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Kinetic Enzymatic Determination of Glycerol in Wine and Beer Using a Sequential Injection System with Spectrophotometric Detection

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A sequential injection system for the automatic determination of glycerol in wine and beer was developed. The method is based on the rate of formation of NADH from the reaction of glycerol and NAD^+ catalyzed by the enzyme glycerol dehydrogenase in solution. The determination of glycerol was performed between 0.3 and 3.0 mmol L^{-1} (0.028 and 0.276 g L^{-1}), and good repeatability was attained (rsd < 3.6%, $n = 5$) for all samples tested. The determination rate was 54 h^{-1}, the reagent consumption was only 0.75 μmol of NAD^+ and 5.4 ng of enzyme per assay, and the waste production was 2.12 mL per assay. Results obtained for samples were in agreement with those obtained with the batch enzymatic method.

KEYWORDS: Glycerol; wine; beer; sequential injection analysis; enzymatic determination; spectrophotometry

INTRODUCTION

Besides ethanol and carbon dioxide, glycerol is a major end product resulting from the fermentation process involving *Saccharomyces cerevisiae*, thus being present in almost all alcoholic beverages and spirits. In wines, glycerol is the most abundant compound after water and ethanol, and it contributes to the taste properties and smoothness of wine (*1*). The usual content in wines is within the 1−15 g L^{-1} range (*2*), but it can be higher for wines produced from "noble rot" grapes, infected by *Botrytis cinerea* (*3*). In beers, glycerol also contributes to the sensory characteristics (*4*), and it can be a carbon source for spoilage bacteria, originating highly volatile acidity and aroma compounds that confer unacceptable flavor to beers (*5*). Hence, the determination of glycerol is important for industrial quality control, requiring fast and reliable methods for routine analysis.

Several methods exploiting chromatographic techniques (*6*, *7*) and/or enzymatic reactions (*8*, *9*) are available. Automated procedures relying on immobilized enzymes have been described in relation to fluorometry *(10, 11)*, spectrophotometry (*12*−*14*), or amperometry (*15*). In the present work, a novel approach for flow-based determination of glycerol is proposed, based on enzyme kinetics, and using glycerol dehydrogenase (GDH) in solution. Quantification of glycerol is based on the rate of formation of NADH from the reaction of glycerol with NAD^+ catalyzed by GDH.

This approach presents advantages when compared to those exploiting immobilized enzymes. For instance, inactivation of the enzyme due to adsorption of polyphenols and dyes has been reported previously (*16*). This situation can be avoided using enzymes in solution as a fresh aliquot of this reagent is applied in each determination. Furthermore, utilization of a kinetic-based approach as proposed in the present work avoids the measurement of sample blank, as the analytical signal is based on the absorbance change during a fixed time period instead of an absolute value. For implementation of the flow-based determination, sequential injection analysis (SIA) was chosen as this novel computer-controlled technique enables the precise control of volume delivery, flow rates, and operation timing (*17*). These characteristics are essential in kinetic-based procedures, also allowing reduction of reagent consumption.

MATERIALS AND METHODS

Reagents and Solutions. All of the solutions were prepared with water from a Milli-Q system (resistivity > 1.8×10^5 Ωm) and chemicals of analytical reagent grade quality.

Glycerol dehydrogenase (EC 1.1.1.6) from *Enterobacter aerogenes* (ref 258555, 20 mg mL^{-1}, 25 units mg^{-1}) and NAD^+ (grade III, 90%, ref 710113) were purchased from Roche. For comparison purposes, lyophilized GDH enzyme from *Cellulomonas* sp. (ref G3512, Sigma, 70% protein, 3.7 mg of solid, 69 units mg^{-1}) and from *E. aerogenes* (ref G4783, Sigma, 5.0 mg of solid, 21 units mg^{-1}) were also used. These enzymes were suspended in 3.2 mol L^{-1} ammonium sulfate

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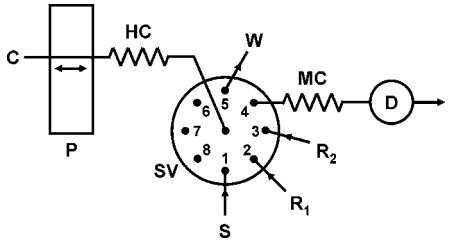

Figure 1. Flow diagram of the SIA system: SV, selection valve; P, peristaltic pump; HC, holding coil; MC, mixing coil; D, spectrophotometer; S, sample or standard; R_1, NAD^+ reagent; R_2, GDH enzyme solution; C, buffer/carrier stream; W, waste.

Table 1. Protocol for the Determination of Glycerol

step	valve position	operation time/s	flow rate/ mL min^{-1}	vol/ μL	description
a	1	2.8	1.9	90	sample/standard aspiration into HC
b	2	0.9	1.9	30	NAD^+ aspiration into HC
c	3	2.8	1.9	90	enzyme aspiration into HC
d	4	15.0	3.8	960	propulsion of HC content toward the flow cell
e	4	30.0			stop period, acquisition of analytical signal
f	4	15.0	3.8	960	system washing

solution with the pH adjusted to 7.5 and stored at 4 °C before preparation of working solutions. Glycerol (G5516) was also purchased from Sigma.

The buffer/carrier solution (C, **Figure 1**) was prepared by dissolving 10.0 g of $KHCO_3$ in 1000 mL of water and adjusting the pH to 9.5 with a 2.0 mol L^{-1} NaOH solution. The enzyme solution (60 μg mL^{-1}) was prepared daily by dissolving 60 μL of commercial enzyme in 20 mL of buffer solution. The 25 mmol L^{-1} NAD^+ water solution was also prepared daily.

The glycerol stock solution, 100 mmol L^{-1}, was prepared by weighing it and dissolving the appropriate amount in the hydrogencarbonate buffer solution. Working standards within the 0.3−3.0 mmol L^{-1} concentration range were also prepared in this buffer solution. Beer and wine samples were diluted with hydrogencarbonate buffer solution.

Apparatus. Solutions were propelled by a Gilson (Villiers-le-Bel, France) Minipuls 3 peristaltic pump, equipped with PVC pumping tubes. Manifold was built up with 0.8 mm i.d. Omnifit (Cambridge, U.K.) PTFE tubing of the noncollapsible wall type. The pump was connected to the central channel of an eight-port electrically actuated selection valve (C15-3118E, VICI, Houston, TX).

A Thermo-Spectronic (Cambridge, U.K.) Helios γ UV−vis spectrophotometer equipped with a thermostatic cell holder and a model 178.710 QS Hellma (Mullheim/Baden, Germany) flow-through cuvette (internal volume = 80 μL, optical path = 10 mm) was used. Wavelength was set at 340 nm. Temperature was maintained by resorting to a thermostatic bath (I. S. Co GTR 190, Milan, Italy) and circulating water through the cuvette holder.

A personal computer based on the 80486 Intel chip, equipped with an Advantech (Taipei, Taiwan) PCL-818L interface card, running a laboratory-made software written in QuickBasic 4.5 (Microsoft), controlled the position of the selection valve, the flow direction, and the rotation speed of the peristaltic pump. Data acquisition was performed at 4 Hz using the same software.

Sequential Injection System. The components of the sequential injection system were assembled as specified in **Figure 1**. The holding coil (HC) was 200 cm long, whereas the mixing coil (MC), located between the selection valve and the detector, was 100 cm long. Other tubes connected to the selection valve were 30 cm long.

The protocol sequence for the determination of glycerol is presented in **Table 1**. First, sample/standard solution, NAD^+, and enzyme solution were aspirated sequentially into the HC. After flow reversal, the stacked zones inside the HC were sent through the MC toward detection. After a preselected period of time, the flow was stopped and acquisition of the analytical signal was performed during a preset time interval. Thereafter, the peristaltic pump was reactivated, and the flow cell was washed by the buffer/carrier solution.

The quantification of glycerol was based on the rate of formation of NADH, which was evaluated by monitoring the absorbance at 340 nm after the flow stop, during a preset period of time. Thereafter, the absorbance values were plotted as a function of time, and the slope of the resulting function was obtained, provided that linearity was verified. It should be emphasized that the slope value reflected the rate of NADH formation. The linearity of the absorbance versus time function was assessed after visual inspection of the obtained graphs and considered to exist when the correlation coefficient was equal or superior to 0.995 ($n > 50$). The analytical curves were obtained by plotting the rate of NADH formation versus concentration of glycerol.

Accuracy Assessment. For comparison purposes, the determination of glycerol in beer and wine samples was also performed according to the usual methods recommended by the Organisation Internationale de la Vigne et du Vin (*9*) and by the European Brewery Convention (*8*), using the commercial test kit "UV-method for the determination of glycerol in foodstuffs and other materials" (ref 10148270035, Roche Diagnostics GmbH, Mannheim, Germany). The absorbance measurements (340 nm) were done after the procedures "determination of glycerol in wines" and "determination of glycerol in beer" which are described in the test kit package.

RESULTS AND DISCUSSION

Development of the Sequential Injection System. Some parameters were fixed, namely, the volume and aspiration sequence of solutions and also the time interval during which the pump was activated before the flow stop period. Because zone overlap is essential to attain suitable mixing conditions in SIA, the solution aspiration sequence was of utmost importance. In the present application, the solutions were drawn into the HC in the following order: 90 μL of sample/standard, 30 μL of NAD^+, and 90 μL of enzyme. This sequence was chosen by taking into consideration that the enzyme should be the last reagent aspirated in order to minimize its dilution and that the plug of solution placed between the other two should be as small as possible to allow suitable overlap of the three original zones. Hence, NAD^+ solution was preferred as its concentration could be raised if necessary, and its volume was reduced to 30 μL.

The time interval during which the pump was activated before reaction monitoring (**Table 1**, step d) was an important parameter in the system design, as it defined the portion of the overlapped zones (sample/NAD^+/enzyme) present in the flow cell during the flow stop step (**Table 1**, step e). This interval was fixed at 15.0 s as the absorbance increase was similar for time periods within 12.5 and 16.0 s (2.0 mmol L^{-1} glycerol, 50 mmol L^{-1} NAD^+, and 20 μg mL^{-1} GDH solutions, pH 9.0).

Dimensioning of other parameters influencing the system performance was carried out by the univariate method. Two aspects were considered: first, the existence of a linear relationship between absorbance and time during the period of signal acquisition as the rate of reaction was calculated from the slope of this graph; second, the influence on the sensitivity, assessed as the rate of reaction value (using a single standard) or as the slope of the calibration curve (rate of reaction versus concentration, when several standards were used).

pH of Carrier/Buffer Solution. The influence of this parameter on the rate of reaction development was investigated within 8.5 and 10.5. Concentrations of glycerol, NAD^+, and GDH solutions were 1.5 mmol L^{-1}, 50 mmol L^{-1}, and 20 μg mL^{-1}, respectively; the temperature was set at 40 °C. A linear relationship between absorbance and time was noted for all tested values of pH during the 60 s of absorbance monitoring (**Figure 2A**). Furthermore, the maximum slope (corresponding

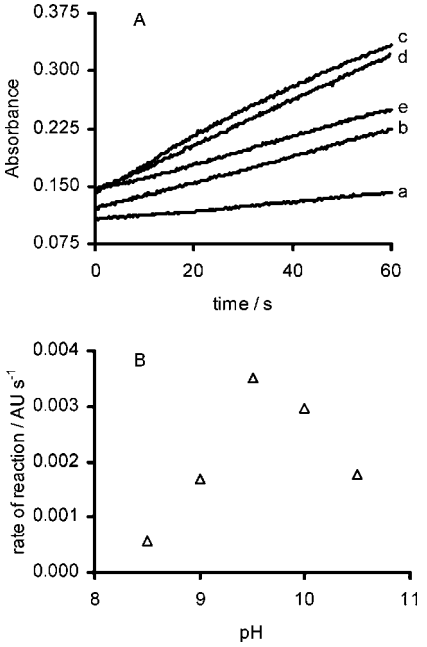

Figure 2. (**A**) Absorbance values obtained for 1.5 mmol L^{-1} glycerol standard during the stop period (**Table 1**, step e) for carrier solution with different pH values: a, 8.5; b, 9.0; c, 9.5; d, 10.0; e, 10.5. (**B**) Rates of reaction (absorbance units s^{-1}) ($n = 3$; RSD < 2.7%) obtained for the same glycerol solution using carrier solution at different pH values.

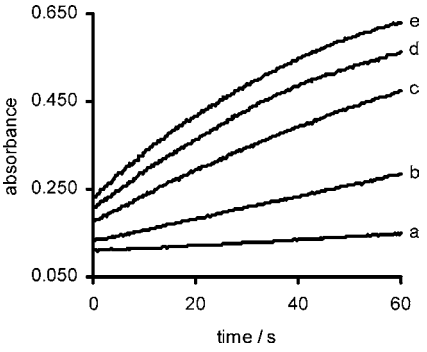

Figure 3. Absorbance values obtained for 2.0 mmol L^{-1} glycerol standard during the stop period (**Table 1**, step e) using different concentrations of enzyme: a, 10 μg mL^{-1}; b, 20 μg mL^{-1}; c, 40 μg mL^{-1}; d, 60 μg mL^{-1}; e, 80 μg mL^{-1}.

to the highest reaction rate) was obtained for a pH of 9.5 (**Figure 2B**), which was chosen for further experiments.

Enzyme Concentration. This parameter was studied between 10 and 80 μg mL^{-1} (0.25 and 2.0 units mL^{-1}) by maintaining the same experimental conditions as described above. The results obtained for 2.0 mmol L^{-1} glycerol standard solution are presented in **Figure 3**; similar results were found for 0.3−3.0 mmol L^{-1} glycerol solutions. For the highest tested enzyme concentrations (60 and 80 μg mL^{-1}), the absorbance/time relationship was not linear during the 60.0 s of signal monitoring. Therefore, the reaction rate was evaluated by considering the values obtained between 0.0 and 30.0 s, providing values of 2.65×10^{-3}, 5.01×10^{-3}, 6.72×10^{-3}, and 7.00×10^{-3} absorbance units s^{-1} for 20, 40, 60, and 80 μg mL^{-1} of enzyme, respectively. The concentration of enzyme was chosen as 60 μg mL^{-1} because when compared to the highest tested concentration, the rate of reaction was similar (≈10% lower) with a 25% reduction in enzyme consumption.

NAD^+ Concentration. The concentration of NAD^+ was studied between 10 and 100 mmol L^{-1}. For a 2.0 mmol L^{-1} glycerol solution, the reaction rates corresponding to 25, 50, and 75 mmol L^{-1} NAD^+ were about 77, 89, and 96% of that obtained for 100 mmol L^{-1} NAD^+ (7.10×10^{-3} absorbance units s^{-1}). For a 10 mmol L^{-1} NAD^+ solution, the relationship between absorbance and time was not linear. Similar results were found for other concentrations of glycerol tested (0.3−3.0 mmol L^{-1}). Considering that the reaction rate values obtained for 25 mmol L^{-1} NAD^+ were only ≈20% lower in

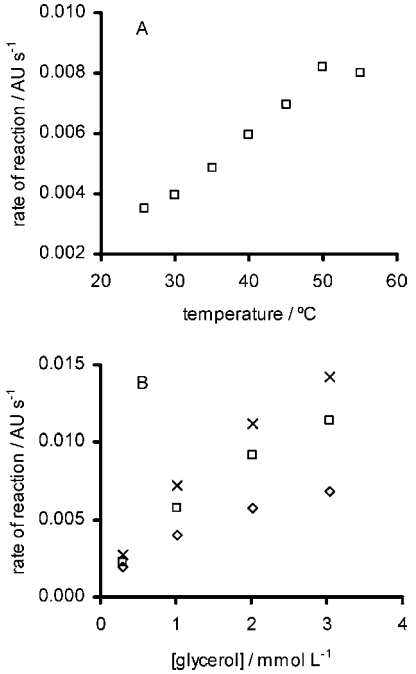

Figure 4. (**A**) Rates of reaction (absorbance units s^{-1}) ($n = 3$; RSD < 2.0%) obtained for 1.5 mmol L^{-1} glycerol standard during the stop period (**Table 1**, step e) at different temperatures. (**B**) Calibration curves established at 30 °C (◇), 40 °C (□), 50 °C (×).

Table 2. Rates of Reaction Obtained for GDH Enzymes from Different Sources Using 1.0 mmol L^{-1} Glycerol Standard Solution: (A) GDH from *E. aerogenes* (Roche); (B) GDH from *E. aerogenes* (Sigma); (C) GDH from *Cellulomonas* Species (Sigma)

cnzyme	concn/ units mL^{-1}	concn/ μg mL^{-1}	rate of reaction[a]/ absorbance units s^{-1}	rel response to A/%
A	1.5	60	$4.69 (\pm 0.05) \times 10^{-3}$	100
B	4.5	214	$0.95 (\pm 0.05) \times 10^{-3}$	20.3
C	1.5	22	$1.41 (\pm 0.05) \times 10^{-3}$	30.0
	3.0	43	$3.23 (\pm 0.03) \times 10^{-3}$	68.9
	4.5	65	$4.38 (\pm 0.02) \times 10^{-3}$	93.5

[a] $n = 3$.

Table 3. Results (Mean Value ± Standard Deviation, in Grams per Liter of Glycerol) Obtained with the Proposed Method (C_p) and the Batch Method (C_r) for the Analyses of Wines and Beers

sample	C_r[a]	C_p[b]	rel deviation/%
table white wine A	5.17 (± 0.02)	4.99 (± 0.08)	–3.5
table white wine B	5.45 (± 0.00)	5.28 (± 0.19)	–3.1
table white wine C	6.51 (± 0.00)	6.56 (± 0.09)	0.8
table red wine A	7.79 (± 0.05)	7.84 (± 0.24)	0.6
table red wine B	4.05 (± 0.03)	4.12 (± 0.12)	1.7
barley based lager beer A	1.55 (± 0.02)	1.59 (± 0.04)	2.6
Trappist beer B	0.95 (± 0.03)	0.94 (± 0.02)	–1.1
barley-based lager beer C	1.72 (± 0.00)	1.77 (± 0.04)	2.9

[a] $n = 2$. [b] $n = 5$.

Table 4. Characteristics of Flow-Based Analytical Systems Developed for Glycerol Determination[a]

	present system	FIA (*10*)	FIA (*11*)	FIA (*12*)	SIA (*13*)	MCFA (*14*)	FIA (*15*)
matrix	wine and beer	wine	wine	wine	wine	wine	alcoholic beverages
detection	S	F	F	S	S	S	E
NAD^+ (μmol/assay)	0.75	7.2	0.4	19.2	0.9	1.22	3.4
sample (μL/assay)	90	150	2000	5	45	44 or 2	170
waste production (mL/assay)	2.12	1.6	>19	16.8	1.54	na	0.76
determination rate (h^{-1})	54	60	6	10	22.5	33	30
rsd (%)	<3.6	0.92	2	2.1	<3.4	1.8	<1.0

[a] FIA, flow injection analysis; SIA, sequential injection analysis; MCFA, multicommuted flow analysis; S spectrophotometry; F, fluorometry; E, electrochemical; na, not available.

relation to those obtained for 100 mmol L^{-1} NAD^+, the first concentration was chosen as a compromise between sensitivity and reagent consumption.

Temperature. The influence of temperature was investigated by varying the temperature of the cell holder (26−55 °C). An increase in this temperature led to a pronounced increase in the rate of the enzymatic reaction up to ≈50 °C (**Figure 4A**). An increase in sensitivity was also observed (**Figure 4B**). However, the temperature should not be increased above the maximum value tested as enzyme deactivation would occur, and the occasional formation of air bubbles would impair measurement. Therefore, the temperature was chosen as 50 °C.

Evaluation of GDH from Different Sources. A comparative study among GDH from *E. aerogenes* supplied by Roche (A) and by Sigma (B) and GDH from *Cellulomonas* sp. (C) purchased from Sigma was performed. Enzyme solutions in a concentration range from 1.5 to 4.5 units mL^{-1} were prepared, and the rate of reaction for the 1.0 mmol L^{-1} glycerol solution was evaluated (**Table 2**). In general, enzymes B and C led to lower reaction rates when compared to enzyme A, considering all concentrations tested. For enzyme B, the absorbance versus time function was linear only for the highest concentration of enzyme tested. For enzyme C, there was an increase in reaction rate values when the enzyme concentration was increased. In fact, a rate similar to that obtained with enzyme A was achieved for the highest concentration tested of enzyme C, indicating that this commercial enzyme could also be applied.

Figures of Merit and Application to Samples. The performance of the proposed sequential injection system for the determination of glycerol in wine and beer was evaluated regarding application range, accuracy, repeatability, and determination frequency. The standards concentration varied between 0.3 and 3.0 mmol L^{-1}; this range was appropriate for determination in wines with 1.5−15 g L^{-1} of glycerol when diluted 50 times or in beers with 0.3−3.0 g L^{-1} when diluted 10 times.

Initially, the analytical curve was obtained by plotting the reaction rate versus glycerol concentration. As this relationship was not linear for the concentration range defined, the Lineweaver−Burk plot (*18*) was applied. The analyte concentration was then calculated through the linear relationship between the inverse of the rate of reaction (1/*V*) and the inverse of the glycerol (substrate) concentration (1/*S*).

To evaluate the accuracy of the proposed system, eight samples of wine and beer were analyzed according to the proposed method (C_p) and the batch method (C_r). Relative deviations of <3.5% were found for all samples (**Table 3**). Furthermore, a linear relationship ($C_p = C_0 + S \times C_r$) was established, described by the equation $C_p = 0.02\ (\pm\ 0.19) + 0.993\ (\pm\ 0.040) \times C_r$, $R = 0.9992$, $n = 8$. The values in parentheses correspond to the limits of the 95% confidence level intervals. From these figures it is evident that the estimated intercept and slope values do not differ significantly from 0 and 1, respectively (*19*).

Repeatability was estimated by calculating the relative standard deviation from five consecutive injections of each sample (**Table 3**); values between 1.4 and 3.6% were obtained. The determination frequency was calculated by considering the time intervals inherent to each step of the protocol sequence (**Table 1**). The time required for all of these operations was 66 s, meaning 54 determinations per hour.

In conclusion, the proposed sequential injection system allowed fast and reliable determination of glycerol in wine and beer samples, with similar or even superior characteristics relative to those inherent to previously developed flow systems relying on immobilized enzymes (**Table 4**). Low reagent consumption was attained (5.4 ng of enzyme; 0.75 μmol of NAD^+) with minimum production of waste per assay (≈2 mL). When compared to the usual batch method, the present system allowed the same determination using only one enzyme (GDH), whereas the batch method needed three (glycerokinase, pyruvate kinase, and lactate dehydrogenase). Furthermore, the determination frequency was enhanced, and this is a worthwhile aspect if one considers that it was possible to perform only six to eight determinations per hour with the batch method.

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