Kinetics of Rebaudioside A Degradation in Buffer Solutions as Affected by UV Light Exposure

by

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Abstract

Rebaudioside A is a natural non-caloric high-potency sweetener extracted from the leaves of *Stevia rebaudiana*. With rebaudioside A use increasing in foods, understanding factors affecting its stability is necessary. The literature contains contradictory data about the photostability of rebaudioside A. In addition, kinetic data are lacking regarding the effect of light on rebaudioside A stability. The objective of this project was to determine the degradation rates of rebaudioside A in buffer solutions as a function of ultraviolet (UV) light intensity.

Six solutions containing rebaudioside A were prepared: 0.1 M sodium phosphate (pH 3 and 7), 0.1 M sodium citrate (pH 3 and 7), and water adjusted to pH 3 and 7. Eleven 3.7-mL glass vials containing 2 mL of each solution were stored at 32.5 ℃ in darkness, under low intensity UV radiation (365 nm, 27 $\mu W/cm^2$), and under high intensity UV radiation (365 nm, 190 $\mu W/cm^2$). Samples were removed at regular time intervals for up to 205 days. Rebaudioside A concentrations were determined using high performance liquid chromatography. Pseudo-first-order rate constants with 95% confidence intervals were calculated for the degradation of rebaudioside A.

Rebaudioside A stability was adversely affected by light exposure. Under dark conditions, rebaudioside A in water, citrate buffer solutions, and phosphate buffer solutions was relatively stable. The degradation rate constants increased significantly ($p<0.05$) with increasing light intensity in all solutions. In both light-protected and light-exposed groups, rebaudioside A in water and citrate buffer solutions was more stable at pH 7 than pH 3, but in phosphate buffer

solutions rebaudioside A was more stable at pH 3 than pH 7. The degradation rate constants of rebaudioside A in phosphate buffer solutions at pH 3 and pH 7 were both significantly ($p<0.05$) higher than the values in water and citrate buffer solutions, suggesting the susceptibility of rebaudioside A degradation in phosphate buffer solutions when protected from light. In darkness, this rapid degradation of rebaudioside A occurring in phosphate buffer at pH 7 was hypothesized to be the result of the dibasic phosphate anion catalyzing its hydrolysis. However, exposure to UV light resulted in rebaudioside A degradation occurring approximately 10 times faster in citrate buffer than phosphate buffer at both pH levels. The highest degradation rate constants occurred in pH 3 and 7 citrate buffers, which were not significantly different ($p>0.05$). The sensitivity of rebaudioside A to UV light was thus greater in citrate buffers than in water or phosphate buffers. The accelerated degradation of rebaudioside A in citrate buffer was hypothesized to result from free radicals generated by Fe (III)-citrate complexes during UV light exposure.

Manufacturers and distributors of beverages containing rebaudioside A must recognize the detrimental effects of light exposure on the stability of rebaudioside A. Appropriate product formulations, packaging, and storage are needed to optimize the shelf life and quality of the rebaudioside A products.

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Table of Contents

Abstract ii

Acknowledgments iv

List of Tables vii

List of Figures ix

Chapter 1: Introduction 1

Chapter 2: Literature Review 4

Reason for high-potency sweeteners 4

Structure of rebaudioside A 4

Extraction and purification 7

Identification and quantification 10

Approval as food additive 15

Application 16

Metabolism 16

Safety studies 19

Healthy effects 22

Stability studies 24

Objective 32

Chapter 3: Materials and Methods 33

Reagents and chemicals 33

Sample preparation 33

Sample analysis 36

Data analysis 37

Chapter 4: Results and Discussion 39

Results 39

Stability of rebaudioside A in darkness 43

Stability of rebaudioside A as a function of light intensity 48

Stability of rebaudioside A: combined effects of pH, buffer type, light intensity 52

Chapter 5: Summary and Conclusion 61

References 63

Appendix 75

List of Tables

Table 4.1 Pseudo-first order rate constants (d^{-1}) with 95% confidence limits for rebaudioside A degradation in solutions at 32.5 ℃ stored under various intensities of UV light. Rate constants have been multiplied by 1000 42

Table 4.2 Estimated half-life (weeks) for rebaudioside A in solutions at 32.5 ℃ calculated using the pseudo-first order kinetic model 43

Table 4.3 Estimated rebaudioside A loss (%) after 1 week storage at 32.5 ℃ calculated with the pseudo-first order kinetic model 43

Table 4.4 Rebaudioside A degradation rate constants (d^{-1}) with 95% confidence limits in mock beverage solutions at 30 ℃ calculated with the data from Prakash and others (2012b) 45

Table 4.5 Rebaudioside A degradation rate constants (d^{-1}) with 95% confidence limits in 0.1 M buffer solutions at various temperatures in darkness using the data from our research and Gong and Bell (2013). Rate constants have been multiplied by 1000 46

Table 4.6 Estimated rebaudioside A loss (%) in 0.1 M citrate and phosphate buffers after 1 week storage at room temperature calculated with the pseudo-first order kinetic model from Gong (2012) 50

Table A1 Concentration of rebaudioside A in pH 3 water at 32.5 ℃ under different UV intensities 75

Table A2 Concentration of rebaudioside A in pH 7 water at 32.5 ℃ under different UV intensities 76

Table A3 Concentration of rebaudioside A in 0.1 M pH 3 phosphate buffer at 32.5 ℃ under different UV intensities 77

Table A4 Concentration of rebaudioside A in 0.1 M pH 7 phosphate buffer at 32.5 ℃ under different UV intensities 78

Table A5 Concentration of rebaudioside A in 0.1 M pH 3 citrate buffer at 32.5 ℃ under different UV intensities 79

Table A6 Concentration of rebaudioside A in 0.1 M pH 7 citrate buffer at 32.5 ℃ under different UV intensities 80

Table A7 Concentration of rebaudioside A in 0.1 M pH 7 citrate buffer at 32.5 ℃ held 10 days under high UV to test reproducibility of methodology 81

Table A8 Concentration of rebaudioside A in 0.1 M pH 3 citrate buffer at 32.5 ℃ held 35 days under high UV exposure with different vial filling volumes 81

List of Figures

Figure 2.1 Structure of rebaudioside A 6

Figure 4.1 Degradation of rebaudioside A in pH-adjusted water at 32.5 ℃ as affected by UV light intensity at 365 nm 40

Figure 4.2 Degradation of rebaudioside A in 0.1 M citrate buffer at pH 3 at 32.5 ℃ as affected by exposure to UV light at 365 nm 40

Figure 4.3 Degradation of rebaudioside A in 0.1 M phosphate buffer at pH 7 at 32.5 ℃ as affected by exposure to UV light at 365 nm 41

Figure 4.4 Degradation of rebaudioside A in 0.1 M buffer solutions at 32.5 ℃ under dark conditions at 365 nm 54

Figure 4.5 Degradation of rebaudioside A in 0.1 M buffer solutions at 32.5 ℃ under high UV exposure (365nm, 190 $\mu W/cm^2$) 54

Figure 4.6 Degradation rate constants of rebaudioside A in 0.1 M pH 3 citrate buffer solution and pH 7 phosphate buffer solution at 32.5 ℃ as a function of UV light intensity at 365 nm 57

Chapter 1: Introduction

Rebaudioside A is a natural high-potency sweetener extracted from the leaves of *Stevia rebaudiana* (Bertoni), which is a herbaceous perennial plant indigenous to Paraguay and Brazil (Jaitak and others 2008). Structurally, rebaudioside A is a diterpene glycoside with a β-glucosyl and a β-glucosyl-(1-3)-sophorosyl residue attached to the aglycon steviol (Chang and Cook 1983). As the second most predominant steviol glycoside (2-4% w/w) in *S. rebaudiana*, rebaudioside A is approximately 250-450 times sweeter than sucrose and appears as a white to off-white powder (Lindley 2006). It has a desirable flavor profile and resembles the taste of sugar closely (DuBois 2000; Pól and others 2007). Considered Generally Recognized As Safe (GRAS) for use as a food ingredient by the U.S. Food and Drug Administration (FDA), rebaudioside A has developed into a commercially viable non-caloric sweetener which is incorporated into foods and beverages (Tarantino 2008; Urban and others 2013). This sweetener has become increasingly popular in recent years due to consumer demand for all-natural and reduced-caloric food products (Clos and other 2008).

When consumed by human beings, rebaudioside A is completely hydrolyzed in the gastrointestinal tract into steviol, which cannot be degraded by intestinal microflora but is excreted primarily in the feces with limited urinary elimination (Gardana and others 2003; Geuns and others 2003). Elicited data from a series of toxicological studies support the safety of long-term rebaudioside A consumption. It does not pose risks of allergic reactions, reproductive toxicity, genotoxicity, mutagenicity or carcinogenicity (Brusick 2008; Curry and others 2008; Urban and others 2015; Nakajima 2000a; Nakajima 2000b; Sekihashi and other 2002; Xili and others 1992). Rebaudioside A is suggested to be acceptable for both diabetic and

phenylketonuria patients (Maki and others 2008). Some positive health effects are also exerted, such as anti-hypertensive effect, dental benefits, hypolipidaemic effect, obesity and overweight control, oxidative protection, and renal function (Chatsudthipong and Muanprasat 2009; Goyal and others 2010; Sharma and Mogre 2007; Shivanna and others 2013).

Loss of rebaudioside A from foods may change their sensory profiles. Therefore, with the commercial development in rebaudioside A, many studies have examined its stability at different conditions, including the effects of moisture content, temperature, pH, interaction with food components, and light exposure. Rebaudioside A is more stable in dry powder, with only 1-2% loss after 2 years storage in polyethylene bags, while in aqueous solution, it is more susceptible to break down via several reaction pathways (Prakash and others 2008). At low temperature, rebaudioside A in solutions can be kept for several months, but its degradation rates and the formation rates of degradation products are accelerated with increasing temperature (Chang and Cook 1983; Gong and Bell 2013; Prakash and others 2012b). The effect of acidity on rebaudioside A stability was evaluated by dissolving rebaudioside A in buffers or mock beverages with different pH values. It was demonstrated that rebaudioside A was less stable in acidic conditions than in neutral systems, with the degradation increasing at lower pH values (Chang and Cook 1983; Prakash and others 2012b; Wölwer-Rieck and others 2010b). However, Gong and Bell (2013) noted that with increasing pH values, degradation rate constants of rebaudioside A in phosphate buffer increased but decreased in citrate buffer. Some studies considering the stability of rebaudioside A in different food matrices showed that rebaudioside A did not affect the food quality or shelf life (Jooken and others 2012; Prakash and others 2008).

The literature contains contradictory data on the stability of rebaudioside A when exposed to light. Chang and Cook (1983) reported significant degradation (18-22%) of rebaudioside A in

citric and phosphoric acid systems after exposure to 3000 langleys of sunlight, but Clos and others (2008) replicated this study and suggested no photodegradation of rebaudioside A occurred in cola and lemon-lime beverages. Good photostability of a mixture of steviol glycosides (containing rebaudioside A) in mock beverages under fluorescent light exposure was reported by Chaturvedula and others (2011). Later, Gong (2012) found the increased degradation of rebaudioside A in pH 3 citrate buffer with light exposure, but no obvious effect appeared in pH 7 phosphate buffer.

Data on rebaudioside A photostability are limited and contradictory. Kinetic data are also lacking regarding the effect of light on the stability of rebaudioside A. Therefore, the objectives of this study were to evaluate the long-term stability of rebaudioside A in different solutions as a function of ultra-violet (UV) light intensity and to determine the corresponding degradation kinetics, in terms of degradation rate constants.

Chapter 2: Literature Review

Reason for high-potency sweeteners

There is an increasing demand for sugar-free or reduced-caloric foods and beverages in the market, due to the increasing rates of obesity, diabetes mellitus (type II) and cardiovascular diseases (Kroger and others 2006; Kunová and others 2014). In the United States, nonnutritive sweeteners are very popular for their taste of sweetness without caloric value. Nowadays, most of the high-potency sweeteners on the market are synthetically made, such as acesulfame-K, aspartame, neotame, saccharin, and sucralose (Pól and others 2007). Besides their benefits, the health effect of artificial sweeteners should be considered. Although the carcinogenicity of artificial sweeteners in human beings has not been proven, some synthetic sweeteners have been linked to cancer in lab animals (Briciu and others 2010). Animal studies have suggested that artificial sweeteners can cause weight gain, brain tumors, bladder cancer and other health hazards (Tandel 2011). Thus, consumers have an increasing interest in the natural sugar substitutes on the market. Numerous natural sweeteners are available and popular among consumers, one being rebaudioside A, which is used as a high-intensity sweetener. It is added in small amounts to replace regular large amounts of sucrose for reduced calories in beverages and some foods. Rebaudioside A and monk fruit extracts are the only natural high-intensity sweeteners on the United States' market.

Structure of rebaudioside A

Rebaudioside A is one of the principle components in leaf extracts of *Stevia rebaudiana* Bertoni (family: Asteraceae), the natural herb indigenous to Paraguay, Brazil, and other South

American regions for over 1,500 years. *S. rebaudiana* is known to be one of the sweetest plants, and thus is cultivated commercially in Asia and Europe. The consumption of *S. rebaudiana* extracts in Japan and Korea is reported to be 200 and 150 tons/year, respectively (Kinghorn and others 2001). Among the more than 30 sweet compounds isolated from *S. rebaudiana*, stevioside (6-10% w/w) and rebaudioside A (2-4% w/w) are the two major ones, while other minor glycosides are present up to 1-2% (w/w) (Makapugay and others 1984; Wölwer-Rieck 2012). Kennelly (2002) states that the yield of rebaudioside A from dried leaves ranges widely from 25 to 54% depending on genotype, cultivation and growing conditions. Accounting for approximately 2% of the mass from dried *Stevia* leaves, rebaudioside A contributes the second most to the *ent*-kaurene diterpene glycosides, the compounds responsible for the sweetness in *S. rebaudiana* (Abudula and others 2004). Rebaudioside A belongs to the ent-13-hyroxykaur-16-en-19-oic acid group characterized by the *ent*-kaurene diterpenoid steviol skeleton (Montoro and others 2013). In the aglycone, a β-D-glucopyranose is attached to the carboxyl group at C19 and a 2,3-substituted β-D-triglucosyl unit at the C13 position (Prakash and others 2012a; Steinmetz and others 2009). Rebaudioside A is a β-D-glucopyranosyl ester: 13-[(O-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside]-13-hydroxy-16-kauren-19-oic acid β-D-glucopyranosyl ester, with the structure presented in Figure 2.1. With the empirical formula of $C_{44}H_{70}O_{23}$ and molecular weight of 967.01 g/mol, rebaudioside A appears as white to off-white powder. It has better dissolution in water than in methanol or ethanol, and the solubility increases with increasing temperatures (1 g in 100 mL water at 25 ℃ in 5 min) (Prakash and others 2008).

Other minor components that contribute to the sweet sensation in *S. rebaudiana* are rebaudiosides B to O, steviolmonoside, steviolbioside, steviol, rubusoside, and dulcoside A and

B (Wölwer-Rieck 2012). However, some evidence shows that rebaudioside B and steviolbioside are not genuine constituents, but products due to artifacts of the extraction and/or isolation procedures (Geuns and others 2003; Kennelly 2002). Rebaudioside B is the partial hydrolysis product of rebaudioside A with hesperidinase (Kohda and others 1976), and steviolbioside is generally believed to be an artifact of alkali treatment of stevioside (Kim and DuBois 1991). Steviolbioside and rebaudioside B are considered to be degradation products of stevioside and rebaudioside A under strong alkaline conditions, respectively (Mizutani and Tanaka 2002).

Of the steviol sweeteners, rebaudioside A resembles the taste of sugar the most closely (Pól and others 2007). It is the sweetest glycoside, tasting approximately 250-450 times sweeter than sucrose (Lindley 2006). It has a desirable flavor profile with superior quality of taste (DuBois 2000); it has a clean sweet taste at low sucrose equivalence (SE) levels, but bitterness

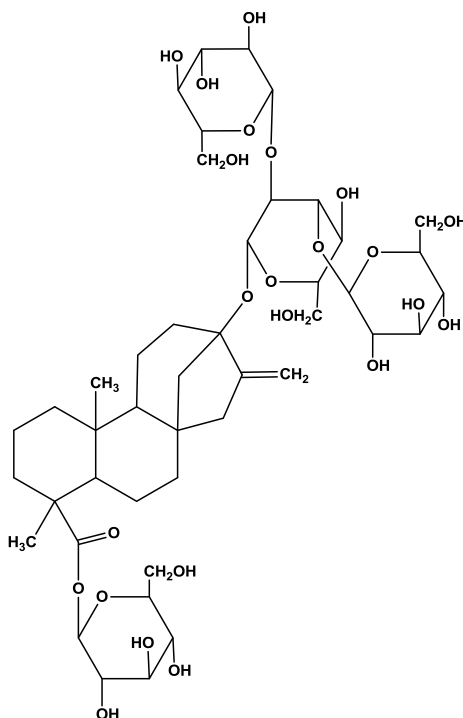

Figure 2.1 Structure of rebaudioside A.

and a black licorice flavor are perceptible at higher SE levels (Prakash and others 2008). It is devoid of sourness, saltiness, savor, metal or other qualities (Prakash and others 2008). Further, rebiana (at 529 mg/L), the commercial product comprised of predominately high-purity rebaudioside A, exhibits both prolonged appearance time and longer extinction time than aspartame (at 531 mg/L) and sucrose (8%) in water at room temperature (Prakash and others 2008). Sweet-taste appearance time represents the time to reach a sweetness response equivalent of 10% sucrose, while extinction time is the time from initial exposure to the stimulus until it is no longer perceived (ASTM International Subcommittee 2015; DuBois 2012). Such sweetness temporal profile is desirable and beneficial in foods and beverages.

Extraction and purification

Rebaudioside A can be obtained from *S. rebaudiana* leaves after a several stage process that includes: extraction, pre-treatment, separation, and purification followed by refining. Sometimes, removal of essential oils, lipids, chlorophyll, and other nonpolar compounds is necessary by pre-treating the leaves with chloroform or hexane (Pasquel and others 2000). After extraction of dried, powdered leaves with hot water or alcohols, precipitation with salt or alkaline solutions proceeds for clarification. The extract is then concentrated and re-dissolved in methanol for crystallization (Pasquel and others 2000). This protocol is the conventional methodology.

Solvent extraction and supercritical fluid extraction (SFE) are two general methods applied in rebaudioside A extraction. The other methods utilized can be categorized into chromatographic adsorption, ion exchange, selective precipitation, and membrane processes (Lemus-Mondaca and others 2012).

Jaitak and others (2009) analyzed the effect of different solvents and binary mixtures on rebaudioside A extraction, including methanol, ethanol, water, methanol:water (80:20 v/v), and ethanol:water (80:20 v/v). After comparing the yields of rebaudioside A, they concluded that due to the enhancement in polarity, the use of a binary system, methanol:water (80:20 v/v) in this case, affords the optimum extration. The utilization of microwaves was also explored in the same study. Jaitak and others (2009) demonstrated the highest yield of rebaudioside A (2.34%) was achieved by utilizing a microwave-assisted extraction procedure with binary solvents, followed by an ultrasound method (1.98%) and conventional cold extraction (1.20%). It is noted that a power level of 80 W and a temperature of 50 ℃ were effective in breaking the analyte-matrix bonds for optimum yield.

SFE is another common technique for rebaudioside A extraction. With higher diffusivity and lower viscosity than conventional solvents, CO_2 is employed as the medium in the SFE procedure (Erkucuk and others 2009). The superiority of SFE over conventional soxhlet extraction is the solvent-free extracts without potential impairment in taste (Kienle 1992). Erkucuk and others (2009) optimized the SFE conditions with response surface methodology, demonstrating that the highest yield of rebaudioside A (17.79 mg/g) was obtained under the extraction conditions of 211 bar, 80 ℃, and the co-solvent, ethanol:water (17.4:82.6 v/v). It was also found that the amount of rebaudioside A extract from supercritical CO_2 was close to that yield with water (22.53 mg/g), with both being higher than that using ethanol extraction (14.84 mg/g).

The purification method generally involves the crystallization of a substantially pure composition. Jackson and others (2011) invented a new method to produce high-purity rebaudioside A with a maximum yield of 99+% purity. The basic embodiment included five

steps: an ethanol solvent formulation stage, a reflux stage, a stirred wash stage, and an ethanol purge followed by a drying stage. After the first refluxing with 1:4 mass to solvent ratio (*Stevia* starting material by mass to ethanol solvent by volume) at 80 ℃ for 1 h with stirring, samples were place on ice for 1 h then filtered over filter paper. The wet solids were then stirred in 100% ethanol for 15 min. The *Stevia* starting material had purity of 82.3%, while the ethanol solvent was a mixture of ethanol and water (92:8 v/v). A second reflux with 1:4 mass to solvent ratio under same conditions proceeded, followed by a stirred wash stage with absolute ethanol and stirred for 15 min. Finally, the wet solids were dried to constant weight at 80 ℃, providing the highest recovery of rebaudioside A at the target purity.

Prakash and others (2007) hold an invention to purify crude rebaudioside A with a single crystallization step. The combination of crude rebaudioside A (80.37%) and solvent mixture (64% ethanol, 21% methanol, and 15% water) at around 1:5 mass to volume ratio was refluxed for 10 min. After cooling the mixture to 22 ℃ for 16 h with stirring, the white crystals were filtered and washed with ethanol:methanol (75:25 v/v) twice. The high-purity rebaudioside A (>99% by HPLC) was obtained by drying the wet solids at 50 ℃ in a vacuum oven overnight (16-24 h) under reduced pressure (20 mm). Rebaudioside A with even higher purity (>99.5% by HPLC) could be obtained through modification of the methodology. In this case, crude rebaudioside A (80.37%) was mixed with ethanol:water (80:20 v/v) at around 1:4 mass to volume ratio, and then refluxed for 30 min instead. After cooling to ambient temperature, the product was filtered and washed twice with ethanol. The drying temperature was changed to 60 ℃, while other conditions were the same. However, the yield of rebaudioside A (39.8%) with this method was lower than the previous one (52%).

Identification and quantification

Several investigators have devised HPLC analytical methods for rebaudioside A both qualitatively and quantitatively. HPLC is well known and widely applied for its good accuracy and precision, rapid analysis time, high sensitivity and specificity, good reproducibility and duration, as well as simple preparation procedure and low solvent usage.

The first successful determination of rebaudioside A with this technique was reported by Hashimoto and Moriyasu in 1978. A general-purpose hydrophilic column, Shodex OHpak M-414 (500 x 4 mm I.D.), was selected together with a refractive index detector. Stevioside and rebaudioside A were well separated and identified by choosing acetonitrile:water (80:20 v/v) as the mobile phase at a flow rate of 0.5 mL/min. Their limit of detection (LOD) was 2 ppm. The linear relationship between amount of sample and peak height or peak area was also noted. Thus, the concentrations of stevioside and rebaudioside A were determined by creating a liner calibration curve with Beer's Law.

Better resolution of rebaudioside A was obtained with size exclusion chromatography by Ahmed and Dobberstein (1982). After soxhlet extraction of dried, powdered *S. rebaudiana* leaves with chloroform for 3 h, two Waters Protein I-125 columns (300 x 7.8 mm I.D.) in series were applied to purify the *Stevia* extracts, followed by 5 h methanol treatment. The extract was evaporated to dryness in *vacuo* and then dissolved in 1-propanol:water (98:2 v/v) for HPLC analysis. A Waters Model 6000 A liquid chromatograph was equipped with two Protein I-125 columns. One-propanol was the eluting solvent ran at 1.0 mL/min, and 210 nm was the wavelength of the ultra-violet (UV) spectrophotometer. Under these conditions, stevioside, rebaudioside C, and rebaudioside A were well separated and quantitated with the LOD of 1.0, 2.0, and 2.0 μg/100 μL, giving retention times (RT) of 43.2, 49.5, and 59.0 min, respectively.

While using the UV detector for rebaudioside A analysis, 210 nm was always the wavelength of choice due to the intense absorption maximum of the carboxylic acid and olefin moieties in steviol aglycone, providing the adequate sensitivity to meet the required quantitation limit of 0.5 mg/L (Clos and others 2008).

Carbohydrate column is another option for rebaudioside A analysis. Chang and Cook (1983) used a μBondpak-carbohydrate analysis column equipped with differential refractometer in their stability investigation of rebaudioside A. Acetonitrile:water (80:20 v/v) was selected as the eluting solvent at 1.0 mL/min.

Shorter analysis time and better baseline separation can be achieved by using an amino (NH_2) column, since *Stevia* glycosides differentiate in the number and type of glycoside moieties attached to the *ent*-kaurene skeleton. RT increases with increasing number of glucose units. Makapugay and others (1984) used Zorbax NH_2 column (5 μm, 250 x 0.4 mm I.D.) with a linear gradient elution to identify 8 *Stevia* sweeteners. The mobile phase, acetonitrile:water (84:16 to 70:30 v/v at pH 5), changed over 15 min at a flow rate of 2.0 mL/min. At ambient temperature, rebaudioside A was separated and eluted at 11.9 min, and the sensitivity was adjusted to 0.04 absorbance units full scale (AUFS). Later, Kitada and others (1989) reported the simultaneous determination of rebaudioside A in foods with normal-phase LiChrosorb NH_2 column (5 μm, 250 x 4.0 mm I.D.). Samples were pre-treated on a reversed-phase Sep-Pak octadecyl siloxane (C_{18}) cartridge to clean up the stevioside, rebaudioside A and C, and dulcoside A in a series of foodstuffs, including beverages, soy sauce, candy, and pickled radish. Then the samples were syringed into the column at 50 ℃, while acetonitrile:water (80:20 v/v) isocratic elution was set at a flow rate of 0.8 mL/min. The RT for rebaudioside A was 14 min with a LOD of 5 ppm. It was noted that efficient separation, determination, and quantitation were achieved without

interferences from other food matrices. Makapugay and others' analytical procedure was later improved by Kolb and others (2001). The optimum solvent extraction condition was determined to be a mixture of ethanol and water (70% w/w) in a 70 ℃ water bath for a period of 30 min. After cooling and filtration, the extract was analyzed by a HPLC system using a Zorbax NH_2 column (5 μm, 250 x 4.6 mm I.D.) with two different elution conditions. In the gradient elution mode, the mobile phase, acetonitrile:water (84:16 to 70:30 v/v, pH 5), changed over 15 min with a flow rate of 2 mL/min. It took more than 15 min for the complete separation of analytes. Compared to the gradient method, the total operation time was reduced to 7 min with the RT for rebaudioside A being 6.097 min in isocratic mode, in which acetonitrile:water (80:20 v/v at pH 5) was used as the eluting solvent and the flow rate was 2 mL/min. The precision for stevioside and rebaudioside A was improved to 2.25% and 3.03%, respectively, with very high recoveries for added standards and a sensitivity of 0.04 AUFS.

However, the NH_2 column is unsuitable for principally aqueous samples since only limited amount of samples (<10 μL) can be injected and water is a strong solvent for NH_2 column (Clos and others 2008). In order to solve the problem of poor LOD and resolution, reversed phase C_{18} column is generally employed in beverages analysis. Rebaudioside A, stevioside, and their metabolites could be well separated and quantitated by using C_{18} column with linear gradient elution of acetonitrile-water mixture (Hutapea and others 1999; Gardana and others 2003). Pól and others (2007) investigated the characterization of steviol glycosides with strong cation-exchange, NH_2, and C_{18} columns. They found that C_{18} column provided the best separation results in single-dimension, but the co-eluting phenomenon existed between rebaudioside A and E, as well as stevioside and rebaudioside B. The full separation and quantification of all *Stevia* glycosides could be achieved with a comprehensive two-dimensional

chromatography (LC x LC) connected to electrospray ionization time-of-flight mass spectrometry. In the LC x LC system, a Waters C18 column (3 µm, 150 x 2.1 mm I.D.) was selected for the first-dimension and Phenomenex Luna NH_2 (3 µm, 50 or 30 x 2 mm I.D.) as the second column, with gradient elution of acetonitrile-water (5:95 to 100:0 v/v within 55 min) and isocratic elution in equal ratio, respectively. It was also noted that the flow rate for the first column should be slow (0.1 mL/min at maximum) and as fast as possible for the second one. However, the identification of the peak with m/z value of 965.4 was impossible, since rebaudioside A and C have the same molecular formula. Pól and others (2007) also suggested the high reliability of LC x LC results due to the better separation effect and the compound determination based on two independent RTs.

A Waters sub-2 µm column, C_{18} HSS column (1.8 µm, 150 x 2.1 mm I.D.), was employed by Gardana and others (2010) for the evaluation of *Stevia* glycosides in ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) mode, while another shorter C_{18} HSS column (1.8 µm, 100 x 2.1 mm I.D.) was selected for steviol quantification. The proposed column was superior in efficiency, analysis time, and resolution than NH_2 and traditional C_{18} columns. Besides, the retention time for tested *Stevia* glycosides and steviol was also reproducible with the UHPLC-MS method.

Wölwer-Rieck and others (2010a) developed an improved HPLC analytical method. The glycoside samples from acetonitrile:water (80:20 v/v) extraction were filtered and further cleaned up with C_{18} solid phase extraction (SPE) cartridges. The SPE clean-up was superior in the limited usage of organic solvents, short analysis time, and the possible detection of glycosides with shorter RT than stevioside. Added into the tube, the sample was washed with water and acetonitrile:water (20:80 v/v) solution. After air-drying, the glycosides were eluted

with acetonitrile:water (80:20 v/v), then injected to the HPLC system. Luna hydrophilic interaction liquid interface chromatography (HILIC) column (5 μm, 250 x 4.6 mm I.D.) was selected with acetonitrile:water (85:15 v/v) eluting at a flow rate of 1.0 mL/min at 36 ℃, while in a second method, the acetonitrile:water (75:25 v/v) mobile phase flowed through a Luna NH_2 100 A column (5 μm, 250 x 4.6 mm I.D.) at the same conditions. Identification of rebaudioside A was conducted by LC-MS detection with electro spray ionization. These two columns showed similar retention patterns with high recovery around 100%. The RT for rebaudioside A using the NH_2 column was shorter, 6.6 min. Although separation with HILIC column took a longer time, 9.7 min, more robust results could be obtained with shorter equilibration times. Resembling the normal-phase mode, HILIC was suitable for compounds with poor retention in reversed-phase HPLC.

During the HPLC analysis of rebaudioside A, detection is performed mostly with UV and MS, but occasionally with pulsed amperometric, charged aerosol and fluorescence detection (Ahmed and others 2002; Clos and others 2008; Minne and others 2004).

Several other techniques are also employed to identify and quantify steviol glycosides. A chemical reaction followed by enzymatic hydrolysis was applied for the quantification of stevioside by Mizukami and others (1982). Total glycoside content was determined with gas chromatography after acid hydrolysis (Sakaguchi and others 1982). Over-pressured thin layer chromatography was applicable for the analytical and preparative separation of glycoside constituents (Fullas and others 1989). Mauri and others (1996) employed capillary electrophoresis to analyze diterpene glycosides, while rebaudioside A and steviobioside were isolated with semi-preparative HPLC. Densitometry was developed by Dacome and others (2005) for analysis of rebaudioside A and stevioside. Jaitak and others (2008) worked on the

quantification of rebaudioside A, stevioside, and steviolbioside with high performance thin-layer chromatography. Later, the conformation of rebaudioside A was confirmed by Steinmetz and Lin (2009) with nuclear magnetic resonance (NMR). LC-ESI-MS/MS was used by Montoro and others (2013) for the determination of six steviol sweeteners.

Approval as food additive

Although steviol glycosides have been approved as sweeteners in a wide range of foods in certain countries, notably Argentina, Brazil, China, India, Indonesia, Israel, Japan, Malaysia, Paraguay, Russia, and South Korea, the powdered *Stevia* leaves and extracts were only used for dietary supplementation and skin care in the United States before 2008 (Carakostas and others 2008; Kroger and others 2006; Lemus-Mondaca and others 2012; Wölwer-Rieck and others 2010a). Some food safety and regulatory agencies have questioned the utilization of steviol glycoside sweeteners in the food industry for several years. In June 2008, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established both temporary specifications and temporary acceptable daily intakes (ADI) for steviol glycosides as 0-4 mg/kg body weight/day with respect to steviol (JECFA 2008). It also specified that the sum of seven steviol glycosides must account for at least 95% of the dried substance, including rebaudiosides A to C, stevioside, steviolbioside, dulcoside A, and rubusoside (JECFA 2008). Later, Food Standards Australia New Zealand (FSANZ) permitted the use the steviol glycosides in specified foods with the same ADI as JECFA in Australia and New Zealand (FSANZ 2008). More recently, in December 2008, purified rebaudioside A from *Stevia* was classified as GRAS by the U.S. FDA (Tarantino 2008). Then, rebaudioside A (marketed as rebiana) was developed to sweeten beverages and some foods, primarily by the Coca Cola Company and Cargill, Inc. (Clos and others 2008). The approval and

regulation of steviol glycosides into food products for European countries was issued by the European Commission in November 2011, in which the ADI was set to 4 mg steviol equivalents/kg body weight/day (European Commission 2011). The steviol glycosides (≥ 95% purity) can be used as food additives, taken as the sum of rebaudiosides D to F and the seven compounds allowed by JECFA, while stevioside and rebaudioside A should be present at a minimum of 75% in the dried basis.

Application

With the approval documents, rebaudioside A is used as an intense sweetener in several commercial foods, including soft drinks, like Coca-Cola Life, as well as cold confectionary, breads, biscuits, delicacies, desserts, sauces, sweet corn, soju, soy sauce, yogurt, dried seafood, candies, ice cream, chewing gum, and others (Gardana and others 2003; Erkucuk and others 2009; Lemus-Mondaca and others 2012). Rebaudioside A is successfully formulated into toothpaste, mouthwash and related products (Erkucuk and others 2009). As a high-potency sweetener, it is also used in blends with other non-caloric sweet enhancers, such as mogroside, monatin, aspartame, acesulfame salts, cyclamate, sucralose, saccharin salts, and erythritol, or in combination with carbohydrates like sucrose, glucose, fructose, and high fructose corn syrup (Prakash and others 2008). Some amino acids with sweet taste are also desirable, including glycine, alanine, and serine (Prakash and others 2008).

Metabolism

In vitro, the human digestive enzymes, α-amylase, pepsin, pancreatin, together with hepatic tissues, are demonstrated to be unable to cleave the rebaudioside A structure

(Ishii-Iwamoto and others 1995; Hutapea and others 1997). Instead, it is partly degraded into steviol by microflora of the colon, and this hydrolysis is necessary for absorption. After being taken up by the gut and transported to the liver, it is converted to steviol glucuronide and excreted in the urine (Jooken and others 2012).

Wingard and others (1980) investigated the metabolism of rebaudioside A in rat intestinal microflora *in vitro*, and suggested that it is degraded into the diterpenoid aglycone, steviol, by microbial flora in the mammalian lower bowel. The complete conversion of rebaudioside A took 6 days on incubation of whole-cell suspensions, while in cell-free extracts the metabolism was much slower, and only 2% of steviol was generated after 7 days. The degradation rate was measured to be 0.4-0.8 mg/h/g of cecal content, and estimated to be greater than 0.4 g/h in the human bowel *in vivo*, by predicting the same metabolic rate in humans. After oral or intracecal administration, steviol was completely absorbed by rats and eliminated through biliary excretion.

A relatively comprehensive study on the metabolism of rebaudioside A by human intestinal microflora was conducted by Gardana and others (2003). Under strict anaerobic conditions, the feces collected from volunteers were homogenized into the incubation medium, followed by the addition of rebaudioside A (90% w/w). After incubation, the transformation process and the selected cultures of microbial groups were analyzed by LC-DAD-MS. They reported that rebaudioside A was completely hydrolyzed into steviol after 24 h, being first hydrolyzed to steviolbioside after an initial lag phase of 6-7 h, and then converted to steviol rapidly. However, the metabolite, steviol, remained unchanged when incubated with the microflora for 72 h, indicating that it could not be degraded by human intestinal microflora. Rebaudioside A also did not affect the microbial composition significantly, but showed weak inhibitory activity on total aerobes and coliforms. Gardana and others (2003) also demonstrated

that of the selected microorganisms, only bacteroides were efficient in hydrolyzing rebaudioside A.

With a Caco-2 cell system, Geuns and others (2003) explored the characteristic absorption and transport of rebaudioside A and steviol. In this system, the permeability of rebaudioside A was very low, due to its hydrophilic property, while the efficient transport of steviol was explained by passive diffusion and carrier-mediated transport. The poor absorption *in vivo* of rebaudioside A might also be attributed to its hydrophilic nature, and the same situation of steviol was speculated to result from the low diffusion out of caecal content. Considering the very low amount of steviol in blood samples, it was also suggested that *in vivo* the steviol was absorbed by compounds in the colon (pH 7-7.5), which was concentrated by withdrawal of water. Nearly all the steviol administrated was then excreted in the feces and urine. Geuns and others (2006) also demonstrated that steviol and its related metabolite, steviol glucuronide, did not accumulate in the human body. Roberts and Renwick (2008) further indicated that the glucose units were successively cleaved from rebaudioside A by microbial hydrolysis, and then the metabolite was glucuronidated extensively to steviol glucuronides, which were subsequently eliminated in the bile and de-conjugated in the lower intestine, followed by excretion in the feces primarily as steviol through hydrolysis.

From a pharmacokinetic study, Wheeler and others (2008) demonstrated that after human consumption, 59% of rebaudioside A was excreted primarily in the urine as steviol glucuronide, which was formed from steviol systematically through rapid first-pass conjugation. Only a small amount of rebaudioside A (0.04%) was excreted as steviol in urine.

Safety studies

Many studies have demonstrated the safety of rebaudioside A consumption. A series of toxicological assessments suggest that rebaudioside A is relatively safe. In addition, studies also indicated it to be non-toxic, non-mutagenic, and non-carcinogenic.

To study sub-chronic toxicity, Wistar rats received rebaudioside A at concentrations of 0, 25,000, 50,000, 75,000 and 100,000 ppm in their diets for 4 weeks, while another group was administered the same sweetener at dietary concentrations of 0, 12,500, 25,000 and 50,000 ppm in a 13-week study. The resulting reduction in body weight gain at high doses was not considerably biologically and toxicologically significant, which was a common confounding effect in dietary toxicity studies administrated with high potency sweeteners (Curry and others 2008). The decreased body weight gain and initial taste aversion were attributed to the low caloric density of the diet and poor palatability initially in the study. In addition, the serum cholesterol and triglyceride levels decreased slightly, which was explained by the altered bile acid homeostasis through the metabolites production. Although there were slight increases in serum urea and creatinine, no other changes in urinalysis parameters were observed. The macroscopic and microscopic evaluation of the kidney also indicated the normal renal function of tested rats. Curry and Roberts (2008) determined the no observed adverse effect levels (NOAELs) of rebaudioside A were 4,161 and 4,645 mg/kg body weight/day for male and female rats, respectively, in their 13-week study, which were around 1000-fold higher than the normal usage of rebaudioside A in human consumption. In another oral toxicity study, Sprague-Dawley rats were administered with 500, 1,000, and 2,000 mg/kg body weight/day of rebaudioside A in a dietary mix. Nikiforov and Eapen (2008) reported that body weight gain decrement occurred at the highest dietary level without other direct malnutritive effect or treatment-related adverse

results. The NOAEL was determined to be higher than 2,000 mg/kg body weight/day, 2,055 and 2,050 for males and females, respectively. No toxicity-associated evidence was shown in either of the oral experiments over the dose-range, suggesting the safe use of high concentration rebaudioside A for human dietary intake.

Another rat study reported by Curry and others (2008) offered evidence about the reproductive safety of rebaudioside A. Han Wister rats were administered high purity rebaudioside A (97%) at dietary level of 0, 7,500, 12,500, and 25,000 ppm for two generations. There are no significant differences in body weight, body weight gain, or food intake for the F0 and F1 generations. No treatment-related effects on the survival and general conditions of F1 and F2 offspring were observed. Without evidence of reproductive or developmental hazards, the NOAEL of rebaudioside A was determined to be 2,048-2,273 mg/kg body weight/day. Other studies on general and reproductive toxicity of rebaudioside A also confirm its safe intake at high dietary levels.

The genetic toxicological potency of rebaudioside A was evaluated with a set of *in vitro* and *in vivo* assays. Rebaudioside A was first reported to lack mutagenic or genotoxic activity with *Salmonella typhimurium* strain TM677 by Pezzuto and others (1985). The consistently negative results from chromosome damage and DNA strand breakage studies also indicated the low potential genotoxic risk of rebaudioside A (Nakajima 2000a; Nakajima 2000b). Additional information was provided from the comprehensive battery test of high purity rebaudioside A (95.6%) in accordance with Organization for Economic Co-operation and Development (OECD) guideline by Williams and Burdock (2009). Three *in vitro* assays conducted at concentrations up to 5,000 μg/ml were used to test the mutagenic activity of rebaudioside A, including Ames test with OECD #471, mammalian chromosome aberration test with OECD #473, and mouse

lymphoma test with OECD #476, but no mutagenic effects of rebaudioside A were reported in any of the strains at any of the doses. Two *in vivo* assays conducted to analyze the genotoxic potency were mouse micronucleus test with OECD #474 and unscheduled DNA synthesis (UDS) test with OECD #486. Although all Naval Medical Research Institute mice administered with rebaudioside A at a dose of 750 mg/kg body weight in the mouse micronucleus test exhibited signs of toxicity, animals at lower concentrations and Wistar rats in the UDS test at all doses selected did not induce any genotoxic effects. Williams and Burdock's study (2009) demonstrated that rebaudioside A was neither mutagenic nor genotoxic, which was in agreement with the JECFA report (JECFA 2004).

Several genetic tests were performed with steviol, the metabolized product of rebaudioside A, with most of them showing no evidence of genotoxic potency (Matsui and others, 1989; Suttajit and others, 1993; Matsui and others, 1996; Oh and others, 1999; Temcharoen and others, 2000; Sekihashi and others, 2002). No measurable DNA damage in liver, stomach, kidney, and other tested tissues was shown in the standard comet assay of steviol administered at doses up to 2,000 mg/kg body weight. (Sekihashi and other 2002). After reviewing the literature concerning the mutagenicity of steviol and its related derivatives, Brusick (2008) questioned the limitation of *in vitro* genotoxic tests in mammalian cells and that the lack of normal DNA repair and replication processes of the *S. typhimurium* strain TM677 used in the assays indicated the mutagenicity of steviol. However, Brusick (2008) concluded that steviol did not pose a risk of genetic damage after human intake.

Deniņa and others (2013) reported an inhibitory effect of rebaudioside A on the growth of *Lactobacillus reuteri* (often used as functional food additive and inhabitant of human gut) *in vitro* in a strain-dependent manner. They noted that the most pronounced

concentration-dependent effect of rebaudioside A on *L. reuteri* strains was on lactic and acetic acid production (Deniņa and others 2013). However, Li and others (2014) suggested rebaudioside A was safe for the gut microbes with little pressure on the growth and composition of total bacteria, enterobacteria and lactobacilli *in vivo*. Kunová and others (2014) reported that the suggested prebiotic effect of rebaudioside A was not confirmed, and the utilization of steviol glycosides as a carbon source by lactobacilli and bifidobacteria was very limited.

The doses of rebaudioside A utilized in toxicity studies were much higher than that of dietary consumption. Based on the scientific studies on absorption, metabolism, and toxicity, the safety of rebaudioside A for human consumption can be affirmed (Nikiforov and Eapen 2008).

Healthy effects

Besides its sweet sensation, rebaudioside A may also provide some health benefits. It is non-caloric since the sugars are connected to each other and to the steviol scaffold by β–glycosidic ester bonds (Jooken and others 2012). It cannot be metabolized to produce energy, thus being helpful for individuals who intend to lose weight by restricting or controlling caloric intake. The substitution of sugar with rebaudioside A is an effective strategy to control obesity and weight gain.

Rebaudioside A is recommended as a treatment against diabetes. According to a long-term study of patients with type II diabetes mellitus (DM), the chronic intake of 1,000 mg/day of rebaudioside A for 16 weeks was well-tolerated, and it did not alter the glucose homeostasis or blood pressure, suggesting the safe intake of rebaudioside A for type II diabetes patients (Maki and others 2008). Curi and others (1986) reported the significantly enhanced glucose tolerance and suppressed plasma glucose levels after the ingestion of *Stevia* extracts by

conducting the glucose tolerance tests before and after the administration. The insulinotropic effect of rebaudioside A was later noted by Abudula and others (2008). It directly stimulated the insulin secretion from pancreatic β-cells by inhibiting the K^+-ATP channels with less risk to cause hypoglycemia. Rebaudioside A benefits the glucoregulation and provides a relatively comprehensive set of mechanisms to counter type II DM together with its complications (Gupta and others 2013).

Rebaudioside A is also helpful for patients with cardiovascular disease. Sharma and Mogre (2007) reported a hypolipidaemic effect of *Stevia* extracts. They found that the levels of cholesterol, triglycerides, and low-density lipoprotein-cholesterol were significantly lowered after the 20 selected hypercholesteric women ingested the *Stevia* extract. A desirable increase in high-density lipoprotein-cholesterol was also observed.

Rebaudioside A is also reported to exhibit some protective activities against oxidative damage. *S. rebaudiana* hot water extracts were found to prevent the oxidation of sardine oil and linoleic acid by scavenging the free radicals and superoxides (Xi and others 1998). In an *in vivo* test conducted by Shivanna and others (2013), obviously increased levels of malondialdehyde, conjugated dienes, and hydroperoxides were observed upon injection of streptozotocin (STZ) in Wistar rat diabetic groups. Their peroxidation level was suppressed significantly if pre-fed with a stock diet containing 4% of *Stevia* leaf powder and polyphenols. The pre-treatment with *Stevia* extracts also maintained the levels of antioxidants in plasma, which were altered significantly with STZ administration. The antioxidant enzymes, such as superoxide dismutase and catalase, were also stimulated to reverse oxidative damage.

Shivanna and others (2013) also demonstrated the renal protective capacity of *Stevia* extracts. Significant reduction in the kidney weight was observed in rats pre-feeding with *Stevia*

leaf powder, while kidney enlargement was induced by STZ treatment. The elevation in glomerular filtration rate from hyperfunctioning kidneys was brought down by 7% with *Stevia* supplementation in STZ-diabetic rats, suggesting the possibility of *Stevia* extracts as a treatment for some renal diseases in diabetic patients.

No potential increased risk of dental caries development was found with the prolonged use of rebaudioside A (Das and others 1992). Instead, rebaudioside A is non-cariogenic and provides some dental benefits through plaque inhibition and cavity reduction (Goyal and others 2010). Rebaudioside A, together with other *Stevia* extracts, can inhibit cariogenic organism aggregation induced by glucan, eliminating the dental decay and gingivitis (Gupta and others 2013).

Other therapeutic benefits of rebaudioside A include but are not limited to anti-hypertensive, anti-inflammatory, anti-tumor, anti-diarrheal, diuretic and immunomodulatory effects (Chatsudthipong and Muanprasat 2009). Furthermore, rebaudioside A is also safe for phenylketonuria patients, since it does not contain the amino acid phenylalanine, making it superior to aspartame.

Stability studies

With its excellent sensory and functional properties, the stability of rebaudioside A in foods has been the subject of numerous scientific studies. Moisture content, temperature and pH are some of the key factors affecting its stability in food systems (Prakash and others 2008).

Effect of moisture

Highly purified rebaudioside A was very stable in dry powder form, with only 1-2% loss recorded after two years storage in polyethylene bags at 25 ℃ and 60% relative humidity

(Prakash and others 2008). Less than 1.5% reduction of rebaudioside A was reported when kept in glass bottles for 65 weeks under the same conditions, while no more than 6.4% loss was shown over the course of 65-week period at 40 ℃ and 75% relative humidity (Prakash and others 2008; Cargill 2008). The slight loss of rebaudioside A was accompanied with increased level of rebaudioside B and the appearance of a compound referred to as DS-1(CC-00219) (Cargill 2008).

Comparatively, rebaudioside A was less stable in aqueous solution, with several reaction pathways, such as isomerization, hydration, and hydrolysis, leading to its degradation (Prakash and others 2008). When dissolved in 0.1 M phosphate buffer at 25 ℃, 50% loss of rebaudioside A was predicted to occur after 590 weeks storage at pH 3.4 and 7, and 59 weeks when the pH was 2.3 (Prakash and others 2008). In pH 3.4 or 7 phosphate buffer held at 25 ℃ for 2 years, approximately 11.5% loss of rebaudioside A is estimated based on the data. Compared to the 1-2% reduction in the dry powder, rebaudioside A in the buffer solution was approximately 10 times less stable than in the powder.

Effect of temperature

Several studies have investigated the effect of temperature on the stability of rebaudioside A. Chang and Cook's work (1983) demonstrated that there were no significant losses of rebaudioside A in citrate (pH 2.6) or phosphate buffer solutions (pH 2.4) kept at 4 ℃ for 4 months, at room temperature (22 ℃) for 3 months, and at 37 ℃ for 1 month. A 3% loss was recorded after rebaudioside A was stored in a citric acid beverage at 60 ℃ for 137 h, while 6% loss was noted in a phosphoric acidified system at the same conditions, without any appreciable degradation products. Heating at 100 ℃ for 13 h resulted in up to 76% loss of rebaudioside A in the citrate solution and up to 87% loss in phosphate solutions. Rebaudioside B and glucose were

recognized as the degradation products after heating at 100 °C for 4 h, resulting from the cleavage of rebaudioside A at the C19 ester linkage. One unknown product and another unknown compound in trace amounts were also detected at 4 h. It was also noted that the concentration of these degradation products increased with increasing heating time.

In an investigation conducted by Wölwer-Rieck and Papagiannopoulos (2008), 0.247 M citrate buffer at pH 3.8 containing rebaudioside A was stored at 4 °C, room temperature (25 °C), and 40 °C for up to 7 days, and at 80 °C for 3 days. No degradation of rebaudioside A was observed under these conditions. In a mock lemon-lime soft drink at 40 °C, 13.7% reduction of rebaudioside A was reported after 26 weeks storage (Prakash and other 2012b). Rebaudioside A was quite stable after the first week, which was consistent with the results in Wölwer-Rieck and Papagiannopoulos' (2008) experiment, in which lemonade samples at pH 3.8 were stored for 7 days. However, Prakash and others (2008) showed that when storage temperature was elevated from 25 °C to 40 °C, the half-life of rebaudioside A in 0.1 M phosphate buffer at pH 4 decreased by approximately 10 fold (one log); if temperature was further elevated to 80 °C, another two log reduction in the half-life was reported.

Mock beverages formulated with rebaudioside A were exposed to various temperatures (5, 20, 30, and 40 °C) for up to 26 weeks in an experiment conducted by Prakash and others (2012b). Six compounds were identified as the degradation products of rebaudioside A. Based on HPLC analysis over various time intervals, it was concluded that the degradation rates of rebaudioside A and the formation rates of degradation products increased as the temperature was elevated. Consistent results were reported by Gong and Bell (2013), in which the stability of rebaudioside A was investigated as a function of temperature (20, 30, and 40 °C). In both phosphate and citrate buffers, greater concentrations of rebaudioside A were lost as temperature increased. In 0.1 M

phosphate buffer at pH 3 and 7, the rebaudioside A degradation rate increased 2.7 – 3.4 fold from 20 °C to 40 °C (Gong and Bell 2013), which was less than the 10-fold increase shown by Prakash and others (2008).

Unpublished data, reported by Prakash and others (2008), noted the good stability of rebaudioside A during high temperature-short time treatment and the subsequent storage of some heat-processed beverage products. Less than 1% loss of rebaudioside A was reported in short-term thermally processed mock beverages at pH 3.2 and 6.5 (Cargill 2008). In addition, no measurable degradation of rebaudioside A was recorded if it was pasteurized in dairy products or baked to at least 390 °F (Carakostas and others 2008).

Overall, rebaudioside A degradation is very slow at low temperatures and increases as temperature increases. However, short term thermal processing does not appear to promote significant degradation.

Effect of pH

In Chang and Cook's research (1983), HPLC and TLC results demonstrated that rebaudioside A broke down faster in two acidic solutions, phosphoric and citric acid beverages, than in a neutral system. They also recognized that this high-intensity sweetener exhibited better stability in the citric acid (pH 2.6) than phosphoric acid solution (pH 2.4), which may partially be explained by the relatively higher pH value in the former system. Later, Prakash and others (2008) found that rebaudioside A in 0.1 M phosphate buffer was most stable at pH 4-8, but became less stable when the pH was decreased to pH 2. However, Wölwer-Rieck and Papagiannopoulos (2008) found that in citrate buffer solutions, rebaudioside A was stable at pH 2 (0.146 M) and 3.5 (0.223 M) at room temperature for a period of 3 days, but 12% degradation occurred at pH 5 (0.459 M) after 3 days. The contradiction in the stability of rebaudioside A

under various pH conditions may be explained by the differences in buffer type and buffer concentration, as experimentally shown by Gong and Bell (2013).

Wölwer-Rieck and others (2010b) investigated the usage of rebaudioside A in three different soft drinks at 80 °C. The pH was set to 2.4 in the caffeinated soft drink, 2.7 in the lemon-lime flavored drink, and 3.5 in the energy drink. Highest degradation of rebaudioside A (around 54%) was observed in the caffeinated soft drink after 72 h storage, while the best stability was found in the energy drink, suggesting that under acidic conditions, the stability of rebaudioside A increased with rising pH values, which was consistent with data from Prakash and others (2008), but inconsistent with data of Wölwer-Rieck and Papagiannopoulos (2008). Through analysis with UV-HPLC and LC-ESI-MS, the corresponding degradation products were identified to be rebaudioside B and steviolmonoside. Wölwer-Rieck and others (2010b) also concluded that rebaudioside A was more stable than stevioside.

In Prakash and others' (2011) research, steviol glycosides were dissolved in mock beverage solutions simulating the commercial formulations but without the flavor components. These beverage systems were cola soft drinks at pH 2.8 and 3.2, lemon-lime soft drink at pH 3.8, and root beer soft drink at pH 4.2. They were all buffered with phosphoric acid and monitored for up to 26 weeks**.** It was demonstrated that under acidic conditions, the steviol glycosides were less stable at lower pH conditions. More recently, Prakash and others (2012b) investigated the stability of rebaudioside A using the same conditions and confirmed that the concentration of degradation products increased with decreasing pH levels after extended storage, without appreciable loss of sweet taste. Up to 60% loss of rebaudioside A was recorded in pH 2.8 cola soft drink kept at 40 °C. The degradation products from rebaudioside A were analyzed with HPLC and determined to be rebaudioside B, rebaudioside F, and nine other acids and esters.

In systematic research reported by Gong and Bell (2013), the stability of rebaudioside A was studied as a function of pH (3, 5, and 7), buffer type (citrate and phosphate buffer) and buffer concentration (0.02 and 0.1 M) for 9 months. They found that in phosphate buffer the degradation rate constants of rebaudioside A increased as pH increased from 3 to 7, with up to 37.3% loss recorded in 0.1 M phosphate buffer at 40 ℃. The degradation was also accelerated by higher buffer concentrations. However, the degradation rate constants in citrate buffer samples decreased with increasing pH values. The loss of rebaudioside A was not pronounced at pH 3 (< 6% loss at 20-30 ℃ in 6 months), without an observable effect of buffer type and buffer concentration. Reported by Gong and Bell (2013), the pseudo-first-order rate constant of rebaudioside A in 0.1 M phosphate buffer at 40 ℃ and pH 3 was 0.000773 d^{-1}, and the corresponding half-life would be 2.2 x 10^4 hours, which was similar to the data shown by Prakash and others (2008). However, at the same temperature, the calculated half-lives of rebaudioside A at pH 5 and 7 in Gong and Bell's (2013) data were lower than the half-life at pH 3, which was inconsistent with the estimated values presented by Prakash and others (2008).

Effect of food matrix

So far, there is no data showing that rebaudioside A will interact with other components of the food matrix or cause browning. Tan and others (1988) reported that no observable browning or caramelization reaction occurred when rebaudioside A was heated at the elevated temperatures used in food processing. Rebaudioside A was considered to remain stable in soft drinks (cola and lemon-lime) and chewing gum for at least 26 weeks, and as a table top sweetener for 52 weeks under the investigated conditions (Prakash and others 2008). According to unpublished data cited in Prakash and others (2008), no sign of decomposition of rebaudioside A was shown in plain yogurt stored at 4 ℃ for 6 weeks. After baking white cake samples at

360 ℉ for 20 min or at 335 ℉ for 25 min, the level of rebaudioside A remaining when stored for 5 days at 25 ℃ and 60% relative humidity was identified to be 99.9% (Cargill 2008). The stability of rebaudioside A was demonstrated in a wide variety of everyday foods and beverages (Prakash and others 2008).

An extensive study considering the stability of steviol glycosides in different food matrices was performed by Jooken and others (2012), including semi-skimmed milk (6 ℃ and 20 ℃ for 20 weeks), soy drink (6 ℃ and 20 ℃ for 20 weeks), fermented milk drink (6 ℃ and 20 ℃ for 20 weeks), ice cream (-18 ℃ for 12 weeks), full-fat yogurt (6 ℃ for 35 days at pH 4.65), skimmed yogurt (6 ℃ for 35 days at pH 4.65), dry biscuit (baked at 185 ℃, and stored for 28 days), and jam (4 ℃ in light, room temperature in light and in the dark for 12 weeks). No systematic variations in rebaudioside A concentrations were noted for all selected food matrices under the specified conditions. Other steviol glycosides also remained stable over the investigation periods, indicating the good stability of steviol glycosides in a diverse range of food categories. Thus, Jooken and others (2012) concluded that the addition of steviol glycosides, including rebaudioside A, would not have adverse effects on the quality or shelf life of food products.

Effect of light exposure

Light is another factor that may have an impact on the stability of rebaudioside A. The photostability was first evaluated by Chang and Cook (1983). Beverage samples containing rebaudioside A were kept in tightly closed glass bottles. After exposure to sunlight (3000 langleys) for 1 week at 10-25 ℃, significant losses of rebaudioside A were observed in both phosphoric acid (pH 2.4) and citric acid (pH 2.6) systems, 22 and 18% respectively, suggesting

the adverse effect of light exposure on the stability of rebaudioside A. However, no other degradation products were noted by HPLC and TLC.

However, contradictory results were reported by Clos and others (2008). By replicating the study conducted 25 years earlier, high-purity rebaudioside A (> 97%) was dissolved in model cola (pH 2.4) and lemon-lime (pH 2.6) beverages, which were stored in 10 oz clear glass bottles with crown closures. Samples were exposed to sunlight until a level of 3000 langleys was reached (around 1 week), with the temperature cycling from 18-23 ℃ at night to 30-34 ℃ during the day. A light-protected group wrapped in aluminum foil was set as the control. In cola and lemon-lime soft drinks, 7.6% and 4.1% loss of rebaudioside A were reported after exposure to light, which compared to 1.5% and < 0.1% in the absence of light, respectively. Degradation was found for light-exposed samples with high mass balance value, and two esters were identified to be the degradation products. However, the same degradation products in similar amounts were also detected in the control groups, suggesting that these products were acid-promoted rather than light-promoted. Thus, Clos and others (2008) claimed that rebaudioside A was photostable and proposed that the differences between analytical methods (sample preparation and chromatographic techniques) resulted in the inconsistent findings between these two duplicated experiments. The size and type of containers used in these two papers were possibly different, and the filling volume was not noted in either paper, which might affect the light exposure on the stability of rebaudioside A.

More recently, the stability of a mixture of steviol glycosides (containing rebaudioside A) under fluorescent light exposure was investigated by Chaturvedula and others (2011). In the mock lemon-lime beverage solution (lacking flavor component) at pH 3.8, the mixture was exposed to fluorescent light (1.2 million lux hours total with a minimum of 200 watt hours/m^2 of

near-UV light) for 2 weeks at 25 ℃ and 60% relative humidity. No considerable changes in concentration were reported for both control and fluorescent light-treated samples with good mass balance. Chaturvedula and others (2011) concluded that the steviol glycoside mixture was relatively stable under the fluorescent light.

The effect of light on the stability of rebaudioside A was also preliminarily studied by Gong (2012), in which comparisons between the degradation rate constants of rebaudioside A in light and dark environments at room temperature were conducted. Approximately 1 – 2 mL of rebaudioside A solutions were placed into 2 mL sterile septum-containing vials. The light groups were expose to ambient room light (scattered sunlight, occasional fluorescent lighting) at room temperature. It was noted that degradation of rebaudioside A was almost ten times faster with light exposure than kept in dark environment in 0.1 M citrate buffer at pH 3. However, the effect of light was not obvious in samples stored in 0.1 M phosphate buffer at pH 7. This preliminary data indicted the need for additional research.

Objective

Because of inconsistent findings regarding the effect of light on rebaudioside A degradation, the subject requires to be evaluated with more data and systematic research. In addition, kinetic data are lacking regarding the photostability of rebaudioside A. The objective of this study is to evaluate the effect of UV light on the degradation rate constants of rebaudioside A in various buffer solutions.

Chapter 3: Materials and Methods

Reagents and chemicals

HPLC grade rebaudioside A (≥ 96%) was purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (CH_3CN) was bought from EMD (Gibbstown, NJ) and 85% phosphoric acid (H_3PO_4) was obtained from Fisher Scientific (Fair Lawn, NJ); both were HPLC grade. Hydrochloric acid (HCl), sodium hydroxide (NaOH), citric acid ($C_6H_8O_7$), sodium citrate ($Na_3C_6H_5O_7.2H_2O$), sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$), and sodium phosphate dibasic anhydrate (Na_2HPO_4) were from Fisher Scientific (Fair Lawn, NJ), all of which were reagent grade. Ultra-pure water (Type I, 18.2 MΩ-cm) was purified with an Elga Purelab Classic DI system from VWS (Berks, UK).

Sample preparation

The bulk solutions used to prepare the individual buffers were made with the following procedures. By adding 9.606 g citric acid into 500 mL water, a 0.1 M solution at pH 2.1 was made, while the 0.1 M sodium citrate solution at pH 8.0 was prepared by adding 14.704 g sodium citrate into 500 mL water. Phosphoric acid (2.885 g) was added into 250 mL water to make a 0.1 M solution at pH 1.68; 13.801 g monobasic sodium phosphate was added into 1 L water for 0.1 M monobasic phosphate solution at pH 4.5. Similarly, by adding 7.099 g dibasic sodium phosphate into 500 mL water, a 0.1 M dibasic phosphate solution at pH 9.1 was made.

Sodium citrate buffer at pH 3 and pH 7 were mixtures of the citric acid solution (pH 2.1) and the sodium citrate solution (pH 8.0) in different ratios. Sodium phosphate buffer at pH 3 consisted of the phosphoric acid solution (pH 1.68) and the sodium phosphate monobasic solution (pH 4.5), while phosphate buffer at pH 7 was prepared from the sodium phosphate

monobasic solution and the sodium phosphate dibasic solution (pH 9.1). All buffer solutions had the same concentration, 0.1 M. Unbuffered aqueous solutions were made by adding HCl or NaOH into ultra-pure water to get a pH of 3 and 7, respectively. Thus, four buffered and two unbuffered solutions were used as experimental solutions.

Approximately 100 mg of rebaudioside A were dissolved into 100 mL of each solution, resulting in a concentration of 100 mg/100 mL (1.0 g/L). In order to minimize potential microbial contamination, each sample solution was filtered through a 0.2 μm sterile nylon syringe filter (25 mm, Fisher Scientific, Ireland) with a PrecisionGlide™ needle (BD) into a 100 mL sealed sterile septum-containing glass bottle. It was then transferred into thirty-three 2-mL sterile septum-containing clear and colorless glass vials (total volume of 3.7 mL each) (Thermo Scientific, Miami, OK) using a new sterile syringe and needle. Volume was controlled to ensure that each vial was half loaded, containing approximately 2 mL sample solution.

For each solution, these 33 vials were evenly divided into 3 groups of 11 vials, which were then placed on their sides into an incubator set at a constant temperature of 32.5 ℃ (90 ℉). The dark group was stored in a paperboard box to block the light exposure. Another group was exposed to UV radiation with an intensity of 190 $\mu W/cm^2$, approximately 86.5 cm (34.2 inches) away from the light source, which was regarded as the "high-intensity group". A third group of vials was also exposed to the UV light at the same distance from the lamp, but the vials were covered by a piece of semi-transparent plastic sheet (plexiglass, 3 mm) to lower the intensity to 27 $\mu W/cm^2$ as the "low-intensity group". The UV light was emitted by a XX-15L bench UV lamp (365 nm, 115 V, 60 Hz, 15 W, UVP, Upland, CA). Both intensities of UV light were measured and recorded weekly with a UVX digital radiometer with light sensor (UVP, Upland, CA). Each vial was labelled according to their pH value (3 or 7), buffer type (C for citrate, P for

phosphate), light intensity (L for darkness, M for low light intensity, or H for high light intensity), and sampling order on the cap as to not interfere with light exposure. For example, “3CL1” represented the first sample to be detected in citrate buffer at pH 3 without UV radiation, while “7PH11” meant the last sample for analysis with high intensity UV exposure treatment in phosphate buffer at pH 7. Because of the high degradation rate, additional samples for 3CM, 3CH, and 7CH were prepared to obtain more data points.

One buffer solution was used to test the reproducibility of the method, which was 0.1 M citrate buffer at pH 7. This solution contained approximately the same amount of rebaudioside A. Six vials containing 2 mL rebaudioside A citrate buffer solution were placed in the incubator under high intensity UV exposure for 10 days and analyzed. The average concentration of rebaudioside A for these 6 samples was 51.38 mg/100 mL, ranging from 49.84 to 52.67 mg/100 mL, with a coefficient of variation of 0.02 or 2%, indicating the good reproducibility of the experiments.

In order to test the pH stability of buffers under different UV exposures, 0.1 M citrate and phosphate buffers at pH 3 and 7 lacking rebaudioside A were prepared. Two mL of buffer solutions were filtered through a 0.2 μm membrane and syringed into septum-containing glass vials. There were 6 vials for each buffer, which were then evenly divided into 2 groups and labelled. The low UV-exposed and high UV-exposed groups were placed in the incubator at 32.5 ℃ under various light exposures as previously specified. The samples were collected after 30 days and the pH value of each vial was measured and recorded.

In order to investigate the influence of filling volume on the UV stability of rebaudioside A, 0.1 M citrate buffer at pH 3 containing rebaudioside A was placed into glass vials at different volumes: 1 mL, 2 mL, and 3.7 mL. Four vials at each filling volume were prepared and placed in

the incubator at 32.5 ℃ under high UV exposure. The samples were collected after 35 days and the remaining concentrations of rebaudioside A were detected with HPLC.

The sampling process was performed up to a 205 day period. Light-exposed groups (both high and low intensity) were sampled around every 10 days, while the light-protected groups were analyzed less frequently, approximately every 20 days. For example, the samples in the 7PM group were collected at day 0, 14, 28, 38, 48, 56, 73, 82, 103, 114 and 124, while the 3WL samples were removed at day 0, 14, 28, 48, 73, 103, 124, 144, 164, 185 and 205. Samples were shaken before sampling, and the exact sampling date was recorded. An aliquot of the sample or standard solution was syringed into a 12 x 32 mm amber target snap-it ID^{TM} vial (Thermo Scientific, Rockwood, TN) and covered with blue snap-it seal (Thermo Scientific, Rockwood, TN). All samples and standards were treated identically to minimize bias.

Sample analysis

The photostability of rebaudioside A was evaluated in different solutions by measuring the concentrations of rebaudioside A in experimental solutions with reversed-phase HPLC. An Agilent (Wilmington, DE) 1200 series HPLC was used for analysis, which includes a binary pump, a temperature-controlled column heater, an autosampler, and a diode array detector. A micro vacuum degasser was also used. The system was controlled using Bruker (Billerica, MA) Hystar 3.1 software. A Phenomenex (Torrance, CA) Luna NH_2 100A column (5 μm, 250 mm x 4.6 mm I.D.) with a Phenomenex security guard NH2 cartridge (4 x 3.0 mm I.D.) was used. The column was held at a temperature of 45 ℃ during the analytical procedure. An isocratic mode was employed in the HPLC system, and the mobile phase consisted of 76.5%:23.5% (v/v) acetonitrile:phosphate buffer. The phosphate buffer was at a concentration of 0.025 M, prepared

by dissolving 3.4 g sodium phosphate monobasic monohydrate and 0.05 g sodium phosphate dibasic anhydrate into 1 L of ultra-pure water. The mobile phase originally at pH 8.4 – 8.5 was modified to pH 7.5 with a phosphoric acid solution. The flow rate was set as 2.0 mL/min with a total run time of 10 min. The injection volume of each sample was 20 µL. Samples were kept at ambient temperatures in the autosampler. The retention time for rebaudioside A was around 7 min. Absorbance was monitored at a wavelength of 210 nm.

Standards were made by dissolving rebaudioside A in ultra-pure water to create a series of concentrations: 2.5, 5, 10, 15, 20, 30, 40, 60, 80, and 120 mg/100 mL. The 80 mg/100 mL standard solution was prepared by dissolving 200 mg rebaudioside A into 250 mL water, while the 120 mg/100 mL solution was made by adding 300 mg rebaudioside A into 250 mL water. Other standard solutions were prepared from serial dilutions of these two standards. Before analysis, new rebaudioside A dilutions were prepared and then analyzed together with the samples at the scheduled intervals.

Standard curves were created with half heights of the rebaudioside A peak at the different concentrations. The peak areas were not used, since a shoulder in the rebaudioside A peak was present in some standards and samples, which brought difficulties to define the resolution and to separate the two peaks. Instead, half heights were utilized in all analysis for better consistency. Using the standard curves, the concentrations of rebaudioside A in the experimental solutions were determined.

Data analysis

A standard curve was created for quantitative analysis by plotting half height against concentration of the rebaudioside A standards. From the half height of the sample solution, the

corresponding rebaudioside A concentration was calculated. The % remaining of rebaudioside A was obtained by dividing the other concentrations by the original amount of rebaudioside A (i.e., at time 0). Degradation curves of rebaudioside A in corresponding experimental solutions were then plotted.

Gong and Bell (2013) showed that pseudo-first-order kinetic model fits the degradation behavior of rebaudioside A. Thus, pseudo-first-order rate constants with 95% confidence intervals were calculated from the slope of the Napierian logarithm of the rebaudioside A remaining concentrations as a function of time. The least-squares linear analysis was applied as described by Labuza and Kamman (1983). The determination of significant differences was analyzed by testing the homogeneity of regression at $p<0.05$, as described by Steel and Torrie (1980). This test was conducted (1) between samples of the same buffer type and pH with different light intensities and (2) between all samples under the same light exposure.

Chapter 4: Results and Discussion

Results

Rebaudioside A degradation was observed in various solutions during storage, both with and without exposure to UV radiation. Figures 4.1-4.3 show representative pseudo-first order kinetic profiles of rebaudioside A degradation. The stability of rebaudioside A in solutions was affected by pH, buffer type, and light intensity. The degradation rate constants associated with the loss of rebaudioside A with 95% confidence intervals, calculated using the pseudo-first order kinetic model, are listed in Table 4.1. Significant differences ($p<0.05$) were determined by testing for the homogeneity of the regression (Steel and Torrie 1980) and are also indicated in Table 4.1.

The half-lives of rebaudioside A in weeks (Table 4.2) were calculated using the pseudo-first order rate constants. Rate constants and half-lives are inversely related such that less stable systems have high rate constants and low half-lives, while more stable systems have low rate constants and high half-lives.

The estimated percentage loss of rebaudioside A after 1 week storage in our research was calculated and appears in Table 4.3. These data will be used to compare with the results of others regarding the photostability of rebaudioside A.

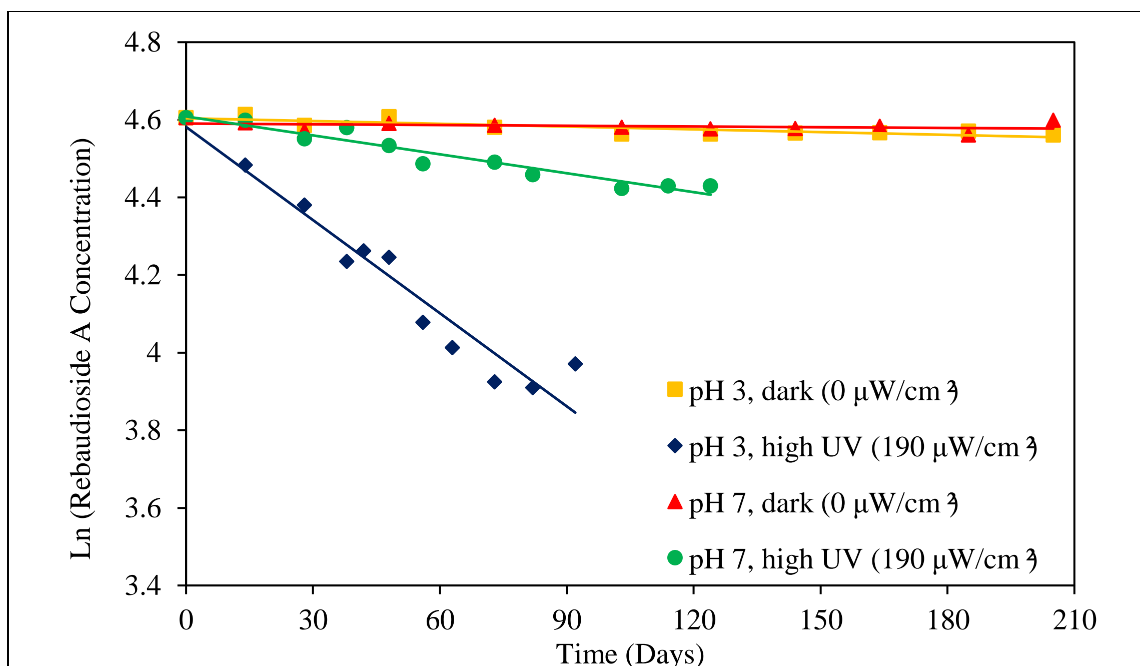

Figure 4.1 Degradation of rebaudioside A in pH-adjusted water at 32.5 °C as affected by UV light intensity at 365 nm.

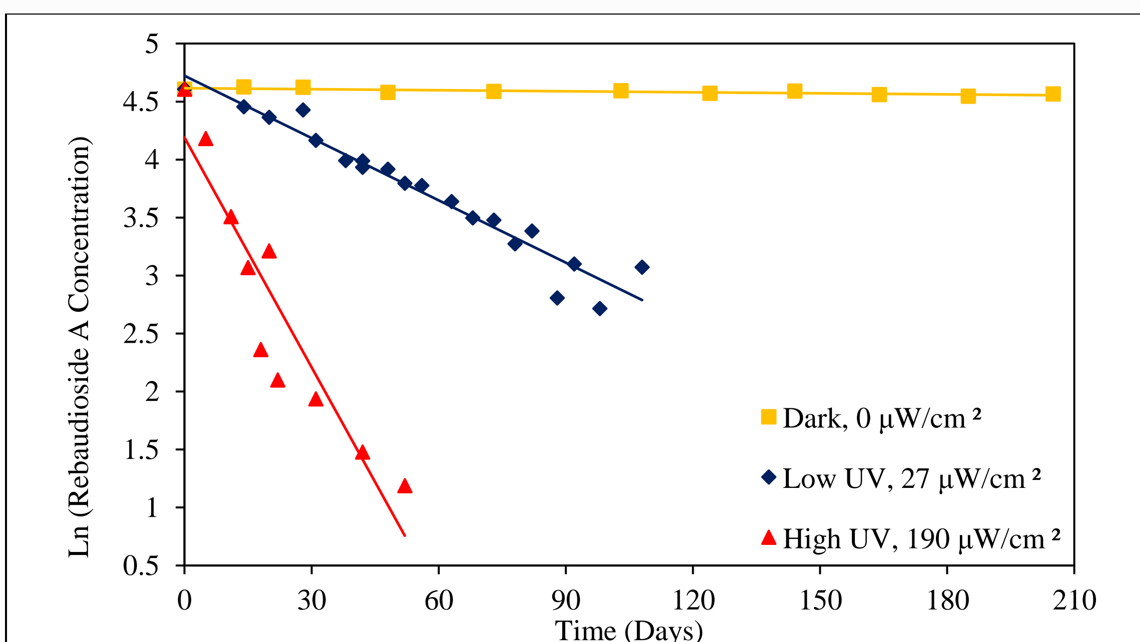

Figure 4.2 Degradation of rebaudioside A in 0.1 M citrate buffer at pH 3 at 32.5 °C as affected by exposure to UV light at 365 nm.

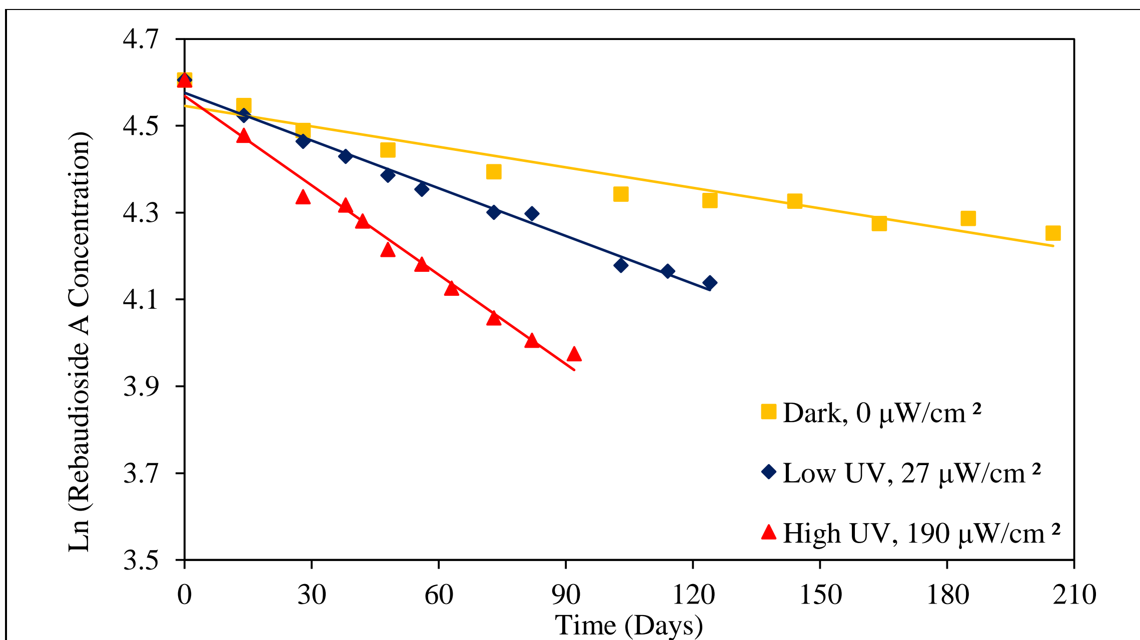

Figure 4.3 Degradation of rebaudioside A in 0.1 M phosphate buffer at pH 7 at 32.5 ℃ as affected by exposure to UV light at 365 nm.

Table 4.1 Pseudo-first order rate constants (d^{-1}) with 95% confidence limits for rebaudioside A degradation in solutions at 32.5 ℃ stored under various intensities of UV light. Rate constants have been multiplied by 1000.

UV intensity	pH 3 water	pH 7 water	pH 3 citrate	pH 7 citrate	pH 3 phosphate	pH 7 phosphate
Dark	0.238±0.112 aA	0.0628±0.123 aB	0.296±0.147 aA	0.105±0.054 aB	0.547±0.092 aC	1.57±0.34 aD
Low	2.29±0.22 bA	0.278±0.359 aB	17.9±2.3 bC	6.07±0.88 bD	1.80±0.39 bE	3.66±0.31 bF
High	8.00±1.56 cA	1.63±0.35 bB	66.0±0.4 cC	62.2±21.1 cC	5.65±1.22 cD	6.86±0.61 cAD

Different capital letters represent significant differences within the same row ($p<0.05$).

Different lower case letters represent significant differences within the same column ($p<0.05$) as a function of UV light exposure.

Table 4.2 Estimated half-life (weeks) for rebaudioside A in solutions at 32.5 ℃ calculated using the pseudo-first order kinetic model

UV intensity	Water pH 3	Water pH 7	Citrate pH 3	Citrate pH 7	Phosphate pH 3	Phosphate pH 7
Dark	416	1,577	335	943	181	63
Low	43	356	5.5	16	55	27
High	12	61	1.5	1.6	18	14

Table 4.3 Estimated rebaudioside A loss (%) after 1 week storage at 32.5 ℃ calculated with the pseudo-first order kinetic model

UV intensity	Water pH 3	Water pH 7	Citrate pH 3	Citrate pH 7	Phosphate pH 3	Phosphate pH 7
Dark	0.2	< 0.1	0.2	< 0.1	0.4	1.1
Low	1.6	0.2	12	4.2	1.2	2.5
High	5.4	1.1	37	35	3.9	4.7

Stability of rebaudioside A in darkness

Table 4.1 lists the rate constants for rebaudioside A degradation in solutions held under dark conditions. The rate constants of rebaudioside A in water and citrate buffers at pH 3 were both significantly ($p<0.05$) higher than the values at pH 7, suggesting that rebaudioside A in these two solutions was more stable in the neutral environment. Under dark conditions, the ratio of the rate constants at pH 3 to pH 7 in water was 3.8, and the ratio was 2.8 in citrate buffers.

Consequently, longer half-lives of rebaudioside A in water and citrate buffer were noted at pH 7 (1,577 and 943 weeks, respectively), compared to at pH 3 (416 and 335 weeks). It was found that rebaudioside A in water was slightly more sensitive to changes of acidity.

However, in phosphate buffers, a significantly ($p<0.05$) greater degradation rate constant for rebaudioside A was found at pH 7 than pH 3, indicating that rebaudioside A was more stable in acidic phosphate buffer solutions when protected from light. The ratios of degradation rate constants of rebaudioside A at pH 3 to pH 7 in phosphate buffers was 0.35 under dark conditions.

Based on the data, it was noted that for light-protected groups, rebaudioside A was less stable in acidic conditions in both water and citrate solutions; in phosphate buffer, more rebaudioside A loss occurred in neutral conditions. Clearly, buffer type was another key factor affecting the stability of rebaudioside A. Dibasic sodium phosphate was used in preparing pH 7 phosphate buffer solutions. The dibasic phosphate anion is believed to contribute to the degradation susceptibility of rebaudioside A in the pH 7 phosphate buffer solution by more effectively catalyzing proton transfers associated with the degradation reactions, as hypothesized by Gong and Bell (2013).

Our results were similar to those reported by Wölwer-Rieck and Papagiannopoulos (2008). After 3 days storage with accelerated temperature of 80 ℃, rebaudioside A in a caffeinated lemonade at pH 2.3 had the highest degradation (48%), while the lowest loss (16%) was observed in an energy drink at pH 5. The beverage systems used in this research were all prepared with citric acid buffers and protected from light. The results suggested that the stability of rebaudioside A mainly depended on pH and increased with rising pH levels when buffered with citrate.

Table 4.4 Rebaudioside A degradation rate constants (d^{-1}) with 95% confidence limits in mock beverage solutions at 30 ℃ calculated with the data from Prakash and others (2012b)

pH value	Pseudo-first order rate constant (d^{-1})
2.8	0.00133±0.000137
3.2	0.000621±0.0000892
3.8	0.000156±0.000126
4.2	0.0000932±0.0000864

Prakash and others (2012b) investigated the stability of rebaudioside A under acidic conditions by detecting the concentrations of rebaudioside A during 26 weeks storage in four commercial soft drinks with pH values ranging from 2.8 to 4.2 modified with trisodium citrate. Using the data presented, the degradation rate constants with 95% confidence intervals were calculated and are listed in Table 4.4. The degradation rate constants at pH 2.8 was approximately 14 times higher than the value at pH 4.2. The results indicated that rebaudioside A in citrate solutions was less stable at lower pH values, which are consistent with our results.

Our findings were generally consistent with the results reported by Gong and Bell (2013) regarding the effect of pH on rebaudioside A. The degradation rate constants of rebaudioside A at temperatures ranging from 20 to 40 ℃ in darkness are listed in Table 4.5, using data from our research and Gong and Bell (2013). They noted that under dark conditions, the degradation rate constants of rebaudioside A increased with increasing pH in phosphate buffer, and degradation was accelerated with increasing storage temperature. The rate constants of rebaudioside A generally decreased as pH increased in citrate buffer at 30 and 40 ℃, which is consistent with our data when samples were kept at 32.5 ℃. However, rebaudioside A degradation rate constants in

Table 4.5 Rebaudioside A degradation rate constants (d^{-1}) with 95% confidence limits in 0.1 M buffer solutions at various temperatures in darkness using the data from our research and Gong and Bell (2013). Rate constants have been multiplied by 1000.

Temperature	0.1 M phosphate buffer		0.1 M citrate buffer	
	pH 3	pH 7	pH 3	pH 7
20 ℃[a]	0.226±0.102	0.953±0.147	0.0102±0.224	0.0604±0.126
30 ℃[a]	0.228±0.0878	1.61±0.12	0.242±0.242	0.133±0.137
32.5 ℃[b]	0.547±0.0921	1.57±0.34	0.296±0.147	0.105±0.0539
40 ℃[a]	0.773±0.147	2.59±0.22	0.930±0.267	0.212±0.147

[a] Gong and Bell (2013); [b] Current data

citrate buffer at pH 7 and pH 3 at 20 ℃ were not significantly different (Gong and Bell 2013). The degradation rate constants of rebaudioside A in pH 7 phosphate and citrate buffers at 32.5 ℃ appear slightly lower than the corresponding values at 30°C from Gong and Bell's (2013) research. Although the rate constants of rebaudioside A stored at 32.5 ℃ were expected to be slightly higher than those of kept at 30 ℃, these corresponding values are very close and by testing the homogeneity of the regression, they were non-significantly different ($p>0.05$). Slight differences in sample composition (pH and buffer concentration) could contribute to these inconsistencies.

As mentioned before, the stability of rebaudioside A in solutions was also affected by the buffer type. In the light-protected groups, rebaudioside A in water and citrate buffers was relatively stable. Degradation rate constants of rebaudioside A in water and citrate buffer at either pH 3 or pH 7 were not significantly different ($p>0.05$). However, rebaudioside A in

phosphate buffers was more susceptible to break down, especially in neutral conditions. A 50% reduction of rebaudioside A would be obtained after 63 weeks storage in the pH 7 phosphate buffer solution, compared to 943 weeks in citrate buffer. In darkness, the degradation rate constant in pH 7 phosphate buffer was significantly ($p<0.05$) higher, being approximately 25 and 15 times larger than the values in water and citrate buffer, respectively.

Long-term rebaudioside A stability in solutions protected from light was conducted by Chang and Cook (1983). Both carbonated citric acid (pH 2.6) and phosphoric acid (pH 2.4) beverages containing rebaudioside A were stored for 4 months. According to the percentage degradation of rebaudioside A provided, the pseudo-first order rate constants with 95% confidence intervals at 37 ℃ were 0.00135 ± 0.000776 and 0.00262 ± 0.00111 d^{-1} for rebaudioside A in citric and phosphoric acid systems, respectively. Chang and Cook (1983) noted greater stability of rebaudioside A in citric than phosphoric acid beverages, which was in agreement with our results under dark conditions. The slightly greater acidity in the phosphoric acid system was another factor that should be taken into consideration.

Our results somewhat agreed with the previous study done by Gong and Bell (2013), noting the apparent effect of buffer type on the degradation of rebaudioside A at pH 5 and 7 in darkness. However, Gong and Bell (2013) concluded that buffer type did not affect the degradation rate constants of rebaudioside A in pH 3 citrate and phosphate buffers at 20 and 30 ℃; a non-significant difference between rate constants in 0.1 M buffers with a pH of 3 at 40 ℃ was also reported. Our results showed that differences regarding buffer type were significant ($p<0.05$) in all light-protected groups except rebaudioside A between water and citrate buffers at both pH levels. As mentioned previously, inconsistencies might be explained by slight differences in sample composition.

Stability of rebaudioside A as a function of light intensity

Under dark conditions, rebaudioside A was relatively stable with limited degradation occurring during the storage period (up to 205 days). The degradation rate constants increased significantly ($p<0.05$) with increasing light intensity in all solutions, as shown in Table 4.1. For example, in pH 3 citrate buffer solutions, the rebaudioside A degradation rate constants were 0.000296 ± 0.000147, 0.0179 ± 0.00227, and 0.0660 ± 0.0204 d^{-1} in dark, low and high UV exposure conditions, respectively; the corresponding kinetic plots were shown in Figure 4.2. The remaining concentrations of rebaudioside A in pH 7 phosphate buffer solutions as a function of time were shown in Figure 4.3, where the effect of UV light is similar.

The pseudo-first order rate constants for rebaudioside A degradation in water stored under high UV light radiation (190 $\mu W/cm^2$) were approximately 34 and 26 times higher than those without light exposure at pH 3 and 7, respectively, whose degradation curves were shown in Figure 4.1. In pH 3 and 7 citrate buffers, rebaudioside A degradation rate constants associated with high UV exposure were about 223 and 592 times higher, respectively, than the values without light exposure. UV exposure increased the rate constants 10 and 4 times in phosphate buffer solutions at pH 3 and 7, respectively.

Another perspective for evaluating stability is through the half-life. The half-lives (in weeks) of rebaudioside A in solutions were calculated and shown in Table 4.2. For example, with high UV radiation, 50% loss of rebaudioside A occurred in less than 2 weeks in pH 7 citrate buffer, while in the light-protected groups, it would take over 900 weeks to degrade 50% of the rebaudioside A, indicating the adverse impact of light on rebaudioside A stability.

The results of this study were similar to those of Chang and Cook (1983) and Gong (2012), suggesting that rebaudioside A was adversely affected by light exposure. Chang and

Cook (1983) reported approximately 20% reduction of rebaudioside A with 1 week sunlight exposure, 18% in citric and 22% in phosphoric acid beverages, respectively. However, much lower amounts of rebaudioside A loss were noted by Gong (2012) using the pseudo-first order rate constant model. With ambient light exposure, only around 0.8% loss of rebaudioside A in 0.1 M pH 7 phosphate buffer was predicted after 1 week storage while 0.3% loss was predicted in 0.1 M pH 3 citrate buffer (Table 4.6). Using the pseudo-first order rate constants in our research to determine the extent of loss (Table 4.3), up to 37% rebaudioside A loss was predicted when exposed to high UV light for 1 week, which was higher than the amount indicated in the other two studies. This highest reduction of rebaudioside A occurred in 0.1 M citrate buffer at pH 3. Other estimated rebaudioside A loss after 1 week storage under different conditions using the pseudo-first order kinetic model are listed in Table 4.3 and ranged from 1.1 to 35% depending upon the solution type. Variability between the extents of rebaudioside A degradation in these three studies was likely due to variations in experimental conditions, such as light source and intensity, container size and material, buffer pH and concentrations, as well as the storage temperature.

The degradation rate constant of rebaudioside A in 0.1 M phosphate buffer at pH 7 stored in darkness (0.00157 ± 0.000343 d^{-1}) was higher than the value of 0.000993 ± 0.000183 d^{-1} provided by Gong (2012), which would be explained by the storage temperature differences. Similarly, the degradation of rebaudioside A in 0.1 M citrate buffer at pH 3 without UV exposure (0.000296 ± 0.000147 d^{-1}) was faster than that of 0.0000547 ± 0.000143 d^{-1} from Gong's (2012) study. In our research, the rate constant in pH 7 phosphate buffer was approximately 5.3 times higher than the value in pH 3 citrate buffer when protected from light, while a much higher difference, 18.2 times, was reported by Gong (2012). The light-protected samples in Gong's

Table 4.6 Estimated rebaudioside A loss (%) in 0.1 M citrate and phosphate buffers after 1 week storage at room temperature calculated with the pseudo-first order kinetic model from Gong (2012)

	pH 3 citrate	pH 7 phosphate
Dark	0.0383	0.693
Ambient Light	0.310	0.823

(2012) research were kept in paperboard box at room temperature (20-25 ℃), while samples in darkness in our study were stored in an incubator at a higher temperature (32.5 ℃). Degradation of rebaudioside A would be accelerated by increasing temperature. Pseudo-first order rate constants of rebaudioside A loss under high and low UV light exposure in 0.1 M citrate buffer at pH 3 were approximately 149 and 40 times greater, respectively, than the rate constant in the same buffer solution in the study conducted by Gong (2012); rate constants under high and low UV radiation in 0.1 M phosphate buffer at pH 7 in our research were around 6 and 3 times higher, respectively, than the values provided by Gong (2012). The higher rate constants in our study would be due to both the higher storage temperature and differences in light source. Sample solutions in Gong's (2012) experiment were placed under ambient room light (scattered sunlight, occasional fluorescent lighting) with relatively lower light intensity as compared with the light provided by the UV lamp (both low and high intensity) in this study. However, Gong (2012) did not indicate the light intensity.

Our findings did not agree with the conclusions of Clos and others (2008) and Chaturvedula and others (2011). Clos and others (2008) claimed that rebaudioside A was stable under light exposure. However, low amounts of rebaudioside A degradation were detected in their light-exposed groups; 7.6% in cola (pH 2.4) and 4.1% in lemon-lime (pH 2.6) soft drinks in

comparison to 1.5% and <1% loss in the control groups, respectively. Chaturvedula and others (2011) noted the relative stability of a steviol glycoside mixture (containing rebaudioside A) stored under the fluorescent light. Differences between our findings and these two conclusions might possibly result from the differences in sample preparations and light exposure conditions. Light-exposed samples in Clos and others' (2008) research were stored in 10 oz glass bottles with crown closures and exposed to sunlight until the intensity reached 3000 langleys, which took around 1 week, while the light-protected groups were stored in the same condition but wrapped in aluminum foil. Chaturvedula and others (2011) stored the mock lemon-lime soft drinks (pH 3.8) in glass bottles, covered with plastic wrap, and placed side-by-side, which were then exposed to a minimum of 1.2 million lux hours and not less than 200 watt hours/m^2 at 25 ℃ for 2 weeks. However, the volume of the glass bottles used for this research was not indicated by Chaturvedula and others (2011).

To examine potential reasons for discrepancies with the rebaudioside A stability data as a function of light exposure, another experiment was conducted to explore the influence of filling volume/container size on its stability. Samples (n=4) were prepared and collected following the procedures described in the Materials and Methods chapter. Starting with 0.1 M citrate buffer at pH 3 containing 100 mg rebaudioside A/100 mL, the average concentrations of rebaudioside A with a volume of 1, 2, and 3.7 mL decreased to 1.17, 2.14, and 72.3 mg/100 mL, respectively, after UV storage for 35 days; the corresponding standard deviations were 0.44, 0.15, and 1.78 (see Table A8 in the Appendix). Based on the data, it was found that the stability of rebaudioside A under light exposure was influenced by the filling volume of the containers, which would correlate with the size of the containers. Greater filling volume would represent a larger container size. The greater the filling volume, the better the stability of rebaudioside A, which

could be explained by water absorbing UV light and consequently exposing the sample to less intense radiation. When the containers were completely filled (3.7 mL in our glass vials), the exposure of UV light to the samples was more limited, and thus less degradation of rebaudioside A was observed. Therefore, less rebaudioside A loss would be expected when kept in larger containers, such as the 10 oz glass bottles used by Clos and others (2008). The inconsistent results reported by Chang and Cook (1983), Clos and others (2008), Chaturvedula and others (2011), and Gong (2012) could be partially explained by the differences in their container sizes, composition, and filling volume.

This study demonstrated that light exposure has adverse effects on the stability of rebaudioside A in solutions, and the degradation of rebaudioside A increases significantly ($p<0.05$) with increasing light intensity. Further research on the identification of degradation products and degradation mechanism are necessary.

Stability of rebaudioside A: combined effects of pH, buffer type, light intensity

The impact of light exposure, pH, and buffer type on the stability of rebaudioside A was investigated by preparing samples in water, citrate buffer, and phosphate buffer at pH 3 and 7 under different light conditions (in darkness, under low-intensity UV light exposure, and under high-intensity UV radiation). Based on the pseudo-first order degradation rate constants listed in Table 4.1, it was noted that light exposure negatively impacted rebaudioside A stability in solutions; the degradation rate of rebaudioside A increased significantly ($p<0.05$) with increasing light intensity, except the degradation in pH 7 water between dark and low UV conditions. It was also shown that fastest loss of rebaudioside A occurred in pH 3 and pH 7 citrate buffers exposed to high UV radiation, while least loss was found in pH 7 water in darkness.

In both light-protected and light-exposed groups, rebaudioside A in water and citrate buffers was more stable at pH 7 than in pH 3, and the degradation rate constants of rebaudioside A increased with increasing acidity and increasing light intensity. However, rebaudioside A in phosphate buffer was found to be more stable at pH 3 than pH 7, regardless the effect of light exposure. The degradation rate constants of rebaudioside A in phosphate buffers increased with increasing pH value and increasing light intensity. Significant differences ($p<0.05$) regarding pH were reported in all solutions, except for rebaudioside A in citrate and phosphate buffers exposed to high UV radiation. In other words, under high UV light exposure, the pH effect went away in both buffer types.

In the light-protected groups, faster loss of rebaudioside A was observed in phosphate buffers, while rebaudioside A in citrate buffers was comparatively stable (Figure 4.4). In dark conditions, the highest degradation rate constant of rebaudioside A was found in phosphate buffer at pH 7, followed by phosphate buffer at pH 3; rebaudioside A in phosphate buffer is less stable at pH 7 because of the catalytic dibasic anion, as mentioned before. Although rebaudioside A was more susceptible to break down in phosphate buffer solutions when protected from light, faster degradation of rebaudioside A was found in citrate buffer solutions when exposed to light (Figure 4.5). With low and high UV light exposure, the degradation rate constants of rebaudioside A in citrate buffers were significantly ($p<0.05$) higher than those of phosphate buffer solution at the corresponding pH levels. When exposed to light, the highest degradation rate constant of rebaudioside A was found in citrate buffer at pH 3, followed by citrate buffer at pH 7; however, the rate constants were not significantly different ($p>0.05$). Thus, buffer type, pH value, and light intensity interact together to affect the rebaudioside A stability in solutions.

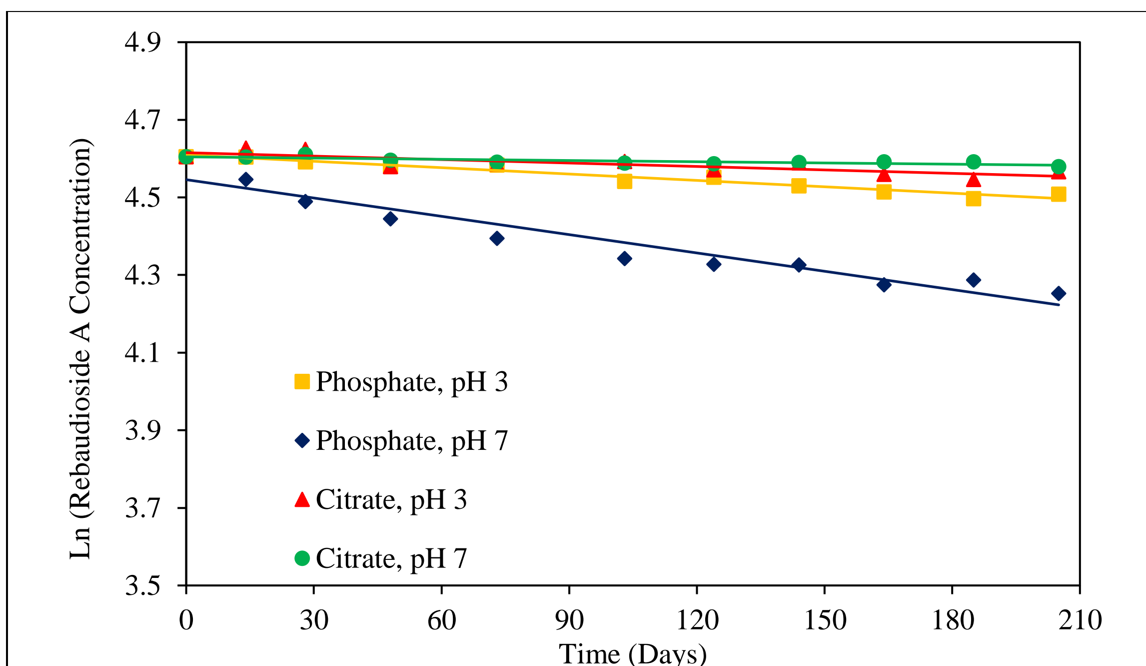

Figure 4.4 Degradation of rebaudioside A in 0.1 M buffer solutions at 32.5 ℃ under dark conditions at 365 nm.

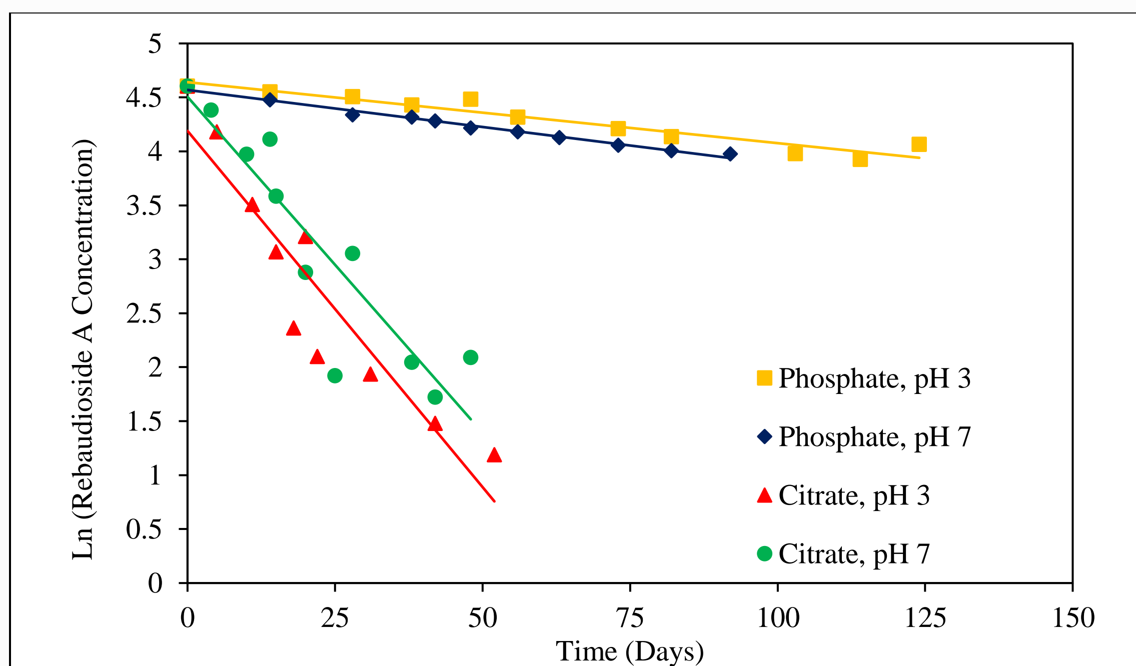

Figure 4.5 Degradation of rebaudioside A in 0.1 M buffer solutions at 32.5 ℃ under high UV exposure (365nm, 190 μW/cm²).

Table 4.2 reported the half-lives for rebaudioside A loss; the half-lives of rebaudioside A in citrate buffers were 5.5 weeks at pH 3 and 16 weeks at pH 7 in low UV light and 1.5 weeks at pH 3 and 1.6 weeks at pH 7 in high UV radiation. The half-life of rebaudioside A in pH 3 citrate buffer under low UV light was even shorter than those in water and phosphate buffers at both pH values under high UV light, where the degradation might be accelerated by the higher UV intensity. Based on the data obtained, it was concluded that rebaudioside A in citrate buffers was extremely sensitive to the change of light intensity.

Our findings were somewhat consistent with the results provided by Gong (2012), which indicated the greater sensitivity of rebaudioside A in citrate buffers. In Gong's (2012) research, the estimated rebaudioside A loss in 0.1 M citrate buffer at pH 3 under ambient light was approximately 8 times higher than the value for rebaudioside A stored in darkness, but in 0.1 M phosphate buffer solutions at pH 7, the degradation in the ambient light sample was only 1.2 times larger than the sample held in darkness (Table 4.6). If compared to the pseudo-first order rate constant of rebaudioside A in 0.1 M citrate buffer at pH 3 stored in darkness, a larger rate constant was found in 0.1 M phosphate buffer at pH 7 (Gong 2012), which was consistent with our result that rebaudioside A in darkness was more stable in citrate than in phosphate buffer solutions. Gong (2012) also reported a greater degradation rate constant in pH 7 phosphate buffer than in pH 3 citrate buffer when exposed to ambient light; she indicated that light did not play an important role in the degradation of rebaudioside A in pH 7 phosphate buffer with the difference between light exposed and protected samples being non-significant ($p>0.05$). However, our results showed rate constants of rebaudioside A loss in pH 3 citrate buffer were both significantly ($p<0.05$) higher than those in pH 7 phosphate buffer under low and high UV exposure. When exposed to low and high UV radiation, the accelerated degradation of

rebaudioside A in both solutions was noted, suggesting the adverse effect of light exposure on the stability of rebaudioside A. Such inconsistencies might be partially explained by the differences in experimental temperature, approximately 10 ℃. Differences in the light source and light intensity could also contribute to the inconsistent results. Rebaudioside A samples were placed under ambient light exposure in Gong's (2012) research, and the light intensity was expected to be very low, at least lower than the low UV intensity in our study. Based on our data, curves showing the rebaudioside A degradation rate constant as a function of UV light intensity for samples kept in pH 3 citrate and pH 7 phosphate buffer solutions cross around 14 $\mu W/cm^2$, as shown in Figure 4.6. The ambient light intensity was probably below the intensity of the cross point; thus Gong (2012) reported a greater degradation rate constant in 0.1 M phosphate buffer solution at pH 7 as compared with the value in the pH 3 citrate buffer solution.

In the study conducted by Chang and Cook (1983), 18% loss of rebaudioside A in a citric acid beverage was reported after 1 week sunlight exposure with 22% loss in a phosphoric acid system. This finding did not agree with our conclusion that rebaudioside A was more susceptible to break down in citrate than phosphate buffer when exposed to light. This inconsistency could be partially explained by the differences in pH. In their research, the citric acid system had a higher pH value (pH 2.6) than the phosphoric acid system (pH 2.4). Since rebaudioside A in citrate buffer was less stable at lower pH values, it would be expected that rebaudioside A stored

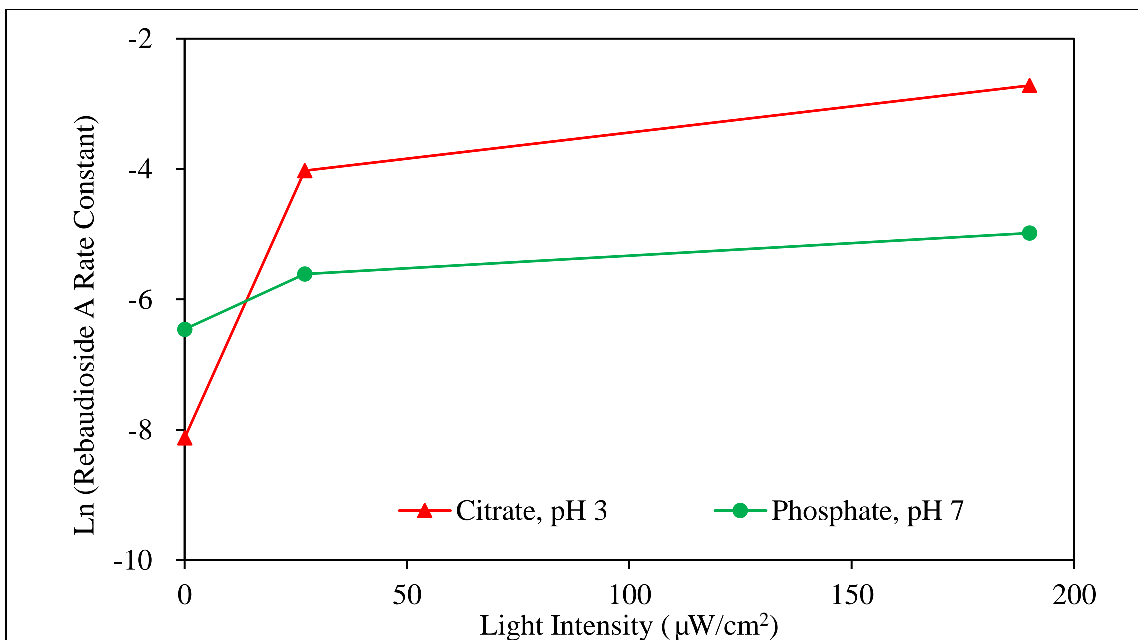

Figure 4.6 Degradation rate constants of rebaudioside A in 0.1 M pH 3 citrate buffer and pH 7 phosphate buffer at 32.5 ℃ as a function of UV light intensity at 365 nm.

in a citric acid system at pH 2.4 might have more degradation than at pH 2.6. It is possible that by increasing the acidity to pH 2.4, the percentage loss of rebaudioside A in the citric acid beverage might be greater than 22%.

Although Clos and others (2008) claimed rebaudioside A was stable when exposed to sunlight, greater percentages of loss were reported in pH 2.4 cola samples (containing phosphoric acid) than in the pH 2.6 lemon-lime samples (containing citric acid) in both light-protected and light-exposed groups. If the pH in lemon-lime beverages was lowered from 2.6 to 2.4, more loss of rebaudioside A would be predicted in both control and sunlight-exposed groups. Thus, it was difficult to compare the loss of rebaudioside A in these two beverages. However, based on our findings, it was estimated that rebaudioside A at pH 2.4 would be more

stable in lemon-lime than cola samples if protected from light, and less stable under light exposure.

Under UV light exposure (both low and high), the reason for the accelerated degradation of rebaudioside A in citrate buffer as compared with its loss in other two solutions is unclear. A hypothesis was put forward that the citrate became "activated" by the UV radiation to cause greater degradation of rebaudioside A, which may be associated with hydroxyl free radicals, HO•, generated from Fe(III)-citrate complexes.

Unknown amounts of iron were noted on the labels of citric acid and sodium citrate compounds. In order to identify and quantify the iron content, 0.1 M citrate buffer solutions at pH 3 and pH 7 were prepared and sent to a chemical-analytical lab for iron analysis via inductively coupled plasma. The iron concentrations for 0.1 M citrate buffer solutions were 0.84 and 0.51 mg/L at pH 3 and pH 7, respectively. Some literature about Fe(III)-photocatalysis were found (Chen and others 2011; Wu and Deng 2000). Citric acid can combine with iron to form Fe(III)-citrate complexes. The complex can photo-oxidize under UV light to generate HO•, which is pH-dependent. As reported by Chen and others (2011), the formation rate of HO• is greater at pH 3 than pH 7 with a Fe(III)-to-citrate ratio of 10:50, but at lower ratios, such as 10:150 and 10:500, more HO• is formed at pH 7 than pH 3. Rebaudioside A has been reported to be a free radical scavenger (Hajihashemi and Geuns, 2013). It is hypothesized that the HO• generated from Fe(III)-citrate complex can accelerate the degradation of rebaudioside A. As the rebaudioside A quenches these free radicals, the rebaudioside A itself is degraded. Thus, rebaudioside A in citrate buffers becomes more susceptible to break down in the light-exposed groups. In other words, the citrate in the presence of Fe(III) is "activated" by light exposure to form reactive radicals.

According to the photochemical reactions from Chen and others (2011), O_2 is present in the pathways leading to the formation of HO•. The O_2 or headspace volume in the vials may therefore impact the reactivity. As mentioned previously, vials containing lower solution volumes showed faster rebaudioside A loss. Because the lower volume may lead to greater exposure to both light and oxygen, more research is needed to clarify their potential roles on rebaudioside A stability.

The accelerated degradation of rebaudioside A in phosphate buffer solution under UV radiation might be explained by another hypothesis associated with the catalysis effect of HO•. Some HO• can be generated from Fe(III)-OH in acidic aqueous solutions under UV light (Wu and Deng 2000). Scavenger rebaudioside A can quench the HO•, thus accelerating the degradation reaction with light exposure. However, the amount of HO• generated from Fe(III)-OH is expected to be lower than Fe(III)-citrate complex, explaining the greater degradation rate constant of rebaudioside A in citrate than phosphate buffer solution under UV radiation.

In darkness, the catalytic effect of citric acid for rebaudioside A degradation is weak, thus rebaudioside A in citrate buffer solutions is relatively stable at both pH 3 and 7. Comparatively, the catalytic effect of dibasic phosphate anion is stronger, resulting in higher loss of rebaudioside A in pH 7 phosphate buffer solutions when protected from light. Under UV exposure, the HO• is produced from the Fe(III)-citrate complex. The degradation of rebaudioside A is very quickly catalyzed by HO• through scavenging activity. Small amounts of HO• are also produced under acidic conditions such as pH 3 phosphate buffer (Wu and Deng 2000). However, Fe(III) in acidic phosphate buffer produces less free radicals than the Fe(III)-citrate complex. The HO• and dibasic phosphate anion together contribute to the increased degradation rate constants of

rebaudioside A in phosphate buffer solutions under UV exposure. The HO• may be more catalytic than the dibasic phosphate anion, which explains why the rate constants increased dramatically in citrate buffer with UV exposure.

Thus, determination and quantification of degradation products of rebaudioside A in our research with HPLC/MS could be the next step to explore the degradation mechanisms. Once the pathways are defined, it may be possible to verify the hypothesis for the accelerated degradation of rebaudioside A in citrate buffer under UV light exposure.

Chapter 5: Summary and Conclusion

Extracted from the leaves of *Stevia rebaudiana*, rebaudioside A is used as a non-caloric high-potency sweetener with increasing application in the food industry. For better use in beverage applications, evaluating and understanding the effect of light exposure on rebaudioside A stability is necessary. This study determined the storage stability of rebaudioside A in buffer solutions as a function of UV light intensity and provided corresponding kinetic data as pseudo-first order degradation rate constants.

Light exposure has adverse effects on the stability of rebaudioside A in solutions. When protected from light, rebaudioside A in water, citrate buffer solutions, and phosphate buffer solutions was relatively stable with limited degradation. In both light-protected and light-exposed groups, rebaudioside A in water and citrate buffers was more stable at pH 7 than pH 3; however, rebaudioside A in phosphate buffer was more stable in acidic conditions regardless the effect of light exposure. The degradation rate constants increased significantly ($p<0.05$) with increasing UV light intensity in all solutions. Under dark conditions, rebaudioside A in water and citrate buffers was more stable when compared with rebaudioside A in phosphate buffer. However, the degradation of rebaudioside A in citrate buffer showed greater sensitivity to light exposure. Upon exposure to light, the degradation rate constants of rebaudioside A in citrate buffer solutions at both pH values became significantly ($p<0.05$) higher than the rate constants in water and phosphate buffer.

The accelerated degradation of rebaudioside A in citrate buffer could be possibly explained by the following hypothesis. Trace amounts of iron in the citrate buffer may create

Fe(III)-citrate complexes, which generates free radicals upon exposure to UV light. The photo-induced radicals then promote very fast degradation of rebaudioside A through scavenging activity. Further research to confirm the Fe(III)-citrate complex's role as the cause of higher degradation rate constants of rebaudioside A in citrate buffer solutions is necessary. The identification and quantification of degradation products of rebaudioside A in different solutions could be done with HPLC/MS; thus degradation pathways or mechanisms could be defined.

This systematic study evaluated rebaudioside A stability as a function of light exposure, pH, and buffer type. It provides manufacturers with data for optimizing shelf life by improving the formulation and packaging of beverage products containing rebaudioside A. Shelf life testing that includes light exposure is recommended to help understand and control the loss of rebaudioside A, and thus loss of sweetness. It is suggested that beverages formulated with rebaudioside A be tested for stability in the actual packaging. Iron content should be determined in beverages containing rebaudioside A, particularly if citric acid is used. Suitable packaging materials should be selected and tested before marketing to protect rebaudioside A beverages from UV exposure, especially for products formulated with citrate. Appropriate storage conditions, including but not limited to temperature and light exposure, should also be considered for optimizing rebaudioside A stability.

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APPENDIX

Table A1. Concentration of rebaudioside A in pH 3 water at 32.5 ℃ under different UV intensities

Dark		Low Intensity		High Intensity	
Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)
0	103.16	0	102.51	0	104.30
14	104.06	14	102.04	14	92.37
28	101.20	28	99.24	28	83.26
48	103.46	38	96.63	38	72.04
73	100.67	48	93.36	42	74.00
103	98.94	56	90.75	48	72.82
124	98.94	73	87.01	56	61.60
144	99.16	82	87.76	63	57.67
164	99.24	103	81.60	73	52.81
185	99.69	114	79.92	82	52.02
205	98.67	124	79.21	92	55.32

Table A2. Concentration of rebaudioside A in pH 7 water at 32.5 ℃ under different UV intensities

Dark		Low Intensity		High Intensity	
Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)
0	105.90	0	101.73	0	102.17
14	104.51	14	99.09	14	101.56
28	102.43	28	101.55	28	96.78
48	104.34	38	101.82	38	99.65
73	103.90	48	102.26	48	95.13
103	103.38	56	101.64	56	90.78
124	102.95	73	102.52	73	91.13
144	103.03	82	100.94	82	88.26
164	103.64	103	100.15	103	85.14
185	101.30	114	100.41	114	85.74
205	105.25	124	94.60	124	85.74

Table A3. Concentration of rebaudioside A in 0.1 M pH 3 phosphate buffer at 32.5 ℃ under different UV intensities

Dark		Low Intensity		High Intensity	
Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)
0	104.10	0	107.04	0	102.70
14	103.99	14	101.96	14	97.37
28	102.71	28	99.60	28	93.17
48	101.75	38	99.71	38	86.13
73	101.85	48	96.18	48	90.82
103	97.68	56	92.22	56	76.94
124	98.75	73	94.04	73	69.22
144	96.50	82	94.46	82	64.33
164	95.00	103	87.93	103	54.85
185	93.39	114	87.40	114	52.01
205	94.46	124	81.99	124	59.93

Table A4. Concentration of rebaudioside A in 0.1 M pH 7 phosphate buffer at 32.5 ℃ under different UV intensities

Dark		Low Intensity		High Intensity	
Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)
0	111.67	0	106.25	0	105.76
14	105.27	14	97.94	14	93.13
28	99.46	28	92.24	28	80.84
48	95.09	38	89.16	38	79.35
73	90.42	48	85.33	42	76.44
103	85.90	56	82.62	48	71.60
124	84.62	73	78.33	56	69.21
144	84.47	82	78.14	63	65.49
164	80.25	103	69.36	73	61.17
185	81.23	114	68.43	82	58.11
205	78.51	124	66.66	92	56.32

Table A5. Concentration of rebaudioside A in 0.1 M pH 3 citrate buffer at 32.5 ℃ under different UV intensities

Dark		Low Intensity		High Intensity	
Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)
0	101.42	0	96.49	0	98.66
14	103.63	0	99.00	0	94.33
28	103.29	14	85.10	5	64.63
48	98.79	20	75.73	11	32.89
73	99.47	28	82.95	15	21.21
103	100.15	31	62.12	18	10.48
124	98.03	38	53.62	20	23.40
144	99.81	42	53.46	22	8.05
164	96.92	42	49.32	31	6.54
185	95.65	48	49.69	42	4.14
205	97.52	52	42.86	52	3.09
		56	43.15		
		63	37.60		
		68	31.87		
		73	32.06		
		78	25.49		
		82	29.14		
		88	15.96		
		92	21.98		
		98	14.58		
		108	20.84		

Table A6. Concentration of rebaudioside A in 0.1 M pH 7 citrate buffer at 32.5 ℃ under different UV intensities

Dark		Low Intensity		High Intensity	
Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)	Time (Days)	Concentration (mg/100 mL)
0	112.23	0	100.06	0	100.26
14	112.13	14	95.66	0	103.23
28	112.81	28	84.83	4	79.44
48	111.15	38	76.47	10	52.92
73	110.66	48	68.72	14	61.26
103	110.37	56	64.93	15	35.93
124	110.17	73	60.71	20	17.87
144	110.57	82	56.13	25	7.02
164	110.76	103	53.05	28	21.24
185	110.76	114	49.62	38	7.75
205	109.39	124	49.09	42	5.62
				48	8.12

Table A7. Concentration of rebaudioside A in 0.1 M pH 7 citrate buffer at 32.5 ℃ held 10 days under high UV to test reproducibility of methodology

Sample	Concentration (mg/100 mL)
1	52.24
2	51.55
3	50.61
4	51.38
5	49.84
6	52.67

Table A8. Concentration of rebaudioside A in 0.1 M pH 3 citrate buffer at 32.5 ℃ held 35 days under high UV exposure with different vial filling volumes

Volume (mL)	Concentration (mg/100 mL)				Mean (mg/100 mL)	Standard Deviation
	Sample 1	Sample 2	Sample 3	Sample 4		
1	1.75	1.04	0.73	1.14	1.16	0.43
2	2.26	2.26	1.96	2.06	2.14	0.15
3.7	72.70	73.41	73.31	69.64	72.27	1.78