

“One-shot” analysis of wine parameters in non-*Saccharomyces* large-scale alcohol reduction processes with one- and two-dimensional nuclear magnetic resonance

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Abstract. Facing climate change in wine industry comprises the implementation of strategies, such as to reduce alcohol in wines, promoted by abnormal increment of sugar levels in wine grapes. The present work discusses the first industrial-scale use of specific yeast strains able to produce wine with reduced alcoholic concentration. Reduction of alcohol content and quantification of key metabolites associated to oenological practice and/or quality were simultaneously measured in a “one-shot” way with proton Nuclear Magnetic Resonance Spectroscopy. Novel relevant metabolites were revealed with the use of a two-dimensional ¹H-¹³C HSQC multipresat correlation spectroscopy, whereas a detailed methodological NMR description is stressed, towards revealing novel resonances within the NMR signature. The use of multitask analytical methods to simultaneously describe alcohol reduction and NMR targeting, completes the portfolio of NMR solutions recently proposed to the World Organisation of Vine and Wine for as well quantify aging and varieties.

1. Introduction

Current oenological practices are focused in searching strategies for reducing alcohol content in wine, as climate changes has provoked an important increase of sugar amounts in must. With the simplest *Saccharomyces* yeast strains anaerobic conditions, fermentation of grapes with increased sugar content will produce wine with high alcohol by volume percentage (%ABV) and thus a product with penalized mouth-feel and/or taint and/or flavour [1,2]. In consequence, an important reduce of market consumption or severe tax government policies, could be expected. Amongst the broad spectrum of viticultural, physical or microbiological processes suggested for alcohol reduction, the concatenated use of non-*Saccharomyces* yeast strains to first aerobically sequester excess of sugar content by respiration, followed by the use of anaerobic *Saccharomyces* strains for final fermentation, has gained attention in the last years [3–5]. Methods comprising the use of several non-*Saccharomyces* yeast strains for assimilating must sugar to produce wine with reduced alcohol content and appropriate organoleptic properties has been extensively reported at lab- medium- and pilot-scales [6–8], but scarcely reported at industrial scale [9]. Finally, analysis of relevant wine parameters of reduced alcohol wines is carried out currently by a set of different enzymatic

colorimetric trials and chromatographic schemes. Basic wine analysis comprises the quantification of primary metabolites such as ethanol, acetic acid, D-glucose, D-fructose, glycerol, lactic acid, malic acid and/or isovaleric acid -each with a particular enzymatic test kit-, that present some disadvantages such as time consuming; they present certain complexity in terms of sample preparation and chemical manipulations, they are costly and require some level of analytical expertise.

The present work proposes a “one-shot” evaluation of basic dealcoholized wine parameters with one- and two-dimensional Nuclear Magnetic Resonance (NMR) fingerprint and profiling [10–12]. Mono-varietal Mexican Cabernet Sauvignon wines with reduced alcohol content, were prepared in a large-scale regime (3.0 Ton grape, final volume of c.a. 2900 L) with non-*Saccharomyces* *Candida zeylina* strain, in co-inoculation with *S. cerevisiae* for final fermentation. Said reducing alcohol content procedure, was compared with at least two alcoholic fermentations at the same scale, carried out respectively with *S. bayanus* or *S. cerevisiae* (CM) strains. Ethanol reduction with simultaneous profiling and quantification of key metabolites such as acetic acid, malic acid, sorbic acid, fumaric acid and shikimic acid were carried out with a recent OIV procedure [10]. Finally, the use of 2D-NMR acquisition schemes are proposed in order to increase

Table 1. Degrees Brix, acidity (pH) and total acidity (T.A.) of selected Cabernet Sauvignon grapes from MX and CM, used for large scale fermentations.

Site	Brix (°)	pH	T.A. (g/L)
MX	23.0	3.68	5.53
CM	23.8	3.65	6.4

Table 2. Microbiological and oenological characteristics of the Non-*Saccharomyces Candida zemplinina* strain, used in sequential inoculation, for reduction of wine alcohol content in the present large-scale trial.

Fermentation temperature	15–26 °C
Fermentation velocity	Slow
Alcohol tolerance	≤10% v/v
SO ₂ resistance	<20 ppm free
Volatile acidity (VA) production	20–30% less than <i>S. cerevisiae</i>
Glycerol production	Very high
H ₂ S production	Very low
SO ₂ production	Very low
Nitrogen need	Not Specified

the number of observables for quantifying efficiency of alcohol reduction, with a set of additional parameters so far not explored.

2. Materials and methods

2.1. Large scale fermentations

Large scale production of wine with reduced Alcohol content (%alc. v/v) was carried out in two different regions: Baja California, Mexico (Monte Xanic, hereinafter called as **MX**) and Parras, Coahuila, Mexico (Casa Madero, hereinafter called as **CM**), using the variety Cabernet Sauvignon, obtaining yields of c.a. 2950 L of wine, using as raw material 3.0 Ton Cabernet Sauvignon grapes. Prior to vinification, used grapes presented the following chemical properties, resumed in Table 1.

Large-scale alcohol reduction were done with the following non-conventional strains (Tables 2–4), whereas results will be compared against a large-scale control process (*Saccharomyces bayanus*, Strain 10N45).

Large scale fermentations herein developed, can be resumed as:

- Standard *Saccharomyces bayanus*, Strain 10N45.
- Co-inoculation Non-*Saccharomyces Candida zemplinina* (Enartis Ferm) + *S. cerevisiae* 10N45
- Inoculation w/*Saccharomyces Bayanus ex uvarum* ES-U42 (Enartis Ferm)
- Inoculation w/*Saccharomyces cerevisiae* (IONYS).

2.2. Nuclear magnetic resonance (NMR) spectroscopy

Sample preparation for NMR studies comprised the addition of 100 µL of a mixture of D₂O and chemical-shift reference sodium 3-(trimethylsilyl)-propionate-2, 2, 3, 3-d₄ (TSP), phosphonate buffer KH₂PO₄ 0.1% and 2%

Table 3. Microbiological and oenological characteristics of the *Saccharomyces Bayanus ex uvarum* ES-U42 strain, used for reduction of wine alcohol content in the present large-scale trial.

Fermentation temperature	15–20 °C (<25 °C for red wines)
Fermentation velocity	Moderate at low temperatures
Alcohol tolerance	≤16% v/v
SO ₂ resistance	High
Volatile acidity (VA) production	Very low (<0.25 g/L)
Glycerol production	Very high
H ₂ S production	Medium
SO ₂ production	Low
Nitrogen need	150 g/hL of YAN
Oxygen need	Medium-low

Table 4. Microbiological and oenological characteristics of the *Saccharomyces cerevisiae* IONYS strain, used for reduction of wine alcohol content in the present large-scale trial.

Fermentation temperature	25–28 °C
Fermentation velocity	Slow and constant
Alcohol tolerance	15.5% v/v
SO ₂ resistance	Not Specified
Volatile acidity (VA) production	Very low
Glycerol production	Very high (up to 15 g/L)
H ₂ S production	Not Specified
SO ₂ production	Low
Nitrogen need	Very high
Oxygen need	NE

NaN₃ to 900 µL of wine sample, whereas pH was finally adjusted to a value of 3.9 for all samples. Samples were finally versed in standard 5 mm NMR tubes.

All spectra were recorded on a Bruker 600 AVANCE III HD equipped with a 5 mm ¹H/D TXI probehead with z-gradient. The following set of NMR experiments were conducted at a temperature of 298 K, stabilizing the temperature with a Bruker VCU flow unit:

- Quantitative one-dimensional proton nuclear magnetic resonance spectra (q-1D-¹H-NMR) used to measure the reduction of alcoholic content in wines were carried out by recording a total of 64 transients, that were collected into 28,844 complex data points, with a spectral width of 20 ppm (12019 Hz), an optimized recovery delay of 5.6 seconds to obtain quantitative signal integration and acquisition times of 1.2 s, produced experimental times of 7 minutes per experiment. No apodization function was used prior to Fourier Transformation.
- 1D-¹H experiments with water-to-ethanol solvent presaturation were carried out as elsewhere reported [12].
- Two-dimensional ¹H-¹³C Heteronuclear Single Quantum Coherence experiment [13] with a home-made water-to-ethanol multipresaturation scheme [12], prior to INEPT polarization transfer [14] were recorded by acquiring 7810 × 256 points with 32

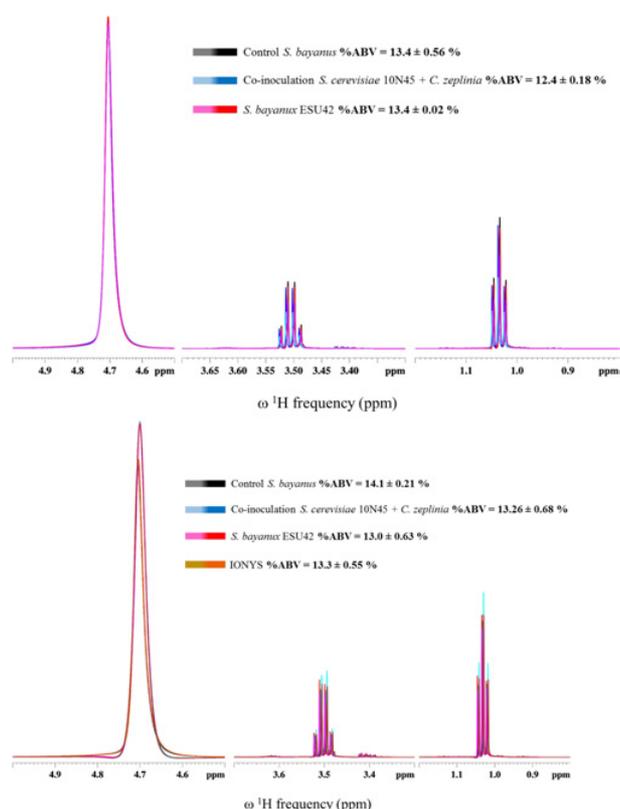


Figure 1. Quantitative one-dimensional proton nuclear magnetic resonance (q-1D-¹H NMR) spectra of large-scale fermented wine samples of MX (top) and CM (bottom) obtained with: standard *S. bayanus* as control (black); Non-*Saccharomyces Candida zeylinia* + *S. cerevisiae* 10N45 (blue); *Saccharomyces Bayanus ex uvarum* ES-U42 (pink) and *Saccharomyces cerevisiae* (IONYS, brown).

scans per transient. Heteronuclear ¹H-¹³C spectral widths were setup respectively at 20 (¹H), 180 (¹³C) ppm. With acquisition times for the direct F2 dimension of 325 ms and a recovery delay of 1 s. produced experimental times of 3 h 18min per HSQC experiment.

3. Results and discussion

Q-1D-¹H-NMR spectra of full set of samples (Fig. 1) were obtained in order to calculate %alc. (v/v) of each fermented product, by means of signal integration of methyl ($\delta = 1.026$ ppm, triplet)-to-methylene ($\delta = 3.497$ ppm, quartet) signals, with respect water signal ($\delta = 4.7$ ppm, singlet). Direct percentage of integrated methylene signal ($\delta = 3.497$ ppm, quartet), with respect integrated H₂O signal ($\delta = 4.7$ ppm, singlet), immediately provides the alcohol percentage of fermented samples, as methylene and water signals present both the same number of observed spins ($I = 2$). Alcohol reduction of each large scale trial, computed by q-1D-¹H-NMR was of around 1% in all cases (see Fig. 3), as expected and verified by cross-check methods. In the other hand, multipresat 1D-¹H-NMR experiments will serve to detect and quantify appropriate metabolites such as acetate (1.9–1.92 ppm), malate (2.45–2.48 ppm), sorbate (5.84–5.87 ppm), fumaric (6.65–6.61 ppm) and shikimic (6.68–6.69 ppm)

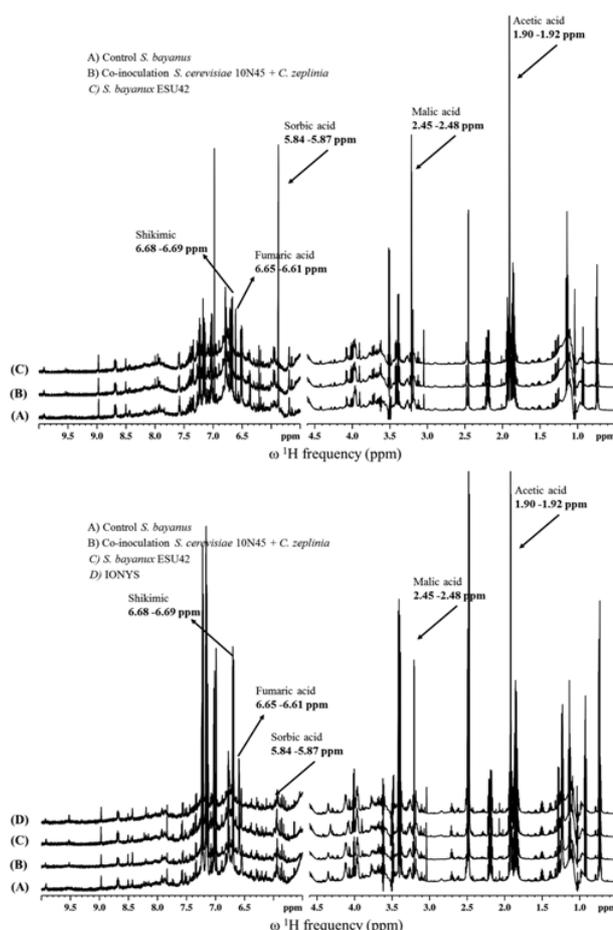
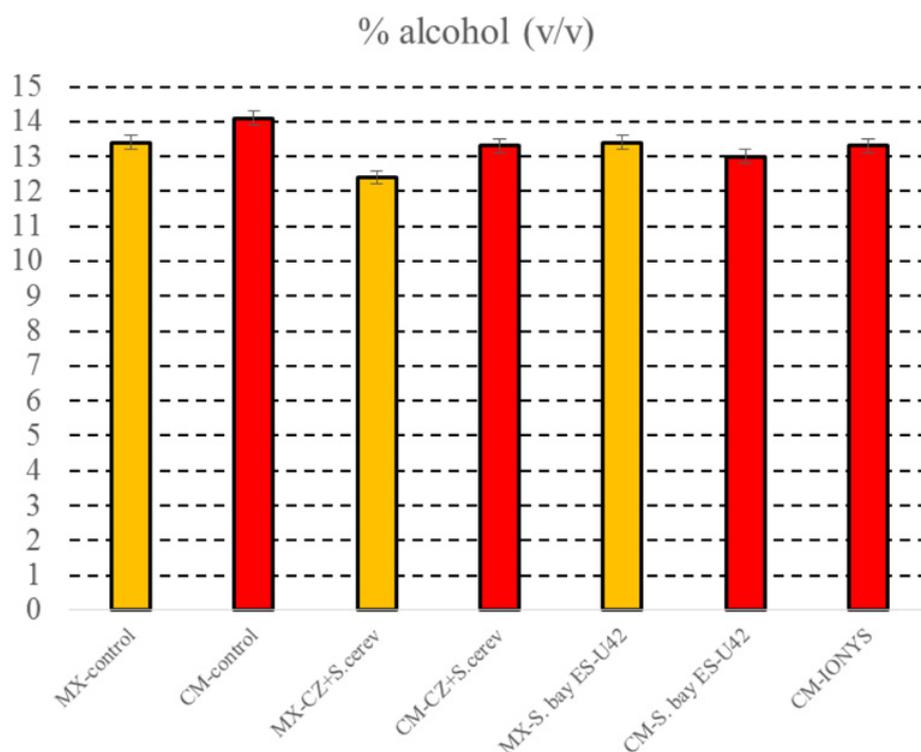


Figure 2. Stacked proton one-dimensional Nuclear Magnetic Resonance spectra (¹H-NMR) with improved water-to-ethanol multipresat. Scheme [12] of Mexican monovarietal Cabernet Sauvignon wines, with different large-scale fermentations (A to D fermentation processes, see Materials and Methods, see as well Figure legends). Top: stacked 1D-¹H NMR from Monte Xanic; Bottom: stacked 1D-¹H NMR from Casa Madero. NMR signature of Mexican Cabernet Sauvignon shows the signal assignment of relevant metabolites prone to be quantified by the PULCON/NMR-OIV method [10, 15].

by the Pulse Length based CONcentration (PULCON) method [15].

Detection and quantification of acetate, malate, sorbate, fumaric and shikimic moieties from the NMR signature is related to the easiness for signal selection of isolated-intense resonances from above mentioned metabolites, even exposed within the q-1D-¹H-NMR spectra, without multipresat scheme (Fig. 1), but at low signal-to-noise ratio. However, novel schemes involving solvent elimination and addition of a second dimension for dispersing encumbered resonances from the 1D-¹H fingerprint will shed light in novel resonances or exposed regions, potentially ready to be quantified with PULCON/NMR procedures. For that it is presented the use of a two-dimensional ¹H-¹³C Heteronuclear Single Quantum Coherence (HSQC) experiments, applying a home-made water-to-ethanol multipresaturation scheme [12] for assigning novel resonances involving relevant metabolites associated to oenological practices or quality control parameters.



Alcohol reduction with respect controls

Site	Non- <i>Saccharomyces</i> <i>Candida zemplinina</i> + 10N45 <i>S. cerevisiae</i>	<i>Saccharomyces</i> <i>Bayanus ex</i> <i>uvarum</i> ES-U42	<i>Saccharomyces</i> <i>cerevisiae</i> IONYS
Monte Xanic	1.0 %	0.0%	–
Casa Madero	0.8%	1.1%	0.8%

	%Alc. (v/v)	pH	TA (g/L)	VA (g/L)	free SO ₂ (mg/L)
MX-control	13.4	4.03	3.94	0.34	22
CM-control	14.1	3.7	5.5	0.49	25
MX-CZ+S.cerev	12.4	4	4.06	0.42	23
CM-CZ+S.cerev	13.3	3.65	5.6	0.49	32
MX-S. bay ES-U42	13.4	4.02	4.73	0.53	27
CM-S. bay ES-U42	13	3.81	5.4	0.56	28
CM-IONYS	13.3	3.64	5.8	0.54	33

Figure 3. Alcohol reduction (with respect standard *S.bayanus* controls), wines' final pH, Total Acidity (T.A.), Volatile Acidity (V.A.) and free sulphites of large-scale fermentation trials.

Set of novel assigned resonances with the use of two-dimensional ¹H-¹³C HSQC are: tyrosine, phenylalanine, fructose, glycerol, glutamine, lactic acid and quercetin. Assignments were done by confirming a ¹H-resonance with its correlation to a specific ¹³C chemical shift. It is worth noting to highlight as example that quercetin proton resonance (6.68 to 6.77 ppm) present a shift as a function of large-scale fermentation scheme. Despite the last, the easiness to identify quercetin relies on the unambiguous

carbon correlations ($\delta^{13}\text{C} = 116$ and 137 ppm) with shifted protons, proving though the advantages of using additional NMR dimensions.

Quantification with PULCON/NMR method consists in referring the signal integral of an unambiguously assigned resonance (or set of them), with respect signal intensity and line width of a known external reference, prepared at chemical conditions close to the sample of unknown concentration. Set method has been proposed

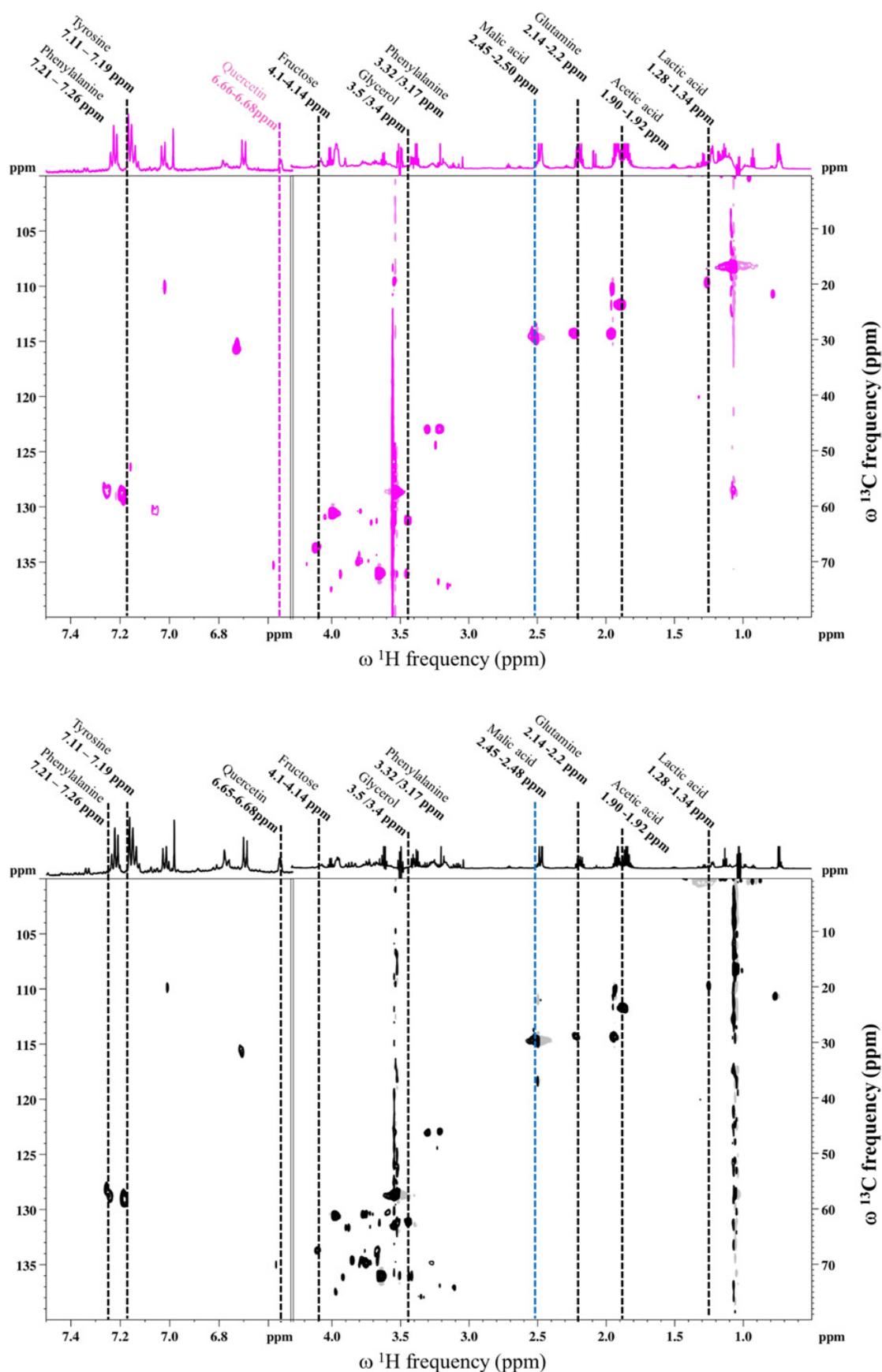


Figure 4. Two-dimensional ^1H - ^{13}C Heteronuclear Single Quantum Coherence (HSQC) experiments, applying a home-made water-to-ethanol multipresaturation scheme [12] used to detect novel metabolites with the addition of a ^{13}C NMR dimension. Magenta: ^1H - ^{13}C HSQC of large-scaled fermented wines with *Saccharomyces Bayanus ex uvarum* ES-U42 strain; Black: ^1H - ^{13}C HSQC of large-scaled fermented wines with standard *Saccharomyces Bayanus* control; Blue: ^1H - ^{13}C HSQC of large-scaled fermented wines with Co-inoculation of Non-*Saccharomyces Candida zemplinina* (Enartis Ferm) with *S. cerevisiae* 10N45 strain. Bottom: ^1H - ^{13}C HSQC pulse program, with the addition of a multipresat module, prior to the INEPT polarization transfer module.

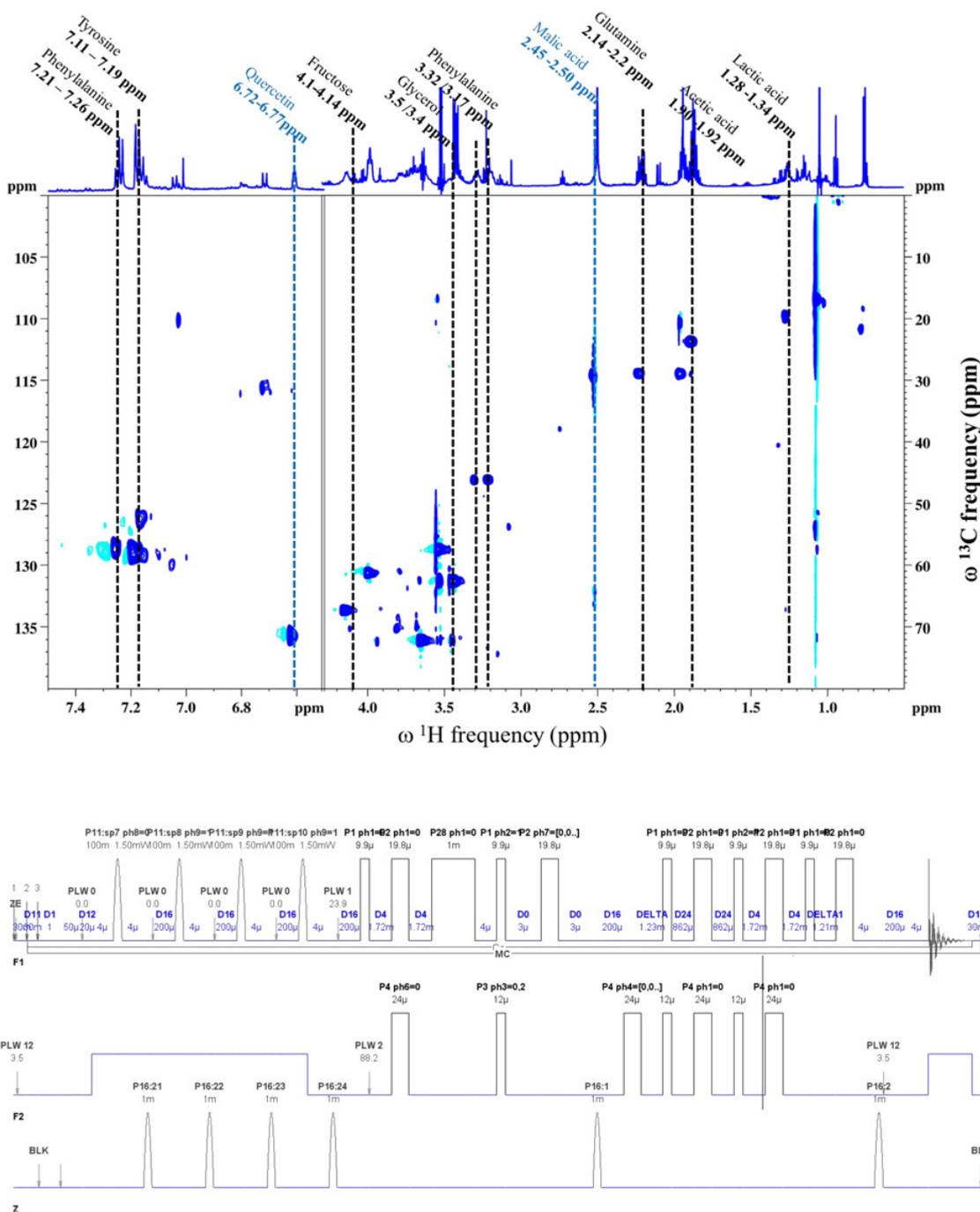


Figure 4. Continued.

for proteins [15] and recently to wine targeting [10], whereas for the later study, 20 mg/L of citric acid was used as external reference [10]. For the present study a set of external references were used for cross-check validation: 650 mg/L of acetic acid; 1436 mg/L of malic acid; 152 mg/L of sorbic acid; 49 mg/L of fumaric acid and 51 mg/L of shikimic acid. All external reference solutions were prepared at similar pH and buffer conditions with respect wine samples (see Materials and Methods). PULCON/NMR targeted analysis is stressed in Fig. 5 and Table 5.

From PULCON/NMR quantifications the following observations arise: At selected large-scale fermentation conditions, control and dealcoholized products present normal mean values of both acetic and shikimic acids. Normal mean values of malic acid were detected for both control and dealcoholized products of only Monte Xanic stocks. Acceptable mean values of fumaric acid were only obtained for dealcoholized wines from Casa Madero, fermented with co-inoculations *S. cerevisiae* 10N45 + *C. zeylinia*. Controls and dealcoholized wines in all cases present low values of sorbic acid (8–15 mg/L for CM

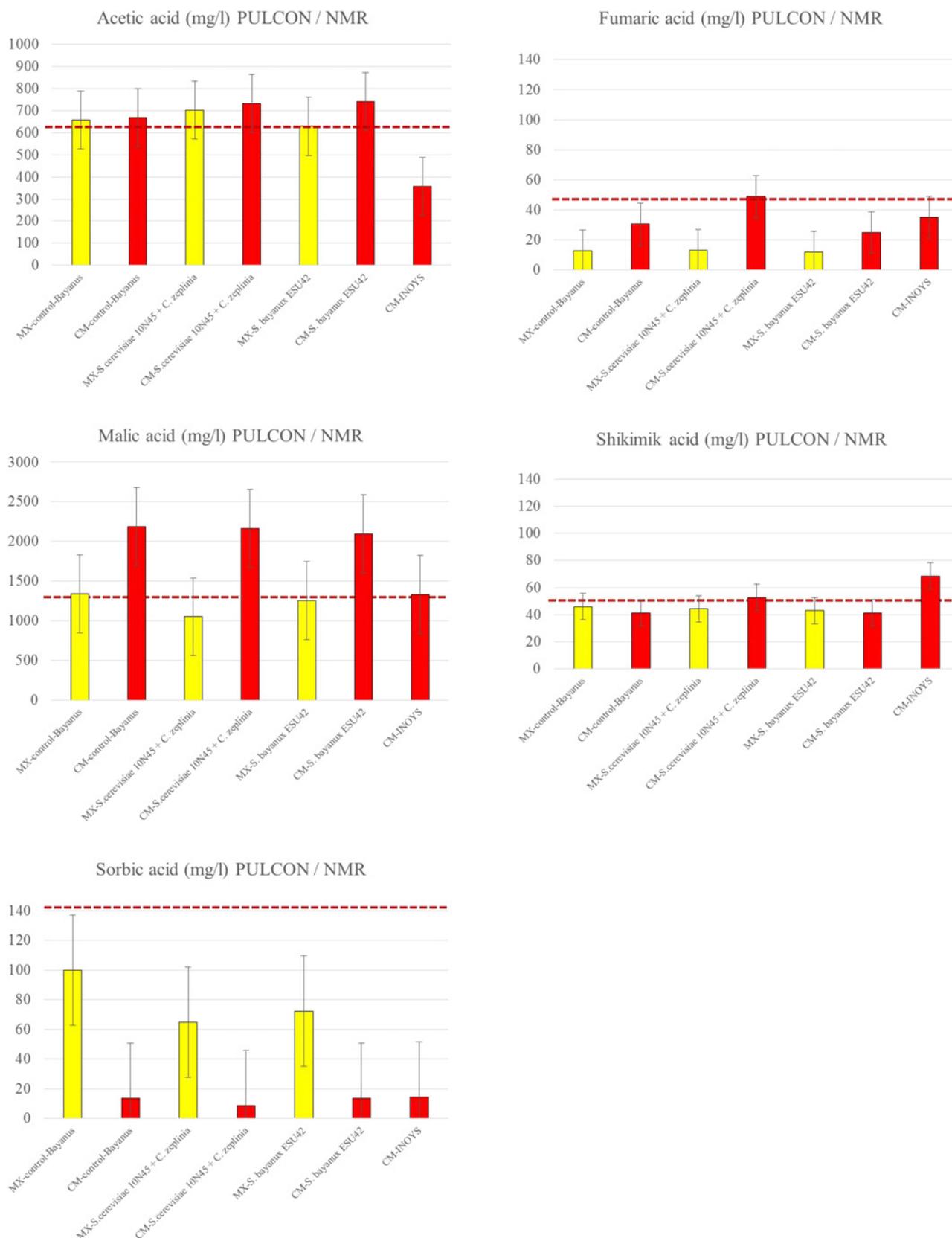


Figure 5. Quantification of acetic acid, malic acid, sorbic acid, fumaric acid and shikimic acid according to the PULCON/NMR method [15]. In all cases, an external reference of each metabolite, with known concentration, has been used for calibration. External standards were prepared in concentrations close to the OIV reference mean value (dotted red line) as follows: [acetic acid] = 650 mg/L; [malic acid] = 1436 mg/L; [sorbic acid] = 152 mg/L; [fumaric acid] = 49 mg/L; [shikimic acid] = 51 mg/L.

Table 5. Quantification of assigned metabolites by PULCON/NMR analysis. Standard deviations per metabolite are as well described.

	Shikimic (mg/L)	Fumaric (mg/L)	Sorbic (mg/L)	Malic (mg/L)	Acetic (mg/L)
MX-control-Bayanus	45.8	12.8	99.9	1338.3	657.3
CM-control-Bayanus	41.3	30.4	13.5	2186.3	668.8
MX- <i>S.cerevisiae</i> 10N45 + <i>C. zephalina</i>	44.3	13.2	64.8	1050.2	703.2
CM- <i>S.cerevisiae</i> 10N45 + <i>C. zephalina</i>	52.6	48.8	8.6	2160.9	732.8
MX <i>S. bayanus</i> ESU42	42.9	12.0	72.4	1256.6	629.2
CM <i>S. bayanus</i> ESU42	41.2	24.8	13.6	2095.4	741.3
CM-INOYS	68.4	35.2	14.4	1333.9	358.2
Desvest	±9.8	±13.9	±37.2	±492.6	±131.4

samples; 64–100 mg/L for MX samples) with respect OIV accepted mean values (150mg/L), suggesting a strong susceptibility of all samples to be unprotected against fungi or bacteria attacks [16].

4. Conclusions

A multitask NMR strategy is herein proposed to analyse alcohol reduction and oenological practice in wines produced with the first large-scale Non-*Saccharomyces* or non-conventional *Saccharomyces* fermentation processes. Standard metabolites were detected and quantified with the PULCON/NMR procedure and quality recommendations can be done as a function of selected fermentation scheme for reducing %alc. (v/v). High-resolution two-dimensional ¹H-¹³C HSQC with solvent multipresat have revealed novel resonances ready to be targeted and quantified with PULCON/NMR method, towards a not known accuracy in wines' quality control.

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