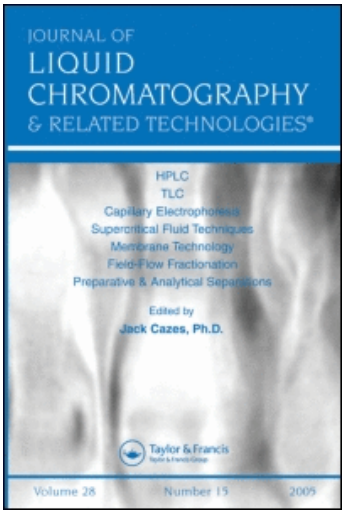

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ANALYSIS OF CARBOHYDRATES BY CAPILLARY ZONE ELECTROPHORESIS WITH ON-CAPILLARY DERIVATIZATION

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ANALYSIS OF CARBOHYDRATES BY CAPILLARY ZONE ELECTROPHORESIS WITH ON-CAPILLARY DERIVATIZATION

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ABSTRACT

Carbohydrates were on-capillary derivatized by *p*-hydrazine-benzenesulfonic acid (BHZ) and separated by capillary zone electrophoresis using 100 mM boric acid (pH 10.2) as running buffer. The derivatization was achieved by keeping the capillary tip introduced with sample in between two zones of labeling reagent at room temperature for 30 min. The detection limit of galactose and glucose were 15.6 and 31.2 μm, respectively, a bit higher than the corresponding values from pre-capillary derivatization (10.5 and 3.6 μM, respectively). Nevertheless the sample consumption was decreased by two to three orders of magnitude compared to the pre-capillary mode. Reproducible quantification of carbohydrates was demonstrated in the concentration range of 0.3–30 mM. The application of this method to determine the glucose in human serum was also present.

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INTRODUCTION

Pre-capillary derivatization is the most commonly used means to improve the detectivity of carbohydrates. However, this technique consumes several microliters of sample for labeling by manual operation, which causes problems in dealing with some biological samples. On-capillary derivatization should be an alternative way to alleviate the problems. In this case, the sample consumption should be less than capillary volume, even down to nanoliter level (1). It can, thus, be verified as a powerful technique for the analysis of samples with very tiny amounts, such as a single cell (2) and even single biological vesicles of attoliter volume (3,4). Dopamine and several amino acids in a single mammalian cell have been determined through the at-inlet labeling technique using naphthalene-2, 3-dicarboxyaldehyde, and CN^- as labeling reagents (2). With the same approach, components in single secretory vesicles obtained from the atrial gland have been assayed by introducing the vesicles into the taper inlet of a separation capillary and on-capillary derivatized (3).

Therefore, various species (amino acids (5–11), peptides (3,11), amines (11–14) proteins (15,16), and a few neurotransmitters (2)) have been analyzed by this method. Recently, oligosaccharides were also determined using 1-phenyl-3-methyl-5-pyrazolone (PMP) as a on-capillary derivative reagent (17), showing that this model is promising and worthy of further development. The key to carry out an on-capillary derivatization is to find out the quick labeling reagent, also, the labeling conditions should be mild and should not disturb the separations; and the excess reagent and/or catalyst should not interrupt the detection and resolution. Similarly, the running buffer used should not affect the labeling reaction. Such reagents are not easy to explore for carbohydrates because the reactions involved are usually slow, and require multisteps and high temperature. However, besides PMP, *p*-Hydrazinebenzenesulfonic acid (BHZ) seems to be explorable. Our previous studies have verified that BHZ can react with carbohydrates in acidified water at room temperature, or higher, within a short period of time (70°C, 10 minutes) (18). It was then tried and positive results were obtained. In this paper, the performance will be discussed.

EXPERIMENTAL

Capillary Electrophoresis (CE)

CE was performed on a P/ACE 5000 system (Beckman, Fullerton, USA). The system was equipped with a PACE Station and controlled by an IBM compatible PC. A capillary tube of fused silica (50 μm id × 60/67 cm, effective/total length, Yongnian Optical Fiber Factory, Hebei China) was used.

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The detection window was made by removing polyimide coating at the position of 7 cm from cathodic end. Prior to each injection, the capillary was sequentially flushed with 0.1 M NaOH, 1 M HNO_3, water, and running buffer for 2, 2, 2, 3 minutes, respectively. The capillary tube was filled with 0.1 M NaOH for overnight storage. The separation buffer was composed of 100 mM boric acid, adjusted to pH 10.4 by addition of KOH pellets. Sample and labeling reagents were introduced by pressure at 0.5 psi. The separation voltage applied was fixed at 23.1 kV. Detection was achieved by UV absorption at 200 nm.

Reagent

p-Hydrazinebenzenesulfonic acid was synthesised in our laboratory (18). *p*-Aminobezenesulfonic acid was diazotized by HNO_2. The diazoate was then reduced by $NaHSO_3$ to produce *p*-hydrazinebenzenesulfonic. The crude product was purified by recrystallization in hot water until its electropherogram showed a single peak. The samples of monosaccharide were purchased from Sigma Chemical Co. All water used was doubly distilled.

The standard sample solution was prepared by mixing an equal volume of 2 M NH_4 Ac buffer (adjust pH by conc. HCl to 4.3) with sugar solution containing 2.0 mM indolylacetic acid (as internal standard). The labeling reagent solutions were made by dissolved BHZ in equivalent solution of sodium carbonate. Both the sample and reagent solution were daily prepared.

On-Capillary Derivatization

Sample carbohydrates were introduced into the capillary tip in between two zones of labeling reagents. To avoid the labeling reagents from diffusing out of the capillary tip, a plug of running buffer was also introduced as the final zone. All zones were introduced with a time program listed in Table 1.

Pre-capillary Derivatization (as Reference)

A 5 μL sample solution was mixed with 2x μL (the value of x was the same as that in Table 1) BHZ solution, in a sealed ampoule. The mixture was allowed to stand for 20 min at room temperature. It was then introduced into the capillary for $(2x + 5)$ s and analyzed immediately by CE.

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Table 1. The Time Program of the On-Capillary Derivatization Process[a]

Step	Function	Duration	Inlet Vial[b]	Outlet Vial
1	Injection	x s	17	1
2	Injection	5 s	18	1
3	Injection	x s	17	1
4	Injection	5 s	15	1
5	Wait	20 min[c]	15	1
6	Separation	15 min	15	1
7	End		15	1

[a] Injection press was 0.5 psi and separation voltage was 23.1 kV.
[b] Vial 1 contains running buffer, 15 running buffer, 17 reagent solution, and 18 sample solution, respectively.
[c] Reaction time required. In other cases, it may vary in between 0 to 60 min.

Determination of Glucose in Human Serum

Human serum was deproteinized according to the reference (19): an aliquot of 10 μL serum was mixed with 15 μL acetonitrile for 15 s and centrifuged at 15,000 g for 1 min. The supernatant was further filtered through a Millipore filter (0.2 μm). The filtrate was diluted with an equal volume of 2 M NH_4Ac–HCl (pH 4.3) buffer containing 2 mM indolylacetic acid. Its on-capillary derivatization was performed according to the aforementioned procedure (Table 1).

RESULTS AND DISCUSSION

Sample-Reagent Mixing

The top factor is how to completely mix the sample with the labeling reagent. A. Taga et al. have discussed in detail the sample/reagent mixing state in a narrow capillary (50 μm id) (15). In their experiments, two solutions of cinnamyl alcohol (C_{A1}) at different concentrations were selected to represent sample and reagent, respectively. The mixing states in the capillary at different standing time or injection periods were, thus, evaluated from the peak shape. This is an effective way to investigate the hydrodynamics in a narrow capillary, but it neglects the influence of some physical property (such as viscosity of the reactant solutions) on the mixing, which leads to deviation of the model system from real samples.

We, thus, designed another way to overcome the problem. The key is to determine the mixing rate by comparing the peak area of on- and pre-capillary

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derivatizations, considering the reactants mixed completely in the pre-capillary labeling mode. Although the zones of labeling reagent and sample will overlap for a while after the application of voltage, the duration will be very short compared to the reaction time required and the resulted overlapping reaction is, thus, negligible. A sandwich mode of reagent -sample -reagent introduction program was developed to gain a higher efficiency of derivatization (5). Running buffer was introduced following the final zone of labeling reagent in order to prevent the reagent from diffusing out of the capillary. The effect of injection time on the mixing was examined by using fucose, glucose, galactose, and arabinose as probes. Figure 1 shows the relationship between injection time and the mixing ratio. When the injection times were kept at 15, 5, and 15 (s) for reagent, sample, and reagent, respectively, the derivatization reaches almost the same level as that of pre-capillary labeling. This implies that the mixing reaches the maximum level.

BHZ Concentration

Figure 2 illustrates the dependence of the relative sugar peak area on BHZ concentration. The curves increase sharply as BHZ increases but level off at the

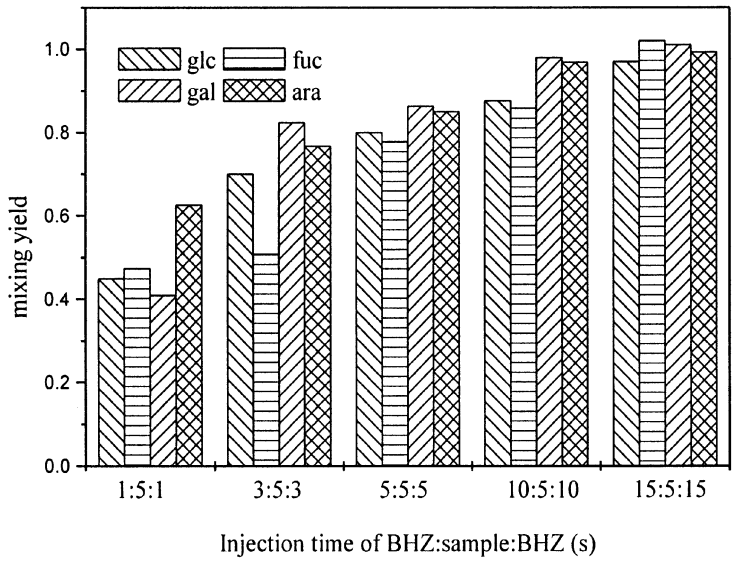

Figure 1. Effect of injection time of reagent and sample on the mixing state of on-capillary derivatization. Conditions: BHZ concentration was fixed at 0.2 M; waiting time was 20 minutes.

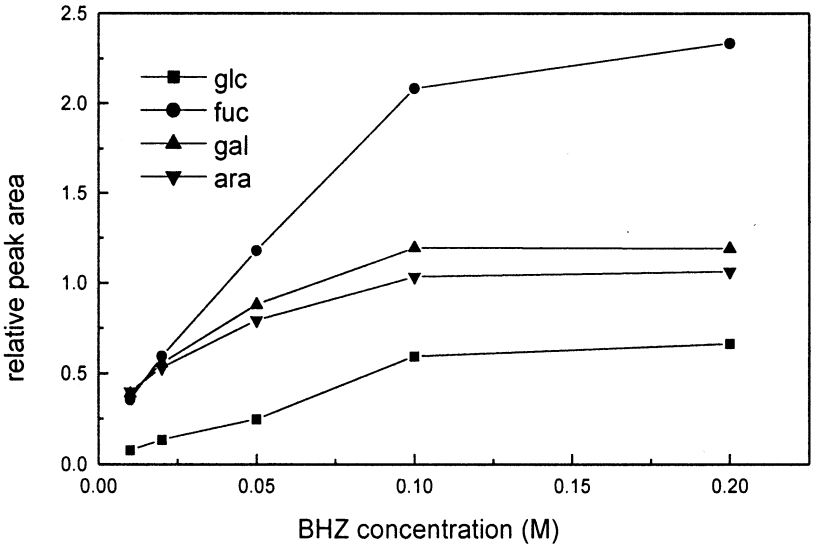

Figure 2. Effect of BHZ concentration on the derivatization. Conditions: the injection time of reagent, sample, running buffer were 10 s, 5 s, 10 s, 5 s, respectively; waiting time fixed at 20 min.

concentration of 0.1 M, except for fucose that still increases with BHZ. This turning point was thought to be the optimum concentration because too much of BHZ interferes with the separation.

Reaction Time

Figure 3 shows the effect of reaction time. It was observed that the relative peak area of monosaccharides reached a turning point after 30 minutes of reactions. The relative peak area continued to increase even after 60 minutes of incubation, but 30 minutes was arbitrarily adopted for economy of time.

pH of the Sample Solution

According to previous experiments (18), the labeling needs acidic condition and the optimum range is in between pH 3–4 for pre-capillary reaction. However, in the on-capillary labeling process, BHZ would precipitate as its acid form and block up the capillary if the pH of sample solution was lower than 4.0. So, the

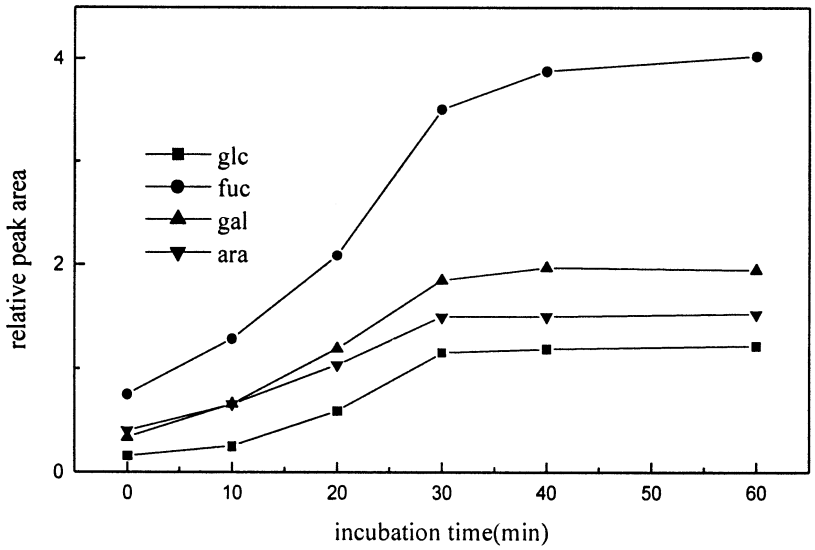

Figure 3. Effect of waiting time on the derivatization. The BHZ concentration was 0.1 M. The other conditions as in Figure 2.

selected pH was slightly apart from the optimum value, namely, at pH 4.3. Because sugars are normally separated in alkaline medium such as borate at pH > 9, higher concentration of the labeling buffer (2 M NH_4AC–HCl) was needed to maintain the pH of the reaction zone. This acidic sample zone slightly disturbed the separation. The electrophoretic current will reach a steady state within 0.5 minutes.

Reaction Temperature

Temperature is another key factor. However, at present the commercial available CE instruments do not allow to heat the tip of the capillary with ease. Additional equipment is required to carry out the incubation at the capillary end. Taga *et al.* overcame the problem by introducing the plugs of sample and reagent solution into the tube at the entrance of the heat portion (17). In order to simplify the procedure, we conducted the derivatization at ambient. BHZ can react with most of the sugars quickly, even at room temperature, according to the previous investigation (18). For example, the yield of fucose sulfophenylhydrazones at 25°C reached about 80% as that at 70°C. For sugars needing higher temperature of derivatization, their reaction yield can also be increased by prolonging the

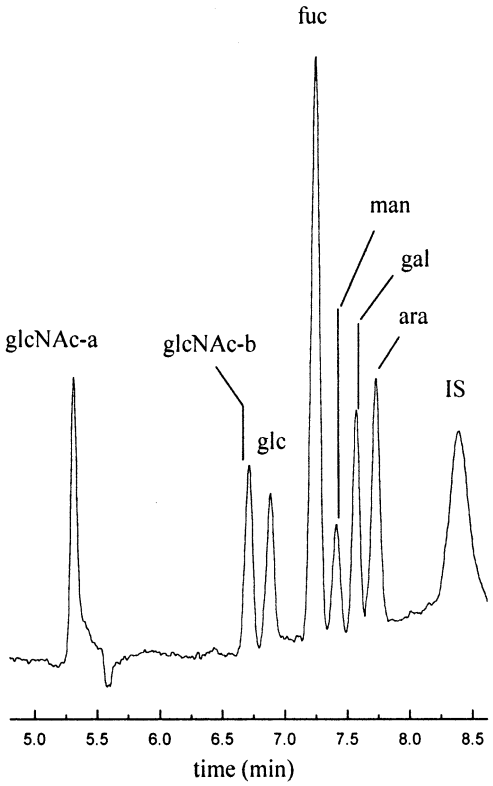

Figure 4. Electropherogram of BHZ derivatized sugars by capillary electrophoresis with on-capillary derivatization. Conditions: the injection time of reagent, sample, and reagent, running buffer were 10 s, 5 s, 10 s, and 5 s, respectively; BHZ concentration, 0.1 M; waiting time, 30 min.

incubating time or increasing the sample amount. Figure 4 depicts the electropherogram of on-capillary labeled monosaccharides together with indolylacetic acid as an internal standard.

Quantitative Features

The new method was shown to be applicable to quantitative analysis. Linear calibration curves of relative peak area vs. sugar concentration have been

measured, in the range of 0.3–30 mM ($R^2 > 0.9997$) of sugars:

$$Y = 0.0895 + 0.10397\ X \text{ (glucose)};$$
$$Y = 0.11043 + 0.02519\ X \text{ (arabinose)};$$
$$Y = 0.0826 + 0.20753\ X \text{ (galactose)};$$
$$Y = 0.1136 + 0.31649\ X \text{ (fucose)};$$

where X is sugar concentration (mM) and Y is the relative peak area of sugar (over 1 mM indolylacetic acid). The linear range is wider than that of pre-capillary labeling (0.1–9.1 mM), but the upper and lower limits of the linear curves increased, respectively. The detection limit was 15.6 μM for galactose, which is close to the value from pre-capillary derivatization (10.3 μM) and 31.2 μM for glucose, which was almost one order of magnitude higher than that of pre-capillary (3.6 μM). The average analytical recovery of glucose spiked in serum samples (50 μL of 10 mM glucose mixed with the same volume serum sample) was 98.1% (n = 5). The relative standard derivation was below 3.4% measured with 5.0 mM sugars.

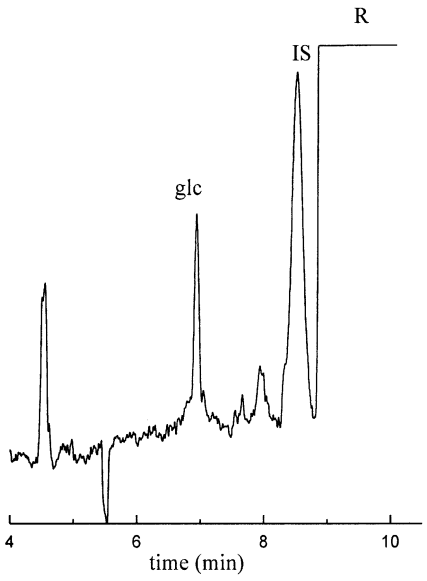

Figure 5. Electropherogram of on-capillary derivatized human serum. The derivatization and analysis conditions were as in Figure 4.

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Table 2. Comparing the Present Method with Automated Analysis of Serum Glucose

Serum Sample	On-Capillary Derivazation (mM)	Automated Analysis (mM)
1	8.41	8.41
2	8.75	9.15
3	9.68	9.51
4	8.93	9.27
5	4.10	4.61
6	5.63	5.52
7	5.15	5.29
8	5.64	5.87

Glucose in Human Serum

The new method has been applied to the determination of glucose in human blood, which is an important task in the clinical laboratory to diagnosis diabetes or hypoglycemia. Figure 5 shows one of the electropherograms from serum samples. The glucose peak was completely separated from other serum components. The glucose peak was identified by spiking the serum with standard glucose. In order to gain precise results, the standard glucose solutions were treated by the same procedure for serum sample. The results were comparable to those obtained from the auto analysis system (PBA-30, Panasonic) based on glucose oxidase reaction, which was the routine assay in clinical (Table 2).

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