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Short Report

Simultaneous High Performance Liquid Chromatographic Analysis of Vitamins B_1, B_2 and B_6 in Royal Jelly

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A geléia real é utilizada como suplemento alimentar, conhecido popularmente como rico em vitaminas B. Os objetivos do presente trabalho foram a determinação simultânea, por cromatografia líquida de alta eficiência, da tiamina (vitamina B_1), riboflavina (vitamina B_2) e piridoxina (vitamina B_6) e a comparação com a indicação da Ingestão Diária Recomendada (DRI). Os valores obtidos variaram de 20 a 171 ng g^{-1} de riboflavina e 408 a 2 188 ng g^{-1} de piridoxina nas amostras de geléia real analisadas. A tiamina não foi detectada. Segundo o "Food and Nutrition Board" (2000), as recomendações para estas vitaminas variam de 0,2-1,4 mg para a tiamina; 0,3-1,6 mg para a riboflavina e 0,1-2,0 mg para a piridoxina, dependendo da idade e sexo. Seguindo essas recomendações, a geléia real não pode ser considerada uma boa fonte dessas vitaminas, uma vez que elas aparecem na ordem de nanogramas por grama na geléia real. O método proposto pode ser utilizado em análises de rotina de geléia real tendo as vantagens de ser simples e rápido.

Royal jelly is used as a food supplement, popularly known as rich in B vitamins. The present work has two objectives: firstly, to apply simultaneous quantitative determination by High Performance Liquid Chromatography of thiamin (vitamin B_1), riboflavin (vitamin B_2) and pyridoxine (vitamin B_6) and secondly to compare the obtained data with the Dietary Reference Intake (DRI) values. The values obtained showed no thiamin, a range from 20 to 171 ng g^{-1} of riboflavin and from 408 to 2 188 ng g^{-1} of pyridoxine in royal jelly. According to the Food and Nutrition Board (2000), the DRI of these vitamins varies from 0.2-1.4 mg for thiamin; 0.3-1.6 mg for riboflavin and 0.1-2.0 mg for pyridoxine, depending on age and sex. According to these recommendations, royal jelly is not a good source of vitamins B_1, B_2 or B_6 as these vitamins appear only on order of ng g^{-1}. The proposed method can be used in routine analysis for royal jelly, having the advantage of being simple, fast and reliable.

Keywords: HPLC, thiamin, riboflavin, pyridoxine, royal jelly

Introduction

Royal jelly is a honeybee's secretion from the hypopharyngeal and mandibular glands (*Apis melifera*), used for the nutrition of honeybee workers, drones and queens. Only the queen receives royal jelly throughout its life.[1] A review of the chemical components of royal jelly in the literature reveals many quantitative discrepancies in the reported moisture, protein and lipid fraction, and many qualitative discrepancies in the reported amino acid and fatty acid values. There are no established identity or quality standards, specially for the vitamin content, although the royal jelly product has been sold as a food supplement, popularly known as a complex nutrient rich in B vitamins.

The recommended USP (United States Pharmacopeia) methods for the determination of water soluble vitamins are tedious and time-consuming, and currently automated methods, although fast and reliable, can assay only a single vitamin in each run. Thus, the development of a rapid and reliable method for the simultaneous liquid chromatographic assay of these vitamins is necessary.[2]

Considering that the composition of Brazilian royal jelly is poorly known, a method for simultaneous determination of B vitamins (B_1, B_2 and B_6) was needed. Once quantified the values were compared with the quantities recommended in the Dietary Reference Intake (DRI).[3]

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Experimental

Materials

Samples of royal jelly were obtained directly from beekeepers from São Paulo State, Brazil, in 1998 and they were frozen until analysis.

Thiamin (Sigma® thiamin hydrochloride T-4625), riboflavin (Sigma® riboflavin R 4500) and pyridoxine standards (Sigma® P-5669) were used to determine the recovery of the determinations and as external standards.

Methods

Simultaneous analysis of vitamins B_1, B_2 and B_6. The method for thiamin, riboflavin and pyridoxine determination using Reversed Phase High Performance Liquid Chromatography Method (RP - HPLC) was based on the Bianchini and Penteado procedure with modifications.[4]

Procedure modifications. For vitamin B_2 (riboflavin) a fluorescence detector at 450 nm for excitation and at 525 nm for emission was used instead of diode array detector at 275 nm; the flow rate was changed from 1.5 to 1.0 mL min^{-1}; in sample preparation, the use step of an ultrasonic bath was changed to the use of a Vortex followed by centrifugation, using an Eppendorf® tube.

Procedure description method

Approximately 0.5 g of royal jelly were transferred to an Eppendorf® tube and 1 mL of 8% TCA (trichloroacetic acid) was added. Each solution was mixed using a Vortex and centrifuged for 5 min at 14,000 rpm in order to precipitate the proteins. Then, the supernatant solution containing the vitamins was separated and filtered through a 0.45 mm membrane (Millex HV filter unit from Millipore®) before injection into the liquid chromatographic equipment.

The Shimadzu® 10 AD-*VP* high performance liquid chromatographic system consisted of a multisolvent pumping system (Shimadzu® Pump LC-10 AD-VP, Kyoto, Japan), an auto injector (Shimadzu® SIL-6B, Kyoto, Japan) with computer software (Shimadzu® CLASS-VP, version 5.03, Kyoto, Japan) and a printer. The detectors were a photodiode array UV-Vis (Shimadzu® SPD-M 10 AVP, Kyoto, Japan), programmed to monitor at 245 nm for thiamin analysis and a fluorescence detector (Shimadzu® RF-10 AXL, Kyoto, Japan) programmed to monitor at 295 nm for excitation and 395 nm for emission for pyridoxine during the first eight minutes and at 450 nm for excitation and at 525 nm for emission for riboflavin analysis during the last seven minutes. The detectors were connected in sequence for the 15 min run.

The chromatography was carried out at room temperature on a Vydac® 5 μm C_{18} reversed phase column (CAT 201TP54) (250 mm x 4.6 mm). The mobile phase used was an isocratic system consisting of a mixture of hexanesulfonic acid, ammonium hydroxide, acetonitrile and water (0.09 : 0.05 : 9.02 : 90.84), with pH adjusted to 3.6 using phosphoric acid. The flow rate was 1.0 mL min^{-1}. All solvents used in the mobile phase were HPLC grade from Merck® and were filtered through a 0.45 μm membrane (Millipore®) and degassed with helium gas for 10 min. The vitamins were identified by comparison of retention times with those of authentic standards, and were quantified by peak area. The purity of the standards was determined by absorbance spectroscopy before use. After the analysis, the column was washed with mobile phase.

Determination of the Quantification Limit (LOQ) and the Detection Limit (LOD)

The determination of the Quantification Limit (LOQ) and the Detection Limit (LOD) were based on previously described procedures.[5,6] The detection limit and the quantification limit were established as the vitamin quantity which gave a peak 3 and 5 times higher, respectively, than the mean of the height of the noise level of the baseline.

Results and Discussion

For each vitamin, stock solutions were prepared with standards of known concentrations which were injected in different volumes in triplicate to obtain the retention times for identification of each peak. Their quantification was made following standard procedures.[7,8] Calibration curves were constructed, where the mass of each vitamin (ng) was plotted versus the average area found for each peak. The detection limits were determined as 66.90 ng mL^{-1} for vitamin B_1, 6.47 ng mL^{-1} for vitamin B_2 and 7.80 ng mL^{-1} for vitamin B_6 while the quantification limits were determined as 111.5 ng mL^{-1} for vitamin B_1, 10.78 for vitamin B_2 and 13.00 ng mL^{-1} for vitamin B_6.

Regarding sample preparation, the proposed method showed a significant decrease in chemical reagents used, which is an important factor concerning residue generation.

Figure 1 shows a typical chromatogram of the thiamin standard. The retention time of thiamin was 10.19 min. Thiamin was not identified in royal jelly samples, because there was no peak at the correct retention time. In order to

check the method, some samples were enriched with vitamin B_1 before the extraction step and the recovery was nearly 100%.

For thiamin determination, a diode array detector was used because vitamin B_1 is not fluorescent as are vitamins B_2 and B_6. So, the use of diode array in sequence with the fluorescence detector made the simultaneous analysis of the three vitamins possible.

Figure 2 shows typical chromatograms of pyridoxine and riboflavin from pure royal jelly samples. The retention time of pyridoxine and riboflavin were 5.52 min and 12.57 min respectively.

For the pyridoxine and riboflavin determination a fluorescence detector was used, which is more sensitive and selective than the diode array UV-Vis detector, which presents more interferences compared to the fluorescence detector.

Comparing the proposed method with literature data, vitamins B_2 and B_6 are generally measured using a fluorescence detector but are not always simultaneously detected in usual HPLC methods.[9-12]

Vitamin B_1 can be detected by an HPLC method using a fluorescence detector when thiamin is oxidized to thiochrome with potassium ferrocyanate in an alkaline medium, preceding the detection.[11,13] When simultaneous analysis of vitamin B_1 and B_2, with derivatization of thiamin is attempted, it has the disadvantage that riboflavin may be destroyed during the oxidation step.[14]

The method proposed in this paper can be used in routine analysis for royal jelly, having the advantage of being simple, fast and reliable. The quantitative results for riboflavin and pyridoxine are presented in Table 1. The results varied from 20.35 to 171.00 ng g^{-1} of royal jelly for riboflavin and from 407.90 to 2187.70 ng g^{-1} of royal jelly for pyridoxine. There are no papers in the literature concerning the determination of these vitamins in royal jelly to compare with these data.

According to Food and Nutrition Board of the Institute

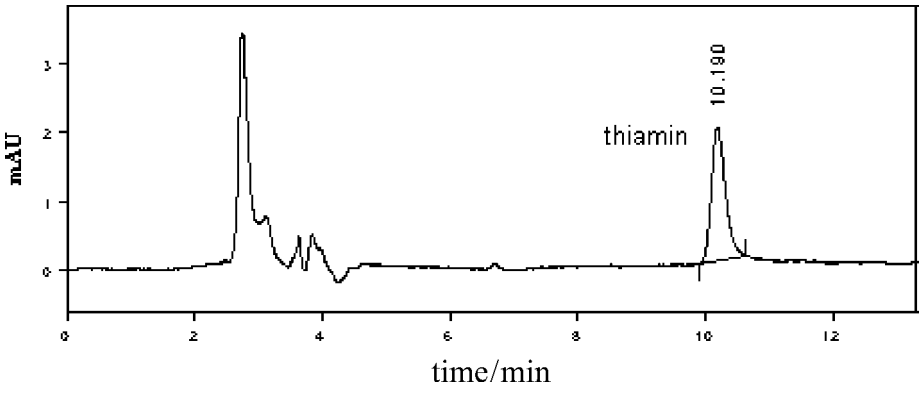

Figure 1. Chromatogram of vitamin B_1 standard (thiamin). Amount and retention time of thiamin were as follows: 111.5 ng g^{-1}; 10.19 min. Conditions are described in the text.

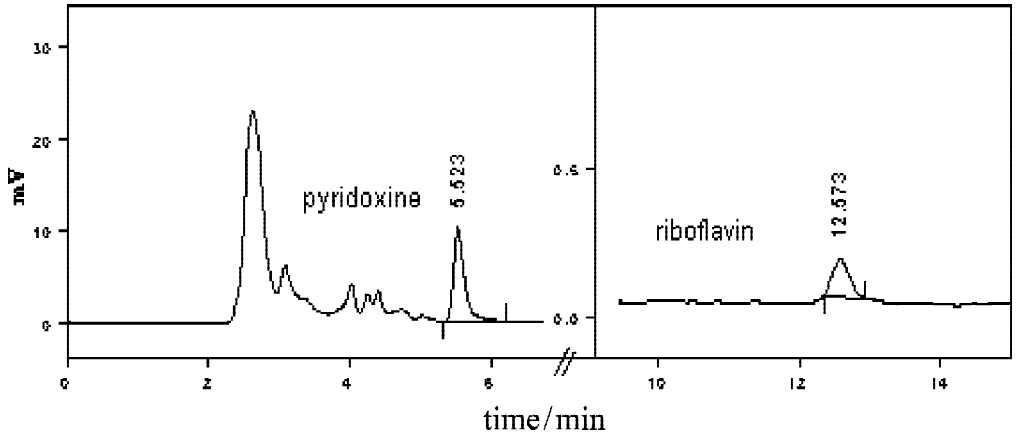

Figure 2. Chromatogram of vitamin B_6 (pyridoxine) and B_2 (riboflavine) of royal jelly samples. Amounts and retention times of vitamins were as follows: 407.90 ng g^{-1}; 5.52 min (B_6); 20.35 ng g^{-1}, 12.57 min (B_2). Conditions are described in the text.

Table 1. Average quantities of riboflavin and pyridoxine in several royal jelly samples (ng g^{-1})

Samples	Vitamin B_2 Average (ng g^{-1})	Vitamin B_2 SD	Vitamin B_6 Average (ng g^{-1})	Vitamin B_6 SD
A	26	0	474	17
B	92	5	663	38
C	88	3	814	86
D	20	4	565	55
E	31	1	408	7
F	134	4	2188	77
G	171	4	543	103

SD = standard deviation.

of Medicine of the National Academy of Sciences[2] the DRI of these vitamins varies from 0.2 to 1.4 mg for thiamin (vitamin B_1); 0.3 to 1.6 mg for riboflavin (vitamin B_2) and 0.1 to 2.0 mg for pyridoxine (vitamin B_6), depending on age and sex. According to these recommendations, royal jelly is not a good source of the vitamins B_1, B_2 or B_6 as these vitamins appear only on order of ng g^{-1} or not at all.

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