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Optimization for the separation of ribonucleotides by capillary electrophoresis at high pH

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Abstract

A versatile method for the rapid capillary electrophoresis (CE) separation of ribonucleotides using a carbonate buffer was optimized. The separation of twelve 5′-ribonucleotides was obtained in 25 min using a voltage of 18 kV and a 30 m*M* sodium carbonate/bicarbonate buffer at a pH of 10. For the separation of the monophosphates or the diphosphates, a lower buffer concentration and pH could be used. However, for the triphosphates a 50 m*M* buffer at pH 11 gave the best peak sharpness and resolution. Therefore, optimal conditions for the separation of the twelve ribonucleotides were a compromise between those best for the mono- and diphosphates and those needed for the triphosphate nucleotides. The sodium carbonate/bicarbonate buffer was inexpensive and easy to prepare. In addition, the high alkalinity of the buffer resulted in a shorter analysis time and the capillary equilibration time with NaOH was minimal compared to an acidic buffer. Since bare capillaries were used, the time and expense of preparing and maintaining coated capillaries was eliminated. © 1997 Elsevier Science B.V.

Keywords: Buffer composition; pH effects; Ribonucleotides; Nucleotides

1. Introduction

The quantitative determination of nucleotides is vital in many areas of biochemical research. Nucleotides control syntheses in DNA and RNA molecules and play a key role in cell metabolism in both plants and animals [1–12]. Nucleotide analogs have been effective in treating AIDS and cancer [1–7]. Other adducts cause mutations in cells and are of interest in cancer research [1–4]. Nucleotide levels in cell pools may help reveal the mechanism of carcinogenesis and lead to more effective treatments in many different types of cancer. Therefore, the ability to quantify nucleotides quickly, inexpensively and accurately is very important.

High-performance liquid chromatography (HPLC) has been routinely used to quantify nucleotides [2–8]. A variety of techniques have been developed using both ion-pairing and ion-exchange modes. HPLC has many advantages for nucleotide analysis. It is reproducible, sensitive, selective and is easily automated. However, analyses may take an hour to perform and the equipment is relatively expensive to run and maintain. Resolution is frequently a problem and a relatively large amount of sample, often up to 100 μl may be required to attain the necessary sensitivity [2–4].

The use of capillary electrophoresis (CE) as an

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alternative to HPLC for the analysis of nucleotides has been investigated [1–16]. CE methods are fast, inexpensive and use minimal sample. Methods have been developed for both capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) modes [1–16]. These methods are very good at detecting nucleotides at a level above 10 m*M*. However, below 1 m*M*, some type of preconcentration is necessary as the sensitivity of CE methods with UV detection is limited by the pathlength of the detection cell. In an effort to increase the sensitivity of these methods, stacking methods, especially whole capillary stacking, are of practical interest [1,4,10,17–20]. As the negatively charged nucleotides flow counter to the electroosmotic flow (EOF), the use of high pH buffers is preferred because faster EOF results in rapid analyses. The equilibration time after conditioning with base is also shorter with basic buffers than with acidic or neutral buffers [19]. As a result, the total analysis time is minimized when high pH buffers are used.

Both borate and carbonate buffers in the pH range of 8.8–9.8 have been investigated for their use in CE procedures for a variety of nucleotide groups [1,4–6,10,12,16]. While borate buffers have been used successfully to separate different nucleotide groups [5,6,16], additives such as cyclodextrins, are often required to obtain adequate resolution [5]. Although the current generated by carbonate buffers is higher than that of borate buffers, better resolution can be obtained without additives [10]. In addition, carbonate buffers are inexpensive, easy to prepare and provide a stable, reproducible EOF. When ammonium carbonate is used, the buffer is volatile and it can be readily used with mass spectrometry (MS) detection [1]. Carbonate buffer has been primarily studied at pH 9.6, the pK_a of the buffer [1,4,10]. However, as the pK_a values of guanosine and uridine nucleosides are 9.4 and 9.7, respectively, the buffer pH had an effect on the selectivity of these compounds [20]. In addition the pK_a values of guanosine and uridine nucleotides are dependent on ionic strength of the buffer [20]. Thus, the effects of pH and buffer concentration on the separation of the mono-, di- and triphosphate ribonucleotides were investigated to determine the optimal conditions for the analyses of these compounds using carbonate buffers.

2. Experimental

2.1. Chemicals

Sodium carbonate, sodium bicarbonate and all nucleotide standards were purchased from Sigma (St. Louis, MO, USA). All buffers and samples were prepared with distilled water purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Materials

The capillary used was cut from bulk stock of polyimide coated 0.75 μm I.D. electrophoresis capillary purchased from Polymicro Technologies (Phoenix, AZ, USA). All buffer solutions were filtered with a 45 μm syringe filter (Fisher Scientific, Eden Prairie, MN, USA).

2.3. Instrumentation

Analyses were performed using a Waters Quanta 4000 CE unit with UV detection at 254 nm (Milford, MA, USA) and a 70 cm capillary with the detection window burned in 10 cm from the detector end. Data were collected on a Spectra-Physics Integrator Model SP4270 (San Jose, CA, USA).

2.4. Buffer preparation

The various buffers were prepared in the following manner. A solution of 100 m*M* sodium carbonate and a solution of sodium bicarbonate were prepared. The pH of the sodium carbonate solution was approximately 11.4 and that of the sodium bicarbonate solution was approximately 8.5. Dilutions of each of these buffers were prepared to give concentrations of 10 m*M*, 20 m*M*, 30 m*M*, 40 m*M* and 50 m*M*. The pH levels used in the study were 9, 9.5, 10, 10.5 and 11 at each of the previously described concentrations. The desired pH at each concentration was obtained by adding sodium carbonate solution to sodium bicarbonate solution while the solution was monitored with a pH meter. Thus the concentration of the carbonate in the solution was constant even at varying pH values. The solutions were filtered with a 45 μm syringe filter and degassed using sonication before use.

2.5. Standard nucleotide preparations

Approximately 5 mg of each of the twelve 5′-ribonucleotides were weighed out into separate glass vials and diluted with 500 μl Milli-Q grade water. The resulting concentration of each nucleotide was approximately 20 m*M*. The nucleotides were then combined into groups of monophosphates, diphosphates or triphosphates and diluted until the concentration of each nucleotide in the solution was between 1 m*M* and 4 m*M*. A mixture of adenosine nucleotides was prepared by combining 25 μl of each nucleotide with distilled water until diluted by a factor of 10. A mixture of all twelve ribonucleotides was prepared by combining 25 μl of each nucleotide. A second mixture of each of the nucleotide pools was prepared by varying the quantity of each nucleotide. The differences between the peak areas at the same migration time on the two electropherograms was used to determine peak identity. These stock nucleotide solutions were kept frozen below 0°C when not in use. A 1:10 dilution of each nucleotide pool was prepared fresh each day from the frozen stock solutions and discarded after it was run.

2.6. Capillary preparation

The capillary was cleaned at the beginning of each day by rinsing the capillary according to the following method. The capillary was first rinsed for 5 min with 1 *M* NaOH, followed by 5 min with Milli-Q water, it was then rinsed for 5 min with the buffer to be used in the analysis. After the capillary was filled with the buffer, the CE was run at a voltage of 18 kV for 30 min to condition the capillary and to warm up the UV lamp.

2.7. CE method

All analyses were run with a voltage of 18 kV and positive polarity. Acetone was used to determine the EOF before each set of analyses were run. The acetone peak coincided with the migration time of a peak from the water in the sample matrix. This water peak was used as a marker for determining the EOF for the remainder of the runs. The nucleotide samples were run consecutively and automatically with a 10 s hydrostatic injection. The combination of adenosine mono-, di- and triphosphates was run six times to determine the reproducibility of the method. After each set of analyses, the capillary was cleaned and conditioned and fresh nucleotide samples were loaded before using a new buffer. The data were collected on an integrator. Concentration and pH ranges were run randomly to avoid any bias that might result from possible column degradation or even conditioning to a certain pH or concentration over time. All data were collected over the course of approximately eight weeks and the same capillary was used for all runs.

3. Results and discussion

3.1. General results

The voltage of 18 kV was selected based on previously optimized separations using carbonate buffer [4]. The buffer range investigated was 10 m*M* to 50 m*M* which is the range most commonly used in CE analyses [15]. As the current increased with increasing voltage, buffer concentrations above 50 m*M* were hampered by outgassing of the buffer caused by Joule heating. The Joule heating resulted in either a loss of current or a decrease in reproducibility. Below a buffer concentration of 10 m*M* the sample capacity was low and peak separation was inadequate. The relative standard deviation (R.S.D.) was less than 1% for the EOF migration times and the migration time of the EOF increased with increasing concentration at all the pH values studied. The EOF migration times with respect to buffer concentration was linear at all pH levels studied. Sodium carbonate/bicarbonate buffer has a pK_a of 9.6. The pH range studied was between 9 and 11 as these values are within approximately ± 1 pK_a unit of the pK_a of the buffer, thus ensuring consistent buffer capacity. The current increased as the buffer became more alkaline, but there was no significant change in the EOF due to pH.

3.2. Separations of the 5′-ribonucleoside monophosphates

The optimal separation for the monophosphates was obtained in 13 min using a buffer concentration of 20 m*M* and a pH of 10 (Fig. 1). Above a pH of 9.5, there is clear separation of all four ribonu-

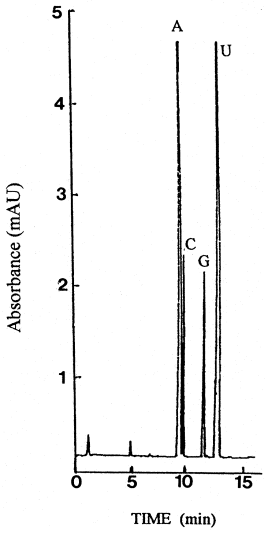

Fig. 1. CE separation of 5′-ribonucleoside monophosphates (~0.01 m*M*). Conditions: 10 s hydrodynamic injection (10 cm); capillary: 70 cm×75 μm I.D. column, 60 cm separation distance; buffer: 20 m*M* sodium carbonate/bicarbonate, pH 9.5; voltage: +18 kV; detection: 254 nm. Peak identification: A=AMP, C=CMP, G=GMP, U=UMP.

cleoside monophosphates with a migration order of AMP, CMP, GMP and UMP. Although the most reproducible separation was obtained at pH 10, the analysis was 25% faster at pH of 9.5 (Fig. 2). At or below a pH of 9.0, the separation of the ribonucleotides was not complete.

At all buffer concentrations above 10 m*M* the ribonucleoside monophosphates were separated as long as the pH was greater than 9.5. Concentrations above 40 m*M* resulted in slightly better resolution between AMP and CMP, but the total analysis time was 22 min, almost twice as long as the separation performed with a 20 m*M* buffer.

3.3. Separations of the 5′-ribonucleoside diphosphates

Baseline separation of all four ribonucleoside diphosphates was achieved in 16 min at pH 10 with a buffer concentration of 20 m*M* (Fig. 3). With buffer concentrations at 30 m*M* and higher there was an increase in migration time when compared to a separation at 20 m*M*; however, increasing the buffer concentration did not affect the migration order or resolution of the diphosphates. Above a pH of 10, there was baseline separation of the four nucleotides with the migration order of ADP, CDP, GDP and UDP. Below pH 10, GDP and ADP comigrated at all buffer concentrations.

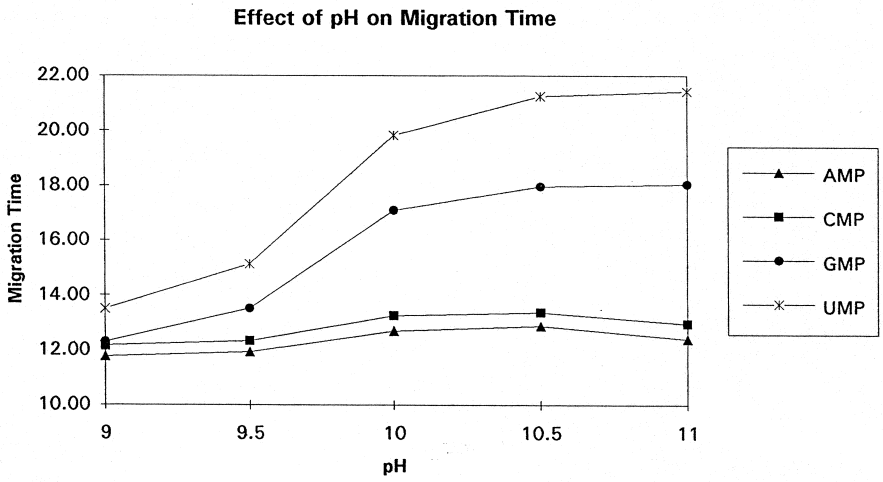

Fig. 2. Effect of pH on migration time for 5′-ribonucleoside monophosphates at a carbonate concentration of 40 m*M*. Conditions: 10 s hydrodynamic injection (10 cm); capillary: 70 cm×75 μm I.D. column, 60 cm separation distance; buffer: 20 m*M* sodium carbonate/bicarbonate, pH 9.5; voltage: +18 kV; detection: 254 nm.

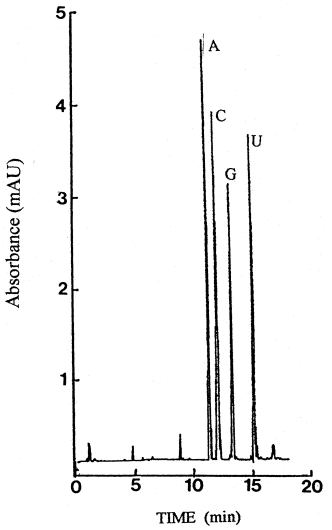

Fig. 3. CE separation of 5′-ribonucleoside diphosphates (~0.01 m*M*). Conditions: 10 s hydrodynamic injection (10 cm); capillary: 70 cm×75 μm I.D. column, 60 cm separation distance; buffer: 20 m*M* sodium carbonate/bicarbonate, pH 10; voltage: +18 kV; detection: 254 nm. Peak identification: A=ADP, C=CDP, G=GDP, U=UDP.

3.4. Separations of the 5′-ribonucleoside triphosphates

The best buffer for separating the triphosphate nucleotides was a buffer concentration of 50 m*M* and a pH of 11 (Fig. 4a). However, adequate separation and peak shape of the nucleotides could be obtained at a buffer concentration of 30 m*M* and a pH of 10. At low buffer concentrations and all pH levels, poor peak shape of all four ribonucleotide triphosphates was observed. An increase in the buffer concentration to 30 m*M* resulted in better peak shape, but the resolution was even higher when a buffer concentration of 50 m*M* was used.

The pH of the buffer was an even more important factor then buffer concentration in triphosphate separations. Below pH 10, overlap of all four triphosphates was observed because the peaks were very broad (Fig. 4b). When the pH of the buffer was increased from 10 to 11, the peaks became sharper and more Gaussian. There was an significant increase in resolution but no significant increase in migration time was observed when the pH was

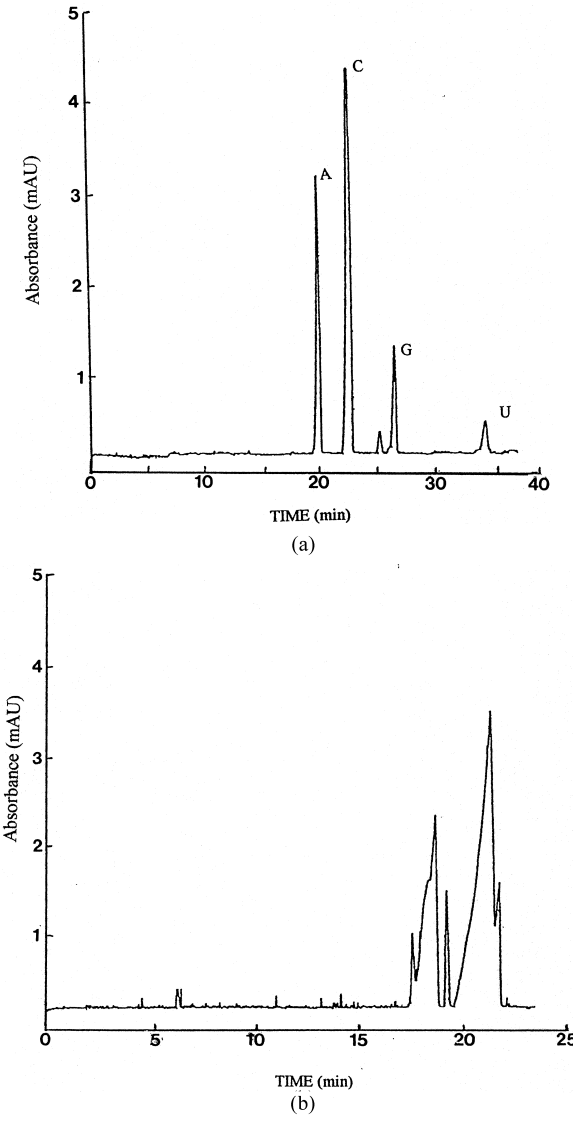

Fig. 4. CE separation of 5′-ribonucleoside triphosphates (~0.01 m*M*). Conditions: 10 s hydrodynamic injection (10 cm); capillary: 70 cm×75 μm I.D. column, 60 cm separation distance; buffer: 50 m*M* sodium carbonate/bicarbonate, pH 10; voltage: +18 kV; detection: 254 nm. (a) Separation at pH 11. A=ATP, C=CTP, G=GTP and U=UTP. The peaks were identified by varying the analyte concentrations. The unidentified peak in (a) is GDP that was present as an impurity in the GTP standard. (b) Separation at pH 9. Peaks could not be positively identified because of the poor resolution and peak shape at pH 9.

increased from 10 to 11; thus the more alkaline buffer is preferred when analyzing nucleoside triphosphates.

3.5. Separation of twelve 5′-ribonucleotides

The best separation of the twelve nucleotides was achieved in approximately 30 min with a 30 m*M* sodium carbonate buffer at a pH of 10 (Fig. 5). The order of migration was AMP, CMP, ADP, GMP, CDP, ATP, UMP, CTP, GDP, GTP, UDP and finally UTP. All the monophosphate and diphosphate nucleotides readily separated at buffer concentrations between 10 m*M* and 50 m*M*. With the triphosphates, buffers with molarities of at least 30 m*M* and a pH of 10 were needed to achieve good peak shape.

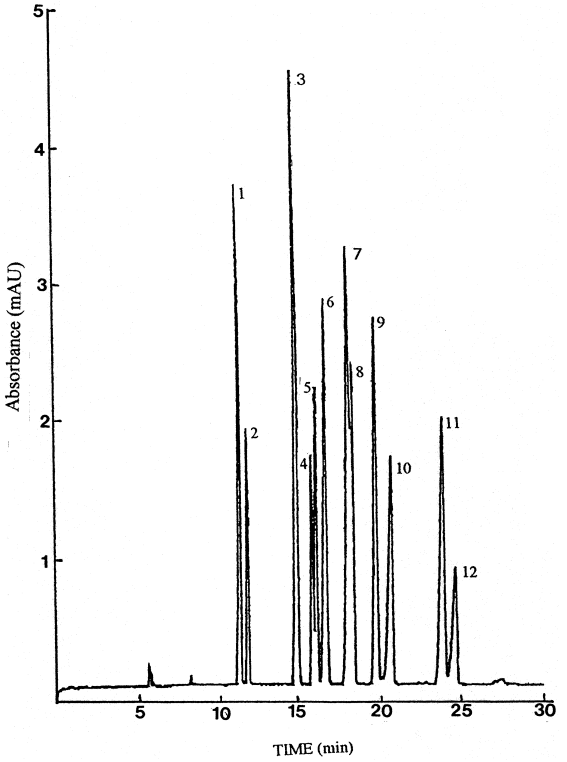

Fig. 5. CE separation of twelve 5′-ribonucleotides (~0.01 m*M*). Conditions: 10 s hydrodynamic injection (10 cm); capillary: 70 cm×75 μm I.D. column, 60 cm separation distance; buffer: 30 m*M* sodium carbonate/bicarbonate, pH 10; voltage: +18 kV; detection: 254 nm. Peak identification: 1=AMP, 2=CMP, 3=ADP, 4=GMP, 5=CDP, 6=ATP, 7=UMP, 8=CTP, 9=GDP, 10=GTP, 11=UDP and 12=UTP.

Although a pH of 11 and a buffer concentration of 50 m*M* provided the optimal peak sharpness and separation of the triphosphates, a compromise in both pH and concentration was necessary to obtain separation of the mono-, di- and triphosphate nucleotides in the same sample. When a buffer concentration of 50 m*M* was used the GDP and GTP comigrated and UDP and UTP were also unresolved. With a buffer concentration of 30 m*M*, baseline separation of the guanosine and uridine di- and triphosphate nucleotides could be achieved and the overlap of the CDP and the ATP that occurred with a buffer concentration of 50 m*M* was minimized.

3.6. Determination of efficiency (N) and reproducibility

The adenosine nucleotides were used as a model to calculate efficiency and reproducibility of nucleotide separations when a 30 m*M* sodium carbonate buffer at a pH of 10 was used. The number of theoretical plates (*N*) determined for AMP, ADP and ATP were approximately $2 \cdot 10^5$.

Migration time reproducibility (R.S.D.) for the adenosine nucleotides at all concentrations and pH levels was under 1.0%. The reproducibilities of the peak areas of the AMP and ADP were approximately 2%. For the ATP the area reproducibility was 4% at the standard conditions of 30 m*M* and a pH of 10. However, at a pH of 11, the area reproducibility for all three nucleotides improved with a R.S.D. of 0.7% for AMP, 0.8% for ADP and 1.6% for ATP. Day-to-day reproducibility for all the adenosine nucleotides was 5% for both migration time and area for a total of twelve runs taken on two consecutive days.

4. Conclusions

Sodium carbonate was found to be an effective buffer for separating 5′-ribonucleotides at high pH. It provided a reproducible current and EOF and was resistant to changes in pH. Because the pH range studied was high, between 9 and 11, the equilibration time needed after rinsing the capillary with sodium hydroxide was only a few min. There was no sign of capillary degradation, such as loss of resolution or reproducibility, during the eight weeks of the study.

The use of high pH buffers in nucleotide analyses has previously been avoided because nucleotides tend to hydrolyze at high pH [2,3,7]. However, there was no sign of degradation of any of the nucleotides in the electropherograms. Thus, the high pH of the buffer did not degrade the nucleotides in the 35 min required for the analysis.

Since buffer concentration and pH cause differences in both selectivity and resolution, both parameters were optimized. The primary effects of the buffer concentration were retention of the analytes and sharpening of the peaks of the nucleoside triphosphates. The mono- and diphosphates were easily separated with sodium carbonate concentrations of 20 m*M*, but a buffer concentration of at least 30 m*M* was needed to obtain good peak shape of the triphosphates. The migration times also increased with increasing buffer concentration as a result of decreased EOF in the capillary.

Separation of all nucleotide groups was more reproducible when the pH level was between 10 and 11. Between pH 9.5 and 10, the day-to-day reproducibility of the guanosine and uridine nucleotides was poor and between pH 9 and 9.5 the migration order of these nucleotides was variable, since the pK_a values for uridine and guanosine are between pH 9.2 and 9.7.

The mono-, di- and triphosphates of a single nucleoside such as adenosine nucleotides were easily separated. The differences in migration were due only to the number of phosphate groups. However, separations of the mono-, di- and triphosphate nucleotides involving mixtures of nucleotides of different bases were more complex. The mechanisms by which the ribonucleotides are separated in CE analyses with buffers at high pH are currently being investigated.

Nucleotide separations at high pH are as reproducible, sensitive and selective as those obtained at low pH [2,3,5,6,9,10,12,16,18]. There are many advantages to using sodium carbonate/bicarbonate and other high pH buffers in CE separations of nucleotides. Bare capillaries can be used, thus eliminating the extra time and expense associated with preparing and maintaining coated capillaries. The carbonate buffer did not degrade the capillary, and the buffer salts are non toxic, readily available and inexpensive. Adequate resolution was obtained without the need for additives. Since the difference in pH between the wash solution of NaOH and the buffer is small, the capillary equilibration time was minimal. Finally, with the recent interest in whole capillary stacking techniques to increase the sensitivity of CE analyses, alkaline buffers are preferred because the EOF is faster in basic solutions than in acidic buffers and the total analysis time is shorter. Therefore, carbonate buffers are a practical convenient alternative to acidic buffers for CE analyses of nucleotides.

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