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Development of Suspended Droplet Microextraction Method for Spectrophotometric Determination of Serum Iron

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ABSTRACT

A facile, selective and sensitive method was devised for the Fe^{3+} quantification in low volume samples, such as bovine serum based on suspended droplet microextraction (SDME). Various process parameters such as concentrations of acid (1.5% hydrochloric acid), complexing agent (0.7% ammonium thiocyanate), quaternary ammonium salt (0.2% Aliquat 336) and extracting solvent (500 µL octanol) were optimised. Ammonium thiocyanate forms water soluble, red coloured, anionic ferric thiocyanate complex $[Fe(SCN)_6]^{3-}$ with Fe^{3+} ions released from the iron–protein complex under an acidic medium. Negatively charged $[Fe(SCN)_6]^{3-}$ complex forms hydrophobic ion associate $Fe(SCN)_6{}^{3-}$–Aliquat $336_3{}^{3+}$ with hydrophilic $NH_4{}^+$ head groups of Aliquat 336 and drives out the formed micelle from aqueous solution along with the iron complex. After stirring, ion associate bonded micelles are separated into a hanging micro droplet of octanol. Red coloured ferric thiocyanate complex in a suspended droplet is solubilised in methanol and Fe^{3+} concentration in serum samples is obtained by recording the spectrophotometric absorbance at 505 nm. The recoveries ranged from 96.2%–98.8% with relative standard deviation (RSD) (%) values from 1.4% to 5.0% at 100–400 ng/mL confirming interference free quantification at optimised conditions. The developed method was linear over the range of 20–1000 ng/mL of Fe^{3+} with a limit of detection of 2.4 ng/mL for the serum matrix. The developed method is applied to various bovine serum samples and Fe^{3+} concentration values ranged from 62.7 to 1582.5 ng/mL. The obtained values were in accordance with the results obtained from the electrothermal atomic absorption spectrometry at 99% confidence level using *t*-test indicating the accuracy of the developed method. The proposed procedure offers various advantages such as enhanced sensitivity of the spectrophotometer towards iron determination, low-cost complexing agent, low sample volume, metal and biological interference free, simplicity and selectivity. Thus, the developed method can be an alternative to the routine spectrophotometric analysis of low volume samples such as serum and other biological fluids.

1 | Introduction

Serum iron is the circulating, transit form of iron that is bound to transferrin, an iron binding protein that transports it throughout the body [1]. The ferric ions are reversibly bound to transferrin and its concentration is in the typical range of 500–1700 ng/mL [2–5]. Serum iron is a helpful biomarker and predictor for many diseases and their progression [6] and functions as a principle,

Abbreviations: Aliquat 336, methyltrioctylammonium chloride; CLSI, Clinical and Laboratory Standards Institute; CPB, cetylpyridinium bromide; CTAB, cetyltrimethylammonium bromide; ET-AAS, electrothermal atomic absorption spectrometry; HCl, hydrochloric acid; ICP-MS, inductively coupled plasma-mass spectrometry; ICP-OES, inductively coupled plasma-optical emission spectrometry; ICSH, International Committee for Standardisation in Haematology; LOD, limit of detection; NH_4SCN, ammonium thiocyanate; SDME, suspended droplet microextraction; TBAP, tetrabutylammonium perchlorate; THAI, tetraheptylammonium iodide; TPTZ, 2, 4, 6-tripyridyl-s-triazine.

clinical test parameter for diagnosing many disease conditions and disorders, such as iron metabolism (iron deficiency, repletion, overload) [6–8], etc. Hence, a simple, fast, cost effective and accurate protocol for quantifying the low levels of iron in serum is essentially needed especially for clinical analysts, who routinely analyze a large number of samples on a day-to-day basis for taking accurate clinical decisions [2, 9, 10]. Fetal bovine serum and calf serum are widely used in commercial animal cell culture media for mimicking the extracellular milieu and vast quantities of serum are utilised for production of vaccines and biological drugs from cell lines [11]. The iron supplemented calf serum is used for the long term proliferation of various populations of fibroblast like, epithelial like and lymphoid cells. The iron is required for different metabolic activities such as oxidation reduction catalysis, oxygen transport, electron transport, energy production, DNA synthesis, etc. [1, 11].

Various analytical techniques, including flame atomic absorption spectrometry (FAAAS) [3, 12], electrothermal atomic absorption spectrometry (ET-AAS) [13, 14], inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) [15], inductively coupled plasma-mass spectrometry (ICP-MS) [1], chromatography [15], voltammetry [16], etc. are frequently utilised for the quantification of iron in different matrices. Out of which, determination of iron at ng/mL level is possible only with ET-AAS and ICP-DRC-MS. Nevertheless, these techniques are limited by complex and laborious sample preparation, matrix interferences, costly operations and instrumentation and analyst expertise [14, 15, 17]. However, enhancement of the sensitivity of the ubiquitous UV–visible spectrophotometer is still a choice of candidates due to its simplicity, low cost instrumentation, ease of operation, etc. [18, 19].

Different chromogenic agents including, 1, 10-phenanthroline [20, 21], bathophenanthroline [18, 22, 23], 2, 4, 6-tripyridyl-s-triazine (TPTZ) [18, 24], ferrozine [18, 25–29], ferene-S [2, 3, 10, 30], 2-(5-nitro-2-pyridylazo)-5-(N-propyl-N-sulphopropylamino) phenol (Nitro-PAPS) [31], etc. were used for the spectrophotometric quantification of Fe^{2+}. Whereas ammonium and potassium thiocyanate [15, 29, 32–34]; potassium ferrocyanide, potassium ferricyanide, salicylic acid [23], sulphosalicylic acid [21, 33], etc. were employed for the spectrophotometric determination of Fe^{3+}. The chromogenic ligand ammonium thiocyanate is widely exploited both for the qualitative and quantitative spectrophotometric quantification of Fe^{3+} in natural water, wine [35], seawater [34, 36], pulses, milk, human blood, urine, rainwater, groundwater [15]; blood serum [32], domestic tap water [37], bioethanol fuel [17] based on red-coloured complex formation. Currently, many ionic liquids are also utilised in the trace level quantification of different metals [38, 39]. The iron panel of the International Committee for Standardisation in Haematology (ICSH) and Clinical and Laboratory Standards Institute (CLSI) recommended kits and methods based on ferrozine or ferene-S as standard procedures for the spectrophotometric estimation of human serum iron, after trichloroacetic acid mediated protein precipitation and thioglycolic acid induced iron reduction [3, 10, 19, 40, 41]. The analytical stripe [37], multi syringe flow injection systems [36], smart phone-based devices [17] and electrochemical sensor-dependent [5] methods detect Fe^{3+} at a higher concentration range of μg/mL. The multi-step, sophisticated flow injection systems mandate either the analyte's online oxidation or reduction step [17, 33, 42]. The Fe^{3+} ion extraction and preconcentration protocols mandate specialised spectrophotometer [15], long waveguide capillary cell with a preconcentration resin [29], costly preconcentration resins [29, 35] and tailored sorbents [34] and suitable for high volume samples such as waters [29, 34, 35, 42, 43] and ethanol [17].

TABLE 1 | Standardised temperature programme used in continuum source ET-AAS for the quantification of iron in serum.

Step	Temperature (°C)	Ramp (°C/s)	Hold (s)
Drying 1	80	6	20
Drying 2	110	5	10
Pyrolysis	900	300	10
Gas adaption	900	0	5
Atomisation	2000	1500	4
Cleaning	2450	500	4

Abbreviation: ET-AAS, electrothermal atomic absorption spectrometry.

In this perspective, the current research aims to devise a simple, selective and sensitive method for the quantification of Fe^{3+} based on suspended droplet microextraction (SDME) without the need of reduction step and reducing environment [1–3, 18, 19, 22, 23, 25–28, 33] employing a much cheaper ammonium thiocyanate complexing agent for low volume samples such as bovine serum and human serum. The proposed method relies on the separation and preconcentration of water-soluble, red-coloured ferric thiocyanate complex from the solution and measurement of absorbance at 505 nm by spectrophotometry. Various process parameters such as concentrations of acid, complexing agent, quaternary ammonium salt and extracting solvent were optimised. The concentration of the Fe^{3+} in various bovine serum samples obtained from spectrophotometry was validated with ET-AAS.

2 | Materials and Methods

2.1 | Instrumentation

The concentration of Fe^{3+} was measured by recording the absorbance at 505 nm using Analytik Jena AG, Specord 200 Plus UV–visible spectrophotometer (Jena, Germany). The iron concentration in treated bovine serum samples was determined employing Analytik Jena AG, Contra AA 700 continuum source ET-AAS (Jena, Germany). A spectral line at 248.327 nm was utilised for iron quantification [3, 13]. The optimised temperature scheme for the iron determination is given in Table 1.

2.2 | Materials

Sub-boiled hydrochloric acid (HCl), sub-boiled nitric acid (HNO_3), 98% sulphuric acid (H_2SO_4), ferrous ammonium sulphate hexahydrate (≥98.5%), ferric chloride hexahydrate (≥ 98.5%), ammonium thiocyanate (NH_4SCN), methyltrioctylammonium chloride (Aliquat 336), octanol,

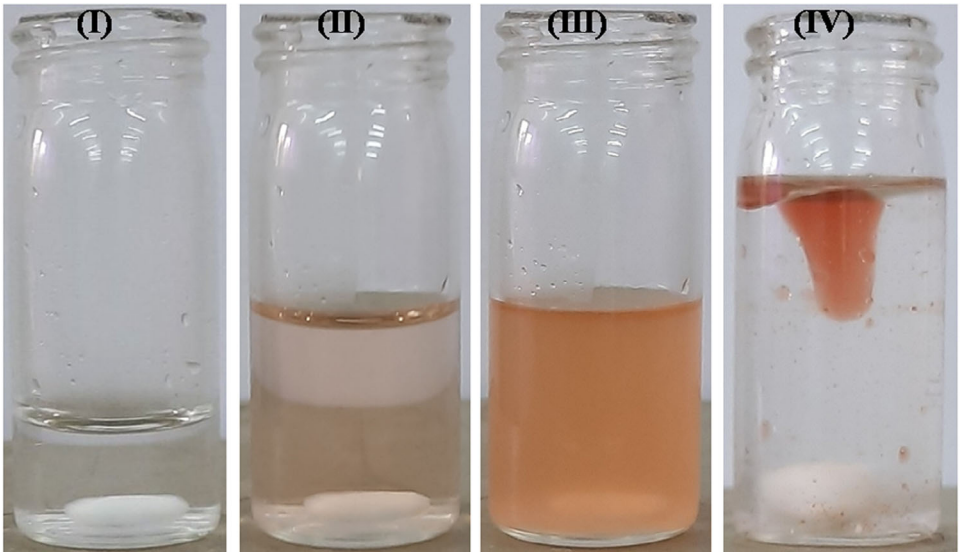

FIGURE 1 | The schematic images showing the steps involved in the suspended droplet microextraction (SDME) of serum iron; (I) serum + HCl, (II) serum + HCl + NH_4SCN, (III) serum + HCl + NH_4SCN + Aliquat 336 + octanol and (IV) scarlet red coloured suspended droplet.

cetylpyridinium bromide (CPB), cetyltrimethylammonium bromide (CTAB), tetrabutylammonium perchlorate (TBAP), tetraheptylammonium iodide (THAI), ethanol (E. Merck, Mumbai, India) of analytical reagent grade were utilised. The Fe^{2+} and Fe^{3+} stock standard solutions (1 mg/mL) was made from ferrous ammonium sulphate hexahydrate and ferric chloride hexahydrate in HCl [5% (v/v)] and H_2SO_4 [5% (v/v)], respectively. The standard running solutions were made by successive dilutions of the stocks. The stock solutions of 10% (w/v) NH_4SCN and Aliquat 336 were made with ultrapure water (Elga Purelab Flex 3 water polishing unit, High Wycombe, UK) and ethanol, respectively. The other metal (Ca^{2+}, Mg^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Al^{3+}, Ni^{2+}, Co^{2+}, Bi^{3+} and Mn^{2+} stock solutions (1 mg/mL) used for interference study were prepared by dissolving their respective nitrate and chloride salts in ultrapure water. The labware (glass vials, micropipette tips) used during this study were cleaned with HNO_3 [10% (v/v)], washed with ultrapure water and the cleaning was repeated with HCl [20% (v/v)]. Commercially available, different fetal bovine serum (Sigma-Aldrich, Bengaluru; Himedia, Mumbai, India) samples (2 mL) were treated with 3 mL of reagent mixture made up of HCl, methanol and chloroform [1:1:1 (v/v)] [3, 23]. The precipitated serum proteins and extracted lipids were centrifugally separated at 10, 000 rpm for 10 min and the deproteinised serum samples were used for iron estimation.

2.3 | Methods

A 100 µL of treated serum was taken in a 15 mL glass tube and 150 µL of sub boiled HCl was added, followed by 700 µL of 10% ammonium thiocyanate, 200 µL of 10% Aliquat 336 and 500 µL of octanol. The volume of the mixture was brought to 10 mL with ultrapure water and thoroughly vortexed under magnetic stirring at 2000 rpm for 10 min. The upper, separated organic layer in the form of a suspended droplet was pipetted out into a fresh vial, solubilised in methanol and the final volume was made into 3 mL (Figure 1). Similarly, spiked Fe^{3+} standards were also maintained in treated serum matrix at varying concentrations of 20–1000 ng/mL. The Fe^{3+} concentration in the dissolved, suspended droplet was measured by spectrophotometry against a methanol blank.

3 | Results and Discussion

In this study, as a miniaturised sample extraction method, SDME was selected for the separation and preconcentration of Fe^{3+} ions from the deproteinised serum matrix. It offers various advantages, including simplicity, low cost, low extraction time, low quantity of extraction solvent, high preconcentration efficiency, analyte preconcentration in low volume, minimal laboratory equipment, applicability to a wide range of polar, non-polar, inorganic and organic analytes, etc. [43–46]. The three essential steps in serum iron determination are the release of bound iron from transferrin, trichloroacetic acid mediated deproteinisation and colour development [1, 22, 47]. The iron extraction and precipitation reagent (HCl + methanol + chloroform) was used for iron dissociation from transferrin, protein precipitation and lipid extraction [3, 5]. The strong, inorganic acid HCl was used as an analyte releasing agent from the matrix. The transferrin bound iron was ultimately released and all the forms of iron were converted into Fe^{3+}. Also, HCl prevents the precipitation of Fe^{3+} in the solution [3, 13, 23, 24, 27, 28, 35]. While the ammonium thiocyanate (NH_4SCN) acts as a chromogenic complexing agent for Fe^{3+} and forms a water soluble, faint red-coloured ferric thiocyanate complex, $[Fe(SCN)_6]^{3-}$ in the acidic medium [15, 17, 36].

Under the influence of high concentration of thiocyanate, the Fe^{3+} ion complexes with thiocyanate ion in a stoichiometric ratio of 1:6 [48, 49]. Further, the negatively charged $[Fe(SCN)_6]^{3-}$ complex forms an ion associate with hydrophilic NH_4^+ head groups of quaternary ammonium salt, Aliquat 336. The hydrophobic ends of the cationic surfactant, Aliquat 336 drive the formed micelles out of the aqueous solution along with the formed iron complex. Upon magnetic stirring, the extracted, driven

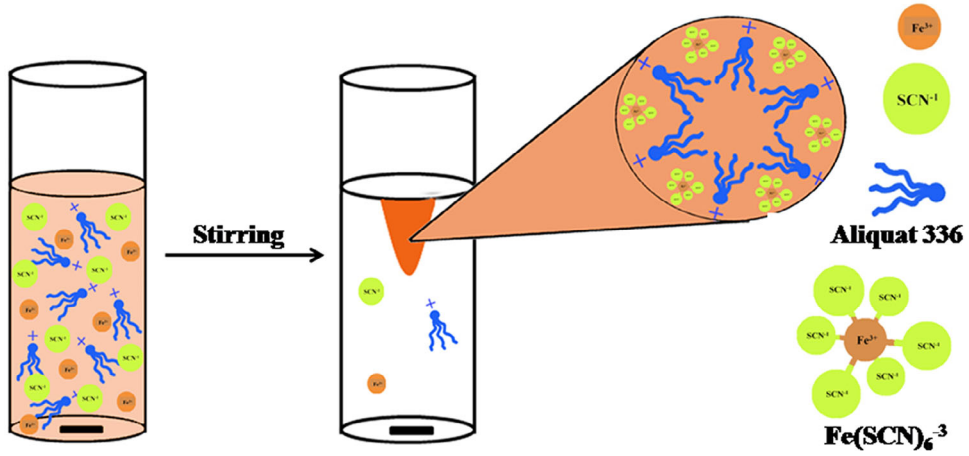

FIGURE 2 | The scheme indicating the underlying mechanism involved in the suspended droplet microextraction (SDME) method used for separation and preconcentration of Fe^{3+} ions from the serum samples.

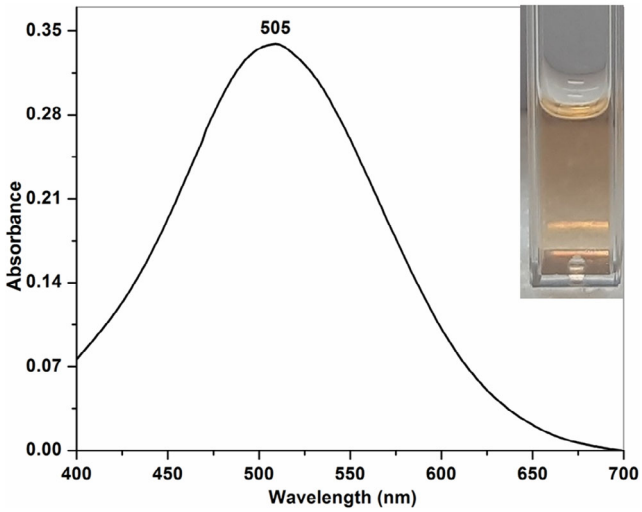

FIGURE 3 | The UV–vis absorption spectrum of the methanol solubilised suspended droplet of ferric thiocyanate complex indicating an absorption peak at 505 nm. Inset: corresponding solution colour.

out micelles are separated into a micro-droplet of the octanol organic layer hanging from the surface (Figure 2) [15]. The characteristic scarlet red-coloured viscous organic phase was pipetted out and homogenised in methanol. The absorption spectrum of the methanol solubilised suspended micro-droplet was recorded spectrophotometrically in the range of 400–700 nm. The solubilised complex showed an absorption peak at 505 nm. The ferric thiocyanate complexes are known for their typical absorption peaks in the range of 490–540 nm, depending upon the local environment (Figure 3) [37, 42]. The concentration of reagents such as HCl, NH_4SCN, Aliquat 336 and octanol on SDME was optimised by measuring the absorbance at 505 nm.

3.1 | Process Optimisation

The influence of HCl concentrations between 0.2% and 5% on the extractive separation of serum iron was studied at known concentrations of Fe^{3+} (400 ng/mL) by keeping the other parameters such as NH_4SCN (1%), Aliquat 336 (0.1%) and octanol (1000 µL) in a final volume of 10 mL (Figure 4a). The extraction increased with an increased concentration of acid from 0.2% to 1%, reached the maximum at 1.5% and then decreased with a further rise in HCl concentration to its maximum level of 5%. Thus, the optimum acid concentration was 1.5% and the results align with earlier studies [17, 36]. At an optimised HCl concentration of 1.5%, the serum iron extraction was followed by varying the NH_4SCN concentration (0.1%–3%) at Fe^{3+} (400 ng/mL), Aliquat 336 (0.1%) and octanol (1000 µL) in 10 mL final volume (Figure 4b). The extraction enhanced from 0.1%–0.5%, reached the highest value at 0.7% and reduced with a further rise in NH_4SCN concentration (1%–3%). From Figure 4b, it is evident that the optimal complexing agent concentration is 0.7%, which is in conformity with earlier studies [15, 36]. Further, the concentration of Aliquat 336 was optimised at HCl (1.5%), NH_4SCN (0.7%), Fe^{3+} (400 ng/mL) and octanol (1000 µL) by varying the Aliquat 336 concentration (0.01%–1%) (Figure 4c). The extraction capacity was enhanced from 0.02% to 0.1%, reached optimum value at 0.2% and decreased further from 0.3%–1% of Aliquat 336 concentrations. In addition, the extraction was studied at HCl (1.5%), NH_4SCN (0.7%), Aliquat 336 (0.2%) and Fe^{3+} (400 ng/mL) at variable volumes of extracting solvent, octanol (100–1000 µL) (Figure 4d). The extraction efficiency increased from 100 to 400 µL, reached an optimal value at 500 µL and further declined at elevated octanol concentrations. Based on the standardisation studies, the optimal conditions for serum iron extraction were found to be HCl (1.5%), NH_4SCN (0.7%), Aliquat 336 (0.2%) and octanol (500 µL).

3.2 | Stability of Ferric Thiocyanate Complex

The stability of the formed ferric thiocyanate complex at optimal conditions [HCl (1.5%), NH_4SCN (0.7%), Aliquat 336 (0.2%), octanol (500 µL)] was determined at 300 ng/mL of Fe^{3+} by recording the absorbance at different time intervals of 30 min for 2 h. The complex was stable for 90 min, beyond which the absorbance was found to decrease at the optimised conditions (Figure 5a). Thus, the stability period of 90 min is sufficient for the analyst to record the serum iron absorbance. Unlike the ferrozine

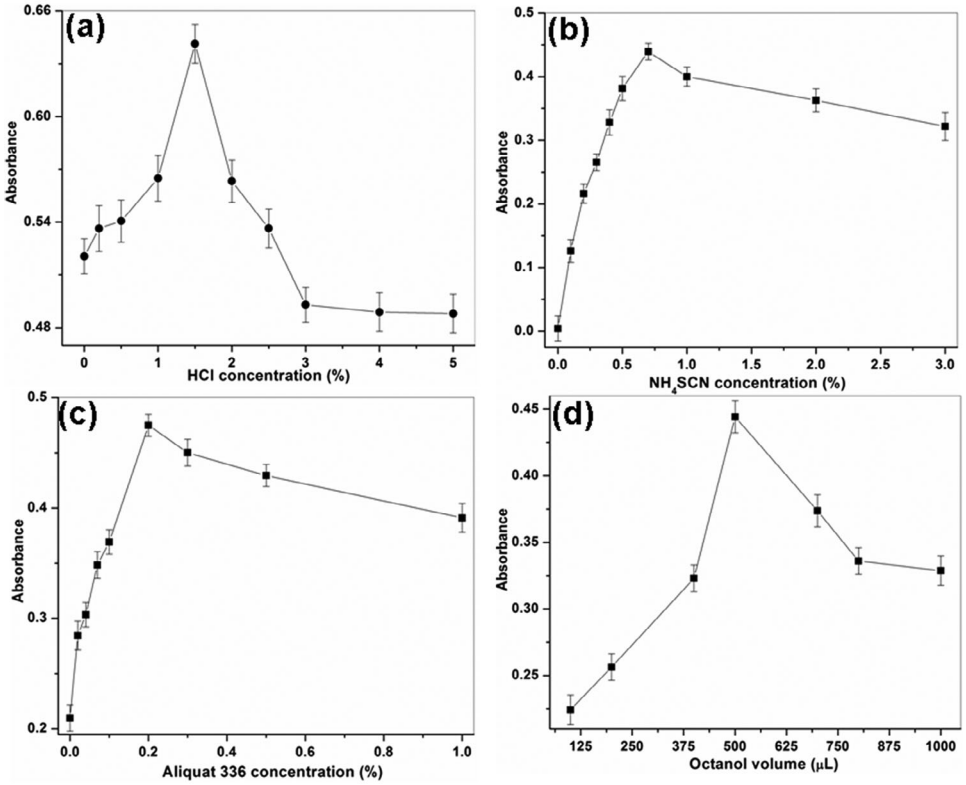

FIGURE 4 | The effect of different parameters on the suspended droplet microextraction of serum iron at variable concentrations of (a) HCl (conditions: 400 ng/mL Fe^{3+}, 1% NH_4SCN, 0.1% Aliquat 336, 1000 μL octanol), (b) NH_4SCN (conditions: 400 ng/mL Fe^{3+}, 1.5% HCl, 0.1% Aliquat 336, 1000 μL octanol), (c) Aliquat 336 (conditions: 400 ng/mL Fe^{3+}, 1.5% HCl, 0.7% NH_4SCN, 1000 μL octanol) and (d) octanol (conditions: 400 ng/mL Fe^{3+}, 1.5% HCl, 0.7% NH_4SCN, 0.2% Aliquat 336).

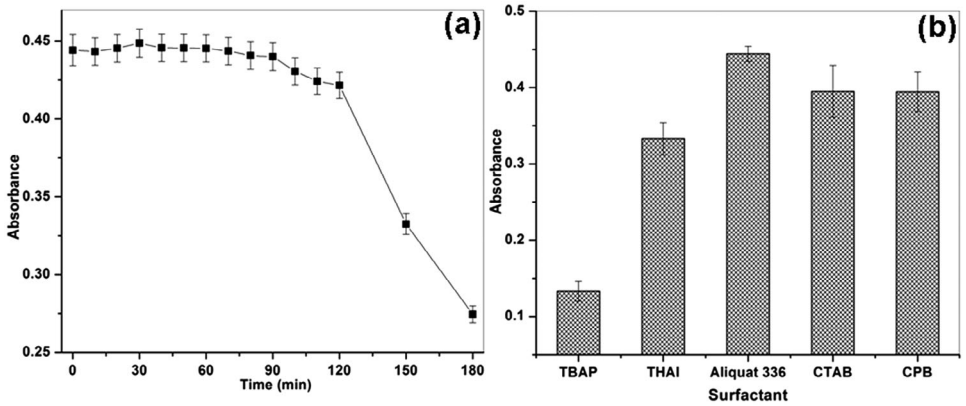

FIGURE 5 | (a) The stability of dissolved ferric thiocyanate complex, in terms of absorbance at 505 nm with time and (b) the effect of different quaternary ammonium salts (0.2%) on the suspended droplet microextraction of serum iron at 300 ng/mL Fe^{3+}, 1.5% HCl, 0.7% NH_4SCN and 500 μL octanol.

and ferene-S based spectrophotometric methods, there is no requirement of reducing environment to stabilise the formed iron complex, which an advantage in the developed SDME method [1–3, 18, 19, 22, 23, 25–28, 33]. However, further studies are needed on the effect of pre-analytical interferences such as sample collection time, sample handling, sample storage, etc. on the analytical performance of SDME towards serum iron quantification. Further, the stability of the ferric thiocyanate complex at 300 ng/mL of Fe^{3+} at optimal conditions was determined by varying the pH (1–14). It was found that beyond pH 1 the complex stability was found to decrease (Figure S1), thus confirming the standardised conditions of acidity for SDME. At standardised conditions and 300 ng/mL of Fe^{3+}, further experiments were done to see the role of different quaternary ammonium salts such as TBAP, THAI, CTAB and CPB on serum iron extraction in comparison with Aliquat 336 at 0.2% concentration (Figure 5b). Aliquat 336 was effective in serum iron extraction among all the salts, as it functions as a phase transfer catalyst, metal extraction agent and

TABLE 2 | Recoveries of Fe^{3+} from treated serum samples using the proposed SDME method ($n = 4$).

Sample	Spiked (ng/mL)	Found (ng/mL) (mean ± SD)	RSD (%)	Recovery (%)	$t_{calculated}$	$t_{critical}$
1	0	62.7 ± 2.5	—	—	—	—
	100	156.6 ± 7.9	5.0	96.2	1.37	3.18
	200	259.6 ± 9.3	3.6	98.8	0.52	3.18
	400	453.0 ± 8.7	1.9	97.9	2.07	3.18
2	0	598.2 ± 11.8	—	—	—	—
	100	687.1 ± 9.9	1.4	98.4	2.20	3.18
	200	784.1 ± 12.2	1.6	98.2	2.28	3.18
	400	980.3 ± 10.5	1.5	98.2	2.44	3.18

Abbreviation: SDME, suspended droplet microextraction.

a substitute for volatile organic solvents. The room temperature ionic liquid reagent is affordable, easier to synthesise and handle; and bestowed with characteristics such as superior chemical and thermal stability; low vapour pressure, non-flammability, recyclability, etc. [50].

3.3 | Selectivity, Reproducibility and Repeatability of the Developed Method

As the thiocyanate ion interacts with various metal ions (Cu^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+}) and forms thiocyanate complexes [35, 51], an interference study was done with treated, deproteinised serum samples to find out the selectivity of the ligand towards Fe^{3+} (400 ng/mL) at optimal conditions. The selected metal ions such as Ca^{2+}, Mg^{2+} (400 µg/mL); Cu^{2+}, Pb^{2+}, Zn^{2+}, Al^{3+} (50 µg/mL); Ni^{2+}, Co^{2+}, Bi^{3+}, Mn^{2+} (20 µg/mL) did not interfere with the serum Fe^{3+} quantification in terms of absorbance at 505 nm, even at elevated concentrations of µg/mL. It is significant to note that the interferences such as Cu, Co and Zn found in ferrozine and ferene-S based spectrophotometric methods [2, 18, 19] had no effect in the current protocol. These findings concur with earlier spectrophotometric methods on Fe^{3+} determination [15, 17, 34, 52]. The effect of various forms of iron (Fe^{2+}, Fe^{3+}) on the SDME method at optimal conditions was studied at variable pH (1–14) and concentration of 300 ng/mL. It was found the Fe^{2+} did not interfere in the extraction of Fe^{3+} across the pH range and the Fe^{2+} extraction was negligible at optimal conditions (Figure S2). Further, the effect of biological interferences (proteins, lipids, bilirubin) on the recovery for the proposed SDME method was studied utilising standard addition method by spiking the treated serum matrix with Fe^{3+} standards between 100 and 400 ng/mL concentration levels [53–55]. The obtained recoveries are reasonable and ranged from 96.2%-98.8% with relative standard deviation (RSD) (%) values varying from 1.4% to 5.0% ($n = 4$). Thus, the results of the Student t-test indicated no biological matrix effect on the recovery of Fe^{3+} in the developed procedure with excellent accuracy with no significant systematic error, confirmed from the smaller $t_{calculated}$ values compared to $t_{critical}$ value (3.18) at 95% confidence level (Table 2). It is important to note that the utilised iron extraction and precipitation reagent (HCl + methanol + chloroform) effectively removes the proteins and lipids from the serum [3, 23] and the residual bilirubin in the treated serum had no effect on iron quantification. The current method is free from metal and biological interferences, thus paving a way for clinical serum analysis.

The reproducibility of the method was assessed by quantifying the iron concentrations in five independently aliquoted serum solutions and the RSD (%) values ranged from 1.2% to 4.3%. At confidence of 95%, the obtained $t_{calculated}$ values were smaller than $t_{critical}$ value (3.18), thus reflecting the satisfactory reproducibility (Table S1). The repeatability of the method was evaluated by intraday (5 times) and interday (5 days) measurements [56–59]. The RSD (%) values ranged from 1.3% to 2.9% for intraday and the acquired $t_{calculated}$ values were smaller than $t_{critical}$ value (3.18) at significance level of $p = 0.05$ conforming the adequate repeatability (Table S2). Also, the acceptable repeatability at interday measurements was confirmed from the RSD (%) range (1.5%–3.5%) and achieved lower $t_{calculated}$ values than $t_{critical}$ value (3.18) at 95% confidence (Table S3). It is important to note that the RSD (%) values calculated for intraday measurements were lower than that of interday measurements. Thus, the robustness of the developed method was demonstrated through the reproducibility and repeatability studies [53–59].

3.4 | Analytical Figures of Merit

The calibration plot achieved with spectrophotometry by extracting and preconcentrating the treated serum samples spiked with Fe^{3+} ions was linear between 20 and 1000 ng/mL. The correlation coefficient and limit of detection (LOD) values were 0.997 and 2.4 ng/mL, respectively (Figure 6). The measurement of Fe^{3+} in different treated bovine serum samples was achieved from standard addition calibration graphs. The Fe^{3+} concentration values achieved for various bovine serum samples ranged between 62.7 and 1582.5 ng/mL and the obtained RSD (%) ranged from 0.8% to 3.9% ($n = 4$). The accuracy of the developed procedure was validated with the results obtained by the determination of Fe^{3+} in the treated serum matrix with ET-AAS, which serves as a reference method [3, 14] and the obtained RSD (%) ranged from 0.8% to 2.9% ($n = 4$). Also,

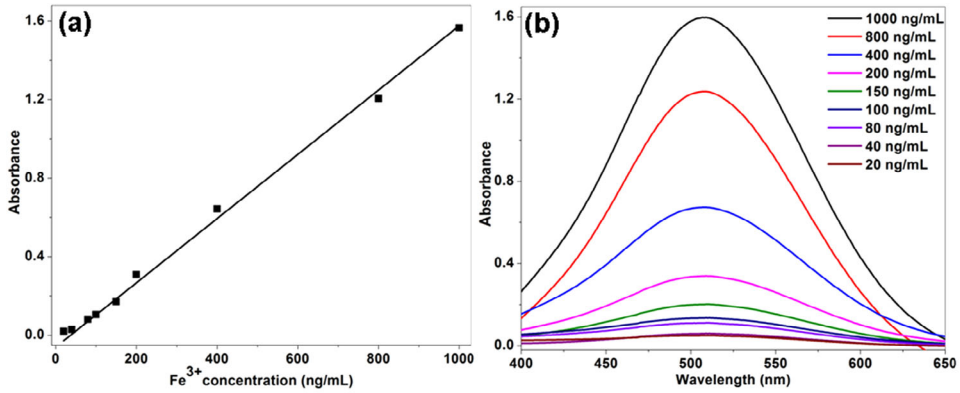

FIGURE 6 | (a) The calibration plot in SDME method showing the absorbance at 505 nm vs. Fe^{3+} concentration in serum and (b) corresponding absorption spectra. SDME, suspended droplet microextraction.

TABLE 3 | Quantification of Fe^{3+} in different treated serum samples based on the developed SDME method and ET-AAS ($n = 4$, Student t-test at 99% confidence level).

Sample	SDME (ng/mL) (mean ± SD)	RSD (%)	ET-AAS (ng/mL) (mean ± SD)	RSD (%)	$t_{calculated}$	$t_{critical}$
1	62.7 ± 2.5	3.9	57.3 ± 1.7	2.9	2.91	3.74
2	598.2 ± 11.8	1.9	567.1 ± 9.6	1.6	3.33	3.74
3	601.2 ± 5.3	0.8	587.7 ± 8.4	1.4	2.21	3.74
4	1409.9 ± 17.4	1.2	1395.1 ± 12.3	0.8	1.13	3.74
5	1582.5 ± 21.6	1.3	1522.5 ± 17.9	1.1	3.49	3.74

Abbreviations: SDME, suspended droplet microextraction; ET-AAS, electrothermal atomic absorption spectrometry.

a statistical comparison was made to validate the agreement between the proposed SDME method and the reference ET-AAS technique. It was noted that the acquired $t_{calculated}$ values were smaller than $t_{critical}$ value (3.74) at 99% confidence significance level ($n = 4$) (Table 3). Thus, the spectrophotometric results concur with the results obtained from the ET-AAS according to the student t-test. The varying level of serum iron concentrations in fetal bovine serum samples is reported in different batches and manufacturers [11].

According to the guidelines of ICSH and CLSI, the serum iron levels can be categorised as Fe overload (>2000 ng/mL), Fe normal (500–1500 ng/mL), Fe depletion (<150–500 ng/mL), Fe-deficient erythropoiesis (< 150 ng/mL) and Fe-deficient anaemia (<100 ng/mL) [40]. The developed SDME method was compared with earlier reported spectrophotometric methods for iron determination in terms of detectable iron form, chromogen, matrix, complex colour, λ_{max}, linear detection range and LOD (Table 4).

In the current study, a linear detection level of 20–1000 ng/mL with an LOD of 2.4 ng/mL was observed for serum samples, which is much superior to almost all the reported spectrophotometric methods. For serum iron determination using ICSH and CLSI recommended ferrozine and ferene-S based detection methods or kits, the Fe^{3+} ions should be reduced to Fe^{2+} employing reducing agents such as ascorbic acid, hydroxylamine hydrochloride, hydroquinone, hydrazine sulphate, sulphite, dithionite, thioglycolic acid, mercaptoacetate, etc. and maintenance of reducing environment is needed [1–3, 18, 19, 22, 23, 25–28, 33]. There is no requirement for a reduction step in the current protocol and the NH_4SCN is much cheaper than ferrozine and ferene-S in terms of cost. Also, highly water-soluble ferrozine complex is challenging to preconcentrate [19]. Standard methods require a serum sample volume of 0.5–0.8 mL for analysis [13, 27, 40] and our method needs only 0.1 mL of sample. Low sample volume is always a gain, as the blood collected from patients in a single shot is simultaneously utilised for multiple pathological and diagnostic tests in laboratories. Especially with paediatric and animal experimentation samples [62] and where the sample collection is an arduous task for mentally challenged children.

The methods based on analytical stripes [37], multi-syringe flow injection systems based on chelating disks [36], smart phone-based devices [17] and paper-based electrochemical sensors [5] offer Fe^{3+} detection at a higher concentration range such as 5–100, 0.1–20, 0.5–10 and 0.4–10 μg/mL, respectively. Also, the methodologies based on multi-step, sophisticated flow injection systems require either the analyte's online oxidation or reduction step [17, 33, 42]. The methods based on separation/extraction and preconcentration of Fe^{3+} ions demand either a specialised spectrophotometer (NanoDrop) [15], long waveguide capillary cell with a preconcentration resin [29], expensive preconcentration resins [29, 35], or custom-synthesised sorbents [34]. Most of the methods are tailored for large volume samples such as

TABLE 4 | Comparison of the developed SDME method with other reported spectrophotometric methods for iron determination.

Detectable iron form	Chromogen	Matrix	Complex colour	λ_{max} (nm)	Linear detection range (ng/mL)	LOD (ng/mL)	Refs.
Fe^{2+}	1, 10-phenanthroline	Different waters	Orange red	510	500–5000	500	[60]
Fe^{2+}	Bathophenanthroline	Freshwater	Red	535	100–8000	800	[18]
Fe^{2+}	TPTZ	Freshwater	Blue	595	100–10,000	20	[18]
Fe^{2+}	Ferrozine	Human serum	Violet	562	100–5000	120	[61]
Fe^{2+}	Ferene-S	Human serum	Blue	595	150–10,000	150	[61]
Fe^{3+}	NH_4SCN	Tap water	Red	540	5000–100,000	—	[37]
Fe^{3+}	NH_4SCN	Seawater	Red	469	100–20,000	200	[36]
Fe^{3+}	NH_4SCN	Bioethanol	Red	480	500–10,000	100	[17]
Fe^{3+}	NH_4SCN	Human blood, milk, pulses, urine, rainwater, groundwater	Red orange	477	50–6000	5	[15]
Fe^{3+}	NH_4SCN	Soil	Red	473	50–3000	—	[52]
Fe^{3+}	NH_4SCN	Rainwater	Red orange	490	30–3000	8	[42]
Fe^{3+}	NH_4SCN	Bovine serum	Scarlet red	505	20–1000	2.4	Current study
Fe^{3+}	NH_4SCN	Natural water, wine	Red	480	10–400	10	[35]
Fe^{3+}	NH_4SCN	River water	Red	480	1–50	0.5	[29]

Abbreviations: LOD, limit of detection; SDME, suspended droplet microextraction.

rainwater [42], seawater [34], natural water [35], river water [29] and bioethanol [17] matrices.

The existing spectrophotometric Fe^{2+} detection techniques quantify at a higher concentration range of μg/mL such as 0.1–5 [61], 0.5–5 [60], 0.1–8 [18], 0.1–10 [18] and 0.15–10 μg/mL [61], respectively with corresponding high LOD of 0.12, 0.5, 0.8, 0.02 and 0.15 μg/mL. The reported Fe^{3+} spectrophotometric detection techniques based on the chromogen NH_4SCN determine at a higher concentration range of 0.03–3 [42], 0.05–6 [15], 0.5–10 [17] and 0.1–20 μg/mL [36], respectively with corresponding high LOD of 0.08, 0.05, 0.1 and 0.2 μg/mL. The system developed in the current study determines Fe^{3+} in serum samples at a sensibly good detection range of 20–1000 ng/mL with an LOD of 2.4 ng/mL. Compared to the other existing spectrophotometric techniques with higher concentration detection levels and higher LOD, the devised method offers substantial improvement in terms of lower linear range (20–1000 ng/mL) and lower LOD values (2.4 ng/mL). It is advantageous in terms of no sample reduction step, low cost complexing agent, low sample volume, metal and biological interference free, simplicity and selectivity. The developed methodology can be utilised to quantify iron in other biological fluids and water samples.

4 | Conclusions

An SDME procedure was devised for the spectrophotometric quantification of serum iron in the linear concentration range of 20–1000 ng/mL of without the need of complex and laborious sample preparation, iron reduction step, high cost instrumentation and analyst expertise [14, 15, 17]. Thus, the developed method can serve as a simple, low cost, metal and biological interference free, selective, low sample volume based alternative for the routine spectrophotometric analysis of serum iron and other biological fluids, especially in resource constrained, small rural hospitals, dispensaries, clinics and diagnostic centres. However, the amenability and feasibility of the reported method towards automation and its robustness in real-world clinical settings should be explored as routinely various samples are analysed for serum iron and other clinical parameters employing high throughput automated biochemical analysers [2, 4, 9, 16, 63] in clinical biochemistry laboratories. Further studies are needed on deployment of certified reference material (CRM) for serum iron for improving analytical performance; pre analytical interferences such as sample collection time, sample handling, sample storage, haemolysis, ethylenediaminetetraacetic acid (EDTA) contamination; multiplexing capabilities of spectrophotometric assays integrating multi analyte platforms in a single measurement, integration with artificial intelligence for data analysis and interpretation, etc.

Author Contributions

Aruna Jyothi Kora: Conceptualisation, methodology, resources, project administration, investigation, data curation, validation, writing–original draft preparation, reviewing and editing. **K. Madhavi**: Conceptualisation, methodology, investigation, formal analysis, data curation, validation, writing–reviewing and editing. **N. N. Meeravali**: Conceptualisation, writing–reviewing and editing.

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Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

Data generated were described and used in the paper.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.