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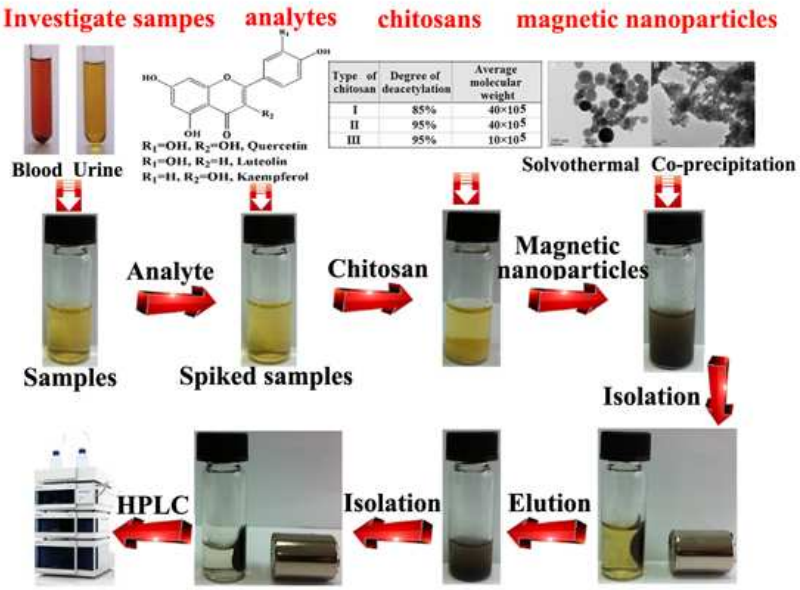

This is the first attempt to use the easy-handle magnetic retrieval of adsorbents extraction technique based on Fe_3O_4 nanoparticles synthesized by solvothermal method in complex biological samples for preconcentration of the natural substances. and study it more comprehensively and systematically. It was notable that the chitosan with different degrees of deacetylation and average molecular weight, Fe_3O_4 nanoparticles synthesized by the the solvothermal and chemical co-precipitation methods were investigated systematically. Especially, a comparative study of the difference between the urine and serum samples was also examined in detail. The strategy not only provided high extraction efficiency and eliminated the time-consuming filtration operation and complex chemical modification but also possessed some remarkable superiorities, for example special affinity and selectivity, short equilibrium time, non-toxicity, low cost and accessibility, biocompatibility.

Magnetic solid-phase extraction based on Fe_3O_4 nanoparticles retrieval of chitosan for the determination of flavonoids in biological samples coupled with high performance liquid chromatography

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Abstract

A novel and facile magnetic solid-phase extraction method (MSPE) based on the two-step magnetic retrieval of chitosan was developed and applied for the first time in bio-matrix samples for the simultaneous extraction and determination of trace flavonoids. A systematic study on the different types of chitosan, Fe_3O_4 nanoparticles (NPs), the analytes and the matrixs were presented. Owing to higher extraction efficiency and capacity for analytes, chitosan with 95% degree of deacetylation and average molecular weight of 1.0×10^6 and the Fe_3O_4 NPs synthesized by the solvothermal method were selected as MSPE materials. Three analytes of luteolin, quercetin and kaempferol can be quantitatively extracted and simultaneously determined coupled with high performance liquid chromatography (HPLC) in urine and serum samples. No interferences were caused by proteins or endogenous compounds. Good linearities ($r^2 > 0.9990$) for all calibration curves were obtained, and the limits of detection (LODs) for quercetin, luteolin, and kaempferol were 1.0, 0.5 and 0.7 ng/mL in urine samples and 10, 2 and 5 ng/mL in serum samples, repestively. Satisfactory recoveries (90.1–106.5%, 91.1–105.5% and 93.5–108.8% for quercetin, luteolin and kaempferol) in biological samples were achieved.

Keywords : Fe_3O_4 nanoparticles, Magnetic solid-phase extraction, Chitosan, Flavonoids, Retrieval.

1. Introduction

Flavonoids, which is one of the largest groups of natural phenols present in medicinal plants, have drawn considerable public attention over several past decades due to their antioxidant activity [1, 2]. Numerous studies have revealed that flavonoids exert positive influence on health owing to alleviating and preventing manifold serious diseases, such as inflammation, cancer, cardiovascular, arteriosclerosis, bleeding, allergy, and swellings [3, 4]. Therefore, Flavonoids are always widely used as remedies because of their biological and physiological importance. In order to investigate different flavonoid consumption and metabolism more efficiently, there is a necessity to develop analytical methods to meet a rapid and sensitive simultaneous measuring of trace-level flavonoids in human fluids. To date, several approaches have been described for the determination of flavonoids in biological samples, including high-performance liquid chromatography (HPLC) coupled with different detectors, such as ultraviolet detector (UV) [5, 6], fluorometric detection (FD) [7], electrochemical detection (ED) [8], liquid chromatography–mass spectrometry (LC–MS) [9] and capillary electrophoresis (CE) [1] and gas chromatography-mass spectrometry (GC–MS) [4, 10]. Even though some of these methods are sensitive and capable of low detection limits, it is unsatisfactory for the quantitative determination of flavonoids because of the interference of complex matrix and their extremely low concentration in body fluids. Therefore, it is crucial for the separation and enrichment of flavonoids prior to determination. However, the traditional pretreatment procedures were always time-consuming and solvent depending. In order to solve the aforementioned problems, it is necessary to develop a practicable enrichment material with high extraction efficiency and time-saving property for the separation and determination of flavonoids.

Nowadays, bioadsorbents have attracted extensive attention owing to some outstanding advantages, such as nontoxic, biodegradable, and biocompatible properities and so on. In particular, chitosan is known as a distinguished bioadsorbents and widely used in many areas[11]. Chitosan is one of the high

performance natural polysaccharide materials and derived from the deacetylation of chitin. Due to the presence of large amounts of amino ($-NH_2$) and hydroxyl (-OH) groups, chitosan exhibits a high adsorption capacity and fast adsorption rate on the substantial amounts of materials, such as dyes, heavy metal ions, phenolic compounds, and so on [12-14]. However, its practical applications is baffled by the two predominant defects existing in the chitosan. On the one hand, the fact that chitosan dissolves in the acid envientment can severely imposed great limittion on the chitosan to be an effective adsorbent [15, 16]. On the other hand, the powdery chitosan is difficult to be separated and recovered except by high speed centrifugation and filtration which is too time-consuming and tedious to meet the high-throughput and fast enrichment and purifcation in biological samples [17].

In order to overcome the above barriers, Magnetic carrier technology (MCT) has been gradually attracted the eyeballs of many scientists and technicians as a rapid and effective technology for magnetic separation[18]. A distinct advantage of MCT is the utilization of magnetic carriers materials. Among the various magnetic carriers, magnetic nanoparticles (MNPs), such as Fe_3O_4 NPs, are promising candidates for carrier technology[19-21]. Hence, special attentions have been directed to combine magnetic nanoparticles (MNPs) with chitosan to make most use of their relative virtues and have potential to solve the existing problems [22, 23]. With the advent of Fe_3O_4 nanoparticles, the chitosan is endowed with magnetic property, which makes sampling and collection easier and faster. However, much time-consuming and arduous work is still dedicated to synthesize Fe_3O_4@chitosan composites compared to their pretreatment processes. Moreover, the adsorption efficiency and capacities of Fe_3O_4@chitosan composites become lower because the synthesis procedure is involved in the reaction of amino and hydroxyl groups, which are expected to play a great part in the adsorption process[24]. In this sense, enforcements are still necessary for developing a new extraction technique offering a simple but effective approach to achieve the extraction. In the present work, a new two-step extraction technique based on magnetic retrieval of chitosan was developed as a rapid and efficient sample preparation method, which could not only avoid tedious procedure involved in the

complex chemical modification but also realize the retrieval and separation of chitosan from dispersion rapidly and effectively. The chitosan substituted for the traditional SPE adsorbent was utilized to extract the analytes in the first process. Then the MNPs was used to retrieve the chitosan enriched with the analytes in the second step [25].

To the best of our knowledge, Some researchers have studied the two-step extraction mode in many matrixs[26-29]. Zhang *et al* have applied the magnetic retrieval of chitosan solid extraction in the green tea beverage samples[25]. Li *et al* used the magnetic retrieval of ionic liquid in environmental water samples[28]. This is the first report to study the mode based on the combination of Fe_3O_4 nanoparticles synthesized by solvothermal method and chitosan in complex biological samples preconcentration of organic compounds. Besides, it is worth noting that the chitosan with different degrees of deacetylation and average molecular weight, Fe_3O_4 nanoparticles synthesized by the different methods were investigated systematically. Especially, a comparative study among different bio-matrix samples and analytes was also examined in detail.

2. Experimental

2.1. Chemicals and materials

All reagents were of analytical reagent grade and used as supplied. Luteolin, quercetin and kaempferol standards were purchased from Sinopharm Chemical Reagent Co., Ltd., China. Their structures were shown in Fig. 1. Chitosan with 100 mesh, 95% degree of deacetylation and average-molecular weight of 1.0×10^6 was supplied by Qingdao Baicheng Biochemical Corp. Other reagents include ferric chloride hexahydrate ($FeCl_3 \cdot 4H_2O$), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), ethylene glycol (EG), diethylene glycol (DEG), sodium acrylate (CH_2=CHCOONa, Na acrylate), sodium acetate (CH_3COONa, NaAc). Water used in all experiments was prepared using a compact ultrapure water system from Ulupure Corporation (Chengdu, China).

2.2. Instruments

The size and distribution of the as-synthesized nanoparticles were studied using a FEI

TecnaiG2 F20 transmission electron microscope (TEM). The surface groups on the nanoparticles were measured with a 8400s FT-IR spectrometer (Shimadzu Corporation, Japan). Phase identification was done by the X-ray powder diffraction pattern (XRD), using X' TRA X-ray diffractometer with Cu Ka irradiation at c = 0.1541 nm. The magnetic properties were studied using a LDJ 19600-1 vibrating sample magnetometer (VSM) operating at room temperature with applied fields up to 10 kOe. The specific surface area of the as-synthesized nanoparticles in the dry state was determined by a multipoint Brunauer–Emmett–Teller (BET) apparatus (3H-2000PS2, Beishide instrument, China). Zeta-potential measurements of two kinds of Fe_3O_4 NPs and the chitosan were performed with Zeta Plus Zeta Potential Analyzer (Brookhaven, USA).

2.3. Preparation of magnetic Fe_3O_4 NPs

Fig. 2 illustrates the whole procedures of two-step magnetic retrieval of chitosan and its application as MSPE adsorbents for simultaneous extraction and preconcentration of targeted analytes in urine and serum samples. The Fe_3O_4 NPs prepared by the solvothermal and chemical co-precipitation methods were used and compared in the MSPE process. Firstly, the following is the solvothermal method preparing Fe_3O_4 NPs[30]. Briefly, 2.4 g $FeCl_3$ •$6H_2O$, 3.4 g CH_2=CHCOONa and 3.4 g NaAc were added into a mixture of ethylene glycol (EG, 22.5 mL) and diethylene glycol (DEG, 22.5 mL) under ultrasonication for about 1 h. The resulting homogeneous black solution was then transferred and sealed into a Teflon-lined stainless-steel autoclave. The autoclave was heated at 200 °C for 10 h, and then cooled to room temperature. After the reaction, the obtained Fe_3O_4 NPs was washed with ethanol and water for several times, and then dried in vacuum at 65 °C for 10 h. The chemical co-precipitation method is as follows [31]. Briefly, 100 ml of 0.02 mol Fe^{2+} and 0.04 mol Fe^{3+} solutions were prepared with deionized water in two beakers, and then transferred to a 500 mL three necked flask together. When the solution was heated to 80 °C, ammonia solution (100 mL) was added dropwise under nitrogen gas protection with vigorous mechanical stirring until the pH was between 10 and 11. After the addition of ammonia, the solution immediately turned black indicating the formation of iron oxide (Fe_3O_4) in the system.

The solution continued to be heated at 80 °C for 2 h, and then the precipitated powders were collected by magnetic separation. The obtained magnetic nanoparticles were washed immediately with deionized water several times. The final product was dried into powder at 40 °C in a vacuum oven.

2.4. Urine and serum sampling

Standard stock solutions of luteolin (1 mg mL^{-1}), quercetin (1 mg mL^{-1}) and kaempferol (1 mg mL^{-1}) were prepared in methanol and then diluted to the desired concentration. Blank urine and serum samples were collected from volunteers in China Pharmaceutical University (Nanjing, China). Appropriate of stock solutions of luteolin, quercetin and kaempferol were spiked to the blank urine and serum solutions. The concentrations of urine samples were prepared with 3.0–2000 ng mL^{-1} of quercetin, 2.0–2000 ng mL^{-1} of luteolin and 2.5–2000 ng mL^{-1} of kaempferol, respectively. While the serum solutions were prepared with 20.0–2000 ng mL^{-1} of quercetin, 10.0–2000 ng mL^{-1} of luteolin and 15.0–2000 ng mL^{-1} of kaempferol, respectively. All solutions were stored at 4 °C.

2.5. SPE based on magnetic retrieval chitosan

Firstly, 2 mL urine samples were added into a 5 ml vessel, the pH of which was adjusted to 5 using 0.01 mol/l phosphoric acid. Secondly, 10 mg chitosan were added into the above solution, and then shaken gently for 30 s. For serum samples, the pH was adjusted to 7 and then 2 ml serum samples were added into another vessel. Secondly, 15 mg chitosan were added into the above solution, and then shaken gently for 30 s, respectively. The obtained mixtures were kept still for 10 min to completely trap the target analytes. Secondly, 2 mg and 3 mg Fe_3O_4 NPs prepared by the solvothermal method were put into the urine and serum tubes respectively, and then under ultrasonication for 1 min, and therefore, Fe_3O_4 NPs retrieval chitosan sorbents were isolated from the solution by placing a strong magnet at the side of the tube. The suspension became limpid after 1 min, the supernatant was decanted, and the collected Fe_3O_4 retrieval chitosan adsorbents were eluted with 1 mL acetonitrile containing 5% HAc under ultrasonication for 2 min (0.5 mL each time and eluted twice). Finally, the eluted solution was collected and then dried under a stream of

nitrogen at 60 °C and redissolved in 500 μL methanol. After filtration through 0.45 μL membrane, 10 μL of the solution was injected into the HPLC system for analysis.

2.6. HPLC analysis of urine and serum sampling

The three flavonoids (luteolin, quercetin and kaempferol) were separated and quantified by using a high performance liquid chromatography with an automatic sampler (Agilent). The analytical column was a ZORBAX Eclipse XDB-C18 column (4.6×150 mm, 5 <mu>m) supplied by Agilent. The mobile phase consisted of methanol-0.2% aqueous phosphoric acid solution (48:52, V/V) and the flow-rate was set at 1 mL/min. Column temperature was 30 °C. The injection volume was 10 μL, and the effluent was analyzed by HPLC equipped with UV detector at 360 nm[32].

3. Results and discussion

3.1. Characterization

TEM images of the Fe_3O_4 NPs prepared by the solvothermal and chemical co-precipitation methods were shown in Fig. 3A and B, respectively. The result shows that the mean diameters of nanoparticles prepared by the solvothermal method were mainly distributed in the range of 100–200 nm, which is smaller than that of the Fe_3O_4 nanoparticles (200-300 nm) prepared by the chemical co-precipitation method. The specific surface area of the Fe_3O_4 NPs prepared by the solvothermal method was found to be 11 m^2 g^{-1}, which was apparently higher than that of the Fe_3O_4 NPs prepared by chemical co-precipitation method (9 m^2 g^{-1}).

As shown in Fig. 4A, the XRD patterns of pure Fe_3O_4 NPs prepared by the solvothermal method and the Fe_3O_4 retrieval chitosan composites were presented. In the 2θ region of 20~70°, six characteristic peaks marked by their indices (220), (311), (400), (422), (511), and (440) (JCPDS card 19-0629 for Fe_3O_4) were observed for pure Fe_3O_4 NPs and the Fe_3O_4 retrieval chitosan composites. Because of the encapsulation by chitosan on the magnetic nanoparticles, the intensity of the peaks decreased slightly from Fe_3O_4 NPs to the Fe_3O_4 retrieval chitosan composites.

Fe_3O_4 NPs prepared by the solvothermal method, chitosan and Fe_3O_4 retrieval chitosan composites were characterized by FT-IR [33, 34]. As observed in Fig. 4B, all displayed many common characteristics in their spectras. As shown in Fig. 4B-a, the

Fe-O characteristic band at 583 cm^{-1} is indicative of Fe_3O_4 NPs. At the same time, the band appeared at 2919 cm^{-1} was the stretching of C-H from methyl group (-CH_2, -CH_3), the peaks at 1076 cm^{-1} (C-O) and 1645 cm^{-1} (N-H) were also the characteristics of chitosan, which were demonstated in the Fig. 4B-b. Moreover, as displayed in Fig. 4B-c, the stretching vibration of C-H band at 2918, 2852 cm^{-1}, the band at 3430 cm^{-1} corresponded to the hydroxyl（-OH) groups and the vibration of N-H in imidazolering at 1470 cm^{-1} appeared in the spectrum of the the Fe_3O_4 NPs retrieval chitosan sorbents, which indicated that chitosan was successfully adsorbed on the Fe_3O_4 NPs.

To enable practical application of MCT, It is critical that the adsorbents should possess sufficient magnetic properties in MSPE application. Fig. 4C shows VSM magnetization curves of Fe_3O_4 NPs prepared by the solvothermal and chemical co-precipitation methods at room temperature. It is found that the maximum saturation magnetization was 64.60 emu g^{-1} for Fe_3O_4 NPs prepared by the solvothermal method and 51.34 emu g^{-1} for Fe_3O_4 NPs prepared by chemical co-precipitation method, respectively. Although the prepared two kinds of Fe_3O_4 NPs could be separated from their dispersion quickly once an external magnetic field was applied, the more time was needed for the Fe_3O_4 NPs prepared by chemical co-precipitation method [35].

The isoelectric point (IEP) is known to be an important character. The charge density is a predominant factor influencing the interaction between Fe_3O_4 NPs and chitosan. The isoelectric point (IEP) of chitosan, Fe_3O_4 NPs prepared by the solvothermal method and Fe_3O_4 NPs prepared by the chemical co-precipitation method were measured under different pH (shown in Fig. 4D). The IEP of Fe_3O_4 NPs prepared by the solvothermal and chemical co-precipitation methods were found to be at pH 3.47 and 3.56, respectively. For chtosan, the IEP was found to be at pH 8.16, which approached previously reported data for them [36, 37].

3.2. Optimization of the analysis conditions

In order to obtain high recovery for three flavonoids while eliminating most of the interference originating from the urine and serum samples, the significant factors

affecting the extraction recoveries of the MSPE-HPLC-UV method including amount of chitosan, the amount of the Fe_3O_4 NPs, pH, the adsorption time, ionic strength and desorption conditions were studied. It is worth noting that this work represents the first attempt to systematically investigated the types of chitosan and Fe_3O_4 NPs in this two-step MSPE procedure.

3.2.1. Effect of the chitosan type

As a bioadsorbent, chitosan exhibits many bio-properities, among which the degree of deacetylation and average molecular weight are two predominant characteristics playing a great part in the adsorption efficiency[38]. In order to investigate the kinds of chitosan, three types of the chitosan possessing different degrees of deacetylation and average molecular weight were evaluated in the urine and serum samples. The results were shown in the Figure 5A and B, which implied that with the same average molecular weight, the chitosan with 95% degree of deacetylation showed stronger adsorption efficiency than that of chitosan with 85% deacetylation degree. This may be attributed to the fact that as the increasing of the degree of deacetylation, there exists more free amino group in the chitosan which could react with the more analytes through hydrogen bond. On the other hand, based on the same degree of deacetylation, the adsorption efficiency of the chitosan with average molecular weight of 1×10^6 is higher than that of chitosan with 4×10^6 average molecular weight, which can be explained that the chitosan with higher average molecular weight dissolves less in the solution due to the higher degree of twisting among the chitosan molecules. Based on the above results which implyed the choose of the chitosan was very important, the chitosan with 95% degree of deacetylation and average molecular weight of 1×10^6 was selected in the following experiment.

3.2.2. Effect of the type and amount of Fe_3O_4 NPs

Magnetic nanoparticles have been used as better adsorbents for their high surface areas and strong magnetism. In the novel SPE mode, Fe_3O_4 NPs acts as carrier to separate the chitosan loading three flavonoids based on the electrostatic attraction and complexation. In the experiment, two kinds of Fe_3O_4 NPs synthesized by the solvothermal and chemical co-precipitation methods were investigated and compared

in urine and serum samples. To find the optimized amount of Fe_3O_4 NPs for the extraction and make a comparative study between the two kinds of Fe_3O_4 NPs, the amount of two kinds of Fe_3O_4 NPs ranged from 0 to 12 mg were tested in the urine and serum samples. As shown in Fig. 6, the extraction recoveries increased with the increasing of Fe_3O_4 NPs, further increasing the amounts of the Fe_3O_4 NPs showed no significant improvement for the recoveries of flavonoids. From the Fig 6A and C in the urine sample and Fig 6B and D in the serum sample, it was inferred that higher extraction effiency and greater adsorption capacity could be obtained both in the urine and serum samples by using the Fe_3O_4 NPs prepared by the solvothermal method, which can be attbrituted to the fact that the Fe_3O_4 NPs prepared by the solvothermal method possess stronger higher saturation magnetization and larger specific surface area compared to the co-precipitation Fe_3O_4 NPs in the urine and serum samples. According to these results, both two kinds of Fe_3O_4 NPs could be successfully used to retrieve the chitosan and exploited for the SPE mode. The Fe_3O_4 NPs of the solvothermal method was selected because fewer amounts of nanoparticle adsorbents could achieve higher extraction efficiency, and therefore, 2 mg and 3 mg of Fe_3O_4 NPs prepared by the solvothermal method were selected and adequate for extracting the three analytes from the urine and serum samples, respectively, in the following study.

3.2.3. Effect of the amount of chitosan

The percentage of the retained three flavonoids is a key parameter depending on the amount of chitosan added. To achieve good extraction recoveries towards the three flavonoids, the amount of chitosan was investigated from 0 to 25 mg in the urine and serum samples, as shown in Fig 7A and B. From the results, it can be concluded that the flavonoids were hardly adsorbed onto the surface of Fe_3O_4 NP**s** in the absence of chitosan, indicating that the Fe_3O_4 NP**s** almost have no enrichment ability towards the three flavonoids. The adsorption ratio of luteolin, quercetin and kaempferol increasesd remarkably with the increasing amount of chitosan added into the solution, then kept invariant relatively. Comparing Fig. 7A and B, more chitosan is required for serum samples than urine samples because more protein or endogenous compound is

presented in the former. Given above findings, 10 mg and 15 mg chitosan were employed as the final addition amount of chitosan in urine and serum samples in the following studies, respectively.

3.2.4. Effect of solution pH

The pH is one of the prime factors to influence the extraction efficiency by affecting both the existing forms of analytes and the charge species and density on the adsorbents surface. In the present study, the isoelectric point (IEP) of Fe_3O_4 NPs and chitosan were around 3.0 and 8.16, relatively, and the effect of pH was investigated by varying the pH values from 3.0 to 10.0 in the urine and serum samples. Figure. 7C and D implied that maximum adsorption performance occurred at pH 5.0 in the urine sample and 7.0 in the serum sample, which is all between the isoelectric point (IEP) of Fe_3O_4 NPs and chitosan. When the pH value was around 3.0, the charge density of Fe_3O_4 NPs surface was very low and most of these functional groups present in chitosan are protonated and presented in positively charged form. The small amout of chitosan was absorbed on the Fe_3O_4 NPs and therefore could not be retrieved enough based on the electrostatic attraction [25]. In addition, the presence of large number of H^+ and H_3O^+ in the aqueous solution may compete with the three flavonoids for adsorption sites available on chitosan. Thus low extraction recoveries were observed at low pH. The high extraction recoveries increased as the increasing of pH varying from 4 to 8, which could be explained by the fact that as the increasing of the pH, there are more free $–NH_2$ and –OH functional groups in chitosan to react with the three flavonoids through hydrogen bond. Morever, chitosan and Fe_3O_4 NPs became the different charged. Therefore, the chitosan could be retrieved enough by the Fe_3O_4 NPs based on the strong electrostatic attraction.

However, when the pH was above 8, the extraction recoveries of the three flavonoids decreased as the increasing of the pH, which is due to the fact that chitosan and Fe_3O_4 NPs became the same negatively charged unfavorable to the retrieval process because of the electrostatic repulsion. In addition, the pKa values of luteolin, quercetin, kaempferol are 7.04, 7.36, 8.09, respectively[39], Under this condition, the three flavonoids were ionized and therefore electrostatic repulsion will occur between the

three flavonoids and negatively charged chitosan surface. For further studies, pH 5.0 and 7.0 were selected as the optimal pH in the urine and serum samples for the rest of experiments, respectively, since sufficient extraction recoveries were achieved of all the three flavonoids at those pH values.

3.2.5. Effect of extraction time

Generally, sufficient time is required to achieve the adsorption equilibrium for the analytes on the adsorbent. In this study, the effect of the extraction time on the extraction efficiency of the three flavonoids was investigated by changing the time from 2 to 30 min under the above optimal condition in the urine and serum samples The results were shown in Fig. 8A and B. The extraction recoveries for all the three flavonoids reached their maxima when the extraction time was increased to 5 min, and prolonged extraction time did not increase the extraction recoveries of the analytes any more, indicating that the extraction equilibrium could be achieved in a very short time. However, a little longer time was beneficial to get good reproducibility. Therefore, the extraction time of 10 min was selected,. which not only enabled these three flavonoids to be completely absorbed on the chitosan, but also ensured good reproducibility.

3.2.6. Effect of ionic strength

Ionic strength, which is examined through the addition of salt, may enhance the partition of analytes, thereby influcing the enrichment performance[16]. To investigate the effect of salt on the proposed method, various concentrations of sodium chloride from 0 to 12% were examined in the urine and serum samples. As shown in the Figure 8C and D, the result indicated that no significant effect on the extraction recovery was observed for luteolin and kaempferol in the whole NaCl concentration range investigated. While, for quercetin a slight decreased extraction recovery was observed with increased concentration of NaCl from start to finish. Therefore, no addition of NaCl into the sample solution was adopted for all the subsequent experiments in urine and serum samples.

3.2.7. Desorption conditions

A suitable desorption solvent plays an important role in MSPE. In the study,

acetonitrile and methanol were studied for the desorption of analytes from the chitosan adsorbents. In the first place, it is found that the analytes could not be desorbed from the adsorbents completely even when desorption time was prolonged to 4h, but the desorption ability of acetonitrile was superior to that of methanol. This phenomenon may be attributed to the fact that the multi-interactions such as hydrogen bonds between the adsorbent and analytes could not be disrupted by the above solvent completely. Therefore, acetonitrile with different contents of acetic acid (1–10%, v/v) was used as the desorption solvent to desorb the three flavonoids. The results indicated that as the increasing of the amount of acetic acid, the recoveries of luteolin, quercetin and kaempferol increased remarkably, but then the adsorption of analytes decreased gradually. Because the addition of acetic acid not only caused the analytes to exist in the molecular form which was prone to be soluble in organic solvent, but also made the chitosan dissolve faster, while excess acetic acid could disrupt the desorption ability of acetonitrile[40]. At last, acetonitrile containing 5% HAc was selected as the final desorption solvents and quantitative recoveries of the flavonoids were achieved with 1 mL acetonitrile containing 5% HAc (0.5 mL every time and washed two times).

3.3. Method validation

Based on the above results, the conditions of the MSPE were as follows: 10 mg chitosan was added into urine samples, and next, 2.0 mg Fe_3O_4 NPs was added to the solution to retrieve the chitosan, 15 mg chitosan was added into serum samples, 3.0 mg Fe_3O_4 NPs was added to the solution to retrieve the chitosan. Extraction time was 10 min, acetonitrile containing 5% HAc was used for the desorption. To evaluate the accuracy and feasibility of the method developed, Fig. 9A and B shown the chromatograms of blank and the spiked urine and serum samples, respectively. The samples were treated with the same proposed SPE before injection to HPLC. The retention time of quercetin, luteolin and kaempferol were 9.57 min, 11.94 min and 17.69 min, respectively. No matrix effects such as the proteins and endogenous components in urine and serum samples were observed, which implies the excellent specificity for the determination of quercetin, luteolin and kaempferol with this novel

two-step MSPE technique.

3.3.1. Linearity, LOD and LOQ

Under the optimized conditions, the quantitative parameters of the proposed method, including linear range, correlation coefficients, precision, limits of detection (LOD), limits of quantification (LOQ) and recovery were evaluated in urine and serum samples, respectively. As shown in Table 1, each analyte exhibited good linearity with correlation coefficient $R^2 > 0.9990$ in the studied range. The LOD of the investigated compounds in urine samples should be was the minimum concentration determined based on a signal-to-noise ratio of 3 (S/N = 3). They were estimated to be 0.50 ng mL^{-1}, 0.10 ng mL^{-1} and 0.20 ng mL^{-1} for quercetin, luteolin and kaempferol in urine samples and 10 ng mL^{-1}, 2.0 ng mL^{-1} and 5.0 ng mL^{-1} for quercetin, luteolin and kaempferol in serum samples, respectively. The LOQ values based on a signal-to-noise ratio of 10 (S/N = 10) were 0.80 ng mL^{-1}, 0.25 ng mL^{-1} and 0.50 ng mL^{-1} for quercetin, luteolin and kaempferol in urine samples and 15 ng mL^{-1}, 5.0 ng mL^{-1} and 8.0 ng mL^{-1} for quercetin, luteolin and kaempferol in serum samples, respectively.

3.3.2. Precision and recovery

The overall performance of the method in terms of the intra-day and inter-day precision and accuracy were evaluated by applying the proposed MSPE to six replicate spiked urine and serum samples at three different concentration levels (low, middle and high quantification concentrations) of the targets in the same day and in three consecutive days, respectively. The relative standard deviations (RSDs) of the intra-day and inter-day precision and accuracy values for spiked urine and serum samples are summarized in Table 2. The RSDs of the intra-daily tests are less than 5.0%, and the RSDs of inter-daily tests are less than 5.4%. These results indicated that the two-step MSPE method has good accuracy and precision.

The recovery of the method was assessed using the standard addition method. Table 3 showed the recoveries calculated after spiking three different concentration levels low, middle and high quantification mean value of three independent determinations. It can be seen that satisfactory recovery ranged from 93.5% to 108.8% and the RSDs of

recoveries ranged from 3.0% to 5.0% were obtained using the proposed method. Thus the method is suitable for analyzing the three flavonoids in biological samples.

3.3.3. Comparison of the present two-step MSPE with other analytical methodologies

The present work established a easy-handle, cheap and accurate two-step extraction mode for the determination of quercetin, luteolin and kaempferol in human urine and serum samples. Table 4 compared some analytical performance of previous reported methods for the determination of the flavonoids. As can be see, the present method exhibited some remarkable strong points from the viewpoint of LOD, linearity, extraction time, recovery, accuracy, and especially the adsorbent used.

Firstly, compared to traditional SPE method [41, 42], our present work not only have lower LODs and wider linear ranges but also possess some attractive merits. An important aspect should be pointed out that the chitosan is low-cost, accessible and environmentally friendly, which turns the method into a potential technique for routine analysis of actual samples. The Fe_3O_4 NPs used in the study possess the stong magnetization characteristics and large specific surface area, which enable that the Fe_3O_4 NPs were favorable for MCT enough to retrieve the chitosan containing the targeted annlytes. With the aid of Fe_3O_4 NPs, the present method greatly simplifies sample pretreatment and eliminates the time-consuming column passing and filtration operation and shows great analytical potential in pretreatment.

Secondly, It is known to us that magnetic mixed hemimicelles solid-phase extraction method is widely used in the sample pretreatment [36, 39, 40]. However, the present method still have some remarkable superiority over it. In the first place, as shown in the table 4, the extraction time of the present method is shorter than that of the magnetic mixed hemimicelles solid-phase extraction method. Because the adsorbent chitosan exhibits several desirable properties such as dispersing ability, high adsorption capacity and fast adsorption rate, which render the method capable of high extraction efficiency and short equilibrium time. Besides, the strong hydrogen bond between the chitosan and the targeted analyst have afforded a high affinity and selectivity toward the three flavonoids, which is superior to the other method.

Accordingly, successful application in the enrichment and determination of the three trace flavonoids from serum and urine samples suggested that the two-step MSPE method based on magnetic carrier technology (MCT) and the chitosan could be a promising alternative for fast and selective extraction of trace amounts of the natural therapeutic substances from biological fluids.

4. Conclusions

In conclusion, a rapid, sensitive and simple two-step SPE method based on the magnetic retrieval of chitosan was the first time to be applied in biological samples for extraction and preconcentration of the active compounds. Three types of chitosan with different degrees of deacetylation and average-molecular weight were investigated. The experimental results have demonstrated that chitosan with 95% degree of deacetylation and average-molecular weight of 1×10^6 was found to be more appropriate for SPE of luteolin, quercetin and kaempferol. Two kinds of magnetic nanoparticles synthesized by solvothermal and chemical co-precipitation methods were compared and the solvothermal method was selected because higher extraction efficiency and greater adsorption capacity was achieved, which was attributed to the higher saturation magnetization and larger specific surface area of Fe_3O_4 NPs prepared by this method.

It is widely known to us that the efficient preconcentration of trace compounds in biological samples remains a challenge. Although some researchers have conducted a preliminary exploration of the extraction mode based on the magnetic retrieval of chitosan, this is the first attempt to apply it in complex biological samples and study it more comprehensively and systematically. Moreover, good linearities and recoveries with serum and urine samples were obtained, which indicates that this proposed method can be successfully applied in the sample preparation of biological samples and will hopefully have high analytical potential for preconcentration of trace analytes from complex samples.

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575

576 ## Figure Captions

577 Fig. 1 Structures of quercetin, luteolin and kaempferol.

578 Fig. 2 Schemes of two-step magnetic retrieval of chitosan and its application as MSPE
579 adsorbents for simultaneous extraction and preconcentration of targeted analytes in
580 urine and serum samples.

581 Fig. 3 TEM images of Fe_3O_4 NPs prepared by the solvothermal method (A) and Fe_3O_4
582 NPs prepared by the chemical co-precipitation method (B).

583 Fig. 4A XRD patterns for Fe_3O_4 NPs prepared by the solvothermal method (a) and
584 Fe_3O_4 NPs retrieval chitosan composites (b). Fig. 4B FT-IR spectra of Fe_3O_4 NPs
585 prepared by the solvothermal method (a), chitosan (b) and Fe_3O_4 NPs retrieval
586 chitosan composite (c). Fig. 4C VSM magnetization curves of Fe_3O_4 NPs prepared by
587 the solvothermal method and chemical co-precipitation method. Fig. 4D
588 Zeta-potential at different pH of chitosan, Fe_3O_4 NPs prepared by the solvothermal
589 method and Fe_3O_4 NPs prepared by the chemical co-precipitation method.

590 Fig. 5 Effect of the types of chitosan on the adsorption of luteolin, quercetin and
591 kaempfero performed in urine samples (A) and serum samples (B). Operation in the
592 batch mode. Concentration of each analyte: 60.0 ng/mL. In urine samples : Amount of
593 chitosan: 10 mg; Amount of Fe_3O_4 NPs prepared by the solvothermal method: 2 mg;
594 pH: 5.0;. In serum samples: Amount of chitosan: 15 mg; Amount of Fe_3O_4 NPs
595 prepared by the solvothermal method: 3 mg; pH: 7.0;

596 Fig. 6 Effect of the type and amount of Fe_3O_4 NPs on the adsorption of luteolin,
597 quercetin and kaempferol in urine samples (A, C) and serum samples (B, D).
598 Operation in the batch mode. Concentration of each analyte: 60.0 ng/mL. In urine
599 samples: Amount of chitosan: 10 mg; pH: 5.0;. In serum samples: Amount of chitosan
600 15 mg; pH: 7.0;

601 Fig. 7A-B Effect of the amount of chitosan on the adsorption of luteolin, quercetin
602 and kaempferol performed in urine samples (A) and serum samples (B). Operation in
603 the batch mode. Concentration of each analyte: 60.0 ng/mL. In urine samples:

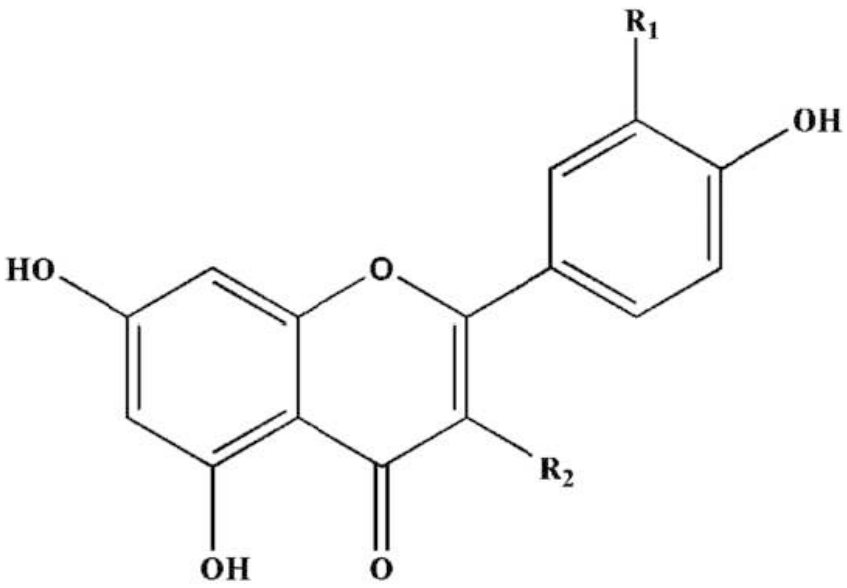
Amount of Fe_3O_4 NPs prepared by the solvothermal method: 2 mg; pH: 5.0. In serum samples: Amount of Fe_3O_4 NPs prepared by the solvothermal method: 3 mg; pH: 7.0;

Fig. 7C-D Effect of pH on the adsorption of luteolin, quercetin and kaempferol in urine samples (C) and serum samples (D). Operation in the batch mode. Concentration of each analyte: 60.0 ng/mL. In urine samples; Amount of chitosan: 10 mg; Amount of Fe_3O_4 NPs prepared by the solvothermal method: 2 mg. In serum samples: Amount of chitosan: 15 mg; Amount of Fe_3O_4 NPs prepared by the solvothermal method: 3 mg;

Fig. 8 (A-B) Effect of extraction time on the adsorption of luteolin, quercetin and kaempferol in urine samples (A) and serum samples (B). (C-D) Effect of ionic strength on the adsorption of luteolin, quercetin and kaempferol in urine samples (C) and serum samples (D).

Fig. 9 HPLC–UV chromatograms of samples with the two-step MSPE procedure: (A) blank urine sample; (B) urine sample spiked with 5 ng mL^{-1} standard solution of three flavonoids; (C) blank serum sample; (D) serum sample spiked with 20 ng mL^{-1} standard solution of three flavonoids.(1. quercetin; 2. luteolin; 3. kaempferol).

621 **Fig. 1.**



R_1=OH, R_2=OH, Quercetin
R_1=OH, R_2=H, Luteolin
R_1=H, R_2=OH, Kaempferol

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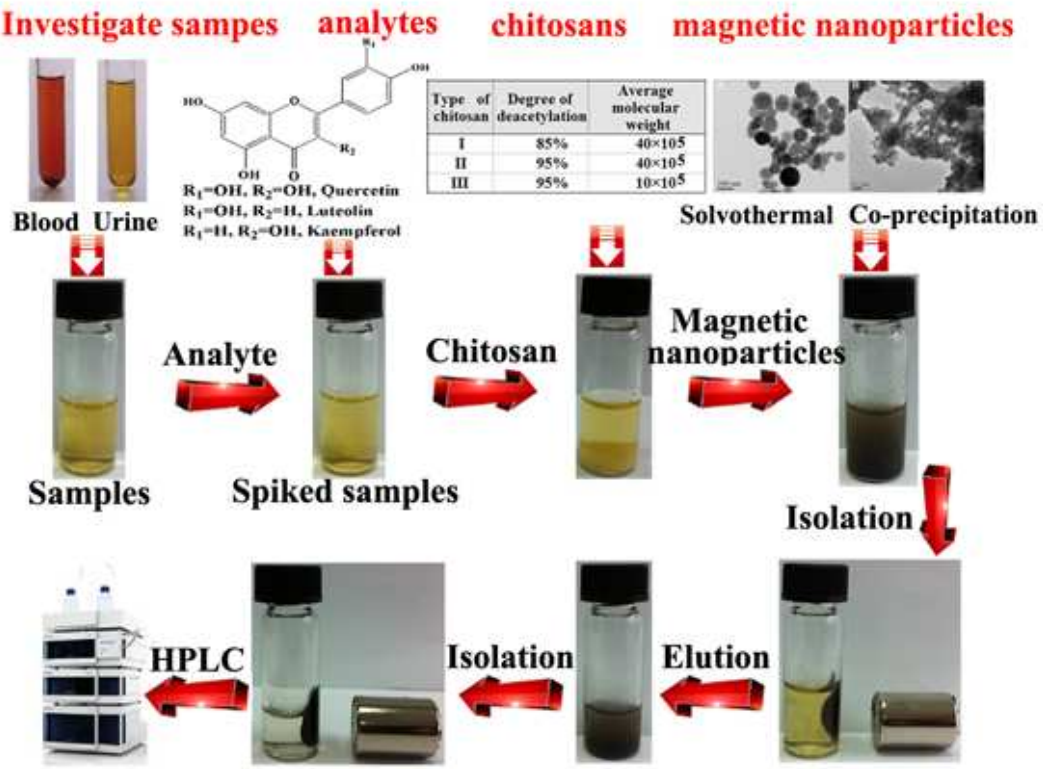
624 **Fig. 2.**



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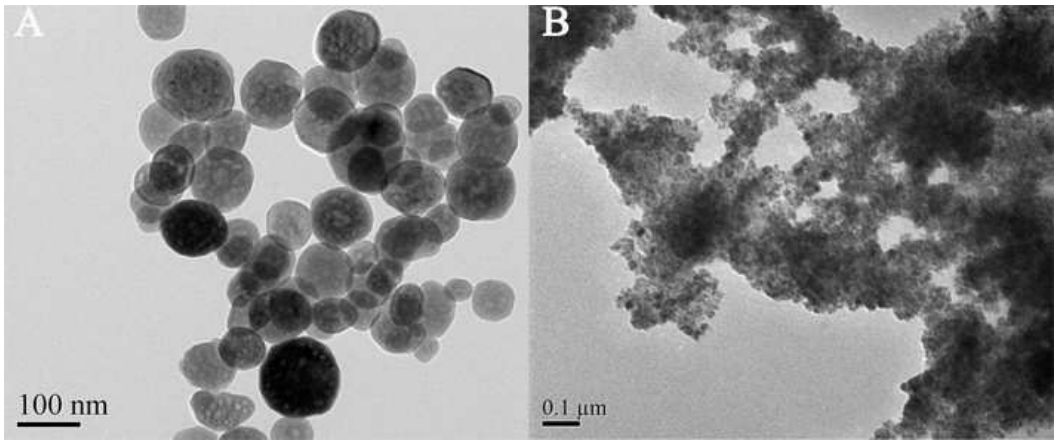
627 **Fig. 3.**



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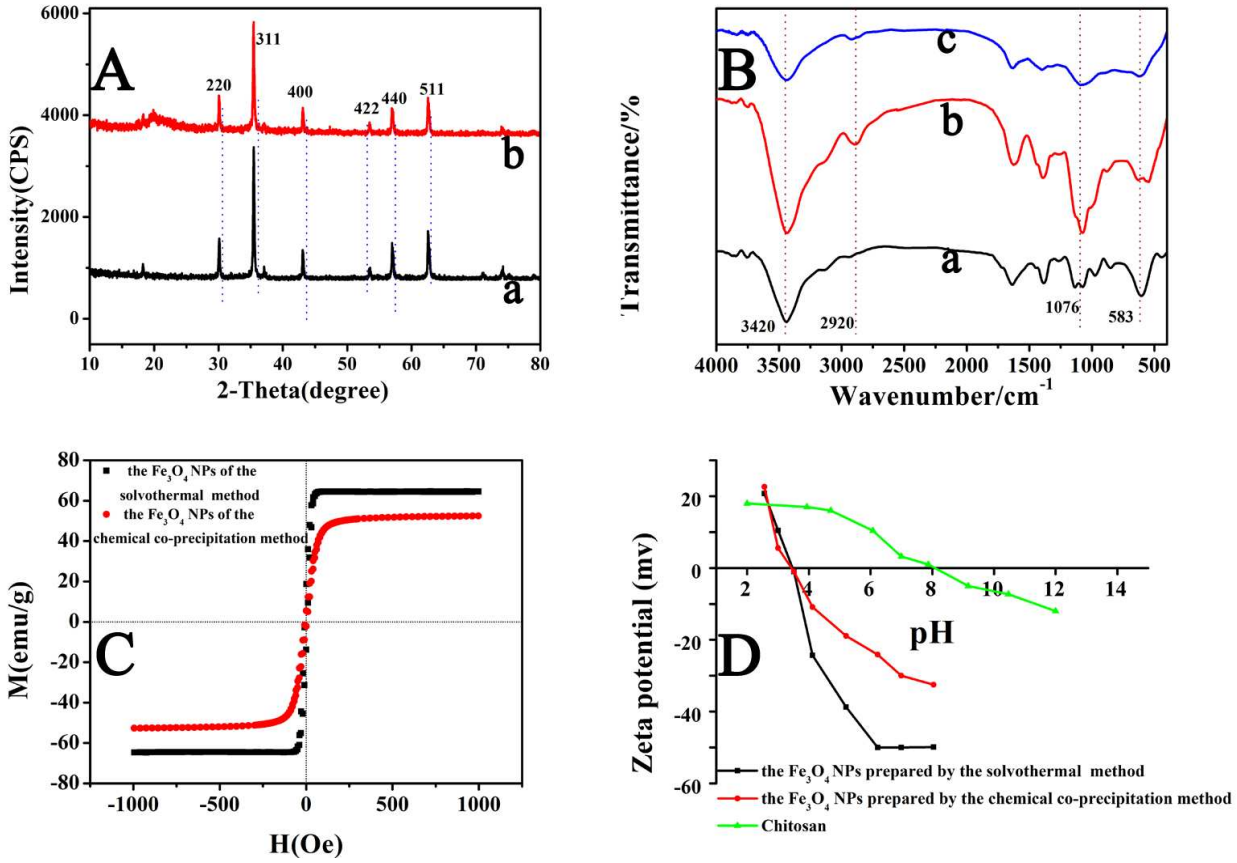
630 **Fig. 4.**



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633 **Fig. 5.**

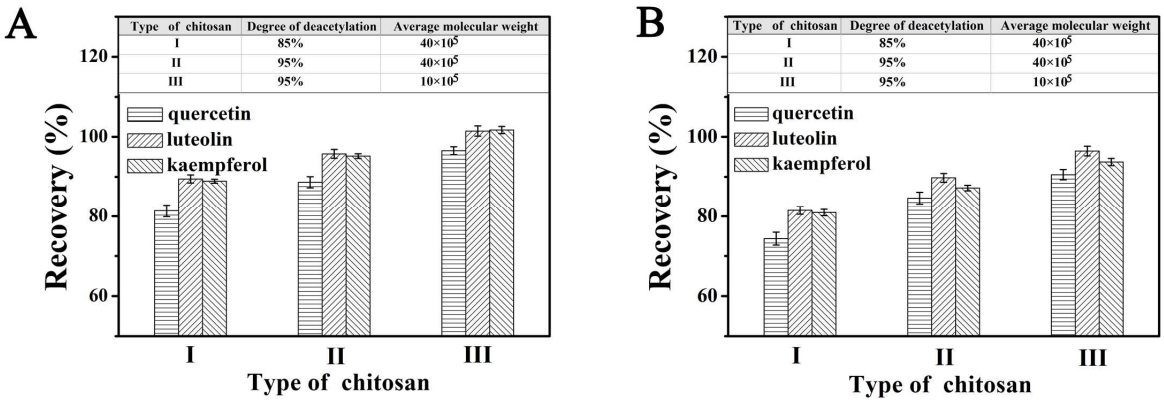

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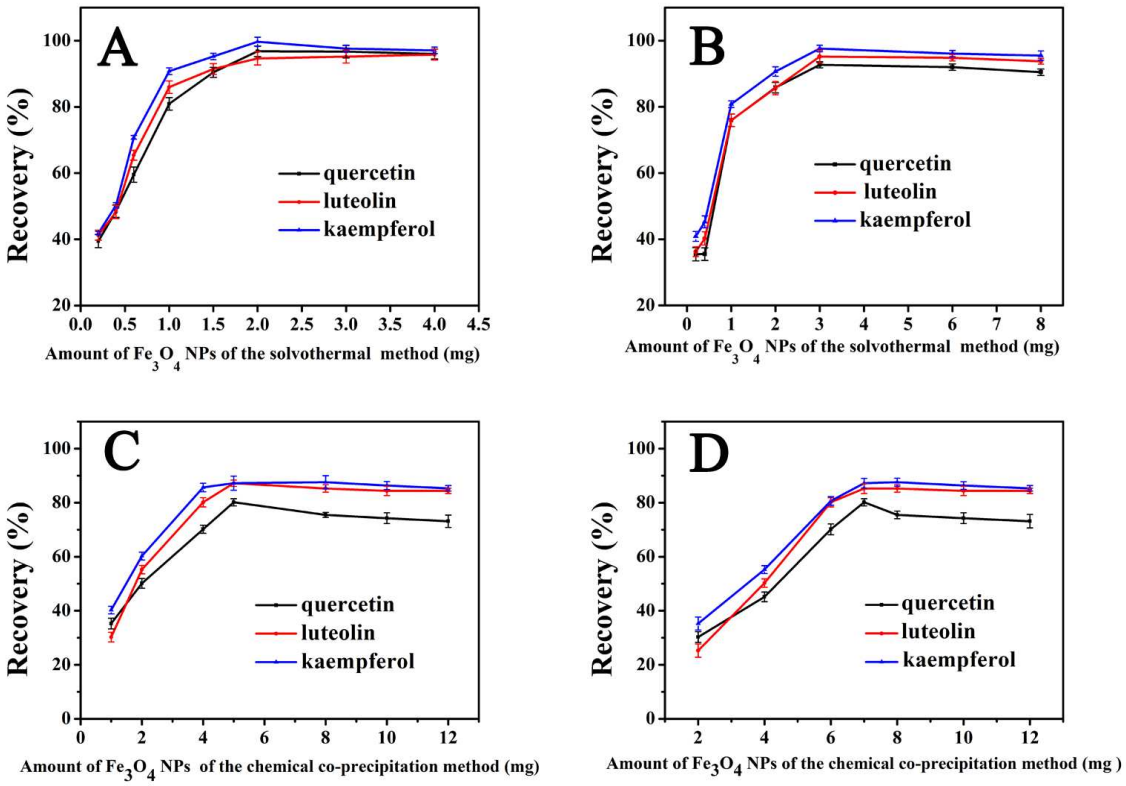
Fig. 6



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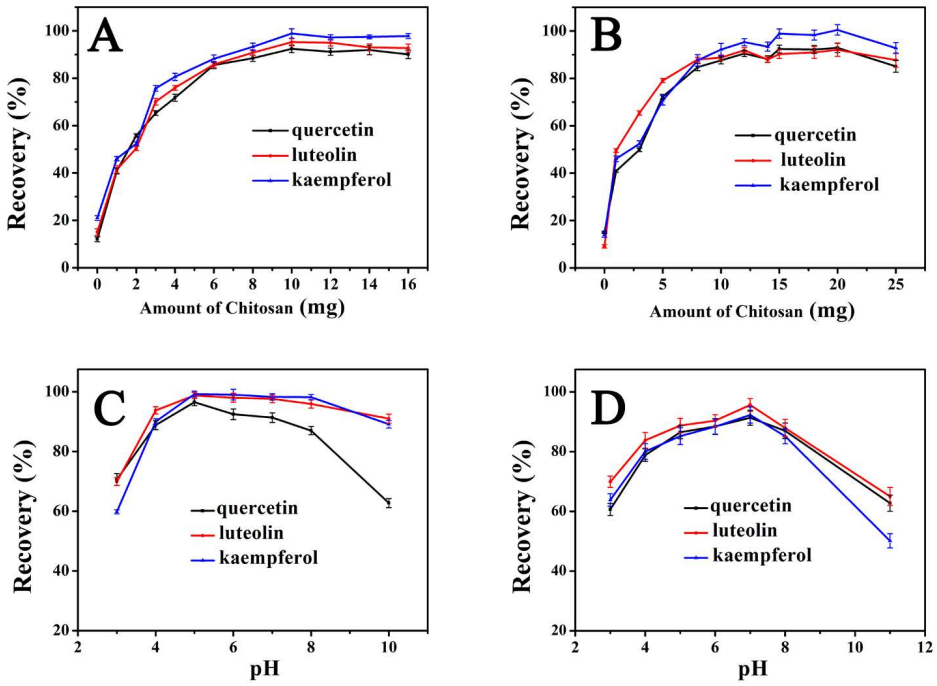
638 **Fig. 7.**



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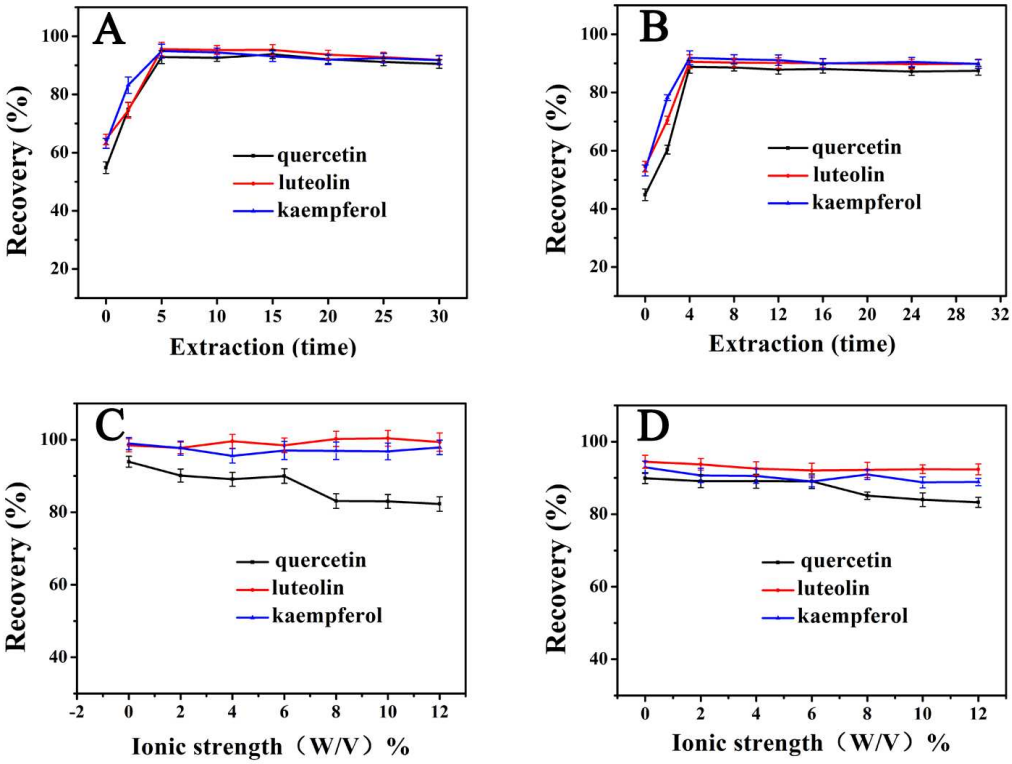
642 **Fig. 8.**



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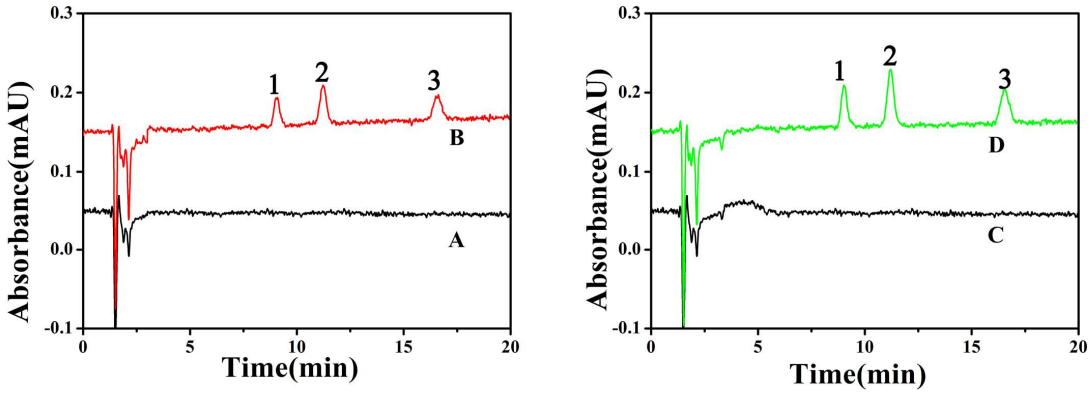
645 **Fig. 9.**



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647

Table 1. Analytical parameters of the proposed method.

Biological Samples	Compound （n=6）	Linear rang (ng ml^{-1})	Linearity (R^2)	LOD (ng ml^{-1})	LOQ (ng ml^{-1})
urine	Quercetin	3.0-2000	0.9995	1.0	2.0
	Luteolin	2.0-2000	0.9994	0.50	1.0
	Kaempferol	2.5-2000	0.9997	0.70	1.3
serum	Quercetin	20-2000	0.9994	10	15
	Luteolin	10-2000	0.9991	2.0	5.0
	Kaempferol	15-2000	0.9992	5.0	8.0

650 **Table 2.Precision and accuracy for detection of three flavonoids in urine and**
651 **serum samples.**

Biological Samples	Compound (n = 6)	Conc. (ng ml^{-1})	Inter-day (n=6)		Intra-day (n=6)	
			Mean accuracy (%)	RSD (%)	Mean accuracy (%)	RSD (%)
Urine	Quercetin	10	94.3	4.6	93.5	3.3
		100	93.6	3.2	93.2	4.2
		1500	93.7	3.5	91.5	3.5
	Luteolin	5	97.1	4.5	95.6	3.4
		100	91.1	2.8	97.8	4.8
		1500	95.2	3.1	98.5	4.1
	Kaempferol	5	98.4	3.6	96.3	4.9
		100	93.5	4.2	97.5	3.5
		1500	98.1	4.5	95.4	4.6
Serum	Quercetin	20	94.5	5.0	93.6	5.4
		200	93.4	3.6	94.5	3.6
		1500	90.1	2.9	97.6	3.0
	Luteolin	10	95.6	4.5	90.1	4.5
		200	96.4	3.8	91.4	3.5
		1500	95.8	2.4	96.5	3.1
	Kaempferol	10	97.4	4.1	90.9	3.8
		200	96.4	4.0	96.8	4.0
		1500	93.4	3.1	97.4	3.1

652

653 **Table 3.Recovery for three flavonoids from blank urine and serum samples.**

Bioligical samples	Compound (n = 6)	Conc. (ng ml^{-1})	Mean recovery (%)	RSD(%)
urine	Quercetin	10	99.0	4.8
		100	93.5	3.0
		1500	106.5	2.5
	Luteolin	5	99.8	4.9
		100	96.6	2.8
		1500	105.5	3.4
	Kaempferol	5	108.8	5.0
		100	98.1	4.1
		1500	100.1	3.6
serum	Quercetin	20	98.7	4.5
		200	105.2	3.6
		1500	100.4	3.7
	Luteolin	10	104.2	5.1
		200	99.7	3.4
		1500	98.4	4.0
	Kaempferol	10	95.8	4.0
		200	97.4	3.0
		1500	102.1	4.5

654

655 **Table 4 Comparison of different methods applied to extract the flavonoids.**

Method	Sample matrix	Recovery (%)	Linear range (ng ml^{-1})	LOD (ng ml^{-1})	LOQ (ng ml^{-1})	RSD (%)	Extraction Time (min)	Ref
HF-LPME-HPLC-UV[a]	Echinophora platyloba DC	92.0–99.0	3.0–500	0.5–7.0	-	3.18-11.00	-	43
UAE-HPLC-UV[b]	The dried celery	72.7–89.5	1×10^2–2.5×10^5	70	-	2.5–4.5	90	44
SPE-HPLC-UV[c]	Plasma and urine	-	4.0–1×10^3	0.35-7	35	1.5–9.4	~45	5
LLE-HPLC–QQ- MS[d]	plant	93.03-98.06	5.0-2×10^3	1.0	5	1.3-3.0	>60	9
SPE- CZE[e]	Flos Lonicer	93-104	8×10^3–1.59×10^5	60	1800	2.57-4.36	>60	42
SPE –UHPLC-UV[f]	urine	70.35–96.58	0.05–5.0	15.4	46.2	3.9-5.0	>60	41
MMHSPE-HPLC-UV[g]	urine	90.1–97.6	0.5–1500	0.1-0.5	0.25-0.8	3.3-5.0	90	39
MSPE-HPLC-UV	urine and serum	91.2-99.7	2.5-2000	0.5-10	1-15	2.5-5.0	10	This method

656 [a] HF-LPME-HPLC-UV: hollow fibre liquid phase microextraction- high-performance liquid
657 chromatography-ultraviolet detector. [b] UAE-HPLC –UV: ultrasonic-assisted extraction-
658 high-performance liquid chromatography- ultraviolet detector. [c] SPE-HPLC-UV: solid phase
659 extraction - high-performance liquid chromatography- ultraviolet detector. [d] LLE -HPLC–QQQ
660 MS: liquid-liquid extraction-high-performance liquid chromatography-triple quadrupole mass
661 spectrometry. [e] SPE- CZE: solid phase extraction-capillary zone electrophoresis. [f] SPE
662 –UHPLC-UV: solid phase extraction -ultra high-performance liquid chromatography- ultraviolet

detector. [g] MMHSPE-HPLC-UV: magnetic mixed hemimicelles solid-phase extraction method-high-performance liquid chromatography-ultraviolet detector.