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Determination of Erythromycin in Milk Samples by Nano Magnetite Hexadecylsilane by LC-Tandem Mass Spectrometry

Abdollah Tavassoli, Seyed Naser Azizi* and Abdolraouf Samadi-Maybodi

Department of Analytical Chemistry, Faculty of Chemistry, University of Mazandaran, Babolsar, Iran

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Erythromycin is one of the typical macrolides which is isolated by the Saccharopolyspora erythraea. It has been listed in WHO essential medicine for improvement of the efficiency of health system. In this study, magnetic iron nanoparticles were coated with cetyl as a non-polar functional group and characterized by various techniques such as infrared spectroscopy, thermal gravimetric analysis, scanning electron microscopy and vibrating sample magnetometer. The as-prepared nanoparticles were used to extract erythromycin from the milk samples. Separation was performed on a pentaflurophenyl column (150 × 2 mm, 3 μm) using a mobile phase consisting 70% acetonitrile and 30% ammonium acetate (10 mM, pH3.5) with a high performance liquid chromatography system coupled with tandem mass spectrometry. The separation was fast and completed in less than 5 min, under the optimized condition. Stable isotope of erythromycin was used as internal standard in the sample preparation and calibration curve. It was found that relative recovery of the method was 92.6%. The proposed method was convenient, and a quick preparation method was achieved using external magnetic field without centrifugation and filtration. The detection limit and coefficient of determination were 2.4 μg l^{-1} and R^2 = 0.9983, respectively. The intra- and inter-day precisions of the proposed method in different levels of spiked sample were in the range of 5.6-8.5% and 8.4-12.5%, respectively.

Keywords: Antibiotic, Erythromycin, Magnetic Fe_3O_4-C16 nanoparticles, Tandem mass spectrometry, Milk

INTRODUCTION

Macrolides are widely used in veterinary medicines for mastitis and chronic respiratory infection [1]. It typically has a large lactone ring in their structure and is used as an effective antibiotic against gram positive bacteria. Moreover, they contain a dimethyl amino group, solved in more polar organic solvents [2] and interrupt the microorganisms by inhibiting protein synthesis by reversibly binding to the 50S subunit of the ribosome. The macrolide includes Erythromycin (ERY), Azithromycin, Clarithromycin, Fidaxomicin, and Telithromycin. The ERY is a typical macrolide, isolated from *Saccharopolyspora erythraea* in 1952 [3]. It is administered through food additives and drinking water to stop the outbreaks, and listed in WHO essential medicine for efficiency in health system [3]. Both irregular consumption and ignorance in the withdraw time result in health hazards such as arrhythmia, allergic reactions, toxic epidermal necrolysis and increased bacteria resistance [4]. For the concerns mentioned, European Union (EU) has set the maximum residual limits (MRLs) for macrolides especially ERY, in different matrices into Annex I of Council Regulation (EEC) No.2377/90 in which 200 and 40 μg l^{-1} are designated for tissue and milk, respectively [5]. Generally, there are several methods for detection ERY such as inhibitory microbial and immune assay techniques, but they lack specificity and takes long time to perform. On the other hand, new development in chromatography techniques such as high-performance liquid chromatography coupled to diode-array detector (HPLC/DAD) or tandem mass spectrometry (LC/MS/MS) resolve the obstacles in traditional methods problems at determination of ERY [6,7]. Nevertheless, the extraction and preparation methods

*Corresponding author. E-mail: azizi@umz.ac.ir

such as solid phase extraction (SPE), and liquid-liquid extraction (LLE) have been improved for multi residues purification of milk matrix [8]. Recently, magnetic solid phase extraction (MSPE) with iron oxide nanoparticles has attracted much attention as a new and enhanced method for solid phase extraction [9]. Moreover, in the last decades, the synthesis of magnetite nanoparticles have been greatly developed by numerous applications in medicine such as DNA extraction, separation and preconcentration of various cations [10] and anions [11]. In addition, iron oxide magnetic nanoparticles have many potential applications such as biocompatible ferro-fluids queues [12], trace quantification in water samples [13], as a substrate used for extraction and separation [14], and solid phase extraction [15,16]. In each of these applications, the nanoparticles must carry specific size, shape, surface and magnetic properties. The advantages of using magnetic iron nanoparticles include: (1) The ease in synthesis of nanoparticles, (2) large surface area based on their nanometric dimensions, (3) The superparamagnetic property that causes these particles to respond to the external magnetic field, (4) Easy and rapid extraction of different species using only an external magnetic field, (5) No need for filtration and centrifugation during the extraction process, (6) Ability to extract large volumes of samples, and (7) Modifying nanoparticles to the level enabling them for selectivity. However, the most important advantage of using magnetic iron oxide nanoparticles is the ability of retrieving particles after magnetic field application and the easy recycling feature with excellent adsorption properties [9-11, 13-16]. Since milk is a complex matrix, some molecules can interfere targeted analytes, thus, in this study, we applied magnetics nanoparticles (MNPs) Fe_3O_4@SiO_2@C16 instead of common preparation, which there is no need to extra steps for removing macromolecules. The synthesized MNPs characteristics were investigated by different techniques such as thermogravimetric curves, scanning electron microscopy (SEM), VSM diagrams and Fourier transform-infrared (FT-IR). Taking into consideration the low MRLs range, improving simple, rugged and effective method with quantifying and confirming capability in detection of multi residues drugs is valuable. Thus, the objective of this study is to developing and validating of novel, easy and quick extraction technique for the first time based on using and application of LC-MS/MS for detection of ERY in milk samples.

MATERIALS AND METHODS

Chemicals and Reagents

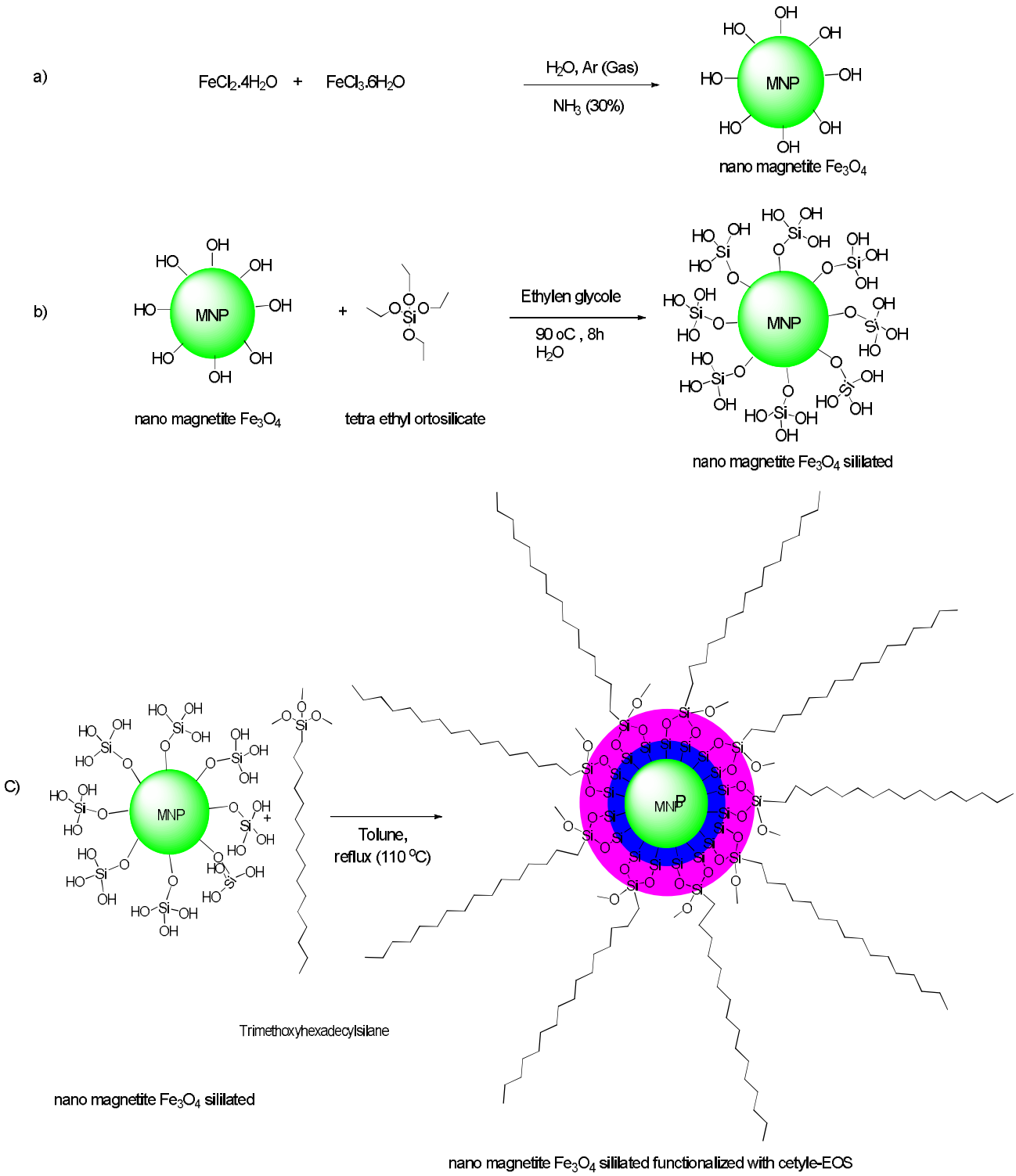
ERY dihydrate and ERY-(N, N-dimethyl-^{13}C, D_3), as internal standards, were purchased from Sigma (St. Louis, MO, USA). $FeCl_2.4H_2O$, $FeCl_3$ $6H_2O$, ammonia solution (32%), hydrochloric acid (37%), tetraethylorthosilane (TEOS) (98%), trimethoxyhexadecylsilane, HPLC grade (methanol, acetonitrile and ethanol), and synthesis grade (toluene and dimethyl formamide (DMF)) were bought from Merck (Darmstadt, Germany). Also, double distilled water was obtained by Milli-Q plus system for sample preparation (Millipore, USA).

Standards and Stock Solutions

Stock solutions of ERY and internal standard were prepared by dilution to 1000 µg ml^{-1} in methanol and were stored at -20 °C. The secondary standard solutions were prepared by dilution of the stock solution with methanol, and individual working standard mixtures were prepared daily by dilution of the secondary standards in water.

APPARATUS

Chromatographic separation was carried out by Flexar model of PerkinElmer liquid chromatographic system (Perkin Elmer, USA) and it was equipped with Binary pump, vacuum degasser, auto sampler and column oven. An Applied Biosystem/AB Sciex 3200 triple Quad MS-MS system (Toronto, Canada) was used for detection of ERY that includes Turbo V electrospray ionization source in the positive mode (ESI+). Multiple reaction monitoring mode (MRM) was performed to obtain data. The precursor ion and two most abundant product ions were chosen for identification, confirmation and quantification of the ERY. The Analyst 1.6.2 software was used for acquisition and quantification. The separation was accomplished with a Pentaflurophenyl column, 150 × 2 mm, 3 µm, protected with a 20 × 2 mm guard column of the same phase that was purchased from Macherey-Nagel (Germany). The mobile phase was 70% of acetonitrile and 30% of 10 mM



Scheme 1. Preparation schemes of the MNPs: a) magnetite/silica composite particles, b) Silica coating of MNPs, c) Cetyl functionalization of silica coated MNPs

ammonium acetate (pH, 3) that operated as an isocratic condition. The flow rate was 0.3 ml min^{-1}, runtime for each injection was 5 min and the injection volume was 20 µl. The optimal parameters of mass spectrometry were tuned with infusion of 1 µg ml^{-1} of analytes by inbuilt syringe pump and the 734.3 m/z ion as the parent ion and the daughter ions of 158.3 m/z and 576.2 m/z were selected as the quantitative and qualitative ions, respectively. Achieved conditions were curtain gas pressure (CUR) at 20, (GS1) at 50, (GS2) at 55 and collision gas at medium pressure. The ion spray voltage was 5500 V, the turbo heater temperature was 600 °C, and the interface heater was turned on. More information such as transitions and tuned parameters are shown in Table 1.

Morphological characterization of Fe_3O_4@SiO_2@C16

The material was characterized and examined by means of VSM (Meghnatis Daghigh Kavir, Iran) for magnetic properties, TGA at the following rate of heating (10 °C min^{-1}) (Thermo Analyze/STA 504 BAHR model, Germany) for thermal gravimetric studies, scanning electron microscopies SEM (EM-3200 model, KYKY company, China) for morphology, and FT-IR with KBr pressed pellets (Tensor 27 spectrometer, Germany) was used to identification of functionalities. Finally, ultrasonic bath from Elma-Hans Schmidbauer Company Elmasonic S 60H model (Germany) was employed for ultrasound aided dispersion of MNPs.

Synthesis of Magnetite Hexadecylsilane Nanoparticles

Preparation of MNPs. Magnetic iron nanoparticles (MNPs) were prepared by the co-precipitation reduction procedure. The amounts of 11.68 g of $FeCl_3.6H_2O$ and 8.3 g of $FeCl_2.4H_2O$ were dissolved in 200 ml of deionized water under argon atmosphere at 85 °C and stirred vigorously, then 20 ml of 30% ammonia solution droplet was added to the solution and the color of the solution was rapidly changed from orange to black and rotated at this temperature for 30 min. The synthesized nanoparticles were washed twice with deionized water and once with 0.02 M sodium chloride solution. Then, they were washed twice with acetone and once with ethanol and dried at 60 °C in the oven [17].

Coating MNPs with silica. To prevent the agglomeration of Fe_3O_4 NPs, they were coated with silica (SiO_2) by sol-gel method as follows: firstly, Fe_3O_4 (10.0 g) MNPs were transferred to a solution containing of ethanol (100 ml), distilled water (10 ml), and 32% ammonia (4 ml) and sonicated for 30 seconds. Thereafter, TEOS (3 ml) was poured into the mixture and further sonicated for 3 h at 60 °C, in order to process the final reaction. The produced Fe_3O_4@SiO_2 MNPs were easily collected with the aid of a magnet and three times washed with 50 ml ethanol/distilled deionized water (30/70 % v/v) per wash and lastly dried at 60 °C for 24 h.

Preparation of hexadecylsilane nanoparticles (Fe_3O_4@SiO_2@C16). 1.2 g of dried silica coated MNPs was suspended in 50 ml of toluene by 15 min sonication. Then, 3 ml of Trimethoxyhexadecylsilane was added to the suspension and the final solution was refluxed at 110 °C and under N_2 atmosphere for 8 h. Then, the cetyl functionalized MNPs were gathered by a super magnet and after three times washing with 50 ml of a solution with ethanol, then washed thoroughly with distilled water and dried at 50 °C in a vacuum oven [18] (Scheme 2).

Sample preparation with Fe_3O_4@SiO_2@C16. The procedure for erythromycin extraction is systematically illustrated in (**Error! Reference source not found.Error! Reference source not found.**). 0.5 ml milk sample was spiked by adding 20 µl of internal standard (1 µg ml^{-1}) to all sample. Spiked samples with final concentration of 1 to 100 µg l^{-1} of erythromycin were prepared in milk. Each time, 30 mg of MNPs was dispersed into the sample under constant vortex for 5 min. Then, the suspension of adsorbed erythromycin-Fe_3O_4@SiO_2@C16 was collected by a magnet placed at the bottom of the vial and rinsed and washed twice with water (250 µl). Then, the captured erythromycin was eluted from Fe_3O_4@SiO_2@C16 by acetonitrile (250 µl) for 5 min. Finally, the preconcentrated analyte *via* MNPs was analyzed by LC-MS/MS instrument.

RESULTS AND DISCUSSION

Characterization of Sorbent

Characterization of the synthesized MNPs (Fe_3O_4, Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2@C16) was performed using SEM, TGA, VSM and FT-IR. The SEM images of silica coated nanoparticles (TEOS-MNPs) and cetyl-TEOS-MNPs adsorbents are shown in the Fig. 1Fig. 1. The SEM imaging

Table 1. MRM Transitions and MS Parameters

Analyte	Retention time (min)	ESI (+/-)	Precursor ions (m/z)	Product ions (m/z)	DP (V)	CE (V)	EP (V)	CXP (V)
Erythromycin	2.1	+	734.3	158.3	41	39	8	4
				576.2	41	27	8	4
Erythromycin-$^{13}CD_3$ (I.S.)	2.1	+	738.3	162.3	46	46	8	4

DP: Declustering potential. CE: Collision energy. EP: Entrance potential. CXP: Collision exit potential.

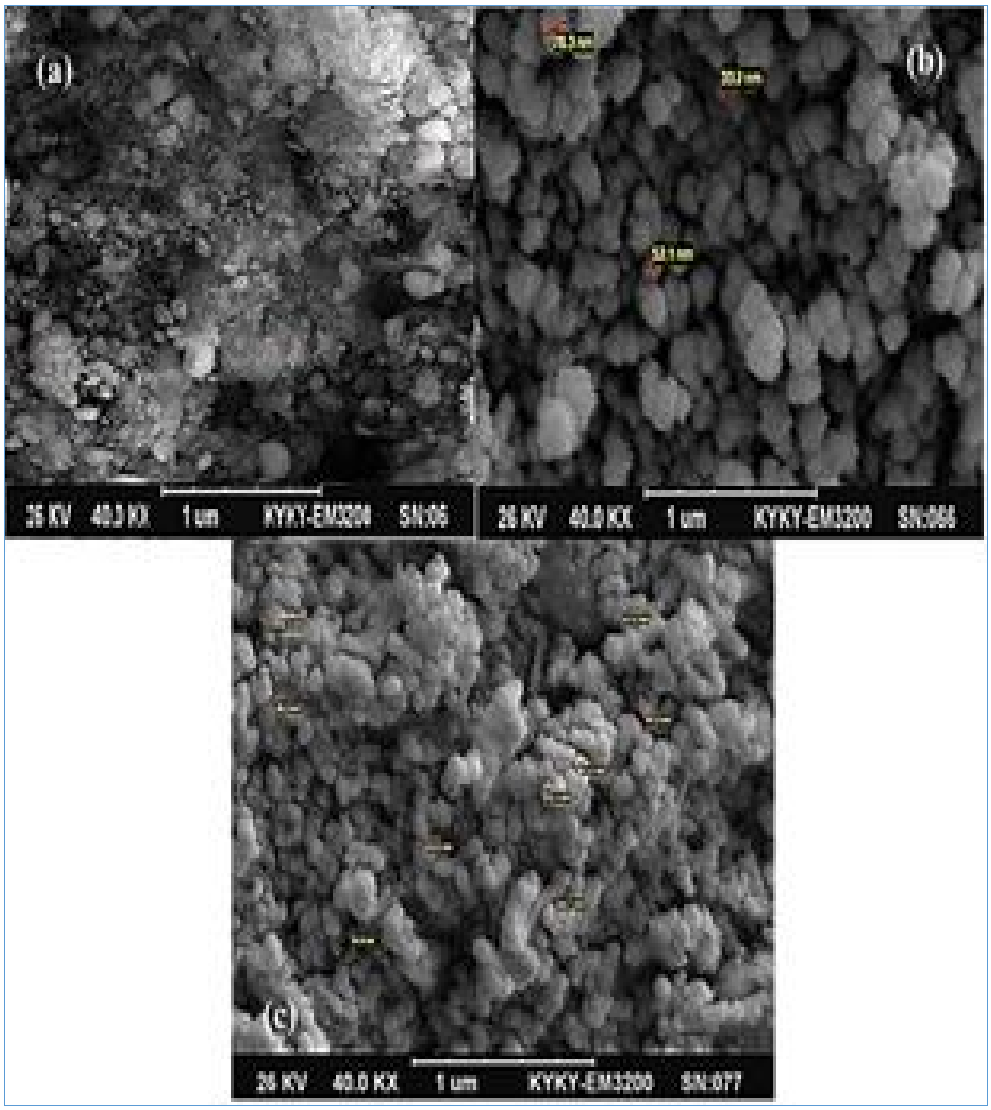


Fig. 1. SEM pictures of a) Fe_3O_4, b) Fe_3O_4@SiO_2, c) Fe_3O_4@SiO_2@C16 magnetic nanoparticles (MNPs).

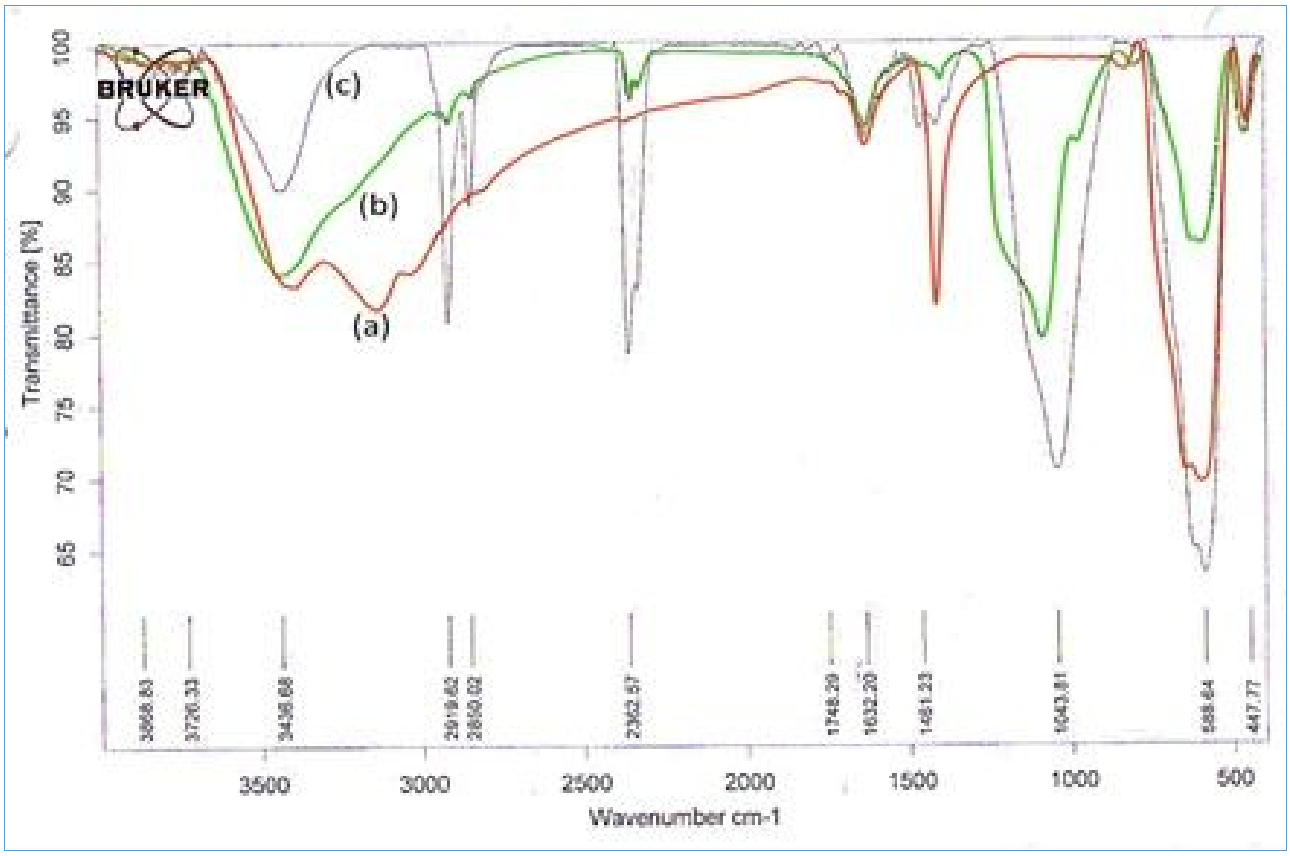


Fig. 2. FT-IR spectra of a) Fe_3O_4, b) Fe_3O_4@SiO_2, c) Fe_3O_4@SiO_2@C16 magnetic nanoparticles (MNPs).

shows spherical entities in the nanometer scale with relatively uniform morphology and a size distribution in the range of 35-70 nm which are evident for the uniform and successful surface modification. Comparison between the sizes of two trials indicates that the size remains unchanged upon functionalization which is assumed to be a result of thin layer formation of organic phase on the surface *via* chemical type bonding.

Meanwhile, FTIR characterization, given in Fig. 2, confirms that functionalization has been successfully performed. The presence of the following bonds can be demonstrated namely aliphatic C-C (800 cm^{-1}), Fe-O (460 and 580 cm^{-1}) [18] , C-C (~1100 cm^{-1}), Si-O-C (1090 cm^{-1}), Si-O-Si (~1035 and 1085 cm^{-1}), C-H (2870 and 2920 cm^{-1}) and O-H (3100-3500 cm^{-1}) from the spectrum of cetylated MNPs [19].

For further illustration of the surface coverage, TGA was done for the functionalized material (can be seen in Fig.), and three different regions observed are attributed to: weight loss at initial temperature due to water removal, chemically formed organic layer decomposition performed in the range of 100-500 °C, and the plateau at very high temperature (>530 °C) pertaining to the stable silica layer coated MNPs. By subtracting water weight, the dried MNPs' organic layer can be estimated as a %weight of 13.4% of total weight.

The VSM diagrams of synthetic compounds showed that the magnetic property decreased with the functionalization of magnetic Fe_3O_4 nanoparticles (Fig. 3). When the magnetic nanoparticles of Fe_3O_4 are coated with silica, the magnetic property is reduced to a small amount. Also, when the cetyl-TEOS-MNPs adsorbent was measured, the magnetic property of the magnetic resonance was reduced, and its magnetization property was reduced compared to its initial composition. However, the final combination still had high magnetism intensity, which was

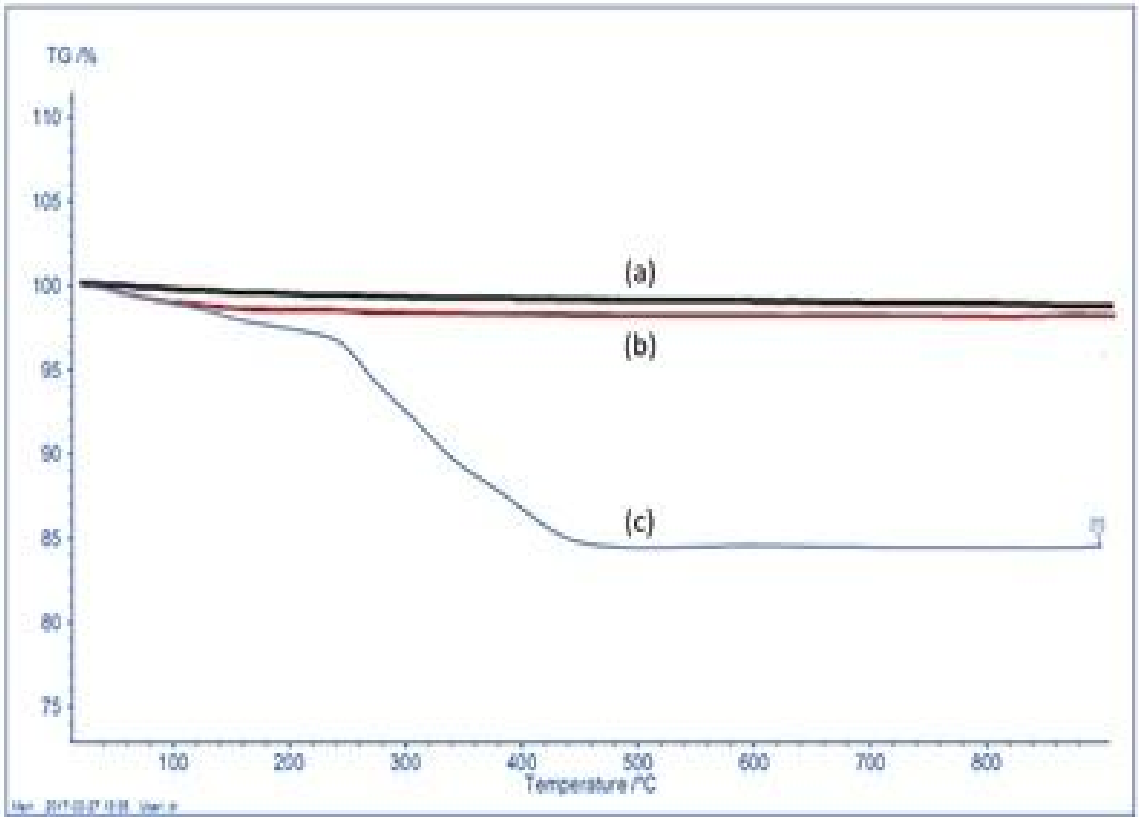


Fig. 3. Thermogravimetric curves of a) Fe_3O_4, b) Fe_3O_4@SiO_2, c) Fe_3O_4@SiO_2@C16 magnetic nanoparticles (MNPs).

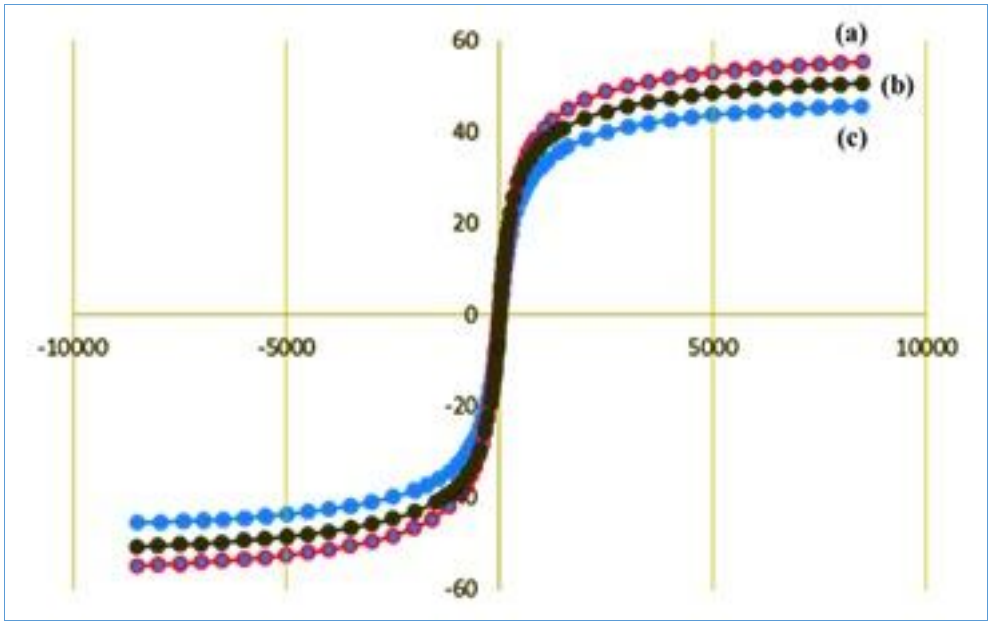


Fig. 4. VSM diagrams of a) Fe_3O_4, b) Fe_3O_4@SiO_2, c) Fe_3O_4@SiO_2@C16 magnetic nanoparticles (MNPs).

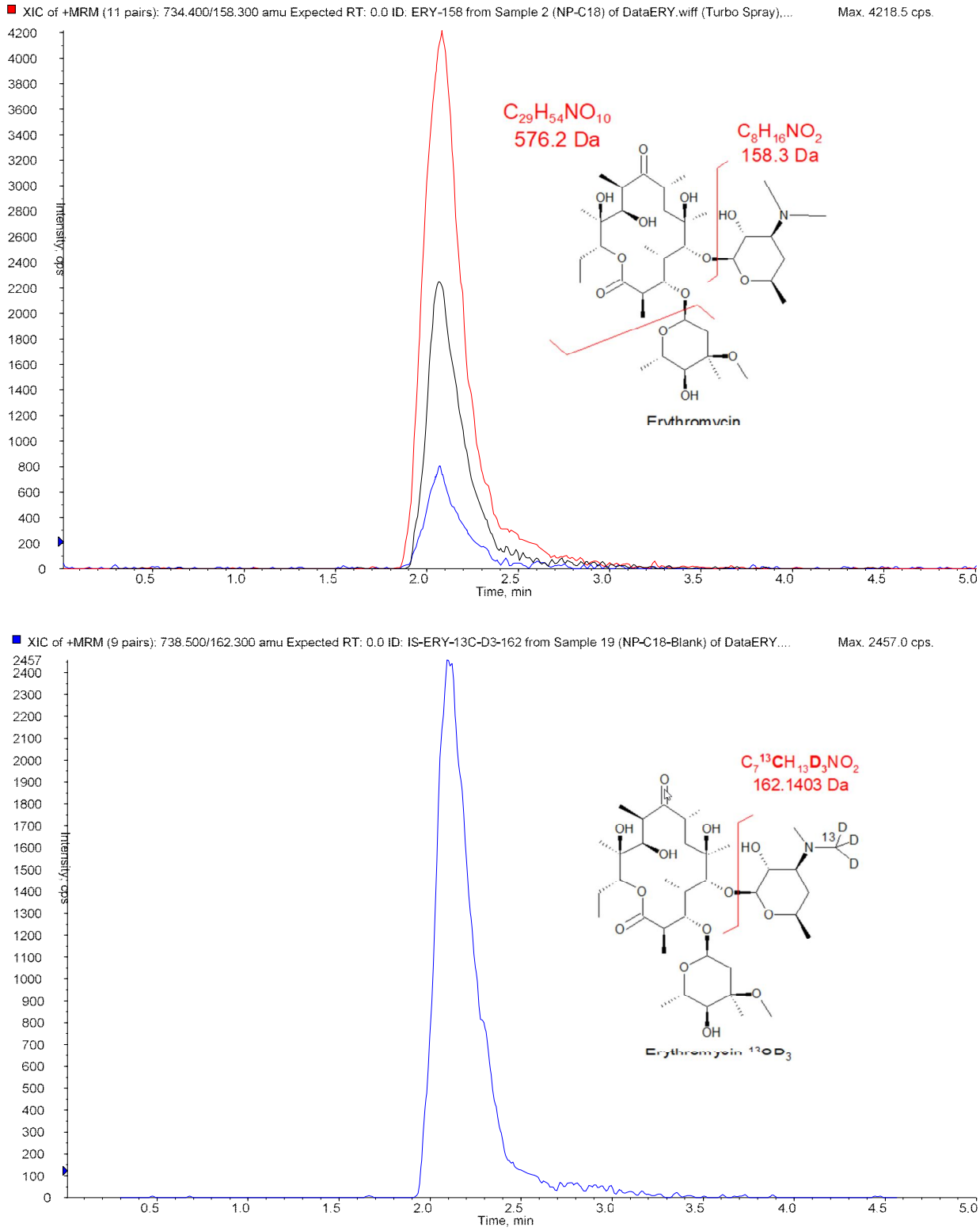


Fig. 4. Chromatograms fortified level 40µg kg^{-1}, A) Erythromycin in fortified sample, B) Erythromycin in Blnak sample.

suitable for further study.

Optimization of Effective Parameters

Amount of MNPs, effects of volume and type of eluting solvent, extraction and eluting time were optimized in the determination of ERY.

Effect of MNPs Quantity

Generally, the higher the surface area, the lesser dosage of adsorbent is required for the satisfactory results. Therefore, the adsorbent dosage was varied in the range of 10-100 mg and it can be seen that the extraction efficiency has increased with the increment before it levels off at 30 mg. Consequently, 30 mg adsorbent was chosen as optimum extent and was applied within further experiments.

Extraction Time and Sample Volume Effect

One of the necessary requirements for a better extraction is the quick analysis to obtain a minimum time needed for equilibration and extraction. Therefore, the effect of the extraction time was evaluated in the range of 1-10 min (Fig. S2). It was found that an adsorption time of 5 min was adequate to get adsorption equilibrium. The high surface area of MNPs could be the possible reason for achieving such a fast extraction process. As a result, the equilibrium state is obtained quickly and adsorption time is very short. This is the most important advantage of this purification method as compared to conventional methods. The effect of sample volume was also investigated, as shown in. The better extraction yield was achieved in the lower amounts of the sample volume, because the matrix effect is minimized in the lower sample volumes. The obtained data was resulted to the selection of 0.5 ml as optimum sample volume due to the increased sensitivity.

Effect of Type, Volume and Eluting Solvents

It is of major importance in the optimization of the separation parameters to select a proper solvent capable of effective elution of the adsorbed analyte with minimum possible volume consumption and least co-elution impurity interfering and damage to the surface of the adsorbent material. Herein, various solvents such as acetonitrile, ethyl acetate, methanol, acetone and chloroform were studied (Fig. S4).

Due to the similarity of the polarity of methanol and acetonitrile to the ERY analyte, a better elution was occurred using acetonitrile, and also acetonitrile removes other interference through the formation of better precipitate [20]. In addition, it was selected as the optimum solvent for elution. Meanwhile, the volume of the eluent was varied in the range of 0.1-1 ml and it was observed that complete elution was ensured at 250 µl (Fig. S5). Likewise, upon varying desorption time from 1 to 10 min, it was seen that 3 min was enough and further stirring had no significant effect on extraction (Fig. S6). Therefore, 250 µl acetonitrile and 3 min elution were selected as optimum throughout this work.

Matrix Effect and Extraction Efficiency

The matrix has important effect on the efficiency of the analyte ionization in the MS instrument. Relative recovery describes the efficiency of the analyte extraction process. The efficiency of the process showed the performance of analyte extraction and ionization. These analytical parameters were evaluated by comparing the various responses of the standards extracted from the enriched matrix, the post-extraction matrix of standards and the standards in pure solution. The average matrix effect at three levels of ERY concentration was 12.7%, and the average relative recovery was 92.6% and the average efficiency of the process was 78.3%. The results, summarized in Table 2, showed that the sample matrix cannot significantly affect the performance of the determination of ERY.

Method Performance

The performance of the method was assessed with investigation of the intra-day and inter-day precision (Table 3). The intra-day precision was assessed based on five replicates determination of the spiked concentration of ERY at three levels within one day. The inter-day precision was assessed based on five replicate determination of the spiked concentration of ERY during a three-day period. The two estimated RSD % precisions (intra and inter-day) were in the range of5.6-8.5% and 8.4-12.5%, respectively. Calibration curve was obtained by linear least-squares regression analysis of the peak area at seven different levels of the ERY concentration and a good coefficient of the

Table 4. Intra- and Inter-day Precision (%RSD) Obtained in Analysis of Selected ERY in Spiked Milk at Fortified Levels (0.5MRL, 1MRL, 1.5MRL)

Analyte	Fortified ($\mu g\ kg^{-1}$)	Between-day		Within-day	
		Mean ± SD ($\mu g\ kg^{-1}$)	RSD	Mean ± SD ($\mu g\ kg^{-1}$)	RSD
ERY	20 (0.5MRL)	18.5 ± 1.7	8.5	19.3 ± 2.5	12.5
	40 (1MRL)	40 ± 2.5	6.2	40.5 ± 3.7	9.2
	60 (1.5MRL)	59.5 ± 3.4	5.6	61 ± 5.1	8.4

Table 4. LOD, LOQ, Calibration Curve, Coefficient of Determination in Analysis of Erythromycin Fortified Milk Samples

Method	Fe_3O_4@SiO_2@C16
LOD ($\mu g\ kg^{-1}$)	2.4
LOQ ($\mu g\ kg^{-1}$)	8.8
Calibration curve	y = 0.0478 × -0.0034
Coefficient of determination (R^2)	0.9983

Table 4. Analyte Recovery, Matrix Effect and Process Efficiency

Analyte	($\mu g\ kg^{-1}$)	RE (%)[a]	ME (%)[b]	PE (%)[C]
Concentration of Erythromycin	20	89	87	76
	40	94	85	78
	60	95	90	81
	Mean	92.6	87.3	78.3

[a]Analyte Recovery was determined by: RE(%) = A/B × 100. [b]Matrix effect was determined by: ME(%) = B/C × 100. [c]Process efficiency was determined by: PE (%) = A/C × 100. A: Standards extracted from fortified matrix; B: standards fortified after post-extraction matrix; C: standards in the pure solution.

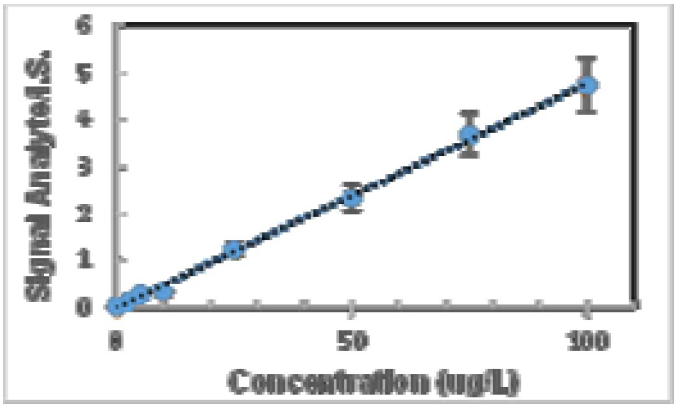


Fig. 5. Calibration curve.

determination was achieved (Fig. 5). As it is summarized in Table 4, method detection limit (MDL) and limit of quantification (LOQ) turned out to be 2.4 and 8.8 μg l^{-1}, respectively, on the basis of signal-to-noise ratio of 3 and 10. Furthermore, the performance and advantages of the proposed method was compared with other studies, and the results are summarized in Table 5.

CONCLUSIONS

This method provides appropriate accuracy, Low MDL, high recovery, good repeatability, sensitivity, and effective determination for ERY using MNPs-assisted separation of ERY. The MNPs are synthesized through simple sol-gel method, which provides more effective separation of ERY from the minimum amounts of samples with high sensitivity. Therefore, the obtained MDL is lower than that of MRL imposed for milk by EU regulation, which affirms that the given proposed method is suitable for the determination of ERY. The high efficiency and extraction outcome of the process can be explained by the possible hydrophobic interaction between C16 coated MNPs and ERY. In addition, the proposed method provides some advantages such as rapid and efficient sample preparation process, while there is no need to filtration and centrifuge (as seen in Table 5). Furthermore, in contrast to the other classical methods, the proposed method is efficient, able to reduce the matrix effects, and able to treat large-volume samples in a short time period. On the other hand, the proposed method was aimed with LC/MS/MS method, leading to increase the sensitivity, and the determination of ERY was confirmed by the tandem mass spectrometry.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version.

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