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Fructose-selective calorimetric biosensor in flow injection analysis

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ABSTRACT

A highly selective, interference free biosensor for the measurement of fructose in real syrup samples was developed. The assay is based on the phosphorylation of D(−)fructose to fructose-6-phosphate by hexokinase and subsequent conversion of fructose-6-phosphate to fructose-1,6-biphosphate by fructose-6-phosphate-kinase. The heat liberated in the second reaction is monitored using an enzyme thermistor. The major advantages of this biosensor are rapid and selective measurement of fructose without the need to eliminate glucose and inexpensive FIA-based, mediator-free calorimetric measurement suitable for regular fructose analysis. This method was optimised for parameters, such as pH, ionic strength, interference, operational stability and shelf life. Good and reproducible linearity (0.5–6.0 mM) with a detection limit of 0.12 mM was obtained. Fructose determination in commercial syrup samples and spiked samples confirmed the reliability of this set-up and technique. The biosensor gave reproducible results with good overall stability for continuous measurements over a period of three months besides a useful shelf life of six months. The method could be used for routine fructose monitoring in food samples.

1. Introduction

D-Fructose (DF), an insulin-independent monosaccharide is widely distributed in fruits and vegetables. Because of its higher sweetening ability compared to sucrose and glucose, DF is used as a diet sweetener in diabetic food [1–3]. In food industry regular monitoring of glucose and fructose is important in process and product control for nutritional purposes. For example monitoring the ripening of fruits and determination of saccharide composition in syrups is very important in quality and health control. Determination of fructose is also important in clinical diagnosis in patients with fructosuria, which results in intolerance to dietary fructose. High fructose levels are seen in blood and excretions of such patients [4–6]. Many types of mono- and disaccharide sensors have been described over the past two decades [3,7–9]. Various methods have been developed for the determination of DF in food and clinical samples, which include optical [10,11] and electrochemical [12] detection systems. Amperometric fructose biosensors based on fructose dehydrogenase (FDH) have been described earlier [9,13–15]. The FDH based amperometric fructose biosensors reported to date though sensitive have very short operational life as well as interferences arising from ferrocene leakage [16–20]. The short operational stability of such sensors renders them unfit for routine analysis. In only one study a shelf life of six months has been reported [15]. There is a need for reliable sensors to measure selectively fructose in food products such as syrup, fruit juice, wine, and in clinical samples including blood serum and seminal fluid [21,22]. The application of biosensors in food analysis and clinical diagnosis is a growing field with increasing demand for reliable sensors. The objective of the present work is to demonstrate the application of enzyme thermistor for selective determination of fructose using immobilised hexokinase and fructose-6-phosphate kinase (F6PK).

DF has been determined so far after elimination of glucose through an enzyme column [23] or by using commercially available kits like the one available from Roche, which is based on hexokinase (HK). The available fructose biosensing methods are few and they are also hampered by the complexity of using three enzymes namely hexokinase (HK), phosphoglucoisomerase (PGI) and glucose-6-phosphate dehydrogenase (G6P-DH) besides the need for coenzymes like ATP and NADP [1,2,24–26]. These assays are time-consuming and DF is measured indirectly. Food industries need rapid, easy to afford and selective methods and tools for deter-

Abbreviations: DF, D(−)fructose; DG, D(+)glucose; FIA, Flow injection analysis; DF6P, D(−) fructose-6-phosphate; F6PK, fructose-6-phosphate-kinase; HK, hexokinase; ET, enzyme thermistor; G6P, glucose-6-phosphate; PGI, phosphoglucoisomerase; CPG, controlled pore glass.

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mination of fructose and glucose. In this paper, we report a practical calorimetric flow injection analysis (FIA) set-up for selective determination of fructose using the enzyme couple hexokinase (HK) and fructose-6-phosphate kinase (F6PK). The method combines the advantages of the inherent enzyme selectivity and the short analysis time, low reagent consumption particularly when the enzymes are immobilised and used in a flow injection analysis (FIA) mode [27], the intrinsic insensitivity of thermal biosensors to the optical and electrochemical properties of the sample. This enables selective measurement of specific compounds in complex mixtures. The enzyme thermistor has been, so far, successfully applied to approximately 40 analytes [28–31].

Besides FDH there is no commercially available enzyme that specifically acts on fructose. We therefore used two sequentially acting enzymes to obtain specificity. The first one, hexokinase (HK) phosphorylates fructose, but also glucose. The second enzyme, fructose-6-phosphate kinase (F6PK), however, acts specifically on the F6P formed in the first reaction. The enzymatic couple, HK and F6PK, applied for the development of the proposed biosensor thus catalyses the following reactions:

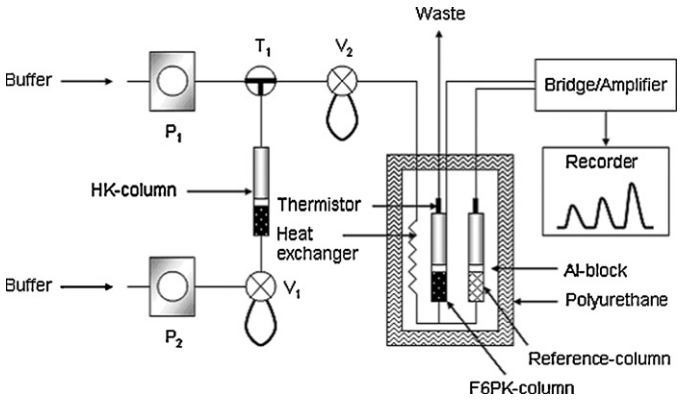
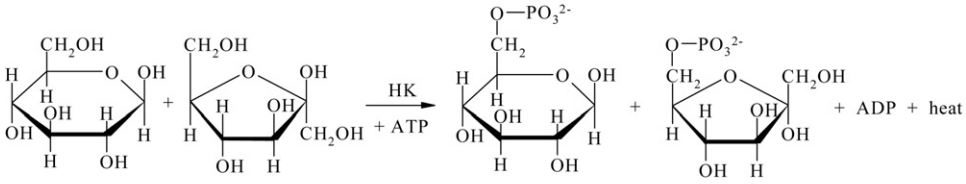
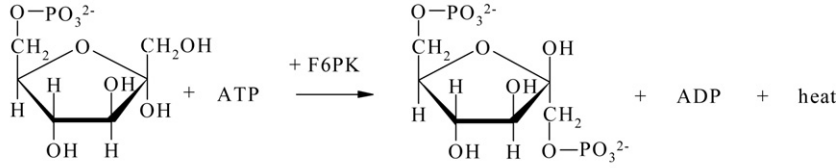


Fig. 1. Experimental set-up of FIA-ET for fructose determination. P_1 and P_2: Peristaltic pumps; V_1 and V_2 are rotary valves. T: junction valve, Column 1: hexokinase immobilised on CPG, column 2: fructose-6-phosphate kinase (F6PK) immobilised on CPG.



β-D-glucose β-D-fructose β-D-glucose-6-phosphate β-D-fructose-6-phosphate



β-D-fructose-6-phosphate β-D-fructose-1,6-biphosphate

F6P from step 1 acts as a substrate for F6PK, which in the presence of adenosine triphosphate (ATP) gives fructose-1,6-biphosphate (F16BP) and adenosine diphosphate (ADP). The heat evolved in the second step is proportional to the amount of fructose phosphorylated in the first step when suitable fructose calibration is done.

2. Experimental

2.1. Materials

Enzyme hexokinase (from yeast overproducer, 450 U mg^{-1}) was obtained from Roche, Germany; fructose-6-phosphate kinase (160 U mg^{-1}, EC: 2.7.1.11, Type VII from *Bacillus Stearothermophilus*), D-fructose-6-phosphate (barium salt), D-glucose-6-phosphate (monosodium salt), adenosine 5′-triphosphate (disodium salt), a 25% aqueous solution of glutaraldehyde and ethanolamine were obtained from Sigma Chemicals Co. (St. Louis, MO USA). D(+)glucose monohydrate, D(−)fructose, magnesium chloride hexahydrate GR grade and Tris (hydroxymethyl)-aminomethane GR grade buffer substance were obtained from Merck, Germany. Amino silanized CPG spherical beads (Trisoperl, size 125–140 μm, pore size 50 nm) were purchased from VitraBio, Germany. All other reagents were of analytical reagent grade.

Standard solutions of D(+)glucose, D(−)fructose, D(+)glucose-6-phosphate, D(+)fructose-6-phosphate and ATP were prepared with 0.1 M Tris–HCl buffer (pH adjusted to 9), filtered through 2 μm filter and degassed in a sonication bath prior to use. Deionised water (18 MΩ) was delivered from a Maxima pure device (Elga, UK). When not in use, solutions were stored at 4 °C. Syrup samples were diluted with 18 MΩ water to reach the linear response of the biosensor.

2.2. Enzyme immobilisation

HK and F6PK were immobilised separately on aminopropyl-CPG beads following the glutaraldehyde activation procedure [32]. Shortly, 1500 U of HK (predialysed overnight in 0.05 M phosphate buffer pH 7.0) were added to 350 mg (wet weight) activated beads in 1 mL of 0.1 M sodium phosphate buffer, pH 7.0. The coupling was allowed to take place overnight at 4 °C with gentle shaking. The remained unreacted aldehyde groups were blocked by adding 50 mg ethanolamine (0.1 M, pH 8). The beads were washed extensively with phosphate buffer and then stored at 4 °C. HK available for the experiment was preserved with some ammonium sulfate. Therefore HK was purified before immobilisation. The other enzyme F6PK was free from such preservative and did not require any purification before immobilisation. The enzyme F6PK was used as received and 250 U were dissolved in 400 μL of phosphate buffer (pH 7.0). This enzyme solution was added to 170 mg of activated CPG in a 1 mL Eppendorf tube, kept for shaking in cold (6–8 h) and followed by 2–3h shaking at room temperature. This helps to remove any adsorbed gas on the mate-

rial. Thus was adopted for experimental measurement conditions using the packed column. Ethanolamine (50 mg, 0.1 M, pH 8) was added to block unreacted aldehyde groups followed by gentle shaking at room temperature for 30–40 min. The beads were washed with 200 mL phosphate buffer (0.1 M) and stored in buffer solution at 4 °C. The immobilised enzymes were separately packed in two special plastic columns, consecutively mounted in the flow of the ET.

2.3. Instrumentation

Fig. 1 shows the experimental set-up of the biosensor described in detail elsewhere [28,29]. It consists of a FIA manifold coupled to ET. In the FIA manifold two peristaltic pumps P_1 and P_2 (Gilson, Minipuls 2, France) generate the flow streams. Junction T, injection valves, V_1 and V_2 (type 50 from Rheodyne, Cotati CA, USA) with 100 μL sample loop were used for sample injections to the enzyme in column 1 (kept outside ET system) and column 2 (inside ET) respectively. PTFE tubing (0.8 mm id) was used for connections. The carrier buffer was 0.1 M Tris–HCl (pH 9 with 7 mM ATP and 10 mM Mg^{2+}). The ET consists of a sensor device and a Wheatstone bridge equipped with a chopper-stabilised amplifier and a recorder. During the operation, the HK column converts DF to F6P, which in turn acts as substrate to F6PK column. The F6PK column is thermostated (30 °C) in an aluminium block insulated with polyurethane foam. This minimizes interference from changes in the environmental temperature. The heat evolved in the enzymatic reaction was detected with a Wheatstone bridge and the signal was amplified and recorded. At maximum sensitivity, the bridge produces a 100 mV change in the recorder signal for a temperature change of 10^{-3} °C. This sensitivity allows substrate concentrations as low as 100 nM to be detected when highly exothermic enzyme reactions are available.

3. Results and discussion

3.1. Optimisation of the FIA biosensor system

3.1.1. Buffer and pH

The activity of the immobilised enzymes and therefore the output signal depends on the buffer pH, ionic strength and presence of divalent metal ions. Thus the activities of the enzymes HK and F6PK were checked with buffers of different ionic strength in the flow system. A constant baseline and a sensitive signal were obtained for Tris–HCl (0.1 M). The response of the enzymatic system (HK/F6PK) was studied by changing pH of the Tris–HCl buffer over the range 7.5–10 for 1 mM DF standard solution. The pH 9.0 at which the maximum signal is recorded (Fig. 2) was used for further studies. The activities of the enzymes HK/F6PK also depend on the presence of divalent metals such as Mg^{2+} and Mn^{2+}. Precipitation was encountered in presence of Mn^{2+} whereas 10 mM Mg^{2+} was sufficient to give a constant background. Therefore Tris–HCl buffer pH 9.0 was supplemented with 10 mM Mg^{2+} in further experiment. An optimal flow rate of 0.6 mL min^{-1} was used to have sufficient contact time between the enzyme and the substrate.

3.1.2. Dependence of ATP concentration

The two phosphorylation reactions must be optimised in order to ensure the complete conversion of the substrates in both reactions and therefore various concentrations of ATP (1–20 mM) were tested. It was observed that the addition of 7 mM ATP (Tris–HCl buffer) to the substrates was sufficient to ensure complete conversion of DF → DF6P and DF6P → F16BP for a linear working range of up to 6 mM DF. The influence of the sample volume on sensor response was studied by varying the sample loop of the ET using 50, 100, 250 and 500 μL loops. As a compromise between the good sensitivity and signal to noise ratio, 100 μL sample loop was used in further experiments (data not shown). The optimum conditions established with the above studies (Tris–HCl pH 9.0, 10 mM Mg^{2+}, 7 mM ATP and flow rate 0.6 mL min^{-1}) were used for the calibration of the system.

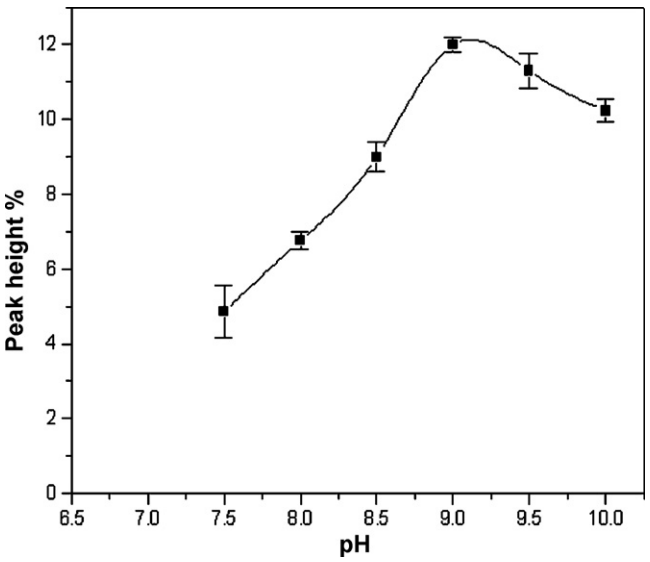


Fig. 2. Effect of pH of the Tris buffer, 0.1 M, 7 mM ATP, 10 mM Mg^{2+}, on the response of integrated FIA sensor for 100 μL of injection of 1 mM DF, flow rate 0.6 mL min^{-1}.

3.1.3. Calibration of the HK column

The immobilised HK column was kept inside the calorimeter and allowed to equilibrate overnight. The buffer was filtered, degassed and pumped via FIA manifold, through P_1, until a constant baseline is achieved. DF standard solution 100 μL (in carrier buffer with 7 mM ATP), were injected through V_1 into the flow stream. Typical thermometric signals were recorded on a recording integrator and plotted as peak height % *vs* DF [mM]. A linear response was obtained (Fig. 3) for the conversion of DF to F6P in the range 0.5–6 mM with a detection limit of 0.12 mM. Linear fit of the data shows good correlation between the DF and DF6P formed (r^2 = 0.99774, S.D. = 0.9%, and P < 0.001). Similarly, the response of HK column to DG stan-

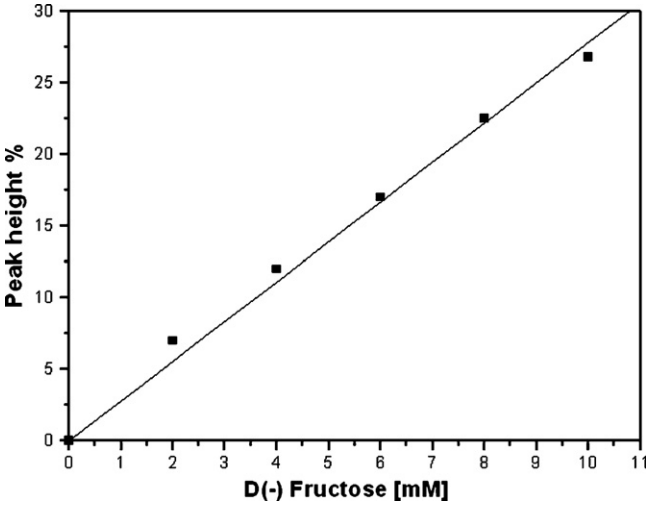


Fig. 3. Calibration plot obtained for DF using hexokinase column, kept inside of the ET. Calibrations were obtained by injecting 100 μL of different concentrations of substrate in the flow. Carrier buffer Tris buffer 0.1 M, pH 9.0, 7 mM ATP, 10 mM Mg^{2+}, flow rate 0.6 mL min^{-1}. The results are average of duplicates. The measurements were performed at 30 °C.

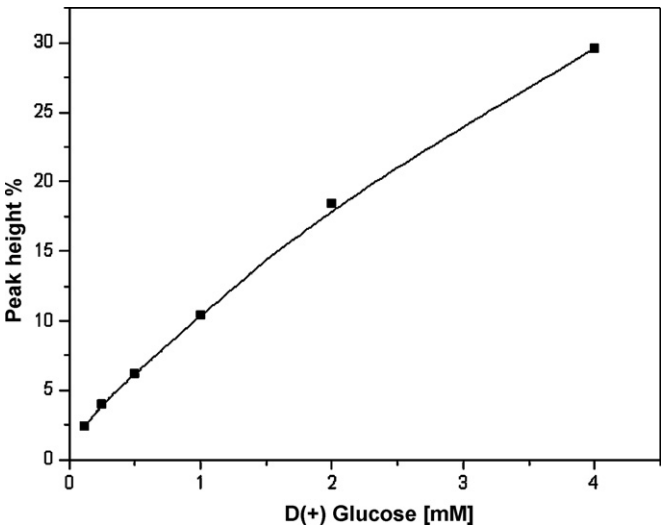


Fig. 4. Calibration plot for linear response of DG using hexokinase column kept inside thermistor. Calibration obtained by injecting 100 μL of different concentrations of D(+)glucose in FIA system.

dard solutions were obtained and plotted against peak height % *vs* DG [mM] as shown in Fig. 4. A linear response was registered up to 6 mM. The signals were comparable to those of DF.

3.1.4. Calibration of F6PK column

The immobilised F6PK column was kept inside the calorimeter to get equilibrium temperature. The buffer was pumped through P_1 till a constant baseline was seen. The response of the enzyme sensor was checked with a synthetic substrate F6P (barium salt) dissolved in carrier buffer with 7 mM ATP. The responses to 100 μL injections were recorded and plotted as peak height % *vs* F6P [mM] as shown in Fig. 5.

3.1.5. Integrated biosensor for fructose

The HK column 1 was kept in a Teflon holder (Fig. 1) outside the ET, and connected to the FIA manifold through junction T_1, while the F6PK column 2 was kept inside the ET. The carrier buffer was pumped through P_1 and valve V_2 until a constant baseline was achieved. DF standard solution was injected through V_2. DF6P produced through HK was collected in the sample loop of V_2 through a T-junction and injected through ET. The signals were recorded and plotted as peak height % *vs* DF [mM], as shown in Fig. 6. The response signal obtained for DF was not equal to the signal obtained for the F6P. Hence the signal for DF through HK column was amplified by recycling the product twice through this column with further addition of ATP. Two cycles with 7 mM ATP ensured 100% conversion of DF to F6P. The ATP volume was reduced by injecting it together with the substrate instead of using it in running buffer. This provided additional economy to the method. The performance of the HK column was further checked by comparing the output signals through the integrated sensor with those obtained by injecting the synthetic substrate F6P directly through the F6PK. A very good correlation, proportional to F6P concentration, was observed. The system is heterogeneous. There is a gradient established wherein the K_m will be higher for the inner enzyme than the outer one. The K_m is of limited use in heterogeneous experimental system like this. The concentration of ATP is the dictating factor, as it also inhibits the enzyme F6PK above the levels reported herein. Thus the ATP concentration was optimised to achieve the two step phosphorylation so that the inhibition by ATP is avoided. For system performance, calibration check can be done.

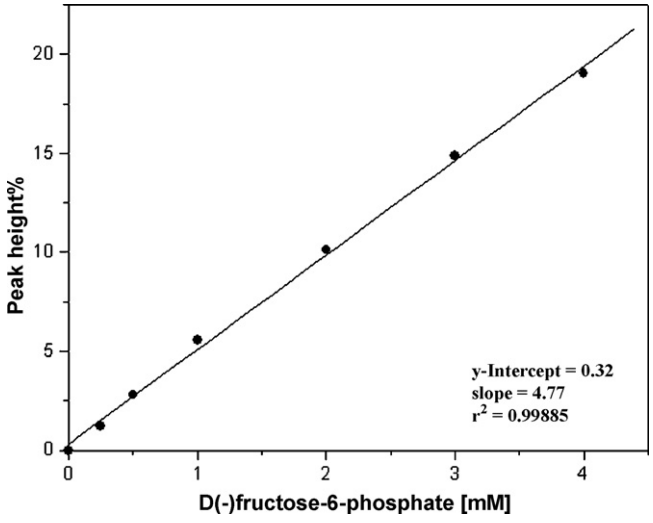


Fig. 5. Calibration plot for linear response of the F6PK sensor (kept inside thermistor) to synthetic substrate F6P. Calibration obtained by injecting 100 μL of different concentrations of F6P in FIA system.

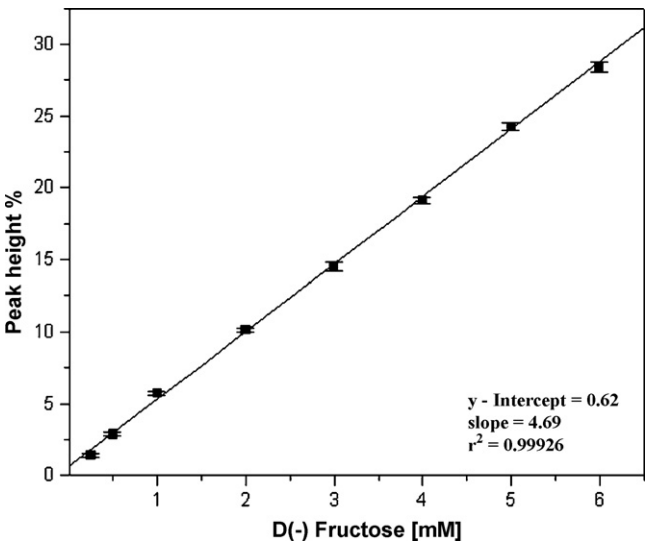


Fig. 6. Calibration plot for linear response of the integrated sensor system (HK and F6PK) to DF substrate. Calibration obtained by injecting 100 μL of different concentrations of DF in FIA system.

3.1.6. Sensitivity, detection limit and dynamic range

Unless mentioned otherwise, all results are based on injections performed in duplicate. A broad linear range, 0.5–6 mM was obtained with the limit of detection being 0.12 mM. This method provided a signal response sensitivity of 4.69% mM^{-1} DF.

3.1.7. Response time and sample throughput

The response of the FIA sensor was highly reproducible with a variation coefficient less than 3%, at 30 °C and it was estimated from 25 repeated injections of 1 mM DF. The conversion of about 100 μL DF to F6P, after two cycles through HK, took about 2.5 min, whereas the response signal for conversion of DF6P to F16BP was 2 min. Therefore, it is possible to analyse about 30 analyses per hour.

3.1.8. Operational stability and shelf life

The stability of the system was checked during approximately 20 days of continuous operation. About 30 measurements were made every day. The operational characteristics and stability of the columns were monitored over a period of 14 months. The oper-

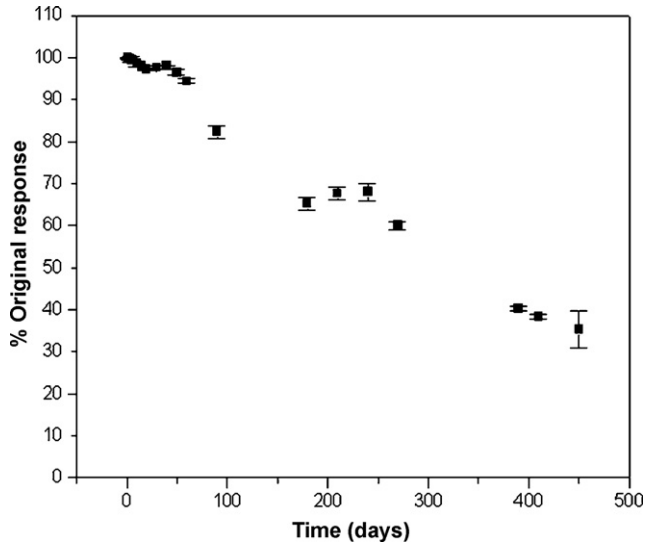


Fig. 7. Stability of the biosensor over 14 months, stored under buffer at 4 °C. Response to 100 μL, 1 mM DF measured over a random interval. The results are average of triplicate measurements.

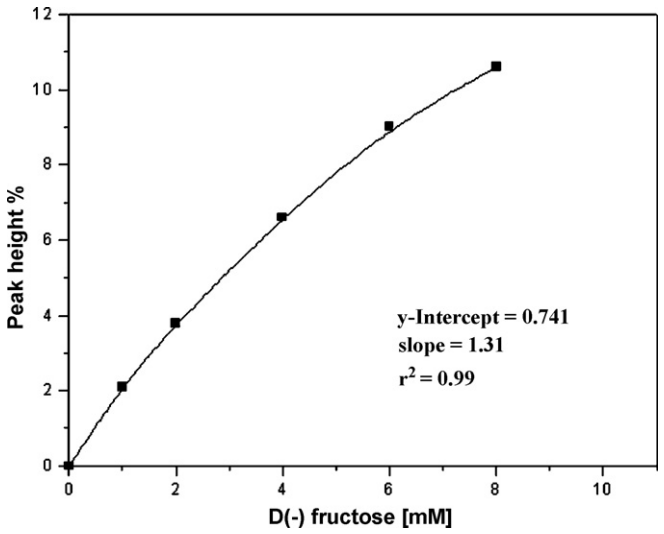


Fig. 8. Calibration plot for linear response of the integrated biosensor (HK and F6PK) to DF substrate after being stored at 4 °C for 14 months. Calibration obtained by injecting 100 μL of different concentrations of DF in FIA system.

ational stability over the 60 days and the stability of the column over 14 months are presented in Fig. 7. The immobilised enzyme presented excellent storage stability. Over the first three months it was actually even used and there was no appreciable loss of activity of the sensor. However, after a period of six months operation it was observed about 25% decay in response. After 14 months, about 71% loss in sensitivity was registered (1.31% mM^{-1} DF, compared to 4.69% mM^{-1} DF). Fig. 8 shows the sensor response obtained during 14th month, linearity 1–4 mM, detection limit 0.5 mM and the calibration equation was $y = 1.31\%\ mM^{-1} + 0.74$, $r^2 = 0.99$. Thus a shelf life of 14 months for the sensor system was ascertained. In between the tests the immobilised enzyme column was stored under buffer at 4 °C.

3.1.9. Specificity study

Various biosensors reported in literature for the determination of fructose are listed in Table 1. Most of these sensors use FDH as the biological recognition element and have good detection range and limits. However, the present work is unique as it determines fructose selectively in the presence of glucose. When determining fructose, glucose is one of the major interfering substances encountered. In practice, elimination of glucose is achieved either by employing enzymes such as glucose oxidase/catalase or by introducing a glucose-eliminating reactor [23]. But the poor selectivity of the second enzyme used for glucose elimination has been a major concern. In the present work, fructose-6-phosphate kinase (F6PK) which selectively phosphorylates F6P but not G6P was used to develop a highly selective thermometric biosensor for fructose. A systematic study was performed to check the selectivity of the enzyme. Series of solutions of DF:DG [mM] in the ratios 1:0.5, 1:1, 1:1.2, 1:4, 1:6 and 1:8 were injected through HK/F6PK and response signal were recorded. The % change in signal was determined by comparing it with the response to DF alone. Similarly, mixtures of F6P:G6P were injected into F6PK column and the % change in signal was determined by comparing the response to DF6P. The results obtained are presented in Table 2. The better selectivity

Table 1
Comparison of the F6PK-ET-FIA biosensor with other reported biosensors and methods for fructose determination.

Detection principle	Enzyme system	Working range	Reference
Thermometric	HK/F6PK	0.5–6 mM	Present work
Amperometric	FDH	25 nM–0.5 mM	[13]
Amperometric	FDH	0.1–0.8 mM, 1.0–5.0 mM	[9,14]
Amperometric	FDH	50 μM–10 mM	[15]
Amperometric	FDH	0.01–0.3 mM	[20]
Redox biosensor	FDH/HRP-HRP/AOX	0.5–20 mM	[34]
Fluorimetric	FDH	1.0–5.5 nM	[35]

HK-hexokinase; FDH-fructose dehydrogenase; HRP-horseradish peroxidase; AOX-alcohol oxidase.

Table 2
Analysis of fructose in real samples, recovery and selectivity studies.

Sample	Real samples[a]		Spiked samples[b]		Selectivity[b]		
	Fructose (M)	Glucose[c] (M)	Fructose (mM)	Recovery (%)	DF:DG/DF6P:DG6P (mM)	Change in signal DF:DG (%)	Change in signal DF6P:DG6P (%)
Dark syrup	0.5 ± 0.02	1.5 ± 0.05	1.0	100.4	1:1	1	1.2
Light syrup	1.8 ± 0.06	2.35 ± 0.08	4.0	100.0	1:2	0	0

F6P – fructose-6-phosphate; G6P – glucose-6-phosphate.
[a] Commercial samples from a production site.
[b] Average of 3 analyses.
[c] Samples analysed separately by a glucose enzyme thermistor.

of the system is further confirmed by selective response to F6P from the mixture of synthetic F6P and G6P and the absence of any appreciable changes in the thermometric response for increasing G6P concentration. This selectivity remained unaltered when the monophosphorylated mixture of DG and DF derived from HK treatment was injected through F6PK. This is the first time that such a fructose biosensor with F6PK as a biological recognition element is demonstrated without the need to eliminate the interfering glucose from the sample. The comparable percentage change in the F6PK-ET observed for the synthetic F6P:G6P mixture and for the DF:DG mixture phosphorylated by HK confirms the selectivity (Table 2). The better selectivity and the sensitivity obtained using the reliable ET-based flow injection analysis makes the present work a novel methodology for rapid fructose monitoring and determination.

3.2. Validation and analysis of real samples

Prior to analysis of real samples, the sensor system was validated by measuring the fructose concentration in buffer spiked with various concentrations of DF. Samples spiked with 0.5–4.0 mM fructose were analysed for the recovery test and select data are given in Table 2. The system offered complete fructose recovery (99.8–100.4%) for the measured concentrations. This is a proof of the accuracy and reliability of the reported biosensor system. Based on the validation data, real fructose syrup samples, obtained from a product site in Sweden, were analysed for fructose content. The syrup samples namely dark syrup (72% sugar) and light syrup (65% sugar) were diluted to match the working range and were directly injected into the system. The glucose content was measured using an ET-based glucose oxidase biosensor described elsewhere [29]. The fructose concentration was found to be 0.5 ± 0.02 M and 1.8 ± 0.06 M respectively in the dark and light syrup. The corresponding glucose concentrations were 1.5 ± 0.05 M and 2.35 ± 0.08 M (Table 2). The concentrations were determined based on 5 repeated analyses. The values obtained for fructose content in these commercial samples were comparable to claimed amount.

3.3. Uniqueness and advantages of the HK/F6PK FIA-ET

The unique advantage of the present thermometric biosensor is its ability to measure fructose even in the presence of glucose owing to the selective phosphorylation of F6P by F6PK. It is also possible to measure glucose and fructose simultaneously by using a sample split valve leading to a glucose oxidase thermistor without the need for a separation step. However, simultaneous determination of glucose and fructose in the same sample by amperometry is difficult as both the sugars show amperometric signal at the detection potential used [19]. With respect to the life of the sensor, HK/F6PK FIA-ET can provide constant signal response for 3 months which is a 3-fold better stability compared to 35 days life time warranted for the FDH based amperometric sensor [33]. The higher stability of the present sensor can be attributed to the covalent immobilisation of the enzyme. The immobilised columns were stored at 4 °C in the refrigerator with buffer. Sodium azide (1 mg) was added to the buffer to counter the microbial growth.

4. Conclusion

A practical and novel fructose-6-phosphatekinase based flow injection thermometric biosensor set-up for fructose determination was developed and demonstrated. This never before used F6PK enzyme as the sensing enzyme has been easily adopted for thermometric measurement. The method was optimised and had been successfully applied to determine fructose in commercial syrup samples. The advantages include low sample requirement, high selectivity, better stability, short analysis time and low consumption of enzymes and coenzymes. The system could analyse fructose in syrups in the presence of glucose without its elimination. The method provided a good practical working range (0.5–6 mM) comparable to other fructose biosensors. Moreover, the improved selectivity and ease of sample handling makes this set-up highly practical for regular analysis. The excellent operational stability and shelf life provide additional cost benefit to this method. An additional application of the above biosensor could be the direct determination of F6P and ATP besides its potential application in process monitoring.

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