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Original Article

Direct separation, detection and quantitation of iron (II) using high performance liquid chromatography and evaporative light scattering detection

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ABSTRACT

This study demonstrates the use of high-performance liquid chromatography (HPLC) equipped with evaporative light scattering detection (ELSD) for the direct detection and quantitation of iron (II) in ferrous sulfate tablets of 65 mg strength, whereas the corresponding United States Pharmacopoeia (USP) monograph is based on a titrimetric technique. After evaluating the effects of buffer concentration and pH, the separation of iron (II) was accomplished using a ZIC-HILIC® peek column with a hydrophilic interaction chromatography (HILIC) mode gradient elution. Typical validation parameters were evaluated to assess the method's quantitative performance for iron (II) which included specificity, accuracy, precision, linearity, stability, and limit of detection. This technique provides a unique and practical alternative method for the accurate quantitation of iron (II) as well as separation from iron (III). Additionally, the detection and separation for cobalt II and Manganese II were also accomplished using the same technique in the presence of Iron II and Iron III.

KEYWORDS: iron (II), iron (III), ascorbic acid, HPLC, ELSD, ZIC HILIC, transition metals.

1. INTRODUCTION

The most common oxidation states of iron are iron (II) and iron (III). Iron shares many properties of other transition metals, including the other group 8 elements, ruthenium, and osmium. Iron forms compounds in a wide range of oxidation states, −2 to +7. Iron also forms many coordination compounds; some of them, such as ferrocene, ferrioxalate, and Prussian blue, have substantial industrial, medical, or research applications [1]. Iron is often determined by spectroscopic techniques like spectrophotometric determination [2], atomic absorption spectroscopy (AAS) [3] and inductively coupled plasma (ICP) spectroscopy [4] but it can also be determined by chromatographic techniques [5-10]. High performance liquid chromatography (HPLC) has been applied for the analysis of iron (II) by employing reversed-phase chromatography with desferrioxamine (DFO) as chelating reagent, where the iron chelates are then detected with an ultraviolet (UV) detector. A highly selective, non-extractive spectrophotometric method uses rapid in-direct determination of iron (II) at trace levels using 2,3,4,5,7-pentahydroxyflavone (morin) as a new spectrophotometric reagent in slightly acidic solution (0.0001-0.0002 M H^2SO_4) [11]. The reaction is instantaneous, and absorbance remains stable for over 24 h (λmax = 415 nm). Hydrophilic interaction chromatography (HILIC) combined with aerosol-based detectors have an extensive application base for the direct separation and direct detection of positive and negative counterions, as well as accurate quantitation [12-18]. Here, in this paper, we used a HILIC column with evaporative light scattering detector (ELSD) for the direct separation, detection, and quantitation of iron (II). Other transition metals such as cobalt (II),

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and manganese (II) are all separated and detected using this method in the presence of iron (II) and iron (III). Typical validation parameters were evaluated to assess the method's quantitative performance for iron (II) which included specificity, accuracy, precision, linearity, stability in sample solvent and limit of detection.

2. MATERIALS AND METHODS

2.1. Chemicals

Acetonitrile (MeCN) was purchased from EMD Sciences Inc. (Gibbstown, NJ). Ammonium formate, formic acid, hydrochloric acid, and L-ascorbic acid were obtained from Millipore-Sigma chemical company (St. Louis, MO). The transition metal analytes, iron (III) chloride was purchased from Alfa Aesar, cobalt (II) acetate, was purchased from Millipore-Sigma chemical company (St. Louis, MO) while manganese (II) chloride was purchased from Fisher chemicals. Iron (II) sulfate heptahydrate standard (99.9%) was obtained from Millipore-Sigma chemical company (St Louis, MO). Ferrous sulfate tablets (65 mg) were purchased from Nature's Truth (New York, NY). Deionized water and nitrogen were from an in-house system from Eli Lilly and Company (Indianapolis, IN).

2.2. Equipment

Chromatographic analysis was performed on an Agilent 1260 HPLC system (Santa Clara, CA) equipped with a binary gradient pump and auto sampler. Detection was performed with an evaporative light scattering detector (ELSD) 1290 from Agilent Technologies (Santa Clara, CA). The ELSD settings were as follows: nitrogen gas at 1.6 L/min, nebulizer temperature and evaporator temperature at 70°C. A ZIC-HILIC® peek column (150 mm x 4.6 mm I.D., 5 µm) from Millipore-Sigma (St. Louis, MO) was used for the separation.

2.3. Standard and sample preparation

Diluent selection for iron (II) determination was extremely critical due to the potential rapid oxidation of iron (II) to iron (III). Iron (II) oxidizes to iron (III) when dissolved directly in water or water: acetonitrile mixtures. The rate of oxidation of iron (II) also depends upon the type of iron salt being used. For example, iron (II) acetate when dissolved in water or water: acetonitrile mixtures oxidize immediately to iron (III) while on the other hand, iron (II) sulfate heptahydrate dissolved in water or water: acetonitrile mixtures were observed in the ratio of ~93% iron (II) and 7% iron (III) (refer to Table 1). Halogenated iron salts would be expected to be more stable, and the pH of the solution also impacts the iron oxidation state [19]. Therefore, a reducing agent, ascorbic acid solution, was added as a diluent for method development to minimize the oxidation. Using ascorbic acid as diluent, less than 1% Iron III was observed in iron (II) sulfate heptahydrate solution in comparison to water or water:acetonitrile mixtures. The iron (II) standards were prepared from iron (II) sulfate heptahydrate by dissolving the standards in 1% acidified ascorbic acid solution. The diluted standard concentration ranged from 1 mg/mL – 3 mg/mL. The sample preparation was accomplished using intact tablets or crushed tablets. The crushed tablet samples were prepared by accurately weighing the sample of crushed powder (so the theoretical content amount would be within the standard range) into a 50 mL volumetric flask. Then 40 mL of diluent was added to the flask containing sample and the sample solution was sonicated for 15 minutes with intermittent shaking. The sample solution was allowed

Table 1. Comparison of iron (II) sulphate stability in Water:MeCN mixtures and ascorbic acid diluent.

Water:MeCN mixture				**Ascorbic acid diluent**	
Time in hrs	**% Area Fe (II)**	**% Area Fe (III)**	**Time in hrs**	**% Area Fe (II)**	**% Area Fe (III)**
0	92.75	7.25	0	99.42	0.58
0.6 hrs	92.28	7.72	1 hr	99.42	0.58
1.5 hrs	92.48	7.52	2 hrs	99.39	0.61
3 hrs	92.38	7.62	3hrs	99.39	0.61
6 hrs	91.19	8.81	6hrs	99.34	0.66

to cool to room temperature and then diluted to volume with diluent and mixed well. For whole tablet analysis, one tablet was added to a 50 mL volumetric flask and then the same procedure as for the crushed tablets was followed. The final solutions were filtered using 0.2 um glass filters prior to injection by HPLC. The standard curve was calculated by least-squares regression analysis of peak area versus concentration. The concentration of iron (II) in the samples was determined by comparing the peak area to the standard curve. The cobalt (II) acetate and manganese (II) chloride samples were diluted as a mixture with a sample solvent comprising 50% acetonitrile and 50% water for selectivity experiments.

3. RESULT AND DISCUSSION

3.1. Method development

The main purpose of this work was to develop a simple, direct, and robust HPLC method for the separation and quantitation of iron II that could easily be integrated for in-process assays or fast analysis using common analytical laboratory equipments. The ZIC-HILIC® peek column was selected based on method development trials that resulted in the best peak shape and selectivity. A starting mobile phase of 80:20 (ACN: buffer) was used with a hold at 70:30 for 10 min and a linear gradient of increasing aqueous composition to 5:95 (ACN: buffer) for 15 minutes to produce the optimized gradient described in section 3.4. The mobile phase flow rate was maintained at 1.0 mL/min with an injection volume of 5 µL.

3.2. Effect of ammonium formate buffer concentration

The effect of increasing the buffer concentration in the mobile phase was evaluated on the retention time for iron (II), cobalt (II), manganese (II) and iron (III). Ammonium formate buffer was used in the range of 150 – 200 mM while maintaining the pH at 2.8 with formic acid. As the buffer concentration increased, the transition metal peaks slightly decreased in retention time as shown in Table 2. Iron (III) did not elute off the column when using the buffer concentration below 150 mM. The peak shape for iron (II), cobalt (II) and iron (III) improved with increasing buffer strength while the manganese (II) peak was affected due to the interference of blank peak.

3.3. Effect of pH

The effect of increasing the pH in the mobile phase was evaluated on the retention time for iron (II), cobalt (II), manganese (II) and iron (III). The pH effect was examined across a range of 2.6 – 3.5 while maintaining the ammonium formate buffer strength at 175 mM. As the pH of the buffer increased, the transition metals peaks did not show a significant shift in retention time except for iron (III) as shown in Table 3. The peak shape of manganese (II) was poor at pH 2.6. The peak shape for iron (III) was also affected by the increase in pH where it was broad and had low response at pH greater than 3.0. Further experiments for iron (II) determined that the optimum pH range for this analyte was 2.8-3.0. The buffer pH can be adjusted in increments of 0.05 within this range to achieve the best desired peak shape for iron (II) and (III), although a recommended starting point is pH 2.8.

3.4. Final gradient conditions

Based on the buffer concentration and pH experiments above, the final gradient conditions were optimized for the ZIC-HILIC® PEEK column. The mobile phase flow rate was set at 1.0 mL/min with an injection volume of 5 uL and a column temperature of 40 °C. Mobile phase A comprised 175 mM ammonium

Table 2. Effect of mobile phase buffer strength at pH 2.8 on the retention time (Minutes).

#	Ammonium formate buffer concentration evaluation			
	Name	Retention Time (in min)		
		150 mM Buffer	175 mM Buffer	200 mM Buffer
1	Cobalt (II)	11.740	11.426	10.947
2	Iron (II)	12.079	11.658	11.221
3	Manganese (II)	12.668	12.602	12.345
4	Iron (III)	13.997	14.247	13.984

formate, pH 2.8, with formic acid (pH can be adjusted in 0.05 increments within a range from 2.8 to 3.0 for best peak shape) and mobile phase B was acetonitrile. The following gradient was employed: initial 80% B with a linear gradient to 70% B in 1 minute, a hold at 70% B for 10 minutes and then a steep gradient to 5% B in 4 minutes (5 minutes equilibration time at the starting conditions before the next injection). The ELSD is non-linear over a wide concentration range and logarithm plots are often needed to obtain calibration curves spanning several orders of magnitude. However, for this iron (II) analysis, the concentration range was small enough to work within the linear range of the detector; thus, a linear fit could be applied. The Agilent ELSD settings with the chromatography conditions described above were maintained as follows: nitrogen gas flow rate 1.6 L/min; nebulizer temperature and evaporator temperature of 70 °C.

4. EVALUATION OF VALIDATION PARAMETERS

4.1. Specificity

Cobalt (II) and manganese (II) are other transition metals which are close in the periodic table to iron and could potentially interfere with iron (II). Therefore, the specificity of the method was determined by analyzing individual preparations of each transition metal to ensure that iron (II) peak was free from any interference. Figure 1 shows the Iron II standard chromatogram while Figure 2 demonstrates the separation of transition metals cobalt (II), manganese (II), iron (II) and iron (III) using the recommended conditions outlined in 3.4.

4.2. Accuracy and precision

Two sample preparation techniques were used to demonstrate the precision of this method using iron

Table 3. Effect of mobile phase pH on the retention time (Minutes) with buffer concentration of 175 mM.

pH condition	Cobalt (II)	Iron (II)	Manganese (II)	Iron (III)
pH 2.6	10.99	11.26	12.60	13.70
pH 3.0	10.64	10.90	11.79	15.10
pH 3.2	10.62	10.90	11.70	16.61
pH 3.5	10.79	11.14	11.87	No elution

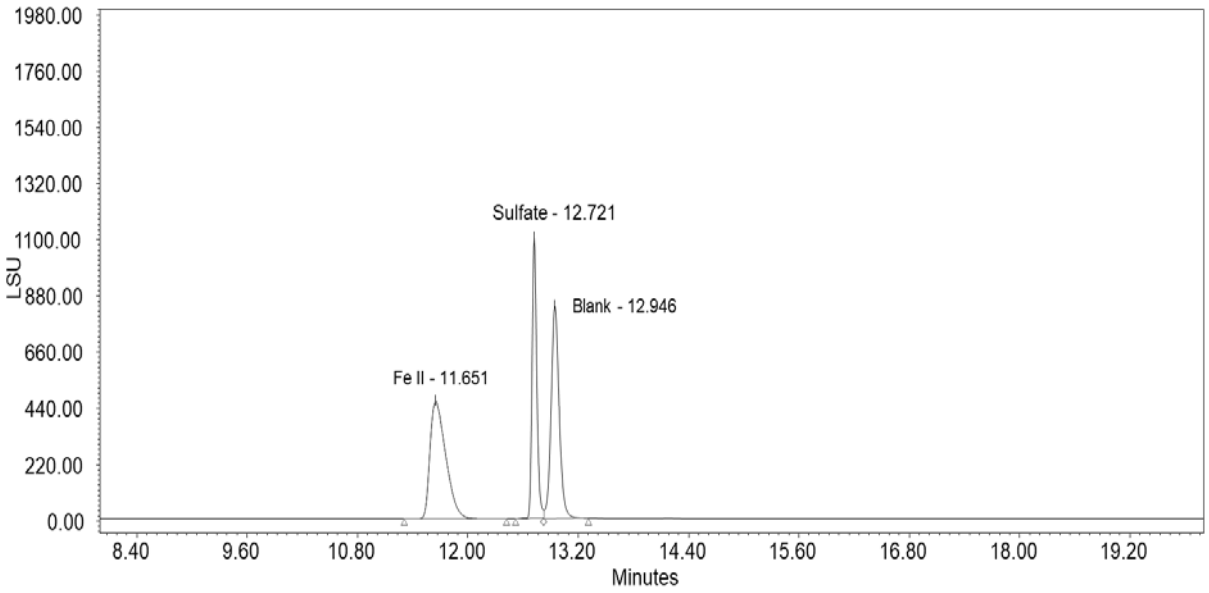

Figure 1. Iron (II) sulfate heptahydrate 2 mg/mL in ascorbic acid diluent.

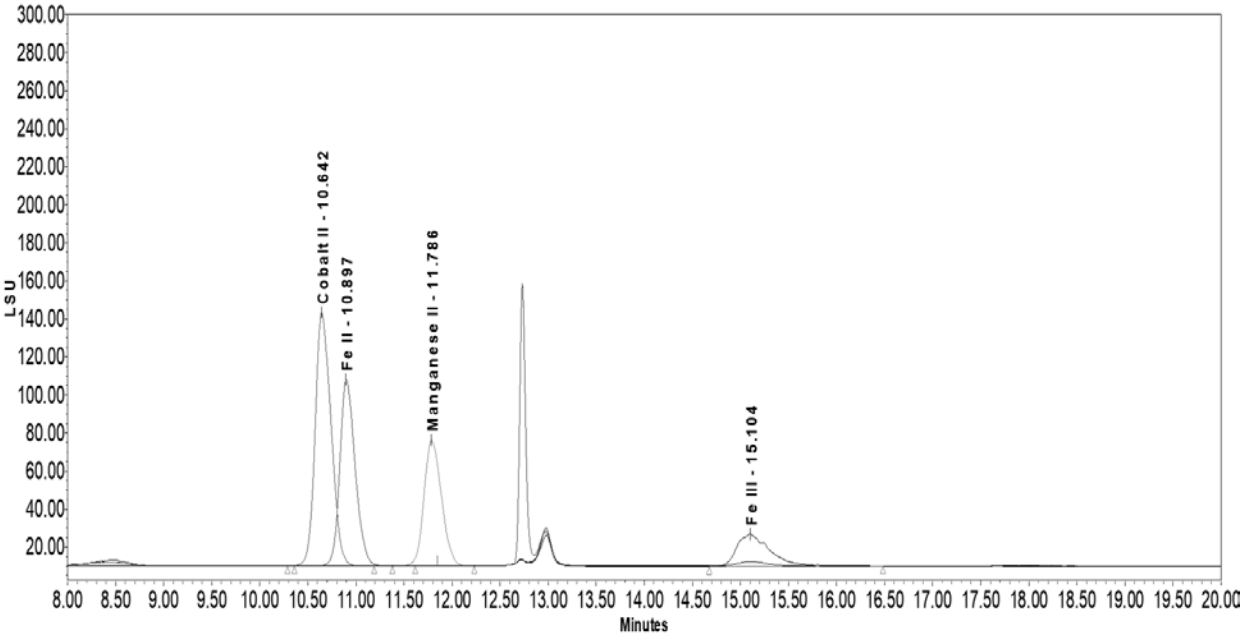

Figure 2. The separation of the transition metals cobalt (II), iron (II), manganese (II) and iron (III) is shown with mobile phase A comprised 175 mM ammonium formate pH 2.8 and mobile phase B acetonitrile with the following gradient employed: initial 80% B with a linear gradient to 70% B in 1 minute, a hold at 70% B for 10 minutes and then a steep gradient to 5% B in 4 minutes (5 minutes equilibration time at the starting conditions before the next injection). The flow rate was 1.0 mL/min with an injection volume of 5 uL and a column temperature of 40 °C.

Table 4. Method precision using iron tablets of 65 mg based on intact tablets.

Injections	Sample Name	RT	Assay in mg/tablet	Assay in %w/w
1	Fe (II)	11.529	66.1	101.7
2	Fe (II)	11.526	64.7	99.6
3	Fe (II)	11.522	67.0	103.1
	Mean	11.525	65.9	
	SD	0.00	1.15	
	RSD	0.03	1.75	

tablets (65 mg) as sample. First sample preparation was based on using the entire tablet as shown in Table 4 and second by crushing the tablets to fine powder and weighing the equivalent tablet weight as shown in Table 5. For quantification, three standard concentrations 1.0 mg/mL, 1.4 mg/mL and 2.0 mg/mL, were used to generate the standard calibration curve. First, five replicate injections of the middle standard solution (1.4 mg/mL iron II sulfate heptahydrate) were injected to determine the reproducibility of the instrument as shown in Table 6. The accuracy of the method was checked by injecting 100% check standard at the end of each sequence and recovery of the check standards were obtained within 97%-102%. Ferrous sulfate tablets are official in United states pharmacopoeia and the specification limit of iron II in ferrous sulfate tablets should not be less than 95.0% and not more than 110.0% as per United states Pharmacopoeia.

4.3. Linearity

The linearity of the method was established by injecting five different concentrations of ferrous sulfate heptahydrate standards. A standard concentration set of 0.8, 1.0, 1.2, 1.4 and 1.6 mg/mL

Table 5. Method precision using iron tablets of 65 mg based on crushed tablets.

Injections	Sample Name	RT	Assay in mg/tablet	Assay in %w/w
1	Fe (II)	11.697	65.6	99.1
2	Fe (II)	11.687	67.2	103.4
3	Fe (II)	11.682	68.8	105.8
	Mean	11.688	67.2	
	SD	0.01	1.60	
	RSD	0.07	2.38	

Table 6. System precision by using 1.4 mg/mL ferrous sulfate heptahydrate standard.

Injections	Sample Name	RT	Response
1	Fe (II)	11.529	5376109631
2	Fe (II)	11.526	5395053713
3	Fe (II)	11.522	5269879219
4	Fe (II)	11.519	5302720451
5	Fe (II)	11.526	5321810128
	Mean	11.5244	5333114628
	SD	0.00	51805731.36
	RSD	0.03	0.97

equivalent to iron (II) was evaluated for the validation purposes while a set of 1.0, 1.4, and 2.0 mg/mL was used for the assays. The Agilent 1290 ELSD consistently had R^2 values of 0.998 or greater. Linear calibration curve was used for the purpose of quantification to calculate the correlation coefficient values based on the slope of the curve.

4.4. Solution stability

Sample solution stability was evaluated quantitatively by initially analyzing the sample preparations and then injecting the sample in separate vials over 8 hours. The sample was found to be stable for 8 hours at room temperature. The assay value after every hour was observed as 99.98, 100.87, 99.25, 101.825, 99.149, 97.79, 96.06 and 100.23. Sample solution stability can be maintained for longer duration if free oxygen within the mobile phase, column and sample solvent is under controlled condition. Decreasing peak areas of iron (II) and increasing peak areas of Iron III can indicate an issue with oxidation in the method parameters.

4.5. Limit of detection

The limit of detection was evaluated by dilution of the standard solutions to the lowest concentration level to obtain a peak signal that was 3 times the baseline noise. The limit of detection for iron (II) was determined to be 25 µg/mL.

4.6. Sample analysis

This work was concluded by analyzing ferrous sulfate tablets of 65 mg strength using this HPLC-ELSD method. The tablet samples were prepared in triplicate so that the theoretical concentration of iron (II) would be approximately 1.4 mg/mL. A three-point standard set (1.0, 1.4 and 2.0 mg/mL) was used to quantitate iron (II) in each tablet sample. As shown in Figure 3, the chromatogram overlays with the standard and sample solvent blank, illustrates no interferences with the iron (II) peak while the standard and Iron II peaks in different salt form samples match the retention times. Tables 4 and 5 show the iron (II) results using this HPLC-ELSD method based on intact tablets and crushed powder.

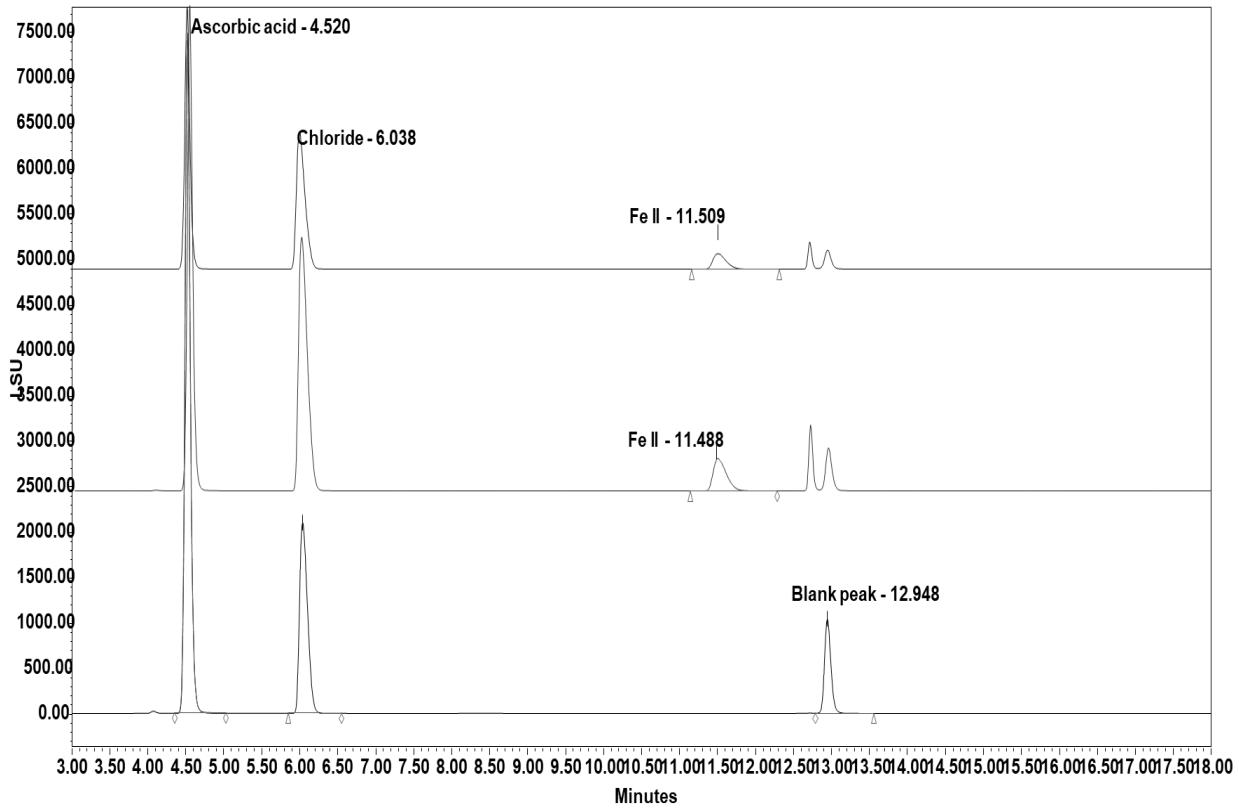

Figure 3. The chromatogram overlays are shown for the sample solvent blank, standard and ferrous sulfate tablet sample of iron (II).

Overall, the results were within the USP specification limits and are also comparable to the label claim of 65 mg.

5. CONCLUSION

This HPLC-ELSD method for iron (II) determination was found to be accurate, specific, reproducible, and precise. Also, the data generated with this HPLC-ELSD technique was found to be comparable to other techniques like titrimetric techniques as mentioned in USP monograph of ferrous sulfate tablets analysis which can also be used to quantitate total iron. This method allows for speciation, specifically determining the amount of iron (II). The practicality of using HPLC-ELSD should not only be limited to the detection and quantitation of Iron (II), but can be applied to other transition metals for their detection and quantitation.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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