Development of a Capillary Zone Electrophoresis Method for the Analysis of Four Extracellular Matrices Commonly Found in Foods with Functional Claims

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Abstract
Quantification of four extracellular matrices commonly found in foods with functional claims was conducted using capillary zone electrophoresis with direct ultraviolet (UV) absorbance detection at 200 nm. The four polymeric compounds, namely, proteoglycan, chondroitin sulfate, hyaluronic acid, and collagen, were separated with 100 mM borate buffer (pH 10.0) using capillaries coated with poly-N,N-dimethylacrylamide on the inner wall, which almost completely suppressed the electroosmotic flow to give reproducible migration times for the analytes. The addition of 5 mM sodium dodecyl sulfate to the running solutions improved the shapes of the peaks and the linearity of the calibration curves. Some commercial products, including the extracellular matrices under investigation, were analyzed with the method developed.

Keywords: Capillary zone electrophoresis; Proteoglycan; Chondroitin sulfate; Hyaluronic acid; Collagen; Foods with functional claims

Introduction
In recent years, people have taken more interest in being healthy. In addition to the ingestion of dietary supplements, mainly vitamins, minerals, and amino acids, many kinds of “foods with functional claims” are being taken on a daily basis. Such foods can be brought to market after research evidence of their inherent health benefits are displayed on the packages, whereas those labeled as “food for specified health use” require more stringent examination by certified national institutions. However, at the very least, quality control should be performed on all supplements to quantify and identify the functional substances in these foods, even if the effects of these ingredients are milder in comparison with their pharmaceutical counterparts. For some ingredients, appropriate methods of quantification need to be developed in order to perform through quality control analyses.

In the present work, a simple capillary electrophoresis (CE) method was developed to quantify four extracellular matrices, namely, proteoglycan (PG), chondroitin sulfate (CS), hyaluronic acid (HA), and collagen. These are often included in foods with functional claims and are used as moisturizing ingredients for cosmetic purposes. PG possesses a unique structure consisting of a core protein and numerous glycosaminoglycan side chains [1], and has garnered attention for its epidermal growth factor properties

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Additionally, glycosaminoglycan was revealed to have some biological activity with oral administration in cartilage aging and the maintenance joint health [3]. In general, these charged matrices are analyzed using conventional slab gel electrophoresis followed by dyeing [4]. Unfortunately, this method of analysis may not be adequate in quantification. For instance, better quantitative data can be obtained for CE from electropherograms carried out by high-performance detectors equipped with flow detection cells.

The unit structure of PG, CS and HA is represented in Fig.1. PG of animal origin has glycosaminoglycan side chains that are typified by CS, which possesses a sulfate group and a carboxyl group in the unit structure. Most CS within an organism exist as parts of PG. HA is also a kind of glycosaminoglycan in that it has only carboxyl functional groups; however, it does not exist as part of PG. The amino acid composition of collagen in a mammalian skin and a fish skin is given as 33.9% and 32.9% of glycine, 10.8% and 12.6% of proline, 11.4% and 10.9% of alanine, and 6.7% and 9.5% of hydroxyproline, and additionally, the aromatic amino acids, namely, histidine, tryptophan, phenylalanine, and tyrosine, are found in proportions 0.5%, 0%, 1.3%, and 0.3%, respectively [5]. This is indicative of collagen’s weak light absorbance at common ultraviolet (UV) wavelengths, i.e., at 254 nm. This wavelength is often employed in conventional analysis. PG, CS, and HA have no chromophores in their structure. On the other hand, CE is sensitive to a shorter detection wavelength, namely, at 200 nm, which ultimately allows for the detection of saccharides, non-aromatic amino acids, and their corresponding oligomers (including those of PG, CS, HA, and collagen) without the need for staining or derivatization. Furthermore, CE has been successfully employed in the separation of biological ionic polymers such as proteins, nucleic acids, and polysaccharides [6-8].

![Fig. 1. Schematic structure of proteoglycan (PG) (a), chondroitin sulfate (CS) (b), and hyaluronic acid (HA) (c).](image)

In this work, capillary zone electrophoresis (CZE) separation is applied to PG, CS, HA, and collagen using detection of UV absorption at 200 nm. Real products, including the four extracellular matrices, were also analyzed with the intention of developing simple quality control protocols for foods with claims of functional health benefits.

### 2. Experimental

#### 2.1. Chemicals and reagents

Proteoglycan (500-1000 kD), chondroitin sodium salt, sodium bio-hyaluronic acid (1200 kD), and type 2 collagen were purchased from FUJIFILM Wako Pure Chemical, Kishida Chemical, Shiseido and from The Collagen Research Center. These were used as standard samples for PG, CS, HA, and collagen to build the present CZE method; and make calibration curves for the four analytes under investigation. Commercial food products, namely, proteoglycan (extracted from salmon nasal cartilage) -containing dietary supplement (Proteoglycan F), HA powder, shark cartilage powder, and a supplement tablet labelled as containing CS, PG, HA and collagen were obtained from ICHIMARU PHARCOS, Kibun Food Chemical, Marine Cartilage, and a food manufacturer. Phthalic acid of an internal standard used for quantification was purchased from Wako Pure Chemical.

Sodium borate (Na2B4O7·10H2O) and sodium dodecyl sulfate (SDS) were obtained from Kishida Chemical and Wako Pure Chemical, respectively. Coating material for the inner capillary wall, namely, poly-N,N-dimethylacrylamide (PDMA), N,N-dimetylacrylamide (1), 3-(trimethoxysilyl)propylmethacrylate (2), N,N,N,N-tetramethyletylenediamine (3), and ammonium peroxodisulfate (4) were purchased from Aldrich [(1), (2)], and nacarai tesque [(3), (4)]. All chemicals were of analytical grade.

#### 2.2. Apparatus

All CE measurements were performed using a Hewlett-Packard 3DCE system. Fused silica capillary tubes (50 µm i.d. and 375 µm o.d.) were purchased from Polymicro technology and used with 600 mm for the bare sample tube and 1100 mm for the sample tube coated with PDMA on the inner wall. Their effective lengths were 510 and 1010 mm, respectively. Capillaries coated with PDMA on the inner wall were prepared using Wan’s method but with a few procedural adjustments applied [9].

#### 2.3. Procedure

To create stock solutions, each standard sample of PG, CS, or HA was dissolved in water and made up to a concentration of 5.0 mg/mL. Only collagen was dissolved in the presence of 10 mM SDS with an accompanying incubation at 37 °C for 30 min because of its poor solubility. The stock solutions were diluted with water to the...
appropriate concentration before analysis. Real samples were dissolved in water and the solutions were filtrated using a 0.45 μm PTFE membrane (Millipore) before analysis.

CZE measurements were performed using a 100 mM borate buffer solution (pH 9.2) or 100 mM borate buffer solutions (pH 10.0) with or without 5 mM SDS. The applied voltage was set at 15 kV (for bare capillaries of 600 mm) or ~30 kV (for PDMA coated capillaries of 1100 mm) where the operating current was indicated as 19 or 30 μA, respectively. Operating temperature was set at 20 °C. Sample solutions were injected with a pressure of 25 mbar for 20 s. The detection wavelength was set to 200 nm.

3. Results and discussion
3.1. CZE separation of the four extracellular matrices

Figure 2 shows CZE separation of four standard analytes for PG, CS, HA, and collagen using 100 mM borate buffer at pH 9.2 with a bare capillary tube. Under such conditions, all four polymeric compounds migrated to the cathode due to strong electroosmotic flow (EOF) and were almost separated out from each other. The four compounds were negatively charged under the present conditions, as shown in Fig. 2. Since HA had a carboxyl group and CS had both a carboxyl and a sulfonate group in their unit structure, both anionic groups would be completely dissociated in the running solutions of this work (pH ≥ 9.2). Therefore, the order of the absolute values for electrophoretic mobility were CS > HA, which was in accordance with the migration order seen in Fig. 2. PG predominantly consisted of CS, and thus, the electrophoretic mobility was close to that of CS. Collagen had the smallest electrophoretic mobility among the four analytes at pH 9.2 and 10.0 (see Fig. 3 (a)) in the absence of SDS in the running solution.

With bare capillaries, the EOF gradually decreased depending on the number of measurements obtained, and finally, PG and CS became not to be detected; this could be attributed to extensive adsorption of any polymeric analytes on the capillary’s inner walls. Some improvement was achieved by washing between each run, but reproducibility in the migration times remained poor despite these efforts.

Next, separations were attempted using capillaries coated with PDMA on the inner walls. Figure 3 shows CZE separations using a PDMA-coated capillary with a 100 mM borate buffer (pH 10.0) in the absence (a) and the presence (b) of 5 mM SDS. PDMA coating suppressed non-specific adsorption of samples and EOF almost completely [9-12]. In the absence of an EOF, migration order in the absence of SDS (Fig. 3(a)) was reversed when compared with the order seen in Fig. 2. The PDMA coating brought marked improvements in migration time reproducibility mainly because of sufficient suppression of the EOF. Changes in the pH of the running buffer solutions from 9.2 to 10.0 resulted in some improvement in peak broadening for CS, as indicated in the change in theoretical plate numbers of the CS peaks from 19.4 (pH 9.2) to 36.0 (pH 10.0). This, in turn, improved separation between CS and PG from Rs = 0.98 to 1.04. Given this, future experiments were conducted at pH 10.0.

![Fig. 2. CZE separation of four standard compounds for the extracellular matrices using a 100 mM borate buffer (pH 9.2, as a running solution) with a bare capillary. Conditions: Applied potential, 15 kV (Operating current, 19 μA); Capillary, a bare one; total length, 600 mm (eff. Length, 510 mm); 0.050 mm i.d, (0.365 mm o.d.); Injection, 25 mbar, 20 s; Temperature, 20 °C; detection, 200 nm. Peak assignment: 0, EOF; 1, collagen; 2, HA; 3, PG; 4, CS.](image1)

![Fig. 3. CZE separation of four standard compounds for the extracellular matrices using a 100 mM borate buffer (pH 10.0, as a running solution) with a PDMA coated capillary in the absence (a), and the presence of 5 mM SDS (b). Conditions: Applied potential, -30 kV (Operating current, 30 μA); Capillary, a PDMA coated one; total length, 110 mm (eff. Length, 101 mm); 0.050 mm i.d, (0.365 mm o.d.); Injection, 25 mbar, 20 s; Temperature, 20 °C; detection, 200 nm. Peak assignment: 1, collagen; 2, HA; 3, PG; 4, CS.](image2)
HA with the running buffer solution containing 5 mM SDS, as shown in Fig. 3 (b). Additionally, the HA peak became narrower in comparison with the peak obtained in the absence of SDS. Furthermore, addition of 5 mM SDS to the running buffer solution improved the linearity of the calibration curves. Analysis of a supplement tablet, however, suffered from interference peaks that appeared in the presence of SDS in the running buffer solution due to the diluting agents. To minimize this effect, the running solution used for analysis of the tablet was kept at a concentration of 100 mM borate buffer (pH 10.0) in the absence of SDS (see below).

All four matrices standards were detected as relatively wide peaks in their respective electropherograms. This meant that complex variations in both the length and partial structure for each of the standards were present, creating wide ranges in the ratios between the hydrodynamic radius and the effective charges. On the other hand, the present CZE method performed excellently during separation of another HA product as shown in Fig. 4. This result indicated that the HA product consisted of isoforms undergoing stepwise changes that influenced the ratio between the hydrodynamic radius and the effective charges, thus suggesting that variations in the number of carboxyl groups and/or sulfate groups that had been removed from the HA chains.

Detection limits (S/N=3) were 0.058, 0.075, 0.038, and 0.009 mg/mL (in the absence of SDS) or 0.045, 0.040, 0.014, and 0.021 mg/mL (in the presence of 5 mM SDS) for CS, PG, collagen, and HA, respectively. Standard variations in the migration times were 0.59, 0.57, 0.51, and 0.50% (n = 5) in the absence of SDS, and 0.24, 0.26, 0.12, and 0.25% (n = 5) in the presence of 5 mM SDS for CS, PG, HA, and collagen, respectively.

### 3.3. Analysis of real samples

Figure 5 shows CZE separations of shark cartilage powder (a) and a PG-containing dietary supplement (Proteoglycan F) (b) using a running solution in the presence of 5 mM SDS, the same as that seen in Fig. 3 (b). Judging from the electropherogram, the cartilage powder consisted of predominantly CS and virtually no PG. On the other hand, in Fig. 5 (b), PG was detected at 21 min as a major component with a peak that was similar to that of the PG sample standard. The amount of PG in the food material sample was estimated to be 21.3% (w/w) and was almost in accordance with the product’s labels (The indicated value is 20%). (Fig. 5 (b)).

Figure 6 shows the results of the supplement tablet which contained CS, PG, HA, and collagen; their respective concentrations were estimated to be 1.83%, 3.25%, 0.14%, and 1.60% (w/w) per tablet. This result was obtained using a running solution in the absence of SDS, as seen in Fig. 3 (a). Serious interference from the large peaks of other excipients caused major overlap with the four analytes in the presence of SDS in the running solution (data not shown). Unfortunately, the separation between CS and PG in Fig. 6 seemed poorer than that in Fig. 3 (a). This could be attributable to larger variations in the structure of PG included in the supplement tablet, which tended to broaden its peak in comparison to the peak of the standard PG (Fig. 3 (a)).

### Table 1. Calibration curves when using (A) the running solution in the absence of SDS or (B) the running solution in the presence of 5 mM SDS in the concentration range from 0.1125 to 1.1250 mg/mL for all of the four analytes.

| Samples | Equations | $R^2$ (n=4) |
|---------|-----------|-------------|
| (A) CS  | $y = 25.54x + 0.100$ | 0.9962 |
| PG     | $y = 31.42x + 2.199$ | 0.9652 |
| HA     | $y = 26.55x + 2.677$ | 0.9807 |
| Collagen | $y = 50.73x - 3.750$ | 0.9788 |
| (B) CS  | $y = 32.88x + 0.541$ | 0.9941 |
| PG     | $y = 58.29x - 2.145$ | 0.9964 |
| HA     | $y = 72.12x - 1.127$ | 0.9883 |
| Collagen | $y = 20.85x + 0.944$ | 0.9975 |
In conclusion, the present CZE method is a simple one with a relatively short analysis time and without the need to derivatize the analytes of interest. This simple method has the potential to be useful in quality control analyses of food items that have functional claims, namely, those that contain CS, PG, HA, and collagen.

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Fig. 5. CZE analysis of products for special dietary use: shark cartilage powder (a) and PG-containing dietary supplement (Proteoglycan F) (b). Conditions: Same as in Fig. 3 (b). Peak assignment: 3, PG; 4, CS.

Fig. 6. CZE analysis of a tablet as a food with functional claims with a PDMA coated capillary. Conditions: Same as in Fig. 3(a). Peak assignment: 1, collagen; 2, HA; 3, PG; 4, CS.