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Flow Injection Spectrophotometric Technique for Determining of Genistein in Pure and Supplements Formulations Through Diazotization Coupling Reaction

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Abstract

Genistein (GEN) is the major isoflavone found in soybeans, has a number of cardiovascular health benefits, postmenopausal syndrome and osteoporosis. A direct flow injection analysis method for estimation of GEN in pure and supplements formulation was suggested. This system is based on a diazotization coupling reaction between procaine penicillin (PR) and GEN in an alkaline medium. The formed orange dye has a maximum absorption at 416 nm. Calibration curve was constructed over different GEN concentrations with a linearity range of 10-100 µg/mL and a detection limit of 1.51 µg/mL. For the FIA technique, all analytical factors were analyzed and optimized. The established method was successfully used to determine GEN in the supplement formulations.

Keywords: Genistein, Flow injection analysis, Spectrophotometry, Procaine penicillin, Diazotization coupling reaction.

تقنية طيف الحقن الجرياني لتقدير الجينيستين في المستحضرات النقية والمكملات من خلال تفاعل ازوتة والازدواج

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الخلاصة

الجينيستين هو مركب ايسوفلايفون موجود في فول الصويا بصورة رئيسية وله العديد من الفوائد الطبية لكثير من الامراض كأمراض القلب والأوعية الدموية وهشاشة العظام ومتلازمة ما بعد انقطاع الطمث . تم اقتراح طريقة تحليل حقن التدفق الجرياني المباشر لتقدير الجينيستين بصورته النقية وفي المكملات الصيدلانية . تعتمد طريقة التقدير على تفاعل الأقتران والأزوتة بين البروكائين بنسلين مادة دوائية تم استعمالها كاشف في التفاعل والجينيستين في وسط قاعدي . حيث تنتج صبغة ذات لون برتقالي وتقاس عند طول موجي 416 نانومتر . تم قياس الأمتصاصية لتراكيز مختلفة من الجينيستين 10-100 ميكروغرام/ مل وكانت حدود الكشف 1.51 ميكروغرام / مل . تم تقييم وتحسين جميع المتغيرات التحليلية المستخدمة في تطبيق الطريقة المحددة بنجاح.

الكلمات المفتاحية:

Introduction

Flavonoids are a type of secondary metabolism molecule that occurs naturally in the world of plants. They are regarded as a quality indication for fruits and medicinal plants, and hence an important component to consider in the development of agricultural and manufactured products. Many publications have been written throughout the years about the extraction and identification of flavonoids [1,2] .Genistein (Figure 1) is an isoflavone that acts as a phytoestrogen and an angiogenesis inhibitor. The chemical name comes from the fact that it was initially isolated in 1899 from the dyer's broom, *Genista tinctoria*. The structure of the chemical was determined to be identical to that of prunetol in 1926. Genistein was quantified by spectral methods [3-6], mass spectrometry (MS) with high performance liquid chromatography (HPLC)[7], UV or electrochemical detection[8–16], gas chromatography-mass spectrometry [17], LC-MS method[18].

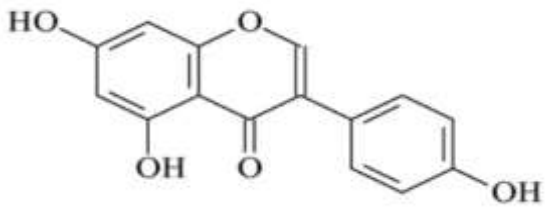

Figure 1. Genistein

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The analysis method used in the present work is flow injection analysis. It's done by inserting a sample plug into a moving carrier stream [19,20]. Detection methods include spectrophotometry, fluorescence spectroscopy, atomic absorption spectroscopy, mass spectrometry, and other experimental methods. FIA techniques have evolved into a wide range of applications. Flow injection's application to real-world tests benefits from automated sample processing, good repeatability, adaptability to micro-miniaturization, chemical containment, waste reduction, and reagent economy in a system that runs at microliter levels [21,22].

Experimental Work

Apparatus

A digital spectrophotometer Shimadzu mini UV-VIS 1240 was used for absorbance equipped with flow cell with 50 µl internal volume and 1 cm of bath length. It was with a peristaltic pump (Shennchen, China) equipped with flexible vinyl tubes of an inner diameter of 0.5 to transfer the solutions. Injection valve from Knauer, Germany was used to provide appropriate injection volumes of standard solutions in addition a reaction coil (RC) made of Teflon with a 0.5 mm inner diameter was used. The manifold with two channels was used to determine GEN spectrophotometric ally . The FIA manifold used was depicted in Figure.(2).

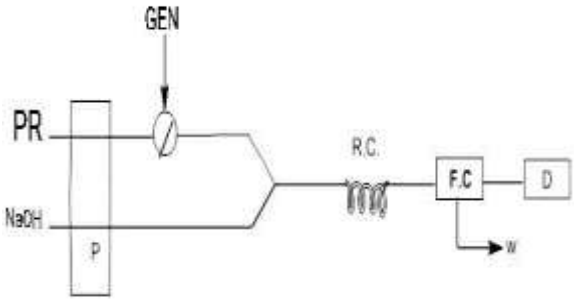


Figure 2. Schematic of FIA manifold for estimated GEN . P: peristaltic pump, F.C: Flow cell, R.C: Reaction coil and W: waste.

Reagents and solutions

Standard GEN 200 µg/ml : Dissolving 0.02 (no. of mol) g of pure GEN in 10 ml 0.5M of NaOH in a small beaker, then transferring the solution to a 100 ml volumetric flask and completing the volume with distilled water to the mark .

Diazotized Reagent solution: A solution of PR 0.003M was freshly prepared by dissolving 0.1712 g (no. of mol) of PR in 25 mL ethanol in beaker and cooling in ice bath. After that,3 ml,1M of HCl was added to it and stirring with cooling , then 0.0207(no. of mol) g of sodium nitrite (Merck) (0.003M) was added with constant stirring. Then transferring the solution to a 100 mL volumetric flask and completing the volume by distilled water to the mark.

Sodium hydroxide (BDH, England) 1M : In a 250 ml volumetric flask, dissolve 10 g of NaOH with distilled water and completed the volume to the mark by distilled water.

Hydrochloride acid (BDH , England) 1M : In a 500 mL volumetric flask, dilute 43.7 mL of 11.44 M concentrated hydrochloric acid with distilled water.

Preparation solutions of GEN pharmaceutical forms: (200 µg/ml) an appropriate number of capsules (10 capsules) were emptied and weighted, and the average weight of one capsule's content was selected, and accurately weighted to equate to 0.02 g of GEN and dissolved in 10 ml NaOH 0.5M. Then transferred to a 100 ml volumetric flask and completed to the mark with distilled water. Serial dilutions were done to prepare the working solutions.

General FIA procedures

Working solutions for the process were made from GEN stock solutions ranging from 10 to 100 $g.ml^{-1}$. A 100µl portion of GEN was injected into a stream of 0.003M procaine solution, then the solution is mixed with 0.1M NaOH in 50cm reaction coil. at a total flow rate of 1.9 $mL.min^{-1}$. At 416 nm, the orange dye absorption was measured.

Results and Discussion

Preliminary studies were indicated that procain penicillin (1 mL of 0.003M) coupled with GEN (1 mL of 200 µg $.ml^{-1}$)in alkaline medium NaOH (1 mL of 0.1M) and formed orange dye can be detected spectrophotometrically. The spectra of colored complex formed are shown in Figure. 3.

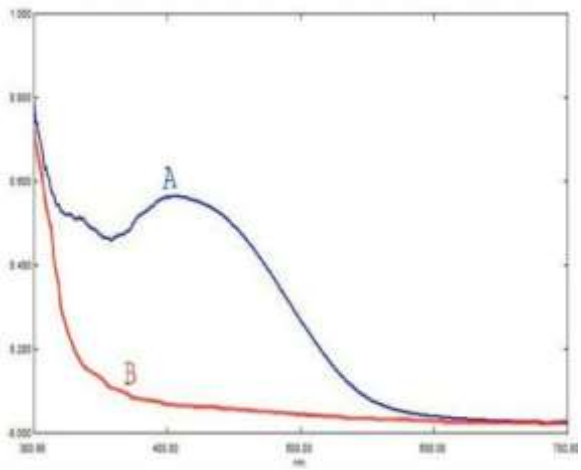
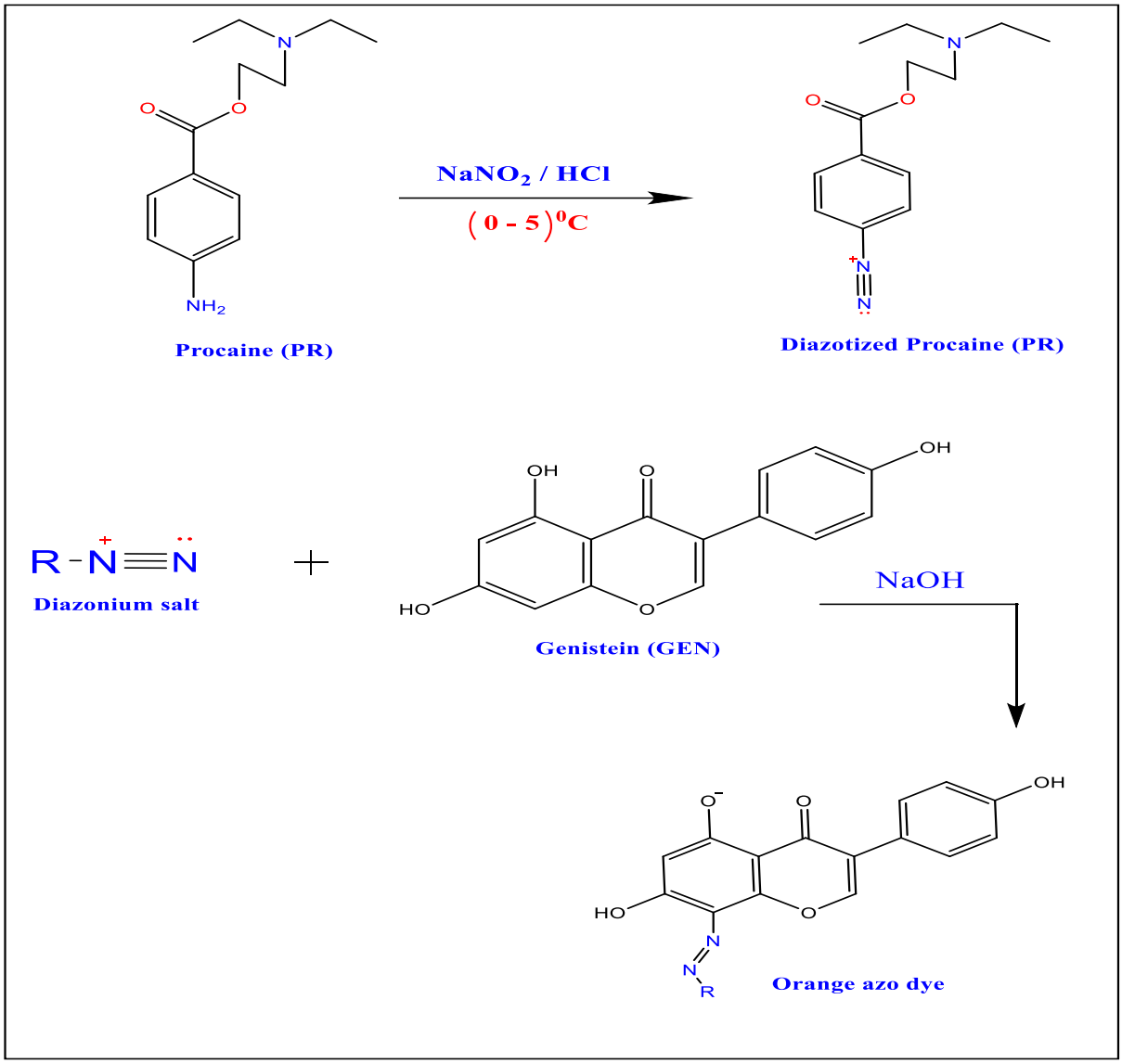


Figure 3. A: Absorption spectrum of azo dye formed when 20 µg.ml^{-1} of GEN coupled with procaine penicillin in alkaline medium, B: Blank.

After chemical and physical variables have been optimized the calibration graph was performed to test the linearity of GEN concentration (triplicate injections). Two stages were necessary to complete the diazotization coupling reaction. PR was initially converted to a diazo compound by reacting with nitrous acid (NaNO2/HCl). The diazonium salt was then coupled with GEN in para position of a poly phenolic molecule, producing orange dye in a basic medium in the second step, The phenolic nature of GEN resulted in fast coupling with diazotized reagent, as shown in Scheme 1.



Scheme 1. Proposed mechanism for diazotization coupling reaction of GEN with Procaine

Optimization of chemical parameters

The chemical and hydrodynamic parameters that could affect the reaction and the stability of the colored formed were studied by changing one variable at a time while keeping the others constant. The experiment was conducted using 20 µg.ml^{-1} of GEN. The chemicals parameters, including the concentrations of reagent, medium, etc. were studied; the optimization of the concentration of (PR) was shown that when the concentration was increased up to 0.003 M, the absorbance increased. with increasing concentration, the absorbance was observed to decrease than it was in the previous concentration .therefore, 0.003 M concentration was selected for further used. The greater concentration leads to increase the blank signal as shown in Figure 4. Preliminary studied for type of alkaline medium reveals (NaOH, KOH and Na_2CO_3) that sodium hydroxide was the suitable base for this method. Different concentrations of NaOH were investigated in the range of (0.07 -0.9 M) and the concentration chosen was 0.1 M. The concentration greater than 0.1 M shown as an inhibitor for sensitivity of colored products, the results obtained are as shown in Figure 5.

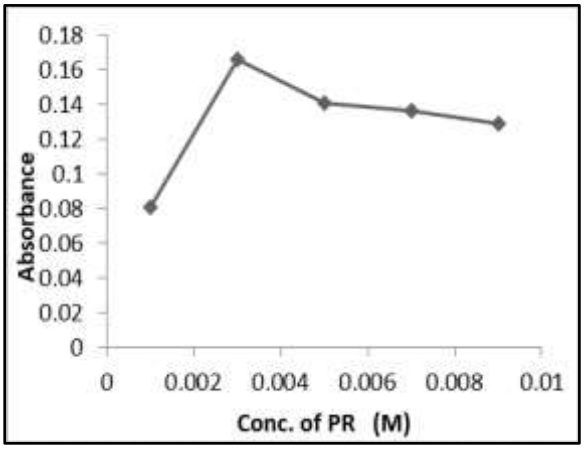


Figure 4. Effect of the reagent concentration

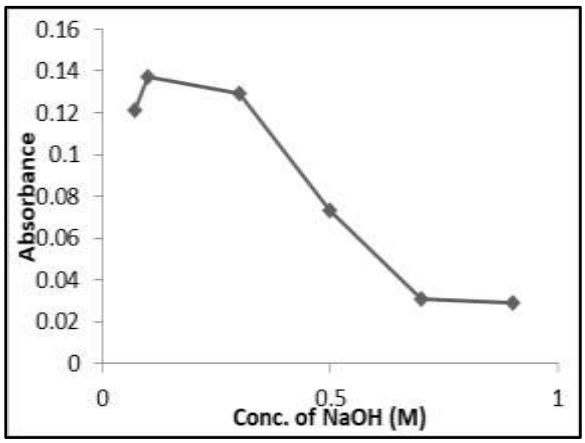


Figure 5. Effect of NaOH concentration

Optimization the physical parameters

Effect of total flow rate

Total flow rate has a great role in FIA system, since it is use to get the best reaction time and has a directed effect on sampling frequency. At higher flow rate the dispersion and reagent consumption were increased , therefore ,the absorbance and sensitivity decreased with increased flow rate. In this study, a flow rate range 0.45-4.95 $mL.min^{-1}$ was used, and the optimum value was 1.9 $mL.min^{-1}$.The current study indicated that the absorbance was decreased above 1.9 $mL.min^{-1}$ flow rate because of dispersion and dilution . The result obtained from flow rate is shown in Figure (6. a).

The effect of the reaction coil length

To enhance the sensitivity of the colored reaction and increase mixing of the reactants, the effect of reaction coil lengths was investigated .The dispersion of reactant zone probably increase with increasing the reaction coil length. Different reaction coil lengths were studied in the range of 25 to 150 cm, and the coil length of 50 cm gave the maximum absorbance, therefore was selected and used in subsequent experiments(Figure 6. b).

The influence of the sample volume injected

Effect of injection volume was investigated with varying sample loops in the range of 75 to 200 µL. The highest absorbance was obtained at 150 µL, and this sample volume was used in subsequent experiments. The obtained result is shown in (Figure 6.c).

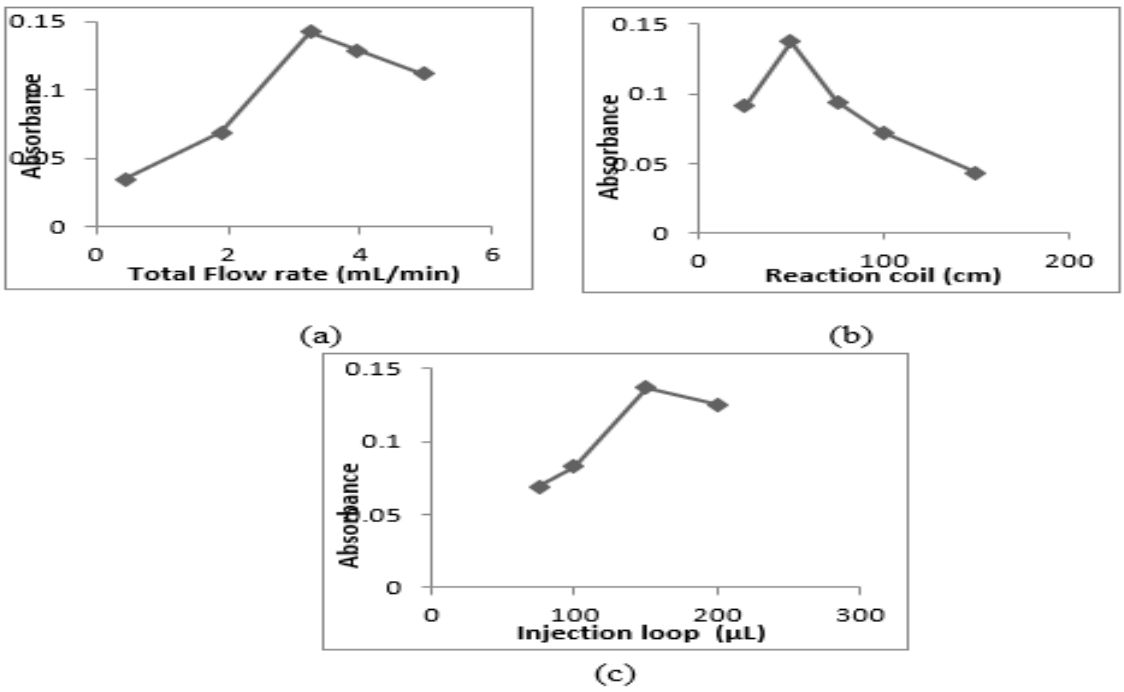


Figure 6 . (a) The effect of total flow rate on reaction absorbance, (b) Effect of reaction coil of the absorbance, (c) Effect of the injection loop

Analytical characteristics

The calibration curve was constructed under optimal conditions for GEN estimation (Table 1). Table 2 shows the values for the calibration curve's intercept, slope, correlation coefficient, and molar absorptivity, as well as values for analytical statistical treatments.

Table 1. Summary of the analytical parameters obtained from calibration graph, which indicated good linearity, highly reproducibility and low limit of detection

Parameters	Value
Conc. of GEN	20 µg mL^{-1}
Conc. of PR	0.003 M
Conc. of NaOH	0.1 M
Total flow rate	1.9 $mL.min^{-1}$
Sample loop	150 µL
Reaction coil	50 cm

Accuracy and precision

To determine the accuracy and precision, three different concentrations of standard solution of Genistein were investigated in five replicates. For each concentration, the accuracy and precision were performed and the relative standard deviation RSD% was obtained with relative error E%. Low values of the RSD% and E% indicated that method gave acceptable results. Table 3 shows the precision and accuracy for the suggested method.

Table 2. Analytical values of the techniques suggested for determinate GEN

Parameter	Value
Regression equation	y = 0.0153 x – 0.1223
Correlation coefficient, r^2	0.9989
Linearity percentage ,R^2 %	99.89
Slope, b (µg mL^{-1})	0.0153
Intercept, a	0.1223
Linearity range (µg mL^{-1})	10 - 100
Standard deviation of the slope, S_b	1.83 X 10^{-4}
Relative standard deviation RSD%	0.7
Recovery range %	99.45 – 100.56
Molar absorptivity , Ɛ (L mol^{-1} cm^{-1})	4.135 X 10^3
LOQ, (µg mL^{-1})	2.67
LOD, (µg mL^{-1})	0.88
Standard deviation of the residuals, $S_{y/x}$	1.66 X 10^{-2}
Sandell،s sensitivity (µg cm^{-2})	0.0654

Table 3 . The precision and accuracy

Present	Found	Error	SD%	*Rec.%	*RSD%
20	19.89	-0.55	0.003	99.45	0.71
40	39.93	-0.17	0.002	99.83	0.27
50	50.28	0.56	0.002	100.56	0.22

*Average of five determinations

Analytical applications (GNS supplements)

The current method was used to determine the amount of GEN in capsules by analyzing three different concentrations (20,40, and 50 µL mL^{-1}) under calibration graph conditions directly.

According to the obtained results shown in Table 4, the small values of calculated RSD% and E% refer to the repeatable and accurate of the suggested method.

Table 4. Application of the proposed method for determining GEN in pharmaceutical formulations by using direct method

Conc. GNS Alternative Medicine Solutions, Inc.USA µg mL^{-1}	Found µg mL^{-1}	Error%	Rec.%	RSD%
20	20.3	1.5	101.5	0.5
40	40.2	0.5	100.5	0.3
50	50.9	1.8	101.8	0.2

As shown in Table 5, the calculated t and F-test values did not outperform the theoretical ones .When the suggested method and the ported UV method were evaluated, the findings revealed that there was no significant difference between them.

Table 5. The t- and F-tests were used to compare the proposed methods to the UV method

Preparation form	Proposed method	Classical UV method
	Recovery %	
GEN (pure)	101.00	99.23
GEN capsule 125 mg	99.27	101.34
t-calculate (t*=4.303)	0.13	
F-calculate (F*=161.4)	1.44	

The influence of foreign compounds was eliminated using standard addition method which applied under calibration curve conditions. Good accuracy and precision was obtained .The results are shown in Figure 7 and Table 6.

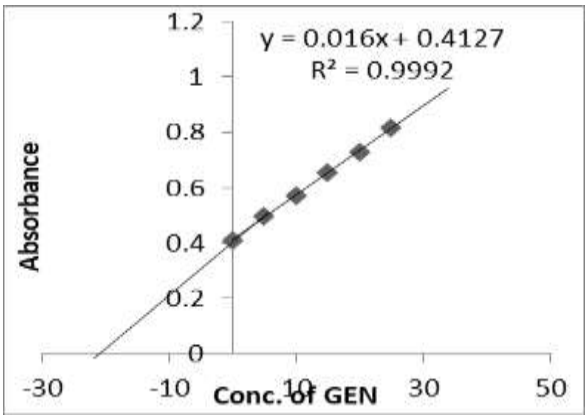


Figure 7. Standard addition method for determination of 20 µg.ml^{-1} of GEN in supplements

Table 6. Application of the proposed method for determining GEN in pharmaceutical formulations by using standard addition method .

Pharmaceutical preparation	Proposed method				Classical UV method			
	Conc. of GEN (µg.mL^{-1})		*Rec.%	*RSD%	Conc. of GEN (µg.mL^{-1})		*Rec.%	*RSD%
	Present	Found			Present	Found		
Genistein capsule 125 mg	15	15.1	100.7	0.5	2	2.03	101.5	1
	20	19.8	99.0	0.6	3	3.02	100.7	0.7

* Average of five determinations

Conclusion

According to the research study, no flow injection methods for estimating GEN in medicinal preparations have been documented. The current study demonstrated that a new spectrophotometric-flow injection approach for determining GEN based on the diazotization coupling reaction using reagent PR at the microgram level was simple, fast, and robust. the suggested method has a high sensitivity, linearity, and cost effectiveness when compared to previous FIA methods and other methods such as LC-MS, HPLC, HPTLC, GCE, and HPLC-UV. The recovery values were satisfactory, indicating the suggested method's excellent accuracy and reproducibility. The T- and F-values proved that the proposed method and the classical method were in agreement. The method was sufficient for routine GEN estimation in pure and pharmaceutical formulations.

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