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Comparison of different flow injection approaches to the automatic determination of enzymatic activity

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Abstract Three configurations based on the principles behind flow injection analysis (FIA) are proposed for the automatic determination of enzymatic activity The proposed approaches are normal, stopped-flow and open–closed FIA The comparative study of the methods developed from these approaches allows the establishment of the scope of the application of each, with inherent advantages and disadvantages

Keywords *Flow injection analysis (FIA), enzyme assay*

Introduction

Flow injection analysis (FIA) has shown its great usefulness in clinical chemistry [1–4], especially, in the development of enzymatic methods involving enzymes as catalysts, both dissolved and immobilized [5] FIA has been less often used for the determination of enzymatic activity but there are some 30 papers showing its usefulness in this area [6]

The resources of the technique become apparent from a quick overview of the literature on enzyme activity determinations The variety of approaches used, show its ability for adaptation to the characteristics of the system in question

The configurations used, range from straightforward two-channel manifolds (used by Yoza *et al* for measuring the enzymatic activity of inorganic pyrophosphatase [7]), to the three-channel system for amyloglucosidase [8], the use of the merging zones mode with halting of the flow at the detector for the fluorescent determination of plasma enzyme [9], the semi-on-line measurement of creatine kinase isoenzyme MB in serum with immunoseparation and electrochemical detection [10], and the use of closed-loop systems [11–14] for glucose oxidase [15] or holding coils to accomplish long incubation times (e g those proposed by Rocks *et al* [16]) for the determination of serum prostatic acid phosphatase

This paper reports three configurations of different complexity and characteristics for this type of determination After experimental study, their advantages and disadvantages are discussed and compared

The enzyme chosen for this study was lactate dehydrogenase, whose clinical range is 210–420 U l^{-1}, and the determination was based on the action of this catalyst on the

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reduction of pyruvate to lactate by NADH followed by monitoring the decrease in absorbance due to the conversion of the coenzyme into the oxidized form at 340 nm

Experimental

Reagents

A stock solution of NADH (10 mmol l^{-1}) was prepared by dissolving 0 1735 g of NADH (Sigma No 8129) in 30% ethylene glycol–water and making up to 25 ml with this mixture A stock solution of pyruvate (50 mmol l^{-1}) was prepared by dissolving 0 5500 g of sodium pyruvate (Sigma No 2256) and making up to 25 ml with 0 1 M diacid potassium phosphate buffer of pH 7 0 The working solutions were prepared by dilution in the same buffer A stock solution of LDH was made by dissolving the contents of a vial of 1000 U (Sigma No L-1254, from rabbit muscle) in 2 ml of 0 1 M diacid potassium phosphate of pH 7 0 All reagents used were p a grade

Apparatus

A Perkin–Elmer Lambda-1 single-beam spectrophotometer equipped with a Hellma 178 12QS flow-cell and connected to a Radiometer REC 80 Servograph recorder was used A four-channel Gilson-Minipuls-2 peristaltic pump with rate selector, two Rheodyne 5041 injection valves (one of them modified to act as selecting valve) a Tecator TM III chemifold, and a Selecta 382-S thermostat were also used A Hewlett–Packard HP-85 microcomputer was employed to collect the absorbance–time data and absorbance values from the maxima and minima and to stop and start the peristaltic pump synchronized with the injection

Manifolds

The configurations used are depicted in Figs 1 and 2

The simplest configuration used is based on normal (n-FIA) and consists of (Fig 1a) a two-channel system Through one of the channels is circulated a buffered pyruvate solution into which the sample is inserted, this channel later merges with that of the coenzyme (NADH) The reaction develops along L_1, the resultant absorbance decrease being recorded as the reaction mixture passes through the detector (Fig 1b)

The second configuration, based on stopped-flow-FIA (sf-FIA), involves the use of the part connected to the above configuration through dashed lines (microcomputer and printer) which, through an active interface, controls the stop and start of the peristaltic pump synchronized with the injection system (Fig 1a) to effect the halting of the flow when the reacting plug is at the photometer flow-cell, in this case, the measured parameter is the absorbance decrease resulting from the reaction taking place during the stop time The length of L_1 is smaller than in the n-FIA configuration, in order to ensure minimal development of the reaction on the arrival of the reacting plug at the detector (Fig 1c)

The third configuration is an open–closed (oc-FIA) system in which the sample is inserted, as above, into a buffered pyruvate solution later merging with the reduced coenzyme channels After passing through selecting valve S V , this is switched and the reacting plug is trapped in the closed circuit including the detector (Fig 2a) As many signals as times the reacting plug is cycled before it is eventually homogenized with the carrier are obtained The multipeak recording (Fig 2b) provides a wealth of information Different types of measurements related to the enzymatic activity can be

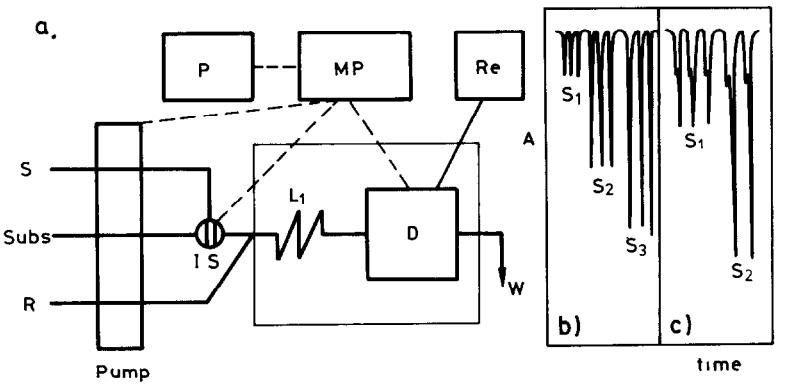

Figure 1
(a) Simple manifold for the determination of enzymatic activity (the units connected through dashed lines are required in the stopped-flow approach, the zone enclosed by the fine line is thermostatted) Recordings are those obtained with the simple (b) and with the stopped-flow (c) manifolds S, Sample, Subs, substrate, R, reagent (NADH), I S , injection system, L_1, reactor, D, detector, W, waste, P, printer, MC, microprocessor, Re, recorder, S_1, S_2 and S_3 are standards

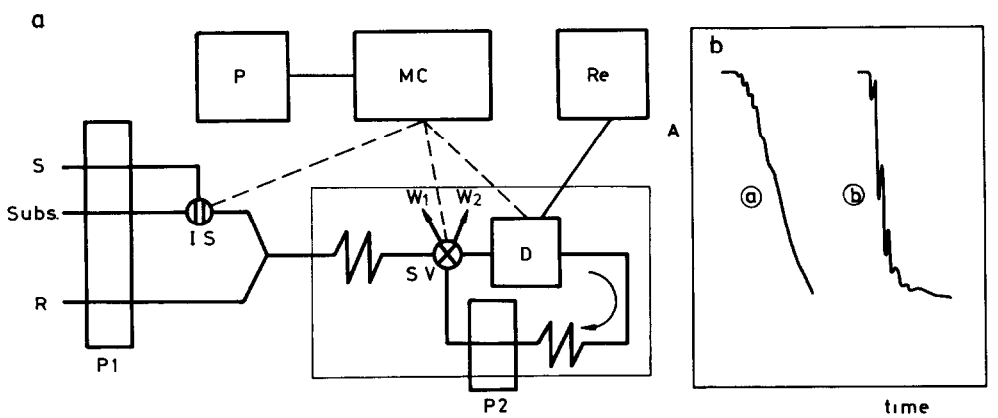

Figure 2
(a) Open–closed configuration for the determination of enzymatic activity (b) Multipeak recording obtained with 0 2 (a) and 2 0 U l^{-1} (b) of enzyme Symbols are as in Fig 1

made (e g measurements of individual maxima or minima, the sum of several maxima or minima, reaction-rate measurements from the absorbance decrease between two consecutive maxima or minima, the sum of such increments or their relation to physical parameters such as process and rate constants, the Michaelis constant, etc

It is necessary to emphasize that, in all cases (fixed-time or reaction-rate measurements) the analytical signal is due to an absorbance decrease measured from a baseline of a high absorbance (fixed-time methods) or from one measured over a preselected interval (reaction-rate methods)

Results and Discussion

The ranges studied and the optimum values of the variables influencing the systems are listed in Tables 1 and 2, respectively

There is a series of common variables which have a similar influence in all three configurations, with very slight or nil divergencies in their optimum values Such variables are the temperature, type and concentration of the buffer, pH, flow-rate and NADH concentration The optimum concentration of coenzyme is that yielding an absorbance of 1 0–0 9 upon passage through the detector, i e the most suitable value for covering a wide range of reaction development without saturation of the detector capacity for the baseline The influence of the injected volume is similar for all three configurations, but while for the open–closed system the variable to be optimized is the ratio of the total volume of the closed circuit to the injected volume, this limitation does not apply in the other two configurations In all cases splitting of the peak (the first peak in the open–closed systems) resulted from injected volumes above those considered as optimum The influence of the reactor length is similar Decreasing the flow-rate resulted in increased peak heights caused by a longer resident time (greater reaction development), but to the detriment of the sampling frequency 1 64 ml min^{-1} was chosen as a compromise

A variable whose behaviour must also be considered is the substrate (pyruvate) concentration The higher optimum concentration (10 mM) for the first two configurations is due to the shorter contact time between the sample–reagent interface, so that

Table 1
Ranges of the variables investigated

Variable	Range
pH (KH_2PO_4, 0 1 M)	5 5–8 5
[Pyruvate] (mmol l^{-1})	0 5–20 0
[NADH] (mmol l^{-1})	0 2–2 0
Flow-rate (ml min^{-1})	0 96–2 56
Reactor length (cm)	20–150
Injected volume (μl)	23 3–250
Temperature (°C)	20–45
Delay time (s)	10–20
Stop time (s)	10–90

Table 2
Optimum values of the variables

Common values	pH	Temperature	Buffer	[NADH]	Flow-rate
	7 0	40°C	KH_2PO_4 0 1 M	1 0 mM	1 64 ml min^{-1}

Non common values	n-FIA	sf-FIA	oc-FIA
[Pyruvate] (mM)	10 0	10 0	2 5
Reactor length (cm)	50	20	
Injected volume (μl)	100	100	200

Characteristic variables (sf-FIA)	Delay time	Stop time
	12 s	60 s

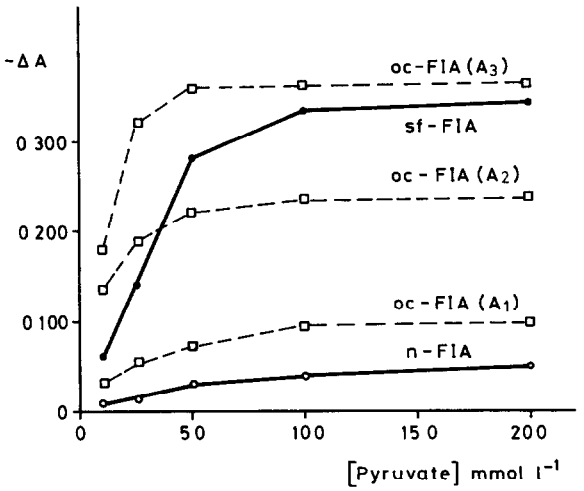

Figure 3
Influence of the pyruvate concentration on the analytical signal obtained by the different approaches

the reagent concentration must be increased to establish a higher gradient, in which the substrate diffusion is faster Figure 3 shows the absorbance decrease obtained with the three manifolds for the same amount of enzyme as a function of the substrate concentration (three different parameters are plotted for the open–closed configuration) The optimum pH was 7 0, pHs below and above this decreased the signal The buffer used (KH_2PO_4) was selected from the literature [17–19]

Increasing temperatures gave rise to greater signals up to 40°C, above which the signal decreased, probably due to the deactivation of the analyte

The time variables of the stopped-flow mode show an optimum value for delay time for 12 s at which the centre of the reacting plug is at the detector The increase of the signal with the stop time was important up to 60 s, above which the increase was so small that it did not compensate for the decrease of the resultant sample frequency

In all cases, the studies have been performed at two enzyme concentrations (0 2 and 2 0 U l^{-1})

Features of the Methods

Under the optimum working conditions for each method, calibration curves were run In Table 3 the values of the intercept, slope and regression coefficients of the different calibration curves and their linear range are summarized The open–closed configuration provides a number of calibration curves (14 from fixed-time measurements and 14 from reaction-rate measurements), so only the most representative are summarized in Table 3

The reproducibility of each method was studied with 11 different samples (concentration 0 5 U l^{-1}) injected in triplicate The results obtained and the sampling frequencies are also given in Table 3

There was no matrix effect for serum because of the high dilution required to perform the determination (1 1000)

Table 3
Features of the proposed methods

Method	Measured parameter	Features of the calibration curves				Coefficient variation (%)	Sampling frequency (h^{-1})
		Intercept	Slope	Regression	Determination range ($U\ l^{-1}$)		
n-FIA	$-\Delta A$	1×10^{-4}	0 0218	0 992	0 5–2 0	1 36	130
sf-FIA	$\Delta A/\Delta t$	−0 1847	0 4689	0 997	0 5–2 0	1 03	40
oc-FIA	A_1	−0 0105	0 113	0 999	0 1–2 0	2 93	110
	A_4	0 0067	0 538	0 998	0 1–1 0	0 81	22
	A'_1	−0 0020	0 046	0 999	0 1–1 5	2 59	70
	A'_4	−0 0066	0 534	0 999	0 1–2 0	1 02	20
	$\sum_1^4 A_i$	0 0160	1 194	0 998	0 1–1 0	0 94	22
	$\sum_1^4 A'_i$	0 0189	1 115	0 999	0 1–1 0	0 94	20
	$\bar{v}'_1$	−0 336	6 333	0 999	0 1–2 0	3 02	110
	$\bar{v}_4$	−0 0051	3 686	0 999	0 1–1 5	1 05	22
	$\bar{v}'_1$	−0 073	1 231	0 999	0 1–1 5	3 50	70
	$\sum_1^4 \bar{v}_i$	0 109	8 011	0 998	0 1–1 5	1 02	22
	$\sum_1^4 \bar{v}'_i$	0 130	6 096	0 998	0 1–1 0	0 60	20

A_n = absorbance of peak n $\bar{v}'_n$ = Increment of absorbance between two minima A'_n = Absorbance of minimum n $\sum_1^4$ = Sum of signals $\bar{v}_n$ = Increment of absorbance between two maxima

Comparison of Results

The three proposed approaches and the features of the methods developed on their basis are compared below

The chief advantage of the normal FIA configuration lies in its straightforward construction and handling, it provides fixed-time measurements, characteristic of conventional FIA, which are affected by the sample matrix, this is of particular relevance in clinical chemistry where only the effect due to interfering reactions, whose rate is slower than that of the main reaction, is diminished or cancelled The stopped-flow configuration required, in addition to the elements used in the n-FIA configuration, an electronic or computerized system for sequencing the stop and start of the peristaltic pump synchronized with the injections, measurements are based on the reaction rate, so they are not affected by the matrix characteristics as to whether the potentially interfering reactions are faster or slower than the main reaction Finally, the open–closed manifold requires a microcomputer and a passive interface for collecting the data from the multipeak recording, while the use of an active interface controlling the switching of the injection and selecting valves is optional Measurements can either be performed at fixed times or can be based on the reaction rate

The widest determination ranges are obtained with the open–closed system, with a determination limit of 0 1 U l^{-1} versus 0 5 U l^{-1} for the first two configurations

The sensitivity of the methods (slope of the calibration curve) is only comparable in those cases where the same type of measurement is performed Fixed-time measurements (n-FIA and oc-FIA) are improved by a factor of up to 60 from the n-FIA to oc-FIA method (sum of the absorbance decrease of the first four peaks), while measurements based on the reaction rate improve their sensitivity by a factor of 17 from the stopped-flow to the open–closed method (measurement of the absorbance decrease between the first two peaks)

The linearity achieved with the three configurations was similar (Table 3)

The reproducibility increases from the n-FIA to sf-FIA method (coefficients of variation 1 36 and 1 05, respectively) In the open–closed approach, this parameter depends on the data from the recording used for the calculation, it ranged between 0 2–3 5, but was close to 1 in most cases

The sampling frequency, a major parameter in routine methods such as those for the determination of enzymatic activity, varied widely for the three methods from 130 h^{-1} for the n-FIA method, to 20^{-1}h for the oc-FIA method with higher sensitivity The stopped-flow method provided a relatively slow sampling rate, typically 40 samples h^{-1}

Conclusions

From the above comparisons, it can be inferred that the use of some of the proposed approaches can solve any problem related to any type of sample (matrix) and any type of enzyme present in the sample

If the sample does not absorb in the measurement zone and the enzyme concentration is high enough, the n-FIA method has excellent features for routine purposes When there is absorption from the sample matrix, fluorimetric or voltammetric detection can be used to monitor the change in the oxidation state of the coenzyme

If the enzymatic activity is small and the sample does not absorb in the measurement zone, the open–closed approach can be used with fixed-time signal measurements,

reaction-rate measurements from these recordings can be used to check the absence of matrix effect If the effect does not exist, it is necessary to use a method based on reaction rate (stopped-flow or open–closed system), the choice depends on the sensitivity, sampling rate and determination limit required

In any case, the open–closed system provides much information (more than other configurations) through the multipeak recording obtained, this information can be manipulated for analytical–quantitative purposes or for the calculation of physical or physico-chemical parameters [11–14]

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