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Simultaneous determination of degree of deacetylation, degree of substitution and distribution fraction of –COONa in carboxymethyl chitosan by potentiometric titration

Xiangping Kong*

College of Chemistry and Pharmacy, Qingdao Agricultural University, Qingdao 266109, Shandong, PR China

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ABSTRACT

A novel method for simultaneously determining the degree of deacetylation (DD), the degree of substitution (DS) and the distribution fraction of –COONa ($\delta_{\text{–COONa}}$) or –COOH ($\delta_{\text{–COOH}}$) in carboxymethyl chitosan (CM-chitosan) was developed by potentiometric titration. The effect of precipitate on the third inflection point, a common problem in the previous method, was eliminated by integrated application of alkaline titration and acidic titration. Calculating formulas of DS, DD and $\delta_{\text{–COONa}}$ considering both the residues (GlcN and GlcNAc) and the carboxymethyl forms (–COONa and –COOH) were proposed. CM-chitosan products in different forms were examined to prove the reliability of the method. The standard deviation of DS, DD and $\delta_{\text{–COONa}}$ of Na salt CM-chitosan ($n = 9$) were 0.109, 3.51% and 4.30%, respectively. As CM-chitosan was confirmed by IR and ^{1}H NMR to be N,O-CM-chitosan, the method is suitable for the parameter determination of N-, O- and N,O-CM-chitosan.

1. Introduction

Chitosan is a β-(1-4)-linked copolymer mainly composed of 2-amino-2-deoxy-D-glucopyranose (GlcN) with a small amount of 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc), whose application is limited owing to the poor solubility in neutral water. Carboxymethyl chitosan (CM-chitosan), the most fully explored water-soluble chitosan derivative, is an important amphoteric ether polymer with wide applications due to its many attractive chemical, physical and biological properties (Elsabee, Morsi, & Al-Sabagh, 2009; Jayakumar et al., 2010; Muzzarelli, 1988). Since there are three reactive sites of the C_2-amino and C_3-, C_6-hydroxyl groups in molecular chains for the carboxymethylation of chitosan, products with different substitution positions including N-, O-, N,O- and N,N-CM-chitosan could be prepared under different reaction conditions and reagents (Ge & Luo, 2005; Jayakumar et al., 2010; Lin & Lin, 2009; Muzzarelli, Ilari, & Petrarulo, 1994; Muzzarelli et al., 2012; Muzzarelli, 1988; Song, Zhang, Gao, & Ding, 2010).

The quality and chemical properties of CM-chitosan products are closely related with the degree of deacetylation (DD) and the degree of substitution (DS) (Chen, Du, Wu, & Xiao, 2002; Kittur, Prashanth, Sankar, & Tharanathan, 2002; Shi, Du, Yang, Zhang, & Sun, 2006). In particular, the water solubility, a key property connected to applications, is strongly dependent on the distribution fraction of –COOH ($\delta_{\text{–COOH}}$) or –COONa ($\delta_{\text{–COONa}}$) in the polymer chain. Thus, an accurate, quick and cost-saving method for the determination of these parameters is crucial to meet the scientific research or industry requirements.

Compared with ^{1}H NMR (Chen & Park, 2003) and infrared spectroscopy (Bidgoli, Zamani, & Taherzadeh, 2010), either with expensive equipments, complicated procedures or extremely dried samples, potentiometric titration seems the most common method for the determination of DS of CM-chitosan due to the simple operation and low cost (Ge & Luo, 2005; He, Si, Han, Liu, & Sun, 2002; Song et al., 2010; Sun & Wang, 2006; Xue, Li, & He, 2009). The titration was usually performed by dissolving the biopolymer in a known excess of HCl, and then titrated with NaOH. The DS value could be calculated from the two inflection points in the titration curve: the first corresponds to the excess of HCl, while the second to the protons of –COOH group. However, only one of the two residues is considered in the reported formulas for DS calculation. Meanwhile, the carboxymethyl groups may exist as $–CH_2COOH$ or $–CH_2COONa$, which are also not fully taken into account. If both the residues and the carboxymethyl forms are to be considered, a third inflection point corresponding to the protons of $–NH_3^+$ and $–NH_2^+CH_2COO^-$ is required. Although Muzzarelli, Tanfani, Emanuelli, and Mariotti (1982) and Liu, Chen, Jin, Sun, and Gao (2004) have given the titration curves of N-CM-chitosan and

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* Tel.: +86 0532 86080442.
E-mail address: kxp2004@163.com

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N,O-CM-chitosan with the third inflection point, respectively, large experimental errors may be caused by the formation of precipitate around the second inflection point. Moreover, there has been no report about DD or $\delta_{\text{-COONa}}$ determination of CM-chitosan by potentiometric titration yet, or even no existing methods including ^{1}H NMR and infrared spectroscopy are capable of determining the three structural parameters simultaneously.

As for this problem, taking N,O-CM-chitosan for example, a novel potentiometric titration method for the simultaneous determination of DS, DD and $\delta_{\text{-COONa}}$ or $\delta_{\text{-COOH}}$ was developed, where the effect of precipitate on the third inflection point was eliminated.

2. Experimental

2.1. Materials

Chitosan (DD > 90%, viscosity < 100 cps) was supplied by Shanghai Lan-Ji Biotechnology Development Co., Ltd., China. Monochloroacetic acid and all the other reagents used in this study were of analytical grade and all solutions were prepared with distilled water.

2.2. Preparation of CM-chitosan

CM-chitosan was prepared based on the method described in the literature (Chen & Park, 2003) with slight modifications. Briefly, 10 g chitosan and 13.5 g sodium hydroxide were added into 100 mL solvent (20 mL water, 80 mL isopropanol) to swell and alkalize for 2 h at 60 °C. Then 15 g monochloroacetic acid dissolved in 20 mL isopropanol was added drop-wise for 30 min. After continued stirring for an additional 4 h at 60 °C, 200 mL of 70% ethyl alcohol was added. The solid was filtered out, rinsed with 70–90% ethyl alcohol to desalt and dewater, and then dried at 60 °C. The product was raw Na salt CM-chitosan.

The blank test (without adding monochloroacetic acid) was carried out simultaneously. The product was designated as A.

The raw Na salt CM-chitosan (2 g) was added into 200 mL distilled water, and filtrated after stirring for 30 min. The CM-chitosan was purified by the following two methods, respectively.

Organic solvent precipitation method: anhydrous ethanol (600 mL) was added into the filtrate. The precipitate was filtered, washed with 70–90% ethyl alcohol for three times, and then dried at 60 °C. The product was Na salt CM-chitosan and designated as B. The product B (1 g) was suspended in 100 mL of 80% ethyl alcohol aqueous solution, and 10 mL of 37% hydrochloric acid was added and stirred for 30 min. The solid was filtered out, rinsed with 70–90% ethyl alcohol to neutral, and dried at 60 °C. The product was the H-form of CM-chitosan (Chen & Park, 2003) and named C.

Isoelectric point precipitation method: 6.0 M HCl was added into the filtrate to adjust its pH to isoelectric point. The precipitate was filtered, washed with 70–90% ethyl alcohol for three times, and then dried at 60 °C. The product marked as D (1.5 g) was dissolved in 100 mL of 0.15 M NaOH aqueous solution, and anhydrous ethanol (200 mL) was added to precipitate the product. Then the product was filtered, rinsed thrice with 70–90% ethyl alcohol, and dried at 60 °C. The product was Na salt CM-chitosan and designated as E.

2.3. Determination of isoelectric point

Isoelectric point was estimated according to the method presented in the literature (Ji & Gao, 2007). Product B (1 g) was dissolved in 100 mL of 0.1 M NaCl aqueous solution, and was divided into several parts in equal volume. Then 0.1 M HCl was added to adjust their pH to 3, 4, 4.5, 5 and 6, respectively, and the solid was separated by centrifugation at 8000 rpm. Next, pH of the supernatant was adjusted to 8 by adding 0.1 M NaOH, and the absorbance was recorded at 220 nm on a SP-754 UV–Vis spectrophotometer (Shanghai Spectrum Instruments Co., Ltd., China). The pH corresponding to the minimum absorbance is the isoelectric point.

2.4. FT-IR spectroscopy

The Fourier Transform Infrared (FT-IR) spectra of chitosan and product B were recorded on a Nicolet IR200 FT-IR Spectrometer (Thermo Fisher Scientific Inc., USA) with a resolution of 4 cm^{-1} from 32 scans at room temperature. Each sample was vacuum-dried at 80 °C for 6 h and then cooled in a desiccator with color silicone. A pellet was formed from 2.5 mg sample and 250 mg of KBr. Data analysis was carried out using Omnic (Windows XP).

2.5. ^{1}H NMR spectroscopy

The sample of product B was dried as described in Section 2.4, and then dissolved in D_2O in a 5 mm diameter tube. 500 MHz ^{1}H NMR spectrum was recorded on a Bruker Avance 500 spectrometer at 25 °C with 32768 data points, 10 kHz spectral width, a 30° pulse, and an acquisition time of 2.0 s.

2.6. Measurement of chitosan DD

The DD of the chitosan and product A were determined by alkalimetry of aquatic industry standards of P.R. China (2005). Accurately disperse chitosan (0.2–0.3 g) into 30 mL of 0.1 M HCl aqueous solution. Stir the mixture until the sample was dissolved completely, and then add 2–3 drops of mixed indicator (0.1% methyl orange and 0.1% aniline blue, with v/v of 1:2). Under continuous stirring, 0.1 M NaOH of the standard solution was added stepwise with alkali-burette until the color of the solution changed from purple to blue-green. The volume of added NaOH solution was recorded and marked as *V*.

The blank test was carried out as mentioned above, where the volume of the added NaOH standard solution was marked as V_0. DD was calculated according to the following equation:

$$DD = \frac{203C(V_0 - V)}{42C(V_0 - V) + m(1 - w)} \times 100\% \tag{1}$$

where *C* is the concentration of sodium hydroxide standard solution (mol/L). V_0 and V are the volumes of sodium hydroxide consumption for blanket test and excessive hydrochloric acid (mL), respectively. 203 and 42 are the molar weight of chitin residue and acetyl group, respectively. *m* is the weight of the sample (mg), and w is the water content of the sample (%), which was determined by the direct drying method of national standards of P.R. China (2010).

2.7. Potentiometric titration experiments

The DD, DS and $\delta_{\text{-COOH}}$ or $\delta_{\text{-COONa}}$ were estimated by potentiometric titration based on the idea mentioned in the literature (Liu et al., 2004). The accurately weighed CM-chitosan (0.24–0.26 g) was dissolved in 25.00 mL of hydrochloric acid solution (0.1 M), and the calculated amount of NaCl (0.1 M) was added to maintain the ionic strength. A standard solution of NaOH and NaCl both 0.1 M was used as the titrant. Alkalimetric curves were recorded by a ZDJ-4A automatic titrator equipped with E-201-C-65 pH electrode (Shanghai Precision & Scientific Instruments Co., Ltd., China). The titrator makes a sound automatically when the titration reaches the end-point, and the corresponding volume is given by the end of the titration. Three replicates were performed for each sample, and manual titration was an exception with five replicates. The factorial differential coefficient figure could be obtained based on the sodium hydroxide volume and the corresponding pH value recorded. The inflection points in this figure corresponded to the sodium hydroxide volume consumed at the end-points of the titration. Hydrochloric acid solution of the same volume was used for blank titration. Considering both the residues (GlcN and GlcNAc) and carboxymethyl forms, the following formulas were proposed to calculate the total DS, DD, $\delta_{\text{-COOH}}$ and $\delta_{\text{-COONa}}$ of CM-chitosan:

$$DS = \frac{203C(V_2 - V_1)}{m(1-w) - 80C(V_2 - V_1) + 22C(V_3 - V_0) + 42C(V_3 - V_2)} \quad (2)$$

$$DD = \frac{203C(V_3 - V_2)}{m(1-w) - 80C(V_2 - V_1) + 22C(V_3 - V_0) + 42C(V_3 - V_2)} \times 100\% \quad (3)$$

$$\delta(\text{–COOH}) = \frac{V_3 - V_0}{V_2 - V_1} \times 100\% \quad (4)$$

$$\delta(\text{–COONa}) = 100\% - \delta(\text{–COOH}) \quad (5)$$

where C is the concentration of sodium hydroxide standard solution (mol/L). V_1 is the volume of sodium hydroxide consumed by excessive hydrochloric acid (mL), and V_2, V_3 are the volumes of sodium hydroxide corresponding to titration terminal of –COOH and –NH_3^+, $\text{–NH}_2^+\text{CH}_2\text{COO}^-$, respectively. V_0 is the volume of sodium hydroxide consumed by hydrochloric acid in the blank test (mL). m is the weight of the sample (mg), and w is the water content of the sample (%). 203, 42 and 80 are the molar weight of chitin residue, acetyl and $\text{–CH}_2\text{COONa}$ group, respectively. 22 is the molar weight difference between $\text{–CH}_2\text{COONa}$ and $\text{–CH}_2\text{COOH}$.

3. Results and discussion

3.1. Position of the carboxymethyl group

3.1.1. FT-IR analysis

FT-IR absorption bands (cm^{-1}) of chitosan were 3450 (O–H stretching and N–H stretching), 2875 (C–H stretching), 1653 (C=O of NH–C=O stretching), 1596 (N–H bending), 1420 (C–H of –CH_2 bending), 1321 (C–N stretching), 1159 (bridge-O-stretching), 1088 and 1035 (C–O stretching). The results are consistent with the FT-IR spectra in the literature (Lin & Lin, 2009; Sun & Wang, 2006). In particular, the C=O band of NH–C=O has often been used to calculate the DD of chitosan (Duarte, Ferreira, Marvao, & Rocha, 2002; El-Sherbiny, 2009; Zhang, Zhang, Ding, Zhang, & Liu, 2011). Thus the clearly visible and weak absorption band at 1653 cm^{-1} indicates that the chitosan is mainly composed of GlcN with seldom GlcNAc.

FT-IR absorption bands (cm^{-1}) of product B were 3450 (O–H stretching and N–H stretching), 2881 (C–H stretching), 1597 (C=O of –COONa antisymmetric stretching), 1412 (C=O of –COONa symmetric stretching), 1323 (C–N stretching), and 1067 (C–O stretching). The characteristic bands are also similar to those in the literature (Lin & Lin, 2009; Sun & Wang, 2006). Compared with the chitosan spectrum, the new absorption bands of –COONa are strong, and the O–H and N–H bands become narrow and weak, both indicating a high carboxymethylation on –OH or –NH_2. Meanwhile, the bands at 1597 cm^{-1} and 1323 cm^{-1} intensify significantly, thus indicating that carboxymethylation has occurred on both the amino and the hydroxyl groups of chitosan (Ge & Luo, 2005). In addition, the bands corresponding to C=O of NH–C=O stretching and N–H bending are overlapped with the much stronger C=O of –COONa antisymmetric stretching, so the FT-IR method seems not suitable to determine the DD of Na salt CM-chitosan. In particular, as the band of C=O of –COONa symmetric stretching is usually used to calculate the DS of CM-chitosan (Bidgoli et al., 2010), the effect of $\delta_{\text{C–H}}$ band of –CH_2 should also be considered especially when the DS is low.

3.1.2. NMR data analysis

Product B, ^{1}H NMR, δ (ppm): 4.66 (1H, H-1A, GlcNAc), 4.5–4.1 (4H, H-6′ and H-3′, $\text{–O–CH}_2\text{–COOD}$), 3.9–3.6 (5H, H3–6), 3.25 (2H, H-2′, $\text{N–CH}_2\text{–COOD}$), 3.15 (1H, H-2D, GlcN), 2.12 (3H, N–CO–CH_3). The resonance of the single proton from H-3′ cannot be distinguished from 4.5 to 4.1 ppm, different from what Chen and Park (2003) reported. The reason may be that the intensity of the resonance from H-6′ is much higher than the one from H-3′ due to a higher reactivity of the hydroxyl group at C_6 than the one at C_3. The resonance signal (3.25 ppm) of the two protons from $\text{N–CH}_2\text{–COOD}$ group can be found in the ^{1}H NMR spectrum of CM-chitosan (Abreu & Campana-Filho, 2005; Muzzarelli et al., 1994; Rinaudo, Dung, Gey, & Milas, 1992), also described by Hjerde, Varum, Grasdalen, Tokura, and Smidsrod (1997). The strong resonances of $\text{O–CH}_2\text{–COOD}$ and $\text{N–CH}_2\text{–COOD}$ confirm a high carboxymethylation on –OH or –NH_2, correlating well with FT-IR results. The integrals of the integrated region of 4.5–4.1 ppm and 3.3–3.2 ppm are 1.00 and 0.62, respectively, thus the ratio of carboxymethylation at the position O- and N- is 62:38.

Both FT-IR and ^{1}H NMR results showed that the products were N,O-CM-chitosan, which is in agreement with the result reported by Sun and Wang (2006), but different from Chen and Park's (2003) result. It may be caused by the longer swelling and alkalization time.

3.2. DD of the chitosan

The average DD of the raw chitosan and product A samples were 84.8% and 85.5% ($n = 6$), respectively. The DD of the chitosan was almost unchanged before and after the reaction, which indicates that the reaction conditions had almost no impact on DD. So the DD of the raw chitosan sample could be used to characterize the CM-chitosan product. In addition, the DD value of the raw chitosan sample was lower than that provided by the manufacturer. This is mainly because only part of the crystal water could be determined by the direct drying method.

3.3. Development of the novel potentiometric titration method

For the titrator, the adjustment range of the pre-control amount on the titration speed is 30–90%, and 50% is usually selected. The typical alkalimetric and first derivative (A–FD) curves of product B determined with the pre-control amount of 50% under the fastest stirring speed are shown in Fig. 1. As can be seen, the first two end-points ($V_1 = 4.05$ mL, $V_2 = 13.49$ mL) were found. The second end-point correlated well with the one from the ZDJ-4A automatic titrator, while the first titration inflection point was too small to be detected by the instrument. The reason may be that the

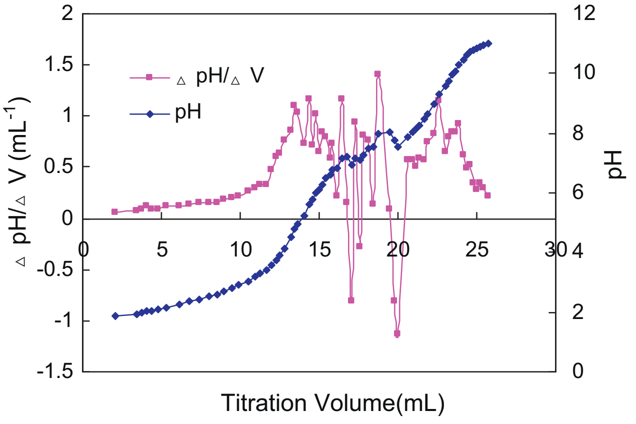

Fig. 1. The typical A–FD titration curves of product B at the titration speed of 50% pre-control amount, three parallel measurements.

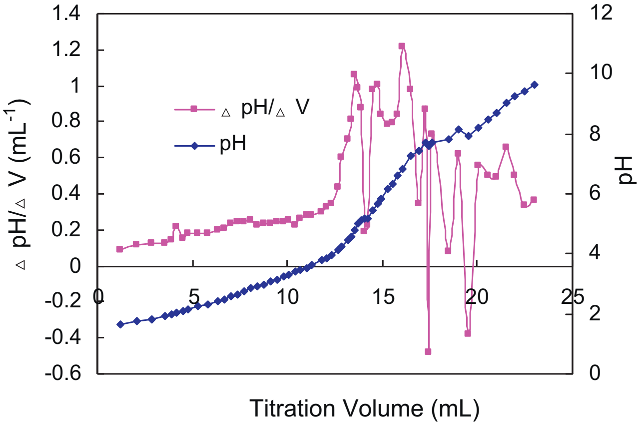

Fig. 2. The typical A–FD titration curves of product B at the titration speed of 30% pre-control amount, three parallel measurements.

acidity of hydrochloric acid with low concentration was not stronger enough than that of –CH_2COOH. It indicates that the concentration of hydrochloric acid used to dissolve CM-chitosan should not be less than 0.1 M. The third end-point occurred only in one of the three parallel experiments at 18.82 mL from the instrument, much lower than V_0 (23.39 mL), inconsistent with the theory of $V_3 \geq V_0$ (Liu et al., 2004). Also, V_3 could not be obtained from Fig. 1 due to the fluctuations in the first derivative curve. Large white precipitate was produced around the second end-point (isoelectric point of about pH 4.5) which led to the slow reaction and the inaccurate data. So the titration speed should be lowered.

The typical A–FD curves of product B determined in the slowest rate of titration (30% of pre-control amount) under the fastest stirring speed are presented in Fig. 2. The results were slightly better than those determined with the pre-control amount of 50%, because the precipitate became flocculent and small. However, Fig. 2 was basically the same as Fig. 1, where the third end-point was not obtained. In addition, it occurred only once at a much lower value of 16.22 mL than V_0 (22.65 mL) from the instrument. So the titration speed should be further lowered.

The next titration was a manual operation. Additions were made at time intervals to ensure accurate recordings, especially in the central part of the pH range. The representative potentiometric titration curves of product B are shown in Fig. 3. Although three end-points (V_1 = 5.30 mL, V_2 = 14.75 mL, V_3 = 20.83 mL) were found, V_3 was still lower than V_0 (21.95 mL). Similarly, V_3 in the other two parallel experiments were 21.80 mL and 21.68 mL, also lower than V_0 (22.65 mL and 22.77 mL), respectively. Meanwhile, there were still two parallel experiments from which the third end-point could not be obtained. Although large amounts of flocculent precipitate around the second end-point were gradually dissolved and the solution eventually turned clear, there were still a lot of precipitate stuck on the electrode, which led to the incomplete reaction and early-occurring end-point. Thus, the third inflection point is still significantly affected by the precipitate even though the titration speed is very slow.

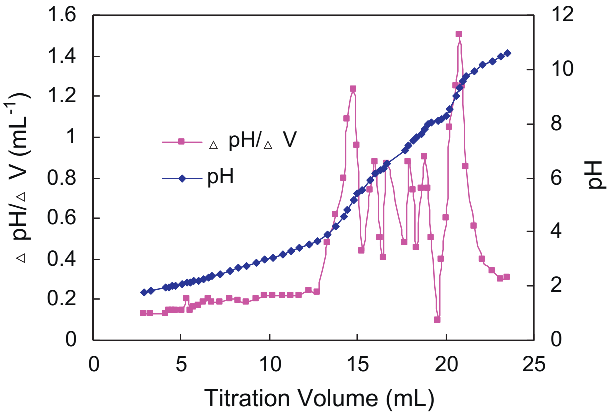

Fig. 3. The typical A–FD titration curves of product B with manual titration, five parallel measurements.

In summary, there were three problems in the existing potentiometric titration experiments. Firstly, the first inflection point was too small to be detected by the ZDJ-4A automatic titrator. Secondly, the second inflection point was affected by the precipitate. The errors caused by these two problems could be reduced by careful operation in accordance with the corresponding experimental procedures. Finally, the third inflection point was seriously affected by the precipitate, which could not be eliminated by adjusting the titration speed.

Since CM-chitosan is only insoluble in the vicinity of isoelectric point, the effect of precipitate on the third end-point could be avoided by operating as follows. Two samples of CM-chitosan with the same quality (0.24–0.26 g) were accurately weighed. One was titrated as described above manually, and V_2 was recorded. Another sample was dissolved in V_2 of sodium hydroxide standard solution (0.1 M), and was titrated with 0.1 M hydrochloric acid solution. At a solution of pH about 7.5, before the precipitate occurred, 0.1 M sodium hydroxide standard solution was added simultaneously with the hydrochloric acid solution to maintain the pH unchanged. After 25.00 mL of hydrochloric acid was consumed, sodium hydroxide was sequentially added stepwise, and V'_3 was recorded when the third titration end-point was reached. Then V_3 could be calculated by $V_3 = V_2 + V'_3$. The typical A–FD titration curves of product B from the proposed method are shown in Fig. 4, and three inflection points are obtained. There are no fluctuations around the third inflection point at the pre-control amount of 50%, indicating no precipitate effect on the third neutralization point.

3.4. Precision and accuracy of the novel method

To estimate the accuracy of the novel method, the parameters of product B were calculated according to the formulas mentioned above, and nine replicates were performed. The results are shown in Table 1. As can be seen, the average δ_{-COONa} of product B was 95.0%, which was in agreement with the fact that product B was primarily Na salt CM-chitosan (Chen & Park, 2003). The DD of product B was slightly higher than that of product A. It may be explained by the slightly lower value of V_2 caused by the formation of precipitate.

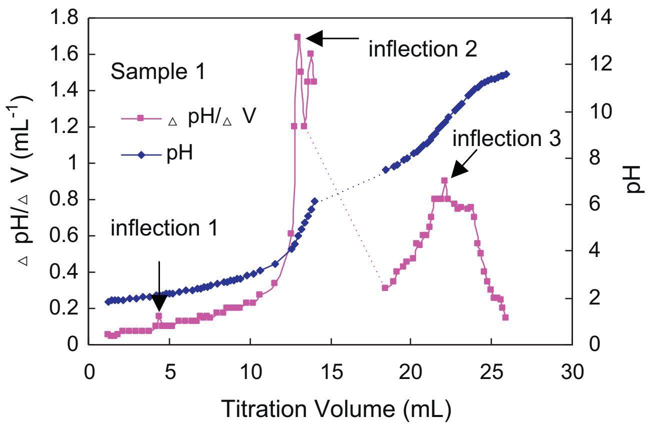

Fig. 4. The typical A–FD titration curves of product B with the novel method at the titration speed of 50% pre-control amount, nine parallel measurements. Dotted lines represented pH maintained at about 7.5, and sample 1 was described in Table 1.

Table 1
The parameters of product B determined by the novel method.

Samples of product B	DS	DD (%)	$\delta_{\text{–COONa}}$ (%)	$\delta_{\text{–COOH}}$ (%)
1	0.917	90.6	90.1	9.9
2	0.880	91.5	91.4	8.6
3	1.149	88.5	91.6	8.4
4	1.164	86.9	94.2	5.8
5	1.051	94.3	94.5	5.5
6	0.975	85.3	99.4	0.6
7	0.902	85.3	102.6	−2.6
8	0.954	85.1	92.2	7.8
9	0.892	84.1	98.7	1.3
Mean	0.987	88.0	95.0	5.0
s	0.109	3.51	4.30	4.30
μ (P^{a} = 0.95)	0.987 ± 0.084	88.0 ± 2.7	95.0 ± 3.3	5.0 ± 3.3

[a] Confidence interval.

Table 2
The parameters of products C, D and E determined by the novel method.

Samples	DS	DD (%)	$\delta_{\text{–COONa}}$ (%)	$\delta_{\text{–COOH}}$ (%)
Product C 1	0.865	89.2	–[a]	145.6
Product C 2	0.841	91.3	–	149.3
Product C 3	0.858	84.8	–	153.7
Mean	0.855	88.4	–	149.5
Product D 1	0.906	91.7	3.8	96.2
Product D 2	1.027	86.3	4.9	95.1
Product D 3	0.929	85.4	−2.7	102.7
Mean	0.954	87.8	2.0	98.0
Product E 1	0.906	86.5	94.1	5.9
Product E 2	0.869	89.0	92.8	7.2
Product E 3	0.987	87.4	94.5	5.5
Mean	0.921	87.6	93.8	6.2

[a] $\delta_{\text{–COONa}} = 0$.

Moreover, as the DD of the raw chitosan sample could be used as the DD of CM-chitosan product (Section 3.2), the DS of CM-chitosan could be calculated according to the following relationship (Bidgoli et al., 2010; Zamani, Henriksson, & Taherzadeh, 2010):

$$DS = \frac{V_2 - V_1}{V_3 - V_2} DD \tag{6}$$

So the novel method was suitable for the simultaneous determination of $\delta_{\text{–COONa}}$, DD and DS. Table 1 showed that the precision of this method was not high, which might be ascribed to the following reasons. Firstly, the relatively large error could be caused by the determination of too many test parameters (V_1, V_2, V_3, V_0, m and w). It could be eliminated by calculating the average value of multiple test results. Secondly, the first titration jump was small, which can lead to large experimental error. Additionally, the measurement of V_2 was influenced by the precipitate, and negative error often occurred. Thus, in order to improve the accuracy of the method, further technological improvements should be explored to solve the last two problems. Products C, D and E were examined to further estimate the accuracy of the method. The results are shown in Table 2. The DS and DD values of products B, C, D and E should be equal, because their preparation method was exactly the same. As can be seen from Tables 1 and 2, however, the DS of product C measured was much lower than that of product B. It could be explained as follows: the $\delta_{\text{–COOH}}$ of product C was much higher than 100%, which indicated that part of $–NH_2$ or $–NHCH_2COOH$ groups existed in the form of $–NH_3^+$ or $–NH_2^+CH_2COOH$. Thus the $C(V_3 - V_0)$ section in formula (2) which represented the –COOH content in the sample became higher, and the value of DS became lower. Additionally, we got a lower end-point of about 1.85 at the first inflection point, while those of products B, D and E were about 2.0–2.2, in good agreement with the literature (Liu et al., 2004; Muzzarelli et al., 1982). It seems that the CM-chitosan hydrochloride could not be accurately measured by this method.

As shown in Tables 1 and 2, the DD values of products B, C, D and E were basically the same, and the DS values of products D and E were basically consistent with that of product B. Product D was isoelectric point resultant. The lost protons of $–CH_2COOH$ were all gained by $–NH_2$ or $–NHCH_2COO^-$ group. Thus the $\delta_{\text{–COOH}}$ of product D should be 100% in theory, which was supported by its experimental value of 98.0% in Table 2. The $\delta_{\text{–COONa}}$ of product E was 93.8%, in accordance with the fact that it was the sodium salt form of product D. Meanwhile it was found that the sodium salt substitution of product E was not complete here. Based on the consistency of the test data, the determination of DD, DS and $\delta_{\text{–COOH}}$ or $\delta_{\text{–COONa}}$ for CM-chitosan by the novel method was reliable.

4. Conclusion

The newly developed potentiometric titration method is an accurate method in determining the DD, DS and $\delta_{\text{–COONa}}$ or $\delta_{\text{–COOH}}$ of CM-chitosan simultaneously, where the effect of precipitate on the third inflection point is eliminated. Since N,O-CM-chitosan used in this study contains both $–NH_3^+$ and $–NH_2^+CH_2COO^-$ in acidic conditions, the novel method is suitable for the parameter determination of N-, O- and N,O-CM-chitosan. But it seems that the CM-chitosan hydrochloride cannot be accurately measured by this method.

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