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Two Spectrophotometric Methods Based on Diazotization Coupling Reaction for Determination of Genistein in Pure and Supplement

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ABSERACT

Genistein (GEN) is one of the predominant dietary isoflavones found in legumes such as soybeans. Genistein has been recommended as an osteoporosis treatment for postmenopausal women and elderly men, with the intention of reducing cardiovascular disease and hormone-dependent malignancies. Therefore, two sensitive and simple methods for quantifying it in the supplements preparation were developed.The first method (A) comprised employing the surfactant Triton X-114 to extract the result of the diazotization reaction with 4-Aminoacetophenone(4AMA) utilizing a cloud point extraction technique. The product was extracted using micelles of a non-ionic surfactant (TritonX-114) and then spectrophotometrically detected at a specified wavelength of 437nm. The linearity of the calibration curve was in the range of 0.25 -2.75 µg/mL of GEN(r = 0.9978) under optimal conditions. The average sample recovery was determined to be between 96 and 101 %. For 2 µg/mL of GEN, the relative standard deviation (RSD) was (0.27%). The second method (B) involved using the spectrophotometric-flow injection methodology for the same diazotization coupling reaction described above, and the orange colored dyewas measured at a wave length of 437 nm.Calibration curve were linear at different concentration range of GEN 10 to 100 µg/mL , relative standard deviation RSD% were 0.23 to 1.21. The proposed methods were applied to determine the presence of GEN in Supplements.

Keywords: Genistein, cloud point extraction, flow injection analysis , spectrophotometry, Triton X-114, 4-Aminoacetophenone.

طريقتان طيفيتان لتقدير الجينستين في الحالة النقية والمكملات من خلال تفاعلات الأزوتة والأزدواج

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الخلاصة

الجينيستين هو أحد أنواع الايسوفلافون الغذائية السائدة الموجودة في البقوليات مثل فول الصويا. تم تطوير طريقتين حساستين وبسيطتين لتقديره في المستحضر الصيدلاني. تضمنت الطريقة الأولى (أ) استخدام تقنية استخراج نقطة سحابة لمنتج تفاعل ازوتة مع 4-أمينو أسيتوفينون (4AMA) باستخدام Triton X-114 كعامل خافض للتوتر السطحي. تم استخلاص المنتج بواسطة مذيلات الفاعل بالسطح غير الأيوني (TritonX-114) ثم تم اكتشافه بالطيف الضوئي عند 437 نانومتر محدد. في ظل الظروف الفضلى ، كان

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المدى لمنحنى المعايرة (0.25–2.75 µg/mL)(r=0.9978) تم تحديد معدل الاسترداد للعينات ما بين 96%–101%كان الانحراف المعياري النسبي (RSD) لـ 0.27% 2 µg/mL أما بالنسبة للطريقة الثانية (ب) فقد اشتملت على استخدام تقنية حقن التدفق الضوئي الطيفي لنفس تفاعل اقتران الازوتة أعلاه وقيست الصبغة البرتقالية اللون عند الطول الموجي ، 437 نانومتر. كانت الرسوم البيانية للمعايرة خطية في نطاق تركيز مختلف من µg/mL 100 - 10 ، وكان الانحراف المعياري النسبي RSD% 0.23 –1.21. تم تطبيق الطرق المقترحة لتقدير GEN في التراكيب الصيدلانية.

Introduction

Flavonoids are a plant polyphenolic category with secondary metabolites present in fruits, vegetables, tea, and herbs as glycosides [1]. They have been demonstrated to exhibit anti-inflammatory, oestrogenic, antioxidant, antiviral, and cytotoxic antitumoral pharmacological properties in humans [2,3]. Genistein Figure (1) Isoflavone is a naturally occurring chemical that belongs to the Isoflavone class of compounds. It's classified as a phytoestrogen and an angiogenesis inhibitor [4]. In various epidemiological investigations and animal testing, it was discovered to have important biological activity in a number of aging-related and hormone-dependent disorders, ranging from cardiovascular disease to osteoporosis to postmenopause symptoms [5 -10]. GEN has been estimated by several methods, including: UPLC-MS/MS, LC-MS and simple and rapid liquid chromatography [11– 13].

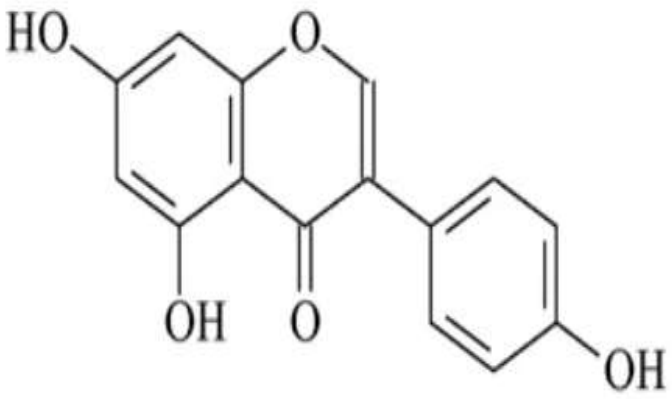


Figure 1: The chemical structure of Genistein

In many matrices, as an alternative to using expensive instruments, the specttrophotometric technique combined with cloud point extraction (CPE) still offers appealing features in regular investigations of metals and chemical compounds [14-18].

The objective of this research is to develop sensitive, selective methods, such as spectrophotometric measurement of genistein in its pure form and supplementation.using Diazotized reagent 4AMA (0.005 M) and base medium NaOH (0.1 M), different procedures (batch-CPE and FIA) were used.

EXPERIMENTAL

Apparatuses and Materials

- Shimadzu UV mini -1240 single beam (UV –Vis spectrophotometer).
- (Cecil, 50 µL internal volume) 1 cm path length matched quartz flow cells.
- A peristaltic pump of two channels (SHENCHEN, China) was used for pumping the reagents and sample solutions.

- Flexible polyvinyl chloride tubes were used for the peristaltic pump and reaction coils with different lengths.
- Sensitive balance (SartoriusBL 210 S).
- A hot-and-cold water bath (Haake, Fe3).
- A Hettich EBA 21 type centrifuge with 50 mL calibrated centrifuge tubes.
- Thermometer Mercury type.

Preparation of Chemicals

Genistein (GEN) (Pharmaceutical grade) was provided from the local commercial suppliers and subjected toanalysis. All reagents used in this study were of analytical reagent grade. Solution of GEN (200 µg/mL). Stock solution of reducing GEN was prepared by dissolving 0.0200 g of GEN in 10 mL of sodium hydroxide (0.5M), which then transferred into volumetric flask of 100 mL, Finally, distilled water was used to dilute the mixture to the desired concentration. Appropriate dilutions in water were used to make the working solution. Diazotized reagent 4-Aminoacetophenon (4AMA) solution (0.005 M) (, purity 99.0%)was freshly prepared by dissolving 0.0675 g of 4AMA in 6 mL ethanol in beaker and cooling in ice bath. After that,4 mL,1M of HCl was added to it and stirring withcooling, then 0.0345 g of sodium nitrite (Merck) (0.005M) was added with constant stirring to form the diazotized reagent. Then transferring the solution to a 100 mL volumetric flask and completing the volume by distilled water to the mark. Triton X-114 (10% v/v) was produced for CPE by dissolving 10 mL of Triton X-114 (purity 99.9%, Fluka) in distilled water and diluting to the desired concentration in a 100 mL volumetric flask, Pharmaceutical samples for genistein were manufactured with the same concentration of pure GEN for both techniques (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. A series . More diluted solutions were prepared by simple dilution with distilled water.

General procedures:

Method A

In a 10 mL volumetric flask, add 1 mL GEN (2µg/mL),1 mL 4AMA solution (0.005 M), 1 mL NaOH (0.1 M), and 3 mL Triton X-114 (% v/v) and stir thoroughly. The contents of the flask were transferred to a 10 ml centrifuging tube and kept at 70 ºC for 20 minutes in the thermostatic bath. Separation of the two phases was accomplished by centrifugation (3500 rpm for 15 minutes), after which they were chilled in an ice bath (in order to increase the viscosity of the surfactant-rich phase). By inverting the tube, the aqueous phase of the experiment was easily poured. The complex's absorbance was measured at 437 nm after the surfactant-rich phase containing it was dissolved in 3 ml of aceton.

Method B

A series of GEN working solution in a range of concentrations 10 to 100 µg/mL,a volume of 20 µg/mL of GEN was injected into the carrier stream of the reagent 4AMA (0.005M), The liquid was then mixed with 0.1 M NaOH and placed in a 100 cm reaction coil.. At 437 nm, the absorbance of the reddish orange product was determined. The FIA manifolds used were displayed in Figure (2).

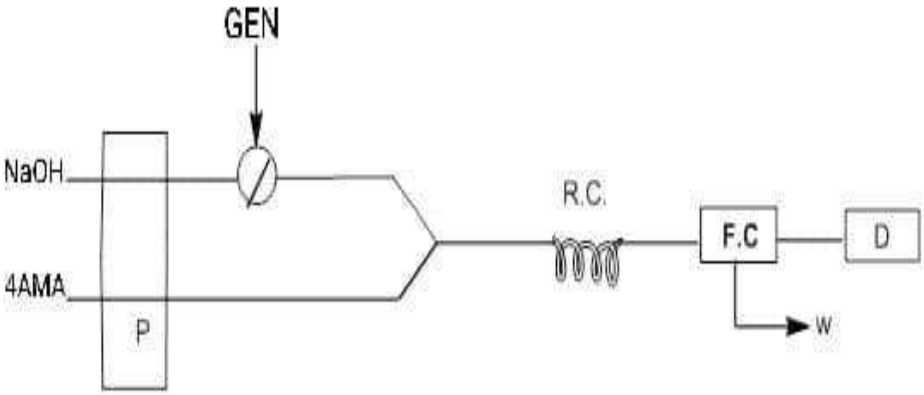


Figure 2: FIA systems for estimated GEN . P: peristaltic pump, F.C: Flow cell, R.C: Reaction coil and W: waste.

RESULTS AND DISCUSSION

The effects of the important parameters that affecting the efficiency for both methods were studied by using a series of experiments. All of the variables that may influence the sensitivity and stability of the colored product were optimized by changing one parameter with time while leaving the others constant. A solution of 200 µg/mL of reduced GEN in a 10 mL final volume (i.e. 1 µg/mL CPE and 20 µg/mL FIA) was used for the optimization all conditions, with measuring absorbance at 437nm against the blank.

Optimization of conditions for method A:

Spectra of absorption Spectrophotometric tests were carried out to investigate the production of a coupling product between GEN and 4AMA in the presence of NaOH (0.1M). The CPE process was used to determine GEN and this reaction was used to accomplish it.

Effect of surfactant volume (Triton X-114)

Triton x-114 is the surfactant used in the CPE technique. Volume of the Triton X-114 (10% v/v) has an important influence on the extraction efficiency under the cloud point extraction method, due to its important effect to increase extraction efficiency through diminish the phase volume ratio. Within the surfactant volume range of (1 - 5 mL) of 10% (v/v) Triton X-114, the effect of Triton X-114 volume on the absorbance of the extracted complex was examined. The results are shown in Figure(3).The greatest intensity was obtained with a volume of 3 mL of 10% Triton X-114, which was employed in all subsequent tests.

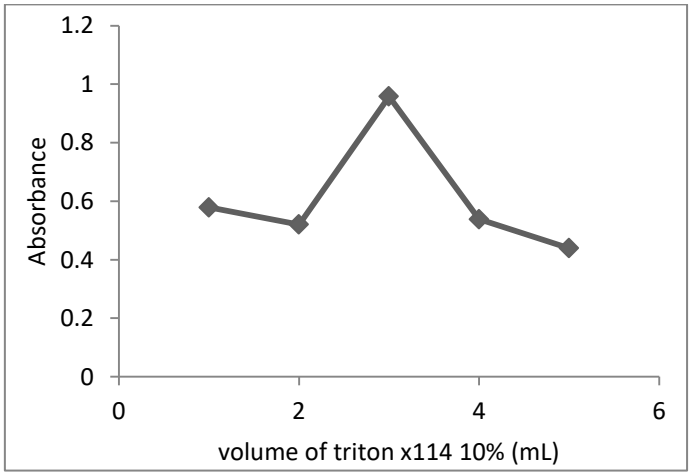


Figure 3: The optimal volume of triton x-114 according to the highest absorbency

Effect of temperature and time of extraction

In order to achieve an efficient phase separation and easy preconcentration, it is essential to optimize the extraction temperature and incubation time. To study the effect of temperature on the extraction of colored product, a varying of the incubation temperature in the range (50 _80ºC) at incubation time for 20 min were applied. Figure(4) shows that at 70°C, the absorbance of the complex was found to be at its maximum. As a result, for future studies, an equilibration temperature of 70 °C was adopted.

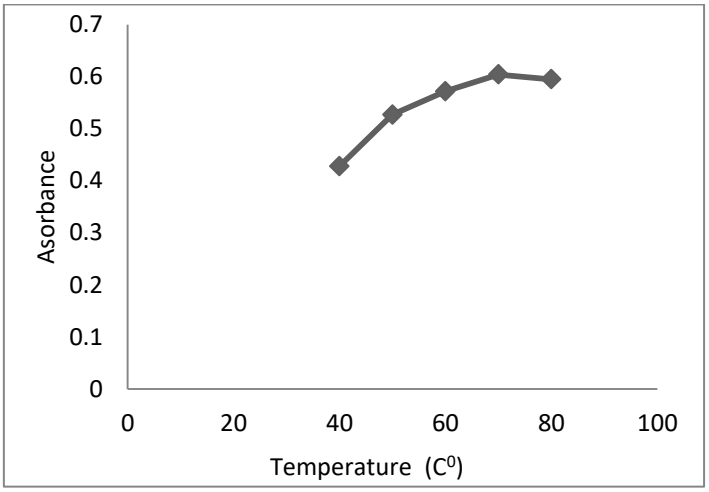


Figure 4: The optimal temperature for method A

CPE method usually needs a sufficient time to reach to the equilibrium between surfactant-rich and aqueous phases. The dependence of extraction efficiency upon incubation time was studied in the range of 10 -30min at 70 ºC. For the separation procedure, a 20-minute incubation time was sufficient. The optimum centrifuge time was determined to be 5.0 minutes since total separation occurred within this time frame and no significant improvements were noticed for longer periods.

Optimization conditions for method B

The chemical and physical variables that influence the suggested method's performance have been investigated. The method's optimal conditions were chosen based on reproducibility, throughput, and sensitivity.

Effect of reagent concentration

Different concentrations of 4AMA in the range of 0.001 to 0.009M were examined. It was found that the maximum absorbance intensity was obtained at 0.005M. Following this concentration, the absorbance of the blank steadily increased, as seen in Fig (5).

Therefore 0.005 M was selected as the optimum reagent concentration

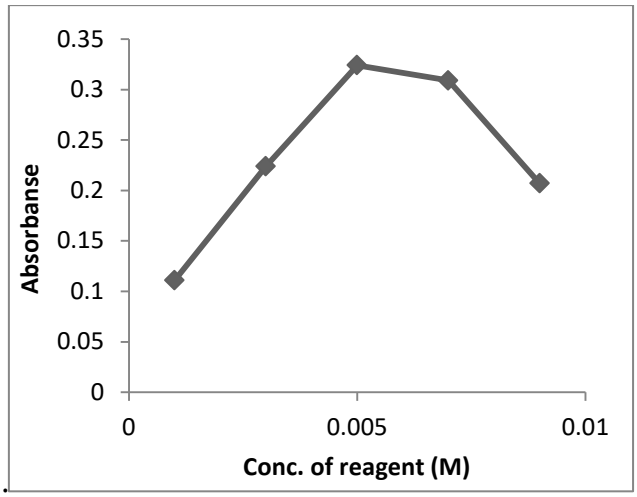
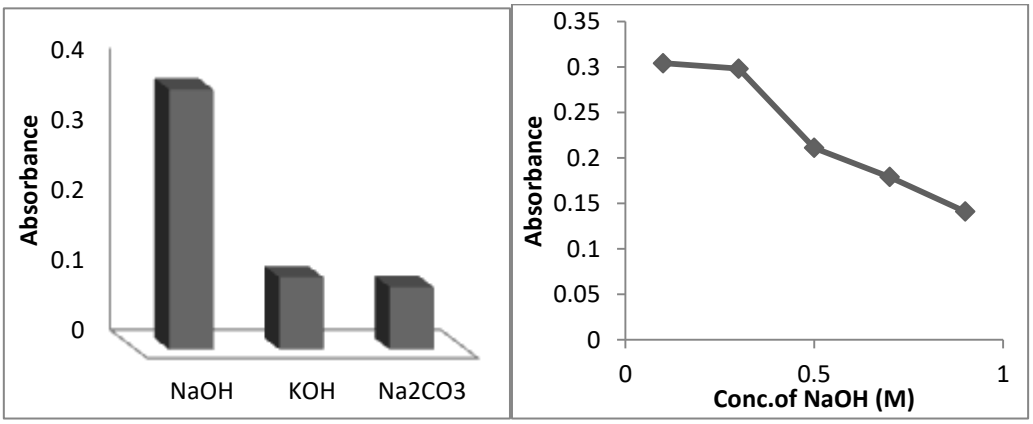


Figure 5: Optimum reagent concentration

Effect of alkaline medium

Preliminary experiments indicated that the orange-colored product was showed more efficiently in an alkaline medium, therefore, different types of basic solutions were examined Figure (6.a). It was found that NaOH (0.1M) was gave the maximum absorbance, stability and sensitivity. Various concentrations of NaOH were studied ranging 0.1 to 0.9 M affecting on the formation of the colored products. Therefore, 0.1 M of NaOH was chosen and used in the subsequent experiments. Figure (6.b) shows the concentration of base.



(a) (b)

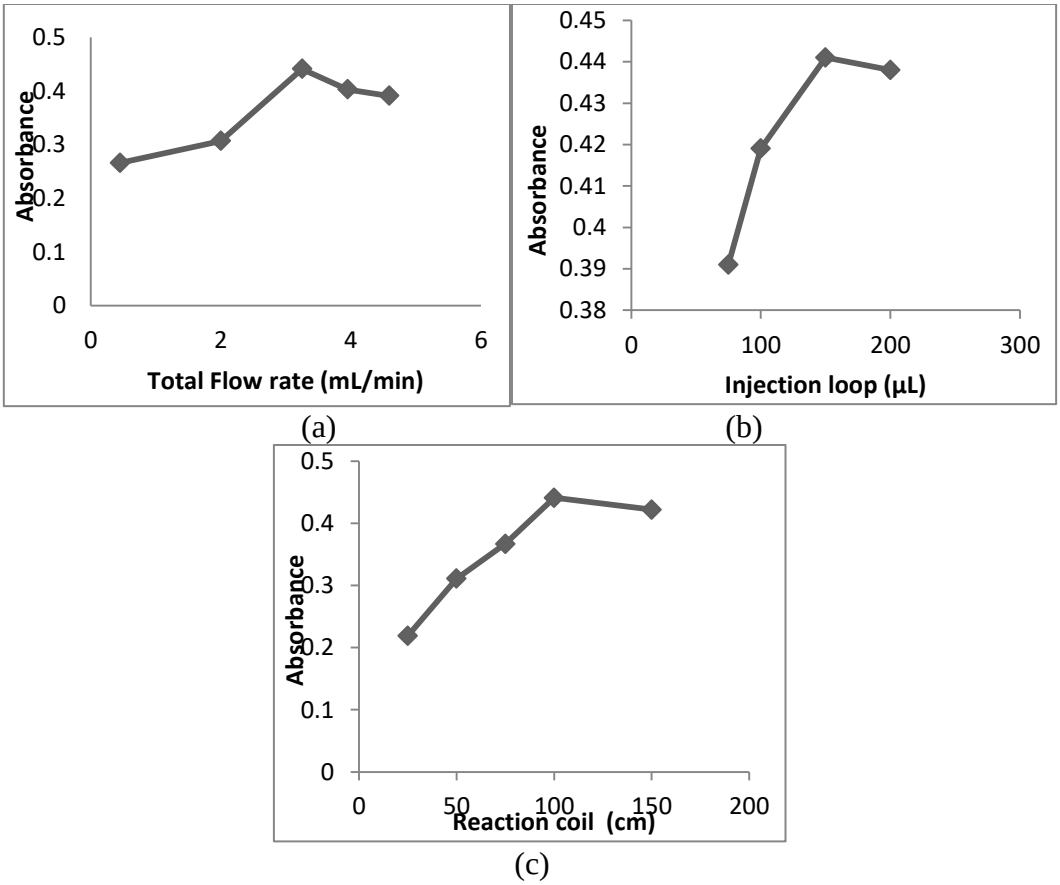
Figure 6: (a) type of base versus the absorbance, (b) Concentration 0f base versus the absorbance

Optimization of Physical parameters

The total flow rate plays an important part in the FIA system since it is used to determine the best reaction time and has a direct impact on sampling frequency. Because the flow rate increased, the dispersion and reagent usage increased as well. As the flow rate increased, the absorbance and sensitivity decreased. In the range of GEN, the influence of flow rate on the sensitivity of the colored reaction product was investigated. the optimum rate of reaction was 3.25 mL /min , Because of dispersion and dilution, the absorbance was reduced above these flow rates. The result of the flow rate calculation is shown in Figure (7.a).

By using sample loops ranging from 75 to 200 µL, the effect of injection volume was investigated, with the highest absorbance achieved at (150 µL) Fig (7. b). After then, the sample volume was used in further studies.

Effect of reaction coil length to enhance the sensitivity of the colored reaction product and increase mixing of the reactants. The dispersion of reactant zone probably increase by using higher reaction coil lengths .Different reaction coil lengths were studied in the range of (25 to 150 cm) Figure(7. c), coil length of 75 cm gave the maximum absorbance for method respectively, therefore were selected and used in subsequent experiments.



(a) (b)

(c)

Figure 7: (a) Effect of total flow rate , (b)Effect of injection loop, (c) Effect of reaction coil of the absorbance

Analytical characteristics

Under optimum conditions for GEN estimation, the calibration curve was constructed. An increasing amount of sample containing (0.25–2.75) µg/mL of GEN was transferred to a series of 10mL standard flasks (covering the range 0.25-2.75 µg/mL), then 1 mL of 0.005 M 4AMA solution, 1 mL of 0.1 M solution of NaOH, and 3 mL of 10% (v/v) Triton X-114 were added and the procedure was carried out. Figure (8) shows the linearity between the measured absorbance and the concentration of the GEN in solution. The least-squares method was used to create the regression equation. The intercept, slope, correlation coefficient, and molar absorptivity values for the calibration curve, as well as the values of analytical statistical treatments, are calculated and given in Table 1.

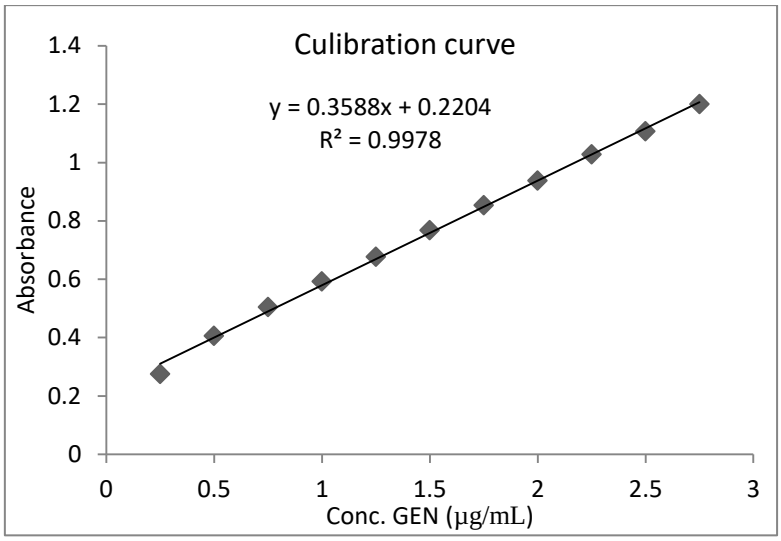


Figure 8: Calibration graph for method A

For method B Under the optimum conditions, the linear calibration graphs were obtained from of GEN standard solutions and prepared by appropriate dilution of the stock solution. The calibration graphs were plotted between the concentrations of GEN against the absorption intensity, Figure(9), which showed that Beer's law was obeyed at the concentration range of 5 to 100 µg/mL with a correlation coefficient of (0.9977).

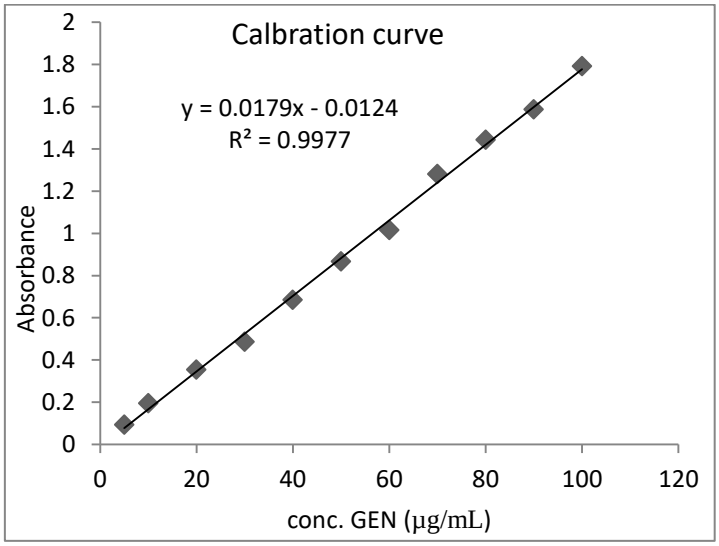


Figure 9: Calibration graph for method B

Table 1: the statistical data of GEN estimation with CPE and FIA

Parameter	Method A	Method B
Regression equation	Y=0.358X+0.220	Y=0.017X+0.012
Correlation coefficient, R^2	0.9978	0.9977
Linearity percentage, $\%r^2$	99.56	99.54
Dynamic rang (µg/mL)	0.25- 2.75	5-100
Slope , b	0.358	0.017
Intercept , a	0.22	0.012
Limit of detection , LOD	0.027	0.582
Limit of quantification, LOQ	0.083	1.76
Molar absorptivity , Ɛ (L mol^{-1} cm^{-1})	96.963 x 10^3	48.373 x 10^2
Sandell's sensitivity, S (µg cm^{-2})	0.0028	0.0559
Preconcentration factor	2.7	---
Enrichment factor	20.5	---
Standard deviation of the intercept. S_a	0.0096	0.3404
Standard deviation of the slope, S_b	0.0056	0.0058
Standard deviation of the residuals, $S_{y/x}$	0.0148	0.5899

Accuracy and precision

To evaluate the accuracy and precision of suggested methods (A and B), two set of different concentrations of GEN solutions were analyzed according to suggested method in five replicates. Table 2 summarizes the analytical values obtained as a result of the procedure. The suggested approach has good accuracy and precision, as indicated by the low percentage RSD values for precision and the accepted values of recovery percent for accuracy

Table 2: Accuracy and precision for both methods A and B

Sample (µg/mL)	Found	Rec.%	RSD%
	method A		
0.5	0.52	104	0.3
1	1.04	104	0.3
1.5	1.53	102	0.1
	method B		
20	20.3	101.5	0.84
40	39.7	99.25	0.6
50	50.2	100.4	0.23

The applications of developed methods in real samples

Solutions of pharmaceutical formulations were prepared as in the prior section. The developed CPE and FIA systems were successfully applied (Table 3) for GEN determination in capsules (SubstitutionalMedicineSolutions, Inc.,125 mg) and by injection of three different concentrations of GEN in FIA under the optimum conditions. Table 4 shows a comparison of GEN determination in pharmaceutical applications by two methods cloud point extraction and the classical UV method.

Table 3: determination of GEN in supplements using direct application for both methods (A,B)

Sample (µg/mL)	Found (µg/mL)	Error%	Rec.%	RSD%
Method A				
1	1.01	1	101	**0.4**
1.5	1.49	-0.7	99.3	**0.4**
2	1.96	-2	98	**0.3**
Method B				
20	20.2	+1	101	**0.84**
40	39.2	-2	98	**0.6**
50	50.2	+0.4	100.4	**0.4**

Table 4: Determination of GEN in supplements using standard addition for CPE and the classical UV methods.

Sample (µg/mL)	Found (µg/mL)	Error%	Rec.%	RSD%
CPE				
0.75	0.77	2.7	102.7	0.9
1	1.01	1	101	0.9
1.25	1.249	-0.08	99.92	0.6
Classical UV				
1	0.98	-2	98	1.1
2	2.03	1.5	101.5	1
3	3.02	0.7	100.7	0.7

Conclusion

The proposed methods, cloud point extraction and FIA-spectrophotometric can be used to determine the concentration of GEN in pure and pharmaceutical formulations with accuracy and precision. CPE is a simple and sensitive method, procedure, as well as a safe and economical method for preconcentration and spectrophotometrically determination of GEN. The validation of the methods yielded favorable results, with linearity, repeatability, and sensitivity all grading well.. The suggested FIA spectrophotometric approach for the determination of GEN in pure and pharmaceutical forms has the benefits of simplicity, speed, and accuracy.

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