Enzymatic determination of ethanol and glycerol by flow injection parallel multi-site detection

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Abstract

A flow injection method was developed for the sequential enzymatic determination of ethanol and glycerol in wines, using immobilised ethanol dehydrogenase and glycerol dehydrogenase, respectively. The enzymes were immobilised separately on alkylaminated controlled pore glass. A multi-site spectrophotometric detection system was used in parallel configuration to monitor the absorbance change in the two independent analytical channels. A 50-fold dilution of the samples was necessary before injection. The working range was between 0.05 and 0.5% (v/v) for the ethanol and between 0.03 and 0.3 g l^{-1} for the glycerol determination, with corresponding detection limits of 2×10^{-3}% (v/v) and 2×10^{-3} g l^{-1}. Relative standard deviations (R.S.D.) (n=9) lower than 2.3% for the ethanol and 2.1% for the glycerol determination were found. For 13 samples of different types of table and Port wines, the results showed good agreement with the corresponding reference procedures; a two level recovery study also showed good accuracy for the developed methods. The sampling rate was 10 h^{-1}, corresponding to 20 determinations per hour.

Keywords: Sequential flow injection; Ethanol; Glycerol; Multi-site detection; Spectrophotometric enzymatic analysis; Wine

1. Introduction

Ethanol is the primary product of yeast fermentation of the grape carbohydrates. The fermentation also leads to different by-products, glycerol being one of the most important. Under normal wine fermentation circumstances the ratio between the concentration of glycerol and ethanol should be approximately 0.07. Deviations from this value might indicate technological alterations during the process or deterioration of the harvested grape [1].

Ethanol is routinely measured during wine production, using well-established methods that usually involve the separation of the analyte from the sample matrix by distillation before chemical or physical measurements, or then using chromatographic methods [2]. However, glycerol is seldom determined in control laboratories [1]. Although enzymatic assays are now available for both determinations [3], the use of these procedures in routine laboratories is still quite limited, probably due to their high cost. The development of automatic methods of analysis associated with enzyme immobilisation could make the enzymatic assays cost effective, which would emphasise their advantages in terms of sensitivity and selectivity.

Some flow injection (FI) systems with immobilised enzymes have been developed for the determination of the two analytes separately in wines. These systems were either based on spectrophotometric [4,5],

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electrochemical [6–10], chemiluminometric [11–14] or spectrofluorimetric [15] detection methods.

Mattos et al. [16] developed a spectrofluorimetric FI manifold for the sequential determination of glycerol and ethanol using parallel injection and a single detector. This method involved an extensive (1:2500) dilution of the samples before injection.

The objective of the present work was to develop a flow injection system for the sequential determination of ethanol and glycerol using immobilised enzyme reactors and relocatable spectrophotometric detection, with the scope of applying minimum sample pre-treatment. The method was based on the following reactions:

$$\text{Glycerol} + \text{NAD}^+ \overset{\text{GlyDH}}{\leftrightarrow} \text{Dihydroxyacetone} + \text{NADH} + \text{H}^+$$

$$\text{Ethanol} + \text{NAD}^+ \overset{\text{ADH}}{\leftrightarrow} \text{Acetaldehyde} + \text{NADH} + \text{H}^+$$

where GlyDH and ADH indicate glycerol dehydrogenase and alcohol dehydrogenase, respectively. The absorbance change caused by the produced NADH permitted the spectrophotometric quantification of the analytes at 340 nm. Multi-site detection [17] allowed to monitor the absorbance signal in two independent analytical channels using a single detector.

2. Experimental

2.1. Instrumentation

The FI system consisted of two Gilson Minipuls 2 and 3 peristaltic pumps, an Unicam UV/VIS spectrophotometer equipped with Hellma 178.712-QS flow cell (internal volume: 18 μl), Omnifit PTFE tubing of 0.8 mm i.d., Omnifit connectors and end-fittings, a diffusion unit [18] with flow channels of 35 mm × 2 mm × 0.5 mm, an injector commutator device [19], T-shaped confluences and a Metrohm E586 Labograph recorder.

2.2. Reagents and solutions

Reagents of analytical grade and deionised water were used.

Alcohol dehydrogenase (300–400 U mg^{-1}, EC1.1.1.1, ref. 102717) and NAD^+ (Grade III, 90%, ref. 710113) were purchased from Boehringer. Glycerol dehydrogenase (65 U mg^{-1}, EC1.1.1.6, ref. G-3512) and aminopropyl glass (average pore size: 500 A; 200–400 mesh, ref. G-4643) were obtained from Sigma.

For the immobilisation of the enzymes, a 0.1 M phosphate buffer (pH 7.0) and a 2.5% glutaraldehyde solution in the phosphate buffer were prepared.

For the flow injection system a carbonate buffer solution was prepared by dissolving 10.0 g of potassium hydrogencarbonate and 10.6 g anhydrous sodium carbonate in 1 l of water and adjusting the pH to 9.5. The aqueous solution of 8 mM NAD was prepared daily.

Mixed working standard solutions in the concentration range of 0.03–0.3 g l^{-1} glycerol and 0.05–0.5% (v/v) ethanol were prepared from absolute glycerol and ethanol reagents.

The wine samples were diluted (1:50) in water before introduction to the system.

2.3. Enzyme immobilisation

Liophilised glycerol dehydrogenase (0.4 mg) and alcohol dehydrogenase (1.4 mg) were immobilised separately on 0.5 g of aminopropyl derivatised controlled pore glass following the procedure described by Masoom and Townshend [20] with some modifications [21]; they remained stable for approximately 2 months.

2.4. Flow injection system

The developed FI system is presented in Fig. 1A.

In the position shown in the figure, the diluted sample was aspirated through the commutator channel to the gas diffusion unit (GDU) where the ethanol diffused to the acceptor stream (Q_1) and filled the loop L_2. At the same time, the sample (L_1) introduced into its carrier stream (Q_2) was mixed with NAD^+ (Q_3) and passed through the enzyme reactor (R_2) where the conversion of glycerol to dihydroxyacetone occurred. When the produced NADH zone reached the flow cell the absorbance increased continuously towards a maximum value, corresponding to the glycerol content of the sample. The commutator was

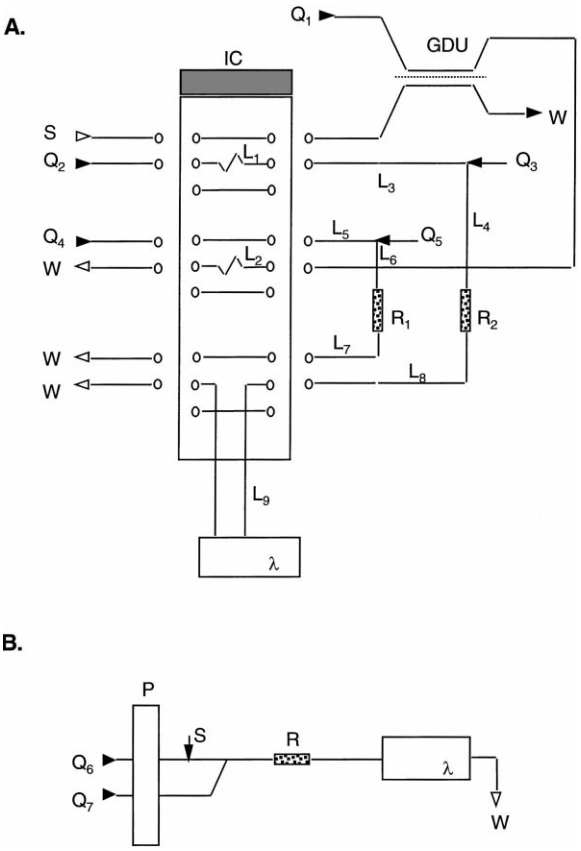

Fig. 1. Manifolds for the sequential determination of ethanol and glycerol in wines by flow injection (FI) analysis (A) and for the preliminary studies (B). IC: injector commutator; S (1.0 ml min^{-1}): sample; Q_i: reagent streams; Q_1 (1.0 ml min^{-1}): 0.1 M carbonate buffer pH 9.5; Q_2 (0.1 ml min^{-1}) 0.1 M carbonate buffer pH 9.5; Q_3 (0.1 ml min^{-1}): 8 mM NAD^+; Q_4 (0.3 ml min^{-1}) carbonate buffer pH 9.5; Q_5 (0.3 ml min^{-1}) 8 mM NAD^+; Q_6: carbonate buffer; Q_7: NAD^+ solution; L_1 and L_2 injection loops; L_1: 5 μl; L_2: 20 μl; L_3=L_4=L_5=L_6=10 cm; L_7=L_8=1 cm; L_9=10 cm; R: immobilised enzyme reactor; R_1: alcohol dehydrogenase reactor; R_2: glycerol dehydrogenase reactor; GDU: gas diffusion unit; P: peristaltic pump; λ: spectrophotometer, 340 nm; W: waste; ▲ indicates the positions where propulsion is applied.

then switched, relocating the detector and injecting the sample in the loop L_2 (previously filled by the Q_1 stream). After mixing with the NAD^+ solution (Q_5), the stream passed through the enzyme reactor (R_1) where the conversion of ethanol to acetaldehyde took place. The absorbance change now recorded corresponded to the ethanol concentration of the sample.

3. Results and discussion

3.1. Study of the flow injection system

The effect of the different parameters such as injection volumes, flow rates, reagent concentrations and reactor dimensions on the performance of the flow injection system was evaluated. Firstly, each analytical channel was studied separately using a simple manifold (Fig. 1B). The results obtained in the ethanol determination described in a previous work [21] were also considered.

The effect of the injection volume on the sensitivity was studied in the range of 5–50 μl. Higher volumes increased sensitivity but also decreased sampling rate. It was found that sample volumes of 15 μl for the ethanol and 25 μl for the glycerol determination yielded satisfactory sensitivity while the sampling rate was still relatively high.

The flow rates also had a large influence on the system performance, since it affected the contact time between the enzymes and the substrates. The flow rate at the enzyme reactors was studied in the range of 0.2 and 1.2 ml min^{-1}. Lower flow rates increased sensitivity and decreased sampling rate. A good sensitivity was obtained for the ethanol at 0.6 and 0.2 ml min^{-1} for the glycerol determination.

The effect of the pH of the carbonate buffer on the glycerol determination was studied in the range of 8–10.5, and maximum sensitivity was obtained at pH 9.5. Since this value was also applicable for the ethanol determination [21] it was selected as a common buffer solution for the two determinations; this allowed to have approximate baseline absorbance values in both analytical channels.

Experiments with various NAD^+ cofactor concentrations were carried out in the range of 2–10 mM. The sensitivity increased with the concentration until 8 mM and then stabilised, thus this solution was used in the FI manifold.

The influence of the immobilised enzyme reactors length was also studied in the range of 5–30 mm (i.d. 3 mm). Longer reactors increased sensitivity but also increased dispersion of the sample zone, and therefore, decreased sampling rate as well as produced a significant back-pressure. Considering these effects, a length of 25 and 20 mm was selected for the glycerol and the ethanol determination, respectively.

After these preliminary studies using the manifold presented in Fig. 1B, application ranges with a good sensitivity were obtained between 10^{-3} and 10^{-2}% (v/v) for ethanol and between 10^{-2} and $0.5\,g\,l^{-1}$ for glycerol determination. Therefore, very different dilutions (1:2500 and 1:30) were necessary to perform the two determinations in the concentration ranges for the wine samples (2.5–25% (v/v) for ethanol and 1.5–15 $g\,l^{-1}$ for glycerol).

As the objective of the work was to develop a system with minimum sample treatment, the first approach was to perform in-line these necessary dilutions. Gas diffusion unit (GDU) has already been proved to be an efficient tool in the ethanol determinations [7,9] for sample dilution and analyte separation. Therefore, the incorporation of a GDU was considered for the ethanol channel, while for the glycerol determination the use of a dialysis unit was tested. However, the passage of glycerol through the dialysis membrane was highly affected by the presence and the concentration of ethanol, therefore, the dialysis process was not applicable under the circumstances for wine analysis. The off-line dilution of the samples was then considered. As this approach does not eliminate the contact of the possible interfering compounds with the enzyme reactor, a minimum injection volume (5 μl) must be used. With this volume and 50 times diluted wine samples no interference of the matrix was found. The final configuration of the system was then set as presented in Fig. 1A.

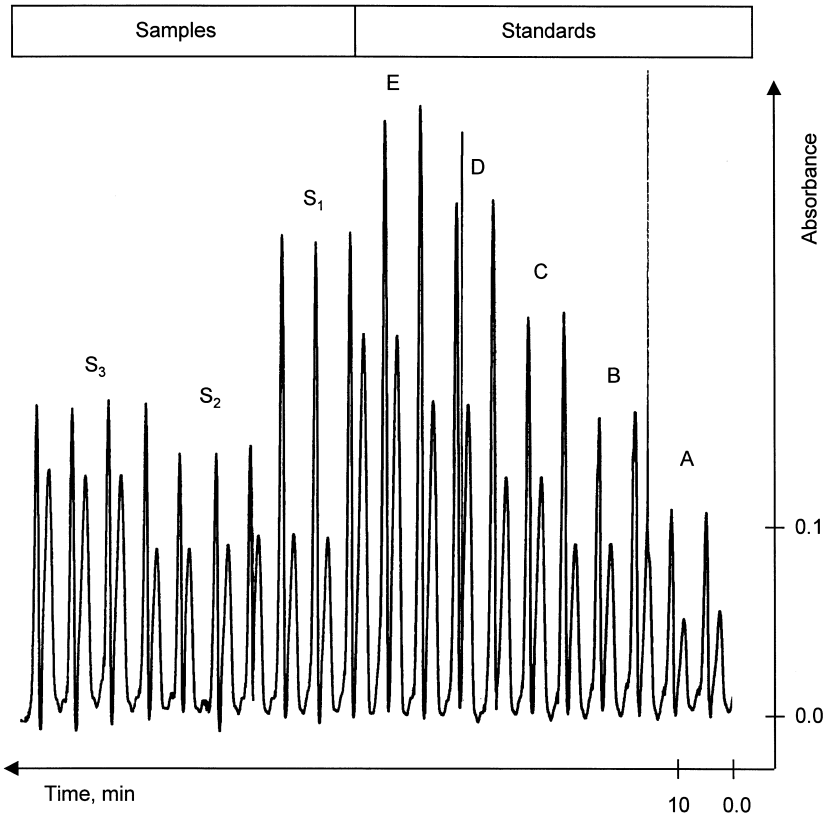

Fig. 2. Recorder output for the sequential determination of ethanol and glycerol content in wines (S_i). The first peak corresponds to the glycerol and the second to the ethanol determination. The composition of the mixed standards were: A, $0.06\,g\,l^{-1}$ and 0.1% (v/v); B, $0.12\,g\,l^{-1}$ and 0.2% (v/v); C, $0.18\,g\,l^{-1}$ and 0.3% (v/v); D, $0.24\,g\,l^{-1}$ and 0.4% (v/v); E, $0.3\,g\,l^{-1}$ and 0.5% (v/v), glycerol and ethanol, respectively.

The flow rates in the GDU (1 ml min^{-1}) were set to obtain good sensitivity for the ethanol determination.

As in both determinations the absorbing species is the same (NADH), a single spectrophotometer could be used; multi-site detection system was applied to relocate the detector between the two analytical channels. Additionally, as the flow-rate in the glycerol channel is very slow, the relocation of the flow cell to the ethanol channel washes out the remaining tail of the plug and so contributes to increase the sampling-rate.

To reduce the dispersion of the NADH zone and the dead volume of the detector, the connections between the enzyme reactors, the commutator and the flow cell (L_7, L_8 and L_9) were kept to a minimum.

Under the final conditions presented in Fig. 1A, a sampling rate of 10 samples per hour, corresponding to 20 determinations per hour, can be achieved in the desired application range, with calibration curves like absorbance=0.0536+0.533×concentration of ethanol and absorbance=0.0155+0.618×concentration of glycerol. Detection limits of 2×10^{-3}% (v/v) and 2×10^{-3} g l^{-1} for ethanol and glycerol determinations were found, respectively, calculated as recommended by IUPAC [22].

3.2. Application to wine analysis

The FI system was applied to the determination of ethanol and glycerol contents of different Portuguese table and Port wines, both red and white. A recorder output is presented in Fig. 2.

For comparison purposes the different wine samples were analysed by reference methodologies, as well. The ethanol content of the samples was measured after distillation by the hydrometric method [23], while for the determination of glycerol the enzymatic kit of Boehringer [3] was used.

Accuracy of the developed method was evaluated by comparison of the results of the FI method with those of the respective reference determinations. Concentrations and respective relative deviation values for 13 wines are summarised in Table 1.

The results showed relative deviations lower than 2.1% for the ethanol and lower than 4.0% for the glycerol determination. A regression line was also used to compare the methods [24]. The resulting equations

Table 1
Results obtained in the analysis of table wine samples by the developed flow injection (FI) method and the reference determinations with the corresponding relative deviation (RD) values

Ethanol determination			Glycerol determination		
FIA[a] % (v/v)	Ref. % (v/v)	RD (%)	FIA[a] (g l^{-1})	Ref. (g l^{-1})	RD (%)
11.0±0.1	11.0	0.0	6.06±0.02	6.20	−2.3
10.8±0.1	10.9	−0.9	7.18±0.0	6.97	3.0
12.2±0.1	12.2	0.0	4.33±0.05	4.26	1.6
11.3±0.1	11.5	−1.7	6.24±0.0	6.39	−2.3
10.7±0.3	10.6	0.94	6.50±0.05	6.38	1.9
9.70±0.1	9.50	2.1	5.07±0.0	5.00	1.4
19.1±0.1	19.1	0.0	2.29±0.01	2.38	−4.0
19.3±0.1	19.4	0.52	3.12±0.15	3.25	−3.9
19.7±0.05	19.6	0.51	4.72±0.07	4.81	−1.9
20.1±0.05	19.9	1.0	4.88±0.09	4.99	−2.2
20.1±0.3	20.1	0.0	4.42±0.09	4.47	−1.1

[a] Average of three determinations and the corresponding standard deviations.

were as follows:

For ethanol,

$$C_{FIA} = -0.175(\pm0.304) + 1.014(\pm0.018)C_{BATCH};\quad R = 0.9997$$

For glycerol,

$$C_{FIA} = -0.135(\pm0.718) + 1.029(\pm0.116)C_{BATCH};\quad R = 0.992$$

where C_{FIA} are the results obtained by the flow injection method and C_{BATCH} are the results obtained by the reference methods. The values in brackets are the limits of the 95% confidence intervals. These values indicated no evidence for systematic differences between the sets of results.

A two-level recovery study was also performed with different type of wines, to assess the applicability of the method in a wider concentration range. The first level corresponded to the addition, in the non-diluted samples, of 5% (v/v) ethanol and 3 g l^{-1} glycerol, and the second level to the addition of 10% (v/v) ethanol and 6 g l^{-1} glycerol. Recovery values obtained are presented in Table 2.

The precision of the FI method was also tested and relative standard deviation (R.S.D.) values of 2.3, 2.3 and 1.8% were found for 20.5, 8.3 and 12.0% (v/v) ethanol concentrations, respectively. For the determi-

Table 2
Results obtained in the recovery (%) study with different types of wines[a]

Wine sample	Ethanol determination			Glycerol determination		
	No addition % (v/v)	First addition	Second addition	No addition ($g\,l^{-1}$)	First addition	Second addition
Table white	8.7	102	94.3	6.97	101	103
Table red	8.3	98.4	95.2	7.65	101	95.8
Table rose	8.5	103	98.7	6.53	105	99.5
Port white	19.9	–	–	4.55	102	102
Port red	19.1	–	–	5.14	105	99.2

[a] First level corresponded to the addition of 5% (v/v) ethanol and $3\,g\,l^{-1}$ glycerol addition, in the non-diluted samples, and the second level to the 10% (v/v) ethanol and $6\,g\,l^{-1}$glycerol, respectively.

nation of the glycerol R.S.D.s of 1.3, 2.1 and 1.4% were obtained for 4.55, 4.47 and $6.69\,g\,l^{-1}$ concentrations, respectively.

4. Conclusions

The features of the proposed methodology make it a good alternative for the simultaneous determination of glycerol and ethanol in wines: the results are comparable to the reference procedures, repeatability is good, and requires minimum sample treatment (dilution).

The manifold configuration developed for this work also emphasises the merits of using multi-site detection to carry out sequential absorbance readings using only one spectrophotometer, thus substantially reducing the cost of the apparatus.

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