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The study of glycerol metabolism in the malolactic fermentation of red wines

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Abstract

Glycerol from wine presents a major importance from at least two different points of view; first, glycerol is considered a test substance, which tests the wine's naturalness; second, glycerol has a very important physiological role on human organism due the esterification of free fat acids from blood, determining a reduction of cholesterol content. In this paper, glycerol was determined quantitatively through the enzymatic method, before and after the malolactic fermentation, for the wine samples inoculated with commercial preparation INOFLORE R (which contains the Oenococcus oeni species) and for the wine samples that were not innoculated at which the malolactic fermentation occurred spontaneously based on the inner microflora. The results of this paper show that the glycerol degradation occurs during the malolactic fermentation especially at wine samples that were not inoculated with malolactic bacteria, at which the malolactic fermentation occurred spontaneously and uncontrolled, exclusively based on the inner microflora. After finishing the malolactic fermentation in controlled conditions, the wines had a normal evolution, fact that confirm us that the glycerol metabolization through controlled malolactic fermentation is only partial and is not accompanied by defects and diseases of wine.

Keywords: malolactic fermentation, glycerol, lactic acid bacteria.

Introduction

Glycerol, also called propanetriol, is the most important by-product of alcoholic fermentation, both in terms of quantity and the influence it exerts on the organoleptic characteristics of wine. It influences positively the taste of wine, giving it soothing tones, finesse, harmony and versatility, because of the softness and sweetness (equal to that of glucose) it has. It is formed at the beginning of the metabolism of sugars by yeasts (via glyceropyruvic fermentation), when the fermentation medium does not yet contain acetic aldehyde. In this situation, under the action of the coenzyme NADH, the triose dihydroxyacetone is converted into glycerol. Depending on the proportions of sugars in musts undergoing fermentation and on the biological and technological factors acting in primary winemaking, glycerol contents in wines range between 5 and 20 g/l (M. GHEORGHIȚĂ & al. [1]). Wines made from grapes attacked by noble rot (*Botrytis cinereae*) carry larger amounts of glycerol, even more than 20 g/l, as is the case of the Cotnari wine – Romania, or Sauternes – France (V. COTEA & al. [2]).

Research has shown that the proportion of glycerol in wine depends on: the initial concentration in sugar of must, the amount of sulfur dioxide used to protect must, the fermentation temperature maintained during the alcoholic fermentation, the yeasts which completed the alcoholic fermentation of the must (POPA [3]).

In case of a more energetic sulfitation, larger amounts of glycerol are formed because part of the acetaldehyde (an acceptor of hydrogen in the final stage of fermentation) conjugates with sulfurous acid, resulting aldehyde-sulfurous acid. In this case, the energy required by yeasts is attained via glyceropyruvic fermentation, resulting in higher amounts of glycerol in wine (KM GROSSMANN & al. [4] G. CALDERON & al. [5]).

Glycerol in wine is of major importance in at least two distinct ways. Firstly, glycerol is considered a test substance that proves the naturalness of wine. Thus, the content of glycerol in wine is in accordance with the level of alcohol in wine and is set at the limit of 6-10 grams per 100 grams of alcohol. It is considered that wine for which the glycerol/alcohol ratio is below 6.5% was fortified, and, when this ratio exceeds 10%, it can be suspected that glycerin was added to it (BADUCĂ CÂMPEANU [6]). Secondly, glycerol in wine has a very important physiological role on the human body, in that it esterifies blood free fatty acids, thus determining a decrease in cholesterol, which thickens blood vessel walls. On the other hand, the consumption of wine rich in glycerol favors the formation of prostacyclin which are agents for the dilation of blood vessels. This explains the fact that in Mediterranean wine countries, where more wine is consumed, vascular mortality is lower than in northern Europe countries (C. ȚÂRDEA & al. [7]).

Glycerol degradation during malolactic fermentation is an evaluation criterion for the oenological assessment of (beneficial and harmful) lactic acid bacteria.

Glycerol catabolism by lactic acid bacteria is detrimental to the quality of wine, on the one hand due to the reduction of its content in wine, and on the other hand because of the resulting metabolic products that lead to a disease known as bitterness in wine. Lactic acid bacteria convert glycerol into β-hydroxypropionaldehyde under the action of glycerol dehydratase. This molecule is the precursor of acrolein whose conjunction with the tannins in wine give bitter substances (N. SAUVAGEOT & al. [8]). Glycerol may also be damaged in another metabolic pathway, in which the first catalysed reaction by glycerol kinase leads to glycerol-3-phosphate. By oxidation to dihydroxyacetone phosphate, this reaction enters the glycolysis flow, reaching pyruvic acid. The end products of this metabolic pathway are the same as the ones resulting on other metabolic pathways from pyruvic acid, in particular acetic acid and acetoin substances (BADUCĂ CÂMPEANU [9]). Glycerol degradation during malolactic fermentation is influenced by the content of fermentable sugars in the medium, the pH of the medium and the lactic acid bacteria strains involved in the fermentation (A. POPA & al. [10]). The biochemical mechanism of glycerol metabolism by lactic acid bacteria is set out in the following figure:

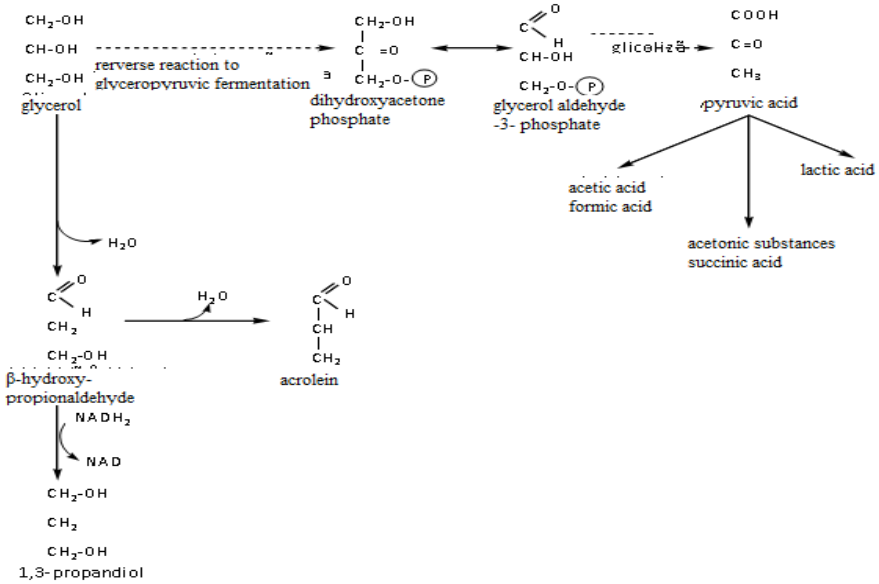

Fig. 1 Glycerol degradation ways by lactic acid bacteria (P. RIBEREAU-GAYON & al. [11])

An almost constant feature of malolactic fermentation observed both in white and red wines is represented by the increase in corpulence and the improvement of aftertaste. Lactic acid bacteria, particularly lactobacilli, are able to metabolise glycerol in whole or in part. (DITTRICH [12]). Comparing 8 wines with and without spontaneous malolactic fermentation, (H. H. DITTRICH & al. [13]) found that 6 wines which underwent malolactic fermentation showed a decrease in glycerol content, which also applied to wine inoculated with *Lactobacillus plantarum* to achieve malolactic fermentation. In similar experiments performed on wines produced under controlled conditions and using *Leuconostoc oenos* starter cultures, no change was noted in the glycerol content of wines (HENICK-KLING T. & al. [14, 15, 16], NIELSEN and RICHELIEU [17]).

For the purposes of this paper, glycerol content was determined before and after malolactic fermentation, both on samples of wine inoculated with the INOFLORE R commercial preparation (containing the *Oenococcus oeni* species) and on uninoculated samples of wine in which malolactic fermentation occurred spontaneously based on indigenous microflora.

Material and method

The wine glycerol content was determined using enzymatic kits supplied by Diamedix Diagnostica, using the enzymatic method set out below (ȚÂRDEA [18], Diamedix Diagnostica Documentation [19]).

Method principle

Glycerol is phosphorylated by ATP to L-glycerol-3-phosphate, the reaction being catalyzed by glycerol kinase (GK).

(1) Glycerol + ATP $\xrightarrow{GK}$ L-glycerol-3-phosphate + ADP

Formed ADP is reconverted to ATP by phosphoenolpyruvate (PEP) using pyruvate kinase (PK), forming pyruvate.

(2) ADP + PEP $\xrightarrow{PK}$ ATP + pyruvate

In the presence of the enzyme L-lactate dehydrogenase (L-LDH), pyruvate is reduced to L-lactate by the enzyme NADH with NADH oxidation to NAD.

(3) Pyruvate + NADH + H^+ $\xrightarrow{\text{L-LDH}}$ L-lactate + NAD^+

Composition of the kit (reagents used)

- container 1 with 2 g coenzyme/buffer, each consisting of: glycylglycine buffer, pH. ca. 7.4; NADH, ca. 7 mg; ATP ca. 22 mg; PEP-CHA, ca. 11 mg; magnesium sulfate.
- container 2 with 0.4 ml suspension consisting of: pyruvate kinase, 240 U; L-lactate dehydrogenase, 220 U
- container 3 with 0.4 ml 34 U glycerol kinase suspension
- Control solution for the analysis of glycerol

The contents of container 1 are dissolved in 11 ml double distilled water. Prior to use, allow to stand at room temperature for 10 minutes. The content of containers 2 and 3 are used as supplied.

Preparation of sample

Clear samples, colorless and practically neutral are used. Volumes up to 2,000 ml directly or after diluting according to the dilution table are used. Turbid samples are filtered. Samples containing CO_2 (by filtering) have to be degassed. Acid samples' pH needs to be adjusted to 8 using KOH or NaOH; let incubate for 15 minutes. Highly colored samples that are used undiluted with polyvinylpyrrolidone (PVPP) or polyamide are treated; e.g., 1 g/100 g. To determine the glycerol content of fermented samples, the sample is placed in a water bath at 80° C for 15 minutes to stop enzymatic reactions. The supernatant is centrifuged and used. The amount of glycerol present in the sample must be between 1 μg and 40 μg. To obtain a significant difference in absorbance, the sample is diluted to achieve a concentration of glycerol between 0.04 and 0.4 g/L.

Table 1 Dilution of wine samples before determining the glycerol content (Diamedix Diagnostica Documentation, [19])

Estimated amount of glycerol per liter	Dilution with water	Dilution factor
<0.4 g	-	1
0.4 – 4.0 g	1 + 9	10
4.0 – 40 g	1 + 99	100
>40 g	1 + 999	1000

The working procedure occurs as in the table below:

Table 2 Working protocol for the determination of glycerol in wine (Diamedix Diagnostica Documentation, [19])

Pipetting	Blank	Sample
Solution 1	1.000 ml	1.000 ml
Sample	-	0.100 ml
Doubly distilled water	2.000 ml	1.900 ml
Suspension 2	0.010 ml	0.010 ml
Mix with a plastic spatula, then read A_1 after ca. 5-7 minutes. Start the reaction by adding:		

Suspension 3	0.010 ml	0.010 ml
Mix with a plastic spatula, wait ca. 5-10 minutes, then read A_2. If the reaction is not over after 15 minutes, read absorbances every 2 minutes until the absorbance decreases steadily in 2 minutes.		

Tthe absorbance differences (A_1-A_2) is calculated for both the blank and the sample. The value obtained for the blank is subtracted from that obtained for the sample, thus resulting the difference in absorbance.

$$\Delta A = (A_1 - A_2)_{sample} - (A_1 - A_2)_{blank}$$

The sample's glycerol concentration is calculated using the formula:

$$c = \frac{3.020 \times 92.1}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A = \frac{2.781}{\varepsilon} \times \Delta A \quad [\text{g glycerol/l sampled solution}]$$

If the sample was diluted during preparation, the result must be multiplied by the dilution factor F.

Results and discussions

After alcoholic fermentation has ended under winery conditions, wines were separated from the yeast, allowed to clarify in a cool place and filtered using sterilized cellulose pads. After filtration, wines were poured into bottles provided with fermentation tanks. Malolactic fermentation was induced by inoculating half the samples with the INOFLORE R bacterial preparation, which contains the *Oenococcus oeni* (*Leuconostoc oenos*) species. The other half of the samples remained uninoculated and malolactic fermentation was initiated under "spontaneous" conditions based on indigenous microflora.

Before starting malolactic fermentation, the glycerol content of wines undergoing malolactic fermentation was determined. Analyses were supplemented also by determinations of the content of alcohol, sugar, malic acid, lactic acid and pH.

Fig. 2 INOFLORE R commercial preparation used for managing malolactic fermentation (supplied by Enzymes&Derivates Romania)

Table 3 Glycerol content of wine samples before malolactic fermentation

Wine sample/wine center	Glycerol concentration (g/l)	Alcohol concentration (% vol.)	Sugar concentration (g/l)	pH	L-malic acid (g/l)	L-lactic acid (g/l)
1. Burgund/ Miniş	7.62	12.2	2.97	3.35	2.07	0.25
2. Pinot noir/ Miniş	6.27	12.3	2.08	3.31	1.85	0.37
3. Merlot/ Recaş	5.26	11.5	5.75	3.19	1.65	0.22
4. Cabernet Sauvignon/ Recaş	4.53	11.2	5.5	3.21	1.24	0.54
5. Burgund/ Recaş	5.28	11.2	5.5	3.18	1.0	0.73

Table 4 Glycerol content of wine samples after malolactic fermentation

Uninoculated wine samples (control)	Glycerol concentration (g/l)	Glycerol metabolism by FML (%)	Alcohol concentration (% vol.)	Sugar concentration (g/l)	pH	L-malic acid (g/l)	L-lactic acid (g/l)
1. Burgund /Miniş	0.31	95.93	12.2	2.66	3.4	traces	1.92
2. Pinot noir/Miniş	1.46	76.71	12.3	1.88	3.4	traces	1.72
3. Merlot/ Recaş	0.39	92.59	11.7	2.32	3.2	traces	1.43
4. Cabernet Sauvignon/ Recaş	0.32	92.93	11.2	2.24	3.3	traces	1.56
5.Burgund/ Recaş	0.10	98.1	11.3	2.27	3.2	traces	1.63
Wine samples inoculated with BMS							
1. Burgund /Miniş	3.91	48.68	12.3	2.56	3.3	traces	2.0
2. Pinot noir/Miniş	6.93	0	12.3	2.05	3.3	traces	1.9
3. Merlot/ Recaş	3.51	33.26	11.6	5.60	3.2	traces	1.65
4. Cabernet Sauvignon/	2.31	49.1	11.3	4.67	3.2	traces	1.81

Recaş							
5.Burgund/ Recaş	3.48	34.09	11.3	4.83	3.1	traces	1.9

Table 3 data show that the glycerol content of wines before malolactic fermentation is between 4.53 g/l for Cabernet Sauvignon (Recaş wine center) and 7.62 g/l for Burgund (Miniş wine center). The Merlot, Cabernet Sauvignon and Burgund wines of the Recaş wine center have more modest glycerol contents due to the fact that musts have had lower sugar contents, and sulfitation was weaker during alcoholic fermentation. Lower concentrations in glycerol in wines obtained at Recaş wine center can also be explained by the use of different species of yeasts in alcoholic fermentation compared with those used by Miniş wine center, involving a different metabolism, glycerol being a metabolite resulting after alcoholic fermentation. Also worth noting is that the malic acid content of Recaş wines is lower (1.0-1.65 g/l), while the lactic acid content is higher (0.22-0.73 g/l), indicating that that malolactic fermentation had already started at the time of the determination.

Table 4 data show that, after the completion of malolactic fermentation, wines have very low concentrations of glycerol, ranging between 0.10 and 6.93 g/l, this indicating a massive metabolism of glycerol during malolactic fermentation. However, there is a difference in terms of glycerol content between malolactic fermented wine samples.

Under an uncontrolled malolactic fermentation, glycerol contents are abnormally low, ranging between 0.10 g/l for the Burgund wine of the Recaş wine center and 1.46 g/l for the Pinot noir wine of the Miniş wine center. The most important metabolism of glycerol occurs under spontaneous malolactic fermentation in the case of Burgund wine from the Recaş wine center, for which the content of glycerol decreases from 5.28 g/l before malolactic fermentation to 0.10 g/L, a value recorded after the completion of the spontaneous malolactic fermentation. For the same type of wine, Burgund, but produced by the Miniş wine center, the glycerol content falls from 7.62 g/l before malolactic fermentation to 0.31 g/l, a value recorded after the completion of the spontaneous malolactic fermentation.

With an uncontrolled malolactic fermentation, glycerol is metabolized almost entirely, the metabolic rate ranging between 76.71 % for the Pinot noir wine of the Miniş wine center and 98.1 % for the Burgund wine produced by Recaş wine center.

In the course of malolactic fermentation under uncontrolled conditions, we notice the appearance of bitterness in wine, which confirms that glycerol metabolism through malolactic fermentation is accompanied by the production of acrolein, the uninoculated wine samples (control) being organoleptically impaired at the end of thespontaneous malolactic fermentation.

The massive metabolism of glycerol during spontaneous malolactic fermentation is explained by the high pH of samples of wine, poor sulfitation, high temperature and the presence of indigenous microflora lactobacilli during fermentation.

On the other hand, for wine samples inoculated with the INOFLORE R commercial preparation (containing the bacterial species of *Oenococcus oeni*), the wines' glycerol content after malolactic fermentation falls near normal limits, ranging between 2.31 g/l for the Cabernet Sauvignon of the Recaş wine center and 6.93 g/l for the Pinot noir made by Miniş wine center. After the malolactic fermentation managed by using selected malolactic bacteria, the Pinot noir wine registers no glycerol concentration decreases, this demonstrating the inappetence of selected malolactic bacteria to degrade glycerol. In the case of the other wines analyzed, too (Burgund-Miniş, Burgund-Recaş, Merlot-Recaş and Cabernet Sauvignon-Recaş), the decrease in the concentration of glycerol during controlled malolactic fermentation are less significant in relation to spontaneous malolactic fermentation, glycerol

being only partially degraded. Given a controlled malolactic fermentation, glycerol is metabolized partially, the metabolic rate ranging from 33.26 % for the Merlot wine of the Recaş wine center to 49.1 % for the Cabernet Sauvignon wine of the Miniş wine center. In addition, the Miniş Pinot noir wine's glycerol content remains unchanged after completion of malolactic fermentation under controlled conditions.

In the course of malolactic fermentation under controlled conditions, the wines have evolved normally from a sensory point of view, which confirms that glycerol metabolism by controlled malolactic fermentation is only partial and is not accompanied by defects and diseases of the wine.

Conclusions

The results of this experiment show that glycerol degradation occurs during malolactic fermentation, especially in wine samples uninoculated with malolactic bacteria, where malolactic fermentation takes place spontaneously and uncontrollably based on indigenous microflora. The concentration of glycerol in malolactic fermented wines is significantly influenced by the microflora (indigenous or selected), but also depends on the value of pH, the concentration of fermentable wine sugars, the temperature and the sulfitation level.

Glycerol degradation during malolactic fermentation should be avoided as it is detrimental to the quality of wine, on the one hand due to its content reduction in wine, and on the other hand because of the resulting metabolic products that lead to the disease known as bitterness in wine.

Partial or total degradation of glycerol during spontaneous malolactic fermentation makes the ratio of glycerol/ ethyl alcohol be below 6.5 %, with the suspicion that these wines have been fortified, though there was no exogenous alcohol intake for these wines. In these cases, the ratio of glycerol/ethyl alcohol is no longer an objective criterion for assessing the naturalness of wine. (POPESCU-MITROI [20]).

Glycerol metabolism during spontaneous malolactic fermentation takes place in a proportion of 76-98%, fermentation degenerating in a defect, while under controlled conditions, the rate of glycerol metabolism by malolactic fermentation is much lower (less than 50 %), wines evolving normally, without the risk of disease and defects, certifying the reduced capacity of the selected malolactic bacteria to degrade glycerol.

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