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EFFECTIVENESS OF HIGHER FATTY ACIDS C_8, C_{10} AND C_{12}, DIMETHYL DICARBONATE AND SULPHUR DIOXIDE FOR INHIBITION OF RE-FERMENTATION AND MALOLACTIC ACTIVITIES IN WINE

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Abstract

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The issue of preventing the re-fermentation and protection against undesirable malolactic fermentation (MLF) in order to safe content of acids in wine is very complicated. In this paper the saturated higher fatty acids (HFA) – C_8, C_{10} and $C_{12,}$ dimethyldicarbonate (DMDC) and sulphur dioxide (SO_2) were tested. The re-fermentation test showed the strongest inhibition power at ratio 2:8, 1:9 and 0:10 as C_8:C_{10} acids – 65 days without re-fermentation. MLF experiments confirmed that addition of SO_2 into the fermenting media causes rapid inhibition of lactic acid bacteria metabolic activity. Malic acid concentrations were proportionally decreasing during 6 days of experiment and at the end the content of this acid varied between 0.16 and 0.22 g/L, the only exception formed a variant with the addition of SO_2 (1.57 g/L of malic acid). After calculation of the average consumption rate of malic acid, the results showed the inhibition power – SO_2 (81.05%) followed by variant of 40 mg/L mixture of HFA (40.76%), a variant of 200 mg/L of DMDC (31.98%) and a variant of 20 mg/L mixture of HFA (12.59%). The addition of HFA can significantly reduce the dosage of other preservatives, especially SO_2. Based on results, this method can be recommend in the production of wines with residual sugar and also wines made from over-mature material to prevent undesirable MLF.

Keywords: yeast inhibition, malolactic fermentation, octanoic acid, decanoic acid, *Saccharomyces cerevisiae*, dimethyl dicarbonate

INTRODUCTION

Malolactic fermentation (MLF) is the second stage of winemaking for most of the red and some white wines. MLF can increase microbiological stability and enhance wine flavor and aroma (BARTOWSKY *et al*., 2002; NEHME *et al*., 2008). A wines undergoing malolactic conversion are cloudy due to the presence of bacteria and cells of dead yeasts, and may have the curious smell of buttered popcorn, due to the production of diacetyl. Wines, especially wines with residual sugar, could be considered as a hostile life medium for micro-organisms. Few of them are able to spoil these wines during storing, therefore causing irreversible organoleptic damages. The last fundamental step of winemaking to stopping and prevention of re-fermentation or start of undesirable MLF is addition of SO_2. The possibility of new fermentation during maturing in barrels or bottle ageing seems to prove the survival of yeasts a long time after the first alcoholic fermentation (DIVOL *et al*., 2006). The issue of preventing or stopping of MLF in order to safe content of acids in wine is very complicated (SON *et al*., 2009; RODRIGUEZ-NOGALES *et al*., 2013). Especially in over-mature material with higher pH where improved growth of lactic acid bacteria can occur. Current methods used in practice, such as cooling and filtration, SO_2 doses or lysozym treating, leading to increasing costs and are undoubtedly very laborious and particularly

unavailable for home winemakers. Separate application of SO_2 is not always wholly reliable, and at high concentrations it leads to quality reduction. Moreover SO_2 may cause allergic reaction and it should be reduced in wine technology in the future (BAROŇ *et al.*, 2011).

Another preservative is dimethyl dicarbonate (DMDC), authorised in the USA up to the cumulative amount of 200 ppm and in Australia up to 200 mg/kg. In Europe, DMDC has just been authorized with the maximum limit of 200 mg/l. DMDC breaks down to form methyl carbamate, carbon dioxide and methanol, which is considered to have practically no toxic effects (HOU *et al.*, 2008). OUGH *et al.* (OUGH *et al.*, 1988) demonstrated that 100 mg/l DMDC sterilized wine completely at pH below 3.8 in the absence of SO_2, even if the initial yeast population was greater than 10^7 cells per milliliter. In the European Union, DMDC is currently authorized for use in unfermented beverages at doses below 250 mg/l. In view of its properties, especially the possibility of reducing the use of SO_2, DMDC is currently being tested with a view to registration in the OIV International Code of Winemaking Practices (COSTA *et al.*, 2008; EDER 2011).

Higher monocarboxylic saturated fatty acids were studied for their inhibitory effect on alcoholic and malolactic fermentation many years ago (VIEGAS *et al.*, 1991; VIEGAS *et al.*, 1995). Some of the higher fatty acids (HFA) with 16 or 18 carbons, C_{16} and C_{18}, are fermentation activators. On the contrary, other HFA with shorter chain, in particular acids, hexanoic C_6, octanoic C_8, decanoic C_{10} and dodecanoic acid C_{12} have fungicidal and antibacterial properties (VIEGAS *et al.*, 1991; VIEGAS *et al.*, 1995; CARRETE *et al.*, 2002). They are made by yeasts themselves during alcoholic fermentation and may contribute to its difficulties in completing the course (SACORREIA *et al.*, 1983; ALEXANDRE *et al.*, 1996). Strong properties to inhibit yeasts and lactic acid bacteria (GARBAY *et al.*, 1995; GUILLOUX-BENATIER *et al.*, 1998) with the current potential for treatment of wine against re-fermentation is likely to offer a mixture of saturated higher fatty acids (HFA) C_8, C_{10} and C_{12} (BAROŇ *et al.*, 2011). HFA are currently not used for inhibition of malolactic fermentation and the possibly prevention of re-fermentation in wine technology and wine storage. Published works demonstrate the properties of HFA. Most experiments were performed in synthetic media, not in real musts or wines.

This paper is aimed on inhibition of malolactic fermentation and the possibility of prevention of re-fermentation in wine technology and wine storage. The aim is to show the use of HFA, which may pose a sparing and safe alternative to this process.

MATERIALS AND METHODS

Re-fermenting test

Wine for re-fermenting test was made from sterilized must with inoculated yeasts *Saccharomyces cerevisiae* (ZYMAFLORE VL1®) and alcoholic fermentation was stopped by cooling and SO_2 addition. Each flask volume 750 ml (n = 2), was treated as outlined in Tab. I. Experiment was performed by room temperature about 22 °C. To determine the effect of HFA on the re-fermentation was selected wine divided into twelve variant (n = 2). Within eleven variant was added a mixture of HFA, one variant served as a control. The bottles were closed only with temporary fermentation stopper and stored at room temperature in order to support of re-fermentation. As a detection of re-fermentation in the bottle was used sensory testing and controlling of carbon dioxide production. Time of re-fermentation was set as the average of two bottles rounded to the day.

Wine for MLF test, each flask volume 750 ml (n = 2), was inoculated with commercial strain of bacteria *Viniflora Oenos* – Christian Hansen

I: *Content of individual HFA in re-fermentation test*

	Higher saturated fatty acids (mg/L)											
Variant	1	2	3	4	5	6	7	8	9	10	11	12
C8	0	10	9	8	7	6	5	4	3	2	1	0
C10	0	0	1	2	3	4	5	6	7	8	9	10

II: *Parameters of treated wines*

Chemical characteristic	Wine for MLF	Wine for re-fermentation	Analytical method
Alcoholic degree %, ethanol (v/v)	13.2	11.8	Distillation
Titratable acidity (g/L tartaric acid)	8.6	7.5	Titration with point of pH 7.0
pH	3.53	3.22	Potentiometer
Residual sugar (g/L)	1.2	18.0	HPLC of sum glucose and fructose
Free SO_2 (mg/L)	4.4	28.4	Ripper method
Total SO_2 (mg/L)	22.6	162.8	Ripper method
Filtered	None	None	

(*Oenococcus Oeni*). For inhibition was used a mixture of HFA C_8, C_{10} (2:8) dissolved in 70% vol. ethanol, with 100 ml ethanol solution containing 10 g of this mixture, C_{12}, DMDC and SO_2.

Analysis of basic parameters was performed as outlined in Tab. II.

Calculation of consumption rates

Malic acid consumption kinetic can be deduced from general consumption kinetics, since most of the available models assume malic acid utilization equal to function of time f_t.

$$\frac{dM_c}{dt} = f_t$$

$$-\frac{dM_c}{dt} = kM_c \qquad (1)$$

$$\ln M_c^t = \ln M_c^0 - kt$$

$$k_i = \frac{\ln \frac{M_c^0}{M_c^{t_i}}}{t_i},$$

where M_c represents concentration of malic acid, M_c^0 and M_c^t represent malic concentration at time zero and t, respectively. Consumption rates k_i induced by used treatments were calculated according to Eq. (1) for each day. Average consumption rate was calculated from individual consumption rates.

Analytical determinations

HPLC estimation of acids and sugars

Must samples were centrifuged (3000 × g; 6 min) and diluted with 10× demineralised water. The estimation was performed by means of IC in the Shimadzu LC-10A system plus the thermostat (column oven) CTO-10ACvp set at 60 °C. The manual injection Rheodyne valve had a loop with the volume of 20 µl. The separation was performed in an isocratic regime with the mobile phase of 2 mM sulphuric acid at the flow rate of 0.75 ml/min in the column Watrex Polymer IEX H form 10 µm; 250 × 8 mm with 10 × 8 mm. Spectrophotometric detection was performed by the DAD detector SPD-MAvp. Organic acids were measured at 210 nm. The quantification of the individual analyses was performed on the basis of external calibration.

Statistical evaluation

The results obtained were statistically analysed using the statistical program STATISTICA 10. Evaluated were the means and standard deviations using ANOVA with subsequent Tukey's test at significance level of $p < 0.01$.

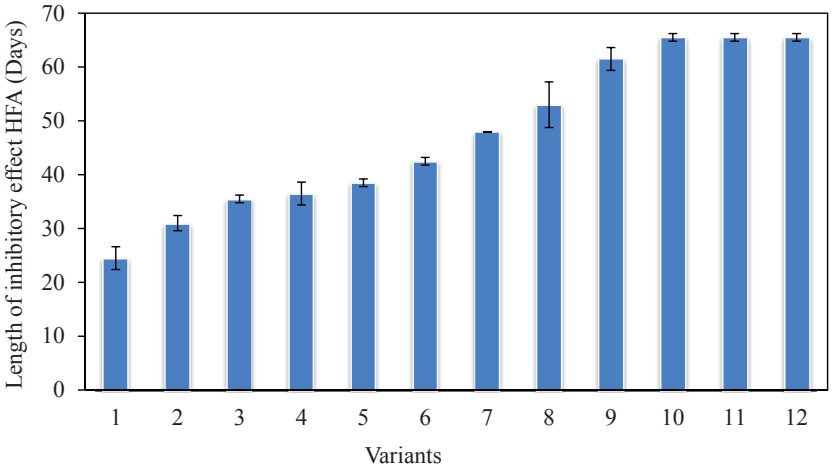
RESULTS AND DISCUSSION

Re-fermentation test

First experiment was carried out with re-fermenting wine (see Materials and Methods). The obtained results (Fig. 1) demonstrate the inhibitory effect of HFA on yeasts initiating the re-fermentation. While the control sample started to re-ferment after 25 days, a sample with addition of HFA (variants 10, 11, 12) resisted few times longer – 65 days. The enhancement of stability against re-fermentation by octanoic and decanoic acid was quantified at different ratios of C_8 and C_{10}. Decanoic acid was found to be more toxic than octanoic acid, which correlates with the higher liposolubility of its undissociated form (VIEGAS *et al.*, 1997; SACORREIA *et al.*, 1986). Results showed the strongest inhibition power of ratio 2:8, 1:9 and 0:10 as C_8:C_{10}. Higher fatty acids dramatically reduce the need of SO_2 addition in stored and bottled wines with residual sugar (BAROŇ *et al.*, 2011).

Inhibition of MLF

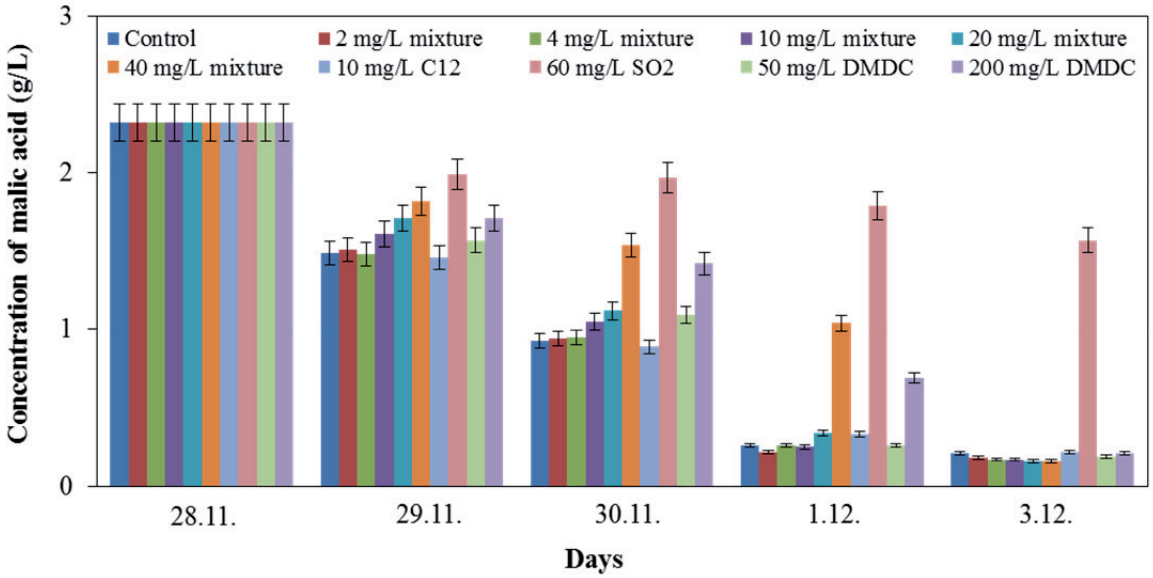
For the MLF experiment the tested wine was inoculated by lactic bacteria (see Materials and Methods). After the beginning of MLF, the wine



1: *The inhibitory effect of HFA for yeasts initiating re-fermentation*

III: *Concentration of lactic, citric and acetic acids in MLF test*

	Lactic acid (g/L)				Citric acid (g/L)				Acetic acid (g/L)			
Days	**29.11.**	**30.11.**	**1.12.**	**3.12.**	**29.11.**	**30.11.**	**1.12.**	**3.12.**	**29.11.**	**30.11.**	**1.12.**	**3.12.**
Control	1.07	1.62	2.07	1.84	0.23	0.19	0.08	0.06	0.40	0.38	0.43	043
2 mg/L mixture	1.29	1.67	1.99	1.83	0.26	0.20	0.08	0.06	0.41	0.40	0.43	0.41
4 mg/L mixture	1.33	1.57	2.10	1.78	0.26	0.19	0.09	0.06	0.43	0.42	0.49	0.41
10 mg/L mixture	1.36	1.58	1.87	1.73	0.30	0.20	0.09	0.08	0.43	0.40	0.45	0.42
20 mg/L mixture	1.17	1.56	1.81	1.77	0.25	0.30	0.09	0.09	0.41	0.42	0.43	0.49
40 mg/L mixture	1.24	1.16	1.62	1.77	0.26	0.27	0.30	0.09	0.39	0.40	0.47	0.49
10 mg/L C12	1.30	1.67	1.84	1.83	0.31	0.27	0.10	0.05	0.44	0.38	0.47	0.44
60 mg/L SO_2	0.89	0.86	0.90	1.01	0.27	0.31	0.31	0.22	0.40	0.40	0.43	0.43
50 mg/L DMDC	1.24	1.65	1.96	1.92	0.24	0.29	0.09	0.07	0.41	0.43	0.49	0.42
200 mg/L DMDC	1.15	1.42	1.68	1.76	0.25	0.25	0.19	0.15	0.41	0.36	0.46	0.46



2: *Content of malic acid in the monitored variants during the six days experiment*

was divided into ten variants (n = 2) and treated by different concentrations of HFA mixture C_8, C_{10} (2:8), individual C_{12}, SO_2 a DMDC at two doses. Mixture of HFA was chosen as the base of re-fermentation test. The evolution of major organic acids was monitored during six days and results are shown in Tab. III and Fig. 2.

Our attention was focused on four important organic acids – malic acid, lactic acid, citric acid and acetic acid. The content of acetic acid in the monitored variants differed minimally. Significant inhibition of MLF occured in the variant with 60 mg/L SO_2, where the lower content (1.01 g/L) of lactic acid was observed and citric acid content was significantly higher (0.22 g/L) (Tab. III). Special attention was paid to the content of malic acid, which significantly affects the taste and quality of wine, while not only its final concentration is important, but also the kinetic of its degradation (UGLIANO *et al.*, 2003; HERNANDEZ-ORTE *et al.*, 2009; EDER, 2011). Malic acid values were proportionally decreasing during the experiment and at the end of the experimental period the content of this acid varied between 0.16 and 0.22 g/L, the only exception was again formed at the variant with the addition of SO_2 (Fig. 2). This was probably due to a greater inhibition of lactic acid bacteria with SO_2.

To have more precise idea about the kinetic of malic acid degradation and so the proportion of inhibition, average consumption rates were calculated (see Materials and Methods). Tab. IV shows data gained from the calculation with standard deviation. After six days the strongest inhibition properties against lactic acid bacteria were investigated in the case of SO_2, which was the only variant where the MLF was definitely stopped after experiment.

The inhibition power was compared and calculated by Tukey's test, $p < 0.01$ (Tab. IV). All the variants were devided into 3 groups. For a simple quantification, the ratio between the average consumption rates and the control variant was calculated in percentages. The MLF experiment showed the highest inhibition power in the case

IV: *Inhibitory power calculated by Tukey's test*

Variant	Inhibition %	Average consumption rate $\bar{k}$	SD
Control	0.00	0.53[a]	0.12
2 mg/L mixture	–3.22	0.54[a]	0.14
4 mg/L mixture	–1.82	0.54[a]	0.12
10 mg/L mixture	3.92	0.51[a]	0.15
20 mg/L mixture	12.59	0.46[ab]	0.13
40 mg/L mixture	40.76	0.31[ab]	0.13
10 mg/L C12	2.20	0.52[a]	0.08
60 mg/L SO_2	81.05	0.10[b]	0.03
50 mg/L DMDC	5.29	0.50[a]	0.14
200 mg/L DMDC	31.98	0.36[ab]	0.09
F		**	

The results were statistically analysed by the ANOVA method and Tukey's test. The letters indicate statistically significant differences determined by Tukey's test; ** $p < 0.01$.

of SO_2 (81.05%) followed by the variant of 40 mg/L mixture of HFA (40.76%), a variant of 200 mg/L of DMDC (31.98%) and the variant of 20 mg/L mixture of HFA (12.59%). The lowest inhibition power was evaluated in the case of 2 and 4 mg/L mixture of HFA (–3.22%, resp. –1.82%), which can be considered as stimulants. The experiment showed a significant inhibition power of HFA and DMDC (EDER 2012). The inhibitory effect of other variants on the lactic acid bacteria was not significant.

CONCLUSIONS

The prevention of re-fermentation and undesirable MLF in order to save the content of residual sugar resp. organic acids in wine is very complicated. HFA were shown as a useful complementary method for SO_2 dosing during wine storage. The procedure uses a mixture of HFA reducing the labor intensity of wines with residual sugar. And according to today's requirements on SO_2 reduction, especially in the case of bio-wines, this mixture could be used very effectively. The most effective can be the addition of HFA in combination with reduced dose of SO_2 in home-winemaking conditions, where it is not possible to use expensive operations commonly used in larger wineries (cooling down, sterile filtration). HFA protect wines containing residual sugar against re-fermentation and they can also effectively increase the wholesomeness of the product.

In the case of the inhibition of lactic bacteria by HFA there was found out that permissible concentrations are not sufficient for stopping the MLF. However, in combination of HFA with SO_2, there could be sufficient synergistic inhibitory effect when using a lower dose of SO_2. Simultaneously the wine can be protected, at least partly, to prevent unwanted MLF.

SUMMARY

This work is aimed on inhibition of wine re-fermentation by mixture of saturated higher fatty acids (HFA) and comparison of HFA – C_8, C_{10} and C_{12}, dimethyldicarbonate (DMDC) and SO_2 efficiency against malolactic activity.

In re-fermentation test was found out the same and the strongest inhibition power of ratio 2:8, 1:9 and 0:10 as C_8:C_{10} acids – 65 days without re-fermentation compared to 25 days as a control variant.

MLF experiment has confirmed that addition of SO_2 into the fermenting media causes rapidly inhibition of lactic bacteria metabolic activity. Malic acid values were proportionally decreasing during 6 days of experiment and at the end was the content of this acid between 0.16 to 0.22 g/L, the only exception formed a variant with the addition of SO_2 (1.57 g/L) where was MLF completely stopped. In the case of HFA and DMDC, kinetic of malic acid degradation showed some inhibition power which was not sufficient to MLF stopping. After calculation of malic acid consumption rate results showed the inhibition power – SO_2 (81.05%) followed by variant of 40 mg/L HFA mixture (40.76%), 200 mg/L DMDC (31.98%) and 20 mg/L HFA mixture (12.59%). Addition of HFA can significantly reduce the dosage of other preservatives especially SO_2. Optimized properties against re-fermentation with the current potential for treatment of wine to lactic acid bacteria is likely to offer a mixture of HFA C_8, C_{10} (2:8) dissolved in 70% vol. ethanol, with 100 ml ethanol solution containing 10 g of this mixture. Such a mixture is prepared in a liquid state and there is unlikely to create a solid phase at low temperatures, which makes it very easy to dose in practice. The advantage of the proposed mix is high fungicidal

activity of C_8 and C_{10} acids, and the inhibitory effect against yeasts and lactic acid bacteria. Secondary purpose of C_8 acid is to increase the solubility of C_{10} acid. Addition of HFA can significantly reduce the dosage of other preservatives such as SO_2.

Described method is now in the Czech Republic three-years testing program under the auspices of the OIV (The International Organisation of Vine and Wine).

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