Flow monitoring of NADH consumption in bioassays based on packed-bed reactors bearing NAD$^+$-dependent dehydrogenases

Determination of acetaldehyde using alcohol dehydrogenase

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Abstract
An enzymatic method for the individual or simultaneous determination of pyruvic acid and acetaldehyde is described. Alcohol dehydrogenase (ADH) was immobilized onto aminopropyl-modified controlled pore glass, which was then used for the construction of packed-bed (PB) reactors. ADH catalyses the reduction of acetaldehyde to ethanol, in the presence of the coenzyme NADH, which is oxidized to NAD$^+$. Photometric measurements in a fully automated flow injection (FI) manifold are used to monitor the decrease of NADH absorbance at 340 nm. The possibility of pyruvate measurements, by combining the above mentioned system with soluble pyruvate decarboxylase (PyDC) is also demonstrated. PyDC catalyses the decarboxylation of pyruvate to acetaldehyde. Analytical parameters such as the buffering system, working pH, flow rate, sample size, and NADH concentration were studied. The interference of various compounds present in real samples was also investigated. Linear calibration graphs over the ranges 0.08–1.25 and 0.04–0.4 mM acetaldehyde were constructed in the presence of 50 mM succinate pH 7.5 and 50 mM phosphate pH 7.0 buffering systems, respectively. A linear calibration graph over the range 0.08–1.25 mM pyruvate was also constructed in the presence of 50 mM succinate pH 7.5 buffer solution. The reactors remain active for more than 6 months under specified storage conditions. The maximal sample throughput is 30 h$^{-1}$ and the R.S.D. of the method is 0.9% for 0.2 mM acetaldehyde ($n = 6$). The suitability of the proposed method for real samples was tested by recovery studies.

Keywords: Flow monitoring; NADH; Alcohol dehydrogenase; Pyruvate decarboxylase; Acetaldehyde; Pyruvate

1. Introduction
In the field of biosensors and enzymatic analysis in general, NAD$^+$-dependent dehydrogenases represent a vast field of applications. Dehydrogenases (more than 300) are selective to substrates of great analytical interest in food, environmental, clinical chemistry and bioprocess applications [1]. Bioassays based on NAD$^+$-dependent dehydrogenases have consequently been the subject of many review articles, where their various applications are presented in respect of the field of application (e.g. clinical, food chemistry), the type of immobilization matrix (e.g. packed-bed (PB) reactors, sol–gel matrices, screen-printed electrodes), or the type of the experimental arrangement where the analysis was performed (e.g. flow injection (FI) analysis, batch methods) [1,2]. Taking as criteria the number of presented applications and the adaptation
of some of them in commercial analyzers [3], we can safely state that PB reactors in flow systems represent the most important example of NAD⁺-dependent dehydrogenase bioassays.

In articles reported so far, the determination of the analyte of interest (substrate) is achieved by monitoring the production of the reduced form of the coenzyme, NADH, in spectrophotometric and spectrofluorimetric approaches by measuring the increase of the absorbance at 340 nm or fluorescence intensity, and in amperometric approaches by measuring the current produced by the oxidation of NADH at a constant potential, commonly in the range 0–0.65 V versus Ag/AgCl [1,2].

The general equation which describes the action of NAD⁺-dependent dehydrogenases is

\[
\text{substrate} + \text{NAD}^+ \leftrightarrow \text{product} + \text{NADH} + \text{H}^+ \quad (1)
\]

where SDH is the dehydrogenase specific to substrate S.

By measuring the concentration of NADH produced during the reaction, the enzyme can be used only for the determination of its major substrate, i.e. acetaldehyde dehydrogenase (AIDH) for acetaldehyde, malate dehydrogenase for malic acid, etc. However, under specific experimental conditions, each enzyme could be potentially used for the determination of both the substrate and the product of a certain enzymatic reaction, i.e. malate dehydrogenase used for both malic and oxaloacetic acids, lactate dehydrogenase for both lactic and pyruvic acids, etc. Acetaldehyde, for example, can effectively be determined by using alcohol dehydrogenase (ADH) and NADH (Eq. (2)), the concentration of the latter being depleted during the enzymatic reaction:

\[
\text{acetaldehyde} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{ADH}} \text{ethanol} + \text{NAD}^+ \quad (2)
\]

This enzymatic pathway is proposed as an alternative to the acetaldehyde dehydrogenase (AIDH)-based methods:

\[
\text{acetaldehyde} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{ADH}} \text{acetic acid} + \text{NADH} + \text{H}^+ \quad (3)
\]

The effectiveness of this concept is well-proven, as the majority of the reference enzymatic methods provided by Boehringer Mannheim (F-kit) utilize dehydrogenases, either for the determination of the substrate or its metabolites [4]. Some typical examples, where the measured parameter is the decrease of the absorbance of NADH, are acetoacetic acid with 3-hydroxybutyrate dehydrogenase, pyruvic acid with lactate dehydrogenase, oxaloacetic acid with malate dehydrogenase, dihydroxyacetone with glycerol dehydrogenase, α-ketoglutaric acid or ammonia with glutamate dehydrogenase, and nitrate with nitrate reductase (coenzyme NADPH) [4]. Moreover, by using the enzymatic reactions catalyzed by NAD⁺-dependent dehydrogenase in both directions (substrate/NAD⁺ or product/NADH), new and versatile enzymatic pathways for various analytes can occur.

The proposed concept of the flow monitoring of NADH depletion for the determination of an analyte, and the combination of such an enzymatic reaction (reversed direction) with another one for the determination of an extra analyte, separately or simultaneously with the first one, is presented here for the individual or simultaneous determination of acetaldehyde and pyruvic acid utilizing pyruvate decarboxylase (PyDC) and ADH.

To the best of our knowledge, this is the first effort which reports on the flow spectrophotometric determination of acetaldehyde by using PB reactors bearing ADH. The possibility for the individual or simultaneous determination of pyruvic acid as well, by combining soluble PyDC with the ADH reactor, is also demonstrated.

2. Experimental section

2.1. Apparatus

All experiments were made by using a photometric unit comprised of a spectrometer equipped with a 2048 pixels CCD (charge coupled device) detector (SD2000, Ocean Optics Inc., FL) and a tungsten light source (LS-1, 360–900 nm, Ocean Optics). Data acquisition was carried out by means of an interface card (ADC 500, Ocean Optics) between the spectrometer and an IBM compatible PC. Data evaluation was done by using the software SpectraWin 4.2 (Top Sensor System BV, The Netherlands). Solutions were pumped through a four-channel peristaltic
pump (Gilson, Minipuls 3, Villiers-le-Bel, France). Sample injections were made with a pneumatically actuated injection valve (Rheodyne, Type 50 Teflon, CA). A Z-type flow-through cell (Shimadzu, Kyoto, Japan), of 8 \( \mu l \) capacity and 1 cm optical path was used throughout. A resident program insures the full control of the pump and the valve, giving to the user the ability to work without manual manipulations [5].

2.2. Chemicals

ADH (alcohol: NAD\(^+\) oxidoreductase; EC 1.1.1.1 from baker’s yeast, lyophilized powder, 356 U mg\(^{-1}\), Cat. No. A-3263) and PyCD (2-oxo-acid carboxylase; EC 4.1.1.1 from baker’s yeast, suspension in 3.2 M (NH\(_4\))\(_2\) SO\(_4\), 100 U/1.4 ml, Cat. No. P-9474) were supplied from Sigma (St. Louis, MO). Aminopropyl controlled pore glass (CPG) (500 Å, 200–400 mesh), NAD\(^+\) (Cat. No. N-7004), NADH (Cat. No. N-8129) and acetaldehyde were also purchased from Sigma. All other chemicals, of analytical-grade, were obtained from Sigma or Merck (Darmstadt, Germany). The NADH solution was freshly prepared in the working buffer. Stock solutions of pyruvic acid (50 mM) was prepared by dissolving 27.5 mg of sodium pyruvate in 5.0 ml of 0.1 M HCl to prevent polymerization. The solution was stored at +4 °C and prepared weekly, owing to its instability [6]. A stock solution of acetaldehyde (0.092 M) was prepared by diluting acetaldehyde in water, standardizing with the bisulphite titrimetric method, and stored at 4 °C [7].

2.3. Preparation of the packed-bed (PB) reactors

The PB reactors were prepared by packing 80 mg of the derivatives in glass columns (2.0 mm i.d. x 30 mm length). The aminopropyl CPG was activated by pumping through the columns a 2.5% (v/v) solution of glutaraldehyde in 50 mM phosphate buffer, pH 7 for 2 h at room temperature and washed with doubly distilled water for another 2 h. The enzyme solution (6 mg of ADH (2100 U) in 12 ml of 50 mM phosphate buffer at pH 7.0) was loaded onto the supporting material by circulating it for 36 h at a flow rate of 0.1 ml min\(^{-1}\) at 4 °C. The enzyme solution was moved along the reactor by reversing the flow at preset time intervals by means of the software [5]. This procedure increases the efficiency of immobilization due to the attack of the available surface of the supporting material from two directions. The support was washed with water (5 min), 50 mM NaCl (60 min), water (60 min) and finally with the immobilization buffer (30 min). The reactor was stored at 4 °C filled with the immobilization buffer.

2.4. Sample preparation

Coffee samples were prepared by diluting 2–4 g of the product to 100 ml with distilled water. Soft drinks, white wine and orange juice samples were prepared by diluting 20 ml of the product with 100 ml of distilled water. Tobacco and tea samples were prepared by
extracting 1–2 g of the product with 50 ml of distilled water. Samples were then filtered through Whatman filter paper and finally diluted to 100 ml of distilled water. All samples were treated with active charcoal (Merck, Cat. No. 2183) for 15 min at 30 °C and finally filtered through a Whatman paper.

2.5. Procedure

The diagram of the FI system is shown in Fig. 1. The carrier (50 mM succinate, pH 7.5 or 50 mM phosphate, pH 7.0) and the NADH (0.4 mM in the carrier solution) streams were continuously pumped at 0.36 and 0.20 ml min⁻¹, respectively, towards the cell until a stable absorbance value was reached (1.2–1.4). Standards or sample solutions of acetaldehyde (appropriate dilutions of the stock solution in the carrier solution) were introduced as short pulses of 120 μl via the loop injection valve.

For pyruvate assays, standard solutions of pyruvate were prepared in the carrier solution, and 2 min before the injection a portion of PyDC (2 U) was added.

The peak height absorbance (decrease of the signal) and transmittance (increase of the signal) were recorded simultaneously (Fig. 2) and the former was taken as a measure of the analyte concentration (better calibration linearity).

Fig. 2. FI peak responses for the determination of (a) 1.0, (b) 0.5, (c) 0.25, and (d) 0.125 mM acetaldehyde as a function of absorbance and transmittance.
3. Results and discussion

3.1. PB reactors bearing ADH

The amount of the immobilized enzyme was determined as protein by measuring the absorbance of the ADH solutions at 280 nm before and after immobilization [8]. The immobilization of ADH on aminopropyl-modified CPG was performed with an efficiency of ca. 85%. In addition, the enzymatic activity of the immobilization solution after the immobilization procedure was determined according to the reference method with ethanol in an alkaline medium [9]. The remaining activity was found to be 187 U. In a previous attempt to load an identical reactor using an immobilization solution of 2 mg of ADH (700 U) in 4 ml of 50 mM phosphate buffer, pH 7.0, the remaining activity was zero.

3.2. Working and flow parameters

Parameters such as buffering systems and working pH, concentration of NADH, sample size and flow rates of the reagents were optimized. The parameter variations were carried out with an acetaldehyde solution of 0.4 mM.

The optimum pH for the activation of ADH has previously been reported to be 8.2–9.5 [10,11], when ethanol was employed as substrate. In this work, where acetaldehyde is employed as a substrate for the immobilized ADH, a wide pH range, from 5 to 8 was tested using phosphate, imidazole and succinate buffering systems. Fig. 3 shows that 50 mM succinate buffer, pH 7.5, and 50 mM phosphate buffer, pH 7.0, are the most suitable. Most of the subsequent work was carried out using succinate. Its selection was based on the fact that succinate is also an excellent buffer medium for PyDC [12], thus allowing the use of the method for the individual (or simultaneous, by signal subtraction) determination of pyruvic acid (see the following sections). The use of phosphate buffer is not appropriate for pyruvate measurements, since phosphate is a major inhibitor of PyDC, even at a low concentration. Phosphate competes with the binding site for pyruvate [12].

Several flow rates in the range 0.10–0.60 ml min$^{-1}$ were applied. An overall flow rate of 0.56 ml min$^{-1}$ reconciles fairly high peaks and satisfactory sampling throughput (30 h$^{-1}$). A sample volume of 120 µl was selected, which balances no peak broadening and a fairly high sensitivity. No influence on the system was observed by positioning a plain reactor after the meeting point of the sample with NADH for better mixing.

Since the sensitivity, accuracy and dynamic range of the method are strictly dependent on the concentration (absorbance) of the NADH, a concentration of 0.4 mM was employed. This concentration is sufficient to give an absorbance of 1.2–1.4.

3.3. Interferences

The behavior of commonly considered interferents normally present in real samples was investigated. All the tested compounds (organic acids, electrolytes and ethanol) were present at concentrations of 2–2000 mM in mixtures containing 0.4 mM acetaldehyde. As expected, the results displayed in Table 1 show that the majority of organic acids do not interfere, indicating that the selectivity of ADH to acetaldehyde is very high. The effect of calcium and potassium ions was also almost nil, indicating that these cations are not considered as activators or inhibitors of the tested enzyme. Results referring to the interference effect...
Table 1

Interference tests on various compounds for the assay of acetaldehyde

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaldehyde (0.4)</td>
<td>100</td>
</tr>
<tr>
<td>Oxalic acid (10)</td>
<td>107</td>
</tr>
<tr>
<td>Butyric acid (5)</td>
<td>106</td>
</tr>
<tr>
<td>Pyruvic acid (2)</td>
<td>102</td>
</tr>
<tr>
<td>Tartaric acid (2)</td>
<td>100</td>
</tr>
<tr>
<td>Malic acid (2)</td>
<td>102</td>
</tr>
<tr>
<td>Citric acid (2)</td>
<td>101</td>
</tr>
<tr>
<td>Glutamic acid (2)</td>
<td>96</td>
</tr>
<tr>
<td>Acetic acid (2)</td>
<td>94</td>
</tr>
<tr>
<td>Lactic acid (2)</td>
<td>96</td>
</tr>
<tr>
<td>Adipic acid (2)</td>
<td>99</td>
</tr>
<tr>
<td>Calcium chloride (10)</td>
<td>103</td>
</tr>
<tr>
<td>Potassium chloride (10)</td>
<td>104</td>
</tr>
<tr>
<td>Ethanol (10)</td>
<td>99</td>
</tr>
<tr>
<td>Ethanol (200)</td>
<td>98</td>
</tr>
<tr>
<td>Ethanol (2000)</td>
<td>59</td>
</tr>
<tr>
<td>Phosphate (5)</td>
<td>100</td>
</tr>
<tr>
<td>Phosphate* (5)</td>
<td>30</td>
</tr>
</tbody>
</table>

In parenthesis, the concentration of the compounds is given in mM. All solutions contain 0.4 mM acetaldehyde and the activity compared to that of plain 0.4 mM acetaldehyde taken as 100%.

*Performing pyruvate measurements; 0.4 mM pyruvate.

of ethanol are of great analytical importance, as it is shown that under the applied conditions (pH 7.5 and 0.4 mM NADH) the reaction is almost quantitative towards the formation of ethanol. In the presence of a 200-fold higher concentration of ethanol no interference was observed, while in the presence of ethanol at a concentration normally found in wine (11%, (v/v)) a 40% decrease of the signal was observed. This means that the method could be applied only in diluted wine samples. Similar results were also obtained in previous studies when AIDH had been utilized [13].

3.4. Calibration and recovery studies

Under the optimum conditions, two calibration graphs, absorbance \( A = f(\text{acetaldehyde/mM}) \), were constructed by applying the least squares method. Using the aminopropyl-modified CPG-ADH reactors, linear relations were obtained between the response and the acetaldehyde concentration in the ranges 0.08–1.25 and 0.04–0.4 mM in the presence of 50 mM succinate, pH 7.5 and 50 mM phosphate, pH 7.0, respectively. Data fitted the equations \( A = 0.05(\pm0.006) + 0.61(\pm0.008) [\text{acetaldehyde/mM}] \) and \( A = -0.03(\pm0.01) + 2.85(\pm0.08) [\text{acetaldehyde/mM}] \), with correlation coefficients, \( r = 0.9997 \) and 0.9972 (\( n = 5 \)) respectively. The detection limits (signal-to-noise ratio of 3) were 30 and 10 \( \mu \text{M} \) acetaldehyde, respectively. The relative standard deviation (R.S.D.) of the system for both buffering systems was 0.9% for 0.2 mM acetaldehyde (six replicate measurements).

The matrix effect was studied for different samples by recovery experiments on a wide variety of real samples such as tobacco, tea, coffee, soft drinks and fresh juices. The recoveries attained are in the range 93–105% as shown in Table 2 and justify the suitability of the method for the flow assay of acetaldehyde in real samples.

3.5. Lifetime

The reactors displayed very good operational and storage stability when kept in the immobilization buffer at 4 °C when not in use. Under the conditions described above, after more than 2000 injections over a period of 6 months, the reactor had retained 90% of its initial activity. Excellent behavior was also observed when reactors were continuously used at room temperature. After about 150 injection over 2
days, no significant loss of the original activity was observed.

3.6. Use of the system for pyruvate detection

As stated above, the use of the reversed NAD$^{+}$-dependent dehydrogenase reactions can promote new enzymatic pathways for the determination of various analytes. The system described so far can potentially be used for the determination of pyruvate by combining the immobilized ADH in the reversed mode (Eq. (2)) and PyDC. PyCD catalyses the decarboxylation of pyruvate to acetaldehyde:

\[
\text{Pyruvic acid} \rightarrow \text{acetaldehyde} + \text{CO}_2
\]

Preliminary experiments were carried out with PyCD immobilized onto aminopropyl-modified CPG, either as a separate PyCD reactor or co-immobilized with ADH into a single reactor. In both cases, however, the efficiencies of immobilization were very poor. The resulting systems showed very low efficiency, poor reproducibility and a remarkable loss (more than 90%) of activity with repeated injections of pyruvate, eliminating the advantages gained from the immobilization of ADH. PyCD is a ternary complex of the apoenzyme with TPP and Mg$^{2+}$, which exhibit a high quasi-irreversible stability [14]. Resolving of the holoenzyme to the apoenzyme and its co-factors, TPP and Mg$^{2+}$, appear to be responsible for the inactivation of the enzyme. The apoenzyme has no decarboxylating activity. The holoenzyme is fully reconstituted by the addition of large amounts of TPP and Mg$^{2+}$ to freshly prepared apoenzyme, however, under the specific conditions (immobilized state), its reconstitution did not occur. Reconstitution of the holoenzyme was attempted by adding various concentrations of MgCl$_2$ (up to 5 mM) and TPP (up to 2 mM) to the carrier stream.

The effect of PyCD on the response of the system was investigated by various enzyme additions (0–3 U per sample)–0.15 mM pyruvic acid standards. The saturation point is 2 U per sample (data not shown). The same profile was also recorded when 0.4 mM pyruvic acid was used. A mixing period of 2 min was found to be adequate for the complete decarboxylation of pyruvate. Termination of air bubble generation is also an indicator of reaction completion.

The effect of pH on the activity of PyCD was re-examined only for the case of succinate buffer (Fig. 3). A small shift of the optimum pH was observed, and subsequent work was carried out at pH 7.0.

Under the optimum conditions, a linear calibration graph: \( A = f ([\text{pyruvate/mM}]) \), over the concentration range 0.08–1.25 mM pyruvate, was constructed by applying the least squares method. Data fitted the equations \( y = 0.04(\pm 0.004) + (0.62 \pm 0.006) \) [pyruvate/mM], with a correlation coefficient, \( r = 0.9998 \) (n = 5). The detection limit (S/N = 3) was 20\( \mu \)M. The R.S.D. of the system was 0.9% the same as for acetaldehyde assay.

4. Conclusions

The flow monitoring of NADH consumption in bioassays based on PB reactors bearing NAD$^{+}$-dependent dehydrogenases is described as an alternative to existing NAD$^{+}$-monitoring methods. This alternative provides the possibility to use a specific dehydrogenase for the determination of both the reactant and its product. In some cases, as the one presented here, some advantages can occur. ADH is more stable and significantly cheaper than AIDH. Moreover, the use of dehydrogenases in this reversed mode can provide new enzymatic pathways for the determination of a wide range of compounds of analytical interest in clinical, environmental and food chemistry.

References