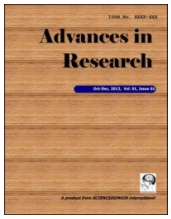

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The Simultaneous Determination of Some Water-Soluble Vitamins in Gum of *Acacia nilotica* by High Performance Liquid Chromatography

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

A rapid, simple and precise method by HPLC (high performance liquid chromatography) has been developed for simultaneous determination of water-soluble vitamins as thiamine(B_1), nicotinamide(B_3), panthotenic(B_5), pyridoxine(B_6) and biotin(B_8) in gum of *Acacia nilotica* using enzymatic hydrolysis. The method uses a C_{18} column (4.6×150 mm, 5µm). Mobile phase such as methanol 0.1M, sodium dihydrogen phosphate (pH = 2.5), (10:90 v/v) is found most suitable for rapid separation and identification of this water –soluble vitamins. Good linearity was observed between the concentration of analytes and peak area (r = 0.9999). Each vitamin was quantitatively determined at its maximum wavelength. Recovery percentages ranged from 97% to 99%.

Keywords: Water; soluble vitamins; gum, Acacia nilotica; HPLC.

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1. INTRODUCTION

Acacia gums have a complex and branched structure, which makes them have good adhesive and cohesive properties. These properties are useful in pharmaceutical preparations. They are used as dental and other adhesives and as bulk laxatives. These hydrophilic polymers are useful as tablet binders, emulsifiers, suspending agents, gelling agents, stabilizers, thickeners, protective colloids and suspending agents keeping tablets [1]. They can also be used as tablet disintegrants [2]. Their adhesive property could be used in the apparatus of colostomies and also in fixing dental prosthesis [3].

For internal use, they help in the preparation of medicines to soothe coughs, diarrhea, dysentery and hemorrhages; for external use, they calm inflammations, so the presence of vitamins in *Acacia* gums is very important since vitamins are essential for human health. [4] As far as we know, other researchers have not reported the presence or absence of vitamins B_1, B_3, B_5, B_6, B_8 in *Acacia* gum, particularly that of *Acacia nilotica*.

These vitamins are very important for the production of energy (B_1), normal growth and development (B_3), the regulation of neurotransmitters (messengers of nerve impulses) (B_5), physical balance and regulation of blood sugar (B_6) and the processing of several products such as glucose and fatty acids (B_8) [5].

Due to the nutritional importance of these vitamins, microbiological assay and several analytical methodologies have been developed for the determination of these substances in food, pharmaceutical supplements and biological fluids [6-10]. There are many analytical methods for performing the assay of vitamins in food, pharmaceutical and physiological specimens such as spectrophotometry [6,11-13] spectrophotoflurorimetry [7], voltammetry [8], the gas chromatography [14-17] and high performance liquid chromatography [18-28].

Normally, it is necessary to determine more than one vitamin; the analytical method must be able to determine multiple components in complex samples, which can lead to interference in chemical analysis.

The aim of this study is to develop a rapid and reliable technique for the simultaneous determination of five water- soluble vitamins (B_1, B_3, B_5, B_6, and B_8) in gum of *Acacia nilotica* by HPLC using the enzymatic hydrolysis.

2. EXPERIMENTALS

2.1 Reagents and Chemicals

Methanol was of HPLC grade. Other chemicals as sodium dihydrogen phosphate (NaH_2PO_4), sodium acetate, sulfuric acid (H_2SO_4) and acetic acid ($C_2H_4O_2$) (Sigma) were of reagent grade. Purified water was obtained from a Millipore Milli-Q system.

Standards of thiamine, nicotinamide, pantothenic, pyridoxine and biotin were purchased from Sigma.

Taka-diastase enzyme from *Aspergillus oryzae* powder, slightly beige was obtained from Sigma. All chemicals and reagents used are of HPLC and were used without further purification. Also, all solutions were filtered through a 0.45 µm membrane (Millipore), protected from light and stored at 4°C.

The mobile phase of the HPLC system was comprised of pure methanol and sodium dihydrogen phosphate NaH_2PO_4 (10:90 v/v).

2.2 Chromatographic Conditions

The HPLC system (Agilent) was equipped with a pump type technology Agilent 1200 series, a vacuum degassing unit model G1322A, a UV-VIS spectrometer to 8 wavelengths, a fluorescence detector (G1321 Agilent 1200 Series), an analytical C_{18} column (Agilent) (4.6 × 150mm, 5µm), During the analysis the column was equilibrated at 30°C and a manual injector uses an injection valve sample seven lane Rheodyne 7725i. The chromatographic peaks were recorded and elaborated automatically by employing a computerized program 'Agilent ChemStation'.

The analyzes were performed by gradient elution of wavelength at room temperature, at a flow rate of 1 mL / min. The total execution time required is less than 20 min.

The program of wavelength changes during elution time for five vitamins determination in gum of *Acacia nilotica* shown in Table 2.

2.3 Standard Solutions

The vitamin stock solution: 100 mg / L were prepared by dissolving 10 mg of each standard in 100 mL of methanol in dark volumetric flasks. These solutions are stable hang at least one month when stored in the dark at 4°C.Working solutions were prepared from stock solutions by appropriate dilution with methanol and protected from light. The following Table 1 illustrates the calibration of the analytical method:

Table 1. Concentration of the standards used for plotting the calibration curve of five vitamins (B_1, B_3, B_5, B_6, B_8)

Vitamins	Concentrations (mg/L)
Thiamine B_1	1.0, 5.0, 10.0, 15.0, 20.0, 30.0
Nicotinamide B_3	1.0, 5.0, 10.0, 15.0, 20.0, 30.0
Pantothenic B_5	1.0, 5.0, 10.0, 15.0, 20.0, 30.0
Pyridoxine B_6	1.0, 5.0, 10.0, 15.0, 20.0, 30.0
Biotin B_8	1.0, 2.0 ,5.0 ,10.0 ,15.0, 20.0, 30.0

2.4 Sample Preparation

1g of gum *Acacia nilotica* which is a fine powder was accurately weighed in a 250 mL erlenmeyer flask, 10mL of sulfuric acid (1N) was added. The mixture was thoroughly shaken, after the pH was adjusted to 4.5 with sodium acetate (2.5M), then 500mg of the enzyme Taka-diastase stirring was added. The solution was incubated at 37°C and protected from light all night. The following content was filtered on 0.45µm filter. Finally, 20 µL of the extract was injected into the HPLC system for analysis.

3. RESULTS AND DISCUSSION

The determination of vitamins in gum of *Acacia nilotica* is a complex analytical problem for several reasons: gum of *Acacia nilotica* is a very complex matrix, vitamins that are micro constituents and vitamins are easily destroyed by strong acids or alkalis, which is why we find that the enzymatic hydrolysis is a good solution for these problems.

First, 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 is shown in Table 2.

Table 2. Program of wavelength changes during elution time for five water-soluble determinations in gum *Acacia nilotica*

Vitamins	Time (min)	Wavelengths (nm)
Biotin (B_8)	0.0 - 2.5	204
Nicotinamide (B_3)	2.6 - 3.8	261
Thiamine (B_1)	3.9 - 4.5	234
Pyridoxine (B_6)	4.6 - 5.0	275
Pantothénic acid (B_5)	5.1- 7.0	210

The mobile phase was composed of methanol and sodium dihydrogen phosphate NaH_2PO_4 (10:90) v/v) for the determination of vitamins B_1, B_3, B_5, B_6, and B_8 in gum *Acacia nilotica*. A study of pH and the proportion of methanol and NaH_2PO_4 were necessary to improve the resolution in the gum of *Acacia nilotica* formulae. When the proportion of methanol is 20%, vitamins are eluted in less than 5 min, but there is an overlap peak of certain vitamins. The pH of the mobile phase is extremely important for the separation of vitamins in order to overcome this problem, a decrease in the proportion of methanol by 10 %, which has the effect of providing a higher resolution but against party, the time of analysis.

A choice of pH = 2.5 implies that most vitamins are of molecular form since the pH is less than the peaks of all vitamins (B_1, B_3, B_5, B_6, B_8). Fig. 1 shows the chromatogram of vitamins B_1, B_3, B_8 in gum of *Acacia nilotica.*

We note from the figure that gum of *Acacia nilotica* contains a wide range of vitamin B_8. The peak of vitamin B_8 was detected at a retention time of about 2.2 to 2.4 min (Figs. 1 and 2), with minor variations on a daily basis due to temperature fluctuations in the laboratory [29]. No other peaks were observed at 204 nm.

All calculations prove that vitamin B_8 is in the order of 12,000 ppm. A part from vitamin B_8, there are vitamins B_1 and B_3 but with low levels. We can conclude that the method gives a good resolution of vitamin B_8.

3.1 Characteristic of the HPLC Method

The proposed method allows the resolution of various forms of vitamin B especially B_8 in gum of *Acacia nilotica* by HPLC with UV detection.

A reliable chromatographic assay requires an acceptable resolution, reasonable retention times and good peak symmetry. Accordingly, in

preliminary studies optimal chromatographic conditions were investigated in gradient elution system with varying wavelengths. The advantage of gradient elution is that the bandwidth can be nearly constant at both early and tardative analytes. Therefore an elution system of five wavelengths has been developed with a beneficial effect on the sensitivity of biotin.

Representative chromatograms with other chromatographic parameters are shown in Figs. 1, 2 and Table 3.

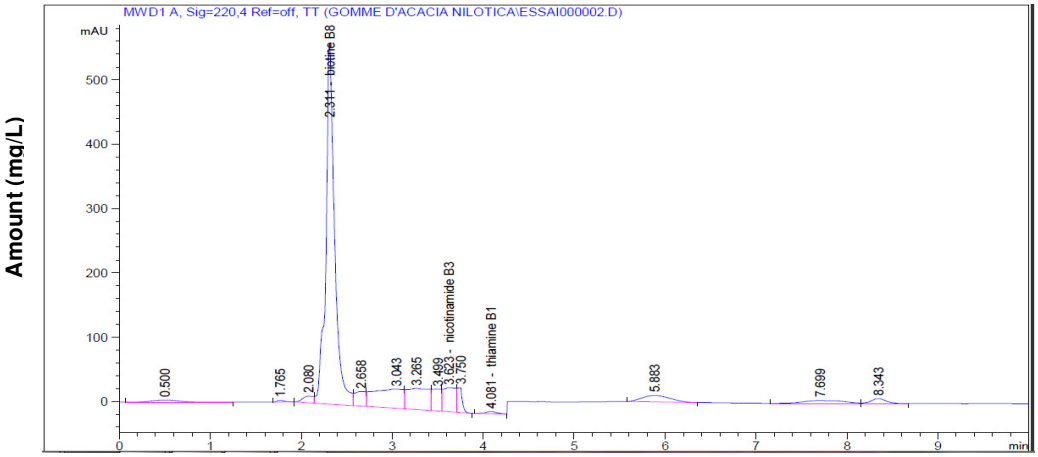

Fig. 1. Typical chromatograms of vitamins

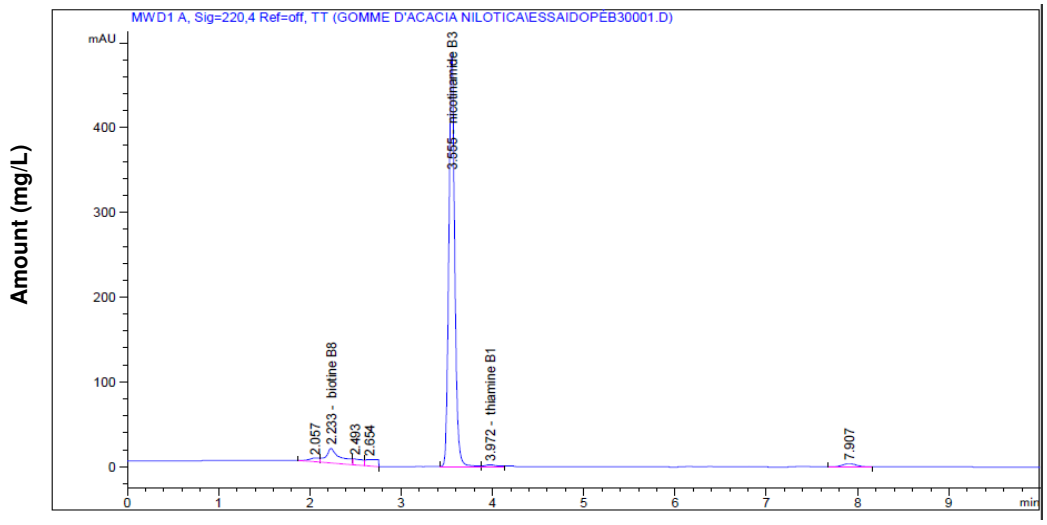

Fig. 2. Typical chromatograms of vitamins

Table 3. High performance liquid chromatographic parameters of the *Acacia gum* separation using the gradient elution system t_0 = tm = 0.56 min

Vitamins	Chromatographic parameters			
	T	t_R (min)	R_S	k'
Biotin B_8	2.306	3.117	1.0	2.412 (B3-B8)
Nicotinamide B_3	3.512	5.27	1.5	2.087 (B1-B3)
Thiamine B_1	4.242	6.575	1.9	3.872 (B1-B8)

t_R: retention time reduced; k': Retention factor; Rs: Resolution factor; T: The asymmetric peak

In practice, care must be taken to keep values k' inferior to 10 for a period of reasonable analysis, values between 2 and 5 are the correct values.

3.1.1 Linearity

Six working solutions were prepared for each analyte whose range is between 1 and 30 mg /L for B_1, B_3, B_5, B_6 and seven solutions between 1 and 30 mg/L for B_8.The analysis was performed in triplicate to determine the linearity of the assay. The regression lines were calculated by the method of least squares of the areas of the peaks relative to the analyte.

The equations corresponding to the five regression analytes were

B_1: y= 25.82754x+6.26753
B_3: y= 33.21959x-1.42661
B_5 : y=7.15590x+5.776 e^{-1}
B_6 : y=13.70389x+9.16444 e^{-1}
B_8: y=11.89793x +2.37982
x: Amount et y: Area

They were consistently linear in the already mentioned range for all compounds.

The linearity was checked by analysis of variance of the regression (Table 4). A value of r above 0.9949 for all vitamins, (P<0.001) except for thiamine with r = 0.9781. The coefficient of determination (r^2) is more than 95.66% for thiamine and 99.66% higher than for others. Six determinations of the same sample were performed to assess the accuracy of the method.

3.1.2 Accuracy and precision

Six determinations of the same sample were performed to assess the accuracy of the method.

The following table (Table 5) illustrates the accuracy of the method for the determination of vitamins B_1, B_3, B_5, B_6, and B_8 in gum of *Acacia nilotica*.

3.1.3 Recovery

The recovery rate was tested by the standard addition procedure. One level was used for each water-soluble vitamin in gum samples (Table 6). Mean recoveries obtained were always satisfactory-higher than 99% for biotin, higher than nicotinamide 98.8 %, and higher than thiamine.

Table 4. Linearity of standard curves of vitamins B_1, B_3, B_5, B_6, and B_8

Vitamins	r	r^2	F^a_{exp}	DF^b	*P*
Biotin (B_8)	0.9949	99.88	21247.5	1.5	(P<0.001)
Nicotinamide(B_3)	0.9998	99.96	165492.10	1.5	(P<0.001)
Thiamine (B1)	0.9781	95.66	100026.5	1.5	(P<0.001)
Pyridoxine (B_6)	0.9998	99.96	27417.53	1.5	(P<0.001)
Pantothénic acid (B_5)	0.9999	99.98	7686.97	1.5	(P<0.001)

F^a (1.5; 0.001) = 6.61. F tab and Fexp are tabulated and exparimental Snedecor's F values, respctivly in ANOVA analysis, DF^b, degrees of freedom

Table 5. Peak area range and concentration, correlation data of the calibration curves and quantification limit of determined vitamins in gum of *Acacia nilotica*

Vitamins	Concentration range (mg/L)	Surface broad peak	Correlation coefficient	Detection limits (mg/L)	Quantification limits (mg/L)
Biotin (B_8)	1-30	59.83426-348.10776	0.9949	0.006	0.022
Nicotinamide (B_3)	1-30	31.45451-1000.048	0.9998	0.008	0.028
Thiamine (B_1)	1-30	18.14213-714.6253	0.9781	0.012	0.042
Pyrodixine (B_6)	1-30	14.31208-409.0012	0.9998	0.002	0.007
Pantotenic acid (B_5)	1-30	8.05888-214.6708	0.9999	0.001	0.0035

Table 6. Study of determining recovery rate by the addition of 500µL of vitamins B_1, B_3 and B_8

N_0 test	Biotin			Nicotinamide			Thiamine		
	Found value (mg/L)	Recovery %	% RSD	Found value (mg/L)	Recovery %	% RSD	Found value (mg/L)	Recovery %	% RSD
1	1994.7	99.0	0.23	5.04	98.8	0.91	$2.81e^{-1}$	97.2	1.26

4. CONCLUSION

In this work, we optimized HPLC conditions for determination of the water –soluble vitamins such as thiamine (B_1), nicotinamide (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin (B_8) in gum of *Acacia nilotica* following sample preparation by enzymatic hydrolys. The chromatographic separation was performed on a C_{18} reverse phase, and vitamins are detected at different wavelengths by UV-visible. This method is rapid, simple, and reliable and saves a significant amount of reagent.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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