Storage Stability of Rebaudioside A in Various Buffer Solutions

by

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Abstract

Rebaudioside A is a non-caloric high intensity sweetener extracted from *Stevia rebaudiana*. For it to be used in the food industry, rebaudioside A needs to be stable during processing and storage. Kinetic data on its long term stability as affected by solution composition are lacking. The primary objective of this study was to evaluate the storage stability of rebaudioside A in various buffer solutions as a function of pH, buffer type, buffer concentration and temperature. The effect of light exposure on rebaudioside A stability was also evaluated.

Rebaudioside A solutions were prepared in 0.02 and 0.1 M phosphate and citrate buffers at pH 3, 5 and 7. Duplicate samples were stored at 20, 30 and 40 °C. Some samples were stored at room temperature under light or dark conditions. Aliquots were removed nine times for approximately nine months. The concentrations of rebaudioside A were analyzed and pseudo-first-order rate constants with 95% confidence intervals were calculated for the loss of rebaudioside A.

In phosphate buffer, the degradation of rebaudioside A was generally faster at higher pH values. The pH effect on rebaudioside A stability was generally reversed in citrate buffer. Rebaudioside A broke down significantly faster in phosphate buffer than in citrate buffer at pH 5 and 7; degradation rates were similar at pH 3. Higher buffer concentrations promoted faster degradation. Rebaudioside A degradation was accelerated by the elevation of temperature. The exposure of light did not have an obvious effect in phosphate buffer at pH 7 while it lowered the stability of rebaudioside A in citrate buffer at pH 3.

For optimum stability of beverages containing rebaudioside A, lower temperatures and lower buffer concentrations are preferred. If the product has a pH value of 5 or 7, citrate buffer is more preferred than phosphate. Dark environments help stabilize rebaudioside A in beverage at pH 3.

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Chapter 1: Introduction

Rebaudioside A is a natural non-caloric sweetener found in *Stevia rebaudiana*, a plant originating in South America and now commercially cultivated in East Asia (Carakostas and others 2008). This steviol glycoside is 200-300 times sweeter than sucrose (Soejarto and others 1983). Although many steviol glycoside can be extracted from *Stevia rebaudiana*, rebaudioside A is most commonly used in foods.

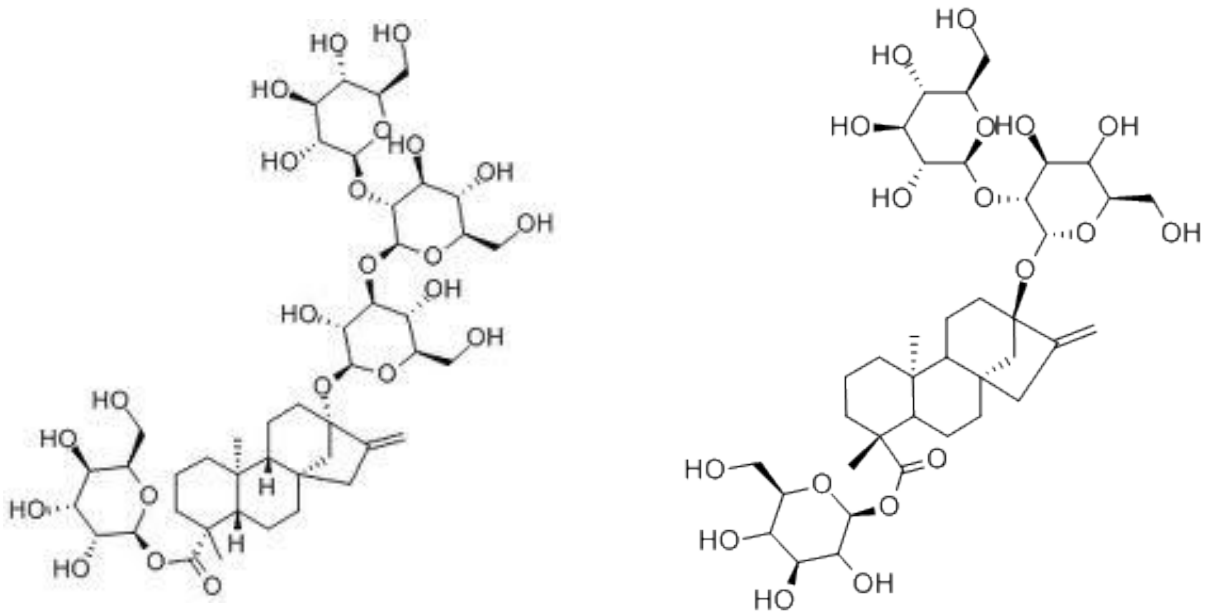
According to numerous toxicity studies, rebaudioside A does not have reproductive toxicity (Curry and Roberts 2008), genotoxicity (Matsui and others 1996), mutagencity (Nakajima 2000) or carcinogenicity (Xili and others 1992). FDA recognized rebaudioside A as generally recognized as safe (GRAS) in a letter dated December 2008 (Tarantino 2008).

Rebaudioside A is starting to be incorporated into beverages and other foods. However, its stability has only been partially investigated. Limited data on this stability indicate pH, temperature and light are important variables for limiting degradation. A systematic study on the effects at buffer type, buffer concentration, pH and temperature has not been reported. Kinetic data (i.e., rate constants) have not been determined. Thus, the objective of this study was to evaluate the long term storage stability of rebaudioside A in different solutions at different storage conditions.

Chapter 2: Literature Review

Introduction

Rebaudioside A is a non-caloric natural sweetener classified chemically as a steviol glycoside, which is extracted and purified from *Stevia rebaudiana* (bertoni). *Stevia rebaudiana* originated from South America and has now been cultivated in Asia (Carakostas and others 2008). *Stevia rebaudiana* extracts consist of 5-10% stevioside, 2-4% rebaudioside A, 1-2% rebaudioside C and other steviol glycosides, like steviolbioside, dulcoside A and rebaudiosides B, D and E (Chabot and Beaulieu 2012). The structures of rebaudioside A and stevioside are shown in Figure 2.1.



Rebaudioside A Stevioside

Figure 2.1 Structures of rebaudioside A and stevioside

Rebaudioside A is a white, crystalline, odorless powder that is freely soluble in water (Carakostas and others 2008). Several steviol glycosides provide sweet tastes but stevioside and rebaudioside A are the predominant sweeteners in *Stevia rebaudiana*. Rebaudioside A is approximately 200 to 300 times sweeter than sucrose when consumed as a 0.4% solution (Soejarto and others 1983). According to some experts, stevioside and rebaudioside C have some bitterness and unpleasant aftertastes while rebaudioside A has a clean aftertaste (Chen and others 1999). Compared to aspartame, rebaudioside A may have wider usage since it can be consumed by people with the metabolic disease phenylketonuria (Grenby 1991). Phenylketonuria (PKU) is a disease where people cannot metabolize phenylalanine, a component of aspartame. Rebaudioside A does not contain phenylalanine and is therefore safe for people with PKU.

Extraction methods

There are ten kinds of steviol glycosides in *Stevia rebaudiana*, with stevioside, rebaudioside A and rebaudioside C being the predominant ones. Rebaudioside A only makes up 2-4% of the dry weight of *Stevia rebaudiana*. Stevia glycosides are extracted mostly with water or methanol. The extraction procedure itself is not complicated, however the separation and purification of rebaudioside A from stevioside, which has a higher concentration and similar chemical structure as rebaudioside A, is more time and energy consuming.

Prakash and others (2007) used hot water (50-60 °C) to extract steviol glycosides from *Stevia rebaudiana* leaves followed by filtration. Adsorption resins with food grade methanol or ethanol were used to retain steviol glycosides in the extracted solution and then it was washed by water. The products were then dried, typically by spray or vacuum drying. The limitation for this extraction method is that it does not intend to separate rebaudioside A and stevioside so further purification is necessary.

Jaitak and others (2008) extracted steviol glycosides with 80% MeOH and 20% H_2O (v/v) for 12 h at room temperature three times. The extract was concentrated under reduced pressure at 50 °C. This method could not extract rebaudioside A alone and further purification was required.

In a later study, Jaitak and others (2009) extracted rebaudioside A and stevioside together from the dry leaves of *Stevia rebaudiana* by ultrasound and microwave-assisted extraction to speed up the process. Microwave-assisted extraction was shown to be rapid and efficient at 50 °C and a power level of 80 W with a high breakage of analyte-matrix bonds yet not too powerful to make rebaudioside A and stevioside adsorb on the raw material surface. Microwave assisted extraction with methanol:water (80:20) only took 1 minute at the optimum condition with almost twice the yield of rebaudioside A compared to cold water extraction for 12 hours at 25 °C and ultrasound extraction for 30 minutes at 35±5 °C. Like the method of Prakash and others (2007), the primary extraction could not separate rebaudioside A from stevioside.

Chen and others (1999) studied the effect of methanol and ethanol used as solvents for the selectivity and enrichment of rebaudioside A. Pyridyl sorbent exhibits higher adsorptive selectivity toward stevioside than rebaudioside A and make the effluent rich in rebaudioside A. Ethanol showed better eluting ability and efficiency but worse selectivity than methanol. They combined the selective adsorption with dynamic chromatographic resolution, whose efficiency was improved by slowing the flow rate and increasing the column length. The concentration of rebaudioside A was enriched by a factor of four under the optimum conditions.

Analysis methods

The concentration of rebaudioside A has typically been determined using HPLC (high-performance liquid chromatography) in recent years. The effects of several parameters, like

column type, column temperature, mobile phase composition and flow rate were studied by many researchers to find the most efficient analytical method.

Kitada and others (1989) used the NH_2 column at a temperature of 50 °C with acetonitrile/water (80:20, v/v) as the mobile phase at a flow rate of 0.8 mL/min. The retention time was 14 minutes for rebaudioside A in pickled radish with 93.2% to 100% recoveries. The retention time was shorter at 36 °C, which means higher column temperatures could shorten the retention time. Kolb and others (2001) also used a NH_2 column and acetonitrile/water (80:20, v/v) mobile phase at pH 5 after fast extraction of rebaudioside A by EtOH: H_2O (70:30, w/w). The flow rate was set as 2.0 mL/min. The precision of this method was the same as traditional gradient HPLC method, which is 200 mL $CHCl_3$ for 3 h and 200 mL MeOH for 5 h, with less sample preparation time and analysis time.

Fan and others (2007) studied the effect of mobile phase composition and NH_2 column temperature on retention time. The flow rate was set as 1.0 mL/min and detection wavelength was 205 nm. With acetonitrile/water (80:20, 82:18, 78:22, v/v) as the mobile phase, they indicated that the retention time was longer with the higher organic composition. According to their study, column temperature (43 °C, 45 °C, 47 °C) did not affect the retention time and peak shape.

Liquid chromatography was also studied to better separate the several stevia glycosides. Comprehensive two-dimensional liquid chromatography was better than single dimension liquid chromatography. The best separation with a single column was obtained by using a C18 column. A combination of a C18 column followed by a NH_2 column could separate all the stevia glycosides from the matrix. The flow rates should be slow for first-dimension separation

(maximum 0.1 mL/min), and as fast as possible while not causing too high pressure in the second dimension columns (Pol and others 2007).

Wolwer-Rieck and others (2010a) used a Luna HILIC analytical column with a mobile phase of acetonitrile/water (85:15, v/v) and a NH_2 column with mobile phase as acetonitrile/water (75:25, v/v). The same flow rate of 1 mL/min and column temperature of 36 °C were applied. Both of the columns showed the same retention pattern and were suitable for the detection of rebaudioside A. The retention times were 9.7 minutes and 6.6 minutes for the HILIC column and NH_2 column, respectively. To extract the rebaudioside A and stevioside, they used another solvent instead of water. Ground stevia leaves were extracted three times in boiling acetonitrile and water (8:2 v/v) for 30 min and centrifuged after cooling to room temperature. To better separate these two compounds, solid-phase extraction was attempted.

Wolwer-Rieck and others (2010b) used a Luna HILIC column at 36 °C with a mobile phase of acetonitrile/water (80:20 v/v) for their HPLC analysis of rebaudioside A in soft drinks. The absorption wavelength for detection was set for 210 nm. The injection volume was 20 µL and the flow rate was 1.0 mL/min. The retention time was 10.5 minutes and the recovery ranged from 95.9% to 109.2%.

To separate rebaudioside A from other steviol glycosides, Liu and others (2011) and Li and others (2012) both used mixed-mode macroporous adsorption resins (MAR). In Liu and others' study, four tyrene divinyl-benzenes with different polarity, particle size and specific surface area, pore size and moisture content were tested. For a single MAR, a larger pore size gave a higher purity of rebaudioside A because stevioside diffused easily into the pores. A larger specific surface area lowered the recovery of rebaudioside A. However, the ideal purity and recovery of rebaudioside A could not be obtained using a single MAR, but a mixture of them

could increase the purity of rebaudioside A from 40.77% to 60.53% after one single run. In Li and others' (2012) study, 19 kinds of tyrene divinyl-benzenes were tested. Some combinations of MAR were able to increase the purity of the obtained product from 60% to 97%.

Metabolism

In anaerobic conditions, like the human digestive system, rebaudioside A degraded into stevioside and then to steviol; bacterial enzymes are not able to cleave it into further products. Steviol can be quickly converted to its glucuronide, and is excreted by the kidneys in humans (Carakostas and others 2008). An *in vitro* experiment showed that stevioside could be degraded completely to steviol after 10 hours under strict anaerobic conditions. Rebaudioside A could also be completely degraded to steviol, but it would take longer, 24 hours, in rats' intestinal microflora (Wingard and others 1980).

Using human intestinal microflora (37 °C for 72 h) from different volunteers, rebaudioside A and stevioside were degraded to steviol; no other derivatives were found. After an initial lag phase of 6-7 h, rebaudioside A was hydrolyzed to steviolbioside and this was rapidly converted to steviol, which remained unchanged during the 72 h incubation. Rebaudioside A showed a weak inhibitory activity on aerobic bacteria and particularly on coliforms (Gardana and others 2003).

In the gastrointestinal track, none of the digestive enzymes from humans or the acidic environment of the stomach were able to degrade stevioside into steviol, and steviol was the only metabolite found in the feces. Very low stevioside concentrations were found in blood plasma (Geuns and others 2007). In the cecum, stevioside was metabolized to steviol by the bacterial flora. Steviol was found in the blood with its maximum concentration occurring after 8 h. In an experiment with rats, little or no stevioside was absorbed into the blood (Nakayama and others

1986). In the colon, steviol was metabolized by bacteria similarly in rats and humans, although the rate of metabolism and uptake in rats appeared to be slightly faster (Koyama and others 2003a,b). Roberts and Renwick (2008) confirmed the previous work on stevioside and demonstrated that rebaudioside A is metabolized in the same pathway as stevioside in both rats and humans.

Although the metabolic pathways of stevioside and rebaudioside A are similar in humans and rats, the excretion of steviol from them is different. The excretion of steviol glucuronide in rats occurs primarily in the feces, while it is mainly eliminated through urinary excretion in humans. This is due to the molecular weight thresholds for biliary excretion being different in humans and rats. In rats, very little steviol is found beyond the portal or biliary systems, while in humans steviol is quickly converted to its glucuronide, which is a stable detoxification product that is quickly excreted by the kidneys (Geuns and others 2006).

Toxicity study

Because rebaudioside A and stevioside have similar chemical structures and metabolism studies indicate that steviol glycosides are metabolized into steviol in the human body, the results from studies regarding about the physiological effects and toxicity of stevioside and steviol may be used to evaluate the safety of rebaudioside A (Roberts and Renwick 2008). Steviol equivalents were used in several studies to compare intake and safety limits. Because the molecular weight of rebaudioside A is three times larger than steviol, the safety limitation of rebaudioside A is about three times higher than the one for steviol when expressed by weight.

A study conducted on mice, rats and hamsters on the acute toxicity of stevioside showed that the LD50 level of steviol was 5.20 g/kg body weight and 6.10 g/kg body weight for male and female hamsters, respectively. For rats and mice, the LD 50 level was as large as 15.0 g/kg

body weight for both genders, which indicates that the hamster was most sensitive to stevioside in this study and that steviol, stevioside and rebaudioside A have no acute toxicity (Toskulkao and others 1997). For comparison, the minimal lethal dose of aspartame in mice, rats and rabbits is greater than 5 g/kg body weight (Molinary 1984).

Macroscopic and microscopic examinations showed that there were no changes to the renal or reproductive systems in rats after 90 days at an oral dose of 25,000 and 50,000 ppm rebaudioside A, which suggests rebaudioside A has virtually no subchronic toxicity. Significant weight loss was observed, which the author concluded was not an adverse side effect but was caused by a lower energy density since rebaudioside A was a diet supplement with no calories (Curry and Roberts 2008).

Research on Sprague-Dawley rats for 90 days showed there were no treatment-related effects on the general condition and behavior of the animals as determined by clinical observations, functional observational battery, and locomotors activity assessments at doses of 500, 1000, and 2000 mg rebaudioside A/kg bw/day (purity 99.5% treatment) (Nikiforov and Eapen 2008).

A study about the toxicity of a *Stevia rebaudiana* extract to the renal system has shown it induced systematic and renal vasodilation, hypotension and diuresis after 40 and 60 days oral administration in Wistar rats. Because the exact extract composition was not shown in this experiment, it is difficult to conclude whether rebaudioside A, stevioside or another compound caused these effects (Melis 1995).

As for the genotoxicity of rebaudioside A and stevioside, several studies were conducted. *In vitro, in vivo*, mutation, chromosome damage, and DNA strand breakage experiments showed no evidence of genotoxic damage relevant to human health (Brusick 2008). In a study with

several mutagenicity tests using bacteria, cultured mammalian cells and mice, stevioside was not shown to be mutagenic (Matsui and others 1996). Nunes and others (2007) showed that 4 mg stevioside/mL in drinking water for 45 days produced DNA breakage in rat blood cells, spleen, liver and brain. Unfortunately, no positive control was provided in this study and the significant elevations of blood cell nuclei number only occurred in week 5, not in the previous 4 weeks. Stomach, colon, liver, kidneys, bladder, lung, brain and bone marrow cells were sampled and tested after 3 and 24 hours of exposure to stevia as high as 2000 mg/kg in mice; these tests produced evidence indicating no increase in DNA damage (Sasaki and others 2002).

Studies have been conducted on the mutagenicity of rebaudioside A and consistent negative results were provided (Brusick 2008; Williams and Burdock 2009). Nakajima's (2000) micronucleus formation experiment on BDF1 mouse bone marrow with 200-2000 mg/kg bw/day for two days suggested rebaudioside A did not have mutagenic toxicity. Rebaudioside A was used in four salmonella strains, where there was no mutagenic response at even the highest level of treatment (Williams and Burdock 2009).

Because the stevia plant as a whole has been used historically as an oral contraceptive in Brazil and Paraguay, there have been questions about its effect on fertility. Older studies, which used a crude stevia aqueous extract, had reported effects on the testes eight, reduction in spermatozoa concentration and other fertility defects while more recent studies, which used purified stevioside, have shown different results. Yodyingyuad and Bunyawong (1991) claimed stevioside showed no toxic effect on developing hamsters (2500 mg/kg bw/day). Both female and male rats were treated during three rounds of mating and neither the fertility, number of offspring nor the reproductive tissue was affected (Yodyingyuad and Bunyawong 1991). Usami and others (1995) verified their results about developmental toxicity at lower intakes (1000

mg/kg bw/day) of stevioside. Curry and others (2008) showed there were no treatment-related effects of rebaudioside A on either F0 or F1 generations up to 25,000 ppm in Wistar rats on reproductive performance (mating performance, fertility, gestation lengths, estrus cycles, or sperm motility, concentration, or morphology). No developmental defects were noticed in the offspring.

Studies have also failed to show any evidence of carcinogenicity. After an oral intake of 85% pure stevioside (600 mg/kg bw/day) for over 24 months, no neoplastic or pre-neoplastic lesions were reported in any Wistar rat tissue (Xili and others 1992). No lesions on any organ or tissue were reported on F334 rats during a 104-week test, which indicated stevioside was not carcinogenic. However, a significant decrease in survival rates was observed in male rats consuming a 5% dose (Toyoda and others 1997). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) used the 970 mg/kg bw/day dose (2.5% dose in male rats) in the previous study to set the temporary ADI for steviol at 12 mg/kg bw/day (Carakostas and other 2008).

The reproductive, carcinogenicity, mutagenicity, and general toxicity studies have demonstrated rebaudioside A and stevioside appear safe at even high dietary intake levels. Based on these results and the historical use of stevia in some cultures, its use in food has been approved by several government agencies, as will be discussed later.

Health effects

Clinical studies about the effect of rebaudioside A on blood pressure and blood sugar levels in healthy humans and patients with hypertension and diabetes have been conducted. Several clinical studies have shown rebaudioside A could offer therapeutic benefits to hypertensive patients.

Chan and others (2000) conducted a study with hypertensive patients who were taken off their antihypertensive medications and treated with stevioside (750 mg/day) or placebo for 12 weeks. The results showed that both systolic and diastolic blood pressure decreased significantly in the stevioside group and this effect persisted during the whole year. Ferri and others (2006) showed no effect of 3.75 mg/kg/day (7 weeks), 7.5 mg/kg/day (11 weeks) and 15.0 mg/kg/day (6 weeks) of a crude steviol glycoside extract on the blood pressure of subjects with mild essential hypertension. This might be due to the different intake levels of stevioside and the fact that the second research used crude steviol glycoside instead of one with higher purity.

Rebaudioside A's effect on patients with hypertension made researchers wonder whether it has any effect on the blood pressure of health people. A study on people with normal blood pressure was conducted with rebaudioside A intakes of 1000 mg/day for 4 weeks. The resting seated systolic blood pressure, diastolic blood pressure, mean arterial pressure, heart rate, and 24-hour ambulatory blood pressure responses in healthy humans were not significantly altered as compared to the placebo group (Maki and others 2008a).

There were numerous studies about the effect of rebaudioside A on diabetic animals and patients. Tests in Goto-Kakizaki rats with type 2 diabetes and normal Wistar rats showed stevioside could suppress the glucagon level and increase the insulin response, which suggested its potential use in diabetes treatment (Jeppesen and others 2002). Abudula and others (2004) showed rebaudioside A stimulated insulin secretion dose-dependently with the presence of extracellular calcium ion in mice and might serve as a potential type 2 diabetes treatment. In type 2 diabetic patients, 16 weeks of consuming 1,000 mg of rebaudioside A daily did not affect glucose homeostasis or blood pressure (Maki and others 2008b). Barriocanal and others (2008) expanded the sample size to both type 1 and type 2 diabetic patients with 750 mg/day steviol

glycosides intake. No significant hemodynamic effects in subjects with or without diabetes mellitus were detected and there was no effect of steviol glycosides on blood lipids (total-, LDL-, HDL-cholesterol).

Food uses and approvals

Extracts of *Stevia rebaudiana* have been used for several years for sweetness in Brazil, Japan, China and Korea (Geuns and others 2003). In Japan and Paraguay, stevia was also consumed as a food and medicine (Carakostas and others 2008). Due to the growing concern of caloric intake, rebaudioside A has been used more as a high intensity sweetener. Its use is rapidly growing in the food industry, especially in beverages. It is used as an ingredient in vitamin water zero, carbonated beverages, yogurt, and orange juice beverages for the sweetness. Rebaudioside A can also be used as a table-top sweetener.

The Joint Expert Committee on Food Additives (JECFA) approved a temporary 0-2 mg/kg bw/d for steviol intake at the 63rd WHO meeting (WHO 2005). At the 69th meeting, ADI values of 0-4 mg/kg bw/day for steviol intake were approved (FAO/WHO 2008), which is equivalent to 0-12 mg/kg bw/day for rebaudioside A. The Food Standards Australia New Zealand (FSANZ) has completed the evaluation of an application of steviol glycoside in food and allowed its use (FSANZ 2008). At least two petitions seeking authorization to use stevioside and steviol glycoside in foods have been submitted since 1989. FDA issued "no objection" letters for the generally recognized as safe (GRAS) notification of rebaudioside A in late 2008. This GRAS approval was based on rebaudioside A being incorporated “under the conditions of its intended use” which would be “largely self-limiting due to its organoleptic properties” (Tarantino 2008).

Stability

The stability of rebaudioside A has been studied for several decades and is important to understand when using it as a sweetener in foods and beverages. The stability of rebaudioside A is affected by the storage form, time, temperature, pH and light exposure.

Chang and Cook (1983) examined the stability of rebaudioside A in various solutions. The concentration of rebaudioside A decreased 31.5% in water at 100 °C for 48 h and 76-87% in acid solutions after 13 hours, depending upon the type of acid. Degradation was faster in phosphoric acid at pH 2.4 than citric acid at pH 2.6. No significant changes were discovered after 4 months at 4 °C, 3 months at room temperature (22 °C) nor 1 month at 37 °C in either citric or phosphoric acid carbonated beverages. They also evaluated the photo stability of rebaudioside A in citric acid and phosphoric acid carbonated beverages. After storing outdoors for a week (3000 langleys of sunlight exposure) at 10-25 °C, rebaudioside A decreased by 22% and 18% in the phosphoric and citric acid beverages, respectively. The buffer concentration and pH were not reported for the carbonated beverages. Rate constants were not calculated from the data.

Using the degradation data presented by Chang and Cook (1983), kinetic plots can be constructed. Figures 2.2 and 2.3 show the degradation of rebaudioside A in the form of pseudo-zero order and pseudo-first order kinetic plots. From the linear fit based on R^2 values, it appears pseudo first order kinetics is more appropriate for modeling the degradation of rebaudioside A.

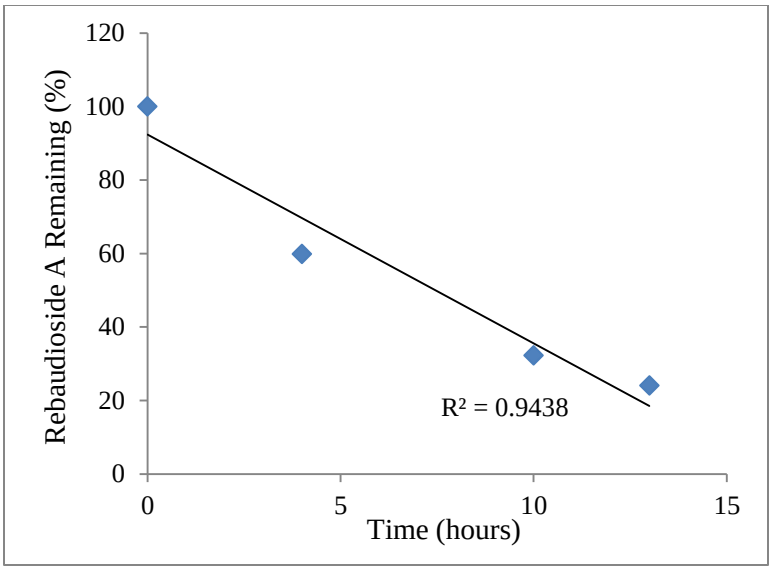


Figure 2.2 Degradation of rebaudioside A at pH 2.6 and 100 °C modeled using pseudo zero order kinetics. Data from Chang and Cook (1983).

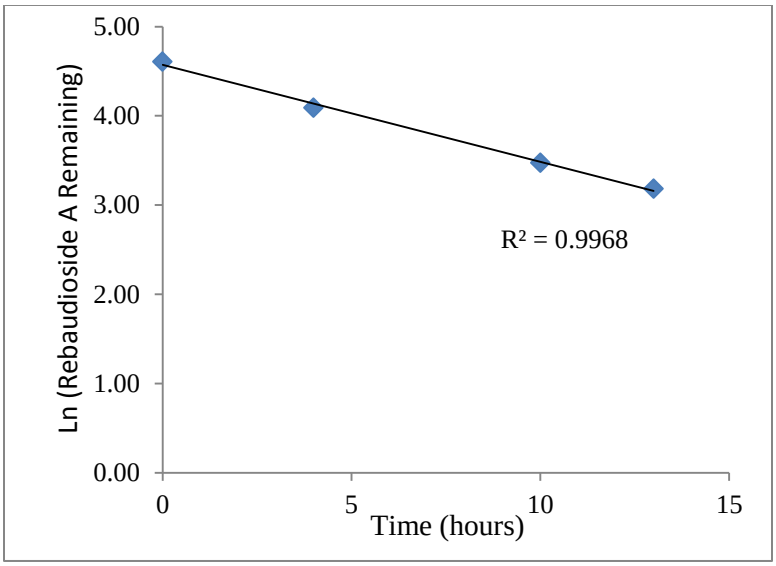


Figure 2.3 Degradation of rebaudioside A at pH 2.6 and 100 °C modeled using pseudo first order kinetics. Data from Chang and Cook (1983).

Kroyer (1999) studied the effect of pH on the stability of stevioside. Stevioside was stable from pH 2-10 at 60 °C for 1 h with only slight losses at pH 2 and 10. Based on the

structural similarities between stevioside and rebaudioside A, the effect of pH on rebaudioside A stability may be similar to that of stevioside.

Clos and others (2008) questioned the photo stability results presented by Chang and Cook (1983) and repeated their experiment. The samples were prepared similarly and stored under similar conditions at slightly higher temperatures (18-34 °C). They concluded that rebaudioside A stability was not affected by light. However, their data actually show 5 times greater rebaudioside A loss when stored in light than darkness.

Prakash and others (2008) reviewed the stability of rebaudioside A in different food environments other than beverages. It was stable in yogurt during pasteurization (190 °F for 5 min) and fermentation as well as for 6 weeks of storage at 4 °C. They had also claimed rebaudioside A was very stable when stored as powder as it only had <5% of loss after 24 months. In the process of baking (350 °F for 20–25 min) and storing (25 °C and 60% RH) of white cake, no significant losses of rebaudioside A were found.

A later study showed the stability of rebaudioside A increased with increasing pH values in a caffeinated soft drink (pH 2.4), a lemon- lime flavored soft drink (pH 2.7) and an energy drink (pH 3.5) at 80 °C, which agreed with data on stevioside (Kroyer 1999). The highest degradation was observed in caffeinated soft drinks after 72 h of storage, while the lowest was in the energy drinks. They also concluded rebaudioside A was more stable against acid hydrolysis compared to stevioside. By using LC-MS, they claimed the degradation products of rebaudioside A were rebaudioside B and steviolmonoside (Wolwer-Rieck and others 2010b).

Prakash and others (2012) studied the degradation products of rebaudioside A in acidic mock beverages, like cola, lemon-lime and root beer soft drinks with pH values from 2.8 to 4.2. The storage temperatures were 5 °C, 20 °C, 30 °C and 40 °C. Degradation was similar at each of

the conditions and occurred after 26-weeks of storage. Excellent mass balance was achieved at all conditions. Rebaudioside A degraded into rebaudioside B and other products faster at lower pH values and high temperatures. The migration of exocyclic double bond between C16/C17 to C15/C16 can form a degradation product and the hydrolysis of the glucose unit at C19 formed rebaudioside B.

Chaturvedula and Prakash (2011) studied the degradation of rebaudioside A and stevioside from acid and alkaline hydrolysis. The structures of degradation products were acquired by NMR, high resolution mass spectral (HRMS) data and comparative spectral data. The alkaline hydrolysis of rebaudioside A conducted by NaOH (2.2 mol/L) yielded rebaudioside B from the cleavage of the β-D-glucopyranosyl unit at the C-19 position while the only product from stevioside was steviolbioside. Acid hydrolysis, applied by H_2SO_4 (5%), could furnish D-glucose from rebaudioside A and acquire the same degradation products.

Objective

Numerous studies have been conducted on the short term stability of rebaudioside A under different conditions. However, no study has actually collected kinetic data to calculate the rate constants for rebaudioside A degradation. The objective of this study was to evaluate the long term stability of rebaudioside A in solutions (in terms of its degradation rate constants) as a function of buffer type, buffer concentration, pH and temperature. The effect of light exposure was also studied as a secondary objective.

Chapter 3: Materials and Methods

Reagents

Rebaudioside A was obtained from Sigma-Aldrich (St. Louis, MO). Sodium phosphate dibasic, sodium phosphate monobasic, citric acid and sodium citrate were acquired from Fisher Scientific (Fair Lawn, NJ). HPLC grade acetonitrile was bought from VWR International (Suwanee, GA).

Sample Preparation

Twelve buffer solutions at different concentrations and pH values were prepared in this study. Six of them were sodium phosphate buffers and the other six were sodium citrate buffers. The buffer concentrations were 0.02 M and 0.1 M, and each was prepared to pH 3, 5 and 7. Each of the 12 buffer solutions was stored at three temperatures to give a total of 36 experimental systems.

The phosphate buffer solutions were made using the following procedure. Phosphoric acid (2.88 g/85%) was added to 250 mL water to make a 0.1 M solution. Similarly, a 0.1 M sodium phosphate monobasic solution was made by adding 6.90 g monobasic sodium phosphate to 500 mL water. A 0.1 M dibasic sodium phosphate solution was also made by adding 7.10 g dibasic sodium phosphate to 500 mL water. These solutions were mixed in varying proportions to yield 0.1 M phosphate buffer solutions at pH 3, 5 and 7. The 0.02 M phosphate buffers were made using the same method, but with one-fifth of the buffer salt amounts.

The citrate buffer solutions were made by mixing appropriate volumes of citric acid and trisodium citrate solutions to acquire pH 3, 5 and 7 buffer solutions. The 0.02 M citric acid solution was prepared by mixing 1.92 g citric acid and 500 mL water, while the 0.02 M sodium citrate solution was made by dissolving 2.94 g trisodium citrate into 500 mL water. The two 0.1 M solutions were made by adding 14.7 g sodium citrate and 9.6 g citric acid to 500 mL water.

Approximately 40 mg of rebaudioside A were dissolved into 100 mL of each buffer solution. The solution was filtered into a 100-mL sterile septum-containing glass bottle using a sterile syringe with a sterile 0.20 µm nylon filter and a sterile needle. Using a new sterile syringe and needle, 1-2 mL aliquots were aseptically transferred into eighteen 2-mL sterile septum-containing vials per experiment. This protocol minimized microbial contamination of the samples.

For the storage stability study, samples were labeled according to their buffer concentration, buffer type, pH and temperature. For example, a 0.1 M phosphate buffer sample at pH 3 and 40 °C was labeled “1p340” while a 0.02 M citric buffer sample at pH 7 and 20 °C was labeled as “02c720”. The samples used for the photo stability study were labeled according to the buffer concentration, buffer type, pH value and storage condition. For example, 0.1 M phosphate buffer at pH 7 stored under light exposure was labeled “1p7-L” and 0.1 M citrate buffer at pH 3 stored in dark was labeled “1c3-D”.

The eighteen vials containing samples at each buffer type, buffer concentration, and pH were placed into each of three incubators set at 20, 30 and 40 °C. Thermometers monitored the internal temperature of the incubators. These samples were stored in darkness and were used to determine the storage stability of rebaudioside A as affected by buffer type, buffer concentration, pH and temperature.

The buffer solutions used for the photo stability component were limited to 0.1 M phosphate buffer at pH 7 and 0.1 M citrate buffer at pH 3. These two solutions were selected to represent extremes in the degradation behavior. These solutions contained approximately the same amount of rebaudioside A as the storage stability experimental groups. These samples were all stored at room temperature. The light exposure groups were placed under ambient room light (scattered sunlight, occasional fluorescent lighting) while the dark protected groups were stored in a paperboard box at the same temperature.

Sampling Procedure

Duplicate samples were removed from storage 9 times for approximately 9 months. For example, the samples in 0.02 M phosphate buffer at 40°C were removed at day 0, 29, 60, 90, 121, 152, 194, 252 and 285. Samples were shaken well before sampling. The exact date of sampling was recorded. An aliquot was removed from each vial using an HPLC syringe, which was injected for analysis.

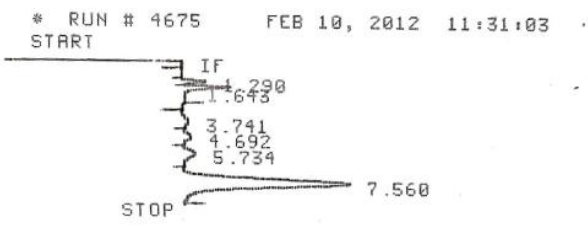
Mold growth was observed in two 20 °C groups, 02p320 and 1c320, after two months of storage. These solutions were remade; approximately 30 mL were placed into 100-mL sterile bottles due to the unavailability of the 2-mL vials. During the same storage periods, aliquots (1 mL) were removed from these bulk solutions through the rubber septum using a sterile needle and syringe for analysis.

Sample Analysis

Rebaudioside A concentrations in the experimental solutions were determined using reverse-phase high performance liquid chromatography (HPLC). Two sets of standard solutions were prepared. Standard solution A was made by dissolving 82.1 mg of rebaudioside A in 100 mL deionized water while solution B was made by dissolving 61.1 mg of rebaudioside A in 100

mL deionized water. Standard solutions were prepared by serially diluting aliquots these solutions three times. The concentrations of experimental samples were acquired by comparing the peak areas to those from the standard solutions. Standard solutions were analyzed every time experimental samples were tested.

The analytical column used was a 250 x 4.6 mm LUNA 5µ amino column (Phenomenex, Torrance, CA) with a corresponding guard cartridge. The column was housed in a column heater set at 45°C. The mobile phase consisted of 77.5/22.5 (v/v) acetonitrile/aqueous buffer at pH 7; the buffer consisted of 0.0008 M monobasic sodium phosphate and 0.0015 M dibasic sodium phosphate. The flow rate was set at 2.0 mL/min. The injection volume was 20 µL, and detection occurred at a wavelength of 210 nm. Data were integrated by a Hewlett-Packard integrator. The retention time for rebaudioside A was around 7.5 min. A sample chromatogram is shown in Figure 3.1. Using the rebaudioside A standard curves every time, the concentrations of rebaudioside A in the experimental solutions were determined.



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AREA%

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Figure 3.1 Chromatograph of rebaudioside A in 0.1 M phosphate buffer at pH 7 and room temperature after 285 days of dark storage (Rebaudioside A eluted at 7.56 min)

Data Analysis

Pseudo-first-order rate constants with 95% confidence intervals were calculated for the loss of rebaudioside A using linear least squares analysis as described by Labuza and Kamman (1983). Significant differences between the rate constants were analyzed by testing the homogeneity of regression at $p<0.05$, as described by Steel and Torrie (1980).

Chapter 4: Results and Discussion

Rebaudioside A does have the potential to breakdown in solutions during storage, as shown by the degradation profiles in Figures 4.1-4.4. The degradation of rebaudioside A was affected by buffer type, buffer concentration, pH and temperature. From the degradation profiles, in the form of pseudo-first order kinetic plots, the rate constants were determined and are listed in Tables 4.1, 4.2 and 4.3. Table 4.4 shows the time required for the rebaudioside A concentration to decrease by 10%. The 10% loss time was calculated from the rate constants in the former tables.

As rebaudioside A degrades, various degradation products are produced. Chang and Cook (1983) reported the formation of rebaudioside B and glucose from the cleavage of the ester linkage of rebaudioside A. Prakash and others (2008) presented a degradation pathway that included rebaudioside B and stevioside as well as a few other derivatives. Wolwer-Rieck and others (2010b) detected rebaudioside B and steviolmonoside as degradation products. Chaturvedula and Prakash (2011) reported the formation of rebaudioside B from alkaline degradation of rebaudioside A. Prakash and others (2012) reported rebaudioside B was formed along with a more prevalent product determined to be 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-gluopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-15-en-19-oic acid β-D-glucopyranosyl ester. In the current study, only rebaudioside A concentrations were determined.

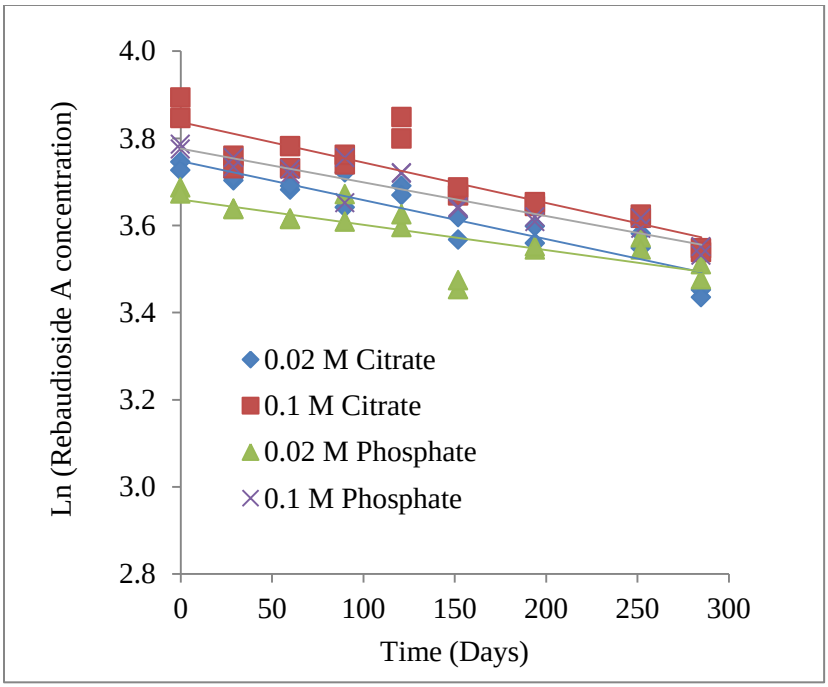

Figure 4.1 Degradation of rebaudioside A in different buffer solutions at pH 3 and 40°C

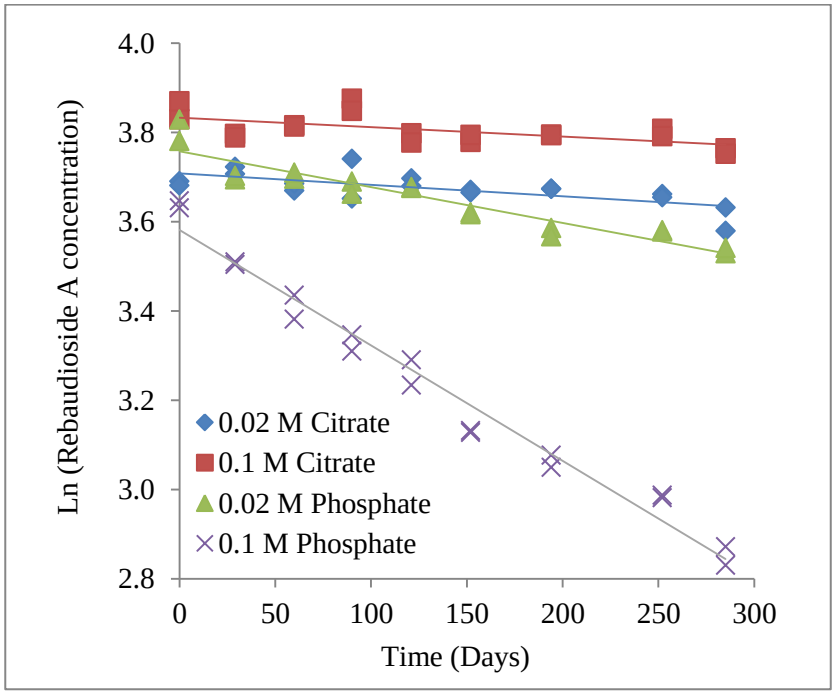

Figure 4.2 Degradation of rebaudioside A in different buffer solutions at pH 7 and 40°C

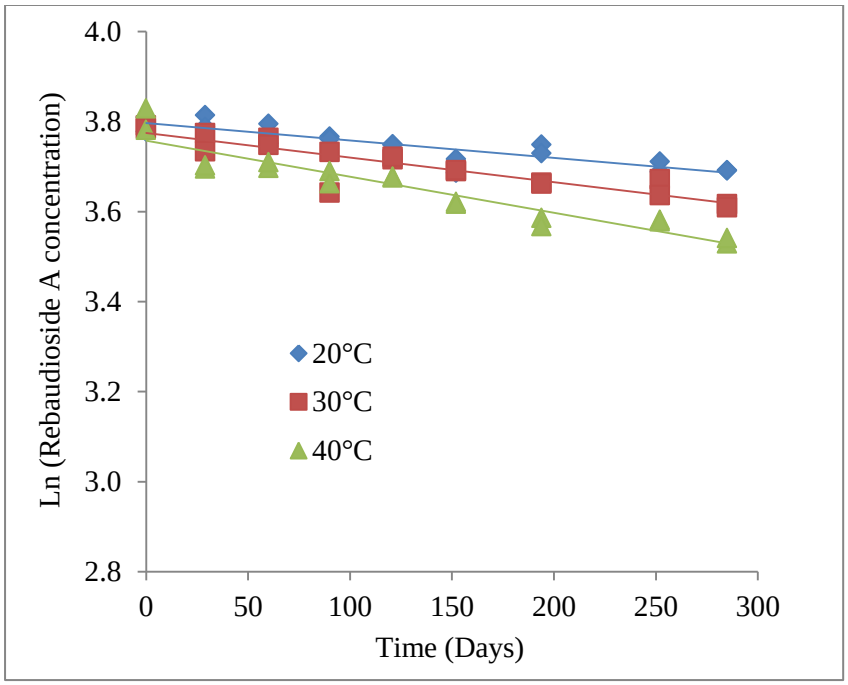

Figure 4.3 Degradation of rebaudioside A at different temperatures in 0.02 M phosphate buffer at pH 7

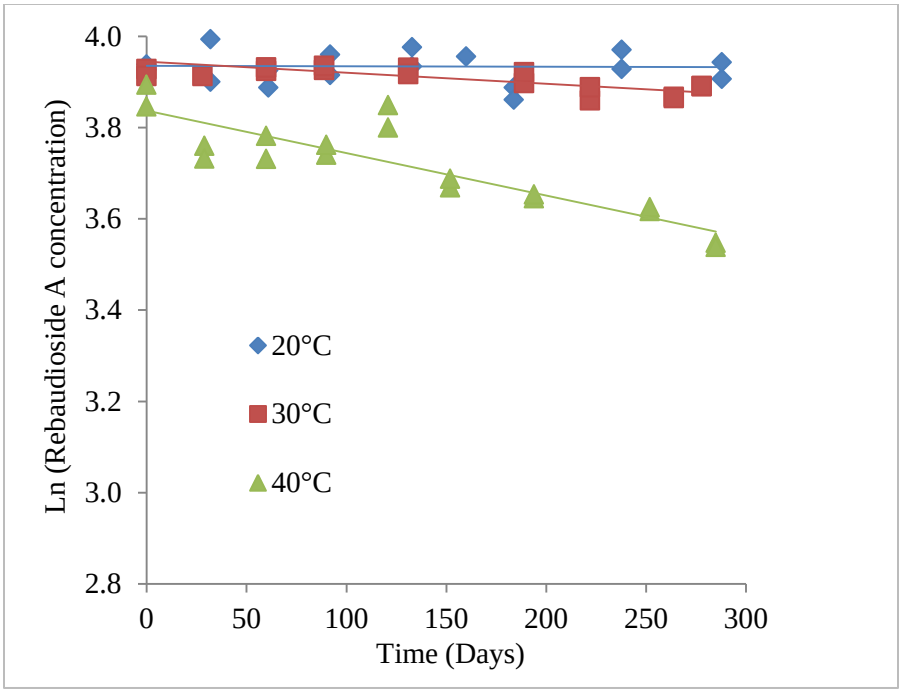

Figure 4.4 Degradation of rebaudioside A at different temperatures in 0.1 M citrate buffer at pH 3

Table 4.1 Pseudo-first rate constants (d^{-1}) with 95% confidence limits for rebaudioside A degradation in buffers stored at 20°C

	pH 3	pH 5	pH 7
0.02 M phosphate	0.0000469±0.000172 aA	0.000301±0.000131 aB	0.000388±0.000109 aB
0.1 M phosphate	0.000226±0.000102 aA	0.000270±0.000115 abA	0.000953±0.000147 bB
0.02 M citrate	0.000138±0.000192 aA	0.000107±0.000186 bcA	0.000247±0.000175 acA
0.1 M citrate	0.0000102±0.000224 aA	0.000109±0.000111 cA	0.0000604±0.000126 cA

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).

Table 4.2 Pseudo-first rate constants (d^{-1}) with 95% confidence limits for rebaudioside A degradation in buffers stored at 30°C

	pH 3	pH 5	pH 7
0.02 M phosphate	0.000286±0.0000837 aA	0.000377±0.000168 aAB	0.000547±0.000137 aB
0.1 M phosphate	0.000228±0.0000878 aA	0.000384±0.000136 aB	0.00161±0.000122 bC
0.02 M citrate	0.000332±0.000109 aA	0.000124±0.000113 bB	0.000137±0.0000972 cB
0.1 M citrate	0.000242±0.000121 aA	0.000243±0.000115 abA	0.000133±0.000137 cdA

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).

Table 4.3 Pseudo-first rate constants (d^{-1}) with 95% confidence limits for rebaudioside A degradation in buffer stored at 40°C

	pH 3	pH 5	pH 7
0.02 M phosphate	0.000579±0.000253 aA	0.000544±0.000241 aA	0.000802±0.000153 aA
0.1 M phosphate	0.000773±0.000147 abA	0.00138±0.000180 bB	0.00259±0.000221 bC
0.02 M citrate	0.000893±0.000192 bA	0.000229±0.0000995 cB	0.000255±0.000140 cB
0.1 M citrate	0.000930±0.000267 abA	0.000581±0.000188 aB	0.000212±0.000147 cC

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).

Table 4.4 Time for 10% rebaudioside A concentration decrease (days)

		pH 3	pH 5	pH 7
20 °C	0.02 M phosphate	2246	350	272
	0.1 M phosphate	466	390	111
	0.02 M citrate	763	985	427
	0.1 M citrate	10329	967	1744
30 °C	0.02 M phosphate	368	279	193
	0.1 M phosphate	462	274	65
	0.02 M citrate	317	850	769
	0.1 M citrate	435	434	792
40 °C	0.02 M phosphate	182	194	131
	0.1 M phosphate	136	76	41
	0.02 M citrate	118	460	413
	0.1 M citrate	113	181	497

Effect of pH

In phosphate buffer, the rebaudioside A degradation rate constants generally increased as pH increased (Tables 4.1-4.3). However in citrate buffer, rebaudioside A degradation rate constants generally decreased as pH increased at 30 and 40 °C and were not significantly different at 20°C. Figure 4.5 shows these combined effects of pH and buffer type in 0.1 M buffer. Clearly, in addition to pH, the buffer type is affecting degradation rates, as will be discussed later.

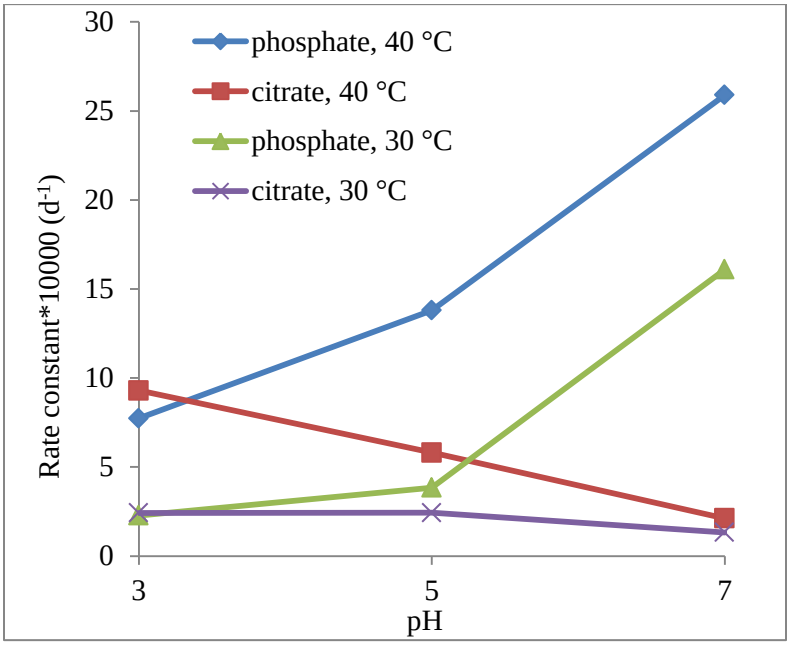

Figure 4.5 Rate constants of rebaudioside A degradation in 0.1 M phosphate and citrate buffer at 30 °C and 40 °C as a function of pH

In the study Chang and Cook (1983) conducted, rebaudioside A had a greater extent of loss in a phosphoric acid solution at pH 2.4 than in a pH 2.6 citric acid solution at 100 °C for 13 h. The differences in degradation could be due to the different pH values, buffer solution compositions, or a combination of both. Their study demonstrates the importance of controlling multiple variables in order to obtain a clear understanding of the factors affecting the reaction rate.

Using data presented by Prakash and others (2012), rebaudioside A degradation rate constants were determined (Table 4.5). At all three temperatures, degradation rate constants decreased as pH increased from 2.8 to 4.2. The buffer solutions they used were made with trisodium citrate and acidified with phosphoric acid. Their results are generally in agreement with those obtained in the current study involving citrate buffer.

Table 4.5 Rate constants (d^{-1}) calculated from the data presented by Prakash and others (2012)

	pH 2.8	pH 3.2	pH 3.8	pH 4.2
20°C	0.0004	0.00007	0.00008	0.00001
30°C	0.0013	0.0006	0.0001	0.00007
40°C	0.0053	0.0025	0.0007	0.0004

Wolwer-Rieck and others (2010b) conducted an accelerated shelf-life study and reported that the concentration of rebaudioside A decreased by 54% in a caffeinated soft drink at pH 2.4 after being stored for 72 h at 80°C while its concentration decreased by 28% in an energy drink at pH 3.5. Although the beverage compositions were not clear, the trend that the stability of rebaudioside A was improved in higher pH environments is again consistent with the citrate buffer data obtained from the current study.

Effect of buffer type and concentration

In low pH environments (pH 3), buffer type did not affect the degradation rates of rebaudioside A at 20 and 30 °C. Rate constants for rebaudioside A degradation in the two 0.1 M buffers were also not different at 40°C (Table 4.1-4.3). Slightly faster degradation was observed in 0.02 M citrate buffer than in 0.02 M phosphate buffer, but the general trend was very little effect of buffer type on the rate constants at pH 3. Likewise, buffer concentration (0.02 M versus

0.1 M) had little to no effect on the degradation rate constants of rebaudioside A at pH 3. These results are not consistent with a previous study where rebaudioside A stored at 22 °C and 37 °C appeared more stable in citric acid than in phosphoric acid beverages (Chang and Cook 1983). The study by Chang and Cook (1983) only had three data points, the number of replicates was not specified, and no statistical analysis was presented making their results questionable.

At higher pH values, an effect of buffer type and concentration became apparent (Tables 4.1-4.3). At pH 5 and 7, rebaudioside A broke down significantly faster in phosphate buffer than in citrate buffer in ten out of twelve experimental group comparisons while the two other groups (0.02 M buffer with pH 7 at 20 °C and 0.1 M buffer with pH 5 at 30 °C) showed the same trend but were not significant different. In addition, higher concentrations of phosphate buffer yielded larger degradation rate constants at pH 7 whereas the concentration of citrate buffer at pH 7 had no effect on the rate constants.

The different effects of buffer type and concentration on rebaudioside A degradation may be due to different hydrolysis mechanisms at the different pH levels. At pH 3, rebaudioside A degradation appears to be occurring via specific acid hydrolysis, where the hydronium ion (H_3O^+) catalyzes the cleavage, irrespective of buffer type and concentration. Therefore, buffer type and concentration have little effect on the degradation rate constant. However at pH 7, there appears to be some general acid-base catalysis occurring. At pH 7, degradation was faster in phosphate buffer than citrate buffer and faster at higher concentrations of phosphate buffer, meaning that buffer species other than H_3O^+ or OH^- were affecting the reaction. The concentration of the phosphate dibasic anion (HPO_4^{-2}) increases as pH increases from approximately pH 5 to 10 (Christian 1980). This anion has been previously linked to enhanced degradation rates for aspartame (Bell and Wetzel 1995), thiamin (Pachapurkar and Bell 2005),

and tagatose (Dobbs and Bell 2010). The role of phosphate buffer as a catalyst was also found in the Maillard reaction (Bell, 1997). It appears that the phosphate dibasic anion is also facilitating the necessary proton transfers to hydrolyze rebaudioside A. Although some degradation products have been identified, the exact degradation mechanisms for rebaudioside A have not been described. Current data suggest the effect of buffer on rebaudioside A degradation depends upon the pH. More research is needed to better understand the role of buffer salts on the mechanism of rebaudioside degradation.

Effect of temperature

As expected, rebaudioside A degradation rate constants increased as temperature increased (Tables 4.1-4.3), except for degradation in 0.02 M citrate buffer at pH 7 and 20 °C. Because this rate constant was larger than in the same system at 30 °C, the activation energy could not be reliably detected.

Activation energy is the energy needed to be overcome in order for a chemical reaction to occur. Higher activation energy means the reaction is more sensitive to temperature changes. In this study, activation energies for rebaudioside A degradation in different solutions were calculated from the slope of Arrhenius plots (Figure 4.6). The activation energies are listed in Table 4.6 and represent the effect of temperature on the stability of rebaudioside A.

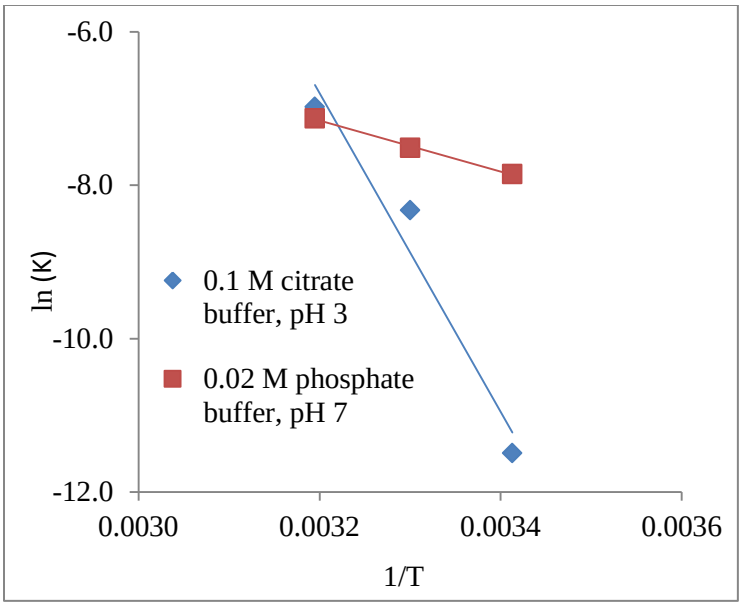

Figure 4.6 Arrhenius plots of rebaudioside A degradation in buffer solutions

Table 4.6 Activation energy (kcal/mol) for rebaudioside A degradation in solution

		pH 3	pH 5	pH 7
phosphate	0.02 M	23	5.4	6.6
	0.1 M	11.1	14.8	9.1
citrate	0.02 M	17	6.9	*
	0.1 M	41.3	15.2	11.5

*unreliable because rate constant at 20 °C was larger than 30 °C

With the exception of degradation in 0.1 M citrate buffer at pH 3, activation energies ranged from 5-23 kcal/mol (Table 4.6). No definitive trends were observed with respect to buffer type, buffer concentration or pH. Potentially different degradation mechanism (specific acid catalysis at pH 3 vs general acid-base catalysis at pH 7) may explain the lack of observable trends. Our values at pH 3 (11-41 kcal/mol) are similar to the 20-34 kcal/mol calculated from previously published data for rebaudioside degradation in mock beverages using citrate and phosphate buffers at pH 2.8-4.2 (Prakash and others, 2012). Other activation energy values

include 15-30 kcal/mol for aspartame degradation (Bell and Labuza 1991) and 12-17 kcal/mol for thiamin degradation (Pachapurkar and Bell 2005). Collecting kinetic data at additional temperatures would improve the reliability of the activation energies.

Effect of light

Light did not appear to play an important role in the degradation of rebaudioside A in phosphate buffer at pH 7 (Table 4.7). On the other hand, rebaudioside A in citrate buffer at pH 3 broke down almost ten times faster under light exposure than that stored in darkness. The results of this study somewhat agree with those of Chang and Cook (1983), who found sunlight enhanced the loss of rebaudioside A. However, they noted the amount of rebaudioside A decreased 18-22%, which is much higher than the amount lost in this study (Table 4.8). The results of this study do not agree with the conclusions made by Clos and others (2008); they claimed rebaudioside A was stable to light exposure. However, as mentioned in the literature review, a closer examination of their data actually reveals that there was five times less rebaudioside A after exposure to light for 1 week. Based on the current data and the two published studies, further research on the effect of light exposure is justified.

Table 4.7 Pseudo-first rate constants with 95% confidence limits (d^{-1}) for rebaudioside A stored under light or dark at room temperature

	0.1 M phosphate pH 7	0.1 M citrate pH 3
light	0.00118±0.000126 a	0.000443±0.000155 a
dark	0.000993±0.000183 a	0.0000547±0.000143 b

Different lower case letters represent significant differences within the same column ($P<0.05$).

Table 4.8 Predicted rebaudioside A loss (%) in 0.1 M buffer solutions after 1 week at room temperature

Storage environment	Percentage loss (%)
Phosphate, pH 7-light	0.82%
Phosphate, pH 7-dark	0.69%
Citrate, pH 3-light	0.31%
Citrate, pH 3-dark	0.04%

Comparison to other sweeteners

Quinlan and Jenner (1990) concluded that sucralose is stable in beverages in regard to temperature, pH and sunlight. There was no loss of sucralose in carbonated cola drinks (pH 2.8) at both 20 °C and 35 °C for 9 months, nor did degradation occur in pH 2.7 and 3.0 cola samples after 26 weeks of storage. Sunlight exposure did not affect the stability of sucralose in both cola and lemon-lime beverages. The stability of rebaudioside A found in the current study is similar to sucralose; very little loss of each occurs in acidic beverages at 20 °C.

Although aspartame degrades much faster than rebaudioside A (Bell and Wetzel 1995), there are some similarities with respect to their behavior. Aspartame was more stable in citrate buffer than in phosphate buffer. Higher concentrations of both buffer solutions accelerated the degradation of aspartame. However, aspartame degradation increased as pH increased irrespective of the buffer type while rebaudioside A degradation increased in phosphate buffer but decreased in citrate buffer as pH increased.

As described above, rebaudioside A stability is equivalent to or better than that of sucralose or aspartame. Another advantage of rebaudioside A is being naturally-derived. As mentioned in Chapter 2, rebaudioside A is also appropriate for individuals with PKU. Overall, rebaudioside A has several advantages compared to the high intensity sweeteners sucralose and aspartame that should allow its expanding use in foods and beverages.

Chapter 5: Summary and Conclusion

Rebaudioside A, a high intensity sweetener, is gaining popularity in the food industry due to its natural classification and zero calories. For better usage in food and beverages, its stability should be evaluated. This study provided kinetic data on the storage stability of rebaudioside A as affected by pH, buffer type and concentration, storage temperature and light exposure.

Rate constants for rebaudioside A degradation were similar at pH 3, regardless of the buffer type or concentration. As pH increased, the rate constants increased for degradation in phosphate buffer, but decreased or stayed the same in citrate buffer. At pH 7, higher phosphate buffer concentrations led to faster rebaudioside degradation rates. Light exposure appears to enhance rebaudioside A degradation in pH 3 citrate buffer, but not in pH 7 phosphate buffer. Beverage formulators should recognize the combined effects of buffer type, buffer concentration, and pH on rebaudioside A stability to optimize the quality of their product during storage. For example, stability of rebaudioside A in beverages would be enhanced by lower concentrations of citrate buffer in light shielded containers.

More studies could be conducted on the light stability of rebaudioside A. Constant temperature and constant light at certain wavelengths could be applied to the experimental groups and the correlation of pH and buffer type could be further studied. More data points at different temperatures could be collected improving the activation energy determination for the degradation of rebaudioside A. Although this study provided information about the storage and photo stability of rebaudioside A, the mechanism was not thoroughly studied and further experiments could be conducted to better understand degradation pathways.

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APPENDIX

Table 1. Concentration of rebaudioside A in 0.1 M citrate buffer at pH 3 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	51.321	50.697
32	49.392	54.225
61	50.650	48.775
92	50.142	52.442
133	51.076	53.288
160	52.226	55.294
184	48.793	47.482
238	50.802	52.974
288	49.719	51.545

Table 2. Concentration of rebaudioside A in 0.1 M citrate buffer at pH 3 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	50.814	50.018
28	50.030	54.672
60	50.590	50.990
89	50.682	51.179
131	50.954	50.222
189	49.271	50.459
222	47.429	48.809
264	47.785	47.667
278	48.903	48.958

Table 3. Concentration of rebaudioside A in 0.1 M citrate buffer at pH 3 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	49.078	46.817
29	42.941	41.734
60	41.721	43.886
90	42.107	43.025
121	44.677	46.928
152	39.192	39.938
194	38.268	38.6
252	37.221	37.520
285	34.721	34.407

Table 4. Concentration of rebaudioside A in 0.1 M citrate buffer at pH 5 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	45.313	45.439
29	45.349	45.619
60	45.860	44.393
90	43.031	43.241
121	45.168	44.927
152	43.303	43.601
194	45.131	45.179
252	42.738	43.640
285	44.147	44.506

Table 5. Concentration of rebaudioside A in 0.1 M citrate buffer at pH 5 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	45.697	46.347
29	45.909	45.651
60	44.267	45.097
90	46.147	43.084
121	44.462	43.970
152	44.317	43.684
194	44.089	45.837
252	44.170	42.922
285	41.417	42.563

Table 6. Concentration of rebaudioside A in 0.1 M citrate buffer at pH 5 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	46.985	46.130
29	44.880	45.714
60	43.184	44.630
90	48.061	45.916
121	44.313	43.532
152	42.415	41.971
194	42.745	43.053
252	41.837	40.773
285	39.587	36.790

Table 7. Concentration of rebaudioside A in 0.1 M citrate buffer at pH 7 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	46.323	45.451
29	46.975	46.729
60	44.569	44.569
90	44.707	43.241
121	45.302	45.203
152	44.330	47.247
194	46.522	45.439
252	45.422	44.803
285	44.362	44.738

Table 8. Concentration of rebaudioside A in 0.1 M citrate buffer at pH 7 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	47.301	45.845
29	47.778	46.433
60	43.203	45.525
90	44.497	44.192
121	45.908	45.231
152	45.241	45.256
194	47.324	45.246
252	44.269	44.209
285	44.708	43.897

Table 9. Concentration of rebaudioside A in 0.1 M citrate buffer at pH 7 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	46.058	47.959
29	44.583	44.21
60	45.441	45.307
90	48.241	46.937
121	44.646	43.711
152	43.763	44.485
194	44.512	44.427
252	45.067	44.355
285	43.143	42.637

Table 10. Concentration of rebaudioside A in 0.02 M citrate buffer at pH 3 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	42.887	42.295
29	43	43.307
60	42.683	41.358
90	41.278	39.89
121	42.01	42.268
152	40.28	40.719
194	42.045	37.03
252	40.321	41.285
285	42.381	42.012

Table 11. Concentration of rebaudioside A in 0.02 M citrate buffer at pH 3 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	41.769	43.006
29	43.954	43.694
60	43.124	40.797
90	40.647	42.131
121	42.106	42.273
152	40.196	40.799
194	40.079	40.662
252	39.671	40.295
285	38.868	39.024

Table 12. Concentration of rebaudioside A in 0.02 M citrate buffer at pH 3 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	42.333	41.554
29	40.565	40.833
60	39.733	40.057
90	38.138	41.324
121	40.081	39.231
152	35.413	37.288
194	36.516	35.142
252	35.92	34.757
285	31.557	31.036

Table 13. Concentration of rebaudioside A in 0.02 M citrate buffer at pH 5 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	38.787	37.846
29	38.203	39.994
60	36.924	37.879
90	37.072	41.016
121	37.263	38.801
152	36.416	36.625
194	37.361	37.644
252	36.567	35.703
285	38.978	38.717

Table 14. Concentration of rebaudioside A in 0.02 M citrate buffer at pH 5 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	45.198	45.810
28	46.348	47.422
60	46.661	46.561
89	47.202	46.050
131	48.071	45.584
189	46.325	44.833
222	45.261	46.454
264	44.652	43.316
278	44.99	45.86

Table 15. Concentration of rebaudioside A in 0.02 M citrate buffer at pH 5 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	37.889	37.308
29	37.985	38.362
60	36.614	37.303
90	37.601	36.689
121	37.327	38.1
152	36.738	36.746
194	36.022	37.334
252	36.578	36.494
285	34.438	34.959

Table 16. Concentration of rebaudioside A in 0.02 M citrate buffer at pH 7 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	40.106	40.359
29	41.556	41.714
60	41.098	40.134
90	38.293	36.88
121	40.712	40.759
152	39.662	38.843
194	40.061	39.943
252	36.314	38.639
285	38.214	38.109

Table 17. Concentration of rebaudioside A in 0.02 M citrate buffer at pH 7 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	39.753	41.199
29	41.706	41.485
60	39.782	40.042
90	38.547	40.699
121	39.983	40.174
152	40.685	40.41
194	39.366	39.91
252	39.351	39.732
285	39.328	38.665

Table 18. Concentration of rebaudioside A in 0.02 M citrate buffer at pH 7 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	40.08	39.721
29	41.407	40.746
60	39.247	39.871
90	38.555	42.142
121	40.343	39.639
152	39.32	39.102
194	39.41	39.426
252	38.936	38.668
285	35.856	37.802

Table 19. Concentration of rebaudioside A in 0.1 M phosphate buffer at pH 3 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	43.812	44.894
29	44.696	44.941
60	43.418	44.57
90	43.111	42.305
121	43.578	43.626
152	41.307	41.732
194	43.36	42.998
252	41.132	41.889
285	42.381	42.012

Table 20. Concentration of rebaudioside A in 0.1 M phosphate buffer at pH 3 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	43.576	42.781
29	43.464	43.106
60	42.632	42.808
90	41.389	41.668
121	41.393	42.114
152	41.776	41.898
194	40.853	43.061
252	40.904	41.734
285	39.983	39.717

Table 21. Concentration of rebaudioside A in 0.1 M phosphate buffer at pH 3 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	43.608	44.089
29	41.84	42.744
60	41.636	41.142
90	38.547	42.739
121	41.286	41.244
152	38.169	38.041
194	37.311	36.918
252	36.341	37.198
285	34.859	34.162

Table 22. Concentration of rebaudioside A in 0.1 M phosphate buffer at pH 5 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	41.765	42.547
29	42.542	42.266
60	40.127	42.183
90	38.866	41.014
121	40.303	40.829
152	39.894	38.745
194	40.494	40.549
252	39.167	39.626
285	38.978	38.717

Table 23. Concentration of rebaudioside A in 0.1 M phosphate buffer at pH 5 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	41.761	40.492
29	42.675	42.109
60	40.21	41.347
90	39.774	40.49
121	39.225	40.439
152	39.036	40.356
194	37.748	41.298
252	37.753	38.187
285	36.209	38.111

Table 24. Concentration of rebaudioside A in 0.1 M phosphate buffer at pH 5 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	40.157	42.73
29	40.093	39.661
60	39.15	38.032
90	38.129	36.638
121	36.362	36.588
152	34.524	34.163
194	32.171	29.292
252	31.251	29.789
285	27.426	28.17

Table 25. Concentration of rebaudioside A in 0.1 M phosphate buffer at pH 7 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	39.126	38.779
29	39.646	39.268
60	37.508	37.499
90	33.912	37.273
121	35.011	35.075
152	32.321	32.969
194	32.928	32.535
252	31.344	30.633
285	30.338	30.607

Table 26. Concentration of rebaudioside A in 0.1 M phosphate buffer at pH 7 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	38.09	38.018
29	36.426	35.932
60	33.21	32.97
90	31.515	32.615
121	30.904	30.556
152	28.985	28.725
194	26.631	26.263
252	26.059	25.077
285	23.757	23.796

Table 27. Concentration of rebaudioside A in 0.1 M phosphate buffer at pH 7 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	37.774	38.377
29	33.446	33.273
60	29.422	31.05
90	27.384	28.407
121	26.867	25.384
152	22.93	22.824
194	21.119	21.698
252	19.831	19.724
285	17.679	16.959

Table 28. Concentration of rebaudioside A in 0.02 M phosphate buffer at pH 3 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	51.688	47.741
32	52.169	52.678
61	52.393	50.876
92	53.978	52.165
133	52.555	50.633
160	54.271	52.468
184	51.423	50.227
238	50.550	50.471
288	51.087	49.771

Table 29. Concentration of rebaudioside A in 0.02 M phosphate buffer at pH 3 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	50.222	51.168
28	49.814	50.809
60	47.821	48.609
89	50.419	49.822
131	48.273	48.839
189	48.266	48.038
222	47.405	47.000
264	45.910	46.199
278	46.762	47.633

Table 30. Concentration of rebaudioside A in 0.02 M phosphate buffer at pH 3 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	39.935	39.362
29	38.021	37.983
60	37.131	37.19
90	36.911	39.305
121	36.438	37.53
152	31.603	32.273
194	34.61	34.872
252	35.599	34.611
285	33.465	32.338

Table 31. Concentration of rebaudioside A in 0.02 M phosphate buffer at pH 5 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	41.765	42.547
29	42.542	42.266
60	40.127	42.183
90	38.866	41.014
121	40.303	40.829
152	39.894	38.745
194	40.494	40.549
252	39.167	39.626
285	38.978	38.717

Table 32. Concentration of rebaudioside A in 0.02 M phosphate buffer at pH 5 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	39.938	40.09
29	41.186	40.357
60	44.495	41.926
90	41.826	39.765
121	39.029	39.431
152	39.502	38.593
194	38.024	38.722
252	38.255	37.372
285	37.183	37.215

Table 33. Concentration of rebaudioside A in 0.02 M phosphate buffer at pH 5 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	41.203	43.043
29	38.714	39.026
60	37.257	38.001
90	41.052	39.1
121	37.347	38.608
152	33.019	35.978
194	36.323	37.604
252	36.333	36.111
285	34.721	34.139

Table 34. Concentration of rebaudioside A in 0.02 M phosphate buffer at pH 7 and 20 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	43.718	45.155
29	45.357	44.167
60	42.599	44.469
90	43.232	43.015
121	42.495	42.444
152	41.144	39.908
194	42.47	41.654
252	39.985	40.91
285	40.107	40.115

Table 35. Concentration of rebaudioside A in 0.02 M phosphate buffer at pH 7 and 30 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	44.315	44.026
29	43.579	41.859
60	43.103	42.44
90	41.773	38.172
121	41.11	41.347
152	40.043	40.135
194	39.021	38.937
252	39.321	37.951
285	37.199	36.942

Table 36. Concentration of rebaudioside A in 0.02 M phosphate buffer at pH 7 and 40 °C

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	46.029	43.875
29	40.263	40.559
60	40.316	40.851
90	40.046	38.964
121	39.569	39.487
152	37.401	37.227
194	36.099	35.436
252	35.928	35.821
285	34.101	34.522

Table 37. Concentration of rebaudioside A in 0.1 M citrate buffer at pH 3 and light exposure

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	45.418	46.106
29	44.015	45.424
60	44.462	44.954
90	43.388	41.325
121	40.360	43.093
152	40.781	40.412
194	41.278	41.582
252	40.630	42.389
285	39.074	40.258

Table 38. Concentration of rebaudioside A in 0.1 M citrate buffer at pH 3 and light protected

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	44.243	45.175
29	43.75	43.54
60	43.702	43.577
90	44.628	41.897
121	44.283	44.191
152	46.755	46.026
194	43.54	43.334
252	44.271	43.98
285	42.504	42.771

Table 39. Concentration of rebaudioside A in 0.1 M phosphate buffer at pH 7 and light exposure

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	38.38	39.067
29	36.099	35.998
60	36.477	36.42
90	35.596	36.248
121	33.211	32.776
152	33.757	31.709
194	30.519	30.724
252	28.163	28.881
285	27.363	27.629

Table 40. Concentration of rebaudioside A in 0.1 M phosphate buffer at pH 7 and light protected

Time (Days)	Concentration (mg/100 mL)	
	sample 1	sample 2
0	37.566	38.197
29	35.196	35.079
60	35.423	36.122
90	35.792	37.783
121	35.604	34.4
152	31.655	33.072
194	31.542	31.275
252	28.973	29.524
285	28.439	28.34