Capillary electrophoresis for the characterization of synthetic polymers

Oudhoff, K.A.

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Chapter 4

Determination of the degree of substitution and its distribution of carboxymethylcellulose by capillary zone electrophoresis


Abstract

A method based on capillary zone electrophoresis (CZE) has been developed to determine the degree of substitution (DS) of carboxymethylcellulose (CMC). Separations were performed with borate buffer (pH 9, ionic strength 20 mM) as background electrolyte in capillaries of 75 μm I.D., with an applied voltage of 10 kV, and for detection UV absorption at 196 nm was measured. The use of an internal standard (phthalic acid) to correct for mobility variations resulted in a strong improvement of the precision of the DS determination. Experiments with indirect UV detection were performed with buffers containing two monitoring ions to study the origin of the peak widths observed. The results of these experiments indicated that the peak widths actually reflect the variation in mobility, and with that of the degree-of-substitution distribution (DSD) of CMC samples. With the proposed method not only the average DS value but also its DSD could be established for technical CMC samples. Experiments carried out with CMC samples fractionated according to molecular size by SEC showed a small but definite effect of the polymer size on the mobilities observed in CZE. Consequently, DS calibration curves will have to be determined for a specific molar-mass range. Since the size effect is small, a classification of CMCs into low, middle, or high MM will be sufficient to obtain accurate data on their DSD.
Chapter 4

Introduction

Carboxymethylcellulose (CMC) is a widely applied cellulose derivative. It is used as thickener, emulsifier or flow controller in a broad range of products including food, textile, paper, paint and pharmaceuticals, and in the oil industry. An important property of CMC is its water solubility, which depends on the degree of substitution (DS) of the CMCs. The DS value of a CMC sample reflects the average number of carboxylic acid groups per glucose unit. CMCs with a DS value above 0.7 are completely water soluble, giving highly viscous solutions [1]. Knowledge of the DS value of CMCs is essential to predict product properties and to qualify technical samples. Also important to know is the dispersity of the substitution. A more narrow distribution improves the quality of CMC products.

Characterization of the DS of CMC is usually carried out by ashing and titration or, alternatively, by acid hydrolysis [2-5] or enzymatic fragmentation [6], followed by the separation of the resulting monomers using anion-exchange chromatography. With these methods the average DS value of the CMC samples can be determined. Although the methods are time-consuming and laborious, as depolymerization of de cellulose backbone is always needed, they are applied in everyday practice. Another technique used to obtain data on the DS value of CMCs is nuclear-magnetic-resonance (NMR) spectroscopy [7-9]. With this technique both the average DS value and the distribution of the substituents over the positions C-2, C-3 and C-6 on the glucose units can be obtained [9]. The latter distribution indicates the reactivity of the hydroxyl groups of the glucose units in the cellulose backbone. Neither with NMR nor with degradation followed by anion-exchange chromatography it is possible to obtain information on the degree-of-substitution distribution (DSD) in (technical) CMC samples.

Capillary zone electrophoresis (CZE) can be used as a highly efficient tool for the separation of a variety of analytes, including inorganic ions, small molecules and (bio)macromolecules. Separations in CZE are based on the migration of analytes under the influence of an applied electric field according to their electrophoretic mobility as determined by their charge-to-size ratio. It has already been shown by Stefansson [10] that the CZE principle is relevant for the characterization of CMC samples. Prior to the injection Stefansson converted the CMCs to fluorescent derivatives to provide for detectability with laser-induced-fluorescence detection. In the CZE system used, the CMCs migrated against the electro-osmotic flow (EOF), with the compounds with the lowest DS values eluting first.
In the work described in this chapter a CZE system with UV detection has been developed and studied to determine the average DS value and DSD of CMC samples. No derivatization or any other preliminary steps were required. It is shown that this CZE system is suitable to characterize (technical) CMCs for industry in every day practice.

**Experimental**

**Chemicals**

CMC samples with average DS values between 0.69 and 1.64 were obtained from Akzo Nobel (Arnhem, The Netherlands). The molar masses (MMs) of the CMCs were all in the range of 200-400 kDa, as determined by the supplier. CMCs were dissolved in sub-boiled demi water at a concentration of 1 g l\(^{-1}\) and were stored at 4°C for at least 16 h. Prior to the analysis phthalic acid (Acros Organics, Geel, Belgium) was added to the samples as an internal standard at a final concentration of 5 μg ml\(^{-1}\). All other chemicals used were of analytical-grade quality and obtained from certified suppliers.

**Capillary zone electrophoresis**

CZE experiments were performed on an HP\(^{3D}\) instrument (Agilent, Waldbronn, Germany). Fused-silica capillaries of 75 μm I.D. obtained from Composite Metal Services (The Chase, UK) were used with a detection window at 45 cm and a total length of 53.3 cm. The phosphate buffer (pH 7.5) consisted of 2.2 mM sodium dihydrogenphosphate and 7.7 mM disodium hydrogenphosphate. Borate buffer (pH 9) was made by dissolving 10 mM disodium tetraborate-decahydrate in sub-boiled demi water. Prior to all injections the capillary was flushed with 0.1 M NaOH and buffer solution, both for two minutes. Following this, a high voltage of 10 kV was applied for one minute and finally the capillary was filled with fresh buffer solution. Samples were injected by a pressure plug of 20 mbar for 5 seconds. Separations were carried out at 35°C. The applied voltage was 10 kV and UV detection was performed at a wavelength of 196 nm with a bandwidth of 8 nm.

Indirect UV detection was carried out with benzenesulfonic acid and picric acid as the monitoring ions, both at concentrations of 4.5 mM, in a background electrolyte (BGE) of 10 mM KOH (1 mM excess). Detection of the displacement of benzenesulfonate and picrate was performed using diode array detection (DAD) at wavelengths of 210 nm and 357 nm, respectively. Other experimental parameters were the same as in the direct detection mode.
Fractionation by size-exclusion chromatography

Size-exclusion chromatography (SEC) experiments were performed using a Waters 410 Alliance 2690 separations module (Milford, MA, USA) combined with a differential-refractive-index detector. The stainless-steel column was a 300 mm x 7.5 mm I.D. aquagel-OH mixed 8 μm column obtained from Polymer Laboratories (Shropshire, UK), and it was thermostated at 30°C. In all experiments the injection volume was 40 μl and the flow rate 1 ml min⁻¹.

SEC separations were carried out using an eluent of 5 mM sodium nitrate (NaNO₃) in sub-boiled demi water. The CMC 1.22 sample was prepared by dissolving the sample in sub-boiled demi water at a concentration of 5 g l⁻¹. Before use, the sample was stored at 4°C for at least 16h. Finally, NaNO₃ was added until the same concentration as in the eluent. This addition was carried out after 16h, because according to a previous publication [11] dissolving of CMC in the presence of salt may result in scaly particles in the solution. Fractionating was carried out with a Waters fraction collector that was connected to the SEC equipment and was controlled by Millenium Software. The interval time was 30 seconds and the total number of runs was 10, yielding six fractions of 5 ml.

After fractionating, a volume of 1 ml of each fraction was placed in an oven at a temperature of 95°C and was pre-concentrated by evaporation to a final volume of 0.1 ml. The pre-concentrated samples were analysed using the CZE system.

Results and discussion

CZE system

A method for the analysis of CMCs by CZE has previously been described by Stefansson [10]. In this work, prior to the injection the CMC samples were derivatized to allow fluorescence detection. Disadvantages of the derivatization are that it is laborious and an extra source of imprecision. Moreover, it cannot be excluded a priori that the derivatization yield depends on the molecular size or DS of the CMC samples. This would influence the CZE separation and thereby the determination of the DSD. We preferred to develop a CZE method without any derivatization or other preliminary step.

Preliminary experiments showed that CMCs could be detected in the direct UV mode at a low wavelength of 196 nm. However, the sensitivity was only moderate. We succeeded in decreasing the detection limit by applying relatively wide capillaries of 75 μm I.D. Still, the injection of CMC samples with high concentrations (1 g l⁻¹) was essential. To prevent sample overloading, we tried
the use of relatively high concentrations of the BGE salt in the buffer solutions. First, a phosphate buffer of pH 7.5 with a final ionic strength of 25 mM was investigated. Electropherograms obtained for a specific CMC sample in various concentrations (0.5 – 5 g l\(^{-1}\)) showed triangular peaks for all the injected samples with the peak top shifting with the sample concentration (Figure 4.1). It is clear that overloading of the system could not be avoided using this buffer composition. A further increase of the ionic strength of the solution was not possible, since the observed current was already quite high (~29 \(\mu\)A at 10 kV).

Another possibility to reduce sample overloading is to change the type of BGE salt. As has been described in the literature the system loadability is a complex function of the mobilities and \(pK_a\) values of analytes and BGE compounds [12]. Better results were expected from a buffer with a better buffer capacity in relation to the conductivity than phosphate. The choice of buffers that could be tested was limited, since most organic BGE salts would show a high background absorbance at the wavelength used for detection (196 nm). We selected a borate buffer (pH 9) with an ionic strength of 20 mM. Symmetrical peak shapes for the CMC samples were observed for concentrations up to 1 g l\(^{-1}\) (Figure 4.2). All further experiments were performed with this borate buffer, using capillaries of 75 \(\mu\)m I.D., sample concentrations of 1 g l\(^{-1}\) and an applied voltage of 10 kV (~33 \(\mu\)A).
Figure 4.2 Separation of CMC 1.22 of (a) 0.5, (b) 1 and (c) 5 g l\(^{-1}\) with a 10 mM borate buffer (pH 9) at a voltage of 10 kV.

The repeatability of the migration times of the analytes is an important issue, since the differences in mobility between CMC samples with different DS values are small. During the development of the CZE conditions it was found that the run-to-run repeatability of the CMC migration times improved when a preconditioning procedure was applied before each injection. The procedure consisted of flushing with a NaOH-solution followed by the borate buffer, both for two minutes. Next, a voltage of 10 kV was applied for one minute. Finally, new in- and outlet vials were put in place and the capillary was filled with fresh buffer solution.

The run-to-run repeatability was investigated from the individual measurements of two selected CMC samples, which were each injected 7 times. From the electropherograms obtained experimentally the apparent mobilities and the electrophoretic (EOF-corrected) mobilities of the CMCs were calculated. As can be seen in Table 4.1 the relative standard deviations (RSDs) of the EOF-corrected mobilities were lower than those of the apparent mobilities. No systematic relationship between the apparent or EOF-corrected mobilities and the order of injections was found. This indicates that, despite the precondition step, there is a random variation in the velocity of the EOF between successive measurements. These results suggest that the velocity of the EOF could also be unstable during a separation run.
To make it possible to correct for the variance of the velocity of the EOF during a run, we added phthalic acid (5 µg ml⁻¹) to the CMC solutions as an internal standard. Phthalate migrated in the borate buffer with a slightly higher mobility than the CMC polyelectrolytes. For the correction of the electrophoretic mobilities of the CMCs a normalization method was applied. The normalized mobilities of the CMC samples were calculated as the sum of their effective mobility plus the difference between the electrophoretic mobility of the internal standard in the sample solution and its average value obtained with solutions without CMC (n=7). The RSDs calculated for the normalized mobilities of the samples were in the order of 0.1% (Table 4.1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Non-corrected RSD (%)</th>
<th>EOF corrected RSD (%)</th>
<th>EOF and IS corrected RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC 1.14</td>
<td>0.45</td>
<td>0.30</td>
<td>0.09</td>
</tr>
<tr>
<td>CMC 1.22</td>
<td>0.56</td>
<td>0.25</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Commercial well-characterized CMC products with an average DS value between 0.69 and 1.64 were available. The optimised CZE conditions were applied to analyse the commercial CMCs. The electropherograms obtained experimentally are shown in Figure 4.3. For clarity, the X-axes were translated into a mobility scale that was normalized using the internal standard (phthalic acid). The CMC 1.64 product contained glycolic acid as an impurity.

Since the mobility of charged compounds in CZE is proportional to their charge-to-size ratio, the mobility is expected to reflect the DS of the CMCs. A plot of the normalized mobility (µ*) as a function of the average DS value is depicted in Figure 4.4. It was found that the mobility of CMCs increased slightly with their DS. The non-linear behaviour obtained for the CMCs with a high charge density might be the result of counter-ion condensation, which has also been mentioned by Gao et al. [13]. Polyelectrolytes with a high charge density have a net charge in an electrolyte solution that is not proportional to their charge density, due to condensation of counter ions close to the polymer backbone [14]. The significantly smaller peak width observed for CMC 1.64 also indicates the occurrence of counter-ion condensation, especially at high-DS values.
Figure 4.3  Electropherograms of (a) CMC 0.69, (b) 0.84, (c) 1.09, (d) 1.16, (e) 1.22 and (f) 1.64 (1 g l⁻¹). The mobility scale of the CMCs is normalized for the electrophoretic mobility of the internal standard phthalic acid (5.5.5 × 10⁻⁹ m² V⁻¹ s⁻¹). Conditions as in Figure 4.2.

Figure 4.4  Plot of the normalized mobility of CMC as a function of the average DS value.
Despite the non-linearity of the plot it is possible to apply it as a calibration curve to characterize CMC products. Since the slope of the calibration curve is low, it is essential to determine the mobility of CMCs precisely. As has already been described, the internal standard (phthalic acid) helps to minimize the random error of the method to 0.1%, which will result in an error in the DS value of about 1%. Using the calibration plot of mobility against DS, the time scale of the electropherogram of CMC 1.09 was translated into a DS scale (Figure 4.5). It was found that the CMC 1.09 product had a DS varying from 0.7 to 1.7, with a polydispersity of 1.04.

![Figure 4.5](image.png)

**Figure 4.5** DS D of CMC 1.09 as obtained from the CZE separation.

**Indirect UV detection for studying the origin of the CZE separation**

Indirect UV detection in CE is generally applied to measure ionic analytes with little or no response with the UV detector. For indirect detection, a strongly UV-absorbing ionic compound (the monitoring ion) is added to the BGE solution. A decrease of the UV signal, caused by the displacement of the monitoring ion by the analyte, will correspond to an increase in the sample concentration. The degree of displacement is given by the transfer ratio. This transfer ratio can be written –at equilibrium- in terms of the Kohlraush functions, as has been described in detail in literature (see, e.g., ref 15). It was found that the transfer ratio depends on the mobilities of the analyte ion, the monitoring ion and its counter ion.

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In this work we studied the origin of the CMC peak widths using an indirect UV detection system with two monitoring ions. In a multiple-ion system the principle of displacement is similar as in a single system. The UV signal detected depends on the displaced concentrations of the monitoring ions by the analyte ions. The displacement of the two monitoring ions will occur in a ratio depending on the mobilities of the ions involved. It is described by:

\[
\frac{\Delta c_A}{\Delta c_B} = \frac{\mu_A}{\mu_B} \times \frac{(\mu_i - \mu_B)}{(\mu_i - \mu_A)} \times \frac{c_A^0}{c_B^0}
\]

(4.1)

where \(\mu_A\), \(\mu_B\) and \(\mu_i\) are the mobilities of the monitoring ions A and B and the analyte ion, respectively, and \(c_A^0\) and \(c_B^0\) are the concentrations of the monitoring ions A and B in the buffer solution. The signs of the charge of the sample ion and monitoring ions are assumed to be the same. By comparing experimentally determined transfer ratios with the theoretical prediction, it can be verified that peak positions and peak widths truly indicate differences in mobilities (DS values) of CMC samples, rather than being caused by artefacts, such as viscosity effects or adsorption phenomena. To obtain the experimental data on the ratio of displacement, it is necessary to measure the transfer ratios of both monitoring ions at specific UV wavelengths.

We used benzenesulfonate (BS) and picrate (P) as monitoring ions. The mobilities of the monitoring ions were obtained in a borate BGE with the same ionic strength (10 mM) as used in the CMC separation. Values of \(-39.8 \times 10^{-9}\) and \(-34.8 \times 10^{-9}\) m² V⁻¹ s⁻¹ were found for benzenesulfonate and picrate, respectively. To measure the ratio of displacement of benzenesulfonate and picrate, UV detection was applied at 210 and 357 nm simultaneously. With a conventional spectrophotometer the molar-absorption coefficients (\(\varepsilon\)) of benzenesulfonate and picrate were measured at these two wavelengths. At 210 nm the \(\varepsilon_0\) values found were \(6.15 \times 10^3\) and \(8.81 \times 10^3\) 1 mol⁻¹ cm⁻¹ and at 357 nm 0 and \(8.78 \times 10^3\) 1 mol⁻¹ cm⁻¹, for benzenesulfonate and picrate, respectively. From the signals obtained at 210 and 357 nm the changes in benzenesulfonate and picrate concentrations and their ratio were calculated.

A set of four CMCs with average DS values of 0.69, 0.84, 1.22 and 1.64 were separated using a buffer solution containing 4.5 mM benzenesulfonic acid and 4.5 mM picric acid dissolved in 10 mM KOH. A voltage of 10 kV was applied. As an example Figure 4.6 shows the two detection signals for CMC 0.69. It was found that the ratio of the displaced concentration of benzenesulfonate
Figure 4.6  Indirect detection signals of CMC 0.69 at (a) 210 nm specific for benzenesulfonate (BS) and (b) 357 nm specific for picrate (P). Data shown (○) reflect the displaced concentration of BS and P over the peak width of CMC 0.69.

Figure 4.7  Ratio of the displaced concentrations of benzenesulfonate (BS) and picrate (P) versus the electrophoretic mobility of CMC 0.69 (○), 0.84 (⊙), 1.22 (△) and 1.64 (□) compared with the predicted values calculated from Eq. 4.1 (-----).
and picrate decreased with increasing mobility over the width of the peak. An overall overview of the ratio between the displaced concentrations and the mobilities for all CMC samples compared with the predicted trend line is depicted in Figure 4.7. It can be seen that the behaviour observed experimentally followed the same trend as the predicted line. The small systematic deviation between experimental results and the prediction may be caused by the non-linear response of UV detection in (cylindrical) capillaries. The sensitivity for the (displacement of the) monitoring ions in this set-up may deviate to some extent from the absorbance values measured in a cuvette. The results of these experiments indicated that the CZE separations of CMCs are based on the charge density of the polyelectrolytes. It also justifies using the peak widths for the determination of the DSD of the CMCs.

Influence of the size of CMCs on the electrophoretic mobility

With the method of indirect UV detection described above it has been shown that the peak widths obtained experimentally reflect the dispersity of mobilities of CMCs. However, it is still possible that the distribution of the size of the polyelectrolytes has an effect on the electrophoretic mobility, rather than variations in the DS. This aspect was studied by CE separations of SEC fractionated samples.

A solution of CMC 1.22 (5 g l⁻¹) was fractionated using an aquagel-OH mixed column and an eluent consisting of 5 mM NaNO₃ dissolved in water. This SEC system was described as very successful for the separation of CMCs when a high salt concentration was used [11]. However, in preliminary experiments it was found that such high salt concentrations in the fractionated samples caused severe peak distortion in the electrophoretic system. For this reason the SEC fractionating experiments were carried out using a lower salt concentration. Six fractions of the CMC 1.22 sample were collected with a fractionation time of 30 seconds. Re-injection of the fractions in the SEC system showed that they contained polyelectrolytes with different average MMs.

The buffer composition used for the CZE separation of the original CMCs (borate buffer, pH 9, ionic strength 20 mM) was also used for the separation of the fractions. Figure 4.8 shows the electropherograms of the six fractions of the CMC 1.22 sample (fraction 1 highest MM; fraction 6 lowest MM). It was found that the electrophoretic mobilities of the CMC fractions increased slightly (0.6%) with increasing fraction number. This behaviour suggests that there is an actual effect of the polyelectrolyte size on the CZE separation. Another possible explanation for the behaviour observed for the CMC fractions is that the charge density of polyelectrolytes with a lower
Determination of the DS and its distribution of CMC by CZE

degree of polymerisation is higher than that of high-MM CMCs. This might be caused by varying reaction activities for CMCs with different polymeric chain lengths. However, such a dispersity in charge density is not to be expected, since the chemical modification of cellulose is performed in the solid state. Another indication of the influence of the size on the CZE separation was obtained with a high-MM CMC of $10^6$ Da. When the normalized mobility of this sample was used in the DS calibration curve, a DS value was calculated that was approximately 10% lower than the value provided by the supplier. This deviation is significantly higher than the imprecision obtained in the repeatability study. These results demonstrated that the effect of the size of the polyelectrolytes could not be fully neglected and that the calibration curves of mobility versus DS value are only valid when standards within a specific molar-mass range are applied. However, the molar-mass effect is relatively small. Commercial CMCs are generally classified as low-, middle- or high-MM products. Calibration according to this classification will be sufficient to determine data on the DS of CMCs accurately.

![Figure 4.8](image)

**Figure 4.8** CZE separations of SEC fractions of CMC 1.22 (1 ~ high MM, 6 ~ low MM). CZE conditions similar as in Figure 4.2.
Acknowledgements

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References