Vineyard calcium sprays shift the volatile profile of young red wine produced by induced and spontaneous fermentation

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Running title:

Vineyard calcium sprays and wine volatile profile

Highlights

- Vineyard calcium sprays shifted wine volatile profile
- Varietal and fermentative metabolites were affected by calcium
- γ-nonalactone was the most susceptible metabolite
- Acetates, alcohols and monoterpenols increased upon calcium treatment
- Calcium reduced volatile phenols, β-damascenone and benzaldehyde

Abstract

Calcium supplements have increasingly been used at pre-harvest stages for improving fruit firmness, aiming at mitigating environmental stress. However, as recent studies demonstrated that calcium modifies ~~biochemical parameters like phenolic content~~the polyphenolic profile of grape berries, we hypothesize in this study that it also affects wine volatile profile. In a two-year study, grapevines cv. “Vinhão” were sprayed with 2% $CaCl_2$ throughout the fruiting season, and musts were prepared at a laboratory scale. Musts from calcium-treated fruits contained higher calcium levels and less anthocyanins. Increased calcium content did not affect the course of fermentation induced with a *S. cerevisiae* starter inoculum, but impacted the course of spontaneous fermentations carried out by endogenous berry microflora. Several compounds associated to varietal and fermentative aromas were largely influenced by the calcium treatment. For instance, volatile phenols decreased, together with β-damascenone, benzaldehyde and γ-nonalactone, while several acetates and alcohols increased. Principal component analysis showed that the volatile profile of control wines produced by spontaneous fermentation substantially differed between replicates, but calcium treatment lowered replicate variability. Volatile profiles were also influenced by the vintage and fermentation type. The shift in wine volatile profile upon calcium treatment may be relevant from an oenological perspective.

Keywords

Acetic acid; Alcoholic fermentation; Lactones; Linalool; Varietal aroma; Vineyard calcium sprays; Wine volatile profile

Abbreviations

GC-FID – Gas chromatography flame ionization detection

GC-MS – Gas chromatography mass spectrometry

PCA – Principal component analysis

SPE – Solid Phase Extraction

1. Introduction

Grapevine production is threatened daily by extreme environmental challenges including high radiation, heat and drought, as opposed to high humidity, intense precipitation and low temperatures, in a context of constant climate change (Jones 2015, del Pozo et al. 2019). Particularly, steady rains before harvest cause grape berry swelling, diluting the flavors and increasing the risk of fruit cracking, leading to spoilage and infection by microorganisms. Calcium supplements have increasingly been used at pre-harvest stages for improving the firmness and robustness of several fruit crops including sweet cherry, apple, strawberry and grapes, in attempts to maintain productivity even in years of unfavorable weather (Wójcik and Lewandowski 2003, Bonomelli and Ruiz 2010, Mao et al. 2017, Torres et al. 2017, Correia et al. 2019, Winkler and Knoche 2019). The benefits of calcium likely arise from its key structural roles in the cell wall and membranes, crosslinking pectin molecules and forming egg-box like structures that strengthen the cell wall and maintain the textural quality of fruits (Hocking et al. 2016). In the initial phases of grape berry development, calcium is actively imported through the xylem to inhibit premature fruit softening and colour changes (Martins et al. 2012). Moreover, it is involved in the resistance to abiotic and biotic stress, such as cold stress and defence against fungi infection through inhibition of fungal polygalacturonases that degrade the plant cell wall (González-Fontes et al. 2017, Aldon et al. 2018).

In line with the numerous roles of calcium in cells, previous studies have reported modifications in °Brix, titratable acidity and color in grape berries, following pre-harvest treatments with calcium (Al-Qurashi and Awad 2013, Ciccarese et al. 2013, Correia et al. 2019). In grape cell cultures, elevated calcium levels were demonstrated to induce a strong repression of anthocyanin biosynthetic pathways, at transcriptional and protein activity levels, decreasing bulk anthocyanin content (Martins et al. 2018). More recently, a targeted metabolomics study unequivocally demonstrated that vineyard calcium sprays affect berry polyphenolic profile, impacting several compounds like malvidin, piceid, ε-viniferin, resveratrol, catechin and quercetins, closely correlating to modifications in the expression of key genes involved in core biosynthetic pathways of secondary metabolism (Martins et al. 2020). These effects are particularly relevant in the grape-growing and winemaking industries as fruit and wine quality greatly rely upon sugar content, color intensity and phenolic richness.

Wine aroma is modulated by complex interactions in its volatile and non-volatile constituents, and can be affected by several factors including climate, soil, cultivar and fruit ripeness degree, besides fermentation conditions such as pH, temperature, aeration, skin contact time and microflora (Teixeira et al. 2013, Belda et al. 2017, Deed et al. 2017, Harrison 2017, Ruiz et al. 2019). Accordingly, previous studies demonstrated that copper-based formulations induced shifts in grape berry metabolomic profile, with consequent modifications in wine volatile compounds including higher alcohols and terpenes (Martins et al. 2014, Martins et al. 2015). In the context of an ongoing project, we aim at understanding the effect of vineyard calcium sprays on biochemical and physical properties of the grape berry, including metabolic profile, firmness and shelf-life. Following ~~previous~~ recent studies showing that calcium ~~may affect phenolic~~affects polyphenolic compounds in grape ~~cells~~berry and in cell cultures (Martins et al. 2018, Martins et al. 2020) and early studies showing that excess calcium may impair yeast growth (Saltukoglu and Slaughter 1983), in this study we addressed the hypothesis that the application of calcium in the vineyards affects the quality traits of young wine, in particular wine volatile profile. In a two-year field trial, grapevines cv. “Vinhão” were sprayed with calcium throughout the fruiting season and musts were prepared at a laboratory scale following the traditional method for red wines. Two types of fermentation were then performed: spontaneous fermentation by endogenous berry microflora, and induced fermentation with a *S. cerevisiae* starter inoculum. The first fermentation type would allow to address the effect of calcium on the behavior of native berry microorganisms during the process of wine-making; in co-existing, these microbes interact with each other in addition to responding to environmental stimuli, and in the course of fermentation the diversity of such populations decreases because only a few species are able to survive the increasingly harsh wine environment (Pinto et al. 2014, Belda et al. 2017, Bartle et al. 2019). Thus, greater variability in the production of volatiles could be expected. In contrast, in musts inoculated with a starter culture, the influence of calcium on fermentative yeast performance could be studied more precisely, as well as its effects on the resulting wine volatile compounds. Biochemical parameters of young wines were initially determined, including °Brix, alcohol content, titratable acidity and anthocyanin content, followed by an extensive characterization of wine volatile profile by Gas chromatography-based techniques. The study contributed to the elucidation of the effect of calcium sprays on wine volatiles underling varietal and fermentative aromas, and allowed to draw conclusions on their suitability from an oenological perspective.

2. Material and Methods

2.1 Vineyard treatments

Field experiments were performed in young grapevines cv. “Vinhão” during the vintages of 2017 and 2018, in a commercial vineyard established in the Portuguese DOC region of ‘Vinhos Verdes’ [coordinates: N41°28'28'' latitude, W8°34'59'' longitude, 165 m altitude]. Grapevine aerial parts were evenly sprayed with a solution of 2% (w/v) $CaCl_{2.}2H_2O$ (Merck KGaA, Darmstadt, Germany) and 0.1% (v/v) Silwet L-77 (Arysta LifeScience Iberia, Lisbon, Portugal) used as surfactant, as previously described (Saftner et al. 1997). Vines were sprayed three times with this treatment throughout the fruiting season, every 30 d, the first performed at the pea size and the last performed 1 week before harvest. Control plants were sprayed with a solution containing the surfactant agent only. Both control and calcium-treated grapevines were cultivated under the same microclimate and subject to the same routine phytosanitary treatments with Topaze® and Ridomil Gold® R WG (Syngenta Portugal LDA., Lisbon, Portugal), according to the suppliers’ instructions.

2.2 Microvinifications

Three mature bunches were randomly harvested from each grapevine, and a total of eighteen bunches per treatment were mixed and crushed. Musts were prepared according to the traditional method for red wines, in a laboratory scale, as described by Martins et al. (2015). Microvinifications occurred in 1 L glass flasks containing 0.5 L of grape juice and 180 g of a mixture of grape berry skins and seeds. Two types of fermentation were performed: spontaneous fermentation by endogenous grape berry microflora, and induced fermentation following inoculation of the musts with a laboratory yeast strain (*Saccharomyces* *cerevisiae* W303, obtained from the Yeast Collection of the Biology Department of University of Minho (strain no. DB 2986)) as described previously (Martins et al. 2015), to obtain unbiased production of volatiles, as wine yeast strains greatly influence wine aromatic traits, being selected according to the winemaker's preferences (Belda et al. 2017). As a first assessment of the effects of calcium on wine volatile profile, two biological replicates per treatment and per fermentation type were performed in the first vintage (2017), and in the following vintage (2018) the study was reproduced with three biological replicates. The musts were incubated in the dark at 24°C and stirred manually once a day. The evolution of fermentation was followed by determination of the °Brix using a H196813 digital refractometer (Hanna Instruments, Póvoa de Varzim, Portugal) and of the alcohol content using a vinometer. The end of alcoholic fermentation was determined as the moment when variations in °Brix and alcohol content ceased.

2.3 Determination of biochemical parameters

Biochemical parameters were determined in musts and wines, before fermentation and at the end of fermentation, respectively. Calcium levels were determined following the method of Spare (1964), the concentration of reducing sugars was determined with the 3,5-dinitrosalicylic acid reagent (Merck KGaA, Darmstadt, Germany) according to the method of Miller (1959), pH was determined using a multi-parameter analyzer Consort C-860, and titratable acidity was determined using an adaptation of the method of Amerine and Ough (1980), as described previously (Martins et al. 2015). Total phenolics were estimated by the Folin-Ciocalteau colorimetry method (Waterhouse et al. 2002, Martins et al. 2018) and total anthocyanins were determined with the differential pH method (Nicoué et al. 2007).

2.4 Quantification of volatile compounds in wine

The quantitative analysis of major volatile compounds was carried out using the method of Ortega et al. (2001). Briefly, 3 x10^{-3} L of wine containing the internal standards -IS- (2-butanol from Merck (Darmstadt, Germany), 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone, and 2-octanol, all of them from Sigma-Aldrich (St. Louis, Mo., USA)) and 7 x10^{-3} L of water were salted with 4.5 g of ammonium sulfate from Panreac (Barcelona, Spain) and extracted with 0.2 x10^{-3} L of dichloromethane from Fisher Scientific

(Loughborough, UK). The extract was then analyzed by GC with FID detection. The area of each analyte was normalized by that of its corresponding IS and was then interpolated in the corresponding calibration plot built by applying exactly the same analytical method as that applied to synthetic wines containing known amounts of the analytes covering the natural range of occurrence of these compounds. Minor compounds were analysed by SPE and GC-Ion Trap-MS analysis, as described by Lopez et al. (2002). Briefly, 50 x10^{-3} L of wine, containing 25 x10^{-6} L of BHA solution (0.01 g of 3-*tert*-butyl-4-hydroxyanisole per g of ethanol, both from Sigma-Aldrich) and 75 x10^{-6} L of a surrogate standards solution (3-octanone, β-damascone, heptanoic acid, and isopropyl propanoate, all of them from Sigma-Aldrich), were passed through a LiChrolut EN (Merck) 0.2-g cartridge at a rate of about 3.3 x10^{-5} L s^{-1}. The sorbent was dried under nitrogen stream (purity 99.999%). Analytes were recovered by elution with 1.3 x10^{-3} L of dichloromethane; 25 x10^{-6} L of an internal standard solution (4-hydroxy-4-methyl-2-pentanone and 2-octanol, both at 0.3 g per g of dichloromethane) were added to the eluted sample. The extract was then analyzed by GC with ion trap MS detection.

2.5 Data handling and statistical analysis

Principal Component Analysis (PCA) of wine volatile data was performed in R software version 3.5.3 using the FactoMineR package 1.4.1 (Lê et al. 2008), and PCA output visualization was achieved with Factoextra package 1.0.5 (Kassambara 2017). Heatmaps of wine volatile data were performed with the ComplexHeatmap package 1.18.1 on Bioconductor 3.9 (Gu et al. 2016). Bar graphs were performed in Prism®6 (GraphPad Software, Inc.). Significant differences between control wines and wines produced from calcium-treated fruits were determined using the Student's *t* test, within each vintage and fermentation type, and marked with asterisks to denote the significance levels: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$. To determine the predicted wine aromatic profiles, volatile compounds were grouped according to their associated aromatic descriptors, and relative values of compounds detected above their odor thresholds were added within each class. The resulting values for each aromatic descriptor were then presented as radar charts in Microsoft Office 365 Excel.

<u>3. Results</u>

The evolution of oenological parameters of wines produced in the vintages of 2017 and 2018 was monitored throughout the fermentation process (**figure 1**). In both vintages, the decrease in °Brix and consequent increase in alcohol content was faster for induced fermentation than spontaneous fermentation. Hence, while induced fermentation finished at day 10, spontaneous fermentations lasted until day 16. In general, wines produced from induced fermentation contained lower °Brix and increased alcohol content than wines produced spontaneously, more evident in the vintage of 2017. The calcium treatment did not significantly influence the progress of induced fermentation. In contrast, calcium affected the course of spontaneous fermentations in both seasons, but in opposite ways; wines produced from calcium-treated berries in 2017 had 1.7 more °Brix and 1.4% less alcohol than control wines, while in 2018 control wines had 1.9 more °Brix and 0.7% less alcohol than wines obtained from calcium-treated fruits (**figure 1**).

Additional biochemical parameters were analysed in unfermented musts (day 0) and in wines at days 10 or 16 corresponding to the end of induced or spontaneous fermentation, respectively. Calcium levels were 1.2-fold higher in musts produced from grape berries treated with calcium than in control musts, in both vintages (**figure 2**), increasing significantly from 0.025 g L^{-1} to 0.028 g L^{-1} in 2017 and from 0.022 g L^{-1} to 0.026 g L^{-1} in 2018. This effect was still observed in wines, more significantly for those produced by induced fermentation (1.2-fold increase, from 0.03 to 0.04 g L^{-1}). Calcium content in wines tended to be higher than in musts, possibly as a result of maceration and release of calcium from the fruit tissues to the soluble fraction. Reducing sugars were 7-30% lower in musts obtained from calcium-treated berries than in control musts (a decrease from ~~300~~181 g L^{-1} to ~~253~~127 g L^{-1} was observed in 2017, and from ~~299~~174 g L^{-1} to ~~280~~163 g L^{-1} in 2018). A 28-40% decrease in reducing sugars upon calcium treatment was still observed in wines produced in 2018, either after spontaneous (5.5 g L^{-1} to 3.3 g L^{-1}) or induced fermentation (4.0 g L^{-1} to 2.9 g L^{-1}). In contrast, in the vintage of 2017 calcium had no significant influence on wine reducing sugars. In general, both the titratable acidity and the pH of musts and wines were not significantly affected by calcium, in comparison to the respective controls, with the exception of wines produced by induced fermentation in the vintage of 2017 where control wines had 1.3-fold more acidity (8 g L^{-1}) than wines produced from calcium-treated fruits (6 g L^{-1}). Moreover, the biological replicates of control wines produced after spontaneous fermentation had great variations in titratable acidity, which oscillated between 6 g L^{-1} and 37 g L^{-1} in both vintages. ~~Estimated total phenolics and total anthocyanin levels increased by up to 4.3-fold during spontaneous and induced fermentation processes, in both vintages, as a result of maceration and release of pigments from the fruit skin to the soluble fraction.~~ Total phenolics in musts and wines were not significantly affected by calcium in the vintage of 2017. However, calcium treatments in the vintage of 2018 caused a 26% reduction in must total phenolic levels (from 1.4 to 1.0 g L^{-1}), and this trend was also observed in wines (± 3.05 g L^{-1} to ± 2.25 g L^{-1}). Total anthocyanins detected in musts decreased by ~20% upon calcium treatment in both vintages; in 2017 a decrease from 0.015 g L^{-1} to 0.012 g L^{-1} was observed, and in 2018 a decrease from 0.029 g L^{-1} to 0.023 g L^{-1} was recorded. Nonetheless, no significant differences were observed in anthocyanin levels of wines regardless of the fermentation type (**figure 2**).

The volatile composition of the wines was analyzed by Liquid-Liquid Microextraction GC-FID and SPE GC-Ion Trap-MS. A total of 64 volatile compounds was quantified, comprising carbonyl compounds, alcohols, acetates, acids, esters, monoterpenols, norisoprenoids, phenols, cinnamates, lactones and vanillin derivatives (**supplementary table S1**). Among the major volatiles detected in wines, found in the range of several mg L^{-1}, were isoamyl alcohol, β-phenylethanol, isobutanol, ethyl acetate, acetoine and acetic acid. In parallel, the most abundant minor volatiles, found in the range of several µg L^{-1}, were ethyl isobutyrate, isobutyl acetate, vanillin, benzaldehyde, 4-vinylguaiacol, 2,6-dimethoxyphenol, 4-vinylphenol, acetovanillone and ethyl vanillate. Principal component analysis (**figure 3**) revealed that the biological replicates among different wine samples clustered closely together, demonstrating the high stability of wine volatiles and the good reproducibility of the wine-making technique. The exception was observed in control wines produced after spontaneous fermentation in both vintages, in which the biological replicates appeared quite dispersed in the score plot, demonstrating a great variability in volatile profiles.

A clear separation between wines produced in the vintages of 2017 and 2018 was highly evident in the score plot (**figure 3**), highlighting a strong contribution of the vintage to the overall volatile profile. The main compounds underlying this effect included (Z)-3-hexenol, butyric acid, methyl vanillinate, ethyl vanillate, acetic acid, isovalerianic acid, m-cresol and guaiacol, which were more prominent in the vintage of 2017, besides isobutanol, acetovanillone, linalool acetate, vanillin, β-ionone, ethyl lactate, ethyl isobutyrate and isoamyl acetate, which were more prominent in the vintage of 2018. The effect of the fermentation type was also evident, as a clear separation was observed for wines produced by induced or spontaneous fermentation (**figure 3**). The main compounds accounting for these differences included isoamyl alcohol, β-phenylethanol, ethyl vanillate, isobutanol, 4-vinylguaiacol, 2,6-dimethoxyphenol, 4-vinylphenol, ethyl acetate, acetoine, acetic acid, ethyl isobutyrate, isobutyl acetate and vanillin. The first three compounds were more prominent in wines produced by induced fermentation, while the last six compounds were more abundant in wines produced after spontaneous fermentation (**supplementary table S1**).

The effect of vineyard calcium treatments on wine volatile profile was also visible in the score plot (**figure 3**), where wines produced from control berries appeared separated from wines produced from calcium-treated fruits. Calcium significantly affected all classes of volatile compounds detected, with the exception of linear ethyl esters (**figure 4**). Although some compounds were affected by calcium in a vintage-dependent manner, others were consistently influenced regardless of the vintage. For instance, calcium led to increased diacetyl levels (2-fold) in wines produced after spontaneous fermentation in the vintage of 2018 but not in 2017. In contrast, benzaldehyde levels decreased by 42-66% upon calcium treatment, in wines produced by induced fermentation in both vintages (**supplementary table S1**). Several acetates increased upon calcium treatment, in wines produced by induced fermentation, namely isoamyl acetate (2.3-fold more than in control wines), isobutyl acetate (by 1.4-fold), butyl acetate (by 1.8-fold) and phenylethylacetate (by 1.5-fold), the effect being more evident in the vintage of 2018. The same was observed for alcohols which increased up to 2.3-fold, namely isobutanol, 1-hexanol, methionol, and benzylic alcohol, although in a manner dependent on the vintage and fermentation type. Acids were differentially affected by the calcium treatment; a sharp decrease of 44% was observed for isobutyric acid and of 27% for hexanoic acid in wines produced by induced fermentation in the vintage of 2018. In the vintage of 2017, calcium treatment resulted in increased levels of octanoic and decanoic acids (up to 2.6-fold), depending on the fermentation type (**figure 4**, **supplementary table S1**). Among branched ethyl esters, only ethyl-2-methylbutyrate was affected by calcium, decreasing by 61% in wines produced by spontaneous fermentation in the vintage of 2018. In contrast, the treatments with calcium led to the formation of diethyl succinate, which was not detected in control wines produced by induced fermentation in the vintage of 2018. The same was observed for linalool acetate in the vintage of 2017. In the vintage of 2018, calcium led to a 7-fold increase in linalool acetate levels, and other monoterpenols namely linalool and α-terpineol were also more abundant (1.7-fold increase), particularly in wines produced by induced fermentation. The levels of β-damascenone belonging to the norisoprenoids class decreased by 12-26% upon calcium treatment in wines produced by induced fermentation in both vintages. The same pattern was observed for α-ionone in the vintage of 2017 (36% decrease), while β-ionone levels increased by 1.6-fold in the vintage of 2018. Calcium treatments resulted in decreased levels in wine phenols, of up to 75%, which included guaiacol, o-cresol, 4-ethyl guaiacol, m-cresol, eugenol, 4-vinylguaiacol, 4-allyl-2,6-dimethoxyphenol. In contrast, E-isoeugenol levels increased by up to 1.3-fold in the vintage of 2017 regardless of the fermentation type. The effect of calcium treatments on wine ethyl dihydrocinnamate were not consistent, varying among vintages and fermentation types. For wines produced by induced fermentation in the vintage of 2018, increased levels of γ-butyrolactone were observed (by 1.2-fold), together with γ-decalactone which was not detected in control wines. In contrast, following spontaneous fermentation, γ-decalactone was only detected in control wines. However, the most significant differences were observed in the levels of γ-nonalactone which invariably decreased upon calcium treatment (up to 62%) regardless of the vintage or fermentation type. Vanillin derivatives were only significantly affected by calcium in wines produced by induced fermentation. Ethyl vanillate decreased by 16-28% in both vintages, while methyl vanillinate decreased only in the vintage of 2018, by 25%. In contrast, acetovanillone increased by 1.7-fold only in wines produced in the vintage of 2017 (**figure 4**, **supplementary table S1**).

As several volatile compounds were detected above their odor thresholds, differences in wine aromatic profile could be predictable. As depicted in **figure 5**, the predicted aromatic traits of wines were mostly dependent on the fermentation type and barely influenced by the vintage. Wines produced by induced fermentation were expected to display essentially fusel and green aromas atributed by alcohols, but also flowery and fruity flavors atributed by acetates, ethyl esters and norisoprenoids (**supplementary table S1**). Less intense oxidative, lactic and cheesy aromas may also be noted given the presence of carbonyl coumpounds and acids above their odor thresholds. Vineyard calcium treatments did not influence the predicted aroma of wines produced by induced fermentation. For wines produced by spontaneous fermentation, the predicted odor diversity was greater than for wines produced by induced fermentation. An accentuation in flowery and fruity notes could be expected, together with a strong vinegar odor attributed by acetic acid, although with lower incidence for wines produced from calcium-treated berries in the vintage of 2018 where acetic acid levels were 10-fold lower than in control wines (**supplementary table S1**). In addition, oxidative and lactic aromas could be more prominent in wines produced from calcium-treated berries, more evidently in the vintage of 2017 (**figure 5**). In the vintage of 2018, lower intensity of cheesy flavors was predicted in these wines.

4. Discussion

Must composition and fermentation conditions are two major factors determining the volatile profile of wines (Belda et al. 2017, Deed et al. 2017). The application of inorganic compounds in vineyards has been proven to modulate grape berry biochemical properties and must composition, with consequences to wine volatile profile (Martins et al. 2014, 2015; Harrison 2017). Hence, viticulturists can act to promote aroma precursors to improve the aromatic profile of grapes and the wine ultimately produced, although agronomic practices do not always have uniform results (Alem et al. 2018). Hence, when implementing new management strategies in viticulture, it is essential to evaluate the efficacy of new supplements and to analyze in each situation the organoleptic traits of the fruits and wines, to achieve optimization of production without detrimental effects in quality. In the present study, the effect of vineyard calcium sprays on fermentation dynamics and volatile profile of young wines cv. “Vinhão” was investigated in two consecutive vintages. This cultivar is one of the most prominent red wine grape cultivar in the DOC region of “Vinhos Verdes” and, due to its economic value, has been a target of different studies focusing on the effects of exogenous mineral-based treatments on berry metabolism and composition (Martins et al. 2014, 2015, 2018). Musts produced from calcium-treated fruits contained higher calcium levels than control musts, and lower amount of reducing sugars and anthocyanins. As calcium is a central secondary messenger interacting with hormone-driven processes that determine berry ripening (Martins et al. 2018), a possible interference with sugar accumulation mediated by ABA signaling could explain the reduced sugar levels in musts from calcium-treated berries. The decreased levels in total phenolics and anthocyanins detected in musts in response to calcium treatment are also in line with previous experiments ~~conducted in grape berry cultured cells~~ (Martins et al. 2018, Martins et al.

2020), notwithstanding the necessary caution when interpreting the results obtained with the Folin-Ciocalteau reagent which is highly reactive towards reducing compounds.

The differences observed in must biochemical parameters upon calcium treatment did not influence the progress of induced fermentation, but likely accounted for the disparities observed during the spontaneous fermentations, suggesting that calcium did not impair sugar degradation by the *S. cerevisiae* strain used to inoculate the musts, but influenced the activity of the endogenous berry microorganisms that carried out spontaneous fermentation, as discussed afterwards. Furthermore, the biochemical parameters of wines were not linearly influenced by the calcium treatment, often varying in a vintage-dependent manner. Wine volatile profiles were also influenced by the vintage, most likely due to differences in the composition of the berries. In line with this hypothesis, in a ~~parallel~~recent study we have performed targeted characterization of berry metabolomic profile and confirmed that both the vintage and the calcium treatment had an influence on berry polyphenolics~~,~~ (Martins et al. 2020), some being consistently affected by calcium regardless of the vintage~~,~~ (including *E*-resveratrol, *E*-ε-viniferin, catechin and malvidin-3-O-glucoside), and others susceptible to the calcium-vintage interaction effect, possibly associated to differences in agrometeorological conditions. These can also influence the varietal aroma directly (Roullier-Gall et al. 2014), and the non-varietal (fermentative) aroma indirectly, by affecting the aminoacidic composition of the must which varies according to the vintage (Hernández-Orte et al. 2002).

Among the volatile compounds consistently affected by calcium, in the present study, were metabolites associated to both varietal and fermentative aromas, the most susceptible one being γ-nonalactone which decreased sharply in both vintages and after both fermentation types. Previous studies demonstrated that the levels of this metabolite correlate linearly with the degree of grape berry maturity, being detected at higher levels in overripe berries, and often associated to vintages with the highest temperatures (Pons et al. 2017). Thus, the results obtained could indicate that calcium may be delaying fruit maturation, preventing the accumulation of excess γ-nonalactone, which can be relevant in the context of ongoing climate change. These observations are in line with the decreased levels of reducing sugars and anthocyanins detected in musts produced from calcium-treated fruits, as discussed elsewhere.

Calcium treatments consistently led to decreased levels in several wine phenols, including guaiacol, o-cresol, 4-ethyl guaiacol, m-cresol, eugenol, 4-vinylguaiacol, and 4-allyl-2,6-dimethoxyphenol. Accordingly, previous studies demonstrated that the application of Bordeaux mixture (a combination of $CuSO_4$ and $Ca(OH)_2$) in cv. “Vinhão” vines led to decreased levels of m-cresol and 4-allyl-2,6-dimethoxyphenol (Martins et al. 2015). In parallel, several alcohols increased in response to calcium treatments in the present study, namely isobutanol, 1-hexanol, methionol, and benzylic alcohol. The ~~later~~latter was also found to increase sharply upon application of Bordeaux mixture, although the remaining alcohols were generally not significantly affected by the treatment (Martins et al. 2015).

In the present study, some volatile classes were only affected by calcium following induced fermentation but not spontaneous, in both vintages. For instance, a general increase in the levels of acetates was found in wines produced from calcium-treated berries after induced fermentation. These aroma-active esters result from the esterification of a higher alcohol with acetic acid, and are formed intracellularly by fermenting yeast cells, diffusing through the cellular membrane into the fermenting medium (Saerens et al. 2008), comprising the most abundant group of esters in industrial fermentations, with a major impact on the fruity flavour of wine (Dzialo et al. 2017). Thus, an increase in specific acetates mediated by calcium treatment could potentially benefit wine aroma. The increased levels in linalool and linalool acetate upon calcium treatment could also favour wine aroma, as these monoterpenols have low perception thresholds, contributing with pleasant lemon, coriander-seed and rose-like notes (Darriet et al. 2012). In line with these results, increased levels of acetates and monoterpenols have been reported previously following application of Bordeaux mixture in cv. “Vinhão” vines (Martins et al. 2015).

Unlike these metabolites, in the present study, the norisoprenoid β-damascenone decreased steadily in wines produced from calcium-treated berries after induced fermentation. Norisoprenoids result of the enzymatic oxidation and cleavage of β-carotene (and other carotenoids) during crushing of grapes, contributing greatly to wine aroma with floral and fruity notes. However, unlike for other C13-norisoprenoid derivatives, it is quite difficult to predict the effect of grape-growing conditions on the concentration of β-damascenone in wine, due to the high complexity of the reaction mechanisms in the berries leading to the formation of this compound (Pons et al. 2017). Nonetheless, having been detected above its odor threshold in the present study, β-damascenone could be considered as a good indicator of the effects of vineyard calcium sprays on the aroma of “Vinhão” wines. The vanillin derivative ethyl vanillate also decreased upon calcium treatment, in line with work by Martins et al. (2015); however, it was not detected in sufficient quantities to predictably influence wine aroma. The same occurred for benzaldehyde, which was present in concentrations below its odor threshold. Lastly, the majority of ethyl esters detected in wines was not affected by the calcium treatment, suggesting that this class of compounds is not susceptible to variations in must composition. The fact that calcium treatment affected several acetate esters but not ethyl esters supports that calcium is only influencing a specific part of the metabolic pathways comprising the formation or degradation of these compounds, which contradicts previous reports suggesting that acetate and ethyl ester production are affected in the same way by the same parameters (Saerens et al. 2008).

In the present study, a few compounds were affected by calcium only in specific conditions, depending on the vintage and fermentation type, including diacetyl, ethyl 2-methylbutyrate, ethyl dihydrocinnamate, diethyl succinate, α-ionone, β-ionone, γ-butyrolactone, methyl vanillinate and acetovanillone; particularly the acids isobutyric and hexanoic decreased, while octanoic and decanoic acids increased, suggesting a less consistent influence of calcium in these volatile classes.

In this study, although the overall wine volatile profile was greatly affected by the vintage besides the fermentation type, the ~~later~~latter almost exclusively defined the predicted aroma of wine, thus the aromatic traits of wines produced by the same fermentation type were highly similar among vintages. These findings are in line with previous reports highlighting that most wine aroma compounds, including those present as precursors, are produced or released during wine fermentation due to microbial activity (Belda et al. 2017, Ruiz et al. 2019). Results further suggested that calcium did not likely influence the aroma of wines produced by induced fermentation. The same was not observed for wines produced after spontaneous fermentations. These wines displayed a wider range of predicted aromatic traits, easily attributed to the high diversity in fermentative microbiota. Grape microorganisms include *Saccharomyces* and non-*Saccharomyces* yeasts, (as well as lactic acid bacteria), of which *Saccharomyces cerevisiae* only occurs at concentrations of less than 10–100 cfu/g berry (Pinto et al. 2014, Belda et al. 2017). In this study, control wines produced after spontaneous fermentation presented highly diverse volatile profiles, together with major disparities in titratable acidity, °Brix and anthocyanin content, likely caused by unpredictable and complex interactions among the must microbial community; however, calcium treatment lowered the variability of these parameters between replicates. In agreement to these results, previous studies demonstrated that the natural yeast microflora is often inadequate to efficiently carry out the fermentative process (De Filippis et al. 2019), often leading to the production of many off flavors (volatile acidity, undesirable sulfur compounds, phenolic off flavors) that have a negative masking effect on wine quality (Tempère et al. 2018). Hence, given the absence of a starter inoculum, the occurrence of alternative fermentations such as acetic and malolactic is highly plausible, as supported by increased levels of acetic acid and ethyl lactate, corroborating the higher titratable acidity levels observed in the present study. The treatment with calcium attenuated the formation of acetic acid by 10-fold in the vintage of 2018, suggesting it may prevent the development of undesired microorganisms, under particular vintage-dependent conditions. In addition, it may hypothetically contribute for stronger oxidative and lactic aromas, attributed by the increased levels of acetaldehyde and acetoine. Ongoing studies comprising the characterization of the grape berry microbiome in response to calcium treatments will help elucidate which microorganisms are more susceptible to calcium, and how their behavior is affected during fermentation. This will complement the present study and allow the establishment of correlations on the effects of calcium on berry metabolome, microbiome and wine volatile profile.

In conclusion, we confirmed the hypothesis that vineyard calcium treatments shift the volatile profile of young wines, affecting compounds associated to both varietal and fermentative aromas, suggesting that it may influence not only grape berry composition but also yeast activity. The levels of several volatile phenols decreased, together with β-damascenone, ethyl vanillate, benzaldehyde and γ-nonalactone, which was the most susceptible metabolite to calcium treatments. In contrast, several acetates, alcohols and monoterpenols increased in wines produced from calcium-treated berries. The effects of calcium on wine biochemical properties and on volatile compounds were often modulated by the vintage and fermentation type. Calcium did not affect the course of induced fermentation, and although modifications were observed in several volatile compounds, we conclude that calcium is not predicted to influence the overall odor of wines produced with a starter inoculum. As for spontaneous fermentations, great variability was observed in volatile profiles of control wines, associated to major disparities in titratable acidity. This variation was not observed in wines produced from calcium-treated berries, suggesting a favorable impact of calcium in fermentations carried out by endogenous berry microflora.

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6. Conflict of Interest

The authors declare no conflicts of interest.

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8. Figure Captions

Figure 1. °Brix and alcohol content in musts obtained from grape berries cv. "Vinhão" treated with calcium during the fruiting season (Ca) or from control fruits (C) in the vintages of 2017 and 2018, throughout the days of induced (+) or spontaneous (-) fermentation. Results are expressed as mean ± SD.

Figure 2. Biochemical parameters in musts and wines obtained from grape berries cv. "Vinhão" treated with calcium during the fruiting season (Ca) or from control fruits (C) in the vintages of 2017 and 2018, following induced (+) or spontaneous (-) fermentation. Results are expressed as mean ± SD and asterisks indicate statistical significance between Ca and the respective control (C) within each sample type, following Student's *t*-test: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

Figure 3. Principal Component Analysis BiPlot of volatile profiles of wines produced from grape berries cv. “Vinhão” treated with calcium during the fruiting season (Ca) or from control fruits (C) in the vintages of 2017 and 2018, following induced (+) or spontaneous (-) fermentation. Biological replicates in the score plot are shown with the same color within each sample type, and the length of the arrows associated to each volatile compound is proportional to its contribution to the overall sample distribution.

Figure 4. Heatmap of the modifications observed in the volatile compounds of wines produced from grape berries cv. “Vinhão” treated with calcium during the fruiting season (Ca) or from control fruits (C) in the vintages of 2017 and 2018, following induced (+) or spontaneous (-) fermentation. Each row represents a metabolite and each column represents a sample type. Values were centred and scaled in the row direction to form virtual colors as presented in the color key, in which the offset was determined by the average values found within the biological replicates of each sample type, and the scaling was defined according to the corresponding standard deviation. Metabolites were grouped according to their volatile classes. Asterisks indicate statistical significance between Ca and the respective control (C) within each sample type, following Student’s *t*-test: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. Compounds that were only detected upon Ca treatment are indicated with a ‘plus’, and compounds not detected upon Ca treatment are indicated with a ‘minus’.

Figure 5. Predicted aromatic profile of wines produced from grape berries cv. “Vinhão” treated with calcium during the fruiting season (Ca) or from control fruits (C) in the vintages of 2017 and 2018, following induced or spontaneous fermentation. Volatile compounds detected above their odor thresholds were grouped according to their aromatic descriptors and log-transformed values for each aromatic trait are shown.

Figure 1

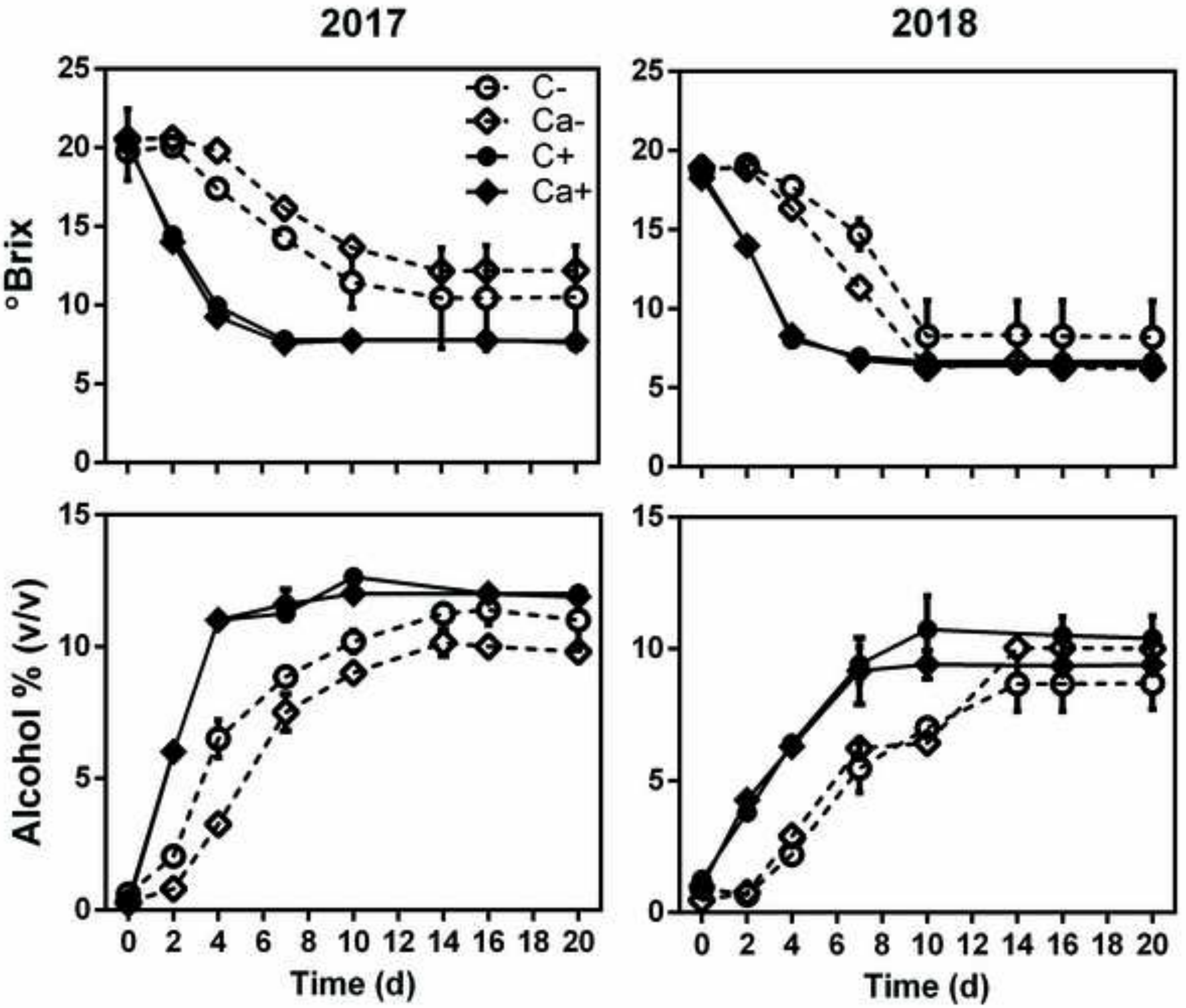
2017
2018
C-
Ca-
C+
Ca+
°Brix
Alcohol % (v/v)
0
5
10
15
20
25
0
5
10
15
0
2
4
6
8
10
12
14
16
18
20
Time (d)
Time (d)

Figure 2

Click here to download high resolution image

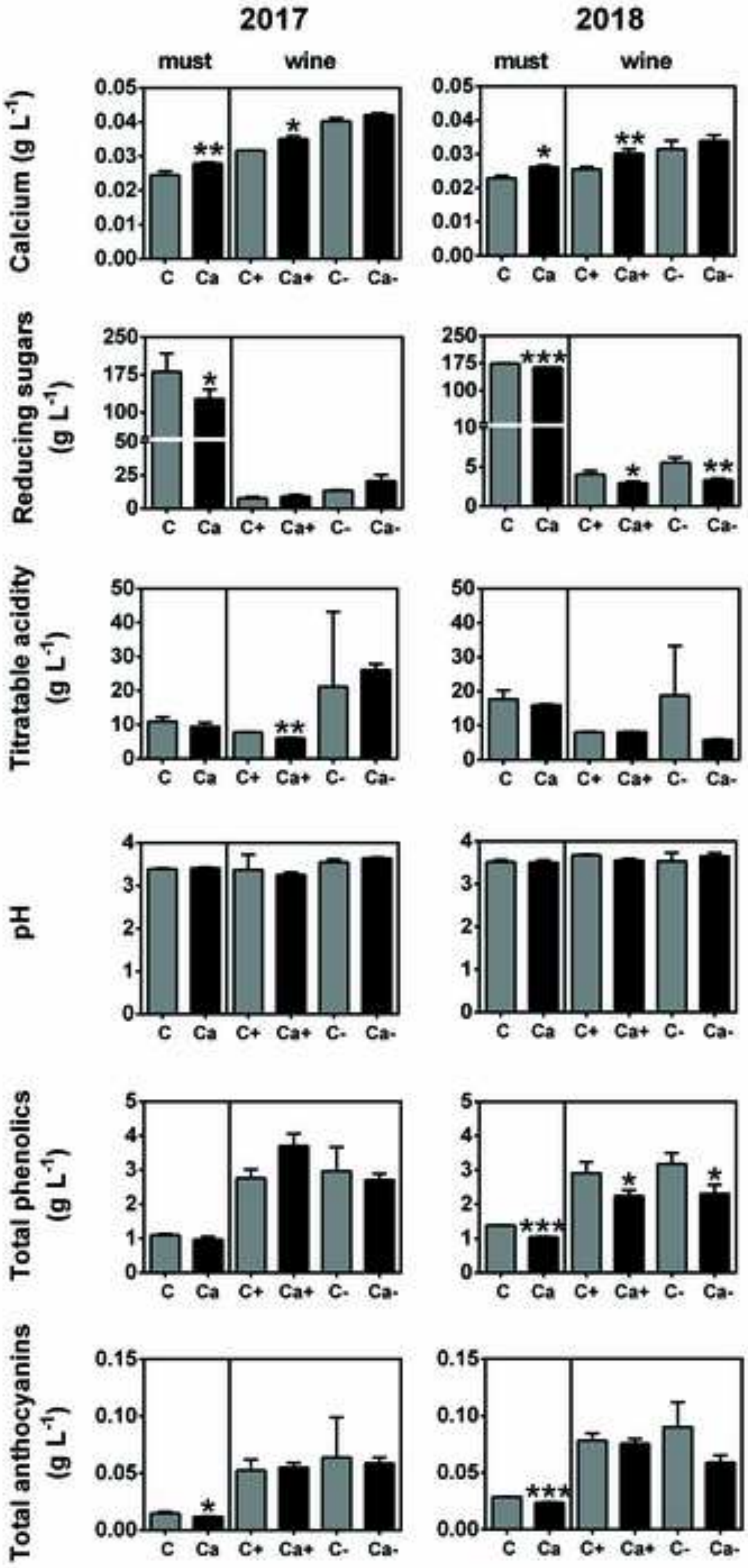

Figure 3

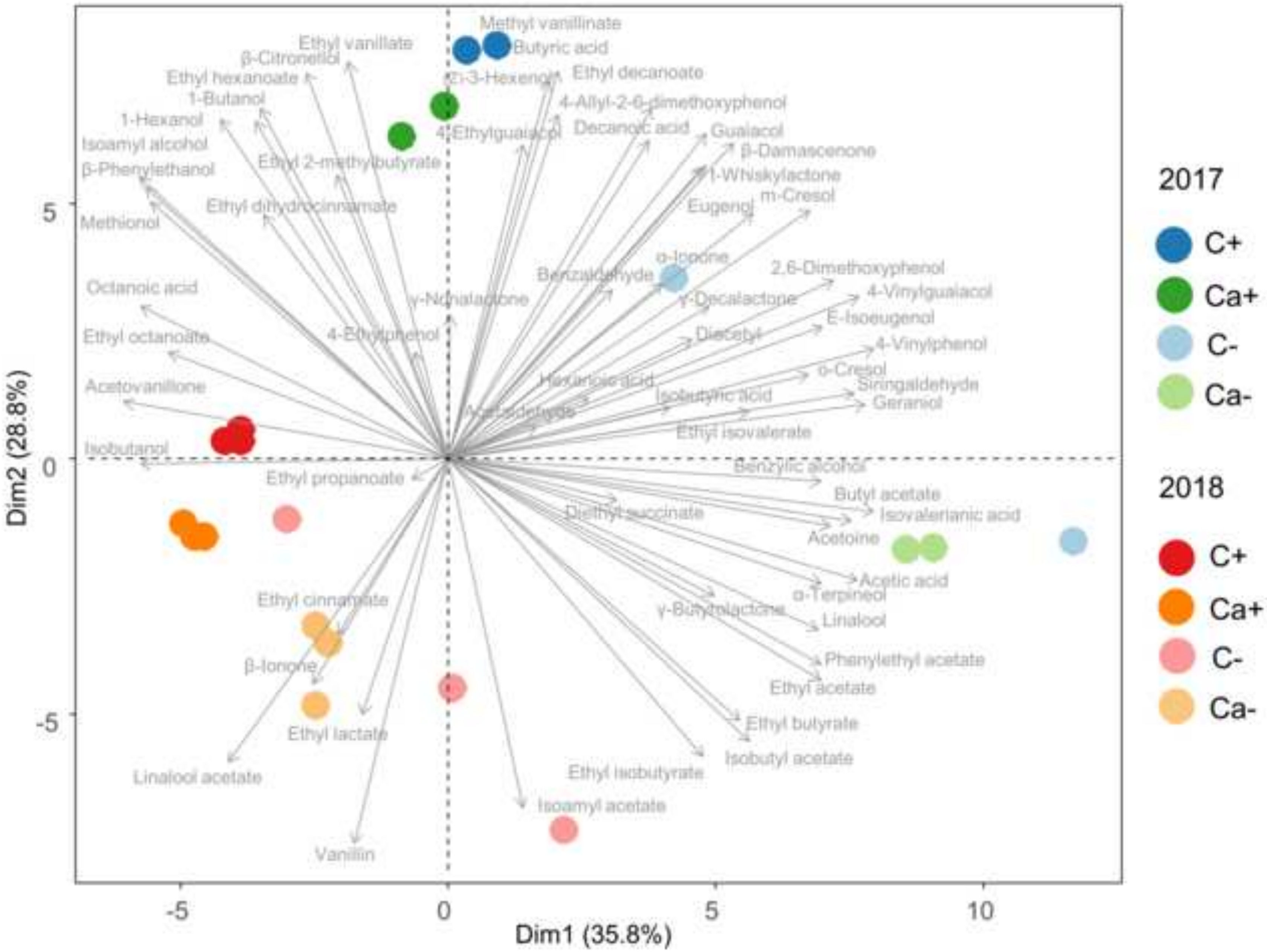
Methyl vanillinate
Ethyl vanillate
Butyric acid
β-Citronellol
Ethyl hexanoate
Z-3-Hexenol
Ethyl decanoate
1-Butanol
4-Allyl-2-6-dimethoxyphenol
1-Hexanol
Decanoic acid
Guaiacol
4-Ethylguaiacol
Isoamyl alcohol
β-Damascenone
Ethyl 2-methylbutyrate
β-Phenylethanol
t-Whiskylactone
m-Cresol
Eugenol
Ethyl dihydrocinnamate
Methionol
α-Ionone
2,6-Dimethoxyphenol
Benzaldehyde
Octanoic acid
4-Vinylguaiacol
γ-Nonalactone
γ-Decalactone
E-Isoeugenol
4-Ethylphenol
Ethyl octanoate
Diacetyl
4-Vinylphenol
o-Cresol
Hexanoic acid
Acetovanillone
Syringaldehyde
Isobutyric acid
Geraniol
Acetaldehyde
Ethyl isovalerate
Isobutanol
Benzylic alcohol
Ethyl propanoate
Butyl acetate
Isovalerianic acid
Diethyl succinate
Acetoine
Acetic acid
α-Terpineol
Ethyl cinnamate
γ-Butyrolactone
Linalool
Phenylethyl acetate
β-Ionone
Ethyl acetate
Ethyl butyrate
Ethyl lactate
Isobutyl acetate
Linalool acetate
Ethyl isobutyrate
Isoamyl acetate
Vanillin
Dim2 (28.8%)
5
0
-5
-5
0
5
10
Dim1 (35.8%)
2017
C+
Ca+
C-
Ca-
2018
C+
Ca+
C-
Ca-

Figure 4

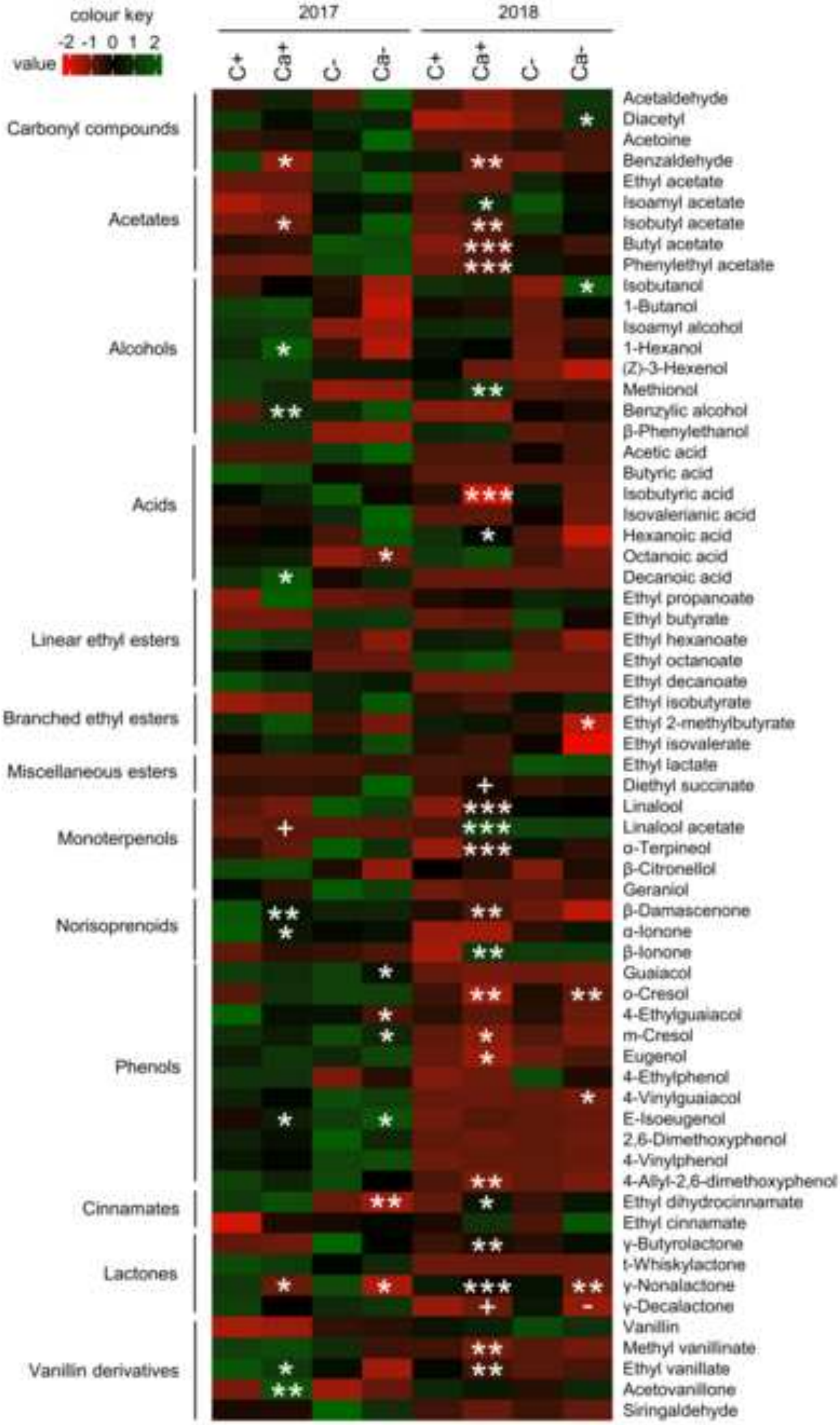
colour key
-2 -1 0 1 2
value
2017
2018
C+
Ca+
C-
Ca-
C+
Ca+
C-
Ca-
Carbonyl compounds
Acetates
Alcohols
Acids
Linear ethyl esters
Branched ethyl esters
Miscellaneous esters
Monoterpenols
Norisoprenoids
Phenols
Cinnamates
Lactones
Vanillin derivatives
Acetaldehyde
Diacetyl
Acetoine
Benzaldehyde
Ethyl acetate
Isoamyl acetate
Isobutyl acetate
Butyl acetate
Phenylethyl acetate
Isobutanol
1-Butanol
Isoamyl alcohol
1-Hexanol
(Z)-3-Hexenol
Methionol
Benzylic alcohol
β-Phenylethanol
Acetic acid
Butyric acid
Isobutyric acid
Isovalerianic acid
Hexanoic acid
Octanoic acid
Decanoic acid
Ethyl propanoate
Ethyl butyrate
Ethyl hexanoate
Ethyl octanoate
Ethyl decanoate
Ethyl isobutyrate
Ethyl 2-methylbutyrate
Ethyl isovalerate
Ethyl lactate
Diethyl succinate
Linalool
Linalool acetate
α-Terpineol
β-Citronellol
Geraniol
β-Damascenone
α-Ionone
β-Ionone
Guaiacol
o-Cresol
4-Ethylguaiacol
m-Cresol
Eugenol
4-Ethylphenol
4-Vinylguaiacol
E-Isoeugenol
2,6-Dimethoxyphenol
4-Vinylphenol
4-Allyl-2,6-dimethoxyphenol
Ethyl dihydrocinnamate
Ethyl cinnamate
γ-Butyrolactone
t-Whiskylactone
γ-Nonalactone
γ-Decalactone
Vanillin
Methyl vanillinate
Ethyl vanillate
Acetovanillone
Siringaldehyde

Figure 5

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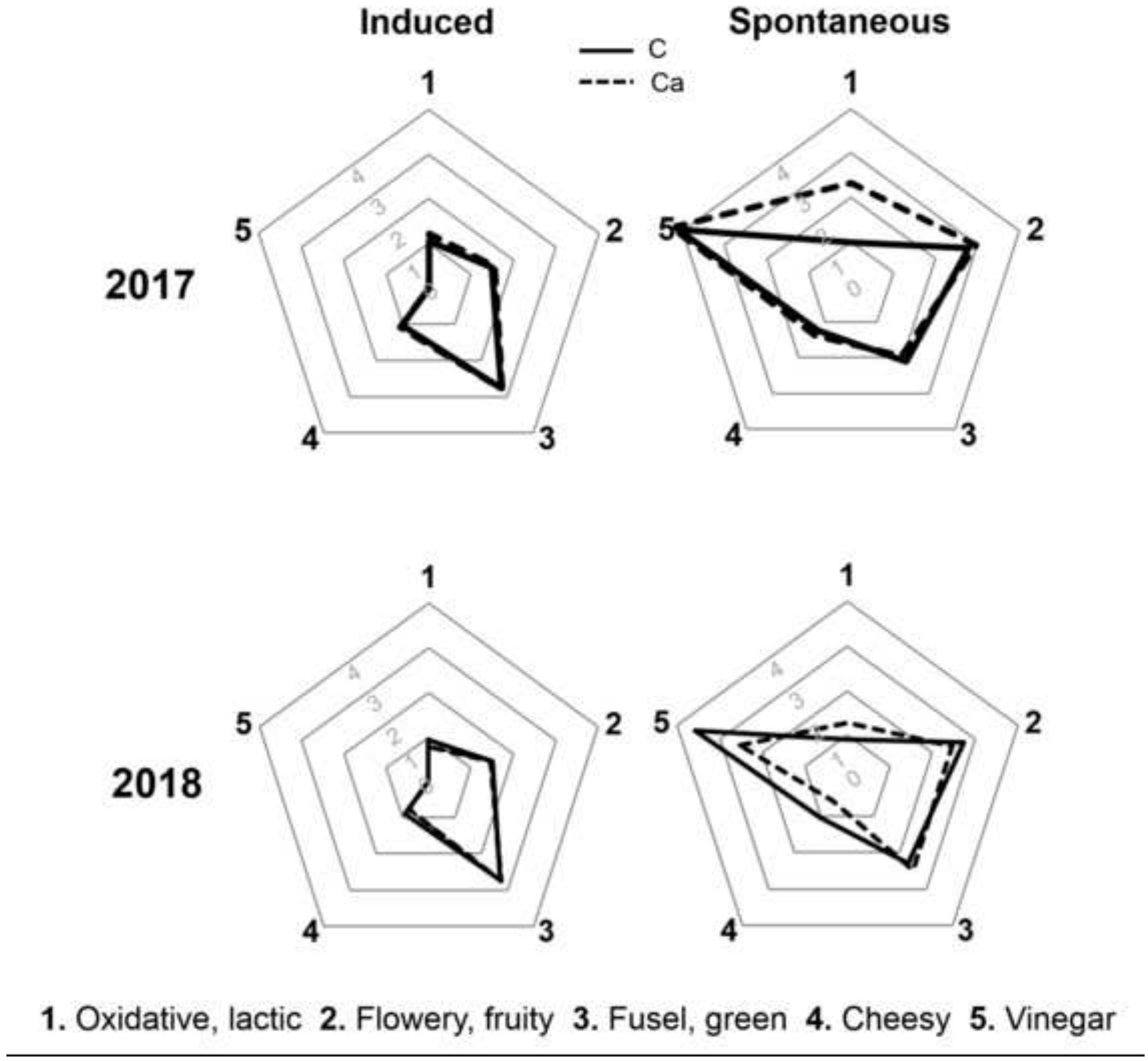
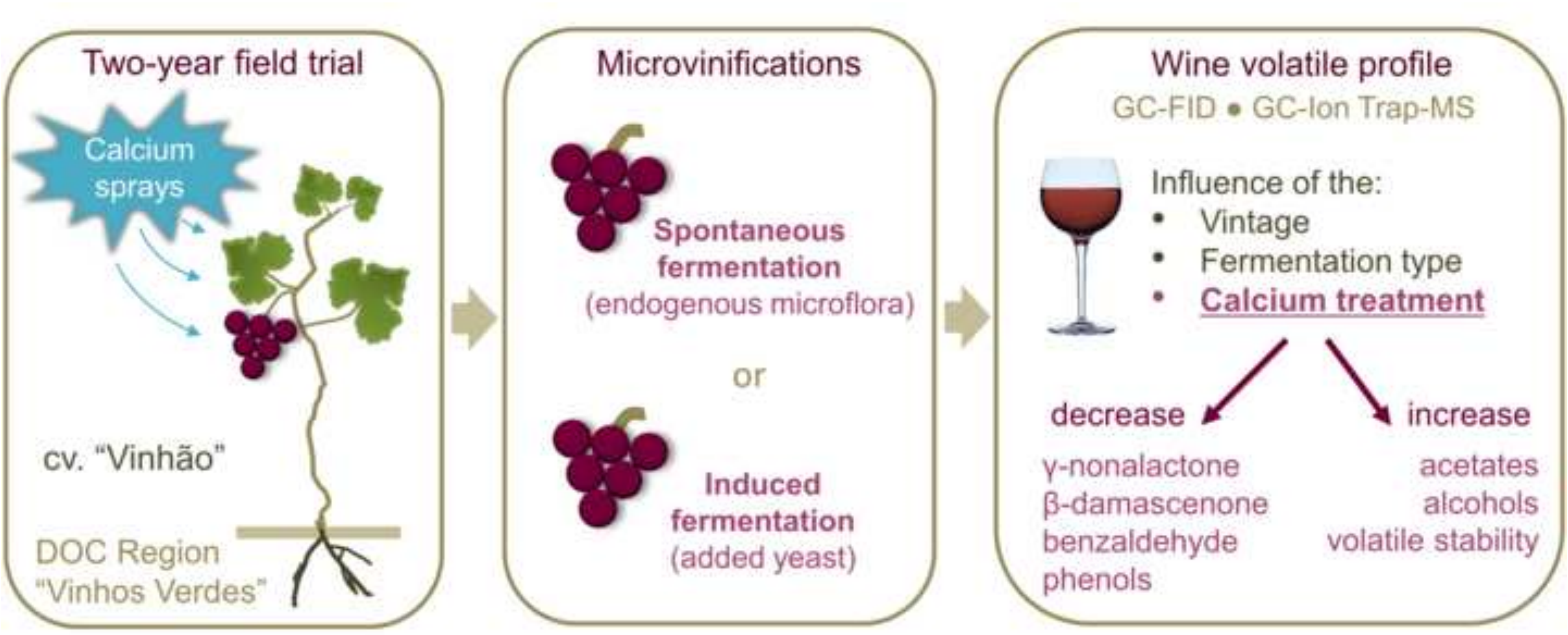


*Graphical Abstract



CRediT author statement

Viviana Martins: Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft. **Ricardo Lopez:** Investigation, Methodology, Formal analysis, Resources, Writing - review & editing. **Ana Garcia:** Investigation. **António Teixeira:** Investigation, Methodology, Formal analysis, Writing - review & editing. **Hernâni Gerós:** Conceptualization, Resources, Funding acquisition, Writing - review & editing.

***Conflict of Interest form**

Declarations of interest: none.