"STUDIES ON EXTRACTION OF STEVIOSIDE FROM DRIED STEVIA (*Stevia rebaudiana*) LEAVES AND IT'S UTILIZATION IN FOOD PRODUCTS"

By

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2005

Dedicated

To

My Beloved Parents

CANDIDATE'S DECLARATION

I hereby declare that the dissertation or part

thereof has not been previously

submitted by me for a

degree of any other

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CERTIFICATE - I

This is to certify that **Mr. KATOLE MOHAN BHAGWAN** has satisfactorily prosecuted his course credits and research work for a period not less than four semesters and that the dissertation entitled **"STUDIES ON EXTRACTION OF STEVIOSIDE FROM DRIED STEVIA (*Stevia rebaudiana)* LEAVES AND IT'S UTILIZATION IN FOOD PRODUCTS"** submitted by him is the results of original research work and is of sufficiently high standard to warrant it's presentation to the examination. I also certify that the dissertation or part thereof has not been previously submitted by him for the award of a degree of any university.

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CERTIFICATE -II

This is to certify that the dissertation entitled **"STUDIES ON EXTRACTION OF STEVIOSIDE FROM DRIED STEVIA (*Stevia rebaudiana)* LEAVES AND IT'S UTILIZATION IN FOOD PRODUCTS"** submitted by **Mr. KATOLE MOHAN BHAGWAN** to the Marathwada Agricultural University, Parbhani in partial fulfillment of the requirements for the degree of **MASTER OF TECHNOLOGY (FOOD SCIENCES)** has been approved by the student's advisory committee after Viva-voce examination in collaboration with the external examiner.

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LIST OF ABBREVIATIONS

Abbreviations	Description
%	Per cent
cm	Centimeter
CRD	Completely Randomized Design
DNSA	Dinitrosalicylic acid
g	Gram
hr.	Hours
Kcal	Kilo calorie
kg	Kilogram
mg	Milligram
min	Minute
ml	Milliliters
mm	Milimeter
N	Normality
°Bx	Degree brix
°C	Degree Celsius
Rs.	Rupees
RTS	Ready to serve
TSS	Total soluble solids
w/w	Weight by weight

INTRODUCTION

CHAPTER – I

INTRODUCTION

Stevia is a perennial shrub that grows up to 1 m tall and has leaves 2-3 cm long. It belongs to the Asteraceae (Compositae) family, which is indigenous to the northern regions of South America. Stevia is still found growing wild in the highlands of the Amambay and Iguacu districts (a border area between Brazil and Paraguay). It is estimated that as many as 200 species of Stevia are native to South America; however, no other Stevia plants have exhibited the same intensity of sweetness as S. rebaudiana. It is grown commercially in many parts of Brazil, Paraguay, Uruguay, Central America, Israel, Thailand and China (Genus, J. M. C., 2003).

For hundreds of years, indigenous peoples in Brazil and Paraguay have used the leaves of stevia as a sweetener. The Guarani Indians of Paraguay call it 'kaa jheé' and have used it to sweeten their yerba mate tea for centuries. They have also used stevia to sweeten other teas and foods and have used it medicinally as a cardiotonic, for obesity, hypertension, and heartburn, and to help lower uric acid levels.

In addition to being a sweetener, stevia is considered (in Brazilian herbal medicine) to be hypoglycemic, hypotensive, diuretic, cardiotonic, and tonic (Jeppesen *et al.,* 2002). The leaf is used for diabetes, obesity, cavities, hypertension, fatigue, depression, sweet cravings, and infections. The leaf is employed in traditional medical systems in Paraguay for the same purposes as in Brazil (Patrick and Massey, 2002).

Europeans first learned about stevia in the sixteenth century, when conquistadores sent word to Spain that the natives of South America were using the plant to sweeten herbal tea. Since then stevia has been used widely throughout Europe and Asia. In the United States, herbalists use the leaf for diabetes, high blood pressure, infections, and as a sweetening agent. In Japan and Brazil, stevia is approved as a food additive and sugar substitute.

Western interest in stevia began around the turn of the nineteenth century, when researchers in Brazil started hearing about a plant with leaves so sweet that just one leaf would sweeten a whole gourd full of bitter yerba mate tea.. It was first studied in 1899 by Paraguayan botanist Moises S. Bertoni, who wrote some of the earliest articles on stevia (in the early 1900s).

Over 100 phytochemicals have been discovered in stevia since it is rich in terpenes and flavonoids. The constituents responsible for stevia's sweetness were documented in 1931, when eight novel plant chemicals called glycosides were discovered and named. Of these eight glycosides, one called stevioside is considered as the sweetest and has been tested to be approximately 300 times sweeter than sugar. Stevioside, comprising 6-18 per cent of the stevia leaf, is also the most prevalent glycoside in the leaf. Other sweet constituents include steviolbioside, rebausiosides A-E, and dulcoside A.

The main plant chemicals in stevia include: apigenin, austroinulin, avicularin, beta-sitosterol, caffeic acid, campesterol, caryophyllene, centaureidin, chlorogenic acid, chlorophyll, cosmosiin, cynaroside, daucosterol, diterpene glycosides, dulcosides A-B, foeniculin, formic acid, gibberellic acid, gibberellin, indole-3-acetonitrile, isoquercitrin, isosteviol, jhanol, kaempferol, kaurene, lupeol, luteolin, polystachoside, quercetin, quercitrin, rebaudioside A-F, scopoletin, sterebin A-H, steviol, steviolbioside, steviolmonoside, stevioside, stevioside a-3, stigmasterol, umbelliferone, and xanthophylls.

The great interest in stevia as a non-caloric, natural sweetener has fueled many studies on it including toxicological ones. The main sweet chemical, stevioside, has been found to be nontoxic in acute toxicity studies with rats, rabbits, guinea pigs, and birds. It also has been shown not to cause cellular changes (mutagenic) or to have any effect on fertility (Toteo *et al.* 1990). The natural stevia leaf also has been found to be nontoxic and has no mutagenic activity. Studies conflict as to the effect of stevia leaf on fertility. The majority of clinical studies show stevia leaf to have no effect on fertility in both males and females. In one study, however, a water extract of the leaf was shown to reduce testosterone levels and sperm count in male rats.

The FDA's position on stevia is somewhat ambiguous. In 1991, citing a preliminary mutagenicity study, the FDA issued an import alert which effectively blocked the importation and sale of stevia in this country. Ironically, this was the year that a follow-up study found flows in the first study and seriously questioned its results.

In September of 1995, the FDA revised its import alert to allow stevia and its extracts to be imported as a food supplement but not as a sweetener. Yet, it defines stevia as an unapproved food additive, not affirmed as GRAS (Generally Recognized as Safe) in the United States (May, A. J; 1996).

The confectionery industry is yet to reap the benefit of stevia, which can replace the usage of sugar as a sweetener, at a relatively comparable cost of Rs.350 per kg of dried leaves of stevia. Stevia can be used in chocolates and candies not only to meet the market demand by the diabetics, but also to harvest the added advantage of this herb that it does not encourage tooth decay. Stevia possessing an anti-microbial property can be used in all the sweets, which is of great fond to the children, as it does not enhance the growth of any bacteria in the teeth, unlike the sugar.

The soft drink manufacturers have introduced several health drinks and many food supplementary beverages, especially for the diabetics. Majority of the food supplementary products for the diabetics emphasizes on the fibre content, protein content etc. The addition of stevia leaves (dried) as such or powder, in such products would not only aid in increasing the sweetness naturally but also helps in rejuvenating the pancreatic gland. Apart from this, Stevia is nutrient rich, containing substantial amount' of protein, calcium and phosphorus.

The leaves of Stevia impart a pleasant flavour apart from increasing the sweetness of the product. As the Stevia leaves 'are used without much process, as direct leaf powder, it can be used in most of the typical Indian dishes like Chakkara pongal, Payasam, Ravaa ladoo, Sauces, Jams, Juices, Pickles, Tea, Coffee, and even herbal tea. Most of these products are available as readymade mixtures in neatly sealed packs. Stevia with them would implant additional acceptable flavour in the mixtures, besides easing the work:

Fresh Juice making is another such area that is still unexplored. The fresh fruit juice can be made cent percent fresh and natural by the addition of this natural sweetener, stevia, instead of cane sugar. The sweet deprived diabetics can relish their favorite sweets with Stevia without any compromise for the sweetness, in addition to the health restoring activity of this herb.

Ground Stevia is excellent when sprinkled lightly over cooking vegetable, and meat, cereals and salads. Besides adding its own sweet taste, it significantly enhances the flavour and nutritional value of the food.

The bakery industry too holds a stand to bring an impact by the usage of Stevia. All cooked and baked food items like puddings, desserts can be sweetened with only very small quantities of Stevia leaf powder as compared to table sugar. Since 50 g of Stevia leaf can replace 1000 g of cane sugar. As the sweetness of stevioside is non-fermenting and the non-browning reaction when cooked, further widens its area of application in baking enhancing the quality and safety of usage with a longer shelf life period. Breads made with Stevia as an ingredient for diabetic customers has proved to show improvement in the texture and softness of the bread and increased shelf life.

Overall, a mere fragment of the leaf is enough to sweeten the mouth for an hour. So Stevia leaves afresh are more than a chewing gum, though Stevia can be used in the making of chewing gums, mints and mouth refreshers.

The soft drink manufacturers in India are yet to exploit the sweetness of this herb by its addition in their product. Though many soft drinks are introduced in the market with the prefix 'dia' and it's meant for the diabetics, the usage of stevia in such products would fetch a greater demand than for the one with artificial sweeteners. Hence in the present study attempts have been made to develop technology to prepare a low calorie food products like cookies and mango fruit beverages(RTS) for energy conscious population by replacing artificial chemical sweetener with natural stevia extract (stevioside) without any loss in nutritive value of prepared products. The present investigation has been undertaken with following distinct objectives such as,

1. To study the physico-chemical characteristics of dried stevia (*Stevia rebaudiana*) leaves.
2. To standardize extraction process of stevioside from dried stevia leaves.
3. To study some of the important characteristics of stevioside with special reference to color, calorific value and solubility behaviour.
4. To evaluate the physico-chemical characteristics of prepared food products.
5. Organoleptic evaluation of prepared food products.
6. To assess the techno-economical feasibility of prepared food products.

REVIEW OF LITERATURE

CHAPTER – II

REVIEW OF LITERATURE

Some of the studies related to extraction of stevioside and its utilization in preparation of low calorie food products in the present investigation are reviewed here under suitable headings.

1. Physico-chemical characteristics of stevia
2. Extraction of stevioside
3. Physico-chemical characteristics of stevioside
4. Product application and formulation with stevioside
5. Safety concerns with stevia

2.1. Physico-chemical characteristics of stevia leaves

Ali (1999) studied sweet compounds extracted from herbs including hernandulcin from lippia dulcis, stevioside from stevia rebaudiana, rubosiside from rubus suavissimus and baiyunoside from phlomis betonicoides, are discussed with particular reference to chemical structure.

Lisistin and Volorik (1999) showed that the chemical composition of stevioside (from stevia rebaudiana) comprising 8 diterpenoid glycosides with a sweet flavour. It's health-giving and sweetening properties were discussed.

Farrar *et al.* (2000) demonstration first report of Verticillium dahliae on stevia (Stevia rebaudiana). In October 1999, stevia plants in a commercial field in California exhibited stunting, leaf necrosis and vascular discoloration. Verticillium dahliae was consistently isolated from diseased root and stem pieces plated on water agar and APDA. This is the first report or V. dahliae on stevia in North America.

Munro *et al.* (2000) studied the effects of a new sweetener, stevia, on performance of newly weaned pigs. A total of 209 purebred Yorkshire newly weaned piglets were used to determine the effect of the sweetener stevia (stevia rebaudiana) at 83.3, 167 or 334 mg kg^{-1} diet on feed consumption (FC), average daily gain (ADG) and feed to gain ratio (F/G) compared with treatments with 5 per cent sucrose and no sweetener (control/ C). The stevia-containing diets did not appear to have detrimental effects on the FC and F/G ratios of the piglets when compared with C. The results indicated only a limited potential for stevia as a feed additive for piglets.

Bondarev *et al.* (2002) studied that growth and development of stevia rebaudiana shoots cultivated in a roller bioreactor and their production of steviol glycosides (SG) were investigated. Owing to the highly favourable conditions of shoot cultivation created in such an apparatus, the intensities of shoot growth and SG production was 1.5-2.0 fold higher than those of shoots grown in tubes. These results indicated a positive correlation between these 2 processes. It is suggested that enhanced SG production may be due to the differentiation of chlorenchyma cells and the formation of specific subcellular structures for glycoside accumulation.

Starratt *et al.* (2002) reported purification and structural characterization of a new steviol glycoside (rebaudioside F, a sweet diterperiod glycoside). Rebaudioside F was obtained from the leaves of a high rebaudioside C-producing line of Stevia rebaudiana; relative levels were 14.4 and 2.7 per cent for C and F-forms, respectively. NMR and MS analyses suggested that rebaudioside F is a steviol derivative with a glucose ester moiety at C19 and a trisaccharide residue consisting of a xylose and 2 glucose units linked via an oxygen residue at C13.

Koyama *et al.* (2003) studied in vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. Although the metabolic pathway in the rat is well known, human intestinal metabolism of stevioside and its related compounds has not been characterized. Intestinal metabolism of a stevia mixture extracted from leaves of Stevia rebaudiana and its alpha-glucose derivative (enzymically modified stevia) was investigated using pooled human faecal specimens and LC-MS to determine structures of the hydrolysis products. Stevia mixture, enzymically modified stevia and other stevia-related compounds (i9ncluding stevioside and rebaudioside A) were incubated with pooled human faecal homogenates for 0, 8 and 24h under anaerobic conditions. Complete degradation of stevia mixture, enzymically modified stevia, stevioside and rebaudioside A was observed after 24 h incubation; steviol was not degraded at all with substrate concentration of 0.08 and 0.2 mg/ml. Steviol appeared to be the hydrolysis product of stevia mixture, stevioside and rebaudioside A after incubation with human intestinal microflora. Results suggested that there are no apparent species differences in the intestinal metabolism of stevia-related compounds between humans and rats.

Papunidze and Kalandia (2003) studied use of stevia (Stevia rebaudiana) in the Georgian food industry is discussed, with reference to : cultivation aspects; sweetening properties; diterpene glucosides in stevia plants from Georgia and food applications (e.g. in tea and other beverages).

Totte *et al.* (2003) showed that the leaves of stevia rebaudiana bertoni produce large amounts of diterprene glycosides, including stevioside, which is 300 x sweeter than sucrose. The entkaurene skeleton of chloroplast deterpene glycosides is formed via the recently discovered 2-C-methyl-D-erthritol-4-phosphate pathway. Enzymes catalyzing the first 2 steps of the this pathway, 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) were characterized. Reverse transcriptase-PCR was used to clone the dxs and dxr cDNA, which comprise open reading frames of 2148 and 1422 nucleotdes, respectively. The cDNA-derived amino acid sequences for DXS and DXR contained 716 and 474 residues, encoding polypeptides of approx 76.6 and 51 kDa, respectively. DXS and DXR both contained an N-terminal plastid targeting sequence and showed high homology to other known plant DXS and DSR enzymes. Furethermore, heterologous expression in Escherichia coli demonstrated that the cloned cDNA encode functional proteins.

2.2. Extraction of stevioside

Zhang Shi Qiu *et al.* (2000) studied membrane based separation scheme for processing sweeteners from stevia leaves. In existing processes, extraction and refining of glycoside based sweetners from stevia leaves involves many process steps including extraction by organic solvents. The purpose of the present study was to develop a process of extraction and refining of sweeteners with reduced number of unit operations and maximization and / or elimination of chemical usage including organic solvents. It was found that water was very effective for extracting glycosides at selected pH and temperatures. It was also shown that a multi-stage membrane process was successfully able to concentrate the glycoside sweeteners. Based on the preliminary results, it appears that bitter-tasting components were washed out from the sweetener concentrate in the nanofiltration process. This work also has demonstrated that a membrane-based separation process for refining glycoside-based sweeteners could e viable and needs to be investigated further.

Hajime *et al.* (1982) reported enzymatic determination of stevioside in stevia rebaudiana. A simple enzymatic method is described for the determination of stevioside in stevia rebaudiana. The method is based on the hydrolysis of stevioside with crude hesperidinase. The reaction is followed by monitoring the production of glucose with a glucose oxidase-peroxidase-2, 2'-azino-di- (3-ethylbenzothiazoline-6-sulfonic acid) system. The results for the stevioside content in *S. rebaudiana* leaves correlate with those obtained by other methods. The stevioside content in *S. rebaudiana* plants showed large variation.

Cardello *et al.* (2003) studied sensory characterization of sweeteners by descriptive analysis and time-intensity analysis. Sensory properties of aspartame, stevia extract and a 2:1 cyclamate/ saccharin mixture, all in aqueous solutions with sweetness equivalent to 10 per cent sucrose, were evaluated with respect to sweetness and bitterness by differences analysis and time-intensity analysis. Parameters considered in these sensory analyses are discussed. Results by both techniques showed that, of the sweetener preparations tested, the resemblance of sensory properties to those of sucrose was best for aspartame and least for stevia extract.

Kitazume and Katabami (2003) described a process for producing a sweetener which has a mild sweetness like sugar while maintaining the high sweetness intensity of a stevia sweetener. It also has a sharp head-taste of sweetness, a sharp after-taste of sweetness, and a reduced bitterness and astringency of after-taste compared to that peculiar to a stevia sweetener. The sweetener contains steviol glucosides comprising beta-1, 4-galactosyl rebaudioside A, having 1-3 beta – 1-3 beta-1, 4 bonded galactosyl groups in a molecule and rebaudioside A.

Yoda *et al.* (2003) reported that the extract obtained from leaves of stevia rebaudiana (stevia) contains a complex mixture of glycosides such as stevioside and rebaudioside A, the sweetest of the stevia glycosides are insoluble CO_2 and soluble in ;mixtures of CO_2 and a polar solvent (e.g. methanol, water); however little research has been conducted on use of supercritical fluid extraction (SFE) to process stevia leaves. Kinetics of SFE from stevia leaves were studied usind a 2 step SFE process (CO_2 extraction under different pressure and temperatue conditions followed by extraction in CO_2 + water). Extracts were analysed for chemical composition by GC-MS and HPLC. Extraction curves for the stevia CO_2 system showed a very similar shape to those reported in similar studies, and were successfully described by the sovova pseudo steady-state model. Yield obtained bythe 2-step process was approx. 1.6% representing a recovery of up to 72 per cent of CO_2 soluble compounds. Major compounds determined in extracts were diterpenes, with austroinulin being the most abundant.

The stevia /CO_2 / water system behaved as expected under extraction conditions of 10 and 16°C and at pressures of 120 and 250 bar; behaviour of this system was unusual at 30°C and 250 bar. The process removed approx. 50 per cent of original stevioside and approx. 72% of rebaudioside A. Results demonstrated that the 2-step process studied is capable of producing extracts having higher sweetener concentration than those produced by conventional processes.

Miotto and Machad (2004) studied a by product of stevia side extraction from stevia rebaudiana leaves, referred to as caramel, shows considerable residual levels of the sweetness stevioside and rebaudioside 'A' and large quantity of leaf components. Removal of the leaf components, which gives the caramel a dark colour, would allow the reuse of this by product as a sweetner. Therefore, the caramel was purified by adsorption using modified Ca- and Mg- based zeolites. Saturation of the adsorbents was tested to evaluate their adsorption capacity, and max. clarification was assessed to determine the max. Purification achieved by zeolite adsorption. Results indicated that the Ca – based zeolite is the more effective adsorbent. Zeolites were able to be reused up to 2x, needing regeneration soon after. The test for max. clarification showed almost clear solutions with high clarification levels (80-90%), but the adsorbents demonstrated high sweetener retention (approx. 70%). This resulted in a process with low sweetner yield. It is suggested, however, that this yield is satisfactory, considering that they by-product, despite being rich in sweeteners, has no current application.

2.3. Physico-chemical characteristics of stevioside

Melis and Sainati (1991) studied the effect of calcium and verapamil on renal function of rats during treatment with stevioside. In a study with rats, the glycoside stevioside, from stevia rebaudiana leaves, produced a fall in systemic blood pressure as well as diuresis and natriuresis. Verapamil tended to increase the renal and systematic effects of stevioside. IN contrast, an infusion of $CaCl_2$ in rats treated with stevioside induced a marked attenuation of the latter compound's vasodilating effects. These data support the lypothesis that stevioside, like verapmil, may act as a Ca antagonist.

Hirata *et al.* (2000) studied analysis of stevia glycosides in stevia products of natural sweetening and evaluation of their chemical quality. TLC and HPLC methods were used to examine stevia extract, powdered stevia and alpha-glucosyltransferase-treated stevia in natural sweetening stevia products; 4 stevia glycosides in these products were analyzed by HPLC. Proportion of total glycosides in all 10 stevia extract products examined amounted to > 80 per cent. Rebaudioside A was detected in 2 products in proportions of > 90 per cent. Total glycosides content in 1 powdered stevia product was 13.6 per cent. Unreacted glycosides in 3 alpha-glucosyltransferase-treated stevia products amounted to 2.8 – 4.9 per cent. On evaluation of the chemical quality of these products (including testing for heavy metals), no food additive products of concern were found.

Jutabha *et al.* (2000) demonstration the effect of stevioside on PAH transport by isolated perfused rabbit renal proximal tubule. Stevioside (from stevia rebaudiana), a non-caloric sweetening agent, was used as a sugar substitute. The present study was designed to explore the direct effect of stevioside on transepithelial transport of p-aminohippurate (PAH) in isolated S2 segments of rabbit proximal renal tubules using in vitro microperfusion. Addition of stevioside (0.45 mM) to either the tubular lumen, bathing medium or both at the same time had no effect on transepithelial transport of PAH. Similarly, 0.7 mM stevioside (maximum solubility in the buffer) when present in the lumen, had no effect on PAH transport. However, this concentration in the bathing medium inhibited PAH transport significantly by about 25-35 per cent. The inhibitory effect of stevioside was gradually abolished after it was removed from the bath. Stevioside (0.7 mM) added to both lumen and bathing medium at the same time produced no added inhi9bitory effect. This concentration had no effect on Na+/K+-ATPase activity or cell ATP content. Stevioside, at a pharmacological concentration of 0.7 mM, inhibited transepithelial transport of PAH by interfering with the basolateral entry step, the rate-limiting step for transepithelial transport of PAH on the luminal side and its reversible inhibitory effect on the basolateral side indicate that stevioside does not permanently change PAH transport and should not harm renal tubular function at normal human intake levels.

Chun-Nin-Lee *et al.* (2001) reported that steviodise, a sweet-tasting glycoside from leaves of stevia rebaudian, has been used for decades in Japan and Brazil as a sweetener. Previous studies have shown that stevioside has an antihypertensive effect in animal models. This study investigated the mechanism of antihypertensive activity of stevioside in spontaneously hypertensive rats. Results indicated that the vsorelaxation effect of stevioside was medicated mainly through Ca 2 + flux inhibition.

Ahmed and Smith (2002) studied determination of stevioside by high-performance liquid chromatography with pulsed amperomatric detection. A novel HPLC method was developed for determination of stevioside in leaves of stevia rebaudiana. An isocratic water-acetonitrile eluent was used with a LiChrosorb NH2 bonded silica column. The effluent was monitored by integrated pulsed amperometry in an alkaline medium (pH>12) after adding NaOH post-column. Peak responses were linear from 1 to 100 mg/l, with a limit of detection of 0.3

mg/l. This method showed greater sensitivity and selectivity than UV detection.

Young-Hae-Choi (2002) studied that optimum conditions were developed for HPLC electrospray ionization (ESI) – MS determination and supercritical fluid extraction (SFE) of stevioside from stevia rebaudiana leaves. In HPLC-ESI-MS analysis of this compound, negative ion mode showed higher sensitivity than both UV and positive ion mode. In developing an alternative extraction method for stevioside using SFE, effects of temperature pressure and percentage of modifier on extraction yield were evaluated. Although sufficient extractability was not obtained by pure CO_2 under any conditions of temperature and pressure, addition of a modifier dramatically improved extraction yield of stevioside, making it comparable to organic solvent extraction. Among modifiers evaluated, a mixture of methanol and water showed greater extraction efficiency than others. Extraction yield by CO_2 methanol water (80:16:4) was 150% of conventional organic extraction. In addition to improving extraction yield, SFE provided higher purity of stevioside in the finaı extract.

Koyama *et al.* (2003) reported reference to the suggestion that stevioside, rebaudioside A and stevia mixture are metabolized to steviol in the human intestine, absorption and hepatic metabolism of steviol and stevia mixture were studied in rats and humans. Rat in vivo experiments using everted gastrointestinal sacs, and in vivo studies in which rats were dosed with steviol or stevia mixture, indicated rapid absorption of steviol but, in in vivo experiments slower absorption of steviol after ingestion of stevia mixture, suggesting metabolism of the components to steviol. In human and rat liver microsomes, steviol was metabolized to hydroxyl metabolites, but steviol clearance was 4x higher in rat than in human microsomes. It is concluded that there are no major species differences and that absorption of steviol in humans should occur in a similar manner to that in rats.

Gardana *et al.* (2003) demonstrated that stevia rebaudiana standardized extracts (SSE) are used as natural sweeteners or dietary supplements in different countries for their content of stevioside or rebaudioside A. These compounds prossess < less than or equal to > 250 x the sweetness intensity of sucrose, and are noncaloric and noncariogenic. This study sought to investigate : the in vitro transformation of stevioside and rebaudioside A after incubation with human microflora; the influence of these sweeteners on human microbial faecal community; and which specific microbial groups preferentially metabolize stevioside and rebaudioside A. Experiments were carried out under strict anaerobic conditions in batch cultures inoculated with mixed faecal bacteria from volunteers. Hydrolysis was monitored by HPLC coupled to photodiode arry and MS detectors. Isolated bacterial strains from faecal materials incubated in selective broths were added to the sweeteners. Results showed that stevioside and rebaudioside A were completely hydrolysed to their aglycon steviol in 10 and 24 h, respectively. Of particular note, the human intestinal microflora was not able to degrade steviol. Furthermore, stevioside and rebaudioside A did not significantly influence the composition of faecal cultures; among the selected intestinal groups, bacteroides was the most efficient in hydrolyzing stevia sweeteners to steviol.

Shu-Wang-Zheng *et al.* (2003) reported the method of improving the quality of taste of natural sweetner. A method is described for improving the flavour of a sweetner, such as stevioside. It involves : mixing a substrate with a predetermined dextrose equivalent with an aqueous solution of stevioside; adding a transferase in a predetermined quantity; and allowing the reaction to proceed for a predetermined time at a predetermined controlled temperature to form a resulting product. Taking production cost and percentage yield of product into consideration, a predetermined set of reaction conditions was established such that a yield of 85% is achieved, and several flavour qualities, e.g. quality of sweetness, pleasantness, aftertaste and similarity to sucrose, are improved.

Gregersen *et al.* (2003) demonstrated the antihyperglycemic effects of stevioside in type 2 diabetic subjects. Stevioside is present in the plant stevia rebaudiana Bertoni (SrB). Extracts of SrB have been used for the treatment of diabetes in, for example, Brazil, although a positive effect on glucose metabolism has not been unequivocally demonstrated. They studied the acute effects of stevioside in type 2 diabetic patients. They hypothesize that supplementation with stevioside to a test meal causes a reduction in postprandial blood glucose. Twelve type 2 diabetic patients were included in an acute, paired cross-over study. A standard test meal was supplemented with either 1 g of stevioside or 1 g of maize starch (control). Blood samples were drawn at 30 minutes before and for 240 mintues after ingestion of the test meal. Compared to control, stevioside reduced the incremental area under the glucose response curve by 18 per cent ($P = 0.013$). The insulinogenic index ($AUC_{i,insulin}/AUC_{i,glucose}$) was increased by approximately 40 per cent by stevioside compared to control ($P < 0.001$). Stevioside tended to decrease glucagons levels, while it did not significantly after the area under the insulin, glucagons-like peptide 1 and glucose-dependent insulinotropic polypeptide curves. In conclusion, stevioside reduces postpradial blood glucose levels in type 2 diabetic patients, indicating beneficial effects on the glucose metabolism. Stevioside may be advantageous in the treatment of type 2 diabetes.

2.4 Production application and formulation with stevioside

Kerzicnik *et al.* (2003) studied food characteristics of recipes using stevia sweetner – A proposed herbal sugar substitute. Stevia rebaudiana is a perennial herb with claimed medicinal and culinary characteristics. It is approximately 300 times sweeter than sucrose (table sugar). Thirty-two volunteer participants evaluated three different food products for the following sensory characteristics: tenderness, aroma, colour, grain size grain size or consistency and overll appearance. Standard food testing evaluation method were used, including ink prints and standing height measurements. Data was analyzed using SPSS Data Software, Mean scores for each of the product characteristics were tested for individual significant differences (P=0.05) using the Friedman Test. In two out of the three products evaluated, recipes using 100 per cent stevia and recipes using 25 per cent / 75 per cent stevia ranked superior in four of five sensory characteristics evaluated (tenderness, aroma, colour, grain size or consistency and overall appearance). However, the type of food product appeared to influence overall evaluation scores. Low sugar diets are used in medical nutrition therapy (MNT) for diabetes, obesity, hyperglycemia, hyperlipidemia, and for the prevention of tooth decay. There is a demand in society for more sugar substitutes in cooked food products. Aspartame (Nutrasweet) disintegrates at higher temperatures and loses its sweetening ability. Likewise, saccharin has limited accessibility because of certain Food and Drug Administration (FDA) restrictions. Study results show that stevia can replace some or all of the sugar (sucrose) in recipes without drastically affecting the visual acceptability or physical characteristics of the food products. Therefore, further studies on the safety of stevia are needed to determine its potential usefulness as a sugar substitute.

Salem and Massoud (2003) reported that use of powdered stevia (Stevia rebaudiana Bertoni) leaves as a non-calorific sweetener in frozen yoghurt was investigated. Forzen yoghurt mixes were prepared in which powdered stevia leaves were used to replace 25-100% of sucrose. Addition of increasing amounts of stevia owder had considerable effects on TS contents but only slight effects on protein contents of frozen yoghurt. The f.p. of friozen yoghurt increased with increasing addition of stevia powder, and sp. Gr. Decreased when all sucrose was replaced with stevia powder. Melting resistance of the product improved with increasing level of stevia sweetener. Replacing sucrose with stevia sweetener reduced the calorific value of frozen yoghurt (33.89 per cent decrease for complete replacement of sucrose), and improved its flavour. It is concluded that replacement of 75 per cent of sucrose by stevia sweetener produces frozen yoghurt with good texture and melting resistance.

Lutz and Richterich (2004) studied confectionery made from herbal mixtures. Sugar confectionery containing herbal extracts and an extract of stevia rebaudiana, which is naturally sweetened, without adding extra calories or increasing the risk of dental caries. The sugar confectionery can be prepared in a range of different forms by mixing herbal extracts with each other and S. rebaudiana before or after thickening.

2.5. Safety concerns with stevia

2.5.1. Chronic subacute toxicity

It has been concluded by Akashi and Yokoyama (1975) that laboratory show containing upto 7.0 per cent w/w stevioside produced no untoward toxic effects, when fed to male and female rats for nearly two months. A subacute toxicity study was carried out on rats using an aqueous extract of *S. rebaudiana* containing about 50 per cent w/w stevioside. Two levels of extract were mixed with laboratory show for feeding studies, allowing each animal to receive either 0.25 g or 0.5 g stevioside in 15 g of feed per day. Animals were fed the experimental diets for 56 days. There were no abnormalities relative to control reported that were dose related, except for a significant decrease in serum lactic dehydrogenase levels. Neither of these two subacute toxicity studies would predict any potential harm on ingestion of *S. rebaudiana* extracts by humans.

2.5.2. Acute toxicity

The toxicology and safety of stevioside used as a sweetener were recently reviewed (Genus, 2002). An acceptable daily intake (ADI) of 7.9 mg stevioside/kg body weight was calculated (Xili *et al.*, 1992). However, this ADI should be considered as a minimum value as the authors did not test concentrations of stevioside higher than 793 mg/kg body weight. Neither those scientific studies where stevia extract or solution of pure stevioside were injected in animals, nor those studies employing perfusion experiments of organs, are considered relevant for the use of Stevia or stevioside as a food additive and are not discussed in this review. Stevioside has a very low acute oral toxicity in the mouse, rat and hamster.

2.5.3. Carcinogenicity

Geuns (2003) studied a weak mutagenic effect of steviol (only 90% purity) in one sensitive *Salmonella typhimurium* TM 677 strain does not mean that stevioside used as a sweetener should be carcinogenic in se, even if the stevioside might be transformed to steviol by bacteria in the colon. Very significant inhibitory effects of stevioside were reported on tumor promotion by 12-O-tetradecanoylphorbol-13-acetate in carcinogenesis in mouse skin

2.5.4. Fertility and teratogenicity

Genus (2003) demonstrated that chicken embryos react very sensitively to administered toxicants. Fertile broiler eggs were injected with stevioside or steviol. At hatch (day 21) and 1 week later no influence of the different treatments could be found on embryonic mortality, body weight of the hatchlings, deformations (e.g. bone, beak and head malformations, abnormal fathering, open vent) or abnormal development of the gonads. The hatchlings developed normally. It was concluded that prenatal exposure to stevioside and steviol was not toxic to the chicken embryo.

MATERIALS AND METHODS

CHAPTER – III

MATERIALS AND METHODS

The present study was carried out at Department of Food Engineering, College of Agricultural Technology, M.A.U., Parbhani during the year 2004-2005. Materials used and methods adopted for the present investigation is presented under suitable headings.

3.1. Materials

3.1.1 Dried stevia leaves

The dried stevia leaves of variety stevia rebaudiana bertoni were procured from village Vambhori, District Ahmednagar at the cost of Rs. 400/kg and dried stevia leaves powder of variety stevia rebaudiana bertoni were procured from The Good Food Company, Mumbai at the cost of Rs. 600/kg.

3.1.2 Materials for mango fruit beverage (RTS) preparation

3.1.2.1 Mango fruits

The mango fruits of variety 'Kesar' having attractive color and pleasant flavour were procured from local market of Parbhani at the cost of Rs. 30/kg.

3.1.2.2 Other raw materials.

Sucrose, Food grade citric acid were purchased from local market of Parbhani.

3.1.3 Materials for cookies preparation

Raw materials required for cookies preparation i.e. maida, sugar, vanaspati ghee and baking powder were procured from Parbhani local market.

3.1.4 Glass bottles

Glass bottles of 250 ml capacity and glass jars of 150 ml capacity were obtained from Department of Food Engineering.

3.1.5 Chemicals and Glassware

Chemicals of analytical grade and sufficient glassware required are available in the P.G. laboratory, Department of Food Engineering, College of Agricultural Technology.

3.2 Methods

3.2.1 Standardization of extraction process for stevioside

3.2.1.1 Preparation of stevia leaves powder

Dried stevia leaves having the moisture content 5.2 per cent were ground by using grinder to get fine powders. This powder contained the leaf veins which causes the bitterness aftertaste. The leaf veins were removed by using sieves having appropriate mesh number. After sieving, the stevia leaves powder was ready for further use.

3.2.1.2 Extraction of stevioside

25 gm of stevia leaves powder were mixed with 500 ml water at room temperature and four different boiling treatments at 100°C for varying times were given as discussed by Simone *et al.* (2002). The details about the extraction treatments given to the stevia leaves powder were as follows.

Table 1. Different treatment for extraction of stevioside.

Sr. No.	Treatment code	Treatment control
1.	E1	Control
2.	E2	Boiling at 100°C for 60 min.
3.	E3	Boiling at 100°C for 70 min.
4.	E4	Boiling at 100°C for 80 min.
5.	E5	Boiling at 100°C for 90 min.

3.2.1.3 Filtration of liquid stevioside

The extract obtained from above treatments were filtered through double lined muslin cloth and stored at refrigeration temperature until its concentration and drying.

3.2.1.4 Concentration and drying of stevioside

Liquid stevioside was concentrated by boiling it at 100°C for 90 minute and dried in hot air oven at 110°C for 6 hours. The dried mass is ground in mixture cum grinder to obtain stevioside powder.

Dried stevia leaves

↓

Fine grinding of stevia leaves

↓

Sieving of ground mass by using sieve of mesh no. 60 → Stevia leaf veins

↓

Stevia leaves powder

↓

Take 25 gm stevia leaves powder and add 500 ml water

↓

E1 : Control
E2 : 60 minutes
E3 : 70 minutes
E4 : 80 minutes
E5 : 90 minutes
→ Boil it at 100°C

↓

Allow it to stand for 8 hr.

↓

Filtering and squeezing the whole mass by using double layered muslin cloth → Stevia leaves powder cake

↓

Liquid stevioside

↓

Passing through charcoal column of height 3 cm (charcoal powder height) and diameter 2 cm for discoloration

↓

Concentrating liquid stevioside by heating it at 80°C for 90 minutes

↓

Oven drying at 105°C for 6 hr.

↓

Fine grinding

↓

Stevioside

Flow sheet 1. Extraction of stevioside from dried stevia leaves (*Stevia rabuadiana*)

3.2.1.5 Stevioside yield

Stevioside yield was determined by using the following formula

$$\text{\% stevioside yield} = \frac{W_1}{W_2} \times 100$$

W_1 = Wt of stevioside extracted

W_2 = Initial wt of stevia leaves powder after sieving (i.e. after removing the stevia leaf veins)

3.2.2 Product development

3.2.2.1 Cookies formulation

Cookies were prepared by using formulation given by Smith (1972).

Recipe

Maida	=	100 g
Sugar	=	65 g
Fat	=	55 g
Baking powder	=	1.5 g

Different formulations were made by replacing sugar with stevioside at different incorporation level.

Table 2. Recipe for preparation of cookies

Sr. No.	Sample code	Maida	Sugar	Stevioside	Fat	Baking powder
1.	A (Control)	100 g	65 g	---	55 g	1.5 g
2.	B	100 g	52 g	0.13 g	55 g	1.5 g
3.	C	100 g	39 g	0.26 g	55 g	1.5 g
4.	D	100 g	26 g	0.39 g	55 g	1.5 g
5.	E	100 g	13 g	0.52 g	55 g	1.5 g
6.	F	100 g	---	0.65 g	55 g	1.5 g

The dry ingredients i.e. maida, baking powder were mixed together. A homogenous paste of fat and sugar was prepared in stainless steel pan at the end of mixing vanilla essence was added to the paste and mixed thoroughly again. the dry mix and homogenous paste of sugar and fat was mixed thoroughly.

The dough so prepared was rolled in a proper shape and thickness (6 mm) and cut into round shape cookies with the help of cutter. These cookies were baked at 200°C for 10 minute.

Maida	Sugar + Vanaspati ghee
↓	↓
Baking powder	Mix thoroughly
↓	↓
Mix thoroughly	Add vanilla essence
↓	↓
Dry mix	Paste

↓

Mix thoroughly the dry mix and paste

↓

Dough

↓

Roll into proper shape and size

↓

Cut with cutter

↓

Bake at 200°C for 10 minute

↓

Cool to room temperature

↓

Pack in polyethylene bags

Flow sheet 2: Cookies preparation

3.2.2.2 Mango fruit beverage (RTS) formulation

Mango fruit beverage was prepared by using following standard recipe

Mango pulp	=	27 g
Sugar	=	50 g
Water	=	375 ml
Citric acid	=	0.75 g

Stevioside was used as a sugar replacer at different concentration to prepare mango fruit beverage (ready-to-serve)

Table 3. Recipe for preparation of mango fruit beverages (RTS)

Sr. No.	Sample code	Mango pulp	Sugar	Stevioside	Water	Citric acid
1.	A (Control)	27 g	50 q	---	375 ml	0.75 g
2.	B	27 g	40 g	0.1 g	375 ml	0.75 g
3.	C	27 g	30 g	0.2 g	375 ml	0.75 g
4.	D	27 g	20 g	0.3 g	375 ml	0.75 g
5.	E	27 g	10 g	0.4 g	375 ml	0.75 g
6.	F	27 g	---	0.5 g	375 ml	0.75 g

Mango pulp (19°Bx) was taken, with water and sugar was added. Citric acid was added to it to maintain acidity. Filter through muslin cloth. It was then pasteurized to temperature 95°C. Then ready to serve mango fruit beverage (Ready – To – Serve) filled in previously sterilized bolters and sealed then.

3.3 Analystical methods

3.3.1 Moisture

It was worked out by weighing 5 g sample accurately and subjected to oven drying at 110°C for 4-5 hrs. Oven dried samples were cooled in desiccators and weighed. The drying was repeated until the constant weights were obtained. The resultant loss in weight was calculated as percent moisture content (A.O.A.C. 1990).

$$\% \text{ moisture} = [W_1 / W_2] \times 100$$

Where,

W_1 = Weight loss in sample

W_2 = Weight of sample

3.3.2 Crude protein

Protein was estimated by microkjeldhal method using 0.5 g of ground sample by digesting with concentrated sulphuric acid at 100°C. Then it was distilled with 40 per cent NaOH and liberated ammonia was trapped in 4 per cent boric acid, using mixed indicator (methyl red : bromocresol green 1:5). Then titrate it with 0.1 N HCl, the per cent of nitrogen was estimated and protein percentage was calculated by multiplying per cent nitrogen with factor 6.25 (A.O.A.C. 1975).

3.3.3 Crude fat

5 g ground sample was weighed accurately in thimble and defatted with petroleum ether in soxhlet apparatus for 6-8 hrs at 70°C. The resultant ether extract was evaporated and crude fat content was calculated (A.O.A.C. 1975).

3.3.4 Total ash

Total ash was determined according to A.O.A.C. (1975). 5 gm sample was weighed into crucible and ignited at low flame till all the material was completely charred. Then it was kept in muffle furnace for 6 hrs at 600°C and further cooled in desiccators and weighed. This was repeated till two consecutive weights were constant and per cent ash was calculated.

3.3.5 Total sugar

Total sugar was determined by the procedure of Wankhede and Tharanathan (1979). 500 mg of sample was taken in big test tube in a ice bath. 2 ml of 72 per cent H_2SO_4 was added to it with gentle stirring for 5 min at ice bath temperature to avoid the burning of sample. Then the volume of the solution was made to (23 ml) with distilled water. The sample was refluxed in water bath at 90 + 5°C for 3 hrs using air condensor. It was then filtered through the Whatman No.1 paper and filtrate was made upto 50 ml volume with deionised water. Total carbohydrate from the filtrate was estimated by Phenol - H_2SO_4 method. Intensity of colour was measured at 480 nm on spectrophotometer. From the standard curve, the concentration of total sugar was calculated as per modified procedure of Wankhede and Tharanathan (1979).

3.3.6 Reducing sugar

Reducing sugar was determined by method described by Plummer (1994) using 3, 5, - dinitrosalicylic acid.

The DNSA reagent was prepared just before use by mixing the stock solutions as reported and 1 ml of the reagent was added to 3 ml of the sugar solution in a test tube. A blank is prepared by adding 1 ml of reagent to 3 ml of distilled water. Each tube was covered with marble and placed in a boiling water bath for 5 min., cooled to room temperature and the extinction at 540 nm was read against the blank. Standard curve of the sugars provided were prepared and used to estimate the concentration of reducing sugars from samples.

3.3.7 Ascorbic acid

Ascorbic acid content was determined by the method given by Ranganna (1995) using 2, 6-Dichloropheno – Indophenol dye using 'visual titration method'.

For standardization of dye, 5 ml of metaphosphoric acid (HPO_3) was added to 5 ml of standard ascorbic acid and it was titrated against the dye till a pink colour observed.

A sample (10 to 20 ml) was taken and volume made-up to 100 ml with 3 per cent metaphosphoric acid and filtered for further analysis. The extract of the sample (2 to 10 ml) was titrated against standard dye to a faint pink end point and ascorbic acid content was calculated by using following formula.

$$\text{Dye factor} = \frac{0.5}{\text{Titre}}$$

$$\text{Per cent Ascorbic} = \frac{\text{Titre x Dye factor x volume madeup}}{\text{Aliquot of extract taken for estimation x Wt. or volume of sample taken for estimation}} \times 100$$

3.3.8 Titrable acidity

Titrable acidity of the dried stevia leaves was measured by preparing it's extract in water and as per the process given by Ranganna (1995) by titrating the sample against 0.1 N sodium hydroxide solution using phenolphthalein indicator.

$$\text{\% titrable acidity} = \frac{\text{Titre x 0.1 x volume madeup x 64}}{\text{Volume of sample taken for estimation x Wt or volume of x 100 sample}} \times 100$$

3.3.9 Total soluble solids

The total soluble solids was determined in terms of degree brix by using hand refractometer.

3.4 Measurement of theoretical energy value

Energy value is determined by using values of crude protein, crude fat and total sugar content of sample and considering that 1 g of protein yields 4 kcal energy, 1 g of fat yields 9 Kcal energy and 1 g carbohydrates yields 4 kcal energy. Total energy value is calculated by adding above three energy values which gives energy value per 100 g of sample.

3.5. Organoleptic evaluation

3.5.1 Organoleptic evaluation of cookies

The organoleptic evaluation of developed cookies was carried out by using 9 point hedonic scale with 8 semi-trained judges with respect to different quality attributes such as color, taste, flavour, aroma, texture and overall acceptability. their average mean values are depicted in Table 10. The used score card and hedonic score is as given bellow.

Organoleptic evaluation score card

Name of evaluator : Date:

Trial No. :

Sample code	**Colour**	**Taste**	**Flavour**	**Aroma**	**Texture**	**Overall acceptability**
A (Control)						
B						
C						
D						
E						
F						

Score card :

9 – Like extremely
8 – Like very much
7 – Like moderately
6 – Like slightly
5 – Neither like nor dislike
4 – Dislike slightly
3 – Dislike moderately
2 – Dislike very much
1 – Dislike extremely

3.5.2 Organoleptic evaluation of mango fruit beverage (RTS)

The organoleptic evaluation of developed mango fruit beverage was carried out by using 9 point hedonic scale with 8 semitrained judges with respect to different quality attributes such as colour, flavour, taste, aroma, mouthfeel and overall acceptability. Their average mean values are depicted in Table 11. The used score card and hedonic scale is given below.

Organoleptic evaluation score card

Name of evaluator : Date:

Trial No. :

Sample code	Colour	Flavour	Taste	Aroma	Mouthfeel	Overall acceptability
A (control)						
B						
C						
D						
E						
F						

Score card :

9 – Like extremely
8 – Like very much
7 – Like moderately
6 – Like slightly
5 – Neither like nor dislike
4 – Dislike slightly
3 – Dislike moderately
2 – Dislike very much
1 – Dislike extremely

3.6 Statistical analysis

The data obtained from various parameters were recorded and statistically analyzed by completely randomized design (CRD) as per method of Panse and Sukhatme (1987).

RESULTS AND DISCUSSION

CHAPTER-IV

RESULTS AND DISCUSSION

The results obtained and relevant information obtained during the present investigation are presented and discussed under suitable heading. The results were discussed in the view of relevant scientific literature available in the country and elsewhere. Unless otherwise stated, the photographs, figures and tables have been inserted in this chapter.

4.1 Chemical composition of stevia leaves

Table 4. Proximate composition of dried stevia leaves

Sr.No.	Parameters	Values
1.	Moisture (Per cent)	5.2
2.	Protein (per cent)	11.5
3	Fat (per cent)	2.1
4.	Total ash (per cent)	5.2
5.	Total sugar (per cent)	1.1
6.	Reducing sugar (per cent)	0.02
7.	Ascorbic acid (mg/100 g)	0.015
8.	Titrable acidity as citric acid (per cent)	0.284

* Each value represents the average of three determinations

Among the different varieties of stevia, the most sweeten variety, stevia rebaudiana bertoni (Plate 1) and dried stevia leaves powder (Plate 2) were analyzed quantitatively for its chemical composition and are presented Table 4. The results revealed that total sugar content was found to be 1.1 per cent which is very less in stevia leaves itself. It is also clear from the results that stivea leaves contains very less amount of fat and reducing sugar which was found to be 2.1

Plate 1. Dried stevia leaves variety *Stevia rebaudiana Bertoni*

Plate 2. Stevia leaves powder variety *Stevia rebaudiana Bertoni*

per cent and 0.02 per cent respectively. Stevia leaves contain higher amount of proteins which was found to be 11.5 per cent.

The results obtained in the present investigation are in good conformity with the results reported by Ruma Roy and Akhtar Khwaja (1993).

4.2 Standardization of extraction process for stevioside from dried stevia leaves

Different extraction process parameter were standardized as per Flow sheet 1 for extraction of stevioside from dried stevia leaves and the byproduct obtained are presented in Plate 3 and Plate 4, while results obtained are presented in Table 5.

Sieves were used for removal of leaf vein which gives bitter aftertaste to extracted stevioside. Hence efforts were made to remove the maximum leaf veins and for this purpose sieve size of 60 mesh number were standardized. The extraction medium used was only water and extraction ratio of stevia leaves powder and water is standardized as 1:20.

Extraction was carried out by boiling stevia leaves powder with water at temperature 100°C for 80 min where the stevioside extraction yield was found to be maximum. Holding time of 8 hrs was found to be sufficient to achieve maximum extraction. Liquid stevioside was passed through the column having 3 cm charcoal height and 2 cm diameter for discoloration. Liquid stevioside was then concentrated by heating at 80°C for 90 min and oven drying was performed at 105°C for 6 hrs. Fine powder was prepared by grinding which yields yellowish white stevioside powder.

Plate 3. Stevia leaves powder cake remained after extraction of stevioside

Plate 4. Stevia leaf veins removed by sieving before stevioside extraction

Table 5. Standardized extraction process parameters for extraction of stevioside from dried stevia leaves

Sr.No.	Process parameters	Standardized values
1.	Sieve size (mesh number)	50
2.	Extraction medium ratio	Stevia leaves powder : water:: 1:20
3.	Extraction temperature (^{o}C)	100
4.	Extraction time (min)	80
5.	Holding time (hrs)	8
6.	Charcoal height in column (cm)	3
7.	Charcoal column diameter (cm)	2
8.	Concentration temperature (^{o}C)	80
9.	Concentration time (min.)	90
10.	Drying temperature (^{o}C)	105
11.	Drying time	6 hrs

* Each value represent the average of three determinations

4.3 Effect of extraction treatments on physcio-chemical characteristics of liquid stevioside

Effect of different extraction treatments on physico-chemical characteristics of liquid stevioside was studied. The results obtained are presented in Table 6.

4.3.1 Stevioside yield

It has been observed from above results that the stevioside yield from boiling stevia leaves powder with water for 80 min was higher than control and other extraction treatments. The stevioside yield from control was found to be 18.95 per cent (stevioside yield was calculated by considering weight of stevioside extracted and initial weight of stevia leaves powder after sieving) and that was from the boiling dried stevia leaves powder with water for 65 min. other extraction treatments for 60 min, 70 min, 90 min were found to be 18.78 per cent, 19.57 per cent

Table 6. Effect of extraction treatments on the physico-chemical characteristics of liquid stevioside

Sr.No.	Liquid stevioside extraction treatments	Stevioside yield (per cent)	T.S.S. (°Bx)	pH	Titrable acidity as citric acid (per cent)	Reducing sugar (per cent)
1.	E1	18.95	5.5	5.55	0.190	0.012
2.	E2	18.78	5.4	5.57	0.189	0.01
3.	E3	19.57	5.6	5.57	0.190	0.011
4.	E4	20.02	5.9	5.56	0.192	0.012
5.	E5	18.25	5.2	5.55	0.192	0.012

* Each value represents the average of three determinations

and 18.25 per cent respectively. However, previous scientist, Yoda *et al.* (2002) reported higher stevioside yield of 23.9 per cent at 120°C boiling temperature by using CO_2 + water. The difference in stevioside yield may be due to the location growing, growing conditions and majority due to use of carbon dioxide with water as extraction medium.

4.3.2 Total soluble solids

From the above results it has been observed that the total soluble solids in boiling stevia leaves powder with water for 80 min was higher than control and rest of other treatments which was found to be 5.9°Bx. As the boiling time increases the total soluble solids increases upto 80 min and again there is decrease in extraction treatment for 90 min which was found to be 5.2 °Bx (Table 6).

4.3.3 Titrable acidity and pH

It is clear from the results presented in Table 6 that there is no significant change in acidity and pH.

4.3.4 Reducing sugar

It is obtained from Table 6 that, reducing sugar for all the extraction treatments were found to be same where for control, it is 0.012 per cent while for extraction for 80 min it was found to be 0.012.

4.4 Physico-chemical characteristics of stevioside

Physico-chemical characteristics of stevioside were studied and are presented in plate 5 and plate 6 while the results obtained were presented in Table 7. The chemical structure of stevioside is presented in Fig 1.

Table 7. Physico-chemical characteristics of stevioside

Sr.No.	Parameter	Values
1.	Color	Yellowish white
2.	Moisture (per cent)	1.4
3.	Protein (per cent)	0.14
4.	Fat (per cent)	0.07
5.	Total ash (per cent)	0.15
6.	Total sugar (per cent)	0.06
7.	Reducing sugar (per cent)	0.012
8.	Ascorbic acid (mg/100 g)	0.01
9.	Titrable acic'ity as citric acid (per cent)	0.192
10.	Stevioside Yield (Per cent)	20.02

* Each value represents the average of three determinations

The results revealed that the color of stevioside was found to be brownish white which indicates the presence of slight coloring component in the crude form of stevioside. Total sugar content and reducing sugar content were found to be 0.06 per cent and 0.012 per cent respectively which directly indicated that stevioside is the non calorie sugar. Though stevia leaves contains 11.5 per cent proteins but from the above results it is clear that proteins are not get extracted with stevioside and which was found to be 0.14 per cent in stevioside.

Fat content of stevioside to be 0.07 per cent where as total ash, titrable acidity and ascorbic acid were found to be 0.15 per cent, 0.192 per cent and 0.01 mg/100 g respectively. Moisture content of stevioside was found to be 1.4 per cent which is very less and it

Plate 5. Liquid stevioside extracted from stevia leaves powder

Plate 6. Stevioside powder

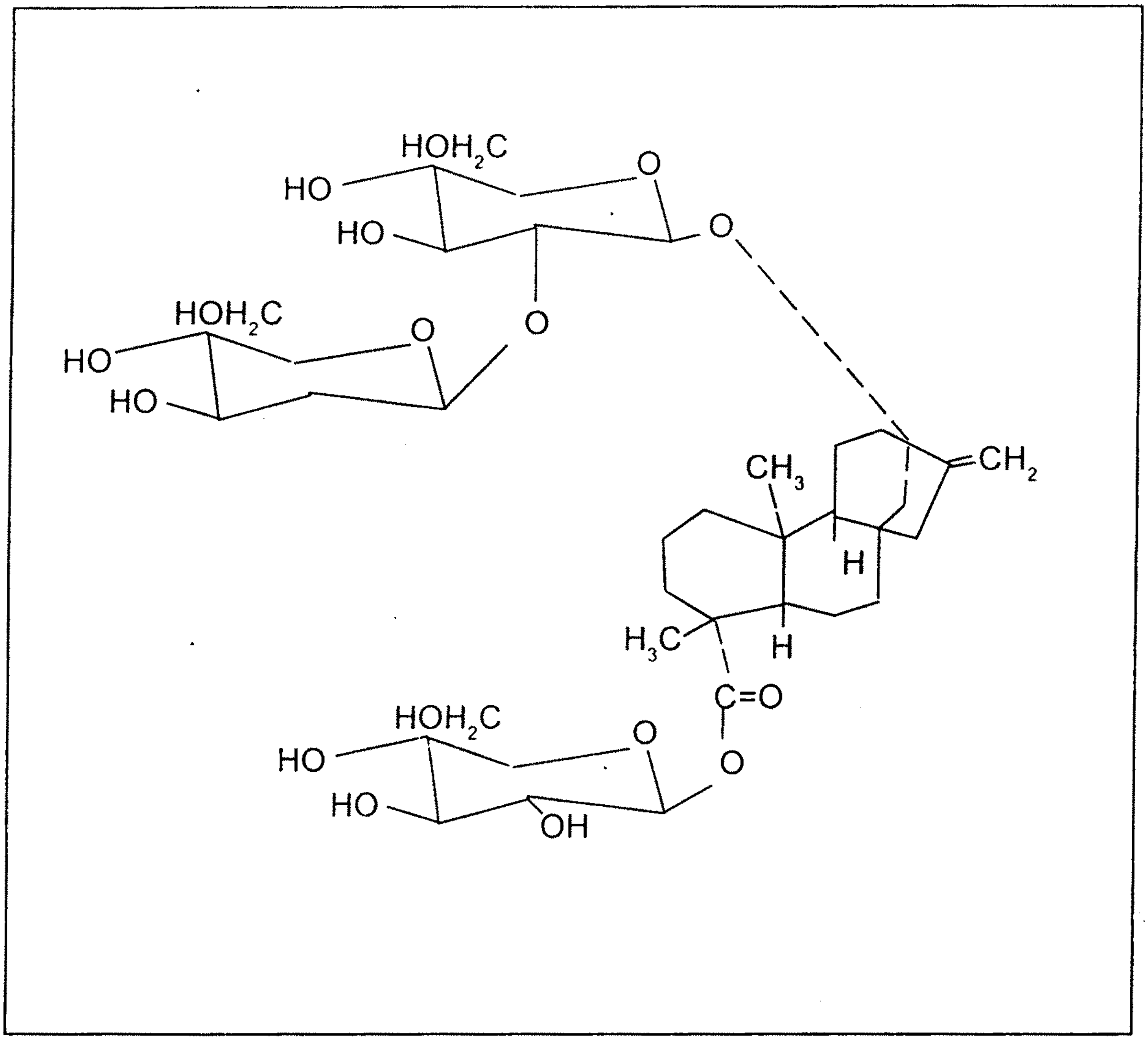

Fig. 1 Chemical Structure of Stevioside

indicates the hygroscopic nature of stevioside. Again results revealed that all the parameter present in stevioside were found to be very less which indicates the purity of stevioside. The results (Table 7) obtained are in good conformity with the results reported by Pederson (1987).

4.5 Physico-chemical characteristics of prepared food products

4.5.1 Physico-chemical characteristics of cookies

Table 8. Physicochemical characteristics of cookies

Sample code	Moisture (per cent)	Protein (per cent)	Fat (per cent)	Total ash (per cent)	Carbohydrate (per cent)
A	4.8	5.35	23.45	0.85	62.64
B	5.0	5.34	23.39	0.86	57.05
C	4.8	5.34	23.41	0.85	51.46
D	4.9	5.35	23.44	0.83	45.87
E	4.8	5.35	23.44	0.84	39.77
F	4.8	5.36	23.45	0.85	33.35

* Each value represents the average of three determination

Physico-chemical characteristics of cookies were studied and presented in plate 7 and plate 8. The results (Table 8.) revealed that there is no significant change in moisture, protein fat and total ash as compared to the values of control and other experimental formulation prepared by replacing sugar with stevioside at different incorporation level. The carbohydrate content of control was found to be 62.64 per cent which is quite higher than other experimental formulation. At 20 per cent replacement of sugar with stevioside the carbohydrate content of was found to be 57.05 where as for 40 per cent, 60 per cent, 80 per cent and 100 per cent replacement of sugar with stevioside, the carbohydrate content were found to be 51.46 per cent, 45.87 per cent, 39.77 per cent and 33.35 per cent respectively.

As incorporation level of stevioside increases by replacing sugar, the carbohydrate content decreases which indicates that

Plate 7. Cookies prepared by replacing sugar with stevioside at different incorporation levels (Sample A, B, C)

Plate 8. Cookies prepared by replacing sugar with stevioside at different incorporation levels (Sample D, E, F)

stevioside reduces carbohydrate content of product with giving equal sweet taste like that of control.

4.5.2 Physicochemical characteristics of mango fruit beverage (RTS)

Physicochemical characteristics of mango fruit beverage (RTS) were obtained and presented in Plate 9 and plate 10 where as results are presented in table 9.

Table 9. Physicochemical characteristics of mango fruit beverage (RTS)

Sample code	Protein (per cent)	Fat (per cent)	Total sugar (per cent)	Reducing sugar (per cent)	Ascorbic acid (mg/ 100 g)	Titrable acidity citric acid (per cent)	T.S.S. (°Bx)
A	0.00	0.00	13.61	12.41	12.13	0.38	13.9
B	0.00	0.00	11.10	10.12	12.11	0.36	11.5
C	0.00	0.00	8.60	7.57	12.08	0.35	8.8
D	0.00	0.00	6.14	5.01	12.09	0.35	6.3
E	0.00	0.00	3.61	2.52	12.11	0.34	3.7
F	0.00	0.00	1.14	0.30	12.10	0.35	1.2

* - Each value represents the average of three determinations

The results revealed that the fat and protein content of control and other experimental formulations of mango fruit beverage (RTS) was not found which indicates absence of fat and presence of negligible amount of protein in mango fruit beverage. Total sugar content of control was obtained to be 13.61 per cent which goes on decreasing as the incorporation level of stevioside increases by replacing sugar. For 20 per cent replacement it was found to be 11.10 per cent while for 40 per cent, 60 per cent, 80 per cent and 100 per cent replacement of sugar with stevioside gives the results 8.60 per cent, 6.14 per cent, 3.61 per cent and 1.14 per cent respectively. This is due to stevioside which is very less in total sugars.

Plate 9. Mango fruit beverage (RTS) prepared by replacing sugar with stevioside at different incorporation levels (Sample A, B, C)

Plate 10. Mango fruit beverage (RTS) prepared by replacing sugar with stevioside at different incorporation levels (Sample D, E, F)

Ascorbic acid and titrable acidity as citric acid were found to be in the range of 12.13 – 12.08 mg/100 g and 0.34 – 0.38 per cent respectively. It was also found that reducing sugar was higher (12.41 per cent) in control than other experimental formulations. As incorporation level of stevioside increases, the reducing sugar decreases and for 100 per cent incorporation, it was found to be 0.30 per cent. Further it was observed that the total soluble solids in control is higher (13.9°Bx) and reduces as the incorporation level of stevioside increases. This is due to the very higher sweetening power of stevioside which replace near about 100 g of sugar with 1 g weight which gives reduction in total soluble solids and at 100 per cent replacement the total soluble solids was found to be 1.2°Bx.

4.6 Organoleptic evaluation of prepared food products

4.6.1 Organoleptic evaluation of cookies

Table 10. Organoleptic evaluation of cookies prepared by replacing sugars with stevioside at different incorporation level.

Sample code	Colour	Flavour	Taste	Aroma	Texture	Overall acceptability
A	7.85	8.14	7.85	7.57	8.00	8.08
B	6.85	7.28	7.28	7.14	7.14	7.28
C	8.00	8.28	8.28	7.71	8.28	8.28
D	8.28	7.57	8.14	7.71	7.57	7.42
E	6.57	6.28	6.42	6.57	6.28	5.64
F	5.28	5.14	5.00	5.42	4.57	5.28
Mean	7.14	7.11	7.16	7.02	6.97	7.00
SE ±	0.41921	0.51066	0.4971	0.44032	0.5238	0.5406
CD at 5% level	1.1602	1.4133	1.3759	1.2186	1.4497	1.496

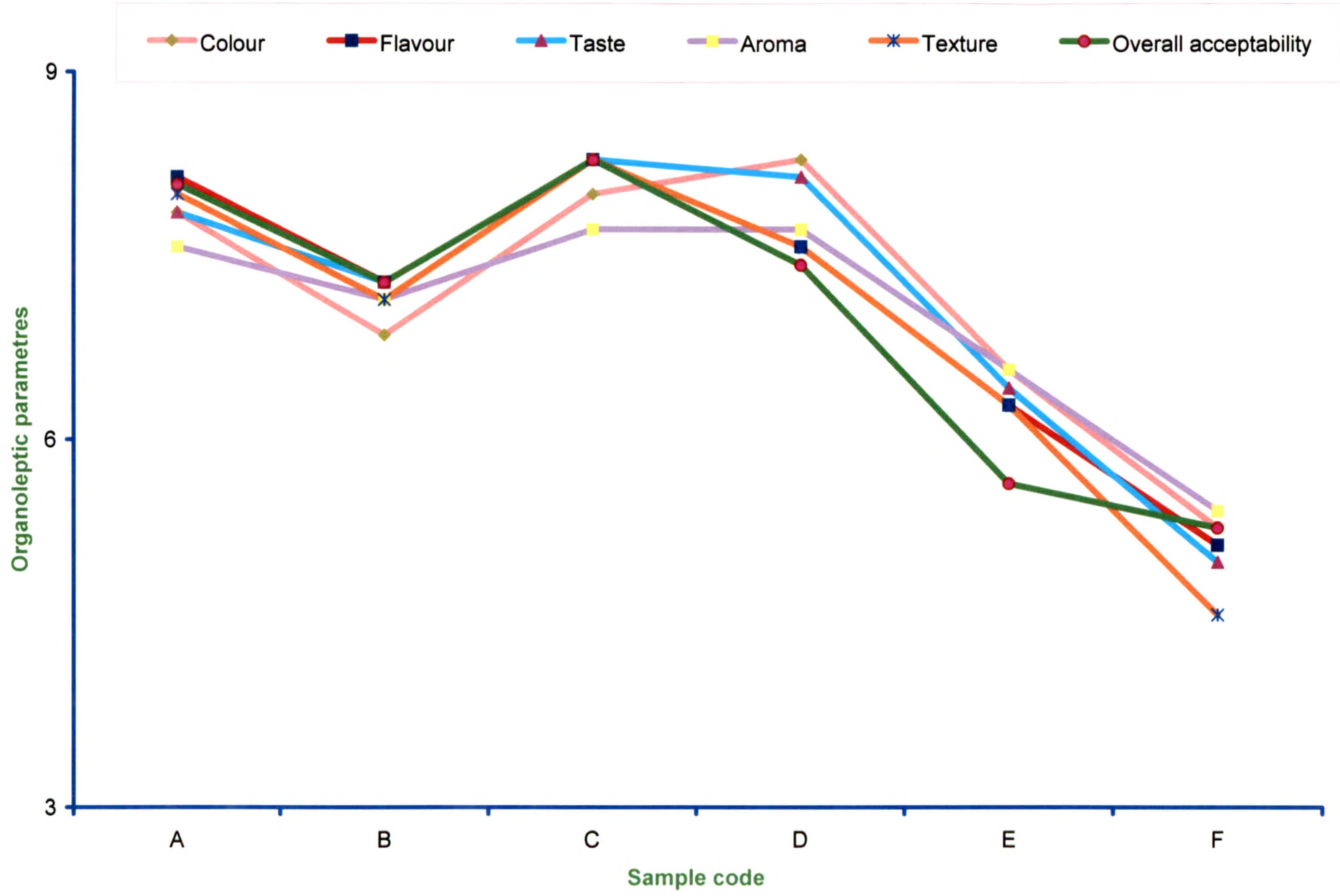

Fig. 2. Organoleptic evaluation for different types of cookies formulations

Where,

A	=	Control sample of cookies prepared by using 100 per cent sugar (sucrose).
B	=	Experimental sample of cookies prepared by using 80 per cent sugar and 20 per cent stevioside.
C	=	Experimental sample of cookies prepared by using 60 per cent sugar and 40 per cent stevioside.
D	=	Experimental sample of cookies prepared by using 40 per cent sugar and 60 per cent stevioside.
E	=	Experimental sample of cookies prepared by using 20 per cent sugar and 80 per cent stevioside.
F	=	Experimental sample of cookies prepared by using 100 per cent stevioside.

Cookies (Plate 7 and Plate 8) prepared with different incorporation level of stevioside were subjected to organoleptic evaluation. The results are presented in Table 10. In case of colour, sample D was significantly superior over the rest of samples and also control A. For flavour and texture, sample C ranked first followed by control sample A. For taste and aroma, sample C again ranked first followed by sample D.

The overall acceptability suggested sample C with significant superiority over remaining samples including control A. In general sample C was the best combination for the organoleptic parameters evaluated.

The evaluation revealed that the incorporation level of 40 per cent stevioside was best suited for cookies.

4.6.2 Organoleptic evaluation of mango fruit beverage (RTS)

Table 11. Organoleptic evaluation of mango fruit beverage (RTS) prepared by replacing sugar with stevioside at different incorporation level.

Sample code	Colour	Flavour	Taste	Aroma	Mouth feel	Overall acceptability
A	8.14	7.71	8.14	8.14	8.14	8.14
B	7.28	6.85	7.28	7.00	7.71	7.21
C	8.42	8.00	8.28	8.14	8.14	8.42
D	8.28	8.42	8.14	8.00	7.85	8.28
E	7.00	6.57	6.57	6.57	6.42	6.28
F	5.85	6.28	5.57	6.28	6.42	6.28
Mean	7.50	7.35	7.33	7.35	7.40	7.53
SE ±	0.3688	0.34667	0.3776	0.3140	0.4298	0.3894
CD at 5% level	1.0208	0.95494	1.0460	0.8691	1.1897	1.0777

Where,

A = Control sample of mango fruit beverage prepared by using 100 per cent sugar (sucrose).

B = Experimental sample of mango fruit beverage prepared by using 80 per cent sugar and 20 per cent stevioside.

C = Experimental sample of mango fruit beverage prepared by using 60 per cent sugar and 40 per cent stevioside.

D = Experimental sample of mango fruit beverage prepared by using 40 per cent sugar and 60 per cent stevioside.

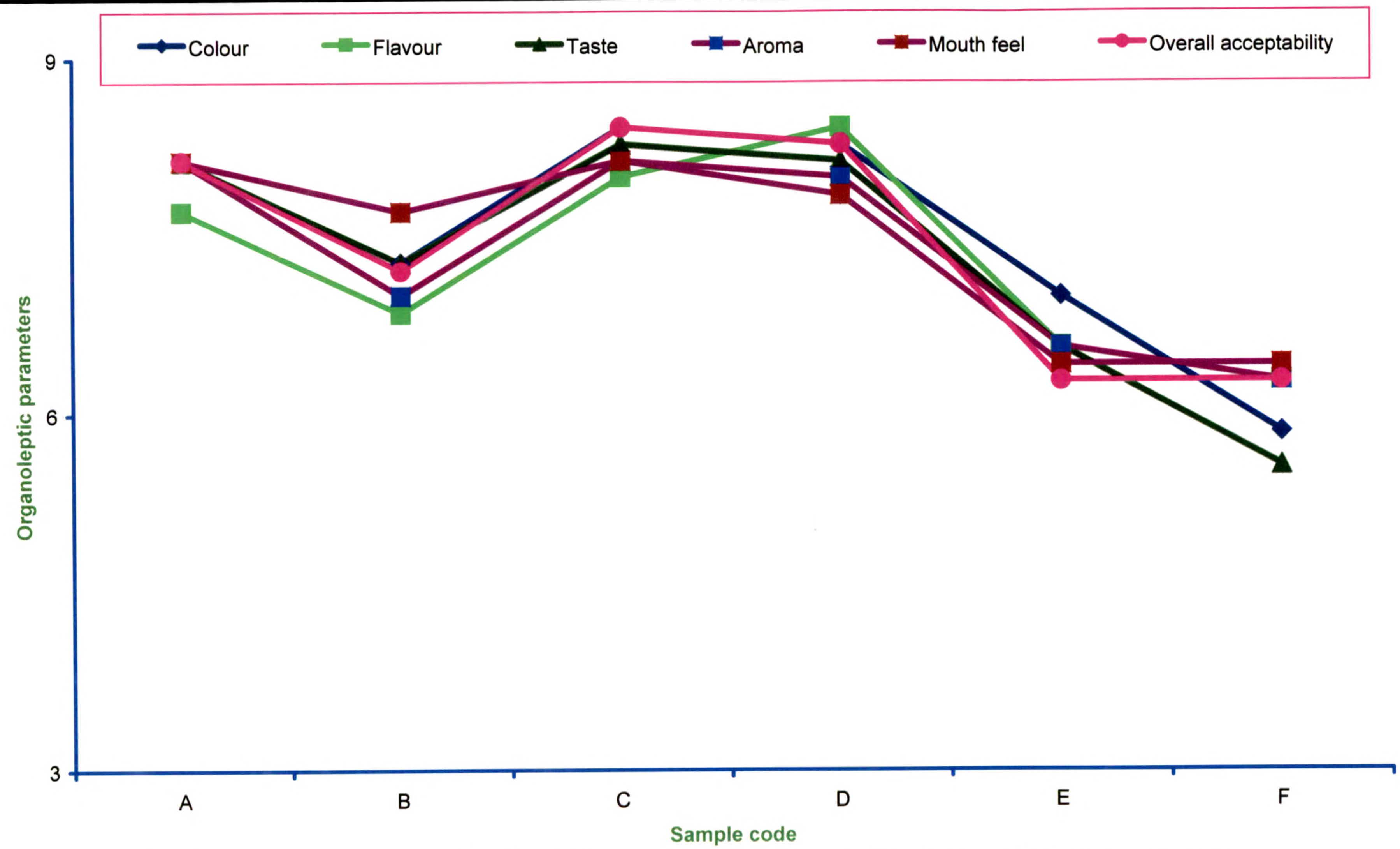

Fig. 3. Organoleptic evaluation for different types of mango fruit beverage (RTS) formulations.

E	=	Experimental sample of mango fruit beverage prepared by using 20 per cent sugar and 80 per cent stevioside.
F	=	Experimental sample of mango fruit beverage prepared by using 100 per cent stevioside.

Mango fruit beverage (Plate 9 and Plate 10) prepared with different incorporation level of stevioside were subjected to Organoleptic evaluation. The results are presented in Table 11. In case of colour sample C was significantly superior over the rest of samples and also control A. For taste, aroma and mouth feel again sample C ranked first followed by control sample A. The Organoleptic scale for flavour indicated sample D was significantly superior over that rest sample studied.

The overall acceptability suggested sample C with significant superiority over remaining samples including control A. In general sample C was the best combination for the organoleptic parameters evaluated.

The evaluation revealed that the incorporation level of 40 per cent of stevioside was best suited for mango fruit beverages (RTS).

4.7 Measurement of theoretical energy value of prepared food products

4.7.1 Theoretical energy value of cookies

The theoretical energy value of cookies are presented in Table 12 and the comparison between the energy value of control and all experimental formulations is presented in Fig.4. Since there was no significant differences in values of fat content and protein content in control as well as in different experimental formulation, the energy value ranges between 210.96 – 211.14 K cal from fat and 21.36 – 21.44 K cal from protein for all formulations including control. But carbohydrate

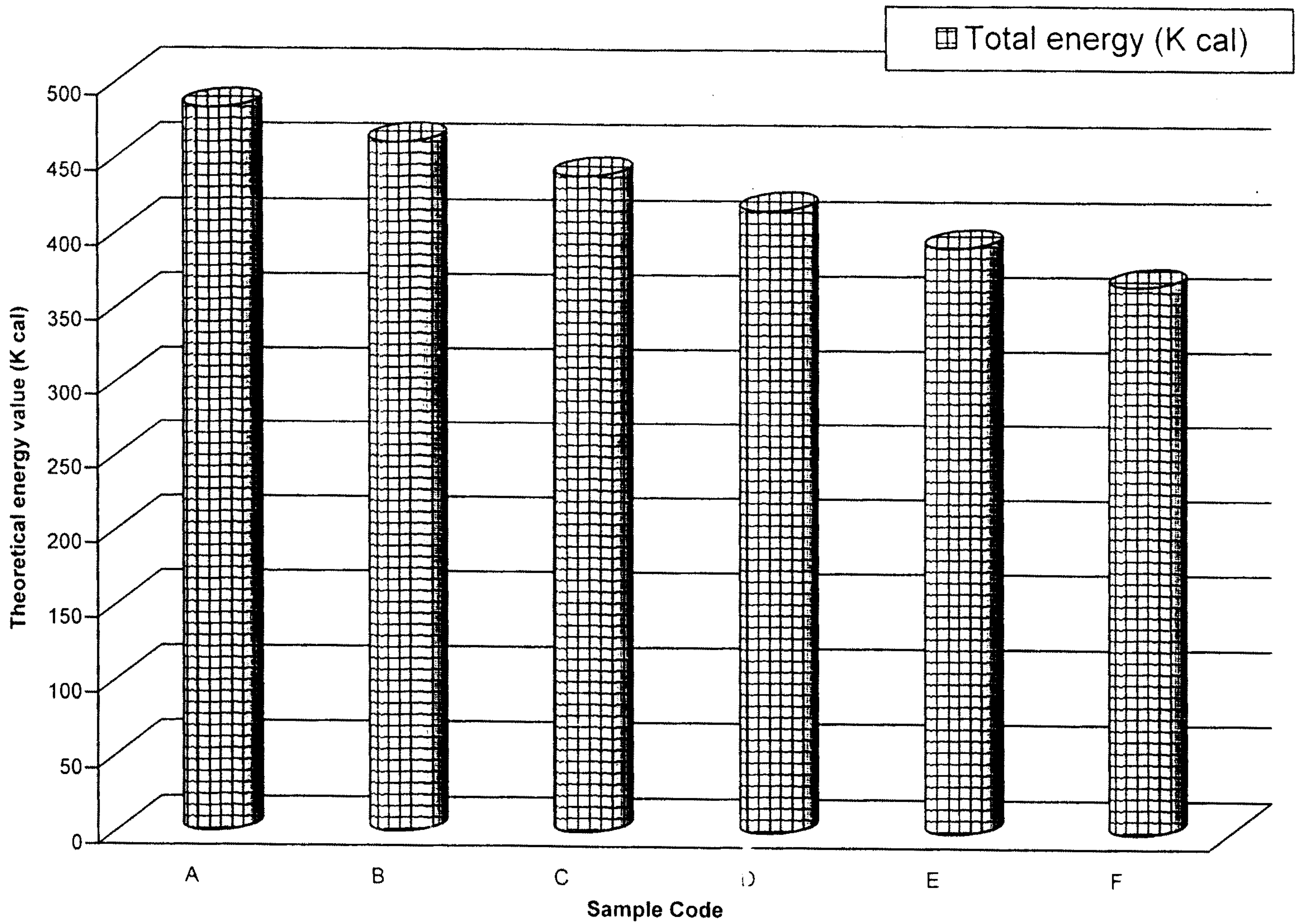

Fig.4 Comparison between the theoretical energy value of control and different experimental formulations of cookies

content of control and different experimental formulations was quite different which makes the different in total energy of cookies. For control energy from carbohydrates was found to be 250.56 K cal which increases total energy value (483.01 K cal) of control sample. As the incorporation level of stevioside increases by replacing sugar, carbohydrate content decreases which reduces total energy value of cookie. At 100 per cent replacement of sugar, energy value was found to be reduced to 365.89 K cal which is less than control by 117.12 K cal. Thus it was clear that, one can prepare low energy cookies by replacing sugar with stevioside without affecting sweet taste.

Table 12. Theoretical energy value of cookies

Sample code	Energy from			Total energy (K cal)
	Protein (K cal)	Carbohydrate (k cal)	Fat (K cal)	
A	21.40	250.56	211.05	483.01
B	21.36	228.05	211.05	460.46
C	21.36	205.84	211.14	438.34
D	21.40	183.48	211.05	415.93
E	21.40	159.08	210.96	391.44
F	21.44	133.40	211.05	365.89

4.7.2 Theoretical energy value of mango fruit beverage (RTS)

The theoretical energy value of mango fruit beverage (RTS) is presented in Table 13 and the comparison between the energy value of control and all experimental formulations is presented in Fig.5. Results revealed that energy value from fat and protein for all samples is zero because fat was found to be absent and protein might be negligible in mango fruit beverage (RTS). Due to higher carbohydrate content in control, the total energy value was found to 54.44 K cal. As the level of incorporation of stevioside increases by replacing sugar, the carbohydrate content decreases which finally decrease the total energy

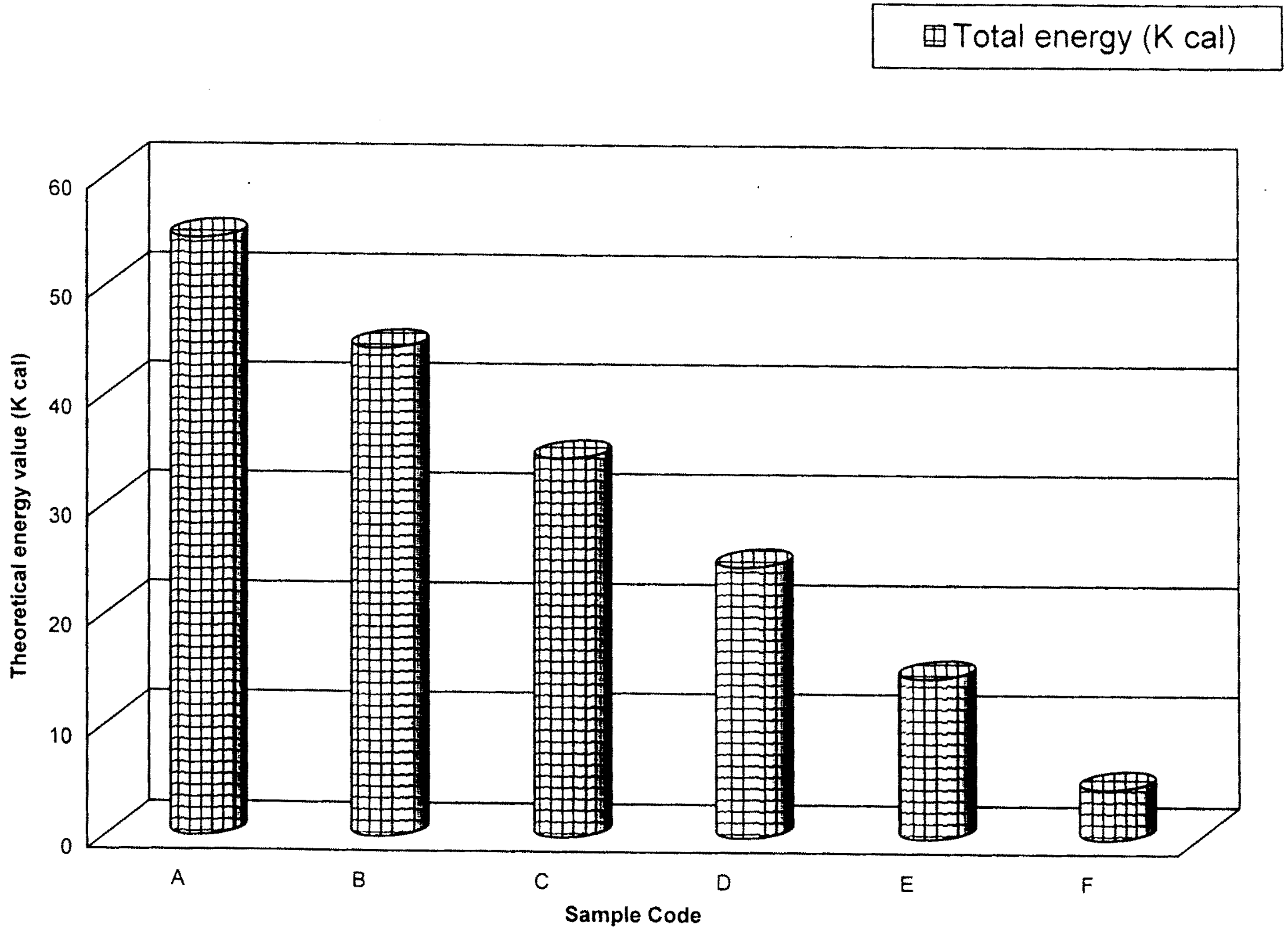

Fig.5 Comparison between the theoretical energy value of control and different experimental formulations of mango fruit beverage

value which was found to 4.56 K cal at 100 per cent replacement of sguar with stevioside. Stevioside reduces the total energy value of mango fruit beverage (RTS) by 49.88 K cal at 100 per cent replacement of sugar.

Table 13. Theoretical energy value of mango fruit beverage (RTS)

Sample code	Energy from			Total energy (K cal)
	Protein (K cal)	Carbohydrate (k cal)	Fat (K cal)	
A	0.00	54.44	0.00	54.44
B	0.00	44.40	0.00	44.40
C	0.00	34.40	0.00	34.40
D	0.00	24.56	0.00	24.56
E	0.00	14.44	0.00	14.44
F	0.00	4.56	0.00	4.56

4.8 Techno-economic feasibility of prepared food products

4.8.1 Techno-economic feasibility of prepared cookies

The data on the cost analysis of prepared cookies are presented in Table 14 and the comparison between the energy value of control all experimental formulations is presented in Fig.4. The above analysis showed that the cost of production of cookies prepared by replacing sugar with stevioside at different incorporation level ranged between Rs. 41.90 – Rs. 42.94 per kg which is slightly higher than the cost of production of control sample (Rs. 41.64 per kg) and this may be due to increased in the cost of stevioside, required to replace the sugar in experimental formulations. However cost of production does not include rent, transport charges, sale commission, local taxes etc.

Table 14. Cost analysis of cookies prepared by replacing sugar with stevioside at different incorporation level

Sr. No.	Ingredients	Rate (Rs. per kg)	Cost (Rs)					
			A	B	C	D	E	F
1.	Maida	20	10.00	10.00	10.00	10.00	10.00	10.00
2.	Sugar	16	5.20	4.16	3.12	2.08	1.04	--
3.	Fat	60	16.50	16.50	16.50	16.50	16.50	16.50
4.	Baking powder	400	3.00	3.00	3.00	3.00	3.00	3.00
5.	Stevioside	2000	---	1.30	2.60	3.90	5.20	6.50
6.	Labour, processing and packaging (20 per cent of production cost	--	6.94	6.94	6.94	6.94	6.94	6.94
7.	Cost /kg (Rs)		41.64	41.90	42.16	42.42	42.68	42.94

4.8.2 Techno economic feasibility of prepared mango fruit beverage (RTS)

The data on the cost anlaysis of prepared mango fruit beverage (RTS) are presented in table 15. The above analysis showed that the cost of production of mango fruit beverage prepared by replacing sugar with stevioside at different incorporation level ranged between Rs. 8.03 – Rs. 8.43 per lit which is higher than the cost of production of control sample (Rs. 7.93 per lit) and this is due to replacement of sugar with stevioside which is costly than sugar and gives equal taste at very less addition (100 g sugar can be replaced by 1 g stevioside). However, cost of production does not include rent, transport charges, sale commission, local taxes etc.

Table 15. Cost analysis of mango fruit beverage prepared by replacing sugar with stevioside at different incorporation level

Sr. No.	Ingredients	Rate (Rs. per kg)	Cost (Rs)					
			A	B	C	D	E	F
1.	Mango pulp	60	4.05	4.05	4.05	4.05	4.05	4.05
2.	Sugar	16	2.00	1.60	1.20	0.80	0.40	---
3.	Stevioside	2000	---	0.50	1.00	1.50	2.00	2.50
4.	Citric acid	300	0.56	0.56	0.56	0.56	0.56	0.56
5.	Labour, processing and packaging (20 per cent of production cost	--	1.32	1.32	1.32	1.32	1.32	1.32
6	Cost /kg (Rs)		7.93	8.03	8.13	8.23	8.33	8.43

SUMMARY AND CONCLUSION

CHAPTER-V

SUMMARY AND CONCLUSION

Sincere efforts were made to explore the potential of stevia (*Stevia rebaudiana* Bertoni). Physicochemical properties of stevia leaves were studied and it was found that stevia leaves contain very less amount of total sugar (1.1 %) as well as reducing sugar (0.02 %).

Different types of extraction treatments were given and the process parameters for extraction of stevioside from stevia leaves were standardized by using water as a extraction medium. Extraction treatment containing boiling of stevia leaves powder with water in the ratio 1:20 at 100°C for 80 min was found to be best which was resulted in high stevioside yield (20.02 pre cent). Color of stevioside was found to be yellowish white and it was highly soluble in water. The results revealed that the total sugar content of stevioside was very less (0.06 per cent).

In the present investigation, attempts were also made to prepare low calorie food products (i.e. cookies and mango fruit beverage) by replacing table sugar (sucrose) with stevioside at different incorporation level. Physicochemical properties of prepared food products were studied where data indicated that there was no significant change in values of protein, fat, ash, titrable acidity and ascorbic acid content amongst all experimental formulations and control whereas difference in total sugar content was observed which makes difference in theoretical energy value of prepared food products. Energy value of cookies in control was observed higher (483.01 Kcal) by 117.12 K cal than experimental formulation (365.89 K cal) where 100 % sugar was replaced by stevioside. Energy value of mango fruit beverage (RTS) in control was again observed higher (54.44 K cal) by 49.88 K cal than experimental formulation (4.56 K cal) 100 % sugar was replaced by stevioside.

The oragnoleptic evaluation of prepared food products revealed that the maximum orangoletic score for most of parameter were obtained for cookies formulation and mango fruit beverage (RTS) formulation which were prepared by replacing 40 per cent sugar with stevioside in both the cases. No significant difference in production cost of control and all experimental formulations was observed in prepared food products which indicates economic feasibility.

Thus it can be concluded that extraction of stevioside by boiling with water in the ratio of stevia leaves powder : water : : 1 : 20 at 100°C for 80 min, gives the maximum stevioside yield (20.02 per cent) which indicated that the stevioside extracted was in crude form of stevioside and it need further purification in order to prepare white stevioside powder. Stevioside can be utilized as the non calorie sweetener in the manufacture of low calorie food products like cookies and mango fruit beverage (RTS) up to the 40 per cent replacement of sugar with good overall acceptability.

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