



# **IsoDrink: Development of an isotonic drink based on thermal water and natural ingredients**

**Maroua Fatma RADHOUANE**

*Dissertation submitted to Escola Superior Agrária de Bragança to obtain the Degree of Master in Biotechnological Engineering, under the scope of the double diploma with l'Université Libre de Tunis*

Supervised by

**Dr. Sandrina Alves HELENO**

**Dr. Maria José ALVES**

**Dr. Ichrak CHARFI**

**Bragança  
2023**

This work was financed by FCT and BPI La Caixa Foundation, within the project ‘Aquaevitae - Água Termal Como Fonte de Vida e Saúde’ - “PROMOVE – O futuro do Interior” call 2020.



*“If you can dream it, you can do it.” — Walt Disney*

## **I dedicate this work:**

### **TO MY DEAR PARENTS: RIM SASSI AND ITEB RADHOUANE**

No dedication can express my respect, eternal love, consideration, and gratitude for the sacrifices you have made for my education and well-being. I would thank you for all the support and love you have given me since I was a child and I hope that your blessing always accompanies me. May this modest work fulfill your wishes, and the fruit of your countless sacrifices, although I can never repay you enough. May God, the Most Highest grant you health, happiness, and long life, and may I never disappoint you.

### **IN MEMORY OF MY GRANDMOTHER: OMI FATMA**

I would have liked so much if you were present. May God have your soul in his holy mercy.

### **TO MY GRANDFATHER AND GRANDMOTHER: SALWA AND HEDI SASSI**

Who accompanied me with their prayers, and their sweetness, may God give them a long life and much health and happiness in their lives.

### **TO MY DEAR AND ADORABLE: EMNA AND YASSINE**

My guardian angels and my faithful companions in the most delicate moments of this mysterious life.

As a token of my brotherly affection, deep tenderness, and gratitude, I wish you a life full of happiness and success, and may God, the Almighty, protect you.

### **TO THE ONE MY SOULMATE**

Who pushes me every day to be a more ambitious person, Thank you so much for always being there for me, and for your unwavering support and boundless love.

### **TO MY SISTERS: SAFA GUETAT AND SYRINE HASNAOUI**

At the friendship that unites us and the memories of all the times we've spent together, I would like to thank you for being in my life and for your support and endless love.

### **To all the members of my family, young and old, to my friends**

Please find the expression of my affection and all my love in this modest work.

## ACKNOWLEDGEMENTS

I would like to thank all the people who contributed to the success of my internship and who helped me during the writing of this report.

I want to thank, first of all, my directors of the training course **Dr. Sandrina Alves HELENO**, and **Dr. Maria José ALVES** doctors at the Polytechnic Institute of Bragança, who accepted me among their adorable team, for the help and advice concerning the missions mentioned in this report, which they brought to me during the different follow-ups. Also for allows me to learn and apprehend a field for which I nurture a certain passion. A vocês um muito obrigada.

I also address my thanks to my thesis director, **Dr. Ichrak CHARFI**, a doctor at l'université libre de Tunis for her patience, her availability, and especially her judicious councils, which contributed to feeding my reflection. Your guidance, expertise, and mentorship have played a pivotal role in shaping my research and helping me navigate the challenges of academic life.

I offer sincere and warm thanks to my training supervisor, **Dr. Tayse Ferreira Ferreira DA SILVEIRA**, the merit of a dissertation belongs certainly to the author, but also to the director who supervises it. In my case, my supervisor was exceptionally supportive and attentive. The trust she placed in me and the moral support she gave me has allowed me to accumulate significant professional and personal experiences that have made me a mature person.

I would also like to thank all the professionals and staff working in the CIMO interviewed for this study, who kindly answered my various questions on the field to facilitate the elaboration of this work.

I address my sincere thanks to all the members of the jury for the honor that they have by accepting to judge this modest work and to share their light.

Finally, I would like to thank all those who have helped me in any way in the elaboration of this work.

## TABLE OF CONTENT

<b>ACKNOWLEDGEMENTS</b> .....	V
<b>LIST OF TABLES</b> .....	VIII
<b>LIST OF FIGURES</b> .....	IX
<b>LIST OF ANNEXES</b> .....	X
<b>LIST OF ABBREVIATIONS</b> .....	XI
<b>ABSTRACT</b> .....	XIII
<b>RESUMO</b> .....	XV
<b>1. Introduction</b> .....	1
<b>2. Thermal water</b> .....	2
<b>2.1 Classifications of thermal water</b> .....	3
<b>2.2 The Benefits and applications of thermal water</b> .....	6
<b>2.3 Chaves thermal water</b> .....	7
<b>3. Isotonic drinks</b> .....	10
<b>3.1 General characteristics</b> .....	10
<b>3.2 The importance of isotonic drinks</b> .....	12
<b>4. Apple juice (<i>Malus domestica</i> borkh)</b> .....	14
<b>5. Hibiscus (<i>Hibiscus sabdariffa</i> L.)</b> .....	15
<b>6 .Objectives</b> .....	17
<b>6.1 General objective</b> .....	17
<b>6.2 Specific objectives</b> .....	17
<b>7. Material and methods</b> .....	18
<b>7.1 Raw Materials</b> .....	18
<b>7.2 Production of hibiscus aqueous extracts</b> .....	19
<b>7.3 Formulation of the isotonic drink</b> .....	20
<b>7.4 Thermal treatment</b> .....	21
<b>7.5 Characterization of the isotonic drinks and stability studies</b> .....	22
<b>7.6 Methods</b> .....	22
<b>7.6.1 pH and total soluble solids</b> .....	22
<b>7.6.2 Color measurement</b> .....	23
<b>7.6.3 Total phenolic compounds</b> .....	23
<b>7.6.4 Total monomeric anthocyanin compounds</b> .....	23
<b>7.6.5 Phenolic compounds by LC-DAD-ESI-MS<sup>n</sup></b> .....	24

7.6.6 Anthocyanin compounds by LC-DAD-ESI-MS <sup>n</sup> .....	24
7.6.7 Mineral composition .....	25
7.6.8 Analysis of individual sugars .....	25
7.6.9 Antioxidant activity .....	26
7.6.10 Anti-inflammatory activity .....	27
7.6.11 Microbiological analysis .....	27
7.7 Statistical analysis .....	28
<b>8. Results and discussion .....</b>	<b>28</b>
8.1 Development of the isotonic drink formulations .....	28
8.2 Characterization of the formulated isotonic drinks .....	30
8.2.1 pH and TSS .....	31
8.2.2 Color measurement .....	32
8.2.3 Non-anthocyanin phenolic compounds .....	33
8.2.4 Anthocyanin compounds .....	34
8.2.5 Antioxidant activity .....	35
8.2.6 Anti-inflammatory activity .....	35
8.2.7 Mineral composition .....	35
8.2.8 Analysis of individual sugars .....	36
8.3 Stability studies at different storage temperatures .....	37
8.3.1 pH and TSS .....	37
8.3.2 Color measurements .....	39
8.3.3 Phenolic compounds .....	40
8.3.4 Anthocyanin compounds .....	44
8.3.5 Antioxidant activity .....	46
8.3.6 Microbiology analysis .....	47
<b>9. Conclusions .....</b>	<b>48</b>
<b>10. Bibliography .....</b>	<b>50</b>
<b>Annexes .....</b>	<b>62</b>

## LIST OF TABLES

<b>Table 1.</b> Classification of thermal natural mineral waters based on fixed residue at 180°C (Nguyen Ba, 2020). .....	3
<b>Table 2.</b> Classification of natural mineral waters based on thermal degrees (Mekebret, 2017). .....	4
<b>Table 3.</b> Classification of natural mineral water based on fixed chemical compounds (Nguyen Ba, 2020; Rémi Parpette, 2021). .....	5
<b>Table 4.</b> Characteristics of the water from the Chaves thermal (Ferreira, 2023; Aires-Barros et al., 1998). .....	10
<b>Table 5.</b> The optimal composition of an isotonic drink (Ruiz and García, 2022). .....	12
<b>Table 6.</b> Description and codes of the studied isotonic drink formulations. ....	21
<b>Table 7.</b> Detailed description of the isotonic drink formulations. ....	29
<b>Table 8.</b> Chemical and physicochemical composition of formulations F1, F2, and F3. 30	
<b>Table 9.</b> Color parameters variation for F1 and F3. ....	39

## LIST OF FIGURES

<b>Figure 1.</b> Termas de Chaves (Chaves, 2021).....	8
<b>Figure 2.</b> The geography emplacement of Chaves in Portugal (Avesso, 2020). ....	9
<b>Figure 3.</b> The importance of an isotonic drink for the human body (Bendaali et al., 2022).....	14
<b>Figure 4.</b> Dried hibiscus flower. ....	18
<b>Figure 5.</b> Thermal water received from Termas de Chaves.....	19
<b>Figure 6.</b> The screw-capped aluminum pouches used to store the isotonic drinks.....	22
<b>Figure 7.</b> The standard isotonic drink F1.....	29
<b>Figure 8.</b> The color of the isotonic drink in F1.....	32
<b>Figure 9.</b> The pH evolution of isotonic drinks at (a) 4 °C and (b) 25 °C. ....	37
<b>Figure 10.</b> Total soluble solids (°Brix) for (a) 4 °C and (b) 25 °C. ....	38
<b>Figure 11.</b> Difference of the color between the beginning and the end of the storage period at 4 °C (a) T=0 (b) T=45. ....	40
<b>Figure 12.</b> Total phenolic compounds in formulations stored at 4 °C and 25 °C during 45 days.....	41
<b>Figure 13.</b> Individual phenolics compounds (a) 4-Caffeoylquinic acid (b) 3-caffeoylquinic acid (c) 3-p-coumaroylquinic acid (d) 4-p-coumaroylquinic acid. ....	43
<b>Figure 14.</b> Difference of total Anthocyanin compounds between 4°C and 25°C.....	44
<b>Figure 15.</b> Individual anthocyanin compounds (a) Delphinidin 3 O-sambubioside (b) Cyanidin 3-O-sambubioside.....	45
<b>Figure 16.</b> Antioxidant activity (ORAC) of formulations at 4°C and 25°C during 45 days. ....	46
<b>Figure 17.</b> The culture medium used (a) DRBC, (b) PCA. ....	48

## LIST OF ANNEXES

<b>Annex 1.</b> Chromatographic and spectra data for non-anthocyanin compounds in the isotonic drink (Formulation F1 and F3). .....	62
<b>Annex 2.</b> Chromatographic and spectra data for anthocyanins in the isotonic drink (Formulations F1 and F3). .....	62

## LIST OF ABBREVIATIONS

3CQA: 3-caffeoylquinic acid

3pCQA: 3-p-coumaroyl quinic acid

4CQA: 4-caffeoylquinic acid

4pCQA: 4-p-coumaroyl quinic acid

5CQA: 5-caffeoylquinic acid

a\*: Red/green coordinate

AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride

b\*: Blue/yellow coordinate

C3S: Cyanidin-3-*O*-Sambubioside

CAA: Cellular Antioxidant Activity

CIMO: Centro de Investigação de Montanha

D3C: Delphinidin-3-*O*-Sambubioside

DRBC: Dichloran Rose Bengal Chloramphenicol

EFSA: European Food Safety Authority

eq: equivalent

F1: Formulation 1 of the isotonic drink

F2: Formulation 2 of the isotonic drink

F3: Formulation 3 of the isotonic drink

g: gram

GAE: Gallic Acid Equivalents

HPLC-RI: High-Performance Liquid Chromatography by Refractive Index

HS: *Hibiscus sabdariffa*

ID: Isotonic Drink

IPB: Instituto Politécnico de Bragança

L\*: Lightness Level

LC-DAD-ESI-MS: Liquid Chromatography coupled to Diode Array Detection and ElectroSpray Ionization tandem Mass Spectrometry

LC-MS: Liquid Chromatography-Mass Spectrometry

LDL: Low-Density Lipoprotein

LOQ: Limit of Quantification

mg: milligram

mL: milliliter  
mOsm: milliosmole  
MS: Mass Spectrophotometry  
MW: Molecular Weight  
Na: No Activity  
NaCl: Sodium Chloride  
ND: No Detected  
°C: Degrees Celsius  
ORAC: Oxygen Radical Absorbance Capacity  
PCA: Plate Count Agar  
pH: Potential of Hydrogen  
ROS: Reactive Oxygen Species  
SFB: Fetal Bovine Serum  
T0: 0 days  
T15: 15 days  
T30: 30 days  
T45: 45 days  
T7: 7 days  
TFA: TriFluoroacetic Acid  
TMA: Total Monomeric Anthocyanins  
TPC: Total Phenolic Compounds  
Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid  
TSS: Total Soluble Solids  
TW: Thermal Water  
µmol: micromole

## ABSTRACT

Isotonic drinks are sport beverages containing mineral salts and carbohydrates of quick absorption, which goal is to replenish electrolytes and energy lost during physical activity. The segment of sport beverages has expanded its market and novel ingredients have been introduced to meet the demand for “*clean-label*” and natural-based products. Thermal water (TW) are natural mineral waters naturally rich in minerals and salts. Particularly, TW from Chaves (Portugal) is rich in sodium, which is the most important mineral in isotonic drinks. Nevertheless, its potential in beverage sectors has been never evaluated. Thus, the aim of this study was to develop an innovative isotonic drink formulation benefiting of TW as source of minerals, apple juice for sugars and antioxidant supply, and an aqueous extract of hibiscus flowers as colorant and source of antioxidants (standard formulation). Two control formulations were also prepared: one with TW but no apple juice, no hibiscus extract (unflavored); and other with no TW, but distilled water instead. The isotonic formulations were developed considering chemical, physico-chemical and sensory parameters (taste, color, sodium content, pH, soluble solids, and sugar content). The final beverages were pasteurized (85 °C/30 seconds) and studied for pH, color, total soluble solids (TSS), minerals, sugars, anthocyanins, phenolic compounds, antioxidant (Oxygen Radical Absorbance Capacity – ORAC) and anti-inflammatory activities using *in vitro* cell-based assays. Moreover, the behavior of these parameters was monitored over 45 days, under 2 storage temperatures 4 °C and 25 °C. The microbial stability of the formulations was also evaluated. The analyses showed that all aspects of the novel isotonic drink align with established parameters for this beverage category. In general, formulations with and without thermal water showed similar chemical, physicochemical and microbiological behavior, suggesting that the addition of this natural resource did not affect the quality characteristics of the beverage. The standard formulation exhibited a pH of  $3.72\pm 0.003$ , a TSS content of  $5.70\pm 0.05$ , and a total sugar content of  $7.93\pm 0.18$  mg/100mL, comprising fructose, glucose and sucrose from apple juice. Furthermore, the beverage was enriched with essential minerals  $87.14\pm 0.89$  mg/100mL, from thermal water. The beverage boasts an attractive red color, attributed to the presence of anthocyanin compounds from the hibiscus extract (majorly delphinidin-3-*O*-sambubioside and Cyanidin-3-*O*-sambubioside) at a total concentration

of  $4.06 \pm 0.03$  mg/100mL. Moreover, the isotonic drink showed antioxidant activity of  $472.72 \pm 3.29$   $\mu$ mol eq. Trolox/100mL (ORAC assay), likely due to phenolic compounds from hibiscus and apple juice. It showed a total phenolic compound content of  $41.36 \pm 1.34$  mg/100mL, predominating chlorogenic acid ( $2.75 \pm 0.10$  mg/100mL), 3-caffeoylquinic acid ( $1.75 \pm 0.20$  mg/100mL), 4-caffeoylquinic acid ( $0.86 \pm 0.17$  mg/100mL), 3- and 4- *p*-coumaroylquinic acids ( $0.11 \pm 0.01$  mg/100mL and  $0.15 \pm 0.03$  mg/100mL). However, none of the formulations showed anti-inflammatory and antioxidant activity in cell-based assays. All the formulations maintained their microbial stability when stored at both 4 °C and 25 °C for a remarkable 45 days. On the other hand, chemical markers, such as color and anthocyanin content, showed a higher reduction in formulations stored at 25 °C compared to those kept at 4 °C. These results indicate that for better sensory outputs, it is recommended to store formulations containing hibiscus extract under refrigeration (with or without TW). Contrarily, unflavored isotonic drinks with TW could be marketed at room temperature. Therefore, this work unveils a novel applicability of TW, and the developed product emerges as an alternative in the sport market, distinguished by the use of natural ingredients.

**Keywords:** sport drink; sport food; minerals; apple juice; hibiscus; natural ingredients; antioxidant; anthocyanin; shelf life; stability.

## RESUMO

As bebidas isotónicas são bebidas desportivas que contêm sais minerais e hidratos de carbono de rápida absorção, cujo objetivo é repor os electrólitos e a energia perdidos durante a atividade física. O setor das bebidas desportivas expandiu o seu mercado e foram introduzidos novos ingredientes para satisfazer a procura de produtos com “*clean label*” e de base natural. As águas termais (TW) são águas minerais naturais ricas em minerais e sais. Em particular, a TW de Chaves (Portugal) é rica em sódio, que é o mineral mais importante nas bebidas isotónicas. No entanto, o seu potencial no sector das bebidas nunca foi avaliado. Assim, o objetivo deste estudo foi desenvolver uma formulação inovadora de bebida isotónica beneficiando da TW como fonte de minerais, sumo de maçã para fornecimento de açúcares e antioxidantes, e um extrato aquoso de flores de hibisco como corante e fonte de antioxidantes (formulação padrão). Foram também preparadas duas formulações de controlo: uma com TW mas sem sumo de maçã, sem extrato de hibisco (nomeadamente sem sabor); e outra sem TW, mas com água destilada. As formulações isotónicas foram desenvolvidas tendo em conta parâmetros químicos, físico-químicos e sensoriais (sabor, cor, teor de sódio, pH, sólidos solúveis e teor de açúcar). As bebidas finais foram pasteurizadas (85 °C/30 segundos) e estudadas quanto ao pH, cor, sólidos solúveis totais (SST), minerais, açúcares, antocianinas, compostos fenólicos, atividade antioxidante (ORAC - Oxygen Radical Absorbance Capacity) e anti-inflamatória, através de ensaios celulares *in vitro*. Além disso, o comportamento destes parâmetros foi monitorizado durante 45 dias, sob 2 temperaturas de armazenamento (4 °C e 25 °C). A estabilidade microbiana das formulações também foi avaliada seguindo normas ISO. As análises confirmaram que todos os aspetos da nova bebida isotónica estão alinhados com os parâmetros estabelecidos para esta categoria de bebidas. No geral, observou-se que todas as formulações (com e sem água termal), apresentam comportamento químico, físico-químico e microbiológico similares, o que indica que a adição de água termal na produção do isotónico não afetou a sua qualidade. A formulação padrão apresentava um pH de  $3,72 \pm 0,003$ , um teor de SST de  $5,70 \pm 0,05$  e um teor de açúcares totais de  $7,93 \pm 0,18$  mg/100mL, composto por frutose, glucose e sacarose do sumo de maçã. Além disso, a bebida foi enriquecida com minerais essenciais ( $87.14 \pm 0.89$  mg/100mL), provenientes da água termal. A bebida apresenta uma cor vermelha atraente,

atribuída à presença de compostos antociânicos do extrato de hibisco (principalmente delphinidina-3-*O*-sambubiosídeo e cianidina-3-*O*-sambubiosídeo) numa concentração total de  $4,06 \pm 0,03$  mg/100mL. Além disso, a bebida isotónica mostrou uma atividade antioxidante de  $472,72 \pm 3,29$   $\mu$ mol eq. Trolox/100mL (ensaio ORAC), provavelmente devido aos compostos fenólicos do hibisco e do sumo de maçã. Apresentou um teor de compostos fenólicos totais de  $41,36 \pm 1,34$  mg/100mL, predominando o ácido clorogénico ( $2,75 \pm 0,10$  mg/100mL), o ácido 3-cafeoilquínico ( $1,75 \pm 0,20$  mg/100mL), o ácido 4-cafeoilquínico ( $0,86 \pm 0,17$  mg/100mL), os ácidos 3- e 4- p-cumaroilquínico ( $0,11 \pm 0,01$  mg/100mL e  $0,15 \pm 0,03$  mg/100mL). No entanto, nenhuma das formulações mostrou atividade anti-inflamatória e antioxidante em ensaios baseados em células. Todas as formulações mantiveram a sua estabilidade microbiana quando armazenadas a 4 °C e 25 °C durante uns notáveis 45 dias. Por outro lado, os marcadores químicos, como a cor e o teor de antocianina, apresentaram uma maior redução nas formulações armazenadas a 25 °C em comparação com as mantidas a 4 °C. Estes resultados indicam que, para obter melhores resultados sensoriais, é recomendável armazenar as formulações que contêm extrato de hibisco sob refrigeração (com ou sem TW). Pelo contrário, as bebidas isotónicas sem sabor com TW podem ser comercializadas à temperatura ambiente. Assim, este trabalho revela uma nova aplicabilidade da TW, e o produto desenvolvido surge como uma alternativa no mercado desportivo, distinguindo-se pela utilização de ingredientes naturais.

**Palavras-chave:** bebida desportiva; alimento desportivo; minerais; sumo de maçã; hibisco; ingredientes naturais; antioxidante; antociânicos; tempo de vida; estabilidade.

## 1. Introduction

Nowadays, it is well recognized that lifestyle, including diet and physical activity, are key factors in health maintenance and prevention of many diseases, such as cancer and cardiovascular pathologies (Sharifi-Rad et al., 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 and Dolle, 2016). ID are sport drinks whose main role is rehydration and replacement of minerals, such as sodium and potassium, and carbohydrates during physical exercise, being essential to maintain water and electrolytes for body homeostasis (Ruiz and García, 2022).

Thermal waters (TW) are all-natural mineral waters with therapeutic applications and health-beneficial effects utilized within a thermal site (Nguyen Ba, 2020). Their diverse chemical composition and mineralization have been associated with various beneficial effects on health, such as the alleviation of symptoms of inflammation-related diseases (Lakhdari and Bouaicha, 2016; Araujo et al., 2017; Silva et al., 2020). Portugal is one of the richest European countries in TW sources with a diversity of properties, and most of them are located in the Northern and central regions (Viegas et al., 2019; Araujo et al., 2017). Among Portugal's thermal waters that are described as mainly rich in trace elements is Chaves water (Pereira et al., 2021).

Although TW has been widely used in pharmaceutical and cosmetic applications, surprisingly its potential in food and beverage formulations is still poorly investigated, despite its natural abundance. Due to the interesting chemical composition of Chaves TW, which is notably rich in sodium that plays a major role in fluid homeostasis (Sadowska, Świdorski, and Laskowski, 2020; Marques and Carreira, 2017; Pereira et al., 2021), we are currently investigating its potential incorporation into isotonic beverages, given the critical role of sodium in such formulations.

Chaves water is considered potable, meaning it is safe for drinking. Locals and visitors often enjoy it as a refreshing and natural beverage, especially due to its unique mineral taste. This exploration aligns with sustainability principles and presents

the opportunity to introduce a unique, locally sourced natural element into isotonic drink production.

Intense physical activity favors the production of reactive oxygen species (ROS), muscle microtrauma, and inflammation (Simioni et al., 2018; Bendaali et al., 2022). Although a certain level of ROS may be beneficial for organism adaptation, highly intense physical activities may evoke an oxidative stress state, which may damage performance (Simioni et al., 2018). Thus, in addition to their basic function, isotonic drinks based on fruit juices and plant extracts have also been increasingly investigated. These products are meant to beyond electrolytes losses during exercises, and provide nutrients and antioxidants, which may contribute to preventing or decreasing adverse effects of ROS due to intense physical activity (Gironés-Vilaplana et al., 2013; Simioni et al., 2018).

Recent studies have consistently demonstrated that consumers are interested in new flavors of isotonic drinks and formulations based on natural ingredients (Świtalski and Rybowska, 2021). In this context, the food and beverage industries are under increasing pressure to provide new, healthy clean-label foods and ingredients that can meet consumers' daily nutritional, functional, and organoleptic requirements. Therefore, this study aimed to design an innovative isotonic drink based on TW, apple juice, and hibiscus tea, which could not only provide electrolytes and rehydration but also afford nutrients and antioxidants to people practicing sports.

## **2. Thermal water**

Thermal waters, also known as hot spring water, are underground natural mineral waters that are distinguished from other waters by their purity, and stable physical and chemical composition and are associated with specific geothermal regions (Lakhdari and Bouaicha, 2016). While mineral water is primarily drinking water, thermal water serves a broader range of applications, particularly in settings for health and wellness treatments (Porowski, 2019). TW is naturally enriched in minerals and trace elements by the Earth's geothermal activity that emerges from the ground at a high temperature typically above 20 degrees Celsius (°C), it's important to note that there are also thermal waters with temperatures below 20°C and still called thermal water, in

contact with the rocks as it crosses before gushing to the surface of the Earth (Porowski, 2019; Nguyen Ba, 2020).

## 2.1 Classifications of thermal water

One key difference between surface water, the water we usually drink, and thermal water, is the presence of minerals. As the water passes through rocks and soil rising from the deep underground springs where it is heated it collects minerals. These minerals dissolve into the water and are present when the water arrives at the hot springs on the surface (Jawadi et al., 2021).

The mineralization of water is characterized by the residue or dry extract in milligrams per liter (mg/L) at 180°C (**Table 1**). The mineral content can range from a few milligrams to a few grams per liter (Nguyen Ba, 2020).

**Table 1.** Classification of thermal natural mineral waters based on fixed residue at 180°C (Nguyen Ba, 2020).

<b>Fixed residue at 180°C</b>	<b>Definition</b>
<b>&lt; to 50 mg/L</b>	very weakly mineralized
<b>between 50 and 500 mg/L</b>	weakly mineralized or oligo-mineral
<b>500 and 1.000 mg/L</b>	moderately mineralized between
<b>1.000 and 1.500 mg/L</b>	mineralized between
<b>&gt; 1.500 mg/L</b>	highly mineralized or rich in mineral salts

The temperature of thermal water is a function of the depth of the original reservoir, the length of the journey, and the speed of the ascent. It is influenced by the mixtures with the surface waters and can thus be variable from one source to another (**Table 2**).

**Table 2.** Classification of natural mineral waters based on thermal degrees (Mekebret, 2017).

<b>Thermal degrees</b>	<b>Definition</b>
<b>Hypothermal waters</b>	They are waters whose temperature at emergence is lower than that of the upper part of the homothermal zone, or the mean interannual temperature of the place of emergence plus 4°C.
<b>Metriothermal waters</b>	They are waters whose temperature at the emergence is lower than 30°C but higher than hypothermal waters.
<b>Mesothermal waters</b>	These are waters whose temperature at emergence is between 30°C and 50°C.
<b>Hyperthermalwaters</b>	these are waters whose temperature at emergence is higher than 50°C and lower than 100°C

The regulatory classification is characterized by the dominant anion at a higher concentration expressed in milligrams/liter (Silva et al., 2020) (**Table 3**):

**Table 3.** Classification of natural mineral water based on fixed chemical compounds (Nguyen Ba, 2020; Rémi Parpette, 2021).

<b>Chemical anion</b>	<b>Definition</b>
<b>Bicarbonate</b>	The bicarbonate content is greater than 600 mg/L.
<b>Sulphate</b>	The sulfate content is higher than 200 mg/L, where the predominant anion is sulfur in the form of sulfate
<b>Sulfurous</b>	Where the sulfur is present in the form of hydrogen sulfide (H <sub>2</sub> S). This component gives these waters their characteristic "rotten egg" smell. They are often hyperthermal and radioactive.
<b>Chloride</b>	The chloride content is greater than 200 mg/L.
<b>Calcium</b>	The calcium content is greater than 150 mg/L.
<b>Magnesian</b>	The magnesium content is greater than 50 mg/L.
<b>Fluorine</b>	The fluorine content is greater than 1 mg/L
<b>Iron</b>	The content of bivalent iron is greater than 1 mg/L
<b>Acidic</b>	The free carbon dioxide content is greater than 250 mg/L
<b>Sodium</b>	The sodium content is greater than 200 mg/L.
<b>Oligo-metallic</b>	Waters with very low mineralization, without predominant minerals.
<b>Particular mineralization</b>	Which contain a rare element with particular properties (arsenic, selenium, copper, etc.).

TW is also classified according to other parameters such as osmotic pressure, pH, and radioactivity (Nguyen Ba, 2020). Thermal water is a highly beneficial natural resource that is worth exploring. It is a versatile and powerful natural remedy with numerous benefits for the skin and overall health. Its unique composition of minerals and trace elements makes it a valuable ingredient in skin care products and a popular treatment for various diseases (Silva et al., 2020; Cacciapuoti et al., 2020). The various therapeutic effects described with thermal therapy have been attributed to its physicochemical

composition, the reason why they are classified as bicarbonate, sodium, chloride, sulfurous, hyposaline, and gasocarbonic waters (Araujo et al., 2017). TW is generally free of contaminants such as nitrates or xenobiotics (Lakhdari and Bouaicha, 2016). They do not undergo any treatment and are used on the thermal site with a constant temperature, mineralization, and flow (Akhtar et al., 2021; Nguyen Ba, 2020).

## **2.2 The Benefits and applications of thermal water**

The uniqueness of the use of TW from the beginning to these days is characterized by a strong established belief in their curative powers in the countries in which they occur. Nowadays this is corroborated by a growing number of balneological healing centers, health resorts, and spas, which are well established in the public health care system in many countries of the world (Porowski, 2019).

Sanitizing, hydrating, rebalancing, and appetite suppressant, TW acts from the inside, and the body benefits directly from its different properties. It can be prescribed as a cure for various pathologies, especially chronic ones, as a complement to medical treatments (Cacciapuoti et al., 2020; Pellegrini et al., 2005). It is beneficial as a treatment for heavy legs, for certain severe dermatological problems (for example eczema and acne), in case of respiratory tract problems, repeated urinary tract infections, digestive problems, rheumatism, depressive and anxiety disorders, and many others (Cacciapuoti et al., 2020; Viegas et al. 2019).

TW is rich in minerals and trace elements with various dermatological indications demonstrated for the treatment of various pathologies, being this way used by the industry for the development of hydrogels, personal care products, incorporation of the production of nanoparticles that allow the controlled release of the drug, sun protection creams (Almeida et al., 2019). They can also be incorporated into some topical formulations prepared with bio-based hydrogel, adding value to the final product and their incorporation in the production of sunscreen creams with antioxidant extracts of flowers (Torres et al., 2021).

Certain thermal waters, also known as hot springs, especially those found in Europe, are not only used for therapeutic swims but also a water cure therapy, under the term "drink cure", sometimes also called "spring treatment", all treatments are collected where thermal water from a mineral spring is drunk for 1 to 6 weeks. The

drinking cure is a method of hydrotherapy, which can be part of the treatment process practiced in a spa (Jungbluth, 2021; Vary, 2015). The special feature of this healthy water is the content of many health-promoting minerals, such as sodium, calcium, or magnesium. Because these ingredients dissolve in the healing water, the body can absorb and use them effectively. Depending on the composition, mineral spring water can help solve a variety of health problems, almost all of them. The thermal drink cure is ideal to rehydrate and remineralize the body in depth. It is used for its diuretic, laxative, and detoxifying action. Hepatic and renal drainage (Jungbluth, 2021; Vary, 2015).

The various therapeutic effects described with thermal therapy have been attributed to its physicochemical composition, and this correlation has been the basis for the indication of the different thermal resorts for different disorders of several vital systems (Araujo et al., 2017).

### **2.3 Chaves thermal water**

The richness of Portugal in TW has been known for a long time and the effectiveness of these waters is well recognized (Rebelo, da Silva, and Rocha, 2015). Portugal has approximately 50 thermal resorts, which display different properties mostly due to their geological variability (Silva et al., 2020). Most Portuguese TW is described as weakly mineralized, sulfurous, and bicarbonated or chlorinated, and sodium-type waters (Araujo et al., 2017).

Many Portuguese springs have been used for medicinal purposes since the earliest times, among them, Chaves thermal springs (**Figure 1**) have a millenarian tradition, and their foundation goes back to the Roman Age, called *Aquae Flaviae* in Roman times.



**Figure 1.** Termas de Chaves (Chaves, 2021).

They are situated in the Northern part of the country and lie in the heart of Trás-os-Montes (**Figure 2**), they are one of Portugal's most important thermal spas, with the hottest waters in Europe (76°C). The exceptional quality of its springs, which form part of the Alto Tâmega thermal axis, explains why *Aquae Flaviae* was one of the most prosperous towns during the Roman period (Smith, 2019; Marques and Carreira, 2017).



**Table 4.** Characteristics of the water from the Chaves thermal (Ferreira, 2023; Aires-Barros et al., 1998).

<b>Parameter</b>	<b>Characteristics of Thermas de Chaves</b>
<b>Temperature</b>	76°C
<b>pH (at 20°C)</b>	6.80
<b>Electrical Conductivity (at 20°C)</b>	2320 $\mu\text{S}/\text{cm}$
<b>Alkalinity (in mg/L CaCO<sub>3</sub>)</b>	1161.0
<b>Total Mineralization</b>	2174 mg/L
<b>Dry Residue (at 180°C)</b>	1744 mg/L
<b>Bicarbonate (HCO<sub>3</sub><sup>-</sup>)</b>	1415 mg/L
<b>Chloride (Cl<sup>-</sup>)</b>	35 mg/L
<b>Sodium (Na<sup>+</sup>)</b>	623 mg/L

Chaves is a notable and unique resource with historical, cultural, and potential industrial significance. Its high mineral content, particularly its sodium richness, its potable quality, and its natural sourcing make it a compelling choice for use in the food and beverage sector, particularly in products like isotonic drinks.

### **3. Isotonic drinks**

#### **3.1 General characteristics**

In recent years, there has been a sharp increase in the trend toward healthy eating and body care, where consuming whole foods and engaging in physical activity are seen as the two main components of a healthy lifestyle. To compensate for the losses of water and electrolytes when we do physical exercise, sports drinks quickly and effectively restore the water and electrolyte balance to a state of equilibrium supporting athletes and active individuals in maintaining optimal performance and well-being (Sadowska, Świdorski, and Laskowski, 2020; Orrù et al., 2018).

Sports drinks are beverages used before, during, and after physical exercises to replace water, electrolytes, and carbohydrates lost during exercise to prevent dehydration and sustain endurance (Sadowska, Świdorski, and Laskowski, 2020; Bendaali et al., 2022). They can be hypertonic if they contain a higher concentration of

sugar and salt than those which are found in the human body with an osmolality of ( $>300$  mOsmol  $\text{kg}^{-1}$ ), isotonic if they contain similar concentrations of sugar and salt as in the human body with an osmolality of ( $275\text{--}300$  mOsmol  $\text{kg}^{-1}$ ), or hypotonic if they contain lower concentrations of sugar and salt ( $<275$  mOsmol  $\text{kg}^{-1}$ ). For adequate hydration, drinks must be isotonic, (Ostrowska et al., 2016; Ruiz and García, 2022; Rowlands, Kopetschny, and Badenhorst, 2022).

Beverages are considered isotonic when they have an osmotic concentration similar to that found in body fluids. This feature allows quick absorption of the drink after ingestion, improving the performance of athletes and preventing muscle fatigue (Porfírio et al., 2019). Isotonic drinks are related to exercise and provide a replacement for the electrolytes, carbohydrates, and other nutrients perspiration removes during physical exertion (Bendaali et al., 2023).

Isotonic drinks are usually made with an optimal composition described in **Table 5**, to achieve optimal hydration, the frequent intake of a combination of water, carbohydrates, and electrolytes in specific amounts is suggested. This optimal composition is based on studies and what sports people need when they practice sports.

The appropriate osmolality for this type of product is defined by the European Food Safety Authority (EFSA), which assumes that it should be at the level of  $300\text{mOsm} \pm 10\%$  ( $270\text{--}330$  mOsm/ kg of water) (EFSA, 2011). In sports drinks, sodium, and potassium content are the most important minerals for replacing the electrolyte loss of the athletes and helping them be absorbed more quickly during training without causing gastrointestinal problems (Bendaali et al., 2023). These drinks should be neither too acidic nor too sweet and not carbonated, they have a pH value close to 3 and 4 (Bendaali et al., 2022), therefore, provided the normal industry hygiene precautions are taken, harmful organisms (pathogens) are not a problem (Bendaali et al., 2022).

Taste, acidity, sweetness, and beverage temperature are features that make isotonic beverages more consumable than water during sports activities, additionally, to counteract the depletion of carbohydrate storage and enhance exercise performance, carbohydrates must be consumed as a source of energy in isotonic beverages (Ruiz and García, 2022).

**Table 5.**The optimal composition of an isotonic drink (Ruiz and García, 2022).

<b>Composition</b>	<b>Definition</b>
<b>Water</b>	The first and most important ingredient is 80%.
<b>Carbohydrates</b>	The form of mono or polysaccharides, the most used are glucose polymers (maltodextrins), fructose, and sucrose. The recommended carbohydrate intake is 6 to 10% /L, i.e. between 60 and 100 g/L.
<b>A natural or artificial source of electrolyte salts</b>	Main ions of sodium, potassium, magnesium, chloride, and calcium and an intake of 460 to 1150 mg/L is recommended.
<b>Vitamins</b>	B1, B2, and B6, C.
<b>Natural juices, colors, and flavors</b>	That improves their organoleptic properties.

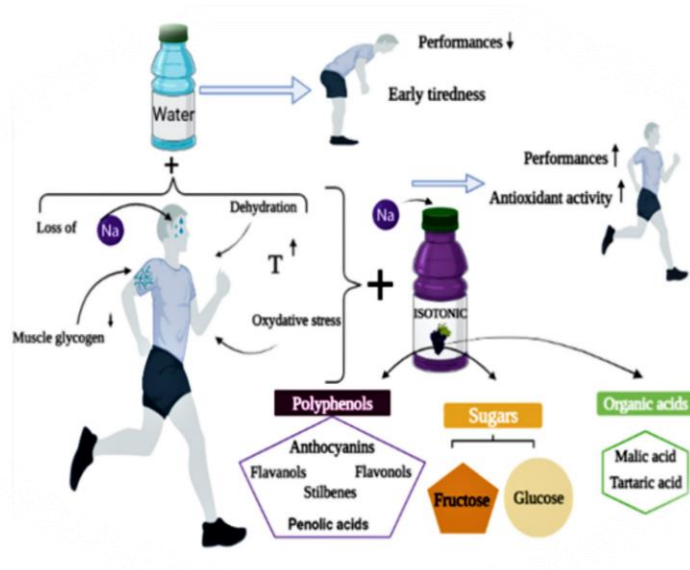
Commercial sports drinks usually contain ingredients such as preservatives, colorants, artificial sweeteners, taste, and flavorings, used to improve palatability and prolong shelf life, but they have been severely questioned for their potential risks to human health (Ruiz and García, 2022). Based on the demand for more natural drinks, there is an interest from the food industry to produce isotonic drinks from fruits, cereals, and other natural ingredients (Pinto, Vilela, and Cosme, 2022).

### **3.2 The importance of isotonic drinks**

The human body is composed of 60-70% water and needs an average of 30 mL of water/kg of body weight which varies according to the metabolism of each person (Riebl and Davy, 2013; Tobias, Ballard, and Mohiuddin, 2022). The athlete must drink at least 1.5 to 2 liters of water per day, more if the effort is intense and/or of long duration as well as in case of strong heat (Judge et al., 2021).

When practicing intense physical exercise for more than an hour, the body loses water, and electrolytes and the glycogen decreases in the liver, same for the blood glucose. The muscles owing to an increase in metabolic rate and increased heat during physical activity may cause dehydration, which influences physical performance and can lead to early tiredness, cognitive changes, sodium deficit, and increases the risk of heat-related diseases (Bendaali et al., 2022) (**Figure 3**).

Therefore, during prolonged physical exercise, it is advisable to counteract the loss of these substances by consuming isotonic sports drinks which require a continuous source of carbohydrates and sodium to provide energy and avoid fatigue (Gironés-Vilaplana et al., 2013). When it comes to staying hydrated during exercise, the choice between water and isotonic drinks can make a difference (**Figure 3**), it can significantly impact performance and well-being. Water, a straightforward and calorie-free option, is effective for basic hydration and helps maintain fluid balance but lacks additional electrolytes and energy replenishment. In contrast, isotonic drinks offer a balanced blend of water, carbohydrates, and electrolytes, making them particularly valuable during intense and prolonged workouts, aiding in rehydration, muscle function maintenance, and energy supply. The choice ultimately depends on individual preferences and the specific demands of the exercise, with water being suitable for shorter and less intense activities, while isotonic drinks are beneficial for more extended strenuous efforts where fluid and electrolyte loss is substantial as (**Figure 3**) shows (Bendaali et al., 2022).



**Figure 3.** The importance of an isotonic drink for the human body (Bendaali et al., 2022).

Depending on its intensity, physical activity may cause physiological alterations such as the production of oxygen-reactive species (ROS), muscle microtrauma, or inflammation. Although a certain level of ROS may be beneficial for organism adaptation, highly intense physical activities may evoke an oxidative stress state, which may damage performance. For this reason, in addition to their basic function, isotonic drinks based on fruit juices and plant extracts that beyond electrolytes provide nutrients, antioxidants, and biological activities - that may contribute to preventing or decreasing adverse effects of ROS due to intense physical activity - have been increasingly investigated (Gironés-Vilaplana et al., 2013). Animal and human studies have shown the benefits of consuming antioxidant-rich beverages during physical exercises in decreasing oxidative stress, reducing inflammatory markers, and improving performance (Simioni et al., 2018). Thus, plant and fruit-enriched isotonic drinks not only provide electrolytes and rehydration, but also afford nutrients, antioxidants, and health benefits to sports. (Orrù et al., 2018).

#### **4. Apple juice** (*Malus domestica* borkh)

Apple, one of the most widely consumed fruits worldwide, is an important part of the human diet as a source of monosaccharides, minerals (zinc, and

iron), dietary fibers (pectin), biologically active compounds such as vitamin C, and phenolic compounds (anthocyanins, flavanols, flavonols) that act as natural antioxidants (Zielinski et al., 2014).

Studies have shown that eating apples regularly stimulates and protects the immune system and helps to fight against the onset of certain diseases, such as cardiovascular disease, cancer, diabetes, and obesity, by neutralizing and eliminating cellular free radicals (Nyamende et al., 2022; Oyenihini et al., 2022). They have also been shown to have beneficial effects on cholesterol, diabetes, overweight, vascular function, blood pressure, lipids, inflammation, and hyperglycemia (Oyenihini et al., 2022). It also brings good hydration (it contains nearly 90% water), which is also essential to the good functioning of the body. Also, it is important to regulate the transit, to help digestion due to its rich in insoluble and soluble fibers (on average 2 g/100g), and the apple is the ally of good intestinal functioning (Bondonno et al., 2017).

Carbohydrates account for >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 and range from 5 to 8, 1 to 4, and 0 to 5% w/w, respectively (Pruksasri, Lanner, and Novalin, 2020; Wosiacki et al., 2009). 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 (Wosiacki et al., 2009). Due to these characteristics, nowadays apple juice is being widely used in the food industry as a natural sweetener.

## **5. Hibiscus (*Hibiscus sabdariffa* L.)**

*Hibiscus sabdariffa* (HS), also known as Roselle, red sorrel, or karkade, is a flowering plant, species of hibiscus that is native to West Africa but is now grown in many parts of the world. It is an annual herbaceous medicinal plant that belongs to the Malvaceae family (Jabeur et al., 2017).

The trumpet-shaped flowers of this plant, which have beautiful red cup-shaped blooms, are what make it so distinctive. These calyxes, are used to make famous herbal teas, to make this beverage all you need to do is steep the dried hibiscus flower calyxes in water. The result is a drink that offers a pleasant balance of sweetness and a refreshing hint of spiciness. It is high in antioxidants and has a spicy, sour flavor

reminiscent of cranberries (Cj and Ej, 2020). People from parts of the globe savor hibiscus tea with its popularity found in North Africa, the Middle East, and Central and South America. Due to its high antioxidant, vitamin, and mineral content, hibiscus is more than just a pretty plant; it also has a variety of other purposes in addition to being used to make tea (Jabeur et al., 2017).

Hibiscus tea is also a source of vitamin C and B, which acts as an antioxidant. Vitamin C plays a role, in supporting the system and maintaining the health of our skin, bones, and other tissues. Research suggests that hibiscus tea offers health benefits. For instance, studies have shown that it can effectively lower blood pressure in individuals with hypertension impacting both diastolic measurements (Jalalyazdi et al., 2019). This positive effect is likely effect, hibiscus tea may contribute to reducing the risk of heart disease and stroke (Chiu et al., 2022).

Antioxidants are essential for shielding our bodies against damage from free radicals that can harm our cells and contribute to the development of illnesses, like cancer, heart disease, and Alzheimer's, they prevent oxidative stress and the hibiscus is a rich source of phenolic compounds, and its consumption is associated with many human health benefits, mainly antioxidant activity (Wang et al., 2014; Maciel et al., 2018). Anthocyanins (such as cyanidin-3-*O*-sambubioside, delphinidin-3-*O*-sambubioside, and cyanidin-3-glucoside), sugars, and other phenolic compounds such as flavonoids like quercetin and hisbiscetrin are all abundant in the extract of *H. sabdariffa* (Morales-Luna et al., 2019). Hibiscus is known to have antibacterial qualities in addition to its antioxidant content. This makes it potentially a natural preservative in food production as it may be able to limit the growth of hazardous bacteria and fungi. This is especially crucial for goods that easily spoil, including jams and fruit juices (Borrás-Linares et al., 2015; Rizkiyah et al., 2023).

For all these advantages Roselle calyxes' day by day, is also becoming one of the intriguing ingredients in food production. The Hibiscus flower, driven by its phytochemical composition and versatile sensory characteristics, is an excellent and attractive addition to many different foods and drink formulations, because of its acidic, tart, and fruity flavor profile (Mahunu et al., 2021). Hibiscus is particularly renowned for its useful qualities and chemical makeup, it has been employed in a variety of products

recently, including beverages, ice cream, sorbet, jams, sauces, and fermented goods (Mahunu et al., 2021).

Furthermore, Roselle calyces appear to be good and promising sources of water-soluble red colorants, it is frequently employed as a natural food coloring agent, giving beverages, baked products, sauces, and other foods a vivid red hue aligning with consumer preferences for natural and health-promoting ingredients (Shruthi and Ramachandra, 2019; Grajeda-Iglesias et al., 2016). The flowers of the hibiscus plant are often used to make natural dyes for fabrics. They can be boiled down to create a vibrant red dye that is commonly used in traditional textiles (Wang et al., 2014).

Hibiscus is predicted to grow in favor of the food industry as customers' interest in organic, plant-based products increases. It is a flexible ingredient that continues to unveil its multifaceted potential, elevating its status as a notable botanical resource in contemporary food science and product development. It can be utilized in a range of products, including our isotonic drink, under its tart, energizing flavor, and vibrant red color, it is an indicator consumers use when anticipating the flavor and taste of food or beverages.

## **6 .Objectives**

### **6.1 General objective**

To develop an innovative isotonic drink based on thermal waters, natural extracts, and ingredients.

### **6.2 Specific objectives**

- Evaluate the potential of thermal waters from Chaves in the development of an innovative isotonic drink.
- Study the phytochemical, chemical and physico-chemical composition of the formulated isotonic drink.
- Study the bioactive properties of the designed isotonic in terms of antioxidant and anti-inflammatory activities.

- Evaluate the chemical and physico-chemical stability (phenolic compounds, sugars, minerals, and color) and microbiological stability of the developed isotonic under commercialization conditions over 45 days.

## 7. Material and methods

### 7.1 Raw Materials

Grounded dried flowers of hibiscus (**Figure 4**) were purchased from local markets in Bragança, Portugal, in the form of tea bags. The contents of the bags were homogenized to create a single, representative sample.



**Figure 4.** Dried hibiscus flower.

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

The thermal water (**Figure 5**) was provided by Termas de Chaves, Chaves, Portugal (Total Mineralization= 2174 mg/L; Dry Residue (at 180°C) = 1744 mg/L; Bicarbonate (HCO<sub>3</sub><sup>-</sup>) = 1415 mg/L; Chloride (Cl<sup>-</sup>) = 35 mg/L; Sodium (Na<sup>+</sup>) = 623 mg/L) (Ferreira, 2023). The pH of the thermal water at the receiving time was 6.7.



**Figure 5.** Thermal water received from Termas de Chaves.

## 7.2 Production of hibiscus aqueous extracts

Aqueous extracts of hibiscus-grounded dried flowers were produced according to the method reported by Cissé et al. 2012, with some modifications. Thermal water was used as the extractor solvent. Briefly, 50 mL of thermal water 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 rpm for 20 min at 10 °C), and the upper phase was filtrated via Whatman paper filter n°1.

As such, this extract presented 2.64 mg/g of total anthocyanin, as measured by the differential pH method described in section 7.6; and 14.13 mg/g of total phenolic compounds evaluated using the Folin Ciocalteu method according to the description in section 7.6. The extracts were directly incorporated into the isotonic drink at different concentrations during the formulation step.

### **7.3 Formulation of the isotonic drink**

The composition of the isotonic beverage was defined after formulation tests, considering sensory (color and taste), sugar and sodium contents, and literature references (Gironés-Vilaplana et al., 2013; Porfírio et al., 2019). The formulation was composed of thermal water, apple juice, and hibiscus aqueous extract obtained in as described in section 7.2.

To achieve the sodium content of commercial isotonic drinks (Gironés-Vilaplana et al., 2013), thermal water was added in all the steps of the formulation, as described below.

Apple juice was added as a source of simple sugars in an amount enough to achieve the sugar content of commercial isotonic drinks (6-10% between 60 and 100 g/L) (Ruiz and García 2022). For that, the individual sugar concentration and the TSS of the commercial apple juice were measured as described in section 7.6, and the necessary dilution of the apple juice was calculated. This process ensures that the final isotonic drink formulation aligns with the acceptable range of sugar content, delivering a product that meets specified taste standards. The dilution of the apple juice was made with thermal water and hibiscus extract.

To determine the concentration of hibiscus in the formulation, different concentrations of hibiscus extract in the isotonic drink were tested and evaluated for color and taste. Firstly, pure hibiscus extract was mixed with apple juice but resulted in unpleasant sensory characteristics due to a high acidity attributed to the hibiscus extract. Then, several dilutions of the pure hibiscus extract (60%, 50%, 40%, 30%, and 20% of the original concentration) were prepared by adding thermal water in the extract, and each of these solutions was combined with the adequate volume of previously prepared apple juice. The extract concentration in the formulation that resulted more satisfactory in sensory terms (taste and color) was selected for the next steps of the study. This was the standard isotonic formulation (F1).

In addition to the standard, one formulation of isotonic drink was prepared containing no thermal water (with distilled water instead), as a control sample (F3). In these samples, the sodium content was adjusted with sodium chloride (PanReac, Milano, Italia) to achieve equivalent concentrations as in F1, and the hibiscus extract was extracted with distilled water (acidified with 0.1% citric acid).

Moreover, a comparable formulation consisting of thermal water only, with no apple juice and no hibiscus extract, was also prepared as another control group (F2). The sugar content was regulated with sucrose. This third formulation aimed to evaluate the potential of preparing unflavored isotonic drinks with thermal water.

A summarized description of the studied isotonic drinks, as well as the identification of each formulation can be found in (**Table 6**).

**Table 6.** Description and codes of the studied isotonic drink formulations.

<b>Formulation code</b>	<b>Description</b>
<b>F1</b>	Thermal water, apple juice, hibiscus extract
<b>F2</b>	Thermal water (0.1% citric acid), sucrose, distilled water
<b>F3</b>	Distilled water, apple juice, hibiscus extract, and sodium chloride.

#### **7.4 Thermal treatment**

After formulated, the isotonic drinks were placed in 100 mL- screw-capped aluminum pouches (**Figure 6**), 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 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).



**Figure 6.** The screw-capped aluminum pouches used to store the isotonic drinks.

### **7.5 Characterization of the isotonic drinks and stability studies**

The formulations were characterized for the following analyses: color, pH, TSS (°Brix), anthocyanins and non-anthocyanin phenolic compounds, individual sugar content, mineral concentration, antioxidant activity using ORAC assay and a cell model antioxidant assay (CAA), and anti-inflammatory activity also using a cell-based assay.

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

### **7.6 Methods**

#### **7.6.1 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).

### 7.6.2 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).

### 7.6.3 Total phenolic compounds

The Folin-Ciocalteu method as described by Singleton, Orthofer, and Lamuela-Raventós (1999) was used after adaptation 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 were expressed as mg of gallic acid equivalents (GAE) per 100 ml of sample.

### 7.6.4 Total monomeric anthocyanin compounds

Total monomeric anthocyanin content was determined using the pH differential method described by Giusti and Wrolstad (2001). Samples were properly diluted in sodium acetate buffer (pH 1.0) and potassium chloride buffer (pH 4.5). The anthocyanin content was calculated according to Equations 1 and 2, and expressed as mg of cyanidin-3-glucoside per 100 mL of sample.

$$A = (A_{510\text{nm}} - A_{700\text{nm}})_{\text{pH } 1.0} - (A_{510\text{nm}} - A_{700\text{nm}})_{\text{pH } 4.5} \text{ (Equation 1)}$$

$$A' = \frac{(A \times \text{MW} \times \text{DF} \times 1000)}{\varepsilon \times l} \text{ (Equation 2)}$$

In which A' = total absorbance, A = absorbance, MW = 449.2 g/mol (molecular weight of cyanidin-3-glucoside), DF = dilution factor, and  $\varepsilon$  = 26.900 L/mol (the molar absorption coefficient).

### **7.6.5 Phenolic compounds by LC-DAD-ESI-MS<sup>n</sup>**

Major individual phenolic compounds were analyzed through high-performance liquid chromatography (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA), according to Bessada et al., 2016, with modifications. The chromatographic separation of the compounds was performed using a Waters Spherisorb S3 ODS-2C18 (3  $\mu$ m, 4.6 mm  $\times$  150 mm, Waters, Milford, MA, USA) column thermostatted at 35  $^{\circ}$ C. The mobile phase was composed of (A) 0.1% formic acid in the water, (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  $^{\circ}$ 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 literature, and the quantification was performed using calibration curves of the corresponded compound. When the respective standard was not available, the calibration curve of the substance with the higher chemical matching was used to quantify the samples. The results were expressed as mg/100mL.

### **7.6.6 Anthocyanin compounds by LC-DAD-ESI-MS<sup>n</sup>**

Major individual anthocyanins were separated on an Aqua C18 (5  $\mu$ m, 4.6 mm  $\times$  150 mm, Waters, Milford, MA, USA) column working at 35  $^{\circ}$ C, using a Dionex Ultimate 3000 UPLC (Thermo Scientific), according to Leichtweis et al., 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 calibration curves of the corresponding compound. When the respective standard was not available, the calibration curve of the substance with the higher chemical matching was used to quantify the samples. The results were expressed as mg/100mL.

#### **7.6.7 Mineral composition**

Minerals were analyzed using Atomic absorption spectrometer (iCET<sup>TM</sup> series, 3000, Thermo Scientific). Potassium (K), sodium (Na), magnesium (Mg) were determined using flame atomization, while iron (Fe) was detected through electrothermal atomization. The samples digestion was performed according to Ang and Lee (2005), with minor modifications. Briefly, 500 mg of the sample was weighted and digested with 9 mL of nitric acid: hydrochloric acid (1:3) for 4h at (105 °C). After cooling, the samples were filtered, and the volume was adjusted to 50 mL. The quantification of the mineral compounds was performed using external calibration. The results were expressed as mg/100mL

#### **7.6.8 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 Zielinsk et al., 2014. The samples were filtered through a 0.22 µm filter membrane in vials and injected for analysis. Sugars were identified by comparing peak retention times with commercial standards, and quantification was performed using calibration curves of the corresponding sugars. The results were expressed is g/100mL.

## 7.6.9 Antioxidant activity

### 7.6.9.1 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) as the reaction medium. The microplates containing 20  $\mu\text{L}$  of the sample or different concentrations of Trolox, 120  $\mu\text{L}$  of fluorescein ( $0.4 \mu\text{g}\cdot\text{mL}^{-1}$ ), and 60  $\mu\text{L}$  of radical AAPH (2,2'-azobis (2-methylpropionamidine) dihydrochloride) ( $108 \text{ mg}\cdot\text{mL}^{-1}$ ) 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 and expressed in  $\mu\text{mol}$  Trolox Equivalent per 100mL of sample.

### 7.6.9.2 Cellular Antioxidant Assay (CAA)

The antioxidant activity in cell lines was determined following the modified method described by Wolfe and Liu, (2007). For this procedure, the cell line used was RAW 264.7 (mouse macrophages), which was maintained in a DMEM culture medium supplemented with L-glutamine, penicillin (100 U/mL), streptomycin (100  $\mu\text{g}/\text{mL}$ ), fetal bovine serum (10%) and non-essential amino acids (2 mM), in an incubator at 37°C with a humidified atmosphere and 5%  $\text{CO}_2$ . After proliferation for 48 hours, cells were washed with HBSS (2x, 100  $\mu\text{L}$ ), and treated with the isotonic drink in 4 dilution scenarios: no dilution, 4, 16, 64, and 256 times in water ( $\text{H}_2\text{O}$ ), which was diluted with 2',7'-dichlorohydrofluorescein (DCFH) prepared with ethanol and diluted with HBSS (50 $\mu\text{M}$ ). The samples were incubated for 1 hour. Then, the cells were washed with HBSS (2x, 100  $\mu\text{L}$ ) and a solution of 2,2 2'-azobis (2-methyl propionamide) dihydrochloride (AAPH) (100  $\mu\text{L}$ ; 600  $\mu\text{M}$ ) 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 dichlorohydrofluorescein and DMEM culture medium were used as a negative control during the assay. The results were given as percent inhibition of cell oxidation.

#### **7.6.10 Anti-inflammatory activity**

The evaluation of the anti-inflammatory activity was performed using the mouse macrophage cell line RAW 264.7 supplemented with heat-inactivated fetal bovine serum (SFB) (10%), glutamine, and antibiotics, and maintained in an incubator at 37°C, with 5% CO<sub>2</sub> and under a humid atmosphere was used for this procedure. The microplate was incubated for 24 hours in the incubator with the previously indicated conditions to allow adequate adherence and multiplication of the cells. After this incubation period, the cells were treated with the different concentrations of the isotonic drink (no dilution, 4, 16, 64, and 256 times with water) and incubated for one hour. We proceeded the stimulation by adding 30 µL of oligosaccharide solution - LPS (1 mL/mL) and incubated for another 24 hours. Ellipsin was used as a positive control and the samples in the absence of LPS as a negative control. The results were presented as described by Sobral et al., (2016) and were calculated by graphing the percentage of inhibition of nitric oxide production versus sample concentration and expressed relative to the concentration of each of the extracts that caused 50% inhibition of nitric oxide production.

#### **7.6.11 Microbiological analysis**

The following microorganisms were analyzed: total aerobic mesophiles, molds, and yeasts to determine the microbial load in the isotonic drink along the shelf life of 45 days at 2 different temperatures 4 °C and 25 °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), after 7 days (T7), after 15 days (T15), after 30 days (T30), and 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: Using the spread plate method, 0.2 mL of each dilution was added 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 in the upright posture at 25 °C for 5 days. After 3 and 5 days of incubation, respectively, yeasts and molds were counted.

### 7.7 Statistical analysis

The analyses were carried out in triplicate, and the results were expressed as mean±standard deviation. The samples were compared using a t-test, Analysis of Variance (ANOVA) followed by Tukey's post-hoc test, or Repeated Measures ANOVA followed by Bonferroni post-hoc test. Samples were considered different when  $p < 0.05$ . The confident interval was set at 95%.

## 8. Results and discussion

### 8.1 Development of the isotonic drink formulations

In the development of the isotonic drink formulation, a series of formulation tests were performed to fine-tune the composition and sensory characteristics of the final product. **Table 7** shows the final formulation of the standard isotonic drink (F1, **Figure 7**), together with the accompanying control samples F2 and F3. The volumes presented were determined based on the desired sugar and sodium contents in the beverage, which must be placed within an acceptable range for an isotonic drink (Ruiz and García, 2022). For F1 and F3, the addition of hibiscus extracts in a concentration of 40% of its original (dilution factor of 2.5) was selected to be applied in the final formulation. This was because it was the one providing the best combination of taste and color (*in-house* evaluation).

**Table 7.** Detailed description of the isotonic drink formulations.

<b>Formulation code</b>	<b>Ingredients</b>	<b>Quantity (mL or g) per 100mL</b>
<b>F1 Standard</b>	Thermal water	33
	Apple juice	45
	Hibiscus extract containing 40% of its original concentration	22
<b>F2</b>	Thermal water (0.1% citric acid)	55
	Distilled water	45
	Sucrose	6.9
<b>F3</b>	Distilled water	33
	Apple juice	45
	Hibiscus extract containing 40% of its original concentration	22
	Sodium chloride	0.0281

NB: the hibiscus extract was produced by adding citric acid.



**Figure 7.** The standard isotonic drink F1.

## 8.2 Characterization of the formulated isotonic drinks

The chemical and physicochemical composition of the formulated isotonic drinks are shown in (Table 8).

**Table 8.** Chemical and physicochemical composition of formulations F1, F2, and F3.

Parameter	F1	F2	F3
pH	3.72±0.003b	5.90±0.07a	3.12±0.01c
TSS (°Brix)	5.70±0.05b	6.50±0.00a	5.60±0.00b
L*	32.35±0.19a	-	30.77±0.45b
a*	24.13±0.27b	-	28.94±0.24a
b*	16.40±0.21b	-	19.92±0.15a
TPC (mg Eq GA/100mL) <sup>1</sup>	41.36±1.34a	ND	39.16±1.09a
TMA (mg/100mL) <sup>2</sup>	4.06±0.03a	ND <sup>3</sup>	3.89±0.05b
<b>Individual anthocyanins (mg/100mL)<sup>5</sup></b>			
Delphinidin 3- <i>O</i> -sambubioside	4.29±0.29a	-	4.14±0.42a
Cyanidin 3- <i>O</i> -sambubioside	1.84±0.14a	-	1.84±0.18a
<b>Individual non-anthocyanins phenolic compounds (mg/100mL)<sup>4</sup></b>			
3-Caffeoylquinic acid	1.95±0.20a	-	1.75±0.12a
3- <i>p</i> -coumaroyl quinic acid	0.11±0.01a	-	0.09±0.003b
4-Caffeoylquinic acid	0.86±0.17a	-	0.61±0.11a
Chlorogenic acid	2.75±0.10a	-	2.21±0.38a
4- <i>p</i> -coumaroyl quinic acid	0.15±0.03a	-	0.09±0.02b
<b>Mineral (mg/100mL)</b>			
Na	31.30±1.78	32.44±1.50	28.10±0.00 <sup>5</sup>
Fe	0.12±0.02	0.08±0.01	-
K	50.81±1.63	1.91±0.09	39.64±1.47
Mg	4.91±0.13	14.06±1.47	-
Total minerals	87.14±0.89	48.55±0.77	67.74±0.73
<b>Sugars (g/100mL)</b>			
Fructose	5.41±0.11a	ND	5.68±0.37a
Glucose	1.57±0.04a	ND	1.61±0.14a
Sucrose	0.95±0.03a	9.21±0.57	1.00±0.02a
Total sugars	7.93±0.18a	9.21±0.57	8.30±0.49a
<b>Antioxidant activity</b>			
ORAC µmol/100mL	472.72±26.45a	ND	473.46±26.60a
CAA <sup>6</sup>	No activity	No activity	No activity
Anti-inflammatory activity	No activity	No activity	No activity

<sup>1</sup>TPC: total phenolic compounds expressed in mg equivalent in gallic acid; <sup>2</sup>TMA: total monomeric anthocyanins; <sup>3</sup>Non detected; <sup>4</sup>LC-MS data of the determined compounds is found in the supplementary material (Annexes); <sup>5</sup>Value according to the amount of exogenous NaCl added; <sup>6</sup>Cell antioxidant assay.

The characteristics of the formulated isotonic drink align with industry expectations and what our body needs when exercising an intensive activities according to National Academy of Medicine (2023), with sodium content and pH levels falling within the typical range for such beverages (Ruiz and García, 2022; Gironés-Vilaplana et al., 2013). In addition, F1 and F3 showed an attractive color satisfactory taste.

### 8.2.1 pH and TSS

For the standard formulation F1, the pH value was  $3.72\pm 0.003$ , F3 has a pH value of  $3.12\pm 0.009$ , while for F2 the pH was  $5.90\pm 0.07$ . For F1 and F3, the lower pH is due to the high acidity of the hibiscus, the citric acid, and the apple juice. The pH values found for F1 and F3 are within the range expected for an isotonic drink, as reported in previous studies (Bendaali et al., 2022). On the other hand, F2 showed a higher pH than that expected for this type of beverage (Bendaali et al., 2022).

The variation in pH levels among the three formulations, despite the addition of the same quantity of citric acid (0.1%), can be attributed to the high level of the hibiscus acidity (Izquierdo-Vega et al., 2020) and to the buffering effect of the thermal water (Wasserfurth et al., 2019). Thermal water can often act as a buffer solution due to its natural mineral content, especially the bicarbonate that can effectively reduce the net acid excretion, which can help maintain a relatively stable pH level in certain situations (Wasserfurth et al., 2019). To confirm this hypothesis and evaluate the buffering potential of the thermal water, a fourth formulation (F4) was prepared exactly as F2, but with distilled water acidified with 0.1% citric acid instead of thermal water. In fact, the pH of this sample was  $2.96\pm 0.05$ , lower than F2, confirming that thermal water may exert buffering effects.

The TSS present in the isotonic drink expresses the amount of total soluble solids present in the product, making it an important ratio for the quality of the final product (Santos, Alves, and Lima, 2013). The TSS values of F1 ( $5.70\pm 0.05$  °Brix) and F3 ( $5.60\pm 0.00$  °Brix) were lower than in F2 ( $6.50\pm 0.00$  °Brix). This TSS level is entirely attributed to the naturally occurring sugars found in the apple juice used in these formulations, without any additional sugar added, and to the naturally present minerals of the thermal water. In contrast, F2 had a higher TSS of  $6.50\pm 0.00$  °Brix. This increase in TSS is a result of the deliberate addition of sugar to these formulations (Porfírio et al.,

2019). According to Sadowska, Świdorski, and Laskowski (2020), the values found in this study are in accordance with the legislation's recommendations for TSS.

### 8.2.2 Color measurement

The designed isotonic drink F1 with natural anthocyanins from the hibiscus flower gives an attractive red color for consumer acceptance as shown in (**Figure 8**).



**Figure 8.** The color of the isotonic drink in F1.

**Table 8** compares the color characteristics of F1 and F3 using  $L^*$ ,  $a^*$  and  $b^*$  values. Despite the use of the same volume of hibiscus extract, F1 and F3 had slightly different colors.

The results observed for the parameter ( $L^*$ ) that represents the luminosity, determine when a producer is presented as light or dark. In this context, F3 exhibits a slightly lower  $L^*$  value ( $30.77 \pm 0.45$ ) compared to F1 ( $32.35 \pm 0.19$ ), indicating that it is darker. The  $a^*$  parameter quantifies the position along the green ( $-a^*$ ) to red ( $+a^*$ ) axis. F3 displays a higher  $a^*$  value ( $28.94 \pm 0.24$ ) compared to F1 ( $24.13 \pm 0.27$ ), indicating that it has a stronger red component. The  $b^*$  parameter represents the position along the

blue (-b\*) to yellow (+b\*) axis. In this case, F3 exhibits a higher b\* value compared to F1, indicating that it has a greater degree of yellowness.

Differences in pH can affect the ionization and interaction of chemical compounds and pigments responsible for color in the products (Khoo et al., 2017). The more the conditions are acidic, the more the anthocyanins appear to the red in color but they turn blue when the pH increases (Khoo et al., 2017).

A lower pH, such as the pH of  $3.12\pm 0.01$  in F3 tends to enhance the intensity of red and yellow colors due to changes in the chemical composition and the ionization of color molecules. As a result, the lower pH in F3 can be scientifically linked to its darker appearance, stronger redness (higher a\*), and increased yellowness (higher b\*) compared to F1 with a pH of  $3.72\pm 0.003$ . This demonstrates the profound impact of pH on the color attributes of these formulations within the context of CIELAB color space analysis (Piovesana, Rodrigues, and Noreña 2019; Kahle, Kraus, and Richling, 2005).

### **8.2.3 Non-anthocyanin phenolic compounds**

Total phenolic compounds were  $41.36\pm 1.34$  mg Eq. GA/100mL for formulation F1, while they were  $39.15\pm 1.08$  Eq. GA mg/100mL for F3 as shown in **Table 8**. There was no significant difference between the total phenolic compounds of both formulations. The use of thermal water in place of the standard water source does not have a significant impact on these characteristics.

Chlorogenic acid was identified as the major phenolic compound, which can be attributed to the presence of hibiscus extract and apple juice. This agrees with the findings of Piovesana, Rodrigues, and Noreña, (2019) and Kahle, Kraus, and Richling, (2005), who reported chlorogenic acid as the predominant phenolic acid in these ingredients with  $2.75\pm 0.10$  mg/100mL for F1 and  $2.21\pm 0.38$  mg/100mL for F3.

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 isotonic drink (Piovesana et al., 2018; Kahle et al., 2005).

In recent years, the sports food industry has been making significant efforts to develop formulations that extend beyond basic nutrition. These formulations aim to provide functional elements that counteract fatigue and exercise-induced oxidative

stress, thereby enhancing performance (Gonçalves et al., 2022). In this context, numerous studies have explored the effects of phenolic compounds, known for their antioxidant and anti-inflammatory properties, on exercise performance (Gonçalves et al., 2022; Kruk et al., 2022; Malaguti, Angeloni, and Hrelia, 2013; Tung et al., 2019). However, the results regarding the impact of these compounds on sports endurance and resistance remain inconclusive (Malaguti, Angeloni, Hrelia, 2013).

#### 8.2.4 Anthocyanin compounds

**Table 8** displays the anthocyanins and non-anthocyanin phenolic compounds present in the isotonic drink. These compounds are attributed to the hibiscus extract and apple juice, aligning with the expected composition found in the literature (Kahle, Kraus, and Richling, 2005; Borrás-Linares et al., 2015).

For the total anthocyanin compounds, F1 has a slightly higher value (4.06 mg/100mL) compared to F3 (3.89 mg/100mL) (**Table 8**). These differences between the total anthocyanin compounds of both formulations could be attributed to natural variations in the extraction process and in the formulation and production process. It is also possible that the type of water used as a solvent (thermal distilled water) could somehow interfere with the extraction efficiency. Thermal water, with its distinct mineral composition, may influence the extraction and stability of anthocyanin compounds, potentially leading to higher anthocyanin content in F1. However, this hypothesis should be further investigated in future studies.

The isotonic drink primarily contained cyanidin-3-*O*-sambubioside and delphinidin-3-*O*-sambubioside, which are the main pigment compounds in hibiscus flowers (Piovesana et al., 2018), with  $4.29 \pm 0.29$  mg/100mL and  $1.84 \pm 0.14$  mg/100mL respectively for F1. On the other hand  $4.14 \pm 0.42$  mg/100mL and  $1.84 \pm 0.18$  mg/100mL for cyanidin-3-*O*-sambubioside and delphinidin-3-*O*-sambubioside, respectively for F3. While both formulations share the presence of the primary anthocyanin compounds, cyanidin-3-*O*-sambubioside, and delphinidin-3-*O*-sambubioside, it is possible that additional anthocyanins are present in differing concentrations, contributing to the observed differences in total anthocyanin content.

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 indicate that the hibiscus extract not only supplied antioxidant compounds but also added an appealing color to the product due to its anthocyanin content, eliminating the need for synthetic colorants.

### **8.2.5 Antioxidant activity**

The antioxidant activity using ORAC method are shown in **Table 8**. It was  $472.730 \pm 26.45$   $\mu\text{mol}/100\text{mL}$  for the F1 and for F3 was  $473.46 \pm 26.61$   $\mu\text{mol}/100\text{mL}$ , and this is likely due primarily to the antioxidant activity of the hibiscus extract (Grajeda-Iglesias et al. 2016). No difference was observed between the antioxidant activity of F1 and F3, and no antioxidant activity was found for F2, showing an insignificant contribution of thermal water from Chaves in this parameter. 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 could investigate the action of digested fractions of the isotonic drink in this cell experimental model of antioxidant activity.

### **8.2.6 Anti-inflammatory activity**

Nowadays, there is a body of evidence in literature showing that exhaustive exercise causes oxidative stress, inflammatory response, and structural damage to muscle cells (Malaguti, Angeloni, Hrelia, 2013). Therefore, a number of studies have investigated the possibility of preventing exercise-induced muscle damage through dietary interventions, for example by consuming compounds with antioxidant or anti-inflammatory properties. However, in the present work, no anti-inflammatory activity was detected when cells (mouse macrophages) were treated with isotonic drinks at any concentration level tested. Further studies should investigate the reasons for the lack of an effect, and also study digested fractions of the isotonic drink in this experimental cell model of inflammation.

### **8.2.7 Mineral composition**

The results of the mineral content are shown in **Table 8**. The total minerals for F1 was  $87.14 \pm 0.89$   $\text{mg}/100\text{mL}$ , it meets the acceptable minerals range for isotonic beverages providing the necessary electrolyte balance according to Ruiz and García, (2022), and Gironés-Vilaplana et al., (2013), with an established concentration of

sodium  $31.30 \pm 1.78$  mg/100mL. The sodium content was a critical parameter under consideration. It is the main electrolyte eliminated in the sweating process during long-term physical exercises (Rowlands, Kopetschny, and Badenhorst, 2022). It plays a major role in the adjustment of the balance of water in the body and transport of carbohydrates and aids the active working muscle to contract and relax (Bendaali et al., 2023). Potassium (K) was the mineral found in the greatest quantity, with concentration of  $50.81 \pm 1.63$  mg/100mL. This mineral creates and preserves constant muscle contraction and nerve impulse, prevents the clotting of blood keeps its pH level, and helps in the storage of carbohydrates in the muscles (Bendaali et al., 2022). F1 contained also a very low amount of iron and magnesium,  $0.12 \pm 0.02$  mg/100mL and  $4.91 \pm 0.13$  mg/100mL, respectively.

These minerals are involved in physiological processes of importance to athletes, such as muscle and heart contraction, oxygen transport and oxidative phosphorylation, enzyme activation, bone health, antioxidant capacities, and immune functions (Orrù et al., 2018).

For F3 adding sodium was  $28.10 \pm 0.00$  mg/100mL and  $39.64 \pm 1.47$  mg/100mL for the potassium, this aligns with industry expectations and commercial isotonic drinks found in the market (Gironés-Vilaplana et al., 2013), and for F2 the differences between it and F1 and F3 were a lower amount of potassium ( $1.91 \pm 0.09$  mg/100mL) and a higher content of magnesium ( $14.06 \pm 1.47$  mg/100mL), with total minerals of  $48.55 \pm 0.77$  mg/100mL, proving the importance of the apple juice and the thermal water in the formulation of the isotonic drink to achieve the adequate amount of minerals.

### 8.2.8 Analysis of individual sugars

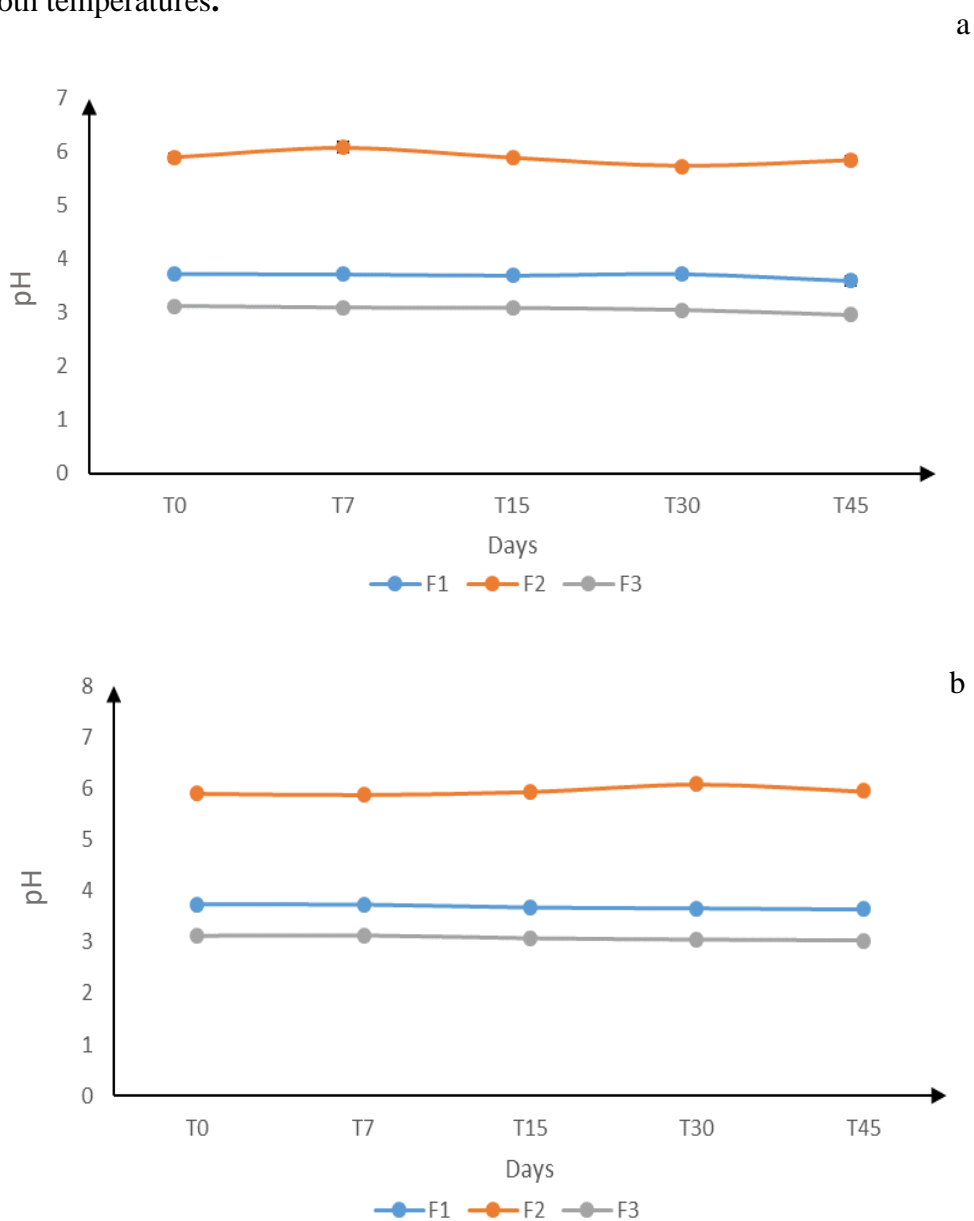
**Table 8** represents the individual sugars presented in the isotonic drinks. The sugars present in F1 and F3 were naturally present initially in the apple juice and traced in hibiscus extract (Edo et al., 2023; Wosiacki et al., 2009). F1 contained  $7.93 \pm 0.18$  g/100mL of total sugars ( $5.41 \pm 0.11$  g/100mL of fructose,  $1.57 \pm 0.04$  g/100mL of glucose, and  $0.95 \pm 0.03$  g/100mL of sucrose) with no significant difference compared to F3 that contained  $8.30 \pm 0.49$  g/100mL of total sugars ( $5.68 \pm 0.37$  g/100mL of fructose,  $1.61 \pm 0.14$  g/100mL of glucose, and  $1.00 \pm 0.02$  g/100mL of sucrose), due the use of the

same volume of apple juice. Therefore, the values found in this study are in accordance with the legislation's recommendations (Ruiz and García, 2022).

### 8.3 Stability studies at different storage temperatures

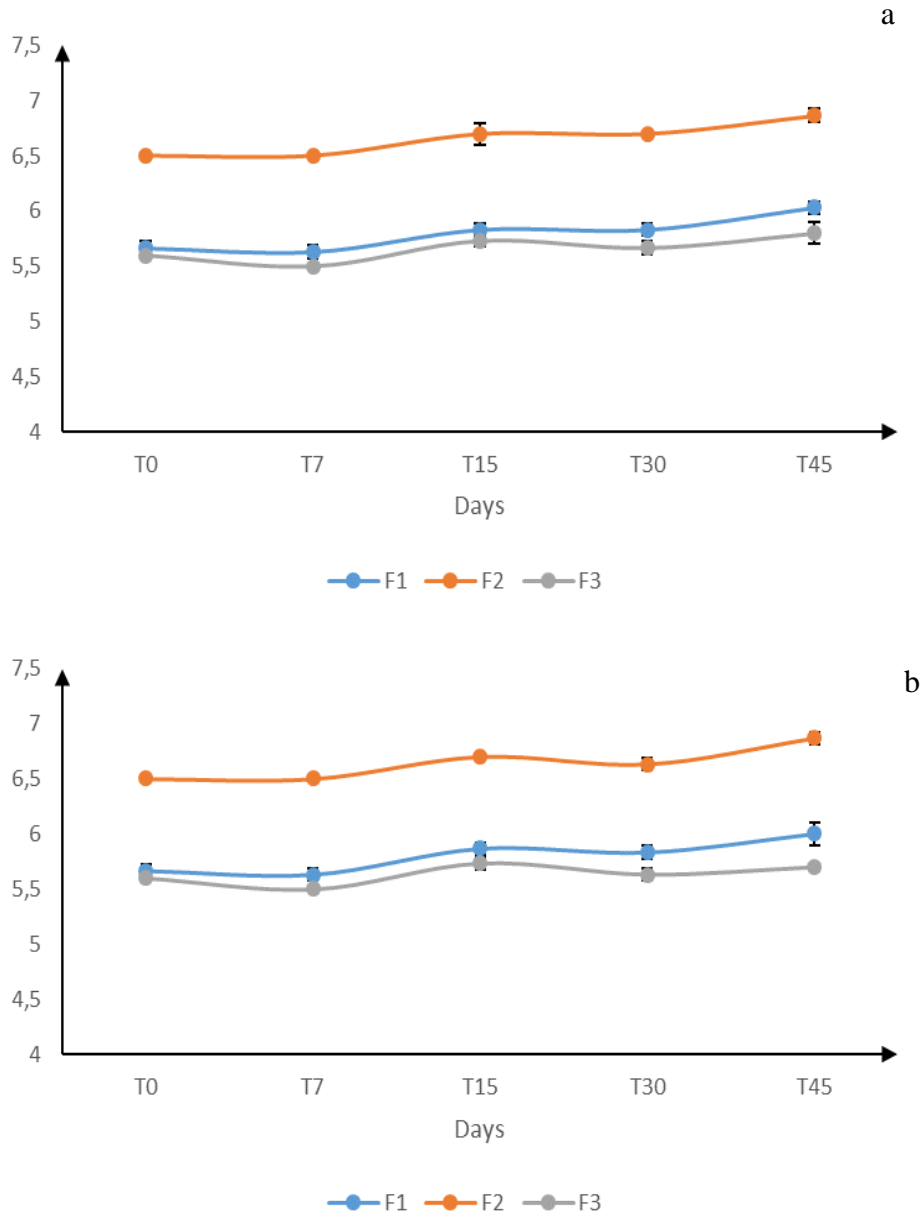
#### 8.3.1 pH and TSS

The pH evolution in the three formulations of isotonic drinks during 45 days is shown in **Figure 9**, for both studied temperatures (4 °C in **Figure 9a** and 25 °C in **Figure 9b**). After 45 days of shelf life, the pH was stable in all three formulations and for both temperatures.



**Figure 9.** The pH evolution of isotonic drinks at (a) 4 °C and (b) 25 °C.

TSS showed a significant but only slight increase ( $p < 0.05$ ), therefore, it was considered stable during the storage of 45 days in all three formulations as shown in **Figure 10** for both temperatures (4 °C in **Figure 10 a** and 25 °C in **Figure 10b**).



**Figure 10.** Total soluble solids (°Brix) for (a) 4 °C and (b) 25 °C.

### 8.3.2 Color measurements

Color measurements during the storage time are shown in **Table 9**. For F1 stored at 4°C no change in L\*, and b\* values was observed between the beginning and the end of the storage period. For F3, a slight but significant increase in L\* and a decrease in b\* was detected. Both formulations showed a reduction in the a\* component of color, implying that by the end of 45 days, the redness of the samples was less intense. Notably, the magnitude of the reduction of this component was higher in F1 than in F3, which could be related with the higher pH of the former causing higher instability of anthocyanins.

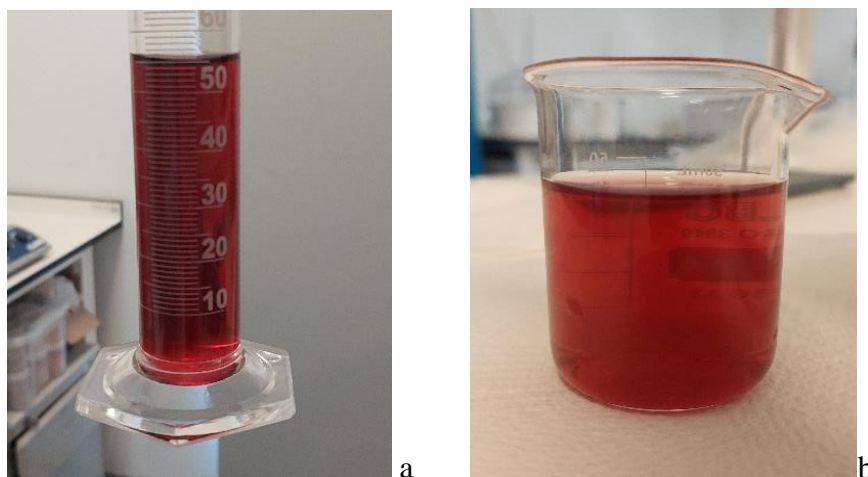
**Table 9.** Color parameters variation for F1 and F3.

Formulation		T0	T7	T15	T30	T45
<b>Storage at 4°C</b>						
<b>F1</b>	<b>L</b>	32.35±0.19a	33.34±0.49ab	33.44±0.31ab	33.15±0.74a	33.20±0.13ab
	<b>a</b>	24.13±0.27b	23.29±0.42a	22.39±0.53a	22.00±0.97a	22.92±0.22a
	<b>b</b>	16.40±0.21a	15.97±0.33a	15.36±0.52a	15.63±0.55a	16.33±0.21a
<b>F3</b>	<b>L</b>	30.77±0.45b	31.59±0.31a	31.44±0.28c	32.19±0.05a	32.17±0.42a
	<b>a</b>	28.94±0.24b	27.74±0.53ab	28.28±0.43a	26.87±0.55a	27.37±0.24a
	<b>b</b>	19.92±0.15a	19.24±0.72ab	19.41±0.31ab	17.64±0.90c	18.36±0.16bc
<b>Storage at 25°C</b>						
<b>F1</b>	<b>L</b>	32.35±0.19a	34.17±0.5a9	33.89±0.28a	33.91±0.78a	34.92±0.36a
	<b>a</b>	24.13±0.27a	20.74±0.55b	19.11±0.93bc	17.24±0.16c	15.26±0.75d
	<b>b</b>	16.40±0.21a	15.54±0.16a	16.24±0.89a	16.50±0.32a	17.58±0.74a
<b>F3</b>	<b>L</b>	30.77±0.45c	31.53±0.34bc	31.80±0.41bc	31.94±0.30b	33.27±0.27a
	<b>a</b>	28.94±0.24a	27.23±0.74b	27.51±0.02b	24.78±0.32c	23.10±0.05d
	<b>b</b>	19.92±0.15a	18.73±0.79ab	18.92±0.17ab	17.51±0.52bc	16.97±0.39c

At 25 °C, both F1 and F3 showed the same trend for L\* and b\* as at 4 °C, but experimented with a sharper decrease in a\*. This may be attributed to the faster degradation of anthocyanins when formulations were stored at 25 °C. During shelf life, the storage temperature plays a crucial role in maintaining the color stability of the isotonic drink containing anthocyanins as mentioned in (Khoo et al. 2017). This phenomenon suggests that at higher temperatures, the drinks become less redness (a\*) and yellowness (b\*), indicating a shift towards a less intense and more faded color profile

due to the degradation of anthocyanin pigments (Khoo et al., 2017). This is attributed to the sensitivity of anthocyanin pigments to temperature (Khoo et al., 2017). At 25 °C, the anthocyanin in the isotonic drink is more prone to degradation and color changes, and this is provided in section 8.3.3.

Consequently, maintaining a lower storage temperature, such as 4 °C, is crucial for preserving the desired color attributes of these isotonic drinks rich in anthocyanin as shown in **Figure 11**.

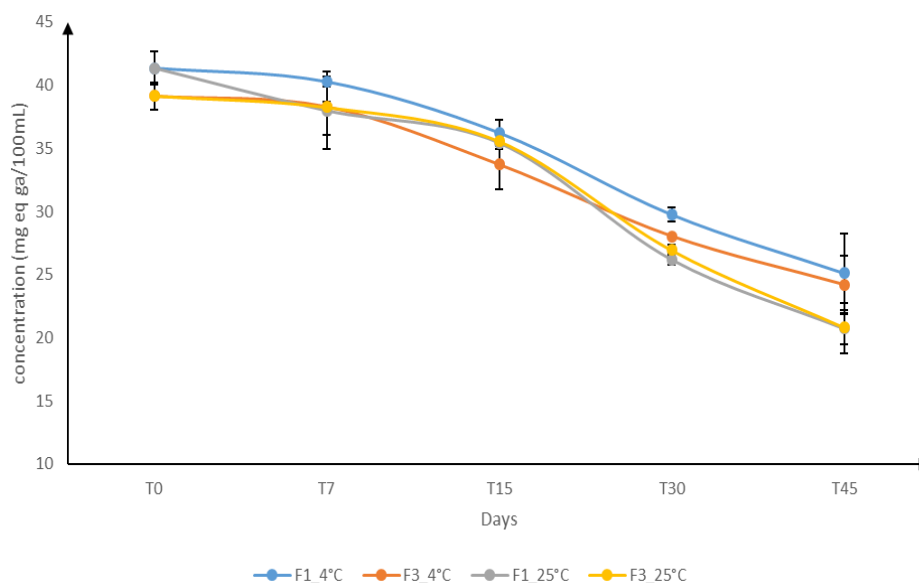


**Figure 11.** Difference of the color between the beginning and the end of the storage period at 4 °C (a) T=0 (b) T=45.

### 8.3.3 Phenolic compounds

Results for phenolic compounds over the storage time are shown in **Figure 12**. As expected, both F1 and F3 stored at 4 °C preserved better the phenolic content compared to those stored at 25 °C. Notably, both fractions contain nearly identical amounts of phenolic compounds by the end of the period of 45 days in both temperatures.

Therefore, the pronounced influence of storage temperature highlights the importance of maintaining a cooler storage environment to effectively preserve the total phenolic compounds and the associated beneficial qualities.



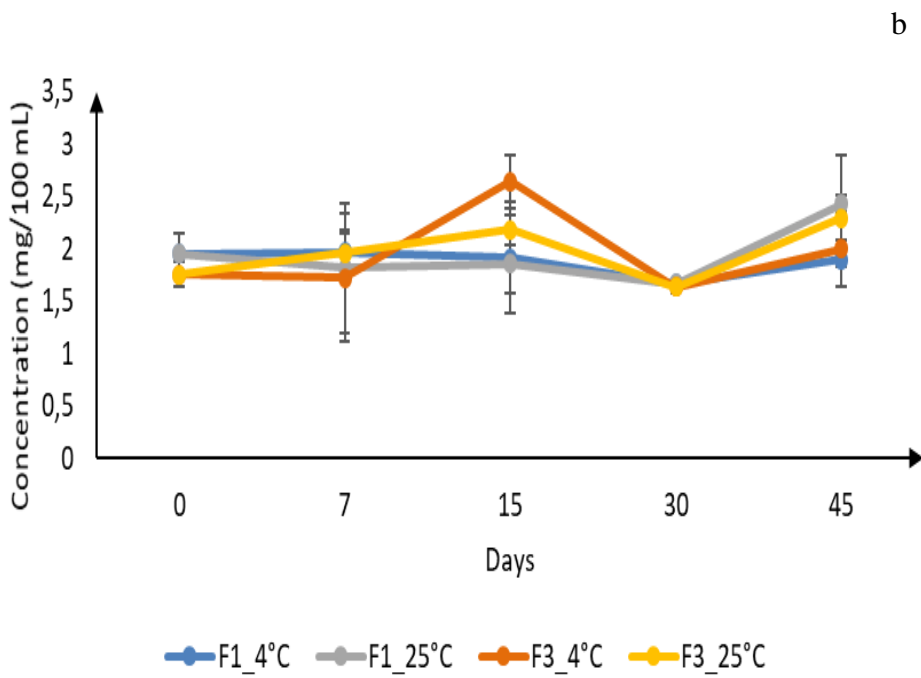
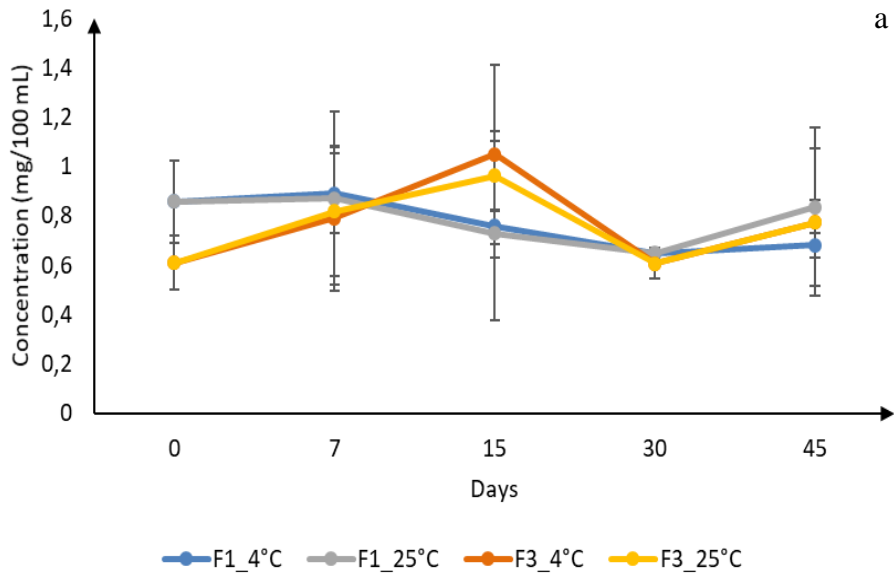
**Figure 12.** Total phenolic compounds in formulations stored at 4 °C and 25 °C during 45 days.

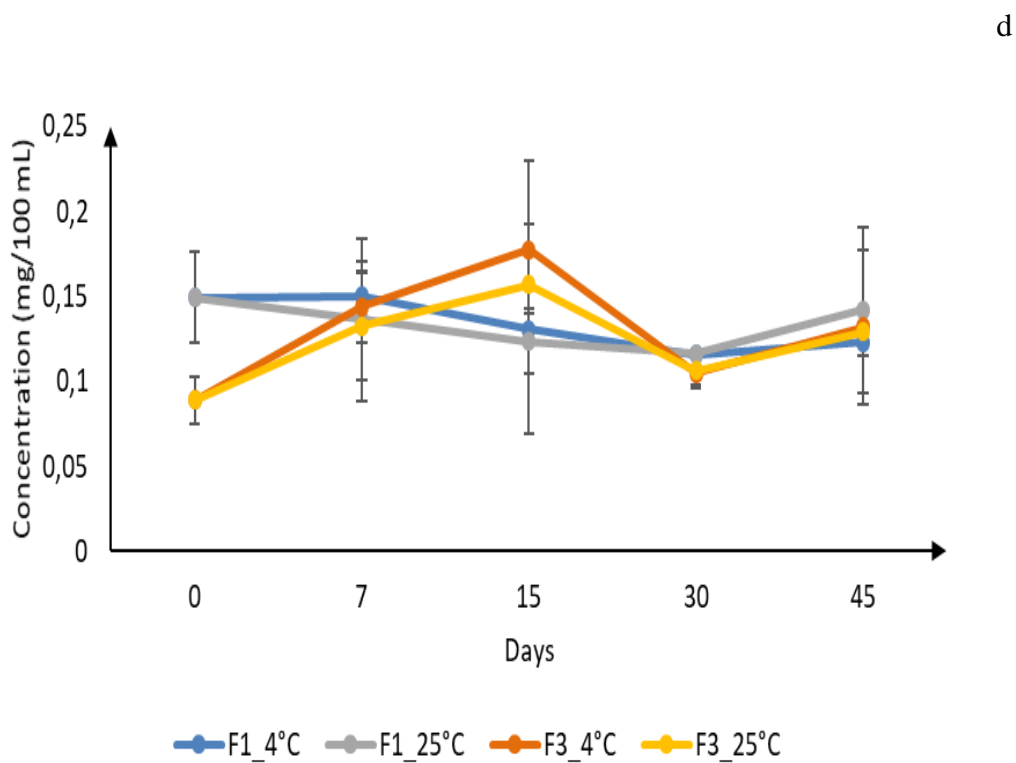
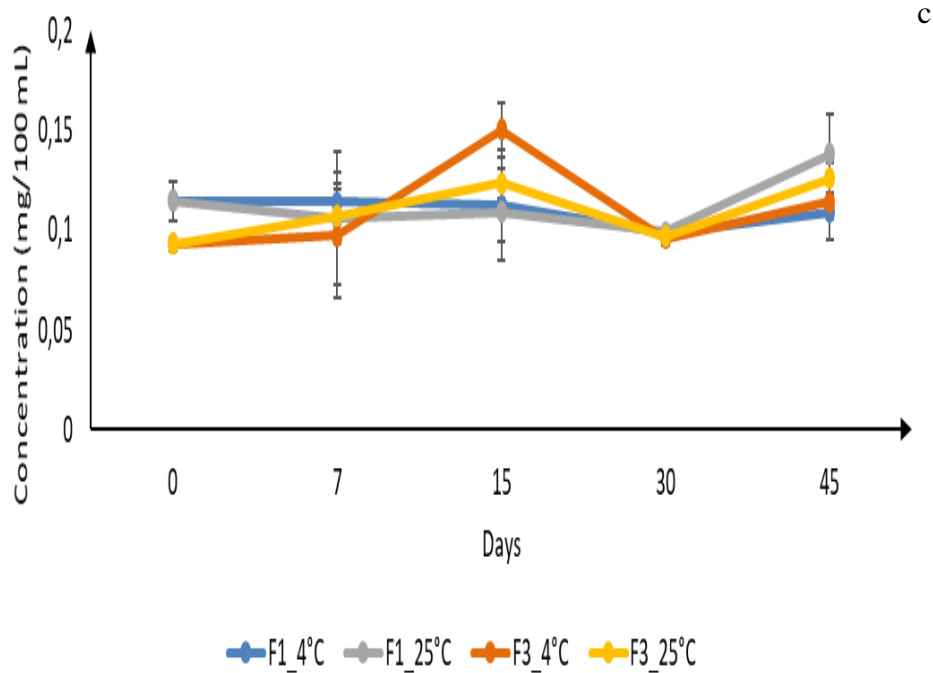
Individual phenolic compounds had a different trend from anthocyanins, as shown in **Figure 13**. In general, they remained stable in all formulations by the end of the studied period ( $p > 0.05$ ), in both temperatures and formulations, regardless the natural variations due to the analysis.

The fact that individual phenolic compounds remained stable in all formulations, by the end of the studied period, regardless of temperature, highlights an interesting contrast with the behavior of anthocyanins. It is important to note that anthocyanins are a subset of phenolic compounds, and their decline in total phenolic content can be attributed to their specific characteristics and sensitivity to factors like temperature and chemical reactions as mentioned in Khoo et al. (2017) study.

This differential stability suggests that while the overall phenolic content remained constant, the decrease observed in total phenolic compounds could be attributed to the decline in anthocyanin levels. Therefore, phenolic contents determined by the Folin–Ciocalteu reagent assay displayed substantially higher concentrations than those determined by HPLC methods due to an overestimation of the real phenolic content, because the reagent reacts not only with phenolics but also with different non-phenolic reducing compounds including tertiary aliphatic amines, tertiary amine-containing biological buffers, amino acids (tryptophan), hydroxylamine, hydrazine, certain purines,

and other organic and inorganic reducing agents (Ikawa et al., 2003). Several studies had similar results, including Gironés-Vilaplana et al., (2014) which designs a new isotonic drink with blackthorn and lemon juice.





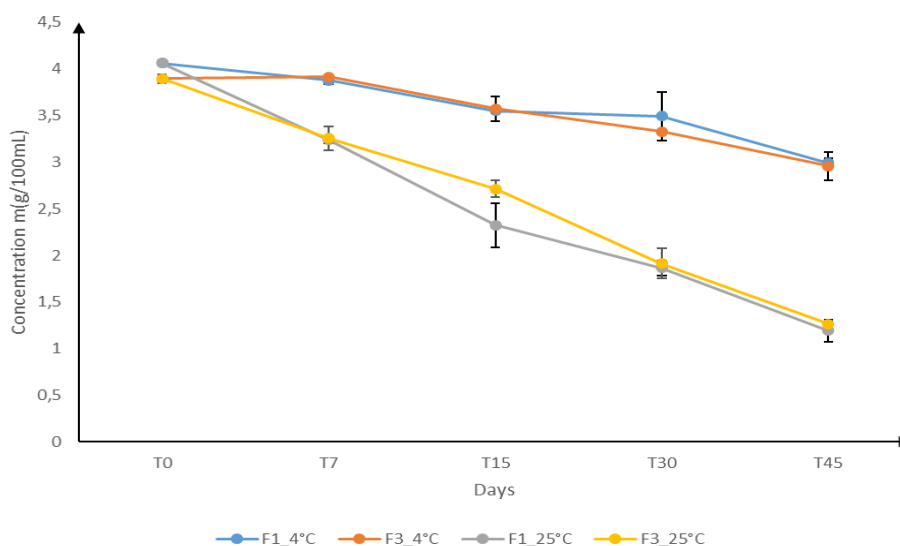
**Figure 13.** Individual phenolics compounds (a) 4-Caffeoylquinic acid (b) 3-caffeoylquinic acid (c) 3-*p*-coumaroylquinic acid (d) 4-*p*-coumaroylquinic acid.

### 8.3.4 Anthocyanin compounds

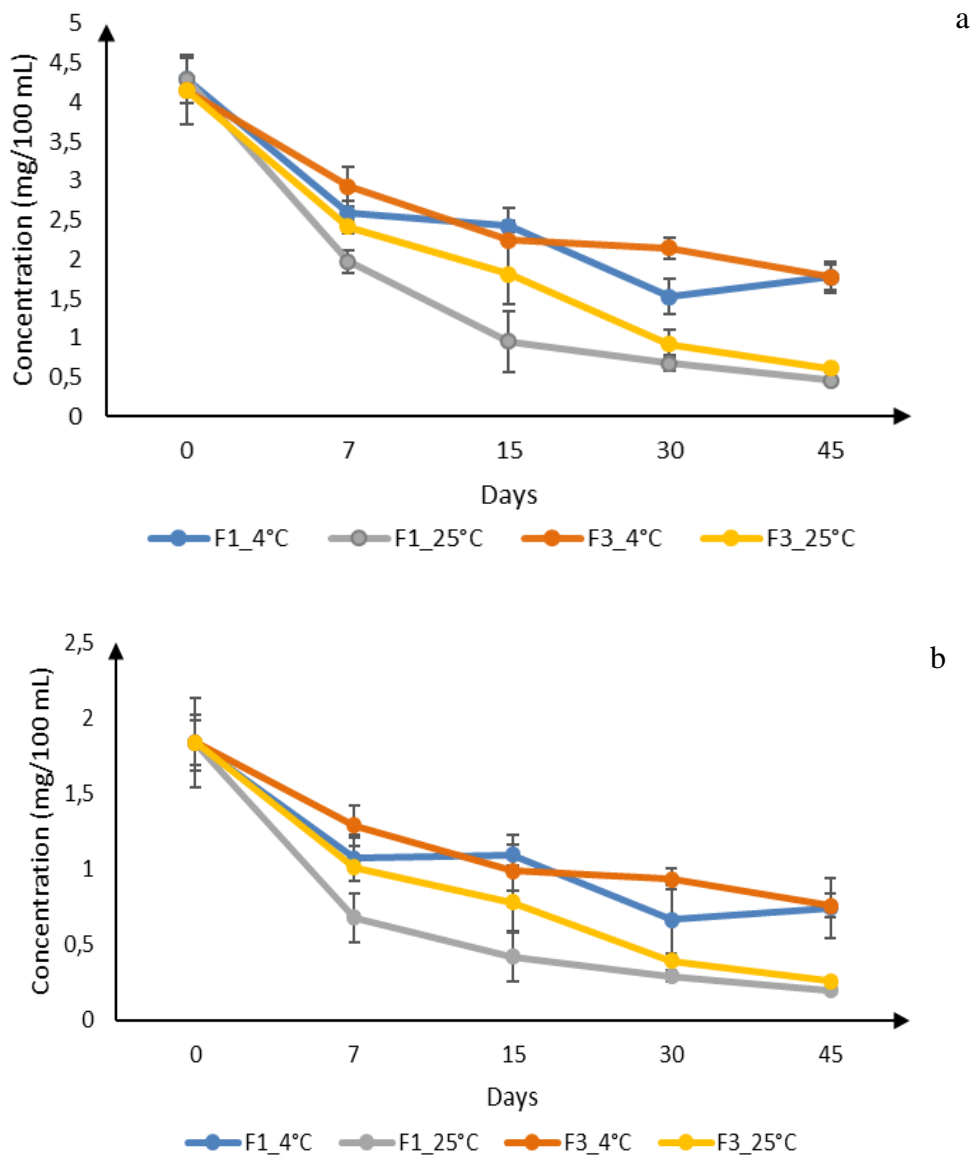
Regarding anthocyanins, the stability of these compounds is influenced during storage by multiple factors including chemical structure, pH, temperature, enzymes, oxygen, light, ascorbic acid, sugars, metals, and pigments (Khoo et al. 2017). In all the tested conditions of formulation and storage temperature occurred a reduction in the total content of anthocyanins (**Figure 14**). Similarly, **Figure 15** shows the evolution of delphinidin 3-*O*-sambubioside and cyanidin 3-*O*-sambubioside during the storage period. In both temperatures and formulations, there was a sharp decrease in these compounds even after 7 days of storage ( $p < 0.05$ ).

As expected, F1 and F3 stored at 4°C preserved better the anthocyanin content (total and individual) compared to those stored at 25°C, retaining more than 70% of the initial total anthocyanin content and 40% when considering these compounds individually at the end of the study period (**Figures 14 and 15**).

Research such as that conducted by (Gironés-Vilaplana et al., 2016), which produced an isotonic drink made of lemon and maqui berry, demonstrated that the anthocyanins of the isotonic beverages stored at 4°C preserved better the anthocyanin content compared to those stored at 25°C. These results also cross with the study of Ndong et al., (2018), which showed how individual anthocyanin compounds from hibiscus can extend the shelf-life at a cooling temperature (4°C) without serious changes.



**Figure 14.** Difference of total Anthocyanin compounds between 4°C and 25°C.



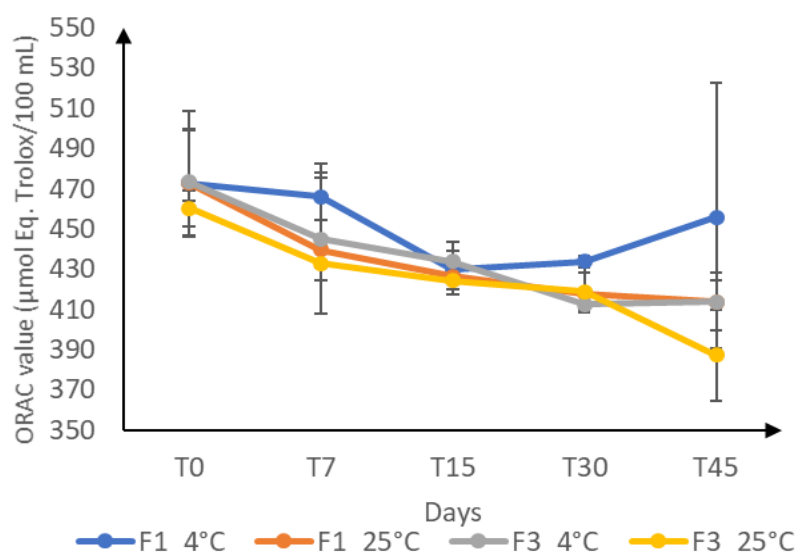
**Figure 15.** Individual anthocyanin compounds (a) Delphinidin 3 *O*-sambubioside (b) Cyanidin 3-*O*-sambubioside.

Therefore, the substantial impact of storage temperature highlights the critical importance of maintaining a cooler storage environment, such as 4°C, to effectively preserve the total anthocyanin compounds and their associated beneficial properties in these samples.

Despite this significant decrease in total and individual anthocyanin contents,  $L^*a^*b^*$  results suggest that the color was still attractive in formulations stored at 4°C during the studied period.

### 8.3.5 Antioxidant activity

**Figure 16** shows the evolution of the antioxidant activity as measured by the ORAC assay.



**Figure 16.** Antioxidant activity (ORAC) of formulations at 4°C and 25°C during 45 days.

According to the results, the antioxidant activity remained stable during the storage time ( $p>0.05$ ) for all the formulations and temperatures, except for F3 at 25°C, regardless the natural variations. This is somewhat unexpected, as anthocyanins sharply reduced after 45 days. Possibly, the fact that the major non-anthocyanin phenolic compounds did not suffer a significant decrease (**Figure 14**) could have contributed to maintaining the ORAC values stable, as they have been positively correlated in previous studies. 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

major 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 (Kruk et al., 2022). For this reason, antioxidant supplementation as well as consumption of sports food rich in antioxidants have been widely spread among sports people (Kruk et al., 2022, Cui et al., 2022). This raises the necessity of constant innovation in this sector, with the development of novel formulations that meet the current demands of sports people. In this context, the present isotonic drink shows promising results, but importantly, ORAC values provide only preliminary results of the antioxidant potential of a food or beverage sample.

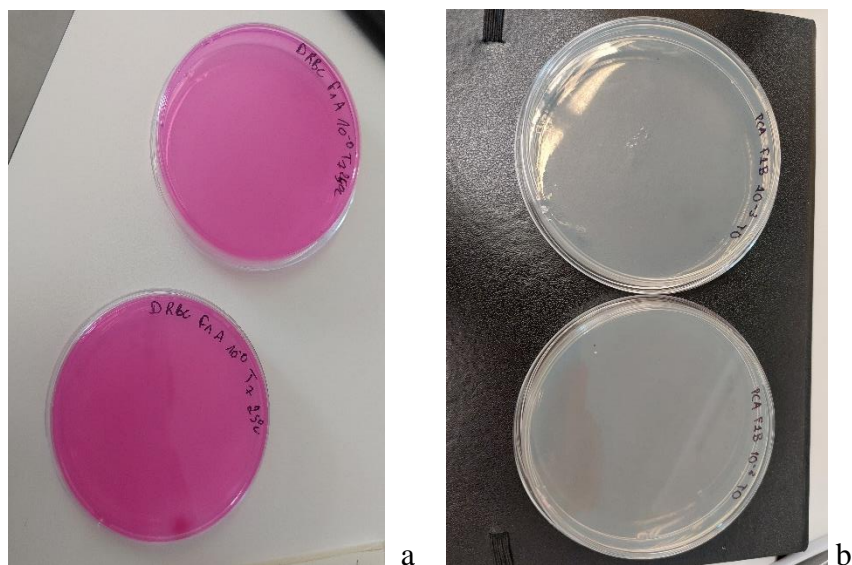
While the cell-based assay tested (CAA) resulted in no activity, further studies with the present isotonic formulation are of paramount importance to validate the observed potential antioxidant activity.

### **8.3.6 Microbiology analysis**

The isotonic drinks did not show any microbiological growth during shelf lifetime at the different temperatures tested (4 °C) and (25 °C), indicating that the pasteurization process, conducted at 85 °C for a duration of 30 seconds, effectively prevented microbial development in the beverages (**Figure 17**). The successful pasteurization of the isotonic drink is evident from the absence of the microorganisms in PCA and DRBC tests, signifying effective microbial control. This outcome not only ensures the safety of the beverage but also preserves its quality, as pasteurization is known for maintaining taste and nutritional attributes.

In addition to pasteurization, the absence of growth is due to the absence of native microflora adapted to the characteristics and conditions of the product (i.e. the acidic pH, and nutrients). 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 pathogenic bacteria, including *Clostridium botulinum*, since the minimum pH for the multiplication of strains varies between 4.6 and 4.8 (Santos, Alves, and Lima,

2013). That makes the product stable during the storage period even under abusive temperature conditions (25 °C).



**Figure 17.** The culture medium used (a) DRBC, (b) PCA.

## 9. Conclusions

The new isotonic drink was designed with thermal waters, hibiscus extract, and apple juice evaluated for their quality parameters, sensorial attributes, and biological characteristics over 45 days of shelf-life under 2 storage temperatures 4 °C and 25 °C. The results indicate that the formulation meets the essential criteria for maintaining electrolyte balance, energy, and antioxidants, and providing the expected sensory experience in isotonic drinks. Notably, these parameters are better preserved when stored at 4 °C with no microbial load during the storage period, emphasizing the importance of cooler temperatures in maintaining the drink's quality.

The hibiscus extract not only supplied antioxidant compounds but also added an appealing color to the product due to its anthocyanin content, eliminating the need for synthetic colorants.

The use of apple juice gave to the beverage, next to the desired amount of the carbohydrate, a special taste. It is a natural source of sugars, which leads to the avoidance of adding sweeteners.

The incorporation of thermal water as a replacement for the conventional water source does not have a significant impact on the characteristics of the isotonic drink, as was shown by studying formulations with and without thermal water. However, it played a crucial role in achieving the desired sodium content, which is essential for an isotonic drink's functionality. This highlights the potential of thermal water as a viable and effective ingredient for enhancing the drink's properties, particularly its electrolyte balance, without significantly altering its other qualities. Moreover, the unflavored isotonic drink studied (F2) complemented the evaluation of the feasibility of thermal water in producing isotonic drinks with different characteristics.

Overall, the standard formulation of the isotonic drink adheres to the anticipated attributes of isotonic drinks, contributing to their potential acceptance in the market.

## 10. Bibliography

- “Annual Report 2011 | EFSA.” n.d. Accessed November 20, 2022. <https://www.efsa.europa.eu/en/corporate/pub/ar11>.
- “Caldas de Chaves.” 2022. In Wikipédia, a enciclopédia livre. [https://pt.wikipedia.org/w/index.php?title=Caldas\\_de\\_Chaves&oldid=64261038](https://pt.wikipedia.org/w/index.php?title=Caldas_de_Chaves&oldid=64261038). Accessed November 5, 2022.
- “Eau Thermale : Minéralité et Autres Composants | Elsevier Enhanced Reader.” n.d. Accessed November 5, 2022. [https://doi.org/10.1016/S0151-9638\(20\)30031-4](https://doi.org/10.1016/S0151-9638(20)30031-4).
- “National Academy of Medicine.” 2023. In *Wikipedia*. [https://en.wikipedia.org/w/index.php?title=National\\_Academy\\_of\\_Medicine&oldid=1168522939](https://en.wikipedia.org/w/index.php?title=National_Academy_of_Medicine&oldid=1168522939).
- Aires-Barros, Luís, José Manuel Marques, Rui Cores Graça, Maria Jose Matias, Cornelis H. Van Der Weijden, Rob Kreulen, and Hermanus Gerardus M. Eggenkamp. 1998. “Hot and Cold CO<sub>2</sub>-Rich Mineral Waters in Chaves Geothermal Area (Northern Portugal).” *Geothermics* 27 (1): 89–107. [https://doi.org/10.1016/S0375-6505\(97\)84483-5](https://doi.org/10.1016/S0375-6505(97)84483-5).
- Akhtar, Naseem, Muhammad Izzuddin Syakir Ishak, Showkat Ahmad Bhawani, and Khalid Umar. 2021. “Various Natural and Anthropogenic Factors Responsible for Water Quality Degradation: A Review.” *Water* 13 (19): 2660. <https://doi.org/10.3390/w13192660>.
- Almeida, Cristina, Ana Madeira, Joana Marto, Angélica Graça, Pedro Pinto, and Helena Ribeiro. 2019. “Monfortinho Thermal Water-Based Creams: Effects on Skin Hydration, Psoriasis, and Eczema in Adults.” *Cosmetics* 6 (3): 56. <https://doi.org/10.3390/cosmetics6030056>.
- Ang, HH and Lee, KL. 2005. Analysis of mercury in Malaysian herbal preparations. *Journal of Biomedical Sciences* 4 (1): 31-36.
- Araujo, A. R. T. S., M. C. Sarraguça, M. P. Ribeiro, and P. Coutinho. 2017. “Physicochemical Fingerprinting of Thermal Waters of Beira Interior Region of Portugal.” *Environmental Geochemistry and Health* 39 (3): 483–96. <https://doi.org/10.1007/s10653-016-9829-x>.
- Avesso, Interior do. 2020. “Radiografias Concelhias: Nelas.” Interior do Avesso. September 18, 2020. <https://interiordoavesso.pt/interior-do-avesso/radiografias-concelhias-nelas/>.
- Bendaali, Yasmina, Cristian Vaquero, Carlos Escott, Carmen González, and Antonio Morata. 2023. “Isotonic Drinks Based on Organic Grape Juice and Naturally Flavored with Herb and Spice Extracts.” *Beverages* 9 (2): 49. <https://doi.org/10.3390/beverages9020049>.
- Bendaali, Yasmina, Cristian Vaquero, Carmen González, and Antonio Morata. 2022.

- “Contribution of Grape Juice to Develop New Isotonic Drinks With Antioxidant Capacity and Interesting Sensory Properties.” *Frontiers in Nutrition* 9. <https://www.frontiersin.org/articles/10.3389/fnut.2022.890640>.
- Bessada, Sílvia M. F., João C. M. Barreira, Lillian Barros, Isabel C. F. R. Ferreira, and M. Beatriz P. P. Oliveira. 2016. “Phenolic Profile and Antioxidant Activity of *Coleostephus Myconis* (L.) Rchb.f.: An Underexploited and Highly Disseminated Species.” *Industrial Crops and Products* 89 (October): 45–51. <https://doi.org/10.1016/j.indcrop.2016.04.065>.
- Bondonno, Nicola P., Catherine P. Bondonno, Natalie C. Ward, Jonathan M. Hodgson, and Kevin D. Croft. 2017. “The Cardiovascular Health Benefits of Apples: Whole Fruit vs. Isolated Compounds.” *Trends in Food Science & Technology, Food Bioactives: From evidence of health benefits to understanding mechanisms*, 69 (November): 243–56. <https://doi.org/10.1016/j.tifs.2017.04.012>.
- Borrás-Linares, I., S. Fernández-Arroyo, D. Arráez-Roman, P. A. Palmeros-Suárez, R. Del Val-Díaz, I. Andrade-González, A. Fernández-Gutiérrez, J. F. Gómez-Leyva, and A. Segura-Carretero. 2015. “Characterization of Phenolic Compounds, Anthocyanidin, Antioxidant and Antimicrobial Activity of 25 Varieties of Mexican Roselle (*Hibiscus Sabdariffa*).” *Industrial Crops and Products* 69 (July): 385–94. <https://doi.org/10.1016/j.indcrop.2015.02.053>.
- Cacciapuoti, Sara, Maria A. Luciano, Matteo Megna, Maria C. Annunziata, Maddalena Napolitano, Cataldo Patruno, Emanuele Scala, et al. 2020. “The Role of Thermal Water in Chronic Skin Diseases Management: A Review of the Literature.” *Journal of Clinical Medicine* 9 (9): 3047. <https://doi.org/10.3390/jcm9093047>.
- Carreño, Ignacio, and Tobias Dolle. 2016. “Food Intended for Sportspeople: The EU’s Regulatory Framework after 20 July 2016.” *European Journal of Risk Regulation* 7 (1): 191–96.
- Chaves, Diário de. 2021. “GALERIA | Abriu hoje ao público o maior Balneário Termal Romano da Península Ibérica.” *Diário de Chaves*. December 21, 2021. <https://www.diariodechaves.pt/album/chaves/album/20211221124011001497.html>.
- Chiu, Hui-Fang, Yi-Ru Liao, You-Cheng Shen, Yi-Chin Han, Oksana Golovinskaia, Kamesh Venkatakrisnan, Chi-Chang Hung, and Chin-Kun Wang. 2022. “Improvement on Blood Pressure and Skin Using Roselle Drink: A Clinical Trial.” *Journal of Food Biochemistry* 46 (10): e14287. <https://doi.org/10.1111/jfbc.14287>.

- Cissé, Mady, Philippe Bohuon, Falillou Sambe, Cheikhou Kane, Mama Sakho, and Manuel Dornier. 2012. "Aqueous Extraction of Anthocyanins from Hibiscus Sabdariffa: Experimental Kinetics and Modeling." *Journal of Food Engineering* 109 (1): 16–21. <https://doi.org/10.1016/j.jfoodeng.2011.10.012>.
- Cj, Etheridge, and Derbyshire Ej. 2020. "Hibiscus Tea and Health: A Scoping Review of Scientific Evidence." *Nutrition and Food Technology: Open Access* 6 (1). <https://doi.org/10.16966/2470-6086.167>.
- Dávalos, Alberto, Carmen Gómez-Cordovés, and Begoña Bartolomé. 2004. "Extending Applicability of the Oxygen Radical Absorbance Capacity (ORAC-Fluorescein) Assay." *Journal of Agricultural and Food Chemistry* 52 (1): 48–54. <https://doi.org/10.1021/jf0305231>.
- Edo, Great Iruoghene, Princess Oghenekeno Samuel, Agatha Ngukuran Jikah, Gift Onyinyechi Oloni, Miracle Nkonyeasuir Ifejika, Oghenechuko Oghenegueke, Samuel Ossai, et al. 2023. "Proximate Composition and Health Benefit of Roselle Leaf (Hibiscus Sabdariffa). Insight on Food and Health Benefits." *Food Chemistry Advances* 3 (December): 100437. <https://doi.org/10.1016/j.focha.2023.100437>.
- Etheridge, Christopher, and Derbyshire EJ. 2020. "Hibiscus Tea and Health: A Scoping Review of Scientific Evidence." *Nutrition and Food Technology: Open Access* 6 (January). <https://doi.org/10.16966/2470-6086.167>.
- Fanali, Chiara, Susanna Della Posta, Alessandra Vilmercati, Laura Dugo, Marina Russo, Tommasangelo Petitti, Luigi Mondello, and Laura de Gara. 2018. "Extraction, Analysis, and Antioxidant Activity Evaluation of Phenolic Compounds in Different Italian Extra-Virgin Olive Oils." *Molecules* (Basel, Switzerland) 23 (12): 3249. <https://doi.org/10.3390/molecules23123249>.
- Ferreira, Fernanda Maria Costa. 2023. "O potencial da água mineral natural termal e engarrafada no desenvolvimento de produtos de panificação de valor acrescentado." masterThesis. <https://repositorio.ucp.pt/handle/10400.14/40726>.
- Gironés-Vilaplana, Amadeo, Pedro Mena, Diego A Moreno, and Cristina García-Viguera. 2014. "Evaluation of Sensorial, Phytochemical and Biological Properties of New Isotonic Beverages Enriched with Lemon and Berries during Shelf Life." *Journal of the Science of Food and Agriculture* 94 (6): 1090–1100. <https://doi.org/10.1002/jsfa.6370>.
- Giusti, M.Mónica, and Ronald E. Wrolstad. 2001. "Characterization and Measurement of

- Anthocyanins by UV-Visible Spectroscopy.” *Current Protocols in Food Analytical Chemistry* 00 (1): F1.2.1-F1.2.13. <https://doi.org/10.1002/0471142913.faf0102s00>.
- Gonçalves, Ana C., Dário Gaspar, José David Flores-Félix, Amílcar Falcão, Gilberto Alves, and Luís R. Silva. 2022. “Effects of Functional Phenolics Dietary Supplementation on Athletes’ Performance and Recovery: A Review.” *International Journal of Molecular Sciences* 23 (9): 4652. <https://doi.org/10.3390/ijms23094652>.
- Grajeda-Iglesias, Claudia, Erika Salas, Nathalie Barouh, Bruno Baréa, Atikorn Panya, and Maria Cruz Figueroa-Espinoza. 2016. “Antioxidant Activity of Protocatechuates Evaluated by DPPH, ORAC, and CAT Methods.” *Food Chemistry* 194 (March): 749–57. <https://doi.org/10.1016/j.foodchem.2015.07.119>.
- Hakeem, Israa J., and Gashaw Tadele Zewudie. 2022. “Antioxidant Effect and Acute Oral Toxicity of Hot Springs.” *Computational Intelligence and Neuroscience* 2022 (September): 4200824. <https://doi.org/10.1155/2022/4200824>.
- Ikawa, Miyoshi, Timothy D. Schaper, Catherine A. Dollard, and John J. Sasner. 2003. “Utilization of Folin-Ciocalteu Phenol Reagent for the Detection of Certain Nitrogen Compounds.” *Journal of Agricultural and Food Chemistry* 51 (7): 1811–15. <https://doi.org/10.1021/jf021099r>.
- Izquierdo-Vega, Jeannett A., Diego A. Arteaga-Badillo, Manuel Sánchez-Gutiérrez, José A. Morales-González, Nancy Vargas-Mendoza, Carlos A. Gómez-Aldapa, Javier Castro-Rosas, Luis Delgado-Olivares, Eduardo Madrigal-Bujaidar, and Eduardo Madrigal-Santillán. 2020. “Organic Acids from Roselle (*Hibiscus Sabdariffa* L.)—A Brief Review of Its Pharmacological Effects.” *Biomedicines* 8 (5): 100. <https://doi.org/10.3390/biomedicines8050100>.
- Jabeur, Inès, Eliana Pereira, Lillian Barros, Ricardo C. Calhelha, Marina Soković, M. Beatriz P. Oliveira, and Isabel C. F. R. Ferreira. 2017. “*Hibiscus Sabdariffa* L. as a Source of Nutrients, Bioactive Compounds and Colouring Agents.” *Food Research International* (Ottawa, Ont.) 100 (Pt 1): 717–23. <https://doi.org/10.1016/j.foodres.2017.07.073>.
- Jalalyazdi, Majid, Javad Ramezani, Azadeh Izadi-Moud, Fereshteh Madani-Sani, Shokufeh Shahlaei, and Shirin Sadat Ghiasi. 2019. “Effect of *Hibiscus Sabdariffa* on Blood Pressure in Patients with Stage 1 Hypertension.” *Journal of Advanced Pharmaceutical Technology & Research* 10 (3): 107–11. [https://doi.org/10.4103/japtr.JAPTR\\_402\\_18](https://doi.org/10.4103/japtr.JAPTR_402_18).
- Jawadi, Hussain Ali, Hasan Ali Malistani, Mohammad Anvar Moheghy, and Jay Sagin. 2021.

- “Essential Trace Elements and Arsenic in Thermal Springs, Afghanistan.” *Water* 13 (2): 134. <https://doi.org/10.3390/w13020134>.
- Judge, Lawrence W., David M. Bellar, Jennifer K. Popp, Bruce W. Craig, Makenzie A. Schoeff, Donald L. Hoover, Brian Fox, Brandon M. Kistler, and Ali M. Al-Nawaiseh. 2021. “Hydration to Maximize Performance and Recovery: Knowledge, Attitudes, and Behaviors Among Collegiate Track and Field Throwers.” *Journal of Human Kinetics* 79 (July): 111–22. <https://doi.org/10.2478/hukin-2021-0065>.
- Jungbluth, Susanne. 2021. “Healing Springs in Karlovy Vary.” *FromPlaceToPlace.Travel* (blog). January 17, 2021. <https://fromplacetoplace.travel/czech-republic/karlovy-vary/karlovy-vary-drinking-cure/>.
- Kahle, Kathrin, Michael Kraus, and Elke Richling. 2005. “Polyphenol Profiles of Apple Juices.” *Molecular Nutrition & Food Research* 49 (8): 797–806. <https://doi.org/10.1002/mnfr.200500064>.
- Karadeniz, Feryal, and Aziz Ekşi. 2002. “Sugar Composition of Apple Juices.” *European Food Research and Technology* 215 (August): 145–48. <https://doi.org/10.1007/s00217-002-0505-2>.
- Khoo, Hock Eng, Azrina Azlan, Sou Teng Tang, and See Meng Lim. 2017. “Anthocyanidins and Anthocyanins: Colored Pigments as Food, Pharmaceutical Ingredients, and the Potential Health Benefits.” *Food & Nutrition Research* 61 (1): 1361779. <https://doi.org/10.1080/16546628.2017.1361779>.
- Kruk, Joanna, Basil Hassan Aboul-Enein, Ewa Duchnik, and Mariola Marchlewicz. 2022. “Antioxidative Properties of Phenolic Compounds and Their Effect on Oxidative Stress Induced by Severe Physical Exercise.” *The Journal of Physiological Sciences: JPS* 72 (1): 19. <https://doi.org/10.1186/s12576-022-00845-1>.
- Lakhdari, Fatna, and Kawther Bouaicha. 2016. “Diagnostic de la qualité des eaux de source et thermales De la Wilaya de Saida-Algérie- Effets thérapeutiques.” <http://e-biblio.univ-mosta.dz/handle/123456789/2310>.
- Leichtweis, Maria G., Carla Pereira, M.A. Prieto, Maria Filomena Barreiro, Ilton José Baraldi, Lillian Barros, and Isabel C.F.R. Ferreira. 2019. “Ultrasound as a Rapid and Low-Cost Extraction Procedure to Obtain Anthocyanin-Based Colorants from *Prunus Spinosa* L. Fruit Epicarp: Comparative Study with Conventional Heat-Based Extraction.” *Molecules* 24 (3): 573. <https://doi.org/10.3390/molecules24030573>.

- M'be, Cho Urielle, Joël Scher, Claire Gaiani, N'Guessan Georges Amani, and Jennifer Burgain. 2023. "Impact of Processing and Physicochemical Parameter on Hibiscus Sabdariffa Calyxes Biomolecules and Antioxidant Activity: From Powder Production to Reconstitution." *Foods* 12 (16): 2984. <https://doi.org/10.3390/foods12162984>.
- Maciel, Laércio Galvão, Mariana Araújo Vieira do Carmo, Luciana Azevedo, Heitor Daguer, Luciano Molognoni, Mereci Mendes de Almeida, Daniel Granato, and Neiva Deliberali Rosso. 2018. "Hibiscus Sabdariffa Anthocyanins-Rich Extract: Chemical Stability, in Vitro Antioxidant and Antiproliferative Activities." *Food and Chemical Toxicology* 113 (March): 187–97. <https://doi.org/10.1016/j.fct.2018.01.053>.
- Mahunu, Gustav, Haroon Elrasheid Tahir, Mildred Osei-Kwarteng, Abdalbasit Mariod, and Joseph Gweyi. 2021. "Food Use of Whole and Extracts of Hibiscus Sabdariffa." In , 123–36. <https://doi.org/10.1016/B978-0-12-822100-6.00009-4>.
- Malaguti, Marco, Cristina Angeloni, and Silvana Hrelia. 2013. "Polyphenols in Exercise Performance and Prevention of Exercise-Induced Muscle Damage." *Oxidative Medicine and Cellular Longevity* 2013: 825928. <https://doi.org/10.1155/2013/825928>.
- Marques, José M., and Paula M. Carreira. 2017. "Questions and Answers Ascribed to Chaves CO<sub>2</sub>-Rich Thermal Waters Conceptual Model (N Portugal): A Review." *Procedia Earth and Planetary Science, 15th Water-Rock Interaction International Symposium, WRI-15, 17 (January)*: 654–57. <https://doi.org/10.1016/j.proeps.2016.12.175>.
- Mekebret, Imane. 2017. "Synthèse hydrogéologique et origine de la salinité des eaux thermo-minérales du littoral oranais par les méthodes géochimiques et isotopiques." Thesis, Université d'Oran2 Mohamed ben Ahmed. <https://ds.univ-oran2.dz:8443/jspui/handle/123456789/2130>.
- Morales-Luna, Elizabeth, Iza F. Pérez-Ramírez, Luis M. Salgado, Eduardo Castaño-Tostado, Carlos A. Gómez-Aldapa, and Rosalía Reynoso-Camacho. 2019. "The Main Beneficial Effect of Roselle (Hibiscus Sabdariffa) on Obesity Is Not Only Related to Its Anthocyanin Content." *Journal of the Science of Food and Agriculture* 99 (2): 596–605. <https://doi.org/10.1002/jsfa.9220>.
- Ndong, Moussa, Ndeye Faye, Joseph Bassama, and Mady Cisse. 2018. "Stability of Concentrated Extracts of Hibiscus Sabdariffa L. Calyx during Storage at Different Temperatures" 12 (December). <https://doi.org/10.5897/AJFS2018.1694>.
- Nguyen Ba, C. 2020. "Eau thermale : minéralité et autres composants." *Annales de Dermatologie*

et de Vénérologie, Eaux thermales, thermes et thermalisme, 147 (1, Supplement): 1S14–19. [https://doi.org/10.1016/S0151-9638\(20\)30031-4](https://doi.org/10.1016/S0151-9638(20)30031-4).

- Nyamende, Nandi E., Gunnar O. Sigge, Zinash A. Belay, Rebogile R. Mphahlele, Ayodeji B. Oyenih, Asanda Mditshwa, Zaharan M. Hussein, and Oluwafemi James Caleb. 2022. “Advances in Non-Thermal Technologies for Whole and Minimally Processed Apple Fruit – A Review.” *Food Bioscience* 50 (December): 102170. <https://doi.org/10.1016/j.fbio.2022.102170>.
- Orrù, Stefania, Esther Imperlini, Ersilia Nigro, Andreina Alfieri, Armando Cevenini, Rita Polito, Aurora Daniele, Pasqualina Buono, and Annamaria Mancini. 2018. “Role of Functional Beverages on Sport Performance and Recovery.” *Nutrients* 10 (10): 1470. <https://doi.org/10.3390/nu10101470>.
- Ostrowska, Aneta, Witold Szymański, Łukasz Kołodziejczyk, and Elżbieta Bołtacz-Rzepakowska. 2016. “Evaluation of the Erosive Potential of Selected Isotonic Drinks: In Vitro Studies.” *Advances in Clinical and Experimental Medicine: Official Organ Wrocław Medical University* 25 (6): 1313–19. <https://doi.org/10.17219/acem/62323>.
- Oyenih, Ayodeji B., Zinash A. Belay, Asanda Mditshwa, and Oluwafemi J. Caleb. 2022. “‘An Apple a Day Keeps the Doctor Away’: The Potentials of Apple Bioactive Constituents for Chronic Disease Prevention.” *Journal of Food Science* 87 (6): 2291–2309. <https://doi.org/10.1111/1750-3841.16155>.
- Packer, Lester, Enrique Cadenas, and Kelvin J.A. Davies. 2008. “Free Radicals and Exercise: An Introduction.” *Free Radical Biology and Medicine* 44 (2): 123–25. <https://doi.org/10.1016/j.freeradbiomed.2007.05.031>.
- Pellegrini, Manuela, Davide Fanin, Yohann Nowicki, Gabriella Guarnieri, Anna Bordin, Diego Faggian, Mario Plebani, Marina Saetta, and Piero Maestrelli. 2005. “Effect of Inhalation of Thermal Water on Airway Inflammation in Chronic Obstructive Pulmonary Disease.” *Respiratory Medicine* 99 (6): 748–54. <https://doi.org/10.1016/j.rmed.2004.11.001>.
- Pereira, Sandra, Jani Silva, Catarina Marques, Ana Maria Galvão, and Maria José Alves. 2021. “Thermalism as A Determinant of Quality of Life and Sleep: A Novel Approach to The Sector Using SF-36v2 and Pittsburgh Index.” *Frontiers in Medical Case Reports*. <https://bibliotecadigital.ipb.pt/handle/10198/23917>.
- Pinto, Teresa, Alice Vilela, and Fernanda Cosme. 2022. “Chemical and Sensory Characteristics of Fruit Juice and Fruit Fermented Beverages and Their Consumer Acceptance.”

Beverages 8 (2): 33. <https://doi.org/10.3390/beverages8020033>.

- Piovesana, Alessandra, Eliseu Rodrigues, and Caciano Pelayo Zapata Noreña. 2019. "Composition Analysis of Carotenoids and Phenolic Compounds and Antioxidant Activity from Hibiscus Calyces (*Hibiscus Sabdariffa* L.) by HPLC-DAD-MS/MS." *Phytochemical Analysis: PCA* 30 (2): 208–17. <https://doi.org/10.1002/pca.2806>.
- Porfírio, Márjorie Castro Pinto, Márcia Soares Gonçalves, Marília Viana Borges, Cristina Xavier dos Santos Leite, Mariana Romana Correia Santos, Andréa Gomes da Silva, Gabrielle Cardoso Reis Fontan, et al. 2019. "Development of Isotonic Beverage with Functional Attributes Based on Extract of *Myrciaria Jaboticaba* (Vell) Berg." *Food Science and Technology* 40 (December): 614–20. <https://doi.org/10.1590/fst.14319>.
- Porowski, Adam. 2019. "Mineral and Thermal Waters." In, 149–81. [https://doi.org/10.1007/978-1-4939-8787-0\\_978](https://doi.org/10.1007/978-1-4939-8787-0_978).
- Pruksasri, Suwattana, Bernhard Lanner, and Senad Novalin. 2020. "Nanofiltration as a Potential Process for the Reduction of Sugar in Apple Juices on an Industrial Scale." *LWT* 133 (November): 110118. <https://doi.org/10.1016/j.lwt.2020.110118>.
- Rebelo, Mariana, Eduardo Ferreira da Silva, and Fernando Rocha. 2015. "Characterization of Portuguese Thermo-Mineral Waters to Be Applied in Peloids Maturation." *Environmental Earth Sciences* 73 (6): 2843–62. <https://doi.org/10.1007/s12665-014-3670-2>.
- Rémi Parpette, Parpette-Bénéteau. 2021. "L'eau minérale naturelle de Jonzac : 'source' de multiples applications," September, 121.
- Riebl, Shaun K, and Brenda M. Davy. 2013. "The Hydration Equation: Update on Water Balance and Cognitive Performance." *ACSM's Health & Fitness Journal* 17 (6): 21–28. <https://doi.org/10.1249/FIT.0b013e3182a9570f>.
- Rizkiyah, Dwila Nur, Nicky Rahmana Putra, Zuhaili Idham, Ahmad Hazim Abdul Aziz, Mohd Azizi Che Yunus, Ibhahm Veza, Irianto, Stanley Chinedu Mamah, and Lailatul Qomariyah. 2023. "Recovery of Anthocyanins from *Hibiscus Sabdariffa* L. Using a Combination of Supercritical Carbon Dioxide Extraction and Subcritical Water Extraction." *Processes* 11 (3): 751. <https://doi.org/10.3390/pr11030751>.
- Rowlands, David S., Brigitte Hani Kopetschny, and Claire E. Badenhorst. 2022. "The Hydrating Effects of Hypertonic, Isotonic and Hypotonic Sports Drinks and Waters on Central Hydration During Continuous Exercise: A Systematic Meta-Analysis and Perspective."

- Sports Medicine (Auckland, N.Z.)* 52 (2): 349–75. <https://doi.org/10.1007/s40279-021-01558-y>.
- Ruiz, Yanelis, and Mario García. 2022. “Isotonic Sports Drinks: Formulation and Physiological Effects of Their Consumption.” *QhaliKay Revista de Ciencias de La Salud ISSN 2588-0608* 6 (September): 73–84.
- Rybczyńska-Tkaczyk, Kamila, Anna Grenda, Anna Jakubczyk, Kaja Kiersnowska, and Marta Bik-Małodzińska. 2023. “Natural Compounds with Antimicrobial Properties in Cosmetics.” *Pathogens* 12 (2): 320. <https://doi.org/10.3390/pathogens12020320>.
- Sadowska, Anna, Franciszek Świdorski, and Waclaw Laskowski. 2020. “Osmolality of Components and Their Application in the Design of Functional Recovery Drinks.” *Applied Sciences* 10 (21): 7663. <https://doi.org/10.3390/app10217663>.
- Santos, Eliana de Souza Marques dos, Rômulo Manoel Alves, and Carla de Souza Lima. 2013. “Elaboração tecnológica e aceitação sensorial de bebida isotônica orgânica da tangerina (*Citrus reticulata* Blanco).” *Revista do Instituto Adolfo Lutz* 72 (1): 87–92.
- Sharifi-Rad, Javad, Célia F. Rodrigues, Farukh Sharopov, Anca Oana Docea, Aslı Can Karaca, Mehdi Sharifi-Rad, Derya Kahveci Karıncaoglu, et al. 2020. “Diet, Lifestyle and Cardiovascular Diseases: Linking Pathophysiology to Cardioprotective Effects of Natural Bioactive Compounds.” *International Journal of Environmental Research and Public Health* 17 (7): 2326. <https://doi.org/10.3390/ijerph17072326>.
- Shruthi, V H, and C T Ramachandra. 2019. *Roselle (Hibiscus Sabdariffa L.) Calyces: A Potential Source of Natural Color and Its Health Benefits CHAPTER 8*.
- Silva, A., A. S. Oliveira, C. V. Vaz, S. Correia, R. Ferreira, L. Breitenfeld, J. Martinez-de-Oliveira, et al. 2020. “Anti-Inflammatory Potential of Portuguese Thermal Waters.” *Scientific Reports* 10 (1): 22313. <https://doi.org/10.1038/s41598-020-79394-9>.
- Simioni, Carolina, Giorgio Zauli, Alberto M. Martelli, Marco Vitale, Gianni Sacchetti, Arianna Gonelli, and Luca M. Neri. 2018. “Oxidative Stress: Role of Physical Exercise and Antioxidant Nutraceuticals in Adulthood and Aging.” *Oncotarget* 9 (24): 17181–98. <https://doi.org/10.18632/oncotarget.24729>.
- Singleton, Vernon L., Rudolf Orthofer, and Rosa M. Lamuela-Raventós. 1999. “[14] Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent.” In *Methods in Enzymology*, 299:152–78. Oxidants and Antioxidants Part A. Academic Press. [https://doi.org/10.1016/S0076-6879\(99\)99017-1](https://doi.org/10.1016/S0076-6879(99)99017-1).

- Smith, Sindy. 2019. "All About Chaves: Your Peace And Tranquility Haven." bePortugal (blog). August 27, 2019. <https://www.beportugal.com/chaves/>.
- Sobral, Filipa, Andreia Sampaio, Soraia Falcão, Maria João R. P. Queiroz, Ricardo C. Calhella, Miguel Vilas-Boas, and Isabel C. F. R. Ferreira. 2016. "Chemical Characterization, Antioxidant, Anti-Inflammatory and Cytotoxic Properties of Bee Venom Collected in Northeast Portugal." *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association* 94 (August): 172–77. <https://doi.org/10.1016/j.fct.2016.06.008>.
- Source, La. 2020. "L'eau thermale, pourquoi?" La Source (blog). September 5, 2020. <https://www.la-source-bio.fr/leau-thermale-pourquoi/>.
- Standards, European. 2003. "BS ISO 15213:2003 Microbiology of Food and Animal Feeding Stuff. Horizontal Method for the Enumeration of Sulfite-Reducing Bacteria Growing under Anaerobic Conditions." <https://www.en-standard.eu>. 2003. <https://www.en-standard.eu/bs-iso-15213-2003-microbiology-of-food-and-animal-feeding-stuffs-horizontal-method-for-the-enumeration-of-sulfite-reducing-bacteria-growing-under-anaerobic-conditions/>.
- Świtalski, Michał, and Agnieszka Rybowska. 2021. "Product Innovation in Isotonic Drinks – Expectations of Tri-City University Students." *Scientific Journal of Gdynia Maritime University*, no. 118/2021 (June): 62–74. <https://doi.org/10.26408/118.05>.
- Team, Auteur La Biotyfull. 2020. "Eau Thermale : Tout savoir sur les bienfaits de l'eau thermale." *Blog Beauté Bio : Notre Top des Astuces Beauté Naturelles !* (blog). May 12, 2020. <https://www.biotyfullbox.fr/cosmetique-bio/soins-visage/conseils-beaute-visage/bienfaits-eau-thermale/>.
- ThriveAdmin. 2017. "HIBISCUS BENEFITS: The Flower Plant That Can Cure Your Hair and Skin Woes." *Simply Organics* (blog). March 2, 2017. <https://www.simplyorganicbeauty.com/hibiscus-benefits-for-hair-and-skin/>.
- Tobias, Abraham, Brian D. Ballard, and Shamim S. Mohiuddin. 2022. "Physiology, Water Balance." In *StatPearls*. Treasure Island (FL): StatPearls Publishing. <http://www.ncbi.nlm.nih.gov/books/NBK541059/>.
- Torres, M. D., P. Fradinho, A. Raymundo, I. Sousa, E. Falqué, and H. Domínguez. 2021. "The Key Role of Thermal Waters in the Development of Innovative Gelled Starch-Based Matrices." *Food Hydrocolloids* 117 (August): 106697.

<https://doi.org/10.1016/j.foodhyd.2021.106697>.

- Tung, Yu-Tang, Ming-Fang Wu, Mon-Chien Lee, Jyh-Horng Wu, Chi-Chang Huang, and Wen-Ching Huang. 2019. “Antifatigue Activity and Exercise Performance of Phenolic-Rich Extracts from *Calendula Officinalis*, *Ribes Nigrum*, and *Vaccinium Myrtillus*.” *Nutrients* 11 (8): 1715. <https://doi.org/10.3390/nu11081715>.
- Vary, Infocentrum města Karlovy. 2015. “What Is Treated in Karlovy Vary.” Karlovy Vary. January 30, 2015. <https://www.karlovyvary.cz/en/what-treated-karlovy-vary>.
- Viegas, Joana, Ana Filipa Esteves, Elsa M. Cardoso, Fernando A. Arosa, Marco Vitale, and Luís Taborda-Barata. 2019. “Biological Effects of Thermal Water-Associated Hydrogen Sulfide on Human Airways and Associated Immune Cells: Implications for Respiratory Diseases.” *Frontiers in Public Health* 7. <https://www.frontiersin.org/articles/10.3389/fpubh.2019.00128>.
- Wang, Hong, Caisheng Qiu, Ling Chen, Arshad Mehmood Abbasi, Xinbo Guo, and Rui Hai Liu. 2019. “Comparative Study of Phenolic Profiles, Antioxidant and Antiproliferative Activities in Different Vegetative Parts of Ramie (*Boehmeria Nivea* L.)” *Molecules (Basel, Switzerland)* 24 (8): 1551. <https://doi.org/10.3390/molecules24081551>.
- Wang, Jin, Xianshuang Cao, Hao Jiang, Yadong Qi, Kit L. Chin, and Yongde Yue. 2014. “Antioxidant Activity of Leaf Extracts from Different *Hibiscus Sabdariffa* Accessions and Simultaneous Determination Five Major Antioxidant Compounds by LC-Q-TOF-MS.” *Molecules* 19 (12): 21226–38. <https://doi.org/10.3390/molecules191221226>.
- Wasserfurth, Paulina, Inga Schneider, Alexander Ströhle, Josefine Nebl, Norman Bitterlich, and Andreas Hahn. 2019. “Effects of Mineral Waters on Acid–Base Status in Healthy Adults: Results of a Randomized Trial.” *Food & Nutrition Research* 63 (December): 10.29219/fnr.v63.3515. <https://doi.org/10.29219/fnr.v63.3515>.
- Wolfe, Kelly L., and Rui Hai Liu. 2007. “Cellular Antioxidant Activity (CAA) Assay for Assessing Antioxidants, Foods, and Dietary Supplements.” *Journal of Agricultural and Food Chemistry* 55 (22): 8896–8907. <https://doi.org/10.1021/jf0715166>.
- Wosiacki, Gilvan, Alessandro Nogueira, Frederico Denardi, and Renato Vieira. 2009. “Sugar Composition of Depectinized Apple Juices Composição de Açúcares Em Sucos de Maçãs Despectinizados.” *Semina : Ciências Agrárias*, January.
- Yang, Jing, Yujie Ren, Shuoyu Chen, Zhiqiang Zhang, Heliang Pang, Xuan Wang, and Jinsuo Lu. 2023. “Thermally Activated Drinking Water Treatment Sludge as a Supplementary

Cementitious Material: Properties, Pozzolanic Activity and Hydration Characteristics.”  
Construction and Building Materials 365 (February): 130027.  
<https://doi.org/10.1016/j.conbuildmat.2022.130027>.

Zielinski, Acácio Antonio Ferreira, Cíntia Maia Braga, Ivo Mottin Demiate, Flávio Luís Beltrame, Alessandro Nogueira, and Gilvan Wosiacki. 2014. “Development and Optimization of a HPLC-RI Method for the Determination of Major Sugars in Apple Juice and Evaluation of the Effect of the Ripening Stage.” *Food Science and Technology* 34 (March): 38–43. <https://doi.org/10.1590/S0101-20612014005000003>.

## Annexes

### Annex 1. Chromatographic and spectra data for non-anthocyanin compounds in the isotonic drink (Formulation F1 and F3).

Peak	Retention time (min)	$\lambda$ (nm)	[M-H] <sup>-</sup>	MS <sup>2</sup>	Assignment
1	13.85	325	353	191, 179, 135	3-Caffeoylquinic acid
2	15.65	311	337	163, 191, 119	3- <i>p</i> -coumaroylquinic acid
3	16.33	325	353	173, 179	4-caffeoylquinic acid
4	16.85	326	353	191, 179	Chlorogenic acid
5	18.92	312	337	173, 163, 109	4- <i>p</i> -coumaroylquinic acid

### Annex 2. Chromatographic and spectra data for anthocyanins in the isotonic drink (Formulations F1 and F3).

Peak	Retention time (min)	$\lambda$ (nm)	[M-H] <sup>-</sup>	MS <sup>2</sup>	Assignment
1	9.89	277, 343, 523	597	303	Delphinidin 3- <i>O</i> -sambubioside
2	13.73	280, 322, 517	581	287	Cyanidin 3- <i>O</i> -sambubioside