Talanta 128 (2014) 386–392

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Electromembrane extraction of salivary polyamines followed by capillary zone electrophoresis with capacitively coupled contactless conductivity detection

Yan Liu, Xiaoli Zhang, Lin Guo, Yang Zhang, Zhuo Li, Ziyao Wang, Mengfei Huang, Chen Yang, Jiannong Ye, Qingcui Chu *

Department of Chemistry, East China Normal University, Shanghai 200241, China

ARTICLE INFO

Article history:
Received 15 January 2014
Received in revised form
22 April 2014
Accepted 29 April 2014
Available online 9 May 2014

Keywords:
Electromembrane extraction
Polyamines
Saliva
Capillary zone electrophoresis
Capacitively coupled contactless conductivity detection

ABSTRACT

Electromembrane extraction (EME) as a novel sample preparation technique was firstly applied for the purification and enrichment of four polyamines mainly present in saliva samples. These four target analytes, putrescine, cadaverine, spermidine, and spermine, were directly determined by CZE with capacitively coupled contactless conductivity detection (CZE-C^4D) after EME procedure. Several factors affecting extraction efficiency, electrophoretic separation, and detection were investigated. Under the optimum conditions, four polyamines were baseline separated within 22 min, exhibiting a linear calibration over three orders of magnitude ($r > 0.999$); the highest enrichment factor could reach 106-fold (for spermidine), and the LODs were in the range of 1.4–7.0 ng mL^{-1}. The proposed EME/CZE-C^4D method has been successfully applied to analyze human saliva samples with recoveries in the range of 78–97%.

1. Introduction

Owing to the low levels of target analytes and the complexity of sample matrix, sample preparation is considered as an important procedure prior to their analyses [1,2]. Good sample preparation should provide not only clean-up, but also enrichment of target analytes prior to their instrumental analysis. In recent years, electromembrane extraction (EME) is proposed as a new concept for analytical sample preparation, firstly introduced by Pedersen-Bjergaard et al. [3]. In this approach, charged analytes are extracted from an aqueous sample (donor phase), through an organic solvent (as a supported liquid membrane, SLM) immobilized in the pores of a thin polymeric membrane, and into a few microlitres of an aqueous solution (acceptor phase). The driving force for the extraction is a dc electrical potential sustained over the SLM. For cationic analytes, the cathode is placed in the acceptor phase; whereas for anionic compounds, the polarity of the electrical potential is reversed, with an anode located in the acceptor phase [3]. Compared with other extraction methods including liquid–liquid extraction, solid-phase extraction and hollow-fiber liquid-phase microextraction, EME could avoid several disadvantages to a certain extent, providing much easier operation, shorter extraction time, or lower consumption of organic solvents and analytical cost [1,4]. Several applications of EME have been published in pharmaceutical, environmental and clinical analyses [5–8].

Saliva is a readily accessible, informative and non-invasive biofluid, making it ideal for the early detection of a wide range of diseases such as cardiovascular, renal, and autoimmune diseases, viral and bacterial infections, and particularly for cancers. Several papers have demonstrated that the concentrations of some species in saliva could be used as the indicators for the diagnoses of physiological and biological conditions [9–11]. For example, biogenic amines in oral cavity are mainly originated from the breakdown of proteins and peptides, and the degradation of precursor amino acids such as ornithine, lysine, and arginine by bacterial decarboxilase enzymes [12–15]. And, the aliphatic polyamines, mainly including putrescine (Put), cadaverine (Cad), spermidine (Spd), and spermine (Spm), are one of the primary non-volatile species present in human oral cavity [16,17], which are probably associated with oral malodour and even oral cancer [9,14,15,18]. For instance, Cooke et al. [14] reported that the mean concentrations of Put and Cad found in saliva of healthy human volunteers, immediately upon waking, are obviously higher than those of post-brushing. Furthermore, Perez [19] found that these polyamine levels in buccodental fluids could increase as a result of

Abbreviations: C^4D, capacitively coupled contactless conductivity detection; EME, electromembrane extraction; ENB, 1-ethyl-2- nitrobenzene; NPOE, 2-nitrophenyl octylether; DEHP, bis (2-ethylhexyl) phosphate; SLM, supported liquid membrane; Spd, spermidine; Spm, spermine; Cad, cadaverine; Put, putrescine

* Corresponding author. Tel./fax: +86 21 5434 0048.
E-mail address: qcchu@chem.ecnu.edu.cn (Q. Chu).

http://dx.doi.org/10.1016/j.talanta.2014.04.079
0039-9140/

some pathological proliferative processes. Therefore, the determination of salivary polyamines is particularly meaningful in both biochemistry and clinical chemistry.

Because of their low volatility and lack of chromophores, derivatization procedure (pre- or post-column) is a commonly used solution for determining these polyamines. Some chromatographic methods such as HPLC combined with UV [20,21], LIF [14,18], and CE with LIF [22], MS [11] have been applied for this purpose, but then, some problems caused by derivatization are also unavoidable such as long reaction time, tedious processes, or side products. Due to its high resolution power, low running cost and environment-friendly factors, CE has gained prominence among the available separation techniques. Now, a few direct analytical methods have been reported for the determination of several biogenic amines in selected food samples based on CE coupled with amperometric detection [23–25], conductometric detection [26], capacitively coupled contactless conductivity detection (C^4D) [27,28] or electrochemiluminescence detection [29]. C^4D introduced by da Silva and Zemann independently in 1998 [30,31] has been considered as an universal detection technique for CE, because of its various advantages including elimination of electrode surface fouling, effective isolation from high separation voltages, simplified detector design and electrode alignment. In our previous work, eight biogenic amines originated from foods have been directly analyzed by CZE with C^4D (CZE-C^4D) method [27].

In this work, EME as a novel sample preparation technique was firstly applied for the extraction of four main polyamines (Put, Cad, Spd, and Spm) in saliva samples, and the extracted solution was directly analyzed by a developed CZE-C^4D method, avoiding complex sample preparation and derivatization procedures. The proposed EME/CZE-C^4D method has been applied to analyze the real human saliva samples from healthy people and patients suffered from oral malodour, dental plaque, or tongue cancer.

2. Experimental

2.1. Materials and reagents

Put (≥ 98.0%), Cad (≥ 99.0%), Spm (≥ 98.0%), and Spd (99.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and they were all used as received. 1-octanol and 18-crown-6 were purchased from J&K Chemical (Shanghai, China), chloride salts (NaCl, KCl, $CaCl_2$, $MgCl_2$, and NH_4Cl) and acetic acid (HAc) were purchased from China National Pharmaceutical Group Corporation (Shanghai, China), and 1-ethyl-2-nitrobenzene (ENB), 2-nitrophenyl octylether (NPOE) and bis (2-ethylhexyl) phosphate (DEHP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical grade, and deionized water with resistivity higher than 18 MΩ cm was used in this work.

2.2. Standard solution and saliva sample preparation

The stock solution of each analyte (1.0 mg mL^{-1}) was prepared with deionized water. A fresh mixed standard solution was prepared daily by diluting the stock solution with running buffer (0.18 mol L^{-1} 18-crown-6/0.50 mol L^{-1} HAc buffer) to the desired concentration. Before use, all solutions were stored in a 4 °C refrigerator. Stock solutions of inorganic cations (Na^+, 0.050 mol L^{-1}; K^+, 0.22 mol L^{-1}; Ca^{2+}, 0.020 mol L^{-1}; Mg^{2+}, 0.020 mol L^{-1}; and NH_4^+, 0.060 mol L^{-1}) were prepared from their corresponding chloride salts. Artificial saliva sample used as donor solution for EME consisted of 0.33 mmol L^{-1} Na^+, 1.5 mmol L^{-1} K^+, 0.13 mmol L^{-1} Ca^{2+}, 0.013 mmol L^{-1} Mg^{2+}, NH_4^+ 0.40 mmol L^{-1} and 0.050 μg mL^{-1} of each polyamine. Saliva samples of healthy people were collected from volunteers at our laboratory, and those of patients suffered from oral malodour, dental plaque, or tongue cancer were obtained from Shanghai Ninth People's Hospital (Shanghai, China). All samples were stored at −20 °C. Before use, each thawed saliva sample was diluted 15-fold with deionized water for EME procedure.

2.3. Electrophoretic conditions

The laboratory-built CZE-C^4D system was employed and described previously [27]. C^4D (ER125) instrument was purchased from eDAQ Pty Ltd. (6 Doig Avenue, Denistone East, NSW 2112, Australia). The excitation frequency was set to 550 kHz and the amplitude to 60 Vpp (peak-to-peak voltage) for C^4D. The effective length of the capillary tube (23.5 μm id × 360 μm od, Polymicro Technologies, Phoenix, AZ, USA) was 88.0 cm to C^4D. Before its first use, the capillary was conditioned by washing with 0.1 mol L^{-1} NaOH for 15 min, deionized water for 15 min, and finally with the running buffer for 15 min. The running buffer was 0.18 mol L^{-1} 18-crown-6/0.50 mol L^{-1} HAc buffer (pH 2.5). The separation voltage was 12 kV and the injection time was 6 s (at 12 kV). All experiments were performed at room temperature. (The typical electropherogram of a standard mixture solution of four analytes was shown in Fig. S1 of 'Supplementary Material'.)

2.4. EME equipment

The equipment for EME mainly consisted of a 8 mL glass vial (Ningbo Yinzhou Hamai Instrument Technology Co., Ltd., Zhejiang province, China) with a screw-cap, two 0.2 mm platinum electrodes (99.95%, Wuhan Gaoss Union Technology Co., Ltd., Hubei Province, China), and a power supply (0–30 V variable voltage and maximum current of 1 A, Shanghai Nuoyi Electronic Technology Co., Shanghai, China). Two holes were drilled through the cap in order to accommodate two 10 μL pipette tips acting as leading channels for the platinum electrodes. The two pipette tips could insulate the platinum electrodes very well and thereby avoid possible short circuit of a power supply. Stirring of the solutions was done by a H01-1B magnetic stirrer (0–1500 rpm, Shanghai Meiyingpu Instrument Manufacturing Co., Ltd., Shanghai, China). Q3/2 Accurel PP polypropylene microporous hollow-fiber membrane (200 μm wall thickness, 600 μm inner diameter, 0.2 μm pore size, 75% porosity; Membrana, Wuppertal, Germany) was cut into 4.5 cm segments, cleaned in acetone and dried prior to use. The one end of the hollow fiber was sealed, and its inner lumen was filled with the acceptor solution using a micro-syringe (with a needle of 0.3 mm outer diameter and 5.5 cm length, Shanghai Guangzheng Medical Instruments Co., Ltd., Shanghai, China) without penetration into the wall of the hollow fiber; otherwise, it would be lost during the extraction. (The illustration of EME device was shown in Fig. S2 of 'Supplementary Material'.)

2.5. EME procedure

The optimization of EME procedure was conducted using a unified standard mixed solution composed of four target polyamines (0.010 μg mL^{-1} each). A volume of 7 mL donor solution was added into the glass vial, and a magnetic stir bar was placed into the glass vial to ensure constant stirring of the donor solution. A one-end-sealed hollow fiber extraction unit was firstly impregnated in the organic solvent (v_{ENB}: v_{DEHP}=30:1) for 5 s to form the SLM, and filled with the acceptor phase (~8 μL, 0.15 mol L^{-1} 18-crown-6/0.50 mol L^{-1} HAc solution). Then the platinum electrode was carefully inserted into the hollow fiber, and the other open end was firmly pulled on the pipette tip acting as a leading channel for the cathode, which was carefully inserted into the donor phase as well as the anode platinum electrode. The magnetic stirrer was switched on to start the extraction at 400 rpm. The EME system was operated at 10 V by a power supply

for 20 min. And then, the magnetic stirrer and power supply were switched off, and the hollow fiber was removed from the sample solution. The acceptor solution in the hollow fiber was withdrawn into a syringe, and was ready for CZE-C^4D analysis.

3. Results and discussion

3.1. Optimization of EME procedure

To investigate the enrichment factor (EF), various parameters, including the type of SLM, the pH of donor and acceptor phases, stirring rate, applied voltage, extraction time, and matrix effect were optimized by a univariate approach. The EF was calculated according to the following equation: $EF = c_{a,final}/c_{d,initial}$, where $c_{a,final}$ was the final concentration of the analyte in the acceptor phase, and $c_{d,initial}$ was the initial analyte concentration within the donor solution.

3.1.1. Selection of organic solvent for EME

The SLM composition plays an important role in EME. Several factors need to be considered in choosing the organic solvent. It is critical that the organic solvents used for EME procedure should have certain polarity, suitable chemical properties to facilitate electrokinetic migration of the analytes and low vapor pressure to prevent loss during the extraction process [32]. Considering all these factors, toluene, 1-octanol, NPOE and ENB were investigated as the suitable SLM. The experimental results showed that the acceptor phase was easily lost when toluene was used as the SLM, and a possible explanation was that the SLM of toluene was unstable in EME process; 1-octanol and ENB could provide slightly higher EFs than NPOE, but in general low EFs of all polyamines could be obtained when only a single organic solvent was used as the SLM, especially for Spm and Spd.

It has been found that addition of hydrophobic ion-pair reagents to SLM would enable better phase transfer and electrokinetic migration of analytes [33]. This is because ion-pair reagents enhance the solubility of the protonated analytes, which aids in the permeation of the analytes at the interface between the sample solution and the SLM. For this purpose, the effects of different quantities of DEHP added to pure 1-octanol and ENB as the SLM were investigated, respectively. In general, although the EFs of all analytes were increased apparently for both ENB/DEHP and 1-octanol/DEHP SLMs, ENB/DEHP could provide much higher EFs than 1-octanol/DEHP. Moreover, ENB could provide more

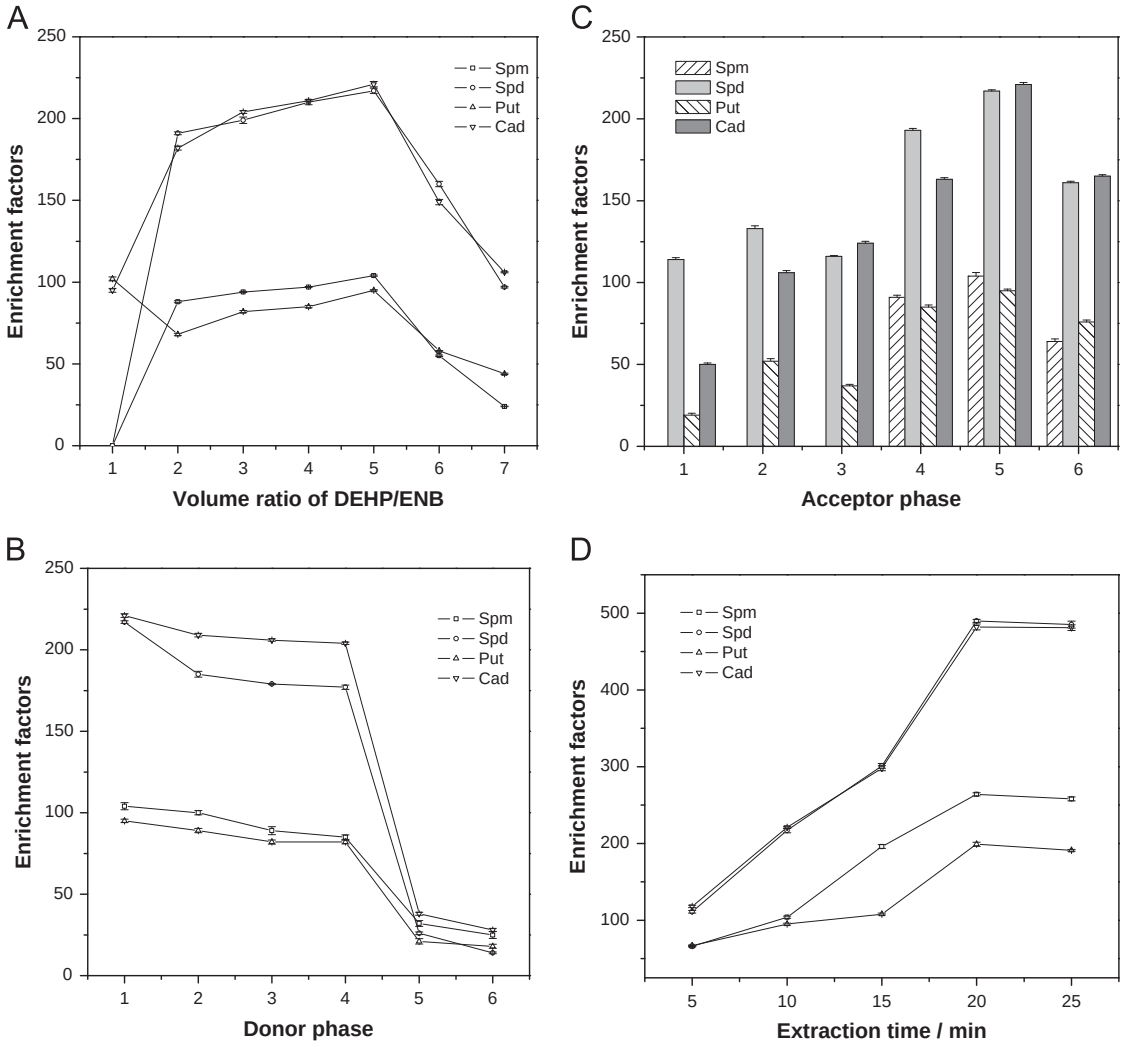

Fig. 1. Effects of (A) volume ratio of DEHP:ENB, (B) donor phase, (C) acceptor phase, and (D) extraction time on EFs of four polyamines. (A) (1) 0:100; (2) 1:80; (3) 1:50; (4) 1:40; (5) 1:30; (6) 1:20; (7) 1:10. (B) (1) neutral deionized water; (2) 0.010 mol L^{-1} HAc solution (pH 3.4); (3) 0.050 mol L^{-1} HAc solution (pH 3.0); (4) 0.10 mol L^{-1} HAc solution (pH 2.9); (5) 0.50 mol L^{-1} HAc solution (pH 2.5); (6) 1.0 mol L^{-1} HAc solution (pH 2.4). (C) (1) 0.25 mol L^{-1} HAc solution; (2) 0.50 mol L^{-1} HAc solution; (3) 1.0 mol L^{-1} HAc solution; (4) 0.50 mol L^{-1} HAc/0.12 mol L^{-1}18-crown-6; (5) 0.50 mol L^{-1} HAc/0.15 mol L^{-1} 18-crown-6; (6) 0.50 mol L^{-1} HAc/0.18 mol L^{-1} 18-crown-6. Optimum EME conditions: SLM: v_{ENB}: v_{DEHP}=30:1; donor phase: neutral solution; acceptor phase: 0.15 mol L^{-1} 18-crown-6/0.50 mol L^{-1} HAc solution; extraction voltage: 10 V; stirring speed: 400 rpm; and extraction time: 20 min.

efficient purification in preventing co-extracting cations across the SLM. As seen in Fig. 1A, the EFs of the analytes were increased with the increasing volume fraction of DEHP except for Put; as the volume ratio of DEHP:ENB reached to 1:30, highest EFs of other three polyamines could be obtained; when the volume ratio was higher than 1:30, increasing the volume fraction of DEHP could cause an obvious decrease in EFs. The strong interaction of the ion-pair complex of target analytes with the organic phase might be responsible for this behavior [33]. Therefore, 1:30 of the volume ratio (ν_{DEHP}: ν_{ENB}) was selected as the SLM for further studies.

3.1.2. Effect of pH in donor and acceptor phase

In this part, the effects of donor and acceptor phase on enrichment efficiency were investigated. Acidic donor and acceptor solutions were investigated for EME in order to be compatible with acidic background electrolyte solution used for CZE-C^4D determination of polyamines. The pKa values of four polyamines are approximately 7.9, 8.4, 10.1, 10.9 (Spm), 8.4, 9.9, 10.9 (Spd), 9.7, 11.2 (Put), and 9.1, 10.3 (Cad), respectively. Since the driving force in EME is an applied electrical potential across the SLM, charged analytes in the donor phase can migrate across the SLM toward the electrode of opposite charge in the acceptor solution. This means that these basic polyamines should have cationic forms in donor solution and could migrate toward the cathode driven by the electrical field. So, the ionization degree of the analytes influenced their transfer from the SLM to the acceptor phase, and the completely ionized forms of analytes could be achieved when the pH value was lower than their pKa values. As shown in Fig. 1B, the effects of various concentrations of HAc solutions (0.010, 0.050, 0.10, 0.50, and 1.0 mol L^{-1}) as well as neutral deionized water on EFs of the target analytes were examined, and neutral water could provide highest EFs for all analytes, thus neutral water was selected as diluent for standard solutions for further investigations.

Besides, the effects of acceptor solution composition on enrichment efficiency were also investigated as shown in Fig. 1C. In considering enrichment efficiency and compatibility with background electrolyte solution used for CZE-C^4D, 0.50 mol L^{-1} HAc solution was selected as basal acceptor solution. Meanwhile, the effects of concentrations of buffer additive 18-crown-6 on EFs of the analytes were also tested. The results showed that the maximum EFs for all polyamines were achieved when the acceptor solution consisted of 0.15 mol L^{-1} 18-crown-6 in 0.50 mol L^{-1} HAc solution. So, 0.15 mol L^{-1} 18-crown-6/0.50 mol L^{-1} HAc buffer was selected as the optimum acceptor phase.

3.1.3. Effect of the applied voltage

In EME, the electrokinetic migration of the analytes across the SLM into the acceptor phase is largely dependent on the applied voltage. So, a series of experiments with extraction voltage ranging from 3 to 30 V was conducted. As expected, the EFs of four analytes were increased with increasing the applied voltage in the range of 3–10 V; when the applied voltage was higher than 10 V, the EFs of the analytes leveled off, and some of them even began to decrease. Therefore, 10 V of extraction voltage was selected for subsequent experiments, which presents a possibility to utilize a common dry cell battery for the EME of these polyamines. (The effects of extraction voltage on EFs of the analytes were shown in Fig. S3A of 'Supplementary Material'.)

3.1.4. Effect of stir rate

Stirring is essential in most diffusion-based microextraction techniques to ensure properly mixing of the sample in order to reduce the thickness of the boundary layer at the interface between sample solution and SLM [3]. So, the effect of stir rate on the extraction efficiency was also investigated in the range of 0–600 rpm. It was found that when the stir rate was lower than 400 rpm, the EFs of the analytes increased with increasing the stir rate because of the convection effect; while EFs decreased at higher rates. A possible reason was that excessive agitation could disturb the directional migration of the analytes toward the cathode. So, stirring at 400 rpm was used for the rest of the experiments. (The effect of stir rate on EFs of the analytes was shown in Fig. S3B of 'Supplementary Material'.)

3.1.5. Effect of extraction time

Extraction time is a key parameter determining the total amount of ions transferred from donor to acceptor phase, provided the extraction voltage is constant during the whole extraction process. To investigate the electrokinetic migration over time, the EFs of the analytes were examined as the extraction time was varied from 5 to 25 min. As shown in Fig. 1D, the EFs of four polyamines were almost linearly increased from 5 to 20 min; as the extraction time was longer than 20 min, the EFs of all analytes leveled off, or even slightly decrease. Considering the enrichment efficiency and analysis time, the EME was performed in 20 min for the subsequent experiments.

Based on above described experiments, following optimum experimental conditions were obtained for EME of polyamines: SLM: ν_{ENB}: ν_{DEHP}=30:1, donor phase: neutral solution, acceptor phase: 0.15 mol L^{-1} 18-crown-6/0.50 mol L^{-1} HAc solution, applied voltage: 10 V, stirring speed: 400 rpm, and extraction time: 20 min. Under the above optimum EME conditions, the EFs of four polyamines could achieve 264-fold (Spm), 490-fold (Spd), 198-fold (Put), and 482-fold (Cad), respectively.

3.1.6. Effect of sample matrix

As we all know, human saliva contained various inorganic cations, mainly including Na^+, K^+, Ca^{2+}, Mg^{2+} and NH_4^+ [34], and their average concentrations in whole unstimulated saliva are 5, 22, 2, 0.2, and 6 mmol L^{-1}, respectively. In the EME process, these inorganic cations to a certain extent could hinder the target analytes transport across the SLM, as these above ions also partially migrated into the lumen. Therefore, under the above optimum EME conditions, the effects of sample matrix on the extraction efficiency were also conducted, and the concentrations of inorganic cations in artificial saliva samples (used as donor

Table 1
The regression equations, linearity and LODs of four polyamines.[a]

Analyte	Regression equation[b]	Correlation coefficient	Linear range (μg mL^{-1})	EF[c]	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Repeatability[a] (n=5, %)
Spm	y=4.1x−1.4	0.9999	0.5–100	80	1.7	5.7	6.3
Spd	y=4.8x+0.3	0.9993	0.5–100	106	1.4	4.7	4.1
Put	y=5.4x +1.4	0.9998	0.5–100	17	7.0	23.3	9.6
Cad	y=6.0x−4.6	0.9991	0.5–100	72	1.6	5.3	4.8

[a] EME/CZE-C^4D conditions were the same as those in Figs. 1 and 2.
[b] In the regression equation, the x value was the concentration of analytes (μg mL^{-1}), the y value was the peak area (mV.s).
[c] The concentrations of inorganic cations in the donor phase were 0.33 mmol L^{-1} Na^+, 1.5 mmol L^{-1} K^+, 0.13 mmol L^{-1} Ca^{2+}, 0.013 mmol L^{-1} Mg^{2+}, and 0.40 mmol L^{-1} NH_4^+, respectively.

solutions) correspond to 15-fold diluted human saliva samples. As expected, there was a clear decrease in the EFs of polyamines as shown in Table 1, however, this method still provide enough sensitivity for the simultaneous determination of the target analytes. Furthermore, as seen from Fig. 2A and 2B, the SLM used in this EME procedure could effectively reduce the migration of most inorganic cations except for K^+, and that four polyamines could be well separated from the co-extraction ions under the optimized CZE-C^4D conditions. Besides, under this EME conditions, other interferences such as anions and neutral molecules present in saliva could have no or ignorable effect on the extraction and electrophoretic separation of the target analytes.

3.2. Method validation

3.2.1. Linearity and LODs of four polyamines

To determine the linearity of four polyamines, a series of the mixed standard solutions containing 0.20 μg mL^{-1} to 0.10 mg mL^{-1} of each analyte was tested. The correlation between peak area and concentration of each analyte was subjected to regression analysis to obtain the calibration equations and correlation coefficients, as listed in Table 1. The results showed an excellent linearity ($r > 0.999$) between peak area and analyte concentration at three orders of magnitude. The LODs and LOQs of four analytes were in the range of 1.4~7.0 ng mL^{-1} ($S/N=3$) and 4.7–23.3 ng mL^{-1} ($S/N=10$), respectively, based on EME procedure. The four polyamines could be pre-concentrated up to 106-fold taking the matrix effects of co-extraction inorganic cations into consideration. Compared with reported methods including HPLC-FD [18], HPLC-UV [20], CE-AD [24], CE-CD [26] and CZE-C^4D [27], the proposed EME/CZE-C^4D method could obtain either equivalent or superior LODs for the target polyamines (Spd: 1.4 ng mL^{-1} vs 7.3–44.3 ng mL^{-1}; Spm: 1.7 ng mL^{-1} vs 8.1–80.9 ng mL^{-1}; Put: 7.0 ng mL^{-1} vs 5.3–176.3 ng mL^{-1}; Cad: 1.6 ng mL^{-1} vs 20.4–204.4 ng mL^{-1}).

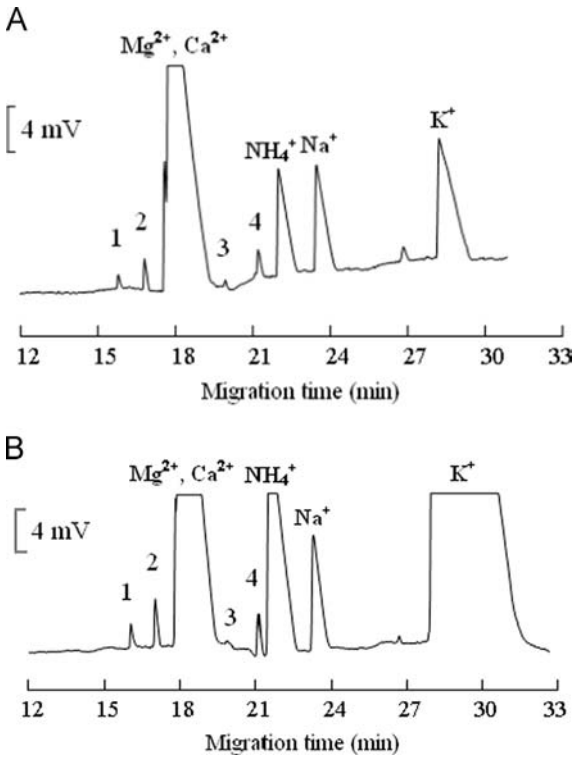

Fig. 2. Electropherograms of acceptor phases of standard mixture solution of four polyamines (A) in deionized water and (B) in sample. Experimental conditions: effective length of the capillary tube, 88.0 cm (23.5 μm id × 360 μm od) for C^4D; excitation frequency for C^4D, 550 kHz; peak-to-peak voltage for C^4D, 60 Vpp; running buffer, 0.18 mol L^{-1} 18-crown-6/0.50 mol L^{-1} HAc buffer (pH 2.5); separation voltage, 12 kV; injection time, 6 s (at 12 kV); peak identification: (1) Spm, (2) Spd, (3) Put, and (4) Cad; concentrations of four polyamines: (A) 0.010 μg mL^{-1}, each; (B) 0.050 μg mL^{-1}, each.

3.2.2. Precision

The reproducibility of the CZE-C^4D method was evaluated by intraday precision and interday precision at three different concentrations (10, 2.0, and 0.5 μg mL^{-1}), respectively. The RSD was used as a measure of precision (as shown in Table S1 of 'Supplementary Material'). The intraday precision was assessed by making seven repetitive injections of the mixed standard solution under the selected optimum conditions. The assay results showed that the RSDs of peak area and migration time were within 1.1%, 1.1% for Spm, 0.8%, 1.2% for Spd, 1.4%, 1.3% for Put and 1.2%, 1.4% for Cad, respectively. Besides, the interday precision was also estimated by making repetitive injections of a mixed standard solution for five consecutive days with three replicates, and the RSDs of peak area and migration time were within 3.4% and 3.2%, respectively. The results indicated that it was feasible to simultaneously determine the polyamines based on the proposed CZE-C^4D method.

Besides, the reproducibility of the EME procedure was also evaluated by making five repetitive extractions of the standard analytes (0.050 μg mL^{-1} each) in the artificial saliva samples under the selected optimum conditions. As shown in Table 1, the RSDs of four polyamines were in the range of 4.1–9.6%, which

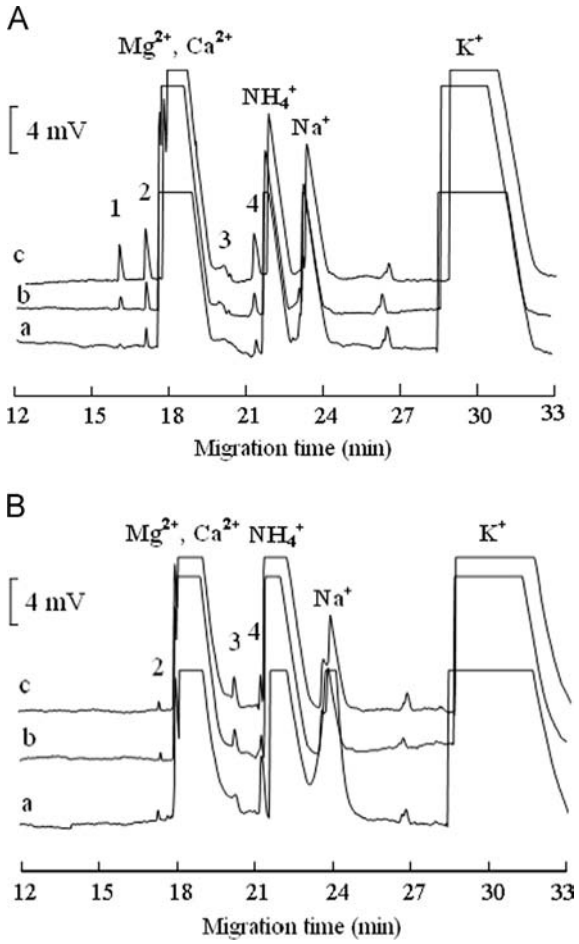

Fig. 3. Electropherograms of (A) healthy volunteer's saliva sample (a. blank saliva sample, b. saliva sample spiked with 0.020 μg mL^{-1} of each polyamine, and c. saliva sample spiked with 0.10 μg mL^{-1} each polyamine) and (B) patients' saliva samples with different oral diseases (a. tongue cancer, b. dental plaque, and c. oral malodour). The EME/CZE-C^4D conditions were the same as in Figs. 1 and 2.

Table 2
Assay results of four main polyamines in human saliva samples ($n=3$).[a]

Saliva samples	Sample time	Spm (ng mL^{-1})	Spd (ng mL^{-1})	Put (ng mL^{-1})	Cad (ng mL^{-1})
Healthy volunteers ($n=5$)	Before-brushing	67.4 ± 45.0	103.6 ± 90.4	1509.8 ± 628.6	1580.2 ± 934.2
	Post-brushing	nd[b]	8.6 ± 19.2	201.0 ± 41.5	113.2 ± 28.1
Dental plaque	Post-brushing	nd	110.4	1921.0	325.5
Oral malodor	Post-brushing	nd	115.7	2480.2	466.9
Tongue cancer	Post-brushing	nd	90.2	1263.4	1669.5

[a] EME/CZE-C^{4}D conditions were the same as those in Figs. 1 and 2.
[b] nd: not detected.

indicated that the EME method could provide relative good repeatability.

3.2.3. Accuracy

To further evaluate the reliability of the proposed method, recovery experiments were also conducted by a standard addition method. Under the optimum conditions, recovery data were determined with the saliva samples of No. 1 healthy volunteer (before brushing) and patient with dental plaque (as shown in Table S2 of 'Supplementary Material'). The average recovery data at the levels of 0.10 μg mL^{-1} and 0.020 μg mL^{-1} were in the range of 78–97% with corresponding RSDs of 2.9–4.2%, which indicated that the EME/CZE-C^{4}D method was sufficiently accurate for the simultaneous determination of the four polyamines.

3.3. Applications to real samples

Under the optimum conditions, the proposed EME/CZE-C^{4}D method was applied for determining four polyamines in human saliva samples collected from different volunteers including healthy human and patients who suffered from oral malodour, dental plaque, and tongue cancer. The typical sample electropherograms of healthy volunteers and patients were shown in Fig. 3A and 3B, respectively; and three electropherograms (as shown in a–c) in Fig. 3A represented blank saliva sample, spiked saliva sample at 0.020 μg mL^{-1} level, and spiked saliva sample at 0.10 μg mL^{-1} level, respectively. By a standard addition method and comparing the migration time of target analytes with those of the mixed standard solution electropherogram (Fig. 2B), four polyamines were determined in different saliva samples. From the sample electropherograms, we can see that the target polyamines could be well separated from the main co-extraction substances in the saliva samples under the optimum experimental conditions.

The detailed data for the tested saliva samples were summarized in Table 2. The assay results showed that the contents of three main polyamines, Put, Cad and Spd, in the saliva samples of patients with oral diseases were significantly higher than those of healthy volunteers. Besides, in terms of healthy volunteers, the contents of four polyamines in saliva samples collected before brushing were 3–4 times higher than those sampled after brushing, which was similar to the assay results found by Cooke et al. [14]. So, the assay results indicated that the polyamines could be recommended as the biomarkers of oral diseases in early non-invasive diagnosis. Besides, the concentrations of Put and Cad obtained in our work were lower than those reported by Cooke [14] and Goldberg et al. [15], and a possible reason was that in their studies, the saliva samples were firstly extracted and then centrifugated, so the assay results were the total contents of both the free amines and the amines bound with proteins and the cellular elements; whereas in our work biological macromolecules could not transfer into SLM of EME, and only the free amines in saliva samples were determined by the proposed EME/CZE-C^{4}D method.

4. Conclusions

This work demonstrated the capability of EME/CZE-C^{4}D method for sensitive determination of four main polyamines in human saliva samples. The LODs of four polyamines obtained by the proposed method were either equivalent or superior to those of most of reported methods; and relatively good recoveries (78–97%) were obtained for the saliva samples. Furthermore, the EME procedure could provide good purification and relatively high extraction efficiency in the analysis of complex biological samples. The proposed method may provide an alternative for early non-invasive diagnosis of several oral diseases. In order to collect statistically significant biological data, further investigations were suggested to discriminate between the pathological state of oral disease patients and physiological conditions of healthy subjects, using the simple, rapid and economic method here reported for the quantification of salivary polyamines.

Acknowledgments

This work was supported by the Natural Science Foundation of China (No. 21205042), the Special Funds for the Development of Major Scientific Instruments and Equipment (No.2011YQ15007205), and the Students Innovative Experimental Project of Shanghai Municipality (No. KY2012-28S).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.079.

References

[1] J. Lee, H.K. Lee, K.E. Rasmussen, S. Pedersen-Bjergaard, Anal. Chim. Acta 624 (2008) 253–268.
[2] K.M. Vamsi, G. Madhavi, V. Ramanasri, J. Chromatogr. Sci. 51 (2013) 619–631.
[3] S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. A 1109 (2006) 183–190.
[4] M.A. Bello-Lopez, M. Ramos-Payan, J.A. Ocana-Gonzalez, R. Fernandez-Torres, M. Callejon-Mochon, Anal. Lett. 45 (2012) 804–830.
[5] N.J. Petersen, K.E. Rasmussen, S. Pedersen-Bjergaard, A. Gjelstad, Anal. Sci. 27 (2011) 965–972.
[6] L. Strieglerova, P. Kuban, P. Bocek, J. Chromatogr. A 1218 (2011) 6248–6255.
[7] K. Alhooshania, C. Basheera, J. Kaurb, A. Gjelstadc, K.E. Rasmussenc, S. Pedersen-Bjergaardc, H.K. Leeb, Talanta 86 (2011) 109–113.
[8] M.R. Payan, M.A.B. Lopez, R.F. Torres, M.V. Navarro, M.C. Mochon, Talanta 85 (2011) 394–399.
[9] C. Streckfus, L. Bigler, M. Tucci, J.T. Thigpen, Cancer Invest. 18 (2000) 101–109.
[10] A. Zinellu, S. Sotgia, E. Pisanu, B. Scanu, M. Sanna, M. Franca Usai, R. Chessa, L. Deiana, C. Carru, Anal. Bioanal. Chem. 398 (2010) 1973–1978.
[11] M Sugimoto, D.T. Wong, A. Hirayama, T. Soga, M. Tomita, Metabolomics 6 (2010) 78–95.
[12] D. Codipilly, I. Kleinberg, J. Breath Res. 2 (2008) 1–10.
[13] H.A. Soini, I. Klouckova, D. Wiesler, E. Oberzaucher, K. Grammer, S.J. Dixon, Y. Xu, R.G. Brereton, D.J. Penn, M.V. Novotny, J. Chem. Ecol. 36 (2010) 1035–1042.
[14] M. Cooke, N. Leeves, C. White, Arch. Oral Biol. 48 (2003) 323–327.
[15] S. Goldberg, A. Kozlovsky, D. Gordon, I. Gelernter, A. Sintov, M. Rosenberg, J. Dent. Res. 73 (1994) 1168–1172.

[16] I. Kleinberg, G. Westbay, Oral Biol. Med. 1 (1990) 247–259.
[17] J. Tonzetich, E. Eigen, W.J. King, S. Weiss., Archs. Oral Biol 12 (1967) 1167–1175.
[18] M. Venza, M. Visalli, D. Cicciu, D. Teti, J. Chromatogr. B 757 (2001) 111–117.
[19] R. Perez, Pract. Odontol. 11 (1990) 59–63.
[20] A.A. Dutov, D.A. Nikitin, M.N. Mishchenko, O.N. Litvintseva, E.N. Ivanova, I. N. Zavorotnykh, E.N. Shishkina, Klinicheskaya Laboratornaya Di. 10 (2009) 27–29.
[21] P. Wang, X.Y. Liu, Chin. J. Biochem. Pharm 29 (2008) 268–270.
[22] T. Tabi, Z. Lohinai, M. Palfi, M. Levine, E. Szoko, Anal. Bioanal. Chem. 391 (2008) 647–651.
[23] Q.C. Chu, Y.Q. Guan, C.H. Geng, J.N. Ye, Anal. Lett. 39 (2006) 729–740.
[24] X.H. Sun, X.R. Yang, E.K. Wang, J. Chromatogr. A 1005 (2003) 189–195.
[25] Q.J. Wang, H. Yu, H. Li, F. Ding, P.G. He, Y.Z. Fang, Food Chem. 83 (2003) 311–317.
[26] F. Kvasnicka, M. Voldrich, J. Chromatogr. A 1103 (2006) 145–149.
[27] W.L. Li, Y.L. Pan, Y. Liu, X.L. Zhang, J.N. Ye, Q.C. Chu, Chromatographia 77 (2014) 287–292.
[28] X.Y. Gong, P.C. Hauser, Electrophoresis 27 (2006) 468–473.
[29] J.F. Liu, X.R. Yang, E.K. Wang, Electrophoresis 24 (2003) 3131–3138.
[30] J.A. Fracassi da Silva, C.L. do Lago, Anal. Chem. 70 (1998) 4339–4343.
[31] A.J. Zemann, E. Schnell, D. Volgger, G.K. Bonn, Anal. Chem. 70 (1998) 563–567.
[32] L. Xu, P.C. Hauser, H.K. Lee., J. Chromatogr. A 1214 (2008) 17–22.
[33] A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, J. Chromatogr. A 1124 (2006) 29–34.
[34] S. Chiappin, G. Antonelli, R. Gatti, E.F. De Palo., Clin. Chim. Acta 383 (2007) 30–40.