DISCRIMINATION AND SENSORY CHARACTERIZATION OF STEVIOL GLYCOSIDES (REBAUDIOSIDE A, D, AND M) BY CONSUMERS AND ELECTRONIC TONGUE

By

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ABSTRACT

DISCRIMINATION AND SENSORY CHARACTERIZATION OF STEVIOL GLYCOSIDES (REBAUDIOSIDE A, D, AND M) BY CONSUMERS AND ELECTRONIC TONGUE

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Rebaudioside (Reb) D and M are the recent focus of the food industry to address the undesirable aftertaste of Reb A, which is the most commonly used steviol glycoside in natural sweetener stevia. The first study evaluated the sensory characteristics of Reb A, D, and M, compared to 14% (w/v) sucrose, using a consumer panel and explored the relationship between 6-n-Propylthiouracil (PROP) taster status (i.e., non-tasters, medium tasters, supertasters) and the perceived intensity of sweet and bitter tastes of the three steviol glycosides. The results showed that Reb D and M had sensory profiles that were closer to sucrose, compared to Reb A, but were associated with negative sensation, such as *artificial,* and Reb M was higher in lingering sweetness than sucrose ($P < 0.001$), which may cause negative perception toward Reb D and M. No significant differences were found among the PROP taster groups on the perceived sweetness and bitterness of Reb A, D, and M, suggesting that supertasters may not report aversive sensations from stevia. The second study was aimed to develop a new protocol for the electronic tongue (E-tongue), which is an analytical instrument for the sensory evaluation of taste, to discriminate stevia leaves for its potential use in stevia breeding programs as a method of testing flavor quality. With the new protocol, the E-tongue successfully separated Reb A, D, and M and discriminated among stevia leaf samples. This suggests that E-tongue has the potential to be used in stevia breeding programs for flavor selection.

This thesis is dedicated to my parents, Xiaodong Tao and Jie Gu.
Thank you for the love and support.

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INTRODUCTION

1. Background

Stevia, which is a natural high-intensity non-nutritive sweetener derived from stevia plant (*Stevia Rebaudiana* Bertoni), has been used by the food industry to respond to the consumers' demand for natural sugar substitutes with low/zero calories. Stevia is the source of many different types of steviol glycosides, which are the sweetening compounds in stevia leaves (Kinghorn, 2002). Stevioside and rebaudioside (Reb) A are the two major steviol glycosides (Kinghorn, 2002) and are the most widely used steviol glycosides on the market (*Mintel Global New Products Database (GNPD)*, 2020). However, many studies reported the bitter and licorice off-taste of stevioside and Reb A (Gwak et al., 2012; Jenner, 1989; Kim et al., 2015; Li et al., 2015; Medeiros et al., 2019; I. Prakash et al., 2008), which pose challenges to consumer acceptance. To address the taste challenges of stevioside and Reb A, the researchers and food industry have investigated Reb D and M, which are two minor steviol glycosides, as they have been shown to elicit significantly less bitterness with better sweetness than Reb A (Hellfritsch et al., 2012; Prakash et al., 2014). Most of the studies investigating sensory characteristics of steviol glycosides were conducted at a relatively low sweetness equivalency related to sucrose (SE) (e.g. at 5-10% SE) (Gwak et al., 2012; Kim et al., 2015; Li et al., 2015; Prakash et al., 2008; Prakash et al., 2014). The sweetness potency of stevia heavily depends on the SE (Prakash et al., 2008), however, little research was done at high concentrations for high-sugar applications such as frozen desserts, which generally contain 13-22% sucrose w/v (Goff, 2015).

Supertasters are a group of people who perceive the intense bitterness from Phenylthiocarbamide (PTC) and 6-n-Propylthiouracil (PROP) bitter-tasting compounds, while

those who barely detect the bitterness of them are classified as non-tasters (Bartoshuk et al., 1994). It has been reported that individuals have different sensitivity to the aftertaste of high-intensity sweeteners (Simons et al., 2008), and thus researchers have long been interested in understanding the relationship between PROP status (e.g. non-tasters vs supertasters) and perceived taste intensities of high-intensity sweeteners. However, the influence of PROP status on the perceived intensity has been controversial. Some researcher found the difference between non-tasters and supertasters in perceiving the bitterness of artificial sweeteners (Bartoshuk et al., 1994; Drewnowski et al., 1997; Zhao & Tepper, 2007), while some did not (Horne et al., 2002; Rankin et al., 2003). Risso et al. (2014) found that the bitter taste receptor for PROP did not predict the bitterness perception of stevioside. However, little research was done to investigate the influence of PROP status on the perceived sweet and bitter taste intensities of the three popular steviol glycosides Reb A, D, and M.

Electronic tongue (E-tongue) is an analytical instrument developed for the sensory analysis of taste, which mimics human sensations and evaluates the taste by analyzing dissolved compounds in a liquid matrix. It has the potential to be used for monitoring quality (Hruškar et al., 2009; Winquist et al., 2005), detecting adulteration (Dias et al., 2009), classification and even predicting attributes of unknown samples (Bleibaum et al., 2002; Dong et al., 2017; Kirsanov et al., 2012; Waldrop & Ross, 2014). Current papers using the E-tongue from AlphaMOS all used old sensor arrays, such as sensor array #1 (Bleibaum et al., 2002; Dong et al., 2017) and #5 (Barnett et al., 2019; Jung et al., 2017; Lee et al., 2019; Lipkowitz et al., 2018; Schlossareck & Ross, 2019; Waldrop & Ross, 2014), but they were discontinued in late 2018. No paper has published using the most updated #6 array to date. Besides, the protocols for E-tongue analysis sequence using old arrays were vague and were different by papers (Bleibaum et al., 2002; Dong

et al., 2017; Lee et al., 2019; Lipkowitz et al., 2018; Schlossareck & Ross, 2019; Waldrop & Ross, 2014).

Stevia plant breeders have worked on developing new varieties of stevia plants with better tasting profile to increase the amount of Reb D and M in the stevia leaves (Watson, 2015). Few breeding programs evaluate the flavor profile of stevia varieties due to the time and cost constraint to train a human panel, however, it is important to know the sensory profile of different stevia varieties to select the desirable ones. High-performance liquid chromatography (HPLC) is one way to determine the steviol glycoside content of stevia leaves (Ahmed & Dobberstein, 1982; Bondarev et al., 2003; Gardana et al., 2010; Hashimoto et al., 1978; Kolb et al., 2001; Makapugay et al., 1984), but the sample preparation and analysis procedure are rather complex (Dong et al., 2017; Kirsanov et al., 2012). If E-tongue could quickly differentiate glycoside profiles of different stevia varieties, it could save a lot of time for breeding programs.

2. Hypotheses and Objectives

2.1. Hypotheses

Researchers hypothesized that there would be significant differences in the sensory profiles of Reb A, D, and M at 14% (w/v) sucrose equivalency. Furthermore, there would not be significant differences between non-tasters, medium tasters, and supertasters on the perceived sweetness and bitterness of the three steviol glycosides. Also, researchers hypothesized the electronic tongue would be able to discriminate steviol glycosides and stevia leaf samples and increase the potential use in stevia breeding programs.

2.2. Objectives

1) Determine sensory characteristics of Reb A, D, and M, compared to 14% (w/v) sucrose, using a consumer panel (Chapter 2).

2) Determine if there is a relationship between PROP taster status and the perceived intensities of three steviol glycosides (Chapter 2).

3) Develop a protocol for E-tongue to discriminate stevia leaf samples for its potential use in stevia breeding programs as a method of testing flavor quality (Chapter 3).

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CHAPTER 1: LITERATURE REVIEW

1. Stevia

1.1. History and Regulation

Stevia Rebaudiana Bertoni is a herbaceous shrub that is native to Paraguay (Brandle *et al.*, 1998). The leaves of stevia, which contain sweetening compound steviol glycoside, has been used to sweeten teas for hundreds of years in Paraguay and Brazil (Brandle *et al.*, 1998; Geuns, 2003). Stevia extract was first commercialized in Japan in the 1970s and was widely used to sweeten food and beverages (Abe & Sonobe, 1977; Akashi, 1977). The use of stevia comes later to western countries. In the United States, FDA approved certain high purity steviol glycosides (>95% pure glycosides) for generally recognized as safe (GRAS) status since 2008, but crude extracts or raw stevia leaf are excluded (FDA, 2018a). In the European Union, the use of steviol glycoside as a sweetener was approved in 2011 (SGS, 2011). Joint FAO/WHO Expert Committee on Food Additives (JECFA) established an acceptable daily intake (ADI) of steviol glycoside up to 4mg/ kg bw/day.

1.2. Metabolism, Chemical Structure and Sweet Potency

Stevia contains zero-calorie because the body does not absorb steviol glycosides. In vitro studies showed that steviol glycosides were hydrolyzed to steviol by human microflora and steviol is not degraded anymore (Gardana *et al.*, 2003; Koyama *et al.*, 2003). Steviol glucuronide (SVG), the major metabolite of steviol, is excreted through urine (Wang *et al.*, 2015).

Stevia leaves contain multiple steviol glycosides. All glycosides share the same steviol backbone (*ent*-13-hydroxykaur-16-en-19-oic acid) (Figure 1). R1 and R2 groups, which contain different numbers of glycosidic molecules, differentiate the glycosides identified in the stevia (Prakash *et al.*, 2008, 2014). The R groups of each steviol glycosides are shown in Table 1.

Generally, the highly branched sugar chains at the R1 site make the glycoside sweeter than those with unbranched chains, the number of glucose molecules attached to the backbone positively correlates to the sweetness of the glycoside, and the rhamnose reduces the sweetness (Kinghorn, Fullas, & Hussain, 1995). For example, Reb D and Reb M have highly branched R1 groups and have five and six glucose molecules attached to the steviol backbone, respectively. Based on Table 1, they are sweeter than most of the glycosides. The relative sweetness of Reb D was higher than any other steviol glycosides, except Reb M (not included in the study) (Hellfritsch *et al.*, 2012). Prakash et al. (2014) listed Reb M as the sweetest steviol glycosides.

Steviol glycosides consist of 4 - 20% of the dry leaves by weight (Geuns, 2003). Stevioside, the most abundant steviol glycosides (4-13% w/w) (Makapugay, Nanayakkara, & Kinghorn, 1984; Momtazi-Borojeni et al., 2017), has been reported to be 150-250 times sweeter than sucrose (Carakostas et al., 2012). Rebaudioside A, the most widely used glycoside (2-4% w/w) (Makapugay et al., 1984; Momtazi-Borojeni et al., 2017), has a sweet potency about 200-300 (Carakostas et al., 2012). The sweet potency has a wide range because it heavily depends on the sweetness equivalency (SE). The sweet potency is higher at low SE levels than at high SE levels. DuBois et al. (1991) reported that the sweet potency of Reb A is about 200 at 6% SE. It is also important to indicate the medium, temperature, and pH because the sweet potency changes when one of the factors changes. The 68% Reb A had a sweet potency of 348 at 5% SE and a 181 at 15% SE in water solution at refrigerated temperature (Wee, Tan, & Forde, 2018). A stevia leaf extract (81% stevioside, 7.7% Reb A, and 0.6% Reb C) had a sweet potency of 97 when pH = 7 and a 109 when pH = 3 in water solutions at room temperature (Cardello, Da Silva, & Damasio, 1999).

Figure 1. Steviol backbone of all glycosides (Geuns, 2003)

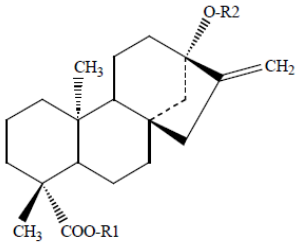


Table 1. R-groups, molecular formulas, molecular weights and potencies of the *Stevia* sweeteners. (Prakash *et al.*, 2014)

Sweetener	R-Groups in Backbone Figure Above		Formula	Molecular Weight (g/mol)	Potency *
	R_1	R_2			
Rebaudioside A	β-glc-	(β-glc)$_2$-β-glc-	$C_{44}H_{70}O_{23}$	967.01	200
Rebaudioside B	H	(β-glc)$_2$-β-glc-	$C_{38}H_{60}O_{18}$	804.88	150
Rebaudioside C	β-glc-	(β-glc, α-rha-)-β-glc-	$C_{44}H_{70}O_{22}$	951.01	30
Rebaudioside D	β-glc-β-glc-	(β-glc)$_2$-β-glc-	$C_{50}H_{80}O_{28}$	1129.15	221
Rebaudioside E	β-glc-β-glc-	β-glc-β-glc-	$C_{44}H_{70}O_{23}$	967.01	174
Rebaudioside F	β-glc-	(β-glc, β-xyl)-β-glc-	$C_{43}H_{68}O_{22}$	936.99	200
Rebaudioside M	(β-glc)$_2$-β-glc-	(β-glc)$_2$-β-glc-	$C_{56}H_{90}O_{33}$	1291.3	250
Stevioside	β-glc-	β-glc-β-glc-	$C_{38}H_{60}O_{18}$	804.88	210
Steviolbioside	H	β-glc-β-glc-	$C_{32}H_{50}O_{13}$	642.73	90
Rubusoside	β-glc-	β-glc-	$C_{32}H_{50}O_{13}$	642.73	114
Dulcoside A	β-glc-	α-rha-β-glc-	$C_{38}H_{60}O_{17}$	788.87	30

glc = glucose; rha = rhamnose; xyl = xylose;

* Potency from Kinghorn, 1999; Prakash, 2008; JECFA, 2008.

1.3. Taste Profile of Steviol Glycosides

Stevioside and Reb A have a clean sweet taste at low SE levels (i.e., SE⩽6), but have an undesirable bitterness and licorice aftertaste at high SE levels (Young & Wilkens, 2007). Stevioside has even stronger bitterness than Reb A, so stevioside is rarely used in consumer products.

Recent focuses on Reb D and Reb M, the two minor glycosides in the stevia plant, show that Reb D and Reb M have a better flavor profiles compared to Reb A. Reb D exhibits a very low level of bitterness, compared to other glycosides (Hellfritsch *et al.*, 2012). Reb D was discovered in the 1970s (Kohda *et al.*, 1976), but Reb M was a more recent discovery (Prakash, Chaturvedula, & Markosyan, 2013). A trained descriptive panel compared the sensory attributes of Reb M and Reb A at 8% SE and reported Reb M had less bitterness and astringency than Reb A (Prakash *et al.*, 2014). Prakash et al. (2014) also reported that Reb M had a similar sweetness level in water but higher sweetness perception in acidified solution (pH 3.2) than Reb A at 8% SE.

1.4. Next Generation Steviol Glycosides (Reb D & M)

Due to the limitation of the bitter aftertaste from Reb A, the food ingredient industry is moving to develop Reb D and M products in recent years to provide better sugar reduction alternatives. Table 2 shows the Reb D and M products currently available. Splenda® stevia sweetener is the only product that is accessible by consumers.

Table 2. Current Reb D and M products.

Product	Company
EverSweet™ (Reb D & M)	Cargill
BESTEVIA® Reb M	Ingredion
BESTEVIA® Reb D	Ingredion
TASTEVA® M	Tate & Lyle
SPLENDA® (Reb D & Erythritol)	Tate & Lyle
AVANSYA™ Reb M	Royal DSM

It is not commercially possible to produce Reb D and M by extracting from the plant currently, because they only constitute approximately 0.4-0.5% by dry leaf weight (Jackson *et al.*, 2009; Prakash *et al.*, 2014). However, the ingredient companies use yeast to convert sugars into Reb D and M via a microbial fermentation process, which makes the production of these desirable rebaudioside more cost-effective and enables the companies to bring them to the market. For example, Avansya™ Reb M sweetener is produced via a genetically modified yeast *Yarrowia lipolytica*, which expresses the same metabolic pathway of steviol glycosides (FDA, 2018b).

Even though the genetically modified yeast is filtered out and the final product is GMO-free, it cannot be labeled as "non-GMO". Besides that, fermentative rebaudioside cannot be labeled as "stevia leaf extract" because it is not plant-derived and can only be labeled as Reb D/M, Rebaudioside D/M, or steviol glycosides (Watson, 2018). From a marketing standpoint, the inability to label as a leaf extract could be a downside because consumers may not be able to associate these terms with stevia. Furthermore, stevia sweetener produced by fermentation may be unattractive to consumers who only want naturally sourced sweetener, said PureCircle (Watson, 2017). Thus companies such as Cargill and PureCircle, are working on breeding stevia with higher levels of Reb D and M (Watson, 2018).

1.5. Food Applications

The leading sources of added sugar in the United States are beverages, desserts, candy, baked goods, and dairy products (HHS & USDA, 2015) and stevia has been used as a sweetener in all of these food categories. Table 3 shows the reported maximum steviol glycosides use-levels in foods (FAO, 2016).

PureCircle (2019) stated the new products sweetened with stevia launched in 2017 increased 11% and then additionally increased by 31% in 2018, based on Mintel Global New Products Database. There was a 36% increase for beverages and 27% for foods containing stevia in 2018. The reason stevia is used more in beverages could be that beverages do not have much texture and mouthfeel. However, in baked goods, the formulators need to consider browning, softness, and all other sensory attributes of the product as sugar not only provides sweetness, but also contributes to texture, mouthfeel, and viscosity (Samuel *et al.*, 2018). In addition, beverages are the top source for added sugar (HHS & USDA, 2015) and also the top concern of added sugar source by consumers (Mintel, 2018).

Table 3. Reported maximum use-level of steviol glycosides (FAO, 2016)

Food type	**Reported maximum use-level (mg/kg)**
Beverages (soft drinks, fruit drinks)	600
Desserts	500
Yogurt	500
Cold confectionery	500
Sauces	1000
Pickles	1000
Delicacies	1000
Sweet corn	200
Bread	160
Biscuits	300

2. Sensory Evaluation

2.1. Consumer Tests

Consumer tests are primarily used to measure consumer acceptance or preference toward products. Representative consumers, generally more than 100, are selected to represent the targeted larger population. Some selection criteria include user group, age, gender, and income level. A 9-point hedonic scale [extremely dislike (1) to extremely like (9)] is a commonly used scale to determine the acceptable level of a sensory attribute of a product or hedonic impression on a product (Peryam & Pilgrim, 1957). A check-all-that-apply (CATA) question, which consumers select terms from a given list, is a simple way to understand consumer perception on certain attributes of the product (Adams et al., 2007; Ares et al., 2010).

Consumers are also capable of evaluating intensities (Moskowitz, 1996; Husson, Le Dien, & Pagès, 2001; Worch, Lê, Punter, 2010; Ares, Bruzzone, & Giménez, 2011). Two consumer panels (n1 = 218, n2 = 124) evaluated 10 attributes of 28 grape/raspberry beverages and the results showed that consumers could discriminate the products and the data was reproducible (Husson *et al.*, 2001). Perfumes were evaluated by naïve consumers (n=103) and a trained panel and both groups gave similar results in terms of discrimination and reproducibility (Worch *et al.*, 2010). Ares et al. (2011) reported that the intensity scores of consumers (n=86) and trained panel are similar when evaluating the five texture attributes of milk desserts.

2.2. Descriptive Analysis

Quantitative descriptive analysis (QDA) method (Stone et al., 1974; Stone & Sidel, 1992) usually consists of a small panel of 8-10 subjects for typical products and the panel is trained to objectively measure sensory attributes of a product. After insensitive training, the trained panel should be able to detect and describe the perceived sensory attributes. A 15-cm line scale is used

to evaluate intensities. Trained panel data can be used to aid in the interpretation of consumer data. Researchers can discover the relationship between product attributes and consumer acceptance by relating the two data and explain why consumers like or dislike a certain product.

Even though the panel is trained to respond like an instrument, they are still humans. Human errors, such as health conditions, emotion, and stress, can affect their ability to smell and taste. Fatigue limits the number of samples that members of a trained panel can taste in once. It is also time-consuming to screen and train panelists.

3. Electronic Tongue

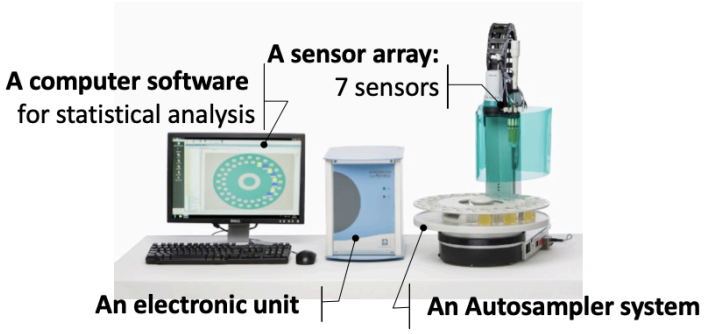
3.1. Electronic Tongue Components

Electronic tongue (E-tongue) is an analytical instrument developed for the sensory analysis of taste. It mimics human sensations and evaluates the taste by analyzing dissolved compounds in a liquid matrix. AlphaMOS (Toulouse, France) is one of the leading E-tongue companies in the world. Its E-tongue includes an autosampler system, seven sensors, a reference electrode, a stirring rod, an electronic unit, and computer software for statistical analysis. The components are shown in Figure 2.

The sensors are potentiometric sensors, which measure the voltage difference between the sensor membrane and the reference electrode and send electric signals to the computer for analysis. The sensors have different coated membranes that give each sensor different selectivity and sensitivity. The most updated sensor array #6 consists of AHS, PKS, CTS, NMS, CPS, ANS, and SCS sensors that each sensor measures multiple tastes. The old sensor array #5 had each sensor measures a single sensation: sweetness (SWS), sourness (SRS), saltiness (STS), bitterness (BRS), umami (UMS), metallic (GPS), and spiciness (SPS), but the production of these sensors was discontinued in late 2018. The old sensory array #1 for soft drinks, beers, and flavors

application and #2 for pharmaceutical formulation were discontinued and replaced by #6 as well. Current papers that used AlphaMOS's E-tongue all used old sensor arrays (Bleibaum *et al.*, 2002; Hruškar, Major, & Krpan, 2009; Waldrop & Ross, 2014; Jung *et al.*, 2017; Barnett, Diako, & Ross, 2019; Lee *et al.*, 2019; Schlossareck & Ross, 2019) and no paper published using #6 array to date.

Figure 2. The components of E-tongue from AlphaMOS (Toulouse, France)



3.2. Benefits and Limitations

One of the benefits of using E-tongue is its rapidity and high throughput of samples. It can evaluate a large number of samples in a relatively short amount of time without fatigue. Moreover, it assures assessors' safety by evaluating potentially dangerous substances or substances that are not allowed for human consumption yet, which strongly benefits the pharmaceutical industry. Besides that, it can be a more reliable sensory evaluation as it is automated and objective.

However, E-tongue also has some limitations. E-tongue can only analyze liquid without particles, so solid samples must be homogenized and extracted in order to analyze. For example, the supernatants of homogenized and centrifuged ground meat samples were used for analysis (Lee *et al.*, 2019). In addition, samples with high fat content could damage the sensors.

Therefore, lipid-free cheese extracts were made to analyze cheese samples (Lipkowitz *et al.*, 2018).

3.3. Food Applications

E-tongue has been used to evaluate many foods in many ways, such as to differentiate coffees (Dong *et al.*, 2017) and milk and yogurts (Hruškar *et al.*, 2009), to detect bitterness and astringency of green tea (Zou *et al.*, 2018), to evaluate apple juices (Bleibaum *et al.*, 2002), wines (Kirsanov *et al.*, 2012), Korean fermented soybean paste (doenjang) (Jung *et al.*, 2017), dry-aged beef (Lee *et al.*, 2019), cheeses (Lipkowitz *et al.*, 2018; Schlossareck & Ross, 2019), and sweet and salt solutions (Waldrop & Ross, 2014; Barnett *et al.*, 2019).

3.4. Statistical Analysis for Electronic Tongue Data

Principal component analysis (PCA) is a visualization plot of the data that presents data in clusters for easier interpretation. It gives information on the similarity and differences among samples. Discrimination index (DI) is determined by how well groups are separated and the size of each group. DI is close to 100 when the groups are clearly separated and the size of each group is small. However, when groups overlap, the DI is negative.

DI is strongly depended on the discrimination power of the sensors, which ranges from 0 to 1. A number closer to 1 means that the sensor has a good discrimination ability on samples and a number lower than 0.5 means that the sensor can hardly differentiate the samples.

4. PROP (6-n-Propylthiouracil) Taster Status

Phenylthiocarbamide (PTC) and 6-n-Propylthiouracil (PROP) are bitter-tasting compounds that have been used to test people's sensitivity to bitter taste. Supertasters are a group of people that are very sensitive to PTC and PROP, while non-tasters can barely detect the bitterness of them. Fox (1932) estimated that about 30% of the Caucasian population is non-

tasters, as the proportion varies among races and ethnicity (Guo & Reed, 2001). Within the 70% tasters, about 45% are medium tasters and 25% are supertasters (Zhao, Kirkmeyer, & Tepper, 2003).

Some studies showed that supertasters perceive not only stronger bitterness, but also other sensory attributes, such as sweetness (Drewnowski, Henderson, & Shore, 1997). Sucrose and saccharin solutions were perceived sweeter by medium and supertasters than non-tasters, (Drewnowski *et al.*, 1997). Zhao and Tepper (2007) found that in addition to bitterness and persistence of bitterness, supertasters perceived more sweetness, aftertaste, thickness, and overall flavor than non-tasters in carbonated soft drinks sweetened with high-intensity sweeteners. A study on coffee showed that supertasters rated the sourness, bitterness, and astringency higher than non-tasters (Masi *et al.*, 2015). However, there were other studies that did not find a relationship between PROP taster status and bitterness and/or sweetness (Horne et al., 2002; Rankin et al., 2003). Risso et al. (2014) investigated the effect of genetic variations on stevioside and found that the bitter taste receptor for PROP did not predict the bitterness perception of stevioside. Little research was done to investigate the effect of PROP taster status on the popular steviol glycosides, such as Reb A, D, and M.

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CHAPTER 2: CONSUMER-BASED SENSORY CHARACTERIZATION OF STEVIOL GLYCOSIDES (REBAUDIOSIDE A, D, AND M)

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Abstract

Rebaudioside (Reb) D and M are the recent focus of the food industry to address the bitter taste challenge of Reb A, which is the most commonly used steviol glycoside in natural sweetener stevia. This study evaluated the sensory characteristics of Reb A, D, and M, compared to 14% (w/v) sucrose, using a consumer panel and explored the relationship between 6-n-Propylthiouracil (PROP) taster status (i.e., non-tasters, medium tasters, supertasters) and the perceived intensity of sweet and bitter tastes of the three steviol glycosides. A total of 126 participants evaluated the intensities of in-mouth, immediate (5 seconds after expectorating), and lingering (1 minute after expectorating) sweetness and bitterness of 0.1% Reb A, D, M, and 14% sucrose and described the aftertaste of the sweeteners by using a check-all-that-apply (CATA) question. The results showed that in-mouth sweetness and bitterness of Reb D and M were not significantly different from sucrose, unlike Reb A which showed significant bitterness. However, Reb D and M showed more intense lingering sweetness than sucrose. The CATA analysis resulted that Reb D and M were closer to positive attribute terms and also to sucrose than Reb A, but Reb D and M were still considered *artificial*, which may cause negative perception. When comparing among PROP taster groups, no significant differences in the perceived sweetness and bitterness of the three steviol glycosides were found. This study generates important information about Reb A, D, and M for the food industry, especially working with products formulated to deliver reductions in sugar using a natural high-intensity sweetener, stevia.

1. Introduction

Artificial sweeteners are widely used in a variety of foods and beverages as a sugar substitute that mimics the effect of sugar on taste without adding calories. However, consumers have a negative perception of artificial sweeteners not only due to aversive sensations such as bitter off-taste [1,2] but also due to potential health risks and demand more natural options [3]. To respond to the consumers' demand for natural sugar substitutes with low/zero calories, the food industry has focused on stevia, which is a natural high-intensity non-nutritive sweetener. Stevia (*Stevia Rebaudiana* Bertoni) is a shrub native to Paraguay, and the leaves of stevia have been used to sweeten teas for hundreds of years in Paraguay and Brazil [4,5]. Stevia is the source of many different types of steviol glycosides, which are the sweetening compounds in stevia leaves [6]. Stevioside and rebaudioside (Reb) A are the major sweet compounds among the steviol glycosides [6] and are the most widely used steviol glycosides on the market according to a Mintel Global New Products Database (GNPD) product search [7]. However, stevioside and Reb A exhibit bitter and licorice off-taste [8–13], which pose challenges to product formulation.

To overcome the taste challenges of stevioside and Reb A, the researchers and food industry have looked into other minor steviol glycosides in the stevia leaves to provide better sugar reduction solutions. Several studies have reported that the two minor steviol glycosides, Reb D and M elicit significantly less bitterness with better sweetness than Reb A and also work well in products without sacrificing the taste [14–19]. Prakash et al. [16] reported that Reb M had less bitterness and astringency than Reb A. Most of the studies investigating sensory characteristics of steviol glycosides were conducted within a specific range of 5-10% sweetness equivalency related to sucrose (SE) [8,10,11,13,16]. Little research was done at high

concentrations for high-sugar applications such as frozen desserts, which generally contain 13-22% sucrose w/v [20], although sweetness potency of stevia heavily depends on the SE [13].

For sensory characterization of food products, sensory descriptive analysis using trained assessors is the most widely used method, but it is time-consuming to train panel. Less time consuming and more flexible methodologies such as check-all-that-apply (CATA) or intensity scales using consumers have been discussed in the last two decades [21]. It has been reported that consumers were capable of evaluating sensory attributes of various products, showing good agreement between consumers and trained assessors in terms of discrimination, reproducibility, and consensus [22–25]. Although Worch et al. [24] found that the trained panel showed greater consensus among each other, the larger sample size of consumers compensated for the higher variability. Moskowitz [26] suggested that a minimum of 40-50 people was needed to get stable averages, and the averages would not be affected by the base size much once the participant number exceeded 80. Ares et al. [27] also indicated that 80 consumers would be sufficient to get stable results when samples had large differences, but caution would be needed if samples had smaller differences or more complex attributes.

CATA is also often used to determine the characteristics of a product from a consumer perspective, which allows the consumers to describe a product by selecting terms from a given list that would match the product [28]. CATA questions have been used for a variety of foods and beverages [28–32], and these studies showed that CATA was a simple way to understand consumer perception on the sensory profile of a product.

Phenylthiocarbamide (PTC) and 6-n-Propylthiouracil (PROP) are bitter-tasting compounds that have been used to test people's sensitivity to bitter taste. Supertasters are a group of people who perceived intense bitter taste from PTC and PROP, while non-tasters barely detect

the bitterness of them [33]. It has been reported that individuals have different sensitivity to the aftertaste of high-intensity sweeteners [34], and thus researchers have long been interested in understanding the relationship between PROP status (e.g. non-tasters vs supertasters) and perceived taste intensities of high-intensity sweeteners. Bartoshuk [35] and Drewnowski et al. [36] found a significant difference between non-tasters and supertasters in the bitterness of saccharin at low concentrations. Zhao and Tepper [37] also suggested that supertasters perceived more bitterness and sweetness than non-tasters in carbonated soft drinks with artificial sweeteners, including sucralose, aspartame, acesulfame-K. However, Horne et al. [38] did not find a relationship between PROP taster status and the sweetness and bitterness of saccharin and acesulfame-K. Rankin et al. [39] failed to find any significant difference in bitterness between supertasters and non-tasters in cola drink sweetened with artificial sweeteners either. Risso et al. [40] looked into the effect of genetic variations on stevioside and found that the bitter taste receptor for PROP did not predict the bitterness perception of stevioside. However, little research was done to investigate the influence of PROP status on the perceived sweet and bitter taste intensities of novel steviol glycosides such as Reb D and M.

The primary objective of this study was to determine sensory characteristics of Reb A, D, and M, compared to 14% (w/v) sucrose, using a consumer panel. A secondary objective was to determine if there is a relationship between PROP taster status and the perceived intensities of the three steviol glycosides.

2. Materials and Methods

2.1. Materials

Sweeteners used in the study were 95% Reb A (ENLITEN® 30000015 High Intensity Sweetener, Ingredion, Westchester, IL), 95% Reb D (BESTEVIA® Reb D stevia leaf sweetener,

Ingredion, Westchester, IL), 95% Reb M (BESTEVIA® Reb M stevia leaf sweetener, Ingredion, Westchester, IL), and sucrose (Smidge & Spoon™, Kroger, Cincinnati, OH). PROP (6-n-propyl-2-thiouracil, #P3755, Sigma-Aldrich, St. Louis, MO), NaCl (Sigma-Aldrich, St. Louis, MO), and filter papers (1.5 dia. cm, VWR Scientific Products, West Chester, PA) were used to make paper disks for supertaster screening.

2.2. PROP Status Determination

The paper disks for PROP status determination were prepared following the method described by Zhao et al. [41]. Blank, NaCl, and PROP disks were prepared. Blank disks were used as the control. NaCl disks were made by placing filter papers in 1.0 mol/l NaCl solution for 30 seconds at room temperature and oven-dried for 1 hour at 121 °C (250 °F). 50-mmol/l PROP solution at boiling temperature was used for PROP disks.

PROP testing and classification were based on Zhao et al. [41] and Zhao and Tepper [37]. Michigan State University SONA Paid Research Pool (https://msucas-paid.sona-systems.com) was used to recruit participants with age between 18 and 55. Participants were instructed to rinse their mouth with water, taste the paper disk for 15 seconds or until the disk is wet, discard the paper disk, and then rate the perceived intensity of the taste on the labeled magnitude scale (LMS). The participants would taste a blank, a NaCl, and a PROP disk in order with a 30-second break in between samples to minimize fatigue and carryover. The set was repeated after a 5-minute break.

The LMS is a 100 mm quasi-logarithmic spacing vertical scale with verbal labels from "barely detectable" to "strongest imaginable" [42]. The scale set up was "no sensation" = 0, "barely detectable" = 1.5, "weak" = 6, "moderate" = 17, "strong" = 35, "very strong" = 52, and "strongest imaginable" = 100 [43,44]. The PROP score of participants was calculated based on

the mean of the two replicates. Because the LMS is not equal in spacing, the difference between two scores when both ratings are at the higher end is less than when ratings are at the lower end. If the difference between two ratings was bigger than 30 mm, or bigger than 40 mm when both ratings were higher than "very strong", the participant would be considered having bad reproducibility and would not be invited to the following water solution testing. Out of 224 participants, 27 were excluded.

Initially, "moderate" or below (≤ 17 mm on the LMS) and "very strong" or above (≥ 52 mm on the LMS) of PROP score were used to group participants into non-tasters and supertasters. The group means and 95% confidence interval were then calculated to set new cut-off scores. The new cut-off score for non-tasters was 10.3 and for supertasters was 70.7. Participants with scores in between were classified as medium tasters. When the PROP score of a participant was at a borderline, the NaCl score was used to help classify the person [41]. A participant would be classified as a non-taster if the person gave a non-taster borderline score and rated NaCl much higher than PROP (~ 30 mm difference on the LMS). When a participant was at the supertaster borderline and gave a much lower NaCl score than PROP, the person would be classified as a supertaster. Out of 197 remaining participants, 25 were identified as non-tasters and 55 were supertasters.

2.3. Subjects Demographics

Following the PROP test, participants were asked to provide some basic demographic information, including age, gender, ethnicity, educational level, weight, height, health condition, consumption frequency of low/zero sugar added products, consumption of sweeteners on a regular basis (at least once a month), and familiarity with stevia.

2.4. Consumer Testing

2.4.1. Samples and Sample Preparation

All solutions were prepared using deionized water and the concentration of the sample is expressed in g/L (w/v). Sucrose at 14% was chosen as the control. Reb A, D, and M at 0.09% were used in a preliminary test (n = 31) to determine the relative sweetness to 14% sucrose. The result showed that 0.09% Reb M were not statistically different from 14% sucrose in sweetness intensity ($P = 0.16$), but there was still a 1.1 difference on a marked 15-cm line scale with descriptors of "not at all" and "extremely" as endpoint anchors. Another preliminary test (n = 65) was then conducted to prove the sweetness equivalency of Reb M to sucrose, using 0.09% and 0.12% Reb M and 10% and 14% sucrose. The result indicated that both 0.09% and 0.12% Reb M were not significantly different from 14% sucrose ($P = 0.34$ and $P = 0.11$, respectively), with 0.09% Reb M closer to 14% sucrose at 0.5 difference on a 15-cm line scale, comparing to a 1.0 difference between 0.12% Reb M and 14% sucrose. Since 0.09% Reb M was again lower in intensity on the scale, Reb M at 0.10% was chosen for the consumer testing. Reb A and D at 0.10% were used to compare the sensory characteristics of the three steviol glycosides at the same concentration. Thus, samples used for the testing were Reb A, D, and M at 0.10%, and 14% sucrose. The consumer test lasted four days and fresh samples were made 1 day before testing each day. 10 ml of each solution was measured into a 1 oz soufflé cup and stored in the refrigerator (4 °C) prior to serving.

2.4.2. Testing Procedure

This study was approved by the University Institutional Review Board of the Michigan State University (East Lansing, MI) [Study ID: STUDY00004019]. SIMS 2000 software (SIMS Sensory Software, Morristown, NJ, USA) was used to create and administer the questionnaire.

Consumers were instructed to rate the sweetness and bitterness intensities of the solutions on a 15-cm line scale three times, which were while the solution was in the mouth, 5 seconds after expectorating it, and 1 minute after expectorating it. Consumers were asked to pinch their nose while holding the solution in the mouth to focus on the taste. The sweet and bitter tastes perceived at this time would be called *in-mouth sweetness and bitterness* throughout this paper. The perceived intensities of sweet and bitter tastes 5 seconds after expectorating would be referred to as *immediate sweetness and bitterness*. A check-all-that-apply (CATA) question on the aftertaste was followed after evaluating the immediate tastes, including terms collected from an open-ended question in the two preliminary tests ($n_1 = 31$ and $n_2 = 65$), asking if the consumers noticed any aftertaste. The term *pleasant* was added to the list as a positive word, and s*picy* was added as an attention check to identify careless respondents and would be removed from the correspondence analysis. The final list of CATA consisted of 15 terms, which were *artificial, bitter, chemical, honey, licorice, metallic, minty, pleasant, pungent, spicy, sweet, tangy, tart, tingling,* and *vanilla*, and the terms were listed in alphabetical order. A 45-second break was enforced after the CATA question, which was before evaluating the sweet and bitter tastes 1 minute after expectorating. The perceived intensities would be considered as *lingering sweetness and bitterness*. Water and crackers were provided as palate cleansers in between samples.

2.5. Statistical Analyses

Data analysis was performed using XLSTAT (AddinSoft, New York, NY). Intensity data were analyzed using a one-factor ANOVA model. For CATA analysis, the frequencies of each attribute were counted. Cochran's Q test was performed for each attribute to compare the difference among samples. Multiple pairwise comparisons using critical difference (Sheskin)

were performed when the attribute was significant ($P < 0.05$). Correspondence analysis (CA) was generated to visually show the relationship between sensory attributes and samples. A two-way ANOVA model was used to determine the effect of PROP taster status, sweetener, and their interaction. Fisher's least significant difference (LSD) post hoc test was performed when $P < 0.05$. Agglomerative hierarchical clustering (AHC) was used as a second way to classify PROP groups. Pearson correlation test was performed, and correlation coefficients were calculated between PROP bitterness and sweet and bitter tastes of Reb A, D, and M combined over time (in-mouth, immediate, lingering sweetness and bitterness).

3. Results

3.1. Participant Characteristics

A total of 126 naïve consumers completed the study, with an average age of 23 ± 1.7 years and an average BMI of 24.7 ± 4.6 kg/m^2 based on self-reported height and weight. None of the participants had heart disease, cancer, or diabetes. The socio-demographics of participants are shown in Table 4. The majority were female (72.2%) and 60.3% of the participants identified themselves as white. Table 5 listed out the responses of sweetener consumption behavior questions. Sucrose (81.0%) and honey (69.8%) were the most commonly consumed sweetener on a regular basis (at least once a month), followed by stevia (19.8%), sucralose (19.0%), and aspartame (19.0%), which were high-intensity sweeteners. Other sweeteners consumed (8.7%) included maple syrup, brown sugar, xylitol, high fructose corn syrup, and acesulfame K. Sixty-seven percent of participants consumed low or zero sugar added products at least once a month. More than half of the participants (54.8%) said they were somewhat or very familiar with stevia.

Table 4. Socio-demographic characteristics of participants (n = 126).

Variable	Definition	Frequency	%
Gender			
	Male	35	27.8%
	Female	91	72.2%
Ethnicity			
	White	76	60.3%
	Hispanic or Latino	5	4.0%
	Asian or Pacific Islander	33	26.2%
	Black or African American	7	5.6%
	Native American or American Indian	0	0.0%
	Other	3	2.4%
	Prefer not to respond	2	1.6%
Education			
	Less than high school	0	0.0%
	High school diploma or GED	29	23.0%
	2-year college degree	4	3.2%
	4-year college degree	50	39.7%
	Graduate degree (Master`s, Doctorate, etc.)	43	34.1%

Table 5. Participants' sweetener consumption behavior (n = 126).

Characteristic	Definition	Frequency	%
Sweetener consumption[1]			
	Agave nectar	16	12.7%
	Aspartame	24	19.0%
	Erythritol	10	7.9%
	Honey	88	69.8%
	Monk fruit extract	9	7.1%
	Saccharin	12	9.5%
	Stevia	25	19.8%
	Sucralose	24	19.0%
	Sucrose	102	81.0%
	Others	11	8.7%
Low/zero sugar added product consumption frequency			
	More than 3 times a week	16	12.7%
	1-2 times a week	29	23.0%
	2-3 times a month	27	21.4%
	Once a month	12	9.5%
	Every other month	6	4.8%
	1-2 times per 6 months	15	11.9%
	Less than once a year	7	5.6%
	Almost never	14	11.1%
Familiarity with stevia			
	Very unfamiliar	25	19.8%
	Somewhat unfamiliar	21	16.7%
	Neutral	11	8.7%
	Somewhat familiar	55	43.7%
	Very familiar	14	11.1%

[1] This is a check-all-that-apply question.

3.2. Sensory Characteristics

3.2.1 Intensities of Sweet and Bitter Tastes

Table 6 summarizes the mean intensity ratings (± SEM) for four sweetener solutions evaluated by all participants. The decreasing trend in sweetness and bitterness intensities from in-mouth to immediate (5 seconds after expectorating the samples) to lingering (1 minute after

expectorating the samples) indicated that consumers followed the directions and evaluated the samples correctly, since a fading in intensity over time was expected.

Table 6. Mean intensity scores (± SEM) of sweetener solutions by participants (n=126).

Sweetener	Sweetness[1]			Bitterness[2]		
	In-mouth	Immediate	Lingering	In-mouth	Immediate	Lingering
Sucrose	8.3 ± 0.3[3] a[4]	7.1 ± 0.3 b	3.6 ± 0.3 b	0.8 ± 0.1 b	0.6 ± 0.1c	0.4 ± 0.1b
Reb A	7.2 ± 0.3 b	6.5 ± 0.3 b	4.3 ± 0.3 b	3.5 ± 0.3 a	3.5 ± 0.3 a	1.6 ± 0.2 a
Reb D	7.8 ± 0.3 ab	7.2 ± 0.3 b	4.5 ± 0.3 ab	1.1 ± 0.2 b	1.3 ± 0.2 b	0.6 ± 0.1 b
Reb M	8.6 ± 0.3 a	8.2 ± 0.3 a	5.3 ± 0.3 a	1.0 ± 0.2 b	0.9 ± 0.1 bc	0.6 ± 0.1 b

[1,2]In-mouth tastes (sweetness and bitterness) were evaluated when the solution was in the mouth; Immediate tastes were evaluated 5 seconds after expectorating the sample; Lingering tastes were evaluated 1 minute after expectorating the sample. [3]Intensities were evaluated on a marked 15-cm line scale anchored with "not at all" to "extremely". [4]Different letters in the same column show the significant differences between sample means at $P < 0.05$ by Fisher's LSD.

The in-mouth sweetness of 14% sucrose and 0.1% Reb M were not significantly different ($P = 0.55$). The in-mouth sweetness of Reb D was slightly lower than sucrose but was still considered to be not different from sucrose ($P = 0.19$). Reb A showed significantly less in-mouth sweetness than Reb M and sucrose ($P < 0.01$ and $P < 0.05$, respectively). Reb M had the highest immediate sweetness among the samples and was significantly different from others. The sweetness of Reb M remained the highest after one minute. The lingering sweetness of Reb M (intensity = 5.3) was higher than Reb D (intensity = 4.5), but the difference was not significant ($P = 0.05$). Reb D was higher in lingering sweetness than sucrose (intensity = 3.6), but it was not significantly different ($P = 0.05$). The participants rated the in-mouth bitterness of sucrose, Reb D, and Reb M around 1, while the rating of Reb A was at 3.5 on a 15-cm line scale. The bitterness of Reb A persisted after 5 seconds (intensity = 3.5). Reb D was perceived to have more immediate bitterness than sucrose ($P < 0.05$), and there was no significant difference in the immediate bitterness between Reb M and sucrose ($P = 0.27$). While the lingering bitterness of sucrose, Reb D, and Reb M was at a minimum, Reb A still had detectable bitterness remaining (intensity = 1.6).

3.2.2. CATA

Table 7 summarizes the total counts of CATA attributes selected by the consumer panel (n=126) to describe the aftertaste of each sweetener solution. The term *sweet* was the most frequently used term, and *spicy* was the least, which were as expected. Significant differences among samples were found in 10 out of 15 attributes ($P < 0.05$). Reb A, D, and M were described as *artificial* more frequent than sucrose. The *bitter* and *chemical* tastes of Reb A were significantly higher than other sweeteners, and fewer participants considered Reb A as *sweet* and *pleasant*. *Honey* and *vanilla* were checked the most for sucrose, followed by Reb D and M, while Reb A was rarely associated with these two terms. *Licorice*, *metallic*, *minty*, *pungent*, *spicy*, *tangy*, *tart*, and *tingling* were rarely selected by participants, with no more than 15 counts for each sample. Among those 8 less-checked terms, *licorice*, *pungent*, *spicy*, *tangy*, and *tingling* were not significantly different among samples.

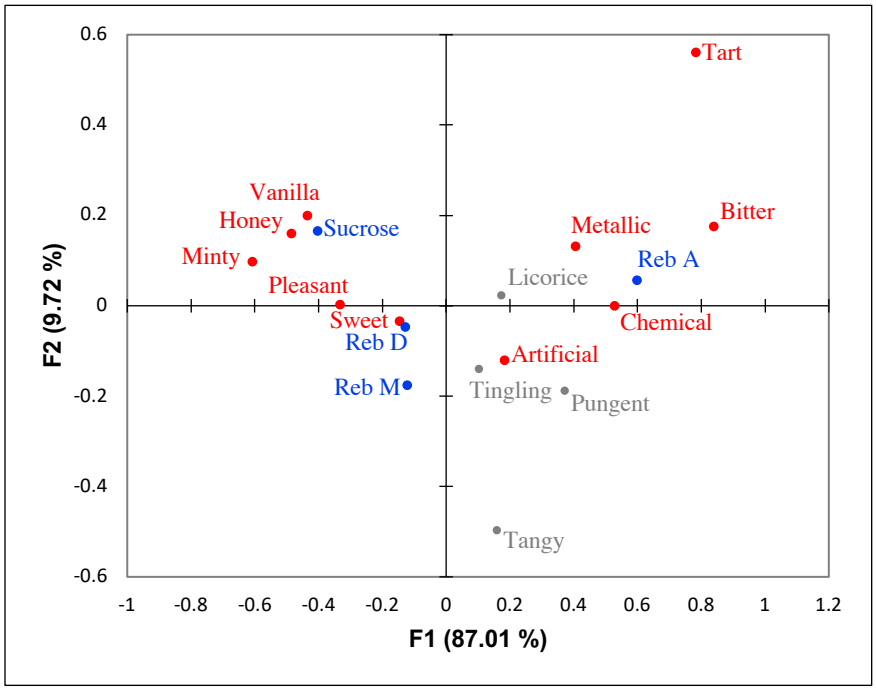
Table 7. Total counts of check-all-that-apply attributes for sweetener solutions.

Attribute	Sucrose	Reb A	Reb D	Reb M
Artificial***	38 b	83 a	64 a	69 a
Bitter***	8 b	66 a	17 b	12 b
Chemical***	9 b	42 a	17 b	18 b
Honey***	41 a	8 c	25 b	24 b
Licoricens	5	8	4	6
Metallic*	6 a	15 a	6 a	6 a
Minty**	7 ab	0 b	9 a	3 ab
Pleasant***	65 a	25 b	49 a	57 a
Pungentns	2	7	2	6
Spicyns	0	0	2	1
Sweet***	110 a	83 b	110 a	114 a
Tangyns	1	5	4	8
Tart*	2 ab	6 a	1 ab	0 b
Tinglingns	4	7	6	7
Vanilla***	27 a	7 b	15 ab	15 ab

* indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, and ns indicates no significant differences among samples. Different letters in the same row indicate the significant differences between sample means at $P < 0.05$ by Critical Difference (Sheskin).

The sensory attributes of sweeteners were summarized visually in Figure 3. The first two dimensions explained 96% of the variation. *Honey* and *vanilla* were associated with sucrose. Reb A was close to *metallic, bitter,* and *chemical*. Reb D and M were similar to each other and were closer to sucrose as compared to Reb A. Reb D and M were mostly associated with the positive words, but *artificial* was between Reb A and Reb D and M.

Figure 3. Correspondence analysis (CA) of sweeteners. Gray color indicates non-significant attributes; Red color indicates significant attributes; Samples are in blue.



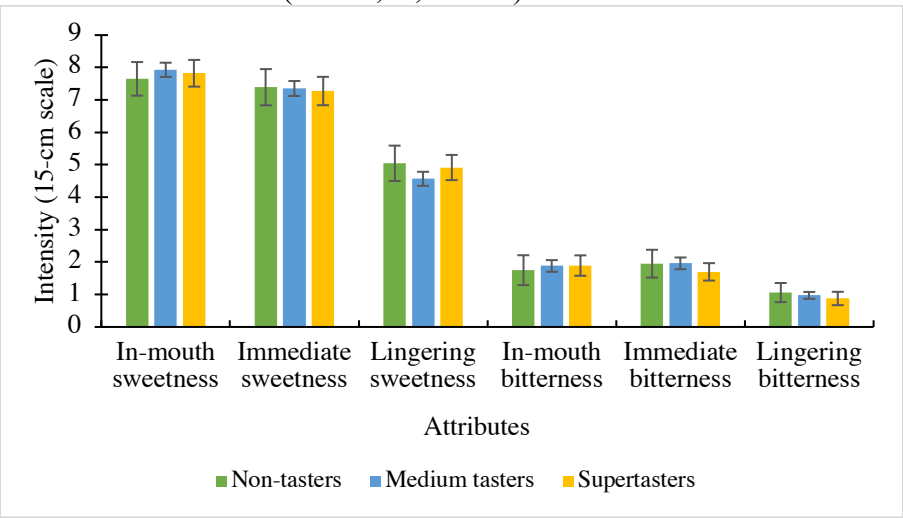
3.3. PROP Bitterness

3.3.1. PROP Taster Groups

Out of 126 participants who completed the consumer test, there were 15 non-tasters, 81 medium tasters, and 30 supertasters. The interaction between taster groups and samples was not significant for all taste evaluations of each Reb A, D, and M solutions. There was also no significant difference when examining the main effect of taster groups on perceived intensity

scores of sweet and bitter tastes of the three sweeteners combined over time (in-mouth, immediate, and lingering) (Figure 4).

Figure 4. The influence of PROP taster groups on the perceived intensities (± SEM), with sweeteners combined (Reb A, D, and M).



Due to the disproportional ratio of people in each taster groups, agglomerative hierarchical clustering (AHC) was used to group people based on their dissimilarity on the PROP rating (data not shown). Three groups were generated with 55, 44, and 30 people, corresponding to low, medium, and high-sensitive clusters, respectively. However, no significant difference in the main effect of clusters was found.

3.3.2. Relationships with Perceived Intensities of Reb A, D, and M

Pearson correlation tests were conducted to determine the association between PROP bitterness and perceived intensities of sweet and bitter tastes of Reb A, D, and M over time (in-mouth, immediate, lingering). No significant relationships existed between PROP bitterness and the rated intensities of the three steviol glycosides ($P > 0.05$).

4. Discussion

The present study investigated the sweetness and bitterness of Reb A, D, and M compared to sucrose at a high sucrose equivalent level (14% w/v) using consumers. To compare with 14% (w/v) sucrose solution, the solution concentration of the three steviol glycosides was determined by two small scale consumer tests as a preliminary test (see 2.4.1 for details). Briefly, the sweetness of 0.1% (w/v) Reb M was proved to be not significantly different from a 14% sucrose solution, and the same concentration was used for Reb A and D to compare the sensory characteristics of the three steviol glycosides at the same concentration. Prakash et al. [16] estimated that Reb M was about 200-350 times sweeter than sucrose, and the sweet potency at 10% SE was calculated to be 159. In the present study, the sweet potency of Reb M at 14% SE was calculated as 140. This is in line with the model from Prakash et al. [16], sweet potency of high-intensity sweeteners tended to decrease as the sucrose sweetness equivalent level increased [45].

The three steviol glycosides showed significant differences in sweetness and bitterness at the same concentration (0.1% w/v). The in-mouth sweetness of Reb D and M at 0.1% were not statistically different from sucrose at 14%, while 0.1% Reb A was less sweet than sucrose. Reb A was significantly less sweet than Reb M as well ($P < 0.01$) but was not significantly different from the in-mouth sweetness of Reb D ($P = 0.24$) with a tendency of being less sweet. Reb D and M were not significantly different in in-mouth sweetness ($P = 0.06$), but there was also a clear tendency of Reb D to be less sweet than Reb M. These results were consistent with the previous studies investigating the sweetness of Reb A, D, and/or M at different concentrations showing that Reb M was the sweetest sweetener and Reb A was the least sweet sweetener among Reb A, D, and M at the same concentration [15,16,46].

The sweetness temporal profile of Reb M was studied by Prakash et al. [16], who compared the sweetness appearance time and extinction time to examine the change in perception over 3 minutes. The sweetness of Reb M elicited later and persisted longer than sucrose at 10% SE in water. The descriptive panel rated the lingering sweetness of Reb M higher than that of sucrose as well [16]. In the present study, Reb M had a similar in-mouth sweetness to sucrose, but the lingering sweetness was significantly higher than that of sucrose, which corresponded with Prakash's finding. Reb A was also found to have a longer extinction time than sucrose [13] and exhibited persistent flavor duration in the mouth [8]. When at a similar sweetness level (i.e., at 8% SE), the lingering sweetness of Reb A and Reb M were not different [16]. Even though, in this study, there was no significant difference in lingering sweetness between sucrose and Reb A (P = 0.12), the lingering sweetness of Reb A became higher than sucrose after being rated less sweet in-mouth, which suggested that if the sweetness of Reb A was at the same level as sucrose, the lingering sweetness might be significantly higher than sucrose. Reb D, like Reb M, also had a similar in-mouth sweetness to sucrose, but had marginally higher lingering sweetness than sucrose (P = 0.05). When comparing Reb D to Reb M, the lingering sweetness of Reb D was marginally less than that of Reb M (P = 0.05) similar to the in-mouth sweetness of Reb D that was almost significantly less than Reb M (P = 0.06). Although the lingering sweetness of Reb M seemed to be stronger than Reb D, it may due to its higher initial sweetness than Reb D.

The bitterness of Reb A stood out among the samples when consumers first tasted the sample and the bitterness continued to be significantly different from others even after one minute. Many other researchers have reported the bitterness of Reb A [13,15]. On the other hand, Reb D and M did not show much in-mouth bitterness and had a similar intensity to sucrose. Even though Reb D exhibited a significantly higher immediate bitterness than sucrose, it was still considered

low. A trained panel did not detect any significant bitter taste of Reb M when comparing to sucrose at 10% SE [16]. Hellfritsch et al. [15] and Ko et al. [47] indicated that Reb D elicited a lot less bitterness than Reb A. Our results confirmed that naïve consumers like trained assessors did not detect much bitterness from Reb D and M.

Based on the total counts of CATA and the CA, Reb A was associated with some negative perception terms, such as *bitter*, *chemical*, and *artificial*. The *bitter* and *chemical* attributes were significantly more selected for Reb A than Reb D, Reb M, and sucrose. The *bitter* attribute was in agreement with the bitterness intensity rating. The bitterness and chemical sensation of Reb A was reported by Fujimaru et al. [48] as well. Significantly less *sweet* was checked for Reb A than the other sweeteners, suggesting that the bitterness and chemical sensation might overshadow the sweetness of it. Reb D and M appeared to have good taste profiles because they were close to positive terms and sucrose. However, even though Reb D and M had low citations for *bitter* and higher citations for *pleasant* than Reb A, many participants still checked *artificial* significantly more frequent than sucrose. Waldrop and Ross [49] reported that consumers did not like stevia because of its association with *artificial* flavor. Thus, the *artificial* attribute may cause negative consumer perception of Reb D and M even though they are natural sweeteners without the aversive bitter aftertaste. Interestingly, the *artificial* attribute was also selected for sucrose by 38 participants. It is not common to drink pure sugar water in daily life, so the participants may not be familiar with the taste of sucrose solutions, and thus might select *artificial* for sucrose solution.

Licorice, *pungent*, *spicy*, *tangy*, and *tingling* were rarely selected by the participants and were not significant to discriminate the samples. Thus, these five terms may not be appropriate terms for consumers to describe the three steviol glycosides, even though *licorice* has been commonly used to describe the aftertaste of Reb A by researchers [10,13,50] and media [18]. The

licorice taste of Reb A did not exhibit at low SE levels, but was elicited at higher SE levels [13], and this was further proved by Reyes et al. [50] that Reb A at 0.1% had more notable *licorice* taste than at 0.012%. In this study, we did not find the correlation between *licorice* and Reb A at 0.1%, since only 8 people out of 126 selected it, which suggested that *licorice* may not be an appropriate term for consumers to describe the aftertaste of Reb A or to discriminate Reb A, D, and M.

The CATA analysis also found that *vanilla* and *honey* were associated with sucrose. A consumer survey showed that honey was the most popular sugar alternative, which was natural, and natural sweeteners were perceived better than artificial sweeteners in general [3]. In this study, those who checked *honey* for steviol glycosides might imply that the sample gave them a sense of natural. As for *vanilla*, Lavin and Lawless [51] showed that an added vanilla flavor enhanced the perception of sweetness in milk, and Wang et al. [52] also indicated a taste-aroma interaction between perceived sweetness and vanilla flavor in skim milk. Vanilla was congruent with sweetness, so participants might choose the term even though the attribute was not presented in the solution.

A secondary objective of this study was to investigate the influence of consumers' PROP taster status on the sweetness and bitterness of Reb A, D, and M. We found that there were no significant differences in the perceived sweetness and bitterness of Reb A, D, and M (in-mouth, immediate, and lingering) among PROP taster groups. Risso et al. [40] reported that there was no correlation between PROP bitterness and stevioside bitterness. Humans have about 25 bitter taste receptors from the taste 2 receptors (hTAS2Rs) gene family [53]. Each receptor responds to different compounds but may have overlapped molecular range [54]. The sensitivity to the bitterness of PROP/PTC is mainly associated with TAS2R38 bitter taste receptor [55,56]. TAS2R4 and TAS2R14 responded to the bitterness of stevioside and Reb A in vitro, while TAS2R38 did

not react [15]. Meyerhof et al. [54] sorted receptors into 4 groups, and both TAR2S4 and TAS2R14 were not in the same group as TAS2R38. The different responses in bitter taste receptors might explain why no relationship was found between PROP taster status and perceived bitterness intensity of Reb A, D, and M.

Some studies suggested that PROP bitterness sensitivity influenced other oral sensations, such as sweetness [36,37,57-61]. Drewnowski et al. [36] found a weak and marginal significant difference in sweetness perception of sucrose and saccharin between PROP tasters and non-tasters, and the difference was more significant at lower concentrations. Allen et al. [59] reported that the sweetness of acesulfame potassium was positively associated with PROP bitterness. A large sample size study ($n > 1,500$) found a weak association between sweetness and PROP bitterness, suggesting that a bigger size sample is required to detect weak association with PROP [60]. A recent study confirmed that PROP bitterness was positively correlated with sweetness of sucrose [61]. However, some of the previous studies also indicated that there was no relationship between PROP sensitivity and sweet taste responsiveness [62-64]. Here, we found no significant differences in perceived sweetness intensity among PROP groups and further, no correlation between PROP bitterness and sweetness of the steviol glycosides. In this study, the test stimuli were singles (i.e., each sweetener solution), but the aftertaste of the three steviol glycosides, especially the sweet-bitter Reb A at a high concentration, might cause difficulties for participants to evaluate intensities of sweet and bitter tastes. Horne et al. [38] reported that sweet-bitter stimuli might be more difficult to evaluate than single taste stimuli due to taste-taste interactions. Expansive, linear, and compressive phases of psychophysical functions could be used to predict how taste stimuli would behave when mixed at low, medium, and high intensity/concentration [65]. For example, perceptual enhancement and suppression has been extensively reported at low

and high intensity/concentration mixtures, corresponding to the expansive phase and compressive phase of the psychophysical function, respectively [65]. Ly and Drewnowski [63] showed a reduced difference in bitterness between PROP taster groups was found when the caffeine solution was sweetened, even though PROP tasters rated caffeine solution without sweetener as more bitter than non-tasters [63]. The perceptual suppression as a result of sweet-bitter interaction at a high intensity/concentration may explain no differences in perceived sweetness intensity among PROP groups and no correlations between PROP bitterness and perceived sweetness of Reb A, D, and M.

One possible limitation of the study was that consumers did not swallow the solution, which limited the number of taste buds utilized for the evaluation. Taste buds are distributed not only in the mouth but also in the throat [66]. Consumers were asked to expectorate the sample to reduce fatigue, however, the swallowing sensation could be different and might impact the perceived intensities. No hedonic question was asked in this study because it might be difficult for naïve consumers to rate the likings of pure sweetener solutions when the solutions were not regularly consumed in daily life. However, no association could be drawn between the negative CATA attributes and the likings of the sweeteners. Another limitation was the disproportional size of PROP groups, which only had 15 non-tasters. The data from non-tasters might be less variable if more non-tasters were recruited.

5. Conclusions

The present study investigated the sensory profile of Reb A, D, and M at 14% SE using a consumer panel, and the influence of PROP taster groups on the perceived sweet and bitter tastes of the three glycosides. Reb D and M had sensory profiles that were closer to sucrose, compared to Reb A, but were still associated with negative sensation, such as *artificial*, which may cause

negative perception toward Reb D and M. The lingering sweetness of Reb D and M was also a concern. The sensory characteristics of Reb A, D, and M in this study can be used as a reference for the food industries working with steviol glycosides in high-sugar applications, such as frozen desserts. Furthermore, there were no significant differences among non-tasters, medium tasters, and supertasters on the perceived sweetness and bitterness of Reb A, D, and M as well as no significant correlations between PROP bitterness and perceived sweet and bitter tastes, suggesting that supertasters who experience more intense taste sensations may not report aversive sensations from stevia. Further studies on the consumer acceptance of Reb A, D, and M are needed to determine if these characteristics would affect the likings of these sweeteners. Since the sweeteners may perform differently in a food matrix than in aqueous solution, more research using steviol glycosides in final food products are needed to determine the sensory profile and acceptance of them.

Author Contributions

R.T. contributed to study design, data collection, data analysis, and manuscript writing. S.C. contributed to study design, data collection, data analysis, and critical revision of the manuscript. S.C. supervised the project.

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 3: NEW PROTOCOL FOR THE #6 SENSOR ARRAY OF ASTREE ELECTRONIC TONGUE TO DISCRIMINATE *STEVIA REBAUDIANA* LEAVES

Abstract

Electronic tongue (E-tongue), which is an analytical instrument for the sensory evaluation of taste, has been used to analyze a variety of foods and beverages. However, a new protocol is needed for the E-tongue analysis sequence and data selection with the most updated #6 sensor array. The objective of this study was to develop a protocol for E-tongue to discriminate *Stevia rebaudiana* leaves for its potential use in stevia breeding programs as a method of testing flavor quality. The E-tongue analysis sequence was set to repeat 6 times to obtain 6 data points and remove the first two and last data points because of sensor error. The run would be repeated, and the second run would be used for analysis. The relative standard deviation (%RSD) needed to be lower than 5% to be considered good for further analysis and the sensor selection was depended on the discrimination power. Rebaudioside (Reb) A, D, and M, which are three steviol glycosides in the stevia leaves, were used for the protocol validation. Seven stevia leaf samples from each of the two stevia breeding programs were analyzed by E-tongue to test its ability to discriminate stevia leaf samples from different lines. The results showed that E-tongue successfully separated Reb A, D, and M and distinguished among stevia leaf samples by using the new protocol, which meant that E-tongue has the potential to be used in stevia breeding programs for flavor selection. Further analyses, such as human panel, are needed to correlate to E-tongue data and determine if E-tongue can be used as a fast and accurate way to determine the flavor profile of stevia leaf samples.

1. Introduction

Electronic tongue (E-tongue) is an analytical instrument developed for the sensory analysis of taste. It mimics human sensations and evaluates the taste by analyzing dissolved compounds in a liquid matrix. AlphaMOS (Toulouse, France) is one of the leading E-tongue companies in the world. Its E-tongue includes an autosampler system, seven sensors, a reference electrode, a stirring rod, an electronic unit, and computer software for statistical analysis. The sensors are potentiometric sensors, which measure the voltage difference between the sensor membrane and the reference electrode and send electric signals to the computer for analysis. The sensors have different coated membranes that give each sensor different selectivity and sensitivity (AlphaMOS, 2020).

E-tongue has been used in foods and beverages in many different ways, such as to differentiate coffees (Dong et al., 2017) and milk and yogurts (Hruškar et al., 2009), to detect bitterness and astringency of green tea (Zou et al., 2018), to evaluate apple juices (Bleibaum et al., 2002), wines (Kirsanov et al., 2012), Korean fermented soybean paste (doenjang) (Jung et al., 2017), dry-aged beef (Lee et al., 2019), cheeses (Lipkowitz *et al.*, 2018; Schlossareck & Ross, 2019), and sweet and salt solutions (Waldrop & Ross, 2014; Barnett *et al.*, 2019). One of the limitations of the E-tongue is that it can only analyze liquid without particles. However, solid samples can still be analyzed by homogenization and extraction. For example, ground meat samples were homogenized and centrifuged to get the supernatants for analysis (Lee et al., 2019). The E-tongue sensors could also be damaged if the sample is high in fat. Therefore, lipid-free cheese extracts were made to analyze cheese samples (Lipkowitz et al., 2018; Schlossareck & Ross, 2019). When E-tongue is used solely, it does not tell much other than differentiating the samples. For example, Hruškar et al. (2009) differentiated 5 different brands of milk and yogurt

samples using E-tongue only and stated that E-tongue could serve as a fast and accurate analysis instrument for evaluation of samples in the dairy industry. On the other hand, when the E-tongue is used with other analytical methods, researchers can correlate quality parameters or sensory data to E-tongue data and even predict attributes (Bleibaum et al., 2002; Dong et al., 2017; Kirsanov et al., 2012; Waldrop & Ross, 2014). For example, Dong et al. (2017) used E-tongue to classify the varieties of 126 roasted coffee beans successfully and the R^2 of the predictive model based on the E-tongue data ranged from 0.879 to 0.933 for calibration set and from 0.855 to 0.892 for prediction set when correlating to flavor quality parameters (pH, total solids (TS), total titratable acidity (TA), total soluble solids (TSS), and TSS/TA ratio). Waldrop and Ross (2014) evaluated sweetener solutions using E-tongue, a trained panel, and consumers and showed that the E-tongue data was strongly correlated with 10 of the 16 intensity scores rated by consumers and trained panel, with R^2 ranging from 0.79 to 0.95, suggesting that E-tongue has a potential to be used to predict sensory characteristics of unknown samples.

Previous studies using AlphaMOS's E-tongue used earlier generation sensor arrays, such as sensor array #5 (Barnett et al., 2019; Jung et al., 2017; Lee et al., 2019; Lipkowitz et al., 2018; Schlossareck & Ross, 2019; Waldrop & Ross, 2014). The sensor array #5 has each sensor measured a single sensation: sweetness (SWS), sourness (SRS), saltiness (STS), bitterness (BRS), umami (UMS), metallic (GPS), and spiciness (SPS), but it was discontinued by the manufacturer in late 2018. The sensory array #1 for soft drinks, beers, and flavors application, which was used by Bleibaum et al. (2002) and Dong et al. (2017), and #2 for pharmaceutical formulation were discontinued and replaced by the most updated sensor array #6 as well. The #6 consists of AHS, PKS, CTS, NMS, CPS, ANS, and SCS sensors that each sensor measures

multiple tastes at the same time to increase measurement accuracy. To our knowledge, no research study to date has used the sensor array #6 yet.

The protocols using earlier generation arrays (sensory arrays #1, 2, 5) were vague in most of the previous studies. Some researchers evaluated samples in triplicate and took the average (Bleibaum et al., 2002; Dong et al., 2017). Waldrop and Ross (2014) ran the samples in triplicate and replicated to get 6 data points, and then removed 3 outliers, which were the first and/or second samplings due to sensor error. Lipkowitz et al. (2018) and Schlossareck and Ross (2019) evaluated the samples in 6 loops but used only 3 data points in the result, assuming the other 3 data points were removed. These studies indicate that the sensors may not be stable all the time. For the new sensory array #6, a new evaluation protocol is needed not only for the E-tongue analysis sequence but also for a data selection basis to improve repeatability and reproducibility.

Stevia, which is a natural high-intensity non-nutritive sweetener, has been the focus of the food industry to respond to the consumers' demand for natural sugar substitutes with low/zero calories. Steviol glycosides are the sweetening compounds in stevia leaves and stevioside and rebaudioside (Reb) A are the major steviol glycosides (Kinghorn, 2002). Plant breeders have worked on developing new varieties of stevia plants with higher Reb A and less stevioside content because stevioside has bitter aftertaste whereas Reb A has better flavor profile (Yadav et al., 2011). However, studies showed that Reb A exhibited bitter off-taste as well (Gwak et al., 2012; Kim et al., 2015; Li et al., 2015; Medeiros et al., 2019). In recent years, Reb D and M, which are two minor steviol glycosides, have been reported to elicit significantly less bitterness with better sweetness than Reb A (Hellfritsch et al., 2012; Prakash et al., 2014). GLG, a company based in Canada, has worked on breeding stevia plants with more Reb D and M contents (Watson, 2015). Few breeding programs evaluate the flavor profile of stevia varieties. It

could be time-consuming to develop a panel as a descriptive panel usually requires 40 to 120 hours of training (Meilgaard et al., 2015). A large number of stevia samples will need to be evaluated. Besides, human panels get sensory fatigue very easily and need to take breaks after evaluating 4 or 5 samples (Gwak et al., 2012; Kim et al., 2015; Waldrop & Ross, 2014). However, it is important to know the sensory profile of different stevia varieties to select the desirable ones. Many studies determined the steviol glycosides content of stevia leaves by high-performance liquid chromatography (HPLC) (Ahmed & Dobberstein, 1982; Bondarev et al., 2003; Gardana et al., 2010; Hashimoto et al., 1978; Kolb et al., 2001; Makapugay et al., 1984), but the sample preparation and analysis were rather complex (Dong et al., 2017; Kirsanov et al., 2012). If E-tongue could quickly differentiate glycoside profiles of different stevia varieties, it could save a lot of time for breeding programs.

Thus, the objective of this study was to develop a protocol for E-tongue to discriminate *Stevia rebaudiana* leaves for the potential of using the E-tongue in stevia breeding programs as a method of testing flavor quality.

2. Samples and Sample Preparation

All sample solutions were made on the day of analysis and kept at room temperature. About 25 ml of each sample was used to fill up the sample beaker to the marked line.

2.1. Part 1: Protocol Validation

Three steviol glycosides were used to validate our new protocol for the #6 sensory array, which were 95% Reb A (ENLITEN® 30000015 High Intensity Sweetener, Ingredion, Westchester, IL), 95% Reb D (BESTEVIA® Reb D stevia leaf sweetener, Ingredion,

Westchester, IL), and 95% Reb M (BESTEVIA® Reb M stevia leaf sweetener, Ingredion, Westchester, IL) at 0.1% (w/v).

2.2. Part 2: Application for Stevia Leaves in Breeding Programs

Dried and ground stevia leaf samples were obtained from the Dr. Bipul Biswas' laboratory in the Department of Agricultural Sciences Academic Department at Fort Valley State University (FVSU) in Georgia and the Dr. Ryan Warner's laboratory in the Department of Horticulture at Michigan State University (MSU). The seven FVSU stevia line samples were selected based on 'cold tolerance', which showed different levels of tolerance to cold in the production field. The MSU stevia line samples were selected from an open-pollinated population. To confirm the E-tongue's ability to discriminate the stevia leaf samples, seven MSU stevia lines (11-223, 11-547, 12-05-140, 12-05-005, 12-11-041, Kenya, Paraguay) were used for this study, which showed the difference in steviol glycoside profiles analyzed following the method described by Shafii et al. (2012).

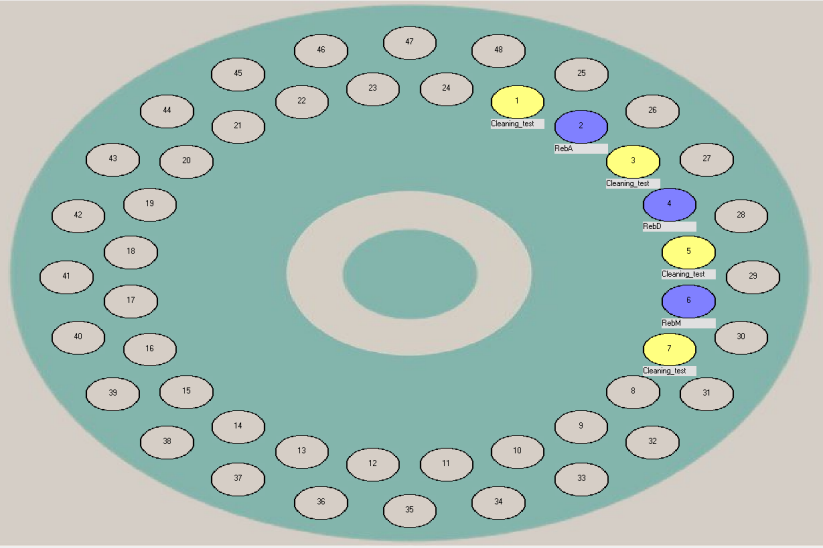
The stevia leaf samples were extracted by the microwave extraction method described by Teo et al. (2009); the microwave power was set at 700 W and the temperature was kept at 100°C for 20 min. The FVSU samples were prepared by placing 0.078 g of stevia leaves in 20 mL of deionized water for extraction and then diluted to 50 ml for analysis. For the MSU samples, 0.2 g of each sample in 20 mL deionized water was used for extraction and then diluted to 50 mL for analysis.

3. E-Tongue Analysis

3.1. Analysis Sequence

The sensors consisting of 7 sensors (AHS, PKS, CTS, NMS, CPS, ANS, and SCS) were immersed in water to hydrate for at least 30 minutes before running the E-tongue. Conditioning, calibration, and diagnostic, using 0.01M hydrochloric acid, sodium chloride, and sodium glutamate standard solutions, were conducted prior to analysis. The analysis sequence was repeated six times to get six data points. The number of beakers with deionized water in between samples for cleaning purposes was depended on the type of samples. One cleaning was sufficient for aqueous solutions, and 3 cleanings would be needed for thicker samples, such as melted ice cream. If too many cleanings were placed in between samples (i.e., 3 cleanings for aqueous solutions), some drift could happen, which would influence the accuracy of the sensors, since the sensors were constantly changing. In this study, one cleaning was used in between steviol glycoside solutions or stevia leaf extract samples. The acquisition time was 120 seconds and the cleaning step was 30 seconds. Figure 5 showed the analysis sequence of protocol validation using Reb A, D, and M on the E-tongue software. The run was replicated, and the second run was used for data analysis since the sensors did not have good stability in the first run.

Figure 5. E-tongue analysis sequence for protocol validation using Reb A, D, and M. Yellow color indicates cleaning steps; Purple color indicates samples.



It is important to note that the sample must be at room temperature for E-tongue to analyze accurately. When possible, it is better to run all the samples in the same sequence because the sensors will not be in the same state. The stirrer should be periodically checked if it stirs properly. If the stirrer rotates slower than normal, adjusting the cable connected to the stirrer should fix the problem.

3.2. E-Tongue Data Analysis

For general data analysis, the first two and last data points were removed due to sensor error, and the 3rd, 4th, and 5th data points were used. Relative standard deviation (%RSD) and discrimination power were used to determine the quality of data. The preciseness of the data was determined by %RSD, which equaled to standard deviation divided by mean and multiply by 100. If the %RSD was less than 5%, the data were considered good. If it was greater than 5%, the samples would either need to be rerun or the sensor with greater than 5% RSD would be deleted, depending on the discrimination power. The discrimination power, which ranged from 0 to 1, indicated 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.

3.3. Statistical Analysis

The data was analyzed using Astree AlphaSoft software (Version 14, AlphaMOS). Principal component analysis (PCA) was generated to visually show the data and discriminate the samples. The discrimination was assessed by the discrimination index and the spread of the samples on the graph (AlphaMOS, 2020). The discrimination index was calculated based on the surface area of groups. When the groupings of the samples were distinct, the discrimination index was calculated using the first equation. When the samples were overlapped, the discrimination index was calculated using the second equation.

1. Di = 100 * [1-[(Surface(A)+Surface(B)+Surface(C)) / (Total Surface)]]
2. Di = - (∑ Intersection Surface / Total surface) * 100

4. Validation of Analytical Protocol

Reb A, D, and M were analyzed 7 times (2 runs on Day 1, 3 runs on Day 2, and 2 runs on Day 3) by using the analysis sequence mentioned in Section 3.1. To compare the 1st and 2nd run each day, the 3rd run on Day 2 will not be discussed further.

4.1. Sensor Stability

The sensors values of the second run on Day 1 were shown in Figure 6. There were differences in sensor values among the 6 data points, especially sensor SCS. Previous studies using sensory array #5 suggested that the first and/or second data points should be removed for data analysis due to sensor instability (Waldrop & Ross, 2014). We also verified that the first two

and the last data points were not stable with sensory array #6, so they were removed and the rest of the three were used for further analysis.

Figure 6. Sensor values of electronic tongue in the second run on Day 1. Blue indicates sample Reb A. Red indicates sample Reb D. Green indicates sample Reb M.

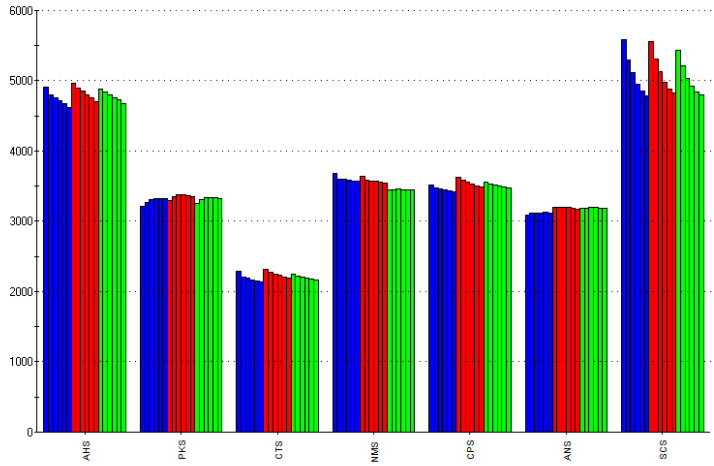


Table 8 shows the %RSD values of the first and second run with selected data points on Day 1. The %RSD of sensor PKS was higher than 5% in the first run, which meant that the first run could not be used for further analysis. However, the second run was improved, in which 6 out of the 7 sensors had %RSD lower than 1%. A huge improvement in sensor PKS and CPS was observed. The comparison of the first and second run in Day 2 and 3 are shown in Table 9 and 10, respectively. Both days showed the same trend that the second run had lower %RSD than the first run. Even though %RSD below 5% is acceptable, our recommendation is to have %RSD less than 1% to get better repeatable data. The discrimination power of the sensors is shown in Table 11. Comparing the second run to the first run of each day, the discrimination power was highly improved in the second run.

Table 8. Relative standard deviation of electronic tongue sensors in the first and second run on Day 1 (using 3rd, 4th, and 5th data points), with Reb A, D, and M samples.

Sensors	First run			Second run		
	Reb A	Reb D	Reb M	Reb A	Reb D	Reb M
AHS	1.28	1.13	0.94	0.65	0.87	0.64
PKS	5.10	5.18	5.78	0.12	0.22	0.04
CTS	0.55	0.27	0.20	0.64	0.78	0.51
NMS	0.07	0.02	0.07	0.20	0.29	0.03
CPS	4.15	3.95	3.68	0.39	0.58	0.42
ANS	0.42	0.35	0.23	0.10	0.23	0.03
SCS	3.33	3.06	2.94	2.11	1.10	1.64

Bold indicates sensors that had big difference (>3.00 % RSD) in %RSD between the two runs.

Table 9. Relative standard deviation of electronic tongue sensors in the first and second run on Day 2 (using 3rd, 4th, and 5th data points), with Reb A, D, and M samples.

Sensors	First run			Second run		
	Reb A	Reb D	Reb M	Reb A	Reb D	Reb M
AHS	0.54	0.57	0.70	0.04	0.11	0.11
PKS	1.40	1.57	1.52	0.18	0.25	0.20
CTS	1.23	1.46	1.45	0.10	0.16	0.08
NMS	0.96	1.27	1.35	0.24	0.20	0.11
CPS	1.01	1.14	1.28	0.15	0.24	0.19
ANS	1.64	1.62	1.56	0.06	0.09	0.09
SCS	0.55	0.67	0.80	0.09	0.03	0.08

Table 10. Relative standard deviation of electronic tongue sensors in the first and second run on Day 3 (using 3rd, 4th, and 5th data points), with Reb A, D, and M samples.

Sensors	First run			Second run		
	Reb A	Reb D	Reb M	Reb A	Reb D	Reb M
AHS	0.88	1.03	0.65	0.44	0.36	0.35
PKS	0.55	0.53	0.41	0.40	0.25	0.26
CTS	1.44	1.14	0.86	0.53	0.40	0.43
NMS	0.69	0.68	0.40	0.98	0.67	0.40
CPS	0.60	0.58	0.47	0.37	0.37	0.39
ANS	0.05	0.13	0.01	0.04	0.07	0.07
SCS	4.00	3.66	3.09	0.05	0.04	0.06

Bold indicates sensors that had big difference (>3.00 % RSD) in %RSD between the two runs.

Table 11. Discrimination power of the 7 electronic tongue sensors on Day 1, 2, and 3.

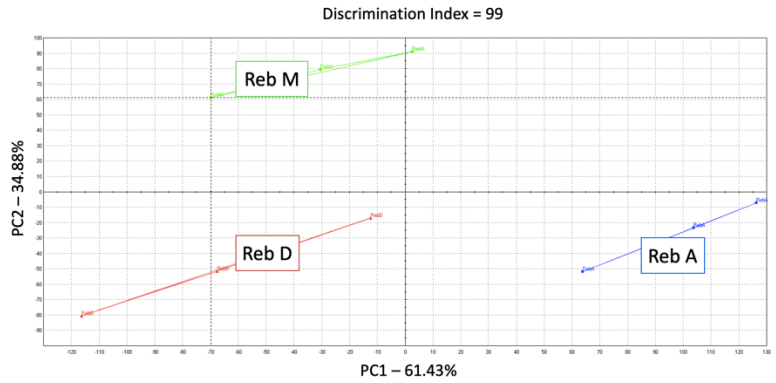
Sensors	Day 1*		Day 2		Day 3	
	First run	Second run	First run	Second run	First run	Second run
CPS	0.057	0.824	0.541	0.851	0.394	0.353
SCS	0.064	0.064	0.471	0.903	0.036	0.984
PKS	0.150	0.949	0.272	0.649	0.231	0.288
AHS	0.200	0.525	0.758	0.978	0.128	0.498
CTS	0.786	0.752	0.405	0.899	0.060	0.373
ANS	0.864	0.982	0.540	0.977	0.989	0.977
NMS	0.996	0.984	0.157	0.983	0.669	0.397

*Compare within the day.

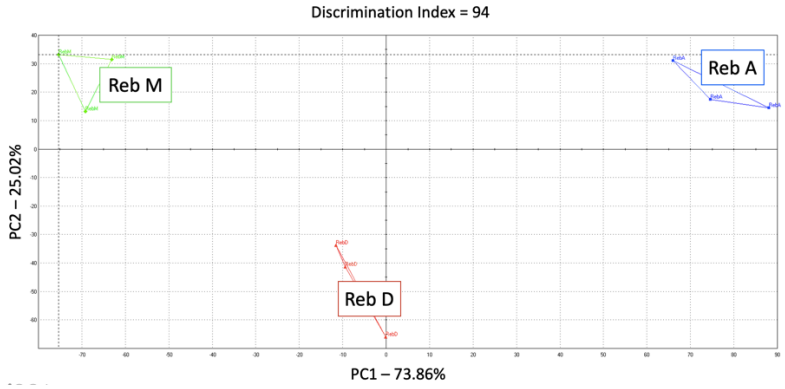
4.2. Discrimination of Reb A, D, and M

Figure 7 (a), (b), and (c) show the PCA of Reb A, D, and M from the second run on Day 1, 2, and 3, respectively. For Figure 7 (a), SCS with a low discrimination power of 0.064 was removed. The first two dimensions explained 96.31% of the variation. The discrimination index was 99, which indicated a good separation among the three steviol glycosides. On Day 2 and 3, 98.88% and 96.10% of the variation were explained by the first two dimensions, respectively. The discrimination index was 94 for both days. The second run on all 3 days showed similar positions of Reb A, D, and M in relation to PC1 and PC2, and the clear separation among Reb A, D, and M was found, which suggests that E-tongue is capable of discriminating Reb A, D, and M.

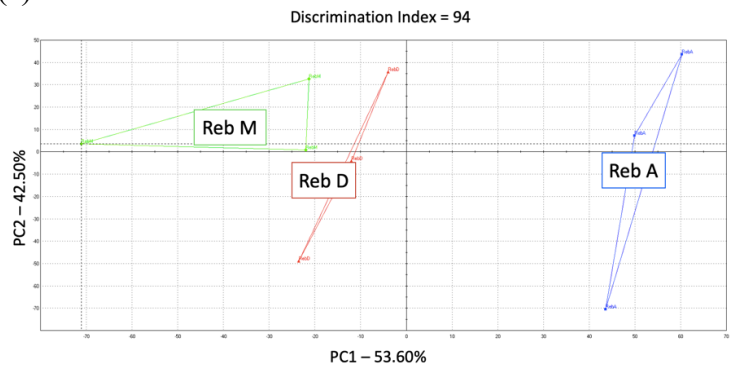
Figure 7. (a) Principal component analysis of steviol glycoside samples in the second run on Day 1 as evaluated by electronic tongue and accounting for 96.31% of the variability. The discrimination index is 99. (b) Principal component analysis of steviol glycoside samples in the second run on Day 2 as evaluated by electronic tongue and accounting for 98.88% of the variability. The discrimination index is 94. (c) Principal component analysis of steviol glycoside samples in the second run on Day 3 as evaluated by electronic tongue and accounting for 96.10% of the variability. The discrimination index is 94.



(b)



(c)



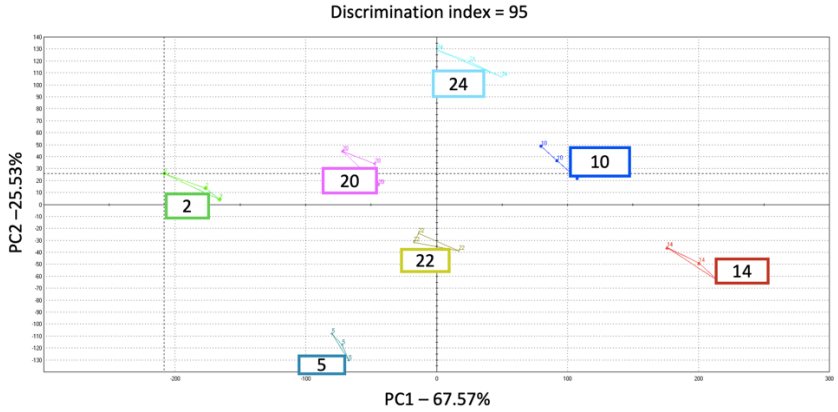
5. Discrimination of Stevia Leaf Samples

Among the seven sensors (AHS, PKS, CTS, NMS, CPS, ANS, and SCS), the CTS sensor had a poor discrimination power when evaluating stevia leaf samples, which suggested that CTS was not capable of distinguishing the stevia leaf samples. Thus, CTS was removed from the analysis.

5.1. Fort Valley State University Samples

Figure 8 shows the PCA of stevia samples from the FVSU. The first two dimensions explained 93.1% of the variation. The discrimination power of the six sensors (AHS, PKS, NMS, CPS, ANS, SCS) ranged from 0.946 to 0.998 and RSD were all below 0.5%; CTS with a low discrimination power of 0.129 was removed. The discrimination index was 95, which indicated that a clear separation among the samples was observed. This application shows the E-tongue's ability to discriminate stevia leaf samples.

Figure 8. Principal component analysis of stevia leaf samples from Fort Valley State University as evaluated by electronic tongue and accounting for 93.10% of the variability. The discrimination index is 95.



5.2. Michigan State University Samples

Figure 9 showed the PCA of stevia line samples from the MSU. The first two dimensions explained 80.9% of the variation. The discrimination power of the six sensors (AHS, PKS, NMS,

CPS, ANS, SCS) ranged from 0.672 to 0.948 and RSD were all below 1%; CTS was removed due to a low discrimination power (0.212). The discrimination index was 90, meaning that the samples were well-separated from each other. Sample Paraguay and 11-223 were grouped together. Kenya and 12-11-041 were together. 12-05-140 and 11-547 were similar and 12-05-005 was closest to them, comparing to other groups.

Figure 9. Principal component analysis of stevia leaf samples from Michigan State University as evaluated by electronic tongue and accounting for 80.86% of the variability. The discrimination index is 90.

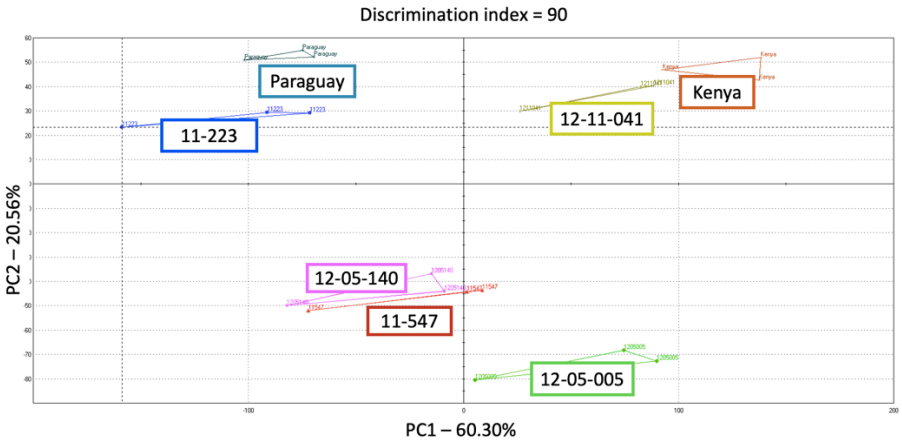


Table 12 shows the steviol glycoside profile of the stevia leaf samples. Agglomerative hierarchical clustering (AHC) was conducted using XLSTAT (AddinSoft, New York, NY) and was used to group stevia lines into 4 groups based on their similarities on the steviol glycoside profiles (Table 13). Both similarities and discrepancies between the clustering methods based on E-tongue and steviol glycosides were observed (Table 13). For example, Paraguay and 11-223 were similar based on PCA acquired from E-tongue but were in different classes according to AHC. This might be due to the different levels of both total glycosides and individual glycoside. 12-05-140 and 11-547 were not in the same class according to the steviol glycoside profile

clustering but were close to each other on PCA. 12-05-005 was relatively close to 11-547 on the PCA and was also grouped with it in AHC based on the steviol glycoside profile.

Table 12. Steviol glycosides profile[1] of stevia leaf samples from Michigan State University.

Samples	Stevioside	Reb A	Reb B	Reb C	Reb D	Reb M	Total
11-223	17.54[2]	9.72	0.33	1.16	0.37	0.00	29.12
11-547	2.99	17.24	0.84	1.42	0.28	0.00	22.78
12-05-005	7.26	62.99	0.00	5.42	5.42	4.25	85.34
Kenya	20.92	109.50	19.24	12.30	5.30	0.00	167.26
12-05-140	10.26	25.57	0.00	4.03	12.39	3.85	56.11
12-11-041	15.95	40.93	3.23	6.47	14.96	4.44	81.56
Paraguay	29.76	37.16	12.40	4.45	0.69	0.00	84.46

[1] Analyzed by QTRAP 3200 mass spectrometer (Shafii et al., 2012)
[2] Amounts are in mg/g**.**

Table 13. Classification of stevia lines based on E-tongue and steviol glycoside profiles.

Samples	E-Tongue[1]	Steviol glycoside profile[2]
11-223	Class 1	Class 1
Paraguay	Class 1	Class 4
Kenya	Class 2	Class 2
12-11-041	Class 2	Class 3
11-547	Class 3	Class 2
12-05-140	Class 3	Class 3
12-05-005	Class 4	Class 2

[1] Classification on the PCA map (Figure 9).
[2] Classification from agglomerative hierarchical clustering

5.3. Future Potential of E-tongue for Flavor Selection in Stevia Breeding Program

It is important to understand not only chemical analysis of steviol glycosides but also sensory data to provide a complete range of flavor information. The chemical analysis by mass spectrometer provides quantitative data of each steviol glycoside that is somewhat predictable for the flavor of stevia. However, because mass spectrometer detects each taste substance separately, it cannot reveal taste-substance interaction (i.e., synergistic and suppression effects), and thus, the overall taste of stevia leaves cannot be fully explained (Kobayashi et al., 2010). Sensory evaluation can provide integrated, direct measurements of flavor, but the sensory evaluation is time-consuming and expensive. Therefore, E-tongue as a quick and simple analytical tool has a

great potential for quick flavor discrimination of stevia lines in the breeding programs since it mimics human taste perception without sensory fatigue. In order to develop a predictive model of stevia flavors in breeding programs using E-tongue, the relationships between chemical analysis, sensory evaluation, and E-tongue should be well understood to correlate the E-tongue data with chemical analysis and human sensory perception.

6. Conclusions

The protocol developed for #6 sensor array worked well to produce more repeatable data for stevia samples. E-tongue successfully discriminated the stevia leaf samples, which was the first step to determine if E-tongue could be used for flavor selection in stevia breeding programs. Further analyses, such as human panels, are needed to evaluate the sensory characteristics of the stevia leaf samples, in order to understand which sample has a better flavor profile such as clean sweetness or less bitterness. E-tongue data could then be correlated to human panel data and determine if E-tongue can be used as a fast and accurate way to determine the flavor profile of stevia leaf samples for the stevia breeding programs.

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CONCLUSIONS

The findings of the first study generate important information about Reb A, D, and M for the food industry. Reb D and M can be considered as good sucrose substitutes due to their similar sensory profiles to the sucrose, comparing to Reb A. However, the use of Reb D and M for products formulated to deliver a reduction in sugar should be careful with the *artificial* attribute and the lingering sweetness of Reb D and M. The sensory characteristics of Reb A, D, and M identified in this study can be used as a reference for the food industries, especially working in high-sugar applications such as frozen desserts. The individual's sensitivity to the perceived intensity of sweet and bitter tastes of the three steviol glycosides was not predicted by the PROP taster groups. This suggests that supertasters who experience more intense taste sensations may not report aversive sensations from stevia. Future studies using steviol glycosides in food matrix are needed to determine the sensory profile and acceptance of them.

The second study provides a new protocol for E-tongue analysis using the most updated sensor array #6 and shows that E-tongue has the potential to be used as an analytical tool for flavor discrimination of stevia lines in the breeding programs. Understanding both the chemical components and sensory data are important to build a complete range of flavor information. Since E-tongue is relatively quick and simple comparing to a human panel, it is important to understand the relationship between E-tongue and human sensory perception in order to develop a predictive model of stevia flavors in breeding programs using E-tongue. Further analyses, such as human panels, are needed to evaluate the sensory characteristics of the stevia leaf samples and correlate to E-tongue data.