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Spectrophotometric Titration of Resorufin and Resazurin with Iron(II) in Strong Phosphoric Acid Medium and Resorufin as a New Redox Indicator in the Reductimetric Titration of Some Metal Ions

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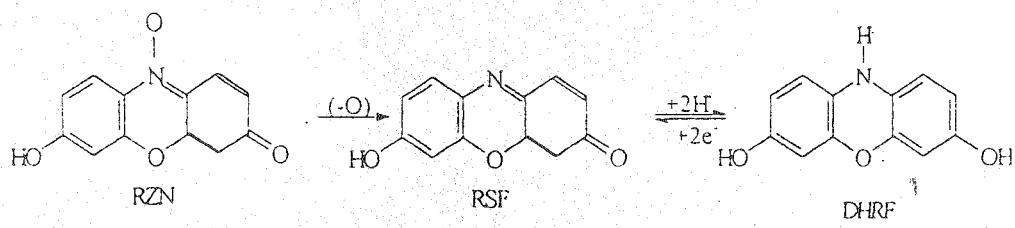
Spectrophotometric titration methods have been developed for the determination of micro-gram quantities of resazurin and resorufin and the analysis of their binary mixture using iron(II) as a reductant in high concentration of orthophosphoric acid (10.5 M) medium. Both resazurin and resorufin are reduced to a blue-green semiquinone (λ_{max} 640 nm) with iron(II) in three electrons and one electron reduction steps respectively. Resorufin has been proposed as a new advantageous redox indicator in the determination of V(V), V(IV), Mo(VI), Cr(VI), Cu(II), Sb(V), As(V), Os(VIII) and Ir(IV) in phosphoric acid medium using the same reductant.

Key Words: Spectrophotometric, Resorufin, Resazurin, Iron(II), Phosphoric acid.

INTRODUCTION

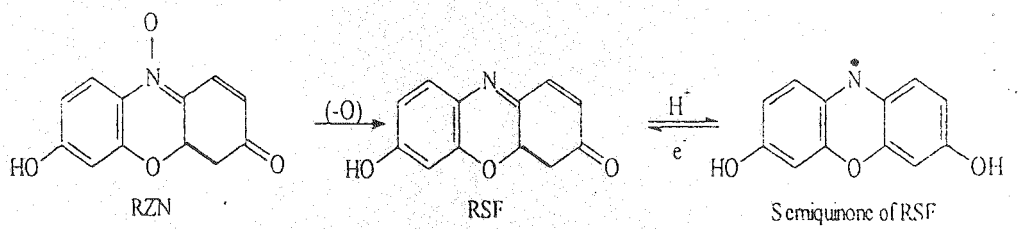
Resazurin (RZN) and resorufin (RSF) are very interesting oxazine groups of dyes and find use in testing the bacterial content of milk[1, 2], sterility tests[3] and also as redox indicators[4–7]. RSF has been employed for the detection of certain organic compounds[8]. Earlier methods for their determination involve the use of only a few reductants like potassium iodide[9], ascorbic acid[10] and titanium(III)[11] and iron(II) in presence of oxalate[12] and pyrophosphate[13]. These methods suffer from several disadvantages. For example, titanium(III) solution is highly susceptible to aerial oxidation and hence requires a special apparatus to prevent oxidation. Titration of the compounds with ascorbic acid must be carried out at higher temperatures. The iodide method of Twigg[9] is tedious and time consuming, since the iodine liberated by treating RZN or RSF with iodide must be extracted with carbon tetrachloride and determined by an iodate method. In one of the iron(II) titration methods[12], the reduction of the compounds is feasible in a narrow pH range, while in the other[13] only RSF can be determined.

As reported by Twigg[9] and confirmed by other investigators[9–12] the reduction of RZN takes place in two stages, first to pink RSF and then to the colourless dihydroresorufin (DHRF) according to the reaction (Eqn. 1).



Equation 1

The first stage of reaction being irreversible, the second stage was reported[9] to be reversible at an inert electrode. However, these authors[9–11] also reported that in strongly acid solution, RZN is reduced to a semiquinone in a 3 electron reduction step as per the equation (Eqn. 2.)



Equation 2

The present paper describes more convenient methods for the determination of microgram quantities of RSF and RZN and analysis of RSF and RZN mixtures spectrophotometrically using iron(II) as a reductont in phosphoric acid medium. The present study enabled the authors to propose RSF as a new advantageous redox indicator in the titration of some metal ions [V(V), V(IV), Mo(VI), Cr(VI), Cu(II), Sb(V), As(V), Os(VIII) and Ir(IV)] with iron(II) in phosphoric acid medium and for analysis of copper in brass.

EXPERIMENTAL

All chemicals were of analytical reagent grade unless otherwise stated and all solutions were prepared in distilled water.

Iron(II) solution: Stock solution of approximately 0.05 M iron(II) in 0.5 M sulphuric acid was prepared from ammonium iron(II) sulphate hexahydrate and standardized[14]. From this stock solution, iron(II) solutions of desired strengths were prepared for use in spectrophotometric as well as in visual titrations.

Titanium(III) chloride solution: An approximately 0.01 M solution of titanium(III) chloride was prepared in 2 M hydrochloric acid and standardized against a standard solution of dichromate[15].

RSF and RZN solutions: Solutions (50 mg/100 mL) of RSF (Aldrich, USA) in water and RZN (Gurr and Co., England) in 0.005 M sodium hydroxide (because of its partial solubility in water) were prepared. The dye solutions were standardized with titanium(III) chloride as described by Ruzicka[11]. From these standard dye solutions a 50×10^{-5} M solution of RSF (for spectrophotometric titrations) and a 6.0×10^{-5} M solution of RZN (for spectrophotometric determination) were prepared.

Metal ion solutions: Approximately 0.05 M solutions of vanadium(IV)[16] (by reduction of vanadium(V) solution with sulphur dioxide); molybdenum(VI)[17] (from molybdic acid); copper(II)[14] (from copper(II) sulphate pentahydrate);

cerium(IV)[18] (from ammonium cerium(IV) sulphate dihydrate in 0.5 M sulphuric acid) were prepared and standardized[16–18]. Approximately 0.025 M solution of vanadium(V)[19] (from NH_4VO_3); antimony(V)[20] (from $Na_2H_2Sb_2O_7{\cdot}4H_2O$ in 1 + 1 HCl medium); arsenic(V)[21] (from $Na_2HAsO_4{\cdot}7H_2O$) were prepared and standardized[19–21]. Approximately 0.01 M solution of iridium(IV)[22] (from sodium hexachloroiridate(IV) hexahydrate) and 0.0025 M solution of osmium(VIII)[22] (from osmium tetroxide) were prepared and standardized[22]. A 0.05 N solution of Cr(VI) was prepared by dissolving an accurately weighed amount of AnalaR potassium dichromate in distilled water.

Reduced products (semiquinones) of RSF and RZN: The reduced products of both RSF and RZN were prepared in 50 mL standard flasks by taking a known aliquot (10 mL) of RSF or RZN solution (each 2.0×10^{-4} M) in a medium containing 10.5 M phosphoric acid, adding 20-fold ratio of iron(II) (4 mL of 0.01 M) and finally diluting to the mark.

Apparatus: Shimadzu double beam spectrophotometer (UV 140.02) with optically matched glass cell of 1 cm path length was used to record the absorption spectra. Glass titration cell of dimension $3 \times 5 \times 6$ cm (3 cm path length) was employed for the spectrophotometric titrations. The cell compartment of the spectrophotometer was modified[13] as described earlier for titration work, since there is no provision in the spectrophotometer for such titrations.

Recommended procedures

A. Determination of RSF: To 5–15 mL of 5.0×10^{-5} M RSF solution taken in the titration cell, 35 mL of orthophosphoric acid are added and diluted to 50 mL (10.5 M). The titration cell is placed in the position of the spectrophotometer and the wavelength set at 510 or at 640 nm (adjusted to zero absorbance with respect to a black). The solution is homogenized by the passage of a rapid stream of purified carbon dioxide gas for about 1 min and the absorbance noted. Iron(II) (2.5×10^{-4} M) solution is added in increments, stirring the solution by passage of purified carbon dioxide gas for about 30 s for each addition and noting the absorbance after stopping the stirring (the carbon dioxide provides an inert atmosphere throughout the titration). The absorbance *vs.* volume of the titrant added is plotted and the point of intersection of the extrapolated lines corresponds to the equivalence point. Some typical results obtained are shown in Table-1.

TABLE-1
SPECTROPHOTOMETRIC TITRATION OF RESORUFIN AND RESAZURIN WITH IRON(II)

Resorufin found (μg)			Resozurin found (μg)		
Reference method[9]	Proposed* method	R.S.D. (%)	Reference method[9]	Proposed* method	R.S.D. (%)
61.36	61.72	0.45	64.05	64.39	0.50
86.14	85.12	0.42	90.43	90.69	0.48
102.66	103.48	0.38	106.76	107.25	0.37
129.80	130.53	0.30	114.29	113.63	0.32
142.78	143.66	0.35	144.44	145.46	0.35
177.00	178.32	0.26	185.88	186.88	0.28

*Average of six determinations

B. Spectrophotometric Determination of RZN: To 5–15 mL of RZN solution [6.0×10^{-5} M] taken in the titration cell, 35 mL of orthophosphoric acid, 3 mL of iron(II) [5.0×10^{-3} M] are added and the solution diluted to 50 mL. The solution is stirred for about 40 s, the stirring is stopped and the absorbance noted at 640 nm. The amount of RZN (or the equivalent semiquinone) was then computed from the calibration curve drawn (for 5–15 mL of RZN solution) under identical conditions. Some typical results obtained are shown in Table-1.

C. Analysis of RZN-RSF mixtures: Total concentration of RZN and RSF in the mixture should not exceed 7.5×10^{-5} M when the solution is diluted to 50 mL. A 10 mL portion of the mixture is titrated with iron(II) (2.5×10^{-4} M) spectrophotometrically in phosphoric acid medium (final concentration 10.5 M) adopting the procedure described for the determination of RSF (procedure A). This titre gives the amount of RSF present in the mixture. The author found that the reduction of RZN by iron(II) in phosphoric acid medium takes place in presence of a large excess of iron(II); hence, RZN does not interfere (the details are furnished under discussion part). Another 10 mL aliquot taken in phosphoric acid medium (final concentration 10.5 M) is treated with 3 mL of iron(II) (5.0×10^{-3} M) under the identical conditions as given for the determination of RZN (procedure-B) (both RZN and RSF are reduced to the semiquinone). The absorbance is measured at 640 mm and compared with the calibration curve drawn (procedure-B) and the total semiquinone equivalent to the total RSF and RZN present in the mixture is computed. Upon subtracting the amount of RSF from the total RZN and RSF, the RZN amount can be computed. Some of the representative results obtained are shown in Table-2.

TABLE-2
DETERMINATION OF RSF AND RZN IN A BINARY MIXTURE

Resorufin (mg) (present in 100 mL) Found (μg)			Resozurin (mg) (present in 100 mL) Found (μg)		
Taken (mg)	Found * (mg)	R.S.D. (%)	Taken (mg)	Found* (mg)	R.S.D. (%)
0.564	0.569	0.57	1.255	1.264	0.52
0.705	0.699	0.52	0.904	0.911	0.50
0.846	0.839	0.49	0.753	0.760	0.49
1.175	1.184	0.32	0.602	0.608	0.35

*Average of 4 determinations

D. Determination of some metal ions in phosphoric acid medium using RSF as a redox indicator: To 2–10 mL of a metal ion solution, (V(V), V(IV), Cr(VI), Mo(VI), Os(VIII), Ir(IV), As(V), Sb(V), Ce(IV), Cu(II)) are added enough orthophosphoric acid and other reagents (Table-4) to give the required final strength near the equivalence point. 0.3 mL of 0.05% (m/v) RSF solution is added and the reaction mixture titrated against 0.05 M iron(II) solution (0.01 M in the case of Os(VIII) and Ir(IV)), while strring the solution by means of a magnetic

stirrer, to the colour transition from red to blue-green. The optimum conditions under which the metal ions are titrated and some typical results obtained by the procedure are shown in Table-4. The osmium(VIII) solution is taken in phosphoric acid cooled in ice water and titrated at 20–25°C because of risk of loss by volatilization[23] at the laboratory temperature (≥ 28°C). The chloride and bromide ions in the determination of As(V) and Sb(V) are found to catalyze the reactions presumably through the formation of a bridged complex[24, 25] involving the halide ion.

F. Analysis of copper in Brass: An accurately weighed sample of the brass in the range 1.0–1.5 g and in the form of drillings is dissolved in 20–25 mL of nitric acid (1 + 1) then 10–15 mL of concentrated sulphuric acid are added and then the mixture is evaporated to fumes. The solution is then cooled, diluted to about 70 mL and filtered. A few mL of bromine water are added to the combined filtrate and washings and the excess bromine boiled off. The solution is cooled, diluted to volume in a 100 mL standard flask and a fraction is analyzed as already described above.. The copper content obtained by the present method (60.15% ± 0.14) (average ± S.D of 6 determinations) is in excellent agreement with that obtained by the standard method[26] (59.8%).

TABLE-3
FORMAL REDOX POTENTIALS OF (RSF)/(SEMIQUINONE) COUPLE AND Fe(III)/Fe(II) COUPLE IN A MEDIUM CONTAINING DIFFERENT CONCENTRATIONS OF PHOSPHORIC ACID (AT 280.1°C)

Concentration of phosphoric acid (M)	Redox potentials (mv ± 5 mV)		
	(RSF)/(Semiquinone)	Fe(III)/Fe(II)	Difference in potential
9.0 M	730	429	311
10.5 M	750	410	340
12.0 M	765	400	365

TABLE-4
DETERMINATION OF METAL IONS WITH IRON(II) USING RSF AS AN INDICATOR

Metal Ion	Optimum H_3PO_4 concentration, M and conditional potential of Fe(III)/ Fe(II) couple	Taken (mg)	Found (mg)	R.S.D (% mg)	Metal ion couple and its condition potential, V (± 5 mv)	Transition potential of RSF, V (± 5 mv)
Vanadium(V)	10–11 M	2.55	2.56	0.40	V(V)/V(III)†	0.763
	0.405 V	6.37	6.41	0.35		
		8.92	8.88	0.32		
		12.75	12.80	0.25		
Vanadium(IV)	10–11 M	5.10	5.13	0.42	V(IV)/V(III)	0.572
	0.405 V	12.75	12.68	0.40	0.700	
		17.85	17.93	0.38		
		25.05	25.11	0.27		

Metal Ion	Optimum H_3PO_4 concentration, M and conditional potential of Fe(III)/ Fe(II) couple	Taken (mg)	Found (mg)	R.S.D (% mg)	Metal ion couple and its condition potential, V (± 5 mv)	Transition potential of RSF, V (± 5 mv)
Chromium(VI)	6–7 M	0.86	0.85	0.39	Cr(VI)/Cr(III)	0.823
	0.432 V	2.16	2.17	0.35	1.255	
		3.02	3.04	0.30		
		4.32	4.33	0.29		
Molybdenum(VI)	11–12M	9.60	9.65	0.45	Mo(VI)/Mo(V)	0.530
	0.388V	24.00	24.11	0.40	0.645	
		33.60	33.70	0.37		
		48.00	47.83	0.30		
Osmium(VIII)	9–10 M	2.37	2.36	0.40	Os(VIII)/Os(IV)	0.654
	Temp. 20–25°C	3.32	3.34	0.38	0.916	
	0.412V	4.57	4.76	0.36		
Iridium(IV)	9–10 M	3.84	3.86	0.50	Ir(IV)/Ir(III)	0.670
	0.412V	9.60	9.55	0.47	0.888	
		13.44	13.50	0.38		
		19.20	19.10	0.29		
Arsenic(V)	10–11 M*	3.75	3.72	0.49	As(V)/As(III)	0.578
	1.5 M HBr	9.37	9.40	0.45	0.782	
	0.412 V	13.12	13.05	0.42		
		18.75	18.68	0.32		
Antimony(V)	10–11 M†*	6.10	6.07	0.45	Sb(V)/Sb(III)	0.560
	2M HCl or	15.25	15.15	0.41	0.748	
	1.0 M HBr	21.35	21.44	0.37		
	0.405 V	30.50	40.50	0.21		
Cerium(IV)	7–8 M	14.00	13.90	0.39	Ce(IV)/Ce(III)	0.845
	0.427 V	35.00	35.20	0.32	1.244	
		49.00	49.22	0.27		
		70.00	70.24	0.21		
Copper(II)	9.10 M	6.30	6.25	0.43	Cu(II)/Cu(I)	0.580
	0.005–0.01M	15.75	15.66	0.30	0.785	
	KCNS	21.95	21.95	0.32		
	0.412V	31.10	31.05	0.23		

*Average of six determinations †could not be measured accurately

RESULT AND DISCUSSION

Absorption Spectra of RZN and RSF: The absorption spectra of RZN, RSF and their reduced products (which is nothing but a semiquinone), iron(II) and iron(III) were recorded in media of varying phosphoric acid concentrations ranging from 9 M (60% v/v) to 12 M (80% v/v) over the range 400–800 nm. The iron solutions have negligible absorbance in the visible region. The absorption spectra of RZN and RSF and their reduced products (or semiquinones) were found to be independent of phosphoric acid concentration in the range 9–12 M. The spectra of semiquinones formed by the reduction of RZN and RSF (each 4.0 ×

10^{-5} M) after final dilution are found to be almost identical overlapping each other. Hence, the spectrum of only one semiquinone along with those of RZN and RSF are shown in Fig. 1.

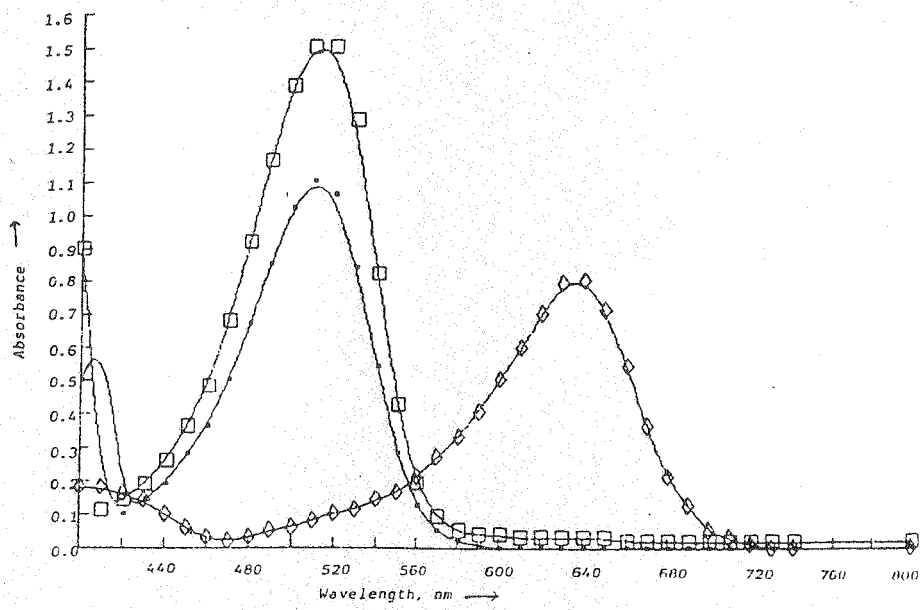


Fig. 1. Absorption spectra of RSF, reduced RSF and RZN

Beer's law and stability of RSF and RZN solution: RSF and RZN solutions obey Beer's law under the recommended titration conditions over the range 1.28–3.72 μg/mL and 1.25–3.54 μg/mL respectively. These dye solutions were found to be stable (studied spectrophotometrically) up to one month in 10.5 M phosphoric acid medium whereas the semiquinone of the dyes was found to be stable towards aerial oxidation up to 6 min (when no excess iron(II) is present). However, in the presence of 20-fold ratio of iron(II) it (semiquinone) is found to be stable for 6 h towards the atmospheric oxygen.

Effect of phosphoric acid concentration: The concentration of phosphoric acid must be 10.0 M or above for a satisfactory titration of both RSF and RZN with iron(II), but while the reduction of RSF with iron(II) is rapid, the rate of reduction of RZN has been found to depend on the concentration of iron(II). Preliminary investigations revealed that RZN does not undergo reduction in 9–12 M phosphoric acid medium even in the presence of 5-fold ratio of iron(II) and even after waiting for longer periods. The reduction of RZN to its semiquinone starts only in the presence of 7-fold ratio of iron(II). However, for a rapid and quantitative reduction of RZN, a 15-fold ratio (mole ratio) of iron(II) in reaction medium (9–12 M H_3PO_4) is found necessary. This difference in behaviour of RZN and RSF with iron(II) in phosphoric acid medium enabled the authors for the analysis of binary RSF-RZN mixtures. From the spectrophotometric titration of RSF with iron(II) it was found that the former is reduced to its semiquinone form in one electron reduction step (mole ratio of RSF to iron(II) found to be 1 : 1). Such a direct evidence could not be obtained in the case of RZN, since, as already stated, the reduction of RZN requires a higher concentration (at least 15-fold excess) of iron(II). However, since the absorption spectra of

the reduction products of RSF and RZN are found to be identical, we concluded that RZN is reduced to its semiquinone form in a 3-electron step according to eqn. (2). Formation of the stable semiquinone from RZN (3 electron reduction step) and RSF (one electron reduction step) in strongly acid solutions was reported by Twigg[9] and Ruzicka *et al.*[11] Michaelis *et al.*[27, 28] reported the formation of stable semiquinones during the reduction of some oxazine and thiazine groups of dyes in strongly acid solutions. All indirect methods (spectrophotometric, potentiometric or visual) to estimate RZN by addition of a known excess of iron(II) and back titrating the excess with oxidants like V(V), Ce(IV) or Cr(VI) did not succeed.

Formal Potentials of Redox Systems: Gopal Rao and Sagi[29] reported that the formal redox potential of iron(III)/iron(II) couple decreases form 684 mV to 400 mV as the concentration of phosphoric acid increases from about 1.00 to 12.00 M. The conditional redox potentials of (RSF)/(semiquinone) couple at different phosphoric acid concentrations in the range 9–12 M were determined by measuring the potential of the system that had been exactly 50% titrated with iron(II) under a carbon dioxide atmosphere and are shown in Table-3. The redox potentials of the (RZN)/(semiquinone) couple could not be measured in the same way, because as already stated RZN requires a 15-fold ratio of iron(II) for its rapid reduction. The redox potentials[29] of iron(III)/iron(II) couple reported at different phosphoric acid concentrations in the range 9–12 M have also been shown in the same table. From these potential data it may be seen that there is enough difference between the redox potential of the (RSF)/(semiquinone) couple and iron(III)/iron(II) couple to allow titration of RSF with iron(II).

In the present communication, the metal ions [V(V), V(IV), Cr(VI), Cu(II), Mo(VI), Os(VIII), Ir(IV), As(V), Sb(V) and Ce(IV)] which are titrated against iron(II) in high phosphoric acid medium and using resorufin as an advantageous redox indicator are rapidly and quantitatively reduced to their corresponding lower oxidation states [V(III), V(III), Cr(III), Cu(I), Mo(V), Os(IV), Ir(III), As(III), Sb(III) and Ce(III) respectively]. In all the titrations, the colour transition of the indicator (resorufin), from red to blue-green is sharp and reversible at the equivalence point which corresponds to the reduction of RSF to its semiquinone form as elucidated above (Eqn. 2). The redox potentials of the reductant system (iron system)[29] and each oxidant system (metal ion system) under the optimum conditions of the titration as reported by the earlier authors[23, 24, 30–35] are presented in Table-4. Since the performance of a redox indicator can be better judged from its transition potential than from its redox potential, the transition potential of RSF was determined as described by Belcher *et al.*[36] during the titration of each metal ion and presented in Table-4. The transition potential of RSF observed in each redox system has been found to range between the redox potential of the corresponding oxidant and reductant (iron) systems, indicating that RSF may function as a satisfactory redox indicator in the present titrimetric procedures. The redox methods so reported for the determination of these metal ions are abundant in literature and beyond the scope of the paper to cite. Nevertheless, a review of the methods can be found in the text books[14, 18, 22, 37] as well as in some of our publications[16, 17, 23, 24, 30, 33–35, 38–45]. In recent times most of the metal ions

are determined spectrophotometrically[46, 47]. However, reductimetric titration methods for the determination of the metal ions with iron(II) in phosphoric acid medium employing thiazine[23, 24, 33, 34, 41, 42] and oxazine[38–40] group of dyes and cacotheline[43–45] as redox indicators were reported from our laboratories.

The advantages associated with the use of RSF as a redox indicator over the above mentioned redox indicators used earlier[23, 24, 33, 34, 38–45] in these methods may be summarized as follows:

1. No inert atmosphere need be maintained during the titrations as in the case of oxazine and thaizine dyes (to prevent the aerial oxidation of the leuco-dyes obtained at the end-point), since the semiquinone (obtained at the end-point) is stable to oxygen of the atmosphere for about 5–6 min, visually.
2. The colour transition of the indicator being from red to blue-green can be easily detected compared to those of the other oxazines (blue or red to colourless) and thiazines (blue to colourless).
3. In some of these determinations[43–45] the indicator solutions are suggested to be added near about the end-point (to prevent partial destruction if added in the beginning), but RSF solution can be added even in the beginning of the titration.
4. RSF solution is found to be quite stable (does not undergo irreversible oxidation) even in the presence of powerful oxidants like vanadium(V), chromium(VI) and cerium(IV).

RZN does not respond satisfactorily in all these determinations because, as already stated, the reduction of RZN requires a high concentration (15-fold reaction) of iron(II).

Interferences:

Large amounts of Mn(II), Pb(II), Zn(II), Sn(IV), SO_4^{2-}, Cl^-, CH_3COO^- and ClO_4^- ions do not interfere. The colour of Cr(III) and Ni(II) does not interfere when less than 0.8 and 4 mg of these ions respectively are present per mL of the titration mixture. W(VI) gives a white precipitate but does not interfere. Nitrate interferes at all concentrations. In view of the non-intereference of other metals normally present in brass, the methods have been applied for the determination of copper in brass.

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