Evaluation of caffeine, vitamins and taurine in energy drinks

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Summary
In this work, a rapid and inexpensive method of high performance liquid chromatography (HPLC) with photodiode array and fluorescence detection was developed to determine ascorbic acid, pyridoxine, thiamine, riboflavin and caffeine simultaneously in energy drinks, using spectrophotometric detection at different wavelengths in a relatively short time. Taurine determination was carried out by HPLC with ultraviolet-visible detector using pre-column derivatization with 2,4-dinitrofluorobenzene. Ultraviolet-visible absorption spectra were used to confirm the identity and purity of pyridoxine, caffeine and riboflavin. Limit of detection and limit of quantification ranged from 0.002 $\mu g \cdot ml^{-1}$ to 2.04 $\mu g \cdot ml^{-1}$ and from 0.008 $\mu g \cdot ml^{-1}$ to 2.12 $\mu g \cdot ml^{-1}$, respectively. Precision (relative standard deviation) was between 1.9 % and 7.8 %. In the energy drinks, caffeine concentration was found to be 252–304 $mg \cdot l^{-1}$, taurine 2180–2750 $mg \cdot l^{-1}$, pyridoxine 13.0–39.8 $mg \cdot l^{-1}$ and riboflavin from not detectable to 16 $mg \cdot l^{-1}$. Ascorbic acid and thiamin were not detected. Labelling of the beverages regarding vitamins, caffeine and taurine contents did not correspond to results of analysis of samples.

Keywords
energy drink; ascorbic acid; vitamins B; caffeine; taurine; high performance liquid chromatography

The term energy drinks has been used for convenience, to encompass a category of beverages that appeared on the European market in recent years. These contain various combinations of substances. The term refers to beverages meant to "boost energy" by stimulating the nervous system [1]. Caffeine is the major active component of these drinks, and the risks derived from its consumption was already extensively studied [1–7]. In addition to caffeine, energy drinks contain high amounts of taurine, B vitamins, particularly pyridoxine (vitamin B_6) and riboflavin (vitamin B_2), simple sugars, electrolytes and/or herbal preparations. The consumption of energy drinks has markedly increased in the past few years, becoming very popular among college students [1, 8–10].

Both scientific community and consumers have a great concern about the increase in the consumption of energy drinks and its potential adverse health effects. The most common reasons for energy drink consumption include counteracting sleepiness and "increasing energy", maintaining alertness while studying or driving, and reducing symptoms of hangover [2–11], to increase the energy level in compensation for lack of sleep, or to mix with alcohol. The effects of single and repeated doses of caffeine consumed within a day on the central nervous system were assessed in adults (sleep, anxiety, perceived exertion during exercise and subjective perception of alcohol intoxication) and children (sleep, anxiety and behavioural changes) [2, 3, 7, 11–14]. According to a recent study of the European Food Safety Authority (EFSA) [9] on energy drinks consumption, 13.3 % of young adults (18–29 years) consume energy drinks at least 4 or 5 times a week with an estimated intake of 4.5 l per month per person [3, 14]. Given the current context of the lack of regulation of energy drinks marketing and availability coupled with their popularity, energy drinks consumption may pose a substantial risk of harm to adolescent health [1, 5, 7, 8, 13–16]. The Directive 2002/67/EC [17], establishes that beverages containing caffeine in excess of 150 $mg \cdot l^{-1}$ must

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include the message "high caffeine content", followed by the amount of caffeine expressed in milligrams per 100 ml, on the label.

The common methods for determination of caffeine, B vitamins and taurine in energy drinks are summarized in Tab. 1 [12, 18–33].

The aim of this study was to determine the actual composition of energy drinks regarding compounds with possible toxic effects. Energy drinks are a new type of beverage, not well defined, whose consumption is increasing considerably. High performance liquid chromatography (HPLC) using photodiode array detector (PDA) and fluorescence (FL) detection was proposed for determination of ascorbic acid, pyridoxine, thiamin, riboflavin and caffeine, because it is simple, the sample is directly injected into the chromatograph and simultaneous determination of several compounds is possible in a relatively short time. Moreover, such methods are widely described in the scientific literature. Taurine determination was achieved by means of HPLC with ultraviolet detection (HPLC-UV) after pre-column derivatization with 2,4-dinitrofluorobenzene, which is also a widely used analytical method.

MATERIALS AND METHODS

Pyridoxine (vitamin B_6) hydrochloride, L-sodium ascorbate, thiamine (vitamin B_1) hydrochloride, riboflavin, caffeine, taurine, sodium hexanesulfonate, sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3) and 2,4-dinitrofluorobenzene (DNFB) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Sodium dihydrogenphosphate (NaH_2PO_4), disodium hydrogenphosphate (Na_2HPO_4) and acetonitrile were obtained from Merck (Darmstadt, Germany). Dimethylsulfoxide (DMSO) was obtained from Fluka (Buchs, Switzerland). Ultrapure water was used for preparation of all solutions (Milli Q System; Millipore, Bedford, Massachusetts, USA).

The concentration ranges of solutions of calibration standards for each analyte were set taking into account specifications declared on the label by manufacturers. pH was measured by using a Crison pH meter (Crison Instruments, Barcelona, Spain). Electrical conductivity of the samples was determined using a Conductivity Meter (Radiometer, Copenhagen, Denmark).

Determination of vitamins and caffeine

The method used was according to Gliszczyńska-Świgło and Rybicka [27] with some modifications. The HPLC system consisted of a PU-2089 Plus quaternary pump (Jasco, Tokyo, Japan), a 50 μl injector Rheodyne 7125NS (Rheodyne, Cotati, California, USA) and a column Kinetex C18 (15 cm × 0.46 cm, particle size 2.6 μm; Phenomenex, Torrance, California, USA), with a PDA Thermo Scientific Spectra System UV8000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a Spectra System FL2000 fluorescence detector (Spectra-Physics, Santa Clara, California, USA) in series. The HPLC system was controlled by ChromQuest software (v. 5.0; Thermo Fisher Scientific). The column temperature was maintained at 30 °C. A gradient of mobile phase consisting of methanol (solvent A) and 0.05 mol·l^{-1} NaH_2PO_4 containing 0.005 mol·l^{-1} hexanesulfonic acid, pH 3.0 (solvent B) was used according to the following program: linear increment starting with 10 % A (3 min), to 20 % A for 3.5 min, to 40 % A for another 3 min, return to 35 % A for 15 min and return to initial conditions within the next 5 min. The flow rate was 0.8 ml·min^{-1}. The eluates were analysed using PDA detector operating at 245 nm and 270 nm, and a fluorescence detector at excitation wavelength of 290 nm and emission wavelength of 390 nm.

Determination of taurine

Taurine was determined by HPLC according to Orth [29].

Derivatization procedure

A 10 mmol·l^{-1} carbonate buffer solution pH 9 was made with $NaHCO_3$ and Na_2CO_3, diluted with water, added hydrochloric acid drop-wise until pH 9 was reached. Into a test tube, 1.0 ml of sample, 2.0 ml of the carbonate buffer, 0.5 ml of DMSO and 0.1 ml of DNFB were added. The solution was shaken for 30 s and placed in a 40 °C water bath for 15 min. Finally, 6.5 ml of phosphate buffer were added to the mixture. The phosphate buffer solution (10 mmol·l^{-1}, pH 6) was prepared with NaH_2PO_4 and Na_2HPO_4 diluted with water and then added NaOH until pH 6 was reached.

The HPLC system was equipped with a PU-1580 quaternary pump (Jasco), a 20 μl injector Rheodyne 7125NS, a column Tracer Kromasil C18 (25 cm × 0.4 cm, particle size 5 μm; Teknokroma, Barcelona, Spain) and an ultraviolet-visible (UV-Vis) detector Spectra-Physics (Santa Clara, California, USA). The HPLC system was controlled by ChromPass Chromatography Data System for Windows (v. 1.7.403.1) software (Jasco). The column temperature was maintained at 33 °C with a SP8792 Controller (Spectra-Physics). Isocratic elution of mobile phase consisting of phosphate

Tab. 1. Methods used in the bibliography for determination of compounds.

Compound	Method	Detection	Reference
Caffeine	Spectroscopy	Ultraviolet-visible	Dobrinas et al. [18] Bhawani et al. [19] Khalid et al. [20]
Caffeine and B vitamins	Derivative spectroscopy	Ultraviolet-visible	Pieszko et al. [21]
Caffeine	Spectroscopy	Fluorescence	Smith et al. [22]
Caffeine	Spectroscopy	Fourier transform-near infrared	Rácz et al. [23]
Caffeine	HPLC	Photodiode array	Nour et al. [24]
Caffeine	HPLC	Ultraviolet-visible	Patil [25]
Caffeine and taurine	HPLC	Ultraviolet-visible	Rai et al. [26]
Caffeine and water-soluble vitamins	HPLC	Photodiode array fluorescence	Gliszczyńska-Świgło and Rybicka [27]
Vitamin B6 and B2	HPLC	Fluorescence	Marti-Andres et al. [28]
Taurine	HPLC	Ultraviolet-visible	Orth [29] Sawabe et al. [30]
Taurine	Spectroscopy	Ultraviolet-visible	Draganov et al. [31]
Taurine	Spectroscopy	Luminescence	El Din and Wahba [32]
Taurine	UPLC	Mass spectrometry	Ricciutelli et al. [12]
Caffeine and taurine	MISER liquid chromatography	Mass spectrometry	Welch et al. [33]

HPLC – high performance liquid chromatography, UPLC – ultra performance liquid chromatography.

buffer (80 %) and acetonitrile (20 %) was used with a flow rate of 1 ml·min^{-1}. The eluate was analysed spectrophotometrically at 360 nm.

Identification and quantification of compounds

Identification of the peaks was conducted by comparison of their retention times (*RT*) to those of corresponding to standards. Additionally, UV-Vis absorption spectra measured using the PDA detector were used to confirm the identity and purity of the compounds. Quantification was conducted using an external standard method. The limit of detection (*LOD*) and limit of quantification (*LOQ*) were calculated as three- and ten-fold signal-to-noise-ratio, respectively. Instrument precision was checked from six consecutive injections of a sample. Results in Tab. 2 demonstrate good linearity in the utilized range, together with *LOD*, *LOQ* and relative standard deviation (*RSD*) values. Five-point calibration curves ($n = 3$) were prepared with aqueous solutions of standards at the levels covering those in the assessed samples.

Sample preparation

Six liquid samples of energy drinks of different producers (marked as ED1–ED6) were purchased in several supermarkets of Santiago de Compostela (Spain) in January 2017. The samples presented different volumes and were packed into aluminium. The main ingredients of the energy drinks can be heterogeneous and varied, i.e. water, carbon dioxide, caffeine, taurine, B vitamins, sugars, glucuronolactone, flavourings, colours, acidity regulators (sodium citrate), preservatives (citric acid, sodium benzoate, benzoic acid, sorbic acid, etc.), emulsifiers, stabilizers, but caffeine is always present. In light energy drinks, as samples ED3 and ED5, sugars are replaced with non-nutritive sweeteners. The liquid energy drinks were analysed directly after ultrasonic degassing for 10 min. For analysis of vitamins, the sample was directly injected in the chromatograph. For caffeine analysis, the drinks were diluted 1:10 with ultrapure water. For taurine analysis, the drinks were diluted 2:10 with ultrapure water and then derivatized. Samples were analysed in triplicate.

RESULTS AND DISCUSSION

In this work, HPLC method with PDA-FL detectors in series was proposed for simultaneous separation and determination of caffeine and principal water-soluble vitamins present in energy drinks, namely, ascorbic acid, pyridoxine, thiamin and riboflavin. Ascorbic acid ($RT = 1.9$ min) and thiamine ($RT = 7.8$ min) were quantified using a PDA detector set at 245 nm; caffeine ($RT = 9.9$ min) and riboflavin ($RT = 10.7$ min) were quantified using a PDA detector set at

270 nm and pyridoxine (RT = 5.4 min) was quantified using a fluorescence detector at an excitation wavelength of 290 nm and emission wavelength of 390 nm.

Riboflavin, although also detectable by fluorescence using an excitation and emission wavelengths of 450 nm and 530 nm, respectively, was quantified using PDA detector at 270 nm due to its higher sensitivity.

Taurine, in basic solution (pH 9) and in presence of DNFB, forms a dinitrophenyl derivative of a bright yellow colour, with an absorption maximum of approximately 360 nm (RT = 5.7 min). DNFB is only slightly soluble in water and the addition of DMSO speeds up the reaction considerably. To further accelerate the reaction, this was performed in a water bath at 40 °C.

Linear correlation coefficients of standard curves for all compounds were not lower than 0.9909. The results are shown in Tab. 2.

The energy drinks analysed had acidic pH (3.25 ± 0.60), similar to those studied by other authors [20, 26, 34]. The acid values, expressed as pH, can give us indirect information about the effect that these energy drinks may have on the teeth enamel, taking into consideration that the critical limit of this parameter is pH 5.5 [34, 35]. The conductivity values were found to be 1.71 ± 0.40 $S \cdot m^{-1}$. These conductivity values indicated important presence of minerals [35].

The validated methods (Tab. 2) were applied to 6 different energy drinks. Tab. 3 shows the declared amount of pyridoxine, caffeine, riboflavin and taurine, and the values determined in this study using the proposed HPLC-PDA-FL and HPLC-UV-Vis methods. Ascorbic acid and thiamine were not detected. UV-Vis absorption spectra measured using the PDA detector were used to confirm the identity and purity of pyridoxine, caffeine and riboflavin.

The found concentrations of caffeine were in the range 252–304 $mg \cdot l^{-1}$, higher than in cola-type beverages. Caffeine is the component always present in these beverages [1]. Generally, the caffeine concentrations determined (Tab. 3) were slightly lower than those indicated on the label but higher than 250 $mg \cdot l^{-1}$.

In the European Union, there is no maximum limit for caffeine intake but, in energy drinks, the information "high caffeine content" is required on the label [17]. EFSA [36] proposed, for a 70 kg adult, a safety level of 3 mg of caffeine per kilogram of body weight (bw) per day, that can be applied to children, although there are no sufficient studies available on the acute effects of this compound about behaviour in children and adolescents. Like for adults, caffeine doses of about 1.4 $mg \cdot kg^{-1}$ bw may increase sleep latency and reduce sleep duration in some children and adolescents, particularly when consumed close to bedtime.

Various studies indicated a relation between the consumption of energy drinks and an increased risk of adverse health effects involving diabetics, cardiovascular diseases and diseases of the central nervous system in a number of case reports, particularly when the drinks were consumed within short periods of time, at high doses and in combination with alcohol and/or physical exercise [3, 4, 6, 7]. High consumption of energy drinks may promote weight gain if the energy intake from consumption of energy drinks is not carefully considered as part of the total daily energy intake [5].

The Committee noted that the possible interactions of constituents of "energy" drinks" had not been well studied and considered that the possible interactions between caffeine, taurine and alcohol might warrant investigation in humans, particularly under conditions of exercise and consequent dehydration through sweating [8, 36–39]. Although energy drinks contain a number of nu-

Tab. 2. Calibration parameters obtained for the analysed compounds.

Compound	Detector	Wavelength [nm]	Linearity range [$\mu g \cdot ml^{-1}$]	r^2	*LOD* [$\mu g \cdot ml^{-1}$]	*LOQ* [$\mu g \cdot ml^{-1}$]	*RSD* [%]
Ascorbic acid	PDA	245	10–50	0.9979	0.003	0.010	3.6
Pyridoxine	FL	290–390	10–50	0.9962	0.002	0.008	2.3
Thiamine	PDA	245	10–50	0.9909	2.04	2.12	7.8
Caffeine	PDA	270	5–50	0.9981	0.005	0.016	1.9
Riboflavin	PDA	270	1–20	0.9922	0.020	0.067	5.6
Taurine	UV-Vis	360	20–200	0.9995	0.910	0.930	2.1

r^2 – coefficient of determination, *LOD* – limit of detection, *LOQ* – limit of quantification, *RSD* – relative standard deviation (6 injections), PDA – photodiode array detector, FL – fluorescence detector, UV-Vis – ultraviolet-visible detector.

Tab. 3. Concentrations of compounds found in the energy drinks.

Sample	Country of origin	Pyridoxine [mg·l^{-1}]		Caffeine [mg·l^{-1}]		Riboflavin [mg·l^{-1}]		Taurine [mg·l^{-1}]	
		Found value	Label value	Found value	Label value	Found value	Label value	Found value	Label value
ED1	Austria	32.0 ± 0.2	20	264 ± 9	320	0.2 ± 0.3	Colour	2700 ± 73	4000
ED2	USA	15.0 ± 1.5	8	286 ± 3	320	11 ± 1	7	2670 ± 97	4000
ED3*	USA	13.0 ± 0.2	8	301 ± 3	320	< *LOD*	Colour	2560 ± 56	4000
ED4	Spain	30.7 ± 3.0	20	252 ± 2	320	3.7 ± 0.5	Colour	2750 ± 47	4000
ED5*	Spain	22.0 ± 0.2	20	304 ± 3	320	2.6 ± 0.3	Colour	2200 ± 50	4000
ED6	Spain	39.8 ± 6.3	ni	254 ± 1	320	16 ± 2	ni	2180 ± 18	4000

Results are based on triplicate analyses.
ED – energy drink, * – ED3 and ED5 were light drinks, ni – not indicated on the label, *LOD* – limit of detection.

trients that are purported to affect mental and/or physical performance, caffeine is the primary ergogenic nutrient in most energy drinks.

Taurine concentrations found in the samples were between 2000–3000 mg·l^{-1}, which was not in accord with the label (4000 mg·l^{-1}). Generally in the energy drinks, there is a higher level of taurine with respect to the daily requirements [36].

The concentration of B vitamins in energy drinks is often higher than the recommended daily intake, and may result in liver or nerve injury [40]. Pyridoxine concentration on the labels of drinks ED1, ED4 and ED5 was 20 mg·l^{-1}, while that on the labels of drinks ED2 and ED3 was 8 mg·l^{-1}. In all drinks analysed, the found values were higher that declared. The same occurred regarding the concentration of riboflavin in ED2. In other drinks, riboflavin was declared to be added as a colorant.

Although the number of drink samples analysed in this study was relatively small (six), the data obtained, coupled to those found by other investigators (with a number of samples between three and ten) [20, 21, 26–30, 34], are important for preliminary studies on the composition of energy drinks. In order to discuss the obtained results, they were compared with those of other authors who analysed energy drinks by HPLC. In general, the concentrations of caffeine and vitamins found in the literature were similar to those of this study, but taurine concentrations were more variable, with variations between different brands and many times with concentrations higher than those reported on the label.

Pieszko et al. [21] found similar concentrations in energy drinks for caffeine (237–317 mg·l^{-1}), lower and less variable concentrations in vitamin B2 (2.5–6.0 mg·l^{-1}), lower concentrations of vitamin B6 (3–19 mg·l^{-1}) and higher concentrations of taurine (2106–4186 mg·l^{-1}). Nour et al. [24] found caffeine concentrations in a similar range (168.2–394.8 mg·l^{-1}), similarly to Gliszczyńska-Świgło and Rybicka [27] for caffeine, pyridoxine and riboflavin, and Rai et al. [26] for caffeine and taurine. Ricciutelli et al. [12] found levels of taurine between 100 mg·l^{-1} and 4500 mg·l^{-1}, Orth [29] has found between 830 mg·l^{-1} and 5000 mg·l^{-1} and Sawabe et al. [30] in the range 932–3153 mg·l^{-1}. Marti-Andres et al. [28] determined vitamins in energy drinks and found differences between the experimental values and the labelled values, the former being higher, similar to Attipoe et al. [39] regarding caffeine concentrations. In general, in many of these drinks, the content of the compounds on the label claim did not correspond to the true value [20, 26, 28, 29, 39] so the labelling of many of these products did not comply with legislation [34]. The majority of energy drink samples were non-compliant in terms of caffeine, vitamins and taurine concentrations.

Many energy drinks contain numerous ingredients. Individual components, such as caffeine, should not be studied separately but rather as mixtures and these products in particular merit further study to demonstrate their safety and potential effects on physical and mental performance [38]. From a review of the literature, it would appear that concerns in the scientific community and among the consumers regarding the potential adverse health effects of the increased consumption of energy drinks are broadly valid. Caffeine consumption in energy drinks can be potentially more toxic than from other beverages, due to the other substances present, and due to aggressive publicity, especially in children and adolescents [41]. Other effects of energy drinks are related to child obesity, attention deficit, hyperactivity, insomnia [8] and dental diseases [20, 34, 42].

An increase in production and consumption of energy drinks [41] coupled to inconsistent ingredient labelling requires more research in humans and animals to evaluate the risk and to set limitations. Therefore, stricter regulation of the sale of energy drinks for children and adolescents is necessary [42, 43].

CONCLUSIONS

Energy drinks are a new beverage, not well defined, whose consumption is increasing considerably and may be related to toxic health effects. The aim of the present study was to develop a chromatographic method for the separation and quantification of caffeine, water-soluble vitamins and taurine, which are compounds present, between others, in this beverage type. A fast and inexpensive HPLC-based method, using PDA and FL detectors, for determination of ascorbic acid, pyridoxine, thiamine, riboflavin and caffeine simultaneously in energy drinks was developed, using different wavelengths, in a relatively short time (20 min including equilibration time). Taurine determination was made by HPLC with UV-Vis detector, with pre-column derivatization with 2,4-dinitrofluorobenzene. A simple preparation of the sample by dilution in water was found to be sufficient. Both methods were validated and applied to six energy drinks. *LOD* ranges from 0.002 $\mu g \cdot ml^{-1}$ (pyridoxine) to 2.04 $\mu g \cdot ml^{-1}$ (thiamine).

In the energy drinks, caffeine concentration was 252–304 $mg \cdot l^{-1}$, taurine concentration was 2180–2750 $mg \cdot l^{-1}$, pyridoxine concentration was 13.0–39.8 $mg \cdot l^{-1}$ and riboflavin was from not detectable to 16 $mg \cdot l^{-1}$. Ascorbic acid and thiamine were not detected.

Although the number of drink samples analysed was small, the data presented in this study provided a preliminarily outline about the content levels of given compounds in energy drinks. This study complemented some previous studies showing that there is a limitation in the control of energy drinks, which however may have potential toxic effects on health. In the majority of energy drink samples, contents of vitamins, caffeine and taurine declared on the label did not correspond to the actual values and, in labelling of many of these products, infringements were observed. Currently, because the consumption of energy drinks is relatively modern, there are no data on their long-term effects on metabolic health. Enforced regulation and restriction of energy drinks for children and adolescent consumption is urgently needed in addition to greater visibility of consumption recommendations. Several countries have enacted measures to regulate the labelling, distribution and sale of energy drinks that contain significant amounts of caffeine. The World Health Organization recommends establishing an upper caffeine limit on all products and educating the public about the risks of mixing alcohol with energy drink consumption especially for young people [8].

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