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Research Article

Solid-Phase Extraction and High Performance Liquid Chromatographic Determination of Folic Acid in Fortified Foodstuffs: A Recommended Process Utilizing a New Strong Anion Exchange Sorbent

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Abstract

A high efficiency solid-phase extraction (SPE) column filled with a silica based strong anion exchange (SAX) sorbent was reported in the current study. The fabricated cartridge has been used for the extraction and pre-concentration of folic acid in different standard and real samples prior to their chromatographic (HPLC-UV) investigating. Wide linear range (LR: 0.02-1.20 μgmL^{-1}), low detection limit (LOD: 0.006μgmL^{-1}), low quantification limit (LOQ: 0.02μgmL^{-1}), good relative standard deviation (RSD: 2.57%) and good relative recoveries (RR ≥96.00%) were attained under the optimum conditions. This cartridge was fabricated with accessible and inexpensive materials and possesses the ability to replace expensive commercial columns. In addition, simple synthesis steps, long service lifetime and great extraction capability were some of the other advantages of the proposed SPE column. The proposed technique not to be affected by the interferences of samples matrices and can be successfully used for the routine analysis of weak acidic substances including folic acid (at low levels).

Keywords: High performance liquid chromatography; Folic acid; Silica based strong anion exchange; Solid-phase extraction

Introduction

Folate (also known as vitamin B9, vitamin M and folacin) is a form of the water-soluble B-vitamins that naturally occurred in many foods [1]. Folate is pointed as an anti-anemia and growth factor and involved in the biosynthesis of DNA, RNA, amino acids, nucleic acids and new red blood cells in body [2]. Consumption of adequate folate during rapid growth ages (especially in infancy and adolescence periods) is also extremely indispensable [3]. In addition, the insufficient intake of folate can raise the risk of colorectal, breast, ovarian, pancreas, brain, lung, cervical, and prostate cancers, stork, neurological disorders, cardiovascular disease and childhood mental retardation [4]. Because it is hard to get enough folate through foods, since 1998, synthetic folate or folic acid (FA) is added to enriched grain products, flour, breads, rice, pasta, cereals, bakery items, cookies, and crackers (according to the U.S. Food and Drug Administration (FDA) law). The recommended daily allowance (RDA) of FA is dependent on age, gender and other parameters. For example, women who are pregnant or might become pregnant should take the right amount of FA (RDA = 400μg/day) before and during pregnancy to prevent miscarriage and neural tube defects [5]. On the other hand, high intake of folate (folic acid) from food sources possesses no toxicity and health risks. However, high amounts of FA may hide the pernicious anemia caused by vitamin B12 deficiency and exacerbate neuropathy in these patients [6]. Moreover, high dosages of intravenous FA or supplemental FA intake can cause seizures or enhance the risk of the certain cancers [7]. Consequently, the European Food Safety Authority (EFSA) and the Japan National Institute of Health and Nutrition (NIDDK) have set the adult UL (Underwriter Laboratories) of FA at 1,000μg/day and 1,400μg/day, respectively [8]. Since, the medicines and supplements comprising the higher levels of FA may be harmful to users, its quantitative analysis with a reliable analytical technique is important. However, the analyses of FA is a great challenge in due to its poor stability under acidic conditions, complex composition, low stability against light and high temperature and very low concentration in natural samples [9]. Up to now, different analytical techniques (especially LC and HPLC methods) have been reported to determine the FA from target samples [10]. Utilizing of a purification, extraction and pre-concentration process can extremely influence on improvement, sensitivity and accuracy of FA analysis methods.

Solid phase microextraction (SPE) is the most frequently used sample preparation technique that utilized for the extraction, purification, cleanup, class fractionation and pre-concentration of trace compounds (including folic acid) from different samples [11]. In the SPE, analytes to be separated are selectively distributed between a solid phase sorbent and a liquid mobile phase [12]. The most applied design of SPE is a polypropylene or glass cartridge-type devices containing a placed solid adsorption phase. A number of polar and non-polar materials can be used as the SPE stationary phases, such as normal/reversed phase, ion exchange, mixed-mode phase, functionalized resins, affinity/immune-affinity sorbents, polymeric sorbents, graphitized carbon and porous carbon materials [13].

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Choosing a suitable sorbent is a crucial step in SPE that ensures the method accomplishment. The sorbent should be selected according to the type and chemical characteristics of the target analyte and the physical properties of the sample matrix [14]. Ion exchange SPE sorbents separate analytes and biological fluids that are charged when in a solution, based on electrostatic interactions between the analytes and the positively/negatively charged spices of the sorbent. Therefore, pH should be set at a value where both stationary phase and sample are charged. Ion exchange SPE sorbents are usually classified as cation exchange (containing strong or weak anionic groups (SAX or WAX)) and anion exchange (containing strong or weak cationic groups (SCX or WCX)). It is important to remember that strong ion exchanges are for weak acidic/basic substances while weak ion exchange is for strong acidic/basic compounds [15].

Given the importance of the FA analysis and despite all of the researches, the need for the development of an efficient extraction and determination method has not been addressed. In addition, since in most cases, measurement of low levels of FA in complex matrices (containing proteins, lipids, etc.) is considered, the sample must be cleaned up before the main analysis. Therefore, the focus of the present study was to synthesize a new silica based strong anion exchange sorbent as the SPE-solid phase and evaluate its HPLC-UV performance in the folic acid extraction and measurement.

Experimental

Chemicals and reagents

Pure folic acid (FA, 97%), high-purity grade silica gel (pore size: 90Å, for column chromatography), methyl iodide (MI, 0.99%) and 3-aminopropyltrimethoxysilane (APTMS, 97%) were purchased from Sigma-Aldrich (St. Louis, USA). HPLC grade water and solvents in were obtained from Merck (Darmstadt, Germany). The other chemicals and reagents were from Merck (Darmstadt, Germany). A stock standard solution of FA at a concentration of 100mgL^{-1} has been prepared in NaOH solution (0.1 N). Working solutions of FA were prepared daily by appropriate dilutions of the stock solution with deionized water. All the prepared solutions were wrapped in an aluminum foil and stored at 4°C. A mixture of potassium phosphate (pH = 6.8): methanol (90:10) at the flow rate of 0.7mLmin^{-1} was used as the HPLC mobile phase.

Apparatus

A Perkin-Elmer chromatographic system (CA, USA) equipped with a binary pump (model 200), ultraviolet detector (model 200) and a Rheodyne six port switching valve was employed for high-performance liquid chromatographic (HPLC) experiments. Total Chrom software was used to acquire and process the spectral and chromatographic data. All separations were achieved on a C18-reversed-phase column (Machery-Nagel, 5µm, 4.6mm i.d.×25cm long, 5µm pore size). VEGA3 Tescan electron microscope (Kohoutovice-Czech Republic), vector 22 (Bruker, Ettlingen, Germany) FTIR spectrometer and UV-spectrophotometer (Uvikon spectrophotometer 999, Italy) were utilized for characterization studies. In addition, pH meter model PHS-3C (Shanghai, China) for pH adjustments, Laboratory hot plate magnetic stirrer (0-1600 RPM, USA), Beckman GS-6 centrifuge (USA), S10H ultrasonic cleaning device with heating (ELMASONIC, S10H, Germany) and Memmert oven (Germany) were also applied. The SPE device was a polypropylene cartridge with appropriate frits at both sides.

All the chromatographic studies were performed at laboratory temperature in isocratic mode and wavelength of UV-detector was set at 280nm.

Preparation of silica based anion exchange sorbent

Activation of silica gel: Silica gel treatment was performed with the refluxing process in a mixture of nitric acid: water (1:1) for 3h and hydrochloric acid: water (1:1) for 6h. Then the mixture was filtered and the obtained precipitate rinsed with water for several times to reach neutral pH. Finally, the activated silica was calcinated at 160°C for 10h [16].

Preparation of SiO_2-APTMS: The preparation of SiO_2-APTMS was performed by introduction of the amino groups of APTMS onto the surface of activated silica [17]. For this purpose, 40.0g of activated silica gel, 50.0ml of APTMS and 150ml of toluene were transferred into a soxhlet apparatus for reflux-extraction at 70°C for 12h. The obtained product was then filtered off and washed with toluene, acetone, methanol and deionized water, respectively. The final product was dried at 50°C.

Preparation of SiO_2-MI: In this step, a mixture of 40g of SiO_2-APTMS and 30ml of MI was added to 200ml of methanol as solvent. The mixture was stirred at laboratory temperature for 2h. Then a solution containing sodium methoxide (12g) and methanol (40mL) was slowly added to the reaction vessel and stirred continuously for 24h. To finish, after rotary evaporation of methanol, the solid sorbent was dried at 50°C and the final silica based SAX product was achieved [18].

A schematic diagram of the synthesis process of the suggested silica based anion exchange sorbent is shown in Figure 1.

SPE-HPLC procedure

At the beginning, 0.3g of the synthesized phase was filled into

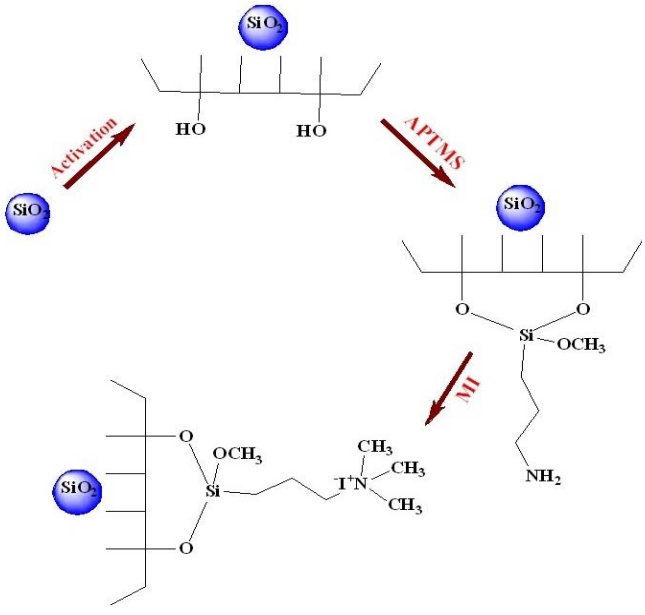

Figure 1: A schematic diagram of the synthesis process of the suggested silica based SAX-sorbent.

an empty polypropylene SPE-cartridge and equilibrated with 5mL each of methanol and deionized water, respectively. Then, 2.5mL of a FA standard/sample solution (at a concentration of 0.5mgL^{-1}) was loaded into the cartridge (flow rate = 3mLmin^{-1}). In the following and after a washing step with ethanol (flow rate = 1.5mLmin^{-1}), the analyte was eluted utilizing 2.5mL of phosphate buffer (pH = 9, flow = 1.5mLmin^{-1}). To finish, 20µL of the obtained solution was injected into a HPLC-UV system for the analysis of target analyte and evaluation of the extraction efficiency.

Real samples preparation

Commercially fruit juices (orange and apple), wheat flour and pasteurized milk were selected as real samples and analyzed by the proposed method. Different procedures had been reported for the preparation of these samples. In this work, fruit juices, wheat flours and pasteurized milks were prepared similar to Novikova (2020), Zhang (2019) and Vincenzetti (2020) studies, respectively [19-21].

Results and Discussion

Characterization of the prepared solid phase

The characterization information of the suggested SAX-sorbent was attained from FESEM and FTIR analysis. As is clear from the FESEM-image of Figure 2A, a relatively smooth surface has been achieved during the silica activation process. Activation of silica generally removes possible contaminations from the surface and activates the silanol groups (-OH). Silanols offer an appropriate surface to interact with other groups via electrostatic interactions or hydrogen bonding [22]. In addition, the silica activation reduces its particle size, enhances the surface area and consequently provides higher efficiency [23]. Nevertheless, after the modification of the activated silica, the sorbent surface was changed and a bumpy structure obtained (Figure 2B). This rough surface proves that the silica particles were well covered with the APTMS and MI materials.

The FTIR spectra of silica gel and prepared SAX-sorbent is also shown in Figure 3A and 3B, respectively. The absorption band at 475cm^{-1} is corresponded to the bending vibration of Si-O-Si in the sorbent, but the silica gel absorption band at 475cm^{-1} presented the bending vibration of Si-O-Si from SiO_4 [24]. The absorption band at 801cm^{-1} is related to the symmetric stretching vibrations of Si-O from Si-O-Si. The absorption band appeared at 1492cm^{-1} of the

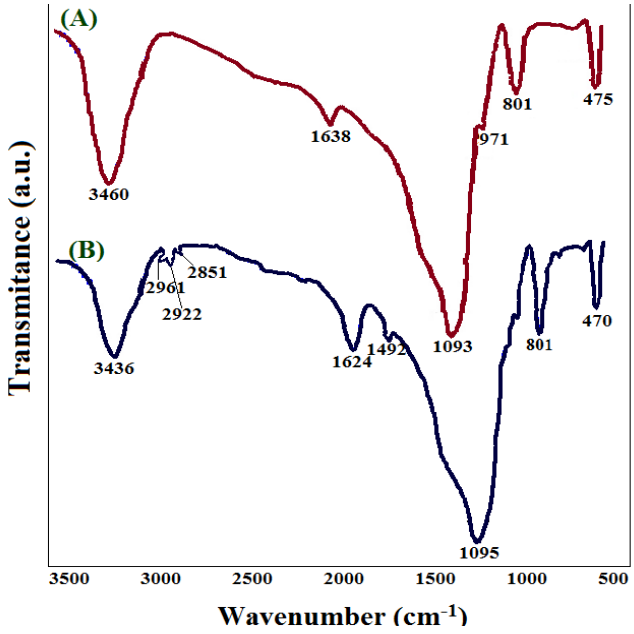

Figure 3: The FTIR spectra of silica gel (A) and the prepared silica based SAX-sorbent (B).

SAX-sorbent spectrum (Figure 3B) indicates the bending vibration of amine groups (N-H primer) [25]. The absorption band at wave number of 1095 and 1093 cm^{-1} is corresponded to the asymmetric stretching vibrations of Si-OH. The new absorption bands appeared in 2961, 2922 and 2851 cm^{-1} in the SAX-spectrum is for the stretching vibration of CH_2 groups. The broad band at about 3436 (and 3460cm^{-1}), followed by a weak band at 1638 (and 1624cm^{-1}) is attributed to the O-H bond stretching vibration of the adsorbed water, and the surface silanol groups [26].

Optimization of the method experimental parameters

Achieving an effective sample preparation technique is usually depended on optimizing different experimental parameters influencing the method efficiency. In the current study, mass of the sorbent, washing and elution solvents, volume of washing and elution solvents, flow rate of loading, washing and elution steps, pH of elution solvent and sample solution were optimized. The intensity

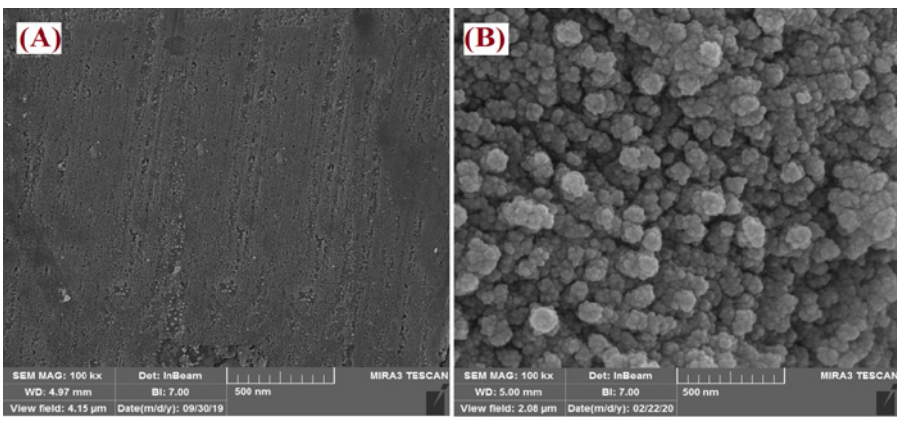

Figure 2: FESEM images of the activated silica gel and the prepared silica based SAX-sorbent.

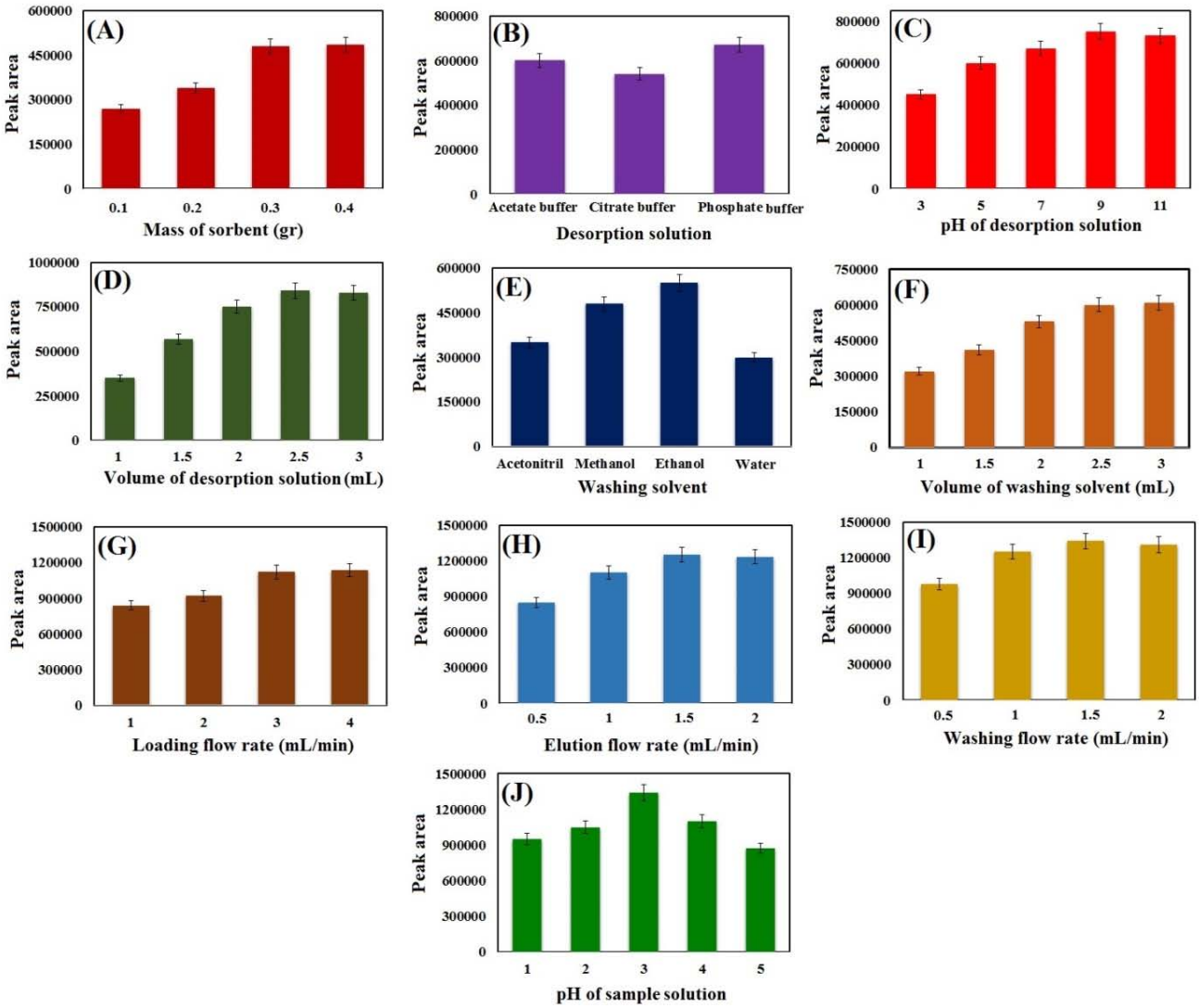

Figure 4: The influence of different experimental factors on the efficiency the suggested silica based-SAX-SPE-HPLC method: The amount of sorbent (A) desorption (elution) solution (B), pH of desorption solvent (C), volume of desorption solution (D), washing solvent (E), volume of washing solvent (F), loading flow rate G), elution flow rate (H), washing flow rate (I) and pH of sample solution (J).

of chromatographic signals was successively improved by the optimization of each of these vital parameters (Figure 4).

The amount of sorbent: Initially, the amount of sorbent that providing sufficient extraction capacity was evaluated by applying different mass of the prepared SAX-phase from 0.1 to 0.4 g. As can be seen in Figure 4A the maximum chromatographic signal was obtained utilizing 0.3g of the sorbent. Therefore, 0.3g was chosen as the optimum mass of the sorbent.

Desorption condition: Desorption (elution) of analytes from the sorbent surface is a vital step in all the SPE-LC methods. An appropriate solvent should be strong enough to elute the analytes from the cartridge completely (or as much as possible). Most SAX-SPE protocols of FA use buffer solutions for desorption step [27]. Therefore, the kind and pH of the buffer solution must be optimized. For this purpose, different solvents includes acetate, citrate and phosphate buffers (pH = 5) were tested for elution step. Phosphate buffer provided the highest recovery (Figure 4B). Then, the appropriate pH of phosphate buffer was examined (in the range of 3-11) and according to the results (Figure 4C), the pH of buffer was adjusted to 9. In addition, the volume of elution solvent as another important factor was also studied by changing the buffer volume from 1.0 to 3.0 mL. Based on the outcomes (Figure 4D), the maximum recovery was obtained utilizing 2.5mL of phosphate buffer.

Washing condition: In the washing step, unwanted interferences co-extracted with the analytes are removed from the adsorbent surface. Therefore, the appropriate washing solution must be strong enough to remove interferences but weak enough to leave target compounds. Deionized water, acetonitrile, methanol and ethanol were tested to find the appropriate washing solvent and finally ethanol was selected results (Figure 4E). In the following, the appropriate volume of ethanol was also investigated in the range of 1.0-3.0 mL. The results have presented that 2.5mL of ethanol is enough for the

Table 1:

Analytical figures of merit / Compound	LR[a] (μgmL^{-1})	R^{2} [b]	LOD[c] (μgmL^{-1})	LOQ[d] (μgmL^{-1})	RSD[e] (%) Intra-day	RSD (%) Inter-day	RSD (%) After one month
Folic acid	0.02-1.20	0.9982	0.006	0.02	1.12	2.57	2.31

a) Linear range
b) Square of correlation coefficient
c) Limit of detection
d) Limit of quantification
e) Relative standard deviation

Table 2: Relative recovery results of FA from real samples utilizing proposed method.

Samples	Added	Found	Recovery (%)
Apple juice	0	0.38	–
	0.5	0.86	96
	1	1.37	99
Orange juice	0	–	–
	0.5	0.52	100.4
	1	0.98	98
Wheat flour	0	1.13	–
	0.5	1.61	96
	1	2.15	102
Pasteurized milk	0	0.93	–
	0.5	1.43	100
	1	1.9	97

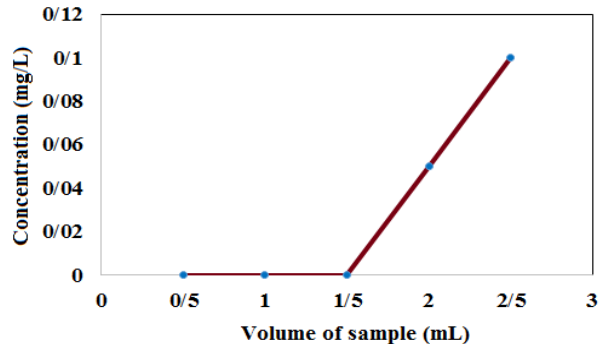

Figure 5: Breakthrough curve of the silica based-SAX-SPE-HPLC method.

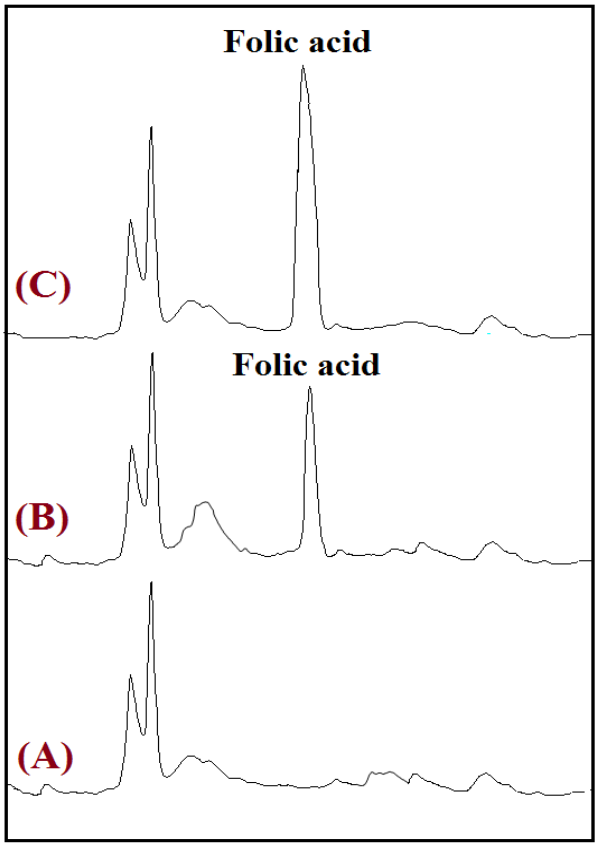

Figure 6: Chromatograms achieved using the presented silica based-SAX-SPE-HPLC method for a commercially apple juice: folic acid-free sample (A), sample contained FA (B) and the same sample spiked with 1.00μgmL^{-1} of standard solution of FA (C).

washing process (Figure 4F).

The flow rate of load, elution and washing steps: Optimization of the flow rate of sample loading, elution and washing steos is performed in order to achieve maximum sample throughput without compromising the method repeatability and robustness. High (or low) flow rates usually reduce the recovery values as the interaction time between the target analytes and the adsorbent is decreased (or extended) [28]. The effect of these parameters on the FA-extraction recovery was investigated in different flow rates. Results have shown that the maximum recovery was achieved by setting the flow rate of loading, elution and washing steps at 3.0, 1.5 and 1.5 mLmin^{-1}, respectively (Figure 4G-4I).

pH of sample solution: One more factor influencing adsorption, extraction and pre-concentration processes is the pH of sample solution. Sample pH may change the chemical form of analytes, affect the interaction between analyte and SPE sorbent and also influence on the SPE sorbents stability [29]. Since FA is a carboxylic acid containing amino groups, the changes in pH can affect its ionization and consequently its adsorption and extraction efficiency. The influence of the sample solution pH was examined in the range of 3-11, utilizing diluted HCl and NaOH. The pH of all the samples was adjusted to 7.0 before starting the SPE procedure (Figure 4J).

Breakthrough volume

Breakthrough volume is one of the main factors that characterize the SPE adsorbent bed. This parameter is defined as the volume of sample that can be loaded on the sorbent bed without losing the analytes [30]. The breakthrough volume is usually estimated by breakthrough curve that is the relationship between the sample concentration and the sample volume passed through the desired sorbent. At the present study, the volume of 2.5mL was obtained from the corresponding breakthrough curve (Figure 5).

Hold-up volume

The hold-up volume is the volume of sample left held-up by a SPE instrument. In other words, the volume of solution maintained by the spaces between the cartridge particles is the SPE hold-up volume. In the SPE process, the volume of conditioning solvent(s) should be at least 6 to 10 times the hold-up volume [BBB]. For this purpose, 10mL of ethanol was passed through the cartridge containing 0.3g of the prepared SAX sorbent and the output solution was collected in a graduated cylinder. Difference between the initial and output volumes was indicated that the hold-up volume of the desired column was 0.7mL.

Adsorption capacity

The adsorption capacity of SPE is the maximum amount of analytes that adsorbed per 1 gram of sorbent [32]. This factor is calculated from the following equation in which q is the equilibrium sorption capacity (mgg^{-1}), C_0 and C_t are the initial and final concentration ($mgmL^{-1}$), G is the mass of sorbent (g) and V is the volume of aqueous solution (mL).

$$q = (C_o\text{-}C_t)/G\times V$$

The adsorption capacity of the prepared SAX phase was estimated based on the concentration of FA before and after the loading 3mL of $3.0mgL^{-1}$ sample solution into the cartridge. The outlet solution was collected in volume intervals of 0.5ml and analyzed with HPLC system. According to the results, $0.156mgL^{-1}$ of the initial solution was not absorbed. Therefore, the adsorption capacity of the prepared sorbent was $28.44mgg^{-1}$.

Method validation

Table 1 is illustrated the analytical figures of merit of the presented silica based-SAX-SPE-HPLC procedure. Good linearity of the calibration curve was achieved at the concentration range of 0.02-1.20 μgml^{-1} with a regression coefficient (R^2) of 0.9982, using the spiked samples. The limit of detection (LOD) and limit of quantification (LOQ) of the method (based on the five repeated analysis of blank samples) were 0.006 and 0.020 μgml^{-1}, respectively. The relative standard deviation (RSD) of the intra-day and inter-day precision were 1.12% and 2.57%, respectively. In addition, a new sorbent was prepared in the same day, under the identical conditions and stored in laboratory ambient for 1 month. In this case, the RSD value was 2.31% and no decrease was happened in the technique efficiency. It is important to note that although the purpose of this study was to develop a high capability technique for FA sample preparation, but the synthesized sorbent could be successfully used for other weak acidic compounds.

Application to commercial real samples

The prepared real samples were initially checked out for the existence/lack of FA. Then, the samples were spiked with the standard solution of FA in two different levels of 0.50 and 1.00 μgml^{-1}. Finally, the samples were analyzed with the presented procedure (based on three repetitive measurements) and were achieved in all cases. The results (Table 2) demonstrated good recoveries (more than 95%) and proved high ability of technique for the determination of FA in real samples.

A chromatogram of a commercially blank apple juice without any FA is presented in Figure 6A. In addition, another chromatograms of a commercially apple juice sample (contained FA) and the same sample spiked with $1.00\mu gmL^{-1}$ of analyte (II) are shown in Figure 6B and 6C, respectively. The matrices compounds were eluted in less than five minutes and FA peak was completely separated from the other peaks, consequently no interference was observed during analysis.

Conclusion

Vitamin B9 plays a vital role in many metabolic procedures and thus is used naturally (folate) or synthetically (folic acid) in fortified foodstuffs and dietary supplements. Shortage or deficiency folate (folic acid) intake is associated with many diseases including severe abnormalities and anemia. Feeding of folate-rich foods is one of the best ways to maintain appropriate folate levels in the human body. Therefore, control of the foodstuffs is essential to ensure the presence of a sufficient amount of this vitamin. Due to the complexity of the matrix of most food samples and also the low amount of folic acid in them, in most cases, it is necessary to perform a sample preparation step before the main analysis. At the present study, a new silica based strong anionic solid phase with high affinity to folic acid was synthesized and used as the SPE-sorbent. The prepared SPE-cartridge was successfully applied to develop a high affinity sample preparation technique for the extraction and pre-concentration of FA prior its HPLC-UV analysis. The suggested technique presented a simple and rapid procedure to the analysis of FA with acceptable precision and recovery values. In addition, the method was not affected by the samples matrix interferences and can be used for routine folic acid (and other weak acidic substances) analysis (particularly at low levels) in laboratories.

Declarations

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Availability of data and material: Adequate and clear descriptions of the applied materials and tools are provided in the materials and method section of manuscript. In addition, the obtained data is clearly justified by mentioning the figures and tables in the manuscript.

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