# Glycerol and Wine Industry

## Glycerol Determination in Grape Must and Wine

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Glycerol is the major fermentation by-product of *Saccharomyces cerevisiae*, which indirectly contributes to the sensory character of wine.[1,2] In dry table wines it is found at concentration of 4-10 $gL^{-1}$ (Table 1) but occasionally may already be present in grape musts infected by moulds (*Botrytis cinerea*).[3,4] The amount of glycerol formed during fermentation is influenced by several factors, such as grape variety, degree of ripeness, fermentation temperature, $SO_2$ concentration, pH of grape must, nitrogen composition, aeration, yeast strain and inoculation level.[5]

Glycerol is a nonvolatile compound, but bigger glycerol concentration contributes significantly to the sweetness, body and fullness of wines, although a concentration of 25.8 $gL^{-1}$ has been proposed as a level at which an increase in viscosity can be perceived.[6] For these reasons,glycerol production is one of the desirable features during grape must fermentation.
Glycerol is also interesting fermentation by-product as a component which should be considered in the selection of wine yeast strains.[23]

When grape must or juice is inoculated with *S. cerevisiae,* ethanol is not immediately produced. Normally, fermentation leads to an excess of energy (ATP) within the cell. This condition will activate enzymes (pyruvate decarboxylase/dehydrogenase[19]) which consequently causes formation of succinate through the TCA cycle and in turn leads to an excess of reduced respiratory nucleotides. As a consequence the excess NADH is formed (Fig. 1) and used in glycerol production as it can be seen from the pathway of glycerol formation (Fig. 2).

Microbiological influence in glycerol formation and consumption (degradation) is observed in wines. It is well established that there is a difference in the amount of glycerol production by various yeast strains during fermentation and this is in correlation with different concentration of glycerol-3-phosphate dehydrogenase in different yeasts.[21] The strong conversion of glycerol to dihydroxyacetone by acetic acid bacteria is widely reported.[24] This may have an important impact to sensory properties of the wine. Certain lactic acid bacteria can metabolize glycerol by dehydration and also influence on quality of wine.

To ensure the quality of wine there are some obligatory analitical methods for the determination of relevant wine constituents, but not for glycerol.[28] From the literature can be seen that glycerol in wine is possible to determine in a number of ways but until recently the procedure was time consuming. The old method was to separate, purify and actually weigh the glycerol. In chemical determination most widely is used colorimetric method published by *Rebelein* in year 1956.[30] Enzymatic methods for glycerol determination are more specific than chemical ones. Flow injection analysis (FIA) using glycerol dehydrogenase and fluorometric determination appears especially usefull for reliable routine analyses because it is specific for glycerol and provides results that are unaffected by preliminary treatment, especially when the sugar content does not exceed 5 $gL^{-1}$ (Fig. 3).[34] Also chemiluminometric determination of glycerol by means of glycerol dehydrogenase and NADH oxidase is often used (Fig. 4).[34]

Gas chromatography determination of glycerol can be made by derivatization of glycerol before analysis to get more volatile compound or very successful by direct analysis of diluted wine samples using Chromosorb 101 column.[15]

Direct analysis of the major organic compounds, including glycerol, in grape must and wine was performed in many articles (Table 2) using anionic- exchange column system (HPLC) and RI/UV detection. HPLC methods have good possibilities for glycerol determination in combination with other wine constituents such as acids and sugars. Simultaneous determination of sugars, acids and glycerol, was made with fully automated sequential injection system with Fourier transform infrared (FT-IR) detection.[57] The detection limit of 0.2 mg $mL^{-1}$ for analysed compounds demonstrates that flow cell-based FT-IR detection is compatible for practical applications in aqueous phase HPLC. A rapid automated method for wine analysis based upon sequential injection (SI)-FT-IR spectrometry were established by *K. Schindler* and coworkers.[68] In comparison with conventional HPLC, the proposed techniques increase the speed of the analysis. The short analysis time together with high reproducibility make the developed method applicable to process control and screening purposes. Average of standard deviation for glycerol was 0.037 $gL^{-1}$.[68]

Application of biosensors for glycerol monitoring during fermentation (Fig. 5)[65] is based on an enzymatic reaction catalyzed by glycerol dehydrogenase and spectrofluorometric detection.[65] Spectrophotometric determination of glycerol in white and red wines was made using glycerol kinase and glycerol-3-phosphate oxidase (Fig. 6) with 1 % accuracy.[73] The optimized biosensors and biosensing systems (Table 3) were used for analysis of glycerol, glucose, and ethanol in wine with detection limit of 0.1-1 mol $L^{-1}$ [82] for glycerol. The main advantage of probe-type biosensor with flow-through sensors is the availability of former for working, both in batch and in continuous approaches, thus making possible their use for *in situ* measurements.Their performance can also be automated when included in a dynamic manifold.

Adulteration of wine by addition of industrial grade glycerol to wine can be detected by GC/MS determination of typical substances added together with industrial glycerol to wine and this are 3-methoxy-propan-1,2-diol (3-MPD), and/or cyclic diglycerols (CycD).[85] Added synthetic glycerol to wine can be determined through isotopic pattern of glycerol. Addition of glycerol from animal sources must be studied through the corresponding $^{2}H$-pattern.[84]

Key words: *Wine, glycerol, glycerol determination*

## Introduction

Glycerol is the major fermentation product of *Saccharomyces cerevisiae* after ethanol and carbon dioxide that can indirectly contribute to the sensory character of wine.[1,2]

Typically it is produced at concentration of 4-10 $gL^{-1}$ in dry table wines, but occasionally, may already be present in grape musts infected by moulds (*Botrytis cinerea*).[3,4] The amount of glycerol formed during fermentation is influenced by several factors, such as grape variety, degree of ripeness, fermentation temperature, $SO_2$ concentration, pH of grape must, nitrogen composition, aeration, yeast strain and inoculation level.[5] The treshold taste level of glycerol is observed at 5.2 $gL^{-1}$ in wine. Glycerol is a nonvolatile compound but contributes significantly to the sweetness, body and fullness of wines, although a mass concentration of 25.8 $gL^{-1}$ has been proposed as a level at which an increase in viscosity can be perceived.[6] For these reasons, glycerol production is one of the desirable features during grape must fermentation.

Table 1 contains ranges and average values of glycerol found in wines of several countries with different analytical methods. For example, the influence of variety, surroundings and wine production on the composition of Austria wines was investigated including glycerol determination (method was not refered).[7]

Therefore it is necessary to know real content of glycerol, and sometimes also concentration of added industrial glycerol for possibile adulteration of wine.

Table 1 — *Mass concentrations of glycerol found in wines from various countries*

Tablica 1 — *Masene koncentracije glicerola nađene u vinima različitih zemalja*

| Country<br>Postojbina | Number of samples<br>Broj uzoraka | Glycerol $gL^{-1}$<br>Glicerol $gL^{-1}$ | | References<br>Literatura |
|---|---|---|---|---|
| | | range<br>raspon | average<br>sred. vrijedn. | |
| Austria | 525 | 4.0-35.7 | 19.85 | 7 |
| France | 71 | 6.59-23.0 | 8. 6 | 8 |
| Germany | 146 | 2.0-20.0 | 7. 7 | 9 |
| Italy | 87 | 3.26-12.40 | 7. 83 | 10 |
| Japan | 42 | 4.90-12.40 | 7. 4 | 11 |
| Japan | 80 | 3.57-9.59 | 7. 1 | 12 |
| USSR | 13 | 1.11-4.32 | 2. 9 | 13 |
| USA | 100 | 1.9-14.7 | 7. 2 | 14 |
| Croatia | 13 | 5.6-10.43 | 7. 75 | 15 |
| Spain | 36 | 2.15-8.16 | 5. 16 | 16 |
| Spain | 21 | 4.23-8.58 | 6. 41 | 17 |
| Croatia | 20 | 5.03-11.0 | 6. 99 | 18 |

## Biochemistry

When grape must or juice is inoculated with *Saccaharomyces cerevisiae*, ethanol is not immediately produced.

Normally, fermentation leads to an excess of energy (ATP) within the cell. This condition will activate pyruvate decarboxylase/dehydrogenase,[19] which consequently causes formation of succinate through the TCA cycle and, in turn, leads to an excess of reduced respiratory nucleotides (Fig.1):

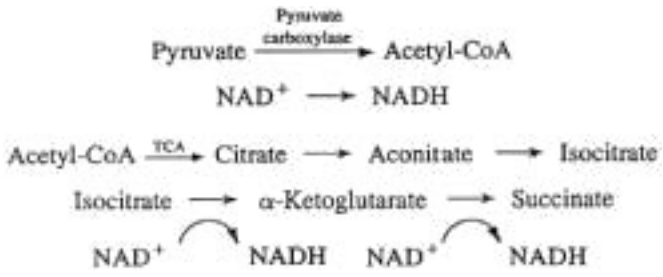



Fig.1 — *Formation of reduced respiratory nucleotides*[19]

Slika 1 — *Nastajanje reduciranih respiratornih nukleotida*[19]

The excess NADH is used to form glycerol. This allows the optimum redox state of the cell to be maintained.

In respiring cells, at the beginning of fermentation, pyruvate decarboxylase and alcohol dehydrogenase activities are not high, both of these enzymes being inducible by glucose.[20] As a consequence, compounds other than ethanol are produced at beginning of grape juice fermentation and these are glycerol, pyruvate, succinate and other organic acids.

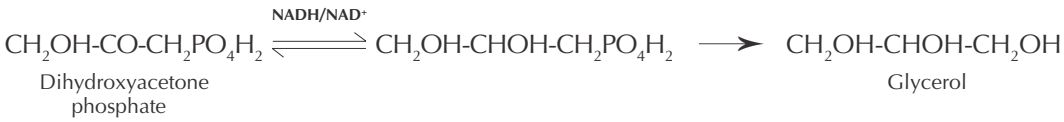



Fig. 2 — *Pathway of glycerol formation as a by-product*[19]
Slika 2 — *Put stvaranja glicerola kao nusproizvoda*[19]

Dihydroxyacetone phosphate can be reduced to glycerol phosphate, converting one molecule of NADH to $NAD^+$ as shown in Fig. 2.

Glycerol phosphate is then dephosphorylated yielding glycerol. While this pathway allows regeneration of $NAD^+$, no ATP is produced. A ratio between pyruvate and glycerol seems to be maintained as these substances are found in equimolar concentration in the medium. Both may be reconsumed later in fermentation. Production of other by-products such as butane-2,3-diole is also facilitated.[18] Red wines usually have greater amounts of glycerol than white ones. This is partially because a higher fermentation temperature is used in the production of red wines.[5]

## Microbiology

It is well established that there is a difference in the amount of glycerol formed by various yeast strains during fermentation. Using identical media and comparable conditions of fermentation even within the species *S. cerevisiae*, amounts of 4.2 to 10.4 $gL^{-1}$ glycerol were observed by different strains.[21] Not more than 0.05 U (unit) of glycerol-3-phosphate dehydrogenase were observed in the normal yeast strain, whereas 0.13-0.21 U were found in the strain that produced large amounts of glycerol. Many factors have been reported to influence the formation of glycerol as earlier was mentioned i.e. oxygen, fermentation temperature and pH.[5] These factors are obviously not very important, particularly when the range of the pH is kept between 2.8 and 5.0.

The total amount of glycerol formed was also influenced by amino acids. In thiamine deficient media a decrease in glycerol formation was observed. Experiments[21] indicate a correlation between the formation of acetaldehyde and glycerol and the production of cell mass that may be of practical interest.

*Sponholz* at al[22] reported that *Kloeckera apiculata* and *Candida stellata* produced significantly more glycerol when compared with *Saccharomyces cerevisiae*.

Glycerol is also interesting fermentation by-product as component which should be considered in the selection of wine strains.[23]

The strong conversion of glycerol into dihydroxyacetone by acetic acid bacteria is widely reported and may have an important impact on sensory properties of the wine.[24]
Certain lactic acid bacteria also may metabolize glycerol by dehydration, producing acrolein and so influencing on organoleptic character of wine. [24]

Investigations of *P. Romano* and coworkers[25] proved that selected strains of apiculate yeasts have ability to produce glycerol and so yield desirable caracteristics to the final product. Despite equal ethanol production in that case, the glycerol/ethanol ratio[26] which should be 1:10, varied significantly, due to a great variability in glycerol produced. Yeasts can also use glycerol as a carbon source during respirative growth in producing sherry wines.[27]

### Determination of glycerol

In spite of diversity of wine for certain substances, concentration ranges are prescribed by law to ensure the quality of wine. Obligatory analytical methods for the determination of relevant analytes, but not for glycerol, are given in a decree of the European Economic Community (EEC).[28] Minimal mass concentration of glycerol in wine in legislation of Croatia is 5 $gL^{-1}$ for wines on the market.[29]

From the literature can be seen that glycerol in wine industry is possible to determine in a number of ways, but until recently the procedure was time-consuming. The old method was to separate and purify and actually weigh the glycerol. Rapid chemical, enzymatic, gas chromatography and liquid chromatography methods make now the analysis simpler and more accurate. Adulteration by addition of industrial grade glycerol to wine can be detected either by determination of typical substances added together with industrial glycerol in wine or on the D/H isotopic ratios as well.

### Chemical determination

The most widely used chemical method is colorimetric method published by *Rebelein*.[30] Sufficient clarification and purification is due to eliminate most interfering substances. The method is also outlined in the official handbook of the OIV.[31] In the official methods of analysis of the AOAC, glycerol is possible to determine by oxidation with dicromate.[32] Determination of glycerol in must, wine and desert wine is also made with colorimetric reaction of glycerol with chinolin.[33]

### Enzymatic determination

The enzymatic methods for glycerol determination can be classified in the three groups.

The first one is based on a series of enzymatic reactions using glycerol kinase in combination with either pyruvate kinase and lactate dehydrogenase or with glycerol-3-phosphate dehydrogenase.[34]

Another class uses glycerol oxidase, which catalyses the reaction:

$$\text{Glycerol} + O_2 \qquad \text{Dihydroxyacetone} + H_2O_2 \qquad (1)$$

As the glycerol oxidase is not commercialy available these methods are not widely used.

The third group of enzymatic methods is based on the reaction of glycerol dehydrogenase shown in the reaction:

$$\text{Glycerol} + NAD^+ \qquad \text{Dihydroxyacetone} + NADH\text{-}H \quad (2)$$

The amount of NADH formed can be monitored either by amperometric[35] or by optical methods. Optical determination of NADH concentrations are based on absorbancy[36] or fluoroscence measurements.[37]

It is also posibile to combine (1) and (2) equotions shown in eq. (3):

$$NADH\text{—}H + O_2 \qquad NAD^+ + H_2O_2 \quad (3)$$

and monitoring $H_2O_2$ by chemiluminometric method in combination with flow injection analysis (FIA).

Schematic diagrams of the FIA system for the fluorometric determination of glycerol via NADH and for the chemiluminometric detection are shown in Fig. 3 and Fig. 4, respectively. With both systems a sample frequency of 40 samples per hour was achived.[36]

The samples of wines analysed by fluorometric (Fig. 3) or chemiluminometric (Fig. 4) method, respectively, were diluted with distilled water to an appropriate concentration prior to analysis.

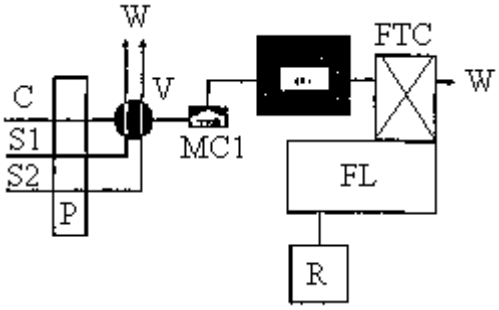



Fig. 3 — *FIA arrangement for the fluorometric determination of glycerol by means of GlyDH. The sample S2, containing glycerol and a solution of NAD⁺ S1, was introduced simultaneously into the carrier stream C by means of an injection valve V. The magnetically stirred mixing chamber MC 1 enhanced the mixing of both streams and the dispersion of the introduced sample. GlyDH immobilised in an enzyme cartridge ER 1 converted glycerol into dihydroxyacetone by reducing NAD⁺ to NADH. A thermostat kept a constant temperature of 30 °C for the enzyme reaction. The produced NADH was detected in a flow-through cell FTC with a fluorometer FL (ex/em = 340/460) and the peaks were recorded by a recorder R. All solutions were pumped into waste W by a pump P.*[34]

Slika 3 — *FIA uređaj za fluorometrijsko određivanje glicerola pomoću GlyDH. Uzorak S2 koji sadrži glicerol i S1 otopina NAD⁺, uvode se istodobno u mobilnu fazu C pomoću injekcijskog ventila V. Ćelija MC1 s magnetskim miješanjem povećava miješanje obiju struja i disperziju uvedenog uzorka. GlyDH imobilizirana u enzimskoj ćeliji ER1 prevodi glicerol u dihidroksiaceton uz redukciju NAD⁺ u NADH. Temperaturu enzimske reakcije od 30 °C održava termostat. Nastali NADH je određen u protočnoj ćeliji FTC s fluorometrom FL (ex/em = 340/460) a pikove bilježi pisač R. Sve otpadne otopine W odvode se pomoću crpke P.*[34]

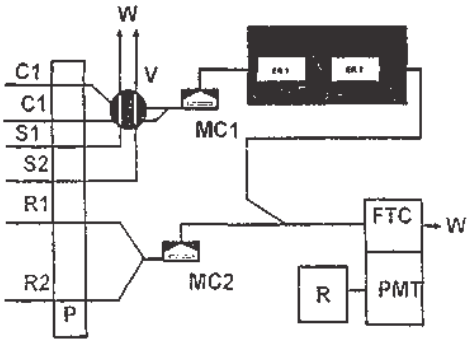



Fig. 4 - *FIA arrangement for the chemiluminometric determination of glycerol by means of GlyDH and NADH oxidase. The sample S1 containing glycerol and a solution of NAD⁺ S2 were introduced simultaneously into the carrier stream C1 by means of an injection valve V. The magnetically stirred mixing chamber MC 1 enhanced mixing of the streams and the dispersion of the introduced sample. The sample passed two subsequent enzyme reactors thermostated at 30°C (black box = thermostat). In the first one ER 1 with immobilised GlyDH, the dehydrogenase reaction took place and NADH was produced. In the second one ER 2, immobilised NADH oxidase oxidised NADH by reducing molecular oxygen to hydrogen peroxide. $H_20_2$ was detected in a flow-through chamber FTC of a luminometer (PMT = photomultiplier tube) by chemiluminescence using luminol and potassium hexacyanoferrate (III), the peaks were recorded by a recorder R. The reagent solutions of luminol R1 and potassium hexacyanoferrate (III) R2 were mixed in the mixing chamber MC2 immediately before the chemiluminescence reaction with $H_20_2$ took place to avoid side reactions. All solutions were pumped (P = pump) into waste W.*[34]

Slika 4 - *FIA uređaj za kemiluminometrijsko određivanje glicerola pomoću GlyDH i NADH oksidaze. Uzorak S1 koji sadrži glicerol i S2 otopina NAD⁺, uvode se istovremeno u mobilnu fazu C1 pomoću injekcijskog ventila V. Magnetski mješajuća ćelija MC1 s magnetskim mješanjem, povećava mješanje struja i disperziju uvedenog uzorka. Uzorak prolazi dva serijski spojena enzimska reaktora termostatirana na 30°C (crna kutija = termostat). U prvom ER 1 s imobiliziranom GlyDH odvija se reakcija s dehidrogenazom i nastaje NADH. U drugom ER 2 imobilizirana NADH oksidaza oksidira NADH reduciranjem molekularnog kisika u vodikov peroksid. Koncentracija $H_20_2$ određuje se u protočnoj ćeliji FTC luminometra (PMT = fotomultiplikatorska cijev) kemiluminiscencijskom reakcijom koristeći luminol i kalijev heksacijanoferat (III), a pikove bilježi pisač R. Otopine luminola R1 i kalijevog heksacijanoferata (III) R2 mješaju se u ćeliji za miješanje MC2 neposredno prije kemiluminoscentne reakcije s $H_20_2$ zbog sprečavanja sporednih reakcija. Sve otpadne otopine odvode se pomoću crpke P u otpad W.*[34]

The detection limit for glycerol was 2.5 x $10^{-5}$ mol $L^{-1}$ with chemiluminiscence measurements and with the fluorometric detection quite the same.[34]

## Gas chromatography determination

Glycerol can be determined by gas chromatography using the proper column and conditions. Early methods used derivatization technique like acetylation [38,39] or silylation [40,41,42] of glycerol. Sometimes it is necessary make wine pretreatments, for example, sweet wines must undergo an ion exchange pretreatment.[41] Using microporous beads, in several investigations,[43,44,45,46] glycerol was directly measured in wine. A promising column was 10 % SP-1000 on Chromosorb W [45] and Chromosorb 101 using gas adsorp-

tion chromatography.[15] Glycerol was directly determined in kiwi wines on classic column Tenax 60/70 mesh.[47] On Chromosorb 101 column varietal differentiation of red wines in the Valencia region in Spain [48] was made on the basis of glycerol determination. The efect of glycerol on the perceived aroma of a model wine and a white wine was studied by capillary gas chromatography.[49] Production of glycerol together with other diols during fermentation with different yeast strains was analysed on capillary column by acetylation of diols.[50]

The detection limit for glycerol in gas chromatography was different depending on method of determination and used instrumentation, including column, preparation of sample etc. Some investigators found = 1.9 % [46], and onother one from 4 % [16] to 4.39 %.[17]

**Liquid chromatography determination**

The isolation and determination of distinct compound or compounds could be solved by high performance liquid chromatography (HPLC) using ion-exchange materials based on polystyrene-divinylbenzene resins in combination with a suitable eluent and specific detection.

There are two groups of possibile detection. The first one is UV spectrophotometric detection for organic acids. Refractiv index (RI) detector is usually used for sugars and glycerol detection at the same time. The another one has a combination of UV spectrophotometric and chromoscope high-speed scanning UV detector together using RI detector [51] with the possibility to determine more then one compound in wine at the same time.

Table 2 – *HPLC systems used for glycerol determination*
Tablica 2 – *HPLC sistemi korišteni za određivanje glicerola*

| Compounds analized<br>Analizirani spojevi | Type of sample<br>Tip uzorka | Sample preparation<br>Obrada uzorka | Column<br>Kolona | Mobile phase<br>Mobilna faza | Detection<br>Određivanje | Reference<br>Literatura |
|---|---|---|---|---|---|---|
| Organic acids<br>Sugars<br>Glycerol | wine<br>vinegar | elution<br>filtration | SPE-$NH_2$ | 0.03 mol $L^{-1}$<br>$H_2SO_4$ | RI | 52 |
| Organic acids<br>Sugars<br>Glycerol | must<br>wine | dilution<br>filtration | ION-300<br>kation-exch | 0.0015 mol $L^{-1}$<br>$H_2SO_4$ | RI | 53 |
| Organic acids<br>Sugars<br>Glycerol<br>Ethanol | must<br>wine | filtration | AminexHP<br>X-87H<br>75 °C | 0.65 mmol $L^{-1}$<br>$H_2SO_4$ | RI/UV | 54 |
| Glycerol<br>Ethanol<br>Acetate<br>Succinate<br>Pyruvate | synthetic<br>medium | without<br>preparation | Aminex<br>HPX-87H<br>45 °C | 8 mol $L^{-1}$ $H_2SO_4$ | UV/RI<br>214nm | 55 |
| Organic acids<br><br>Ethanol<br>Sugars<br>Glycerol | wine | filtration | ION-300<br>71 °C | 0.01 mol $L^{-1}$<br>$H_2SO_4$ | UV/RI<br>210nm | 56 |
| Carbohydrates<br>Organic acids<br>Glycerol<br>Ethanol | wine | filtration | polystyrene-<br>dyvinylbenz-<br>ene | 0.005 mol $L^{-1}$<br>$H_2SO_4$ | FT-IR* | 57 |
| Glycerol<br>propane-1,3-<br>diol | glycerol bio<br>conversion | cetrifugation<br>filtration<br>dilution | Aminex<br>HPX-87H<br>35 °C | 0.01 mol $L^{-1}$<br>$H_2SO_4$ | RI | 58 |
| Glucose<br>Glycerol<br>Methanol | synthetic<br>media | filtration | Aminex<br>FAO<br>60 °C | 0.01 mol $L^{-1}$<br>$H_2SO_4$ | RI | 59 |
| Sugars<br>Glycerol<br>Ethanol<br>Organic acids | wine | filtration | Bio-Rad<br>HPX-87H<br>65 °C | $w$ = 0.065 %<br>$H_3P0_4$ | U/IR | 60 |
| Acids<br>Glycerol | sherry<br>vinegar | without<br>preparation | Fast fruit<br>juice column<br>> 55 °C | $w$ = 0.025 %<br>$H_3P0_4$ | UV/IR | 61 |

*FT-IR-Fourier transform infrared spectroscopy

Direct analysis of the major organic components, including glycerol, in grape must and wine was performed in many articles (Table 2). Kation exchange column system with high performance liquid chromatography (HPLC) and RI/UV detection usually is used. The statistical analysis showed good agreement in accuracy and precision between HPLC and conventional volumetric, distillation and enzymatic methods compared in investigated case.[62]

Using sample preparation on intermediate anion exchange resin, separating was employed to the normally co-eluting compounds like fructose and malic acid [63] in combination with glycerol detection.

Fig 5. shows details of the practical implementation of developed sensor and used application. Elution time was very short (1 min) and volume of 500 l of eluent was sufficient for quantitative removal of monitored product.

Glycerol was monitored with HPLC during biological aging of fino sherry by *P. Martinez* and coworkers.[66]

Characterization of red wine and differences between grape varieties was made by determination of glycerol and organic acids by HPLC and volatile compounds by GC.[67]

Simultaneous determination of sugars, acids, and glycerol was made with fully automated sequential injection system with Fourier transform infrared (FT-IR) detection (HPLC-IR). The short analysis time together with high reproducibility makes the developed method applicable to process control and screening purposes. Average of standard deviation for glycerol was 0.037 g $L^{-1}$.[68]

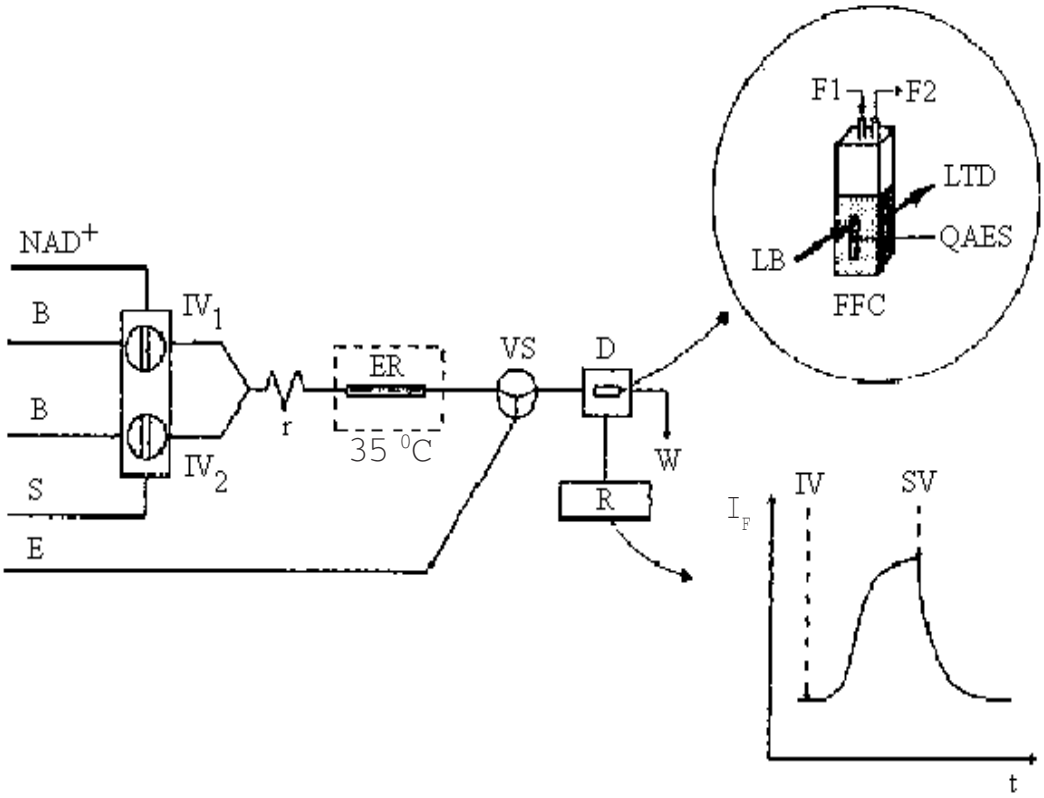



Fig. 5 — *Flow injection manifold for the implementation of the method for glycerol based on an enzymic reaction and integrated retention/spectrofluorometric detection; B, buffer; S, sample; E, eluent; IV 1, 2 injection valve; SV, switching valve; ER, enzymic reactor; r, coil; D, spectrofluorometric detector; FFC, fluorometric flow-cell; F1, flow in; F2, flow out; LB, light beam; LTD, light to the detector; QAES, QAE sephadex;* $\boldsymbol{I_F}$*, fluorescence intensity;* ***t****, time and W, waste*[65]

Slika 5 — *Protočno ubrizgavajuči umnoživač za određivanje glicerola zasnovan na enzimskoj reakciji i integriranom zadržavanju / spektrofluorometrijskom određivanju; B, pufer; S, uzorak; E, eluent; IV 1,2 ubrizgavajući ventil; SV, ventil za isključivanje; ER, enzimski reaktor; r, spirala; D, spektrofluorometrijski detektor; FFC, fluorometrijska protočna ćelija; F1, dotok; F2, izljev; LB, svjetlosna zraka; LTD, svjetlo prema detektoru; QAES, QAE sefadeks;* $\boldsymbol{I_F}$*, intenzitet fluoroscencije;* ***t****, vrijeme i W otpad*[65]

Glycerol concentration during grape juice fermentation by *S. cerevisiae* Y7 at 20 °C in the presence of $w = 150 \cdot 10^{-6}$ of sulfites with no agitation was HPLC analysed using Aminex HPX-87H Bio-Rad column and refractive index monitor. [64]

A flow-through sensor based on transient retention of the product of the reaction of glycerol with $NAD^+$ catalysed by glycerol dehydrogenase was described by *P. Canizares* and coworkers.[65] Obtained results were compared with conventional method based on reversed-phase HPLC with refractometric detection and flow-cell packed with QAE-Sephadex weakly anionic resin.

For studying retention times as a function of column temperature HPLC-FT-IR, new and versatile tool for direct determination of the main components of wine: sugars, alcohols, acids and glycerol in standard solution and in wine, showed the potential possibility of flow cell-based application.[57]

Glycerol overproduction is possible by engineered *S. cerevisiae* wine yeast leading so to substantial changes in by-product formation together with stimulation fermentation rate in stationary phase.[69]

Using HPLC method glycerol was determined during fermentation of Sauvignon blanc with good correlation between investigated parameters.[70]

It can be seen that HPLC methods have good possibilities for glycerol determination in combination with other wine constituents such as acids and sugars.

## Application of biosensors for glycerol monitoring during fermentation

As the most important secondary product of alcoholic fermentation, glycerol can be monitored during wine fermentation on the basis of two different biosensors, each based on a different detection technique.

One is a probe-type amperometric biosensor (A) based on immobilization of glycerol kinase and glycerol-3-phosphate oxidase on specific membranes with a detection limit of 5 x $10^{-7}$ mol $L^{-1}$.[65]

A fluorometric flow-through biosensor (B) is another one posibility for glycerol detection. Method is based on the based on amperometric detection used the biocatalyst immobilised on polymeric support,[71] isotiocyanate controlled glass[72] or amino-cellufine[80] in the case of glycerol dehydrogenase and glycerol-3-phosphate oxidase.[65] Adenosine triphosphate (ATP) and glycerol has been determined enzymaticaly using an automated FIA system[73] (Fig. 6). Each run started automatically by initiating the injection valve with the PC. Glycerol was determined in white and red wines after volume 1 : 5000 dilution. The enzymes glycerophosphate oxidase (GPO) and glycerol kinase (GK) have been immobilized on non-porous glass and packed in two bioreactors coupled in series, thus allowing the determination of glycerol in the Single Bed String Reactor (SBSR) in the range 4-70 μmol $L^{-1}$.[73]

The low limit of quantification, 1 μmol $L^{-1}$ for glycerol with 1 % accuracy, shows that the proposed method offers many significant improvements over conventional methods.

The reactions taking place during this procedure are:

$$\text{Glycerol} + \text{MgATP} \xrightarrow{\text{GK}} \text{—GP} + \text{ADP}$$

$$\text{-GP} \xrightarrow[O_2]{\text{GPO}} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{AAP} + \text{DCPS} \xrightarrow{\text{PO}} H_2O_2 + \text{Red color}$$

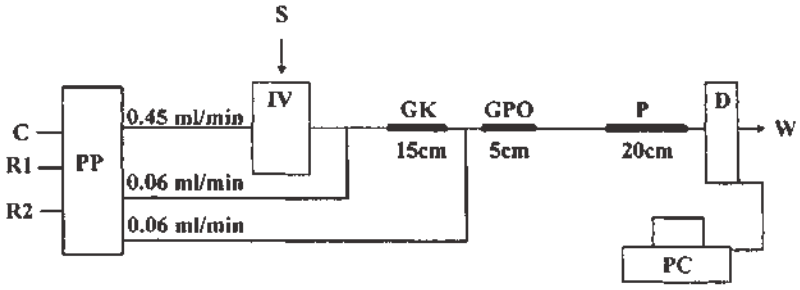



Fig. 6 – *Experimental set-up and FIA manifold, PP: peristaltic pump, GK: SBSR with immobilised GK, C: Carrier (Tris.HCl 0.05 mol $L^{-1}$ pH 8.2), IV: injection valve, GPO:SBSR with immobilised GPO, D: detector, S:sample (Glycerol in Tris. HCl 0.05 mol $L^{-1}$ pH 8.2), P: Plain SBSR, R1: Reagent 1 (ATP+$MgCl_2$ in Tris.HCl 0.05 mol $L^{-1}$ pH 7.0), PC: Personal Computer, R2: Reagent 2 (ATP+ DCPS in Tris. HCl 0.05 mol $L^{-1}$ pH 7.0)* [73]

Slika 6 – *Eksperimentalno podešavanje i FIA umnoživač, PP: peristaltska pumpa, GK: SBSR s imobiliziranom GK, C: Nosač (Tris. HCl 0.05 mol $L^{-1}$ pH 8.2), IV: injektorski ventil, GPO:SBSR s imobiliziranom GPO, D: detektor, S: uzorak (glicerol u Tris. HCl 0.05 mol $L^{-1}$ pH 8.2), P: Plosnati SBSR, R1: Reagens 1 (ATP+$MgCl_2$ u Tris.HCl 0.05 mol $L^{-1}$ pH 7.0), PC: osobni kompjutor, R2: reagens 2 (ATP+ DCPS u Tris.HCl 0.05 mol $L^{-1}$ pH 7.0)* [73]

simultaneous injecting of sample and coenzime through a bioreactor packed with glycerol dehydrogenase immobilized on controlled-pore glass[76] before reaching a flow-cell packed with QAE-Sephadex, which retained the reduced form of the coenzyme for luminescence detection.

The biosensing systems for the determination of glycerol (Table 3) proposed so far have based on continuous work in an automatic or semi automatic manner. Those systems

Better ranges were achieved when flourometric detection was used: 0.3-3 mmol $L^{-1}$ [74], and with photometric detection 0.3-300 mol $L^{-1}$.[75]

A collaborative study of the enzymatic determination of glycerol based on the use of glycerol kinase, pyruvate kinase and lactate hydrogenase with measurement of the NADH consumed from the decrease in the absorbance at 340 nm, showed $_s$ of 0.7 % and 2.3 %, respectively.[79]

Amperometric biosensors for the determination of glucose, fructose, ethanol and glycerol were used to monitor alcoholic fermentation during red wine production in industrial-scale plants.[81] Measurements of the four marker analytes were carried out at-line by sampling the must from the fermentors tanks. However, all biosensors assembled in a flow-injecion analysis manifold can be used for on-line monitoring by adding an appropriate sampling device to the fermentors.[81] This approach is promising for the detection and prevention of unwanted metabolic changes during alcoholic fermentation.

possible their use for *in situ* measurements.Their performance can also be automated when included in a dynamic manifold. The main advantage of flow-through biosensors is their capacity for the retainment of a higher amount of the biochemical species in the flow-cell.

One of the most significant advantages of continuous biosensing systems as compared with flow-through biosensors is the availability of using a great variety of materials as support for the biochemical compound. The amount of the biochemical compound-solid support con-

Table 3 – *Glycerol monitoring in wine by biosensors (A) and biosensing systems (B)*

Tablica 3 – *Glicerol promatran u vinu biosenzorima (A) i biosenzornim sistemima (B)*

| Type<br>Tip | Technique<br>Tehnika | Biomaterial<br>Biomaterijal | Features<br>Izvođenje | Reference<br>Literatura |
|---|---|---|---|---|
| 1. A | Amperometry | Glicerokinase / glycerol-3-phosphate oxidase / Immobilization | Batch measurements | 71 |
| 2. A | Fluorometry | Dehydrogenase / Immobilization | F. I. flow-through biosensor Integrated retention / detection | 65 |
| 3. A | Fluorometry | Dehydrogenase / Immobilization | F. I. | 74 |
| 4. A | Photometry | Dehydrogenase / Immobilization | F. I. | 75 |
| 5. A | Fluorometry | Dehydrogenase / Immobilization | F. I. | 76 |
| 6. A | Amperometry | Dehydrogenase / Immobilization | F. I. graphite electrode | 72 |
| 7. B | Amperometry | Dehydrogenase / Immobilization | F. I. | 80 |
| 8. B | Amperometry | Glycerol kinase / glycerol oxidase / Immobilization | F. I. on-line monitoring | 73 |
| 9. B | Photometry | Kinase / in solution | Collaborative study | 79 |
| 10. B | Fluorometry | Dehydrogenase / NADH oxidase Immobilization | F. I. in series bioreactors | 34 |
| 11. B | Chemiluminometry | Kinase / oxidase / Immobilisation | F. I. luminol, co-immobilization | 77 |
| 12. B | Chemiluminometry | Dehydrogenase / oxidase Immobilisation | F. I. luminol, co-immobilization | 78 |
| 13. A | Amperometry | Glycerol kinase Glycerol-3-phosphate oxidase | F.I. / Pt electrode | 81 |
| 14. A | Amperometry | Glycerol dehydrogenase | F. I. graphite electrode | 82 |
| 15. A | Amperometry | Glycerol dehydrogenase | F. I. modified graphite electrode | 83 |

By *M. Niculescu* and *coworkers*[82,83] optimized biosensors were used in analysis of glycerol together with glucose and ethanol.These studies illustrate the successful use of biosensors for the detection of glycerol with detection limit of 0.1 mol $L^{-1}$.The developed biosensors are suitable for the close control of alcoholic fermentation of wine in industrial mass production.

The main advantage of probe-type as compared with flow-through sensors is the availability of the former for working, both, in batch and in continuous approaches, thus making

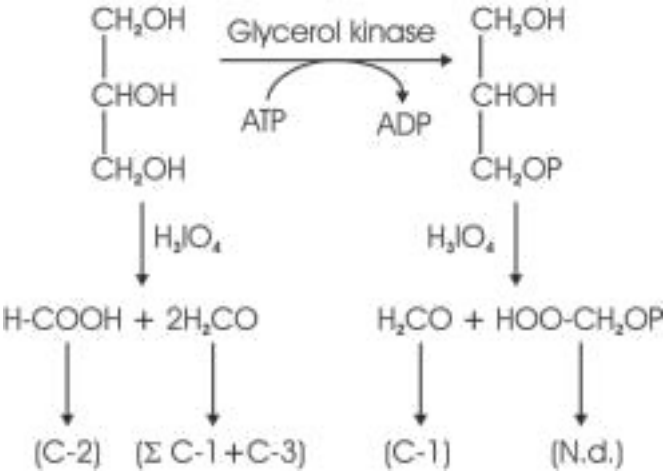
jugate is not limited by the dimensions of the flow-cell, and the reactor can be of the required size, thus endowing the method with higher sensitivity if required. In addition, the immobilization step and the packing on an appropriate column is easier than the construction of a flow-through biosensor, as also is the exchange when the loss of activity makes it unsuitable. Crucially depending on the type of wine, the bioreactor location is very easy to change in the continuous system. The demonstrated better performance of separately immobilized biocatalyst[71] is easy to implement in continuous biosensing systems.

## Methods for determination of industrial added glycerol

An illegal addition of glycerol to wine could be detected on the basis of isotopic correlations, either of global $^{13}C$-values or of isotopic patterns. Intermolecular isotopic balances exist in complementary positions of natural products originating from the same source. Fixed isotopic correlations and complementary patterns have been found.[84]

Isotopic patterns of different molecules from the same precursor are correlated to each other. Behind a metabolic branching point, an isotope effect may lead to a depletion in a given position of one product, and this has as a consequence a corresponding enrichment in the other product. Recently is found a kinetic and an equilibrium isotope effect on the reaction, which is probably responsible for the $^{13}C$-enrichment in position 3 and 4 of glucose. Assuming that, due to the triose phosphate isomerase reaction, a scrambling between position 3 and 4, 2 and 5, 1 and 6 of glucose occurs in trioses. The same was expected for glycerol, an immediate descendent of dihydroxyacetone phosphate.

Degradation of glycerol with periodic acid for positional $^{13}C$ analysis can be seen in the reaction which gave formic acid and formaldehyde:[84]




Phosphorylation takes place only in position 3 of the molecule.

This position is identical to the originally phosphorylated position 1 of dihydroxyacetone phosphate.The results obtained for glycerol from various natural origins indicate a dramatic relative $^{13}C$-depletion in position 1, while the delta-values in positions 2 and 3 and even their differences are, at least in the case of plant products, only slightly changed as compared to the corresponding values of glucose.The observed global depletion of natural glycerol relative to its precursor is obviously solely due to a depletion in position 1. Dihydroxyacetone phosphate is the primary precursor of any other compound outside the cloroplast of plants.

Synthetic glycerol showed stastistical $^{13}C$-pattern of depletion, and therefore it will certainly be possible to prove the addition of synthetic glycerol to wine taking into account the large delta $^{13}C$-value difference between the global value and position 1 or between the values for position 1 and 2. The proof of an addition of glycerol from animal sources will not be easy, because only smaller differences exist.[84] Further studies will therefore include the corresponding $^{2}H$-pattern of glycerol from different sources.

The components 3-methoxy-propan-1,2-diol (3-MPD) and/or cyclic diglycerols (CycD) are not naturally occuring substances in grapes or wine according to both current literature as well as the results of *U. Lampe* and coworkers[85] showed. Neither they are formed during the enological process, nor significant amounts are brought in by admitted processing agents. Therefore the detection of 3-MPD and/or CycD is proof of an illegal addition of industrial grade glycerol to wine after used GC/MS quantification.

## Conclusion

1. Enzymatic methods for glycerol determination are more specific than chemical ones.

2. With HPLC more constituents of wine (including glycerol) can be monitored at the same time than with GC or enzymatic methods.

3. The fluorometric procedure appears especially useful for reliable routine analyses, because it is specific for glycerol and provides results that are unaffected by preliminary treatment, especially when the sugar content does not exceed 5 g $L^{-1}$.[41]

4. With consideration of some basic rules, ion chromatography with resin-based materials is powerful analytical technique in the food and beverage industry, but it is necessary to know sample matrix, separation behaviour of the different ion-exchange columns (eluent concentration, temperature, flow-rate, counter ion), chemical properties of analyte substances and simultaneously eluted compounds under distinct conditions. Using ion chromatography glycerol can be very good detected with resin-based ion-exchange columns.

5. On-line measurement with biosensors in real wine samples illustrate the successful use of immobilized enzymes for the glycerol determination with detection limit of 0.1 mol $L^{-1}$.[82]The developed biosensors are suitable for the close control of alcoholic fermentation of wine industrial mass production[81] and for the detection and prevention of unwanted metabolic changes during alcoholic fermentation.

6. Adulteration of wine is possible to determine through isotopic pattern of glycerol, only when synthetic glycerol is added to wine. Addition of glycerol from animal sources must be studied through the corresponding $^{2}H$-pattern.

7. GC/MS detection of 3-MDP and/or CycD is also proof of an illegal addition of industrial grade glycerol to wine.

## List of simbols
## Popis simbola

HPLC – high performance liquid chromatography
– visokoučinkovita tekućinska kromatografija
GC – gas chromatography
– plinska kromatografija
$NAD^+$ – nicotinamide adenine dinucleotide
– nikotinamid adenin dinukleotid
NADH – nicotinamide adenine dinucleotide, reduced
– nikotinamid dinukleotid, reducirani
ATP – adenosin triphosphate
– adenozin trifosfat
TCA – three carboxylic acids
– trikarbonske kiseline
FIA – flow injection analysis
– protočno injekciona analiza
FL – fluorometer
– fluorometar
GlyDH – glycerol dehydrogenase
– glicerol dehidrogenaza
FTC – flow-through cell
– protočna ćelija
PMT – photomultiplier tube
– fotomultiplikatorska cijev
W – waste
– otpad
MC – mixing chamber
– ćelija za miješanje
P – pump
– crpka
R – recorder
– pisač
ER – enzymic reactor
– enzimski reaktor
V – injection valve
– injekcijski ventil
UV – ultraviolet
– ultraljubičasto
RI – refractiv index
– refraktivni indeks
IV – injection valve
– injekcijski ventil
SV – switching valve
– ventil za isključivanje
S – spectrofluorometric detector
– spektrofluorometrijski detektor
FT-IR – Fourier transforming infrared spectroscopy
– Fourier transformacijska infrared spektroskopija
(SI)-FT-IR – sequential inection Fourier transforming infrared spectroscopy
– uzastopno injektiranje Fourier transformacijskom infrared spektroskopijom
FFC – fluorometric flow-cell
– fluorometrijska protočna ćelija
FI – fluoroscence intensity
– intenzitet fluoroscencije
B – buffer
– pufer
S – sample
– uzorak
E – eluant
– eluent
F1 – flow in
– dotok
F2 – flow out
– izljev
LB – light beam
– svjetlosna zraka
LTD – light to detector
– svjetlo prema detektoru
*t* – time
– vrijeme
MS – mass spectroscopy
– masena spektroskopija
3-MDP – 3-methoxy-propan-1,2-diol
– 3-metoksi-propan-1,2-diol
CycD – cyclic diglycerols
– ciklički digliceroli

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**SAŽETAK**

**Glicerol i industrija vina**
**Određivanje glicerola u moštu i vinu**

*Đ. Šehović, V. Petravić i V. Marić*

Proizvodnja vina uključuje strogo praćenje (i kontrolu, ako je potrebno) niza veličina tijekom dužeg razdoblja. Poželjno je određivanje sastojaka, posebno glicerola od primitka sirovine (grožđa) do punjenja konačnog proizvoda u boce. Složenost priprave vina zahtijeva točne, brze, selektivne i osjetljive metode.

Da bi se prosudilo da li je uzorak vina prirodan ili ne, važno je znati, uz druge sastojke, i koncentraciju glicerola. Također treba utvrditi eventualni dodatak glicerola radi poboljšanja organoleptičkih svojstava i povećanja ekstrakta u vinu.

Kao najvažniji sporedni proizvod alkoholne fermentacije glicerol je jedan od najvrijednijih sastojaka vina. U enološkim laboratorijima njegovo određivanje trebalo bi biti dio uobičajenih analiza. Rutinske metode određivanja glicerola uključuju oksidaciju, esterifikaciju, formiranje etera, uparavanje itd., a sve su dugotrajne. Tradicionalne analitičke tehnike kao visokoučinkovita tekućinska kromatografija (HPLC) i plinska kromatografija (GC) rasprostranjene su metode odvajanja i identifikacije glicerola i drugih poliola. Trajanje analize je dugo, primjenjuju se skupi instrumenti, a ponekad je potrebna i prethodna obrada uzorka. Od svih analitičkih metoda, enzimsko određivanje glicerola pokazalo se najosjetljivije i najspecifičnije.

Kombinacijom enzimske reakcije glicerola s $NAD^+$-om koju katalizira glicerol dehidrogenaza i protočnog senzora povećava se osjetljivost, odnosno moguće je detektirati nižu koncentraciju glicerola nego primjenom drugih metoda, a analiza je brža. Osim za uzorke vina, ta metoda može se u principu primijeniti i na druge tipove uzoraka u kojima je potrebno odrediti glicerol (farmaceutski, prehrambeni i klinički uzorci).

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