



# Use of capillary electrophoresis with dual-opposite end injection for simultaneous analysis of small ions in saliva samples from wrestlers undergoing a weight training program



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## ABSTRACT

Capillary electrophoresis–capacitively coupled contactless conductivity detection (CE–C4D), conducted using an in-house-developed polyvinyl alcohol (PVA)-coated capillary system, was applied for the simultaneous analysis of small anions and cations in saliva samples from wrestlers undergoing a weight training program. Use of the PVA capillary for CE provided good reproducible ion separation with minimization of the electroosmotic flow and suppression of protein adsorption onto the capillary wall. Four cations and eight anions were separated in 12 min, using a background electrolyte of 20 mM MES/20 mM histidine and 18-crown-6 ether (pH 6) at 20 kV. The relative standard deviations ( $n=5$ ) of the migration times and peak areas were <1% and <8%, respectively. The detection limit at a signal-to-noise ratio of 3 ranged from 1.6 to 10  $\mu\text{M}$ . Using the optimized CE–C4D system, we investigated the correlations between the concentrations of salivary ions and cortisol, which is commonly used as a stress marker. Analysis of saliva samples from ten wrestlers, who were attempting rapid weight loss before a competition, showed the following trends: (1) all ion concentrations, except for  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ , and  $\text{Cl}^-$ , increased between the first and last days of weight loss; (2)  $\text{Mg}^{2+}$  increased to 166% (from 0.50 mM to 1.4 mM) between the first and last days of weight loss, being the highest increase of all the ions; and (3)  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{NO}_3^-$ , and  $\text{SCN}^-$  levels were strongly correlated ( $P<0.05$ ) with cortisol. The CE–C4D rapidly produced useful data on saliva ion contents, with good ion recovery as determined by the standard addition method (89–110%).

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## 1. Introduction

For medical tests, the collection of small sample volumes is preferred, as this reduces patient stress. Human saliva is attractive for use as a biological sample because sample volumes of approximately 1–5 mL can easily be obtained using non-invasive techniques, and the samples can complement or replace blood

and urine samples. In previous studies, the concentrations of cortisol [1–18],  $\alpha$ -amylase [13–15,19,20], testosterone [16,21], immunoglobulin A [22–26], lysozyme [22], and inorganic/organic ions [18,27–34] in saliva samples have been used as markers for various stresses related to behavioral disorders, child development, sleep and circadian rhythms, smoking, plaque control, etc. [1–34].

In this work, the behavior of inorganic ions and small organic anions commonly found in human saliva was observed using capillary electrophoresis (CE). CE has been employed for the analysis of ions in samples of human saliva and other bodily fluids, such as serum and urine [35,36]. The inorganic ion species in saliva can easily be analyzed by CE because they are present at relatively high concentrations ( $10^{-5}$ – $10^{-2}$  M) [18,27–34,37]. Several researchers have studied the influence of inorganic cations and anions on human health using CE analyses, but many of these reports have only analyzed the cations or anions individually.

**Abbreviations:** 18C6, 18-crown-6 ether; BGE, background electrolyte; C4D, capacitively coupled contactless conductivity detection; CE, capillary electrophoresis; ECLIA, electrochemiluminescence immunoassay; EOF, electroosmotic flow; FS, fused silica; His, histidine; LOD, limit of detection; LOQ, limit of quantification; MES, 2-(*N*-morpholino)ethanesulfonic acid;  $\mu_{\text{eo}}$ , mobility of electroosmotic flow; MRA, multiple regression analyses; PVA, polyvinyl alcohol; RSD, relative standard deviation; S/N, signal-to-noise ratio.

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Therefore, our goal was to develop a CE method for the simultaneous analysis of common cations and anions in human saliva samples and to identify stress marker ions by comparing the variations in ion levels with those of typical stress markers.

The dual-opposite end injection method was used for the simultaneous CE analysis of cations and anions in samples. This injection method, which was reported by Kubáň et al. [38,39], Padarauskas et al. [40,41], and Nehmé et al [42], introduces the sample into both ends of the capillary, allowing detection near the center.

For biological CE analyses, a chemically coated capillary is typically used instead of an uncoated fused-silica (FS) capillary, to prevent the adsorption of high-molecular-weight matrices, such as proteins, to the capillary walls. Many studies have employed dynamically or chemically coated hydrophilic surfactants (neutral or cationic) and polymers to the inner surfaces of FS capillaries [43–50]. In this study, polyvinyl alcohol (PVA)-coated capillaries were used because it was easy to synthesize the PVA coating on the capillary walls, and the coating could effectively suppress protein adsorption to the capillary, thereby allowing for the stable separation of the anions and cations [49,51,52].

The human saliva samples used in this study were collected from wrestlers who were undergoing rapid weight loss for approximately one week before a wrestling tournament. During weight loss, individuals are often injured because of excessive weight training and mental stress [53,54].

Under optimized background electrolyte (BGE) conditions, the CE–capacitively coupled contactless conductivity detection (C4D) system was used to measure variations in common ions in saliva samples collected during three different periods of the wrestlers' training: the normal condition before the training camp (day 1), the first day of the weight loss period (day 2), and the last day of weight loss (day 3).

Variations in the ionic species in the saliva samples were compared with those of cortisol, a representative stress marker [1–18]. Evaluations were performed using multiple regression analysis (MRA), which is a statistical analysis method used to describe the correlation of two or more explanatory variables to an objective variable [55]. MRA was useful for exploring which saliva ions (the explanatory variables) were related to the secretion of cortisol (the objective variable) into body fluids [56,57].

This paper reports the usability of the developed CE–C4D system for the (1) simultaneous analysis of cations and anions in saliva samples from wrestlers, and (2) analysis of the variations in ion concentrations between the first and last days of weight loss, in order to identify ions related to stress indicators such as cortisol.

## 2. Experimental

### 2.1. Apparatus

CE experiments were performed using an HCZE-30PNO.25 high-voltage power supply (Matsusada-Precision, Tokyo, Japan) and a TraceDec capacitively coupled contactless conductivity detector (C4D, Innovative Sensor Technologies GmbH, Ebnat-Kappel, Switzerland). Data were analyzed using the TraceDec Monitor software. An FS capillary column (total length, 1 m; internal diameter (i.d.), 75  $\mu\text{m}$ ) was purchased from GL-Science (Tokyo, Japan).

### 2.2. Reagents and samples

All reagents used as standards and BGE components were obtained from Wako (Osaka, Japan). Standard samples were prepared from 0.1 M stock solutions using analytical-grade materials. Pure water was used as an electroosmotic flow (EOF) marker. Each standard sample was prepared by mixing  $\text{NH}_4\text{NO}_3$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{MgSO}_4$ , and  $\text{CaCl}_2$  with formic, malic, acetic, and lactic acids.

The BGE was composed of 2-(*N*-morpholino) ethanesulfonic acid (MES) and histidine (His) buffer (pH 6). To selectively separate  $\text{K}^+$  from  $\text{NH}_4^+$ , 18-crown-6 ether (18C6) was added to the BGE solution at a concentration of 0.1–2.5 mM [58]. Pure water was used throughout. All solutions were filtered with a 0.2  $\mu\text{m}$  cellulose acetate filter before use.

### 2.3. Preparation of PVA capillary

Chemical binding of PVA onto an uncoated FS capillary was performed according to the procedures described by Steiner and Hassel [49] and our research team [51,52]. First, 1 mL of vinyltrichlorosilane was deposited onto the inner surface of an argon gas-filled, uncoated capillary at 120 °C for 18 h. The vinylsilanized capillary was washed with methylbenzene, dichloromethane, and methanol and then dried in a constant temperature oven (25 °C). Thereafter, a solution of vinyl acetate in ethyl acetate was flowed into the capillary. Polymerization was allowed to proceed at 80 °C for 24 h. After the capillary ends were cut, the highly viscous polymerization medium was washed out using ethyl acetate. The polyvinyl acetate was converted into PVA through hydrolyzation in the presence of a sodium methylate solution in methanol. The resulting PVA-coated capillary was 80 cm in length (75  $\mu\text{m}$  i.d.).

### 2.4. Instrumentation

An uncoated FS capillary was conditioned with 2 min washes using 0.1 M sodium hydroxide, water, and BGE. The PVA capillary was conditioned for 2 min with the BGE used for electrophoresis. Samples were injected into the capillary using the dual-opposite end method [38,39], which in preliminary tests had proven easier to implement than other injection methods. The CE device used in this study is illustrated in Fig. S1 in Supplementary material. Each sample was introduced into the capillary by electrical injection. Cations and BGE were injected for 5 s using an applied voltage of 10 kV at the anode, while anions were injected for 5 s using an applied voltage of –10 kV at the cathode. Constant voltages of 20 kV were applied to drive EOF and ion separation. All CE experiments were performed at 25 °C.

### 2.5. Conductometric background levels and electroosmotic mobility

The conductometric background levels of PVA-coated and uncoated FS capillaries filled with BGE were compared using the CE–C4D system. These levels were recorded for 10 min with the C4D detector, which was positioned 30 cm from the capillary anodic end (total length, 80 cm). The position of the detector had been previously adjusted to optimize the separation of analyte ions (Fig. S2 in Supplementary materials).

To evaluate the performance of the PVA capillary, the electroosmotic mobility ( $\mu_{\text{eo}}$ ) was measured using a general method that is commonly employed in CE [59]. The voltage applied across the capillary was 20 kV. The total length of the capillary was 80 cm and the capillary length from the inlet to the detector was 30 cm. Water was used as the EOF marker. The BGE solution was 20 mM MES/His at pH 6.

Prior to the ion analysis experiments, the conductometric background of the prepared PVA capillary was compared with that of an uncoated FS capillary. The conductometric background level of the PVA capillary, filled with 20 mM MES/20 mM His (pH 6), was  $155 \pm 11 \mu\text{S}$  ( $n = 5$ ), whereas that for the uncoated FS capillary was  $177 \pm 13 \mu\text{S}$ , indicating that the PVA coating did not significantly affect the C4D conductometric response.

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