Chromatographic determination of major physiologically active components in energy drinks and sports aids commercialized in Costa Rica

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Summary

Energy drinks marketed as providing mental and physical spur, usually contain caffeine as a stimulant, and may contain sugar, other sweeteners, acidulants and taurine. A total of 12 samples were analysed for taurine, caffeine, sugar profile and citric acid. Then, two different liquid chromatography methods were validated for taurine, sugars and citric acid (the latter two determined simultaneously). For taurine, a sensitive fluorescence detection-based method with limit of detection (*LOD)* 5.68 ng·ml^{-1} resulted in relative standard deviation (*RSD*) < 6 % and recovery 89.3–111.0 % for four spiking levels, concentrations being adjusted closely to the label. For sugars and citric acid, a ligand exchange-based method using refractive detection rendered *LOD, RSD* and accuracy values (recoveries) of 0.0896–0.1993 g·l^{-1}, 3–4 % and 94.9–118.1 %, respectively. Sugar levels in beverages ranged from 100 % to 210 % of declared values. For citric acid, values from 2.60 g·l^{-1} to 9.35 g·l^{-1} were determined. Finally, an accredited method with *LOD* of 0.016 μg·ml^{-1} was used to assess caffeine concentration. Values 222.06–722.49 μg·ml^{-1} were higher than in similar products and a half of the drinks had significantly higher values than those reported on the label. Some other discrepancies with the label were also found.

Keywords

beverage; energy drink; chemical analysis; taurine; caffeine; sugar profile; organic acid

Energy drinks (commonly carbonated beverages or soft drinks) are any of various types of beverages considered a source of verve. These formulations usually contain stimulant compounds such as caffeine and may contain sugar, other sweeteners, acidulants, herbal extracts or amino acids. Some of these components are found commonly in foods, though usually in lesser concentrations. For example, taurine and D-glucuronolactone are natural ingredients in food (such as bovine milk, shellfish or poultry) and are normal human metabolites [1–3].

Despite being a common trait in energy drink formulations, taurine effects as a stimulant have been contested and are still today subject of criticism and controversy [4]. For example, an effect relationship was not established between the consumption of taurine and "immune system protection", "metabolism processes," contribution to normal cognitive function, maintenance of normal cardiac function, maintenance of normal muscle function or delay in the onset of physical fatigue during exercise [5]. Notwithstanding, no direct association or causation between energy drink consumption and mental health issues has been demonstrated [6].

Energy drinks are frequently marketed with declarations that suggest an increase in mental and physical spur, providing a short-term boost to mood and performance, there is an increase in the intake of energy drinks and sports aids for some specific age groups (e. g. adolescents) [7]. Worrisomely, some labelling guidelines require simply declaring the presence of stimulants such as caffeine [8].

In terms of risk assessment, the taurine's

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No Observed Adverse Effect Level (NOAEL) had been set at 1 000 mg per kilogram of body weight per day [1]. Put into perspective, to achieve these values, a 60 kg person should drink almost 60 cans of a 4 000 $\mu g \cdot ml^{-1}$ energy drink. Hence, a sufficient margin of safety exists for mean- and high-level regular consumers of energy drinks, drinking on average 125 ml (0.5 cans) and 350 ml (1.4 cans) per person per day, respectively. Then, exposure to taurine and D-glucuronolactone at these levels is not a safety concern [1].

Interestingly, taurine has been concluded to be tolerated by all animal species up to 2 $g \cdot kg^{-1}$ feed and the safe level in humans is 6 g per person per day (i. e. 100 $mg \cdot kg^{-1}$ body weight per day; EC Regulation 2018/249 [9]). On the other hand, US FDA has recorded taurine as Generally Recognized As Safe (GRAS; Notice GRN No 586) at 3 000 $mg \cdot d^{-1}$ established as the practical safe level for human dietary exposure [10]. This amount corresponds to four cans a day for beverages containing 4 000 $\mu g \cdot ml^{-1}$. Also, CODEX STAN 192-1995 "General Guidelines for Food Additives" revised 2019 [11] includes guidelines for such drinks. Finally, no regulations are in force in Costa Rica pertaining to the maximum concentrations (or which levels should be considered acceptable in these products) for any of the analytes assayed in this study (RTCR 436:2009 [12] and Decree 30256 [13]).

In the case of sugars in sweetened drinks worldwide, some countries have implemented or tried to implement policies, with a varying degree of success, to reduce sugar intake, primarily through drinks [14]. Other countries followed, including Latin-American ones (like Costa Rica) [15]. In Costa Rica, legislation is focused on children. For example, beverages containing over 6 g of sugars per package are not allowed to be sold in schools [14]. Elevated taxation for sweetened drinks has also been used as a strategy to reduce sugar consumption [16].

Finally, citric acid (E330) is a widespread additive used in carbonated drinks as acidity regulator, Additionally, it adds aroma and enhances flavour [17]. Inclusion of this additive is bound by the guidelines established under EC Regulation 1333/2008 [18] and US FDA Code of Federal Regulations [19].

On the other hand, caffeine is a natural constituent of coffee and cocoa beans, tea leaves, kola nuts and guaraná. However, they are also used at much higher levels in energy drinks [2, 3, 20]. Intake of 6 mg caffeine per kilogram can maximize physical performance in sports with high endurance demands [21]. This would mean that for a person weighing 60 kg, performance improvement could be achieved by consuming 2–6 cans of the drink, considering that each serving contains 55–180 mg caffeine. For example, Red Bull beverage contains 80 mg caffeine in a 248 ml can. Energy drinks with a high caffeine concentration are legally required to be labelled as such. For example, EU Regulation 1169/2011 (enforced since 2014) [22] contains provisions regarding the labelling of beverages with added caffeine in a concentration higher than 150 $\mu g \cdot ml^{-1}$.

Consumption of energy drinks has already raised health concerns, including fatalities, in young people ingesting energy drinks either in very large amounts (a case of someone drinking 1 420 ml was reported), in combination with physical exercise or, more often, with alcohol. The risks of extensive or chronic consumption of these products were comprehensively described previously in European countries [23]. On the other hand, consumers expect that the products they acquire: a) are safe and their quality is guaranteed, b) information provided on the label helps them make informed decisions about the purchase and c) the label is not misleading. As energy drink consumption increases worldwide and is widespread, it is relevant that the sugar and caffeine content of the energy drinks is communicated to consumers.

In Costa Rica, as in other countries, the consumption of this type of beverage has been growing steadily in recent years. For example, Costa Ricans consumed 3.6 million litres in 2010 and this volume increased to 10 million litres in 2015, representing a local brand of energy drink of 41.5 million USD that year [24]. Additionally, 5.0 % of the population shows interest in energy drinks, approximately 57 % of them being aged from 21 to 30 and classified as having a high socio-economic level [24].

In terms of analysis, taurine and caffeine are amongst the most studied. Data on minor components of energy drinks, such as minerals and water-soluble vitamins, were reported as well [25, 26]. However, sugar content, sugar profile and citric acid levels were not verified.

Herein we present performance parameters of two analytical methods. The first is based on reverse phase chromatography and fluorescence detection for taurine, another is based on ligand exchange and refractive index detection for simultaneous analysis of the drinks' sugar profile and the most abundant organic acid used within formulations. The methods once validated were used to assess these components (along with caffeine) in energy drinks and sports aids available on the market.

MATERIALS AND METHODS

Reagents

Taurine (≥ 99 %), caffeine (ReagentPlus), saccharose (≥ 99.5 %), glucose (analytical standard), fructose (≥ 99 %), citric acid (99 %), sulfuric acid (ACS reagent, 95.0–98.0 %), methanol (HPLC grade, ≥ 99.9 %), boric acid (ACS reagent, ≥ 99.5 %), 2-mercaptoethanol (≥ 99.0 %) sodium dihydrogen phosphate (monohydrate, EMPARTA ACS reagent), phthaldialdehyde (for fluorescence, ≥ 99.0 %, HPLC) and acetonitrile (gradient grade, ≥ 99.9 %) were all purchased from Millipore Sigma (Burlington, Massachusetts, USA). Ultrapure water (type I, 0.055 $\mu S \cdot cm^{-1}$ at 25 °C, 5 $\mu g \cdot l^{-1}$ total organic carbon) was obtained using an A10 Milli-Q Advantage system and an Elix 35 system (EMD Millipore, Burlington, Massachusetts, USA).

Sampling and general sample treatment

Samples were selected in significant supermarkets and convenience store chains in Costa Rica. Criteria for selection included a) beverage or product labelled as a sports aid or energy drink or similar and b) taurine or caffeine reported on the nutritional label. All available brands were included in the selection. A total of $n = 12$ samples were assessed for all the analytes above. Two different production batches were sampled and tested per product. Sampled commercial brands of the drinks and sports aids included Monster Energy (Monster Beverage, Corona, California, USA), Maxxx Energy/Jet (Florida Ice and Farm Company, Lorrente de Flores, Heredia, Costa Rica), Battery (Sinebrycoff Brewery, Helsinki, Finland), Focusaid (Life Aid Beverage, Santa Cruz, California, USA), Raptor (Fabrica de Bebidas y Gaseosas Salvavidas, Guatemala, Guatemala), Red Bull (Red Bull, Fuschl am See, Austria), 226ERS Sport Gummies/Energy Drink (226ers Sports Things, Alicante, Spain), Essential Amino Energy (Optimum Nutrition, Downers Grove, Illinois, USA), OCA (Beliv, Miami, Florida, USA), Raze Energy (REPP Sports, Longwood, Florida, USA) and Rooster Natural Booster (San José, Costa Rica). Club soda (Canada Dry, Dr. Pepper/Seven Up, Plano, Texas, USA) was used as a matrix blank for all assays.

Liquid samples were all subjected to ultrasound treatment by FS60H device (Fisher Scientific, Waltham, Massachusetts, USA) to degas and were sifted through a syringe filter Acrodisc (regenerated cellulose membrane, pore size 0.45 μm; Pall, New York, New York, USA). The samples were diluted, according to analyte concentration and analytical approach (see below), with ultrapure water when necessary. Samples sold as a gummy or powder were entirely dissolved in 100 ml and 500 ml water, respectively, before further treatment.

HPLC analysis

Taurine pre column derivatization

Taurine derivatization was carried out as follows: 10 μl to 100 μl of beverage was mixed with 130 μl borate buffer (50 $mmol \cdot l^{-1}$, pH 10), 10 μl freshly prepared *o*-phtalaldehyde (OPA) reagent (10 mg phthaldialdehyde dissolved in 500 μl in ethanol, 20 μl of 2-mercaptoethanol added and made up to volume with borate buffer in a 10 ml volumetric flask) and sufficient water to complete 500 μl in a high performance liquid chromatographic (HPLC) vial (Agilent Technologies, Santa Clara, California, USA) for injection. One microliter of the resulting mixture was injected into the HPLC system (see below).

Taurine analysis

Analysis was performed using a reverse-phase chromatography using a 1260 Infinity system (Agilent Technologies) equipped with a quaternary pump (G1311B), a fluorescence detector (G1321B), an autosampler (G7129A), a thermostatic column compartment (G1316A) and an analytical column (Zorbax Eclipse AAA, particle size 3.5 μm, 4.6 mm × 75 mm, PN USYP007331, Agilent Technologies). The solvent system consisted of a 40 $mmol \cdot l^{-1}$ NaH_2PO_4 buffer adjusted to pH 7.8 (S9638, ACS, 98 % pure, solvent A (Merck Millipore, Burlington, Massachusetts, USA) and acetonitrile-methanol-water (45 : 45 : 10, solvent B). Gradient mode was as follows: 0 % B at 0 min, 0 % B at 1 min, 57 % B at 9.8 min, 100 % B at 10 min, 100 % B at 12 min, 0 % B at 12.5 min and 0 % B at 16 min at a constant flow of 2 $ml \cdot min^{-1}$. The fluorescence detection system was set at 340 nm (excitation) and 450 nm (emission). To assess taurine concentration, a six-point calibration curve was prepared from 1.00 $\mu g \cdot ml^{-1}$ to 10.00 $\mu g \cdot ml^{-1}$. Column compartment was kept at 40 °C during analysis.

Determination of caffeine

Caffeine was assessed by a modified version of the method based on the work by Srdjenovic et al. [27]. The method was previously validated and ISO/IEC 17025 accredited using a Shimadzu system (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector (SPD-M20AV), column compartment (CTO-20A), autosampler (SIL-20A HT) and a quaternary pump (LC-20AT).

A Zorbax Eclipse C18 column (150 mm × 4.6 mm, 5 μm particle size; Agilent Technologies) was used to perform the separation. Considering the average previously reported amount of caffeine present in the beverages (approximately 160 mg [28]), the samples were diluted 10-fold in a ready-to-inject HPLC vial and 5 μl of the resulting mixture was injected into the system. An isocratic method using methanol-water (24 : 76) at 1 ml·min^{-1} was used, column temperature was kept at 25 °C. Detection was performed at a wavelength of 272 nm. To assess the concentration of caffeine, an eight-point calibration curve was prepared from 2 μg·ml^{-1} to 160 μg·ml^{-1}. Under these conditions, caffeine exhibited a retention time of 6.967 min, a calibration curve was represented by Eq. 1, limit of detection (*LOD*) was 16 ng·ml^{-1} and limit of quantification (*LOQ*) was 53 ng·ml^{-1}.

$$y = (1.47 \times 10^5 \pm 9.00 \times 10^2)x - \\ -(2.67 \times 10^3 \pm 7.11 \times 10^2) \quad (1)$$

Simultaneous determination of saccharose, glucose, fructose and citric acid

The system used to assess sugars and citric acid was similar to that mentioned above. For this particular analysis, a refractive index detector 10A (Shimadzu) was used. A ligand exchange column Hi-Plex Ca (300 mm × 7.7 mm, 8 μm particle size, PN PL1170-6810, Agilent Technologies) was used to separate all analytes at a constant flow of 0.4 ml·min^{-1} of 8.5 mmol·l^{-1} sulfuric acid as a mobile phase, at 25 °C and an injection volume of 10 μl. Considering the concentration of sugars and citric acid present in the samples and the sensitivity of detection, samples were filtered and injected directly into the system. To assess the concentration of each of the analytes, a seven-point calibration curve was prepared from 1 g·l^{-1} to 50 g·l^{-1} as a mixture of the four compounds.

Statistical analysis and method validation parameters

Horrat Ratio (*HorRat*) was based on repeatability, the ratio among the experimental relative standard deviation of repeatability (*RSDr*) and its calculated counterpart. Using a modified Horwitz equation, the predicted relative standard deviation ($PRSD_x$) of the measurand was calculated [29],

$$PRSD_x = 2C^{-0.5} \quad (2)$$

where C represents the analyte content in mass fraction.

A Food Analysis Performance Assessment Scheme-certified reference material (FAPAS FCFA29-DRN14) was used as a quality control during analysis for all analytes tested. Expanded uncertainties were reported with a coverage factor of $k = 2$, which indicates approximately 95.4% confidence. Methods were validated according to International Council for Harmonisation (ICH) [30].

RESULTS AND DISCUSSION

Performance of taurine determination method

A method based on HPLC with fluorescence detection was successfully adapted for the analysis of carbonated beverages supplemented with taurine. Overall, the method performance is adequate and fits to the purpose intended. Notwithstanding, fluorescence detection imparts very high sensitivity to analysis [31], the concentrations within the energy drink formulations do not require such a feature. Therefore, some beverages had to be diluted as much as 400-fold to achieve concentrations within the operating range of the detector. This means that energy drink monitoring and surveillance can be performed by HPLC equipped with a photodiode array (PDA) or variable wavelength detector (VWD), which are widespread. Noteworthy, some precision parameters (expressed as *HorRat*) lied below the common empirical threshold of 0.5 (Tab. 1). Low values of *HorRat* are indicative of a procedure with very few pre-treatment steps and show that the method was performed by an operator with technical expertise. For concentrations in the high range, experimental *RSD* and *HorRat* values of 2.3–11.3 % and 0.39–1.88, respectively, imply that simple precision is in line with what is predicted (i. e. $PRSD_x$ = 6.0 %). The calculated blank- and matrix-matched recoveries did not differ significantly ($p < 0.05$) from each other. These values lied between 89.3 % and 111.0 %, which was in line with what is expected for the given concentration levels, i. e., 90.0–107.0 % for 0.01 μg·ml^{-1} and 0.1 μg·ml^{-1}. These validation data are in line with those reported for other pre-column derivatization techniques using 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) [31] and OPA-sulfite [32].

Determination of taurine

Most previous approaches used Sanger's reagent, 2,4-dinitrofluorobenzene (DNFB), to determine taurine (Tab. 2). However, signals of the unreacted reagents and reaction by-products were observed in the chromatograms and, usually, addition of organic solvents (dimethyl sulfoxide) or temperature increase are needed to hasten the reaction [33]. A similar scenario occurs for

Tab. 1. Performance parameters of the method for taurine determination using pre-column derivatization and HPLC.

Linearity

Retention time [min]	Working range [$\mu g \cdot ml^{-1}$]	Calibration curve equation ($n = 3$)	r	r^2	*LOD* [$\mu g \cdot ml^{-1}$]	*LOQ* [$\mu g \cdot ml^{-1}$]
6.08 ± 0.21	1.00–10.00	$y = (7.07 \times 10^6 \pm 1.97 \times 10^4)x - 7.14 \times 10^5 \pm 1.13 \times 10^5$	0.9986	0.9972	5.68×10^{-3}	17.20×10^{-2}

Repeatability and reproducibility

	Matrix	*RSD* [%]	*HorRat*
Intra-day precision ($n = 3$)	Energy drink	2.3	0.4
	Powdered sport aid	10.9	1.8
	Gummy sport aid	4.1	0.7
Inter-day precision ($n = 6$)	Energy drink	3.9	0.6
	Powdered sport aid	11.3	1.9
	Gummy sport aid	3.3	0.5

Accuracy

Matrix	Concentration level [$\mu g \cdot ml^{-1}$]	Recovery [%]
Blank	2 000	103.9
	200	92.9
	20	97.4
	2	89.3
Energy drink	2 000	100.5
	200	89.7
	20	89.3
	2	90.1
Powdered sports aid	2 000	96.2
	200	94.2
	20	96.4
	2	93.8
Gummy sports aid	2 000	111.0
	200	96.1
	20	96.1
	2	100.0

r – coefficient of correlation, r^2 – coefficient of determination, *LOD* – limit of detection, *LOQ* – limit of quantification, *RSD* – relative standard deviation, *HorRat* – Horwitz ratio.

4-fluoro-7-benzofurazan (NBD-F) pre-column derivatization [34]. In contrast, the OPA derivate forms readily. However, derivatization with OPA generates an adduct with limited stability. The kinetics of its degradation may be needed to study (or at least tested) before considering using this method for routine analysis.

Taurine values declared in the drinks were found to be in line with those obtained experimentally (Tab. 3). The exception was a sample labeled K (a powdered sports aid containing taurine), in which its concentration was 1.5-fold higher than that declared. Worrisomely, samples D and E did not state how much taurine was contained or lacked the indication of taurine content. Both issues demonstrate flaws during formulation and the need for stricter monitoring. Other authors also previously indicated label inconsistencies regarding taurine [35, 36]. For example, Lage-Yusty et al. [26] indicated considerable differences when comparing results of taurine analysis with data declared on the label, always finding lower values. Analogously, Orth [33] found that one third of the samples contained lower concentrations than declared on the label (e. g. detected 830 $\mu g \cdot ml^{-1}$ versus the declared 1 000 $\mu g \cdot ml^{-1}$). In contrast, Omer et al. [31, 32] did not find major discrepancies between the label and the experimental values with taurine determined compared to the declared amount ranging from 94.7 % to 102.0 %. Sawabe et al. [34] found a similar scenario in five beverages with those percentages ranging from 92.9 % to 105.1 %.

Performance of the analytical method for sugars and organic acids

Precision parameters of the analytical method for sugars and citric acid were below those ex-

Tab. 2. Chromatographic conditions and parameters of methods for taurine determination.

Ref.	Equipment	Derivatizing agent	Mobile phase	Flow [ml·min^{-1}]	Column	Wavelength [nm]	*T* [°C]	Run time [min]	*LOD* [μg·ml^{-1}]	*LOQ* [μg·ml^{-1}]
[26]	LC-UV-Vis	DNFB	A: Phosphate buffer B: ACN (80 : 20)	1	Tracer Kromasil C18, 250 mm × 4.0 mm, 5 μm (Teknokroma, Barcelona, Spain)	360	33	20	0.910	0.930
[31]	LC-PDA	NBD-Cl	A: ACN B: TCA at 1 ml·l^{-1} (70 : 30)	0.8	Intersil ODS-3, 250 mm × 4.6 mm, 5 μm (Sigma-Aldrich, St. Louis, Missouri, USA)	472	25	NS	0.296	0.987
	LC-FLD					λ_{ex} 472 λ_{em} 530			6.16×10^{-2}	20.50×10^{-2}
[32]	LC-PDA	OPA and sodium sulfite	A: ACN B: TCA at 1 ml·l^{-1} (70 : 30)	0.8	Intersil ODS-3, 250 mm × 4.6 mm, 5 μm (Sigma-Aldrich, St. Louis, Missouri, USA)	298	25	NS	0.109	0.141
[33]	LC-UV-Vis	DNFB	A: Phosphate buffer 0.01 mol·l^{-1}, pH 6.00 B: ACN	1	Alltech Econocshpere C18, 150 mm × 4.6 mm, 3 μm (Thermo Fisher Scientific, Waltham, Massachusetts, USA)	360	40	19	NS	NS
[34]	LC-PDA	NBD-F	A: Phosphate-citrate buffer, pH 5.40 B: 10 mmol·l^{-1} ammonium tetrabutyl and ACN (7 : 3)	1	L-column ODS, 150 mm × 4.6 mm, 5 μm (Chemical Evaluation and Research Institute, Tokyo, Japan)	470	40	10	0.15	0.50
[35]	LC-UV-Vis	DNFB	A: H_2O-MeOH (60 : 40) B: ACN	1	Zorbax Eclipse Plus C18, 150 mm × 4.6 mm, 5 μm (Agilent Technologies, Santa Clara, California, USA)	360	40	15	NS	NS
[36]	LC-UV-Vis	DNFB	A: Phosphate buffer B: ACN (80 : 20)	1	Kinetex XB-C18, 150 mm × 4.6 mm, 5 μm (Phenomenex, Torrance, California, USA)	360	NS	10	2.19	7.32
This study	LC-FLD	OPA	A: 40 mmol·l^{-1} NaH_2PO_4 buffer, pH 7.8 B: ACN-MeOH-H_2O (45 : 45 : 10) starting at 0 %	2	Zorbax Eclipse AAA, 75 mm × 4.6 mm, 3.5 μm (Agilent Technologies, Santa Clara, California, USA)	λ_{ex} 340 λ_{em} 450	40	16	5.68×10^{-2}	17.20×10^{-2}

ACN – acetonitrile, DNFB – 2,4-dinitrofluorobenzene, FLD – fluorescence detector, LC – liquid chromatography, *LOD* – limit of detection, *LOQ* – limit of quantification, λ_{ex} – excitation wavelength, λ_{em} – emission wavelength, MeOH – methanol, NBD-Cl – 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole, NBD-F – 4-fluoro-7-benzofurazan, NS – not specified, ODS – octadecylsilane, OPA – *o*-phtaladehyde, PDA – photodiode array detector, *T* – temperature, TCA – trichloroacetic acid, UV-Vis – ultraviolet-visible spectrophotometric detector.

Tab. 3. Results of analysis of taurine in commercial samples of energy drinks and sports aids.

Sample	Taurine			
	Concentration declared on the label [$\mu g \cdot ml^{-1}$]	Concentration determined [$\mu g \cdot ml^{-1}$]	U_x [$\mu g \cdot ml^{-1}$]	Share of the declared value [%]
Energy drinks with declared taurine				
A	4 000	4 314.09 ± 31.71	233.27	108.0
B	4 000	3 890.24 ± 578.43	210.36	97.0
C	2 000	2 027.84 ± 98.33	107.98	101.0
Energy drinks with undeclared taurine use				
D*	Not declared	3 390.63 ± 191.44	183.34	Not applicable
E	Not declared	333.73 ± 31.13	18.05	Not applicable
F	Not declared	$< 5.68 \times 10^{-3}$	–	Not applicable
Energy drinks with no taurine use				
G*	Not declared	$< 5.68 \times 10^{-3}$	–	Not applicable
H	Not declared	$< 5.68 \times 10^{-3}$	–	Not applicable
I	Not declared	$< 5.68 \times 10^{-3}$	–	Not applicable
Powdered sports aids				
J	Not declared	75.12 ± 5.46	4.06	–
K	252	379.84 ± 70.45	20.54	150.0
Gummy sports aids				
L	268	273.02 ± 3.45	14.76	101.0

Concentration is expressed as mean ± standard deviation, mean values resulting from two different production batches, each batch measured in triplicate.
* – products D and G were declared to contain artificial sweeteners, U_x – absolute uncertainty.

pected for concentrations ranging from 10 $g \cdot l^{-1}$ to 100 $g \cdot l^{-1}$ (*RSD* 3.0–4.0 % is required, Tab. 4). As expected, the most variation occurred at the gummy sports aid analysis with *RSD* of 15.9–22.8 %. However, accuracy was adequate for all matrices tested, which primarily lied in the expected range of 97.0–103.0 %. Acceptable accuracy percentages at spiking of both the club soda and the energy drink demonstrated that the remainder of the ingredients did not interfere with the analysis even when it was performed using a non-selective detector such as refractive index detector. Sensitivity-wise, *LOD* values experimentally obtained were much lower than those previously described elsewhere (Tab. 5). However, this fact does not represent a constraint, as sugars are present in very high concentrations in these drinks. Differences in sensitivity between our method and others may be due to different electronics, calculation methods or column performance, among other non-exclusive reasons.

Finally, organic acid and sugar determination in most food analyses is performed separately using two different detectors (Tab. 5). In most circumstances, organic acids and sugar analysis in foods are usually used to profile fruits, wines, plants or juices [37–40]. However, in the case of energy drinks, the concentrations in which both sugars and citric acid are added allow simultaneous detection using refractive index detector. At the same time, the relative capacity of the detector to prevent saturation allows the samples to be injected directly after degassing. Other organic acids can also be detected using this approach (e. g. sorbic acid, ascorbic acid), although only some formulations contain them [28].

Determination of sugars

In regular energy drinks, we found total sugar concentrations ranging from 19.87 $g \cdot l^{-1}$ to 151.69 $g \cdot l^{-1}$ (Tab. 6). This would mean that consumption of a sole can of approximately 248 ml provides 4.70–37.92 g sugar, equivalent to approximately 10.0–77.0 % of the recommended daily value. This is a troublesome fact if considered that energy drinks are consumed in addition to typical diets. Similar to soft beverages, unrestricted energy drinks intake will aid in overconsumption of sugars, a key contributor to the growing obesity, prediabetes and type 2 diabetes [41]. A possible response to this issue may be that the most common drinks on the market will be sweetened artificially. Nevertheless, safety and health effects of such substances remain controversial [42]. In this regard, the artificially sweetened drinks analysed were in accordance with their label, as no sugars were detected using our approach except

Tab. 4. Method performance for simultaneous analysis of sugars and organic acids using refractive index detection.

Linearity

Parameter	Retention time [min]	Working range [g·l^{-1}]	Calibration curve (n = 3)	r	r^2	*LOD* [g·l^{-1}]	*LOQ* [g·l^{-1}]
Saccharose	13.56	1–50	$y = (1.41 \times 10^5 \pm 4.30 \times 10^1)x - (1.23 \times 10^4 \pm 1.05 \times 10^3)$	1.00	1.00	0.1039	0.3149
Glucose	15.60		$y = (1.49 \times 10^5 \pm 8.90 \times 10^1)x - (7.83 \times 10^3 \pm 2.17 \times 10^3)$	0.9999	0.9998	0.0896	0.2715
Fructose	23.32		$y = (1.26 \times 10^5 \pm 7.40 \times 10^1)x - (1.27 \times 10^4 \pm 1.80 \times 10^3)$			0.1682	0.5097
Citric acid	20.57		$y = (1.44 \times 10^5 \pm 8.2 \times 10^1)x - (1.10 \times 10^4 \pm 2.01 \times 10^3)$			0.1993	0.6038

Repeatability and reproducibility

	Matrix	Analyte	*RSD* [%]	*HorRat*
Intra-day precision (n = 3)	Energy drink	Saccharose	0.3	0.08
		Glucose	0.3	0.08
		Fructose	0.5	0.13
		Citric acid	2.2	0.56
	Powdered sports aid	Citric acid	1.4	0.36
	Gummy sports aid	Saccharose	0.4	0.11
		Glucose	0.8	0.20
		Fructose	0.2	0.05
		Citric acid	9.8	2.46
Inter-day precision (n = 6)	Energy drink	Saccharose	1.2	0.30
		Glucose	0.5	0.13
		Fructose	0.5	0.13
		Citric acid	1.9	0.48
	Powdered sports aid	Citric acid	5.4	1.35
	Gummy sports aid	Saccharose	15.9	2.64
		Glucose	22.8	3.80
		Fructose	16.2	2.70
		Citric acid	3.2	0.80

Chromatographic performance

Parameter	Working range [g·l^{-1}]	Theoretical plates	Retention factor (k')	Selectivity (α_s)	Resolution (R_s)	Tailing factor	Asymmetry
Saccharose	10–50	621.92	0.293	1.012	–	0.911	0.908
	0.1–5	996.81					
Glucose	10–50	256.74	0.485	1.659	1.132	2.167	4.272
	0.1–5	293.64					
Fructose	10–50	702.41	1.221	1.271	1.832	0.907	0.854
	0.1–5	1 077.49					
Citric acid	10–50	805.63	0.960	2.016	2.437	0.903	0.898
	0.1–5	1 645.67					

Accuracy

Matrix	Nominal recovery level [g·l^{-1}]	Experimental recovery [%]			
		Saccharose	Glucose	Fructose	Citric acid
Blank	40	104.8	114.3	105.6	102.8
	16	109.1	112.8	104.6	103.6
	8	107.2	109.3	101.5	101.7
	5	109.1	110.5	102.5	103.7
	2	116.1	118.1	108.0	113.1
Energy drink	40	94.9	106.0	101.8	101.7

r – coefficient of correlation, r^2 – coefficient of determination, *LOD* – limit of detection, *LOQ* – limit of quantification, *RSD* – relative standard deviation, *HorRat* – Horwitz ratio.

Tab. 5. Chromatographic conditions and parameters of methods for determination of sugars and organic acids in beverages.

Ref.	Equipment	Mobile phase	Flow [ml·min^{-1}]	Column	T_C [°C]	T_D [°C]	Run time [min]	*LOD* [g·l^{-1}]			
								Saccharose	Glucose	Fructose	Citric acid
[37]	LC-RID LC-DAD	Isocratic, 5.0 mmol·l^{-1} H_2SO_4	0.50	Aminex HPX-87H, 300 mm × 7.8 mm, 5 μm (Bio-Rad Laboratories, Hercules, California, USA)	55		22	NS	0.160	0.070	0.030
[38]	LC-RID	Isocratic, 10.0 mmol·l^{-1} H_2SO_4	0.40	Nucleosil CHO 682 Pb, 300 mm × 7.8 mm, 4 μm (Sigma-Aldrich, St. Louis, Missouri, USA)	85		50	0.001	NS	NS	NS
[39]	LC-RID	Isocratic, 4.0 mmol·l^{-1} H_2SO_4	0.50	Hi-Plex H, 300 mm × 7.7 mm, 8 μm (Agilent Technologies, Santa Clara, California, USA)	70	50	20	NS	0.021	0.050	0.021
[40]	LC-RID LC-DAD	Isocratic, 8.0 mmol·l^{-1} H_2SO_4	0.60	Aminex HPX-87H, 300 mm × 7.8 mm, 5 μm (Bio-Rad Laboratories, Hercules, California, USA)	45		20	0.014	0.001	0.006	0.0001
This study	LC-RID	Isocratic, 8.5 mmol·l^{-1} H_2SO_4	0.40	Hi-Plex Ca, 300 mm × 7.7 mm, 8 μm (Agilent Technologies, Santa Clara, California, USA)	25	40	35	0.1039	0.0896	0.1682	0.1993

DAD – diode array detector, LC – liquid chromatography, *LOD* – limit of detection, NS – not specified, RID – refractive index detector, T_C – temperature of column, T_D – temperature of detector.

Tab. 6. Results of analysis of sugars and citric acid in commercial energy drinks and sports aids.

Sample	Saccharose		Glucose		Fructose		Citric acid		Total sugars		
	c [g·l^{-1}]	U_x [g·l^{-1}]	*c* [g·l^{-1}]	U_x [g·l^{-1}]	*c* [g·l^{-1}]	U_x [g·l^{-1}]	*c* [g·l^{-1}]	U_x [g·l^{-1}]	Concentration declared on the label [g·l^{-1}]	Concentration determined [g·l^{-1}]	Share of the declared value [%]
Energy drinks with declared sugar content											
A	48.95 ± 0.15	3.22	50.61 ± 0.48	8.69	32.43 ± 0.22	1.59	9.35 ± 0.28	0.81	108.0	132 ± 0.56	121.0
C	20.11 ± 6.74	1.32	22.06 ± 6.74	3.79	24.01 ± 7.28	1.17	3.51 ± 0.45	0.31	60.0	66.18 ± 7.27	110.0
D	64.88 ± 6.18	4.26	39.61 ± 3.80	6.80	26.20 ± 0.67	1.28	9.29 ± 0.16	0.81	112.5	130.70 ± 10.67	116.0
F	4.87 ± 1.36	0.32	3.83 ± 2.18	0.66	18.33 ± 0.24	0.90	2.60 ± 0.13	0.23	25.3	27.04 ± 0.58	106.0
G	< 0.1039	–	2.76 ± 0.19	0.47	17.11 ± 0.27	0.84	< 0.1682	–	19.7	19.87 ± 0.46	100.0
H	40.17 ± 5.36	2.64	54.63 ± 3.10	9.39	56.89 ± 1.22	2.78	7.08 ± 2.05	0.62	72.0	151.69 ± 9.69	210.0
Energy drinks with no sugar added (artificial sweeteners)											
B	< 0.1039		2.01 ± 0.04	0.34	< 0.1993		8.90 ± 0.39	0.77	Null	2.00 ± 0.04	–
E	< 0.1039		< 0.0896		< 0.1993		3.93 ± 0.25	0.34	Null	0	–
I	< 0.1039		< 0.0896		< 0.1993		4.40 ± 0.06	0.38	Null	0	–
Powdered sports aids											
J	< 0.1039		< 0.0896		< 0.1993		3.06 ± 0.67	0.27	Null	0	–
K	< 0.1039		< 0.0896		< 0.1993		3.31 ± 0.23	0.29	Null	0	–
Gummy sports aids											
L	7.18 ± 0.55	0.47	1.34 ± 0.31	0.23	0.76 ± 0.01	0.04	< 0.1682		10.1	9.28 ± 0.87	92.0

Concentration is expressed as mean ± standard deviation, mean values resulting from two different production batches, each batch measured in triplicate.
c – concentration, U_x – absolute uncertainty.

for the beverage labeled as B, in which glucose was found at 2.01 $g \cdot l^{-1}$ (Tab. 6).

Noteworthy, at least one third of the total sugar in drink formulations was fructose, a sugar readily metabolized by an insulin-independent way and producing minor increase in glycemia [43]. For example, for beverages labeled as H, the relative proportion of saccharose, glucose and fructose was 26.5 %, 36.0 % and 37.5 %, respectively (Tab. 6). Interestingly, two samples, H and A, exceeded considerably (i. e. at 121.0 % and 210.0 %, respectively) the declared sugar concentration. Both these values even surpassed the ± 20 % tolerance allowed in the food industry regarding the declaration of content. This is a curious result demonstrating that sugars were present in much higher concentrations than other components in the drinks. In terms of calories, most energy from the drinks comes in the form of sugars. Considering the amount of added sugars in these products, some countries have initiated programs to reduce the content of this ingredient [44].

Determination of citric acid

Similar to sugars, high sodium intake has been related to disease and its intake has been recommended to be below 2.3 $g \cdot d^{-1}$ [45]. Interestingly, in most of the energy drinks examined, the sole input of sodium comes in the form of sodium citrate [28]. Hence, measuring citrate routinely can indirectly provide information on the amount of sodium present in beverages. In the case of the drinks tested, mostly all of them contained citric acid as an acidulant, concentrations ranging from 2.60 $g \cdot l^{-1}$ to 9.35 $g \cdot l^{-1}$. The highest concentration represents 27.50 mmol (632.5 mg) sodium per a portion of 248 ml.

Determination of caffeine

Caffeine, as a stimulant, has an impact on physical and cognitive performance, but its reported effects vary in magnitude [46]. Most analytical approaches developed for soft drinks include simultaneous determination of caffeine and other food additives (Tab. 7). For example, Aşçı et al. [47] determined preservatives, colourants and caffeine in energy drinks with values below regulatory thresholds. Also, Turak et al. [48] used an approach similar to ours, as they simultaneously analysed ascorbic acid and caffeine in soft drinks. The concentration of caffeine determined in the sole energy drink that they examined (145 $\mu g \cdot ml^{-1}$) was considerably lower than those found in the energy drinks surveyed herein (i. e. 222.06–722.49 $\mu g \cdot ml^{-1}$, Tab. 8).

Tab. 7. Chromatographic conditions and parameters of methods for determination of caffeine in energy drinks.

Ref.	Equipment	Mobile phase	Flow [ml·min^{-1}]	Column	Wavelength [nm]	*T* [°C]	Run time [min]	*LOD* [$\mu g \cdot ml^{-1}$]	*LOQ* [$\mu g \cdot ml^{-1}$]
[26]	LC-PDA	A: MeOH B: 0.05 mol·l^{-1} NaH_2PO_4 containing 0.005 mol·l^{-1} hexanesulfonic acid, pH 3.0	0.8	Kinetex C18, 150 mm × 4.6 mm, 2.6 μm (Phenomenex, Torrance, California, USA)	270	30	25	0.005	0.016
[27]	LC-UV-Vis	A: H_2O:THF 1 g·l^{-1}, pH 8 B: ACN (90 : 10)	0.8	Zorbax Eclipse XDB-C8, 150 mm × 4.6 mm, 5 μm (Agilent Technologies, Santa Clara, California, USA)	273	25	9	0.07	0.20
[35]	LC-UV-Vis	A: H_2O B: MeOH (60 : 40)	1	Zorbax Eclipse Plus C18, 150 mm × 4.6 mm, 5 μm (Agilent Technologies, Santa Clara, California, USA)	275	40	5	NS	NS
[47]	LC-UV-Vis	A: 0.025 mol·l^{-1} HAOc/AOc- B: ACN	1	Inertsil ODS-3V, 250 mm × 4.6 mm, 5 μm (GL Sciences, Tokyo, Japan)	230	NS	10	0.19	0.63
[48]	LC-PDA	A: ACN B: 0.2 mol·l^{-1} H_3PO_4	0.25	BEH C18, 100 mm × 2.1 mm, 1.7 μm (Waters, Milford, Massachusetts, USA)	273.6	50	14	1.44	4.78
[49]	LC-UV-Vis	A: MeOH B: H_2O (28 : 72)	1	Nucelosil HD 100 RP-18, 150 mm × 4.6 mm, 5 μm (Macherey Nagel, Düren, Germany)	272	NS	NS	1	5
This study	LC-PDA	A: MeOH B: H_2O (24 : 76)	1	Zorbax Eclipse Plus C18, 150 mm × 4.6 mm, 5 μm (Agilent Technologies, Santa Clara, California, USA)	272	25	10	0.016	0.053

ACN – acetonitrile, HAOc/AOc- – acetic acid/acetate ion, LC – liquid chromatography, *LOD* – limit of detection, *LOQ* – limit of quantification, MeOH – methanol, NS – not specified, ODS – octadecylsilane, PDA – photodiode array detector, *T* – temperature, THF – tetrahydrofurane, UV-Vis – ultraviolet-visible spectrophotometric detector.

Tab. 8. Results of analysis of caffeine in commercial samples of energy drinks and sports aids.

Sample	Caffeine			
	Concentration declared on the label [$\mu g \cdot ml^{-1}$]	Concentration determined [$\mu g \cdot ml^{-1}$]	U_x [$\mu g \cdot ml^{-1}$]	Share of the declared value [%]
Energy drinks declaring caffeine content				
A	300	386.22 ± 35.34	21.36	129.0
B	336	363.32 ± 70.57	20.09	108.0
C	156	222.06 ± 52.79	12.28	142.0
D	333	417.23 ± 36.85	23.07	125.0
E	634	664.27 ± 62.43	36.73	105.0
F	338	428.85 ± 38.65	23.72	126.0
G	262	404.13 ± 38.62	22.35	154.0
H	320	367.85 ± 41.78	20.34	115.0
I	700	722.49 ± 8.09	39.95	103.0
J	286	387.91 ± 50.39	21.45	135.0
Powdered sports aids				
K	Not declared	< 0.016		Not applicable
Gummy sports aids				
L	Not declared	< 0.016		Not applicable

Concentration is expressed as mean ± standard deviation, mean values resulting from two different production batches, each batch measured in triplicate.
U_x – absolute uncertainty..

On the other hand, that was approximately a third of the concentration that the authors found in commercial iced tea preparations. Similarly, Lage-Yusty et al. [26] found concentrations of caffeine ranging from 252 $\mu g \cdot ml^{-1}$ to 304 $\mu g \cdot ml^{-1}$ when energy drinks were assessed in Spain. Hence, some energy drinks sold in Costa Rica significantly ($p < 0.05$) surpass standard concentrations found in energy drinks elsewhere. In other Latin-American countries, the caffeine concentration in energy drinks formulations has been demonstrated to be equivalent to some coffee and mate preparations [49]. Noteworthy, caffeine intake from energy drinks must be added to the amount of caffeine from coffee consumption, which is relatively high in these countries [50]. For example, if a 60 kg person consumes daily two cups of coffee and a can of energy drink A (237 ml at 95 mg each) and two bottles of cola (600 ml, 57.48 mg each), he or she would consume a total of 397 mg caffeine (6.62 mg per day per body weight). Noteworthy, from all analytes tested, most overdosage cases were found for caffeine (up to 54 % over the expected level, Tab. 8). Six of 12 samples were found in this situation.

CONCLUSIONS

This study provides information on concentration of physiologically active compounds in energy drinks and sports aids commercialized in Costa Rica together with information on useful analytical methods to determine them. Using the proposed methods, sample pre-treatment is minimal and all the important components can be determined with relative ease, using the instrumentation commonly found in laboratories, especially those dealing with food analysis. We suggest that main features of energy drinks are included in surveillance or monitoring programs. Regional and international limits must be set as maxima. Better quality control and reduction of excess consumption of the active substances may have positive health implications. Even though the energy drink market is targeted to a specific age group and people with a certain socio-economic level, we contest that reducing most of the main components should be considered and strict monitoring of labels should be performed. Hence, enforced regulation and restriction of energy drinks for children and adolescent consumption is urgently needed in addition to greater visibility of consumption recommendations. Finally, as energy drinks are fashionable, long-term data on their health and metabolic effects are still lacking and should be obtained through targeted research.

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