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Sensitive determination of anions in saliva using capillary electrophoresis after transient isotachophoretic preconcentration

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ABSTRACT

A transient isotachophoresis–capillary electrophoresis (tITP–CE) system for the determination of minor inorganic anions in saliva is described. The complete separation and quantification of bromide, iodide, nitrate, nitrite, and thiocyanate has been achieved with only centrifugation and dilution of the saliva sample. In-line tITP preconcentration conditions, created by introduction of the plugs of 5 mM dithionic acid (leading electrolyte) and 10 mM formic acid (terminating electrolyte) before and after the sample zone, respectively, allowed the limits of direct UV absorption detection (at 200 nm) to be up to 50-fold improved as compared with CE without tITP. As a result, nitrate and thiocyanate were still detectable at 4.6 and 3.8 $\mu g\,l^{-1}$, respectively, in 1000 times diluted saliva. The daily variations of anionic concentrations in saliva samples taken from a smoking health volunteer were discussed based on the results of tITP–CE analysis. It was confirmed that the thiocyanate concentration in saliva noticeably increased after smoking. This is apparently the first report on simultaneous quantification of more than four anionic salivary constituents using CE.

1. Introduction

Biological sample analyses represent an application field where the benefits of capillary electrophoresis (CE) as a tool for inorganic ion analysis increasingly render it the status of the method of choice [1,2]. This is due to the technique's good assets in bioanalytical applications such as conducting multicomponent analyses in a simple, high-speed and cost-efficient way and in a miniaturized format, minor impact of the separation system on the original speciation of analytes, modest requirements on sample clean up, etc. In particular, one can witness a welcome situation when a large proportion of inorganic biofluid constituents are amenable to reliable CE analysis [2]. Of various biological fluids, blood serum and urine undoubtedly dominate the area. On the other hand, analyses of other types of fluids, one of which, saliva, is a matter of concern in the present work, received comparatively limited consideration.

Saliva is the watery secret produced in the mouth of humans which carries a variety of important compounds, including a group of inorganic anions (chloride, phosphate, and bicarbonate are the most abundant constituents). Early CE work on analyses for inorganic anions revised by one of co-authors [1] encompassed only a few saliva applications. In recent years, CE with direct UV detection was in most cases applied to the determination of salivary levels of nitrate and nitrite as established indicators of nitric oxide metabolism [3–8] or thiocyanate as a marker of exposure in smokers [4,5]. In order to alleviate the matrix interferences some authors used a sample deproteinization step that involved denaturation of salivary proteins with acetonitrile [5] or sodium hydroxide [6]. Otherwise, a zwitterionic surfactant (i.e. *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) added to the background electrolyte may reduce protein–wall interaction and thus improve analyte resolution, peak symmetry, and reproducibility [4]. However, only prominent salivary anions could be really detected and quantified because of limited detectability of basic CE setup, with limits of detection typically in the (low) micromolar range. An ambitious attempt to resolve this challenge was recently made by the group of Padarauskas [9]. Their method implies derivatization of cyanide into the respective nickel complex, followed by extraction from human saliva into a single microdrop of appropriate solvent placed in the headspace of the sample solution. However, such preconcentration approach is only amenable for volatile analytes.

The goal of the work presented here has been to design a more versatile preconcentration procedure to be in-line incorporated into the CE system for signal enhanced detection for a wider range of salivary anions. In the previous work from this laboratory, meth-

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ods of enhancement of the analytical performance of CE regarding sensitivity and the number of biologically relevant analytes have been explored [10–12]. The strategy consisted in using transient isotachophoresis (tITP) preconcentration by taking advantage of occurring a high matrix anion or cation that acts as leading ion (so-called sample-induced tITP [13–15]). Particularly, the suitability of the concept has been successfully demonstrated for the enrichment of inorganic anions from serum and urine samples prior to CE analysis [10]. This paper describes a tITP–CE system that affords the simultaneous determination of five anions, including trace bromide and iodide, in saliva. The first part of the work was devoted to exploring and optimizing tITP conditions. A rational combination of externally introduced leading and terminating electrolytes (LE and TE, respectively) was proposed to accommodate the target analytes. The latter part involved demonstrating the figures of merit and utility of the developed method in the analysis of saliva samples. In addition, the results obtained were examined from the viewpoint of daily variations.

2. Experimental

2.1. Chemicals

Hydroxypropylcellulose (HPC) was from Tokyo Kasei (Tokyo, Japan), dithionic acid was the product of Kanto Kagaku (Tokyo, Japan), and 6-aminocaproic acid and formic acid were purchased from Katayama Kagaku (Osaka, Japan). Ultrapure hydrochloric acid (20%, v/v) used for preparation of the running electrolyte was from Tama Chemicals (Kanagawa, Japan). All solutions were prepared in deionized water obtained from a Millipore purification system (Tokyo, Japan). Anion stock solutions ($1.0\,g\,l^{-1}$) of nitrate, nitrite, bromide, iodide, and thiocynate were prepared from sodium salts (analytical grade) in deionized water, and appropriate dilutions were made to the proper concentrations.

2.2. Sample preparation

Saliva samples taken from a health volunteer with smoking habit were collected in the centrifugal filter unit (cat. no. UFC30SV00 from Millipore) and centrifuged at 5000 rpm for 60 min. The filtrated sample (around 2.0 ml) was divided into four portions and stored at −20 °C. Immediately before analysis, the samples were thawed and diluted with deionized water. Oral cleaning was done before every sampling.

2.3. Capillary electrophoresis

CE analyses were performed on a CAPI-3200 instrument (Otsuka Electronics, Osaka, Japan) equipped with a variable wavelength UV detector (set at 200 nm) and a negative power supply, applying a constant voltage of −30 kV at the injection end of the capillary (migration current around 10 μA). The temperature of the capillary cartridge was maintained at 25 °C. Sample and tITP-supporting electrolytes were injected hydrodynamically at 50 kPa at a specified time. Separations were performed in the 100 cm long, 75 μm i.d. fused-silica capillary, with an effective length of 87.7 cm. Before use, new capillaries were washed with 1 M sodium hydroxide, water, and the separation electrolyte for 10 min. The capillary was flushed with water for 2 min and running electrolyte for 2 min prior to each run. The optimized separation electrolyte consisted of 5 mM HCl with 0.1% (m/v) HPC at pH of 4.4 adjusted with 6-aminocaproic acid. LE (5.0 mM dithionic acid, pH 7.2) and TE (10 mM formic acid, pH 4.4 adjusted with 6-aminocaproic acid) were hydrodynamically (negative pressure) introduced into the capillary before and after the sample injection, respectively. The identity of the peaks was confirmed using standards added to the sample.

3. Results and discussion

3.1. Optimization of the CE separation

In our previous published work [10], optimal CE separation of a similar selection of biofluid anions was achieved with an acidic chloride-based electrolyte system. A high concentration of sodium chloride (250 mM) was necessary to incorporate in order to match serum or urine matrix salinity, as well as to ensure a certain sample stacking effect and to reduce the electroosmotic flow. In addition, cetyltrimethylammonium chloride was used for selectively decreasing the mobility of iodide. This was essential to have iodide moving well after chloride that served as a leading ion for tITP focusing. Also, the surfactant helped prevent matrix protein adsorption onto the capillary walls.

In contrast, saliva is one of few body fluids that contain no high matrix chloride (typically ≥5 mM). Therefore, to adapt the above background electrolyte to saliva analyses, the chloride concentration was reduced to 5 mM. However, under such conditions the electroosmotic flow was fairly high to detect highly mobile target anions ($>60\times10^{-9}\,m^2\,V^{-1}s^{-1}$) within a reasonable time. To suppress it HPC was added to the separation electrolyte. The polymer dynamically coats the capillary wall and hence may also protect it against adsorption of proteins and other matrix constituents (in the same way as the surfactant does). The optimal resolution of the five anions was obtained at pH 4.4, as shown in Fig. 1. Thus, 5 mM HCl with 0.1% (m/v) HPC at pH of 4.4 was used as the separation electrolyte for the rest of this work. The separation voltage was maintained at −30 kV (which is the highest

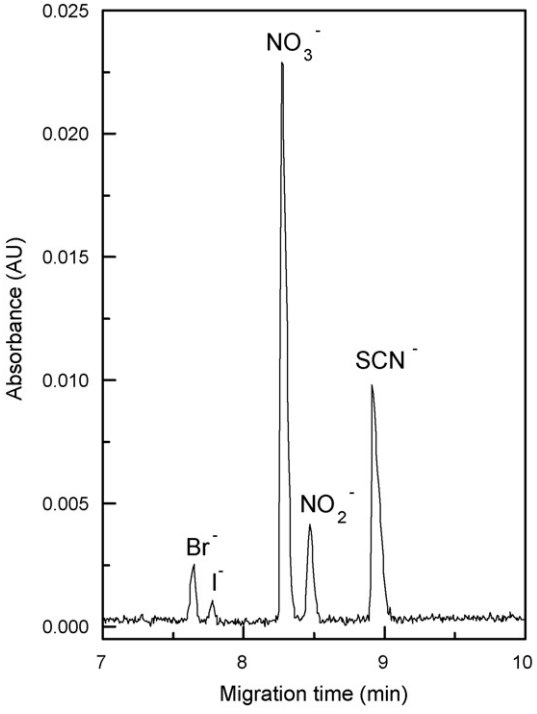

Fig. 1. Electropherogram of a 10-fold diluted saliva sample obtained without tITP preconcentration. Conditions: capillary, fused-silica, 100 cm × 75 μm i.d.; separation electrolyte, 5 mM HCl, 0.1% (m/v) HPC, pH 4.4; sample injection, negative pressure at 50 kPa for 30 s; applied voltage, −30 kV; UV detection at 200 nm. Sampling: 1 h after lunch.

Table 1
Figures of analytical merit

Analyte	Detection limit ($\mu g\,l^{-1}$)	Repeatability (R.S.D., %)[a]		Linear range ($mg\,l^{-1}$)	Correlation coefficient	Average concentration ($mg\,l^{-1}$)[c]
		Migration time	Peak area[b]			
Br^-	3.0	0.28	3.4	0.1–0.5	0.992	5.4 ± 1.4
I^-	5.9	0.28	4.9	0.05–0.5	0.991	0.4 ± 1.0
NO_3^-	4.6	0.29	1.5	1–10	0.991	19.7 ± 15.8
NO_2^-	6.4	0.30	2.7	0.5–5	0.962	3.5 ± 5.3
SCN^-	3.8	0.28	1.0	1–10	0.999	44.5 ± 14.8

[a] The saliva was 100-fold diluted ($n = 3$).
[b] Not corrected for migration time.
[c] For 10 samples taken every 2 h in 1 day and monitored after a 10-fold dilution.

value available with the CE instrument employed) where migration times are minimized while baseline noise is not significantly increased.

3.2. Optimization of tITP preconcentration conditions

Inorganic anions in saliva significantly differ in concentrations. Indeed, nitrate, nitrite, and thiocyanate present from mid- to low-$mg\,l^{-1}$ level and thereby can be determined by CE directly [3–8,16,17], without preconcentration other than field-amplified sample stacking. On contrary, bromide and especially iodide occur in saliva in much lower concentrations, and their accurate quantification is only feasible after substantial sample enrichment. As follows from a report by Sádecká and Polonský [18], ITP can work as a preconcentration tool for salivary anions. However, when applied to such analyses in a genuine ITP mode, it suffers from moderate sensitivity (limits of detection ranged 25–127 $\mu g\,l^{-1}$; cf. data of Table 1) and reduced accuracy (because of a specific, stepped character of electropherograms), requires comparatively large sample volumes, and uses less versatile, conductivity detection method. Therefore, in this study the preference was given to ITP, temporarily operating before zone electrophoresis separation (i.e. tITP), followed by UV absorbance detection.

When analyzing high-salinity biological samples, tITP stacking can most straightforwardly be accomplished by exploiting the matrix chloride to play the role of a leading ion [15]. Then, one needs only a suitable slowly migrating anion to be added to the sample or injected as a separate zone so as to accommodate the target anions into the tITP range. However, saliva does not fulfil the condition of sample self-stacking as its chloride (or any other fast anionic component) does not exceed considerably the analyte concentrations.

In order to solve this challenge, an alternative supporting anion whose mobility is greater than that of any sample ions had to be chosen so that the ITP state is created at the initial stage of CE separation. As a matter of fact, there are very few leading-type stackers that satisfy this mobility criterion in the case of inorganic ion analyses, and one of these is dithionite. Its actual mobility depends on the ionic strength and pH and reaches as high as $96.4 \times 10^{-9}\,m^2\,V^{-1}\,s^{-1}$ [19]. Importantly, at a concentration of 5 mM, dithionite has higher mobility than bromide and iodide (as well as the matrix chloride) do, and hence it could be used as leading ion. Taking into account that temporarily ITP focusing is ensured at a sufficiently high molar amount of the stacker [13], the latter was optimized by increasing the loading time at a fixed concentration of dithionite (5 mM), and 10 s was chosen as optimum from the viewpoint of detectability and resolution. Larger loadings did not result in substantially higher peak heights but in a shorter migration of the analytes under CE mode. This may impair the resolution. For instance, the peaks due to bromide and iodide turned out to be overlapped at a 20 s injection time of LE as a consequence of too long ITP time.

Another important consideration for optimization of the tITP performance was the concentration ratio of a stacker (dithionite) to a destacker, i.e., the salivary chloride which can operate against tITP stacking. For efficient focusing, this ratio is known to be large enough [14], and sample dilution affords a means of controlling it. It was found that saliva samples should be diluted at least 10-fold to guarantee electromigrational sharpening effect and in addition, the conditions of field-amplified stacking at the initial stage of separation. Therefore, the following tITP–CE analyses were carried out with ≥100-fold diluted saliva samples.

As the terminating ion to bracket the mobilities of analytes on the low-mobility side, formate was selected as an anion with suitably low mobility ($46 \times 10^{-9}\,m^2\,V^{-1}\,s^{-1}$ at pH 4.4) and not occurring in saliva. In a combination with the highly mobile dithionite, formate was found to work fairly effective to focus isotachophoretically the analyte anions. Since increased amounts of terminator usually lead to higher concentration factors [20], its molar amount was kept reasonably high by loading a 10 mM solution of TE for 30 s. When larger amounts of formate were tried, the peak heights of only nitrate and thiocyanate continue to be markedly increasing but at the expense of enlarged migration

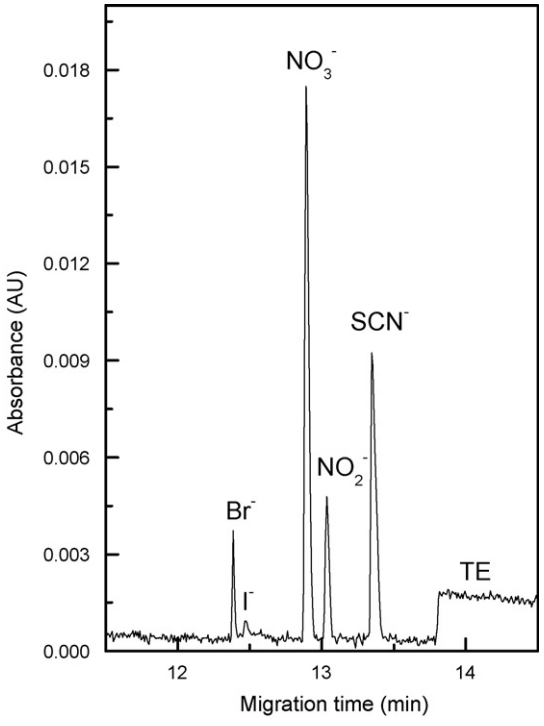

Fig. 2. tITP–CE response of a 100-fold diluted saliva. Conditions: LE, 5 mM dithionic acid, pH 7.2; TE, 10 mM formic acid, pH 4.4; injection time, 10, 30, and 30 s for LE, sample, and TE, respectively. Other conditions are as given in Fig. 1.

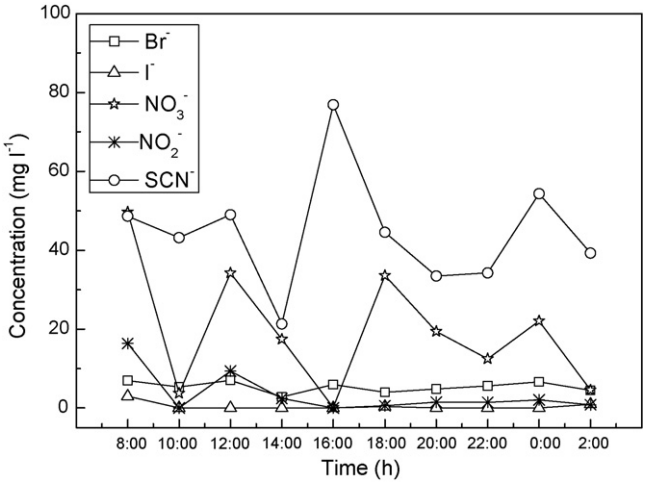

Fig. 3. Time-dependent changes in concentrations of anions monitored by tITP–CE. Conditions are as given in Fig. 2.

times of all anions, and also the stacker appears very close to thiocyanate.

Fig. 2 demonstrates the separation/detection performance for 100-fold diluted saliva under optimized tITP system conditions.

3.3. Limit of detection, calibration range, and precision thresholds

The limits of detection of anions under examination, assessed as three times signal-to-noise ratio, are listed in Table 1. Owing to tITP sample enrichment, the detectability was superior to that of most of previously reported CE methods [3,4,6] and comparable to the data of Tanaka et al. [5]. Notably, none of the mentioned reports takes account of bromide and iodide determination. As a striking result, all anions except for iodide can be detected in saliva yet after 500-fold dilution. Precision measurements performed with a 100-fold diluted saliva sample indicated excellent repeatability of migration time (R.S.D. <0.3%). The R.S.D.s for peak area were below 5% (Table 1). All above demonstrates that tITP–CE is reliable and effective to enhance the determination of inorganic anions in saliva.

3.4. Applicability of the developed method

Five anions were determined in 10-fold diluted saliva samples taken from the smoking volunteer, and the results on concentration variations during 1 day of monitoring are summarized in Fig. 3. The average concentrations of anions are also shown in Table 1. Some preliminary conclusions can be drawn as based on the daily variations of salivary anionic concentrations. First, the nitrate levels are clearly related to stimulus, tending to increase after having meal. Also obvious is an increase in thiocyanate concentration after smoking. On the other hand, other anions of interest experience virtually no alterations in concentration. In order to elucidate how the salivary analyte concentrations would reflect people's habits, activities, and health conditions, further research is anticipated.

4. Conclusions

A combination of tITP and CE holds great promise for carrying out ionic analyses with the enhanced sensitivity. This is mostly due to its in-line realization (with no necessity to modify conventional CE instrumentation) and the potential of selectively increasing the analyte concentration regardless of the sample conductivity. In this work, these merits of tITP–CE have been shown for the determination of a number of biologically relevant anions in saliva that required an explicit modification of the tITP system. A proper selection of the leading anion and its amount, rather than relying on the major sample component as the transient leader, allowed for a good compromise between the UV detection signals, resolution, and analysis time. Although this system has been demonstrated specifically with inorganic anions, it can be translated to other salivary analytes, which will permit this tITP–CE method to be used in clinical laboratories.

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