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# Analysis of chitin oligosaccharides by capillary electrophoresis with laser-induced fluorescence

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## Abstract

A method, using capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection for analyzing chitin oligosaccharides is described. Chitin oligosaccharides were derivatized with 9-aminopyrene-1,4,6-trisulfonate (APTS) via reductive amination at 37 °C for 16 h (optimized conditions). The APTS–chitin oligosaccharides were analyzed using either an acidic citric acid–phosphate buffer or an alkaline borate buffer. The effects of buffer types, buffer pH values, and buffer concentrations on the separation were examined. The analytes were successfully separated by using a pH 4.6 citric acid–phosphate within 19 min. The APTS-derivatized chitin monosaccharide (D-glucosamine) migrated first. The analytes were also completely separated by using a pH 9.0 borate buffer within 24 min. Moreover, the specificity of enzyme digestion on chitin polysaccharides using the optimized APTS labeling procedure and the CE–LIF method was demonstrated.

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## 1. Introduction

Chitin is the *N*-acetylated product of chitosan and is found in the shells of crabs and shrimps, and as cell wall components of most fungi, molds and yeasts [1,2]. These compounds have extensive applications, in the biomedical field, in personal care products, biotechnology, and in food products [3]. Oligomers with a degree of polymerization (DP) of about 6 are potentially useful components of medical materials [4]. Chitin–oligosaccharides and related derivatives, which are amino polysaccharides, have distinctive properties including a variety of biological activities and the fact that they are biodegradable.

In order to obtain bioactive oligosaccharides, chemical and biocatalytical procedures are applied to depolymerize the polysaccharides to oligosaccharides. However, an enhanced efficiency and specificity can be obtained by the use of an enzymatic degradation procedure. Consequently, interest in these bioactive compounds has increased considerably in recent years. Thus, the determination of chitin oligomers becomes important in the complete characterization of chitin polysaccharides and other glycoconjugates.

Carbohydrates generally do not contain chromophoric or fluorophoric groups and, as a result, the determination of these compounds is a challenging topic. Several analytical techniques for the separation of different carbohydrates have been reported [5–14]. However, capillary electrophoresis (CE) [15–27] has attracted a considerable amount of attention recently because of its high sensitivity,

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rapid analysis time, and high resolution. CE in conjunction with indirect detection is a universal method and can be used for carbohydrates analysis [18,19]. However, the disadvantages of limited background electrolyte, unsatisfactory sensitivity, and resolution of complex carbohydrates has limited its development in this area. Generally, in order to obtain more sensitive detection and to enlarge the differences between adjoining carbohydrate molecules, a chromophoric or fluorophoric reagent is introduced into carbohydrates by derivatization. The major derivatization mechanisms are based on reductive amination with various labeling reagents such as 4-aminobenzonitrile, 6-aminoquinoline, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CB-QCA) [20–27]. Such derivatives can then be detected by UV and/or fluorescence methods.

Due to the inherently high sensitivity of laser-induced fluorescence (LIF), it is currently in heavy use for the detection of labeled carbohydrates after high-performance CE separation. In addition, LIF is characterized by its good specificity and a large linear dynamic range. A recent development in the fluorescent labeling of carbohydrates was the introduction of 9-aminopyrene-1,4,6-trisulfonate (APTS) for the CE–LIF of mono- and oligosaccharides [28–32]. Due to its extensive aromaticity and the larger number of fused rings, APTS–carbohydrate derivatives were observed in a substantially higher molar absorptivity and quantum efficiency than most of the commonly used fluorophore carbohydrate derivatives. In addition, the presence of negatively charged functional groups seems to enhance the separation of mono- and oligosaccharides.

In this study, a CE–LIF method was developed for analyzing chitin–oligosaccharides (1–6 DP) derivatized with APTS via reductive amination. Results from three different labeling methods are compared. The electrophoretic separation was carried out under positive and negative high voltages. The effects of buffer types, buffer pH values, and buffer concentrations on the quality of the separation are discussed. The optimized capillary zone electrophoresis (CZE)–LIF methods were employed to analyze the specificity of hydrolytic enzymes that act on chitin–polysaccharides.

## 2. Experimental

### 2.1. Apparatus

The instrument used in these studies was a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a laser-induced fluorescence detector using an 8-mW 488-nm argon-ion laser as the excitation source. The fluorescence emission was collected with a bandpass filter of  $520 \pm 20$  nm. A personal computer using the P/ACE MDQ software program was used to control the instrument. Data analyses were performed using the P/ACE MDQ software. A 50- $\mu$ m I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA) was used at a total length of 60.2 cm (50 cm to the detector). The capillary column was assembled in a cartridge format. The temperature of the capillary tube during electrophoresis was maintained at 25 °C by means of an MDQ thermostating system. The electrophoretic separation was performed with an applied voltage of 25 kV at either positive or negative polarity. Samples were pressure injected at 3.5 kPa.

### 2.2. Chemicals

APTS,  $\text{Na}_2\text{HPO}_4$ , boric acid and glacial acetic acid were purchased from Fluka (Buchs, Switzerland). Borax, *N*-acetylglucosamine and sodium cyanoborohydride ( $\text{NaBH}_3\text{CN}$ ) were obtained from Sigma (St. Louis, MO, USA). The chitin oligosaccharides, including di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose, tetra-*N*-acetylchitotetraose, penta-*N*-acetylchitopentaose, and hexa-*N*-acetylchitohexaose, were purchased from Seikagaku (Tokyo, Japan). Citric acid was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Water was purified in a Milli-Q water system (Millipore, Bedford, MA, USA) and filtered through a 0.22- $\mu$ m filter.

### 2.3. Derivatization of chitin oligosaccharides with APTS

All oligosaccharides were dissolved in water and

stored at  $-20^{\circ}\text{C}$  prior to use. The procedure for the reductive amination of chitin oligosaccharides with APTS followed previously described protocol [28–32]. Briefly,  $10\ \mu\text{l}$  of a  $10\text{-mg/ml}$  oligosaccharide solution in a  $500\text{-}\mu\text{l}$  Eppendorf tube was mixed with  $2\ \mu\text{l}$  of  $0.2\ \text{M}$  APTS in  $15\%$  glacial acetic acid and  $10\ \mu\text{l}$  of freshly prepared  $1\ \text{M}$  aqueous sodium cyanoborohydride. Reaction mixtures were incubated at  $37$ ,  $55$ , and  $75^{\circ}\text{C}$  for  $16$ ,  $2$ , or  $1\ \text{h}$ , respectively. The APTS-derivatized oligosaccharide samples were suitably diluted with  $100\ \text{mM}$ ,  $\text{pH}\ 10.2$  borate buffer and stored under  $-20^{\circ}\text{C}$  without any pretreatment procedure prior to the CE separation. The two enzymatically digested chitin oligosaccharides samples were gifts from Professor Y.-K. Lee in our department. The chitin substrate concentration in the enzymatic digestion incubation was  $5\%$  (w/v).

#### 2.4. Capillary electrophoresis procedures

The procedure used to condition a new capillary involved treatment with  $1.0\ \text{M}$  NaOH, water,  $0.1\ \text{M}$  NaOH, and water for  $30\ \text{min}$  sequentially. For the electrophoretic separation using borate buffer at positive polarity, the capillary was washed between runs with  $0.1\ \text{N}$  NaOH and then water ( $3\ \text{min}$  of high pressure rinsing at  $20\ \text{p.s.i.}$ ;  $1\ \text{p.s.i.}=6894.76\ \text{Pa}$ ), followed by reconditioning with the running buffer, typically for  $3\ \text{min}$ . The electrophoretic separation using acidic citric acid–phosphate buffer was under negative high voltage. The capillary was washed between runs only with water for  $3\ \text{min}$  in this case.

### 3. Results and discussion

The introduction of a charged fluorophore into a carbohydrate molecule increases detection sensitivity and also supplies an additional charge to a neutral carbohydrate molecule, thereby permitting its electrophoretic separation by CE. Fig. 1 shows the reaction scheme for the derivatization of chitin oligosaccharides with APTS by reductive amination. The reaction mechanism involves the reaction of the primary amino group of APTS with the reducing end of the chitin oligosaccharides to form Schiff base. The intermediate is subsequently reduced to a more

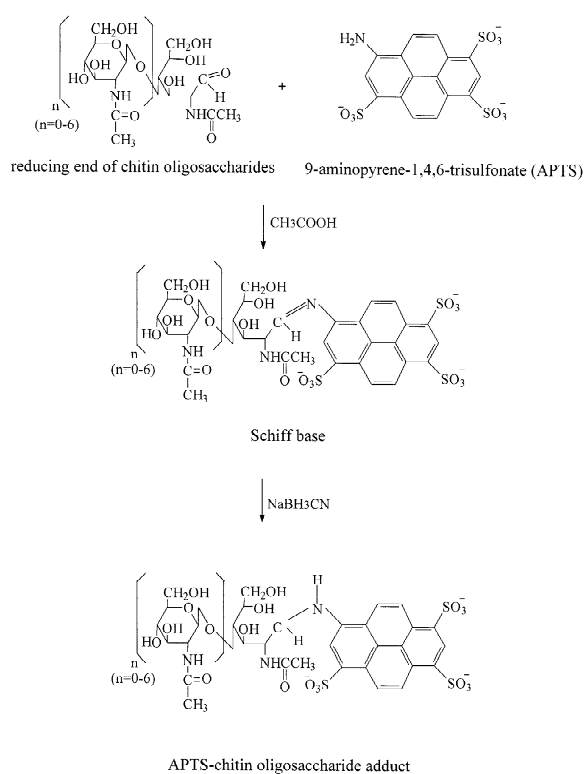


Fig. 1. Reaction scheme for the derivatization of chitin oligosaccharides with APTS followed by reductive amination.

stable secondary amine by  $\text{NaBH}_3\text{CN}$ . As shown in Fig. 1, even in the low pH buffer, the APTS–chitin oligosaccharide molecules contain negative charges, which are contributed to the three-sulfonate groups on APTS.

Figs. 2 and 3 illustrate the effect of buffer pH on the electrophoretic separation of the APTS–chitin oligosaccharides. The ionic strength of each buffer was adjusted so as to be approximately equal. In acidic citric acid–phosphate buffer solutions, the electrophoretic mobility of the APTS–chitin oligosaccharides toward the outlet (anode) is provided by negatively charged sulfonate groups under the negative applied voltage. These electrophoretic mobilities are significantly higher than the electroosmotic flow (EOF) toward the cathode. Consequently, the migration sequence of analytes to the anode is based on their apparent electrophoretic mobility. The APTS–chitin monosaccharide with the highest charge-to-

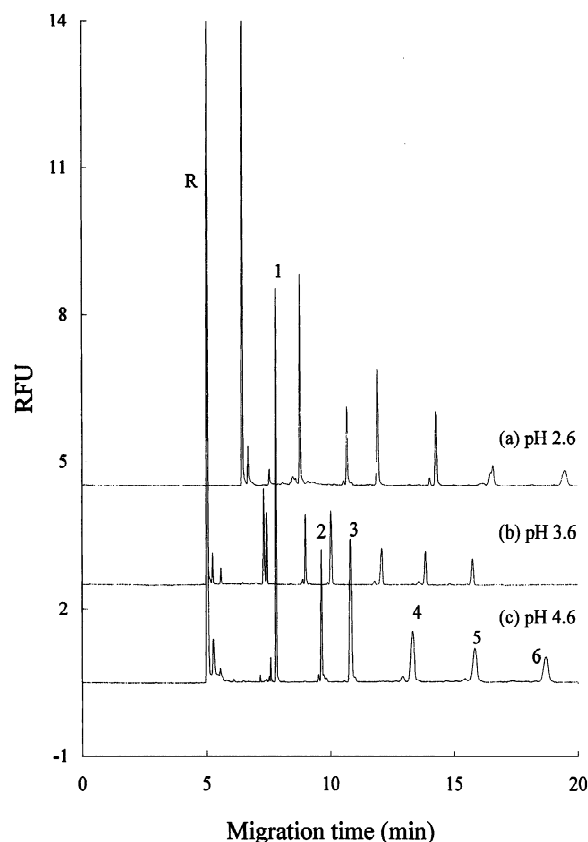


Fig. 2. Separation of APTS-derivatized chitin oligosaccharides using (a) pH 2.6, (b) pH 3.6, and (c) pH 4.6 citric acid–phosphate buffers. Conditions: light source: 488 nm argon-ion laser, 8.0 mW; emission bandfilter:  $520 \pm 20$  nm; buffers: citric acid–phosphate; sample injection: 5 s; applied voltage:  $-25$  kV; outlet: anode; capillary temperature:  $25^\circ\text{C}$ .

mass ratio migrated first. The shortest separation time was achieved using the pH 3.6 buffer. The results indicate that the degree of dissociation of the three sulfonate groups of APTS is repressed in the pH 2.6 buffer. The APTS–chitin monosaccharide peak is split into two peaks when the pH 3.6 buffer is used, indicating that the derivative was unstable in this buffer. Consequently, of the acidic buffers examined, a citric acid–phosphate buffer at pH 4.6 was selected for the analysis of the APTS–chitin oligosaccharides.

The larger APTS–chitin oligosaccharides migrated faster than the smaller oligosaccharides in alkaline phosphate or borate buffer solutions as shown in

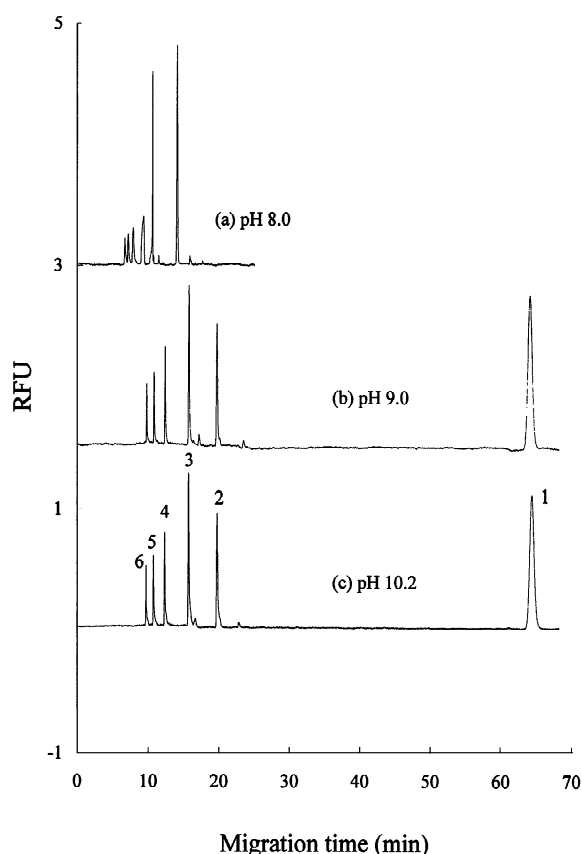


Fig. 3. Separation of APTS-derivatized chitin oligosaccharides using (a) pH 8.0 phosphate buffer, (b) pH 9.0 borate buffer, and (c) pH 10.2 borate buffer. Conditions: applied voltage: 25 kV; outlet: cathode. Other conditions are the same as those in Fig. 2.

Fig. 3. The EOF toward the outlet (cathode) is significantly faster than the electrophoretic mobilities of the APTS–chitin oligosaccharides, even when complexed with  $\text{B}(\text{OH})_4^-$  toward the anode. Therefore, all analytes migrated toward the cathode. The migration sequence of APTS–chitin oligosaccharides was reversed compared with the data shown in Fig. 2. Although, the fastest separation was achieved using a pH 8.0 phosphate buffer, its separation efficiency was not adequate. This is probably due to the absence of complexing interactions with  $\text{B}(\text{OH})_4^-$ . All the APTS–chitin oligosaccharides were effectively separated in both pH 9.0 and 10.2 buffers with similar electrophoretic velocities. However, the entire separation time was extended to 66 min with these high ionic strength buffers. Since the sepa-

ration was adequate using the pH 9.0 borate buffer, it was selected for further improvement with respect to reducing the separation time.

Figs. 4 and 5 display the effects of the buffer concentrations on the CE separation of APTS–chitin oligosaccharides. The effect of buffer concentration on the apparent electrophoretic mobilities of these derivatives was not obvious in acidic citric acid–phosphate (pH 4.6) buffer as shown in Fig. 4. The analytes were completely separated within 19 min. It appears that the EOF was approximately the same in these buffers. Nevertheless, the separation time was significantly affected in the pH 9.0 borate buffer as shown in Fig. 5. The apparent electrophoretic mobilities of the APTS–chitin oligosaccharides were decreased with increasing concentration of borate

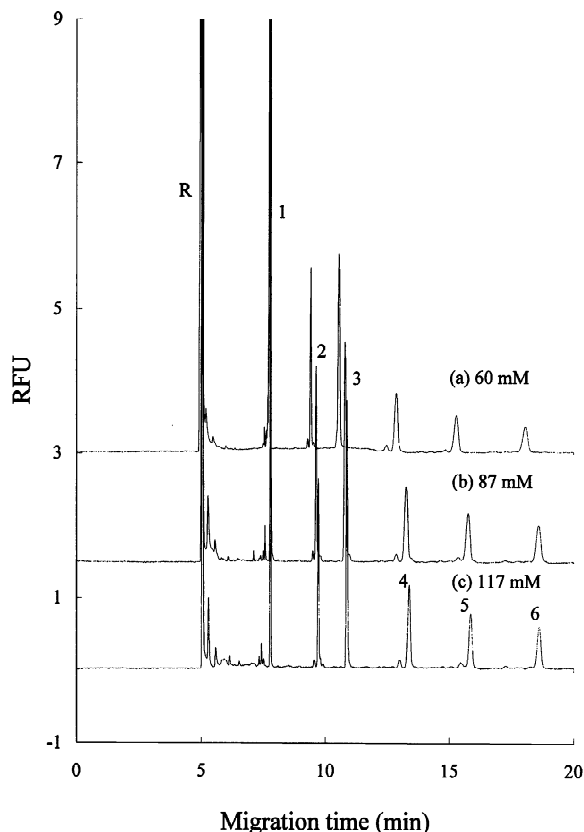


Fig. 4. Effect of citric acid–phosphate buffer concentration on the CE separation of APTS-derivatized chitin oligosaccharides. Conditions: buffer: pH 4.6 citric acid–phosphate buffer with different concentrations (a) 60 mM, (b) 87 mM, and (c) 117 mM. Other conditions are the same as those in Fig. 2.

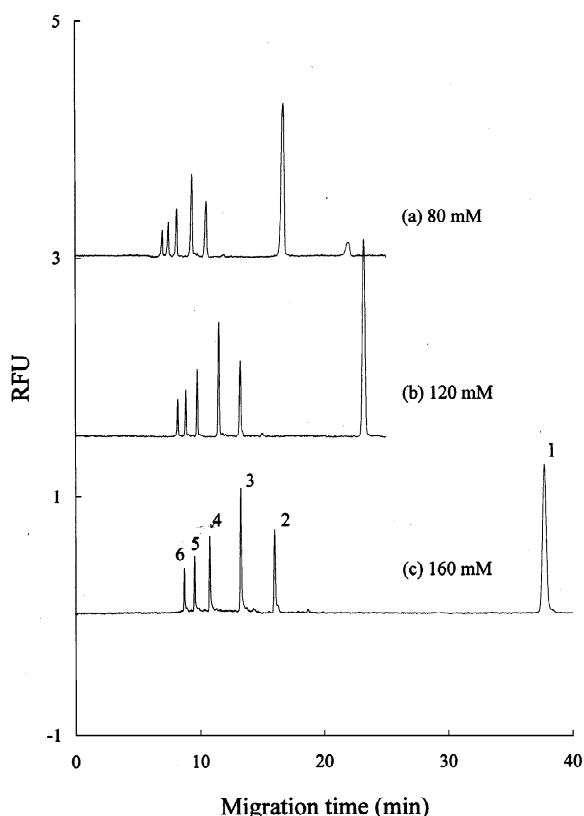


Fig. 5. Effect of borate buffer concentration on the CE separation of APTS-derivatized chitin oligosaccharides. Conditions: buffer: pH 9.0 boric acid–borax buffer with different concentrations (a) 80 mM, (b) 120 mM, and (c) 160 mM. Other conditions are the same as those in Fig. 3.

buffer. This is likely due to a decrease in EOF and/or an increase in the extent of anionic borate complexation. All the analyte peaks were adequately resolved using borate buffers. While considering buffer capacity and separation time, 87 mM citric acid–phosphate buffer and 120 mM borate buffer were selected for the subsequent analysis of enzymatically degraded of chitin polysaccharides.

The efficiency of the APTS derivatization reaction with chitin was evaluated for different reaction temperatures and reaction times using the optimized separation conditions. Table 1 summarizes the peak area ratio of APTS–chitin oligosaccharides based on different APTS–carbohydrate derivatization methods [28–30]. The experimental results indicate that the most effective labeling reaction was at a temperature

Table 1  
The peak area ratio of APTS-derivatized chitin oligosaccharides based on three derivatization methods

Chitin oligosaccharides	Peak area ratio					
	37 °C (16 h)		55 °C (2 h)		75 °C (1 h)	
	A	B	A	B	A	B
Monomer	2.71 (0.11)	1.98 (3.27)	1.98 (0.67)	1.00 (1.12)	1.00 (0.71)	1.00 (1.31)
Dimer	2.64 (1.26)	1.96 (2.50)	1.96 (0.32)	0.98 (0.24)	1.00 (0.89)	1.00 (1.21)
Trimer	2.79 (0.10)	1.78 (1.89)	1.78 (0.50)	1.21 (0.11)	1.00 (0.09)	1.00 (2.28)
Tetramer	2.92 (0.53)	1.74 (2.76)	1.74 (0.51)	1.22 (0.65)	1.00 (0.81)	1.00 (2.64)
Pentamer	2.91 (0.66)	1.84 (2.40)	1.85 (0.20)	1.17 (0.70)	1.00 (0.25)	1.00 (1.74)
Hexamer	2.63 (0.39)	1.87 (2.62)	1.87 (2.01)	1.14 (0.92)	1.00 (0.64)	1.00 (1.23)

Peak area ratio=peak area (37, 55, or 75 °C)/peak area (75 °C) and RSD (%) of triplet derivatizations. A=CE using acidic pH 4.6, 87 mM citric acid–phosphate buffer; B=CE using alkaline pH 9.0, 120 mM boric acid–borax buffer.

of 37 °C for 16 h. The reaction times were shortened at elevated temperature of 55 or 75 °C. However, the reaction efficiencies were decreased. The RSDs for reaction efficiency at 37 °C were less than 3.27%.

Table 2 lists the average migration times, RSDs for the migration time and detection limits of six APTS–chitin oligosaccharides under the optimized separation conditions. The RSDs for the migration time were less than 0.96%. The detection limits of these analytes were in the range of 5.1–16 amol for the acidic citric acid–phosphate buffer and from 6.3 to 17 amol for the alkaline borate buffer.

Fig. 6 shows an electropherogram of APTS-derivatized chitin oligosaccharides from an enzymatic digestion using 87 mM pH 4.6 citric acid–phosphate buffer. Two different enzymes were employed to degrade the chitin polysaccharides to oligosaccharides. According to this figure, the major chitin oligomer products of enzyme A digestion were dimer and traces of trimer oligosaccharides. Chitin trimer, tetramer and pentamer were produced when enzyme

B was used. These analytes were confirmed by the use of spiked standards in the samples. The APTS-derivatized chitin oligosaccharides were adequately separated by the CZE–LIF method. Fig. 7 displays electropherograms of APTS-derivatized chitin oligosaccharides from an enzymatic digestion using a 120-mM pH 9.0 borate buffer. These APTS-derivatized chitin oligosaccharides were also adequately separated. Peak identification was further verified by a comparison of the results from two different separations.

In summary, a CE–LIF method was developed for the analysis of chitin–oligosaccharides, after derivatization with APTS. The optimized derivatization conditions involved heating the reaction mixture at 37 °C for 16 h. The APTS-derivatized chitin–oligosaccharides were adequately separated in both pH 4.6 citric acid–phosphate buffer and pH 9.0 borate buffer. The APTS–chitin monosaccharide with the highest charge-to-mass ratio migrated first in the acidic buffer and the migration order of analytes was

Table 2  
Average migration times, reproducibilities, and the detection limits of six APTS-derivatized chitin oligosaccharides

Chitin oligosaccharide	Migration time (min)		RSD (%)		Detection limit (amol)	
	A	B	A	B	A	B
Monomer	7.74	23.10	0.82	0.86	8.4	17
Dimer	9.65	13.19	0.39	0.54	12	14
Trimer	10.81	11.49	0.39	0.51	5.1	6.3
Tetramer	13.25	9.76	0.73	0.44	13	8.2
Pentamer	15.74	8.84	0.84	0.36	14	9.2
Hexamer	18.57	8.19	0.96	0.35	16	9.4

A=Using acidic pH 4.6, 87 mM citric acid–phosphate buffer ( $n=5$ ). B=Using alkaline pH 9.0, 120 mM boric acid–borax buffer ( $n=5$ ).

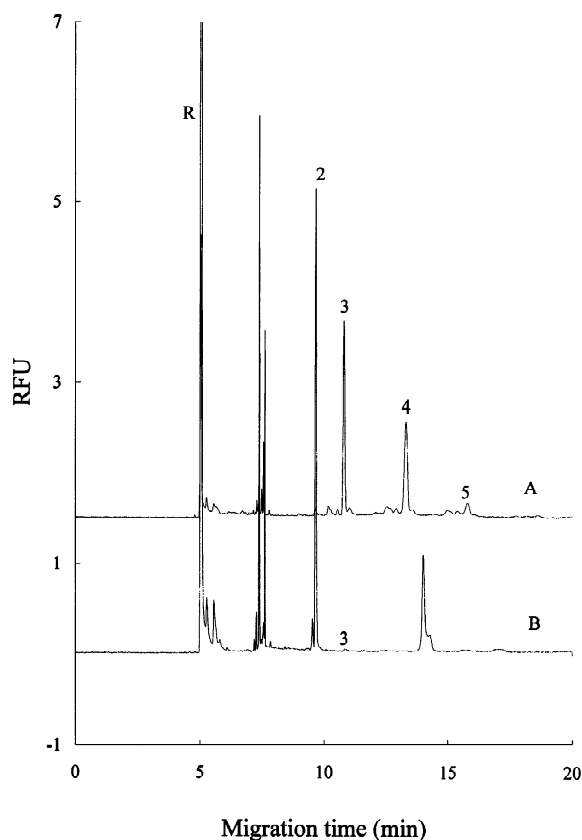


Fig. 6. Electropherogram of APTS-derivatized chitin oligosaccharides from two different enzymatic digestions. Conditions: buffers: 87 mM citric acid–phosphate buffer, pH 4.6. Other conditions are the same as those in Fig. 2.

reversed in the alkaline buffer. The detection limits of APTS-derivatized chitin–oligosaccharides (1–6 DP) ranged from 5.1 to 17 amol. Chitin–polysaccharides, depolymerized by chitinase digestion, were adequately analyzed using the optimized APTS labeling procedure and the CZE–LIF method. Enzyme specificity in the digestion of chitin–polysaccharides was also investigated.

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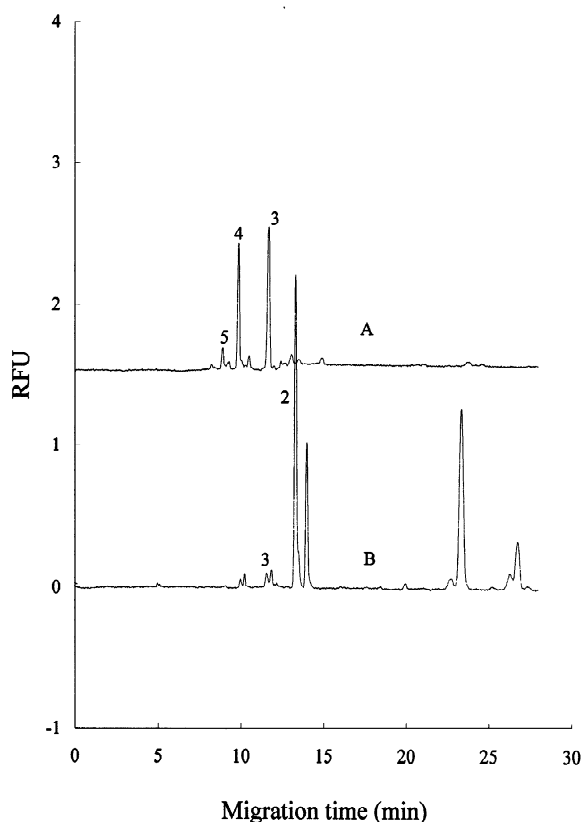


Fig. 7. Electropherogram of APTS-derivatized chitin oligosaccharides from two different enzymatic digestions. Conditions: buffers: 120 mM boric acid–borax buffer, pH 9.0. Other conditions are the same as those in Fig. 3.

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