Autoecological role of steviol glycosides in *Stevia rebaudiana* Bertoni

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ABSTRACT

Stevia rebaudiana Bertoni is a shrub native to Paraguay now a commercial source of the natural and non-calorific sweeteners of the steviol glycoside class. In stevia leaves, stevioside and rebaudioside A are the major steviol glycosides (SGs), occurring at about 10 and 3-5% of the leaf dw respectively. The large amount stored in the stevia leaves is interesting from an ecological perspective considering that the biosynthesis of SGs results from the up-regulation of a biosynthetic pathway shared with gibberellic acid (GA). The disparity in typical amount of GA (1.2 $\mu g.kg^{-1}$ leaf fw) and SGs (10% leaf dw) in a leaf suggests a biochemical investment in SG synthesis which merits an eco-physiological role within the stevia plant.

This thesis hopes to initiate this research area by looking at possible physiological roles in carbon storage, osmoregulation, mammalian herbivory and insect deterrence. The role of stevioside and rebaudioside A in carbon storage and osmoregulation was not observed given the relatively stable leaf concentration which was unresponsive to short periods of light and water treatments. Rather change in stevioside and rebaudioside A leaf concentration was gradual and related to the leaf and plant ontogeny with the highest leaf concentration occurring in the top immature leaves. It seems that synthesis occurs mostly in top young leaves with the SGs stored until leaf aging. The build-up of SGs seems to be ecologically beneficial with observed deterrence towards the generalist insect, *Valanga irregularis*, a grasshopper native to Australia. The leaves also encouraged general mammalian herbivory with the domestic guinea pigs, *Cavia porcellus*, with possible application in animal feed fortification.

Complementary to the ecophysiological investigations, methods for quantification of stevioside and rebaudioside A have also been developed with High Performance Liquid Chromatography (HPLC) as the main reference method. Quick analytical methods with minimal sample preparation

were also developed for possible estimation of stevioside and rebaudioside A in field application. Near Infrared Spectroscopy (NIRs) was found useful for estimating the total amount of stevioside and rebaudioside A in dry ground leaves (R = 0.87, RMSECV = 1.63). Potential transfer of the NIRs method to a handheld equipment would find commercial use in quality assessment of leaf harvests managed by stevia manufacturers. Anthrone colorimetry was also useful in measuring the amount of stevioside and rebaudioside A in solution (up to 0.06 μmol with R^2 = 0.993 and RMSEC = 0.002 μmol for stevioside and R^2 = 0.996, RMSEC = 0.002 μmol for rebaudioside A) and would be useful in quick estimates of liquids containing the sweetener such as beverage application.

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An introduction to the growth, commercial context and cultivation of *Stevia*

An introduction to the stevia plant

Stevia rebaudiana (Asteraceae), commonly called stevia, is a perennial shrub which grows up to 70-80 cm in cultivation (Soejarto 2002). Leaves have an opposite arrangement at 2-4 cm internode length and can be elliptic or oblanceolate with serrate edges depending on variety (Soejarto 2002). The stevia inflorescence usually contains five florets, and produces achenes (Southward, Kitchen *et al.* 2004).

S. rebaudiana was rediscovered in 1887 by Dr. M.S. Bertoni who noted the use of its leaves by native Indians and Mestizos in Paraguay to sweeten their tea (Lewis 1992). It is endemic to Paraguay, specifically to the highlands of Amambai near the source of the River Monday, between 25 and 26°S and at an elevation of 500-1500 m above sea level (Sumida 1973; Lewis 1992; Brandle, Starratt *et al.* 1998). Natural populations have also been found in the watershed of the Ypane River in Paraguay at an elevation of 200 m (Shock 1982). These regions experience a temperature range of -6 to 43 °C, with an average of around 23 °C, and an average annual rainfall of 1.4 m (Shock 1982; Brandle and Rosa 1992). The shrub grows in environments with shallow water tables such as marsh edges and grasslands. The soil is typically infertile, either acid sand or muck (drained humus from swamps), with pH 4-5 (Shock 1982), and plants surrounding the habitat of stevia grow

best in terra roxa and latosol soilsf with low phosphate content (Sumida 1973). In its natural environment, the perennial shrub can grow as high as 50-60 cm within 3-5 years (Brandle and Rosa 1992; Midmore and Rank 2002).

The genus *Stevia* includes around 200 species. Of these, only 110 species have been examined for leaf sweetness. Apart from *S. rebaudiana*, *S. phlebophylla* is the only other species found to have sweet leaves and a related high stevioside content (Kinghorn 1987). *S. phlebophylla* is now believed to be extinct (Kinghorn 1987). Another plant outside the stevia genus has sweet leaves because of a compound similar to stevioside. *Rubus suavissimus* (Rosaceae) contains rubusoside (at up to 5% leaf dw) but its relative sweetness is only half that of stevioside in stevia (Tanaka, Kohda *et al.* 1981; Ohtani, Aikawa *et al.* 1992).

The endemic population of stevia is now limited because of cattle grazing, agronomic activities and transplant of stevia for commercial growth (Soejarto 2002). This is unfortunate, as observation of the species at the original location could have revealed more information on stevia's interaction with its natural environment. Further, given the range in stevioside concentration noted in cultivated genotypes, additional variation could have also existed in the wild populations which may or may not change with respect to environmental conditions.

Stevia as a commercial commodity

Stevia, the sweetener

Stevia leaves contain 'natural' sweeteners, possessing nil calorific value. The sweet taste is due to steviol glycosides (SG), which have a sweetening power comparable with other artificial sweeteners. Stevioside and rebaudioside A, which are the main sweet compounds, are usually estimated to be 250-300 times sweeter than sucrose (at 0.4% solution) (Bridel and Lavieille 1931; cited in Dacome, Da Silva *et al.* 2005). Of the two main SGs,

rebaudioside A has a cleaner taste and is preferred over stevioside which gives a slightly bitter aftertaste (Brandle 1998). The sweetening ability of a sweetener is usually determined by comparing the taste of a sweetener solution with a sucrose solution. The equi-sweet concentration of stevia may vary with a sucrose reference because of panelist variation or difference in the sensory test method (Tunaley, Thomson *et al.* 1987; Cardello, Da Silva *et al.* 1999). Nonetheless, the sweetening performance of stevia is comparable with other commercial sweeteners such as aspartame, acesulfame and saccharin (Tunaley, Thomson *et al.* 1987; Cardello, Da Silva *et al.* 1999).

Stevia leaf extract containing mostly stevioside has long been available as an herbal remedy or 'dietary supplement', sold in health food stores, in developed countries. The first official and commercial use of stevioside as a food ingredient occurred in Japan in the 1970s, driven by a desire to avoid artificial sweeteners (Midmore and Rank 2002). Stevioside was then produced commercially in China to supply the sweetener to the Japanese market in the 1980s, although successful cultivation of the stevia plant (as source of the sweetener extract) has also been reported in sub-tropical Thailand and Indonesia and northern latitude of Canada (Cargill ; Midmore and Rank 2002). Back then, the stevia sweetener was only distributed on a limited basis as a food supplement with its pending approval for use as a food ingredient. The growth of the stevia market became exponential with a series of approvals of the stevia sweetener as a food ingredient in Australia (Daniels 2008; FSANZ 2008), followed by the U.S. (JECFA 2009). In Europe, France became the first country to allow use with a limited two-year approval granted in September 2009 (Halliday 2009). A positive opinion on stevia was likewise announced by EFSA in 2010 with a determined 'Allowable Daily Intake (ADI)' of 4 mg/kg body weight/day(EFSA 2010). This series of approvals has expanded the stevia market, which is now projected to grow up to $2 billion by the end of 2011 (Anonymous 2009). More recently, stevia sweetener with higher rebaudioside A content is promoted for its cleaner taste profile without the licorice aftertaste. Examples of high rebaudioside A

sweeteners include Truvia (Anonymous 2010) and PureVia (Anonymous 2010) which were developed in partnership with Coca-Cola and PepsiCo, attesting to the aggressive efforts of food manufacturers to claim this budding market.

Commercial leaf extraction

Amongst the plant organs, the leaves have the highest concentration of SGs at approximately 10% dw. In practice, the whole shoot is harvested, typically with a cutter bar set at about 5-10 cm above the ground (M.G.R. 1982; cited in Midmore and Rank 2002), air dried and then milled at about 30 mesh size (Dobberstein and Ahmed 1982). The SGs are extracted from ground stevia leaves through solvent extraction, purification and subsequent crystallization. Extraction and purification would usually involve a series of solvents with differing polarities to remove impurities. The sweet compounds are then separated from each other usually through chromatography wherein the compounds are separated depending on their polar interaction with the chromatographic column and the mobile phase.

One method recommended the use of low-polarity solvents such as chloroform to remove the less polar components in the extract (Dobberstein and Ahmed 1982). The SGs are then extracted with a second solvent with higher polarity than the first solvent, such as methanol. The eluant is then allowed to dry by evaporation and dissolved in a third solvent of intermediate polarity between the first and second solvent, such as 1-propanol, to allow it to pass through an HPLC (high performance liquid chromatography) column. A preferred column with a polar oxygen-containing organic stationary phase was then used to separate the SG components (Dobberstein and Ahmed 1982). In another patented method (Kutowy, Gower *et al.* 1999), water was used as the extraction solvent with the impurities filtered out based on molecular size. The first microfiltration had a limit of 2-3 kDa and the subsequent nanofiltration had a limit 200-600 Da (Kutowy, Gower *et al.* 1999). Another method, recommended initial water extraction at 65-100 °C, solvent evaporation under vacuum and subsequent extraction in methanol to

obtain an extract that consist mostly of stevioside and rebaudioside A (Morita, Fujita *et al.* 1978). Pure rebaudioside A is then obtained by allowing the effluent to pass through a silica gel column to separate it from stevioside. Effluent is then re-dissolved in 1:1 volume of water : n-amyl alcohol and evaporated in vacuum condition (Morita, Fujita *et al.* 1978). The method patented by Coca-cola to increase the concentration of crude rebaudioside A involved mixing with 75-80% organic solvent (possibly alcohol, acetone or acetonitrile with a ratio of 4-10 parts solvent and 1 part crude rebaudioside A) with heating at about 20 to 70 °C for up to 8 hours (Prakash, Alpharetta *et al.* 2007). After cooling to room temperature for 24 h, rebaudioside A is crystallized from the solution by seeding with pure rebaudioside A crystals (at about 1% of the total rebaudioside A in the slurry solution) at 18 to 35 °C (Prakash, Alpharetta *et al.* 2007).

Agronomic productivity

As the use of stevia as a sweetener has grown in popularity, so has the cultivation of the stevia plant. Production is predominantly based in China, India and South America. Agronomic practices for maximum biomass yield have been documented extensively. Recommendations for stevia propagation and cultivation are summarized below, but practices are yet to be documented extensively in the public domain. Similarly, availability of genotypes with enhanced SG level is limited with limited material in the public domain on selection for high SG (Brandle 1998).

Stevia and its propagation

Reproductive system and seed production

Stevia may flower profusely with more than 500 inflorescences observed in one plant (Southward, Kitchen *et al.* 2004). The inflorescence usually contains five florets which then ripen to produce achenes (Southward, Kitchen *et al.* 2004). Cold temperature (5.5-14.2 °C) is not ideal for seed

maturation, increasing the maturation period beyond 5 weeks and decreasing the percentage of flowers producing full seeds from 50 to 25% of the total inflorescence (Southward, Kitchen *et al.* 2004).

S. rebaudiana has been reported to be self-incompatible, with a selfing range of 0-0.5% and an out-crossing rate of 0.7 to 68.7% (Katayama, Sumida *et al.* 1976; cited in Brandle, Starratt *et al.* 1998). However experimental details were not reported and it is possible that low germination rate was due to inappropriate pollination conditions rather than self-incompatibility. Goettemoeller and Ching (1999) reported a high germination rate for seed produced by both self and cross pollination. Manual transfer of pollen between stevia flowers produced seed with a 93% germination rate, while pollination induced by wind or bee introduction was less effective (germination rate of 68 and 78% respectively).

Typically diploid (X = 11), the chromosome morphology in the genus *Stevia* has been reported to display pericentric inversions which is considered to contribute to evolutionary diversity (Frederico, Ruas *et al.* 1996). *S. rebaudiana* seeds is reported to be genetically heterozygous, consistent with an outcrossing breeding system (Tamura, Nakamura *et al.* 1984). Consequently, seedlings from the same seed set of a plant can have varied levels of stevioside (CV = 24%) and rebaudioside A (CV = 32%) (Tamura, Nakamura *et al.* 1984).

Propagation - seed

Seeds are crowned with a collection of hairs. These hairs aid in wind dispersal of the seed, but can also aid attachment to passing animals (personal observation), and so also favor animal dispersal. The seeds are light and weigh about 0.15-0.30 g for every one thousand seeds (Carneiro 1990; cited in Brandle, Starratt *et al.* 1998).

Seed germination is relatively poor unless under favourable conditions (cited in Midmore and Rank 2002). Optimum temperature for seed germination is reported to be 20 °C (Kawatani, Kaneki *et al.* 1977) to 25 °C (Southward, Kitchen *et al.* 2004). Even so, stevia seeds germinate poorly and develop slowly into seedlings (Rank and Midmore 2006).

Seed viability can be estimated by color, with dark seeds having a germination rate of 50% and light seeds being completely sterile (Monteiro 1980; cited in de Oliveira, Forni-Martins *et al.* 2004). Sterility of the light seeds is attributed to agamospermy or the asexual production of seeds (de Oliveira, Forni-Martins et al. 2004). Seed viability declines when excess rain occurs during pollination (Brandle, Starratt *et al.* 1998). Germination rate of seed also decreases by 50% after three years of storage at 0 °C (Brandle, Starratt *et al.* 1998). Rate of germination for different cultivars has not been documented.

After germination, seedlings are best clipped before field transplant to minimize transplant shock. Seedlings clipped to a height of 12-15 cm prior to transplant yielded higher leaf dw (913 $kg.ha^{-1}$) in comparison to unclipped seedlings of the same cultivar (465 kg/ha) (Tonello, DeFaveri *et al.* 2006). Moreover, a higher seedling mortality rate was observed for unclipped seedlings (21.4%) compared to clipped seedlings (9.7%) during the fourth harvest (Tonello, DeFaveri *et al.* 2006).

Seedlings from germinated seeds demonstrated higher heterogeneity (e.g. 4.4 % stevioside leaf dw ± 1.0, CV = 24%) than plants propagated asexually (by stem-tip culture) from the same parent line (e.g. 4.9 % stevioside leaf dw

± 0.5, CV = 10%) (Tamura, Nakamura *et al.* 1984). Given the heterogeneity of SG levels in seedlings from the same seed set, methods for stem cutting and tissue culture propagation have been developed to produce more consistent stevia lines. SG uniformity in stevia lines and labour costs are major considerations in selecting the appropriate method for commercial application.

Propagation - stem cutting

Stem cutting produces genetically homogeneous lines with similar stevioside or rebaudioside A leaf concentration (stevioside mean = 5.7%, rebaudioside A mean = 5.9%, CV = 5-6% in both cases; from a seed-germinated population with a CV of 24 and 32%, respectively) (Tamura, Nakamura *et al.* 1984).

Stem cuttings from the upper half of the shoot have higher propagation success than the lower half of the shoot (Shock 1982; Bogor 1988). Top cuttings also exhibited optimal vegetative growth (plant height and branch number) resulting in high biomass yield (leaf dw) (Murayama, Kayano *et al.* 1980). A 15 cm length cutting was reported to result in better growth than that of a 7.5 cm cutting, a result ascribed to the higher level of storage reserves in the longer cuttings (Chalapathi, Thimmegowda *et al.* 1999).

Dipping the tip of stem cuttings into high concentrations of growth regulator (1000 ppm indole-3-butyric acid (IBA)) did not improve sprouting rate of stem cuttings relative to the control (Chalapathi, Thimmegowda *et al.* 1999). Effect of lower concentrations was not investigated although another study used a lower concentration of IBA (600 ppm) to promote root growth (Bogor 1988).

Propagation – tissue culture

Tamura *et al.* (1984) reported that micropropagation from stem-tip cultures also yields lines with consistent SG levels (stevioside mean = 5.6%, rebaudioside A mean = 5.3%, CV = 6-7% in both cases; from a parent population with CV of 24 and 32%, respectively).

Typically, shoot growth is induced in stem tissue explants by addition of growth regulators to the Murashige and Skoog (MS) growth medium. Sivaram and Mukundan (2003) reported 6-benzyladenine (BA) (8.87 μM) with indole-3-acetic acid (IAA) (5.71 μM) was most effective in inducing shoot growth in explants of the shoot apex, node and leaf sections. In comparison, kinetin (2.32-13.93 μM) only promoted shoot growth in shoot apex and nodal explants (Sivaram and Mukundan 2003). Root induction in shoot apex, node and leaf sections was optimal in half strength MS medium supplemented with 4.90 uM of indole-3-butyric acid after 30 days of culture (Sivaram and Mukundan 2003).

Explants can also be regenerated from inter-nodal, nodal and leaf explants with the addition of 2,4-dichlorophenoxyacetic acid (3 $mg.L^{-1}$) to the MS medium (Uddin, Chowdhury *et al.* 2006). Of the three segments however, callus formation was earliest and most profuse in the inter-nodal sections (Uddin, Chowdhury *et al.* 2006).

Biomass yield and SG content of tissue cultures can be improved by optimizing the sucrose concentration of the MS medium used in cultivating the shoot primordia. Within the range of 0-9% sucrose, the MS medium with 3% sucrose produced the highest shoot fw of 70 $g.L^{-1}$ (g tissue/L medium volume), with the control (0% sucrose) yielding only 40$g.L^{-1}$ of shoot fw (Akita, Shigeoka *et al.* 1994). At 3% concentration, sucrose was also more effective than glucose and fructose in improving yield. While glucose, fructose and sucrose (all at 3% w/v) addition resulted in similar levels of total dry mass of around 20mg each for seedlings derived from the meristems and juvenile shoots of intact plants, the SG content quadrupled with 3% sucrose (stevioside, rebaudioside A and rebaudioside C at 0.65, 0.2 and 0.1% dw, respectively) while the SG content only doubled for seedlings grown in mediums with 3% glucose and 3% fructose (stevioside, rebaudioside A and rebaudioside C at 0.3, 0.1 and 0.05% dw, respectively) (Bondarev, Reshetnyak *et al.* 2003). It should be noted that typical SG levels in shoots of stevia tissue cultures are lower compared to leaves of fully developed stevia plants and may be attributed to the limited leaf tissue development inside the

bioreactor. However, the tissue cultures can be acclimatized in soil and the increase in concentration potentially translated into higher SG concentration in fully developed stevia plants (Akita, Shigeoka et al. 1994).

For mass propagation of tissue cultures, a 500 litre bioreactor was filled with a MS medium fortified with 3% sucrose, 0.1 $mg.L^{-1}$ NAA, 1 $mg.L^{-1}$ BA and half strength inorganic salts (KNO_3, NH_4NO_3 and $CaCl_2 2H_2O$) (Akita, Shigeoka *et al.* 1994). A total yield of 64.6 kg (FW) of shoot was achieved after 3 weeks from a 460 g (FW) shoot inoculums after 3 weeks, with shoot proliferation being evident within 1 week of suspension (Akita, Shigeoka *et al.* 1994).

Once plantlets are generated, soil type is critical to seedling survival after transplant. Under greenhouse condition of 40% shade and 60-70% humidity, the highest survival rate was observed in coco-peat at 75%, followed by vermiculite at 25% and soil at 20% (Sivaram and Mukundan 2003). Combinations of the three media achieved survival rates below 60%. Plants were reported to be completely hardened after 30 days of transplant.

Stevia cultivation

In cultivating stevia, the primary practical goal is to manipulate conditions in order to maximise above ground biomass yield, although the real goal is to maximise SG yield. Biomass yield has been found to differ with seasonal changes in daylength, watering regime, nutrition level and planting density although effect on SG leaf content was usually not documented. Recommendations on crop timing and other cultivation protocols should be adopted in context of local conditions to achieve optimal results.

Seasonal changes in daylength and crop cycle

The growth cycle of *S. rebaudiana* varies with latitudinal location because of its photoperiod sensitivity. Valio and Rocha (1966) reported that a photoperiod below 13 h induces flowering in a controlled environment. In its native habitat in Paraguay (23°S, 56°W), vegetative growth occurs between October and December with shortening days inducing flowering which occurs between January and March (Shock 1982). As flowering proliferates, vegetative growth slows down and crown regrowth shortens after cutting (Shock 1982). Based on a daylength calculator, the October to December period would correspond to 12 to 13.5 h daylight which then decreases from 13.3 to 12.7 h daylight from January to March (http://aa.usno.navy.mil/cgi-bin/aa_rstablew.pl). The rest of the year would experience around 10 to 12 h daylight.

In southern Ontario, Canada (48°N), annual production of stevia is reported to start with 6 to 7 weeks of seedling growth in heated greenhouses followed by field transplant in mid to late May (Brandle, Starratt *et al.* 1998). Vegetative growth peaks from July to mid September which corresponds to periods of 14 to 18 h daylight, followed by flowering and annual harvest which occurs under 6 to 13 h daylight (http://aa.usno.navy.mil/cgi-bin/aa_rstablew.pl).

Using the study of Valio and Rocha (1966) and the flowering behavior observed in Paraguay and Canada, the critical period for daylength occurs at around 13-14 h. The variation in daylength sensitivity of different plants to different photoperiods suggests photoperiod sensitivity to be genetically based. Investigation on the genetic basis of photoperiod sensitivity is lacking but can be commercially beneficial if flowering can be delayed to extend the vegetative growth and increase the leaf and SG yield of stevia plantation.

Water requirement

A few studies have attempted to quantify the water requirement of the stevia plant. In Indonesia, plant water use was measured to be 2.33 $mm.day^{-1}$

(0.023 $ML.ha^{-1}$) in a field experiment wherein soil water content was measured to be within the range of 43% (pF 2.54 or -0.03 MPa) - 47.6% (pF 2.0 or -0.01 MPa) (Goenadi and Bogor 1983). In Italy, a micro-lysimeter experiment observed fluctuations in water consumption within an 80 day period with a maximum water intake of 5.44 $mm.day^{-1}$ (0.054 $ML.ha^{-1}$) (Fronza and Folegatti 2003). Fluctuations in water consumption were attributed to the dry climate of Pisa and water loss/evaporation from the silt/clay soil surface (51% sand, 39% silt and 10% clay). The difference in water intake observed is most likely influenced by weather which changes the rate of soil water evaporation as well as soil type. Documentation of stevia water use under different weather and soil conditions is minimal and further studies of the water relations of stevia would be useful to improve production yield, in terms of (1) biomass production, (2) SG content of leaves, and (3) relative proportions of the different SGs.

Nutrition

In a field experiment with a density of 100,000 plants/ha, addition of 40, 20 and 30 kg of N, P and K (i.e. 0.4, 0.2 and 0.3 g N, P, K per plant) improved leaf biomass in both first and ratoon crops grown in an alfisol (Chalapathi, Shivaraj *et al.* 1997; Chalapathi, Thimmegowda *et al.* 1999). Another experiment extrapolated an optimal dose of 175 g of farmyard manure or 200 mL of 6.25% (v/v) liquid organic fertilizer (Goenadi and Bogor 1984). The N, P and K ratio and application rate of this organic fertiler, calculated from elemental analysis of the farmyard manure (1.1% N, 0.5% P_2O_5, 0.4% K_2O and liquid organic fertilizer: 18.3% N, 2.9% P_2O_5, 2.7% K_2O), was similar to the recommendation of Chalapathi, Shivaraj *et al.* (1997) (calculation not shown).

Other researchers, working on an andosol soil, recommended a higher proportion of P & K (at 0.4, 0.4 and 0.6 g N, P and K, respectively per plant) achieved through application of 1 g per plant each of urea, triple superphosphate and muriate of potash (Angkapradipta, Warsito *et al.* 1986; Angkapradipta, Warsito *et al.* 1986). The difference in recommended NPK

amount may be attributed to the soil used in the experiment, as andosol soils were reported to be phosphorus deficient (Takahashi and Anwar 2007). Increased P dosage to plants growing on andosol soils would alleviate growth retardation expected in phosphorus deficient stevia (Lima Filho and Malavolta 1997(a)). Indeed leaf yield per plant was doubled with application of triple superphosphate (1 g.plant^{-1})(Angkapradipta, Warsito *et al.* 1986).

Leaf uptake of N, P and K increased with addition of microbial inoculants to soil (Das, Dang *et al.* 2007). A combination of *Azospirillum*, vesicular arbuscular mycorrhiza and phosphate solubilizing bacteria was reported to result in at least 50% increase in N, P and K uptake and a 44% increase in biomass yield over control. A small increase in leaf uptake of N, P and K was also observed when stevia was planted in a ridge and furrow method (151, 7.0 and 123 kg.ha^{-1} of N, P and K) instead of the flat bed method (148, 6.8 and 120 kg.ha^{-1} of N, P and K) (Chalapathi, Shivaraj *et al.* 1997).

Leaf uptake of macro and micronutrient also change with stage of plant development (Lima Filho, Malavolta et al. 1997(b); Lima Filho, Malavolta et al. 1997(c)). Macronutrient leaf content more than doubled during seed production (% dw before flowering: N 0.6, P 0.07, K 0.5, Ca 0.1, Mg 0.03, S 0.03; during seed production: N 1.3, P 0.2, K 1.3, Ca 0.4, Mg 0.08, S 0.09) and the same pattern was observed in micronutrient leaf content (% dw before flowering: B 8.9 x 10^{-4}, Cu 2.6 x 10^{-4}, Fe 0.01, Mn 0.002, Zn 1.3 x 10^{-4}; during seed production: B 0.002, Cu 7.6 x 10^{-4}, Fe 0.03, Mn 0.005, Zn-3.3 x 10^{-4}).

Correlations between macro and micronutrient leaf content were observed although monitoring was only done during the vegetative growth stage (Lima Filho and Malavolta 1997(d)). In particular, levels of N and P, P and Cu and P and Fe were positively correlated, while N and K, N and Zn, K and Mg and K and S displayed a negative correlation. Mg and Zn and B and Zn displayed both behaviours depending on leaf concentration.

Symptoms of mineral deficiency have been described for *S. rebaudiana,* although images of the symptoms have not been documented (Lima Filho

and Malavolta 1997(a)). N deficiency was accompanied by smaller, generally chlorotic, leaves. K deficient plants had yellowish, curling or necrotic old leaves. Ca deficiency was manifested in leaf spots, chlorosis and necrosis with some wilting in the upper stem. Mg deficient plants exhibited interveinal chlorosis in some older leaves. B deficiency resulted in roots that were thick and dark, with short branches. P deficiency resulted in an overall growth decline of the plant. B and Zn toxicity were also characterized, the former having brown spots at margins and tips of mature leaves prior to necrosis and the latter having necrotic spots in mature leaves followed by wilting and plant death.

Only severe Ca deficiency was found to reduce the concentration (% dw) of glycoside in the shoots (Lima Filho, Malavolta et al. 1997).

Plant density

As plant density is increased, individual plant biomass yield is typically decreased while total biomass yield (per ha) is increased up to some optimum density. Thus, while Murayama and co-workers (1980) reported leaf biomass per plant to decrease with increasing plant density (18 g at 83,000 plants.ha^{-1}, 12 g at 166,000 plants.ha^{-1}), Katayama and co-workers (1976; cited in Brandle, Starratt *et al.* 1998) observed an increase in leaf biomass per unit land area as plant density was increased from 83,000 to 111,000 plants.ha^{-1} in Japan (data not shown). In California, optimal plant density for biomass yield was reported at 190,000 plants.ha^{-1}on the basis of higher mortality rates in densities of 380,000 plants.ha^{-1} (yield data not reported) (Shock 1982). In Indonesia, densities of up to 208,000 plants/ha displayed an increasing trend in total biomass yield (Basuki and Sumaryono 1990). Variations in optimal density range may be attributed to differences in soil nutrition, temperature and stevia variety. While the plantation density from previous studies can serve as a guide, an optimal planting density should be determined for each commercial plantation. Unfortunately, the effect of planting density on leaf stevioside and rebaudioside A content has not been reported.

Other agronomic factors

A number of workers have considered the effect of planting methods and row orientation on the biomass yield of *S. rebaudiana.* Field cultivation technique had marginal effects on harvest yield, with a ridge and furrow method having slightly more leaf yield (2.70 tonne dry leaf/ha) than a flat bed method (2.59 tonne dry leaf.ha plants.ha^{-1}) (Chalapathi, Shivaraj *et al.* 1997).

Mountain slope location has also been reported to affect biomass yield of *S. rebaudiana* (Goenadi 1987). Stevia grown on the lower slope position had higher dry mass yield (474 kg shoot dw.ha plants.ha^{-1}) than those grown in the middle and upper slope sections (328 and 154 kg.ha plants.ha^{-1} respectively), although yield difference declined after the first two years. While soil nutrient level was reported to be similar at the three slope sections, moisture availability was not monitored.

Harvest protocol

Stevia typically stays productive for a total of 3 years with the first harvest occurring around 4-5 months after planting. Subsequent harvests are then done every 3 months (Rayaguru and Khan 2008). After 3 years, it is recommended to grow a second generation of crops from cuttings (Rayaguru and Khan 2008). The relative SG yield per unit biomass has not been reported in terms of main and ratoon crops.

To maximize the overall biomass yield of a plantation, an optimal harvest height of 3-5 cm above soil level was recommended for high fresh and dry biomass yield (43 and 6 metric tons.ha^{-1}, accumulated over five subsequent harvests) (Tonello, DeFaveri *et al.* 2006). Cutting the stems to 1-2 cm stubble resulted in lower fresh and dry biomass yield (16 and 1.5 metric tons/ha) (Tonello, DeFaveri *et al.* 2006). Furthermore, the use of the low harvesting scheme resulted in some plant death after the third harvest, because of increased susceptibility to *Septoria* fungal infection.

After pruning the stems, stevia leaves are usually dried under the sun for about 12 hours (Rayaguru and Khan 2008). Longer drying time is not recommended and extending to up to 3 days can cause stevioside content to deteriorate by up to 33%. After drying, the leaves are separated from the stem by hand or by using a mechanical thresher. The gathered leaves are then crushed using a grinder of about 30 mesh (Dobberstein and Ahmed 1982) in preparation for extraction of SGs (Rayaguru and Khan 2008).

Crop problems – Weeds, Disease, Insects

Management of weed infiltration, disease and insect infestation on stevia has been documented to minimize crop damage in commercial cultivation (Katayama, Sumida *et al.* 1976 cited in Basuki and Sumaryono 1990; Brandle, Starratt *et al.* 1998; Tonello, DeFaveri *et al.* 2006). Unfortunately, effect on SG levels were not reported.

Weeds

Weed infiltration in stevia plantations is exacerbated by slow seedling establishment (Midmore and Rank 2002). The application of the following pre-emergent herbicides applied through mechanical incorporation or pre-plant watering have also been recommended (Tonello, DeFaveri *et al.* 2006):

- Fusilade® (active ingredient: fluazifop-p butyl as butyl ester, http://dkt.net.au/msdsfiles/fusilade%20wg.pdf)
- Dual Gold® (active ingredient: S-metolachlor, http://www.syngenta.com/en/products_services/fact_sheets/dual_gold_window.html)
- Flame® (active ingredient: imidiazilones, http://www.dpi.qld.gov.au/cps/rde/xchg/dpi/hs.xsl/26_4257_ENA_Print.htm)
- Striker® (active ingredient: Oxyfluorfen, http://www.agric.wa.gov.au/content/hort/vit/bulletin_4719_part2c.pdf).

Post-harvest herbicides such as Basta® were also explored although concentration needs to be adjusted to minimize phytotoxic effects on the stevia crop (Tonello, DeFaveri *et al.* 2006). Trifluralin was reviewed as a well tolerated herbicide but extent of efficacy was not mentioned (Katayama, Sumida *et al.* 1976; cited in Brandle, Starratt *et al.* 1998).

As an alternative to use of herbicides, black plastic mulch for weed control has been explored and found to increase leaf dw (13 tons/ha), compared to plantations without plastic mulch (10 tons.ha^{-1}) (Basuki and Sumaryono 1990). Planting density in both cases was at 208,000 plants.ha^{-1}.

Disease

Fungal diseases are more prevalent under moist conditions, and most hostile towards young seedlings (Rank 2004). *Sclerotinia sclerotiorum* and *Septoria steviae* were detected in stevia plants in Canada (Lovering 1996; Chang, Howard *et al.* 1997), *Verticillium dahliae* in California (Farrar and Davis 2000), *Alternaria steviae* in Japan in 1982 (Ishiba, Yokoyama *et al.* 1982). Other fungal diseases known to attack stevia are *Phytophthora* and *Rhizoctonia stevia* (Rank 2004). The effect of such diseases on leaf SG concentration has not been reported.

While treatments for these diseases are yet to be documented, one researcher recorded the alternate spraying of Dithane MZ and Folicur fungicides on a weekly basis (Tonello, DeFaveri *et al.* 2006) to control the occurrence of *Septoria* in *S. rebaudiana*.

Insects

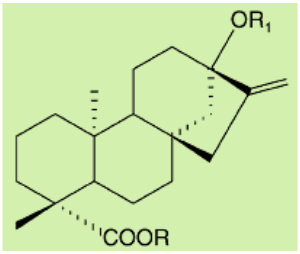
Stevia demonstrate an apparent natural resistance to insect pests, with few incidents of significant insect damage reported. Coleoptera, Lepidoptera and Orthoptera have been reported to occur in field plantations of *S. rebaudiana* in Paraguay (Fuente 2001) while *Thrips imaginis* have been encountered in *S. rebaudiana* planted in Australia (Tonello, DeFaveri *et al.* 2006). For thrips, application of Success® was effective in controlling the insect (Tonello,

DeFaveri *et al.* 2006). As for the Coleoptera, Lepidoptera and Orthoptera, the only recommendation is the precautionary measure of distancing plantations from cucurbitaceas, leguminous and gramineous species (Fuente 2001).

Conclusion

Stevia is a new crop, growing quickly in demand as source of a natural sweetener. Stevia cultivation will most likely increase given the commercial interest in the secondary metabolities, SGs. Given the likely narrow genetic base used in commercial cultivation and the declining natural stevia population, and the lack of reports on the effect of agronomic practices on SG yield, as opposed to biomass, it is timely to consider the ecophysiological role of SG within the stevia plant.

Chapter 2



The Intrigue of Stevia:

A literature review on the synthesis and ecophysiological roles of steviol glycosides in the Stevia plant

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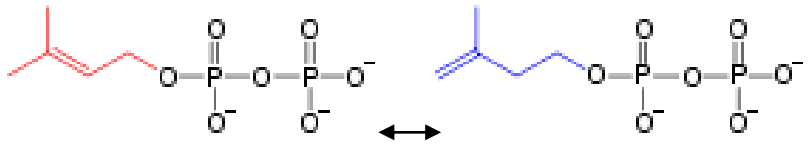
Introduction on secondary metabolites

Within the last 50 years, knowledge of plant secondary metabolites has increased extensively. Initial efforts resulted in an encyclopaedia of “natural products” which were of commercial interest to the pharmacological industry (Hartmann 2007). With only about 20-30% of higher plants investigated, the number of documented secondary metabolites has already reached tens of thousands (Wink 1999). In general, secondary metabolites have been categorized into nitrogen-based and carbon-based metabolites. Nitrogen-containing secondary metabolites include alkaloids, amines, cyanogenic glycosides, glucosinolates and non-protein amino acids (Wink 1999). Carbon-based secondary metabolites without nitrogen include terpenes, flavonoids, polyacetylenes, polyketides and phenylpropanoids (Wink 1999). The chemical structure of these “natural products” has been documented and the biosynthetic pathways and spatial distribution of key enzymes has been also defined (Hartmann 2007). The genetic control of these pathways is now under consideration within the research area of metabolomics (Fiehn 2002). Keen observation of herbivore-plant interactions pointed to an ecological role for certain secondary metabolites (Hartmann 2007). Such work opened the arena of chemoecology or physioecology which looked further into the physiological role of secondary compounds within the plant in relation to its environment (Hartmann 2007).

SGs are secondary metabolites of *S. rebaudiana* that are found mostly in the leaves. The SG chemical structure and biosynthetic pathway have been well studied but its physiological role in the plant remains to be described.

SG chemistry

SGs belong to the terpene group which is one of the biggest groups of plant secondary metabolites (Gershenzon and Kreis 1999). Terpenes consist of five-carbon isoprene units (Fig. 2.1) and are named after the number of units of ten carbon atoms (Gershenzon and Kreis 1999). For example, monoterpenes, diterpene and triterpene would correspond to 10, 20 and 30-carbon units (Gershenzon and Kreis 1999).



Dimethylallyl pyrophosphate (DMAPP) Isopentyl pyrophosphate (IPP)

Figure 2. 1. Two interchangeable structures of the biological isoprene units that function as building blocks for mono-, di- and triterpenes

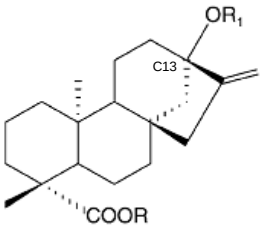
SG is categorized as a diterpene with its central aglycone structure consisting of 20 carbon atoms (Fig. 2.2). The central aglycone structure, called steviol, is shared amongst the nine types of SGs in stevia leaves (Fig. 2.2). These nine variants differ in terms of the number of glucose units attached to the C13 and C19 position of the aglycone (Dacome, Da Silva *et al.* 2005). The presence of a sugar unit or a carboxyl group at C19 and a sugar or hydroxyl group at C13 is considered essential for the glycoside to be perceived as sweet (Brandle and Telmer 2007). Sweetness intensity varies amongst the SGs, with rebaudioside A being the sweetest, followed by stevioside (Brandle and Telmer 2007).

Of the nine SGs found in leaves of *S. rebaudiana*, stevioside generally occurs in the highest amount in leaf tissue (9.1% dw), followed by rebaudioside A (3.8%), rebaudioside C (0.6%) and dulcoside (0.3%) (Brandle, Starratt *et al.* 1998). However, the ratio of these SGs varies with plant variety (Brandle, Starratt *et al.* 1998) and plant tissue (Bondarev, Sukhanova *et al.* 2003). To date only the ratios of stevioside, rebaudioside A and rebaudioside C in stevia plant organs have been investigated (Bondarev, Sukhanova *et al.* 2003).

C19

The family of steviol-derived sweeteners from *Stevia rebaudiana*

Diterpenic glycosides	R	R_1	Relative sweetening power[a]
Rebaudioside-A	**β-Glc**	β-Glc^2—β-Glc^1 (3: β-Glc)	**350–450**
Rebaudioside-B	H	β-Glc^2—β-Glc^1 (3: β-Glc)	300–350
Stevioside	**β-Glc**	**β-Glc^2—β-Glc^1**	**250–300**
Rebaudioside-E	β-Glc^2—β-Glc^1	β-Glc^2—β-Glc^1	250–300
Rebaudioside-D	β-Glc^2—β-Glc^1	β-Glc^2—β-Glc^1 (3: β-Glc)	200–300
Steviolbioside	H	β-Glc^2—β-Glc^1	100–125
Rebaudioside-C	β-Glc	β-Glc^2—α-Rha^1 (3: β-Glc)	50–120
Dulcoside	β-Glc	β-Glc^2—α-Rha^1	50–120
Rebaudioside-F	β-Glc	β-Glc^2—β-Xyl^1 (3: β-Glc)	nd



Glc, glucose; Rha, rhamnose; Xyl, xylose; nd, not determined. Bold label indicates the main glycoside pair focused in this paper.
[a] Respect with sucrose = 1.

Figure 2. 2. The family of steviol derived sweeteners is based on an aglycone unit with two variable groups (R, R_1) (Dacome, Da Silva *et al.* 2005)

SG biosynthetic pathway

Much work has been done in characterizing the general biosynthetic mechanism of the terpenes. Two general pathways are recognized, the mevalonate pathway and the relatively recent glyceraldehyde phosphate-pyruvate pathway (Gershenzon and Kreis 1999). These pathways differ in the precursor compounds involved in the synthesis of the five-carbon isopentyl diphosphate (IPP) which is the basic building block of terpenoids. With the mevalonate pathway, biosynthesis of IPP begins with acetyl-CoA. With the glyceraldehyde phosphate-pyruvate pathway, IPP precursors include pyruvate and glyceraldehyde 3-phosphate (Gershenzon and Kreis 1999). The IPPs then combine to form larger terpenoid skeletons such as geranyl diphosphate (ten carbons), farnesyl diphosphate (fifteen carbons) and geranylgeranyl disphosphate (20 carbons) which become the skeletal framework of monoterpenes, sesquiterpenes and diterpenes respectively (Gershenzon and Kreis 1999).

The biosynthesis of SG follows the glyceraldehyde phosphate-pyruvate pathway, having pyruvate and glyceraldehyde 3-phosphate as precursors (Totte, Charon *et al.* 2000). As diterpenes, SGs originate from the geranylgeranyl diphosphate (GGPP) (Fig. 2.3) (Gershenzon and Kreis 1999). The pathway leading from GGPP to SG is shared with gibberellic acid (GA) (Fig. 2.2) (Richman, Gijzen *et al.* 1999).

The synthesis of SGs from GGPP starts with the cyclization of *GGPP (geranylgeranyl diphosphate)* with involvement of **CPS** *((-)-copalyl diphosphate synthase)* and **KS** *((-)-kaurene synthase)* followed by carboxylation with **KO** *(kaurene oxidase)* to form kaurenoic acid (Brandle and Telmer 2007). The pathway for SGs then branches off from that of GA, with the hydroxylation of kaurenoic acid by **KAH** (*kaurenoic acid 13-hydroxylase)* to form steviol (Brandle and Telmer 2007). Sugar molecules are then added to the steviol backbone with the help of **UGTs** *(UDP-glycosyltransferases)* to form the different SGs (Brandle and Telmer 2007).

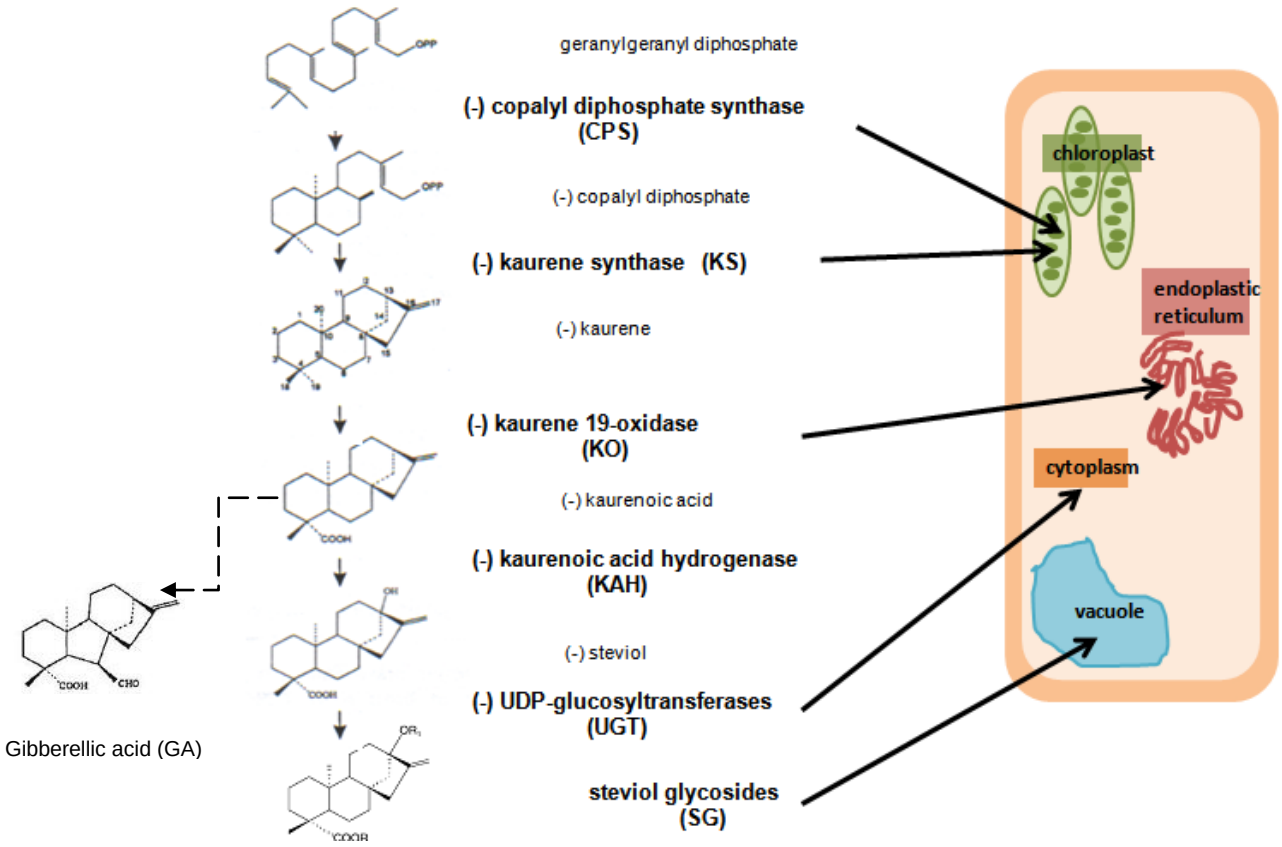


Figure 2. 3. Illustration of common biosynthetic pathway shared by steviol and GA synthesis and the subcellular location of key enzymes based on the illustration of Richman et al (1999).

Although common steps are shared in the biosynthesis of SGs and GA, the activity levels of CPS and KS enzymes relative to leaf maturity are different for SG and GA synthesis. In GA synthesis, expression of KS and CPS genes is normally higher in young than in mature leaves. However, in SG synthesis, expression of KS and CPS genes is higher in mature stevia leaves (Richman, Gijzen *et al.* 1999). Given the large difference in the typical amount of SGs (10% leaf dw) and GA (1.2 μg.kg^{-1} leaf fw) in leaves, it is postulated that the synthesis and accumulation of SGs and GA must be separated either temporally and/or spatially (Alves and Ruddat 1979; Richman, Gijzen *et al.* 1999). A duplicate KS gene was identified in *S. rebaudiana* leaves and further work is needed to trace its role in the spatial or temporal separation of SG and GA synthesis (Richman, Gijzen *et al.* 1999).

Spatial location of SG biosynthesis

Change in tissue SG content with chloroplast development is consistent with a role for chloroplasts in SG synthesis. An increase in stevioside, rebaudioside A and rebaudioside C content was observed with the differentiation of suspension cultures to morphogenic callus and shooted morphogenic callus (Bondarev, Reshetnyak *et al.* 2001). Increase was most evident with stevioside leaf content (387 μg.g dw^{-1} in shoot morphogenic callus, 60 μg.g dw^{-1} in morphogenic callus and 15 μg/g dw in suspension culture) and a similar trend was observed in rebaudioside A and rebaudioside C (Bondarev, Reshetnyak *et al.* 2001). The increase in SG in shoot material was attributed to the presence of well-developed chloroplasts in mesophyll cells of the shoots which were absent in the dedifferentiated cells of the callus in the suspension culture (Bondarev, Reshetnyak *et al.* 2001). Indeed, a stevia leaf chloroplast ultrastructure can be related to leaf SG level. Leaves of greenhouse-grown plants that possessed well-developed chloroplasts had a high SG content (25.4 mg g^{-1} dw), compared to *in vitro* plants (3.3 mg g^{-1} dw), etiolated *in vitro* plants (0.28 mg g^{-1} dw), green callus (0.06 mg g^{-1} dw) and etiolated callus (trace amounts) (Ladygin, Bondarev *et al.* 2008). A similar trend was observed for rebaudioside A and rebaudioside C (at lower levels). Apart from the ultrastructure evidence, the chloroplast was also predicted to be a probable location for KS by using a program, PSORT (Nakai and Horton 1999; Humphrey, Richman *et al.* 2006). PSORT narrows down the possible cellular location of a protein by considering the source of the protein (whether it is from a gram-positive bacteria, gram-negative bacteria, yeast, animal or plant) and the full-length sequence of amino acids in the protein. Based on the semblance in protein sequence, the cellular location is then shortlisted (Nakai and Horton 1999; Humphrey, Richman *et al.* 2006).

The spatial location of the KS enzyme was traced by working with an Arabidopsis KS gene that was identified through a genomic subtraction of a wildtype and a mutant that is unable to produce the KS enzyme (Sun and Kamiya 1994). The KS RNA transcript from this isolated gene was labeled

with ^{35}S-methionine/cysteine and then incubated inside the pea chloroplasts. With the addition of 0.1% Triton x-100, the chloroplast walls were disrupted with subsequent disappearance of the KS enzyme confirming that the KS enzyme was stored inside the chloroplast walls. A follow-up work by Richman *et al*. (1999) with KS and CPS enzymes of stevia confirmed the subcellular location in the mesophyll cells of stevia leaves. Location was traced via *in situ* hybridization with CPS and KS genes that were obtained using antisense RNA probes.

The subcellular location of KO (kaurene 19-oxidase), which transforms kaurene to kaurenoic acid, was traced through GFP (green fluorescent protein) fusions (Humphrey, Richman *et al.* 2006). A KO transcript was isolated from the expressed sequence tag (EST) of stevia leaves by gene homology. Functionality of the KO transcript was confirmed by incubating with kaurene in vitro to form kaurenoic acid. Cellular location was then traced by bombarding a KO-GFP fusion to onion epidermal cells and tobacco leaf cells. A fluorescent web-like pattern observed under a confocal microscope was indicative of an endoplasmic reticulum (ER) location. To confirm the ER location, KO was fused with a cyan fluorescent variant (CFP) and then co-bombarded with an ER-targeted sequence fused with a yellow fluorescent variant (YFP). The nearly identical fluorescent pattern of the CFP and YFP fusions supported the ER location of the KO enzyme.

The cellular location of KAH, which hydroxylates kaurenoic acid to steviol, remains controversial (Hanson and White 1968; Kim, Sawa *et al.* 1996). The transcript of the supposed isolated gene for KAH unexpectedly produced fructose biphosphate aldolase (FBPA) instead of steviol when incubated with kaurenoic acid (Kim, Sawa *et al.* 1996). Furthermore, the purified enzyme used to describe the KAH gene was not tested for enzyme activity (Kim, Sawa *et al.* 1996). This and the proposed chloroplast location of KAH by Kim et al (1996) contradicts the ER location of the previous enzyme (KO). Thus, further work on both the sequence identification and the subsequent tracing of subcellular location of KAH is needed.

UGTs, similar to the KO enzyme, have been traced at a cellular level using a GFP fusion technique (Humphrey, Richman *et al.* 2006). Three UGT transcripts (UGT85C2, UGT74G1 and UGT76G1) were isolated using a homology based screening of the Stevia EST. *In vitro* activity of UGTs in both soluble and microsomal fractions of the leaf protein extract was tested by incubating with the different SG substrates (steviol, steviol monoside, steviol bioside, rubusoside, stevioside and rebaudioside B). Only the soluble fraction of the leaf protein extract indicated some activity as indicated by the transformation of the SG substrates to a final SG product. To trace the location, an onion bulb epidermis was bombarded with particles carrying GFP fusions of the three UGTs, which resulted in tissue that expressed into a diffuse fluorescence pattern that was characteristic of a cytoplasmic location. Given the co-localization of the UGTs, possible molecular interaction was checked by looking at the fluorescence resonance energy transfer. Results suggested that no aggregation or molecular interaction occurs between the UGTs.

The SG product is believed to be stored in the chlorenchyma vacuole after production (Brandle and Telmer 2007). A close look at the morphology of leaf cell vacuoles of stevia revealed a change in structure as the leaf ages (Mu-zuan, Wei-lian *et al.* 1983). The vacuoles, which are small and translucent in “young” leaves, then transform into morphologically specialized vacuoles with granules and vesicle inclusions. These inclusions decompose and disappear in “older” leaves. The presence of granules in the vacuole was related to the stevioside content of the leaf although the chemical nature of the granules, presumably crystalline SG, remains to be determined (Mu-zuan, Wei-lian *et al.* 1983). The mechanism of SG transfer from cytosol to vacuole also remains to be determined (Brandle and Telmer 2007).

Brandle and Telmer (2007) graphically summarized the locations of enzymes involved in SG synthesis (Fig. 2.2). Cyclization of geranylgeranyl diphosphate (3) produced from pyruvate (1) and glyceraldehyde 3-phosphate (2) occurs in the chloroplast. KS then transforms GGDP (3) to kaurene (4) in preparation for its carboxylation (5) and hydroxylation (6) into the steviol

precursor. This series of transformation occurs in the endoplasmic reticulum. Steviol is then transformed into SGs by UGTs within the cytosol, after which SGs are transported into the vacuole for storage.

Distribution of SG in leaf and plant organs

SGs are not uniformly distributed throughout the plant. For example, Zaidan *et al.* (1980) reported that stevioside is found mostly in the leaves (6.88% dw) followed by the flowers (2.88% dw). Stevioside was not detected in the roots most likely due to the low sensitivity of the descendent chromatography method that was used. Rebaudioside A and C were also not measured in that experiment (Zaidan, Dietrich *et al.* 1980). Bondarev *et al.* (2003) reported that leaves contain the highest concentration of SGs (sum of stevioside, rebaudioside A and rebaudioside C) (3.4% dw), followed by the flowers (0.7% dw), stems (0.25% dw), seeds (0.2% dw) and roots (0.15% dw). However, these results are unusual in that the overall level of SG is low compared to the typically reported levels of 10% dw.

Bondarev and co-workers (2003) also reported that the proportion of the various SG varied by plant organ. The flowers and seeds mainly contained stevioside, the roots had approximately equal proportions of stevioside, rebaudioside A and C, and the stem had approximately equal amounts of stevioside and rebaudioside A. However, the relative ratios of the SGs may change with variety and growth conditions. These observations should be repeated with different stevia lines before a generalization is made.

The proportion of SG in leaves has also been reported to vary with leaf developmental stage and age by Bondarev et al (2003). On a dw basis, young developing leaves were found to contain more SGs than middle mature and senescent leaves. Top, middle and senescent leaves sampled from clone 0 possessed a stevioside concentration of 1.3%, 1.2% and 0.8% dw, respectively, while maintaining similar proportions of the three main SGs (stevioside, rebaudioside A and rebaudioside C). The observations of Kang and co-workers (1981) are also consistent with this trend, in that lower

canopy leaves (less than 20 cm from the ground) had lower stevioside content than leaves 20 cm above the ground. Rebaudioside A and rebaudioside C content was not monitored in the experiment.

The relative paucity of published reports on the distribution of SG within the plant is surprising, given its commercial relevance. Further replicated work on leaf SG at different canopy levels (top, middle and bottom) at different stages of plant ontogeny is warranted.

SG and photoperiod

In comparison to primary metabolites, secondary metabolites exhibit high plasticity under selection pressure (Hartmann 2007). However, the responsiveness of SG synthesis to environmental factors has not been fully reported except in the context of photoperiod, as discussed below.

Day length and stevia biomass

Vegetative growth of stevia is enhanced under conditions that delay or inhibit flowering, such as photoperiods longer than the critical photoperiod (around 13 h) of this short day (SD) plant. Consequently, total biomass yield per plant and per ha increase under long days (LD). Indeed, Metivier and Viana (1979) observed an increase in biomass per plant under LD conditions, with plants possessing 35 pairs of leaves after 145 days from planting while short day (SD) plants only had 26-30 leaf pairs. Furthermore, LD plants maintained a greater height increment of 0.88 $cm.d^{-1}$, in contrast to SD plants in which the initial height growth rate of 0.16 $cm.day^{-1}$ increased to 0.6 $cm.d^{-1}$ only after bolting. Specific leaf weight (dw basis) of LD plants was also higher than that of SD grown plants (LD 1.79 $mg.cm^{-2}$, SD 1.24 $mg.cm^{-2}$). In addition, leaves from SD plants have higher fw than leaves of LD plants given the same leaf dw (e.g. SD leaves with 160 mg fw and LD leaves with 40 mg fw will both have a dw of 20 mg). Leaf shape also varied, with LD plants having ovate leaves, while SD plants have smaller, round leaves (Metivier and Viana

1979). Yermakov and Kochetov (1996) also reported an increase in vegetative biomass with photoperiods up to 16 h given illumination of 50 and 100 W m^{-2}, and with photoperiods of up to 18 h with the lower illumination level. Presumably the increase in biomass yield was related to the extended vegetative growth because of delayed flowering although flowering behavior was not described in the English abstract (Yermakov and Kochetov 1996).

LD and leaf SG concentration

Long daylengths increase not only leaf mass per plant and per area, but leaf SG concentration. Stevioside concentration of dry leaf biomass was observed to be at maximum (90 mg.g dw^{-1}) between flower bud formation and flower opening, with a decrease noted after flowering (to 78 mg.g dw^{-1}) (Kang and Lee 1981). Bondarev and co-workers (2003) also observed a decline in stevioside concentration of the mature leaves of 'clone 28' as the buds began to flower (from 10 mg.g dw^{-1} stevioside to a negligible amount) although clone 0 displayed the opposite trend (increasing from 13 $mg.g^{-1}$ to 21 $mg.g^{-1}$ dw as the buds began to flower). As Bondarev and co-workers (2003) worked only on two clone types, studies on the variation of leaf SG at the onset of flowering across a number of clones and varieties are warranted.

LD and photoperiod sensitivity

Although a photoperiod of 14 h has been recommended to extend the vegetative state of stevia, the responsiveness to daylength varies as demonstrated by the flowering behaviours of the plant. Zaidan *et al.* (1980), observed three flowering behaviours amongst a line of stevia plants which originated from Paraguay in the early 1960s. One set of plants flowered under 8, 10 and 12, but not 14 h photoperiod while a second set flowered in all four photoperiods. Both sets had the highest percentage of plants flowering in the 12 h treatment. The variation in photoperiod sensitivity is presumably due to a genetic difference, considering that the plants were germinated from achene seeds of the plants originating from Paraguay.

However, genetic variability was not tested. A third set consisting only of one plant was an exception, flowering under 10 and 12 h but not 8 h exposure. The non-flowering behaviour of this plant at 8 h daylength was perhaps related to the physiological maturity of the plant during the exposure resulting in a non-induction of flowering.

Photoperiod sensitivity was also reported to change with plant maturity. At least four pairs of leaves per stem are needed for the plant to be sensitive to day length although only 20% of plants with four leaf pairs flowered under SD treatment (less than 13 h) (Valio and Rocha 1966). Stems with 6 pairs of leaves per stem displayed 100% flowering among plants treated with the same light conditions.

The number of SD cycles also affects the extent of flowering in stevia. A minimum of 2 SD cycles (each cycle lasting for 24h) are needed to induce flowering in 40% of the treated plants. The extent of flowering can be increased to 90% with 4 SD cycles while 6 SD cycles was reported to result in 100% flowering (Valio and Rocha 1966).

Observations on histochemical and morphological changes of transitional shoot apices under SD treatment are consistent with a requirement for 5 SD cycles. Increase in mitotic activity and RNA/protein concentration in transitional apices occurred mostly after the 5th SD cycle (8 h photoperiod) (Monteiro and Gifford Jr 1988; Monteiro and Gifford Jr 1988). The same phenomenon was observed in the apex of *Chenopodium rubrum* which required a minimum of 3 SD cycles for induction of flowering (Seidlova 1974). Low RNA levels in the apex of *C. rubrum* increased only after the 3rd SD cycle. Apices exposed to less than 3 SD cycles underwent incomplete reproductive differentiation and reverted back to vegetative growth (Seidlova 1974).

LD and light intensity

Surprisingly only one published study alluded to the effect of light intensity on SG level. Zaidan *et al.* (1980) reported that a higher leaf stevioside

concentration was achieved in plants grown under natural lighting than in plants grown with greenhouse lighting. In particular, SD plants (8h) under natural light had nearly four times more stevioside in the leaves than the greenhouse counterparts, with light intensity, leaf size and leaf SG concentration undocumented (Zaidan, Dietrich *et al.* 1980). The correlation between leaf SG concentration and light intensity requires further work considering that the plants used for the greenhouse and field experiment were derived from achene seeds which would be genetically variable. A comparison of SG concentration in relation to light intensity would be more useful if plants from tissue cultures or stem cuttings were used. A genetic comparison of photoperiod sensitive plants would also be commercially interesting to allow breeding of plants that can be grown vegetatively for a longer period of time.

SG ecophysiology

While high concentration of SG in stevia leaves has prompted a wide range of studies given the commercial value of SG as a natural, non-calorific sweetener, the question of the eco-physiological role played by SGs in the stevia plant has not been addressed. With SG content at about 10% of the leaf dw, the resource allocated to SG synthesis hints at a beneficial role to the ecological fitness of the plant. This thesis will consider roles that SGs may play in stevia ecology, including its role as an energy reserve, osmoregulator, insect deterrent and herbivore attractant. A premise to this study is that the available stevia phenotypes are not greatly different to wild type stevia in terms of SG content (around 10% dw), and that the wild type populations have not faced selective pressure on SG content from humans.

SG as energy reserve

Higher plants are characterised by the storage of photosynthate as starch, as opposed to glycogen in the Rhodopyhyta and laminarin in the Phaeophyta. However, some higher plants also accumulate storage reserves of oils, lipids

or proteins, although this storage is more typical of seeds than leaves. CAM plants store organic acids in leaf tissue, as part of the temporal separation of light reactions and Calvin cycle reactions (Upmeyer and Koller 1973; Kalt-Torres, Kerr *et al.* 1987; Du, Nose *et al.* 2000). Other plants accumulate high concentrations of other secondary metabolites for roles other than a carbon or energy reserve. Galactomannans in seeds such as *Senna tora/obtusifolia* occur at 15% by dw and aids in hydrating the seed during germination (Cunningham 2000).

SGs, at around 10% of leaf dw, represent a significant energy investment by the plant. If the turnover rate is sufficiently high, SG could function as an energy reserve to the plant. This suggestion is parallel to that of Seigler and Price (1976) for secondary metabolites in plants such as peppermint (monoterpenes), *Marrubium vulgare* (sesquiterpenes), tomato (tomatin) and tobacco (nicotine). The level of these compounds was observed to vary with photosynthetic rate.

The level of certain secondary compounds has been observed to decline in seeds during germination. For example, cyanolipid which occur at 15% dw of the seeds of Mexican buckeye, *Ungnadia speciosa*, is depleted as a nitrogen source after 3 days of seed germination (cited in Seigler and Price 1976). (e.g. cyanolipids in Mexican buckeye, *Ungnadia speciosa*, Seigler, unpublished; and alkaloids in legume seeds (cited in Seigler and Price 1976)). A decline in secondary compounds has also been observed in leaves of seedlings during pollination. In particular, dhurrin in leaves of *Sorghum* species, was found to plateau and decline after pollination and seed production suggesting its role as a nitrogen source during seed production (cited in Seigler and Price 1976).

The levels of SGs in *S. rebaudiana* leaves has been reported to increase before flowering, followed by a decline after flowering, (Kang and Lee 1981; Bondarev, Sukhanova *et al.* 2003). However, studies on SG as an energy or carbon reserve have not yet been reported in the literature.

SG as an osmoregulator

Secondary metabolites are also known to accumulate under conditions of water stress, acting in tissue osmoregulation. No single osmolyte has been identified to accumulate across all plant species. Instead a variety of compounds, including proline, glycine, betaine, polyols and non-reducing sugars, act as osmoregulators, allowing plants to adjust to water stress (Hare, Cress *et al.* 1998). For example, growth improvements under water and salt stress were observed in transgenic tobacco (*Nicotiana tabacum*) that is able to accumulate twice as much proline as unaltered tobacco (cited in Hare, Cress *et al.* 1998). Similarly, transgenic *Arabidopsis thaliana* with low proline leaves were reported to be hypersensitive to osmotic stress (Nanjo, Kobayashi *et al.* 1999). A role for SGs as a possible osmoregulator in *S. rebaudiana* remain to be investigated.

SG as a determinant of vertebrate feeding preference

Throughout evolution, plants have adapted a diverse range of mechanisms for seed dispersal which include chemical attraction for herbivory, clinging structures for sticking to furs or feathers, 'wings' for aerial transport and resistance to sinking for miles of travel on water (Howe and Smallwood 1982). The achene seeds of stevia have a crown of hair which aid in the aerial transport of the seeds while also acting as a clinging structure on herbivores that feed on stevia leaves. Possibly, the sweet taste of SG found in stevia leaves serve as an attractant for herbivores which would help distribute seeds, attached on the herbivore's fur, within a wider area for seed germination.

Overall, a sweet taste is preferred among herbivores while a bitter taste is usually rejected beyond a tolerance level (Jacobs, Beauchamp *et al.* 1978). Harada and co-workers (1993) observed an attraction to stevioside for some aquatic animals namely, *Haliotis discus* (black abalone), *Misgurnus anguilllicaudatus* (oriental weatherfish) and *Seriola quinqueradiata* (juvenile yellowtail). Crumpled gauze dipped in different concentrations of pure stevioside solution was placed in the test compartment and the number of

animals that remained (for black abalone and oriental weatherfish) or entered the compartment (for yellowtail) were noted. The attraction was most likely due to the sweet taste of stevioside as sugar feeds had the same effect.

Limited studies were encountered involving both stevia and mammalian herbivory feeding, with pigs preferring sweetened feed over the control (unsweetened feed) (Munro, Lirette et al. 2000). However, Soejarto's (2002) observation on cattle grazing of stevia indicates possible mammalian feeding possibly encouraged by the sweet taste of SGs on stevia leaves. Increased vertebrate hebivory may provide an ecological advantage in terms of increased dispersal of stevia seed, or of increased grazing pressure on stevia plant competitors.

SG as a determinant of invertebrate feeding preference

SGs may act to deter insect-herbivory. Metivier and Viana (1979) reported that *Epicauta adomaria* (Coleoptera) exhibited dislike for stevia leaves when presented alongside leaves of *Capsicum*, *Amaranthus*, *Emilia* and *Lycopersicum*. The beetles distinguished and rejected *S. rebaudiana* leaf discs from a mixture of leaf discs and also rejected *S. rebaudiana* when presented in isolation. However, this study did not demonstrate whether the SG concentration of the stevia leaves influenced insect feeding behaviour. Other leaf attributes, such as leaf water and fibre content, may have influenced feeding behaviour.

In contrast to observations by Metivier and Viana (1979), members of the Coleoptera, along with a few other insects from the Lepidoptera and Orthoptera orders, were found feeding on stevia leaves in a field experiment (Fuente 2001). However, these observations are insufficient to test the hypothesis that SG play a role in insect deterrence. Possibly the insects were feeding on leaves of low SG content, or possibly the level of insect pressure was high, leading to a high level of herbivory.

In a more tightly controlled experiment, Nanayakkara and co-workers (1987) explored the feeding deterrent properties of SGs and their chemical

derivatives to aphids (*Schizaphis graminum*) belonging to the Homoptera order. Aphids were introduced to an aqueous aphid diet containing the SGs and feeding behaviour was determined by comparing the number of aphids feeding on separate test and control diets. When the number of aphids feeding on the test compound at a known concentration was less than 50% of the control group, that level of compound was defined as having deterrence properties. Feeding deterrence was observed for steviol (150 ppm) and three SG derivatives, dihydrosteviol A (140 ppm), 15-oxosteviol (130 ppm) and isosteviol (115 ppm). Stevioside and rebaudioside A had minimal feeding deterrent activity at concentrations as high as 650 ppm, although this concentration is much lower than the usual leaf content. For example, a typical amount of 10% dw SG would be equivalent to 2% fw SG assuming a leaf dw to fw ratio of 0.2. Assuming that the fw is 80% water, 2% fw SG is then equivalent to 2 g/100 g FW or 2 g/80 mL water, or 25,000 ppm.

Among the SG derivatives identified by Nanayakkara *et al.* (1987) to have insect deterrent properties, only steviol occurs naturally in *S. rebaudiana* leaves (at about 1% dw in the bulk tissue, or 0.1% FW, equivalent to 1,000 ppm) (Dacome, Da Silva *et al.* 2005). Steviol has been demonstrated to be mutagenic with *Salmonella typhimurium* TM677 (Geuns 2004), although similar work with insects has not been undertaken.

It is possible that the SG pathway evolved within stevia to provide insect deterrence, in the form of steviol. While steviol is found in lesser amounts than stevioside and rebaudioside A in the leaf, it is possible that human preference for the sweet taste of SG compounds has resulted in selective propagation of lines that have more SG and less steviol. Alternatively, accumulation of SG may be a metabolic penalty required to maintain steviol levels at a certain level.

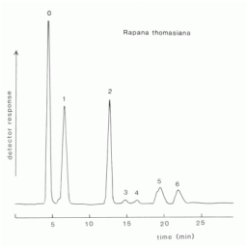
However, the deterrent effects of secondary compounds may be specific to a group of insects. In an unpublished study of Klocke (cited in Nanayakkara, Klock *et al.* 1987), larvae of *Heliothis virescens* Fabr. and *Aedes aegypti* L. (yellow fever mosquito) were not deterred from an *in vitro* diet containing

steviol (500 ppm and 40 ppm respectively). The question of a role in insect deterrence for steviol and SGs remains open.

Conclusion

The eco-physiological role of SGs in the stevia plant is unknown. In the following chapters the results of experiments addressing this issue are presented, testing the hypotheses that stevioside and rebaudioside A in the leaves of *Stevia rebaudiana* play a role in either C-storage, osmoregulation or herbivory alteration, Given the dependence of such work on techniques for measurement of SG, attention is also given to methods development, focusing on the development of simpler methods.

Chapter 3



Methods development:

Steviol glyocoside extraction and quantification using High Performance Liquid Chromatography (HPLC)

Abstract

Water was found to be an effective 'green' alternative as an extraction solvent for the estimation of steviol glycoside (SG) content of stevia leaf, with an extraction effectiveness similar to 70% ethanol. A solid phase extraction (SPE) protocol for use with an ethanol extract was nonetheless revisited and improved, achieving a recovery rate of 83 and 100% for stevioside and rebaudioside A, respectively. However, for water extracts, adequate resolution of both stevioside and rebaudioside A peaks was achieved without the SPE clean-up step. Therefore this step was removed, although at the cost of column life. HPLC injection volume was optimised at 5 μL, and acetonitrile concentration in the mobile phase optimised at 80%. Using a UV detector, a linear response was achieved for the range 0 – 1 $g.L^{-1}$ stevioside and rebaudioside A. Repeated injection allowed calculation of a minimum practical quantitative limit (PQL) of 0.061% stevioside and 0.14% rebaudioside A (leaf dw), based on a double extraction of 0.1g of dry ground leaf with 5 mL of water.

Introduction

Methods for quantification and characterisation of SGs in stevia leaves include gas and liquid chromatography, thin-layer chromatography, IR and NIR spectrophotometry, capillary electrophoresis and electrophoretic techniques (1986; Kedik, Fedorov *et al.* 2003; Dacome, Da Silva *et al.* 2005). Novel methods such as droplet counter-current chromatography have also been considered for isolation of SGs from plant extracts (Kinghorn, Nanayakkara *et al.* 1982). Of these methods, that based on high performance liquid chromatography (HPLC) is the most widely adopted, with variations in extraction, separation and detection of compounds. The popularity of HPLC seems to depend on the method's capability of separating compounds into peaks through automated sample injection and control of the mobile phase composition.

The HPLC method involves injection of a sample through a HPLC column via a liquid mobile phase. As the sample elutes through the stationary phase (column) with the mobile phase, compounds in the sample are partitioned between the stationary column and the mobile phase, with compounds that interact more strongly with the stationary phase displaying a longer retention time. Separation conditions (selection of mobile and stationary phases) must be optimised to achieve a clear separation of compounds reaching the detector (typically an absorbance, refractive index or electrochemical detector). Furthermore the composition of the mobile phase may be kept constant (isocratic scheme) or variable (gradient scheme) depending on the desired separation of the sample between the stationary and mobile phase. To optimise peak resolution, methods for sample extraction, compound separation and compound detection must be optimised.

Stevia leaf analysis typically begins with leaf extraction using dry ground leaves and heated alcohol (Kolb, Herrera *et al.* 2001) or water (Kitada, Sasaki *et al.* 1989; Bovanova, Brandsteterova *et al.* 1998; Kedik, Fedorov *et al.* 2003; Pol, Ostra *et al.* 2007). Some authors advocate purification of

extracts using solvent or solid phase separation (Kitada, Sasaki *et al.* 1989; Bovanova, Brandsteterova *et al.* 1998; Vanek, Nepovim *et al.* 2001). A solid phase extraction (SPE) method using Sep-Pak C18 cartridges was developed for quantification of stevioside, rebaudioside A, rebaudioside C and dulcoside in food items such as beverages, soy sauce, candy, pickled radish and tea to remove ingredients that co-elute with the SG peaks (Kitada, Sasaki *et al.* 1989; Bovanova, Brandsteterova *et al.* 1998; Vanek, Nepovim *et al.* 2001). An SPE Strata NH_2 column was used with leaf water extracts (Kedik, Fedorov *et al.* 2003), and Hearn (2006) developed a clean-up protocol which utilized both an ion-exchange (XC) cartridge and NH_2 cartridge for leaf ethanol extracts.

Regarding the stationary phase, c/hromatographic separation can be achieved using either normal or reverse phase columns. With the normal phase technique, polar compounds are retained by polar functional groups in the column such as amines (Kitada, Sasaki *et al.* 1989; Bovanova, Brandsteterova *et al.* 1998; Kolb, Herrera *et al.* 2001; Kedik, Fedorov *et al.* 2003; Dacome, Da Silva *et al.* 2005). In contrast, the reverse phase technique employs a stationary phase column that retains less polar compounds; an example being the C_{18} column (Hutapea, Toskulkao *et al.* 1999; Vanek, Nepovim *et al.* 2001). Good resolution of SG peaks has been achieved for both types of stationary phase with the only difference being the order of elution, with the most polar SG (rebaudioside A) eluting last in the amino column, but first with the C_{18} column. In addition, a few specialized columns with custom-made functional groups such as protein columns (Protein I-125) have been used in reverse phase mode to separate SG peaks (Ahmed and Dobberstein 1982 (a); Ahmed and Dobberstein 1982 (b)).

Regarding the composition of the mobile phase, one approach is to keep the mobile phase composition constant throughout the sample elution (isocratic elution). With isocratic elution, different solvents have been used with different types of columns. Ahmed *et al.* (1982 (a)) used pure 1—propanol as mobile phase to separate the peaks of stevioside, rebaudioside C and rebaudioside A, sequentially, from a chloroform-based leaf extract (flow rate

1 mL.min^{-1} with two Protein I-125 as stationary columns). Kitada et al (1989) used 80:20 acetonitrile:water with an NH_2 column (flow rate of 0.8 mL.min^{-1}) to elute dulcoside A, stevioside, rebaudioside C and rebaudioside A consecutively from extracts of beverage, soy sauce, candy and pickled radish. Bovanova et al (1998) used 68:32 methanol:water (flow rate 0.9 mL.min^{-1}) with a C_{18} column to quantify the stevioside peak from stevia water extracts and stevioside-containing tea/juice using the C_{18} column. Alternatively, the mobile phase composition can be gradually altered during the separation event through gradient elution which facilitates the separation of adjacent or overlapping peaks. With gradient elution, acetonitrile at different concentrations have been used. Kedik et al (2003) used a five-step change in acetonitrile composition (from 87 to 82.5% for 12 min, to 79% for 8 min, to 5% for 5 min, then a ramp up to 87% over 1 minute, and finally maintain at 87% for the last 3 min) to quantify the stevioside peak of leaf water extracts (flow rate of 1.5 mL.min^{-1}, NH_2 column). Hutapea et al (1999) changed the acetonitrile composition from 30% (for the first 20 min) to 65% (for the next 20.1 min) then back to 30% (for the last 9.9 min) to separate steviol and the steviol components in the urine and faeces of force-fed hamsters (flow rate of 1 mL.min^{-1}, C_{18} column). Vanek et al (2001) increased the acetonitrile composition from 15 to 50% within a 30 min sample runtime (flow rate of 1 mL.min^{-1}, C_{18} column) to separate and quantify the stevioside peak from water extracts of stevia leaves and stevia-based teabags.

Detection of SGs eluting from the chromatography column is typically based on UV absorbance. Maximum absorbance of the SGs occurs at around 200 nm (Kedik, Fedorov et al. 2003; Hearn and Subedi 2006) although absorbance at 210 nm is usually used, given that maximum light transmittance through fused quartz cells occurs around this wavelength (Owen 2000). Derivatization of SGs to form a strong chromophore has also been explored to enhance absorbance at 258 nm. For example, Ahmed et al (1980) attempted hydrolysis of stevioside and rebaudioside A, followed by esterification of the C-19 with p-bromophenacyl bromide to produce chromophoric phenacyl esters that absorb at 258 nm. While this method

enhanced the resolution of stevioside and rebaudioside A in the chromatogram, rebaudioside D and E were converted to the stevioside and rebaudioside esters, making the distinction between stevioside/rebaudioside D and rebaudioside A/E derivatives impossible. Detection may also involve electrical or physical characteristics achieved after SG derivatization. Use of pulsed amperometric detection (PAD) was made possible by ionizing the SGs after column separation with the addition of an alkaline sodium hydroxide (Ahmed and Smith 2002). Detection of SGs through light scattering of solutes post-vaporization have also been explored with an evaporative light scattering detector (ELSD) (Mourey and Oppenheimer 1984; Tateo, Escobar Sanchez *et al.* 1999). These latter techniques offer the advantage of lower detection limits relative to the standard UV absorbance method.

SG quantification by HPLC analysis has involved either normal or reverse phase method, depending on preference of column and mobile phase chemistry. Overall, published methods mostly employ the use of either C_{18} or NH_2 columns with occasional use of specialized columns (e.g. Protein I-125 column) (Ahmed and Dobberstein 1982 (a)). Use of either isocratic or gradient elution is capable of achieving a good peak resolution. UV-Vis detection remains the most popular form of SG detection, although other modes of detection (e.g. PAD and ELSD) may gain wider adoption in the future (Mourey and Oppenheimer 1984; Tateo, Escobar Sanchez *et al.* 1999).

The extraction method and HPLC conditions decribed by Hearn and Subedi (2009) employs ethanol extraction, sample clean-up with SPE cartridge and chromatography using an amine column and a UV-Vis detector, similar to a range of other published studies (see above). For this thesis work, this method was adopted after optimisation. Extraction yield was compared between ethanol (70%) and water. The SPE protocol for an ethanol extract was also revisited and appropriate adjustments were made to improve recovery rate. The HPLC protocol was revisited to achieve simplification

while maintaining a limit of detection (LOD) and a limit of quantitation (LOQ) suitable for leaf SG analysis.

Materials and Methods

Leaf extraction

Hearn and Subedi (2009) extracted ground dry leaves (1.0 g) with 10 mL of 70% ethanol in a single step. This single pass extraction methodology was compared to sequential extraction using two extractions of 5 mL. A bulk lot of mixed fresh leaves, and a bulk lot of dried leaves were prepared. Leaves were dried at 65 °C for 24 h in a ventilated oven. Samples were drawn from these lots using a sampling procedure designed to obtain representative samples. Fresh leaf samples were ground using a mortar and pestle prior to extraction. Extraction of ground fresh or dry leaf material (0.1 g of each) was undertaken using either 70% v/v ethanol or water (10 mL). Samples were placed in a 70 °C water bath with agitation for 30 min during extraction. Extractions were replicated three times.

The supernatant was separated by centrifugation (at 3500 rpm) and from the combined pool of supernatant, a 2 mL aliquot was filtered through a 0.45 µm nylon filter before transferring to a vial for HPLC analysis. Aliquots of the extracts were analysed using HPLC at days 0, 3 and 8 post extraction.

Recovery of stevioside in a leaf water extract was assessed. A leaf extract of known stevioside content was spiked with 0.1 mL, 0.2 mL and 0.5 mL of 1 $g.L^{-1}$ stevioside after the leaf extract has gone through the cleaning procedure. The purpose of the spiking was to determine whether the matrix of the water extract, post-clean up, contains enzymes that will significantly break down some of the added SG. Recovery was quantified by comparing the theoretical and actual stevioside content of the spiked samples.

HPLC

The HPLC conditions were based on a previous work by Hearn and Subedi (2009). Briefly, a Zorbax NH_2 column (250 x 4.6 mm, 5μm) was used with an Agilent Zorbax High Pressure Reliance Cartridge guard column (12.5 x 4.6mm, 5μm). The mobile phase used was an 80/20 acetonitrile/water buffer solution (pH 5, 100 mL of 0.02M glacial acetic acid with 200 μL of 0.1 M NaOH) with flow rate of 1 $mL.min^{-1}$. Sample injection volumes of either 5 or 10 μl were employed, and HPLC signals were monitored at 210 nm in reference to 360 nm, with slit width of 4 nm. A typical chromatogram is presented (Fig. 3.1).

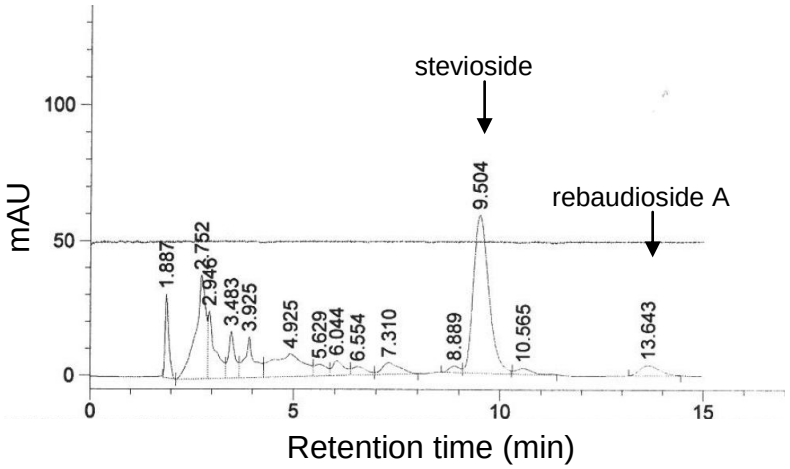


Figure 3. 1. Typical chromatogram of leaf water extract of dry ground stevia leaves with stevioside and rebaudioside A retention time of 9.504 and 13.643 min respectively. Samples were eluted through an amino column using an 80:20 acetonitrile:water mobile phase (pH 5).

Different acetonitrile concentrations (40, 60, 70, 80 and 85%) at pH 5 were tested for use as a mobile phase. The retention time of 2.5 mM rebaudioside A was assessed with each mobile phase.

Standard (aqueous) solutions of stevioside and rebaudioside A (0.125, 0.25, 0.5, 1 $g.L^{-1}$) were used in calibration of the method. Repeatability of injection was tested through seven consecutive sample injections of 0.2 $g.L^{-1}$ of stevioside and rebaudioside A. Limit of detection (LOD) was set at two times the standard deviation while limit of quantitation (LOQ) was set at seven times the standard deviation of the seven consecutive injections.

Solid Phase Extraction (SPE) protocol

A clean-up protocol for the leaf ethanol extract (Hearn and Subedi 2006) which uses two SPE catridges, Strata XC (Phenomenex) and Strata NH_2 (Phenomenex), was re-evaluated and readjusted to maximize SG recovery rate. The original method involved filtering a 1 mL aliquot of the leaf extract using a 0.45 μm nylon membrane and a non-sterile syringe to physically remove particulates that may clog the amine column during HPLC runs. The filtrate was then passed through a non-conditioned XC cartridge with approximately 0.3 mL bed volume and a non-conditioned NH_2 cartridge with a similar bed volume of 0.3 mL (Fig. 3.2). The NH_2 cartridge was then washed with 1 mL of acetonitrile (Fig. 3.2). The cartridge was subsequently dried to remove remaining solvents and the retained SGs were then eluted with 1 mL water (Fig. 3.2).

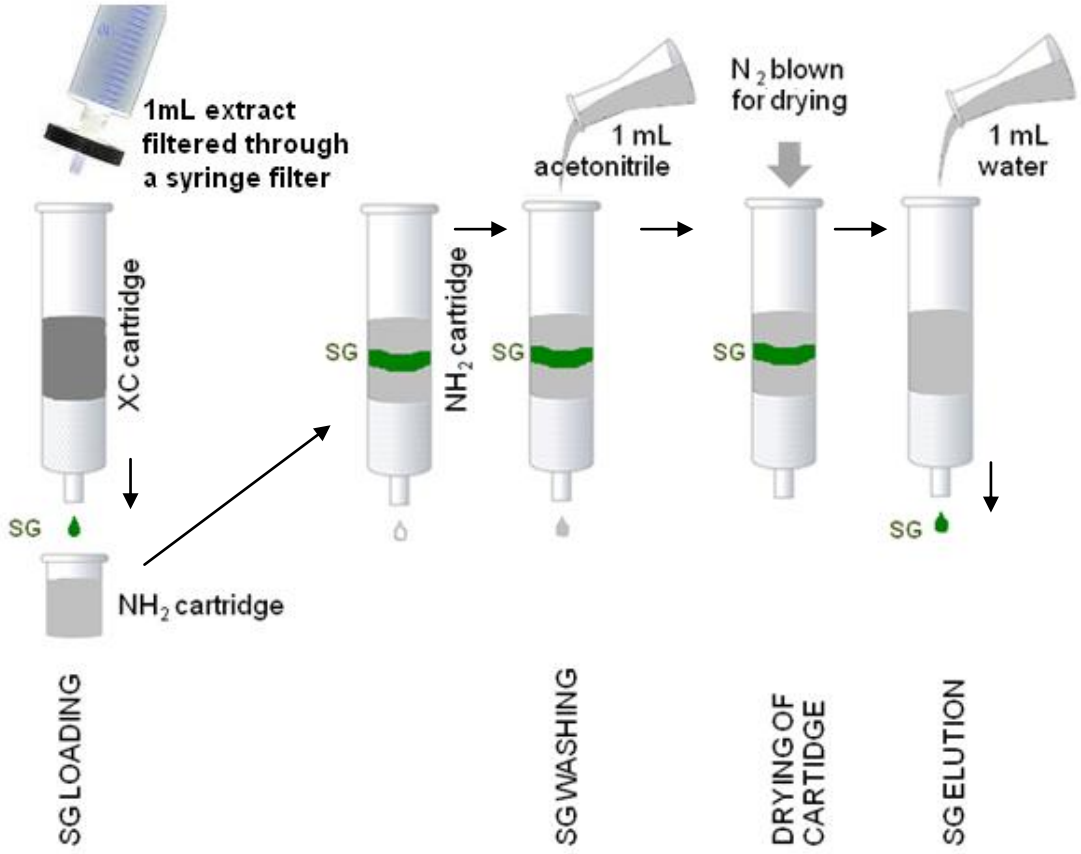


Figure 3. 2. Original clean-up protocol for ethanol (70%) leaf extract using a cation SPE cartridge (Strata XC) and an amine SPE cartridge (Strata NH2). Method was based on the protocol recommended by Hearn (2006).

Recovery of stevioside and rebaudioside A after filtration was quantified by comparing the HPLC peak areas of unfiltered and filtered standards as follows:

$$\text{Recovery} = \frac{\text{HPLC peak area (filtered standard)}}{\text{HPLC peak area (unfiltered standard)}} \times 100 \qquad \text{(eq. 3. 1)}$$

Initial work on quantifying recovery rate with the XC and NH_2 cartridges was done with a 1 $g.L^{-1}$ stevioside because of the limited amount of standards available. Confirmation of recovery with other stevioside (0.125, 0.25, 0.5, 1 $g.L^{-1}$) and rebaudioside A standards (0.125, 0.25, 0.5, 1 $g.L^{-1}$) were performed once recovery of the 1 $g.L^{-1}$ stevioside standard was optimised.

Results

Leaf Extraction

Stevioside extraction from both fresh and dry leaf was over 50% higher when water was used in a single 10 mL extraction, in comparison to 70% ethanol (Fig. 3.3). A two stage extraction protocol (two 5 mL solvent washes) of the same bulk leaf material also achieved higher extraction yields when water was used as the extraction solvent, compared to 70% ethanol (estimated leaf stevioside for water extract of 9.02% w/dw ± 0.12, n = 3, compared to 5.69% ± 0.08, n = 3 for ethanol-based extraction). Based on these results, water extraction is preferred over 70% ethanol extraction.

Stevioside extraction was similar for dry and fresh leaf samples, for samples analysed within hours of extraction (Fig. 3.3). However, a 15% decline in stevioside levels of fresh leaf water extract was noted over 8 days of storage, while that of dry leaf extracts were stable over this period. Presumably the 70 °C extraction temperature did not inactivate enzymes involved in the catabolism of the SGs, while the process of oven drying did inactivate these enzymes. SG in the ethanol extract of both dry and fresh ground leaves were similar (Fig. 3.3). Presumably, the drying process (for the dry ground leaves) and the use of ethanol (for the fresh ground leaves) inactivated enzymes to a similar extent resulting in comparable SG content after 8 days of storage of the extract.

However, rebaudioside A extraction was higher in ethanol (1.87% w/dw ± 0.36, n = 3) than in the water extract (level not detectable) in a single 10 mL extraction (Fig. 3.3) and also in a double extraction with 5 mL of ethanol (6.21% ± 0.09, n = 3) than water (5.69% ± 0.08, n = 3).

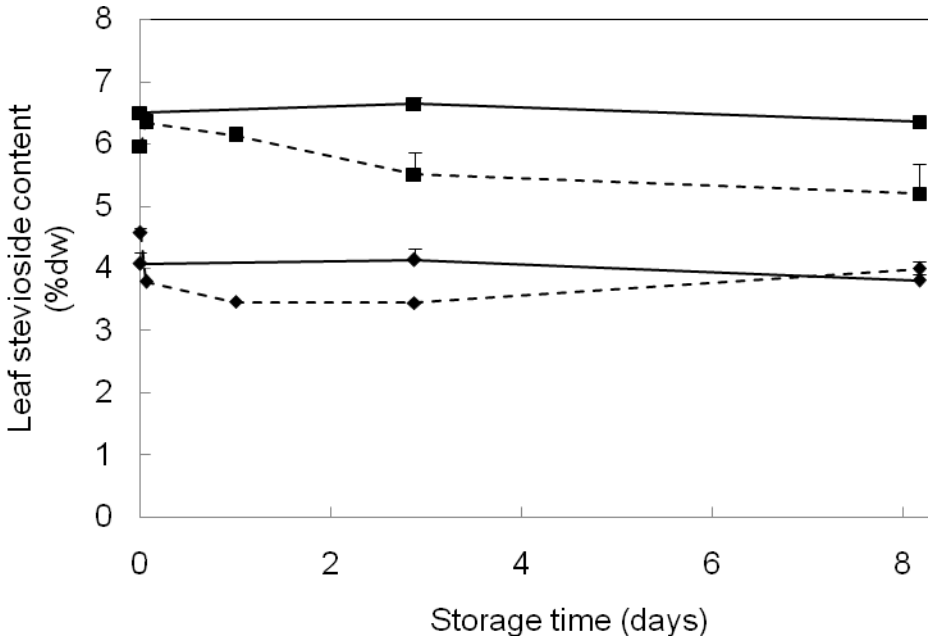


Figure 3. 3. Estimated leaf stevioside content (% dw basis) for extraction using two types of leaf formats: dry (—) and fresh (- - -) ground leaves and two types of extraction solvent: water (■) and 70% ethanol (◆) in a single 10 mL extraction protocol. Data points represent a mean (n = 3) and associated standard error of mean.

Peak shape of the sample chromatogram varied with the extraction solvent used. Standards of stevioside and rebaudioside A (1 $g.L^{-1}$) prepared in water were slightly broader than those prepared in 70% ethanol (Fig. 3.4).

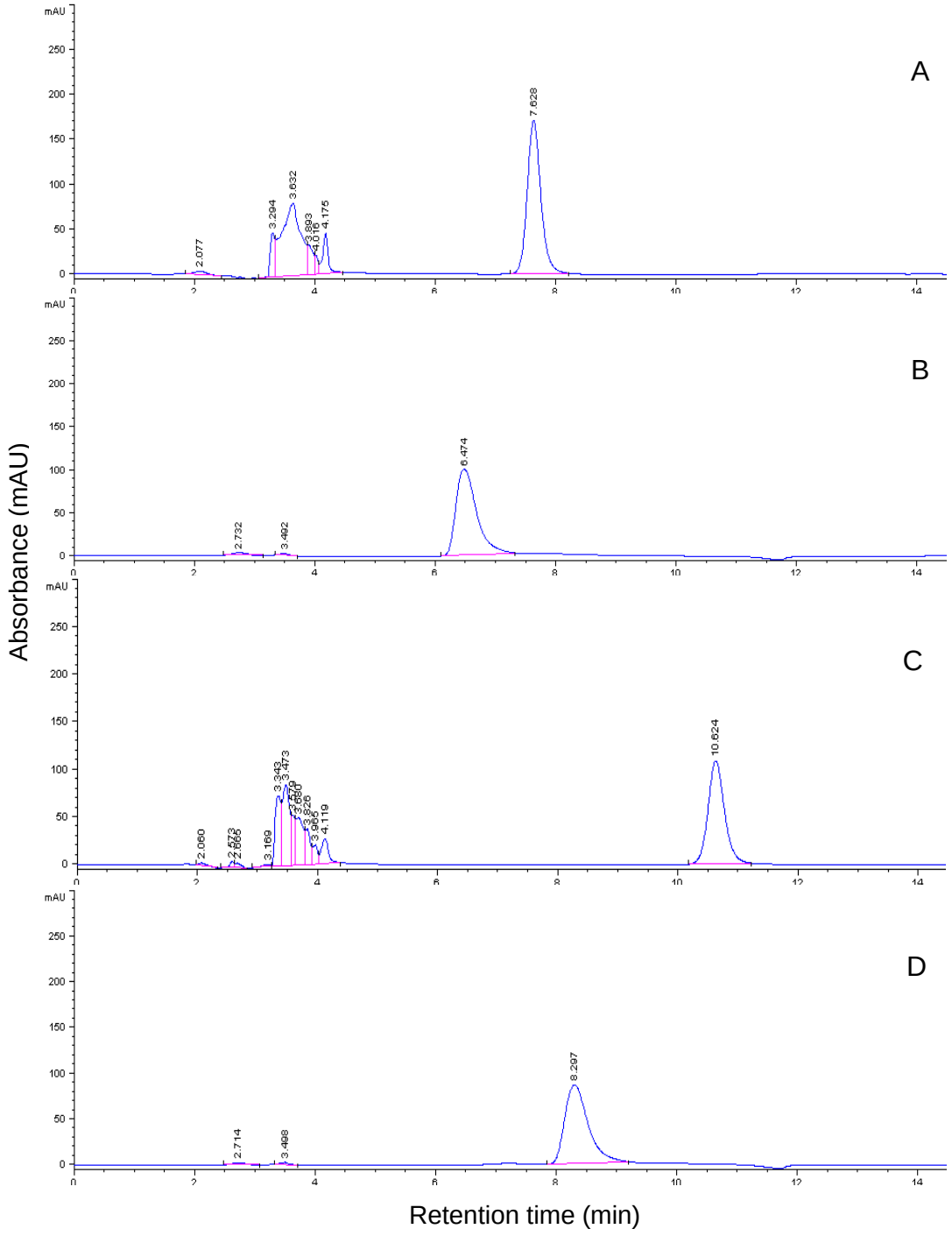


Figure 3. 4. HPLC chromatogram of 1 g.L^{-1} stevioside in (A) 70% ethanol (peak area = 2695) and (B) water (peak area = 2403) and 1 g.L^{-1} rebaudioside A in (C) 70% ethanol (peak area = 2089) and (D) water (peak area = 2339). Injections were done on separate days and with a different mix of mobile phase.

The recovery of stevioside added to a leaf water extract was quantified. A leaf extract with 0.4983 $g.L^{-1}$ stevioside (pre-determined by HPLC analysis) was spiked with addition of 0.1, 0.2 and 0.5 mL of 1 $g.L^{-1}$ stevioside (representing a 10, 20 and 40% increase on sample stevioside level, respectively), with a 100, 91 and 89% recovery rate, respectively, achieved.

HPLC Calibration

Given the broadening of the peaks observed when water was used as a solvent, a smaller injection volume (5 μL) was tested. Injection volume did not impact peak area, which was related to the amount of analyte, as expected (Fig. 3.5).

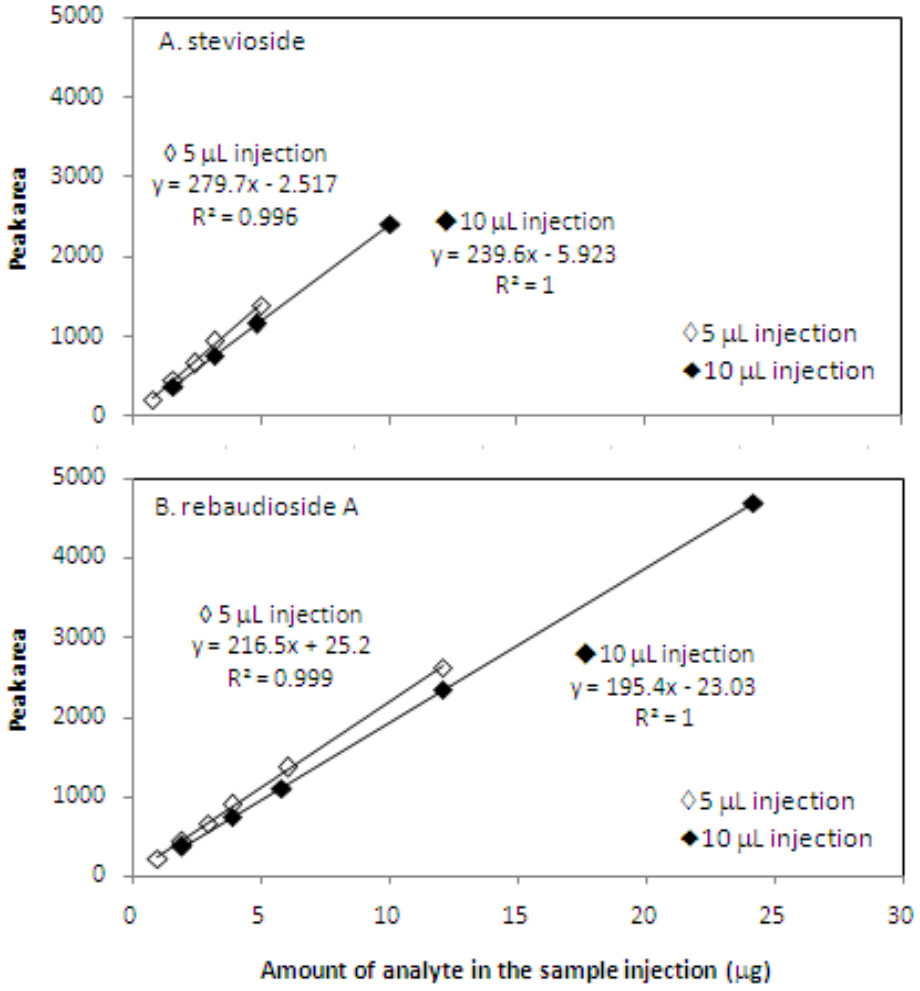


Figure 3. 5. Peak area plotted against amount of (A) stevioside and (B) rebaudioside A delivered in two injection volumes: 5 μL (◊) and 10 μL (◆). Samples were water based.

However the correlation coefficient of the regression of peak area to the amount of analyte decreased with extended concentration ranges (Fig. 3.6 and Table 3.1). The correlation coefficient was optimal up to a maximum concentration of 10 $g.L^{-1}$ stevioside (5 μL injection volume) (R^2 = 0.993). Extending the calibration range to 20 $g.L^{-1}$ of stevioside was associated with a decrease in correlation coefficient (R^2 = 0.952).

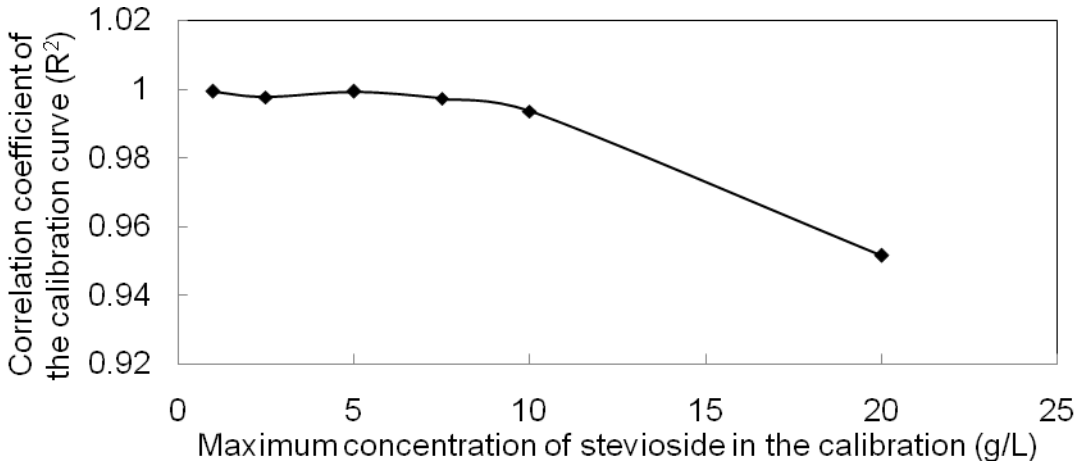


Figure 3. 6. Correlation coefficient of linear regression of peak area to stevioside concentration, in relation to maximum concentration of stevioside in the calibration set. Sample injection volume of 5 μL.

Table 3. 1. Slope and correlation coefficient of linear regression of peak area to stevioside concentration, in relation to the range of stevioside concentrations used in the calibration set (data of Fig. 3.6).

Stevioside Concentration Range ($g.L^{-1}$)	**Slope**	**R^2**
0-20	1680	0.9516
0-10	2247	0.9936
0-7.5	2406	0.9973
0-5	2561	0.9994
0-2.5	2615	0.9977
0-1	2913	0.9994

The repeatability of the HPLC measurement was quantified by running seven consecutive HPLC measurements (5 μL injections) of the 0.2 $g.L^{-1}$ of rebaudioside A and stevioside standards. The standard deviation for the rebaudioside A measurement was 0.0020 $g.L^{-1}$, with coefficient of variation of 1.19%, while that of the stevioside measurement was 0.0009 $g.L^{-1}$, with a coefficient of variation of 0.55%. The limit of detection (LOD) for rebaudioside A, set at twice the standard deviation, was 0.0040 $g.L^{-1}$, while the limit of quantitation (LOQ), set at seven times the standard deviation, was 0.014 $g.L^{-1}$. The LOD for stevioside was 0.0017 $g.L^{-1}$ and the PQL was 0.0061 $g.L^{-1}$.

Based on the extraction ratio of 0.1 g dry ground stevia leaf in 10 mL solvent, the detection limits of stevioside (0.0061 $g.L^{-1}$) and rebaudioside A (0.014 $g.L^{-1}$) are equivalent to 0.061% (w/w, dw basis) stevioside (leaf dw) and 0.14% (w/w) rebaudioside A (leaf dw). These limits are well below the typical leaf concentration of 7-10% stevioside and 3-5% rebaudioside A (leaf dw), making the method fit for measuring SG content in leaf extracts.

Mobile phase and retention time

Overall, the retention time increased with an increase in the acetonitrile content of the mobile phase above 70% (Fig. 3.7).

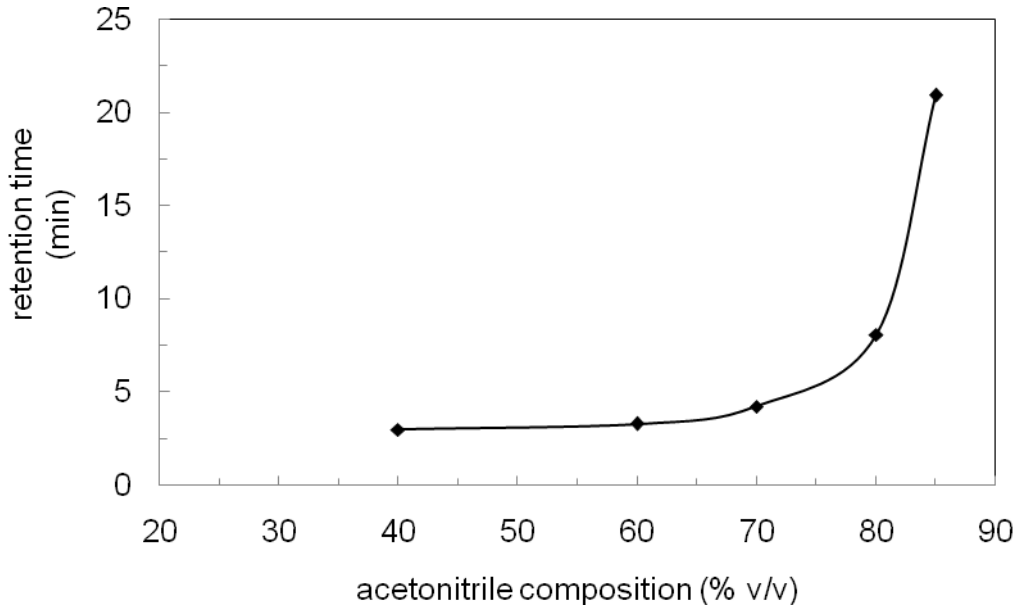


Figure 3. 7. Change in retention time of rebaudioside A with mobile phase composition.

SPE Protocol

XC Cartridge Clean-Up

The HPLC peak height and areas for stevioside were linearly correlated up to a concentration of 10 $g.L^{-1}$ stevioside for both unfiltered and XC cartridge filtered standards (Fig. 3.8). Recovery rate of standard through the XC cartridge was around 100% (Table 3.2).

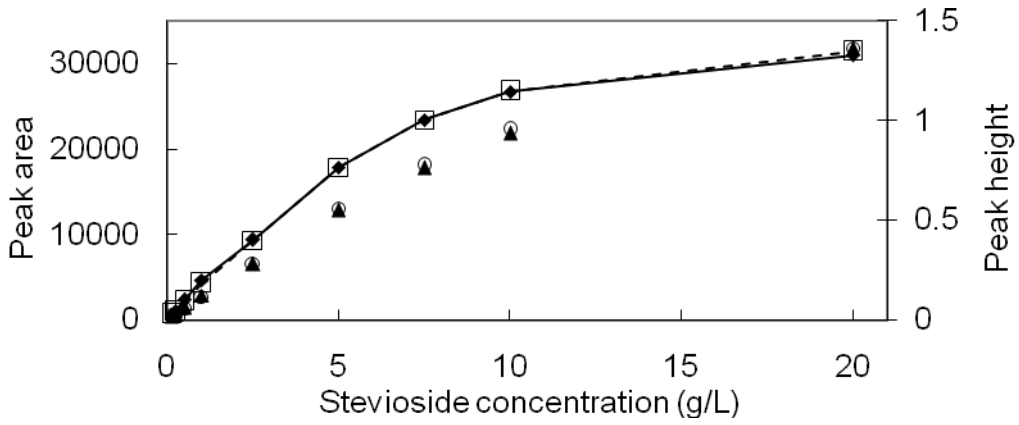


Figure 3. 8. HPLC peak area (▲,○) and peak height (—◆— , - -□- -) of stevioside standards (10 μL injection of 10, 7.5, 5, 2.5, 1, 0.5, 0.25 and 0.125 $g.L^{-1}$ in 70% EtOH) that were unfiltered (shaded symbol) and filtered (open symbol), respectively with an XC cartridge.

Table 3. 2. Comparison of HPLC peak areas of filtered and unfiltered stevioside standards. XC cartridge was used to filter the standards

Stevioside concentration ($g.L^{-1}$) ($g.L^{-1}$)	Peak Area (unfiltered)	Peak Area (filtered with XC) cartridge)	% Recovery
10	21842.1	22244.8	101.8
7.5	17797.4	18075.7	101.6
5	12864.4	13046.9	101.4
2.5	6536.0	6609.5	101.1
1	2906.1	2759.2	94.9
0.5	1464.9	1396.7	95.3
0.25	672.5	656.3	97.6
0.125	378.2	426.5	112.8
		AVE	**100.8**
		STD DEV	**5.6**

NH_2 Cartridge Clean-Up

A recovery rate of only 19% (± 2.74, n = 5) was observed for a 1 mL aliquot of 1 $g.L^{-1}$ stevioside standard when following the original sample clean-up protocol (Fig. 3.1). An attempt was made to dry the acetonitrile wash completely from the cartridge bed to prevent dilution of the SGs in the retained acetonitrile which might lower SG concentration and recovery rate. Dry N_2 gas was delivered into the top of the cartridge for 1, 5, 10 and 30 min, but these treatments did not change the amount of retained solvent which remained constant at about 0.32 g. Use of a reduced pressure to draw dry gas through the cartridge was more effective, with retained solvent mass decreased to 0.23 g after 5 min of treatment.

After incorporating the cartridge drying step into the protocol, the total volume of the acetonitrile wash and the water eluant were increased to 2 and 2.4 mL respectively (as recommended by Phenomenex). This increase resulted in an improved recovery, of 35.3% (± 0.19, n = 3) of applied steviodide. A further increase in the volume of the final water wash (from 2.4 to 3 mL) did not improve the recovery. However, a subsequent wash with 1 mL of ethanol (70%) recovered another 34.2% of the applied stevioside.

Thus the nature of the final elution solvent was critical in improving the recovery rate. Ethanol (70%) was more effective than water in eluting stevioside from the cartridge such that a final wash with 3 mL of water resulted in only 27.7% recovery while washing with 3 mL of 70% ethanol yielded a 74.2% recovery (Fig. 3.9).

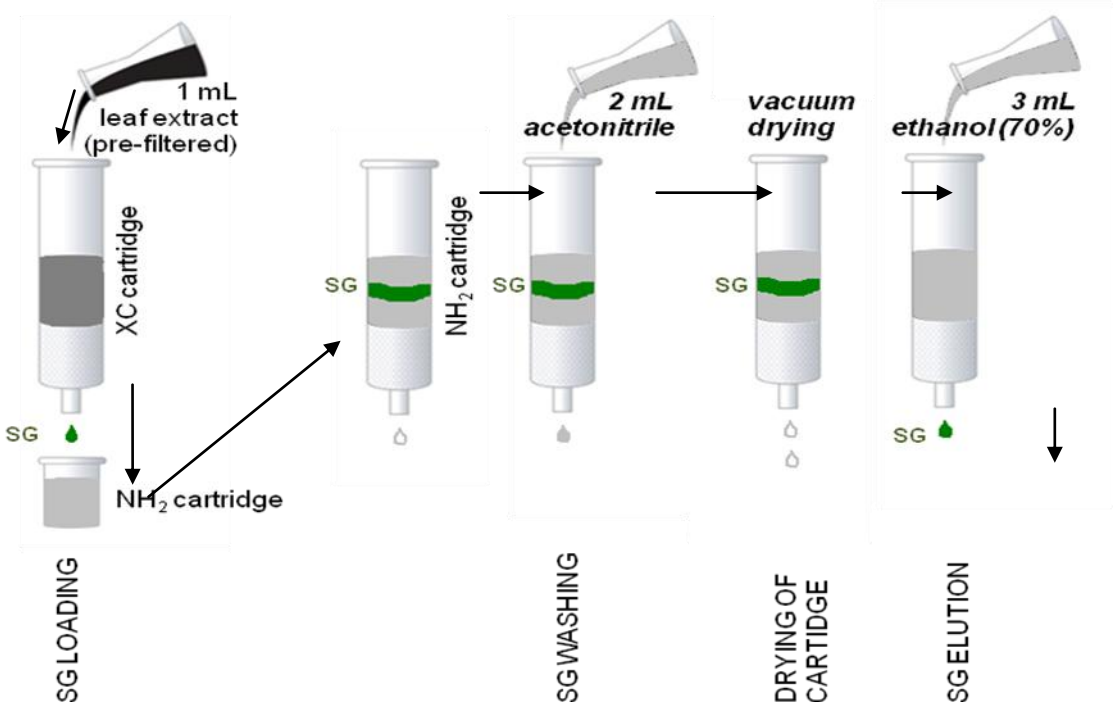


Figure 3. 9. Revised clean-up protocol for SG standards and leaf extracts. Volume of acetonitrile wash was increased from 1 to 2 mL. Air drying of acetonitrile was replaced with vacuum drying. Final water eluant was replaced with 70% ethanol.

A recommendation (Niron Van, Phenomenex, pers. comm.) was made to pre-condition the cartridge with 3 mL ethanol (100%) before sample loading (Fig. 3.10). This step improved the recovery of the added 1 $g.L^{-1}$ stevioside to 100%.

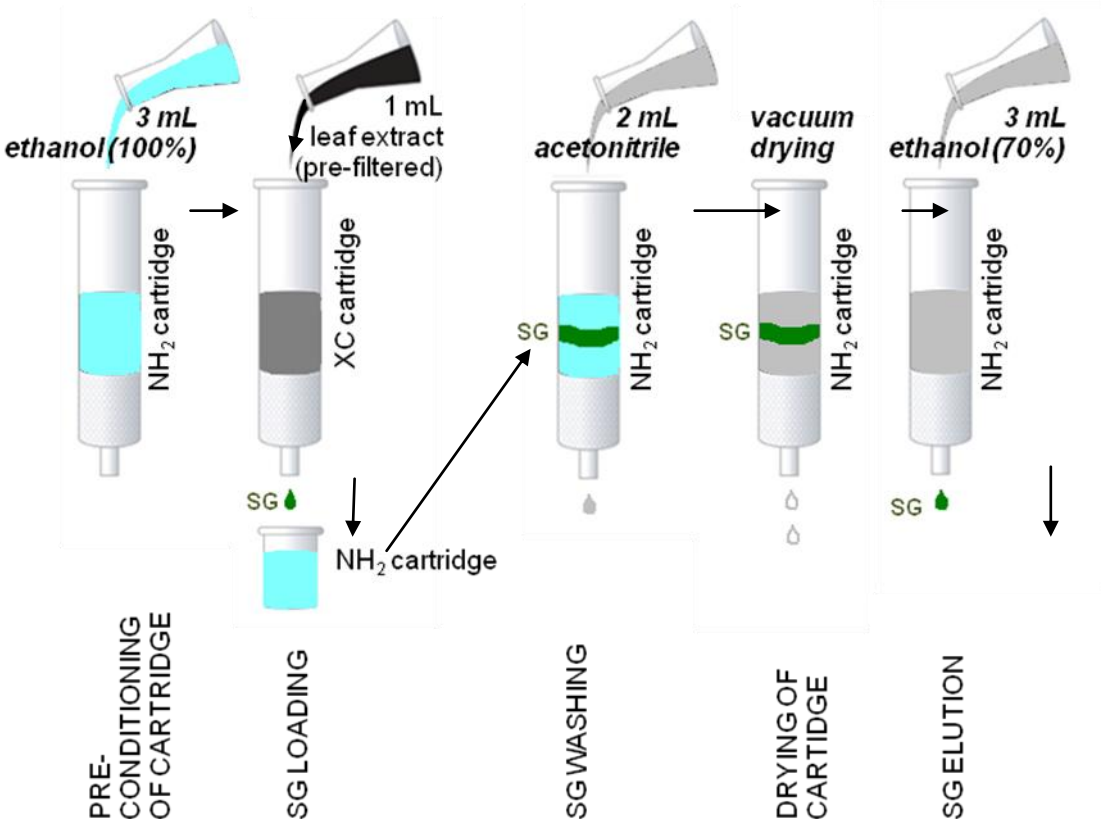


Figure 3. 10. Final clean-up protocol for SG standards and leaf extracts. A pre-conditioning step for the NH2 cartidge was included.

To confirm the improved recovery in the final clean-up protocol (Fig. 3.10), standard solutions of both stevioside and rebaudioside A (1, 0.5, 0.25, 0.125 $g.L^{-1}$) were processed. Recovery of stevioside and rebaudioside A was 84.5% ± 1.8 and 100% ± 1.9, respectively (Fig. 3.11).

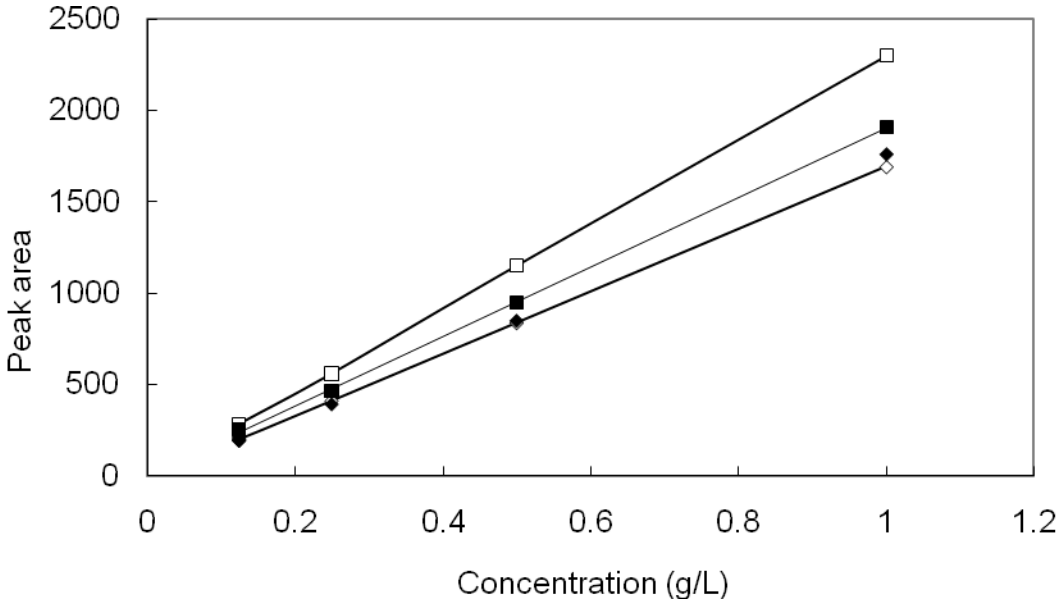


Figure 3. 11. Peak area of ethanol-based standards (0.125, 0.25, 0.5 and 1 g.L^{-1}) of stevioside (filtered ■ and unfiltered —□—) and rebaudioside A (unfiltered —◊— and filtered ◆) using the final SPE clean-up protocol.

The new protocol was also tested with a stevia leaf extract. A 100% recovery for both stevioside and rebaudioside A was observed although the amount of stevioside (2.4% dw) and rebaudioside A (2.9% dw) in the plant matrix was relatively low (with stevioside and rebaudioside A concentration in the extraction solution of 0.24 and 0.29 g.L^{-1}, respectively). However, when 1 mL of the same leaf extract was amended with 0.5 mL of 1 g.L^{-1} stevioside and rebaudioside A, recovery of only 57% of stevioside and 86% of rebaudioside A was achieved.

Discussion

Extraction solvent

The original method of Hearn and Subedi (2009) involved leaf extraction in 70% ethanol. Ethanol is useful for extract preservation, however water has also been used in leaf extraction methods with the advantage of minimizing extraction of a range of organic leaf components. However, water can be used alone in heated extraction (Bovanova, Brandsteterova *et al.* 1998; Kedik, Fedorov *et al.* 2003), as a co-solvent in supercritical fluid extraction (Pol, Ostra *et al.* 2007) or more recently as water in super-critical state for pressurized hot water extraction (Ong, Cheong *et al.* 2006). With commercial extraction, water is recommended as a highly polar solvent for initial extraction of dry ground stevia leaves (Kutowy, Gower *et al.* 1999; Payzant, Laidler *et al.* 1999).

Higher extraction of stevioside from leaf material was achieved using water at 70 °C than with ethanol, consistent with the higher solubility of stevioside in water (0.13%) than dioxane or alcohol, although solubility level for the organic solvents is not detailed in the Merck Index (Merck 1996) or in MSDS sheets (Chemwatch). Solubility of rebaudioside A in water (0.8%) is higher than stevioside, and thus efficiency of extraction of rebaudioside A using water was expected to be at least as high as for stevioside.

The marginally lower rebaudioside A content in the water (5.69%) over the ethanol (6.21%) extract was perhaps influenced by trace amounts of inactivated enzyme which was present in levels high enough to lower the rebaudioside A concentration by less than 1%.

Although some previous reports involving water extraction of SGs from stevia leaf have involved boiling (Bovanova, Brandsteterova *et al.* 1998; Kedik, Fedorov *et al.* 2003), the current extraction temperature was kept at 70 °C to avoid SG degradation, as reported at high temperatures (80 and 100 °C) and low pH (<2.6) conditions (Chang and Cook 1983; Kroyer 1999). Crystalline stevioside has also been observed to degrade at temperatures beyond 120 °C (Kroyer 1999).

The slight change in peak shape with the change of the extraction solvent to water can be explained by the relative polarities of ethanol and water in relation to the stationary amine sites within the stationary column. A 10 μL injection of sample might appear to contain a minimal amount of solvent compared to the total volume of mobile phase flushed through the column over 15 minutes of sample run-time. However, a 1 $mL.min^{-1}$ flow rate of mobile phase represents a flow of 20 $\mu L.second^{-1}$. In this context the solvent added with a 10 μL sample injection is significant. With this volume, the polarity of the solvent in the injected sample (water or ethanol) influences the partition of the SGs between the stationary column and the mobile liquid phase. Water is more polar than amines, maintaining the SGs in solution, and thus resulting in a wider elution peak. In contrast, ethanol is less polar than water, allowing the SGs to interact more strongly with the stationary column, resulting in narrower elution peaks.

Given the slight peak broadening in the water extract, the sample injection volume was reduced from 10 μL to 5 μL, to avoid overlapping of adjacent peaks.

The detection limit (PQL) of 0.0061 $g.L^{-1}$ stevioside and 0.014 $g.L^{-1}$ of rebaudioside A, is equivalent to a detection limit of 0.061 and 0.14% leaf dw, respectively, for the extraction ratios and conditions employed. This limit is acceptable for the analysis of leaf SG concentration, typically 8-10% w/dw stevioside and 3-5% w/dw rebaudioside A. Further, a calibration range of 0.1-1 $g.L^{-1}$ stevioside and rebaudioside A is sufficient to cover the typical

range of leaf SG content (given the extraction ratios (0.1 g of dry ground leaves in 10 mL water) and conditions employed.

The drop in correlation coefficient as the maximum concentration of stevioside is increased beyond 5 $g.L^{-1}$ is consistent with the solubility limit of 0.13% stevioside in water. Indeed when the concentrated stevioside standards (1, 2.5, 5, 7.5, 10 and 20 $g.L^{-1}$) were kept in storage at 4 °C, white crystalline precipitates started to form at the bottom of the container.

Mobile phase and retention time

The partitioning of stevioside and rebaudioside A between the mobile liquid phase and the stationary amine column is influenced by the extent of hydrogen bonding either with the water component of the mobile phase or the amine groups in the column. As the acetonitrile content in the mobile phase decreases, more water molecules that are capable of hydrogen bonding flow through the column. Consequently, the analytes are “pulled” more strongly by the mobile phase resulting in earlier elution and thus narrower peaks with earlier retention time. Conversely, an increase in acetonitrile content allows stronger H-bonding interaction with the column resulting in longer retention time and broader peaks that represent a more staggered elution from the column.

An 80% acetonitrile mobile phase was optimal for achieving good peak resolution. Increasing the acetonitrile content to 85% resulted in wider peaks with poor resolution even though retention time was extended to 21 min. Given that the shift in retention time was most steep within the 80-90% acetonitrile range, the mobile phase was accurately prepared in large batches, which were then stored to avoid shifts in retention time due to variation in mobile phase composition.

Leaf extract purification and recovery rate

Hearn and Subedi (2006) used an SPE cartridge for leaf extract 'clean up' prior to HPLC analysis for SGs. The Strata XC cartridge bed contains sulfonic acid groups which will retain charged compounds such as chlorophyll A and B, while allowing SGs to pass. In contrast, the Strata NH_2 cartridge consists of amine groups which retained SGs through hydrogen bonding while allowing the other leaf components with weaker hydrogen bonding to pass through.

Additional steps were incorporated with the NH_2 filtration to condition the cartridge bed and to ensure that the analyte is only eluted at the last step. First, the NH_2 cartridge was pre-conditioned with 100% ethanol to fully solvate the chromatographic bed and to remove any trapped air in the bed volume. Pure ethanol was chosen because it had a similar nature with the sample solvent (70% ethanol), but was less polar. By loading the cartridge with pure ethanol, which was less polar than the amino groups in the cartridge bed, the SGs are retained in the stationary bed, thus minimizing undesired elution during sample loading.

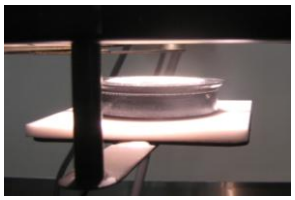
Second, acetonitrile was chosen for the washing step because it is less polar than ethanol and than the amine groups in the cartridge. This ensured that only non-polar components in the extract were eluted. Vacuum drying after washing minimized the retained amount of acetonitrile which could potentially dilute the analyte and result in decreased recovery.

Third, water as the final elution solvent was changed to 70% ethanol. In a separate trial, a cartridge washed with 3 mL water was consecutively washed with 1 mL of water and 1mL of 70% ethanol. From the 3 mL water wash, about 28% of the SG in the filtered sample was recovered. No additional SGs were eluted by the subsequent 1 mL water wash while more SGs were recovered from the subsequent 1 mL ethanol (70%) wash (additional 34% of the SG in the filtered sample). Indeed, when the final 3 mL water eluent was replaced by 3 mL ethanol (70%), the SG recovery increased to 74%. Water is more polar than 70% ethanol, and therefore should act as a stronger

solvent, flushing SGs from the column. However, the residual acetonitrile in the cartridge may have made the cartridge bed less miscible to pure water. Ethanol (70%), being less polar than water, should be more miscible in this matrix, allowing a closer interaction with the retained SGs, and thus resulting in a higher recovery rate.

The amount of the final elution solvent was increased from 1 to 2.4 mL and eventually to 3 mL. The recommended solvent volume is at least four times the bed volume, to ensure that the cartridge bed was fully equilibrated with a solvent. Given a bed volume of the NH_2 cartridge of 300 μL (Phenomenex), a 2.4 mL elution volume is recommended. Increasing the volume of the final ethanol (70%) wash to 3 mL and incorporating a cartridge pre-conditioning step, using 3 mL of pure ethanol increased the recovery of the stevioside and rebaudioside A standards to 83 and 100% respectively.

In succeeding chapters, use of the SPE cartridges was discontinued from the leaf water extract preparation because good HPLC peak resolution was achieved with the unfiltered samples. Removal of the SPE cleanup shortened the sample preparation time and decreased the short term cost of each sample analysis. However, the use of an SPE cartridge will reduce column contamination with leaf extract impurities. Without the use of the SPE cartridges, the theoretical plates in a new amine column were reduced by half after 150 HPLC runs. If this is accepted as the end of the useful life of the column, then column cost per sample analysed was \$1500/150 = \$10. When SPE cartridges were used in sample preparation, column costs per sample were decreased (assuming capacity for 1,000 samples when samples were cleaned, column cost per sample would be \$1500/1000 = \$1.50), but additional cost for the cartridges (\$4 per analysis) and for labour (20 min per sample, costed at \$20/h, \$7/sample), for a total cost of \$12.50 per sample.



Chapter 4
Quantification of steviol glycosides using Near Infrared Spectroscopy
I. Estimation of rebaudioside A in aqueous solution

Abstract

Near infrared spectroscopy was considered as a rapid assessment alternative to HPLC for measurement of rebaudioside A in water solution (0.005 to 0.08% (w/v) range), towards the practical application of in-line quality control on the level of rebaudioside A sweetener. A transmission optical configuration was used. Solution temperature and matrix were varied, the latter through inclusion of sucrose. Partial Least Squares (PLS) regression models were developed using second derivative spectra, with optimal model performance within the wavelength range of 1600-1726 nm (R_{CV} = 0.990, RMSECV = 0.049 % (w/v)).

Abbreviations: R_{CV} – Correlation of cross validation, RMSEC – Root mean square error of calibration, RMSECV – Root mean square error of cross validation, SD – Standard deviation.

Introduction

Stevia is a perennial herb that contains around 10% dw of steviol glycosides (SG) in its leaf tissue. SGs are natural sweetners, being approximately 300 x sweeter than sucrose on a weight basis. They are a class of compounds

which are defined by the presence of a steviol backbone, with different numbers of glucose moieties attached. The dominant SGs in stevia are stevioside and rebaudioside A, with higher rebaudioside A desired, as stevioside has an after-taste. The rebaudioside A ; stevioside ratio in stevia leaf is therefore of interest.

Approvals for use of stevia in food products contain limits on the maximum content for various product, e.g. 115 mg/kg (for liquid milk), and 160 mg/kg (for soft drink) of steviol equivalent (FSANZ 2008), as steviol itself has a toxicity. These limits have been based on a safety limit of 4 mg/kg body weight and the estimated frequency of food intake of each food type. Manufacturing procedures should ensure strict compliance with these limits. However, the small amount of the sweetener required, in contrast to other ingredients, increases the probability of an error in addition. A practical in-line method for estimating total SG content in a commercial product would be useful in ensuring compliance to this regulation.

Gas and liquid chromatography, thin-layer chromatography, IR spectrophotometry and capillary electrophoresis are among the techniques that have been explored for SG measurement (Yoshida 1986; Kedik, Fedorov *et al.* 2003; Dacome, Da Silva *et al.* 2005). Such laboratory-based analyses are accurate but involve considerable sample preparation and have practical limitations for use in in-line testing. With the recent approval of the stevia sweetener as a food ingredient by FSANZ (2008), USFDA (JECFA 2009) and more recently, EFSA (2010), stevia will be more commonly used in manufactured food products with low sugar claims.

Near Infrared spectroscopy (NIRs) is a promising technique for non-invasive estimation of SG concentration in liquid solution. For example, quantification (Multiple Linear Regression-MLR and Partial Least Squares Regression-PLS calibrations) of individual sugars in fruit juice samples has been reported, using both reflectance spectroscopy with a spray-dried on fiberglass filter technique, or transmission spectroscopy using clarified solution in a 1 mm pathlength cell (Dambergs, Esler *et al.* 2004).

Two published studies exist with respect to the use of NIRs for the determination of SG in stevia leaf material. Nishiyama and Alvarez (1992) attempted the estimation of stevioside content in ground dry stevia leaves using a calibration set that consisted of 64 samples scanned using a reflectance optical geometry over the 1100-2500 nm range using a Trebor-70 monochromator spectrometer, with a prediction result for a non-independent set of 30 samples of R^2 = 0.83, SEP = 1.24% dw. Hearn and Subedi (2009) also worked with reflectance spectra of dry ground stevia leaves, reporting calibration results based on the second derivative spectra (2042-2240 nm window) of R^2 = 0.93, RMSECV = 0.68% dw for total SG, and R^2 = 0.80, RMSECV = 0.8% dw (2246-2290 nm) for stevioside.

Several limitations to these studies are apparent. The wavelength regions were chosen on an empirical basis (ie. various regions tested, and the best result adopted). The calibration models were not validated with an independent set of samples. Also, as a limited sample set was used, in which the ratio of component SGs will be relatively similar, it is not possible to comment on the utility of the NIRs technique to measure stevioside and rebaudioside A per se, in addition to total SG content.

The aim of the current study was therefore to contribute to an understanding of the spectroscopy of the SGs, providing a foundation for future applications of the NIRs method in SG analysis.

Materials and Methods

Chemicals

Stevioside and rebaudioside A standards were obtained from Wako Reagents P/L. Crystalline sodium chloride and starch (AnalaR grade), and sucrose (ACS Reagent grade) were obtained from BDH Chemicals and Sigma Aldrich P/L respectively. The commercial stevia sweetener, Soolite (99% rebaudioside A) was donated by Sanitarium. Acetonitrile (E

Chromasolv for HPLC), as used for the HPLC mobile phase, was obtained from Sigma-Aldrich.

Spectroscopy

Spectra were acquired using an Antaris Nicolet FTNIR spectrometer. Liquid samples were scanned using an interactance mode, using a B screen filter, with a built-in gold flag as reference. Crystalline samples were scanned using a transmission mode with air as reference. Each spectrum was taken with 8 cm^{-1} resolution, using 2 x gain within the 800-2500 nm region, and each spectrum was averaged from a total of 64 scans. Background spectra were averaged from 128 scans and taken every hour during a continuous spectral run. The spectral scale was converted from wavenumber to wavelength, for convenience.

Samples of crystalline stevioside (0.0402 g), rebaudioside A (0.0486 g), sodium chloride (0.1659 g), starch (0.0823 g) and sucrose (0.0924 g) were placed inside a small glass tube (50 mm height x 6 mm diameter), filled to a height of 7-8 mm. Samples were scanned once at room temperature.

Solutions of pure rebaudioside A (0.005, 0.01, 0.02, 0.04, 0.06, 0.08% (w/v)) and of the commercial stevia sweetener, Soolite (99% rebaudioside A) (0.01, 0.02, 0.04, 0.06, 0.08, 0.125, 0.25, 0.5, 0.75, 1% (w/v) were scanned in triplicate. About 1 mL of the solution was placed in a standard flat glass vial with a path length of 1 mm. Sample temperature was monitored during scanning and kept constant at 40 °C (± 1 °C). Triplicate scans were considered as individual spectra (not averaged) during calibration.

Soolite standards were also scanned at 23, 29 and 50 °C. In addition, the solution matrix was varied with the addition of sucrose. Different sucrose solutions (aq) (1%, 10% and 20% w/v) were used instead of water to produce additional sets of Soolite standards (at 0.01, 0.02, 0.04, 0.06, 0.08, 0.125, 0.25, 0.5, 0.75 and 1% w/v). This series of samples was scanned at a fixed temperature of 40 °C. Solutions scanned at different temperatures and

solution matrix were limited to Soolite standards because of the limited amount of pure stevioside and rebaudioside A standards that was available.

Difference spectra were obtained from the raw absorbance spectra by subtraction of an adjusted water absorbance spectrum from the spectra of each solution. The adjustment of the water absorbance spectrum was proportional to the amount of water in the solution, as estimated from the density of the solution relative to pure water. Specific absorbance spectra were then obtained from the difference spectra by dividing each spectral point by the solution concentration.

HPLC

HPLC analysis was performed using an Agilent 1100 and a Zorbax NH_2 column (250 x 4.6 mm, 5 µm) connected to an Agilent Zorbax High Pressure Reliance Cartridge guard column (12.5 x 4.6 mm, 5 µm). Mobile phase was 80% acetonitrile (pH 5) buffered with 100 mL of 0.02 M glacial acetic acid and 200 µL of 0.1 M sodium hydroxide (aq) for every 500 mL of total mobile phase solution. Flow rate was at 1 $mL.min^{-1}$ and injection volume was at 5 µL. The UV detector was set at 210 nm with 360 nm as reference signal and slit at 4 nm.

HPLC analysis verified the stated composition of the materials used in this study (data not shown).

Chemometrics

Unscrambler v 9.1 (Camo, Sweden) was used for partial least square regression analysis of the NIR spectra. Full leave one out cross validation was implemented and the calibration set was left intact with no outliers removed. An in-house window selection program based on Matlab and Eigenvector PLS tool box (Guthrie, Walsh *et al.* 2005) was also used to select the optimal wavelength regions for the calibration model. The PLS regression model was validated using the prediction module of Unscrambler. A minimum model performance of $R = 0.87$ ($R^2 = 0.75$) was sought, as at this

level RMSECV is half population SD, allowing sorting of the population into two grades.

Results

NIR spectral features

The spectra of crystalline stevioside and rebaudioside A were similar to each other, with both possessing features at 1200, 1450, 1900, 2100 and 2300 nm, although absorbance was lower overall than starch and sucrose (Fig. 4.1).

The NIR spectrum of crystalline NaCl was relatively featureless, with only a slight feature at 1950 nm (Fig.1). Spectra of crystalline starch and sucrose possessed major features at around 1200, 1500 and 1900-2100 nm. An absorption feature at 1900 nm was present in the starch sample that was not present in the sucrose sample, while features at 1420 and 1700 in the sucrose spectra were not apparent in that of starch (Fig. 4.1).

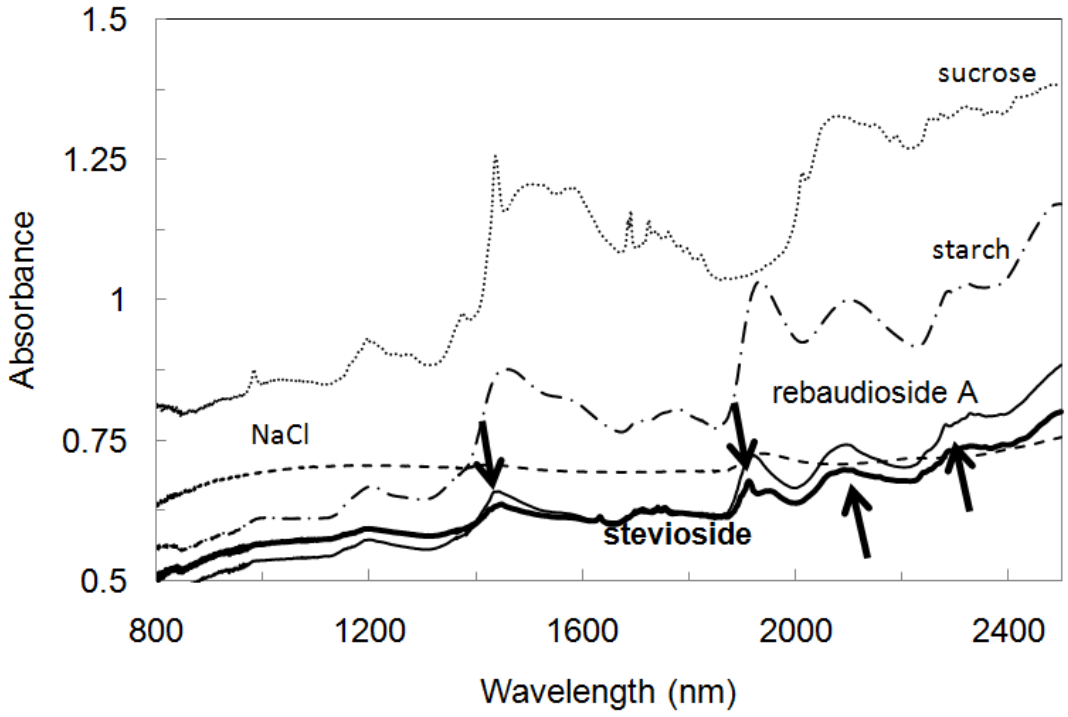


Figure 4. 1. NIRs spectra of powdered crystalline samples of stevioside (**—**), rebaudioside A (—), sucrose (····), starch (-·-·-) and salt (- - -) using a transmission optical geometry (Antaris FTNIR unit). Arrows point to major features of the SGs.

In aqueous solution, the contribution of rebaudioside A (as Soolite) to the absorbance spectrum was not obvious, given the dominance of water related peaks around 1450, 1990 and 2450 nm (Fig. 4.2A). Even the second derivative of the absorbance spectra (Fig. 4.2D) did not reveal small peaks possibly obscured by the water absorbance. Following subtraction of the water spectrum, absorption peaks apparently associated with rebaudioside A were evident at 1440, 1990 and 2450 nm, with peak height varying with Soolite concentration (Fig. 4.2B). Division of these 'difference spectra' by the concentration of Soolite in the solution yielded 'specific absorbance' spectra (Fig. 4.2C).

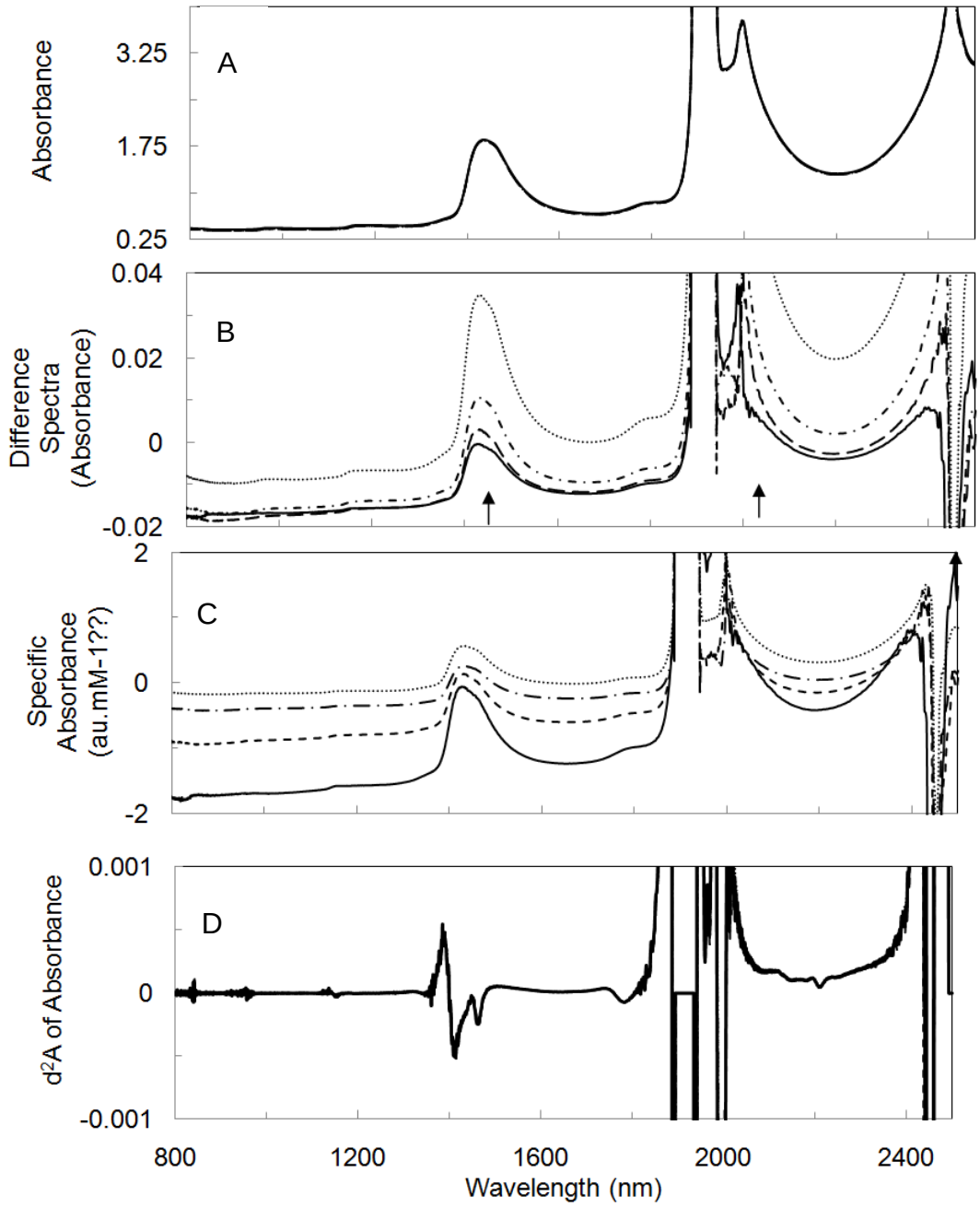


Figure 4. 2. Raw absorbance spectra (A), difference spectra (to water) (B), specific absorbance spectra (C) and d^2A spectra (D) of rebaudioside A (Soolite) standards (0.01% (—), 0.02% (– – –), 0.04% (–·–·–) and 0.06% (····)) scanned at a fixed temperature of 40 °C. Air was used as reference. Spectra were acquired using the Antaris FTNIR unit.

The identified absorbance peaks at 1440, 1990 and 2450 nm were observed to shift with variations in sample temperature and solution matrix. A shift to the left (to shorter wavelengths) was observed for spectra of all aqueous solutions with an increase in temperature (as seen in spectra of pure water; Fig. 4.3). The same trend was observed with aq. solutions of stevia powder (Soolite) (0.01, 0.02, 0.04, 0.06, 0.08, 0.125, 0.25, 0.5, 0.75, 1% (w/v), data not shown). Peak shift (at 1440 and 1990 nm) to shorter wavelengths was also observed with the addition of sucrose to the solution matrix (Fig. 4.3, 4.4). The same trend was observed in the difference spectra (data not shown).

Given the sensitivity of the spectra to changes in temperature and solution components, Partial Least Squares Regression calibration models between spectra and rebaudioside A concentration were initially developed for each matrix condition (Table 4.1). At a fixed temperature, calibration statistics of R_{cv} = 0.84 and RMSECV = 0.002 % (w/v) were achieved using a set of rebaudioside A solutions of limited concentration range and the wavelength range 1600-1726 nm. Using Soolite solutions of a greater concentration range, calibration statistics of R_{cv} = 0.995 and RMSECV = 0.047 % (w/v) was achieved using the 1600-1726 nm range (the optimal window identified, see below). The 1600-1726 nm window also supported models with R_{cv} > 0.99 for Soolite in data sets in which sample temperature and solution sucrose content were varied, although the number of factors used in the model increased (from 1-2 to 3-4). Inclusion of spectra of pure water at a range of temperatures did not degrade model calibration statistics.

The optimal wavelength window (1600-1726 nm) was identified based on minimum RMSECV and RMSEC with use of a reasonable number of PLS factors using a window selection program (Fig. 4.5). This result was confirmed by comparison to models developed using the 800-1100 nm and 1100-2490 nm regions (Table 4.1).

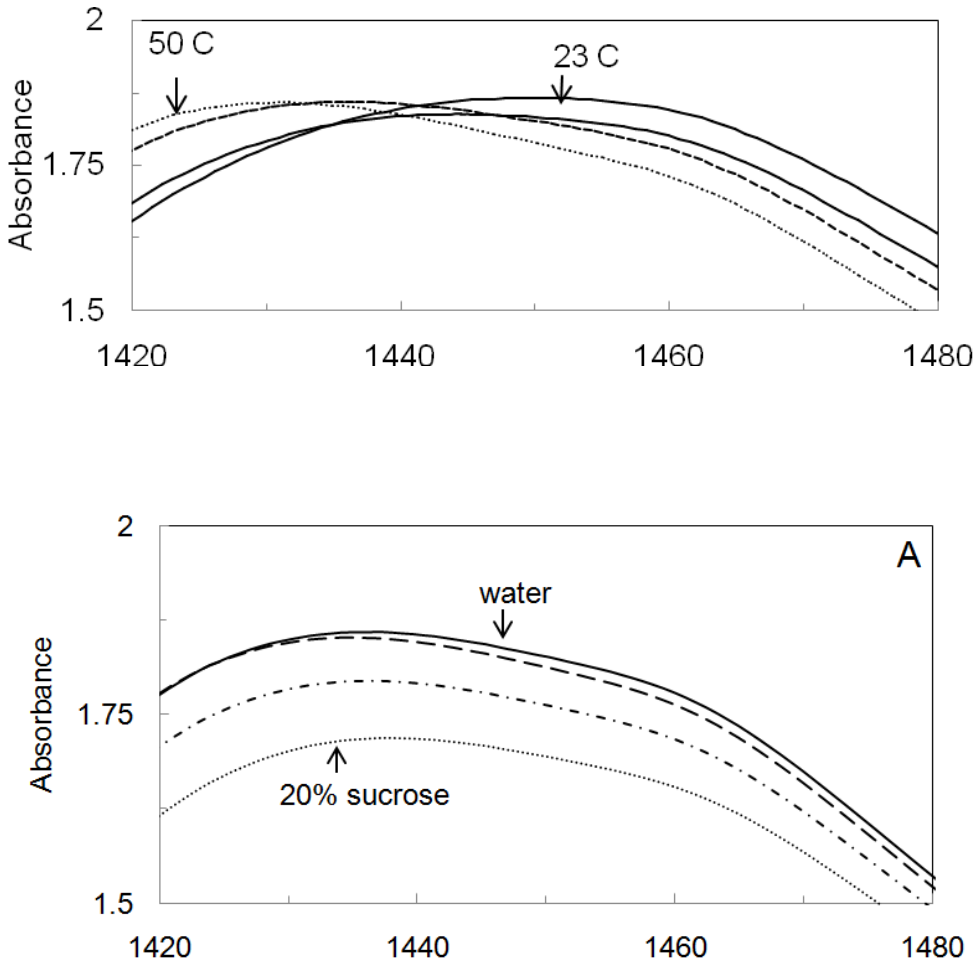


Figure 4. 3. Absorbance spectra (around 1400 nm) of water at (**A**) 23 °C (**—**), 29 °C (– – –), 40 °C (– · –) and 50 °C (····), and (**B**) at 40 °C, but in the presence of sucrose at 1% (– – –), 10% (– · – · –) and 20% w/v (····).

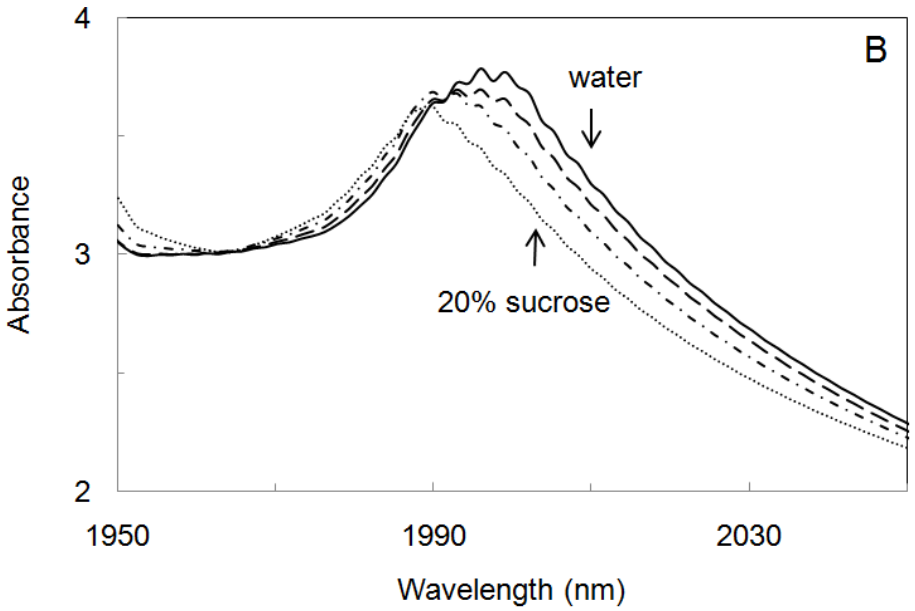


Figure 4. 4. Absorbance spectra (and 2000 nm) of water at 40 °C with sucrose content of 1% (– – –), 10% (– · – · –) and 20% (····).

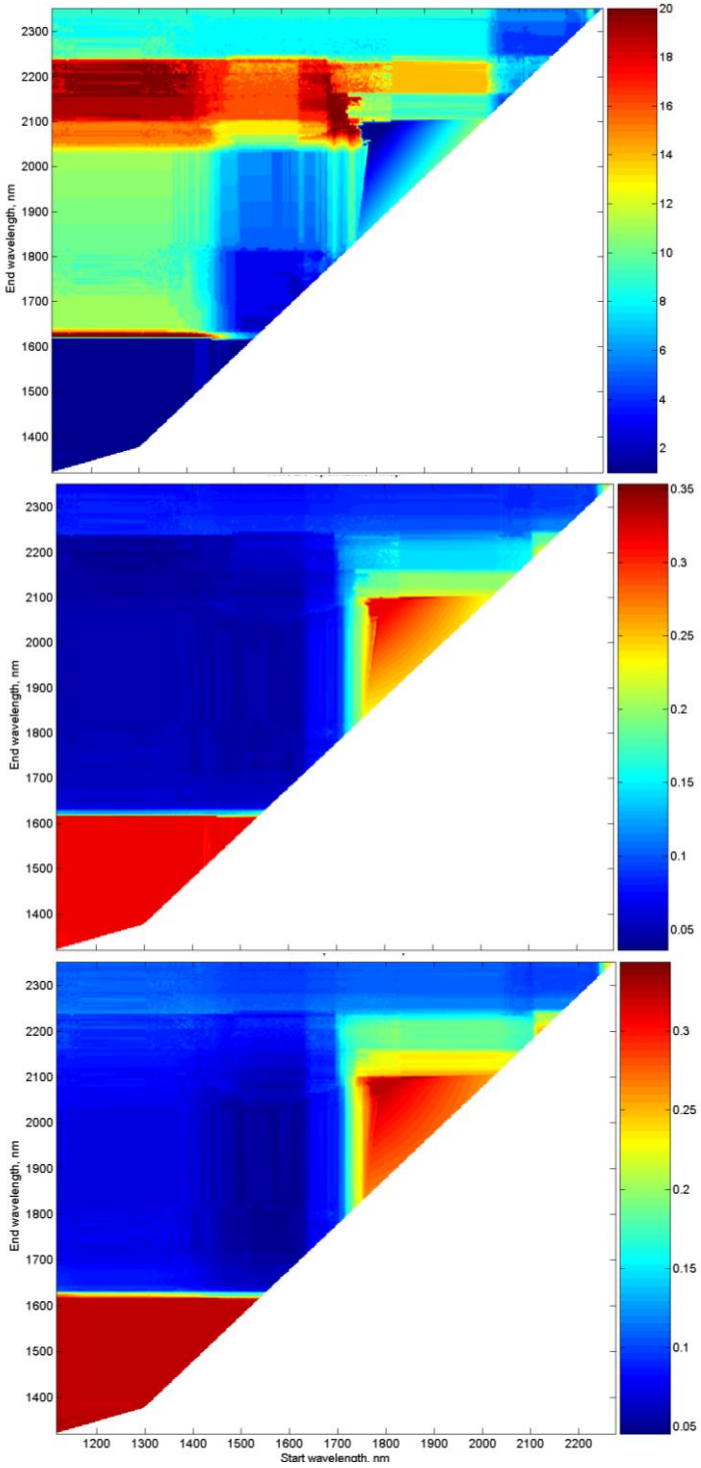


Figure 4. 5. Calibration model statistics for PLS regression models of Soolite in aqueous solutions, based on various wavelength regions (start wavelength displayed on the x axis, end wavelengths on the y axis) – (A) Number of PLS factors, (B) RMSEC, and (C) RMSECV. The bar to the right of each panel is a colour scale for the respective attribute. Second derivative of absorbance data of Soolite at 12 concentrations, a range of temperatures and in a matrix of several sucrose concentrations was used (ie. data set of Table 4.1E).

Table 4. 1. PLSR calibration statistics for models of (A) rebaudioside A and (B-E) Soolite in solution, based on different wavelength ranges (using an Antaris FTNIR instrument). The standard deviation of the rebaudioside A set was 0.004% w/v, while that for Soolite was 0.32% (12 concentrations: 0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.125, 0.25, 0.5, 0.5, 0.75 and 1% w/v). Samples of set A, B and D were at 40 °C. Samples of set C were at 23, 29, 40 and 50 °C. Samples of set D contained 1, 10 and 20% sucrose present in the solution. The units of RMSECV are % w/v. The number of principle components used in the PLS regression are given for only the models developed using the 1600-1726 nm window. Results of models in which R > 0.87 are in bold font.

	N	**800-1100 nm**		**1100-2490 nm**		**1600-1726 nm**		
		R_{CV}	**RMSECV**	**R_{CV}**	**RMSECV**	**#PC**	**R_{CV}**	**RMSECV**
A: Reb A	7	-0.62	*0.003*	0.63	*0.003*	1	0.84	*0.002*
B: Soolite	33	-0.76	*0.47*	-0.30	*0.52*	**1**	**0.995**	***0.047***
C: Soolite (varied temp)	41	-0.12	*0.36*	0.16	*0.31*	**2**	**0.995**	***0.047***
D: Soolite (varied sucrose)	99	0.33	*0.35*	-0.15	*0.33*	**3**	**0.990**	***0.049***
E: Soolite (varied temp and sucrose)	158	-0.05	*0.34*	-0.09	*0.32*	**3**	**0.990**	***0.049***

Discussion

Interpretation of NIR spectra

The NIR spectrum of crystalline NaCl was featureless, as expected for this molecule (Miller 2001), except a small feature interpreted as water present within the crystal. Several features were visible in the spectra of crystalline sucrose and starch. In crystalline material, a specific molecular order is maintained, with regular intra-molecular distances, such that spectra are expected to display sharper features than amorphous material. For example, the spectrum of crystalline sucrose displayed a characteristic spike at 1440 nm. The 1440 nm feature represents a non-H-bonded form of OH group within the sucrose crystal structure (Miller 2001). The overall increase in absorbance with increasing wavelength is interpreted as an effect of scattering, with the finer stevioside material demonstrating a higher level of scattering than seen with the larger grain sizes of the sucrose and starch used in this trial. The SG features may be attributed to a CH second overtone (around 1200 nm), an OH first overtone and/or a CH first overtone (around 1450 nm), an OH combination (around 1900 nm) and OH and CH combination bands (around 2100 and 2300 nm).

The narrow features observed with the crystalline ma l were not present in aqueous solutions. Indeed, the absorbanc C eatures of water overwhelmed those of the sugars in the raw spectra, with major features at 1900 and 1200 nm ascribed to a first and second overtone of an O-H combination band, and at 1470 and 970 nm, to a first and second overtone of O-H stretching, respectively.

Subtraction of the water peak allowed visualization of those areas of the spectrum influenced by the presence of the SG, rebaudioside A (as Soolite). However, this spectral change represents both absorption by the sugar directly, and the influence of the sugar on the vibrational characteristics of water, presumably through an impact on the extent of H bonding between

water molecules. If water were not so affected by the addition of the sugar, division of the difference spectra by the concentration of sugar present would yield a constant 'specific absorbance' spectrum. In practice, more change in this spectrum was observed with small additions of sugar than with larger additions, e.g. around 1440 nm. Thus this absorption feature is a combination of absorption by O-H groups within rebaudioside A directly, and the impact of rebaudioside A on the spectra of water, with low rates of addition causing more disruption to the order of H bonding (and thus change in the absorption spectrum) than larger additions. These multiple influences are also evidenced in the second derivative spectrum.

Change in temperature also effects a change in the H bonding induced order of water molecules. A shift of the water absorption peak to shorter wavelengths was observed, as expected for a condition that results in decreased H bonding, favouring those water species with less structure, and thus tighter O-H bonds which require more energy to elicit vibration (Miller 2001; Golic, Walsh et al. 2003; Subedi 2007). That the water 'peaks' are effectively composite peaks, representing water in different degrees of H-bonding, may be evidenced by the fine detail of the 1990 nm peaks, with multiple peaks resolved by the FTNIR. A similar shift to shorter wavelengths was observed with the addition of sugars for the 1440 and 1990 nm spectral features, consistent with the disruption of the ordered state of water by addition of solute.

For a NIRs based prediction of rebaudioside A in a solution of constant matrix composition (e.g. constant sucrose) and constant temperature, it is immaterial whether the absorption features described above are directly due to absorption by rebaudioside A, or due to the indirect effect of rebaudioside A on water absorption features. In practical use, however, neither temperature nor matrix are constant, and thus a robust prediction is expected for a model based on absorption features of the analyte of interest, rather than those of water.

Rebaudioside A as Soolite was used, as sufficient quantities of pure stevioside or rebaudioside A were not available. This prevented studies involving varied ratios of the two SGs. However, there was little difference in the spectra of crystalline samples of either material, suggesting that it may be difficult to quantitatively estimate these chemicals when mixed in varying proportions. Further work on this issue is required.

Wavelength region selection

Given the influence of temperature and sample matrix, both parameters should be considered when developing a robust NIR based calibration for rebaudioside A. A data set including variation in temperature and matrix (in the form of sucrose levels) was used in a 'brute force' (empirical) method of wavelength region selection, in which a model was developed for every combination of start and end wavelengths between 1100 and 2400 nm. The wavelengths regions giving minimal SEC values are further narrowed by consideration of RMSECV values, and by the number of factors in the model (Fig. 4.5). A region starting between 1450 and 1650 and ending between 1700 and 2050 was identified as meeting these criteria, with the best model obtained for the region 1600 – 1726 nm. This wavelength window selection supported a PLS regression model of rebaudioside A content in aqueous solution with R of 0.995 and RMSECV of 0.049% w/v, despite inclusion of a high variation in solution temperature and matrix sucrose content. This wavelength region is well away from those associated with –OH features.

Note that this wavelength region recommendation is specific for the spectroscopic conditions employed in this study. In vibrational spectroscopy, fundamental bands with high absorption coefficients and narrow bandwidth occur in the infrared region for typical organic bonds (O-H; C-H), with a series of overtone bands of decreasing intensity and increased bandwidth located at shorter wavelengths, ie. through the near infrared (1100-2500 nm) and Herschel (750-1100 nm) regions of the spectrum. Thus an optical arrangement configured for measurement of a third order overtone band, for example, will not be suitable for measurement of a first order overtone band, for which much higher absorbances will occur. Conversely, if conditions are optimized for the first overtone band, signal strength in the shorter wavelength region will be very low. As such, signal to noise ratio is expected to be poorer for data in these regions.

Conclusion

The results presented are promising towards the application of rapid assessment of aqueous extracts of leaf material using NIRS, rather than HPLC.

The next logical step is to trial use of NIR to estimate SG concentration of crude leaf water extracts as well as ground stevia leaves, using leaf material varying in the stevioside : rebaudioside A ratio. Further work with portable NIR spectrometers is recommended. Ideally instrumentation would utilize the Herschel wavelength range (700 – 1050 nm), as the Si detectors used for this region are cheaper than the detectors used for longer wavelengths. Also, biological sample absorptivity is low in the Herschel region, enabling use of longer path-length optical geometries.

Acknowledgments

We thank John Ashton of Sanitarium for provision of Soolite.

Chapter 5
Quantification of total steviol glycosides using Near Infra-Red spectroscopy II. Stevia leaf

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Abstract

Near Infra-Red spectroscopy was explored as a non-invasive alternative to HPLC for estimation of the steviol glycoside (SG) concentration of stevia leaves in fresh, dry and dry ground format using three bench (InSight, NIRS 6500 and Antaris FTNIR) and one portable (Phazir) NIR instrument. Optimal calibration results were obtained with dry ground leaves (R = 0.87, RMSECV = 1.63) within the wavelength window of 1520-1804 nm, allowing segregation of leaf samples into low and high SG. Use of portable NIR equipment operating within this region is feasible.

Abbreviations: R_{CV} – Correlation of cross validation, RMSECV – Root mean square error of cross validation, SD – Standard deviation.

Introduction

With the recent approval of stevia as a food ingredient by FSANZ (2008), USFDA (JECFA 2009) and more recently, EFSA (2010), stevia leaf is fast becoming a commodity, but the value of the commodity is determined by its SG content. Gas and liquid chromatography, thin-layer chromatography, IR spectrophotometry and capillary electrophoresis are among the techniques that have been explored for SG measurement (Yoshida 1986; Kedik,

Fedorov *et al.* 2003; Dacome, Da Silva *et al.* 2005). Such laboratory-based analyses are accurate but involve considerable sample preparation and have practical limitations for field use. The HPLC method involves four hours of sample preparation and eight hours of actual HPLC runtime for a batch of 30 samples. Thus a non-invasive method for SG determination involving minimal sample preparation would be very useful to the industry, with potential application in or near field to assess SG content of different stevia genotypes in breeding programs, or SG content of crops in context of maturity or agronomic practice, or at later stages in the supply chain, for example to facilitate payment on SG content as well as biomass, or to verify SG content of manufactured product.

Near Infra-Red spectroscopy (NIRs) is a promising technique for non-invasive estimation of SG leaf concentration. NIRs is based on the premise that absorption of near infrared radiation by specific functional groups in a given compound is proportional to the amount of the compound of interest. NIRs is used widely in the food and agriculture industry to quantify, for example, cereal nutritional value in terms of dietary fiber, protein, sugar, fat and energy (Kays 2004), protein in forage (Roberts, Stuth *et al.* 2004), oil and protein content in oilseeds (Dyer 2004) and sugar content of intact fruits (Walsh, Guthrie *et al.* 2000).

Nishiyama and Alvarez (1992) attempted the estimation of SG content in ground dry stevia leaves using a calibration set that consisted of 64 samples scanned over the 1100-2500 nm range using a reflectance optical geometry using a Trebor-70 monochromator spectrometer. Models were developed by stepwise multiple regression wherein the best first pair of wavelengths was selected based on the corresponding correlation coefficient and standard deviation of the calibration. Four additional wavelengths were then selected using forward stepwise regression. Of three data processing formats considered, the best calibration model was obtained for first derivative spectra (R^2 = 0.904, SD = 0.96), followed by second derivative spectra (R^2 = 0.902, SD = 0.95), while the model based on raw spectra [log (1/reflectance)] (R^2 = 0.866, SD = 1.12) gave the poorest result. The calibration based on the

first derivative spectra utilized data at wavelengths 1380, 1660, 1816, 2276, 2300 and 2404 nm. The robustness of the calibration was tested using an 'independent' prediction set consisting of 30 samples. However, while the prediction samples were different from the calibration samples, both sets were sourced from the same 'set' of samples (same group of plants). This resulted in a very similar profile of stevioside content between the calibration group (3.16-14.36%, x = 8.85% ± 2.98 (SD)) and the validation group (4.06-13.46%, x = 8.68% ± 2.87 (SD)). The prediction (R^2 = 0.83, SEP = 1.24%) was therefore over-optimistic in terms of expected performance of this technique with truly independent data sets.

In a more recent work with stevia (Hearn and Subedi 2009), dry ground stevia leaves were scanned in reflectance mode using a NIRS 6500 unit over the wavelength range of 400-2500 nm. A chlorophyll absorption feature dominated the visible region (400-700 nm), while the spectra in the 1100-2500 nm region was very similar to that of Nishiyama and Alvarez (1992). Calibrations from a set of 33 samples were developed for total SGs (sum of stevioside, rebaudioside A and rebaudioside C) (4.3 - 11.1% dw, x = 8% ± 1.8 (SD)) and stevioside content (2.7 - 8.0% dw, x = 5.0% ± 1.4 (SD)). For total SG, calibration based on the second derivative spectra was optimal (R^2 = 0.93, RMSECV = 0.68% dw) within the 2042-2240 nm window. Calibration for stevioside alone was not as good (R^2 = 0.8, RMSECV = 0.8% dw) within the wavelength region identified as optimal (2246-2290 nm), while calibration statistics for rebaudioside A and rebaudioside C were not shown. Unfortunately, the calibration model was not validated with an independent set of samples.

The selection of instrumentation for a given application also requires consideration (Walsh, 2009). A need for low cost and portable instrumentation, for example, demands consideration of silicon photodiode based technology, if the wavelength region serviced by this detector type (to approximately 1050 nm) is appropriate to the application. For longer wavelength ranges, InGaAs or PbS detectors are usually employed. Wavelength resolution of the optical bench also deserves consideration. For

many applications in NIRs a resolution (FHWM) of 10 nm or greater is adequate, but in some instances a greater resolution is advantageous. Fourier transform NIRs instruments provide much higher resolution than scanning grating or diode array instruments. The optical geometry of the light source – sample – detector is another major factor, with reflectance, partial transmittance (interactance) or full transmission optics as the typical alternatives. A reflectance geometry may be appropriate for ground samples, for example. With only two published studies on the use of NIRs to measure SG content of stevia leaf, both of which employ scanning grating instrumentation and reflectance optics, and lack a prediction exercise using an independent sample set, it is timely to further consider this methodology. Further work is required to explore the robustness of this method for total SG as well as for assessment of the component SGs. Further, while better NIRs-based predictions are expected with dry ground samples, the utility of the method with dry intact (non-ground) leaf and with fresh leaf should be explored, as minimal sample preparation allows for field use.

In the current study, spectra were collected of leaf material in different formats (fresh intact leaf, dry intact leaf and ground dry leaf) using several NIRs instruments, differing in wavelength range and resolution, sample-detector-light source optical geometry, and portability. The adage of 'no prediction without interpretation, and no interpretation without prediction' is adopted.

Materials and Methods

Chemicals

Stevioside and rebaudioside A standards were obtained from Wako Reagents P/L. Acetonitrile (E Chromasolv for HPLC) as used for the HPLC mobile phase was obtained from Sigma-Aldrich.

Spectroscopy - instrumentation

Samples were scanned using five instruments.

1) The 'Insight' unit employed an interactance optical geometry (Greensill and Walsh 2000) over the 400-1100 nm wavelength region using a 100W tungsten halogen lamp as light source, and a Zeiss MMS1 (silicion photodiode) detector system with pixel resolution of approx. 3 nm and wavelength resolution of approx. 10 nm. A built-in white tile was used as a reference. The set-up was a prototype of the in-line Insight (www.cvs.com.au) and handheld Nirvana (www.intspec.com) spectrometers. Sample integration time was set to achieve a signal strength of around two thirds maximum, with 60, 200 and 180 ms used for fresh, dry intact and dry ground stevia leaves, respectively. For ground stevia leaves (0.5 g) presented in a petri dish, sample integration time was set at 70 ms. The spectral resolution of this system is reported to be around 10 nm.
2) The silicon photodiode MMS1 detector system in the configuration described above was replaced with a Zeiss InGaAs diode array based detector operating over the range 900-1668 nm wavelength region. Sample integration time was set at 50 ms.
3) A NIRSystems 6500 (FOSS P/L) was used with samples scanned in reflectance mode using a spinning cup accessory over the 400-2500 nm wavelength region (scanning grating, silicon photodiode and PbS detectors). Each spectrum was an average of 32 scans acquired at 1x gain, with each spectrum referenced against the built-in tile of the unit. The spectral resolution of this system is reported to be around 10 nm.
4) A FTNIR Antaris Nicolet was used with samples scanned using a reflectance mode (integrating sphere) over the 800-2500 nm region, using the built-in gold flag as the reference. Each spectrum was an average of 64 scans, acquired with 2 x gain. Spectral resolution was 8 cm^{-1}. Background spectra was averaged from 128 scans and taken every hour. For the convenience of comparison with the other

systems, data from this system is expressed on wavelength rather than a wavenumber scale.

5) The Phazir Polychromix (http://www.polychromix.com/) offers a handheld format with two instrument ranges available (1000-1800 nm and 1600-2400 nm), with pixel resolution of 8 nm and optical resolution of 12 nm. These units use a micromechanical device (MEMS) as a wavelength filter, with a single detector element (InGaAs or presumably PbS).

Experiment 1: leaf water content

Four stevia leaves were floated on water for 3 hours to maximize leaf hydration and turgidity. Excess water was wiped off the leaves which were then placed on a petri dish for leaf scanning in reflectance mode using the integrating sphere accessory of the FTNIR Nicolet Antaris unit. Leaf weight was noted before each scanning, allowing later calculation of the water content. Leaves were initially allowed to dry at room temperature for 41 h, after which the leaves were placed in the oven at 45 °C for 29 h until a constant dw was obtained. The leaves were kept on the petri dish throughout the dehydration period and were handled carefully so that minimal leaf movement occurred throughout the test period. Spectra were acquired at room temperature at intervals during the drying process.

Experiment 2: leaf SG content

For the calibration exercise, leaves were obtained from a total of 105 stevia plants which were grown both inside and outside a glasshouse in Rockhampton, Queensland under natural light. Three stevia varieties were used, Fengtian (50 plants), 99-8 (37 plants) and Shoutian (18 plants). Harvested leaves were kept inside plastic tubes with water and stored in an ice box to ensure leaf freshness.

Spectra of these samples were acquired in several sample formats, with spectra acquired using the three lab-based NIR units (Insight with with Zeiss

MMS1 spectrometer, NIRS 6500 and FTNIR Antaris). Hydrated fresh leaves were first placed inside a standard NIRS 6500 spinning cup with the abaxial leaf surface touching the quartz window of the cup. After scanning using all three instruments, leaves were removed from the cup with minimal disturbance of the layers and transferred to a petri dish for drying in the oven at 65 °C for two days. Once fully dried, leaves were again pressed inside the spinning cup and scanned. The dried leaf samples were then ground using mortar and pestle. About 1 g of the ground dry sample was repacked in the spinning cup accessory. About 0.1 g of dry leaf sample was then subsampled, and used for HPLC analysis of the SG content.

As a follow-up activity, 30 ground dry leaf samples (being those samples with more than 0.5 g available) were scanned using the Insight unit equipped with either an InGaAs array or an MMS-1 spectrometer. Because of the limited amount of samples left after HPLC analysis, about 0.5 g of dry ground stevia leaves was placed in a 40 mm diameter glass petri dish and then compressed using a 35 mm diameter petri dish cap. The petri dishes were held in a dessicator between packing and scanning to minimize moisture absorption.

For a validation exercise, dry ground leaf material was obtained from 55 plants of varieties different to that used in the calibration exercise. The samples were supplied by external commercial groups, and were of unknown varieties, and ground using different procedures to the calibration exercise. Plants were grown overseas, probably in China and Paraguay and certainly in different field conditions. Samples were supplied and scanned only in dry ground format using the four instruments noted above. Of the 55 samples used, 47 samples were also scanned by an external group, using a Phazir handheld unit.

HPLC as reference method

To quantify the amount of stevioside and rebaudioside A in the scanned leaves, leaf water extracts were analyzed using HPLC. About 0.1 g of the

ground sample was mixed with 5 mL of milli-Q water and then held in a shaker water bath at 70 °C for 30 min. The ground leaf pellet was separated from the water extract by centrifugation, with the aqueous extract poured into a separate container before proceeding with a second round of 5 mL water extraction. From the combined 10 mL water extract, a 2 mL aliquot was filtered through a 0.45 µm filter before transferring to a vial for analysis using High Performance Liquid Chromatography (HPLC).

HPLC analysis was performed using an Agilent 1100 and a Zorbax NH_2 column (250 x 4.6 mm, 5 µm) connected to an Agilent Zorbax High Pressure Reliance Cartridge guard column (12.5* 4.6 mm, 5 µm). Mobile phase was 80% acetonitrile (pH 5) buffered with 100 mL of 0.02 M glacial acetic acid and 200 µL of 0.1 M sodium hydroxide (aq) for every 500 mL of total mobile phase. Flow rate was at 1 $mL.min^{-1}$ and injection volume was at 5 µL. The UV detector was set at 210 nm with 360 nm as reference signal and slit at 4 nm.

Chemometrics

Unscrambler v 9.1 (Camo, Sweden) was used for partial least square regression analysis of the NIR spectra and the stevioside and rebaudioside A leaf content quantified through HPLC. Full leave one out cross validation was implemented and the calibration set was left intact with no outliers removed. An in-house window selection program based on Matlab and Eigenvector PLS tool box (Guthrie, Walsh *et al.* 2005) was also used to select the optimal wavelength regions for the calibration model. The PLS regression model was validated using the prediction module of The Unscrambler (Camo P/L). A minimum model performance of R = 0.87 (R^2 = 0.75) was sought, as at this level RMSECV is half population SD, allowing sorting of the population into two grades (from the relationship $R^2 = 1 – (RMSECV/SD)^2$).

Results

Experiment 1: leaf water content

Reflectance spectra of fresh leaf collected using the FTNIR unit were dominated by water absorption peaks, evident at 960, 1200, 1450, 1950 and above 2400 nm (Fig. 5.1). As the leaves dehydrated, initially at room temperature and then in an oven, the water peaks declined in intensity and minor absorption peaks became apparent at 1822, 2150 and 2300-2400 nm region (black arrows) (Fig. 5.1). Spectral change was observed even after just 1 h of drying at room temperature (data not shown).

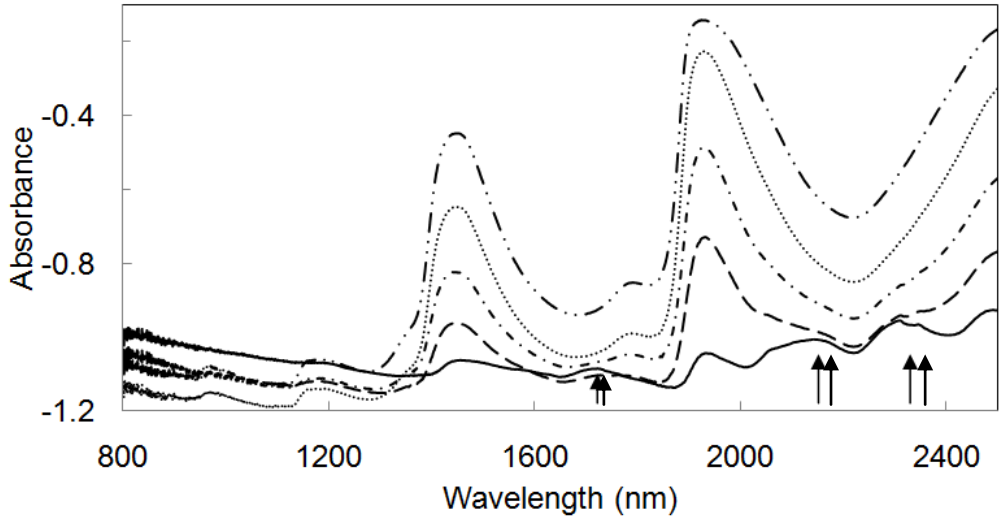


Figure 5. 1. NIR reflectance spectra (log 1/R) of stevia leaves using an FTNIR Nicolet Antaris unit. Leaves were dried to 33% RWC at room temperature and then dried to a steady state weight in an oven at 45 °C. Leaves were scanned at 100% (– ··–), 53% (·····),15% (–·–), 5% (– – –) and 0% (—) relative water content (RWC). RWC was measured by weight comparison relative to initial weight of the fresh leaves before dehydration. Arrows indicate minor peaks that appear in fully dried intact leaves.

Reflectance spectra of fresh leaf collected using the NIRS6500 scanning monochromator unit were similar to that collected using the FTNIR. However the available wavelength range was extended into the visible spectrum, with an absorption peak associated with chlorophyll, evident at 680 nm (Fig. 5.2).

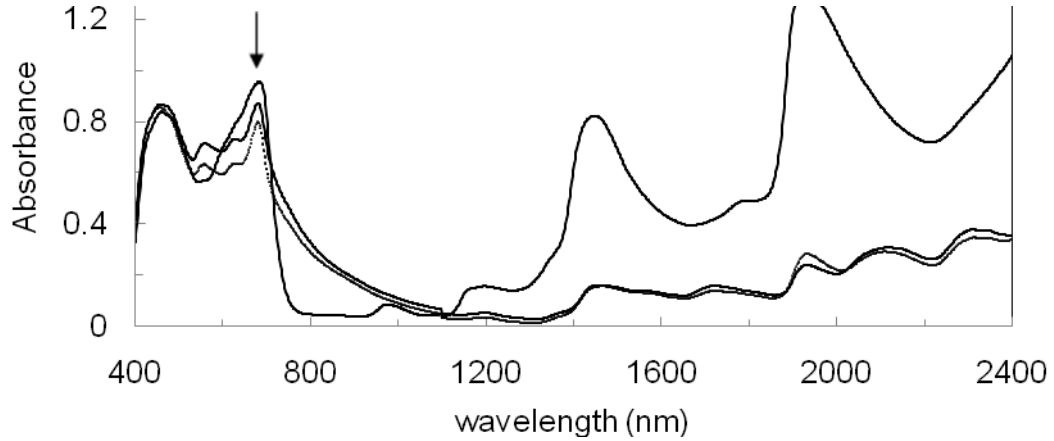


Figure 5. 2. NIRs reflectance spectra of fresh (**—**), dry (- - -) and dry ground (·····) stevia leaves using the NIRS 6500 unit. Arrow indicates chlorophyll absorption peak.

Experiment 2: leaf SG content

Partial least squares regression models were developed on leaf stevioside and rebaudioside A content, initially using four wavelength windows for second derivative spectra of fresh, dry and ground dry stevia leaves (a visible wavelength region, 400-700 nm; a SW-NIR region, 700-1100 nm; a NIR region, 1100-2500 nm; and a combined region, 700-2500 nm). Population statistics for the groups employed in this exercise are presented in Table 5.1.

Of the three leaf formats, the calibration statistics for total SG concentration were best when the model involved spectra of dry ground leaves, followed by dry leaves and then fresh leaves (Table 5.2). The trend was consistent across all wavelength regions and the three spectrophotometers used. Of the four wavelength regions considered, the 1100-2500 nm window was optimal (Table 5.2). An extension of the 1100-2500 nm window into the SW-NIR region (700-2500 nm) did not improve model performance. Calibration models developed using the SW-NIR region (700-1100 nm) alone were poor.

Table 5. 1. Population statistics (sample size, mean and standard deviation) of total SG, stevioside and rebaudioside A content in leaf material (% w/w) of populations used for the calibration and validation of models for each NIR instrument. The same samples were used for all leaf formats and instruments, with differences in sample size due to loss of sample or spectra. A subset of samples were available for the FTNIR work.

Sample set	Leaf Format	Instrument	Total SG			Stevioside		Reb A	
			n	mean	SD	mean	SD	mean	SD
Calibration	Fresh	Insight-MMS1	105	9.6	*3.4*	7.3	*2.6*	2.3	*1.8*
		NIRS 6500	105	9.6	*3.4*	7.3	*2.6*	2.3	*1.8*
		FTNIR	52	9.0	*2.7*	7.0	*2.2*	2.0	*1.4*
	Dry	Insight-MMS1	91	9.7	*3.3*	7.3	*2.5*	2.4	*1.8*
		NIRS 6500	91	9.7	*3.3*	7.3	*2.5*	2.4	*1.8*
		FTNIR	56	8.8	*2.7*	6.8	*2.2*	2.0	*1.4*
	Dry Ground	Insight-MMS1	83	9.8	*3.4*	7.3	*2.6*	2.5	*1.9*
		NIRS 6500	91	9.7	*3.3*	7.3	*2.5*	2.4	*1.8*
		FTNIR	56	8.8	*2.7*	6.8	*2.2*	2.0	*1.4*
Prediction	Dry Ground	NIRS 6500 & FTNIR	55	10.0	*1.7*	4.4	*2.3*	5.7	*2.2*

Table 5. 2. Cross validation statistics (correlation coefficient, R_{CV}, and root mean square error of cross validation, RMSECV) for a PLS regression models based on total SG as determined by HPLC and four wavelength ranges of d^2(log 1/R) spectra collected with three instruments, and using the same leaf material in three formats. Units of RMSECV are % w/v. Results for models with R >= 0.87 are in bold. *FTNIR wavelength windows were 800-1100 nm and 1100-2490 nm. Results with a R > 0.86 are bolded.

Leaf Format	NIR Instrument	400-700 nm		700-1100 nm*		1100-2500 nm*			700-2500 nm	
		R_{CV}	RMSECV	R_{CV}	RMSECV	PC	R_{CV}	RMSECV	R_{CV}	RMSECV
Fresh	InSight-MMS1	0.10	*3.34*	0.31	*3.15*		-	-	-	-
	NIRS 6500	0.40	*3.05*	0.66	*2.68*		0.67	*2.53*	0.63	*2.61*
	FTNIR	-	-	0.36	*2.64*		0.54	*2.32*	0.58	*2.22*
Dry	InSight-MMS1	0.44	*3.14*	0.49	*2.93*		-	-	-	-
	NIRS 6500	0.52	*2.84*	0.56	*2.79*		0.79	*2.07*	0.77	*2.12*
	FTNIR	-	-	0.2	*2.79*		0.78	*1.73*	0.52	*2.34*
Dry Ground	InSight-MMS1	-0.16	*3.54*	0.37	*3.18*		-	-	-	-
	NIRS 6500	0.54	*2.79*	0.56	*2.76*	**9**	**0.87**	***1.63***	0.82	*1.87*
	FTNIR	-	-	0.05	*3.00*	**2**	**0.86**	***1.39***	0.65	*2.09*

Calibration model performance with respect to stevioside and rebaudioside A content was also optimal with dry ground leaves, relative to dry intact leaf or fresh leaf, although the correlation coefficients were not as high as that obtained with total SG (Table 5.3), and not acceptable for practical use.

Only a satisfactory calibration model R of 0.87, for sorting a population into two groups, was achieved even with the analysis of total SG content of dry ground leaf material using the 1100-2500 nm range.

Table 5. 3. Calibration statistics (correlation coefficient, R_{CV} and root mean square error of cross validation, RMSECV) for models of stevioside and rebaudioside A (% w/dw) of the d^2(log 1/R) spectra of dry ground stevia leaf. Calibration was made using the window of 1100-2500 nm. Spectral data was common to Table 5.2.

						1100-2500 nm	
Leaf format	**Attribute (% w/w)**	**NIR Instrument**	**n**	**mean**	**SD**	**R_{CV}**	**RMSECV**
Dry Ground	stevioside	NIRS 6500	91	7.33	*2.54*	0.67	*1.87*
		FTNIR	56	6.80	*2.20*	0.78	*1.36*
	rebaudioside A	NIRS 6500	91	2.42	*1.83*	0.42	*1.69*
		FTNIR	56	1.97	*1.42*	0.33	*1.36*

To optimize the model developed for the 1100-2500 nm window, different data processing methods were trialed, with models based on log of the inverse of reflectance (log 1/R) spectra, first and second derivative spectra of log 1/R, and second derivative spectra with multiplicative scatter correction (MSC). Model correlation coefficients of R = 0.86, 0.83 and 0.76 were achieved with second derivative, first derivative and the raw spectra, respectively (Table 5.4). The data pre-treatment of second derivative was adopted for all subsequent analyses.

Table 5. 4. Calibration statistics (correlation coefficient, R_{CV} and root mean square error of cross validation, RMSECV) for models of total SG (% w/dw) of spectra of dry ground stevia leaf, using different spectral pre-processing methods. Calibration was made using the window of 1100-2500 nm.

		log 1/R		d(log 1/R)		d2(log 1/R)		d2(log 1/R) + MSC	
Leaf Format	**Instrument**	**R_{CV}**	**RMSECV**	**R_{CV}**	**RMSECV**	**R_{CV}**	**RMSECV**	**R_{CV}**	**RMSECV**
Fresh	NIRS 6500	0.58	*2.76*	0.61	*2.69*	0.67	*2.53*	0.68	*2.50*
	FTNIR	0.57	*2.31*	0.62	*2.18*	0.54	*2.32*	0.52	*2.35*
Dry	NIRS 6500	0.72	*2.32*	0.76	*2.15*	0.79	*2.07*	0.80	*2.00*
	FTNIR	0.75	*1.90*	0.76	*1.78*	0.78	*1.73*	0.78	*1.70*
Dry ground	NIRS 6500	0.76	*2.16*	0.83	*1.85*	**0.87**	***1.63***	**0.87**	***1.61***
	FTNIR	0.80	*1.67*	0.86	*1.40*	**0.86**	***1.38***	**0.87**	***1.35***

The regression coefficients of the PLS model developed for the 1100-2490 nm window were examined in an attempt to identify wavelength regions carrying little information relevant to SG concentration. Features in the plot of coefficients (Fig. 5.3) were sharp, preventing a clear recommendation of such regions. The highest coefficients occurred at 1422, 1634, 1896 and 2242 nm.

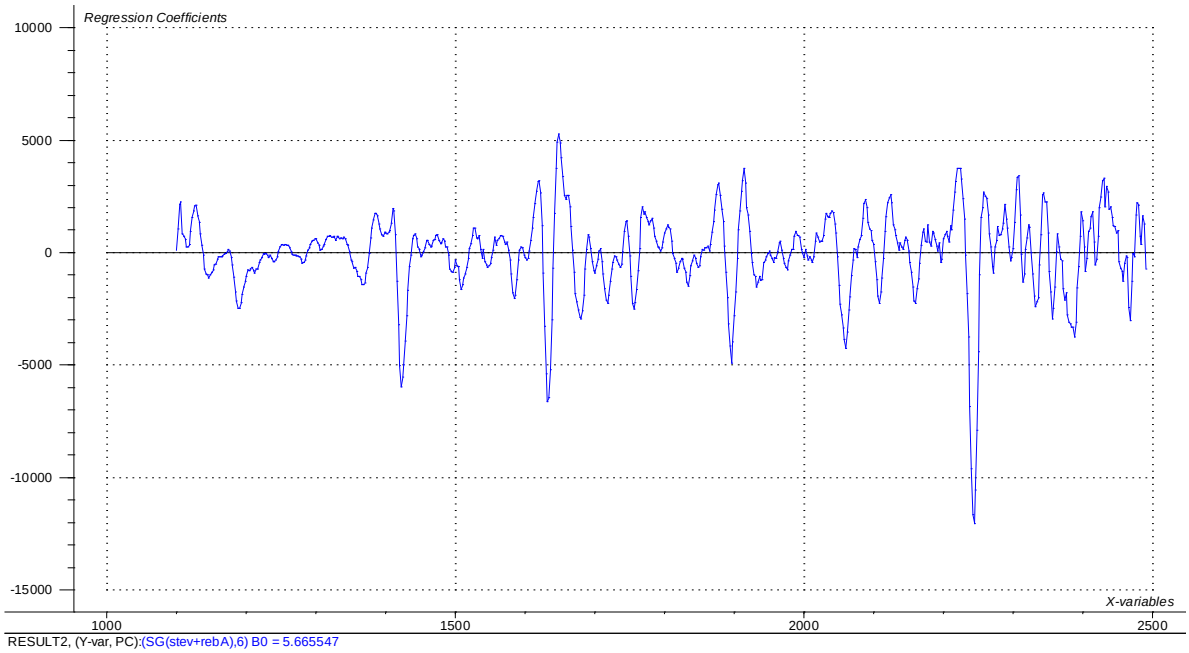


Figure 5. 3. PLS regression coefficients for the model based on NIR6500 spectra (1100-2500 nm region) of dry ground stevia leaves.

A 'brute force' method involving development of a PLS model for every combination of start and end wavelengths between 1100 and 2450 nm was used to empirically identify the best wavelength region to use. Lowest RMSECV and RMSEC values, with reasonable number of PLS factors, was achieved using a wavelength region beginning between 1450 and 1500 nm and ending around 2250 nm. Minimum RMSECV was achieved using the region 1520-1804 nm (Fig. 5.4).

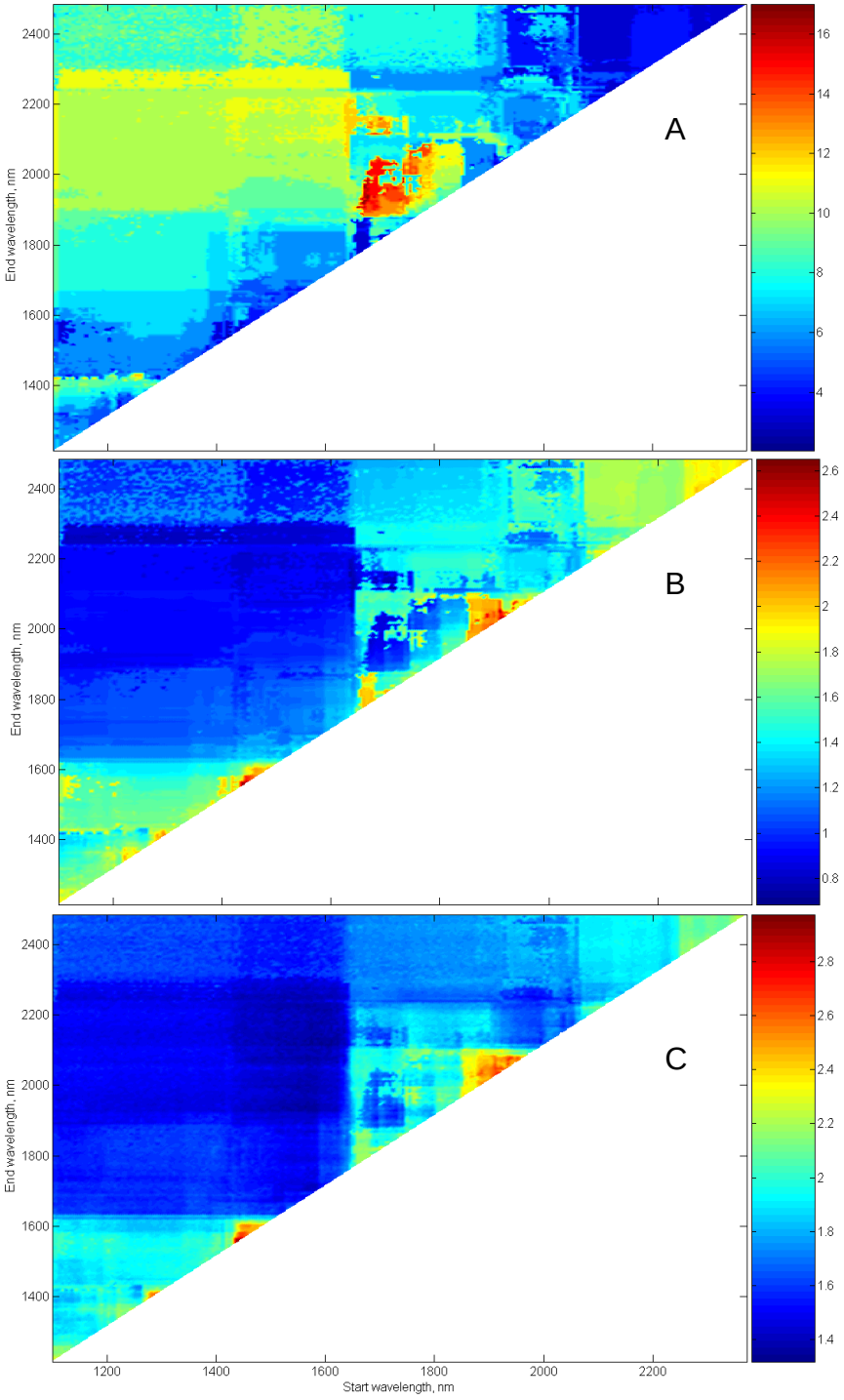


Figure 5. 4. Comparision of different wavelength regions used in PLS regression models on total SG content, based on d2(log1/R) spectra of dried ground leaf, collected with the NIR6500 instrument. Results for (A) Number of PLS factors, (B) RMSEC and (C) RMSECV re presented for PLS regression models with different start (x axis) and end (y axis) wavelengths for total SG concentration of dry ground leaf material. Bar to right of each panel is a colour scale for the respective attribute (number of factors, RMSEC and RMSECV).

PLSR model validation

A totally independent set of 55 stevia samples were scanned as dry ground powder and used as a prediction set. Calibration models based on second derivative spectra of the NIRS 6500 and FTNIR Nicolet Antaris units were used in prediction of the total SG, stevioside and rebaudioside A content of the independent samples, using two wavelength regions (1100-2500 nm and 1520-1804 nm).

Overall, the prediction statistics for total SG were improved when the model was based on the narrowed region of 1520-1804 nm, relative to that for the 1100-2500 nm region (Table 5.5). However, while prediction of total SG was good in the 1520-1804 nm window, prediction for stevioside and rebaudioside A content was poor (Table 5.5).

Table 5. 5. Summary of correlation (R) and root-mean-square-error-of-prediction (RMSEP) for the prediction of total SG in two wavelength windows, and stevioside and rebaudioside A leaf concentration (% dw) in one wavelength window, of 55 independent leaf samples. Prediction was based on the calibration set of the NIRS 6500 (n = 91) unit and the FTNIR Nicolet Antaris (n = 56) unit reported in Tables 4.3 and 4.4. Bold numbers correspond to R and RMSEP of the best prediction of total SG.

Instrument	1100-2500 nm		1520-1804 nm					
	Total SG		Total SG		Stevioside		RebA	
	R	RMSEP	R	RMSEP	R	RMSEP	R	RMSEP
NIRS 6500	0.63	5.73	**0.85**	**1.08**	0.08	4.19	0.26	3.33
FTNIR Nicolet Antaris	-0.03	5.46	0.74	2.58	0.21	5.82	0.70	3.44

Overall, the prediction statistics for total SG, stevioside or rebaudioside A were improved when the model was based on the narrowed region of 1520-1804 nm, relative to that for the 1100-2500 nm region (Table 5.5). However, prediction of stevioside and rebaudioside A content was unacceptable for product grading, using either instrument and either window (Table 5.5), at

least for this population. For total SG, the R of the best model achieved (with the NIRS 6500; 0.85) was nearly, but still not adequate to allow sorting of the population into the two categories, low SG and high SG (criterion of 0.87 set).

Comparison of the result of models based on the 1520-1804 nm region using the NIRS 6500 instrument (R = 0.85) and the FTNIR unit (R = 0.74) is not strictly valid, in that while the mean values for total SG, stevioside and rebaudioside A were comparable between the two calibration sets, more samples, with greater variability, were used with the NIRS 6500 based model (Table 5.1).

The prediction test was harsh in the sense that the validation set was very independent of the calibration set, being samples of different genotype, grinding method, SG range etc. For example, the poor prediction result for stevioside and rebaudioside A may be partly ascribed to the difference in the mean values of stevioside and rebaudioside A between the calibration set and prediction sets (Table 5.1). The calibration set had higher levels of stevioside than rebaudioside A while the reverse was true for the prediction set. Further, the range of the stevioside and rebaudioside A values of the calibration and prediction set also did not overlap.

A less rigorous approach for testing the prediction performance was also adopted, in which samples from the original calibration and prediction sets were combined and randomly allocated into two sets which were then used interchangeably either as a new calibration or prediction set. Both sets were comparable in terms of the mean values of total SG, stevioside and rebaudioside A (Table 5.6). An R > 0.87 was achieved in prediction of total SG for both instruments using the 1502-1804 nm window, and for the NIR6500 unit in the 1100-2500 nm region (Table 5.7). Prediction of stevioside and rebaudioside A was still unacceptable (R < 0.87), except for the case of rebaudioside A using FTNIR spectra (within the 1520-1804 nm region).

Table 5. 6. Population statistics of the first and second half of the combined calibration and prediction set for the NIRS 6500 and FTNIR Nicolet Antaris unit.

			Total SG		Stevioside		Reb A	
NIR Instrument	Sample set	n	x (mean)	SD	x (mean)	SD	x (mean)	SD
	1	73	9.79	*2.8*	6.04	*2.8*	3.76	*2.80*
	2	73	9.93	*2.9*	6.41	*2.8*	3.52	*2.3*
NIRS 6500	1 & 2 combined	146	9.86	*2.8*	6.22	*2.8*	3.64	*2.5*
	1st half	56	9.41	*2.4*	5.57	*2.6*	3.85	*2.5*
	2nd half	55	9.40	*2.4*	5.66	*2.5*	3.75	*2.7*
FTNIR Antaris	1 & 2 combined	111	9.41	*2.4*	5.61	*2.5*	3.80	*2.6*

Table 5. 7. Prediction statistics for total SG, stevioside and rebaudioside A using a prediction set of unique spectra, but of the same population that the calibration set was drawn from. Results for the NIRS 6500 and FTNIR Nicolet Antaris are presented. Results for models with R > 0.87 are in bold.

		1100-2500 nm		**1520-1804 nm**					
	Calibration Set	**Total SG**		**Total SG**		**Stevioside**		**Reb A**	
Instrument		**R**	**RMSEP**	**R**	**RMSEP**	**R**	**RMSEP**	**R**	**RMSEP**
NIRS 6500	1	**0.89**	***1.31***	**0.88**	***1.40***	*0.76*	*2.06*	0.68	*1.76*
	2	**0.89**	***1.32***	**0.87**	***1.46***	*0.74*	*1.89*	0.80	*1.76*
FTNIR Antaris	1	0.84	*1.31*	**0.88**	***1.17***	*0.77*	*1.64*	**0.87**	***1.35***
	2	0.85	*1.26*	**0.87**	***1.25***	*0.85*	*1.4*	0.84	*1.37*

Another exercise was undertaken to explore the use of relatively low cost, field portable instrumentation, towards enabling in field measurements. The Zeiss MMS1 (400-1100 nm) and the Zeiss InGaAs (900-1700 nm) detectors were used alternately with the optical assembly and electronics of the Insight unit. A total of three calibration models were developed for comparison. Integration times were set to optimize signal strength (and thus signal to noise ratio). Model 1 was based on a subset of the original calibration samples (n = 30), model 2 was based on a subset of the prediction samples (n = 47) and model 3 was based on the combination of these samples (n = 77) (Table 5.8).

Calibration statistics were superior for the 900-1668 nm range obtained using the InGaAs detector relative to the shorter wavelength ranges, however the model R did not exceed 0.87 for any of the independent variable (total SG, stevioside or rebaudioside A) (Table 5.8).

Table 5. 8. Calibration statistics (correlation coefficient, R, and root-mean-square-error-of-prediction, RMSEP) for models based on subset samples from the original calibration set (Model 1), the original prediction set (Model 2) and their combination (Model 3). Models were developed from spectra collected using the Insight (Zeiss MMS1) and the Insight (InGaAs) unit. RMSECV units are % w/w (dw ground leaf).

					InSight - MMS				InSight - InGaAs	
					400-700 nm		700-1100 nm		900-1668 nm	
Attribute	**Model**	**n**	**Mean**	**SD**	**R**	**RMSECV**	**R**	**RMSECV**	**R**	**RMSECV**
Total SG	1	30	9.06	*2.68*	0.58	*2.35*	0.58	2.15	0.78	*1.68*
	2	47	9.76	*1.58*	0.18	*1.57*	0.64	1.26	0.82	*0.89*
	3	77	9.49	*2.09*	0.18	*2.07*	0.46	1.92	0.71	*1.54*
Stevioside	1	30	7.06	*2.34*	-0.39	*3.24*	0.48	2.04	0.55	*2.00*
	2	47	3.59	*1.18*	0.3	*1.13*	0.17	1.17	0.21	*1.16*
	3	77	4.94	*2.41*	0.68	*1.91*	0.67	1.81	0.69	*1.75*
Reb A	1	30	2.00	*1.49*	0.03	*1.91*	0.48	1.29	0.11	*1.51*
	2	47	6.17	*1.98*	0.01	*2.08*	0.45	1.87	0.7	*1.45*
	3	77	4.54	*2.72*	0.54	*2.47*	0.72	1.91	0.79	*1.68*

Discussion

Wavelength region selection

A robust model capable of prediction of SG content of stevia leaf material is of considerable practical value. Ideally instrumentation would utilize the Herschel wavelength range, as the Si detectors used for this region are cheaper than the detectors used for longer wavelengths. Also, biological sample absorptivity is low in the Herschel region, enabling use of longer pathlength optical geometries.

The potential for NIRs to be used in assessment of SG content of fresh leaves in the field (i.e. with no sample preparation) is expected to be impacted negatively by several factors: (a) the overlay of the prominent water absorption peaks, particularly as leaf water content varies in the fresh product; (b) shifting of the water peaks associated with temperature; (c) the overlay of absorption features due to other materials in the leaf (e.g. sucrose). The NIRS 6500 calibration statistics for total SG content of ground stevia leaves (R = 0.87, RMSECV = 1.63) was superior to that of dry intact leaves (R = 0.79, RMSECV = 2.07) and that of fresh leaves (R = 0.67, RMSECV=2.53). The spectra of dry intact leaves were 'simplified' by the removal of water. Grinding the dry leaves allowed the sample to be presented homogeneously.

In practice, models of SG content in fresh leaf material were unacceptable using any of the instrument - wavelength range trialed. However, there is promise for further optimization of sample geometry and wavelength range (within 1100-2500 nm). An attempt was made in this direction with the use of the Zeiss MMS NIR unit, however the upper limit of 1700 nm on this instrument is not optimal for this application. Other potential instrumentation is discussed below.

Good model results were obtained with dry leaf material using both the NIR6500 and the Antaris FTNIR instruments, but only with the longer

wavelengths. However, in the current study, a single set of acquisition conditions was implemented when acquiring spectra across a broad wavelength range (with the Foss 6500 and Thermo Nicolet) instruments. These conditions were optimized for the 1440 nm OH band (in solutions and in fresh leaf material; absorbance around 1 unit), with the 1900 nm feature allowed to go 'off scale' (i.e. level at light received at detector is not measurable, resulting in 'saturated' absorbance). These conditions were not optimal for signal at wavelengths below 100 nm. Admittedly, however, the third overtone features in the Herschel region are typically broad and overlapped, with 'cleaner' information available at longer wavelengths.

Spectra of the shorter wavelength regions was also collected using a MMS1 (Si photodiode array) and a MMS NIR (InGaAs photodiode array) using InSight interactance optics, with acquisition conditions optimized for these wavelength regions. Model performance was also not acceptable using these units.

The 400-700 nm (visible) region was considered in case of a secondary correlation, e.g. between chlorophyll, which has an absorption at around 420 and 680 nm (Gitelson, Buschmann *et al.* 1999) and SG. However, model performance using this region was poor, with evidently little relationship between SG and chlorophyll concentration.

PLSR model statistics were slighter better for the 700-1100 nm region, relative to the 400-700 nm region (Table 5.2). However, only the 1100-2500 nm region supported models with R >0.85. The optimal window for modeling of total SG content of dry ground stevia leaf identified using a PLS regression models of all regions in a Matlab-based window-selection program was 1520-1804 nm. This result contrasts to the wavelengths selected through multiple linear regression by Nishiyama and Alvarez (1992) (1380, 1660, 1816, 2276, 2300 and 2404 nm) for stevioside concentration of dry ground leaf, and the 2042 to 2240 nm optimal window for total SG content identified by Hearn and Subedi (2009) using iterative PLSR. Correlation was optimal

using the second derivative spectra, consistent with the report of Nishiyama and Alvarez (1992) and Hearn and Subedi (2009).

The current study is based on a greater sample base (n = 91) compared to the earlier studies. The 1520-1804 nm region corresponds to absorption of methyl groups (CH_3, CH_2, CH) in the first overtone region, most likely due to the peripheral glucose units attached to the aglycone structure of stevioside (MW = 804.87 $g.mol^{-1}$) and rebaudioside A (MW = 967.01 $g.mol^{-1}$).

The 1520-1804 nm window was robust in predicting the total SG of an independent sample set. Prediction of stevioside and rebaudioside A was not as good in the same region and may have been influenced by the difference in the mean values of stevioside and rebaudioside A between the calibration and the prediction set. This, being an extreme case, demonstrates the necessity for a diverse population of samples that covers a wide range of stevioside and rebaudioside A concentration. Indeed, when the calibration and prediction set were combined and re-distributed evenly to two sets with comparable stevioside and rebaudioside A concentration (Table 5.7), prediction of one group using the other as a model resulted in a much better prediction performance. With the varying levels of stevioside and rebaudioside A, total SG would seem to be a more stable value for NIRs calibration. For practical application, the calibration set should be expanded to include stevia leaves of different varieties, growth conditions and sample grinding methods, with the aim of improving the robustness of the model in prediction of new sample sets.

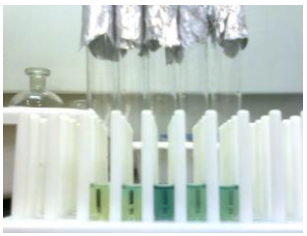
The NIRS 6500 and FTNIR Antaris are not appropriate for field use, in terms of cost and portability. However, the lower cost and more portable InSight-MMS1 and MMS NIR do not span the 1520-1804 nm window. The Phazir Polychromix (http://www.polychromix.com/) offers a handheld format with two instrument ranges available (1000-1800 nm and 1600-2400 nm), with pixel resolution of 8 nm and optical resolution of 12 nm. These units use a micromechanical device (MEMS) as a wavelength filter, with a single detector element (InGaAs or presumably PbS). Specifications on signal to

noise or repeatability are not given, however a RMSECV of 0.5 Brix is given for a model based on spectra of an intact apple.

Conclusion

Calibration of total SG in ground leaf material was optimal within the 1100-2500 nm region particularly at the 1520-1804 nm window. Further work with a portable format NIR spectrometer capable of working in this wavelength region, such as the Polychromix, is recommended.

Chapter 6



Methods development:

Steviol glycoside quantification using anthrone colorimetry

Abstract

The feasibility of using the anthrone reaction for the estimation of steviol glycosides (SG) in stevia leaf extracts was explored by developing calibrations for sucrose and glucose as representative sugars present in stevia leaves and stevioside and rebaudioside A as SGs. Calibrations were linear up to 0.06 μmol for stevioside (R^2 = 0.993, RMSEC = 0.002 μmol) and rebaudioside A (R^2 = 0.996, RMSEC = 0.002 μmol), respectively. However, the absence of specific absorption peaks for stevioside and rebaudioside A within the 200-800 nm made the anthrone reaction impractical for use on stevia leaves.

Introduction

The traditional methods for measurement of SGs (SG) such as HPLC involve time-consuming sample preparation, purification, and analysis and all the costs associated with equipment and labor. To assist in quality control programs (e.g. to pay growers on SG content, rather than leaf biomass), a rapid, inexpensive method for SG estimation is desirable. Near infrared spectroscopy is a candidate technique, of particular relevance to direct estimation on fresh or ground leaf material (as explored in Chapter 4 & 5).

Alternatively, while still requiring SG extraction, a simple colorimetric method would be of practical interest to the industry.

Colorimetry can be used for quantification of sugar molecules which are weakly detected in the UV-Vis region for lack of a chromophoric structure. In practice, the sugar molecules are first cleaved to monosaccharides then dehydrated with loss of water molecules in strong acid to form furfural (if pentose) or 5-hydroxy methyl furfural (if hexose), which reacts with a colorimetric reagent, such as anthrone or phenol-sulphuric acid reagent, to form a colored solution (Fig. 6.1) (Ahmed 2005). With the anthrone reagent, a blue-green color is formed, while with the phenol-sulfuric acid reagent, a yellow-gold solution is produced (Yemm and Willis 1954; Dubois, Gilles *et al.* 1956; Ahmed 2005). The color change is then quantified by measuring the absorbance of the solution at a specific wavelength. With the anthrone reagent, maximum absorbance is at 620 nm while with the phenol-sulphuric acid, maximum absorbance is at 490 nm (Nielsen 2003).

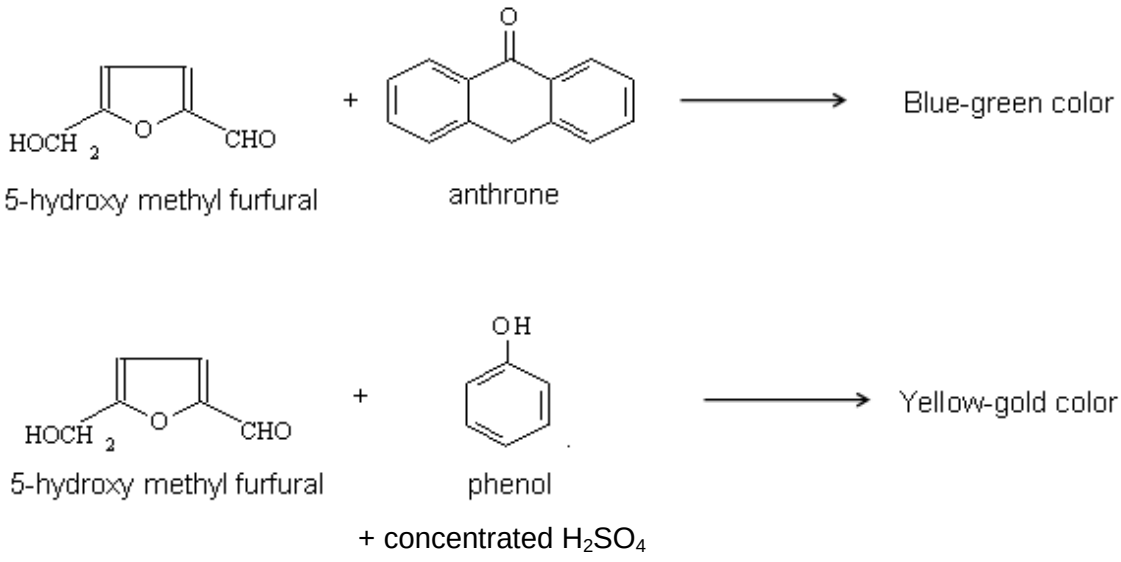


Figure 6. 1. Colorimetric reaction with anthrone and phenol of 5-hydroxy methyl furfural derived from sugar dehydration.

Generally, the phenol-sulfuric acid test is preferred over the anthrone test when quantifying pentose sugars because of the more stable color change. Bailey (1958) attributed the less stable color of pentose sugars with anthrone to the presence of excess anthrone which destroys the chromophore

complex, reducing the color change. Bailey (1958) recommended use of a low concentration of anthrone (0.01% w/v) to quantify samples containing 20-100 μg pentose sugars (e.g. D-xylose, L-arabinose and D-ribose).

Calibration with sugar standards is undertaken to determine the concentration range within which a linear absorbance is observed, with each type of sugar having a characteristic color development per unit of sugar mass (i.e. slope of Absorbance – sugar concentration regression). Critical to the color development are the duration of the reaction period, the temperature at which colorimetric reaction occurs as well as the composition of the reagent to ensure comparable colour development in standards and samples. With the phenol-sulfuric acid reagent, Buysse and Merckx (1993) were able to achieve equal color absorbance for sucrose, glucose and fructose standards diluted in 80% ethanol (v/v) by mixing with 28% (w/w) phenol reagent and allowing to stand at room temperature for 15 min. The calibration was linear within the concentration range of 20-80 μg mL^{-1} (Buysse and Merckx 1993). With the anthrone reagent, Bonting (1954) was able to quantify both glucose and fructose at microgram quantities (0.2 to 2.0 μg) by using different reaction conditions. Standard solutions containing both glucose and fructose (up to 50 μg mL^{-1} each) were quantified not only for total sugars but for individual sugars by allowing 25 μL of the standards to react with 50 μL of 0.2% anthrone reagent in both room temperature and boiling temperature (100 °C) for 10 min. Color change in the former was used to quantify fructose while color change in the latter was used to quantify both glucose and fructose (Bonting 1954).

The use of phenol-sulfuric acid to assess SG concentration has been explored by Nishiyama and co-workers (1991), with these authors advocating the use of phenol-sulfuric acid test (Dubois, Gilles *et al.* 1956) for estimation of leaf SG. A positive correlation (TC = 0.93 ST + 7.83) between the concentration of stevioside (ST; $mg.L^{-1}$) determined by HPLC and soluble carbohydrates (TC) (expressed in glucose units; $mg.L^{-1}$), as determined by phenol-sulfuric acid test, was observed. The leaves had an average

composition of 25.3% w/w carbohydrates (colorimetric assay) and 15.4% stevioside (HPLC assessment) (leaf dw basis) (Nishiyama, Kusumoto *et al.* 1991). Thus for the phenol-sulfuric acid test to be used in assay of leaf SG content, leaf soluble sugars would need to remain constant. The assumption of a constant pool of leaf soluble carbohydrates is, however, unlikely, with leaf sugar content typically varying diurnally in response to photosynthetic conditions and source-sink balance (further explored in Chapter 6 and 7).

Differences in the absorption spectra of anthrone-SG complexes compared to complexes involving the simple sugars could allow use of the colorimetric assay for assessment of SG in simple extracts of stevia leaf. However, no reports of the absorption spectra of the reaction complexes were found in the literature.

Methodology

Anthrone reagent and standards preparation

Pure standards were used for calibration (D-glucose anhydrous Pronalys reagent from Biolab; sucrose ACS reagent from Biolab; and stevioside, from Wako). For rebaudioside A, a commercial sweetener, Soolite^{TM} (99% rebaudioside A), was used in place of a pure standard. Other chemicals used for the anthrone reagent include anthrone lab reagent (Ajax Chemicals), analytical grade sulphuric acid (AnalR BDH Laboratory supplies) and analytical grade ethanol (LabServe Pronalys from Biolab).

Crystalline anthrone (0.4 g) was dissolved in 16 mL of ethanol and 60 mL of deionized water. Concentrated sulfuric acid (200 mL) was then added slowly into the solution while immersed in an ice bath, and then mixed until the anthrone crystals were completely dissolved, in approximately 3-5 minutes.

Aqueous standard solutions of glucose (0.2, 0.3, 0.4, 0.6, 0.7, 0.9 and 1.1 mM), sucrose (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM), stevioside (0.05, 0.1, 0.2, 0.4, 0.6 mM) and rebaudioside A (0.05, 0.1, 0.2, 0.4, 0.8 mM) were prepared.

Anthrone assay

With the glucose and sucrose standards, a 200 μL aliquot of sample was mixed with 1 mL of the anthrone reagent in a 5 mL test tube. With the SG standards, only 100 μL aliquot was needed. After vortex mixing, test tubes were covered with foil and immersed in a boiling water bath for 7 min. Solutions were then quickly cooled to room temperature by placing in a water bath. Tubes were given a final vortex mix before dispensing to a 1 mL cuvette, with absorbance assessed at 620 nm relative to the anthrone reagent alone using a CaryWin UV-Vis spectrophotometer. An absorption spectrum (200-800 nm) was acquired of anthrone complexes of equimolar standards (0.4 mM) of glucose, sucrose, stevioside and rebaudioside A.

Results and Discussion

The absorbance of the anthrone complexes of glucose (R^2 = 0.991, RMSEC = 0.007, SD = 0.07) and sucrose (R^2 = 0.993, RMSEC = 0.003, SD = 0.04), stevioside (R^2 = 0.993, RMSEC = 0.002, SD = 0.02) and rebaudioside A (R^2 = 0.996, RMSEC = 0.002, SD = 0.03) were linear up to the maximum level of analyte tested (0.22, 0.12, 0.06 and 0.06 μmol respectively) (Fig. 6.2).

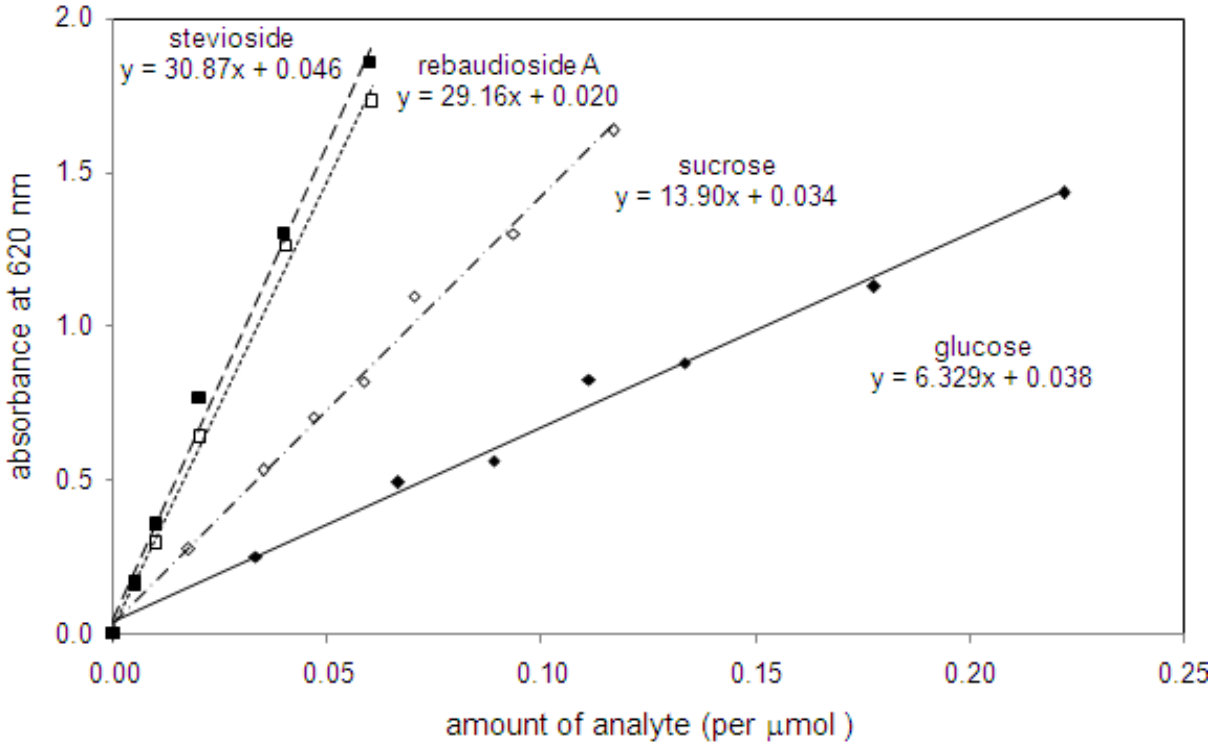


Figure 6. 2.. Absorbance at 620 nm for anthrone complexes of stevioside (--■--) (R^2 = 0.993), rebaudioside A (---□---) (R^2 = 0.996), sucrose (--◇--) (R^2 = 0.993) and glucose (—◆—) (R^2 = 0.991) absorbance at 620 nm after reacting with the anthrone reagent. Each absorbance data was averaged from triplicate runs. X axis was expressed as amount of analyte in µmol present in the assay.

The extent of colour development ($A_{620\ nm}$) per µmol of analyte was greatest in stevioside and rebaudioside A, followed by sucrose then glucose (Fig.6.2). The difference in colour intensity can be ascribed to the number of hexose molecules in the analyte. In sucrose, two hexose units (glucose and fructose) are present and cleaved before reacting with the furfural derivative which then absorbs at 620 nm. This resulted in twice as much absorbance relative to the same molar quantity of glucose. With rebaudioside A, a total of four glucose units were cleaved from the steviol structure which then reacted with the anthrone reagent (Dacome, Da Silva *et al.* 2005). Indeed absorbance of an equimolar amount of rebaudioside A resulted in an absorbance at 620 nm which was four times stronger than the glucose standard (Fig. 6.2). However, the absorbance of stevioside (which has 3 glucose unit attached to the steviol compound) was similar if not slightly higher than rebaudioside A. This

result is contradictory to the lesser number of glucose units in stevioside (3 glucose units per molecule).

The maximum amount of 0.06 μmol stevioside and 0.06 μmol rebaudioside A in the anthrone calibration is sensitive enough to measure the typical amount of stevioside and rebaudioside A in stevia leaves. For example, stevioside (MW = 804.87 g/mol) and rebaudioside A (MW = 967.01 g/mol) in a 100 μmol aliquot of the extract would be equivalent to 1.24 and 0.5 μmol, assuming a concentration of 10% and 5% leaf dw respectively. In both cases, an extraction protocol of 1 g of stevia leaves in 10 mL of water solvent is used. The leaf extract would even have to be diluted to 1/20th of the original concentration to fall within the 0.01 to 0.06 μmol calibration range.

However, the practicality of use of the anthrone reagent for analysis of SGs in leaf extracts depend on a lack of interferences, and particularly the ability of the method to differentiate between SGs and of simple sugars found in stevia leaves. Full scans (from 200-800 nm) of representative leaf sugars (sucrose and glucose) were obtained and compared with that of stevioside and rebaudioside A (Fig. 6.3). The absorption spectra of stevioside and rebaudioside A were, however, similar to that of the dominant leaf sugars, glucose and sucrose. All four compounds possessed an absorbance peak at 620 nm. Indeed, in testing the glucose concentration of two stevia leaf extracts from Chapter 7 with the anthrone reagent, measurements were slightly higher (by 44 and 86% for two samples) than that determined by enzymatic starch assay. Presumably, overestimation was due to colorimetric reaction of residual SGs that were left in the leaf pellets used for starch digestion.

Absorbance was proportional to the number of glucose units in the sugar molecule with sucrose (0.7448 AU) having about twice as much absorbance as glucose (0.3025 AU) while rebaudioside A (1.3327 AU) was four times that of glucose. Again, the result for stevioside (1.2827 AU) was higher than expected for its glucose content.

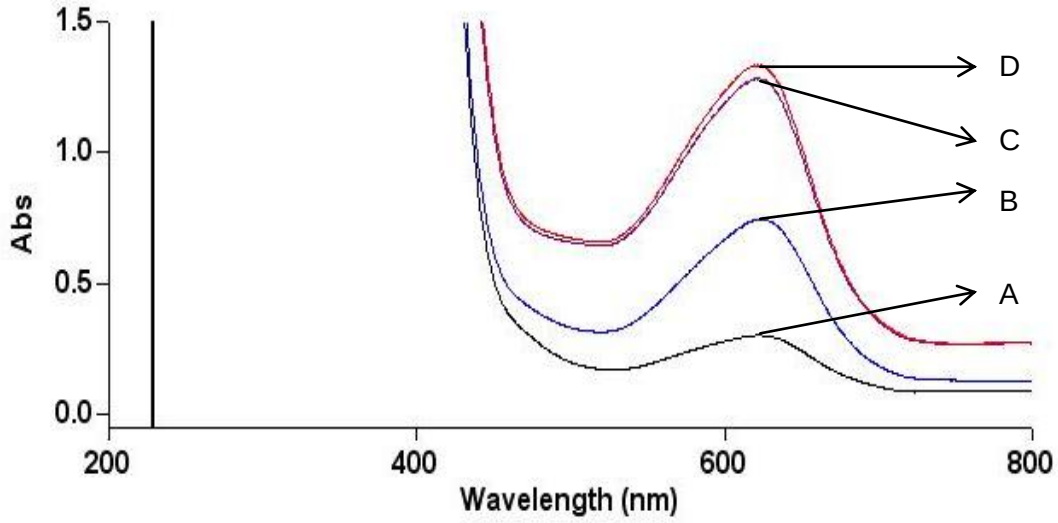


Figure 6. 3. Absorption spectra (200-800 nm) of anthrone complexes with (A) 0.03 μmol glucose, (B) 0.04 μmol sucrose, (C) 0.04 μmol stevioside and (D) 0.04 μmol rebaudioside A present in a standard assay condition.

The sensitivity of the anthrone method to SG relative to simple sugars, and the high level of stevioside and rebaudioside A within the leaf, favour the use of anthrone for quick SG estimation. However, this suggestion is viable only if the diurnal and seasonal variation in simple sugar concentration in leaves is low in absolute terms, relative to leaf SG content. Sucrose leaf concentration was observed to be more than double (from 4.8 to 13.2% dw) within 3 h of monitoring (Chapter 7) while starch concentration followed a diurnal cycle (Chapter 8). Therefore, use of anthrone reagent to determine SG in crude leaf extracts is not practical.

Conclusion

A linear increase in absorbance at 620 nm for anthrone complexes with stevioside and rebaudioside A was observed with samples containing up to 0.06 μmol of analyte. The colorimetric response of both SG was higher than that of sucrose and glucose making it a useful method for estimating SG concentration in samples with constant sugar concentration. With leaf samples, however, change in sugar concentration relative to a diurnal cycle of photosynthate production and transport makes the use of the anthrone reagent impractical for leaf SG estimation. Variation of assay conditions (e.g. temperature and composition of anthrone reagent) to achieve differential colour development with SG over simple sugars could be further explored.

Chapter 7

Rate of turnover of the steviol glycoside pool

Abstract

Change in steviol glycoside (SG) concentration with respect to leaf age and plant ontogeny was investigated by comparing stevioside and rebaudioside A leaf concentration of (upper) immature, (middle) fully expanded and (bottom) mature leaves of stevia plants of early vegetative to mid reproductive stages. In addition, a pulse-chase labeling experiment was conducted to investigate the rate of turnover of SGs in stevia leaves with respect to labeled soluble sugars in stevia leaves. SG concentration decreased with leaf age. SG turnover was slow with no ^{14}C labeling detected within a 24-hour chase period in either immature or mature leaves. These results are consistent with a slow rate of synthesis of SG from recent photosynthate, occurring predominantly in young leaves, with maintenance of this pool in mature leaves and a small loss in senescing leaves.

Introduction

SGs are secondary metabolites that are found in the leaves of *Stevia rebaudiana* Bertoni at about 10% leaf dw, although small amounts are also found in the flower, stem and roots (Bondarev, Sukhanova *et al.* 2003). The concentration of SG in leaf tissue has been observed to increase during the vegetative phase of the plant. Kang and Lee (1981) observed maximum stevioside concentration of dry leaf biomass during flower bud formation, but

before flower opening (at 90 mg.g dw^{-1}). After flowering, leaf stevioside declined to 78 mg.g dw^{-1} (Kang and Lee 1981). As a short day plant, stevia is induced to flower under short photoperiods (less than 14 h daylight) (Zaidan, Dietrich *et al.* 1980). Therefore, extension of the vegetative phase under daylength longer than 14 h should result in the maintenance of higher SG leaf concentration as a consequence of delayed flowering and possibly enhanced photosynthetic conditions.

SG leaf concentration differs spatially within the plant canopy. Bondarev et al (2003) demonstrated a difference in total SG leaf concentration between leaves from the top, middle and bottom canopy of a stem (at 13, 12 and 8 mg/g dw) for one clone. Kang and co-workers (1981) also observed this trend, showing that higher leaves in the canopy (above 20 cm from the ground) had higher stevioside content than leaves in the lower canopy (below 20 cm height). Rebaudioside A and rebaudioside C content was not monitored.

However the studies mentioned above fail to differentiate immature and mature leaves. If SG concentration is different with respect to leaf physiological age, then the relative proportion of these tissue will be relevant to SG concentration of leaves from different levels of the canopy or different plant ontogenetic stages.

The biochemical pathway of SG biosynthesis is considered to be localized in the chlorenchyma (Fig. 2.3). The first two enzymes, CPS and KS, of the pathway are localized to the chloroplasts (abbreviations defined in Fig. 6.1) (Richman, Gijzen *et al.* 1999). The subsequent reaction step is catalysed by KO in the endoplasmic reticulum (Fig. 2.3) (Humphrey, Richman *et al.* 2006). The subcellular location of the enzyme of the fourth step, KAH, is likely also to be in the endoplasmic reticulum (Hanson and White 1968; Kim, Sawa *et al.* 1996), although this remains controversial, with a chloroplast location proposed by Kim et al (1996). The pathway to the point of steviol formation is common to that of GA. Glucose units are added to steviol with the help of UGTs within the cytoplasm, producing the different SGs. The SGs are then

transported from the cytosol to the vacuole for storage (Fig. 2.3) (Brandle and Telmer 2007).

Although the pathway of SG synthesis in the leaf mesophyll cells has been quite well documented, the turnover of the SG pool in relation to leaf ontogeny has not yet been explored. The degradation pathway of SGs also remain to be defined.

The difference in SG concentration with leaf canopy section observed by Bondarev, Sukhanova et al (2003) suggests that SG synthesis is most active in young leaves, with storage in the vacuole as the leaf matures and eventual catabolism or simple loss (leaching) from the leaf as the leaf senescences. This scenario assumes a low turnover rate of the SG pool.

To test this hypothesis, a two-step approach was employed. Firstly, leaf SG concentration was assessed in relation to leaf age and plant developmental stage. Secondly, a (30 min) pulse - (24 h) chase labeling experiment was undertaken for both immature and mature leaves, with 14-C label distribution to sugars and SGs assessed.

Materials and Methods

SG comparison of leaf canopies

A total of 10 mature stevia plants (seven Fengtian and three 99-8 variety) chosen for their range of leaf SG concentration were allowed to regrow from a ratooning event. Plants were maintained inside a growth cabinet under controlled conditions (14 h light/10 h dark cycle, 25 °C temperature and 80% relative humidity) and were fertilized weekly with Aquasol (Hortico) using the recommended dilution (16 g in 10 L water). Plants were watered by an overhead sprinkler every 12 h for a duration of 10 minutes.

For each sampling event, leaves and buds/flowers (when present) were sampled from a single stem of each plant at different stages of the plant's ontogeny. Immature (upper), most recently expanded (middle) and mature

(bottom) leaves were distinguished from each other by leaf size comparison. Fully expanded leaves in the mid-section of the stem were assigned as middle leaves and leaves from above and below this section were assigned as immature (upper) and mature (bottom) leaves, respectively. Mature leaves of the stem were slightly rounder around the edges and slightly smaller than middle leaves. If any mature leaves were discoloured or shriveled they were separated into a senescent category. Immature leaves varied in leaf size. Both buds (during early reproductive stage) and flowers (during mid reproductive stage) were collected from each plant.

Samples were dried for 1-2 days at 65 °C before grinding using a microbead beater machine (Mini-Beadbeater-96, Biospec Products Inc USA). Samples were placed inside a 2 mL microtube (Sarstedt) with 3 pieces of chrome steel beads (3.2 mm in diameter) for rapid shaking for 1 min. SGs were then extracted from a 0.1 g aliquot of ground sample by adding 5 mL of milli-Q water and placing the solution in 70 °C shaking water bath for 30 min. Ground leaf pellet was separated from the water extract by centrifugation. Aqueous extract was poured into a separate container before proceeding with a second round of 5 mL water extraction. A 2 mL aliquot from the combined 10 mL water extract was filtered through a 0.45 μm filter before transferring to a 2 mL HPLC vial for HPLC analysis.

HPLC analysis was performed using an Agilent 1100 and a Zorbax NH_2 column (4.6 x 250 mm, 5-micron) connected to an Agilent Zorbax High Pressure Reliance Cartridge guard column (4.6 x 12.5 mm, 5-micron). The mobile phase was 80% acetonitrile (pH 5) buffered with 100 mL of 0.02 M glacial acetic acid and 200 µL of 0.1 M sodium hydroxide (aq) for every 500 mL of the mobile phase mix. Flow rate was 1 $mL.min^{-1}$ and injection volume was 5 µL. The UV detector was set at 210 nm using 360 nm as reference signal, and slit width at 4 nm. HPLC was calibrated with stevioside and rebaudioside A standards at different concentrations (0.2, 0.4, 0.6 and 1.25 mM).

^{14}C labeling

A total of 8 stevia plants were labeled with $^{14}CO_2$. The shoots of each plant were enclosed within a clear plastic bag (sealed around the stem with a 3M^{TM} sticky putty). Inside the plastic bag was a 1 mL vial containing 200 μl of saturated citric acid. A 100 μL aliquot of radioactive sodium bicarbonate (925 MBq in 12.5 mL solution) was injected through the plastic bag into the vial. The plants were kept inside the bag for 30 min afterwhich the plastic bags were removed.

Four plants (AM group) were labeled at 9 am and maintained under natural light for 24 h. Another four plants (PM group) were labeled at 4 pm and kept under natural light for 1.5 h before being placed inside a dark growth cabinet for 22.5 h. In each case the four plants were assigned to two pairs (i.e. two pairs of two plants).

Immature and mature leaves were sampled from each plant on six occasions (0.5, 1, 1.5, 2, 5 and 24 h after labeling). Small (28 mm^2) and large (123 mm^2) disc borers were used to sample tissue of known area from young and mature leaves respectively. Both leaf discs and leaf cutoffs were immediately placed separately into eppendorf tubes in liquid N_2 prior to storage at -70 °C. The lids of these tubes were perforated with a small hole to allow pressure venting. On three sampling events (0.5, 1.5 and 5 h from labeling), discs were combined for each pair of plants (i.e. two samples of immature and mature leaves, respectively). On the other three sampling events (1, 2 and 24 h from labeling), samples from all four plants within a group were combined. Thus a total of 18 samples were processed for each of the two labeling groups (AM and PM).

Carbohydrate and radioactivity measurement

Leaf cut-offs were defrosted from storage and dried for two days at 60 °C, with the dw measured. Each sample was oxidized and the labeled $^{14}CO_2$ trapped with 10 ml of carbon dioxide absorbent (Carbo-Sorb E, Perkin

Elmer). The Carbosorb was then flushed into a counting vial along with 10 ml of scintillation fluid (BCS GE Healthcare Life Sciences) for count analysis.

Total soluble count was determined from extracts of the leaf discs. Following addition of 1 mL of 80% ethanol to the eppendorf tubes, samples were ground manually using an eppendorf pestle. The tubes were then sealed and placed in 60 °C water bath for 1 h with intermittent mixing using a vortex mixer. After decanting the solvent, the pellet was washed twice with 0.5 mL of 80% ethanol. The first extract and subsequent wash were recombined and evaporated at 40 °C. The dehydrated extract was reconstituted with 650 μL of deionized water, then filtered through a cation (generated from an SP Sephadex suspension – Sigma Aldrich) and anion (generated from a QAE Sephadex suspension – Sigma Aldrich) exchange column followed by two additional 650 μL water injections to rinse the original container. The columns were then washed four times with 650 μL of water. A 50 μL aliquot of the extract was mixed with 1 mL scintillant (BCS GE Healthcare Life Sciences) for measurement of total soluble count using a scintillation counter. Remaining extract was then stored frozen at -80 °C before carbohydrate analysis.

The cation and anion exchange columns were prepared by soaking 50 g of the resin in 1 L of the appropriate solvent which was replaced five times within 2 days (SP Sephadex in 0.5 M ammonium sulphate and 0.5 M QAE Sephadex in sodium formate respectively). The resin was then rinsed with formic acid (5% formic acid for the cation suspension and 10% formic acid for the anion suspension) and 3 bed-volumes of water before final storage in 2% formic acid. About 1 mL of the suspension was injected into a plastic cartridge for use as a polymeric exchange column.

Total count from oxidized leaves and leaf extracts were measured using a Tri-Carb® Liquid 95 Scintillation Analyzer (Model 2910TR, Perkin Elmer). A conversion factor from cpm to dpm was determined using a set of standards with another scintillation counter (Wallac 1414 Liquid Scintillation Counter) with an established quench curve. When sample count rate was too high (i.e.

saturation reading) on the scintillation counter, samples were re-analysed in the scintillation counter using a 1:4 or 1:10 dilution of the original sample.

Individual sugar count and concentration

The radioactivity of individual sugars and SGs were determined separately, using a 10 μL aliquot of the disc extract and a Dionex ICS 3000 in both cases.

Sugar labeling was measured by separating the soluble sugars using a PA1 column and 8 mM NaOH as mobile phase (flow rate of 1 $mL.min^{-1}$), in combination with a photo-amperometric detector (PAD) calibrated with sugar standards (sucrose, glucose, fructose, myo-inositol, trehalose and galactose at 1, 5, 10, 15, 20, 35, 50 and 75 $\mu g.ml^{-1}$). Stevioside and rebaudioside A labeling was measured using an amine column (Prevail) flushed with 85% acetonitrile mobile phase (flow rate of 1 $mL.min^{-1}$) and a UV-Vis detector calibrated with inositol standards (1, 5, 10, 15, 20, 35, 50 and 75 $\mu g.ml^{-1}$). Stevioside standards run with the amine column were partly retained, with only 12% recovery observed for a 10 μL injection of 50 $mg.mL^{-1}$ stevioside standard. Radioactivity was measured downstream of the chemical detector using a Raytest Ramona 2000 with a glass solid scintillant cell (0.225 μl volume). The radioacitivity detector was calibrated with radioactive sucrose (10,000, 25,000, 50,000, 75,000, 100,000 and 150,000 dpm / 10μl).

Only samples with detected sugar labeling were measured for carbohydrate concentration by reinjecting a 10 μL aliquot of the disc extract which was diluted ten times. Stevioside was also measured qualitatively in the diluted aliquot using calibrations of the inositol peak.

Results and Discussion

Leaf SG concentration in relation to leaf age

Leaf SG concentration decreased with position of the leaf along the stem (i.e. with leaf age) (Fig. 7.1). SG was present in flower buds and flowers, but only at 15 and 6%, respectively, of the levels in immature leaf tissue (Fig. 7.1).

This distribution is consistent with either of two hypotheses: (a) that SG is synthesised predominately in developing leaves, with storage in mature leaves and loss in senescing leaves; or (b) synthesis is balanced by catabolism of SG in mature leaves, and catabolism exceeding synthesis in senescent leaves. The former possibility is reminiscent of terpinol formation in *Melaleuca alternifolia*, wherein the essential oils are produced with the formation of lysigenous glands (oil is formed by cells that lyse, releasing their contents in an apoplastic space contained within the oil gland) in the developing leaf. The mature leaf cannot form further glands, and so oil content is constant with leaf age, or decreasing if leaf area increases (decreasing the density of glands) or with release of oil (List, Brown et al. 1995). However, SG is produced and stored in vacuoles in the chlorenchyma, and presumably loading of vacuoles is retained in mature leaves.

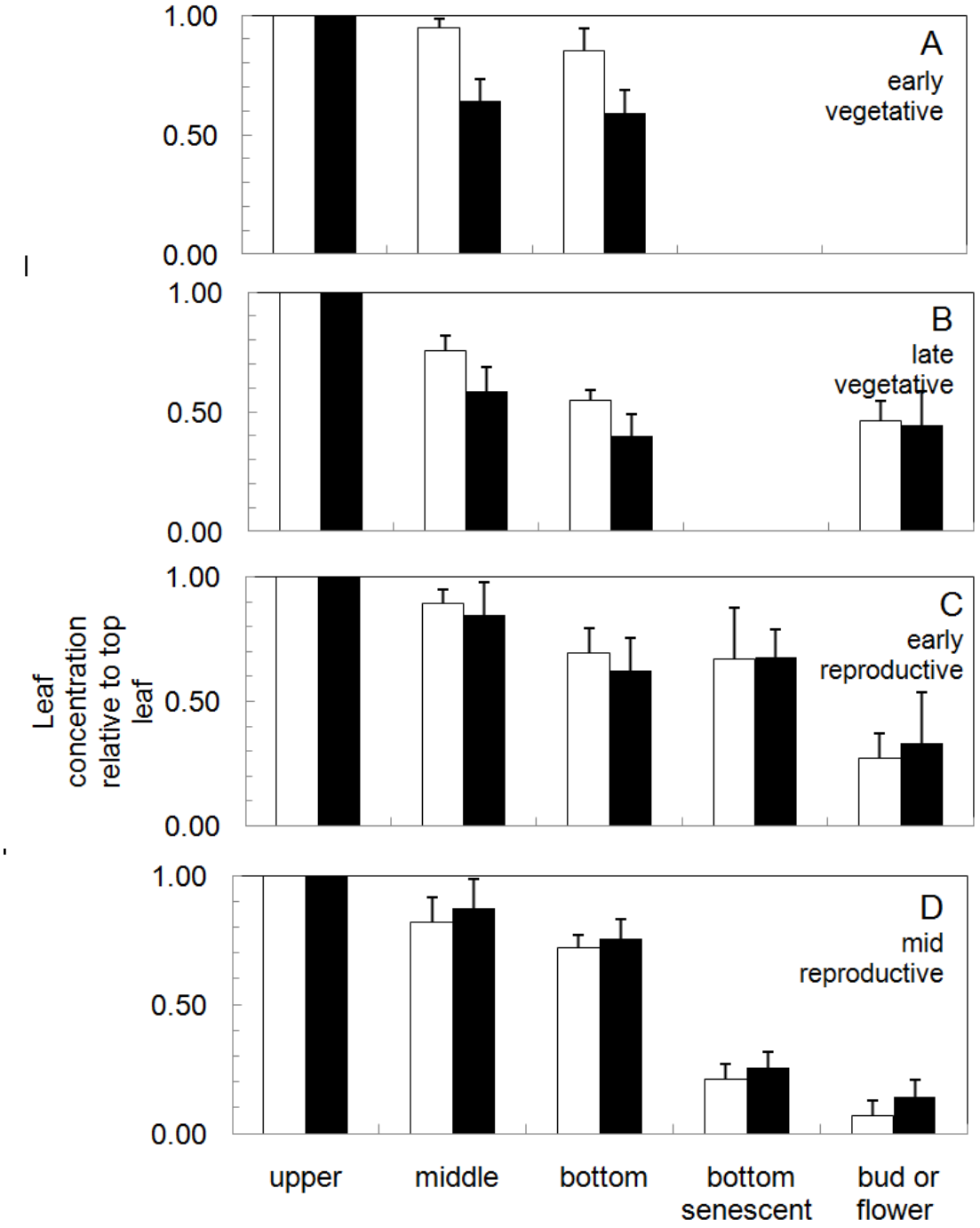


Figure 7. 1. Leaf concentration (% total SG w/w, dw basis), expressed as a proportion of that of upper immature leaves, with the upper (immature leaves), middle (most recently expanded leaves) and bottom (mature) of stems sampled from 10 stevia plants at each of four stages of plant ontogeny (A-early vegetative, B-late vegetative, C-early reproductive, D-mid reproductive) (n = 35 per leaf physiological status, with only five plants sampled at the early reproductive – flower bud stage). Senescent leaves (n = 10) and buds (n = 8) or flowers (n = 8) were sampled during the reproductive stage of the plant. Error bars correspond to standard error (SE). White and black bars represent stevioside and rebaudioside A concentration respectively.

Pulse-chase experiment

To assess the rate of SG pool turnover in immature and mature leaves a ^{14}C pulse chase exercise was attempted.

Label as ^{14}C fed as CO_2 to the shoot of a C3 plant under photosynthetic conditions is expected to be incorporated primarily through the Calvin Benson pathway, and also, to a much lower extent, via other carboxylation reactions (e.g. PEP carboxylase). This label will redistribute in both chemical and spatial form during the chase period. Label typically appears quickly (within hours) into sucrose and starch pools, with remobilization of the labeled starch pool to support leaf export functions during night periods (e.g. Walsh, Vessey *et al.* 1987). Total label in a given organ is determined by the balance between loss through respiration, and import and export from the leaf (primarily via phloem transport). Immature leaves may show an increase in label during the chase period, if label import exceeds label respiration and export.

An initial labeling experiment using one plant was undertaken. Label was apparently identified as stevioside within 6 h of the label event (detected by HPLC connected to a radioactivity detector, data not shown), leading to the design of the current experiment (24 h chase period). However later characterization of the HPLC chromatographic system revealed that the radioactive peak identified as stevioside was really sucrose and that the original chromatographic conditions used was not effective in eluting the injected stevioside standard (N.B. Standards of stevioside and reb A were only available after the initial label experiment and so retention time of the SG standards associated with the HPLC chromatographic system used were only documented after the initial labeling experiment). For the reported experiment, replication per sampling event was low owing to the complexity of sample processing. Instead, samples were taken at multiple time points per plant to allow consideration of time series trends. A treatment on time of labeling in relation to photoperiod was also included to assess the turnover of the SG pool under non-photosynthetic conditions (darkness).

Total label in leaf (around 400-600 Bq.mg dw^{-1}, as measured using tissue oxidation of leaf 'cut offs') demonstrated, at best, a slight decrease over the 24 h chase period in both immature and mature leaf, and for AM and PM feed events (Fig. 7.2A). Evidently respiration of label was negligible, and there was no significant net export or net import of label. The apparent increase in leaf total label of the PM feed group between 1.5 and 3 h post pulse event probably represents sampling variation. This increase could be interpreted as an import of label into leaves, however this is not expected for mature leaves. Label in the soluble pool (measured of the soluble extract of leaf discs at around 100-200 Bq.mg dw^{-1}) was approximately 20% of the total leaf label (Fig. 7.2B). There was no marked change in the total soluble label during the chase period for either immature or mature leaves, in either AM or PM feed events (Fig. 7.2B), even 48 h from initial labeling (with the label in the leaf soluble pool of young and mature leaves at 668 and 494 Bq.mg dw^{-1}, respectively, data not included in the graph). If export of soluble carbohydrate were occurring through the dark periods, it was either of amounts insignificant to the size of the leaf soluble pool, or it was derived from sources of unlabeled carbon within the leaf.

Amongst the sugars tested, only sucrose was labeled to detectable levels within the 24 h period although the level of radioactivity was below the lower limit of the calibration range (10,000 dpm / 10 μL). While the data were corrected for using a single point factor from the lowest radioactive standard used, the values were presented here only for qualitative comparison of label distribution amongst the sugars (Table 7.1).

In the AM group, radioactivity in sucrose was detected transiently in mature leaves (at very low levels, 17 Bq.mg dw^{-1}; data not shown). Label was not detectable in the sucrose or glucose pools of young leaves at any sampling event. Similarly, SGs were not labeled within the 24h period. This result is surprising, given use of 7.4 MBq of label per plant, with incorporation of 400-600 Bq.mg dw^{-1} into leaf tissue measured. Presumably the label was rapidly processed into insoluble pools (e.g. starch).

In the PM labeled group, the label in the sucrose pool of mature leaves was measurable, decreasing during the chase period (from 116 ± 13 Bq.mg dw^{-1} at 0.5 h from labeling to 15 ± 0.35 Bq.mg dw^{-1} at 5 h from labeling) (Fig.7.2B). Radioactivity was also weakly detected in the glucose pool of young and mature leaves (data not shown). The SG pool was not measurably labeled during the 24 h chase event in either immature or mature leaf, or AM or PM feed events.

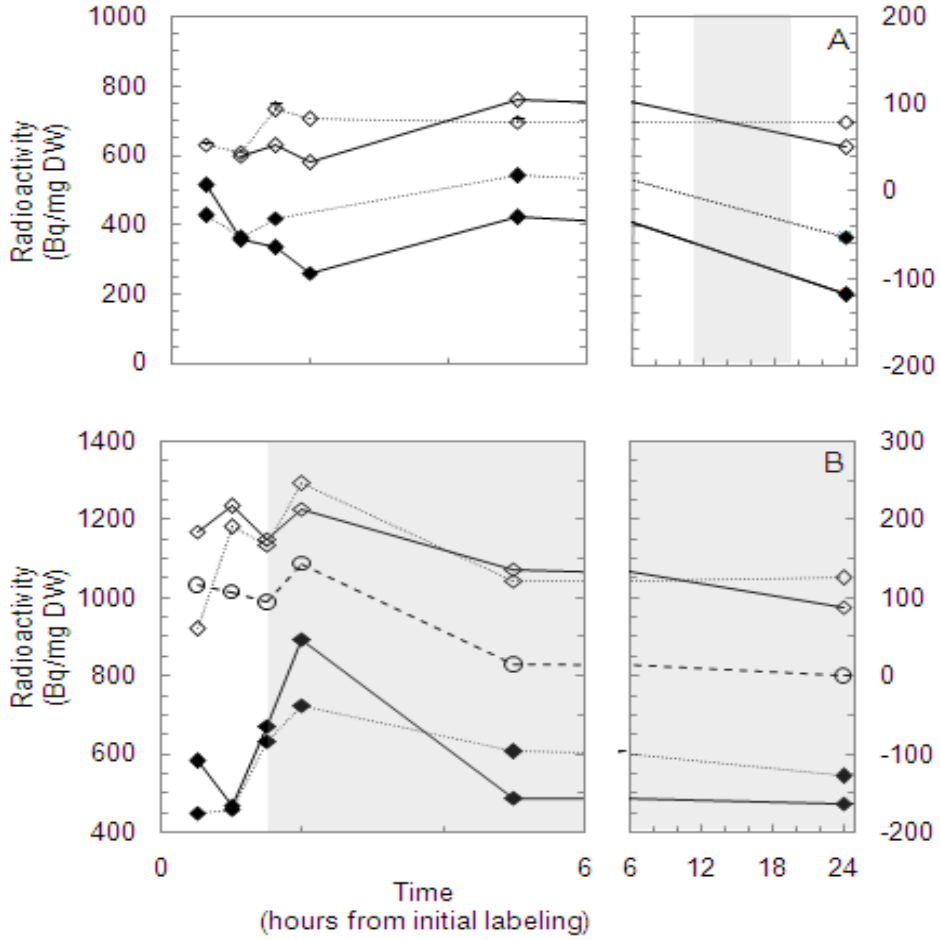


Figure 7. 2. Time course of total leaf ^{14}C count (◆) (left scale) and total soluble ^{14}C count (◇) (right scale) in young (····) and mature (—) stevia leaves following a 30 min pulse feed of $^{14}CO_2$. Leaves were sampled (n = 2 at 0.5, 1.5 and 5 hrs from initial labelling, otherwise n = 1) from plants labelled at 9 am (A) or 4 pm (B). Shaded areas correspond to dark periods. Sucrose radioactivity (- - ○ - -) was also included for the mature leaves from the PM group.

Table 7. 1. Summary of sugar and stevioside content of leaves that had labeled sucrose. Leaves were sampled from plants labeled at 9 am (AM) and 4 pm (PM) of the same day. The PM group was kept in extended dark period after 1.5 h from initial labeling. Data with ± SE were averaged from two analysis (n=2).

Leaf type (label group)	**Time** (hours)	**sucrose** (% dw)	**glucose** (% dw)	**fructose** (% dw)	**stevioside** (% dw)	**myo-inositol** (% dw)	**trehalose** (% dw)	**galactose** (% dw)
young leaf (9 AM)	0.5	12.0	2.3	1.6	9.1	1.5	0.2	0.0
mature leaf (9 AM)	1.5	4.8	0.7	0.6	3.7	0.6	0.1	0.1
	2	7.6	2.2	1.3	3.8	0.8	0.1	0.0
	5	13.2	8.5	3.2	5.5	0.9	0.1	0.0
young leaf (4 PM)	1	10.3	2.2	1.4	3.7	1.0	0.1	0.1
	1.5	7.2 (± 0.2)	0.8 (± 0.4)	0.6 (± 0.03)	4.0 (± 0.02)	1.2 (± 0.2)	0.05 (± 0.05)	0.1 (± 0.01)
	2	5.4	0.2	0.5	4.8	1.3	0.0	0.2
mature leaf (4 PM)	0.5	7.3 (± 1.3)	2.2 (± 0.4)	1.1 (± 0.1)	2.9 (± 0.2)	0.8 (± 0.1)	0.1 (± 0.00)	0.04 (± 0.04)
	1.5	9.0 (± 1.3)	0.9 (± 0.2)	1.0 (± 0.2)	2.7 (± 0.6)	0.9 (± 0.2)	0.1 (± 0.01)	0.1 (± 0.01)
	2	7.9	0.9	0.9	2.5	0.8	0.1	0.1
	5	9.1 (± 1.7)	1.4 (± 0.3)	1.0 (± 0.1)	3.3 (± 1.0)	0.9 (± 0.2)	0.1 (± 0.01)	0.1 (± 0.01)

The major soluble sugars/sugar alcohols measured in leaf extracts were sucrose, stevioside, glucose, fructose, myo-inositol, trehalose and galactose,

in approximate order of abundance, and not corrected for the retention of stevioside by the column (Table 7.1). There was no evidence of diurnal fluctuation in pool size (e.g. mature leaf data, 4 PM group, Table 7.1), unlike the fluctuation seen in starch levels (Chapter 8). The maximum sucrose concentration measured was 12% w/w (leaf dw basis), with an average around 9% w/w. Thus sucrose represents a significant C and energy reserve within stevia leaves. Presumably this sucrose is stored with SG in chlorenchyma vacuoles (Scofield, Ruuska et al. 2009).

Viana and Metivier (1980) reported stevia leaf soluble sugar content to decrease from 1500 to 900 $\mu g.leaf^{-1}$ as the leaf expanded from 4 to 8 cm^2 (to full expansion) and then increase back to 1600 μg in older leaves (sugar levels assessed in the topmost 7 apical leaves which were observed to have a 4 day difference in chronological age between each leaf). Expressed on a dw basis, the level of soluble sugars decreased from 6 to about 4% (60 to about 40 $\mu g\ mg^{-1}$) during leaf expansion, with a concentration of (3-4% (30-40 $\mu g\ mg^{-1}$). The change in sugar concentration was attributed to the source-sink relationship of young and mature stevia leaves, with young leaves importing sugars from mature leaves. Higher concentrations of sugars (approx. two fold higher) were measured in the current study, a result ascribed to better photosynthetic conditions. The current study, however, was not designed to compare the sugar levels of immature and mature leaves, with inadequate sampling and a sampling procedure that will blur any difference (leaves up to fully expanded were sampled as immature leaves). In summary, the results of the current study are consistent with that of Viana and Metivier (1980).

That the SG pool was not labeled is indicative of a slow rate of turnover of this pool, however an estimation of SG metabolic turnover is not possible without introduction of label to the pool and a measure of decrease over time. To achieve this aim, increased labeling of the shoot (e.g. 10 fold increase in ^{14}C label) and an increased chase period (e.g. daily samples of immature and mature leaves over 10 days) is recommended. An alternative

to a pulse-chase experiment would be a long term steady state labeling using $^{13}CO_2$ (e.g. Kouchi and Yoneyama 1984) which would allow label to be incorporated through low rate biosynthetic pathways.

Conclusion

SG leaf concentration is highest in immature (top) leaves with fully expanded (middle) and mature (bottom) leaves having only about 75 and 55% respectively, of the SG concentration in the immature leaves, and no labeling of the SG pool was achieved during a 24-hour chase following a 30-minute pulse feed per plant with 7.4 MBq ^{14}C as CO_2. These results are consistent with the interpretation that SG synthesis occurs primarily in developing stevia leaves, with a low turnover rate in mature leaves. As such, it is predicted that the SG pool of mature leaves will not vary in response with water stress or change in photosynthetic conditions, other than indirectly through effects on leaf weight / area.

Chapter 8
Do steviol glycosides act either as a carbon storage pool or in osmoregulation in stevia leaves?

Abstract

Steviol glycosides (SG) are present in wild type *Stevia rebaudiana,* at levels of approx. 10% dw, prompting a consideration of the autoecological role played by these compound in terms of energy (C) storage or osmoregulation. While the leaf starch pool was observed to change diurnally with respect to the light cycle (from 3.29% to 0.73% leaf dw between dusk and dawn), and also to increase under constant light treatment (from 1.53% to 6.25% leaf dw), the leaf glucose (2-4% leaf dw) and SG pools were relatively constant. The same trend was observed during exposure to elevated CO_2 (800 ppm), with starch increasing (from 10 to 15% leaf dw), while SG pool size was constant. When plants were subjected to increasing water stress over several days, increase in leaf sap osmolality was observed in the leaves of a severely stressed group (from -1 to -3 MPa, after 2 days of treatment) while stevioside and rebaudioside A leaf concentration were relatively constant. These results are not consistent with a role for SG as either a C store or as an osmoregulator in the *Stevia* plant.

Introduction

SGs have achieved public attention as a ‘natural’ sweetner, having a sweetening value of 300X (weight basis) that of sucrose. SGs are secondary metabolites, generally categorized as diterpenes, that comprise about 10%

of the leaf dw of *Stevia rebaudiana*. There are at least eight SG structures, all similar in that they share steviol as a common aglycone backbone, but differing in the number of glucose units attached to the C13 and C19 of the molecule (Dacome, Da Silva *et al.* 2005). Of the these eight SGs, stevioside generally occurs in the highest concentration (9.1% w/w, dw basis) in *S. rebaudiana*, followed by rebaudioside A (3.8%), rebaudioside C (0.6%) and dulcoside (0.3%) (Brandle, Starratt *et al.* 1998), with these concentrations indicative of that of the wild (Paraquay) population, with total SG occurring at about 10% of leaf dw. However, the concentration of SG in the stevia leaves is quite variable between genotypes, with some having low stevioside content (3.4% dw) compared to others (6.88% dw) (Zaidan, Dietrich *et al.* 1980; Bondarev, Sukhanova *et al.* 2003), and current breeding programs are attempting to increase the total, and the relative proportion of rebaudioside A.

The SG biosynthetic pathway is an up-regulation of a biosynthetic pathway shared with GA, branching at kaurenoic acid, with hydroxylation by **KAH** (*kaurenoic acid 13-hydroxylase)* to form steviol (Brandle and Telmer 2007). Subsequent glucose additions catalysed by UGTs *(UDP-glycosyltransferases)* then form the SGs, which are stored in the vacuole of chlorenchyma cells (Brandle and Telmer 2007).

The disparity between the typical levels of GA (1.2 ug/kg leaf fresh weight; Alves and Ruddat 1979; Richman, Gijzen et al. 1999) and SGs (c. 100 $g.kg^{-1}$) is intriguing. Presumably the high metabolic investment by the stevia plant into SGs confers some form of ecological fitness. The variability of SG leaf concentration with respect to ecophysiological roles in the stevia plant remains to be described. Mooney (1972) summarized the role of a range of secondary metabolites as including energy (carbon) storage, (osmo)regulation, attractants, and deterrents. SG biosynthesis can be linked to the general carbon cycle summarized by Mooney (1972). This paper will explore the possible role of SGs, present predominately in the form of stevioside and rebaudioside A, as an alternative carbon store parallel to starch and soluble sugars, and as possible osmoregulatory compounds.

Photosynthate is generally translocated from photosynthetic organs to the various sink organs in the form of sucrose, with re-mobilisable storage occurring typically in the form of sucrose or starch, and also as lipids or protein in some species. The proportion of C flux diverted to storage will depend on photosynthetic conditions and plant C status. For example, the leaves of sugar cane (Du, Nose *et al.* 2000), maize (Kalt-Torres, Kerr *et al.* 1987) and soybean (Upmeyer and Koller 1973) store up to about 15 – 35 $mg.dm^{-2}$ starch during a light period. Starch reserves in soybean leaves were noted to vary between 14% dw and 6% dw under a 16 h photoperiod, deplete to 3% dw within 48 h of darkness, and to increase to 17% dw after 48 h of continual light (Walsh, Vessey *et al.* 1987).

In addition to the build-up of carbon-based primary metabolites such as sucrose and starch, accumulation of secondary metabolites under conditions of excess photosynthate is predicted by the carbon-nutrient balance hypothesis (Hamilton, Zangerl *et al.* 2001; Lerdau 2002). For example, high and low concentrations of total phenol glycosides was correctly predicted for cottonwood plants placed under high light and shaded environments, respectively, for 20 days (Crone and Jones 1999). Similarly, the concentration of salidroside, a phenol glycoside in the leaves of seedling European white birch (*Betula pendula* Roth) maintained at different CO_2 levels (350, 700, 1050 and 1400 ppm) for four months was noted to increase with elevated CO_2 level (up to 1050 ppm) (Lavola and Julkunen-Tiitto 1994). High CO_2 and fertilizer levels were synergistic in increasing both leaf sugars (sucrose, glucose and fructose) and phenolics (proanthocyanidin and myricitin) in European white birch (*Betula pendula* Roth), up to the highest level of 1400 ppm CO_2 and 100 kg ha^{-1} fertilizer (Lavola and Julkunen-Tiitto 1994). Similarly, total phenolics in leaves of hay scented fern were found to be positively correlated with light levels when eight field sites with varying light levels were compared (Dustin and Cooper-Driver 1992).

The allocation of energy derived from photosynthesis within a plant is a strategic process that will determine the plant's competitiveness in its environment and its reproductive success (Mooney 1972). The impact of

altered photosynthetic conditions on SG concentration has not yet been reported, with such information useful in exploring the possible role of SGs in leaf energy storage.

Alternatively, SGs may play a role in the stevia plant's adaptation to water stress. In its original habitat in Paraguay, the stevia shrub grows in environments with shallow water tables which are usually moist but not inundated (Shock 1982). Such environments may expose the stevia plant to wetting and drying cycles, and the stevia plant may have evolved adaptations to cope with water stress conditions. The plant has no marked xerophytic morphological features, such as leathery leaves or a thick cuticle or epidermal hairiness. Thus, any adaptation is presumably limited to mild water stress.

Plant cells maintain turgor in the face of variation in plant water potential through osmoregulation. Osmoregulation prevents cellular dehydration by increasing osmotic potential through osmolyte build-up which then minimizes outflow of cellular fluids and thus maintains cell turgidity (Hare, Cress *et al.* 1998). There is no single compound that acts in osmoregulation within a given species or across different plant species. For example, in fully expanded sorghum leaves, the doubling of leaf osmotic potential (from -0.25 to -0.49 MPa) with a decline in shoot water potential (from -0.85 MPa to -1.3 MPa) in adapted leaves was associated with higher leaf concentrations of sugars (sucrose, glucose and fructose) and potassium and chloride ions relative to control leaves (Jones, Osmond *et al.* 1980). In mature cotton leaves, adaptation to water stress (at leaf water potential of -1.8 MPa) was accompanied by an increase in glucose, sucrose and starch concentration (Ackerson 1981). Ten-day old seedlings of *A. thaliana* subjected to salt stress (86 mM NaCl) increased in free proline concentration from 46 to 276 μg.g fw^{-1} after 24 h of exposure (Verbruggen, Villarroel *et al.* 1993). Barley plants increased in betaine levels (from 2 to 22 μmol.g^{-1} fw) with increasing salinization (0-300 mM NaCl) (Grumet and Hanson 1986). Furthermore, barley with high betaine levels (18-24 μmol.g^{-1} dw or 2 μmol.g^{-1} fw based on maximum dw to fw ratio of 100 mg.g^{-1}) had constantly lower solute potential

(0.1 MPa) than a low-betaine population (12-16 μmol.g^{-1} dry wt) (Grumet and Hanson 1986).

An increase in concentration of a number of different compounds removes the need for a large change in the concentration of any individual compound. In fully expanded sorghum leaves, for example, an overall increase in total sugars relative to control leaves (54 mM, equivalent to a -0.14 MPa decrease in osmotic potential) was responsible for only 29% of the overall change in osmotic potential (-0.49 MPa) of stressed leaves (Jones, Osmond *et al.* 1980). The rest of the osmotic potential adjustment was attributed to an increase in inorganic ions, potassium carboxylate, total free amino acids and sugar phosphates (Jones, Osmond *et al.* 1980). The varied osmolytes can contribute to other beneficial roles in the plant's physiology, such as maintaining redox balance and protecting against hypoxia (Hare, Cress *et al.* 1998; Yancey 2005).

In stevia leaves with 90% w/w moisture and a 10% w/w stevioside concentration (based on leaf dw), the concentration of stevioside (MW 804.87 g/mol; ionization constant = 1) in leaf 'sap' would be 124 mM, equivalent to -0.31 MPa (Van't Hoff equation), assuming that stevioside is distributed uniformly through the soluble components of the leaf. Thus stevioside alone could not account for tolerance of water stresses greater than -0.34 MPa. Further, for SGs to play a role in osmoregulation, large fluctuations in the size of these pools within the leaf would be required.

In the current exercise, the change in leaf concentration of stevioside and rebaudioside A, as representative of SG concentration, under different light and CO_2 levels as well as different degrees of water stress were monitored to explore the possible role of SG in carbon storage and osmoregulation of stevia leaves.

Materials and Methods

Plant material and growth conditions

A total of 55 stevia plants from two varieties (23 from 99-8 variety and 32 from Fengtian variety), at mid-vegetative stage, were randomly selected from a population that was maintained and watered automatically inside a glasshouse under natural light. The plants were transferred to growth cabinets (Contherm Phytotron) to acclimatize under 600 μmol m^{-2} s^{-1} PAR, 25 °C, 65-80% relative humidity and photoperiod cycle of 14 h light / 10 h dark. Plants were each maintained in a 4 L pot using a medium comprised of 45% sand, 30% peat and 25% composted pine bark.

During the acclimatization period, the plants used in the CO_2 and water stress experiment were sprayed with Eco-oil and a persimillis predator introduced to control a red spider mite population. Liquid fertilizer (Aquasol^{TM}) (dilution of 16 g fertilizer in 10 L water) was also applied weekly (250 mL for each pot).

Photoperiod experiment

Thirty plants were equally divided into three light treatments. Plants in the control group were placed in a single growth cabinet and continued under a 14 h light / 10 h dark photoperiod cycle. The other twenty plants were placed in a second growth cabinet which was lit constantly at 600 μmol $m^{-2}s^{-1}$. Ten of these plants were kept under a black plastic shroud (at 1 μmol $m^{-2}s^{-1}$; but well ventilated to maintain ambient temperature). All plants were watered each morning and afternoon and fertilized weekly with 500 mL of a complete fertilizer (16 g of Aquasol, Hortico P/L, in 10 L water).

The photosynthetic rate of a single mature leaf of each plant (i.e. n = 10 per treatment) was monitored daily up to the fifth day of treatment. Leaf greenness of each plant was measured at the first and fifteenth day of treatment (average of 3 randomly selected mature leaves from each plant).

Leaf sampling from the last day of acclimatization up to the second day of treatment was undertaken at the end of each light and dark period. Subsequent samplings were undertaken at the end of the light period until the fifth day of treatment.

CO_2-enrichment experiment

A total of 20 plants were equally distributed into two CO_2 treatments. A control group of 10 plants was kept in the acclimatization growth cabinet (at ambient CO_2 levels), while a second group of 10 plants was transferred to a growth cabinet (Conviron P/L) equipped with CO_2 control, and maintained at a higher CO_2 level (800 ppm). Plants were watered daily in the afternoon with 500 mL of water per pot and fertilized weekly with Aquasol ^{TM} (Hortico) (16 g in 10L water).

Photosynthetic rate of the plants were monitored daily up to the fifth day of treatment by taking the photosynthetic rate of single mature leaves from stems selected for leaf sampling.

Leaves were sampled on Day 0 and Day 4 of the treatment period.

Water stress experiment

A total of 15 plants were distributed into three water treatments. The control and mild water stress groups were irrigated daily with 500 mL and 150 mL water, respectively, while the severe water stress group was deprived of water throughout the experiment.

Progression of water stress was monitored through daily measurement of soil moisture, leaf gas exchange parameters, and plant water potential (total water potential as indexed by Scholander bomb, and leaf sap osmolality).

In vitro leaf chemical analyses

About four to six middle mature leaves, selected downwards from the youngest fully expanded leaf, were taken from a branch of each plant at each

sampling period. For the water stress experiment, leaves were sampled from the stem cutting used for the measurement of stem water potential. Leaf area and fw of stevia leaves was recorded immediately after harvest before placing the leaves inside a fan-forced oven at 65 °C for 48 h to obtain the leaf dw. Specific leaf weight was calculated as the ratio of leaf dw to leaf area.

Stevioside and rebaudioside A concentration were measured of water extracts of 0.1 g of a composite of four to six dried ground stevia leaves. The ground leaf pellets derived from the water extraction were used for starch analysis of samples from the photoperiod and CO_2 experiment.

Dry ground stevia leaves (0.1 g) were extracted using two cycles of water extraction, each involving 5 mL of milli-Q water, with agitation in a 70 °C water bath for 30 min. Insoluble material was separated from the water extract by centrifugation (3500 rpm) for 5 min. A 2 mL aliquot from the combined 10 mL water extract was filtered through a 0.45 μm filter before transferring to a 2 mL HPLC vial for SG analysis. A 200 μL aliquot of the supernatant was also assayed for glucose content.

HPLC analysis was performed using an Agilent 1100 equipped with an Agilent Zorbax High Pressure Reliance Cartridge guard column (4.6 x 12.5 mm, 5-micron) in series to a Zorbax NH_2 column (4.6 x 250 mm, 5-micron). The mobile phase was 80% acetonitrile (pH 5), with addition of 200 mL of 0.02 M glacial acetic acid and 400 μL of 0.1 M sodium hydroxide (aq) per litre of total mobile phase. Flow rate was at 1 $mL.min^{-1}$ and injection volume was at 5 μL. The UV detector was set to a slit width of 4 nm, and measurement at 210 nm, using absorbance at 360 nm as a reference. The system was calibrated with stevioside and rebaudioside A standards using concentrations of 0, 0.2, 0.4, 0.6 and 1.25 mM.

Ground leaf pellets derived from the leaf water extraction were prepared for starch analysis by autoclaving the pellets in 25 mL of milli-Q water at 121 °C for 1 hour. A 1 mL aliquot of the supernatant was then mixed with 1 mL of

starch assay reagent (Sigma-Aldrich) containing 50 $U.mL^{-1}$ of amyloglucosidase (*Aspergillus niger*) to convert the starch into glucose units, and placed in a water bath at 60 °C for 15 minutes and then cooled quickly to room temperature. A 200 μL aliquot of the digested sample was incubated with 1 mL of glucose assay reagent (involving hexokinase and glucose-G-phospho dehydrogenase, with substrates ATP and NAD; Sigma, SA-20) for 15 minutes at room temperature to convert the glucose units to an equimolar amount of NADH. Absorbance at 340 nm was measured as an index of NADH content, relative to a blank solution (without addition of the glucose assay enzyme). From the corrected absorbance values, both starch and glucose concentration (expressed as % leaf dw) were determined. A check of assay procedure was undertaken by analysis of a starch standard (540 mg/L). The analysed value was 98.8% of the reference value.

Other measures of plant physiolgy

Leaf gas exchange was assessed using the broad leaf chamber of an infrared gas analyzer (LCA-4, ADC). Measurements were made daily, three hours from the onset of daylight, of single mature leaves (three per plant) of material subsequently harvested for chemical analysis.

Leaf greenness was assessed of three randomly selected mature leaves per plant using a Minolta Chlorophyll Meter (SPAD-502). Leaf area was obtained of leaves harvested for SG and carbohydrate content, with area determined using a Delta-T SCAN (version 2.04nc, Delta-T Devices Ltd 1996).

In the water stress experiment, soil volumetric moisture content was measured daily three hours from the start of photoperiod, and before watering, with readings averaged from three random measurements in each pot. Soil moisture was measured using the Hydrosense Portable Soil Moisture System (http://www.campbellsci.com.au/hydrosense). Stem water potential was assessed following the gas exchange measurement, using a Scholander pressure chamber (Soil Moisture Equipment, Model 3005)

(harvest of stem cuttings with two pairs of fully expanded leaves, with each plant sampled once daily).

Leaf sap osmolality was measured using a Wescor vapour pressure osmometer (Model 5520), calibrated using NaCl standards. Osmotic potential was derived from the osmolality readings using the Van't Hoff equation (assuming an ionization constant = 1). Leaf solute potential was measured from 2 to 4 mature leaves remaining on the branch immediately following harvest of a part branch for use in the Scholander pressure chamber. Leaves were immediately frozen with liquid N_2, and later thawed, with sap extracted by pressing the leaves inside a 10 mL syringe. Osmolality was measured of a 10 μL sample of the sap. The remaining sap was stored and frozen in eppendorf tubes for later HPLC measurement of stevioside and rebaudioside A concentration (of 1:10 diluted samples).

Data analysis

The two cultivars were not significantly different in terms of the attributes assessed, and data is presented of the combined population. A one-way analysis of variance (SPSS 17.0) was employed with leaf starch concentration and leaf specific weight data (photoperiod experiment). Statistical significance is reported at a 95% confidence level.

Results and Discussion

Photoperiod experiment

Leaf carbon dioxide exchange rate was relatively constant at 10 μmol m^{-2} s^{-1} for the control and the continuous light treatment over the 5 d of monitoring (Fig. 8.1), while exchange rates of plants held in darkness were negative, as expected (at -5 to -1 μmol m^{-2} s^{-1}). Thus end-product inhibition of photosynthesis was not evident in the continuous light treatment, a result which may reflect the relatively low light levels provided within the growth cabinet.

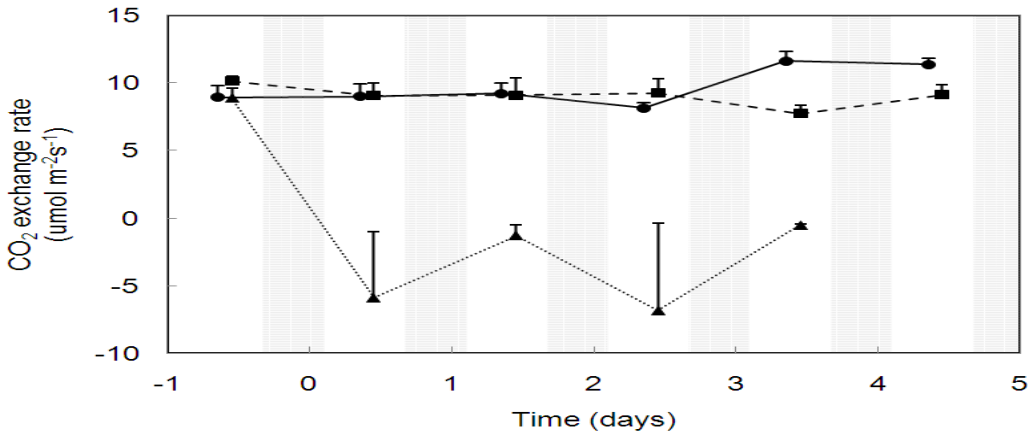


Figure 8. 1. Average leaf CO_2 exchange rate of plants placed under control diurnal light (—●—), constant light (- -■- -) and constant dark (· · ▲ · · ·) conditions. Data points represents the mean with associated SEM (n = 10). Shaded area corresponds to dark period for the control group.

Following 6 d of treatment imposition, a slight increase in leaf greenness (SPAD) was noted for both control and light group relative to readings taken just prior to treatment imposition, while a decrease was observed in the dark group (data not shown). A decrease in chlorophyll content is expected for a continuous dark treatment of this duration.

The starch content of stevia leaves (at 4.58% dw or 15.42 mg dm^{-2}) was low compared to that of some species (e.g. 14% dw in soybean leaf, Walsh, Vessey *et al.* 1987). It was of similar size to the glucose pool (2-4%) and less than the SG pool (5-8% stevioside and 0.5-1.5% rebaudioside A).

The observed changes in starch concentration were consistent with a role for starch as a leaf C store. For example, after five days of continual light treatment, leaf starch content had increased to 6.25% dw or 26.65 mg dm^{-2}, while that of plants maintained in darkness decreased to effectively 0% (0.18% dw or 0.49 mg dm^{-2}) (Fig. 8.2A) ($F_{(4,9)} = 2.649$, $p = 0.096$). The build-up in starch leaf content under continual light was also reflected in the leaf specific weight (Fig. 8.2B).

A decrease in the size of the starch pool was observed during dark periods in the 14/10 h light/dark treatment (Fig. 8.2A). Between dusk and dawn of Day -

1, leaf starch content decreased from 3.29% to 0.73%, and then increased to 1.74% at the end of the subsequent light period of Day 1 (Fig. 8.2A). Using the leaf specific weight ($g.mm^{-2}$) of the analysed leaf samples, the observed diurnal change in starch was equivalent to 8.39 and 3.69 mg dm^{-2} at the end and beginning of the photoperiod, respectively. This change of 4.7 mg dm^{-2}, or 165 mmol C m^{-2}, can be compared to the measured photosynthetic rate of 10 μmol m^{-2} s^{-1}, equivalent to 36 mmol m^{-2} h^{-1}, or 504 mmol m^{-2} d^{-1} (14 h photoperiod), i.e. the increase in the starch pool represents approx. 33% of daily C uptake through photosynthesis.

The change in starch level (3.7-8.4 mg dm^{-2}) noted through a photoperiod in mature stevia leaves was less than that observed in sugar cane leaves (15 mg dm^{-2}), maize leaves (35 mg dm^{-2}) and soybean leaves (30 mg dm^{-2}) (Upmeyer and Koller 1973; Kalt-Torres, Kerr *et al.* 1987; Du, Nose *et al.* 2000). The low carbohydrate content of stevia leaves may be in part genetic, but is likely to be largely due to the relatively low light intensity used (600 μmol m^{-2} s^{-1} PAR at leaf level, compared to 50,000 lux (equivalent to 1,700 μmol m^{-2} s^{-1} PAR assuming a metal halide lamp) for the soybean result, 1500 μmol m^{-2} s^{-1} PAR for the sugarcane result, and natural light inside a glass house for the maize result) (Upmeyer and Koller 1973; Kalt-Torres, Kerr *et al.* 1987; Du, Nose *et al.* 2000). Indeed the maximum leaf CO_2 exchange rate of sugarcane leaves under 1500 μmol m^{-2} s^{-1} PAR was 35 μmol m^{-2} s^{-1}, (Du, Nose *et al.* 2000), while the stevia leaves achieved a rate of 10 μmol m^{-2} s^{-1} at 600 μmol m^{-2} s^{-1} PAR.

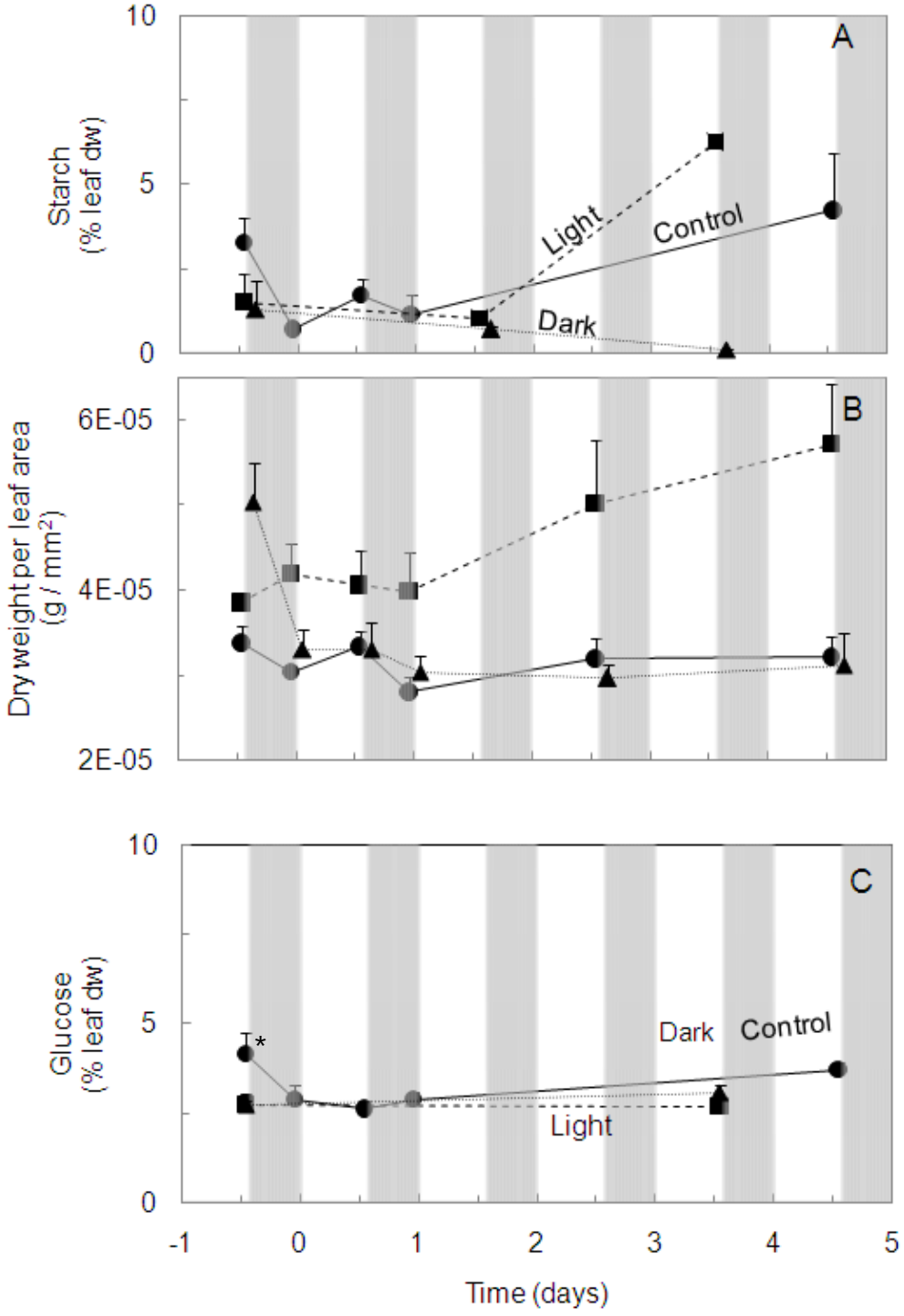


Figure 8. 2. Change in leaf starch content (% dw) (A), leaf specific weight (g mm^{-2}) (B) and leaf glucose content (% dw) (C) of stevia plants maintained under a 14h / 10h photoperiod (—●—), constant light (- -■- -) and constant dark (· · ▲ · ·). Plot of dark group is shifted to the right by 0.1 days. The dark period of the control group is shaded. Data points represent an average, with associated SEM. One outlier was removed from the control data point (*).

In sugarcane and maize leaves, the diurnal change in starch was accompanied by a smaller change (approximately 15%, on a weight per unit leaf area basis) in sucrose leaf concentration (Kalt-Torres, Kerr *et al.* 1987; Du, Nose *et al.* 2000) (2.5 mg dm^{-2} sucrose *cf* 15 mg dm^{-2} starch in sugarcane; 5 mg dm^{-2} sucrose *cf* 35 mg dm^{-2} starch in maize). Glucose and other hexoses in sugarcane and maize leaves also followed the diurnal pattern of sucrose but with a smaller net increase by the end of the light period. In sugarcane leaves, glucose increased by 1.5 mg dm^{-2} (10% of the starch increase) (Du, Nose *et al.* 2000). In maize leaves, hexose sugars increased by 1.8 mg dm^{-2} (5% of the net starch increase (Kalt-Torres, Kerr *et al.* 1987).

With the stevia leaves, glucose leaf content was relatively constant at around 2-4% of leaf dw across all treatments (Fig. 8.2C). Acting in primary metabolism, the glucose pool presumably has a high turnover rate, but is maintained by the degradation of the starch storage pool. Given the relatively small observed change in the starch pool it may be that the draw on leaf carbohydrate pools was relatively modest, such that glucose levels were maintained, in contrast to the reported change for maize and sugarcane.

Stevioside and rebaudioside A leaf concentration on both a leaf dw and a leaf area basis were also constant across all treatments (Fig. 8.3). This evidence of a constant pool size runs counter to the suggestion of a role for SG as a short term C reserve by the plant.

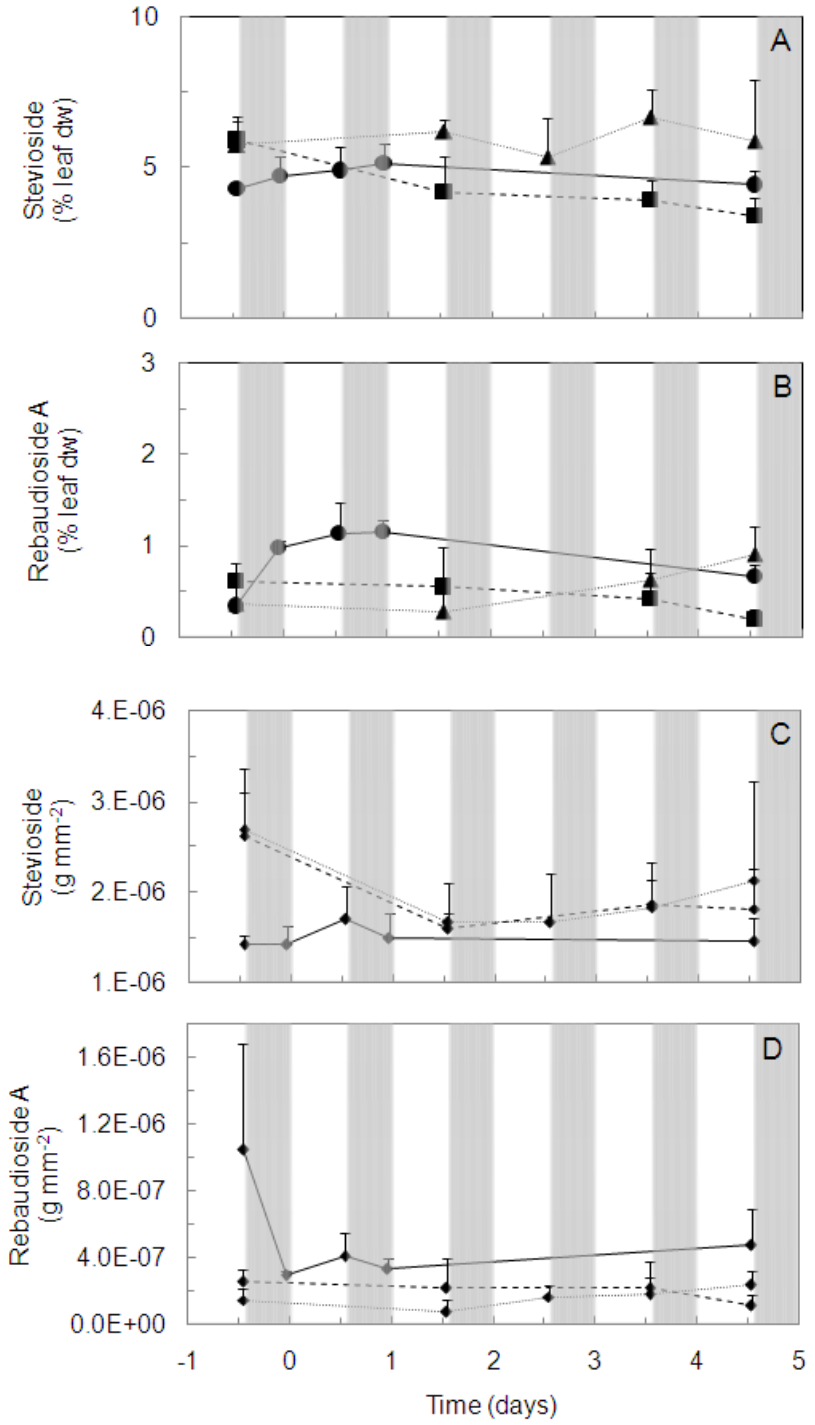


Figure 8. 3. Change in leaf SG concentration of stevia plants maintained under a 14h/10h photoperiod (—●—), constant light (- -■- -) and constant dark (···▲···). Leaf SG concentration was expressed either as % dw stevioside (A), % dw rebaudioside A (B), g stevioside / mm^2 leaf area (C) or g rebaudioside A / mm^2 leaf area (D). Control leaves were sampled from the same set of plants throughout the treatment period (n = 4) while leaves from the light and dark group were each sampled from a set of plants (n = 7) which were divided into two subsets (n = 4 or n = 3) and sampled alternately on Day 2, 4 and 5. Data points represent an average with associated SEM. The dark period of the control group is shaded.

CO_2-enrichment experiment

The carbon dioxide exchange rates of plants in this experiment were lower than those of the photoperiod experiment (approx. 6 *cf.* 10 μmol m^{-2} s^{-1} for control plants), while starch levels were higher (approx. 10% dw cf. < 5% dw). Taken together, these values indicate that the plants used in this experiment possessed a higher C status. Possibly plants used in the photoperiod duration experiment were still acclimating to the lower light levels of the growth cabinet (having been transferred 10 days earlier from a glasshouse), or were still recovering from the damage of a prior infestation of spider mite. Alternatively, the low photosynthetic rate may be a consequence of an unintended elevation of CO_2.

The CO_2 treatment of 800 ppm was chosen as an approximate doubling of ambient CO_2 levels (expected at approx. 360 ppm). However, ambient CO_2 levels within the 'control' growth cabinet were closer to 600 ppm. This is ascribed to an unintended recirculation of exhaust gases from the elevated CO_2 cabinet within the room containing the growth cabinets. Nevertheless an overall elevation of 200 ppm CO_2 was effected, relative to the control group (Fig. 8.4A). The increase in CO_2 levels was not associated with a consistent increase in the leaf CO_2 exchange rate (about 5-7 μmol m^{-2} s^{-1} for both groups) (Fig. 8.4B). The lack of photosynthetic response to elevated CO_2 is explained as due to an overall decrease in stomatal conductance in the treated plants, such that internal CO_2 levels were maintained (Fig. 8.4C). A significantly higher substomatal CO_2 level was recorded in favour of the CO_2 enriched group only on day 4 (Fig. 8.4D).

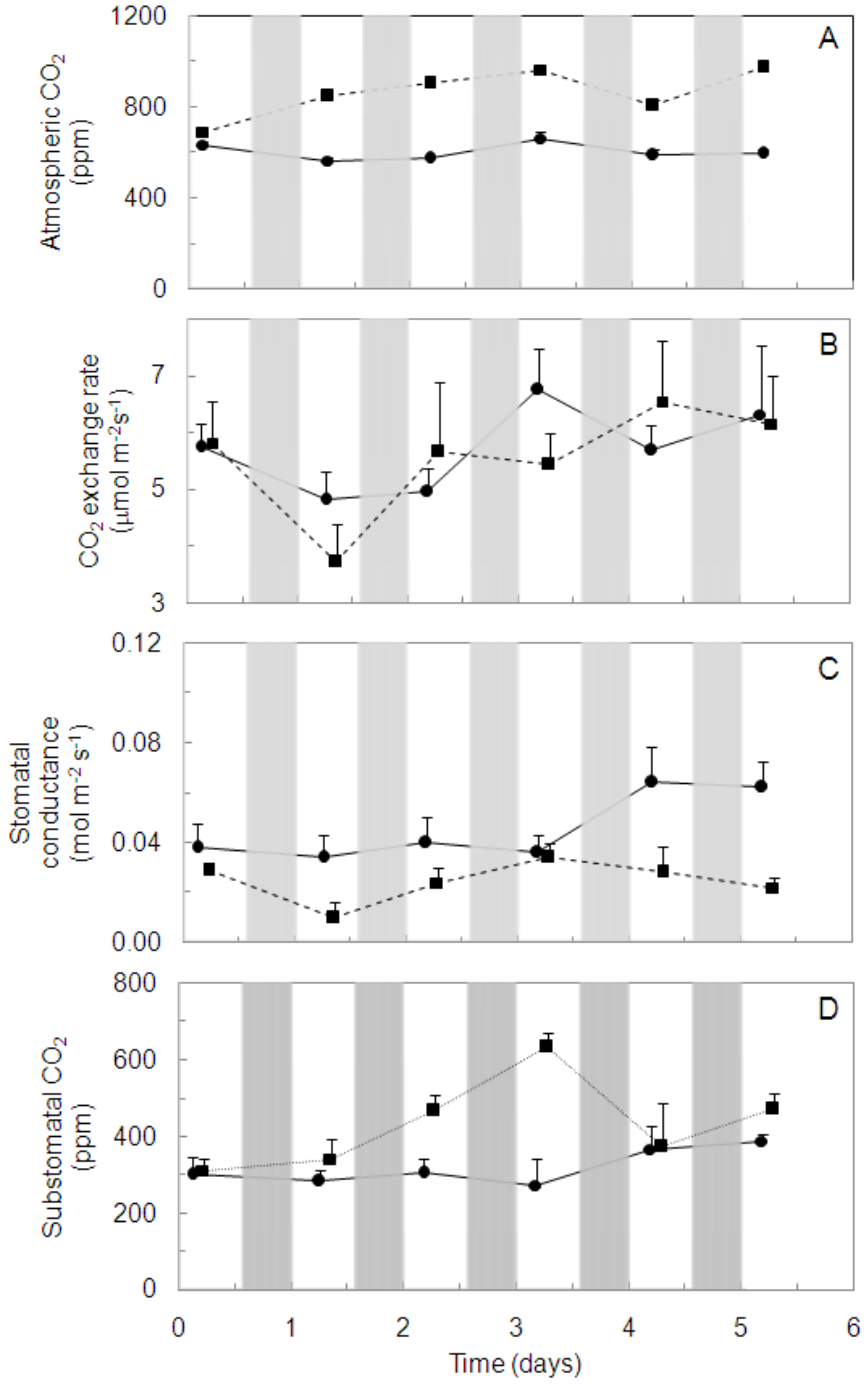


Figure 8. 4. Average atmospheric CO_2 level (A), leaf CO_2 exchange rate (B), leaf substomatal conductance (C) and leaf substomatal CO_2 level (D) of stevia plants placed in either ambient (600 ppm) (—●—) or elevated (800 ppm) (- -■- -) CO_2 environment (n = 5, error bar = SE).

While there was little evidence from the instantaneous gas exchange measures of increased CO_2 fixation with CO_2 enrichment, an overall

significant increase in leaf starch content was observed at elevated CO_2 (increasing from 11% leaf dw at treatment start to 16% leaf dw starch on the fourth day of treatment with elevated CO_2), while that of the control group remained constant (at about 11-12% leaf dw starch) (Fig. 8.5A).

To investigate this possibility that a response to elevated CO_2 occurred over a shorter time frame following treatment imposition than the sampling schedule employed, the leaf carbon dioxide exchange rate of a randomly selected mature leaf of two plants was monitored on an hourly basis following transfer to the growth cabinet with elevated CO_2. An abrupt increase in leaf photosynthetic rate was evident up to 7 hours of treatment, after which photosynthetic rate declined (data not shown). It is concluded that the elevated starch levels noted after 4 days of CO_2 treatment were developed within the first day of treatment.

Unlike starch, stevioside and rebaudioside A leaf content remained constant over the treatment period (Fig. 8.5B & 8.5C), a result consistent with that of the photoperiod trials. It is concluded again that SGs do not function as a short term C reserve within the plant.

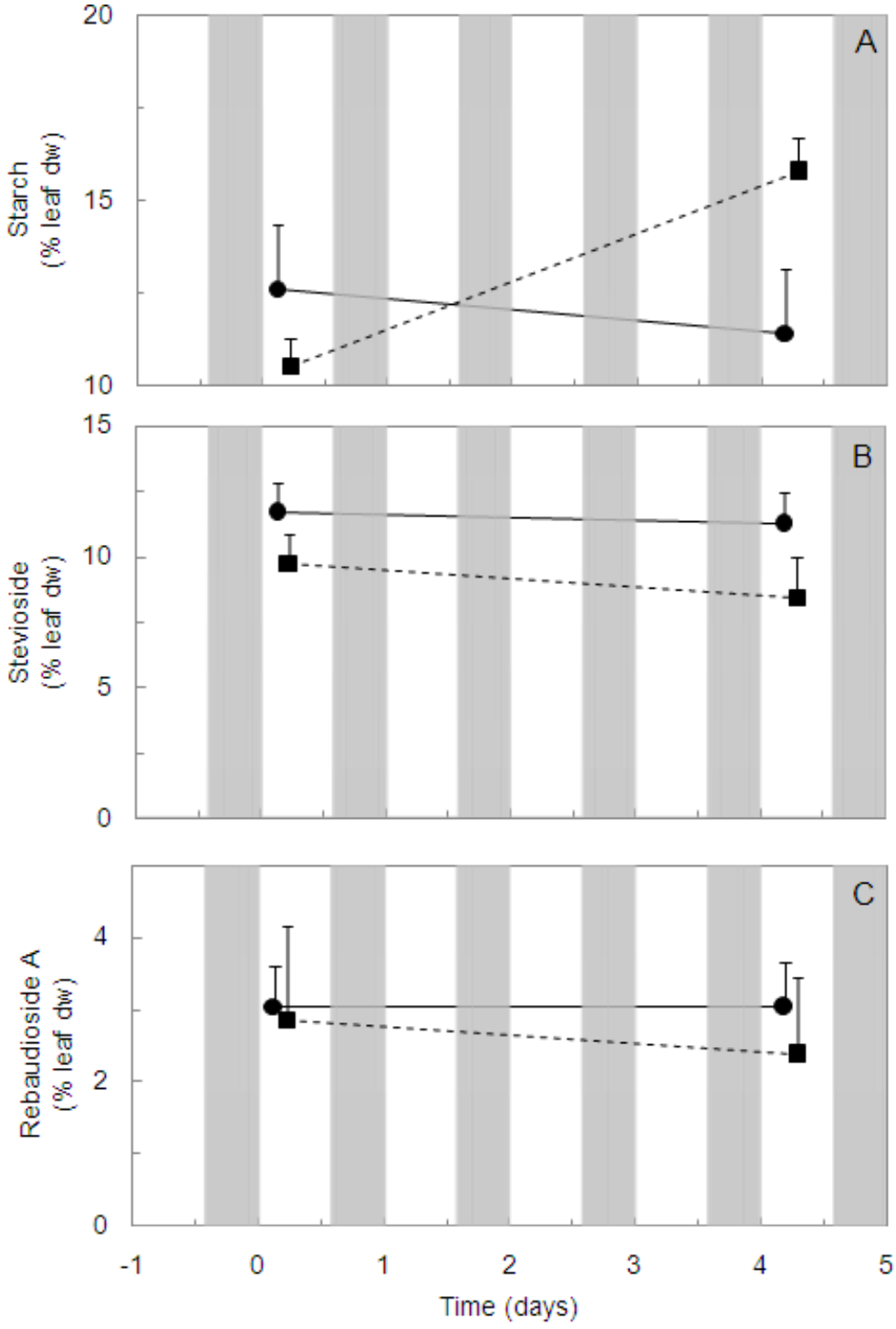


Figure 8. 5. Change in leaf concentration (expressed as % leaf dw) of starch (A), stevioside (B) and rebaudioside A (C) averaged from plants placed under 600 ppm CO_2(—●—) or 800 ppm CO_2 (- -■- -) (n = 5, error bar = SE). The dark period of the diurnal photoperiod (14 h light / 10 h dark) is shaded.

Water stress experiment

Different rates of dehydration were implemented by varying the amount of watering, instead of subjecting plants to a number of dehydration cycles. The water content of the soil was maintained at approximately 55% v/v in the control treatment, and decreased over approximately 7 and 3 days to 12% in the mild and severe stress treatments, respectively (Fig. 8.6C). The level of soil water content in the control treatment was higher than field capacity of the soil mix (32.3% ± 2.5% v/v; n = 4).

A decrease in soil moisture content preceded the decrease in the leaf gas exchange activity. Carbon dioxide exchange rates and stomatal conductance were similar between plants of all treatments prior to and during the first day of treatment (at approx. 9 μmol m^{-2} s^{-1}, and 0.08 mol m^{-2} s^{-1}, respectively) (Fig. 8.6). Leaf photosynthetic rate decreased abruptly during the second day of denial of water (severe treatment), decreasing overall from approx 10 to 2 μmol m^{-2} s^{-1}. As expected, a more gradual decrease was observed in the mild water stress treatment (Fig. 8.6). However, plants of the control (well watered treatment) demonstrated a decrease in leaf carbon dioxide exchange rates (from approximately 10 to 5.5 μmol.m^{-2}.s^{-1}), similar to that of the mild stress treatment (Fig. 8.6). Similar trends were observed in leaf stomatal conductance (Fig. 8.6). The decrease in leaf activity of the control group is ascribed to imposition of a soil water regime above field capacity, presumably resulting in a level of root anoxia.

Curiously, the highest shoot water potential was < -1 MPa, even in well watered plants. The high level of stored osmotically active solutes may contribute to this phenomenon, with the plant maintaining a lower water potential as part of a feedback loop that provides homeostasis on cell turgor. (as calculated in the Introduction, a 10% SG content is equates to an osmotic potential of -0.34 MPa).

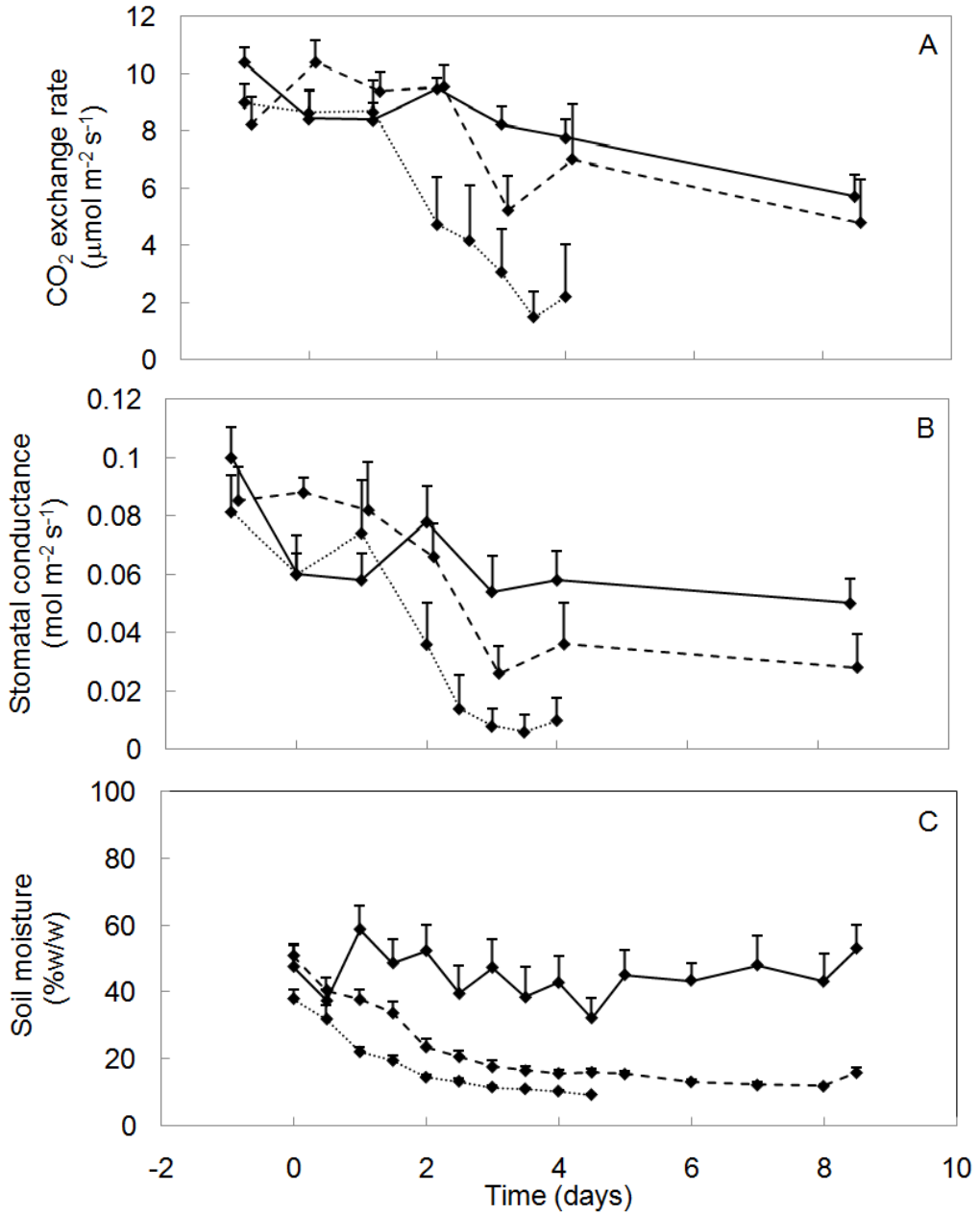


Figure 8. 6. Mature fully expanded leaf photosynthetic rate (A) and leaf stomatal conductance (B) and soil moisture (C). One leaf was randomly chosen from each plant and measured for leaf photosynthetic rate using the broad leaf chamber. Leaves from the control group (—◆—), the mild water stress group (--◆--) and the severe water stress group (…◆…) were sampled daily, with data points representing the mean (n = 5) and associated SEM. Soil moisture of each plant was based on 3 soil moisture readings around the root area (mean, n = 15, presented with associated SEM).

Shoot water potential was maintained at a constant level over 9 days in control and mild stress treatments, while the severe stress treatment demonstrated a decrease in shoot water potential during the third day of treatment (Fig. 8.7). Water stress was evidenced by leaf wilting, occurring from Day 2 to Day 4 in the severe treatment and from Day 5 to Day 8 in the mild treatment. A slight decrease in leaf sap osmotic potential over 9 days was noted for plants of the control and mild stress treatments (to approximately -1.5 MPa), while a dramatic decrease in osmolality (from -1 to -3 MPa) was noted during the fourth day for the severe stress treatment (leaf water potential at -2.87 MPa, Fig. 8.7). This decrease was at least partly attributable to a loss of leaf water content, with leaf dw to fw ratio constant in control and mild stress treatments, but doubling by day 4 in the severe stress treatment (Fig. 8.7).

However, the concentration of stevioside and rebaudioside A in the leaf sap extract did not significantly increase during the time of imposition of any of the treatments (Fig. 8.8). Concentration of stevioside and rebaudioside A in the dry ground leaf samples was also constant during the treatment, except for an increase at day 4 for the severe treatment (Fig. 8.9).

Thus the extent of osmoregulation in stevia leaf during the imposed treatments was minor, with no evidence of a role for SGs. Future studies might consider the role of glucose or sucrose in osmoregulation occurring in stevia, as in cotton leaves (Ackerson 1981).

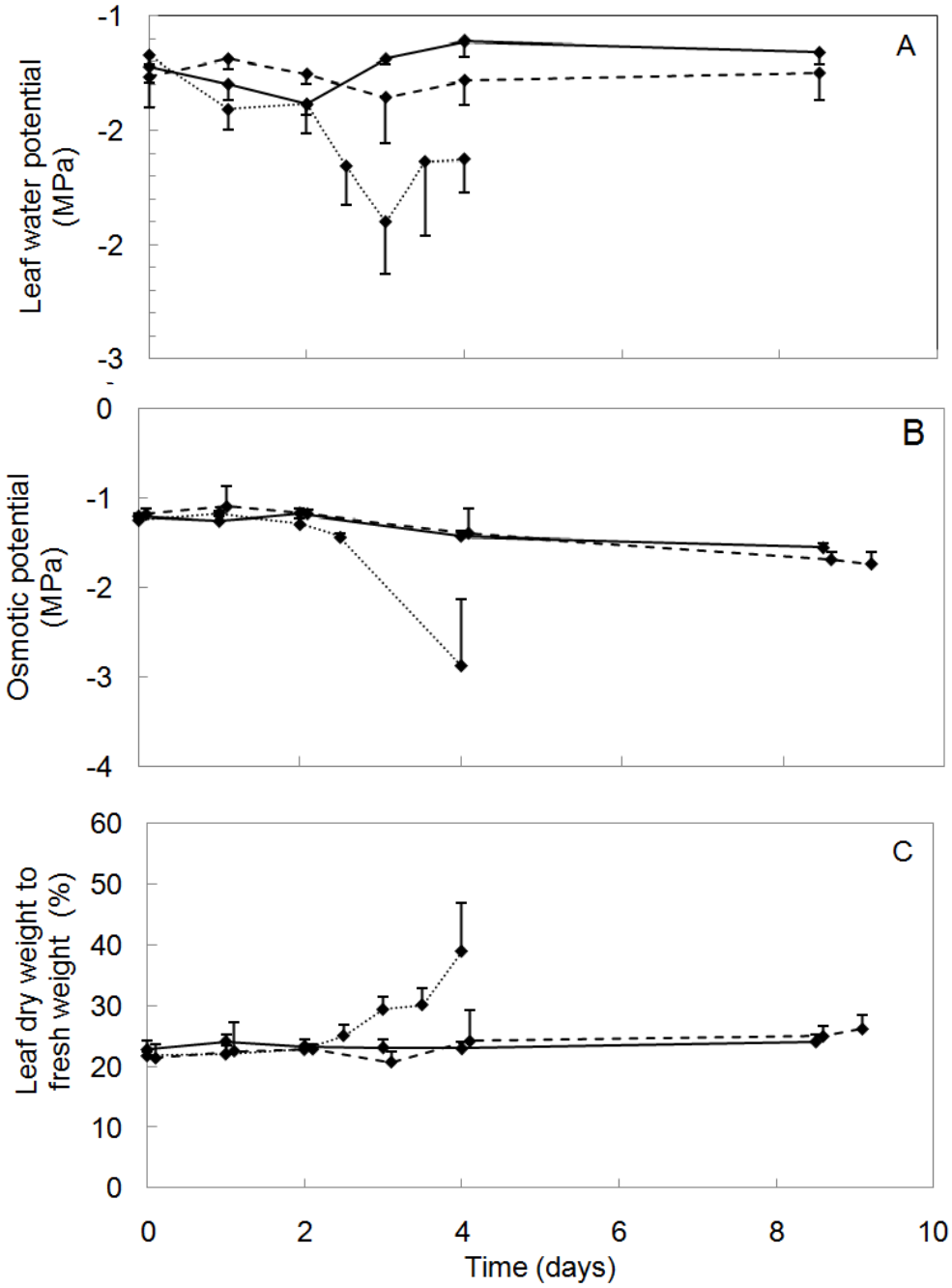


Figure 8. 7. Shoot water potential (A), leaf osmotic potential (B) and leaf dw to fw ratio of plants in the control group (—◆—), the mild water stress group (- -◆- -) and the severe water stress group (···◆···) (n = 5, error bar = SE).

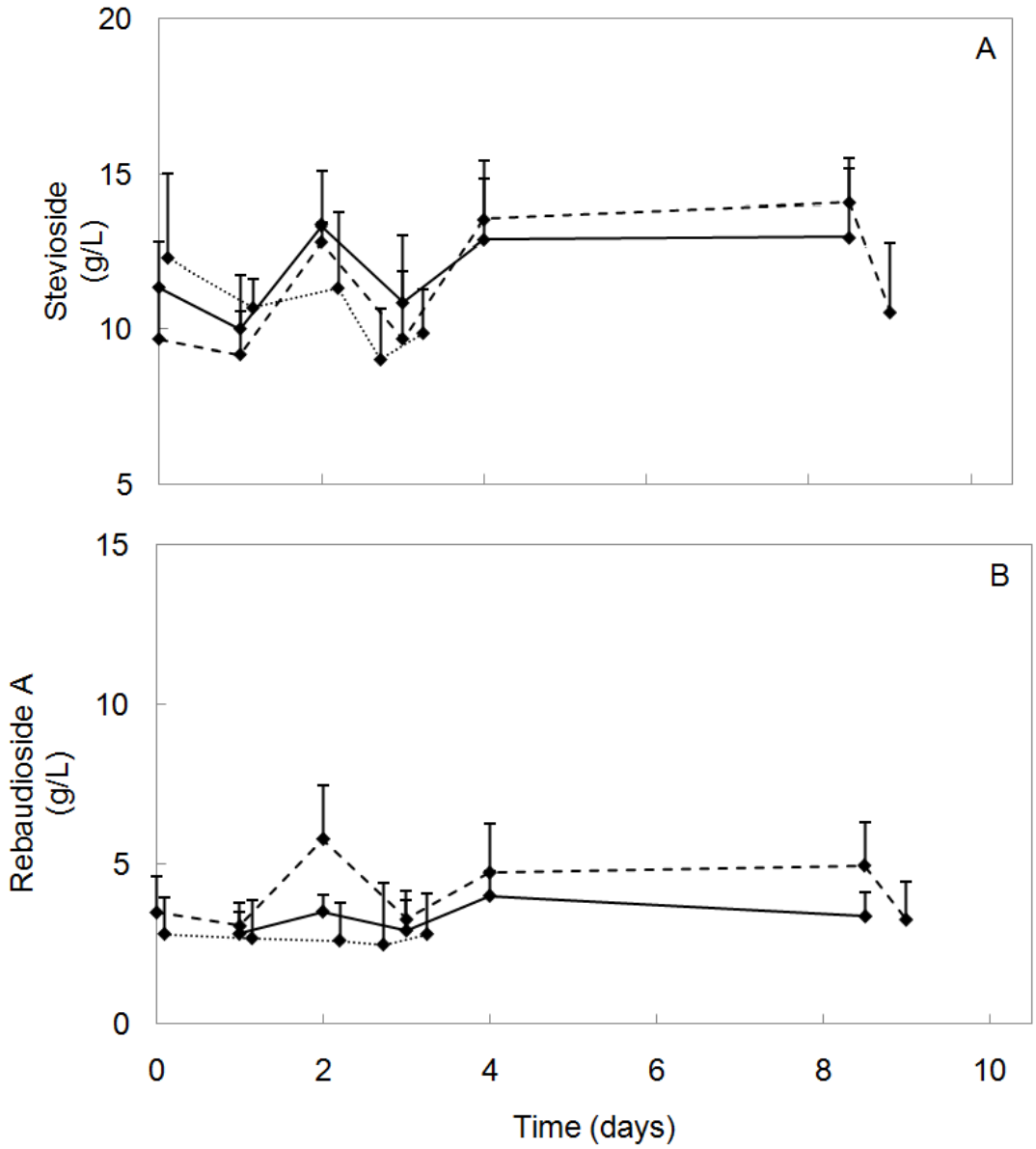


Figure 8. 8. Stevioside (A) and rebaudioside A (B) concentration in leaf sap extract (extracts as used for measurement of osmolality). Sap from aliquot was diluted in water (50 μL extract mixed with 450 μL water) and analysed by HPLC. Sap was obtained from leaves randomly sampled from plants in the control group (—◆—), the mild water stress group (- -◆- -) and the severe water stress group (···◆···) (n = 5 except for mild Day 1 & 4 and severe Day 0 with n = 4; error bar = SE).

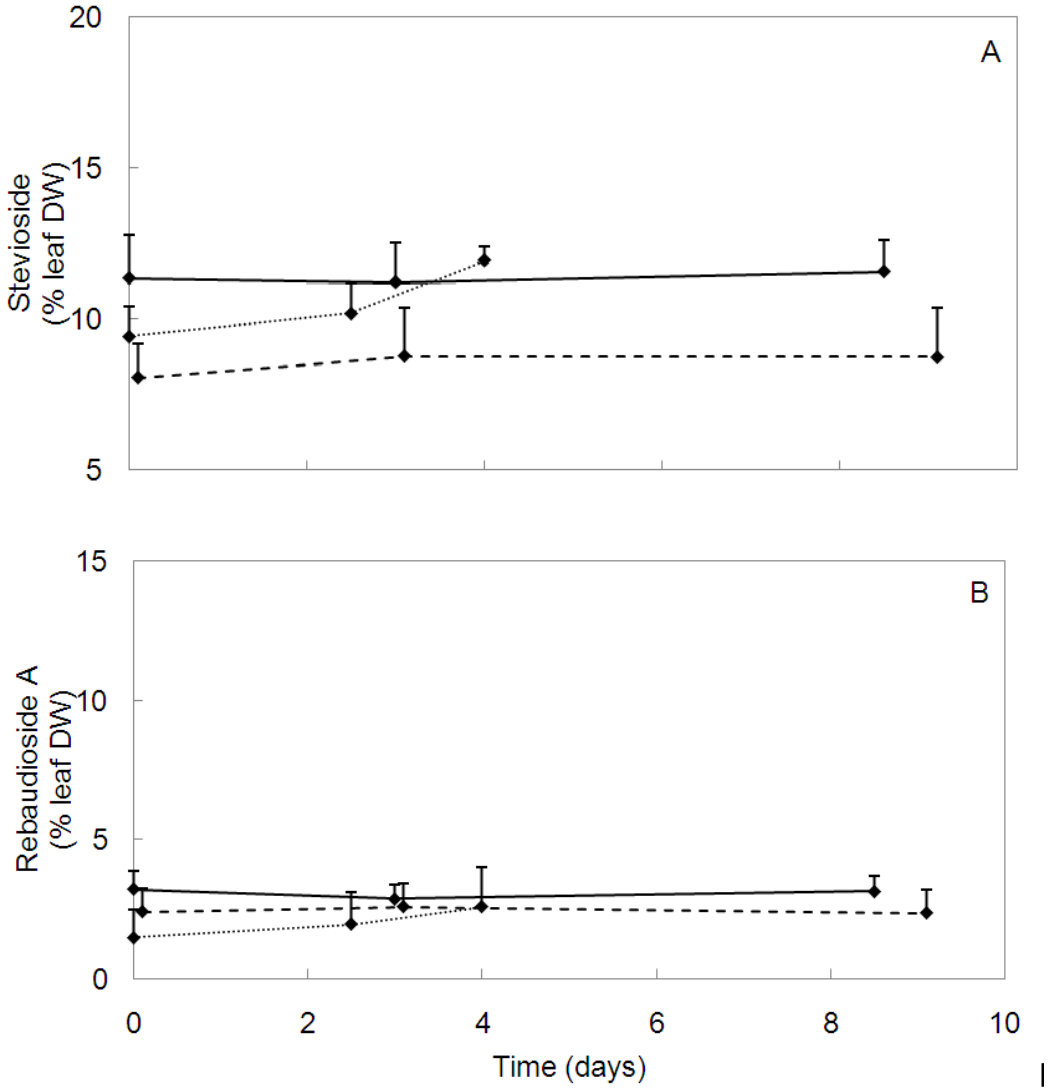


Figure 8. 9. Stevioside (A) and rebaudioside A (B) concentration of mature fully expanded leaves based on a dw basis. Leaves were randomly sampled from plants in the control group (—◆—), the mild water stress group (– –◆– –) and the severe water stress group (···◆···) (n = 5 except for severe Day 4 n = 4, error bar = SE).

A role for SGs in energy storage or osmoregulation?

As a secondary metabolite, SG is predicted to increase by the carbon-nutrient balance on the premise that the excess C accumulated under constant light or elevated CO_2 will be redirected to secondary metabolism. This was not observed.

The carbon-nutrient balance hypothesis in its raw form is rather naive with respect to biochemical regulation, in that simple substrate availability alone is assumed to drive the production of secondary metabolites (Hamilton, Zangerl *et al.* 2001). This assumption disregards stringent regulation in the biosynthetic pathways of secondary metabolites (Hamilton, Zangerl *et al.* 2001). Indeed, increase in the level of all metabolites under conditions of C excess would disrupt cell homeostatic function.

A useful consideration of this issue is provided by Haukioja, Ossipov et al (1998). In a meta-analysis involving 211 woody plant studies, the effect of fertilizer levels on phenolics and terpenoids as subgroups of secondary metabolites was inspected. Based on the carbon-nutrient balance hypothesis, secondary metabolites are expected to drop in concentration with the addition of fertilizers, as growth stimulated by the fertilizers will consume more carbon store and limit C allocated for secondary metabolism (Hamilton, Zangerl *et al.* 2001). From the meta-analysis, phenolic concentration responded to fertilizer treatment in accordance with the hypothesis, but terpenoids did not. Haukioja et al (1998) attributed the difference in responsiveness of terpenoids and phenolic pools to their different biosynthetic pathways. Phenolics stem off from the shikimate pathway which competes with growth requirements by having phenylalanine as a common precursor for the synthesis of both proteins and phenylpropanoids (Haukioja, Ossipov *et al.* 1998).

SG synthesis diverges from the MEP pathway for terpene synthesis (Totte, Charon *et al.* 2000). Although the remaining steps of the MEP pathway remains to be defined, a common biosynthetic pathway with GA has been determined (Gershenzon and Kreis 1999). The up-regulation of SG synthesis from this common pathway indicates a highly regulated mechanism influenced by factors other than the C availability.

A conclusion was reached that SGs do not act as osmoregulatory agents. Several factors mitigate against this assessment. Firstly, only mature leaves were assessed in the current exercise, but osmoregulation has been

reported to decrease with leaf age (O'Neill 1983; Morgan 1984). For example, in strawberry (*Fragaria virginiana*), middle-aged leaves (leaf 5) adapted to 2-3 cycles of water stress (-2.0 MPa leaf water potential) by lowering leaf osmotic potential, while older bottom leaves were unable to adjust and thus senesced under water stress conditions (O'Neill 1983). Young elongating wheat leaves that have not yet emerged from the sheath were more adaptable to water stress (at -2.7 MPa water potential) and were able to retain higher water content (300% of leaf dry matter) than fully expanded wheat leaves which contained less water (100% of leaf dry matter) under the same condition (Munns, Brady *et al.* 1979).

Second, the ability of the plant to withstand water stress, and to osmoregulate, may be increased by cycling stress conditions. In the current study only one 'cycle' was imposed. For example, leaves of potted strawberry plants, *Fragria virginiana*, exhibited leaf osmotic potential which was 0.2 MPa lower than control during the first stress cycle, but 1.0 MPa lower than the control leaves after 2 cycles of 7-10 days water deprivation (O'Neill 1983). A similar behaviour was observed in a field experiment with strawberry plants (*F. xannanasa*), wherein the leaf osmotic potential of the 'dry group' dropped by 0.2 to 0.4 MPa relative to the 'wet group' after 8 months of intermittent cycles of water stress (Save, Penuelas et al. 1993). Similarly, adaptation to water stress in mature cotton leaves was most evident after four cycles of 2-day stress (Ackerson and Herbert 1981).

A gradual stress conditioning through cycles of stress interspersed by recovery periods may trigger stronger osmoregulatory response as observed previously in strawberry and cotton leaves (Ackerson 1981; Ackerson and Herbert 1981; O'Neill 1983). Further investigations on stevia osmoregulation might be conducted using stress cycles with a maximum water stress below -1.9 MPa (stem water potential) which was previously observed to be the permanent wilting point of stevia (Gautam-Kafle, unpublished).

Conclusion

SGs, in the form of stevioside and rebaudioside A, do not seem to function as an alternative carbon store and osmoregulator given the relatively stable SG concentration in contrast to the responsive change in starch leaf content with respect to light and CO_2 levels. As SG synthesis involves an upregulation of the GA biosynthetic pathway, SG synthesis seems to be driven by factors other than the short term (days) nutrient/resource availability and water stress. A possible role of SG in altering herbivory patterns will be considered in future experiments.

Chapter 9

A role for steviol glycosides in mammalian herbivory feeding preference?

Abstract

Guinea pigs were used in choice feeding trials, being presented with choices between a control feed and feed amended to contain 5% sucrose, 0.02, 4 or 10% stevia leaf. Animal body weight, water and feed consumption were monitored on a daily basis for the three day feeding events. An increase (39% above the control) in total feed intake was observed at high levels of steviol glycoside (SG) amendment of feed (10% stevia leaf w/w mixed with standard feed). Encouragement of general herbivory may provide ecological fitness to *Stevia rebaudiana* if it is more tolerant of grazing pressure than other plants in its environment. Improvement in feed intake may have commercial implication for use of stevia as an additive in stock feeds.

Introduction

Taste and smell influence feeding behaviour. In mammals, taste receptors for sweet, salt, sour and bitter taste have been described, with these sensors guiding feeding behaviour to select foods rich in soluble carbohydrates and amino acids, and to avoid foods containing toxic compounds or undergoing decay (Harborne 1982).

Taste preference is typically gauged through behavioral “two-bottle choice tests”, i.e. between plain water and an aqueous solution of tastant compounds, or comparative ‘standards’ such as glucose or sucrose for

sweet taste, quinine or sucrose octa-acetate for bitter taste, acetic acid or citric acid for sour taste or tannic acid for astringent taste (Jacobs, Beauchamp *et al.* 1978; Harborne 1982). Tastants may also be mixed with ground feed which are then presented as an alternative feed in choice feed tests (Jacobs, Beauchamp *et al.* 1978; Harborne 1982; Munro, Lirette *et al.* 2000). More coarsely, the natural product containing the tastant may be presented as the alternative feed.

Mountain gorillas in the African Congo are known to feed mainly from 29 species out of a hundred angiosperm species present in their habitat (Harborne 1982). These selected plants are of different families, but all lack condensed tannins (Bates-Smith and Metcalfe 1957; Harborne 1982). Similarly, the colobus monkey, *Colobus guereza*, will reject plant parts with over 0.2% dw tannin (Oates and Swain 1977; Harborne 1982). Most of the trees in the native habitat of the black colobus, *Colobus satonas,* have leaves with relatively high levels of tannin, and this monkey is observed to feed only on rare deciduous trees, supplemented with plant seeds, thus avoiding food with higher tannin levels (McKey, Waterman *et al.* 1978; Harborne 1982). Harborne (1982) infers from this body of work that primates have a general preference for plant material with minimal tannin content.

However, while taste may be used to detect positive and negative features of a food source and cause learned preference or aversion of a given food source by taste association, it is also possible for taste preference to be influenced by secondary correlations with other attributes of the sample that affect a behavioral influence. For example, physical discomfort triggered by ingestion will result in conditioned taste aversion of the flavour associated with that food source (Provenza, Pfister *et al.* 1992). Lithium chloride causes temporary nausea upon intake and when associated with a flavor results in subsequent rejection of that particular flavor, as documented for goats (Kimball and Billings 2007), sheep (Burrit and Provenza 1990) and guinea pigs (Lichtenstein and Cassini 2001).

Taste preference may also be influenced by taste sensitivity, which varies between species and also between individuals (Hagstrom and Pfaffmann 1959; Brouwer, Hellekant *et al.* 1973; Hellekant and Danilova 1996). Responsiveness to a given taste stimulant can be quantified through measurement of electrophysiological signals from the chorda tympani following the exposure of the tongue to different concentrations of a tastant compound (Sato 1985). Taste sensitivity has been demonstrated to vary between rats, hamsters, gerbils, guinea pigs and macaque monkeys, with the latter having the closest behavior to humans (Beidler, Fishman *et al.* 1955; Hagstrom and Pfaffmann 1959; Noma, Goto *et al.* 1971; Brouwer, Hellekant *et al.* 1973; Noma, Sato *et al.* 1974; Jakinovich Jr. and Oakley 1975; Jakinovich Jr. 1981; Vasquez and Jakinovich Jr. 1993). Given a sucrose solution (0.5 M) as a common stimulus, the chorda tympani response to sucrose (relative to 0.1 M NaCl) was greatest in hamsters (0.75), followed by guinea pigs (0.62) and then rats and cats (0.21) (Beidler, Fishman *et al.* 1955).

SGs are of commercial interest for their perceived sweetness, that is, they elicit a response from the 'sweet' taste receptors of the mouth, with a response approximately 300 times that of sucrose (on a weight basis) (Inglett 1981). SGs comprise about 10% of stevia leaf dw, conferring a level of sweetness that may act in either herbivory attraction or deterrence towards the leaf. Herbivore deterrence has an obvious ecological benefit, while attraction may serve to aid seed dispersal or to reduce the fitness of neighbor competitor plant species through associated grazing. To date, no study of herbivory of stevia in its native habitat have been documented, although cattle grazing of stevia in Paraguay has been observed (Soejarto 2002). Munro *et al.* (2000) considered the addition of commercial stevia powder into solid feeds to encourage intake of solid feeds in weaning pigs. Pigs were randomly assigned to either a standard control feed, a sucrose-mixed feed (5% w/w) or a feed mixed with a commercial stevia sweetener (at 83.3, 167 or 334 mg kg^{-1} feed, equivalent to 2.5%, 5% and 10% w/w sucrose using the human equivalence taste of 300 times sweeter than sucrose). Only

the intake of sucrose amended feed was significantly higher than the control feed, with the intake of the stevia-mixed feeds comparable with the control. Thus there is no evidence that SG influences herbivory.

The indigenous mammalian herbivores of the Andes region of Colombia, Ecuador and Chile includes the cavies species, *Cavia aperea* (Gade 1967) (Fig. 9.1). As the domestic guinea pig (*Cavia porcellus*) is the closest tamed relative of the wild specie (e.g. *Cavia aperea*), it was considered to be an appropriate animal model to test for herbivore preference to stevia leaf. Taste preference of wild and domestic guinea pigs has been previously investigated through choice experiments between water and different tastant solutions such as glucose and saccharin (for the sweet taste), sodium chloride (for the salty taste), citric acid (for the sour taste) and quinine sulfate and sucrose octaacetate (for the bitter taste) (Jacobs and Beauchamp 1977; Jacobs 1978). Overall, guinea pigs demonstrated a preference for the sweet taste (solutions of 0.2 M and higher glucose were significantly preferred over water within a solution range of 0.0016 - 0.8 M, $p<0.01$, while saccharin was significantly preferred at 0.0008 M – 0.016 M, $p<0.01$). Guinea pigs tolerated the bitter quinine sulfate (0.00016 M - 0.00124 M) and rejected the bitter sucrose octaacetate at concentrations higher than 0.001 M while salty (concentrations greater than 0.5 M sodium chloride) and sour (for all tested concentrations, 0.00025 M - 0.063 M citric acid) solutions were rejected (Jacobs and Beauchamp 1977; Jacobs 1978).

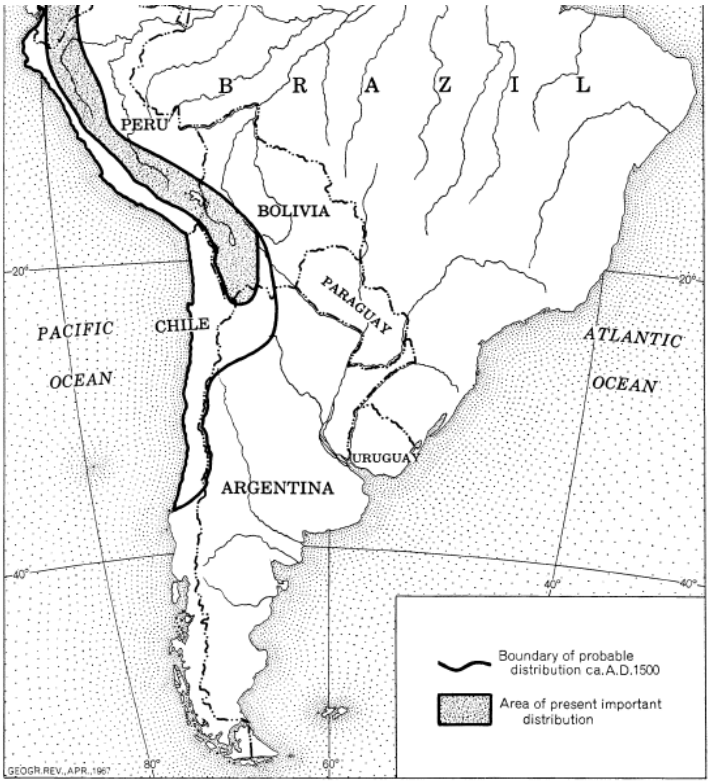


Figure 9. 1. Distribution of guinea pigs in its native habitat (Gade 1967)

In this chapter, guinea pigs were employed as an animal model for possible herbivory attraction encouraged by the sweet taste of stevia leaves. Guinea pigs were given a choice of either a standard guinea pig feed or a feed sweetened with either sucrose (5% w/w) or with stevia leaf (at 0.02%, 4% or 10% w/w). Preference was assessed through the absolute and proportional amounts of consumption of the two feeds.

Materials and Methods

Cavia porcellus preference towards stevia leaf was tested through a series of two-choice feed experiments using either a standard guinea pig feed amended with crushed stevia leaf or a standard feed mixed with sugar as an alternative feed compared to standard guinea pig feed. Preference was

measured by comparing the intake of both feeds during each test period. Guinea pig weight was also measured daily.

Animal selection and culture

A total of 18 male guinea pigs (*Cavia porcellus*) of different breeds and sizes were acquired from a local pet shop. The 18 guinea pigs were assigned to two feeding groups, with each group having 9 guinea pigs. Male guinea pigs were selected to eliminate the effect of the estrous cycle on the feeding behavior. The animals were initially kept in pairs for five days to help transition to the new environment, after which the guinea pigs were placed individually in cages. Cages were arranged in a circular formation and guinea pigs were referred to according to the cage number (Fig. 9.2). Adjacent cages were separated by wire mesh that allowed a level of interaction between animals. Each wire-bottomed cages had a dimension of 38 cm x 90 cm x 29 cm, with an enclosed section and an open section in which feed was presented (Fig. 9.2). The enclosed section was lined with cardboard and shredded paper to serve as bedding for the guinea pigs. In the open section, two feeding dishes were placed against opposite sides of the cage, with a water bottle placed centrally on the end wall of the cage (Fig. 9.2). The feeding dish was made out of a disposable plastic dish as base and a pot (12 cm diameter x 13 cm height) as side walls of the dish. A 9 x 9 cm square window was cut through the pot to allow the guinea pigs access while feeding. The dish was screwed on a 10 x 10 x 1 cm wooden platform to prevent the tipping of the dish during feeding. The base of the feeding dish was lined with cardboard to allow collection of feed that was spilled during feeding. The cardboard base was divided into two by a cardboard folding to distinguish spills from each feeding dish.

Room temperature was maintained at 25 °C, light was maintained at 12 h light / 12 h dark diurnal cycle and classical music was played at low volume.

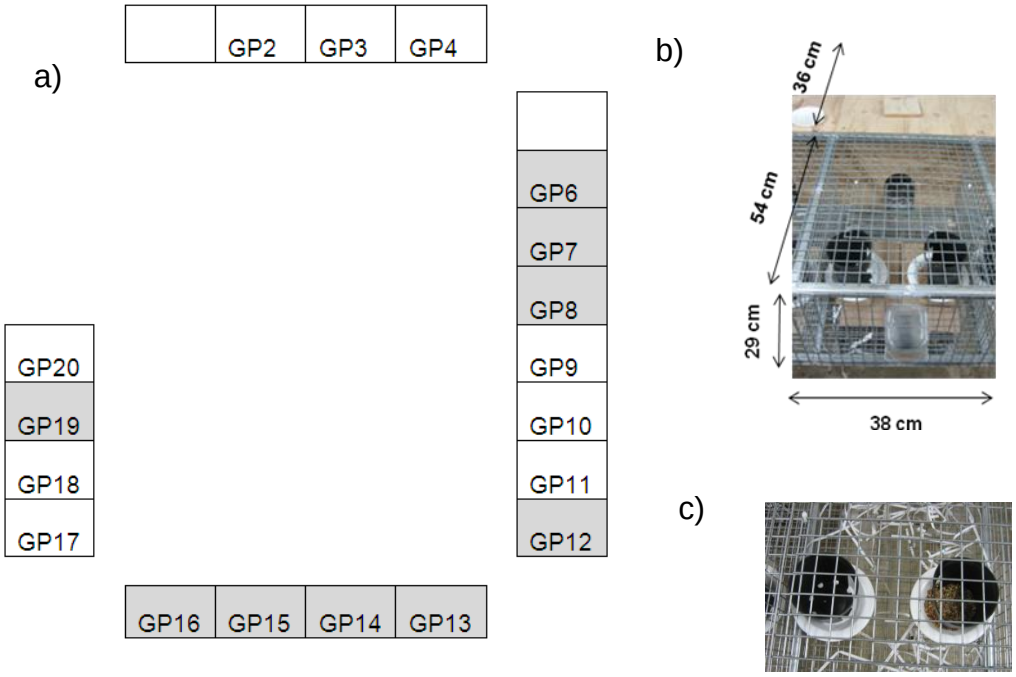


Figure 9. 2. Cage assignment of guinea pigs and their arrangement inside the feeding room (a) with layout of feeding area (b) and pairs of feeding dish (c) in detail. Shaded cages (in panel a) belong to the second feeding group while unshaded cages belong the first feeding group.

Feed preparation and SG amendment

Feed acquired from a local pet shop consisted of a mix of pellets, grains and lucerne hay. To allow for homogenous mixing with the stevia leaves, both the standard feed and the stevia leaf material was ground through a hammer mill (Talon garden shredder) to pass a 2 mm mesh. For the 4% w/w stevia feed mix, 500 g of ground stevia leaf was mixed with 12 kg of ground standard feed, and re-ground to assist in homogenization. For the 10% stevia feed mix, 2 kg of the 4% w/w stevia feed mix was mixed with additional 120 g of ground stevia leaf. For the 0.02% stevia feed, the 4% stevia mix was milled finely using a Retsch Centrifugal Grinding Mill (0.2 mm mesh size). Then 4 g of the fine powder was sprinkled and mixed onto 70 g of the standard feed.

To avoid particle size differences between sugar crystals and the ground standard feed, sugar was added to the feed by spraying a sugar solution (1000 g of table sugar dissolved in 3 L of water) onto the ground standard feed (20 kg). To ensure homogeneous distribution of the sugar solution, the

standard feed was thinly layered on a horizontal surface during spraying. The mix was then allowed to dry in a ventilated oven at 40 °C for 24 hours. After drying in the oven, the feed was passed through the mill before storage.

The ground stevia leaf material was sampled and assessed for SG content prior to mixing with the standard feed pellet. The stevia leaves contained stevioside (7.9% dw) and rebaudioside A (1.8% dw). The stevioside content of the stevia leaf amended feeds was calculated on a % w/w basis (Table 9.1).

	Types of stevia-amended feed		
	stevia 0.02%	stevia 4%	stevia 10%
stevia leaf content (% w/w)	0.02	4	10
stevioside (% w/w)	0.002	0.316	0.79
sucrose equivalence (% w/w)	0.2	37 %	92%

Table 9. 1. Summary of stevioside concentration in the stevia-mixed feeds based on HPLC leaf analysis of the ground stevia leaves (7.9% stevioside and 1.8% rebaudioside A). Sucrose equivalence ratio to stevioside was assumed to be 1 : 117 (see Table 9.2).

Feeding schedule

The feeding schedule (Fig. 9.3) began with a two-week acclimatization phase. During the acclimatization phase, standard feed was placed in only one of the two feeding dishes. This dish was randomly chosen every day to avoid development of a spatial preference by the animals. After the acclimatization phase, a series of test periods (T) of three days each was imposed. During each test period, the standard feed and the test feed were presented in separate feeding dishes, with the position of the two feeding dishes switched daily to avoid spatial bias. Test periods were separated by control periods (C) of at least 7 days duration.

The daily feeding schedule started at 10.30 am, with 1 h taken for weighing and refilling of feeds and water containers, and weighing of animals of each feeding group. The order of cage monitoring was kept constant within the 1

hour period to minimize any effect on the feeding behavior. The proportion of feed sampling from each dish relative to the total feed intake was calculated as an indicator of feed preference during the test periods. The first test (T1) involved a choice between the standard feed and the feed mixed with stevia leaves (at 4% of the total dw). The second test (T2) was a choice between the standard feed and the feed mixed with stevia leaves (at 10% of the total dw). The third test (T3) was a choice between the standard feed and the feed mixed with sucrose amounting to 5% of the total feed weight. Test 4 (T4) was a choice between the standard feed and the feed mixed with sucrose (at 5% of the total dw; i.e. a repeat of T3). Test 5 (T5) involved a choice between the standard feed and the feed mixed with stevia leaves (at 0.02% of the total dw). The last comparison (T6) was a choice between the sucrose feed as used in T4 and the stevia feed as used in T5.

Tests 1 to 3 were conducted on a group of guinea pigs consisting of 9 male American breed guinea pigs which were presented with three consecutive sets of choice tests (Fig. 9.3). During the subsequent control period, symptoms of weight loss were observed in some guinea pigs and so a decision was made to discontinue use of these animals. Tests 4 to 6 were conducted on a second group, consisting of 3 American and 6 Abyssinian male guinea pigs which were presented consecutively with another three sets of choice tests (Fig.9. 3).

Feeding Group 1 9 guinea pigs Average guinea pig weight: 450 g	**Feeding Group 2** 9 guinea pigs Average guinea pig weight: 600 g
Acclimatization period (14 d)	Acclimatization period (14 d)
T1 test period (3 d) control feed *cf* Stevia feed (4% w/w)	**T4** test period (3 d) Control feed *cf* Sucrose feed (5% w/w)
Control period (7 d)	Control period (8 d)
T2 test period (3 d) Control feed *cf*. Stevia feed (10% w/w)	**T5** test period (3 d) Control feed *cf* Stevia feed (0.02% w/w)
Control period (10 d)	Control period (15 d)
T3 test period (3 d) Control feed *cf*. Sucrose feed (5% w/w)	**T6** test period (3 d) Sugar feed (5% w/w) *cf* Stevia feed (0.02% w/w)

Figure 9. 3. Feeding scheme for the two feeding groups (n = 9 animals each). Position of the feeding dish was randomly switched daily to minimize any spatial preference among the guinea pigs. % w/w refers to percentage of ground stevia leaf or sucrose included in the standard diet.

The overall design of the experiment was submitted to and approved by the CQU Animal Ethics Committee (A09/05-247) headed by Dr. Andrew Fenning as AEC chair.

Statistical analysis

The daily proportion of feed intake from the two feeding dishes was calculated for each animal. A modified chi-square test was used to quantify the normal distribution curve of the feeding behavior of the guinea pigs during the control periods (McKillup 2005). Assuming a non-preference between the two feeding dishes, a 50:50 sampling ratio from both dishes is expected. Using this assumption, the daily chi-scores for each guinea pig were calculated for all days included in the control period. For example, for a 40:60 ratio of feed sampling between dish A and B, the chi-square score of the guinea pig on that particular day is calculated as:

$$\chi^2 = \frac{(40-50)^2}{50} + \frac{(60-50)^2}{50} = 4 \qquad \text{(eq. 9. 1)}$$

The daily chi-scores of all animals for the control days (C) (n = 202) were then tabulated and ranked. The topmost 5% of the chi-scores for the control period were then regarded as significant deviations from a 50:50 sampling of the two feeding dishes and adopted as a criterion for significant difference. Thus test days with chi-scores below the criterion value were interpreted to indicate a significant preference for the feed with the higher proportion of feed intake.

The average daily intake of the control feed, the test feed and the sum of both during the test periods were compared using a one-way anova analysis using SPSS v 17.0.

Results

Feeding preference

The guinea pigs in both feeding groups maintained a steady weight varying by about +/- 4% over the entirety of the trial periods (60-70 days) (Fig. 9.4 and 9.5). A few animals lost weight beyond this range, which was interpreted

as a sign of health deterioration, and led to exclusion of these animals from the analysis. For group 1, guinea pigs in cage 3 and 18 were excluded while for batch 2, guinea pigs in cage 6 and 7 were excluded. This reduced the number of replicates per feeding group from 9 to 7.

In the first feeding group, most of the guinea pigs consumed the control and test feed equally, regardless of the type of test feed (4% stevia, 10% stevia, 5% sucrose). Three (GP 11, 17 and 20) of the seven guinea pigs exhibited a significant rejection of the test feed ($\chi^2 > 56.25$), although only on some days (Fig. 9.6). GP11 was cautious and initially rejected the 4 and 10% stevia feed, but consumed the test feed on later days in some test periods (T1 and T2). GP 17 likewise ignored the 4% stevia feed on the first day of introduction but then consumed the stevia-mixed feeds on subsequent days. The same cautiousness was demonstrated by GP 17 towards the 5% sucrose feed on the first test day (T3), which was overcome in the following 2 days. GP 20 also demonstrated a slight avoidance of the 4% stevia for only one day, after which both control and test feed were consumed.

In the second feeding group, the majority of animals exhibited equal preference for the control and test feed except for three (GP 8, 12, 16), which exhibited a slight preference ($\chi^2 > 51.02$) for the sweetened feed (Fig. 9.7). GP 12 consistently preferred the 5% sucrose feed over the control feed for the three consecutive test days (T4), while GP 16 preferred the 5% sucrose feed during the 1st and 3rd test days. GP 8 demonstrated one day of preference for the 0.02% stevia feed. When given a choice between the sucrose (5%) and the stevia (0.02%) feed (T6), equal sampling of both dishes was observed.

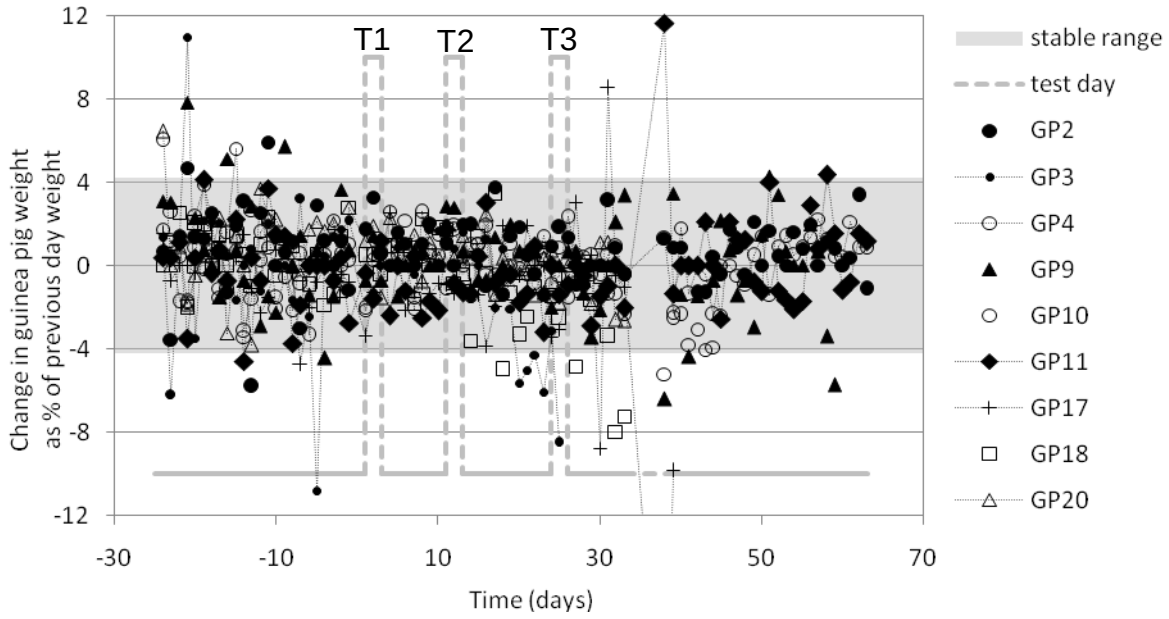


Figure 9. 4. Feeding group 1: Percentage change in guinea pig weight relative to weight measurement from the previous day (T1 - standard feed *cf* 4% stevia feed, T2 - standard feed *cf* 10% stevia feed, T3 - standard feed *cf* 5% sucrose feed). Shaded area indicates ± 4% deviation from initial weight.

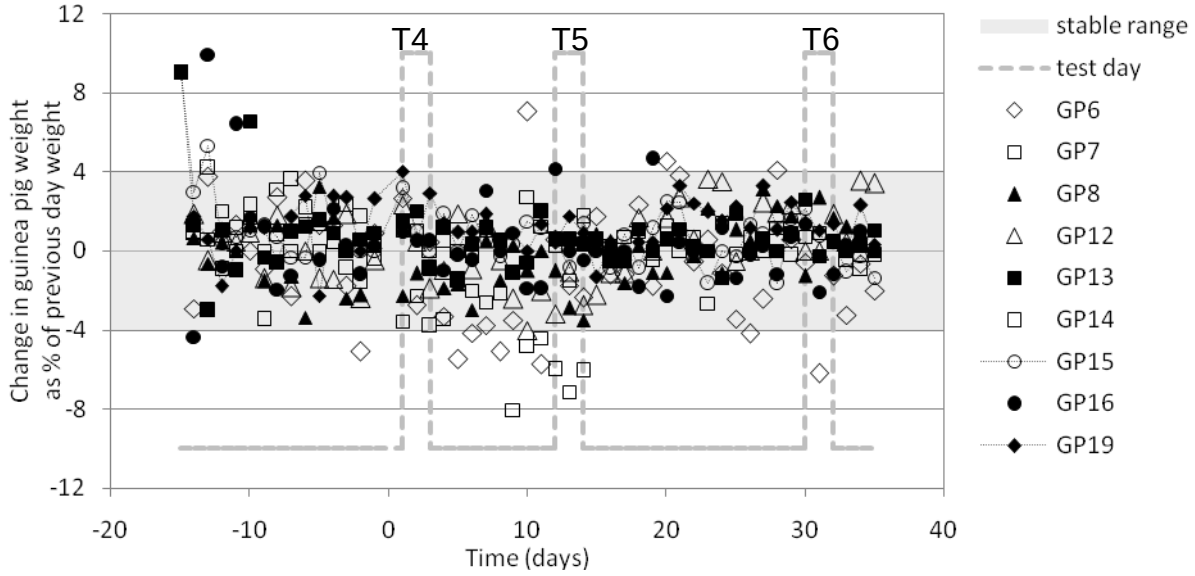


Figure 9. 5. Feeding group 2: Percentage change in guinea pig weight relative to weight measurement from the previous day (T4 - standard feed cf 5% sucrose feed, T5 - standard feed cf 0.02% stevia feed, T6 – 5% sucrose feed cf 0.02% stevia feed).

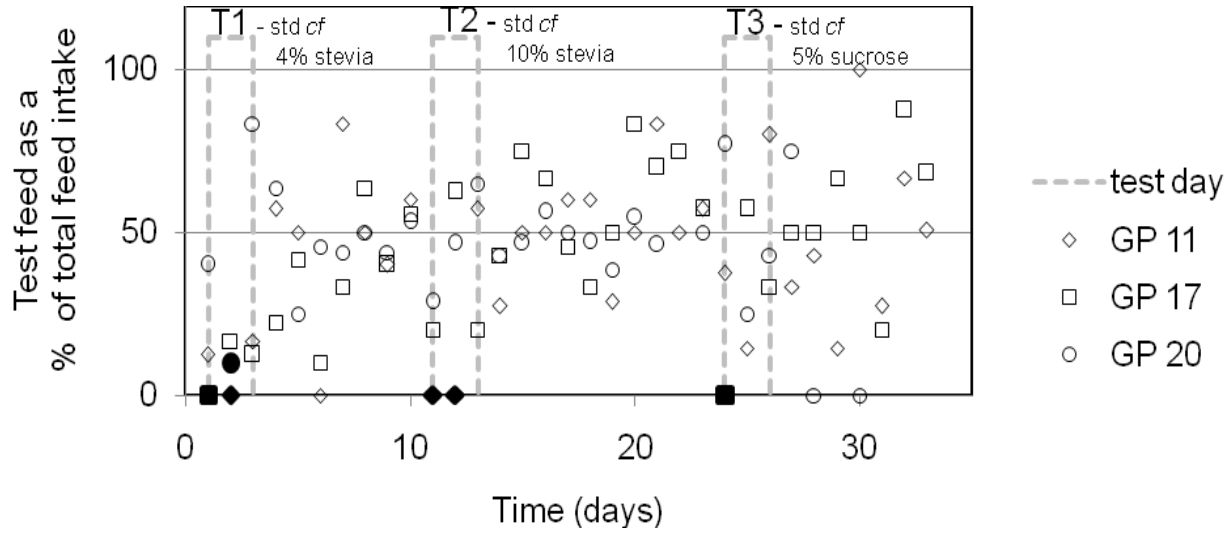


Figure 9. 6. Feeding group 1: Intake of sucrose or stevia feed expressed as a percentage of the total feed intake of animals (GP 11 – 520 g, GP 17 – 520 g and GP 20 – 420 g) which demonstrated a significant deviation from a 50:50 feed sampling behavior. Feeding behavior is plotted for the three test periods (T1 - standard feed *cf* 4% stevia feed, T2 - standard feed *cf* 10% stevia feed, T3 - standard feed *cf* 5% sucrose feed) and intervening control periods. Bold symbols indicate significant deviation from a 50:50 sampling ($\chi^2 > 56.25$).

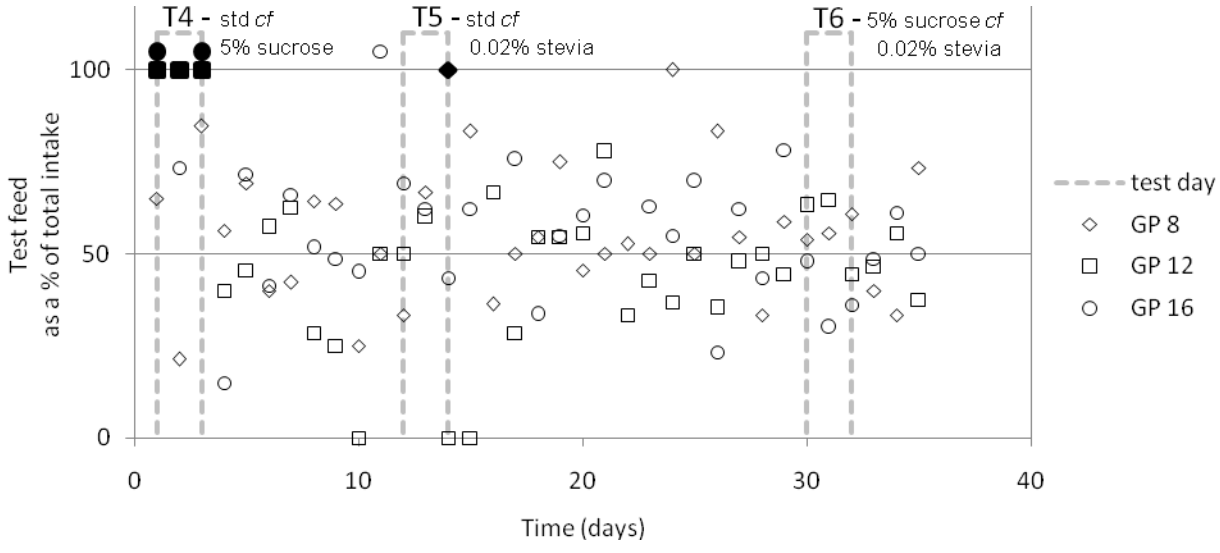


Figure 9. 7. Feeding group 2: Intake of sucrose or stevia feed expressed as a percentage of the total feed intake of animals (GP 8 - 750 g, GP 12 - 390 g and GP 16 – 878 g) which demonstrated a significant deviation from a 50:50 feed sampling behavior. Feeding behavior is plotted for the three test periods (T4 - standard feed *cf* 5% sucrose feed, T5 - standard feed *cf* 0.02% stevia feed, T6 – 5% sucrose feed *cf* 0.02% stevia feed) and intervening control periods. Bold symbols indicate

significant deviation from a 50:50 sampling (χ^2 > 51.02). Data for GP16 was shifted upwards by 5% to avoid overlap of data points.

Water intake was relatively stable across the entire treatment duration for both the first and second feeding group, except for a 3-day control treatment window in the second feeding group (Fig. 9.8). The change in drinking behavior was most likely due to the temporary fortification of the control feed with lucerne hay (8 g) for 9 days which began just after the end of the T5 treatment, to help alleviate the stress condition observed in some animals within the first feeding group. After the 9 days of fortified food, the group was returned to the standard control feed for another 6 days before proceeding with the last test feed (T6). Within this period, normal drinking behavior resumed (Fig. 9.8B).

Although an equal preference between the control feed and the test feed was generally exhibited, the total feed intake (expressed as a percentage of body weight) increased significantly during treatment T2 (10% stevia) relative to all other treatments ($F(2,1) = 5.463$, $p = 0.008$) (Fig. 9.9). The increase in total intake was due to a significant increase in intake of the 10% stevia feed ($F(2,1) = 5.725$, $p = 0.006$) and a marginal, but not significant, increase in the intake of the control feed ($F(2,1) = 2.428$, $p = 0.101$) (Fig. 9.9). With the second feeding group, the amount of feed intake was similar for both the control and test feed during the two test periods, T4 and T5 (Fig. 9.9).

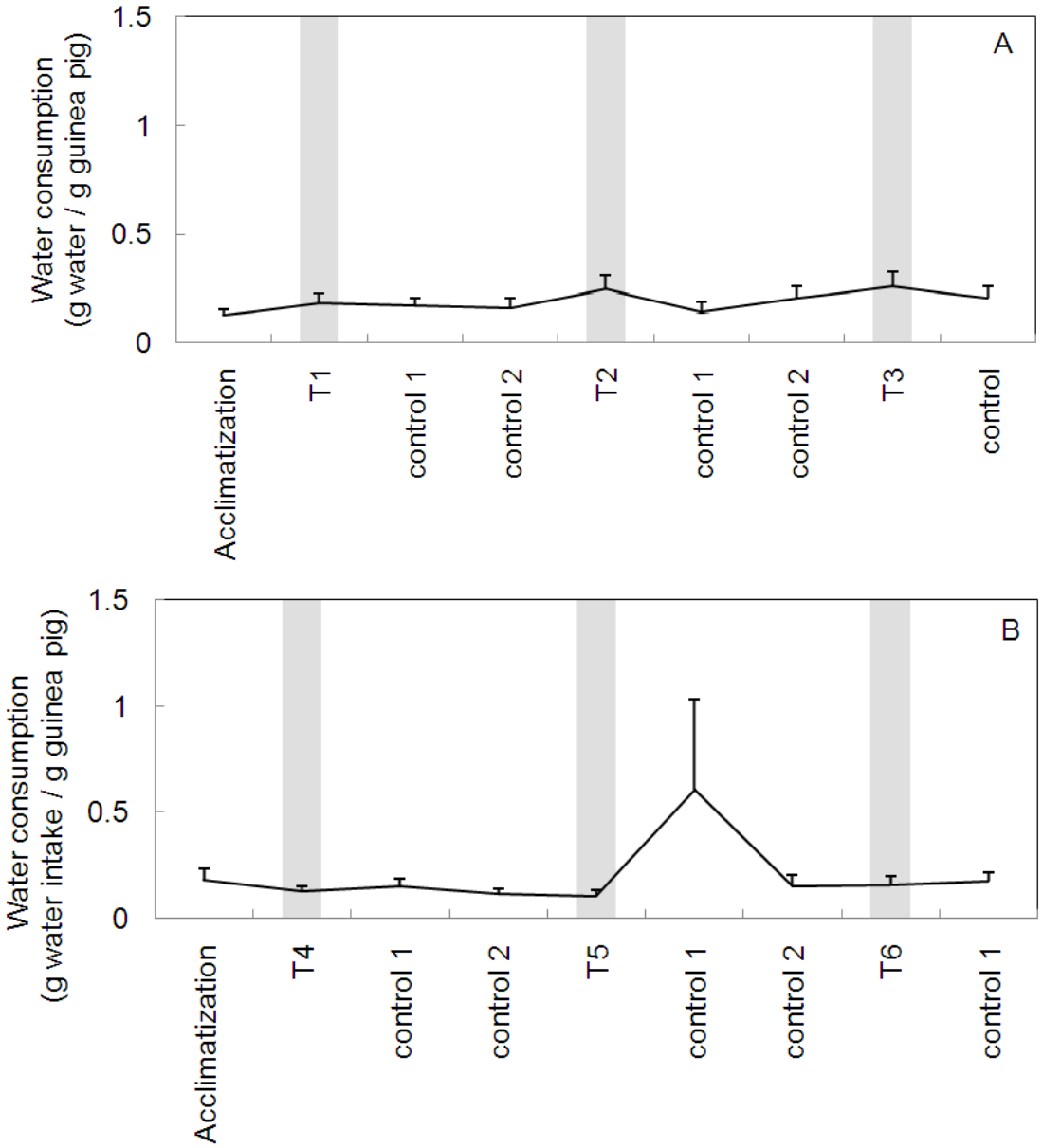


Figure 9. 8. Average water intake of each guinea pig expressed relative to body weight from (A) feeding group 1 and (B) and feeding group 2. Each point represents an average for seven guinea pigs over a three day period (n = 21) and associated SEM. Shaded area represent test periods).

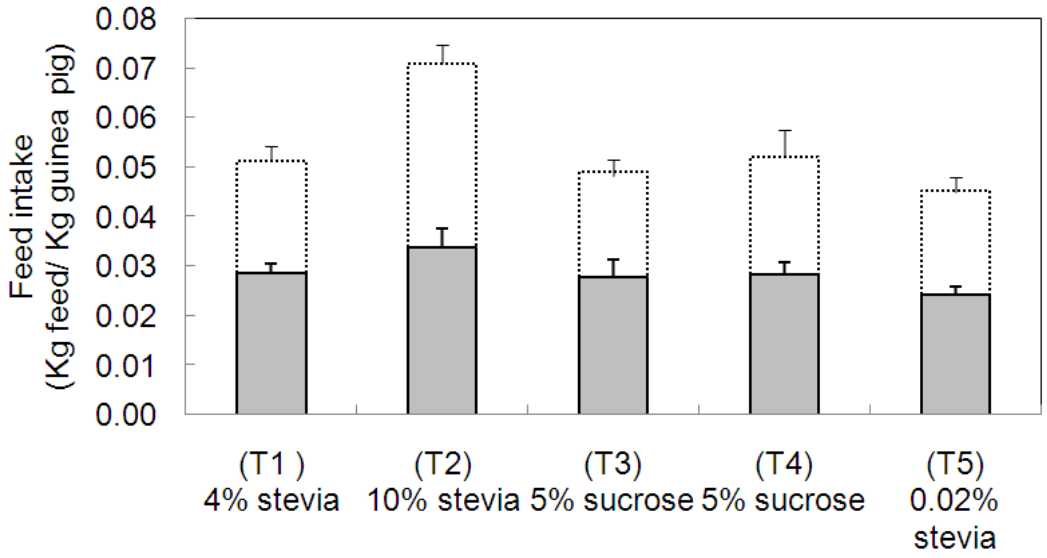


Figure 9. 9. Average daily intake of the control feed (dark bar) and the test feed (white bar) during five test periods (**T1**: control feed *cf* 4% stevia feed of group 1, **T2**: control feed *cf* 10% stevia feed of group 1, **T3**: control feed *cf* 5% sucrose feed of group 1, **T4**: control feed *cf* and 5% sucrose of group 2, **T5**: control feed *cf* 0.02% stevia feed of group 2). Feed intake was expressed as the ratio of feed weight to guinea pig weight (n = 5; vertical bars: standard error of total feed intake). Guinea pigs that exhibited preference on some days (GP 11, 17 and 20 from feeding group 1 and GP 8, 12 and 16 from feeding group 2) were excluded from the averaging so that only the feeding behavior of guinea pigs with equal preference was considered.

Discussion

Animal health

The steviol derivative of SGs has toxicological effects (Geuns 2004). In studies on mice, rats and hamsters, stevioside was not lethal at a high dose of 15 $g.kg^{-1}$ body weight ingested over a 14-day period (Tsokulkao, Chaturat *et al.* 1997; Geuns 2004). However, steviol was lethal to hamsters at an LD_{50} of 5.2 and 6.5 $g.kg^{-1}$ body weight for male and female respectively, while rats and mice had higher tolerance to steviol (LD_{50} > 15 $g.kg^{-1}$ body weight) (Tsokulkao, Chaturat *et al.* 1997; Geuns 2004).

Of animals in the first feeding group, a total of six guinea pig fatalities occurred within a five week span following the 6 weeks of feeding tests (T1, T2 and T3). Of the six guinea pig fatalities, one was autopsied by a

veterinarian, with pathological and histological testing of the intestine, lung, heart and liver tissues. The laboratory report indicated inflammation of the mesenteric fat which was attributed to possible dietary deficiency of antioxidants, a fatty change in the liver which may be consistent with anorexia, a pulmonary oedema in the lungs and stomach erosion possibly due to stress.

The long duration of the feeding trials (almost 3 months), during which time the guinea pigs were confined individually in a cage, may have aggravated stress in these social animals. With the second feeding group, the animals were provided with a daily dose of vegetables in addition to the dry feed. The second series of tests was conducted for almost 2 months and only one fatality was observed.

Based on the average daily intake of the stevia-amended feed illustrated in Fig 9.9 (0.023 kg of 4% stevia feed / kg body weight and 0.037 kg of 10% feed / kg body weight), the amount of equivalent steviol intake from the 4 and 10% stevia feed mix was 29 and 119 mg / kg body weight. Conversion was done using the stevioside concentration of the stevia-amended feed (see Table 9.1) and the molecular weight of steviol (318 g/mol) and stevioside (805 g/mol). Using the LD_{50} of 5.2 g.kg body weight for male hamsters as a conservative estimate, the maximum daily steviol equivalent consumed by the guinea pigs (119 mg.kg body weight from the 10% stevia feed), is 44 times lower than the hamster LD_{50}. Even if the cumulative amount of steviol equivalents from both stevia feeds (4 and 10%, which were separated by a 7 d control feeding event) was considered, the total steviol equivalent (444 $mg.kg^{-1}$ body weight) from the 3-day exposure to the 4% stevia feed (87 $mg.kg^{-1}$ body weight) and the 10% stevia feed (357 $mg.kg^{-1}$ body weight) would still be below the hamster LD_{50} of 5.2 $g.kg^{-1}$ body weight.

In comparison, a sub-acute toxicity study on hamsters with a maximum daily dose of 2.5 $g.kg^{-1}$ body weight of stevioside over a four month period (120 days) had no adverse effect on the growth or reproduction of hamsters or their succeeding two generations of offspring was observed (Yodyingyuad

and Bunyawong 1991). This level is six times higher than the cumulative (maximum) steviol intake of the guinea pigs in this experiment (444 $mg.kg^{-1}$ body weight).

The difference in adverse health effect relative to SG dosage (expressed in steviol equivalence) between this chapter and the aforementioned hamster experiment (Yodyingyuad and Bunyawong 1991) may be attributed to the fact that the SGs consumed by the guinea pigs were taken in combination with other secondary metabolites found in the dry stevia leaves. Synergism has previously been observed among secondary metabolites wherein mixtures were found to be more toxic than the expected additive effect of individual compounds. For example, the binary mixture of trans-anethole/thymol, trans-anethole/a-terpineol, a-terpineol/eugenol and thymol/citronellal were deterrent at lower concentrations (expressed in $\mu g/cm^2$) than when presented individually to tobacco cutworm, *Spodoptera litura* (Hummelbrunner and Isman 2001; Wittstock and Gershenzon 2002). The antifungal effect of α-solanine and α-chaconine, extracted from potato, also displayed synergism against *Ascobolus crenulatus*, *Alternaria brassicicola*, *Phoma medicaginis* and *Rhizoctonia solani* when mixed in a wide range of mixtures with as little as 10% of each compound (Fewell and Roddick 1993; cited in Gershenzon and Dudareva 2007). Possibly, the fatality of some of the guinea pigs may be caused by the synergistic effect of secondary metabolites in the stevia leaf. Further work on feeding with guinea pigs should be conducted using standard solutions of SG in pure or in combination should be explored to confirm possible deterrent effects on guinea pigs with measurements of LD_{50}.

SG sweetness relativity

The electrophysiological response of Mongolian gerbil taste buds to different sweet solutions has been extensively documented in terms of the electrical signal strength from the chorda tympani nerve as the taste buds are exposed

to different sweet solutions (Beidler 1954; Jakinovich Jr. and Oakley 1975; Jakinovich Jr. 1976; Jakinovich Jr. 1981; Vasquez and Jakinovich Jr. 1993). Molar equivalents of the sugars based on chorda tympani responsiveness of gerbils are summarized in Table 9.2. Sucrose is perceived by gerbils to be sweeter than glucose, while the sweeteners saccharin, stevioside and rebaudioside A were perceived to be sweeter than sucrose although at a lesser magnitude than human taste perception (Inglett 1981). Reported electrophysiological tests on guinea pigs are limited only to sucrose (0.5 M) and two sweet proteins, monellin and thaumatin (0.02%), with only sucrose eliciting an electrical response on the chorda tympani nerve (Brouwer, Hellekant *et al.* 1973). Reports of guinea pigs chorda tympani response in relation to saccharin, stevioside and rebaudioside A were not encountered.

	Ratio	Basis	Reference
sucrose : glucose	1 : 0.5	glucose solution (0.5M) elicits only half the CT signal of sucrose solution (0.5M)	(Jakinovich Jr. and Oakley, 1975)
sucrose : saccharin	1 : 4	3.9 x 10-2 M sucrose eliciting a response similar to 9.9 x 10-3 M saccharin	(Jakinovich Jr., 1981)
sucrose : stevioside	1 : 117	based on the Kd values of sucrose (0.07) and stevioside (0.0006) which were obtained from the plot of CT responsiveness vs molar concentration of sucrose and stevioside	(Jakinovich Jr., 1976; Vasquez and Jakinovich Jr., 1993)
sucrose : reb A	1 : 117	based on the Kd values of sucrose (0.07) and reb A (0.0006) which were obtained from the plot of CT responsiveness vs molar concentration of sucrose and reb A	(Jakinovich Jr., 1976; Vasquez and Jakinovich Jr., 1993)

Table 9. 2. Summary of equivalent ratio of sucrose to other sweet compounds based on the electrophysiological response of the chorda tympani (CT) of the Mongolian gerbil based on literature (Beidler 1954; Jakinovich Jr. and Oakley 1975; Jakinovich Jr. 1976; Jakinovich Jr. 1981; Vasquez and Jakinovich Jr. 1993). Kd is the dissociation constant by Beidler's (1954) taste theory to summarize the linear relationship between the concentration of the tastant compound and the available site of taste receptors.

Given the lack of available data on guinea pigs, the sweetness equivalence derived from the gerbil electrophysiological tests will be used with the assumption that the taste bud receptors of gerbils and guinea pigs are similar. This assumption is however taken with caution given the documented difference in taste response between animals (Beidler, Fishman *et al.* 1955) A sweetness equivalence of 117 (Table 9.2) implies that the

treatments of 0.02, 4 and 10% stevia in amended feeds had sucrose equivalency of 0.2, 37 and 92%. In retrospect the 0.02% stevia amendment was low, and ideally a 0.5% amendment (sucrose equivalency of 5%) could have been employed, to match the sucrose test feed.

Feeding preference

When the 5% (w/w) sucrose feed was presented with the 0.02% (w/w) stevia feed (0.2% sucrose equivalency) in the second feeding group, equal sampling was observed. Thus while the guinea pigs were presumably able to perceive the difference between the 5% sucrose and 0.02% stevia treatments, this perception did not influence feeding preference.

Preference for the 5% sucrose feed was shown over control feed, but only in some cases. Deterrence for the high concentration (4, 10% stevia) feed was shown over control feed, again only in some cases, however an increase in total feed consumption occurred with the 10% stevia treatment (39% increase relative to mean of T1 treatment). There was no impact on water consumption associated with the various test feeds. Thus the impact on animal feeding behavior was relatively mild, despite the use of levels of stevia that must have been detectable to the animals.

The current trial employed a trial duration of three days, with daily measurements. It is possible that in the current trial an initial short-term preference for the stevia-mixed feed may be obscured. For example, Jacob (1977; 1978) demonstrated guinea pig preference for solutions sweetened with glucose relative to saccharin and water using a two-bottle choice set-up with trials lasting only 4 hours. However, the longer feeding period and the use of solid feeds rather than liquids have more relevance to a consideration of an ecological role in influencing herbivory behaviour. Further work could occur to address the issue of short term preference, although such behavior is not relevant to the ecological role of herbivory to stevia. A choice test using water solutions of stevioside and rebaudioside A (as representative SG) and sucrose would be of interest to compare the feeding behavior with the pellet-

mixed feed. A range of concentrations should be used to allow a qualitative comparison of stevioside, rebaudioside A and sucrose sweetness as perceived by guinea pigs. A complementary electrophysiological study would quantify the sweetness equivalence in terms of the responsiveness of the chorda tympani of guinea pigs to the different solutions.

Some animals were cautious in sampling the stevia feeds. This result may be due to the presence of other components in the ground stevia leaves that were perceived organoleptically by the guinea pig. For example, the few guinea pigs that were cautious toward sampling of the stevia-containing feed may have been sensitive towards stevioside which is perceived by humans to have a bitter aftertaste. Guinea pigs have demonstrated a mild reduction of feed intake of water solutions due to addition of quinine and sucrose octaacetate even though guinea pigs were generally tolerant of bitter compounds (Jacobs 1978; Nolte, Mason *et al.* 1994). Further work could employ the stevia sweetening agents directly (stevioside compared to rebauside A) as feed amendments.

Commercial application in animal feeds

The increase in total feed consumption for 10% stevia amended feeds is interesting, implying a role for stevia amendment in animal feeds to increase growth rates. This potential has been considered previously by Munro *et al.* (2000), who report a study of weaning pigs which were presented solely with either a control feed, a sucrose-amended feed (5% w/w) or a feed mixed with commercial stevia sweetener (98% stevia purity, composition of stevioside and rebaudioside A not indicated). at three levels (83.3, 167 and 334 mg stevia sweetner.kg^{-1} feed; i.e. 0.008%, 0.02% and 0.03% w/w). Pigs assigned to the sucrose feed (at 5% w/w) consumed more feed (0.546 ± 0.021 kg pig^{-1} d^{-1}) than pigs assigned to the control feed (0.444 ± 0.021 kg pig^{-1} d^{-1}) or the stevia feed at any of the three stevia sweetner concentrations (at 334 mg.kg^{-1} feed, consumption was 0.473 ± 0.021 kg pig^{-1} d^{-1}) (Munro, Lirette *et al.* 2000).

Munro *et al.* (2000) estimated the stevia amended feed (334 mg stevia sweetener.kg^{-1} feed) to have twice the sweetness level of the 5% (w/w) sucrose test feed, based on the assumption that pigs perceive SGs to be 300x sweeter than sucrose (as for humans) (Inglett 1981; Munro, Lirette *et al.* 2000). However, in an electrophysiological study using the chorda tympani response, the pig was only 124 times more sensitive to stevioside (30 pulse/0.9 mM stevioside) than to sucrose (80 pulse/0.3 M sucrose) (Hellekant and Danilova 1996). Using this conversion factor, the amended feeds would have a sweetness equivalent to 1, 2 and 4% (w/w) sucrose feed. Thus, at least the feed amended with 83.3 and 167 mg stevia may not have been perceived to be as sweet as the 5% w/w sucrose feed, thus explaining the similar feed intake of the stevia feed with the control feed (Munro, Lirette *et al.* 2000).

At the levels of stevia incorporated in the feed (0.2, 4 and 10% w/w), there is little evidence that steviol gylcosides cause preferential herbivory. However, at a higher rate of stevia amendment (10% w/w), a 39% total increase in feeding was observed. The animals were apparently encouraged to eat the unsweetened control feed as well as the stevia-amended feed as the average intake of both feeds increased marginally.

Such encouragement in feeding would increase the ecological fitness of stevia if it is more tolerant of herbivory than its environmental competitors. However, at least for large herbivores, the reverse appears to be true, given Soejarto's (2002) remark on the decline of stevia in plant communities experiencing cattle grazing. The increase in total intake with the feed amended with stevia leaf at 10% (w/w) (or 7900 mg.kg^{-1} of pure SG), contrary to Munro (2000) (no increase for pig's presented with 334 mg pure SG.kg^{-1} feed), shall encourage other workers to trial stevia amendment for feeds of various stock and poultry.

Conclusion

Addition of SG to feed at a significant level seem to encourage overall feed as observed by the overall increase in intake of both control and 10% stevia test feed. This observation may have potential application in animal feed amendment. Health complications in guinea pigs may have occurred due to synergistic effect of other secondary metabolites found in the dry stevia leaves that were added to the amended feed. Further work with solutions of SG is necessary to check both the safety and sweetness suitability of SG in guinea pigs.

Acknowledgements

This work was undertaken in compliance with the CQUniversity animal ethics policy (A09/05-247). The assistance of Dr. Andrew Fenning with issues on animal culture, health and ethics, and A/Prof. Steve McKillup for statistics advice is also acknowledged.

Chapter 10

A role for steviol glycosides in insect- feeding deterrence towards the grasshopper *Valanga irregularis* and the red spider mite *Tetranychus urticae*?

Introduction

A range of secondary metabolites in plants have been identified as either insecticidal or feeding-deterrent to insects, with a range of the compounds belonging to the terpenoid family (Jacobson and Crosby 1971; Mabry and Gill 1979). The monoterpenic pyrethroids, which are extracted from the dried flowers of chrysanthemum (*Chrysanthemum cinerariaefolium*), are insecticidal with the bioactive compounds (pyrethrin I, pyrethrin II, cinerin I, cinerin II, jasmolin I and jasmoline II) occurring at about 1.8-2.5% of the flower dw (Matsui and Yamamoto 1971; Mabry and Gill 1979; Morris, Davies et al. 2006). dw. Monoterpenes found in the wood of western red cedar, *Thuja plicata*, are termiticidal, with β-thujaplicin (at about 0.51% of the volatile extract) effective against *Hylotrupes bajulus* (Cerambycidae) and methyl thujate (at abouto 0.17% of the volatile extract) potent against the larvae of *Reticulothermes flavipes*, black carpet beetle, furniture carpet beetle and the case making moth (Becker 1965; Arndt 1968; cited in Mabry and Gill 1979; Mah 1984). Diterpenes such as kaurenoic acid and trachylobanoic acid, occurring at about 5% of the sunflower (*Helianthus annuus*) floret dw, inhibit the growth rate of sunflower moth (*Homeosoma electellum*) and tobacco budworm (*Heliothis virescens*) by 50% when placed in artificial diets containing 1% of both kaurenoic acid and trachylobanoic

acid (Elliger, Zinkel et al. 1976; Waiss, Chan et al. 1977; Mabry and Gill 1979).

Other compounds function as deterrents by discouraging intake of either leaf or artificial diets. The bitter sesquiterpene lactones of *Vernonia* (Compositae) leaves, at about 0.5 to 1% of the leaf dw, have been observed to deter feeding of four Lepidopteran species in both leaf and artificial diets (Burnett Jr., Jones *et al.* 1974). In a choice feed set-up, fall (*Spodoptera frugiperda*), southern (*S. eridania*) and yellow-striped (*S. ornithogalli*) armyworms and the saddle-back caterpillar (*Sibine stimulea*) preferred Vernonia leaf discs that lacked glaucolide-A (*Vernonia flaccidifolia*) over those containing glaucolide-A (*V. glauca* and *V. gigantea*) (Burnett Jr., Jones *et al.* 1974). The southern armyworm also ate less of the artificial feed that was made of ground, freeze-dried leaves (*V. flaccidifolia)* as more glaucolide-A powder was added (Burnett Jr., Jones *et al.* 1974). Triterpenes have also been reported to be deterrent to acridids, with azadirachtin (from *Azadirachta indica*), at about 0.01% dw of neem tree leaves, being avoided by the desert locust *Schistocerca gregaria* when impregnated as an artificial feed on glass fibre discs (at 0.001 ppm) (Mabry and Gill 1979; Cottee, Bernays *et al.* 1988; Mordue, Nisbet *et al.* 1996).

The amount of insecticidal or deterrent metabolites in plants may occur in either minute or large amounts. 1,8-Cineole, which is a volatile monoterpenic oil, occurs at about 90% of the leaf dw of *Melaleuca* leaves (Suzuki 1999) but is lethal at minute levels against rice weevil *Sitophilus oryzae* and red flour beetle *Tribolium castaneum* (23.5 and 15.3 $\mu L.L^{-1}$ of air respectively) (Southwell 1999; Lee, Choi *et al.* 2001; Lee, Annis *et al.* 2004; Hammer, Carson *et al.* 2006). The large amount of oils in the leaves plays a role not only in insect deterrence but also in the plant's fire ecology wherein the flammable leaf oil vis-à-vis the spongy outer bark insulates the epicormic trunk buds from the heat increasing its fire adaptability (Schmitz, Simberloff *et al.* 1997; Turner, Center *et al.* 1998).

The diterpene SGs, which occur at a high concentration of about 10% of the leaf dw, may also play a role in insect deterrence. SGs are widely regarded as non-toxic and as a sweet compound, SGs may influence feeding behaviour of mammalian herbivores (Harborne 1982). However, the aglycone steviol is toxic at relatively low concentrations and a safe daily intake for humans (2 $mg.kg^{-1}$ body weight) has been recommended (Geuns 2004).

To date, the influence of the diterpenic SGs on insect feeding preference has received little attention. The most systematic documentation of the impact of SGs on insect feeding behaviour involved artificial feed testing with aphids (*Schizaphis graminum*) (Nanayakkara, Klock *et al.* 1987). Feeding deterrence at low concentrations was observed for steviol (150 ppm), isosteviol (115 ppm), and three chemical derivatives of steviol (130-200 ppm), while stevioside deterrence was at a higher concentration (650 ppm) and rebaudioside A was acceptable even at the highest concentration (750 ppm) (Nanayakkara, Klock *et al.* 1987). While work on aphids and SG artificial feeds is interesting, the feeding mechanism of aphids via stylus access to the phloem does not simulate the sampling of SGs stored in the vacuole of the mesophyll cells of the leaf tissue (Brandle and Telmer 2007).

Instead, work with an insect that feeds on the whole leaf tissue is more practical to investigate the role of SG in insect deterrence. To date, only observational accounts have been made on the non-preference of *Epicauta adomaria* (Coleoptera) towards stevia leaf, as indicated by avoidance of stevia leaf discs when mixed with leaf discs of *Capsicum*, *Amaranthus*, *Emilia* and *Lycopersicum* (Metivier and Viana 1979).

Investigation of the feeding behavior of grasshoppers towards stevia leaves would be interesting, because the chewing of grasshoppers through the leaf with their mandibles would ensure that the SGs in the leaves are properly consumed. Furthermore, grasshoppers are generalist herbivores that feed on a wide variety of grass and broad leaf plants (Dadd 1963)). While the feeding behavior of grasshoppers is influenced by factors such as physical and

nutritional properties of the leaf, familiarity with the plant as well as deterrent effects of leaf metabolites, grasshoppers generally eat a good variety of leaves making them an ideal insect model for sampling of SGs in stevia leaves (Dadd 1963).

Studies on the feeding behavior of the generalist grasshopper towards stevia leaves were not encountered although sensitivity to other leaf secondary metabolites has been investigated. *Schistocerca americana* was reported to avoid phenolics (coumarin, umbelliferone, salicin), alkaloids (gramine, nicotine) and terpenoids (geraniol, abeitic acid, ursolic acid) which were either artificially presented in capsules, gelatine microcaps or spray-dried on leaves. The tested metabolite concentrations were equivalent to either the maximum natural leaf concentration or one-fifth of the natural leaf concentration (Bernays 1991). In another study, *Locusta migratoria* exhibited 50% reduction in feeding compared to intake of a control disc when presented with glass fiber discs impregnated with artificial solutions of alkaloids (nicotine, quinine and tomatine), phenolics (salicin and umbelliferone), sulphur compounds (sinigrin and allylisothiocyanate) and triterpenoid (azadirachtin) that were equivalent to the natural leaf concentration of about less than 1% (leaf dw) (Cottee, Bernays *et al.* 1988). However, sensitivity towards the metabolite varied with species. For example, *S. gregaria* had higher tolerance for allylisothiocyanate, quinine, salicin, sinigrin, tomatine and umbelliferone, tolerating a level exceeding the natural leaf concentration, while *L. migratoria,* had low levels of tolerance (Cottee, Bernays *et al.* 1988). In contrast, *S. gregaria* was very sensitive towards azadirachtin at a low concentration of 0.00001% leaf dw, while this compound was tolerated by *L. migratoria* (Cottee, Bernays *et al.* 1988).

Only one study was encountered on the feeding behavior of the Australian grasshopper, *Valanga irregularis*, that was used in this chapter. *V irregularis* was observed to avoid leaves of *Citrus limon* when presented alongside three other leaves (*Bauhinia blakeanas*, *Hibiscus rosa-sinensis* and *Prunus domestica*) (Freeland 1975). Furthermore, pre-feeding with only *C. limon* resulted in an overall decline in feeding amount when all four leaves were

subsequently presented (Freeland 1975). Deterrence was possibly related to the alkaloid content and volatile oils in *C. limon,* although concentration of these secondary metabolites was not quantified in the experiment (Freeland 1975).

This chapter revisits the impact of SG on insect feeding preference by looking at the feeding behaviour of *V. irregularis*, towards stevia leaves. Stevia leaf damage by red spider mite (*Tetranychus urticae)* was also passively investigated relative to leaf SG concentration to estimate the influence of SGs on spider mite feeding.

Materials and Methods

Grasshopper material

Native Australian grasshoppers (*V. irregularis*) were harvested from Poison peach trees (*Trema tomentosa*) in a coastal vineforest community in Yeppoon within the time period of March to April of 2010. The harvested grasshoppers varied in instar maturity. To work around this limitation, grasshoppers were sorted by weight. The light weight grasshoppers were mostly 1st to 3rd instar nymphs with an average start weight of 0.3 g (range of 0.1 - 0.4g). The heavy weight grasshoppers were mostly 4th and 5th instar nymphs with an average weight of 0.9 g (range of 0.5 – 1.3g).

Leaf material

Stem cuttings of the Poison peach tree (from which the grasshoppers were harvested) were regularly collected and kept fresh by maintaining the cut stem in water, and by covering the cutting with a plastic bag to increase the relative humidity. Stem cuttings of stevia were cut from a single plant which was maintained inside a growth chamber under controlled conditions (14 h light/10 h dark, 80% humidity, 25 °C). From the leaf analysis in Chapter 6, the stevioside and rebaudioside A concentration of top and middle leaves of the stevia plant used, was about 10-15% dw and 1-2% dw, respectively.

Cage set-up

Grasshoppers were maintained in groups of three inside inverted cylindrical jars (12 cm diameter x 12 cm height). Jars were covered at the bottom with a cardboard base (10 cm length x 10 cm width x 2 cm height) (Fig. 10.1). The cardboard base was lined with a filter paper and perforated in the centre to allow the leaf cuttings to sit in water throughout the experiment. The ceiling of the inverted jar was also symmetrically perforated to allow air circulation inside the jar.

A total of 20 jars, arranged in a 4 x 5 matrix, were available for grasshopper incubation (Fig. 10.1). Five feeding trials were conducted within the period March to April 2010, re-using the jars.

(a) (b)

Figure 10. 1. Feeding trial set-up: (a) side view, (b) top view. Feeding chambers were arranged in a 4 x 5 matrix, with 3 grasshoppers of similar size placed inside each jar. Leaves inside the jar were changed according to the assigned treatment during the test period.

The jars were maintained in 12h/12h light/dark cycle under controlled temperature (25 °C day/20 °C night), initially inside a growth cabinet and later within an AC-controlled room (when the temperature control of the growth chamber failed).

Feeding experiment

Grasshoppers were initially acclimatized for 3 days (Day 0 to 3), feeding them with the same trema leaves from which they were harvested. After the acclimatization period, the grasshoppers were maintained with the same leaves for another three days, which served as the control period (Day 4-6). A 3-day test period (Day 7-9) followed wherein grasshoppers were randomly assigned one of the three diets: (a) ***control***: trema leaves only, (b) ***choice***: trema and stevia leaves or (c) ***no choice***: stevia leaves only. After the test period, the grasshoppers were returned to the trema leaf diet for a 5-day post-control period (Day 10-14).

Leaf weight was not monitored in the experiment because of the daily variations in the fw of the stem cuttings. Instead, feeding activity was measured through weight comparison of the grasshoppers relative to their starting weight at Day 0 (eq. 10.1). The weight comparison assumes that a healthy, feeding grasshopper will increase or maintain its original weight. The total grasshopper from each jar was measured at the end of the acclimatization (Day 3), control (Day 6), test (Day 9) and post-test (Day 14) period. The total weight of the grasshoppers from each jar was also monitored daily during the acclimatization period. Grasshoppers that didn't exhibit an overall weight increase during the three day acclimatisation period were eliminated from the trial. In instances where a grasshopper died during a feeding period, only the weights of the live grasshoppers were averaged.

$$\text{\% change in average grasshopper (ghop) weight per jar} = \frac{\dfrac{\text{current total ghop weight}_{alive}}{\text{current \# ghop alive}} - \dfrac{\text{start total ghop weight}_{alive}}{\text{start \# ghop alive}}}{\dfrac{\text{start total ghop weight}_{alive}}{\text{start \# ghop alive}}} \times 100 \quad \text{(eq. 10. 1)}$$

Faecal weight was also used as an indirect measure of feeding with more faecal waste expected after feed digestion. The faecal weight for each jar was referenced to the faecal weight on Day 3 (eq. 10.2). Also, grasshoppers that died in the middle of the treatment were included in the total count of grasshopper which was used to average the faecal weight per jar. Calculation for average faecal weight is summarized as:

$$\% \text{ change in average faecal weight per jar} = \frac{\frac{\text{current total faecal weight}}{\text{current \# ghop in the jar}} - \frac{\text{day 3 total faecal weight}}{\text{day 3 \# ghop in the jar}}}{\frac{\text{day 3 total faecal weight}}{\text{day 3 \# ghop in the jar}}} \times 100 \quad \text{(eq. 10. 2)}$$

Mortality test

Some mortality was noted during the feeding trails, however if it was not clear if this was associated with ingestion of stevia tissue, starvation, or another reason. To characterise the timeline for mortality through starvation, grasshoppers from the feeding test were re-acclimatized with trema leaves for two weeks to normalize their feeding behaviour. Fourteen grasshoppers were then randomly selected and individually placed inside a jar. Grasshoppers were initially given trema leaves for the first three days (Day 0-3) and then either starved (control group, no food source) or presented only with stevia leaves (stevia group) for the next three days (Day 4-6). Grasshopper weight was measured on Day 0, 3 and 6 while faecal weight was measured on Day 3 and 6. Change in grasshopper weight was expressed relative to initial weight while faecal weight was plotted as a ratio relative to grasshopper weight.

Spider mite feeding experiment

Stevia plants maintained inside a growth cabinet (600 umol $m^{-2}s^{-1}$), 25 °C, relative humidity of 85% and 14 h light/10 h dark cycle) were attacked heavily by red spider mites (*Tetranychus urticae*). To investigate a possible correlation between leaf damage and SG leaf concentration, leaf damage was evaluated on a scale of 1-5 (with 1 being the least damaged) and leaf SG concentration was then evaluated by HPLC. Leaf samples were also sent for SEM imaging to investigate the extent of leaf damage caused by the spider mites.

Results

Feeding experiment

Although grasshoppers were randomly allocated to the three feeding regimes, there was some difference in the growth rates of the treatments groups in the first three days. For the group destined for a continued diet of trema leaves for three days, lightweight grasshoppers displayed a 20% increase in weight (Fig. 10.2), while heavyweight grasshoppers maintained their start weight (Fig. 10.2). For the group destined for a diet of trema-stevia choice or stevia only, lightweight grasshoppers maintained weight (Fig. 10.2), while heavyweight grasshoppers demonstrated a 10% increase in weight on the trema-stevia choice diet and maintained weight on the stevia only diet (Fig. 10.2). However, the succeeding three days (Day 3-6) allowed the grasshopper weight to stabilize and reach a plateau before the test period (Day 6-9) (Fig. 10.2).

After 3 d on a stevia only diet (i.e. by Day 9), a slight drop in weight was observed for both the light weight (Fig. 10.2) and the heavy weight grasshoppers (Fig. 10.2). Following transfer to a trema diet, a weight increase was observed in both the light and heavy weight grasshoppers (Fig. 10.2).

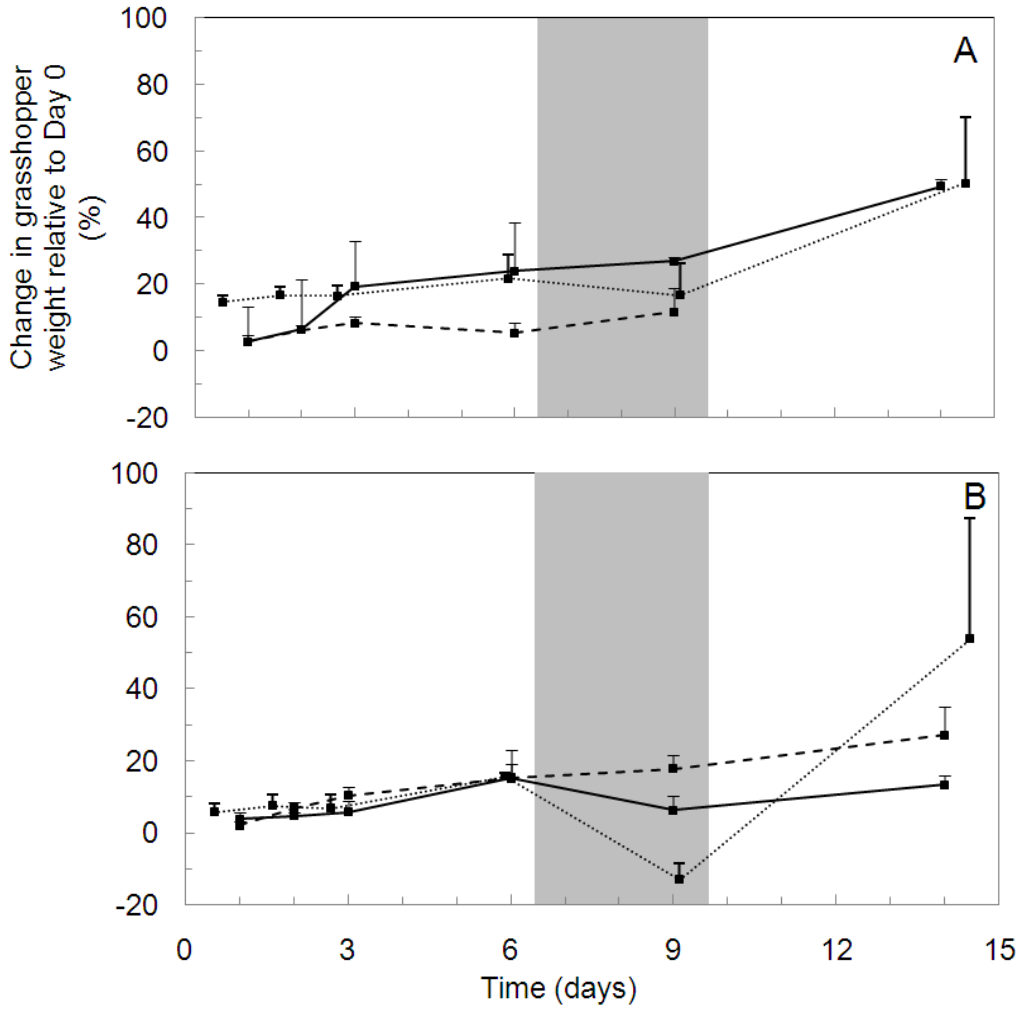


Figure 10. 2. Change in weight of small (average start weight of 0.3 g) (A) and large grasshoppers (average start weight of 0.9 g) (B) relative to Day 0 over a three day period. Grasshoppers were placed in a trema only control (—■—) (n = 5 for small and n = 2 for large grasshopper), trema-stevia choice (– –■– –) (n = 5 for small and n = 5 for large grasshopper) or stevia only (···■···) (n = 7 for small and n = 4 for large grasshopper) feeding regime during the test period (shaded area) (vertical bars: standard error). Weight after the test period was measured after 5 days from the last test date.

A significant decrease in faecal weight occurred only in the stevia only treatment (Fig. 10.3).

An increase in mortality rate was also observed in the stevia only treatment with many of the grasshoppers dying at the end of the test period. The increase in mortality rate was higher with the light weight but was also evident with the heavy weight grasshoppers (Fig.10.4).

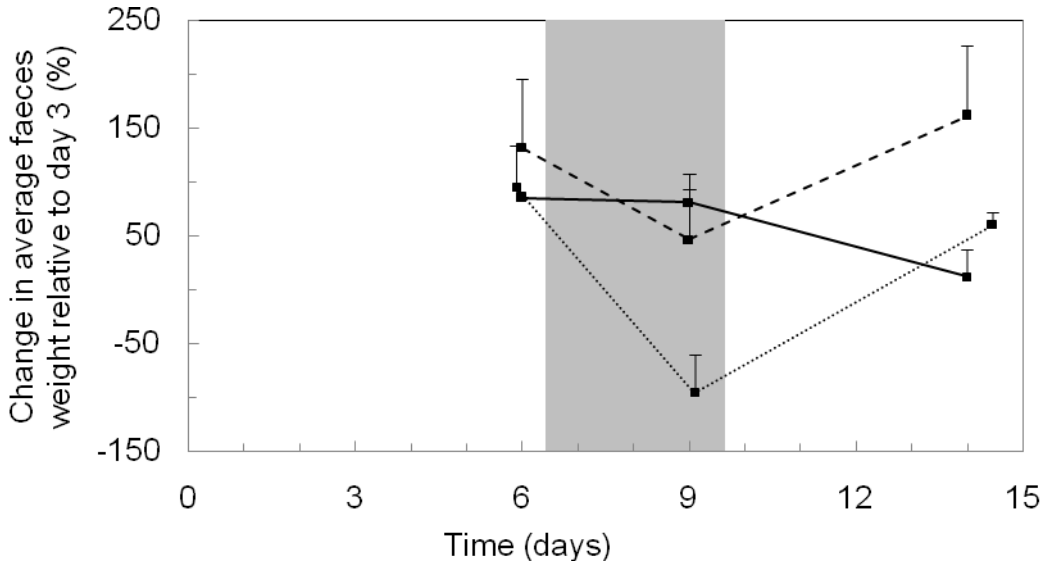


Figure 10. 3. Change in average faecal weight produced over a three day period by large grasshoppers (average start weight of 0.9 g) relative to Day 3. Grasshoppers were placed in a trema only control (—■—) (n = 2), trema – stevia choice (‑ ‑■‑ ‑) (n = 5) or stevia only (···■···) (n = 4) feeding regime during the test period (shaded area) (vertical bars: standard error). Faecal weight after the test period was monitored 5 days after the last testing.

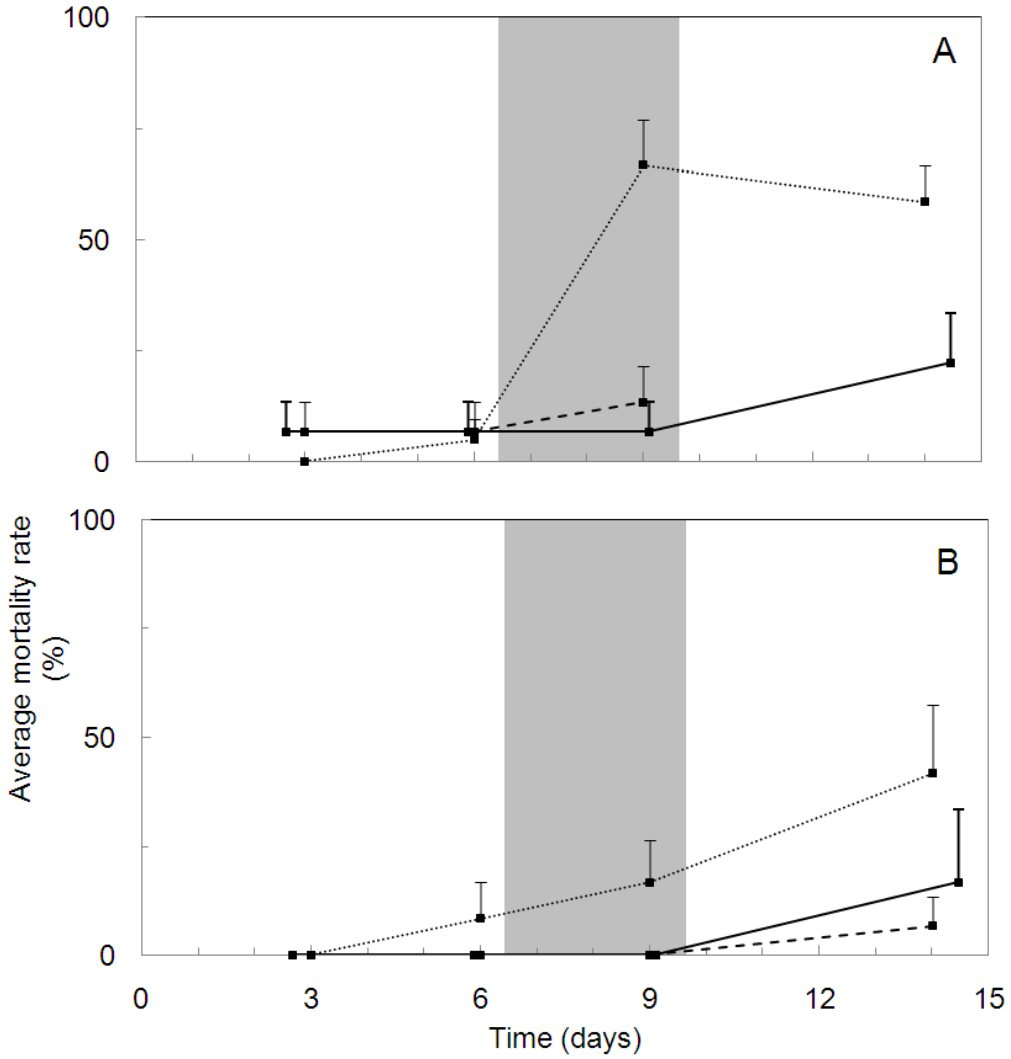


Figure 10. 4. Change in mortality rate of small (average start weight of 0.3 g) (A) and large grasshoppers (average start weight of 0.9 g) (B) over a three day period. Grasshoppers were placed in a trema – only (—■—) (n = 5 for small and n = 2 for large grasshoppers), trema – stevia choice (- -■- -) (n = 5 for small and n = 5 for large grasshoppers) or stevia only (···■···) (n = 7 for small and n = 4 for large grasshoppers) feeding regime during the test period (shaded area) (vertical bars: standard error).Live grasshoppers were counted after 5 days from the final test day.

Mortality test

After three days of starvation, three (of seven) grasshoppers had died while three days of stevia diet resulted in one (of seven) deaths. Rate of decline in grasshopper weight (Table 10.1) and faecal production (Table 10.2) was comparable between the control and stevia-fed group.

Table 10. 1. Average change in grasshopper weight per jar relative to initial grasshopper weight from Day 0. Grasshoppers were either starved (control) or given stevia leaves during the test period (Day 3 to 6) (n = 7, standard error indicated).

	Average change in grasshopper weight per jar expressed as a percentage relative to the initial weight in Day 0	
	Day 3	Day 6
Control	-7.2331 (± 2.2994)	-18.1446 (±1.7356)
Stevia	0.6261 (±2.2350)	-16.5662 (±2.3073)

Table 10. 2. Average faecal weight per jar of grasshoppers that were either starved (control) or given stevia leaves during the test period (Day 3 to 6) (n=7, standard error indicated).

	Average faecal weight of grasshoppers in each jar	
	Day 3	Day 6
Control	0.1184 (±0.0286)	0.0052 (±0.0038)
Stevia	0.2025 (±0.0421)	0.0754 (±0.0186)

Spider mite feeding experiment

Substantial variation in population density of red spider mite was noted amongst stevia leaves sampled from two stevia plants. However, the extent of red spider mite damage was not correlated with the leaf SG concentration (Fig. 10.5).

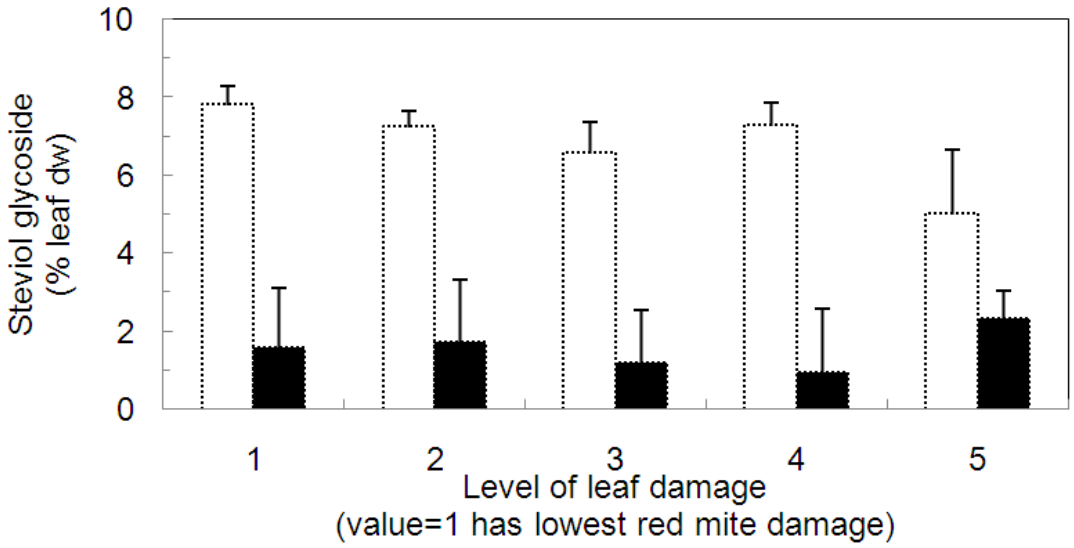
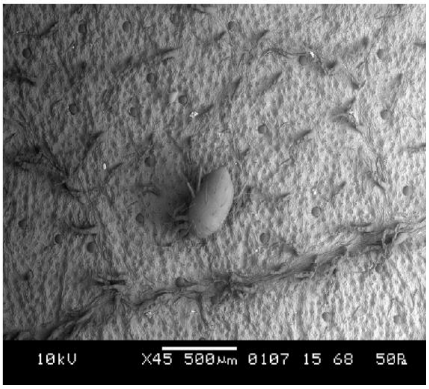


Figure 10. 5. Relationship between extent of leaf damage and leaf concentration (% leaf dw) of SG expressed either as stevioside (white bar) or rebaudioside A (black bar). Leaf damage was qualitatively evaluated and ranked from 1-5 (with 1 as least damaged and 5 as most damaged). A total of 29 leaf samples from two plants were analysed.

A close look at the leaf surface using a scanning electron microscope (SEM), indicates feeding of the red spider mite through the leaf epidermis (Fig. 10.6).

(a)



(b)

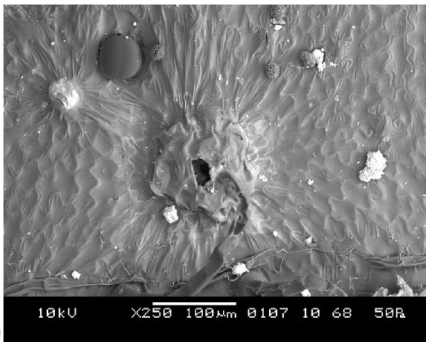


Figure 10. 6. SEM Image (10 kV / 14 mm) of a red spider mite on a fresh stevia leaf (a) and leaf damage on stevia leaf after red spider mite attack (b).

Discussion

V. irregularis avoided feeding on stevia leaf, as evidenced by a decrease in faecal weight produced over the test period for animals on a stevia only diet, and by a decrease in grasshopper weight during the test period, at least for the heavyweight insects. Further, a qualitative examination of the stevia leaves from the stevia only treatment indicated avoidance to the point of near starvation of the grasshoppers, with little or no leaf feeding damage evident, and some stem damage evident. Feeding on stems may be a strategy to

reduce intake of SG, as stems contain a much lower SG concentration than leaves.

The mortality rate over the test period was higher for grasshoppers on the stevia only diet. Although it is tempting to assume that fatality was caused by the toxic effects of SG ingestion, the follow-up mortality test indicated that death may be attributed to starvation.

Cottee et al (1988) noted that a few secondary metabolites (allylisothiocyanate, azadirachtin, nicotin, quinine, salicin, sinigrin, tomatine and umbelliferone) acted as a feeding deterrent in artificial diets supplied to the grasshopper species *L. migratoria* and *S. gregaria*, although the level of deterrence was different between these species.

The current study is the first consideration of the deterrent effects of stevia leaves on the Acrididae family although further work with artificial feeds containing SGs at different concentrations is necessary to confirm that SG leaf concentration is one of the main factors influencing the feeding behavior of *V. irregularis*.

Furthermore, the difference in sensitivity amongst Acrididae species towards SGs should be evaluated, just as the responsiveness of *L. migratoria* and *S. gregaria* differed for other secondary metabolites (Cottee, Bernays *et al.* 1988). A feeding trial with insects that are sympatric with stevia in its native Paraguay should also be considered as this would give a more significant evolutionary perspective on the deterrence of SGs toward insects. Lepidopteran and orthopteran insects observed in stevia cultivations (Fuente 2001) should be considered for future experiments on potential feeding deterrence.

Avoidance of stevia leaf discs by *Epicauta adomaria* (Coleoptera) when presented alongside leaf discs of *Capsicum*, *Amaranthus*, *Emilia* and *Lycopersicum* was reported by Metivier *et al.* (1979), while acceptance of *in vitro* diet containing steviol (500 ppm and 40 ppm respectively) by *Heliothis virescens* Fabr. and *Aedes aegypti* L. (yellow fever mosquito) (unpublished

study of Klocke cited in Nanayakkara, Klock *et al.* 1987) may represent variation in insect responsiveness. Difference in feeding behaviour may allow certain insect species to avoid the SGs or their precursors by feeding on the stem or by feeding on leaf tissues other than the chlorenchyma. Red spider mites induce leaf damage by puncturing through the cuticle and epidermis layer with their stylets allowing selective damage of palisade and spongy mesophyll cells (Mothes and Seitz 1982). With this feeding mechanism, the spider mites were possibly able to avoid the SGs and their precursors leaving them undeterred. Future investigation on SG herbivory should also consider the feeding mechanism of the insect considered.

Conclusion

Deterrence of SGs towards *V. irregularis* was demonstrated through avoidance of stevia leaves in a no-choice set-up. Avoidance was accompanied by a decline in grasshopper weight, faecal amount and increased mortality rate. A closer look at the cause of mortality seemed to indicate that grasshoppers died because of starvation rather than the toxic effect of the stevia leaves. Future work with different concentrations of SG artificial feed will help confirm and define the level of sensitivity of *V. irregularis* towards SGs. Other insects with different feeding mechanism should also be considered if a holistic ecological perspective of SG's deterrence on insect herbivory is to be defined.

Summary

The role of SG, at approximately 10% of the leaf dw, in wild type *Stevia rebaudiana* is unknown, although given the metabolioc cost of such a reserve to the plant, presumably there is a role played in ecological fitness. SGs, in the form of stevioside and rebaudioside A, were demonstrated to decrease with leaf age but increase with plant age up to the beginning of the flowering stage (Chapter 6). A 24 h pulse-chase experiment with intact stevia plants confirmed that SG turnover was slow with no label detected in stevioside and rebaudioside A within a 24h period (Chapter 6). In contrast to starch pool size, SG pool size was stable under increased or decreased photosynthetic conditions (through manipulation of light duration and CO_2 level), a result indicating that SGs do not act as a C-store (Chapter 7). Leaf SG concentration was also stable within a single cycle of water stress, a result inconsistent with a role of SGs as an osmoregulating agent (Chapter 8). The relatively stable concentration of SGs in stevia leaves within a short-term period indicates a genetic rather than environmental influence on SG levels.

SG may act in affecting herbivore feeding behavior. An Australian grasshopper species (*V. irregularis*) was observed to avoid stevia leaves under controlled conditions when given either a choice between leaves of stevia and Poison peach tree or in a no-choice set-up with only stevia leaves available (Chapter 10). With domestic guinea pigs, stevia leaves encouraged general herbivory with an overall increase in feed intake with an increase in leaf composition (from 4 to 10% w/w) in the standard feed (Chapter 9).

The improvement of methodology to measure SG, particularly with minimal sample preparation, was a second aspect investigated by this thesis. The traditional high performance liquid chromatography (HPLC) method, used as a reference method, was optimized for throughput speed, although at the cost of column life. An anthrone colorimetry method was explored as an alternative to HPLC, which demonstrated potential use for quantification of

stevioside and rebaudioside A in a constant solution matrix (Chapter 5). Near infrared spectroscopy (NIRs) was found useful for both solutions and dry ground stevia leaves with practical application in categorizing leaf harvest into either high or low total SG concentration.

The use of NIR for field estimation of SG quality in stevia leaf harvests remains to be explored further. Use of available handheld units, such as the Phazir, should be tested using a variety of ground leaf samples from different species and with different concentrations to develop a commercially-viable calibration.

The insect-deterrent property of stevia leaves is interesting and may benefit stevia growers with their line selection. Future experiments involving artificial feed diets of stevioside and rebaudioside A will help define the threshold limit of *V. irregularis* and better clarify their taste sensitivity. Deterrence towards other commercially damaging insects should also be considered.

The general encouragement of herbivory in domestic guinea pigs suggests practical application in animal feed fortification. Level of detection for each animal considered should be defined to ensure effective encouragement of feeding in guinea pigs. Further work with respect to the use of stevia leaf powder to encourage feeding in commercial animal production systems can be expected, mainly when tannin-rich plants are involved.

With the increasing agronomic value of stevia leaves as source of a natural sweetener, research in maximizing its economic value has become more relevant. Our investigation on the ecophysiological role of SGs (as stevioside and rebaudioside A) in the stevia plant, highlighted a role in insect deterrence and has hopefully encouraged future research on the other possible ecological roles of SGs. This and the NIRs methodology proposed will hopefully allow growers to maximize SG concentration in the intact plant before harvest.

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