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Sequential flow injection analysis of complex systems using calorimetric detection

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Abstract

The application of the calorimetric detection seems to be an interesting possibility for the determination of single substrate components in mixtures or for the identification of substrate systems. The flow injection technique is used for a sequential initialisation of different enzyme catalysed reactions. The method is developed by use of a conventional flow-through calorimeter and demonstrated for a ternary system containing glucose, urea and penicillin G. More effective working conditions are realised by application of our IC calorimeters. First results from investigations with a flow-through IC calorimeter are communicated. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Enzyme catalysed reactions; Mixtures of substrates; Quantitative analysis; Sequential flow injection technique; Calorimetric detection; IC calorimeters

1. Introduction

The combination of the universally usable calorimetric detection with the high specificity of enzymatic catalysis promises miscellaneous applications. Thermodynamic and kinetic parameters, enzyme activities and analyte or inhibitor concentrations are successfully investigated by use of this combination. For a more global application and as a consequence of the price and availability of the substances the use of immobilised enzymes or/and of miniaturised calorimetric methods seems to be a necessary prerequisite for further investigations.

In former publications, we presented our developments of miniaturised calorimeters with integrated circuits for the heat power detection (IC calorimeters). The reliable functioning in the batch and also in the flow through mode was verified with different well known chemical reactions. Numerous investigations of enzyme catalysed reactions were carried out with very small sample volume (volume $v < 6\ \mu l$) or with enzymes immobilised on different carriers [1–7].

Special problems arise from the determination of single components of real, multi-component mixtures or from the aim to identify such a system. For successful experiments, we propose to realise the following procedure:

- The measurements should be carried out using an IC calorimeter with the mixing of the liquid flows from two inlet channels. Into one of the inlet channels continuously the buffered substrate solution flows, while into the substrate stream into the second channel enzyme solution pulses can be injected in a defined time interval (principle of the sequential flow injection analysis).

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- It is difficult to execute the sequential determination of different analytes or the identification of a mixture by use of a number of different enzyme pulses combined with a change of the applied buffer system. Therefore, a selection of a common usable buffer and a control of the different enzymatic reactions in this buffer is an important task (chemical system parameterisation).
- The cross sensitivities of the used enzymes at the chosen conditions must be proved. The cross sensitivity is of different importance for the determination of single components and system identification, respectively.

The aim of an earlier publication was to test the practicality and reliability of the proposed method of a sequential flow injection analysis for calorimetric investigations of enzymatic reactions by use of a conventional flow mixing calorimeter SETARAM Micro-DSC II. The connection between the chemical parameters on one hand and the calorimetric detection limits on the other hand were studied using single components and also binary systems. Furthermore, the application of an flow-through IC calorimeter, developed in our institute, was successfully tested first by the investigation of an enzyme catalysed reaction.

In the present publication, the results of investigations of enzymatic multi-component systems by use of the IC mixing calorimeter are given. The Micro-DSC II was applied once more for the selection and determination of the influence of uniform chemical conditions on the catalytic activity of the used different enzymes. The results of measurements using a ternary system are given in the following section.

2. Experimental

2.1. Micro-DSC II

Most of the measurements, which are discussed in the following, were carried out by means of a conventional calorimeter Micro-DSC II from SETARAM with a circulation mixing vessel (31/1530) [8]. This vessel has two separate inlet channels for simultaneous injection of two liquids and one outlet channel. Special obstacles in the flow channel ensure complete mixing of the inlet flows in the vessel. To inject the liquids we used two peristaltic pumps ‘‘Perimax 12’’ (Fa. Spetec). For baseline into both inlet tubes a permanent flow of substrate solution was injected. Then the substrate flow into one of the inlet was interrupted by a pulse of enzyme solution using a sample loop. The flow rate for each channel was 10.5 ml min^{-1}, the sample loop had a volume of 1 ml and all measurements were carried out at 25 °C.

The detected peak heights and peak areas correspond to the maximum heat power and the pulse heat amount. The slope of the signal parameter versus substrate concentration is interpreted as analytical sensitivity of the method for the particular substrate.

2.2. IC calorimeter

The basic part of the miniaturised flow-through calorimeter is a monolithic integrated silicon thermopile chip manufactured by Xensor Integration (Delft, The Netherlands) [5,6]. Fig. 1 depicts the scheme of the calorimeter and the flow chamber. The chamber is a plastic disk with a thickness of 2 mm, which is glue-bonded onto the surface of the silicon chip. The disk contains a cylindrical hole with a volume of approximately 20 μl. Capillaries of stainless steel serve as inlet and outlet for the liquid flow. To improve the thermostating of the input liquid flows, the connecting tubes are contacted to the chip carrier and the surrounding aluminium block. The measurements were carried out at room temperature. To inject the liquids we used a syringe pump ‘‘sp250i’’ (World Precision Instruments). Into one of the inlet tubes a permanent flow of substrate solution was injected. The second inlet was alternately switched between substrate and enzyme solution. The flow rates for both channels were equal and the detected baseline shift (change of the thermopile voltage) correlates to the heat power. The given analytical sensitivity for a substrate means the slope of the baseline shift versus substrate concentration.

2.3. Substances

The following enzymes we used: glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*; approximately 180 U mg^{-1} (Biozyme); catalase (EC 1.11.1.6) from *Aspergillus niger*; lyophil., approximately 2000 U mg^{-1} (Serva); invertase (EC 3.2.1.26) from yeast,

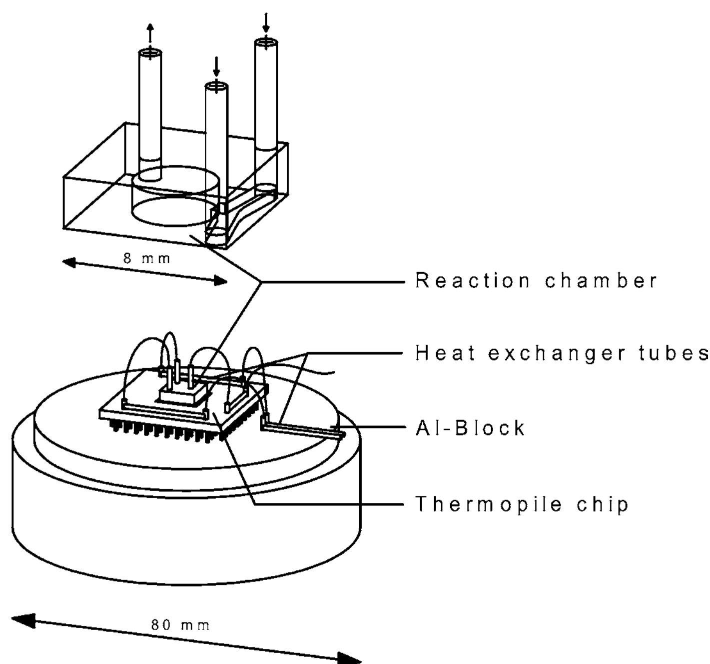


Fig. 1. Scheme of the IC calorimeter and the flow chamber.

approximately 380 U mg^{-1} (Boehringer Mannheim); hexokinase (EC 2.7.1.1) from yeast overproducer, lyophil., approximately >70 U mg^{-1} (Boehringer Mannheim); penicillinase (β-lactamase) (EC 3.5.2.6) from *Bacillus cereus*, lyophil., 44.6 U mg^{-1} (Serva); urease (EC 3.5.1.5) from Jack bean, lyophil., 324 U mg^{-1} (Serva).

The substrate substances D(+)-raffinose pentahydrate, D(+)-glucose and D(−)-fructose (Fluka), sucrose, penicillin G potassium salt and urea (Merck), ATP-Na_2-salt (Serva) were of grade "p.a." or "extra pure".

All other chemicals were of analytical grade or higher purity.

3. Results

3.1. Results of the measurements using the Micro-DSC II

For analysing a mixture of various substances using a sequential flow injection analysis technique, it is desirable to carry out all reactions under the same reaction conditions regarding buffer (buffer substances, concentration, pH value, cofactors), flow rate and temperature. In practice this can be difficult. Problems with required cofactors, inhibition effects, stability and activity of enzymes and cofactors can arise. Moreover, buffer, flow rate and temperature have an influence on the measurable heat power.

Therefore, it is required to find such conditions under which as many reactions as possible are practicable—not necessarily with the maximum of reaction rate for each reaction. To find a good compromise, first, we have to know or find out the optimal reaction conditions regarding the measurable heat power for each enzyme reaction, which we intend to use.

That means, in a first step the enzyme reaction with only a single analyte in the substrate solution has to be investigated for each substrate.

The influences of temperature and flow rate were discussed in former works [5,7].

3.1.1. Single analyte detection

In the scope of a former publication [5], we reported about these reaction systems.

1. Inversion of sucrose catalysed by invertase (EC 3.2.1.26).
2. Oxidation of β-D-glucose catalysed by glucose oxidase (EC 1.1.3.4), combined with decomposition

of the resulting hydrogen peroxide by catalase (EC 1.11.1.6).
3. Splitting of maltose and sucrose catalysed by α-glucosidase (EC 3.2.1.20).
4. Phosphorylation of D-glucose and D-fructose catalysed by hexokinase (EC 2.7.1.1) at presence of ATP^{4-}.

   Now we will add our latest findings about system (2) and the following three systems.
5. Decomposition of raffinose catalysed by invertase (EC 3.2.1.26).
6. Decomposition of penicillin G catalysed by penicillinase (EC 3.5.2.6).
7. Hydrolysis of urea catalysed by urease (EC 3.5.1.5).

3.1.1.1. System 2, glucose and GOD/catalase. This reaction was already described in detail [5]. Due to the comparatively high flexibility regarding the pH value and the buffer substances, for a multi-substrat analysis the conditions for this reaction can be adapted to the requirements of the other reactions to a large extent.

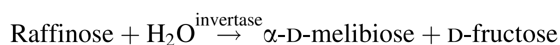
We completed the data of sensitivity at various buffers (see Table 1 and Fig. 2). The optimal buffer for this reaction is the phosphate buffer with pH 6.9. Using it, the highest sensitivity is determined. In acetate buffer with pH 4.6, a slightly lower sensitivity and also a lower linear concentration range were found. Applying Tris-buffer with pH 8.0 we observed the lowest sensitivity, but the widest linear range.

The discussed linear concentration ranges vary between 1.5 and 2.0 mmol l^{-1}. They were mainly limited by the available amount of oxygen in the solution.

3.1.1.2. System 5, raffinose and invertase. The enzyme invertase, we used for the detection of the disaccharide sucrose [5], catalyses also the decomposition of the trisaccharide raffinose. In conformity with the chosen reaction conditions for the sucrose inversion we used acetate buffer (0.1 M, pH 4.6) and an invertase concentration of 90 U ml^{-1}.

Although the molar reaction enthalpy is equal, we found a lower sensitivity, but a wider linear range for raffinose than for sucrose, because raffinose is converted with a lower reaction rate [9].

A summary of the reaction conditions (buffer, enzyme concentration), the observed linear concentration ranges and the sensitivities in these ranges are given in Table 1.



$$\text{Raffinose} + H_2O \xrightarrow{\text{invertase}} \alpha\text{-D-melibiose} + \text{D-fructose}$$

3.1.1.3. System 6, penicillin G and penicillinase. The enzyme penicillinase (β-lactamase) was applied for quantitative analysis of penicillin G.

Table 1
Summary of molar reaction enthalpies, used buffer systems, concentrations of enzyme, observed range of linearity and the peak height related sensitivities for the investigated reaction systems (buffers: (A) 0.1 M acetate, pH 4.6; (B) 0.1 M phosphate, pH 6.9; (C) 0.1 M Tris–HCl, pH 8.0; (D) 1 M Tris–HCl + 0.1 M $MgCl_2$, pH 8.0)

Substrate	Enzyme(s) and cosubstrates	Buffer	$\Delta_R H$ [5,9] (kJ mol^{-1})	Enzyme concentration[a] (U ml^{-1})	Linear range up to (mmol l^{-1})	Sensitivity[b] (μW (mmol $l^{-1})^{-1}$)	Uncertainty of sensitivity[c] (%)
Sucrose	Invertase	A	−15	90	≈15	−8.9	2
Raffinose	Invertase	A	−15	90	>30	−1.42	3
Glucose	GOD/catalase	A	−225	270/250	1.5	−196	2
		B	−225	270/250	1.8	−234	1
		C		270/250	2	−163	4
Maltose	α-Glucosidase	B	−4	100	40	−0.75	5
Sucrose	α-Glucosidase	B		100	≈40	−2.5	5
Fructose	Hexokinase + ATP	D	−63[d]	150	>20	−24.1	3
Glucose	Hexokinase + ATP	D	−74[d]	150	5	−71.8	3
Urea	Urease	B	−59	810	20	−159.0	0.4
Penicillin G	Penicillinase	B	−77	4.5	1.2	−142	4

[a] In some cases for unit definition by the manufacturers other substrates, buffers and/or temperatures were used.
[b] In the linear range.
[c] S.D. of the slope of the calibration graph (linear regression).
[d] Including protonation of buffer.

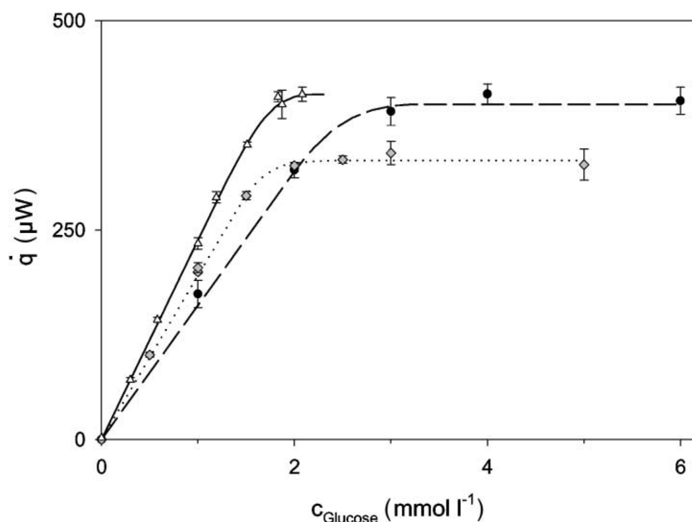


Fig. 2. Dependence of the maximum thermal power on the glucose concentration for GOD catalysed glucose oxidation coupled with the splitting of H_2O_2 catalysed by catalase in 0.1 M Tris-buffer pH 8.0 (●/dashed line), 0.1 M acetate buffer pH 4.6 (◇/dotted line) and 0.1 M phosphate buffer pH 6.9 (△/solid line) [Micro-DSC].

Due to the instability of penicillin G against acids and bases it is advisable, to work under nearly neutral pH conditions. We chose the phosphate buffer with pH 6.9. The substrate solutions were prepared as fresh as possible, and the store flask was ice-cooled during the measurements.

After the heat power signal for an enzyme pulse reached the maximum, the signal remained high for a long time (several hours). We could prove that the penicillinase settles in the flow channel, and because of their catalytic effect a nearly constant heat power signal results. By addition of pulses (using the same sample loop like for the enzyme pulses) for rinsing or cleaning with a surfactant this ‘‘immobilisation’’ could be eliminated (see Fig. 3). We applied a sequence of two pulses of a solution of 20 mmol l^{-1} sodium dodecyl sulfate (SDS) in water at an interval of 3 min, followed by one pulse of water after approximately

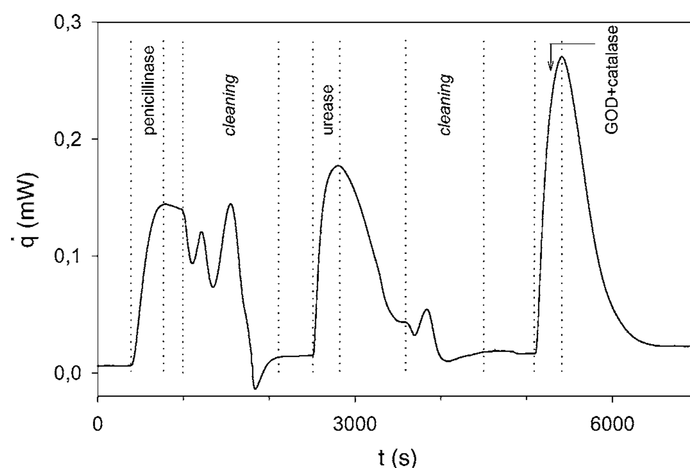
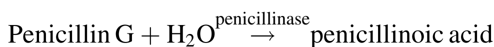


Fig. 3. Multi-substrate analysis: thermal power signals generated by sequential pulses of penicillinase, urease and GOD/catalase into a solution of 1 mmol l^{-1} penicillin G, 1 mmol l^{-1} urea and 1 mmol l^{-1} glucose [Micro-DSC].

10 min. The cleaning sequence should be started only after the maximum peak height was observed.

A relatively low enzyme concentration of $<20\ \mathrm{U\ ml^{-1}}$ was sufficient to obtain a maximum heat power, which is equivalent to the expected value for complete conversion. Only for enzyme concentrations up to approximately $5\ \mathrm{U\ ml^{-1}}$ the effect of immobilisation could eliminate by simple rinsing steps during an ongoing measurement. For all following measurements we chose a penicillinase concentration of $4.5\ \mathrm{U\ ml^{-1}}$.

Under these conditions the dependence of the maximum heat power on the penicillin G concentration is found to be linear up to approximately $1.2\ \mathrm{mmol\ l^{-1}}$ (see Table 1). For higher concentrations the sensitivity decreases and the scatter of data is high. An evaluation of the peak area was not possible because of the described immobilisation phenomenon.



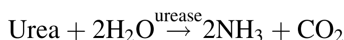
$$\text{Penicillin G} + H_2O \xrightarrow{\text{penicillinase}} \text{penicillinoic acid}$$

3.1.1.4. System 7, urea and urease. For detecting urea we used the enzyme urease.

With the prospect of using urea hydrolysis in combination with the penicillinase reaction for a multi-substrate analysis and it is the optimal medium for this reaction as well [9], we chose the phosphate buffer (0.1 M, pH 6.9).

We determined an optimal enzyme concentration of $810\ \mathrm{U\ ml^{-1}}$. The heat power signal did not reach the baseline after the enzyme pulse in this case too. But the effect is not so strong as obtained for penicillinase. So a single cleaning pulse of a solution of $5\ \mathrm{mmol\ l^{-1}}$ SDS (in water) is enough, to eliminate the disturbance.

In the tested range up to $20\ \mathrm{mmol\ l^{-1}}$ urea we found a strictly linear correlation between the substrate concentration and the maximum heat power. The scatter of the data is relatively low and, therefore, the error of the sensitivity calculated by linear regression is low too (see Table 1).



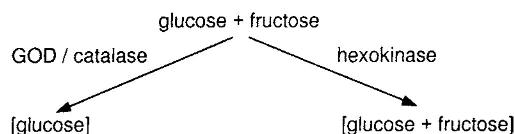
$$\text{Urea} + 2H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

3.1.2. Multi-analyte mixtures (Micro-DSC)

In the next step, we mixed two or three of the investigated substrates and attempted to determine their concentrations by means of the sensitivities calculated from the calibration graphs for the single substrates.

We have already reported about the mixtures of sucrose and glucose (analysis without any problems), maltose and sucrose (not possible), glucose and fructose (possible, but with little problems). Now we will present some more data for the mixture of (1) glucose + fructose, and the results for combinations of (2) sucrose + raffinose and (3) glucose + urea+ penicillin G.

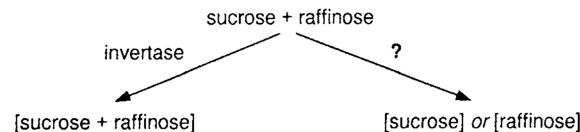
3.1.2.1. System 1, glucose and fructose.



The enzyme hexokinase catalyses the phosphorylation of both saccharides. Under the selected reaction conditions (1 M Tris-buffer pH 8.0 containing 0.1 M $MgCl_2$, $10\ \mathrm{mmol\ l^{-1}}$ ATP^{-4}, $150\ \mathrm{U\ ml^{-1}}$ hexokinase), we had found that the measured heat power for mixtures of these saccharides does not correspond to simple addition of the single contributions, which can be calculated from the calibration graphs. Moreover, it seems impossible to use a kinetic model for calculation of the fructose concentration from the detected heat power [5].

For any glucose concentration we found a linear correlation between the maximum heat power and the fructose concentration. This can be supported now by more data (Fig. 4a). The points of intersection with the axis of ordinate are equivalent to the calibration for pure glucose solutions. The slopes of the straight lines also depend on the glucose concentrations (Fig. 4b). So it should be possible to calculate the fructose concentration from the heat power signal, if the glucose concentration is known.

3.1.2.2. System 2, sucrose and raffinose.



We tested mixtures of sucrose and raffinose in the concentration ranges of up to $15\ \mathrm{mmol\ l^{-1}}$ sucrose and $50\ \mathrm{mmol\ l^{-1}}$ raffinose. For either saccharide we found

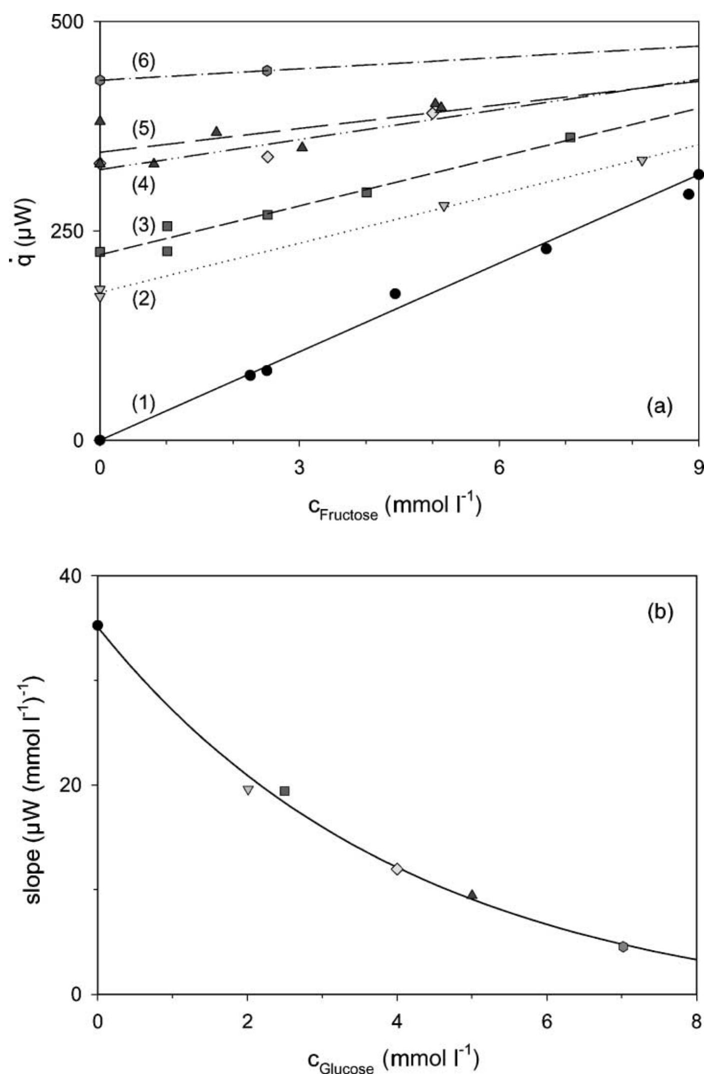


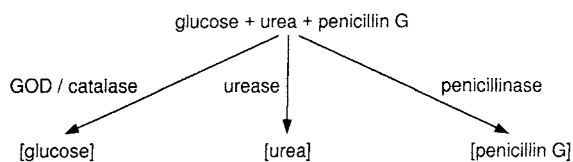
Fig. 4. (a) Dependence of the maximum thermal power on the fructose concentration at glucose concentrations of 0 mmol l^{-1} (1), 2.0 mmol l^{-1} (2), 2.5 mmol l^{-1} (3), 4.0 mmol l^{-1} (4), 5.0 mmol l^{-1} (5) and 7.0 mmol l^{-1} (6) for the hexokinase catalysed phosphorylation of glucose and fructose in mixtures [Micro-DSC]; (b) correlation between the slope of the straight lines from Fig. 4a and the glucose concentration.

a linear increase of the maximum heat power ($\dot{q}$) with the substrate concentration (c_i) at constant concentration (c_j) of the other saccharide. The parameters (a, b) of the linear equations depend on the latter (Fig. 5a and b).

$$\dot{q} = a_i c_i + b_i, \quad \text{if } c_j = \text{constant} \quad \text{and} \quad a_i = f(c_j), \quad b_i = f(c_j)$$

So, if the concentration of one of these two saccharides can be determined by means of another reaction, the concentration of the second one should be calculable from the heat power signal of the invertase catalysed reaction.

3.1.2.3. System 3, Glucose, urea and penicillin G.



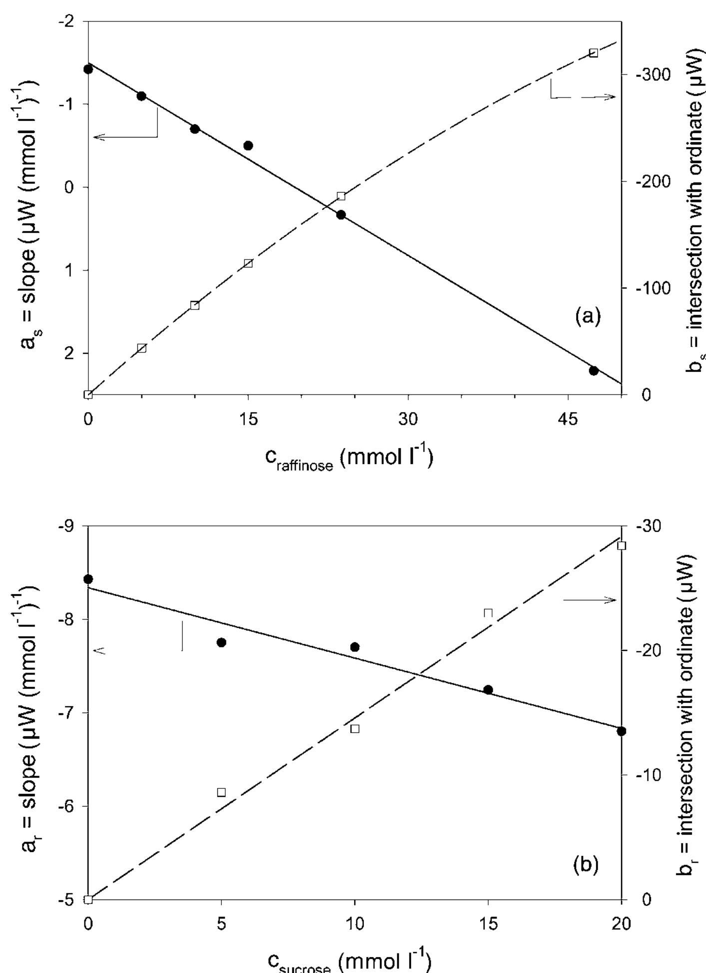


Fig. 5. (a) Correlation between the calibration parameters a_s (●/solid line), b_s (□/dashed line) and the raffinose concentration c_r for the invertase catalysed decomposition of sucrose and raffinose in mixtures with *known raffinose concentration* [Micro-DSC]; (b) correlation between the calibration parameters a_r (●/solid line), b_r ((□/dashed line) and the sucrose concentration c_s for the invertase catalysed decomposition of sucrose and raffinose in mixtures with *known sucrose concentration* [Micro-DSC].

Mixtures containing glucose, urea and penicillin G in different proportions were prepared in phosphate buffer pH 6.9 and the store flask was ice-cooled during the measurements, because of the reasons described above for the special reactions. Because there are no cross activities, the quantitative analysis of these three substrates was practicable without problems. In Fig. 3, an example for the resulting heat power versus time curves is shown. Considering the limits of error the experimentally determined concentrations agree very well with the applied concentrations (see Table 2). The given errors consist of the standard deviations of repetitive measurements and the error of the sensitivity calculated from calibration graphs by linear regression.

3.2. Result of first investigations using the IC calorimeter

As first test system the mixture of glucose and sucrose was chosen. All of the solutions were prepared in acetate buffer pH 4.6 and the enzyme concentrations correspond to the data given in Table 1.

In analogy to the procedure, we described for the Micro-DSC II, we first determined the sensitivities

Table 2
Results of analysing glucose, urea and penicillin G in mixtures

Substrate	Applied substrate concentration ($mmol\ l^{-1}$)	Dilution	Determined substrate concentration ($mmol\ l^{-1}$)
Glucose	0.99	–	1.01 ± 0.06
Urea	1.03	–	1.01 ± 0.02
Penicillin G	0.99	–	0.96 ± 0.05
Glucose	10.01	0.1	10.55 ± 0.34
Urea	4.93	–	4.93 ± 0.03
Penicillin G	0.50	–	0.56 ± 0.04
Glucose	0.51	–	0.53 ± 0.01
Urea	5.02	–	5.04 ± 0.05
Penicillin G	0.30	–	0.34 ± 0.03

Table 3
Comparison of the parameters of the calorimetric arrangements

	Micro-DSC	IC calorimeter
Volume of reaction chamber (μl)	≈180	20
Flow rate (typical) (μl/min)	170	30
Detection limit (μW)	5–10	2
Enzyme pulse volume (μl)	≥1000	≥45
Enzyme pulse length (s)	≥350	≥90
Degree of mixing (%)	100	<40
Glucose + GOD/catalase		
Sensitivity ($\mu W\ (mmol\ l^{-1})^{-1}$)	196	2
Linear range ($mmol\ l^{-1}$)	0–1.5	1 to >10
Resolution ($mmol\ l^{-1}$)	<0.1	1
Sucrose + invertase		
Sensitivity ($\mu W\ (mmol\ l^{-1})^{-1}$)	8.9	0.2
Linear range ($mmol\ l^{-1}$)	0–15	10–70
Resolution ($mmol\ l^{-1}$)	1	5

using single substrate solutions. Fig. 6 shows the calibration graph for the sucrose inversion in comparison to that received by means of the Micro-DSC II. It is to be seen that the scatter of the data is markedly higher for the IC calorimeter, but the linear range is noticeable expanded. The investigation of the oxidation of glucose catalysed by GOD/catalase yields similar results. For numerical values and a summary of some typical parameters of the two different calorimetric arrangements see Table 3.

The volume of the reaction chamber of the IC calorimeter is approximately only a tenth of that of the Micro-DSC and the used flow rate is between 5 and 10 times lower. In the result, on the one hand, the volume of enzyme solution or the time, which is needed to produce the maximum signal height, strongly decreases. On the other hand, the mixing efficiency and residence time are lower and, hence, the degree of conversion decreases [5]. Moreover, the limit of detection, which is caused by the signal noise and other random disturbances of the baseline, could not decreased so markedly. Therefore, we found an

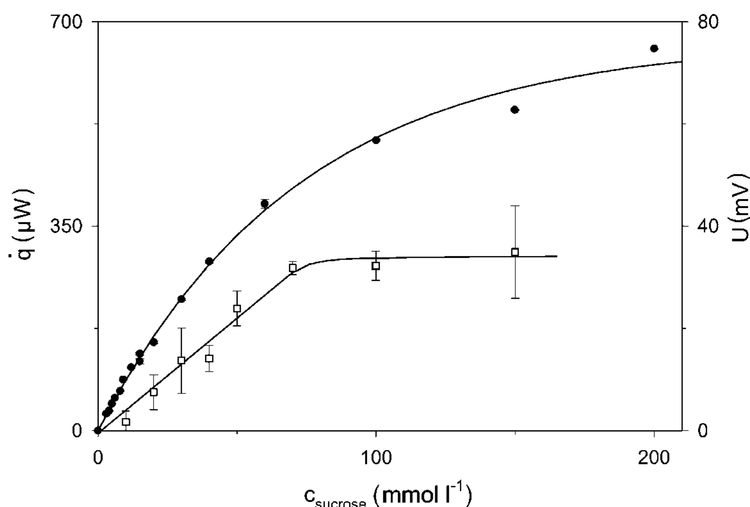


Fig. 6. Comparison of the of the obtained concentration dependency of the signals for the invertase catalysed splitting of sucrose by means of the Micro-DSC (●) and the IC calorimeter (□).

expanded linear range, but a poorer resolution (factors 5–10).

4. Conclusions

The results of the investigations by means of the Micro-DSC II and the IC calorimeter show, that a sequential flow injection analysis technique with independent substrate and toggled enzyme solution flow, combined with calorimetric detection, is well applicable. The proposed method is an alternative to the well-known devices with immobilised enzymes and offers a high degree of flexibility.

We used a calorimeter Micro-DSC II from SETARAM to investigate the influence of reaction conditions on the heat power signals, intended to optimise the conditions for the sequential flow injection analysis. We demonstrated the procedure by the quantitative analysis of a ternary mixture of glucose, urea and penicillin G.

As a first example of use for our IC calorimeter, the analysis of binary mixtures containing glucose and sucrose was tested. As expected, the measurement time and the consumption of substances could decreased strongly.

The miniaturisation of the calorimeter opens great possibilities for the extension of the enzyme and substrate base, not only regarding the determination of single components, but also for the identification of complex liquids.

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