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Full Length Research Article

Spectrophotometic Determination of Caffeine and Vitamin B_6 in Selected Beverages, Energy/Soft Drinks and Herbal Products

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ABSTRACT

In this study, a simple, sensitive and reproducible spectrophotometric technique has been developed and validated for the determination of caffeine and vitamin B_6 in beverages, energy/soft drinks and herbal products. The determination of caffeine and vitamin B_6 in the respective samples were carried out at maximum (λ_{max}) absorbance of 272 and 290 nm respectively. The method was validated in terms of linearity, sensitivity (limit of Detection (LOD) and limit of Quantification (LOQ), accuracy (% Recovery), precision (relative standard deviation). The method was linear from (4-20 µg/ml and 50 - 250 µg/ml with r^2 of 0.9991 and 0.9996 for vitamin B_6 and caffeine respectively. The accuracy of the method ranged from 99.48 - 101.42% for caffeine and 99.94% - 102.35% for vitamin B_6. The detection limit and quantification limit were 0.192 µg/ml and 0.640 µg/ml for vitamin B_6 while 0.0155 µg/ml and 0.0518 µg/ml was obtained for caffeine. The method for the two analytes was found to be precise as the percentage relative standard deviation was below 5%. Therefore, the method proposed in this study is rapid, suitable and can be used as a quality control index for caffeine and vitamin B_6 in beverages, energy/soft drinks and herbal products in industries.

Keywords: Caffeine, Vitamin B_6, Beverages, Energy/Soft drinks, Herbal products, Spectrophotometry.

INTRODUCTION

Vitamin B_6 and caffeine are the most versatile compounds in the sense that almost every human being is exposed to these compounds via beverages and energy drinks (Andrews *et al.,* 2007). The popularity of these drinks is due to the fast-acting energy boost it gives consumers through caffeine, vitamins, carbohydrates, and other ingredients such as taurine (Sather and Vernig, 2011).Although the consumption of caffeine and vitamins are recommended in certain amounts, over-consumption of these ingredients could potentially be harmful (Leacock *et al.,* 2011).

Vitamin B_6 is a water-soluble vitamin that functions as a coenzyme in the metabolism of amino acids, protein and the maintenance of body cells (Cimpoiu *et al.,* 2005). Vitamin B_6 is present as pyridoxine hydrochloride in the multi-vitamin pill and energy drinks. Pyridoxine is a cofactor of several enzymes that catalyze decarboxylations, transaminations and racemizations of amino acids in the human body (Lehne *et al.,* 2001). Vitamin B_6 is widespread in nature especially in foods of both plant and animal origin with meats, vegetables and nuts having the highest concentrations. Therefore, deficiency of vitamin B_6 is not common in humans but when it is consumed at a level above the safe recommended upper limit (100 mg for adult) leads to neurological damage and disorders (Niraimathi *et al.,* 2015). However, in order to meet the normal/acceptable range of 100 mg of pyridoxine per day as proposed by the United States Food and Nutrition Board, humans must acquire Vitamin B_6 from nutrient intake (Medline, 2011).

Caffeine is a xanthine alkaloid and is used as a diuretic and a stimulant in the central nervous system (Wanyika *et al.,* 2010; Gerald *et al.,* 2014). It is absorbed and distributed throughout the body by the circulation of blood flow to a final

destination within the brain (Seifert *et al.*, 2011). Besides being a stimulant and diuretic, there are a variety of unpleasant side effects when its consumption exceed the recommended limit of more than 250 mg per day and this include nausea, vomiting, restlessness, anxiety, depression, tremors and difficulty sleeping (Ortega-Barrales *et al.,* 1998). The normal/acceptable range of caffeine for an average adult is 250 mg per day (Wikoff *et al.,* 2017).

Many methods exist in the literature for the determination of caffeine and vitamin B_6 in various sample mixtures. Some of these methods include UV-visible spectrophotometry (Dobrinas *et al.,* 2013), gas chromatography/mass spectrometry (Muller *et al.,* 2014), micellar electrokinetic chromatography (Meinhart *et al.,* 2010), voltammetry (Švorc *et al.,* 2012) and high performance liquid chromatography (HPLC) (Srdjenovic *et al.,* 2008). Of the above methods, HPLC has become one of the most commonly used analytical methods. However, HPLC procedures often require expensive solvents and sample pre-treatment stages. Thus, spectrophotometric determination in UV-vis region is less expensive, follows a simple procedure, and gives a high accuracy and reproducibility results (Atomssa and Gholap, 2011).

Therefore, this research work focused on the validation of a simple and specific UV spectrophotometric technique for the quantification of caffeine and vitamin B_6 contents in some common beverages, energy/soft drinks and herbal products with the objective to establish a quick and reproducible method for the routine determination of caffeine and vitamin B_6 in these products.

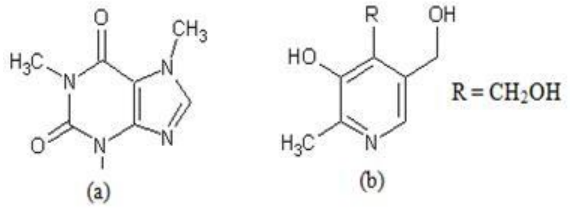

Figure 1: Structure of Caffeine (a) and Vitamin B_6(b)

MATERIALS AND METHODS

Description of Samples Used

Ten (10) commercial samples (Table 1) made up of two commonly consumed soft drinks, two popular herbal mixtures, two beverages and four commonly consumed energy drinks. All samples used were purchased from local grocery stores in Ijebu-ode, Ogun State, Nigeria.

Table 1: Samples Selected for Caffeine and Vitamin B_6 Estimation

S/no	Sample	Sample code	State of the sample
1	Soft drink	SOF 1301	liquid
2	Soft drink	SOF 1302	liquid
3	Herbal formulation	HER 1304	liquid
4	Herbal formulation	HER 1305	liquid
5	Beverage	BEV 1307	solid
6	Beverage	BEV 1308	solid
7	Energy drink	ENE 1309	liquid
8	Energy drink	ENE 1310	liquid
9	Energy drink	ENE 1311	liquid
10	Energy drink	ENE 1312	liquid

Chemicals and Reagents

Caffeine and pyridoxine hydrochloride reference standard were obtained from Sigma Aldrich (Milan, Italy), dichloromethane and Hydrochloric acid were all obtained from Merck Ltd (Darmstadt, Germany). Double distilled water was used for preparing all solutions.

Equipment

A JENWAY- SPEC/6400, 520 ×330 × 180 mm: Rs 232 output, band width of 5 nm Scanning Visible Spectrophotometer with recording unit and matched set of 1 cm quartz cuvettes were used for this study. Samples were weighed using a Shimadzu-AUX-220model digital electronic balance.

Preparation of Standard Solutions

One mg/ml stock standard of caffeine was prepared by dissolving 198.2 mg of caffeine in 200 ml purified water. Working standards were prepared by pipetting 2.5, 5, 7.5, 10 and 12.5 ml aliquots of the stock standard solution into separate 50.0 ml volumetric flasks and diluting to volume with deionized water to prepare the final working concentrations of 50, 100, 150, 200 and 250 µg/ml. Similarly, solution of Vitamin B_6 was prepared by weighing 20 mg of the standard then dissolved in 0.1 N HCl in a 100 ml volumetric flask and made up with the same solvent to produce 200 µg/ml stock solution. The stock solution was further diluted to obtain 20 µg/ml from which working standards were prepared by pipetting 10, 20, 30, 40 and 50 ml aliquots into separate 50.0 ml volumetric flasks and diluting to volume with distilled deionized water to prepare the final working concentration of 4, 8, 12, 16 and 20 µg/ml. The absorbance of each solution was measured at 272nm and 290 nm respectively for caffeine and vitamin B_6. The absorbance values were then plotted against concentrations to generate a calibration curve. All analyses were performed in triplicate.

Extraction of Caffeine and Vitamin B_6 from the Samples

Caffeine

Exactly 200 ml boiling distilled water was added to each of two 250 ml beakers containing 1 g solid (BEV 1307-1308) or 200 ml of samples in liquid form (SOF 1301-1302, HER 1304-1305 and ENE 1309-1312). The mixtures was stirred for 30 seconds using a magnetic stirrer and allowed to cool to room temperature. A 50 ml aliquot of the each solution was placed separately in a separating funnel and 25 ml of dichloromethane was added to extract the caffeine by inverting the funnel at least three times, venting the funnel after each inversion. The dichloromethane layer was decanted to a clean flask and the extraction procedure was repeated twice. The principle of this procedure is based on the increased solubility of caffeine in dichloromethane (140 mg/ml) compared to boiling water (22 mg/ml) (Atomssa and Gholap, 2011). The absorbance of the dichloromethane phase was measuredat 272 nm. The caffeine levels of the samples were extrapolated from the prepared standard curve as described.

Vitamin B_6

Twenty milligram of solid (BEV 1307-1308) or 60 ml of liquid samples (SOF 1301-1302, HER 1304-1305 and ENE 1309-1312) was added into a 125 ml Erlenmeyer flask, filtered and degassed by sonicating for five minutes. A 10 ml aliquot of the degassed sample was placed in each of five 100 ml volumetric flasks which were then made up using 0.1 N HCl after which absorbance was measured at 290 nm.

The final caffeine and vitamin B_6 contents per milligram of all the samples determined were then calculated using equation 1.

$$\text{Analytecontent (mg)} = \text{concentration (µg/ml)} \times \frac{(Total\ sample\ volume\ (ml))^2}{Measured\ sample\ volume\ (ml)} \times 1000 \quad (1)$$

Method Validation

The experiment was carried out according to the official specifications of Global Quality Guidelines

2002 (Global Quality Guideline, 2002) and international conference on harmonization (FAO, 1997). The method used was validated based on the following parameters: suitability, specificity, range and linearity, sensitivity (LOD and LOQ), accuracy and precision.

Accuracy

Recovery experiments were carried out by spiking a known amount of caffeine and vitamin B_6 to pre-analysed samples at three different concentrations and the percentage recovery is calculated using equation (2).

$$\frac{w}{z} \times 100 \qquad (2)$$

Where, w is amount of analytes (caffeine and vitamin B_6) taken while z is amount of analytes found.

Precision

Method precision was evaluated by determining the intra-day and inter-day relative standard deviation of the measured concentrations of caffeine and vitamin B_6. The reproducibility (intra-day precision) and repeatability of system (inter-day precision) was checked by measuring the absorbance of different concentrations of standard solution on the same day and on different days respectively under the same experimental conditions.

Linearity

Linearity of the methods was determined by constructing calibration curves from the absorbance of standard solutions of caffeine and vitamin B_6 at different concentrations level.

Sensitivity

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated from the calibration lines that defined linearity, using the Long and Winefordner criterion as expressed in equations (3) and (4).

$$LOD = \frac{3\,S}{a} \qquad (3)$$

$$LOQ = \frac{10\,S}{a} \qquad (4)$$

Where *a* is the slope of the calibration line and S is the standard deviation of response.

Statistical Analysis

All presented data are means ± standard error of the mean of three independent measurements using statistical package for social sciences software, version 18 (SPSS Inc., Chicago, IL, USA) Test of significance was done at $p < 0.05$. Origin software version 18 was used for the plotting of bar graphs while Microsoft excel software 2013 was used for plotting of calibration curves.

RESULTS AND DISCUSSION

Linearity and Sensitivity

Absorbance responses of standard vitamin B_6 and caffeine were significantly linear from 4 – 20µg/ml and 50µg/ml- 250µg/ml respectively. Therefore, the regression model represents the data correctly. The slopes of the standard calibration lines for both caffeine and vitamin B_6 were 0.006 and 0.002 respectively. Thus, the method used is sensitive enough to detect both analyte at low concentrations of 0.006 and 0.002 µg/ml (Figures 2 and 3). The obtained LOD and LOQ values for caffeine and vitamin B_6 were 0.0155 µg/ml and 0.0518 µg/ml, 0.192 µg/ml and 0.640 µg/ml respectively.

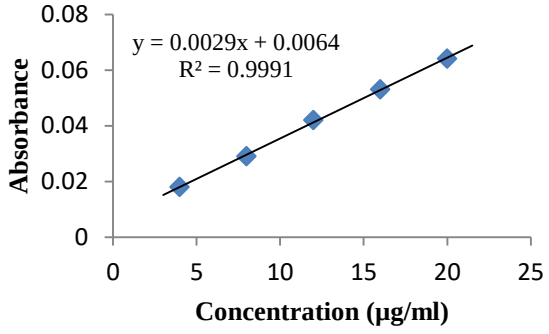

Figure 2: Standard Calibration curve obtained from absorbance of Vitamin B_6 standard solutions.

Precision

As presented in Table 2, the intraday and inter-day relative standard deviation (RSD) values of the system were less than 2 for the two analytes. This shows the reproducibility of the values obtained from the instrument with little or no interferences.

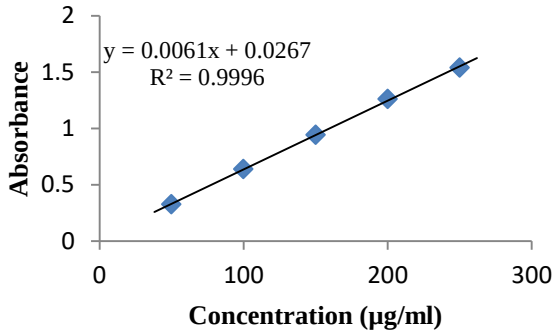

Figure 3: Standard Calibration curve obtained from absorbance of Caffeine standard solutions

Accuracy

The result of accuracy study is presented in Table 3. Data on recovery studies conforms with expected values for quantitative estimation of caffeine and vitamin B_6 in the samples as the statistical parameters are within the acceptance range (RSD < 2.0).

In this work, we present a simple validated and reliable spectrophotometric technique for determination of caffeine and Vitamin B_6 in beverages, energy/soft drinks and herbal products. The technique was rapid and easy to perform. Moreover, the method is sensitive enough to detect analyte in the presence of sample matrices even at a low concentration due to good analytical parameters like linearity, sensitivity (LOD and LOQ), and precision with a good recovery studies (accuracy) Table 4. Dobrinas *et al.* (2013) use molecular absorption spectra in the visible and ultraviolet region of spectrophotometric method for caffeine analysis in tea, coffee and other beverages, some of the solvents used are not environmental friendly and also not cost effective. The sensitivity in terms of LOD and LOQ are not sensitive enough when compared to the present study and accuracy in form of recovery was not determined although, the precision of the method is less than 2. Niraimathi *et al.* (2015) introduced 1st and 2nd derivative spectrophotometry using methanol as a solvent for estimation of vitamin B_6 in pharmaceutical oral dosage form by UV-spectrophotometry.

Table 2: Intra- and inter-day instrument precision validation for spectrophotometric determination of caffeine and vitamin B_6

	Intraday		Interday	
Analyte	Mean±SD	RSD (%)	Mean±SD	RSD (%)
Caffeine	25.62 ± 0.008	0.56	25.59±0.009	0.70
Vitamin B6	2.62 ± 0.002	1.52	2.60± 0.003	1.65

Table 3: SpectrophotometricMethod Accuracy for Determination of caffeine and vitamin B_6 extracted from Selected Samples

Amount of Sample spiked (µg/ml)	Amount of caffeine found (µg/ml)	Recovery± RSD (%)	Amount of vitamin B_6 found(µg/ml)	Recovery± RSD (%)
50	49.74	99.48± 1.24	49.98	99.96± 1.92
100	99.78	99.78± 1.02	99.94	99.94± 1.73
200	202.83	101.42± 0.98	204.70	102.35± 1.54

RSD = relative standard deviation

This method is based on the simultaneous use of 1st and 2nd derivative spectrophotometry ratio spectra and measurements of derivative ratio analytical signals corresponding to the crossing points of wavelengths. Although the method gave a good precision and accuracy but the whole procedure is complex and not as simple as the present study and also the sensitivity of the method is not determined. The second and third order derivative spectrophotometric method was also used for the determination of caffeine in cola, coffee, and tea (Alpdogan *et al*., 2002). This method was applied without any separation. Apart from the complexity of the method, the sample matrices may interfere with the analyte which may have effect on the results. The summary of comparison of the present analytical assay with other reported methods in the literature is presented in Table 4. The validated method was used to determine the concentration of caffeine and vitamin B_6 in selected beverages, energy/soft drinks and herbal products samples. Figures 4 and 5 presents the milligram amounts of extracted caffeine and vitamin B_6 from respective samples. Amount of Caffeine is higher in ENE 1311 (118.30± 0.73 mg), followed by energy drink ENE 1310 (88.25± 1.29 mg). The lowest level of caffeine is found in HER 1304 (33.78± 0.026 mg). The amount of vitamin B_6 is higher in HER 1305 (31.13 ± 0.037 mg) while the least amount of vitamin B_6 was found in ENE 1311 (2.5 ± 0.16 mg).

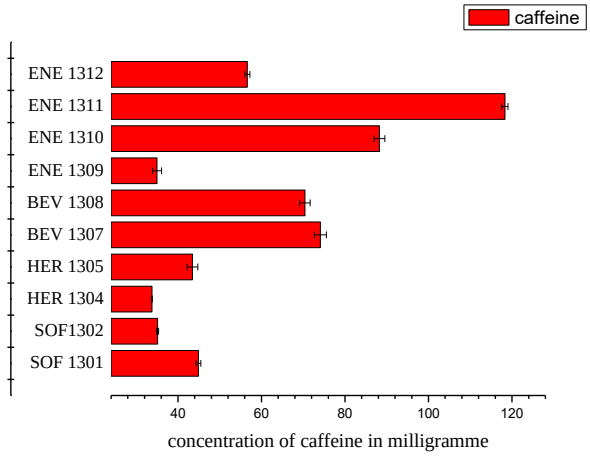

Figure 4: Concentration of Caffeine in the samples

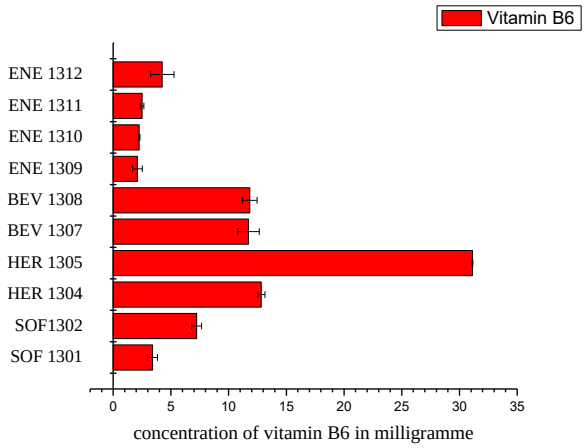

Figure 5: Concentration of Vitamin B_6 in the samples

CONCLUSIONS

In conclusion, the UV spectrophotometric method for vitamin B_6 and caffeine in the selected samples has a detection and quantification limits of 0.192 µg/ml and 0.640 µg/ml and 0.0155 µg/ml and 0.0518 µg/ml respectively with method linearity ranging from 4-20 µg/ml and 50 - 250 µg/ml which showed reliability of the method on quantification of the analytes present in the samples. UV spectrophotometric analyses could be a potential and valuable tool for industrial quality control of caffeine and vitamin B_6 in beverages, energy drinks and herbal formulations

Table 4. Comparison of the present analytical assay with other reported methods for the determination of caffeine and vitamin B_6using spectrophotometric technique

Analytical Parameters	Present Method	Dobrinas *et al.,* 2013	Amos-Tautua *et al.,* 2014	Niraimathi *et al.,* 2015	AbdulKadir, 2010
Method	simple spectrophotometric	spectrophotometric	uv-spectrophotometry	1st and 2nd derivative spectrophotometry	spectrophotometric
Solution	Dichloromethane, HCl	H_2SO_4, $Zn(CH_3COO)_2$, $K_4[Fe(CN)_6]$	carbon teterachloride	Methanol	Diazotized p-Nitroaniline
Analyte	caffeine and Vitamin B_6	caffeine	Caffeine	Vitamin B_6	Vitamin B_6
λ max (nm)	272 and 290	273.5	270	291	480
Linearity range (µg/ml)	50 -250& 4-20	3-18 mg/L	10-60	10-40	5-500 µg/25 ml
LOD and LOQ (µg/ml)	0.026,0.496 and0.192, 0.640	0.85 & 1.52 mg/L	_____	_____	_____
Acuracy (%)	104-112	_____	_____	99.15-100.85	99.63-100.29
Precision	< 2	< 2	_____	< 1	≤ ± 0.98
Sample	beverages, energy/soft drinks & herbal products	Tea, coffee & other beverages	Soft & energy drinks	Pharmaceuticals (tablets)	Pharmaceuticals (tablets)

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