Impact of phenylalanine on *Hanseniaspora vineae* aroma metabolism during wine fermentation

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Abstract. *Hanseniaspora vineae* exhibits extraordinary positive oenological characteristics contributing to the aroma and texture of wines, especially by its ability to produce great concentrations of benzenoid and phenylpropanoid compounds compared with conventional *Saccharomyces* yeasts. Consequently, in practice, sequential inoculation of *H. vineae* and *Saccharomyces cerevisiae* allows to improve the aromatic quality of wines. In this work, we evaluated the impact on wine aroma produced by increasing the concentration of phenylalanine, the main amino acid precursor of phenylpropanoids and benzenoids. Fermentations were carried out using a Chardonnay grape juice containing 150 mg N/L yeast assimilable nitrogen. Fermentations were performed adding 60 mg/L of phenylalanine without any supplementary addition to the juice. Musts were inoculated sequentially using three different *H. vineae* strains isolated from Uruguayan vineyards and, after 96 h, *S. cerevisiae* was inoculated to complete the process. At the end of the fermentation, wine aromas were analysed by both gas chromatography–mass spectrometry and sensory evaluation through a panel of experts. Aromas derived from aromatic amino acids were differentially produced depending on the treatments. Sensory analysis revealed more floral character and greater aromatic complexity when compared with control fermentations without phenylalanine added. Moreover, fermentations performed in synthetic must with pure *H. vineae* revealed that even tyrosine can be used in absence of phenylalanine, and phenylalanine is not used by this yeast for the synthesis of tyrosine derivatives.

Keywords: non-*Saccharomyces*, fermentation, mixed cultures, benzenoids, phenylpropanoids, assimilable nitrogen.

1. Introduction

Wine producers are interested in new wine styles and differentiation of their products in the global market; therefore, the use of new yeast strains able to confer improved sensory properties to wine is a trend in oenology (Jolly et al., 2014; Mas et al., 2016; Pretorius, 2020). In addition, there has also been a loss of diversity in winery microbiota due to the standardization of commercial *S. cerevisiae* strains selected during the last decades for fast and complete fermentation performance, but forgetting the aroma complexity valuable for wine consumers (Carrau et al., 2020).

Several yeast species referred in general as non-*Saccharomyces* have been proposed to be inoculated with *S. cerevisiae*. Mixed cultures can be performed sequentially, adding first the non-*Saccharomyces* strain to increase some pleasant aromas and, after some hours, *S. cerevisiae* strains can be inoculated to ensure complete sugar consumption. When wines are produced from musts with a high sugar concentration, specially above 10% of potential alcohol, *S. cerevisiae* is added due to its increased tolerance to ethanol (Martin et al., 2018; Medina et al., 2013), that is accumulated in the extracellular medium throughout the process (Pina et al., 2004).

Hanseniaspora vineae is a non-*Saccharomyces* species commonly found in the early stages of wine fermentation. The aromatic properties conferred by *H. vineae* result in wines with increased fruity and flowery notes (Martin et al., 2018; Valera et al., 2021). The aromatic profile of these wines has revealed the presence of enhanced levels of 2-phenylethyl acetate and 2-phenylethanol (Valera et al., 2021; Viana et al., 2011), both derivatives of phenylalanine metabolism, and some benzenoids originated from the other two aromatic amino acids, tyrosine and tryptophan. Also, *H. vineae* is characterised by its high capacity for acetylation compared with *S. cerevisiae* and other *Hanseniaspora* species (Carrau et al., 2023) According to the whole genome sequence study of *H. vineae*, this species presents a high copy number of acetyl transferases (Fig. 1) that could be conferring it this specific property (Giorello et al., 2019).

Interestingly, grape musts with a low assimilable nitrogen concentration stimulate the production of benzenoids by *H. vineae* (Martin et al., 2016a; Carrau et al., 2023). In fact, addition of an inorganic phosphate, such as diammonium phosphate salt, causes a reduction of these aromatic compounds. However, the addition of a benzenoid precursor such as phenylalanine can increase the production of benzenoids and phenylpropanoids to obtain enhanced positive aromas (Zhang et al., 2020). Grape juices from different viticultural regions contain a full amino acid profile that will vary and depend on many aspects of soil, grape cultivar and level of maturity. Phenylalanine is the aromatic amino acid with the largest concentration range in grapes of different countries between 3 and 138 mg/L (Bell and Henschke, 2005). For example, in Australia, the grape variety Pinot Noir contains an average of 5 mg/L and Chardonnay up to 22 mg/L (Henschke and Jiranek, 1993; Treeby et al. 1998).

In *S. cerevisiae*, it has been reported that the alternative use of phenylalanine or tyrosine, influences the production of the derived correspondent higher alcohols (phenylethanol and tyrosol) (Cordente et al., 2021). However, in feeding experiments it was not possible to detect the interconversion of phenylalanine derivatives into tyrosine derivatives and vice versa (Payet et al., 2016; Robinson et al., 2021). No studies were found about these alternatives in *H. vineae* or other *Hanseniaspora* species. Also, it is noteworthy that higher alcohols can be synthetised not only from amino acids in the medium but also de novo from sugars using the synthetic and degradative routes of the Erlich pathway (Ayrapaa, 1965; Hazelwood et al., 2008).

This study investigates the impact of phenylalanine concentration in grape musts and its potential influence on aroma biosynthesis, aiming to understand its role in shaping wine quality. Mixed cultures of *H. vineae* and *S. cerevisiae* were inoculated sequentially in Chardonnay must with a moderate assimilable nitrogen content. The increase of phenylalanine concentration and its impact on the aroma profile of three different strains of *H. vineae* was compared with that in a natural grape must control. Also, the possible interconversion of phenylalanine and tyrosine aroma derivatives in *H. vineae* was analysed using feeding experiments in a synthetic grape must.

2. Materials and methods

2.1. Yeast strains

Non-*Saccharomyces* strains used in this work were *H. vineae* T02/05AF (Vineae HV205 Oenobrands, France), *H. vineae* M12/196F and *H. vineae* T02/19AF from our strains collection, all of which were isolated from Uruguayan vineyards. These strains are able to produce enhanced fruity and flowery aromas that impact in final wines quality (Carrau et al., 2023). A known commercial strain of *S. cerevisiae* was used as control ALG804 (Oenobrands, France). Yeast strains were grown on solid Yeast Peptone Dextrose medium (2% glucose, 2% peptone, 1% yeast extract, 1.5% agarose all these products were purchased from OXOID, Hampshire, United Kingdom) before preparing the pre-cultures for fermentation.

2.2. Fermentation conditions

Chardonnay musts were sterilised with dimethyldicarbonate (Velcorin®, Lanxess, Cologne, Germany) at a final concentration of 200 mg/L and stored at 4 °C for three days until it was used. Grape must was analysed for physicochemical parameters (sugars, nitrogen, pH, malic acid, tartaric acid, total acidity) by near infrared spectroscopy using a OenoFoss™ (FOSS, Hilleroed, Denmark).

Yeast Assimilable Nitrogen (YAN) content in the must was 150 mg N/L, with a sugar concentration of 149 g/L, total acidity was 3.9 g/L and 3.6 of pH.

Before starting the fermentation, the must was filtered through a sterile 0.45 µm pore diameter membrane, and every yeast strain was grown overnight in 50 mL of Chardonnay must in Erlenmeyer flasks at 25 °C, shaken at 150 rpm. These *H. vineae* pre-cultures were used to inoculate 120 mL of Chardonnay juice to obtain a total final population of 1×10^6 cells/mL according to counts performed under the microscope using a Neubauer chamber. Fermentations were carried out in 125 mL Erlenmeyer flasks with a silicone cap adapted with a Pasteur valve for CO_2 release. Fermentations were carried out in triplicate for each treatment and strain, with or without addition of 60 mg/L of phenylalanine to the Chardonnay juice. This concentration was defined according to the average reported level of phenylalanine in grape musts of different regions (Bell and Henschke, 2005). Erlenmeyer flasks were maintained in batch static conditions at 20 °C and shaken once every 24 h throughout the experiment.

S. cerevisiae overnight pre-culture was inoculated 96 h after *H. vineae* inoculation. The final concentration inoculated was 1 × 10^6 cells/mL for each fermentation. Together with the sequential addition of *Saccharomyces*, must in fermentation was supplemented with 500 mg/L of yeast extract (Oxoid, Hampshire, United Kingdom) to add available nitrogen for the yeasts avoiding the use of inorganic salts of nitrogen that reduce the production of benzenoids (Martin et al., 2016a) and 0.5 mg/L of thiamine (Sigma, St. Louis M.O., USA) to ensure yeast growth.

Fermentation kinetics were evaluated daily by weight loss of the flasks and expressed as CO_2 grams per 100 ml of medium. Fermentations were analysed until the weight remained stable for 48 h. Additionally, presence of the two strains inoculated and contamination control were evaluated at day 4 and 10 of the fermentation process by plating on Wallerstein Laboratory Nutrient (WLN) Medium (Oxoid, Hampshire, UK). Wines were also analysed for physicochemical parameters (ethanol, volatile acidity) by near infrared spectroscopy using a OenoFoss™ (FOSS, Hilleroed, Denmark).

Final wines were centrifuged at 3000 rpm for 8 min to remove yeast cells and stabilised with 50 mg/L of sodium metabisulphite (J.T.Baker, Xalostoc, Mexico). Then, 50 mL of each wine was analysed for volatile compounds and the rest of the volume was used for sensory analysis.

2.3. Sensory analysis

Sensory aroma description was performed by a panel of eight independent wine experts 3 oenologists and 5 professional sommeliers (4 women and 4 men). Wines samples (30 mL) were served at 20 °C in clear, tulip-shaped 250 mL wine glasses (ISO 3591, 1977) covered with a watch glass, and were identified with random three-digit codes. Six samples were evaluated for aroma characteristics in each session. Experts were required to rate secondary aroma terms (microbiological, spicy, nutty, floral, dry vegetable, fruit, tropical fruit, citrus fruit, liquor) using a 10-point intensity scale (Gámbaro et al., 2001). At the same time, wines were also subjected to three triangular tests for each *H. vineae* strain to differentiate the addition of phenylalanine to the must.

2.4. Chemical characterization of aroma

Wine samples (50 mL) were extracted using an Isolute ENV+ cartridge (International Sorbent Technology Ltd., Mid Glamorgan, UK) packed with 1 g of a highly cross-linked styrene–divinylbenzene (SDVB) polymer, as described previously (Boido et al., 2003). Adsorption and posterior elution with dichloromethane were performed, and after N_2 concentration, chemical analysis was carried out by gas chromatography coupled to mass spectrometry (GC-MS) in a Shimadzu-QP 2010 ULTRA mass spectrometer (Tokyo, Japan) equipped with a Stabilwax capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Restek Corporation, Bellefonte, PA, USA).

Wine aromas were identified by comparison of their Linear Retention Indices (LRI) with pure standards and values calculated by means of a C9-C30 saturated n-alkane homolog series prepared with isolated alkanes (Sigma Aldrich Inc., St Louis, MO, USA). Additionally, mass spectral fragmentation patterns were compared with those stored in commercial and our own databases (McLafferty, 2005; Mondello, 2015; Adams, 2007). Internal standards for quantification were 1-heptanol at 230 ppm and 2-octanol at 230 ppm in methanol solution.

2.5. Fermentations with isotopically labelled amino acids

Experiments designed to detect interconversion between phenylalanine and tyrosine derivatives during the fermentation in *H. vineae* were carried out in a synthetic must with a composition similar to that of grape must, as previously described (Carrau et al., 2008). Fermentation medium with 100 mg N/L of YAN: sum of nitrogen equivalents of the amino acids and ammonium salts (not including proline) was prepared. Amino acid concentrations were used based on those determined in different grape must analysis (Henschke and Jiranek, 1993). Different combinations of tyrosine and phenylalanine were tested (Table S1) and using either isotopically marked L-phenylalanine-(phenyl-$^{13}C_6$) (Merck Group, Germany) or L-tyrosine-(phenyl-$^{13}C_6$) (Merck Group, Germany). Synthetic must with unlabelled Phe and Tyr was used as control (9 mg/L Phe and 1.2 mg/L Tyr), also must with labelled Phe-$^{13}C_6$ and Tyr, must with labelled Tyr-$^{13}C_6$ and Phe, must prepared with 10 mg/L of labelled Phe-$^{13}C_6$ that did not contain Tyr and must with 11 mg/L of labelled Tyr-$^{13}C_6$ which did not contain Phe. The pH of each medium was adjusted with HCl to a final pH of 3.5. Equimolar concentrations of glucose and fructose were added to reach 200 g/L and mixed vitamins and salts were added as previously described (Carrau et al., 2008). Ergosterol (10 mg/L) was the only lipid supplement added. The must was filtered through a sterile 0.22 µm pore diameter membrane. Erlenmeyer flasks containing 75 mL of must were inoculated with 1 × 10^6 cells/mL of *H. vineae* M12/196F previously reported as the highest producer of bencenoids (Martin et al., 2016b) and fermentations were carried out in triplicate for each condition for 10 days.

Samples of 50 mL were subjected to aroma extraction by Isolute ENV+ cartridge and analysed by GC-MS as detailed above. Isotopically labelled compounds were quantified by ion fragment comparison.

2.6. Statistical analysis

The sensory and analytical results were gathered and subjected to statistical analysis. Volatile quantifications were compared by ANOVA carried out with STATISTICA 7.0 software (StatSoft, Tulsa, OK, 1984–2005). Mean rating, Tukey and Fisher significant differences were calculated.

3. Results and discussion

3.1. Effects of phenylalanine concentration in Chardonnay wines aroma

Wine fermentations were completed after 13 days, at which point less than 5 g/L of sugars was detected in final wines. The ethanol content of all samples was around 9% (v/v) and volatile acidity was less than 0.5 g of H_2SO_4/L in all treatments (Table 1).

The kinetic behaviour was similar in all the fermentations, independent of yeast strain and phenylalanine addition. The concentration of *H. vineae* colonies at day 4 was around 2 × 10^7 ufc/mL in all the treatments (Table S2), no contamination with other yeasts was detected. Also, *H. vineae* strains were recovered on WLN plates after 10 days of fermentation in a concentration of 10^6 ufc/ml although most of the colonies (5 × 10^7 ufc/ml) corresponded to *S. cerevisiae*.

Table 1. Chemical characterization of final wines. Average and standard deviation from triplicates were calculated. Different letters represent significant differences according to a Tukey test.

	Sugars (g/L)		Ethanol (% v/v)		Volatile acidity (g H_2SO_4/L)	
Strain	Control	Phe added	Control	Phe added	Control	Phe added
Hv02/05AF	4.3 ± 2.5	2.5 ± 0.8	8.9 ± 0.0	8.9 ± 0.0	0.4 ± $0.0^{a,b}$	0.3 ± $0.1^{a,b}$
Hv12/196F	3.4 ± 1.8	3.1 ± 2.0	8.9 ± 0.0	9.0 ± 0.1	0.4 ± $0.1^{a,b}$	0.3 ± 0.0^{a}
Hv02/19AF	2.3 ± 0.3	5.5 ± 2.1	9.0 ± 0.1	8.9 ± 0.0	0.5 ± 0.1^{b}	0.3 ± 0.1^{a}

Wine samples evaluated from must with added phenylalanine had a greater complexity of aromas, as described by the panel, compared to those from Chardonnay must with no phenylalanine added (Fig. 2A). Secondary descriptors attributed by the panel of experts revealed an increased floral and fruity character in musts with added phenylalanine for the three strains of *H. vineae* used (Fig. 2B).Also, the panel considered those wines with added phenylalanine more pleasant, and aromatic compared with those made with the original Chardonnay.

A triangular test showed that six of the eight wine experts were able to distinguish as different those samples with phenylalanine added to Chardonnay must fermented with *H. vineae* T02/05AF. Samples with phenylalanine added were considered different with 5% significance. In contrast, those fermented with *H. vineae* T02/19AF and M12/196F with added phenylalanine were distinguished only by three experts in triangular tests.

Regarding aroma analysis, significant differences in several compounds were observed among strains, as well as in response to the addition of phenylalanine. *H. vineae* T02/05AF presented a reduced content of 1-butanol and 3-hydroxy-2-butanol (acetoin) when phenylalanine was added to the must (Table 2). Both compounds were detected in concentrations below 150,000 µg/L which is their reported odour threshold (Fariña et al., 2015; Romano and Suzzi, 1996) so their influence in wine aroma might be not remarkable. However, they could be precursors of other aroma compounds able to contribute to wine bouquet such as diacetyl (Romano and Suzzi, 1996). Besides that, the strain *H. vineae* M12/196F showed less 3-methyl-1-butanol (isoamyl alcohol) when phenylalanine was added, but surpassed, in any case, the odour threshold for this compound in wine that is 30,000 µg/L (Fariña et al., 2015).

The highest concentration of 2-phenylethanol was detected in *H. vineae* T02/19AF when phenylalanine was added. In fact, all the strains presented a significantly higher concentration of this compound when phenylalanine was added to the must. With this addition, the odour threshold (15,000 µg/L) was reached by the three strains, in contrast to the results obtained for the original must. This is in agreement with previous studies using a lower phenylalanine concentration (Martin et al., 2016b).

Table 2. Aroma compounds (µg/L) detected by GC-MS in Chardonnay wines produced by three *H. vineae* strains and *S. cerevisiae* ALG804. Linear retention index based on a series of n-hydrocarbons reported according to their elution order on Carbowax 20 M. Average and standard deviation from triplicates were calculated. Different letters represent significant differences according to a Tukey test.

		***H. vineae* 02/05AF**		***H. vineae* 12/196F**		***H. vineae* 02/19AF**	
Compound	**LRI**	**Control**	**Phe added**	**Control**	**Phe added**	**Control**	**Phe added**
Alcohols							
2-Methyl-1-propanol	1067	7421^{a} ± 818	4814^{a} ± 315	16517^{b} ± 5012	7890^{a} ± 621	7264^{a} ± 514	7626^{a} ± 1343
1-Butanol	1128	490^{c} ± 136	280^{a,b} ± 66	539^{c} ± 216	394^{b,c} ± 135	269^{a,b} ± 58	174^{a} ± 15
3-Methyl-1-butanol	1187	124987^{a} ± 11290	141420^{a} ± 56795	333465^{b} ± 119309	178239^{a} ± 59386	137173^{a} ± 4839	252754^{a} ± 19253
3-Ethoxy-1-propanol	1389	139 ± 62	172 ± 154	253 ± 69	313 ± 76	150 ± 26	95 ± 82
2-Ethyl-1-hexanol	1453	1306 ± 132	2567 ± 707	3206 ± 687	752 ± 79	974 ± 84	4340 ± 3887
1-Hexanol	1384	220 ± 38	287 ± 134	86 ± 149	212 ± 11	155 ± 13	85 ± 73
2,3-Butanediol	1526	nd	nd	135 ± 234	194 ± 78	197 ± 40	101 ± 145
Methionol	1716	436^{b} ± 194	336^{a,b} ± 216	407^{b} ± 78	274^{a,b} ± 75	173^{a,b} ± 74	100^{a} ± 91
3-Acetoxy-1-propanol	1756	288 ± 56	664 ± 548	744 ± 227	798 ± 238	221 ± 384	705 ± 308
2-Phenylethanol	1906	6226^{a} ± 1178	20834^{b} ± 1047	13812^{a} ± 5061	34358^{b,c} ± 1176	11293^{a} ± 2709	42773^{c} ± 15373
Tyrosol	3012	241^{a} ± 50	330^{a} ± 149	375^{a,b} ± 128	557^{a,b} ± 72	736^{b,c} ± 260	1377^{c} ± 378
Tryptophol	3514	244 ± 34	233 ± 163	228 ± 269	91 ± 79	473 ± 269	785 ± 675
Esters							
Ethyl octanoate	1027	nd	nd	nd	nd	79 ± 72	98 ± 92
3-Methylbutyl acetate	1126	258^{a,b} ± 119	153^{a} ± 145	2192^{c} ± 1533	858^{a,b,c} ± 288	1525^{a,b,c} ± 805.16	1928^{b,c} ± 649
Ethyl lactate	1341	40 ± 7	5 ± 9	nd	nd	nd	28 ± 25
2-Phenylethyl acetate	1813	14773^{a} ± 1434	60775^{b} ± 9913	19444^{a} ± 6815	59546^{b} ± 14275	6152^{a} ± 221	18432^{a} ± 6009
Tyrosol acetate	2995	2255^{a} ± 670	4548^{b} ± 1857	2561^{a} ± 770	4295^{b} ± 372	2584^{a} ± 256	3604^{a,b} ± 1197

Tryptophol acetate	3405	7755^{a}	±	970	16749^{b}	±	4582	$12401^{a,b}$	±	4446	$14033^{a,b}$	±	3668	8246^{a}	±	210	$12293^{a,b}$	±	5910
Fatty acids																			
2-Methylpropanoic acid	1588	1692	±	847	2290	±	177	767^{a}	±	13	1137	±	239	nd^{a}			41^{a}	±	71
Heptanoic acid	1918	nd			nd			nd			nd			444	±	12	nd		
Octanoic acid	2070	nd			nd			nd			nd			119	±	20	248	±	43
Other compounds																			
3-Hydroxy-2-butanone	1270	744^{c}	±	187	331^{b}	±	195	61^{a}	±	106	nd^{a}			nd^{a}			nd^{a}		
3-Hydroxy-2-pentanone	1330	8389	±	2924	4150	±	1227	1867	±	604	667	±	66	275	±	12	854	±	61
Y-Butyrolactone	1620	223	±	54	119	±	38	43	±	74	58	±	61	172	±	17	61	±	53

In *H. vineae* T02/196AF and *H. vineae* T02/05AF strains, the addition of phenylalanine caused a significant increase in 2-phenylethyl acetate. *H. vineae* T02/19AF presented no significant differences in 2-phenylethyl acetate between the control and the treatment with addition of phenylalanine, although the mean value was higher with this treatment. However, the sum of 2-phenylethanol and 2-phenylethyl acetate is significantly higher with the addition of phenylalanine for this strain (Fig. 3). It is remarkable that 2-phenylethyl acetate is an aroma produced during fermentation, being part of the fermentative bouquet. In young wines these compounds increase the aromatic complexity of the final product (Martin et al., 2018). Benzyl alcohol, other derivative of phenylalanine commonly detected in *H. vineae* during the fermentation (Martin et al., 2018) was present in some of the samples but in such low concentration that did not allow their quantification. Also, tyrosol acetates produced by strains Hv02/05 and Hv12/196AF and tryptophol acetate from strain Hv02/05AF present differences in concentration depending on the concentrations of phenylalanine in the must and these differences are maintained regarding to the sum of alcohols and acetates (Fig. 3).

Enhanced acetylation capacity of *H. vineae* has been widely described (Martin et al., 2018; Valera et al., 2021; Carrau et al., 2023) and it was proposed that the high copy number of acetyl transferases in the genome of this species (Giorello et al., 2019) could be explaining the difference of acetate esters production compared with *Saccharomyces* Acetate esters present, in general, lower odor thresholds compared with their corresponding alcohols. Therefore, the presence of increased acetates levels is positive to enhance wine bouquet. Moreover, both benzenoids, 2-phenylethanol and 2-phenylethyl acetate, are associated with a floral aroma, generally described as roses although it is present in essential oils of other flowers such as carnation and orange blossom (Panda, 2010). Tryptophol and tyrosol acetates have been related with fruity and flowery descriptors in concentrations ranging 5 to 10mg/L although their odor thresholds have been not characterised yet (Valera et al., 2021).

3.2. Phenylalanine and tyrosine use by H. vineae metabolic pathways

As commented before, tyrosol acetate concentrations were significantly higher in *H. vineae* mainly with T02/05AF and *H. vineae* M12/196F strains to which phenylalanine had been added. Mean tyrosol values increased with this treatment but were not statistically significantly different to those of the control in any strain. These results suggest that *H. vineae* might be using some of the Phe to synthetize tyrosine derivatives. An increase in the content of phenylalanine derivatives was expected when this amino acid was added to the must (Martin et al., 2018; Zhang et al., 2020), but not of the tyrosine derivatives. Although according to Cordente et al. (2021) there is a relationship between higher alcohols production and the alternative use of tyrosine or phenylalanine by *S. cerevisiae*, studies using isotopically labelled phenylalanine have reported no interconversion between phenylalanine derivatives and tyrosine derivatives in *S. cerevisiae* and vice versa (Payet et al., 2016; Robinson et al., 2021).

To investigate the possible interconversion of these amino acids and their derived alcohols and acetates in *H. vineae*, fermentations were performed in synthetic must. Five conditions were used: must with Tyr-$^{13}C_6$ without Phe, must with Phe-$^{13}C_6$ without Tyr, musts with one of the two amino acids labelled and both unlabelled Tyr and Phe, which was used as a control. The other amino acids were present in the medium and, in all cases, the total YAN was adjusted to 100 mg/L to increase the production of these amino acid-derived compounds according to Martin et al. (2016a). There were no differences in aroma composition regarding phenylalanine and tyrosine derivatives (Table 3) but the labelling of these compounds presented a different pattern.

Table 3.- Concentration (µg/L) of phenylalanine and tyrosine derivatives in different synthetic grape musts

	2-Phenyletyl acetate	Benzyl alcohol	2-Phenylethanol	Tyrosol acetate	Tyrosol
Phe+Tyr	13342±3320	175±42	11551±1045	2057±507	189±46
^{13}Phe+Tyr	13163±1757	257±46	8512±1203	2246±164	157±52
^{13}Tyr+Phe	12649±2244	218±38	11022±258	1925±111	122±12
^{13}Phe	11235±1952	132±24	6927±756	1953±423	154±43
^{13}Tyr	6700±694	111±27	5510±1166	1228±58	132±26

Most of the aromas were not isotopically labelled, probably due to the time spent by the fermentation process. Other synthetic routes are involved in the synthesis of higher alcohols de novo such as the Erlich pathway (Ayrapa et al., 1965; Hazelwood et al., 2008). The compounds 2-phenylethanol, 2-phenylethyl acetate and benzyl alcohol presented up to 15% of labelling, in those wines produced with Phe-$^{13}C_6$ must without tyrosol (Fig. 4). This labelling is higher (35%) when tyrosine is also present in the medium. Conversely, low proportions of tyrosol and tyrosol acetate were labelled, and there were no significant differences to the control found. However, tyrosol and its corresponding acetate were labelled comparatively in wines produced with Tyr-$^{13}C_6$ must. But in these conditions, benzyl alcohol were slightly more labelled, and significantly different to the control must regarding the mass fragments studied when phenylalanine was not present in the medium (Table 4).

Table 4. Fragments analysed for labelling comparison in each compound.

	Molar mass (g/mol)		**Fragment used for comparison**	
	unlabelled	^{13}C	unlabelled	^{13}C
2-Phenylethyl acetate	164.20	170.20	104	110
Benzyl alcohol	108.14	114.14	108	114
2-Phenylethanol	122.16	128.16	122	128
Tyrosol acetate	180.20	186.20	120	126
Tyrosol	138.16	144.16	107	113

4. Conclusion

Some selected non-*Saccharomyces* yeasts are interesting to the winemaking industry due to the increased aroma properties that they confer to the final product. Wines elaborated with *H. vineae* present significantly higher concentrations of phenylpropanoid flavour compounds than those made with *S. cerevisiae*. Therefore, the concentration of phenylalanine in grape juice has a direct correlation with these compounds that enhance the floral and fruity characteristics that are typical within benzenoids. An increase of this amino acid at standard natural levels did not cause any negative sensory effect on the wine aroma, but significantly increase concentrations of the aromatic higher acetates phenylethanol, tyrosol and tryptophol acetates in some strains. The floral and fruity descriptors that are significantly increased in the final Chardonnay wines might be correlated to the overall increase of benzenoids and other acetates from the aromatic higher alcohols. This finding substantiates the potential alternative of using yeast lysate preparations as a nitrogen source for live cells, thereby increasing amino acids in the grape must. This approach avoids the use of ammonium salts, which has been reported to decrease the formation of benzenoids in both *Saccharomyces* and *H. vineae*. Future studies could focus on designing specific nutrients enriched in phenylalanine, aiming to enhance the aroma profile in the final wines. Moreover, although phenylalanine and its derivatives are not converted into tyrosine-derived aromas in *H. vineae*, interestingly when tyrosine is added some conversion is detected to benzyl alcohol. This fact appears not to happen in *Saccharomyces*.

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Figure captions:

Fig. 1. Biosynthesis of 2-phenylethanol and 2-phenylethyl acetate in yeasts. Genes involved and their corresponding copy numbers are specified for *H. vineae* (yellow) and *S. cerevisiae* (orange) in the different reactions.

Fig. 2. Sensory characteristics of wines elaborated from Chardonnay must (green) and must with phenylalanine added (orange) fermented with three different *H. vineae* strains. A) Secondary aroma description for each strain. B) Absolute frequency of descriptors considered fruity or floral among the terms used by the panel. Different letters represent significant differences according to a Tukey test.

Fig. 3. Sum of alcohols and their corresponding acetates derived from aromatic amino acids obtained in two different conditions. Different letters represent significant differences according to a Fisher LSD test.

Fig. 4. Labelling results. Percentage of labelled fragments in those compounds containing labelled Tyr-$^{13}C_6$ and Phe-$^{13}C_6$ compared with the corresponding proportion of the same fragment sizes in control synthetic wine without labelling. Bars represent standard deviation.

Table S1. Concentration of amino acids and inorganic ammonium source in the synthetic must media.

Amino acid	**Phe+Tyr** (mg/L)	**^{13}Phe+Tyr** (mg/L)	**^{13}Tyr+Phe** (mg/L)	**^{13}Phe** (mg/L)	**^{13}Tyr** (mg/L)
PRO	60.3	60.3	60.3	60.3	60.3
ALA	12.1	12.1	12.1	12.1	12.1
ARG	90.4	90.4	90.4	90.4	90.4
ASN	18.1	18.1	18.1	18.1	18.1
ASP	42.2	42.2	42.2	42.2	42.2
GLN	24.1	24.1	24.1	24.1	24.1
GLU	60.3	60.3	60.3	60.3	60.3
GLY	6.0	6.0	6.0	6.0	6.0
HIS	18.1	18.1	18.1	18.1	18.1
ILE	24.1	24.1	24.1	24.1	24.1
LEU	36.2	36.2	36.2	36.2	36.2
LYS	30.1	30.1	30.1	30.1	30.1
MET	18.1	18.1	18.1	18.1	18.1
PHE	9.0	0	9.0	0	0
PHE-$^{13}C_6$	0	9.0	0	10	0
SER	48.2	48.2	48.2	48.2	48.2
THR	42.2	42.2	42.2	42.2	42.2
TRP	12.1	12.1	12.1	12.1	12.1
TYR	1.2	1.2	0	0	0
TYR-$^{13}C_6$	0	0	1.2	0	11
VAL	24.1	24.1	24.1	24.1	24.1
Inorganic amonium source	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
DAP	60.3	60.3	60.3	60.3	60.3

Table S2. Colony forming units (cfu/mL) obtained by WLN plating of *S.cerevisiae* and *H. vineae* in each treatment throughout the fermentation (day 4 and day10). Average and standard deviation from triplicates were calculated.

	Hv02/05AF			Hv12/196F			Hv02/19AF		
	Day 4	Day 10		Day 4	Day 10		Day 4	Day 10	
	Hv	Hv	Sc	Hv	Hv	Sc	Hv	Hv	Sc
Control	$2x10^7±5x10^6$	$1x10^6±4x10^5$	$5x10^7±4x10^7$	$2x10^7±1x10^7$	$2x10^6±8x10^5$	$5x10^7±3x10^7$	$2x10^7±1x10^7$	$1x10^6±2x10^5$	$5x10^7±2x10^7$
Phe added	$2x10^7±4x10^6$	$2x10^6±2x10^5$	$6x10^7±2x10^7$	$2x10^7±4x10^6$	$1x10^6±4x10^5$	$6x10^7±3x10^7$	$2x10^7±1x10^7$	$1x10^6±7x10^5$	$5x10^7±3x10^7$

Fig1.tif

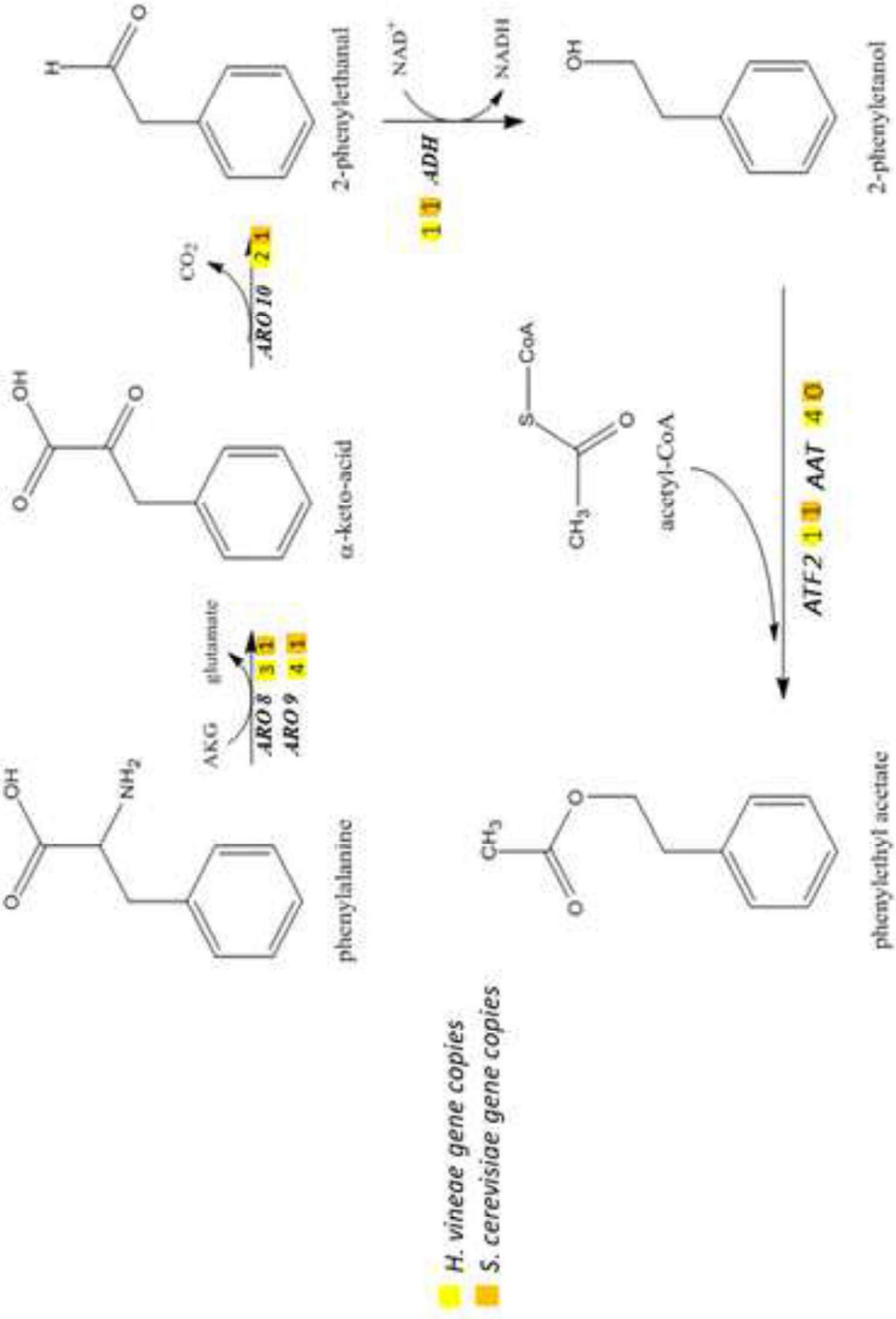
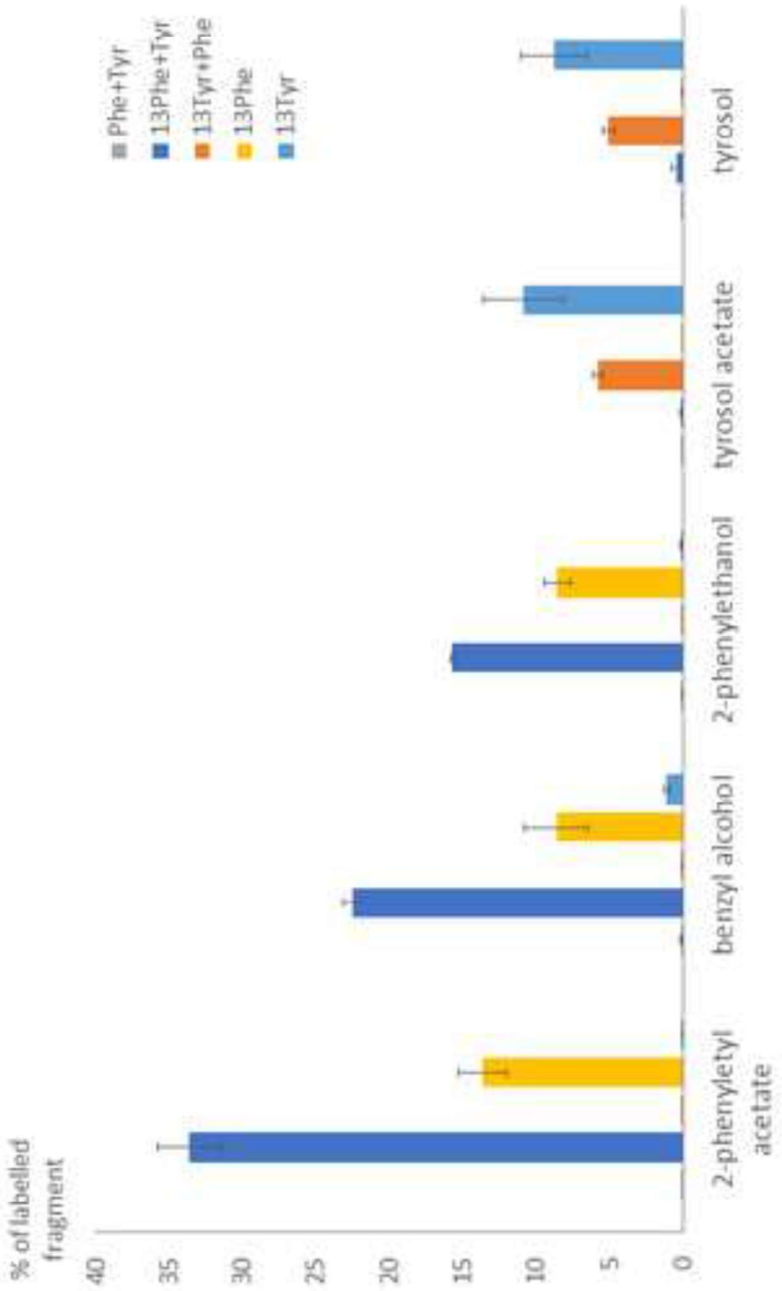


Fig 1

Fig 4

Fig 4.tif



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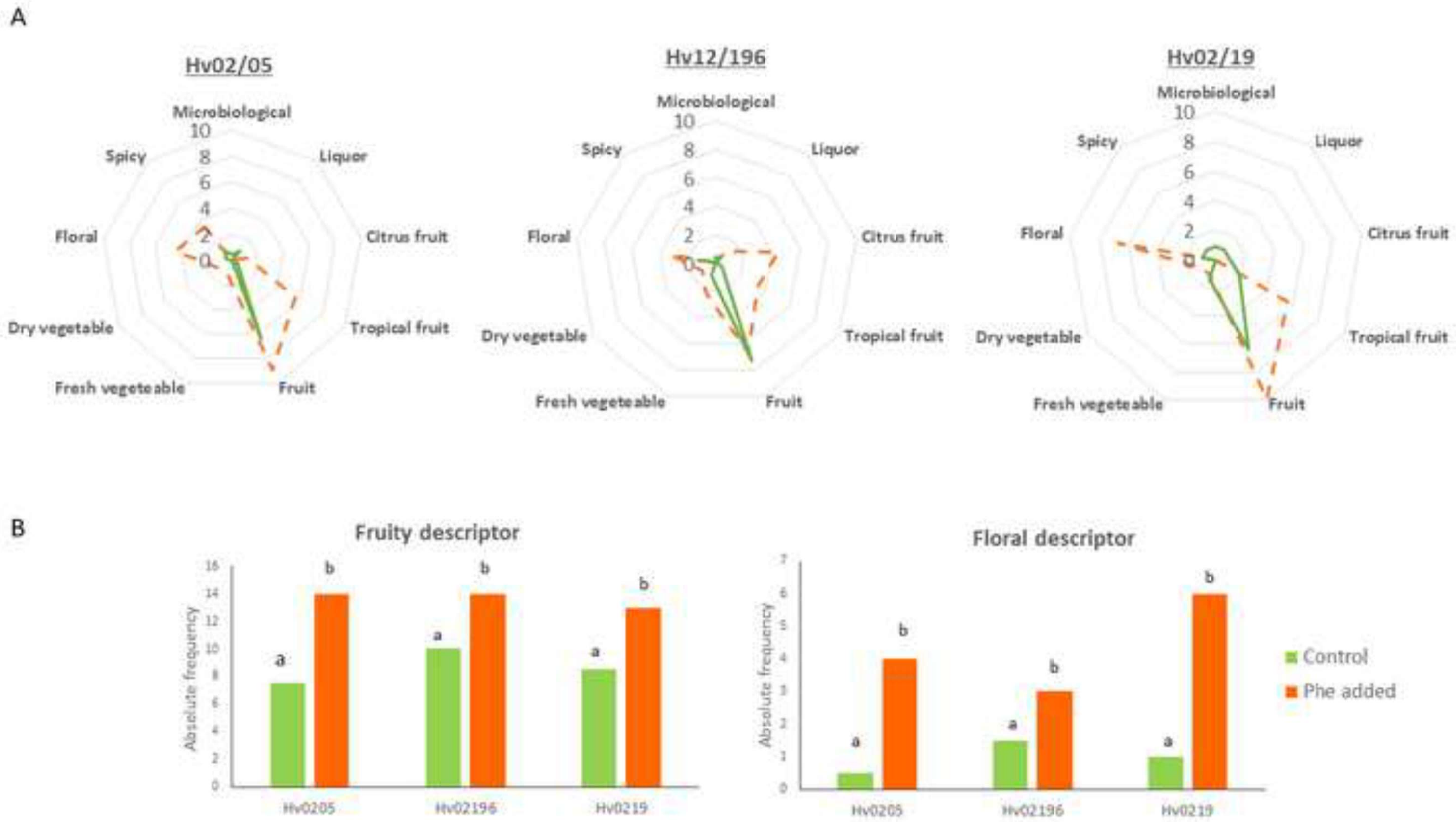
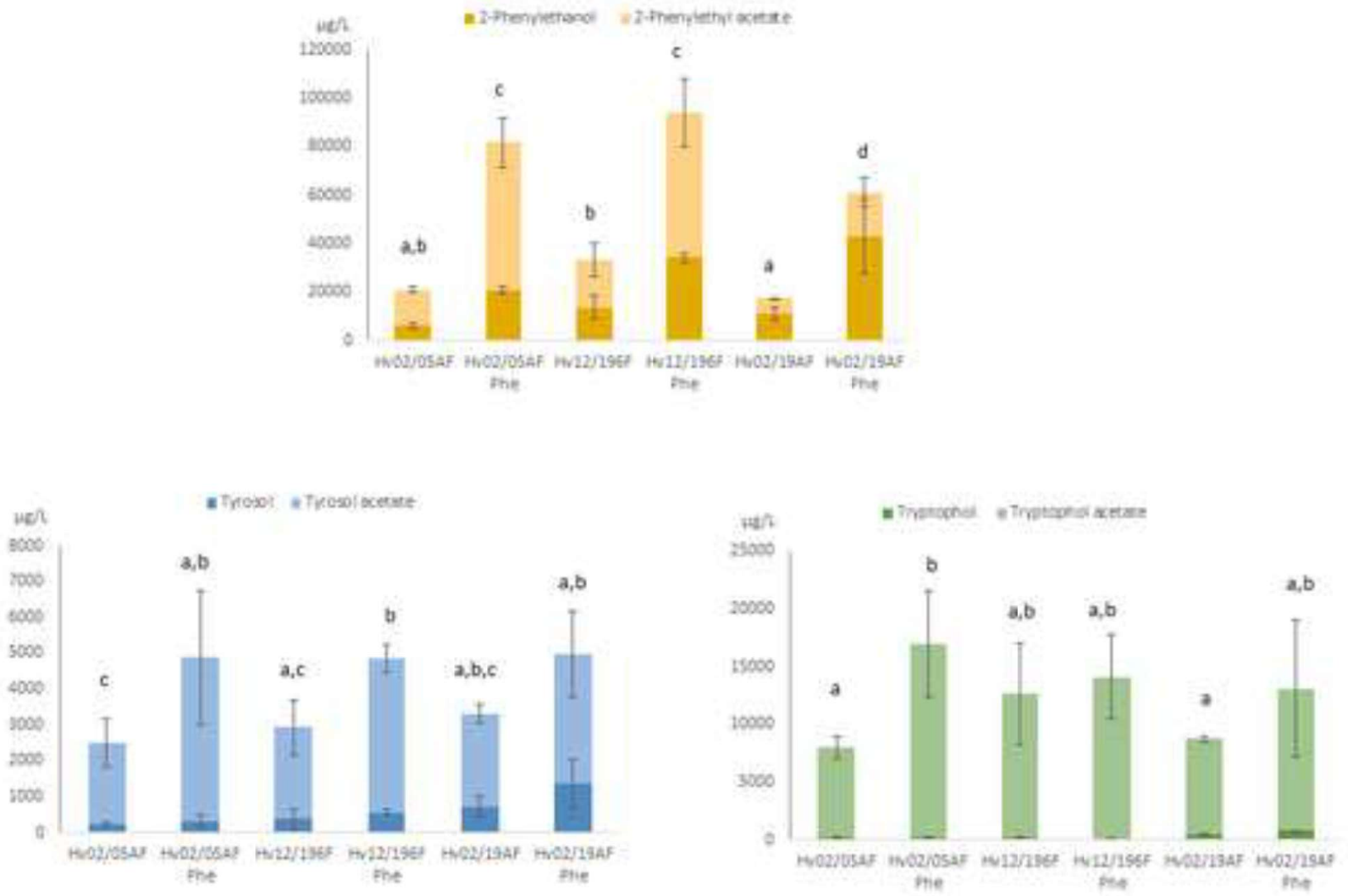

A
Hv02/05
Hv12/196
Hv02/19
Microbiological
Liquor
Citrus fruit
Tropical fruit
Fruit
Fresh vegeteable
Dry vegetable
Floral
Spicy
10
8
6
4
2
0
B
Fruity descriptor
Floral descriptor
Absolute frequency
0
2
4
6
8
10
12
14
16
1
3
5
7
a
b
Hv0205
Hv02196
Hv0219
Control
Phe added

Fig 3

Fig 3.tif



Declaration of competing interest

All authors of the manuscript declare no conflict of interest.