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Eurasian Journal of Analytical Chemistry, 2018, 13(5), em40
ISSN:1306-3057
Research Paper https://doi.org/10.29333/ejac/93422

High Performance Liquid Chromatographic Methods for Analysis of Taurine in Energy Drinks after Pre-column Derivatization

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Received 16 April 2018 ▪ Revised 26 May 2018 ▪ Accepted 20 June 2018

ABSTRACT

Simple and efficient high performance liquid chromatography (HPLC) with photodiode array (PDA) and fluorescence (FLD) detection methods have been validated for determination of taurine in energy drinks. These methods are based on pre-column derivatization of taurine with 4-chloro-7-nitrobenzo-2-oxa-1,3- diazole (NBD-Cl) at alkaline medium to form colored fluorescent product. In the both validated HPLC methods, the derivatization product is separated on Intersil ODS-3 analytical column with acetonitrile and 0.1% trichloroacetic acid (30:70, v:v) as mobile phase. The eluted derivative is detected at 472 nm for HPLC-PDA and 472 nm/530 nm (Ex/Em) for HPLC-FLD. The methods were validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy (recovery). Good linearities were achieved for taurine (r^2> 0.9998 and 0.9993) in the concentration range of 5-50 mg L^{-1} and 5-50 µg L^{-1} for HPLC-PDA and HPLC-FLD respectively. The LODs were 0.296 and 0.616 × 10^{-3} mg L^{-1} for HPLC-PDA and HPLC-FLD respectively. The precision for peak area were 0.78 and 0.61% for HPLC-PDA and HPLC-FLD respectively. Recoveries of taurine ranging from 92-103.3%), (n = 3) were obtained. The validated method was successfully applied for the determination of taurine in some selected energy drinks available in local markets.

Keywords: taurine, NBD-Cl, HPLC, energy drinks, derivatization

INTRODUCTION

Energy drinks are type of non-alcoholic functional beverage that increase alertness and enhance the psycho-physiological responses in human [1]. The main ingredients of energy drinks are caffeine, taurine, vitamins, carbohydrates, and other ingredients such as tyrosine, citicoline and guarana [2]. These compounds stimulate brain activity, memory and attention [3].

Taurine (2-aminoethanesulfonic acid) is a free sulfur-containing β-amino acid that distributes widely in biological fluids and tissues of many mammalians [4]. Taurine is one of the most common free amino acid naturally present in the diet [5,6]. It is the main component of many energy drinks as a tonic medicine [7]. Taurine has several essential physiological functions such as; anti-inflammatory properties, formation of bile salts, acts as an antioxidant, membrane stabilization, and osmosis, liver detoxication by binding harmful substances, protection against glutamate cytotoxicity, neurotransmitter and a modulator of intracellular ions levels [8, 9]. Studies have shown that high concentration of taurine can induce side effects such as; declining heart rates while arterial blood pressure is increased (with caffeine), hypoglycemia, dizziness, diarrhea, peptic ulcer, influence defects in nerve blood flow and dehydration [6, 10]. The combination of taurine with caffeine, amino acids and guarana in energy drinks increases the risk of side effects [6]. Hence, it is essential to control the maximum limits of taurine in beverage and food. The European Authority for Food Safety reported that, acute health and fatal problems occurred due to

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the high consumption of energy supplements [3]. Therefore, development and validation of accurate analytical methods for analysis of taurine in energy drinks is very important issue. Many analytical methods have been described for the measurement of the amount of taurine in food. The Majority of these methods based on high-performance liquid chromatography (HPLC) with different detection systems. Due to the lack of chromophoric group in taurine molecule, most of the chromatographic methods of its analysis involves pre- or post-column derivatization to enhance its detectability using ultraviolet (UV), visible or fluorometric detection. HPLC with electrochemical, refractive index detection and mass spectrometry [7, 11] were also reported. Methods using techniques such as capillary electrophoresis [12], nuclear magnetic resonance spectroscopy (H1-NMR) [13] , UV-spectrophotometric methods [10,14] and spectrofluorimetric [15] were also found in the literature. However, most of these methods have drawbacks, such as they require expensive equipment and derivatizing reagent [7,9] or have low sensitivity [12,14].

Several pre-column derivatizing agents have been used for the determination of taurine such as 2,4-dinitrofluorobenzene (DNFB) [9] , *o*-phthalaldehyde (OPA) [16] , 4-(5,6-dimethoxy-2-phthalimidinyl)-2-metho and xyphenylsulfonyl chloride [17] and fluorescamine [18]. However these derivatizing agents have some drawbacks such as time-consuming [9,12]. DNFB method either applied tedious time-consuming procedures or offered low sensitivity values [9]. The major problem with OPA derivative is its instability, which affect the performance of the HPLC method [16].

4-(5,6-Dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride and fluorescamine methods required complicated pretreatment procedure [17]. 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) is an extremely sensitive labeling agent that have been used for the determination of primary and secondary amino acids and some drugs [12]. NBD-Cl react amino group to form stable condensation colored products [19]. NBD-Cl has advantages such as low cost, it can be used for fluorescent labeling of amino acids and UV detection, and this reagent produces low number of byproducts [20].

Most of the reported methods for taurine determination used expensive and complicated instrumentations that are not available in most developing countries quality control laboratories. For this reason, the development and validation of simple detection procedures for taurine in energy drinks are mandatory. HPLC with PDA and FLD detection is general applicable and available in many analytical laboratories.

In Sudan there are a few works done in the area of determination of taurine in energy drinks. Therefore, in this research paper a simple and accurate HPLC with PDA and FLD methods after pre-column derivatization with NBD-Cl were validated and applied for quantification of taurine amount in some selected energy drink samples available in local markets in Khartoum state.

MATERIALS AND METHODS

Samples

A total of five samples of energy drinks namely red bull, tornado, kratingdeang, bison and tiger were purchased from local supermarkets in Khartoum, Sudan. All samples were stored at refrigerator until opened for analysis and the content of taurine was measured compared to the labeled amount in the samples.

Standards, Reagents and Materials

Taurine purity (≥99 %) and 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) purity 98% were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium chloride was obtained from CDH (New Delhi, India). Potassium hydroxide pellets was from Lab Tech Chemical (India). Boric acid was purchased from VWR International (Leuven, Belgium). Methanol (99.8%) was supplied by chem-lab NV (Belgium). Water was purified with Daihan Lab Tech (Kyonggi, Korea). Acetonitrile HPLC grade was purchased from Duksan (Korea). Trichloroacetic acid was from Scharlau (European Union). Hydrochloric acid (37%) was purchased from Sham Laboratory (Addra, Syria).

Preparation of Standards and Solutions

Stock solution of taurine at concentration of 1000 mg L^{-1} was prepared by dissolving 0.05 g taurine in distilled water in a 50 mL volumetric flask and made up to the mark with distilled water.It was appropriately diluted to prepare intermediate standard solutions at concentrations of 500 mg L^{-1} and 0.5 mg L^{-1} for HPLC-PDA and HPLC-FL detection, respectively.

NBD-Cl solution (0.05%) was prepared by dissolving 0.0125 g of the reagent in methanol then transferred to a 25 mL volumetric flask and the volume completed to the mark with methanol. This solution was stable for one week when kept in refrigerator in amber container bottle.

The borate buffer (5 mM) was prepared by dissolving 0.31 g boric acid and 0.29 g of sodium chloride in 80 mL of distilled water and the pH adjusted to 10 with 1.0 M potassium hydroxide then the volume was completed to 100 mL with distilled water.

Samples Preparation

An aliquot of 20 mL of each sample was poured into a 100 mL beaker and degassed for 30 min in a Bandelin Sonorex ultrasonic bath (Berlin, Germany). Then the pH was adjusted to pH 7.0 potassium hydroxide (1.0 M). The samples were diluted with distilled water until the concentrations of taurine were 200 mg L^{-1} and 0.2 mg L^{-1} for HPLC-PDA and HPLC-FL, respectively based on the labeled amount. Then 0.5 mL of the samples were subjected to derivatization.

Pre-column Derivatization with NBD-Cl of Taurine Standard and Taurine in Energy Drink Samples

The derivatization procedure was done according to previously reported method by Mohamed et. al., 2017, with some modifications. Varied volumes (0.1-1.0 mL) were transferred from intermediate standard solutions (500 mg L^{-1} and 0.5 mg L^{-1}) for HPLC-PDA and HPLC-FL detection, respectively into a series of 10 mL volumetric flasks.An aliquots of 0.5 mL of each diluted sample were transferred to 10 mL volumetric flasks. Then the volumes of standards and samples were adjusted to about 1.0 mL with distilled water. Following addition of 1.0 mL of borate buffer (pH 10) and 1.0 mL of 0.05% NBD-Cl solution. The mixture was heated in IKA thermostatically controlled water bath (WERKE GmbH, Co. KG, Germany) at 70 °C for 35 min in the dark. The reaction was terminated by cooling in ice water. Then 0.2 mL of HCl was added to adjust the pH to 3.5 to prevent the analytical column from damage. Then the volume was brought to 10 mL with distilled water. The mixture was filtered through a 0.45 µm cellulose acetate syringe filter from Thermo Scientific (Mexico) into a HPLC auto sampler vial for analysis.

High Performance Liquid Chromatography (HPLC) Analysis

The separation of taurine derivative was carried out on Waters HPLC with PDA detector (Waters, Milford, MA) and Shimadzu HPLC with fluorescence detector (HPLC-FLD) (Shimadzu Corporation, Kyoto, Japan). The HPLC-PDA consisted of a Waters alliance 2695 gradient separations module equipped with auto sampler and column oven, a Waters 2996 Photodiode array detector. All data were processed using Empower software (Waters, Milford, MA). The HPLC-FL equipped with Prominence LC-20 AD pump, DGU- 20 A3R degassing unit, Prominence SIL-20A autosampler, Prominence CTO-20 A column oven, CBM-20A Communications bus module and RF-20 A fluorescence detector. All data were processed using LC solution software (Shimadzu Corporation, Kyoto, Japan). The chromatographic separation was done using an Intersil ODS-3 (250 mm× 4.6 mm, 5 µm) column (GL Sciences Inc., Japan). The separation was performed at ambient temperature. The mobile phase consisting of acetonitrile and 0.1% trichloroacetic acid (30:70, v:v) with flow rate of 1.0 mL/min and injection volume of 20 µL. The wavelength of PDA detector was 470 nm and the excitation and emission wavelengths of fluorescence detector were 470 nm and 530 nm, respectively.

RESULTS AND DISCUSSION

NBD-Cl is an electroactive halide labeling reagent, which have been used for derivatization of some primary and secondary amine and amino acids. Taurine molecule does not have a chromophore, it has to be derivatized enhance its detectability [19]. In this study, the non-fluorescent NBD-Cl reagent reacted with taurine in the presence of borate buffer (pH 10.0), producing a stable yellow colored adducts. The suggested reaction pathway between taurine and NBD-Cl was shown in **Figure 1**.

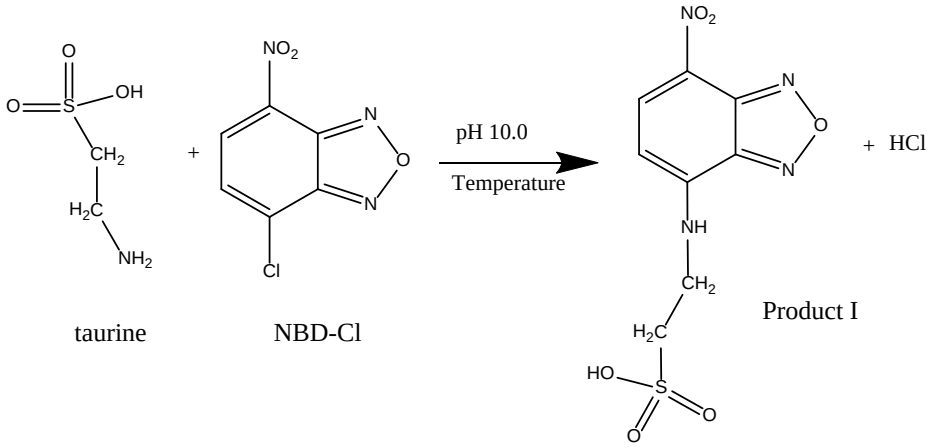

Figure 1. Reaction pathway of taurine with NBD-CL

Table 1. Equations for external calibration curves, regression coefficient, limit of detection (LOD) and limit of quantification (LOQ) for taurine derivative with NBD-Cl using HPLC-PDA and HPLC-FL

Parameter	HPLC-PDA	HPLC-FLD
Concentration range (mg L^{-1})	5-50	0.005-0.05
Equation	Y= 103011x + 33685	Y= 5061.1x + 160.11
Regression coefficient (r^2)	0.9998	0.9993
LOD (mg L^{-1})	0.296	0.616 ×10^{-3}
LOQ (mg L^{-1})	0.888	1.847 ×10^{-3}

Validation of the Methods

To ensure that this method for determination of taurine after derivatization with NBD-Cl was applicable to real samples, several basic analytical parameters were evaluated, including linearity, LOD, LOQ, precision (intra-day and inter-day repeatability) (RSD%) and accuracy (recovery). All these parameters are determined for both HPLC-PDA and HPLC-FLD analysis.

Linearity

The calibration curves were constructed by plotting taurine derivatives peak areas against concentrations of taurine. For HPLC-PDA measurement, linearity was studied in the concentration range of 5.0-50 mg L^{-1}. As shown in **Table 1** good linearity was obtained for taurine derivative with correlation coefficient (r^2) of 0.9993 (n=6). The concentration range of taurine that was used for HPLC-FLD analysis was 5.0-50 µg L^{-1}. The results show a good linear relationship with coefficient of 0.9998 (n=6).

Limit of detection and limit of quantification

The limit of detection (LOD) and the limit of the quantification (LOQ) were calculated using the external standard calibration curve. The LOD was established using LOD = 3.3×(s/S) and the LOQ = 10×(s/S), where s is the standard deviation of the intercept and S is the slope of the curve. The LOD and LOQ of the methods were estimated to be 0.295, 0.888 mg L^{-1} and 0.615, 1.847 µg L^{-1} for HPLC-PDA and HPLC-FLD, respectively. It was found that HPLC-FLD is more sensitive 480 times than HPLC-PDA analysis. Hence this derivatization method with HPLC-FLD analysis can use for determination of taurine at trace level.

Precision

The precision of this method was tested by intra-day repeatability and inter-day reproducibility as RSD%. The intra-day repeatability was studied by performing six successive injections of 10 mg L^{-1} and 10 µg L^{-1} taurine derivative for HPLC-PDA and HPLC-FLD, respectively. The inter-day reproducibility was determined by nine consecutive injections of the same concentration of intra-day repeatability. As summarized in **Table 2** the RSD of the peak area and retention time for intra-day repeatability and inter-day reproducibility were less than 0.78, 1.34% and 0.25, 0.71%, respectively, which indicate that the precision of the method is satisfactory.

Table 2. Intraday and interday precisions for the determination of taurine derivative with HPLC-PDA and HPLC-FL

Parameter	HPLC-PDA		HPLC-FLD	
	Peak area	Retention time	Peak area	Retention time
Intraday precision (RSD%) (*n*=6)	0.78	0.25	0.61	0.13
Interday precision (RSD%) (*n*=9)	1.34	0.71	1.13	0.52

Table 3. Percentage recovery (n = 3) for determination of taurine in energy drink samples

Sample	HPLC-PDA				HPLC-FLD			
	Sample content (mg L^{-1})	Added amount (mg L^{-1})	Found (mg L^{-1})	Recovery% ± SD (*n*=3)	Sample content (µg L^{-1})	Added amount (µg L^{-1})	Found (µg L^{-1})	Recovery% ± SD (*n*=3)
Tornado	5.0	5.0	10.21	99.7 ± 0.05	10.0	10	20.13	98.2 ± 0.13
	5.0	20.0	25.74	102.2 ± 0.65	10.0	15	25.05	98.2 ± 0.13
	5.0	40.0	45.33	100.2 ± 0.41	10.0	20	30.06	98.9 ± 0.01
Krating-daeng	5.0	5.0	10.20	102.4 ± 0.13	10.0	10	19.87	96.6 ± 0.08
	5.0	20.0	25.26	100.9 ± 0.43	10.0	15	25.12	99.4 ± 0.20
	5.0	40.0	44.68	99 ± 0.15	10.0	20	29.8	98.4 ± 0.06
Bison	10.0	5.0	15.14	99.9 ± 0.25	10.0	10	19.99	99.9 ± 0.06
	10.0	20.0	30.17	100.1 ± 0.59	10.0	15	25.05	98.2 ± 0.13
	10.0	40.0	49.30	97.7 ± 0.80	10.0	20	30.06	98.9 ± 0.01
Tiger	10.0	5.0	15.0	100.1 ± 0.27	10.0	10	20.08	91.9 ± 0.09
	10.0	20.0	29.94	99.7 ± 0.30	10.0	15	25.11	94.7 ± 0.09
	10.0	40.0	49.21	97.8 ± 0.71	10.0	20	30.22	97 ±0.05
Red Bull	10.0	5.0	15.03	103.3 ± 0.14	10.0	10	20.11	99.9 ± 0.15
	10.0	20.0	30.27	102 ± 0.20	10.0	15	25.11	100 ± 0.19
	10.0	40.0	49.6	99.2 ± 0.49	10.0	20	29.93	99 ±0.07

Accuracy (recovery)

For the recovery study, all energy drink samples (red bull, tornado, kratingdeang, bison and tiger) with known taurine concentration were spiked with taurine at levels (5, 20 and 40 mg L^{-1}) and (10, 15 and 20 µg L^{-1}) for HPLC-PDA and HPLC-FL analysis, respectively. Good percentage recoveries (92–103.3%), RSD (0.145-2.53%) were obtained for both HPLC-PDA and HPLC-FLD methods as in **Table 3**.

Application of the method

The validated methods were assessed by analyzing a total of five energy drinks contain taurine (i.e., red bull, tornado, kratingdeang, bison and tiger). **Figure 2** (A-D) presents the chromatograms of taurine derivative (10 mg L^{-1}), Tornado sample (10 mg L^{-1}) and taurine derivative (30 µg L^{-1}) Tornado sample (30 µg L^{-1}), determined by HPLC-PDA and HPLC FL, respectively. As shown in **Table 4**. The obtained concentrations of analyzed energy drinks were found to be not significantly different from the concentration values in the labels. The percentage recovery was less than (106%), this indicate the high accuracy of the two proposed methods (HPLC-PDA and HPLC-FLD) for determination of taurine in energy drinks. The concentrations of taurine in energy drink samples that obtained by the two proposed methods were statistically compared with each other using T and F-tests (**Table 4**). The results obtained showed there was no significant difference between these values at the 95% confidence level this indicated similar accuracy and precision in the two methods.

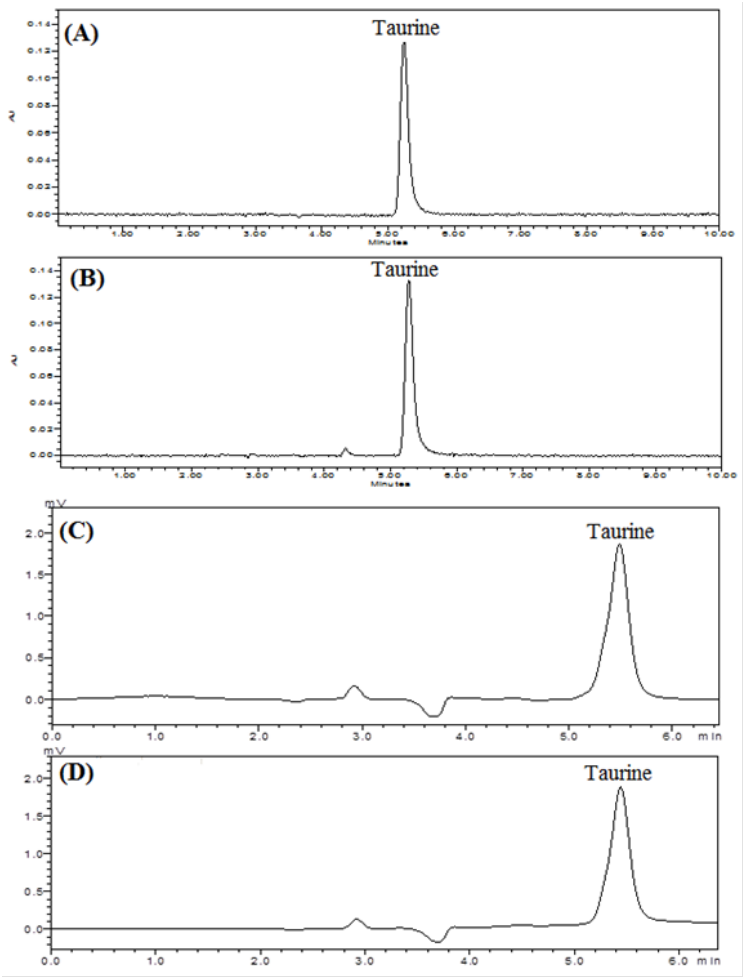

Figure 2. Chromatograms of taurine derivative (A) taurine std. derivative (10 mg L^{-1})by HPLC-PDA, (B) taurine derivative in Tornado sample(10 mg L^{-1}) by HPLC-PDA (C) taurine std. derivative(10 µg L^{-1}) by HPLC-FLD, (D) taurine derivative in Tornado sample(10 µg L^{-1}) by HPLC-FLD

Table 4. Comparison between measured taurine contents in this study and labeled amount of taurine in energy drink samples and values of T and F tests

Sample	Labeled amount in (mg L^{-1})	HPLC-PDA		HPLC-FLD		T	F
		Conc. mg L^{-1} ± SD[a]	Recovery (%)	Conc. mg L^{-1} ± SD	Recovery (%)		
Tornado	100	106 ± 2.12	106	103 ± 0.63	103	1.92	11.31
Kratingdaeng	4000	4005± 3.54	100.1	4002 ± 5.66	100	0.63	0.39
Bison	4000	4008 ± 2.83	100.3	3990 ±7.07	99.7	3.34	0.16
Tiger	3200	3199± 2.12	99.9	3202 ± 1.41	100	1.67	2.26
Red Bull	4000	3996 ± 4.24	99.9	3992 ± 2.83	99.8	1.11	2.24

[a] n=2

Analytical Performance Comparison of Present Work with other Reported Studies

Comparison of the LODs or LOQs obtained with the present work of pre-column derivatization of taurine with NBD-Cl followed by HPLC-PDA and HPLC-FLD detection with the LODs achieved with other high performance chromatographic methods using different derivatization procedures are shown in **Table 5**. Compared with the previously reported HPLC methods for the determination of taurine, the mobile phase used in this proposed method (acetonitrile: trichloroacetic acid) is simpler than the majority of mobile phases used in reported HPLC methods. Most of the mobile phases used in previous HPLC methods for taurine analysis are containing buffer solutions or ion pair reagents which are needed long time to wash them out the analytical column . The sensitivies of the validated methods are higher than the results obtained using HPLC-UV/VIS pre-column derivative with fluorescamine [18], HPLC-PDA direct analysis [22] and direct HPLC with evaporative light scattering detector (ELSD) analysis [23]. The LOD of current HPLC-PDA is comparable with previous HPLC-UV/VIS pre-column derivative with *O*-phtha-aldehyde/ 2-mercaptoethanol [24].

Table 5. Comparison of HPLC conditions, LOD and LOQ of present work with other HPLC methods for taurine analysis published in literature

Chromatographic method	Mobile phase	Column	Derivtization agent	Detection method	LOD & LOQ (mg L^{-1})	Ref
HPLC-UV/VIS pre-column derivative	Tetrahydrofuran-acetonitrile-phosphate buffer (15 mM, pH 3.5) (4:24:72)%	Bondclone C-18 (300 x 3.9 mm, l0 µm)	Fluorescamine	Fluorescence	LOQ: 5.0	[18]
HPLC-UV/VIS with pre-column derivative	Disodium hydrogenphosphate-citric acid (PH 5.4) containing 10mM tetrabutylammonium bromide: acetonitrile (70:30)%	ODS, (150 x 4.6 mm, 5µm)	4-fluoro-7-nitrobenzofurazan (NBDF)	UV-Visible	LOQ: 0.50	[7]
HPLC-UV/VIS pre-column derivative	phosphate buffer (0.05M, pH: 5.3):Methanol (60:40)%	C18 (S10 ODS_2)	O-phtha]aldehyde/ 2-mercaptoethanol	UV-VIS	LOD: 0.3	[24]
HPLC/ESI-MS Direct analysis	Gradient between (A) 5 mM heptafluorobutyric acid and (B) methanol	Spherigel- C18 silica 5µm	-	MS	LOD: 0.01	[11]
HPLC-PDA Direct analysis	orthophosphoric acid :acetonitrile (60:40)%	Inertsil ODS (250 x 4.6 mm, 5 µm)	-	PDA	LOD: 2.76	[22]
HPLC- ELSD Direct analysis	Methanol: water (70:30)%	Astec apHeraTM NH_2 polymer (150 x 2.1 mm, 5µm)	-	ELSD	LOD: 2.0	[23]
HPLC-PDA pre-column derivative	Acetonitrile : 0.1% trichloroacetic acid (30:70)%	Intersil ODS-3 (250× 4.6 mm, 5 µm)	NBD-Cl	PDA	LOD: 0.296 LOQ:0.888	Present work
HPLC-FLD pre-column derivative	Acetonitrile : 0.1% trichloroacetic acid (30:70)%	Intersil ODS-3 (250 × 4.6 mm, 5 µm)	NBD-Cl	Fluorescence	LOD: 0.62×10^{-3} LOQ:1.85×10^{-3}	Present work

CONCLUSIONS

This current work described validation of two simple HPLC methods based on pre-column derivatization with NBD-Cl and detection with PDA and FLD for the quantification taurine in energy drinks. The methods provide good linearities, precision and recoveries. The methods were applied to determine of taurine level in five frequently consumed energy drink samples i.e. red bull, tiger, bison , kratingdaeng and tornado. The obtained concentrations of taurine measured by HPLC-PDA and HPLC-FLD are comparable and do not significantly differ for the taurine amount written in Label of the energy drinks by manufactures. It was found that HPLC-FLD is more sensitive than HPLC-PDA and could be used for determination of taurine at trace level in biological samples.

ACKNOWLEDGEMENTS

All the authors would like to thank the Arab-German Young Academy of Sciences and Humanities (AGYA) for research funding. The financial support from Ministry of Higher Education & Scientific Research, Commission of scientific research and Innovation is gratefully acknowledged.

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