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Determination of inorganic anions in saliva by electroosmotic flow controlled counterflow isotachophoretic stacking under field-amplified sample injection

Haixia Ren[a,b], Hongdeng Qiu[a], Xiaojing Liang[a], Xusheng Wang[a], Shengxiang Jiang[a,*]

[a] Key Laboratory of Chemistry of Northwestern Plant Resources, CAS and Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, China
[b] University of the Chinese Academy of Sciences, Chinese Academy of Sciences, Beijing 100049, China

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ABSTRACT

Under a strong counter-electroosmotic flow, five salivary inorganic anions, bromide, iodide, nitrite, nitrate and thiocyanate were determined by field-amplified sample injection in combination with isotachophoretic stacking. Separation and concentration conditions were investigated. A terminating electrolyte, 5 mM borate, was added in the sample. Under the optimized conditions, Br^-, I^- and SCN^- were concentrated online using 150 mM HCl–Tris buffer at pH 7.8 in a bare fused capillary, providing more than ten thousand of sensitivity enrichment compared with normal injections. The relative standard deviations (RSDs, $n = 5$) were less than 1% in migration times, 8% in peak areas. Using direct UV detection at 200 nm and 226 nm, the limits of detection (LODs, S/N = 3) were of 0.002–0.01 μM. Unfortunately, NO_2^- and NO_3^- could be observed in purified or deionized water. Therefore, a low dilution factor was applied to saliva samples. Due to the matrix effect, samples were injected in a shorter time, and standard addition method was applied to determine all the five inorganic anions in saliva. The RSDs of the migration times and peak areas were in a range of 0.2–0.4% and 3.0–4.0%, respectively. The LODs were 0.2–2.0 μM. The salivary levels of the anions obtained were in accord with the reference data. The external standard method can not be adapted to real samples due to biases caused by electrokinetic injection and errors from high dilutions

1. Introduction

Capillary electrophoresis is a powerful analytical tool for the analysis of ionic and neutral compounds, such as small ions [1–3], drugs [4], particles, cells and microorganisms. In capillary electrophoresis, samples can be delivered onto column hydrodynamically or electrokinetically [5]. Electrokinetic injection (EKI) is advantageous for mobile components and able for viscous sample and gel electrophoresis [6].

EKI is first demonstrated as a sample stacking technique by Chien and Burgi in 1991 [7], and theoretically explained by Chien [8]. Analytes were enriched when the conductivity of the sample is much lower than that of the background electrolytes (BGE). It is also called field-amplified sample injection (FASI), allowing hundreds or thousands of improvements [9,10].

During the last decades, EKI stacking methods, combined with solid phase extraction [11,12], pH conjunction [13,14], sweeping and isotachophoresis (ITP), have achieved extremely high sensitivity enrichments. For example, cation – or anion – selective exhaustive injection – sweeping, can achieve up to million-fold sensitivity increase [15]. A combination of EKI and ITP, one of the most powerful preconcentration techniques, has demonstrated over 100,000-fold enrichment [16,17]. The terminating electrolyte (TE) is either located after the sample zone, or added in samples. As there is bias for low mobility component, a low concentration of TE is usually added into the sample [18]. Hence, an ITP state is generated during EKI, where compounds migrate at a velocity the same as the leading ion, rather than its own electrophoretic mobility. Based on this fact, million fold sample stacking was achieved with a microchip under electroosmotic flow suppression condition [19]. Later, Breadmore and Quirino have further developed the method, a strong electroosmotic flow (EOF) controlled counterflow isotachophoretic stacking boundary system, where extremely long injection time can be applied [20], with an enrichment factor up to 100,000-fold [17].

Incredible improvements have been made in these studies. However, few of them have involved its application in biological samples.

* Corresponding author. Tel.: +86 931 4968266; fax: +86 931 8277088.
E-mail address: sxjiang@lzb.ac.cn (S. Jiang).

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Saliva contains many important substances, including electrolytes, mucus, antibacterial compounds and various enzymes. Determination of inorganic anions in saliva is important in diagnosis of physiological and biological conditions of an individual. Br^- is a sedative hypnotic. I^- is involved primarily in the synthesis of thyroid hormones. They are substrates of lactoperoxidase, which plays a vital role in innate immune system [21,22]. NO_2^- and NO_3^- can be reduced to NO and other bioactive nitrogen oxide species [23]. The SCN^- is a detoxification product of cyanide in the liver, and is considered to be a marker of distinguishing between smokers and nonsmokers [24]. Besides, it is a substrate of peroxidase [22,24], an inhibitor on NO formation [25]. Many methods have been developed to determine NO_2^-, NO_3^- [26] and/or SCN^- [24] in saliva, such as potentiometric sensors [27], ion chromatography [28–30], capillary ion chromatography [31], short end injection [32], capillary electrophoresis [33]. In order to reduce protein adhesion, micellar electrokinetic chromatography [34] or BGE additive was applied for saliva analysis. Besides, the capillary can be either coated with polymer layers [35] or washed with SDS in capillary zone electrophoresis [36]. Few studies report the concentration of salivary I^-. Even though direct injection of saliva was applied, I^- could not be detected in most cases [37]. A high level of I^- was reported by capillary ITP [38]. Xu et al. [39] described a more sensitive transient isotachophoretic method for the separation and quantification of these five inorganic anions in saliva.

Under strong counter EOF, the mobilities of Br^-, I^-, NO_2^-, NO_3^- and SCN^- are higher than the EOF, which ensures detection of these anions in negative polarity. In this study, we are aimed to apply the counterflow isotachophoretic stacking to determination of these anions in saliva.

2. Materials and methods

2.1. Chemicals and reagents

Tris(hydroxymethyl)aminomethane (Tris) and KBr were supplied from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was obtained from Bio Basic Inc. (Toronto, Canada). MES was purchased from Amresco (Solon, USA). Boric acid was from Fengyue Chemicals Co., Ltd. (Tianjin, China). NaI, $NaNO_2$ and KNO_3 were from Shanghai Chemical Reagent General Works (Shanghai, China). KSCN was obtained from Beijing Chemical works (Beijing, China). All analytes and other chemicals were of analytical grade.

BGEs were prepared by dilution of HCl to 150 mM, and adjusted to pH 7.8 with Tris. The TEs were prepared from MES, HEPES and boric acid at a concentration of 100 mM, and the pH was adjusted to 7.8 with Tris.

2.2. Standards and sample preparation

Stock standard solutions of 10 mM KBr, 10 mM NaI, 10 mM $NaNO_2$, 10 mM KNO_3 and 10 mM KSCN were prepared in purified water. Working standard solutions were diluted in TE as required.

Saliva samples were collected from a healthy volunteer. 2 mL saliva was diluted with acetonitrile to 4 mL. The mixture was centrifuged at 8000 rpm for 10 min and filtered through a 0.45 μm membrane filter.

2.3. Capillary electrophoresis

Experiments were performed on An Agilent CE system (Agilent Technologies, Beijing, China) equipped with a UV DAD utilizing ChemStation (Rev.A 09.03). Detection was monitored at 200 and 226 nm. The capillary cartridge was thermostated at 20 °C. Electrophoresis was carried out at −20 kV.

A fused capillary (Hebei Yongnian Country Reafine Chromatography, China) of 50 μm i.d. with a length of 68.5 cm, 60 cm to detector, was used for separation. Before use, the capillary was flushed (≈930 mbar) with methanol (10 min), 1 M HCl (20 min) and 1 M NaOH (20 min). Between runs, the capillary was rinsed with 0.1 M NaOH for 2 min followed by 3 min BGE.

The analysis was performed by external standard and standard addition methods. For the former method, 1.6 mL standard solution was used for injection at −10 kV, 200 s. In addition, 10 μL saliva–acetonitrile mixture was diluted to 5 mL in 5 mM borate for injection, giving a high dilution factor of 1000 for saliva. For the latter one, 500 μL saliva–acetonitrile was mixed with 50 μL 100 mM borate and 500 μL standard solutions, and diluted to 1 mL. An aliquot of 200 μL mixture was then injected at −10 kV, 15 s.

3. Results and discussion

3.1. Optimization of separation conditions

A strong EOF to the cathode can be generated in the fused-silica capillary. Under counter flow conditions, only anions with mobility greater than the EOF can migrate into the capillary towards the anode.

The pH value has little effect on ionization of the inorganic acid in a pH rang 7–8.6. Supplementary Fig. 1 shows the influence of pH on effective mobility and EOF. Anions showed the highest electrophoretic mobilities at pH 7.8, where the maximum peak efficiency was observed (Supplementary Fig. 2) The EOF was almost constant over this pH range. Therefore, this decrease is referred to interactions between the five anions with the background electrolyte, such as ion paring with Tris. The resolution between Br^- and I^- increased as the increase of pH. However, almost constant resolution was observed between NO_2^- and NO_3^- over the pH range. The separation pH was compromised at pH 7.8 in the following study.

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The UV spectra were tested from 190 to 250 nm. The I^- has a maximum absorption at 226 nm. The Br^-, NO_2^-, NO_3^- and SCN^- exhibit higher absorption at shorter wavelength. As the background absorption of Tris is considered, 200 nm and 226 nm were selected for detection.

The BGE concentration plays an important role in peak heights. The concentration of the buffer was studied in a range of 50–200 mM. Peak heights increased with the increase of concentrations from 50 to 150 mM, while decreased at a concentration larger than 150 mM. Larger conductivity difference between BGE and sample, higher stacking improvement will be obtained during EKI. Besides, the stacking factors are dependent upon the concentration of the leading in ITP process. Therefore, higher concentration of the BGE favours the concentration of the anions. However, high ionic strength of the BGE generates high current, leading to a significant Joule heating effect. Thus, the concentration was set at 150 mM in the following studies.

3.2. Counterflow isotachophoretic stacking

When the conductance of the sample is much lower than that of the BGE, ions move faster than that inside the BGE during EKI, resulting in a stacked zone at the inlet of the capillary, in the form of FASI. When an amount of TE is added into the sample and the capillary is filled with leading electrolyte, an ITP boundary will be generated, where the anions migrate at a velocity of the leading ion. As the mobilities of bromide and iodide are higher than that