Determination of thiols following their separation by CZE with amperometric detection at a carbon electrode

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Abstract

The amperometric detection (AD) employing a carbon disk electrode as a working electrode to determine the thiol compounds, including cysteine (CYS), glutathione (GSH), 6-thiopurine (TP), and methimazole (MMI), following their separation by capillary zone electrophoresis (CZE) is described in this paper. The detection potential was chosen at $+1100$ mV and all analytes exhibit good response at this potential on the carbon disk electrode. The reproducibility, linearity, and recovery were evaluated under the optimum conditions. The four analytes can be baseline resolved within 20 min and the detection limits reached about 10^{-6} mol/l of magnitude. The method was applied to the separation and determination of the actual thiol samples and the analytical results were satisfactory. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Capillary zone electrophoresis; Amperometric detection; Thiols; Cysteine; Glutathione; Methimazole; 6-Thiopurine

1. Introduction

Thiols have been of continuing interest for many years because of their important role in several biological processes. It is of clinical, biological, and pharmaceutical importance to determine these compounds in biological fluids and tissues [1,2]. Thiols play important roles in drug metabolism and protein synthesis, as well as having been used as radio-protective agents and antibiotics. Examples of important thiols include cysteine (CYS), glutathione (GSH), 6-thiopurine (TP), and methimazole (MMI). CYS is an amino acid that plays a critical role in protein synthesis and structure. GSH plays an important role in drug metabolism and toxicity. TP is widely used as a clinical agent in a therapy of human leukemia and as an immunosuppressive drug. MMI is a drug used in the treatment of human hyperthyroidism, and an illness caused by thyroid gland hyperfunction. However, these compounds are used to cure diseases of human beings and are also harmful to human health at the same time.

Many analytical methods of thiol compounds include spectrophotometry [3,4], mass spectrometry [5], gas chromatography [6], and electroanalysis [7–9] have been reported. The more procedures of thiols using liquid chromatography

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(LC) have been reviewed [10] in 1985. Recently, LC was continuously used in the separation of thiols [11–14]. But most of these analytical procedures for thiols involve some of derivatization followed by separation by a LC method with various detection techniques because of the absence of strong chromophores or fluorophores. Derivatization is not required in electrochemical detection (ED) by using chemically modified electrodes due to thiols are often of a high overpotential at bare electrodes [15–18].

With capillary electrophoresis (CE) being introduced in the analytical field, it has already become a powerful analytical technique in pharmaceutical and biomedical aspects [19,20]. The separation and determination of thiols by CE with UV [21,22] or fluorescence detection [23] has been described. O'Shea and Lunte [24,25] developed an off-column ED method for the determination of various thiol compounds following their separation by CE. They used a Nafion joint to decouple the electric field and amalgamated gold [24] or cobalt phthalocyanine modified carbon paste electrodes [25] for detection. Huang and Kok described a cobalt phthalocyanine modified conductive carbon cement electrodes were tested for the determination of underivatized CYS compounds separated by CE in Ref. [26].

In this paper, a method of separation and determination of thiols by CE with amperometric detection (AD) at a bare carbon disk electrode in near neutral solutions differing from most of other procedures, most of which were processed in acidic medium, is reported. Although these analytes exhibit a relatively high overpotential, this method may be yet applied to analyze thiols in pharmaceutical and human serum samples because of the high separation efficiency of CE. And it demonstrates good reproducibility and practicality.

2. Experimental

2.1. Working electrode

A carbon disk electrode with 500 μm diameter, which was made using pencil lead (produced by Shanghai Shangyuan Stationery Factory, Shanghai), was employed as a working electrode which was prepared according to a previously described method [27]. Briefly, the electrode was constructed by introducing a pencil lead into a glass pipette until it protruded from the pipette tip (one end of pipette, about i.d. 0.1 cm). Epoxy was used at the tip of the pipette to seal the carbon rod, then put in the air to dry naturally. Before use, the surface of the working electrode was gradually polished with emery sand paper, sonicated in deionized water, and finally positioned carefully opposite the capillary outlet with the aid of a micromanipulator to minimize the gap between the electrode tip and the capillary outlet.

2.2. Apparatus

A laboratory-constructed CE system equipped with wall-jet AD [28] was employed in the research work. The separations were carried out in a 75 cm long, o.d. 360 μm, i.d. 25 μm, polyimide-coated fused silica capillary (Polymicro Technologies, Phoenix AZ) with the help of a 30 kV high voltage power supply (Shanghai Institute of Atomic Nucleus Research, Chinese Academy of Science). The injector electrode was kept at high positive voltage, the detector electrochemical cell at ground. Samples were all injected electrokinetically, applying 28 kV for 10 s.

Cyclic voltammograms were performed on a CHI 832 electrochemical system (CH instrument, USA). A potentiostat (ZF-3, Shanghai Second Component Factory, China) was used to supply a constant potential to the working electrode in the electrochemical cell, which is composed of a platinum auxiliary electrode, a carbon disk working electrode and an Ag/AgCl (KCl 3 mol/l) reference electrode. A picoammeter (WD-1, Shanghai Yanzhong Instrument Factory, China) was utilized to amplify the electrochemical current and a chart recorder (XWTD-164, Shanghai Dahua Instrument Factory, China) was used to obtain the electropherograms.

2.3. Reagent and procedures

CYS, GSH (reduced form), TP, and MMI, biochemical-reagent grade, were purchased from the Institute of Pharmaceutical and Biological of China. All other chemicals were of analytical reagent grade and obtained from local commercial sources. MMI tablets were purchased from local drug store (Shanghai, China). Blood serum was supplied by a local hospital of Shanghai. Stock solutions of standard analytes, especially for CYS and GSH, were prepared daily in double distilled water and stored in refrigerator at about 5°C and then diluted to the desired concentration with 20 mmol/l, pH 7.4 phosphate buffer solution. For MMI tablets, five tablets were finely ground, then one fifth of the powder of one tablet was accurately weighed and dissolved in the same way. The serum sample was diluted 5-fold with the running buffer solutions and then it was centrifugalized, filtered and immediately subjected to injection. Before use, all solutions were filtered through a 0.22 μm polypropylene filter film, and then thoroughly degassed under ultra sonication. In addition, all experiments were performed in ambient temperature (about 20°C).

3. Results and discussion

3.1. Selection of potentials applied to the working electrode

Because the potential applied to the working electrode greatly affected the electrode response of analytes, it is very necessary to select a suitable potential when AD acted as a detection method in CE. Fig. 1 demonstrates the hydrodynamic voltammograms at carbon disk electrode for GSH, CYS, TP, and MMI. Fig. 1 illustrates that the hydrodynamic voltammograms of the analytes are similar in the range of the applied potential from $+375$ to $+1275$ mV. When the potential is lower than $+375$ mV, the peak current is relatively small, especially for GSH and TP. While the potential is greater than $+375$ mV, the response current of the four analytes increase with the increase of the potential applied to the working electrode. In starting, the oxidation current of GSH increases slowly, whereas the ones of the other three analytes increase more obvious compared to the response of GSH. When the potential reached $+775$ mV, the increase value of the peak currents change relatively slow up to $+975$ mV for CYS. Then its currents begin to increase clearly. For the other three analyzed compounds, the peak currents rise with the increase of the potential range from $+375$ to $+1275$ mV. Unlike oxidation currents of some compounds [29] at the carbon disk electrode, the peak currents of the studied thiol compounds have not reached a maximum value under the experimental conditions. When the potential reached $+1175$ mV, the base currents begin to increase rapidly. This made the measurements and the records more difficult on the apparatus employed in the experiments. Simultaneously, the noise increased promptly in this condition. All these made the determination of four thiol compounds impossible. At the same
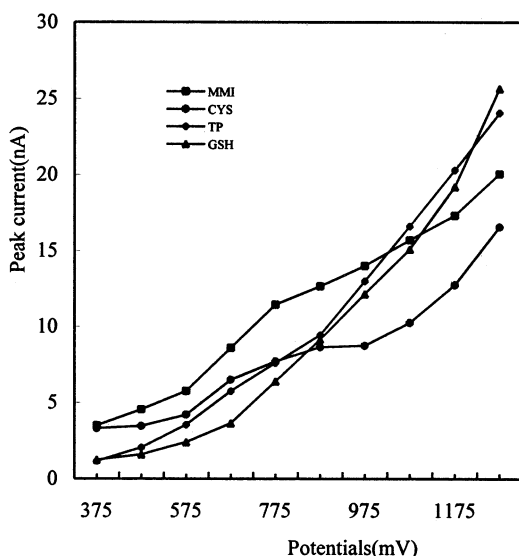

Fig. 1. The hydrodynamic voltammograms obtaining from the experiment for all analytes. Fused-silica capillary, 25 μm i.d. × 70 cm; working electrode, 500 μm carbon disk electrode; separation medium, 20 mmol/l; pH 7.4 PB solution; separation voltage, 27 kV; injection, 25 kV × 10 s. The concentration of cysteine (CYS), glutathione (GSH), 6-thiopurine (TP), and methimazole (MMI) was 1×10^{-4}, 2×10^{-4}, 1×10^{-4}, and 5×10^{-5} mol/l, respectively. Here, dashed line was used to denote the base current.
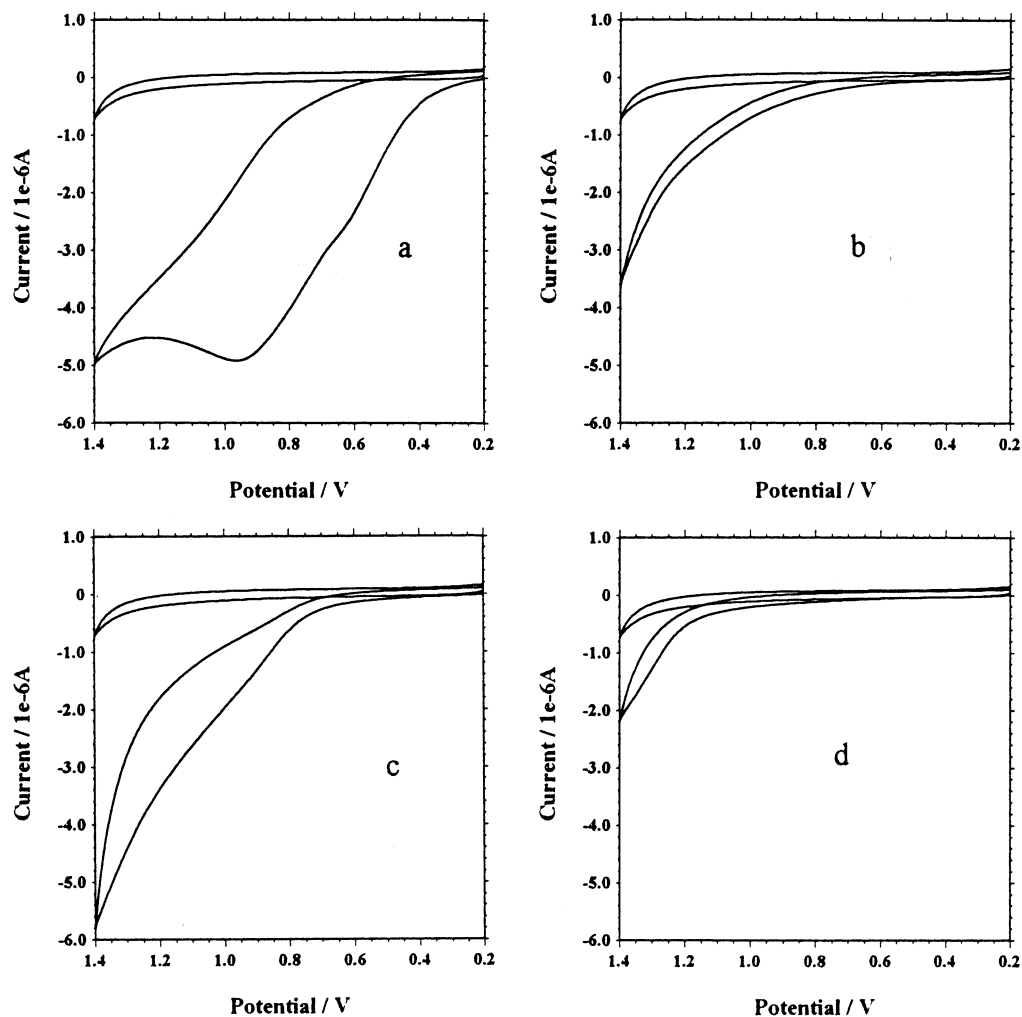
Fig. 2. The cyclic voltammograms of four analytes at carbon disk electrode. Supporting electrolyte, 20 mmol/l, pH 7.4 PB solution; reference electrode, Ag/AgCl (3 mol/l KCl); auxiliary electrode, platinum wire. (a) Methimazole (MMI); (b) cysteine (CYS); (c) 6-thiopurine (TP); (d) glutathione (GSH).

time, it can be seen from their cyclic voltammograms (Fig. 2) that the MMI has a very obviously irreversible oxidation peak at + 950 mV. But for the other three analytes, the oxidation current of TP has a clear increase at about + 1000 mV, the oxidation current of CYS take second place, the oxidation current of GSH was the smallest but yet observed. Considering the results of the Figs. 1 and 2 simultaneously, + 1100 mV was selected as the optimum working potential, where the base current is not too high and the S/N ratio is relatively large, whereas the working electrode can maintain both constancy and reproducibility in a longer time. Under the selected optimum conditions the carbon disc electrode gives a good response for CYS, TP, MMI, and GSH for at least 3 months.

3.2. Selection of pH value of the running buffer

It is noticeable that the running buffer solutions act as an important factor for the separation

efficiency of CE. Most reports previously about the separations of thiol compounds, by using both liquid chromatography [14] and CE [25,26], were achieved in the acidic solutions. In order to make the separation and determination conditions accord with the human physiological conditions, the dependence of the migration behavior of the four analytes on pH value was measured with phosphate buffer solution in the pH range 7.0–8.2 and the results were demonstrated in Fig. 3. It is seen from the figures that the migration times of the analytes are greatly affected by the pH value of the background electrolyte. When pH 7.0, the differences of the migration time for CYS and TP or CYS and MMI are so small that their electrophoretic peaks can not be fully separated on baseline. And the migration time of GSH is relatively long. With the increase of the pH value to 7.4, the separation effect is the most ideal. Under this pH value, all analyzed compounds can not only be resolved on baseline but their migration times are also relatively small. When pH value reaches 7.8, the migration times of MMI and GSH decrease with the increase of pH value, whereas the ones of CYS and TP begin to increase at the same conditions. And the increased value of the CYS is greater than that of TP. This made the separation of two compounds

Fig. 3. Effect of pH on the peak current of the analytes. Working potential,: +1100 mV (vs. Ag/AgCl); other conditions as in Fig. 1.

very difficult. In this condition, the peak shape begins to change very poorly. When pH arrived at 8.2, peaks of TP and GSH overlapped into one single peak, and the peak of CYS is very near to this peak. This result made the three analyte's separation impossible. In the range of pH examined, the peak currents of MMI, TP, and GSH almost maintain constants except for CYS. In addition, the concentrations of the running buffer solutions also affected the migration behavior of the analyte. In these experiments, the 20 mmol/l, pH 7.4 phosphate buffer solution was chosen as the running buffer solutions.

3.3. Effect of separation voltage

In order to select the most optimum separation voltage, the dependence of the migration velocities of the solutes on the electrical field strength was examined under various applied voltages in the running buffer solution of 20 mmol/l, pH 7.4 phosphate solutions. The curves of retention time versus applied voltage indicated, as expected, that the electric field strength increased and the electroosmosis decreased correspondingly with the increase of separation voltage. This leads to a decrease in the migration time of the solutes. But the relative retention time of each analyte does not change obviously and does not influence the resolution of the analytes. When the separation voltage was too low, the analysis time would be too long, and the samples in the capillary would diffuse resulting in a broadening of the peaks in the electropherogram. Considering the higher efficiency and to save analysis time, a separation voltage of 27 kV was employed to accomplish a good compromise.

To sum up the discussions described above, the 20 mmol/l, pH 7.4 phosphate buffer solution, a separation voltage of 27 kV as the optimum separation conditions and a potential of +1100 mV as the determination potential were elected. Under the optimum conditions, the identical electropherogram for a standard mixture of four thiol compounds are shown in Fig. 4. The four analytes could be obviously separated on baseline within 19 min.
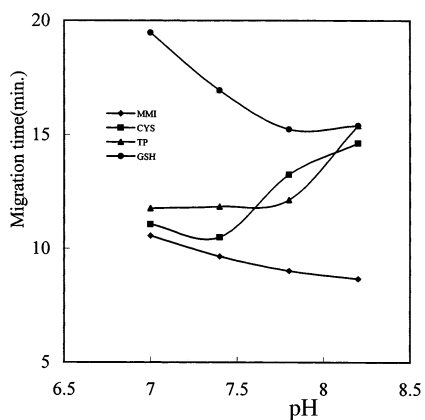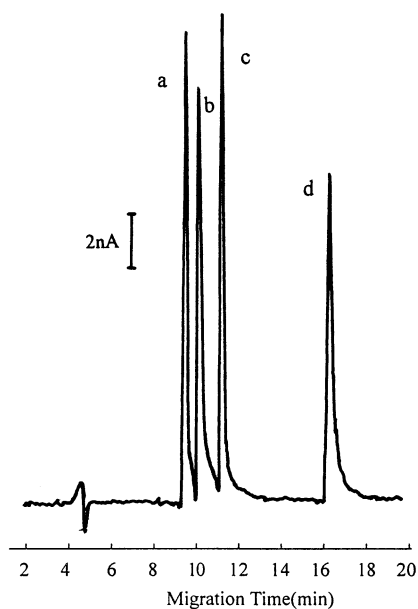
Fig. 4. Typical electropherograms obtained in the chosen optimum conditions for the analytes. (a) Methimazole (MMI) $(5 \times 10^{-5}$ mol/l); (b) cysteine (CYS) $(1 \times 10^{-4}$ mol/l); (c) 6-thiopurine (TP) $(1 \times 10^{-4}$ mol/l); (d) glutathione (GSH) $(2 \times 10^{-4}$ mol/l). Working potential, $+1100$ mV (vs. Ag/AgCl); other conditions as in Fig. 1.

3.4. Analytical characteristics of the method

In order to determine the linearity for CYS, TP, MMI, and GSH in capillary zone electrophoresis (CZE)-AD at the carbon disk electrode, a series of the synthetic standard mixture of four analytes were examined. The detection limits were evaluated on the basis of a S/N ratio of 3. The results obtained by using this method, including regres-

sion equations, linear range, correlation coefficiency, and detection limits of all analytes under the optioned conditions were summarized in Table 1. It is a coincident that the data obtained from the method compared with the results by LC [30]. But this method shows the merits are more simple and convenient than LC. It is seen that all these data obtained from the procedure are satisfactory.

3.5. Applications and recovery

3.5.1. Purity analysis of the MMI tablet

The active component of the MMI tablet was separated and detected according to the procedure described previously. The electropherogram of the MMI tablet sample was shown in Fig. 5A. Only one single peak is obtained. The peak height increases with the increasing of the MMI quantity by the added standard. The content of MMI in the tablet can be determined and the assay result was listed in Table 2. The results obtained from this method are consistent with the one of 4.82 ± 0.18 mg tested by LC [30]. At the same time, this result also agrees quite well with the labeled amount. It is clear that this method can be applied to the determination of the drug purity as an effective approach.

3.5.2. Serum sample analysis

Another application of this method was to determine the CYS in serum and the electropherogram were given in Fig. 5B. This analysis was achieved in the procedure described above. The assay results obtained were summarized in Table

Table 1
The regression equations and detection limits[a]

Compounds	Regression equation, $Y = bX + a$[b]	Correlation coefficiency	Linear range (mol/l)	Detection limits [c] (mol/l)
CYS	$Y = 173.67X + 0.36$	0.9996	$1.0 \times 10^{-3} \sim 2.5 \times 10^{-6}$	1.0×10^{-6}
GSH	$Y = 74.78X + 0.05$	0.9998	$1.0 \times 10^{-4} \sim 5.0 \times 10^{-6}$	2.5×10^{-6}
MMI	$Y = 141.78X + 2.70$	0.9999	$5.0 \times 10^{-4} \sim 1.0 \times 10^{-6}$	8.0×10^{-7}
TP	$Y = 129.60X + 1.57$	0.9977	$5.0 \times 10^{-4} \sim 2.5 \times 10^{-6}$	1.0×10^{-6}

[a] CZE-AD conditions as in Fig. 4.
[b] Where Y and X are the peak current (nA) and concentration of the analytes (mmol/l), respectively.
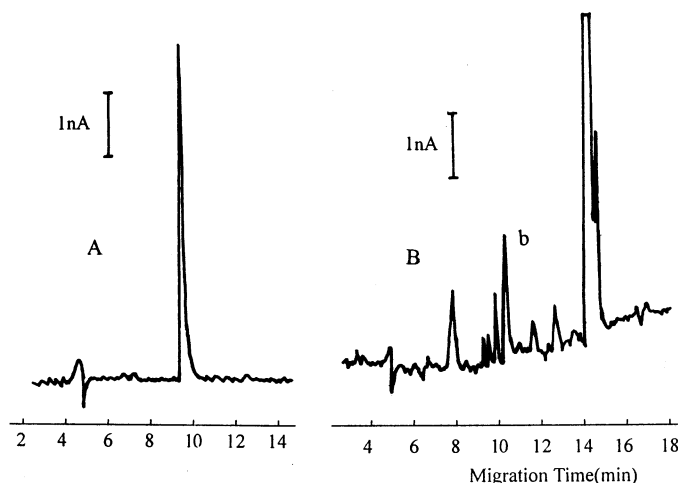[c] The detection limits are estimated on the basis of a S/N ratio of 3.

Fig. 5. The electropherograms of samples. (A) The methimazole (MMI) tablet; (B) the serum sample: the peak b is the electrophoresis peak of cysteine (CYS). Working potential: +1100 mV (vs. Ag/AgCl); other conditions as in Fig. 1.

Table 2
Purity analysis of the methimazole (MMI) tablet $(n = 3)^a$

Compound	Found amount (mg)	Label claim (mg)	Percent (%)	R.S.D. (%)	Percent of Ref. [30] (%)
MMI	4.86	5.0	97.20	2.87	96.4 ± 3.6

[a] CZE-AD conditions as in Fig. 4.

Table 3
Analytical results of serum sample $(n = 3)^{a,b}$

Compound	Detected amount (mol/l)	Total value (mol/l)	RSD (%)	Recovery(%)
CYS	1.28×10^{-5}	6.40×10^{-5}	3.79	95.4

[a] CZE-AD conditions as in Fig. 4.
[b] The values are caculated from the regression equations and are the average of three times of determination.

3. It can be seen, from the results, that a concentration of 6.40×10^{-5} mol/l for CYS is obtained and this value is within the normal range of CYS for healthly people. Additionally, it is clear that some other oxidation current peaks exist in the electropherograms. These phenomena may be a result from the undetermined analytes existing in the serum sample. But, all these peaks do not interfere with the determination of CYS. All these were attributed to the good separation efficiency of this procedure.

To evaluate the feasibility of this method, the recovery was determined by adding the standard of all thiols in the first sample under the optimum conditions in the experimental. The results were given in Table 4 and these recovery values were found to be in 93–103% for the analytes. These results demonstrate that this procedure employed in the analysis of thiol compounds is feasible.

4. Conclusions

The analytical results shown that the method used for the separation and determination of the thiols is feasible for both simple and complicated

Table 4
Recovery of the determination of thiols in this method ($n = 3$)[a,b]

Compounds	MMI sample (mol/l)	Artificial sample (mol/l)	Added amount (mol/l)	Found amount (mol/l)	Recovery (%)	R.S.D. (%)
CYS	Free	2.98×10^{-5}	1.00×10^{-5}	3.91×10^{-5}	93.0	3.57
GSH	Free	5.03×10^{-5}	2.00×10^{-5}	7.08×10^{-5}	102.5	2.13
MMI	1.69×10^{-5}	2.56×10^{-5}	1.00×10^{-5}	3.53×10^{-5}	97.0	1.36
TP	Free	2.49×10^{-5}	1.00×10^{-5}	3.47×10^{-5}	98.0	2.87

[a] CZE-AD conditions as in Fig. 1.
[b] The values are calculated from the regression equations and are the average values of three times of determination.

samples. Although there are some impurity peaks in the electropherograms, this phenomenon does not affect the determination of the analyzed CYS in the serum sample because of the high separated efficiency of CE. Therefore, it is due to a few of merits such as reproducibility, stability, simpleness etc. compared with some other detection methods such as the electro-polymerized modified electrodes as detectors in CE. It is a useful technique for the analysis of actual samples.

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