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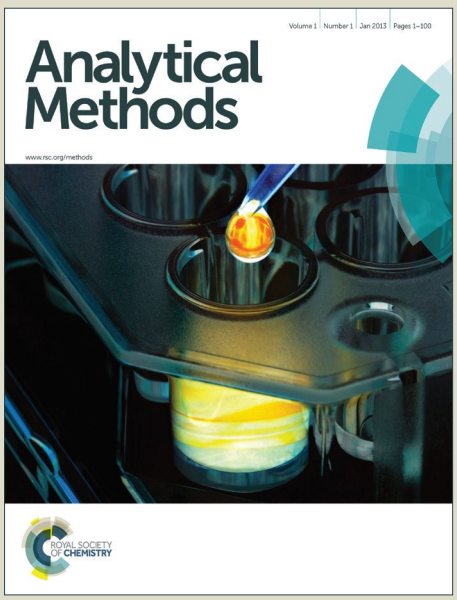

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ARTICLE

Preparation of a new restricted access molecularly imprinted hybrid adsorbent for the extraction of folic acid from milk powder samples

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The synthesis of a new hybrid molecularly imprinted polymer (MIP) combined with restricted access (RAM) has been based on random free-radical polymerization and sol–gel process. From competitive adsorption studies by using RAM-MIP and RAM-NIP with folic acid and structurally similar molecules (caffeine, 4-aminobenzamide or paracetamol), relative selectivity coefficients (*k*) higher than one unit were achieved, indicating good recognition selectivity for the folic acid. The percentage of BSA protein exclusion was higher for the RAM-MIP (55.3±2.0) as compared to MIP (35.9±2.5%). The solid phase extraction (SPE) procedure was performed by loading the acid extract from milk powder samples previously submitted to saponification and acidification with 10% trichloroacetic acid (TCA) until pH 1.5, by means of 100 mg of RAM-MIP packed into SPE cartridges. The elution step was carried out by using a mixture of acetonitrile: 0.266 mol L^{-1} acetate buffer at pH 5.7 (15:85, v/v), the same composition of mobile phase of HPLC. The intra-day precision (n=10) of the procedure assessed as relative standard deviation (RSD) was of 4.7 and 4.1% for the respective concentrations of 20.0 and 150.0 μg L^{-1}. The applicability of the method was attested by analysis of different brands of milk powder samples fortified with folic acid, as well as by high recovery percentages 95.0-108.4% obtained upon addition and recovery tests. The cleanup process accomplished by RAM-MIP was so efficient that very few remaining matrix components were detected in the eluate by high performance liquid chromatography.

Introduction

Folic acid (FA) is a water-soluble vitamin (B9) of great clinical importance to human health. Some studies have shown that the deficiency of folic acid in the diet may be related to chronic diseases, such as heart attack, gigantocytic anemia, cerebrovascular diseases, epilepsy, certain types of cancer and mainly those concerned with malformation of fetus during pregnancy.[1-3] Therefore, FA has been recommended to be incorporated as a vitamin supplement to human health, especially to reduce the risk of heart diseases and the incidence of neural tube defects during the embryo development in pregnant women.[2] European Union (EU) recommend the daily intake of FA as being 400 μg d^{-1} for adults.[4] In 1998, in the United States of America, the Food Drug Administration (FDA) issued a regulation for the fortification of FA in wheat flour, rice, bread and corn meal as being 140 μg $100g^{-1}$, while in Brazil, the National Health Surveillance Agency (ANVISA) determined the fortification of FA in wheat flour and corn meal with FA as being 150 μg $100g^{-1}$.[5,6] One should note that in Brazil, lacteous products, chocolate and powder milk samples have been increasingly fortified with FA as a way of compensating the losses of vitamin during processing or simply to increase the nutritional value, once this food is classified as deficient in vitamins C, D and those of the B group.[7] Therefore, the development of appropriate analytical methods for quality control of food samples fortified with FA is of paramount importance. However, as milk samples are complex matrices constituted by proteins, carbohydrates, minerals, lipids and vitamins, the determination of FA in these samples represents a difficult task. Thus, sample treatment methods have been mandatory prior to analysis even using high liquid performance chromatography (HPLC), aiming at removing interferences and/or at improving the detectability of the technique. Some methods based on optical biosensors and voltammetric sensors modified with carbon nanotubes and ionic liquid have also been developed for folic acid determination in milk samples, but similarly to HPLC they are prone to interferences from matrices components, thus needing prior sample treatment, using enzymatic hydrolysis and extraction of FA with organic solvent, respectively.[8,9] Therefore, owing to its reliability and excellent analytical performance for vitamins determination, HPLC is still the choice technique for folic acid determination. Some treatments of milk samples prior to HPLC analysis involving the extraction of FA strongly bonded to proteins by using alkaline hydrolysis followed by precipitation of proteins with strong acids[10], such as hydrochloric acid[11] trichloroacetic acid[12] have been reported. In some applications, the alkaline hydrolysis can be omitted, since it is recommended for milk samples with high content of lipids. Nevertheless, at the same time that acids have been used for protein precipitation, it has been noticed the appearance of

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impurities and instability of baseline of chromatographic determination.[13] Therefore, the unwanted interference may be overcome by using highly selective solid phase extraction (SPE) methods based on affinity column for extensive sample extraction and clean-up.[14] Despite the efforts on this direction, these materials are still very expensive and difficult to obtain in the laboratory. In this context, the synthesis of polymers possessing specific recognition centre and their applications in highly selective SPE methods is increasingly being exploited for solving some drawbacks of molecular recognition systems based on biological molecules, such as instability under wide pH range, low life-time and high acquisition cost.[15] These polymers are recognized as molecularly imprinted polymers (MIPs) and are designed by polymerization in the presence of a template molecule to be imprinted, resulting in trapping template in highly cross-linked polymer matrix. After template removal from the polymer by dissolution or evaporation, the polymer will reveal binding sites possessing a shape and an arrangement of functional groups corresponding to that of the template.[16] Despite their outstanding features, very few attempts have been investigated for the synthesis of MIPs aiming at the extraction of folic acid most likely due to large size of the molecule, which can hinder the creation of selective binding sites in the MIP.[17] At the best of our knowledge, the only studies addressed to the synthesis of MIP selective to folic acid are dedicated to the development of electrochemical voltammetric sensors based on electropolymerization[18], composites[19] as well as extractor devices based on fiber-molecularly imprinted polymer with differential pulse cathodic stripping voltammetric determination,[20] but neither of them were applied to folic acid determination in milk samples, which clearly demonstrates that the studies of this nature still remain incipient.

According to the aforementioned, the present study deals with synthesis of a new restricted access molecularly imprinted poly(methacrylic acid- trimethylolpropane trimethacrylate)/SiO_2 hybrid adsorbent for the cleanup/extraction of folic acid from milk samples. Apart from the contribution of this study regarding the synthesis of MIP for folic acid, in the present study the properties of restricted access material (RAM) ascribed by insertion of hydrophilic groups bonded at the outer surface of polymer together with the properties of hybrid materials have been combined for the first time, in one unique adsorbent. Some studies have already demonstrated the outstanding features of water-compatible RAM-MIP based on organic polymers for the selective recognition/extraction of drugs and sample cleanup of biological matrices.[21] Regarding the preparation of hybrid molecularly imprinted polymers by means of combination of random free-radical polymerization followed by sol–gel process, one should note that only recently this approach has been proposed for MIP synthesis. The studies have been devoted to the extraction of oxytetracycline in milk[23], lincomycin in aqueous medium[24] and cholesterol in milk.[25] In the case of folic acid extraction from milk samples, the use of hybrid material is particularly attractive, once this molecule is fairly soluble in acid medium, which justifies the presence of organic phase in the polymer to assure chemical stability to the adsorbent. Yet the inorganic phase assures better textural and morphological properties and low swelling effect to the hybrid material.[26]

For the synthesis of hybrid RAM-MIP, methacrylic acid (MAA) has been used as organic monomer, folic acid as template, vinyltrimethoxysilane (VTMS) as coupling agent and 2,2´-azobis-isobutyronitrile (AIBN) as initiator. Tetraethoxysilane (TEOS) was used as inorganic monomer, and (3-glycidyloxypropyl)trimethoxy-silane was used as modifier agent with posterior opening of epoxy ring to impart hydrophilic properties of material in the extraction of folic acid. Upon synthesis and characterization by scanning electron microscopy (SEM) and textural data, a solid phase extraction procedure using RAM-MIP was developed for the extraction and determination of folic acid in milk samples by HPLC-DAD. The results of RAM-MIP applicability in milk powder samples have been compared to the corresponding blank polymer (RAM-NIP), the imprinted polymer without restricted access (MIP) and commercial adsorbent material (modified octadecyl silica-C_{18}).

Experimental

Chemicals

Methacrylic acid (MAA, 99.5%), trimethylolpropane trimethacrylate (TRIM, 90%), 2,2'-azoisobutyronitrile (AIBN, 98%), Tetraethoxysilane (TEOS, 98%), (3-glycidyloxypropyl)-trimethoxysilane (GPTMS, >98%), vinyl trimethoxysilane (VTMS, ≥97.5 %), folic acid (FA, ≥97 %), methanol (HPLC grade ≥ 99.9%), acetonitrile (99.0%), Ethanol (99.5%), hydrochloric acid (37%), acetic acid glacial (99.85%), caffeine (CF) (99%), paracetamol (PAR) (>98%), and 4-aminobenzamide (AB) (98%) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich® (Steinheim, Germany) and used as received. Potassium hydroxide and trichloroacetic acid (TCA, >99%) were purchased from Vetec® (São Paulo, Brazil). All the solutions were prepared in deionized water from a water purification system Milli-Q® (Bedford, USA). The water used to prepare the mobile phase was obtained from the purification system Milli-Q® and filtered through a 0.45 µm nylon membrane daily. The water and solvents used in mobile phase were also degassed using an ultrasonic Bath model USC 1400 (Marconi®, Piracicaba, Brazil). Modified octadecyl silica-C_{18} (40 - 60µm) was acquired from Allcrom (São Paulo, Brazil).

Equipments and chromatographic analytical conditions for folic acid determination

Chromatographic separations were carried out using a Shimadzu® (Kyoto, Japan) HPLC system series LC-20AD/T LPGE KIT consisting of CLC-ODS column (250 mm x 4.6 mm id, 5 µm in particle size) and a guard column Phenomenex (4.0 mm x 3.0 mmm i.d., 5 µm in particle size), a 7725i manual injector with a 20 µL loop, (Rheodyne®, California, USA), a CTO-10AVP column oven and a LC-20AT controller. The peak purity was determined on a photodiode-array detector (PDA) and monitored at λmax 281 nm. Chromatographic separation was performed in gradient mode using the mobile phase acetonitrile (A)/0.266 mol L^{-1} acetate buffer (pH 5.7) (B). Initial proportion of the mobile phase was A/B (15:85, v/v) and an elution gradient (15:85 to 24:76, v/v) was used during 8.5 min of the analysis. The final proportion was then held for another 21.5 min. The flow rate was operated at 0.5 mL min^{-1} and the injection volume was 20 µL. The temperature of chromatographic separation (25˚C) was controlled by using a CTO-20A column oven. The SPE procedure was performed using a manifold system (Bio-Rad) with a capacity for 12 cartridges, coupled to a vacuum pump (Marconi MA 2057). The pH of solution was measured with a combined glass electrode and a Metrohm 827 pH lab digital pH meter (Herisau, Switzerland). The morphological features of RAM-MIP and RAM-NIP were evaluated by scanning electron microscopy (SEM), using a microscope JEOL JSM-6360 LV equipped with dispersive energy microscopy. Before analysis, the polymers were

coated with a thin layer of gold, using a Bal-Tec MED 020 equipment, in order to minimize charging under the incident electron beam. Average pore sizes and volumes of the polymers were estimated by the Barrett-Joyner-Halenda (BJH) method based on nitrogen adsorption experiments using a Quantachrome Nova 1200e automatic instrument coupled to an automatic gas analyzer (all from Quantachrome). The specific surface area (S_{BET}) was estimated from nitrogen adsorption isotherms according to the multipoint Brunauer-Emmett-Teller (BET) method.

Synthesis of RAM-MIP

The synthesis of MIP containing restricted access was based on the precipitation method with modification according to literature data.[27] Firstly, 2.0 mmol of folic acid (0.8828g) were dissolved in 100 mL of 3.0 mol L^{-1} HCl and stirred during 30 min. In the next step, 300 mL of acetonitrile and 12.0 mmol of MAA were added to the mixture and stirred for another 30 min. Then, 24.0 mmol of TRIM (7.112 g), 2.94 mmol de VTMS (0.436 g) and 600 mg of AIBN were added to the mixture. A chain structure pre-polymer was formed by radicalar copolymerization of MAA and VTMS in the presence of AIBN and folic acid as template molecule. The mixture was than purged with argon gas for 5 min, the flask was sealed, and submitted to heating at 60°C for 24 h in bath oil. The obtained polymer was filtered and dried at 60° C for 24 h. In the next step, 10.05 g of organic polymer were suspended in 300 mL of ethanol under stirring followed by addition of 52 mmol of TEOS, 48 mmol of GPTMS and 26.0 mL of 1.0 mol L^{-1} NaOH. The hydrolysis, condensation and co-condensation reactions of inorganic phase were kept at room temperature during 24 h, thus making it possible to obtain the hybrid polymer. The obtained material was then filtered under vacuum and dried at 60 °C for 48 h. In order to open the epoxide ring grafted on the hybrid surface polymer and impart the hydrophilic properties to the material, 500 mL of 0.18 mol L^{-1} HCl solution were stirred with 10 g of hybrid polymer at 60°C in a thermostatic bath for 36 h.[28] This material was named RAM-MIP. To remove folic acid and excess of reagents from the polymeric matrix of hybrid material, the RAM-MIP was submitted to an extraction procedure with Soxhlet system using a mixture of methanol:acetic acid (90:10 v/v) until no template could be detected by HPLC-DAD. In order to evaluate the influence of chemical modification with GPTMS and posterior opening of epoxy ring and the imprinting effect created in the hybrid polymer, MIP and RAM-NIP were prepared in a similar way, except for addition of GPTMS and template in the synthesis, respectively. Additionally, an organic MIP was also synthesized to compare with the performance of hybrid MIP for FA extraction. This polymer was synthesized similarly as described for RAM-MIP, except by addition of TEOS and GPTMS. Figure 1 shows a schematic representation of the RAM-MIP synthesis.

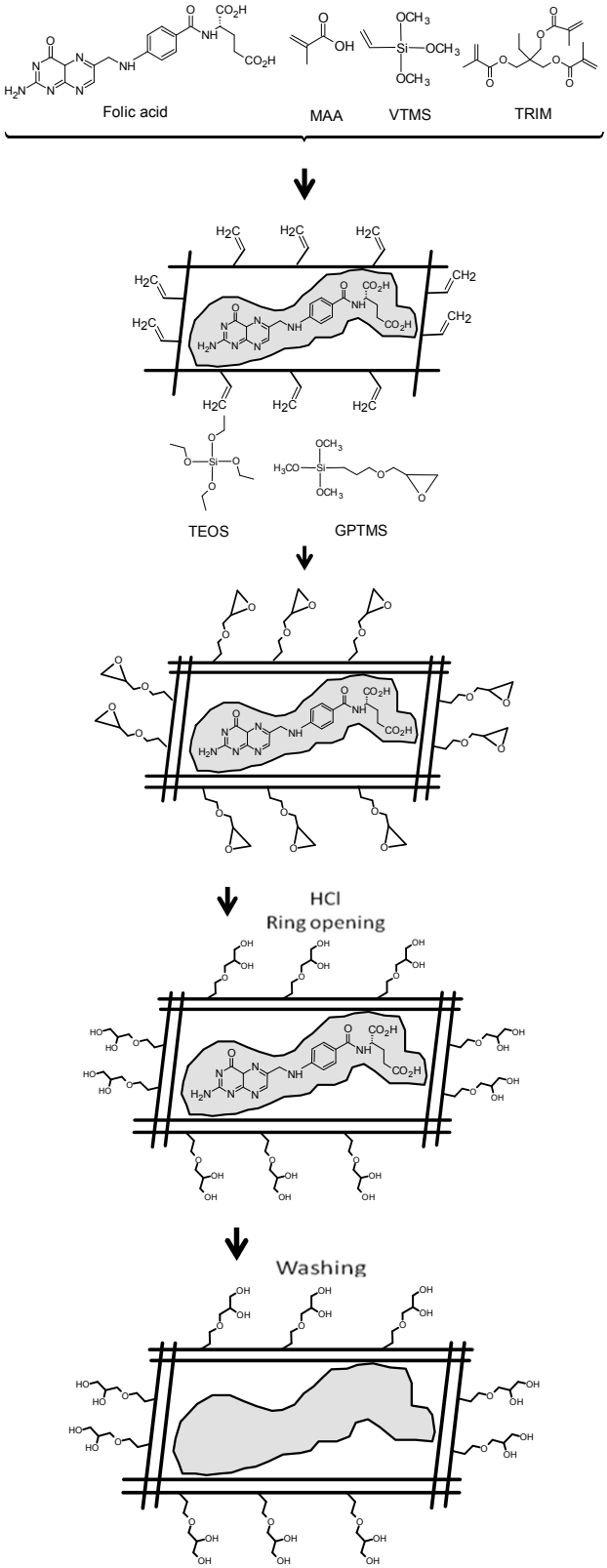


Figure 1. Schematic synthesis of restricted access molecularly imprinted poly(methacrylic acid-trimethylpropane trimethacrylate)SiO_2 hybrid adsorbent

Selectivity in competitive adsorption on RAM-MIP and RAM-NIP

In order to check the selectivity of the restricted access molecularly imprinted hybrid adsorbent, competitive adsorption studies of folic acid/caffeine, folic acid/paracetamol and folic acid/4-aminobenzamide were carried out. The experiment was conducted as follows: in a batch procedure, 50 mg of RAM-MIP or RAM-NIP were added to a 40.0 mL of binary solution at 10.0 mg L^{-1} concentration, whose pH was adjusted to 1.5 with a pseudo-buffer solution of 0.01 mol L^{-1} KCl/HCl. The suspensions were stirred for 390 min using a magnetic stirrer. After centrifugation, the supernatant solutions were filtered through a 0.45 µm nylon (GVS, New York, USA) filter and analyzed by UV-Vis spectrophotometry at 281, 272, 243 and 221 nm, respectively, for folic acid, caffeine, paracetamol and 4-aminobenzamide. Simultaneous determination of folic acid and the structurally similar concomitant compounds to folic acid was possible by using the deconvolution process of obtained spectra using the ORIGIN® program (version 8.0). The distribution (K_d), selectivity (k) and the relative selectivity (k') coefficients were calculated by the following equations:[29]

$$K_d = \frac{(C_i - C_f)}{C_f}\frac{V}{M}, \quad (1)$$

where C_i and C_f are the initial and final (supernatant) concentration of folic acid and concomitants (mg L^{-1}), V is the solution volume (mL), and M is the polymer (RAM-MIP or RAM-NIP) mass (mg);

$$k_{RAM-MIP} = \frac{K_d(FA)}{K_d(concomi\tan t)} \quad (2)$$

$$k_{RAM-NIP} = \frac{K_d(FA)}{K_d(concomi\tan t)} \quad (3)$$

$$k' = \frac{k_{RAM-MIP}}{k_{RAM-NIP}} \quad (4)$$

Evaluation of exclusion properties of macromolecule by RAM-MIP and MIP

Firstly, an amount of 100.0 mg of RAM-MIP or MIP was filled into a conventional SPE (solid phase extraction) cartridge capped with fritted polyethylene disks at the top and bottom. The cartridge was placed in a manifold system (Bio-Rad) coupled to a vacuum pump (Marconi MA 2057). Before use, 10.0 mL of water was passed through the cartridge for conditioning. To test the exclusion properties of RAM-MIP, BSA was chosen as macromolecule and the experiment was carried out by loading an aliquot of 2.0 mL of a 1.2 g L^{-1} BSA solution at pH 1.5 at a flow rate of 0.5 mL min^{-1} through polymers. The percentage of BSA exclusion was determined by the ratio (absorbance of BSA in the effluent from cartridge/ absorbance of original concentration of BSA) and the result was multiplied by 100%. The absorbances were determined by UV-Vis spectrophotometry at 277 nm and the experiments were performed in triplicate. Upon BSA loading (2.0 mL) through the cartridge, 1.0 mol L^{-1} NaCl solution was percolated through cartridge in sequence for cleaning and removing the BSA still retained in the polymer.

Sample preparation and SPE procedure

Milk powder samples were acquired from local supermarkets in Londrina city (Brazil). Before performing the SPE procedure, folic acid was firstly extracted from the samples. For this task, an amount of sample was dissolved in 10.0 mL of deionized water and mixed with 7.5 mL of 0.1 mol L^{-1} KOH. The mixture was sonicated for 10 min, followed by addition of 10% trichloroacetic acid (TCA) until pH 1.5. The mixture was then transferred to volumetric flask of 25.0 mL, whose volume was made up with deionized water. Upon this procedure, the solution was centrifuged for 10 min at 1600 rpm and 10.0 mL of the supernatant were subjected to SPE procedure. It was performed in three steps as follows: 1- conditioning of cartridge containing 100.0 mg of RAM-MIP by using 10.0 mL of deionized water; 2- percolation of 10.0 mL of sample; 3 – elution with 1.0 mL of the mixture acetonitrile:0.266 mol L^{-1} acetate buffer at pH 5.7 (15:85, v/v), the same composition of mobile phase of HPLC. In this procedure, no washing step was required. The eluate was directly injected in the chromatographic system and the peak of folic acid at retention time of 8.42 min was monitored at λmax 281 nm. The extractions were performed in triplicate.

Determination of figures of merit and application of method in different brands of milk powder samples

The limits of detection (LOD) and quantification (LOQ) of method were determined according to IUPAC recommendation, being as three and ten times, respectively, the relative standard deviation (RSD) of ten blank measurements divided by the slope of the analytical curve constructed for the solid phase procedure.[30] The preconcentration factor was calculated as the ratio between the slopes of the analytical curve obtained with and without folic acid extraction by SPE procedure. The precision of SPE procedure was assessed by intra-day repeatability (n=10) for folic acid concentration of 20.0 e 150.0 µg L^{-1} and inter-day using four replicates (n=4) obtained for the same concentrations of FA and analyzed in two different days. Four brands of fortified milk powder samples were analyzed by the proposed method containing different amounts of folic acid. The accuracy of the method was assessed by adding a known amount of folic acid to the sample followed by recovery tests.

Results and discussion

Characterization of polymers

SEM images of RAM-MIP and RAM-NIP are shown in Figure 2. As one can see from images, the hybrid polymers present spherical particles with high degree of aggregation and diameter in nanoscale. The morphological feature of hybrid polymers is a consequence of the synthesis adopted. In the polymerization method, the reaction of functional monomer and cross-linking agent in the presence of large porogenic solvent avoids the coalescence of particles. Additionally, as no morphological differences were observed between RAM-MIP and RAM-NIP, any differences on folic acid adsorption and exclusion of BSA macromolecule would not be attributed to the morphological

features. Instead, the differences will be attributed to the imprinted sites and the restricted access.

As it is very well known, the adsorption process is greatly dependent on the textural data of polymers. Herein we have determined the average pore diameter (nm) of RAM-MIP, RAM-NIP and MIP, whose obtained results were found to be 3.04, 3.32 and 3.35, respectively. As observed, the pore diameters of polymers are very similar to each other and, according to IUPAC definition, the obtained materials are considered mesoporous (2-50 nm)[31], a desirable condition for the adsorption of molecules.

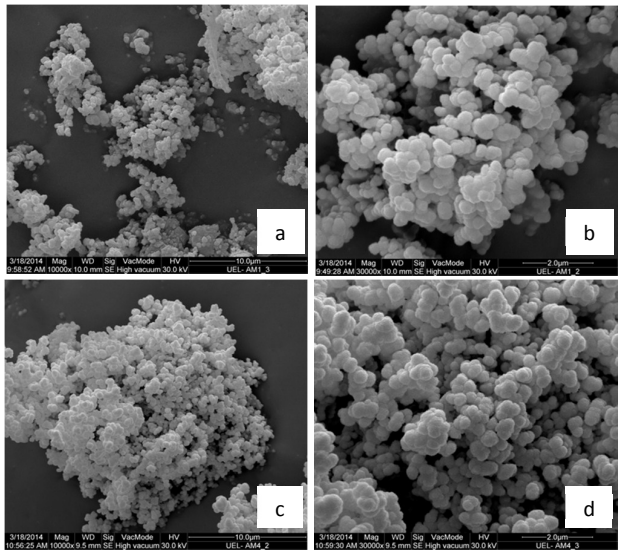


Figure 2. Scanning electron microscopy of (a, b) RAM-MIP and (c,d) RAM-NIP.

The pore volume ($cm^3\ g^{-}$) and surface area ($m^2\ g^{-1}$) obtained for the RAM-MIP, RAM-NIP and MIP were found to be 0.039, 0.114, 0.103, and 17.5, 61.0 and 55.3, respectively. The differences between RAM-MIP and RAM-NIP can be attributed to the presence of template in the synthesis, which can increase the solubility of template-monomer in the porogenic solvent, and as a consequence, makes the removal of solvent from the interstices of polymer more difficult, thus giving rise to a lower pore volume and lower surface area. Regarding the MIP, the RAM-MIP has lower surface area and lower pore volume most likely due to the chemical modification with (3-glycidyloxypropyl)trimethoxysilane (GPTMS) that takes place inside pores, which justifies the decrease of pores volume and surface area. Once again, in a similar way to those results observed from morphological data, any differences on folic acid adsorption and exclusion of BSA macromolecule will be, in fact, ascribed to the imprinted sites created during polymer synthesis and restricted access, without dependence of textural data.

Influence of pH on folic acid adsorption by RAM-MIP

A set of aliquots of 40.0 mL of folic acid solution containing 10.0 mg L^{-1} were stirred for 60 min with 50 mg of RAM-MIP. The pH of set solutions was adjusted between 1.5-10.4. For pH adjustment, buffer solutions at 0.01 mol L^{-1} concentration were used as follows: KCl/HCl for pH 1.5, CH_3COONa/CH_3COOH for pH 4.4 and 5.4, phosphate for pH 6.4 and 7.4, and NH_4^+/NH_3 for pH 8.4, 9.4 and 10.4. After stirring, the mixture was centrifuged at 2000 rpm for 10 min; the supernatants were filtered through a 0.45 µm Nylon® membrane and evaluated by UV absorbance at 281 nm. As observed from Figure 3, the adsorption of folic acid increases with decreasing sample pH. Under very acid medium, the carboxylic groups in the methacrylic acid and folic acid molecule are in its protonated form, thus making easier the interaction of monomer with template, most likely through hydrogen bonding interactions and/or London dispersion forces. Thus, taking into account the obtained results, all further adsorption studies were carried out at pH 1.5.

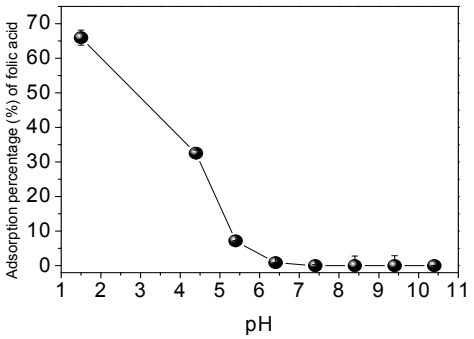


Figure 3. Influence of pH on folic acid adsorption by RAM-MIP

Selectivity in competitive adsorption on RAM-MIP and RAM-NIP

The distribution (K_d), selectivity (k) and the relative selectivity (k') coefficients of folic acid with respect to caffeine, paracetamol and 4-aminobenzamide using RAM-MIP and RAM-NIP are summarized in Table 1.

Table 1. Distribution (K_d), selectivity (k) and relative selectivity (k') coefficients for the competitive adsorption of folic acid and structurally similar molecules on RAM-MIP and RAM-NIP

Molecules	K_d (mL mg^{-1})		k		k'
	RAM-MIP	RAM-NIP	RAM-MIP	RAM-NIP	
FA	979.8	890.6			
	1247.2	920.4			
	1173.3	867.1			
CAF	55.5	91.7	17.6	9.7	1.8
PAR	277.2	313.9	4.5	2.9	1.5
AB	35.2	113.5	33.3	7.6	4.4

FA = folic acid, CAF = caffeine, PAR = paracetamol, AB = 4-amino-benzamide

Comparing RAM-MIP and RAM-NIP it can be observed for the binary mixtures FA/CAF, FA/PAR and FA/AB higher K_d values for folic acid with respect to other molecules. The higher adsorption of folic acid by RAM-MIP in competitive adsorption clearly demonstrates the imprinting effect created in the RAM-MIP, mainly if one considers the presence of molecules with smaller structure than the folic acid, where an easier mass transfer towards the binding sites owing to the absence of steric hindrance would naturally be expected. These results reflect in higher selectivity coefficients (k) for RAM-MIP when compared to RAM-NIP and, as a consequence, relative selectivity coefficients (k) higher than one unit, thus confirming once again the good recognition selectivity for the folic acid by RAM-MIP.

Evaluation of exclusion properties of macromolecule by RAM-MIP and MIP

Figure 4 shows the exclusion percentage (%) of BSA by RAM-MIP and MIP. The better exclusion property of RAM-MIP for macromolecule BSA confirms the chemical modification of MIP with GPTMS agent and posterior opening of the epoxy ring, ensuring the formation of a hydrophilic surface containing diol groups, very efficient to prevent or diminish the adsorption of proteins.[32]

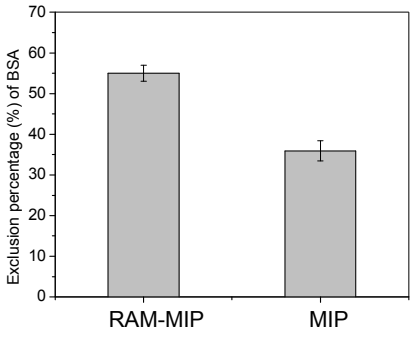


Figure 4. Exclusion percentage (%) of BSA by RAM-MIP and MIP.

Optimization of extraction procedure of folic acid from milk powder samples

RAM-MIP has been ideally designed to reduce the number of steps in the pretreatment of complex matrices containing high amount of proteins, such as milk samples. Therefore, the use of RAM-MIP for direct extraction of folic acid in milk powder samples was herein investigated. The samples were dissolved in deionized water and loaded into a column containing RAM-MIP. However, as the adsorption of folic acid occurs at pH 1.5, no retention of folic acid was observed at the natural pH of milk sample. Therefore, new approaches involving saponification and acidification were evaluated for the folic acid extraction from milk powder samples. In these procedures, the quantitative extraction of folic acid is crucial for the development of a reliable analytical methodology. Four extraction procedures were evaluated as follows:

1 –Saponification of fatty acids in sample with 7.5 mL of 0.1 mol L^{-1} KOH and sonication for 10 min with posterior addition of 10% trichloroacetic acid (TCA) solution until pH 1.5. The mixture was transferred to a 25.0 mL volumetric flask and the volume was made up with deionized water. Next, the solution was centrifuged for 10 min at 1600 rpm and 5.0 mL of the supernatant were filtered through a 0.45 µm nylon membrane, heated to 60°C until dryness, dissolved in the mobile phase and injected in the chromatography system.

2 – The extraction procedure was performed in a similar way to procedure 1, except for changing 10% trichloroacetic acid (TCA) for 0.5 mol L^{-1} HCl.

3 - The extraction procedure was performed in a similar way to procedure 1, except for the absence of the saponification step with 7.5 mL of 0.1 mol L^{-1} KOH, which was replaced by 7.5 mL of deionized water.

4 - The extraction procedure was performed in a similar way to procedure 2, except for the absence of saponification step with 7.5 mL of 0.1 mol L^{-1} KOH, which was replaced by 7.5 mL of deionized water.

The results of the four extraction procedures are summarized in Table 2. It can be seen that quantitative extraction (94.3± 6.8%) of folic acid from milk powder sample was achieved by using the mixture of 0.1 mol L^{-1} KOH and 10% trichloroacetic acid (TCA), being these conditions employed for further experiments.

Table 2. Results of folic acid extraction (%) from milk powder samples using different procedures

Extraction procedures	Extraction percentage of folic acid (%)
1- KOH + TCA	94.3 ± 6.8
2- KOH + HCl	82.3 ± 5.6
3- TCA	73.8 ± 0.6
4- HCl	79.9 ± 2.5

0.1 mol L^{-1} KOH; TCA= 10% trichloroacetic acid (m/v); 0.5 mol L^{-1} HCl. Results are expressed as mean value ± standard deviation based on three replicates (n=3).

Optimization of SPE procedure

The optimization of SPE procedure was carried out by using the acid extract (pH 1.5) as described in the extraction procedure (1) summarized in Table 2. The effect of the washing solvent and the type and volume of elution solvent were investigated (Table 3). Firstly, the acid extract was loaded into RAM-MIP cartridges previously conditioned with deionized water, yielding retention of 80.3 ± 1.3% of folic acid. Then, aliquots (10.0 mL) of different solvents or mixtures were evaluated in the washing procedure for removing interferences without removing folic acid. After the washing step, 10.0 mL of the mixture methanol:0.01 mol L^{-1} acetate buffer at pH 5.7 (50:50, v/v) was employed in the elution step.

Table 3. Percentage of folic acid removal (%) from RAM-MIP cartridges using different washing solvents

Washing solvents	Percentage of folic acid removal (%)	P	α	β
Acetone	53.0 ± 0.2	5.1	0.06	0.38
Dichloromethane:acetone (1:1, v/v)	19.0 ± 3.5	-	-	-
Chloroform	9.1 ± 0.4	4.1	0.43	0.00
Dichloromethane:ethanol (1:1, v/v)	6.4 ± 0.5	-	-	-
Acetic acid (0.1 mol L^{-1})	-	6.0	0.54	0.15
Dichloromethane	-	3.1	0.27	0.00
Hexane	-	0.1	-	-
Without washing	-	-	-	-

P= Polaritiy; α- acidicity; β- Basicity. Results are expressed as mean value ± standard deviation based on three replicates (n=3).

As summarized in Table 3, in acetone, the desorption of folic acid was much higher most likely due to its higher basicity making the interaction with folic acid more favorable. The mixture of dichloromethane: acetone (1:1, v/v) was able to desorb 19.0% of folic acid due to the presence of acetone, once the dichloromethane is less polar than acetone and more acid, which justifies its inability for removing folic acid when used alone. Both chloroform and dichloromethane do not exhibit basicity; however due to its higher polarity, chloroform removes 9.1% of folic acid. For the mixture dichloromethane:ethanol (1:1, v/v) only a low

percentage of folic acid removal was observed (6.4%), as result of absence of basicity by the dichloromethane. Hexane is unable to desorb folic acid indeed by its non polar feature. As observed from the results, it clearly seems that the chemical and physical properties including polarity and acidicity/basicity play an important role on the removal of folic acid from MIP-RAM. Additionally, the pH of sample is another important factor for the removal of folic acid as evidenced by the use of 0.1 mol L^{-1} acetic acid. In acid medium, the hydrogen bonding interactions between folic acid and monomer are maintained, thus justifying the non removal of folic acid from MIP-RAM.

On the basis of the above results, dichloromethane, hexane and 0.1 mol L^{-1} acetic acid solution would be the best washing solvents in the SPE procedure. However, the organic solvents were not efficient to remove interferences from the sample (data not shown) and the use of acetic acid solution gave rise to severe swelling effect in the cartridge packed with RAM-MIP precluding its use in the SPE procedure. Therefore, further experiments were carried out without including the washing step.

Table 4 shows the data of percentage of folic acid elution (%) using different elution solvents and mixtures.

Table 4. Percentage of folic acid elution (%) from RAM-MIP cartridges using different elution solvents (10.0 mL)

Solvents	Percentage of folic acid elution (%)	P	α	β
Ethanol	82.0 ± 0.9	4.3	0.39	0.36
Isopropanol	83.4 ± 3.1	3.9	0.22	0.35
Ethyl acetate	12.0 ± 0.5	4.4	0.00	0.45
Acetonitrile/0.266 mol L^{-1} acetate buffer (15:85) (pH 2.8)	86.2 ± 4.1	-	-	-
Acetonitrile/0.266 mol L^{-1} acetate buffer (15:85) (pH 5.7)	99.7 ± 1.9	-	-	-
Methanol:0.01 mol L^{-1} acetate buffer (50:50) (pH 5.7)	96.6 ±5.5			

P= Polaritiy; α- acidicity; β- Basicity. Results are expressed as mean value ± standard deviation based on three replicates (n=3).

As seen in Table 4, the differences of ethanol, isopropanol and ethyl acetate for eluting folic acid can be ascribed to the protic conditions of the alcoholic solvents, able to disrupt the hydrogen bonding interactions between folic acid and methacrylic acid. As regards the mixtures, it was observed that the larger the pH, the better the elution of folic acid. Such result can be explained as already mentioned, by disrupting of hydrogen bonding interactions between folic acid and monomer, once the methacrylic acid exists, at pH 5.7, on its deprotonated form (pKa = 4.66).[33] The quantitative elution of folic acid was found to be achieved by using the mixture acetonitrile/0.266 mol L^{-1} acetate buffer (15:85) (pH 5.7) or methanol:0.266 mol L^{-1} acetate buffer (50:50) (pH 5.7). However, the mixture acetonitrile/0.266 mol L^{-1} acetate buffer (15:85) (pH 5.7) was chosen as elution solvent, the same composition of mobile phase of HPLC, thus avoiding the drawbacks concerning the evaporation of elution solvent.

The chromatogram of milk acid extract submitted to the optimized SPE procedure using RAM-MIP is shown in Figure 5. Comparing the chromatogram of direct injection of acid extract in the chromatographic system with the acid extract submitted to the SPE procedure, we note clearly the great benefit of RAM-MIP as adsorbent for sample cleanup and removal interferences. The milk acid extract was also submitted to SPE procedure using the RAM-NIP, MIP and modified octadecyl silica-C_{18} (Figure 6a).

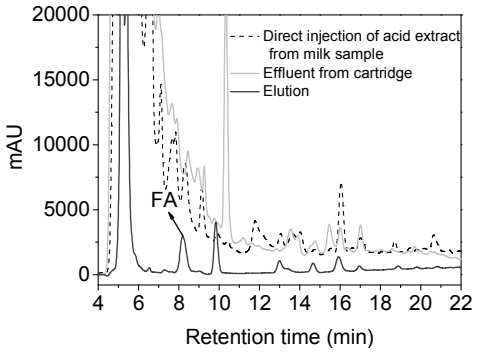
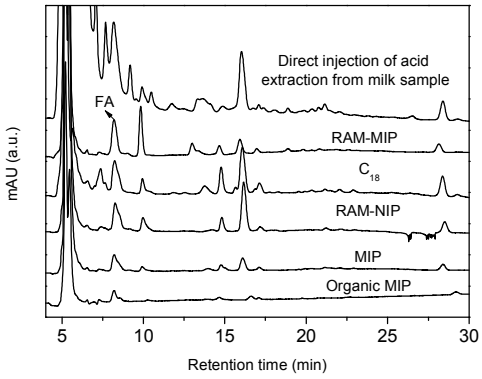
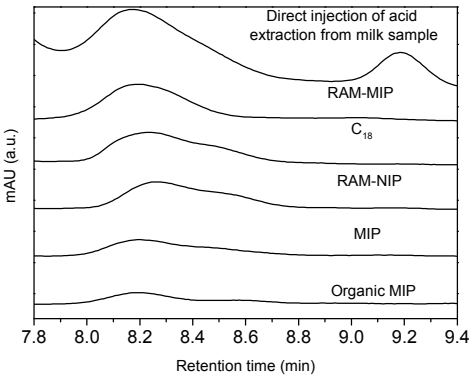


Figure 5. Chromatograms of direct injection of acid extract from milk sample, effluent from cartridge and elution after submitting the acid extract to SPE procedure using RAM-MIP.



(a)



(b)

Figure 6. Chromatograms of direct injection of acid extract from milk sample and and elution after submitting the acid extract to SPE procedure using RAM-MIP, C_{18}, RAM-NIP, MIP and organic MIP (a) and magnification of peak region of FA (b)

Compared to direct injection of acid extract from milk samples, cleaner chromatograms were obtained when using the adsorbents in SPE procedure. However, the peak area obtained for FA using RAM-MIP was higher than the one achieved for MIP and RAM-NIP. The lower peak area observed for the organic MIP,

confirms the great benefits of hybrid MIP for the extraction of FA. One should note that, the separation profile of FA using RAM-MIP was somewhat similar to that achieved for C_{18}. Nevertheless, a more detailed observation of chromatographic profile (Figure 6b), shows clearly that the use of RAM-MIP positively influences on peak profile of FA yielding an appropriated peak resolution. In addition to this finding, in comparison to C_{18}, the use of RAM-MIP allowed better cleanup of sample in the first times (4-6.5 minutes) of chromatographic separation, where sugars are most probably detected, thus indicating the good property of RAM-MIP to exclude macromolecules in real samples.

Determination of figures of merit and application of method in different brands of milk powder samples

In order to improve the detectability of folic acid, and to reduce the consumption of solvent, different volumes of elution were evaluated (0.5-3.0 mL). All studied volumes were found to be enough for complete elution of folic acid; however, as a compromise between the detectability and precision, the volume of 1.0 mL was herein chosen. Thus, under optimized condition, the analytical curve was built using folic acid standard solutions (blank up to 250 μg L^{-1}) submitted to SPE procedure. The linear equation obtained was found to be response (mAU) = 315.34[FA] + 1220.20 (r=0.999), while the linear equation obtained without SPE procedure was: response (mAU) = 143.36[FA]-1083.57 (r= 0.999). From these curves a preconcentration factor (ratio of slopes) of 2.2 times and limits of detection and quantification of 1.45 e 4.83 μg L^{-1}, respectively, were obtained. The intra-day precision of SPE procedure was found to be very satisfactory since low relative standard deviations (RSD) of 4.7 and 4.1% were obtained, respectively, for folic acid concentrations of 20.0 and 150.0 μg L^{-1} (*n*=10). For the inter-day precision assessed in two consecutive days the relative standard deviations (RSD) were found to be 2.57 and 0.88%, for the respective folic acid concentrations of 20.0 and 150.0 μg L^{-1}. The SPE method was applied to determine folic acid in different brands of fortified milk powder samples. As observed in Table 5, the folic acid content declared by manufacture was very similar to that determined by the proposed method.

Table 5. Application of SPE method for milk powder sample cleanup and folic acid determination

Milk samples	Declared value (μg)	Folic acid added (μg)	Amount found (μg)	Recovery (%)
A	120*	0	119.1 ± 1.1	-
		120	244.1 ±1.1	104.2
B	37**	0	35.5 ± 2.1	-
		37	70.7 ± 1.3	95.0
C	110***	0	110.1 ± 1.2	-
		110	229.3 ± 1.3	108.4
D	130***	0	123.3 ± 3.0	-
		130	259.6 ± 6.6	104.8

* μg of FA in 25 g of sample; ** μg of FA in 28.5 g of sample; *** μg of FA in 100 g of sample. The results are expressed as mean value ± S.D based on three replicates.

For sample B and D, the obtained results for folic acid were slightly lower than the declared value probably due to the losses of vitamin during storage, as has already been very well documented.[7] Addition and recovery tests were performed to assess the accuracy of SPE method, and, as observed, very good recoveries were obtained (95.0 to 108.4%), which confirm the applicability of the method for real samples with accuracy. Another beneficial feature of the new RAM-MIP is its high reusability (relative standard deviation, RSD of 4.9%) because the cartridge packed with material was used more than 100 times for the extraction of folic acid in real samples without loss of adsorption capacity. Besides the high life-time of RAM-MIP and the good cleanup performance of sample, Table 6 shows the great performance in terms of limit of detection for the proposed method regarding the other ones for the folic acid determination in milk samples

Conclusions

From the results it was demonstrated that the new adsorbent synthesized in this work, based on restricted access molecularly imprinted poly(methacrylic acid- trimethylolpropane trimethacrylate)/SiO_2 hybrid adsorbent (RAM-MIP) can be considered an attractive material for the cleanup/extraction of folic acid from milk samples. From the physical characterization using SEM and textural data associated to selectivity data, it was clearly demonstrated the imprinting effect created in the RAM-MIP. RAM-MIP adsorbs, in general, the highest amounts of folic acid and less structurally similar molecules (caffeine, 4-aminobenzamide or paracetamol) than RAM-NIP. The chemical modification of hybrid polymer with (3-glycidyloxypropyl)trimethoxysilane and posterior opening of the epoxy ring significantly improved the exclusion properties of macromolecules of MIP. For final remarks and future outlook, we can infer that hybrid molecularly imprinted polymer containing restricted access shows outstanding analytical potentiality in cleanup/extraction of analytes in complex sample, once the chromatographic profile for FA separation was better when compared to RAM-NIP, MIP and commercial adsorbent material (modified octadecyl silica-C_{18}). Thus, studies concerning the synthesis of new hybrid RAM-MIP include a broad research field that can be greatly explored aiming at improving and/or developing of new analytical methods.

Table 6. Comparison of some published methods for the folic acid determination in milk samples using different techniques and sample preparation methods

Methods of sample preparation	Technique	LOD (μg L^{-1})	Ref.
Precipitation	HPLC/ corona-charged aerosol detector	5800	34
Precipitation	HPLC/ultraviolet	50	35
Centrifugation	Indirect immunoassay	16.5	36
Centrifugation	Electrochemistry/MWCNT with platinum nanoparticles	22	37
SPE using sol-gel β-lactoglobulin columns	HPLC/Ultraviolet	179	38
SPE using RAM-MIP	HPLC/Ultraviolet	1.45	This study

LOD = limit of detection, MWCNT = multiwall carbon nanotubes

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GRAPHICAL ABSTRACT