occurred, cell use of nitrogen was reduced because the TCA cycle was also reduced, thus there was a less need of nitrogen for aminoacidic synthesis. The content of polyphenols and anthocyanins was significantly lower in AIR samples, while the NM samples exhibited significantly higher content than the one of the other macerated samples (Table 1). The reduction of these

compounds in all the samples compared to the initial value, is probably due to an oxidation process occurring at higher extent overall in air. Indeed, consequences of hypoxia – induced oxidative stress, depend on tissue and/or species (i.e. their tolerance to anoxia), on membrane properties, on endogenous antioxidant content and on the attitude to induce the response in the antioxidant system (Blokhina et al, 2003). We can assume that, before all the berry tissue become anoxic in technical gas macerated samples, the oxidation process occurs due to the residual oxygen content inside the berry and provokes the loss of antioxidant compounds. The case of higher content of these compounds in NM sample compared to the other samples, might be related to the ethanol content, thus more extraction, as already observed by Pace et al. (2014) and Hernandez-Jimenez et al. (2012).

Concerning wines, at the end of fermentation (Table 2), the AIR sample had lower alcoholic content, and significantly higher residual sugars, titratable and volatile acidities. This wine also showed the lowest content in anthocyanins and polyphenols. Wine from the NM, NOM, and CM showed similar but, in some cases, significantly different values in alcohol, titratable and volatile acidities and a higher concentration of polyphenols and anthocyanins than the one in the AIR sample; in the NM wine, anthocyanins content was higher than in the CM sample. The lower content of titratable acidity in technical gas macerated wines than in AIR macerated one is correlated to the lower content of tartaric acid, but also to the one of acetic acid. The different concentration in polyphenols and anthocyanins affected the wine colour of the AIR wine which had the lowest value of intensity and the highest in tonality and, among the technical gas macerated wines, the NM showed the highest colour intensity value (Figure 1, top). The NM, CM, NOM samples differed greatly from the sample in AIR maceration as evidenced by the ΔE which was the highest in CM wine. The spatial arrangement of the samples on the colour plane, shows that the color of NM, CM, NOM wines tends more to intense red tone with purple hue, while the AIR wine has less intense ruby red color (Figure 1). This is also highlighted by the angle h*, which is significantly lower (lower tonality) with respect to the three other wines (Figure 1, bottom).

Also, the glycerol was significantly higher in the macerated wines, especially in CM, and NOM (Table 2). The values were surprising because similar to that measured in Amarone wine vinified from dehydrated grapes (Bellincontro et al., 2016). In our case, the used yeast is a great producer of glycerol but while in the grapes after maceration, glycerol was present only in AIR sample, after vinification the highest concentrations were found in the CM, NOM, and NM wines, respectively. Probably, the pH difference could have an effect of yeast metabolism, indeed, the high pH value has slightly positive effect on glycerol formation in beverages fermentation process (Arroyo-López et al., 2010). The positive effect is probably because of the increased activity of aldehyde dehydrogenase at higher pH values. This step generates extra NADH which require reoxidization via glycerol synthesis in the absence of oxygen which is our case (Wang et al., 2001).

3.2 VOCs characterization

A total of 20 esters, 15 alcohols including 2 terpinols, 6 acids and 4 further miscellaneous VOCs (Table 3), were detected in the wines. All the macerated wines had a greater content of VOCs classes (except for acids) than the AIR sample. The CM wine showed the highest content in esters mainly due the high concentration in ethyl lactate and being characterized by the highest concentration in isoamyl acetate (1405.88 µg/L), ethyl lactate, and diethyl succinate. Globally the CM wine shows significant higher concentrations of those esters that have been reported as the most impacting for the fruity character of wine: ethyl 3-methylbutyrate, ethyl octanoate and even ethyl 2- methylbutyrate and ethyl hexanoate compared to the other macerated samples. Considering that these molecules can also act synergistically each other, and that the CM wine also has the highest level of diethyl succinate - a further compound with a potential additive effect - a significant sensory impact on the fruity character of CM compared to the other wines, cannot be excluded (Waterhouse et al., 2016). It is believed that precursor concentrations (medium chain fatty acids = MCFA) are the limiting factor for ethyl ester synthesis, because the addition of MCFA to the fermentation medium resulted in higher ethyl ester production (Hu et al., 2018) but the formation of MCFA ethyl ester is more complicated; it is dependent on the concentration of the two substrates (the acyl-CoA component and ethanol) and the total activity of the enzymes that are involved in the synthesis and

hydrolysis of the MCFA ethyl esters (Liu et al., 2019). We found a higher content of MCFA esters as well as a higher content of MCFA as precursor in the CM wine. In contrast, the NM wine had the highest content in n-propyl acetate (69.19 µg/L) and ethyl 3-hydroxybutyrate (35.60 µg/L). The partial presence of oxygen in the maceration atmosphere (NOM) induced the production of β-phenethyl acetate in the significant highest content, while the AIR macerated wine contained the highest content in ethyl 2-methyl-butyrate, ethyl hexanoate, ethyl pyruvate, hexyl acetate, ethyl vanillate, and ethyl lactate (1691.24 µg/L).

The total alcohol content was the highest in technical gas macerated wines especially the NM and CM (Table 3). As usual, 3-Methyl-1-butanol, 2-Methyl-1-butanol, and phenethyl alcohol were the alcohols with the highest concentrations, but they were significantly higher in the NM and CM wines. 3-(Methylthio)-1-propanol was higher in the NM wine than in CM one. The macerated grapes of the CM and NM wines had the highest content in YAN, and this could affect the formation of branched alcohols during the yeast fermentation. Alcohols of varietal origin, namely linalool and citronellol, resulted more related to the macerated samples; especially citronellol showing doubled levels compared to the one of the AIR wine.

As for esters and alcohols, also volatile fatty acids (MCFA) were globally more concentrated in the CM wine compared to all the other samples (Table 3). The concentration in the NM was the lowest. Medium-chain fatty acids formation entails the activity of acetyl-CoA carboxylase and the fatty acid synthase and the genes ACC1 and FAS1, which are involved in the synthesis of medium-chain fatty acyl-CoA (MCFA-CoA), and their up-regulations, are usually accompanied with the high production of MCFAs (Furukawa et al., 2003). Low concentration of UFA (unsaturated fatty acids such linoleic or linolenic acids) increases the formation of MCFA (Liu et al., 2019). Thus, the lowest amount of these compounds found in the NM and the highest content in the CM wine, suggests that the two types of maceration act directly on fatty acid membrane synthesis and degradation. Indeed, the concentration of cis-3-Hexen-1-ol in the CM wine was lower than in the NM one, and also, 1-Hexanol content was lower but not significantly, leading to suppose an oxidation of UFA. Octanoic acid (caprylic acid) is the main responsible for this difference in the volatile acid content, but a similar trend has been detected also for isovaleric, hexanoic, and decanoic acids, all showing double levels in the CM wine compared to the NM one. The trend observed for acetic acid confirms the results reported in Table 2, suggesting a sluggish fermentation in the AIR sample (and also in the NOM where air was provided temporarily) which, also, the significantly highest production of 3-Hydroxy-2-butanone (acetoin) could be linked to (Cheynier et al., 2010). The technical gas macerated samples also showed higher concentrations of butyrolactones, especially the NM and NOM ones.

3.3 E-nose evaluation

Aromatic patterns of each wine detected by the eight E-nose sensors were employed in a multivariate calculation which was performed after applying a mean center pre-processing to the raw data. In detail, a principal component analysis (PCA), and a Cluster Analysis by the Ward's method were computed and the obtained results were reported in Figure 2, top and bottom respectively.

In relation to the PCA, three principal components (PCs) described most of the 95% of the residual variance: 75.12% (PC1), 14.32% (PC2), and 7.72% (PC3), respectively (Figure 2, top). The graphical scoreplot describes the good separation among the wine scores representing the four maceration processes, and the significance of their clusterization is better evincible through the graphical dendrogram reporting the hierarchical segregation (Figure 2, bottom). The first cluster attributed to the NOM wine presents the strongest distance from the other wines which are, at first hierarchical stage, grouped in a unique cluster. A relative sub-separation is ascribable to the AIR wine which, in turn, is segregated from the last small cluster represented by the NM and CM wines. E-nose measurements appears to detect, as more similar, the aromatic patterns released by the two wines produced under completely anoxic maceration (NM and CM).

A cluster analysis (always by the Ward's method) performed on the VOC measurements revealed some interesting partial overlapping of the results obtained by GC-MS and E-nose methods of aromatic profile investigation. The two-way dendrogram associated to the heat-map reported in Figure 3 is quite clear in explaining it. VOC measurements, if combined in a multivariate approach, influenced a sample separation where AIR wine is hierarchically segregated from a second cluster which includes CM, NM, and

NOM wines. Within this group, NM wine is close to NOM one, while CM wine looks a little far from them. In terms of VOC influence on the wine sample clustering (appreciable by the heat-map), hexyl acetate, ethyl pyruvate, ethyl vanillate, acetic acid, and acetoin present the relative higher impact on AIR sample. CM sample is quite significantly dominated by 1-butanol, n-decanoic acid, ethyl decanoate, and hexanoic acid. Ethyl iso-butyrate seems to evidence a great influence only on one of the triplicate samples related to NM group, while a more diffuse impact is associated to ethyl 3-hydroxybutyrate, to n-propyl acetate, and to cis-3-hexen-1-ol. Finally, NOM wine, in its segregation, is influenced by 2,3-butanediol, ethyl acetate, 1-octanol, beta-phenylethyl acetate, and ethyl methyl succinate.

4. Conclusions

For the first time a comparison of the CM with other types of anoxic or hypoxic situation has been tested. Wine from NM has similar features to the one of CM but higher content in anthocyanins and slightly in polyphenols. The CM wine showed the highest content in ester and volatile acid classes, while the NM wine the highest in alcohols, due to isobutyl alcohol and phenethyl alcohol. The NM provided grape at the end of maceration with higher extraction of polyphenols and anthocyanins. Thus, this technique can be useful to produce aromatic wine with different profile compared to CM, but with high rate of environment and economic sustainability.

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Figure & Table Captions

Table 1. Enochemical characteristics of Gamay teinturier grape at the beginning (harvest) and at the end of maceration period (7 days). CM = carbonic maceration, NM = nitrogen maceration, NOM = nitrogen/AIR maceration, AIR. The data are expressed as mean ± SD of the berry juices from 3 glass jars, each gas maceration. Different letters in the row of each parameter refer to significant differences (Tukey, $p \leq 0.05$). n.d. = not detected.

Table 2. Enochemical parameters of wines at the end of both fermentations, alcoholic and malolactic. CM = carbonic maceration, NM = nitrogen maceration, NOM = nitrogen/AIR maceration, AIR. The data are expressed as mean ± SD of the wines from 3 jars, each gas maceration. Different letters in the row of each parameter refer to significant differences (Tukey, $p \leq 0.05$). n.d. = not detected.

Table 3. Volatile esters, alcohols, acids and other miscellaneous compounds detected in wines at the end of the alcoholic and malolactic fermentations. AIR, NM = nitrogen maceration, NOM = nitrogen/AIR maceration, CM = carbonic maceration. The data are expressed as mean ± SD of the wines from 3 jars, each gas maceration. Different letters in the row of each compound refer to significant differences (Tukey, $p \leq 0.05$). n.d. = not detected.

Figure 1. Colour parameters of wines (top), and the spatial arrangement of the samples on the colour plane (bottom) at the end of both fermentations, alcoholic and malolactic. CM = carbonic maceration, NM = nitrogen maceration, NOM = nitrogen/AIR maceration, AIR. The data are expressed as mean ± SD of the wines from 3 jars, each gas maceration. Different letters in the row of each parameter refer to significant differences (Tukey, $p \leq 0.05$).

Figure 2. Scoreplot (top) representing the results of principal component analysis (PCA) and dendrogram (bottom) derived from cluster analysis computation. Both statistic rielaborations were performed on E-nose measurements re-ported in triplicate. NM = nitrogen maceration, NOM = nitrogen/AIR maceration, CM = carbonic maceration, AIR

Figure 3. Two-way dendrogram and heat-map obtained from the cluster analysis (by the Ward's method) performed on the VOCs GC-MS detected after their autoscaling pre-treatment. NM = nitrogen maceration, NOM = nitrogen/AIR maceration, CM = carbonic maceration, AIR.

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495 **TABLES**

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Grape parameters	Units	Harvest	CM	NM	NOM	AIR
Alcohol	% V/V	n.d.	0.82 ± 0.04 b	1.20 ± 0.08 a	0.98 ± 0.06 b	0.26 ± 0.06 c
Sugars	g/L hexoses	213.7 ± 0.7 a	190.0 ± 0.2 bc	177.1 ± 0.6 d	182.8 ± 0.6 cd	179.7 ± 0.8 d
pH		3.41 ± 0.02 a	3.37 ± 0.02 a	3.36 ± 0.03 a	3.35 ± 0.03 a	3.21 ± 0.01 b
Titratable acidity	g/L tartaric acid	8.10 ± 0.03 a	6.68 ± 0.13 b	6.42 ± 0.08 b	6.49 ± 0.07 b	8.02 ± 0.07 a
Volatile acidity	g/L acetic acid	0.07 ± 0.02 c	0.22 ± 0.06 b	0.24 ± 0.05 b	0.19 ± 0.02 b	0.53 ± 0.02 a
Malic acid	g/L	1.70 ± 0.03 a	1.26 ± 0.07 b	1.14 ± 0.09 b	1.20 ± 0.06 b	1.17 ± 0.04 b
Tartaric acid	g/L	6.02 ± 0.03 b	5.82 ± 0.06 c	5.47 ± 0.04 d	5.51 ± 0.05 d	6.23 ± 0.08 a
Citric acid	g/L	0.27 ± 0.04 a	0.27 ± 0.03 a	0.23 ± 0.02 a	0.21 ± 0.02 a	0.14 ± 0.03 b
YAN	mg/L	253 ± 5 a	112 ± 10 b	101 ± 10 b	85 ± 5 c	63 ± 2 d
Glycerol	g/L	n.d.	n.d.	n.d.	n.d.	0.7 ± 0.1
Gluconic acid	g/L	n.d.	n.d.	n.d.	n.d.	0.52 ± 0.12
Total anthocyanins	mg/L malvidin	1712 ± 55 a	1576 ± 67 b	1599 ± 84 b	1588 ± 40 b	1175 ± 72 c
Total polyphenols	mg/L gallic acid	3225 ± 102 a	2947 ± 81 c	3059 ± 102 b	2838 ± 78 d	2347 ± 93 e

497 **Table 1**

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Wine parameters	Units	CM	NM	NOM	AIR
Alcohol	% V/V	11.16 ± 0.08 a	11.13 ± 0.09 a	11.02 ± 0.08 a	9.5 ± 0.04 b
Sugars	g/L hexoses	1.89 ± 0.09 c	2.18 ± 0.05 b	1.92 ± 0.07 c	3.57 ± 0.08 a
pH		3.14 ± 0.02 b	3.23 ± 0.01 a	3.17 ± 0.01 b	2.99 ± 0.01 c
Titratable acidity	g/L tartaric acid	8.39 ± 0.04 bc	8.29 ± 0.08 c	8.50 ± 0.09 b	9.04 ± 0.06 a
Volatile acidity	g/L acetic acid	0.22 ± 0.02 c	0.31 ± 0.02 b	0.28 ± 0.03 b	0.55 ± 0.02 a
Lactic acid	g/L	1.04 ± 0.06 a	1.14 ± 0.03 a	1.10 ± 0.02 a	1.16 ± 0.05 a
Tartaric acid	g/L	5.67 ± 0.06 bc	5.60 ± 0.04 c	5.76 ± 0.06 b	6.65 ± 0.05 a
Citric acid	g/L	0.22 ± 0.02 a	0.19 ± 0.01 a	0.19 ± 0.02 a	0.07 ± 0.01 b
Total extract	g/L	32.36 ± 0.12 bc	32.23 ± 0.11 c	32.49 ± 0.14 b	36.70 ± 0.13 a
Ash	g/L	3.01 ± 0.01 b	3.05 ± 0.01 b	3.03 ± 0.02 b	3.23 ± 0.03 a
Glycerol	g/L	8.63 ± 0.02 a	8.24 ± 0.04 b	8.57 ± 0.03 a	6.71 ± 0.06 c
Gluconic acid	g/L	n.d.	n.d.	n.d.	0.65 ± 0.02
Total anthocyanins	mg/L malvidin	1397 ± 42 b	1481 ± 21 a	1408 ± 32 b	836 ± 16 c
Total polyphenols	mg/L gallic acid	2695 ± 67 bc	2732 ± 66 ab	2593 ± 92 c	2027 ± 95 d

Table 2

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Esters	CM (µg/L)	NM (µg/L)	NOM (µg/L)	AIR (µg/L)
Ethylisobutyrate	46.11 ± 38.90 ns	62.69 ± 31.20 ns	27.23 ± 1.00 ns	16.45 ± 23.00 ns
n-Propylacetate	24.17 ± 1.87 c	69.19 ± 3.89 a	38.10 ±5.00 b	39.75 ± 1.09 b
Ethyl 2-methylbutyrate	230.72 ± 8.07 b	59.26 ± 6.98 c	136.87 ± 10.99 c	384.06 ± 33.00 a
Ethyl 3-methylbutyrate	12.13 ± 1.09 a	n.d.	7.50 ± 1.00 ab	5.63 ± 1.00 b
Isoamyl acetate	1405.88 ± 6.98 a	1049.04 ± 11.12 b	696.20 ± 50.54 c	553.17± 18.00 c
Ethyl hexanoate	365.41 ± 30.54 b	252.35 ± 10.66 c	261.39 ± 13.34 c	433.18 ± 11.87 a
Ethyl pyruvate	39.29 ± 1.90 c	55.73 ± 2.87 b	40.00 ± 3.33 c	83.28 ± 1.99 a
Hexyl acetate	7.28 ± 1.45 b	8.56 ± 0.99 b	6.37 ± 1.22 b	19.93 ± 1.99 a
Ethyl lactate	1470.54 ± 76.99 ab	1152.16 ± 41.67 b	1168.60 ± 70.87 b	1691.24 ± 80.98 a
Ethyl octanoate	412.40 ± 7.87 a	266.65 ± 14.21 c	315.61 ± 11.54 bc	383.26 ± 24.00 ab
Ethyl 3-hydroxybutyrate	24.77 ± 2.34 b	35.60 ± 1.09 a	11.67 ± 1.00 c	6.41 ± 4.20 c
Isoamyl lactate	23.15 ± 1.34 a	11.26 ± 2.87 bc	13.79 ± 2.87 b	10.56 ± 1.23 c
Ethyl methyl succinate	5.91 ± 1.67 b	8.09 ± 1.34 b	17.82 ± 2.44 a	n.d.
Ethyl decanoate	96.67 ± 3.56 a	59.33 ± 8.89 b	70.14 ± 3.45 ab	69.96 ± 2.76 b
Diethyl succinate	260.73 ± 32.32 a	191.39 ± 10.56 b	239.26 ± 30.23 ab	135.92 ± 8.54 c
β-Phenethyl acetate	542.82 ± 41.02 c	769.31 ± 30.22 b	1003.45 ± 91.20 a	246.14 ± 13.00 d
Ethyl 3-hydroxyhexanoate	7.65 ± 1.53 a	n.d.	6.19 ± 0.19 a	n.d.
Diethyl malate	43.67 ± 2.98 a	32.16 ± 4.54 ab	38.73 ± 1.78 a	24.75 ± 3.30 b
Ethyl hydrogen succinate	444.26 ± 109.09 ns	311.73 ± 87.21 ns	467.00 ± 12.00 ns	128.12 ± 7.99 ns
Ethyl vanillate	97.70 ± 4.35 b	107.26 ± 6.54 b	91.06 ± 21.09 b	165.61 ± 5.00 a
Total	**5561.26**	**4501.76**	**4656.98**	**4269.39**

Alcohols				
2-Methyl-1-propanol (Isobutyl alcohol)	2932.25 ± 424.00 ab	3925.94 ± 376.53 a	3188.66 ± 341.65 ab	1579.40 ± 210.00 b
1-Butanol	48.94 ± 6.87 a	27.71 ± 2.09 ab	27.63 ± 4.19 b	38.21 ± 5.02 ab
3-Methyl-1-butanol (Isoamyl alcohol)	45449.09 ± 1340.21 a	45499.58 ± 3297.45 a	36352.07 ± 2400.01 b	21221.54 ± 1166.30 c
4-Methyl-1-pentanol	25.65 ± 2.43 a	17.07 ± 4.02 ab	16.22 ± 0.91 ab	9.32 ± 1.00 b
3-Methyl-1-pentanol	39.51 ± 0.90 a	18.37 ± 1.00 b	18.50 ± 1.29 b	10.33 ± 1.00 c
1-Hexanol	836.91 ± 11.32 a	850.17 ± 37.11 a	639.06 ± 33.64 b	412.95 ± 11.21 c
cis-3-Hexen-1-ol	4.15 ± 0.29 c	11.09 ± 1.01 a	7.65 ± 1.32 b	n.d.
4-Methyl-2-pentanol	130.47 ± 8.21 a	75.65 ± 5.67 ab	94.12 ± 11.32 ab	49.80 ± 1.98 b
β -Linalool [t]	6.00 ± 0.88 a	n.d.	4.91 ± 1.10 a	n.d.
1-octanol	13.39 ± 1.19 b	14.48 ± 1.11 b	20.75 ± 1.23 a	12.75 ± 2.00 b
3-(Methylthio)-1-propanol	65.58 ± 4.88 b	105.96 ± 8.43 a	79.34 ± 3.98 b	19.91 ± 2.09 c
β -Citronellol [t]	88.77 ± 7.02 a	77.07 ± 7.67 a	83.18 ± 2.99 a	37.28 ± 2.77 b
Benzyl alcohol	34.27 ± 4.23 a	38.19 ± 5.88 a	41.84 ± 5.10 a	n.d.
Phenylethyl alcohol	19677.80 ± 146.73 ab	20996.32 ± 609.24 a	18792.81 ± 313.51 b	10211.00 ± 78.99 c
2.3-butanediol	18.53 ± 1.67 b	22.35 ± 1.76 b	34.56 ± 3.43 a	21.50 ± 1.20 b
Total	**69372**	**71651**	**59402**	**33623**

Acids and other compounds				
Acetic acid	79.30 ± 10.22 b	75.33 ± 6.98 b	124.80 ± 25.23 b	228.51 ± 11.00 a
Isobutyric acid	62.59 ± 11.32 ns	59.14 ± 0.19 ns	61.34 ± 10.87 ns	38.90 ± 21.00 ns
Isovaleric acid	143.32 ± 8 a	65.32 ± 8 b	79.81 ± 2 b	52.56 ± 2 b
Hexanoic acid	525.29 ± 1 a	238.81 ± 3 d	363.70 ± 8 c	406.48 ± 11 b
Octanoic Acid	1744.34 ± 100 a	839.10 ± 77.10 c	1291.83 ± 27.34 b	1779.98 ± 91.09 a
n-Decanoic acid	362.18 ± 61.09 a	161.16 ± 36.11 d	290.83 ± 10.28 bc	252.53 ± 32.12 c
Total	**2917**	**1439**	**2212**	**2759**
5-Oxotetrahydrofuran-2-carboxylic acid [t]	72.52 ± 3.46 a	55.00 ± 2.98 b	70.05 ± 4.68 a	28.05 ± 2.02 c
3-Hydroxy-2-butanone (acetoin)	95.57 ± 6.90 b	65.60 ± 3.32 c	82.93 ± 3.54 bc	172.48 ± 7.09 a
γ -Butyrolactone	189.60 ± 8 b	312.29 ± 20 a	328.35 ± 8 a	151.26 ± 4 b
γ -Nonalactone [t]	18.73 ± 1.79 a	n.d.	21.52 ± 2.34 a	n.d.

Table 3

FIGURES

Wine Colour Parameters	CM	NM	NOM	AIR
Intensity	3.16 ± 0.02 b	3.28 ± 0.02 a	3.23 ± 0.03 a	1.95 ± 0.02 c
Tonality	0.44 ± 0.02 b	0.42 ± 0.02 b	0.45 ± 0.01 b	0.70 ± 0.01 a
L*	-5.33 ± 0.03 a	-3.92 ± 0.02 c	-4.95 ± 0.05 b	2.04 ± 0.06 d
a*	22.11 ± 0.06 c	20.75 ± 0.12 d	24.92 ± 0.11 b	25.90 ± 0.05 a
b*	-5.31± 0.09 d	-4.80 ± 0.08 c	-3.78 ± 0.08 b	5.51 ± 0.09 a
C*	22.75 ± 0.08 c	21.00 ± 0.08 d	25.22 ± 0.09 b	26.47 ± 0.05 a
h*	6.05 ± 0.02 a	6.08 ± 0.03 a	6.11 ± 0.02 a	0.22 ± 0.01 b
ΔE*	13.66 ± 0.12 a	12.96 ± 0.14 b	11.72 ± 0.13 c	-
ΔL*	7.37 ± 0.04 a	5.96 ± 0.04 c	6.99 ± 0.05 b	-

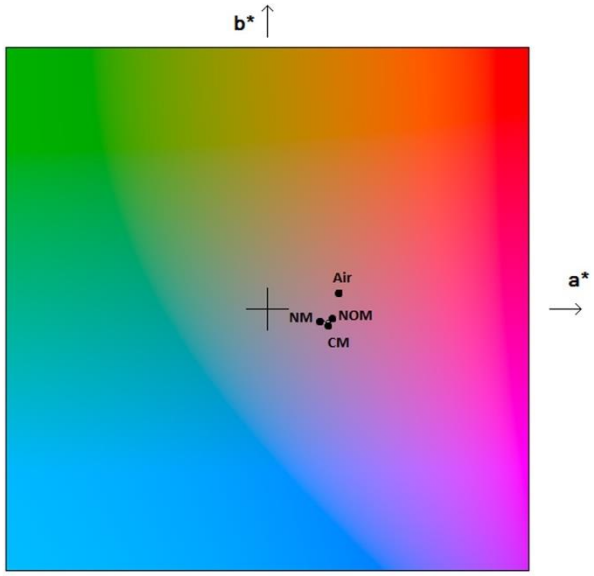
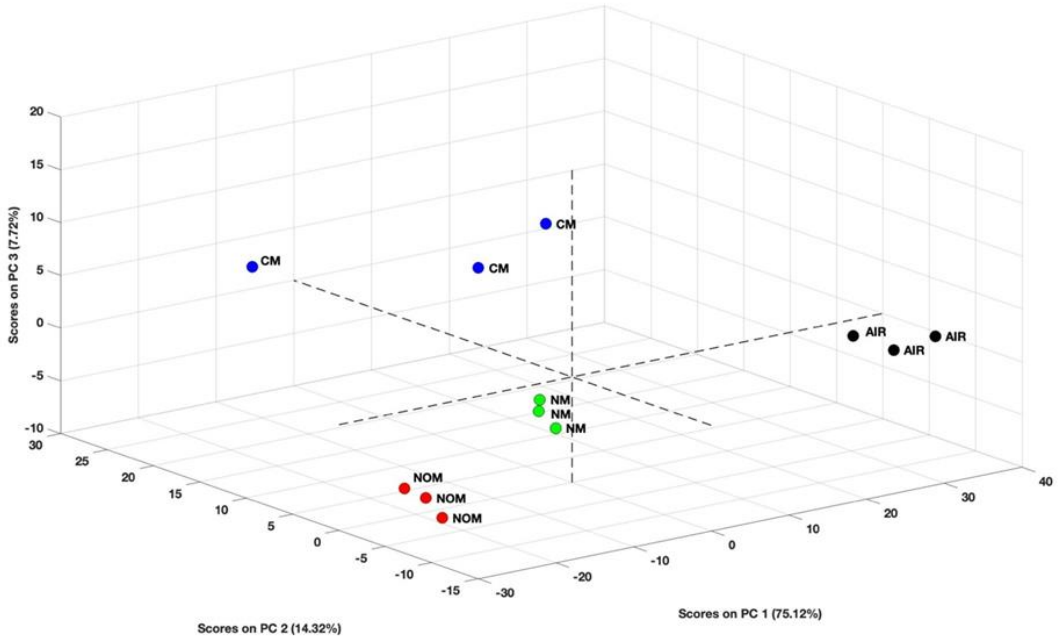


Figure 1


Scores on PC 3 (7.72%)
Scores on PC 2 (14.32%)
Scores on PC 1 (75.12%)
CM
CM
CM
AIR
AIR
AIR
NM
NM
NM
NOM
NOM
NOM

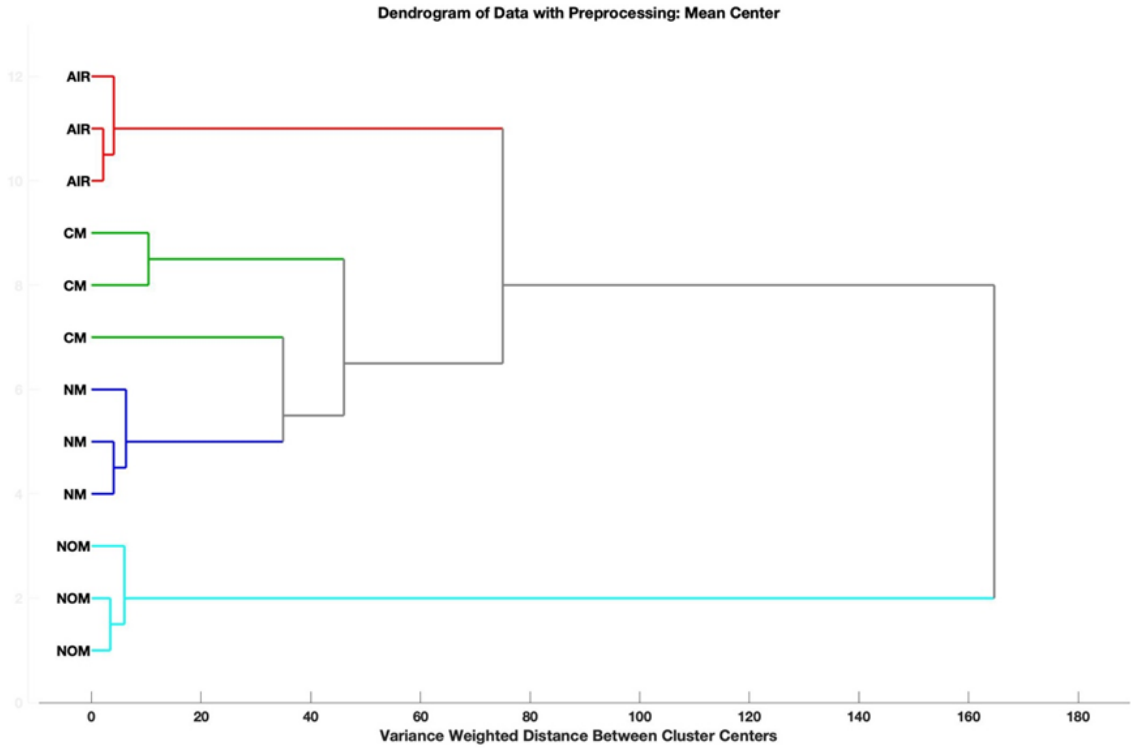

Dendrogram of Data with Preprocessing: Mean Center
AIR
AIR
AIR
CM
CM
CM
NM
NM
NM
NOM
NOM
NOM
Variance Weighted Distance Between Cluster Centers

Figure 2

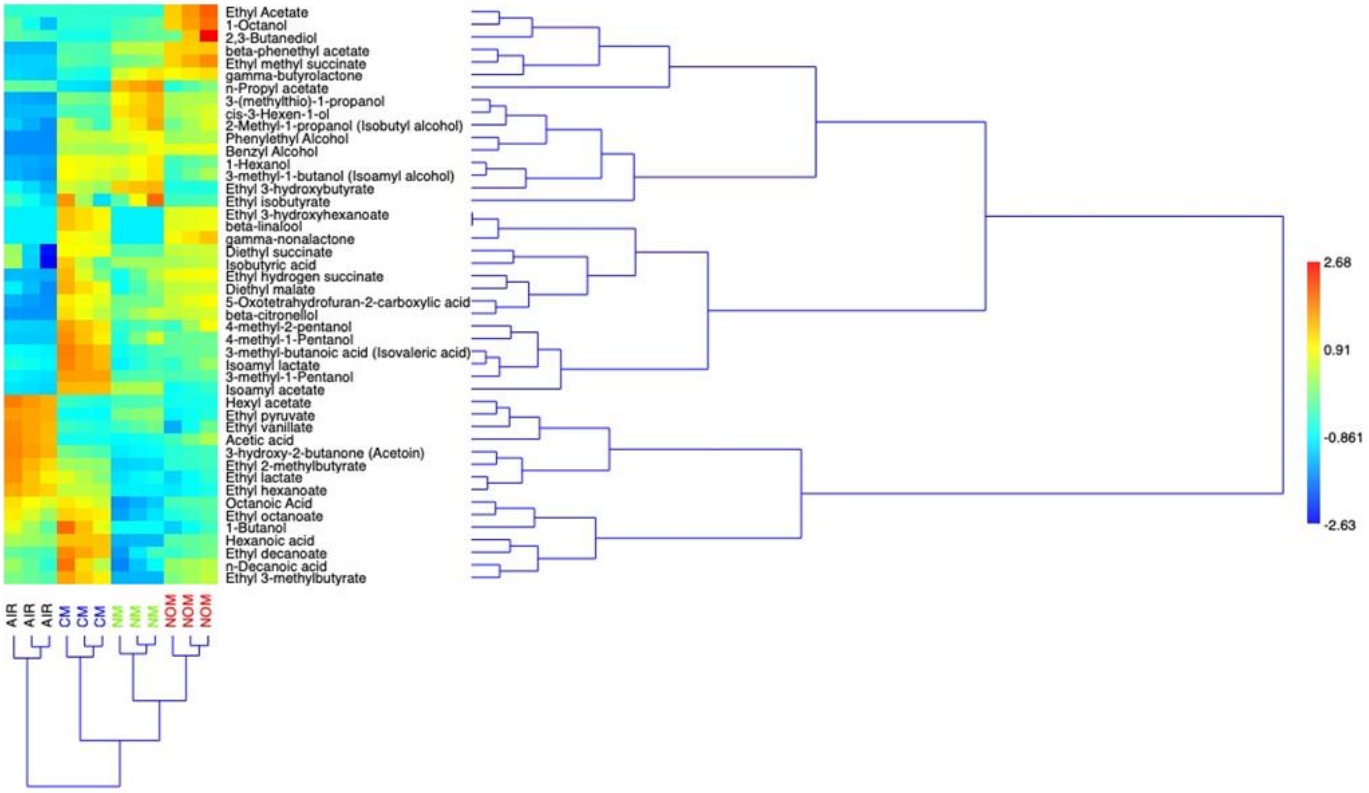

Ethyl Acetate
1-Octanol
2,3-Butanediol
beta-phenethyl acetate
Ethyl methyl succinate
gamma-butyrolactone
n-Propyl acetate
3-(methylthio)-1-propanol
cis-3-Hexen-1-ol
2-Methyl-1-propanol (Isobutyl alcohol)
Phenylethyl Alcohol
Benzyl Alcohol
1-Hexanol
3-methyl-1-butanol (Isoamyl alcohol)
Ethyl 3-hydroxybutyrate
Ethyl isobutyrate
Ethyl 3-hydroxyhexanoate
beta-linalool
gamma-nonalactone
Diethyl succinate
Isobutyric acid
Ethyl hydrogen succinate
Diethyl malate
5-Oxotetrahydrofuran-2-carboxylic acid
beta-citronellol
4-methyl-2-pentanol
4-methyl-1-Pentanol
3-methyl-butanoic acid (Isovaleric acid)
Isoamyl lactate
3-methyl-1-Pentanol
Isoamyl acetate
Hexyl acetate
Ethyl pyruvate
Ethyl vanillate
Acetic acid
3-hydroxy-2-butanone (Acetoin)
Ethyl 2-methylbutyrate
Ethyl lactate
Ethyl hexanoate
Octanoic Acid
Ethyl octanoate
1-Butanol
Hexanoic acid
Ethyl decanoate
n-Decanoic acid
Ethyl 3-methylbutyrate
AIR
AIR
AIR
CM
CM
CM
NM
NM
NM
NOM
NOM
NOM
2.68
0.91
-0.861
-2.63

Figure 3