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# Analysis of natural carbohydrate biopolymer-high molecular chitosan and carboxymethyl chitosan by capillary zone electrophoresis

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**Abstract**

Chitosan and its derivatives have been widely used in many fields for their various function, there were some difference in bioactivities and applications between them. In this paper, a direct capillary zone electrophoresis (CZE) method without any pre-treatment was developed for separation of chitosan and its derivative, a water soluble chitosan called carboxymethyl chitosan (CM-chitosan), which was proved to be rapid and effective with satisfactory resolution. Optimization of conditions including pH, concentration of the buffer, and length of column were investigated, showing that when employing 50 mM sodium phosphate at pH 2.0 and untreated fused silica capillary, high-molecular weight chitosan and CM-chitosan were baseline separated with ultraviolet (UV) detector with satisfactory repeatability and excellent linear responses. Therefore, this method could be applied to separate and identify chitosan and CM-chitosan in biological samples and commercial products.


*Keywords:* Chitosan; Caboxymethyl chitosan; Capillary zone electrophoresis; Separation; Analysis

## 1. Introduction

Chitosan is made by linear β-(1 → 4) –linked monosaccharides and a polysaccharide with structure similar to cellulose. The difference of chitosan from cellulose is that chitosan is composed of 2-amino-deoxy-β-D-glucan combined with glycosidic linkages (see Fig. 1a). It is obtained from deacetylation of chitin that is the second most abundant natural polysaccharide after cellulose and abundantly available in marine crustaceans. Chitosan is a class of cationic polysaccharides under acidic condition, representing some special properties compared with other polysaccharides and many other natural polymers. The inherent biodegradability, good biocompatibility, which do not elicit adverse reactions when in contact with human cells, and the speciality of bringing drugs to their target selectively because of its recognition by tumor cells made many scientists all over the world devote attention to chitin and chitosan (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). Now chitosan and its derivatives had found wide applications in food, agriculture, cosmetics, health care, biotechnological, wastewater treatment and pharmaceutical industries (Agnihotri, Mallikarjuna, & Aminabhavi, 2004; Agullo, Rodriguez, Ramos, & Albertengo, 2003; Hejazi & Amiji, 2003; Muzzarelli et al., 2001).

Chitosan usually has high molecular weight and strong network of intermolecular or intramolecular hydrogen bonds. Its poor solubility in water and common organic solvents has so far limited its widespread utilization. As a result, there have been many publications about the methods to enhance the solubility of chitosan, one of which was derivatization. For example, its solubility can be dramatically enhanced by introducing the carboxymethyl groups to the chitosan. The structure of CM-chitosan is similar to

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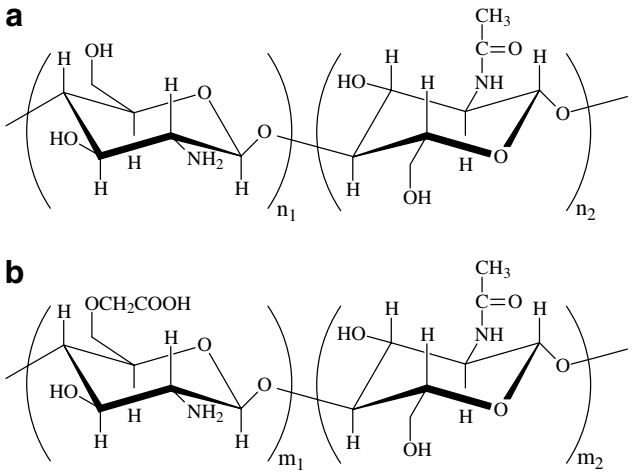


Fig. 1. Structures of (a) chitosan and (b) CM-chitosan.

amino acids with amino group and carboxyl group in the molecule, and the difference from chitosan is the carboxymethyl group linked to the nitrogen or oxygen atom (see Fig. 1b). Its good water-solubility, non-cytotoxicity and good bioactivity as functional biomaterial made CM-chitosan an important derivative(Chen, Wang, Liu, & Park, 2002; Lin, Liang, Chung, Chen, & Sung, 2005; Liu, Qiu, Chen, Peng, & Du, 2005). Chitosan and CM-chitosan have been widely used in chemical, pharmaceutical, food, agriculture and cosmetic industries, and the minor difference in molecular structure of them may affect their various chemical and physical properties, such as solubility, viscosity and chemical reactivity and bioactivity (Liu, Guan, Yang, Li, & De Yao, 2001). Since CM-chitosan is derivatized from chitosan, and CM-chitosan used in various products often contains chitosan as one of impurities, which affect the quality of related products. It is also needed to quantitatively determine them for monitoring the completeness of the derivative reaction. Consequently, it is very important to separate and determine chitosan and its derivatives for understanding therapeutic and bioactive effects of drug and the continuing development of more safe and effective drugs and food additives.

Nowadays, many chromatographic methods have been utilized to analyze chitin and its derivatives. Pyrolysis–gas chromatography (Py–GC) with good sensitivity and separation efficiency has been applied to determine the degree of acetylation of chitin and chitosan (Sato et al., 1998) and the degree of substitution in chitin derivatives (Sato et al., 2002), and it has also been used for the quantitative and qualitative analysis of the chitin in fossil arthropods (Bierstedt, Stankiewicz, Briggs, & Evershed, 1998). However, Py-GC is an indirect method unable to distinguish polysaccharide from oligosaccharide and monosaccharide. A colorimetric method for quantitative determination of chitosan based on reaction with ninhydrine without any pre-treatment such as hydrolysis of solution was described by Sabina Prochazkova (Prochazkova, Varum, & Ø´stgaard, 1999), but this method was highly dependent on the fraction of acetylation of the chitosan and could not distinguish chitosan from its derivatives, too. High performance liquid chromatography (HPLC) is another important separation method widely used for analysis of carbohydrates, and chitosan was quantitatively determined after acidic hydrolysis of chitosan to glucosamine (Eikenes, Fongen, Roed, & Stenstrom, 2005; Zhu, Cai, Yang, & Su, 2005). However it is very difficult to analyze chitosan without hydrolysis because of large molecule and high viscosity, resulting in clogging the column. Furthermore, HPLC often needs longer time for column re-equilibration. Comparing with the methods discussed above, capillary electrophoresis (CE) is a powerful technique with recent notable accomplishment, and there are many publications on the analysis of carbohydrates, such as monosaccharides and oligosaccharides (El Rassi, 1999). The obvious advantages of CE are simplicity, high efficiency, good selectivity, and short analysis times, while various separation modes can be selected for different analytes. CE had been applied to separate a mixture of chitin and chitosan oligosaccharides (Beaudoin, Gauthier, Boucher, & Waldron, 2005; Wang & Hsieh, 2002), and high-molecular chitosan(Ban, Choi, Ryu, & Yoo, 2001; Liu, Shirota, & Novotny, 1991). Although there are many reports on the analysis of chitin or chitosan, the separation and determination of chitosan and CM-chitosan by CE have not been reported up to now, to our best knowledge.

In this paper, the analytical method for high molecular weight chitosan and CM-chitosan was investigated by using capillary zone electrophoresis (CZE), a most often used mode of CE. We optimized many important parameters affecting the separation, including pH and concentration of buffer, temperature and applied voltage, the inner diameter and length of capillary column, achieving a baseline separation of chitosan and CM-chitosan under the optimal conditions. This method can be used to identify the chitosan or CM- chitosan at health care products.

## 2. Experimental

### 2.1. Chemicals

The chitosan standard ($M\eta = 1{,}500{,}000$ and degree of deacetylation is 75%) was purchased from KATOKICHI Co. Ltd. Japan, and CM-chitosan ($M\eta = 29{,}000$; degree of deacetylation is 84% and degree of substitution is 91%) standard was purchased from Koyou Chemical Industrial Co. Ltd. Japan. Chitosan health care product was purchased from Jinan Haidebei Marine Bioengineering Co. Ltd. China. Phosphoric acid, sodium hydroxide, sodium phosphate, triethylamine were analytical-grade reagents purchased from Beijing Reagent Company, China. All other inorganic reagents used were analytical grade, and organic reagents as additives were chromatographic grade. All the solutions were prepared in redistilled water and filtered with 0.45 μm membrane before use.

### 2.2. Apparatus and conditions

All experiments were performed on an Agilent 3D CE system equipped with air-cooling and a diode array detector (Agilent Technologies, Palo Alto, CA, USA), and an untreated fused-silica capillary column (Polymicro, USA) of 48.5 cm × 50 μm ID (40 cm effective length) was used with applied voltage of 15 kV at 25 °C. Detection wavelength was set at 193 nm. The capillary was conditioned prior to the first use by rinsing with 1.0 M NaOH for 15 min and then rinsing with 0.1 M NaOH for 15 min, at last rinsing with water for 20 min. At the start of each sequence, the capillary column was washed with buffer for 5 min. Between runs, the capillary was rinsed for 2 min with running buffer. Between workdays, the capillary was conditioned by flushing 0.1 M NaOH for 10 min and water 10 min. Samples were introduced into the capillary by hydrodynamic injection for 10 s at 50 mbar.

### 2.3. Preparation of buffer, samples, and standard solutions

All sodium phosphate buffers at different concentrations were adjusted to pH 2.0 with phosphoric acid. The mixed stock solution was prepared by dissolving 75 mg CM-chitosan and 15 mg chitosan in 0.1 M HCl into 5.0 mL volumetric flask. Then, the stock solution was diluted with 0.1 M HCl to obtain tested standard solutions for calibration and the concentration was ranged from 15 to 0.75 mg/mL for CM-chitosan. Both of the buffer and sample solution were filtered through a 0.45 μm membrane filter and degassed by ultrasonic apparatus before use. The preparation method of health care product was same to that of the standard solution.

## 3. Results and discussion

In our work, CZE mode was established based on a series of optimizations, including buffer pH, the concentration of sodium phosphate, some modifiers, capillary column, temperature, and applied voltage.

### 3.1. Optimization of buffer pH

Two kinds of buffers, sodium borate buffer (pH 8–10) and phosphate buffers (pH 1.5–3.0) were selected to test at the beginning for the separation of chitosan and CM-chitosan The result indicated that pH value had great effect on the separation efficiency. Under basic condition, the analytes can not be eluted out the capillary and the current was easily broken off because the chitosan compound will lose its positive charge at basic pH and its solubility is obviously decreased, resulting in high viscosity and the jam in the capillary. Under acidic condition, especially pH value below 3, the separation was obviously improved, because the electroosmotic flow (EOF) was dramatically decreased, and the analytes were partially charged. Furthermore, the positive charge of them was increased with the decreasing of pH, which could accelerate their migration towards the cathodic end of capillary. From Fig. 2 it can be seen that with the increasing of pH, the peak width of CM-chitosan had been broadened, and with the decreasing of pH the current and resolution were increased. The reason is that the more phosphoric acid was added to the buffer for the lower pH, leading to higher current because of high ionic strength. On the other hand, positively charged analytes at lower pH migrated faster, resulting shorter migration time, which reduced the resolution although narrower peaks were observed. Taking the resolution and current into account, pH 2.0 was selected for further optimization.

### 3.2. Optimization of buffer concentration

Sodium phosphate concentration was investigated in the range of 25–100 mM in the buffer system at pH 2.0, 25 °C, and 15 kV applied voltage. It was demonstrated that, with

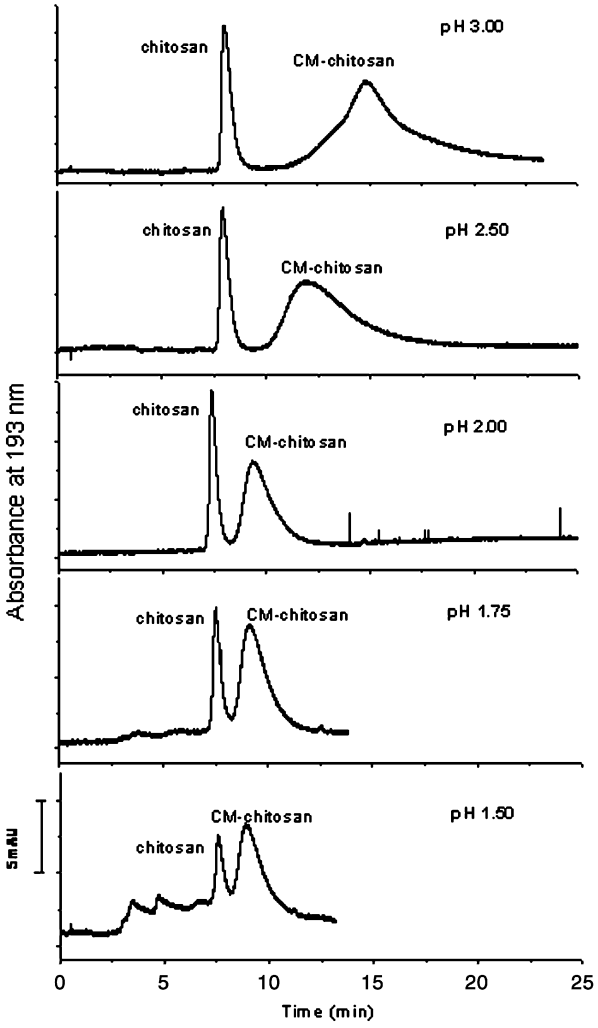


Fig. 2. The effect of buffer pH on peak shape. Separation conditions: 50 μm × 48.5 cm untreated fused-silica capillary (40.0 cm effective), 50 mM sodium phosphate, capillary temperature of 25 °C, applied voltage of 15 kV, UV detection at 193 nm. The concentration of chitosan and CM-chitosan was 1.0 and 5.0 mg/mL, respectively.

the increasing of sodium phosphate concentration, the resolution of chitosan and CM-chitosan was not changed obviously. Additionally as expected, the migration time was reduced with the decreasing of buffer concentration. Comprehensively considering the resolution, peak shape, current and the migration time, 50 mM of sodium phosphate buffer was confirmed to be the optimal condition.

### 3.3. Selection of organic and other modifiers

As above mentioned, the peaks of chitosan and CM-chitosan were dramatically broadened, thus we further attempted to use some additives to improve the separation efficiency. Different proportions of methanol, acetonitrile, 2-propanol were respectively investigated, showing that little improvement was obtained. The reason probably relied on the fact that both of chitosan and CM-chitosan have poor solubility in these organic solvents. In addition, the ultraviolet absorbance of these organic solvents at 193 nm elevated the baseline, reducing detection ability. Furthermore we added triethylamine to the buffer for improving the peak shape, because the better result can be obtained in the report of (Ban et al., 2001). However, the broaden peak of CMC was observed for the use of triethylamine. Then, we did not add any modifier to the running buffer for this separation.

### 3.4. Optimization of applied voltage and temperature

The applied voltage was optimized from 15 to 30 kV in 50 mM sodium phosphate buffer at pH 2.0, indicating that higher applied voltage can not improve the separation efficiency but cause current breaking. Therefore, the lower voltage of 15 kV was finally used.

The temperature of capillary was optimized from 10 to 35 °C, illustrating that the separation efficiency was a little bit improved at lower temperature. The migration time was shortened at higher temperature, but the current was broken off easily because the bubble was generated inside the capillary. Finally, 25 °C was selected for our separation.

### 3.5. Selection of capillary inner diameter and length

In order to improve the resolution and sensitivity of this method, we compared the capillaries with different inner diameters (50 and 75 μm). The results showed that the capillary with 75 μm ID generated so high current and caused the current broken. Furthermore, we also studied the effects of capillary length from 38.5 to 48.5 cm on migration time and peak shape of chitosan and CM-chitosan, indicating that, with the increasing of the length the current was decreased because of reduced strength of the electric filed, while the migration time was increased as expected. The resolution was improved with longer capillary, and the theoretical plate number for both analytes did not show obvious difference between different lengths of capillaries. 48.5 cm was proved to be suitable for this separation. Therefore, 50 μm and 48.5 cm was selected for the further study.

### 3.6. Method evaluation

Since chitosan and CM-chitosan investigated are high-molecular weight polymer and are not volatile, they cannot be directly separated by HPLC and GC, and some complicated pretreatment procedures were inevitable. Compared with the HPLC and GC, the proposed CZE method was simple and effective to separate and analyze chitosan and its derivative under the following conditions: 50 mM sodium phosphate (pH 2.0) under 25 °C and 15 kV applied voltage (see Fig. 3a). Under these optimized conditions, chitosan and CM-chitosan can be completely separated within 15 min, but they did not be separated in previous method (Ban et al., 2001). Furthermore, the better repeatability can be obtained for this simple buffer. For quantitatively determination of these analytes, a series of standard samples were analyzed and the calibration curves of peak area (A) vs. amount of CM-chitosan and chitosan (c) are as flowing:

For chitosan: $A = 267.78c - 0.8013$ ($r = 0.9978$)

For CM-chitosan: $A = 139.34c + 43.39$ ($r = 0.9996$)

It can be see that the linearity was satisfactory with a correlation coefficient ($r$) greater than 0.99. As shown in

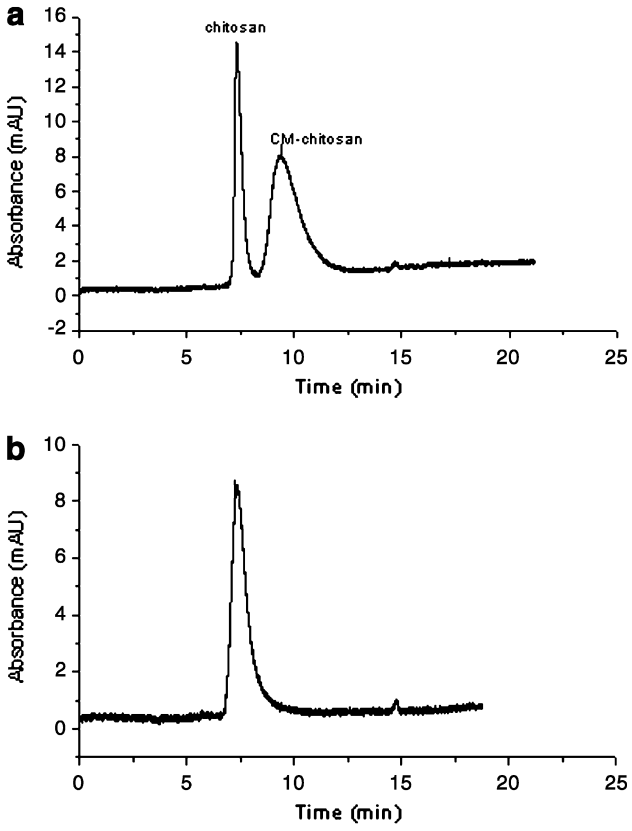


Fig. 3. (a) Typical electropherogram of chitosan and CM-chitosan. (b) Real sample: health care product with chitosan. Separation conditions: 50 mM sodium phosphate (pH 2.0), the other conditions were the same as in Fig. 2.

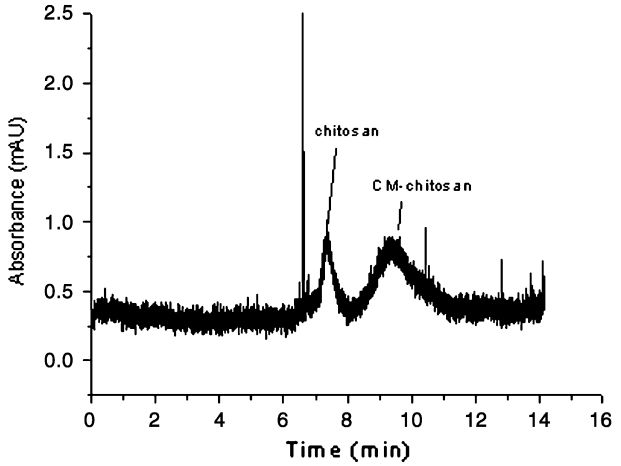


Fig. 4. Detection limits of chitosan and CM-chitosan. Separation conditions were the same as in Fig. 3. The concentration of chitosan and CM-chitosan was 0.04 mg/mL and 0.2 mg/mL, respectively.

Fig. 4, the detection limits (at a signal-to-noise ratio of 3) for chitosan and CM-chitosan were found to be 0.04 mg/mL and 0.2 mg/mL, respectively. As for repeatability, the relative standard deviation (RSD) for migration time and peak area of chitosan were 0.58% ($n = 5$) and 1.17% ($n = 5$), respectively, and those of CM-chitosan were 0.49% and 1.89%, respectively, which is better than that of Ban's work (Ban et al., 2001). The drawback of this method was the wider peak of CM-chitosan, resulting in poor detection sensitivity. The reason is perhaps that the carboxyl groups in CM-chitosan can form hydrogen bond with the Si–OH groups on the wall of capillary, causing the analytes absorbed onto the capillary wall. We will investigate the coating procedure for the capillary to overcome the absorption and improve the column efficiency in the future.

### 3.7. Analysis of real sample

The identification and quality control of chitosan and its derivations is necessary for their different bioactivities and applications in food additive, health care products and clinical studies. In order to demonstrate the usefulness of this method, a health care product which purchased in commercial market was analyzed. This real sample was dissolved in 0.1 M HCl, and analyzed by CE after filtered by a 0.45 μm filter. Fig. 3b shows the electropherograms of the health care product, indicating that this product was mostly composed of chitosan.

## 4. Conclusion

In this work, separation and determination of chitosan and CM-chitosan has been achieved with 50 μm × 48.5 cm untreated fused-silica capillary under CZE mode using 50 mM sodium phosphate under 25 °C temperature and 15 kV applied voltage with UV detection at 193 nm, without any pre-treatment. Although the detection sensitivity it is not satisfactory, it can be used for the analysis of chitosan and CM-chitosan in routine analysis. This method is simple, fast and cost-effective, and overcome the drawbacks of HPLC and GC to some extend. In the future, we will investigate the enrichment method to improve the sensitivity for determination of chitooligosacchrides and polysacchrides.

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