Iranian Journal of Analytical Chemistry
Volume 12, Issue 1, March 2025(11-21) Original Research Article

مجله ایرانی شیمی تجزیه
دوره ۱۲، شماره ۱، فروردین۱۴۰۴(۱۱-۲۱)

Development of a Core–Shell Nanomagnetic Agarose-Based Solid-Phase Extraction Method Modified with Quercetin for Ultrasensitive Determination of Remdesivir in Serum via HPLC

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Received: 25 December 2024 **Accepted:** 28 February 2025
DOI: 10.30473/ijac.2025.74833.1322

Abstract
A magnet-enhanced solid-phase extraction technique utilizing spherical core–shell nanomagnetic agarose particles was devised to extract remdesivir from serum samples. These nanomagnetic agarose particles underwent activation through the epichlorohydrin method and were subsequently modified using quercetin dihydrate as a ligand to facilitate remdesivir extraction. To quantify the target analyte, high-performance liquid chromatography (HPLC) was employed following preconcentration via the developed method. The influence of various analytical variables, including pH, ionic strength, magnet passes, and adsorbent quantity, was systematically examined and optimized using a multivariate central composite design approach. Under optimal conditions, five consecutive analyses demonstrated a remdesivir recovery rate of 99.4%, with a relative standard deviation of 3.66%. The method's detection limit (3σ) for remdesivir was determined to be 0.027 mg L^{-1}. This extraction technique was successfully validated for the quantification of remdesivir in serum samples

Keywords
Remdesivir; Solid-phase extraction; Nanomagnetic agarose particles;Quercetin dehydrate.

1. INTRODUCTION
Magnetic nanoparticles, particularly Fe_2O_3, have gained significant traction in various scientific and technological domains, including biomedical imaging, biological applications, targeted drug delivery, and advanced extraction techniques. Their broad utility stems from exceptional characteristics such as nanoscale dimensions, large surface area, and biocompatibility. To enhance stability and enable functionalization, these nanoparticles can be encapsulated with diverse inorganic and organic coatings, including silica, carbon-based materials, metal oxides, noble metals, polyvinylpyrrolidone, gelatin, dextran, chitosan, and agarose, among others [1]. Various synthesis routes such as co-precipitation [2], chemical decomposition [3], sonochemical processes [4], and microwave-assisted techniques have been employed to fabricate iron-based magnetic nanoparticles with tailored properties [5]. Agarose is a transparent and hydrophilic polysaccharide polymer known for its chemical inertness, which makes it highly adaptable across various scientific applications [6]. Its hydroxyl groups enable functionalization with different chemical entities, broadening its versatility. Due to these characteristics, agarose has been widely utilized in extraction methods such as solid-phase extraction [7], affinity chromatography [8], and electrophoresis [9], as well as in drug delivery systems [10] and optical sensor development [11]. Magnetic solid-phase extraction (MSPE) using magnetic particles (MP) as adsorbents has gained attention [12]. Target analytes are adsorbed onto the surface of the MP and then separated from the aqueous solution by an external magnetic field. Subsequently, the target analytes are eluted from the adsorbent for further measurement. Proper

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separation of analytes depends on the chosen adsorbent. The selection of a suitable adsorbent depends on the nature of the sample being tested. The polarity of the analytes, their solubility, and the composition of the complex matrix are also important [13]. These characteristics can affect the strength of the analyte's interaction with the adsorbent. Also, choosing the appropriate solvent for eluting the analytes is important, and their elution strength should be considered in the extraction process and final measurement. The use of MP simplifies the sample preparation process compared to conventional SPE techniques, as MSPE does not require packing a column with the adsorbent, and phase separation can be performed quickly and easily by applying an external magnetic field [14].

The use of magnetic nanoparticles in SPE significantly reduces the time required for analysis by decreasing the number of steps in the extraction process and enabling simultaneous separation and enrichment of analytes, and facilitates the separation of the adsorbent with adsorbed analytes on the surface using an external magnetic field. Furthermore, MSPE can reduce the use of organic solvents and thus the formation of toxic and hazardous waste, in accordance with the principles of green chemistry [15]. The use of ionic liquids(ILs) and magnetic ionic liquids (MILs) in solid-phase extraction (SPE) methods has garnered significant attention in analytical chemistry. Ionic liquids, due to their unique properties such as low melting points, high solubility, and non-volatile nature, serve as effective and green alternative solvents for extracting various compounds [16,17]. Moreover, magnetic ionic liquids can be easily separated using magnetic fields, making the extraction process faster and more efficient. These characteristics lead to improved accuracy and sensitivity in analytical measurements while allowing for higher recovery rates of the target analytes. Overall, the integration of ILs and MILs enhances the performance of SPE techniques, providing a valuable approach in modern analytical methodologies [18].

Magnetic particles, spanning both nanometer and micrometer scales, play a crucial role in biological, biochemical, and analytical chemistry applications. In recent years, their ability to concentrate diverse biological, organic, and inorganic compounds has garnered increasing interest. The inherent magnetic properties of these adsorbents facilitate efficient and straightforward analyte separation, simplifying the process for researchers. Consequently, this technique has been widely acknowledged as an accessible, cost-effective, and environmentally sustainable separation method, requiring minimal use of organic solvents.

Since this extraction technique is often applied directly to samples with intricate matrices, the process duration remains efficient and practical. The magnetic adsorbent particles are gathered using an external magnetic force, ensuring that sample texture has minimal impact on extraction efficiency, except in cases where it influences the interaction between analytes and active surface groups during adsorption [19].

Remdesivir emerged as a novel advancement in antiviral drug development, aimed at targeting RNA viruses with high efficacy. Utilizing sophisticated chemical methodologies, it was engineered to specifically combat viruses that replicate rapidly [20]. During the 2014 Ebola outbreak in Africa, remdesivir was evaluated as a potential therapeutic agent. While preliminary studies demonstrated effectiveness in animal models, the results in human trials were not satisfactory, prompting further investigations into treatments for other viral infections [21].

Following the emergence of the COVID-19 pandemic, researchers promptly initiated studies to assess remdesivir’s potential efficacy against SARS-CoV-2. In January 2020, Gilead announced its investigation into the drug’s effects on COVID-19 patients. By February 2020, initial clinical trial findings from China indicated remdesivir’s potential as a treatment for the disease. These studies suggested that the drug could shorten recovery time by approximately three days and lower the probability of intensive care unit admission. In October 2020, remdesivir became the first antiviral drug to receive official approval for COVID-19 treatment in the United States.Several analytical techniques have been developed for remdesivir quantification, including HPLC, UV spectrophotometry [22,23], HPLC-MS/MS [24], and fluorometry [25].

In this research, the exceptional properties of agarose and the quercetin ligand were employed to functionalize iron magnetic nanoparticles, yielding nanoparticles with outstanding characteristics for the extraction, separation, and subsequent quantification of the antiviral drug remdesivir (an anti-COVID-19 agent) in serum samples. This methodology incorporated an innovative automated device designed for the Microextraction by Packed Sorbent (MEPS) technique, which was executed without the application of specific mechanical forces. The final analysis was performed using High-Performance Liquid Chromatography (HPLC). The remarkable efficiency and rapidity in the extraction and separation processes represent significant advantages of this approach.

2.EXPERIMENTAL

2.1. Reagents and materials

DOI:10.30473/ijac.2024.72409.1309

Agarose, epichlorohydrin, anhydrous iron (III) chloride, ethanol, ammonia, methanol, and disodium phosphatase were purchased from Merck. In this process, a standard working stock solution is first created by dissolving a specific amount of remdesivir in methanol. After that, this stock solution is diluted in specific ratios to prepare various working solutions. Hydrochloric acid (HCl) and sodium hydroxide (NaOH) with a concentration of 2 M are used to control and adjust the pH of the solutions.

These steps are performed to achieve precise and controlled concentrations for subsequent experiments and to ensure reliable results. In this regard, attention to accuracy in measuring and diluting solutions is of particular importance so that the results obtained from the research are based on valid scientific and experimental standards.

2.2. Apparatus

A smartline 1000 model HPLC (Knauer) liquid chromatography instrument was used for the separation and measurement of compounds. The functional groups of the synthesized adsorbents were recorded using an 8400 S model FT-IR (Shimadzu) Fourier transform infrared spectrophotometer with the KBr pellet method. Also, a scanning electron microscope with energy-dispersive X-ray analysis (SEM-EDX) model (FE SEM, TESCAN MIRA3 LMU) was used to examine the surface structure of the particles and analyze the constituent elements and their distribution. A HANNA Microprocessor pH Meter 211p was used for pH measurement. Additionally, a water bath shaker (Memmert WB14, Germany) was used for stirring the solutions.

2.3. HPLC analysis

In this study, a wavelength of 246 nm was used to identify and examine the remdesivir peak using a UV detector. Peak identification was performed by comparing their retention times with standard values.

For quantitative measurement, 20 microliters of standard solutions with known concentrations were injected into the HPLC system, and the corresponding calibration curve was plotted. The obtained peak area for the target compound was placed in the equation of the line obtained from the calibration curve to calculate its concentration. As the mobile phase, a mixture of water and acetonitrile with a ratio of 55:45 (v/v) was used.

The mobile phase flowed through the column at a constant rate of 1 ml per minute. After the completion of each run, the column was given 5 minutes until the next injection to allow the mobile phase to equilibrate. Also, the time between two injections continued until a stable baseline was reached.

The chromatogram obtained under these conditions is shown in Figure 1. As is clear from the chromatogram, the peak corresponding to the compound was recorded at 4.13 minutes, and for each injection, 25 microliters of the sample, which was diluted 20 times with the mobile phase, were injected into the HPLC system to fill the 20 microliter loop.

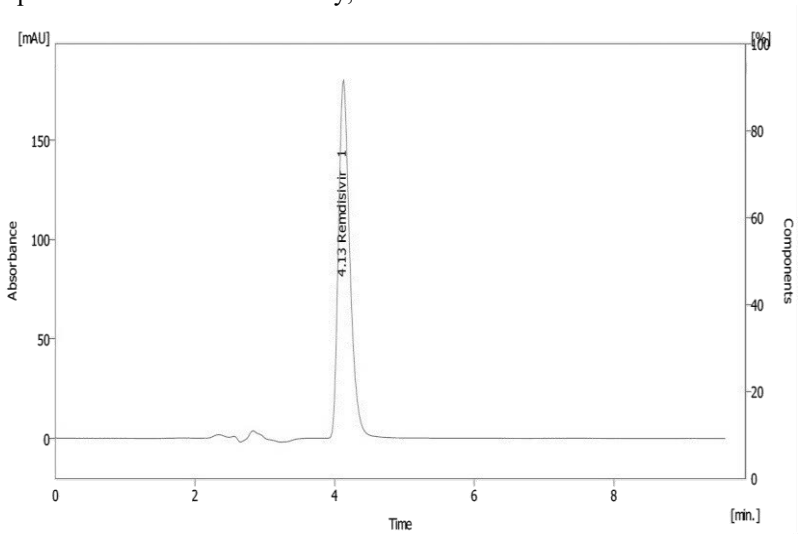

Fig. 1. Chromatogram obtained from direct injection of Remdesivir into the HPLC

2.4. Synthesis of Fe3O4magnetic nanoparticles

DOI: 10.30473/ijac.2025.74833.1322

Initially, 50 ml of distilled water was placed in an ultrasonic bath to remove oxygen. Then, 0.324 g of FeCl3 and 0.198 g of FeCl2.4H2O were added to this solution. After 7 minutes, the mixture was transferred to a two-necked flask. This flask, along with its contents, was placed in an oil bath for reflux conditions. At this stage, after 20 minutes, 5 ml of ammonia was slowly added to the mixture, resulting in the formation of a black solution. The resulting black precipitate was kept at 80°C under argon gas flow and reflux conditions for 90 minutes. Finally, the produced nanoparticles were separated using a permanent magnet, washed several times with distilled water, and then dispersed in water.

2.5. Synthesis of agarose-coated Fe3O4

After washing the nanoparticles to remove unreacted materials from their surface, these nanoparticles were first suspended in 50 ml of water. In the next step, 0.05% agarose, which had gelled at 80°C, was added to the solution containing the iron nanoparticles. To complete the nanoparticle coating process, this mixture was gently stirred for approximately two hours. Finally, the coated nanoparticles were collected using a magnetic field and used in subsequent research steps.

2.6. Activation of agarose coating

The activation of the agarose membrane was carried out using the epichlorohydrin method with some modifications. In the first step, epichlorohydrin was used to increase the strength of the particles and create suitable conditions for the chemical attachment of the desired ligand. For this purpose, 4 ml of 2 M sodium hydroxide solution was added to the nanoparticle mixture, followed by the addition of epichlorohydrin.

This mixture was placed in a water bath shaker for approximately 2 hours to ensure complete surface modification reaction. In each step of this process, an external field is used to separate the particles, which helps improve the membrane's performance and facilitates the achievement of desired properties.

2.7. Chemical attachment of ligand onto activated particles

Following surface activation, nanoparticles undergo modification with an appropriate ligand. To achieve this, a ligand solution composed of quercetin dihydrate in ethanol at a 0.01 M concentration is initially prepared, followed by pH adjustment using sodium hydroxide [26]. The nanoparticles are then introduced into this solution and subjected to continuous agitation in a water bath shaker for 48 hours to ensure effective surface functionalization. Subsequently, the modified nanoparticles are preserved in a mixture containing 20% ethanol and water and stored under refrigeration until further application.

2.8. Extraction of remdesivir using magnet assisted magnetic solid phase extraction method (MMSPE)

To extract and pre-concentrate remdesivir from serum samples, the magnetic stirring solid-phase extraction method was employed. In this procedure, 5 mL of a 5 ppm remdesivir solution is placed in a designated container, followed by the addition of 330 microliters of adsorbent. A magnet is positioned beside the sample container, inducing movement in the adsorbent particles within the analyte solution. Lowering the magnet causes the particles to adhere to the container's wall near the magnet, while lifting it results in their redistribution throughout the solution. After 13 cycles of back-and-forth magnet motion, the adsorbent interacts with the analyte, facilitating the extraction process. Subsequently, the remdesivir bound to the adsorbent is rinsed with 450 microliters of acetonitrile for five minutes. Finally, the obtained solution is transferred to analytical instruments for quantification.

2.9. Characterization of the adsorbents

Figure 2 compares the FT-IR spectra of two different samples: pure iron oxide nanoparticles, and also magnetic agarose particles obtained from the synthesis of these compounds. Among these, the presence of characteristic bands related to agarose in the spectrum of magnetic agarose particles clearly indicates that these particles have been successfully combined with the agarose coating. This confirms the preservation of the structural characteristics of agarose during the synthesis process.

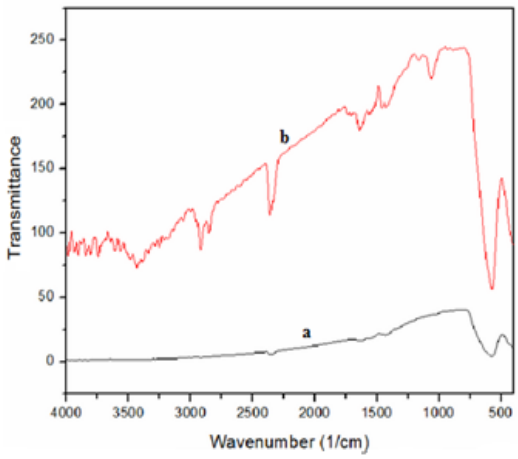

Fig. 2. FT-IR spectra of (a) Fe3O4 and (b) Fe3O4@Agarose.

The magnetization curves of Fe3O4 and Fe3O4@Agarose nanocomposites recorded by

DOI:10.30473/ijac.2024.72409.1309

VSM at room temperature is shown in Fig. 3. The saturation magnetization for Fe3O4 and Fe3O4@Agarose is 65.74 and 55.52 emu/g, respectively. This decrease in magnetization, which is often observed for nanoparticles, may be related to the contribution of agarose shells surrounding magnetite nanoparticles. However, the magnitude of this decrease is not large enough to seriously affect the application of nanoparticles in magnetic separations.

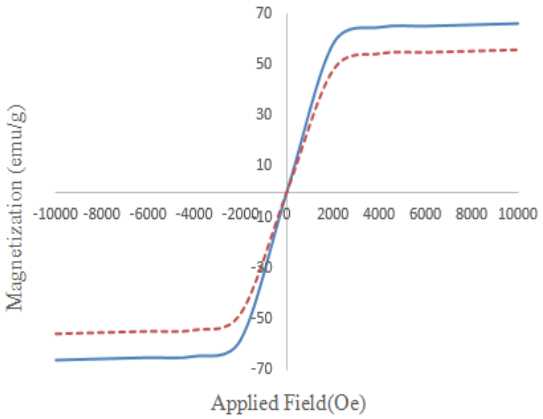

Fig. 3. Magnetic curves of the (_____) Fe3O4 and (--------) Fe3O4@Agarose MNPs.

Additionally, a scanning electron microscope (SEM) was utilized to meticulously examine the size and shape of the nanoparticles. The analysis revealed that the nanoparticles predominantly exhibit a spherical morphology. The average size of the magnetic nanoparticles is approximately 17 to 25 nanometers (Fig. 4). These structural characteristics play a crucial role in nanomaterial applications, as their shape and size directly influence their physical and chemical properties. Consequently, detailed SEM observations provide essential insights into nanoparticle behavior and functionality across various systems.

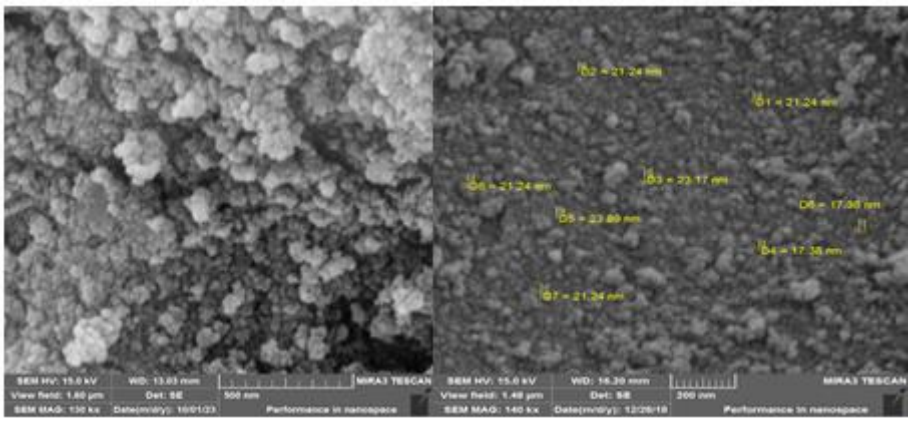

Fig. 4. SEM images of agarose and ligand-coated iron oxide nanoparticles

3. RESULTS AND DISCUSSION

3.1. Selection of the type and amount of elution solvent

To separate remdesivir from the adsorbent surface, it is necessary to select an elution solvent that does not damage the adsorbent surface. In this regard, various solvents including ethanol, acetonitrile, acetone, and methanol were investigated. Numerous experiments were conducted using each of these solvents as the elution solvent to evaluate their effectiveness on remdesivir. The results of these tests are shown in Figure 5 and a comparison between the efficiency of different solvents is presented. Based on the findings, acetonitrile was selected as the best option for the elution solvent because it has a high ability to separate remdesivir and does not damage the adsorbent surface. Based

DOI: 10.30473/ijac.2025.74833.1322

on the results obtained, acetonitrile was selected as the elution solvent.

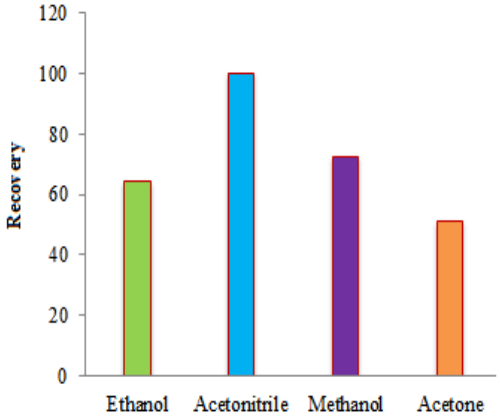

Fig. 5. Comparison of extraction rates using different washing solvents

The amount of elution solvent in the MMSPE extraction method is directly related to the amount of adsorbent.

The volume of the elution solvent should be such that it can elute all the remdesivir from the surface of the adsorbent, and also not be too large, which would reduce the preconcentration factor. Different volumes of acetonitrile were studied and investigated. Based on the results presented in Figure 6, volumes lower than 350 µl were not sufficient for complete elution of the adsorbent from remdesivir, and a significant decrease in extraction efficiency is observed at a volume of 150 microliters.

After 500 µl, further increase in solvent volume had no effect on extraction efficiency, and this is because all the remdesivir has been separated from the surface of the adsorbent. A volume of 500 µl of acetonitrile was selected as the optimal amount and used in subsequent steps.

To investigate the parameters affecting the elution of Remdesivir after optimizing the factors affecting extraction, the one-factor-at-a-time optimization method was used as a simple method to investigate the factors affecting the elution of the analyte. In this simple method, one factor is considered variable in each experiment while the others are kept constant.

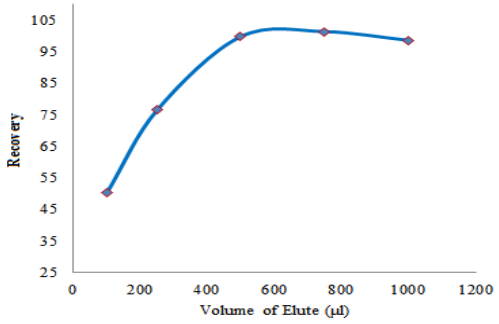

Fig. 6. Effect of elution solvent volume on efficiency

3.2. Optimization of parameters affecting remdesivir extraction using the MMSPE method

In this study, the multivariate optimization method was applied, distinguishing itself from the univariate approach by simultaneously emphasizing factor interactions. This real-time consideration of multiple variables enhances efficiency and accelerates the optimization process. Furthermore, it enables the simultaneous refinement of several parameters, leading to more comprehensive and effective outcomes.

The response surface method can examine the interaction between different factors, and finally, statistical analysis is performed using the charts provided by this method. In this research, the response surface method was used to investigate different factors. In this method, after introducing 4 main factors, 5 levels were considered for each of these factors, and the experimental design was performed using MINITAB 16.1.1 software. The amount of adsorbent, analyte solution pH, magnet passes, and ionic strength effect were the four variables in the design. The high and low levels for each variable are given in Table 1 based on the results of some preliminary experiments.

Table 1. Selected factors and levels considered for each factor in the response surface methodology

Factor	Factors levels	
	Low	High
Solution pH	4	8
Amount of adsorbent (mg)	250	350
Magnet passes	8	16
Ionic Strength (%)	2	6

DOI:10.30473/ijac.2024.72409.1309

Table 2 presents the designed experiments alongside the measured values for each variable, expressed in terms of absorption efficiency. This table allows for a detailed examination of experimental conditions and their impact on extraction efficiency. The recorded results illustrate variations in efficiency across different conditions, offering valuable insights into the correlation between variables and absorption behavior. Additionally, optimal adsorption conditions for remdesivir were systematically analyzed. Following the execution of the designed experiments, key optimization points were identified and predicted. These optimal conditions are outlined in Table 3 under predefined parameters.

Table 2. Designed experiment conditions and corresponding response values obtained

No.	pH	Amount of adsorbent	Magnet passes	IS	%Recovery
1	2	300	12	4	32.60
2	6	300	12	0	47.50
3	6	300	12	4	85.65
4	8	250	8	2	50.32
5	4	350	8	6	47.92
6	8	350	8	2	59.23
7	8	350	16	2	67.34
8	6	400	12	4	87.98
9	4	350	16	6	67.91
10	4	250	8	6	76.13
11	6	300	12	4	87.14
12	4	250	8	2	34.56
13	4	350	16	2	69.34
14	8	250	16	6	56.98
15	4	250	16	6	66.40
16	6	300	12	4	86.78
17	6	300	20	4	44.12
18	6	300	12	4	88.26
19	8	250	16	2	38.97
20	10	300	12	4	48.12
21	6	200	12	4	31.93
22	8	350	8	6	44.69
23	6	300	12	4	89.27
24	6	300	12	8	50.43
25	8	350	16	6	29.73
26	4	250	16	2	41.54
27	6	300	4	4	26.91
28	6	300	12	4	88.78
29	4	350	8	2	45.87
30	6	300	12	4	87.59
31	8	250	8	6	32.54

Table 3. Optimal points obtained by the response surface model using MINITAB software for Remdesivir

pH	6
Adsorbent	330
IS	3.5
Magnet passes	13

The 3D surface plot illustrates the correlation between response values and varying factor levels, with Figure 7, presenting a clear visualization of each response level. This graph represents a dependent variable in relation to two remdesivir parameters. Similarly, contour plots yield comparable results, providing an effective way to identify maxima, as depicted in Figure 8. This analytical approach enhances comprehension of how variable fluctuations influence the overall outcome.

DOI: 10.30473/ijac.2025.74833.1322

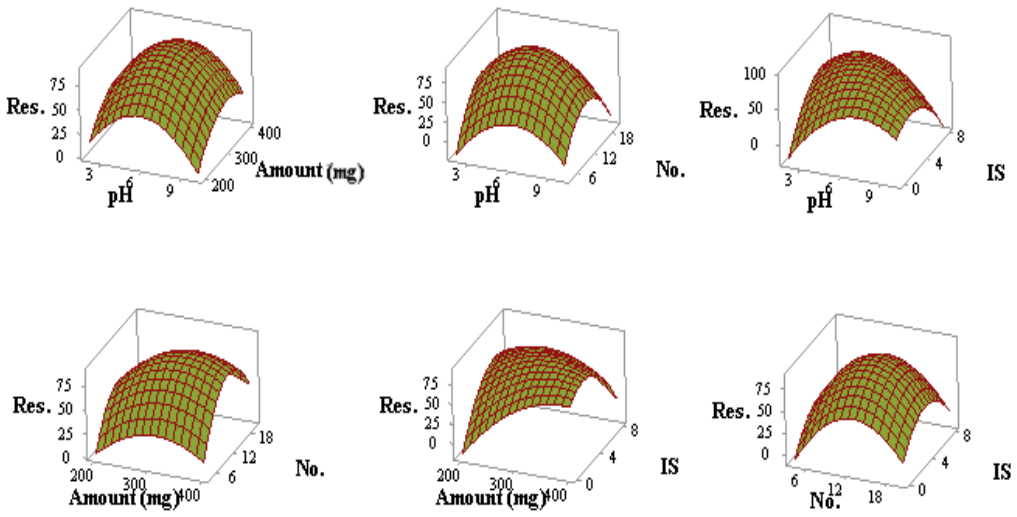

Fig. 7. Plots of response surface methodology with the central composite design obtained for remdesivir

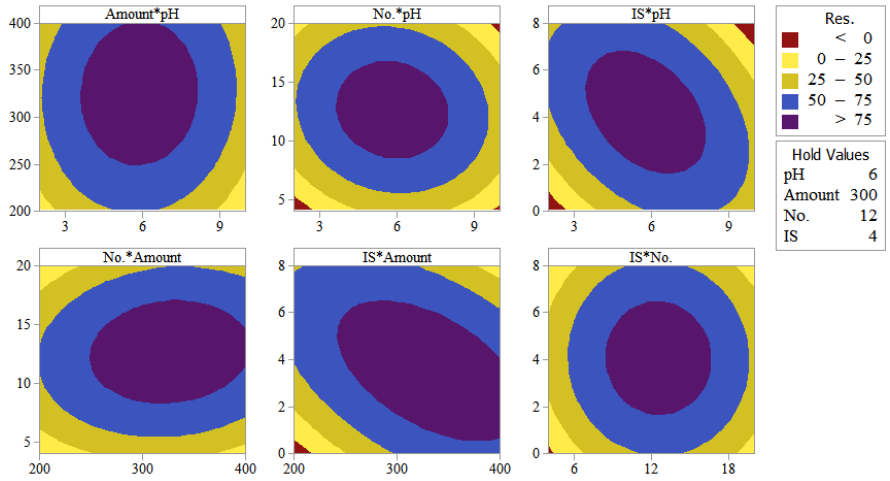

Fig. 8. Contour plots of recovery of the remdesivir

3.3. Analytical performances

Following optimization of the factors for the developed method and based on the results obtained during the optimization process, the calibration curve demonstrated strong linearity, with determination coefficients reaching 0.999 (Table 4). The repeatability of the method was investigated by measuring the remdesivir standard sample 5 times under optimal conditions (adsorbent amount 330 µg, 13 cycles(magnet passes), pH=6, and salt concentration 3.5%). Under these conditions, an efficiency of 99.4% and a relative standard deviation of 3.66 were calculated for the measurement of remdesivir after separation by the method and absorbance measurement. The limit of detection of the method was calculated to be 0.027 mg L−1[27].

The limit of detection (LOD), limit of quantification (LOQ), linear dynamic range (LDR), correlation coefficient (R2), and regression equation for remdesivir in serum samples analyzed by MMSPE method compared to several other methods are presented in Table 4. As can be seen, our proposed method shows very good performance. Low detection and quantification limits, wide linear dynamic range, and high recovery percentage are notable features of this method.

DOI:10.30473/ijac.2024.72409.1309

Table 4. Comparison of performance parameters of the proposed method with several established methods

Method performance parameters	UV method	HPLC method	Spectro Fluorescence Method	Present Method MMSPE
Linear dynamic range (µg mL−1)	10.0 – 60.0	10.0 – 60.0	0.1-1.1	0.081 – 40.0
Limit of detection (µg mL−1)	3.000	2.400	0.010	0.081
Limit of quantification (µg mL−1)	9.000	7.300	0.030	0.027
R2	0.999	0.999	0.99	0.999
Recovery (%)	99	99	95	99.4
RSD (%)	3.5	3.5	3.2	3.66

3.4. Extraction and measurement of remdesivir in real samples by MMSPE method

To investigate whether the desired adsorbent is applicable to real samples, the method was used to determine remdesivir in various serum samples. Acetonitrile was used for serum deproteinization. First, one milliliter of acetonitrile was added to the serum and allowed to stand for 10 minutes for complete deproteinization. The precipitated proteins settle at the bottom of the container. To completely separate the precipitated proteins, the solution was centrifuged at 6000 rpm for 10 minutes. The spiked serum solution was placed under optimal conditions. All three samples were free of remdesivir, so they were spiked with remdesivir at a concentration of 5 ppm, and 5 milliliters of it were taken for extraction and measurement of remdesivir by the mentioned method and measured under optimal conditions. Table 5 shows the results.

Table 5. Results obtained from the extraction of remdesivir from serum samples spiked with 5 ppm using the MMSPE method under optimal conditions

Sample	Before spike	After spike
S1	-	4.98
S2	-	4.83
S3	-	5.02

4. CONCLUSION

The MMSPE technique enhances solid-phase extraction by magnetically stirring adsorbent particles without relying on mechanical force, making it well-suited for agarose-based magnetic adsorbents. The proposed synthesis method for magnetic adsorbents is a straightforward, single-step process that yields particles with optimal properties for compatibility with the intended device. The agarose layer binds to magnetic iron oxide nanoparticles via hydrogen bond interactions, ensuring favorable physical characteristics in the adsorbent. By optimizing the translational movement of adsorbent particles within the sample solution, this approach accelerates equilibrium attainment in the extraction process, leading to efficient and rapid extraction performance compared to conventional stirring methods.

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DOI:10.30473/ijac.2024.72409.1309

توسعه یک روش استخراج فاز جامد مبتنی بر آگاروس نانومغناطیسی هسته-پوسته اصلاح‌شده با کوئرستین برای تعیین حساس و سریع رمدسیویر در سرم با استفاده از HPLC

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تاریخ دریافت: ۱۴۰۳/۱۰/۵ **تاریخ پذیرش:**۱۴۰۳/۱۲/۱۰

چکیده:
یک روش استخراج فاز جامد مغناطیسی به کمک آهنربا بر اساس ذرات آگارز نانومغناطیسی کروی هسته-پوسته توسعه داده شد و برای استخراج رمدسیویر در نمونه‌های سرم مورد استفاده قرار گرفت. ذرات آگارز نانومغناطیسی با روش اپی کلروهیدرین فعال شده و با دی هیدرات کوئرستین به عنوان لیگاند برای استخراج رمدسیویر عامل‌دار شدند. برای تعیین رمدسیویر هدف، پس از پیش تغلیظ با این روش، از HPLC استفاده شد. تأثیر پارامترهای مختلف تحلیلی مانندpH ، قدرت یونی، عبور آهنربا و مقدار جاذب بر استخراج رمدسیویر بررسی و با روش طراحی کامپوزیت مرکزی چند متغیره بهینه شد. پنج آنالیز تکرار شده در شرایط بهینه منجر به بازیابی ۹۹/۴٪ با انحراف استاندارد نسبی ۳/۶۶٪ برای رمدسیویر شد. حد تشخیص روش (3σ) برای آنالیت ۰/۰۲۷ میلی‌گرم در لیتر بود. این روش با موفقیت برای تعیین رمدسیویر در نمونه‌های سرم به کار گرفته شد.

کلید واژه ها
رمدسیویر; استخراج فاز جامد; ذرات آگاروس نانومغناطیسی; دی هیدرات کوئرستین

DOI: 10.30473/ijac.2025.74833.1322