Determination of Ethanol in Wines by Flow Injection Spectrophotometry Using Gas-Diffusion and an Immobilized Enzyme Reactor

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A flow injection analysis (FIA) system for the spectrophotometric determination of ethanol in wines using an immobilized alcohol dehydrogenase enzyme reactor was developed. A gas diffusion unit was used to achieve a large dilution and to separate ethanol from the matrix. This way, the contact of the sample with the reactor was avoided and possible interferences in the spectrophotometric measurements were significantly reduced. The alcohol dehydrogenase enzyme was immobilized on alkylaminated controlled pore glass, and the consumption of the NAD cofactor was minimized (0.6 µmol per assay) by using the merging zone technique. The detection limit was 0.4% (v/v). Good precision was achieved, with relative standard deviations less than 2.2% (n = 9). For 20 samples of different types of table and Port wines, the results showed good agreement with the CIV usual procedure, the relative deviation being less than 4.4%. Thirty determinations per hour can be carried out within the ethanol concentration range of 5% to 25% (v/v), without any sample pretreatment.

KEY WORDS: flow injection, ethanol, immobilized alcohol dehydrogenase, spectrophotometry, wine

The determination of ethanol is probably the most frequently performed analysis in wine laboratories, since it is important for process and quality control. Standard methods [15,18] involving distillation or gas chromatography are laborious and time consuming. Therefore, in the last few years, efforts have been made on the automation of the ethanol determination in wines, mainly by using the flow injection analysis (FIA) technique. Several manifolds were described using electrochemical [3,5,10,14,16,20], spectrophotometric [6,7,12,22], fluorimetric [11], chemilumimetric [23], and enthalpimetric [17] detection. Most of these procedures were based on enzymatic reactions involving either alcohol oxidase [5,10,14,23] or alcohol dehydrogenase [6,7,11,20,22]. Some of these systems presented the disadvantage of requiring sample pretreatment before injection, or then made use of detection systems that are not widely available in wine laboratories.

In this work, a flow injection system for the determination of ethanol in wine was developed using an immobilized alcohol dehydrogenase reactor and an in-line separation/dilution system. The methodology was based on the enzymatic conversion of ethanol to acetaldehyde with the reduction of NAD to NADH. The latter was detected spectrophotometrically at 340 nm.

The use of immobilized rather than soluble enzymes reduces enzyme consumption and also may improve stability [21]. To minimize the consumption of the cofactor NAD, the merging zones technique [1] was applied. This way, only the necessary amount per sample was consumed. A gas-diffusion unit was incorporated to separate the analyte from the complex wine matrix, thus avoiding reactor fouling and interferences in the absorbance measurements. It also provided the necessary extensive sample dilution to adjust the concentration to the limited range of spectrophotometric enzymatic determinations [4].

Materials and Methods

Reagents and solutions: Alcohol dehydrogenase (300 to 400 U mg⁻¹, EC 1.1.1.1, ref. 102717) and NAD (GRADE III, 90%, ref. 710113) were purchased from Boehringer (Manheim, Germany). Aminopropyl glass (average pore size: 500 Å; 200–400 mesh, ref. G-4643) obtained from Sigma (St Louis, MO, USA) was used. All other reagents were analytical grade.

For the immobilization of the ADH, a 0.1 M phosphate buffer solution (pH 7.0) and a 2.5% glutaraldehyde solution (Merck, Schuchardt, Germany) in the phosphate buffer were prepared. The phosphate buffer was obtained by adding 500 mL of 0.1 M NaH₂PO₄, 290 mL of 0.1 M NaOH, adjusting the pH to 7.0, and diluting to 1 L with deionized water.

In the flow injection systems, a carbonate buffer (pH 9.0) containing 18.8 g sodium hydrogencarbonate and 2.12 g anhydrous sodium carbonate in 1 L of water was used. A 3 mM and a 4 mM NAD solution were prepared daily by dissolving the corresponding amount of solid in the carbonate buffer. Working standard solutions of ethanol were prepared daily in the range of 5% to 25% (v/v) by rigorous dilution of absolute ethanol.

Immobilization of the alcohol dehydrogenase on aminopropyl glass and reactor preparation: The procedure was similar to that described by Masoom
and Townshend [9] for glucose oxidase and by Ruz et al. [19] for alcohol dehydrogenase, except leaving the salanization step out. This was not necessary as we have used a Sigma controlled pore glass (CPG) suitable for direct attachment of proteins, in which the salanization step had been previously carried out (50 to 125 μm primary amine per gram of glass).

Three mL of glutaraldehyde (2.5% in 0.1 M phosphate buffer of pH 7.0) was added to 0.5 g of aminopropyl glass in a vessel where nitrogen was bubbled to remove oxygen. The reaction was allowed to proceed for one hour at room temperature with brief deoxygenation every 10 minutes for the first 30 minutes. The glass was washed with deionized water through a glass filter (pore size 4). The activated glass was added to 3 mL of 0.1 M phosphate buffer (pH 7.0) containing 15 mg of ADH. Nitrogen was bubbled through and the suspension agitated gently for four hours at 4°C. The glass was then successively washed with cold deionized water and phosphate buffer. The immobilized enzyme was stored wet at 4°C in a tightly closed tube.

Afterwards, eight reactors were prepared, consisting of a piece of Gilson (Villiers-le-Bel, France) PVC pump tubing (i.d. 3.5 mm, length 1.5 cm), filled by means of a syringe with the immobilized enzyme dispersed in the phosphate buffer. Ordinary dishwashing foam was placed at both ends of the reactor to entrap the controlled pore glass. When not in use, the reactors were kept at 4°C in the phosphate buffer at pH 7.0.

**Apparatus:** The FIA system consisted of Gilson Minipuls-3 peristaltic pumps, two Rheodyne (Cotati, CA, USA) 5020 six port internally coupled injection valves, an Unicam (Cambridge, UK) UV/Vis spectrophotometer equipped with a Hellma (Müllheim/ Baden, Germany) 178011 flow cell (internal volume 30 μL) and a Gilson N1 recorder.

A gas diffusion unit [8] with straight flow channels (2 mm wide, 0.5 mm deep, and 3.5 cm long) was used. The gas diffusion membranes tested were made of polytetrafluoroethylene (PTFE) (TBA, UK) and polyvinylidene fluoride (PVDF) (MILLIPORE, Bedford, MA, USA) GVHP 09050.

All the tubing connecting the different components of the FIA system were made of Omnifit (Cambridge, UK) PTFE tubing with 0.8 mm i.d., and Gilson end-fittings and connectors.

For the reference ethanol determination, a distillation apparatus and Denis Alcoholmeters (Arnouville, France), were used, as it is described for the usual methods of the OIV [18].

**Flow injection systems:** Two different flow injection systems were used, as shown in Figure 1. A simple manifold (A) was used for the optimization of the operational conditions of the immobilized enzyme reactor. A small volume (15 μL) of diluted ethanol standard (in the range of 0.005% to 0.05% (v/v) of ethanol) was injected into the stream of carbonate buffer, which merged with the buffer stream containing the cofactor NAD at the concentration α. The plug was led to the enzyme reactor and subsequently to the spectrophotometer [I] (wavelength set at 340 nm).

System B was developed for the determination of ethanol in wine samples. The samples or standards and the cofactor solution were injected simultaneously in Vᵢ and Vᵢ₊₁, respectively. The sample was diluted by the water stream Q₁ in β and was directed to the gas diffusion unit. The ethanol diffused to the acceptor buffer stream (Q₃) to meet the injected NAD zone. Therefore, the flow parameters were adjusted in a way that the two zones reached the membrane at the same time. The zone was led to the enzyme reactor (CPG-ER) and afterwards to the flow cell of the spectrophotometer [I]. The ethanol concentrations of the wine samples were obtained by interpolation in a previously established calibration curve in the range of 5% to 25% (v/v).

**Results and Discussion**

**Study of the enzymatic reaction using a two-channel flow injection manifold:** Some preliminary experiments were carried out to improve the immobilization of the enzyme on CPG, and also to establish the reaction conditions for the determination of ethanol. Therefore, the enzyme activity of the supernatant solution was measured during the immobilization. The conditions for this assay were: 75 μM ethanol and 1.2 mM NAD in 0.2 M carbonate buffer (pH 9.0). The rate of formation of NADH was monitored at 24°C and at 340 nm and was correlated with the enzyme activity. The results showed that most of the enzyme was bound after 15 minutes in contact with activated CPG, the overall efficiency of the immobilization being 95.3%. These results were in agreement with those of Brochet and Bucholcz [2].
Subsequently, the immobilized ADH reactor was placed in a two-channel manifold (Fig. 1A) to study the effect of the pH, NAD concentration and the flow rate, on the immobilized enzyme activity and on the reactor operational stability.

The effect of the pH was studied in the range of pH 8 to 10, using a 0.2 M carbonate buffer. The sensitivity of the measurement increased with the pH, but for pH values higher than 9.5, the stability of the enzyme reactor decreased very rapidly. Therefore, a buffer with a pH 9.0 was used for further experiments. The peak heights, as well as the sensitivity, increased with the concentration of the NAD cofactor (in the range of 2 to 10 mM NAD), however, not at the same rate. Considering the cost of the cofactor, a low concentration (4 mM NAD) was used to study the stability of the enzyme reactor. The increase of the flow rate from 0.8 to 2 mL min⁻¹ did not significantly change the sensitivity, but with flows over 1.2 mL min⁻¹ the repeatability decreased. Therefore, a 1.2 mL min⁻¹ flow rate was used as a compromise between good sampling rate and reactor stability. Under these conditions, the operational stability of the enzyme reactor was assessed, and the results showed that no significant loss of sensitivity occurred in the first three weeks.

**Development of a flow injection system for wine analysis:** The configuration of the manifold was designed to allow determinations without any wine pretreatment. Due to the probable influence of the wine matrix on the enzymatic reactor and on the spectrophotometric detection, it would be advantageous to separate the analyte from the sample. To do so, a gas-diffusion unit was incorporated in the manifold. This way, the hydrophobic membrane only allowed the volatile compounds to diffuse to the acceptor stream, thus providing additional selectivity. Since the gas-diffusion process only provides a low yield, it also provided the necessary adjustment of the ethanol concentration in the acceptor channel that was transported to the enzyme reactor. To decrease the NAD consumption, a second injection valve was included, so that the merging zones principle could be implemented.

The influence of the different parameters were then studied to allow the determination of between 5% and 25% (v/v) ethanol, with good sensitivity, low reagents consumption and a fair determination rate. To begin with, the flow rate and the pH of acceptor stream were set to the values found in the previously described study of the enzymatic reaction.

In order to minimize the effect of the sample matrix on the gas-diffusion process, and also to adjust the ethanol concentration, a diluting water stream and a 30-cm long mixing tube was connected to confluence β, and the sample injection volume was minimized. Volumes in the range of 10 to 50 μL were tested, and a value of 25 μL was selected, since volumes lower than 25 μL caused decreased repeatability when standards containing high concentration (20% and 25% (v/v)) of ethanol were injected.

Regarding the study of the gas-diffusion process, two flow channel lengths (3.5 and 7 cm) and two membrane materials (PTFE and PVDF) were tested. In order to achieve a higher dilution, a 3.5-cm-long straight line flow channel was selected. When the PTFE membrane was used, a pronounced membrane bulging occurred, probably due to the back pressure produced by the enzyme reactor in the acceptor channel. Therefore, the more resistant PVDF membrane was applied, and then no significant bulging was observed.

The parameters of the flow system were set to implement the merging zones technique. The arrival of the NAD plug over the gas-diffusion membrane was synchronized to the arrival of the sample zone, by changing the injection volume and the length of the L₁₃ tube. The use of a 200-μL injection volume and a 28-cm-long tube assured that the time that the NAD zone spends over the membrane is sufficient to receive the ethanol diffusing to the acceptor stream.

The concentration of the injected NAD solution was studied in the range 1 to 6 mM. A 3 mM NAD solution was chosen, as it provided sufficient sensitivity (A = 0.125+0.74 × 10⁻³ [Et-OH]-3.92 × 10⁻⁴ [Et-OH]², R = 0.9957), similar to one obtained by using a 2 mM NAD solution flowing continuously in the acceptor stream. This way the consumption of the NAD solution was reduced to 0.6 μmol per assay, compared to the 4 nmol per assay of the continuous approach, and the 6 nmol per assay consumption of the batch enzymatic procedure. If the system is only used in the range of table wines (up to 14% (v/v)), the injection of 2 mM NAD is sufficient to achieve good sensitivity; this way the NAD consumption can be further decreased.

With the optimized system, a detection limit of 0.4% (v/v) ethanol [13] was obtained. A sampling rate of 30 determinations per hour was also achieved.

**Application to wine analysis:** The developed system was applied to determine the ethanol content of different Portuguese table and Port wines, both red and white. A typical recorder output is presented on Figure 2.

To assess the quality of the FIA results, 20 wines were also analyzed by the usual aeroetric method of the OIV [18], and the results compared in terms of accuracy (Table 1).

The results showed relative deviations between the two methods lower than 4.4%. When paired t-test was performed on the data, a t value of 0.644 was obtained, the comparison with the t = 0.05, d.f. = 19) = 2.093 indicates no significant difference for the mean concentrations obtained by the two methods. Additionally, a regression line was used as well to compare the two methods [13]. The resulting equation is the following:

\[ C_{\text{fi}} = 0.164 (±0.458) + 0.991 (±0.030) C_{\text{OIV}} ; R = 0.938 \]

where \( C_{\text{fi}} \) was the result obtained by the flow injection method and \( C_{\text{OIV}} \) was the result obtained by the OIV method. The values in brackets are the limits of the 95% confidence intervals. These values also indicated no evi...
Fig. 2. Recorder output obtained with the developed flow injection method for the determination of ethanol in different wine samples.

Table 1. Results obtained in the determination of ethanol in 20 wine samples by the developed flow injection and the OIV method, and the corresponding relative deviations (RD).

<table>
<thead>
<tr>
<th>Sample n°</th>
<th>OIV method (v/v%)</th>
<th>FIA (v/v%)</th>
<th>RD (%)</th>
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dence for systematic difference between the two sets of results.

The precision of the developed flow injection determination was assessed at three levels of the application range from nine consecutive injections of three different wines, and the relative standard deviations obtained were 1.4%, 1.1%, and 2.2% at 7.02%, 12.06%, and 18.57% (v/v) of mean ethanol concentration, respectively.

Conclusions

The method developed here allows one to determine ethanol in a wide variety of wines, from young table wines to fortified wines, in the range of 5% to 25% (v/v) ethanol, with good accuracy and precision and without requiring any sample pretreatment. The experimental setup is simple to assemble, easy to operate, and the detection system is usually available in wine laboratories. A sampling rate of 30 determinations per hour is advantageous when compared to the recommended methods [15,19]. It should also be emphasized that the introduction of the gas-diffusion unit allowed one to reduce the interference from the sample matrix on the spectrophotometric detection and, on the other hand, provide extensive and reproducible dilution. The reagent consumption, both the enzyme and the cofactor is low. The reactor lifetime (3 weeks) is long enough for routine applications.

Literature Cited


