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More sensitive way to determine iron using an iron(II)–1,10-phenanthroline complex and capillary electrophoresis

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Abstract

Iron is one of the major metal species of concern in many samples, such as in serum, foods, drinking waters, etc. In this paper, we present a more sensitive way to determine the iron concentration in water solutions by using an iron(II)–1,10-phenanthroline complexing system with high-performance capillary electrophoresis, and have applied this method to the determination of the levels of iron in serum samples. The technique uses ammonium acetate–acetic acid (50 m*M* NH_4Ac–HAc, pH 5.0) as a running buffer, and the detection wavelength is set at 270 nm instead of 508 nm. This new approach enhances the molar absorbance of the Fe(II)–1,10-phenanthroline complex by about eight-fold compared with that obtained at 508 nm. By combining the larger light output of the deuterium (D_2) lamp and the lower noise level at 270 nm, the sensitivity was improved at least twenty-fold compared to that at 508 nm. The detection limit for iron(II) is lower than 5×10^{-9} *M*, which has never been reached by reported spectrophotometric methods or with the recently published HPCE method. The effects of pH, buffer concentration and operation voltages on the sensitivity and resolution are also discussed. The signal response is linear over two orders of magnitude ($r^2=0.995$) and the iron recovery for samples reached 99–101%. The technique described here is much more sensitive, fast and simple and is suitable for determining trace amounts of iron in biological, food, water and other samples.

Keywords: Iron; 1,10-Phenanthroline

1. Introduction

Determination of the concentration of iron is of great importance in environmental and biological sciences. It has been reported that iron might be a limiting micronutrient of biological productivities for many ocean organisms [1]. It was also reported that the photoreduction of iron(III) to iron (II) is a major source of hydroxyl radical (OH·), which is an important radical in environmental photochemistry [2]. In humans and animals, iron is one of the most important trace metals. An appreciable number of human disease are related to iron deficiency or to disorders of iron metabolism. Therefore, accurate determination of the levels of iron in environmental and biological samples is critically important for environmental studies, for human nutrition and for health studies.

A lot of work has been carried out to determine the concentration of iron. Over two decades, more than 50 different methods for determining the concentration of iron in serum, in addition to other

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systems, have been reported [3]. In order to measure the concentration of iron, quite a few chromogenic reagents, such as 1,10-phenanthroline [4], bathophenanthroline sulfonate [5] and ferrozine [6], have been adopted to form complexes with iron so that an UV–Vis spectrophotometer can be used for detection and quantitation. Unfortunately, since there is no separation procedure involved, copper, cobalt, nickel and other metal ions in the mixtures can also form complexes with these complexing reagents, often resulting in serious interferences when these chromogens are used in the colorimetric analysis of iron, especially for serum samples. Therefore, a high degree of error is present in the results obtained using these methods.

In recent years, there has been a lot of interest in the use of high-performance capillary electrophoresis (HPCE) for the separation of small molecules, peptides, proteins and DNA fragments [7], due to its great advantages of high resolution and low sample consumption. HPCE has been successfully used for separating metal ions in real samples in environmental, food, agricultural [8–11] and biological chemistry [12–14].

To date, UV–Vis absorbance is still the most commonly used detection technique in capillary electrophoresis. However, due to the short light path length, the detection limits for HPCE are restricted to about 10^{-5}–10^{-6} *M* [15], which is a serious problem for the determination of ultra-trace components. Some publications have reported a decrease in the detection limit using a laser [16], a Z-shaped window cell [17], a preconcentration method [18] and a larger diameter capillary as the detection window [19]. In most cases, the detection sensitivity can be enhanced by derivatizing the analytes with a molecule that has a higher absorptivity [20].

For the determination of iron by capillary electrophoresis, one publication reported that iron (Fe^{2+}) can be separated by HPCE and determined using indirect UV absorbance detection with a detection limit of 10^{-5} *M* iron [21], which is not satisfactory for a lot of environmental and biological samples that have a much lower concentration of iron, such as rain water, lake water and human serum. In this paper, we report a new sensitive way for determining iron by HPCE with UV absorbance detection. Iron (Fe^{2+}) is complexed with 1,10-phenanthroline, the Fe(II)–1,10-phenanthroline complex was separated from 1,10-phenanthroline and other interfering molecules by HPCE, and the absorbance is detected at 270 nm. The absorbance of the complex at this wavelength is analyzed and verified using a spectrophotometric method, resulting in greater sensitivity. The detection sensitivity for iron is enhanced to 5×10^{-9} *M*, which is sufficient to detect the concentration of iron in serum [22], environmental water samples [23] and could also be used for other biological samples.

2. Experimental

2.1. Reagents and samples

All reagents used were of analytical or ACS certified grade. Deionized water (D.I. water) was prepared with a Milli-Q system (Millipore, Bedford, MA, USA). All glassware and plasticware was washed carefully with 20% nitric acid and D.I. water. Ammonium acetate (NH_4Ac) with 0.5 ppm Fe, 1,10-phenanthroline and ferrous ammonium sulfate ($Fe(NH_4)_2{\cdot}(SO_4)_2{\cdot}6H_2O$) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Acetic acid (HAc) and sodium hydroxide were obtained from Aldrich (Milwaukee, WI, USA). The serum samples were collected from student volunteers in Northeast Missouri State University.

2.2. Preparation of the background electrolyte

The background electrolyte (BGE) solutions for HPCE were prepared by dissolving a certain amount of NH_4Ac in 80 ml of D.I. water (the amount of NH_4Ac is based on the concentration needed), adjusting the pH to the desired value with 5% HAc, and then diluting to 100 ml with D.I. water. The BGE was filtered and degassed before use.

2.3. Preparation of standard Fe(II)) and Fe(II)–1,10-phenanthroline standard solutions

A 1.00 m*M* Fe^{2+} stock solution was prepared by dissolving 39.2 mg of $Fe(NH_4)_2{\cdot}(SO_4){\cdot}6H_2O$ in 70 ml of D.I. water and diluting to 100 ml. The 0.1 m*M*

Fe^{2+} standard solution could be obtained by further dilution of the above-prepared stock solution.

A 0.100-ml volume of the Fe^{2+} standard solution (0.100 m*M*), 0.500 ml of BGE with different pH values and concentrations, and 0.800 ml of 1,10-phenanthroline (1.0 m*M*) were added sequentially to a 10-ml volumetric flask and were diluted to the mark with D.I. water, then the mixture was shaken well. These solutions were used to study the effects of pH, BGE concentration and operating voltage.

The standard Fe(II)–1,10-phenanthroline solutions that were used for making calibration curves were the same as above except that the BGE buffer solution was 50 m*M* NH_4Ac–HAc with a pH of 5.00.

2.4. Serum sample treatment

The procedures for serum sample treatment were similar to those described in [24] with minor modifications. Serum samples (1 ml) were obtained and the proteins were precipitated with 0.5 ml of 20% trichloroacetic acid (TCA) and removed by centrifugation. In order to prevent the occlusion of iron within the precipitates, the precipitate was washed with 0.5 ml of 10% TCA twice more and combined with the supernatants. The combined supernatant was extracted twice with 1 ml of diethyl ether to remove excess TCA. Then the iron (Fe^{3+}) in the supernatant was reduced to Fe^{2+} by adding 0.2 ml of 0.25% thioglycolic acid. The solution was transferred to a 10-ml volumetric flask and 0.500 ml of NH_4Ac–HAc (50 m*M*, pH 5.00) and 0.800 ml of 1,10-phenanthroline (1.0 m*M*) were added and then the solution was diluted to the mark with D.I. water for HPCE analysis.

2.5. Equipment

A DU-70 spectrophotometer (Beckman, Arlington Heights, IL, USA) was used to characterize the properties of the Fe^{2+}–1,10- phenanthroline complex system. A quartz cell with a path length of 1 cm was chosen for scanning and single wavelength measurements.

The HPCE system with an UV detector was purchased from ISCO (Lincoln, NE, USA; Model 3850). A positive high voltage was applied to the capillary end where the injection was made. A DataJet computing integrator (Spectra Physics, Mountain View, CA, USA) was used to collect the data. The capillary column (Polymicro Technologies, Phoenix, AZ, USA) used for separation was 60 cm long with an inner diameter of 75 μm, the polymer coating was burned off at 35 cm from the injection end to form a detection window. An UV detector was used for detection and the detection wavelength was set at 270 nm.

2.6. HPCE analysis

A new capillary column was pretreated as described previously [25]. The sample solution was injected electrokinetically at 5 kV for 20 s and the electrophoresis was carried out at 12.5 kV. The data were collected and processed using the DataJet integrator.

2.7. Quantitation

A standard calibration curve was used to quantify the concentration of iron (Fe^{2+}) and a standard addition method was used to verify the reliability of the method. For example, after the first injection, to determine the concentration of iron in serum or in other environmental samples, a very small volume (e.g., 10 μl) of standard Fe^{2+} solution was added to the sample and injected to the HPCE column for reanalysis, after which the relative recovery was calculated.

2.8. Spectrophotometric method

The spectrophotometric method developed by Valcour et al. [24] was used for comparative purposes with our HPCE method. Briefly, ferrozine was used as a complexing reagent and chromophore to form a complex with iron(II). The absorbance was measured at 562 nm and quantification of the level of iron in serum was obtained using a linear calibration curve method.

3. Results and discussion

3.1. Characterization of the Fe(II)–1,10-phenanthroline complex system

It is well known that Fe(II) can react with 1,10-phenanthroline to form an orange–red complex with a maximum molar absorptivity of 11 100 at 508 nm [26]. Based on Beer's law:

$$A = \epsilon \cdot b \cdot c = \log(I_o/I)$$

Better sensitivity may be achieved by increasing ϵ (absorptivity) and I_o (intensity of the light source). Since most HPCE instruments are equipped with a deuterium (D_2) lamp as a light source (and the wavelength range of the D_2 lamp is from 180 to 370 nm with a maximum intensity at about 270 nm [27]), it would be ideal to detect the absorbance of the Fe(II)–1,10-phenanthroline complex at this wavelength range. Using a D_2 lamp as a light source (at 508 nm) for measuring the absorbance of the Fe(II)–1,10-phenanthroline complex is much less ideal.

As shown in Fig. 1B, a mixture of Fe(II) and 1,10-phenanthroline in NH_4Ac–HAc buffer has three absorbance peaks, as determined by spectrophotometric scanning. In addition to the absorbance peak at 508 nm (peak 3), which is the result of the absorbance of the Fe(II)–1,10-phenanthroline complex ($Fe(o\text{-ph})_3^{2+}$) [28], two greater absorbance peaks show up at $\lambda_{max} = 270$ nm (peak 2) and 226 nm (peak 1). When a pure 1,10-phenanthroline solution was scanned on a spectrophotometer, these two peaks do show up at 226 and 270 nm (no absorbance at 508 nm) (as shown in Fig. 1A), which means that 1,10-phenanthroline itself does absorb at 226 and 270 nm. When a series of Fe(II) solutions of different concentrations were added to a 1,10-phenanthroline solution (the 1,10-phenanthroline concentration was fixed at 100 μM), the absorbances at 508 nm will increase with increasing concentrations of Fe(II), which reaches a maximum when the molar ratio of 1,10-phenanthroline to Fe(II) is three, which is comparable with the reported results [28]. Our experiment has proved that the absorbance at 270 nm is proportional to the concentration of 1,10-phenanthroline in a free 1,10-phenanthroline solution. Based on the normal prediction, if the absorbance at 270 nm was only determined by 1,10-phenanthroline and not by the Fe(II)–1,10-phenanthroline complex, the absorbance at 270 nm (peak 2) should decrease when the concentration of Fe(II) increases, due to the decrease in the concentration of free 1,10-phenanthroline (consumed by forming a complex with Fe(II)). However, our experiment has proven that this is not true. If 1,10-phenanthroline was fixed while the concentration of Fe(II) was increasing, the absorbance at 270 nm (peak 2) increased (as shown in Fig. 1C). Two series of experiments have been done, one in which the concentration of 1,10-phenanthroline was maintained at 200 μM while the iron(II) concentration was changed from 2 to 24 μM

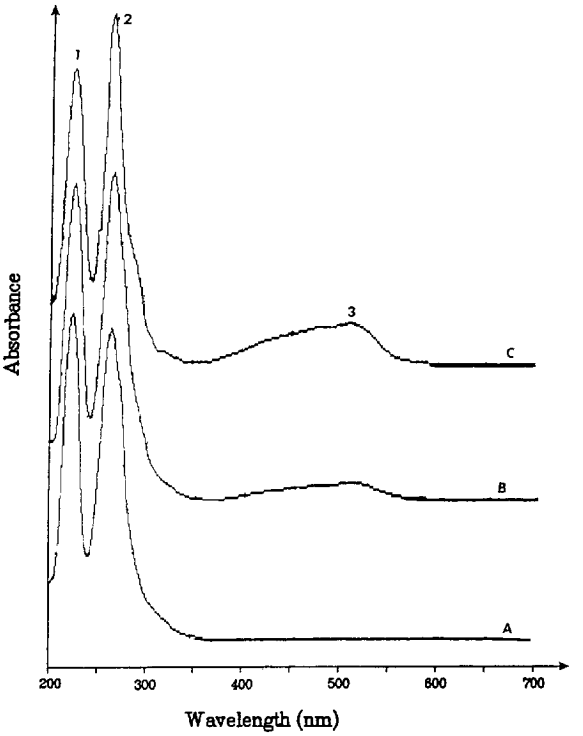

Fig. 1. Spectrophotometric scans of 1,10-phenanthroline and Fe(II)–1,10-phenanthroline solutions. Graph A, a spectrophotometric scan of 80 μM 1,10-phenanthroline; Graph B, a spectrophotometric scan of a mixture of 10 μM Fe(II) and 80 μM 1,10-phenanthroline; Graph C: a spectrophotometric scan of a solution containing 25 μM Fe(II) and 80 μM 1,10-phenanthroline. Peak identification: peak 1, 1,10-phenanthroline; peak 2, 1,10-phenanthroline and Fe(II)–1,10-phenanthroline complex; peak 3, Fe(II)–1,10-phenanthroline complex. Buffer: 50 mM NH_4Ac–HAc, pH 5.0.

(twelve points were taken) and the other in which the concentration of 1,10-phenanthroline was maintained at 80 μM while the iron(II) concentration was changed from 3 to 26 μM (ten points were taken). If the Fe(II)–1,10-phenanthroline complex does not absorb at 270 nm, the absorbance of the system should decrease from 3 to 36% for the first experiment and from 11.25 to 97.50% for the second experiment. Actually, the absorbance of the system increases with the increase of iron(II). This means that the Fe(II)–1,10-phenanthroline complex also absorbs at 270 nm and has a higher molar absorptivity, ϵ, than has 1,10-phenanthroline. The decrease in absorbance due to the decrease in the concentration of free 1,10-phenanthroline is compensated for by the absorbance of the Fe(II)–1,10-phenanthroline complex, which has a larger molar absorptivity, ϵ. It has been calculated from a series of Fe(II) concentrations and the corresponding absorbances that the molar absorptivity, ϵ, for 1,10-phenanthroline is 24 000 at 270 nm and that the molar absorptivity of the Fe(II)–1,10-phenanthroline complex is 86 000, which means that the sensitivity can be enhanced about eight times at 270 nm compared to that at 508 nm, due solely to the larger ϵ. However, to date the determination of the Fe(II)-1,10-phenanthroline complex by measuring the absorbance at 270 nm has not been reported in the literature. The spectrophotometric method cannot be used due to the non-separation between Fe(II)–1,10-phenanthroline complex and 1,10-phenanthroline itself. With HPCE, the Fe(II)–1,10-phenanthroline complex can be easily separated from 1,10-phenanthroline. Therefore, the sensitivity can be greatly improved by setting the detection wavelength of HPCE at 270 nm, due to the greater ϵ. In combination with the greater light output of the D_2 lamp and the better S/N ratio at 270 nm, the sensitivity can be improved even further.

3.2. Optimizing conditions for HPCE analysis

3.2.1. pH effect

A buffer system should be chosen so that good separation of the sample's components and good sensitivity can be achieved. The achievement of separation in HPCE has been theoretically studied by different authors [29,30]. Practically, the sensitivity is closely related to peak height and peak width, and resolution is closely related to the distance between two neighboring peaks [29]. It is obvious that for the same peak area, the higher the peak and the smaller the base peak width, the more sensitive the system will be, generally leading to better resolution. In our experiment, we used 50 mM NH_4Ac–HAc as the buffer system and studied the system at different pH values; the results are shown in Fig. 2. From the figure we can see that higher resolution can be obtained for the separation of the Fe(II)–1,10-phenanthroline complex and 1,10-phenanthroline at higher pH values (pH>6), but that the peak shape is not good. In addition, the signal-to-noise ratio (S/N) becomes worse. When the pH is below 3.5, the Fe(II)–1,10-phenanthroline complex and 1,10-phenanthroline are not well separated. Within pH values of 4.5–5.5, the separation was good and the peak shapes were excellent, with higher S/N ratios. Peak 2 is the Cu(II)–1,10-phenanthroline complex peak (Cu always exists in serum samples and in environmental water samples). Therefore, a buffer pH of 5 was selected as the running buffer for future experiments.

3.2.2. Operating voltage

The effects of the operating voltage on the separation and sensitivity were investigated. A series of experiments were carried out and proved that the sensitivity and separation are optimal within the voltage range of 10–15 kV. Therefore, 12.5 kV was selected as the running voltage.

3.2.3. Buffer concentration

Buffer concentrations ranging from 40–80 mM NH_4Ac–HAc (pH 5) were investigated. As the buffer concentration was increased, the separations improved. However, the current increased dramatically with increasing buffer concentration. This results in greater Joule heating, which would cause a deterioration in the peak shape and might lead to decomposition of the sample. Thus, 50 mM NH_4Ac–HAc was chosen as the running buffer for the separation.

3.3. Linearity and sensitivity

Linearity in the determination of Fe(II) was studied and the linear range was found to cover approx. two orders of magnitude (i.e., from 0.1 to 10

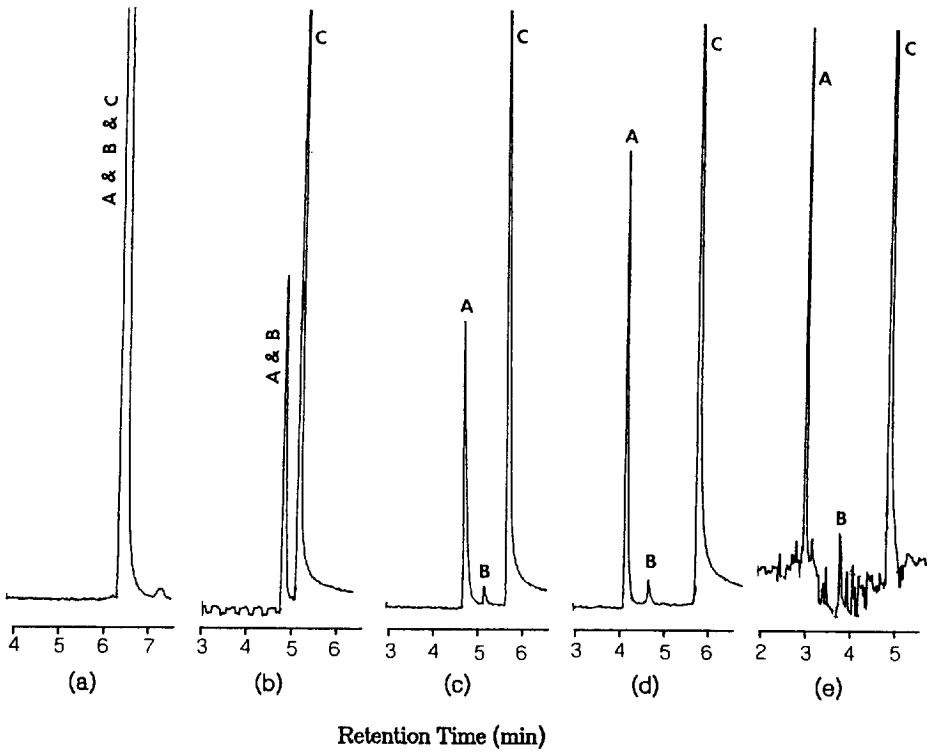

Fig. 2. The influence of the pH of the BGE solution on the separation of 1,10-phenanthroline and the Fe(II)–1,10-phenanthroline complex in HPCE. BGE used: 50 m*M* NH_4Ac–HAc. The concentration of 1,10-phenanthroline used is 80 μM (in D.I. water), concentration of iron(II) is 1.0 μM (in D.I. water). The pHs studied are (a) pH 3; (b) pH 3.95; (c) pH 4.5; (d) pH 5.0 and (e) pH 6.8. Injection, 20 s at 5 kV. Electrophoresis was carried out at +12.5 kV in a 60-cm (75 μm I.D.) capillary. Detection wavelength, 270 nm. Peak identification: A, the Fe(II)–1,10-phenanthroline complex; B, the Cu(II)–1,10-phenanthroline complex; C, 1,10-phenanthroline.

μM). The detection limit was lower than 5×10^{-9} *M* Fe(II) with a *S/N* value of five. With total of sixteen points, the coefficient of regression was 0.995. This allowed us to determine the level of iron in human serum precisely, even of the serum sample was diluted over ten fold (the iron level in normal human serum is 11–35 μM [29]). As a comparison, the linear range was also determined for Fe(II) using by HPCE at 508 nm, and was found to be from 5 to 80 μM (figure not shown), with a detection limit of 3.0×10^{-6} *M* iron. Based on the slope ratio of linear responses of iron signals at 270 nm over 508 nm, the sensitivity was improved at least twenty-fold at 270 nm compared to that obtained at 508 nm and the detection limit was improved even more (about 600 times). This improvement is due to the large molar absorptivity, ϵ, the higher light output, I_0, and the higher *S/N* ratio at 270 nm.

3.4. Determination of iron in human serum and its recovery

Using a spectrophotometric technique [3,4,24], serum can only be diluted a little by the addition of the necessary reagent and the chromogen and the precipitate cannot be washed [31–33], due to the low sensitivity of the detection. As stated in Section 2, the serum concentration of iron has been diluted ten-fold before the HPCE analysis and still gives a big signal for quantitation. This is critically important because during the serum treatment the precipitate can be washed and the occlusion problem can be solved. Also, the interference from other metal ions, such as copper, would be eliminated in our HPCE method because they can be well separated from the iron signal. The quantitative data obtained for the determination of iron by HPCE are

Table 1
Quantitative data for the determination of iron in serum and the relative recovery of iron

Sample	Fe(II) in sample (μM)	Standard Fe(II) added (μM)	Fe(II) level by HPCE (μM)	Recovery (%)
Blank 1	0	2.25	2.27	100.9
Blank 2	0	2.75	2.74	99.6
Serum 1	1.05	1.27	2.10	90.5
Serum 2	1.75	0.89	2.48	94.0
Serum 3	1.38	1.09	2.50	101.2
Serum 4	2.03	0.74	2.74	98.9

HPCE conditions are: Running buffer, 50 mM NH_4Ac–HAc, pH 5.0; Injection of samples, 20 s at 5.0 kV. Electrophoresis was carried out at 12.5 kV and all serum samples were diluted ten-fold.

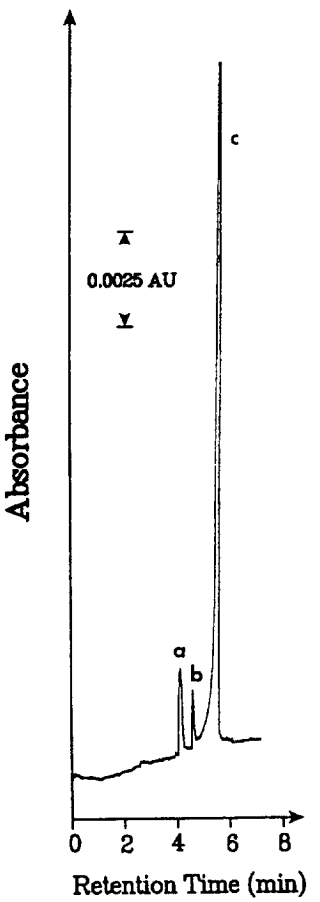

Fig. 3. Representative electropherogram of a typical serum sample diluted ten times. All electrophoretic conditions and peak identifications are the same as those in Fig. 2, except that 50 mM NH_4Ac–HAc buffer, pH 5, was used as the BGE. The iron concentration in this serum sample was 31.4 μM.

shown in Table 1. A representative electropherogram for serum iron determination is shown in Fig. 3. The recovery of iron was found to be 90–100% for the total concentration of iron (including serum iron and standard addition of iron). By comparing the spectrophotometric method [24] (which uses ferrozine as a chromophore) with our HPCE method for the same serum sample, the results from HPCE were found to be 6.0% lower than those obtained with the spectrophotometric method. The reason for this may be that the copper interference cannot be eliminated in the spectrophotometric method. The results shows that the HPCE method is excellent for use in the determination of trace concentrations of iron in human serum and in other biological samples.

4. Conclusion

A new, sensitive, method for the determination of iron by HPCE was developed. The sensitivity has been improved at least twenty fold compared to the normal method which measured the absorbance of Fe(II)–1,10-phenanthroline at 508 nm. The detection limit is lower than 5×10^{-9} M iron. This will be an excellent method for the determination of trace amounts of iron in serum [22] and in environmental water samples [23].

Acknowledgments

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