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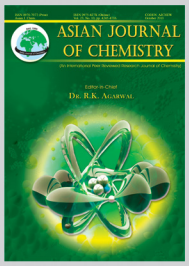

Simultaneous Determination of Four Water-Soluble Vitamins in Chinese Jujube (*Ziziphus jujuba* Mill.) by High-Performance Liquid Chromatography

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A simple, fast and accurate liquid chromatography method for simultaneous separation and determination of vitamin B_1 (thiamine), B_3 (nicotinic acid), B_6 (pyridoxine) and B_2 (riboflavin) in Chinese jujube (*Ziziphus jujuba* Mill.), was developed and validated. The chromatographic separation was achieved on a Venusil XBP-C_{18} column at 25 ºC, using a gradient of water with 0.1 % phosphoric acid (pH 2.3) and acetonitrile at a flow rate of 0.8 mL/min. An ultra-violet detector (UV) was used to monitor the eluent at 270 nm with a total analysis time of 0.5 h. The method provided low limits of detection (LOD) and limit of quantification (LOQ), good linearity, accuracy and precision and had been successfully applied to the determination of water-soluble vitamins in five Chinese jujube cultivars. These studies could be vital interest for evaluating nutritional quality of Chinese jujube during production and at the end of shelf life.

Key Words: High-performance liquid chromatographic method, Water-soluble vitamins, Chinese jujube (*Ziziphus jujuba* Mill.)

INTRODUCTION

Vitamins, a diverse group of organic compounds found in small amounts in foods, are designated as indispensable nutrients because they cannot be synthesized by the body and are required to support health and well being[1]. These compounds, depending on their solubility, are classified as fat-soluble and water-soluble[2]. The major part of the water-soluble vitamins comprises the B-complex and vitamin C[3]. Of B-complex, vitamin B_1 (thiamine), B_3 (nicotinic acid), B_6 (pyridoxine) and B_2 (riboflavin) plays different specific and vital functions in metabolism. Lack of them can cause serious diseases in human being such as anorexia, muscle weakness, pellagra beriberi, glossitis, seborrheic dermatitis and so on[1,4-7]. A well-balanced diet supplies all of the required vitamins[8]. Consuming fruits and vegetables rich in vitamins and vitamin-supplemented foods have been growing in popularity in human especially among consumers as a means of preventing or retarding illnesses such as hyperlipidemia, hypertension, obesity and cardiovascular diseases, which are common in industrialized countries[9].

Chinese jujube (*Ziziphus jujuba* Mill.), a native and medicinal plant of China, belongs to the genus *Ziziphus* (Rhamnaceae) and is widely distributed in China and South Korea with a total growing area of over 1,600,000 hectares[10]. Its fruit is mainly consumed fresh and dehydrated (dried fruit), or processed into candies, jam, juice, wine, syrup, canned food, or vinegar[11]. Chinese jujube contains numerous naturally occurring compounds including polysaccharides[12], alkaloids[13], flavonoids[14], phenolic acids[15], saponins[16], fatty acids[16,17] and vitamins[18,19]. As to vitamins, present research showed that Chinese jujube is rich in vitamin C (ascorbic acid and dehydroascorbic acid), of which ascorbic acid content (200-800 mg/100 g) is even higher than that in kiwi fruit and dehydroascorbic acid (20-120 mg/100 g) is 10-100 times more than that in common fruits, such as strawberry, apples, orange, banana, papaya, mango, lemon and so on or 1-10 times more than the common vegetables including green bean, tomato, green pepper and cabbage[10]. However, there is a lack of knowledge of the other water-soluble vitamins, especially the important B-complex vitamins in Chinese jujube. Being a potential source of vitamins, it is important to determine the content of B-complex vitamins in Chinese jujube.

Determination of vitamins is often a challenging task due to their instability. Most of them are particularly susceptible to many factors including temperature, moisture, oxygen, light, pro-oxidants, reducing agents and pH[9,20,21].

In the past decades, water-soluble vitamins were determined generally by traditional analytical methods involving enzymatic, microbiological and chemical procedures[22]. These

methods, which are tedious and time-consuming, involve pre-treatment of the sample through complex chemical, physical or biological reactions to eliminate the interferences commonly found, followed by individual methods for each different vitamin[23,24].

Today, high-performance liquid chromatography method (HPLC) with appropriate detection systems is regarded as the most convenient method for the evaluation of water-soluble vitamins. Several methods had been published dealing with the simultaneous determination of water-solution vitamin compounds[25-27]. However, most of these methods are mainly applied for analyzing less complex matrices including multivitamin syrup and tablets or various fortified food products which contained significant amounts of added vitamins (enriched foodstuff)[8,26,28-32]. They are usually inapplicable to fruit of Chinese jujube with a complex matrix for which an accurate chromatographic isolation of the different vitamin compounds and adjacent peaks, often present in small amounts, is difficult. Moreover, currently an economical simultaneous technique for determination of several vitamin compounds in Chinese jujube fruit is not available.

In this study, a simultaneous HPLC method for determination of vitamin B_1, B_3, B_6 and B_2 in fruit of Chinese jujube was firstly developed and validated for evaluating its nutritional quality during production and at the end of shelf life.

EXPERIMENTAL

The vitamin B_1, B_3, B_6 and B_2 standards were acquired from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The HPLC grade solvents (methanol, acetonitrile, sodium hexanesulfonate) and the guaranteed grade reagents (phosphoric acid, potassium dihydrogen phosphate, potassium phosphate, acetic acid, potassium acetate) were purchased from Tianjin Guangfu Chemical Company (Tianjin, China). For chromatographic analysis, ultrapure water of 18 MΩ/cm resistivity was purified using a Milli-Q system (Millipore, USA). The five cultivars of Chinese jujube including 'Jinsixiaozao', 'Lianxianmuzao', 'Xiangzao', 'Linzedazao', 'Nanjingzao' analyzed in this study were collected from Taigu of Shanxi Province, China. Fruits were harvested at their physiological maturity stage. After collected, all fruits were stored at -70 ºC until the analyses were carried out.

The chromatographic measurements were carried out with an Agilent 1200 model HPLC system (Waldbronn, Germany) equipped with a vacuum degasser, a quaternary pump, an automatic injection system (0-100 µL), an ultra-violet detector (UV) and a temperature control compartment. Prepared samples were centrifuged with a CT14RD centrifuge (Tianmei, China) and were concentrated with a RE-52A rotary evaporator (Zhenjie, China).

Chromatographic conditions: For chromatographic analysis, the column used for chromatographic separation was Agela Venusil XBP-C_{18} (250 mm × 4.6 mm, 5 µm). The mobile phase adopted was acetonitrile (A) and 0.1 % phosphoric acid aqueous solution (B) (v/v) using a linear gradient elution of 0-2 % A at 0-10 min, 2-20 % A at 10-20 min, 20 % A at 20-25 min. Re-equilibration duration was 8 min by using the starting condition before injection of the next individual sample. The flow-rate was kept at 0.8 mL/min all the times. The column was operated at 25 ºC. The injection volume was 20 µL. The eluent was monitored at 270 nm for all analytes.

Sample preparation: Chinese jujube fruit samples were cutted into small pieces. A portion of 2.5 g of fruit was added to 10 mL of 0.1 % aqueous solution of phosphoric acid. The mixture was homogenized by grinding, transferred to a 25 mL volumetric flask and diluted to volume. After centrifuged at 10,000 g (refrigerated at 4 ºC) for 10 min, an aliquot of 10 mL of the supernatant was evaporated to dryness. And the residue was dissolved in 2 mL of 0.1 % aqueous solution of phosphoric acid. The final solution was used to estimate vitamin B_1, B_3, B_6 and B_2.

All samples were prepared in triplicate. To prevent the loss of vitamin, all operations were performed in the absence of direct sunlight, using amber glassware.

Standard solutions: Stock standard solutions were prepared by accurately weighing proper quantities of vitamin B_1, B_3, B_6, B_2 and dissolving in 25 mL of 0.1 % aqueous solution of phosphoric acid to give concentrations of 350, 400, 460 and 200 mg/L, respectively. Working mixed standard solutions were prepared by accurately mixing respective stock standard solution with a 1:1:1:1 ratio to the required concentrations and then diluted to appropriate concentration ranges for the establishment of calibration curves. Quantification was performed by external standard method using the vitamin B_1, B_3, B_6 and B_2 standards. The standard solution was kept in the dark at 4 ºC and was prepared daily.

RESULTS AND DISCUSSION

Chromatography optimization: The chromatographic separation conditions were optimized to assure a good separation of the analytes and potential impurities. At the same time it is necessary to shorten the analysis time, stabilize retention of all analytes and cause the least damage to HPLC system.

In this study, the tested columns included Agilent Eclipse XDB-C_{18} (150 mm × 4.6 mm, 5 µm), Elite Hypersil BDS-C_{18} (250 mm × 4.6 mm, 5 µm), Agela Promosil C_{18} (250 mm × 4.6 mm, 5 µm), Agela Venusil MP-C_{18} (250 mm × 4.6 mm, 5 µm) and Agela Venusil XBP-C_{18} (250 mm × 4.6 mm, 5 µm). And the optimization procedure of the mobile phase for each tested columns included the composition of the mobile phase: acid type (phosphoric or acetic acid), buffer type (phosphoric or acetic buffer), the ion-pair reagent (sodium hexanesulfonate), organic modifiers (methanol or acetonitrile), pH of the mobile phase (from 2.0-6.0), flow rate (from 0.6-1.2 mL/min) and temperature (from 20-40 ºC).

As results of these experiences, the HPLC methods have been improved for the separation of polar vitamins by using ion paring agent. But, it isn't adopted because these reagents often display stability problems such as sudden decrease of retention time and poor reproducibility of the separation. A gradient elution technique involving acid or salts buffer mobile phases was applied to achieve a good separation of the four vitamins. The better chromatograms obtained on different tested columns were shown in Fig. 1. The baseline separate

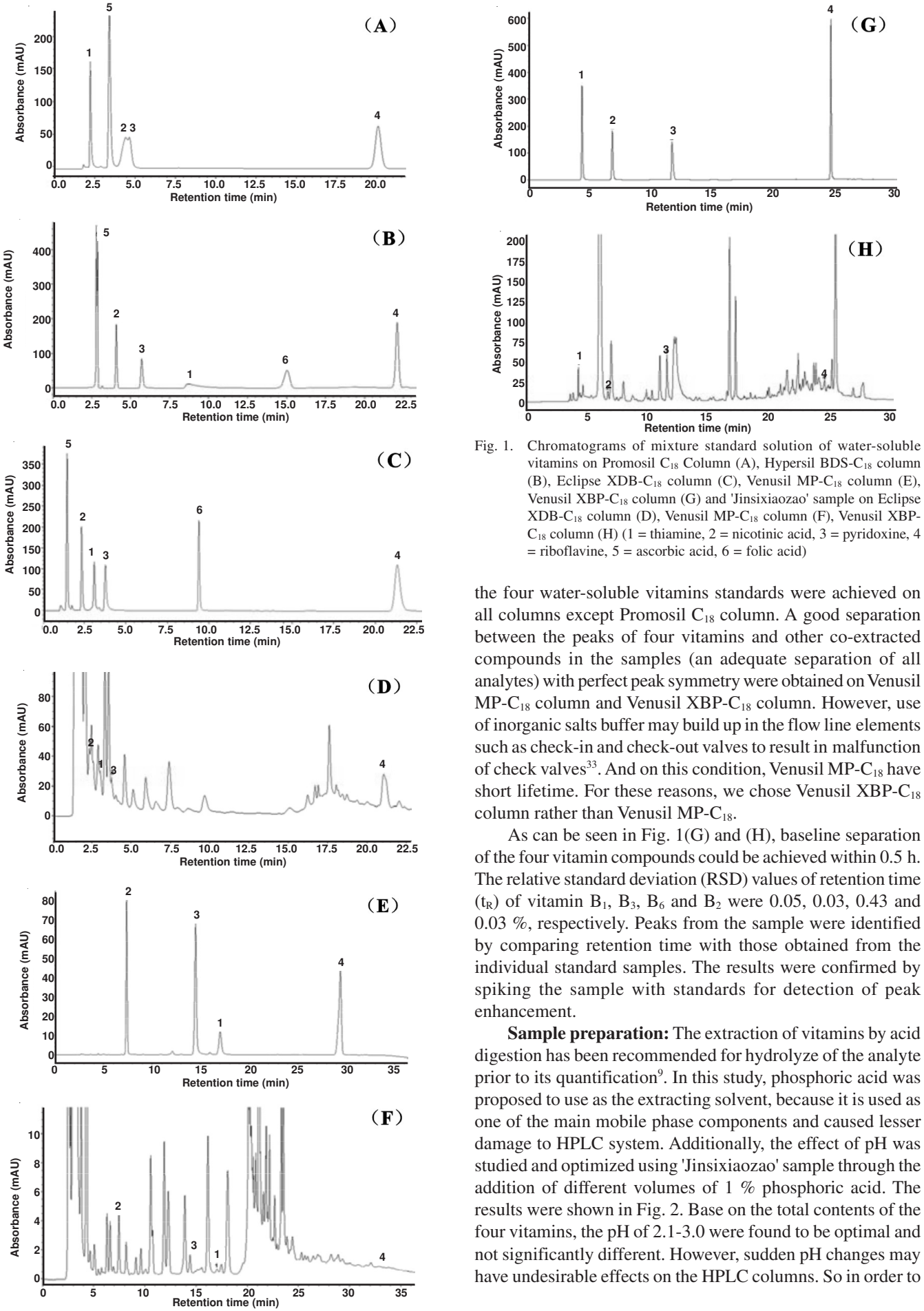

Fig. 1. Chromatograms of mixture standard solution of water-soluble vitamins on Promosil C_{18} Column (A), Hypersil BDS-C_{18} column (B), Eclipse XDB-C_{18} column (C), Venusil MP-C_{18} column (E), Venusil XBP-C_{18} column (G) and 'Jinsixiaozao' sample on Eclipse XDB-C_{18} column (D), Venusil MP-C_{18} column (F), Venusil XBP-C_{18} column (H) (1 = thiamine, 2 = nicotinic acid, 3 = pyridoxine, 4 = riboflavine, 5 = ascorbic acid, 6 = folic acid)

the four water-soluble vitamins standards were achieved on all columns except Promosil C_{18} column. A good separation between the peaks of four vitamins and other co-extracted compounds in the samples (an adequate separation of all analytes) with perfect peak symmetry were obtained on Venusil MP-C_{18} column and Venusil XBP-C_{18} column. However, use of inorganic salts buffer may build up in the flow line elements such as check-in and check-out valves to result in malfunction of check valves[33]. And on this condition, Venusil MP-C_{18} have short lifetime. For these reasons, we chose Venusil XBP-C_{18} column rather than Venusil MP-C_{18}.

As can be seen in Fig. 1(G) and (H), baseline separation of the four vitamin compounds could be achieved within 0.5 h. The relative standard deviation (RSD) values of retention time (t_R) of vitamin B_1, B_3, B_6 and B_2 were 0.05, 0.03, 0.43 and 0.03 %, respectively. Peaks from the sample were identified by comparing retention time with those obtained from the individual standard samples. The results were confirmed by spiking the sample with standards for detection of peak enhancement.

Sample preparation: The extraction of vitamins by acid digestion has been recommended for hydrolyze of the analyte prior to its quantification[9]. In this study, phosphoric acid was proposed to use as the extracting solvent, because it is used as one of the main mobile phase components and caused lesser damage to HPLC system. Additionally, the effect of pH was studied and optimized using 'Jinsixiaozao' sample through the addition of different volumes of 1 % phosphoric acid. The results were shown in Fig. 2. Base on the total contents of the four vitamins, the pH of 2.1-3.0 were found to be optimal and not significantly different. However, sudden pH changes may have undesirable effects on the HPLC columns. So in order to

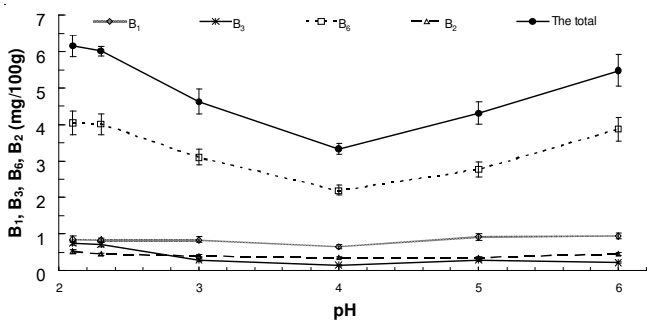

Fig. 2. Effect of the pH on the extraction efficiency of four vitamin compounds

accord with pH of mobile phase, the 0.1 % aqueous solution of phosphoric acid (pH 2.3) was opted as extracting solvent.

Linearity, limit of detection and limit of quantification: Standard mixtures were prepared to study the linearity, the limit of detection (LOD) and limit of quantification (LOQ) of the method. The linearity was investigated for each compound by plotting peak-area (y) against the concentration of each reference compound (x, mg/L). The correlation coefficients of all the calibration curves were found to be higher than 0.9998 (Table-1). The calibration plots for the four vitamins showed good linear relationships. Absorbance responses of vitamin B_1, B_3, B_6 and B_2 were significantly linear up to 70, 80, 92 and 40 mg/L, respectively. To evaluate the sensitivity of the method, the limits of detection (LOD) and limits of quantification (LOQ) for each vitamin were calculated with corresponding standard solution on the basis of signal-to-noise ratio (S/N) of 3 and 10, respectively. As shown in Table-1, LODs of the four vitamins varied from 2.29 ng/mL (vitamin B_3) to 23.00 ng/mL (vitamin B_6), while LOQs ranged from 8.00 ng/mL (vitamin B_3) to 92.00 ng/mL (vitamin B_6).

TABLE-1
REGRESSION EQUATION, CORRELATION COEFFICIENTS (R^2), LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ) OF THE HPLC METHOD

Compound	Regression equation	R^2	LOD (ng/mL)	LOQ (ng/mL)
B_1	y = 42.468x-17.497	0.9999	11.26	56.32
B_3	y = 27.955x-1.2138	0.9999	2.29	8.00
B_6	y = 25.482x-9.8398	0.9999	23.00	92.00
B_2	y = 95.707x-22.084	0.9998	16.00	40.00

y is the peak-area and x is the concentration (mg/L).

Accuracy, precision and repeatability: The accuracy was calculated on the recovery of known amounts of analyte, spiking analyte in 'Jinsixiaozao' samples. To evaluate the per cent recovery, standard mixtures at two nominal concentration levels, 100 and 200 % of the estimated initial amount of four vitamins, were spiked into 'Jinsixiaozao' samples and extracted in triplicate. The recovery percentages for four vitamins were above 97 % at all nominal concentration levels (Table-2). The intermediate precision was determined by evaluating inter-day and intra-day injections variation. The intra-day variation was determined by analyzing the six replicate samples within one day and inter-day variation was determined on 3 consecutive days. The RSD values of the intra-variations for four vitamins were not more than 2 % and the inter-variations were not more than 5 % (Table-2). Repeatability of the extraction procedure was determined by repeating the extraction procedure six times using the same 'Jinsixiaozao' sample and analyzed by HPLC. The results were shown in Table-2. The RSD values of peak-area for four vitamins were no more than 5 %.

The results indicated that this method is accurate, sensitive and reproducible. A useful quantitative method was provided for determination of the four vitamins in Chinese jujube.

Stability: The stability of vitamin standard preparation and sample solution was fully evaluated at 4 and 25 ºC for 96 h. The standard preparation and sample solution ('Jinsixiaozao') of four vitamin compounds were found to be stable for at least 24 h at 4 ºC and the remaining ranged from 99.02-101.91 %. All solutions could keep stable for no less than 4 h at 25 ºC and the remaining ranged from 99.14-101.78 %.

These results indicated that many samples could be processed at one time within 24 h at 4 ºC or 4 h at 25 ºC, which would also compensate for the shortcoming of relative long analysis time (about 0.5 h) of this assay.

Analysis of Chinese jujube samples: The developed optimized method was used for quantification of four vitamin compounds in five cultivars of Chinese jujube. The results were shown in Table-3. There were remarkable differences among the contents of the four vitamins in five cultivars of Chinese jujube. The contents of vitamin B_1, B_3, B_6 and B_2 in different cultivars varied from 0.77 mg/100 g ('Xiangzao') to 1.87 mg/100 g ('Nanjingzao'), 0.71 mg/100 g ('Jinsixiaozao') to 3.27 mg/100 g ('Nanjingzao'), 1.41 mg/100 g ('Linzedazao') to 15.57 mg/100 g ('Lianxianmuzao') and 0.40 mg/100 g ('Linzedazao') to 1.44 mg/100 g ('Nanjingzao'), respectively. On the average, a 50 g portion of Chinese jujube fruit studied would provide about all of an adult's daily need of vitamins B_1, B_6 and B_2 recommended by Food and Nutrition Board, National Research Council[34]. And a 500 g portion of Chinese jujube fruit studied would provide about all of an adult's daily need of vitamin B_3. So, Chinese jujube could be considered as good sources of

TABLE-2
RECOVERY, INTERMEDIATE PRECISION AND REPEATABILITY OF THE HPLC METHOD (n ≥ 3)

Compound	Recovery (%)				Intermediate precision (% RSD)		Repeatability (% RSD)
	Spiked level 1		Spiked level 2				
	Mean	RSD (%)	Mean	RSD (%)	Intra-day	Inter-day	
B_1	97.38	1.85	102.37	2.44	0.66	1.21	2.16
B_3	100.80	0.26	100.61	2.64	1.17	1.53	4.73
B_6	105.54	3.74	102.73	2.82	0.58	4.31	3.92
B_2	97.51	2.10	102.48	1.11	0.91	4.80	4.08

Spiked level 1 = 100 % of the estimated initial amount of four vitamin compounds in 'Jinsixiaozao' samples. Spiked level 2 = 200 % of the estimated initial amount of four vitamin compounds in 'Jinsixiaozao' samples.

TABLE-3
DETERMINATION OF FOUR VITAMINS IN FIVE CULTIVARS OF CHINESE JUJUBE

Cultivar	Vitamin compounds means content (mg/100g)				
	B_1	B_3	B_6	B_2	Total
Jinsixiaozao	0.84 ± 0.02	0.71 ± 0.03	4.03 ± 0.16	0.45 ± 0.02	6.02 ± 0.13
Lianxianmuzao	1.60 ± 0.05	1.84 ± 0.09	15.57 ± 0.72	0.80 ± 0.03	19.82 ± 0.74
Xiangzao	0.77 ± 0.04	1.95 ± 0.08	5.76 ± 0.29	0.56 ± 0.03	9.04 ± 0.29
Linzedazao	1.65 ± 0.05	1.68 ± 0.07	1.41 ± 0.05	0.40 ± 0.02	5.15 ± 0.15
Nanjingzao	1.87 ± 0.03	3.27 ± 0.14	7.22 ± 0.29	1.44 ± 0.07	13.79 ± 0.37

vitamin B_1, B_6 and B_2. Further studies on their contents in more cultivars of Chinese jujube are of great importance for selection and breeding of plant cultivars rich in vitamins.

Conclusion

A simple, rapid and accurate HPLC method was developed and this is the first report of simultaneous determination of vitamin B_1, B_3, B_6 and B_2 in Chinese jujube fruit by HPLC. The HPLC method using 0.1 % aqueous solution of phosphoric acid as the main mobile phase component, might cause lesser problem to HPLC system than salt buffer and ion paring agent and still have satisfactory separation. The proposed method provided good linear, sensitivity, repeatability, precision and accuracy and was successfully applied to the determination of water-soluble vitamins in fruit of five Chinese jujube cultivars. The results showed that Chinese jujube were very good sources of vitamin B_1, B_6 and B_2.

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