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Analysis of water soluble vitamins in energy drinks by HPLC

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Abstract

Reversed phase high-performance liquid chromatography with diode array detection (HPLC-DAD) was utilized to analyze the ingredients of various dietary supplements. Dietary supplements are classified by the Food and Drug Administration (FDA) as foods rather than drugs, and as such have very different labeling laws. Specifically, we tested several types of "vitamin water", 5-hour energy, and multi-vitamin supplements to determine if the advertised contents were actually present at the levels specified. Using our method, we were able to simultaneously analyze the B-vitamins present. It was determined that several of the energy drinks contained B-vitamins that were not in agreement with their label claims.

Introduction

Energy drinks are beverages offering metabolic stimulation through B-complex vitamins and central nervous stimulation through caffeine. The B-vitamins are a group of water soluble vitamins commonly found in many unprocessed food and supplements. They help to speed up the metabolism, aid the formation of red blood cells, and improve the immune system by aiding in the formation of antibodies.[1] The B vitamins studied here were: B1 – thiamine, B3 (PP) – nicotinamide, niacin, or nicotinic acid, B6 – pyridoxine, B5 – pantothenic acid, B9 – folic acid, and B2 – riboflavin. The structures of the B vitamins studied can be seen in Figure 1.

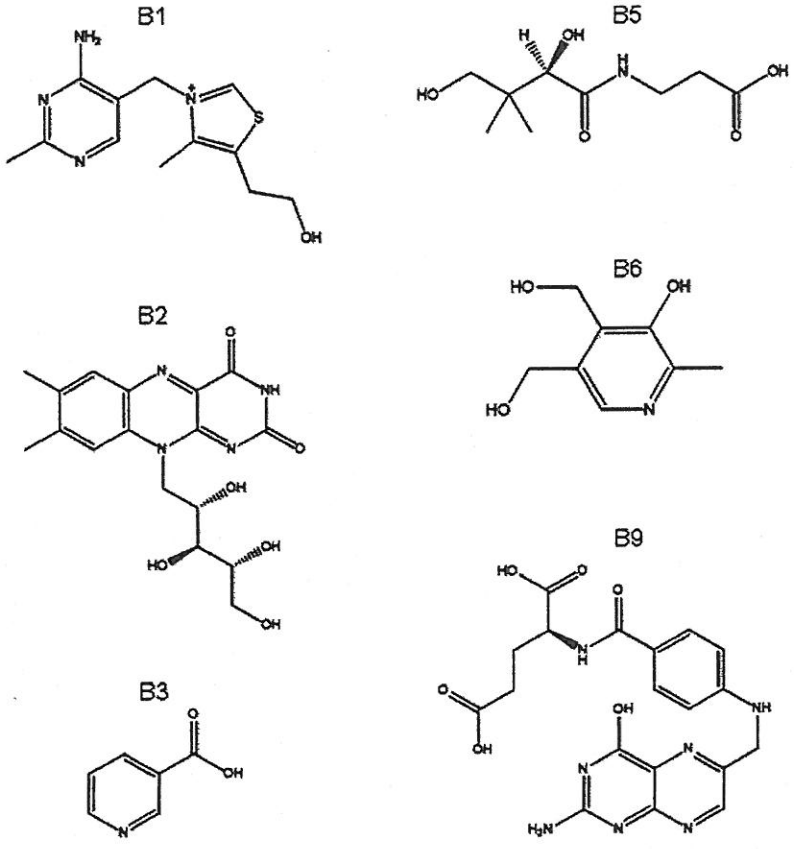

Figure 1. Structure of B vitamins

Table 1 highlights the benefits of the various B vitamins and why they are a necessary part of a healthy diet. It also shows the possible harmful side effects that coincide with deficiencies of B vitamins. Because the B vitamins are water-soluble, they are considered to be safe because any excess amounts are excreted quickly in the urine. However, side effects have been reported when consuming high levels of B vitamins and also when taken with other supplements and drugs.[2]

Table 1. Information about B vitamins.[1]

Vitamin	Use	Deficiency
B1	generates energy; aids in the digestion of carbohydrates	weakness & feeling tired; nervous irritability; insomnia; the loss of appetite
B2	Necessary for carbohydrate, fat & protein metabolism; aids in the formation of antibodies and red blood cells; maintains cell respiration	May result in sluggishness; bloodshot eyes; digestive disturbances; trembling
B3	helps metabolize protein, sugar, & fat; reduces high blood pressure; increases energy through proper utilization of food	May result in mental depression, nervousness, irritability, fatigue, insomnia, headaches, loss of appetite
B5	Participates in the release of energy from carbohydrates, fats & protein; aids in the utilization of vitamins	May lead to restlessness, retarded growth, dizzy spells
B6	Necessary for synthesis & breakdown of amino acids, the building blocks of protein; aids in fat and carbohydrate metabolism; aids in the formation of antibodies	May result in nervousness, insomnia, loss of muscular control, anemia, muscular weakness
B9	Necessary for DNA & RNA synthesis; essential to the formation of red blood cells; aids in amino acid metabolism	May result in gastrointestinal disorders, anemia, Vitamin B-12 deficiency, pre-mature gray hair

Official methods of analysis are based on microbiological assays that are tedious and sometimes non-specific.[3] These outdated analyses depended upon preparation of a growth medium (either an agar or a broth) with a set amount of foodstuff and an unknown quantity of vitamins. Researchers then compared the rate of growth of the microorganisms with unknown quantities of vitamins present to the rate of growth of the microorganism samples of known vitamin concentrations. They then hoped that their procedure would show them how available the vitamin was in the food sample to the microorganism they were studying.[4] These assays are now considered to be outdated due to the cost of the assay and because they are especially time-consuming because each vitamin must be analyzed separately and the growth media is typically incubated for 48 hours.[5] Using high performance liquid chromatography (HPLC) allows for the analysis of a multi-component mixture simultaneously.

There is not much data available on vitamins in dietary supplements because the variation in label claims and the actual concentrations has not been studied extensively.[6] The FDA requires that every dietary supplement have a label identifying each ingredient contained in the product. Items not listed under the "supplement facts" section may be included as "other ingredients."[7] These dietary supplements are regulated as foods as opposed to drugs. Therefore, manufacturers of dietary supplements are only required to report the minimum contents of nutrients, and the actual concentrations could be much higher than stated.[6] The FDA does acknowledge that dietary supplements can be harmful, and consumers are at increased risks when dietary supplements are combined, taken with medications, or taken in large quantities.[8] The FDA requires that drug manufacturers be much more extensive when labeling their

products. Drug labels must contain warnings about possible harmful combinations, discuss all symptoms of any adverse reactions, and list conditions under which the medicine should not be taken.[9] Consumers would benefit from more thorough labeling requirements for dietary supplements as the demand for analytically verified data on ingredients continues to grow.

The Dietary Supplement Ingredient Database (DSID) was developed by federal agencies in order to quantify the various ingredients in dietary supplements. The main focus of the DSID is on adult multivitamin/minerals (MVMs), followed by categories including antacids, calcium, other single minerals, vitamin E, vitamin C, and B vitamins.[10] Independent studies have been done on adult MVM products using validated HPLC methods to provide data for the DSID. However, dietary supplements like energy drinks are not yet included on the DSID.[11] Other HPLC methods are being performed to validate concentrations of different vitamin tablets.[12] Our purpose was to study the stability of B vitamins in energy drinks using an HPLC method that is faster than microbiological assays and previously published HPLC methods.[3]

High Performance Liquid Chromatography

HPLC is used for the separation of different compounds in a mixture, in this case water-soluble vitamins, based on the varying polarities of each component. By using HPLC instruments, one is able to identify and quantify the different components of a sample. The analyte and mobile phase are pumped through a long column until they

finally reach the detector. The detector used on our HPLC instrument is a diode array detector (DAD). The DAD is a UV-visible spectrometer that identifies compounds based on their UV-visible spectrum. Quantification is achieved by analyzing the difference in the amount of light absorbed by the analyte when compared to the amount of light absorbed by the solvent.[13] Figure 2 shows the pathway an analyte must travel in a modern HPLC instrument. The solvent reservoirs contain the mobile phase. The high pressure pump moves the mobile phase through the system at pressures up to 1000 psi. The column is packed with small particles that are coated with the stationary phase.

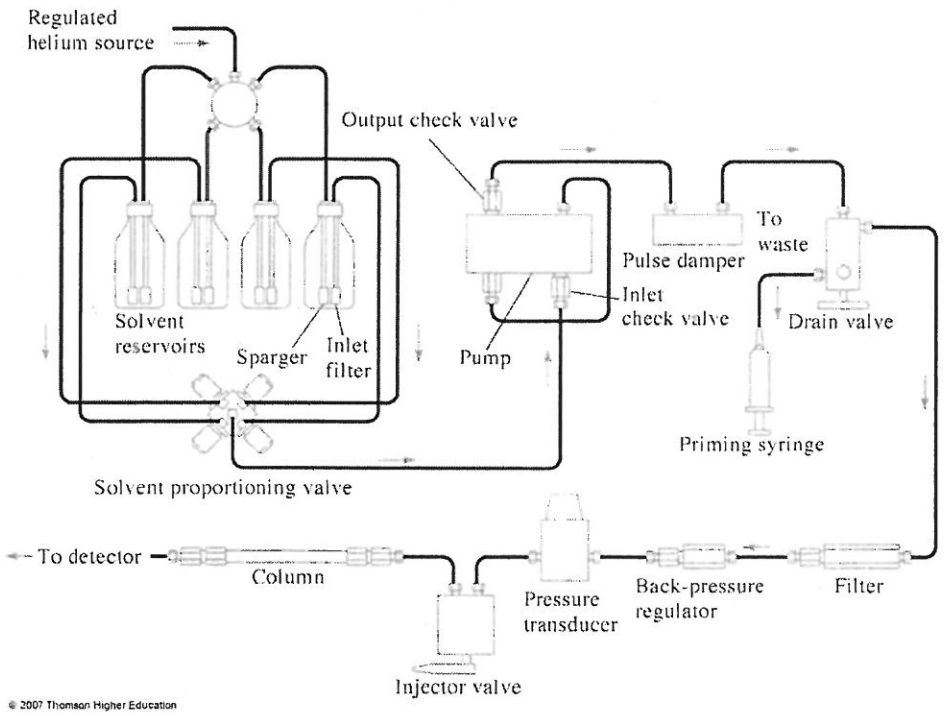

Figure 2. Schematic of an HPLC instrument.[14]

Reversed phase liquid chromatography (RPLC) is the most widely used of the different types of HPLC.[13] Reversed phase specifies that the stationary phase is less polar than the solvent. The mobile phase is RPLC is usually a mixture of aqueous buffer and methanol or acetonitrile. In RPLC, the most polar molecules are eluted first, followed by molecules with decreasing polarity, eluting the least polar molecules last. Using isocratic mobile phases, where the concentrations of each component of the mobile phase remain constant throughout the separation, can result in extremely lengthy retention times and excessively large gaps between peaks. In order to decrease the retention times of some of the less polar components, the organic phase (acetonitrile or methanol) of the mobile phase can be increased throughout the separation and will not affect the resolution of the more polar components. This method is referred to as gradient chromatography.

Experimental

Instrumentation

The instrument used for this research project was a Shimadzu Prominence HPLC-DAD system. The stationary phase in the HPLC was octadecylsilane (C18) on 5 µm particles. The column was 150 mm in length and 4.6 mm in diameter. The flow rate was 1 mL/min. The mobile phase consisted of 0.035 M formic acid and acetonitrile with a gradient of 2-50% acetonitrile in 10 min following a 2 minute hold of 2% acetonitrile at the beginning of each run.

Standard Preparation

The multi-vitamin stock solution was prepared by adding different amounts of vitamins B2, B9, B1, B6, B3 (PP), B5, and 100 mL of water to a 250 mL volumetric amber flask. The solution was shaken vigorously and 10 mL of 2 M NaOH was added to help dissolve the vitamins. Phosphate buffered saline (PBS) solution (125 mL) was added to the flask and the solution was diluted to the mark with water. The concentrations of vitamins B2 and B9 in the stock solutions were 125 ppm, B1, B6, and B3 (PP) were 250 ppm, and B5 was 1000 ppm. PBS was prepared by adding 8.06 g NaCl, 0.20 g KCl, 1.20 g $H_2PO_4^-$, and 2.30 g HPO_4^{2-} to a 1000 mL volumetric flask and diluting with H_2O.

A set of standards was prepared from the multi-vitamin stock solution in order to obtain a set of calibration curves for each vitamin. The set of standards made it possible to determine the amount of the various B vitamins present in the energy drink samples tested. Standards were prepared from 0.12 – 100 ppm by diluting the appropriate amount of stock solution in 10 mL volumetric flasks with PBS and transferring to autosampler vials using a syringe and 0.45 µm nylon filters. Table 2 shows the concentration of each B vitamin in the stock solution and the calibration range of the standards prepared.

Table 2. Multivitamin stock solution and standard concentrations.

Vitamin	Stock Concentration (ppm)	Calibration Range (ppm)
B1	250	0.25 – 25
B2	125	0.12 – 12.5
B3	250	0.25 – 25
B5	1000	1 – 100
B6	250	0.25 – 25
B9	125	0.12 – 12.5

Energy Drink Preparation

The fresh drink samples were prepared as 1:10 dilutions by adding 1.0 mL of drink sample, 5.0 mL of PBS, and 0.25 mL of 2 M NaOH to a 10 mL volumetric flask and diluting with H_2O. The drinks were prepared to have the same matrix as the standards at a 1:10 dilution. The energy drinks tested were 5-Hour Energy, Amp Energy, Rockstar Juiced, Sobe Lifewater, and Sobe Lizard Lava. Diluted drink samples were tested without manipulation. Each energy drink sample was also exposed to heat (60 °C) and UV light (365 nm) for 3 hours. After exposing the drink samples to heat and light, 1:10 dilutions of each sample were prepared in the same way the fresh samples were prepared. UV-spectra for each vitamin were obtained from a chromatogram of the stock solution.

Results and Discussion

Based on the UV spectra of the vitamins shown in Figure 2, it was not possible to detect all of the vitamins with a single wavelength. Chromatograms of B1, B2, B3 (PP), B6, and B9 were observed at 280 nm. Vitamin B5 was observed at 210 nm because it had a much higher signal than at 280 nm.

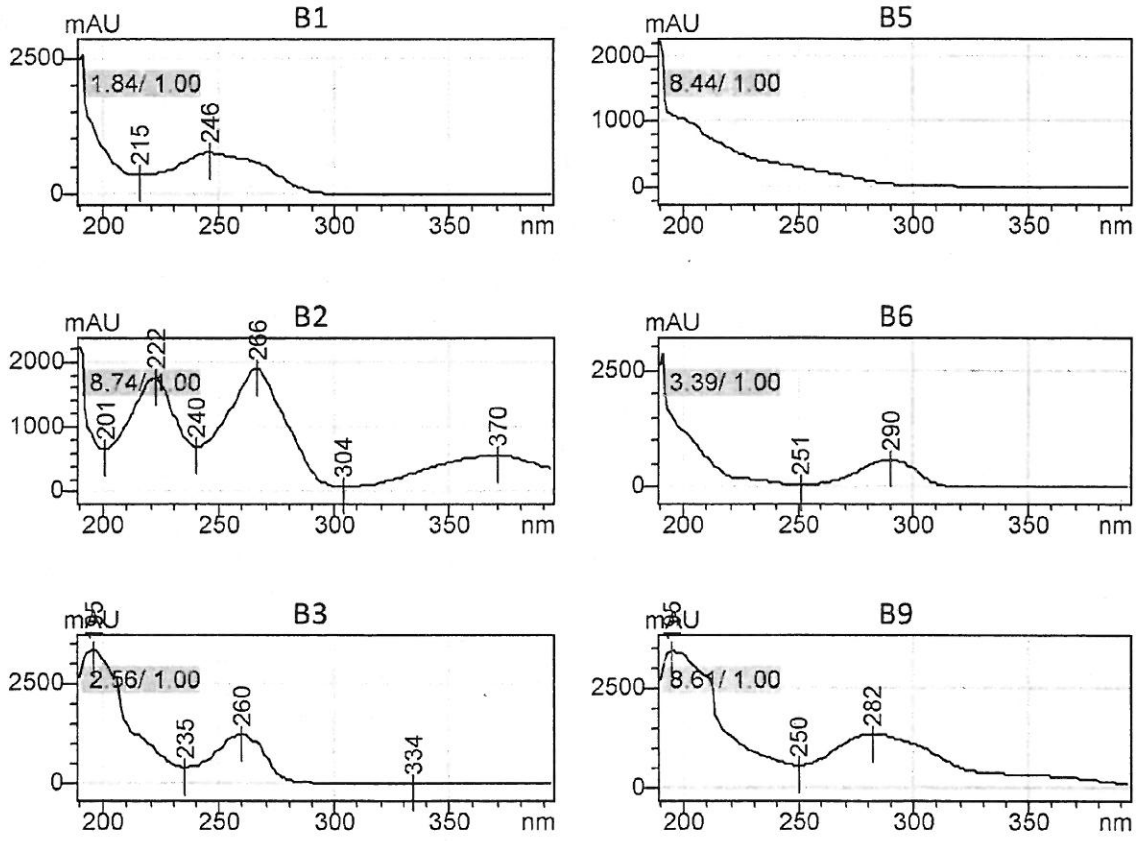

Figure 3. UV spectra of the B-vitamins

Calibration Curves

Figure 4 shows the calibration curves obtained from the set of standards of the multivitamin stock solution. Obtaining calibration curves from a set of standards with known concentrations allows for the quantification of unknown concentrations of the B vitamins in the energy drink samples. The correlation between the peak area and the concentration of an analyte is determined in order to calculate the concentration of an unknown. Vitamin B5 could not be quantified due to a high level of noise or interference at 210 nm. The UV cutoff for acetonitrile is 190 nm[15], so therefore it makes quantification at 210 nm difficult. The calibration curve for vitamin B5 shown in Figure 4 shows poor correlation between the peak area and the standard concentration.

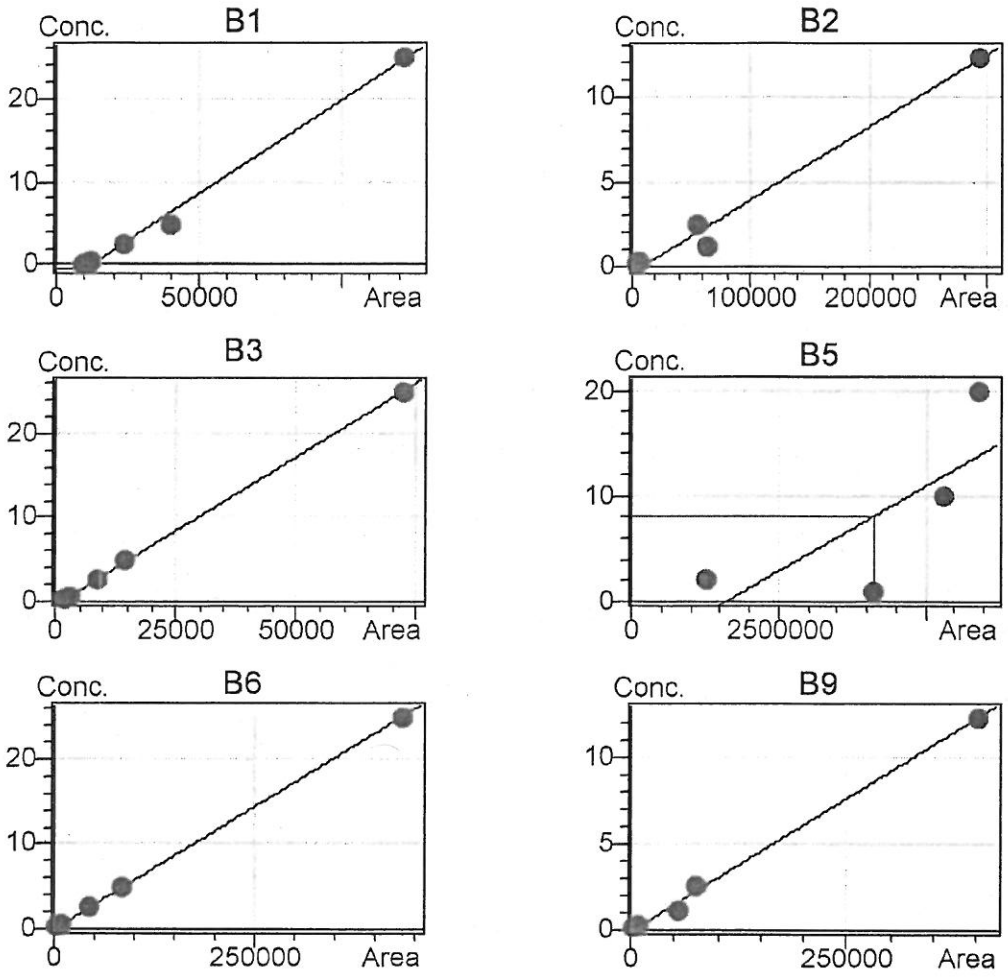

Figure 4. Calibration curves for B vitamin standards.

Chromatograms

The chromatograms collected after method development was complete show good separation between peaks. The peaks were identified based on the retention times and the spectra taken from the multivitamin standards. The chromatograms for

the different energy drink samples can be compared to the standard mixture with the highest concentration.

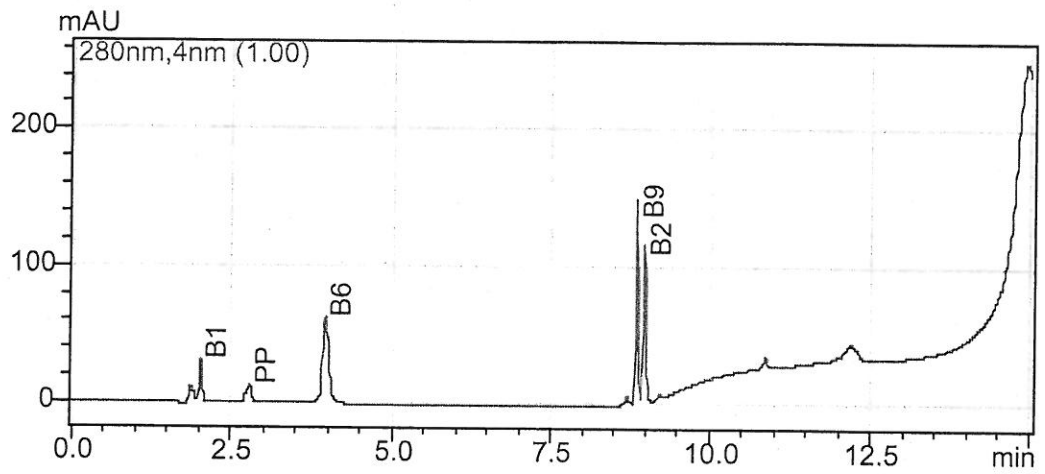

Figure 5. Chromatogram of standard mixture.

Each peak has a narrow peak width and is baseline resolved. Vitamin B5 elutes immediately before B9 but is very small because it does not absorb very well at 280 nm, as seen in its spectrum in Figure 2.

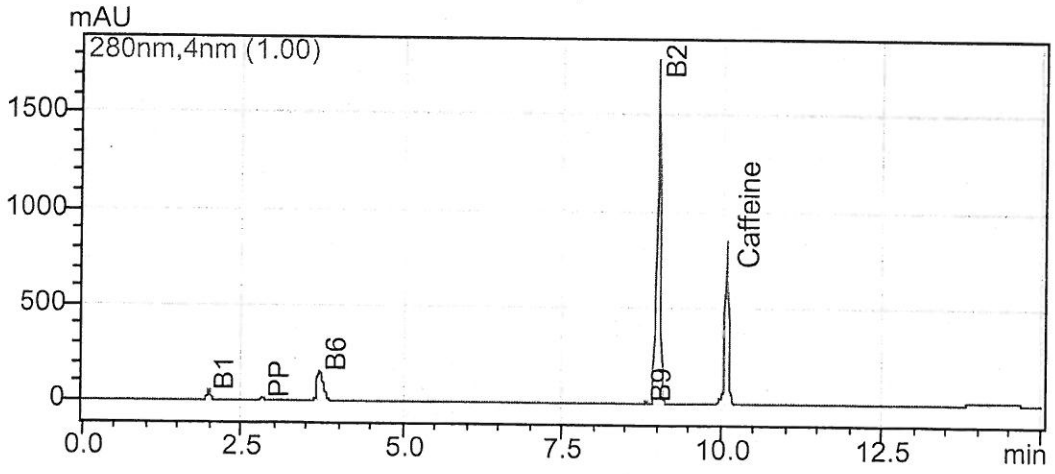

Figure 6. 5-hour Energy.

Figure 6 shows the chromatogram of the unmanipulated 5-Hour Energy drink. Five different B vitamins can be seen in this chromatogram. These vitamins are eluted in the same order and nearly the same time as those in the standard chromatogram. Each peak has a narrow bandwidth and is baseline resolved. Caffeine elutes around 10 minutes. Both vitamin B2 and caffeine are much higher than vitamins B1, B3 (PP), and B6.

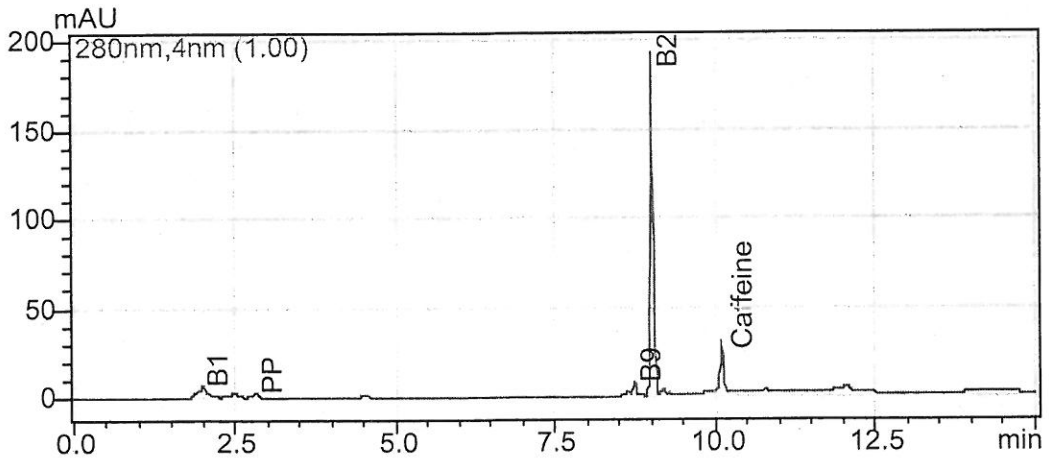

Figure 7. Amp Energy

Figure 7 shows the chromatogram of Amp Energy. Vitamins B1, B3 (PP), B9, and B2 are shown in this chromatogram with similar retention times to the standard chromatogram. Vitamin B2 had a much higher absorbance than the other B vitamins present, but it is still relatively small when compared to the 5-Hour Energy sample.

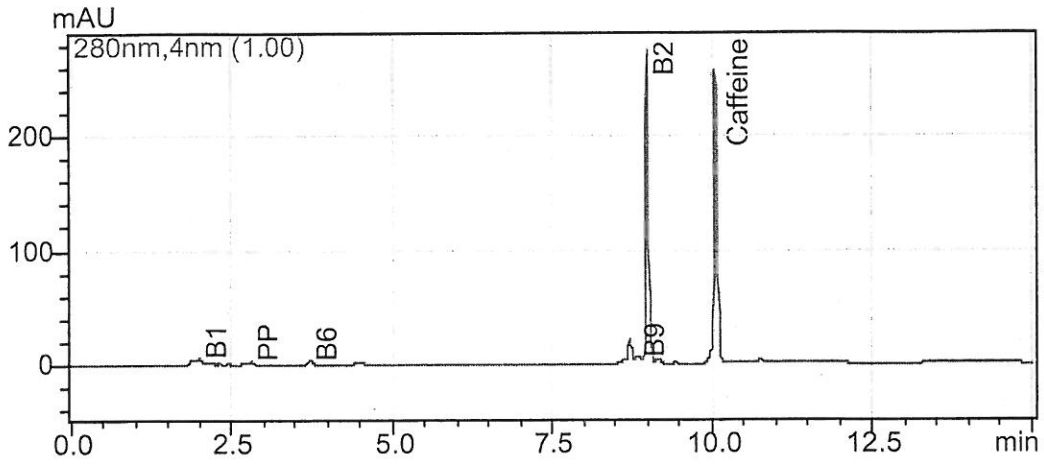

Figure 8. Rockstar Juiced

Figure 8 shows the chromatogram of Rockstar Juiced. Vitamins B1, B3 (PP), B6, B9, and B2 all appear in this chromatogram and have similar retention times to the standard chromatogram. Vitamin B2 also showed strong absorbance, and was also similar to the AMP Energy sample but much lower than the amount found in the 5-hour Energy sample. It is interesting to note the difference in the size of the caffeine peak in these three beverages. Although caffeine was not quantified as part of these experiments, it is clear that the Rockstar and Amp Energy had much lower levels of caffeine than 5-Hour Energy.

Table 3. Results for 5 Hour Energy, Amp, and Rockstar.

Vitamin	Energy Drink	Expected Amount (ppm)	Measured Amount (ppm)	% difference
B1	5-Hour Energy	N.L.*	49	N.A.**
	Amp	N.L.	14	N.A.
	Rockstar	N.L.	12	N.A.
B2	5-Hour Energy	N.L.	252	N.A.
	Amp	7	20	186
	Rockstar	N.L.	29	N.A.
B3	5-Hour Energy	508	21	96
	Amp	84	4	95
	Rockstar	84	6	93
B6	5-Hour Energy	678	62	91
	Amp	N.L.	0	N.A.
	Rockstar	8	2	75

*Not listed
**Not Applicable

The 5-Hour Energy drink sample tested had much less vitamins B3 and B6 than what the label claimed. There were significant amounts of vitamins B1 and especially B2 detected although the label did not claim these vitamins to be present. The Amp Energy and Rockstar Juiced drink samples also contained higher amounts of vitamins B1 and B2 than what the label claimed. There was also much less vitamin B3 present than what was presented on the labels.

Conclusions and Future Work

Further research could be done on this project to provide more analytically validated data on water-soluble vitamin concentrations in energy drinks and other dietary supplements. More B vitamins should be studied, including cyanocobalamin (vitamin B12) and biotin (vitamin B7). Although five different energy drinks were tested, many more are available to customers. Other dietary supplements will be tested including tablet and powder formulations. Vitamin B5 could possibly be standardized by using a different wavelength for analysis or by using a different mobile phase. It would also be interesting to quantify caffeine in various energy drink samples because these quantities are not listed on the labels.

The Dietary Supplement Ingredient Database (DSID) should consider adding data on energy drinks and other types of food and beverages that people take as dietary supplements. Products such as these are prevalent on grocery shelves and may be consumed instead of vitamins in tablet form. Analyzing the vitamin contents of other energy drinks would be beneficial to the consumer, and a simple, rapid HPLC method makes the analysis of these products accessible to any analytical laboratory.

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