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Solid-Phase Extraction of Florfenicol from Meat Samples by a Newly Synthesized Surface Molecularly Imprinted Sol–Gel Polymer

S. Sadeghi · M. Jahani

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Abstract A new molecularly imprinted polymer (MIP) as a solid-phase sorbent for selective extraction of florfenicol (FF) was prepared by combination of the surface molecular imprinting technique with the sol–gel process. The FF-imprinted silica sorbent was prepared using FF as template, 3-aminopropyltriethoxysilane as functional monomer and tetraethoxysilicate as cross-linker on the silica gel support. The non-imprinted silica (NIP) was synthesized in the same way without addition of FF. The MIP was evaluated as a sorbent in column extraction approach for extraction of FF from aqueous solutions followed by spectrofluorometric determination. The influence of certain variables including the sample pH, the sample volume, the sample flow rate, the type of eluent, and its flow rate on the extraction efficiency of FF was assessed. The prepared FF-MIP silica sorbent showed higher adsorption capacity (64.9 mg g^{-1}) and significant selectivity than the corresponding NIP (11.5 mg g^{-1}). The FF-MIP-based solid phase extraction method was successfully applied to the separation and determination of FF from fish and chiken meat samples under the optimized extraction conditions.

Keywords Florfenicol · Molecularly imprinted polymer · Sol–gel · Solid-phase extraction · Food samples

Introduction

Florfenicol (FF) and chloramphenicol (CAP) belong to a broad spectrum antibiotic family widely used in the veterinary medicine. This group of antibiotics can inhibit the bacterial enzymes which are necessary for protein synthesis. According to their toxic side effects, and the risk of drug residues in animal tissues, their clinical applications are strictly controlled and many countries including China, USA, and member states of European Union (EU) prohibited the use of these drugs (Zhang et al. 2008; Xie et al. 2011; Kinsella et al. 2009; Hayes et al. 2003).

Florfenicol as a synthetic analogue of CAP is proposed to replace CAP and now is widely used in veterinary medicine. Many countries established the maximum residue levels (MRLs) for veterinary residues in animal-based food stuffs. In the European community, the MRL for the sum of the FF and its metabolites was set in the range of 100–250 μg kg^{-1} (Zhang et al. 2008; Alechaga et al. 2012). Thus, it is important to develop analytical methods for determining FF present in animal products. To quantify the trace level of FF and its related analogous in animal tissue matrices, several analytical techniques such as gas chromatography (Nagata and Oka 1996), high-performance liquid chromatography with detections of mass spectrometry (Malik et al. 2010; Zhang et al. 2008), fluorescence spectroscopy (Xie et al. 2011), UV–Vis spectrophotometry (Koc et al. 2009; Feng and Jia 2009; Anadon et al. 2008), and capillary electrophoresis (Kowalski et al. 2005) have been used. But these methods need to sample preparation and cleanup procedures before analysis (Zhang et al. 2008; Li et al. 2006), and sometimes, an additional cleanup step with a commercial solid-phase extraction (SPE) sorbent is required.

The use of the molecularly imprinted polymers (MIPs) as selective sorbents for solid-phase extraction has been one of the most promising technical applications of MIPs in the last decade and most of the recent studies have been focused on the extraction of the analytes of interest from biological samples (Caro et al. 2006; Ma et al. 2012; Stoilova et al. 2013). In the preparation of the MIPs, a template molecule is linked to the suitable monomer(s) containing proper functional group(s)

S. Sadeghi (✉) · M. Jahani
Department of Chemistry, Faculty of Science, University of Birjand, Birjand, Iran
e-mail: ssadeghi@birjand.ac.ir

and then copolymerization occurs in the presence of the cross-linker. The initial complex between the template and monomer molecules forms by covalent or non-covalent bonds. In some cases, preparation of MIPs is relatively easy and inexpensive and they can be used for many times without losses in their specific characteristics. Their selectivity arises from the used specific synthetic approach. The selectivity of MIPs has been exploited in solid-phase extraction (MISPE), so that MISPE allows not only the analyte to be preconcentrated but also the other compounds present in the sample matrix to be removed. Most of the studies performed have focused on extraction of compounds from biological samples and few studies related to the extraction of compounds from edible animal tissue samples (Benito-Penˇ et al. 2008; Guo et al. 2008; Hu et al. 2013; Shi et al. 2012; Chen et al. 2009, 2011).

Due to the high cross-linking nature of MIPs, extraction of the template located at the interior of the polymer material in bulk polymerization method is quite difficult. This problem can be solved by surface imprinting in which the imprinted templates are located at the surface or in the proximity of the polymer surface. As a result, the binding capacity enhances and mass-transfer and binding kinetics becomes faster. Several strategies have been examined for surface imprinting such as immobilized template (Yang et al. 2005), initiator or supporting matrix (Qin et al. 2009), and controlled living free radical polymerization (CLRP) (Wang et al. 2006). Among the support particles which have been used in the surface imprinting process, activated silica gel shows promising characteristics due to the nature of non-swelling of silica, stability in the acidic or basic conditions, and high thermal resistance (Fang et al. 2014; Chen et al. 2011; Dıaz-Garcıa and Laıno 2005).

Despite emerged interests in the imprinting techniques, to the best of our knowledge, no surface imprinted polymer has been reported for the separation and determination of FF, so far. The purpose of the present work is synthesis of a new FF-MIP sorbent by surface imprinting technique as a solid-phase sorbent for selective sorption of trace amount of FF in food samples prior to determination by spectrofluorometry. In this study, the FF was used as the template, 3-aminopropyltriethoxysilane (APTES) as functional monomer, and tetraethoxysilicate (TEOS) as cross-linker agent in tetrahydrofuran (THF) as polymerization solvent. Under the optimized conditions, the imprinted polymer was applied as a sorbent to extract FF from spiked chicken and fish meats. Moreover, the selectivity of the prepared MIP was evaluated by determination of FF in chicken meat sample coupled with HPLC analysis. The results showed that the MIP could effectively recognize FF in complex matrices. The developed MISPE coupled with fluorescence detector offered an alternative method for clean up and determination of FF, carrying the benefit of simplicity, low cost, and sufficient sensitivity, without any large volumes of organic solvents or further treatment for removing the matrix interferences.

Experimental

Chemicals and Reagents

FF was donated from Erfan Darou Pharmaceutical Company (Tehran, Iran). CAP was purchased from Sigma-Aldrich Corporation (Missouri, USA). APTES and TEOS were purchased from Merck (Darmstadt, Germany) and used as the functional monomer and cross-linker agents, respectively. Silica gel (70-230 mesh, Merck) was used as the support and was activated by hydrochloric acid. Methanol (MeOH), acetonitrile (MeCN), THF, acetic acid (HOAC), ethylacetate, and other organic solvents were purchased from Merck (Darmstadt, Germany) and used without any purification.

A stock FF solution was prepared by dissolving an appropriate amount of FF in doubly distilled water (DDW) and stored in the dark place at 4 °C. The work or standard solutions were freshly prepared before use by dilution from the stock solution.

Apparatus and Equipments

A molecularly imprinted polymer solid-phase extraction (MISPE) system was used for preconcentration of the FF. The MISPE columns were prepared by filling the imprinted or non-imprinted sorbents into SPE cartridges prepared by empty polypropylene clinical syringes (5 cm×10 mm i.d.) with two high density polyethylene frits at each end. The FF solution was passed through the MISPE column at a constant flow rate by an EYELA SMP-23S peristaltic pump (Tokyo Rikakikai Co, Japan). A RF-5301PC spectrofluorometer (Shimadzu, Japan) equipped with a xenon discharge lamp and 1 cm quartz cells was used for the determination of FF. Peristaltic pumps were used to deliver all solutions and PTFE tubing (0.8 mm i.d.) was used to connect the flow system. Infrared spectra (4,000–600 cm^{-1}) of the synthesized MIP and NIP in KBr were recorded by a Perkin-Elmer 781 infrared spectrometer (PerkinElmer Ltd, Buckinghamshire, England). All the absorption spectra of FF were recorded on a 2501 PC UV–Vis spectrophotometer (Shimadzu, Japan) by wavelength scanning from 220 to 350 nm at room temperature. The chromatography test was performed with a Shimadzu chromatography system (Tokyo, Japan) consisted of a Model LC-10A VP HPLC pump and a SPD-10A VP UV detector. A Branson 1510R-MTH ultrasonic bath (BRANSON ultrasonic corporation, Danbury, USA) and a Hettich EBA-20 centrifuge (DJB Labcare Ltd., England) were used in pretreatment of meat samples. DDW (18.0 MΩ cm^{-1}) was obtained from an Aqua Max ultra pure water system (Young Lin, Korea). All pH measurements were performed by use of a pH/ion meter Schott CG 843 (SCHOTT Instruments GmbH, Mainz, Germany) with an uncertainty of 0.1 mV.

Preparation of FF-Imprinted and Non-imprinted Silica Sorbents

The silica gel surfaces were activated by combination of 10 g of the silica gel with 100 mL of 6 mol L^{-1} HCl. The resultant mixture was refluxed under stirring for 10 h. The solid product was filtered and washed repeatedly with DDW to neutrality and dried at 110 °C for 12 h.

To prepare the FF-imprinted silica sorbent using a sol–gel reaction, FF (0.4764 g) and APTES (0.48 mL) were dissolved in THF (5 mL) and stirred for 30 min at 45 °C. Then, activated silica gel (0.15 g) and TEOS (1.18 mL) were added to this mixture and stirred for another 20 min. After addition of ammonia to the mixture as catalyst (0.3 mL of 0.1 mol L^{-1}), the sol was stirred for 15 min to form the sol–gel polymer and then was incubated at 60 °C in an oil bath for 15 h to obtain high cross-linking density polymer. The solid resultant product was isolated from the solution by centrifugation at 6,000 rpm for 10 min and washed with ethanol. To remove the template molecules from the imprinted sol–gel material, it was refluxed with MeOH for 24 h. The product was isolated by filtration and washed with DDW for several times and dried in an oven at 70 °C for 24 h (Duan et al. 2011). The non-imprinted polymer was prepared in the same way without addition of FF. Figure 1 shows the scheme of stepwise preparation of the FF molecularly imprinted sol–gel polymer.

Determination of FF

All fluorescence intensities were assayed at room temperature (25±1 °C) by spectrofluorometry using excitation wavelength at 232±1 nm and emission wavelength at 290±1 nm (or scanning from 220 to 350 nm). Both the excitation and emission slits were set at 5 nm.

Rebinding Experiments

The steady-state binding capacity of the MIP or NIP to FF was measured in a static (batch) mode. For this, 50 mg of MIP or NIP silica sorbents was mixed with FF solution (10 mL) at different concentrations (10–900 mg L^{-1}). The mixtures were mechanically shaken for 7 h at room temperature and then filtered through 0.45-μm membrane filters and the concentration of the unbound FF in the supernatants was determined. The adsorption capacity (q, mg g^{-1}) was calculated as the following:

$$q = \frac{(C_0 - C_e)v}{w} \quad (1)$$

where C_0 and C_e are the initial and final concentrations of the analyte in the solution, and v and w are the volume of solution (L) and the mass of the polymer (g), respectively.

MISPE Procedure

SPE cartridges were packed with the 150 mg of MIP or NIP dry particles. Then, the cartridges were rinsed and pretreated with 5 mL of MeOH/H_2O (50 %, v/v) to remove residual contaminations and then washed with DDW. Afterwards, 15 mL of FF solution at appropriate concentration with pH= 6.0 was introduced onto the MIP cartridge at a flow rate of 0.5 mL min^{-1}. The cartridge was rinsed with 2 mL of DDW to remove nonspecific retained analyte and then eluted with

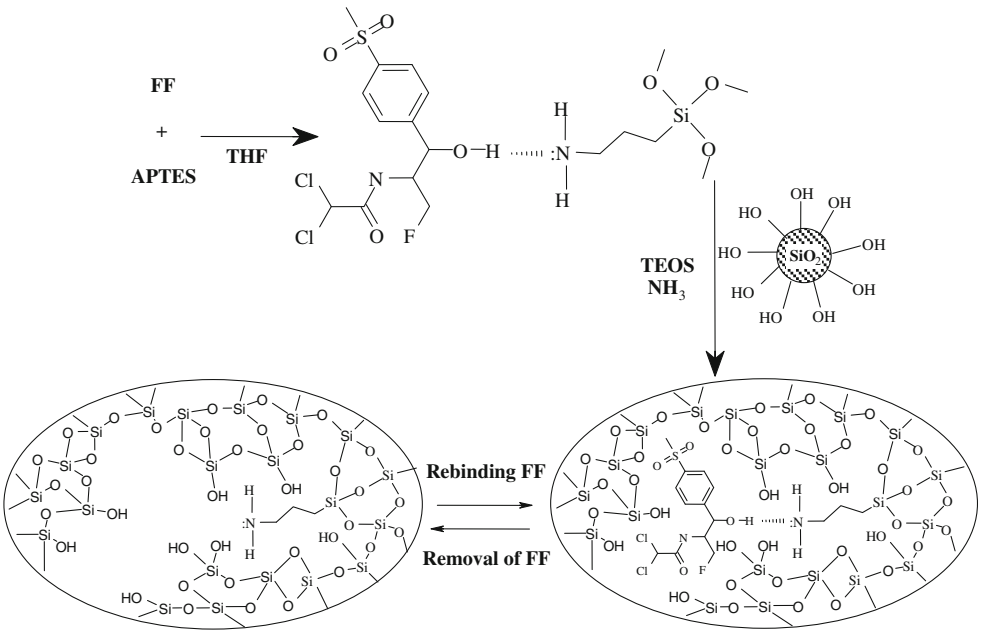

Fig. 1 Scheme of the FF molecularly imprinted sol–gel preparation

2.5 mL of ammonium acetate solution containing 30 % (*v*/*v*) MeOH/H_2O at a flow rate of 0.5 mL min^{-1}. The eluate was analyzed for determination of the adsorbed FF by spectrofluorometry.

Selectivity of the MISPE Column

Selectivity of the FF-MIP silica sorbent was performed in column method. After pre-conditioning of the SPE columns with MeOH/H_2O solution (50 %, *v*/*v*), 15 mL binary solutions containing 0.5 mg L^{-1} of FF and coexisting substances at different concentrations was introduced onto the MIP or NIP-SPE columns at a flow rate of 0.5 mL min^{-1}. The columns were washed with 2 mL of DDW and eluted with 2.5 mL of ammonium acetate solution containing 30 % (*v*/*v*) MeOH/H_2O and the eluate was analyzed for determination of FF.

Sample Collection and Pretreatment

Tissues (chicken and fish) used in this study were purchased from a local food supermarket. The tissue samples were chopped and homogenized and then 1 g of ground tissue was weighed into a 50 mL polypropylene vessel. The samples were spiked with FF at three levels namely, 3.0, 4.0, and 5.0 μg g^{-1} and then were diluted with 6 mL of ethylacetate solution (83 %, *v*/*v*). The mixture was homogenized in an ultrasonic bath for 10 min and centrifuged at 6,000 rpm for 5 min and the supernatant was introduced to a 10-mL polypropylene centrifuge tube. The extraction step was repeated and the two supernatants were combined. After evaporation of the combined supernatant to dryness at 60 °C under a gentle stream of nitrogen, the residue was dissolved in 10 mL of DDW and then 5 mL *n*-hexane was added and vigorously mixed. After centrifugation at 6,000 rpm for 10 min, the hexane layer was discharged. The water-based phase was filtered through a 0.45-μm filter, and after pH adjustment, the sample was passed through a SPE column and the eluted solution for FF content was analyzed spectrofluorometrically by the standard addition method (Feng and Jia 2009).

Results and Discussion

Preparation of the FF-MIP

In the non-covalent approach for the synthesis of MIPs, a preliminary complex forms between the template molecule and the functional monomer and afterwards this complex was polymerized with the aim of the cross-linker. Often, creation of nonspecific pores by solvent in polymeric matrix in the polymerization process reduces the specificity of the resultant polymer for the recognition of the template. The sol–gel process in imprinting of a template can be considered as an alternative method to reduce this problem. Selective binding in non-covalent imprinting is due to ionic interactions or hydrogen bonding, so the right selection of functional monomers is a critical step for successful molecular imprinting. In this study, APTES was selected as the functional monomer which can form stable complex through hydrogen bonding interactions of hydroxyl group of FF with amine group of APTEs in the pre-polymerization mixture.

To understand the recognition mechanism, the interactions between the template and the functional monomer was studied by means of spectrophotometry. Individual solutions of the FF, APTES, and their mixture were prepared in the THF. FF concentration is approximately 68 g L^{-1} in the polymerization step, but in such high concentrations appropriate absorption spectra cannot be recorded. For comparison, in mixture of FF and APTES, a solution was prepared containing 5 mg L^{-1} FF and 10 mg L^{-1} with regarding the same concentration ratio of template (FF) to monomer (APTES) as polymerization conditions. After equilibrating the mixture, the UV–Vis spectra were recorded in the 220–500-nm region. The changes in absorbance of the solution were determined at 280 nm, at which APTES has no significant absorption. As presented in Fig. 2a, the absorption values of FF (a, 5 mg L^{-1}) and APTES (b, 10 mg L^{-1}) at 280 nm were 0.8611 and 0.069, respectively, while the maximum absorbance of the FF-APTES mixture (c) at this wavelength was 2.141. This absorbance value was much higher than the numeral summation of FF and APTES absorbances (d). This difference resulted from the interaction of –OH group of FF and N-H group of the APTES in pre-polymerization mixture. In addition with increasing of the FF concentration in the solution, the absorbance became stronger. Thus, the residual amino group of APTS at the surface of silica particles may also more contribute to the interaction with FF resulting in a high density of recognition sites in the imprinted polymer.

The solvent has a substantial effect in the non-covalent imprinting process. It must dissolves the constituent of the polymerization mixture specially the template, meanwhile solvents with lower polarity have less effect on the hydrogen bonding interactions between the template and monomer. In this study, the solubility of FF in several common solvents was examined and the THF with lower polarity and appropriate solubility was selected as the polymerization solvent.

In preparation of the MIP sol–gel material, TEOS acts as an accelerator of the condensation process in the imprinting process and contributes to a higher rigidity of the siloxan network around the template. TEOS was hydrolyzed by aqueous ammonia according to the Stöber method (Stöber et al. 1968). The gelation reaction takes place between the hydroxyl groups at the surface of silica and produces a FF-APTES/TEOS MIP–sol layer at the surface of the silica particles. The

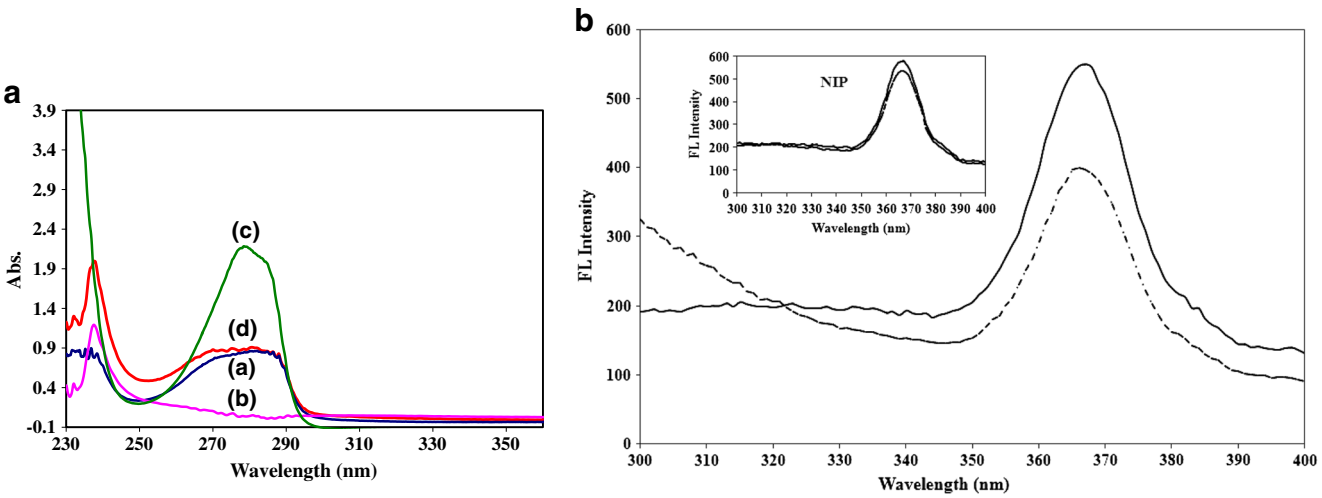

Fig. 2 **a** The UV–Vis absorption spectra of (**a**) FF (5 mg L^{-1}), (**b**) APTES (10 mg L^{-1}), (**c**) the mixture of FF and APTES (1:2, *v*/*v*), and (**d**) the sum of absorption values for FF and APTES in THF; **b** the fluorescence emission spectrum of the of the FF solution employed in rebinding experiment for imprinted and non imprinted sol–gel: (*solid line*) before and after (*broken line*) uptake the FF

removal of the template generates cavities with recognition sites that are complementary to the shape, size, and chemical functionality of FF molecule. In addition, the rigidity of the silica matrix stabilizes these recognition sites.

The fluorescence emission spectra of the FF-imprinted silica and non-imprinted polymer sorbents before and after uptake of FF are presented in Fig. 2b. The standard FF solution has an emission peak at 290 nm, but the FF-imprinted silica sorbent shows an emission peak at 368 nm. It is expected that particular active recognition sites at the imprinted sol–gel created by FF is responsible for the adsorption of the FF and the spectral changes. These interactions in the non-imprinted polymer sorbent were lower than that of the FF-imprinted silica sorbent.

Characteristic of the Infrared Spectra

The activated silica-gel and the leached MIP and NIP were characterized by their IR spectra. As can be seen in Fig. 3, the most absorption bands for the activated silica around 950 and 1,100 cm^{-1} represented the stretching vibrations of the Si–O–Si and Si–O–H groups. The absorption band at 3,450 and 1,620 cm^{-1} are assigned to the OH vibration frequencies, while the band around 800 cm^{-1} resulted from Si–O vibration. Compared with the IR spectra of pure silica (Fig. 3a), the FF-imprinted silica (Fig. 3b) displayed clearly the characteristic vibration frequency peaks of the N–H group at around 1,530 cm^{-1} and C–H group around 1,410 and 2,920 cm^{-1}, indicating that the silica-modified polymer has been successfully coated with APTES. The –OH groups in silica at 3,330 cm^{-1} were absent in the MIP silica sorbent.

The absorption bands at about 3,300 cm^{-1} in Fig. 3b belong to NH_2 group in APTES that appeared after removal of the template molecule from MIP. Due to random incorporation of functional monomers in the polymer matrix of NIP which is the origin of non-selective binding sites, these absorption bands could not be seen clearly in Fig. 3c.

Specificity Evaluation of the MIP

Metronidazole (MN) and the structural analogue of FF namely, CAP, were selected to investigate the selectivity of the MIP. FF is a fluorinated derivative of CAP, which has fluorine and methyl sulfonyl groups substitute for the nitro and hydroxyl groups in CAP. Fifteen milliliters of standard solutions of each compound with 0.5 mg L^{-1} (pH 5.4) was individually percolated through the prepared MIP cartridges, and the capacity of the cartridges was calculated according to Eq. (1). The

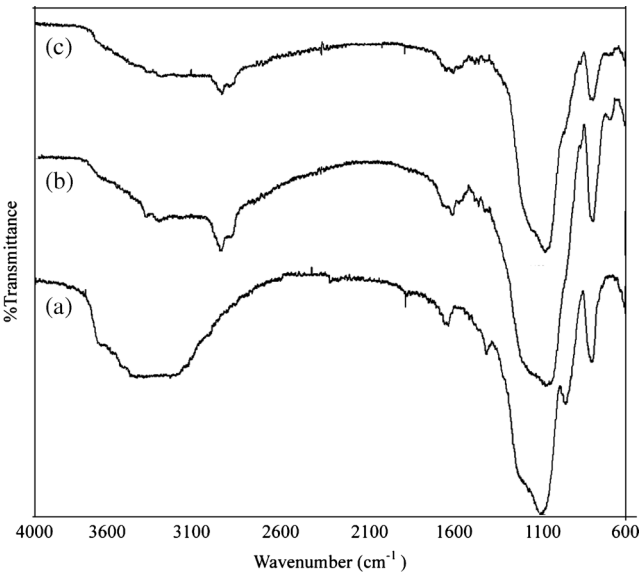

Fig. 3 The IR spectra of the activated silica gel (**a**), imprinted (**b**), and non-imprinted (**c**) sorbents

recovery was only 25.3 % for CAP and 12.9 % for MN on the MIP cartridges, while the recovery was 95.0 % for FF. The results demonstrated that the MIP had higher ratio of capacity for the adsorption of FF than MN and CAP (QMIP, FF/QMIP, CAP=4.4; QMIP, FF/QMIP, MN=6.6), indicating that MIP had a superior specific adsorption capacity to FF compare to its structurally related compounds. Thereby, the designed MIP in this study with high selectivity could be used for separation and enrichment of FF.

Kinetic Uptake of FF by the FF-Imprinted Sol–Gel Material

To evaluate the kinetics of recognition of FF by the imprinted material in a static extraction mode, the uptake amounts of FF from 10 mL of 10 mg L^{-1} FF solution by 50 mg of the imprinted or non-imprinted silica sorbents were examined at different times (5–600 min). As can be seen in Fig. 4a, the loading half-time ($t_{1/2}$), defined as the time required for reaching 50 % of the sorbent total loading capacity, determined from the resulting adsorption capacity–time plot found to be 250 min for the MIP silica material, whereas the non-imprinted particles needed longer time. It seems that the adsorption rate was faster at the early stage of adsorption probably because of easy occupation of imprinted sites on the surface of the polymer of the FF molecules onto the imprinted cavities at surface of the MIP material. After that, the FF molecules penetrate to the internal cavities that take more time, resulting in slower adsorption as already reported in literature (see, e.g., Song et al. 2009; Gao et al. 2007). However, much faster kinetics has been reported (Sadeghi and Jahani 2013).

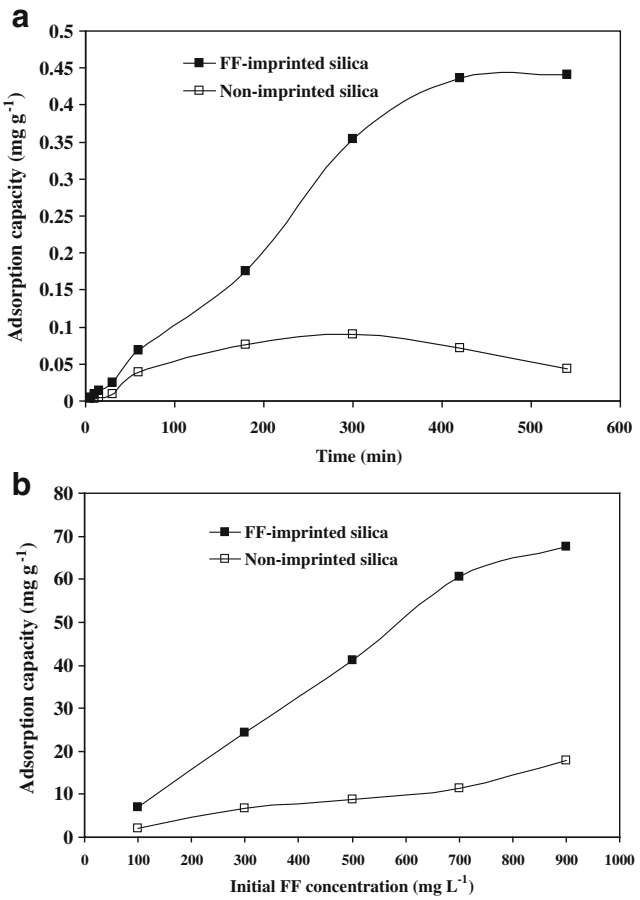

Fig. 4 The comparison of molecular recognition properties: the kinetic uptake of 10 mg L^{-1} FF solution at pH 6 (**a**) and the rebinding amount of FF at different concentrations (**b**) by 50 mg MIP and NIP silica sorbents

Evaluation of Static Adsorption Capacity

The rebinding amount of FF by the MIP and NIP was determined by incubation of 50 mg of sorbents with various FF concentrations and measuring the difference between the initial and the residual amount of FF in the solution. The results are depicted in the adsorption isotherm curve in Fig. 4b. It illustrates that the adsorption capacity of the MIP for FF is significantly greater than that of the NIP (60.5 vs. 11.5 mg g^{-1}), which further demonstrates that the MIP has effective imprinted sites for the FF molecule. The imprinted factor which is defined as the ratio of the adsorption capacities of the MIP to the NIP was calculated as 5.26.

The MIP adsorption isotherm of the FF-imprinted silica material was interpreted by the Langmuir and Freundlich isotherm models. The Langmuir isotherm model describes monolayer adsorption based on homogeneous and identical adsorption sites equivalent which is described in linearized expression of Eq. (2):

$$\frac{C_e}{q_e} = \frac{C_e}{q_{max}} + \frac{1}{q_{max} K_L} \tag{2}$$

where C_e is the equilibrium concentration of FF (mg L^{-1}) in supernatant phase, q_e is the equilibrium adsorption capacity (mg g^{-1}) at given FF concentration, q_{max} is the maximum sorption capacity (mg g^{-1}) of the adsorbent, and K_L (L mg^{-1}) is the equilibrium adsorption constant related to the affinity of the adsorption sites. Meanwhile, the Freundlich isotherm model considers heterogeneous adsorption sites and is presented in linear form as Eq. (3):

$$\log q_e = \log K_F + \frac{1}{n} \log C_e \tag{3}$$

where K_F (L g^{-1}) and n are constants related to adsorption capacity and heterogeneity factor in Freundlich isotherm. The parameters and the correlation coefficients calculated from the corresponding models were given in Table 1. According to the obtained results, both the adsorption models fit the experimental data well, but heterogeneous binding model showed slight improvement for adsorption of FF. It may be attributed

Table 1 The parameters of Langmuir and Freundlich isotherms for adsorption of FF by the FF-imprinted silica sorbent

Langmuir			Freundlich		
R^2	K_L (L mg^{-1})	q_{max} (mg g^{-1})	R^2	K_F (mg g^{-1})	n
0.9682	9.12×10^{-4}	64.93	0.9991	77.85	0.75

to the fact that there are sites with low and high binding energies to adsorb/release the template. Thus, the Freundlich model may be describe as the adsorption of FF onto the sorbent.

Optimization of the MISPE Procedure

The FF-imprinted silica as a SPE sorbent was used in the MISPE of FF in column method. Various parameters affecting the extraction efficiency of the polymer including sample acidity, sample flow rate, sorbent weight, and type of the eluent were optimized.

Effect of Sample pH

Taking into account the chemical structure of the template molecule and presence of specific functional groups at the active sites in the MIP, the dependency between extraction efficiency and sample pH would be expected. The influence of the sample pH on the adsorption of 15 mL of 0.5 mg L^{-1} FF solution was evaluated in the pH range of 2–9 with MISPE columns packed with 100 mg of dry polymer. Figure 5a shows the effect of pH on the adsorption of FF by imprinted and non-imprinted silica materials. It can be seen that the adsorption capacities of FF on both materials were increased significantly with the increase in pH below pH 5, but then levelled off at pH levels 5.0–6.0. The specific sites for FF binding on the polymeric backbone are amino groups incorporated at the surface of the silica particles and in the inside of the polymer cavities which can be protonated at low pH values (≤pH 5). The decrease in the adsorption capacity at low pH values can be attributed to the competitive binding of H_3O^+ and FF to the amine groups. On the other hand, at higher pH values, the extraction decreased because a high fraction of FF was present in its neutral form (pK_a 9.03), but amino groups were deprotonated which decreased the interactions and lowered the extraction efficiency. The maximum FF adsorption was obtained at pH 6.0.

Effect of Sample Flow Rates

The proper interaction of the target molecule with the specific binding sites in the MIP needed to appropriate sample flow rate that can enhance retention of the target molecule on the MIP column. In this study, 15 mL of 0.5 mg L^{-1} FF solution at pH=6.0 was passed through the MISPE column packed with 100 mg of MIP or NIP at different flow rates (0.25–2.0 mL min^{-1}). The results are presented in Fig. 5b. As expected, a low loading flow rate benefits the sufficient interaction of FF with the selective binding sites and higher extraction efficiency was obtained. The retention amount of analyte gradually reduced at high flow rates. Therefore, the maximum extraction efficiency was achieved when the sample flow rate was fixed at 0.5 μL min^{-1}.

Influence of the Washing Step

It is well known that the target molecule could be retained on the MIP by selective and nonspecific interactions. In order to reduce the nonspecific interactions, a washing step is implemented with a solution that has moderate elution strength to not disrupt the specific interactions between the imprinted polymer and FF and thereby retaining FF in the imprinted polymer. After loading of 15 mL of 0.5 mg L^{-1} FF solution (pH 6.0) onto SPE column at flow rate of 0.5 mL min^{-1}, a washing step was performed with 2 mL of several solvents such as HOAC/H_2O (1 %, *v*/*v*), MeOH/H_2O (1 %, *v*/*v*), and DDW. The retained amount of FF on the column was determined spectrofluorometrically and the best result was obtained using 2 mL of DDW.

Effect of the Sorbent Weight

In the next step, MIP columns containing different amounts of the dry sorbent (50–200 mg) were prepared and preconditioned. The efficiency of these columns was evaluated in adsorption of 15 mL of 0.5 mg L^{-1} FF solution (pH 6.0) at a flow rate of 0.5 mL min^{-1}. As illustrated in Fig. 5c, the adsorption capacity increased when increasing the sorbent weight until 150 mg and after that, not significant improvement was seen in adsorption capacity. It is evident that with increase of the sorbent weight, the higher adsorption sites were accessible to uptake FF and resulted in an increase in the adsorption efficiency.

Selection of the Eluent

To obtain high enrichment factor, the effect of different eluents on recovery of the FF from the polymeric materials with minimal amount of the eluent was investigated. For this, the column containing 150 mg of dry polymer was prepared and pre-conditioned. The concentration of FF was maintained at 0.5 mg L^{-1} and percolated onto the column at flow rate of 0.5 mL min^{-1}. Figure 5d compares the achieved recoveries of FF after eluting the column with 2.5 mL of MeOH/H_2O or MeCN/H_2O at a flow rate of 0.5 mL min^{-1}. It can be seen that although MeOH with higher polarity than MeCN showed high recovery, but the improvement of the elution strength

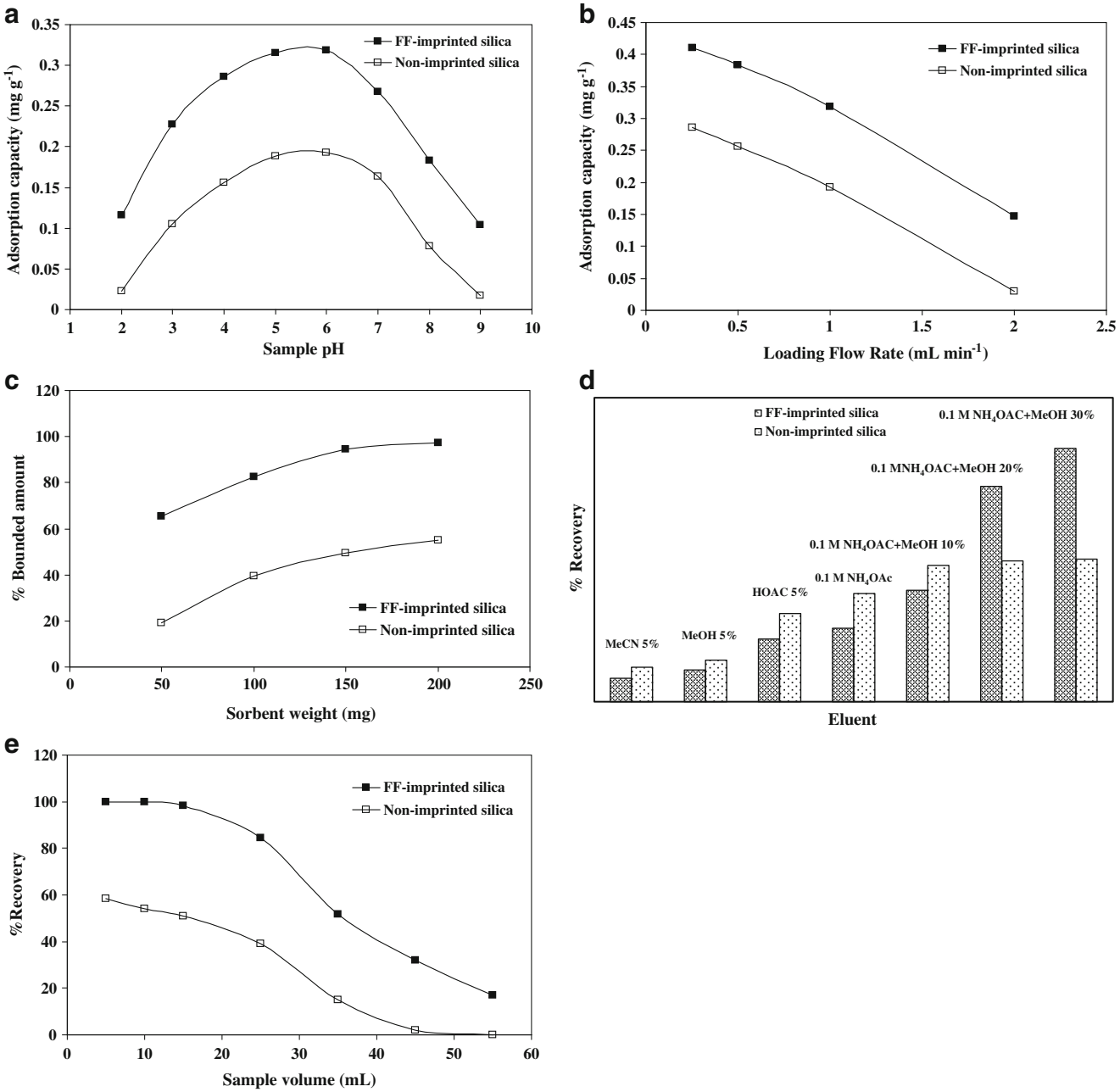

Fig. 5 **a** The effect of sample pH on the extraction efficiency of FF by MIP and NIP silica sorbents; **b** dependence of extraction efficiency of FF on the sample loading flow rate; **c** influence of weight sorbent on the extraction efficiency of FF; **d** the effect of the eluent on the recovery percentage of FF; **e** the FF recoveries as a function of the sample volume (conditions: sample volume=15 mL, FF concentration=0.5 mg L^{-1} at pH 6.0; elution: 2.5 mL of NH_4OAC 0.1 mol L^{-1}+MeOH 20 % at a flow rate of 0.5 mL min^{-1})

using a suitable base could be more effective due to high interruption of the hydrogen bonds between the template and the binding sites on the imprinted silica. By considering the silica nature of the sorbent and its deterioration at high pH media, a 0.1 mol L^{-1} ammonium acetate solution at pH=8.0 was tested. The results showed that by addition of 0.1 mol L^{-1} ammonium acetate to 30 % (*v*/*v*) MeOH/H_2O eluent, the most recovery of FF was obtained.

Sample Breakthrough Volume

The sample volume that influences the magnitude of analyte retention is known as the breakthrough volume. An enrichment step of the samples is required in order to achieve the low FF concentration levels detection. For this purpose, different volumes from 5 to 55 mL containing a total amount of 7.5 μg FF were introduced on to the SPE cartridges and the retained analyte was eluted with MeOH/H_2O (30 %, *v*/*v*) containing

0.1 mol L^{-1} NH_4OAC. As seen in Fig. 5e, with increasing sample volume beyond 15 mL, a high decrease in retention of FF on the column was observed probably owing to increase in physical adsorption of FF on the column and lost it in the washing step. For a 15-mL sample, the process resulted in a nominal enrichment factor of 6.

Reusability and Repeatability of the MIP-SPE Column

The stability of the MIP toward the SPE of FF was investigated by using an FF-imprinted silica cartridge for five adsorption–desorption cycles under the optimized conditions. After each usage of the cartridge, it was regenerated with MeOH/H_2O (50 %, *v*/*v*) solution and again was used to SPE process. The recovery of FF after the second cycle of regeneration was 96 % and after five cycles it was 91.8 %. These results indicate to good stability of the FF-MIP material. The repeatability of the MIP was explored by using five extractions of 0.5 mg L^{-1} FF solution at pH 6. The relative standard deviation was 4.5 %.

Selectivity

MIP cartridges were pre-conditioned and all MISPE experiments were performed under optimized conditions. The binary solutions containing 0.5 mg L^{-1} of FF and different concentration of the coexisting substances were prepared and the influence of the coexisting substance on the fluorescence intensity of the FF before and after the MISPE was studied. The results are presented in Table 2. As can be seen, the presence of Na^+, K^+ ions, and some investigated amino acids have no significant effects on the FF recoveries up to 1,000 and 100 mg L^{-1}, respectively. Although CAP is a structural analogue of the FF, but the existence of CAP in the sample insignificantly affected the recovery of FF up to 5 mg L^{-1}. Meanwhile, the recoveries of 90 and 85 % were found for FF in the mixture of this compound with 5 mg L^{-1} metronidazole and erythromycin, respectively. These results provide evidence that the specific recognition sites are mainly complementary to the template in terms of their size and shape, indicating good selectivity of the synthesized MIP for cleanup and preconcentration of FF.

Determination of FF in Meat Samples

The effects of the matrix on the uptake of FF were investigated by MISPE of meat samples. At first, the blank meat samples were analyzed by HPLC for the residue of FF. Blank chromatogram showed no peak at FF retention time. Thus, the proposed method was applied to the extraction of FF from spiked fish and chicken samples. The FF contents in spiked meat samples were found using standard addition method. The calibration curves for FF in the chicken and fish matrix were constructed using FF concentrations of 1–7 and 2–8 μg mL^{-1}, respectively, with correlation coefficients higher than 0.99. The chicken and fish meat samples were spiked at three levels of FF: 3.0, 4.0, and 5.0 μg g^{-1}. A blank and three

Table 2 The recovery percentages of FF by the FF-imprinted silica sorbent in the presence of different concentrations of coexisting substances

Coexisting substance	Concentration ratio (coexisting substance/FF)	Change of fluorescence intensity of FF (%)
L-lysine	10	−4.4
	100	−6.8
	250	−21.7
Glutamine	10	−0.8
	100	−1.9
	250	−19.3
Alanine	10	−1.2
	100	−3.4
	250	−9.7
Chloramphenicol	1	−1.3
	2	−7.8
	4	−6.2
	8	−10.9
Metronidazole	1	−4.8
	2	−4.2
	8	−4.6
	10	−11.6
Erythromycin	1	−1.5
	2	−1.4
	4	−11.3
Na^+	100	2.8
	250	−4.5
	1,000	−8.5
K^+	100	−1.1
	250	−2.7
	1,000	−2.7

Table 3 Application of FF-imprinted silica sorbent in extraction of FF from spiked meat samples

Sample	Added (μg g^{-1})	% Recovery	% RSD
Chicken	3	96.6	1.0
	4	95.4	2.3
	5	85.6	5.2
Fish	3	95.3	3.0
	4	93.9	2.9
	5	87.4	3.9

samples at each concentration per species were analyzed for FF. The accuracy of the method was calculated as the percent recovery of FF in each sample. The precision of the method was calculated as the relative standard deviation for each concentration level (%RSD). The accuracy ranged from 85.6 to 96.6 % with precision varying from 1.0 to 5.2 % for all spiked levels (Table 3). The limit of detection of the method calculated based on signal-to-noise ratio of 3 was 0.58 and 0.28 μg g^{-1} in chicken and fish meat matrices, respectively. In accordance with the results, the prepared MIP sorbent showed good performance for trace concentration levels of FF in real samples with appreciable selectivity and accuracy.

To validate the established method in real complex samples, the MISPE cartridge was used for SPE of FF in chicken sample before HPLC analysis. SPE provides a simple effective extraction and purification method for complex sample matrixes. The chicken meat samples were spiked with standard FF solution at three different concentration levels. The FF content in the spiked samples was analyzed by HPLC on a C_{18} column (4.6×250 mm) with a flow rate of 0.5 mL min^{-1} at room temperature. The mobile phase was a mixture of MeCN/H_2O (30:70, *v*/*v*) and was filtered through a 0.45-μm filter prior to use. The UV detector was operated at 224 nm. The spiked chicken meat samples were processed according to

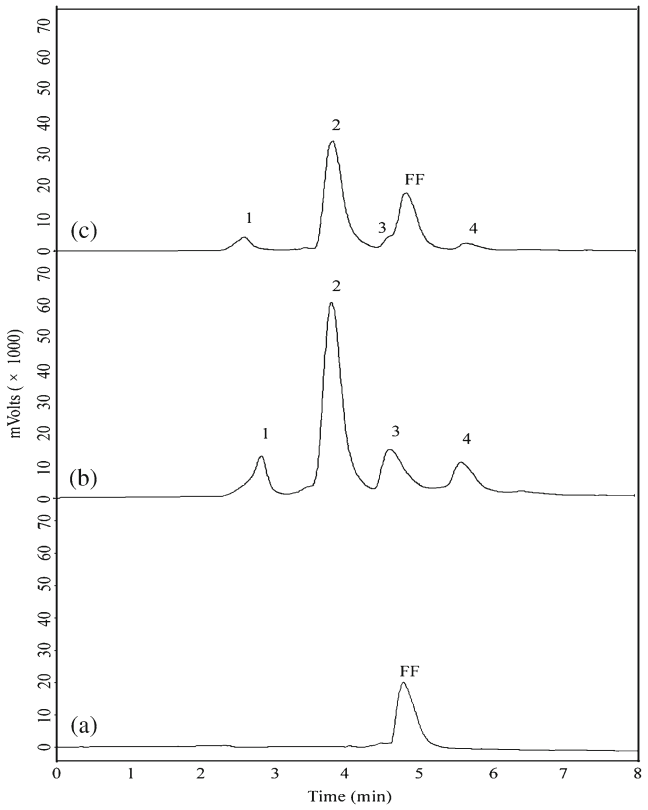

Fig. 6 The recorded HPLC chromatograms of **a** direct injection of 3 mg L^{-1} of FF standard solution; **b** direct injection of chicken meat sample extract; and **c** chicken meat sample extract spiked with 3 mg L^{-1} of FF with a cleanup of MISPE

Table 4 The main features of the developed method and other reported MISPEs for determination of FF

Analyte	MIP synthesis method	Extraction technique/ Detection system	The spiked FF concentration	LOD	%Recovery	%RSD	Matrix	Capacity mg g^{-1}	Ref.
FF	Bulk	FIA-MISPE/ fluorescence	5.0–25.0 μg/mL	0.34 μg/mL	96.8–100	3.5	Liver, pork, chicken, fish	0.104	Ge et al. (2010)
Fenicols	Bulk	MISPE-HPLC/UV	1.5–4.5 μg/kg	0.102 μg/kg	94.5–98.0	3.2	Shrimp	–	Shi et al. (2012)
FF	Precipitation	MISPE/UV	50 μg/g; 50 μg/g; 20 μg/g	–	88.9; 93.5; 96.2	3.6; 2.5; 3.2	Fish; Chicken; Honey	4.32	Sadeghi and Jahani (2013)
FF	Surface imprinting	MISPE/ fluorescence	3.0–5.0 μg/g	0.58 μg/mL; 0.28 μg/mL	85.6–96.6; 87.4–95.3	1.0–5.2; 2.9–3.9	Fish; Chicken	64.9	This work

FIA flow injection analysis

the recommended procedure and the results of analysis recorded in Fig. 6. Figure 6a shows the chromatogram of FF standard solution which is identified with a retention time at 5.9 min. In comparison with the chromatogram of direct injection of chicken meat extract in Fig. 6b, an obvious enhancement of the FF peak height was observed after MISPE in Fig. 6c; meanwhile, the other matrix peak intensities (peak nos. 1–4) were substantially reduced, indicating the remarkable preconcentration ability of the prepared MIP.

Comparison of the Developed Method with Other MISPE Methods for FF

For the comparison of the developed method with methods reported earlier in the literature based on MISPE (Sadeghi and Jahani 2013; Shi et al. 2012; Ge et al. 2010), the main features are listed in Table 4. As it is obvious, the developed method exhibits the best capacity value among other methods based on MISPE with different detections. In addition, the performance of the developed MISPE is comparable to the reported MISPEs in terms of the accuracy and precision.

Conclusions

In this study, a new FF-MIP was prepared by surface imprinting polymerization and characterized by dynamic absorption experiments. The MIP sorbent had high rebinding ability for FF. Good precision and accuracy of the MISPE column for FF in real spiked samples demonstrated the feasibility of the prepared MIP for FF extraction. The sample preparation protocol could be simplified by using FF-MIP column before HPLC analysis without further sample cleanup.

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Conflict of Interest Susan Sadeghi declares that she has no conflict of interest. Moslem Jahani has received the research financial support from the University Birjand Research Council. The animal issues were provided from the local supermarket and this article does not contain any studies with special animal tissues.

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