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FIA– Spectrophotometric methods for the determination of Naringenin in supplements and urine samples using diazotization coupling reactions

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Abstract

Direct FIA methods for estimation of Naringenin (NG) in different samples were proposed. These methods are based on diazotization coupling reactions of two reagents: method (A) p-chloroaniline (PCA) and method (B) procaine hydrochloride (PRH) with NG in basic medium. Yellow dyes with maximum absorption at 416 and 415 nm were formed respectively. Calibration curves were constructed over different NG concentrations, linearity was from 1- 70 and 1- 40 μgmL^{-1} with detection limits of 0.55 and 0.24 μgmL^{-1} for (A) and (B) respectively. All analytical variables involved in the FIA procedure were evaluated and optimized. The established methods were successfully applied for the determination of NG in its supplements and urine samples.

Keywords: Flow injection analysis, Naringenin, spectrophotometric, procaine hydrochloride, diazonium salts, p-chloroaniline.

طرائق الحقن الجرياني الطيفية لتقدير النارنجنين في نماذج المكملات و الادرار باستخدام تفاعلات الازوتة والازدواج

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الخلاصة

تم اقتراح طرق حقن جرياني مباشرة لتقدير النارنجنين في نماذج مختلفة. تعتمد هذه الطرق على تفاعلات الازوتة والازدواج في الوسط القاعدي لكاشفين مع النارنجنين: الطريقة الاولى (A) مع الباراكلوروانيلين والطريقة الثانية (B) مع البروكائين هيدروكلورايد في الوسط القاعدي، انتجت الطريقتين صبغة صفراء تعطي اعلى قمة امتصاص عند 416 و 415 نانومتر على التوالي. مدى الخطية لمنحنيات المعايرة لتراكيز مختلفة من النارنجنين كانت من 1− 70 و من 1− 40 مايكروغرام / مل مع حدود كشف 0.55 و 0.24 مايكروغرام/ مل للطريقة (A) وللطريقة (B) على التوالي. تم تحديد المتغيرات المثلى المتضمنة في طريقة الحقن الجرياني. كما تم تطبيق الطرق المعمول بها بنجاح لتقدير النارنجنين في نماذج المكملات و الادرار.

1. Introduction

Naringenin (NG), chemically (2, 3, -dihydro-5, 7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), is one of the most diets consumed flavonoids. This constituent can be found in fruits, vegetables, and beverages. It has various ranges of biological functions such as antioxidant, blood lipid- lowering, anti-inflammatory and anti- carcinogenic [1-5]. NG has been determined using

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different methods like a spectrophotometric [6], electrophoresis [7- 8] and chromatographic methods [9].

Flow injection analysis (FIA) system offers many advantages: increase sample throughput, decrease reagent consumption, reduce waste production, inexpensive equipment and simplicity [10].

In the present study, PCA and PRH were diazotized and coupled with NG in an alkaline medium to yield colored products can be monitored directly at room temperature (25^0C) with maximum absorption at 416 and 415 nm respectively. Normal FIA technique presenting a simple, fast and cheap assay procedure for estimation of NG in pure, supplements and urine samples. The suggested methods are available for application in laboratories with simple devices.

2. Experimental

2.1. Apparatus

Shimadzu UV-VIS 260 digital double beam spectrophotometer recording used for spectral and absorbance measurements equipped with flow cell of 50 µL internal volume and 1 cm path length. A peristaltic pump (Shennchen, China) supplied with a flexible vinyl tubing of 0.5 mm internal diameter to transport the carrier solution. Injection valve (Knauer, Germany) was employed to provide appropriate injection volumes of standard solutions. In addition, reaction coil (RC) of Teflon material with an internal diameter of 0.5 mm was used. Two-channel manifolds were employed for the FIA spectrophotometric determination of NG.

2.2. Reagents and solutions

Standard NG (Carl Roth, Germany). 500 µg mL^{-1} of NG was prepared by dissolving 0.05 g of pure NG in 100 ml of ethanol.

Diazotized p-chloroaniline (DPCA) DPCA (BDH, M. Wt= 127.57 g/mol) and **Diazotized procaine hydrochloride** (DPRH) (SDI, Samara, Iraq, M.Wt= 272.78 g/ mol). Solutions of (0.003M) were prepared daily by dissolving 0.0382 g of PCA in 20 mL ethanol and 0.0818 g of PRH in 20 mL distilled water in 100 mL volumetric flasks and cooling in ice bath. In a separated beakers a volume of 2 mL of (1M) HCL adding to 0.0207 g of sodium nitrite (0.003M) with stirring and cooling. The mixture was added to PCA and PRH to form the diazotized reagents. After five minutes the volume made up to the mark with distilled water. The reagents were leave to stabilize for 15 minutes before use.

Sodium hydroxide (BDH) 1M Prepared by dissolving 10 g of NaOH with distilled water in a 250 mL volumetric flask then the volume was completed to the mark with distilled water.

Hydrochloride acid 1M (BDH). This solution was prepared approximately by diluting 43.7 mL of 11.44 M of concentrated hydrochloric acid with distilled water in 500 mL volumetric flask.

Solutions of supplements (500 µg mL^{-1}) an appropriate number of supplements capsules (Alternative Medicine Solutions, Inc., 250 mg) were emptied and weighted and the average weight of the content of one capsule was taken. An accurate weight equivalent to 0.05 g of NG was taken and dissolved in ethanol. The solution was filtered into 100 mL volumetric flask, the residue was washed with ethanol and completed to the mark with the same solvent. More diluted solutions were prepared by simple dilution.

Preparation of urine samples [11]: 5 mg of pure NG transferred into 25 mL volumetric flask and then dissolved in 5 mL of ethanol. A volume of 12.5 mL of urine (supplied from healthy volunteers) was added, the volume was completed to the mark with ethanol. The content was mixed well. Two different concentrations were analyzed as a described method, against a reagent blank.

2.3. General FIA procedures

Working solution of NG in a range of (1– 70) µg mL^{-1} for method (A) and (1– 40) µg mL^{-1} for method (B) were prepared from stock solutions.

For method (A); 100 µL portion of NG was injected into the stream of 0.1 M NaOH and was then combined with a stream of 0.003 M DPCA with a total flow rate of 1.8 mL/min and the reagents mixing in 50 cm reaction coil. The yellow dye absorbance was measured at 416 nm.

For method (B); 100 µL portion of NG was injected into the stream of the mixture of 0.1 M NaOH and 0.003 M DPRH solution with a total flow rate of 1.8 mL/min. The reagents were mixed in 25 cm reaction coil. The yellow dye absorbance was measured at 415 nm.

The FIA manifolds used were shown in Figure-1 (a, b).

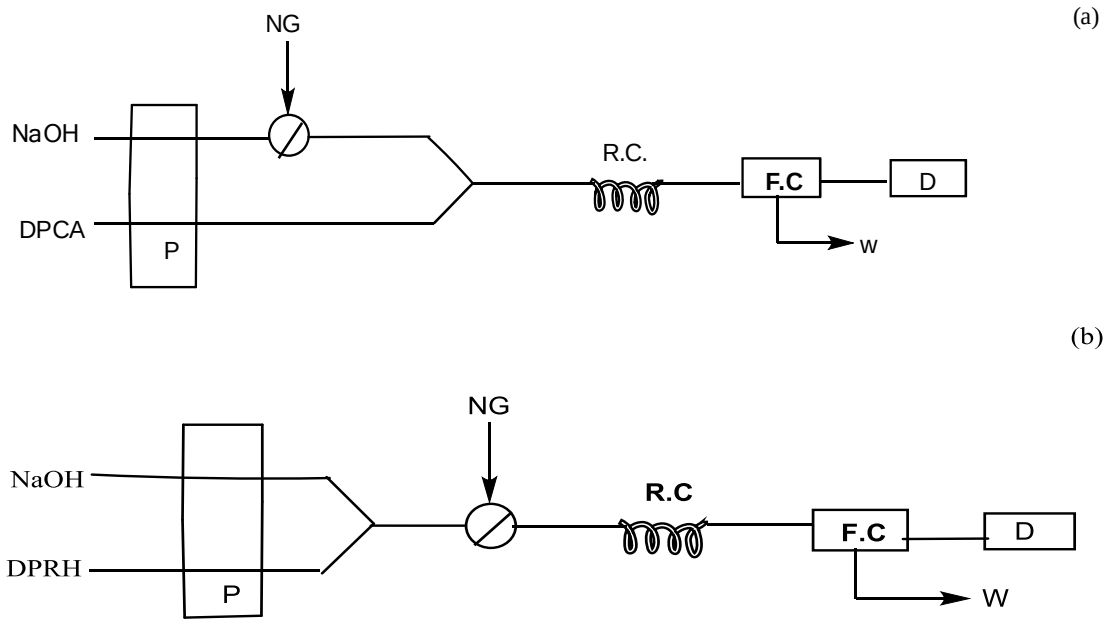
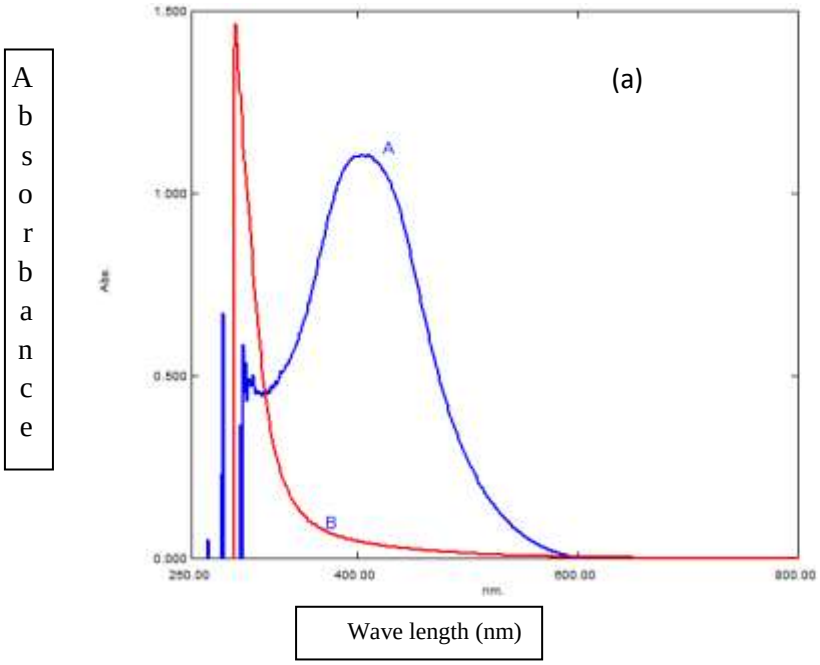


Figure 1-Schematic diagram of FIA systems, (a): Method (A) and (b): Method (B), whereas. P: peristaltic pump, F.C: Flow cell, R.C: Reaction coil and W: waste.

3. Results and discussion

Preliminary studies were indicated that DPCA and DPRH coupled with NG in alkaline medium and formed yellow dyes can be detected spectrophotometrically. The spectra of aqueous solutions are shown in Figure-2(a, b).



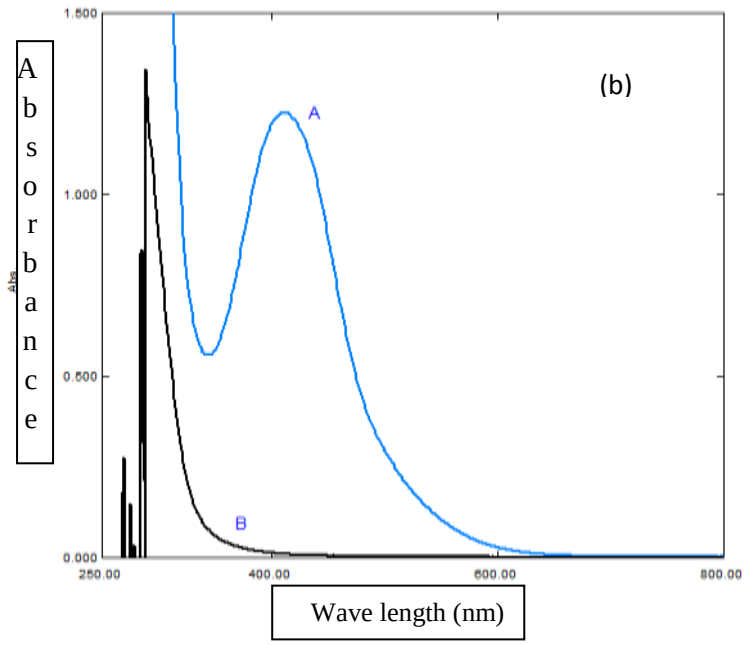
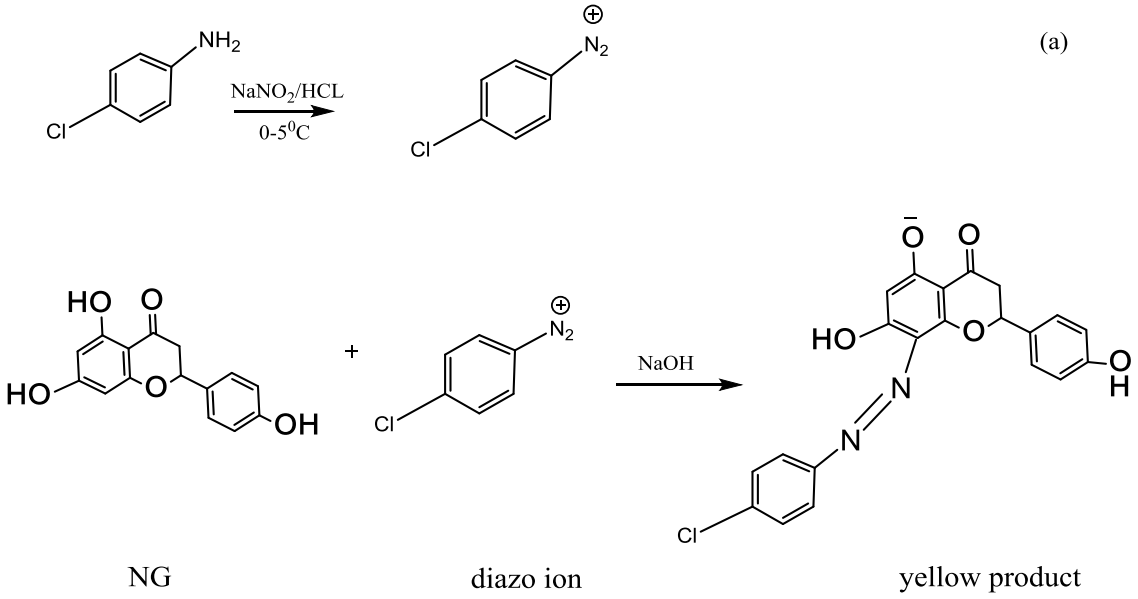


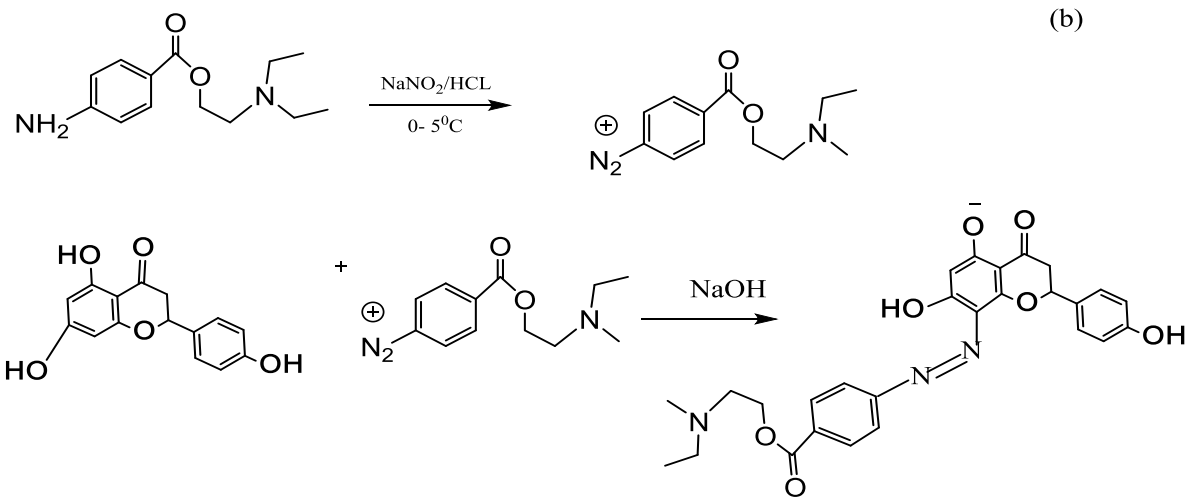
Figure 2- (a) Absorption spectrum of azo dye formed when 8 µg mL^{-1} of NG coupled with DPCA (a) and DPRH (b) in alkaline medium (A) against the blank, (B) the reagents blank against distilled water.

Scheme 1 shows the proposed reaction for the determination of NG which involving the following [12]:

1. The reaction of PCA or PRH with sodium nitrite in acidic medium to produce the diazonium ion.
2. The coupling of NG with diazonium ion in an alkaline medium to produce a yellow compound which was monitored at 416 and 415 nm. The Job's method [13] applied to investigate the stoichiometry of the reaction between diazotized reagents and NG and the results indicated that the ratio product 1:1 (PCA: NG and PRH) were formed.

The chemical and physical parameters that could affect the reaction and the sensitivity of the colored formed were optimized by altering one variable in time while keeping others constant. The experiments carried out using 20 µg mL^{-1} of NG for both methods.





NG diazo ion Yellow prodect

Scheme 1-A Probable mechanism for the diazo coupling reaction of NG with the reagents, (a): method (A), (b) method (B).

3.1. Optimization of chemical parameters

The chemicals parameters that involved in the reaction including the concentrations of diazotized reagents and sodium hydroxide were studied. The optimization of the concentrations of the DPCA and DPRH were shown that the absorbance increased for the concentration up to 0.003 M and after this concentration, the absorbance decreased, therefore this concentration was selected for further used Table-1. Method (A) and method (B) were carried out in alkaline medium and the preliminary studied reveals that sodium hydroxide was the suitable base for both methods. The concentration of NaOH was investigated in the range of (0.03 -0.4 M) for methods (A) and (B), and the concentration of 0.1 M was chosen for both methods, the results obtained are as shown in Table-2.

3.2. Optimization of physical parameters

The physical parameters studied were reaction coil, injection volume, and total flow rate. The reaction coil length was studied in the range from (0- 250 cm), the results indicated that reaction coil length of 50 cm for method (A) and 25 cm for method (B) gave maximum absorbance and therefore were selected. Higher lengths perhaps increase the dispersion of reactant zone. The influence of injection volume was investigated with varying sample loops in the range of (75- 250 µL), and the volumes 150 µL and 100 µL were selected for method (A) and (B) respectively. To study the effect of the total flow rate different rates were used in the range of (0.4– .9 mL/min), the flow rates that accomplished the compromise between sampling rate and sensitivity were chosen, which was 1.8 mL/min method for (A) and 2.8 mL/min for method (B). Figure-3, shows the results obtained from the physical conditions studies.

Table 1- Effect of reagent concentration.

Reagent concentration (M)	Absorbance	
	Method (A)	Method (B)
0.001	0.397	0.383
0.002	0.412	0.516
0.003	**0.441**	**0.555**
0.005	0.420	0.454
0.007	0.401	0.411
0.009	0.311	0.38

Table 2-Effect of NaOH concentration.

NaOH concentration of (M)		Absorbance	
		Method (A)	Method (B)
0.03		0.201	0.361
0.05		0.341	0.467
0.1		**0.441**	**0.555**
0.2		0.35	0.432
0.3		0.22	0.311
0.4	0.163	0.269	

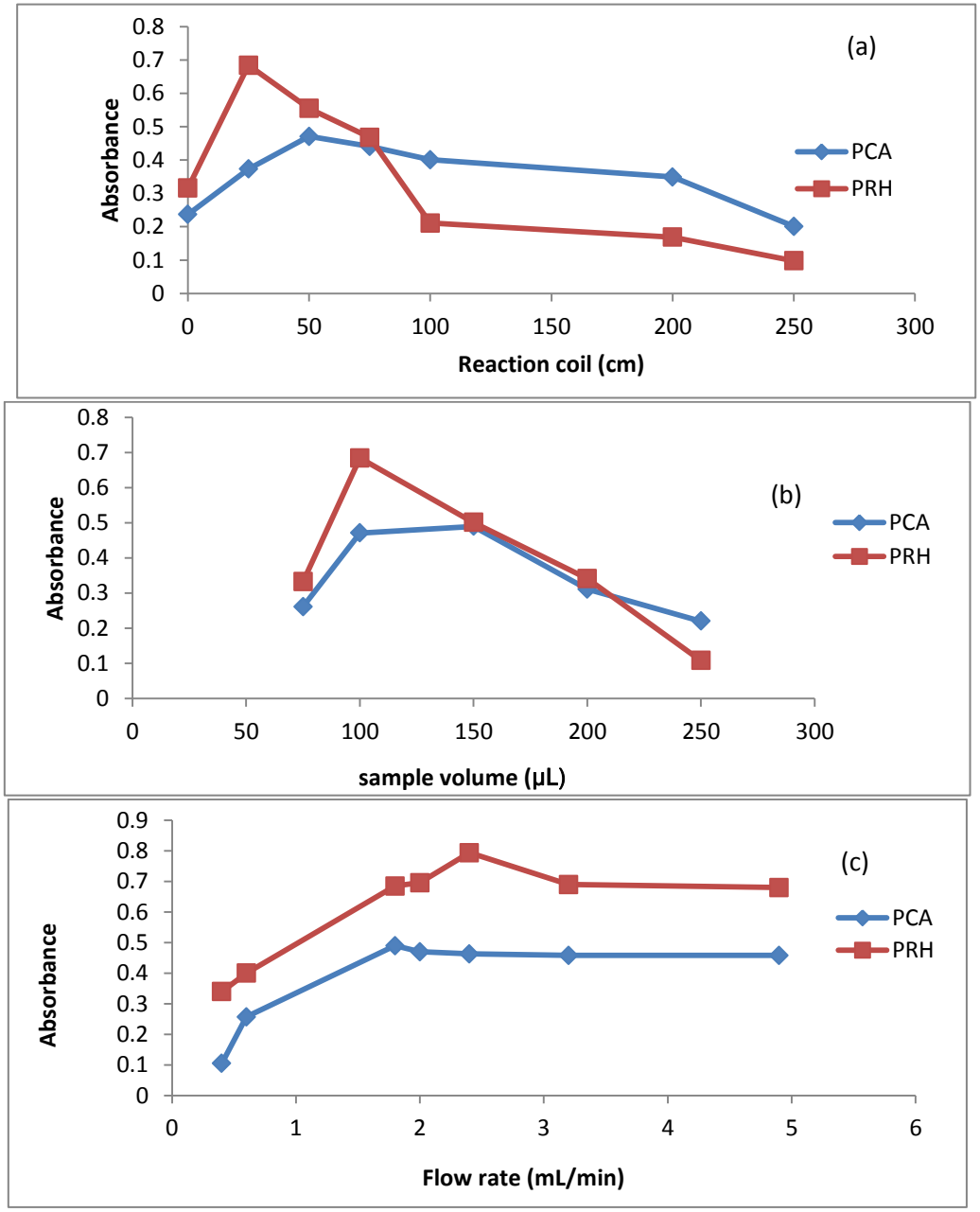


Figure 3-Effect of physical parameters: (a) Effect of reaction coil, (b) Effect of sample volume, (c) Effect of total flow rate.

3.3. Analytical characteristics

After optimized all, the chemical and physical variables the calibration graphs were performed to test the linearity of NG concentrations (triplicate injections). Table-3 summarizes the analytical parameters that obtained from calibration graph, which indicated good linearity and low limits of detection for both methods. Good precision and highly reproducible were obtained for both methods (Table-4). The time required for the appearance of the maximum absorbance was 40 sec and 30 sec for method (A) and method (B), respectively.

Table 3-Analytical values of the suggested methods for determinate of NG.

Parameter	Value for method (A)	Value for method(B)
Regression equation	Y=0.0185x+ 0.1135	Y=0.0392x+ 0.0091
Correlation coefficient, r	0.9992	0.9995
Linearity range (µgmL^{-1})	1-70	40
Molar absorptivity (Lmol^{-1}cm^{-1})	5036.81	5880.81
Throughput (Sample/h)	90	120
Limit of detection (µg mL^{-1})	0.55	0.25
Limit of quatification (µg mL^{-1})	1.83	0.83

Table 4-Accuracy and precision of the proposed methods.

Method	Conc. of NG (µg/mL)		Rec. %	RSD %
	present	found		
Method (A)	10	10.081	100.810	0.379
	20	20.351	101.756	0.939
	30	30.081	100.270	0.913
Method (B)	15	14.757	98.384	0.423
	20	20.0229	100.115	0.257
	25	25.528	102.112	0.735

The influence of foreign compounds was eliminated using standard addition method which applied under calibration curve conditions. Good accuracy and precision were obtained. The results were shown in Figure -(4,5) and Table-5.

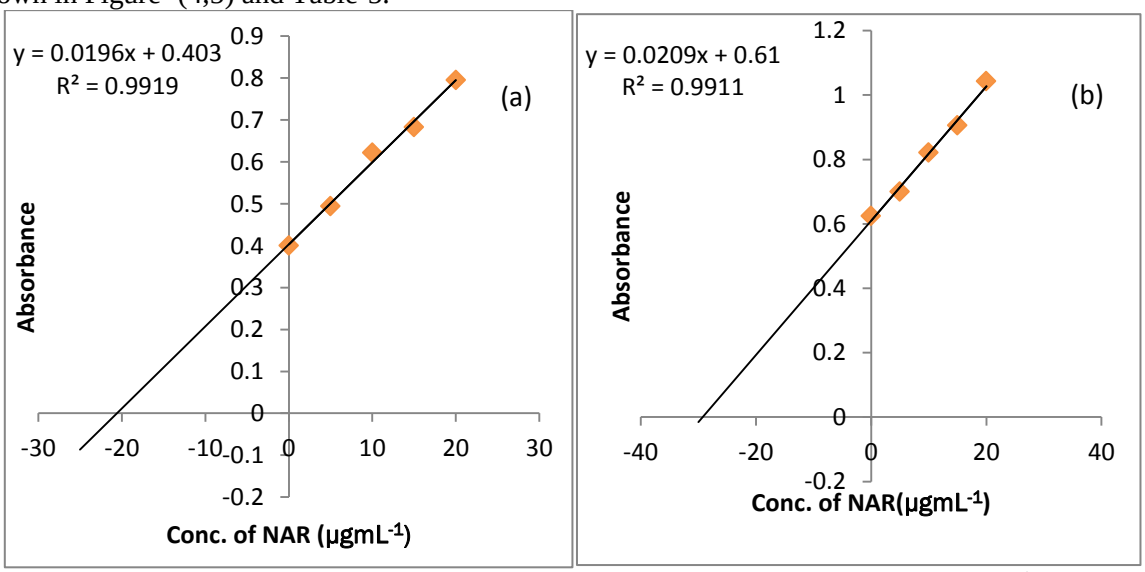


Figure 4- Standard addition method for determination NAR method (A): (a) 20 µg mL^{-1}, (b) 30 µg mL^{-1}.

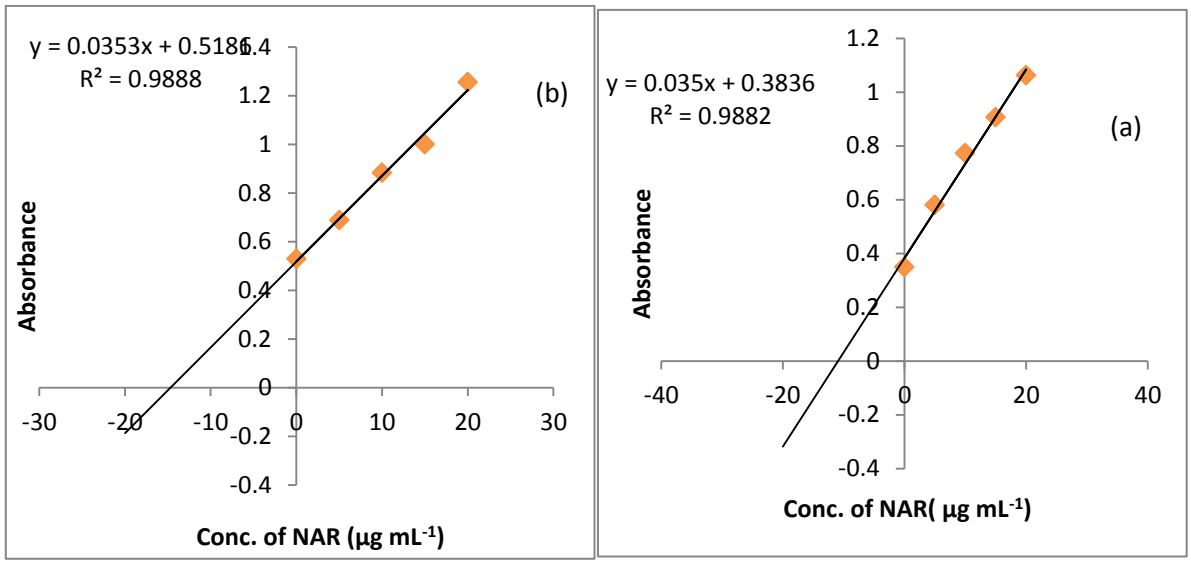


Figure 5- Standard addition method for determination NAR method (B): (a) 10 µg mL^{-1}, (b) 15 µg mL^{-1}.

Table 5- Application of the proposed methods for determination of NG in supplements by using standard addition method.

Proposed method	Supplements	Conc. Of NG (µg/mL)		Rec. %*	RSD %*
		Present	Found		
Method (A)	Naringenin capsule 250 mg (alternative medicine solutions, Inc. USA)	20	20.418	102.092	0.112
		30	29.856	99.522	0.113
Method (B)	Naringenin capsule 250 mg (alternative medicine solutions, Inc. USA)	10	9.971	99.714	0.203
		15	14.986	99.906	0.134

*Average of five determinations.

The proposed methods were also applied directly to analysis supplements capsules containing NG (Table-6). The methods were applied directly for human urine samples, the recovery study was performed with samples spiked with two different concentrations of NG for each method and the results revealed that urine's constituents did not interfere in the methods (Table- 7). The results obtained were compared with those obtained by UV method [14] using t-test and F - test within 95% confidence level [15]. The results listed in Table -8, shows that the calculated value of (t) is less than the critical value of (t) which means that no significant difference between the two suggested methods and the classical method for determination of NG.

Table 6-The direct applications of proposed methods in supplements samples.

Proposed method	Applications	Conc. of NG (µg/mL)		Rec. %*	RSD %*
		Present	Found		
Method (A)	Naringenin (capsule 250 mg)	10	9.973	99.729	0.237
		20	20.297	101.487	0.145
		30	30.514	101.712	0.956
Method (B)	Naringenin (capsule 250 mg)	10	10.027	100.271	0.191
		15	15.172	101.145	0.236
		20	19.862	99.311	0.152
		25	25.080	100.321	0.151

*Average of five determinations.

Table 7-The direct applications of proposed methods in urine samples.

Proposed method	Applications	Conc. of NG (µg/ mL)		Rec. %*	RSD %*
		Present	Found		
Method (A)	Urine	20	19.270	96.351	1.094
		30	28.721	95.766	0.548
Method (B)		10	9.589	95.893	0.184
		20	19.283	96.416	0.277

Table 8-The statistical comparison between the suggested methods with the classical method using t- and F- test.

Pharmaceutical preparation	Method (A)		Method (B)		Classical method [14]	
	Rec. %	RSD %	Rec. %	RSD %	Rec. %	RSD %
Pure NG	100.928	0.624	100.204	0.347	100.229	0.679
Naringenin capsule 250 mg	100.255	0.4467	100.579	0.1927	100.5694	0.804
t- Calculate (theoretical= 4.303)	0.509				1.008	
F- Calculate (theoretical = 161.4)	3.922				13.835	

The degree of freedom for F= n_1+ n_2 . 2 = 2, the degree of freedom for t= n_1-1 = 1.

4. Conclusion

FIA technique is a rapid, simple and economic determining method. This paper described the development of sensitive, robust and accurate spectrophotometric methods for estimation of NG based on diazotization coupling reaction using the two reagents p-chloroaniline and procaine hydrochloride (which are cheap, available and stable reagents). The value of recoveries indicated a high precision and accuracy with low limits of detection for methods. The proposed methods are agreed with the classical method. The average recovery method (A) was 100.97 and for method (B) was 100.20, therefore the method is suitable for determination of NG in supplements and biological samples.

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