Food Chemistry Advances 5 (2024) 100823

Contents lists available at ScienceDirect

Food Chemistry Advances

journal homepage: www.elsevier.com/locate/focha

Development, characterization and stability of a novel sport drink based on thermal water, apple juice and hibiscus

Maroua Fatma Radhouane [a,b], Tayse F.F. da Silveira [a,*], Jessica Ribeiro [a], Paula Rodrigues [a], Rafaela Guimarães [c], Ricardo Calhelha [a], Filipa Mandim [a], Ichrak Charfi [b,d], Isabel C.F.R. Ferreira [a], Maria José Alves [a,c], Lillian Barros [a], Sandrina A. Heleno [a]

[a] CIMO, LA SusTEC, Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[b] Private University of Tunis, Avenue Kheireddine Pacha, Tunis, Tunisia
[c] AquaValor - Centro de Valorização e Transferência de Tecnologia da Água – Associação, 5400-342, Chaves, Portugal
[d] Laboratory of Innovation and Valorization for Sustainable Food Industry, Higher school of food industries of Tunis, University of Carthage, Avenue Alain Savary, Tunis, Tunisia

ARTICLE INFO

Keywords:
Sports beverage
Natural mineral water
Minerals
Apple juice
Hibiscus
Stability
Phenolic compounds

ABSTRACT

Isotonic drinks are sports beverages that replenish minerals and rapidly absorbable carbohydrates. We proposed to surpass traditional formulations of isotonic drinks by integrating thermal water, which remains unexploited as a source of electrolytes in isotonic despite the high mineral content. The novel formula combined thermal water with apple juice and hibiscus extract (as sources of sugars and color, respectively, and bioactive compounds). Through comprehensive analysis of chemical, physicochemical, and microbiological properties over 45 days at 4°C and 25°C, the results unveiled an isotonic drink with satisfactory characteristics for this product category: pH of 3.72, 7.93 mg/100 mL of total sugars, and 148.30 mg/100 mL minerals, predominantly sodium (34.99 mg/100 mL) and potassium (73.20 mg/100 mL). It showed significant phenolic compounds, notably chlorogenic acid (2.75±0.10 mg/100 mL), and antioxidant activity (472.72 μmol Trolox eq/100 mL). It maintained a vibrant red hue derived from hibiscus anthocyanins (delphinidin 3-*O*-sambubioside and cyanidin 3-*O*-sambubioside, totaling 4.06±0.03 mg/100 mL), and demonstrated microbiological stability throughout the storage period in both temperatures studied. Therefore, by harnessing the potential of thermal water, this study paves the way for a novel approach to developing sports beverages and can serve as a model for countries rich in thermal springs.

1. Introduction

Nowadays, it is well recognized that lifestyle, including diet and physical activity, are critical factors in health maintenance and prevention of many diseases, such as cancer and cardiovascular pathologies (Sharifi-Rad & Rodrigues, 2020). This has led to a growing interest in developing food products with health-promoting properties. Moreover, people are increasingly inclined to do sports, which leads to the consumption of sport-related products, such as food supplements and isotonic drinks (ID) (Carreño & Dolle, 2017). ID is a sports drink consumed during intense physical exercise, whose primary role is rehydration and replacement of carbohydrates and minerals, such as sodium and potassium.

Commercially available ID is predominantly composed of artificial flavors and dyes, added sugars (glucose, sucrose), minerals, and synthetic vitamins, which goes in the opposite direction of the current consumer's requirements for healthy, clean-label, and all-natural based foods and ingredients (Gironés-Vilaplana, Mena, Moreno, & García-Viguera, 2014; Tomczyk, Zaguła, & Dżugan, 2020). This raises the need to look for alternative ingredients and develop innovative ID products. Recent studies have shown that consumers are open to innovation in sports foods and interested in new flavors of isotonic drinks and formulations based on natural ingredients (Cui et al., 2022).

Thermal waters (TW) are natural mineral waters whose diverse chemical composition and mineralization have been associated with various beneficial effects on health, such as alleviating symptoms of inflammation-related diseases (Silva et al., 2020). Portugal is one of the wealthiest European countries in TW sources, most located in the Northern and central regions, such as the one in Chaves (*Supplementary Fig. 1*) (Silva et al., 2020).

* Corresponding author.
E-mail address: tayse.silveira@ipb.pt (T.F.F. da Silveira).

https://doi.org/10.1016/j.focha.2024.100823
Received 6 June 2024; Received in revised form 25 September 2024; Accepted 29 September 2024
Available online 30 September 2024

TW has been included in many medicinal and cosmetic formulations, but its potential in food and beverage formulations has yet to be explored. Due to the interesting chemical composition of Chaves TW, which is notably rich in sodium, potassium, magnesium, and other minerals that play a significant role in fluid homeostasis (Sadowska, Świderski, & Laskowski, 2020), it was hypothesized that TW could be successfully incorporated to produce an ID without the need of artificially added minerals.

Hibiscus sabdariffa L., also known as Roselle, is a flower whose calyxes make herbal teas, which are highly appreciated for their pleasant taste. Hibiscus has also been broadly addressed for its health-promoting effects due to the presence of anthocyanins and other antioxidant compounds (Arce-Reynoso, Mateos, Mendivil, Zamora-Gasga, & Sáyago-Ayerdi, 2023). Additionally, due to anthocyanin pigments, hibiscus may be a promising source of water-soluble red colorants with the potential to be applied in food products to replace synthetic dyes (Adeyi et al., 2022).

Apple (*Malus domestica* borkh.), one of the most widely consumed fruits worldwide, is an essential part of the human diet as a source of monosaccharide, dietary fibers, and phenolic compounds (Kahle, Kraus, & Richling, 2005; Zielinski et al., 2014). Carbohydrates account for more than 98% of the total soluble solids in apple juice. The main sugars are fructose, glucose, and sucrose, with an approximate ratio of 6:3:2 (Pruksasri, Lanner, & Novalin, 2020). Three commercially available sweeteners that approximate the carbohydrate composition of apple juice are fully inverted beet/cane sugar, high fructose corn syrup, and hydrolyzed inulin syrup. Due to these characteristics, apple juice is now a natural sweetener (Stavale, Assunção Botelho, & Zandonadi, 2019).

Previous studies have used tea, fruit juices, or extracts as a flavoring or color agent in ID formulations (de Rosso & Mercadante, 2007; Gironés-Vilaplana et al., 2014; Porfírio et al., 2020). However, no studies have utilized fruit juices as a natural sweetener and hibiscus as a coloring ingredient in ID. Therefore, this study aimed to design an innovative ID based on TW, combining attractive sensory characteristics and potential health benefits of apple juice and hibiscus aqueous extract. The data obtained herein may pave the way for an innovative approach to producing ID drinks. They can encourage novel strategies for sustainable exploration of TW by other countries rich in thermal springs across the globe.

2. Material and methods

2.1. Standards and reagents

Acetonitrile (99.9% purity) and formic acid of LC-MS grade were acquired from Fisher Scientific (Lisbon, Portugal). Chlorogenic acid, *p*-coumaric acid, and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). Micro (Fe, Cu, Mn, and Zn) and macroelements (Ca, Mg, Na, and K) standards (>99% purity), as well as $LaCl_2$ and CsCl (>99% purity), were purchased from Merck (Darmstadt, Germany). Delphinidin-3-*O*-rutinoside was obtained from Extrasyntèse (Genay, France). Folin reagent was from Panreac (Barcelone, Spain), and sodium carbonate, monobasic, and dibasic potassium phosphate were from Merck Millipore (Darmstadt, Germany). Sodium acetate and potassium chloride were obtained from Thermo Scientific (Waltham, MA, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Raw materials

Grounded dried hibiscus flowers were purchased as tea bags from local markets in Bragança, Portugal. The contents of the bags were homogenized to create a single, representative sample (approximately 50 g) and were used in this manner for the experiments.

Pasteurized commercial apple juice was obtained from local markets in Bragança, Portugal. The juice was centrifuged (6000 x g for 20 min at 10°C) to have a clarified apple juice and used in this condition for the formulations. The juice contained 16.76±0.73 g/100 mL of total sugars (11.83±0.56 g/100 mL of fructose, 2.95±0.10 g/100 mL of glucose, and 1.96±0.09 g/100 mL of sucrose). The pH was 3.5, and the total soluble solids (TSS) was 12.6 °Brix. As declared on the label, the apple juice contained no significant amounts of sodium and fiber.

The TW was provided by Termas de Chaves, Chaves, Portugal, with the following major compounds: K = 61 mg/L; Na = 614 mg/L, Ca = 23 mg/L; Mg = 4,9 mg/L; Fe = 0.67 mg/L, Zn = 0.04 mg/L; Cu = 0.01 mg/L; Mn = 0.01 mg/L. The pH of the thermal water at the receiving time was 6.7.

2.3. Production of hibiscus aqueous extracts

TW was used as the extractor solvent for the production of the aqueous hibiscus extract. Prior optimization experiments were conducted using the initial conditions reported by (Cissé et al., 2012). These experiments investigated the effect of the time and solid-to-liquid ratio on the extraction of total monomeric anthocyanins and total phenolic compounds (data not shown). The best extraction condition was chosen based on the results and is described as follows. Briefly, 50 mL of TW acidified with 0.1% citric acid was added to 1.5 g of hibiscus flowers and maintained at room temperature, protected from light, with magnetic agitation for 43 minutes. After the extraction, the samples were centrifuged (6000 x g for 20 min at 10°C), and the upper phase was filtrated via Whatman paper filter n°1. As such, it yielded 2.64 mg/g of total anthocyanins, as measured by the differential pH method described in Section 2.7, and 14.13 mg GAE/g of total phenolic compounds evaluated using the Folin-Ciocalteu method according to the description in Section 2.7. The extracts were then directly incorporated into the isotonic drink at different concentrations during the formulation step.

2.4. Formulation of the isotonic drink

The ID comprised TW, apple juice, and hibiscus aqueous extract obtained as described in Section 2.3. The proportion of each ingredient was defined through formulation tests considering sensory properties (color and taste), sugar and sodium contents, and literature references (Gironés-Vilaplana et al., 2014; Porfírio et al., 2020), as described below.

The amount of TW in the formulation was set to achieve the sodium content of commercial ID (290 to 500 mg/L) (Porfírio et al., 2020), as this element is the most important in this type of beverage for being the primary electrolyte in the bloodstream (Sadowska et al., 2020).

The amount of apple juice was determined based on the sugar content of commercial ID (between 60 and 100 g/L). For that, the individual sugar concentration and the TSS of the commercial apple juice were measured as described in Section 2.7, and the necessary dilution of the apple juice to deliver the desired concentration of sugars was calculated.

Different concentrations were tested and evaluated for color and taste to determine the concentration of hibiscus aqueous extract in the formulation. Pure hibiscus extract was tested, but it resulted in unpleasant sensory characteristics due to the high acidity attributed to hibiscus. Then, several dilutions of the pure hibiscus extract (60% to 20% of the original concentration) were prepared. The extract concentration in the formulation that resulted in more satisfactory sensory terms (taste and color) was selected for the study, and more satisfactory sensory terms (taste and color) were chosen for the next steps. The dilution of the hibiscus extract was carried out with TW. Once ready, this formulation was called F1 (Table 1).

One control formulation was prepared with distilled water instead of TW, named F2 (Table 1) for comparison purposes. In these samples, hibiscus was extracted with distilled water (acidified with 0.1% citric acid), and all the necessary dilutions described above for F1 were made with distilled water. The sodium content was adjusted with sodium

Table 1
Description and codes of the studied isotonic drink formulations.

Formulation code	Description ingredients	Quantity (mL or g) per 100 mL
F1	Thermal water	33
	Hibiscus extract[1]	22
	Apple juice	45
F2	Distilled water	33
	Hibiscus extract[1]	22
	Apple juice	45
	Sodium chloride	0.0281

[1] Final concentration of the extract in the beverage: 40% of the original concentration.

chloride (PanReac, Milano, Italia). A flowchart of the designed ID's production and the composition of F1 and F2 can be found in Fig. 1 and Table 1, respectively.

2.5. Thermal treatment

After formulation, the IDs were placed in 100 mL screw-capped aluminum pouches and pasteurized. For pasteurization, 60 mL of each beverage was placed in the pouches and fixed in a thermostatic water bath at (85 °C). The temperature at the core of a control pouch pack was monitored using a digital thermometer. When the temperature was reached (85 °C), time counting was triggered using a chronometer up to 30 seconds. Afterward, pasteurization was interrupted by immersing the pouches in an ice water bath at (4 °C) until (25 °C).

2.6. Characterization of the isotonic drinks and stability studies

The ID was characterized for the following analyses: color, pH, TSS (°Brix), total phenolic compounds (TPC), total monomeric anthocyanins (TMA), individual anthocyanins, and non-anthocyanin phenolic compounds by LC-DAD-ESI-MS^n, free sugars content, mineral concentration, antioxidant activity using Oxygen Radical Absorbance Capacity (ORAC) assay and a cell model antioxidant assay (CAA).

For the stability studies, the ID was stored at room temperature (25°C) or in the fridge at 4°C for 45 days. Three pouches of each temperature were collected on days 0, 7, 15, 30, and 45 for color, pH, TSS, TPC, TMA, anthocyanins and non-anthocyanin phenolic compounds, antioxidant activity by ORAC assay, and microbial load.

2.7. Methods

2.7.1. pH and total soluble solids

The pH was measured using a calibrated digital pH meter (HI 99161, Hanna Instruments, Woonsocket, RI, USA), and TSS was determined as °Brix using a digital refractometer (Milwaukee MA871, Rocky Mount, North Carolina, USA).

2.7.2. Color measurement

The color was measured using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan) previously calibrated against a standard white tile. The CIE L* (lightness), a* (greenness-redness), and b* (blueness-yellowness) color space values were recorded using Spectra Magic Nx software (version CM-S100W 2.03.0006). Using L*a*b* data,

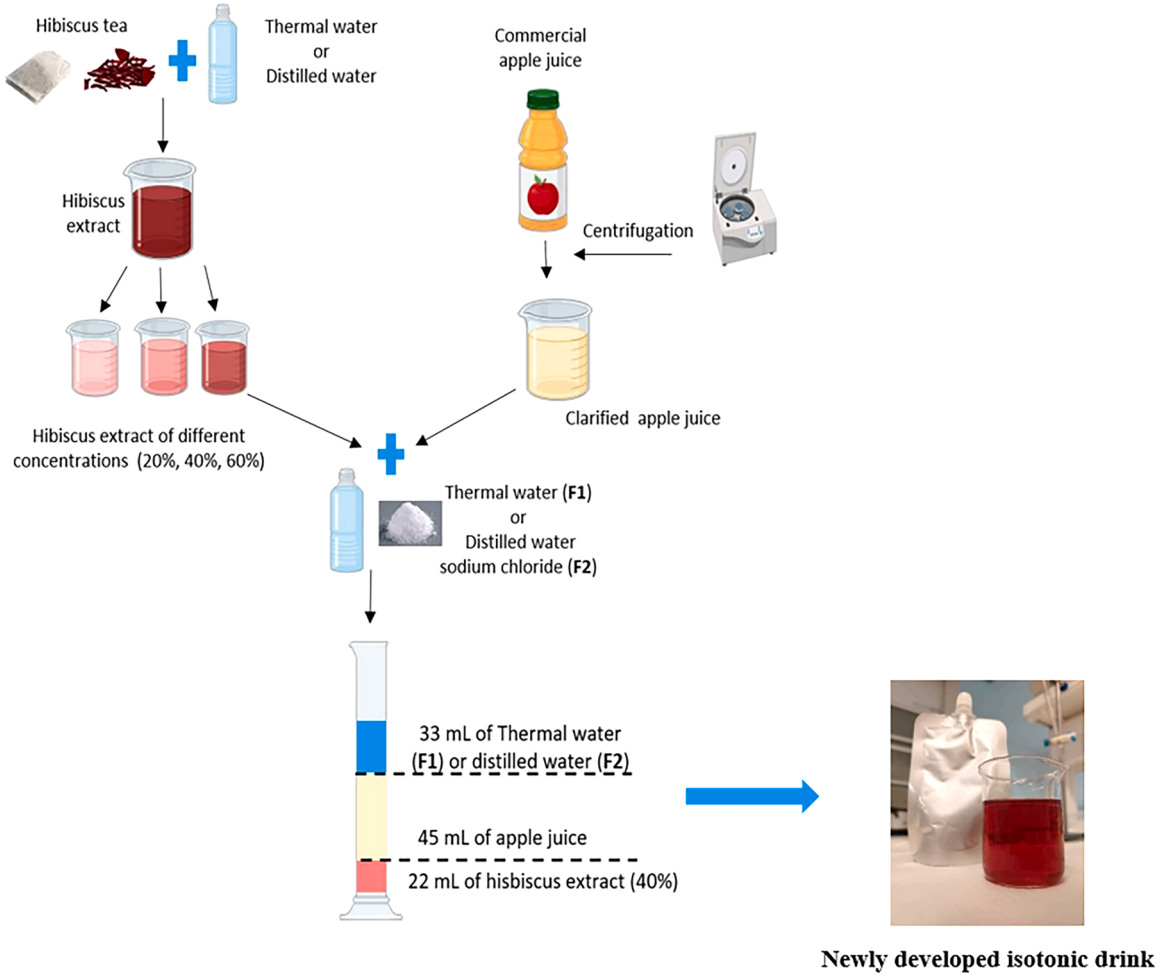

Fig. 1. Flowchart of the production of the newly developed isotonic drink.

ΔE* was calculated according to the following equation (de Rosso & Mercadante, 2007)

$$\Delta E^{*}: \left[(\Delta L^{*})^{2} + (\Delta a^{*})^{2} + (\Delta b)^{2}\right]^{1/2}$$

2.7.3. Total phenolic compounds

The Folin-Ciocalteu method, as described by Singleton and Rossi (1965), was adapted to a microplate reader (Spectro star nano ELX800 BMG LABTECH, Ortenberg, Germany). The samples or the gallic acid standard were set to react with the Folin reagent for 5 minutes, and then sodium carbonate (7.5%) was added to the mixture. After 2 hours, the blue color intensity of the samples was measured at 760 nm. Results are expressed as mg of gallic acid equivalents (mg GAE) per 100 ml sample.

2.7.4. Total monomeric anthocyanin compounds

Total monomeric anthocyanin (TMA) content was determined using the pH differential method described by Giusti and Wrolstad (2001). Samples were diluted adequately in sodium acetate buffer (pH 1.0) and potassium chloride buffer (pH 4.5) to achieve absorbances between 0.1 and 1.0. They were incubated for 15 minutes, and read at 510 and 700 nm in a SPECTROstar Nano Microplate Reader (BMG Labtech, Ortenberg, Alemanha). The anthocyanin content was calculated by the following formula, where $A = (A_{510nm} - A_{700nm})_{pH\ 1.0} - (A_{510nm} - A_{700nm})_{pH\ 4.5}$, MW = 449.2 g/mL (molecular weight of cyanidin-3-glucoside), DF = dilution factor, and ε = 26.900 L/mol (the molar absorption coefficient of cyanidin-3-glucoside). The results are expressed as mg of cyanidin-3-glucoside per 100 mL sample.

$$TMA = \frac{(A \times MW \times DF \times 1000)}{\varepsilon \times 1}$$

2.7.5. Anthocyanin compounds by LC-DAD-ESI-MS^n

Major individual anthocyanins were separated on an Aqua C18 (5 μm, 4.6 mm x 150 mm, Waters, Milford, MA, USA) column working at 35°C, using a Dionex Ultimate 3000 UHPLC (Thermo Scientific), according to Leichtweis, Pereira, and Prieto (2019). The gradient elution was: (A) 0.1% trifluoroacetic acid (TFA) in water, (B) acetonitrile, 10% B for 3 min, from 10 to 15% B for 12 min, 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min, from 30 to 35% B for 5 min, and from 35 to 10% B for 10 min, using a flow rate of 0.5 mL/min. Detection was carried out in the DAD using 520 nm and in a mass spectrometer (Linear Ion Trap LTQ XL, Thermo Scientific) equipped with an ESI source in positive mode. Nitrogen served as the sheath gas (50 psi); the system was operated with a spray voltage of 4.8 kV, a source temperature of 320°C, and a capillary voltage of 14 V. The full scan covers the mass range from *m/z* 100 to 1800. The collision energy used was 35 (arbitrary units). Anthocyanins were identified by comparing their retention times, UV–Vis, and mass spectra with those obtained from standard compounds, when available, or with data provided in the literature, and the quantification was performed using a calibration curve of delphinidin-3-*O*-rutinoside. The results are expressed as mg/100 mL.

2.7.6. Non-anthocyanin phenolic compounds by LC-DAD-ESI-MS^n

Major individual phenolic compounds were analyzed through high-performance liquid chromatography (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA), according to Bessada, Barreira, Barros, Ferreira, and Oliveira (2016). The chromatographic separation of the compounds was performed using a Waters Spherisorb S3 ODS-2C18 (3 μm, 4.6 mm × 150 mm, Waters, Mil-ford, MA, USA) column thermostatted at 35°C. The mobile phase was composed of (A) 0.1% formic acid in the water and (B) acetonitrile, with the following gradient elution: 0% B (5 min), 15% B to 20% B (5 min), 20–25% B (10 min), 25–35% B (10 min), 35–50% B (10 min), using a flow rate of 0.5 mL/min. Detection was carried out with a diode array detector (DAD) at 280, 330 nm, and 370 nm and connected with a Linear Ion Trap LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with an ESI source working in negative mode. For MS detection, nitrogen served as the sheath gas (50 psi); the system operated with a spray voltage of 5 kV, a source temperature of 325°C, and a capillary voltage of −20 V. The full scan covered the mass range from *m/z* 100–1800, and the collision energy used was 35 (arbitrary units). The phenolic compounds were identified by comparing their retention times, UV–Vis, and mass spectra with those obtained from standard compounds, when available, or with data provided in the literature, and the quantification was performed using calibration curves of chlorogenic acid (for caffeoylquinic acid derivatives) and *p*-coumaric acid (for *p*-coumaroyl derivatives). The results are expressed as mg/100 mL.

2.7.7. Mineral composition

Minerals were analyzed using Atomic absorption spectrometry with air/acetylene flame Perkin Elmer PinAAcle 900T Atomic Absorption Spectrophotometer (Perkin Elmer, Waltham, MA, USA). To analyze calcium (Ca) and magnesium (Mg), the samples were diluted with the appropriate volume of 1 g/L lanthanum chloride solution. Standard Ca and Mg solutions were prepared by properly diluting the commercial standard solutions (1000 mg/L) with 1 g/L lanthanum Chloride solution. For iron (Fe), Manganese (Mn), Copper (Cu), and Zinc (Zn) analysis were performed directly on the sample. Standard solutions were prepared by diluting the respective commercial solution (1000 mg/L) using a 5% HNO_3 solution. The results are expressed as mg/100 mL.

2.7.8. Analysis of individual sugars

Individual sugars were determined by High-Performance Liquid Chromatography (Knauer, Smartline system 1000, Berlin, Germany) coupled to a refractive index (RI) detector, according to Barros, Carvalho, and Ferreira, (2010), Zielinski et al. (2014). The samples were filtered through a 0.22 μm filter membrane in vials and injected for analysis. The column used was a 250 mm × 4.6 mm, 5 μm, Eurospher 100-5 NH2 with a 5 mm × 4 mm guard column of the same material (Knauer, Berlin, Germany), operating at 30°C. The mobile phase was acetonitrile/ water 70:30 (v/v) at a 1 mL/min flow rate. Sugars were identified by comparing peak retention times with commercial standards, and quantification was performed using calibration curves of the corresponding sugars. The results are expressed as g/100 mL.

2.7.9. Antioxidant activity

2.7.9.1. Oxygen radical absorbance capacity (ORAC). The tests were performed according to the method described by Dávalos Gómez-Cordovés and Bartolomé, 2004) using a microplate reader FLUOstar Omega (BMG LABTECH, Ortenberg, Germany). Fluorescein was used as the fluorescent probe, and potassium phosphate buffer (pH 7.4, 75 mM) was used as the reaction medium. The microplates containing 20 μL of the sample or different concentrations of Trolox, 120 μL of fluorescein (0.4 μg/mL), and 60 μL of radical AAPH (2.2′-azobis (2-methylpropionamidine) dihydrochloride) (108 mg/mL) was subjected to reading every 1 min for a total of 80 min (emission and excitation wavelength of 485 nm and 520 nm, respectively), under controlled temperature at 37 °C. ORAC results were determined using a regression equation relating to Trolox concentrations and the net area under the fluorescein kinetic decay curve. They are expressed in μmol Trolox equivalent per 100 mL of sample (Trolox eq/100 mL).

2.7.9.2. Cellular antioxidant assay (CAA). The antioxidant activity in cell lines was determined following the modified method described by de la Fuente et al. (2022). For this procedure, the cell line used was RAW *264.7* (murine macrophages) commercially acquired from European Collection of Authenticated Cell cultures (ECACC 91062702), which was maintained in a DMEM (Dulbecco′s Modified Eagle′s Medium) culture medium supplemented with L-glutamine, penicillin (100 U/mL),

streptomycin (100 μg/mL), fetal bovine serum (10%) and non-essential amino acids (2 mM), in an incubator at 37°C with a humidified atmosphere and 5% CO_2 (Heal Force CO_2 Incubator, Shanghai Lishen Scientific Equipment Co, Ltd.). The cells were used only when they had 70 to 80% confluence and were seeded. A 70,000 cells/mL density solution was prepared and transferred (300 μL) to black microplates with clear bottoms (SPL Lifesciences). After proliferation for 48 hours and the cells being confluent, the cells were washed with HBSS (Hanks Balanced Salt Solution, 2x, 100 μL) and treated with the isotonic drink in 4 dilution scenarios: no dilution, 4, 16, 64, and 256 times in water (H_2O), which was diluted with 2′,7′-dichlorohydrofluorescein (DCFH) prepared with ethanol and diluted with HBSS (50 μM). After incubation for 1 hour under the conditions mentioned above, the cells were washed with HBSS (2x, 100 μL) and a solution of 2,2 2′-azobis (2-methyl propionamide) dihydrochloride (AAPH) (100 μL; 600 μM) was added. Fluorescence was read every 5 minutes for 1 hour (Biotek FLx800 microplate reader) at 485 nm excitation and 538 nm emission. Quercetin was used as the positive control, and dichlorohydroflurescein and DMEM culture medium were used as the negative control during the assay. The results are given as percent inhibition of cell oxidation.

2.7.10. Microbiological analysis

The following microorganisms were analyzed: total aerobic mesophiles, molds, and yeasts to determine the microbial load in the isotonic drink for 45 days at room temperature (25°C) or fridge temperature (4°C). The sample preparation follows the procedure described in the International Organization for Standardization (Standards, 2003). These analyses were performed immediately after the isotonic drink preparation (T0), on seven days (T7), on 15 days (T15), on 30 days (T30), and on 45 days (T45).

Total aerobic mesophiles were determined using the incorporation sowing approach, which involved placing 1 mL of each dilution of the material in a Petri dish and then adding 15 mL of Plate Count Agar (PCA), incubated for 72 hours at 30°C while being held inverted. Plates with 15–300 colonies were used for counting (Limit of Quantification (LOQ) = 1 log (Colony Forming Units) CFU/g).

Molds and yeasts: The spread plate method added 0.2 mL of each dilution to Petri dishes containing 15 mL of Dichloran Rose Bengal Chloramphenicol (DRBC). The counting was done on plates with fewer than 150 colonies (LOQ = 1.7 log CFU/g), which were incubated upright at 25°C for five days. After 3 and 5 days of incubation, respectively, yeasts and molds were enumerated.

2.7.11. Statistical analysis

All the analyses were conducted in triplicate, and the results were expressed as mean±standard deviation. The samples were compared using a *t*-test or Repeated Measures ANOVA followed by a Bonferroni post-hoc test. Samples were considered different when $p<0.05$ at a 95% confidence level. Statistica 7.0 was used to treat the data.

3. Results and discussion

3.1. Development of the isotonic drink formulation

In the development of the ID formulation, a series of formulation tests were carried out to fine-tune the composition and sensory characteristics of the final product. Table 1 and Fig. 1 show the final formulation's composition and appearance. The volumes presented were determined based on the beverage's desired sugar and sodium contents, which must be placed within an acceptable range for an ID. The addition of hibiscus extracts to achieve a final concentration of 40% of its original was selected to be applied in the final formulation (Fig. 1). This was because it provided the best combination of taste and color (*in-house* evaluation).

3.2. Characterization of the formulated isotonic drink

The chemical and physicochemical composition of the formulated isotonic drink is shown in Table 2.

The pH (Table 2) was within the typical range for isotonic drinks, as reported in previous studies (Bendaali, Vaquero, González, & Morata, 2022; Gironés-Vilaplana et al., 2014). Notably, F1 showed a higher ($p<0.05$) pH value (3.72±0.01) compared with the control formulation (F2, 3.12±0.01) despite the use of the same ingredients (0.1% citric acid). This difference could be attributed to a buffering effect from TW due to its natural mineral content, especially the bicarbonate that can effectively reduce net acid excretion (Wasserfurth et al., 2019).

TSS (Table 2) is an essential parameter for the quality of the final product of isotonic drinks (Santos, Alves, & Lima, 2013). The TSS values of F1 were 5.70±0.05 °Brix and F2 5.60±0.01 °Brix. These values are slightly lower than those in previous studies (6.10-8.10 °Brix).

Table 2 also shows the free sugar composition of the ID, which originated primarily from apple juice. The total concentration of free sugars was 7.93±0.18 g/100 mL (F1), which was comparable ($p>0.05$) with F2 (8.30±0.49 g/100 mL). As expected, fructose was the primary sugar found in the ID, followed by glucose and sucrose, aligning with previous findings for apple juice (Dussling, Steingass, Dreifke, Will, & Schweiggert, 2024). The typical carbohydrate concentrations in ID may vary between 6 and 8% and can achieve up to 10% (Tomczyk et al.,

Table 2
Chemical and physicochemical composition of the newly developed isotonic drink.

Parameter	F1	F2
pH	3.72±0.01[a]	3.12±0.01[b]
TSS (°Brix)[1]	5.70±0.05[a]	5.60±0.01[a]
L*	32.35±0.19[a]	30.77±0.45[b]
a*	24.13±0.27[b]	28.94±0.24[a]
b*	16.40±0.21[b]	19.92±0.15[a]
TMA (mg/100 mL)[2]	4.06±0.03[a]	3.89±0.05[b]
TPC (mg Eq GA/100 mL)[3]	41.36±1.34[a]	39.16±1.09[a]
ORAC (μmol/100 mL)[4]	472.72±26.45[a]	473.46±26.60[a]
CAA[5]	No activity	No activity
Sugars (g/100 mL)		
Fructose	5.41±0.11[a]	5.68±0.37[a]
Glucose	1.57±0.04[a]	1.61±0.14[a]
Sucrose	0.95±0.03[a]	1.00±0.02[a]
Total sugars	7.93±0.18[a]	8.30±0.49[a]
Minerals (mg/100 mL)		
Na	34.99±2.30[a]	18.74±0.24[b]
K	73.20±3.20[a]	49.6±1.81[b]
Ca	9.90±1.16[a]	8.54±1.05[a]
Mg	26.95±4.83[a]	24.51±3.38[a]
Mn	2.17±0.18[a]	2.37±0.13[a]
Fe	0.64±0.03[a]	0.60±0.04[a]
Zn	0.42±0.04[a]	0.40±0.01[a]
Total minerals	148.30±3.16	114.05±3.83
Individual anthocyanins (mg/100 mL)[6]		
Delphinidin 3-*O*-sambubioside	4.29±0.29[a]	4.14±0.42[a]
Cyanidin 3-*O*-sambubioside	1.84±0.14[a]	1.84±0.18[a]
Individual non-anthocyanins phenolic compounds (mg/100 mL)[6]		
3-caffeoylquinic acid	1.95±0.20[a]	1.75±0.12[a]
3-*p*-coumaroyl quinic acid	0.11±0.01[a]	0.09±0.01[b]
4-caffeoylquinic acid	0.86±0.17[a]	0.61±0.11[a]
Chlorogenic acid	2.75±0.10[a]	2.21±0.38[a]
4-*p*-coumaroyl quinic acid	0.15±0.03[a]	0.09±0.02[b]

Measurements express mean±standard deviation. Different lowercase letters in a row indicate significant differences between samples at a 95% confidence level. [1]TSS: total soluble solids, [2]TMA: total monomeric anthocyanins

[3] TPC: total phenolic compounds expressed in mg gallic acid equivalent/g sample

[4] Oxygen radical absorbance capacity

[5] Cell antioxidant assay, with Quercetin as positive control (95.3 ± 4.6% oxidation inhibition at 0.3 μg/mL)

[6] LC-MS data of the determined compounds is found in the *Supplementary Tables 1 and 2*.

2020). A study by Szwedziak (2015) indicated that the content of carbohydrates in isotonic drinks declared by manufacturers ranged from 2.7 to 6.7 g/100 ml. Hence, the sugar content identified in this product slightly exceeds the specified range; nevertheless, it remains within the acceptable limits for this product category.

The mineral content followed the same tendency. As can be observed in Table 2, TW contributed mainly to the supply of sodium (34.99±2.30 mg/100 mL and 18.74±0.24 mg/100 mL, respectively) and potassium (73.20±3.20 mg/100 mL vs. 49.6±1.81 mg/100 mL, respectively). Porfírio et al. (2020) determined the content of minerals in commercially available isotonic drinks and found values between 29 and 50 mg/100 mL of sodium and 10 and 12.5 mg/100 mL of potassium. Consequently, the ID created with TW has the potential to provide customers with the appropriate amount of electrolytes without the necessity of adding exogenous mineral compounds, as is currently done.

The concentration of the other minerals was revealed to be strongly influenced by the presence of apple juice or hibiscus extract. The data showed that adding fruit and herbal extracts enriches the ID with minerals beyond what is provided synthetically in commercial formulations. Tomczyk et al. (2020) reported a similar conclusion, who studied ID formulations added with honey, plant matrices, and jabuticaba (*Myrciaria jabuticaba*) extracts, respectively.

Regarding the color (Table 2), the results revealed that the incorporation of TW in the formulation affected this parameter to some extent, as F1 showed higher values of L* (32.35±0.19) and lower values of a* (24.13±0.27) and b* (16.40±0.21) than F2 (L*= 30.77±0.45, a*= 28.94±0.24, b*= 19.92±0.15). In summary, this data indicates that F1 was lighter (lower L*) and presented weaker redness (lower a*) and a greater degree of yellowness than F2. The slight color differences could be related to the pH value observed, which was higher in F1 than in F2. The color of anthocyanins is highly dependent on pH, and the more acidic the conditions are, the more the anthocyanins appear red (Khoo & Azlan, 2017).

Table 2 also shows phenolic compounds (total and individual) present in the ID. These compounds are attributed to the hibiscus extract and apple juice, aligning with the expected composition found in the literature (Kahle et al., 2005; Piovesana, Rodrigues, & Noreña, 2019). For total phenolic compounds, no significant difference was found between F1 and F2 (41.36±1.34 and 39.15±1.08 mg GA/100 mL, respectively) (Table 2). Concerning individual non-anthocyanin phenolic compounds, chlorogenic acid was the primary phenolic compound (2.75±0.10 mg/100 mL for F1 and 2.21±0.38 mg/100 mL for F2), which can be attributed to the presence of hibiscus extract and apple juice. This agrees with the findings of Kahle et al. (2005), Piovesana et al. (2019), who reported chlorogenic acid as the predominant phenolic acid in these ingredients. Other hydroxycinnamic acid derivatives, such as caffeoyl and *p*-coumaroyl quinic acid isomers, have also been identified in hibiscus and apple juice, explaining their presence in the formulated ID (Kahle et al., 2005; Piovesana et al., 2019). The chromatographic and mass spectrometry data of the quantified compounds can be found in *Supplementary Tables 1* and *2*.

For the total monomeric anthocyanins, F1 had a slightly higher value than F2 (4.06 mg/100 mL and 3.89 mg/100 mL, respectively). Regarding individual anthocyanins, the ID primarily contained comparable concentrations ($p>0.05$) of delphinidin-3-*O*-sambubioside (4.29 ±0.29 mg/100 mL for F1 and 4.14±0.42 mg/100 mL for F2) and cyanidin-3-*O*-sambubioside (1.84±0.14 mg/100 mL for F1 and 1.84±0.18 mg/100 mL for F2), which are the main color compounds in hibiscus flowers (Piovesana et al., 2019).

Finally, concerning the antioxidant activity using the ORAC method (Table 2), it was 472.730±26.45 μmol Trolox eq/100 mL and 473.46 ±26.61 μmol Trolox eq/100 mL for F1 and F2, respectively, with no difference between them ($p>0.05$). On the other hand, none of the formulations showed antioxidant activity when tested in the cell-based assay using RAW-264.7 cell lines. Further studies should be conducted to investigate the lack of action of the ID in this cell's experimental model of antioxidant activity.

These data imply that TW allowed the production of an ID in concordance with the requirements for this beverage category and had no influence on the content of phenolic compounds or the antioxidant activity in the developed beverage.

In recent years, the sports food industry has been making significant efforts to develop formulations that extend beyond essential nutrition (sugars and electrolytes). These formulations aim to provide functional elements that mitigate weariness and exercise-induced oxidative stress, enhancing performance (Gonçalves, Gaspar, & Flores-Félix, 2022). The data suggest that due to apple juice and hibiscus extract, the produced isotonic drink fulfills this requirement by supplying bioactive substances such as phenolic compounds. Numerous studies have explored the effects of phenolic compounds, known for their antioxidant and anti-inflammatory properties, on exercise performance (Gonçalves et al., 2022; Malaguti, Angeloni, & Hrelia, 2013). However, the results regarding the impact of these compounds on sports endurance and resistance *in vivo* remain inconclusive (Malaguti et al., 2013).

The growing demand for clean-label products and reduced use of synthetic compounds has driven the industry to seek natural solutions for food additives. The results suggest that, in addition to contributing antioxidant compounds, the hibiscus extract imparted an attractive color to the product, thanks to its anthocyanin content, eliminating the need for synthetic colorants. As a result, the developed isotonic drink aligns with current key industry and consumer preferences.

Furthermore, given that apple and hibiscus are low-cost and widely available raw materials, the product shows strong potential for scale-up. However, future studies are needed to validate the feasibility of large-scale production, including the logistical considerations of using TW as an electrolyte source. In addition to Portugal, other European countries like Germany, France, and Spain possess springs abundant in sodium, making them suitable for ID production. Hence, the findings presented here may encourage innovative strategies to explore thermal sources in these countries and worldwide.

3.3. Stability studies at different storage temperatures

3.3.1. pH, TSS, and color

The pH and TSS evolution in the ID during 45 days for both studied temperatures. The pH (*Supplementary Fig. 2a*) was stable for both temperatures. TSS (*Supplementary Fig. 2b)* showed a significant but only slight increase ($p<0.05$); therefore, it was considered stable during the storage period.

Color is one of the most important food quality characteristics for capturing consumer preference. Color measurements during the storage time at both temperatures are shown in Table 3. Both formulations showed a reduction in a* value over 45 days at 4°C (from 24.13±0.27 to 22.92±0.22 in F1 and from 28.94±0.24 to 27.37±0.24 in F2), implying a slim decrease in redness of the samples. Likewise, at 25°C, F1 and F2 showed lower a* values on day 45, varying from 24.13±0.27 to 15.26 ±0.75 (F1) and from 28.94± to 23.10±0.05 (F2). As can be noticed, the magnitude of the reduction of a* was remarkably greater at 25°C compared with that observed at 4°C (Table 3), indicating that the red color of the beverages degraded more intensely when stored under this condition.

de Rosso and Mercadante (2007) observed the decrease of a* values associated with a gradual degradation of red color in ID made with açaí or acerola extracts stored under light for 25 days at 20°C. The authors concluded that redness was closely related to anthocyanin content, and reductions in this parameter expressed anthocyanin degradation (de Rosso & Mercadante, 2007). The high sensitivity of anthocyanins to temperature variations could explain the distinct decrease rates of a* value at the different monitored temperatures (Jang & Koh, 2023).

The parameter ΔE* (Table 3) denotes the total color difference between the initial sample and after 45 days of storage. According to the data, the ID stored at 4°C had ΔE*=1.53±0.33 (F1) and 2.63±0.57 (F2),

Table 3
Color parameters of the newly developed isotonic drink.

Formulation		T0	T7	T15	T30	T45	ΔE
Storage at 4°C							
F1	**L**	32.35±0.19^{a}	33.34±0.49^{ab}	33.44±0.31^{ab}	33.15±0.74^{a}	33.20±0.13^{ab}	1.53±0.33^{b}
	a*	24.13±0.27^{b}	23.29±0.42^{a}	22.39±0.53^{a}	22.00±0.97^{a}	22.92±0.22^{a}	
	b*	16.40±0.21^{a}	15.97±0.33^{a}	15.36±0.52^{a}	15.63±0.55^{a}	16.33±0.21^{a}	
F2	**L**	30.77±0.45^{b}	31.59±0.31^{a}	31.44±0.28^{c}	32.19±0.05^{a}	32.17±0.42^{a}	2.63±0.57^{a}
	a*	28.94±0.24^{b}	27.74±0.53^{ab}	28.28±0.43^{a}	26.87±0.55^{a}	27.37±0.24^{a}	
	b*	19.92±0.15^{a}	19.24±0.72^{ab}	19.41±0.31^{ab}	17.64±0.90^{c}	18.36±0.16^{bc}	
	Storage at 25°C						
F1	**L**	32.35±0.19^{a}	34.17±0.59 ^{a}	33.89±0.28^{a}	33.91±0.78^{a}	34.92±0.36^{a}	9.34±0.90^{a}
	a*	24.13±0.27^{a}	20.74±0.55^{b}	19.11±0.93^{bc}	17.24±0.16^{c}	15.26±0.75^{d}	
	b*	16.40±0.21^{a}	15.54±0.16^{a}	16.24±0.89^{a}	16.50±0.32^{a}	17.58±0.74^{a}	
F2	**L**	30.77±0.45^{c}	31.53±0.34^{bc}	31.80±0.41^{bc}	31.94±0.30^{b}	33.27±0.27^{a}	
	a*	28.94±0.24^{a}	27.23±0.74^{b}	27.51±0.02^{b}	24.78±0.32^{c}	23.10±0.05^{d}	7.98±0.67^{b}
	b*	19.92±0.15^{a}	18.73±0.79^{ab}	18.92±0.17^{ab}	17.51±0.52^{bc}	16.97±0.39^{c}	

Measurements express mean±standard deviation. Different lowercase letters in a row indicate significant differences between samples at a 95% confidence level. For ΔE, Different lowercase letters in the column mean significant difference at a 95% confidence level.

while at 25°C, these values were 9.3±0.94 and 7.98±0.67, respectively. As reported by de Rosso and Mercadante (2007), Gonnet (2001), the color difference of anthocyanin-rich products can be easily perceived by human eyes when ΔE*>10, which connotes extensive anthocyanin degradation. Hence, the data indicate that the ID showed no visual color change in either storage temperature. This suggests that after 45 of storage, the color would still be attractive and would not adversely affect consumer preferences. However, it was noticeable that color preservation was more effective under cold storage temperatures, and extended storage periods at 25°C could potentially lead to product rejection due to color fading.

Overall, these results provide evidence that the primary factor controlling the longevity of color is the storage temperature. At the same time, incorporating TW in the ID formulation had a minor impact on the stability of color parameters compared to the control sample.

3.3.2. Phenolic compounds

3.3.2.1. Anthocyanins. The behavior of anthocyanins throughout the storage time is shown in Fig. 2**.** The total content of these compounds over 45 days (Fig. 2**a**) was reduced ($p<0.05$) at both tested storage temperatures. Similarly, Figs. 2**c and** 2**d** reveal a substantial decline in delphinidin 3-*O*-sambubioside and cyanidin 3-*O*-sambubioside concentration during the storage time, regardless of the temperature ($p<0.05$).

As expected, storage at 4°C was more effective in preserving anthocyanins (total and individual) than at 25°C, in which samples showed a sharper decrease since day 7 ($p<0.05$). On day 45, at 4°C, F1 and F2 maintained 70% of the original total anthocyanin content and 40% of individual components, respectively, compared to just 30% and 12% at 25°C (Fig. 2).

The reported drop is consistent with research conducted by Gironés-Vilaplana et al. (2016), who found that after 40 days of storage at 18–22°C, total anthocyanins in isotonic drinks, including berries and lemon, decreased by around 50%. Moreover, the authors also reported retention between 60% and 80% of individual anthocyanins, such as delphinidin 3-*O*-sambubioside and cyanidin 3-*O*-sambubioside, in the isotonic beverages stored at 7°C compared with less than 10% of retention at 37°C. These results also cross with the study by Ndong, Ndeye, Joseph, and Mady (2018), which showed how individual anthocyanin compounds from hibiscus can extend the shelf-life at a cooling temperature (4°C) without profound changes.

Additionally, Fig. 2 shows that when the samples were stored at 25°C, the incorporation of TW impacted the stability of individual anthocyanins (Figs. 2**c and** 2**d**). Under this storage condition, F1 depicted a faster degradation of delphinidin 3-*O*-sambubioside and cyanidin 3-*O*-sambubioside than F2, as from day 7, these compounds were in lower concentrations in F1 ($p<0.05$). No effect of TW was observed for the other storage conditions and total anthocyanins ($p>0.05$). The accelerated degradation of individual anthocyanins in F1 stored at 25°C but not at 4°C may be related to the higher pH of this sample compared with F2, combined with the high sensitivity of anthocyanins to heat (Jang & Koh, 2023). The color and chemical structure of anthocyanin molecules are significantly influenced by environmental pH and temperature variations. As these parameters rise, the structural integrity of anthocyanins becomes increasingly vulnerable, leading to their easy degradation (Li, Linli, Yang, & Chen, 2023).

Overall, these results concord with the color data (Section 3.2), in which anthocyanins' higher content and stability reflect a more intense and stable red color (a*).

3.3.2.2. Non-anthocyanin phenolic compounds. Results for non-anthocyanin phenolic compounds over the storage time are shown in Fig. 2. Likewise, total anthocyanins and phenolic compounds undergo acute reduction on day 45 ($p<0.05$, Fig. 2**b**) regardless of the storage temperature. No difference in the degradation rate was observed when the beverages were kept at 4°C or 25°C, incorporated or not with TW ($p>0.05$) (Fig. 2**b**).

Individual phenolic compounds had a different progress, as shown in Figs. 2**e-i**. Generally, they remained stable in all formulations throughout the studied period ($p>0.05$) in both temperatures and formulations. In a study conducted by Gironés-Vilaplana, Huertas, Moreno, Periago, and García-Viguera (2016), the developed ID with maqui, lemon, açaí, and blackthorn showed a comparable behavior for caffeoylquinic and *p*-coumaroyl quinic acids derivatives, remaining stable over 70 days stored at 18-22°C (Fig. 2).

3.3.4. Antioxidant activity

According to the results (Fig. 2), the antioxidant activity showed a trend of reduction during the storage time, but it was not significant ($p>0.05$), except for F2 at 25°C ($p<0.05$). This is somewhat unexpected, as anthocyanins sharply reduced after 45 days. The fact that the major non-anthocyanin phenolic compounds did not suffer a significant decrease (Fig. 2) could have contributed to maintaining the ORAC values stable, as they have been positively correlated in previous studies (Fanali et al., 2018). For example, (Wang et al., 2019) reported a high positive correlation (0.926 and 0.970) between ORAC, total individual phenolic compounds, and chlorogenic acid (the primary non-anthocyanin compound here), but a lower correlation between this assay and flavonoids (0.764).

Recent research has found increased generation of reactive oxygen species, free radicals, and reactive nitrogen species during acute physical exercise that can lead to oxidative stress and reduce physical performance. For this reason, antioxidant supplementation and the consumption of sports food rich in antioxidants have been widely spread

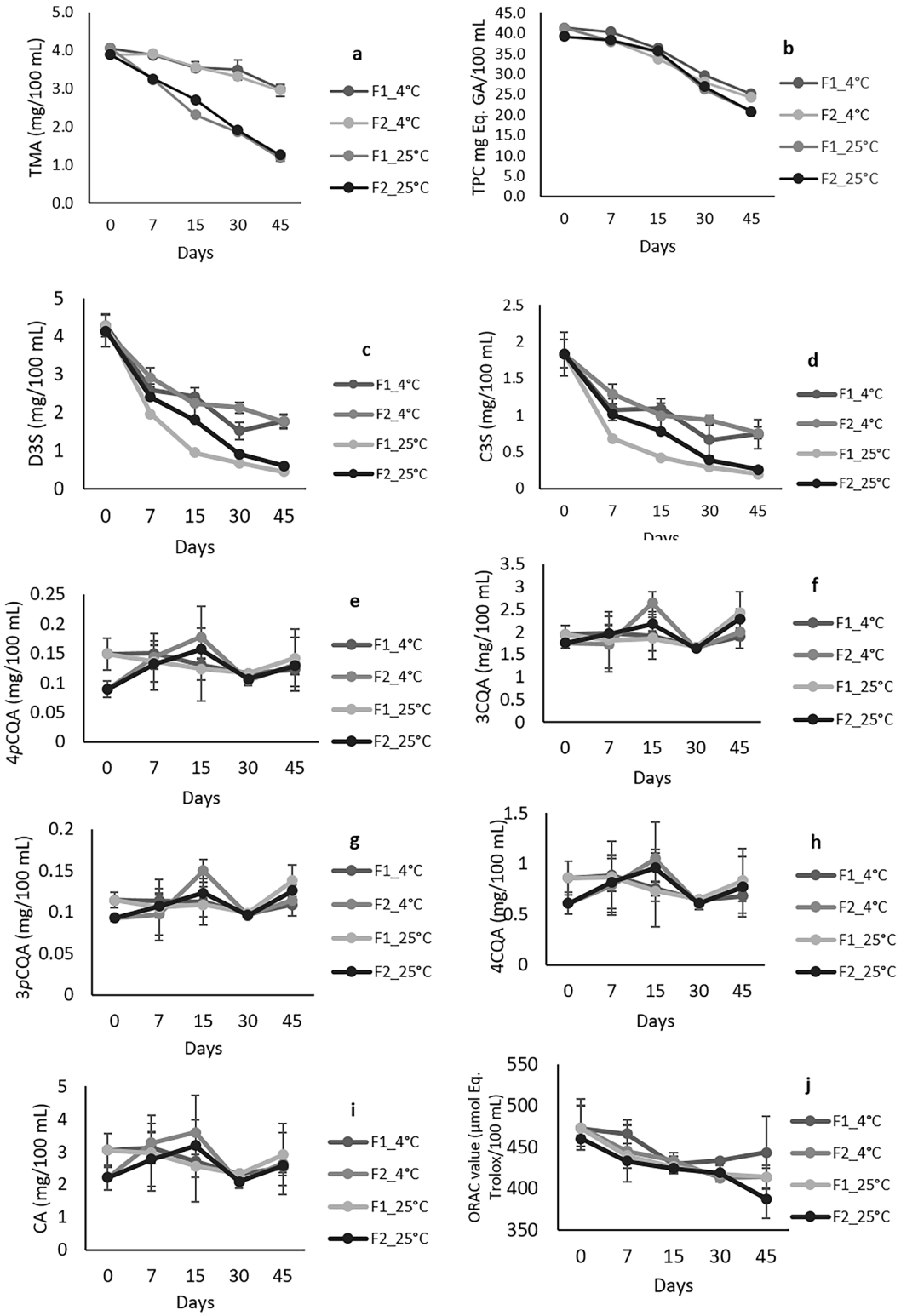

Fig. 2. Phenolic compounds and antioxidant activity of the isotonic drinks over 45 days of storage at 4°C and 25°C. TMA: Total monomeric anthocyanins (a); TPC: total phenolic compounds (b); D3S: delphinidin-3-O-sambubioside (c); C3S: cyanidin-3-O-sambubioside (d); 4pCQA: 4-p-coumaroylquinic acid (e); 3CQA: 3-caffeoylquinic acid (f); 3pCQA: 3-p-coumaroylquinic acid (g); 4CQA: 4-caffeoylquinic acid (h); CA: chlorogenic acid (i); antioxidant activity measured through the ORAC assay.

among sports people (Cui et al., 2022; Malaguti et al., 2013). This raises the necessity of ongoing innovation in this sector, with the development of novel formulations that meet the current demands of sports people. In this context, the present ID shows promising results, but importantly, ORAC values provide only preliminary results of the antioxidant potential of a food or beverage sample. While the cell-based assay tested (CAA) resulted in no activity, further studies on the developed ID's antioxidant activity are paramount to validating the observed potential antioxidant activity.

3.3.5. Microbiology

Table 4 shows microbiological results for F1 and F2 stored at 4°C and 25°C. The developed beverage showed no microbial growth during the studied period under both storage temperatures. This could be the result of a successful pasteurization process (85°C/30 sec) and due to the absence of native microflora adapted to the characteristics and conditions of the product (the acidic pH), as reported by Gironés-Vilaplana et al. (2016) for isotonic beverages with pH 2.8 and pasteurized at 80-85°C for 6 seconds. The pH values obtained for the beverages were lower than 4.0. These values ensure the safety of the beverage by making it inhospitable to the proliferation of most spoiling and pathogenic microorganisms since the minimum pH for the multiplication of strains varies between 4.6 and 4.8 (Gironés-Vilaplana et al., 2016). That made the product stable during storage, even under abusive temperature conditions (25 °C).

Table 4
Viable plate counts of the microorganisms' analysis (mesophilic microorganisms, yeasts, and molds) of the newly developed ID.

Temperature	Samples	Time (days)				
		0 (CFU/mL)	7 (CFU/mL)	15 (CFU/mL)	30 (CFU/mL)	45 (CFU/mL)
4°C	Total aerobic mesophiles					
	F1	<10	<10	<10	<10	<10
	F2	<10	<10	<10	<10	<10
	Yeast & molds					
	F1	<10	<10	<10	<10	<10
	F2	<10	<10	<10	<10	<10
25°C	**Mesophilic microorganisms**					
	F1	<10	<10	<10	<10	<10
	F2	<10	<10	<10	<10	<10
	Yeast & moulds					
	F1	<10	<10	<10	<10	<10
	F2	<10	<10	<10	<10	<10

4. Conclusions

A unique bio-based ID was created using TW, hibiscus extract, and apple juice. Quality parameters, chemical and physicochemical and microbiological stability were examined for 45 days at either 4°C or 25°C. The results demonstrated that the formulation met the essential criteria of providing electrolytes and simple sugars, revealing the feasibility of TW being used as a novel element in sports beverage preparation. The hibiscus extract supplied antioxidant compounds and added an appealing color to the product due to its anthocyanin content. Notably, the drink was microbiologically and physicochemically stable throughout 45 days under both storage temperatures studied. The best storage condition for color and anthocyanins preservation was at 4°C. Non-anthocyanin phenolic compounds and the antioxidant activity of the ID also remained stable over the studied period. In conclusion, making an ID with TW is possible without adding artificial colorants, flavorings, and sweeteners. Additionally, the results show that TW is a promising novel ingredient for the sports beverage industry.

As a final remark, several limitations of this study should be noted, which will guide future research. These include: i) conducting comprehensive sensory analyses, ii) assessing the feasibility of large-scale production, and iii) investigating bioaccessibility, bioavailability, and *in vivo* effects to determine whether the antioxidants provided by the beverage can exert a beneficial impact during intense physical exercise.

CRediT authorship contribution statement

Maroua Fatma Radhouane: Formal analysis, Data curation, Conceptualization. **Tayse F.F. da Silveira:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jessica Ribeiro:** Formal analysis. **Paula Rodrigues:** Writing – review & editing, Methodology, Data curation. **Rafaela Guimarães:** Writing – review & editing, Formal analysis. **Ricardo Calhelha:** Formal analysis, Data curation. **Filipa Mandim:** Writing – review & editing, Formal analysis, Data curation. **Ichrak Charfi:** Supervision. **Isabel C.F.R. Ferreira:** Writing – review & editing, Resources, Methodology, Funding acquisition. **Maria José Alves:** Resources, Project administration, Funding acquisition. **Lillian Barros:** Resources, Project administration, Methodology, Funding acquisition. **Sandrina A. Heleno:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors are grateful to the Fundação "La Caixa" and to FCT for the financial support through Project "Aquae Vitae - Água Termal como fonte de vida e Saúde", Promove Mobilizador "O futuro do Interior", call 2020. The authors are also grateful to the Foundation for Science and Technology (FCT, Portugal) for support through national funds FCT/MCTES (PIDDAC) to CIMO: UIDB/00690/2020 (DOI:10.54499/UIDB/00690/2020) and UIDP/00690/2020 (DOI: 10.54499/UIDP/00690/2020); and SusTEC (LA/P/0007/2020, DOI: 10.54499/LA/P/0007/2020). S. Heleno thanks FCT for her employment program–contract CEECIND/03040/2017, and L. Barros also thanks FCT through the institutional scientific employment program–contract for her contract. T.F.F. da Silveira, thanks to Fundação "La Caixa", within the project titled 'Aquae Vitae' for her research contract. R. Guimarães acknowledges national public funding through "Investimento RE-C05-i02 – Missão Interface N.° 01/C05-i02/2022", a project supported under the PRR (www.recuperarportugal.gov.pt), and financed by the European Union/Next Generation EU.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.focha.2024.100823.

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