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Original article

Capillary electrophoresis with laser-induced fluorescence detection of main polyamines and precursor amino acids in saliva

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ABSTRACT

A hollow-fiber liquid-phase microextraction (HF-LPME) method has been developed for the purification and preconcentration of biogenic polyamines and their precursor amino acids in human saliva. Putrescine (Put), cadaverine (Cad), spermidine (Spe), ornithine (Orn), lysine (Lys), and arginine (Arg) were determined by the CE-LIF detection after microextraction. Several factors that affect extraction efficiency, separation, and detection were investigated. Under the optimum conditions, six analytes could achieve baseline separation within 30 min, exhibiting a linear calibration at three orders of magnitude ($r^2 > 0.998$); the obtained enrichment factors of HF-LPME were between 19 (for Orn) and 218 (for Cad), and the LODs were in the range of 0.0072–0.26 nmol/L. The proposed HF-LPME/CE-LIF method has been successfully applied for the sensitive analyses of the real-world saliva samples collected from healthy volunteers and different patients with oral diseases, providing a potential method for primary non-invasive diagnosis of some oral diseases.

1. Introduction

Organic compounds with amino functional groups are very important biomolecules, and the quantitative analysis of these compounds plays a critical role in many fields including clinical diagnostics, food analysis, and biochemical research [1,2]. In particular, amino acid and biogenic amine analyses have become one focus of international research; and several analytical approaches such as HPLC [3] or CE [4,5] coupled to a variety of detectors have been developed for this purpose. CE, because of its electrophoretic separation mechanism, is particularly suited to analyzing charged amines or amino acids. However, direct detection of these compounds is rather difficult due to their low volatility and lack of chromophores. In practice, analyte derivatization (pre-, on- or post-column) has played an important role to overcome these challenges, and the LIF detection offers especially outstanding sensitivity in a variety of detectors [6]. A number of fluorescence reagents have been proposed for amino compounds derivatization. Several reviews describing the derivatization reagents and procedures for CE-LIF detection have been published [4,7,8]. Generally, naphtalene-2,3-dicarboxyaldehyde, *o*-phthaldialdehyde, 9-fluorenylmethyl chloroformate, 3-(4-carboxybenzoyl)quinoline-2- carboxaldehyde, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole, and fluorescein isothiocyanate (FITC), *etc.* have been reported. Among these, FITC is one of the most suitable and widely used reagents for derivatization of primary and secondary amine groups, and the resultant products are highly fluorescent.

Saliva is a readily accessible and informative biofluid, making it ideal for the early detection of a wide range of diseases including cardiovascular, renal, and autoimmune diseases, viral and bacterial infections and, more importantly, cancer [9–11]. In recent years, several research groups have tried to analyze some amino compounds in human saliva based on CE coupled with LIF [1,12–15], mass spectrum [10], or contactless conductivity detection [16]. Because of its easy sample purification, lack of interference, and low organic solvent consumption, hollow-fiber liquid-phase microextraction (HF-LPME) [17] has gained attention in the field of sample preparation. HF-LPME is a microextraction process based on mass transfer through the supported liquid membrane formed by an organic solvent within the fiber holes of the wall, with extraction taking place in the porous hollow fiber lumen. The acceptor phase is not in direct contact with the sample solution, thus avoiding the solvent loss that happens easily in single-drop microextraction and dispersive liquid-phase microextraction. Moreover, as macromolecules, granular impurities *etc.* are not transferable across the fiber wall holes, HF-LPME also has a more prominent sample purification function compared with solid-phase microextraction and liquid-phase microextraction methods mentioned above, hence expanding the scope of

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analytical substrates. As a result, it can be used for direct analyses of samples with complex matrices.

In the present work, a novel method of HF-LPME/CE-LIF combining with FITC derivatization, has developed for the first time to determine three biogenic polyamines (putrescine (Put), cadaverine (Cad), and spermidine (Spe)) and their amino acid precursors (ornithine (Orn), lysine (Lys), and arginine (Arg)) in non-invasive saliva samples. The parameters involved in derivatization, electrophoretic separation and enrichment factors (EFs) of the targeted analytes were optimized. This method took advantage of the efficiency, reproducibility, ultra-small sample volume of CE, the high sensitivity of LIF and the enrichment capability of HF-LPME. So this detection method that we developed has the superiority of low LOD (nmol) and less injection volume. The proposed method has been applied to analyze real-world saliva samples from healthy volunteers, and patients suffering from different oral diseases including halitosis, gingivitis, dental plaque, tongue cancer or gingival cancer.

2. Experimental

The laboratory-built CE-LIF system was employed and described previously [18]. A fused-silica capillary (25 μm i.d. × 360 μm o.d., Polymicro Technologies, Phoenix, AZ, USA) was used for the separation, and the effective capillary length was 90 cm for LIF. LIF detector (TriSepTM-2100LIF, Unimicro (Shanghai) Technologies Co., Ltd., Shanghai, China) was used in this work. The excitation wavelength of LIF detector was 483 nm, and the emission central wavelength was 520 nm.

Put (≥98.0%), Cad (≥99.0%), Spe (≥98.0%), Orn (>98.0%), Lys (99.0%), Arg (99.0%), and FITC (99.0%) were purchased from Sigma–Aldrich (St. Louis, MO, USA); poly(sodium-*p*-styrenesulfonate) (PSS) and cetrimonium bromide (CTAB) were purchased from China National Pharmaceutical Group Corporation (Shanghai, China), and they were all of analytical grade and used as received. The stock solution of each analyte (1.0 mmol/L) was prepared with doubly distilled water, and that of FITC (1.0 mmol/L) was prepared with acetone; and all stock solutions were stored in a refrigerator at 4 °C. A fresh, mixed standard solution was prepared daily by diluting the stock solution with a NaOH solution (300 mmol/L) to the desired concentrations.

Saliva samples of healthy volunteers were collected randomly from students in our laboratory, and those of patients suffered from halitosis, gingivitis, dental plaque, tongue cancer or gingival cancer were collected in Shanghai Ninth People's Hospital (Shanghai, China). All samples were stored at −20 °C. To a 1.5 mL micro-tube with 500 μL thawed saliva sample containing analytes of interest was added 500 μL of acetonitrile. The mixture was mixed thoroughly, centrifuged (High-speed desktop centrifuger, Flying Pigeon TGL-16C, Suzhou Bozhao Electronics Co., Ltd., Suzhou, China) for 15 min at 10,000 rpm, and then filtered through 0.22 μm nylon filters. An appropriate amount of filtrate was derivatized by adding 180 μL of FITC (1.0 mmol/L) as a derivatization reagent, 360 μL of borate buffer (pH 9.23, 200 mmol/L) and ultra-pure water to a total volume of 1 mL in a 1.5 mL centrifuge tube. After gentle mixing, the reaction mixture was incubated in the dark at room temperature for 12 h. The derivatization ratio of FITC/analyte of 3:1 was chosen as a good compromise considering the peak responses of all analytes and peak interference generated from FITC. (The effect of the derivatization ratio on the peak areas of the analytes was shown in Fig. S1 in Supporting information.) All the samples were repeatedly injected three times for regression analysis and error analysis. Before extraction, the reactants were stored at 4 °C in darkness.

Q3/2 Accurel PP polypropylene microporous hollow-fiber membrane (200 μm wall thickness, 600 μm inner diameter, 0.2 μm pore size, 75% porosity) were obtained from Membrana (Wuppertal, Germany). The hollow fiber was cut into segments with a length of 5 cm, and the detailed preparation can be found in our previous work [19]. The optimization of dynamic three-phase HF-LPME procedure was conducted using a unified standard solution (10.0 nmol/L). An 8 mL of mixed standard solution (the pH value of the donor phase was adjusted with HCl to the final concentration of 5 mmol/L) was placed in a 10 mL beaker, and a small stirring bar was placed in the solution to ensure efficient stirring during the extraction, which was covered with aluminum foil to prevent targeted analytes from photodecomposition and evaporation. At the same time, the magnetic stirrer was switched on to start the dynamic extraction at 500 rpm. After a prescribed time, the magnetic stirrer was switched off and the hollow fiber was removed from the sample solution. One end of the hollow fiber was cut carefully with a sharp blade, and the needle tip of a microsyringe was carefully inserted into the hollow fiber. The acceptor solution in the hollow fiber was withdrawn into the syringe, and was ready for the CE-LIF analysis. Each piece of hollow fiber was used only for a single extraction.

3. Results and discussion

To identify the optimum electrophoretic conditions, several factors including the pH and concentration of the running buffer, buffer additives, separation voltage and injection time were investigated, respectively. Under the optimum conditions, six analytes could be well separated with the main coexisting interference compounds in saliva samples in 12 g/L PSS (3 mmol/L) CTAB (80 mmol/L) $Na_2B_4O_7$-NaOH buffer (pH 12.35) at the separation voltage of 22 kV within 30 min; and the injection time was 8 s (at 22 kV).

The type of organic solvent, compositions of both donor and acceptor phases, stirring rate and extraction time were also investigated in order to obtain good extraction efficiency for the three-phase HF-LPME procedure. In three-phase HF-LPME, the type of organic solvent plays an important role in the extraction efficiency and the analyte preconcentration. So, the commonly-used organic solvents such as 1-octanol and toluene have been examined in this work. The experimental results showed that 1-octanol as the extraction solvent can offer the best enrichment characteristics for the targeted analytes and exhibited good reproducibility. The compositions of both donor and acceptor phases are also very important parameters affecting the extraction efficiency in HF-LPME. The pH value of the donor phase is adjusted to deionize the analytes, while the acceptor phase is adjusted to ionize them. Therefore, the concentrations of HCl as the donor phase and NaOH as the acceptor phase were studied, respectively. As shown in Fig. 1a and b, when the 5 mmol/L HCl and 300 mmol/L NaOH were used as donor media and acceptor media, respectively, the extraction process could provide the highest EFs. Some research has shown that addition of salt may improve the extraction efficiency by decreasing the aqueous solubility of the organic analytes. So, the effect of salt concentration was investigated in the range of 50–250 g/L as shown in Fig. 1c, and 200 g/L was selected as the concentration of sodium chloride in this experiment. Besides, the appropriate enhancement of the stirring rate can promote the diffusion of donor phase and the transfer of targeted analytes, and then shorten the extraction time; when the stirring rate was higher than 500 rpm, vortex phenomenon disrupted the organic-phase membrane and resulted in the loss of EFs. So, 500 rpm was selected as the optimum stirring rate in this work. Finally, the effect of extraction time on EFs of the targeted analytes was also investigated as shown in Fig. 1d. In order to balance the extraction efficiency and analysis time, 4 h was chosen as the optimum time in this work.