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Kinetic- spectrophotometric Method for the Determination of Naringenin in Pure and Supplements Formulations

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Abstract:

Simple, cheap, sensitive, and accurate kinetic- spectrophotometric method has been developed for the determination of naringenin in pure and supplements formulations. The method is based on the formation of Prussian blue. The product dye exhibits a maximum absorbance at 707 nm. The calibration graph of naringenin was linear over the range 0.3 to 10 µg ml^{-1} for the fixed time method (at 15 min) with a correlation coefficient (r) and percentage linearity (r^2%) were of 0.9995 and 99.90 %, respectively, while the limit of detection LOD was 0.041 µg ml^{-1}. The method was successfully applied for the determination of naringenin in supplements with satisfactory results.

Key words: Kinetic method, Naringenin, Prussian blue, Spectrophotometric.

Introduction:

Naringenin (NAR) (2,3- dihydro-5,7-dihydroxy -2-(hydroxyphenyl)-4H-1-benzopyran-4-one),(Fig .1) is a flavonoid found in different fruits and vegetables and exhibits a wide range of bioactive effect for human health such as anti-inflammatory, antioxidant, anticarcinogenic and it reduces blood lipid (1-4). Different Spectrophotometric (5), chromatographic (6) and electrophoresis (7) methods have been reported to determining NAR.

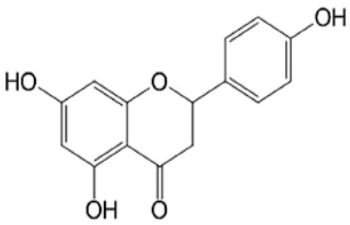

Figure 1.Chemical structure of naringenin

The kinetic method has been developed since the 1950s, and until nowadays it has a special interest which can be a credit to the progress made in the principles, in understanding the chemicals and instrumentation and in the analytical applications (8). The kinetic spectrophotometric methods offer many advantages over traditional spectrophotometric method such as high selectivity because with the reaction time the evolution of the absorbance is measured, low detection limit (9), simplicity and rapidly (10) due to avoiding some experimental steps such as extraction or filtration. Several kinetic methods have been reported for the determination of different pharmaceuticals in drugs (11-14).

The Prussian blue (PB) was synthesized for the first time around the year 1706 (15). The PB formation has a classical applied for detected Fe (II) by using potassium hexacyanoferrate, the blue precipitates obtained from mixing Fe (III) with $[Fe(CN)_6]^{-4}$ or Fe (II) with $[Fe(CN)_6]^{-3}$, the both give $Fe^{+3}_4[Fe^{+2}(CN)_6]_3.15\ H_2O$ compound, and in spite of Prussian blue is prepared from cyanide salts, but it is nontoxic because the cyanide groups are tightly bound to Fe (16).PB has many useful applications basically as pigments, inks, paints, laundry bluing. Since it is ynthesized until nowadays PB was used for the determination of many pharmaceuticals and compounds preparations (17, 18).

This work reports the use of easy and accurate kinetic -spectrophotometric method for the determination of NAR in supplement formulations. The method was based on the formation of PB the reaction between NAR with the mixture of Fe(II) and Fe(III) ,where NAR reduces Fe (III) ion to Fe (II) which reacts with $K_3[Fe(CN)_6]$ to produce the Prussian dye, fixed time and initial rate methods were adopted for this work. The method was validated and the effect of different experimental conditions on the chemical reaction speed were investigated to yield information about the mechanism of the reaction.

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Materials and Methods:

Apparatus

Digital double-beam recording spectrophotometer (Shimadzu UV-VIS 260. Kyoto. Japan) was used for spectral and absorbance monitored.

Chemicals, Standards and Supplements Preparations

All chemicals and reagents were of analytical grade.

Naringenin(NAR) Stock Solution (100 µg ml^{-1})

A stock solution (100 µg ml^{-1}) was daily prepared by dissolving 0.01 g of pure NAR (Carl Roth, Germany, ≥95.0%) in a sufficient amount of ethanol and the volume was completed to 100 mL in a volumetric flask with the same solvent. Work solutions were prepared by appropriate dilution of the stock solution with distilled water.

Ferric Chloride Solution (0.02 M)

This solution was prepared daily by dissolving 0.3244 g of ferric chloride (BDH, England, 97.0%) in 0.1 M of HCl (BDH, England, 36%) in 100 mL volumetric flask and the volume was filled up to the mark with the same solvent.

Potassium Ferricyanide Solution (0.02 M)

This solution was daily prepared by dissolving 0.6586 g of potassium ferricyanide(Hopkin &Williams, England,99.0%) in 100 mL of distilled water into a volumetric flask.

Hydrochloric Acid (0.1 M)

HCl 0.1M prepared by diluting 4.3706 mL of 11.44 M of concentrated hydrochloric acid with distilled water in 500 ml volumetric flask.

Supplements Solution

(100 µg ml^{-1}): an appropriate number of 250 mg supplements capsules (supplied from Alternative Medicine Solutions, Inc., USA) were emptied and wighed and the average weight of the content of one capsule was taken. An accurate weight equivalent to 0.01 g of pure NAR was taken and dissolved in ethanol into a 100 ml volumetric flask; the residue was washed with ethanol and the volume was made up to the mark with the same solvent, the flask then was shaken and filtered. More diluted solutions were prepared by simple dilution with distilled water.

General Procedure

Initial -rate Method

A series of standard NAR solution (100µg ml^{-1}) in the range of ((0.3µg mL^{-1} to 20µg mL^{-1}) were transferred into 10 mL volumetric flasks, and then 0.75 mL 0f 0.02 M ferric chloride solution and 1 mL of 0.02 M of potassium ferricyanide solution were added. The mixture was diluted to the mark with distilled water and the absorbance was measured immediately at 707 nm (at 25°C) as a function of time against a reagent blank. The initial rates of the reaction (k) at different concentrations were calculated from the slope tangents of the absorption time curve. Calibration graph was obtained by plotting the logarithms of the reaction initial rate (log k) against the logarithms of NAR molar concentrations (log C).

Fixed – time Method

In this procedure, the absorbance was measured at a fixed time of 15 min and plotted against the final concentrations of NAR**.**

Results and Discussion:

Preliminary Investigations

The reaction between NAR and the mix of ferric chloride and Potassium Ferricyanide yielded colored dye, the absorption spectra for the reaction product was recorded between 300 - 1100 nm (Fig .2). The maximum absorption of the product was at λ_{max} 707 nm against the reagent blank which gave minimum absorption at the same wavelength (potassium ferricyanide with acidic ferric chloride) which has a maximum absorbance at 420 due to the formation of ferric hexacyanoferrate complex.

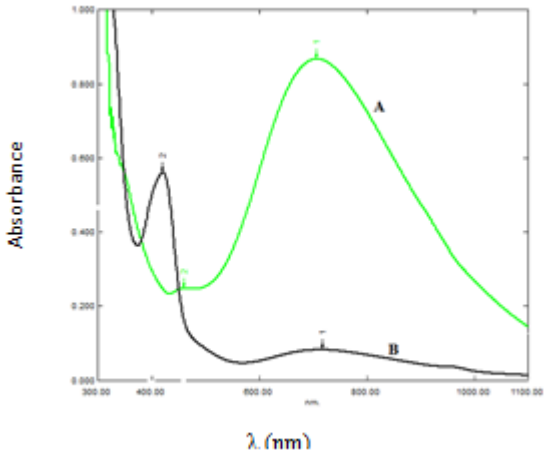

Figure 2. Absorption spectra of (A) product obtained by reaction of NAR (5 µg ml^{-1}) with $FeCL_3$ and $K_3Fe(CN)_6$ against reagent blank, (B) reagents blank against distilled water.

Optimization of the Experimental Conditions

The effect of various variables on the absorption intensity and the color development of the colored product were studied with care.

Effect of volume of K_3 [Fe (CN) $_6$]

The effect of different volumes of 0.02 M K_3 [Fe (CN) $_6$] was examined. The amount of K_3[Fe (CN) $_6$] ranging from 0.25 to 2 mL,(Fig. 3a) shows that the absorbance reached a maximum with increasing volumes of K3[Fe (CN) $_6$] up to 1 mL, above this volume the absorbance is stable. 1 mL was enough for the reaction, this volume was used in further works.

Effect of HCl Concentration

Hydrochloric acid was used as a solvent for ferric chloride, whereas Fe (III) can be used as an oxidant in acidic solution. While in neutral and alkaline medium Fe (III) forms a precipitate. The effect of different concentrations of HCl (which used to dissolved and prepared 0.02 M of $FeCL_3$) was investigated. The results shown in Fig. 3b reveal that 0.1 M of HCl gave the maximum absorbance above this concentration the absorbance value was decreased; therefore 0.1 M was used in the subsequent experiments.

Effect of Volume of $FeCL_3$

The effect of volume of $FeCL_3$ was investigated using different volumes of 0.02 M $FeCL_3$ in the range of (0.25- 2) mL, the maximum absorbance was obtained when 0.75 mL of $FeCL_3$ was used, after this volume the absorbance was almost constant, and 0.75 mL was sufficient for the best color development and to oxidant all NAR, therefore this volume was used for the next experiments (Fig. 3c).

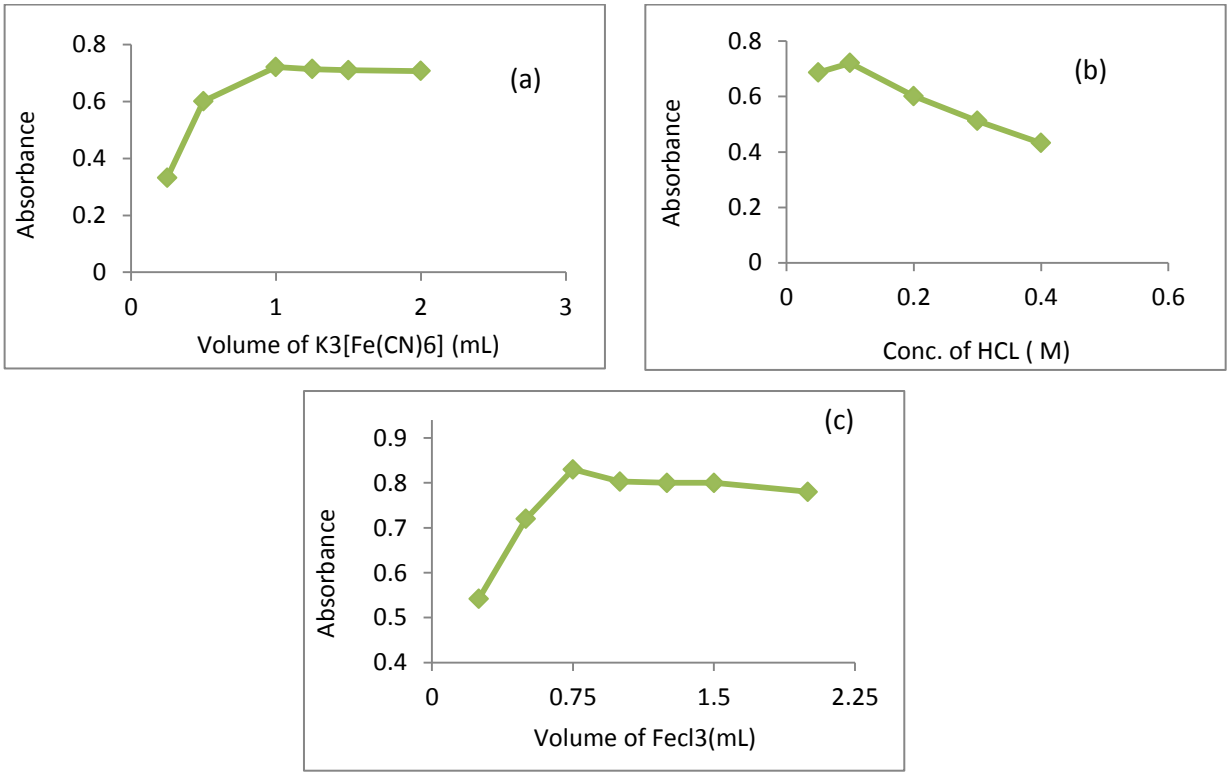

Figure 3. The absorbance against (a) volume of K_3Fe (CN) $_6$, (b) Concentration of HCL, (C) volume of $FeCL_3$.

Effect of Order of Addition and Temperature

Different orders of addition were performed. It was found that the best order of addition is $FeCL_3$ added to NAR solution followed by the addition of K_3 (Fe (CN) $_6$). NAR reduced ferric ion in $FeCL_3$ to ferrous which then reacts with K_3 (Fe (CN) $_6$) to form PB complex, (Fig .4a).

The effect of temperature on the absorbance intensity was tested with different temperatures (5, 25 and 70 °C). Room temperature (25 °C) gave the best absorbance; therefore the subsequent experiments were carried out at this temperature (Fig .4b).

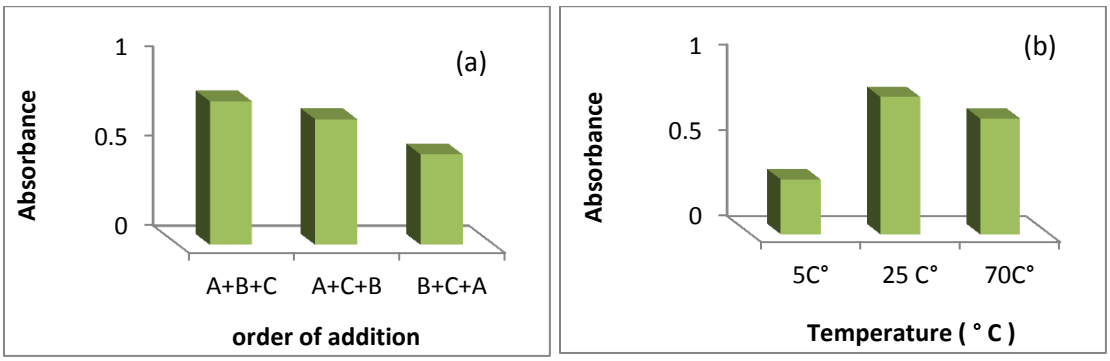

Figure 4. The absorbance against (a) different order of addition whereas; A, NAR; B, $FeCL_3$; C, $K_3Fe(CN)_6$), (b) Temperature

Stoichiometric Relationship

The stoichiometry of the reaction was determined by the mole ratio (19) and continuous variation (Job's method) (20) methods. The results (Fig .5) indicated that the existence of 1:2 (NAR: Fe (III)).

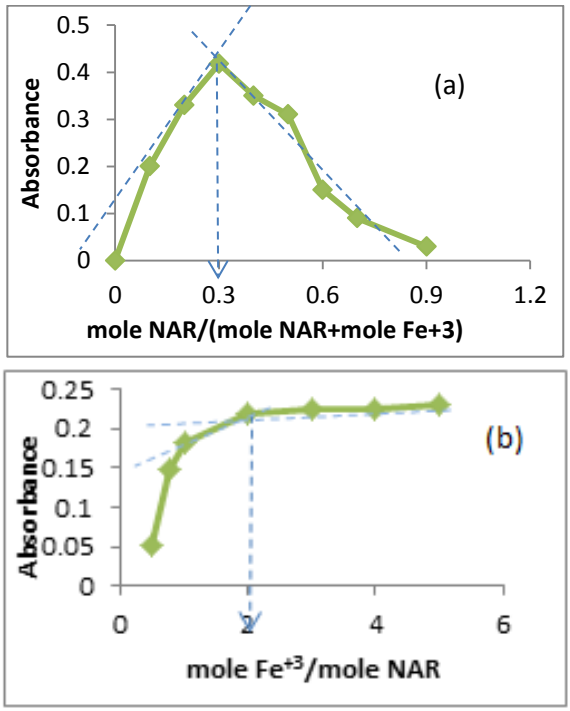

Figure 5. (a) Job's method, (b) mole ratio method

Reaction Mechanism

In this reaction, acidic ferric chloride is reduced to ferrous by NAR then reacts with potassium ferricyanide to form Prussian blue complex with the maximum absorbance at 707 nm against the reagent blank (potassium ferricyanide with acidic ferric chloride) the reaction occurs as following (16):

$$NAR + 2Fe^{+3} \longrightarrow 2Fe^{+2} + NAR \text{ (oxidant)}$$

$$Fe^{+2} + K_3[Fe(CN)_6] \longrightarrow K\,Fe[Fe(CN)_6]$$

Prussian blue(16)

Quantitation Methods

Initial -rate Method

Under the optimum reaction conditions summarized in Table 1. The initial rate would follow a pseudo-first order rate constant, and would obey the following equation:

$$k = \Delta A/\Delta t = k' C^{n} \ldots\ldots\ldots (1)$$

Where *k* is the reaction rate, *A* is the absorbance, *t* is the measuring time, *k'* is the pseudo- the first-order rate constant, *C* is the molar concentration of the NAR and *n* is the order of the reaction. The absorbance time graph for the reaction of varying concentration of NAR with a fixed concentration of $K_3[Fe(CN)_6]$ and $FeCL_3$, (0.02 M) for both was performed for 40 min at intervals of 5 min starting from 3 min, the absorbance was recorded at each time interval. The method was carried out in presence of excess $K_3[Fe(CN)_6]$ and $FeCL_3$ solutions, therefore a pseudo-first order reaction condition was achieved out with respect to the concentration of the reagents. The kinetic plots are all sigmoid in nature and the initial rate of reaction was obtained by measuring the slopes ($\Delta A/\Delta t$) of the initial tangent to the absorbance-time curves at different concentrations of the NAR (Fig6). The constructed calibration graph showed a linear relationship over the concentration range of 0.3 - 20 µg $mL^{-1}$for NAR as shown in Fig.7. Taking the logarithms of the above equation (1) is transformed into:

$$\text{Log } k = \text{Log } \Delta A/\Delta t = \text{Log } k' + n \text{ Log [NAR]} \ldots (2)$$

Regression of log (rate) versus log [NAR] gave the regression equation:

$$\text{Log (k)} = 3.3851 + 1.01 \log C \ (r^2 = 0.9985)$$

Hence, k = 2427.17 min^{-1} = 40.45 sec^{-1} and the reaction is first order (n = 1.01) with respect to NAR concentration. The value of *n* 0.9985 (≈ 1) in the regression equation confirmed that the reaction was first order. However, under the reaction condition, the concentration of reagents was 20 fold than that of NAR which indicate the Pseudo first order.

Table 1.The optimum conditions for the determination of NAR.

Parameters	Range studied	Optimum value
λ max (nm)	300- 1100	707
Volume of $K_3Fe(CN)_6$ (mL)	0.25- 2	1
Concentration of HCL (M)	0.05- 0.4	0.1
Volume of $FeCl_3$ (mL)	0.25- 2	0.75
Order of addition	NAR, $K_3Fe(CN)_6$, $FeCl_3$	NAR, $FeCl_3$, $K_3Fe(CN)_6$
Temperature (° C)	5,25,70	25

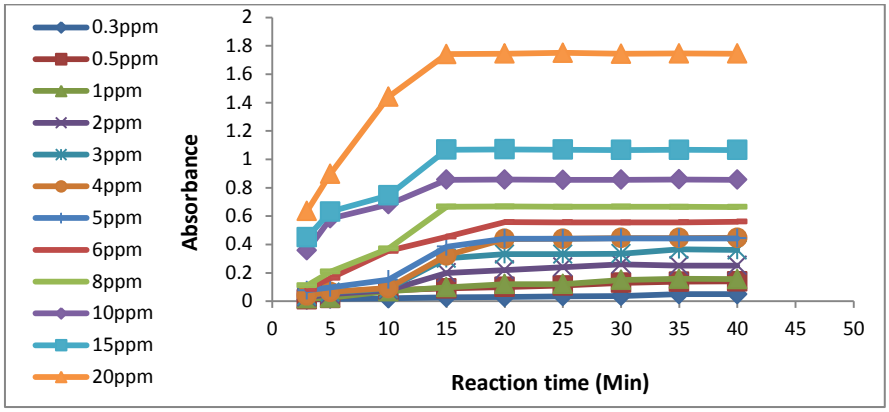

Figure 6. Absorbance versus time graph for NAR

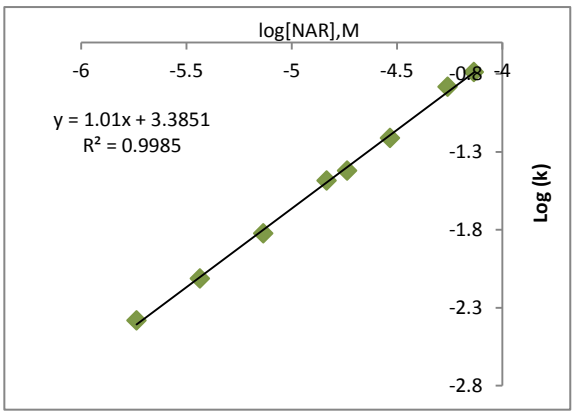

Figure 7. log (k) versus log [NAR] graph

Fixed – time Method

The absorbance for different concentrations of NAR was measured at a pre-selected fixed time at 707 nm. The recorded absorbance at a fixed time of (3, 5, 10, 15, 20, 25, 30, 35 and 40 min) plotted versus initial concentration of NAR (Fig 8). Table 2 summarized the values of correlation coefficients, regression equations and linear ranges. It is clear that the slope increases with time and the most acceptable values of *r* and the intercept were obtained for a fixed time of 15 min which was therefore chosen as the most suitable time interval for the measurement of NAR.

Table 2. Regression equations for NAR for different concentrations at different fixed time

Reaction time(min)	Regression equation	Correlation coefficient	Linear range (µg mL^{-1})
3	A= 0.358x +0.0854	0.9931	5- 20
5	A= 0.0505 x+ 0.1314	0.9927	3- 15
10	A= 0.057x+ 0.0001	0.9904	0.5- 10
15	**A= 0.1522x+ 0.0434**	**0.9995**	**0.3- 10**
20	A= 0.1506x +0.0963	0.9954	0.3- 10
25	A= 0.0775x+ 0.0712	0.9935	0.5- 10
30	A= 0.0762x+ 0.1055	0.9993	1- 15
35	A= 0.0709x + 0.1078	0.9991	1- 15
40	A= 0.0759x+ 0.0943	0.9984	1- 10

It is clear that the slope increase with time for NAR and the most acceptable values of the linear range, the correlation coefficient, and the intercept were obtained for a fixed time of 15 min, therefore, this fixed time was chosen for the estimation of NAR in pure form over the range of 0.3 - 10 µg ml^{-1}. The analytical values for the calibration graph are assembled in Table 3. The small values of the statistical parameters such as (S_a), (S_b) and ($S_{y/x}$) indicated the high precision of the method. The high values of the correlation coefficients of the regression equations indicated good linearity over the working concentration ranges.

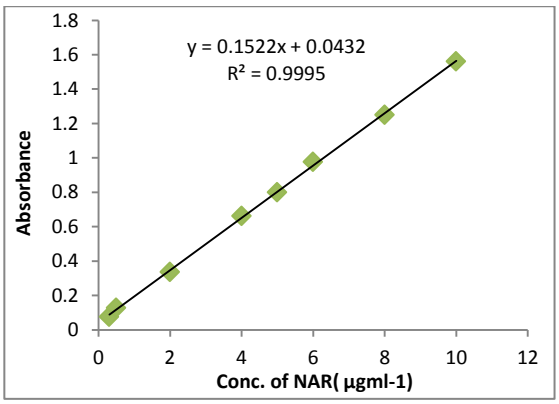

Figure 8. Calibration graph to a fixed method

Table 3.Analytical values of statistical treatments for the calibration graphs for fixed time method.

parameter	value
Regression equation	Y= 0.1522x + 0.0432
Linearity range (µg ml^{-1})	0.3- 10
Correlation coefficient, r	0.9995
Linearity percentage, r^2 %	99.9000
Slope, b (mL μg^{-1})	0.1524
Intercept, a	0.0419
Standard deviation of the residuals, $S_{y/x}$	0.0137
Standard deviation of the slope, S_b	0.0015
Standard deviation of the intercept, S_a	0.0082
Molar absorptivity, ε (L mol^{-1} cm^{-1})	41437.9720
Sandell's sensitivity, S (µg cm^{-2})	0.0066
Limit of detection, LOD (µg ml^{-1})	0.0410
Limit of quantification, LOQ (µg ml^{-1})	0. 1320

Accuracy and Precision

The accuracy and precision of the proposed method for the estimation of NAR were studied by the analysis of five replicates of three different concentration of NAR by both initial - rate and fixed - time methods. The result shows good accuracy and precision (Table 4).

Table 4.The accuracy and precision of the initial rate and fixed time methods

Method	Conc. of NAR(µg ml^{-1}) present	Conc. of NAR(µg ml^{-1}) found *	E %	Rec. %	RSD %
Initial rate	4	3.928	- 1.80	98.200	0.843
	5	5.047	+ 0.94	100.940	1.034
	6	6.074	+ 1.233	101.233	1.00
Fixed time	4	3.988	- 0.300	99.700	0.353
	5	4.983	- 0.340	99.660	0.614
	6	6.0265	+ 0.441	100.441	0.567

* Average of five determinations

Applications to Supplements Formulations

The proposed initial rate and fixed time methods were successfully applied to determine NAR in supplements by the analysis of three different concentrations of NAR using the analytical procedure directly and by standard addition method. The results in Table 5 and Table 6 prove good accuracy and precision and absence of interference.

In order to evaluate the quality and competence of the proposed method, the results which were obtained from initial rate and fixed methods were statistically compared with UV method (21) using the t- test and F- test at 95 % confidence level in respect to the accuracy and precision (22). No significant difference was found between the theoretical and calculated values of the proposed and the classical (UV) method which indicate that the proposed methods are applicable to the analysis of NAR in supplements (Table 6).

Table 5. Standard addition method of NAR by proposed initial rate and fixed methods

Method	Supplements	Conc. of NAR(µg ml^{-1}) Present	Conc. of NAR(µg ml^{-1}) Found *	E %	Rec. %	RSD%
Initial rate	NAR,250mg (Alternative Medicine Solutions, Inc.USA)	4	3.934	-1.650	98.350	0.998
		5	5.091	+1.820	101.820	0.436
		8	8.016	+0.200	100.200	0.802
Fixed time		4	3.995	-0.125	99.875	0.744
		5	5.009	+0.180	100.180	0.488
		8	7.959	-0.512	99.488	0.450

* Average of five determinations

Table 6. Direct application for the proposed initial rate and fixed methods for determination of NAR in supplements.

Method	Supplements	Conc. of NAR(µg ml^{-1}) present	Conc. of NAR(µg ml^{-1}) Found *	E %	Rec. %	RSD %	t- value [b]	F-value [b]
Initial rate	NAR,250mg (Alternative medicine solutions, Inc.USA)	4	4.006	+ 0.150	100.150	0.998	1.846	1.116
		5	5.024	+ 0.480	100.480	1.050		
		8	7.901	-1. 237	98.763	1.478		
Fixed time		4	4.012	+ 0.300	100.300	0.239	0.419	3.341
		5	5.039	+ 0.780	100.780	0.501		
		8	8.049	+ 0.613	100.613	0.279		

*Average of five determinations, [b] The theoretical values of t and F at 95% confidence level are 4.303 and 161.4, respectively.

Conclusion:

This work describes a simple kinetic spectrophotometric method based on the formation of Prussian blue to estimate NAR, in addition, the easy applicability of the initial rate and fixed time methods in estimation of NAR in pure and supplements. The proposed method is sensitive, cheap and does not require complicated steps for implementation. Furthermore, using the spectrophotometer method which is usually available in all quality control laboratories rather than the expensive methods encourages the application of the proposed method in the routine estimation of NAR. The proposed method is superior to previously reported kinetic methods as it does not require heating neither pH control, this method offers the advantage of carrying out the analysis with speed, cost-effective with keeping the accuracy, low detection limit, and sensitivity. The method showed good agreement with the compared UV method and noninterference in the estimation method. Table7 summarizes the comparison between the proposed kinetic method with some available colorimetric and kinetic methods.

Table7. Comparison of the proposed kinetic method with some available colorimetric and kinetic methods

Method	λ_{max} nm	Linear range (µg ml^{-1})	Correlation coefficient	LOD (µgml^{-1})	Remarks	Ref.
Kinetic spectrophotometric method for determination NAR, the reaction based on the reduction of Cu (Π) to Cu (I) by NAR.	450	0.15 – 1.5	0.9988	0.063	Using Britton – Robinson buffer pH 8.6, required heating at 45 C for 50 seconds, limitation linearity	(23)
Ultraviolet spectrophotometric method	290	2- 12	0.9982	0.06	NAR dissolved in methanol and sonicated for 15 minutes then completed to the mark, less sensitive method.	(24)
Ultraviolet- visible spectrophotometric method	287.49	5- 25	0.9990	0.187	Less sensitivity	(5)
Kinetic spectrophotometric method for determination NAR based on Prussian blue formation	707	0.3 - 10	0.9995	0.041	Simple, sensitive, cheap, not require heating or pH control steps	Present work

Conflicts of Interest: None.

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طريقة حركية - طيفية لتقدير النارنجنين النقي وفي المكملات

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الخلاصة:

طورت طريقة حركية – طيفية سهلة ، رخيصة ، حساسة ومضبوطة لتقدير النارنجنين بشكله النقي وفي المكملات. تستند الطريقة على تكوين الصبغة البروسية ، الصبغة الناتجة تعرض اعلى امتصاص عند 707 نانوميتر. منحنى المعايرة لمادة النارنجنين كان خطيا للمدى من 0.3- 10 مايكروغرام / مل لطريقة الوقت الثابت (عند 15 دقيقة) مع معامل ارتباط ونسبة خطية 0.9995 و 99.90 % على التوالي، بينما حد الكشف كان 0.041 مايكروغرام / مل . طبقت الطريقة بنجاح لتقدير النارنجنين في المكملات وبنتائج مرضية.

الكلمات المفتاحية : طريقة حركية، نارنجنين، صبغة بروسية، طيفية.