Application of Polypyrrole (Ppy) nanofibers in Solid Phase Extraction of samples before the determination of vitamin B families in infant powder

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Abstract: In this study, a kind of core/sheath Polystyrene/Polypyrrole (PS/PPy) electrospun nanofibers was prepared and used as the solid phase extraction (SPE) adsorbent. Primary extraction of target analytes, three kinds of vitamin B (VB2, riboflavin; VB9, folic acid; VB12, cobalamin), was carried out by loading samples onto the column along with appointed boric acid compound reagent, and then the column should also be rinsed with the same reagent solution before elution. The boric acid compound was applied as complexing reagent to retain as much of three analytes as possible on the column based on the multi interaction between three vitamins and the boronate affinity reagent, thus improving hydrophobicity of targets and adsorption efficiency through loading and rinsing steps. Analytical parameters include linearity r^2> 0.99; limit of detection (LOD) ranged from 0.05 to 0.093μg/ml; Intraday and Interday precision of the method, 0.59%-12.1% and 0.64%-14.5%, respectively; the spiked extraction recoveries ranged from 84.9% to 111.7% in the infant powder samples. A Ppy nanofibers SPE column coupled with HPLC-UV was established for assay of elements in infant powder. The levels of three vitamin Bs in three brands of real milk powder samples were determined and the results were compared with the prescription. Results showed that the analysis method in the paper were reliable to be used in the determination of vitamin B families in infant powder. .
Keywords: vitamin B families; Polypyrrole; electrospun nanofibers; boric acid compound; packed-fiber solid-phase extraction (PFSPE).

1. Introduction

The vitamin B families are water-soluble vitamins required as coenzymes for enzymes essential for cell function often involved in neurotransmitter production and fatty acid metabolism etc. Small amounts of vitamin B families are required to maintain good health while it is well known that lack of them should cause serious diseases[1, 2]. Especially for folic acid (B9) and vitamin B12 , their possible involvement in the development of mental disturbance for children has been a subject of scientific investigation for the past decade[3]. Vitamin B2 is widely distributed and fairy important for child's development outcomes[4]. The food safety of infant powder is of great importance, because the insufficient intake of nutrients from infant powder will seriously affect the growth and development of the baby.

The water soluble vitamins are unstable and of strong polarity, hard to enrich and concentrate[5]. During the last decades, there has been an increasing interest for the simultaneous determination of vitamins. But most of the method were individually identified or with limited results in terms of lengthy sample preparation steps and method's robustness and reproducibility[6, 7]. Among them, high performance liquid chromatography (HPLC) techniques show some merits in view of simplicity and speed[8] [9]. For the reason that HPLC system has a high requirement of sample purity, so the pretreatment procedure to pre-clean the sample is very necessary. But most conventional methods add inorganic buffers or ion pairing reagents into the eluents[10][11] [12][13] which cause some anomalies. Solid-phase extraction (SPE) is a cost-effective technique through adsorb-desorb process with micro-amount of inorganic solvent involved, and it could not only extract traces of targets from samples, but also remove the interfering components of the complex matrices. The material used in SPE in the market mostly rely on their superficial area to adsorb targets[7] [14] which is not suitable for polar compounds. While, nano-scale materials in solid-phase extraction, an adsorbent having large surface area-to-volume ratios and easily chemically modified surface, is most prospectively for overcoming these problems. Some modified nanomaterials has been reported as the extraction reagent to extract a variety of B-vitamins[13] [15]，Owing to their large surface area

to volume ratio, electrospun nanofibers facilitate the miniaturization of SPE when they are packed into a mini column as the sorbent beds.

In recent years, conductive polymers have attracted great attention for the extraction of polar compounds due to special characteristic. The main advantage of using conducting polymers in SPE is that the charge of the sorbent can readily be controlled by oxidation and reduction of the polymers[16]. Previous study have developed a packed-fiber solid-phase extraction (PFSPE) method using Polypyrrole (Ppy) as adsorbent to extract VB2, VB9, VB12 in the urine sample and adding diphenylboronic acid 2-aminoethylester (DPBA) in loading and rinsing steps as complex reagent to improve hydrophobicity of targets and adsorption efficiency[17]. DPBA is a kind of boric acid compound, which has been verified that it could complex with compounds containing multi oxhydryl or amidogen groups because it can integrate or break with dihydroxyl and amidogen groups in different pH condition[17]. The materials or reagents made from boric acid compound has been widely used in sensing, separation and self-assembly, especially in separation of compounds[18]. This paper took more profound discussion about whether this method could be modified and applied into quantifying detection of three kinds of vitamin Bs in infant powder which is another real sample with complicated matrix. The levels of three vitamin Bs in three international famous brands of milk powder samples were determined, and the results were compared with the prescription. The method were convenient, green and cost effective, compatible to be applied in food testing industry.

2. Materials and methods

2.1 chemicals

Methanol of HPLC grade, standards of vitamin B families (B2, riboflavin; B9, folic acid; B12, cobalamin) and DPBA (diphenylborinic acid 2-aminoethyl ester), boric acid, 3-pyridine boric acid were purchased from Aladdin Industrial Corporation (USA). Sodium heptanesulfonate and other chemical reagents were purchased from Nanjing Chemical Reagent Co., Ltd (analytical grade). The polypyrrole (Ppy) nanofibers packed solid phase extraction column were purchased from Dongqi Bio-technology Co., Ltd Suzhou, China. Triple distilled deionized water was used throughout.

2.2 Apparatus and chromatographic conditions

The HPLC system SHIMADZU LC-20AD High Performance Liquid Chromatography (Shimadzu Corporation, Japan) and SPD-10AD UV Detector (Shimadzu Corporation, Japan) was used for analytical determinations. The parameters of the instrument, flow rate 1 mL / min; column temperature 25℃; the injection volume for the samples 20 μL; UV detection wave length 280 nm. Separation column was VP-ODS Shimadzu C18 (5 μm, 250 mm × 4.6 mm) chromatographic column. Separation of vitamins was carried out by 20% methanol and 80% aqueous phase which composed of 5 mmol/L sodium heptanesulfonate solution（adjust pH to 3 with H_3PO_4）and 60 mmol/L KH_2PO_4. The mobile phase was filtered through 0.45 μm microporous membrane twice and degassed under ultrasound for 15min.

2.3. Preparation of standard solutions

Standard stock solution of VB12 (1 mg/ml) were prepared by dissolving an appropriate amount of the compound in water and stored in the dark at 4℃. VB2, VB9 was dissolved in 10% Na_2CO_3 solution, and then diluted with water before use. 2 mg/ml stock solution of DPBA, Boric acid and 3-pyridine boric acid was prepared by dissolving an appropriate amount of the compound in 200μL methanol and then diluted with distilled water to 5 mL. The prepared solution was stored in the dark at 4℃.

In addition, spiked infant powder samples used for the recovery studies were prepared by adding appropriate volume of standard solution into 0.1 g/mL dissolved infant powder (formula). The final spiked VBs concentrations for infant powder solution were 0.1 μg/mL、1 μg/mL and 5 μg/mL.

2.4. The PFSPE procedure

The nanofibers should be activated before use. The extraction columns were conditioned by washing with 100 μl of methanol, followed by 100 μl of deionized water. After that, 500 μL of sample solution and 50 μL boric acid solution was loaded into the cartridge and pushed through the pre-conditioned nanofibers by air pressure forced by a gas-tight plastic syringe (10 ml), which was fitted to the top of the extraction cartridge. Then the column should be rinsed with 100 μL boric acid solution for three times to get rid of interference from real samples.The analytes retained on the nanofibers were quantitatively eluted with 100 μl of eluant solution. The flow rate was carefully controlled in a slow dropwise manner during the adsorption and desorption procedures. Finally, 20 μL of elute was injected onto the HPLC column via an auto-sampler. All operations were performed at room temperature.

2.5. Method Validation

2.5.1. Linearity and sensitivity

The stock solution containing the mixture of standards (each VB concentration was 1 mg/ml) was spiked into 5 mL 0.2 g/mL dissolved infant powder and then diluted them to 10 mL with distilled water, the spiked concentration of each target ranged from 0.05 to 50 μg/ml (n = 3 replicates per concentration). The solution were pretreated with PFSPE to obtain a calibration curve. The value of primary dissolved infant powder sample were also tested. The calibration curves were constructed by plotting spiked peak area minus primary ones versus concentration for each individual VB. Each point of the calibration curve corresponds to the mean value from three independent replicate injections.

The limits of detection (LOD) and quantification (LOQ) of individual analyte were obtained by injecting successively eluent which was from standard solutions and pretreated with PFSPE, and then calculated according to previous method[19] based on a signal-to-noise ratio (S/N) of 3 for the LOD and of 10 for the LOQ.

2.5.2. Spiked Recovery

The standard stock of vitamin B mixture (each VB concentration was 1 mg/ml) was spiked into 5 mL 0.2 g/mL dissolved infant powder solution and then diluted them to 10 mL with distilled water and got spiked solution with final concentration of each target (0.1 μg/mL, 1 μg/mL and 5 μg/mL; n = 3 replicates per concentration). The spiked sample were precipitated with the magnesium hydroxide co-precipitation method (as shown in 3.1.1) and then went through PFSPE procedure, the average concentrations calculated from the standard curve equation were recorded as Cs. The blank infant powder solution was also tested and got average concentrations as Cb. The spiked recoveries of each spiked concentration were determined as ((Cs-Cb)/the spiked concentrations)×100%.

The intra-day and inter-day precision and accuracy were evaluated for formula samples spiked with VBs (n = 5) at three different concentrations of every element according to the above pretreatment method. The inter-day precision was evaluated on five successive days. The method precision was reported as the %CV of repeatability at the concentration of each analyte. A calibrator series was freshly prepared for every run and the means for preparation of solutions was described above.

3. Results and Discussion

3.1. Optimization of sample protein precipitation and PFSPE procedure

3.1.1 Optimization of sample protein precipitation

The precipitation method for dairy product are generally sorted into three kinds: precipitate with organic solvent, precipitate with acid solvent, and precipitate with saline solution. For the extraction of polar compounds from dairy products, researchers have applied acetonitrile[20], chloroform[21], trichloroacetic acid[22], perchloric acid[23], magnesium sulfate[24], calcium chloride[25], etc. Referred to the methods above, the paper discussed the influence of protein precipitation method to the recovery of targets after PFSPE. The concrete precipitation process were as below.

Chloroform precipitate: 1 g of milk powder were accurately weighed and dissolved in 10 mmol/L ammonium acetate solution to make 10 mL of sample solution. Then 2 mL chloroform were added to 2 mL separated sample solution and vibrated well. The mixture solution was centrifuged at 15000 rpm/min for 10 min, then sediment was discarded and supernatant was gathered to another 10 mL polypropylene centrifuge tube.

Acetonitrile precipitation: 1 g of milk powder were accurately weighed and dissolved with distilled water to 10 mL. Then 2 mL sample was separated and added another 2 mL acetonitrileinto it. After vibrating, the sample was centrifuged at 3000 rpm/min for 10 min and the sediment was discarded and supernatant was gathered to another 10 mL polypropylene centrifuge tube. Rejoin 4 ml chloroform to draw acetonitrile out and transfer the upper supernatant to another tube for PFSPE.

The magnesium hydroxide co-precipitation: 1 g of milk powder were accurately weighed and dissolved with distilled water to 10 mL. Then 50 μL 10% magnesium sulfate solution were added in 2 mL of separated sample. The pH of the solution was adjusted to netural by the addition of 1mol/L NaOH. The sample was vibrated well and centrifuged at 1500 rpm/min for 5 min and the sediment was discarded and supernatant was gathered.

Calcium phosphate co-precipitation: 1 g of milk powder were accurately weighed and dissolved with distilled water to 10 mL. Then 150 μL 2 mol/L calcium chloride solution were added into 2 mL of separated sample. After vibrating, another aliquot of 150 μL 0.5 mol/L KH_2PO_4 solution (pH=6.0) was added. The sample was centrifuged at 13300 rpm/min for 5 min and the sediment was discarded and supernatant was gathered.

Trichloroacetic acid precipitation: 1 g of milk powder were accurately weighed and dissolved with distilled water to 10 mL. Then 200 μL 60% trichloroacetic acid solution were added into 2 mL of separated sample. After

vibrating, the sample was centrifuged at 10000 rpm/min for 5 min and the sediment was discarded and supernatant was gathered, the pH of the supernatant was adjusted to 5 ～6 by 1 mol/L NaOH.

Perchloric acid precipitation: 1 g of milk powder were accurately weighed and dissolved with distilled water to 10 mL. Then 200 μL 60% perchloric acid solution were added into 2 mL of separated sample. After vibrating, the sample was centrifuged at 10000 rpm/min for 5 min and the sediment was discarded and supernatant was gathered, the pH of the solution was adjusted to 5 ～ 6 by 1 mol/L NaOH.

The precipitation methods above were all carried out before PFSPE and the peak area of targets were compared respectively (shown as Figure.1.). The results showed that the organic solvent adding would cause massive loss of targets especially VB2. While the saline solution co-prepicitation method retained the targets most in the eluent. Above all, it would be best to choose saline co-prepicitation method such as the magnesium hydroxide or calcium phosphate to pretreat the milk powder samples and it is more suitable for the PFSPE process.

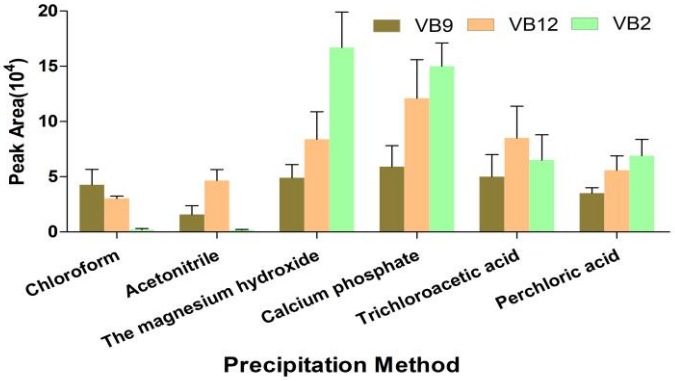

Figure.1. The milk powder were handed to different precipitation method of getting rid of the protein in the sample. After different precipitation management, the sample went through PFSPE in the paper, and the peak area of three VBs in the eluent were compared.

3.1.2 Optimization of eluent composition

The π-π interactions, hydrogen bonding, polar functional groups. etc which equipped by Ppy material are easy to break or become invalid under acid pH condition, as well as the complexation formed by boric acid compound and targets in the sample are also sensitive to low pH matrix. It is reasonable to adjust eluent to acid, and referred to the recipe of previous study[17], the eluent composition were optimized (shown as Figure.2.). Previous study took 80% aqueous phase and 20% methane acid as eluent (methane acid: methanol: aqueous phase = 2 : 2 : 6, v/v/v), and in this paper, the content of methane acid and methanol was optimized further. Figure.2A shows optimized the ratio of methane acid should be added into the eluent, and finally 20% volume ratio of methane acid was still selected to be added. Figure.2B shows optimized the ratio of methanol should be added into the eluent, and finally 10% volume ratio of methanol was selected to be added. Therefore the best recipe of eluent was methane acid: methanol: aqueous mobile phase at a volume ratio of 2:1:7 (v/v/v).

3.1.3 Optimization of boric acid solution

The research about the reaction between boric acid compound and dioxhydryl or amidogen group have been well summarized by Liu[18]. They studied that the PKa value of boric acid compound is a critical factor to the complexation and generally when the pH of reaction matrix is equal or greater than the PKa value of the boric acid compound, the complexation can form and otherwise uncapable to complex. The paper select three kinds of boric acid compound (DPBA, PKa=8.03±0.1; boric acid, PKa=9.24; 3-pyridine boric acid, PKa=4.4;) with different PKa value to add them into the samples pH neutral and tested their effect on the adsorption efficiency of VB targets onto Ppy nanofibers (shown as Fig 3). Result indicated that the DPBA were obviously superior to the other two. Thus, DPBA was selected and the concentration of DPBA was determined as 2 mg/mL (shown as Figure.3.B).

The chromatographic condition was optimized to provide preferable separation as soon as possible. The chromatograms of targets in standard solution were shown in Fig. 4A. All analytes were separated and identified very well at 280 nm. The chromatogram of sample supernatant was shown in Fig. 4B; the chromatogram of sample that dealt with PFSPE without adding of any boric acid solution and rinsing steps was shown in Fig. 4C, and dealt with PFSPE with adding of DPBA solution and rinsed with DPBA solution was shown in Fig. 4D. Although the target compounds were observed in the chromatogram of supernatant without any other treatment,

the targets were of poor identity and accompanied with lots of impurity peaks. After PFSPE with Ppy nanofibers without adding of boric acid compound and rinsing steps (shown in Figure 4C), the impurity peaks were still massive but obviously the analytes were concentrated and the signals were strengthened. While as in Fig. 4D, the supernatant was handled to PFSPE with adding of DPBA solution and rinsing steps, it was seen that all the targets were apparent and easy to distinguish, and the signals of analytes in the chromatogram were also magnified.

3.2 Method Validation

3.2.1. Linearity and sensitivity

The parameters of the calibration functions including LOD, LOQ, linearity, and calibration range are shown in Table 1. Calibration curves showed a good linearity ($r^2 \geq 0.99$) in wide range of concentrations. Overall, the data signifies it is feasible to detect VB9, VB12 and VB2 in samples with different levels of analytes. The LOD ranged from 0.05 μg/ml to 0.093 μg/ml. LOQ values from 0.17 μg/mL to 0.31 μg/mL. Therefore, the developed method provide referable sensitivity for the detection of these analytes in infant powder samples.

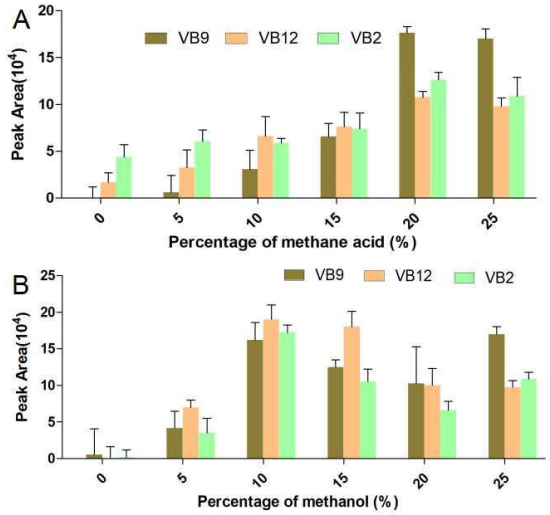

Figure.2. The optimization of eluent composition condition: (A) The ratio of methane acid in the eluent; (B) The ratio of methanol in the eluent.

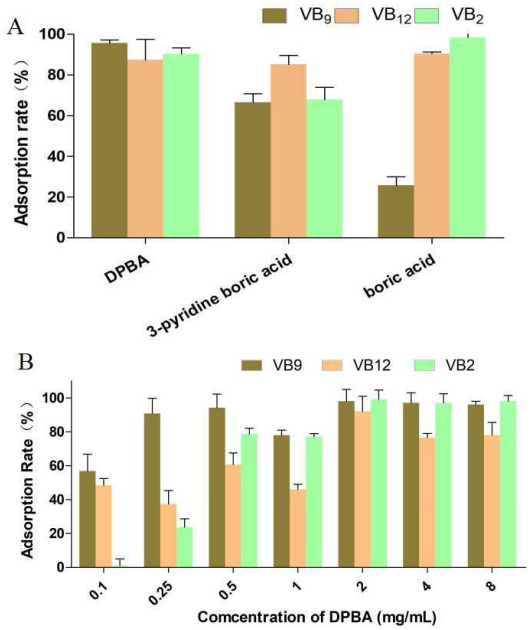

Figure.3. (A) Effect of boric acid compounds on the extraction for spiked water samples; (B) Effect of different concentrations of DPBA added into the samples.

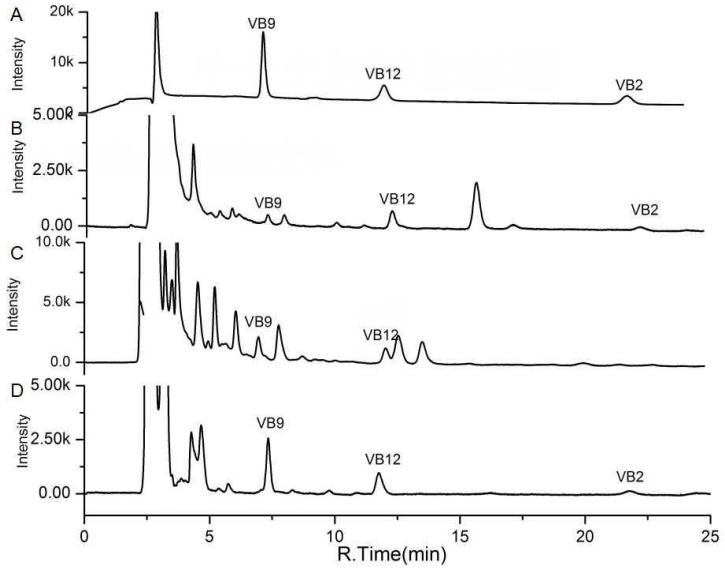

Figure.4. (A) Chromatograms of spiked water sample (1 µg/mL) without extraction; (B) Sample supernatant percipitated by magnesium hydroxide method; (C) Infant powder sample extracted by PFSPE without adding of boric acid compound and rinsing steps; (D) Infant powder sample extracted by PFSPE with adding of DPBA solution and rinsing with DPBA solution for three times.

Table 1. Analytical parameters for the HPLC method: linearity, R^2, and limits of detection (LOD) and quantification (LOQ).

Analytes	Linear range (µg/mL)	R^2	LOD (µg/mL)	LOQ (µg/mL)
VB9	0.5-50	0.996	0.093	0.31
VB12	0.5-50	0.990	0.056	0.19
VB2	0.5-50	0.999	0.05	0.17

3.2.2. Recovery and repeatability

The estimated recovery percentages for each VB target spiked at different concentrations are shown in Table 2. The recovery values ranged from 84.90% to 111.7% (for low, medium and high concentrations of formula samples) indicating a good recovery of these compounds in infant powder samples. The intra-day precision and the inter-day precision were from 0.64% to 14.5%, which met test requirements of food samples. Overall, the proposed method was sensitive enough to quantify the three analytes extracted from infant powder.

Table 2. Recovery, intraday and interday repeatability expressed as %CV (n = 5).

Analytes	Spiked concentration (ug/mL)	Spiked recovery (%)	Intraday (%CV)	Interday (%CV)
VB9	0.1	111.7	0.59	7.6
	1	84.90	6.9	5.6
	5	91.41	7.6	0.64
VB12	0.1	111.1	5.5	8.2
	1	87.09	3.0	7.3
	5	109.2	12.1	3.9
VB2	0.1	108.4	7.1	12.3
	1	101.3	5.6	14.5
	5	96.00	6.4	3.1

3.3 Detection of VB9, VB12 and VB2 in infant powder

The researchers purchased three different international famous brands of infant powder on the market (Brand I; Brand II; Brand III) and analyzed in order to check the applicability of the method for real food samples. Concentrations found after triplicate analysis are summarized in Table 3 and the data showed that the analyzed value were generally higher than the labelled value which indicated qualified quality and reliable assay.

Table 3: Concentration of VB9, VB12, VB2 in real infant powder samples. Values are expressed as the mean value ± SD.

	Brand I（μg/100g）		Brand II（μg/100g)		Brang III（μg/100g)	
	Analyzed value	Brand labelled	Analyzed value	Brand labelled	Analyzed value	Brand labelled
VB9	67.81±1.87	67	149±2.35	65	239.59±4.3	110
VB12	2.9±1.23	1.9	3.16±1.76	1.5	3.26±0.8	2.4
VB2	1654.68±134.5	1309	756.41±121.2	600	1704.08±157.1	1230

4. Conclusion

The proposed Ppy nanofiber-based solid-phase extraction coupled with HPLC-UV method was applied to determine three important VB families (VB9, VB12 and VB2) in infant milk powder. The boric acid compound was served as complexation and retention reagent in loading and rinsing steps in PFSPE procedure, respectively. The developed method can concentrate the targets in real samples without evaporation stage, avoiding losses of the the unstable VBs as well as improving the sensitivity and selectivity. The new method was applied successfully to the measurement of VBs in infant powder, and the tested content of every ingredient was equal or higher than that labeled. The method has potential to be adopted for the monitor of real food samples on the market.

Acknowledgements

This study was supported by the National Science Foundation of China (No.81172720, No. 81673230), the Social Development Research Program of Science and Technology department of Jiangsu Province (No. BE2016741).

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