

**MESTRADO**

Química Farmacêutica

# **Development of Pharmaceutical Formulations Containing Green Tea Catechins Extracted with Natural Ionic Liquids**

Tiago Filipe Pereira Alves

**M**

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## **Development of Pharmaceutical Formulations Containing Green Tea Catechins Extracted with Natural Ionic Liquids**

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Work developed under the supervision of:

Professor Victor Armando Pereira de Freitas

PhD, Full Professor of Chemistry and Biochemistry, Faculty of Science, University of Porto

Professor Maria Helena dos Anjos Rodrigues Amaral

PhD, Assistant Professor of Pharmaceutical Technology, Faculty of Pharmacy, University of Porto

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## ABSTRACT

Considering the therapeutic potential of the phenolic groups in green tea, mainly catechins, in diseases of cognitive development, this work aimed to prepare new pharmaceutical formulations especially aimed at children with this type of problems.

In this work, catechins were extracted with natural solvents available on the market, such as agave syrup, honey, maple syrup and stevia (option for diabetics). Solvents with a naturally sweet taste were used to mask the taste and astringency of catechins when administered orally. These solvents demonstrated a good ability to extract these polyphenolic compounds from green tea.

The extracts obtained were then used in the preparation of an oral pharmaceutical formulation (syrup) with potential action on cognitive development diseases and which would improve the effectiveness and stability of these extracts rich in catechins. Subsequently, stability tests were carried out, which demonstrated satisfactory results after 90 days of storage at 4 °C.

After the development of syrups containing green tea polyphenolic compounds extracted with natural solvents, they were subjected to an in vitro static digestion simulation. At the end of the in vitro static digestion simulation, the presence of polyphenolic compounds was detected in the samples. Then, passage through caco-2 cells, which mimic the cell wall of the small intestine, was simulated. The results of this study allowed us to conclude that the constituents of the syrup were collected from the basolateral side, showing the ability to permeate through the intestinal cell wall, reaching the bloodstream. However, to deepen knowledge about these compounds, it will be necessary to carry out more studies.

**Keywords:** Catechins; Cognitive Development; Pharmaceutical Formulations; Bioavailability.

## RESUMO

Tendo em conta o potencial terapêutico dos grupos fenólicos do chá verde, principalmente as catequinas, em doenças de desenvolvimento cognitivo, este trabalho teve como objetivo a preparação de novas formulações farmacêuticas especialmente direcionadas a crianças com este tipo de problemas.

Neste trabalho, as catequinas foram extraídas com solventes naturais disponíveis no mercado, como o xarope de agave, o mel, o xarope de ácer e a stevia (opção para os diabéticos). Foram utilizados solventes com sabor naturalmente doce, numa tentativa de mascarar o sabor e a adstringência das catequinas quando administradas por via oral. Estes solventes demonstraram uma boa capacidade de extração destes compostos polifenólicos do chá verde.

Os extratos obtidos foram então utilizados na preparação de uma formulação farmacêutica oral (xarope) com potencial ação em doenças de desenvolvimento cognitivo e que permitisse melhorar a eficácia e a estabilidade destes extratos ricos em catequinas. Posteriormente, foram efetuados testes de estabilidade, os quais demonstraram resultados satisfatórios ao fim de 90 dias de armazenamento a 4 °C.

Após o desenvolvimento dos xaropes contendo os compostos polifenólicos do chá verde extraídos com solventes naturais, estes foram sujeitos a uma simulação de digestão estática *in vitro*. No final da simulação de digestão estática *in vitro* foi detetada a presença de compostos polifenólicos nas amostras. De seguida, simulou-se a passagem através de células caco-2, que mimetizam a parede celular do intestino delgado. Os resultados deste estudo permitiram concluir que os constituintes do xarope foram recolhidos do lado basolateral, mostrando capacidade de permeação através da parede celular intestinal, alcançando a corrente sanguínea. Contudo, para aprofundar o conhecimento a respeito destes compostos, será necessário realizar mais estudos.

**Palavras-chave:** Catequinas; Desenvolvimento Cognitivo; Formulações Farmacêuticas; Biodisponibilidade.

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## ABBREVIATIONS

AcOEt – Ethyl Acetate

ASD – Autism Spectrum Disorder

CNS – Commercial Natural Solvent

DLS – Dynamic Light Scattering

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl Sulphoxide

(E)C – (Epi)Catechin

(E)CG – (Epi)Catechin Gallate

EDTA - Ethylenediaminetetraacetic Acid

(E)GC – (Epi)Gallocatechin

(E)GCG – (Epi)Gallocatechin Gallate

FBS - Heat-inactivated Fetal Bovine Serum

GT – Green Tea

GT25S750H<sub>2</sub>O\_25\_ T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with 2.500:7.500 of Stevia:Water (1:3 w/w) at 100 °C

GT50S50H<sub>2</sub>O\_25\_ T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with Stevia:Water (1:1 w/w) at 100 °C

GT50S50H<sub>2</sub>O\_25\_ T<sub>100</sub>\_Dark - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Stevia:Water (1:1 w/w) at 50 °C in Dark Condition

GT50S50H<sub>2</sub>O\_25\_ T<sub>100</sub>\_Light - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Stevia:Water (1:1 w/w) at Room Temperature in Light Condition

GT50S50H<sub>2</sub>O\_25\_ T<sub>100</sub>\_Temperature - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Stevia:Water (1:1 w/w) at 100 °C in Temperature (40 °C) Condition

GT75S25H<sub>2</sub>O\_25\_ T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with Stevia:Water (3:1 w/w) at 100 °C

GTH<sub>2</sub>O\_25\_ T<sub>amb</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Water at Room Temperature

GTH<sub>2</sub>O\_50\_ T<sub>amb</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Water at Room Temperature

GTH<sub>2</sub>O\_50\_ T<sub>50</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Water at 50 °C

GTH<sub>2</sub>O\_50\_ T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Water at 100 °C

GTM\_25\_ T<sub>amb</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Honey at Room Temperature

GTM\_25\_ T<sub>50</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Honey at 50 °C

GTM\_25\_ T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Honey at 100 °C

GTM\_50\_ T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Honey at 100 °C

GTP – Green Tea Powder

GTS\_25\_ T<sub>amb</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Stevia at Room Temperature

GTS\_25\_ T<sub>50</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Stevia at Maple Syrup 50 °C

GTS\_25\_ T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Stevia at 100 °C

GTS\_25\_ T<sub>100</sub>\_Dark - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Stevia at 50 °C in Dark Condition

GTS\_25\_ T<sub>100</sub>\_Light - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Stevia at Room Temperature in Light Condition

GTS\_25\_T<sub>100</sub>\_Temperature - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Stevia at 100 °C in Temperature (40 °C) Condition

GTS\_50\_T<sub>amb</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Stevia at Room Temperature

GTS\_50\_T<sub>50</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Stevia at 50 °C

GTS\_50\_T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Stevia at 100 °C

GTXA\_25\_T<sub>amb</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Maple Syrup at Room Temperature

GTXA\_25\_T<sub>50</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Maple Syrup at Maple Syrup 50 °C

GTXA\_25\_T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Maple Syrup at 100 °C

GTXA\_25\_T<sub>100</sub>\_Dark - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Maple Syrup at 50 °C in Dark Condition

GTXA\_25\_T<sub>100</sub>\_Light - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Maple Syrup at Room Temperature in Light Condition

GTXA\_25\_T<sub>100</sub>\_Temperature - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Maple Syrup at 100 °C in Temperature (40 °C) Condition

GTXA\_50\_T<sub>amb</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Maple Syrup at Room Temperature

GTXA\_50\_T<sub>50</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Maple Syrup at 50 °C

GTXA\_50\_T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Maple Syrup at 100 °C

GTXAG\_25\_T<sub>amb</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Agave Syrup at Room Temperature



GTXAG\_25\_ T<sub>50</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Agave Syrup at 50 °C

GTXAG\_25\_ T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Agave Syrup at 100 °C

GTXAG\_25\_ T<sub>100</sub>\_Dark - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Agave Syrup at 50 °C in Dark Condition

GTXAG\_25\_ T<sub>100</sub>\_Light - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Agave Syrup at Room Temperature in Light Condition

GTXAG\_25\_ T<sub>100</sub>\_Temperature - Green Tea Polyphenolic Compounds Extracted with 10.000 g of Agave Syrup at 100 °C in Temperature (40 °C) Condition

GTXAG\_50\_ T<sub>amb</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Agave Syrup at Room Temperature

GTXAG\_50\_ T<sub>50</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Agave Syrup at 50 °C

GTXAG\_50\_ T<sub>100</sub> - Green Tea Polyphenolic Compounds Extracted with 5.000 g of Agave Syrup at 100 °C

HBSS - Hanks' Balanced Salts Solution

HPLC – High-Performance Liquid Chromatography

HPLC-DAD - High-Performance Liquid Chromatography – Diode Array

HPLC-ESI-MS - High-Performance Liquid Chromatography – Electrospray Ionization – Mass Spectrometry

LY - Lucifer Yellow

MME – Multiple Microemulsion

MME\_H<sub>2</sub>O\_5 - Multiple Microemulsion with Water and 5.0 % of Tween® 80

MME\_H<sub>2</sub>O\_7.5 - Multiple Microemulsion with Water and 7.5 % of Tween® 80

MME\_GTS\_50\_ T<sub>100</sub>\_5 - Multiple Microemulsion with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Stevia at 100 °C and 5.0 % of Tween® 80

MME\_GTS\_50\_T<sub>100</sub>\_7.5 - Multiple Microemulsion with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Stevia at 100 °C and 7.5 % of Tween® 80

MME\_GTXA\_50\_T<sub>100</sub>\_5 - Multiple Microemulsion with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Maple Syrup at 100 °C and 5.0 % of Tween® 80

MME\_GTXA\_50\_T<sub>100</sub>\_7.5 - Multiple Microemulsion with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Maple Syrup at 100 °C and 7.5 % of Tween® 80

MME\_GTXAG\_50\_T<sub>100</sub>\_5 - Multiple Microemulsion with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Agave Syrup at 100 °C and 5.0 % of Tween® 80

MME\_GTXAG\_50\_T<sub>100</sub>\_7.5 - Multiple Microemulsion with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Agave Syrup at 100 °C and 7.5 % of Tween® 80

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NR - Neutral Red Solution

PBS - Phosphate Buffered Saline

PD-BG – Prodelphinidin B-4 3'-O-Gallate

PI – Polydispersity Index

RPM – Revolutions Per Minute

SSRI – Selective Serotonin Reuptake Inhibitors

Syrup\_GTS\_50\_T<sub>100</sub>\_Lot1\_Dark - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Stevia at 100 °C and Lot 1 in Dark Condition

Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2\_Dark - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Stevia at 100 °C and Lot 2 in Dark Condition

Syrup\_GTS\_50\_T<sub>100</sub>\_Lot1\_Light - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Stevia at 100 °C and Lot 1 in Light Condition

Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2\_Light - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Stevia at 100 °C and Lot 2 in Light Condition

Syrup\_GTS\_50\_T100\_Lot1\_Temperature - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Stevia at 100 °C and Lot 1 in Temperature (4.0 °C) Condition

Syrup\_GTS\_50\_T100\_Lot2\_Temperature - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Stevia at 100 °C and Lot 2 in Temperature (4.0 °C) Condition

Syrup\_GTXAG\_50\_T100\_Lot1\_Dark - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Agave Syrup at 100 °C and Lot 1 in Dark Condition

Syrup\_GTXAG\_50\_T100\_Lot2\_Dark - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Agave Syrup at 100 °C and Lot 2 in Dark Condition

Syrup\_GTXAG\_50\_T100\_Lot1\_Light - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Agave Syrup at 100 °C and Lot 1 in Light Condition

Syrup\_GTXAG\_50\_T100\_Lot2\_Light - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Agave Syrup at 100 °C and Lot 2 in Light Condition

Syrup\_GTXAG\_50\_T100\_Lot1\_Temperature - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Agave Syrup at 100 °C and Lot 1 in Temperature (4.0 °C) Condition

Syrup\_GTXAG\_50\_T100\_Lot2\_Temperature - Syrup with Green Tea Polyphenolic Compounds Extracted with 5.000 g of Agave Syrup at 100 °C and Lot 2 in Temperature (4.0 °C) Condition

TGA – Thermogravimetry

TLC – Thin Layer Chromatography

ZP – Zeta Potential



# **CHAPTER 1:**

# **INTRODUCTION**



## CHAPTER 1 – INTRODUCTION

### 1.1. Green Tea

#### 1.1.1. Green Tea: An Overview

Green tea is one of the world's oldest and most beloved beverages, dating back thousands of years. Green tea, which originated in China, has long been prized not only for its delicious taste but also as a health and well-being elixir.<sup>1, 2</sup>

The history of green tea begins in ancient China during the Shang Dynasty (2737 B.C.). According to legend, tea was discovered by accident by Emperor Shen Nong, also known as the "Father of Agriculture" and a renowned herbalist. While he was resting under a tree, a breeze blew some wild tea leaves into a boiling pot of water that was within his reach. The aroma and flavor of the resulting infusion pleased the emperor, and thus green tea was born.<sup>1-5</sup>

Because tea was regarded as a valuable medicinal plant in ancient Chinese culture, it was initially used for its medicinal properties. Tea became popular among the Chinese nobility and upper classes as a stimulating and energizing beverage over time. Over the centuries, various Chinese dynasties learned about tea and its medicinal properties. During the Zhou Dynasty, tea was popular for medicinal and ritualistic purposes (1050-256 B.C.).<sup>1, 5, 6</sup>

During the Tang Dynasty (618-906 A.D.), tea gained social and cultural significance, spreading beyond China's borders to Japan via Buddhist monks studying in China. The tea ceremony became an important part of Japanese culture, and tea culture developed uniquely in Japan. Japanese Buddhist monks studying in China brought tea seeds with them, and tea plantations were established in Buddhist temples. The tea ceremony, known as Chanoyu or Chado in Japan, evolved from Chinese tea preparation and consumption practices. Green tea became an essential part of daily life in China during the Song Dynasty (960–1279 A.D.), and tea production and trade expanded rapidly.<sup>1, 5, 7, 8</sup>

Tea trade expanded significantly during the Ming and Qing dynasties (1368-1644 A.D. and 1644-1911 A.D.), establishing commercial routes known as the "Tea and Horse

Road" that connected China to Asia and the Western world. Green tea ascended in value as a result of its flavor and health benefits. The tea trade was critical in developing commercial and cultural relations between China and other Asian countries through maritime trade.<sup>1, 5, 7, 8</sup>

Green tea gained worldwide popularity in the twentieth century as people began to appreciate its distinct flavors and health benefits. Many of the associated benefits of green tea, such as antioxidant, anti-inflammatory, and properties that can support metabolism and cardiovascular health, have been confirmed by modern scientific studies.<sup>1, 9</sup>

Green tea is now grown in countries other than China and Japan, including India, Sri Lanka, Indonesia, Portugal, and others. The beverage is still valued for its long history, diverse flavors, and potential health benefits. Green tea's history connects us to centuries of tradition and culture, while remaining relevant and appreciated in modern times.<sup>1, 9, 10</sup>

### 1.1.2. Tea Manufacturing Process

Tea is one of the most popular beverages in the world, with a long history and a wide range of flavors and aromas. Tea, which is derived from the *Camellia sinensis* plant, is produced in various regions around the world and can be divided into several main categories, each with distinct characteristics.<sup>11</sup>

Green tea is distinguished by its bright green color and fresh, vegetal flavor. To prevent enzymatic oxidation, tea leaves are rapidly fixed in the production of Green Tea, usually with steam or a hot pan. The rolling process is gentle, retaining the original shape of the leaves, which are then dried to ensure color and natural properties are retained. Green tea is popular due to its antioxidant properties and low caffeine content.<sup>11-13</sup>

Black Tea, on the other hand, is known for its dark amber color and robust flavor. The leaves in this tea are completely oxidized, allowing enzymes to transform the chemical compounds in the leaves. This produces a more robust and full-bodied beverage. The leaves are rolled and fermented after oxidation before drying. Black tea is a high-caffeine beverage with a wide range of blends and flavorings.<sup>11, 13, 14</sup>

In terms of oxidation, Oolong Tea falls somewhere between Green Tea and Black Tea. Because the leaves are partially oxidized, the flavor becomes more complex and varied. Oolong Tea is made by withering, fixation, rolling, and fermentation, with oxidation



controlled to achieve the desired levels of taste and aroma. Oolong Tea comes in a variety of flavors, ranging from light and floral to full-bodied and fruity.<sup>15-17</sup>

White tea, on the other hand, is the least processed type of tea. The leaves are harvested while still young, usually before the leaves fully open, and are processed minimally. White tea is produced by withering and drying without the use of fixation or oxidation. This straightforward method preserves the leaves' natural characteristics, resulting in a delicate, light, and subtly flavored beverage. The antioxidant properties of white tea are well known.<sup>15, 16</sup>

The various teas on the market are the result of various manufacturing processes that transform the leaves of the *Camellia sinensis* plant into distinct beverages. Each type of tea has a distinct profile, ranging from the freshness of Green Tea to the robustness of Black Tea, the complexity of Oolong Tea, and the delicacy of White Tea.

### 1.1.3. Composition

Tea is an ancient beverage and one of the most popular around the world, valued for its distinct organoleptic properties and potential health benefits. Tea's chemical composition is quite complex and intriguing, as it results from the interaction of various compounds found in the *Camellia sinensis* plant's leaves.

Tea contains polyphenolic compounds, caffeine, amino acids, vitamins, minerals, carbohydrates, and volatile compounds, all of which have distinct sensory and nutritional properties.<sup>18, 19</sup>

The main antioxidants found in tea, such as catechins, are polyphenols, which contribute to its color, taste, and aroma. Because of their ability to combat oxidative stress and protect the body from free radical damage, these antioxidants have been the subject of scientific research.<sup>18, 19</sup>

Caffeine, though in smaller amounts than in coffee, is a significant component of tea. It is responsible for providing a mild stimulant effect, which aids in the improvement of alertness and concentration.<sup>18, 19</sup>

Furthermore, amino acids such as theanine play an important role in tea flavor, particularly in varieties such as Green Tea and White Tea. Theanine is known to promote

relaxation and well-being, which can help balance out the stimulating effects of caffeine.<sup>18, 19</sup>

Other constituents, such as vitamins A, B, C, and E, are present in smaller amounts but still contribute to tea's nutritional value. Tea also contains minerals such as manganese, potassium, and fluorine, which contribute to daily dietary intake.<sup>12, 18, 19</sup>

The aromas of tea are caused by volatile compounds, which vary depending on the type of tea and cultivation and processing conditions. These compounds give the tea a variety of flavors and fragrances, making it a popular beverage among many people.<sup>19, 20</sup>

GT dry leaf contains approximately 36% polyphenols, 3% caffeine, 1.5 % theanine, 15% protein, 6.5 % lignin, 25% carbohydrates, 0.5 % chlorophyll, and 1.5 % organic acids.<sup>9, 21-23</sup> The major polyphenolic compounds found in GT are catechins, which are represented in Figure 1 as (+)-catechin (C), (-)-epicatechin (EC), (+)-gallocatechin (GC), (-)-epigallocatechin (EGC), (+)-catechin gallate, (-)-epicatechin gallate (ECG), (+)-gallocatechin gallate and (-)-epigallocatechin gallate (EGCG).<sup>9, 21-28</sup>

Catechins, as a flavonoid, can donate hydroxyl groups of their structure, demonstrating an excellent antioxidant capacity through free radical scavenging ability that is stronger than vitamin C and E.<sup>9, 29</sup>

The fermentation process is the primary cause of thearubigin and theaflavin formation, and despite the fact that the fermentation process in GT is blocked at the beginning, some of these compounds can be detected in GT leaves.<sup>21, 30</sup>

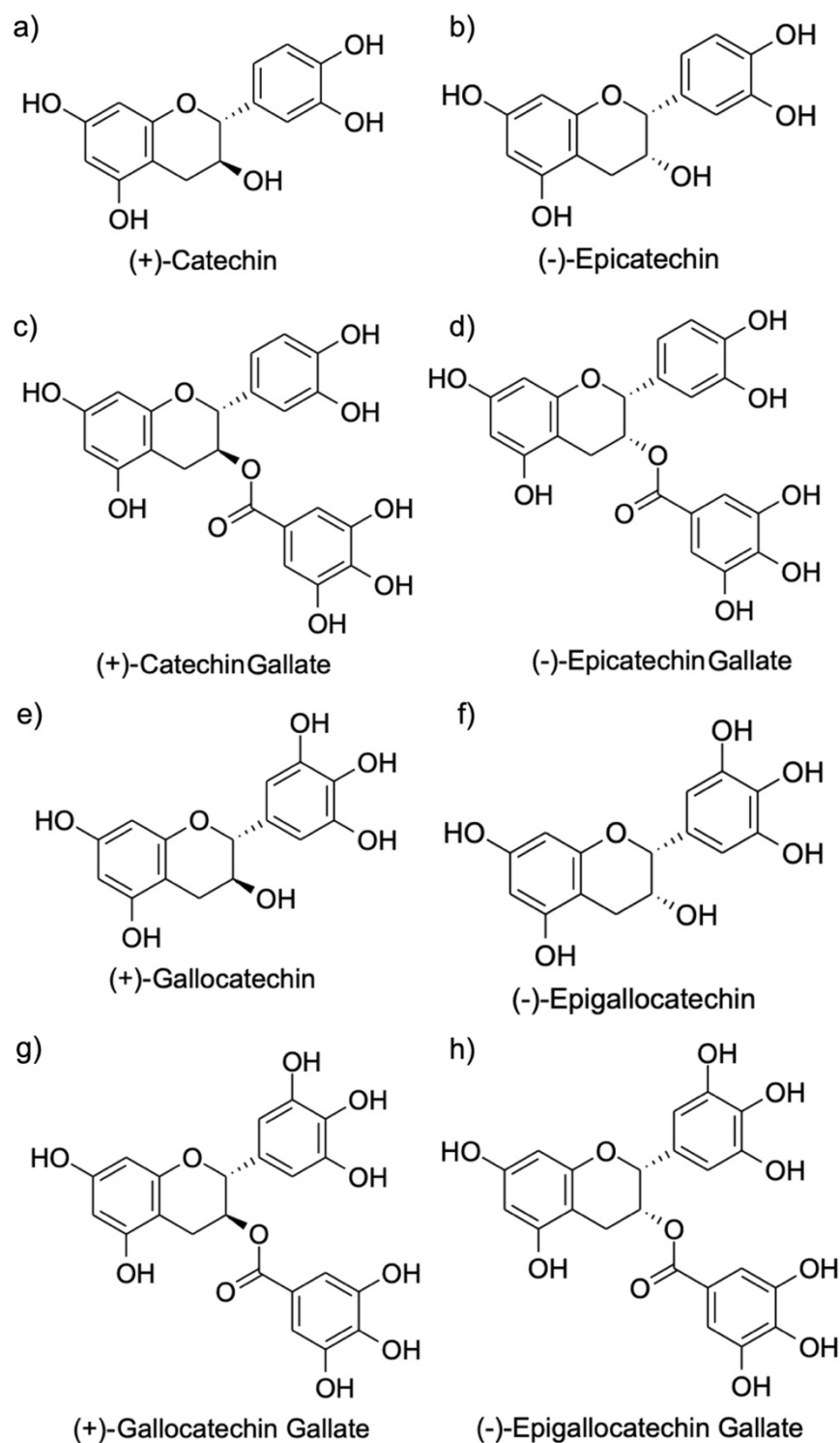


Figure 1 - Chemical structure of major catechins present in GT. a) - (+)-Catechin; b) - (-)-Epicatechin; c) - (+)-Catechin Gallate; d) - (-)-Epicatechin Gallate; e) - (+)-Gallocatechin; f) - (-)-Epigallocatechin; g) - (+)-Gallocatechin Gallate; and h) - (-)-Epigallocatechin Gallate.

### 1.1.4. Applications

Green tea has established a reputation for itself in the food industry thanks to its nutritional properties as well as its popularity among consumers looking for healthy and functional options. This ancient Asian beverage has been widely incorporated into a variety of food products over the years, becoming a versatile and valued ingredient in the global gastronomic scene.

One of the primary reasons for green tea's growing presence in the food industry is its high antioxidant content. Green tea polyphenols and catechins have shown strong antioxidant potential, assisting in the protection of processed foods against oxidation and degradation caused by free radicals, thereby extending shelf life and improving product stability.<sup>11, 31, 32</sup> Furthermore, the antioxidant capacity of green tea has caught the interest of the food industry as a way to boost the nutritional value of specific products. Green tea extracts, for example, can provide an additional boost of antioxidants in foods such as energy bars, breakfast cereals, or functional beverages, making these products more appealing to health-conscious consumers. Green tea leaf infusion can be used to make a variety of ready-to-drink beverages, ranging from iced teas to natural energy drinks. Furthermore, the inclusion of green tea in ice creams, desserts, chocolates, and confections can impart a distinct flavor and aroma that will appeal to the most discerning palates.<sup>9, 31, 32</sup> Because of its antimicrobial and antioxidant properties, green tea has also been used as a natural preservative in foods and beverages. Because of this ability, green tea is an appealing alternative to synthetic preservatives, meeting consumer demand for more natural and additive-free food products.<sup>33</sup>

Green tea has found a prominent place in the cosmetics industry, in addition to its food applications. Green tea has become a highly valued ingredient in personal care and beauty products due to its high concentration of antioxidants, vitamins, and bioactive compounds, providing a natural and effective approach to promoting skin and hair health and beauty. Green tea polyphenols have demonstrated remarkable effectiveness in neutralizing free radicals and protecting the skin from oxidative stress and premature aging. Green tea's antioxidant properties make it an important ingredient in anti-aging products, moisturizers, and lotions, restoring radiance and firmness while reducing the appearance of fine lines and wrinkles.<sup>9, 31, 34</sup> It is also known for its anti-inflammatory and soothing properties, making it ideal for sensitive or irritated skin. Green tea extracts are frequently used in skincare products such as toners, masks, and cleansers to soothe and balance the skin, promoting a healthy and revitalized appearance.<sup>9, 31</sup> Promote skin hydration and

reduce UV-ray damage, and strengthen the hair, making green tea an appealing option for hair care products.<sup>35, 36</sup> Is also used in the cosmetics industry as a natural fragrance and aroma ingredient. Green tea's volatile compounds impart a fresh and pleasant aroma, making it a popular choice in perfumes, body lotions, and bath products.<sup>9, 31</sup> In addition to its skin and hair benefits, green tea is known for its purifying and astringent properties, making it an important ingredient in acne and excessive skin oil control products.<sup>9, 31, 37</sup>

Green tea, which has long been prized for its therapeutic and cultural properties, has recently gained prominence in the pharmaceutical industry as a valuable source of bioactive compounds and nutrients with potential health benefits. Green tea's medicinal properties have sparked scientific research and the incorporation of extracts into pharmaceutical formulations, resulting in a diverse range of products that go beyond its traditional use as a beverage.<sup>11</sup> Due to its rich chemical composition is one of its main draws for the pharmaceutical industry. Its polyphenols, particularly epigallocatechin gallate (EGCG), have received extensive research for their potential antioxidant and anti-inflammatory properties, which may aid in the protection of cells and tissues from oxidative damage and inflammation. These properties make green tea extracts a promising option in pharmaceutical products for oxidative damage protection, inflammation reduction, and potential prevention of chronic diseases such as cardiovascular disease, diabetes, cancer, and neurodegenerative diseases.<sup>10, 11, 34</sup> Green tea's antioxidant activity has also been studied in terms of cardiovascular health. According to research, the compounds found in green tea may help to improve cholesterol levels, lower blood pressure, and promote vascular health, all of which may be beneficial in the context of cardiovascular disease prevention.<sup>10, 34</sup> Another area of interest is green tea's potential role in metabolism regulation and body weight control. Green tea contains catechins and caffeine, which can increase thermogenesis and fat oxidation, making it a researched option for weight management and aiding in weight loss diets.<sup>10, 34</sup> Has also been studied for its neuroprotective properties, owing to the presence of theanine, an amino acid known for its relaxing properties. These potentially neuroprotective effects have been studied in terms of neurodegenerative disease prevention and cognitive enhancement.<sup>34, 38</sup>

Green tea's use in medicine and the pharmaceutical industry extends beyond medications and includes nutritional supplements and natural health products. Green tea extract supplements have grown in popularity among consumers seeking to improve their overall health and well-being.

## 1.2. Cognitive and Neurodegenerative Diseases

Cognitive and neurodegenerative diseases are a group of medical conditions that affect the functioning of the brain and, as a result, the affected individuals' cognitive ability and nervous function. Memory, language, attention, reasoning, and learning capacity are all affected by these diseases, which cause a progressive decline in mental abilities.<sup>38</sup>

Alzheimer's disease, Parkinson's disease, and Autism Spectrum Disorder (ASD) are among the most common diseases, but there are other, lesser-known conditions that also fall into this category.<sup>39, 40</sup>

Although the exact cause of these diseases is unknown, genetic, environmental, and other factors may all play a role in their development. Understanding the underlying mechanisms of these diseases has been the focus of scientific research in order to develop effective treatments and, ideally, find a cure.<sup>41, 42</sup>

### 1.2.1. Autism Spectrum Disorder (ASD)

Kanner described the first source of information on Autism Spectrum Disorder (ASD) in 1943, where he closely observed several children's behaviors, stating how these "conditions differ so markedly and uniquely from anything reported so far." Kanner only described these atypical behaviors and did not provide a definitive definition of ASD. Even after seven decades, Kanner's initial description of the primary characteristics of ASD remains relatively consistent: deficits in social communication behaviors and repetitive and unusual sensory-motor behaviors. However, scientific research has disproved the notion that ASD is a rare behavioral disorder in children, and it is now recognized as a lifelong condition.<sup>43</sup>

The human brain is one of the most complicated and delicate organs on the planet. The more information the brain receives, the more synapse connections it forms, making brain research more difficult. ASD is a neurological disorder that affects the brain in a variety of ways as a result of abnormal connections between brain regions (Figure 2).<sup>44</sup>

ASD is more common in Caucasian children than in African, American, or Hispanic children, and boys are more susceptible to this condition than girls due to X-chromosome

mutations. Because girls have a homologous pair of chromosomes, XX, these mutations are recessive and will only be expressed if a recessive homozygote exists. Boys have a different pair of chromosomes, XY, and these mutations are more easily expressed, resulting in a PTCHD1 gene microdeletion (Figure 3).<sup>44, 45</sup>

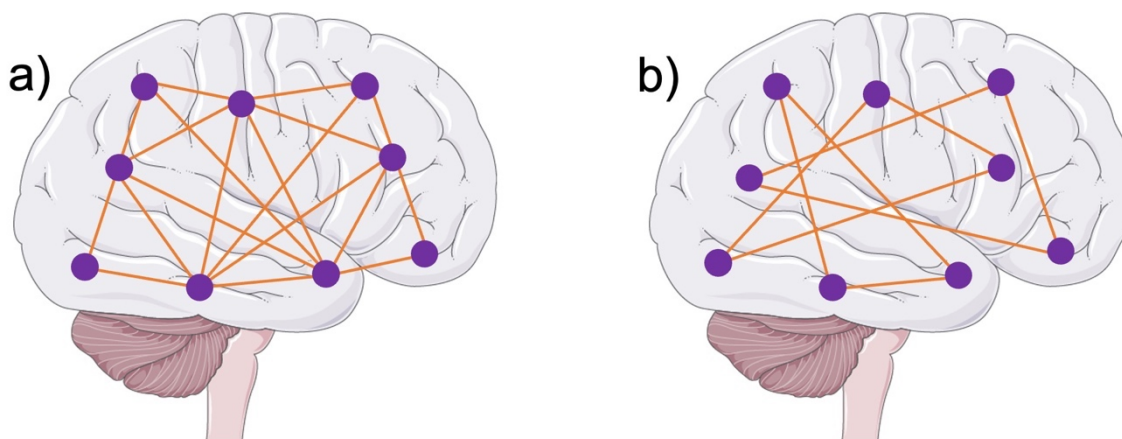


Figure 2 - Brain interactions between different zones. a) Normal brain and b) autistic brain. Adapted from Bhat 2014.

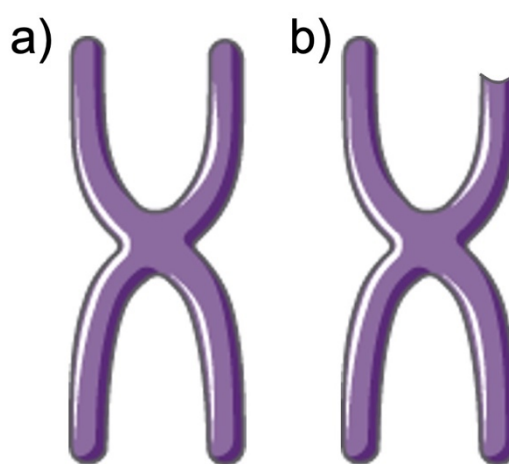


Figure 3 - Microdeletion of the chromosome lead to neurodevelopmental disorder. a) Normal chromosome and b) mutation chromosome. Adapted from Bhat 2014.

Autism is linked to other neurodevelopmental disorders and is linked to environmental factors and medical conditions. For example, in the literature, ASD has been linked to Rett syndrome, a neurodevelopmental disorder in which previously acquired skills regress, and it can present with a variety of symptoms such as slowed head growth, loss of purposeful hand movements, often replaced by repetitive stereotypical movements, breathing and gait abnormalities, and loss of speech. This disorder was linked to mutations in the X-linked gene methyl-CpG-binding protein 2 (MECP2), a ubiquitously expressed

transcriptional regulator, and while it was previously thought to affect only females, male carriers with the MECP2 phenotype and mutations are now known to be affected as well.<sup>43, 44, 46-49</sup>

#### 1.2.1.1. Autism Spectrum Disorder Specifiers and Diagnosis

The *Diagnostic and Statistical Manual, Fifth Edition*, categorizes Autism Spectrum Disorder (ASD) into three levels: "Requiring support," "Requiring substantial support," and "Requiring very substantial support." The first level is associated with deficits in social communication, difficulty in initiating conversations, atypical answers, decreased social interest, and difficulty in making friends. The second level, "Requiring substantial support," describes patients with marked deficits in social communication, limited initiating conversation, reduced answers, and limited narrow specified interests. The third level, "Requiring very substantial support," is considered the higher classification for ASD, characterized by difficulties in verbal and non-verbal communication, limited initiation socials, minimal communication, and social overture.<sup>46</sup>

Severity specifiers are used to describe the actual symptomatology in social communication, but the recognition of severity can be variable. The "with or without accompanying intellectual impairment" specifier is used to understand the intellectual profile of a child or adult with ASD, and separate tests of verbal and non-verbal capacities are necessary for diagnosis. The "with or without accompanying language impairment" specifier is used to identify patients who cannot include legible discourse or single words or phrased speech, while the "without" specifier is used to assess fluency and complete sentences.<sup>46</sup>

The "associated with a known medical or genetic condition or environmental factor" specifier is used when a patient has a known disease like Rett syndrome, Down syndrome, or epilepsy, and a known history of environmental exposure. Other neurodevelopmental, mental, or behavioral diseases, such as attention-deficit/hyperactivity disorder, anxiety, depression, and bipolar, should also be noted in the diagnostic.<sup>46</sup>

The stages where the disorder becomes obvious are associated with individual characteristics and environment. The disorder manifestation depends on the severity of the autistic condition, level of development, and chronic age, making the spectrum term necessary. ASD disorder englobes old terms used for autism disorder, such as early infantile disorder, Kanner's autism, and Asperger's disorder.<sup>46</sup>



### 1.2.1.2. Approved Medicines

Currently, there is no direct therapy for ASD treatment, but psychotropic medications are used to relieve co-occurring problems such as aggression, hyperactivity, and anxiety without interfering with ASD core symptoms. The medications approved for ASD treatment include Risperidone and Aripiprazole (Figure 4), which are used for aggression, self-injurious, and severe tantrums. Risperidone is well-tolerated at the gastrointestinal level and has mild side effects, such as sedation, appetite increase, fatigue, dizziness, and drowsiness. Aripiprazole, on the other hand, has milder side effects, including weight gain, fatigue, somnolence, gastrointestinal symptoms, and motor restlessness.<sup>50</sup>

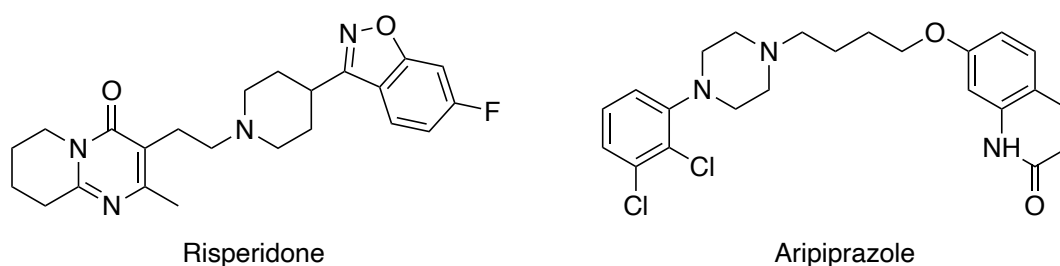


Figure 4 - Chemical structure of Risperidone and Aripiprazole.

Selective serotonin reuptake inhibitors (SSRIs) like Fluoxetine, Escitalopram, Fluvoxamine, Paroxetine, and Sertraline (Figure 5) have potential benefits for ASD symptoms, but they have side effects like hyperactivity, hypomania, agitation, and disinhibition. Tricyclic antidepressants have been used in the treatment of ASD symptoms, but their therapeutic and side effects have been contradictory.<sup>50</sup>

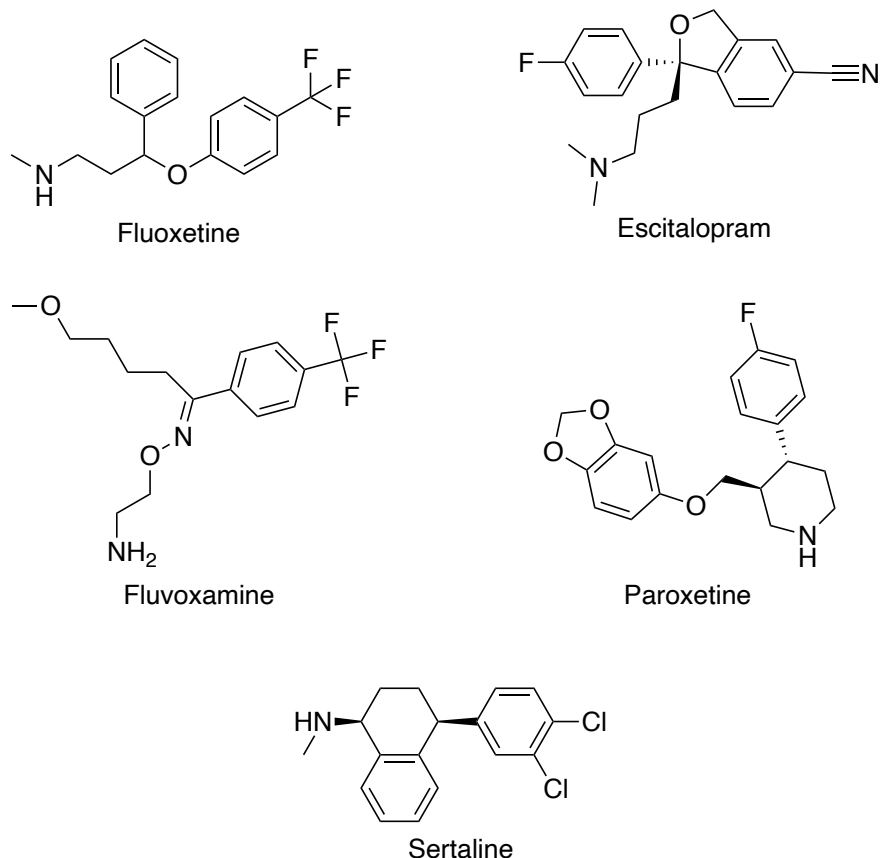


Figure 5 - Chemical structure of Fluoxetine, Escitalopram, Fluvoxamine, Paroxetine, and Sertraline.

Anticonvulsants like Valproate (Figure 6) have shown efficacy in treating irritability and repetitive behavior in children. Acetylcholinesterase inhibitors like Rivastigmine, Donepezil, and Galantamine (Figure 7) have shown improvement in sleep patterns and overall ASD behaviors in ASD children, but they also have side effects like verbal or behavior regression, headaches, irritability, tremor, rash, vomiting, gastrointestinal problems, and sedations.<sup>50</sup>

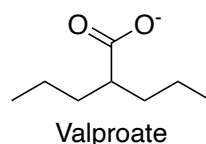


Figure 6 - Chemical structure of Valproate.

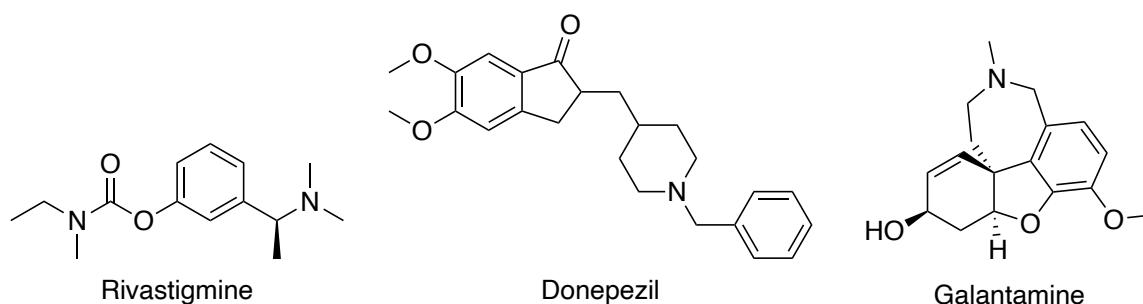


Figure 7 - Chemical structure of Rivastigmine, Donepezil, and Galantamine.

Central nervous system stimulants like Ritalin (Figure 8) are used to treat attention deficit disorder, narcolepsy, and attention deficit hyperactivity disorder, but they have side effects like loss of appetite, weight loss, nervousness, nausea, and vomiting.<sup>50</sup>

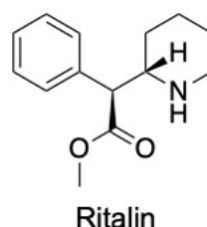


Figure 8 - Chemical structure of Ritalin.

### 1.2.2. Alzheimer's Disease

Alzheimer's disease is a progressive and irreversible neurodegenerative disease that primarily affects memory, cognition, and behavior. This pathology is named after Alois Alzheimer, who described it for the first time in 1906. It is the most common type of dementia in older people and has a significant impact on the quality of life of patients, their families, and caregivers.<sup>51, 52</sup> This disease progresses slowly and insidiously, affecting areas of the brain associated with memory and learning at first. As it progresses, it affects other cognitive domains such as language, reasoning, and motor skills. Furthermore, as the disease progresses, the individual's behavior and personality may change.<sup>51, 53</sup>

Although the exact cause of Alzheimer's disease is unknown, there is evidence that a combination of genetic, environmental, and lifestyle factors may play a role in its progression. A distinctive neuropathological feature of the disease is the presence of amyloid plaques and neurofibrillary tangles in the brain.<sup>54, 55</sup>

Unfortunately, there is no cure for Alzheimer's disease at this time. The primary goal of treatment is to alleviate symptoms, improve the patient's quality of life, and assist caregivers in managing the challenges that patients face.

Alzheimer's disease is becoming an increasingly important public health issue as the world's population ages. As a result, the pursuit of a better understanding and management of this disease becomes critical in confronting the challenges associated with this devastating condition.

#### **1.2.2.1. Alzheimer's Disease Diagnosis**

A detailed clinical evaluation is required to diagnose Alzheimer's disease, which includes a review of the patient's medical history, family history, and a thorough analysis of the presented cognitive and behavioral symptoms. Other medical conditions that may be contributing to the symptoms, such as nutritional deficiencies, thyroid issues, or infections, must be ruled out.<sup>52, 56</sup>

The presence of progressive cognitive decline that interferes with the patient's daily activities is one of the key criteria for diagnosing Alzheimer's disease. Recent memory loss, difficulty finding words, disorientation in time and space, and changes in judgment and social behavior are all possible symptoms.<sup>52, 56, 57</sup> Brain imaging studies (such as computed tomography or magnetic resonance imaging) and neuropsychological tests can also be used to support the diagnosis and assess the patient's level of cognitive impairment.<sup>58, 59</sup> It is important to note, however, that the definitive diagnosis of Alzheimer's disease can currently only be confirmed post-mortem, via a neuropathological analysis of the brain to identify distinguishing features such as amyloid plaques and neurofibrillary tangles.<sup>58, 60</sup>

#### **1.2.2.2. Approved Medicines**

Alzheimer's disease is becoming an increasingly important public health issue as the world's population ages. As a result, the pursuit of a better understanding and management of this disease becomes critical in confronting the challenges associated with this devastating condition.<sup>58, 59</sup>

Alzheimer's disease medications are classified into two types: cholinesterase inhibitors and NMDA receptor antagonists (N-methyl-D-aspartate). Both classes of medications are designed to treat neurotransmitter deficiencies in the brain, particularly acetylcholine, which is essential for cognitive function and memory.<sup>58, 61, 62</sup> Cholinesterase inhibitors, such as donepezil, rivastigmine, and galantamine (Figure 9), work by preventing the enzyme cholinesterase from breaking down acetylcholine. This helps to increase acetylcholine levels in the brain and, in some cases, may temporarily improve cognitive and behavioral symptoms in patients with mild to moderate Alzheimer's disease.<sup>58, 61, 62</sup>

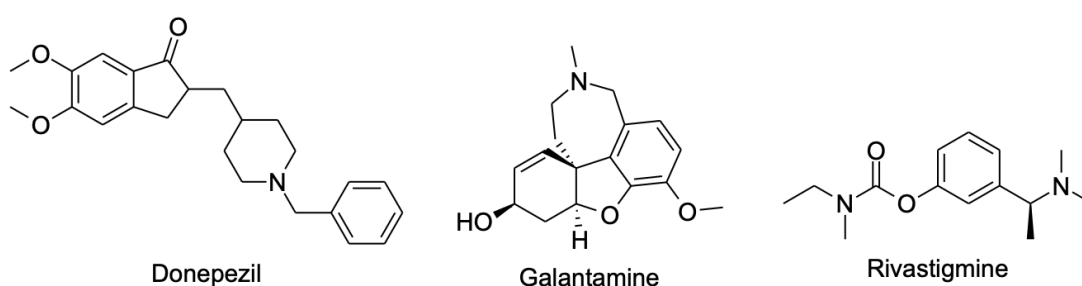


Figure 9 - Chemical structure of Donepezil, Galantamine, and Rivastigmine.

NMDA receptor antagonists, on the other hand, like memantine (Figure 10), work by inhibiting the excessive activity of the neurotransmitter glutamate, which has been linked to excitotoxicity and cell death in the brains of Alzheimer's patients in later stages of the disease. Memantine is approved to treat moderate to severe Alzheimer's disease.<sup>58, 62, 63</sup>

It is critical to note that the medications currently approved for the treatment of Alzheimer's disease do not reverse the disease's progression or provide a permanent cure. They may help control symptoms for a while, but the disease progresses over time.

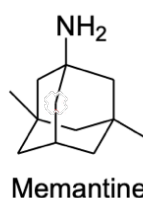


Figure 10 - Chemical structure of memantine.

### 1.2.3. Parkinson's Disease

Parkinson's disease is a chronic and progressive neurological disorder that primarily affects the motor system of the brain. It is named after the British James Parkinson, who described it in detail for the first time in 1817.<sup>64, 65</sup> It is primarily characterized by the gradual degeneration of nerve cells responsible for producing dopamine in the substantia nigra, a brain region. Dopamine is a critical neurotransmitter involved in controlling body movements and mood regulation.<sup>64, 65</sup> As dopamine-producing cells degenerate, imbalances in the neural circuits responsible for movement control occur, resulting in Parkinson's disease symptoms. Resting tremors, muscle rigidity, bradykinesia (slow movements), and difficulties with motor coordination are among the symptoms.<sup>64, 65</sup>

Although the exact cause of Parkinson's disease is unknown, it is thought to be a combination of genetic and environmental factors that contribute to its development. Age is also a risk factor, with most cases occurring in people over the age of 60.<sup>64, 65</sup>

While there is no cure for Parkinson's disease at this time, there are therapeutic approaches that can help relieve symptoms and improve patients' quality of life. Medication to increase dopamine levels in the brain, as well as physical rehabilitation therapies and, in some cases, surgery, are common treatments.<sup>64, 65</sup>

Parkinson's disease is a severe condition that can have a significant impact on the lives of patients and their families. Ongoing research into this disease is critical for the development of more effective treatments and, ideally, the eventual discovery of a cure.

#### 1.2.3.1. Parkinson's Disease Diagnosis

The accurate diagnosis of Parkinson's disease is critical for ensuring appropriate treatments and improving patients' quality of life. This diagnosis, however, remains difficult because there is no specific test or definitive biomarker that can confirm the presence of the disease.<sup>66, 67</sup> Parkinson's disease's early symptoms can be subtle and easily confused with the natural aging process or other neurological conditions. Resting tremors, muscle rigidity, bradykinesia, and postural instability are all classic symptoms of the disease. However, these symptoms can also be seen in other pathologies, so a differential diagnosis is required to rule out other possibilities.<sup>38, 42, 66</sup>

The clinical criteria obtained from the patient's history, detailed neurological examination, and assessment of the presented symptoms are used to make the diagnosis of Parkinson's disease. Motor sign analysis and the patient's response to specific medications, such as levodopa, can also be used to aid in the diagnosis.<sup>42, 66</sup> Furthermore, neuroimaging techniques can help rule out other diseases with similar symptoms. It is important to note, however, that neuroimaging does not always provide a definitive diagnosis of Parkinson's disease because structural brain changes can be found in a variety of neurological conditions.<sup>66, 67</sup>

### 1.2.3.2. Approved Medicines

The medications approved for the treatment of Parkinson's disease are intended to address the brain's dopaminergic deficiency, which is a key feature of the disease. Dopaminergic agonists like pramipexole (Figure 11), which directly activate dopamine receptors and mimic the action of this neurotransmitter, are among the most approved medications. Another important class is MAO-B inhibitors such as safinamide and selegiline (Figure 12), which reduce dopamine degradation in the brain, thereby extending its action and improving dopaminergic function.<sup>67, 68</sup>

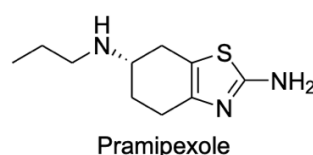


Figure 11 - Chemical structure of pramipexole.

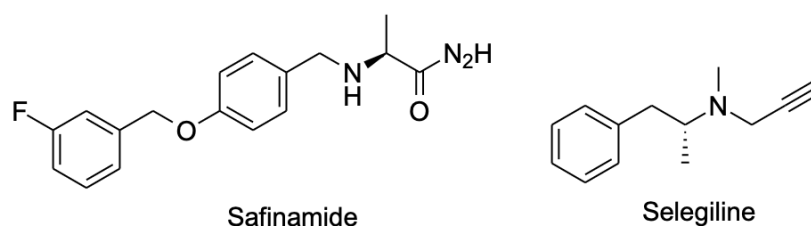


Figure 12 - Chemical structure of safinamide and selegiline.

Other medications include levodopa (Figure 13), a dopamine precursor that is converted into dopamine in the brain, providing the body with the required amount of this neurotransmitter. Although levodopa is one of the most effective Parkinson's disease treatments, its long-term efficacy may be limited by motor complications such as dyskinesias.<sup>42, 67</sup>

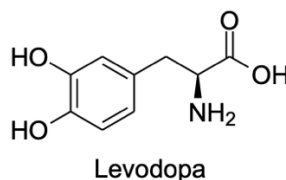


Figure 13 - Chemical structure of levodopa.

It is critical to emphasize that, while approved medications can provide relief to patients, there is currently no cure for Parkinson's disease. Furthermore, some patients may experience long-term complications as a result of continued use of certain medications, emphasizing the need for more advanced therapeutic approaches.

### 1.3. Drugs Administration

A drug delivery system is a formulation or device that allows for the introduction of a therapeutic substance into the body and improves its efficacy and safety by controlling the rate, time, and release of drugs in the body.

Drugs can be administered in the body via several anatomical routes like gastrointestinal system, parenteral, transmucosal, nasal, and pulmonary, and the route of administration chosen depends on the patient's condition, the disease, the desired effect, and the product available.<sup>69</sup>



### 1.3.1. Oral Drug Delivery

The oral route is the most popular and widely accepted method of drug administration due to its ease of administration and widespread acceptance. However, this route has limitations, such as problems with absorption into the bloodstream, permeation through biological membranes, and solubility.<sup>69, 70</sup> Some drugs cannot pass the epithelial membrane cells, and others are insoluble at certain pH levels, reducing bioavailability. The metabolism of oral drugs can also be difficult, as they are driven into the liver, which can suffer variable metabolism, making the drug more hydrophilic and easier to excrete or produce active metabolites.<sup>69, 70</sup> Additionally, oral administration can interact with the flora and mucous line of the gastrointestinal tract, causing irritation. Despite these issues, the oral route remains a preferred method of administration, and extensive investigation have been made to improve drug formulations.<sup>69, 70</sup>

## 1.4. Objectives of this Work

Green tea has numerous benefits and applications, including regular consumption, active packaging, cosmetics, dietary supplements, and even medications. Green tea extracts, particularly the catechin's class, have shown promising results in the treatment cognitive and neurodegenerative diseases. Recent research on green tea extract has shown behavioral and memory improvements in patients, as well as *in vitro* tests for diseases such as Alzheimer's, Autism Spectrum Disorder, and Parkinson's.

Given the promising results of green tea consumption in the stabilization of patients with cognitive and neurodegenerative diseases, the goal of this work is to develop an oral formulation that, in addition to its potential benefits, can mask the bitter and astringent taste of the polyphenolic compounds extracted from green tea, so that its administration does not result in a negative consumer experience due to its flavor. The intention is to mask the bitter and astringent taste of green tea polyphenolic compounds with commercial natural solvent sweeteners (CNS) such as agave syrup, honey, maple syrup, and stevia, by directly extracting the polyphenolic compounds from green tea into their matrices, being these extractions characterized and their stability evaluated.

Following extraction, the CNS will be tested in oral formulations to ensure a stable and effective oral formulation. As a result, *in vitro* assays will be required to validate the bioaccessibility and bioavailability of green tea polyphenolic compounds in the prepared formulations.

# **CHAPTER 2:**

# **MATERIAL AND METHODS**



## CHAPTER 2 – MATERIAL AND METHODS

### 2.1. Green Tea

The green tea leaves were grinded to guarantee the homogeneity of the sample and also to ensure reliable analysis results. Dried green tea leaves withdrawn from Lot 45/42 on 13/02/2020, were grinded with a blade grain miller Retsch ZM200 (Germany) at 10000RPM.

### 2.2. Commercial Natural Solvents

The Commercial Natural Solvents used in this work were bought in a supermarket. Agave syrup was obtained from Origens Bio, Portugal, maple syrup from Trader Joe's®, USA, honey from Mel de Flores, Spain, and stevia from Canderel, United Kingdom.

#### 2.2.1. Thermal analysis- Thermogravimetry (TGA)

STA7200RV from Hitachi Instruments, Inc. (Tokyo, Japan) was used to perform the thermal stability studies (Thermogravimetry (TG)). Solid (~4 mg) and liquid samples were placed in an open aluminium crucible. Nitrogen atmosphere with a purge gas flow of 200.0 mL·min<sup>-1</sup> was created in the dynamic experiments. Analysis was carried out from 25 to 600 °C at 10 °C·min<sup>-1</sup> heating rate ( $\beta$ ). Three replicates were carried out for each thermogram, with estimated uncertainties of  $\pm 0.5$  °C. The thermal analysis was performed in FCUP|DQB – Lab&Services.

## 2.3. Green Tea Polyphenolic Compounds Extraction

To make possible the realization of the work in this chapter, the equipment in Table 1 was used.

Table 1 - List of equipments used in this chapter.

| Equipment                     | Producer                | Model                                | Country        |
|-------------------------------|-------------------------|--------------------------------------|----------------|
| Analytical Balance            | Kern & Sohn             | ABT 120-5DM                          | Germany        |
| Semi-analytical Balance       | Kern & Sohn             | 440-45 N                             | Germany        |
| Centrifuge                    | Dynamica Scientific Ltd | Velocity 14R Refrigerated Centrifuge | United Kingdom |
| Minispin Plus Microcentrifuge | Eppendorf               | WD 7079                              | Portugal       |
| Magnetic Stirrer              | IKA®                    | RET basic                            | Portugal       |
| Temperature Sensor            | IKA®                    | ETS-D4 fuzzy                         | Portugal       |
| Oven                          | Memmert                 | U10                                  | Germany        |

### 2.3.1. Traditional Method

Mimicking a traditional tea preparation, 3x 0.2500 g of green tea powder was dissolved in 3x 10.0 mL of distilled water. One of the samples was heated at 100 °C for 15 min; other sample was heated at 50 °C for 15 min; and the third sample was left at room temperature also for 15min. A 3x 0.2500 g of green tea powder was dissolved in 3x 5.0 mL of distilled water at the same three temperature conditions and time of extraction, for compound extraction comparison purposes.

### 2.3.2. Commercial Natural Solvents Media

Commercial Natural Solvents (CNS) were used as a naturally sweet medium for the extraction of polyphenolic compounds from green tea. A similar procedure as that described

in section 2.3.1. was used. Briefly, an amount of 0.2500 g of green tea powder (GTP) was weighted and mixed with 10.000 g and 5.000 g of each CNS.

The 4 CNS used: agave syrup, honey, maple syrup, and stevia; were placed at 3 different conditions: room temperature and in a silicon bath at 100 °C and 50 °C, under stirring for 5 minutes. Then, the weighed green tea powder was added and left under stirring for further additional 15 minutes. All sample's insoluble solids were filtered using a conventional-tea filter bag. Next, the remaining solution was centrifuged at  $21.000 \times g$  (9 ACC, 25°C) for 15 minutes.

## **2.4. Green Tea Polyphenolic Compounds Characterization**

### **2.4.1. High-Performance Liquid Chromatography - Mass Spectroscopy (HPLC - MS)**

All extraction samples were analyzed by HPLC-ESI-MS performed in a Finnigan Surveyor Plus HPLC fitted with a PDA Plus detector, an auto-sampler Plus and a LC quaternary pump plus coupled to a Finnigan LCQ Deca XP Plus mass detector equipped with an ESI source and a linear ion trap quadrupole. The stationary phase was a Thermo Finnigan Hypersil Gold column (150 x 4.6 mm i.d., 5 mm) at 25°C. The mass spectrometer was operated in the negative-ion mode with source, with a capillary temperature of 275 °C and capillary voltages of 4.5 kV. The mass spectra were recorded between 150 and 2000 m/z.

The mobile phase was composed by solvent A, 1% (v/v) formic acid from ChemLab (Belgium), and solvent B, 100% (v/v) acetonitrile from ChemLab (Belgium). The flow rate was 0.50 mL/min and the gradient method started with a linear gradient ranging from 90% A to 65% A in 50 min, then reaching 100% B in 5 min, a final isocratic gradient of 100% B during 7 min and a final re-equilibration isocratic gradient of 90% A for 5 min.

### 2.4.2. High-Performance Liquid Chromatography – Diode Array (HPLC - DAD)

All extraction samples were analyzed with a HPLC VWR Hitachi Chromaster (column oven: 5310; pump: 5160; auto sampler: 5260) equipped with a detector DAD 5430. 30  $\mu$ L of sample were injected in a Merck column of reverse phase C18 ODS (column dimensions: 150 x 4.6 mm, 5  $\mu$ m, d.i), the solvents and gradient used were the same described previously for HPLC-MS analysis. All the chromatograms were obtained with a wavelength of 280 nm, 30 °C, flow rate of 0.50 mL/min.

The calibration curve for (-)-epicatechin and the study of the repeatability of this method were obtained as follows: a concentration range of 2.00 to 0.0625 mg mL<sup>-1</sup> was used, and each sample was injected in triplicate. Unknown concentrations were determined from the regression equation ( $y = 6E6 x - 8E6$ ;  $r^2 = 0.9551$ ).

### 2.4.3. Density and Viscosity

The density and viscosity tests were performed with an Anton Paar, densitometer model DMA 4500M, and viscosimeter model Lovis 2000 ME, respectively. Both tests were executed in FCUP|DQB – Lab&Services. Each measurement was conducted in triplicate at a temperature range of 80 °C to 20 °C.

### 2.4.4. Rheology

For the assays with CNS and CNS with GT polyphenolic compounds extracted, a stress-controlled rotational rheometer ARG2, TA Instruments (USA), with 25 mm parallel plates and a gap varying between 0.55 and 1.02 mm, was used. Oscillatory assays and flow curves at different shear rates were performed between 0.1 and 300 s<sup>-1</sup>.



## 2.5. Stability of Extracted Green Tea Polyphenolic Compounds

### 2.5.1. High-Performance Liquid Chromatography – Diode Array (HPLC - DAD)

All the stability assays were performed using the same equipment and methods described in the section 2.4.2.

### 2.5.2. Colorimetry

The sample was placed in a disposable cuvette and the colorimetric assays were performed with a spectrophotometer from Agilent model Cary 60 UV-Vis (USA).

## 2.6. Development of an Oral Microemulsion with Green Tea Extracted with Commercial Natural Solvents

Table 2 - List of raw materials used in this chapter.

| Raw Materials         | Producer | Country  | Lot        |
|-----------------------|----------|----------|------------|
| Glycerin              | LabChem  | Portugal | GLU158492  |
| Oleic Acid            | Acofarma | Spain    | 190034-O-2 |
| SPAN <sup>®</sup> 80  | Acofarma | Spain    | 215742-N-1 |
| Tween <sup>®</sup> 80 | Acofarma | Spain    | 222722-N-1 |

Table 3 - List of equipment used in this chapter.

| Equipment               | Producer                         | Model   | Country |
|-------------------------|----------------------------------|---------|---------|
| Semi-analytical balance | Adventurer <sup>™</sup><br>OHAUS | ARC 120 | China   |

| Equipment                 | Producer          | Model               | Country |
|---------------------------|-------------------|---------------------|---------|
| Stirring Plate            | IKA®              | C-MAG HS 7          | Germany |
| Ultra-turrax              | Heidolph          | Kinematica Pt 45-80 | Swiss   |
| Sonicator                 | Sonic Vibra-cell™ | VCX 130             | USA     |
| Water Purification System | MERCK             | Direct-Q® 3UV-R     | Germany |

7.60 g of water and 7.60 g of the CNS with GT polyphenolic compounds were mixed in a beaker mixture with magnetic stirring at 1250 RPM. A mixture of 5.00 g of SPAN® 80 and 15.00 g of oleic acid was prepared in a second beaker with magnetic agitation at 1250 RPM. The third beaker was heated at 50 °C to improve the dissolution of 5.00 g of Tween® 80 in 54.80 g of water and, after that, 5.00 g of glycerin was added.

The aqueous phase contained in the first beaker was added to the oil phase contained in the second beaker with agitation at 8000 RPM for 2 minutes using an ultra-turrax IKA (Germany) to form the W/O emulsion. After 2 minutes of agitation, the aqueous phase contained in the third beaker was added to the W/O emulsion, followed by 2 minutes of ultra-turrax agitation at 8000 RPM, forming the W/O/W multiple microemulsion (MME).

The oral microemulsion was prepared using the excipients described in Table 2.

### 2.6.1. Accelerated Stability by Centrifugation

The accelerated stability tests of oral microemulsions were performed using an Eppendorf AG centrifugate model 5804 (Germany) at 3500 RPM for 30 minutes, and if phase separation did not occur, the samples were centrifuged for another 30 minutes.

### 2.6.2. Dynamic Light Scattering (DLS)

The samples were placed in a DLS cuvette and the measurements were performed using a DLS ZetaPALS Brookhaven Instruments (EUA).

## 2.7. Development of a Syrup with Green Tea Extracted with Commercial Natural Solvents

Table 4 - List of raw materials used in this chapter.

| Raw Materials   | Producer | Country  | Lot        |
|-----------------|----------|----------|------------|
| Glycerin        | LabChem  | Portugal | GLU158492  |
| Sodium Benzoate | Acofarma | Portugal | 216192-J-1 |
| Xanthan Gum     | Acofarma | Portugal | 230512-I-1 |

Table 5 - List of equipment used in this chapter.

| Equipment                 | Producer             | Model           | Country |
|---------------------------|----------------------|-----------------|---------|
| Semi-analytical balance   | Adventurer™<br>OHAUS | ARC 120         | China   |
| Stirring Plate            | IKA®                 | C-MAG HS 7      | Germany |
| Water Purification System | MERCK                | Direct-Q® 3UV-R | Germany |

A 0.8 % (w/w) xanthan gum solution was prepared at 80.0 °C with magnetic stirring at 1250 RPM. Then, this solution was allowed to cool to room temperature before resting overnight in the refrigerator to remove air bubbles.

For the syrup preparation 54.60 g of the xanthan gum solution previously prepared, 5.00 g of glycerin, 0.20 g of sodium benzoate, and 40.20 g of CNS containing GT polyphenolic compounds extracted were mixed with magnetic stirring at 1250 RPM, until complete homogenization.

The syrup was prepared using the raw materials described in Table 4.

## **2.8. Stability of Syrup with Green Tea Extracted with Commercial Natural Solvents**

### **2.8.1. High-Performance Liquid Chromatography – Diode Array (HPLC - DAD)**

All the stability assays were performed using the same equipment and methods described in the section 2.4.2.

### **2.8.2. Colorimetry**

The equipment and procedure were described in the previous section 2.5.2.

### **2.8.3. Rheology**

Rheology of syrup formulations was performed using a Kinexus Lab+ (United Kingdom) rheometer model KNX2110.

To measure the viscosity, about 2.0 mL of syrup was placed in the rheometer, and a shear rate range of  $0.1 \text{ s}^{-1}$  to  $100 \text{ s}^{-1}$  was performed with 10 samples per decade, and a flow curve was plotted.

## 2.9. *In vitro* Assays

### 2.9.1. Infogest Static *in vitro* Simulation

The assays of digestion simulation were performed following the Infogest protocol.<sup>71</sup>

Tables 6 and 7 shows the raw material and equipment used in this section.

Table 6 - List of raw materials used in this chapter.

| Raw Materials                         | Producer | Country  | Lot       |
|---------------------------------------|----------|----------|-----------|
| Bile Extract Porcine                  | SIGMA    | Portugal | SLBR0091V |
| Calcium Chloride Anhydrous            | Scharlau | Spain    | 11386701  |
| Lipase from Porcine Pancreas, Type II | SIGMA    | Portugal | 155K0681  |
| Pancreatin from Porcine Pancreas      | SIGMA    | Portugal | SLBM0006V |
| Pepsin, from Porcine Gastric Musocsa  | SIGMA    | Portugal | BCBG3305V |

Table 7 - List of equipment used in this chapter.

| Equipment          | Producer                | Model                                | Country        |
|--------------------|-------------------------|--------------------------------------|----------------|
| Analytical Balance | Kern & Sohn             | ABT 120-5DM                          | Germany        |
| Centrifuge         | Dynamica Scientific Ltd | Velocity 14R Refrigerated Centrifuge | United Kingdom |

For the Infogest static *in vitro* simulation, two solutions of HCl and NaOH 1 M, and a solution of CaCl<sub>2</sub> 3M were prepared, and SGF, SIF, and SSF were prepared in accordance with described by Infogest protocol.<sup>71</sup> The solutions of lipase, pepsin, pancreatin, and bile were prepared with a final activity and concentration of 60 U/mL, 2000 U/mL, 100 U/mL, and 10mM respectively. Initially, the solutions of SGF, SIF, and SSF were pre-warm in the controlled bath with agitation at 60 RPM and 37 °C. 1.0000 g of each sample was measured into a falcon, then 800.0 µL of SSF, 5.00 µL of CaCl<sub>2</sub> 3M, and 195.00 µL of water MilliQ were added to the sample and left incubated in the controlled bath with agitation at 60 RPM and 37 °C for 2 minutes. To the previous solution, 1600.0 µL of SGF was added and the pH was adjusted to 3.0, then, 1.00 µL of CaCl<sub>2</sub> 3M and 100.00 µL of lipase and pepsin solutions were added to the solution and a final volume of 4.00 mL completed with water MilliQ, after the sample was incubated in the previous conditions for

2 hours. After that time, 1700.0  $\mu\text{L}$  of SIF was added to the solution and the pH was adjusted to 7.0. After the pH adjustment, 8.00  $\mu\text{L}$  of  $\text{CaCl}_2$  3M, 1000.0  $\mu\text{L}$  of pancreatin solution, and 500.0  $\mu\text{L}$  of bile solution were added to the solution and the volume final of 8.00 mL completed with water MilliQ and left to incubate at the previous conditions for 2 hours.

At the end of the incubation, the sample was placed in a bath at 100.0  $^{\circ}\text{C}$  for 5 minutes for enzyme activity inactivation, and then 4.00 mL of a solution of ethanol and methanol (1:1 v/v) previously prepared and pre-cooled at -20.0  $^{\circ}\text{C}$  was added to promote the proteins precipitation. To separate the protein precipitated, the sample was placed in a centrifuge at 13000 RPM for 10 minutes at 4.0  $^{\circ}\text{C}$ . After, the ethanol and methanol (1:1 v/v) solution was evaporated with a rotary evaporator, the remaining solution was transferred to a syringe filled with C18 and washed under vacuum with 900.0 mL of water MilliQ to lower the concentration of salts in the solution. In the end, 100.0 mL of methanol was used to clean the compounds trapped in the C18, and the methanol solution was collected. 10.0 mL of water MilliQ was added to the methanol collected and put in a rotary evaporator until methanol fully evaporated from the solution. The final solution was lyophilized.

### 2.9.2. Transwell™ Assays

Tables 8 and 9 shows the raw material and equipment used in this section.

Table 8 - List of raw materials used in this chapter.

| Raw Materials                                                                                                                                 | Producer        | Country |
|-----------------------------------------------------------------------------------------------------------------------------------------------|-----------------|---------|
| 3-(4,5-dimethylthiazol-2-yl)-<br>2,5-diphenyltetrazolium<br>bromide (MTT)                                                                     | Invitrogen      | USA     |
| Corning® Transwell™ 12-<br>well inserts with a<br>polycarbonate membrane<br>(1.12 $\text{cm}^2$ surface area, 0.4<br>$\mu\text{m}$ pore size) | Corning Coaster | USA     |
| Dimethyl sulphoxide<br>(DMSO)                                                                                                                 | Fluka           | Spain   |
| Dulbecco's modified<br>Eagle's Medium (DMEM)-                                                                                                 | Sigma-Aldrich   | Spain   |

| Raw Materials                                        | Producer           | Country |
|------------------------------------------------------|--------------------|---------|
| high glucose without L-glutamine and sodium pyruvate |                    |         |
| Glutamine                                            | Biowest            | France  |
| Hanks' Balanced Salts Solution (HBSS)                | Sigma-Aldrich      | Spain   |
| Heat-inactivated fetal bovine serum (FBS)            | Biowest            | France  |
| Lucifer yellow (LY)                                  | Sigma-Aldrich      | Spain   |
| Neutral red (NR) solution                            | Sigma-Aldrich      | Spain   |
| Penicillin–streptomycin antibiotic solution          | Gibco Laboratories | USA     |
| Phosphate buffered saline (PBS) tablets              | PanReac Applichem  | Spain   |
| Sodium pyruvate solution                             | Sigma-Aldrich      | Spain   |
| Transferrin                                          | Sigma-Aldrich      | Spain   |
| Trypsin-0.5%                                         |                    |         |
| ethylenediaminetetraacetic acid (EDTA) solution      | Gibco Laboratories | USA     |

Table 9 - List of equipment used in this chapter.

| Equipment         | Producer               | Model                    | Country |
|-------------------|------------------------|--------------------------|---------|
| Microplate Reader | Molecular Devices, LLC | FlexStation 3 Multi-Mode | EUA     |
| Voltohmeter       | Bedford                | Millicell® ERS-2         | EUA     |

### 2.9.2.1. Cell Culture and Seeding

Caco-2 cells were cultured to confluence in 75-cm<sup>2</sup> flasks containing DMEM cell culture medium supplemented with 10 % FBS, 1 % penicillin–streptomycin, 1 % human transferrin, 1 % sodium pyruvate and 1 % glutamine in a humid atmosphere of 5 % CO<sub>2</sub> and at 37 °C. The culture medium in the flask was changed every 2-3 days until the cells reached about 85 % confluency at which time the cells were plated. Briefly, flask medium was removed, and the Caco-2 cells were washed with PBS. Then, trypsin–0.5 % EDTA was

added, and the flask incubated for about 5 minutes, with agitation 2–3 times to encourage cell detachment. Following complete cell detachment, cells were then homogeneously resuspended in culture medium. Cell counting was made using a hemocytometer (Neubauer chamber) and cells were seeded at density of 100,000 cells/cm<sup>2</sup> on 12-Transwell™ inserts and 48-well plates, or at density of 150,000 cells/cm<sup>2</sup> in 96-well plates, according to the type of assay performed. The 96-well plate was incubated for 3 days (to reach cell confluence) while remaining plates were incubated and cultured for 21 days with medium refresh every 2-3 days until experiments. The cells used in all experiments were taken between the 29<sup>th</sup> and 32<sup>nd</sup> passages.

### 2.9.2.2. Cytotoxicity Evaluation

Samples's cytotoxicity was assessed by two distinct methods: MTT reduction assay and NR uptake assay. The MTT reduction assay is a colorimetric assay used as an indicator of cellular metabolic activity once the principle of the method is based on the activity of NAD(P)H-dependent oxidoreductase enzymes in metabolically active cells to reduce the yellow tetrazolium salt MTT to water-insoluble purple formazan crystals.<sup>72</sup> The NR uptake assay is used to estimate cell viability in culture. The estimation of viable cells is based in the ability of living cells to incorporate and bind the weakly cationic neutral red dye in their lysosomes.<sup>73</sup> In both assays absorbance of the control cells was considered as 100 %.

The undifferentiated cells (initial cytotoxicity screening) seeded in 96-well plates were treated with samples with dilutions of 0, 1:2, 1:3, 1:6 and 1:10 (in HBSS) and incubated for 2 hours. The cells were then incubated for an additional 1 hour in culture medium containing MTT solution at final concentration of 0.45 mg/mL. Following removal of the culture medium, the formazan crystals were dissolved using DMSO. The absorbance was then measured at 570nm using the microplate reader.

The differentiated cells previously seeded in 48-well plates were treated as described for the MTT reduction assay with sample dilution of 1:6 and 1:10 for further confirmation of the initial cytotoxic screening in the same conditions of the bioavailability assay (21 days of differentiation). The cells were then incubated for an additional 1 hour in culture medium containing NR solution at a final concentration of 50 µg/mL. Following removal of the culture medium, the NR destain solution (1 % glacial acetic acid solution, 50 % ethanol, 49 % distilled water) was added to each well and the absorbance was measured at 540 nm using a microplate reader.



### 2.9.2.3. Transepithelial Electrical Resistance (TEER)

Manipulation of the cell monolayers seeded in the Transwell™ inserts was conducted under sterile conditions (in the laminar flow cabinet) with culture medium replaced by fresh medium every 2–3 days until bioavailability experiments. The TEER value of each insert was measured simultaneously to medium replacement, using a Millicell® ERS-2 voltohmmeter equipped with a chopstick electrode pair, over the 21 days of differentiation. Average of TEER values for cell monolayers were around 1200  $\Omega \cdot \text{cm}^2$  (two independent experiments).

At the day of the experiments, culture medium was removed and HBSS (further supplemented with 1 % transferrin) pre-warmed to 37 °C was added to the apical (0.5 mL) and basolateral (1.5 mL) sides to wash the cell monolayers. Upon the washing step, new HBSS was added to the inserts to measure the TEER values ( $t_0$ ). Then, the HBSS was removed from the apical compartment and the non-cytotoxic dilution of 1:10 of each sample was loaded into triplicate to the apical wells and incubated at 37 °C for 2 hours. At the end of incubating period, the samples from both apical and basolateral sides were collected and stored at -20 °C for further analysis. New HBSS was added to the inserts to measure the TEER value ( $t_2$ ).

### 2.9.2.4. Characterization of Basolateral Samples

#### 2.9.2.4.1. High-Performance Liquid Chromatography - Mass Spectroscopy (HPLC - MS)

All samples were analyzed by HPLC-ESI-Q-Orbitrap-MS. The analyses were performed in a HPLC Vanquish (Thermo Fischer Scientific, Bremen, Germany) coupled to an Orbitrap Exploris 120 mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) controlled by Orbitrap Exploris Tune Application 2.0.185.35 and Xcalibur 4.4.16.14. The capillary voltage of the electrospray ionization source (ESI) was set to 3.5 kV and 2.5 kV for positive and negative mode. SRM mode was used for all possible compounds. The capillary temperature was 300 °C. The sheath gas, auxiliary gas, and sweep gas flow rate were at 50, 10, 1 (arbitrary unit as provided by the software settings). The resolution of MS scan

was 60 000. Data-dependent MS/MS was performed on HCD using nitrogen as gas with collision energy settings of 30 V. The m/z range were 100-1000 Da.

The elution gradient was performed as the same described in section 2.4.1.

#### **2.9.2.4.2. High-Performance Liquid Chromatography – Diode Array (HPLC - DAD)**

All the samples were analyzed in a Merck-Hitachi Elite LaChrom HPLC-DAD on a 150 x 4.6 mm i.d., 5 µm pore size reversed-phase C18 column (Merck) thermostated at 25°C (Merck-Hitachi Column Oven L-2300). Detection was carried out at 280 nm using a diode array detector (Merck-Hitachi Diode Array Detector L-2455). The method used was the same as described earlier.

The calibration curve for (-)-epicatechin was obtained using a concentration range of 1.300 to 0.000688 mg mL<sup>-1</sup> was used and each sample was prepared in duplicate and injected in triplicate. Unknown concentrations were determined from the regression equation ( $y = 1E8 x + 1E6$ ;  $r^2 = 0.9987$ ).

All results were expressed as mean ± standard deviation and presented as mg mL<sup>-1</sup> equivalents of (-)-epicatechin.

The elution gradient was performed as the same described in section 2.4.1.

### **2.10. Statistical Analyses**

The data were analyzed by ANOVA with Bonferroni post-test using the software package GraphPad Prism, CA, USA, version 9.5.0 for Mac.

# **CHAPTER 3:**

## **RESULTS AND DISCUSSION**



## CHAPTER 3 – RESULTS AND DISCUSSION

### 3.1. Thermogravimetry of Commercial Natural Solvents

TGA is an analytical technique used to investigate the thermal decomposition of materials. It provides valuable information about samples mass changes as a function of temperature, allowing for quantitative and qualitative analysis of its constituents.<sup>74, 75</sup> TGA works by measuring the mass variation of a sample as it is heated or cooled in a controlled environment. A small amount of the sample is placed on a thermogravimetric balance, a sensitive microbalance that records mass variations during the experiment (Figure 14).<sup>74, 75</sup>

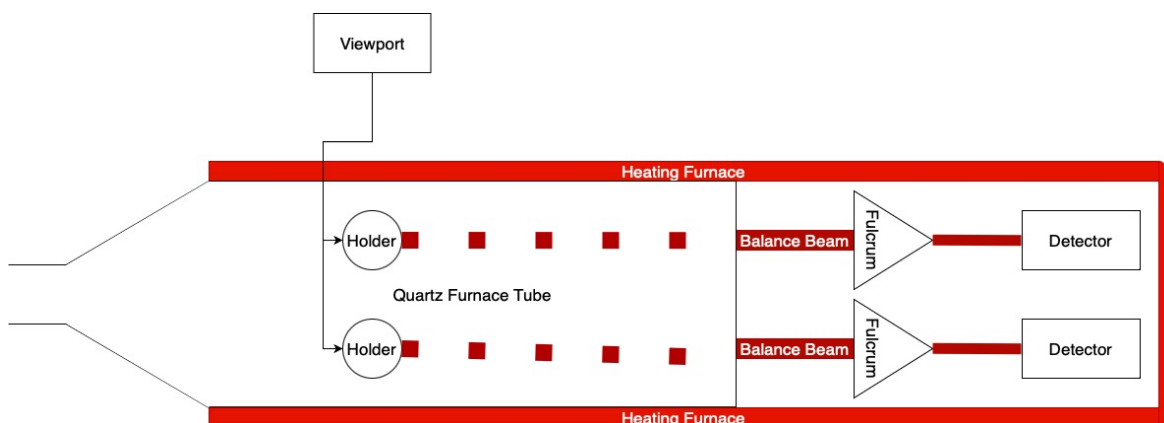


Figure 14 - Thermogravimetry schematic process.

Thermal reactions such as solvent evaporation, decomposition of organic or inorganic compounds, oxidation or reduction of materials, and other processes occur during heating. These reactions cause mass variations in the sample, which the thermogravimetric balance records.<sup>74, 76</sup>

TGA data is frequently represented in a thermogravimetric plot, which plots mass variation against temperature. This curve can be used to identify mass loss events, calculate decomposition temperatures, and estimate the amount of each component in the sample.<sup>74, 75</sup> This technique is widely used in many fields, including chemistry, materials science, pharmacology, and environmental analysis. It is useful in the study of the thermal stability of substances, polymer characterization, organic and inorganic compound analysis, moisture content determination, and other applications.<sup>76</sup>

Figure 15 shows the TG curves and DTG curves for thermal decomposition of the CNSs.

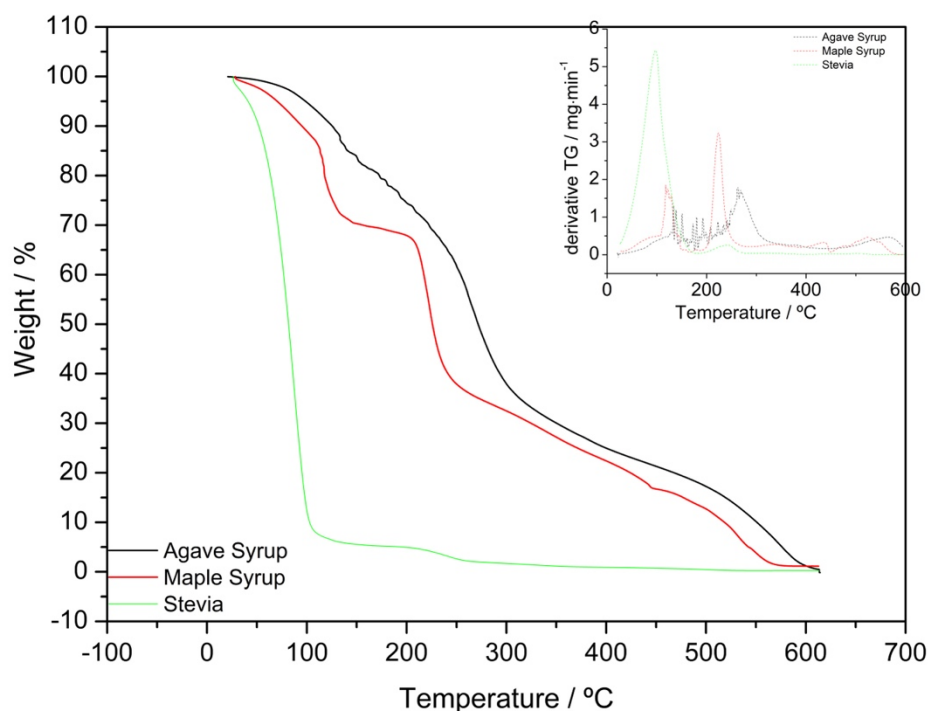


Figure 15 - TG curves and DTG (insert graphic) curves for thermal decomposition of agave syrup, maple syrup, and stevia.

In Figure 15 it is possible to see that stevia started the degradation at the initial temperature range and lost more than 90 % of the initial weight until reach 100 °C. This early degradation is probably due to the major component in stevia CNS: water, as described in stevia label; which was evaporated until 100 °C, after a couple of compounds degradation occurs, as shown in Figure 16. In this case, this variation of weight % can be due to the steviol glycosides from stevia and other compounds used as preservatives and stabilizers. Agave and maple syrup demonstrated more gradual weight loss, with more than 50 % weigh lose after 200 °C. Agave syrup showed degradation of several compounds between 150 °C and 250 °C, and that can be confirmed in the insert graph (DTG). Table 10 shows the initial temperature of degradation ( $T_{\text{onset}}$ ) and temperature of degradation of the various components in each CNS ( $T_{\text{peak}}$ ).

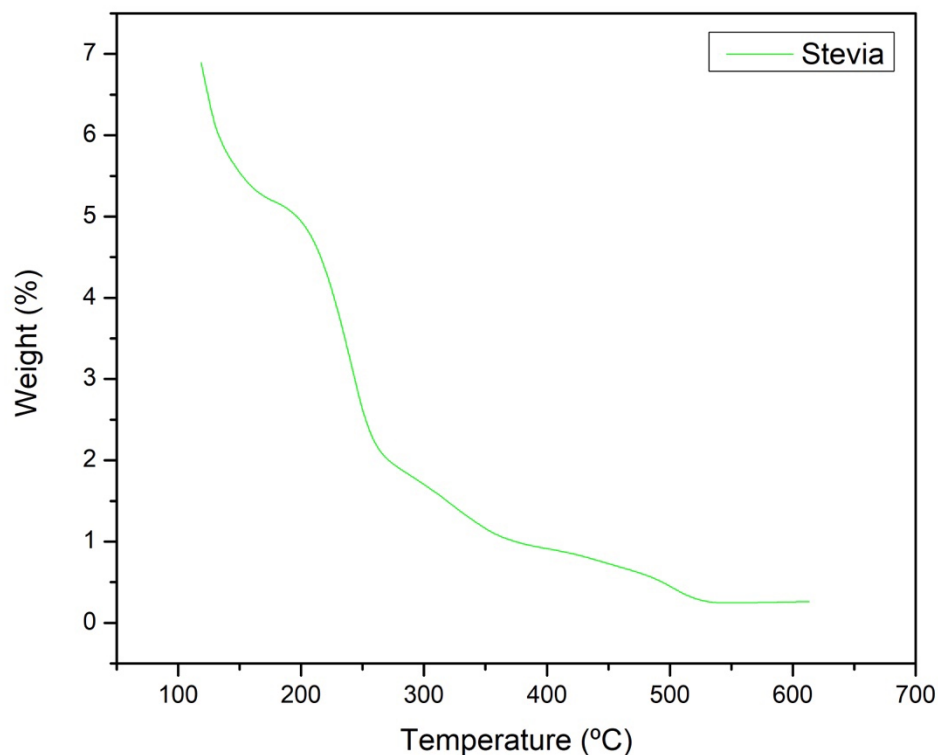


Figure 16 - TG curves for thermal decomposition of stevia after 100 °C.

Table 10 - Thermal decomposition parameters for agave syrup, maple syrup, and stevia.

| <b>Commercial<br/>Natural Solvent</b> | <b>T<sub>onset</sub> (°C)</b> | <b>T<sub>peak 1</sub> (°C)</b> | <b>T<sub>peak 2</sub> (°C)</b> | <b>Δm (%)</b> |
|---------------------------------------|-------------------------------|--------------------------------|--------------------------------|---------------|
| Agave Syrup                           | 69.32                         | 136.96                         | 265.88                         | 99.5          |
| Maple Syrup                           | 91.82                         | 116.57                         | 223.43                         | 98.8          |
| Stevia                                | 208.60                        | 97.86                          | 242.13                         | 99.7          |

Assuming that mass variation until 100 °C in stevia is due to water loss, the T<sub>onset</sub> value was measured considering the values in Figure 16 being the degradation of the rest of the compounds at 208.60 °C (Table 10). In the case of agave and maple syrup, the temperature of the initial degradation was 69.32 °C and 91.82 °C, respectively (Table 10). With these assays, we can understand if the CNSs demonstrate degradation at the extraction temperature utilized.

## 3.2. Green Tea Polyphenolic Compounds Extraction and Characterization

### 3.2.1. Optimization of the procedure

The extraction of GT polyphenolic compounds was initially performed using the traditional method, with the addition of GT powder to the CNS and stirring for 15 minutes,<sup>28</sup> and the different conditions received an abbreviation code, as shown in Table 11.

Table 11 - Codification between the condition of extraction of the GT polyphenolic compounds and the attributed code by authors.

| The ratio between Liquid and Solid | Extraction Medium | T (°C)           | Name attributed by authors             |
|------------------------------------|-------------------|------------------|----------------------------------------|
| 5.000:0.2500                       | Water             | Room Temperature | GTH <sub>2</sub> O_50_T <sub>amb</sub> |
| 5.000:0.2500                       | Water             | 50               | GTH <sub>2</sub> O_50_T <sub>50</sub>  |
| 5.000:0.2500                       | Water             | 100              | GTH <sub>2</sub> O_50_T <sub>100</sub> |
| 10.000:0.2500                      | Water             | Room Temperature | GTH <sub>2</sub> O_25_T <sub>amb</sub> |
| 10.000:0.2500                      | Water             | 50               | GTH <sub>2</sub> O_25_T <sub>50</sub>  |
| 10.000:0.2500                      | Water             | 100              | GTH <sub>2</sub> O_25_T <sub>100</sub> |
| 5.000:0.2500                       | Agave Syrup       | Room Temperature | GTXAG_50_T <sub>amb</sub>              |
| 5.000:0.2500                       | Agave Syrup       | 50               | GTXAG_50_T <sub>50</sub>               |
| 5.000:0.2500                       | Agave Syrup       | 100              | GTXAG_50_T <sub>100</sub>              |
| 10.000:0.2500                      | Agave Syrup       | Room Temperature | GTXAG_25_T <sub>amb</sub>              |
| 10.000:0.2500                      | Agave Syrup       | 50               | GTXAG_25_T <sub>50</sub>               |
| 10.000:0.2500                      | Agave Syrup       | 100              | GTXAG_25_T <sub>100</sub>              |
| 5.000:0.2500                       | Honey             | Room Temperature | GTM_50_T <sub>amb</sub>                |
| 5.000:0.2500                       | Honey             | 50               | GTM_50_T <sub>50</sub>                 |
| 5.000:0.2500                       | Honey             | 100              | GTM_50_T <sub>100</sub>                |
| 10.000:0.2500                      | Honey             | Room Temperature | GTM_25_T <sub>amb</sub>                |
| 10.000:0.2500                      | Honey             | 50               | GTM_25_T <sub>50</sub>                 |
| 10.000:0.2500                      | Honey             | 100              | GTM_25_T <sub>100</sub>                |
| 5.000:0.2500                       | Maple Syrup       | Room Temperature | GTXA_50_T <sub>amb</sub>               |
| 5.000:0.2500                       | Maple Syrup       | 50               | GTXA_50_T <sub>50</sub>                |
| 5.000:0.2500                       | Maple Syrup       | 100              | GTXA_50_T <sub>100</sub>               |
| 10.000:0.2500                      | Maple Syrup       | Room Temperature | GTXA_25_T <sub>amb</sub>               |



| The ratio between Liquid and Solid | Extraction Medium         | T (°C)           | Name attributed by authors                   |
|------------------------------------|---------------------------|------------------|----------------------------------------------|
| 10.000:0.2500                      | Maple Syrup               | 50               | GTXA_25_ T <sub>50</sub>                     |
| 10.000:0.2500                      | Maple Syrup               | 100              | GTXA_25_ T <sub>100</sub>                    |
| 5.000:0.2500                       | Stevia                    | Room Temperature | GTS_50_ T <sub>amb</sub>                     |
| 5.000:0.2500                       | Stevia                    | 50               | GTS_50_ T <sub>50</sub>                      |
| 5.000:0.2500                       | Stevia                    | 100              | GTS_50_ T <sub>100</sub>                     |
| 10.000:0.2500                      | Stevia                    | Room Temperature | GTS_25_ T <sub>amb</sub>                     |
| 10.000:0.2500                      | Stevia                    | 50               | GTS_25_ T <sub>50</sub>                      |
| 10.000:0.2500                      | Stevia                    | 10               | GTS_25_ T <sub>100</sub>                     |
| 7.500:2.500:0.2500                 | Stevia:Water<br>(3:1 w/w) | 100              | GT75S25H <sub>2</sub> O_25_ T <sub>100</sub> |
| 5.000:5.000:0.2500                 | Stevia:Water<br>(1:1 w/w) | 100              | GT50S50H <sub>2</sub> O_25_ T <sub>100</sub> |
| 2.500:7.500:0.2500                 | Stevia:Water<br>(1:3 w/w) | 100              | GT25S75H <sub>2</sub> O_25_ T <sub>100</sub> |

After the GTXA 25\_ T<sub>100</sub> extraction, due to the high concentration of sugar in the solution, sugar crystals solidified. The low amount of water evaporated, resulting in the precipitation of the sugar in the solution, which is common in the food industry.<sup>77</sup> To prevent this undesirable crystallization, a reflux condenser system was used, minimizing water loss through evaporation. All extractions were performed using the same reflux condenser system.

After minimizing the water loss, filtration of the GT powder from the CNSs was required. Initially, a traditional GT net was used to remove the largest particles from the CNSs, and then the extract was centrifuged at 21,000 g (9 ACC, 25(C) for 15 minutes,<sup>28</sup> but, due to the viscosity increase in the combination of the GT polyphenolic compounds with the already high viscous agave syrup, honey, and maple syrup solvents, it was necessary to adapt the method to achieve a better elimination of the GT powder particles. The traditional green tea net was replaced by a tea bag with lower porosity, decreasing the powder in the solution, and the filtered solution was centrifuged at 21.000 g (9 ACC, 25C) for 15 minutes. This methodology resulted in a significantly decrease of the extraction yield in the GTXAG\_25\_ T<sub>amb</sub>, GTXAG\_25\_ T<sub>50</sub>, GTXAG\_50\_ T<sub>amb</sub>, GTXAG\_50\_ T<sub>50</sub>, GTM\_25\_ T<sub>amb</sub>, GTM\_25\_ T<sub>50</sub>, GTM\_25\_ T<sub>100</sub>, GTM\_50\_ T<sub>amb</sub>, GTM\_50\_ T<sub>50</sub>, GTM\_50\_ T<sub>100</sub>, GTXA\_25\_ T<sub>amb</sub>, GTXA\_25\_ T<sub>50</sub>, GTXA\_50\_ T<sub>amb</sub>, and GTXA\_50\_ T<sub>50</sub>, with higher viscosity,

so these extracts will be disregarded for future phases of pharmaceutical formulation preparation. The remaining conditions produced a good extraction yield.

After optimizing the extraction and filtration procedures, the characterization procedure must be optimized as well. (-)-Epicatechin (Figure 17) was defined as a calibration curve due to being a compound representative of polyphenolic compounds in GT with a good answer from the equipment at different concentrations.

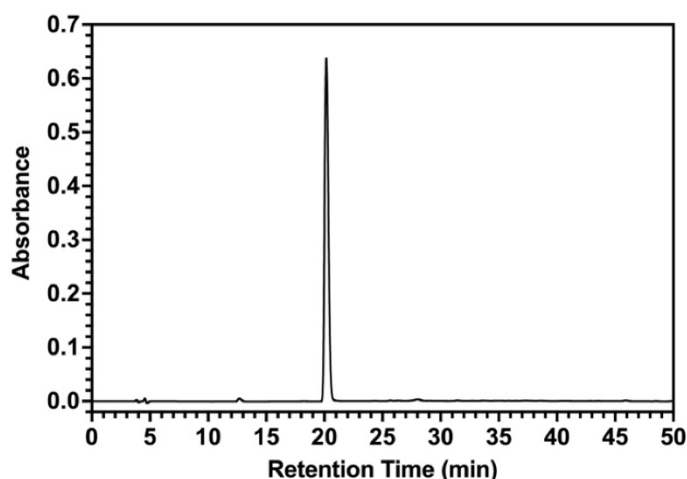


Figure 17 - (-)-Epicatechin chromatography obtained through HPLC-DAD analyses.

At this point it is necessary to understand how to overcome the obstacles to quantitative and qualitative analysis of the extracted GT polyphenolic compounds. Because some CNS, such as agave syrup, honey, and maple syrup, have a high viscosity, and the incorporation of GT polyphenolic compounds in the solvents increases that viscosity. A direct injection in the HPLC-DAD equipment can result in an inaccurate measurement of the volume injected and even obstruct the solvent lines. To address this issue, a liquid-liquid extraction using ethyl acetate (AcOEt) was performed. This liquid-liquid extraction is intended to remove the GT polyphenolic compounds from the CNS matrix, allowing quantitative and qualitative analysis. Following the extraction, the AcOEt was evaporated to dryness, and 500  $\mu\text{L}$  of water were added and placed in ultrasound to facilitate solubilization. Finally, the solution was injected into the HPLC-DAD, and the results indicated that a proper extraction of the GT polyphenolic compounds from the CNS matrix had not been achieved (Figure 18).

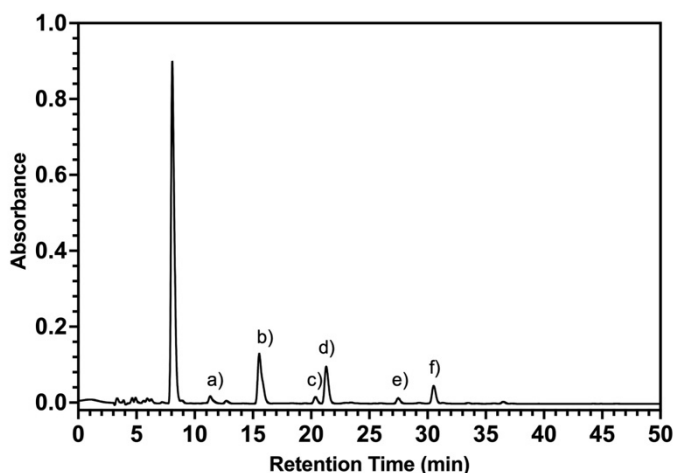


Figure 18 - Typical chromatography obtained through HPLC-DAD with liquid-liquid extraction of the polyphenolic compounds in the CNS. a) (E)GC; b) PD-BG; c) (E)C; d) (E)GCG; e) Quercetin-deoxyhexoside-hexoside-hexoside; and f) (E)CG.

An internal standard was used to test a new approach, so, some compounds were tested with a concentration of 1.00 mg/mL and injected into an HPLC-DAD to see the chromatography profile.

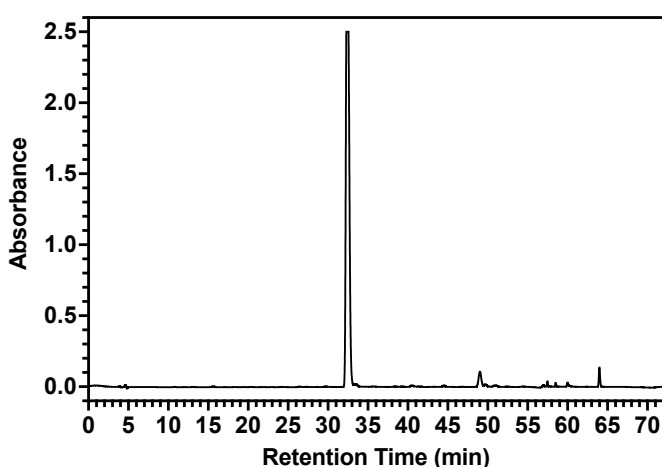


Figure 19 - (+)-Taxifolin chromatography obtained through HPLC-DAD analyses.

After analyzing all the chromatograms, the Ascorbic Acid showed a poor response in terms of retention time and equipment, the Acid Gallic had an inappropriate retention time, and the 2,3-Dihydroxybenzoic Acid, 4-Hydroxybenzoic Acid, and Gentisic Acid showed a poor response from the equipment for the concentration of 1 mg/mL, so (+)-Taxifolin (Figure 19) was chosen as a compound with a retention time that did not interfere with the retention time of the GT polyphenolic compounds and had a good response from the equipment.

(+)-Taxifolin with a concentration of 0.50 mg/mL was prepared and injected in HPLC-DAD with GTXAG\_25\_T<sub>amb</sub> (1:1 v/v), but the results obtained suggested that the problem was due to an unsuitable volume injection of the sample into the equipment. In Figure 20 we can see the changes in the equipment's answer in different analyses using the same sample.

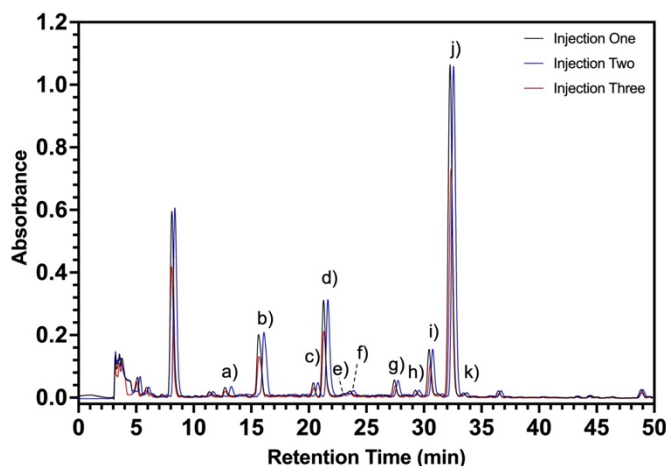


Figure 20 - GTXAG\_25\_T<sub>amb</sub> with (+)-Taxifolin (1:1 v/v) chromatography obtained through HPLC-DAD. a) – (E)GC; b) – PD-BG; c) – (E)C; d) – (E)GCG; e) – Gallic Acid; f) - Apigenin-6-C-pentoside-8-C-hexoside; g) - Quercetin-deoxyhexoside-hexoside-hexoside; h) - Quercetin-3-O-rutinoside; i) – (E)CG; j) – (+)-Taxifolin and k) - Kaempferol 3-rutinoside.

To determine whether a different volume was injected into the equipment, the weight of the vial before and after the injection was measured in four HPLC-DAD sample injections, and the results are shown in Table 12.

Table 12 - Weight variation in a vial after injection into HPLC-DAD.

| Measure No. | Vial Weight (g) | Injection No. | Weight Variation (g) |
|-------------|-----------------|---------------|----------------------|
| 1           | 3.14712         | 1             | 0.04720              |
| 2           | 3.09992         | 2             | 0.01300              |
| 3           | 3.08692         | 3             | 0.04567              |
| 4           | 3.04125         | 4             | 0.00519              |
| 5           | 3.03606         |               |                      |

As we can see in table 12, there is a significant difference in the vial weight variation after the injections in the HPLC-DAD. For example, the difference in weight variation between injections 1 and 4 is 0.04201 g, which is higher than the weight variations in injections 2 and 4.

Knowing what the problem was, a system of needle and line cleaning was performed after each HPLC-DAD analysis. The sample analysis was intercalated with a deionized water sample, and the dilution of 50% of the CNS samples was maintained, but instead of the internal standard dilution, only deionized water was used. The results indicated that the commutation of the problem was not achieved in some extractions with specific CNS and under specific conditions. To try a better accuracy injection, the HPLC-DAD method was modified, and the thermostat temperature was set at 30.0 °C, lowering the viscosity of the samples in the study. This alteration in the method demonstrated better volume injection results. However, in some conditions this alteration showed to be insufficient. As a result, the analysis of the extractions with an extraction ratio of 0.2500 g of GTP to 10.000 g of maple syrup does not have reproducible results (Figure 21), and the extractions with honey demonstrated a bad answer from the equipment and non-reproducible results (Figure 22) in all ratios and temperature conditions, indicating a risk of blocking the equipment's line systems. Following these assays, the quantitative and qualitative analyses will take place under the following conditions represented in Table 13:

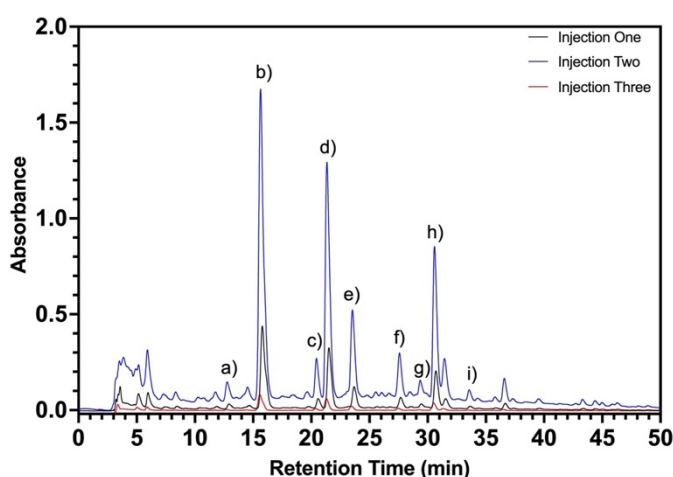


Figure 21 - GTXA\_25\_T<sub>100</sub> with water (1:1 v/v) chromatography obtained through HPLC-DAD. a) – (E)GC; b) – PD-BG; c) – (E)C; d) – (E)GCG; e) - Apigenin-6-C-pentoside-8-C-hexoside; f) - Quercetin-deoxyhexoside-hexoside-hexoside; g) - Quercetin-3-O-rutinoside; h) – (E)CG; and i) - Kaempferol 3-rutinoside.

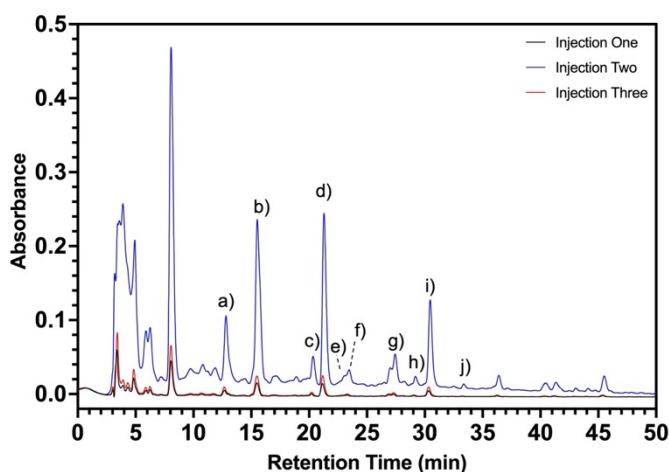


Figure 22 - GTM<sub>50</sub>T<sub>100</sub> with water (1:1 v/v) chromatography obtained through HPLC-DAD injection three. a) – (E)GC; b) – PD-BG; c) – (E)C; d) – (E)GCG; e) – Gallic Acid; f) - Apigenin-6-C-pentoside-8-C-hexoside; g) - Quercetin-deoxyhexoside-hexoside-hexoside; h) - Quercetin-3-O-rutinoside; and i) – (E)CG.

Table 13 - Advanced conditions for quantitative and qualitative assays.

| Advanced conditions                                    |
|--------------------------------------------------------|
| GTH <sub>2</sub> O <sub>50</sub> T <sub>amb</sub>      |
| GTH <sub>2</sub> O <sub>50</sub> T <sub>50</sub>       |
| GTH <sub>2</sub> O <sub>50</sub> T <sub>100</sub>      |
| GTH <sub>2</sub> O <sub>25</sub> T <sub>amb</sub>      |
| GTH <sub>2</sub> O <sub>25</sub> T <sub>50</sub>       |
| GTH <sub>2</sub> O <sub>25</sub> T <sub>100</sub>      |
| GTXAG <sub>50</sub> T <sub>amb</sub>                   |
| GTXAG <sub>50</sub> T <sub>50</sub>                    |
| GTXAG <sub>50</sub> T <sub>100</sub>                   |
| GTXA <sub>50</sub> T <sub>amb</sub>                    |
| GTXA <sub>50</sub> T <sub>50</sub>                     |
| GTXA <sub>50</sub> T <sub>100</sub>                    |
| GTS <sub>50</sub> T <sub>amb</sub>                     |
| GTS <sub>50</sub> T <sub>50</sub>                      |
| GTS <sub>50</sub> T <sub>100</sub>                     |
| GTS <sub>25</sub> T <sub>amb</sub>                     |
| GTS <sub>25</sub> T <sub>50</sub>                      |
| GTS <sub>25</sub> T <sub>100</sub>                     |
| GT75S25H <sub>2</sub> O <sub>25</sub> T <sub>100</sub> |
| GT50S50H <sub>2</sub> O <sub>25</sub> T <sub>100</sub> |
| GT25S75H <sub>2</sub> O <sub>25</sub> T <sub>100</sub> |

Due to non-reproducible results and risks associated with the equipment, all ratios of 0.2500 g of GTP to 10.000 g CNS were discarded for quantitative and qualitative studies, with the exception of stevia CNS, which was still possible to inject in HPLC-DAD without the risk of obstruction after the extractions, so they will be characterized as a comparison model to the 0.2500 g of GTP to 5.000 g of stevia. Apart from this condition, all honey CNS extractions were discarded due to their extremely high viscosity.

### **3.2.2. High-Performance Liquid Chromatography - Mass Spectrometry (HPLC-MS)**

The High-Performance Liquid Chromatography coupled with Electrospray Ionization Mass Spectrometry (HPLC-ESI-MS) technique is a powerful and sensitive analytical method that is widely used in various fields, such as chemistry and pharmacology in food analysis, drug discovery, and natural product research. It is a sophisticated approach that combines the benefits of High-Performance Liquid Chromatography (HPLC), which performs efficient chemical compound separation, with the sensitivity and accuracy of identification capabilities provided by mass spectrometry and electrospray ionization.<sup>78-80</sup>

The soft ionization technique ESI is commonly used in mass spectrometry. In ESI, the sample is nebulized into small, electrically charged droplets. These droplets are then dehydrated, resulting in ions that can be analyzed using MS. ESI is particularly useful for analyzing polar and labile compounds.<sup>81, 82</sup> MS is a detector that uses a mass-to-charge ratio ( $m/z$ ) to identify the components separated by HPLC (Figure 23). MS can detect both positive and negative ions, allowing it to identify a wide range of compounds.<sup>81, 83</sup> The HPLC-ESI-MS technique can detect compounds at low concentrations, making it extremely useful for analytical applications. This technique identifies and quantifies compounds in complex samples such as food, drugs, and natural products.<sup>81</sup>

Polyphenolic compounds are found in a variety of foods, including fruits, vegetables, tea, and wine. The HPLC-ESI-MS technique separates and identifies distinct subclasses of polyphenolic compounds, including flavonoids, phenolic acids, and tannins.<sup>28, 78, 84</sup> Because polyphenolic compounds are tendentially acidic compounds, dissociation is easier than protonation in this case. However, using the negative ionization mode with formic acid can result in the presence of adducts and dimerization at the source of the equipment.<sup>85</sup>

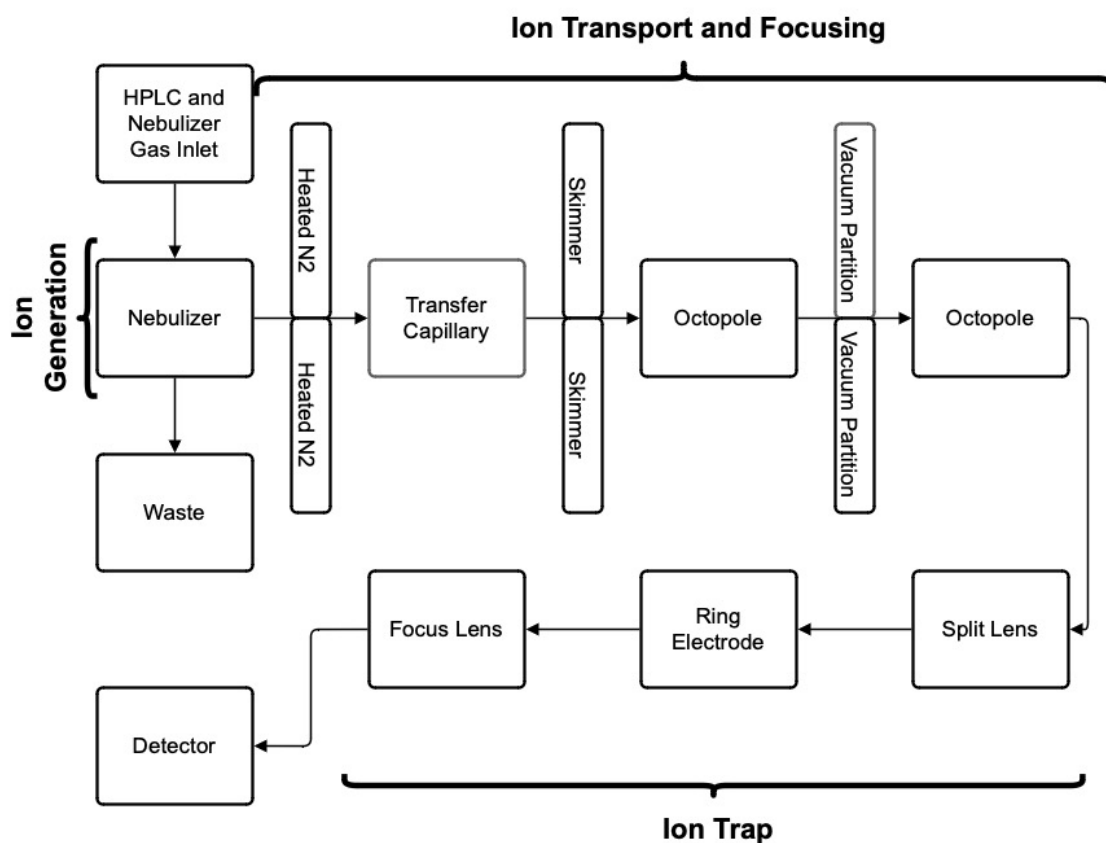


Figure 23 - ESI-MS schematic process.

The GT extracts were analyzed using HPLC-ESI-MS, and the different polyphenolic compounds are shown in Table 14.

Table 14 - GT Polyphenolic compounds identified by HPLC-MS in extracts.

| Peak No. | Compound                                    | [M-H] <sup>-</sup> (m/z) | MS <sup>2</sup> (m/z)                   | MS <sup>3</sup> (m/z)              |
|----------|---------------------------------------------|--------------------------|-----------------------------------------|------------------------------------|
| 1        | (E)GC                                       | 305                      | 261; 221; <b>179</b> ;<br>165; 137      | <b>163</b> ; 151; 135              |
| 2        | PD-BG                                       | 761                      | 609; <b>593</b> ;<br>575; 423           | <b>575</b> ; 423                   |
| 3        | (E)C                                        | 289                      | <b>245</b> ; 205; 179                   | 227; <b>203</b> ; 187              |
| 4        | (E)GCG                                      | 457                      | 331; 305; 287;<br>269; <b>169</b>       | 169; <b>125</b>                    |
| 5        | Gallic acid                                 | 169                      | <b>125</b>                              | 107; 97; 83; <b>81</b> ;<br>79; 69 |
| 6        | Apigenin-6-C-<br>pentoside-8-C-<br>hexoside | 563                      | 545; 503; 473;<br><b>443</b> ; 383; 353 | 425; 383; <b>353</b> ;<br>335      |



| Peak No. | Compound                                  | [M-H] <sup>-</sup> (m/z) | MS <sup>2</sup> (m/z) | MS <sup>3</sup> (m/z)                |
|----------|-------------------------------------------|--------------------------|-----------------------|--------------------------------------|
| 7        | Quercetin-deoxyhexoside-hexoside-hexoside | 771                      | 609; <b>301</b>       | 273; 257; <b>179</b> ; 151           |
| 8        | Quercetin-3-O-rutinoside                  | 609                      | 343; <b>301</b>       | 273; 257; <b>179</b> ; 151           |
| 9        | (E)CG                                     | 441                      | <b>289</b>            | <b>245</b> ; 205; 179                |
| 10       | Kaempferol 3-rutinoside                   | 593                      | <b>285</b>            | 267; <b>257</b> ; 241; 229; 213; 197 |

(E)C - (Epi)Catechin, (E)GC - (Epi)Gallocatechin, (E)CG - (Epi)Catechin gallate, (E)GCG - (Epi)Gallocatechin gallate, PD-BG - Prodelphinidin B-4 3'-O-gallate.

Several compounds, including flavan-3-ol, flavonoids, and tannins, have been identified in GT extracts with CNS, as shown in Table 3. The major catechins in the flavan-3-ol subclass have been identified. Peak 1 was identified as (E)GC due to the deprotonated ion at m/z 305 and the main MS<sup>2</sup> fragmentation ion at m/z 179, both of which correspond to the literature.<sup>28, 86</sup> (E)C was identified in peak 3 as an m/z 289 corresponding to the deprotonated ion, and the ion fragmentation pattern agrees with that described in the literature, with an MS<sup>2</sup> majority ion m/z 245 as expected.<sup>28, 86, 87</sup> Peak 4 m/z 915 corresponds to a probable dimeric of (E)GCG with an m/z 457 and an MS<sup>2</sup> with the expected major fragmentation ion of m/z 169.<sup>28, 86</sup> The final, (E)CG m/z 441, demonstrated a probable dimeric in the source m/z 883 and an MS<sup>2</sup> fragmentation consistent with the literature.<sup>28, 86, 88</sup>

Four probable flavonoids were identified, Apigenin-6-c-pentoside-8-c-hexoside in peak 6, with a deprotonated ion at m/z 563, and the same pattern of MS<sup>2</sup> fragmentation described in the literature, with the majority fragment at m/z 443.<sup>89, 90</sup> Peak 7 contained a deprotonated ion with an m/z 771 that was identified as a possible Quercetin-deoxyhexoside-hexoside-hexoside with an MS<sup>2</sup> and MS<sup>3</sup> majority fragmentation of m/z 301 and m/z 179, as described in the literature.<sup>91</sup> Quercetin-3-O-rutinoside was identified in peak 8 in agreement with what has been described in the literature, as a deprotonated ion of m/z 609 with major ion fragmentation in MS<sup>2</sup> and MS<sup>3</sup> of m/z 301 and m/z 179.<sup>92, 93</sup> The final possible flavonoid found was Kaempferol 3-rutinoside in peak 10, which had a deprotonated ion m/z 593 and the same ion fragmentation pattern as described in the literature.<sup>94, 95</sup>

A probable tannin and phenolic acid were identified in peak 2 with a deprotonation ion  $m/z$  of 761, identified as PD-BG, and gallic acid was identified in peak 5 with a deprotonation ion  $m/z$  of 169, both of which are ion fragmentation patterns consistent with what has been described in the literature.<sup>28, 96, 97</sup>

### 3.2.3. High-Performance Liquid Chromatography – Diode Array (HPLC - DAD)

HPLC-DAD (High-Performance Liquid Chromatography with Diode Array Detection) is a popular analytical technique for separating, identifying, and quantifying compounds in complex samples.<sup>98, 99</sup> The Diode Array Detector (DAD) is a detector that includes a series of light-sensitive silicon diodes. Because each diode absorbs light at a different wavelength, detection is possible across a wide range of the electromagnetic spectrum. This provides a significant advantage over traditional detectors, such as a single UV-visible detector, because the DAD can provide additional information about a sample's composition.<sup>100</sup> The sample is introduced into a liquid chromatography system, and the components of the sample are separated on a chromatographic column in HPLC-DAD. The different components elute at different rates, depending on their interactions with the column's mobile and stationary phases. The eluted components interact with the emitted light from the diodes as they pass through the diode array detector, and the absorption at different wavelengths is recorded (Figure 24).<sup>100, 101</sup>

The acquisition of an absorption spectrum for each chromatographic peak is enabled by detection at multiple wavelengths. Based on their specific absorption characteristics, these spectra can be used to identify the components present in the sample. Furthermore, HPLC-DAD allows for component quantification because absorption intensity is proportional to the concentration of the analyzed substance.<sup>100, 101</sup> Due to the non-polar nature of the polyphenolic compounds, HPLC-DAD analyses are performed using reverse phase columns, with a non-polar stationary phase (C18) and a polar mobile phase, facilitating the separation of the different compounds due to their different affinity for the stationary phase.

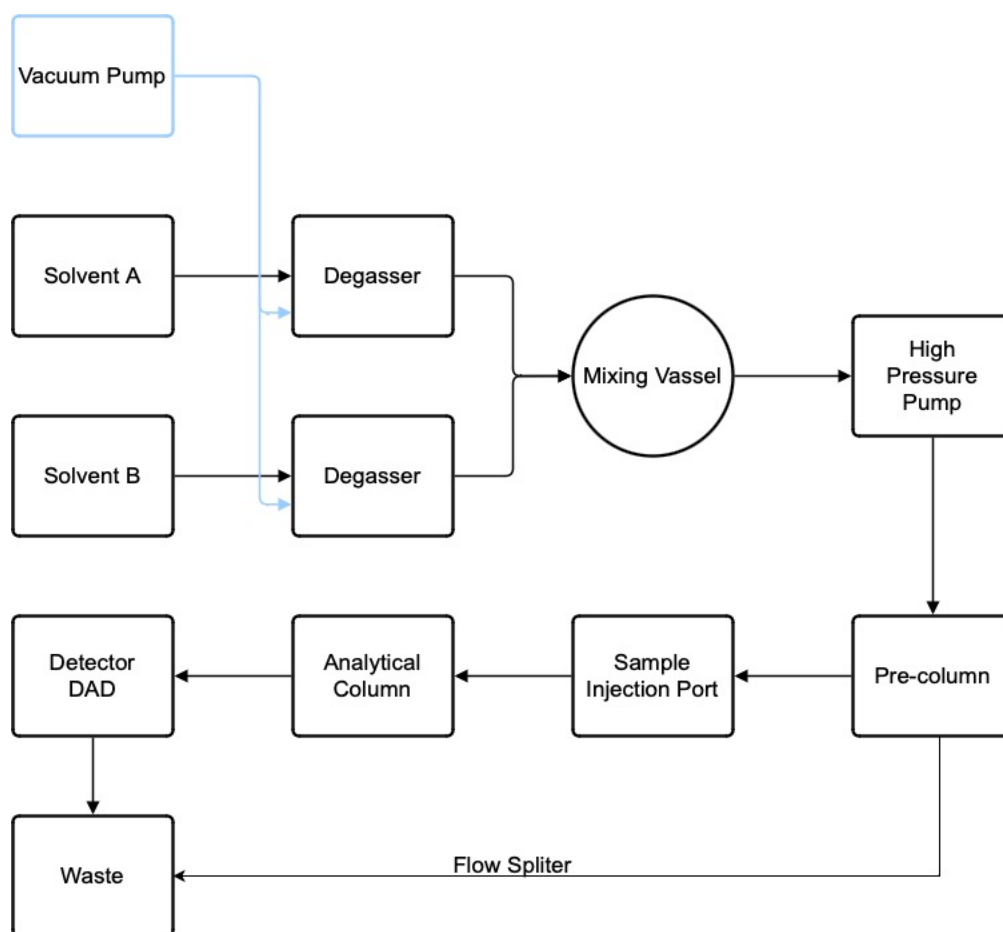


Figure 24 - HPLC-DAD schematic process.

Table 15 - Concentration of polyphenolic compounds in extractions using CNS and water.

Value expressed as mean  $\pm$  standard deviation; Same superscripts (a-i) indicate not significant differences ( $p > 0.9999$ ) among the values in the column values.

| Sample/<br>Compound                    | Concentration [FT]/g.L-1 eq. (-)-Epicatechin |                                  |                                    |                                |                                  |                                             |                                                       |                                  |                                  |                                  |
|----------------------------------------|----------------------------------------------|----------------------------------|------------------------------------|--------------------------------|----------------------------------|---------------------------------------------|-------------------------------------------------------|----------------------------------|----------------------------------|----------------------------------|
|                                        | (E)GC                                        | PD-BG                            | (E)C                               | (E)GCG                         | Gallic acid                      | Apigenin-6-C-<br>pentoside-8-C-<br>hexoside | Quercetin-<br>deoxyhexosid<br>e-hexoside-<br>hexoside | Quercetin-3-O-<br>rutoside       | (E)CG                            | Kaempferol 3-<br>rutoside        |
| GTH <sub>2</sub> O_50_T <sub>amb</sub> | 1.947 <sup>a</sup> $\pm$ 0.003               | 5.60 <sup>a,b</sup> $\pm$ 0.01   | 2.348 <sup>a,b</sup> $\pm$ 0.004   | 6.00 <sup>a,b</sup> $\pm$ 0.02 | 1.414 <sup>a</sup> $\pm$ 0.002   | 1.545 <sup>a</sup> $\pm$ 0.002              | 2.181 <sup>a-c,e,f</sup> $\pm$ 0.007                  | 1.803 <sup>a</sup> $\pm$ 0.001   | 3.65 <sup>a,b</sup> $\pm$ 0.02   | 1.581 <sup>a</sup> $\pm$ 0.001   |
| GTH <sub>2</sub> O_50_T <sub>50</sub>  | 1.989 <sup>a</sup> $\pm$ 0.003               | 6.16 <sup>a,c,d</sup> $\pm$ 0.02 | 2.491 <sup>a</sup> $\pm$ 0.003     | 6.30 <sup>a</sup> $\pm$ 0.02   | 1.4267 <sup>a</sup> $\pm$ 0.0006 | 1.582 <sup>a</sup> $\pm$ 0.001              | 2.290 <sup>a-c,e,f</sup> $\pm$ 0.005                  | 1.960 <sup>a</sup> $\pm$ 0.002   | 3.89 <sup>a,c</sup> $\pm$ 0.01   | 1.6595 <sup>a</sup> $\pm$ 0.0009 |
| GTH <sub>2</sub> O_50_T <sub>100</sub> | 2.129 <sup>a</sup> $\pm$ 0.007               | 9.14 <sup>e</sup> $\pm$ 0.04     | 2.641 <sup>a</sup> $\pm$ 0.001     | 10.11 <sup>c</sup> $\pm$ 0.04  | 1.460 <sup>a</sup> $\pm$ 0.006   | 1.825 <sup>a</sup> $\pm$ 0.001              | 2.902 <sup>a,d,f,h</sup> $\pm$ 0.007                  | 2.015 <sup>a</sup> $\pm$ 0.001   | 6.12 <sup>d,e</sup> $\pm$ 0.02   | 1.696 <sup>a</sup> $\pm$ 0.003   |
| GTH <sub>2</sub> O_25_T <sub>amb</sub> | 1.669 <sup>a</sup> $\pm$ 0.002               | 4.51 <sup>f</sup> $\pm$ 0.02     | 1.882 <sup>a,b</sup> $\pm$ 0.002   | 4.39 <sup>d,e</sup> $\pm$ 0.02 | 1.385 <sup>a</sup> $\pm$ 0.002   | 1.454 <sup>a</sup> $\pm$ 0.001              | 1.828 <sup>b,c</sup> $\pm$ 0.004                      | 1.576 <sup>a</sup> $\pm$ 0.001   | 2.78 <sup>b,f</sup> $\pm$ 0.01   | 1.462 <sup>a</sup> $\pm$ 0.002   |
| GTH <sub>2</sub> O_25_T <sub>50</sub>  | 1.662 <sup>a</sup> $\pm$ 0.003               | 4.94 <sup>b,f</sup> $\pm$ 0.03   | 1.915 <sup>a,b</sup> $\pm$ 0.004   | 4.31 <sup>d</sup> $\pm$ 0.03   | 1.381 <sup>a</sup> $\pm$ 0.001   | 1.4587 <sup>a</sup> $\pm$ 0.0003            | 1.851 <sup>b,c</sup> $\pm$ 0.005                      | 1.633 <sup>a</sup> $\pm$ 0.002   | 2.83 <sup>b,f</sup> $\pm$ 0.01   | 1.486 <sup>a</sup> $\pm$ 0.001   |
| GTH <sub>2</sub> O_25_T <sub>100</sub> | 1.8364 <sup>a</sup> $\pm$ 0.0009             | 6.56 <sup>c,d</sup> $\pm$ 0.01   | 2.163 <sup>a,b</sup> $\pm$ 0.002   | 7.41 <sup>f</sup> $\pm$ 0.01   | 1.402 <sup>a</sup> $\pm$ 0.003   | 1.754 <sup>a</sup> $\pm$ 0.002              | 2.480 <sup>a,b,e,f,h</sup> $\pm$ 0.004                | 1.7736 <sup>a</sup> $\pm$ 0.0004 | 4.847 <sup>c,g</sup> $\pm$ 0.007 | 1.5607 <sup>a</sup> $\pm$ 0.0002 |
| GTXAG_50_T <sub>amb</sub>              | 1.3771 <sup>a</sup> $\pm$ 0.0004             | 1.801 <sup>g,h</sup> $\pm$ 0.006 | 1.403 <sup>b,c</sup> $\pm$ 0.001   | 1.88 <sup>g</sup> $\pm$ 0.01   | 1.3405 <sup>a</sup> $\pm$ 0.0002 | 1.3607 <sup>a</sup> $\pm$ 0.0003            | 1.420 <sup>c</sup> $\pm$ 0.002                        | 1.3677 <sup>a</sup> $\pm$ 0.0003 | 1.585 <sup>h</sup> $\pm$ 0.005   | 1.3489 <sup>a</sup> $\pm$ 0.0003 |
| GTXAG_50_T <sub>50</sub>               | 1.6043 <sup>a</sup> $\pm$ 0.0004             | 4.315 <sup>f</sup> $\pm$ 0.005   | 1.7744 <sup>a,b</sup> $\pm$ 0.0008 | 4.57 <sup>d,e</sup> $\pm$ 0.02 | 1.372 <sup>a</sup> $\pm$ 0.002   | 1.497 <sup>a</sup> $\pm$ 0.002              | 1.838 <sup>b,c</sup> $\pm$ 0.004                      | 1.5246 <sup>a</sup> $\pm$ 0.0002 | 2.85 <sup>b,f</sup> $\pm$ 0.01   | 1.423 <sup>a</sup> $\pm$ 0.001   |
| GTXAG_50_T <sub>100</sub>              | 2.059 <sup>a</sup> $\pm$ 0.001               | 10.65 <sup>i</sup> $\pm$ 0.02    | 2.595 <sup>a</sup> $\pm$ 0.003     | 11.36 <sup>h</sup> $\pm$ 0.03  | 1.4365 <sup>a</sup> $\pm$ 0.0007 | 1.954 <sup>a</sup> $\pm$ 0.003              | 3.041 <sup>a,e,f,h</sup> $\pm$ 0.002                  | 1.896 <sup>a</sup> $\pm$ 0.009   | 6.86 <sup>d</sup> $\pm$ 0.02     | 1.651 <sup>a</sup> $\pm$ 0.002   |
| GTXA_50_T <sub>amb</sub>               | 1.42 <sup>a</sup> $\pm$ 0.01                 | 2.47 $\pm$ 0.02                  | 1.491 <sup>b,c,d</sup> $\pm$ 0.006 | 2.3 <sup>g</sup> $\pm$ 0.1     | 1.351 <sup>a</sup> $\pm$ 0.001   | 1.964 <sup>a</sup> $\pm$ 0.004              | 1.522 <sup>c</sup> $\pm$ 0.004                        | 1.391 <sup>a</sup> $\pm$ 0.001   | 1.75 <sup>h</sup> $\pm$ 0.03     | 1.393 <sup>a</sup> $\pm$ 0.003   |
| GTXA_50_T <sub>50</sub>                | 1.795 <sup>a</sup> $\pm$ 0.004               | 6.51 <sup>a,c,d</sup> $\pm$ 0.02 | 2.098 <sup>a,b</sup> $\pm$ 0.001   | 6.06 <sup>a,b</sup> $\pm$ 0.05 | 1.384 <sup>a</sup> $\pm$ 0.002   | 1.576 <sup>a</sup> $\pm$ 0.008              | 2.054 <sup>b-e</sup> $\pm$ 0.008                      | 1.6041 <sup>a</sup> $\pm$ 0.0004 | 3.48 <sup>a,b</sup> $\pm$ 0.02   | 1.4715 <sup>a</sup> $\pm$ 0.0006 |
| GTXA_50_T <sub>100</sub>               | 2.0403 <sup>a</sup> $\pm$ 0.0006             | 10.478 <sup>i</sup> $\pm$ 0.006  | 2.564 <sup>a</sup> $\pm$ 0.002     | 9.79 <sup>c</sup> $\pm$ 0.05   | 1.3987 <sup>a</sup> $\pm$ 0.0007 | 2.005 <sup>a</sup> $\pm$ 0.004              | 2.729 <sup>a,b,e,f,h</sup> $\pm$ 0.007                | 1.816 <sup>a</sup> $\pm$ 0.002   | 5.71 <sup>e,g</sup> $\pm$ 0.03   | 1.6017 <sup>a</sup> $\pm$ 0.0007 |
| GTS_50_T <sub>amb</sub>                | 1.99 <sup>a</sup> $\pm$ 0.02                 | 8.1 $\pm$ 0.3                    | 2.43 <sup>a,d</sup> $\pm$ 0.03     | 9.2 <sup>c</sup> $\pm$ 0.3     | 1.44 <sup>a</sup> $\pm$ 0.01     | 1.711 <sup>a</sup> $\pm$ 0.008              | 2.90 <sup>e-h</sup> $\pm$ 0.07                        | 1.93 <sup>a</sup> $\pm$ 0.02     | 5.5 <sup>e,g</sup> $\pm$ 0.2     | 1.65 <sup>a</sup> $\pm$ 0.01     |
| GTS_50_T <sub>50</sub>                 | 2.02 <sup>a</sup> $\pm$ 0.04                 | 9.1 <sup>e</sup> $\pm$ 0.2       | 2.49 <sup>a</sup> $\pm$ 0.07       | 9.5 <sup>c</sup> $\pm$ 0.3     | 1.46 <sup>a</sup> $\pm$ 0.02     | 1.79 <sup>a</sup> $\pm$ 0.04                | 3.1 <sup>f,h</sup> $\pm$ 0.1                          | 2.00 <sup>a</sup> $\pm$ 0.04     | 6.1 <sup>d,e</sup> $\pm$ 0.2     | 1.69 <sup>a</sup> $\pm$ 0.02     |
| GTS_50_T <sub>100</sub>                | 2.10 <sup>a</sup> $\pm$ 0.01                 | 10.82 <sup>i</sup> $\pm$ 0.09    | 2.59 <sup>a</sup> $\pm$ 0.02       | 11.3 <sup>h</sup> $\pm$ 0.1    | 1.49 <sup>a</sup> $\pm$ 0.01     | 1.86 <sup>a</sup> $\pm$ 0.01                | 3.26 <sup>h</sup> $\pm$ 0.04                          | 2.04 <sup>a</sup> $\pm$ 0.01     | 6.8 <sup>d</sup> $\pm$ 0.1       | 1.72 <sup>a</sup> $\pm$ 0.01     |
| GTS_25_T <sub>amb</sub>                | 1.623 <sup>a</sup> $\pm$ 0.002               | 4.68 <sup>b,f</sup> $\pm$ 0.05   | 1.780 <sup>a,b</sup> $\pm$ 0.007   | 5.29 <sup>b,e</sup> $\pm$ 0.01 | 1.375 <sup>a</sup> $\pm$ 0.003   | 1.487 <sup>a</sup> $\pm$ 0.004              | 2.029 <sup>b-d,g</sup> $\pm$ 0.006                    | 1.560 <sup>a</sup> $\pm$ 0.005   | 3.28 <sup>a,b</sup> $\pm$ 0.02   | 1.454 <sup>a</sup> $\pm$ 0.002   |
| GTS_25_T <sub>50</sub>                 | 1.670 <sup>a</sup> $\pm$ 0.003               | 5.9 <sup>a,c</sup> $\pm$ 0.3     | 1.87 <sup>a,b</sup> $\pm$ 0.02     | 6.2 <sup>a,b</sup> $\pm$ 0.2   | 1.383 <sup>a</sup> $\pm$ 0.004   | 1.548 <sup>a</sup> $\pm$ 0.007              | 2.20 <sup>a-g</sup> $\pm$ 0.02                        | 1.63 <sup>a</sup> $\pm$ 0.01     | 3.9 <sup>a,c</sup> $\pm$ 0.1     | 1.493 <sup>a</sup> $\pm$ 0.008   |
| GTS_25_T <sub>100</sub>                | 1.748 <sup>a</sup> $\pm$ 0.007               | 6.9 <sup>d</sup> $\pm$ 0.1       | 1.98 <sup>a,b</sup> $\pm$ 0.01     | 7.5 <sup>f</sup> $\pm$ 0.1     | 1.413 <sup>a</sup> $\pm$ 0.006   | 1.619 <sup>a</sup> $\pm$ 0.007              | 2.37 <sup>a-g</sup> $\pm$ 0.02                        | 1.678 <sup>a</sup> $\pm$ 0.007   | 4.51 <sup>a,c</sup> $\pm$ 0.07   | 1.520 <sup>a</sup> $\pm$ 0.004   |

| Sample/<br>Compound                              | Concentration [FT]/g.L-1 eq. (-)-Epicatechin |                            |                              |                            |                            |                                             |                                                       |                              |                            |                             |
|--------------------------------------------------|----------------------------------------------|----------------------------|------------------------------|----------------------------|----------------------------|---------------------------------------------|-------------------------------------------------------|------------------------------|----------------------------|-----------------------------|
|                                                  | (E)GC                                        | PD-BG                      | (E)C                         | (E)GCG                     | Gallic acid                | Apigenin-6-C-<br>pentoside-8-C-<br>hexoside | Quercetin-<br>deoxyhexosid<br>e-hexoside-<br>hexoside | Quercetin-3-O-<br>rutinoside | (E)CG                      | Kaempferol 3-<br>rutinoside |
| GT75S25H <sub>2</sub> O<br>_25_ T <sub>100</sub> | 1.3837 <sup>a</sup> ± 0.0003                 | 1.401 <sup>h</sup> ± 0.005 | 1.407 <sup>b</sup> ± 0.003   | 3.845 <sup>d</sup> ± 0.007 | 1.376 <sup>a</sup> ± 0.006 | 1.530 <sup>a</sup> ± 0.003                  | 2.06 <sup>b-d,g</sup> ± 0.01                          | 1.552 <sup>a</sup> ± 0.007   | 2.24 <sup>f,h</sup> ± 0.01 | 1.460 <sup>a</sup> ± 0.004  |
| GT50S50H <sub>2</sub> O<br>_25_ T <sub>100</sub> | 1.384 <sup>a</sup> ± 0.002                   | 1.391 <sup>h</sup> ± 0.007 | 1.415 <sup>b</sup> ± 0.001   | 3.90 <sup>d</sup> ± 0.06   | 1.376 <sup>a</sup> ± 0.002 | 1.528 <sup>a</sup> ± 0.004                  | 2.09 <sup>a-d,g</sup> ± 0.02                          | 1.585 <sup>a</sup> ± 0.004   | 2.26 <sup>f,h</sup> ± 0.02 | 1.467 <sup>a</sup> ± 0.002  |
| GT25S75H <sub>2</sub> O<br>_25_ T <sub>100</sub> | 1.380 <sup>a</sup> ± 0.001                   | 1.383 <sup>h</sup> ± 0.005 | 1.4152 <sup>b</sup> ± 0.0005 | 3.71 <sup>d</sup> ± 0.04   | 1.374 <sup>a</sup> ± 0.002 | 1.511 <sup>a</sup> ± 0.004                  | 2.02 <sup>b-d,g</sup> ± 0.02                          | 1.573 <sup>a</sup> ± 0.004   | 2.19 <sup>f,h</sup> ± 0.02 | 1.459 <sup>a</sup> ± 0.003  |

Table 15 shows the quantification results of polyphenolic compounds extracted from GT under various extraction conditions, taking into account the ratio of GT to CNS, as well as the CNS itself, and the effect of temperature on the extractions, with the traditional water extraction method used as a reference. It is possible to see that, in the same concentration and CNS used, temperature influences the extraction capacity of each solvent. Understanding that increased temperature increases the extraction capacity of each CNS and water, there is a significant increase in some GT polyphenolic compound concentrations, as in the case of PD-BG, (E)GCG, and (E)CG, where significant differences in the extraction capacity were demonstrated for most of all temperature increases.

When comparing the differences in extraction capacity using different GT to CNS ratios, it is clear that, as the GT to CNS ratio increases, so does the extraction capacity, probably owing to the higher gradient of polyphenolic compounds available. This increase is visible in both GT polyphenolic compound extractions using water and stevia at different ratios. In some cases, the extraction at 100 °C with a lower GT to CNS ratio is comparable to the extraction at 50 °C using the same solvent, however, at a higher GT to CNS ratio, some GT polyphenolic compounds are extracted in lower concentration than compared to the same condition but at lower GT to CNS ratio, we can see that in PD-BG and (E)CG extractions with GTH<sub>2</sub>O\_25\_T<sub>100</sub> and GTH<sub>2</sub>O\_50\_T<sub>50</sub>.

Furthermore, the extractions with different percentages of water and stevia does not show significant differences in extraction capacity when compared to each other, and in general, demonstrated a lower extraction capacity when compared to the GTS\_25\_T<sub>100</sub> condition.

When all of the different concentrations of all compounds in each extraction condition are analyzed, it is possible to see that GTS\_50\_T<sub>100</sub> demonstrated the higher extraction capacity, with higher concentrations of half of the GT polyphenolic compounds identified (PD-BG, Gallic Acid, Quercetin-deoxyhexoside-hexoside-hexoside, Quercetin-3-O-rutinoside, and Kaempferol 3-rutinoside), and no significant differences in the other five compounds when compared to the condition that extracted the compound in higher concentrations.

Comparing the extraction capacity of GTH<sub>2</sub>O\_50\_T<sub>100</sub> and GTH<sub>2</sub>O\_25\_T<sub>100</sub> it is possible to see that (E)C, (E)GCG, and (E)CG were extracted in more concentration when compared to the reported by Nkhili E. *et al.* and Perva-Uzunalic A. *et al.* Extracted more concentration of (E)GC when compared to Perva-Uzunalic A. *et al.* and Gallic acid and Quercetin-3-O-rutinoside when compared to Nkhili E. *et al.* The method development by

the authors requires much less extraction time than the used by Nkhili E. *et al.* and Perva-Uzunalic A. *et al.*<sup>23, 102</sup>

### 3.2.4. Viscosity and Density

The determination of sample viscosity and density is an essential part of analyzing various materials, ranging from liquids and gases to solids. These physical properties are critical in chemical, petrochemical, pharmaceutical, food, and beverage industries, among others. Viscosity is the resistance of a fluid to flow, whereas density is the mass per unit volume of a substance.<sup>103-107</sup>

Several techniques for identifying and quantifying these properties have been developed over the years, each with its own set of advantages and limitations. Viscometry is one of the most common and widely used techniques. There are various types of viscometers available, including capillary viscometers, cone and plate viscometers, and rolling-ball viscosimeters. These instruments determine a sample's viscosity by measuring the time it takes to pass through an orifice or the resistance it provides to the movement of a rotating element.<sup>108</sup>

The rolling-ball viscosimeter measures the rolling time of a ball through a sample using a laser system. The equipment measures the time it takes the ball to travel a few distances, and the relationship between the mass of the ball, temperature, inclination degree, and velocity of the ball in the sample (using the distance and time relation) providing information about the sample viscosity (Figure 25).

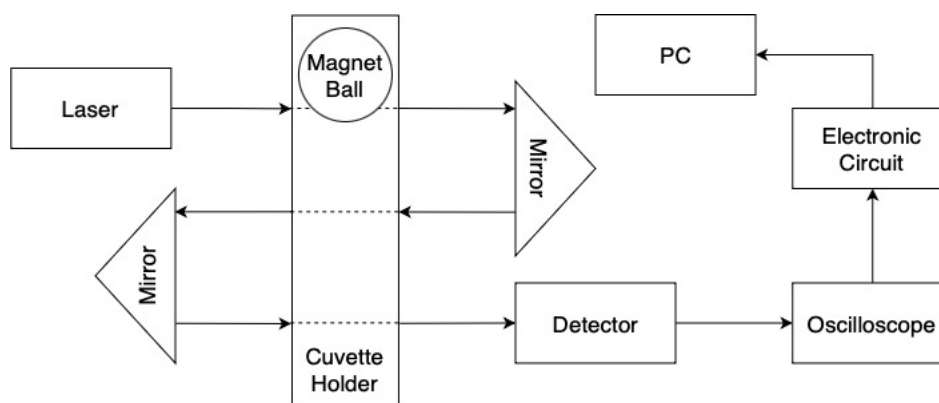


Figure 25 - Viscosimeter schematic process.

Densitometry, which involves determining the density of a substance, is another widely used technique. Density can be measured using a variety of methods, including TLC densitometry, oscillating U-tube, and electronic densitometers. These devices are based on Archimedes' principle, which connects an object's density to the buoyant force it experiences when immersed in a liquid.<sup>109-111</sup> The liquid sample is placed in a U-shaped tube in the oscillating U-tube technique. A vibrating probe is inserted into the tube, which can cause oscillations in the liquid inside the tube. The operation is based on measuring the probe-liquid system's resonance frequency. The mass of the liquid inside the tube, which is directly related to its density, influences the resonance frequency. The lower the measured resonance frequency, the higher the density of the liquid (Figure 26).

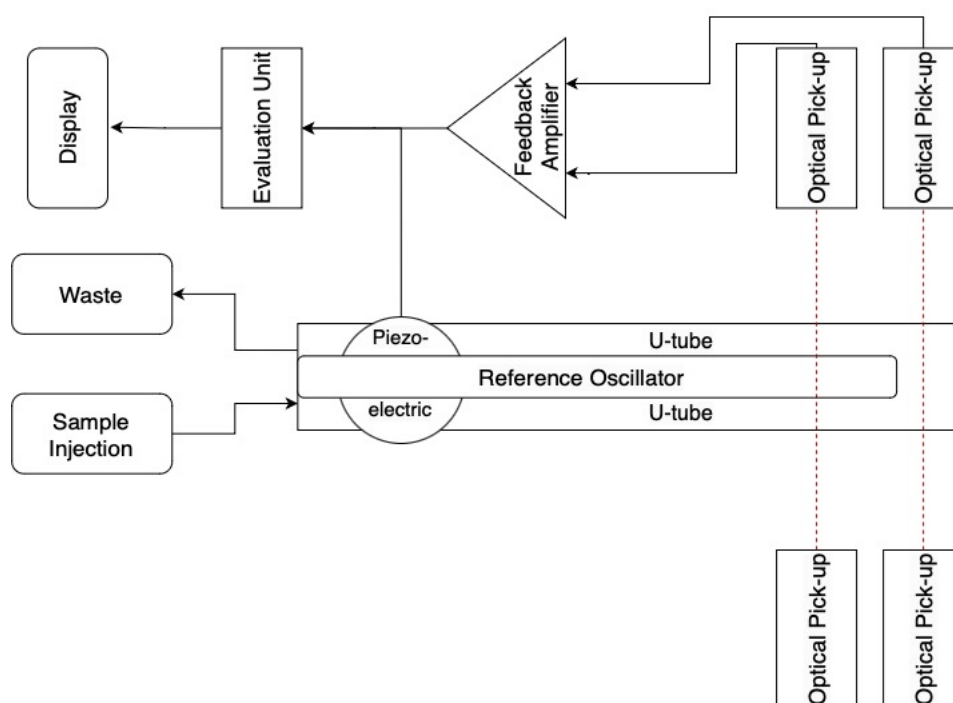


Figure 26 - Densitometer schematic process.

These viscometry and densitometry techniques provide valuable information about the physical properties of materials and are critical in the characterization and quality control of products in a variety of industrial sectors. Viscosity and density are linked to a substance's physical and chemical properties.

Viscosity, for example, is influenced by molecular structure, molecule interactions, and temperature. The fluidity of a liquid or the flow capacity of a fluid can be determined by measuring its viscosity. This is especially important in industrial processes where the viscosity of substances such as paints, polymers, lubricating oils, cosmetics, and food must



be controlled to ensure the appropriate consistency and desired performance of the final product.<sup>107, 112-114</sup>

The density of a substance, on the other hand, is proportional to its mass and volume. Density is an important property in material characterization because it can reveal information about a substance's composition, purity, and degree of compactness. Density measurement is widely used in industries such as chemical and pharmaceutical, where it is critical to ensure that the materials produced meet the desired specifications.<sup>106, 115</sup>

As a result, by determining a sample's viscosity and density, one can gain insights into the physical and chemical properties of materials, which are critical for characterization, quality control, and process optimization. This data enables appropriate adjustments and interventions to ensure that products meet desired standards while also contributing to innovation and advancement in a variety of industrial sectors.

Table 16 showed the results of the viscosity and density assays for higher viscosity conditions at three different temperatures of analysis (80.00 °C, 50.00 °C, and 25.00 °C).

Table 16 - Temperature-dependent density and viscosity values of the different extraction conditions of GT polyphenolic compounds investigated.

| Name                      | T (°C) | Density (g/cm <sup>3</sup> ) | Viscosity (mPa.s) | Observations    |
|---------------------------|--------|------------------------------|-------------------|-----------------|
| GTXA_50_T <sub>amb</sub>  | 80.00  | 1.30405                      | 14.69             | Valid           |
|                           | 50.00  | 1.32306                      | 49.61             | Valid           |
|                           | 25.00  | 1.33726                      | 224.2             | Valid           |
| GTXA_50_T <sub>50</sub>   | 80.00  | 1.31912                      | 20.35             | Filling warning |
|                           | 50.00  | 1.33819                      | 78.11             | Filling warning |
|                           | 25.00  | 1.35251                      | 403.2             | Filling warning |
| GTXA_50_T <sub>100</sub>  | 80.00  | 1.32440                      | 30.62             | Valid           |
|                           | 50.00  | 1.34363                      | 136.6             | Filling warning |
|                           | 25.00  | 1.350804                     | 846.4             | Filling warning |
| GTXAG_50_T <sub>amb</sub> | 80.00  | 1.35660                      | 27.65             | Valid           |
|                           | 50.00  | 1.38099                      | 148.2             | Filling warning |
|                           | 25.00  | 1.39929                      | >QL               | Ball blocked    |
| GTXAG_50_T <sub>50</sub>  | 80.00  | 1.35491                      | 31.94             | Filling warning |
|                           | 50.00  | 1.37932                      | 184.2             | Filling warning |
|                           | 25.00  | 1.39773                      | >QL               | Ball blocked    |
| GTXAG_50_T <sub>100</sub> | 80.00  | 1.38637                      | 71.91             | Filling warning |
|                           | 50.00  | 1.41101                      | 550.3             | Filling warning |

| Name                    | T (°C) | Density (g/cm <sup>3</sup> ) | Viscosity (mPa.s) | Observations    |
|-------------------------|--------|------------------------------|-------------------|-----------------|
|                         | 25.00  | 1.42930                      | >QL               | Ball blocked    |
| GTM_50_T <sub>amb</sub> | 80.00  | 1.38603                      | 128.8             | Timeout         |
|                         | 50.00  | 1.40960                      | 1159              | Filling warning |
|                         | 25.00  | 1.42676                      | >QL               | Ball blocked    |
| GTM_50_T <sub>50</sub>  | 80.00  | 1.38594                      | 100.1             | Filling warning |
|                         | 50.00  | 1.40828                      | 865.8             | Filling warning |
|                         | 25.00  | 1.42504                      | >QL               | Ball blocked    |
| GTM_50_T <sub>100</sub> | 80.00  | 1.39159                      | 122.6             | Filling warning |
|                         | 50.00  | 1.41375                      | 1164              | Filling warning |
|                         | 25.00  | 1.43052                      | >QL               | Ball blocked    |

>QL – Over quantification limit of the equipment.

The results revealed that the viscosity of the samples and the presence of air bubbles in the matrix of the CNS were the major problems for the assay's performance. Except for the GTXA\_50 conditions, all other conditions at the assay temperature of 20 °C demonstrated that samples have a higher viscosity value than the capacity of the equipment measure. To overcome these issues, a rheological study was conducted to better understand the CNS's behavior.

Using the GTXA\_50\_T<sub>amb</sub> condition, which has three different conditions in a valid condition, a viscosity and density plot was performed (Equation 1 and 2 respectively) to allow for value extrapolation to 20 °C, the average temperature during the season.

$$\mu = -3.7221 \times T + 288.47$$

Equation 1 - Calibration curve for GTXA\_50\_T<sub>amb</sub> viscosity in g/cm<sup>3</sup>.  $\mu$  - Viscosity;  $T$  – Temperature;  $r^2 = 0.8338$ .

$$\rho = -6 \times 10^{-4} \times T + 1.3483$$

Equation 2 - Calibration curve for GTXA\_50\_T<sub>amb</sub> density in g/cm<sup>3</sup>.  $\rho$  - Density;  $T$  – Temperature;  $r^2 = 0.9999$ .

At 20 °C, the values for density and viscosity from Equations 1 and 2 are 1.33630 g/cm<sup>3</sup> and 214.0 mPa.s, respectively. However, due to the low  $r^2$  associated with Equation 1, the viscosity value demonstrated lower viscosity than at 25 °C, implying that more detailed evaluation was required to make some conclusions possible.

### 3.2.5. Rheology

#### 3.2.5.1. Viscosity

Rheology is a fundamental technique in the study of matter's deformation and flow in response to applied forces. It enables detailed analysis of material mechanical behavior, particularly when subjected to different tension, shear, and deformation conditions. It is a technique used in many scientific and engineering fields to study the behavior of complex materials such as polymers, fluids, pastes, and suspensions when subjected to controlled forces and deformations. The term "rheology" is derived from the Greek words "rheos" (flow) and "logia" (study of). It basically includes measuring the mechanical and rheological properties of the materials.<sup>116-118</sup>

Rheometers are the equipment used to conduct these rheological analyses. They are intended to subject the material to various force and deformation conditions, allowing the collection of important data such as viscosity, elasticity, shear stress, and other parameters that characterize the material's response to these forces (Figure 27). This data is useful in a variety of industries, including food, cosmetics, plastics, petroleum, and pharmaceuticals, because it helps with the development of more efficient products and the optimization of manufacturing processes.<sup>116, 117</sup>

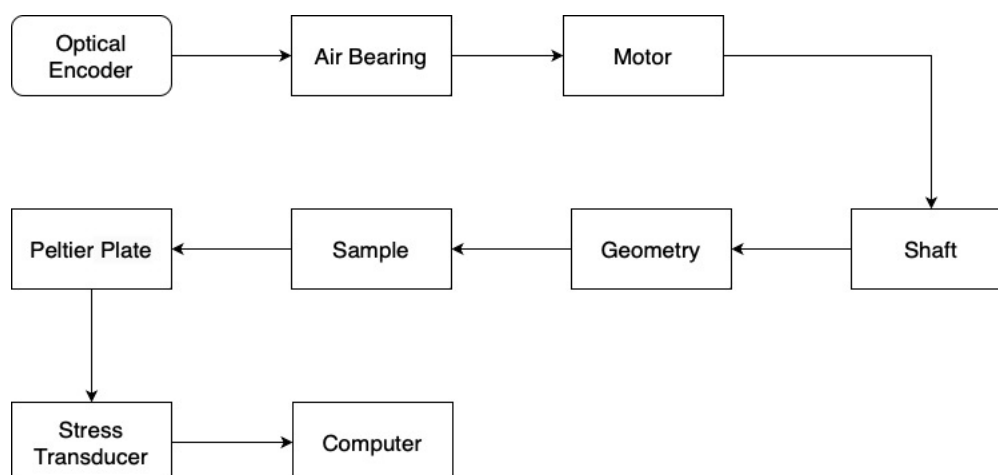


Figure 27 - Rheometer schematic process.

The following Figures shows the flow curves of all CNS and GT polyphenolic compounds extracted with the CNSs.

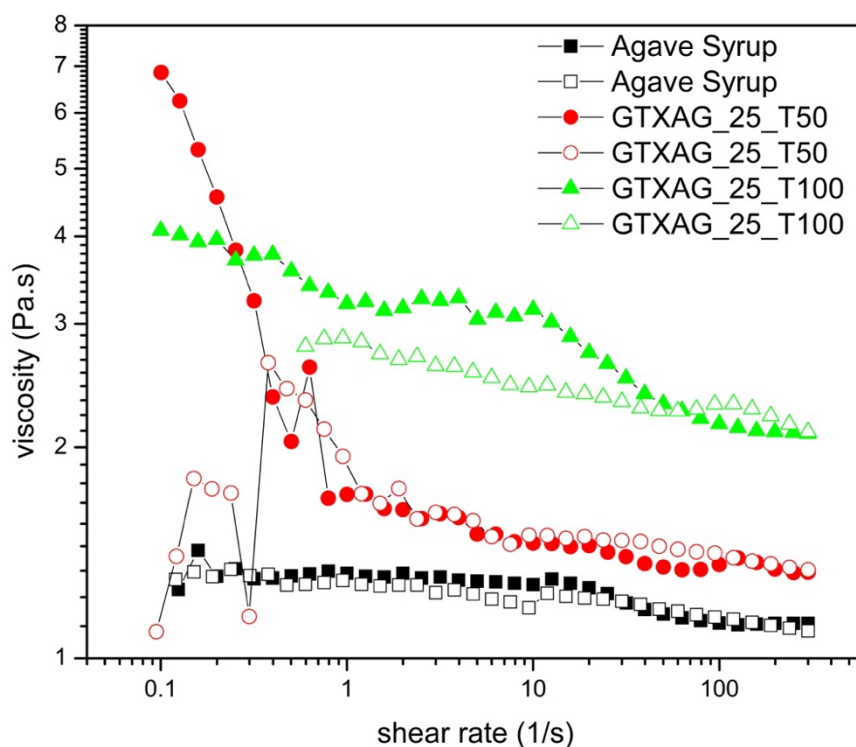


Figure 28 – Flow curve of agave syrup and extractions of GT polyphenolic compounds with 10.000 g of agave syrup at 50 °C and 100 °C. Full symbol means increasing of shear rate and open symbol means decreasing of shear rate.

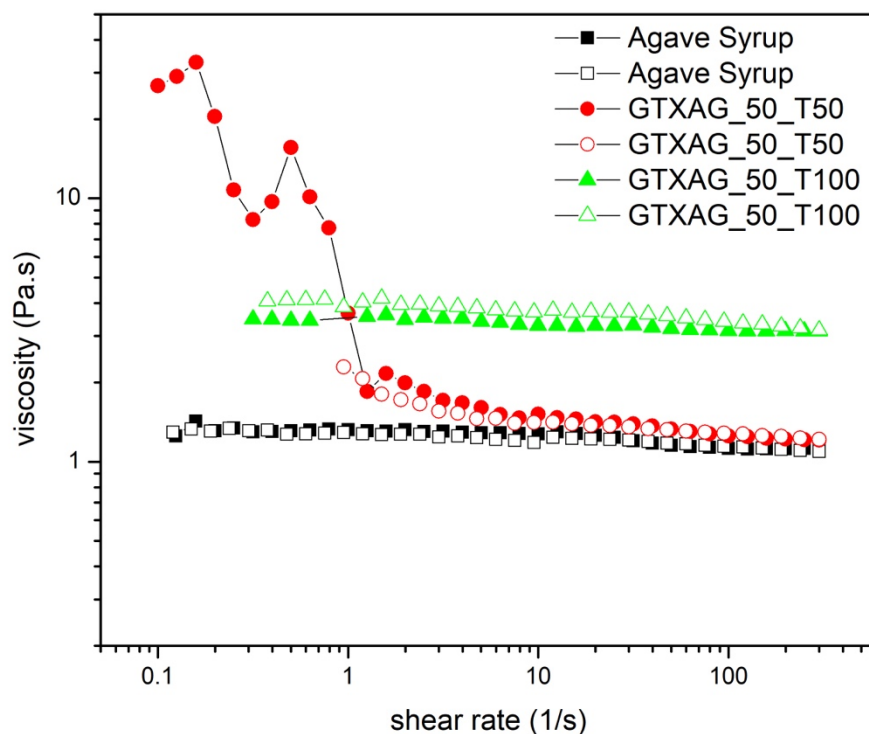


Figure 29 - Flow curve of agave syrup and extractions of GT polyphenolic compounds with 5.000 g of agave syrup at 50 °C and 100 °C. Full symbol means increasing of shear rate and open symbol means decreasing of shear rate.

Figure 28 shows the flow curves of agave syrup and the extraction of GT polyphenolic compounds using a 10.000 g agave syrup to 0.250 g of GT ratio. It is possible to see that different viscosities were observed between the samples. Where GTXAG\_25\_T<sub>50</sub> demonstrated the higher value of initial viscosity (0.1 1/s) of 6.856 Pa.s and agave syrup showed the lower value of initial viscosity of 1.254 Pa.s. The viscosity of agave syrup, GTXAG\_25\_T<sub>50</sub>, and GTXAG\_25\_T<sub>100</sub> decrease with the increase of shear rate, being more significative in GTXAG\_25\_T<sub>50</sub>. Also, with the decrease in shear rate, the apparent viscosity in agave syrup showed no variation when compared to the values in the increase of shear rate, maintaining the structure of the sample over the shear rate variations. GTXAG\_25\_T<sub>50</sub> showed no variations until 1/s shear rate, maintaining the structure of the sample until that shear rate, and GTXAG\_25\_T<sub>100</sub> has not followed this trend.

GTXAG\_50\_T<sub>50</sub> had a higher viscosity (Figure 29) than all other agave syrup extractions (Figures 28 and 29), with a viscosity value at 0.1 1/s of shear rate of 26.77 Pa.s, while GTXAG\_50\_T<sub>100</sub> had a viscosity value of 2.885 Pa.s. In this case (Figure 29), only GTXAG\_50\_T<sub>50</sub> had a markable decrease in viscosity value over the increase of shear rate. In the decrease of shear rate, GTXAG\_50\_T<sub>100</sub> showed no variations in viscosity values when compared with the increase of shear rate, maintaining the structure of the sample. The same trend was verified in GTXAG\_50\_T<sub>50</sub> until 1/s shear rate.

Figure 30 shows the shear rate values of honey extractions at 100 °C in both honey ratios. Extractions with honey at 50 °C and CNS honey have viscosities that exceed the equipment's limit of quantification, so no rheological studies have been performed. GTM\_25\_T<sub>100</sub> and GTM\_50\_T<sub>100</sub> had problems at higher shear rates, and GTM\_50\_T<sub>100</sub> had problems at low shear rates. Aside from reaching higher shear rates than in GTM\_25\_T<sub>100</sub>, in GTM\_50\_T<sub>100</sub>, the equipment was unable to proceed with the measurements after stopping at a higher shear rate. The initial viscosity at 0.1 1/s is 35.10 Pa.s to GTM\_25\_T<sub>100</sub> and 46.14 Pa.s to GTM\_50\_T<sub>100</sub>.

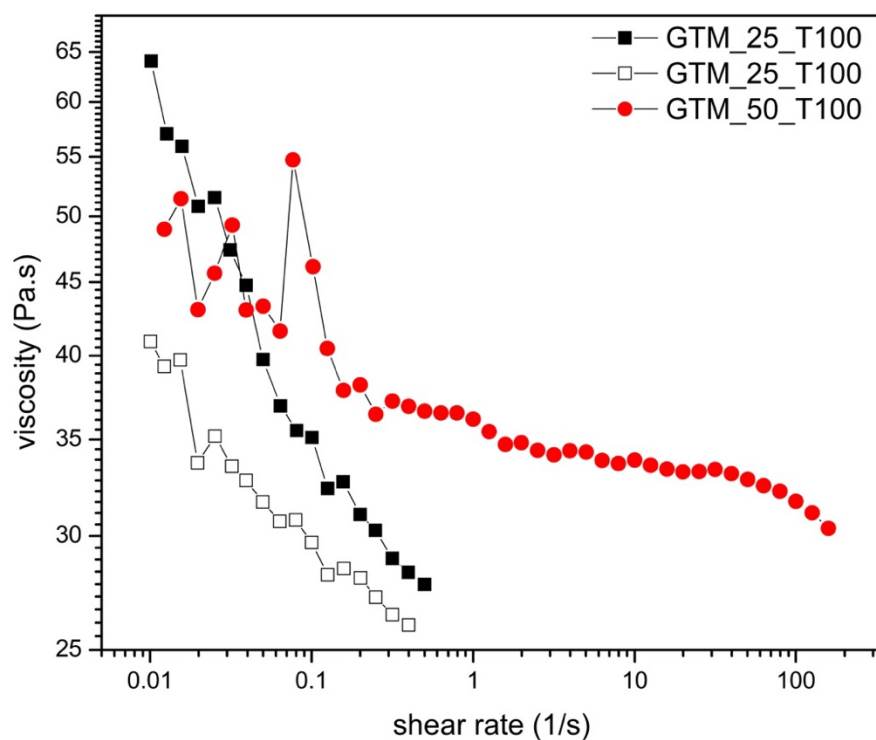


Figure 30 - Flow curve of extractions of GT polyphenolic compounds with 5.000 g and 10.000 g of honey at 100 °C. Full symbol means increasing of shear rate and open symbol means decreasing of shear rate.

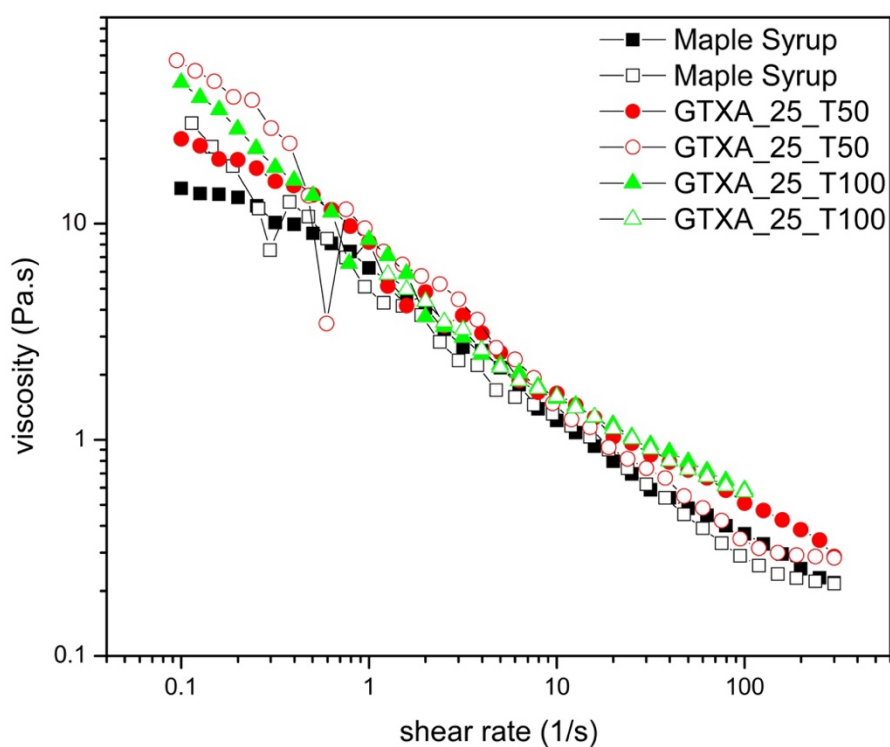


Figure 31 - Flow curve of maple syrup and extractions of GT polyphenolic compounds with 10.000 g of maple syrup at 50 °C and 100 °C. Full symbol means increasing of shear rate and open symbol means decreasing of shear rate.

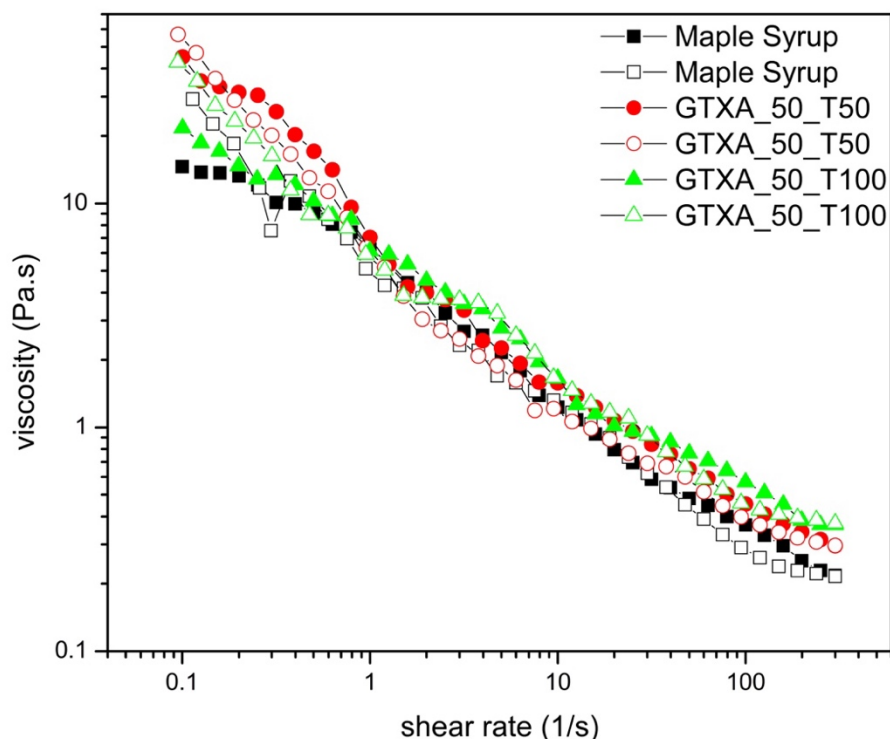


Figure 32 - Flow curve of maple syrup and extractions of GT polyphenolic compounds with 5.000 g of maple syrup at 50 °C and 100 °C. Full symbol means increasing of shear rate and open symbol means decreasing of shear rate.

In Figure 31 it is possible to see that GTXA\_25\_T<sub>100</sub> has a higher value of apparent viscosity at 0.1 1/s of 44.84 Pa.s, and maple syrup has a lower apparent viscosity of 14.57 Pa.s at the same shear rate. GTXA\_25\_T<sub>50</sub> at 0.1 1/s has an apparent viscosity of 24.70 Pa.s. All the samples showed a decrease in viscosity value with the increase in shear rate. Until 1/s shear rate, in decreasing shear rate, all the samples showed no variations in viscosity values when compared to increasing shear rate viscosities values, maintaining the structure of the sample until that.

At 0.1 1/s shear rate, GTXA\_50\_T<sub>100</sub> (Figure 32) showed the higher value of apparent viscosity of 21.74 Pa.s, and GTXA\_50\_T<sub>50</sub> an apparent viscosity of 14.57 Pa.s. Also, all the samples showed a decrease viscosity value over the increase of shear rate. In decreasing shear rate, GTXA\_50\_T<sub>50</sub> showed the better capacity to maintaining the structure of the sample, with no variations in viscosities values in increasing and decreasing shear rate.

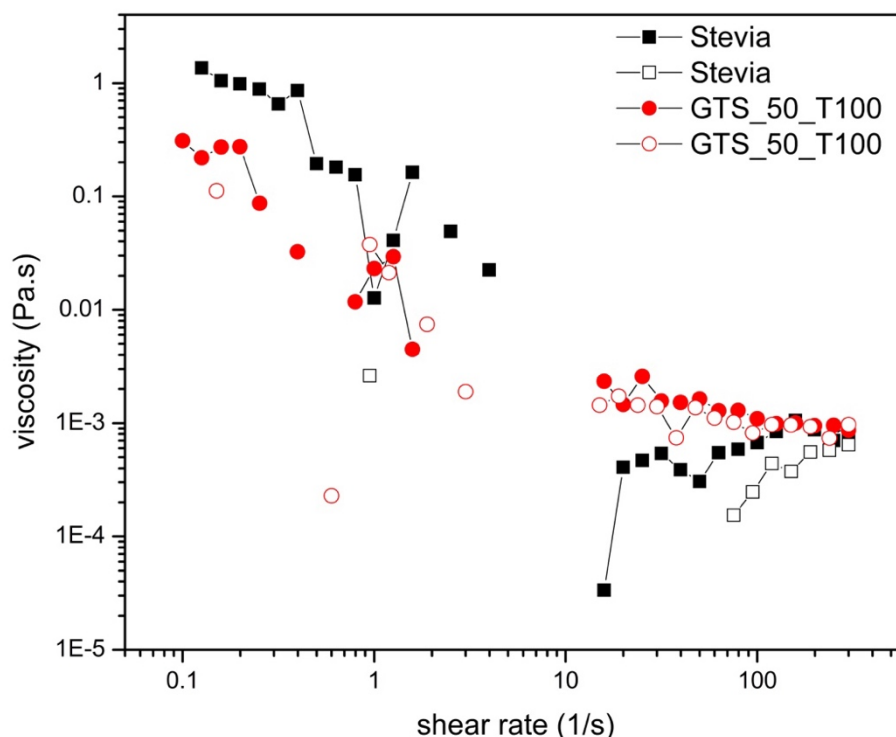


Figure 33 - Flow curve of stevia and extractions of GT polyphenolic compounds with 5.000 g of stevia at 100 °C. Full symbol means increasing of shear rate and open symbol means decreasing of shear rate.

Stevia and all GT polyphenolic compound extractions with stevia showed low viscosity, mostly above the quantification limit of the equipment, but the analysis revealed many perturbations (Figure 33). To overcome this problem, studies using a capillary rheometer was necessary. Stevia and GTS\_50\_T<sub>100</sub> had viscosities of 1.3650 Pa.s and 0.3096 Pa.s at 0.1 1/s shear rate, respectively.

### 3.2.5.1. Oscillatory Frequency Sweep

The following figures represent the oscillatory frequency sweep for all samples. To better understand the typical comportment of the samples. The  $G'$  value showed a solid appear comportment and  $G''$  value showed a liquid appear comportment.



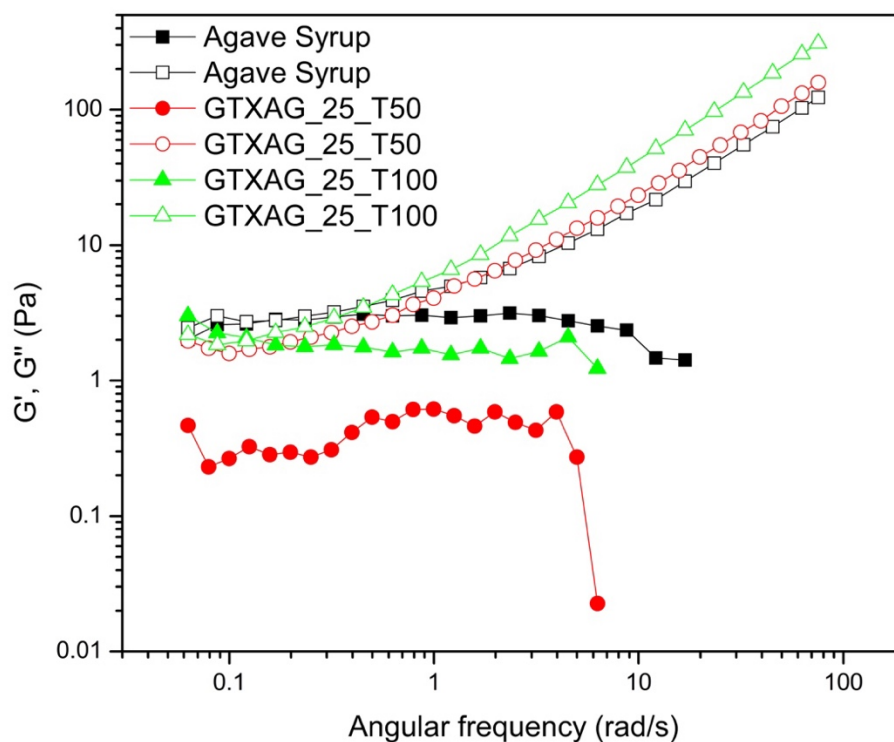


Figure 34 – Oscillatory frequency sweep of agave syrup and extractions of GT polyphenolic compounds with 10.000 g of agave syrup at 50 °C and 100 °C. Full symbol means  $G'$  and open symbol means  $G''$ .

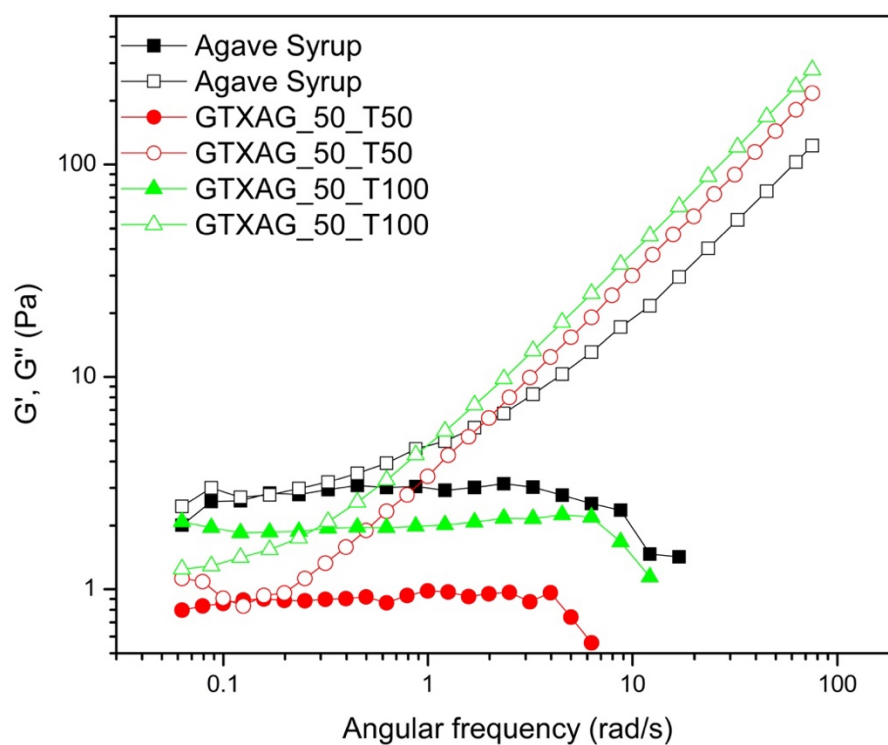


Figure 35 – Oscillatory frequency sweep of agave syrup and extractions of GT polyphenolic compounds with 5.000 g of agave syrup at 50 °C and 100 °C. Full symbol means  $G'$  and open symbol means  $G''$ .

The  $G'$  and  $G''$  values for all agave syrup analyses are shown in Figure 34. Agave syrup CNS and GTXAG\_25\_T<sub>50</sub> exhibit liquid comportment at all frequencies with a  $G''$  values higher than  $G'$  in all frequencies. However, at higher frequencies, the  $G'$  values disappear due to the extremely low solid comportment. In the case of GTXAG\_25\_T<sub>100</sub>, no differences were observed between  $G'$  and  $G''$  at low frequencies, but as the frequency increases, the comportment changes to the sample appearing liquid with  $G''$  higher than  $G'$ , and as with agave syrup and GTXAG\_50\_T<sub>50</sub>, the value of  $G''$  is too low and exceeds the quantification limit of the equipment at higher frequencies.

In low frequencies, all samples in Figure 35 showed initially liquid comportment with a value of  $G''$  higher than the  $G'$ , however, at 0.1 rad/s for agave syrup and GTXAG\_50\_T<sub>50</sub>, and 0.2 rad/s for GTXAG\_50\_T<sub>100</sub> no differences between the values of  $G'$  and  $G''$  were registered. After that, all the samples exhibit liquid comportment with a higher value of  $G''$  than  $G'$ . At higher frequencies, all samples showed extremely low solid comportment, as shown in Figure 35, and the equipment did not register those values.

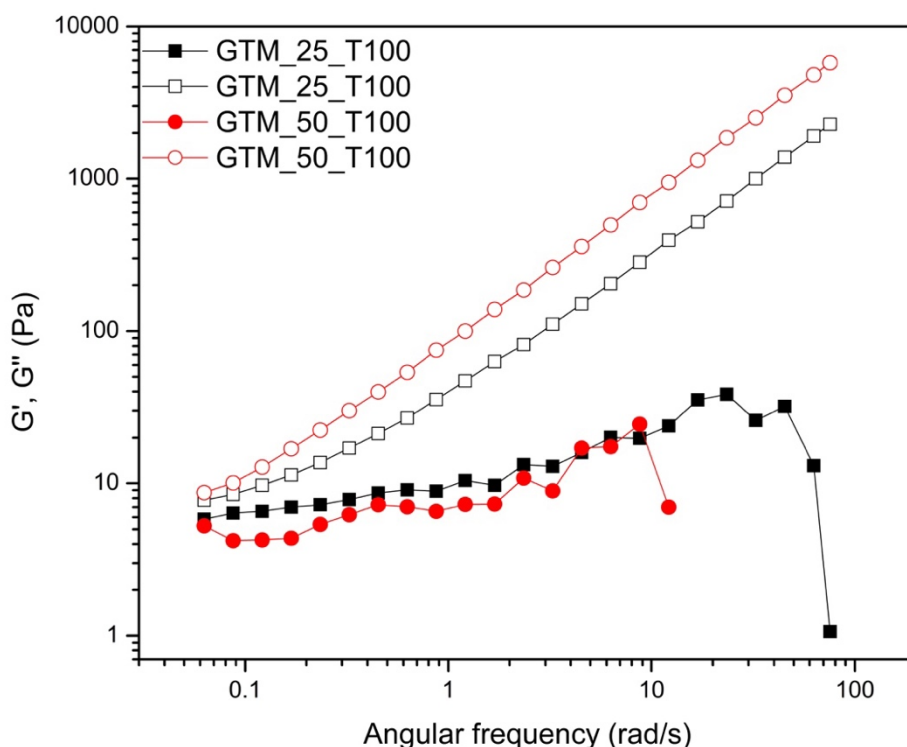


Figure 36 – Oscillatory frequency sweep of extractions of GT polyphenolic compounds with 5.000 g and 10.000 g of honey at 100 °C. Full symbol means  $G'$  and open symbol means  $G''$ .

GTM\_25\_T<sub>100</sub> and GTM\_50\_T<sub>100</sub> (Figure 36) appear liquid comportment at all frequencies with a higher value of  $G''$  than the  $G'$  value. Maintaining the trend, at higher frequencies, the  $G'$  value is lower than the sensibility of the equipment.

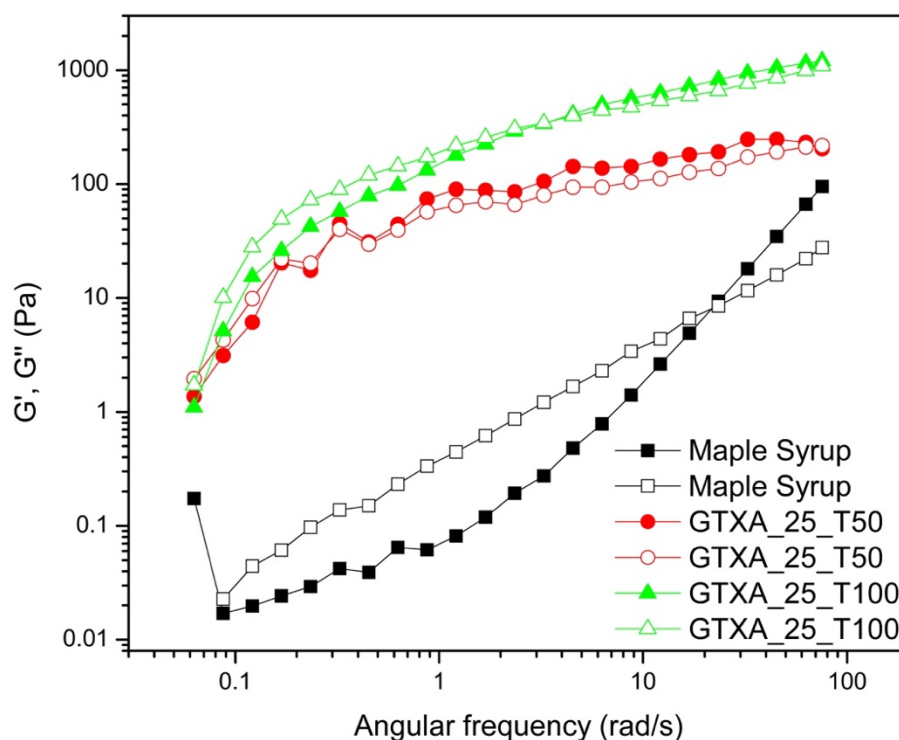


Figure 37 – Oscillatory frequency sweep of maple syrup and extractions of GT polyphenolic compounds with 10.000 g of maple syrup at 50 °C and 100 °C. Full symbol means  $G'$  and open symbol means  $G''$ .

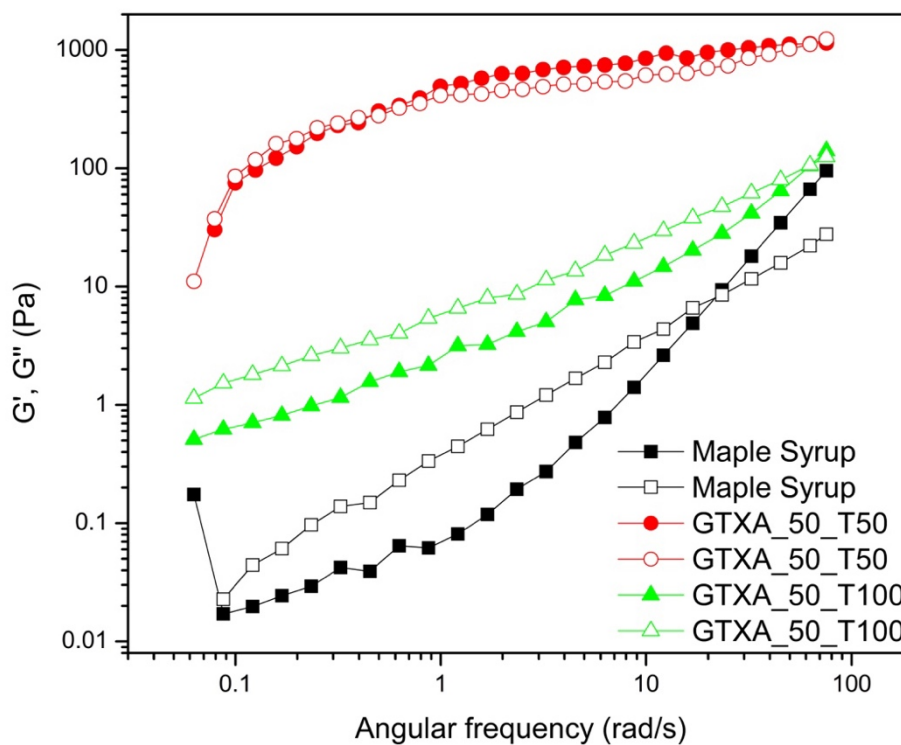


Figure 38 – Oscillatory frequency sweep of maple syrup and extractions of GT polyphenolic compounds with 5.000 g of maple syrup at 50 °C and 100 °C. Full symbol means  $G'$  and open symbol means  $G''$ .

Oscillatory frequency sweep analyses for maple syrup and maple syrup with GT polyphenolic compounds (Figure 37) show that GTXA\_25\_T<sub>50</sub> and GTXA\_25\_T<sub>100</sub> have in most frequencies no differences between the values of  $G'$  and  $G''$ . Maple syrup exhibits majority liquid comportment at low frequencies with  $G''$  values higher than  $G'$  values, and solid comportment in higher frequencies with a higher value of  $G'$  than  $G''$ .

GTXA\_50\_T<sub>50</sub> (Figure 38) exhibits no differences between  $G'$  and  $G''$  values in most frequencies. GTXA\_50\_T<sub>100</sub> (Figure 38) appears liquid in the majority of frequencies with  $G''$  higher than  $G'$ , yet at higher frequencies, no differences were registered between  $G'$  and  $G''$ .

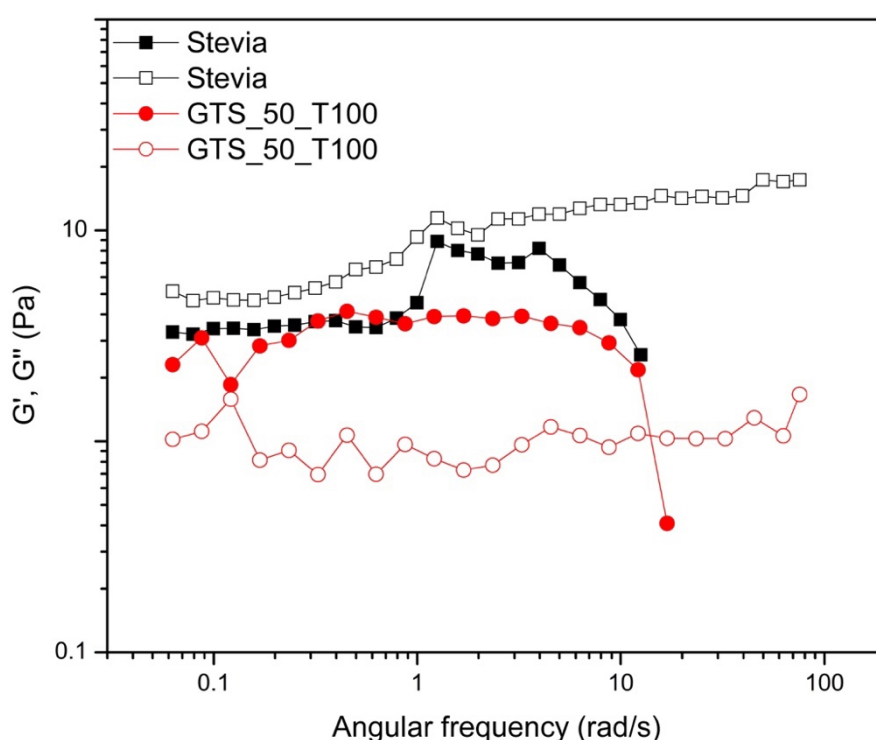


Figure 39 – Oscillatory frequency sweep of stevia and extractions of GT polyphenolic compounds with 5.000 g of stevia at 100 °C. Full symbol means  $G'$  and open symbol means  $G''$ .

In stevia analysis (Figure 39),  $G'$  and  $G''$  values showed many interferences, and in agreement with those discussed in the previous section, the utilization of a capillary rheometer is necessary for better performance and results accuracy.

### 3.2.6. Final Review

It was possible to tentatively identify 10 distinct compounds in the GT polyphenolic compounds extractions: 4 monomeric flavan-3-ol being (E)GC, (E)C, (E)GCG, and (E)CG; 4 flavonoids: Apigenin-6-C-pentoside-8-C-hexoside, Quercetin-deoxyhexoside-hexoside-hexoside, Quercetin-3-O-rutinoside, and Kaempferol 3-rutinoside; 1 dimeric flavan-3-ol: PD-BG; and 1 phenolic acid: Gallic acid.

When all extraction conditions are compared, it is possible to conclude that 0.250 g of GT to 5.000 g of CNS ratio extracts more polyphenolic compounds from GT than extractions with 0.250 g of GT to 10.000 g of CNS ratio, and that GTS\_50\_T<sub>100</sub> has a high extraction power.

It is possible to conclude that honey and stevia CNS and the CNS with GT polyphenolic compounds need more studies of rheological proprieties due to the extremely high viscosity of honey and low viscosity for stevia due to the sensibility limitations of the equipment used. For a better comparison of viscosities values, Table 17 shows the viscosities of all analyses at 0.1 1/s.

Table 17 - Viscosity values from CNS with and without GT polyphenolic compounds extracted at 0.1 1/s.

| Sample                    | Viscosity (Pa.s) |
|---------------------------|------------------|
| Agave Syrup               | 1.254            |
| GTXAG_25_T <sub>50</sub>  | 6.856            |
| GTXAG_25_T <sub>100</sub> | 4.080            |
| GTXAG_50_T <sub>50</sub>  | 26.77            |
| GTXAG_50_T <sub>100</sub> | 2.885            |
| GTM_25_T <sub>100</sub>   | 35.10            |
| GTM_50_T <sub>100</sub>   | 46.14            |
| Maple Syrup               | 14.57            |
| GTXA_25_T <sub>50</sub>   | 24.70            |
| GTXA_25_T <sub>100</sub>  | 44.84            |
| GTXA_50_T <sub>50</sub>   | 14.57            |
| GTXA_50_T <sub>100</sub>  | 21.74            |
| Stevia                    | 1.365            |
| GTS_50_T <sub>100</sub>   | 0.3096           |

Table 17 shows that GT polyphenolic compounds extracted increased the viscosity of agave and maple syrup when compared to the CNS without GT polyphenolic compounds extracted. However, this increase in viscosity was not verified in stevia. Therefore, more accurate tests were required in this case. Extractions at 50 °C increase viscosity more significantly than extractions at 100 °C in agave syrup. The opposite was noticed in maple syrup.

In oscillatory frequency sweep assays, all showed a trend of predominant liquid compartment appearance or no differences between the  $G'$  and  $G''$  values. More accurate studies in honey and stevia samples are required.

### **3.3. Green Tea Polyphenolic Compounds Extracted Stability**

#### **3.3.1. High-Performance Liquid Chromatography – Diode Array (HPLC-DAD)**

HPLC-DAD is an excellent technique for separation and qualitative analyses; it has advantages and disadvantages, and its operation is described in Section 3.2.3. This technique was used on a more qualitative level in 3.2.3. to understand what the different extraction capacities of the different extraction conditions are, but in this section, it will be extremely useful to understand the degradation of the individual GT polyphenolic compounds in the different CNS used for these stability assays.

The CNSs were exposed to three different conditions: light, dark and temperature. Light can cause photodegradation and chemical reactions that lead to the breakdown of polyphenolic compounds. Light radiation can also promote the oxidation of polyphenolic compounds, resulting in a loss of antioxidant capacity, and can also cause compound isomerization. Dark condition allows to understand the influence of light in the degradation of GT polyphenolic compounds. Even without direct exposure to light, degradation and oxidation of GT polyphenolic compounds continues to occur, primarily due to naturally present enzymes in solution, where the absence of light does not inhibit their activity and the spontaneous degradation process of polyphenolic compounds due to optimal pH and temperature conditions. The final condition in the study is temperature; a long-term

exposure at 40 °C was performed to see if any thermal decomposition occurs as a result of the exposition to the temperature.

A relation code between the CNS with GT polyphenolic compounds extracted and the condition in the study was created and shown in Table 18.

Table 18 - Correlation between the condition of extraction of the GT polyphenolic compounds under light, dark, and temperature conditions and the attributed code by authors.

| Extracted Medium              | Condition Tested    | Name attributed by authors                               |
|-------------------------------|---------------------|----------------------------------------------------------|
| Agave Syrup                   | Light               | GTXAG_25_T <sub>100</sub> _Light                         |
| Agave Syrup                   | Dark                | GTXAG_25_T <sub>100</sub> _Dark                          |
| Agave Syrup                   | Temperature (40 °C) | GTXAG_25_T <sub>100</sub> _Temperature                   |
| Maple Syrup                   | Light               | GTXA_25_T <sub>100</sub> _Light                          |
| Maple Syrup                   | Dark                | GTXA_25_T <sub>100</sub> _Dark                           |
| Maple Syrup                   | Temperature (40 °C) | GTXA_25_T <sub>100</sub> _Temperature                    |
| Stevia                        | Light               | GTS_25_T <sub>100</sub> _Light                           |
| Stevia                        | Dark                | GTS_25_T <sub>100</sub> _Dark                            |
| Stevia                        | Temperature (40 °C) | GTS_25_T <sub>100</sub> _Temperature                     |
| Stevia and Water<br>(1:1 w/w) | Light               | GT50S50H <sub>2</sub> O_25_T <sub>100</sub> _Light       |
| Stevia and Water<br>(1:1 w/w) | Dark                | GT50S50H <sub>2</sub> O_25_T <sub>100</sub> _Dark        |
| Stevia and Water<br>(1:1 w/w) | Temperature (40 °C) | GT50S50H <sub>2</sub> O_25_T <sub>100</sub> _Temperature |

As a result, Figures (40, 41, and 42) correspond to the stability studies conducted with the agave syrup CNS.

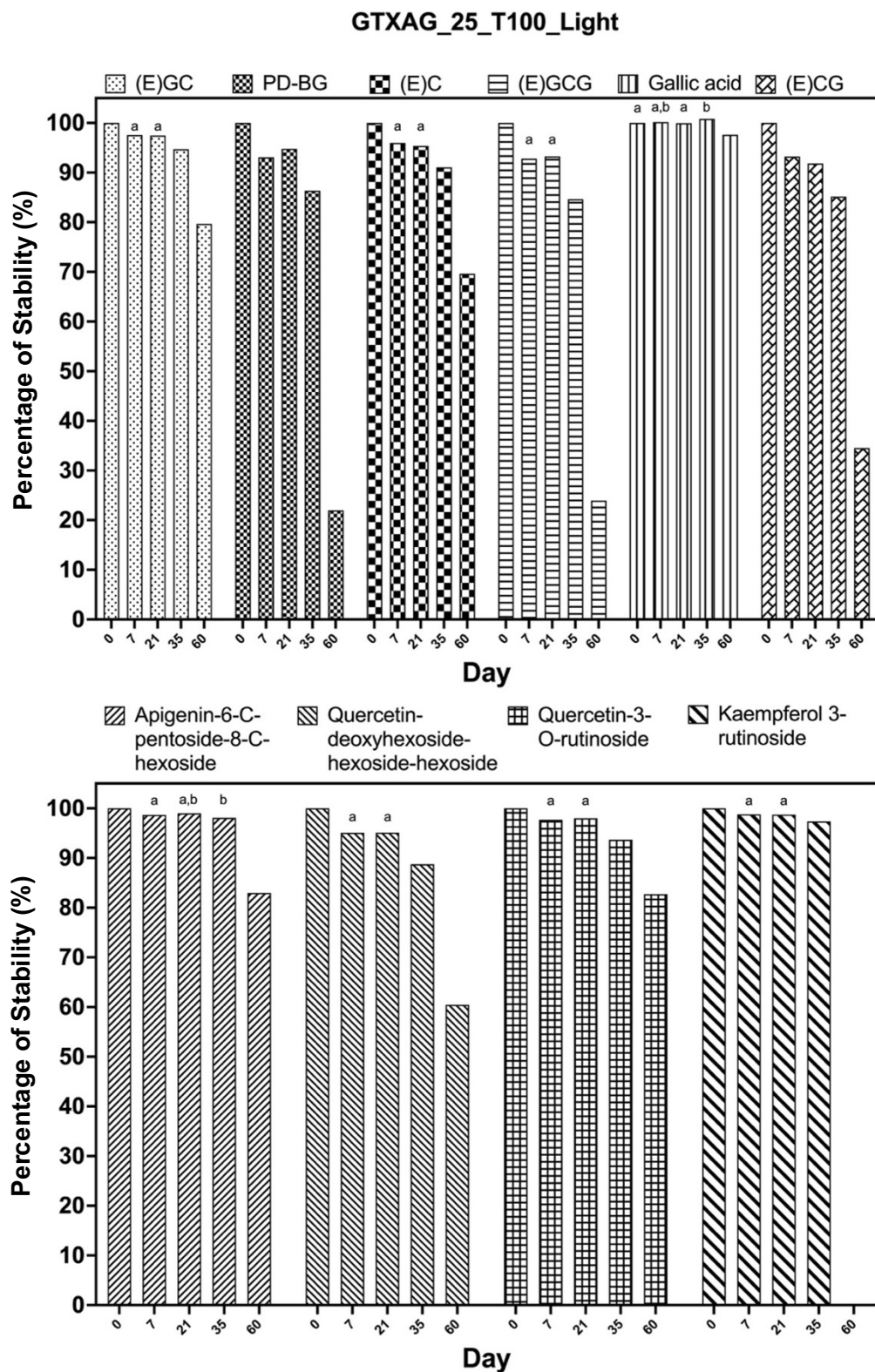


Figure 40 - Percentage of stability of each polyphenol compound in stability assay using GTXAG\_25\_T100\_Light condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.



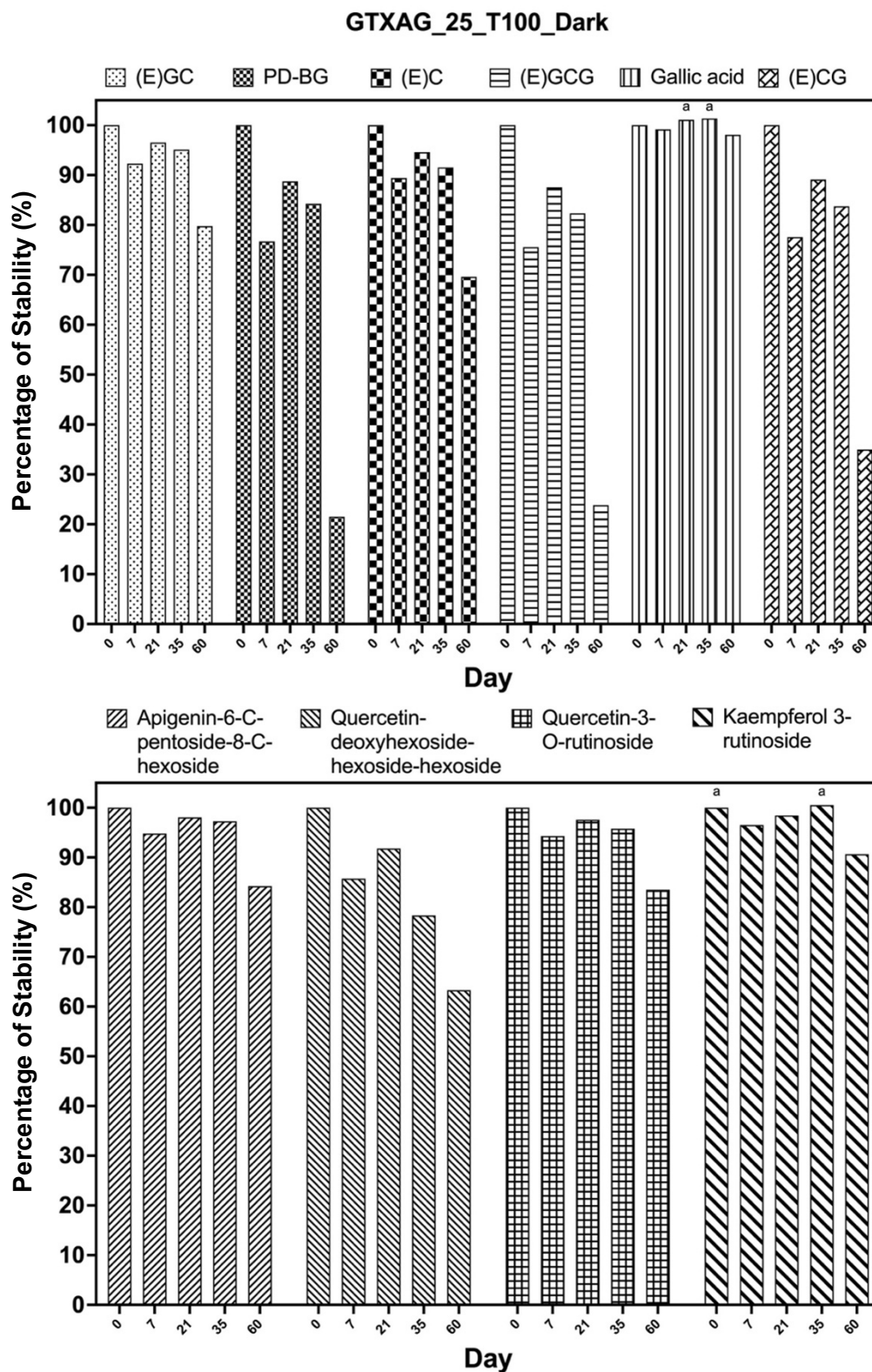


Figure 41 - Percentage of stability of each polyphenol compound in stability assay using GTXAG\_25\_T100\_Dark condition. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

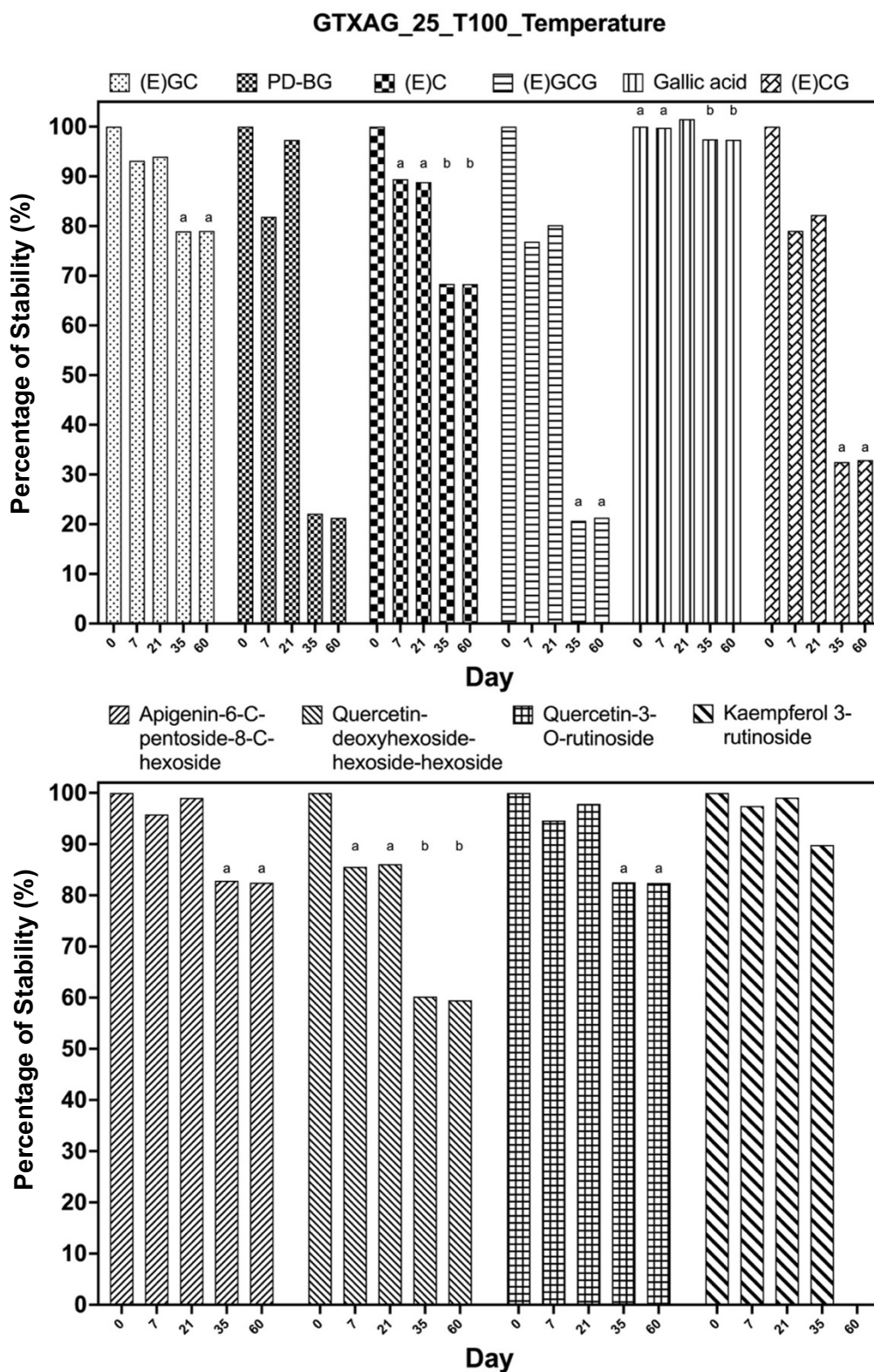


Figure 42 - Percentage of stability of each polyphenol compound in stability assay using GTXAG\_25\_T100\_Temperature condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

Only kaempferol 3-rutinoside was completely degraded after 60 days in Figure 40; with the exception of PD-BG, (E)GCG, and (E)CG, all of the other GT polyphenolic compounds demonstrated stability greater than 50% (Figure 40). Gallic acid is a component of other polyphenol compounds with more complex structures, so its formation can occur due to the degradation of some of those complex structures. Similarly, gallic acid, (E)GC, apigenin-6-C-pentoside-8-C-hexoside, and quercetin-3-O-rutinoside demonstrated excellent stability, with a stability percentage of around 80% after 60 days (Figure 40). PD-BG and (E)GCG, in addition to kaempferol 3-rutinoside, demonstrated lower compound stability, with a stability percentage of around 20% at the end of 60 days (Figure 40).

After 60 days, PD-BG and (E)GCG demonstrated stability percentages close to 20% (Figure 41), and gallic acid, (E)GC, apigenin-6-C-pentoside-8-C-hexoside, and quercetin-3-O-rutinoside demonstrated identical behavior as has been reported in GTXAG\_25\_T100\_Light results. On the other hand, kaempferol 3-rutinoside demonstrated the opposite behavior and, after 60 days, had not been degraded and demonstrated a stability percentage close to 90% (Figure 41), indicating that the absence of light significantly increased the stability of kaempferol 3-rutinoside, suggesting that it was extremely sensitive to degradation by light.

The results obtained in the GTXAG\_25\_T<sub>100</sub>\_Temperature condition (Figure 42) suggest a faster degradation of the GT polyphenolic compounds in that condition, with results similar to those described in light and dark conditions (Figures 40 and 41, respectively), but results were obtained in 35 days, whereas in light and dark conditions, it takes 60 days to reach the same stability percentage values. After 60 days, kaempferol 3-rutinoside was completely degraded, indicating that temperature is also a significant aging factor in the degradation of kaempferol 3-rutinoside in this CNS condition of extraction.

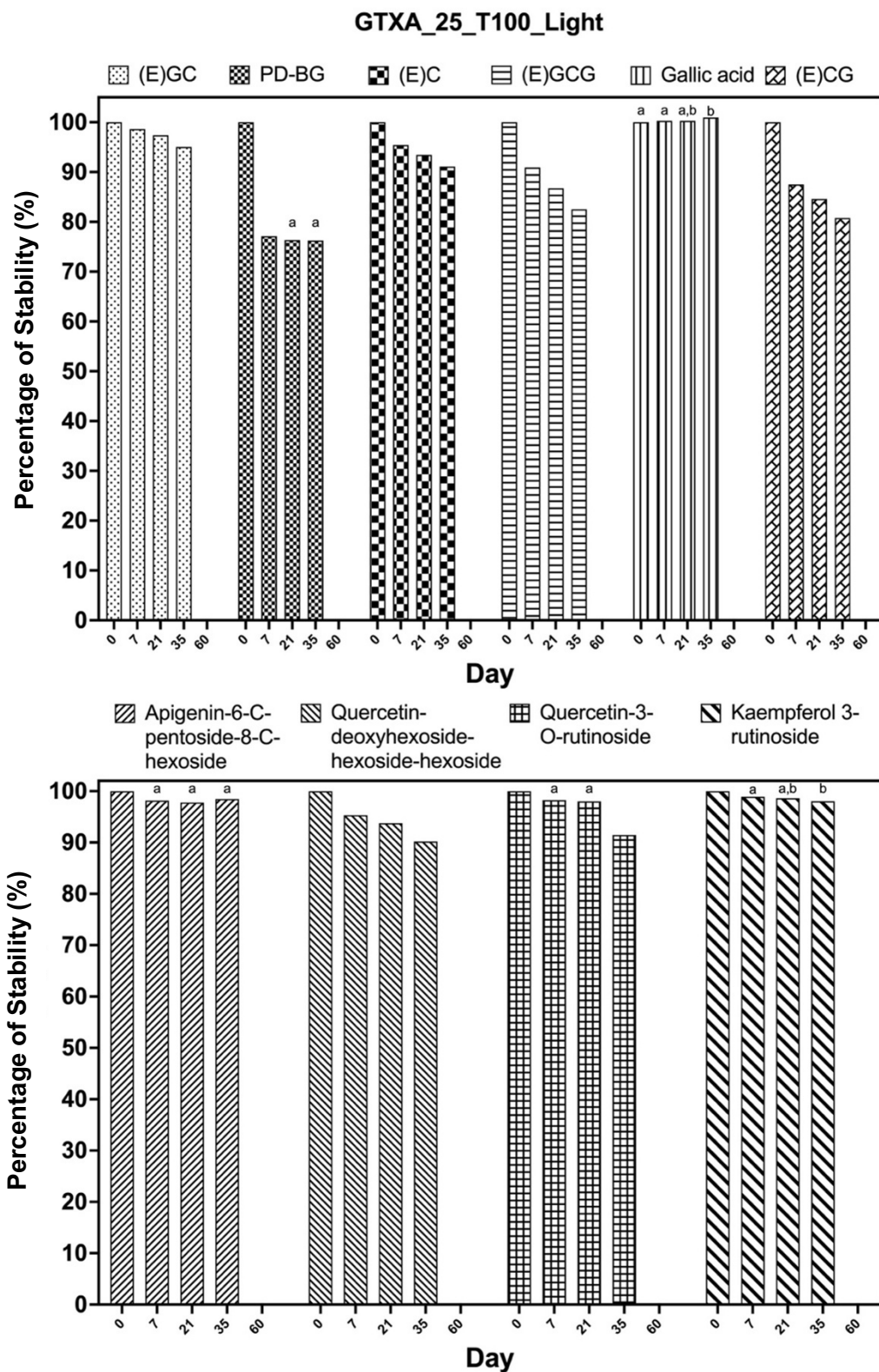


Figure 43 - Percentage of stability of each polyphenol compound in stability assay using GTXA\_25\_T<sub>100</sub>\_Light condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

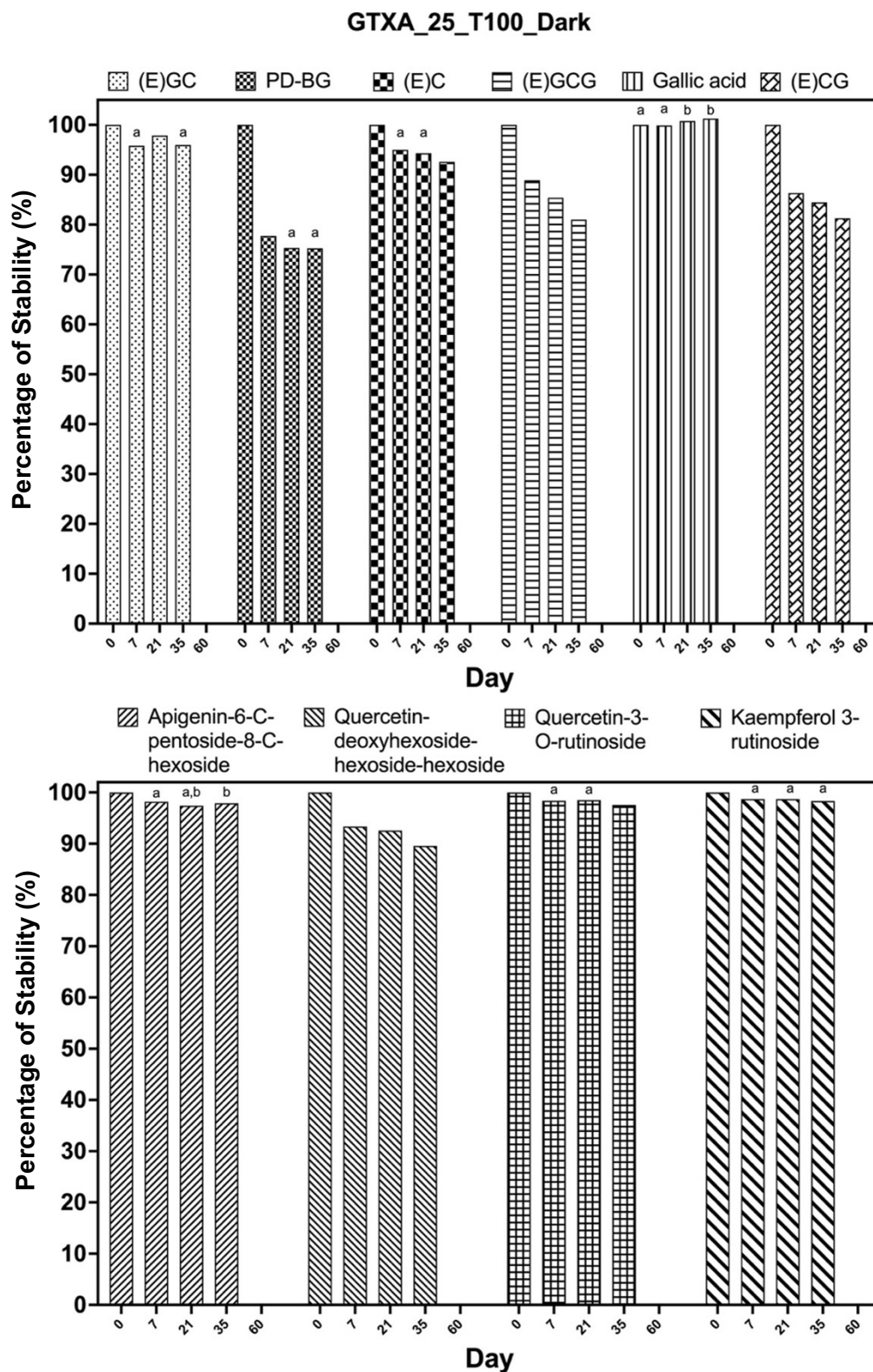


Figure 44 - Percentage of stability of each polyphenol compound in stability assay using GTXA\_25\_T100\_Dark condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

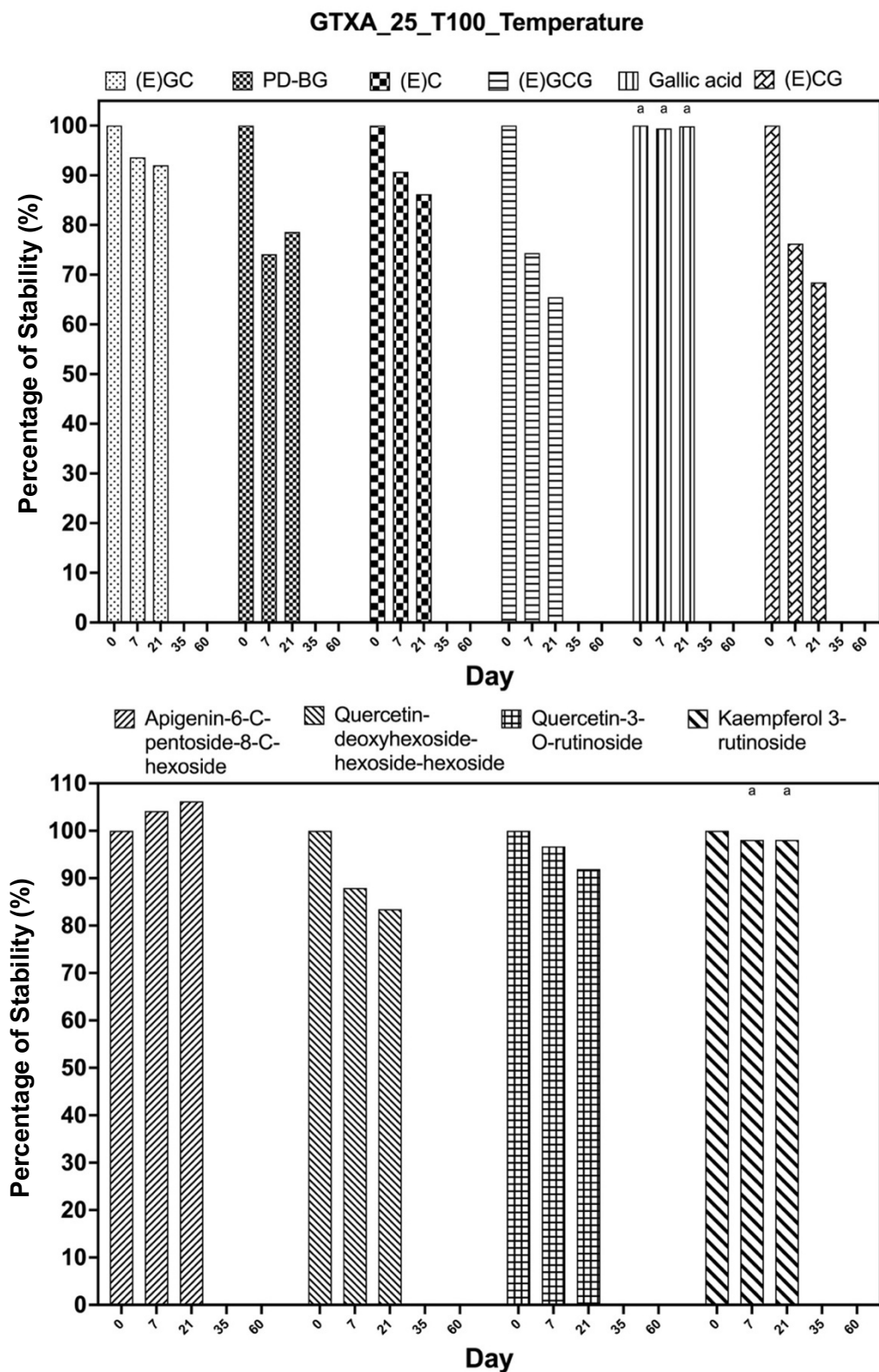


Figure 45 - Percentage of stability of each polyphenol compound in stability assay using GTXA\_25\_T<sub>100</sub>\_Temperature condition. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

GTXA\_25\_T<sub>100</sub>\_Light (Figure 43) showed similar stability until 35-day assays when compared to GTXAG\_25\_T<sub>100</sub>\_Light (Figure 40) and GTXA\_25\_T<sub>100</sub>\_Dark (Figure 44) when compared to GTXAG\_25\_T<sub>100</sub>\_Dark (Figure 41) at the same 35 days. However, after 60 days, a fungal colony was detected in the extraction samples (Figure 46).

In contrast to the other two degradation conditions with the same CNS, but consistent with what was previously stated, the degradation of GT polyphenolic compounds is faster at higher temperatures (Figure 45). Due to the probable loss of water in the CNS, the extracted sample solidified after 35 days (Figure 46).

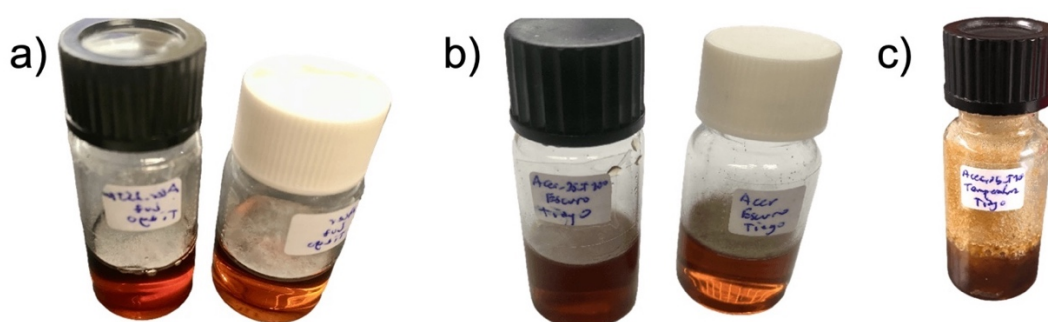


Figure 46 – Fungal colony detected in a) GTXA\_25\_T<sub>100</sub>\_Light and b) GTXA\_25\_T<sub>100</sub>\_Dark; after 60 days, and c) GTXA\_25\_T<sub>100</sub>\_Temperature solidified after 35 days.

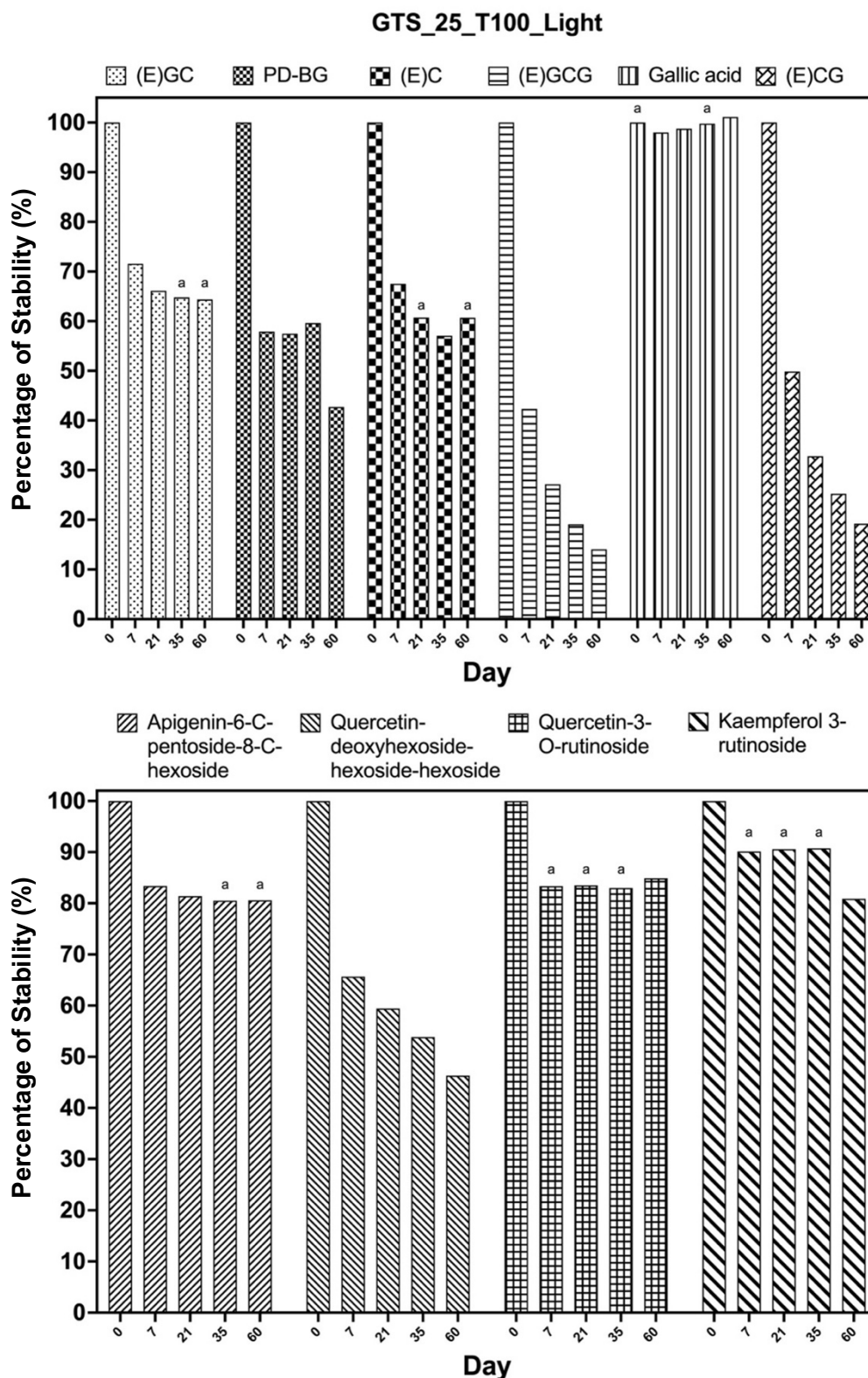


Figure 47 - Percentage of stability of each polyphenol compound in stability assay using GTS\_25\_T100\_Light condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.



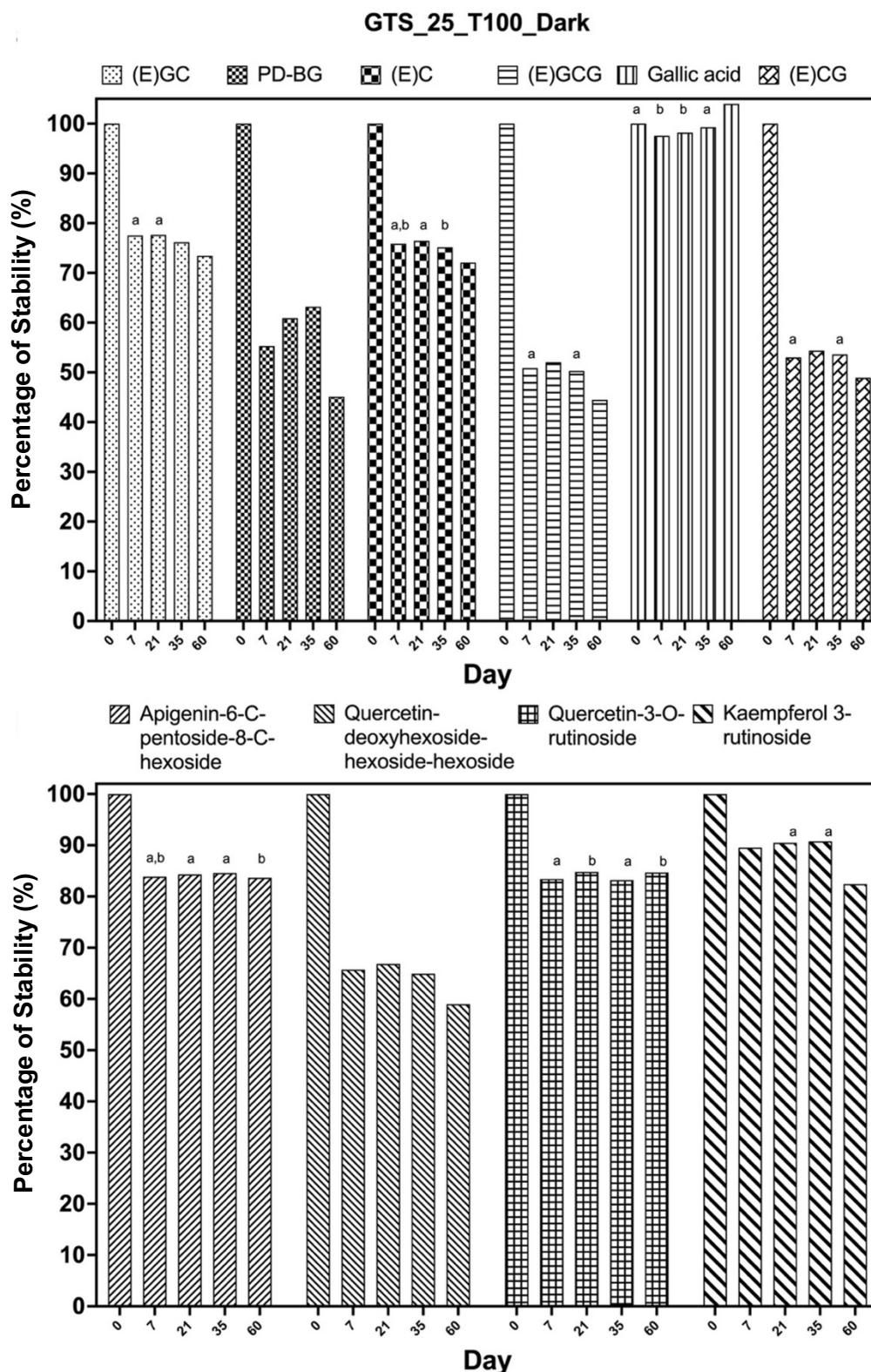


Figure 48 - Percentage of stability of each polyphenol compound in stability assay using GTS\_25\_T100\_Dark condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

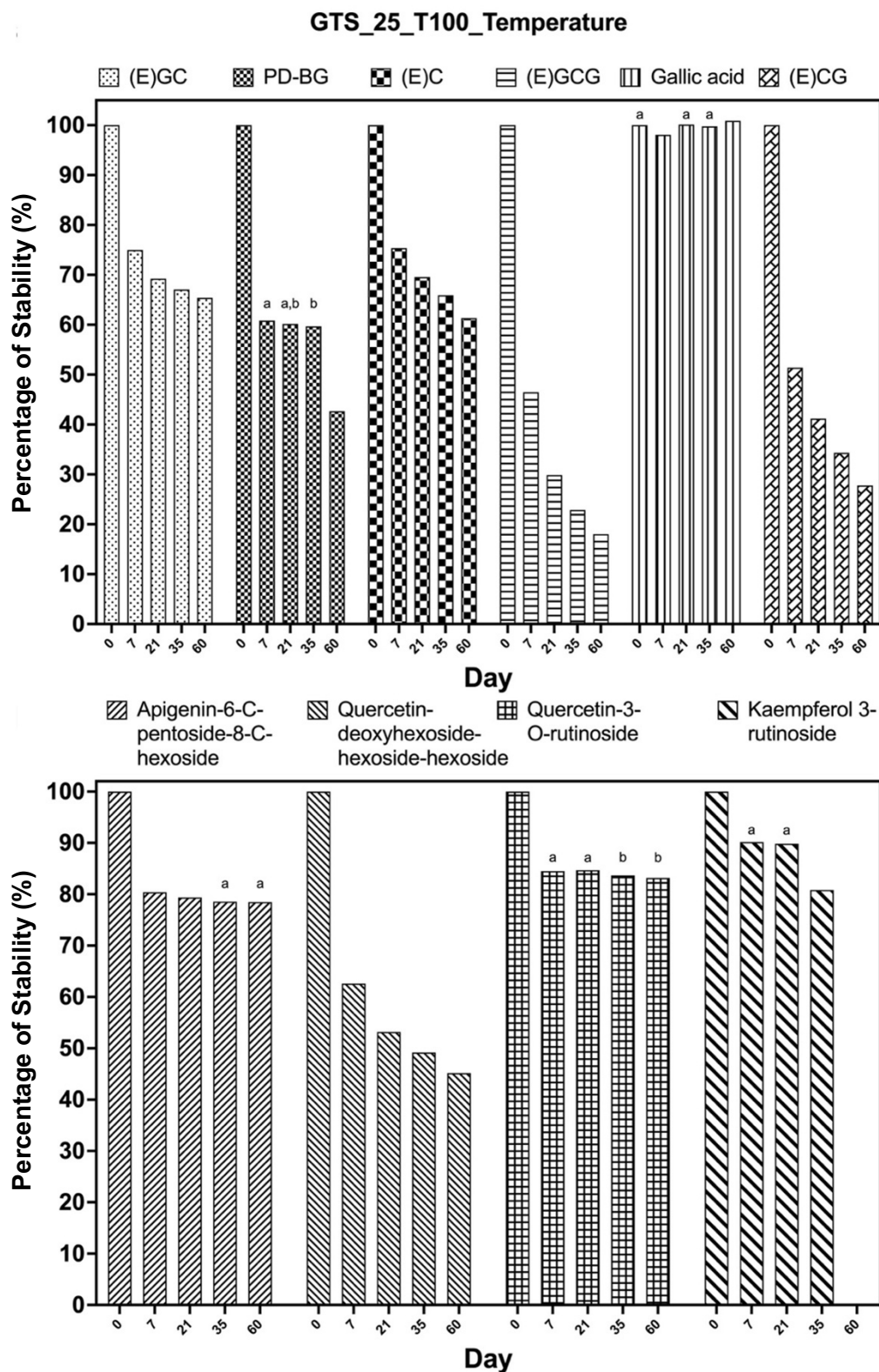


Figure 49 - Percentage of stability of each polyphenol compound in stability assay using GTS\_25\_T100\_Temperature condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

Figure 47 depicts the results obtained under GTS\_25\_T<sub>100</sub>\_Light conditions over 60 days. In comparison to the extracts with agave syrup (GTXAG) and maple syrup (GTXA), a faster degradation occurs in the first 7 days. After 60 days, (E)GCG and (E)CG showed a lower percentage of stability of around 15 % and 20 %, respectively. However, in comparison to GTXAG\_25\_T<sub>100</sub>\_Light, PD-BG demonstrated better stability, with approximately 40% degradation after 60 days. Once again, gallic acid's higher stability can be attributed to its formation via the degradation of more complex structures, as well as (E)GC and (E)C, whose percentage stability remains greater than 50 % after 60 days and can be obtained via the degradation of more complex structures. After 60 days, kaempferol 3-rutinoside showed good stability, indicating that the CNS matrix can interfere with the stability of GT polyphenolic compounds.

In comparison to the previous conditions, the GTS\_25\_T<sub>100</sub>\_Dark condition (Figure 48) demonstrated a higher percentage of stability for PD-BG, (E)GCG, and (E)CG. After 60 days, PD-BG and (E)GCG had a lower percentage of stability (above 40 %), about twice as stable as GTXAG\_25\_T<sub>100</sub>\_Dark. The remaining GT polyphenolic compounds demonstrated a percentage of stability similar to GTXAG\_25\_T<sub>100</sub>\_Dark.

GTS\_25\_T<sub>100</sub> (Figure 49) degraded similarly to GTS\_25\_T<sub>100</sub>\_Light, with the exception of kaempferol 3-rutinoside, which was completely degraded after 60 days, indicating a higher sensitivity to temperature degradation in this CNS.

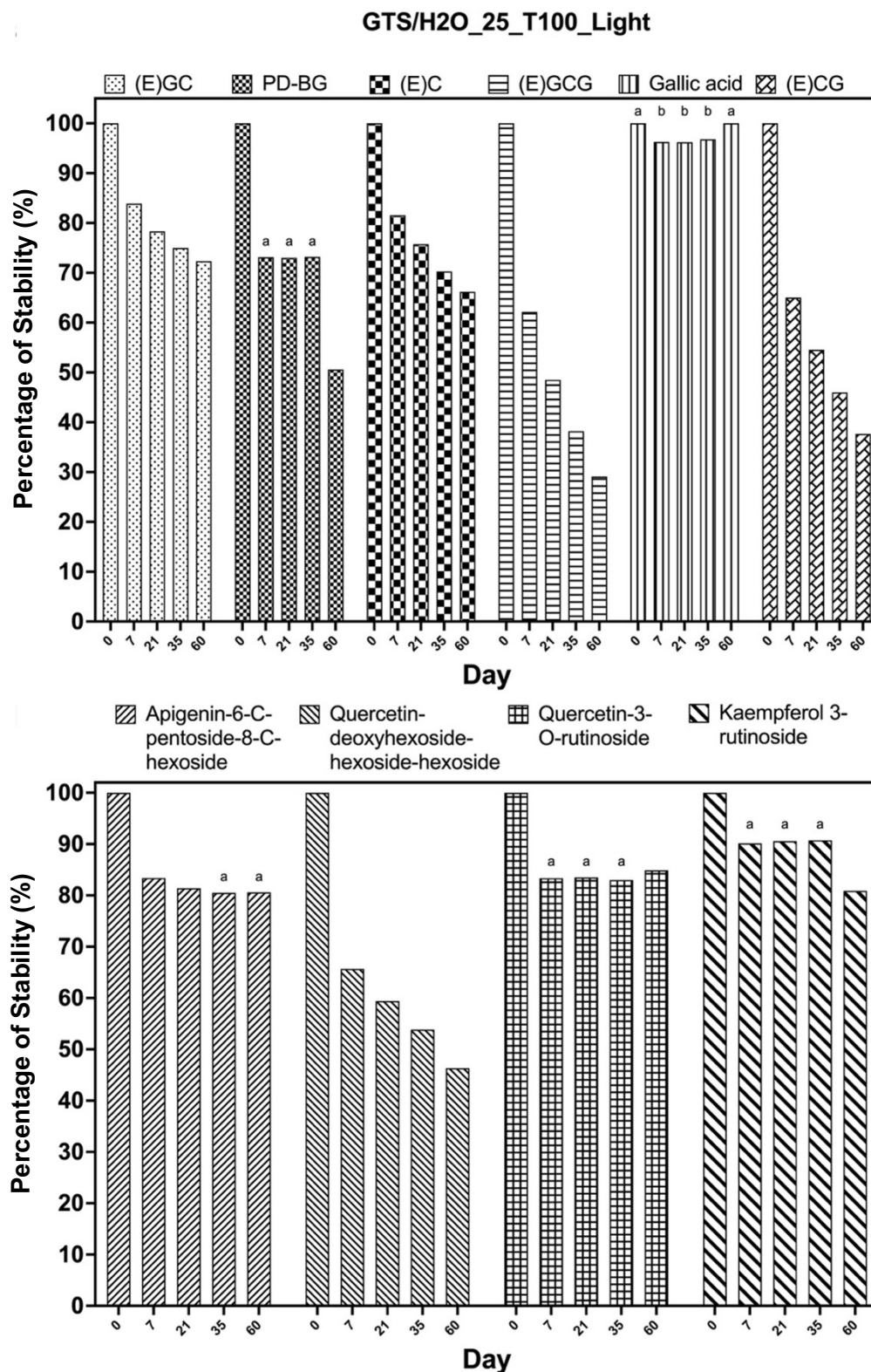


Figure 50 - Percentage of stability of each polyphenol compound in stability assay using GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Light condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

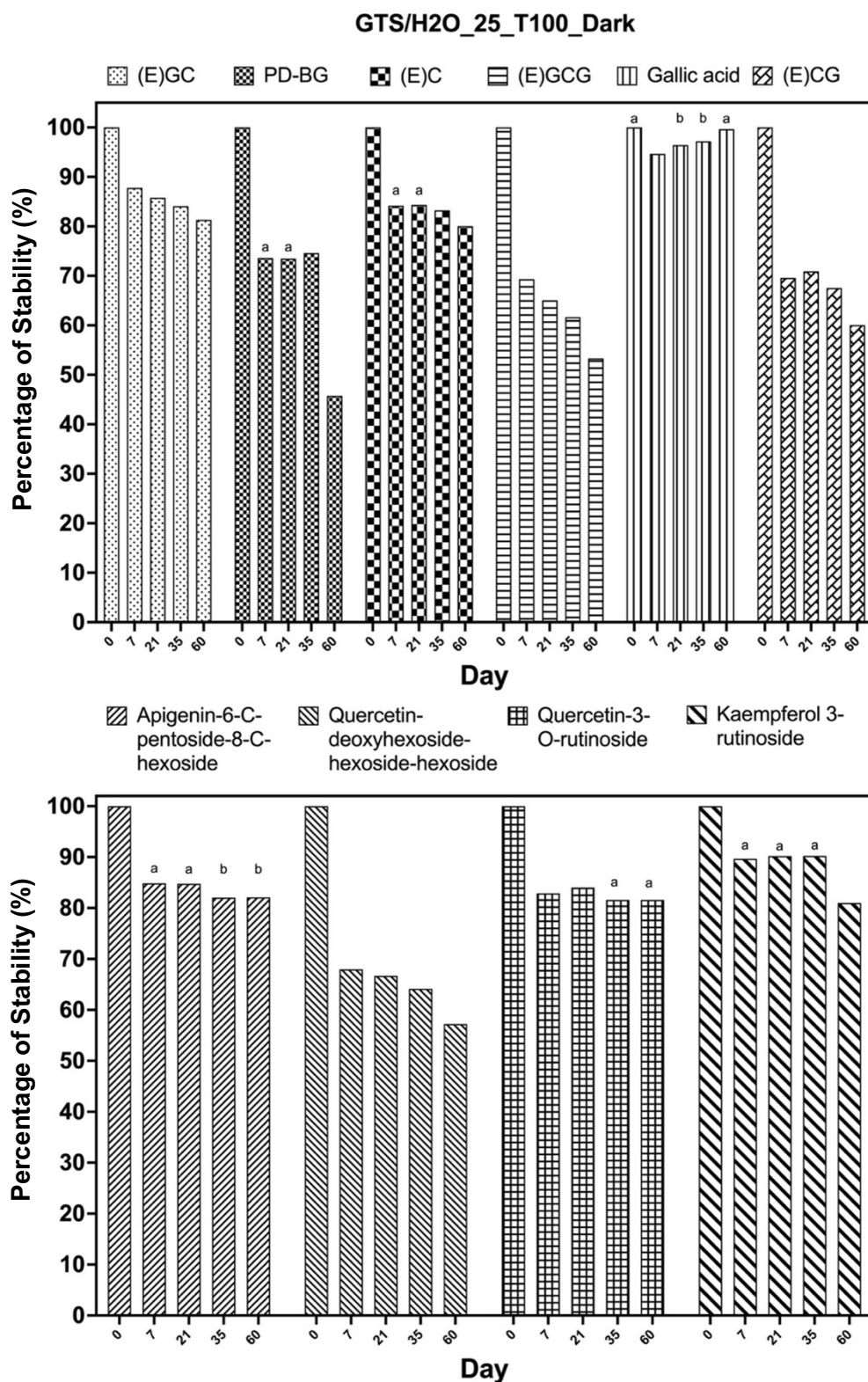


Figure 51 - Percentage of stability of each polyphenol compound in stability assay using GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Dark condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

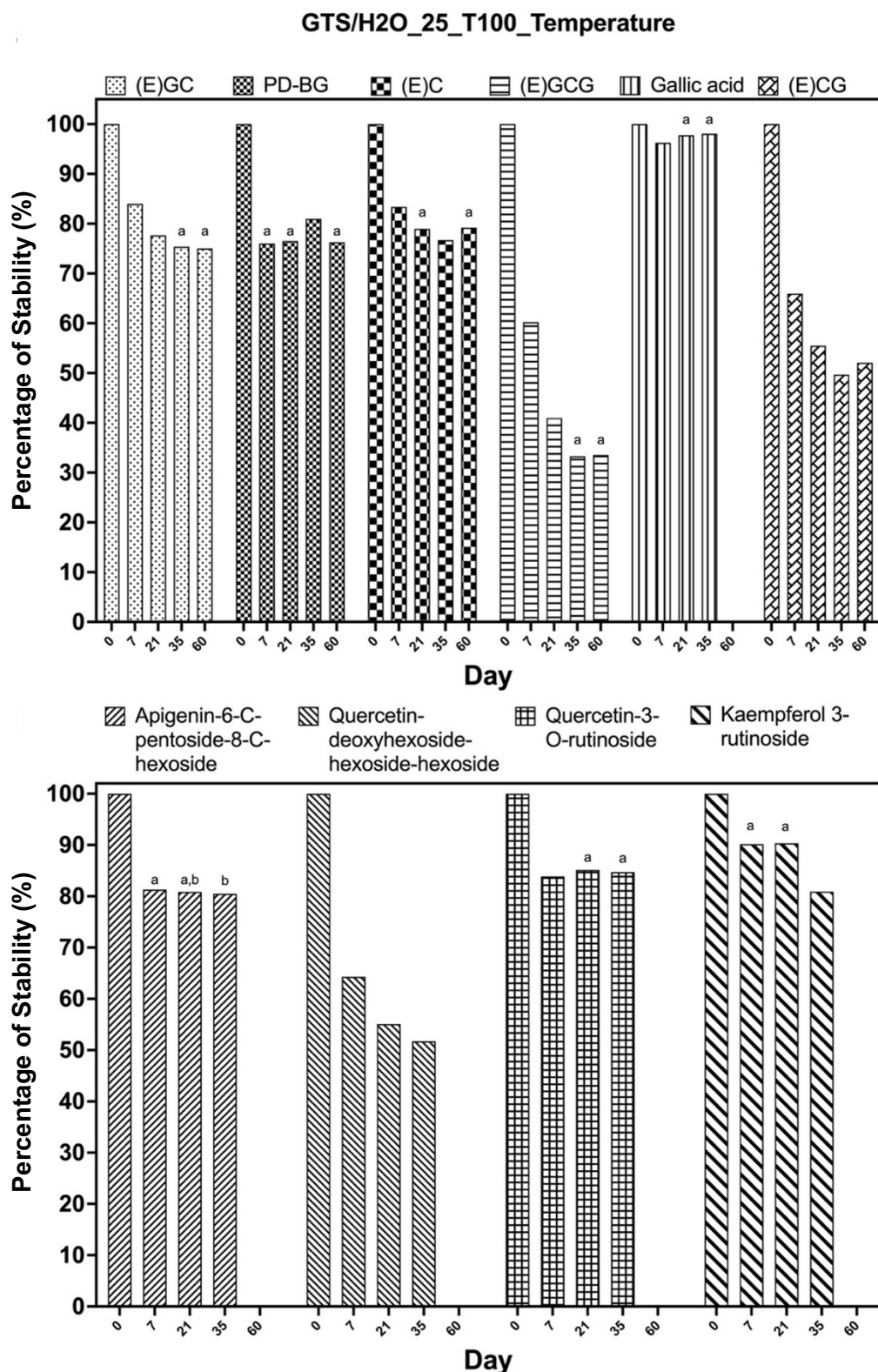


Figure 52 - Percentage of stability of each polyphenol compound in stability assay using GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Temperature condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

In agreement with the stability assays with stevia alone, this stevia and water (1:1 w/w) mixture (Figure 50) demonstrated better stability for the PD-BG, (E)GCG, and (E)CG compounds, with a percentage of stability around 50 % in the case of PD-BG and higher than 20 % for (E)GCG and (E)CG. After 60 days of light aging, the other GT polyphenolic compounds showed the same percentage of stability as the other conditions under light. Furthermore, kaempferol 3-rutinoside has not been degraded and has a stability percentage of around 80 %.

Analyzing the GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Dark condition results (Figure 51), it is possible to see that only the PD-BG compound had a percentage of stability lower than 50%, and six of the GT polyphenolic compounds ((E)GC, (E)C, gallic acid, apigenin-6-C-pentoside-8-C-hexoside, quercetin-3-O-rutinoside, and kaempferol 3-rutinoside) showed a percentage of stability around or even higher than 80 % after 60 days.

Figure 52 depicts the results of the GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Temperature analysis, which revealed an unexpected result. GT polyphenolic compounds with lower percentages of stability, such as PD-BG and (E)CG, demonstrated in this case a percentage of stability over 50 %. GT polyphenolic compounds, on the other hand, such as gallic acid, apigenin-6-C-pentoside-8-C-hexoside, and quercetin-3-O-rutinoside, which typically have a higher percentage of stability, were completely degraded after 60 days. Different targets of degradation can be observed, possibly with the higher water content in the sample and the temperature influence.

The sample had solidified nine days after the day 60 stability test (Figure 53), possibly due to the low water content of the sample and the elevated degradation of kaempferol 3-rutinoside, which contributed to a higher concentration of sugar in the sample and led to crystallization.

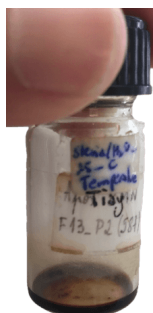


Figure 53 – GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Temperature solidified after 69 days.

### 3.3.2. Colorimetry

Colorimetry is a branch of science concerned with the measurement and description of colors. It is essential in many disciplines, including materials science, design, the textile industry, and printing. Colorimetry is a technique that allows for the objective evaluation of colors in order to accurately characterize and communicate them.<sup>119-121</sup>

The CIELAB color space, developed by the International Commission on Illumination (CIE), is one of the most widely used notations in colorimetry. The CIELAB notation is based on the human visual system's perceptual characteristics and provides a three-dimensional representation of colors.<sup>122</sup> Colors are described using three main components:  $L^*$ ,  $a^*$ , and  $b^*$ . The  $L^*$  component represents the color's lightness or brightness, which ranges from 0 (black) to 100 (white). The  $a^*$  component describes the color's position along the red-green axis, with positive values representing red tones and negative values representing green tones. The color's position along the yellow-blue axis is described by the  $b^*$  component, with positive values indicating yellow tones and negative values indicating blue tones.<sup>122, 123</sup> The CIELAB notation is especially useful because it enables objective measurements and comparisons of different color samples. It is device-independent and can describe colors outside of the visible spectrum.

The ( $L^*$ ;  $C^*$ ;  $h^\circ$ ) color metric system is an extension of the CIELAB system that provides additional information about color characteristics in addition to the  $L^*$ ,  $a^*$ , and  $b^*$  components. The  $C^*$  component, chroma (Equation 3), in this system represents the color's intensity or saturation, indicating how vibrant or diluted it is. Higher  $C^*$  values denote more saturated colors, while lower values denote less saturated colors.<sup>124</sup>

$$C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

Equation 3 – Chroma ( $C^*$ ) parameter calculation.

The hue angle is represented by the  $h^\circ$  component, which describes color in terms of its angular position in the color space. It has a scale of 0 to 360 degrees, with 0 representing pure red, 90 representing a pure yellow, 180 representing pure green, and 270 representing pure blue. The hue angle can be used to differentiate different color hues, allowing for a more precise analysis of chromatic variation.<sup>122-124</sup> The ( $L^*$ ;  $C^*$ ;  $h^\circ$ ) color metric system provides a more comprehensive and detailed description of colors. This notation, in conjunction with the CIELAB notation, improves the ability to describe and communicate



colors by providing a more complete and objective representation of their visual properties, which are normally measured by a colorimeter or a spectrophotometer.

A spectrophotometer is a scientific device that measures the intensity of light at various wavelengths. It is especially useful in colorimetric studies, where color is an important property to assess. A spectrophotometer's basic operation is based on the Beer-Lambert law, which describes the relationship between a substance's concentration in a solution and the amount of light absorbed by that substance. According to this law, the absorbance of a solution is proportional to the concentration of the substance present.<sup>125,</sup>

126

Light from a source is directed onto the sample under study to perform a colorimetric analysis. This light is directed to a detector after passing through the sample. At various wavelengths, the spectrophotometer measures the amount of light transmitted or absorbed by the sample (Figure 54).<sup>127</sup>

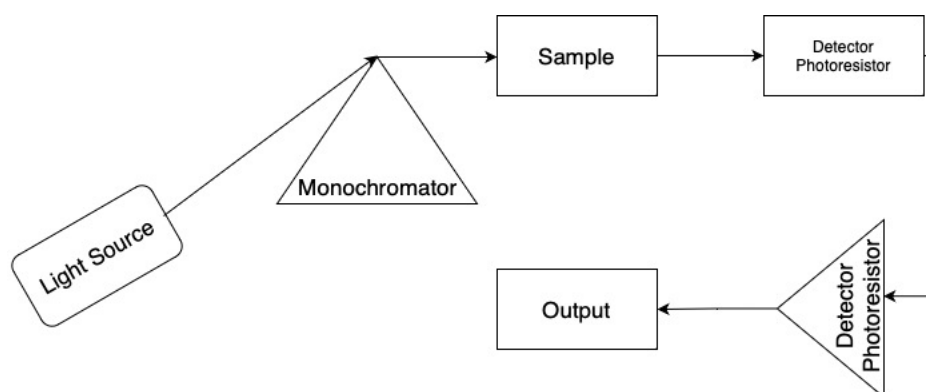


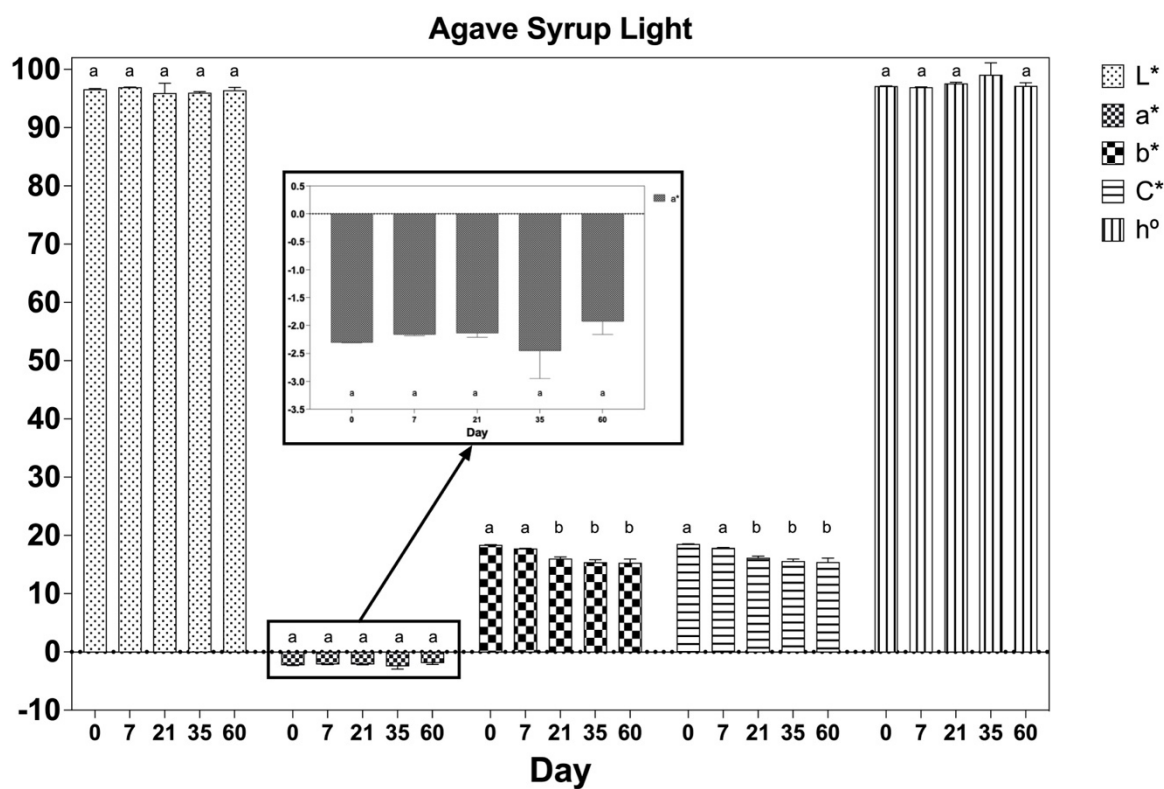
Figure 54 - Spectrophotometer schematic process.

The spectrophotometer creates an absorption or transmittance spectrum by measuring the intensity of light at each wavelength. This graph depicts how much light is absorbed or transmitted by the sample at various wavelengths.<sup>127</sup> It is used in colorimetric studies to determine the concentration of a colored substance in a solution by determining the intensity of color, which is important in fields such as chemistry, biochemistry, environmental sciences, and food analysis.<sup>128, 129</sup>

A colorimetric study was conducted to gain a better understanding of the changes in the sample's stability assays, and the results are shown below. An attribution was performed taking into account the following division of values (Table 19).

Table 19 - Relation between a data range and author's attribution.

| Range       | Attribution  |
|-------------|--------------|
| 0  to  30   | Low          |
| 30  to  40  | Low Average  |
| 40  to  60  | Average      |
| 60  to  70  | High Average |
| 70  to  100 | High         |

Figure 55 - Colorimetric stability assay using Agave Syrup in Light condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

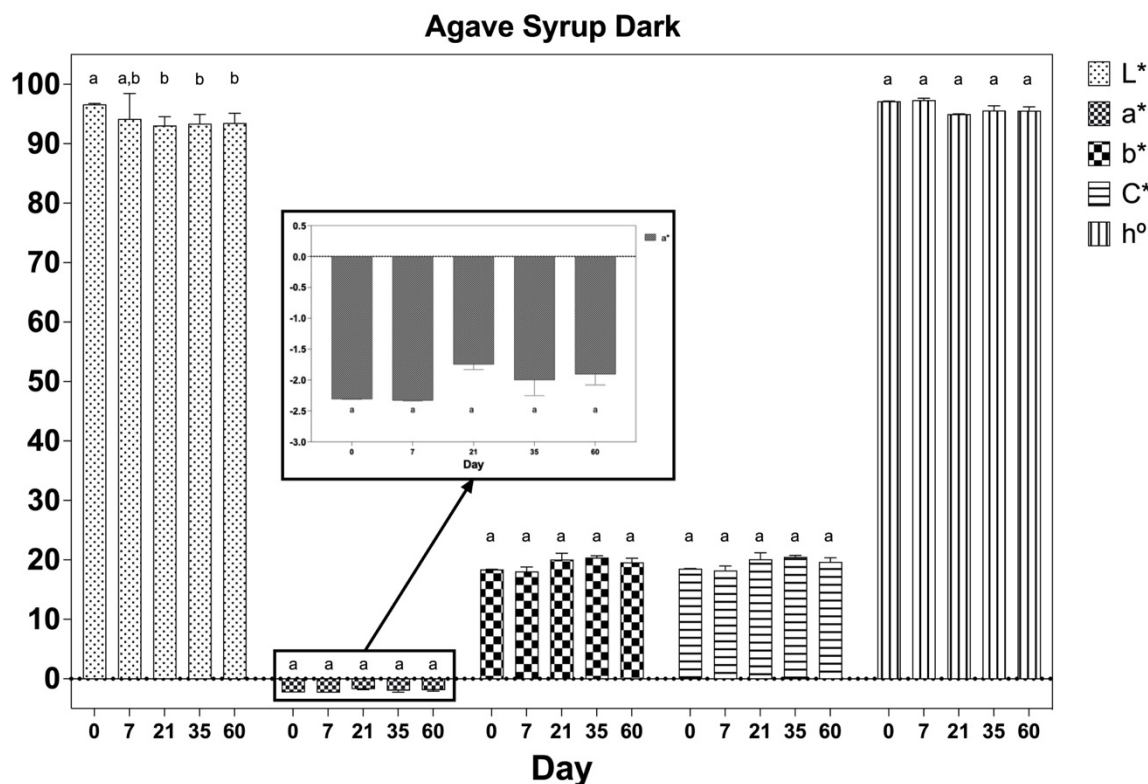


Figure 56 - Colorimetric stability assay using agave syrup in dark condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

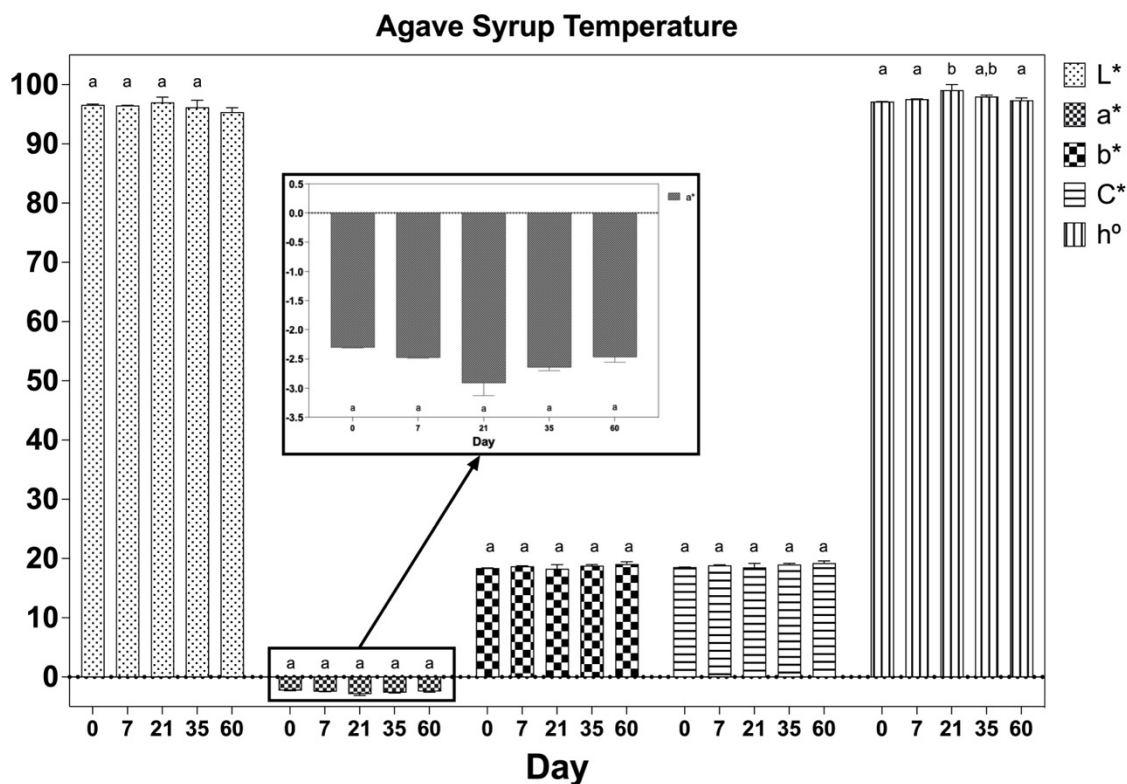


Figure 57 - Colorimetric stability assay using Agave Syrup in Temperature condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

Figure 55 shows the variations in  $L^*$ ,  $a^*$ ,  $b^*$ ,  $C^*$ , and  $h^\circ$  parameters over 60 days for Agave Syrup exposed to light.  $L^*$  and  $a^*$  had no significant differences in these parameters after 60 days, whereas  $b^*$  and  $C^*$  had significant differences after 21 days, but no more significant differences had been registered until day 60. Only the hue angle ( $h^\circ$ ) showed significant differences in the stability assays performed on day 35.

After 21 days, agave syrup in the dark (Figure 56) showed a significant decrease in the  $L^*$  (lightness) parameter, but no further significant variation was observed until day 60. The remaining color parameters show no significant variations under dark conditions.

As shown in Figure 57, the  $L^*$  parameter showed a significant difference after 60 days. The  $a^*$ ,  $b^*$ ,  $C^*$ , and  $h^\circ$  parameters did not show any significant differences over the 60-day period, but the  $h^\circ$  parameter did show some significant differences on day 21.

After 60 days, the color of agave syrup under light, dark, and temperature conditions remained consistent, with high lightness, low green and yellow tones, low saturation, and close to pure yellow.

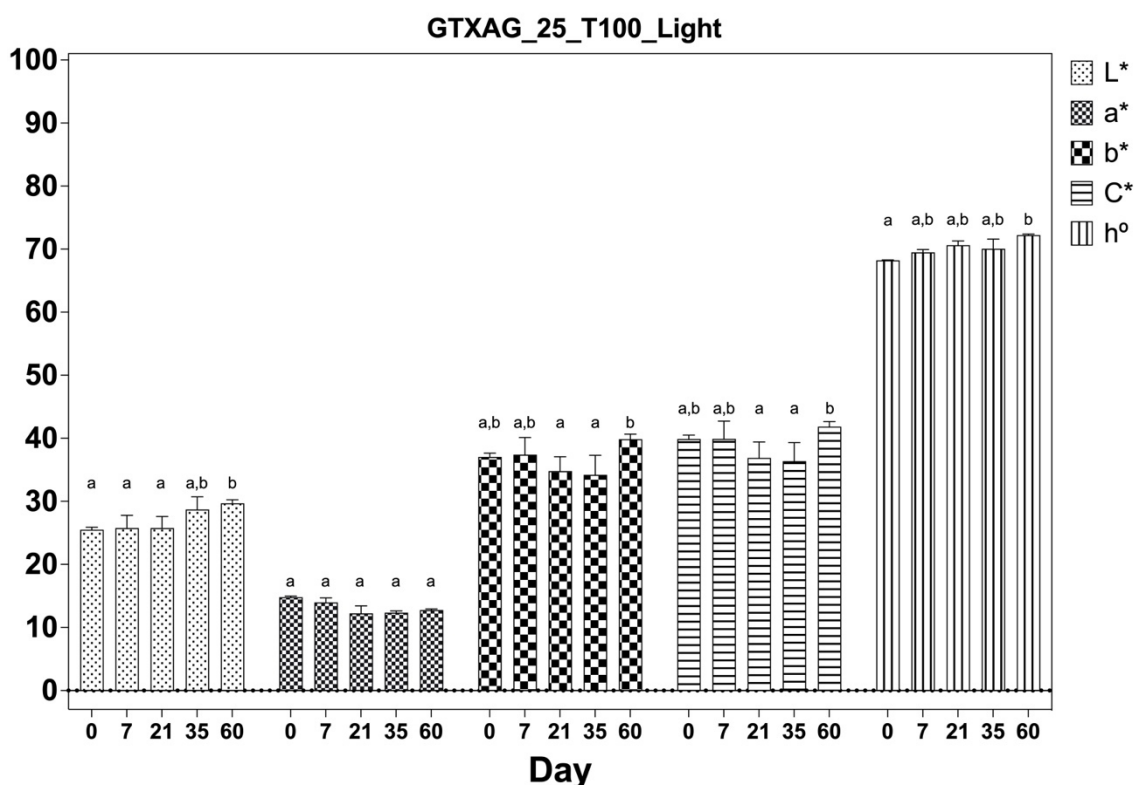


Figure 58 - Colorimetric stability assay using GTXAG\_25\_T<sub>100</sub>\_Light condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

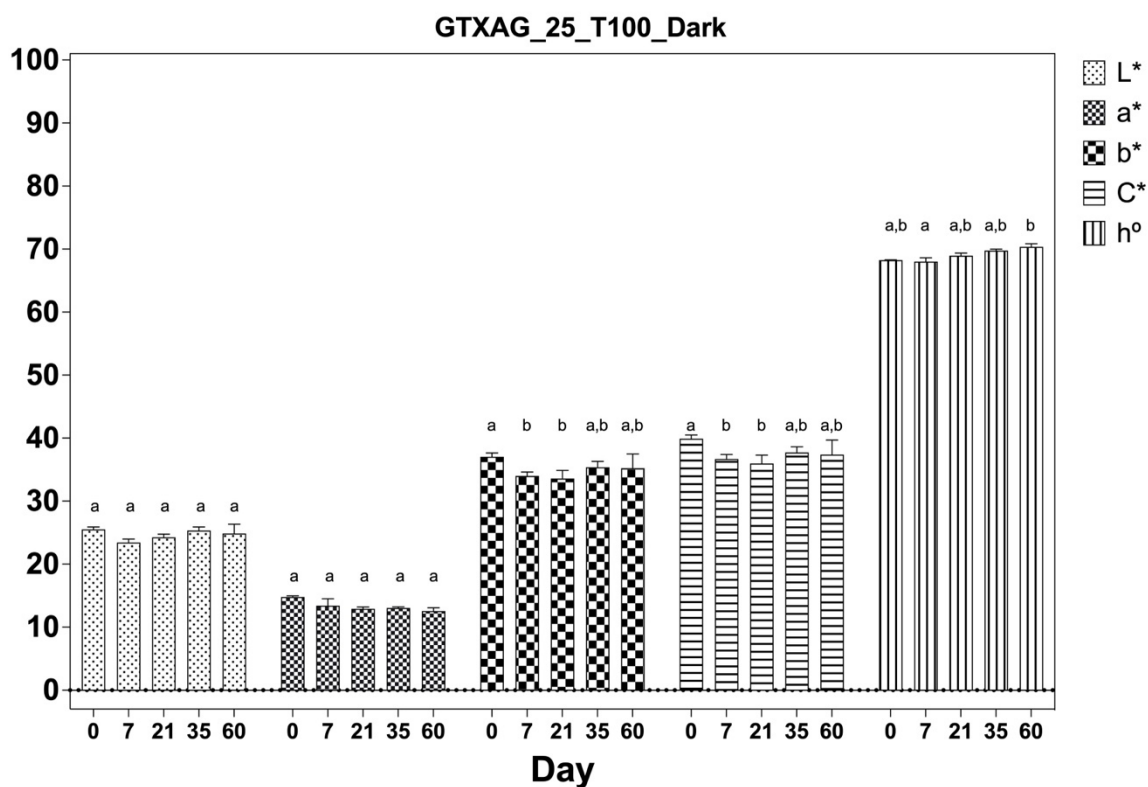


Figure 59 - Colorimetric stability assay using GTXAG\_25\_T<sub>100</sub>\_Dark condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

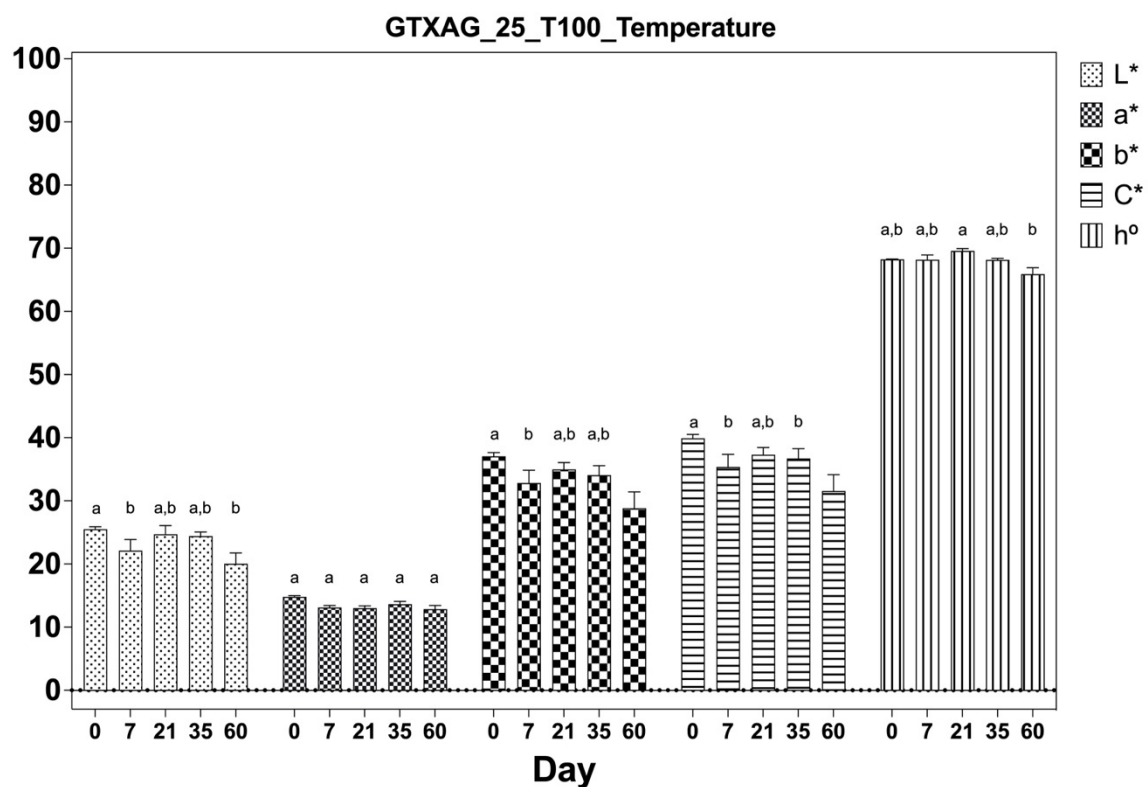


Figure 60 - Colorimetric stability assay using GTXAG\_25\_T<sub>100</sub>\_Temperature condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

After 60 days,  $L^*$  showed significant differences, with an increase in lightness in the GTXAG\_25\_T<sub>100</sub>\_Light condition (Figure 58). There were no significant differences in the  $a^*$  parameter, and the  $b^*$  and  $C^*$  parameters showed similar variation over the 60 days, with a significant difference in the assays performed on days 21 and 35 compared to the assay performed on day 60. Over the last 60 days, the  $h^\circ$  parameter has increased significantly.

Over 60 days, no significant differences were observed in the GTXAG\_25\_T<sub>100</sub>\_Dark condition (Figure 59). However, with the exception of  $L^*$  and  $a^*$  parameters, the others demonstrated significant variation in assays on days 7 and 21 compared to the assay on day 0 for the  $b^*$  and  $C^*$  parameters, and in the assay on day 7 compared to the assay on day 60 for the  $h^\circ$  parameter.

With the exception of the  $a^*$  and  $h^\circ$  parameters, all other parameters for the GTXAG\_25\_T<sub>100</sub>\_Temperature condition showed significant differences after 60 days (Figure 60).  $L^*$  showed a significant decrease in lightness after 60 days,  $b^*$  was diverted to more blue tones, with a significant decrease after 60 days, and saturation, like the other variation, showed a significant decrease after 60 days. In the  $a^*$  parameter, no significant variations were observed across all assays performed, and in the  $h^\circ$  parameter, although no significant variations were observed after 60 days, a significant variation was observed between the assays performed on days 21 and 60.

GTXAG\_25\_T<sub>100</sub>\_Light and GTXAG\_25\_T<sub>100</sub>\_Dark had lower lightness, low average yellow and low red tones, low average saturation, and was located between a pure red and a pure yellow. Some statistical differences in sample appearance are registered over the 60 days but the attribution at the day 0 is still the same.

GTXAG\_25\_T<sub>100</sub>\_Temperature had low lightness, low yellow and low red tones, low average saturation, and appeared as a mix of red and yellow. Over the course of 60 days, there were significant differences in the appearance of the sample when compared to day 0.

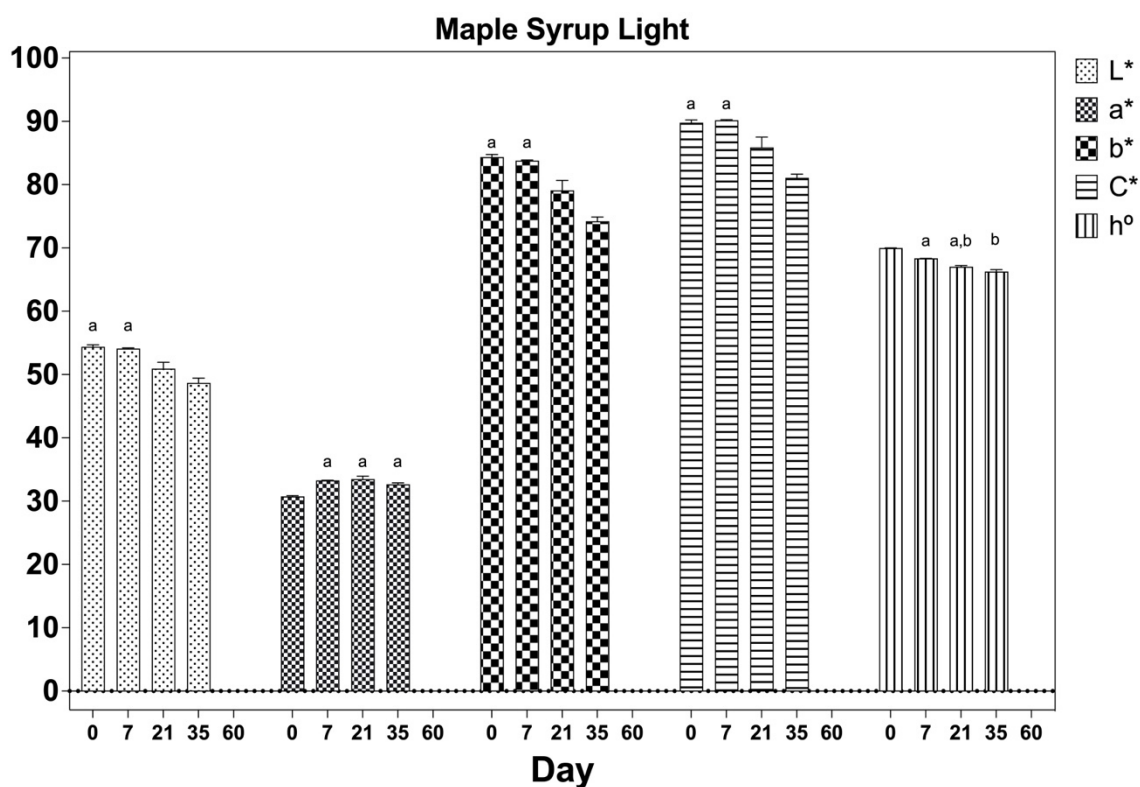


Figure 61 - Colorimetric stability assay using Maple Syrup in Light condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

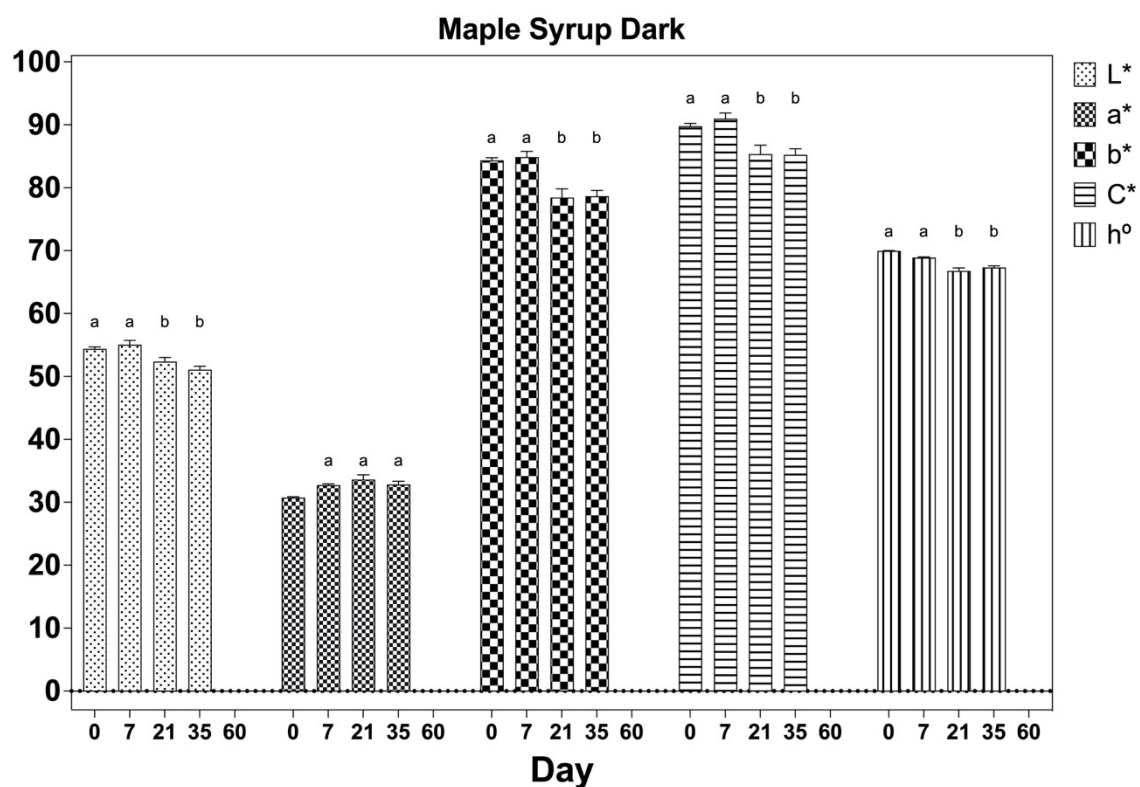


Figure 62 - Colorimetric stability assay using Maple Syrup in Dark condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.



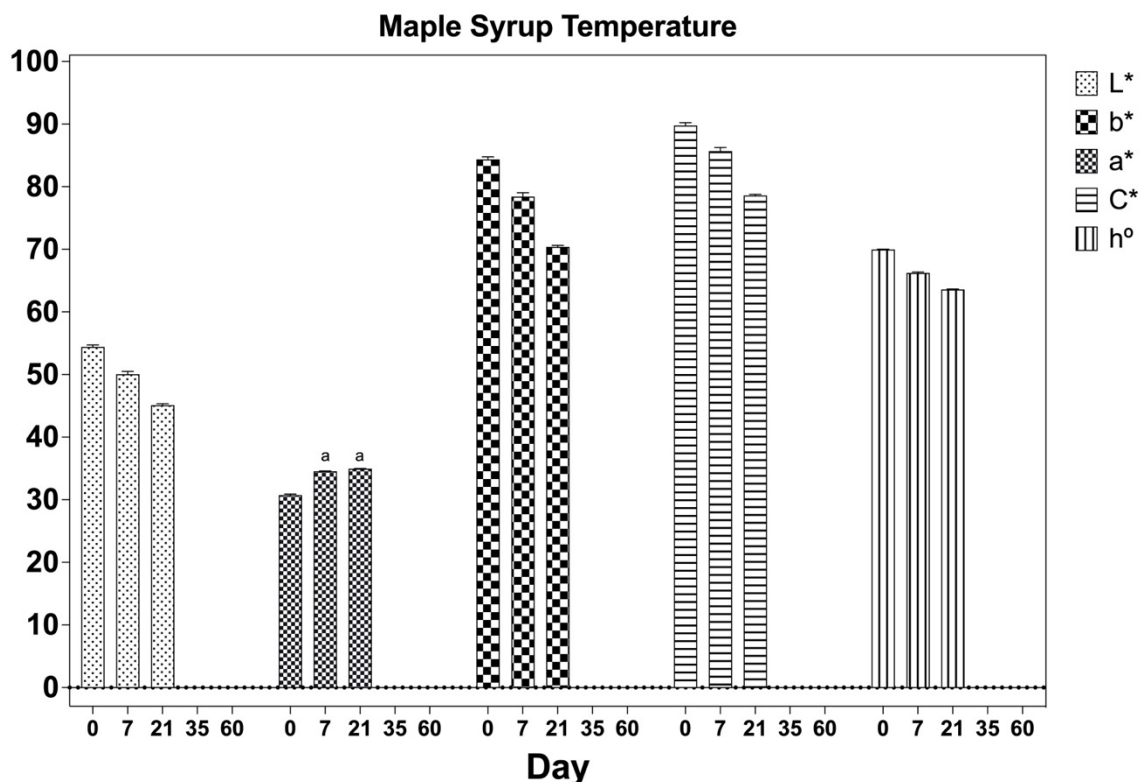


Figure 63 - Colorimetric stability assay using Maple Syrup in Temperature condition. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

After 35 days, all color parameters studied in maple syrup under light conditions (Figure 61) showed significant differences. As previously stated, a fungal colony was identified in the GTXA\_25\_T<sub>100</sub>\_Light condition after 60 days, and no further tests using maple syrup and light conditions were performed.

Over the course of 35 days, the maple syrup under dark conditions (Figure 62) showed significant variations in all color parameters. Similarly to maple syrup under light conditions, a fungal colony was identified in GTXA\_25\_T<sub>100</sub>\_Dark after 60 days, and no further studies using maple syrup and dark conditions were conducted.

As previously stated, GTXA\_25\_T<sub>100</sub>\_Temperature had solidified after 35 days, and no further assays using maple syrup and temperature conditions were performed. Significant differences in all color parameters were found in maple syrup after 21 days under temperature condition (Figure 63).

Maple syrup after 21 days under temperature condition and 35 days under light and dark conditions has an average lightness, high yellow and low average red tones, high saturation, and a color between a pure red and a pure yellow. The samples at day 0 are the



same appearance attribution but significant statistical differences in the appearance of the sample were registered.

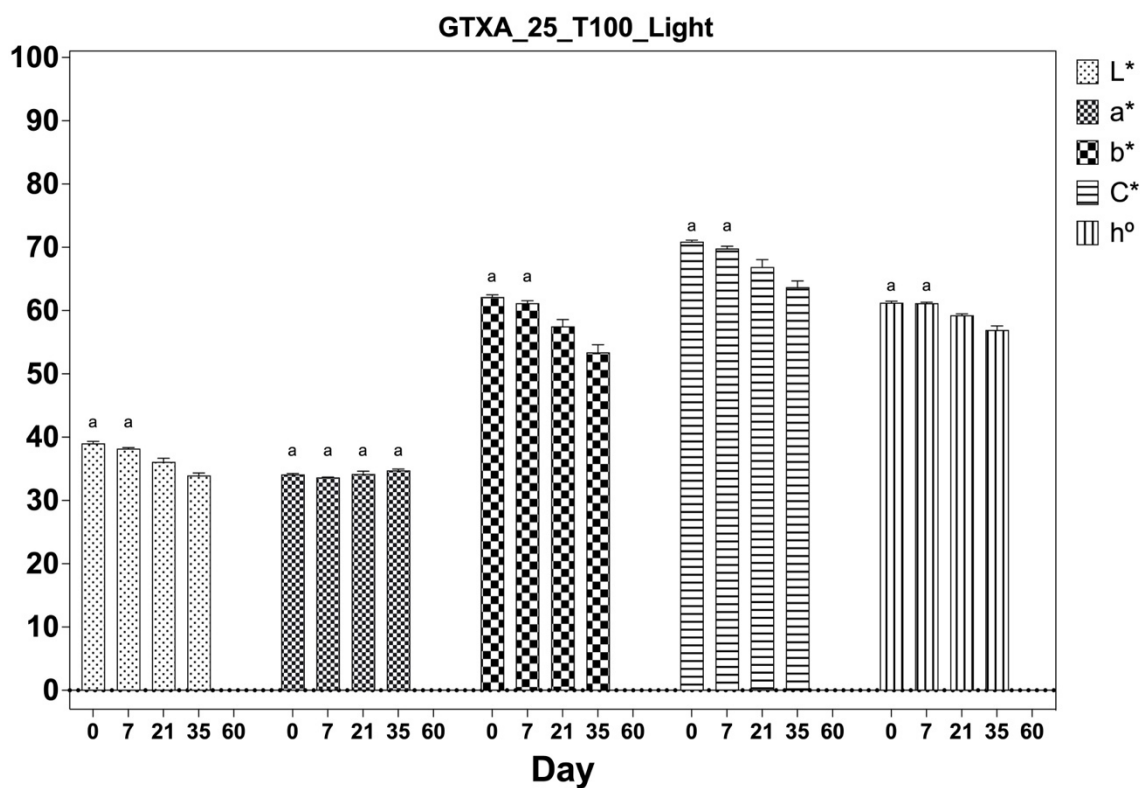


Figure 64 - Colorimetric stability assay using GTXA\_25\_T<sub>100</sub>\_Light condition. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

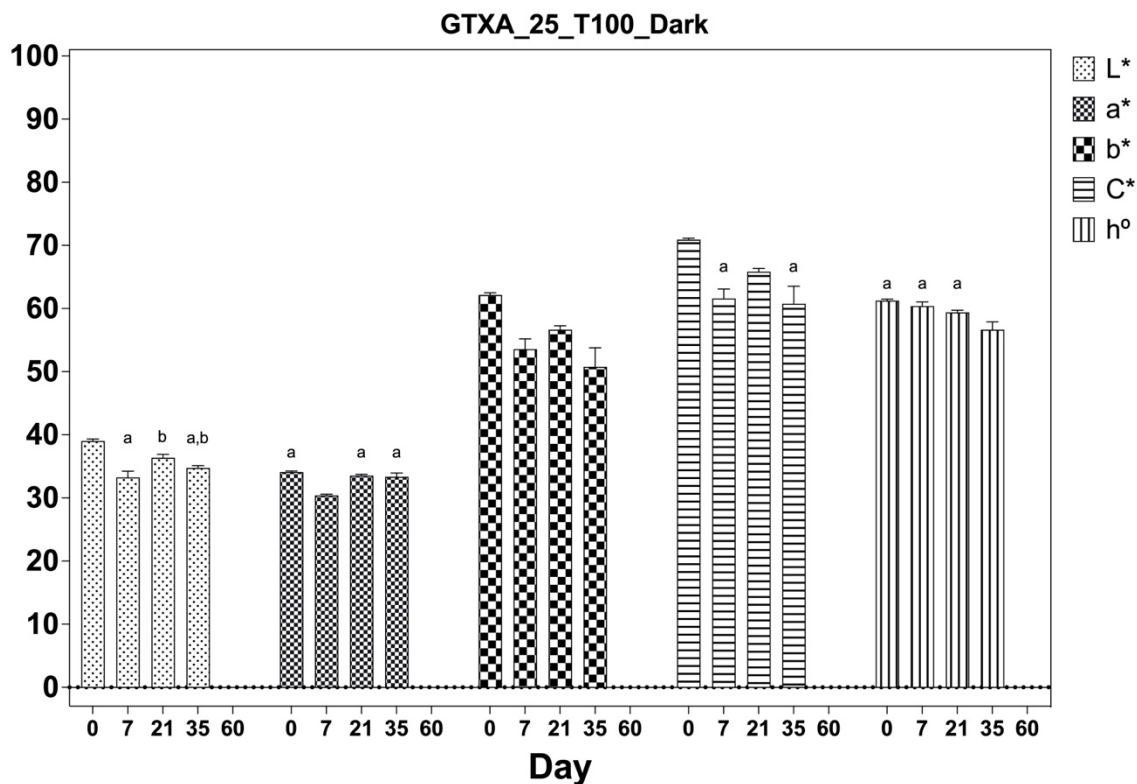


Figure 65 - Colorimetric stability assay using GTXA\_25\_T<sub>100</sub>\_Dark condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

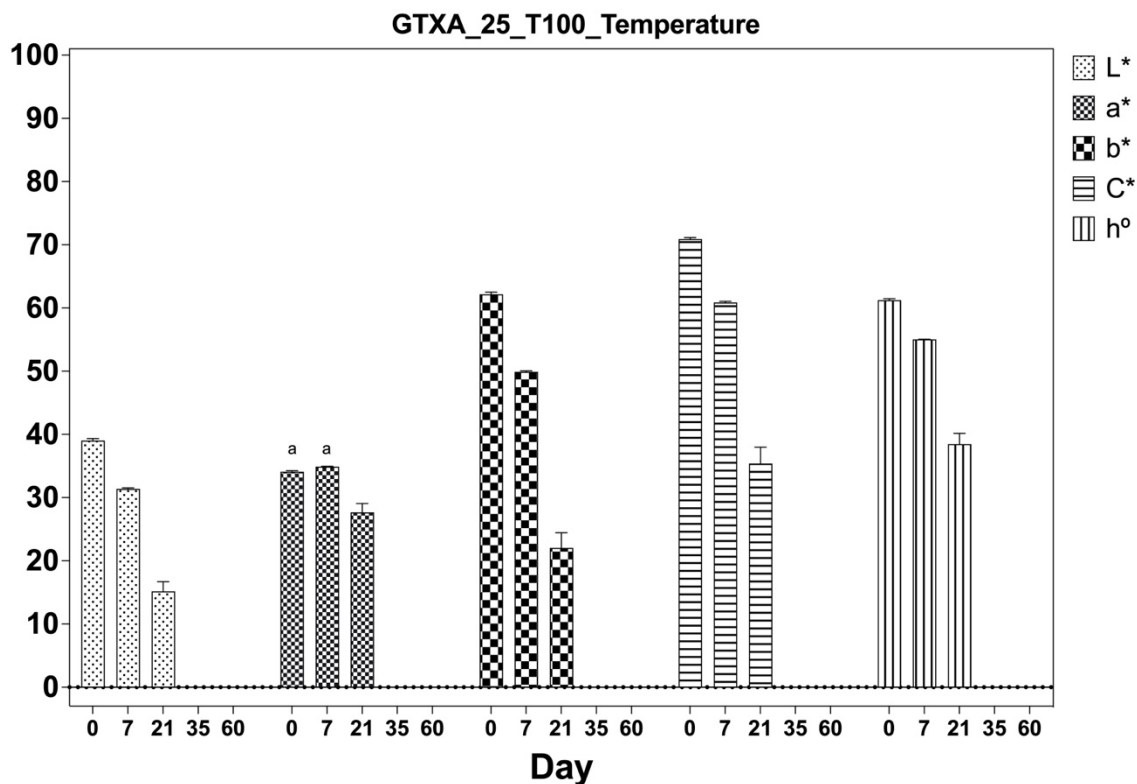


Figure 66 - Colorimetric stability assay using GTXA\_25\_T<sub>100</sub>\_Temperature condition. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

Due to the discovery of fungal colonies on day 60, the evaluation of color variation is performed using values up to day 35. The parameters  $L^*$ ,  $b^*$ ,  $C^*$ , and  $h^\circ$  in GTXA\_25\_T<sub>100</sub>\_Light (Figure 64) followed a similar pattern, with a significant decrease in parameter values after 35 days. The  $a^*$  parameter, on the other hand, showed no significant variation over the 35-day period.

Figure 65 depicts the results obtained for GTXA\_25\_T<sub>100</sub>\_Dark colorimetric stability assays. It is possible to see that a significant variation had been registered in  $L^*$ , with a decrease in the lightness value over 35 days, and a significant variation had been registered in  $a^*$  on the assay performed on day 7, but no significant differences had been registered on the assay performed on days 21 and 35. The  $b^*$ ,  $C^*$ , and  $h^\circ$  parameters all showed a significant decrease in value over the course of 35 days.

After 21 days, all parameter values in the GTXA\_25\_T<sub>100</sub>\_Temperature condition (Figure 66) decreased significantly.

GTXA\_25\_T<sub>100</sub>\_Light and GTXA\_25\_T<sub>100</sub>\_Dark have a low average lightness, average yellow and low average red tones, a high average saturation, and a color that was intermediate between a pure red and a pure yellow. GTXA\_25\_T<sub>100</sub>\_Temperature on the other hand, has a low lightness, low yellow and red tones, a low average saturation, and a color that is a low average between pure red and pure yellow. The attribution day 0 have some differences attributed with low average lightness, high average yellow and low average red tones, high saturation, and color between a pure red and a pure yellow.

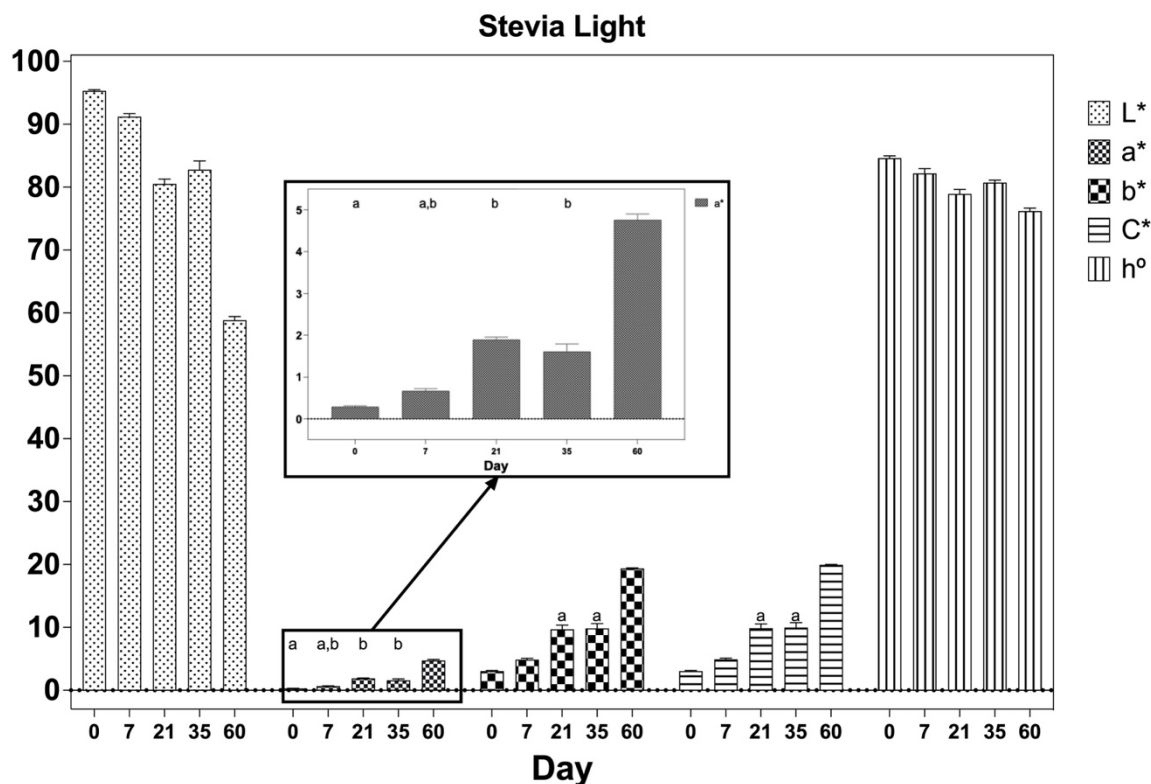


Figure 67 - Colorimetric stability assay using Stevia in Light condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

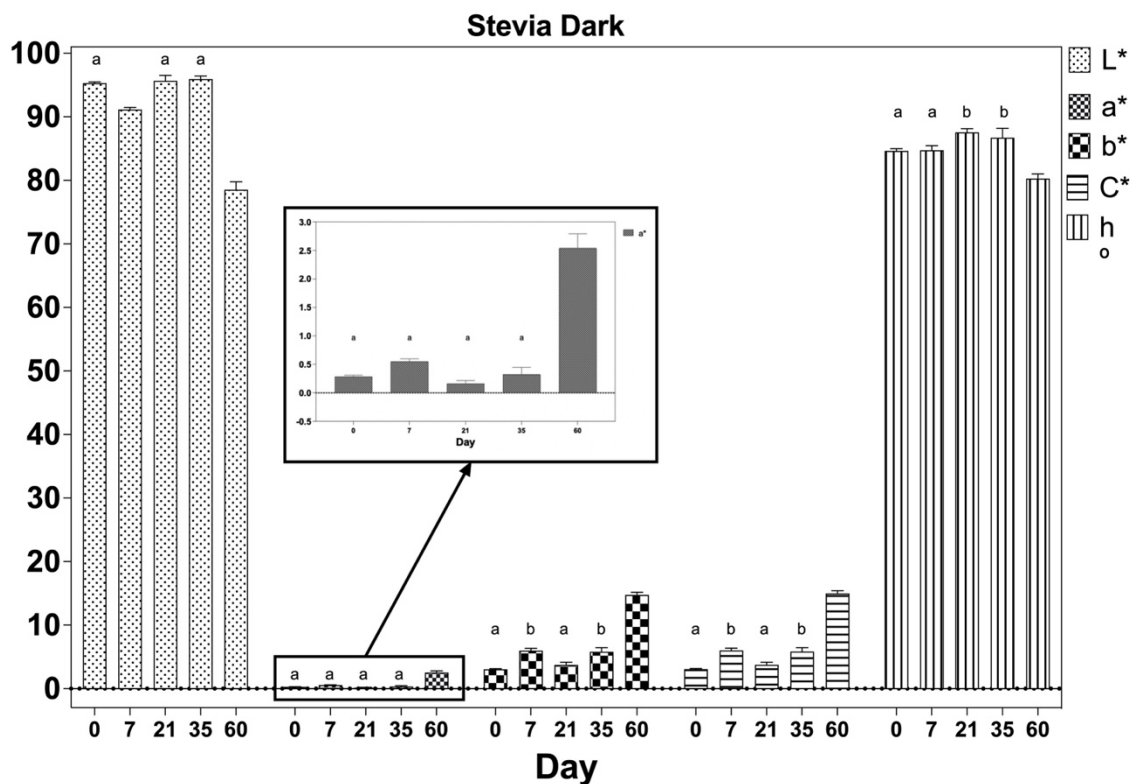


Figure 68 - Colorimetric stability assay using Stevia in Dark condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

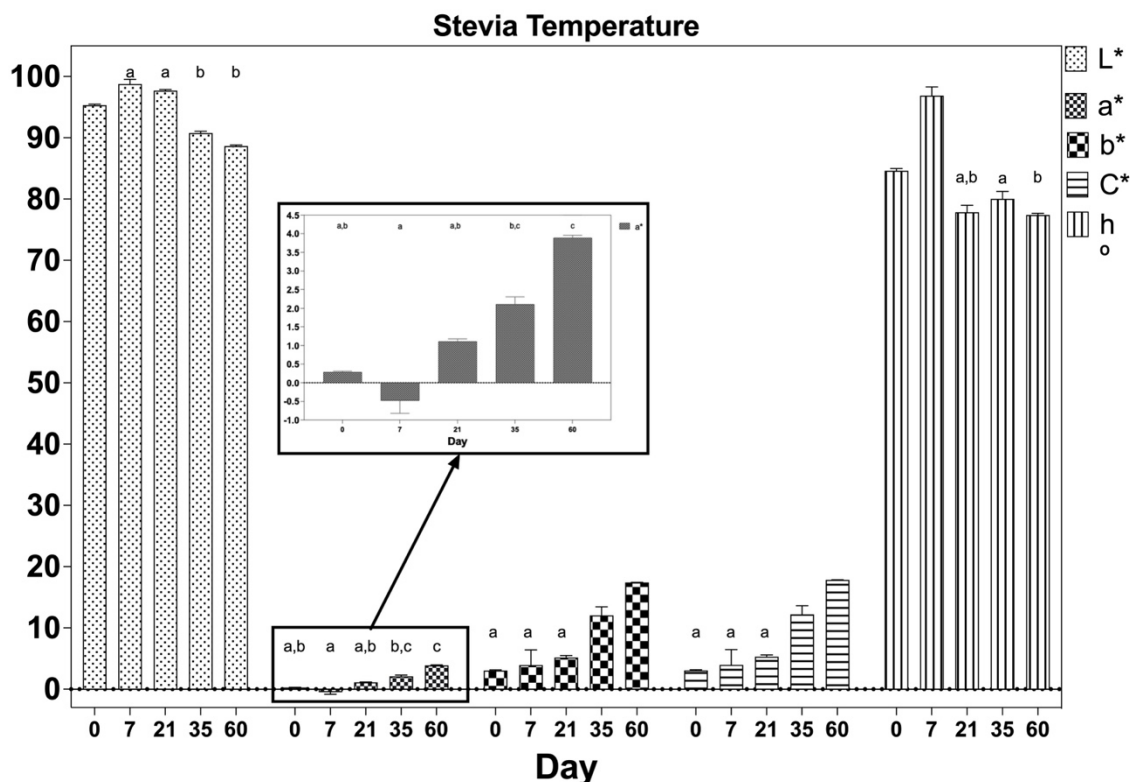


Figure 69 - Colorimetric stability assay using Stevia in Temperature condition. Same superscripts (a-c) indicate not significant differences ( $p > 0.9999$ ) among the column values.

After 60 days, there were significant differences in all color parameter values for stevia in light condition (Figure 67).

When compared to stevia in light condition, stevia in dark condition (Figure 68) showed less variation in all color parameter values. However, significant differences in all color parameter values were observed after 60 days.

Stevia under temperature (Figure 69) demonstrated significant variation in all color parameter values after 60 days.

Stevia under light condition had an average lightness, low yellow and red tones, a low saturation, and a color near to a pure yellow. Significant differences were observed after 60 days when compared to day 0.

The stevia samples under dark and temperature conditions had a high lightness, low yellow and red tones, low saturation, and a color near to a pure yellow. The sample on day 0 had the same appearance, but there were significant statistical differences.

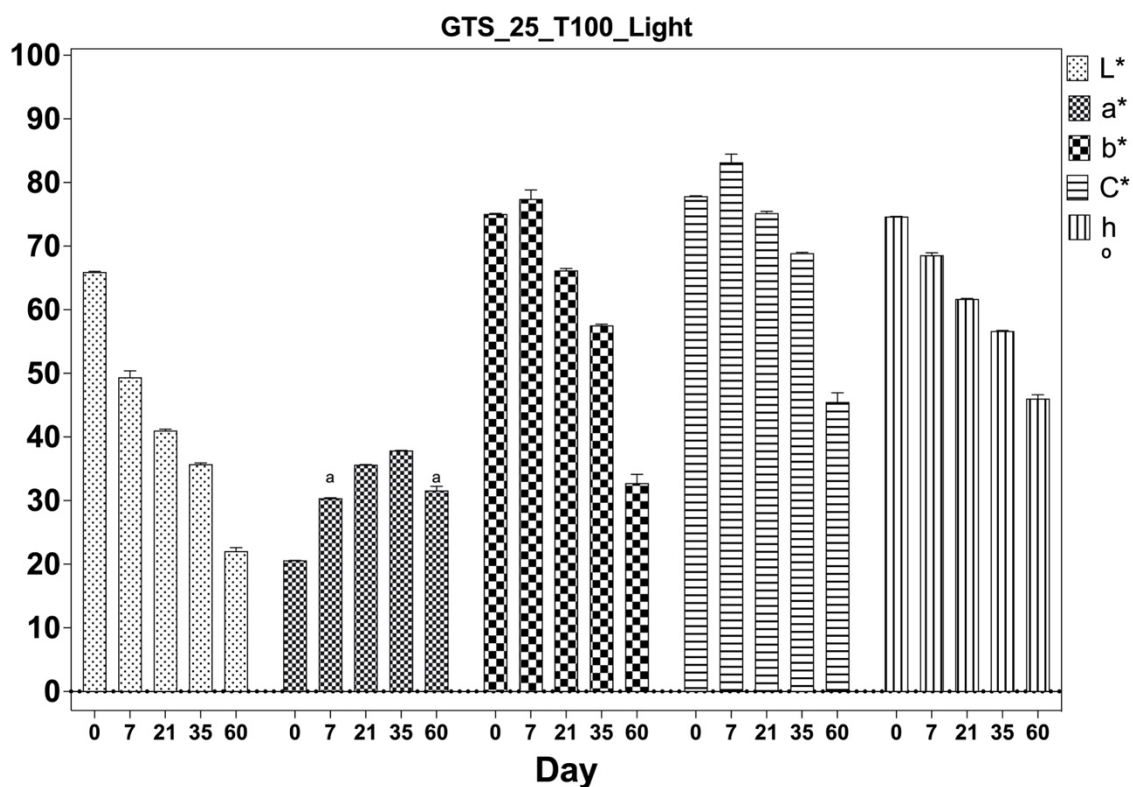


Figure 70 - Colorimetric stability assay using GTS\_25\_T<sub>100</sub>\_Light condition. Same superscripts (a-c) indicate not significant differences ( $p > 0.9999$ ) among the column values.

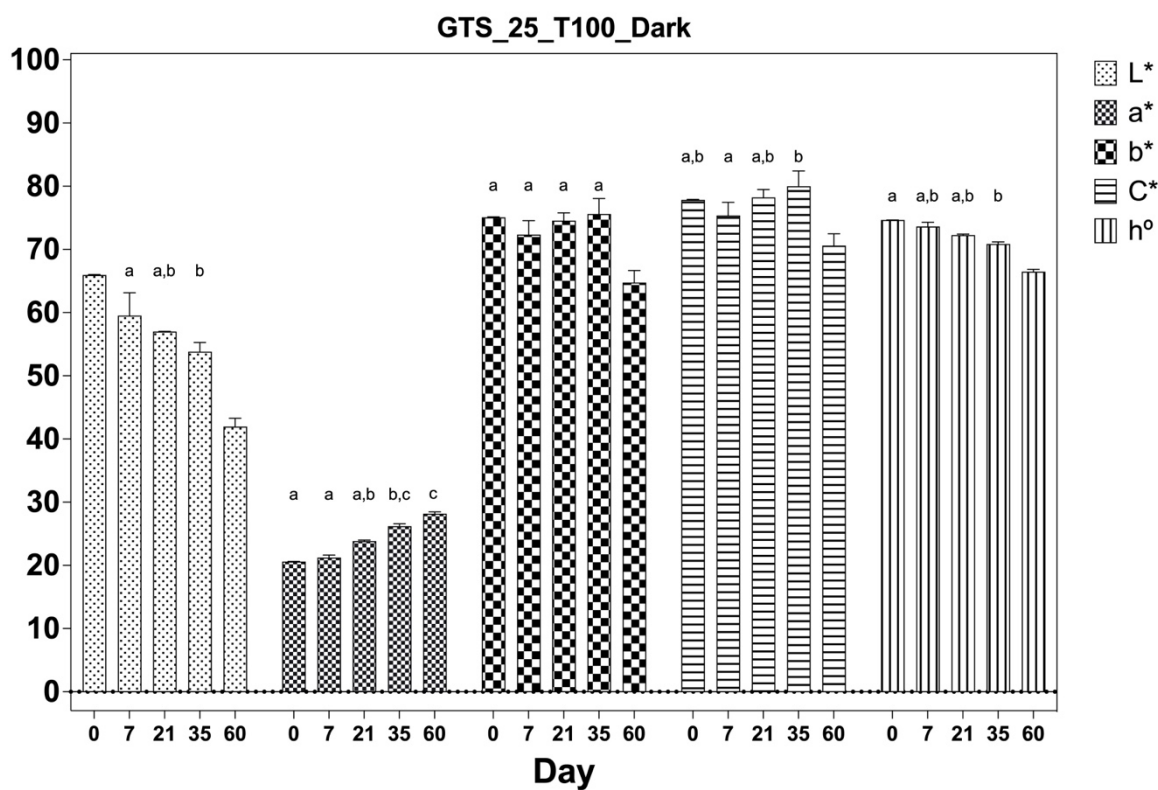


Figure 71 - Colorimetric stability assay using GTS\_25\_T<sub>100</sub>\_Dark condition. Same superscripts (a-c) indicate not significant differences ( $p > 0.9999$ ) among the column values.

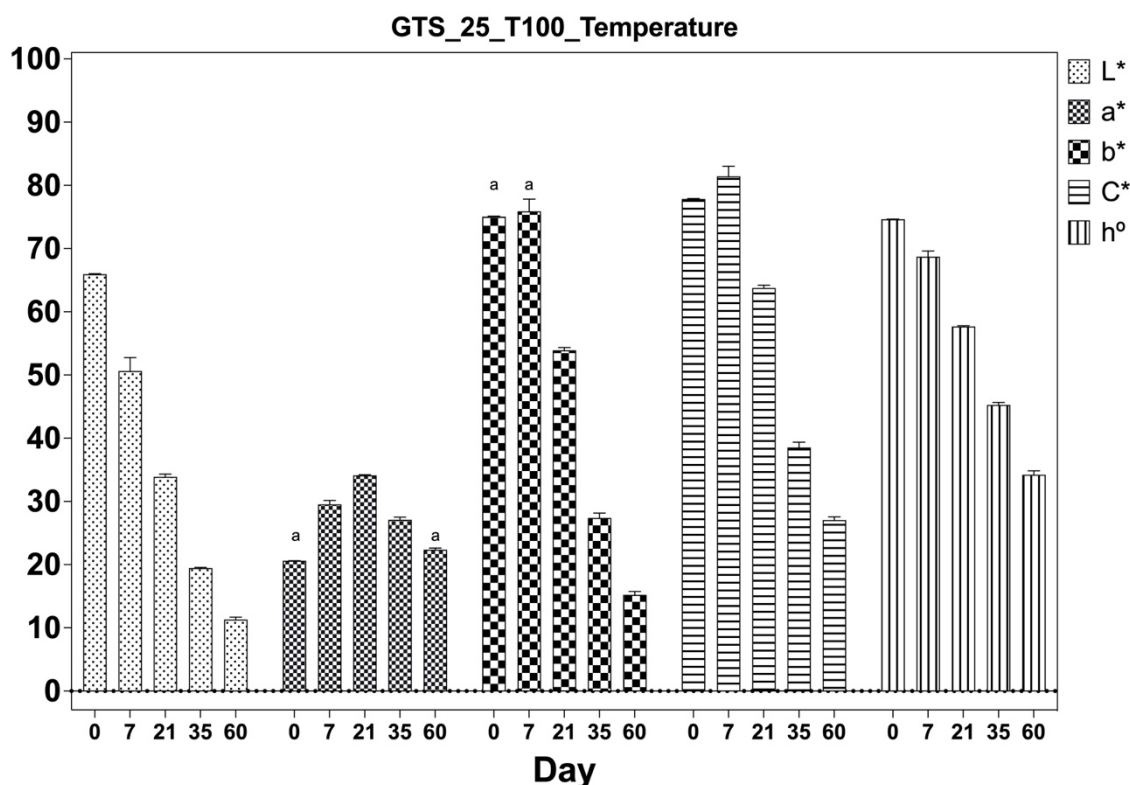


Figure 72 - Colorimetric stability assay using GTS\_25\_T<sub>100</sub>\_Temperature condition. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

Figure 70 shows that all color parameter values of GTS\_25\_T<sub>100</sub>\_Light changed significantly over the course of 60 days.

When GTS\_25\_T<sub>100</sub>\_Dark (Figure 71) was compared to the same in temperature condition, lower differences were registered; however, significant differences in all color parameter values were registered after 60 days.

Color parameter values in GTS\_25\_T<sub>100</sub>\_Temperature (Figure 72) vary significantly over the course of 60 days. With the exception of the a\* parameter, all of the parameters showed significant differences between days 0 and 60. However, significant variations occur between these days in the a\* parameter.

Initially all samples displayed high average lightness, high yellow and low red tones, high saturation, and color ranging between a pure red and a pure yellow.

After 60 days, there was a significant difference in GTS\_25\_T<sub>100</sub>\_Light with average lightness, high yellow and low red tones, average saturation, and color between a pure red and a pure yellow, GTS\_25\_T<sub>100</sub>\_Dark with the color was between a pure red and a pure yellow, with average lightness, high average yellow and low red tones, and high saturation,



and GTS\_25\_T<sub>100</sub>\_Temperature with had low lightness, yellow and red tones, saturation, and a low average color between a pure red and a pure yellow.

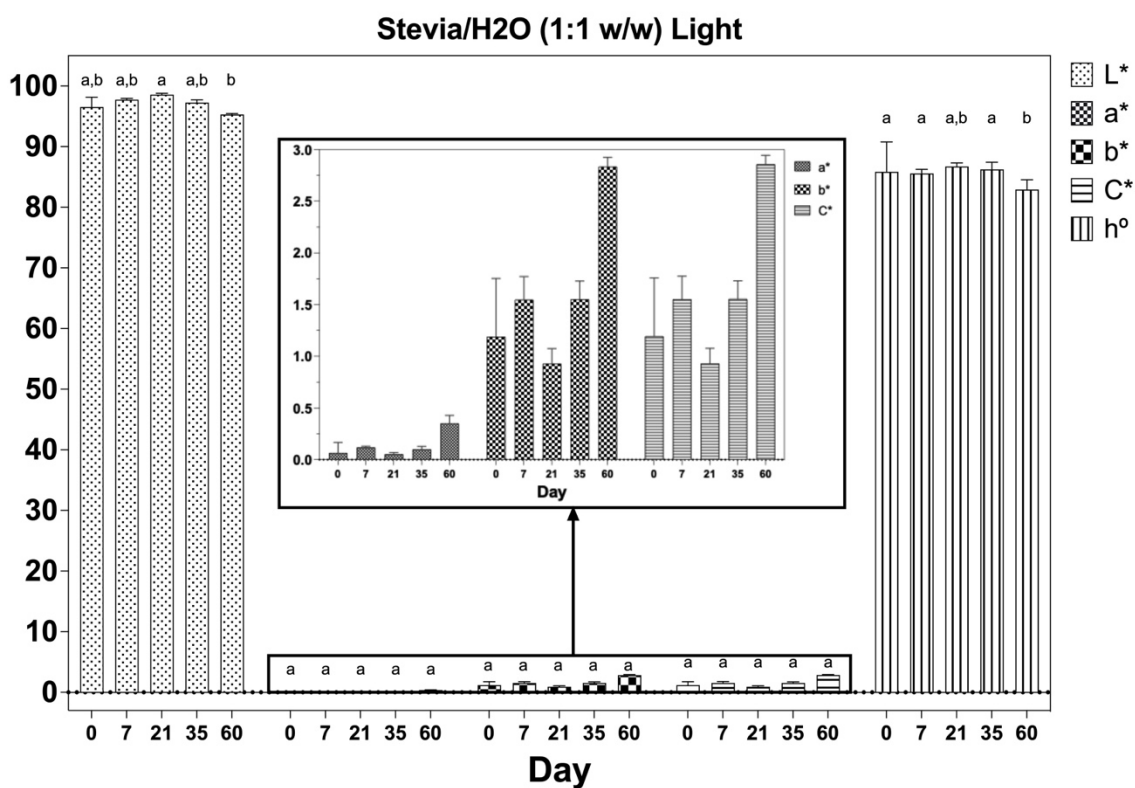


Figure 73 - Colorimetric stability assay using Stevia/H<sub>2</sub>O (1:1 w/w) in Light condition. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.



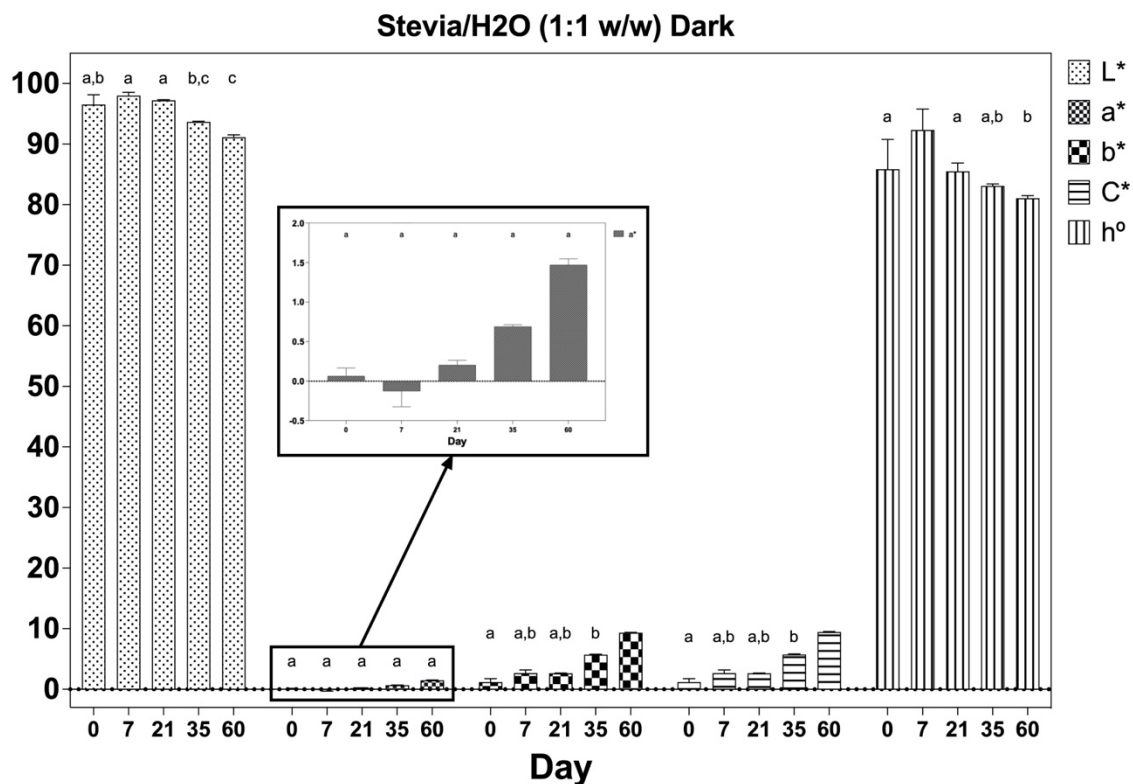


Figure 74 - Colorimetric stability assay using Stevia/H<sub>2</sub>O (1:1 w/w) in Dark condition. Same superscripts (a-c) indicate not significant differences ( $p > 0.9999$ ) among the column values.

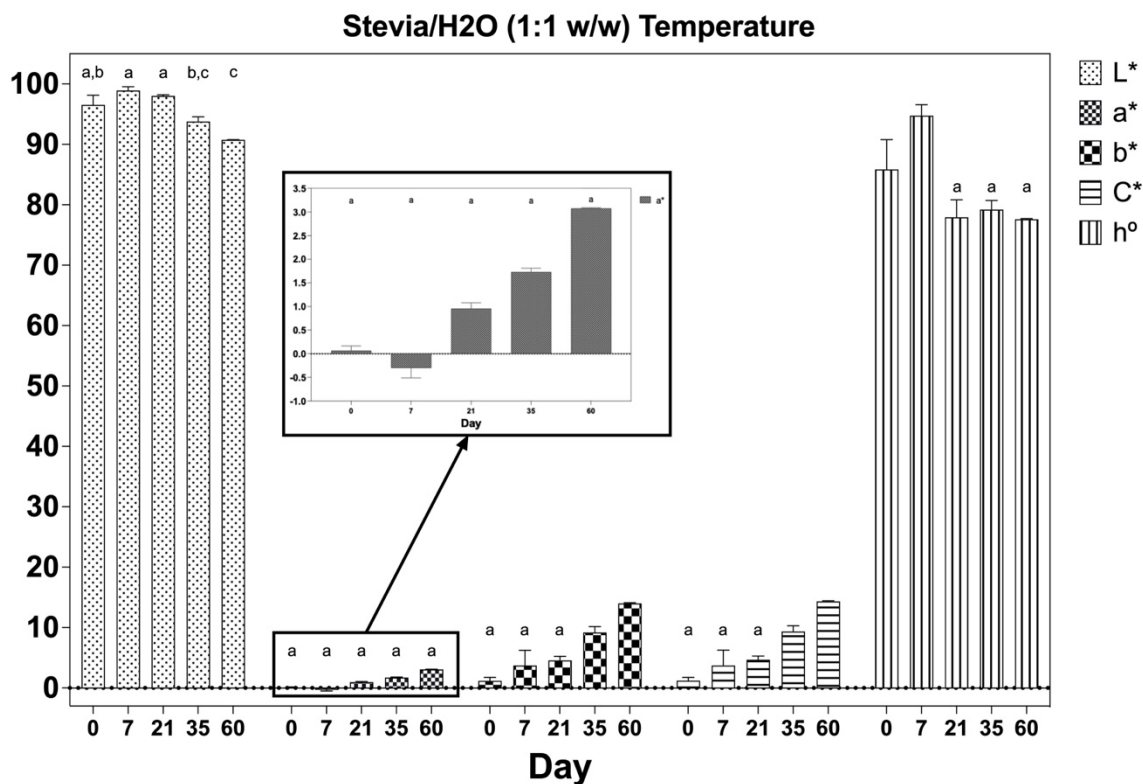


Figure 75 - Colorimetric stability assay using Stevia/H<sub>2</sub>O (1:1 w/w) in Temperature condition. Same superscripts (a-c) indicate not significant differences ( $p > 0.9999$ ) among the column values.

Stevia/H<sub>2</sub>O (1:1 w/w) under light condition (Figure 73) showed no significant differences in the L\* parameter between days 0 and 60, but there are some significant differences between these days. Over the course of 60 days, there were no significant differences in the a\*, b\*, and C\* parameters. The h° parameter showed significant differences over the course of 60 days.

With the exception of the a\* parameter, all other color parameter values differed significantly between days 0 and 60 for stevia/H<sub>2</sub>O (1:1 w/w) in dark conditions (Figure 74).

Except for the a\* parameter, all color parameter values showed significant differences in temperature condition between days 0 and 60 for Stevia/H<sub>2</sub>O (1:1 w/w) (Figure 75).

Despite the differences over the 60 days in all conditions, days 0 and 60 have the same attribution with high lightness, low yellow and red tones, low saturation, and color near to a pure yellow.

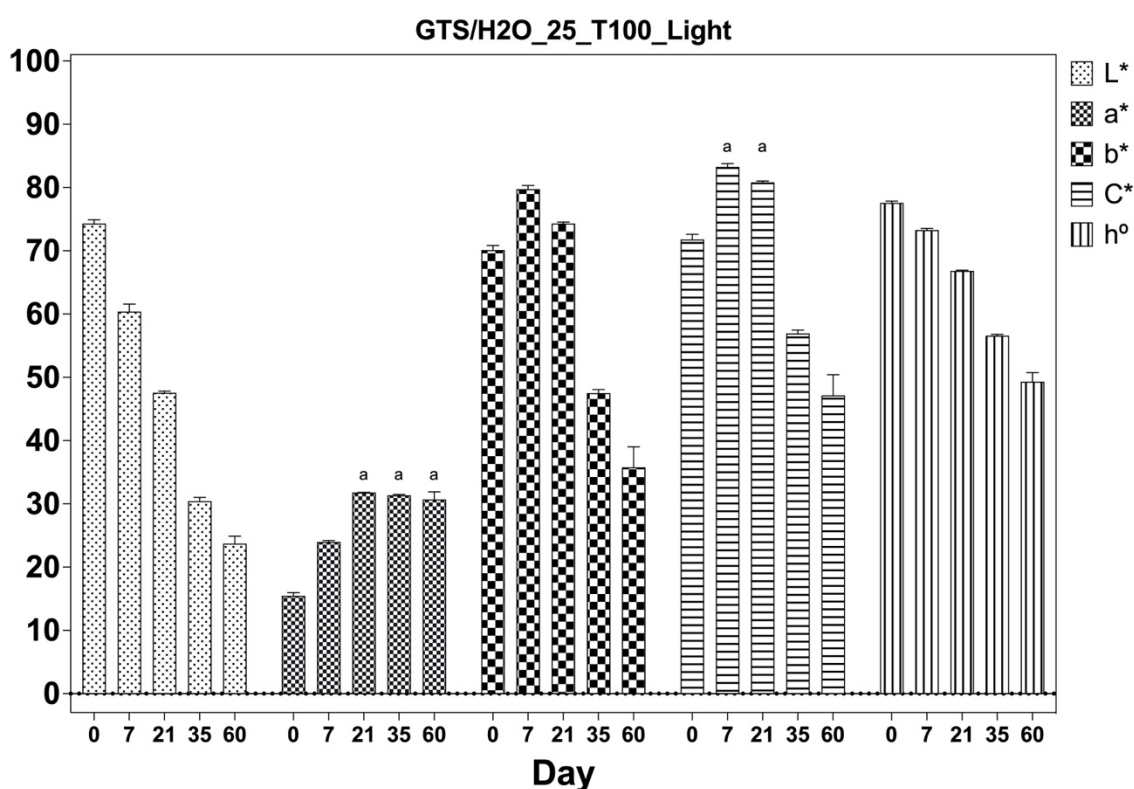


Figure 76 - Colorimetric stability assay using GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Light condition. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

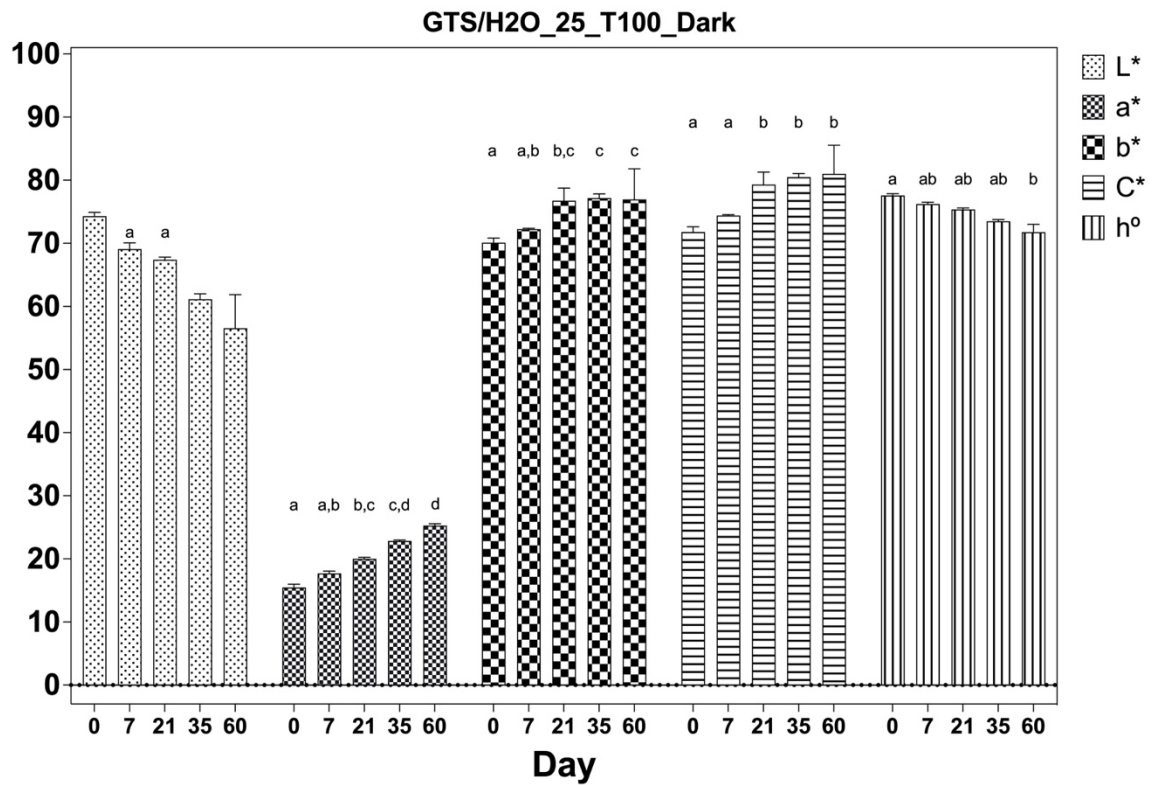


Figure 77 - Colorimetric stability assay using GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Dark condition. Same superscripts (a-d) indicate not significant differences ( $p > 0.9999$ ) among the column values.

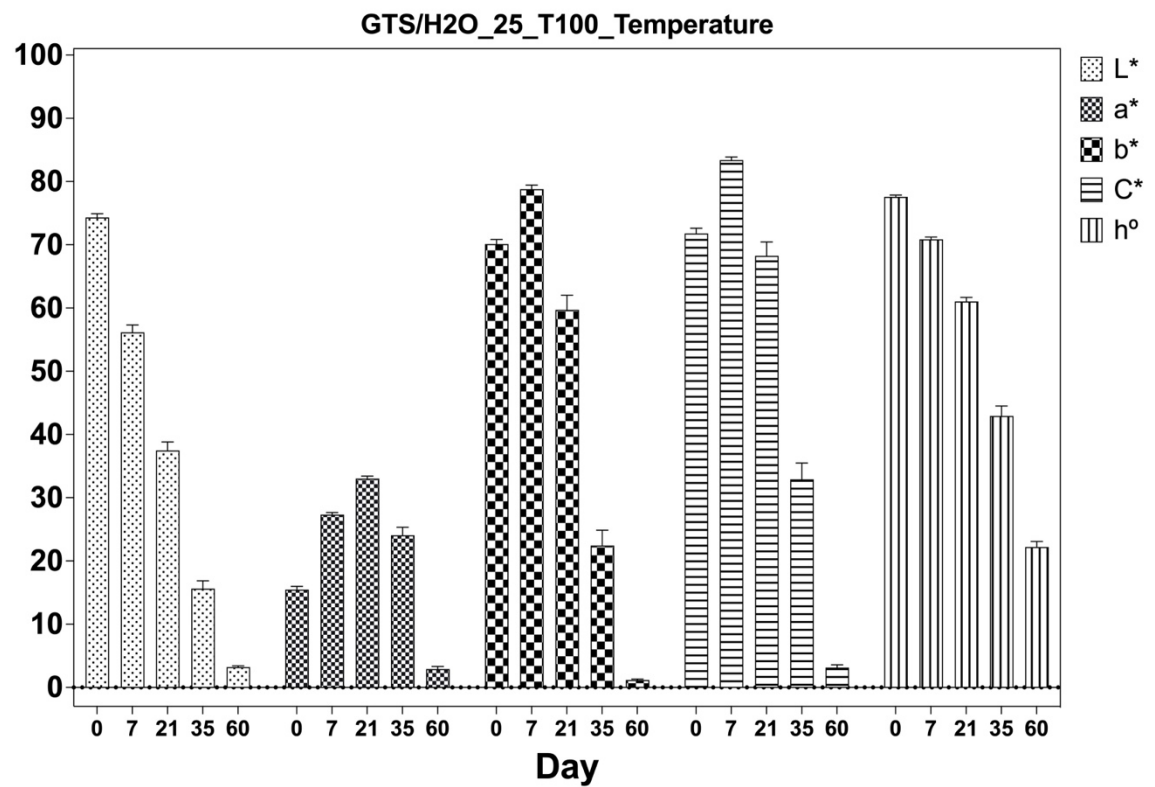


Figure 78 - Colorimetric stability assay using GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Temperature condition.

Over 60 days, all of the color parameter values in the GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Light, GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Dark, and GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Temperature conditions (Figure 76, 77, and 78, respectively) showed significant variations.

Initially, the samples displayed high lightness, high average yellow and low red tones, high saturation, and color between a pure red and a pure yellow. After 60 days, GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Light was low lightness, low average yellow and red tones, average saturation, and average color between a pure red and a pure yellow, GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Dark displayed color between a pure red and a pure yellow, with average lightness, high yellow and low red tones, and high saturation, and GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Temperature showed low lightness, low yellow and red tones, low saturation, and a color close to a pure red.

### 3.3.3. Final Review

The main comparisons between the different conditions studied in both assays have been discussed in section 3.3.1. and 3.3.2. and were represented and summarized in Tables 20 and 21, respectively, for a better comparison of the results. For each condition, the number of compounds with a final percentage of stability between five major intervals was counted: 0-30 %, 30-50 %, 50-70 %, 70-80 %, and 80-100 %, as well as the number of compounds degraded and the day that the compounds were detected as degraded.

Table 20 – Table summarizing the overall comparison of the various conditions in HPLC-DAD assays.

| Condition                                 | Day | Degraded | No. of Compound |              |              |              |               |
|-------------------------------------------|-----|----------|-----------------|--------------|--------------|--------------|---------------|
|                                           |     |          | 0-30<br>(%)     | 30-50<br>(%) | 50-70<br>(%) | 70-80<br>(%) | 80-100<br>(%) |
| GTXAG_25_T <sub>10</sub><br>o_Light       | 60  | 1        | 2               | 1            | 2            | 1            | 3             |
| GTXAG_25_T <sub>10</sub><br>o_Dark        | -   | 0        | 2               | 1            | 2            | 1            | 4             |
| GTXAG_25_T <sub>10</sub><br>o_Temperature | 60  | 1        | 2               | 1            | 2            | 1            | 3             |
| GTXA_25_T <sub>100</sub><br>Light         | 60  | 10       | -               | -            | -            | -            | -             |

| Condition                                                | Day | Degraded | No. of Compound |              |              |              |               |
|----------------------------------------------------------|-----|----------|-----------------|--------------|--------------|--------------|---------------|
|                                                          |     |          | 0-30<br>(%)     | 30-50<br>(%) | 50-70<br>(%) | 70-80<br>(%) | 80-100<br>(%) |
| GTXA_25_T <sub>100</sub> _Dark                           | 60  | 10       | -               | -            | -            | -            | -             |
| GTXA_25_T <sub>100</sub> _Temperature                    | 35  | 10       | -               | -            | -            | -            | -             |
| GTS_25_T <sub>100</sub> _Light                           | 60  | 1        | 2               | 2            | 1            | 0            | 4             |
| GTS_25_T <sub>100</sub> _Dark                            | -   | 0        | 0               | 2            | 2            | 2            | 4             |
| GTS_25_T <sub>100</sub> _Temperature                     | 60  | 1        | 2               | 2            | 2            | 1            | 2             |
| GT50S50H <sub>2</sub> O_25_T <sub>100</sub> _Light       | -   | 0        | 1               | 3            | 1            | 1            | 4             |
| GT50S50H <sub>2</sub> O_25_T <sub>100</sub> _Dark        | -   | 0        | 0               | 1            | 3            | 0            | 6             |
| GT50S50H <sub>2</sub> O_25_T <sub>100</sub> _Temperature | 60  | 5        | 0               | 1            | 1            | 3            | 0             |

According to Table 20, the dark condition obtained more polyphenolic compounds with percentage stability greater than 50 % after 60 days: seven for GTXAG\_25\_T<sub>100</sub>\_Dark, eight for GTS\_25\_T<sub>100</sub>\_Dark, and nine for GT50S50H<sub>2</sub>O\_25\_T<sub>100</sub>\_Dark assays, demonstrating an agreement between the different CNS as the aging condition with better results.

The maple syrup did not complete the 60-day assays, so it was designated as the CNS with lower stability. Agave syrup and stevia performed better as CNS in all aging conditions, with 2 of 30 (6.67 %) and 2 of 30 (6.67 %) GT polyphenolic compounds degraded, respectively, and 19 of 30 (63.33 %) and 18 of 30 (60.00 %), respectively, GT polyphenolic compounds with stability greater than 50 % after 60 days.

Table 21 - Table summarizing the overall comparison of the various conditions in colorimetric assays.

|                                                          |          |     | No. of parameters,<br>comparing between<br>days 0 and 60, with: |                            |
|----------------------------------------------------------|----------|-----|-----------------------------------------------------------------|----------------------------|
| Condition                                                | Degraded | Day | Different<br>attributes                                         | Significant<br>differences |
| Agave Syrup in Light                                     | No       | -   | 0                                                               | 2                          |
| GTXAG_25_T <sub>100</sub> _Light                         | No       | -   | 0                                                               | 2                          |
| Agave Syrup in Dark                                      | No       | -   | 0                                                               | 1                          |
| GTXAG_25_T <sub>100</sub> _Dark                          | No       | -   | 0                                                               | 0                          |
| Agave Syrup in Temperature                               | No       | -   | 0                                                               | 1                          |
| GTXAG_25_T <sub>100</sub> _Temperature                   | No       | -   | 1                                                               | 3                          |
| Maple Syrup in Light                                     | Yes      | 60  | -                                                               | -                          |
| GTXA_25_T <sub>100</sub> _Light                          | Yes      | 60  | -                                                               | -                          |
| Maple Syrup in Dark                                      | Yes      | 60  | -                                                               | -                          |
| GTXA_25_T <sub>100</sub> _Dark                           | Yes      | 60  | -                                                               | -                          |
| Maple Syrup in Temperature                               | Yes      | 35  | -                                                               | -                          |
| GTXA_25_T <sub>100</sub> _Temperature                    | Yes      | 35  | -                                                               | -                          |
| Stevia in Light                                          | No       | -   | 1                                                               | 5                          |
| GTS_25_T <sub>100</sub> _Light                           | No       | -   | 5                                                               | 5                          |
| Stevia in Dark                                           | No       | -   | 0                                                               | 5                          |
| GTS_25_T <sub>100</sub> _Dark                            | No       | -   | 2                                                               | 5                          |
| Stevia in Temperature                                    | No       | -   | 0                                                               | 5                          |
| GTS_25_T <sub>100</sub> _Temperature                     | No       | -   | 4                                                               | 4                          |
| Stevia/H <sub>2</sub> O in Light                         | No       | -   | 0                                                               | 1                          |
| GT50S50H <sub>2</sub> O_25_T <sub>100</sub> _Light       | No       | -   | 1                                                               | 5                          |
| Stevia/H <sub>2</sub> O in Dark                          | No       | -   | 0                                                               | 4                          |
| GT50S50H <sub>2</sub> O_25_T <sub>100</sub> _Dark        | No       | -   | 1                                                               | 5                          |
| Stevia/H <sub>2</sub> O in Temperature                   | No       | -   | 0                                                               | 4                          |
| GT50S50H <sub>2</sub> O_25_T <sub>100</sub> _Temperature | No       | -   | 4                                                               | 5                          |

Table 21 shows the colorimetric assay comparisons, and it is clear that GTXAG\_25\_T<sub>100</sub>\_Dark demonstrated better stability in colorimetric assays with no significant differences and different attributes after 60 days. For agave and stevia CNSs, dark aging produced better stability results after 60 days. Agave demonstrated superior stability across all comparisons, with 1 of 30 (3.33 %) different attributes exhibiting

significant differences between days 0 and 60. Stevia/H<sub>2</sub>O (1:1 w/w) performed better than Stevia CNS, with 6 of 30 (20.00 %) and 12 of 40 (40.00 %) different attributes, and 24 of 30 (80.00 %) and 29 of 30 (96.67 %) significant differences between days 0 and 60, respectively.

When the results of both assays are compared, it is clear that agave syrup CNS had better stability in both, while stevia CNS and stevia/H<sub>2</sub>O had better results in the HPLC-DAD stability and colorimetric assays, respectively. However, when the overall conditions were considered, stevia CNS demonstrated the second-best stability over 60 days.

### **3.4. Development of an Oral Microemulsion with Green Tea Extracted with Commercial Natural Solvents**

#### **3.4.1. Optimization of the Procedure**

The formulation of the oral microemulsion was optimized with the goal of creating a multiple microemulsion system. A multiple emulsion is a colloidal system made up of several dispersed phases in a continuous medium. It is made by combining at least two types of emulsions, usually an oil-in-water (O/W) emulsion and a water-in-oil (W/O) emulsion, as well as a surfactant and a co-surfactants (Figure 79).<sup>130-132</sup>

The presence of distinct regions or domains of different emulsion types within the formulation is the defining feature of a multiple emulsion. Each emulsified phase contains different types of surfactants and co-surfactants that help reduce interfacial tension and promote emulsion formation and stabilization.<sup>131-133</sup>

Because of this colloidal structure, a multiple emulsion is thermodynamically stable and highly efficient in solubilizing different components, such as lipophilic and hydrophilic substances. Furthermore, the presence of multiple emulsified phases can provide benefits such as controlled release of active substances, improved chemical and physical stability, and improved bioaccessibility and bioavailability.<sup>131, 132</sup>

Multiple emulsions have received a lot of attention in the pharmaceutical, cosmetic, and food industries because of their ability to improve drug solubility, improve transdermal

absorption of active compounds, and make it easier to formulate more stable and effective products.<sup>131, 134</sup>

In summary, a multiple emulsion is a complex colloidal formulation made up of multiple emulsified phases that provides benefits in terms of solubility, stability, and controlled release of active substances.

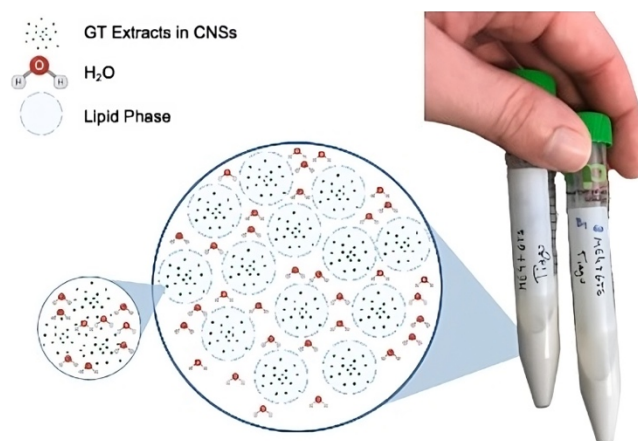


Figure 79 - Schematic illustration of a multiple emulsion.

To achieve the goal of producing a stable multiple microemulsion (MME), first, the MME's stability was tested regarding phase separation by replacing the GT polyphenolic compounds extracted in CNSs by water, and the first assay was carried out under the conditions shown in Table 22. The MME was prepared using magnetic agitation at 1250 RPM. In the MME\_1 formulation, the GT extract and water were the internal aqueous phase, Span<sup>®</sup> 80 was the water-in-oil surfactant and oleic acid was the oily phase. The external aqueous phase was composed by purified water containing Tween<sup>®</sup> 80 (oil-in-water surfactant) and glycerin (co-surfactant).

Table 22 - Percentual composition of MME\_1.

| Constituents                                       |                        | Percentage (%) |
|----------------------------------------------------|------------------------|----------------|
| <b>W/O<br/>(40.2 %)</b>                            | GT Compounds Extracted | 7.6            |
|                                                    | Purified Water         | 7.6            |
|                                                    | Span <sup>®</sup> 80   | 2.5            |
|                                                    | Oleic Acid             | 22.5           |
| <b>External<br/>aqueous<br/>phase<br/>(59.8 %)</b> | Tween <sup>®</sup> 80  | 2.5            |
|                                                    | Glycerin               | 2.5            |
|                                                    | Purifier Water         | 54.8           |



After successfully preparing MME\_1 (Table 22), this formulation was centrifuged to investigate its stability. MME\_1 demonstrated phase separation after 30 minutes of centrifugation at 3500 RPM.

To avoid phase separation, an increase of Span® 80, Tween® 80, and glycerin was added from 2.5 % to 5.0 %. To balance these increases, oleic acid was proportionally decreased. The composition of MME\_2 was presented in the following table (Table 23).

Table 23 - Percentual composition of MME\_2.

|                                                                      |  | Constituents           | Percentage (%) |
|----------------------------------------------------------------------|--|------------------------|----------------|
| <b>W/O</b><br><b>(35.2 %)</b>                                        |  | GT Compounds Extracted | 7.6            |
|                                                                      |  | Purified Water         | 7.6            |
|                                                                      |  | Span® 80               | 5.0            |
|                                                                      |  | Oleic Acid             | 15.0           |
| <b>External</b><br><b>aqueous</b><br><b>phase</b><br><b>(64.8 %)</b> |  | Tween® 80              | 5.0            |
|                                                                      |  | Glycerin               | 5.0            |
|                                                                      |  | Purifier Water         | 54.8           |

After 30 minutes of centrifugation at 3500 RPM, the MME\_2 showed phase separation.

Aside from the changes in the formulation composition, an ultrasound was used for 10 minutes at 70 % amplitude to better homogenize the MME\_2 intending to increase its stability. Then, the MME\_2 was centrifuged at 3500 RPM for 60 minutes and there was no phase separation.

Despite the demonstrated stability concerning phase separation, MME\_2 did not have the desired oily phase droplet size. To address this issue and achieve lower droplet sizes, magnetic stirring at 1250 RPM was replaced by ultra-turrax agitation at 8000 RPM for 2 minutes during the formation of W/O and W/O/W emulsions. The MME\_2 showed no phase separation after 60 minutes of centrifugation at 3500 RPM, and a desired oily phase droplet size.

In addition to MME\_2, another formulation (MME\_3) with a higher percentage of Tween® 80 (Table 24) was used in subsequent trials in the next sections to verify the effect of increasing this surfactant on MME characteristics.

Table 24 - Percentual composition of MME\_3.

| Constituents                  |                        | Percentage (%) |
|-------------------------------|------------------------|----------------|
| <b>W/O</b><br><b>(35.2 %)</b> | GT Compounds Extracted | 7.6            |
|                               | Water                  | 7.6            |
|                               | SPAN 80                | 5.0            |
|                               | Oleic Acid             | 15.0           |
| <b>O/W</b><br><b>(64.8 %)</b> | Tween 80               | 7.5            |
|                               | Glycerin               | 5.0            |
|                               | Purifier Water         | 52.3           |

After the MME optimization, it was necessary to decide how to quantify the GT polyphenolic compounds in the formulation, so a couple of organic solvents were tested to understand if they can destabilize the MME\_2 allowing the release of the GT polyphenolic compounds and their quantification. Liquid-liquid extraction of MME with petroleum ether, hexane, and chloroform was performed. 1.00 mL of MME was initially diluted in 5.00 mL of water, and then 1.00 mL of this solution was added to 4.00 mL of the organic solvent. The organic and aqueous phases have their solvents evaporated and the remaining powder has been solubilized with 0.200 mL of water and injected in HPLC-DAD.

The injections showed, except for the hexane, no capacity to destabilize de MME. The Figure 80 shows the chromatogram obtained with hexane liquid-liquid extraction where it is possible to see the GT polyphenolic compounds extracted from the MME.

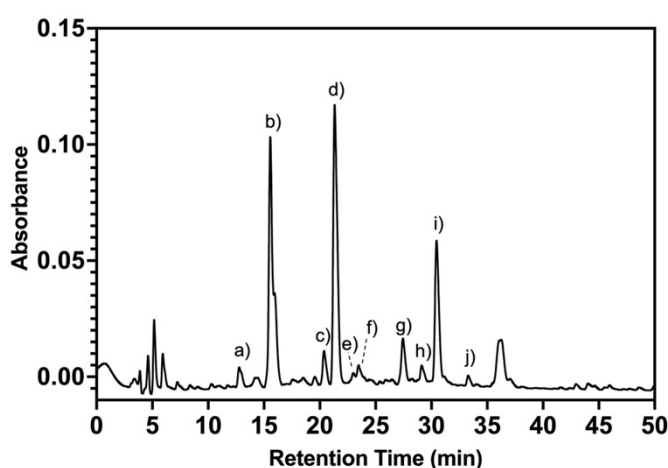


Figure 80 – Liquid-liquid extraction of MME with hexane chromatography obtained through HPLC-DAD analyses. a) – (E)GC; b) – PD-BG; c) – (E)C; d) – (E)GCG; e) – Gallic Acid; f) - Apigenin-6-C-pentoside-8-C-hexoside; g) - Quercetin-deoxyhexoside-hexoside-hexoside; h) - Quercetin-3-O-rutinoside; i) – (E)CG; and j) - Kaempferol 3-rutinoside.

### 3.4.2. Accelerated Stability by Centrifugation

Multiple microemulsions are complex systems made up of several phases, and their stabilization is critical because phase separation can occur due to thermodynamic fluctuations, resulting in droplet coalescence and aggregation. Phase separation assays are used to evaluate and comprehend the stability of these systems.<sup>131</sup>

These assays involve applying controlled external stresses to the multiple microemulsions, such as temperature variation, pH, electrolyte concentration, or surfactant addition. These systems' responses to applied stresses are observed and analyzed. Droplet size, polydispersity, viscosity variation, and turbidity can all be measured to determine the system's stability.<sup>135, 136</sup>

There are also assays that involve vigorous agitation of multiple microemulsions at high rotations per minute (RPM). These tests are referred to as centrifugation or high-speed agitation tests.<sup>135</sup> Multiple microemulsions are subjected to centrifugal forces produced by high-speed rotation in these tests. The rapid and intense agitation produces a field of forces that mimics the extreme stress conditions that these systems may encounter during industrial processing or handling.<sup>135</sup>

The high RPM agitation in centrifugation assays speeds up the processes of coalescence and phase separation, revealing instabilities that would otherwise go undetected. Segregated phases can be identified and analyzed by interrupting the assay at a specific time.<sup>135</sup>

These assays allow for a more precise evaluation of the stability of multiple microemulsions, providing information about the robustness of formulations under extreme conditions. Based on the results of these assays, adjustments can be made to the formulations or stabilizing agents can be added to enhance the resistance of multiple microemulsions to phase separation.

In conclusion, high RPM agitation assays are a valuable tool in the study of multiple microemulsions, allowing for a thorough understanding of their stability and providing relevant data for the development of more stable and effective formulations.

The relationship between the code attributed by the authors and the condition used in the MME under study is shown in Table 25.

Table 25 - Correlation between the condition used in the MME and the code attributed by the authors.

| Sample                    | Tween® 80 (%) | Name attributed by authors         |
|---------------------------|---------------|------------------------------------|
| H <sub>2</sub> O          | 5.0           | MME_H <sub>2</sub> O_5             |
| H <sub>2</sub> O          | 7.5           | MME_H <sub>2</sub> O_7.5           |
| GTXAG_50_T <sub>100</sub> | 5.0           | MME_GTXAG_50_T <sub>100</sub> _5   |
| GTXAG_50_T <sub>100</sub> | 7.5           | MME_GTXAG_50_T <sub>100</sub> _7.5 |
| GTXA_50_T <sub>100</sub>  | 5.0           | MME_GTXA_50_T <sub>100</sub> _5    |
| GTXA_50_T <sub>100</sub>  | 7.5           | MME_GTXA_50_T <sub>100</sub> _7.5  |
| GTS_50_T <sub>100</sub>   | 5.0           | MME_GTS_50_T <sub>100</sub> _5     |
| GTS_50_T <sub>100</sub>   | 7.5           | MME_GTS_50_T <sub>100</sub> _7.5   |

To verify the effect of the utilization of GT polyphenolic compounds extracted using CNSs in the formulation stability, all formulations (Table 25) were tested to accelerated stability by centrifugation, and demonstrated no phase separation after 60 minutes.

### 3.4.3. Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) is a non-invasive optical technique used to investigate the physical properties of particles suspended in solutions. Nanoparticles, microparticles, proteins, and even viruses are examples of these particles. DLS provides useful information about average particle size, size distribution, polydispersity, and structural properties.<sup>137, 138</sup>

DLS is based on the measurement of the light scattering caused by the Brownian motion of particles in solution (Figure 81). When a light beam collides with particles moving at random, the light is scattered at various angles. The fluctuation rate of the scattered light signal is proportional to the particle size in the solution. The particle size distribution can be determined through mathematical analysis of these fluctuations.<sup>137, 138</sup>

In addition to particle size, DLS can provide information about colloidal suspension stability, molecular interactions, diffusion coefficients, and particle hydrodynamic properties.<sup>137, 138</sup> The zeta potential is an important parameter in the study of colloids and dispersed systems.<sup>139</sup>

The zeta potential describes the magnitude of electrostatic repulsion between suspended particles and is related to their surface electrical charge. A high zeta potential indicates strong electrostatic repulsion between particles, which leads to greater colloidal suspension stability. A low or near-zero zeta potential, on the other hand, can cause particle aggregation, resulting in flocs or aggregates. This aggregation can cause problems such as particle sedimentation or changes in the solution's physical and rheological properties.

<sup>139, 140</sup> The zeta potential is also related to particle-surface interactions and particle-particle interactions in biological systems. It is critical to the development of stable formulations in pharmaceutical, cosmetic, and food industries. <sup>139, 140</sup>

In summary, Dynamic Light Scattering (DLS) is a powerful technique for determining particle size and size distribution in solution, as well as providing information about their stability and interactions. The zeta potential, when measured in conjunction with DLS, provides information about particle surface electrical charge and influences colloidal system stability and behavior.

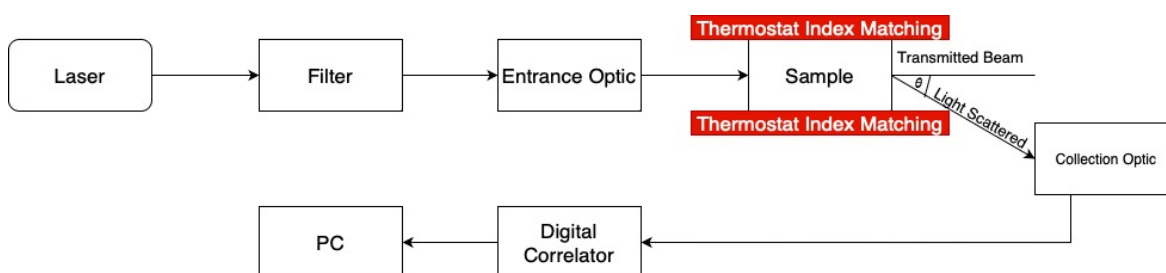


Figure 81 - DLS schematic process.

The values for particle size, polydispersity index (PI), and zeta potential (ZP) for all formulations tested are shown in the table below.

Table 26 - Results of particle size, PI, and zeta potential measured for all oral formulations.

| Formulation                        | Particle Size<br>(nm) | PI                       | Zeta Potential<br>(mV) |
|------------------------------------|-----------------------|--------------------------|------------------------|
| MME_H <sub>2</sub> O_5             | 227 ± 24              | 0.22 <sup>a</sup> ± 0.02 | -19 <sup>a</sup> ± 4   |
| MME_H <sub>2</sub> O_7.5           | 230 ± 9               | 0.19 <sup>a</sup> ± 0.03 | -28 ± 3                |
| MME_GTXAG_50_T <sub>100</sub> _5   | 266 ± 10              | 0.19 <sup>a</sup> ± 0.02 | -31 <sup>b</sup> ± 2   |
| MME_GTXAG_50_T <sub>100</sub> _7.5 | 264 ± 10              | 0.18 <sup>a</sup> ± 0.02 | -32 <sup>b</sup> ± 2   |
| MME_GTXA_50_T <sub>100</sub> _5    | 314 ± 20              | 0.19 <sup>a</sup> ± 0.03 | -20 <sup>a</sup> ± 4   |
| MME_GTXA_50_T <sub>100</sub> _7.5  | 279 ± 10              | 0.23 <sup>a</sup> ± 0.02 | -23 ± 2                |
| MME_GTS_50_T <sub>100</sub> _5     | 306 ± 17              | 0.19 <sup>a</sup> ± 0.01 | -25 <sup>c</sup> ± 4   |
| MME_GTS_50_T <sub>100</sub> _7.5   | 318 ± 8               | 0.22 <sup>a</sup> ± 0.01 | -26 <sup>c</sup> ± 2   |

Table 26 shows that increasing the Tween<sup>®</sup> 80 percentage had no significant effect on most of the parameters studied. This percentage increase resulted in less desirable results in some of the compared formulations, such as MME\_H<sub>2</sub>O and MME\_GTS\_50\_T<sub>100</sub>,

or did not show a significant upgrade in comparison to the formulation with a lower Tween<sup>®</sup> 80 percentage.

MME\_GTXAG\_50\_T<sub>100</sub>\_7.5 showed better results in all the parameters tested. However, MME\_GTXAG\_50\_T<sub>100</sub>\_5 produced comparable results with no significant differences in the PI and zeta potential parameters. MME\_GTXAG\_50\_T<sub>100</sub> and MME\_GTS\_50\_T<sub>100</sub> performed better across all parameters, with values close to those defined as having good stability, namely particle size less than 300 nm, PI less than 0.2, and zeta potential greater than |30| mV.

#### 3.4.4. pH

The pH of a substance indicates its acidity or alkalinity. It is critical in many fields, including the pharmaceutical industry. The concentration of hydrogen ions (H<sup>+</sup>) in a solution determines pH, which is measured on a scale of 0 to 14. Lower values indicate increasing acidity, while higher values indicate increasing alkalinity.<sup>141</sup>

The significance of pH in pharmaceutical formulations comes from the fact that many active ingredients and excipients used in medications are pH sensitive. Many drugs' pharmacological activity is influenced by the acidity or alkalinity of the medium in which they are present.<sup>142</sup> Additionally, pH affects the physical and chemical stability of a pharmaceutical formulation. Within certain pH ranges, some substances may decompose, lose efficacy, or produce toxic products. As a result, it is critical to carefully control the pH of medications during development, manufacturing, and storage.<sup>142</sup> Furthermore, pH can influence drug absorption in the body. The gastrointestinal tract, for example, has different pH values in different regions. Understanding the pH in these areas is critical for developing oral formulations that ensure proper drug absorption.<sup>143</sup>

In summary, pH is important in pharmaceutical formulations because it affects medication stability, efficacy, and absorption. To ensure the quality, safety, and effectiveness of pharmaceutical products, precise pH control is required.

Setting aside the formulations with a higher percentage of Tween<sup>®</sup> 80 because they did not provide significant advantages over the 5.0 % Tween<sup>®</sup> 80 formulations, the pH of these formulations was measured, and the results are shown in Table 27.

Table 27 - Results of pH measured for all oral microemulsion formulations.

| Formulation                      | pH          |
|----------------------------------|-------------|
| MME_GTXAG_50_T <sub>100</sub> _5 | 5.28 ± 0.01 |
| MME_GTXA_50_T <sub>100</sub> _5  | 5.14 ± 0.02 |
| MME_GTS_50_T <sub>100</sub> _5   | 4.22 ± 0.02 |

The formulation with GT polyphenolic compounds extracted with stevia (MME\_GTS\_50\_T<sub>100</sub>\_5) was the most acidic (4.22), while the formulation with GT polyphenolic compounds extracted with agave syrup (MME\_GTXAG\_50\_T<sub>100</sub>\_5) was the least acidic (5.28).

### 3.4.5. Final Review

Following the optimization of the production of the oral microemulsions and the successful completion of the first formulation stability tests, new prolonged stability tests are required, both at the formulation level as a whole and, also at the level of the polyphenolic compounds extracted from GT. Assays to confirm each formulation's bioavailability are also required.

However, before carrying out these tests, it was found that it was necessary to take an excessive amount of the prepared formulations in order to administer the catechin dose indicated in the reference article.<sup>144</sup> To address this issue, a novel method of extracting and dissolving GT polyphenolic compounds in CNSs was necessary. As a result, no further assays using the oral microemulsion were performed. Instead, a new syrup formulation for young children administration was developed incorporating GT polyphenolic compounds extracted with CNSs.

## 3.5. Development of a Syrup Formulation with Green Tea extracted with Commercial Natural Solvents

### 3.5.1. Optimization of the Procedure

Syrups are widely used in the pharmaceutical industry to alleviate symptoms such as, for example, coughing and pain. They are made up of a variety of active ingredients and additives, each of which contributes to the final product's effectiveness and stability. The active ingredients are responsible for the syrup's therapeutic action and can vary depending on the treatment goal. A cough syrup, for example, may contain antitussive ingredients that suppress coughing, whereas a pain syrup may contain analgesics that reduce pain sensation.<sup>145</sup>

Syrups, in addition to the active ingredients, contain a liquid base, which is typically made up of water, glycerin, or sugar solutions. The liquid base is critical to the stability and palatability of the syrup, providing the necessary viscosity and facilitating medication administration. The liquid base is chosen based on factors such as the solubility of the active ingredients and patient preference.<sup>145</sup>

To improve the appearance, taste, and shelf life of syrups, additives such as preservatives, flavorings, and colorants can be added. Preservatives help to prevent the growth of microorganisms and extend the shelf life of the product, whereas flavorings and colorants can make the syrup more enjoyable to taste and visually appealing.<sup>145</sup>

Syrup development necessitates a careful balance of therapeutic efficacy, physical and chemical stability of the product, and patient acceptance. Aspects such as active ingredient solubility, component compatibility, physicochemical interactions, and ease of administration during the formulation process must be considered.<sup>146, 147</sup> Furthermore, to ensure the quality, safety, and efficacy of syrups, government regulations and pharmaceutical industry guidelines must be followed. This includes stability studies, purity tests, and adhering to good manufacturing practices.<sup>148</sup>

To summarize, syrup development is a complex process that includes the selection of appropriate active ingredients, liquid bases, and additives. Precise formulation aims to ensure therapeutic efficacy, product physical and chemical stability, and patient



acceptance. Compliance with regulations and industry guidelines is critical to ensuring the quality and safety of the syrups developed.

In this case, the solution with the active substances is naturally rich in sugar, so the base for the syrup was already made, and in addition to the solutions of CNSs with GT polyphenolic extract, glycerin (humectant), xanthan gum solution (thickener), and sodium benzoate (preservative) are added to create a syrup formulation, the percentage in the solution of each syrup component is shown in Table 28.

Table 28 - Percentual composition of Syrup\_1.

| <b>Constituents</b>        | <b>Percentage (%)</b> |
|----------------------------|-----------------------|
| GT Compounds Extracted     | 40.2                  |
| Glycerin                   | 5.0                   |
| 0.2 % Xanthan Gum Solution | 54.6                  |
| Sodium Benzoate            | 0.2                   |

After preparing the syrup, a low viscosity was observed, which was outside of the normal parameters associated with syrup viscosity, so the concentration of the xanthan gum solution was increased to increase the viscosity of the syrup. The amount of the xanthan gum solution was increased to 0.4 %. It was also verified that the desired viscosity was still not achieved, so a 0.8% xanthan gum solution was added, keeping the same proportion of each component in the syrup preparation. After a new syrup preparation, the desired viscosity was obtained, and the final syrup composition is shown in Table 29.

Table 29 - Percentual composition of final Syrup.

| <b>Constituents</b>        | <b>Percentage (%)</b> |
|----------------------------|-----------------------|
| GT Compounds Extracted     | 40.2                  |
| Glycerin                   | 5.0                   |
| 0.8 % Xanthan Gum Solution | 54.6                  |
| Sodium Benzoate            | 0.2                   |

## 3.6. Stability of Syrup Formulation with Green Tea Extracted with Commercial Natural Solvents

### 3.6.1. pH

As previously stated, pH is an important factor in pharmaceutical formulations because it has an impact on their stability, efficacy, and absorption, as well as on their physical and chemical stability. To ensure quality, safety, and effectiveness, pH must be controlled during development, manufacturing, and storage. Understanding the pH in various regions is critical for creating oral formulations that ensure proper drug absorption.

In the syrup stability tests, two lots of each syrup formulation were prepared, and Table 30 demonstrated the relationship between the code name assigned by the authors and the CNS used, the lot information, and the condition exposed.

Table 30 - Correlation between the syrup, lot, and extraction medium of the GT polyphenolic compounds under light, dark, and temperature conditions and the attributed code by authors.

| Extraction Medium | Lot | Condition            | Name attributed by authors                        |
|-------------------|-----|----------------------|---------------------------------------------------|
| Agave Syrup       | 1   | Light                | Syrup_GTXAG_50_T <sub>100</sub> _Lot1_Light       |
| Agave Syrup       | 2   | Light                | Syrup_GTXAG_50_T <sub>100</sub> _Lot2_Light       |
| Agave Syrup       | 1   | Dark                 | Syrup_GTXAG_50_T <sub>100</sub> _Lot1_Dark        |
| Agave Syrup       | 2   | Dark                 | Syrup_GTXAG_50_T <sub>100</sub> _Lot2_Dark        |
| Agave Syrup       | 1   | Temperature (4.0 °C) | Syrup_GTXAG_50_T <sub>100</sub> _Lot1_Temperature |
| Agave Syrup       | 2   | Temperature (4.0 °C) | Syrup_GTXAG_50_T <sub>100</sub> _Lot2_Temperature |
| Stevia            | 1   | Light                | Syrup_GTS_50_T <sub>100</sub> _Lot1_Light         |
| Stevia            | 2   | Light                | Syrup_GTS_50_T <sub>100</sub> _Lot2_Light         |
| Stevia            | 1   | Dark                 | Syrup_GTS_50_T <sub>100</sub> _Lot1_Dark          |
| Stevia            | 2   | Dark                 | Syrup_GTS_50_T <sub>100</sub> _Lot2_Dark          |
| Stevia            | 1   | Temperature (4.0 °C) | Syrup_GTS_50_T <sub>100</sub> _Lot1_Temperature   |
| Stevia            | 2   | Temperature (4.0 °C) | Syrup_GTS_50_T <sub>100</sub> _Lot2_Temperature   |

In this case, the temperatures of storage were room temperature and 4.0 °C (normal condition of syrup storage), with the goal of determining whether that condition can promote better stability in syrup formulations. As previously described in section 3.2.1, even without direct exposure, degradation and oxidation of syrup formulations can occur due to enzymes in solution and optimal pH and temperature conditions. The following Table shows the pH values of the syrup formulations studied.

Table 31 - pH values of the different syrup formulations investigated.

| Formulation                                               | Day 0                      | Day 30                       | Day 60                     | Day 90                     |
|-----------------------------------------------------------|----------------------------|------------------------------|----------------------------|----------------------------|
| Syrup_GTXAG_5<br>0_T <sub>100</sub> _Lot1_Light           | 6.06 <sup>a</sup> ± 0.01   | 5.42 <sup>d,e</sup> ± 0.04   | Fungal Colony<br>Detected  | -                          |
| Syrup_GTXAG_5<br>0_T <sub>100</sub> _Lot2_Light           | 6.02 <sup>a</sup> ± 0.03   | 5.31 <sup>d,f</sup> ± 0.05   | Fungal Colony<br>Detected  | -                          |
| Syrup_GTXAG_5<br>0_T <sub>100</sub> _Lot1_Dark            | 6.06 <sup>a</sup> ± 0.01   | 5.3 <sup>d,f</sup> ± 0.4     | Fungal Colony<br>Detected  | -                          |
| Syrup_GTXAG_5<br>0_T <sub>100</sub> _Lot2_Dark            | 6.02 <sup>a</sup> ± 0.03   | 5.42 <sup>d,e</sup> ± 0.02   | 4.94 <sup>f</sup> ± 0.06   | 5.24 <sup>d</sup> ± 0.03   |
| Syrup_GTXAG_5<br>0_T <sub>100</sub> _Lot1_Tem<br>perature | 6.06 <sup>a</sup> ± 0.01   | 5.9 <sup>a,h</sup> ± 0.2     | 5.6 <sup>e</sup> ± 0.1     | 5.79 <sup>a</sup> ± 0.03   |
| Syrup_GTXAG_5<br>0_T <sub>100</sub> _Lot2_Tem<br>perature | 6.02 <sup>a</sup> ± 0.03   | 6.0 <sup>a,h</sup> ± 0.1     | 5.62 <sup>e</sup> ± 0.06   | 5.96 <sup>a</sup> ± 0.09   |
| Syrup_GTS_50_<br>T <sub>100</sub> _Lot1_Light             | 4.07 <sup>b,j</sup> ± 0.04 | 3.87 <sup>b,c</sup> ± 0.05   | 3.70 <sup>c,g</sup> ± 0.03 | 4.05 <sup>b,j</sup> ± 0.03 |
| Syrup_GTS_50_<br>T <sub>100</sub> _Lot2_Light             | 3.96 <sup>b,c</sup> ± 0.03 | 3.87 <sup>b,c,g</sup> ± 0.06 | 3.64 <sup>g,i</sup> ± 0.03 | 4.00 <sup>b,j</sup> ± 0.02 |
| Syrup_GTS_50_<br>T <sub>100</sub> _Lot1_Dark              | 4.07 <sup>b,j</sup> ± 0.04 | 3.92 <sup>b,c,g</sup> ± 0.02 | 3.73 <sup>c,i</sup> ± 0.02 | 4.08 <sup>b,j</sup> ± 0.03 |
| Syrup_GTS_50_<br>T <sub>100</sub> _Lot2_Dark              | 3.96 <sup>b,c</sup> ± 0.03 | 3.88 <sup>b,c,g</sup> ± 0.02 | 3.70 <sup>c,i</sup> ± 0.04 | 4.00 <sup>b,j</sup> ± 0.01 |
| Syrup_GTS_50_<br>T <sub>100</sub> _Lot1_Tempe<br>rature   | 4.07 <sup>b,j</sup> ± 0.04 | 4.1 <sup>b</sup> ± 0.1       | 3.9 <sup>b,c,i</sup> ± 0.1 | 4.30 <sup>i</sup> ± 0.1    |

| Formulation                            | Day 0                      | Day 30                   | Day 60                     | Day 90                     |
|----------------------------------------|----------------------------|--------------------------|----------------------------|----------------------------|
| Syrup_GTS_50_                          |                            |                          |                            |                            |
| T <sub>100</sub> _Lot2_Tempe<br>rature | 3.96 <sup>b,c</sup> ± 0.03 | 4.09 <sup>b</sup> ± 0.09 | 3.9 <sup>b,c,i</sup> ± 0.1 | 4.22 <sup>b,j</sup> ± 0.04 |

Same superscripts (a-i) indicate not significant differences ( $p > 0.9999$ ) among all values.

Analyzing the results obtained in pH assays for 90 days (Table 31), it is possible to see that, with the exception of syrup formulations stored in the dark with GT polyphenolic compounds extracted in agave syrup, all the conditions showed no significant variation of pH when compared between the lots. Syrup formulations with GT polyphenolic compounds extracted in agave syrup showed less stability to the formation of fungal colonies, so this suggests that a higher percentage of sodium benzoate or the association of another preservative may be necessary. Between the days 0 and 90, syrup formulations with GT polyphenolic extracted in stevia and in agave syrup under temperature conditions showed no significant differences, aside this these formulations showed no significant differences between the lot's values, demonstrating a stability after 90 days.

### 3.6.2. High-Performance Liquid Chromatography – Diode Array (HPLC - DAD)

Figure 82 shows the percentage of stability of each polyphenolic compound from GT over 90 days of Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot1 in light, dark, and temperature of 4.0 °C conditions, as before mentioned in light and dark conditions, fungal colonies were detected on day 60 assays. In temperature condition, in exception of PD-BG, (E)GCG, and (E)CG, all the other compounds demonstrated stability over 50 % on day 90 assay.

Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot2 (Figure 83) shows that in exception of Quercetin-3-O-rutinoside and Kaempferol 3-rutinoside, all the other compounds in dark condition showed significantly less stability over the 90 days when compared to temperature condition. In exception of (E)GCG and (E)CG, all the other compounds in temperature of 4.0 °C condition showed stability over 50 % on day 90 assay.

Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot1 (Figure 82) and Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot2 (Figure 83) showed that (E)GC, (E)C, Gallic Acid, Apigenin-6-C-pentoside-8-C-hexoside,

Quercetin-3-O-rutinoside, and Kaempferol 3-rutinoside demonstrated stability higher than 70 % over the 90 days.

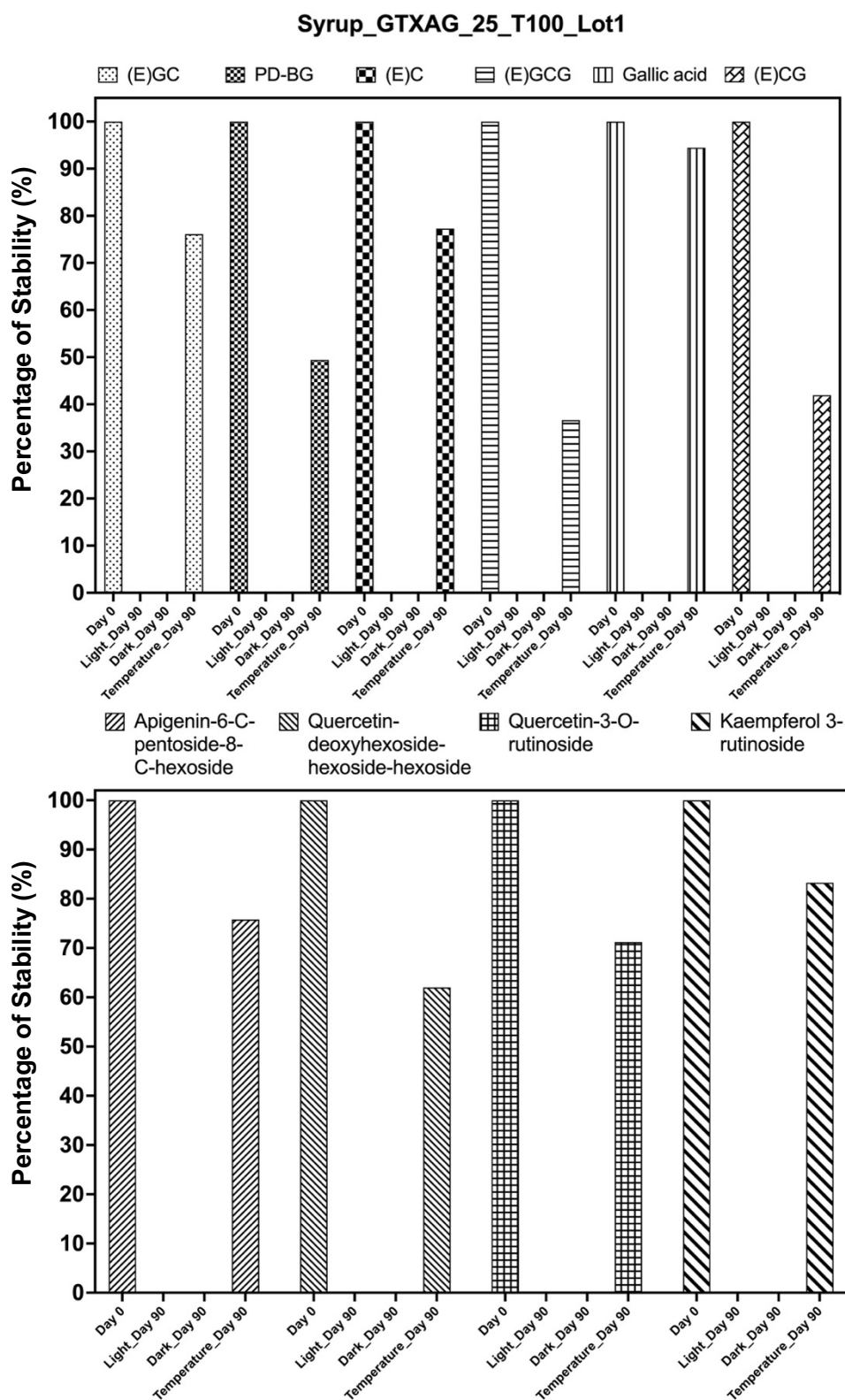


Figure 82 - Percentage of stability of each polyphenol compound in stability assay using Syrup GTXAG\_50\_T100\_Lot1.

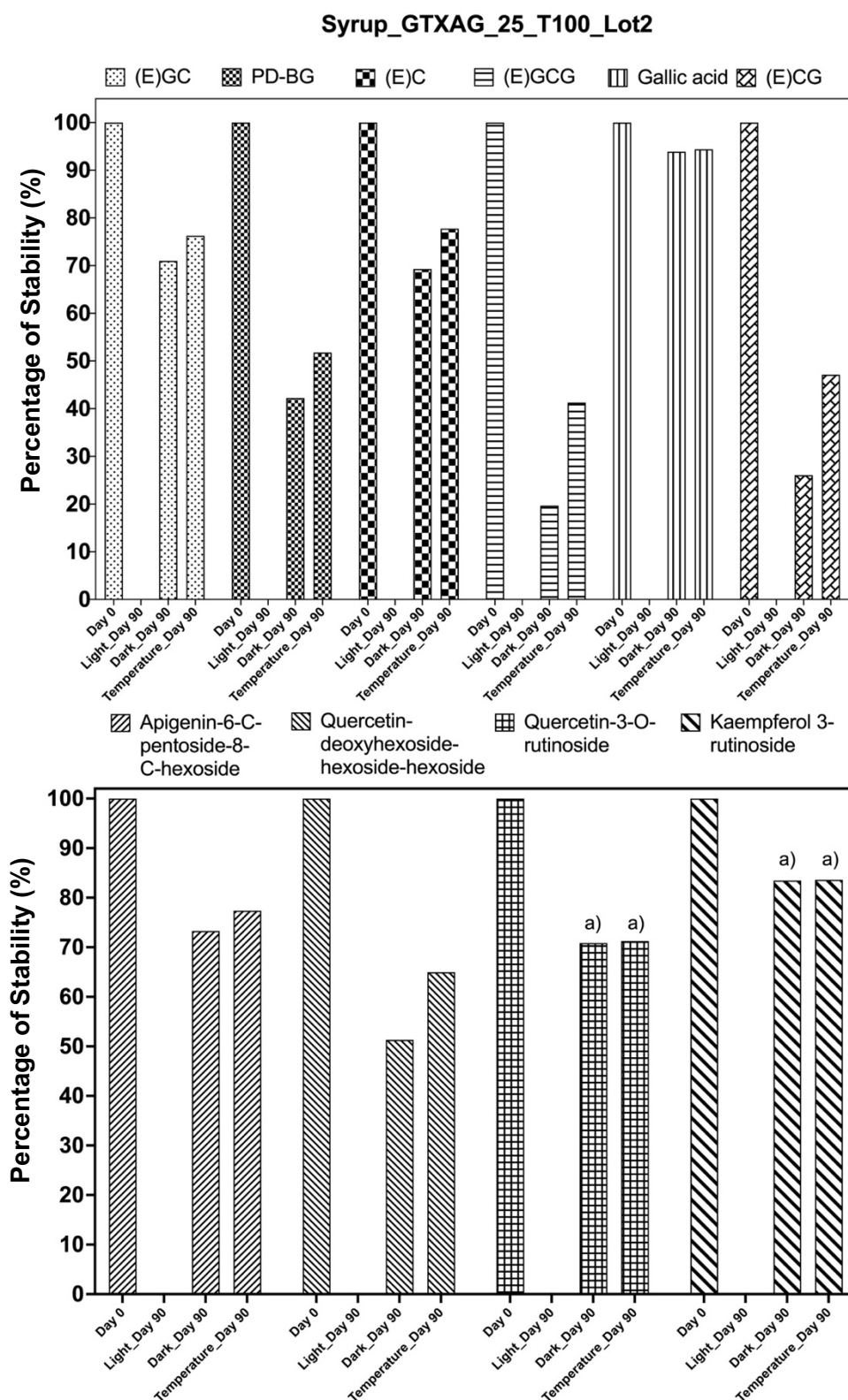


Figure 83 - Percentage of stability of each polyphenol compound in stability assay using Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

Figure 84 shows the stability of all compounds in Syrup\_GTS\_50\_T<sub>100</sub>\_Lot1 over 90 days. Comparing the different aging conditions, temperature condition demonstrated better stability than light and dark conditions in all compounds, with the exception of (E)GC and Quercetin-3-O-rutinoside when compared with light condition and PD-BG and Gallic acid when compared to dark condition.

Similar to the observed in Syrup\_GTS\_50\_T<sub>100</sub>\_Lot1 (Figure 84), Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2 (Figure 85) under temperature condition demonstrated better stability over 90 days when compared to the other aging condition, however, some differences occur between the lots. When compared to light condition, Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2 under 4 °C showed no significative differences observed in (E)GC, and when compared to dark conditions no significative differences were observed in Quercetin-3-O-rutinoside.

Syrup\_GTS\_50\_T<sub>100</sub>\_Lot1 (Figure 84) and Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2 (Figure 85) showed that under temperature condition all compounds showed stability higher than 50 % over the 90 days, and in all aging conditions (E)GC, Gallic acid, Apigenin-6-C-pentoside-8-C-hexoside, and Kaempferol 3-rutinoside showed stability higher than 70 % on day 90 assays, (E)C demonstrated stability over 70 % in dark and temperature condition, and (E)CG and Quercetin-deoxyhexoside-hexoside-hexoside in temperature condition.

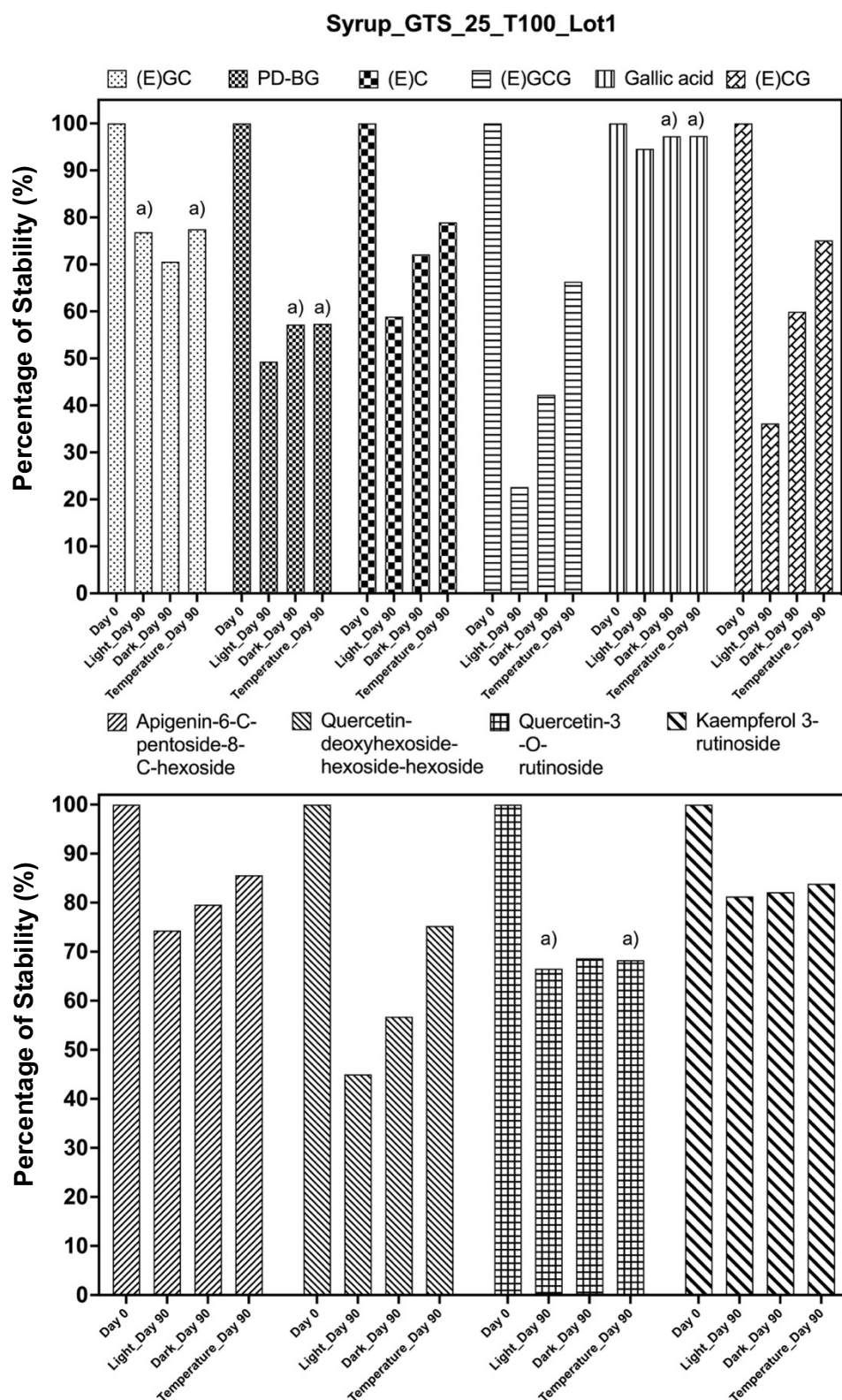


Figure 84 - Percentage of stability of each polyphenol compound in stability assay using Syrup\_GTS\_50\_T<sub>100</sub>\_Lot1. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.



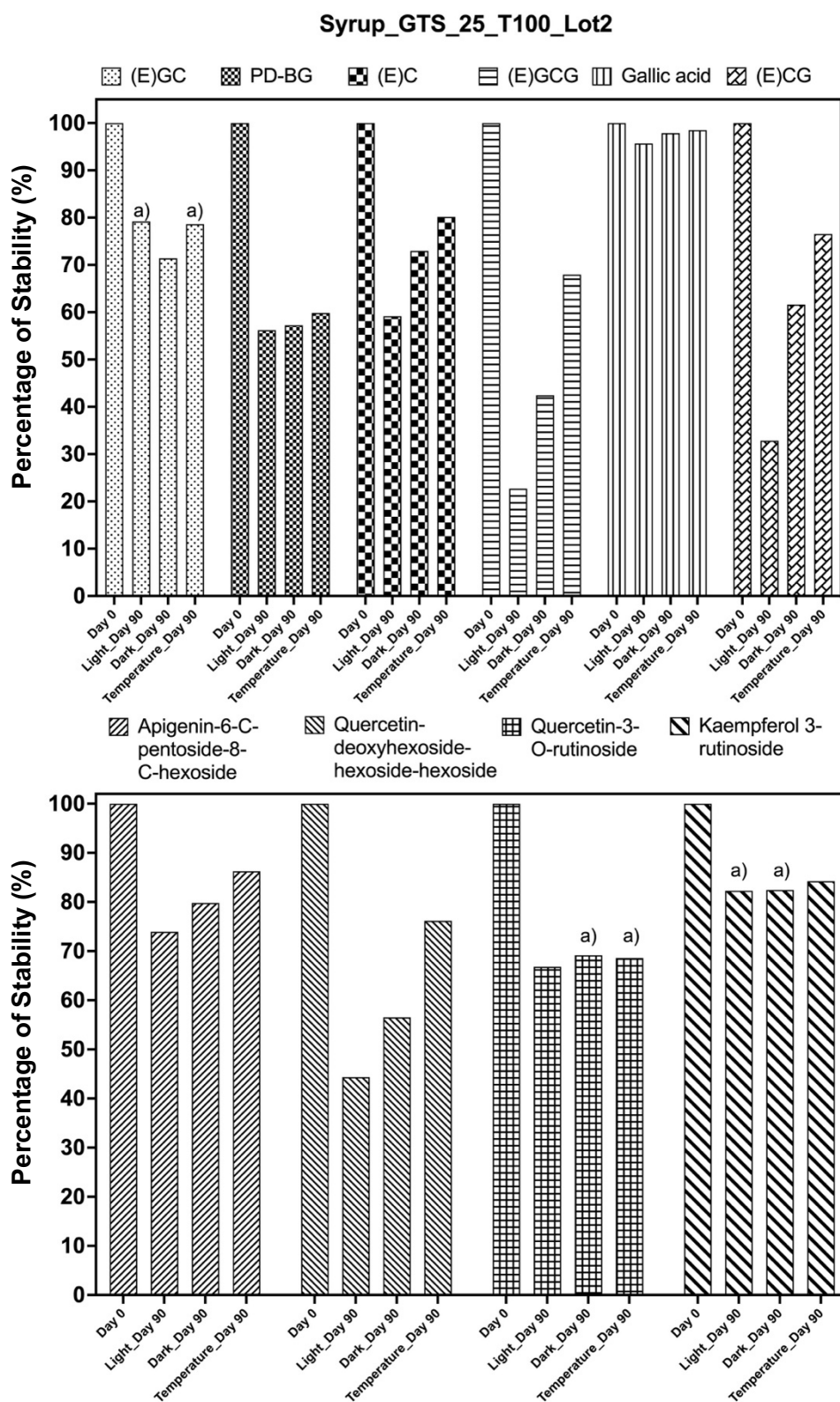


Figure 85 - Percentage of stability of each polyphenol compound in stability assay using Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

### 3.6.3. Colorimetry

Similar to the section 3.3.2. colorimetric assays were performed in the syrup formulations over 90 days to understand the changes in the samples color in different aging conditions. The colorimetric assays were carried out in Syrup\_GTXAG\_50\_T<sub>100</sub> and Syrup\_GTS\_50\_T<sub>100</sub> stored under light, dark, and at a temperature of 4 °C. Figures 86-91 shows the results of the colorimetric stability assays of Syrup\_GTXAG\_50\_T<sub>100</sub>.

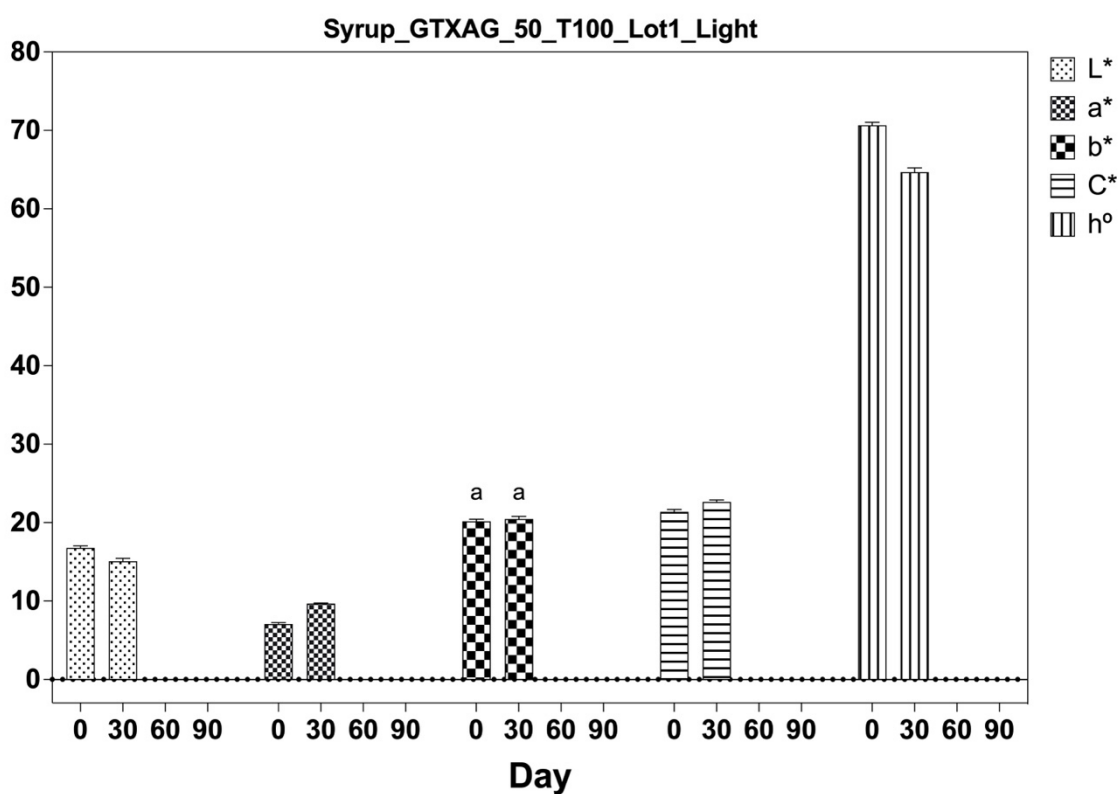


Figure 86 - Colorimetric stability assay using Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot1\_Light. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

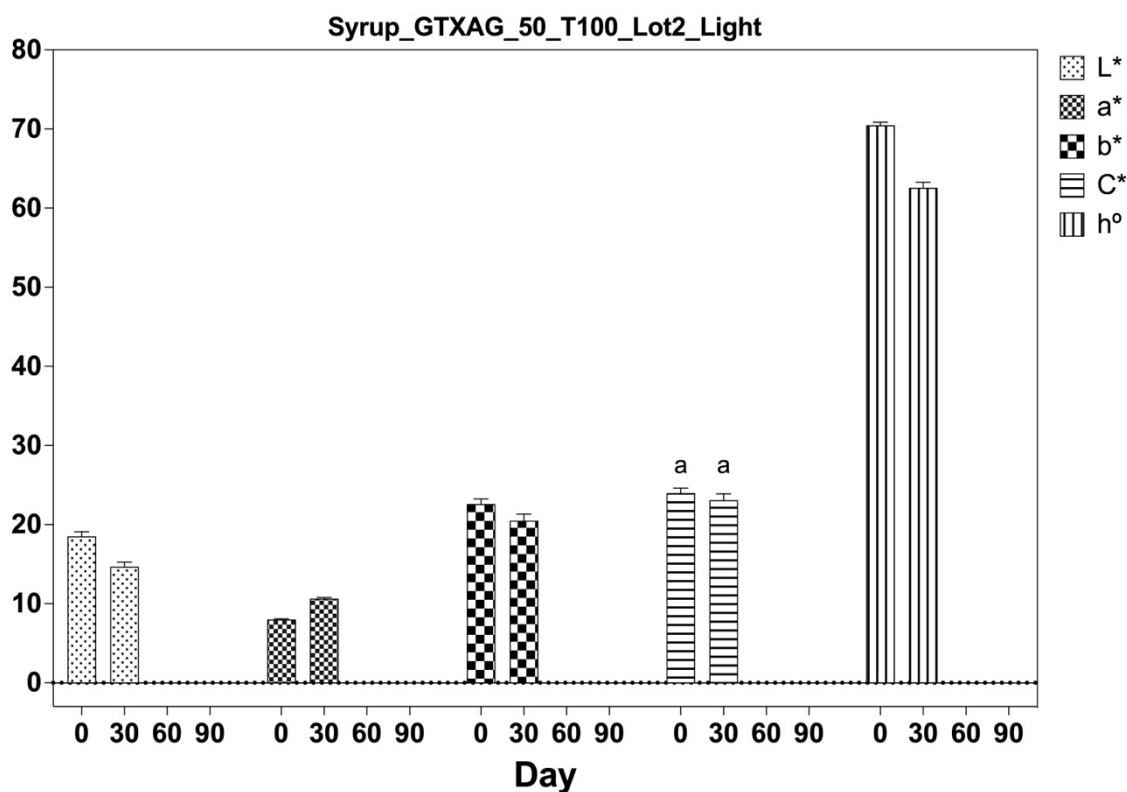


Figure 87 - Colorimetric stability assay using Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot2\_Light. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

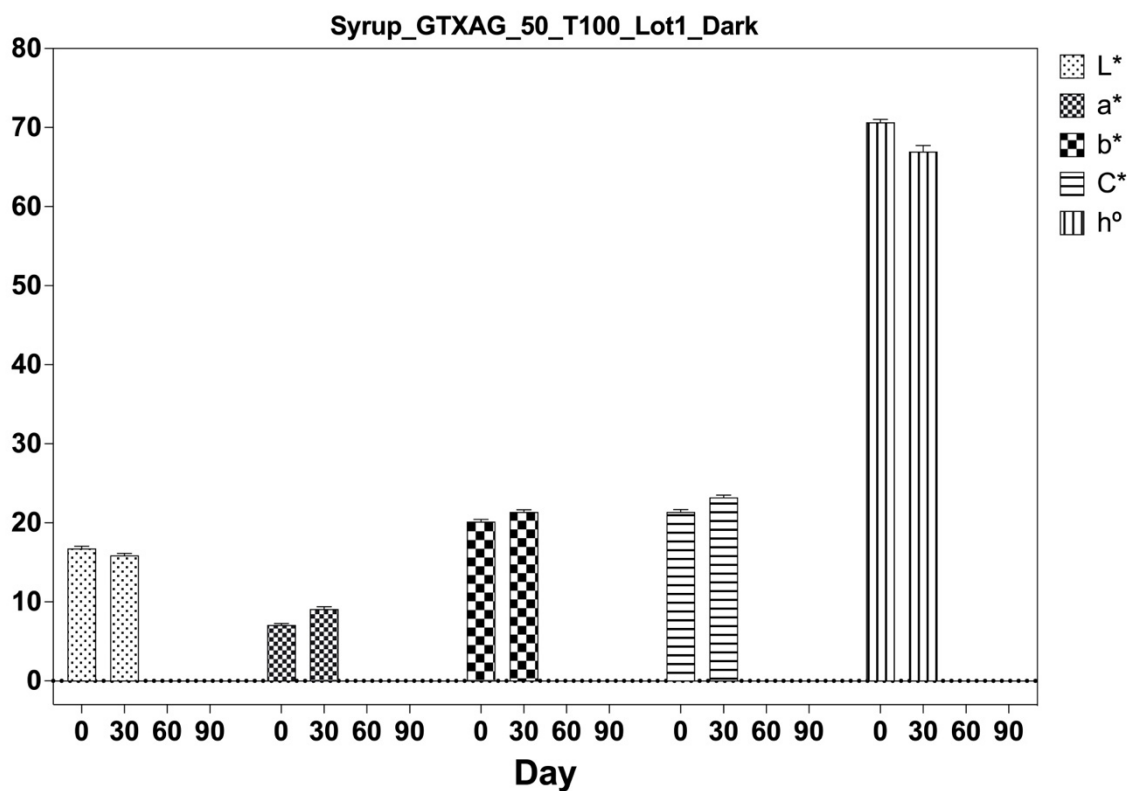


Figure 88 - Colorimetric stability assay using Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot1\_Dark.

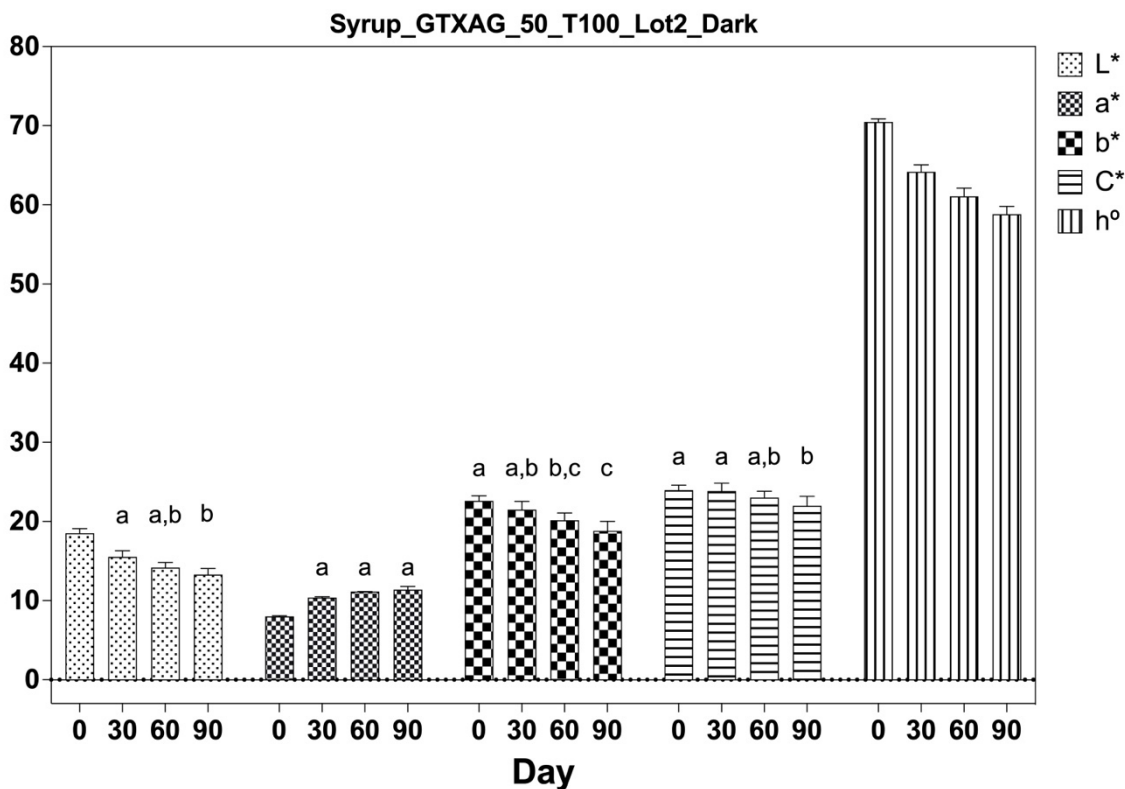


Figure 89 - Colorimetric stability assay using Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot2\_Dark. Same superscripts (a-c) indicate not significant differences ( $p > 0.9999$ ) among the column values.

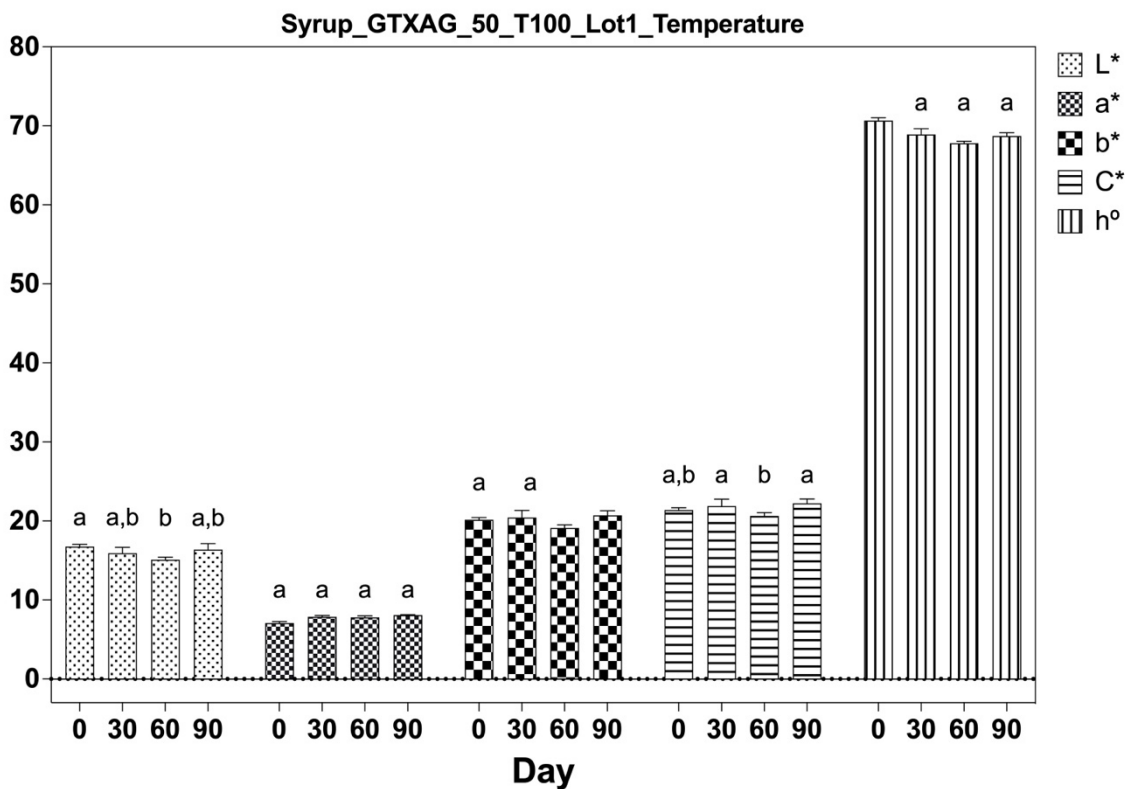


Figure 90 - Colorimetric stability assay using Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot1\_Temperature. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

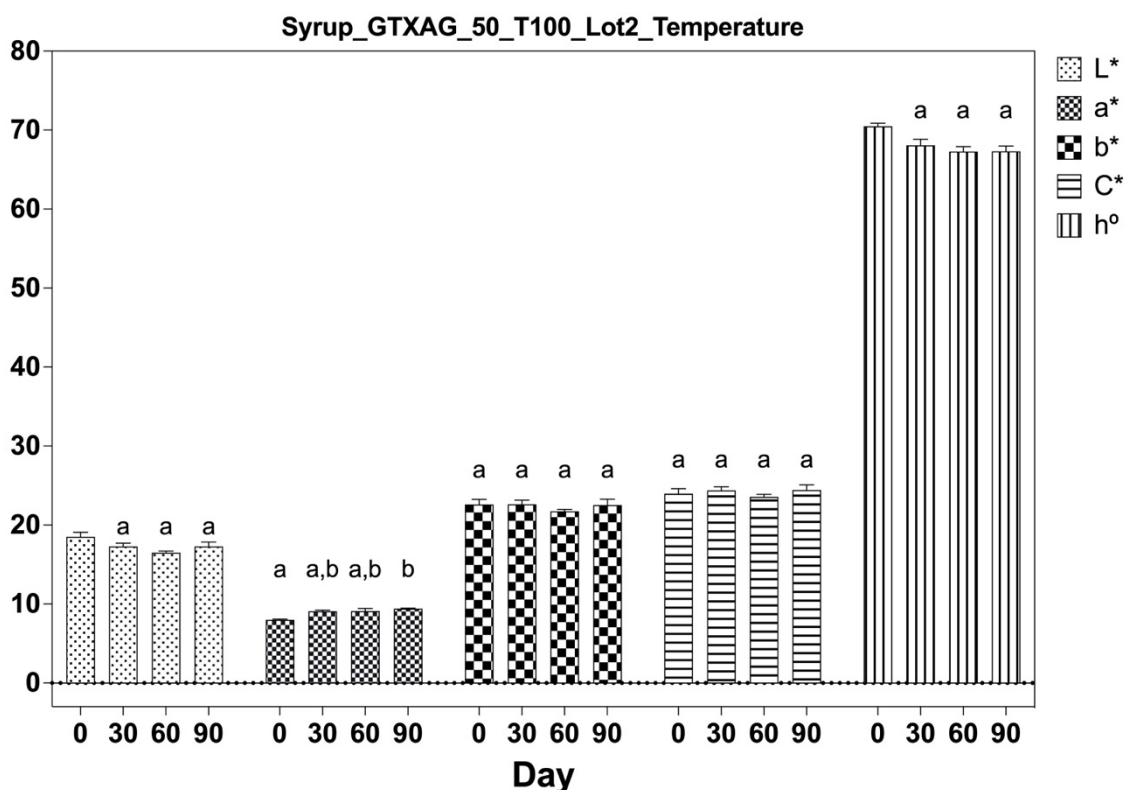


Figure 91 - Colorimetric stability assay using Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot2\_Temperature. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

Figures 86 and 87 show the colorimetric assays performed to Syrup\_GTXAG\_50\_T<sub>100</sub> under light conditions, both lots showed fungal colonies on day 60. Under dark condition only lot 2 (Figure 89) reach the day 90, demonstrating good stability in the b\* and C\* parameters over the 60 days, and no significant differences in the rest of the color parameters compared between days 30 and 60. Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lote1\_Dark (Figure 88) showed fungal colonies on day 60. Syrup formulations under a temperature of 4°C showed, in general, a good stability over the 60 days. Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lote1\_Temperature (Figure 90) showed no significant differences between days 0 and 90 in the L\*, b\* and C\* parameters, and no significant differences between days 0, 30, and 60 in the a\* parameter. The case of Syrup\_GTXAG\_50\_T<sub>100</sub>\_lote2\_Temperature (Figure 91) demonstrates no significant differences between days 0 and 90 in the b\* and C\* parameter, and no significant differences between days 30 and 90 in the L\*, a\*, and h° parameters.

In all cases, besides the statistical differences after 60 days, all demonstrated low lightness, low red and yellow tones, low saturation, and color near to a pure yellow.

Figures 92-97 shows the results of the colorimetric stability assays of Syrup\_GTS\_50\_T<sub>100</sub>.

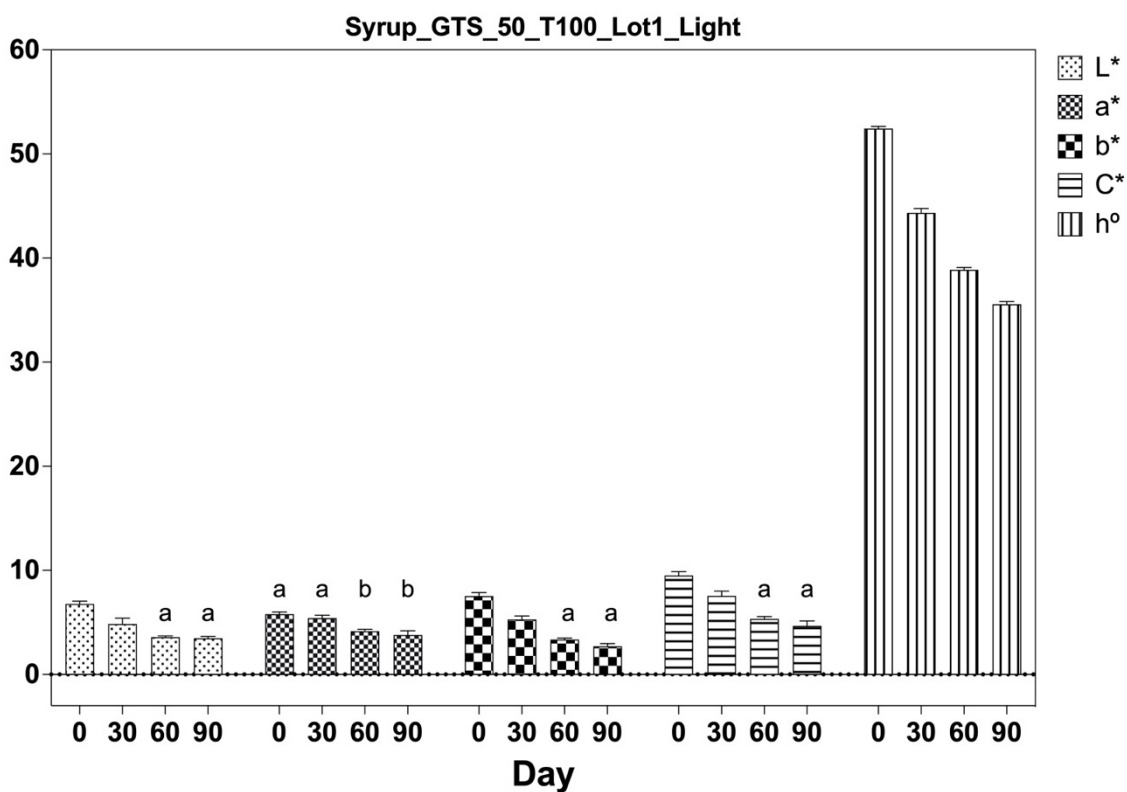


Figure 92 - Colorimetric stability assay using Syrup\_GTS\_50\_T<sub>100</sub>\_Lot1\_Light. Same superscripts (a) indicate not significant differences ( $p > 0.9999$ ) among the column values.

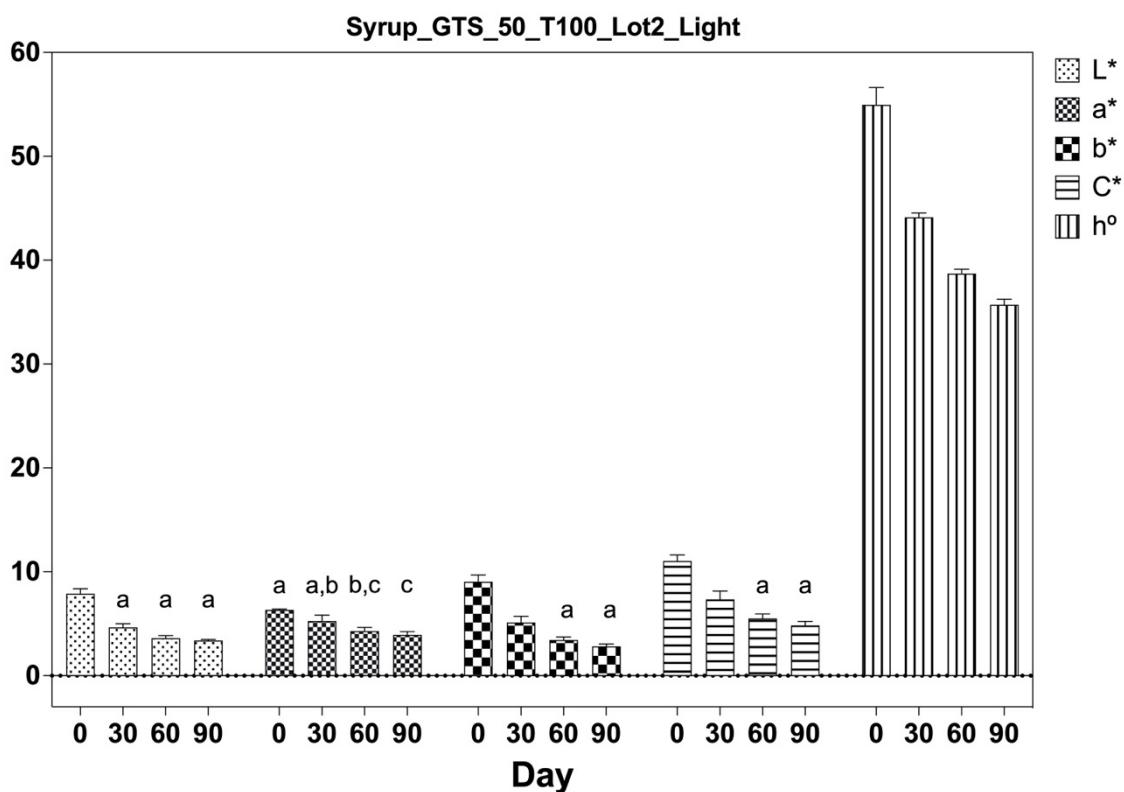


Figure 93 - Colorimetric stability assay using Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2\_Light. Same superscripts (a-c) indicate not significant differences ( $p > 0.9999$ ) among the column values.

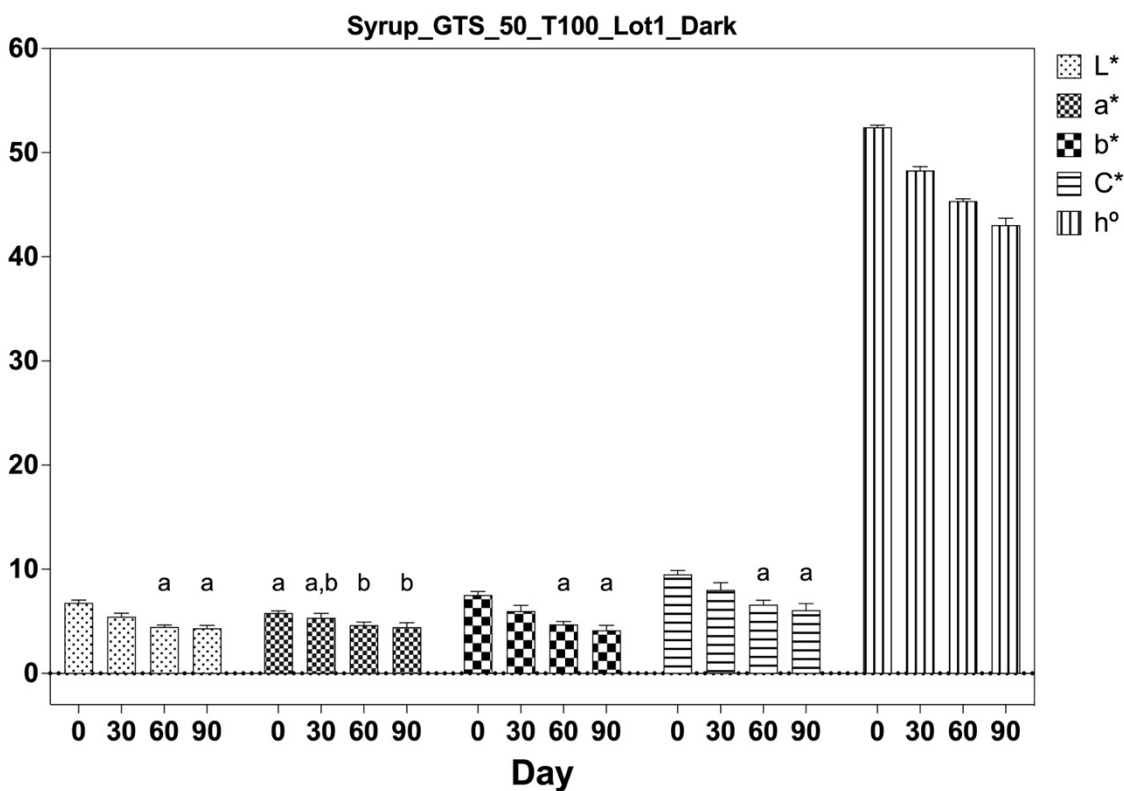


Figure 94 - Colorimetric stability assay using Syrup\_GTS\_50\_T<sub>100</sub>\_Lot1\_Dark. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

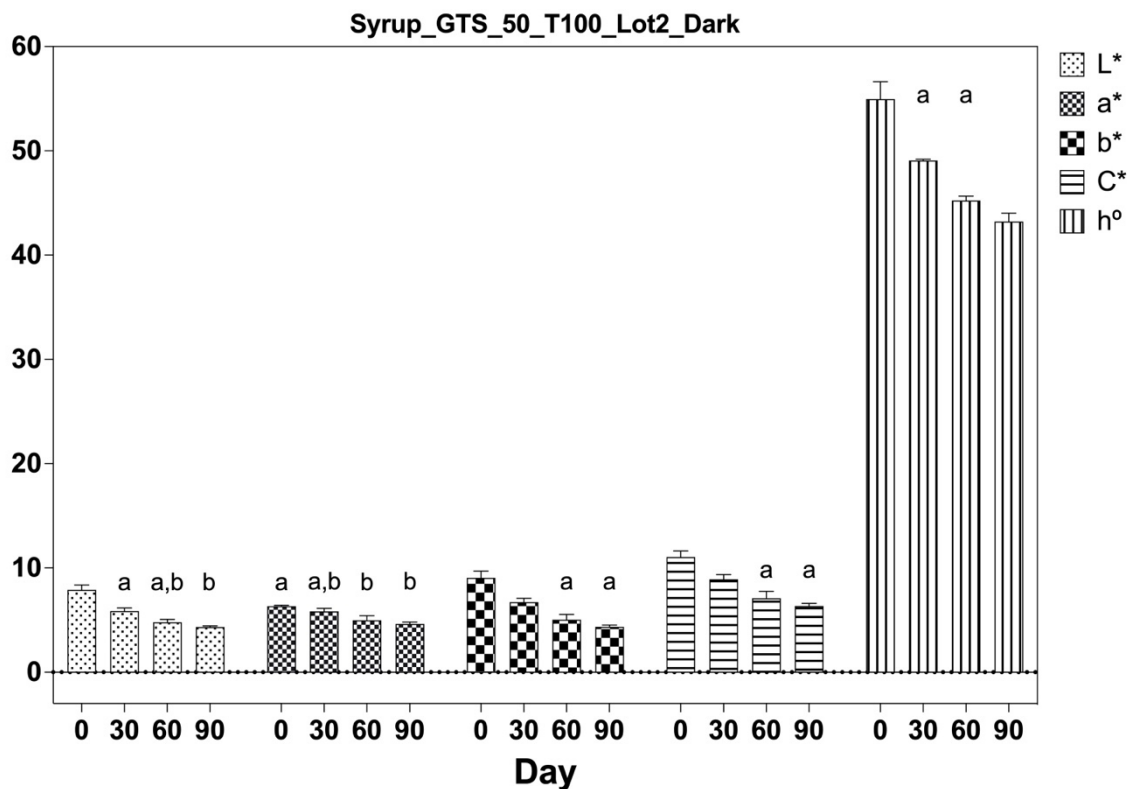


Figure 95 - Colorimetric stability assay using Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2\_Dark. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

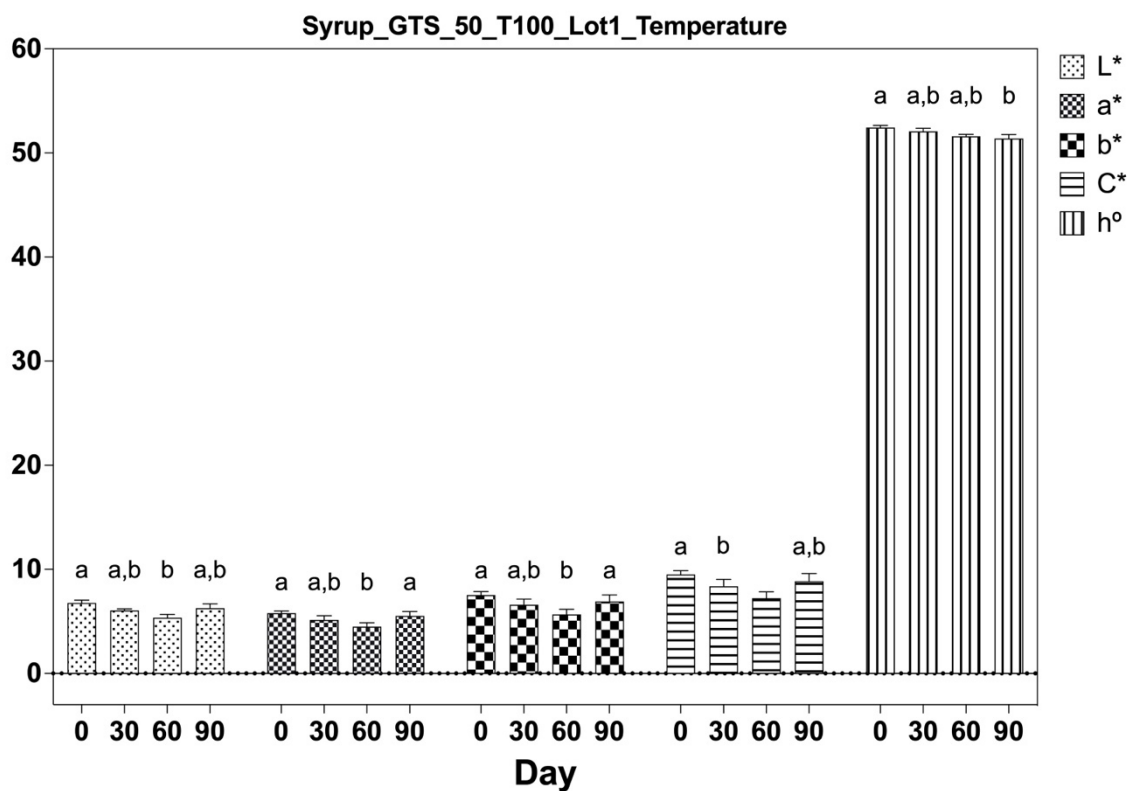


Figure 96 - Colorimetric stability assay using Syrup\_GTS\_50\_T<sub>100</sub>\_Lot1\_Temperature. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.



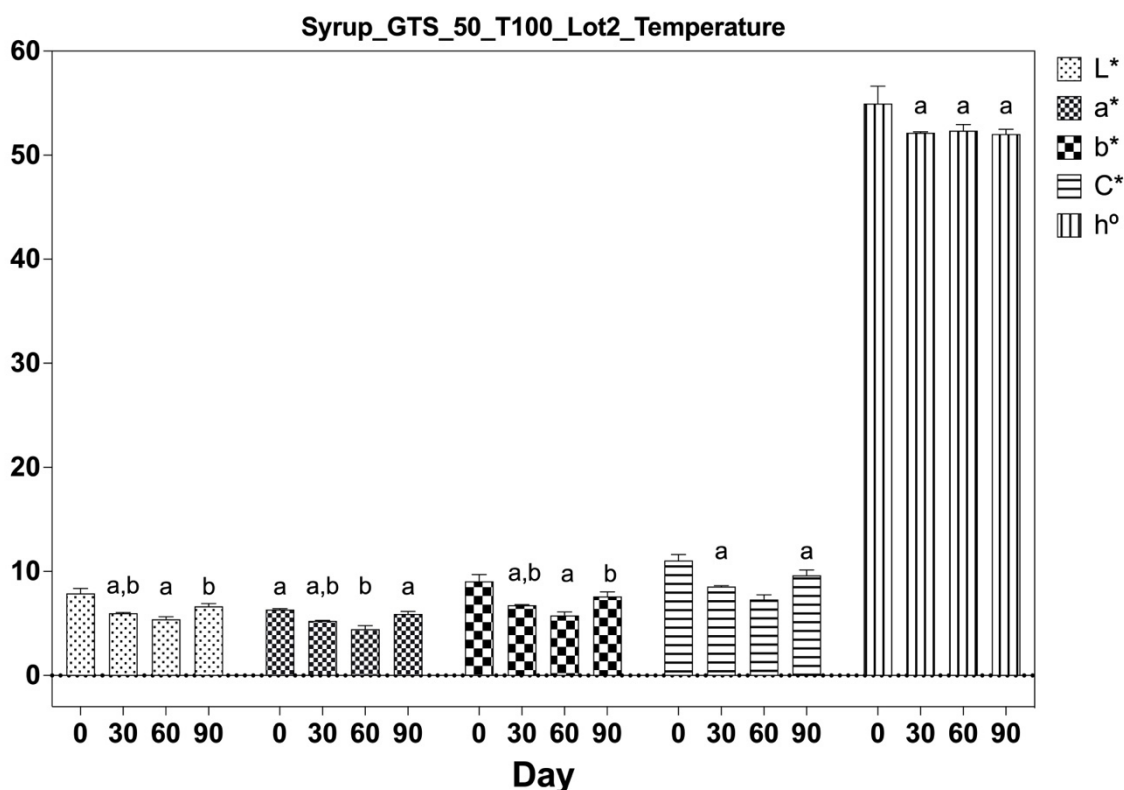


Figure 97 - Colorimetric stability assay using Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2\_Temperature. Same superscripts (a,b) indicate not significant differences ( $p > 0.9999$ ) among the column values.

Figures 92 and 93 shows that Syrup\_GTS\_50\_T<sub>100</sub> under light condition have significant differences in all parameters after 90 days. Syrup\_GTS\_50\_T<sub>100</sub>\_Lot1\_Dark (Figure 94) showed no significant differences between days 0 and 30 and days 30, 60, and 90 in the a\* parameter. Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2\_Dark (Figure 95) showed no significant differences between days 30 and 60, and between 60 and 90 in the L\* parameter, the a\* parameter showed no significant differences between days 0 and 30, and between 30, 60, and 90 days, and b\* and C\* parameters showed no significant differences between days 60 and 90. Under a temperature of 4°C Lot 1 (Figure 96) showed no significant differences between days 0 and 90 in the L\*, a\*, b\* and C\* parameters. On the other hand, Syrup\_GTS\_50\_T<sub>100</sub>\_Lote2\_Temperature (Figure 97) showed no significant differences between days 30 and 90 in the a\* parameter and between days 30 and 90 in the L\*, a\*, b\*, and C\* parameter.

In all cases, the samples showed low lightness, low yellow and red tones, low saturation, and a color between a pure red and a pure yellow.

### 3.6.4. Rheology

Rheology is an important technique for studying the deformation and flow of materials in response to forces. It enables detailed analysis of material mechanical behavior under a variety of conditions, including tension, shear, and deformation. Rheometers collect data on mechanical and rheological properties of materials, such as viscosity, elasticity, and shear stress, as mentioned in section 3.2.5.1.

Figures 98 and 99 show the rheological behavior of all syrups stored in different conditions.

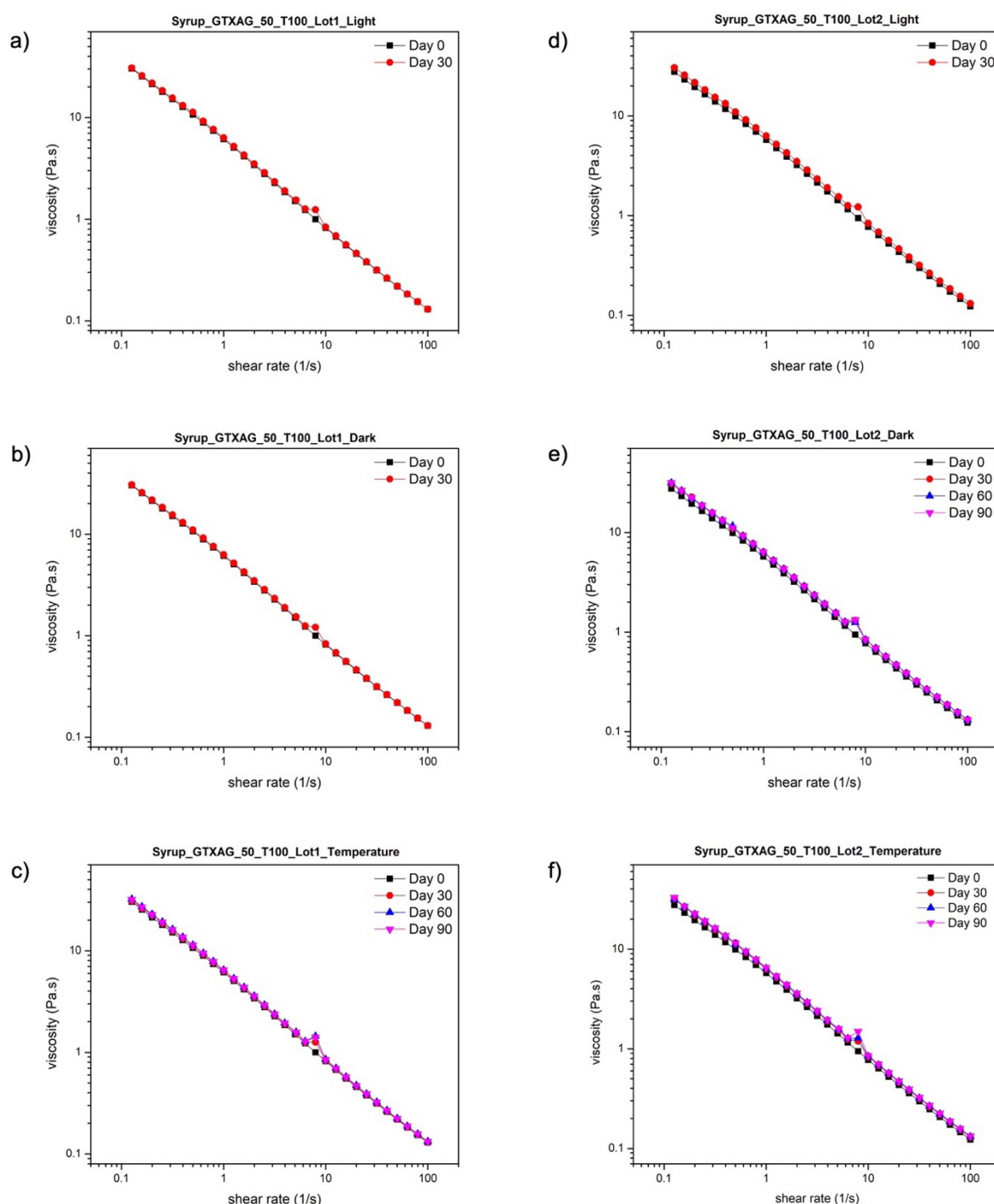


Figure 98 - Flow curves of all Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lote1 and Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lote2 stored 90 days in different conditions.

As mentioned in section 3.6.3, Syrup\_GTXAG\_50\_T<sub>100</sub> stored in Light conditions (Figure 98 a and d) and Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot2\_Dark (Figure 98 b) showed fungal colonies on day 60; therefore, in these cases tests on days 60 and 90 were not performed. However, in all tests performed with Syrup\_GTXAG\_50\_T<sub>100</sub> (Figure 98), a non-Newtonian,

pseudoplastic behavior (decrease in viscosity with shear rate increase) was verified, however, some variations occurred in some specific shear rate values.

In the case of samples that did not suffer contamination, it was verified that the viscosity values remained constant over the 90 days of storage.

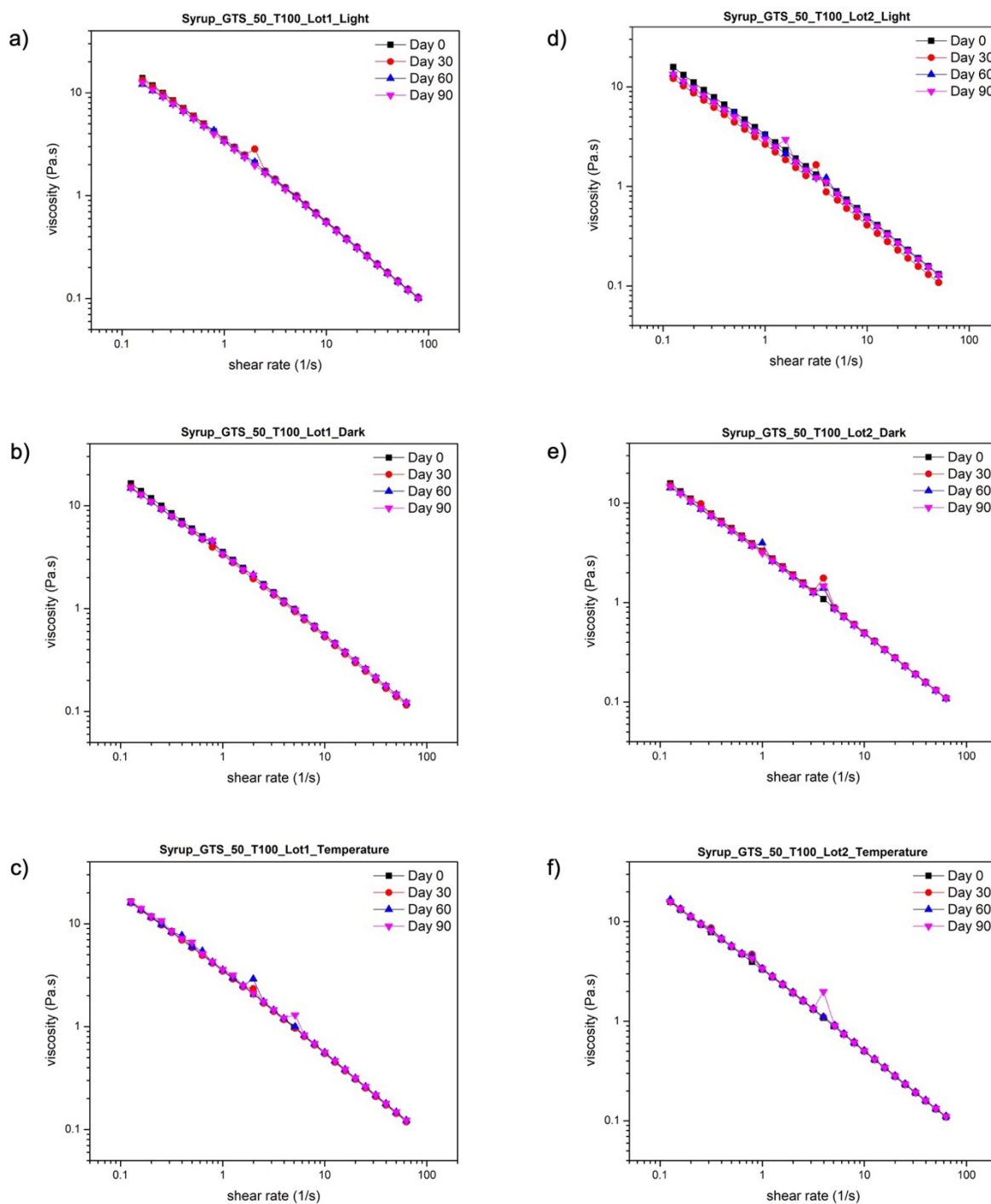


Figure 99 - Flow curves of all Syrup\_GTS\_50\_T100\_Lote1 and Syrup\_GTS\_50\_T100\_Lote2 stored 90 days in different conditions.

Similar to the Syrup\_GTXAG\_50\_T<sub>100</sub> (Figure 98), all Syrup\_GTS\_50\_T<sub>100</sub> (Figure 99) demonstrated a non-Newtonian, pseudoplastic behavior, and the viscosity values remained unchanged over 90 days of storage in different conditions; however, some differences were observed at some shear rate values. All viscosities at 0.1 1/s shear rate were recorded and shown in Table 32 for all 90 days.

Table 32 - Viscosity values of Syrup\_GTXAG\_50\_T<sub>100</sub> and Syrup\_GTS\_50\_T<sub>100</sub> stored in different conditions over 90 days.

| Sample                                            | Viscosity (Pa.s) |       |       |       |
|---------------------------------------------------|------------------|-------|-------|-------|
|                                                   | Day              |       |       |       |
|                                                   | 0                | 30    | 60    | 90    |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot1_Light       | 39.00            | 39.60 | -     | -     |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot2_Light       | 35.80            | 39.60 | -     | -     |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot1_Dark        | 39.00            | 39.18 | -     | -     |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot2_Dark        | 35.80            | 39.88 | 39.72 | 40.24 |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot1_Temperature | 39.00            | 39.97 | 40.71 | 40.58 |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot2_Temperature | 35.80            | 39.75 | 40.8  | 40.85 |
| Syrup_GTS_50_T <sub>100</sub> _Lot1_Light         | 21.46            | 19.87 | 17.53 | 18.41 |
| Syrup_GTS_50_T <sub>100</sub> _Lot2_Light         | 20.36            | 15.67 | 17.26 | 17.31 |
| Syrup_GTS_50_T <sub>100</sub> _Lot1_Dark          | 21.46            | 19.18 | 18.58 | 18.89 |
| Syrup_GTS_50_T <sub>100</sub> _Lot2_Dark          | 20.36            | 19.57 | 18.47 | 18.85 |
| Syrup_GTS_50_T <sub>100</sub> _Lot1_Temperature   | 21.46            | 20.53 | 20.67 | 21.31 |
| Syrup_GTS_50_T <sub>100</sub> _Lot2_Temperature   | 20.36            | 20.74 | 20.62 | 20.43 |

Table 32 shows that the Syrup GTXAG\_50\_T<sub>100</sub> samples showed higher viscosity values than the Syrup\_GTS\_50\_T<sub>100</sub> samples, and these values slightly increase over the time. All Syrup\_GTS\_50\_T<sub>100</sub> stored under different conditions, on the other hand, demonstrated lower viscosity values after 90 days when compared to the initial values. Overall, syrups kept their viscosities over 90 days when stored at a temperature of 4°C.

### 3.6.5. Final Review

After 90 days of stability assays under light, dark, and temperature of 4.0 °C of syrup formulations with agave syrup and stevia CNSs with GT polyphenolic compounds extracted

showed that Syrup GTXAG\_50\_T<sub>100</sub>\_Lot1\_Light, Syrup GTXAG\_50\_T<sub>100</sub>\_Lot2\_Light, and Syrup GTXAG\_50\_T<sub>100</sub>\_Lot1\_Dark showed fungal growth. It is suggested that controlled temperature is important for the preservation of syrup formulations containing agave syrup. In the case of stevia syrup formulations, all conditions reached 90 days without fungal colonies being observed.

Except for the formulations discarded due to the appearance of fungal colonies, in pH assays, all syrup formulations, with the exception of Syrup GTXAG\_50\_T<sub>100</sub>\_Lot2\_Dark, showed no significant differences when compared to the pH value measured at day 0 assays.

Table 33 was represented, for each condition, the number of compounds with a final percentage of stability between five major intervals counted: 0-30 %, 30-50 %, 50-70 %, 70-80 %, and 80-100 %, as well as the number of compounds degraded and the day that the compounds were detected as degraded.

Table 33 - Table summarizing the overall comparison of the various conditions in HPLC-DAD assays.

| Condition                                         | Degraded | Day | No. of Compound |           |           |           |            |
|---------------------------------------------------|----------|-----|-----------------|-----------|-----------|-----------|------------|
|                                                   |          |     | 0-30 (%)        | 30-50 (%) | 50-70 (%) | 70-80 (%) | 80-100 (%) |
| Syrup GTXAG_50_T <sub>100</sub> _Lot1_Light       | Yes      | 60  | -               | -         | -         | -         | -          |
| Syrup GTXAG_50_T <sub>100</sub> _Lot2_Light       | Yes      | 60  | -               | -         | -         | -         | -          |
| Syrup GTXAG_50_T <sub>100</sub> _Lot1_Dark        | Yes      | 60  | -               | -         | -         | -         | -          |
| Syrup GTXAG_50_T <sub>100</sub> _Lot2_Dark        | No       | -   | 2               | 1         | 2         | 3         | 2          |
| Syrup GTXAG_50_T <sub>100</sub> _Lot1_Temperature | No       | -   | 0               | 3         | 1         | 4         | 2          |
| Syrup GTXAG_50_T <sub>100</sub> _Lot2_Temperature | No       | -   | 0               | 2         | 2         | 4         | 2          |
| Syrup GTS_50_T <sub>100</sub> _Lot1_Light         | No       | -   | 1               | 3         | 2         | 2         | 2          |
| Syrup GTS_50_T <sub>100</sub> _Lot2_Light         | No       | -   | 1               | 2         | 3         | 2         | 2          |

| Condition                                       | Degraded | Day | No. of Compound |              |              |              |               |
|-------------------------------------------------|----------|-----|-----------------|--------------|--------------|--------------|---------------|
|                                                 |          |     | 0-30<br>(%)     | 30-50<br>(%) | 50-70<br>(%) | 70-80<br>(%) | 80-100<br>(%) |
| Syrup_GTS_50_T <sub>100</sub> _Lot1_Dark        | No       | -   | 0               | 1            | 4            | 3            | 2             |
| Syrup_GTS_50_T <sub>100</sub> _Lot2_Dark        | No       | -   | 0               | 1            | 4            | 3            | 2             |
| Syrup_GTS_50_T <sub>100</sub> _Lot1_Temperature | No       | -   | 0               | 0            | 3            | 4            | 3             |
| Syrup_GTS_50_T <sub>100</sub> _Lot2_Temperature | No       | -   | 0               | 0            | 3            | 3            | 4             |

It is possible to see in Table 33 that syrup formulations in temperature conditions demonstrates better stability results over 90 days. Besides this, formulations with stevia CNS showed better results in the compound's stability when compared to the formulations with agave syrup CNS in all conditions. Syrup\_GTS\_50\_T<sub>100</sub>\_Lot1\_Temperature and Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2\_Temperature showed stability higher than 50 % in all compounds over the 90 days, being Syrup\_GTS\_50\_T<sub>100</sub>\_Lot2\_Temperature the condition with better results.

Table 34 showed the comparison in colorimetric assays between days 0 and 90 of all syrup formulations prepared of differences in attributes and statistical significant differences of L\*, a\*, b\*, C\*, and h° parameters.

Table 34 - Table summarizing the overall comparison of the various conditions in colorimetric assays for syrup formulations with CNS with GT polyphenolic compounds extracted.

| Sample                                            | Degraded | Day | No. of parameters,<br>comparing between<br>days 0 and 60, with: |                              |
|---------------------------------------------------|----------|-----|-----------------------------------------------------------------|------------------------------|
|                                                   |          |     | Different<br>attributes                                         | Significative<br>differences |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot1_Light       | Yes      | 60  | -                                                               | -                            |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot2_Light       | Yes      | 60  | -                                                               | -                            |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot1_Dark        | Yes      | 60  | -                                                               | -                            |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot2_Dark        | No       | -   | 0                                                               | 5                            |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot1_Temperature | No       | -   | 0                                                               | 2                            |

|                                                   |          |     | No. of parameters,<br>comparing between<br>days 0 and 60, with: |                              |
|---------------------------------------------------|----------|-----|-----------------------------------------------------------------|------------------------------|
| Sample                                            | Degraded | Day | Different<br>attributes                                         | Significative<br>differences |
| Syrup_GTXAG_50_T <sub>100</sub> _Lot2_Temperature | No       | -   | 0                                                               | 3                            |
| Syrup_GTS_50_T <sub>100</sub> _Lot1_Light         | No       | -   | 0                                                               | 5                            |
| Syrup_GTS_50_T <sub>100</sub> _Lot2_Light         | No       | -   | 0                                                               | 5                            |
| Syrup_GTS_50_T <sub>100</sub> _Lot1_Dark          | No       | -   | 0                                                               | 5                            |
| Syrup_GTS_50_T <sub>100</sub> _Lot2_Dark          | No       | -   | 0                                                               | 5                            |
| Syrup_GTS_50_T <sub>100</sub> _Lot1_Temperature   | No       | -   | 0                                                               | 1                            |
| Syrup_GTS_50_T <sub>100</sub> _Lot2_Temperature   | No       | -   | 0                                                               | 4                            |

Table 34 shows that all formulations did not exhibit different color attributes after 90 days of assays. However, in statistical comparisons among the color parameters studied, the syrup formulations at 4.0 °C temperature showed fewer differences when compared to light and dark conditions.

In rheological tests, syrup formulations containing stevia at 4.0 °C temperature condition showed less variation in viscosity after 90 days when compared to the initial viscosity measured. In stevia syrup formulations, formulations under dark condition have lower viscosity variation over 90 days when compared to formulations under light condition. Syrup\_GTXAG\_50\_T<sub>100</sub>\_Lot1\_Temperature showed lower viscosity variation over the 90 days in comparison to the other syrup formulations with agave syrup.

### 3.7. *In vitro* Assays

*In vitro* assays are crucial in scientific research because they allow the investigation of a wide range of biological and physiological phenomena in a controlled laboratory setting. These methods have proven to be especially useful in studying interactions between



chemicals, drugs, and human body cells. Caco-2 cells are among the most commonly used cell lines in *in vitro* assays.<sup>149, 150</sup>

Caco-2 cells, which were originally isolated from a human colon adenocarcinoma, are widely used in *in vitro* studies as a model for intestinal cells. The gastrointestinal tract is essential for nutrient absorption, pathogen defense, and interaction with foreign substances such as drugs. Caco-2 cells, as a result, provide a valuable experimental platform for studying compound absorption, transport, and metabolism in an environment similar to the lining of the small intestine.<sup>151, 152</sup>

At the end stage of static *in vitro* simulated digestion assays, all the samples were analyzed by HPLC-DAD, and it is believed that polyphenolic compounds remained in the samples, although, some differences in the chromatographic pattern were detected.

After 21 days in the culture medium, Caco-2 cells showed a TEER value (Figure 100) of 1199.19  $\Omega \cdot \text{cm}^2$ , higher than the value described by Srinivasan B. *et al.*<sup>153</sup> for gastrointestinal tract models, and by Speer J. E. *et al.*<sup>154</sup> for *in vivo* intestine membrane values. However similar to the values described by Markell L. K. *et al.*<sup>155</sup>.

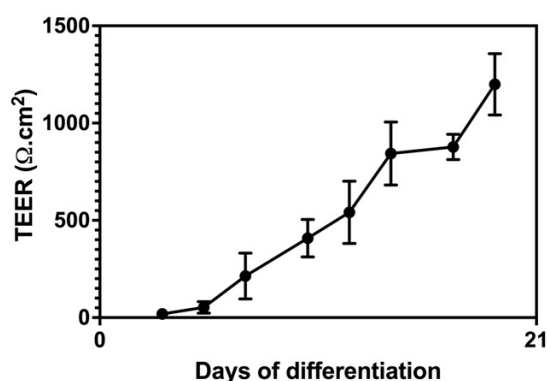


Figure 100 - TEER values of Caco-2 cell over 21 days of differentiation.

In the cytotoxic evaluation using the MTT reduction assays, the 1:10 dilution showed better results when compared to the control group for Syrup\_GTXAG\_50\_T<sub>100</sub> (Figure 101 a)), Syrup\_GTS\_50\_T<sub>100</sub> (Figure 101 b)), and water (Figure 101 c)) after the static *in vitro* simulated digestion using the Infogest protocol.

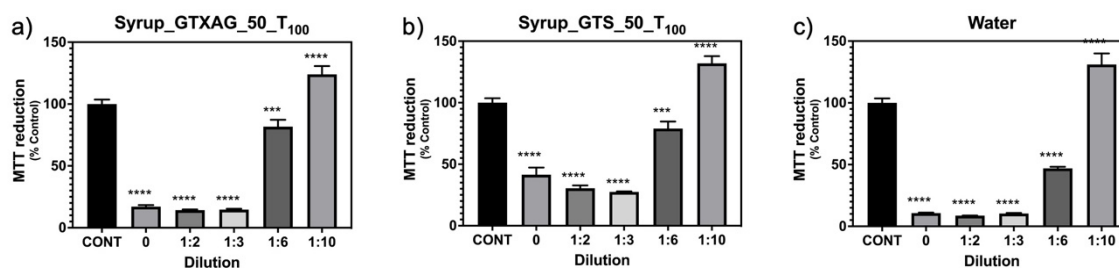


Figure 101 - Cytotoxic evaluation using the MTT reduction assays for a) Syrup\_GTXAG\_50\_T<sub>100</sub>, b) Syrup\_GTS\_50\_T<sub>100</sub>, and c) water after the static *in vitro* simulated digestion.

A second cytotoxic evaluation was performed with neutral red, and the results suggested that the 1:10 dilution did not have significant differences with the control group for Syrup\_GTXAG\_50\_T<sub>100</sub>, Syrup\_GTS\_50\_T<sub>100</sub>, and water (Figure 102) after the static *in vitro* simulated digestion. Due to these higher values in 1:10 dilution, a supplementary assay with natural red was performed to verify the viability of the results.

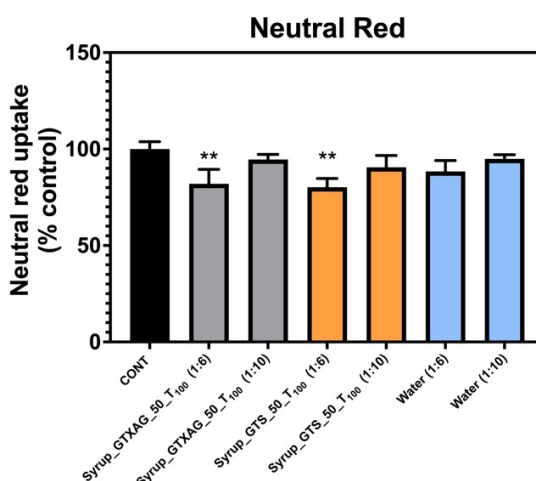


Figure 102 – Cytotoxic evaluation using the neutral red assay for Syrup\_GTXAG\_50\_T<sub>100</sub>, Syrup\_GTS\_50\_T<sub>100</sub>, and water after the static *in vitro* simulated digestion.

After identifying the necessary dilution for non-cytotoxic dose administration for the Caco-2 cells, the Syrup\_GTXAG\_50\_T<sub>100</sub>, Syrup\_GTS\_50\_T<sub>100</sub>, and water after the static *in vitro* simulated digestion were placed in the cell for 2 hours, and the TEER value for each one was verified after 2 hours (Figure 103).

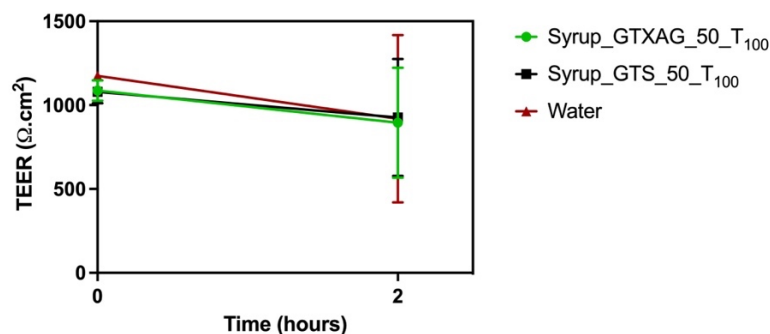


Figure 103 – Variation of TEER values of Caco-2 cell measured for Syrup\_GTXAG\_50\_T<sub>100</sub>, Syrup\_GTS\_50\_T<sub>100</sub>, and water after the static *in vitro* simulated digestion.

After 2 hours, all the samples have no significative differences in TEER values when compared to the value initially measured demonstrating that the cell line was no committed during the assay performing.

### 3.7.1. Characterization of Basolateral Samples

The basolateral samples were analyzed by HPLC-DAD and HPLC-ESI-MS to allow both quantification and identification of the compounds. However, the linear trap ESI-MS equipment was not able to provide the required ionization to allow the proper identification of all compounds found in the DAD detector. To try to overcome this problem, the samples were injected in another HPLC-ESI-MS with an orbitrap. With the analysis perform with this HPLC-ESI-Q-Orbitrap-MS equipment, it is believed that (E)GC, PD-BG, (E)C, (E)GCG, gallic acid, Quercetin-3-O-rutinoside, (E)CG, and Kaempferol 3-rutinoside (Figure 104 a), b), c), d), e), f), g), and h), respectively) have reached the basolateral site. Furthermore, since HPLC-ESI-Q-Orbitrap-MS allows the analysis using positive and negative ionization mode at the same time, it was possible to find out that caffeine reached the basolateral site.

Figure 104 shows that the majority of the compounds mentioned before, were only detected during the washing stage of the HPLC gradient used. This suggests the necessity of an adaptation of this gradient, increasing the time with the less polar organic solvent to make a better and faster separation of the compounds. In addition to that, the use of a longer chromatographic column, in other words, the increase of the number of theoretical plates can promote a better separation the compounds in solution.

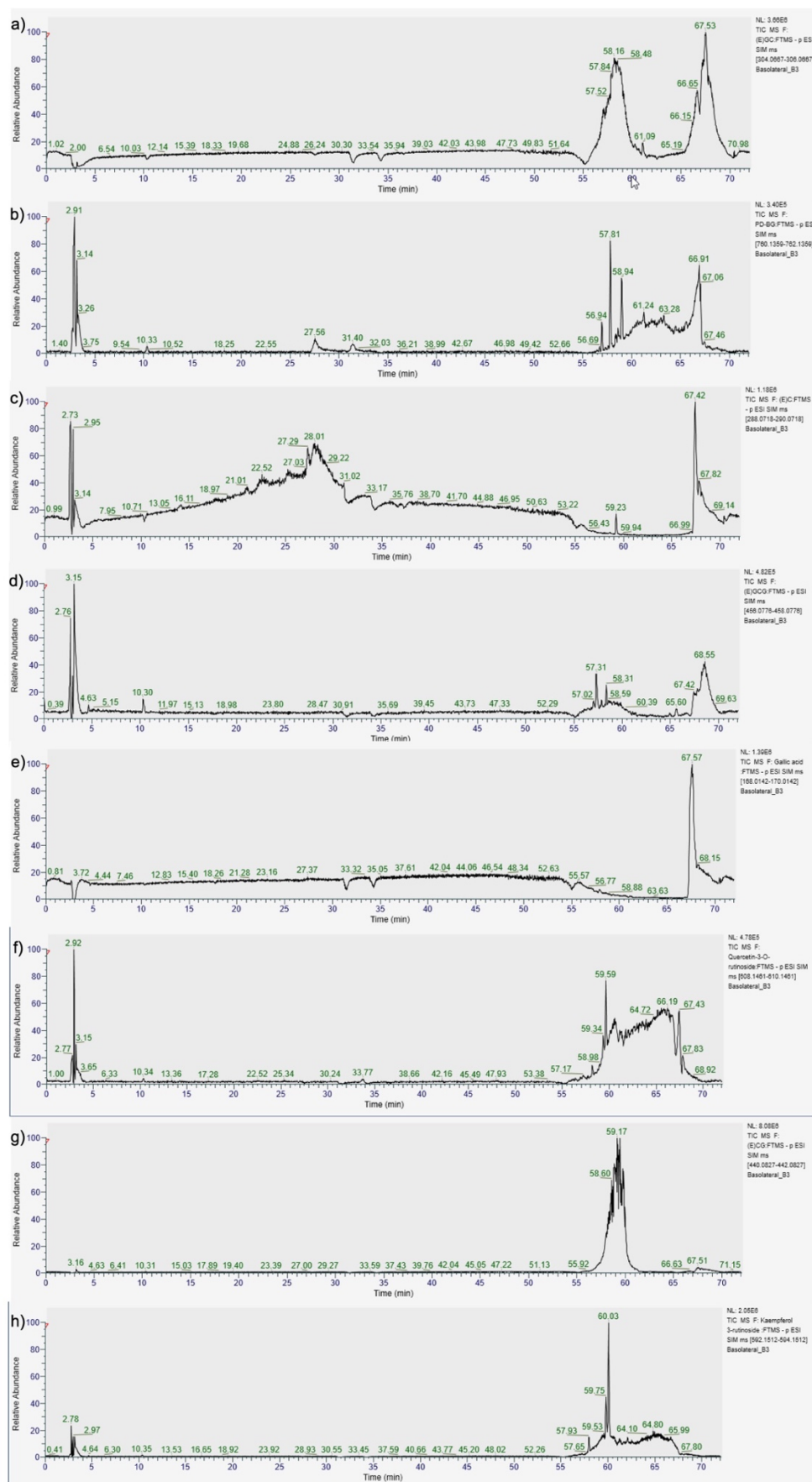


Figure 104 – Chromatograms obtained by tSIM for a) (E)GC, b) PD-BG, c) (E)C, d) (E)GCG, e) gallic acid, f) Quercetin-3-O-rutinoside, g) (E)CG, and h) Kaempferol 3-rutinoside.

Table 35 represents the values of the exact mass of each compound using PubChem online database, and the exact mass obtained by the HPLC-ESI-Q-Orbitrap-MS analysis.

Table 35 - Comparison between the exact mass of each polyphenolic compound and the corresponding mass obtained in the analysis.

| Compound                 | Exact Mass (g/mol) | Identified Mass (g/mol) |
|--------------------------|--------------------|-------------------------|
| (E)GC                    | 306.07             | 306.22                  |
| PD-BG                    | 762.62             | 762.30                  |
| (E)C                     | 290.08             | 290.91                  |
| (E)GCG                   | 458.08             | 458.69                  |
| Gallic acid              | 170.02             | 169.84                  |
| Quercetin-3-O-rutinoside | 610.15             | 610.33                  |
| (E)CG                    | 442.09             | 442.26                  |
| Kaempferol 3-rutinoside  | 594.16             | 594.40                  |

Taking into account the values of the mass identified, and the values of the exact mass of each compound, it is possible to see some differences in the decimal values. These differences may be due to the isotopic elements, leading to a pattern peak splitting corresponding to the number of isotopic elements different<sup>156</sup>, or lack of equipment calibration with proper standards.

### 3.7.2. Final Review

It is believed the possible identification of (E)GC, PD-BG, (E)C, (E)GCG, gallic acid, Quercetin-3-O-rutinoside, (E)CG, and Kaempferol 3-rutinoside in basolateral samples, demonstrating stability after the static *in vitro* simulated digestion, and capacity to pass through an *in vitro* Caco-2 cell barrier, simulating the intestinal membrane, to reach the bloodstream.

However, optimizations in the chromatographic procedure are necessary to a better understanding.



# **CHAPTER 4:**

## **CONCLUSION AND FUTURE PERSPECTIVES**





## CHAPTER 4 – CONCLUSION AND FUTURE PERSPECTIVES

This work resulted in the successful optimization and extraction of GT polyphenolic compounds directly in various ratios of CNS: agave syrup, honey, maple syrup, and stevia.

The main catechins from GT ((E)C, (E)CG, (E)GC, and (E)GCG) were successfully extracted and identified in the CNSs, as well as four flavonoids (Apigenin-6-C-pentoside-8-C-hexoside, Quercetin-deoxyhexoside-hexoside, Quercetin-3-O-rutinoside, and Kaempferol 3-rutinoside), one tannin, and phenolic acid (PD-BG and Gallic acid, respectively).

When compared to the CNS value without GT polyphenolic compounds extracted, the extractions at 100.0 °C and with 5.000 g of CNS ratio showed a higher capacity of extraction of GT polyphenolic compounds and, in most cases, a lower variation in the viscosity value. Due to the high viscosity of honey CNS, the assays could not be performed accurately. Due to its low viscosity, the stevia CNS rheologic assay requires a new evaluation with a capillary rheometer.

Considering the identification, characterization, and rheological studies for the CNS with GT polyphenolic compounds extracted, it is possible to conclude that the honey CNS exhibited high viscosity, making the assays difficult to perform. Extractions with 10.000 g of CNS ratio showed more variations in viscosity in some cases than extractions with 5.000 g of CNS ratio, as well as lower GT polyphenolic compounds. As a result, honey CNS extractions and the 10.000 g CNS ratio were discarded for formulation assays.

The stability assays for CNS with GT polyphenolic compounds extracted under aging conditions revealed that, when compared to direct exposure to light and 40 °C temperature, the extractions showed better stability in the color parameters and appearance variation and in the GT polyphenolic compounds extracted in the absence of light, being the most stable extractions with agave syrup and stevia CNSs.

Aside from optimizing MME production, a low GT polyphenolic compound content in the formulation necessitates a new evaluation of the extraction process, and due to the time required, a new oral formulation was performed.

So, successful syrup formulations using agave syrup and stevia CNSs were prepared and demonstrated that when stored at 4.0 °C, no significant statistical differences in pH values were detected after 90 days, and in colorimetric assays, no different color

attributes were detected besides the significant statistical differences in some color parameters studied. After 90 days, syrup formulations containing stevia CNS and stored at 4.0 °C showed a slight variation in viscosity. In terms of compounds stability, syrup formulations showed better stability under low temperature conditions, whereby the syrup formulations with stevia CNS showed compound stability over 50 % in all compounds after 90 days.

Overall, the stability of the parameters studied over 90 days was better under a 4.0 °C temperature condition than under light or dark conditions.

Regarding the *in vitro* studies, it is estimated the possibility to identify polyphenolic compounds in basolateral samples. However, future optimization of the chromatographic procedure is fundamental, along the use of other techniques for compound identification, such as NMR, to a better understanding of the compounds with the capacity to reach the basolateral site.

After the identification of the compounds present in the basolateral site, a quantitative analysis is required to understand their concentration. Also, in a therapeutical approach, it is important to understand if the concentration of caffeine in the basolateral site follows the legally authorized.

Another important step to be addressed is to run an intensive study of all the modifications in the compounds of the syrup samples during each static *in vitro* simulated digestion phase (oral, gastric, and intestinal), to understand the degradation promoted by enzymes and/or chemical variations. Also, the creation of a database of the products obtained in each phase, and the intestinal barrier simulated with Caco-2 cells, using standard compounds, would be crucial.

If the therapeutic concentration is achieved *in vitro*, the final goal would be to run more detailed *in vivo* tests to confirm that GT polyphenolic compounds in syrup formulations have effective therapeutic concentrations, and in which terms they can interfere with the disease symptoms.

In the case of MME formulations, a review of the extraction process will be required to try to achieve the required concentration in the formulation, possibly through non-direct extraction in the CNSs.

# **CHAPTER 5:**

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Tiago Filipe Pereira Alves  
FFUP  
2022/2023

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