



New Spectrophotometric Methods for Estimation of Diosmin in Pharmaceutical Formulations Using Batch and FIA Systems

Mayasa Mansour Mohammed*, Sadeem Subhi Abed

Department of Chemistry, College of Science, University of Baghdad, Baghdad, Iraq

Received: 30/1/2021

Accepted: 23/5/2021

Abstract

A new attempt is made to determine diosmin (DIO) in its pure form and in dietary supplements by using spectrophotometric flow injection analysis (FIA) assay method conjugated with batch method. The analysis was achieved depending on the oxidative coupling reaction with N, N-dimethyl-p-phenylenediamine (DMPD) to form a green dye which is measured at wavelength of 677 nm. The tested methods were found to be economical, delicate, precise and sturdy. The validation variables of the batch and FIA methods gave linearity in the determination range of DIO (1-35) $\mu\text{g/mL}$ and (5-120) $\mu\text{g/mL}$ demonstrated calibration graphs with linearity coefficient values of $r^2=0.9989$ and $r^2=0.9991$, respectively. Limits of quantitation (LOQ) values were found to be (0.8463 and 1.022) $\mu\text{g/mL}$, while limits of detection (LOD) were (0.2539 and 0.3067) $\mu\text{g/mL}$ for the two methods, respectively. The precision for the developed methods denoted by relative standard deviation (RSD %), were 0.386 and 0.55 %, while the accuracy based on recovery values (Rec %) were 100.273 and 100.24, respectively. The relative error (RE %) was less than 1% for the batch method and (1.1%) for the FIA method. The values of these parameters were observed to fall within the specified accepted limits; therefore, the tested methods seem to be adequate for the analysis of DIO in pharmaceutical preparations.

Keywords: Flavonoids, diosmin, coupling, flow injection analysis, N, N-dimethyl-p-phenylenediamine.

طرق طيفية جديدة لتقدير الدايسمين في المستحضرات الصيدلانية باستخدام أنظمة الدفعة والتحليل بالحقن الجرياني

مياسه منصور محمد* ، سليم صبحي عبد

قسم الكيمياء، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

تم اجراء محاوله لتقدير الدايسمين (DIO) في شكله النقي وفي المكملات الغذائية بواسطة طريقة فحص طيفية جديدة للتحليل بالحقن الجرياني مقترنة بطريقه الدفعة. يتم اجراء التحليل اعتمادا على تفاعل الازدواج التأكسدي لتكوين صبغة خضراء تم قياسها عند الطول الموجي 677 نانوميتر. الطرق المختبرة اقتصادية، حساسة، دقيقه وقوية. أعطت متغيرات التحقق لطريقتي الدفعة والتحليل بالحقن الجرياني الخطية عند مدى تقدير الدايسمين (1-35) و(5-120) مايكروغرام/مل وبرهنت قيم معامل الخطية لمنحنيات المعايرة $r^2=0.9989$ and $r^2=0.9991$ على التوالي. وجدت قيم حدود الكمية (LOQ) لتكون (0.8463

*Email: mayasa.mansour93@yahoo.com

(0.2539 and 0.3067) (LOD) (1.022 and مايكروغرام/مل بينما كانت حدود الكشف
 مايكروغرام/مل للطريقتين على التوالي. دقة الطرق المطوره يشار اليها بالانحراف القياسي النسبي (RSD %)
 كانت % (0.386 and 0.55) بينما التوافق يعتمد على قيم الاستعادية (Rec %=100.273 and)
 100.24 على التوالي. كان الخطأ النسبي (RE %) اقل من (1%) لطريقة الدفعة و (1.1%) لطريقة FIA
 لوحظ ان قيم هذه المعلمات تقع ضمن الحدود المقبولة المحدده لذلك يبدو ان الطرق المختبرة مناسبة لتحليل
 الداوسمين في المستحضرات الصيدلانية.

1. Introduction

Flavonoids are a crucial group of phenolics phytochemicals with slight systemic poisonousness that are abundant in vegetables and fruits. They are considered as the most prevalent type of plant secondary metabolites, and participate the basic skeleton of (C6-C3-C6) [1-5]. Additionally, they exist in two different forms, namely the aglycones (free form) and the glycosides (the bonded form with the sugar molecule)[6]. In general, the taste and colour of food are conferred by flavonoids, which also play a role in the prevention of fat oxidation and the protection of enzymes and vitamins [7]. The antioxidant constituents of flavonoids have the capability of slowing or constraining the oxidation process caused by reactive oxygen species (ROS) and free radicals (FR) in the body [8]. Diosmin (DIO: C₂₈H₃₂O₁₅) belongs to a family of flavonoids that usually exists in most fruits and other plants, mostly of the Citrus spp. DIO is an adjusted form of hesperidin, also named as “diosmetin 7-O-rutinoside” [9-15]. Diosmin was reported to exhibit significant activities in promoting venous tone [16]. Furthermore; it has several advantageous effects against experimental renal and hepatic injuries [17, 18] with hepatocarcinogenesis [19], myocardial infarction [20], as well as diabetes mellitus [21]. The coupling reaction is one of the most important tools in organic synthesis [22]. In 1859, Berthelot was the first whom recorded the oxidative coupling reaction which was also known as indophenol reaction [23]. This reaction involves the emission of two electrons from the reactant then receives by an oxidant. It typically comprises the association of two or more organic substances in the presence of an oxidizing agent. These substances are oxidized to form effective intermediates. It is also a vital organic reaction that has extensive applications in analytical chemistry, with simple and extremely sensitive methods [24]. Different approaches have been developed for determination of pharmaceuticals in different matrices, based on oxidative coupling reaction like amoxicillin, Paracetamol, salbutamol sulphate and pyridoxine hydrochloride [25-28]. Nonetheless, there is no FIA-spectrophotometric technique was reported for determining DIO through the oxidative coupling reaction. For that reason, efforts were given in the current study for validation and development of an analytical method that is suitable to determine DIO in pharmaceutical formulations.

2. Experimental part

2.1. Apparatus

In the batch method, the absorbance measurements were carried out using a PD-303 spectrophotometer (APEL, JAPAN) with silica cells of 1cm. A digital single beam recording spectrophotometer, Shimadzu UV mini-1240 (Shimadzu, Kyoto, Japan) was employed for spectral and absorbance measurements using flow cells of quartz (Cecil) with 1 cm path length and 50 µL internal volumes for FIA method. A peristaltic pump of three channels (SHENCHEN, LabM1 model, China) was used to transport the sample solutions and reagents. The Injection valve (KNAUER, Germany) of 6-ways with different sample loops was applied to provide suitable injection volumes of sample and standard solutions. Flexible tubes of teflon material with (0.5 mm) internal diameter were utilized for different lengths of reaction coil (25-200) cm. A plastic syringe with 1 mL volume was used to inject the sample solutions and reagents.

2.2. Reagents and chemicals

The used chemicals of analytical-reagent grade, also the commercial sources of DIO supplements were from (Nutrition Greenlife, USA), as well distilled water was used in all experiments.

2.2.1. Sample preparation

DIO stock solution (200 µg/mL)

This solution (Carl Roth GmbH & Co. Kg) was prepared by dissolving 0.02 g of the pure DIO in 10 mL of NaOH (0.5M) then completed to the mark with distilled water in 100 mL volumetric flask. The prepared solution remain for two weeks.

Dietary supplement solutions (200 µg/mL)

Ten capsules of commercial DIO (Nutrition Greenlife, USA ,750 mg) were precisely weighed, then the average weight of the contents was taken to obtain the weight of one capsule. A portion of DIO powder which equivalent to 0.02 g of DIO (pure drug) dissolved in 10 mL of 0.5 M of NaOH. The solution was mixed well, shaken and filtered to 100 mL volumetric flask. Then the volume was completed with distilled water to the mark. The other dilutions required in this study were prepared with distilled water.

2.2.2. Reagents preparation

N, N-dimethyl-p-phenylenediamine (0.005 M)

This solution (Kanto chemical CO, Japan) was prepared daily for both of the suggested methods. Approximately 0.1045 g of DMPD was dissolved in a little volume of distilled water, then the volume is made up with the same solvent to 100 mL volumetric flasks.

Oxidants (0.005) M

The aqueous solutions of these oxidants were prepared by dissolving 0.0534 g of NaIO₄, 0.0675 g of K₂S₂O₈, 0.0823 g of K₃[Fe(CN)₆], and 0.0535 g of KIO₃ in 50 mL of distilled water.

Sodium periodate (0.003) M

The oxidant solution used in the FIA method was prepared by dissolving (0.0641 g) of sodium periodate (B.D.H, U.K) in a suitable amount of water in 100 mL volumetric flask.

Ammonium hydroxide (1 M)

This stock solution was prepared through diluting 18.7 mL of NH₄OH (B.D.H, U.K) in distilled water to the mark in 250 mL volumetric flask. Further suitable dilutions were prepared using distilled water.

2.3. General procedure

2.3.1. General batch procedure

A series of DIO solutions over the concentration range (1-35) µg/mL were prepared in flasks. A solution of 1 mL NaIO₄ was transferred, then a standard solution of DIO (200 µg/mL), about 1.5 mL of (0.005 M) DMPD, and 0.5 mL of NH₄OH (0.1 M) were added to each flask. The reactants were diluted with distilled water to the mark and then mixing well. At ambient temperature, the absorbance of the green product was measured in the wavelength of 677 nm after 3 min. A concentration of 20 µg/mL of DIO solution was used to optimize all the variables.

2.3.2. General FIA procedure

The carrier stream containing DMPD (0.005 M) was pumped by the peristaltic pump and reacted with 100 µL of the injected DIO (50 µg/mL). The resulting solution was combined with NaIO₄ (0.005 M) over a T-link. The solution was then combined with NH₄OH (0.1 M) through another T-link at flowrate of 3.4 mL/min, and mixed in 50 cm reaction coil to provide efficient mixing between all solutions. The green product was measured at 677 nm.

3. Results and discussion

3.1. Absorbance spectrum

The development of the formed dye was achieved under optimal reaction parameters. The

spectra of the formed product and the reagent blank are recorded between (390-900) nm. The starting investigation proved that the green dye has the maximum absorption at wavelength of 677 nm when measured against the reagent blank, the same results were obtained currently as illustrated in Figure 1.

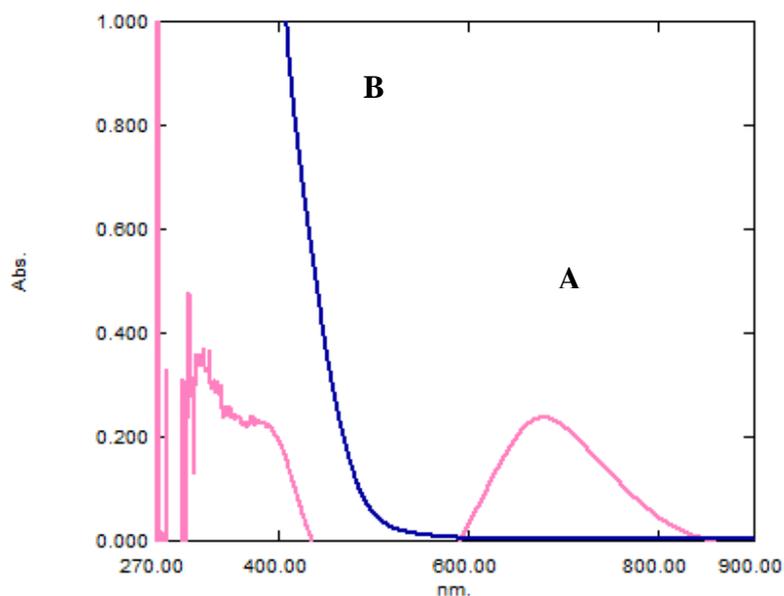
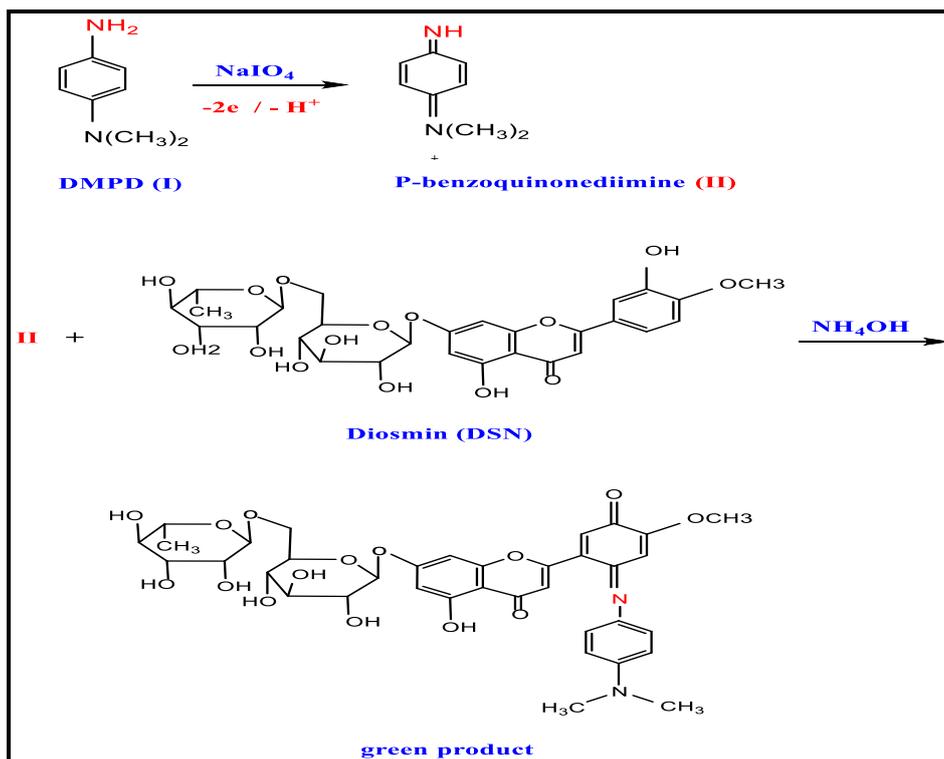


Figure 1- Absorption spectrum (A) 5 µg/mL of the formed product measured versus the reagent blank (B) the reagent blank measured versus distilled water.

3.2. The probable mechanism of the reaction product

Under the optimum conditions, the developed reaction should have occurred based on the oxidation of DMPD with sodium periodate. DMPD loses one proton and two electrons to form an active coupling species (electrophilic intermediate). The electrophilic substitution takes place for DMPD intermediate with DIO at slight basic medium to yield the colored product. Scheme (1) displays the proposed mechanism of the reaction [29]. The continuous variation and mole ratio methods were applied to establish the composition of the formed complex among each DIO and DMPD. In order to investigate the stoichiometry of the oxidative coupling reaction, equimolar concentrations of DIO and DMPD (5×10^{-3} M) were used. The absorbance of the green product was measured at 677 nm against the corresponding reagent blank. The results confirmed the ratio (1:1) of DMPD to DIO is formed for the current reaction.



Scheme 1-Pathways for the proposed mechanism of oxidative coupling reaction with DMPD

3.3. Optimization of reaction parameters for batch method

The experimental parameters affecting mostly the stability and development of the green product were carefully studied. This study tested the factors that impact the performance of the current batch method by the univariate method. Different volumes of DMPD (0.005) M ranging from 0.5-3 mL were studied to examine the effect of the volume of the coupling reagent on the sensitivity of the forming product. The greatest intensity was found to be at a volume of 1.5 mL of DMPD, which was necessary for the color development of the green product with minimal value of the blank. Hence, 1.5 mL of DMPD used in the subsequent experiments. NaIO_4 was found as an advantageous oxidative agent in the coupling reaction. Various oxidants, including $\text{K}_2\text{S}_2\text{O}_8$, NaIO_4 , KIO_3 , and $\text{K}_3[\text{Fe}(\text{CN})_6]$ were also examined; nevertheless, none showed highest absorbance than sodium periodate. The impact of altered volumes of NaIO_4 solution on the highest absorbance was investigated by varying the volume of NaIO_4 between 0.5-3 mL. The results revealed that the absorbance of the green dye increases with increasing NaIO_4 volume up to 1 mL. Over that volume the absorbance is reduced with highest value of the blank, as a result this volume was chosen for the next experiments. Initial studies revealed that the presence of the base in the reaction mixture was required for developing the oxidative coupling product in order to become more intense and stable. Accordingly, (0.1) M of different basic solutions such as KOH , Na_2CO_3 , NaOH and NH_4OH were tested to determine the best basic medium. The oxidation of DIO was accomplished in ammonium hydroxide medium with greatest color intensity and was sufficient for the reaction to proceed. To obtain the optimum volume of ammonium hydroxide solution, various volumes of NH_4OH solution were examined, in the range of 0.25-2 mL, while fixing the other variables. The volume of 0.5 mL of ammonium hydroxide found to be adequate to give the greatest absorbance and selected for further usage. Under the optimal conditions of the proposed method, four different orders of addition of DIO and the reagent were experimented. The optimum results and highest absorbance were obtained with addition order of (NaIO_4 +DIO+R+B) which designated for

reaction completion. The effect of the reaction time on the stability of the resulting green product was as well studied; the experimental results indicated that the product was formed intensively and immediately. The colour intensity was stable and sharply increased after 3 min; hence, 3 min was chosen as the optimum developing time and kept fixed for at least 30 min. In order to examine the effects of temperature on the formed dye product of the current method, different temperatures 5, 25 and 55°C were studied to achieve the oxidative coupling reaction. The stability of the developed green product was decreased at higher temperature (55 ° C) in the water bath. Also, the low temperature (5° C) caused a decrease in the sensitivity and absorbance with slowing down the reaction. For that reason, the reaction must be completed at 25 ° C and chosen for further used.

3.4. Optimization of reaction parameters for FIA method

In order to select the design of manifold under the starting FIA conditions, four manifolds of three-channels were adopted to achieve various reaction paths of DIO with DMPD in the presence of sodium periodate in basic medium, in addition to achieving a good sensitivity of the present method. The manifold illustrated in Figure 1 has a good repeatability, minimum values of dispersion, and maximum absorbance; therefore, it was selected for the next experiments.

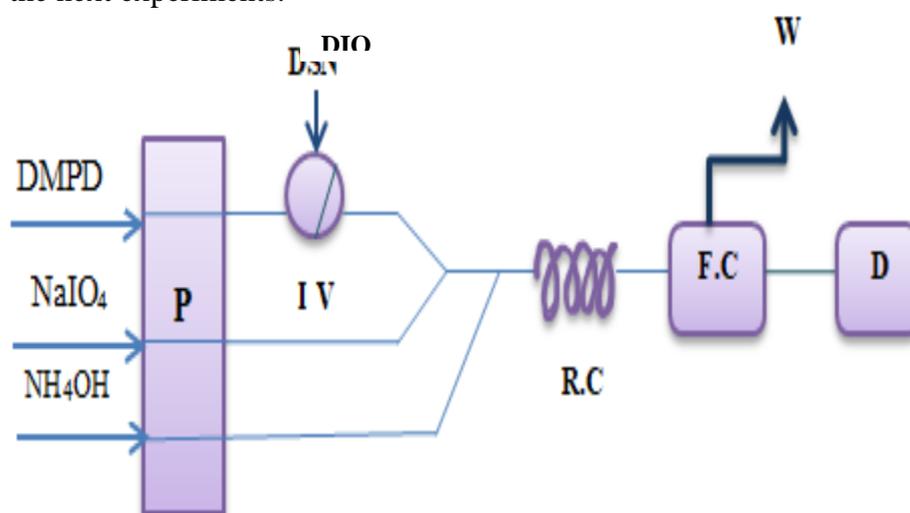


Figure 1-Manifolds used in FIA method for determination of DIO with DMPD in the presence of NaIO₄ in basic medium; P, Peristaltic pump; I.V, Injection valve; F.C, Flow cell; R. c; Reaction Coil ; D , Detector ; W, Waste .

3.4. 1.Effect of chemical variables

Altered concentrations of DMPD in the range of 0.001-0.015 M were studied and optimized, while fixing the other parameters. The concentration of 0.005 M DMPD developed the green product to highest intensity and provided the greatest absorbance to the suggested FIA method. Then, the absorbance of the reaction product started to decrease. On the other hand, a high blank signal was observed with the further increase in concentration to more than 0.005 M, and so 0.005 M of DMPD was considered to be optimum to further use, as illustrated in Figure 2,a. To examine the impact of the type of oxidizing agent on the green product, 0.005 M of different oxidants such as K₃ [Fe (CN)₆], NaIO₄, K₂S₂O₈, and KIO₃ were examined. It was found that NaIO₄ is necessary for the reaction between DIO with DMPD in the presence of ammonium hydroxide medium Figure 2,b. Different concentrations of NaIO₄, over the range of 0.001-0.01 M , were tested to select the suitable concentration of the oxidant. An increase in absorbance was noticed as NaIO₄ concentration increased up to 0.003 M , followed by a decrease in absorbance with higher concentrations because of the further oxidation .Figure 2,c

indicates the highest absorbance with 0.003 M of NaIO_4 , which was accordingly chosen for the following experiments. In accordance with the initial spectrophotometric investigation regarding to the influence of basic medium on the absorbance of the formed dye, ammonium hydroxide was found to be necessary for increasing the stability and developing the green product; therefore, various concentrations of ammonium hydroxide at the range (0.03-0.2) M were tested in oxidation solution. It was observed in Figure 2,d that the reaction implemented at the concentration of 0.1M of NH_4OH with highest absorbance and sensitivity. Hence, it was used throughout the study.

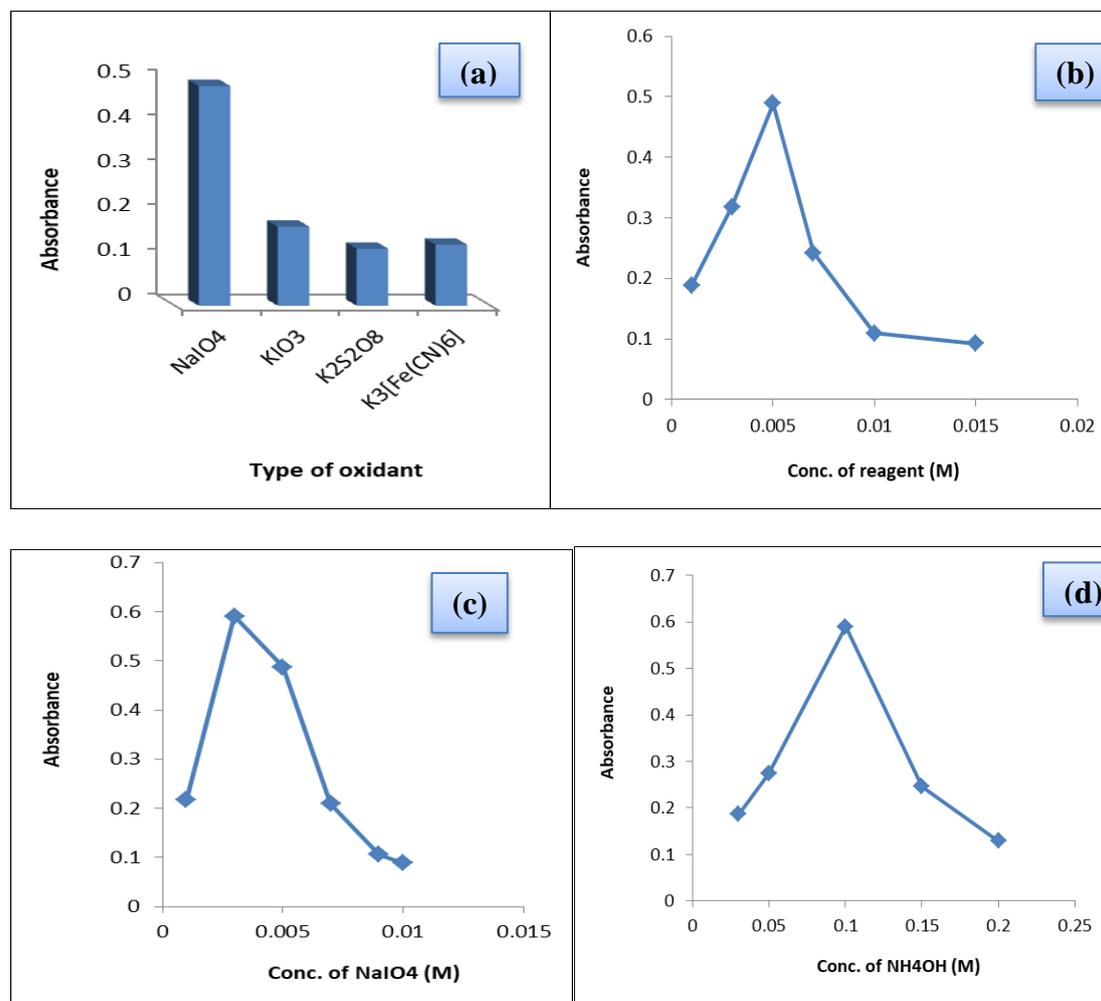


Figure 2- The Effect of (a) DMPD concentration ;(b) oxidant type; (c) concentration of NaIO_4 ; (d) concentration of NH_4OH on the absorbance

3.4. 2.Effect of physical variables

In order to select the most suitable flow rate, different flow rates were investigated on analytical signals at the range of 2-6.4 mL/min. The maximum sensitivity and best absorbance were obtained at flow rate of 3.4 mL/min and decreased slightly with higher flow rates, as illustrated in Figure 3,a.The high speed causes dispersion and insufficient time to mix the reactants which leads to a significant decrease in absorbance. On the other hand, the sampling rate decreases at lower rate. Consequently, a flow rate of 3.4 mL/min was achieved the compromise between sensitivity and sampling rate, thus, was chosen as the optimal value. The sensitivity of the reaction product is affected by coil length of the reaction. For that reason the effect of altered coil lengths (0-200) cm was tested in the same experimental

conditions designated above. The present study displayed that 50 cm coil length has a good precision and best absorbance, as can be seen from Figure 3,b. Higher lengths consume large volume of standards, and increase the dispersion as well as require longer time to accomplish the analysis. The absorbance was decreased continually beyond 50 cm coil length due to the resulting dilution from dispersion. Thus, 50 cm coil length which was selected. The volume of injected sample was varied by altering the sample loop length in the injection valve between 75-250 μL , whilst the other parameters remained constant. A noticeable increase in absorbance was found within the range as the injection volume was increased up to 100 μL , then the response decreased to larger injection volumes. Figure 3,c illustrates the highest sensitivity was obtained with a loop of 100 μL , which was chosen as the optimum loop for the recommended method. The sampling frequency was studied by calculating the time recorded from the instant of the injection until the appearance of the maximum signal; it was observed that the calculated time was 56 sec under the optimal conditions; hence, the sample throughput found to be 64 sample / hour for the current FIA method. Under the optimum manifold, the dispersion was also investigated in three streams of reactants which were passed through it. The dispersion value found as 1.430, which indicates that no convection and diffusion dispersion were obtained in the developed system.

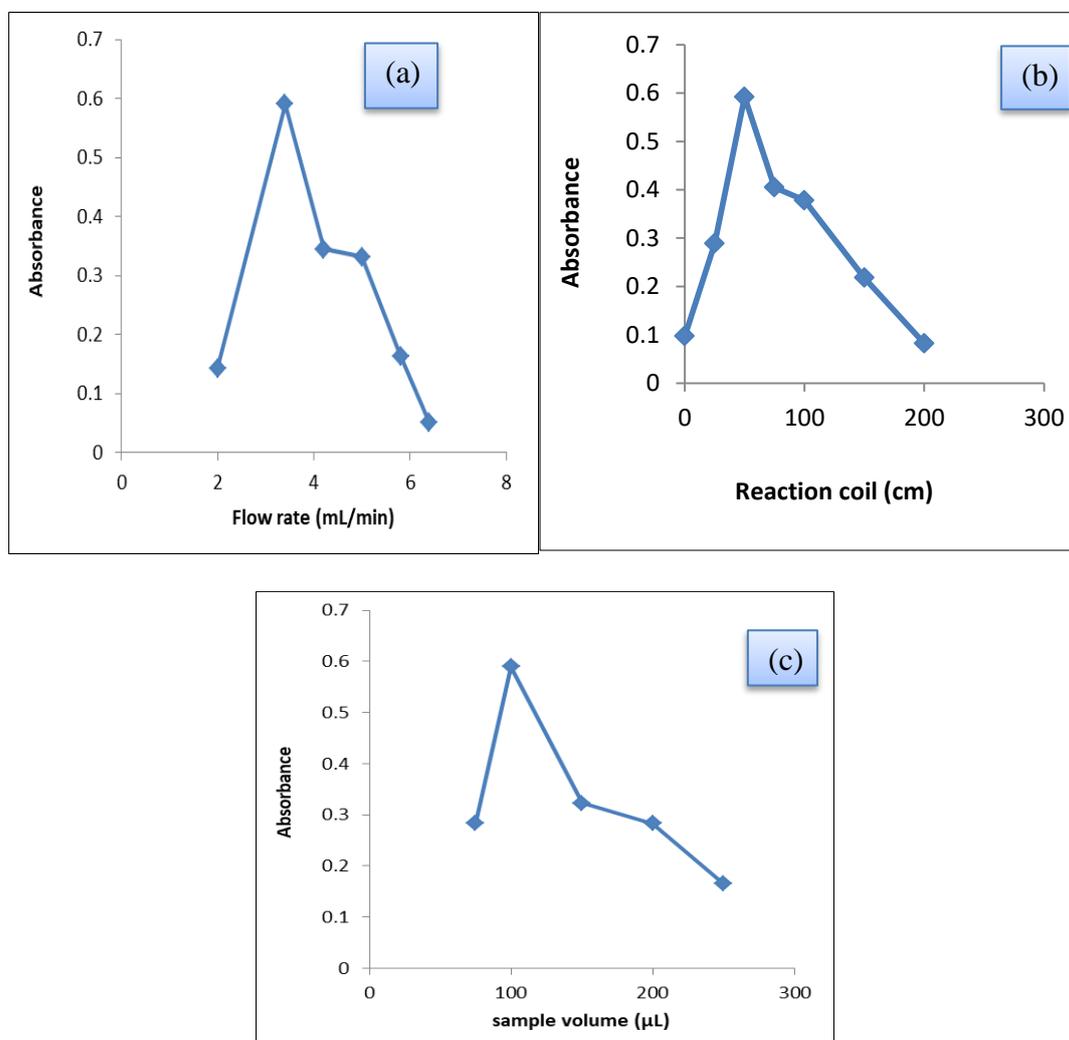


Figure- 3 The Effect of (a) reaction flowrate ;(b) coil length of the reaction; (c) sample volume on the absorbance

3.5. Analytical characteristics

After optimizing all the reaction parameters for the batch and FIA methods, the calibration graphs were accomplished by adding or injecting variable amount of DIO standard solution. The linearity of DIO concentrations was tested in three replicates and over the range 1-35 $\mu\text{g/mL}$ for the batch method and 5-120 $\mu\text{g/mL}$ for the FIA method; the absorbance was recorded at 677 nm versus the reagent blank. Table 1 summarizes the statistical treatments and the analytical data for the calibration graphs, which demonstrate the high precision and best sensitivity for the present batch and FIA methods.

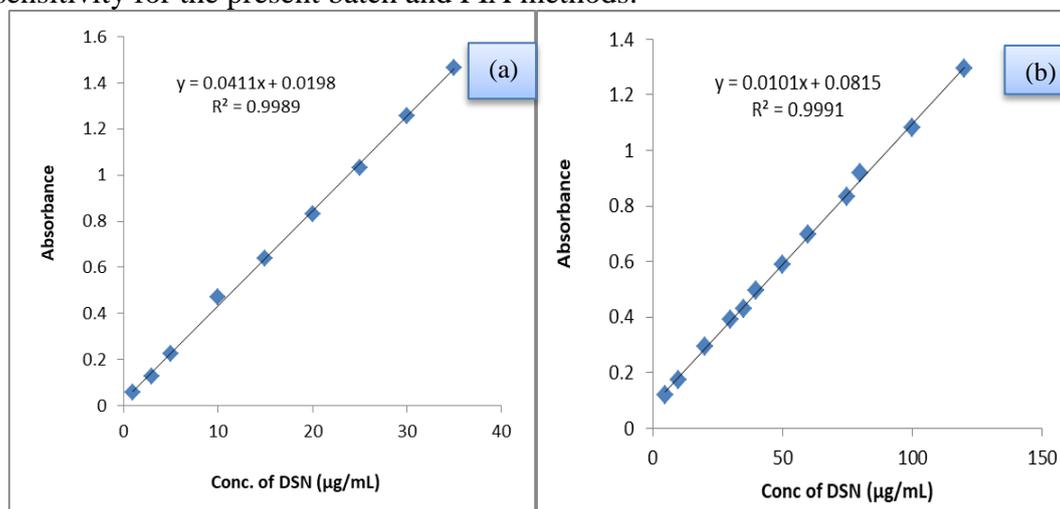


Figure 4- calibration graph of DSN for a: batch method, b: FIA system

Table 1- Analytical data and regression variables of the current methods

Parameters	Value of batch method	Value FIA method
Regression equation	$Y=0.0411x + 0.0198$	$Y=0.0101x + 0.0815$
Molar absorptivity ($\text{L mol}^{-1}\text{cm}^{-1}$)	2.5011×10^4	6.1460×10^3
Linearity coefficient, r^2	0.9989	0.9991
Linearity range ($\mu\text{g/mL}$)	1-35	5-120
Sandell's sensitivity ($\mu\text{g cm}^{-2}$)	0.0243	0.0990
LOQ ($\mu\text{g/mL}$)	0.8463	1.022
LOD ($\mu\text{g/mL}$)	0.2539	0.3067
standard deviation of the residuals $S_{y/x}$	1.761×10^{-2}	1.152×10^{-2}
Standard deviation of intercept, S_b	5.070×10^{-4}	9.667×10^{-5}
Standard deviation of slope, S_a	1.001×10^{-2}	6.035×10^{-3}

3.6. Accuracy and precision

The accuracy and precision for the proposed batch and FIA methods were investigated and analyzed at three altered concentrations in five replicates for each concentration. The highest values of relative standard deviation were found to be 0.697% and 0.862%, whereas the relative error values were between -0.94 to 0.95 % and -1.00 to 1.08 % for batch and FIA methods, respectively. These satisfactory results are listed in Table 2, which indicate the reasonable values of accuracy and precision.

3.7. Pharmaceutical applications

In order to examine the possibility of applying the current methods for the quantitative determination of DIO in capsules. Different concentrations of pharmaceuticals containing DIO were analyzed directly in (five replicates) for each concentration. The assay results in Table 2 were in agreement with the declared content. At 95% confidence level, an effective comparison was done for the recovery values between the suggested methods and the reported UV method. The values of the students "t-test and F-test" were calculated to confirm the performance for the present methods. Table 2 reveals that the calculated values of "t-test and

F-test" do not exceed the critical values. From the results of comparison, no significant differences were observed in accuracy and precision between the theoretical and calculated values of the two methods.

Table 2-Accuracy and precision with analytical applications of the proposed methods

Pharmaceutical preparation	Batch method					FIA method				
	Conc. of DSN $\mu\text{g/mL}$		%RE	%Rec	%RSD	Conc. of DSN $\mu\text{g/mL}$		%RE	%Rec	%RSD
	Take n	found *				Taken	found *			
DSN (pure)	5	5.04	0.82	100.82	0.69	30	30.32	1.08	101.08	0.86
	15	15.14	0.96	100.96	0.29	50	50.32	0.65	100.65	0.52
	20	19.81	-0.95	99.05	0.18	75	74.24	-1.00	98.99	0.27
DSN capsules (Nutrition Greenlife, USA ,750 mg)	5	5.01	0.24	100.24	0.85	30	30.40	1.35	101.35	0.59
	15	15.09	0.66	100.66	0.42	50	50.20	0.41	100.41	0.45
	20	19.83	-0.82	99.17	0.18	75	73.69	-1.74	98.25	0.31
t-test	0.0744		t**=4.303 ($n_1+n_2-2=2$)			*=average of five analysis ** = t and F-test theoretical values at 95% confidence level				
	0.185									
F-test	5.322		F**=161.4 ($n_1-1=1,(n_2-1)=1$)							
	4.807									

4. Conclusion

The oxidative coupling reaction is one of the most momentous reactions employed to analysis of drugs. Its applications for the spectrophotometric determination of diosmin in pure or dietary supplements were developed by the batch and FIA methods. The current methods were sensitive, efficient and showing low detection limit. In addition , no reports on the use of FIA and spectrophotometric analysis of diosmin with DMPD in the presence of NaIO_4 in basic medium are available. The suggested methods have several advantages as the usage of low-cost equipment, accuracy and easiness. To compare the batch with FIA methods, the latter is more suitable than the earlier method due to a good recovery, wider range of linearity to calibration graph, and higher speed for analysis (throughput of 64 sample/ hour). The precision and the rapidity of analysis for the present FIA system are appropriate to the quality control of supplements containing diosmin replacing the expensive chromatographic methods. The comparison of current and classical methods to diosmin determination is completed with the statistical methods which obviously reveals the validity. The widespread applicability of the novel methods in pharmaceutical formulations was established via the assay of diosmin on the trace concentration level .

5. Acknowledgments

The authors thank the department of chemistry at university of Baghdad for the help provided in this work.

References

- [1] C. R. Ferraz, T. T. Carvalho, M. F. Manchope, N. A. Artero, F. S. Rasquel-Oliveira, V. Fattori, *et al.*, "Therapeutic potential of flavonoids in pain and inflammation: mechanisms of action, pre-clinical and clinical data, and pharmaceutical development," *Molecules*, vol. 25, pp. 762, 2020.
- [2] R. Pei, X. Liu, and B. Bolling, "Flavonoids and gut health," *Current Opinion in Biotechnology*, vol. 61, pp. 153-159, 2020.

- [3] A. Adamczak, M. Ożarowski, and T. M. Karpiński, "Antibacterial activity of some flavonoids and organic acids widely distributed in plants," *Journal of Clinical Medicine*, vol. 9, pp. 109, 2020.
- [4] D. Seleem, V. Pardi, and R. M. Murata, "Review of flavonoids: A diverse group of natural compounds with anti-Candida albicans activity in vitro," *Archives of oral biology*, vol. 76, pp. 76-83, 2017.
- [5] L. M. Cordenonsi, R. M. Sponchiado, S. C. Campanharo, C. V. Garcia, R. P. Raffin, and E. E. S. Schapoval, "Study of flavonoids presente in Pomelo (Citrus máxima) by DSC, UV-VIS, IR, 1H and 13C NMR and MS," *Drug Analytical Research. Porto Alegre*. Vol. 1, no. 1 (2017), pp. 31-37, 2017.
- [6] C. Rodríguez-García, C. Sánchez-Quesada, and J. J. Gaforio, "Dietary flavonoids as cancer chemopreventive agents: An updated review of human studies," *Antioxidants*, vol. 8, pp. 137, 2019.
- [7] S. Izzo, V. Naponelli, and S. Bettuzzi, "Flavonoids as Epigenetic Modulators for Prostate Cancer Prevention," *Nutrients*, vol. 12, pp. 1010, 2020.
- [8] S. Pitaro, L. Fiorani, and N. Jorge, "Potencial antioxidante dos extratos de manjeriçao (Ocimum basilicum Lamiaceae) e orégano (Origanum vulgare Lamiaceae) em óleo de soja," *Revista Brasileira de Plantas Mediciniais*, vol. 14, pp. 686-691, 2012.
- [9] S. Shabani and M. A. Mirshekar, "Diosmin is neuroprotective in a rat model of scopolamine-induced cognitive impairment," *Biomedicine & Pharmacotherapy*, vol. 108, pp. 1376-1383, 2018.
- [10] V. V. Prabhu, D. Sathyamurthy, A. Ramasamy, S. Das, M. Anuradha, and S. Pachiappan, "Evaluation of protective effects of diosmin (a citrus flavonoid) in chemical-induced urolithiasis in experimental rats," *Pharmaceutical biology*, vol. 54, pp. 1513-1521, 2016.
- [11] C. Caristi, E. Bellocco, V. Panzera, G. Toscano, R. Vadalà, and U. Leuzzi, "Flavonoids detection by HPLC-DAD-MS-MS in lemon juices from Sicilian cultivars," *Journal of agricultural and food chemistry*, vol. 51, pp. 3528-3534, 2003.
- [12] F. I. Kanaze, C. Gabrieli, E. Kokkalou, M. Georgarakis, and I. Niopas, "Simultaneous reversed-phase high-performance liquid chromatographic method for the determination of diosmin, hesperidin and naringin in different citrus fruit juices and pharmaceutical formulations," *Journal of pharmaceutical and biomedical analysis*, vol. 33, pp. 243-249, 2003.
- [13] F. R. Marín and J. A. Del Río, "Selection of hybrids and edible Citrus species with a high content in the diosmin functional compound. Modulating effect of plant growth regulators on contents," *Journal of agricultural and food chemistry*, vol. 49, pp. 3356-3362, 2001.
- [14] A. Noorafshan, S. Karbalay-Doust, and F. Karimi, "Diosmin reduces calcium oxalate deposition and tissue degeneration in nephrolithiasis in rats: a stereological study," *Korean journal of urology*, vol. 54, pp. 252-257, 2013.
- [15] S. Srinivasan and L. Pari, "Ameliorative effect of diosmin, a citrus flavonoid against streptozotocin-nicotinamide generated oxidative stress induced diabetic rats," *Chemico-Biological Interactions*, vol. 195, pp. 43-51, 2012.
- [16] P. C. Lyzengr Williamson K, "Micronized purified flavonoid fractions. A review of its use in chronic venous insufficiency, venous ulcers and haemorrhoids," *Drugs*, vol. 63, pp. 71-100, 2003.
- [17] M. U. Rehman, M. Tahir, A. Q. Khan, R. Khan, A. Lateef, O. O. Hamiza, *et al.*, "Diosmin protects against trichloroethylene-induced renal injury in Wistar rats: plausible role of p53, Bax and caspases," *British journal of nutrition*, vol. 110, pp. 699-710, 2013.
- [18] M. Tahir, M. U. Rehman, A. Lateef, R. Khan, A. Q. Khan, W. Qamar, *et al.*, "Diosmin protects against ethanol-induced hepatic injury via alleviation of inflammation and regulation of TNF- α and NF- κ B activation," *Alcohol*, vol. 47, pp. 131-139, 2013.
- [19] M. Tahir, M. U. Rehman, A. Lateef, A. Q. Khan, R. Khan, W. Qamar, *et al.*, "Diosmin abrogates chemically induced hepatocarcinogenesis via alleviation of oxidative stress, hyperproliferative and inflammatory markers in murine model," *Toxicology letters*, vol. 220, pp. 205-218, 2013.
- [20] S. S. Queenthy and B. John, "Diosmin exhibits anti-hyperlipidemic effects in isoproterenol induced myocardial infarcted rats," *European journal of pharmacology*, vol. 718, pp. 213-218, 2013.

- [21] H. H. Arab, S. A. Salama, H. A. Omar, E.-S. A. Arafa, and I. A. Maghrabi, "Diosmin protects against ethanol-induced gastric injury in rats: novel anti-ulcer actions," *PloS one*, vol. 10, pp. e0122417, 2015.
- [22] T. Zhang, N.-X. Wang, and Y. Xing, "Advances in Decarboxylative Oxidative Coupling Reaction," *The Journal of organic chemistry*, vol. 83, pp. 7559-7565, 2018.
- [23] Y. Liang, C. Yan, Q. Guo, J. Xu, and H. Hu, "Spectrophotometric determination of ammonia nitrogen in water by flow injection analysis based on NH₃-o-phthalaldehyde-Na₂SO₃ reaction," *Analytical chemistry research*, vol. 10, pp. 1-8, 2016.
- [24] G. P. Gorumutchu, V. N. RATNAKARAM, and S. Malladi, "Determination of Riociguat by Oxidative Coupling Using Visible Spectrophotometry," *Oriental Journal of Chemistry*, vol. 35, pp. 48-53, 2019.
- [25] A. M. Aljeboree, "Spectrophotometric and Colorimetric Determination of Pharmaceutical by Oxidative Coupling Reaction: A," *analytical methods*, vol. 31, p. 32, 2020.
- [26] M. Q. Al-Abachi and H. Hadi, "Flow injection-spectrophotometric determination of amoxicillin based on its oxidative condensation with 4-aminoantipyrine," *IJS*, vol. 50, pp. 8-15, 2009.
- [27] M. Q. Al-Abachi and S. Subhi, "Flow injection-spectrophotometric determination of salbutamol sulphate and pyridoxine hydrochloride using 2, 4-dinitrophenylhydrazine," *Iraqi J. Sci*, vol. 54, pp. 6-16, 2013.
- [28] M. Q. Al-Abachi, S. A. Al-Safi, and H. S. Al-Ward, "Spectrophotometric Kinetic Methods for the Determination of Paracetamol in Pure Form and Pharmaceutical Preparations," *Iraqi Journal of Science*, vol. 56, pp. 2704-2717, 2015.
- [29] M. Q. Al-Abachi, H. Haddi, and A. M. Al-Abachi, "Spectrophotometric determination of amoxicillin by reaction with N, N-dimethyl-p-phenylenediamine and potassium hexacyanoferrate (III)," *Analytica chimica acta*, vol. 554, pp. 184-189, 2005.