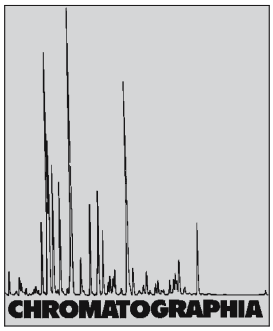
# CE Determination of 2-(9-Carbazole)ethyl Chloroformate-Labeled Oligopeptides




**2008**, *67*, 245–251

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Received: 4 June 2007 / Revised: 24 October 2007 / Accepted: 5 November 2007
Online publication: 12 December 2007

## Abstract

A pre-column derivatization method for sensitive determination of oligopeptides, using the tagging reagent 2-(9-carbazole)ethyl chloroformate (CEOC-Cl) followed by capillary electrophoresis (CE) with diode-array detection, has been developed. Maximum yield close to 100% were observed when a three to fourfold molar excess of reagent was used at pH 9.0–10.0. Excess reagent was extracted with *n*-hexane–ethyl acetate 9:1–10:1 (*v*/*v*); this enabled direct analysis using CE with no significant disturbance from the major fluorescent reagent degradation by-products. The effects on the results of buffer pH and of SDS and organic modifier concentrations were examined. Good baseline resolution in the separation of five CEOC-peptides was achieved with a 48.5-cm total length (effective length 40 cm) 50-μm inner diameter capillary column.

## Keywords

Capillary electrophoresis
2-(9-Carbazole)ethyl chloroformate (CEOC-Cl)
Oligopeptides
Derivatization

## Introduction

Capillary electrophoresis (CE) is a rapidly expanding field of analytical chemistry. Because of highly efficient peak separation and short analysis time, in the last decade CE has become a popular tool for determination of a variety of compounds. In several publications it has been reported that the technique is suitable for separation of peptides [1, 2]. Because most peptides have neither natural UV absorption nor fluorescence, pre-column or post-column chemical derivatization is necessary to increase detection sensitivity and to improve selectivity in HPLC [3, 4], on electrophoretic microchips [5], or in CE [6–9]. Although several different types of tagging reagent have been developed for amino functional groups [10–16] there have also been many reports describing their various shortcomings. For example, the *o*-phthaldialdehyde (OPA) method results in high sensitivity and selectivity [17, 18], but is limited to primary amino compounds only. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) [19] has been developed for determination of primary and secondary amino compounds. Data reported previously indicate the reagent is approximately 50% decomposed in 25 min in methanol–water solution exposed to daylight [20]. The reagents 9-fluorenylmethyl chloroformate (FMOC) [21, 22], 1-(9-fluorenyl)ethyl chloroformate (FLEC) [23], and 2-(9-anthryl)ethyl chloroformate (AEOC) [24] have also been developed for derivatization of amino functional groups for chiral or


DOI: 10.1365/s10337-007-0475-4
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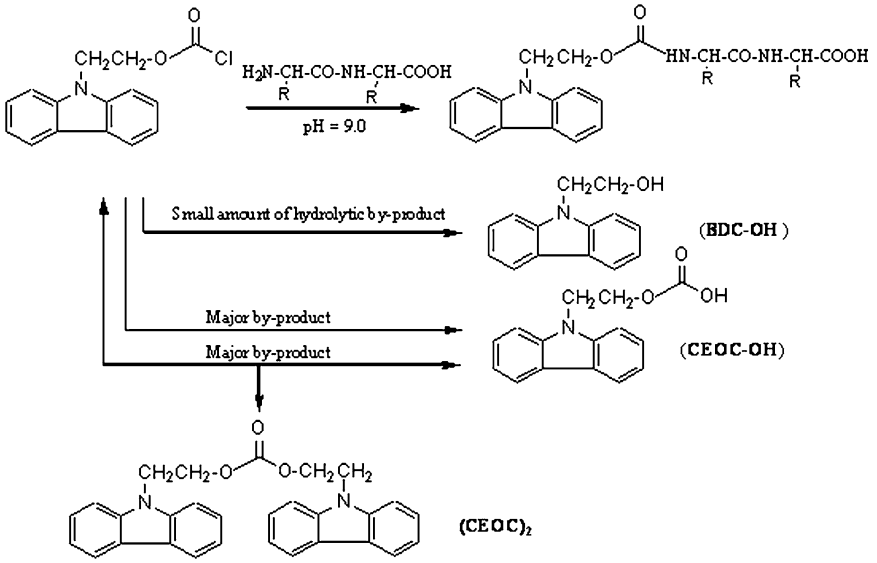


**Fig. 1.** Schematic illustration of the derivatization of peptides with 2-(9-carbazole)ethyl chloroformate (CEOC-Cl)

non-chiral separation by CE or LC. More recently, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) has been developed as a popular pre-column derivatization reagent for determination of amino compounds by CE [25] or LC [26, 27], with satisfactory results. Detection limits for early-eluted amino compounds are, however, usually higher than for those eluted later [26].

On the basis of its ultraviolet (UV) absorption and fluorescence (FL), the chromophore in 9-fluorenylmethyl chloroformate (FMOC) was replaced by a carbazole functional group, which resulted in a sensitive derivatizing agent CEOC-Cl. CEOC-Cl has been found to be easily accessible, very stable, and suitable for labeling of amino compounds, as we have described elsewhere [28]. In the work discussed in this paper the CE separation of peptides using CEOC-Cl as labeling reagent was studied to determine the optimum electrophoretic conditions for routine operation and to determine the effects on the analysis of buffer pH, analysis time, and SDS concentration. Under the optimized conditions, separation of five CEOC-Cl-peptides was achieved in less than 6 min. The linearity, detection limits, and precision of the procedure were determined. To the best of our knowledge this is the first report of the use of CEOC-Cl for separation of peptides by CE.

# Experimental

## Chemicals

The peptide standards $(Gly)_2$, $(Gly)_3$, $(Gly)_4$, $(Gly)_5$, and $(Gly)_6$ were purchased from Sigma (St Louis, MO, USA). HPLC-grade acetonitrile was purchased from Yucheng Chemical Reagent Company (Shandong Province, China). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Borate buffer was prepared from 0.2 mol $L^{-1}$ boric acid solution adjusted to pH 9.0 with 4 mol $L^{-1}$ sodium hydroxide solution prepared from sodium hydroxide pellets.

CEOC-Cl was synthesized as reported elsewhere [28]. A solution ($1.0 \times 10^{-3}$ mol $L^{-1}$) of the derivatizing reagent was prepared by dissolving 2.74 mg 2-(9-carbazole)ethyl chloroformate in 10 mL anhydrous acetonitrile prepared by distilling dried HPLC-grade acetonitrile from $P_2O_5$. The standard peptides for CE analysis at individual concentrations of $5.0 \times 10^{-5}$ mol $L^{-1}$ were prepared by dilution the corresponding stock solutions ($1.0 \times 10^{-3}$ mol $L^{-1}$) of each peptide with 0.2 mol $L^{-1}$ borate buffer (pH 9.0). When not in use, all standards were stored at −4 °C in a refrigerator.

## Derivatization Procedure

An aqueous solution (20–30 µL) of the peptides was placed in a vial then 200 µL 0.2 mol $L^{-1}$ borate buffer (pH 9.0) and 100 µL CEOC-Cl solution in acetonitrile were added consecutively. The solution was shaken for 1 min and left to stand for 10 min at room temperature. After derivatization, the mixture was extracted with hexane–ethyl acetate 9:1–10:1 (*v*/*v*) to remove excess reagent. The aqueous phase was transferred to another conical vial and adjusted to a final pH in the range 6.0–6.5 by addition of an appropriate amount of 36% acetic acid. The derivatized sample solution was then used directly for analysis. The derivatization process is shown in Fig. 1.

## Capillary Electrophoresis

Capillary electrophoresis was performed with an Agilent HP-3D system equipped with a diode-array detector (DAD). Derivatives were separated in a 50-µm inner diameter fused-silica capillary column of total length 48.5 cm, effective length 40-cm. The CE system was controlled by HP Chemstation software. All capillary tubing was purchased from Yong Nian Chemical Factory (Hebei Province, China).

A new capillary column was activated by washing consecutively with 0.1 mol $L^{-1}$ hydrochloric acid (20 min), 0.1 mol $L^{-1}$ sodium hydroxide (30 min), and deionized water (30 min). At the beginning of each working day, the capillary was washed with 0.1 mol $L^{-1}$ HCl for 10 min, 0.1 mol $L^{-1}$ NaOH for 10 min, and running buffer for 20 min. Before each analysis, the capillary was rinsed consecutively with 0.1 mol $L^{-1}$ NaOH (2 min), water (1 min), and running buffer (5 min). Sample was loaded on to the column by pressure injection for 5 s at 50 mbar. The running buffer

was 36 mmol $L^{-1}$ borate buffer (pH 9.0) containing of 3.0% acetonitrile. Column temperature was 25 °C. A constant potential of 25 kV was applied during analysis. Diode-array detection was performed at 254 nm.

## Results and Discussion

### Stability of CEOC and CEOC-Peptide Derivatives

When a solution of CEOC in anhydrous acetonitrile was stored at 4 °C for 2 weeks, derivatization yields for peptides were no different from those obtained with freshly prepared CEOC acetonitrile solution. When the labeled peptide derivatives were stored at room temperature in darkness for 24 h careful examination of the reaction mixture by CE indicated no obvious degradation had occurred. When anhydrous methanol and acetonitrile solutions of the labeled peptide derivatives were stored at 4 °C in darkness for one week CE analysis showed normalized peak areas varied by less than 2.3%.

### Ultraviolet Absorption of CEOC and Its Peptide Derivatives

2-(9-Carbazole)ethyl chloroformate (CEOC-Cl) has a carbazole functional group in its molecular backbone; this is an excellent property for UV detection. Introduction of one carbazole group into each peptide molecule endows the derivative with strong ultraviolet absorbance. The ultraviolet absorption of CEOC was reported previously by our laboratory [28]. The UV absorption maximum is at 254 nm. The molar absorption coefficient ($\varepsilon$) in acetonitrile is $2.34 \times 10^4$ L $mol^{-1}$ $cm^{-1}$. For quantitative comparison of UV absorbance, the representative CEOC-$(Gly)_3$ derivative ($1.0 \times 10^{-5}$ mol $L^{-1}$) was applied to a small preconditioned Sep-Pak $C_{18}$ cartridge, purified with 4 mL methanol and 5 mL water, and eluted with 30% aqueous acetonitrile. Careful examination of the UV absorption indicated that CEOC and its peptide derivatives have the same absorption intensities at 254 nm. The UV responses of the unlabeled oligopeptides were also investigated by CE. The results indicated that the five unlabeled oligopeptides $(Gly)_2$, $(Gly)_3$, $(Gly)_4$, $(Gly)_5$, and $(Gly)_6$ had very weak absorption at 214 nm. The ratios of the UV responses of the labeled oligopeptides to the UV responses of the unlabeled oligopeptides, $I_{254}/I_{214}$, were 12:1. Chemical derivatization is, therefore, necessary to increase detection sensitivity and improve selectivity in the CE analysis of peptides.

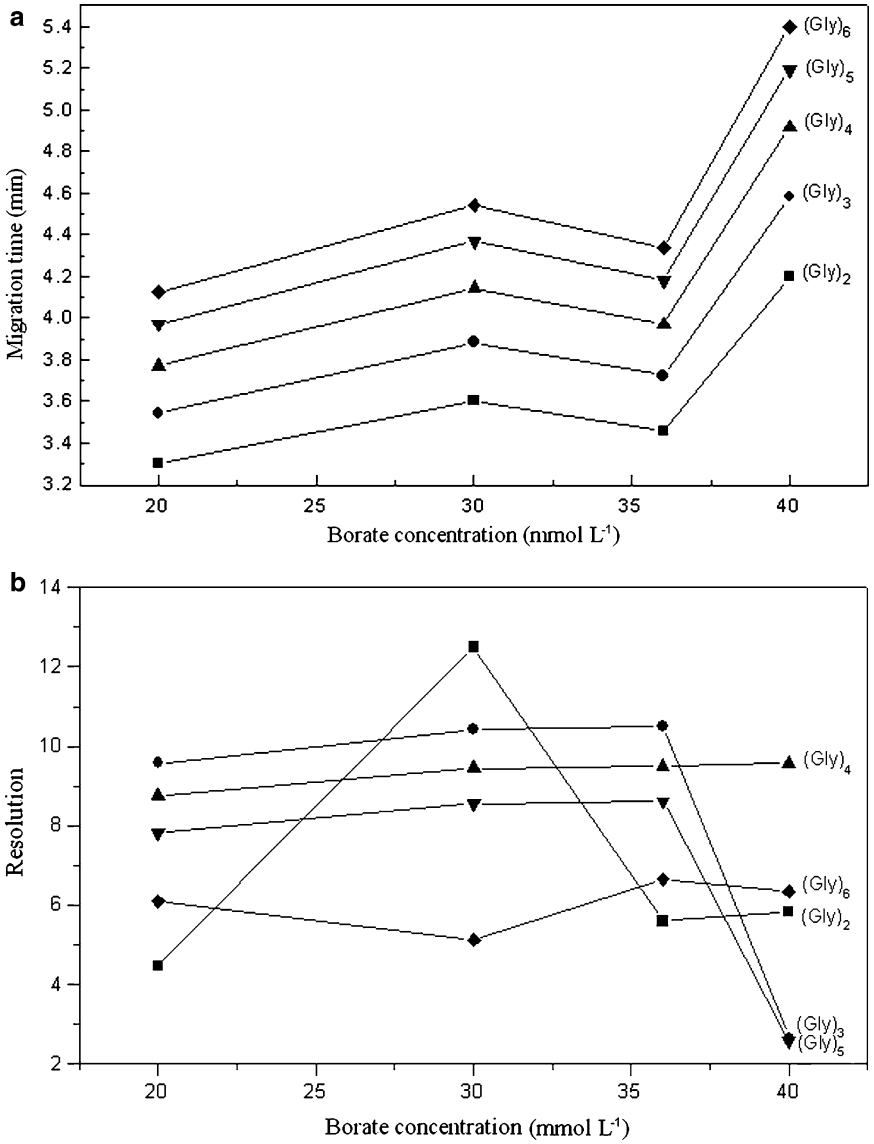


**Fig. 2.** Effect of borate buffer concentration on (**a**) migration time and (**b**) resolution. Conditions: fused-silica capillary (48 cm to the detector); 5-s injection at 50 mbar; running buffer pH 9.0; 3% acetonitrile as organic modifier; operating potential 25 kV; temperature 25 °C

### Optimization of Derivatization

Reaction of CEOC-Cl reagent with the peptides occurs in aqueous solution at room temperature under basic conditions. When the reaction is complete, excess reagent and hydrolyzed by-products (CEOC-OH) are removed by extraction with hexane–ethyl acetate

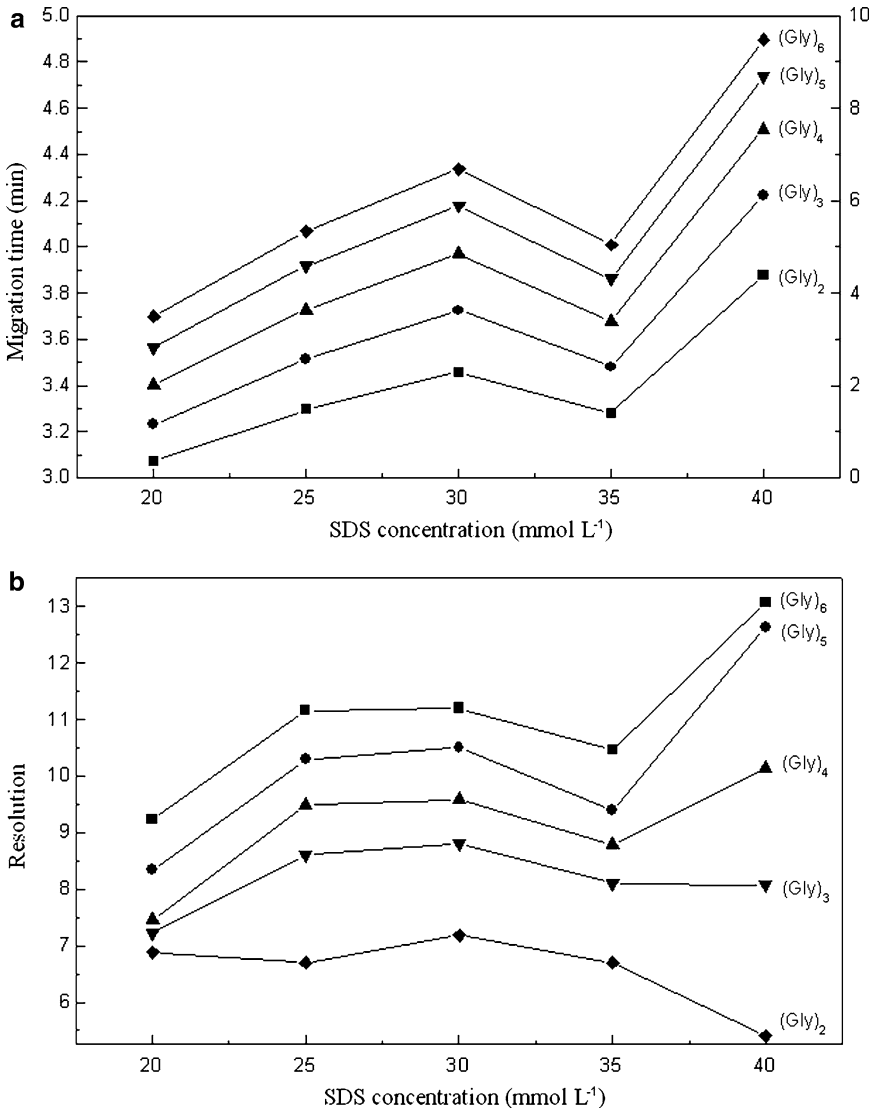


**Fig. 3.** Effect of SDS concentration on migration time (**a**) and resolution (**b**) of five CEOC-peptides by CE. Conditions were the same as for Fig. 2 except for variation of the SDS concentration

9:1–10:1 (*v*/*v*). Derivatization of peptides with CEOC-Cl can be achieved within 10 min at room temperature. Yields were not usually affected when the derivatization time was >10 min. The results indicated the speed of derivatization of peptides with CEOC-Cl was similar to that with FMOC or AEOC previously reported [22, 24]. The effect of CEOC-Cl concentration on the derivatization yield was investigated for the peptide derivatives. The results indicated that the UV intensity of the CEOC derivatives increased with increasing amount of reagent. Constant intensity was achieved by addition of three to fourfold molar excess of reagent to the peptides; increasing the excess of reagent beyond this amount had no significant effect on yield. With as little as twofold molar excess of the reagent, derivatization of the peptides was incomplete. One side-reaction is reaction of the reagent with its own hydrolysis product (BDC-OH) to give bis-(2-(9-carbazole)ethyl carbonate $(CEOC)_2$. Other side-reactions include hydrolysis of the reagent and formation of 2-(9-carbazole)ethanol (CEOC-OH) a major by-product (Fig. 1). The structures of these by-products have previously been reported by our laboratory [28]. Removal of excess reagent and by-products is a key step in establishing an efficient procedure. On extraction with hexane–ethyl acetate 9:1–10:1 (*v*/*v*) excess reagent CEOC-Cl and by-products $(CEOC)_2$ and CEOC-OH are easily removed from the reaction solution, because of their high hydrophobicity. Extraction of the by-product BDC-OH is, unfortunately, not efficient, because of its low hydrophobicity. Complete extraction of BDC-OH can be achieved with hexane–ethyl acetate 5:1–8:1 (*v*/*v*), but slight loss (ca 5–12%) of the derivatives is also observed. Fortunately, the presence of a small amount of BDC-OH does not interfere with the separation of the peptide derivatives after extraction with hexane–ethyl acetate 9:1–10:1 (*v*/*v*). The basic medium may facilitate derivatization of the peptides with CEOC-Cl. Borate buffer was found to be the best choice. The effect of pH on the derivatization reaction was investigated with 0.2 mol $L^{-1}$ borate buffer in the pH range 8.5 to 10.5. The maximum derivatization yields were achieved in the pH range 9.0 to 10.0.

## CE Operating Conditions

### *Effect of Buffer Concentration*

Several types of buffer, including carbonate, phosphate, and borate buffers, at concentrations of 15–50 mmol $L^{-1}$ (pH 9.0) were examined to compare the separation efficiency and peak-to-peak resolution of the five CEOC-peptides. The results indicated that reasonably good separation of the CEOC-peptides was achieved with borate buffer. Several different borate buffer concentrations (20, 25, 30, 35, and 40 mmol $L^{-1}$) were examined. It was found that separation of the five CEOC-peptides was very sensitive to borate buffer concentrations from 20 to 40 mmol $L^{-1}$. Separation

efficiency was greatly improved by increasing the buffer concentration from 20 to 30 mmol $L^{-1}$, and the order of elution of the peptides remained the same. The results are shown in Fig. 2a. It is apparent from Fig. 2 that with gradually increasing buffer concentration from 30 to 40 mmol $L^{-1}$ the migration time became longer and resolution deteriorated (Fig. 2b). It seems that the migration times of the CEOC-peptides are loosely associated with the borate group either by hydrogen bonding or complex formation. The true mechanism will require further investigation. With higher buffer concentration substantial Joule heating affects separation efficiency and results in increased migration time. Taking both the shorter run-time and the higher zeta potential into consideration, the borate buffer concentration selected was 30 mmol $L^{-1}$.

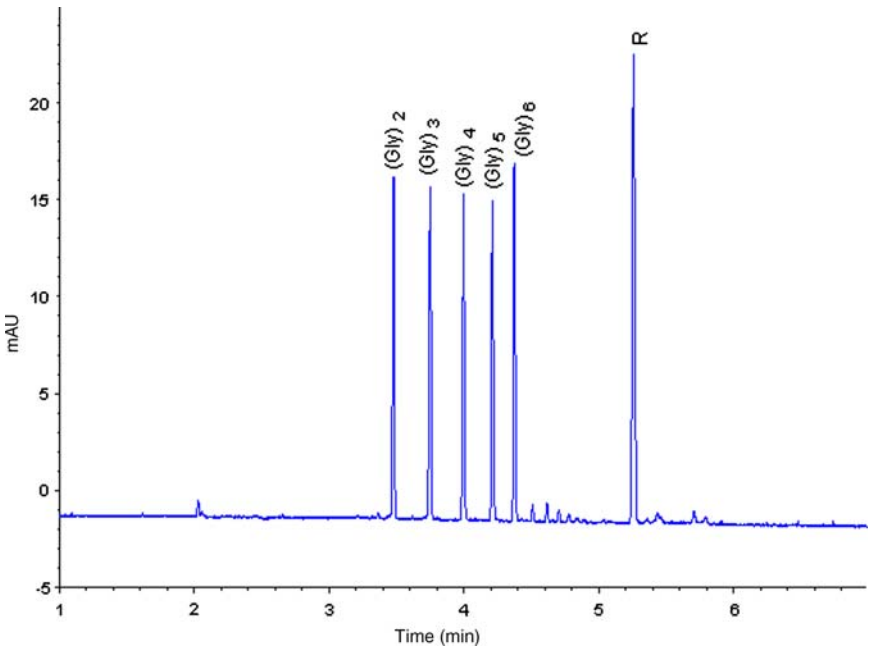


**Fig. 4.** Electropherogram obtained from CEOC-peptide standards separated by CE, with diode-array detection at 254 nm. Conditions: fused-silica capillary, total length 48.5 cm, effective length 40 cm (50 mm i.d.); 5-s injection at 50 mbar; running buffer pH 9.0; SDS (30 mmol $L^{-1}$); acetonitrile 3% as organic modifier; operating potential 25 kV; temperature 25 °C. R reagent peak

### *Effect of Buffer pH*

It is well known that buffer pH is always very important in the separation of ionizable analytes with similar isoelectric points, because it determines the extent of ionization of each analyte and also affects the surface of the capillary wall. In this study the effect of pH on the separation was investigated in the range 8.0 to 9.5 using borate buffer solutions as background electrolytes. The migration time decreased slightly with increasing buffer pH. As buffer pH was increased further from 9.0 to 9.5, however, a slight increase in migration time was observed and for buffer pH > 9.0, a substantial decrease in resolution for each CEOC-peptide was observed. Taking both short run-time and good separating power into consideration, an optimized pH of 9.0 was used in this study.

### *Effect of Surfactant Concentration*

The effect of SDS concentrations on resolution of the CEOC-peptides was also investigated. In this experiment, five CE running solutions containing from 20 to 40 mmol $L^{-1}$ SDS were prepared in borate buffer (36 mmol $L^{-1}$) at the optimum pH (9.0) containing 3% acetonitrile. The effects of SDS on migration time and resolution of the five peptides are shown in Fig 3a, b. It is apparent the migration times of the solutes increase with increasing SDS concentration. Increasing the concentration of SDS does not affect the order of elution of the CEOC-peptides and resolution of the CEOC-peptides increases with increasing SDS except for a slight decrease at 35 mmol $L^{-1}$ SDS. Although much better resolution could be obtained with 40 mmol $L^{-1}$ SDS, the running time was increased by ca 30% compared with that with 30 mmol $L^{-1}$ SDS, probably because the increase in the concentration SDS increases the conductivity of the running buffer and increases the surface charge of the capillary wall, which increases retention of the solutes. To achieve the shortest analysis time and good resolution of the CEOC-peptides, 30 mmol $L^{-1}$ was selected as the optimum concentration of SDS.

### *Effect of Organic Modifier*

Addition of an organic modifier is important because it improves the viscosity of the medium, the dielectric constant, and the structure of the electric double layer at the capillary wall. In this study, a variety of additives, for example *iso*-propanol, methanol, and acetonitrile were examined. After a series of experiments we found acetonitrile was most effective at separating all the CEOC-peptides. Subsequent experiments were performed to optimize the concentration of acetonitrile in the electrolyte solution. The effects of acetonitrile at concentrations of 1, 2, 3, 5, and 6% on separation efficiency and migration times for each CEOC-peptide derivative were investigated with 36 mmol $L^{-1}$ borate buffer at pH 9.0. Addition of acetonitrile to the running electrolyte increased the flow-rate, partially because the ionic strength was reduced. Addition of acetonitrile also made the electrolyte more polar, which changed the partitioning equilib-

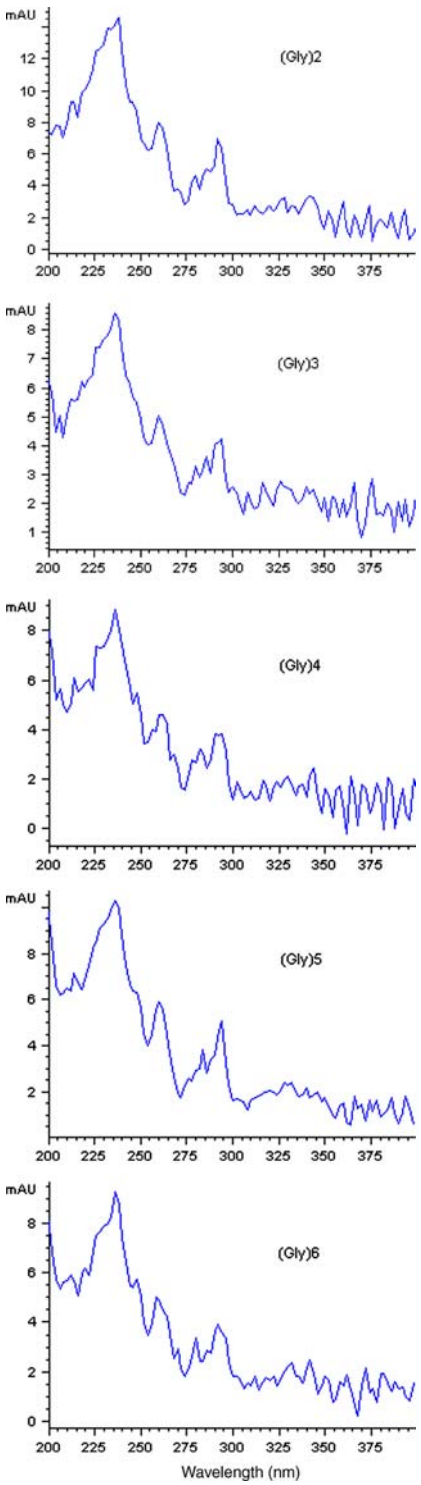


**Fig. 5.** UV spectra obtained for the five CEOC-labeled oligopeptides by use of the online diode-array detector

rium and thus affected selectivity and resolution. When acetonitrile concentrations higher than 5.0% were used, resolution of $(Gly)_2$ and $(Gly)_3$ decreased substantially. When 6% acetonitrile was used, $(Gly)_2$ and $(Gly)_3$ co-eluted and the high concentration of acetonitrile resulted in a noisier baseline. Taking both short run-time and good peak shape into consideration, 3% acetonitrile was adopted.

### *Effects of Temperature and Separation Potential*

The effect of capillary temperature on resolution of the CEOC-peptide derivatives was investigated. The results indicated that resolution was not much improved by increasing the temperature from 15 to 35 °C, although a slight decrease in migration times was observed, probably because the increased working temperature reduced the viscosity of the medium. To reduce the effect of Joule heating, the capillary working temperature was set at 25 °C. The effect of working potential on migration time was obvious compared with that of capillary temperature. Migration time and resolution of CEOC-peptides decreased substantially when the working potential was increased from 15 to 35 kV. In this study a potential of 25 kV was selected.

On the basis of these data the optimum conditions for separation of CEOC-peptides were: 36 mmol $L^{-1}$ borate buffer (pH 9.0), 30 mmol $L^{-1}$ SDS, 3% acetonitrile, working potential 25 kV, temperature 25 °C. Figure 4 shows the separation of the CEOC-peptide derivatives by CE under these conditions. UV spectra obtained for the five labeled oligopeptides by use of the diode-array detector are shown in Fig. 5.

### *Repeatability, Detection Limits, and Linearity for CEOC-Peptides*

A standard containing 50 μmol $L^{-1}$ CEOC-peptides was prepared for examination of method repeatability for migration time and peak area. The results are shown in Table 1. The RSDs of peak area and migration time were, respectively, from 0.014 to 0.026 and from 0.12 to 0.21. To determine precision and accuracy at low and high concentrations six replicate analyses ($n = 6$) of 1.0, 5.0, 10.0, and 50 μmol $L^{-1}$ CEOC-peptides were performed. The target values for intra- and interassay mean accuracy and precision of each CEOC-peptide were less than ±8.4% of the expected concentration.

Detection limits are an important consideration when the components of biological matrixes are analyzed, particularly the analytes of interest are present at low or trace concentrations. The detection limits calculated for injection of 1.0 μmol $L^{-1}$ CEOC-peptides (at a signal-to-noise ratio of 3:1) were from 0.19 μmol $L^{-1}$ for $(Gly)_2$ to 1.07 μmol $L^{-1}$ for $(Gly)_6$ (Table 2). Linearity was established over a 400-fold concentration range by analysis of serial dilutions of standard solutions containing from 0.25 to 100 μmol $L^{-1}$ of the CEOC-peptides. For all the CEOC-peptides response was a linear function of concentration over this range, with correlation coefficients >0.9902 (Table 2). Linear relationships for higher concentrations were not tested as they were over linear response range for all the peaks.

## Conclusions

A rapid CE method for separation of CEOC-peptides has been demonstrated for the first time. The method enables simple preparation of the peptide derivatives. The accuracy and repeatability of the method are good. After extraction with hexane–ethyl acetate hydrolysis products of the reagent did not interfere with the separation. Our investigations showed that resolution and migration times of the CEOC-peptides were very sensitive to buffer concentration and pH, and that addition of acetonitrile to the running buffer was necessary for sepa-

ration of all the CEOC-peptides. Analysis of the five derivatized CEOC-oligopeptides takes less than 6 min.

**Table 1.** Repeatability of migration time and peak area for the five CEOC-peptides

| RSD% | $(Gly)_2$ | $(Gly)_3$ | $(Gly)_4$ | $(Gly)_5$ | $(Gly)_6$ |
|---|---|---|---|---|---|
| Migration time | 0.014 | 0.026 | 0.016 | 0.016 | 0.015 |
| Peak area | 0.126 | 0.183 | 0.206 | 0.126 | 0.171 |

**Table 2.** Linearity, detection limit, and correlation coefficients for the five CEOC-peptides (the concentration injected ranged from 2.5 to 50 μmol $L^{-1}$)

| Peptide | $Y = A + BX$ | $r$ | Detection limit (μmol $L^{-1}$) |
|---|---|---|---|
| $(Gly)_2$ | $Y = 0.163 + 127.24X$ | 0.9908 | 1.23 |
| $(Gly)_3$ | $Y = 0.148 + 129.36X$ | 0.9934 | 1.55 |
| $(Gly)_4$ | $Y = 0.166 + 123.69X$ | 0.9924 | 1.71 |
| $(Gly)_5$ | $Y = 0.110 + 114.40X$ | 0.9902 | 3.68 |
| $(Gly)_6$ | $Y = 0.172 + 125.00X$ | 0.9954 | 3.61 |

$Y$ peak area, $X$ concentration (μmol $L^{-1}$)

## Acknowledgments

The National Science Foundation under grant #20075016 and the "Wester Light" program of talent cultivation of The Chinese Academy of Sciences (CAS) supported this research.

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