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Journal of Chromatography A, 687 (1994) 141–148

JOURNAL OF CHROMATOGRAPHY A

Determination of carbohydrates, sugar acids and alditols by capillary electrophoresis and electrochemical detection at a copper electrode

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First received 1 July 1994; revised manuscript received 12 August 1994

Abstract

Capillary electrophoresis combined with electrochemical detection at copper electrodes has been shown to provide a simple and sensitive method for the direct analysis of samples containing a wide range of carbohydrate compounds including simple sugars, sugar acids and alditols. In this approach, both the separation and the detection required the use of a strongly alkaline medium whose hydroxide content could be varied to optimize conveniently the migration times and resolution obtained. Detection consisted of a direct oxidation that required no derivatization and yielded detection limits at or below the fmol level for most of the carbohydrate species.

1. Introduction

Because carbohydrates are the most abundant class of organic compounds in nature and are found universally distributed among plants, animals and microorganisms, the development of analytical methods for their determination is an increasingly important research area. However, despite extensive efforts, carbohydrate analysis still presents a challenge, especially for complex, natural samples. First, resolution of the many closely related carbohydrate compounds possible in a sample poses a problem that is difficult, if not impossible, for most applicable separation techniques. To date, high-performance liquid chromatography (HPLC) has most commonly been employed for this purpose. Second, carbohydrate compounds generally do not possess chromophores which absorb appreciably at accessible UV–visible wavelengths and therefore are not readily detected by conventional spectrophotometric methods unless rigorous and time-consuming derivatization is first carried out. Until recently, electrochemical detection (ED) played only a very limited role in carbohydrate detection. This is due to the fact that, although carbohydrates can be oxidized by numerous chemical reagents, they are not normally electroactive at carbon electrodes, which are the most commonly used working electrodes in ED. Over the past decade, oxidation at platinum and gold electrodes has gained some popularity for the detection of underivatized carbohydrates [1,2]. However, because of electrode fouling caused by adsorption of the sugar oxidation products, the applied potential for these elec-

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0021-9673/94/$07.00
SSDI 0021-9673(94)00783-7

trodes must be continuously pulsed for stable, long-term response to be possible.

Very recently, two developments have occurred which have provided attractive new tools for carbohydrate determination. First, capillary electrophoresis (CE) has become firmly established as a separation technique which, compared to HPLC, offers extremely high separation efficiency [3]. For example, for a 50 cm long capillary tube, the number of theoretical plates typically reaches 100 000 or more, which is about 10 times higher than that for a typical 25 cm long HPLC column. As a result, much higher resolution and much greater separating power for carbohydrate samples is potentially available with CE. Second, investigations using Cu electrodes [4–10] have shown that carbohydrates can be oxidized facilely at these surfaces at constant applied potential both in bulk solution and in HPLC detection schemes. This permits less expensive, less complicated, but still highly sensitive detection of these species. Moreover, it has been found that amperometric detection at Cu electrodes can be employed to detect not only carbohydrates themselves but also many related compounds including alditols and aldonic, aldaric and uronic acids [4]. Therefore, CE's high efficiency coupled with convenient, sensitive and dependable detection at Cu electrodes would seem to represent an ideal analytical tool for the determination of carbohydrates and their derivatives. Although the Cu electrode detection systems always require strongly alkaline conditions —which drastically limits the stationary phase options when used as HPLC detectors— high pH levels are perfectly compatible with the fused-silica capillaries used in CE.

To this point, only a few reports describing the use of CE–ED with metallic electrodes for the separation and determination of carbohydrate compounds have been published. In these studies, both pulsed amperometric detection at Au electrodes [11] and constant potential detection at Cu electrodes [12,13] have been successfully employed for sensing purposes following the CE separation. However, the analytes included consisted primarily of simple mono- and disaccharides, with little attention paid to important derivatives such as alditols and acidic sugars. In view of the varying charges exhibited by all these compounds under the high pH conditions required for oxidation at the metallic electrodes, CE–ED appeared to present a logical and attractive analysis approach for samples containing not only simple sugars but also many of their important derivatives. In an attempt to realize this possibility and thereby expand the analytical tools available for carbohydrate-related compounds in complex samples, we report here the capabilities of CE–ED with Cu electrodes for the determination of alditols and aldonic, uronic and aldaric acids as well as ordinary sugars. Such analyses, which often present problems when the separation is carried out by liquid chromatography [6,14–17], were in fact able to be accomplished in an extremely straightforward manner by a simple CE–ED procedure.

2. Experimental

2.1. Reagents

Stock solutions of all sugars, alditols and sugar acids (purchased from Sigma and Aldrich) were prepared fresh daily in deionized water. Just prior to use, samples to be injected were prepared from these stock solutions by dilution to the desired concentration with the NaOH solution used as separation electrolyte. Experiments involving enzymatic oxidation of glucose were conducted by adding 1.0 mg of glucose oxidase (Type X, purchased from Sigma) to a stirred pH 5.3 phosphate buffer solution (200 ml, room temperature) containing 1.0 m*M* β-D-glucose and 0.50 m*M* glucitol (which was used as an internal reference).

2.2. Apparatus

All CE experiments were performed on a laboratory-built instrument with a 30 kV high-voltage power supply (Model 30B, Hipotronics, Millerton, NY, USA) and an 80 cm length of 25 μm I.D. $\times$ 360 μm O.D. fused-silica capillary

(Polymicro Technologies, Phoenix, AZ, USA). In order to protect the operator from accidental exposure to high voltages, the entire capillary, the electrolyte reservoirs, and all electrodes were enclosed in a Plexiglass box equipped with a safety switch wired to shut down the power supply whenever the box was opened. In addition, the outlet end of the capillary was always maintained at ground. A 1-in.-diameter (1 in. = 2.54 cm) plastic vial served both as the cathode compartment of the CE instrument and as the electrochemical cell for the CE detection. Before insertion into the vial through a small slot cut into its side, the outlet and of the capillary was made as flat as possible by use of a fiber cleaver (Newport, Irvine, CA, USA). The electrophoresis medium was always just an NaOH solution; NaOH concentrations used ranged from 25 to 250 m*M*. To minimize the effect of CO_2 pickup from the atmosphere, NaOH solutions were replaced daily. Sample injection was carried out by electromigration by immersing the inlet of the capillary in the sample solution and applying a high voltage for a suitable time period.

The design and performance of the ED system employed have been described previously [13]. The specific working electrode used was a 100-μm-diameter Cu magnet wire (Newark Electronics, Chicago, IL, USA) whose side areas were covered with a non-conductive coating. The procedure for construction of these working electrodes was detailed previously [13]. The working electrode was arranged in a wall-jet configuration in which the Cu wire was inserted into the cathode compartment of the CE instrument and then positioned up against the capillary outlet with the help of an Oriel (Stratford, CT, USA) Model 14901 micropositioner. In this configuration, the CE effluent impinged directly onto the disk-shaped Cu electrode and then flowed radially outward across its surface. Also placed into this compartment were an Ag/AgCl (3 *M* NaCl) reference electrode and a platinum wire counter electrode. Control of the applied potential and measurement of the resulting current were carried out with a Bioanalytical Systems (West Lafayette, IN, USA) Model LC-4B amperometric detector.

3. Results and discussion

3.1. Nature of the CE separation

The electrochemical behavior of carbohydrates at metallic Cu electrodes in strongly alkaline solution has been reported previously [4,5,18,19]. The range of carbohydrate compounds that can be oxidized usefully at the Cu electrode has been shown to include mono- and oligosaccharides, both reducing and non-reducing, and many related compounds such as alditols, aldonic acids, uronic acids and aldaric acids, with the primary molecular feature needed for oxidative response being the presence of multiple aliphatic hydroxyl groups [19]. Of course, if CE–ED is to be effective for the identification and quantitation of any of these compounds in authentic samples, the CE system employed must be effective in separating them from one another and from other oxidizable species present in the sample matrix. Fortunately, CE's extremely high separation power can usually meet this requirement. In the work presented here, this was accomplished with a strongly basic separation medium (25–250 m*M* NaOH) which served both to maintain the activity of the Cu electrode for ED detection and to maintain all the analytes in anionic form for the differential migration required for CE separation.

The electropherograms shown in Fig. 1 for sample mixtures containing glucose and galactose as well as their respective alditol and acidic sugar derivatives illustrate the nature of the separation that can be achieved by means of CE. In both cases, the order of elution observed (alditol, aldose, aldonic acid, uronic acid, aldaric acid) was exactly that expected on the basis of the compounds' pK_a values and resulting ionic character in the 50 m*M* NaOH electrophoresis medium employed. Because the polarity of the voltage applied across the capillary was set so that the detector end was negative compared to the injection end, cationic species migrated toward, and anions away from, the capillary exit. Thus, it was expected that the nearly uncharged alditols (pK_a values ca. 13.6) would elute first

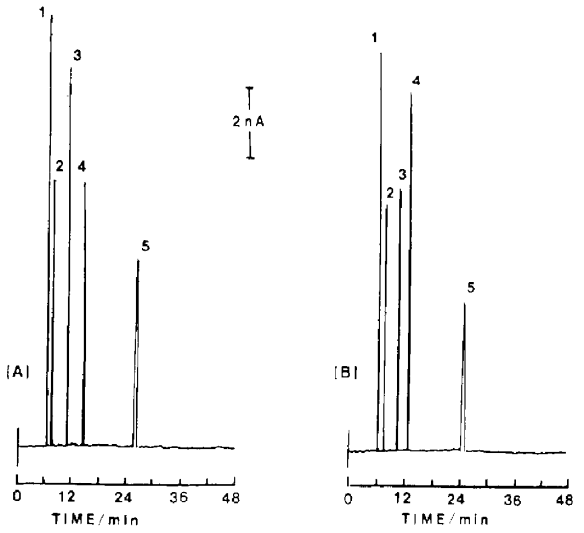

Fig. 1. Electropherograms of glucose (A) and galactose (B) families. Labeled peaks in (A) correspond to glucitol (1, 250 μM), glucose (2, 250 μM), gluconic acid (3, 500 μM), glucuronic acid (4, 1 mM) and glucaric acid (5, 2 mM). Labeled peaks in (B) correspond to galactitol (1, 250 μM), galactose (2, 250 μM), galactonic acid (3, 500 μM), galacturonic acid (4, 1 mM) and galactaric acid (5, 2 mM). Electrophoresis medium: 50 mM NaOH; working electrode: 100-μm Cu disk at +0.60 V vs. Ag/AgCl; separation voltage: 30 kV; injection by electromigration (30 kV for 3 s).

and the di-anionic aldaric acids should take the longest time. The specific migration times seen for the glucose family as well as the average charges calculated for these pH conditions are shown in Table 1.

An interesting aspect of the CE separations in Fig. 1 is that, because of the high efficiency of the CE approach and the different charge character of the various carbohydrate derivatives, the different classes of compounds were relatively easy to resolve from one another. This is not always the case with HPLC-based methods [6,14–17]. A more challenging analytical task involves the resolution of individual members of each class from one another —e.g., the determination of one aldonic acid in the presence of another. Significantly, it appears that the CE–ED approach presents very useful capabilities for this application as well. Shown in Fig. 2, for instance, is a single electropherogram obtained for a sample mixture containing all ten glucose and galactose species from Fig. 1. Exactly the same CE conditions were employed for this separation as earlier except that the separation voltage was decreased from 30 to 15 kV in order to facilitate the resolution of glucitol and galactitol. Apart from these two isomers whose pK_a values differ by only about 0.1 [20], the other sample components were comparatively easy to resolve completely. Of course, the decrease in CE potential had the expected effect of lengthening the time required for the separation.

An alternative approach, which offers the possibility of fine-tuning the separation further, consists of adjusting the pH of the electrophoresis medium so as to adjust the net charge on the very weakly acidic hydroxyl groups. Increasing the pH can be of special utility, for example, for alditols and simple sugars to increase the

Table 1
Migration times and estimated charges for glucose derivatives

	Glucitol	Glucose	Gluconic acid	Glucuronic acid	Glucaric acid
Migration time (min)	6.6	8.1	10.8	12.9	26.4
pK_a [a]	13.6	12.3	3.86	–[c]	3.77, 6.08
Molecular mass (g/mol)	182.2	180.2	196.2	194.1	212.2
Charge[b]	−0.11	−0.65	−1.0	−1.65[c]	−2.0

[a] From Refs. [20–22].
[b] Charges were calculated from the compounds' pK_a values listed above for the pH 12.7 electrophoresis medium.
[c] The pK_a values for glucuronic acid are not available; calculation is based on the assumption that, because of the structural similarities, these values are similar to those of gluconic acid (3.8) and glucose (12.3).

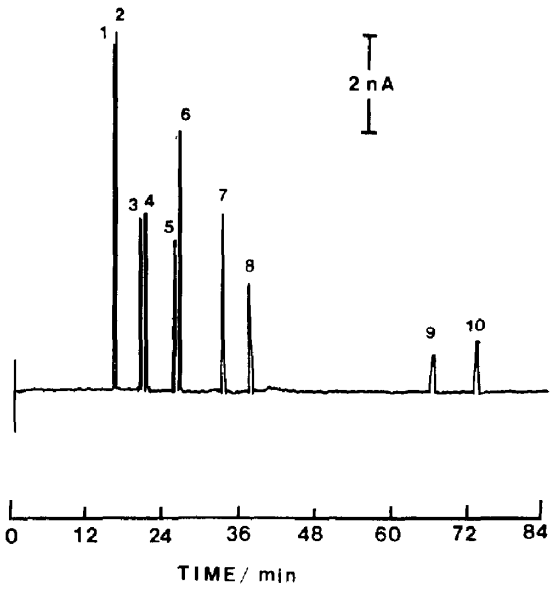

Fig. 2. Electropherogram of a mixture of glucose and galactose families. Labeled peaks correspond to galactitol (1, 125 μM), glucitol (2, 125 μM), galactose (3, 125 μM), glucose (4, 125 μM), galactonic acid (5, 250 μM), gluconic acid (6, 250 μM), galacturonic acid (7, 500 μM), glucuronic acid (8, 500 μM), galactaric acid (9, 1 mM) and glucaric acid (10, 1 mM). Electrophoresis medium: 50 mM NaOH; working electrode: 100-μm Cu disk at +0.60 V vs. Ag/AgCl; separation voltage: 15 kV; injection by electromigration (15 kV for 5 s).

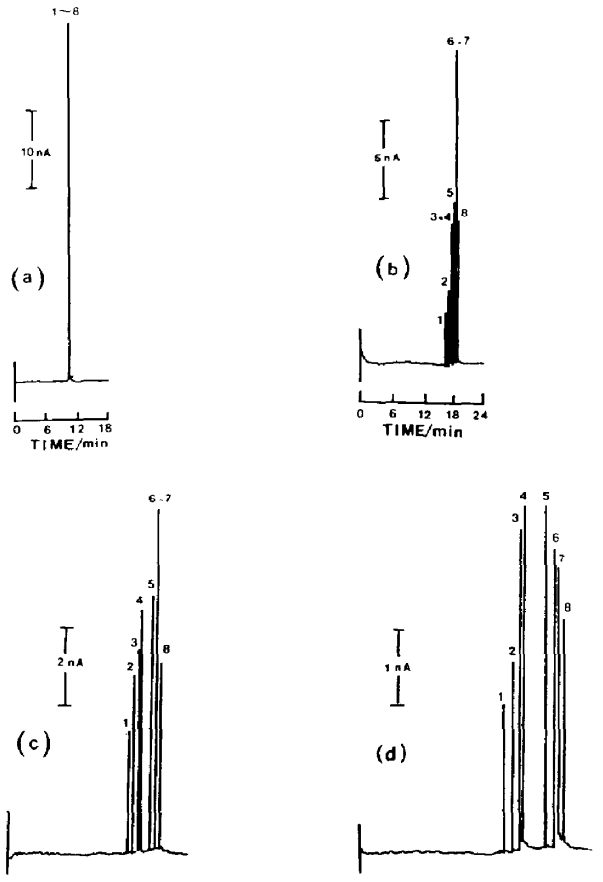

Fig. 3. Electropherogram of alditol mixture. Labeled peaks correspond to ethylene glycol (1, 1 mM), glycerol (2, 500 μM), inositol (3, 160 μM), erythritol (4, 250 μM), galactitol (5, 250 μM), adonitol (6, 250 μM), glucitol (7, 250 μM) and mannitol (8, 250 μM). Electrophoresis media: (a) 25 mM NaOH, (b) 0.10 M NaOH, (c) 0.20 M NaOH and (d) 0.25 M NaOH. Working electrode: 100-μm Cu disk at +0.60 V vs. Ag/AgCl; separation voltage: 15 kV; injection by electromigration (15 kV for 3 s).

migration times and improve the separations obtained for these compounds. This is illustrated clearly in Fig. 3 which shows the electropherograms obtained for a mixture of eight different alditols at four different NaOH concentrations, all other CE conditions remaining the same. While no separation at all occurred when the electrophoresis medium was 25 mM NaOH (Fig. 3a), the separation improved as the OH^- level was increased, with complete resolution of the mixture achieved at NaOH concentrations of 250 mM and above (Fig. 3d). Because the pK_a values for alditols are typically 13–14, highly basic conditions are required for significant dissociation and differential electrophoretic migration to occur. Otherwise, the alditols are nearly neutral in charge and, due to the electroosmotic flow, emerge from the capillary at practically the same time. Of course, because of their greater anionic character under the high NaOH conditions of Fig. 3d, simple sugars and the acidic sugars remained on the capillary much longer than the alditols and would therefore present no problem for the alditol analysis.

3.2. Analytical applications

The analytical results for a test mixture containing all ten members of the glucose and

Table 2
Analytical performance of Cu electrode CE–ED for glucose and galactose compounds

Peak	Compound	Detection limit (fmol)	Linear range	No. of theoretical plates	R.S.D. (%)[a]
1	Galactitol	0.5	0.5 μM–1 mM	145 000	4.9
2	Glucitol	0.5	0.5 μM–1 mM	147 000	5.8
3	Galactose	1	1 μM–1 mM	154 000	5.5
4	Glucose	1	1 μM–1 mM	166 000	5.2
5	Galactonic acid	2	2 μM–1 mM	151 000	8.2
6	Gluconic acid	1.5	1.5 μM–1 mM	155 000	6.0
7	Galacturonic acid	3	3 μM–2 mM	150 000	7.7
8	Glucuronic acid	4	4 μM–2 mM	180 000	8.9
9	Galactaric acid	8	8 μM–4 mM	71 000	7.0
10	Glucaric acid	6	6 μM–4 mM	81 000	7.8

CE–ED conditions as in Fig. 2.
[a] These numbers represent the relative standard deviations for four identical injections.

galactose families are summarized in Table 2, and analogous results for a sample containing only alditols are shown in Table 3. For most of these compounds, peak heights varied linearly over a roughly three-order-of-magnitude range with detection limits (signal-to-noise ratio 3) near or below the fmol level. The reproducibility and stability of the CE–ED system were quite reasonable in practice. Repeated injections of the same analyte typically exhibited relative standard deviations in the 5–7% range —which was essentially the reproducibility of the electrokinetic injection process employed here. The Cu electrode surfaces were generally used for periods of a month or more without replacement or any special treatment. Over the course of two weeks of continuous use in the CE–ED apparatus, only a gradual decrease in electrode response (less than 10%) was observed. Finally, separation efficiencies were generally greater than 150 000 theoretical plates; the exceptions to this were galactaric and glucaric acids which had very long migration times under the particular CE conditions in effect.

These results are noteworthy in one additional respect. The ED system employed here was based on a wall-jet configuration [13] which allows the use of much larger electrodes than has normally been the case for CE–ED. This makes electrode construction and alignment a compara-

Table 3
Analytical performance of Cu electrode CE–ED for alditols

Peak	Alditol	Detection limit (fmol)	Linear range	No. of theoretical plates	R.S.D. (%)[a]
1	Ethylene glycol	19.2	16 μM–4 mM	148 000	4.6
2	Glycerol	2.4	2 μM–2 mM	166 000	10.2
3	Inositol	0.38	0.32 μM–0.64 mM	158 000	5.5
4	Erythritol	0.6	0.5 μM–1 mM	164 000	6.7
5	Galactitol	0.6	0.1 μM–1 mM	190 000	5.0
6	Adonitol	0.6	0.5 μM–1 mM	209 000	6.5
7	Glucitol	3	0.5 μM–1 mM	215 000	6.3
8	Mannitol	4	0.5 μM–1mM	180 000	5.9

CE–ED conditions as in Fig. 3d.
[a] These numbers represent the relative standard deviations for six identical injections.

tively simple process and greatly enhances the stability and reproducibility of the detector response. As a result, the CE–ED method described is not difficult to implement technically. This characteristic should make the approach attractive for many applications involving the analysis of carbohydrates and related compounds in a wide variety of complex natural samples. Two specific examples are illustrated below.

Fig. 4 shows a typical electropherogram obtained at a Cu electrode for a commercial apple juice (Carolina Gold, 100% natural). The only sample pretreatment prior to injection was dilution by a factor of 300 with the running electrolyte solution. The electropherogram exhibits four well-separated peaks whose migration times match those of glucitol, sucrose, glucose and fructose standards. Based on the calibration curves obtained for glucitol and glucose, the contents of these two compounds in the original apple juice were estimated to be 1.55 ± 0.05 and 12.36 ± 0.62 mg/ml, respectively. The separation obtained here was much more efficient than that reported by HPLC [14] where the same components were observed for apple juice but the resulting peaks were not able to be resolved completely.

It is well known that glucose oxidase (GOx) catalyzes the oxidation of β-D-glucose to form gluconic acid and hydrogen peroxide. Because both glucose and gluconic acid peaks can be monitored very effectively by the Cu electrode CE–ED approach, the enzymatic process can thus be followed conveniently. Fig. 5 shows three different stages of such a GOx oxidation. In Fig. 5a, the enzyme had not yet been added to the system which consisted of 200 ml of pH 5.3 phosphate buffer containing 0.5 m*M* glucitol (used as an internal standard) and 1.0 m*M* glucose. Therefore, as expected, both glucitol (peak 1, 6.6 min) and glucose (peak 2, 8.2 min) were readily detected; and no other peaks were seen. Fig. 5b shows the electropherogram obtained for a sample after GOx had been added to the system for 5 min. As expected, the glucitol peak remained unchanged, while that for glucose decreased. In the meantime, two new peaks (peaks 3 and 4), resulting from the formation of the gluconic acid and hydrogen peroxide products of the glucose oxidation, appeared at 10.9 and 24.0 min, respectively. After 25 min, the resulting electropherogram (Fig. 5c) shows the glucitol peak to remain unchanged, that for glucose to become even smaller, and those for gluconic acid and hydrogen peroxide to be larger. After about 90 min, when the glucose peak had vanished completely, the enzymatic oxidation was obviously complete. Such simultaneous peak-height monitoring of glucose as well

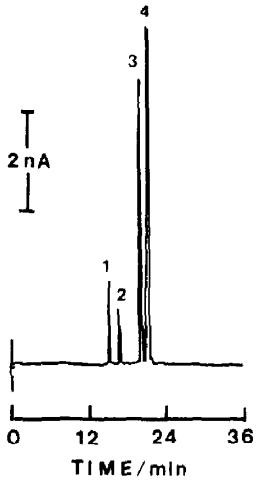

Fig. 4. Electropherogram of apple juice. Peaks: 1 = glucitol; 2 = sucrose; 3 = glucose; 4 = fructose. CE–ED conditions as in Fig. 2.

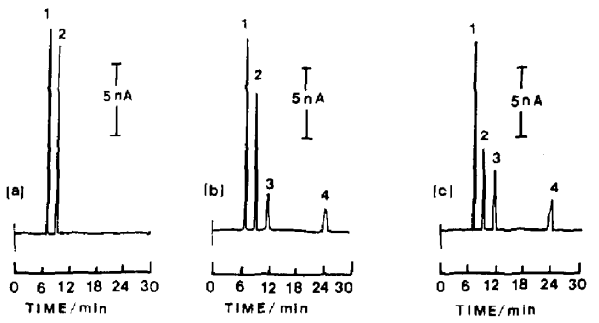

Fig. 5. Electropherograms obtained during different stages of enzymatic oxidation of D-glucose. Peaks: 1 = glucitol; 2 = glucose; 3 = gluconic acid; 4 = hydrogen peroxide. Electropherograms a, b and c correspond to samples taken 0, 5 and 25 min after adding glucose oxidase, respectively. CE–ED conditions as in Fig. 1.

as its enzymatic oxidation products could provide useful information in studying the kinetics of GOx-catalyzed reactions and, more importantly, in process monitoring and control applications. Extension of the approach to other enzyme systems involving carbohydrate metabolism should be straightforward.

Acknowledgement

This work was supported by the National Science Foundation through grant EHR-9108764 of the Kentucky Advanced EPSCoR Program.

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