

# Development of a Capillary Electrophoresis Method for the Characterization of Collagens in Cartilage Tissue

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## Abstract

The use of capillary electrophoresis (CE) for the separation of peptides specific to type I and type II collagen is evaluated. The aim of this work is to develop a method to characterize cartilage, cartilage repair tissue, and tissue engineered cartilage. The analysis is dependent on the cleavage of collagen into constituent peptides by cyanogen bromide. A number of these peptides are specific to the collagen type. CE is evaluated for the separation of these specific peptides using uncoated and coated capillaries over a wide range of pH and buffer concentrations. Separation of peptides specific to type I and type II collagen is achieved using a Supelco CElect N capillary and a 100mM phosphate buffer at pH 6. Meniscal cartilage is characterized using this method. The proportion of type I collagen to type II collagen corresponds well with that reported by others and indicates the potential of this method for the characterization of cartilage.

## Introduction

Collagen quantitation is valuable in assessing the repair mechanism and regimes in cartilage and also in the assessment of tissue engineered cartilage. Articular cartilage contains predominantly type II collagen, whereas meniscal cartilage contains a mixture of type I and type II collagen, the levels of which are dependent upon location.

Conventional collagen typing analysis by cyanogen bromide (CNBr) cleavage of the tissues followed by densitometry of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the cyanogen bromide peptides (CB peptides) is semiquantitative at best, has low sensitivity, and is labor intensive (1–2). Black et al. (3) developed a qualitative high-performance liquid chromatography (HPLC) method to analyze bovine collagens I, II, and III. Harwood et al. (4) reported a “semiquantitative” method for the analysis of collagens I and III in tissues. Miller et al. (5) reported the analysis of collagens I, III, and V in noncartilaginous human tissue samples by cation exchange

chromatography and HPLC. A capillary electrophoresis (CE) method was described by Deyl and Miksik (6) for the analysis of collagens I, II, V, IX, and XI using uncoated fused-silica capillaries at pH 9. Deyl et al. (7) also described a sodium dodecyl sulphate capillary gel electrophoresis (SDS-CGE) method for the separation of collagen type I chain polymers. The method used a 4% non-cross-linked polyacrylamide gel. The CE analysis of CNBr-cleaved collagen I peptides was reported by Novotna et al. (8). The separation was performed in a phosphate buffer at pH 2.5 to prevent solute–capillary wall interactions. Deyl et al. (9) described a method for the quantitation of collagen types I, III, and V in tissue using CE. Separation was performed in an uncoated fused-silica capillary with an acidic phosphate buffer to prevent solute–capillary wall interactions. This method was used to characterize the percentage of these collagens in tissue samples.

This work describes the development and validation of a novel method for the characterization of collagen within cartilage tissue by CE using a coated capillary. It has been developed for the separation and quantitation of type I and type II collagen specific peptides resulting from the digestion of tissues with CNBr.

## Experimental

### Materials

Glacial acetic acid, orthophosphoric acid, and sodium hydroxide (1N) were obtained from BDH Laboratory Chemicals Division (Poole, Dorset, U.K.). Monobasic sodium phosphate was obtained from Sigma UK (Poole, Dorset, U.K.).

Collagen I and collagen II were obtained from Sigma. Ovine tendon (which is comprised of mainly type I collagen), ovine articular cartilage (which is comprised of mainly type II collagen), and meniscal cartilage were obtained from two-year-old sheep from a local abattoir.

### Apparatus

CE analysis was carried out using a Hewlett-Packard (Stockport, Cheshire, U.K.) HP 3D CE fitted with an HP diode array detector. All experiments were performed at 25 kV and 20°C.

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Samples were introduced by hydrodynamic injection. Ultraviolet (UV) detection was at 200 nm.

### Sample and capillary preparation

Samples were cleaved in CNBr, freeze-dried, and stored at  $-20^{\circ}\text{C}$  until required. Prior to analysis, samples were re-suspended in 1% (v/v) acetic acid at a concentration of 1 mg/mL and incubated at  $60^{\circ}\text{C}$  for 1 h.

Uncoated fused-silica capillaries were washed with NaOH (1N for 10 min), deionized water (10 min), and run buffer (40 min) prior to initial analysis. Between subsequent runs, the capillary was washed with run buffer (5 min), 0.1M  $\text{H}_3\text{PO}_4$  (2 min), and run buffer (7 min).

Coated capillaries were washed with deionized water and run buffer (10 min each) prior to initial use. After initial conditioning, coated capillaries were washed with run buffer for 8 min between each analysis.

### Evaluation of different capillaries

Two capillaries were evaluated for this work: an uncoated fused-silica capillary (50- $\mu\text{m}$  i.d., 54-cm effective length) and a Supelco (Sigma) CElect N coated column (75- $\mu\text{m}$  i.d., 60-cm effective length). In both cases, CNBr-cleaved ovine tendon was injected hydrodynamically (20 s, 50 mbar) and run in 50mM dibasic sodium phosphate adjusted to pH 2.5 with NaOH.

### Evaluation of buffer

Samples of CNBr-cleaved ovine articular cartilage and ovine tendon were analyzed using a Supelco CElect N (75- $\mu\text{m}$  i.d.) coated capillary. The optimum pH of the separation was determined by adjusting the pH of the sodium phosphate buffer between 2.5 and 7.0 using NaOH.

Collagen type I was analyzed on this column. The pH of the buffer was kept at pH 6.0 while its concentration was varied from 25 to 100mM.

### Determination of collagen types I and II in tissue

CNBr-cleaved samples were analyzed using a Supelco CElect N (75- $\mu\text{m}$  i.d.) coated capillary using 50mM phosphate at pH 6.0 as run buffer.

A mixture of ovine tendon and ovine cartilage was run to assess the possibility of resolving collagen type I and type II marker CB peptides. CNBr-cleaved Sigma standard collagen I and II were analyzed in a range of concentrations using the aforementioned conditions to establish calibration curves, against which known mixtures of cartilage and tendon were quantitated. Two control Sigma standards, collagen type I and collagen type II (approximately 200  $\mu\text{g}/\text{mL}$ ), were quantitated to assess the reproducibility of the method.

Accurate concentrations of the mixtures and the standards were established using a total collagen assay and a total protein assay (Micro BCA, Pierce, Chester, Cheshire, U.K.).

## Results and Discussion

### Evaluation of different capillaries

To minimize protein-capillary wall interaction, the uncoated fused-silica column was evaluated at pH 2.5. Samples of CNBr-

cleaved ovine tendon (mainly collagen type I) were analyzed using these conditions. The separation achieved with this method was poor. After 3 runs, the reproducibility was lost, and resolution severely deteriorated. This effect was also reported by Deyl et al. (9).

The capillary was replaced with a Supelco CElect N coated capillary, and a typical separation of ovine tendon by CE at pH 2.5 using 50mM phosphate buffer is shown in Figure 1. This separation corresponds reasonably well with the separation reported by Deyl et al. (9) The earlier-eluting collagen CB peptides correspond well to the assignments by Deyl et al.; however, the use of this column seems to have improved the resolution of the later-eluting peaks, revealing more CB peptides.

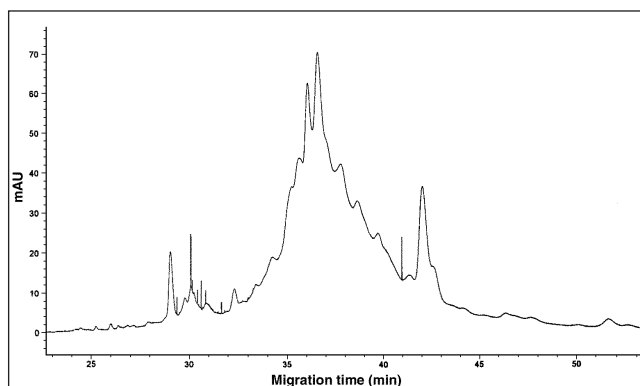
The analysis at pH 2.5 using the coated capillary was repeated up to 40 times before loss of reproducibility and capillary contamination became a problem. It is believed that the acidic buffer was slowly removing the capillary coating.

CNBr-cleaved ovine articular cartilage (mainly collagen type II) was run using the coated capillary with phosphate buffer at pH 2.5. Separation was poor, and no specific CB peptides unique to cartilage or tendon could be identified, ruling out the use of these conditions for typing collagen types I and II in tissues.

### Buffer evaluation

Use of the coated column allowed the use of 50mM phosphate buffer between pH 2.5 and pH 6.0 without potential absorption problems. The effect this has on the separation of CNBr-cleaved ovine tendon (type I) and ovine articular cartilage (type II) samples can be seen in Figure 2. Even a small change in the pH of the buffer can have a large effect upon the separation. A similar effect was seen for the ovine tendon (type I) sample. The most effective separations for both the CNBr cleaved tendon and cartilage were achieved using phosphate buffer at pH 6.0. The electropherograms indicated specific peptides for tendon and cartilage. At higher pH values, detail was lost in the separation for both samples.

A sample of CNBr-cleaved collagen type II was analyzed in 25, 50, and 100mM phosphate buffer, all adjusted to pH 6.0. Electropherograms for each of these analyses are shown below (Figure 3). At a buffer concentration of 25mM, all resolution of



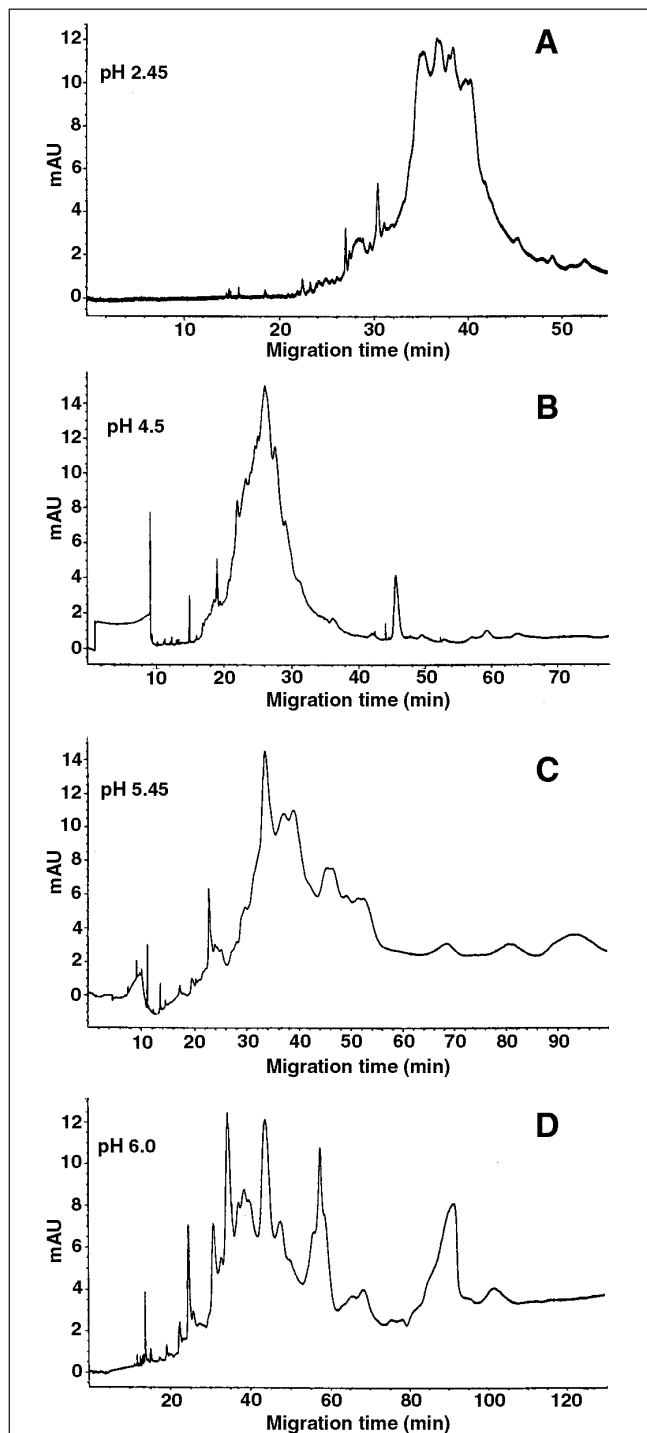
**Figure 1.** Electropherogram of CNBr-cleaved ovine tendon (collagen type I) at pH 2.5. CE conditions: Sulpelco CElect N capillary (54 cm  $\times$  75- $\mu\text{m}$  i.d.); hydrodynamic injection, 20 s and 50 mbar; buffer, 50mM sodium phosphate; running voltage, 25 kV at  $20^{\circ}\text{C}$ ; UV detection, 200 nm.

the CB peptides was lost. This may be due to an "ionic strength" effect or to the inability of the run buffer to stabilize the pH of the peptides while they migrate through the capillary. With the 50mM phosphate buffer, the separation was good; however, there still appeared to be an unresolved peak comigrating with the collagen II CB marker peptide. This peptide was fully resolved from

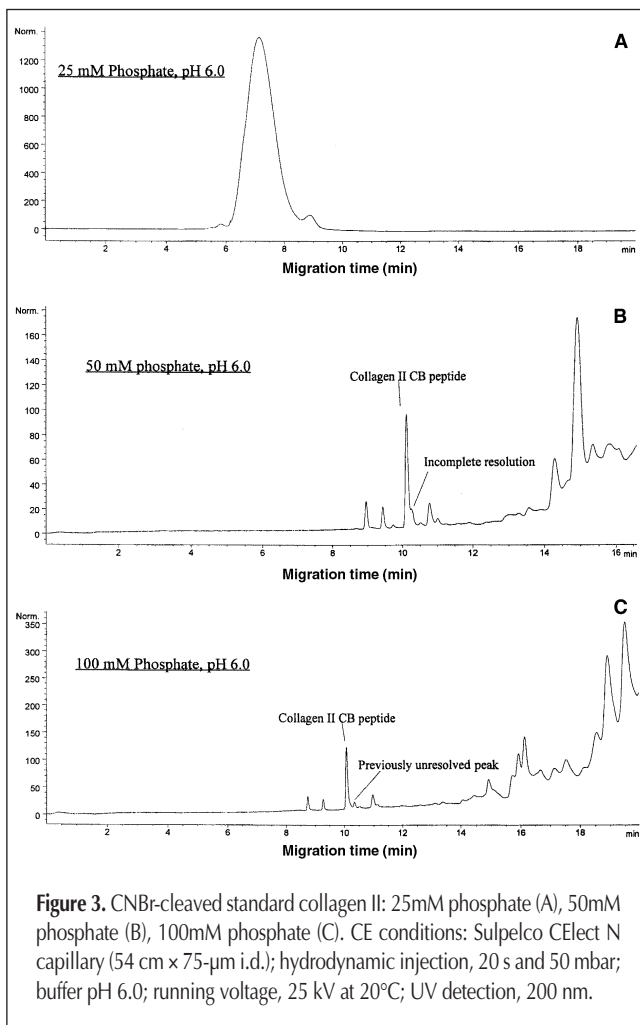
the CB marker peptide when the concentration of the buffer was increased to 100mM.

### Determination of collagen types I and II in tissue

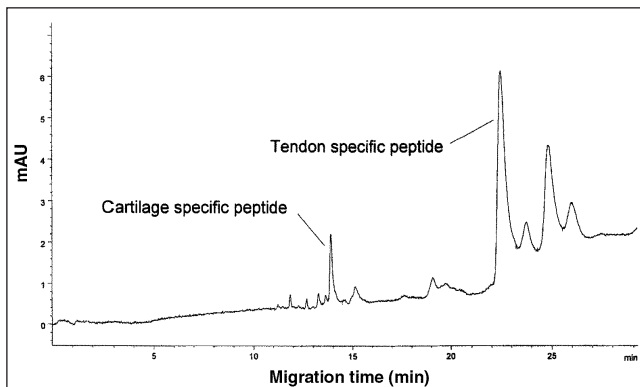
Using the Sulpeco coated column with the phosphate buffer at pH 6.0, specific peptides were observed for cartilage and tendon.



**Figure 2.** Effect of pH on the separation of CNBr-cleaved ovine articular cartilage (collagen type II): pH 2.45 (A), pH 4.5 (B), pH 5.45 (C), pH 6.0 (D). CE conditions: Sulpeco CElect N capillary (54 cm x 75- $\mu$ m i.d.); hydrodynamic injection, 20 s and 50 mbar; buffer, 50mM sodium phosphate; running voltage, 25 kV at 20°C; UV detection, 200 nm.

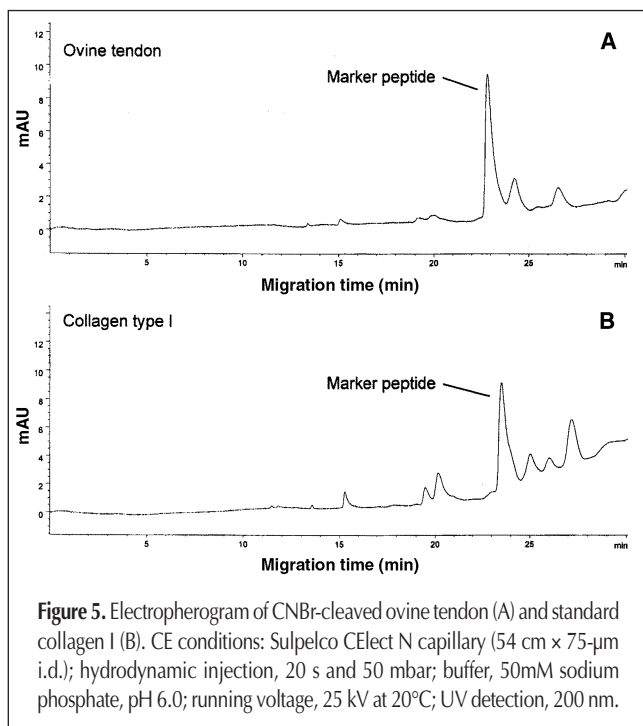


**Figure 3.** CNBr-cleaved standard collagen II: 25mM phosphate (A), 50mM phosphate (B), 100mM phosphate (C). CE conditions: Sulpeco CElect N capillary (54 cm x 75- $\mu$ m i.d.); hydrodynamic injection, 20 s and 50 mbar; buffer pH 6.0; running voltage, 25 kV at 20°C; UV detection, 200 nm.

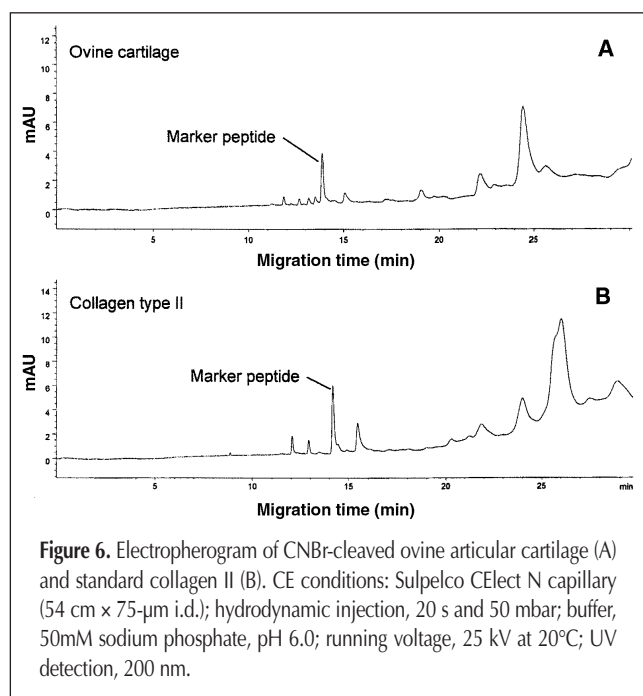


**Figure 4.** Mixture (50:50) of CNBr-cleaved ovine tendon and ovine articular cartilage. CE conditions: Sulpeco CElect N capillary (54 cm x 75- $\mu$ m i.d.); hydrodynamic injection, 20 s and 50 mbar; buffer, 50mM sodium phosphate, pH 6.0; running voltage, 25 kV at 20°C; UV detection, 200 nm.

Figure 4 shows the electropherogram obtained for the analysis of a 50:50 mixture of CNBr-cleaved tendon and cartilage. To establish that the CB marker peptides are cleavage products of collagen type I and collagen type II, standard collagen I and collagen II were analyzed. Figures 5 and 6 clearly show that the CB peptide specific to tendon (retention time, 4.1 min) is a collagen I cleavage peptide and that the CB peptide specific to cartilage (retention time, 23.6 min) is a collagen II cleavage peptide. In both cases, the migration times of the tissue marker CB peptides corresponded well to those of the collagen marker CB peptides, and UV spectral analysis showed that both pairs of peaks had iden-



**Figure 5.** Electropherogram of CNBr-cleaved ovine tendon (A) and standard collagen I (B). CE conditions: Sulpelco CElect N capillary (54 cm  $\times$  75- $\mu$ m i.d.); hydrodynamic injection, 20 s and 50 mbar; buffer, 50mM sodium phosphate, pH 6.0; running voltage, 25 kV at 20°C; UV detection, 200 nm.



**Figure 6.** Electropherogram of CNBr-cleaved ovine articular cartilage (A) and standard collagen II (B). CE conditions: Sulpelco CElect N capillary (54 cm  $\times$  75- $\mu$ m i.d.); hydrodynamic injection, 20 s and 50 mbar; buffer, 50mM sodium phosphate, pH 6.0; running voltage, 25 kV at 20°C; UV detection, 200 nm.

tical UV spectra. A range of CNBr-cleaved Sigma collagen I and II standards were then analyzed. Calibration curves were constructed from the peak areas of the collagen I and II marker CB peptides in the 100–1100  $\mu$ g/mL range. The correlation coefficients  $r$  were calculated to be 0.995 and 0.994, respectively. The control standards for both collagen I and II gave a relative standard deviation of less than 5%. The purity of these standards was confirmed by a total collagen assay (hydroxyproline assay), but because this assay is not sensitive to the type of collagen, contamination is possible from other collagen types. Validated specific collagen standards are not available. For this reason, absolute quantitation was not possible, but a relative determination against the Sigma standards was performed. To test the accuracy of this approach, mixtures containing known quantities of CNBr-cleaved tendon and cartilage samples were then analyzed, and the concentration of collagens I and II were determined from the calibration curves. The results are summarized in Table I.

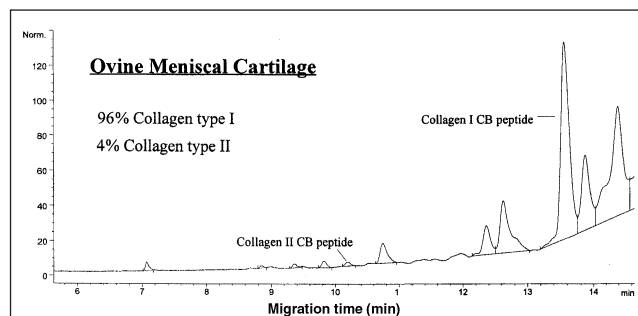
The accuracy of this method in these initial analyses compares well with those reported for the SDS-PAGE method (2).

**Table I. Summary of Results for Collagen Determinations of Known Mixtures of CNBr-Cleaved Ovine Tendon and Ovine Articular Cartilage**

Actual composition tendon:cartilage (%)	Determined composition collagen I:collagen II (%)
32:68	33:67
52:48	57:43
21:79	24:76
81:19	77:23

**Table II. Relative Amounts of Collagen I and II Found in Ovine Meniscus**

Sample	Collagen I (%)	Collagen II (%)
1	88	12
2	96	4
3	92	8
4	93	7



**Figure 7.** Electropherogram of CNBr-cleaved ovine meniscal cartilage. CE conditions: Sulpelco CElect N capillary (54 cm  $\times$  75- $\mu$ m i.d.); hydrodynamic injection, 20s and 50 mbar; buffer, 50mM sodium phosphate, pH 6.0; running voltage, 25 kV at 20°C; UV detection, 200 nm.

### Analysis of meniscal cartilage

Samples of ovine meniscal cartilage were analyzed, and the collagen I and II content was quantitated relative to Sigma collagen preparations. Figure 7 shows the electropherogram obtained for the analysis of the meniscus sample. The collagen type I and II CB marker peptides are shown.

Table II shows the relative amounts of collagen I and collagen II as a proportion of the total. These values correspond well with those found by others (10) and indicate the potential of this method.

### Conclusion

The separation of CNBr-cleaved peptides specific to type I and type II cartilage using a coated capillary and a phosphate buffer at pH 6 was described. Uncoated capillaries were found to be of limited use. Resolution was rapidly lost after a few runs even when using a low pH that minimizes the adsorption of peptides onto the capillary wall.

The use of a coated capillary minimizes adsorption problems and enables the optimization of the buffer pH. Using a Supelco CElect N coated column at pH 6, a known mixture of type I and type II collagen was analyzed, and the results corresponded well with actual compositions of the mixture. Meniscal cartilage was characterized, and the proportion of type I collagen and type II collagen corresponded well with those found by other workers.

This CE method offers an improvement over previous approaches in the quantity of sample required and quantitation of collagen types within a tissue. It shows good promise for the accurate analysis of cartilage, repair cartilage, and tissue engineered cartilage and offers possibilities for the analysis of biopsies in human studies.

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