Electronic Tongue Analysis of Major and Minor Steviol Glycosides and Their Application in Foods

by

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Abstract

The electronic tongue (E-tongue) is a taste-sensing analytical device that simulates the human tongue. It has been predominantly adopted in food industries as a tool for taste evaluation. Significantly, some products causing carry-over effects to human panels due to strong aftertastes, such as stevia, would need an analytical approach like E-tongue to assess tastes, especially for analysis of large numbers of samples. Stevia, a natural sweetener, contains major and minor steviol glycosides with different taste characteristics. Rebaudioside (Reb) A, the major steviol glycoside, is the most widely used in the food industry, but it provides a bitter aftertaste. Minor steviol glycosides (i.e., Reb D and M) display a similar taste profile to sugar with a significantly less bitter aftertaste, but their contents in the leaves are low. Therefore, this study examined the potential of E-tongue to find an optimal ratio between major and minor steviol glycosides to resolve both the bitter taste of Reb A and low concentrations of Reb D and M. This study verified a protocol for the E-tongue analysis with the most updated sensors for stevia samples. Also, it was found that some of the mixtures between Reb A and Reb M showed comparable taste profiles to a single steviol glycoside, Reb M. However, human panel data would be needed to confirm the findings. The second study evaluated sensory characteristics of Reb A, D, and M in ice cream using regular ice cream consumers (*n=92*) as minor steviol glycosides have been little studied for food applications. The results confirmed that these minor steviol glycosides might resolve the bitter aftertaste often associated with Reb A in food applications, and they might be able to act as sole sweeteners without affecting sensory qualities.

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Table of Contents

Abstract 2

Acknowledgments 3

List of Tables 7–8

List of Figures 9–10

List of Abbreviations 11

Chapter 1. Introduction 12–14

Chapter 2 Literature Review 15–37

2.1 E-tongue 15

2.1.1 Basic principles 16

2.1.2 General application of E-tongue 16–17

2.1.3 Types of sensors from Alpha MOS 17–18

2.1.4 E-tongue sensor array #6 and its application 18–20

2.1.5 Statistic for E-tongue analysis 20–21

2.1.6 Advantages and disadvantages of E-tongue 21

2.2 Stevia, A Natural High Intensity Sweetener 22

2.2.1 History and background 22

2.2.2 Stevia plant characteristics and plant cultivation 22–23

2.2.3 Steviol glycosides, the sweet compounds 24–25

2.2.4 Major and minor Steviol glycosides 26

2.2.5 Health benefits 26–27

2.2.6 Safety of stevia 27

2.2.7 Stevia as a sugar substitute 28

Reference 29–37

Chapter 3 Determination of sensory characteristics of mixtures of steviol glycosides using an electronic tongue 38–62

Abstract 38

1. Introduction 39–42

2. Materials and Methods 42–45

2.1 Material 42

2.2 Solution preparation 42

Part 1: Validation of the protocol for sensor array#6 for the mixtures of..... steviol glycosides 42–43

Part 2: E-tongue analysis of the mixtures of steviol glycosides (Reb A and M) At iso-sweetness equivalent to sucrose 43

2.3 Astree E-tongue 43–44

2.4 E-tongue analysis 44–45

2.5 Statistical Analysis 45

3. Results 45–56

3.1 Part 1: Validation of the protocol for sensor array#6 for the mixtures of ..... Steviol glycosides 46–51

3.2 Part 2: E-tongue analysis of the mixtures of steviol glycosides (Reb A And Reb M at iso-sweetness) 51–56

4. Discussion 56–59

5. Conclusion 59

References 60–62

Chapter 4 The Effect of Steviol Glycosides on Sensory Properties and Acceptability of Ice Cream 63–92

Abstract 63

1. Introduction 63–66

2. Materials and Methods 67–70

2.1 Materials 67

2.2 Ice cream Preparation 67–68

2.3 Panel Recruitment 68

2.4 Sample Preparation 68

2.5 Testing Procedure 69–70

2.6 Statistical Analysis 70

3. Results 70–79

3.1 Participant's Characteristics 70–73

3.2 Sensory Analysis of Ice cream 74–79

4. Discussion 79–84

5. Conclusion 85–86

References 87–92

Conclusion 93

List of Tables

Table 1 Main steviol glycosides presented in Stevia (S. rebaudiana) along with their glucose moieties structures and sweet potencies 25

Table 2 Ratios between 0.1 % (w/v) of Reb D and Reb M 43

Table 3 Ratios between 0.1 % (w/v) of Reb A and Reb M 43

Table 4 The temperature and time for (0.1 % w/v) major and minor types steviol glycosides (Reb A, D, and M) solutions to turn clear..... 45

Table 5 The relative standard deviation (%RSD) of electronic tongue sensors was obtained from samples on Day 1 (Table 1) 47

Table 6 The relative standard deviation (%RSD) of electronic tongue sensors was obtained from samples on Day 2 (Table 1) 48

Table 7 The relative standard deviation (%RSD) of electronic tongue sensors was obtained from samples on Day 3 (Table 1) 48

Table 8 The relative standard deviation (%RSD) of electronic tongue sensors was obtained from samples on Day 1 (Table 2) 52

Table 9 The relative standard deviation (%RSD) of electronic tongue sensors was obtained from samples on Day 2 (Table 2) 53

Table 10 The relative standard deviation (%RSD) of electronic tongue sensors was obtained from samples on Day 3 (Table 2) 54

Table 11 The functionality of each ingredient for ice cream formulation and caloric values. ... 68

Table 12 Demographic and socioeconomic characteristics of the consumer panelists (n=92) .. 71

Table 13 Ice cream consumption behaviors by the consumer panel (n=92)..... 72

Table 14 Familiarity in various sweeteners selected by consumer panel (n=92)….....73

Table 15 Consumption of low/zero sugar products and zero-calorie sweeteners by consumer panel (at least once a month) (n=92)……...73

Table 16 The means (± Standard Error) overall liking, the attributes liking, and the purchase intent of sucrose, Reb A, D, and M ice cream samples (n=92)…….. 74

Table 17 The means (± Standard Error) sweetness and bitterness intensities rated by the consumer panel for sucrose, Reb A, D, and M ice cream samples (n=92)…….75

Table 18 Aftertaste attributes selected by the consumer panel for sucrose, Reb A, D, and M ice cream (n=92)……76

List of Figures

Figure 1 Autosampler system of Alpha MOS Electronic Tongue 16

Figure 2 Electronic tongue (Alpha MOS, Toulouse, France) 18

Figure 3 Stevia plant and its flowers 23

Figure 4 Steviol glycosides structure (a) core structure; (b) Rebaudioside A;

(c) Rebaudioside M 25

Figure 5 The sequence for samples used for Part 1 and 2 displayed on Alpha MOS

Software: Blue indicates cleaning vial (Deionized water); Yellow indicates samples 44

Figure 6 The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 1)

from Day 1 second run. 50

Figure 7 The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 1)

from Day 2 second run. 50

Figure 8 The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 1)

from Day 3 second run. 51

Figure 9 The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 2)

from Day 1 second run. 55

Figure 10 The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 2)

from Day 2 second run 55

Figure 11 The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 2)

from Day 3 second run. 56

Figure 12 Correspondence analysis (CA) of each ice cream. Blue indicates samples;

Red indicates significant attributes; Grey indicates not significant attributes 78

Figure 13 The means overall liking (± standard error) of each ice cream between female

and male participants. Green indicates sucrose ice cream; Blue indicates Reb M ice cream; Red indicates Reb D ice cream; Black indicates Reb A ice cream..... 79

List of Abbreviations

AHC	Agglomerative Hierarchical Clustering
CA	Correspondence Analysis
CAGR	Compound Annual Growth Rate
CATA	Check-All-That-Apply analysis
DI	Discrimination Index
E-tongue	Electronic Tongue
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
PCA	Principal Component Analysis
Reb	Rebaudioside
RSD	Relative Standard Deviation
SE	Standard Error

1. Introduction

The electronic tongue (E-tongue) (Alpha MOS, Toulouse, France) is an analytical instrument that has been used to mimic the human tongues' taste perception using chemometric methods and the artificial intelligence (Coisek and Wróblewski 2011; Podrażka et al. 2017). This potentiometry E-tongue is comprised of an autosampler system (stirring rod, reference rod, and seven sensors), α-Astree (electronic unit), and computer software (Alpha MOS, Toulouse, France). When obtaining data, it measures the difference in electronic potential between the sensor and the reference electrode (Wang et al. 2021), and it will create a change in the membrane potential resulting in potentiometric measurement of the sensor (Tao 2020; Wang et al. 2021)). It has been used in product discriminations (Jung et al. 2017), pharmaceuticals to hinder the release of bitter taste molecules in drugs (Legin et al. 2004), formulation development (Lorenz et al. 2009), and determination of spoilage in foods (Paup et al. 2021), but these research articles used the old versions of E-tongue (i.e., sensor array #1, 2 or 5), some of which the manufacturer discontinued production. E-tongue sensor array #5 was one of the older versions of E-tongue sensors that Alpha MOS developed. The sensor array consisted of 7 sensors, and each was responsible for detecting a specific taste (SRS = sourness, BRS= bitterness, SWS = sweetness, UMS = umami, STS = saltiness, GPS and SPS = general purposes).

Alpha MOS launched a new version known as sensor array #6. Wang et al. (2021) used this sensory array to predict the bitterness intensity in the tablet at three different concentrations (3.33, 1.66, and 1.00 w/v %). Tao (2020) developed a protocol of E-tongue using three steviol glycosides solution (0.1 w/w % of Reb A, D, and M) and used it to discriminate stevia leaf extracts. The results showed that E-tongue successfully discriminated the three steviol glycosides and seven

stevia leaf extract samples. However, to date, no studies used E-tongue to analyze mixtures at different steviol glycoside ratios to compare the intensities of sweetness and bitterness.

Stevia (*Stevia rebaudiana*) is a plant that originated in Paraguay that has been used as a sweetening agent and dietary supplement over the decade (Carakostas et al. 2008; Lemus-Mondaca et al. 2012; Castro-Muñoz et al. 2022). Its sweet compounds, steviol glycosides, are about 150-230 times sweeter than regular table sugar with no-caloric intakes (Gibson et al. 2014; IFST 2019; Khalid et al. 2021). Stevia became commercially available in the U.S. in 2009. Since then, it has become one of the most popular natural sweeteners used by many companies to incorporate into food and beverage products (Savita et al. 2004; Ahmad et al. 2020). According to EMERGEN RESEARCH published in 2022, the stevia market in 2021 was approximately USD 650 million, and it is expected to have an annual growth rate of 8.7 % in 2028. Thus, many researchers have investigated various applications of stevia and determined sensory characteristics of stevia-sweetened products (e.g., bakery and dairy products) (Alizadeh et al. 2014a; Ahmad et al. 2020). Currently, stevia is frequently combined with sugar and/or other high-intensity sweeteners such as erythritol in food products as the mixtures of stevia and other sweeteners were more acceptable to consumers than sole stevia due to its strong bitter aftertaste, especially in high sugar food products such as ice cream and frozen desserts (Alizadeh et al. 2014a; Alizadeh 2014; Li et al. 2015).

In the stevia leaves, the two major steviol glycosides are stevioside and rebaudioside (Reb) A, and these are most widely used in the food industry. However, negative aftertastes (i.e., bitter and licorice aftertastes) have been found in food products sweetened with stevia (Prakash Chaturvedula et al. 2011; Gwak et al. 2012; Kim et al. 2015). Researchers have investigated other types of steviol glycosides found in the leaves to address the undesirable aftertaste of the major steviol glycosides. Several studies have proven that minor glycosides (e.g., Reb D or Reb M)

provide better sensory characteristics than Reb A (Prakash et al. 2014; Watson 2015; Neuwirth 2020; Tao and Cho 2020) and have more similar taste profiles to sugar (Prakash et al. 2014). These minor glycosides are called next-generation premium stevia sweeteners because of their superior taste quality (Prakash et al. 2014; Olsson et al. 2016). However, to the authors' knowledge, there is no research that compares sensory characteristics of food products that are sweetened with major and minor steviol glycosides in high-sugar food applications.

Thus, the objectives of this research are to:

- Validate protocol for the E-tongue with sensor array #6, the most recently updated version of sensors to analyze mixtures of steviol glycosides (Chapter 3).
- Determine the discrimination ability of E-tongue for mixtures of steviol glycosides and steviol glycosides at the iso-sweet equivalent to 5 % sucrose (Chapter 3).
- Evaluate the preference between the major steviol glycoside (Reb A) and the minor steviol glycosides (Reb D and M) in a high-sugar product application using a consumer panel (Chapter 4).
- Investigate sensory characteristics of ice creams sweetened with Reb A, D, and M (Chapter 4).

2. Literature Review

2.1 Electronic Tongue

2.1.1 Basic principles

The electronic tongue (E-tongue) is an analytical instrument compromised of sensor arrays capable of detecting non-volatile compounds in liquid replicants with human taste. Well-known companies that manufacture electronic tongues are SA402B and TS-5000Z (Intelligent Sensor Technology Inc., Atsugi-shi, Kanagawa, Japan), The Multiarray Chemical Sensor (McScience Inc., Suwon, Korea), Sensor System (St. Petersburg, Russia), and Astree II (Alpha MOS, Toulouse, France) (Podrażka et al. 2017). The common types of E-tongues include potentiometry, voltammetry, and impedance spectroscopy (del Valle 2017), with voltammetry and potentiometry as the most common types. The voltammetry electronic tongue comprises a working electrode and one reference electrode. When measuring samples, the voltage is applied to the working electrode, which causes the current to generate due to the reduction and oxidization of the analytes (Winquist 2008; Tan and Xu 2020). The potentiometry electronic tongue (e.g., Alpha MOS Electronic tongue) is composed of an autosampler system (seven sensors, a reference electrode, a stirring rod) (Figure 1), an electronic unit (α-Astree), and computer software for statistical analysis (Figure 2). The electrode is measured when the equilibrium state is reached (Winquist 2008). When analyzing the solutions, these sensors detect taste molecules, measure differences in voltage between the sensor membrane and the reference electrode, and then send electric signals (potentiometric measurement) to the computer (del Valle 2017; Jiang et al. 2018).

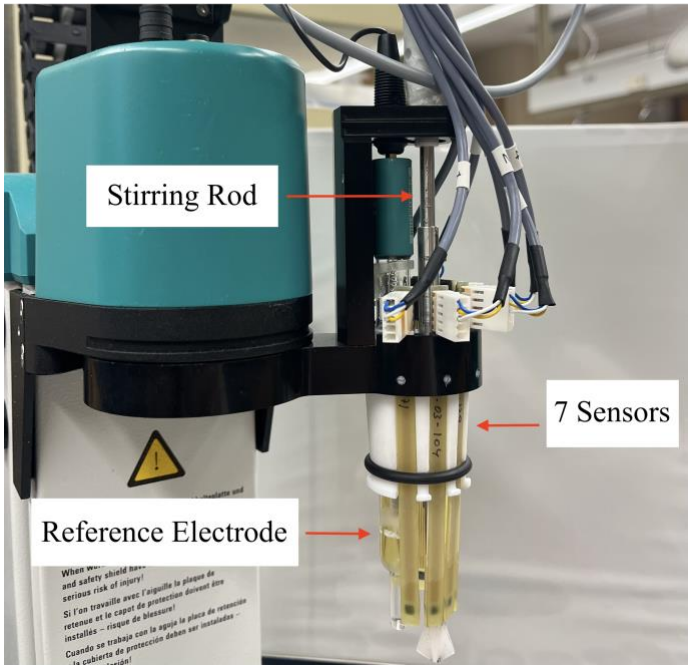


Figure 1. Autosampler system of Alpha MOS Electronic Tongue

2.1.2. General application of E-tongue

The emergence of E-tongue has caught the attention of many industries, including the food and beverage industry, and it has been a promising easy-to-handle tool for different applications. This novel instrument can be a replacement for a human-trained panel as it provides results in a rapid time frame at a low cost, is capable of handling process control at an industrial scale, and is essential for analyzing toxic/hazard samples (Podrażka et al. 2017). At first, E-tongue was mainly used in the food and beverage industry (Latha and Lakshmi 2012) to quantify the taste intensity level (Habara et al. 2004; Fujita et al. 2010; Titova and Nachev 2020), to differentiate product samples (e.g., brands, cultivars) (Bett-Garber et al. 2001; Beullens et al. 2008; Blanco et al. 2015; Śliwińska et al. 2016; Huang et al. 2017; Lasekan and Hussein 2018) and to detect food spoilage (Winquist et al. 1998; Sim et al. 2003). This tool was later utilized in other fields, such as pharmaceuticals (Di Natale et al. 2000), as it could evaluate taste-masking efficiency in tablets,

analyze the stability of medicine, and evaluate the taste of drugs (Legin et al. 2004; Baldwin et al. 2011; Latha and Lakshmi 2012).

2.1.3. Types of sensors from Alpha MOS

The potentiometry E-tongue was developed by Alpha MOS (Toulouse, France), and the company has developed four sensor arrays (#1, 2, 5, and 6) (Baldwin et al. 2011; Alpha MOS). The sensor arrays include 7 sensors that detect taste molecules (Figure 2). From previous studies, it was found that sensor arrays #1 and #5 were mainly used for food and beverage products as well as food spoilage detection (Beullens et al. 2008; Rudnitskaya et al. 2009; Apetrei et al. 2010), while sensor array #2 was mainly used for pharmaceuticals. Sensor #1 consisted of sensors ZZ, BA, BB, CA, GA, HA, and JB (Alpha MOS) and sensor array #2 consisted of ZZ, AB, BA, BB, CA, DA, and JE. Although sensor arrays #1 and #2 were applied in different fields, their protocols were similar to each other. These sensor arrays were mainly used to discriminate samples from different taste intensity levels or samples from different varieties (Bett-Garber et al. 2001; Baldwin et al. 2011; Xu et al. 2013; Choi et al. 2014; Huang et al. 2017; Lasekan and Hussein 2018). On the other hand, sensor array #5 contained SRS, BRS, SWS, UMS, STS, GPS, and SPS. Each of the sensors was responsible for specific tastes, which were sourness, bitterness, sweetness, umami, and saltiness, respectively. GPS and SPS served as calibration sensors (Alpha MOS). These sensor arrays were used to predict the taste profiles of food samples without correlating with human panel data. However, some of these sensor arrays (e.g., sensor array #5) were discontinued by the manufacturer, and the most recently developed sensor array #6 replaced these older versions (Tao, 2020). This array #6 consists of AHS, PKS, CTS, NMS, CPS, ANS, and SCS sensors.

However, Tao (2020) found there was no specific sensor that was responsible for detecting particular tastes such as sweetness or bitterness. Also, the author removed one of the sensors (CTS)

for stevia samples due to its poor discrimination power. Hence, the author recommended that the E-tongue sensor data should be correlated with human panel data to identify specific taste profiles.

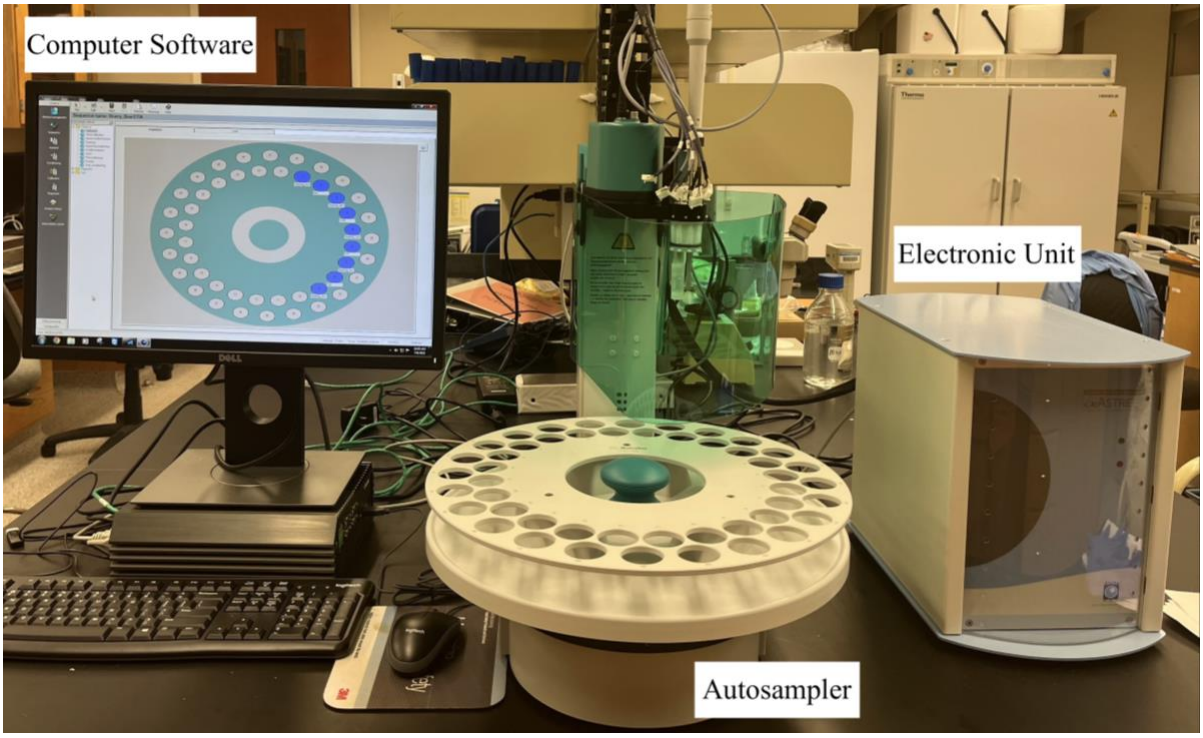


Figure 2. Electronic tongue (Alpha MOS, Toulouse, France)

2.1.4 E-tongue sensor array #6 and its application

To the authors' knowledge, there are only three articles (Tao, 2020; Wang et al. 2021; Zhu et al. 2022) using Alpha MOS E-tongue with sensor array #6 while most other studies using Alpha MOS were with the previous sensor arrays (#1, #2 or #5). In the most recent study by Zhu et al. (2022), the authors claimed AHS, CTS, NMS, ANS, and SCS specifically responded to sour, salty, umami, sweet and bitter tastes, respectively, while PKS and CPS worked as general purpose. Zhou et al. (2022) adopted E-tongue to explore relationships between umami intensity and umami sensor from E-tongue in 14 different Chinese commonly consumed food. All solid food samples were homogenized, centrifuged, and diluted if the samples were at high concentrations. All samples were run in seven replicates at room temperature and were run for three consecutive days. The

authors claimed umami flavor is correlated to sensor NMS. However, the NMS sensor was negatively correlated with trained panelist data (i.e., perceived umami intensity) and equivalent umami concentration. Other than NMS, this study could indicate that each sensor may measure multiple taste profiles.

Another study was conducted by Wang et al. (2021). The authors claimed only three of seven sensors were responsible for specific tastes (AHS = sourness, CTS = saltiness, and NMS = umami), while PKS, CPS, ANS, and SCS were general-purpose sensors. Wang et al. (2021) demonstrated the use of E-tongue as a tool to detect bitter taste-making for instant-dissolve tablets. The authors acquired three different concentrations of levetiracetam (3 g, 1.5 g, and 0.9 g) in 90 mL of water. Each sample was measured three times with no sensor removal. The results showed that E-tongue was able to discriminate between three samples and was able to identify different concentrations.

There is another study that used sensor array #6 for the stevia samples (Tao 2020), where the author claimed, each sensor was used to measure multiple tastes. The author used E-tongue as a tool for a testing profile in three steviol glycosides solutions (0.1% w/v of Rebuadioside (Reb) A, D, and M). The samples were run for six trials (two runs per day for three consecutive days). The first two and the last data were removed, so only three runs were used (#3, 4, and 5). When analyzing data from E-tongue sensor array #6, the author suggested that two values should be considered to monitor the sensor performance: relative standard deviation (%RSD) and discrimination power of the sensors. These values are crucial to determine the precision of the data. %RSD is measured by $(\frac{\text{Standard Deviation}}{\text{Mean}} \times 100)$, in which a value of 5 % or below was considered good. The discrimination power indicated the discrimination ability of the sensors on samples which ranged from 0 to 1. A number closer to 1 meant that the sensor could separate the

samples. The results showed that E-tongue was able to discriminate three steviol glycosides samples successfully. The second data of each day was found to be more acceptable than the first data. Moreover, Tao (2020) suggested removing sensor CTS when evaluating stevia leaf as it had a poor discrimination power. From the three studies above, there is no general protocol for sensor array#6 as the three articles use slightly different protocols. Thus, it is important to develop and validate a protocol before using the Alpha MOS E-tongue with sensor array #6.

2.1.5 Statistics for E-tongue analysis

Alpha MOS E-tongue software processes the E-tongue data based on multivariate analysis to provide analytic results from more than one outcome variable to conduct pattern recognition (Tan and Xu 2020; Zaukuu et al. 2020). Principal component analysis (PCA) is one of the multivariate analyses E-tongue provides. It performs a linear transformation and reduces the dimensionality into two-dimensional space (Wang et al. 2021; Zaukuu et al. 2020). It is widely used as a primary classification technique (Tan and Xu 2020). This allows easy and comprehensive analysis as it visually shows the pattern of products, highlighting the differences and similarities of the products.

Clustering analysis is another important method for pattern recognition and machine learning (Zhou et al. 2017)**.** Many researchers have used clustering methods to group the same food product but differ in concentration, ingredients, and cultivars (Bett-Garber et al. 2001; Huang et al. 2017; Lasekan and Hussein 2018). There are two main clustering methods which are partitional and hierarchical. Partitional clustering organizes patterns into a small number of clusters (Fred and Leitão 2000), while hierarchical clustering nests data partitions in a hierarchical structure in which the output presents in a dendrogram (a tree-like diagram) (Fred and Leitão 2000)**.** Agglomerative hierarchical clustering (AHC) is a subpart of hierarchical clustering, and it could

offer more clustering results than partitional algorithms (Zhou et al. 2017). PCA and AHC are found to summarize and explain large datasets statistically and visually.

2.1.6 Advantages and disadvantages of E-tongue

Humans, as measuring instruments, are quite variable over time, inconsistent among themselves, and are highly prone to bias (Meilgaard et al. 2016). Moreover, human panels experience sensory fatigue easily and thus, need to take a break (30 – 45 s between samples for a consumer panel) after evaluating four or five samples (Lucak and Delwiche 2009). Therefore, researchers have been looking for alternative methods to replace sensory evaluation with human panels. For example, E-tongue mimics the human tongue to analyze tastes. It is considered a low-cost tool, easy to handle, and able to measure the samples in a rapid time (Legin et al. 2004; Gallardo et al. 2005; Bataller et al. 2012). It also provides higher selectivity and lower detection limits than the human panel (Baldwin et al. 2011).

One of the disadvantages of E-tongue is that it can only analyze product samples in the liquid phase because the electrodes need to be washed with solvents to minimize the effect (Ciosek and Wróblewski 2011). Hence, when analyzing a solid product, additional steps are required to transform it into a liquid sample. These sensors are also sensitive to temperature, and the manufacturer recommends keeping samples at the room temperature. Another limitation found by Rudnitskaya (2018) was that the sensors could be prone to adsorbing specific components after signal acquisition, causing interference in the data acquisition of other samples. Moreover, aside from sensory array #5, other papers used E-tongue to correlate with other detection methods such as (high-performance liquid chromatography or gas chromatography-mass spectrometry) or corresponded data with the consumer panel.

2.2 Stevia, A Natural High-Intensity Sweetener

2.2.1 History and background

Stevia (*Stevia rebaudiana*) is a native South American plant from northeast Paraguay. Leaves of stevia contain sweet compounds known as steviol glycosides which were determined in 1952 (Lemus-Mondaca et al. 2012). Stevioside and rebaudioside A (Reb A) are the most abundant steviol glycosides found in stevia leaves which provide about 200 – 300 times sweeter taste than regular table sugar (Goyal et al. 2010; Kumar et al. 2011). Stevia has been used as a natural sweetening agent and medicine in many countries for decades, including Japan, Brazil, and Paraguay (Koyama et al. 2003). Japan is the most notable country in Asia to adopt stevia since the 1970s as a replacement for saccharin (Brouns et al. 2012). The use of stevia has gained popularity as a natural zero-calorie sweetener and was approved in the U.S. by the Food and Drug Administration (FDA) in 2008 and approved in Europe by the European Union in 2011 (Libik-Konieczny et al. 2021).

2.2.2 Stevia plant characteristics and plant cultivation

The stevia plant is a shrub that belongs to the sunflower family (*Asteraceae*) (Figure 3). It can grow up to 1 m tall with the leaves serrated growing opposite along the stems (Kumar et al. 2011; Le Bihan et al. 2020). Stevia grows in sub-tropical climates but does not tolerate cold weather (below 9 °C) (Lemus-Mondaca et al. 2012). It grows during spring and summer, making them an annual or biannual crop, and can be used in rotation with other plants. The leaf harvest starts before the production of flowers because the glycoside content in the leaves decreases after flowering (Huber and Wehner 2021).

Currently, stevia is mainly cultivated in South America and Asia, but production has been expanded to other countries around the world, including Canada and the U.S (Sivaram and

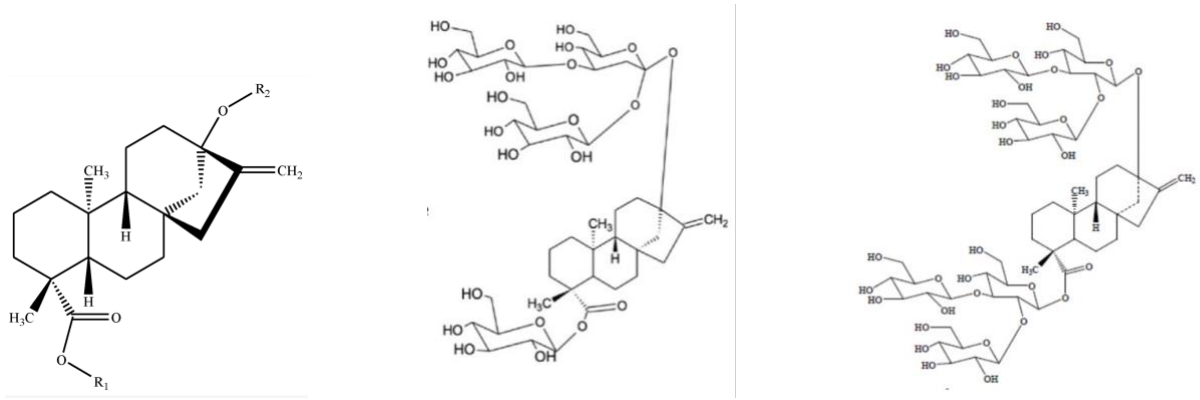
Mukundan 2003; Megeji et al. 2005). Since it was approved in 2008 in the U.S., farmers and ingredient companies have started crop cultivation in the southeastern part as a crop rotation with tobacco because they share some of the same technology and equipment (Koehler 2018). Stevia is seeded and grown in the float tray, which is also used for tobacco production, and then transplanted to the field (Shew 2012). Tobacco was an important crop produced in the Southern U.S. (Novotny et al. 2015), but the decline of the market has reduced the profits of farmers as well as the economy (Bialous and Glantz 2018). Hence, stevia has brought attention to these farmers and also the stevia industry. The first project began in Bertie County, North Carolina in 2011. Initially, the development of producing stevia with a higher content of Reb A was the primary aim of plant breeders (Yadav et al. 2011) because highly purified Reb A was found to have a better taste quality than stevia extracts. In 2017, PureCircle, the world's leading producer of stevia sweeteners, introduced the Starleaf project, which aims to cultivate stevia to contain a more desirable taste of steviol glycosides (e.g., Reb M) (Shoup 2018).

Figure 3. Stevia plant and its flowers
Source: Petruzzello, 2022 (Link: https://www.britannica.com/plant/stevia-plant)

2.2.3 Steviol glycosides, the sweet compounds

Dried leaves of stevia contain about 7 – 15 % steviol glycosides (Carakostas et al. 2012). There are about 40 different steviol glycosides that have been identified (Purkayastha et al. 2016; Samuel et al. 2018; EFSA Panel on Food Additives and Flavourings (FAF) et al. 2020). Each of the steviol glycosides shows a distinct sweet taste profile due to the different chemical structures (Purkayastha et al. 2016). The steviol glycoside structure has a steviol core in the center, and different numbers and types of glucose moieties (as well as different types of linkage) are attached to C-13 and C-19 positions of the steviol (Purkayastha et al. 2016; Samuel et al. 2018; Libik-Konieczny et al. 2021) (Figure 4 [a]) (Table 1).

Stevioside (100 – 270 times sweeter than sugar) and Reb A (150 – 320 times sweeter than sugar) (Figure 4b) are the most abundant steviol glycosides, which are found in dried stevia leaves at around 4 – 13 % and 2 – 4 %, respectively (Table 1) (Goyal et al. 2010; Samuel et al. 2018; Peteliuk et al. 2021). Although stevioside and Reb A are the main sweet compounds, recent research has focused on the minor steviol glycosides such as Reb D and M (Figure 4 [c]) (Prakash Chaturvedula et al. 2011; Prakash et al. 2014). The minor steviol glycosides, Reb D and M, are both 200 – 300 times sweeter than sugar but are found in only about 0.1 – 0.2 % (Prakash et al. 2014; Neuwirth 2020). These minor steviol glycosides have more glucose moieties attached to the steviol core than Reb A, providing more sweetness potency and less bitterness aftertaste than the major steviol glycosides.



(a) (b) (c)

Figure 4. Steviol glycosides structures (a) core structure; (b) Rebuadioside A; (c) Rebaudioside M

Source: Prakash et al. (2014) (Link: https://doi.org/10.3390/foods3010162); Peteliuk et al. (2021) (Link: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8600158/); Momentazi et al. (2017) (Link: https://www.ingentaconnect.com/content/ben/cpd/2017/00000023/00000011/art00006)

Table 1. Main steviol glycosides presented in Stevia *(S. rebaudiana)* along with their glucose moieties structures and sweet potencies.

Steviol glycoside	Rgroup at C19	Rgroup at C13	Sweet Potency	Content in dried leaf (%w/w)
Stevioside	β-glc-	β-glc-β-glc-	150 – 300	4 – 13
Rebaudioside A	β-glc-	$(\beta\text{-glc})_2$-β-glc-	200 – 400	2 – 4
Rebaudioside B	H	$(\beta\text{-glc})_2$-β-glc-	30 – 150	< 0.3
Rebaudioside C	β-glc-	(β-glc, α-rha-)-β-glc-	50 – 120	1 – 2
Rebaudioside D	β-glc-β-glc-	$(\beta\text{-glc})_2$-β-glc-	200 – 300	< 0.3
Rebaudioside E	β-glc-β-glc-	β-glc-β-glc-	200	< 0.3
Rebaudioside F	β-glc	(β-glc, β-xyl)-β-glc-	200	< 0.3
Rebaudioside M	$(\beta\text{-glc})_2$-β-glc-	$(\beta\text{-glc})_2$-β-glc-	250	< 0.3
Steviolbioside	H	β-glc-β-glc-	90 – 125	N/A
Rubusoside	β-glc-	β-glc-	110 – 114	N/A
Dulcoside A	β-glc-	α-rha-β-glc-	30 – 85	0.4 – 0.7

glc = glucose; rha = rhamnose; xyl = xylose;
Source: Prakash et al. 2014; Ashwell 2015; Samuel et al. 2018; Németh and Jánosi 2019; Purkayastha and Kwok 2020

2.2.4 Major and minor steviol glycosides

Due to more desirable taste profiles than the major steviol glycosides (i.e., stevioside and Reb A), researchers have investigated other minor steviol glycosides (i.e., Reb D and Reb M) despite presenting in small quantities. Both Reb D and Reb M provide similar sweetness properties to sucrose as compared to the major steviol glycosides (Watson 2015). They are found to bring less astringency and bitterness taste attributes than Reb A at the same concentration (Allen et al. 2013; Prakash et al. 2014), making them more interested in the food and beverages industries as well as research areas. Tao and Cho (2020) used a consumer panel to evaluate the sensory characteristics of Reb A, D, and M at the same concentration and compared them with sucrose as a control. Both minor steviol glycosides and sucrose solutions reported no significant differences in the in-mouth sweetness and bitterness, while Reb A solution had the lowest intensity of sweetness and the highest intensity of bitterness among the samples. Due to the fact that the minor glycosides had better taste, there has been great interest in increasing the concentrations of these minor glycosides in stevia leaves and developing improved stevia varieties (Watson 2015; Shoup 2018).

2.2.5 Health benefits

Stevia provides zero-caloric intake. When consuming stevia, the steviol glycoside compounds hydrolyze into steviol and quickly release glucose moieties to be utilized as an energy source (Samuel et al. 2018). Steviol is then excreted in this form through urine and/or feces (Atteh et al. 2011). Stevia has been found to provide health benefits including reduced blood sugar levels (Anton et al. 2010; Hazali et al. 2014) and a decreased risk of dental caries (de Slavutzky 2010; Basu 2014). It is also recommended for diabetics to consume as a replacement for sugar (Ashwell 2015; Marcinek and Krejpcio 2016).

Although the consumption of stevia is safe for everyone, the European Food Safety Agency (EFSA) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established the acceptable daily intake (ADI) of steviol glycosides of 4 mg or kg of body weight per day (Carakostas et al. 2012; Gupta et al. 2017; EFSA Panel on Food Additives and Flavourings (FAF) et al. 2020).

Aside from using stevia as a food additive, stevia has also been used in the pharmaceutical industry as it provides many therapeutic properties (Chatsudthipong and Muanprasat 2009; Marcinek and Krejpcio 2016) due to the other constituents of the leaves such as minerals, vitamins, phenolic compounds, and alkaloids (Christaki et al. 2013; Khalid et al. 2021). Previous studies have found that stevia has anticancer activity on breast cancer cells (Khare and Chandra 2019), significantly reduces total cholesterol (Hossain et al. 2011; Brijesh et al. 2016), and plays an important role in preventing liver cirrhosis in rats (Sudha et al. 2017).

2.2.6 Safety of stevia

Despite the health benefits stevia offers, the safety of stevia still should be considered because steviol glycosides are poorly absorbed both in humans and rats (Carakostas et al. 2008; Ahmad et al. 2020; Han 2020). So far, researchers have not found any negative correlations between health and stevia consumption, but the ADI of stevia should still be considered. Moreover, many countries only accept high-purity stevia extracts to be used as a food additive in food and beverages (Samuel et al. 2018; Castro-Muñoz et al. 2022). For instance, in Europe, the European Union (EU) approved stevia extracts for not less than 95 % of steviol glycosides (EU, 2011), and in the U.S., stevia was approved as generally recognized as safe (GRAS) for 95 % of higher purified steviol glycosides extract in 2008 (FDA, 2008).

2.2.7 Stevia as a sugar substitute

Sweeteners are one of the most important ingredients in the food industry (Kim et al. 2017). However, consumers have become more health conscious and concerned about the amount of sugar consumed with the products they regularly consume because they are more aware that a high intake of sugar has caused several health problems, including obesity (Anton et al. 2010; Drouin-Chartier et al. 2019). Pawar et al. (2013) claimed continued consumer demand for low/zero-calorie sweeteners would be expected to increase more development of natural sweeteners. Furthermore, Roman et al. (2017) highlighted the concept of naturalness which played an important factor for consumers. Thus, there has been a trend to replace sucrose in food products with non-nutritive sweeteners, particularly from natural sources (Luo et al. 2019; Ahmad et al. 2020). Due to the trend, stevia has gained popularity among other artificial high-intensity sweeteners (e.g., saccharin, aspartame) as it is natural. Many studies continue to explore ways to use stevia-based sweeteners and investigate their sensory characteristics, including beverages (Goyal et al. 2010; Bordi et al. 2016), dairy products (Alizadeh et al. 2014b; Narayanan et al. 2014), and cakes (Bijarnia et al. 2017).

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Chapter 3

Determination of Sensory Characteristics of Mixtures of Steviol Glycosides Using Electronic Tongue

Abstract

Many industries use an electronic tongue (E-tongue) for taste evaluation. Especially the food industry adopted this novel technology to detect food spoilage and assess tastes in a rapid timeframe. There has been an effort to improve the taste quality of stevia by reducing bitter aftertaste from major steviol glycosides (rebaudioside [Reb] A and stevioside) and producing a similar taste profile to sugar. Minor steviol glycosides (Reb D and M) have been reported to have faster sweetness on-set without the significant bitter aftertaste. However, the concentrations of the Reb D and M are so low, making the production cost extremely high. Therefore, there has been an increasing interest in determining an optimal ratio between major and minor steviol glycosides to have a better-tasting stevia product at a reasonable production cost. In this study, E-tongue was used to assess the tastes of steviol glycosides and their mixtures instead of descriptive sensory panels to save time and money. This study first validated the protocol for E-tongue sensor array #6 (the most updated version of the sensors) developed in the previous study that the data from second run obtained from each day provided the most useful information. The %RSD was lower than in the first run, and the discrimination power was more homogeneous. This study also confirmed that E-tongue could be used as a quick tool to discriminate and group the different ratios of the steviol glycoside mixtures. However, human assessors are still needed to find an optimal ratio based on their preferences.

1. Introduction

Humans can detect five main basic tastes (i.e., bitter, sweet, salty, sour, and umami); many researchers and the food industry have practiced the use of a human sensory panel (trained or untrained) for taste evaluations on many food products (Jiang et al. 2018). However, there are some disadvantages to using human panels as they may introduce biases, can be expensive, and can be affected by flavor carryover and sensory fatigue (Meilgaard et al. 2016; Paup et al. 2019). Therefore, much effort has been made to develop instruments and devices to replace human sensory panels, especially in routine analysis or evaluation of a large number of samples (Wang and Liu 2019). One of the novel technologies that can resolve the issues with human panels is E-tongue.

The E-tongue is an intelligent instrument that was developed to mimic the human tongue to analyze the tastes of food products in liquid form. It has been used to discriminate and quantify a wide range of products, including foods (Sim et al. 2003; Beullens et al. 2008; Baldwin et al. 2011; Ciosek and Wróblewski 2011; Blanco et al. 2015) and pharmaceuticals (Baldwin et al. 2011; Choi et al. 2014; Wang et al. 2021). Alpha MOS (Toulouse, France) is one of the leading companies in the world that manufacture potentiometric E-tongue, which has the most common type of chemical sensor. Alpha MOS E-tongue consists of an autosampler system (stirring rod, reference rod, and sensor array), α-Astree (electronic sensor unit [mV]), and computer software (Alpha MOS). When obtaining data, it measures the difference in voltage between the sensor membrane and the reference electrode. It will then send electric signals to the computer software (Tao 2020; Wang et al. 2021). The sensor array consists of seven sensors, and each sensor has different coated membranes enabling each sensor to have different selectivity and sensitivity (Alpha MOS, 2020). Alpha MOS has manufactured several different versions of the sensor arrays

(#1, 2, 5, and 6). Each version of the sensor array has its unique applications. For example, it was found that sensor arrays #1 and #5 were mainly used for food and beverage products as well as food spoilage detection, while sensor array #2 was used primarily for pharmaceuticals.

Sensory array #6 is a relatively new developed E-tongue in the field since it has been only a few years in the market. Only a very limited number of studies used the new sensor to determine the taste profiles of products (Tao 2020; Wang et al. 2021; Zhu et al. 2022). Tao (2020) developed a protocol for sensor array #6 to determine if E-tongue could discriminate different types of steviol glycosides at the same concentration (0.1 w/v %) and stevia leaf extracts from different irrigation systems. The author found that E-tongue could quickly discriminate these stevia samples, which could be used to separate the taste profiles for the breeding program as it produced repeatable data for the stevia sample. However, to use this type of novel technology, there are some challenges to be addressed before applying it to real-world problems, as little research has been done: 1) Protocols for E-tongue should be developed and validated for each type of product because the sensors have different selectivity and sensitivity, depending on product matrix; 2) E-tongue can only analyze liquid solutions; and 3) E-tongue itself cannot give much information other than classification, especially with the sensory array #6 unless it is compared or correlated with other methods (e.g., consumer panel and detection methods) (Tao 2020). However, many researchers believe that E-tongue still has the potential to replace human panels (Legin et al. 2003; Gallardo et al. 2005; Bataller et al. 2012), and it can be an alternative method for analyzing taste profiles of products, especially with strong carry-over effects due to significant aftertastes such as stevia.

Stevia (*Stevia rebaudiana*) is a plant that has been referred to as the sweet leaf of Paraguay, sweet herb, candy leaf, and honey yerba (Ranjan et al. 2011) due to its high-intensity sweet taste profile (200 – 350 times sweeter than sugar)(Carakostas et al. 2008; Lemus-Mondaca et al. 2012;

Prakash et al. 2014; Ahmad et al. 2020). Its sweet compounds are known as steviol glycosides (Carakostas et al. 2008; Lemus-Mondaca et al. 2012). To date, there are over 40 different types of steviol glycosides that have been discovered (Purkayastha et al. 2016; EFSA Panel on Food Additives and Flavourings (FAF) et al. 2020). The predominant types of steviol glycosides are stevioside and rebaudioside A (Reb A) (Carakostas et al. 2008). Reb A was initially found to have a better sweet taste than stevioside, making it the first commercial steviol glycoside launched in the marketplace (Neuwirth 2020). However, both stevioside and Reb A were later found to exhibit bitter and licorice off-taste (Gwak et al. 2012; Hellfritsch et al. 2012; Kim et al. 2015), which has become a challenge for food industries. Hence, researchers have been studying the minor type of steviol glycosides (i.e., Reb D and M) as they have higher sweetness intensity (up to 350 times sweeter) and provide less bitter aftertaste than other steviol glycosides (Hellfritsch et al. 2012; Prakash et al. 2014; Neuwirth 2020). Many findings have shown that minor steviol glycosides (Reb D and M) provide better sensory characteristics and are more similar in taste profile to sugar (Allen et al. 2013; Prakash et al. 2014; Tao and Cho 2020). However, these minor steviol glycosides naturally occur in small concentrations (0.01 – 0.02 % [w/w]), making their cost higher. According to Research and Market in 2022, the stevia market is projected to reach USD 965.82 million by 2028, and its compound annual growth rate (CAGR) is expected to grow at 8.7 % from 2021 to 2028. Due to the increase in demand for stevia production, researchers are seeking ways to develop the best-tasting stevia that minizine off-flavor (i.e., licorice, lingering, and bitter) as taste evaluation is very important in the foods (Kobayashi et al. 2010). One of the strategies to develop better-tasting stevia products would be the development of optimal ratios of different types of steviol glycosides by characterizing taste profiles of a wide range of mixtures of steviol glycosides using both human panels and instruments (e.g., E-tongue). In addition, several

ingredient companies (e.g., PureCircle and Cargill) and researchers started several projects to increase the concentration of minor steviol glycosides through breeding programs (Watson 2015). E-tongue may be used in these breeding programs as a tool to analyze a wide range of stevia samples within a rapid time frame at a low cost.

To evaluate the taste profiles of steviol glycosides and their mixtures, the protocol of the E-tongue sensor array #6 should be validated before conducting the experiments because the selectivity and sensitivity of the sensors will depend on the types of samples. Thus, the objectives of this study are 1) to validate the protocols of sensory array #6 for the mixtures of steviol glycosides (Reb D and M at 0.1 w/v %) and 2) to investigate the discrimination ability of E-tongue using the mixtures of steviol glycosides (0.020 % Reb A and 0.015 % M) at the iso-sweetness intensity at 5% of sucrose.

2. Materials & Methods

2.1 Materials

Steviol glycoside solutions were made using high purity 95 % Reb A, Reb D, and Reb M from SweeGen, Inc (Santa Margarita, CA) and deionized water.

2.2 Solution Preparation

Part1: Validation of the protocol for sensor array #6 for the mixtures of steviol glycosides

Steviol glycosides (0.12 g of Reb D and M) were combined with 120 mL of deionized water. The solution was heated on the hot plate with constant stirring until homogenized. The solution was cooled down until it reached room temperature (~25 °C) before analysis. Next, each sample was pipetted according to the ratio guide (Table 1). The final volume was adjusted to 25 mL for each sample.

Table 1. Ratios between 0.1 % (w/v) of Reb D and Reb M.

	mL (%)	
Sample	Reb D	Reb M
RD	25 (100.0 %)	0 (0.0 %)
RM	0 (0.0 %)	25 (100.0 %)
RD20RM10*	20 (66.6 %)	10 (33.3 %)
RD10RM20*	10 (33.3 %)	20 (66.6 %)
RDRM15*	15 (50.0 %)	15 (50.0 %)
RD12.5RM17.5*	12.5 (41.6 %)	17.5 (58.3 %)
RD17.5RM12.5*	17.5 (58.3 %)	12.5 (41.6 %)

* 5mL of the solutions is discarded.

Part 2: E-tongue analysis of the mixtures of steviol glycosides (Reb A and M) at iso-sweetness equivalent to sucrose.

The iso-sweetness of Reb A and M equivalent to 5 % sucrose was found to be at 0.02 % and 0.015 % (w/v), respectively (SweeGen Inc, California, USA). To further determine E-tongue's discrimination ability, Reb A (0.02 %), Reb M (0.015 %), as well as blends between Reb A and M (Table 2) were used for this study. Steviol glycosides (0.024 g Reb A and 0.018g M) were combined with 120 mL of deionized water. The same method as *Part 1* was followed.

Table 2. Ratios between 0.1 % (w/v) of Reb A and Reb M.

	mL (%)	
Sample	Reb A	Reb M
RA	25 (100.0 %)	0 (0.0 %)
RM	0 (0.0 %)	25 (100.0 %)
RA20RM10*	20 (66.6 %)	10 (33.3 %)
RA10RM20*	10 (33.3 %)	20 (66.6 %)
RARM15*	15 (50.0 %)	15 (50.0 %)
RA12.5RM17.5*	12.5 (41.6 %)	17.5 (58.3 %)
RA17.5RM12.5*	17.5 (58.3 %)	12.5 (41.6 %)

* 5mL of the solutions was discarded.

2.3 Astree E-tongue

A potentiometric E-tongue (Alpha M.O.S, Toulouse, France) was used for the taste profiling of steviol glycoside solution samples (Table 1). Sensor array #6 consisted of seven different sensors AHS, PKS, CTS, NMS, CPS, ANS, and SCS. AHS, CTS, and NMS are responsible for sourness, saltiness, and umami sensors respectively, while PKS, CPS, ANS, and

SCS are general-purpose sensors. Prior to the analysis, E-tongue performed conditioning, calibration, and diagnostic cycles, using 0.01M hydrochloric acid, sodium chloride, and sodium glutamate standard solutions. The solutions were pipetted into a glass vial for analysis. In between samples, deionized water was placed. Figure 1 shows the sequence of the analysis used for both *Part 1* and *Part 2*. Sensors were placed into each sample for 120 seconds to obtain taste measurements and were rinsed with deionized water for 10 seconds after each sample measurement. Each sequence was run for three consecutive days for two runs each day. Therefore, a total of six replications were conducted. The first two and last replicates were removed from data analysis.

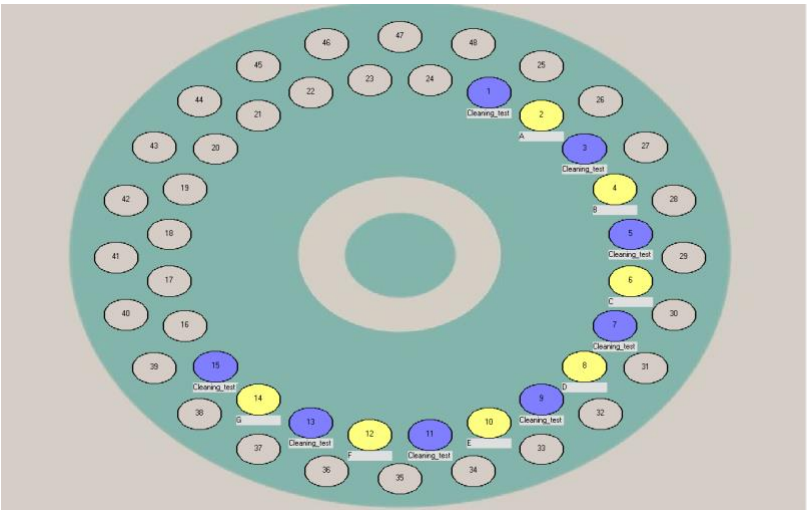

Figure 1. The sequence for samples used for Part 1 and 2 displayed on Alpha M.O.S. software; Blue indicates cleaning vial (Deionized water); Yellow indicates samples.

2.4 E-tongue analysis

Relative standard deviation (%RSD) and discrimination power are two main values to determine the quality of data (i.e., precision and reproducibility). %RSD measures the precision of the data which is calculated by ($\frac{\text{Standard Deviation}}{\text{Mean}} \times 100$). If the %RSD value of each sensor was less than 5 %, the data were considered good (Zheng and Keeney, 2006; Tao, 2020). If it was greater than 5 %, the samples would either need to be rerun or the sensor that has a greater than 5

% RSD would be deleted, depending on the discrimination power. The discrimination power, which ranged from 0 to 1, indicates the discrimination ability of the sensors on samples. A number closer to 1 meant that the sensor could separate the samples, while a number lower than 0.5 meant that the sensor could hardly differentiate the samples. If one of the sensors had poor discrimination power when analyzing the samples, the sensor would be removed from the analysis.

2.5 Statistical Analysis

All samples were measured six times using E-tongue (Alpha MOS, Toulouse, France). Principal component analysis (PCA) was conducted to determine if E-tongue could discriminate different mixtures of steviol glycosides (Table 1 and 2). A discrimination index (DI) was also included on the PCA map. The higher index number indicated less similarity between samples or groups (Zheng and Keeney 2006). Agglomerative hierarchical clustering (AHC) was used to group the blends with similar taste profiles into categories on different levels in the form of a dendrogram (Lê and Worch 2018) (XLSTAT software, Addinsoft, New York, USA)

3. Results

Table 3 shows the time and temperature it took to dissolve 0.1 % w/v of three types of steviol glycosides. Reb A, major type of steviol glycosides, was the fastest to dissolve at room temperature (~23 °C). On the other hand, both minor steviol glycosides, Reb D and M, required heat and a longer time to dissolve in the deionized water. However, Reb M required less time and temperature than Reb D (3 minutes at 40 °C vs 5 minutes at 65 °C).

Table 3. The temperature and time for (0.1 % w/v) major and minor types steviol glycosides (Reb A, D, and M) solutions to turn clear.

Stevia	Weight (g)	Temperature (°C)	Time (min)
Reb A	0.12	23	< 1
Reb D	0.12	65	5
Reb M	0.12	40	3

3.1. Part1: Validation of the protocol for sensor array #6 for the mixtures of steviol glycosides

Tables 4, 5, and 6 show the %RSD and discrimination power from a total of six replicates of Reb D and M (samples A and B, respectively) and their blends (samples C to G) which were collected for three consecutive days. A similar trend occurred on Day 1 and Day 2. In the first run of both days, some sensors were removed from the analysis because %RSD values were higher than 5 %. On Day 1, sensor CPS was removed and on Day 2, sensors NMS, CPS and SCS were removed. On the other hand, all %RSD values on Day 3 in both runs were below 5%. Therefore, none of the sensors were removed which were considered good data. The discrimination power also played a role together with %RSD. The discrimination power with values above 0.5 were considered good, but preferable if they were closer to 1. The discrimination power on the second run from all data collections was found to provide more uniform values (at around 0.9) than the first run. Thus, this confirmed that E-tongue could provide better discrimination capability throughout the runs from Day 1 to Day 3 and especially on the second run from each day.

Table 4. The relative standard deviation (%RSD) of electronic tongue sensors obtained from samples on Day 1 (Table 1).

Run1		%RSD						
Sensors	Discrimination Power	RD	RM	RD20 RM10	RD10 RM20	RDRM15	RD12.5 RM17.5	RD17.5 RM12.5
AHS	0.976	1.721	1.247	1.662	0.700	1.086	2.079	1.033
PKS	0.954	3.504	3.939	2.768	2.546	1.740	1.741	2.123
CTS	0.829	3.405	3.740	2.747	1.815	1.245	0.648	2.073
NMS	0.912	2.974	3.197	2.229	1.626	1.337	0.529	1.723
CPS	0.920	6.134	6.286	4.808	5.540	3.485	1.204	3.601
ANS	0.963	1.942	1.766	1.399	1.781	1.648	0.741	1.695
SCS	0.973	1.049	1.386	0.689	0.370	0.264	0.572	0.338
Run 2								
Sensors		RD	RM	RD20 RM10	RD10 RM20	RDRM15	RD12.5 RM17.5	RD17.5 RM12.5
AHS	0.931	1.817	1.447	0.837	1.178	1.034	0.878	0.465
PKS	0.953	2.427	2.071	3.020	1.625	1.525	1.426	1.502
CTS	0.964	1.807	1.387	2.093	1.031	1.007	0.9207	1.080
NMS	0.956	2.657	2.397	3.466	1.264	1.683	1.562	1.648
CPS	0.948	4.874	4.530	7.743	3.379	3.178	3.017	4.092
ANS	0.611	2.918	2.355	2.084	1.959	2.128	1.427	1.415
SCS	0.879	2.799	2.696	3.497	2.271	2.219	2.057	2.211

Bold means the sensors were removed from the analysis because the %RSD was higher than 5 %

Table 5. The relative standard deviation (%RSD) and discrimination power of electronic tongue sensors obtained from samples on Day 2 (Table 1).

Run 1		%RSD						
Sensors	Discrimination Power	RD	RM	RD20 RM10	RD10 RM20	RDRM15	RD12.5 RM17.5	RD17.5 RM12.5
AHS	0.922	2.318	1.701	2.147	1.977	1.746	0.856	1.516
PKS	0.687	4.358	4.382	3.971	8.075	3.845	4.067	3.562
CTS	0.721	3.379	3.344	3.045	6.450	3.127	3.143	2.810
NMS	0.715	5.501	5.508	5.062	11.571	5.233	5.055	4.628
CPS	0.691	8.749	9.593	8.524	23.239	8.651	9.033	7.773
ANS	0.887	1.810	1.898	2.393	1.798	1.802	1.252	1.757
SCS	0.626	5.150	5.396	4.936	9.417	5.088	5.110	4.578
Run 2								
Sensors		RD	RM	RD20 RM10	RD10 RM20	RDRM15	RD12.5 RM17.5	RD17.5 RM12.5
AHS	0.909	2.109	1.410	1.147	0.493	0.427	0.826	0.794
PKS	0.968	0.982	0.857	1.514	2.129	0.961	1.632	0.380
CTS	0.985	0.389	0.162	0.849	1.240	0.475	0.970	0.004
NMS	0.991	0.220	0.138	0.871	1.596	0.409	0.888	0.419
CPS	0.986	1.170	0.889	1.927	3.593	1.164	2.977	0.123
ANS	0.958	1.405	1.256	1.144	0.572	0.382	0.681	1.199
SCS	0.908	1.909	1.654	2.695	3.421	2.371	2.837	1.449

Bolded means the sensors were removed from the analysis because the %RSD was higher than 5 %

Table 6. The relative standard deviation (%RSD) and the discrimination power of electronic tongue sensors obtained from samples on Day 3 (Table 2).

Run 1				%RSD				
Sensors	Discrimination Power	RD	RM	RD20 RM10	RD10 RM20	RDRM15	RD12.5 RM17.5	RD17.5 RM12.5
AHS	0.317	2.365	2.501	1.617	2.257	2.380	2.475	2.300
PKS	0.662	1.358	1.088	1.331	4.238	4.716	4.750	1.793
CTS	0.742	0.673	0.573	0.746	3.139	3.507	3.368	0.969
NMS	0.836	0.462	0.193	0.412	4.099	4.740	3.889	0.433
CPS	0.756	1.506	1.512	1.490	7.302	8.293	8.514	2.577
ANS	0.765	2.323	2.438	1.665	1.836	2.101	2.671	2.075
SCS	0.671	2.263	2.458	2.433	6.434	7.027	7.293	3.257
Run 2								
Sensors	Discrimination Power	RD	RM	RD20 RM10	RD10 RM20	RDRM15	RD12.5 RM17.5	RD17.5 RM12.5
AHS	0.948	0.464	0.515	0.336	0.454	0.763	0.753	0.672
PKS	0.814	1.301	0.698	0.723	0.972	0.952	0.872	1.192
CTS	0.975	0.206	0.114	0.136	0.210	0.328	0.269	0.466
NMS	0.918	0.797	1.539	0.939	0.866	0.485	0.499	0.255
CPS	0.991	0.194	0.317	0.832	0.249	0.153	0.032	0.278
ANS	0.966	0.277	0.289	0.224	0.338	0.405	0.447	0.328
SCS	0.951	0.811	0.526	0.494	0.705	0.837	0.689	0.869

Figures 2, 3, and 4 show PCA of response signals on the electronic tongue and AHC analysis of the second run from Days 1, 2, and 3, respectively. All PCA data from Days 1, 2, and 3 explained high variations, which were at 99.47 %, 98.84 %, and 94.79 %, respectively. When the PCA is above 80 %, it was proved to be the most informative in the samples (Wang et al. 2021). This meant that most of the information for each sample was obtained (i.e., less square error occurred).

On Day 1, PC1 explained 98.59 %, and PC2 explained 0.87 % of the variations. The discrimination index of the second run was -32. This was due to overlap in some solutions, which meant E-tongue could not discriminate some of the solutions. When looking at Figure 2 (b), which was the AHC analysis, the data was able to create two cluster groups. The blue group contained samples RD20RM10 and RD17.5RM12.5, and the red group contained samples RD, RM, RD10RM20, RDRM15, and RD12.5RM17.5

On Day 2, PC1 and PC2 were explained at 97.50 % and 1.33 % of the variations, respectively (Figure 3 [a]). The discrimination index was less negative as compared to Day 1 (-32 vs. -0.3). This meant E-tongue improved its discrimination property with samples of stevia blends. The PCA visually showed better separation between samples, but there were still some overlaps between ratio samples. The AHC analysis (Figure 3 [b]) created two clusters but was slightly different from Day 1. The Blue group included samples RD, RM, and RD17.5RM12.5G. In contrast, Red group included samples RD20RM10, RD10RM20, RDRM15, and RD12.5RM17.5.

Lastly, Figures 4 (a) and (b) show PCA and AHC from Day 3. PC1 and PC2 explained 84.56 % and 10.23 % of the variations, respectively (Figure 4 [a]). The discrimination index was at -1 as there were some solutions that overlapped, which still meant E-tongue could not separate some of the samples. However, the AHC analysis (Figure 4 [b]) increased from two to three

clusters. This indicated an improvement of E-tongue sensors since the AHC was able to create more clustering groups. The Blue group only contained sample RD20RM10. The red group included solutions RM and RD12.5RM17.5. Lastly, the green group contained samples RD, RD17.5RM12.5, RD10RM20, and RDRM15.

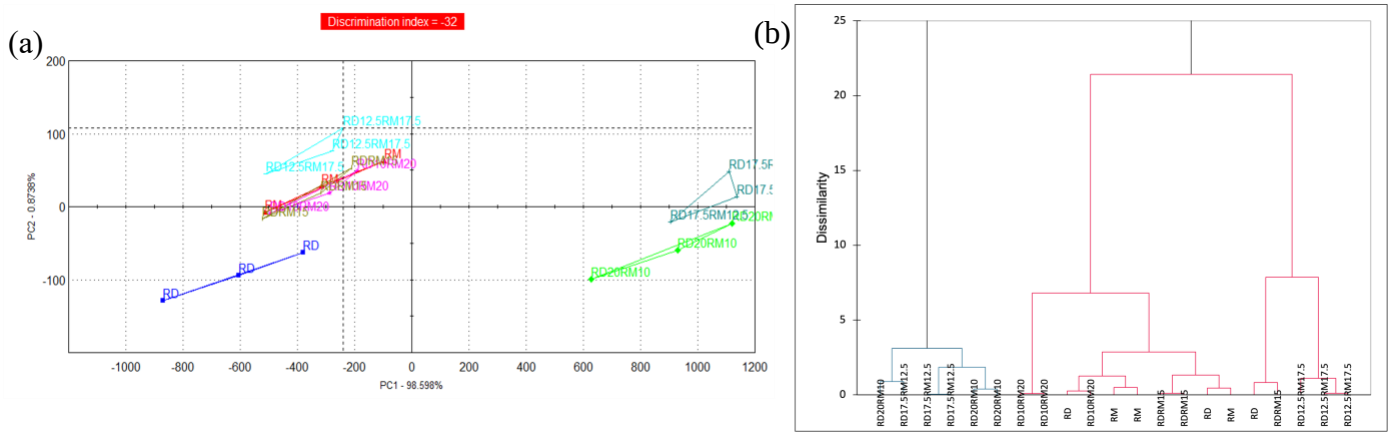

Figure 2. The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 1) from Day 1 second run.

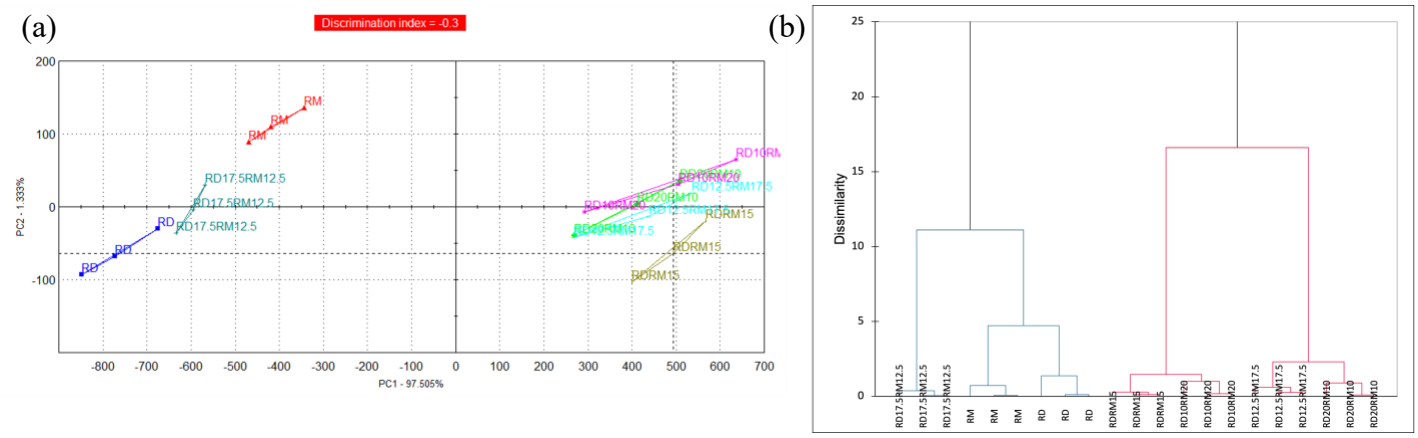

Figure 3. The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 1) from Day 2 second run.

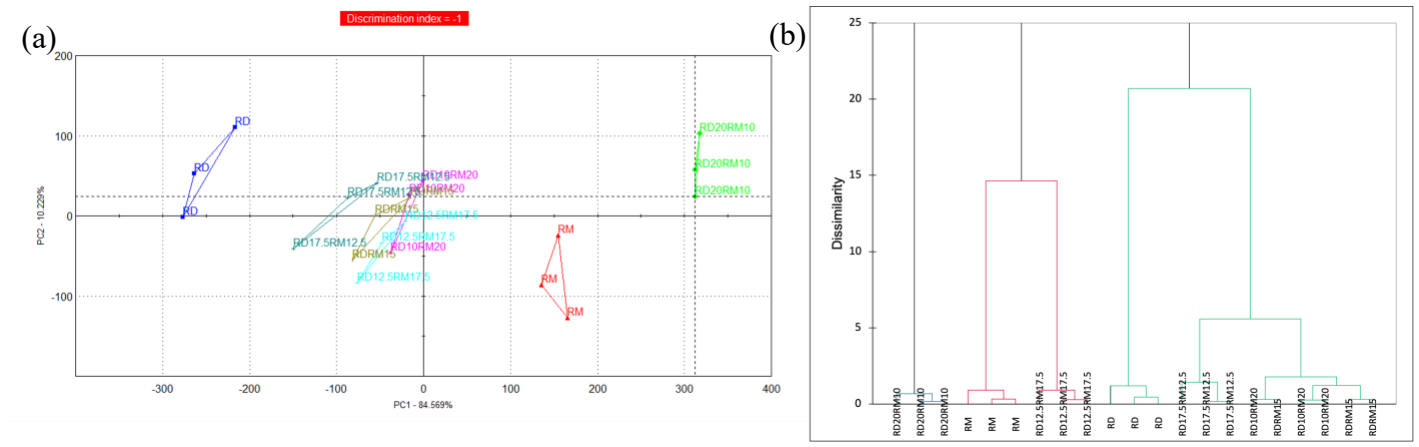

Figure 4. The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 1) from Day 3 second run.

3.2. Part 2: E-tongue analysis of the mixtures of steviol glycosides (Reb A and M) at iso-sweetness equivalent to sucrose.

Tables 7, 8, and 9 show the %RSD and discrimination power of E-tongue sensors obtained from Days 1, 2, and 3. The same trends occurred in this data which were comparable to results from Part 1. In run 1 of both Day 1 and Day 2, there were some sensors removed from the analysis due to high % RSD while none of the sensors were removed on Day 3 (%RSD values were ≤ 1). On Day 1, sensors AHS, NMS, CPS, and SCS were discarded while on Day 2, sensors AHS and ANS were removed. In addition, discrimination powers obtained from the second run on each day were more homogeneous than in the first run. Therefore, runs from Day 3 was found to be the most useful as there's no sensors removed especially on the second run.

Table 7. The relative standard deviation (%RSD) and discrimination power of electronic tongue sensors obtained from samples on Day 1 (Table 2).

Run 1		%RSD						
Sensors	Discrimination Power	RA	RM	RA20 RM10	RA10 RM20	RARM15	RA12.5 RM17.5	RA17.5 RM12.5
AHS	0.238	7.189	6.278	6.288	6.230	5.992	5.557	4.712
PKS	0.288	3.466	3.620	4.048	10.167	4.640	4.851	5.224
CTS	0.241	2.430	2.660	3.053	8.249	3.660	3.803	4.153
NMS	0.165	2.925	3.358	3.940	11.006	4.751	4.922	5.294
CPS	0.346	5.404	5.962	6.485	20.248	8.263	8.611	9.384
ANS	0.793	4.353	4.922	5.046	4.324	4.878	4.663	4.232
SCS	0.192	2.731	2.925	3.122	7.640	3.799	3.930	4.211
Run 2								
Sensors	Discrimination Power	RA	RM	RA20 RM10	RA10 RM20	RARM15	RA12.5 RM17.5	RA17.5 RM12.5
AHS	0.921	1.863	1.165	2.148	2.495	1.501	0.864	1.419
PKS	0.993	0.527	0.421	1.024	0.489	0.474	0.203	0.151
CTS	0.985	0.919	0.732	1.029	0.444	0.529	0.203	0.309
NMS	0.962	1.740	1.568	1.338	1.040	1.151	0.500	1.302
CPS	0.986	1.831	1.530	2.316	0.914	1.164	0.394	0.942
ANS	0.978	1.050	1.658	1.201	1.964	1.015	0.905	1.006
SCS	0.981	0.405	0.445	0.987	0.631	0.907	0.663	0.689

Bolded means the sensors were removed from the analysis because the %RSD was higher than 5 %

Table 8. The relative standard deviation (%RSD) and discrimination power of electronic tongue sensors obtained from samples on Day 2 (Table 2).

Run 1		%RSD						
Sensors	Discrimination Power	RA	RM	RA20 RM10	RA10 RM20	RARM15	RA12.5 RM17.5	RA17.5 RM12.5
AHS	0.546	7.814	6.342	6.494	6.916	5.795	6.275	5.901
PKS	0.053	1.580	2.201	1.608	2.373	1.841	1.668	2.356
CTS	0.118	0.623	0.721	0.959	1.095	1.098	0.797	1.303
NMS	0.831	0.611	0.449	0.116	0.0845	0.265	0.382	0.274
CPS	0.820	0.307	0.377	0.447	0.582	0.602	0.537	0.632
ANS	0.727	5.507	4.838	5.202	4.107	4.456	5.415	4.533
SCS	0.248	0.660	0.774	0.838	0.977	0.919	1.047	0.978
Run 2								
Sensors	Discrimination Power	RA	RM	RA20 RM10	RA10 RM20	RARM15	RA12.5 RM17.5	RA17.5 RM12.5
AHS	0.878	1.663	1.700	2.695	2.216	2.702	2.827	1.718
PKS	0.824	1.351	1.351	1.307	0.856	0.742	1.037	1.142
CTS	0.824	0.748	0.760	0.682	0.507	0.344	0.545	0.397
NMS	0.691	0.281	0.264	0.484	0.559	0.786	0.163	0.408
CPS	0.946	0.211	0.095	0.717	1.200	1.450	0.3726	0.263
ANS	0.835	3.959	2.855	4.552	1.751	2.210	2.355	1.541
SCS	0.787	0.872	0.806	0.777	0.580	0.523	0.715	0.590

Bold means sensors were removed due to RSD value below 5 %

Table 9. The relative standard deviation (%RSD) and discrimination power of electronic tongue sensors obtained from samples on Day 2 (Table 2).

Run 1		%RSD						
Sensors	Discrimination Power	RA	RM	RA20 RM10	RA10 RM20	RARM15	RA12.5 RM17.5	RA17.5 RM12.5
AHS	0.847	0.986	1.186	2.580	2.277	2.413	2.668	4.385
PKS	0.540	1.452	1.630	2.662	1.748	2.149	1.290	0.814
CTS	0.816	0.303	0.268	0.787	0.354	0.738	0.579	0.634
NMS	0.663	0.881	1.288	0.924	1.527	0.789	0.773	1.023
CPS	0.938	0.116	0.219	0.191	1.048	0.273	0.295	0.317
ANS	0.837	1.001	0.697	1.840	1.694	1.799	2.224	3.588
SCS	0.549	0.707	0.729	0.745	0.596	0.926	0.778	0.657
Run 2								
Sensors	Discrimination Power	RA	RM	RA20 RM10	RA10 RM20	RARM15	RA12.5 RM17.5	RA17.5 RM12.5
AHS	0.987	0.498	0.991	0.129	0.713	0.574	0.763	0.335
PKS	0.819	1.064	0.943	0.765	0.900	0.714	0.605	0.951
CTS	0.916	0.613	0.355	0.367	0.337	0.287	0.389	0.077
NMS	0.919	0.312	0.244	0.203	0.279	0.222	0.143	0.720
CPS	0.981	0.632	0.477	0.389	0.336	0.319	0.529	0.415
ANS	0.982	0.567	1.188	0.001	0.643	0.461	0.519	0.540
SCS	0.752	1.201	1.098	1.067	0.883	0.907	1.085	0.999

Bold means sensors were removed due to RSD value below 5 %

Figures 5 (a)(b), 6 (a)(b), and 7(a)(b) show PCA and AHC on run 2 obtained from days 1,2 and, 3. The PCA were 97.58 %, 87.75 %, and 91.48 %, respectively. On Day 1, PC1 and PC2 explained 95.16 % and 2.42 % of the variation. The discrimination index was at 84 %. Therefore, in this run E-tongue could discriminate against all samples. However, the AHC graph created two main cluster groups. The red group contained RARM15 and RARM10 and the blue group contained RA12.5RM17.5, RA10RM20, RA17.5RM12.5, RA, and RM.

On Day 2, PC1 and PC2 were explained at 74.23 % and 13.52 %, respectively. However, the discrimination index was at -14 as there were some overlaps between RA, RM, and RA12.5RM17.5 meaning that E-tongue could not discriminate between these three solutions. Moreover, in the AHC analysis, these three solutions were grouped in one cluster (Red), while the rest of the solutions were in the blue group.

Lastly, on Day 3, PC1 and PC2 explained 78.14 % and 13.34 % of the variation with the discrimination index at -8. This confirmed that E-tongue could not discriminate some of the samples. In the AHC analysis, the same trend occurred similarly to *Part 1*. It was able to increase the cluster into three clusters where RA, RM, RA20RM10, and RA17.5RM12.5 were in the green group. RA12.5RM17.5 was in the blue group. RA10RM20 and RARM15 were in the red group. Moreover, AHC analysis from Day 1 to Day 3 agreed that RA17.5RM12.5 was clustered in the same group at RA and RM.

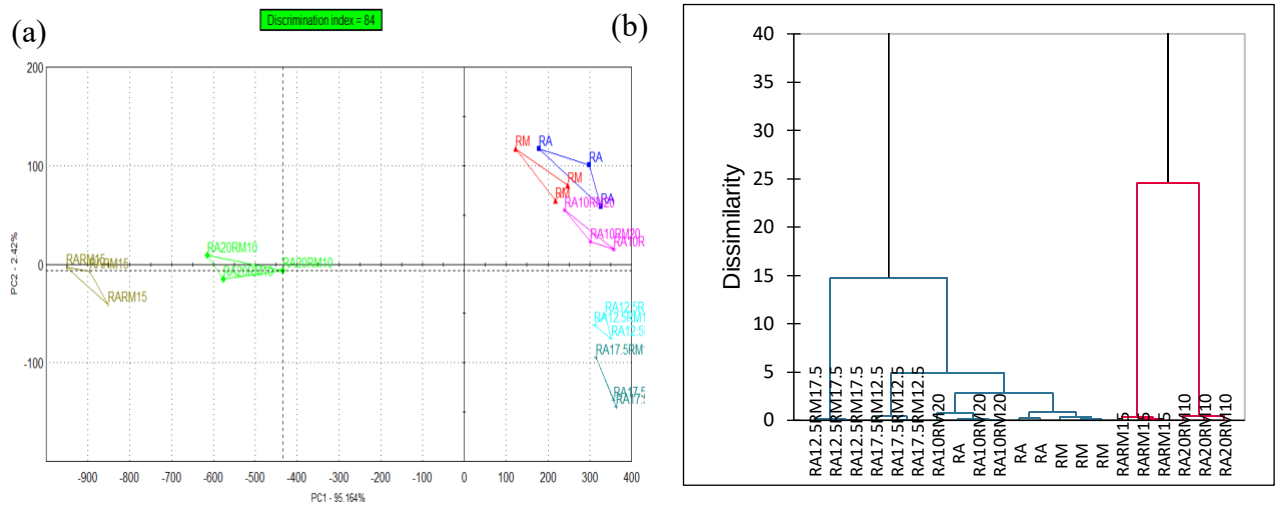

Figure 5. The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 2) from Day 1 trial 2.

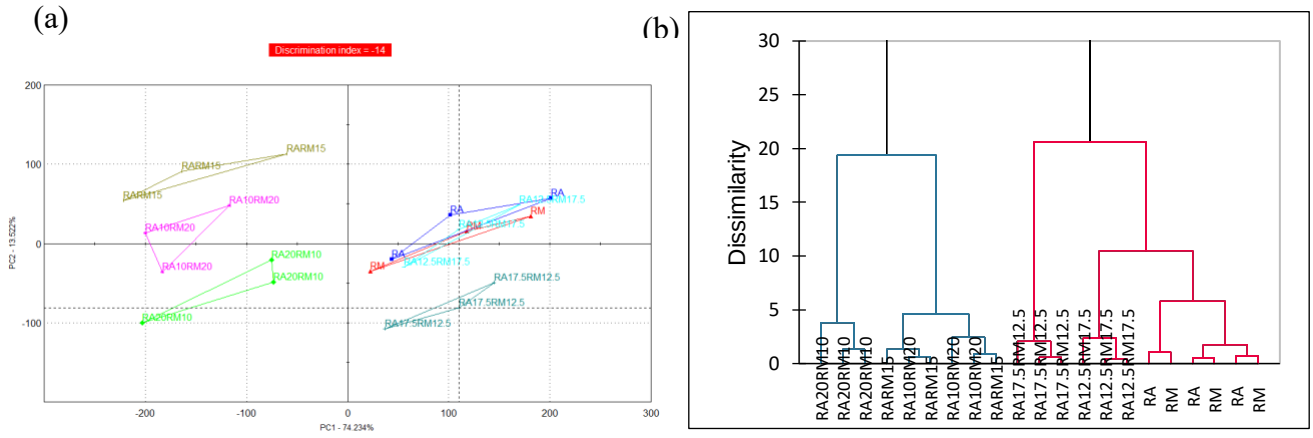

Figure 6. The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 2) from Day 2 trial 2.

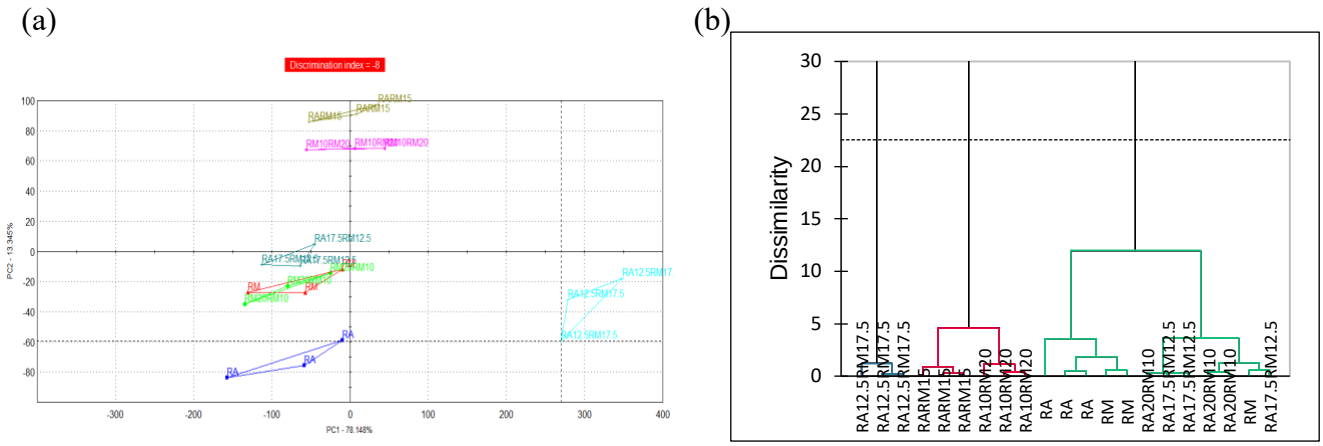

Figure 7. The PCA (a) and AHC (b) of minor steviol glycosides solution (Table 2) from Day 3 trial 2.

4. Discussion

Prior to the experiment, both major and minor steviol glycosides (Reb A, D, and M) were dissolved in deionized water. Reb A, major steviol glycoside, was found to require less time and heat for a solution to turn clear, whereas Reb D and M were the opposite. Although they were all steviol glycosides, each of these samples (Reb A, D, and M) had a different molecular weight (g/mol) which were 967.01, 1129.15, and 1291.3, respectively (Prakash et al. 2014). Thus, the molecular weight could affect the diffusion properties of each sample (2018). In addition, the low abundance of these steviol glycosides may reduce their solubility properties as compared to Reb A (Dong and Yang 2020). Prakash et al. (2015) reported the aqueous solubility (25 °C) of minor steviol glycosides, which included Reb D and Reb M. The result reported the solubility was at ~0.04 % and ~0.26 %, respectively (Prakash et al. 2014). Another factor that could influence this was the moieties compounds which could affect the solubilization (Celaya et al. 2016). Reb A only has one glucose moiety attached to C-19, while Reb D has two and Reb M has three (Libik et al. 2021). However, there's no clear relationship between the solubility and glucose moieties in minor

steviol glycosides (i.e., Reb D has a smaller number of glucose moieties at position C-19 as compared to Reb M). This phenomenon may cause by the steric hindrance and the molecular structure of Reb D (Gunawardena 2021).

This study confirms that the protocol from the previous study that used extracts of stevia leaves (Tao 2020) can be used to differentiate mixtures of steviol glycosides. The data in the second runs obtained from three consecutive days provided the most precise and accurate information because %RSD values were at around 4 % or less (Zheng and Keeney 2006; Woertz et al. 2010; Tao 2020), and the discrimination power was close to 1. Tao (2020) mentioned that sensor CTS should be removed from the analysis when evaluating stevia solutions due to the low value in discrimination power (less than 0.5). However, there were no issues with this sensor in both *Part 1* and *Part 2*. This phenomenon could occur because sensor CTS is claimed to be responsible for saltiness taste. However, Zhou et al. (2022) claimed that a single sensor from sensor array #6 could not define a specific taste intensity of samples. The authors found negative correlations between food samples and NMS sensor even though it was claimed to detect umami taste.

In the first part of the experiment, E-tongue was used to analyze different ratio solutions (Table 1) against Reb D and Reb M as an anchor. The result showed that E-tongue could not discriminate some of the mixtures from these two minor glycosides. Although Reb M was found to provide sweeter intensity than Reb D, there were some attributes that both steviol glycosides were found to provide comparable taste intensity (Tao and Cho, 2020; Prakash et al. 2014). In addition, Reb D and M, as well as the ratios, were used at the same concentration (0.1 % w/v). Hence, this could cause E-tongue not to be able to distinguish these two solutions from the blends and group them together into one cluster (Figures 2 [b] and 3 [b]). The PCA from Day 1 and Day

2 showed Reb D, and Reb M plotted close to each other, but there was no overlap between these two solutions. On Day 3, the PCA was clearly separated from one another (Figure 4 [b]). Thus, the AHC analysis was able to separate Reb D and Reb M into two different groups. Moreover, if there were no ratio blends in the analysis, E-tongue could discriminate between these two solutions (Reb D and M). Tao (2020) analyzed three different steviol glycosides solutions (Reb A, D, and M) at the same concentration (0.1 % w/v) and showed good discrimination ability of the E-tongue with sensory array #6 for all three consecutive days.

In the second part, E-tongue was used to determine if it could be used to discriminate Reb A and M when their concentrations were at iso-sweetness of 5 % sucrose along with stevia blends (Table 2). The data were obtained similar to *Part 1*. The % RSD and discrimination power from E-tongue on the second run showed great reproductivity and precisions of the data. Thus, it was confirmed that the second run should be used for data analysis. The results found that E-tongue could discriminate Reb A (RA; 0.020 % w/v) and M (RM; 0.015 % w/v), although these solutions were at the same level of sweetness. However, E-tongue could not discriminate these anchors (RA and RM) from the stevia blends (Table 2). In addition, the ACH cluster analyses also confirmed this because RA and RM were grouped in the same cluster (Figures 5 [b], 6 [b], and 7 [b]). Blend RA17.5RM12.5 was grouped in the same cluster with RA and RM for all three consecutive days (Figures 5 [b], 6 [b], and 7 [b]). RA12.5RM17.5 were grouped in the same cluster as those three solutions for the first two days (Figures 5 [b] and 6 [b]). However, it was in a different group on day 3 (Figure 7 [b]). This could mean a blend (RA17.5RM12.5) could be used to have a similar taste profile equivalent to 5 % sucrose. Blends RARM15 and RA20RM10 were plotted close to each other on PCA graphs and were in the same clusters on Day 1 and Day 2, but on Day 3, they were in different groups.

One of the limitations of this study was there no data from other detection methods to determine the taste profiles of steviol glycosides other than discrimination of the mixtures. Although, Reb M was found to provide a similar taste to sucrose, having a taste panel data to describe the attributes of each solution would be more helpful and informative.

5. Conclusions

This study was to validate the protocol for sensor array #6 that was previously developed for stevia extract. The previous protocol suggested removing one of the seven sensors to produce repeatable data, but this study found that all sensors would be needed to discriminate the steviol glycoside mixtures. The results of this study confirmed that E-tongue can be used for the analysis of taste profiles of steviol glycosides and their mixtures. E-tongue has the potential to be used in a wide range of applications in the food industry because of its benefits in analyzing data in a rapid time frame. However, when running samples, it is preferable to conduct it with other quick assay methods not only to discriminate the samples but also to characterize the taste profiles of the samples. Moreover, it is recommended to conduct reference data, which previously correlated with human panel data so that this would help characterize unknown samples as E-tongue sensor measure multiple tastes simultaneously.

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Chapter 4

The Effect of Steviol Glycosides on Sensory Properties and Acceptability of Ice Cream
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Abstract

There has been a challenge overcoming bitter aftertaste of stevia, a natural non-caloric sweetener. Recent research focuses on investigating various types of steviol glycosides, the sweet compounds in stevia leaves, as they exhibit different sensory characteristics. This study determined sensory properties and acceptability of ice cream sweetened solely by three steviol glycosides, Rebaudioside (Reb) A, D, and M (0.09 % w/v) against sucrose as a control (14 % w/v). Ice cream consumers (n=92) rated overall and attributes liking, determined sweetness and bitterness intensities, and described aftertastes of each sample using Check-All-That-Apply. The liking scores of Reb D and M ice cream were significantly higher than Reb A. Among the three glycosides, only Reb M showed the sweetness comparable with that of sucrose. Consumers perceived aftertaste of Reb D and M as more *sweet*, *pleasant*, *creamy*, and *milky* while Reb A as more artificial and chemical. Reb D and M were also plotted close to sucrose in the correspondence analysis graph, meaning their aftertaste characteristics were similar to that of sucrose. The present study highlights that Reb D and M have clearly better taste and provide better perception to consumers than Reb A, which is the most widely used glycoside in food industry.

1. Introduction

According to the American Heart Association (AHA), the daily-recommended consumption of sugar should be no more than 36 g for men and no more than 25 g for women. The average American adult consumes twice the daily amount of recommended sugar (Vreman et al. 2017; Ricciuto et al. 2021) leading to the development of chronic diseases and diabetes (Stanhope

2016; Vreman et al. 2017; Castro-Muñoz et al. 2022). in consumers has resulted in food industries investing in finding alternatives to sucrose in the form of high-intensity sweeteners (HIS). HIS approved by the U.S. Food and Drug Administration (FDA) include six artificial sweeteners (saccharin, aspartame, acesulfame potassium (Ace-K), sucralose, neotame, aspartame) and two natural sweeteners, stevia (steviol glycosides) and monk fruit (Luo Han Guo fruit extract) (Nutrition 2020). These HIS are used in very small amounts while providing low to almost negligible caloric content to food and beverages (Saraiva et al. 2020). However, the effects of consuming artificial HIS on health and metabolism are not well-established (Schiffman et al. 1987; Whitehouse et al. 2008). Some studies found no adverse effect on diabetics' blood glucose after they consumed artificial sweeteners (Grotz et al. 2003), but others claimed consumption of artificial sweeteners could trigger a small rise in insulin levels (Pepino 2015), negatively change gut bacteria (Suez et al. 2014) and have a positive association with obesity (Suez et al. 2014; Ruanpeng et al. 2017; Pearlman et al. 2017). These contradictory results have negatively impacted consumers' perceptions of artificial HIS (Gibson et al. 2014) and increased interest in natural sweetener options such as stevia (Román et al. 2017). Not only is stevia a natural non-caloric HIS, but it also lowers the glycemic index (GI) (Alizadeh et al. 2014) and blood glucose levels (Alizadeh et al. 2014). In addition, Anton et al. (2010) discovered consumption of stevia significantly helped to reduce the food intake of consumers as compared to sucrose ($P < 0.01$).

Many food companies have started to introduce stevia into their food and beverage products to promote healthy options to consumers (Narayanan et al. 2014; Vreman et al. 2017). Stevia has been widely used in beverage drinks more commonly than other HIS (e.g., aspartame) (Boldt 2019) because of the advantages over sucrose and artificial sweeteners. According to Statista Research Department (Wunsch 2021), the global market value of stevia was predicted to

reach over USD 770 million by 2022. Furthermore, according to Allied Market Research in 2020, the global market of stevia is also predicted to reach about USD 1.2 billion by 2026 with a compound annual growth rate (CAGR) of 8.0 % from 2019 to 2026. Moreover, natural HIS options, including stevia, have been utilized in dairy products as it grows in popularity among consumers and food industries. For instance, ice cream, a popular dessert in the U.S. (Lin 2012), typically contains an average of 15 % sucrose (Qamar et al. 2018). The demand for natural sweetener ice cream products has become a challenge to the industry, leading companies to launch healthy options for consumers, such as low-fat, low-sucrose, or no-sucrose-added products (Malochleb 2018). Currently, brands like Halo-Top and Enlightened, utilize either stevia extract (a mixture of steviol glycosides) or pure Reb A with other caloric or non-caloric sweeteners. However, none of the companies use stevia as a sole sweetener.

Stevia (Stevia rebaudiana) is a South American plant that has up to 300 times the sweetness of sucrose (Goyal et al. 2010; Kumar et al. 2011). Its natural constituents of the stevia leaves, steviol glycosides, have been considered generally recognized as safe (GRAS) in the U.S. since 2008 (Nutrition 2020). Leaves of stevia contain sweet compounds, known as diterpene glycosides or more commonly as steviol glycosides (Prakash Chaturvedula et al. 2011; Peteliuk et al. 2021), with more than 40 steviol glycosides identified (Prakash Chaturvedula et al. 2011; Purkayastha et al. 2016; EFSA Panel on Food Additives and Flavourings (FAF) et al. 2020; Libik-Konieczny et al. 2021). Most of them lack relevant sweetness data except for the following 11 types: stevioside, rebaudioside A (Reb A), Reb B to F (Starratt et al. 2002; Savita et al. 2004), M (Prakash et al. 2014; Peteliuk et al. 2021), steviolbioside (Prakash et al. 2014), Rubusoside (EFSA Panel on Food Additives and Flavourings (FAF) et al. 2020; Peteliuk et al. 2021) , and dulcoside A (EFSA Panel on Food Additives and Flavourings (FAF) et al. 2020; Peteliuk et al. 2021). Moreover, these steviol

glycosides are currently approved in the European markets (2013). The major types of steviol glycosides are stevioside (110 – 270 times sweeter than sucrose) (EFSA Panel on Food Additives and Flavourings (FAF) et al. 2020; Peteliuk et al. 2021) and Reb A (150 and 320 times sweeter) (Kochikyan et al. 2006; Libik-Konieczny et al. 2021; Peteliuk et al. 2021). However, these major steviol glycosides have been found to provide a significant bitter aftertaste (Prakash et al. 2011 ; Purkayastha et al. 2016; Libik-Konieczny et al. 2021). Thus, many researchers have investigated various steviol glycosides (Prakash et al. 2014; Watson 2015) and it was found that minor types of steviol glycosides such as Reb D and M would exhibit different levels of sweetness and bitterness from the major types of steviol glycosides. Reb M displays fast sweetness onset, reduces the non-sweet taste, and results in less lingering bitterness when compared to Reb A (Prakash et al. 2014). Similarly, Reb D elicits significantly less bitterness than Reb A at similar levels of sweetness using trained panelists (Allen et al. 2013). In addition, Tao and Cho (2020) found both Reb D and M showed better taste characteristics than Reb A (e.g., less bitterness) in water solution at 0.09 % (w/v) using a consumer panel. They also found that the aftertaste descriptors of Reb D and M were close to sucrose. However, no study to date has investigated the sensory properties of Reb D and M compared to Reb A in food matrices.

In this research study, we investigated sensory characteristics and acceptability of ice cream sweetened solely with Reb A, D, and M. Using a consumer panel, consumer perceptions of ice cream sweetened with minor glycosides (Reb D and M) and the major glycoside (Reb A) were compared to sucrose-sweetened controls. Furthermore, we investigated if Reb A, D, and/or M could be used as a sole sweetener in high sucrose applications without compromising sensory quality.

2. Materials and Methods

2.1. Materials

The materials used to produce ice cream samples were purchased from a local grocery store: heavy cream (Horizon Organic, Broomfield, CO, USA), non-fat dry milk (Kroger, Cinciannati, OH, USA), vanilla extract (Spice Island, B&G Foods Inc, Parsippany-Troy Hills, NJ, USA), polydextrose (Litesse, DuPont, Wilmington, DE, USA), and sucrose (Smidge & SpoonTM, Kroger, Cincinnati, OH, USA). The steviol glycosides used in the ice cream for the study were high purity (95 %) Reb A, Reb D, and Reb M from Sweegen (Santa Margarita, CA, USA).

2.2. Ice Cream Preparation

This study wanted to evaluate the sensory characteristics between steviol glycosides at the same concentration in the high sucrose food application. Reb A, D, and M were used at 0.09 % (w/v) in the ice cream formulation. The 0.09 % concentration was chosen because Reb M was initially found to have a similar sweetness level as the 14 % sucrose (w/v) from the previous study by Tao and Cho (2020) which is within the sweetness level range for frozen desserts and ice cream. Table 1 shows ice cream formulations and indicates the functionality of each ingredient used in this study. The dry ingredients (nonfat dry milk, polydextrose, and sucrose or Reb A, D, or M) were first blended in the mixer (KitchenAid, St. Joseph, MI, USA) until they were homogenized, followed by the addition of warm water (~43 °C). Next, heavy cream and vanilla extract were added with continuous stirring until the mixture was homogenized. The ice cream mixture was aged for one hour at 4 °C and then place in the ice cream maker for one hour (Cuisinart, Stamford, CT, USA). The ice cream was transferred into a plastic container (64 oz) and stored in a walk-in

freezer at - 20 °C. Table 1 also shows the caloric values of each ice cream, which was based on 80 g or 2/3 cups (i.e., ice cream serving size). It was generated using Genesis R&D Supplement Formulation & Labeling Software (ESHA Research, Oak Brook, IL, USA).

Table 1. The functionality of each ingredient for ice cream formulation and caloric values.

Ingredients	Functionality	Sucrose (g)	Stevia (Reb A, D, M) (g)
Heavy cream	Mouthfeel texture (Alvarez et al. 2005)	400.0	400.0
Non-fat dry milk	Texture and flavor (Alvarez et al. 2005)	140.0	140.0
Water	Solvent (Qamar et al. 2018)	650.0	650.0
Vanilla extract	Flavoring agent	5.0	5.0
Sucrose	Sweetener	203.0	0.0
Reb A, D, M	Sweetener	0.0	1.3
Polydextrose	Bulking agent (Nath et al. 2015)	50.0	245.0
Total		1448.0	1441.3
Calories per serving[1] (80.0 g or 2/3 cup)		150.0	120.0

[1] The caloric values were generated by Genesis R&D Supplement Formulation & Labeling Software (ESHA Research, Oak Brook, IL)

2.3. Panel Recruitment

Consumer panelists who consume ice cream (at least 2 – 3 times per month) and zero-calorie sweeteners (at least once a month) were recruited from Auburn University (18 – 65+ years old). The pre-survey was performed using Qualtrics online survey software (Qualtrics, LLC, Provo, UT) including the consumption behavior of HIS and the frequency of ice cream consumption.

2.4. Sample Preparation

All ice-cream samples were made two days before the test. A day before the test, a scoop (~30 g) of ice cream was transferred into a 2-oz plastic soufflé cup labeled with 3-digit random coded numbers. They were stored in a walk-in freezer (-20 °C).

2.5. Testing Procedure

This study was approved by the University Institutional Review Board of Auburn University (Auburn, AL) (Protocol #: 21-204 EX 2104). RedJade sensory science software (RedJade Sensory Solutions LLC, Redwood City, CA) was used to collect data during the entire testing. After confirming the served sample code matched the code on the screen, the panelists were asked to taste a spoonful of the ice cream sample (less than 1/2 of the serving cup) to evaluate overall liking and attributes liking (appearance, flavor, texture/mouthfeel, and aftertaste) of the sample using a 9-point hedonic scale (1= Dislike extremely, 9 = Like extremely). Next, they were instructed to take another spoon of the same sample and swallow it to evaluate the aftertaste of each sample. For the aftertaste, they were asked to rate the intensities of sweetness and bitterness using a 15 cm-line scale (0 = Not at all sweet/bitter, 15 = Extremely sweet/bitter) and then to choose aftertaste descriptors using check-that-all-apply (CATA) analysis. The listed attributes for CATA included *Artificial, Metallic, Milky, Buttery, Chemical, Bitter, Spicy, Vanilla, Honey, Minty, Pleasant, Tart, Sweet,* and *Spicy*. This study used 11 terms from a previous study conducted by Tao and Cho (2020) who evaluated the aftertaste of stevia solutions. Terms *Buttery*, *Creamy*, and *Milky* were added to describe the flavor attributes of ice cream. Lastly, the term *Spicy* was used as an attention check. The purchase intent question using a 5-point Likert scale (1= Definitely would not buy, 5 = Definitely would buy) was asked at the end of each sample. A 30-second break was enforced before receiving the next sample. During the break, water and unsalted crackers were also provided as palate cleansers. After evaluating all four samples, consumer behavior and demographic questions were asked, including Low/No sugar product consumption behavior, the familiarity of zero-calorie sweeteners (i.e., aspartame, ace-k, erythritol, monk fruit, saccharin, stevia, sucralose, and xylitol), health-related questions (health conditions of the panelists and their

family and diet) and demographic questions (i.e., age, gender, height, weight, education level, ethnicity, and household income).

2.6. Statistical Analysis

Data analysis was performed using XLSTAT (AddinSoft, New York, NY, USA). The sensory evaluation questions and the sweetness and bitterness intensities were determined by two-way analysis of variance (ANOVA) with a 95 % confidence level ($P < 0.05$) and Tukey's (HSD) tests treating ice cream samples as a fixed effect and the consumer panel as a random effect. To determine if there was an interaction effect between the overall liking score of each ice cream and gender, data were also analyzed using two-way ANOVA with one interaction effect (fixed effects: ice cream sample and gender). Cohran's Q test was used to analyze the check-all-that-apply (CATA) to determine any significant differences between ice cream samples. Correspondence analysis (CA) was used to show the relationship between sensory attributes and samples.

3. Results

3.1. Participants' Characteristics

A total of 92 participants who consumed ice cream at least 2-3 times per month completed the study. The age range was between 18 to 65 years old with the average body mass index (BMI) of 26.0 ± 5.3 kg/m^2. Table 2 shows the socioeconomic status of the panelists. We recruited female and male participants (59.8 % and 40.2 %, respectively). The majority variables of the panel are consumers aged between 18-25 years old (51.1 %) with the highest education level at the graduate degree (38.0 %). Therefore, most panelists received household incomes under USD 30,000 (72.8 %). Lastly, the majority of participants were White or Caucasian (70.6 %).

Table 2. Demographic and socioeconomic characteristics of the consumer panelists (n=92).

Variable	Definition	Participant (n)	Frequency (%)
Gender			
	Female	55	59.8
	Male	37	40.2
Age			
	56 - 65	4	4.4
	46 - 55	3	3.3
	36 - 45	5	5.4
	26 - 35	33	35.9
	18 - 25	47	51.1
BMI (Mean ± Standard Deviation)		26.0 ± 5.3 kg/m^2	
Education level			
	Graduate degree (Master's, Doctorate, etc.)	35	38.0
	4-year college degree	28	30.4
	2-year college degree	5	5.4
	High School diploma or GED	24	26.1
Household income			
	Over $ 80,000	4	4.4
	$ 50,000 to $ 79,999	7	7.6
	$ 30,000 to $ 49,999	14	15.2
	Under $ 30,000	67	72.8
Ethnicity			
	Asian or Pacific Islander	11	12.0
	Black or African American	2	2.2
	Hispanic or Latino	13	14.1
	White or Caucasian	65	70.6
	Prefer not to say	1	1.1

Table 3 shows ice cream consumption behaviors of the panelists. Over 80 % of the consumer panel consumed ice cream at least once a week, and more than 90 % of them purchased ice cream at least once a month. However, only 31.5 % of the ice cream consumers purchased low or no sugar ice cream within the past six months.

Table 3. Ice cream consumption behaviors by the consumer panel (n=92).

Variable	Definition	Participants (n)	Frequency (%)
Frequency of ice cream consumption			
	2-3 times per month	16	17.4
	Once a week	33	35.9
	2-3 times per week	37	40.2
	More than 3 times per week	6	6.5
Frequency of ice cream purchase			
	Once every 2 or 3 months	6	6.5
	Once a month/every four weeks	17	18.5
	Once every 2 or 3 weeks	50	54.4
	Once a week or more often	19	20.7
Low or no sugar ice cream purchase within the past six months			
	Yes	28	30.4
	No	59	64.2
	Don't remember	5	5.4

Table 4 shows how many different types of sweeteners the consumer panel could recognize. They were required to select each sweetener from 'Very unfamiliar' to 'Very familiar'. Among all-natural sweeteners, stevia was picked the most for 'Very familiar', while monk fruit was picked the most for 'Very unfamiliar' (41.3 % and 4.3 %, respectively). For the artificial sweeteners, the consumer panel frequently selected 'Very familiar' for sucralose (33.7 %) and 'Very unfamiliar' for Ace-K (72.8 %).

Table 4. Familiarity in various sweeteners selected by consumer panel (n=92).

Low/zero sugar sweeteners	Familiarity, n (%) Very unfamiliar	Somewhat unfamiliar	Neutral	Somewhat familiar	Very familiar
Artificial sweeteners					
Acesulfame-K	67(72.8%)	14(16.3%)	4(4.3%)	3(3.3%)	4(4.3%)
Aspartame	25(28.3%)	9 (9.8%)	3(3.3%)	29(31.5%)	26(28.3%)
Erythritol	62(68.5%)	10(10.9%)	5(5.4%)	10(10.9%)	5(5.4%)
Saccharin	22(26.1%)	7(7.6%)	10(10.9%)	26(28.3%)	27(30.4%)
Sucralose	17(18.5%)	7(7.6%)	5(5.4%)	32(34.8%)	31(33.7%)
Natural sweeteners					
Monk Fruit	57(62.0%)	10(10.9%)	8(8.7%)	13(14.1%)	4(4.3%)
Stevia	17(18.5%)	3(3.3%)	4(4.3%)	30(32.6%)	38(41.3%)
Xylitol	45(48.9%)	11(12.0%)	9(9.8%)	16(18.5%)	11(12.0%)

Table 5 shows consumption behaviors of low/zero sugar products and various sweeteners by the consumer panel. A total of 52 (56.6 %) participants consumed low/zero sugar foods and/or beverages at least once a month. Out of 92 participants, only 28.3 % consumed stevia at least once a month. This shows that they were not frequent stevia users although the majority of participants were very familiar with stevia among all artificial and natural sweeteners.

Table 5. Consumption of low/zero sugar products and zero-calorie sweeteners by consumer panel (at least once a month) (n=92).

Variables	Consumption frequency, n (%) Yes	No	Don't know
Low/zero sugar foods/beverages	52(56.6 %)	37(41.3 %)	3(3.3 %)
Artificial sweeteners			
Acesulfame-K	0(0.0 %)	57(62.0 %)	35(38.0 %)
Aspartame	16(17.4 %)	52(56.5 %)	24(26.1 %)
Erythritol	6(6.5 %)	49(53.3 %)	37(40.2 %)
Saccharin	11(12.0 %)	59(64.1 %)	22(23.9 %)
Sucralose	23(25.0 %)	46(50.0 %)	23(25.0 %)
Natural sweeteners			
Monk Fruit	3(3.3 %)	61(66.3 %)	28(30.4 %)
Stevia	26(28.3 %)	43(46.7 %)	23(25.0 %)
Xylitol	11(12.0 %)	48(52.2 %)	33(35.9 %)

3.2. Sensory Analysis of Ice Cream

Table 6 summarizes the means (± Standard Error (SE)) of overall liking, attributes liking, and the purchase intent of each ice cream sample evaluated by the ice cream consumers (n=92).

Table 6. The means (± Standard Error) overall liking, the attributes liking, and the purchase intent of sucrose, Reb A, D, and M ice cream samples (n=92).

Ice cream	Liking Score[1]					Purchase Intent[2, ***]
	Overall[*]	Appearance[*]	Flavor[***]	Texture/ Mouthfeel[*]	Aftertaste[***]	
Sucrose	7.6 ± 0.13 [a]	7.5 ± 0.12 [a]	7.7 ± 0.12 [a]	7.3 ± 0.16 [a]	7.4 ± 0.13 [a]	3.7± 0.12 [a]
Reb A	5.4 ± 0.19 [c]	6.7 ± 0.14 [b]	5.2 ± 0.19 [c]	6.1 ± 0.17 [c]	4.3 ± 0.23 [c]	2.1 ± 0.11 [c]
Reb D	6.4 ± 0.16 [b]	6.9 ± 0.17 [ab]	6.2 ± 0.17 [b]	6.4 ± 0.17 [bc]	5.5 ± 0.19 [b]	2.6 ± 0.11 [b]
Reb M	6.6 ± 0.18 [b]	7.1 ± 0.13 [ab]	6.5 ± 0.19 [b]	6.7 ± 0.14 [ab]	5.6 ± 0.21 [b]	2.8 ± 0.12 [b]

[1] The liking scores were evaluated on a 9-point hedonic scale (1 = Dislike extremely, 9 = Like extremely); [2]The purchase intent was evaluated on a 5-point Likert scale (1=Definitely would not buy, 5 = Definitely would buy); [a,b,c] values in the same column show the significant differences between sample means at $P < 0.05$ by Tukey's (HSD). [*] indicates $P < 0.05$; [***] indicates $P < 0.001$.

Sucrose ice cream received scores of over 7.0 (moderately like) on a 9-point hedonic scale and was significantly higher than all three steviol glycosides in overall liking ($P < 0.05$) and every attribute liking ($P < 0.001$) except for appearance and texture/mouthfeel liking. There were no differences between minor steviol glycosides (Reb D and M) and sucrose ice cream on the appearance liking ($P = 0.063$ and $P = 0.183$, respectively). In the texture/mouthfeel liking, Reb M and sucrose ice cream were found not to be significantly different from one another ($P = 0.052$), but the P-value is close to the significance level ($P < 0.05$). Although Reb M ice cream received slightly higher liking scores than Reb D ice cream in every category, they were not significantly different (Table 6). There were significant differences in hedonic impressions between major steviol glycoside (Reb A) and minor steviol glycosides (Reb D or Reb M). Reb A ice cream was significantly liked less than Reb D and M in every category except texture/mouthfeel and appearance liking. All steviol glycoside ice cream showed similar scores in appearance liking with the range of 6.9 ± 0.67. In the purchase intent score, sucrose ice cream received 3.7 on a 5-point

Likert scale which is close to 4 ‘Probably buy’. The purchase intent of Reb A was close to 2 ‘Probably would not purchase’ (i.e., 2.1) and Reb D and M ice cream samples were rated significantly higher than Reb A ($P < 0.001$), which were close to 3 ‘Might or might not purchase’ (i.e., 2.6 and 2.8, respectively). Reb M ice cream received the highest purchase intent score among the steviol glycoside ice cream although there was no significant difference between Reb D and M ($P = 0.49$).

Next, the participants were asked to put each sample on their tongues for 5 seconds and then swallow the sample. Immediately after swallowing, they were asked to determine the intensities of sweetness and bitterness using a 15-cm intensity line scale. Table 7 shows the means (± SE) of sweetness and bitterness intensities of ice cream samples.

Table 7. The means (± Standard Error) sweetness and bitterness intensities rated by the consumer panel for sucrose, Reb A, D, and M ice cream samples (n=92).

	Intensity[1]	
Ice cream	Sweetness***	Bitterness*
Sucrose	10.3 ± 0.24 [a]	1.6 ± 0.27 [c]
Reb A	7.9 ± 0.40 [b]	5.4 ± 0.37 [a]
Reb D	8.0 ± 0.29 [b]	2.9 ± 0.33 [b]
Reb M	9.8 ± 0.30 [a]	2.6 ± 0.36 [bc]

[1]Intensities measured immediately after swallowing on a 15-cm line scale (0 = Not at all sweet/bitter, 15 = Extremely sweet/bitter). [a,b,c] values in the same column show the significant differences between sample means at $P < 0.05$ by Tukey’s (HSD). [*]indicates $P < 0.05$; [***] indicates $P < 0.001$.

Among all three steviol glycosides, Reb M ice cream received the highest sweetness intensity and showed comparable sweetness to that of sucrose ice cream ($P = 0.609$). Reb A and D ice cream samples were both significantly less sweet than sucrose and Reb M ice cream ($P < 0.001$). Moreover, Reb M and sucrose ice cream received bitterness intensity with no significant difference ($P = 0.175$), but the intensity score of Reb M ice cream was higher than sucrose ice cream (i.e., 2.6 and 1.6 on a 15-cm line scale, respectively). Reb A ice cream received the highest bitterness score among all the samples ($P < 0.001$). There was no significant difference in the

bitterness intensity between Reb D and M ice cream (P = 0.853). The participants rated the bitterness intensity of Reb D and M at 2.9 and 2.6 respectively, while Reb A received 5.4 on a 15-cm line scale. Table 8 shows the aftertaste attributes selected for each ice cream sample by consumer panelists (n=92).

Table 8. Aftertaste attributes selected by the consumer panel for sucrose, Reb A, D, and M ice cream (n=92)

	Ice Cream			
Attributes[1]	Sucrose	Reb A	Reb D	Reb M
Artificial ***	7 ^{a}	54 ^{c}	47 ^{bc}	35 ^{b}
Bitter ***	2 ^{a}	37 ^{b}	14 ^{a}	10 ^{a}
Buttery^{ns}	22	18	17	27
Chemical***	1 ^{a}	26 ^{c}	12 ^{b}	10 ^{ab}
Creamy***	62 ^{c}	32 ^{a}	50 ^{bc}	45 ^{ab}
Honey^{ns}	5	4	6	4
Metallic**	2 ^{a}	14 ^{b}	5 ^{ab}	8 ^{ab}
Milky***	62 ^{b}	30 ^{a}	49 ^{b}	58 ^{b}
Minty^{ns}	1	1	0	0
Pleasant***	51 ^{c}	9 ^{a}	28 ^{b}	29 ^{b}
Sweet***	72 ^{c}	39 ^{a}	53 ^{ab}	62 ^{bc}
Tart*	2 ^{a}	13 ^{b}	6 ^{ab}	5 ^{ab}
Vanilla***	84 ^{b}	54 ^{a}	64 ^{a}	64 ^{a}

[1]The listed terms for CATA analysis; ^{a,b,c} values in the same column show the significant differences between sample means at $P < 0.05$ by Critical Difference (Sheskin). * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$ and ^{ns} indicates no significant differences among samples.

Reb D and M ice cream samples received no significant difference from each other in each aftertaste term (Table 8). The terms including *bitter*, *metallic*, *milky*, and *tart* were used to describe Reb D, Reb M, and sucrose ice cream. Interestingly, for the term, artificial, Reb D ice cream was chosen by 12 more panelists than Reb M (47 vs 35 for Reb D and M, respectively), but there was no significant difference between them. However, the term, *artificial* was used to describe Reb A significantly more than Reb M (54 vs 35, respectively) ($P < 0.0001$), but there was also no significant difference between Reb A and Reb D (54 vs 47, respectively). The result from the term

sweet of all ice cream samples was complementary to a 15-cm line intensity scale in that sucrose and Reb M ice cream were chosen by most panelists (72 vs 62, respectively) and Reb A and D ice creams were chosen the least frequent with this term (39 vs 53, respectively). The term *bitter* was chosen the most with Reb A ice cream by a consumer panel (37) which was similar to the bitterness intensity scale (Table 7). However, for the term *bitter*, Reb D (14) was described similarly to both sucrose and Reb M ice cream (2 and 10, respectively). Reb A ice cream was described more frequently with negative terms than other ice cream samples including *bitter*, *metallic*, and *tart*. However, all three steviol glycosides shared *metallic*, *vanilla,* and *tart* terms with no significant difference ($P < 0.05$). Three terms which were not significantly different from each other were *buttery*, *honey*, and *minty*.

The sensory attributes of sweeteners were summarized visually in Figure 1. The first two dimensions explained 96.96 % of the variation. Terms *pleasant*, *vanilla*, *sweet*, and *creamy* were associated and chosen with sucrose more than all three steviol glycosides. Reb A was close with more negative terms including *metallic*, *bitter*, *chemical*, and *tart*, while Reb D and M were mostly associated with positive words. Moreover, both minor steviol glycosides were plotted close to each other and were closer to sucrose when compared to Reb A.

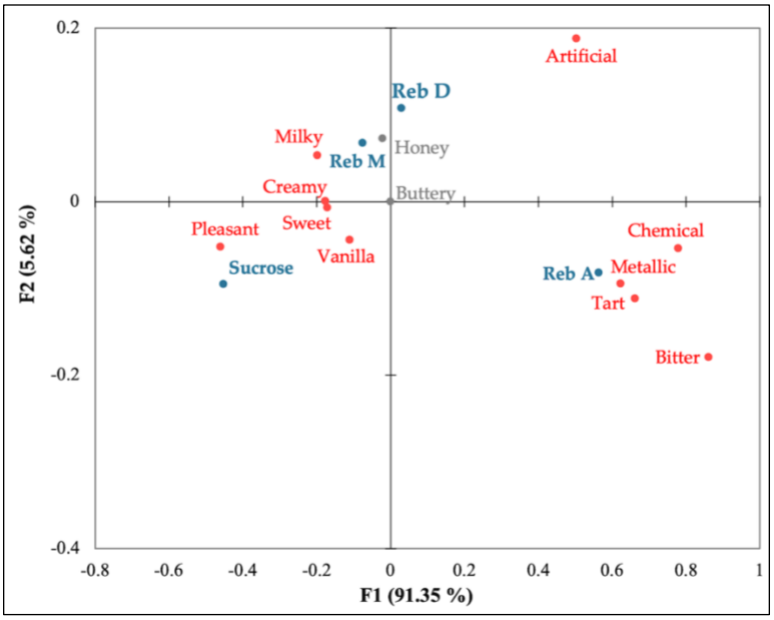

Figure 1. Correspondence analysis (CA) of each ice cream. Blue indicates samples; Red indicates significant attributes; Grey indicates not significant attributes

In this study, male (n=37) and female panelists (n=55) were later found to rate overall liking of the ice cream differently from each other. Figure 2 indicates that male panelists gave mean overall liking scores of all steviol glycoside ice cream (i.e., Reb A, D, and M) lower than sucrose ice cream (5.6, 6.2, 6.2 vs 7.7, respectively). On the other hand, female panelists gave both sucrose and Reb M ice cream a similar liking score of 7-points (7.6 and 7.0, respectively), followed by Reb D (6.5), and Reb A (5.3).

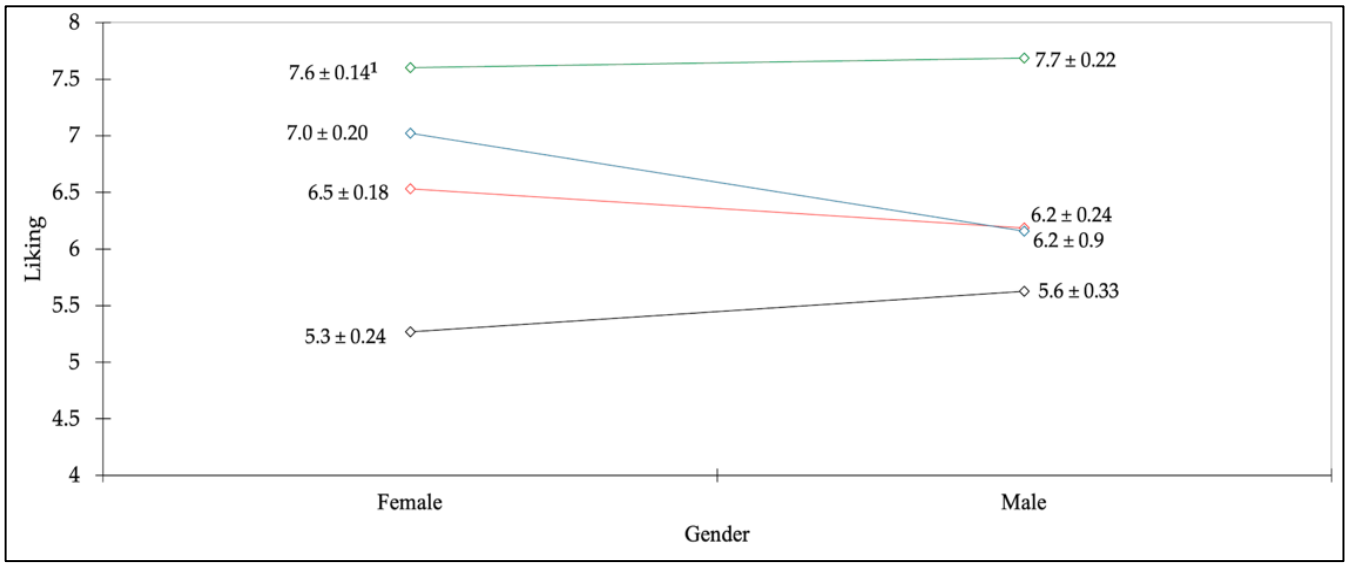

Figure 2. [1]The means overall liking (± standard error) of each ice cream between female and male participants. Green indicates sucrose ice cream; Blue indicates Reb M ice cream; Red indicates Reb D ice cream; Black indicates Reb A ice cream.

4. Discussion

4.1 Sensory Evaluation

This study examined the sensory quality of three different steviol glycosides (0.09 % w/v) in ice cream and compared them against sucrose ice cream (14 % w/v) as a control. Stevia has different physiochemical properties than sucrose (Alizadeh et al. 2014), one of which is that it lacks a bulking agent. Thus, this negatively affects the texture of stevia ice cream samples. Therefore, we used polydextrose as a bulking agent when using stevia instead of sucrose in ice cream formulations (Table 1). Polydextrose has a variety of functional properties with potential health benefits, making it a great additive in various food products (Nath et al. 2015). Not only does polydextrose aid in enhancing ice cream texture, it also acts as a fat replacer to improve the appearance and the mouthfeel of the ice cream (Alvarez et al. 2005). This allows the ice cream made with stevia to acquire some sensory characteristics similar to those of sucrose (i.e., appearance and texture/mouthfeel attributes). However, polydextrose contains 1 Kcal per gram,

which adds additional calories to stevia ice cream samples. Despite small differences in caloric intake between sucrose and stevia ice cream (150 vs. 120 Kcal, Table 1), this ice cream formulation with stevia is suitable for people with diabetes who are looking for an ice cream option with no sugar while having a similar texture/mouthfeel as regular ice cream. Polydextrose, similarly to stevia, does not affect blood glucose levels (Anderson et al. 2009; Canfora and Blaak 2015). Additionally, the human body does not metabolize stevia, meaning we obtain no calories from consumption (Alizadeh et al. 2014; Samuel et al. 2018; Han 2020).

The results from Table 6 show significant differences in overall liking and attribute liking scores among stevia ice cream samples (Reb A, D, and M) at 0.09 % (w/v) and sucrose ice cream samples at 14 % (w/v). When comparing Reb D and M (the minor steviol glycosides) with Reb A (the most widely used steviol glycoside in the food industry), Reb A ice cream was least preferred by panelists; it was given the lowest score among samples in all hedonic liking scores. The consumer panel preferred minor steviol glycosides over the major steviol glycoside, except in appearance and texture/mouthfeel attributes ($P < 0.001$). Reb D and Reb M ice creams scored at around six points (Like slightly) in all hedonic liking scores except aftertaste liking, which were both at around five points (Neither like nor dislike). They both shared similar scores and showed no significant difference, but the consumer panel showed a slightly higher preference for Reb M over Reb D ice cream. Moreover, Reb M and sucrose ice creams shared more similar attributes than other steviol glycosides (i.e., appearance and texture/mouthfeel). According to (Everitt 2009), a mean liking score of seven or higher on a nine-point hedonic scale is acceptable for sensory quality. Even though the replacement of sucrose by high-intensity sweeteners can negatively alter the perception of bitter and sweet taste (Cardello et al. 1999), we found that these minor steviol glycosides were nearly as good as sucrose ice cream. The mean purchase intent was scored the

least with Reb A ice cream, followed by Reb D and M ice creams, then sucrose ice cream. This pattern was reflected in overall liking and attribute liking scores (Table 6). The nine-point hedonic scores and five-point Likert score from this study confirmed the better effect of utilizing minor steviol glycosides (especially Reb M) as sucrose substitutes, rather than Reb A, in food matrices. While many studies have developed ice cream formulations with different ratios of stevia and other sweeteners, few studies have incorporated formulations using purely stevia. Alizadeh et al. (2014) used five different ratios of sucrose and stevia in ice cream and compared them against the control (sucrose only) using a five-point intensity score (zero = uncharacterized intensity and five = very strong intensity). One of the ratios, 9.3 g sucrose and 0.04 g stevia, was found to receive high liking scores in taste, texture, and overall liking, among four other different ratios. However, the control still maintained the highest liking scores for flavor, taste, and mean liking scores. The authors found that the substitution of sucrose with stevia negatively affected the liking scores of panelists (Alizadeh et al. 2014). This assumes that panelists do not prefer the product with stevia. To address this point, Alizadeh et al. (2014) used nearly pure steviol glycosides to test consumer acceptability in ice cream, using a purification rate of 90 %. McCollum (Foodnavigator-Usa.com, 2009) claimed that a high percentage of purity of stevia indicated purer extraction, which brings a sweeter taste and hinders the bitter aftertaste of steviol glycosides. In another study, Velotto et al. (2021) used solely >98 % Reb A stevia extract powder and compared it against sucrose (control) at 26.1 % in both traditional (1.0 % Reb A) and vegan ice cream (1.5 % Reb A) samples. The results showed that both traditional and vegan ice cream sweetened with stevia received significantly higher scores than sucrose samples in sweet taste/flavor and overall taste attributes ($P < 0.05$). Thus, a high-purity stevia extraction method could mitigate negative aftertastes (i.e., bitter and lingering) found in stevia, especially Reb A at a high concentration. This could be the

potential reason explaining the consumer panel's preference for minor steviol glycoside ice cream over Reb A ice cream.

On the sweetness and bitterness intensity scales (Table 7), the consumer panel gave Reb M and sucrose ice creams similar scores, with no significant difference ($P = 0.220$ and $P = 0.175$, respectively). This could explain why Reb M received the highest hedonic and Likert scores among all steviol glycoside samples, even though Reb D and M ice cream samples received bitterness intensity scores of 2.9 and 2.6, with no significant difference ($P < 0.05$). A study done by Jung et al. (2021) found that Reb D (0.0209 %) and Reb M (0.0190 %) exhibited a similar bitterness aftertaste, with no significant difference ($P < 0.05$). In addition, Tao and Cho (2020) found that Reb D and M solutions were not significantly different in terms of in-mouth and lingering sweetness ($P = 0.05$) at the same concentrations as this study. However, their consumer panelists were able to distinguish Reb D from Reb M because the Reb M solution provided the highest immediate sweet taste among other samples.

Thus, in this study, Reb M ice cream was found to be sweeter than either Reb A or D ice creams. The sweet taste attribute of steviol glycosides is dependent on the functional group (R-groups) at positions C-13 and C-19 of the steviol core, on which different types of sweet molecules are attached (Libik-Konieczny et al. 2021; Peteliuk et al. 2021). The main difference between Reb A and Reb D and Reb M is the number of glucose molecules positioned at the C-19 (Libik-Konieczny et al. 2021). Reb A only has one glucose moiety, while Reb D has two and Reb M has three. This makes both minor steviol glycosides provide a sweeter taste and a less bitter aftertaste than Reb A (Gwak et al. 2012; Watson 2015; Libik-Konieczny et al. 2021). This finding corresponds with the bitterness intensity score being highest for Reb A among the three steviol glycoside ice cream samples (Table 7). The chemical compounds of the different steviol glycosides

may affect the taste in both solutions (Prakash et al. 2014; Libik-Konieczny et al. 2021) and food matrices encountered by the consumer panel.

In the CATA analysis (Table 8), consumer panelists selected every aftertaste term for Reb D and M ice creams with similar frequencies to one another. They were described with several positive attributes such as *milky*, *vanilla*, and *pleasant*, and they were also plotted close to sucrose in the corresponding analysis. Reb A ice cream was on the opposite side of sucrose ice cream, with more negative attributes such as *bitter* and *metallic* (Figure 1). All steviol glycoside ice cream samples were described with the term *metallic* and *tart*, although Reb D and M were chosen less frequently than Reb A. However, Tao and Cho (2020) found that there was no significant difference with the term *metallic* between all steviol glycosides (Reb A, D, and M) and the sucrose solution. In this study, the term *artificial* was selected to describe all three steviol glycoside ice creams more than sucrose ice cream but was used to describe Reb M ice cream significantly less than Reb A and D ice creams ($P < 0.0001$). On the other hand, Tao and Cho (2020) found that there was no significant difference between all steviol glycoside solutions (Reb A, D and M) at the same concentration, and the sucrose solution was least frequently chosen among the samples ($P < 0.001$). Although steviol glycoside extracts are known for their sweetness, many elicit undesirable aftertastes, including bitterness (Allen et al. 2013).

In this study, we confirmed that these minor glycosides give a significantly less bitter aftertaste than Reb A in food matrices, especially at high-sucrose concentrations. The result for Reb A ice cream from CATA with the term bitter corresponded to the bitterness intensity rating on a 15-cm line scale (Tables 7 and 8). Many research and food industries have been investigating the sensory analysis of the minor steviol glycosides because they provide more sweet and less

bitter taste profiles (Prakash et al. 2014; Olsson et al. 2016). Although we observed that these minor steviol glycosides contained some negative terms, they were more positively associated with sucrose ice cream than Reb A ice cream. Our CATA analysis revealed that consumer panelists associated Reb M and sucrose with the term *sweet* with similar frequency. Although Reb D and M shared similar scores in every aftertaste descriptor, the consumer panel selected the sweet attribute with similar frequency for both sucrose and Reb M ice creams (72 and 62, respectively). This supports previous findings suggesting that Reb M has the highest sweetness intensity compared to other steviol glycosides (Prakash et al. 2014; Watson 2015; Tao and Cho 2020).

There was no interaction between male and female preferences for ice creams sweetened with steviol glycosides versus sucrose (n = 37 and n = 55, respectively). The *P*-value of the interaction effect was 0.06, which is slightly greater than 0.05, the significance level, even though the *P*-value could be changed with the unequal sample size of the panelists for each gender. However, it is important to note that female participants clearly showed a higher preference for the Reb D and M ice creams than male participants did (Figure 2). This may be because sugar-free products are more popular among female participants than males and as such they are more familiar with the taste of zero-calorie sweeteners. Several studies have shown that women tend to choose healthier food choices than men (Lattimore and Halford 2003; Wardle et al. 2004), making them a targeted consumer for ice cream sweetened with stevia. Therefore, it would be interesting to further investigate the gender differences in terms of preferences and perceptions of stevia-sweetened products.

One possible limitation of this study could be the participants' household incomes. There are many factors that affect the consumer's purchase intent, one of which is the

Price (Guinard et al. 2000). The majority of participants (~70 %) earned an income of less than $ 30,000 because a majority of participants were still in college (four-years college and graduate degree). Consumers were asked if they were willing to purchase ice cream samples for $ 4.99 per pint using a five-point Likert scale. Therefore, the skew of this demographic might affect the purchase intent score. Another limitation of this study could be that we only used a pure vanilla flavor in this study. If strawberry or chocolate flavors were used in the ice cream, the stronger flavors might be able to mask the unpleasant aftertastes of stevia and might increase liking scores.

5. Conclusion

This study has confirmed that minor steviol glycosides, Reb D and M, had positive effects on the acceptability of zero sugar ice cream when compared with Reb A, the major steviol glycoside. Although sucrose ice cream received the highest liking scores among ice cream samples, these minor steviol glycosides received overall liking scores between 'like slightly' and 'like moderately', which were significantly higher than Reb A. Furthermore, the aftertaste characteristics of Reb D and M were comparable to sucrose ice cream. Interestingly, only Reb M was found to provide a sweet taste profile similar to sucrose, but there was no significant difference in flavor liking of Reb D and M ice cream. It is, thus, suggested that Reb D and M can be used as a natural non-caloric sweetener option to replace sucrose without adding bitter aftertaste even in high sugar applications such as ice cream or frozen desserts. Further studies are needed to commercially produce Reb D and M though since their small quantities in the stevia leaves. Breeding for increased concentrations of these minor glycosides in stevia plants would be beneficial to increase the supply of the desired glycosides. Also, identifying an optimal combination of different steviol glycosides to use both major and minor steviol glycosides to

accommodate the bitter aftertaste issue from Reb A and small extractable quantities of Reb D and M.

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5. Conclusions

The findings of the first study validate the protocol of the E-tongue sensor array #6 to be used as a tool to discriminate major and minor steviol glycosides (Reb A, D, and M), which are currently being used in the food industry. The results confirmed that this novel technology has the potential to be used as a quick tool to detect differences among a variety of mixtures of steviol glycosides to find an optimal ratio that has a better-tasting stevia product at a reasonable cost. However, other testing methods (e.g., sensory panel and/or chemical analysis methods) should be conducted and correlated with E-tongue data to determine accurate descriptive terms and the chemical compounds that are responsible for specific taste characteristics.

The results from the second study confirmed that minor steviol glycosides (Reb D and M) provide superior taste profiles in ice cream, which may potentially replace Reb A, the most abundant type steviol glycoside in the stevia leaves and the most widely used stevia in the food industry. Reb M had slightly better sensory characteristics than Reb D in that it showed similar sweetness intensity to sucrose and contained more positive aftertaste descriptive terms. However, both Reb D and M may be used as the sole sweetener in high-sugar food applications, while Reb A should be combined with other sweeteners or intense flavors to mask the negative aftertastes.

From this study, instrument measurements and sensory analysis were found to be valuable tools to assess sensory characteristics and consumer acceptability of stevia.