

**DETERMINATION OF SEVEN WATER-SOLUBLE
VITAMINS IN TARHANA, A TRADITIONAL TURKISH
CEREAL FOOD, BY HIGH-PERFORMANCE
LIQUID CHROMATOGRAPHY**

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ABSTRACT

A reversed-phase high-performance liquid chromatographic procedure has been developed for determination of water-soluble vitamins (ascorbic acid, niacin, panthothenic acid (vitamin B₅), pyridoxine (vitamin B₆), thiamine (vitamin B₁), folic acid, and riboflavin (vitamin B₂)) in tarhana samples, a traditional Turkish cereal food. Sample-treatment entails SPE with Sep-Pak C₁₈ (500-mg) cartridges. The water-soluble vitamins were analyzed by HPLC on a Discovery C-18 150 mm × 4.6 mm column with 0.1 mol L⁻¹ KH₂PO₄ (pH 7.0)–methanol, 90:10, as mobile phase (0.7 mL min⁻¹) in isocratic mode. Identification of compounds was achieved by comparing their retention times and UV spectra with those of standards stored in a data bank. The detection limits ranged from 0.1 to 0.5 mg L⁻¹. The accuracy of the method was tested by measuring average recovery; values ranged between 96.51 and 99.40%.

INTRODUCTION

Tarhana, a traditional Turkish food, is prepared by mixing wheat flour, yoghurt, yeast and a variety of cooked vegetables (tomatoes, onions, green pepper etc.), salt and spices (mint, paprika) followed by fermentation for one to seven days [1]. In the central and eastern part of the Turkey, one or more ingredients such as milk, soybean, lentil, chickpea, corn flour, and egg are also added. [1,2]. Tarhana has an acidic and sour taste with a strong yeast flavour and also a good source of proteins, vitamins, and minerals and is therefore used largely for feeding children and elderly people [1,3]. Vitamins are a broad group of organic compounds that are minor, but essential, constituents of food required for normal growth, self-maintenan-

ce, and functioning of human and animal bodies. These compounds can be classified in two main groups – water-soluble and fat-soluble vitamins. Among the B group of water-soluble vitamins, both thiamine (B₁) and pyridoxine (B₆) are important. They play different specific and vital functions in metabolism, and their lack or excess produces specific diseases. Vitamins are relatively unstable and can be lost during processing and storage of food [4,5]. Because of the critical role of vitamins in nutrition and their relative instability, qualitative and quantitative analyses are important issues and a challenging task for food manufacturers. HPLC is the preferred technique for vitamin separation, because of its high selectivity [6]. In addition, solid-phase extraction is necessary before HPLC to remove interfering components [7].

The literature contains several methods, including microbiological assay, for determination of vitamins in tarhana [8,9] but there is no report of simultaneous determination of water-soluble vitamins in tarhana. In this paper, a method for simultaneous determination of seven water-soluble vitamins by HPLC with diode-array detection is reported. The proposed method is simple and rapid and it is possible to identify and simultaneously determine seven water-soluble vitamins in less than 25 min with only one injection.

EXPERIMENTAL

Materials

The tarhana samples studied were obtained from local markets (Ankara, Turkey).

Equipment

Liquid chromatography was performed with a Shimadzu (Kyoto, Japan) system consisting of a column oven (model CTO-10ASVP), a UV–visible diode-array detector (model SPD-M10 Avp), a degasser (model DGU 14A), and a liquid chromatography pump (model LC-10AT-VP); Shimadzu software was used to calculate peak areas. The sample (20 µL) was injected into the HPLC with a syringe (Hamilton, Reno, NV, USA). The HPLC column used was a reversed-phase Discovery C₁₈ (150 mm × 4.6 mm, 5 µm; #504955) from Supelco (Bellefonte, PA, USA).

Reagents

Methanol (HPLC grade) and K₂HPO₄ (extra pure) were obtained from Merck (Darmstadt, Germany). Water used in all the experiments was

doubly distilled and deionised. The vitamin standards (ascorbic acid, niacin, pantothenic acid, pyridoxine, thiamine, folic acid, and riboflavin) were of analytical-reagent grade from Sigma (Sigma–Aldrich, Deisenhofen-Germany) and were not further purified. Stock and standard solutions of water-soluble vitamins were prepared in mobile phase. Five different concentrations of each standard were used to prepare the calibration plot. These solutions were sonicated and stored in dark glass flasks, to protect them from light, and kept under refrigeration. A calibration plot was prepared for each vitamin. Correlation coefficients for ascorbic acid, niacin, pantothenic acid, pyridoxine, thiamine, folic acid, and riboflavin on the basis of plots of concentration ($\mu\text{g mL}^{-1}$) against peak area (mAU) were found to be >0.999 .

Sample Preparation (Solid-Phase Extraction, SPE)

Tarhana consists of many components that cause chromatographic interferences with vitamins. For this reason the sample treatment proposed consists of SPE with Sep-Pak C₁₈ (500 mg) cartridges that enable separation of water-soluble vitamins and remove most of the interfering components. Four parts of deionised water (20 g) were added into one part of tarhana (5 g) (dilution factor, F , = 5). The mixture was homogenized using a homogenizer at medium speed for 1 min. The homogenized samples were centrifuged for 10 min at 14×10^3g (Sigma, Bioblock Scientific 2-16). The SPE method of Cho et al. [7] was used for the extraction of water-soluble vitamins. The stationary phase was flushed with 10 mL methanol and 10 mL water adjusted to pH 4.2 to activate the stationary phase. Homogenized and centrifuged tarhana (10 mL) was the loaded. Acidified water was prepared by adding a 0.005 M HCl solution drop by drop with stirring until the pH reached a predetermined value. The sample was eluted with 5 mL water (pH 4.2) then 10 mL methanol at a flow rate of 1 mL min^{-1} . The eluent was collected in a bottle and evaporated to dryness. The residue was dissolved in mobile phase. Before HPLC analysis all samples were filtered through $0.45 \mu\text{m}$ pore size FP 30/45 CA-S filters (Schleicher and Schuell, Darmstadt, Germany) at 7 bar max. Samples (20 μL) of solutions of the water-soluble vitamins were injected into the HPLC column.

Methods

The column eluate was monitored with a photodiode-array detector at 265 nm for vitamin C, 234 nm for thiamine, 266 nm for riboflavin, 324 nm for pyridoxine, 282 nm for folic acid, 204 nm for biotin, 261 nm for niacin, and 204 nm for pantothenic acid. The mobile phase was filtered

through a 0.45- μm membrane and degassed by sonication before use. The mobile phase was 0.1 mol L⁻¹ KH₂PO₄ (pH 7)–methanol, 90:10. The flow-rate was 0.7 mL min⁻¹. The column was operated at room temperature (25°C). Chromatographic peak data were integrated up to 39 min. Identification of compounds was achieved by comparing their retention times and UV spectra with those of standards stored in a data bank. Concentrations of the water-soluble vitamins were calculated from integrated areas of the sample and the corresponding standards.

Recovery of Water-Soluble Vitamins

Tarhana samples containing known amounts of ascorbic acid, niacin, pantothenic acid, pyridoxine, thiamine, folic acid, and riboflavin were spiked with two addition levels of standard ascorbic acid, niacin, pantothenic acid, pyridoxine, thiamine, folic acid, and riboflavin to determine the recovery. Six determinations were performed for each level of addition.

RESULTS AND DISCUSSION

The isocratic separation published by Supelco [10] was modified by increasing the concentration of KH₂PO₄ from 0.05 mol L⁻¹ to 0.1 mol L⁻¹, reducing the solvent flow rate from 1.0 to 0.7 mL min⁻¹, reducing the column temperature from 35 to 25°C and setting detection at 265 nm for vitamin C, 234 nm for thiamine, 266 nm for riboflavin, 324 nm for pyridoxine, 282 nm for folic acid, 204 nm for biotin, 261 nm for niacin, and 204 nm for pantothenic acid. In addition, KH₂PO₄ content was reduced from 99% to 90%, and so methanol content was increased from 1% to 10%.

To the best of our knowledge there are no published research studies about HPLC determination of water-soluble vitamins in tarhana. We analyzed tarhana samples using a Discovery C-18 reversed phase HPLC column, 15 cm \times 4.6 mm i.d., 5 μm particles, to determine the water-soluble vitamins. Because each of these water-soluble vitamins has its own maximum absorbance at a different wavelength, the detection wavelengths were set at the wavelength of maximum absorption for each vitamin to obtain higher sensitivity.

For separation and determination of water-soluble vitamins in tarhana, the best results were obtained with 0.1 mol L⁻¹ KH₂PO₄ (pH 7)–methanol mobile phases. When the proportion of methanol was more than 10%, the vitamins ascorbic acid, niacin, pantothenic acid, pyridoxine, thiamine, and folic acid eluted in less than 14 min (within 5 min) but resolu-

tion was very poor. In contrast, riboflavin eluted at 32 min and resolution of riboflavin was very good only when the proportion of methanol was more than 10%. We used a mobile phase containing 10% methanol, which increased the total analysis time but enabled better resolution than other compositions. Also, riboflavin was detected when the mobile phase contained 10% methanol. Thus, only riboflavin can be detected in tarhana samples when the proportion of methanol was more than 10%. On the other hand, ascorbic acid, niacin, pantothenic acid, pyridoxine, thiamine, folic acid, and riboflavin eluted and were detected well when the mobile phase contained 10% methanol.

The pH of the KH_2PO_4 was a critical factor for separation of the vitamins. KH_2PO_4 at pH from 3 to 7 was studied and all water-soluble vitamins were well resolved only at pH 7.0. When the pH of KH_2PO_4 was different from 7, however, the resolution was poor because ascorbic acid, niacin, pantothenic acid, pyridoxine, thiamine, and folic acid eluted within 5.0 min.

Changing the temperature from 20 to 35°C reduced the retention times slightly but had no effect on the quantitative results.

Figure 1 shows the chromatogram obtained from tarhana by use of KH_2PO_4 (pH 7)–methanol, 80:20, as the mobile phase. Resolution is poor

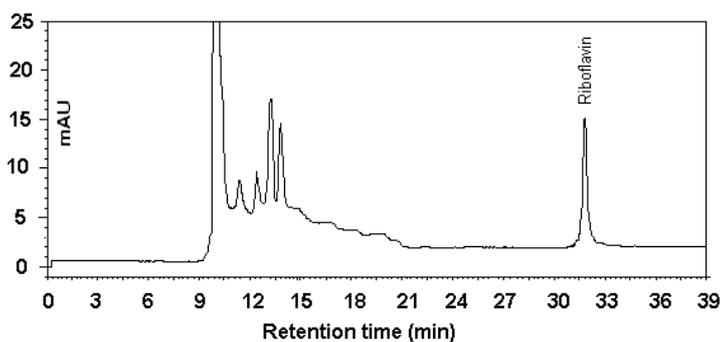


Fig. 1

Separation of water-soluble vitamins by isocratic elution with KH_2PO_4 (pH 7)–methanol, 80:20, as mobile phase.

except for riboflavin when the methanol content of the mobile phase increased from 10% to 20%. Figure 2 shows a typical chromatogram obtained from a tarhana sample when the 0.1 mol L^{-1} KH_2PO_4 (pH 7)–methanol, 90:10, was used as mobile phase. As shown in Fig. 2, good separation can be achieved in 37 min. It is apparent from the chromatogram that as-

corbic acid, niacin, pantothenic acid, pyridoxine, thiamine, folic acid, and riboflavin are separated well. Identification of the compounds was achieved by comparing their retention times and UV spectra with those of standards stored in a data bank.

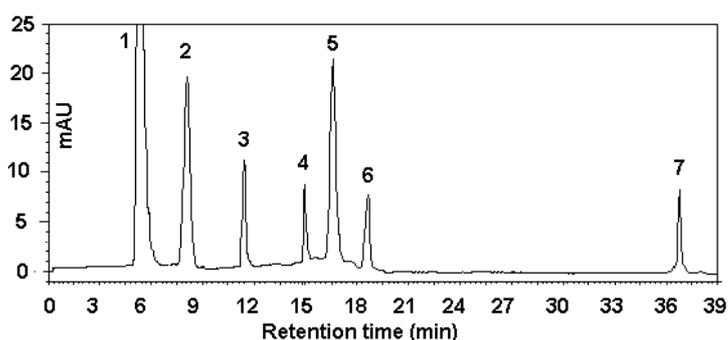


Fig. 2

Separation of water-soluble vitamins by isocratic elution with KH_2PO_4 (pH 7)–methanol, 90:10, as mobile phase. Peaks: 1. ascorbic acid, 2. niacin, 3. pantothenic acid, 4. pyridoxine, 5. thiamine, 6. folic acid, 7. riboflavin

Analytical Characteristics of the HPLC Method

Linearity and Detection Limits

As seen from Table I, an r value >0.9985 was obtained for all vitamins. Coefficients of determination (r^2) were greater than 99.67% for all vitamins. The detection limits ($S/N = 3$) [11] ranged from 0.1 to 0.5 mg L^{-1} .

Table I

Linearity of standard curves and detection limits for the seven water-soluble vitamins

Vitamin	Linear range (mg L^{-1})	R	r^2	Detection limit (mg L^{-1})
Ascorbic acid	5.0–200.0	0.9992	99.90	0.1
Niacin	10.0–200.0	0.9996	99.93	0.1
Panthenic acid	1.0–30.0	0.9989	99.83	0.5
Pyridoxine	0.5–30.0	0.9990	99.82	0.1
Thiamine	1.0–50.0	0.9985	99.69	0.5
Folic acid	0.5–20.0	0.9992	99.67	0.1
Riboflavin	1.0–40.0	0.9998	99.94	0.2

Precision

Six determinations of the same tarhana sample were performed using the same reagents and apparatus to evaluate the method precision in tarhana. The precision of the method for determination of seven water-soluble vitamins in tarhana is shown in Table II.

Table II

Precision of method for determination of water-soluble vitamins in tarhana

Vitamin	Mean \pm S.D. ^a (mg kg ⁻¹)	R.S.D. (%)
Ascorbic acid	17.8 \pm 0.67	2.63
Niacin	12.4 \pm 0.54	3.12
Panthenic acid	5.3 \pm 0.28	3.54
Pyridoxine	0.38 \pm 0.08	5.62
Thiamine	4.7 \pm 0.20	3.30
Folic acid	0.40 \pm 0.09	4.98
Riboflavin	1.8 \pm 0.17	4.41

^aMean \pm standard deviation

Recovery

Recovery was tested by the standard addition procedure. Two addition levels were used for each water-soluble vitamin in tarhana samples (Table III). Six determinations were performed for each addition level. Mean recoveries obtained were always satisfactory – higher than 99% for niacin, higher than 98% for ascorbic acid, pantothenic acid, pyridoxine and thiamine, higher than 97% for riboflavin, and higher than 96% for folic acid. The average recoveries of ascorbic acid, niacin, pantothenic acid, pyridoxine, thiamine, folic acid, and riboflavin in tarhana were 98.77%, 99.40%, 98.28%, 98.74%, 98.40%, 96.51%, and 97.96% respectively, for added levels of two different concentrations.

CONCLUSION

A new method is proposed for separation and quantification of seven water-soluble vitamins – ascorbic acid, niacin, pantothenic acid (vitamin B₅), pyridoxine (vitamin B₆), thiamine (vitamin B₁), folic acid, and riboflavin (vitamin B₂). SPE proved an effective tool for isolation of the se-

Table III

Recovery of method for determination of water-soluble vitamins in tarhana

Vitamin	Initial content (mg kg ⁻¹)	Content after addition (mg kg ⁻¹)		Recovery (%)	
		Level I ^a	Level II ^b	Mean ± S.D. ^c	R.S.D. (%)
Ascorbic acid	17.8	37.22 ± 0.28	57.36 ± 0.30	98.77 ± 0.62	0.75
Niacin	12.4	22.34 ± 0.20	32.17 ± 0.19	99.40 ± 0.54	0.60
Panthenic acid	5.3	10.05 ± 0.16	15.11 ± 0.14	98.28 ± 0.60	0.74
Pyridoxine	0.38	0.86 ± 0.08	1.37 ± 0.10	98.74 ± 0.71	1.1
Thiamine	4.7	9.49 ± 0.12	14.67 ± 0.13	98.40 ± 0.52	0.76
Folic acid	0.40	0.86 ± 0.05	1.36 ± 0.10	96.51 ± 1.08	1.33
Riboflavin	1.8	3.72 ± 0.10	5.69 ± 0.11	97.96 ± 0.85	0.96

^a20 mg for ascorbic acid, 10 mg for niacin, 5 mg for pantothenic acid and thiamine, 0.5 mg for pyridoxine and folic acid and 2 mg for riboflavin

^b40 mg for ascorbic acid, 20 mg for niacin, 10 mg for pantothenic acid and thiamine, 1 mg for pyridoxine and folic acid and 4 mg for riboflavin

^cMean ± standard deviation

ven water-soluble vitamins and HPLC enabled rapid, accurate, and reliable determination of the compounds with recoveries ranging from 96.51 to 99.40%. The isocratic mobile phase used for the vitamins resulted in high detection limits and good resolution within a minimum analysis time of 37 min.

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