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# Microwave-accelerated derivatization for capillary electrophoresis with laser-induced fluorescence detection: A case study for determination of histidine, 1- and 3-methylhistidine in human urine

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ABSTRACT

The feasibility of microwave-accelerated derivatization for capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection was evaluated. The derivatization reaction was performed in a domestic microwave oven. Histidine (His), 1-methylhistidine (1-MH) and 3-methylhistidine (3-MH) were selected as test analytes and fluorescein isothiocyanate (FITC) was chosen as a fluorescent derivatizing reagent. Parameters that may affect the derivatization reaction and/or subsequent CE separation were systematically investigated. Under optimized conditions, the microwave-accelerated derivatization reaction was successfully completed within 150 s, compared to 4–24 h in a conventional water-bath derivatization process. This will remarkably reduce the overall analysis time and increase sample throughput of CE-LIF. The detection limits of this method were found to be 0.023 ng/mL for His, 0.023 ng/mL for 1-MH, and 0.034 ng/mL for 3-MH, respectively, comparable to those obtained using traditional derivatization protocols. The proposed method was characterized in terms of precision, linearity, accuracy and successfully applied for rapid and sensitive determination of these analytes in human urine.

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## 1. Introduction

Laser-induced fluorescence (LIF) is one of the most sensitive detection techniques, but most analytes lack intrinsic fluorescence under excitation of readily available laser wavelengths, ranging from red to blue. Derivatization reactions, which intended to label the analytes with suitable fluorogenic or fluorescent reagent to produce fluorescent adducts, are vital for developing capillary electrophoresis (CE) methods with LIF detection [1,2]. Derivatization can be performed at different stages of a CE analysis, i.e. before, during and after the electrophoretic separation, which usually referred to as pre-, on- and post-column derivatization [3–6]. The exact stage of derivatization depends on many factors including the derivatization reagents, the physicochemical properties of analytes, the number of samples to be analyzed, the reaction speed and, in some cases, other specific reasons for a particular analysis. In CE, the most frequently used mode is pre-column derivatization due to the difficulty in implementing the other two. The pre-column mode also has little restriction on the derivatization reaction conditions [7–11]. Both fluorogenic and fluorescent reagents can be employed, and extreme conditions (such as elevated temperature or long reaction time) can be adopted without deteriorating the following CE separation. Possibly because of this, many pre-column derivatization methods are tedious and time consuming, making it incompatible with the high analysis speed offered by CE. Under these situations, the bottleneck that limits the sample throughput is sample preparation rather than CE separation. Therefore, it is highly desirable to develop novel techniques for accelerating those slow derivatization reactions without any compromise of their merits.

In recent years, the use of microwave to accelerate chemical reactions in organic synthesis has attracted a considerable amount of attention due to its fascinating properties over conventional heating [12–16]. Controlled microwave heating has been used to dramatically shorten reaction time, increase product yields and reduce unwanted side reactions. In addition to organic synthesis, microwave irradiation has also shown great application potential in analytical chemistry, mainly in the area of sample preparation [17,18], such as microwave-assisted extraction [19–24] and digestion [25–29]. Studies have been performed on microwave-assisted derivatization, but their purpose is, however, to enhance the volatility of analytes for gas chromatography or mass spectrometry analysis [30–33]. Recently, Momenbeik and Khorasani [34] reported a pre-column microwave-assisted derivatization protocol for analysis of sugars by reversed-phase high-performance liquid chromatography with UV detection, and observed that the derivatization reaction of sugars with *p*-nitroaniline was complete within

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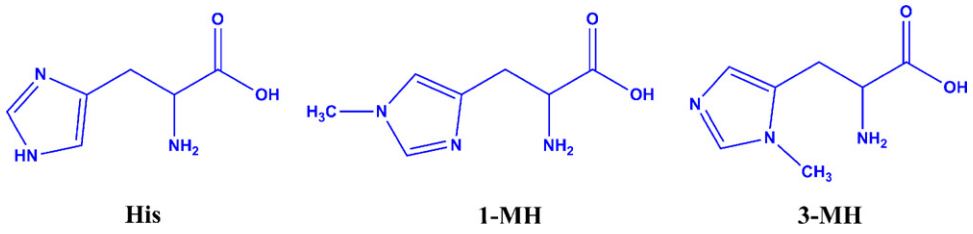


**Fig. 1.** Chemical structures of histidine (His), 1-methylhistidine (1-MH) and 3-methylhistidine (3-MH).

5 min under a 600 W microwave irradiation. Up to now, to the best of our knowledge, microwave-assisted derivatization for CE-LIF is not reported.

Fluorescein isothiocyanate (FITC) is a popular fluorescent derivatization reagent. It possesses good reactivities with primary and secondary amines, providing the derivatives with high fluorescence quantum yield. The widely available blue lasers, including the argon-ion laser (λ 488 nm) and solid-state laser (λ 473 nm) can excite strong fluorescence from FITC-derivatized analytes and thus it is compatible with many commercially available LIF detectors. Also, it is much cheaper than other similar reagents. These features of FITC have made it a widely used labeling reagent for sensitive analysis of amino compounds including amino acids [35–37], peptides and proteins [38–41], pharmaceuticals [42,43] and pesticide residues [44–47], with detection limits of $10^{-9}$–$10^{-12}$ M. However, the derivatization kinetics of amino compounds with FITC is rather slow, 4–24 h at room temperature [48], far beyond the time scale for a CE separation, which is complete in several minutes. Therefore, FITC was selected as the derivatization reagent to evaluate the feasibility of microwave-accelerated derivatization in this study.

Separation and determination of 1-methylhistidine (1-MH) and 3-methylhistidine (3-MH) is of continuous interest due to their clinical significance as metabolites [49–52]. These two species are produced from the methylation on the 1- and 3-position, respectively, on the imidazole moiety of histidine molecules in the course of post-translation modifications of actin and myosin after their proteolysis, by which 3-MH is liberated and is no longer metabolized, thus removing from the body unchanged in urine [50–52]. Therefore, 3-MH can be used as an indicator of the degree of degradation of skeletal muscle proteins, reflecting the state of human health. However, proteolysis is not the only source of 3-MH, because significant amounts of 3-MH can be obtained from the diet, in particular from fatty nutrients. Endogenic 3-MH can be distinguished from the exogenic one by detecting 1-MH, which is not formed in humans, but is common in other animals. To monitor proteolysis, the level of 1-MH must be determined together with that of 3-MH, because the 1-MH content in urine correlates well with the 3-MH amount introduced with fatty nutrients [50–52]. In the present study, derivatization of His, 1-MH and 3-MH with FITC under microwave irradiation heating was investigated for their rapid and sensitive CE-LIF detection. Experimental conditions for microwave-accelerated derivatization and subsequent CE separation were systematically optimized. The results suggest that the microwave heating can greatly speed up the derivatization process of these three analytes and the proposed method has been used for the fast analysis of His, 1-MH and 3-MH in human urine samples by CE-LIF.

## 2. Materials and methods

### 2.1. Materials

Histidine (His), 1-methylhistidine (1-MH) and 3-methylhistidine (3-MH) were obtained from Sigma, and their structures are shown in Fig. 1. Fluorescein isothiocyanate (FITC) was a product of Aldrich. Human urine samples were collected from two healthy volunteers, a female and a male. All other chemicals used were of analytical grade and used without further purification. Distilled water was used throughout all experiments.

### 2.2. Solutions and sample preparation

Stock standard solutions of His, 1-MH and 3-MH with the concentration of 300 μg/mL were prepared by dissolving appropriate amounts of these analytes in distilled water. All standard solutions were stored at 4 °C in a refrigerator. 10 mM FITC stock solution was prepared in acetone, stored at −18 °C, and diluted to the desired concentration with acetone before use.

A buffered solution of pH 9.4, consisting of 20 mM $Na_2CO_3$ and 20 mM $NaHCO_3$, was used for derivatization. The running buffers employed for separation were prepared from a stock solution of 100 mM sodium tetraborate and acetonitrile (ACN). All buffers were adjusted on a pH meter (Shanghai Precision & Scientific Instrument, China) to the desired pH with 1.0 and 0.1 M HCl or NaOH solutions as necessary, and filtered through a 0.45 μm cellulose acetate membrane prior to use.

Human urine samples were collected in the early morning and stored immediately at 4 °C. Before the derivatization, the urine samples were first centrifuged for 10 min and filtered through 0.45 μm membranes. The derivatized urine was directly injected into the capillary without further treatment for analysis.

### 2.3. Derivatization procedures

Unless stated otherwise, the microwave-accelerated derivatization was performed as follows: 10 μL of analyte solution, 165 μL of derivatization buffer, and 25 μL of 2 mM FITC solution were added sequentially into a 1.5 mL microcentrifuge vial. After thoroughly mixed, the microcentrifuge vial was sealed by a small piece of Parafilm and placed in a domestic microwave oven (700 W, Qin-Dao HaiEr) together with a conical flask containing 20 mL water as shown in Fig. 2. The derivatization reactions were conducted under microwave irradiation for 150 s following general microwave cooking procedure. After cooling to ambient temperature, the reaction mixture was diluted by 20 times with distilled water for CE analysis. For urine samples derivatization was completed following the same procedure. Blank contrasts were prepared in the same way except no analyte was added to the reaction mixture.

Conventional water-bath derivatization was carried out by incubating the reaction mixtures of the same composition as described above in 1.5 mL microcentrifuge vials in a water bath (set at 20 °C or at the boiling temperature) for given periods. All other operations were same as for microwave-accelerated derivatization described above.

### 2.4. Apparatus and electrophoretic procedures

All experiments were performed on a P/ACE MDQ system (Beckman Coulter, Fullerton, USA) equipped with a LIF detection system

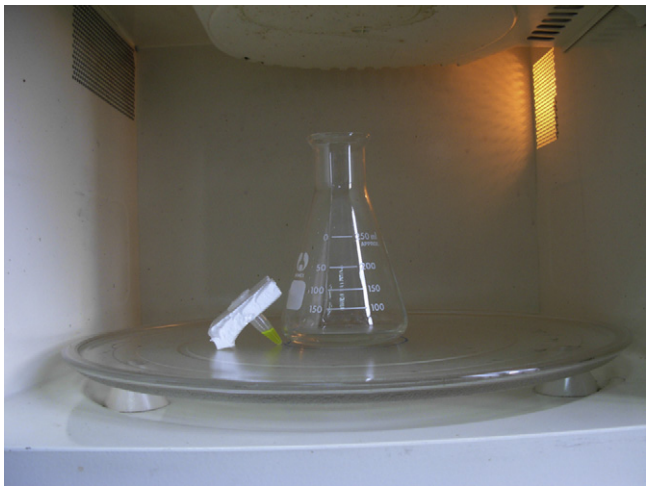

**Fig. 2.** Optimum position of sample microcentrifuge vial and conical flask containing water in the microwave oven chamber.

and controlled by 32 Karat™ software (Version 7.0). The excitation light from an argon ion laser (3 mW) was focused on the capillary window by means of a fiber-optic connection. 488 and 520 nm band-pass filters were used as excitation and emission filter, respectively. A piece of uncoated fused-silica capillary (Yongnian Ruifeng Chromatogram Equipments, Yongnian, Hebei, China) with dimensions of 75 μm I.D. (375 μm O.D.) × 50.2 cm length (effective length 40 cm) was accommodated in a cartridge configured for LIF detection. Prior to initial use, the capillary was preconditioned by rinsing consecutively with methanol for 5 min, distilled water for 2 min, 1.0 M HCl for 5 min, distilled water for 2 min, 0.1 M NaOH for 15 min, distilled water for 2 min, running buffer for 15 min and, finally, equilibrated at 20 kV with running buffer for 20 min.

Typically, the derivatized analytes were separated using the optimized running buffer: 22 mM sodium tetraborate, 32% (v/v) ACN, pH 10.5. The sample was introduced into the capillary by hydrodynamic injection using a pressure of 3.45 kPa for 3 s. Additional experimental details are described in figure legends or as indicated in the text. All separations were conducted with a constant voltage of 25 kV, and the capillary was thermostated at 25 ± 0.1 °C with a liquid coolant system. To maintain a good reproducibility, the capillary was rinsed sequentially with distilled water for 5 min, 0.5 M HCl for 5 min, distilled water for 2 min and running buffer for 5 min at the beginning of each experimental session. Between two electrophoretic runs, a rinse-cycle of 0.1 M NaOH for 2 min, distilled water for 2 min, 0.5 M HCl for 2 min, distilled water for 2 min and running buffer for 2 min was employed.

## 3. Results and discussion

### 3.1. Optimization of microwave-accelerated derivatization conditions

A domestic microwave oven is one of the most robust appliances nowadays; it is an ideal tool for rapid heating of small amounts of samples. Its most important advantage is that heating can uniformly occur inside the "microwave active" substances, the aqueous solution in this case. The interaction between electromagnetic wave and reactants may also lend an exceptional effect on the chemical reactions [53]. Although it is possible to "heat" about 200 μL of liquid, as long as the heating time is not too long, running of a microwave oven empty or with such small amount of "load", that is, substance which can be heated, may cause damage of its magnetron, the essential part of a microwave oven. For this reason, in our preliminary experiments, a conical flask containing certain amounts of water was placed in the microwave oven to avoid such damage. Since the spatial distribution of a microwave is not uniform inside the cooking chamber, a rotary tray is often used for uniform heating of food. Surprisingly, even with rotation of the derivatization vial along with the rotary tray, the exact position of the vial and conical flask containing water still has significant effect on derivatization results. With a series of tests, it was found that the optimum derivatization results were obtained when the sample vial and the water container were fixed at the center of rotary tray, as shown in Fig. 2.

#### 3.1.1. Effect of derivatization buffer concentration and pH

The derivatization reaction of amino compounds with FITC is usually carried out in an alkaline medium, this is ascribed to the efficient deprotonation of amine groups at an alkaline condition. In the present study, a $Na_2CO_3$ and $NaHCO_3$ mixture (1:1, molar ratio) was selected as the derivatization buffer and its concentration was investigated in the range of 5–30 mM. The results indicated that the fluorescence intensity of derivatives increased gradually as the buffer concentration increased from 5 to 20 mM. Beyond 20 mM, there was a significant chance that the microcentrifuge vial lid may burst, causing the solution to splash, probably due to the sudden boiling of the solution. Hence, we selected 20 mM $Na_2CO_3$–$NaHCO_3$ solution as derivatization buffer. Even at this concentration, the microcentrifuge vials used as the derivatization vessels were sealed by a piece of Parafilm to avoid splashing issues during the microwave treatment. Then, the influence of buffer pH varied from 9.0 to 10.0 on derivatization was studied. The results indicated that the fluorescence intensity of derivatives changed slightly over all the buffer pH studied, and a relatively better result was obtained in the buffer pH of 9.2–9.6. Accordingly, a solution of 20 mM $Na_2CO_3$–$NaHCO_3$ at pH 9.4 was applied as the derivatization buffer.

#### 3.1.2. Effect of microwave irradiation power and time for derivatization

The microwave irradiation intensity is one of critical factors influencing derivatization efficiency. To find the optimum derivatization condition, the microwave irradiation with different power (280–700 W, by setting cooking power) was investigated first with constant derivatization time of 120 s. The results showed that the fluorescence intensity of the FITC labeled analytes increased monotonically with irradiation power. So 100% of power output, i.e. 700 W, was used for all further studies.

The subsequent optimization of microwave irradiation time (30–210 s) for derivatization was performed to obtain the maximum fluorescence intensity of the analytes. It is apparent from Fig. 3 that the fluorescence responses increased as the irradiation time increased from 30 to 150 s, and longer exposure time above 150 s would produce a slight decrease in the fluorescence response. Thereby, the microwave irradiation time of 150 s was applied for further studies.

#### 3.1.3. Effect of water usage in the microwave oven

The amount of water used for protecting the microwave oven has been proven to have a significant influence on derivatization efficiency. Fig. 4 showed the effect of the amount of water ranged from 0 to 100 mL. The results indicate that the maximum fluorescence response of analyte derivatives was obtained at 20 mL of water, further increase of water volume steadily decreased analyte fluorescence. The later can be easily explained because more water consumes more microwave energy, but the behavior of analyte fluorescence intensity at a water volume less than 20 mL is hard to explain. This unexpected behavior might be related with

**Table 1**
Results of regression analysis on calibration curves and the detection limits.

| Analyte | Regression equation | | Correlation coefficient | Linear range (ng/mL) | Detection limits (ng/mL) |
|---|---|---|---|---|---|
| | Slope | Intercept | | | |
| His | $9.59 \times 10^5$ | $-2.97 \times 10^6$ | 0.997 | 1–250 | 0.023 |
| 1-MH | $9.52 \times 10^5$ | $-2.95 \times 10^6$ | 0.998 | 1–250 | 0.023 |
| 3-MH | $6.43 \times 10^5$ | $-1.65 \times 10^6$ | 0.998 | 1–250 | 0.034 |

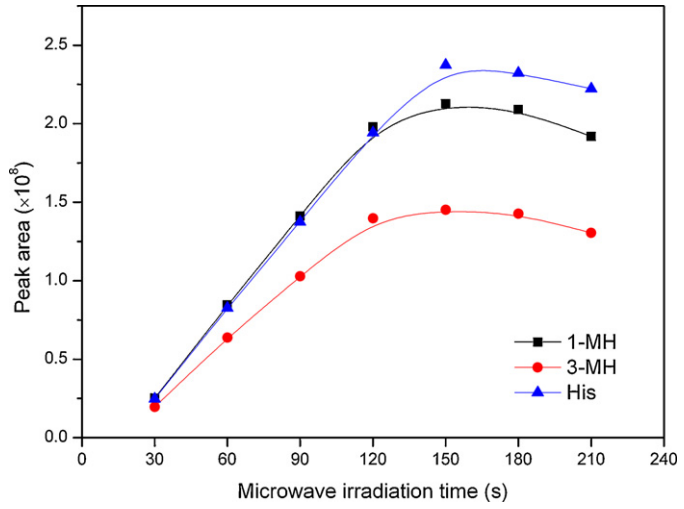


**Fig. 3.** Effect of irradiation time for microwave-accelerated derivatization. Derivatization conditions: 20 mM $Na_2CO_3$–$NaHCO_3$ solution at pH 9.4, 1.5 mM FITC, reaction under microwave irradiation at 700 W for 30–210 s, water amount of 20 mL. Separation conditions: 22 mM sodium tetraborate, 32% (v/v) ACN, pH 10.5 and an applied voltage of 25 kV.

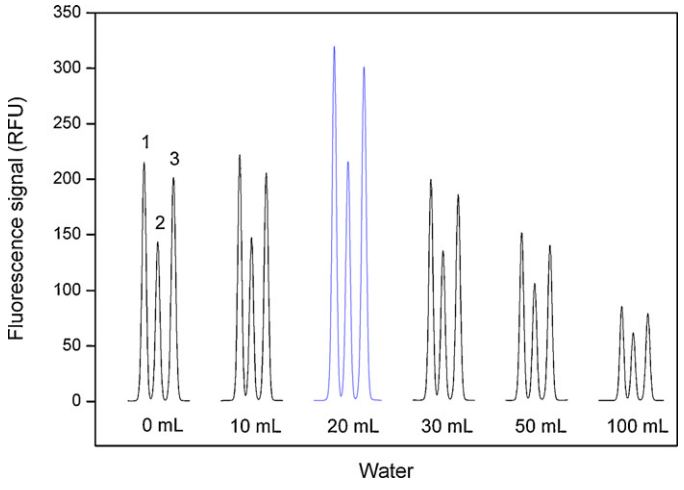


**Fig. 4.** Effect of water amount for microwave-accelerated derivatization. Derivatization conditions: 20 mM $Na_2CO_3$–$NaHCO_3$ solution at pH 9.4, 1.5 mM FITC, reaction under microwave irradiation for 150 s at 700 W, the volume of water ranged from 0 to 100 mL. Separation conditions as in Fig. 3 and peak identification: 1, 1-MH; 2, 3-MH; 3, His.

the microwave spatial distribution inside the cooking chamber. Therefore, 20 mL water was used for all further derivatization.

#### 3.1.4. Effect of FITC concentration

This derivatization reaction is concentration dependent, and increasing the reagent concentration would improve the derivatization efficiency. In the present study, the effect of FITC concentration on derivatization efficiency was investigated in the range of 0.5–5 mM. The experimental results illustrated that the fluorescence response of selected analytes increased with increasing FITC concentration and leveled off when it was higher than 2 mM. In addition, excess FITC may increase the baseline noise and deteriorate the signal-to-noise ratio. So, it is reasonable to choose 2 mM of FITC as the optimal reagent concentration.

### 3.2. Separation of FITC labeled His, 1-MH and 3-MH

After derivatization with FITC, the presence of the big fluorophore attached to the analytes would reduce the differences in the structure and charge of the derivatives and increase the burden of separation by CE. Various running buffers, including phosphate, acetate and Tris, combining with several additives, including SDS, sodium cholate, Tween-20, and β-CD were attempted for better separation efficiency, but no significant improvement on peak resolutions was achieved with those combinations. Borate buffer with ACN as electroosmotic flow (EOF) modifier gave the best separation of these FITC labeled analytes. Parameters including tetraborate concentration (10–30 mM), ACN concentration (10–40%, v/v) and buffer pH (10.0–11.0) on the separation were investigated systematically. The results suggested that the efficient separation were obtained with 22 mM sodium tetraborate, 32% (v/v) ACN, pH 10.5 and an applied voltage of 25 kV.

### 3.3. Linearity, detection limits and precision

The linearity, detection limits and precision of this method were measured and summarized in Table 1. The data implied that an excellent linear relationship is attainable over the concentration range studied (1–250 ng/mL) with correlation coefficients above 0.99. The detection limits, defined as three times the signal-to-noise ratio, were 0.023 ng/mL for His, 0.023 ng/mL for 1-MH, and 0.034 ng/mL for 3-MH, respectively, which indicate high sensitivity. The precision of the proposed method were expressed as the relative standard deviation (RSD) for migration time and peak area, with 2.52% and 5.27% for His, 2.35% and 5.58% for 1-MH, 2.42% and 5.09% for 3-MH ($n = 6$), respectively.

### 3.4. Application

The microwave-accelerated derivatization method was applied for the analysis of His, 1-MH and 3-MH in human urine. The representative electropherograms for the selected standards and urine samples are depicted in Figs. 5 and 6, and the quantitative results are listed in Table 2. The recoveries were evaluated with three addition levels and the average values were in the range of 90.2–107.3%. Interestingly, the two urine samples have visible differences in the determination results of His, 1-MH and 3-MH. However, the number of samples is not enough to offer a physiological explanation.

To verify the efficiency of microwave-accelerated derivatization, a comparison was made between the present results and that performed at ambient temperature (20 °C) and boiling water (100 °C) for 150 s. The typical electropherograms are shown in Fig. 5a and b. It can be seen that in the later two cases, the labeling efficiency is much lower than that of microwave-accelerated derivatization. There is no apparent difference between electropherograms of those derivatization methods except peak heights, which implies that microwave has little influence on the selectiv-

**Table 2**
Determination results of His, 1-MH and 3-MH in human urine samples.

| Analyte | Female urine | | Male urine | |
|---|---|---|---|---|
| | Content (μg/mL) | Recovery (%) | Content (μg/mL) | Recovery (%) |
| His | 77.3 | 102.9 | 264.4 | 107.3 |
| 1-MH | 27.6 | 97.6 | 68.0 | 95.4 |
| 3-MH | 24.9 | 92.9 | 53.8 | 90.2 |

Data are means of three analyses.

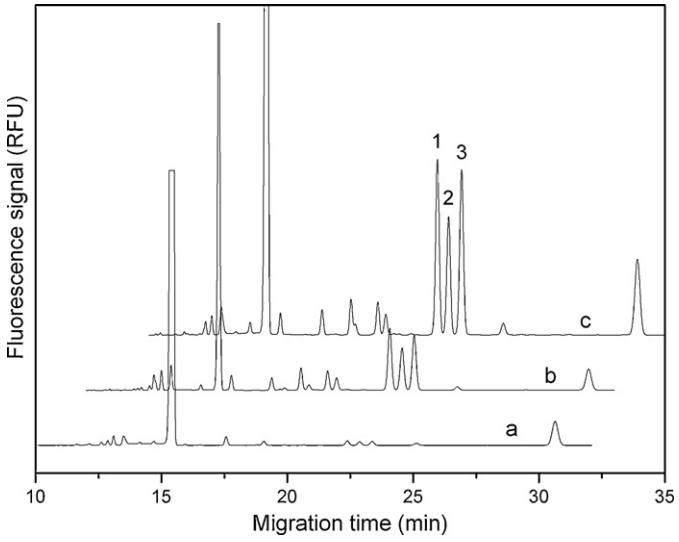


**Fig. 5.** Comparison of different derivatization methods: (a) Derivatization at ambient temperature (20 °C) for 150 s; (b) derivatization in a boiling water-bath (100 °C) for 150 s; (c) derivatization in a microwave oven for 150 s at 700 W. Separation conditions and peak identification as in Fig. 4.

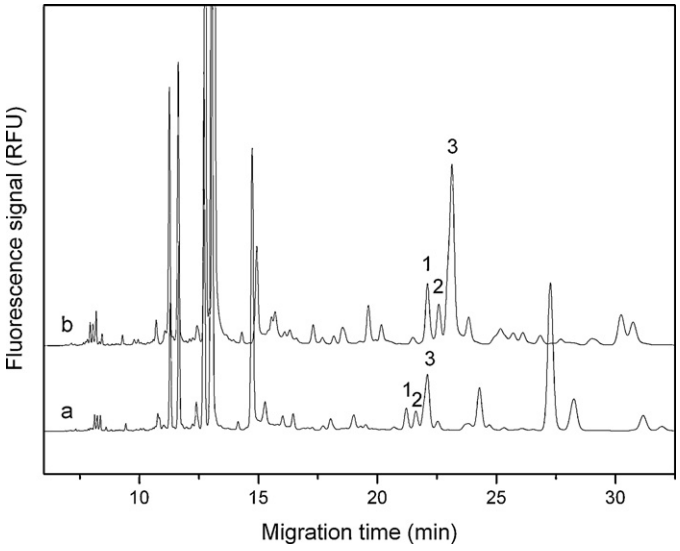


**Fig. 6.** Typical electropherograms of human urine samples collected from the (a) female and (b) male volunteer under the optimum microwave-accelerated derivatization and separation conditions. Peak identification as in Fig. 4.

ity of the derivatization reaction. However, we noticed that the peak heights of the selected analytes gradually increased with time and plateaued after 4 h when derivatization was performed at room temperature. The maximum peak heights can be 1.5–2.2 times higher than that obtained under optimized condition with microwave acceleration if a same FITC concentration was used. Decomposition of FITC at elevated temperature may be responsible for the lower peak heights obtained with microwave, but this will not compromise the advantage provided by the microwave acceleration in practical analysis because of the excellent sensitivity of LIF. The detection limits obtained with microwave are comparable to that from traditional derivatization protocols. Comparing with other chromatographic and electrophoretic methods, the proposed method is far more advantageous over traditional UV [49–51] and contactless conductivity detection [52,54] for the assay of His, 1-MH and 3-MH with regard to sensitivity.

## 4. Conclusions

Using microwave-accelerated derivatization, a rapid and sensitive CE-LIF analysis method for His, 1-MH and 3-MH in human urine was developed. Comparisons with the conventional derivatization method of FITC, which normally takes 4–24 h, 150 s of derivatization time is rather compatible to the sample throughput of CE. Although there is rapid derivatization reaction and reagent available, like naphthalene-2,3-dicarboxaldehyde (NDA), these types of reagents may have shortcomings like expensive, instable, and highly toxic substance (KCN), has to be involved during the labeling process, etc., the proposed method is advantageous and may have great clinical significance because of its speed, sensitivity and analysis cost. On the other hand, a special designed microwave chamber with higher efficiency can be easily incorporated into a CE sampling system, so it is possible to build on-line derivatization system with those labeling reagents with slow reaction under normal condition.

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