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Analytical Determination of Diosmetin 7-O-Rutinoside through Reversed Flow Injection Analysis with Brady's Reagent and $NaIO_4$ as Oxidizing Agent

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Abstract

Diosmetin 7-O-rutinoside (DSM) is an important pharmaceutical compound requiring reliable quantification methods. This study aims to develop and optimize a spectrophotometric procedure using reverse flow injection analysis (rFIA) with 2,4-dinitrophenylhydrazine (DNPH) as a derivatizing agent in an alkaline medium. Preliminary experiments were conducted to optimize reaction conditions and establish suitable parameters for determining DSM compounds. The rFIA technique was used to automate the derivatization process, and absorbance was measured spectrophotometrically. The optimized rFIA method enabled sensitive and selective detection of the DSM compound through its reaction with DNPH. The approach demonstrated practicality for pharmaceutical analysis, avoiding the need for expensive chromatographic instrumentation or toxic solvents. All numerical results for the maximum wavelength of the reaction at 549 nm, the applicability of Beer-Lambert's law (y= 0.0107x+0.0162), the linearity range (2-100 µg/mL), the sensitivity of the method, the limit of detection (2.412 µg/mL), the quantum limit of detection (11.372 µg/mL), and the stability constant recorded 0,9995. The developed rFIA-spectrophotometric method provides a simple, cost-effective, and environmentally friendly alternative for routine quality control of DSM compounds in pharmaceutical laboratories.

Keywords: Brady's reagent, Diosmetin 7-O-rutinoside, Pharmaceuticals analysis, Reverse flow injection analysis, UV-Vis spectrophotometry.

1. Introduction

Diosmetin 7-O-Rutinoside (DSM) is a phenolic compound containing a flavonoid moiety, which is O-glycosidically linked to a carbohydrate moiety at the C7-position[1]. One potential bioactive compound is DSM, which has attracted considerable scientific interest due to its potent antioxidant and anti-inflammatory effects. It fights oxidative stress, which damages cells and tissues during the normal aging process and is linked to health problems associated with growing old, not to mention chronic diseases[2]. The anti-inflammatory action of DSM is involved in regulating biochemical pathways related to immuno-logical processes, which justifies its use as a pharmaceutical for therapeutic purposes in cardiovascular interventions and in the treatment and prevention of venous insufficiencies, as well as other inflammatory diseases[3,4]. The IUPAC name of DSM is 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl), which reflects its complex molecular structure[5,6]. This nomenclature specifies the stereochemistry and functional groups involved in DSM's pharmacological activities. Being a glycoside of a flavonoid, it is linked to sugar moieties on the flavone scaffold, thereby increasing its solubility and bioavailability. This framework is relevant to the antioxidant, anti-inflammatory, and medicinal effects of this agent[7-9]. Its chemical structure has a molar mass of 608.549 g/mole and a molecular formula of $C_{28}H_{32}O_{15}$. It has selective solubility, dissolving in DMSO and NaOH but being insoluble in

water, which could affect the formulation and pharmacological applications. Different quantitative determination of DSM by flow injection analysis (FIA) technique used normal flow injection analysis for determining DSM through charge transfer interaction with 4-methylamino phenol[10], FIA assay method conjugated with batch method, the analysis was achieved depending on the oxidative coupling reaction with N, N-dimethyl-p-phenylenediamine[11], Multisyringe flow injection analysis (MSFIA) systems in the determination of metals, metalloids, organic and inorganic compounds in several kind samples employing spectroanalytical techniques[12], FIA is a highly efficient and automated analytical technique used for the rapid determination of various analytes. When coupled with spectrophotometric detection, FIA based on diazotization-coupling reactions offers a simple, sensitive, and cost-effective method for quantifying pharmaceutical compounds that possess a primary aromatic amine or can be derivatized to form one[13]. The 2,4-dinitrophenylhydrazine (DNPH), or Brady's reagent, is sold[14]; its chemical formula is $2NHNH_2C_6H_3(NO_2)_2$, which contains two ortho- and para-nitro substituents[15], as opposed to hydrazine. It is a dense solid with colors ranging from orange to red[16]. The range of assay procedures, including coupling reactions, diazotization, and oxidative coupling[17], was employed to determine various chemicals quantitatively by spectrophotometry. As a result, rapid detection and monitoring tools are required to prevent their potential adverse effects on ecosystems and human health. One of the most popular FIA methods is reverse flow injection analysis (rFIA), which differs from standard FIA in that the reagent is injected into the flowing sample stream rather than the sample being delivered into a reagent-containing carrier solution. Following injection of the sample, the system can be returned to the FIA standard configuration[18,19]. The method has advantages in reducing reagent use and preventing waste discharge; it is therefore an excellent one economically and environmentally. Reagent–sample mixing efficiency is enhanced with rFIA, thereby increasing analytical sensitivity. Another great characteristic of this technique is its ability to load different reagents into the same sample stream, which permits multiple analyses to be performed easily with just one sample[20]. This study aims to establish a scientific basis for the further development and application of the DSM compound in the pharmaceutical field by systematically evaluating its chemical constituents. This directly leads to the validation or application of the quantitative analytical method used in this study.

2. Materials and Methods

2.1. Preparation of DSM stock standard

To obtain a concentration of 20 µg.mL^{-1}, 0.0200 g of the DSM compound was dissolved in 10 mL of 0.5 M NaOH to prepare a stock solution (**Figure 1**). The final volume was obtained by diluting the solution with 100 mL of DW. To prevent photochemically induced degradation, the stock solution was then left in the dark for two weeks. Under the preparation and storage conditions, this guarantees that we are dealing with a reliable and consistent solution for future analytical methods and experimental tests on a durable medium[21].

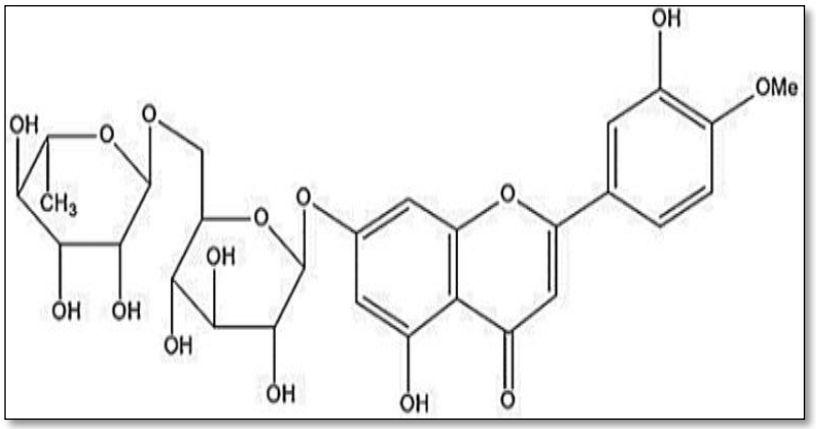

Figure 1. DSM chemical structure[22]

2.2. Preparation of DSM tablets

An analytical balance was used to weigh ten commercial DSM pills (750 mg each, Nutrition Green Life, USA). The mean weight of total tablets was calculated to know the actual quantity of DSR present in each dose. To achieve complete solubilization, 10 mL of 0.5 M NaOH solution was used to dissolve an amount of unaltered DSM powder equivalent to 0.0200 g of pure DSM. The solution obtained was further filtered to remove insoluble excipients and particulate matter. A 100 mL volumetric flask containing the filtrate was filled with distilled water to the appropriate level. This produced a stock solution with a defined concentration, which was then diluted with distilled water to prepare all of the study's working solutions[23].

2.3. The 2,4-dinitrophenylhydrazine

In the presence of 2 mL of concentrated sulfuric acid, 0.0991 g of 2,4-dinitrochlorobenzene and hydrazine sulfate reacted to generate DNPH. This reaction led to a final solution of 0.005 M in the presence of potassium acetate (KOAc), as shown in **Figure 2**. The final solution was transferred to a 100 mL graduated flask to ensure proper dilution and homogeneity. The final total volume was adjusted by adding distilled water up to the calibration mark. This operation resulted in a reagent solution for further experimentation and analysis, yielding consistent results across all measurements in this study[24].

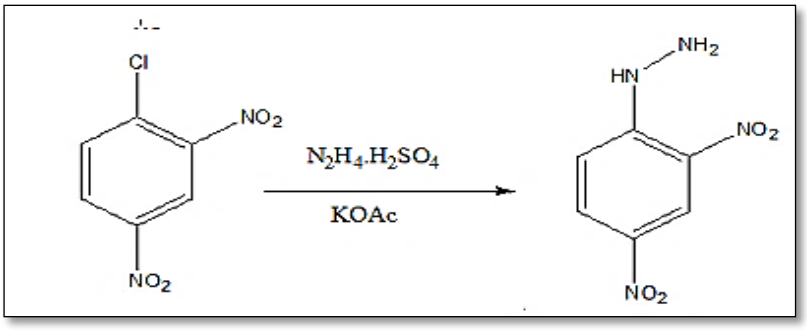

Figure 2. Preparation of DNPH reagent[25]

2.4. Solution of sodium periodate

An accurately weighed amount (0.4278 g, 0.02 M) of sodium periodate in a small volume of DW was transferred to a 100 mL volumetric flask and diluted to the mark with DW[26].

2.5. Apparatus

2.5.1. UV –Vis spectrophotometer

A Shimadzu UV mini-1240 UV–Visible single-beam spectrophotometer (Shimadzu Corporation, Japan) was used to record absorbance spectra for both nFIA and rFIA procedures. Under these experimental conditions, precise and reproducible measurements were carried out using a 50-µL-volume flow-through cell with a 1-cm optical path length. This setting allows the rule of concentration to apply in small-volume samples, enhances detection sensitivity, reduces reagent requests, and provides consistent spectral response across analyses[27].

2.5.2. Flow injection system

For accurate and controllable fluid flow, an adjustable peristaltic pump (Shennchen, Lab N1, China) with four independent channels was used to deliver the reagent and sample solutions into the system. An automated reagent injector system (loop injector: 6 positions) from Knauer (Germany) that carries a set of loops with varying inner diameters, intended for different volumes to optimize analysis parameters, was used in this study. The FIA absorbance readings were obtained using 50-µL volumes and 1-cm-path-length-matched quartz flow cells. The system allowed accurate and repeatable quantification. The rFIA system consists of a peristaltic pump, an injection valve, PTFE tubing, a reaction coil, and a detector such as a UV–Visible spectrophotometer equipped with a flow cell. In this system, the reagent is injected into a continuously flowing sample stream, allowing rapid mixing and reaction within the reaction coil. The resulting signal is then measured by the detector and recorded by a data acquisition system

for quantitative analysis, thereby providing optimized sensitivity, robustness, and overall reliability of the analytical procedure for routine applications[28,29].

3. Results

The interaction between DNPH and the DSM compound was confirmed by examination of the absorbance spectra of the oxidative coupling reactions. In the current study, 1 mL of standard DSM solution (20 ppm) was mixed with 1 mL of 0.02 M sodium periodate and 2 mL of 0.5 M NaOH. Two mL of test DNPH reagent (0.005 M) were then added, producing a purple product. The mixed solution was then diluted with distilled water to a constant volume (25 mL) in a conical flask to reach a uniform concentration and best measurement conditions. A UV-Vis spectrophotometer was used to analyze the absorption spectrum of the colored product.

3.1. General rFIA method

DSM compound was quantified using a two-channel manifold system designed to optimize reaction conditions, thereby enhancing the absorbance signal generated when DSM compound interacts with oxidized DNPH in an alkaline medium containing sodium periodate. The impact of several physical and chemical parameters on the intensity of the resulting color was assessed for the rFIA process. Optimal conditions were investigated using a one-factor-at-a-time approach, with the other factors held at their levels, as shown in **Table 1**.

Table 1. Initial conditions in terms of chemistry and physics

Parameter	Value
DSM compound	20 µg mL^{-1}
Oxidant Types	$FeCl_3$, $NaIO_4$ $K_2S_2O_3$, $K_3[Fe(CN)_6]$ and $(NH_4)_2Ce(NO_3)_6$)
Conc of $NaIO_4$	0.02 M
Conc of DNPH	0.005 M
Flow rate	20 mL/min
Sample Volume	150 µL
Reaction Coil	75 cm

3.2. Study of manifold design

For a survey or experimental study to yield accurate and consistent results, the appropriate design needs to be selected. Under experimental conditions, the rFIA method employed a double-line reaction manifold design to maximize reaction efficiency, as shown in **Figure 3**.

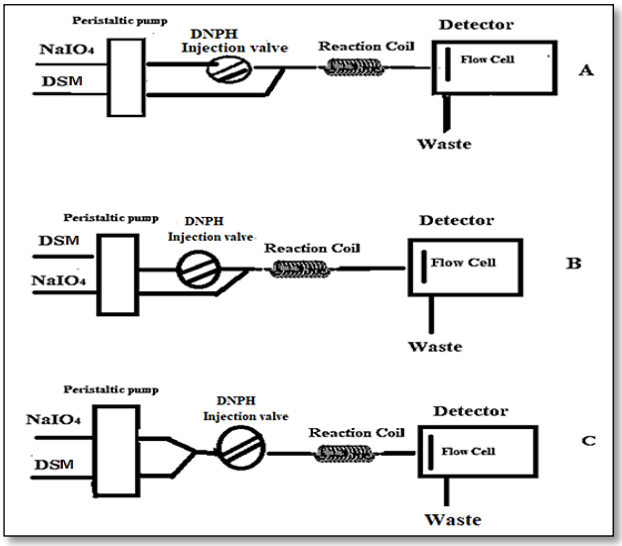

Figure 3. Diagram of rFIA manifolds used in the oxidative coupling experiment to determine the DSM compound

The rFIA system utilized two-channel manifolds with A, B, and C routes, as shown in **Table 2**. In this setup, a 150 µL sample containing 50 µg/mL DSM was injected, while a 75 cm reaction coil delivered the reagent, 0.005 M DNPH, at a flow rate of 20 $mL.min^{-1}$. This design promoted

efficient mixing, fast reaction kinetics, and high sensitivity and reproducibility in absorbance measurements. **Table 2** presents the differences among the three manifolds used to summarize variations in the collected data and experimental values across different levels, interpreting experimental findings sensitive to study design.

Table 2. The reverse flow injection system's three designs under study

Manifold	Absorbance
A	0.572
B	0.356
C	0.188

3.3. Effect of 2,4-dinitrophenylhydrazine reagent concentration

Statistical analysis was applied to the chemical optimization of the reaction between the DSM compound and the DNPH reagents. To assess its influence on reaction efficiency and the stability of the resulting colored product, DNPH concentrations ranging from 0.001 M to 0.01 M were evaluated. To indicate the whole data and in comparison(s) between and within the groups, the general average was calculated from this data set. This enabled quantification of performance for each group member relative to the average, facilitating a more valid interpretation of the results. The experiments were performed using the one-factor-at-a-time method, in which parameters were held fixed while others were controlled, and each was varied in turn. Several chemical factors, including reagent concentration and medium components, were screened systematically, as shown in **Figure 4**.

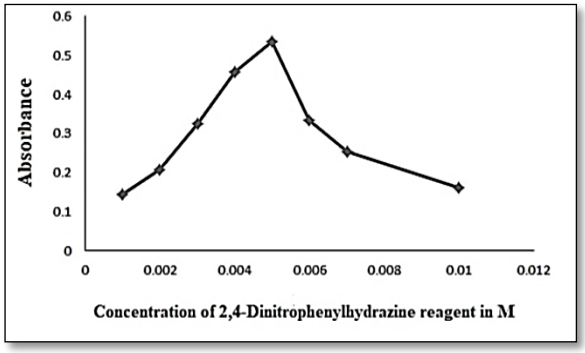

Figure 4. DNPH concentration's effect yields 0.005 M

3.4. Effect of types of oxidizing agents

A 0.02 M concentration of each oxidizing agent, $FeCl_3$, $NaIO_4$, $K_2S_2O_3$, $K_3[Fe(CN)_6]$, and $(NH_4)_2Ce(NO_3)_6$, was systematically evaluated to determine their effectiveness in facilitating the reaction, as shown in **Figure 5**. The study assessed differences in reaction rates, product formation, and stability, providing insights into the most suitable oxidant for the experimental conditions. Various concentrations of $NaIO_4$ were also examined in the range of 0.005-0.06 M, as shown in **Figure 6**.

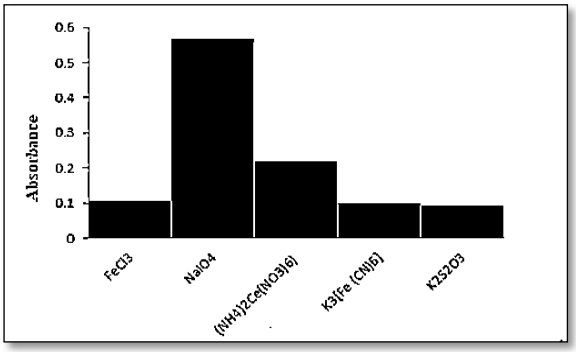

Figure 5. The impact of oxidation on use

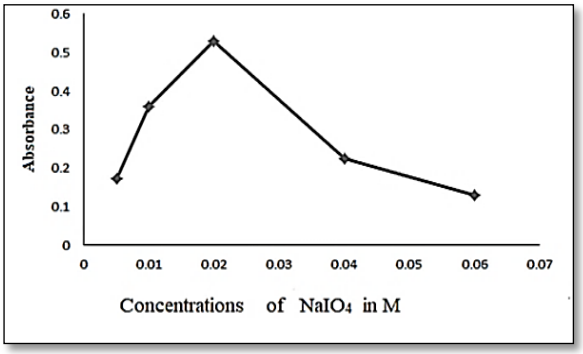

Figure 6. The concentration of $NaIO_4$ (0.002 M)

3.5. Optimization of rFIA physical parameters

In the range of [1-4.5 mL.min^{-1}], the impact of total flow rate on the sensitivity of the colored reaction product was examined. The results showed that the highest absorbance was achieved at a total flow rate of 3 mL.min^{-1} (**Figure 7**), and this setting was used in all subsequent testing. By varying the sample loop length, the injected sample volume was adjusted between 60 and 200 µL. According to the data, the highest absorbance was observed for the 150 µL-injected sample (**Figure 8**).

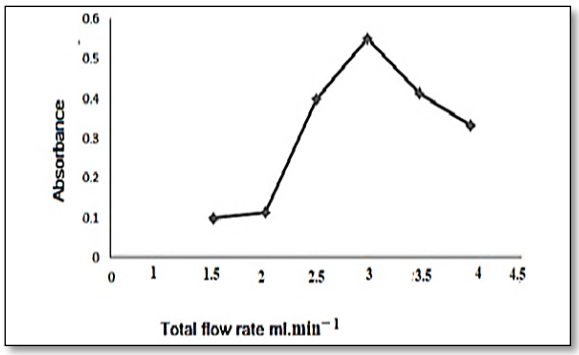

Figure 7. Flow rate's effects

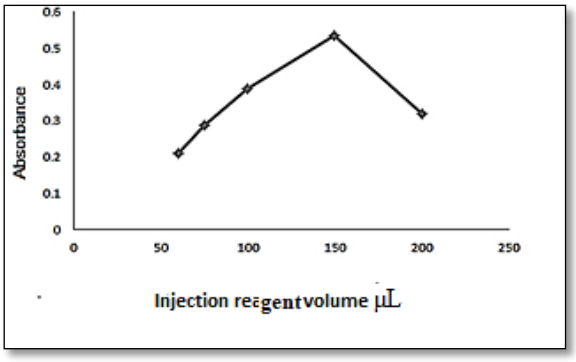

Figure 8. Injection reagent volume effects

The length of the reaction coil used in the flow injection analysis system is a crucial factor affecting the sensitivity of the purple reaction product. Coil lengths ranging from 25 to 250 cm were systematically evaluated to determine their impact on reaction efficiency and absorbance intensity, as illustrated in **Figure 9**. It was indicated that the highest absorbance value (>), which represents good mixing and reaction kinetics in producing the colored product, was achieved using a 75 cm coil. Based on the effects of various experimental parameters on the absorbance intensity of the colored reaction product, **Table 3** displays the optimum conditions for the proposed rFIA method.

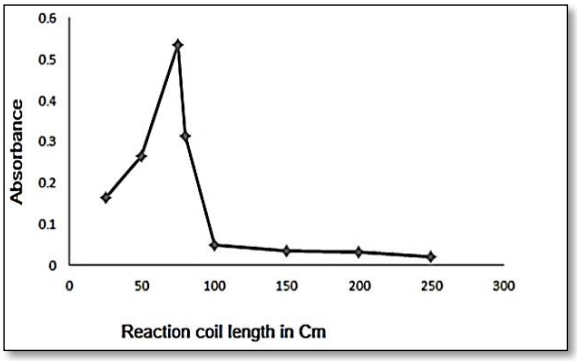

Figure 9. Coil lengths' effects

Table 3. The ideal conditions of the method

Parameters	Range	Optimum
Conc. of DNPH M	0.001-0.01	0.005
Conc. of $NaIO_4$ M	0.005-0.06	0.02
Flow rate, mL/min	0.25-3.5	3
Reaction coil length, cm	25-250	75
Injected sample volume, µL	60-200	150

The calibration curve was derived, and the system's sampling rate was determined using these optimal parameters to achieve maximum sensitivity and repeatability. These factors were used to accurately determine the DSM, thereby providing reliable analytical quality and repeatability of absorbance measurements and serving as the basis for all subsequent experimental analyses and validations in this study.

3.6. Calibration curve

To estimate the principal analytical parameters of merit for this method, a typical calibration curve was established for the DSM compound. The absorbance of the DSM compound was recorded in the concentration range (2–100 ppm), as shown in **Table 4**. The DSM compound was used as the standard for constructing a linear calibration curve (**Figure 10**; linear range determined using the DSM compound under optimized experimental conditions). The total DSM compound content in the samples was then calculated from the calibration plot using the linear regression equation (Y = 0.107X + 0.0162, R = 0.9995), with results expressed as DSM (mg) equivalents per gram of dried material. Statistical parameters were determined in accordance with established statistical principles to ensure reliability and validity.

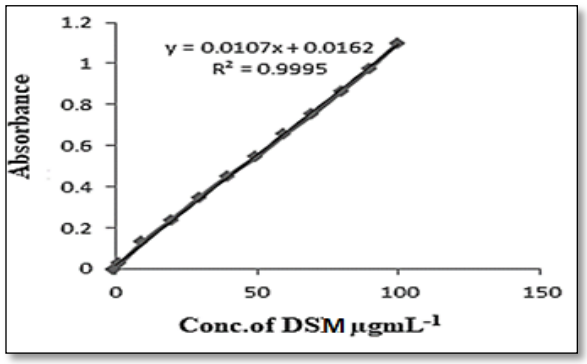

Figure 10. DSM calibration curve

Table 4. Values of the analytical techniques proposed for determining DSM compound

Parameter	Value for method
Λ_{max} (nm)	549
Regression equation	y = 0.0107x + 0.0162
Correlation coefficient, r	0.9995
Linearity range (µg.mL^{-1})	2-100
Molar absorptivity (Lmol^{-1} cm^{-1})	7253.96
Specific absorptivity (ml /g.cm)	433.2
Limit of detection (µg mL^{-1})	2.412
Limit of quantification (µg mL^{-1})	11.372
Standard deviation of the residuals , Sy/x	0.244
Standard deviation of slope, Sb	0.265
Standard deviation of intercept, Sa	0.279

Results from additional testing of the suggested method's precision and accuracy are shown in **Table 5**, which shows that the technique yields precise and repeatable readings. The proposed method for determining the DSM compound was validated using standard DSM solutions prepared at 20 µg/mL under optimal experimental conditions; accuracy and precision were evaluated. To evaluate method reliability and repeatability, the rFIA technique was applied to six DSM concentrations, with five measured in duplicate to assess control uniformity. Some analytical parameters, such as percent error (E%), percent recovery (Rec%), and RSD (%), were calculated and summarized in **Table 5**. The results show that the method performs with high precision (low RSD values) and excellent accuracy (recovery between 80% and 100%).

Table 5. Precision and accuracy of the suggested approach

Pure standard	Conc. of DSM (µg/mL)		Rec. %	RSD %	E %
	Present	Found			
DSM	20	20.21	100.21	2.1	0.21
	30	30.10	100.10	1.87	0.1
	40	39.72	100.28	1.22	0.28
	50	50	100	0.36	0.00
	60	60.10	100.1	0.24	0.1
	70	70.45	100.45	0.61	0.453

3.7. Pharmaceutical applications

Using the recommended rFIA Method, **Table 6** presents the results of DSM estimation in a single pharmaceutical form: DSM [(10 capsules) 750 mg of DSM Nutrition Green Living, USA], administered at three distinct 750 mg DSM doses, with five duplicates. The analytical method for determining the total DSM content in various pharmaceutical products, based on a spectrophotometric assay following complex formation with DNPH in an alkaline medium, was validated in accordance with the requirements of the studies.

Table 6. Direct implementations of the suggested approach in the supplemental sample

Pharmaceutical form	Conc. of DSM (µg/mL)		Rec. %	RSD %	E %
	Present	Found			
DSM (750 mg)	20	20.10	100.1	0.30	0.1
	30	30.17	100.17	0.6	0.17
	40	40.09	100.10	0.32	0.1
	50	50.27	100.27	0.48	0.27
	60	60.27	100.27	0.5	0.27
	70	70.18	100.18	0.22	0.18

3.8. The product's stoichiometry using the conventional addition technique

In the next step, the calibration curve's conditions were used to perform the standard addition procedure. It can be concluded from the values of the primary parameters that good accuracy and precision are achieved. The findings were displayed in **Table 7** and **Figure 11**. All steps were tested under the same sample-preparation conditions: a reaction time of 25 min at room temperature, in the absence of direct sunlight.

Table 7. Utilizing the conventional addition technique in conjunction with the suggested approach to determine the DSM compound in the supplement

Pharmaceutical form	Conc. of DSM (µg/mL) compound		Rec. %	RSD %
	Present	Found		
DSM capsule 750 mg (Nutrition Greenlife, USA)	10	10.09	100.09	0.83
	20	20.16	100.60	0.68
	25	25.08	100.08	0.54

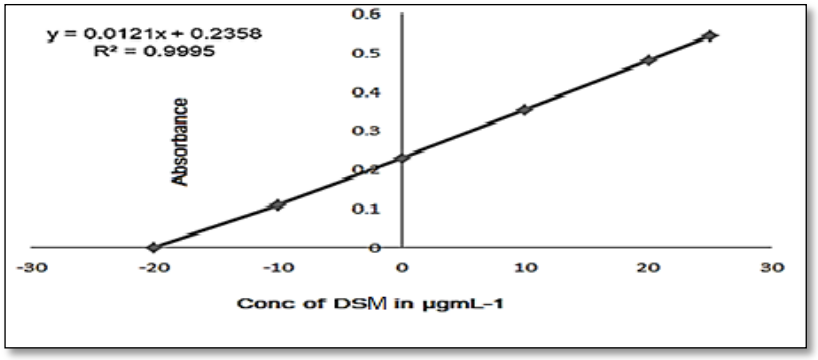

Figure 11. DSM compound technique 20 µg mL^{-1} is the standard addition method for the determination

3.9. Hypothesized oxidative coupling reaction mechanism

The mechanism in **Figure 12** for DSM compound detection involves using DNPH as a reagent in an oxidative coupling reaction, implemented via the rFIA technique, as indicated by the experimental data. During this process, DSM reacts with oxidized DNPH under controlled alkaline conditions to form a colored product. The product has an absorbance maximum at 549 nm, which serves as the analytical signal for quantitative determination, based on the proposed mechanism of oxidative coupling between the DSM and DNPH.

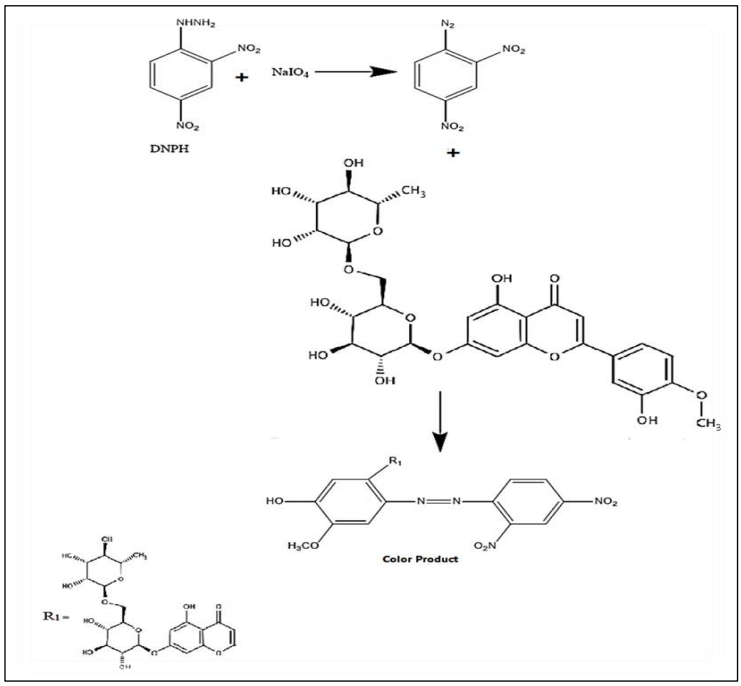

Figure 12. The oxidative coupling process of the DSM and its proposed mechanism

4. Discussion

Reaction of reagent DNPH with the oxidant sodium periodate to produce diazonium ion, which couples in a second step with DSM in an alkaline medium to form an orange product that has the highest absorption at the wavelength of 549 nm[30]. DSM quantification was performed using rFIA coupled with UV–Vis spectrophotometry, owing to the conjugated aromatic system, which exhibits strong absorption bands in both the ultraviolet and visible regions[31]. The DSM, a flavonol that differs mainly in being used as a standard in drug determination, is one kind of natural product and widely spread in the plant kingdom, which possesses keto groups and hydroxyl groups. These groups allow the formation of stable complexes with DNPH, thereby increasing detection sensitivity. The proposed method, a combination of oxidative coupling and FIA-UV-Vis, is reliable, sensitive, and reproducible for the determination of DSM in pharmaceutical samples and provides a direct application for monitoring flavone-based compounds in analytical systems. Under carefully monitored experimental conditions, this method increased the sensitivity and accuracy of DSM compound measurements. Significant effort was devoted to improving experimental conditions to identify the most suitable factors for determining the DSM compound. The [A] manifold design route was used in all subsequent applications because it yielded the highest absorbance. The relationship among the three paths[32] is shown in the result. All experiments were carried out in the presence of 50 µg/mL DSM compound. The data indicated that maximum global reaction performance occurred at a DNPH concentration of 0.005 M, where both the colored product stability and absorbance intensity were highest, also indicative of a good kinetic pace for the reaction. Therefore, a DNPH concentration of 0.005 M was selected for all subsequent experiments to ensure reproducibility and reliable analytical performance[33]. Sodium periodate was chosen as an appropriate oxidant for this procedure because it exhibited a greater reaction than the other agents examined, as shown in Figure 6. Various concentrations of $NaIO_4$ were also examined in the range of 0.005-0.06 M; however, because of its sensitivity to colored products, 0.02 M was selected[34]. Total flow rate of 3 $mL.min^{-1}$, 150 µL-injected sample. Based on this result, a coil length of 75 cm was used throughout to obtain reliable, reproducible, and ultra-sensitive measurements of the reaction product. The calibration and validation studies demonstrate that the developed procedure is suitable for quantitatively determining the DSM compound in matrices with high sensitivity and

precision[35]. The result shows that the rFIA–UV–Vis method is applicable for quantitatively determining DSM in different samples, yielding reproducible, reliable, and accurate values[36]. The developed method's good performance under these conditions makes it suitable for routine analyses and for confidence in the reported values for both research and quality control. The standard addition procedure provides good accuracy and precision because it involves successive interactions that form the chromophore complex. This mechanistic understanding supports the method's sensitivity and specificity for detecting DSM across various sample matrices[37].

5. Conclusion

The present study demonstrated the successful analytical determination of the DSM compound using reverse flow injection analysis, based on its reaction with Brady's reagent in the presence of sodium periodate as an oxidizing agent. The developed method produced a measurable colored product that could be determined spectrophotometrically with good sensitivity and accuracy. The proposed method is simple, rapid, and cost-effective compared with many conventional analytical techniques. Furthermore, the results confirmed the applicability of this method for determining DSM compounds in pharmaceutical preparations, making it a suitable approach for routine analysis in quality control laboratories.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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