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MULTISTEP DETERMINATION OF ENZYME ACTIVITY BY FLOW INJECTION AND SEQUENTIAL INJECTION ANALYSIS. ASSAY OF AMYLOGLUCOSIDASE

Elo Harald Hansen, Bodil Willumsen, Solveig K. Winther and Helle Drabøl

Chemistry Department A, Building 207, The Technical University of Denmark, DK-2800 Lyngby, Denmark

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Summary—A multi-point assay for determination of the activity of amyloglucosidase (AMG) by FIA and SIA is described. The assay is based on two consecutive reactions that are mutually incompatible. Both the FIA and SIA procedures allow these two reactions to be completely separated, whereby each of the processes can be individually optimized with respect to operational parameters. Special emphasis is placed on comparing the performance and applicability of the two procedures for this type of assay.

With the expanding commercial production and application of enzymes there is an increasing demand for reliable, fast and simple methods for determining enzyme activity. In the fermentation processes where the enzymes are produced, or in subsequent industrial processes where their selectivity is exploited for converting specific substrates into useful components, their activity must be carefully monitored in order to achieve optimal reaction conditions. Likewise, when enzymes are to be incorporated into commercial products (*e.g.* washing powders) it is a requirement that their contents are conscientiously administered and accurately declared.

The activity of an enzyme can be assessed by determining the rate of specific reaction that the enzyme catalyzes under specific conditions. For the simple enzymatic conversion of a substrate S to a product P, the overall reaction rate is given by the Michaelis–Menten equation:

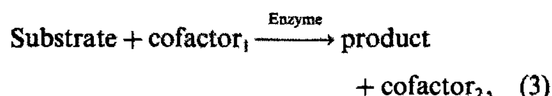
$$d[P]/dt = (k_2[E]_0[S]_t)/([S]_t + K_m), \quad (1)$$

where K_m is the Michaelis–Menten constant, $[E]_0$ is the total concentration of active enzyme, and k_2 is the rate constant for the rate-determining step. From equation (1), the reaction rate depends on both enzyme and substrate concentrations. However, if the conditions are manipulated so that $[S]_t \gg K_m$ (*i.e.* there is a surplus of substrate) the above expression reduces to:

$$d[P]/dt = k_2[E]_0, \quad (2)$$

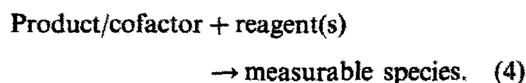
that is, the reaction becomes pseudo-zero-order with respect to the substrate. The reaction rate is then directly proportional to the concentration of the enzyme and hence its activity. This condition must be fulfilled in any enzyme activity assay.

In measuring enzyme activity, advantage can sometimes be taken of the fact that most enzymatic reactions also involve the interaction of appropriate cofactors:



$$\text{Substrate} + \text{cofactor}_1 \xrightarrow{\text{Enzyme}} \text{product} + \text{cofactor}_2, \quad (3)$$

that is, the activity can be quantified either by determining the product or a cofactor generated (or, possibly, a cofactor consumed). If none of the constituents in the enzymatic reaction are directly measurable, one of them might be determined by an accompanying indicator reaction:



$$\text{Product/cofactor} + \text{reagent(s)} \rightarrow \text{measurable species.} \quad (4)$$

No matter how the enzymatic activity is assessed, it is imperative to use a multi-point (kinetic) assay. This has been discussed in detail previously.[1] An instrument widely used for enzyme activity determination in clinical analysis is the centrifugal analyzer that allows the simultaneous multi-point assay of 30 samples.[2,3] Another well suited technique is stopped-flow FIA, where a suitable section of the injected and

dispersed sample zone is arrested within the observation area of the detector and monitored over a fixed period of time.[4] However, if such 'one-step' approaches are to be used, it is a necessary condition that one of the constituents in the enzymatic reaction can be measured directly, or that the coupled indicator reaction is compatible with and much faster than the enzymatic degradation reaction. If the indicator reaction is slower, or incompatible with the degradation reaction, an alternative avenue must be used.

We encountered exactly such a problem when trying to determine the activity of cellulase. The attempt to solve this particular problem gave the impetus to design a generic FIA system for enzyme activity determination. Based on a multi-point assay approach, this system should be able to handle all types of enzymatic reactions, irrespective of the individual reaction rates and particulars of the reactions involved. Such a system, the concept of which was detailed in a previous communication,[1] is schematically outlined in Fig. 1. It comprises two subsystems—one handling the enzymatic degradation reaction and the other one handling the ensuing derivatization chemistry—the two individual subsystems communicating via a common valve. In the enzymatic degradation unit the enzyme and substrate are mixed in the reactor vessel (mixing chamber) at time zero and via pump 1 made to communicate with the valve. In order for the solution in the conduits to be identical at all times to the solution in the reactor vessel, the pumping rate (V) has to be maintained at a relatively high level (of the order of 1.5–2 ml/min). At preselected times, the

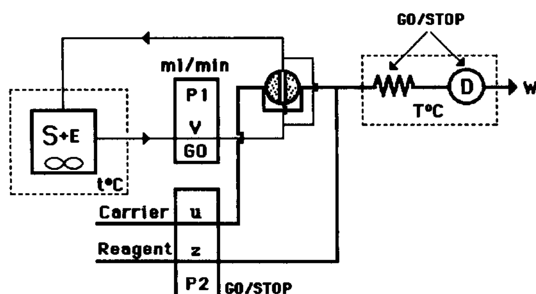
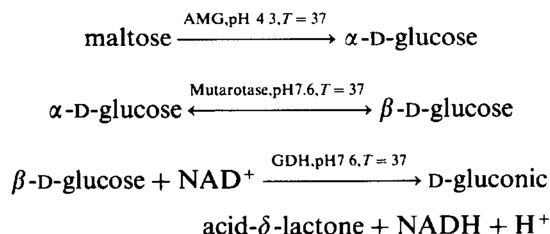


Fig. 1. Integrated FIA system as used for enzyme activity determinations, comprising two entirely separated subsystems communicating via a common valve (V). The subsystem at left is the enzyme reactor where substrate (S, in large excess) and enzyme (E) react, while the chemical derivatization subsystem is depicted to the right. Sample manipulation in the latter subsystem might comprise stopped-flow either outside or within the detector. For further details, see text.

injection valve is switched to the inject position and an aliquot of the sample solution is introduced by pump 2 into the FIA manifold where it is mixed in confluence with suitable reagent(s) and carried towards the detector via a reaction coil. This approach secures that each segment of the sample gradient is thoroughly mixed with reagent. To ensure that the content of the reactor vessel is left intact at all times, it is imperative that the volume of the reactor vessel is much larger than the injected volume so that even eight to 10 withdrawals do not cause alterations to any appreciable extent. For this reason, the volume of the rector vessel was fixed at 25 ml, that is, 500 times larger than the injected volume.[1] Because the indicator reaction manifold is entirely separated from the degradation entity, it is possible to manipulate the sample in this part independently of the degradation part. The sample might either be stopped outside the detector in order to gain increased reaction time without increasing the dispersion (as employed for the cellulase activity determination[1]), or it can be stopped within the detector itself where the reaction can be monitored by the stopped-flow approach (as dealt with in this communication). The most essential feature of the described system is, that by allowing several samples to be withdrawn from the enzyme degradation chamber at prefixed intervals, it yields a multi-point determination of the enzyme activity.

It is the aim to determine in this communication the activity of amyloglucosidase (AMG). AMG is widely used in the food industry, in areas ranging from starch saccharification for alcohol production to manufacture of high-dextrose syrup and fabrication of sorbitol (a sweetening agent for diabetes patients). There is a need for an assay during the fermentation process by which AMG is produced as well as in the finished product. Presently, AMG is assayed by a single-point method using an AutoAnalyzer.[5] The assay developed here adapts the reactions from the AutoAnalyzer procedure to FIA/SIA, yielding a multi-point determination. The assay is based on the following reactions:



As shown, AMG degrades maltose to α-D-glucose units at pH 4.3. None of the constituents of this reaction can be monitored directly, and, therefore, it is necessary to apply an indicator reaction. The indicator reaction is based on determination of glucose in a second enzymatic reaction using glucose dehydrogenase (GDH) at pH 7.6. This reaction consumes NAD^+ and generates NADH, the latter of which can be measured optically at 340 nm. However, as GDH only degrades β-D-glucose, the α-D-glucose must first be converted to β-D-glucose. This is accomplished by the simultaneous presence of mutarotase. The indicator reaction generating NADH is fairly slow and has previously been shown to be superbly fitted to be monitored using stopped-flow.[6]

The AMG assay was performed both in a sequential injection analysis (SIA) system (Fig. 2) and in the generic FIA system described above. Because of hardware simplicity and ruggedness, SIA has previously been shown to be well suited for process monitoring.[7-10] It is thus potentially useful for the given purpose. SIA, which in essence is a subclass of FIA, is based on time-based, sequential aspiration of well-defined sample and reagent zones into a holding coil by means of a directional (selector) valve. The flow is then reversed and the zones are forwarded via a reactor coil

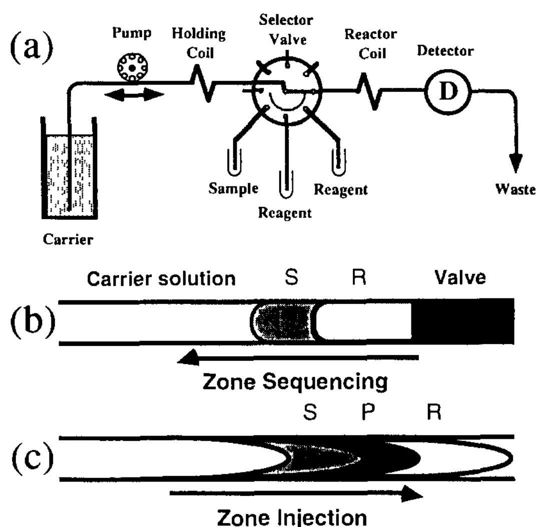


Fig. 2. (a) SIA system consisting of a selector valve, a peristaltic pump and a detector, interconnected with tubing. In the example shown, a sample and two reagents are clustered around the multi-position valve. (b) Zone sequencing when a sample zone (S) and a reagent zone (R) are aspirated into the holding coil. (c) The flow is reversed and the zones penetrate into each other, creating a product zone (P).

to the detector. During these steps the sample and reagent zones will penetrate into each other, and in the region of mixing a product will be formed.

This communication will, in addition to describing the assay of amyloglucosidase by SIA and FIA, focus on the applicability of SIA for enzyme activity measurements, and compare and discuss the advantages and disadvantages of the two approaches for these types of assays. Special emphasis will be placed on the mixing patterns of the two approaches, that is, while the individual segments of the sample gradient in FIA are mixed completely with the reagent this is not the case in SIA, and for that reason the latter approach entails special precautionary measures.

EXPERIMENTAL

Reagents

All reagents were of analytical grade quality, and distilled, degassed water was used throughout. The medium for the enzyme degradation procedure was, for both systems, a diluted maltose solution in an acetate buffer of pH 4.3. In the FIA system, a 27.8 mM maltose solution was used, prepared by dissolving 9.52 g of maltose (Kock and Light, <0.1% glucose) in 100 ml 0.05*M* acetate buffer (pH 4.3) and diluting to 1 l. with water. In the SIA system, the substrate solution was made to contain 50 g/l. in 0.02*M* acetate (*cf.* Results and Discussion). In the initial experiments for optimizing the FIA derivatization chemistry, glucose standards (in 0.005 acetate buffer of pH 4.3) in the concentration range 0.5–10 m*M* were used. They were prepared from a stock solution of 100 m*M*, made by dissolving 1.9817 g of D(+)-glucose monohydrate (Sigma) in 100 ml water. The glucose dehydrogenase reagent used for the indicator reaction was a commercial kit (Gluc-DH Enzyme Mixture, Merck) consisting of a vial containing glucose dehydrogenase, mutarotase and NAD^+ to be dissolved in an accompanying 0.12*M* phosphate buffer of pH 7.6 with a content of 0.15*M* NaCl. For the FIA assay, a stock solution was prepared by taking 3.8 g of the enzyme mixture and dissolving it in 100 ml of buffer. For actual use, this solution was diluted 10 times with phosphate buffer. For the SIA system, the solution used was made by dissolving 3.0 g of enzyme mixture in 50 ml of 0.24*M* phosphate buffer of pH 7.6 (*cf.* Results and Discussion).

The amyloglucosidase (AMG) enzyme (1,4-alpha-D-glucan glucohydrolase [EC3.2.1.3]), from a selected strain of *Aspergillus niger* produced by submerged fermentation; activity 196.4 AGU/g) was supplied by Novo-Nordisk A/S, Copenhagen, and used as received. A stock solution containing 10 AGU/ml was prepared by dissolving 5.0918 g of enzyme in 100 ml of water. Aliquots of this stock solution were frozen until used. For actual enzyme activity assays, an appropriate amount was dissolved in water and added to the reactor vessel of each of the two systems to yield final concentrations of 0.05, 0.1, 0.2, 0.3, and 0.4 AGU/ml. For use in the FIA system, in which a total volume of 25 ml was used, the enzyme solutions were prepared 25 times stronger and 1.0 ml added to the reactor vessel which initially contained 24.0 ml substrate-buffer medium. In the SIA system, the SIA instrument was also used to load the reactor vessel prior to each series of experiments, that is, substrate solution (200 μl) and enzyme solution (60 μl) were aspirated sequentially into the reactor vessel in which they were diluted three times with water (carrier solution, *cf.* the scheme depicted in Table 1).

Apparatus

The components of the FIA system (Fig. 1) were to a large extent identical to those described previously.[1] The reactor vessel, which consisted of a Perspex container of a volume of *ca.* 50 ml equipped with a water-thermostatted jacket, contained in each experiment a total volume of 25.0 ml of solution. The container was fitted with a magnet and placed on a stirring table. As mentioned above, 24.0 ml of 27.8 m*M* maltose substrate in 0.005*M* acetate buffer (pH 4.3) were initially added to the reactor. When the buffered substrate had attained the preset temperature (37°), the enzyme was added. Samples were thereafter withdrawn at preselected times. The solution of the well stirred enzymatic degradation vessel and the FIA manifold were served by two peristaltic pumps (Mini-S-840, Ismatec, Switzerland; for optimal operation parameters, see Results and Discussion and Fig. 6). All connecting lines and the 100 cm reaction coil, which was made as a knotted reactor and emerged into a second water-thermostatted bath (37°), consisted of 0.5 mm i.d. microline tubing. The detector was an HP8452 Diode-Array spectrophotometer (PDA) equipped with a Hellma 18 μl flow-through cell with a path length of 10 mm thermostatted at 37° (all enzymatic reactions were executed at this temperature because Novo-Nordisk A/S prefer to perform their assays at 37°). The operation of the pumps and the injection valve were facilitated via a computer by means of a home-made program. Data retrieval were made by means of the program facilities of the PDA-instrument and subsequently handled separately by the program

Table 1. SIA procedure adopted for AMG measurement where the sample line is purged prior to each analysis and the sample zone of enzyme is sandwiched between two long zones of reagent. The flow is stopped and monitored at the top of the sample peak

Event no.	Pump	Valve	Duration (*sec*)	Description of event
Mixing chamber is filled				
1	Rev	6	10.0	Aspirate substrate
2	Fwd	3	30.0	Pump to mixing chamber
3	Rev	7	3.0	Aspirate AMG enzyme
4	Fwd	3	12.0	Pump to mixing chamber
5	Off	3	5.0	Let equilibrate
Five samples are withdrawn from mixing chamber and treated in indicator system				
6, 15, 24, 33, 42	Rev	3	3.0	Purge sample line
7, 16, 25, 34, 43	Fwd	5	6.0	Flush to waste
8, 17, 26, 35, 44	Rev	2	5.0	Aspirate GDH reagent
9, 18, 27, 36, 45	Rev	3	2.0	Aspirate sample
10, 19, 28, 37, 46	Rev	4	5.0	Aspirate GDH reagent
11, 20, 29, 38, 47	Off	Home	0.6	Wait for valve to turn*
12, 21, 30, 39, 48	Fwd	Home	16.5	Forward to detector
13, 22, 31, 40, 49	Off	Home	15.0	Stop of flow
14, 23, 32, 41	Fwd	Home	15.0	Flush to waste
50	Fwd	Home	45.0	Flush thoroughly

Abbreviations: Rev, reversed; Fwd, forward; Home, directional valve connected to port 1 (reaction coil).

*This operation was necessary because of the rather slow computer used in this investigation. Under normal circumstances it will be superfluous.

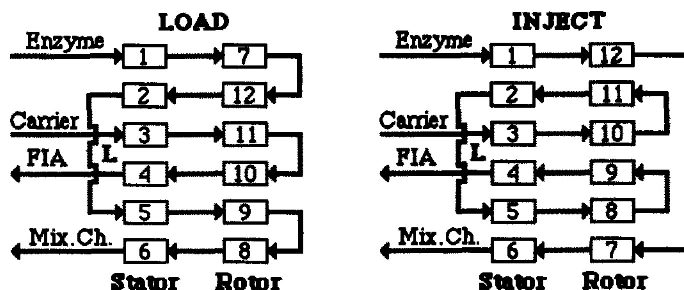


Fig. 3. Configuration of the six-port rotary injection/directional valve used in the FIA system. Numbers 1–6 refer to the ports of the stator, while numbers 7–12 are the ports located at the rotor.

QuattroPro. The communicating injection valve was a 6-port rotary valve with individual access to all ports. It is important that the circulation of the solution of the enzyme degradation subsystem is maintained uninterrupted at all times, that is, irrespective of whether the injection valve is in the inject or load positions. This was effected by the valve configuration shown in Fig. 3.

The SIA system used was a commercial Alitea MIS-1 System (Alitea, U.S.A.). It comprised an Alitea-XV peristaltic pump, a 10-port electrically actuated multi-position directional valve and suitable hardware for computer control. As shown in Fig. 4, the necessary reagents were clustered around the multi-position valve along with the mixing chamber for the enzymatic degradation reaction. The mixing chamber was a small, narrow glass vessel (5 ml) equipped with a stirring bar, placed in a thermostatted water bath (37°) which in turn was placed on a magnetic stirring table. The mixing chamber

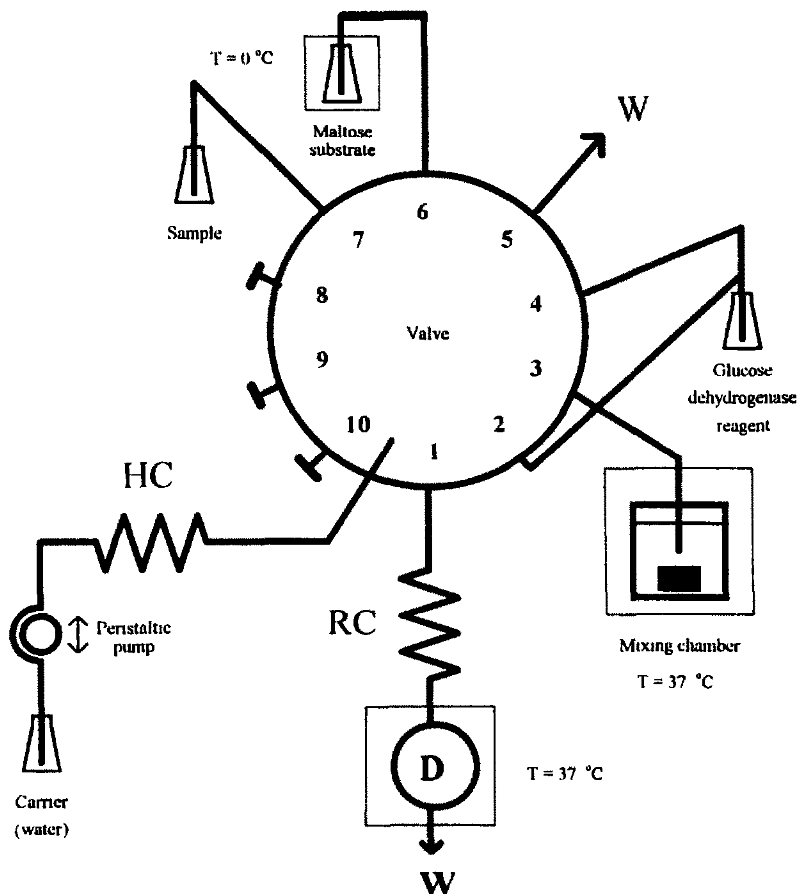


Fig. 4. Configuration around the SIA valve for amyloglucosidase activity assay. Ports 8, 9 and 10 are closed (inoperative). D, Detector; W, waste. Thin lines indicate temperature controlled areas.

was placed as close as possible to the valve in order to minimize the length of the non-thermostatted sample line. The holding coil (HC, 1.2 m) and reactor coil (RC, 45 cm) consisted of 0.76 mm i.d. PTFE tubing. Both coils were designed as Serpentine II reactors.[11] The reagent and sample lines were made of the shortest possible pieces of 0.5 mm i.d. PTFE tubing. The volume of the tube from the valve to the mixing chamber was particularly crucial, because this line has to be purged between sampling. For this reason, it was made to consist of a short (3 cm) piece of 0.5 mm i.d. PTFE tubing to which was attached 15 cm of a 0.38 mm i.d. PTFE tubing (total volume 23 μl). The applied pump tube of the peristaltic pump (used both for aspirating sample and reagent solutions, and later for propelling them forward through the analytical system) was Aliprene (Alitea, USA) with an i.d. of 0.5 mm and a wall thickness of 1.6 mm. The pump was set at 499% rpm which at 220 V gave 50 rotations per min. The resulting flow rate was approximately 1.2 ml/min, or 20 μl/sec. The MIS-1 system was controlled by a 8086 computer, which was fitted with an ADA 1100 data acquisition board. For control of pump and valve the FIALab program version 2.0 (Public Property, University of Washington, U.S.A.) was used. The detector was in this instance also an HP8452 Diode-Array spectrophotometer equipped with a thermostatted Hellma 18 μl flow-through cell with a path length of 10 mm. A 8086 computer furnished with an HPIB board was used for data acquisition and handling.

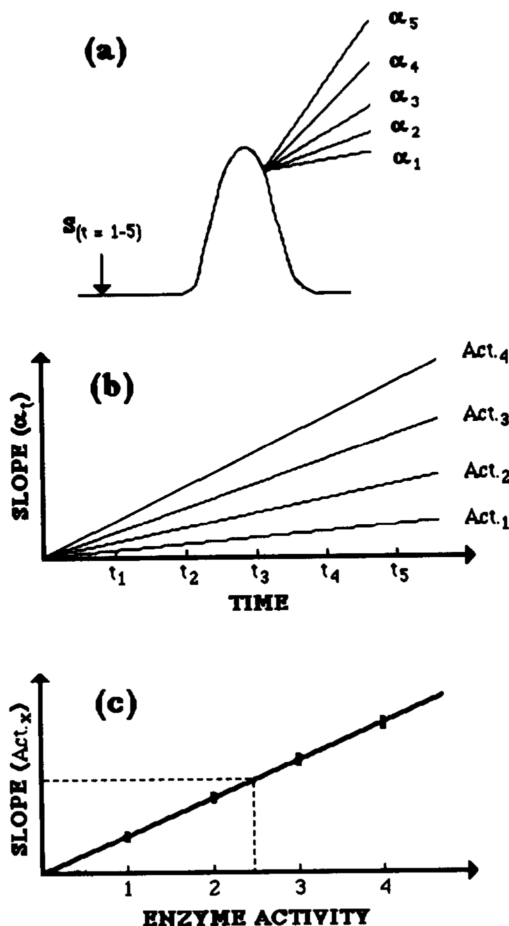


Fig. 5. Principal approach for multi-point determination of AMG activities. (a) Readouts for an enzyme activity standard where five sample aliquots are withdrawn during the monitoring period. Each sample is measured by stopped-flow over a fixed period of time, yielding progressively higher readouts, that is, slope (α_1–α_5). (b) Plot of the slopes (α_t) of the individual enzyme activity standards against times of withdrawal, yielding straight lines the slopes of which are a measure of the individual enzyme activities (Act.$_x$). (c) Final calibration curve constructed on the basis of the slopes determined in (b). The dotted lines refer to the assay of an unknown sample.

RESULTS AND DISCUSSION

Principles of assay

For both systems used, the approach for establishing a calibration curve, as based on multi-point measurement of each enzyme activity, is identical and visualized in Fig. 5a–c. In each experiment of a specific enzyme activity (Fig. 5a), sample aliquots are intermittently (here signified at times t_1 to t_5) withdrawn from the reactor vessel/mixing chamber and injected into the derivatization manifold. The analytical readout is based on monitoring the chemical reaction by the stopped-flow approach by arresting a fixed segment (corresponding to a fixed delay time as measured from the time of injection) of the sample/reagent mixture within the detector, that is, measuring the change of concentration of NADH generated over a fixed period of time (*i.e.* the slope). As the enzymatic degradation reaction in the mixing chamber is progressing, increasingly higher concentrations of glucose are produced, which in turn result in increasing analytical readouts, that is, in increasing slopes (α_1 to α_5; for convenience, the readouts for t_1 to t_5 are superimposed on each other in Fig. 5a). For each enzyme activity the individual slopes are then plotted against time (Fig. 5b), resulting in straight lines of different slopes depending on the enzyme activity monitored. Based on the slopes of these lines the calibration curve can finally be constructed (Fig. 5c). The enzyme activity of a given (un-

known) sample of AMG, as monitored by the scheme in Fig. 5a, can then via a slope/time curve (Fig. 5b) readily be determined from the calibration plot in Fig. 5c (indicated by the dotted lines).

The described calibration procedure is rather time consuming (the analysis time is 5–10 min per sample, which for five samples totally requires approximately 1 h) and, therefore, it would be preferable if calibrations could be limited to less than daily. For that reason it was an integral part of this investigation to ascertain how often it is necessary to perform individual calibrations.

AMG assay with the FIA system

As mentioned earlier, it is essential that the sample solution circulating in the external loop of the enzyme degradation subsystem at all times is representative of the composition of the solution in the reactor vessel. Therefore, the loop should be made as short as physically possible and the circulation rate should be maintained at a fairly high level. In the previously reported assay of cellulase[1] a circulation rate of 1.75 ml/min was found sufficient and, therefore, it was also used in this case. The injected volume was fixed at 50 μl. Since the volume of the reactor vessel was 25 ml, that is, 500 times larger than the injected volume, even eight to 10 withdrawals did not alter the contents of the reactor vessel to any appreciable extent. There is also another reason that the ratio between the two volumes should be high. As seen in Fig. 6, the return of the valve from the inject-position to the load-position will for each cycle imply that a volume of carrier, equal to the volume of sample solution removed, is added to the reactor vessel. As a result, the volume of the reactor vessel will remain constant, yet the solution in it continuously becomes slightly diluted with carrier. However,

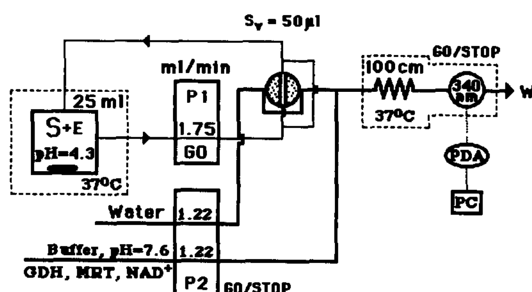


Fig. 6. Operational parameters for the ultimately adopted generic FIA system.

since the volume of the reactor vessel is much larger than the metered injection volume, this dilution can either be neglected or compensated for. Principally, it might seem reasonable to make the carrier identical to that of the buffer employed in the reactor vessel (acetate buffer, pH 4.3). However, since the subsequent chemical indicator reaction proceeds at a higher pH value, any appreciable buffer capacity in the carrier would be unwarranted. For that reason water was selected as carrier, and the buffer capacity in the reactor vessel was fixed at such a level (0.005*M* buffer concentration) that on one hand the pH would be maintained constant during the enzymatic degradation procedure, and on the other the buffer content of the injected sample volume would not affect the maintenance of the pH value of the derivatization reaction (pH 7.6). The temperature for the enzymatic degradation procedure was set to 37° (which, as stated above, is the temperature preferred by Novo-Nordisk A/S for all their enzymatic assays), that is, all substrate/buffer solutions added to the reactor vessel were thermostatted to this temperature prior to enzyme addition. The level of maltose in the mixing chamber (27 m*M*, corresponding to 10 g/l.) should be sufficient, considering that the Michaelis–Menten constant, K_m, for AMG is of the order of 2×10^{-3}M.[12]

The chemical derivatization system, in which the aspirated sample is injected into a carrier of water and subsequently mixed with the reagent stream, was initially optimized by means of injections of glucose standards in the concentration range 0.5–10 m*M* in order to simulate the glucose concentration levels expected in the actual assays. The finally adopted values for the pumping rates are those stated in Fig. 6. The mixing coil (100 cm, made as knotted reactor) served to ensure efficient radial mixing of sample and reagent in each segment of the established gradient prior to entering the flow cell where the sample was stopped and monitored. At the top of the FIA gradient the concentration of sample (glucose) is maximum while the reagent concentration (GDH, mutarotase and NAD^+), due to the confluence configuration employed, is constant all along the gradient. The sensitivity of measurement will, therefore, be highest in the gradient-segments close to the peak maximum, but at the corresponding high concentration of glucose it might be difficult to maintain pseudo-first-order reaction conditions with respect to glucose and

pseudo-zero-order reaction conditions with respect to reagent concentrations, especially when higher AMG activities are monitored. Furthermore, the length of the stop-period will also influence whether these conditions can be fulfilled during the time required for each sample. Based on the initial glucose experiments, and supplemented by the actual AMG assays, it was found that optimal performance was achieved by stopping the sample for 10 sec at a position close to and past the peak maximum, which with the pumping rates and manifold configuration used, corresponded to a delay time from the time of injection of 17.0 sec. For the AMG-activities measured, which were in the range of 0.05 to 0.4 AGU/ml, it was with these operational parameters that it was possible to maintain strictly first-order reaction conditions for glucose in the derivatization system for a period of at least 10 min (corresponding to 10 sample aspirations), whilst also maintaining pseudo-zero-order reaction conditions during the stopped-flow sequence for up to *ca.* 20 sec.

The performance of the system is illustrated in Fig. 7, which at the top shows signal readouts for five different enzyme activities (0.05, 0.1, 0.2, 0.3, and 0.4 AGU/ml) at times 0–10 min, and at the bottom the ensuing calibration graph. As shown, the outputs for the individual enzyme activities generally showed depressed values for the first two aspirations, followed by a linear increase in the signal/time outputs. For the highest activity there is at times 9 and 10 min a tendency towards depletion of reagent. Therefore, and because the number of readouts up to time 8 is adequate, the evaluation of the slope of the individual response/time curves, and as used for establishing the final calibration curve, are in all instances based on the outputs recorded from times 2–8 min (*i.e.* 7 points on the regression line).

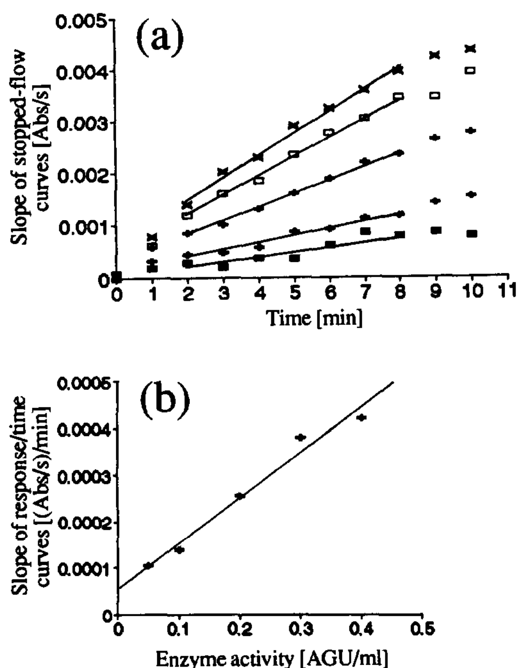


Fig. 7. (a) Response/time curves for 0.05, 0.1, 0.2, 0.3 and 0.4 AGU/ml AMG standards as obtained by the FIA system. Each curve is the result of a single run. (b) Calibration curve for the FIA AMG method. The regression coefficient (R) is 0.988

Generally, the response/time curves, as correlated to straight lines (in the time frame 2–8 min) had, except for the lowest AMG activity, correlation coefficients (R) better than 0.98, and the relative standard deviations (RSD) were in most cases less than 3–5% and in all cases better than 7%. For the 0.05 AGU/ml activity the correlation coefficient varied between 0.98 and 0.85 over different days, and the RSD was between 10 and 25%. The regression coefficient for the calibration curve (Fig. 7b), as based on all the activities measured, was 0.988. The repeatability of the method was tested by analyzing one of the standards (0.2 AGU/ml) three times in a row on two different days by the same operator. The RSD-values for these two series were 1.5 and 1.9%, respectively, which is very satisfactory. The reproducibility of the calibration curve between days was tested by repeating the calibration at days 1, 8 and 15. The results are listed in Table 2, from which it appears that the slope does change with time, which over the recorded period corresponded to an RSD value of *ca.* 5%. While a change of this order might be acceptable and adequate for practical assays, it is, however, significant that the individual values of the calibration points vary non-systematically as a function of time (as reflected in the R-values of the individual calibration curves). Therefore, the calibration curve can be used for extended periods of time provided the found tolerances are acceptable. Furthermore, it should be noted from Table 2 that the standard deviation of the slope is well below the value of the slope. Normally it holds, that if the standard deviation of the slope is less than half the value of the slope, the estimates produced are relatively precise.[14]

In this context it has been pointed out (C. Ridder, personal communication) that because each point in the calibration curve is based on successive linear regressions it will most likely

Table 2. Reproducibility of the calibration curve of the FIA system as obtained on 3 different days spaced over a period of 15 days*. The calibrations are in all cases based on single runs of samples containing 0.05, 0.1, 0.2, 0.3 and 0.4 AGU/*ml*, respectively

Days of calibration	1	8	15
Slope of cal. curve [((Abs/s)/*min*)/(AGU/*ml*)]	9.00 E-04	9.74 E-04	9.90 E-04
SD of slope	6.83 E-05	8.66 E-05	1.56 E-04
y-Intercept [(Abs/s)/*min*]	4.83 E-05	5.60 E-05	5.27 E-05
SD of intercept	1.68 E-05	2.13 E-05	3.84 E-05
SD of estimate (s_{yx})	1.96 E-05	2.48 E-05	4.47 E-05
Regression coefficient (R)	0.991	0.988	0.965
Mean value of slopes		9.54 E-04	
SD		4.8 E-05	
RSD		5.0%	

*Note added in revision: at day 77, after the system had been closed down for several weeks, measurements for the enzyme activities of 0.2 and 0.3 AGU/ml were made. The values of the slope of the two time/response curves were 2.90 E-04 and 2.82 E-04, respectively. Thus, both of these values fitted well with the calibration curves recorded previously, which supports that the calibration curve appears to be reasonably stable over extended period of time.

lead to an accumulation of errors due to the general estimation problem inherent in the least squares methods (outliers in data curves will, for instance, have great impact on the estimates of the slopes). The comparison of the calibration curves from different days clearly shows that the errors are non-systematic. Therefore, the errors in the method do not only originate from laboratory manipulations, but rather from the calculation of the slopes. Thus, an improved algorithm will probably result in increased precision and day-to-day reproducibility. The utilization of multivariate calibration, which can relate the measured FIA-signals directly to enzyme activity, thus appear preferable, and investigations in this direction are currently being undertaken at this laboratory.

AMG assay with the SIA system

With the SIA system all solution handling, including filling the mixing chamber, is automated. Each analysis sequence is initiated by aspirating appropriate amounts of substrate/buffer and enzyme solutions into the mixing chamber which is equilibrated to the desired temperature by the thermostat (37°). Samples are then at prefixed, regular intervals aspirated into the holding coil along with a zone of reagent and forwarded into the detector via the reaction coil. As in the FIA system, the analytical readout is based on stopped-flow measurements. Contrary to the FIA system [where the withdrawal of a sample from the mixing chamber implies that it is replaced by an equal volume of carrier stream (water), and hence the volume of the mixing chamber must be much larger than the aspirated sample volume], sampling from the mixing chamber in the SIA system does not dilute the contents of the mixing chamber. Therefore, the volume of the mixing chamber can be made much smaller; in fact, a volume of a few ml will suffice, since that is enough to allow six to eight samples to be withdrawn in each analysis sequence. For this reason, the liquids aspirated into the mixing chamber will fairly rapidly obtain the desired temperature. The sample volume used here was 40 μl (corresponding to 2 sec injection time) as this was the smallest volume that could be injected with sufficient precision.[10]

When making the initial experiments with the SIA system, several problems were encountered. First, with the chosen sample injection time it was not possible to use the buffer concentrations applied in the FIA system, because the overlap of the acetate-buffered sample zone and the phosphate-buffered GDH/mutarotase zone was not sufficient to ensure the optimal pH value (pH 7.6) for the indicator reaction. Secondly, there were difficulties due to formation of Schlieren patterns because of differences in ionic strength of the two solutions. Both of these problems are associated with the ways that SIA operates: while solutions in the FIA system are mixed via confluence, and, therefore, it is fairly easy to obtain efficient radial mixing in each segment of the sample/reagent zone (*e.g.* by using a knotted reactor as in the FIA system above), the zones of different solution in SIA are stacked next to each other and only gradually mixed on their way to the detector.

It is essential to ensure that the pH for both the enzymatic degradation reaction and the indicator reaction are rigorously controlled and

maintained. Therefore, the concentration of acetate buffer for the degradation reaction cannot be lowered indiscriminately. By performing batch experiments and monitoring the pH during the enzymatic degradation, it was found (for the enzyme activities to be measured, *i.e.* 0.05–4.0 AGU/ml) that the concentration of acetate buffer could not be allowed to be reduced to much less than 0.01*M* (to keep a constant pH value, it should at least be higher than 0.007*M*). With this concentration of acetate fixed it was necessary to increase the buffering capacity of the phosphate buffer used in the indicator reaction; the concentration was thus increased from 0.12*M* (the one used in the kit from Merck, and applied in the FIA system) to 0.24*M*. The problems associated with Schlieren pattern was solved by stopping the zone in the detector, whereby the Schlieren pattern would gradually die out. This implied, however, that the first 7–8 sec of the 15 sec of stopped-flow could not be used. However, during the last 8 sec linear response-time curve (indicating a constant development of NADH) was obtained.

An important aspect to take into consideration when using the SIA approach is the sample line connecting the mixing chamber and the directional valve. While the sample loop in conventional FIA is automatically purged with the new sample while the previous one is being analyzed (or as in the FIA system used here, the sample is replaced by carrier, *i.e.* water), the sample line in SIA still contains the sample from the previous analysis when a new analysis cycle is to be started. There are two ways to overcome this problem. One is to start each analysis cycle by purging the sample line with the new sample and pumping the old sample to waste. Preferably, the sample line should be swept with three times its own volume to avoid carry-over.[13] Subsequently, the holding coil should be swept with three times this volume of carrier. This method is relatively time-consuming. Another way to overcome the problem is to inject a very large sample zone, of which only the last part is used for analysis. The latter method has been used successfully when performing SIA with a piston pump.[9] This method is only feasible if reagent(s) are injected after the sample (closest to the valve). In practice this means that this method is only applicable for single reagent chemistries.

Both approaches were tested in this investigation, keeping in mind that at the point of stopping the sample/reagent zone in the detector a surplus of reagent must be present to ensure pseudo-zero-order reaction conditions with respect to GDH and first-order reaction conditions with respect to the generated glucose, besides which the pH must be maintained at 7.6. In the less time consuming method (no purging prior to analysis), the flow had to be stopped at a very steep sample concentration gradient in order to ensure such conditions. This gave an unacceptable repeatability presumably because the peristaltic pump was not able to stop at exactly the same point of this gradient every time. In contrast, when the sample line was purged prior to analysis, the sample could be injected between two reagent zones and the proper reagent/sample ratio could be ensured while stopping the flow at the sample peak. This method gave by far the best repeatability and thus, even though it was more time consuming, was applied in the following. Furthermore, by testing the relative size of reagent and sample zones it was found that with a sample injection time of 2 sec (corresponding to sample volume of 40 μl) a significant improvement of the reagent to sample ratio could be obtained by increasing the reagent injection time by up to 5 sec (corresponding to 100 μl). The finally adopted procedure (described in Table 1) yielded a reagent/sample ratio of 2.2.

Using this procedure, five different AMG enzyme activities were then monitored. The response/time curves (top) and the resulting calibration curve (bottom) are shown in Fig. 8.

As for the FIA method, the repeatability of the method was tested. This was effected by analyzing each of the five enzyme standards, 0.05, 0.1, 0.2, 0.3 and 0.4 AGU/ml, five times in a row on the same day by the same operator. The results are presented in Table 3. All response/time curves correlated to straight lines that had correlation coefficients (R) in most cases larger than 0.99, and in all cases larger than 0.89. The relative standard deviations (RSD) found were between 5 and 11% and, as expected, highest for the lowest enzyme activities. The reproducibility of the calibration line between days, tested four times over a period of 24 days, is listed in Table 4. As seen, the RSD is in the order of 9%, with the slopes of the individual calibrations curves varying nonsystematically with time. Yet, the R-values of the individual calibration curves are in all cases better than 0.99, which implies that the SIA approach operates very satisfactorily over comparatively shorter periods of time. By compar-

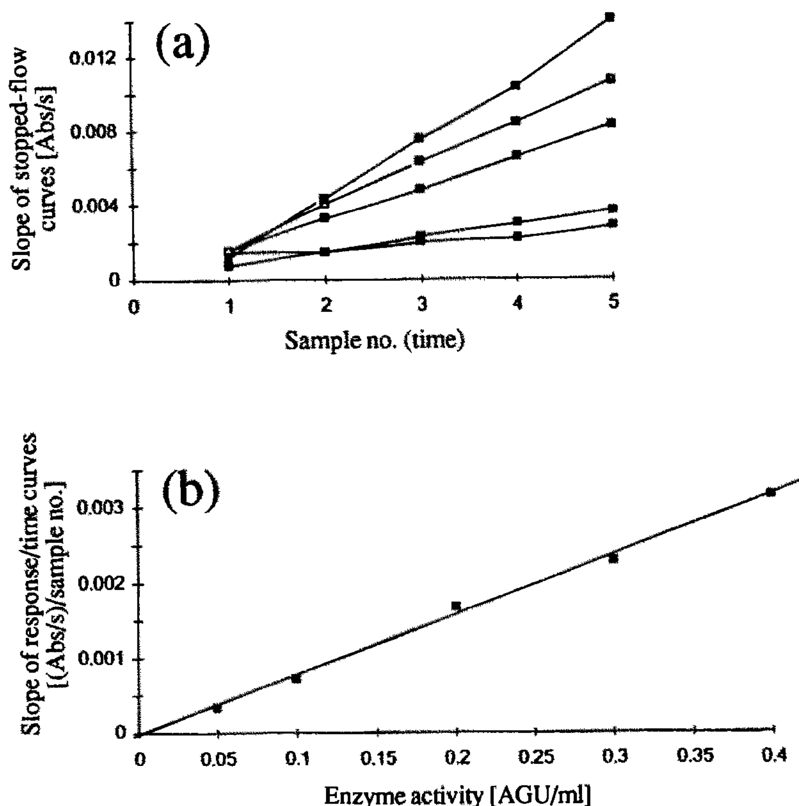


Fig. 8. (a) Response/time curves for 0.05, 0.1, 0.2, 0.3 and 0.4 AGU/ml AMG standards as obtained by the SIA system. Each curve is the average of five analyses. (b) Calibration curve for the SIA AMG method. The curve is linear throughout the dynamic range ($R = 0.988$).

ing the performances of the FIA and SIA systems, it is observed that both the repeatability and the reproducibility parameters were better with the FIA system than with the SIA system (especially considering that the values reported for the FIA system all are based on single runs while those of the SIA system in several instances are the average of multiple runs). Although the response/time curves for FIA relied on seven readouts while those for SIA only comprised five points, a likely reason is that all metering of sample and reagent volumes in the SIA system is time-based, that is, any irregularities in the pumping speed of the peristaltic pump will be reflected in the analytical result, especially over extended periods of time. However, with intermittent calibration, and the saving in time that this implies, the accuracy of both methods should in most instances suffice for practical measurements. If better accuracy is required, it will be necessary to recalibrate more often.

Table 3. Within-day repeatability of the SIA system. Each standard was analyzed five times in a row on the same day by the same operator

AMG concentration [*AGU/ml*]	%RSD of slope of response/time curves
0.05	11.0
0.1	12.2
0.2	7.2
0.3	8.2
0.4	4.9

The SIA approach was finally tested on a matrix simulating the fermentation samples used at Novo-Nordisk. Unfortunately, it was not possible to obtain actual samples from the company because these contained special strains of bacteria that the company did not want to release. Neither the specific conditions of the fermentation process under which AMG is produced, nor typical concentration of the enzyme in fermentation samples could be revealed. Consequently, it was decided to imitate real AMG fermentation samples by dissolving known amounts of AMG standards in a fermentation broth from another fermentation. The broth (whole broth), which was obtained from The Institute of Biotechnology at this university, was from a fermentation completed the previous day. It had produced the glucose polymer dextran by means of the lactic acid bacterium

Table 4. Reproducibility of the calibration curve of the SIA system as obtained on 4 different days spaced over a period of 24 days. The calibrations are in all cases based on single runs of samples containing 0.05, 0.1, 0.2, 0.3 and 0.4 AGU/*ml*, respectively

Days of calibration	1	5	16	24
Slope of cal. curve [((Abs/*s*)sample no.)/(AGU/*ml*)]	8.00 E-03	9.29 E-03	9.34 E-03	8.05 E-03
SD of slope	3.02 E-04	4.68 E-04	4.08 E-04	2.21 E-04
y-Intercept [(Abs/*s*)/sample no.]	−4.80 E-05	4.25 E-05	2.37 E-05	8.71 E-06
SD of intercept	7.42 E-05	1.15 E-04	1.00 E-04	5.43 E-05
SD of estimates (s_{yx})	8.64 E-05	1.34 E-04	1.17 E-04	6.32 E-05
Regression coefficient (R)	0.998	0.988	0.997	0.999
Mean value of slopes	8.67 E-03			
SD	7.45 E-04			
RSD	8.6%			

Leuconostoc mesenteorides 'Sikal', using the enzyme dextransucrase. Presumably, most of the dextran and microorganisms had been removed by centrifugation. Sucrose (4%) had been used as the carbon source and soy milk as the nitrogen source. The fermentation broth was cloudy and a white precipitate appeared when it was left unstirred. The main content of the broth were white soy proteins plus sugar residues, lactic acid and trace amounts of ethanol. Sucrose is formed by the condensation of α-D-glucose and β-D-fructose via an 1,2-alpha-linkage. As AMG only cleaves 1,4- and 1,6-alpha-linkages, the fructose is not a potential substrate for AMG. However, if some sucrose were to have been broken down to glucose and fructose, the glucose formed would not pose any serious problem in the ensuing stopped-flow measurement, but merely result in a somewhat higher background signal. This is a benefit of using a multi-point assay.

In order to give a realistic picture of the performance of the assay on fermentation samples, the fermentation broth was diluted 12 and 120 times, respectively. The two samples were then prepared to contain a final concentration of 0.2 AGU/ml, that is, a level in the middle of the standard curve established above. Three standard additions were performed on each sample giving final AMG concentrations of approximately 0.25, 0.30 and 0.35 AGU/ml. The concentration of AMG in the original sample were calculated by applying linear regression to the four points thus obtained with the calibration curve depicted in Fig. 8b. The results obtained are shown in Table 5, which reveal that the method indeed is capable of handling 'more complicated' media. Furthermore, the readouts, especially for the 12-times diluted sample, did, in fact, reveal that fairly high background levels of glucose were present, yet reliable values of the AMG-content could still be determined [the background level of glucose is automatically subtracted from the response/time curve (*cf.* Fig. 8a)]. Another benefit of a multi-point assay is that it is readily feasible to check whether pseudo-zero-order reaction conditions with respect to enzyme and coenzyme in the enzymatic derivatization process are fulfilled.

As seen in Figs 7 and 8, reliable multi-point assays might, in fact, be obtained with less than five to seven withdrawals of each enzyme activity. Thus, in many instances three to four withdrawals should, at the expense of slightly lower precision, suffice for both procedures tested, which in turn will reduce the time required to perform the individual assays and establish the calibration curves.

Comparison of FIA and SIA for multi-point enzyme activity assays

The SIA approach has four features which are advantageous in the present context. First, it is a fully automated system which also comprises the aspiration of sample (AMG) and substrate (maltose) into the mixing chamber. Second, because the sample/substrate solution in the mixing chamber does not become gradually diluted with carrier stream as in the FIA approach, the mixing chamber can be made much smaller and hence smaller volumes of

Table 5. Recovery of AMG activity in a fermentation medium as determined by the SIA system directly (one measurement) and after three standard additions. The fermentation liquid was diluted 120 and 12 times, respectively, with distilled water. Activities are expressed in AGU/*ml*

Dilution factor	120	12
True AMG activity	0.2	0.2
AMG activity found directly	0.2052	0.1896
Recovery	102.6%	94.8%
AMG activity found by standard addition	0.2027	0.2003
Recovery	101.4%	101.1%

sample and substrate can be applied and shorter times can be allowed for the contents of the mixing chamber to reach the desired temperature. Third, because the ensuing indicator reaction is based on injection of discrete, small zones of sample and reagent, it consumes less reagent per assay compared to the FIA system, where the reagent is pumped continuously. However, there is nothing to hinder the FIA system being modified either to operate discontinuously (*i.e.* only made to pump when a sample is in the derivatization manifold), or that the reagent be injected as a discrete zone to be mixed in confluence with the sample. Fourth, the SIA system is also simpler, because it only requires one pump and, therefore, appears well suited to practical applications.

However, SIA does also have some disadvantages for the given assay as compared with FIA. Of these the partial mixing of the sample and reagent is very likely the most serious one, as detailed above in connection with the optimization of the SIA system (formation of Schlieren patterns). The repeated purging of the sample line from the mixing chamber to the selector valve prior to aspiration of a fresh sample is somewhat cumbersome, and, although readily automated, it increases the overall analysis time. Also, the precision of SIA, furnished with a peristaltic pump, appears over extended periods of operation to be somewhat inferior to FIA because of its reliance on time-based injection of all sample/reagent zones. While SIA seems to function acceptably well in systems which only require a single reagent for the derivatization chemistry (as the one presented herein), it is possibly difficult or impossible to adapt it for chemistries which require more than two reagent additions, which on the other hand is readily adaptable to the FIA system. Although one could think of incorporating a small mixing device into the chemistry manifold of the SIA system, it would not only make it more complicated but also destroy or degenerate the generation of the gradient to be exploited in the analytical evaluation. Finally, it should be added that although the small volume of the well-stirred reactor vessel is an advantage for the assay described here, it would be a distinct disadvantage if the system were to be used for the determination of immobilized enzymes. Procedures for applying the FIA system for these types of assays are currently under investigation.

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