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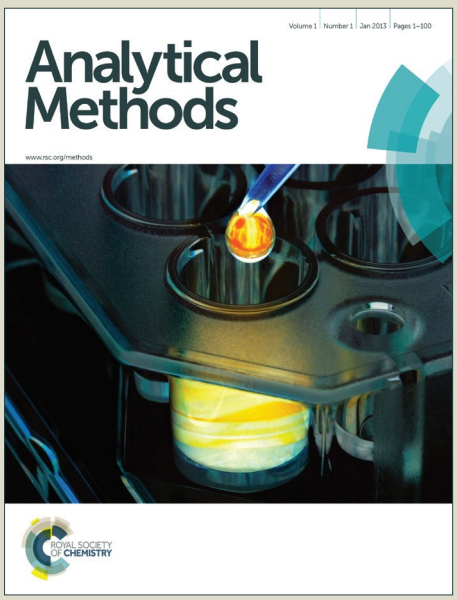

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ARTICLE

Highly sensitive detection of copper(I) and copper(II) in cell specimens by CE-UV with a Large-volume sample stacking

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A sensitive, rapid and simple CE-UV method with large-volume sample stacking (LVSS) and polarity switching has been developed and validated for the analysis of copper(I) and copper(II) simultaneously. Benefitted from the combination of LVSS method, a limit of detection (LOD) of 30 nM was obtained which was much lower than current detection methods. Several factors, such as the concentration and pH of the background solution, injection time and pressure, stacking voltage and inversion electric current were systematically optimized. Under the optimum conditions, the detection limits of copper(I) and copper(II) were improved nearly 1000 times compared with the conventional methods by CE-UV, which made it possible to determine trace copper in cell specimens. Eventually, the proposed method was successfully applied to detect the copper(I) and copper(II) in spiked cell samples with good recoveries.

1. Introduction

As a micronutrient, copper is responsible for the proper functioning of several enzymes and related physiological processes[1]. Copper levels are considerably important in the process of autophagy, which is a cellular pathway involved in protein and organelle degradation with an astonishing number of connections to human disease and physiology[2]. For example, high serum copper levels were found in diverse hematologic and lymphatic malignancies, such as leukemia[3-6], Hodgkin's disease[6-8] and malignant lymphomas[9-11]. Furthermore, on one hand it was found that the misregulation of copper concentration inside the human brain leads to neurodegeneration, cardiovascular disorders, and Menkes and Wilson's diseases[12, 13]. On the other hand, copper complexes could also efficiently inhibit proteasome activity and target multiple pathways in cancer cells[14]. As such, monitoring the concentration of copper is important which in turn would have significant implications in human health.

So far, many methods by using different detection techniques have been proposed for Copper(II) determination such as ICP-AES[15], AAS[16], MS[17] and CE-UV[18]. However, a few studies indicated that copper(I) is present at higher concentrations in cells. Due to the high affinity of certain metalloregulatory proteins for copper(I), it is difficult to measure and monitor changes in the concentration of copper(I) inside cells[13]. Therefore, a number of fluorescent probes for copper(I) have been developed and successfully performed in biological media[13, 19]. But these methods require a pre-synthetic procedure, expensive equipment and costly reagents, and they can only detect copper(I) under specified conditions which limited their wide applications.

Unfortunately, there are few works about the simultaneous detection of copper(I) and copper(II). Bicinchoninic acid disodium (BCA) salt was used as a complexation with copper(I) or copper(II) in many works[20, 21], so the BCA-copper complexes could be used for the determination of copper(I) or copper(II) simultaneously. The only reported in the reference with a LOD for copper(I) and copper(II) were 3.0 and 2.5 mg/mL , respectively[22]. Therefore, more conveniently operated, cost-and-time effective as well as highly sensitive methods are still highly required to be developed for the analysis of copper(I) and copper(II).

CE-UV has been considered as a fast, efficient and cost-effective analytical separation method, and it has already become a powerful analytical technique in pharmaceutical and biomedical aspects. Nevertheless, the restricted injection volumes in CE method unavoidably cause relative lower sensitivity, and consequently limit its widespread applications. To overcome this limitation, the online sample pre-concentration is an efficient approach such as large-volume sample stacking (LVSS)[23, 24] or field-enhanced sample injection (FESI)[18, 25].

As shown in Fig.1, LVSS is a technique which allows sample injection of up to the full capillary volume, so that maximum sensitivity can be obtained. First, a large plug of sample was introduced hydrodynamically into the capillary, and then a high voltage is applied that is reversed in polarity compared to that used for the separation to push the sample matrix out of the capillary. The analytes were moved due to their electrophoretic mobility in the background electrolyte (BGE) boundary and finally the polarity of the high voltage is reversed to bring the separation conditions back to normal for the analysis[23, 26]. In this work, we developed a method to separate and detect copper(I) and copper(II) rapidly and

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conveniently with the advantages of low detection limit, fast analysis time and wide linear range.

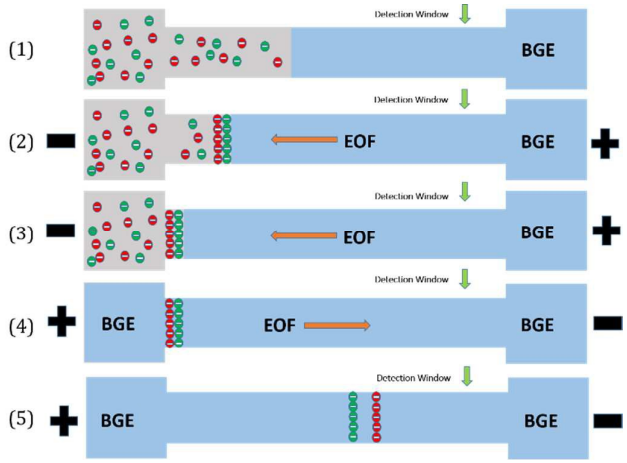

Fig.1 Schematic diagram of a preconcentration mechanism with LVSS. (1) Low-conductivity sample is injected; (2) a negative polarity voltage is applied, and the analytes are stacked at the boundary between the sample plug and BGE plug; (3,4) when the sample matrix has been almost completely removed out of the capillary, the polarity was switched to normal mode (5) the analytes were separated by capillary zone electrophoresis(CZE).

2. Materials and Methods

2.1 Apparatus and Reagent

All experiments were performed with a P/ACE MDQ instrument coupled with a diode-array UV detector (Beckman Coulter, USA). Instrument control and data acquisition were carried out using 32Karat software (Version 8.0). A 50.2 cm (40.0 cm to detector) uncoated fused-silica capillary tubing with 50 μm i.d and 364 μm o.d was obtained from Polymicro Technologies (USA).

Bicinchoninic acid disodium, hydroxylamine hydrochloride, copper sulfate and sodium acetate were purchased from Sigma (USA). All the water used was the commercial purified water Wahaha (Hangzhou, China). The disulfiram-copper solution was prepared by copper acetate and disulfiram with a ratio of 1:2. The BCA-copper(II) solution was prepared by copper acetate and BCA with a ratio of 1:2 and BCA-copper(I) solution was reduced from BCA-copper(II) by the right amount of hydroxylamine hydrochloride[20]. All the chemicals and reagents are of analytical grade and use without further purification.

2.2 CE procedure

The stock solution of disulfiram-copper, BCA-copper(I) and BCA-copper(II) with the concentration of 0.10 M were stored in the refrigerator at 4℃. A series of standard working solutions were further prepared by diluting the stocking solutions with deionized water. Acetate buffer was selected as the running buffer and prepared by adjusting to the required pH with 1.0 M NaOH and 1.0 M CH_3COOH, and then diluted to the required concentrations with deionized water.

Prior to experiment, new capillaries were pre-treated by sequentially rinsing with 1.0 M HCl for 10 min, then with deionized water for 10 min, and finally running buffer for 15 min. To improve the reproducibility, the capillary was flushed with running buffer for 3 min so as to maintain an active and reproducible inner surface between each run. The voltage applied in the separation was + 20 kV. The capillary was held at 25 ℃ and the wavelength of the UV detector was maintained at 240 nm.

A reverse electrode polarity-stacking mode was used in the capillary in LVSS. The sample was injected and a negative polarity was applied while the gradually increasing current was monitored carefully. The current was cut off manually when it reached 99% of the initial current value that was obtained by filling the capillary thoroughly with the running buffer at -8 kV. Then the polarity of the power supply was changed to positive and the voltage was applied to separate the stacked analytes under normal CE separation conditions. The length of sample plug, the time and voltage of switched polarity were studied and optimized in this work. All measurements were carried out at least three times.

2.3 Preparation of sample

HeLa cells were cultured in complete DMEM, which contained 10% horse serum, 10% fetal bovine serum, 100 IU mL^{-1} penicillin-G and 50 mg mL^{-1} streptomycin solution. The cells were maintained in 100 mm dishes at 37 ℃ in a humidified incubator with CO_2-air (5:95) until harvesting for the experiment. When harvesting, the DMEM was drawn out from the culture dishes, and the dishes were rinsed twice with pure water in order to keep a lower ionic strength of sample matrix. The cells were fragmented by ultrasonic and then centrifuged at 8000 rpm for 5 min. Finally, the cell sap was diluted 100-fold with deionized water to decrease the redox effect and spiked with 50 nM BCA-copper(I) and BCA-copper(II). The prepared samples should be tested within 8 h.

3. Result and Discussion

3.1 Separation condition optimization

The disulfiram-copper and BCA-copper were tried as analytes and it was found that disulfiram-copper(I) and disulfiram-copper(II) couldn't be separated well in different condition compared with BCA-copper. So this part would be around BCA-copper(I) and BCA-copper(II) to discuss. Running buffer plays a key role in CE because of its effects on the zeta-potential, the electroosmotic flow (EOF) as well as the overall charge of the analytes, and then finally affects the migration time and the separation of the analytes. In this work, the buffer could enhance the complexation effect between BCA and copper as another feature. After different attempts, sodium acetate was proved to be the best choice. On the contrary, when the phosphate buffer and borate buffer were tried, the signal of BCA-copper disappeared. We considered that either phosphate or borate has a great influence on the BCA and copper ion complexation. The complexes of Cu-BCA are stable

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during separation, and it is not necessary to use BCA as additive to BGE.

3.1.1 The effect of pH of buffer. First, the effect of pH is conventionally investigated in CZE. The BCA-copper(I) and BCA-copper(II) samples were introduced into the capillary at a low pressure of 0.5 psi for 5 s in the optimization experiment. Fig.2 shows the separation of the BCA-copper(I) and BCA-copper(II) under different pH in the range of 7.2–9.7 in acetate buffer. The pH range is out of the buffer capacity because the background electrolyte here played as two very important roles. On one hand, BCA which was negatively charged can complex with copper ions very well only in neutral or weakly basic buffer. And on the other hand, just acetate can assist the complexation between copper ions and BCA. The result indicated that the migration time of the BCA-copper(I) and BCA-copper(II) samples was getting shorter with the decrease of buffer pH from 9.7 to 7.2. However, the peak heights decreased when the buffer pH was changed from 8.0 to 7.2. This could be explained by the fact that the BCA and copper had a better complexation effect at 8.0. Considering that the separation resolution at pH 8.0 was adequate, the pH value of the buffer was finally chosen as 8.0.

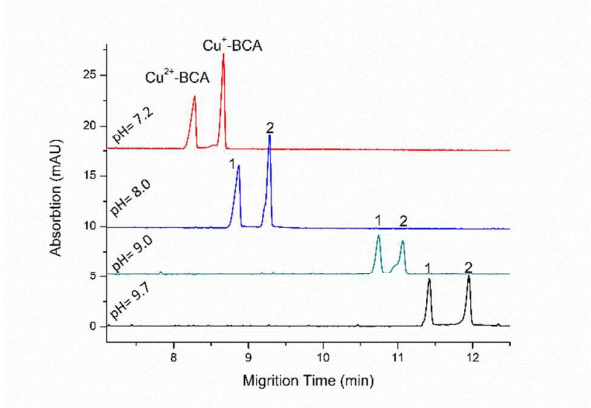

Fig.2 Effect of the pH of buffer on the separation resolution. Concentration (μM): BCA-copper(I), 50; BCA-copper(II), 50. Experimental conditions: 20 mM acetate buffer; injection by pressure, 0.5 psi; injection time, 5s; separation voltage, +20 kV. Peak identities: 1, BCA-copper(II); 2, BCA-copper(I).

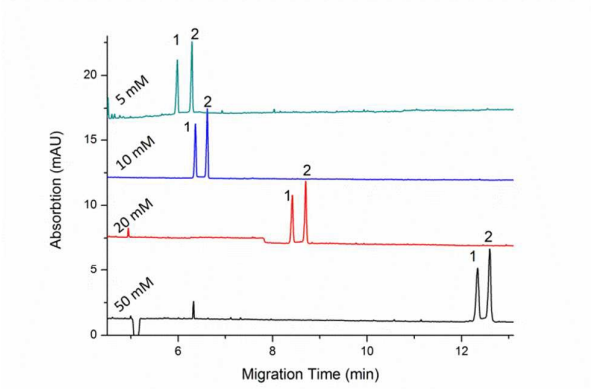

Fig.3 Effect of the concentration of buffer on the separation resolution. Concentration (μM): BCA-copper(I), 50; BCA-copper(II), 50. Experimental conditions: pH=8.0 acetate buffer; injection by pressure, 0.5 psi; injection time, 5s; separation voltage, +20 kV. Peak identities: 1, BCA-copper(II); 2, BCA-copper(I).

3.1.2 The effect of the concentration of buffer. The ionic strength of buffer affecting on solute mobility and separation efficiency was investigated. This effect was studied by varying the acetate buffer concentration from 2 to 50 mM. The results obtained are shown in Fig 3. It was found that increasing ionic strength resulted in increased analysis time quickly. We also found that under the concentration of 5 mM, the buffer system was unstable with poor reproducibility. In order to set up a fast and efficient method, 10 mM was chosen as the practical concentration.

3.2 LVSS condition optimization

Considering the sensitivity is relatively low when utilizing an online UV detector, we have proposed an on-line concentration procedure as a way of tracing enrichment in CE for the analysis of copper(I) and copper(II). Different on-line concentration methods have been tried such as FESI and LVSS. Experiment results showed that the enrichment efficiency of LVSS (1000 times) is much higher than that of FESI (10 times), due to that the negatively charged complexes will be brought out of the capillary by the reversed EOF in the injection process of FESI. In LVSS with polarity switching, a large sample plug with low conductivity was hydrodynamically introduced into the capillary and a negative voltage was applied. Furthermore, in LVSS, apart from usual variables, it is necessary to consider two significant parameters that affect CE separation: the size of plug sample and the current inversion value. In this sense, the following CE-LVSS optimization was carried out.

3.2.1 The optimization of the inversion electric current. During stacking procedure, the application of moderate voltage is a better strategy, and lower stacking voltage can ensure sufficient stacking time to thoroughly remove the sample matrix from the capillary without any loss of the analytes. In this work, the stacking voltage was attempted as -5 kV, -8 kV and -10 kV. Experiment results show little difference among the three conditions, at last the stacking voltage was selected as -8 kV, which produced the stable current of 2.70 μA. Under normal circumstances, most of the sample matrix was pushed out when the electric current reached 95% of the normal electric current during separation in LVSS method.

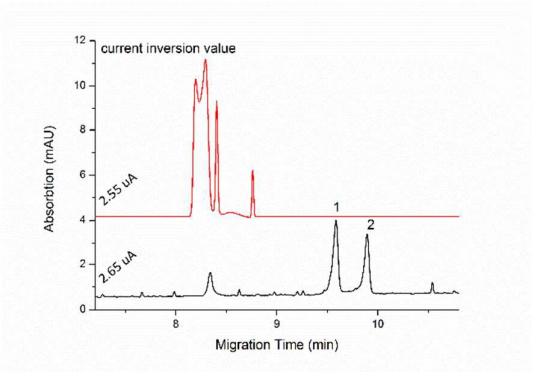

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Fig.4 Effect of inversion electric current on stacking efficiency. Concentration (nM): BCA-copper(I), 250; BCA-copper(II), 250. Experimental conditions: pH=8.0 acetate buffer; injection by pressure, 0.5 psi; injection time, 8 min; stacking voltage, -8 kV; separation voltage, +20 kV; separation current, 2.70 μA. Peak identities: 1, BCA-copper(II); 2, BCA-copper(I).

However, in this work, the applied voltage should be switched back to its normal polarity when the electric current reached 2.65 μA (98%) or higher. In Fig.4, It was found that if the current inversion value was 2.55 μA (95%), the peak would broaden seriously. Thus, a very long inversion time may result in a loss of part of the analytes, especially those with higher mobility. So controlling the inversion current between 98%-99% of the normal electric current is the key point of this experiment.

Table 1 Results of regression analysis on calibration, the detection limits and enhancement factors with LVSS.

Component	Calibration Curve	Correlation coefficient	Intraday RSD (%, *n*=5)		Detection limit (nM)	Linear range (nM)	Enhancement factor
			migration time (min)	peak area (mAU s)			
BCA-copper(I)	*y*=46.73*x*-1316	0.9998	0.41	12	28	30-500	1000
BCA-copper(II)	*y*=44.82*x*-1231	0.9996	0.28	15	26	30-500	1000

Table 2 Results for the determination of the copper(I) and copper(II) in cell sap.

Sample	Component	Original (nM)	Added (nM)	Found (nM)	RSD (%, *n*=5) peak area (mAU s)	Recovery (%)
Cell sap	copper(I)	-	50	54	9	108
		-	100	103	12	103
	copper(II)	-	50	48	11	96
		-	100	94	7	94

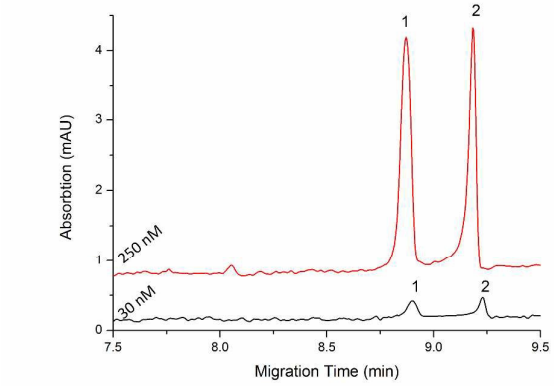

Fig.5 Electropherograms of standard sample of BCA-copper(I) and BCA-copper(II). Experimental conditions: pH=8.0 acetate buffer; injection by pressure, 0.5 psi; injection time, 8 min; stacking voltage, -8 kV; separation voltage, +20 kV; separation current, 2.70 μA. Peak identities: 1, BCA-copper(II); 2, BCA-copper(I).

3.2.2 The effect of water plug length. To determine the highest sample plug length to be injected without degrading the separation profile by LVSS, different sample plugs were tested. To improve the detection limits, the injection time was varied between 1 min and 8 min, and injection pressure was varied between 0.5 psi and 2 psi. The peak area of analytes increases as both of the injection time and injection pressure increases, but higher injection pressure would lead to the undulating volume of the sample plugs. For that reason, 8 min was applied as optimized injection time by applying the injection pressure of 0.5 psi. The length of injection zone was calculated from the velocity of the liquid in the capillary at 0.5 psi using a neutral marker (benzene). Under this pressure, the flow rate of sample solution was determined to be 3.7 cm/min using 50.2 cm capillary, and the sample volume injected during 8 min corresponded to 59% of the whole capillary.

Under the optimal conditions obtained for LVSS, the result of regression analysis on calibration, the detection limits and enhancement factors are summarized in Table 1. The linearity of the response was established from five calibration levels corresponding to 30, 50, 100, 250 and 500 nM of each analyte. Calibration curves were established by considering the relative corrected peak areas as a function of the analyte standard concentration. The sensitivity was greatly enhanced by 1000-fold in terms of LODs compared with the reported method[22]. Fig.5 shows the electropherogram obtained for a standard sample containing the analytes at a concentration of 30 nM and 250 nM with the LVSS-CZE procedure.

3.3 Application

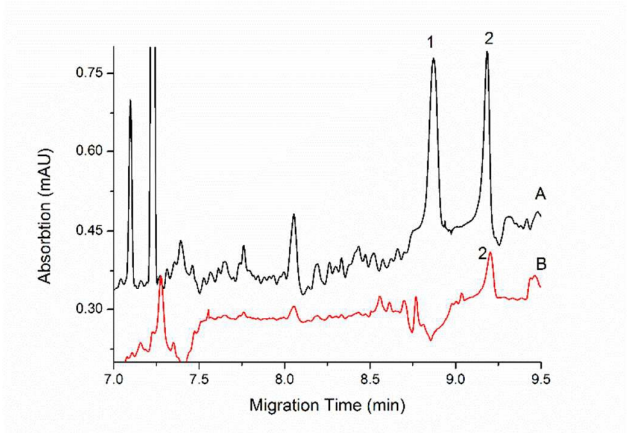

Fig.6 Electropherograms of the copper(I) and copper(II) in cell sap. (A) The cell sap was diluted 100-fold with deionized water to decrease the redox effect and spiked with 50 nM BCA-copper(I) and BCA-copper(II). (B) Directly detection of the BCA-copper(I) and BCA-copper(II) in the cell sample. Experimental conditions: pH=8.0 acetate buffer; injection by pressure, 0.5

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psi; injection time, 8 min; stacking voltage, -8 kV; separation voltage, +20 kV; separation current, 2.70 μA. Peak identities: 1, copper(II); 2, copper(I).

The cell samples were analyzed under the optimum condition. In order to validate the accuracy of this method, the recoveries of the spiked samples were measured by this CE method. Thanks to its high sensitivity, we could dilute the sample solutions 100-fold with deionized water before spiking with BCA-copper(I) and BCA-copper(II) in order to decrease the redox influence of the analytes. The electropherograms of the blank sample and the spiked sample are shown in Fig 6A. The quantitative results and the recoveries of this method are listed in Table 2.

We also tried to detect the copper(I) and copper(II) directly in the cell sample. The cells were cultured in complete DMEM, which contained 1 mM $CuSO_4$, 10% horse serum, 10% fetal bovine serum, 100 IU mL^{-1} penicillin-G and 50 mg mL^{-1} streptomycin solution. But we could only found a small peak of BCA-copper(I) in the electropherogram shown in Fig 6B. We think there are several reasons such as that the protein in the cell would be reduced to BCA-copper(II), or the binding capacity of BCA and copper was not strong than that of copper-protein, or it may be due to the lack of detection limit. In order to truly realize the detection of the concentration of transitional copper ion in the cell, it needs further study.

Concluding remarks

A sensitive, rapid and simple CE-LVSS method for the simultaneously analysis of copper(I) and copper(II) has been developed. It is the first time using the online concentration technique LVSS enhancement procedure in simultaneously detection of copper(I) and copper(II). Compared to CZE and the reported method, the proposed method has much higher stacking efficiency for the copper(I) and copper(II) with extremely low detection limit 30 nM. Results of this study show a great potential for this method to be a useful tool for the rapid and sensitive determination of copper(I) and copper(II) in cell sap. Compared with other copper analysis methods, it has such advantages as simplicity, easy operation, low cost, and high sensitivity enhancement.

Acknowledgements

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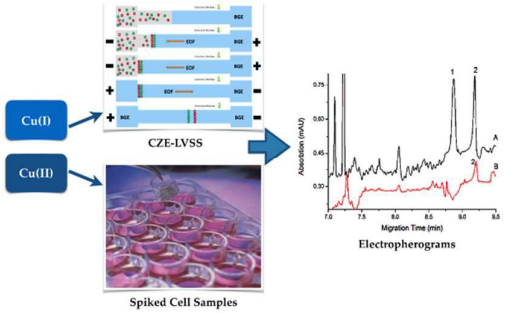

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