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Simultaneous Determination of Water and Fat-Soluble Vitamins in Pekmez Samples by High Performance Liquid Chromatography Coupled with Diode Array Detection

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In the present work, a RP-HPLC procedure has been applied for the determination of water-soluble vitamins (riboflavin, nicotinamide, pantothenic acid, pyridoxine hydrochloride, pyridoxal, folic acid, ascorbic acid) and fat-soluble vitamins (*trans*-retinol, α-tocopherol) in pekmez samples, a tradional Turkish concentrate fruit food. The sample treatment proposed consists of a solid-phase extraction with Sep-Pak C_{18} cartridges that allow the seperation of fat-soluble vitamins, which were retained on the sorbent, from water-soluble vitamins. After isolation of the two fractions, the water-soluble vitamins were analyzed by HPLC on an ACE 5 C_{18} (250 × 4.6 mm, 5 μm) analytical column using 0.1 mol L^{-1} KH_2PO_4 (pH 7.0)-CH_3OH as mobile phase. The chromatographic analysis of the fat-soluble vitamins was carried out after their sequential elution with CH_3OH-CCl_4 from C_{18} sorbent, on the above column. Afterwards, the fat-soluble vitamins were analyzed on an ACE 5 C_{18} analytical with isocratic mobile phase consisting of CH_3OH-CH_3CN at a flow-rate of 2 mL min^{-1}. Identification of compounds was achieved by comparing their retention times and UV spectra with those of standards stored in a data bank. The accuracy of the method was tested obtaining an average recovery ranging between 91 and 110 %.

Key Words: Water-soluble vitamins, Fat-soluble vitamins, HPLC, Pekmez.

INTRODUCTION

Pekmez is the one of the most common and traditional food product in Turkey and produced in almost every region of the country[1]. Fruits containing high sugar like mulberry, plum, apple and mainly grape are used as raw material in the production of pekmez[2]. Pekmez containing no additives is not only a very nutritious and natural famous food but also a good energy and carbohydrate source due to its sugar content up to 50-80 %. The average energy value[3] is *ca.* 280 kcal/100 g.

Vitamins are a diverse group of compounds, both chemically and analytically, because they comprise a range of biomolecules whose common properties residue

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solely in the fact that they are essential dietary components. These compounds are needed in relatively small amounts to sustain life and good health[4]. Furthermore, a significant association between plasma L-ascorbic acid and initial non-verbal intelligence quotient (IQ) has been found in the boys[5].

These compounds can be classified in two main groups: water-soluble and fat-soluble vitamins. Among water-soluble vitamins, the vitamin B group including thiamine (vitamin B_1), pyridoxine (vitamin B_6) and cyanocobalamine (vitamin B_{12}) are the most important. Each member of the B-complex has a unique structure and performs unique functions in the human body[6]. The main source of vitamins for human beings is from foods[7]. The loss of the naturally present vitamins in foods can be related to the intensity of food processing and to the duration of food storage.

Increasing interest in good eating habits in human and also animals, has meant greater awareness of the vital role that vitamins play in growth and health. In addition, the presence of fruits and vegetables in the daily diet and the consume of vitamin-supplemented and preserved foods, has substantially increased. Vitamins of the B group are easily excreted from human body with biological fluids. Therefore, they cannot be accumulated and the hypervitaminosis risk is practically absent while the deficiency, especially of vitamin B_{12} in the elderly, is more common[8,9]. In addition, together with a possible loss through chemical reactions, vitamins might be leached during storage and processing of food[10]. In this sense, it is extremely important to have available preparations to replace the possible lack of the vitamins in daily diet which is why multi-vitamin pharmaceuticals are becoming widely employed[6]. Consuming too much or too little of these essential micronutrients can harm health, so estimates of intake must be accurate. Dietary supplements are widely consumed and frequently measured in various types of nutrition studies. Information on the vitamin contents of supplements is needed for sound estimates of exposures to them, to calculate total nutrient intakes and to understand the associations between exposures, health and diseases[11]. These facts, together with the introduction of food labelling regulations, lead to a need for very powerful analytical separation techniques for the quality control of these complex preparations, as well as of foods, beverages and medications. This has stimulated research on accurate and efficient analytical methods for the determination of vitamins which is problematic because of their instability and the complexity of the matrices in which they are usually analyzed[12].

Traditional methods for vitamin determination require the analysis of each vitamin individually by widely differing physical, biological and chemical methods, including colorimetric, fluorometric, spectrophotometric and titrimetric techniques[13]. The choice of method usually depends on the accuracy and sensitivity required and the interferences encountered in the sample matrix[14].

Nowadays, several papers have been published concerning the separation and quantification of vitamins by more simple methodologies. High-performance liquid chromatographic (HPLC) techniques allow rapid separation and quantification of

vitamins in food using ion-exchange columns and UV or flurometric detection. HPLC provides rapid, sensitive and accurate methods for vitamin determination and have advantages of solvent economy, easy coupling with other techniques and that small amounts of sample are required[15-17].

In addition, during the last decade there has been an increasing interest for the simultaneous determination of vitamin. Thus, methods such as capillary electrophoresis[18], micellar electrokinetic chromatography[19], micellar liquid chromatography[20] and liquid chromatography (LC) have been developed. Amongst these methods, liquid chromatography appears promising due to the improvements of both stationary phases and chromatography equipments.

In this paper, a simultaneous determination of water-soluble vitamins (riboflavin, nicotinamide, pantothenic acid, pyridoxine hydrochloride, pyridoxal, folic acid, ascorbic acid) and fat-soluble vitamins (*trans*-retinol, α-tocopherol) in pekmez samples is reported. Solid-phase extraction is applied as an isolation method of the two fractions of the vitamins (water-soluble and fat-soluble) in case both of them exist in the same sample. In addition, in this research, it was aimed to determine the levels of vitamins (water and fat-soluble) in the marketed pekmez products.

EXPERIMENTAL

Standards of water-soluble vitamins (riboflavin, nicotinamide, pantothenic acid, pyridoxine hydrochloride, folic acid, L-ascorbic acid) and fat-soluble vitamins (*trans*-retinol, α-tocopherol) were purchased from sigma Aldrich (St. Louis, MO, USA). Potassium dihydrogen phosphate of analytical grade purity chemical, HPLC-grade acetonitrile and methanol were from Merck (Darmstsadt, Germany). The vitamin stock standard solutions at 100 μg mL^{-1} were prepared in mobile phase or methanol (fat-soluble vitamins) and stored in the darkness at 4 ºC. The working standard solutions were prepared daily mixing of the stock standard solutions in appropriate proportions and diluting with mobile phase or methanol, if necessary. All solutions were filtered through a 0.45 μm Teflon membrane filters (Agilent Technology) prior to HPLC analyses.

An Agilent 1100 liquid chromatographic system (Germany) was equipped with a vacuum degasser (G1379A, serial # JP 40717565), a qual pump (G1311A, serial # DE 43628803), an auto sampler (G1313A, serial # DE 43626922) a column thermostat (G1315B, serial # DE 43623145))and a UV-Vis diode array dedector (G1315B, serial # DE 43623145) working at 191-949 nm.

Chromatographic conditions

Water-soluble vitamins: A reversed-phase ACE 5 C_{18} (250 × 4.6 mm, 5 μm) column was used for the separation of water-soluble vitamins at room temperature. The mobile phase of the HPLC system consisted of a 0.1 mol L^{-1} KH_2PO_4 at pH 7.0 (solvent A)-methanol (solvent B). Analyses were carried out isocratically (90:10) at room temperature at a flow rate 0.9 mL min^{-1}. The column eluate was monitored with a UV-Vis diode-array dedector at 265 nm for ascorbic acid, 266 nm for riboflavin,

324 nm for pyridoxine, 282 nm for folic acid, 261 nm for niacin and 204 nm for pantothenic acid. The total run time required was less than 10 min. Identification of compound was achieved by comparing their retention times and UV spectra with those of standards stored in a data bank. Concentrations of water-soluble vitamins were calculated from integrated areas of the sample and the corresponding standards.

Fat-soluble vitamins: A reversed-phase ACE 5 C_{18} (250 × 4.6 mm, 5 μm) column was used for the separation of fat-soluble vitamins (*trans*-retinol, α-tocopherol) at room temperature. The mobile phase of the HPLC system consisted of a CH_3OH (solvent A)-CH_3CN (solvent B). Analyses were carried out isocratically (95:5, v/v) at room temperature at a flow rate 2.0 mL min^{-1} and with a 20 μL loap. A total run time of 10 min is necessary for the elution of the two vitamins.

Sample preparation (solid phase extraction, SPE): The SPE strategy generally comprises the isolation (and concentration) of the analytes from a complex matrix by adsorption onto an appropriate sorbent, the removal of interfering impurities by washing with a suitable solvent system system and then the selective recovery of the retained analytes with a modified solvent system of suitable elution strength. If necessary, this process can be modified by selection of sorbent and solvent systems, so that the interfering components are retained by a sorbent and the analytes are then recovered in the eluate.

The sample is dissolved in a polar solvent and then the solution is passed through the reversed-phase cartridge; polar organic compounds will pass through the sorbent, while less polar constituents will be retained. A solvent of intermediate polarity is used to wash off the material that is more polar than the one retained. Then, this elueted with a less polar solvent, leaving the hydrophobic material retained on the sorbent[6].

Pekmez consists of many components that cause chromatographic interferences with vitamins. For this reason, the sample treatment proposed consists of SPE with Sep-Pak C_{18} (500 mg) cartridges that enable separation of water-soluble vitamins from fat-soluble vitamins and remove most of the interfering components.

Pekmez samples were weighed (5 g), placed in a centrifuge tube and dissolved in 20 g of deionized water. The mixtures were homogenized using a homogenizer (Poltron PT-MR 2100) at medium speed for 1 min. The homogenized samples were centrifuged for 10 min at 14×10^3 g (Elektromag M 4812 M). The modified SPE method[21] was used for the separation between the two groups of vitamins. Solid-phase extraction cartridges (SPE C_{18} AR 3 mL) were conditioned immediately prior to use with 1000 μL of methanol followed by 1000 μL of water adjusted of pH 4.2. Then, a 500 μL aliquot of sample was applied to the column. The fat-soluble vitamins passed unretained. The water-soluble vitamins were collected into volumetric flasks and evaporated to dryness. The residue was dissolved in mobile phase (0.1 mol L^{-1} KH_2PO_4 pH 7.0-CH_3OH, 90:10). Then, the column was washed with one column volume of deionized water (pH 4.2) and one column volume of methanol in order to remove all the water-soluble vitamins from the column. The column was dried

and the fat-soluble vitamins were eluted by applying 2 mL of methanol followed by 2 mL of chloroform collecting them into 10 mL volumetric flasks and diluted to volume with methanol. All the solutions were allowed to gravity flow and filtered through the 0.45 μm teflon membrane filters and stored below 4 ºC, protected from light prior to HPLC analyses before injection on the reverse-phase column.

RESULTS AND DISCUSSION

A simultaneous analysis of water-soluble and fat-soluble vitamins by chromatographic methods is difficult due to their completely different physical and chemical properties[22]. In the present paper, a simultaneous determination of water-soluble vitamins (ascorbic acid, nicotinic acid, folic acid, riboflavin, pantothenic acid, pyridoxine) and fat-soluble vitamins (*trans*-retinol, α-tocopherol) in pekmez samples are attempted. Solid-phase extraction is used as a pre-treatment technique of biological matrices and also as an isolation method of the two fractions of vitamins (water- and fat-soluble vitamins), in case the two fractions of vitamins exist in the same sample. Afterwards, we attempted to analyze water- and fat-soluble vitamins by high-performance liquid chromatography coupled with a diode array dedector (HPLC-DAD).

Water-soluble vitamins: Separation of water-soluble vitamins accomplished within 10 min in present method. Standards of the vitamins were chromatographed separately in order to determine the retention time for each of them. A standard mixture of vitamins was then analyzed as a mixture and chromatographic conditions were optimized to maximise peak resolution. The obtained chromatogram of a standard mixture is shown in Fig. 1a. The results of recovery test for the solid phase extraction are assembled in Table-1. The recovery rates are well over 90 % in general.

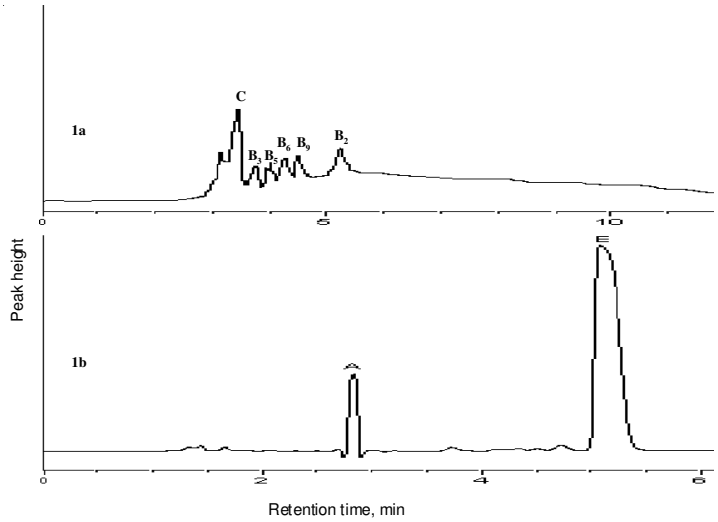

Fig. 1. HPLC chromatogram of standard vitamin mixture (1a, water-soluble vitamins; 1b, fat-soluble vitamins)

TABLE-1
RECOVERY RATES OF WATER-SOLUBLE VITAMINS IN SPIKED PEKMEZ BY SOLID PHASE EXTRACTION AT THREE DIFFERENT SOLUTE CONCENTRATIONS BASED ON COMPARISON OF PEAK AREAS BETWEEN THE EXTRACT AND STANDARD*,**

Standard	Vitamin	Concentration (mg L^{-1})	Recovery (%)
1	B_2	5.00	103 (3.8)
	B_3	5.00	99 (3.6)
	B_5	5.00	101 (2.6)
	B_6	5.00	97 (1.9)
	B_9	5.00	105 (4.6)
	C	5.00	96 (4.6)
2	B_2	25.0	96 (2.1)
	B_3	25.0	101 (4.6)
	B_5	25.0	91 (1.1)
	B_6	25.0	94 (1.9)
	B_9	25.0	96 (4.7)
	C	25.0	93 (3.1)
3	B_2	50.0	98 (3.2)
	B_3	50.0	92 (2.8)
	B_5	50.0	94 (3.3)
	B_6	50.0	97 (2.9)
	B_9	50.0	98 (4.4)
	C	50.0	91 (5.5)

*Standard deviations are in parentheses.
**Four replicate measurements for each concentration.

Scan analysis of standard vitamins was performed to check the optimum conditions for the detection. Wavelengths were changed according to the elution time of each vitamin, as in shown in Table-2. The mobile phase used is based on the phases on the phases described by Ekinci *et al.*[23] for determination of water-soluble vitamins in pekmez samples.

TABLE-2
PROGRAM OF WAVELENGTH CHANGES DURING ELUTION TIME FOR WATER-SOLUBLE VITAMIN DETERMINATION IN PEKMEZ SAMPLES

Vitamin	Time (min)	λ_{max} (nm)
Ascorbic acid	0.0-3.3	265
Nicotinamide	3.5-3.8	282
Pantothenic acid	3.9-4.0	204
Pyridoxine	4.1-4.3	324
Folic acid	4.4-4.5	282
Riboflavin	4.8-5.2	266

Fig. 1 shows a typical chromatogram obtained from pekmez sample when the 0.1 mol L^{-1} KH_2PO_4 (pH 7)-CH_3OH, 90:10, was used as mobile phase. As shown in Fig. 1, good separation can be achieved in 10 min. It is apparent from the chro-

matogram that ascorbic acid, niacin, pantothenic acid, pyridoxine, folic acid and riboflavin are separated well. Integration of the separated peak areas was done with the integrator or the computer programme and each vitamin was determined using the peak area-concentration relationship obtained in the standardization step. The sample peaks were identified by comparing both the relative retention times and the UV-Vis spectrum of each one with those of the standard reference vitamins. These results are summarized in Table-3.

TABLE-3
FOUND CONCENTRATIONS OF WATER-SOLUBLE VITAMINS AND FAT SOLUBLE VITAMINS PEKMEZ SAMPLES (mg Kg^{-1}, n = 3)

Fruit molasses (pekmez)	C	B_2	B_3	B_5	B_6	B_9	A	E
Mulberry (home made)	78±3	45±4	nd	20±0.5	nd	24±4	nd	nd
Mulberry (Sitoglu plant)	77±5	27±4	nd	23±0.2	10±0.2	24±4	nd	nd
Apricot (Sitoglu plant)	94±4	nd	22±1	5±0.5	nd	15±2	102±2	nd
Grape (Sitoglu plant)	30±2	nd	31±0.5	60±0.3	nd	19±1	99±3	nd
Grape (Koska plant)	34±2	15±0.3	12±0.3	6±0.3	nd	12±1	58±1	nd
Date (Sitoglu plant)	134±5	nd	45±0.4	nd	50±3	24±3	nd	nd
Juniper (Sitoglu plant)	150±5	86±2	64±1	86±3	20±1	72±4	nd	nd
Juniper (Taç plant)	56±2	nd	27±1	43±3	6±0.5	25±2	nd	nd
Harnup (Sitoglu plant)	70±4	nd	25±1	10±0.7	6±0.2	10±1	64±2	6±1
Harnup (Taç plant)	113±3	nd	66±1	25±0.2	17±1	16±2	41±0.3	nd
White zile (Özkale plant)	36±2	nd	17±1	14±0.3	nd	20±2	5±0.3	nd

nd: not detected.

Fat-soluble vitamins: The same procedure described for the calibration of the method for the water-soluble vitamins was followed for fat-soluble vitamins as well. Separation of the fat soluble vitamins was carried out by isocratically elution with methanol and acetonitrile (95:5, v/v). CH_3OH-CH_3CN (95:5) at 285 nm gave good resolution of the vitamin A (t_R ~ 2.8 min) and E (t_R ~ 5 min) in less than 10 min as can be seen in Fig. 1b. The sample peaks were identified by comparing both the relative retention times and the UV-Vis spectrum of each one with those of the standard reference vitamins. The fat-soluble vitamins levels of the pekmez samples are shown in Table-3.

Analytical characteristic of the HPLC method

Repeatability: Complete analysis was performed in triplicate on water-soluble vitamins and fat soluble vitamins to calculate the average deviations as a measure of chromatographic reproducibility. The relative standard deviations obtained in the analysis of the vitamins are presented in Table-4.

Linear range: Linearity was obtained in the range of the standard concentration for each vitamin (Table-5). A series of five solutions at low and high concentration levels were prepared, each solution was injected three times and the regression was calculated by the method of least-squares. Peak areas were calculated and results interpolated on the calibration graph for each vitamin.

TABLE-4
QUANTIFICATION OF WATER-SOLUBLE AND FAT-SOLUBLE VITAMINS

Vitamin	Vitamin content (mg L^{-1})	Mean* (mg L^{-1})	SD (mg L^{-1})	RSD (%)	Recovery (%)
C	80.00	72.80	3.27	4.50	91
B_2	43.00	43.25	2.62	6.06	101
B_3	35.00	34.60	0.80	2.32	99
B_5	30.00	29.19	0.96	3.30	97
B_6	18.00	18.17	0.98	5.40	101
B_9	26.00	25.85	2.36	9.13	99
A	62.00	61.52	1.13	1.84	99
E	11.00	10.55	0.66	6.26	96

*Complete analysis was performed in triplicate.

TABLE-5
LINEARITY AND DETECTION LIMITS FOR
WATER-SOLUBLE AND FAT-SOLUBLE VITAMINS

Vitamin	Linear range (mg L^{-1})	R^2	Detection limit (mg L^{-1})
C	8.05-54.505	0.9992	4.78
B_2	6.34-30.500	0.9997	5.13
B_3	6.82-17.430	0.9998	4.84
B_5	5.98-59.560	0.9982	5.04
B_6	5.64-52.640	0.9994	4.86
B_9	9.07-76.580	0.9997	5.26
A	5.09-104.70	0.9987	4.45
E	5.28-17.360	0.9996	4.10

Limits of detection: The detection limits were assessed using internal standards. Those are considered to be the quantities that are producing a signal of peak height three times the size of background noise. The obtained values are shown in Table-5.

Precision: Method precision was determined by measuring repeatability (between-day precision or time-different intermediate precision) for each vitamin at the same concentration levels using the same reagents and apparatus. The variation coefficients varied from 1.8 to 9.1 %.

Conclusion

Six water-soluble vitamins (riboflavin, nicotinamide,pantothenic acid, pyridoxine hydrochloride, pyridoxal, folic acid, ascorbic acid) and two fat-soluble vitamins (*trans*-retinol, α-tocopherol) were separated into two fractions by means of solid-phase extraction and subsequently analyzed by HPLC. Solid-phase extraction proved to be an effective tool for performing adequate separation of the two groups of vitamins while HPLC provided a fast, accurate and reliable method for their determination with recoveries ranging from 91 to 101 %. The developed method was further applied to pekmez samples. The binary eluent system used for water-soluble and the isocratic eluent system used for fat-soluble vitamins provide good separation,

high selectivity and resolution within a minimum analysis time of 10 min for the fraction of water-soluble vitamins and in less than 10 min for the fraction of fat soluble vitamins. The simplicity of the procedure should make it highly desirable for quality control of multi-vitamin products in food industries.

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