HPLC Analysis of Vitamin B3 and B6 in 5-hour Energy Drinks

Melvin Crew

Dr. Diane Nutbrown
Science Senior Seminar
The Neighborhood Academy
April 30, 2021

INTRODUCTION

Energy drinks have become increasingly popular since their introduction in the mid-1980s as highly caffeinated soft drinks (e.g. Jolt), and they have evolved to contain a number of vitamins and amino acids (*1*) to appeal to consumers as products that potentially increase mental awareness and physical performance (*2*). In 2012, while most beverages had declining sales in the United States, energy drinks had reached $8 billion, an increase of 19% in a year (*3*). In 2018, energy drinks generated $13.5 billion, representing a 29.8% increase in sales (*4*). According to The National Center for Complementary and Integrative Health, men between the ages of 18 and 34 consume the most energy drinks, with one third of teens between 12 and 17 drinking them regularly (*2*). These energy drinks typically contain caffeine and taurine, as well as a variety of B vitamins, including B2, B3, B6, and B12. The concentrations of these ingredients may not be explicitly stated on the label of the energy drink. Our research project focused on determining the concentrations of vitamins B3 and B6 in 5-hour energy drinks.

B Vitamins

Energy drinks often contain B vitamins, because these vitamins help cells grow and reproduce, send messages through your nerves to your brain, and break down foods that were eaten into energy (*5*). B vitamins are water-soluble, making them easy to add to beverages; the numbers next to the B are mostly the order in which scientists discovered this class of vitamins. Vitamin B3 occurs in two forms, niacin and nicotinamide (Figure 1). Vitamin B6 can be found in three forms: pyridoxine, pyridoxamine, and pyridoxal (Figure 2). These different forms are usually interchangeable because of their close similarities in how they work. For instance, when niacin is consumed it is converted into nicotinamide, but it is still a form of niacin in your body (*6*). Since both of these have the same health benefits in the body, niacinamide is usually preferred because it does not have the same effects as niacin like flushing of the skin (*7*). Niacinamide is usually the form of vitamin B3 that is found in animal products like meat and poultry, and nicotinic acid, another name for base niacin, can be found in plants such as seeds, and green vegetables (*7*).

The recommended daily allowance (RDA) for B vitamins vary between sexes and a serving of a typical energy drink often exceeds this amount. For instance, vitamin B3 has a RDA of 16.0 mg for men and 14.0 mg for women, although it changes to 18.0 mg due to needs for women during pregnancies (*5*). Vitamin B6's daily recommendation is 1.3 mg for both men and women, but this increases as they grow older (*8*). Companies use large amounts of these vitamins to increase how much your body produces energy from fats and other potential benefits like better moods or higher mental alertness (*9*). Energy drinks like Monster, 5-Hour Energy and Redbull have almost double the amount on their labels than the

RDA has listed. The paper warns about how some cases have caused some people to not walk due to high levels of pyridoxine (vitamin B6) and how some energy drink cans even have 40 mg per can. (*10*).

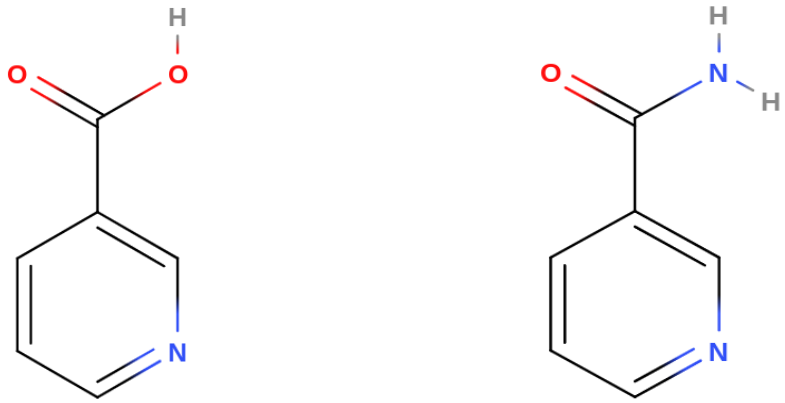

Figure 1. Molecular structures of Niacin (left) and Nicotinamide (right).

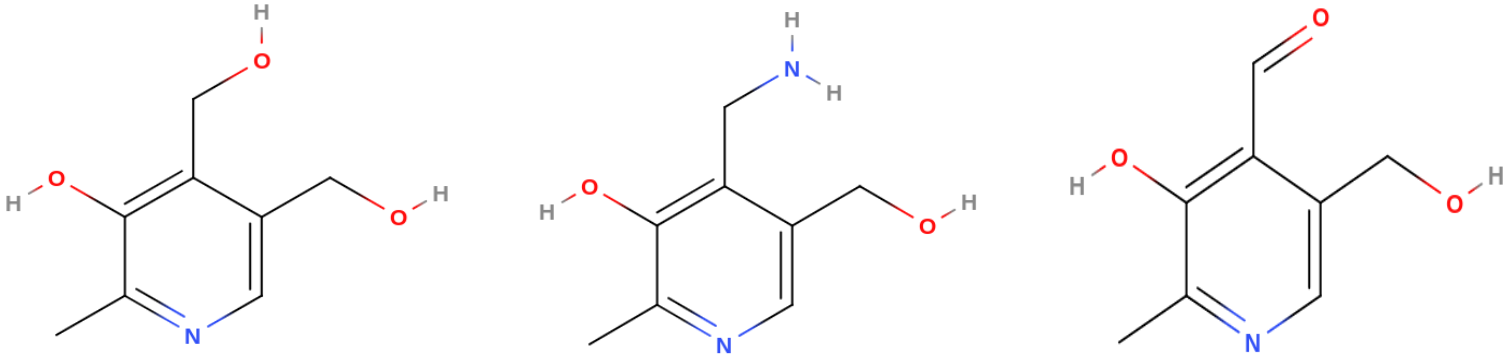

Figure 2. Molecular structure of Pyridoxine, pyridoxamine and pyridoxal, left to right respectively.

Previous Research

Although concentrations are not always listed on the labels of energy drinks, those concentrations may be measured in a laboratory using techniques such as high-performance liquid chromatography (HPLC). Two papers have been published in the *Journal of Chemical Education* reporting laboratory experiments that can be performed by students in university analytical chemistry labs. Leacock, et al. focused on the presence of vitamin B6 in Red Bull, Monster, Rockstar, and Full Throttle (*11*). Marri-Andres, et al. used HPLC with fluorescence detection to study both vitamin B6 and vitamin B2 in three flavors of Monster energy, Red Bull, El Corte Inglés, and Hacendado (*12*). Both papers use an

isocratic method for their experiment with an acetonitrile-phosphate buffer (50 mM pH 3.0) in 2011, and methanol-phosphate buffer (50 mM, pH 3.0) in 2015 for their mobile phase. The flow rate in both experiments were set at 1 mL/min. The 2011 paper used a 292 nm absorbance to find their results while the one in 2015 initially used a 296 and 390 nm until 3 min where they changed the wavelengths to 450 and 530 nm, respectively. This change was made for a better sensitivity for Riboflavin. Both papers used the standard addition method for their analysis. In the 2011 paper, they were finding the concentrations of vitamin B6 and Caffeine, noting that Red Bull actually has 5 mg of the vitamin which is over 250% of the RDA. The paper in 2015 researched the concentrations of vitamin B6 and B2 to provide awareness to younger adults and teens of what they are drinking.

Several other published methods for measuring B vitamins with HPLC were compared prior to choosing parameters for our own experiment. A promising protocol developed by Gliszczynska-Swiglo and Rybicka allows for the simultaneous determination of concentrations of caffeine and all B vitamins excluding vitamin B7. The paper used unnamed energy drinks for their samples. A methanol:phosphate buffer was used in this paper in a gradient method starting at 10:90 to 40:60. (*13*). Although vitamin B6 has been measured in Redbull (*11*) and vitamins B3 and B6 have been quantified in 15 different energy products: Drinks and Supplements (*13*), this will be the first study to compare concentrations of vitamins B3 and B6 in 5-hour energy. We were originally going to be using the standard addition method, like in the JCE research done in 2011, but adapt the HPLC parameters to follow Food Analytics Method's research. In figure 4 below (figure 3 of the Food Anal. paper) the absorption maxima of nicotinamide (vitamin B3, left) and pyridoxine hydrochloride (vitamin B6, right) occurred at 260 and 290 nm, respectively.

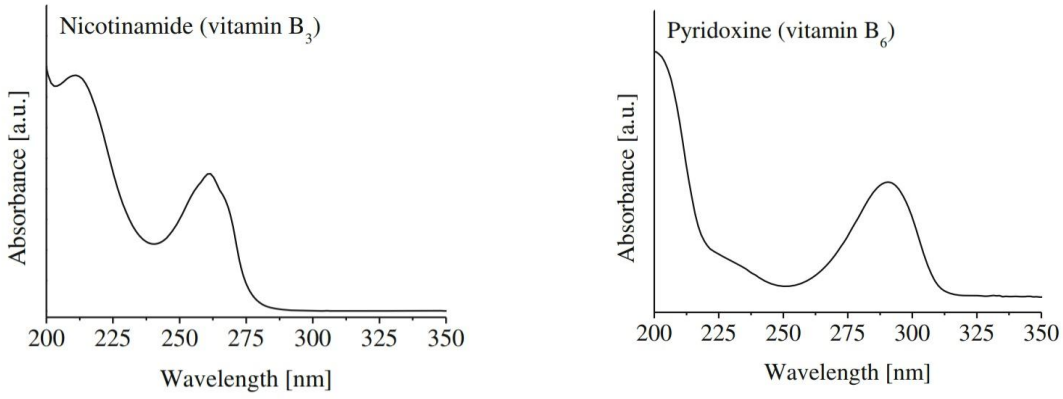

Figure 4. UV absorption spectra of nicotinamide (vitamin B3, left) and pyridoxine (vitamin B6, right) taken from Anna Gliszczyńska-Świgło and Iga Rybicka. (*13*)

Four articles were looked at for the experiment and offered some takes on vitamins. "Reversed-Phase HPLC Separation of Water-Soluble Vitamins on Agilent ZORBAX Eclipse Plus Columns" by Anna Glinko, Michael J. Bozym, et al. is an experiment that focused on separating water soluble vitamins and developing an efficient method (*14*). The experiment uses a 0-30% gradient method using Methanol:NaH_2PO_4 (pH 2.5)as the mobile phase at a rate of 1 mL/min. Three different injection sizes were used, 5 µm, 5 µL 3.5 µm, 3.3 µL 1.8 µm, 1,7 µL, to match the column size. Different sizes were used after noting how the size affected sensitivity. The detection was set at 220 nm for the analysis. The article titled "Liquid Chromatography" was written by Andrew P. Zdzieblo and Wilhad M. Reuter (*15*). They focus on separating all of the B vitamins, separating the two forms of vitamin B3 (Niacin and Niacinamide). Acetonitrile:Ammonium formate buffer (5 mM, pH 4.8 – 4.9) was the mobile phase used in the paper as a 100-70% gradient. The parameters used a 0.6 mL/min flow rate with a 214 nm detection for both vitamins B3 and B6. The mobile phase was injected in a volume of 3 µL. This experiment separated Niacin and Niacinamide. "HPLC Analysis of Water-Soluble Vitamins (B2, B3, B6, B12, and C) and Fat-Soluble Vitamins (E, K, D, A, and *β*-Carotene) of Okra" by Rokayya Sami, Yang Li, et al. is an experiment on fat and water soluble vitamins in Okra, a plant grown in Africa and Asia that is high in vitamins (*16*). For analyzing vitamins, methanol was used as a solvent with a detection of 325 nm for vitamin A, 265 nm for vitamin D3, 290 nm for vitamin E, and 244 nm for vitamin K3. The flow rate was maintained at 1 mL/min and samples injected in 20 µL. "The Analysis of Water and Fat Soluble Vitamins by HPLC-DAD" focuses on making a detailed analysis of water and fat soluble vitamins using a Diode Array Detector HPLC (*17*). The mobile phase consisted of a 10:90 Acetonitrile:Phosphate buffer (pH 5.2) solvent, at a 260 nm detection level.The samples were injected in 10 µL volumes at a 0.8 mL/min flow rate. Every article had good separation between vitamin B3 and B6, the Perkinelmer having a better separation between both forms of vitamin B3.

High-Performance Liquid Chromatography (HPLC) has been the method of choice for analyzing B vitamins, so it is instructive to briefly review this technique. HPLC is a type of chromatography to separate and analyze mixtures. Chromatography refers to the method of dissolving a solute in a solvent called the mobile phase to carry it through the stationary phase (*18*). The mobile phase is able to pass through and separate compounds of a liquid based on the polarity of its components (*19*). The two ways we can separate compounds is by making the stationary phase nonpolar or polar (Reverse-phase or normal-phase). A polar stationary phase is called normal phase because, originally in column chromatography, a method before HPLC machines, the phase was polar and made of silica or alumina. The stationary phase is held within a column of the HPLC machine. This holds the material that is nonpolar or polar (Labcompare). In a reverse-phase HPLC the column is typically made of silica compounds of the substance separate because the more nonpolar it is the slower it will move, meanwhile

it is the opposite for a normal-phase HPLC (*19*). This is because when the components of the mixture match polarities with the stationary phase, it will interact more with it and want to stick to it, causing it to move slower through the stationary phase compared to compounds with opposite polarity due to similar intermolecular forces of attraction (*20*).

A Hypersil BDS C18 column will be used, comparatively to the Nova-Pak C18 column and µBondapak C18 cartridge guard column, 5 µm, 150 x 4.6 mm Kromasil C18 column, and 250x4.60 mm 5 µm Phenomenex C18 column in the other researches.

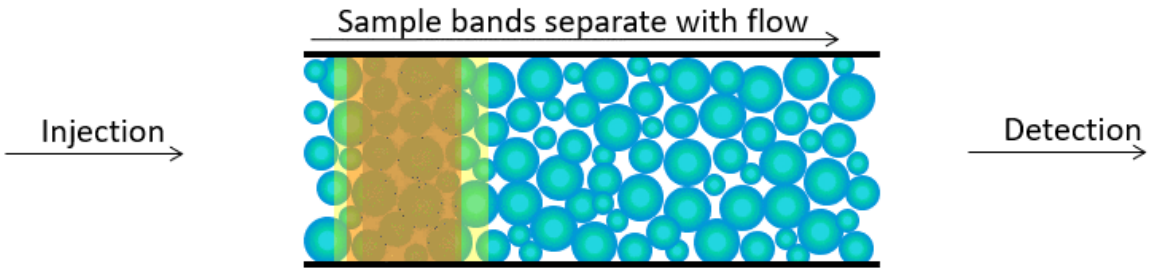

Figure 3. Magnified look into the process inside a column (*18*).

EXPERIMENTAL

Chemicals and Equipment

Rockstar Revolt, 5-hour energy and Monster energy drinks were purchased from a local retailer. Nicotinamide was purchased from Sigma Chemical Company and pyridoxine hydrochloride (>98.0%) was purchased from Tokyo Chemical Industry; these were used to make standards of Vitamin B3 and Vitamin B6, respectively. HPLC grade acetonitrile and HPLC grade methanol (99.9%) were purchased from VWR Chemicals. Water was retrieved from Regis University. HPLC analysis was completed using an Agilent 1100 HPLC with autosampler, multi-wavelength UV-Vis detector, and fluorescence detection (DAD). A Kinetex 5 µm EVO C-18 100 Å LC Column (150 x 4.6 mm, H17-395435) was used. The HPLC was operated using Chromeleon software.

Preparation of Standards

In our experiment, we are using the external standard method to find the concentrations of two different analytes. Nicotinamide (0.1285g) and Pyridoxine (0.1271g) was dissolved in 1 mL of Acetonitrile to produce a 1250 ppm stock solution. The 1250 ppm samples were diluted with the pH 4 phosphate buffer and filled to volume to make a 5, 10, 20, 30, 40 and 50 ppm sample. For each product, six different samples were produced.

Preparation of Samples

The energy drinks were degassed by the instrument on the HPLC machine. [Note any dilution of energy drinks and then state the volume injected

HPLC Analysis

The experiment was performed on our behalf by Dr. Lynetta Mier at Regis University in Denver, CO. The parameters used for this were taken from the labs by Andrew P. Zdzieblo and Wilhad M. Reuter (*15*), with a modification from Chromatography Today (*17*). I injected 20 µL of each sample into the column. This was also used in a Gradient method, but instead we used acetonitrile instead of methanol, we also changed the buffer to have a pH of 4 to balance the Nicotinamide due to it having a higher pKa of 3.35. The gradient started with 100% Acetonitrile to a linear ramp to 70:30 Phosphate:buffer (pH 4) and back down to 100% Acetonitrile. The flow rate during this process was 0.6 mL/min. The samples were monitored at 215 and 253 nm in the experiment to detect Vitamin B3 and B6, respectively with a 4 nm bandwidth.

RESULTS

The standard curve for vitamin's B3 and B6 are given in Figure 5 and the results from our HPLC analysis are given in Tables 1 and 2. The equation of the line of best fit for each set of vitamin standards was used to calculate the concentration of injected samples by substituting the area of the vitamin peak in the chromatogram for y and solving for x, which is the concentration of the injected sample. Because the original solutions had been diluted to prepare these injected samples, the concentrations were multiplied by a factor of 10 to account for that dilution and find the concentration in the original 5-hour Energy samples. This concentration was then multiplied by the volume contained in a bottle of 5-hour Energy (57 mL) to find the mass in grams of the vitamins in a single bottle. This mass was then multiplied by a thousand to convert to matching units (mg) and shown in the following tables. Table 1 summarizes the results from each trial for the concentration of vitamin B3. Table 2 summarizes the results from each trial for the concentration of vitamin B6.

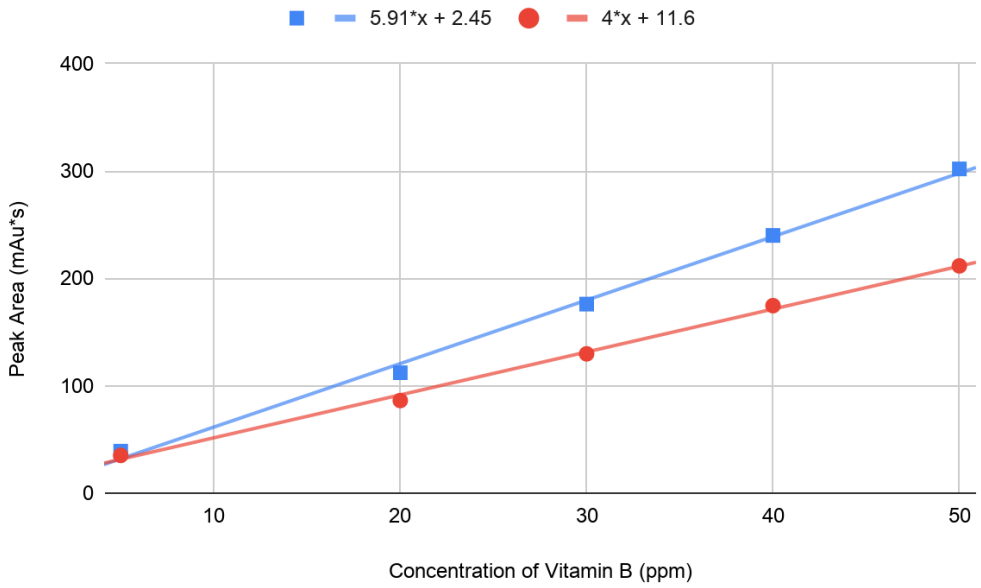

Figure 5. This calibration plot includes standards of vitamin B3 (red circles) and vitamin B6 (blue squares). The equation of the lines of best fit for each set of standards is included.

Table 1. HPLC data and calculated results for Vitamin B3 in 5-hour Energy

Source	Trial	Peak Area (mAu*s)	Conc. in Injected Sample (ppm)	Conc. in Original Sample (ppm)	Mass of Vit. B3 in Volume Sample
Reg. Berry	1	223.3	52.94	529.4	30
	2	225.7	53.52	535.2	31
	3	224.6	53.26	532.6	30
Xtra Berry	1	137.6	31.50	315.0	18
	2	139.8	32.04	320.4	18
	3	138.7	31.78	317.8	18
Reg. Grape	1	218.9	51.83	518.3	30
	2	223.4	52.96	529.6	30
	3	220.0	52.11	521.1	30
Xtra Grape	1	128.2	29.16	291.6	17
	2	130.9	29.83	298.3	17
	3	127.3	28.93	289.3	16

Table 2. HPLC data and calculated results for Vitamin B6 in 5-hour Energy

Source	Trial	Peak Area (mAu*s)	Conc. in Injected Sample (ppm)	Conc. in Original Sample (ppm)	Mass of Vit. B6 in Volume Sample
Reg. Berry	1	453.0	76.24	762.4	44
	2	467.1	78.61	786.1	45
	3	488.5	82.25	822.5	47
Xtra Berry	1	232.3	38.89	388.9	22
	2	226.8	37.96	379.6	22
	3	230.7	38.61	386.1	22
Reg. Grape	1	489.33	82.37	823.7	47
	2	467.0	78.60	786.0	45
	3	458.1	77.10	771.0	44
Xtra Grape	1	224.8	37.63	376.3	21
	2	217.7	36.42	364.2	21
	3	228.5	38.24	382.4	22

Table 3. Statistical Analysis Results for Vitamin B3

Source	Avg. Mass of Vit. B3 from sample	Standard Deviation	T-test P-Value	Conclusion	Confidence Interval (95%)
Reg. Berry	30	0.58	0.42	Same	(28.56, 31.44)
Xtra Berry	18	0	N/A	Different	N/A
Reg, Grape	30	0	N/A	Same	N/A
Extra Grape	17	0.58	0.00020	Different	(15.56, 18.44)

Table 4. Statistical Analysis Results for Vitamin B6

Source	Avg. Mass of Vit. B6 from sample	Standard Deviation	T-test P-Value	Conclusion	Confidence interval (95%)
Reg. Berry	45	1.5	0.026	Different	(41.53, 49.13)
Xtra Berry	22	0	N/A	Different	N/A
Reg, Grape	45	1.5	0.026	Different	(41.53, 49.13)
Extra Grape	21	0.58	0.00032	Different	(19.89, 22.77)

DISCUSSION

The data taken from the experiment concludes that the mass of vitamins in the bottle are different from that present on the labels, except for the regular strength drinks for vitamin B3. Information on the labels of each version of 5-hour energy was taken from CVS and Amazon, which provided the info from the labels and/or pictures of the label. When comparing the table there are two major differences between the results and the measure of vitamins B3 and B6 on the label of 5-hour energy's. On the label, it states that the extra strength versions of the drinks generally have the same amount of vitamin B6 in each bottle, but there is 10 mg extra of vitamin B3 in the extra strength drinks, according to our analysis the drinks have an average of 45.33 mL for vitamin B6 and 30 mL for vitamin B3 in regular strength. Extra Strength bottles contain 21.33-22 mL for vitamin B6 and 17-18 mL for vitamin B3 on average. Observing the results of the experiment shows that there are not only different amounts of vitamins stated, the extra strength drinks actually contain less vitamins than the regular version. In addition a 95% confidence interval (CI) was used in order to see how different the labels are from the results. The vitamin B6 in the regular strength energy drinks are not actually that far from the CI, and are actually within the parameters for a 99% CI. So while this is different than the labels, you would need more samples for better results. For the extra strength versions with both vitamins the labels are completely different than the parameters for the CI, not even close to a 99% CI. Even with this being such a big difference I would suggest taking more samples before accusing the company of false labeling.

In the process of doing the experiment we ran into a few issues, causing some changes in the parameters to get a better and clear analysis. The first issue in the experiment that made adjustments with flow rate and the mobile phase was the retention times to make it take longer. Making the retention time longer in the experiment means that we can separate components of the energy drink further so they do not interfere with our measure of our vitamins. The next thing that was changed in the experiment was the method and mobile phase for it. The first method was a 10-40% Methanol gradient Mobile Phase with methanol:phosphate buffer (pH 3) and was switched to a gradient mobile phase with 100-70% Acetonitrile to phosphate buffer (pH4). If the conditions were changed the results would be different based on the changes we did during our experiment. A lot of small differences can cause our components to separate more or less. I am skeptical of the concentrations that are lower than the label because the ones that were lower typically are from the extra strength drinks. The extra strength brought an assumption that the ingredients would be higher in concentration than the regular strength.

REFERENCES

1. *FastEnergyDrink,* Energy Drinks: How Did They Originate and Why Are They Good? https://www.fastenergydrink.com/en/energy-drinks-how-did-they-originate-and-why-are-they-good/#:~:text=It%20is%20believed%20that%20the,the%20name%20of%20Jolt%20Cola (Accessed April 30, 2021)
2. Energy Drinks, 2018. National Center for Complementary and integrative Health (NCCIH). https://www.nccih.nih.gov/health/energy-drinks (Accessed April 30, 2021)
3. Talpos, Sara. Energy Drinks are popular among teens, despite being loaded with stimulants. Is that something to worry about? *In the Energy Drink Market, Advertising and Science Collide* [Online], June 26, 2019. https://undark.org/2019/06/26/kids-energy-drinks/ (Accessed April 30, 2021)
4. Harfmann, Barbara. Energy Drinks Continue Steady Growth. *Beverage Industry* [Online] August 9, 2019 https://www.bevindustry.com/articles/92310-energy-drinks-continue-steady-growth (Accessed April 30, 2021)
5. Pressman, Alan H. *The Complete Idiot's Guide to Vitamins & Minerals*. Alpha (Penguin), 2007.
6. Niacin vs. Niacinamide (Inositol Hexanicotinate) *Martin's Wellness Compounding Pharmacy [Online]* https://martinswellness.com/wellness-connection/niacin-vs-niacinamide.html (Accessed April 30, 2021)
7. Walle, Gavin Van de. Niacinamide: Benefits, Uses and Side Effects. *Healthline* [Online] November 16, 2018 https://www.healthline.com/nutrition/niacinamide#what-it-is (Accessed April 30, 2021)
8. Pyridoxine HCL. *WebMD* [Online], https://www.webmd.com/drugs/2/drug-5427/pyridoxine-vitamin-b6-oral/details (Accessed April 30, 2021)
9. Yunusa, I; Ahmad, IM; Energy- Drinks: Composition and Health Benefits, *AJOL* [Online] **2011** *4*(2), 186-191. https://www.ajol.info//index.php/bajopas/article/view/75303 (Accessed April 30, 2021)
10. Blackburn, K.; Warren, K.; A case of peripheral neuropathy due to pyridoxine toxicity in association with NOS energy drink consumption (P4.043) *Neurology* [Online] **2017**, 88 (16 Supplement) P4.043; https://n.neurology.org/content/88/16_Supplement/P4.043 (Accessed April 30, 2021)
11. Leacock, R.E.; Stankus, J.J.; Davis, J.M. Simultaneous Determination of Caffeine and Vitamin B6 in Energy Drinks by High-Performance Liquid Chromatography (HPLC). *J. Chem. Educ.*[Online] **2011**, *88(2),* 232-234. https://pubs.acs.org/doi/abs/10.1021/ed100146s (accessed

December 11, 2020).

12. P. Martí-Andrés; L. Escuder-Gilabert; Y. Martín-Biosca; S. Sagrado; M.J. Medina-Hernandez. Simultaneous Determination of Pyridoxine and Riboflavin in Energy Drinks by High-Performance Liquid Chromatography with Fluorescence Detection *J. Chem. Educ.* **2015**, 92, 903-906. https://pubs.acs.org/doi/abs/10.1021/ed500544h (Accessed April 30, 2021)
13. Gliszczyńska-Świglo, Anna; Rybicka, Iga. Simultaneous Determination of Caffeine and Water-Soluble Vitamins in Energy Drinks by HPLC with Photodiode Array and Fluorescence Detection. *Food Anal. Meth.* [Online] **2014**, *8*, 139-146. https://link.springer.com/article/10.1007/s12161-014-9880-0 (Accessed April 30, 2021)
14. Glinko, Anna; Bozym, Michael J.; Owens, Michelle L.; Usher, Karyn M.; Majors, Ronald E.. Reversed-Phase HPLC Separation of Water-Soluble Vitamins on Agilent ZORBAX Eclipse Plus Columns, *Agilent* [Online], **2008**. https://www.agilent.com/cs/library/applications/5989-9313EN.pdf (Accessed April 30, 2021)
15. Zdzieblo, Andrew P.; Reuter, Wilhad M.. The Qualitative and Quantitative Analysis of Water-Soluble B Vitamins by HPLC-PDA in Various Multivitamin Tablets, *Perkinelmer* https://www.perkinelmer.com/lab-solutions/resources/docs/APP_Analysis-of-Water-Soluble-B-Vitamins-by-HPLC-PDA.pdf (Accessed (April 30, 2021)
16. Sami, Rokayya; Li, Yang; Qi, Baokun; Wang, Shengnan; Zhang, Qiaozhi; Han, Feifei; Ma, Ying; Jing, Jing; Jiang, Lianzhou. HPLC Analysis of Water-Soluble Vitamins (B2, B3, B6, B12, and C) and Fat-Soluble Vitamins (E, K, D, A, and β-Carotene) of Okra (*Abelmoschus esculentus*). *JOC* [Online], **2014**, 1-6 https://www.hindawi.com/journals/jchem/2014/831357/ (Accessed April 30, 2021)
17. The Analysis of Water and Fat Soluble Vitamins by HPLC-DA, 2020. Chromatography Today https://www.chromatographytoday.com/news/hplc-uhplc/31/scion-instruments/the-analysis-of-water-and-fat-soluble-vitamins-by-hplc-dad/52162 (Accessed April 30, 2021)
18. High Performance Liquid CHromatography (HPLC) Basics, *Shimadzu Scientific[Online] https://www.ssi.shimadzu.com/products/liquid-chromatography/knowledge-base/hplc-basics.html (Accessed April 30, 2021)*
19. Ask A Scientist Staff, What is Chromatography and How Does it Work? *ThermoFIsher Scientific* [Online] October 17, 2019 https://www.thermofisher.com/blog/ask-a-scientist/what-is-chromatography/ (Accessed APril 30, 2021)

20. Waters. How Does High Performance Liquid Chromatography Work? www.waters.com/waters/en_US/How-Does-High-Performance-Liquid-Chromatography-Work?/nav.htm?cid=10049055&locale=en_US. (Accessed April 30, 2021)