ADDIS ABABA UNIVERSITY

ADDIS ABABA INSTITUTES OF TECHNOLOGY

SCHOOL OF CHEMICAL AND BIO ENGINEERING

PRODUCTION, CHARACTERIZATION AND OPTIMIZATION AN OF STEVIA SWEETENER FROM STEVIA LEAF

A Thesis submitted to the school of graduate studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering Process Engineering Stream

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PROCESS ENGINEERING STREAM

This is to certify that the thesis prepared by Yohannes Dilnessa, entitled: Production of stevia sweetener from stevia leaf and submitted in partial fulfillment of the requirements for the Degree of Master of Science in process Engineering, complies with the regulations of the university and meets the accepted standards with respect to the originality and quality.

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List of Abbreviations

ADI: Accepted daily intake

AIM: Alternative investment market

ANOVA: Analysis of Variance

ARC: Aids-related complex

BW: Body weight

CFU: colony factor unit

EFSA: European Food Safety Association
FAO: Food and Agriculture Organization

Fcal: the calculated F-value from the data

FDA: food and drug administration

FRG: farmers research group

GDP: Gross Domestic Product

GRAS: Generally Recognized as Safe

JECFA: Joint Expert Committee on Food Additives

m_{mix1}: is the mass the mixture of the stevia sweetener and water produced in run 1 during the extraction process

m_{mix2}: is the mass of the mixture of the stevia sweetener and water produced in the optimal run extraction process

m_{rs1}: is the mass of residue produced in run1 during the extraction process

m_{rs2}: is the mass of residue produced in the optimal run extraction process

MS_A: mean square abuot factor A (temperature)

MS_{AB}: mean square about intraction of factor A and B (temperature and residence time)

MS_{AC}: mean square about intraction of factor A and C (temperature and material ratio)

MS_B: mean square abuot factor B (residence time)

MS_{BC}: mean square about intraction of factor B and C (residence time and material ratio)

MS_C: mean square abuot factor C (material ratio))

MS_E: mean square about error

m_{st1}: is the mass of the stevia powder used for run 1 during the extraction process

m_{st2}: is the mass of the stevia powder used for the optimal run extraction process

m_{w1}: is the mass of water (solvent) used for run1 during the extraction process

m_{w2}: is the mass of water (solvent) used for the optimal run extraction process

reb B: Rebaudioside B

reb C: Rebaudioside C

reb D: Rebaudioside D

reb E: Rebaudioside E

Reb-A: rebaudioside-A

S.: stevia

SCFE: supercritical fluid extraction

S_{Gst}: Specific gravity of the liquid stevia sweetener

SS_A: sum square about factor A (temperature)

SS_{AB}: sum square about interaction of factor A and B (temperature and residence time)

SS_{AC}: sum square about interaction of factor A and C (temperature and material ratio)

SS_B: sum square about factor B (residence time)

SS_{BC}: sum square about interaction of factor B and C (residence time and material ratio)

SS_C: sum square about factor C (material ratio)

SS_E: sum square about error

SS_T: sum square about total

St : Stevioside

WHO: World Health Organizatio

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Abstract

This study has been carried out to extract the stevia sweetener, sugar substitute, from the leaf of stevia plant. Solvent extraction of the leaf using hot water was investigated. The main and interaction effects of temperature, extraction time and material ratio (stevia leaf: water) were examined. In the main effects investigation all the main factors had a significant effects on the yield of stevia sweetener. But in the interaction effects, only temperature with material ratio and extraction time with material ratio had a significant effect on the yield of the sweetener. The interaction effects of temperature with extraction time (residence time) had no any significant effects on the yield of stevia sweetener. The two factor interaction model was developed. In this model the term which had no significant effects had been reduced. This term was the interaction effect of temperature with residence time $(\tau\beta)_{ij}$. Different analysis of the data such as analysis of the variance, hypothesis testing and predicted R-squared also enforce this idea. The p-value was 0.1270 which was greater than type one error 0.05. The F-calculate for this term was 1.74 that was less than F-tabulate 1.90. The predicted R-squared was 60.10% that was far from the adjusted R-squared 87.49%. These different analysis leads to the reduction of the interaction effect of temperature with residence time from the model. The model adequacy also investigated by normal probability plot of residuals and plot of residual verses predicted value. Both normal probability plot as well as residual verses predicted value plots showed that the model was acceptable. Optimum conditions for the extraction process were optimized. The optimal treatments of each factor to maximize the yield were, for the temperature 50°C, three hours for the extraction time and 1:10 material ratio. The maximum yield that was obtained at these optimum conditions was 49.90 %. The two methods report form and ramp form of optimization showed the same result or the same conditions.

1. Introduction

A sugar substitute is a food additive that duplicates the effect of sugar in taste, usually with less food energy. Some sugar substitutes are natural and some are synthetic. Those that are not natural are, in general, called artificial sweeteners**.** Now days there are so many sugar substitute are available. Among these stevia sweetener is the most popular one. It is produce from stevia plant Phillips (1987).

Stevia is a South American herb used as a natural sweetener for centuries. The stevia plant is currently growing in the demonstration field of the Ethiopian Agricultural Research Institute at the federal and regional level specifically at wendogenet, bishoftu and holeta. It is therefore, important to characterize and extract the stevia sweet so that the plant became commercially viable. Stevia plant is a bush that can grow from a foot to 4 feet tall. It is probably best known as a source of natural sweeteners. In fact, native people in South America have used stevia as a sweetener for hundreds of years. But the leaves are also used to make medicine. The leaves of the Stevia rebaudiana plant have a refreshing taste, zero calories and zero carbs. Stevia was originally available as a "dietary supplement" in the U.S. It wasn’t allowed as a “food additive” until 2008.

Stevia is also available as a sweetener in Japan, South Korea, Malaysia, Taiwan, Russia, Israel, Mexico, Paraguay, Uruguay, Venezuela, Columbia, Brazil, and Argentina. Stevia is a plant that contains natural sweeteners that are used in foods. Researchers have also evaluated the effect of chemicals in stevia on blood pressure and blood sugar levels. Stevia has become a major source of high-potency sweetener for the growing natural food market. Although it can be helpful to anyone, there are certain groups who are more likely to benefit from its remarkable sweetening potential. These include diabetic patients, those interested in decreasing caloric intake, and children. A white crystalline compound (stevioside) found in the leaf of the plant is a natural herbal sweetener with no calories and is over 100-300 times sweeter than table sugar. Stevia contains high levels of sweetening glycosides including stevioside which, in lab studies, have been found to possess antimicrobial and antifungal properties Tsanava et al. (1991).

1.1 Background of the study

In 1900, the Paraguayan chemist Ovidio Rebaudi, after whom Bertoni named the plant, studied the major characteristics of stevia. The Stevia rebaudiana Bertoni contains a complex mixture of labdanediterpenes, triterpenes, stigma sterol, tannins, volatile oils, and eight diterpenenic glycosides: stevioside, steviobioside, dulcoside, and rebaudiosides A, B, C, D, and E. The most abundant substances are stevioside and rebaudioside A. Of the stevia glycosides rebaudioside A is the sweetest and the most stable. Rebaudioside E is as sweet as stevioside, and rebaudioside D is as sweet as rebaudioside A, while the other glycosides are less sweet than stevioside (Cramer and Ikan, 1987).

A combined process involving a solid/liquid extraction step, followed by a liquid/liquid-purifying step, is traditionally used to extract the glycosides from stevia. There are several hypotheses in regard to the source of the bitter aftertaste of stevia glycosides. Phillips (1987) described a European patent held by the Stevia Company, which attributes the bitter aftertaste to the presence of essential oils, tannins, and flavonoids. Soejarto et al. (1983) believed that the sesquiterpene lactones are responsible for the bitter aftertaste. Tsanava et al. (1991) suggested that caryophyllene and spathulenol contribute decisively to the aftertaste. Nevertheless, as pointed out by Phillips (1987), stevioside and rebaudioside A are partially responsible for the aftertaste, even though the contribution of rebaudioside A is significantly less than that of stevioside.

Tan et al. (1988) hold a Japanese patent for the production of stevia glycosides by supercritical fluid extraction (SCFE) with CO_2 and a cosolvent. Methanol, ethanol, and acetone were used as cosolvents. The purification step is accomplished by adsorption. Kienle (1992) holds a similar patent in the USA. Pasquel et al. (1999) studied the SCFE of the non-glycoside fraction of stevia leaves.

The stevia leaves are extracted with hot water or alcohols. In some cases, the leaves are pretreated with nonpolar solvents such as chloroform or hexane to remove the essential oils, lipids, chlorophyll, and other nonpolar substances.

As of 2011, natural sweeteners made from extract of the stevia plant have taken about 10 per cent of the US consumer market for table-top sugar substitutes, only nine months after being approved by the US Food and Drug Administration. The rapid growth of products produced by Cargill and Merisant underlines the potential for natural sweeteners in a market that could amount to $700m in five years, according to a report by New York-based analysts at Rabobank. Cargill's Truvia and Merisant'sPureVia natural sweetener brands are made with rebaudioside-A (Reb-A), an extract of the stevia plant that was approved for public consumption by the US FDA in 2009. Cargill, the agrobusiness conglomerate, developed Truvia in a joint venture with Coca-Cola, the soft drinks company, but decided to expand beyond its traditional food ingredients business to sell the sweetener directly to consumers. PureVia was developed by Merisant with PepsiCo in partnership with PureCircle, an AIM-listed processor that is the world's largest processor of stevia.

1.2 Statement of the problem

A significant number of the world population including our country is currently suffering from diabetes because of the high sugar content in their blood. This has lead to different health problems such as kidney failure, high blood pressure and others. It is because of this very serious health problem that many synthetic sweeteners are developed in use in many parts of the world where Ethiopia is a part. In such content it will be appropriate and relevant to find natural sweetener product, among which stevia sweetener is one of them. It is therefore, the outcome of this research on the extraction of stevia sweetener can contribute to the development of commercial product that can substitute the sugar aimed at tackling the health problem that can come from such sugar and other sugar substitute.

Ethiopia now a day imports large amount of sugar substitute such as saccharin for different food industry. This sugar substitutes have different health problems beyond the economic disadvantage of the country. When we consider about stevia sweetener, there is no any study which indicates about the health problem of it. As a result locally producing this important sugar substitute has both economical and health advantage.

1.3 Significant of the Study

Introducing this valuable plant and it’s product to our country has enormous advantage in different area such as economically, in health and so on.

The product of this sugar substitute, stevia sweetener, overcomes the problem that comes from sugar and other sugar substitute. The high quality of the product, more than 90 % pure stevia sweetener is very expensive in the world market. The price of the products accounts around US $50-100. If the concerned body and the society as well will do enormous effort, the product has the potential to improve our economy like coffee and other agricultural product of the country. It also reduce the imported other forms of sugar substitute like saccharin which is not recommended for health.

1.4. Objectives

1.4.1 General objectives

The main objective of this research was to produce calories free sugar substitute or stevia sweetener from stevia plant.

1.4.2 Specific objective

- To characterize the stevia plant
- To determine optimum parameters of the extraction processes such as temperature, amount of solvent, residence time
- To conduct statistical analysis of the factors or parameters of the extraction process
- To characterize the stevia sweet as to its suitability in substituting sugar
- To modeling the data obtained from laboratory experiment

2. Literature Review

2.1 What is Stevia rebaudiana Bertoni?

Stevia, botanically known as Stevia rebaudiana (Bertoni) is a sweet Herb and it is one of 154 members of the genus stevia that produce sweet steviol glycosides. They grow primarily in the Amambay mountain range of Paraguay but over 200 various species of stevia have been identified around the globe. In its native state it is a perennial herb, living 3 – 5 years, with variable appearance up to about 0.7 meters tall(Belem, PA, Brazil (1994).

Stevia belongs to: Class: Magnoliopsida(Dicotyledons); Order: Asterales,Family: Asteraceae or Compositae. Stevia is one of the 950 genera of the Compositae (Asteraceae).Stevia rebaudiana has many commonly known Synonyms: Sweet herb, sweet leaf, sugar leaf and honey leaf or simply Stevia(Elsevier, New York, p.45 (1987)).

Stevia rebaudiana is the only species at present which possesses an inordinate ability to sweeten. Its common form is known as stevioside, a fine white powder extracted from the leaves of the plant. The leaves are mid green and intensely sweet. Its leaves contain approximately 10% of steviosides which are intensely sweet compounds (150 to 300 times sweeter than sugar). The leaves have been traditionally used for hundreds of years in Paraguay and Brazil to sweeten local teas, medicines and as a ‘sweet treat’. The plant bears greenish cream flowers in autumn. It is a plant that is native to South America. It is probably best known as a source of natural sweeteners. In fact, native people in South America have used stevia as a sweetener for hundreds of years. Dry leaves of this plant are 30 times sweeter than sugar with Zero calories (Campinas, SP, Brazil (1999).

2.2 Background of stevia

Stevia rebaudiana (Bertoni) was ‘rediscovered’ by Europeans in Paraguay in 1888 by Dr M.S. Bertoni. He later botanically described and named the plant (in 1905) in honor of Paraguayan chemist Dr Rebaudi. There are now known to be more than 150 Stevia species but this is the only one with significant sweetening properties; other species do contain other biochemicals of interest (Supercritical Fluids 14, No. 3, 235 (1999).

Stevia is a plant indigenous to mountainous regions of Brazil and Paraguay. For centuries, this herbal sweetener has been used by native cultures to counteract the bitter taste of various plant-based medicines and beverages. The Guarani Indians of Paraguay have used this potent sweetener in their green tea for generations. The name they designated for stevia leaves was "sweet herb." In addition, these native peoples have historically used stevia as a digestive aid and a topical dressing for wounds and other skin disorders (Belem, PA, Brazil (1994).

In the sixteenth century, Europeans became aware of the herbal sweetener through the Spanish Conquistadors. In the late 1880s, Moises S. Bertoni, director of the College of Agriculture in Asunción, Paraguay, became extremely intrigued by the stevia plant. Its reputation was that it was so sweet that even just a small leaf part could sweeten an entire container of mate tea. Bertoni wanted to find out if this was true. After several years of studying the plant, he wrote about it in a local botanical publication. In 1905, Bertoni published an important article about the incredible sweetening power of the stevia plant, which he considered superior to sugar and extremely marketable (Campinas, SP, Brazil (1999).

It is native to a relatively small area of eastern Paraguay (on the Brazilian border) where its leaves have been used by the local Guarani Indians as a sweetener for many hundreds of years. They especially use it in the local green tea ("Mate" tea – Ilex sp.), as well as with otherwise unpalatable medicinal and other drinks.

The first stevia crop was harvested in 1908 and subsequently, stevia plantations sprang up in South America. In 1921, the American Trade Commissioner to Paraguay, George S. Brady, wrote that although the herb is an extraordinary sweetener with remarkable properties, little had been done to commercially cultivate the plant. He suggested that stevia may be an ideal sugar product for diabetics and strongly advised that American companies pursue its importation.

The sweet principle was first isolated in 1909 and only in 1931 was the extract purified to produce stevioside, the chemical structure of which was established in 1952 as a diterpene glycoside (Belem, PA, Brazil (1994).

In 2006, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) announced a temporary accepted daily intake (ADI) of stevioside of up to 5.0mg/kg body weight (BW). In September 2009, the French Government (via interministerial decree) became the first government in the European Union (EU) to approve Stevia extracts consisting of at least 97% Rebaudioside A (Reb A) as food and beverage sweeteners. Following the recent U.S. FDA recognition of high purity Reb A from key producers as "Generally Recognized as Safe (GRAS)", the global market is now looking forward to approval from the European Food Safety Association (EFSA).

These sweeteners impart cultivation of stevia was started in Paraguay in 1964. Currently it is being cultivated in Japan, Taiwan, Philippines, Hawaii, Malaysia, Kenya in Africa and overall

South America for food and pharmaceutical products. Today, the global interests in stevia have increased and the spread of the stevia phenomenon will not be limited to a certain countries. As part of this increased interest a number of reviews have been undertaken in various countries including: Brazil, Japan, Canada, India, Georgia, Germany, Russia, Czech Republic, Korea, Mexico, Sweden and California. It was also included in a 1992 review of possible new crops for Western Australia (Campinas, SP, Brazil (1999).

The main stevioside producing countries are China and Paraguay with adjacent parts of Brazil. China is the main supplier to Japan, who are the main commercial producers and users of steviosides. Paraguay/Brazil is the main center for the production and distribution of Stevia products direct to consumers via the health food and herbal product outlets and by direct mail order sales around the world.

Stevia was first brought to the attention of the U.S. government in 1918 for the U.S. Department of Agriculture. Stevia is cultivated and used in food elsewhere in East Asia, including in China (since 1984), Korea, Taiwan, Thailand, India and Malaysia. It can also be found in Saint Kitts and Nevis, in parts of South America (Brazil, Colombia, Peru, Paraguay, and Uruguay), and in Israel. China is the world's largest exporter of Stevioside.

2.3 Review on the production of stevia sweetener

Steviol glycosides can be isolated and purified from the leaves of the stevia plant and can be used to sweeten foods and beverages and used as tabletop sweeteners. Rebaudioside A is one of the steviol glycosides purified from the leaf of the stevia plant. Other steviol glycosides include stevioside, rebaudiosides B, C, D, F, steviolbioside, rubusoside, and dulcoside A.

The glycosides are extracted from the leaves using either water or alcohol and membrane filtration. Because it evaporates, no alcohol remains in the finished product. The liquid becomes clear rather than green because the extraction process removes the chlorophyll, and white glycosides remain. No bleach or other chemical whiteners should be used, according to the Sweet Leaf Company.

The stevia leaves are extracted with hot water or alcohols. In some cases, the leaves are pre-treated with non polar solvents such as chloroform or hexane to remove the essential oils, lipids, chlorophyll and other non polar substances. The extract is clarified by precipitation with salt or alkaline solutions (Midmore and Rank, 2006). The extract is concentrated and redissolved in methanol for crystallization of the glycosides. The crystals are formed almost by pure stevioside.

2.3.1 Subcritical Fluid Extraction of Stevia Sweeteners from Stevia plant

Appropriate amounts of accurately weighed dry leaves and stevioside standard were used throughout the work. The dry leaves were totally ground before extraction. Stevioside standard solution (500 ppm) was prepared with methanol. The standard solution (1 mL) was spiked into the glass wool, and the solvents were evaporated before extraction. The samples (including dry leaves and the spiked ones) were manually placed into the extraction cell. Extracts were collected in 3 mL of organic solvents in a 5 mL volumetric flask. The restrictor was immersed below the surface of the collecting solvent, and the flask was covered with a piece of Nescofilm (Nippon Shoji, Kaisha, Osaka, Japan).

Collection was performed at room temperature. After collection, when acetonitrile and water were used as the modifiers, the extracts were diluted with the same modifier in a 10-mL volumetric flask, filtered, and then analyzed by CE. When methanol was used as the organic modifier, the extract solutions were evaporated, and the residues were dissolved in the solution (acetonitrile-water, 80:20, v/v) and analyzed by CE. A suspension of the residue in water was washed with ether and then extracted with butanol. The organic phase was evaporated, and the residue was recrystallized from methanol, giving stevioside.

2.3.2 Supercritical fluid extraction of stevia sweetener from stevia plant

The experimental unit used was that described by Pasquel et al. (1999) for the pretreatment of stevia leaves.

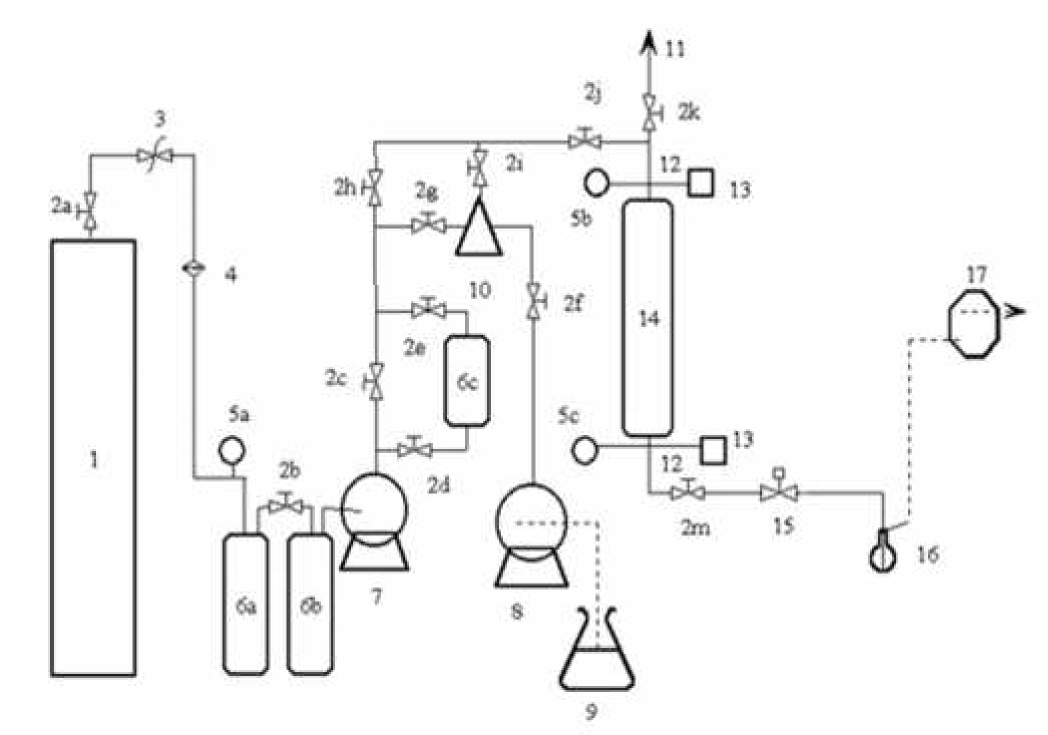


Figure 2.1: Diagram of the experimental unit (pasquel et at,1999). 1) solvent reservoir, 2) Valves, 3) Retention valve, 4) Filter, 5) Manometer, 6) Surge tank, 7) Solvent tank, 8) Cosolvent pump, 9) Cosolvent reservoir, 10) Mixer, 11) Relief pump, 12) Thermocouple, 13) Temperature indicator, 14) Fixed bed extractor, 15) Micrometring, 16) Collector flask, 17) Flow totalizer

Experimental Procedure: SCFE

The mass of solid used varied from 69.10^{-3} to 82.10^{-3} kg. The triturated solid was packed inside the extraction cell (SS 316, with a length of 0.375 m and an inside diameter of 0.0283 m). The extraction cell was adapted to the SCFE unit and the heating and/or cooling system was turned on. Once the system reached a temperature of 30^{o}C (approximately 3 hours), valves 2a, 2b, 2c, and 2h were opened. As soon as the system pressure reached 200 bar, valves 2j, 2m, and micrometering valve 15 were opened. The extracts were collected in 20mL glass flasks. An adsorption column containing Porapak Q (80 /100 mesh, Waters Associates Inc., USA) was adapted to prevent losses of volatile substances in the pretreatment step at the solvent outlet. The solvent flow rate was continuously monitored. Samples of the extract were collected every hour. Pretreatment was carried out at 200 bar, 30^{o}C, and an average solvent flow rate of $4.82.10^{-5}$ kg/s for a period of 12 hours. The extraction cell containing the pretreated stevia leaves was stored in a domestic refrigerator.

For extraction of the glycosides, the extraction cell was readapted in the SCFE unit. The experimental procedure was similar to the one described above. Samples of the extract were collected every 30 minutes and the total extraction time was 12 hours. The experimental runs were conducted at 120 and 200 bar at 16, 30, and 45°C. The cosolvents used were 9.5% (molar) water, ethanol, or an equimolar mixture of water and ethanol. Because the experiments were very long (12 hours for the pretreatment, 12 hours for the glycoside extraction plus setup time), the experimental plan was a fractional factorial design and only one-third of the total was selected.

Stevia leaves subjected to the SCFE pretreatment and stevia leaves with no pretreatment were used. The method described by Alvarez and Couto (1984) and Goto (1997) was used. One liter of boiling water was added to fifty grams of stevia leaves. The infusion was kept at room temperature (25 to 30°C) for one hour. The aqueous extract was vacuum filtered. In a separation funnel the aqueous extract was mixed with isobutyl alcohol (Merck P.A., 99.99%) maintaining the 40:60 (v/v) proportion. The system was allowed to rest until complete phase separation was achieved. The butanolic extract was centrifuged at 3500 rpm (Solvall, RT 600D) for 15 minutes. The extract was heated up to 80°C, and percolated through a bed of activated carbon (1 g of activated carbon for every 100 mL of extract). The extract was concentrated in a rota-evaporator (Tecnal, TE 120) and allowed to rest for 24 hours to achieve crystallization of the glycosides. The crystals were washed with methanol (Merck P.A. 99.9%) and dried in an air-circulating oven. The crystallization mother liquor was concentrated and extracted with acetone (Merck P.A., 99.8%). The crystals were washed with anhydrous acetone and dried in an air-circulating oven.

2.4 uses of stevia

The established uses for Stevia products cover all those of artificial low-calorie (non-sucrose) sweeteners, aspartame as well as most other purposes for which sugar can be used. The primary use is as a sweetener to enhance the palatability of foods and drinks.
Unlike aspartame, Stevia sweeteners are heat stable to 200°C, are acid stable and do not ferment, making them suitable for use in a wide range of products including baked/cooked foods. In some food uses its lack of bulk makes it unsuitable to replace all of the sugar in recipes, such as confectioneries, icings etc. In addition to sweetening foods stevia extracts can increase the palatability and attractiveness (enjoyment) of food through enhancement of flavours and odours. Stevia products also have beneficial uses as herbal and medicinal products and for some more unusual uses, e.g. in tobacco products. Stevia leaf extracts exhibit significant antiviral and antimicrobial activity. For example, fermented aqueous extracts of stevia leaves have exhibited strong antimicrobial, antibacterial and antifungal activity towards a wide range of pathogenic bacteria, including enter hemorrhagic Escherichia coli, without affecting normal intestinal flora. Hot water extracts of stevia leaf inhibit the replication of human rotavirus in vitro by blocking viral attachment to cells.

Stevia contains high levels of sweetening glycosides including stevioside which have been found to possess antimicrobial and antifungal properties. It is not only a ‘natural’ calorie free product but also has other advantages over the currently used chemical artificial sweeteners, such as saccharine, aspartame and cyclamate. In addition, the inclusion of stevia leaf extracts in the diet has been associated with antihyperglycemic, insulinotropic, glucagonostatic, hypotensive, anticariogenic, antiviral, antimicrobial, anti-inflammatory, immunostimulatory and chemopreventative responses (Campinas, SP, Brazil (1999).

Some Uses for Stevia Products and Extracts

- **Food and Culinary Uses**

Table top sweetener – for tea, coffee etc, Soft drinks, cordials, fruit juices, Ice-creams, yoghurts, sherbets, Cakes, biscuits, Pastries, pies, baking, Jams, sauces, pickles, Jellies, desserts, Chewing gum, Candies, confectioneries, Sea-foods, vegetables, Weight-watcher diets, Diabetic diets, Flavour, colour and odour enhancers, A source of antioxidants, Alcoholic beverage enhancer (aging agent and catalyst).

- **Medicinal Uses**

Toothpaste, mouthwashes – plaque retardant/caries preventor, Skin care – eczema and acne control, rapid healing agent, Diabetic foods and weight loss programs, Hypertension treatment and blood pressure control, Calcium antagonist, Bactericidal agent, Pill and capsule additive to improve, Tobacco additive and flavourant, Production of plant growth regulators (potential use).

2.5 Suitable environment for stevia plant

The natural habitat of Stevia is semi-humid subtropical with 1,500 – 1,800 mm of rain and temperature extremes of minus 6^{o} C to plus 43^{o} C. It naturally grows in low lying areas on poor sandy acidic soils adjacent to swamps, and so is adapted to and requires constantly wet feet or shallow water. Under cultivation and on more fertile soils, the mature plant can be larger, up to 1.8m with up to 20 branches per plant. Stevia has been successfully taken to a wide range of climatic locations around the world and apparently grown successfully, although often by using vegetative propagation methods and seedling establishment in a greenhouse before planting in the field (Cramer and Ikan, 1987).

Soils of the natural habitat are generally low fertility, acidic sands (pH 4 – 5) with shallow water tables and little organic matter, where plants grow to 0.6 – 0.7 meters in height. Under cultivation on better, more fertile soils, growth can increase to 1.0 meters and even to 1.8 meters. Soils should be well drained but with reasonable water holding capacity and preferably with pH 5 – 7; alkaline soils should be avoided. A variety of soil types, ranging from course textured sands to well drained loams are suitable for growing stevia. Sweetness is intensified by cooler temperatures and short days; however, sugar levels decline after flowering. The first harvesting can be done four to five months after planting. Subsequent harvesting can be done every three months, for three to five consecutive years.

In warmer climates where some plant growth is possible for most of the year (and plants do not die over winter) more than one harvest per year is normal. In Paraguay/Brazil three harvests a year are normal, often with a cleaning harvest after winter used for vegetative propagation

purposes. In mid-summer harvest intervals can be less than two months. In India four or five harvests a year are possible and in Indonesia up to seven harvests have been possible per year.
Stevia is much more adaptable than it’s herbaceous nature would suggest. We have found the following components have resulted in successful plantings in many locations.

Stevia is especially sensitive to water in the initial field planting times. This is compounded by rapid evaporation of water from the open field conditions –mulching is a major assistance. Organic material >5%, Soil Temperature less 35°C is suitable for the plant. Significant reduction in growing at temperatures higher than 35°C. Again mulching is a major assistance with reducing soil temperatures. Sunlight: varies with latitude and altitude but shade is often required for early root development in the nurseries. Minimize stress through proper soil preparation and proper plant preparation for going to the field and during initial establishment

The plant has been successfully grown under a wide range of conditions from its native sub-tropics to Thailand and Indonesia and the cold northern latitudes of Leningrad and north China and Canada. As an annual crop in Canada, it is suggested that 50 hectares of stevia could produce sweetener equivalent to $1 million of sugar which in Australia would require 240 hectares of cane to grow, i.e. productivity in terms of sweetness equivalent per hectare is high.

2.6 Stevia in Ethiopia

A research project entitled “Participatory Development of Quality Seedlings for Stevia (*Stevia rebaudiana* Bertoni L.) at Sembero Rogicha and Dawile Kebele, South Eastern Ethiopia” has been undertaken by W/Genet ARC with the support of farmers research group(FRG)II project.

The overall objective of the project was to generate quality seedling production technologies for stevia as lack of such technologies appears to have been a vivid gap in utilization and extended popularization of the aforementioned herbs.

FRG members at seedling nursery

Figure 2.2: FRG members at seedling nursery at Wondo Genet

It perpetuate asexually via stem cutting. Therefore, propagation studies aimed at identifying economical size of cutting and cutting positions required for best establishment were conducted in 2011. The activities were conducted with two FRGs at Sembero Rogicha and Dawile Kebele on farmer's nursery sites.

The result of stevia studies revealed that, top cuttings with three nodes demonstrated significantly higher values of survival rate (82.5 %), number of branches/seedling(7), number of leaves/ branch (15) and number of leaves/ seedlings (56) and lowest values of those parameters were recorded for bottom cuttings for stevia. The FRG member farmers have also found consistent result that stevia cutting taken from top position containing three nodes could be recommended for the development of quality stevia seedlings under good nursery management.

2.7 Constitutes of stevia sweetener

The leaves are found to contain a complex mixture of eight sweet diterpene glycosides, including stevioside, steviolbioside, rebaudiosides(A,B,C,D,E) and dulcoside A. Rebaudioside E is as sweet as stevioside, and rebaudioside D is as sweet as rebaudioside A, while the other glycosides are less sweet than stevioside. The components responsible for the sweet properties of the plant are glycosides of steviol, primary stevioside (ent-13-hydroxykaur-16-en-18-oic acid), which is 250 to 300 times sweeter than sucrose and rebaudiosides A and C. Rebaudioside A, or Reb A, is the second most abundant component of Stevia rebaudiana leaf. Steviol and isosteviol are metabolic components of stevioside. Non-sweet elements include the labdane diterpenes, triterpenes, sterols and flavonoid glycosides.

These extracts contain several sweet tasting diterpenoid glycosides of the aglycone, steviol, including stevioside (300-fold sweeter tasting than sucrose), rebaudioside A (reb A; 250- to 450-fold sweeter), reb B (300- to 350-fold sweeter), reb C (50- to 120-fold sweeter), reb D (250- to 450-fold sweeter), reb E (150- to 300-fold sweeter), steviobioside (100- to 125-fold sweeter), dulcoside A (50- to 120-fold sweeter), isosteviol and dihydroisosteviol. The relative sweetness of these diterpenoid glycosides appears to reflect differences in the carbohydrate residues at the 13 and 19 carbons of the common steviol aglycone backbone.

Powdered Stevia extracts with 40–50 percent Sweet glycosides (more than 100 times sweeter than sugar) and Powdered Stevia extracts with 85–97 percent Sweet glycosides (200-300 times sweeter than sugar).

Among the sweeteners, there are glycosides extracted from the dry leaves of a native plant called Stevia rebaudiana (Bert.) Bertoni (Asteraceae), or simply Stevia. Its leaf has several sweetener glycosides such as stevioside, rebaudioside A, B, C, D, E and dulcosides A and B. Those present in larger quantity are stevioside (5-10%), rebaudioside A (2-4%), rebaudioside C (1-2%); others are present in smaller concentrations.

The relative sweetness of the two main glycosides, Stevioside (St) and Rebaudioside A (R-A) are well established. Stevioside traditionally makes up the majority of the sweetener (60 – 70% of the total) and is assessed as being 110 – 270 times sweeter than sugar. It is also responsible for the after-taste sometimes reported (licorice taste). Rebaudioside A is usually present as 30 – 40% of total sweetener and has the sweetest taste, assessed as 180 – 400 times sweeter than sugar with no after-taste. The ratio R-A/St is the accepted measure of sweetness quality – the more R-A the better. If R-A is present in equal quantities to St (or more), it appears that the after-taste is eliminated. The minor glycosides are considered to be less sweet, 30 – 80 times sweeter than sugar.

There are suggestions that the stems and flowers could contain some of the compounds which may give Stevia some of its other properties, especially flavour enhancers (flavanoids), odour enhancers and organoleptic substances. Enzymatic modification (breakdown) of stevioside to produce a flavonoid – rubusoside – is possible. The structure, development and chemical content of Stevia roots have also received attention, often associated with culturing procedures.

Data taken from the Joint FAO/WHO Expert Committee on Food Additives (JECFA) revealed the following

Table 2.1: nutritional content of the glycosides (stevia sweetener)

Nutrition	Amount
calories	0.0 cal/100g
Calories from fat	0.0 cal/100g
Total fat	0.0 g/100g
Saturated fat	0.0 g/100g
Trans fat	0.0 g/100g
Cholesterol	0.0 g/100g
Sodium	1.0mg/100g
potassium	0.0 g/100g
Carbohydrate	1.0 g/100g
Dietary fiber	0.0 g/100g
sugars	< 1.0 g/100g
Protein	0.0 g/100g
Calcium	0.0 g/100g
Iron	0.0 g/100g

Table 2.2: Analytical standards of the stevia sweetener

Chemical data	Amount
Stevioside and rebaudioside (% wt/wt,dry basis)	>80.0
Other steviol glycoside (% wt/wt)	<15.0
Moisture (%)	<6.0
Ash (%)	<0.1
Specific rotation (degree)	-30.0 to -38.0
Lead (ppm)	<1.0
Arsenic (ppm)	<1.0

Table 2.3: microbiological data for the glycoside

Microbiological data	Amount
Standard plat count (CFU/g)	<1000
Total yeast and mold (CFU/g)	<100
Total coliforms (MPN/g)	Negative
E.coli	Negative
Salmonella	Negative

Source: www.pyurebrands.com

2.8 Chemistry of glycosides (stevia sweetener)

The two main glycosides are Stevioside (St), traditionally 5 – 10% of the dry weight of the leaves, and Rebaudioside A (R-A), being 2 – 4%; these are the sweetest compounds. There are also other related compounds including Rebaudioside C (1 – 2%) and Dulcoside A & C, as well as minor glycosides, including flavonoid glycosides, coumarins, cinnamic acids, phenylpropanoids and some essential oils.

Stevioside is a glycoside of the diterpene derivative steviol (ent-I 3-hydroxykaur-I 6-en-19-oic acid). Steviol glycosides are natural constituents of the plant Stevia rebaudiana Bertoni, belonging to the Compositae family.

The leaves of S. rebaudiana Bertoni contain eight different steviol glycosides, the major constituent being stevioside (triglucosylated steviol), constituting about 5-1 0% in dry leaves. Other main constituents are rebaudioside A (tetraglucosylated steviol), rebaudioside C, and dulcoside A. The predominant sweetener components of stevia extracts have been identified as stevioside and Reb A. The chemical identities and key chemical identifiers for the four major components are shown below

Common Name: Stevioside

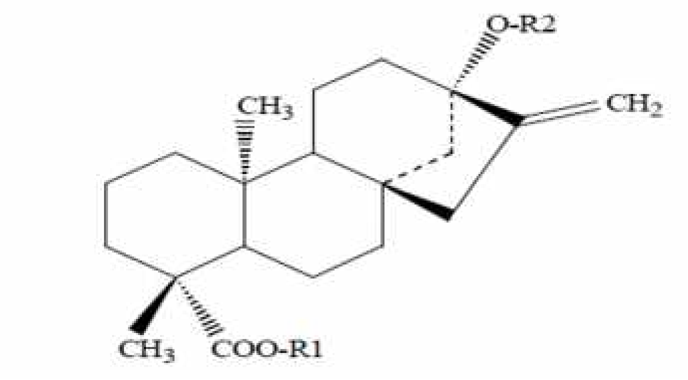
Chemical Name:13-[2-o-beta-D- glucopyranosyl-beta-D- glucopyranosyl)oxy]kaur-16-en-18-oic acid, f3-D-glucopyranosyl ester

Chemical formula: $C_{38}H_{60}0_{18}$

Formula Weight: 804.88

CAS Number: 5781 7-89-7

Chemical Structures:



Where: R_1 = β-glucose

R_2 = β-glucose- β-glucose (2 → 1)

Common Name: Rebaudioside A

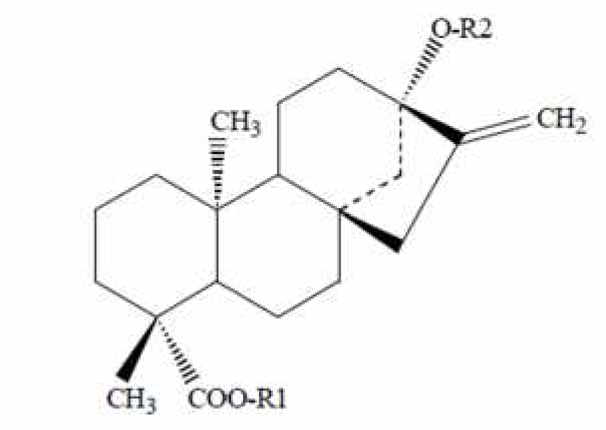
Chemical Name: 13-[2-o-beta-D- glucopyranosyl-3-O-beta-D- glucopyranosyl-beta-D-glucopyranosyl] kaur-6-en-8 oic cid,beta-D-glucopyranosyl ester

Chemical Formula: $C_{44}H_{70}0_{23}$

Formula Weight: 967.03

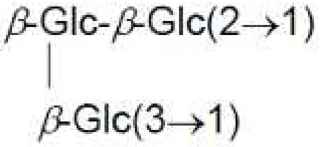
CAS Number: 58543-1 6-1

Chemical Structures:



Where: R1 = β-glucose

R2 =



$$\begin{array}{l}\beta\text{-Glc-}\beta\text{-Glc}(2\to1)\\ \;|\\ \beta\text{-Glc}(3\to1)\end{array}$$

Glc stands for glucose

Common Name: Rebaudioside c

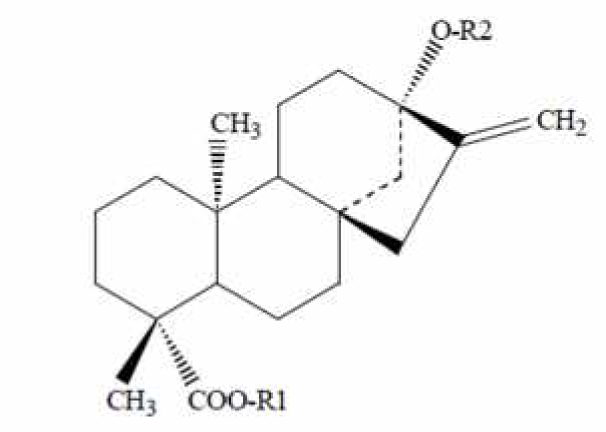
Chemical Name: 13-[2-O-α-L-rhamnopyranosyl-3-O-β-D-glucopyranosyl-β-D glucopyranosyl)oxy] kaur-6-en-18 oic cid, β-D-glucopyranosyl ester

Chemical Formula: $C_{44}H_{70}0_{22}$

Formula Weight: 951.03

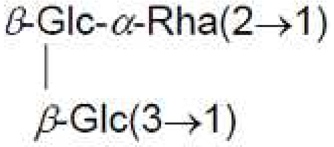
CAS Number: 63550-99-2

Chemical Structures:



Where: R_1 = β-Glc

R_2 =



$$\begin{array}{l}\beta\text{-Glc-}\alpha\text{-Rha}(2\rightarrow 1)\\ |\\ \beta\text{-Glc}(3\rightarrow 1)\end{array}$$

Glc and Rha stands for glucose and rhamnose sugar moieties respectively.

Common Name: Dulcoside A

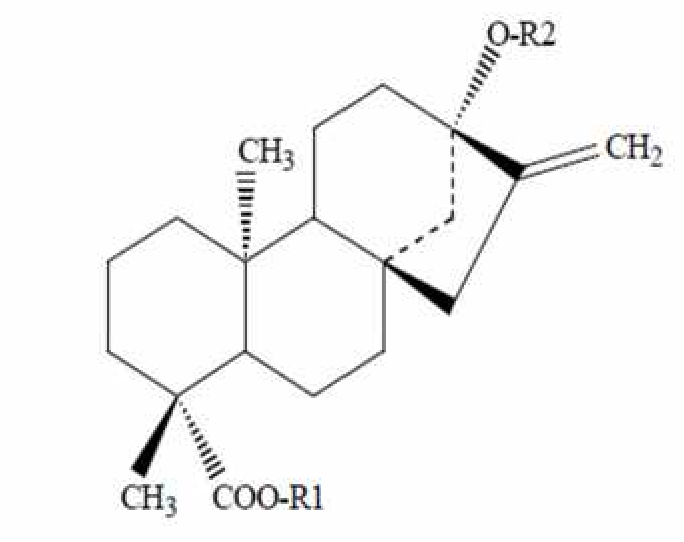
Chemical Name: 13-[2-O-α-L-rhamnopyranosyl-β-D-glucopyranosyl] kaur-16-en-18 oic acid, β-D-glucopyranosyl ester

Chemical Formula: $C_{38}H_{60}0_{17}$

Formula Weight: 788.88

CAS Number: 64432-06-0

Chemical Structures:



Where: $R_1 = \beta$-Glc

$R_2 =$ β-Glc-α-Rha ($2 \rightarrow 1$)

Glc and Rha stands for glucose and rhamnose sugar moieties respectively.

2.9 Stability and Shelf life of stevia sweetener

Stevioside is a stable molecule over the pH range 3 – 9, but rapidly decomposes at pH levels greater than 9 under otherwise comparable conditions. Unlike aspartame, Stevia sweeteners are heat stable to 200^{o}C, are acid stable and do not ferment, making them suitable for use in a wide range of products including baked/cooked foods. Glycosides are sweeteners of natural origin, stable to a wide range of pH and heat.

Chang and Cook (1983) tested the stability of pure stevioside and Reb A in carbonated phosphoric and citric acidified beverages and reported some degradation of both sweetening components after 2 months of storage at 37^{o}C; however, there was no significant change at room temperature or below following 5 months storage of stevioside and 3 months storage of Reb A.
They also reported that exposure to 1 week of sunlight did not affect stevioside, but it did result in approximately 20% loss of Reb A. Heating at 6O^{o}C for 6 days resulted in 0 - 6% loss of sweeteners.
Detailed stability testing was conducted by Merisant on Reb A as a powder, as a pure sweetener in solution, and on both cola-type and citrus carbonated beverages. No degradation was detected when the powder was stored at 105°C for 96 hours, and it was concluded that the powder was

stable when stored for 26 weeks at 40^{o}C with relative humidity of 75-25%. When considering Merisant's results of the stability investigations, there was considerable degradation noted after 13 hours at 100°C for carbonated beverage solutions and pure sweetener solutions (Merisant, 2008).The extent and rate of degradation is dependent on pH, temperature, and time.

Stevia in the raw has a shelf life of approximately four years when stored under cool and dry conditions. Exposure to high humidity and/or temperatures may result in caking, but the product is still safe to consume (www.steviaintheraw.com).

3. Materials and Methods

3.1. Raw Materials

The stevia leaves were taken from Debre Zeyit (Bishoftu) from Ato Girma Mogess farm. The leaves were dried with the sun light for twenty four hours.

Figure 3.1: The stevia leaf

The dried leaves were crushed into the powder form.

Figure 3.2: The powder form of stevia leaf

In this process hot water was applied. The reasons were that water was preferable than other types of solvent were,

1. The sweetener becomes more natural when we use water as a solvent

2. There is no further complicated process to separate the other chemical from the stevia sweetener. Though this is possible there is no totally separation of stevia sweetener from other chemical.

3.2 Equipment used for the extraction process

The main equipment that was used for the separation of the glycoside from the leaf process was incubator shaker. This equipment was used for not only the separation of the glycosides but also used to adjust different process parameters such temperature, residence time.

Figure 3.3: Incubator shaker

The other equipment that were used for the process were different types of flasks, different types of beakers, vacuum filter, filter paper, centrifuge, balance, thermometer and water bath for more concentrated of the sweetener.

The water bath was adjusted at 85°C and used to concentrate the mixture of stevia sweetener by evaporation of the water for two hours.

Figure 3.4: Water Bath

3.3 Response variable and factors selection of the process

The dependent (response) variable for this experiment was the yield of the stevia sweetener extracted from the leaf of stevia plant. The yield of the extraction process was depends on several conditions. Among these the extraction process parameters were the main one that was why this paper is mainly focuses on the extraction process factors or parameters. There were many factors which affects the extraction of the stevia sweetener from the leaf of the stevia plant. These are temperature, residence time, the ratio of stevia leaf powder with water, particle size, type of solvents and.

From literature review as a reference, among several factors some of them are investigated. These were temperature, residence time and ratio of the stevia leaf with solvent (distilled water). These three important parameters had different levels. Temperature was investigated in five levels 40°C, 45°C, 50°C, 55°C, 60°C. Residence time also considered in five levels that was one hour, two hours, three hours, four hours and five hours. The third independent variable which was ratio of stevia leaf powder with that of solvent (water) was investigated in two levels 1:5 and 1: 10 ratio.

If the number of independent variable was several for the given experiment then, the conclusion drawn from the analysis would be incorrect. As a result as much as possible large numbers of factors in the experiment are not advisable that was why in this experiment considered only the factors that would affect the product (stevia sweetener) highly. This does not mean that the rest of the independent variable does not affect the yield of product both quantitatively and qualitatively. It is obvious that there are so many predictors that will affect the response variable but taking the major one is the great task of the experimenter.

3.4 The design of the experiment

As it was stated above the variable of interest in this research was temperature, residence time and the ratio of stevia leaf with the solvent. The variables also considered in different levels and their interaction effects on the yield of the stevia sweetener. To investigate such like variables, factorial design is advisable and the most efficient. By the factorial design, in each complete trial or replication of the experiment all possible combination of the levels of the factors is investigated. If the levels of the factors are two we can use 2^k factorial design or if the levels of the factors are three we can use 3^k factorial design but in this experiment the levels of the factors extends to five as a result general factorial design was applied.

3.4.1 Model development of the data took from the laboratory experiment

The statistical model for the general factorial design of three factors with two factor interaction as follows

$$y_{ijkl} = \mu + \tau_i + \beta_j + \gamma_k + (\tau\beta)_{ij} + (\tau\gamma)_{ik} + (\beta\gamma)_{jk} + \varepsilon_{ijkl} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3.1$$

$i = 1, 2, \ldots, a$; $j = 1, 2, \ldots, b$; $k = 1, 2, \ldots, c$ and $l = 1, 2, \ldots, n$

where,

y_{ijkl} is the response variable in this case the yield of the stevia sweetener

μ is the grand mean

τ_i is treatment one effect on the response variable in this case temperature effect on the yield of the stevia sweetener.

β_j is treatment two effect on the response in this case residence time effect on the yield of the stvia sweetener.

γ_k is treatment three effect on the response in this case ratio of solvent: stevia leaf effect on the yield of the stevia sweetener.

$(\tau\beta)_{ij}$ is the interaction effect of temperature and residence time on the yield of the sweetener

$(\tau\gamma)_{ik}$ is the interaction effect of temperature and ratio of solvent: stevia leaf on the yield of the sweetener

$(\beta\gamma)_{jk}$ is the interaction effect of residence time and ratio of stevia leaf :solvent on the yield of the sweetener

ε_{ijkl} is the random error term

Since temprature had five levels in the experment; the residence time also had five levels and the ratio had two levels but there was no replication in the experment the value of a and b equals to five, the value of c equals to two and the value of n equales to one.Where a, b and c are the levels of the parametrs temprature,time and raio respectivelly.

The layout of this factorial experment data looke like as follows.The arrangment of the factors can be arrenged as the interest of the researcher.

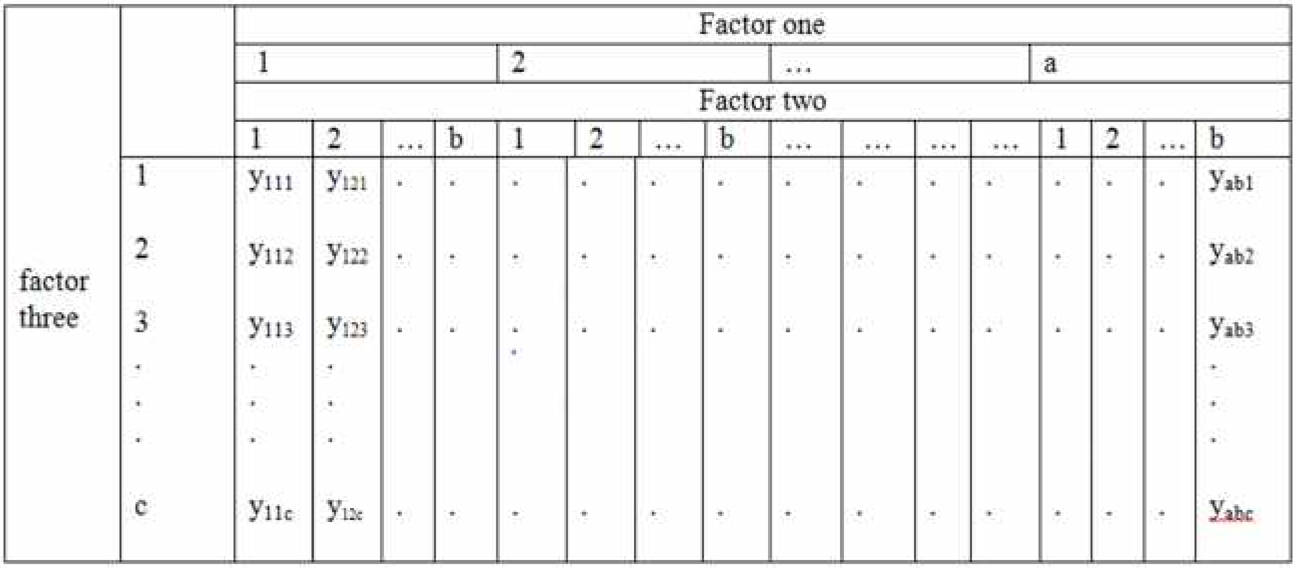


Table 3.1 the arrengment of the factors and data

		Factor one															
		1				2				…				a			
		Factor two															
		1	2	…	b	1	2	…	b	…	…	…	…	1	2	…	b
factor three	1	y_{111}	y_{121}	.	.	.	.	.	.	.	.	.	.	.	.	.	y_{ab1}
	2	y_{112}	y_{122}	.	.	.	.	.	.	.	.	.	.	.	.	.	y_{ab2}
	3	y_{113}	y_{123}	.	.	.	.	.	.	.	.	.	.	.	.	.	y_{ab3}
	.	.	.														.
	.	.	.														.
	.	.	.														.
	c	y_{11c}	y_{12c}	.	.	.	.	.	.	.	.	.	.	.	.	.	y_{abc}

3.4.2 The Analysis Of Variance (ANOVA)

The analysis of variance (ANOVA) table looks like as follows. Let us denote the factor temprature A, residence time B and ratio C.

Table 3.2 The analysis of variance table for the experment

Source of variation	Sum of square	Degree of freedom	Mean square	F_O
Temprature (A)	SS_A	a-1	$MS_A = \frac{SSA}{a-1}$	$F_A = \frac{MSA}{MSE}$
Residence time (B)	SS_B	b-1	MS_B	$F_B = \frac{MSB}{MSE}$
Ratio of stevia leaf: water(C)	SS_C	c-1	MS_C	$F_C = \frac{MSC}{MSE}$
AB	SS_{AB}	(a-1)(b-1)	MS_{AB}	$F_{AB} = \frac{MSAB}{MSE}$
AC	SS_{AC}	(a-1)(c-1)	MS_{AC}	$F_{AC} = \frac{MSAC}{MSE}$
BC	SS_{BC}	(b-1)(c-1)	MS_{BC}	$F_{BC} = \frac{MSBC}{MSE}$
Error	SS_E	abc(n-1)	MS_E	
Total	SS_T	abcn-1		

Usually, the analysis of variance computations would be done using a statistical software package. However, the manual computing formulas are occasionally useful.

The total sum of square is found as

Since there is no replication, $SS_T = \sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{k=1}^{c} yijk - \frac{y2...}{abc}$ ……………...…………3.2

where, y_{ijk} is the yield of stevia obtaind during the experment i^{th} level of factor A,the j^{th} level of factor B and the k^{th} level of factor C.

$$y^2... = \left(\sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{k=1}^{c} yijk\right)^2$$

The sums of squares for the main effects are found in the following formulas

$SS_A = \frac{1}{bc}\sum_{i=1}^{a} y2i.. - \frac{y2..}{abc}$ ……………………………………………………………..….3.3

where, $y_{i..} = \sum_{j=1}^{b}\sum_{k=1}^{c} yijk$

$SS_B = \frac{1}{ac}\sum_{j=1}^{b} y2.j. - \frac{y2...}{abc}$ ……………………………………………………...…. 3.4

where, $y_{.j.} = \sum_{i=1}^{a}\sum_{k=1}^{c} yijk$

$SS_C = \frac{1}{ab}\sum_{j=1}^{b} y2..k - \frac{y2...}{abc}$ ……………………………………………………………..3.5

where, $y_{..k} = \sum_{i=1}^{a}\sum_{j=1}^{b} yijk$

To compute the interaction sum of square,

$SS_{AB} = \frac{1}{c}\sum_{i=1}^{a}\sum_{j=1}^{c} yij. - \frac{y2...}{abc} - SS_A - SS_B$ ……………………………………………3.6

Where, $y_{ij.} = \sum_{k=1}^{c} yijk$

$SS_{AC} = \frac{1}{b}\sum_{i=1}^{a}\sum_{j=1}^{c} yi.k - \frac{y2...}{abc} - SS_A - SS_C$ ……………………………………………..3.7

Where, $y_{i.k} = \sum_{j=1}^{b} yijk$

$SS_{BC} = \frac{1}{a}\sum_{j=1}^{b}\sum_{k=1}^{c} y.jk - \frac{y2...}{abc} - SS_B - SS_C$ ……….………………………………..3.8

The error sum of square may be found by substracting the sum of squares for each main effect and interaction from the total sum of squares

$SS_E = SS_T - SS_A - SS_B - SS_C - SS_{AB} - SS_{AC} - SS_{BC}$ - ……...…………………………………..…3.9

3.4.3 Hypothesis Testing

The procedure of testing the hypothesis of the main effects and interaction effects on the yield of stevia sweetener as follows.

$H_o: \tau_1 = \tau_2 \ldots = \tau_a = 0$; (no main effect of facor one)

V_S

H_1: $\tau_i \neq 0$ for at least one i; (there is an effect on the yield)

Reject H_o, if F calculate (F_{cal}) greater than F tabullate (F α,a-1,N-1) else accept H_o

F_{cal} found from the software output or can be calculated by manually

$F_{\alpha,a-1,N-1}$ found from the F table

Reject H_o means the factor has significant effect on the yield else the factor has no effect on the yield.

Proceed in this way to the rest main and interaction effects.

Like this experment when interactions are present factorial design is necessarly to avoid misleading conclution. Finally, factorial designs allow the effects of a factors to be estimated at several levels of the other factors, yeilding conclusions that are valid over a rang of experimental conditions.

3.5 The extraction process of the glycosides from the leaf of stevia plant

The powder form of the stevia leaf was mixed with distilled hot water in 250 ml of flasks at different process parameter levels. The temperature of water was at 40^oC, 45^oC, 50^oC, 55^oC and 60^oC. The second variable, residence time in five levels at one hour, two hours, three hours, four hours and five hours. The last parameter which was the ratio of powder stevia with water was feed at two level, 1:5 and 1:10 ratio.

After the process parameter satisfied for each run the extract and residue mixture would take from the incubator shaker and cooled for thirty minutes. Then the mixture was vacuum filtered using filter paper to separate the sweetener from the residue. Using centrifuge, further clarification of the stevia sweetener was performed. The pure extract which has dark brown color bottled and placed at room temperature.

3.6 Characterization of the product

3.6.1 Determination of specific gravity

It is the measure of the density of the substance relative to the other substance. In this case the reference substance was water and the process of determining the value was as follows.

$$\text{Specific gravity of the liquid stevia } (SG_{st}) = \frac{\text{density of liquid stevia}}{\text{density of water}} \quad \ldots\ldots\ldots 3.10$$

3.6.2 Determination of moisture content of the product

The proximate analysis were done using the method of Association of Official Analytical Chemists (AOAC, 2000). Moisture content of the powder extract was determined by oven drying method after placing the sample in the oven and drying it to constant weight (AOAC, 2000 official method 925.10). Then, the moisture content was estimated by weight difference using the following formula.

$$\%\ \text{Moisture} = \frac{w2 * 100}{w1} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3.11$$

Where: W1= weight of the powder feed into the oven

W2= loss of weight

3.6.3 Determination of total ash content of the product

The ash content was determined using the following procedure. Dish containing sample were placed in the hot plat and char to remove the smoke for a hour. Then the charred material placed in to the furnace at the temperature 550^{o}C for 5 hours so that the organic matter will be burnet and removed.

The percentage of total ash (dry basis) was calculated using the following formula:

$$\text{Total Ash } (\%) = \left[\frac{W2}{W1}\right] * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3.12$$

Where:

W1 = weight (g) of the dried sample material

W2 = weight (g) of the ash

3.6.4 Determination of crude fat content of the product

The fat content was determined by the soxlet method. Samples were added in to extraction thimbles. There were two steps soaking and extracting. In the soaking step the fat was solubilized using diethyl ether for two hours in the soxlet extraction chamber then the samples fat extracted in the soxhlet extraction chamber for two hours. The content in the extraction flasks was removed from the extraction chamber and placed in the drying oven and dried for 15 minute. Finally, the fat content was calculated using the following formula.

Crude Fat content (%) = $\frac{Wf}{Ws}*100$ ……………………………………..…………...…3.13

Where: w_f = weight of extracted fat

w_s = weight of powder stevia sweetener

3.6.5 Determination of crude Protein content of the product

Protein content was determined by Kjeldahl method according to (AOAC, 2000) using the official method (920.87). All nitrogen was converted to ammonia by digestion with a mixture of concentrated sulfuric acid. The ammonia released after alkalinized with sodium hydroxide is steam distilled into boric acid and titrated with sulfuric acid. The crude protein content was estimated using the following formula.

Total Nitrogen (%) = N $*\frac{V1-V2}{W}$ * 14.07 *100……………………………………….……..3.14

Where: V_1 = volume in ml of standard sulfuric acid solution used for the test material titration

V_2 = volume in ml of standard sulfuric acid solution used in the titration for the blank determination

N = normality of standard sulfuric acid (0.1)

W = weight (g) of the sample material

The universal conversion factor was used to convert percent N to percent crude protein. Most proteins contain 16%N, so the conversion factors is 6.25 (100/16 = 6.25).

protein (%) = 6.25* percentage of nitrogen

3.6.6 Determination of crude fiber content of the product

There are two types procedure to determine the fiber contents of the product. If the fat content is greater than 10 %, then it should be defatted using hexane by cooled extraction for 10 minute. If the fat content less than 10 % this procedure is not necessarily. Using sulfuric acid the component of the product except fiber will digest at 37°C in the fibber Tec (Foss) for 37 minute. Then it will wash three times using distilled water. And again the same procedure will apply using sodium hydroxide. When it removes from the fibber Tec the remaining component is ash and fiber. After that it dry in the oven at 130°C for two hours and measure the weight (w_1) then it place to furnace and stay for 3 hours at 550°C and measure the weight (w_2).

The percentage of total fibber was calculated using the following formula:

$$\text{Percentage of total fibber} = \frac{w1-w2}{w} * 100 \quad \ldots\ldots 3.15$$

Where: w_1= the average weight of oven dry sample

w_2 = the average weight of sample after furnace

w = the average weight of sample of stevia sweetener powder

3.6.7 Determination of carbohydrates content of the product

The total carbohydrate (%) of the sample by mass obtained as follow.

$$\text{Total carbohydrate (\%)} = 100 - [M + A + F + P + Fi] \quad \ldots\ldots 3.16$$

Where: P = the mass % of protein F = the mass % of fat

A = the mass % of ash M = the mass % of moisture

Fi = the mass % of fibber

4. Results and Discussion

In this section output of the results obtained from solvent extraction of stevia sweetener from stevia rebaudnia bertoni leaf using hot water were discussed. The effects of main and interaction factors and the optimum levels of the parameters were also investigated. The model of the output data was also developed and the adequacy of it was investigated.

4.1. Results obtained from solvent extraction of stevia sweetener

The extraction parameters were very important to produce both a good quality and a reasonable amount of stevia sweetener. As a result to achieve these objectives different parameters with different levels were used during the extraction process. The parameters were temperature, residence time and ratio of stevia leaf powder with solvent (water). By using literature reviews as spring point and taking into consideration the levels of temperature were 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C, 60^{o}C. The second important factor and that was necessarily investigated was residence time and its levels were one hour, two hours, three hours, four hours and five hours. The ratio of stevia leaf with that of solvent was the other vital factor that was investigated during the laboratory experiment. This variable considered in two levels, these were 1:5 and 1:10 ratio.

Since all interaction of the level of factors should be considered in factorial design, there were fifty runs could be performed during the laboratory experiment that could be found by multiplying the levels of the factors.

The laboratory experimental outputs in different variable with different levels were recorded in the following table. The yield was obtained by dividing the liquid stevia extract by the crude mixture, the mixture of stevia leaf with solvent (water).

Table 4.1: Process conditions and yield (%) of the stevia sweetener during the laboratory experiment

		Factor three (Ratio of stevia poweder with solvent unit less)									
		1:5					1:10				
		Factor two (Residence time in hours)									
		1	2	3	4	5	1	2	3	4	5
Factor one (temprature in ^{o}C)	40	46.91	47.42	47.81	47.82	47.8	47.27	48.00	48.88	48.85	48.85
	45	46.99	47.60	47.69	47.69	47.64	48.01	48.09	49.01	49.01	49.00
	50	47.59	47.74	47.85	47.80	47.80	48.11	48.16	49.90	49.88	49.88
	55	47.57	47.62	47.82	47.77	47.72	48.10	48.21	49.85	48.29	48.27
	60	47.55	47.57	47.61	47.60	47.60	47.72	47.88	49.82	48.25	48.25

From simple observation from the tabulated data we can observe that, in the first factor (temperature) high yield was observed around the level of fifty degree Celsius, in the second factor (residence time) high yield was observed around the level of three hours and in the third factor (ratio of stevia powder with solvent) high yield was observed at the level of 1:10 ratio.

As we have observed from the raw data of the table 4.1 the highest extraction yield was 49.9 % and obtained at the temperature level of $50^{o}C$, the residence time at the level of 3 hours and at 1:10 material ratio. Whereas the lowest extraction yield was 46.91% obtained at the temperature level of $40^{o}C$, the residence time at the level of one hour and at 1:5 material ratio.

4.2 Characterization of the product

4.2.1 The specific gravity value of the product

It is the measure of the density of the substance relative to the other substance. In this case the reference substance was water and the process of determining the value was as follows.

- Thirty milliliter of liquid stevia sweetener was measured and became 29.8 gram
- The same volume of water was measured and it became 30 gram
- Specific gravity of the substance equals to density of the substance divided by the density of the reference substance

  Hence,

  Using equation 3.10

  Specific gravity of the liquid stevia $(SG_{st}) = \dfrac{\text{density of liquid stevia}}{\text{density of water}}$

  Density of liquid stevia $= \dfrac{mass\ of\ liquid\ stevia}{volume\ of\ liquid\ stevia} = \dfrac{29.8\ g}{30\ ml} = 0.9967$ g/ml

  Density of water $= \dfrac{mass\ of\ water}{volume\ of\ water} = \dfrac{30\ g}{30ml} = 1$ g/ml, and it well known

$$SG_{st} = \frac{\text{density of liquid stevia}}{\text{density of water}} = \frac{0.9967\ g/ml}{1\ g/ml} = 0.9967$$

SG_{st} = 0.9967, it is unit less quantity

4.2.2 The moisture value of the product

- The mass of the powder feed to the oven was 2 gram (W_1)
- The mass of oven dried powder was 1.88 gram

Here, mass loss during drying (W_2) = 2 gram – 1.88 gram = 0.12 gram

Using equation 3.11 the percent of moisture became

% Moisture = $\frac{0.12}{2} * 100$ = 6 %

The moisture content of the powder extract was 6 percent.

4.2.3 The total ash value of the product

- 1 gram of powder of stevia sweetener (w_1) was measured in each two crucible apparatus and burnet
- The ash measured and became 0.8659 gram and 0.8657 gram for run 1 and run 2 respectively the average 0.0.8658 gram ash was taken (w_2)

$$\text{Total Ash (\%)} = [\frac{W2}{W1}] * 100$$

$$\text{Total Ash (\%)} = [\frac{0.8658\ gram}{1\ gram}] * 100 \quad = 86.58\ \%$$

Therefore the total ash percentage of the stevia sweetener powder in mass becomes 86.58 percent.

4.2.4 The crude fat value of the product

- 1 gram of powder stevia sweetener measured and added in three extraction flasks
- The extracted fat measured for the three runs became 0.011gram, 0.018 gram, and 0.0166 gram and the average became 0.0152 gram

$$\text{Crude Fat content (\%)} = \frac{0.0152\ gram}{1\ gram} * 100 = 1.52\ \%$$

The crude fat content of the product is 1.52 percent.

4.2.5 The crude protein value of the product

- 0.5 gram of stevia powder was measured for each tube and placed in the two tube and 6 ml of sulfuric acid added to each and digested or to solubilized the protein for 12 hours in the hod at 370^{o}C temperature
- 3 gram of the mixture of copper sulfate, potassium sulfate and hydrogen peroxide added and heated at 370^{o}C for 4 hours in the hod to facilitate the reaction
- Finally titration would perform and volume of standard sulfuric acid solution used for the test material titration were 1.3 ml and 1.5 ml and took the average 1.4 ml (v_1) and the average volume of the black solution was 0.2 ml (v_2).

Using equation 3.14

Total Nitrogen (%) = N *$\frac{V1-V2}{W}$ * 14.07 *100

Total Nitrogen (%) = (0.1)($\frac{mol}{1000\ ml}$)* ($\frac{1.4\ ml-0.2\ ml}{0.5\ g}$) * 14 g/mol *100 = 0.336 %

Protein (%) = 6.25* percentage of nitrogen

protein (%) = 6.25* 0.336 = 2.1 %

4.2.6 The crude fibber value of the product

- 1 gram of stevia sweetener powder (w) was measured and added to two crucibles and placed in the fibber Tec. After acid and basis digestion, distilled water wash and oven dried measure the weight of the sample and became 0.2600 gram and 0.2400 gram for sample one and two respectively and took the average 0.25 gram (w_1) then after 3 hours in the furnace at 550^{o}C measure sample one and two and became 0.2424 gram and 0.2212 gram and took the average 0.2318 gram (w_2) then using equation 3.15 the percentage of the total fibber became

Percentage of total fibber = $\frac{w1-w2}{w}$*100

Percentage of total fibber = $\frac{0.2500\ g-0.2318\ g}{1\ g}$ *100 = 1.82 %

The total percentage of fibber of the product became 1.82 percent.

4.2.7 The carbohydrate value of the product

The total carbohydrate percentage became

100 – (6 + 86.58 + 1.52 + 2.10 + 1.82 +) = 1.98

The total carbohydrate content in mass of the product is 1.98 percent.

4.3 Effects of the factors on the yield of the stevia sweetener

The effects of the factors on the yield of the stevia sweetener were investigated and the optimal levels of the factors were determined here.

4.2.1 Effect of temperature on the yield of stevia sweetener

Figure 4.1 showed the yield of stevia sweetener increased gradually with rise in temperature that ranges from 40ºC to 45ºC and drastically with rise in temperature 45^{o}C to 50^{o}C but, when the temperature became increased above 50^{o}C, the yield showed decreased. It may be due to the greater speed of the molecule movements in higher temperature so that sweetener diffused more quickly from the solution to the feed carrier.

Temperature effects on extraction are dual. Higher temperature can accelerate the solvent flow and thus increase the stevia sweetener. Higher temperature can also decrease the fluid density that may reduce the extraction efficiency (Guo-Qing *et al*., 2005). High temperature also affects the purity of the stevia sweetener recovered in the extract because at higher temperature more unwanted compounds of the leaf were extracted. Hence, it was found that 50ºC was the optimum temperature for extracting the stevia sweetener from the leaf of stevia.

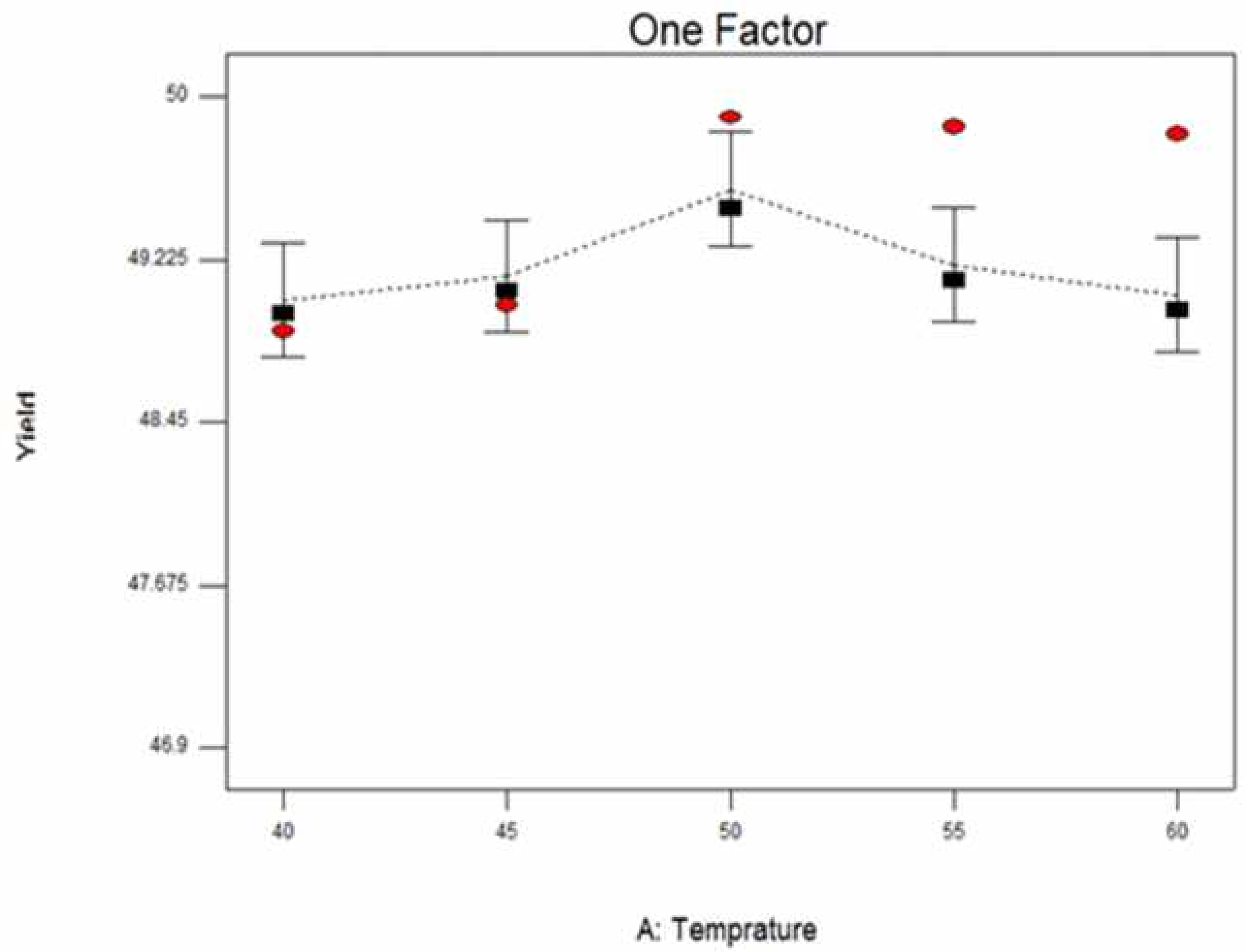


Figure 4.1: The effects of temperature on the yield of stevia sweetener at residence time 3 hours and material ratio 1:10.

4.2.2 Effect of residence time on the yield of stevia sweetener

The other important factor that highly influences the yield of the stevia sweetener was residence time (extraction time). The influence of residence time on the yield of stevia sweetener extraction is shown in the figure below.

The yield of stevia sweetener increased gradually with rise in residence time that ranges from one hour to two hours and rapidly with rise in residence time two hours to three hours but there was no any increment on the yield exceeding the extraction time more than 3 hours. Hence, it was found that three hour was the optimum extraction time for extracting the stevia sweetener from the leaf of stevia.

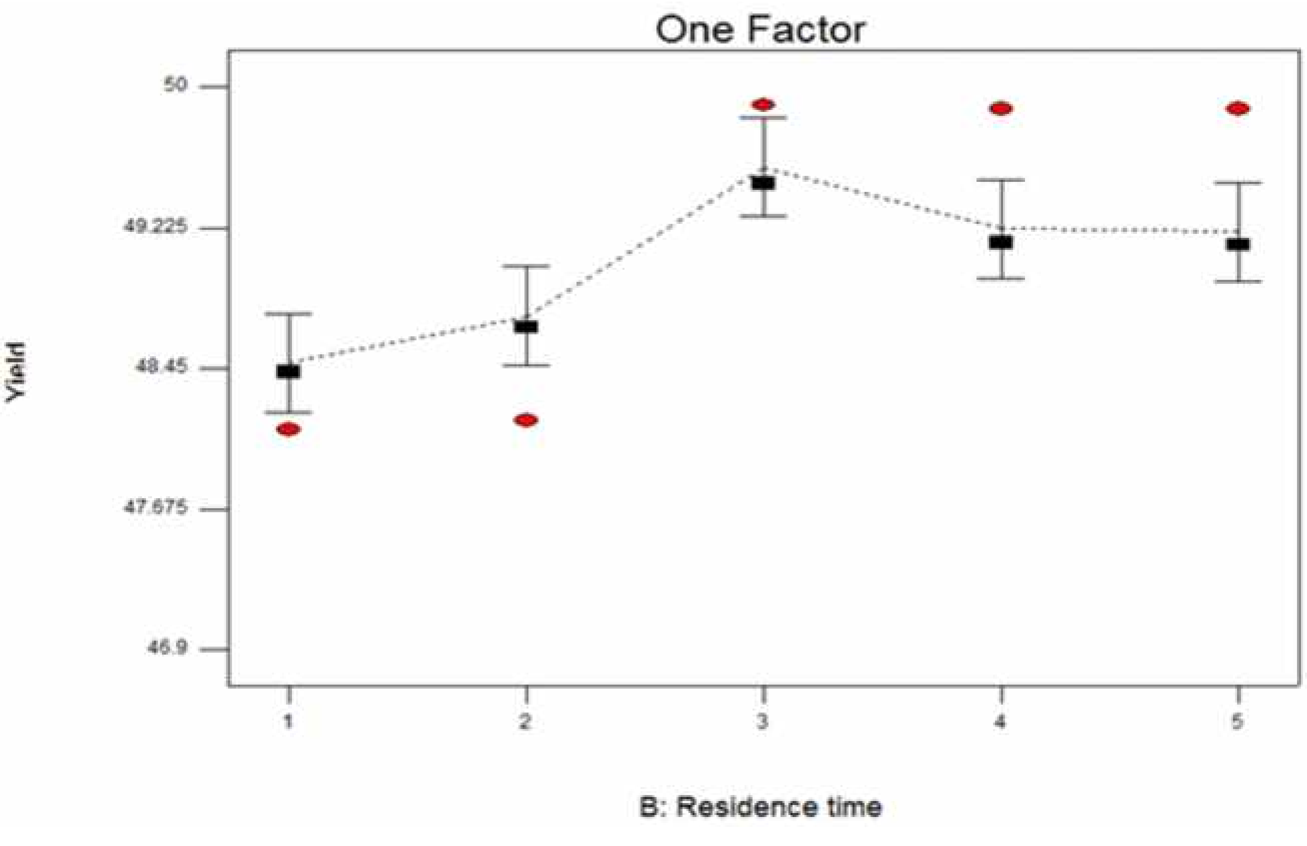


Figure 4.2: The effects of residence time on the yield of stevia sweetener at temperature 50°C and material ratio 1:10.

4.2.3 Effect of the ratio of stevia leaf with solvent (water) on the yield of stevia sweetener

The third important factor that was highly affects the yield of the stevia sweetener was the ratio of stevia leaf with that of solvent (water). Figure 4.3 also shows the effect of the ratio on the stevia sweetener. When we increase the material ratio from 1:5 to 1:10 the yield of the product increase from 47.85 percent to 49.9 percent keeping temperature and residence time constant. As a result the optimal level of the ratio was 1:10 than 1:5.

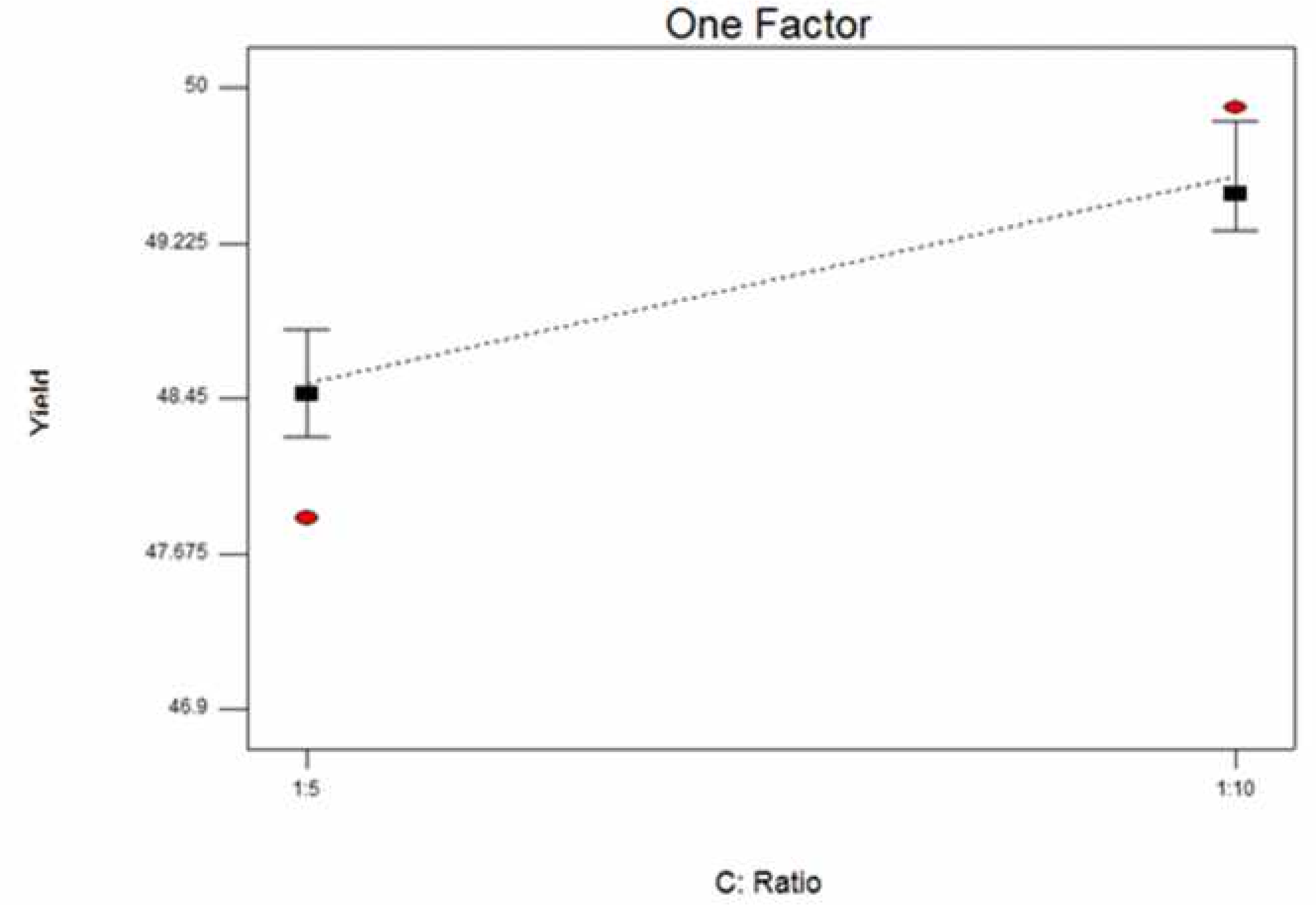


Figure 4.3: The effects of ratio of material on the yield of stevia sweetener at temperature 50°C and residence time 3 hours

4.2.4 The interaction effect of temperature with residence time on the yield of stevia sweetener.

As we have seen from figure 4.4 there were twenty five possible interactions of the two factors at a constant level of factor C (ratio of material) but any one of treatment of factor B (residence time) couldn't gave a maximum yield throughout the level of factor A (temperature). Consequently, the interaction effects of factor A (temperature) with factor B (residence time) on the yield of stevia sweetener was insignificant.

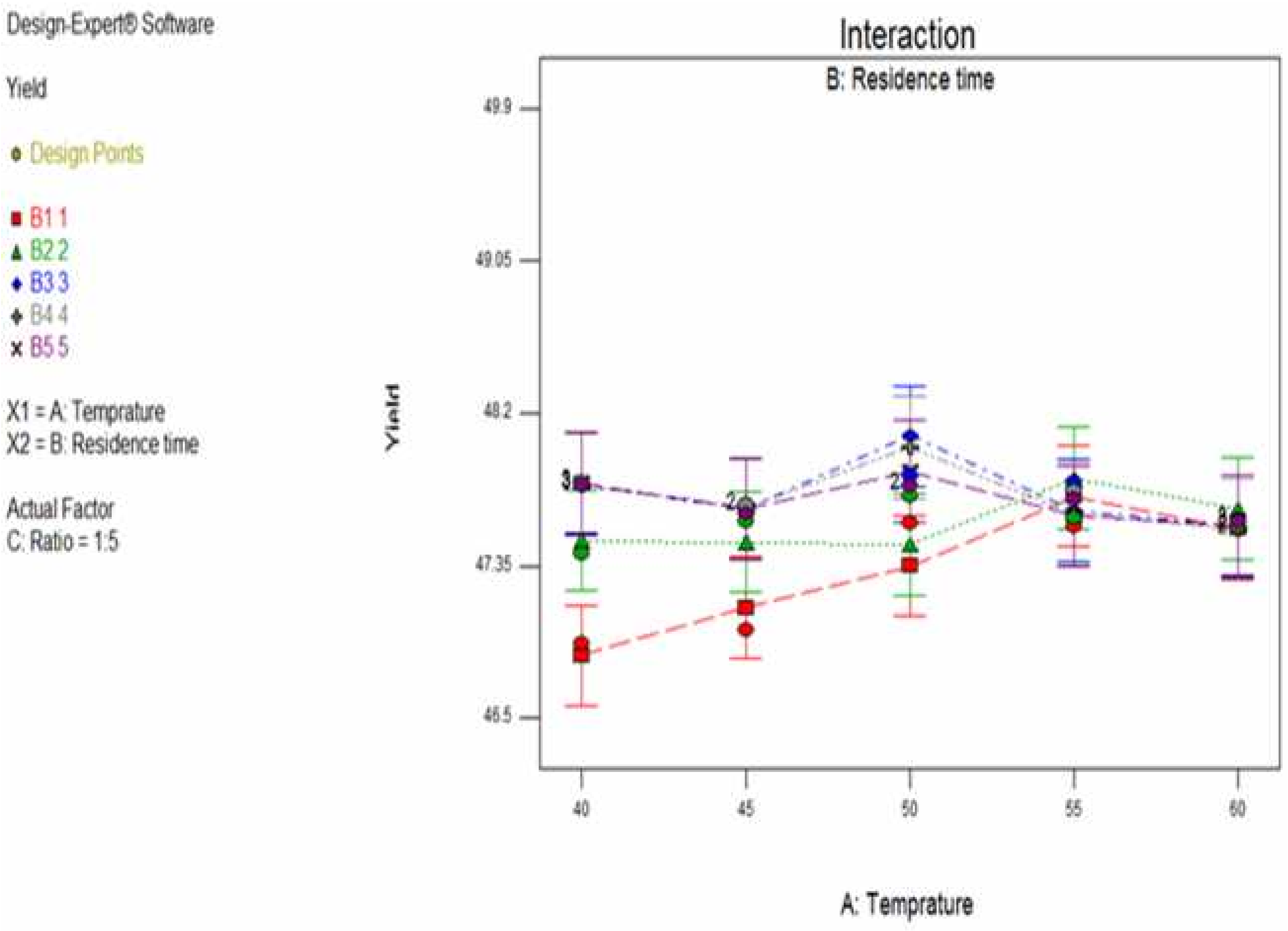


Figure 4.4: The interaction effects of temperature with time on the yield of stevia sweetener

4.2.5 The interaction effect of temperature with ratio of material on the yield of stevia sweetener.

Figure 4.5 showed that the interaction effect of temperature with material ratio on the yield of stevia sweetener. Here, there would be ten possible interactions in a constant level of residence time. From the figure we observed that all treatments of factor A (temperature) could give a maximum yield throughout a single level of factor C (material ratio) than the other level of factor C. As a result there was an interaction effect of factor A (temperature) and factor C (material ratio) on the yield of stevia sweetener. The highest yield also obtained at the interaction of treatment 1:10 of ratio and 50^{o}C of temperature at constant residence time, three hours.

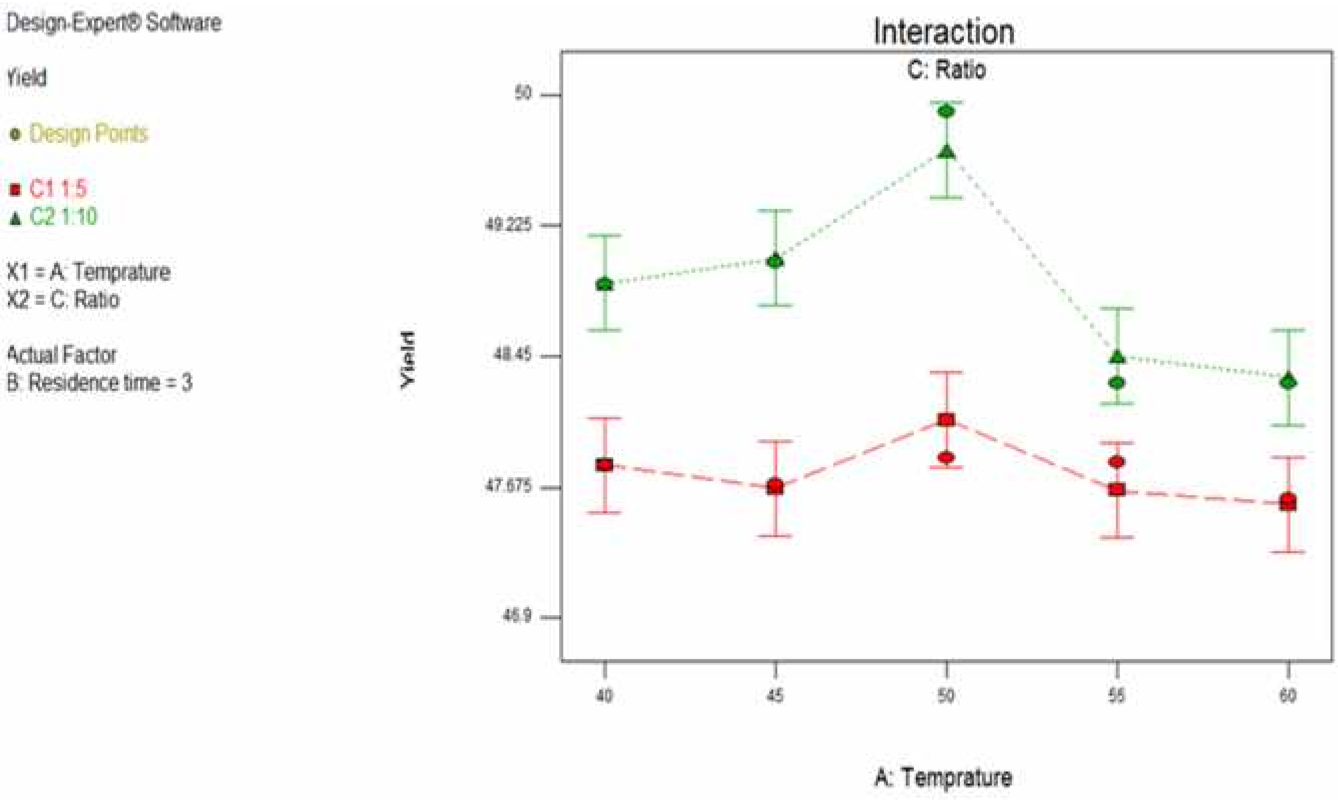


Figure 4.5: The interaction effects of temperature with ratio on the yield of stevia sweetener

4.2.6 The interaction effect of residence time with ratio of material on the yield of stevia sweetener.

The interaction effects of residence time with ratio of material on the yield of stevia sweetener showed in the following figure. The figure showed there was significant interaction effect on the yield because when we observed the figure at 1:10 level of factor C (material ratio) there was maximum yield than the other level of factor C throughout all treatment of factor B (residence time) and the highest yield was obtained at the interaction of three hours of factor B (residence time) and 1:10 material ratio.

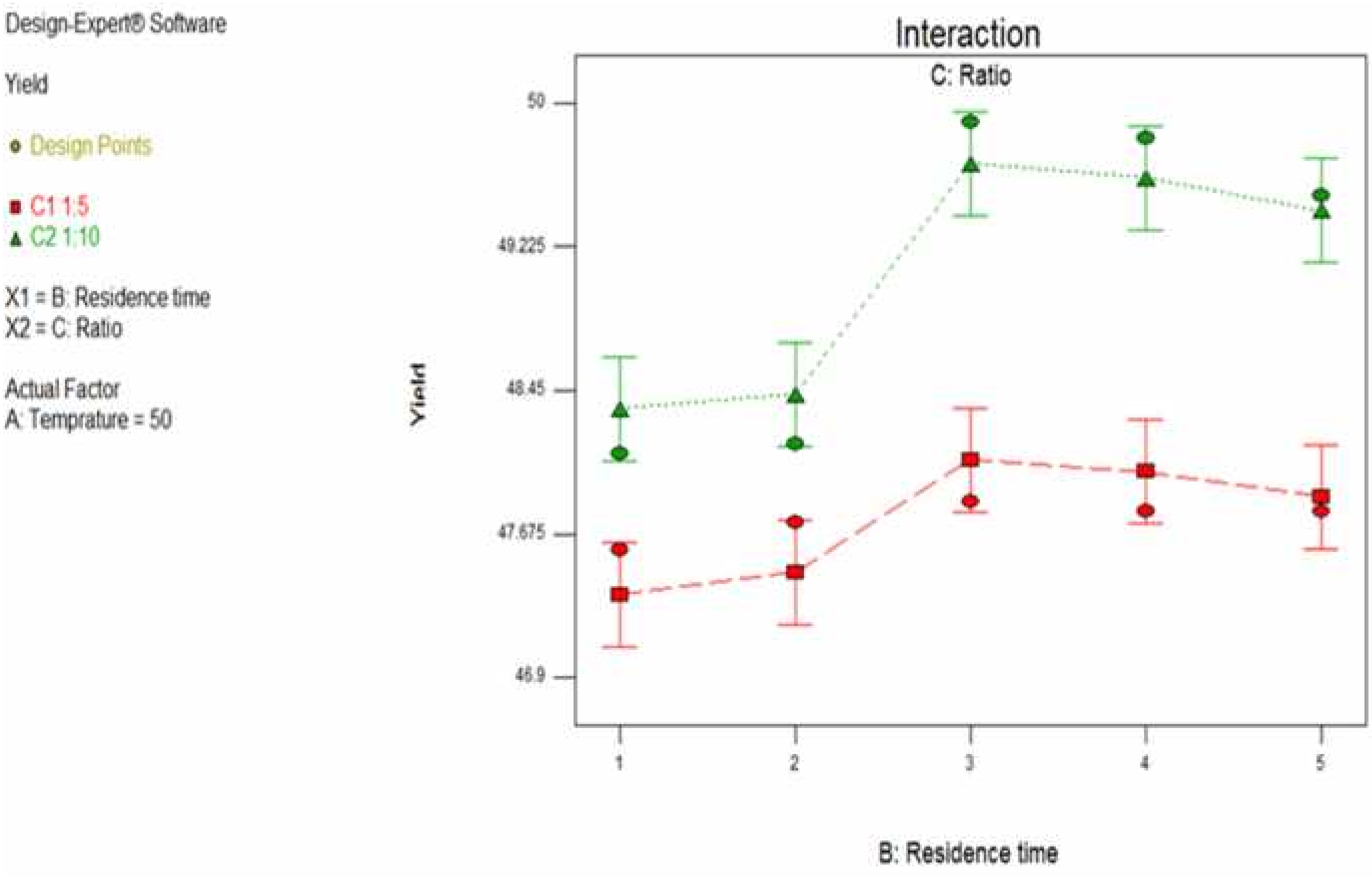


Figure 4.6: The interaction effects of residence time with ratio on the yield of stevia sweetener

4.3 Analysis of Variance for the data (ANOVA)

The study was interested in testing the hypotheses of no main effect for factor *A*, no main effect for *B*, no main effect for factor C, no *AB* interaction effect, no AC interaction effect and no BC interaction effect on the yield of stevia sweetener. The analysis of variance **(ANOVA)** would be used to test these hypotheses. The analysis of variance (ANOVA) can be computed manually using the formula discussed in the previous chapter but different software also can be computing. For this cause design expert software were used and the output of the data was discussed. The following table shows the analysis of variance for the data. Using α = 0.05

Table 4.2: Analysis of variance

Source of variation	Sun of square	df	Mean square	F- value	P- value
model	19.44	33	0.59	11.38	< 0.0001
A-Temperature	2.04	4	0.51	9.87	0.0003
B-Residence time	4.32	4	1.08	20.89	< 0.0001
C- Ratio	9.18	1	9.18	177.33	< 0.0001
AB	1.48	16	0.093	1.79	0.1270
AC	1.33	4	0.33	6.45	0.0027
BC	1.07	4	0.27	5.19	0.0071
Residual (error)	0.83	16	0.052		
Total	20.26	49			

4.4 Hypothesis testing of the main and interaction effects

4.4.1 Hypothesis testing of the temperature effects on the yield of stevia sweetener

H_o: $\tau_1 = \tau_2 = \tau_3 = \tau_4 = \tau_5 = 0$ Vs (no temperature effect on the yield)

H_1: $\tau_i \neq 0$; for at least one i (the was temperature effect on the yield)

F_{cal} from the ANOVA table became 9.87

$F_{\alpha,a-1,N-1}$ ($F_{0.05,4,49}$) from F distribution table became 2.57

Decision: since F_{cal} (9.87) greater than $F_{0.05,4,49}$ (2.57) we reject Ho implies accept H_1

Conclusion: The effect of temperature on the yield of the stevia sweetener was significant. The p-value from the ANOVA also enforces this conclusion because the p-value of the temperature from the ANOVA became 0.0003 which was less than the type one error (0.05). In this case we can also reject the null hypothesis (H_o).

4.4.2 Hypothesis testing of the residence time effects on the yield of stevia sweetener

H_o: $\beta_1 = \beta_2 = \beta_3 = \beta_4 = \beta_5 = 0$ Vs

H_1: $\beta_j \neq 0$; for at least one j

F_{cal} from the ANOVA table became 20.89

$F_{\alpha,b-1,N-1}$ ($F_{0.05,4,49}$) from F distribution table became 2.57

Decision: since F_{cal} (20.89) greater than $F_{0.05,4,49}$ (2.57) we reject Ho implies accept H_1

Conclusion: The effect of residence time on the yield of the stevia sweetener was significant. The p-value from the ANOVA also enforces this conclusion because the p-value of the residence time from the ANOVA became less than 0.0001 which was less than the type one error (0.05). In this case we can also reject the null hypothesis (H_o).

4.4.3 Hypothesis testing of the material ratio effects on the yield of stevia sweetener

H_o: $\gamma_1 = \gamma_2 = 0$ Vs

H_1: $\gamma_k \neq 0$; for at least one k

F_{cal} from the ANOVA table became 177.33

$F_{\alpha,c-1,N-1}$ ($F_{0.05,1,49}$) from F-distribution table became 4.04

Decision: since F_{cal} (177.33) greater than $F_{0.05,1,49}$ (4.04) we reject Ho implies accept H_1

Conclusion: The effect of material ratio on the yield of the stevia sweetener was significant. The p-value from the ANOVA also enforces this conclusion because the p-value of the material ratio from the ANOVA became 0.0001 which was less than the type one error (0.05). In this case we can also reject the null hypothesis (H_o).

4.4.4 Hypothesis testing of the interaction effects of temperature with residence time on the yield of stevia sweetener

H_o: $(\tau\beta)_{11} = (\tau\beta)_{12} = (\tau\beta)_{13} = (\tau\beta)_{14} = (\tau\beta)_{15} = (\tau\beta)_{21} = \ldots = (\tau\beta)_{55} = 0$
Vs

H_1: $(\tau\beta)_{ij} \neq 0$; for at least one interaction of i and j

F_{cal} from the ANOVA table became 1.79

$F_{\alpha,(a-1)(b-1),N-1}$ ($F_{0.05,16,49}$) from F distribution table became 1.90

Decision: since F_{cal} (1.79) less than $F_{0.05,16,49}$ (1.90) we accept Ho implies reject H_1

Conclusion: The interaction effect of temperature with residence time on the yield of the stevia sweetener was insignificant. The p-value from the ANOVA also enforces this conclusion because the p-value of the interaction effect of temperature with residence time from the ANOVA became 0.1270 which was greater than the type one error (0.05). In this case we can also accept the null hypothesis (H_o). Here this interaction term should reduce from the model.

4.4.5 Hypothesis testing of the interaction effects of temperature with material ratio on the yield of stevia sweetener

H_o: $(\tau\gamma)_{11} = (\tau\gamma)_{12} = (\tau\gamma)_{21} = (\tau\gamma)_{22} = (\tau\gamma)_{31} = (\tau\gamma)_{32} = \ldots = (\tau\gamma)_{52} = 0$ Vs

H_1: $(\tau\gamma)_{ik} \neq 0$; for at least one interaction of i and k

F_{cal} from the ANOVA table became 6.45

$F_{\alpha,(a-1)(c-1),N-1}$ ($F_{0.05,4,49}$) from F distribution table became 2.57

Decision: since F_{cal} (6.45) greater than $F_{0.05,4,49}$ (2.57) we reject Ho implies accept H_1

Conclusion: The interaction effect of temperature with material ratio on the yield of the stevia sweetener was significant. The p-value from the ANOVA also enforces this conclusion because the p-value of the temperature from the ANOVA became 0.0027 which was less than the type one error (0.05). In this case we can also reject the null hypothesis (H_o).

4.4.6 Hypothesis testing of the interaction effect residence time with material ratio on the yield of stevia sweetener

H_o: $(\beta\gamma)_{11} = (\beta\gamma)_{12} = (\beta\gamma)_{21} = (\beta\gamma)_{22} = (\beta\gamma)_{31} = (\beta\gamma)_{32} \ldots = (\beta\gamma)_{52}$ Vs

H_1: $(\beta\gamma)_{jk} \neq 0$; for at least one interaction of j and k

F_{cal} from the ANOVA table became 5.19

$F_{\alpha,(b-1)(c-1),N-1}$ ($F_{0.05,4,49}$) from F distribution table became 2.57

Decision: since F_{cal} (5.19) greater than $F_{0.05,4,49}$ (2.57) we reject Ho implies accept H_1

Conclusion: The interaction effect of residence time with that of material ratio on the yield of the stevia sweetener was significant. The p-value from the ANOVA also enforces this conclusion because the p-value of the interaction effect of residence time with that of material ratio from the ANOVA became 0.0071 which was less than the type one error (0.05). In this case we can also reject the null hypothesis (H_o) and accept the alternative hypothesis (H_1)

4.5 Two factor interaction statistical model of the data

From equation 3.1 we the following

$$y_{ijkl} = \mu + \tau_i + \beta_j + \gamma_k + (\tau\beta)_{ij} + (\tau\gamma)_{ik} + (\beta\gamma)_{jk} + \varepsilon_{ijkl}$$

Since there was no replication during laboratory experiment, the subscript l did not exist. From the above analysis i.e hypothesis testing and analysis of variance, the $(\tau\beta)_{ij}$ term would not significant as a result this term should reduce and the model became as follows.

$$y_{ijk} = \mu + \tau_i + \beta_j + \gamma_k + (\tau\gamma)_{ik} + (\beta\gamma)_{jk} + \varepsilon_{ijk}$$

But the error term (ε_{ijk}) could not estimate both manually and using different software so the final reduced model should be

$y_{ijk} = \mu + \tau_i + \beta_j + \gamma_k + (\tau\gamma)_{ik} + (\beta\gamma)_{jk}$ estimated as follows

Yield (y_{ijk}) = $48.05 - 0.091A_1 + 0.021A_2 + 0.37A_3 - 0.086A_4 - 0.47B_1 - 0.22B_2 + 0.26B_3 + 0.24B_4 + 0.43C - 0.4A_1B_1 - 0.1A_2B_1 - 0.11A_3B_1 + 0.34A_4B_1 -0.02A_1B_2 - 0.003A_2B_2 - 0.25A_3B_2 - 0.17A_4B_2 + 0.12A_1B_3 +0.014A_2B_3 + 0.19A_3B_3 -00.17A_4B_3 + 0.14A_1B_4 + 0.014A_2B_4 + 0.14A_3B_4 - 0.17A_4B_4 - 0.019A_1C + 0.12A_2C + 0.24A_3C - 0.16A_4C - 0.17B_1C - 0.19B_2C + 0.13B_3C + 0.12B_4C$

Where A stands for τ, B for β and C for γ

Coefficient of determination (R^2)

The coefficient of determination (R^2) became 95.91%. This implies that the 95.91 % of the total variability of the response variable (yield) was explained by the independent variable temperature, extraction time, and material ratio.

The predicted R-squared became 60.10% and the adjusted R-squared 87.49%. Since the predicted R- squared was far from the adjusted R-squared, different measurement should be taking such as the removal of outliers, transformation of the response data and reduction of the model. But there were no outlier in the data so the removal of the outlier was not the solution. When we considered the ratio of the response data, it was 1.064 which was less than 10 transformations of the response data was not recommended. As a result model reduction was the solution to overcome the problem.

From the analysis of variance table interaction of temperature with residence time term was unnecessary in the model therefore the model reduced to

Yield (y_{ijk}) = $48.05 - 0.091A_1 + 0.021A_2 + 0.37A_3 - 0.086A_4 - 0.47B_1 - 0.22B_2 + 0.26B_3 + 0.24B_4 + 0.\ 43C - 0.019A_1C + 0.12A_2C + 0.24A_3C - 0.16A_4C - 0.17B_1C - 0.19B_2C + 0.13B_3C + 0.12B_4C$

4.6 Model Adequacy Check

Before the model implemented for different applications it should satisfy different criteria such as the normal distribution of the error term (residuals) and so on. Unless the model should not satisfy these criteria it is not advisable to use the model for different purpose rather other methods are applied to adjust the model in desirable way.

4.6.1 Normal probability plot of the residuals

The normal probability plot of the residuals showed that, it follows almost a straight line which implies residuals are approximately normally distributed that satisfy the most important assumption in any model.

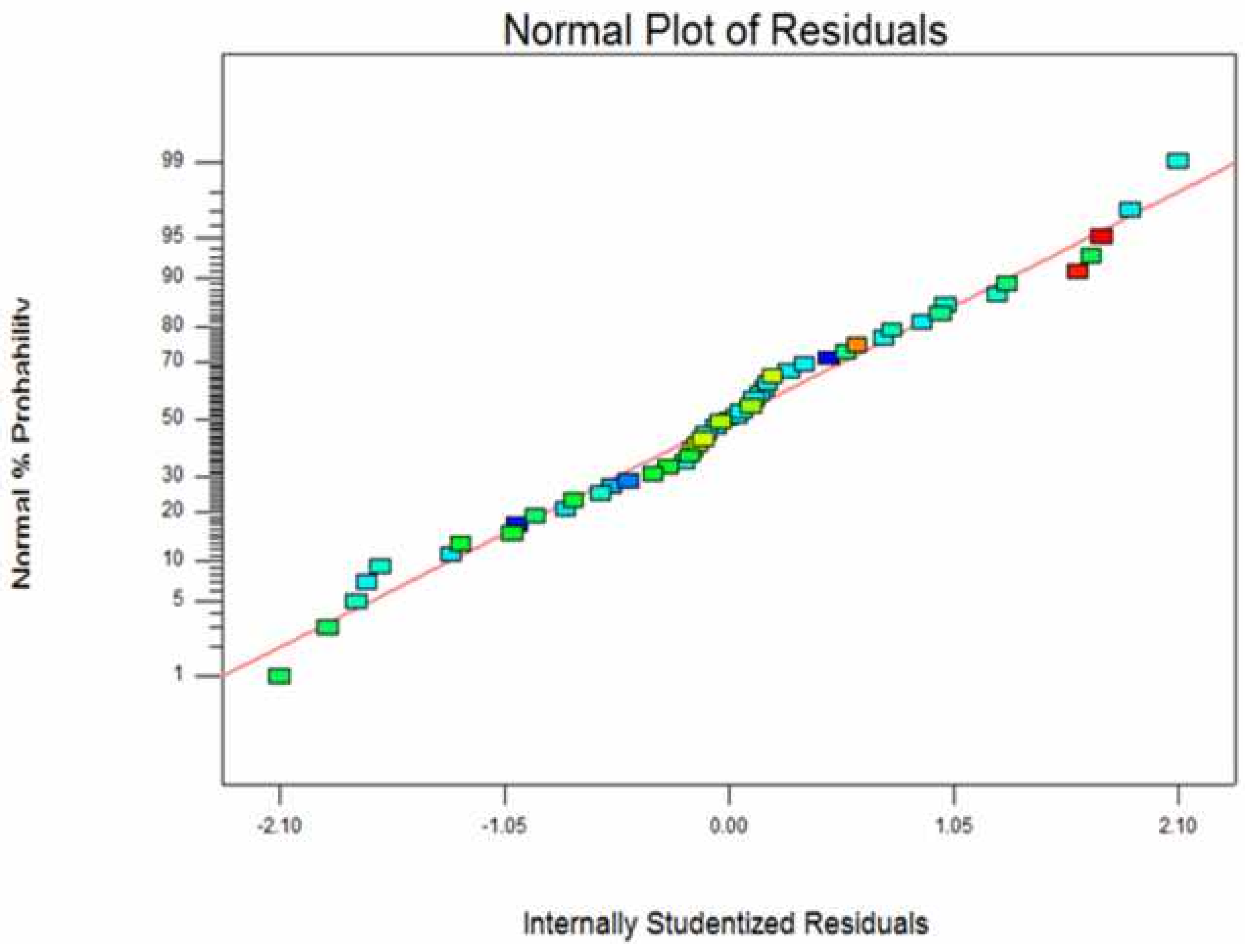


Figure 4.7: Normal probability plot of residuals

4.6.2 Plot of Residuals verses predicted values

Figure 4.8 showed that the plot of residuals verses predicted value did not follow any pattern, it is random it implies that the model is adequate.

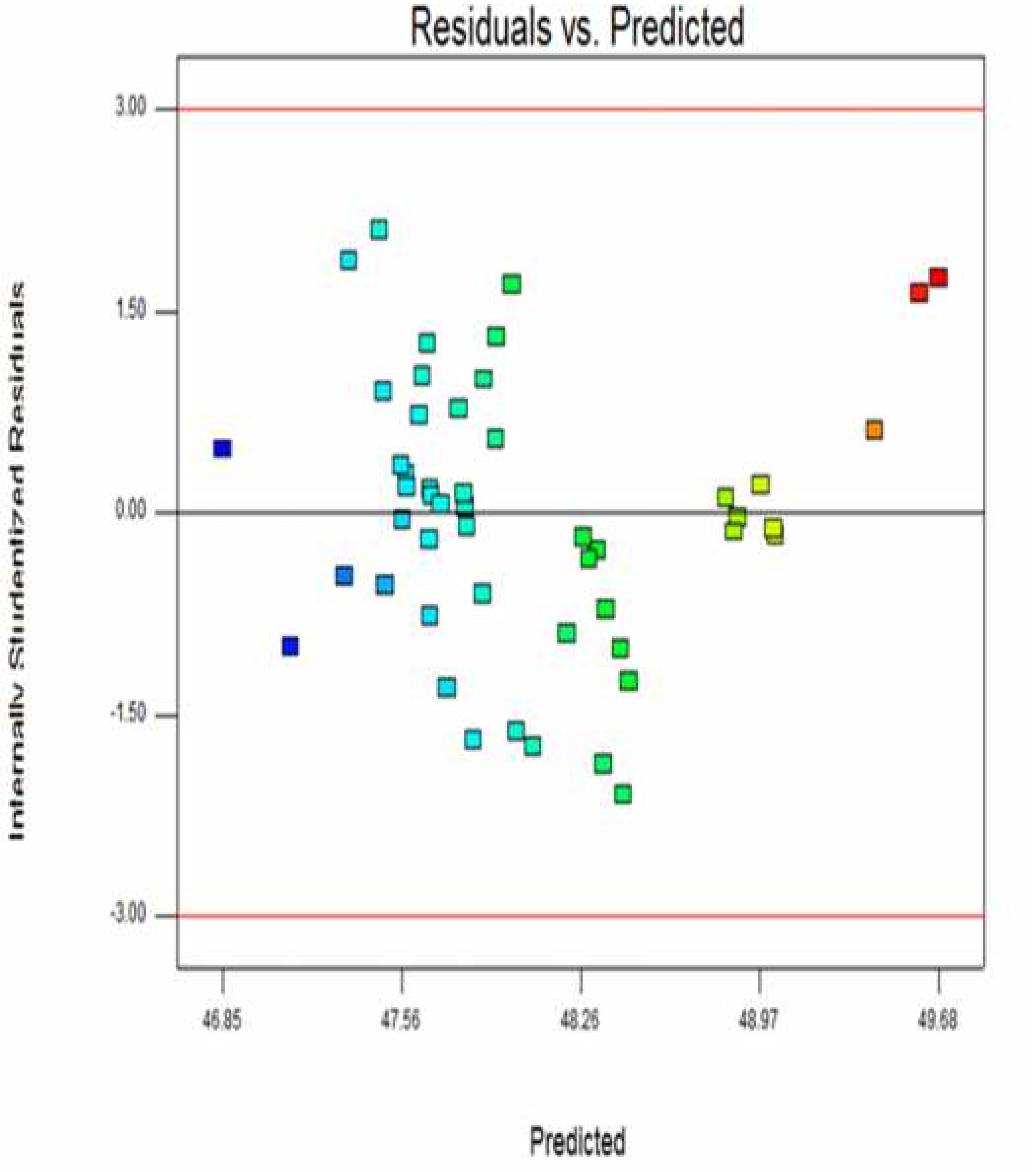


Figure 4.8: Plot of residuals Vs predicted values

4.7 Optimization of the yield of stevia sweetener

The objective here was to obtain maximum yield in the given interval of the investigated independent variables. Using design expert software the maximum yield of stevia sweetener was achieve at the combination of the third level of the first factor (50^oC), the third level of the second factor (3 hours) and the last level of the last factor (1:10) and the desirability equal to 0.925. The second highest yield obtained at the combination of the third level of the first factor (50^oC), the fourth level of the second factor (4 hours) and at the last level of the third factor (1:10) and the desirability equal to 0.900.

The minimum yield of the stevia sweetener obtained at the combination of the first level of the first factor (40^oC), the first level of the second factor (1 hour) and the first level of the last factor (1:5) and the desirability was equal to 0.069. The other analysis such as main and interaction effect analysis also enforce this conclusion.

The following data took from design expert output. Among many possible combinations, ten of the highest yield offering combination would take.

Table 4.3: Ten highest yields offering possible combination of the treatments in report form

Number	Temperature	Residence time	Ratio	Yield	Desirability
1	50	3	1:10	49.6756	0.925
2	50	4	1:10	49.5996	0.900
3	50	5	1:10	49.4226	0.840
4	45	3	1:10	49.0316	0.710
5	45	4	1:10	49.0256	0.708
6	45	5	1:10	48.9736	0.690
7	40	3	1:10	48.8846	0.660
8	40	4	1:10	48.8686	0.655
9	40	5	1:10	48.8366	0.644
10	40	5	1:10	48.8366	0.644

The desirability less than 70 % combination of factors were not advisable. As a result in the above table the first five possible combinations of the factors gave maximum yield.

The highest yield offering possible combination of the treatments in ramps form for the top two possible combination were the following and it also strength the above expression (report form). The following figure showed the first high yield offering combination of the treatments.

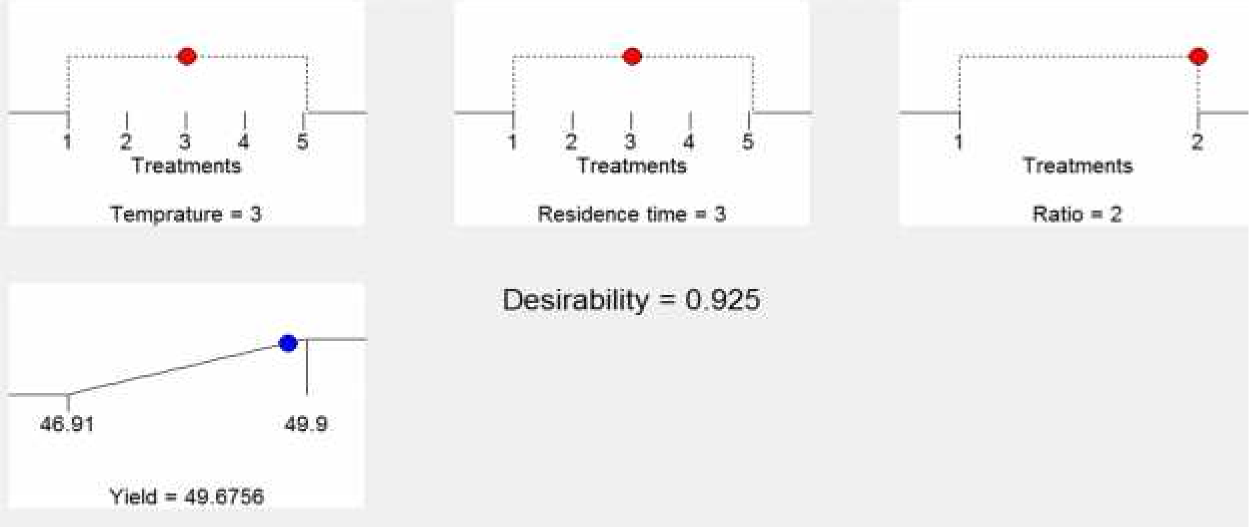


Figure 4.9: The highest yield offering combination of the treatments

The second highest yield obtained at the combination of treatments 50°C for the temperature, four hours for the residence time and 1:10 material ratio like in the report form discussion.

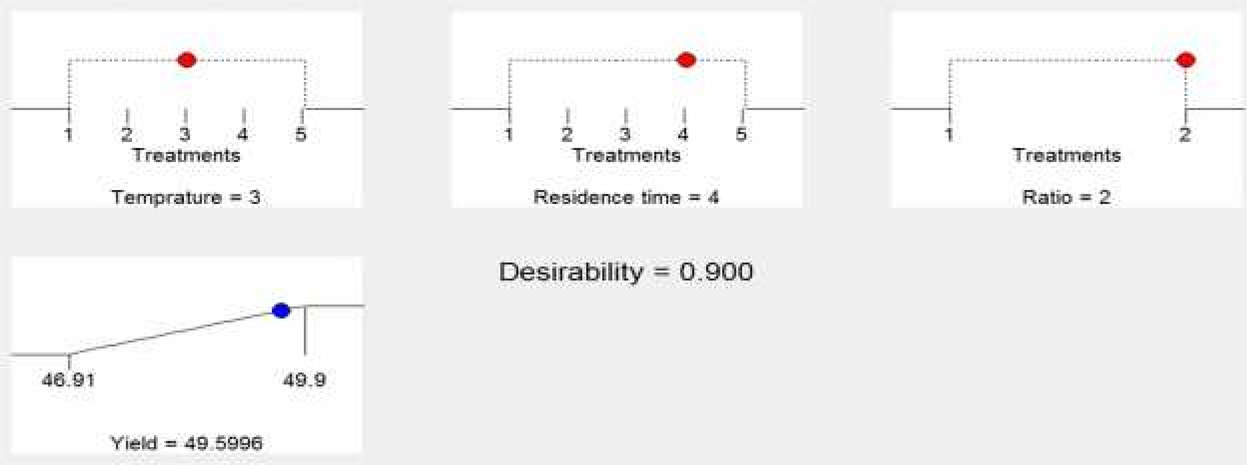


Figure 4.10: The second highest possible combination of treatments

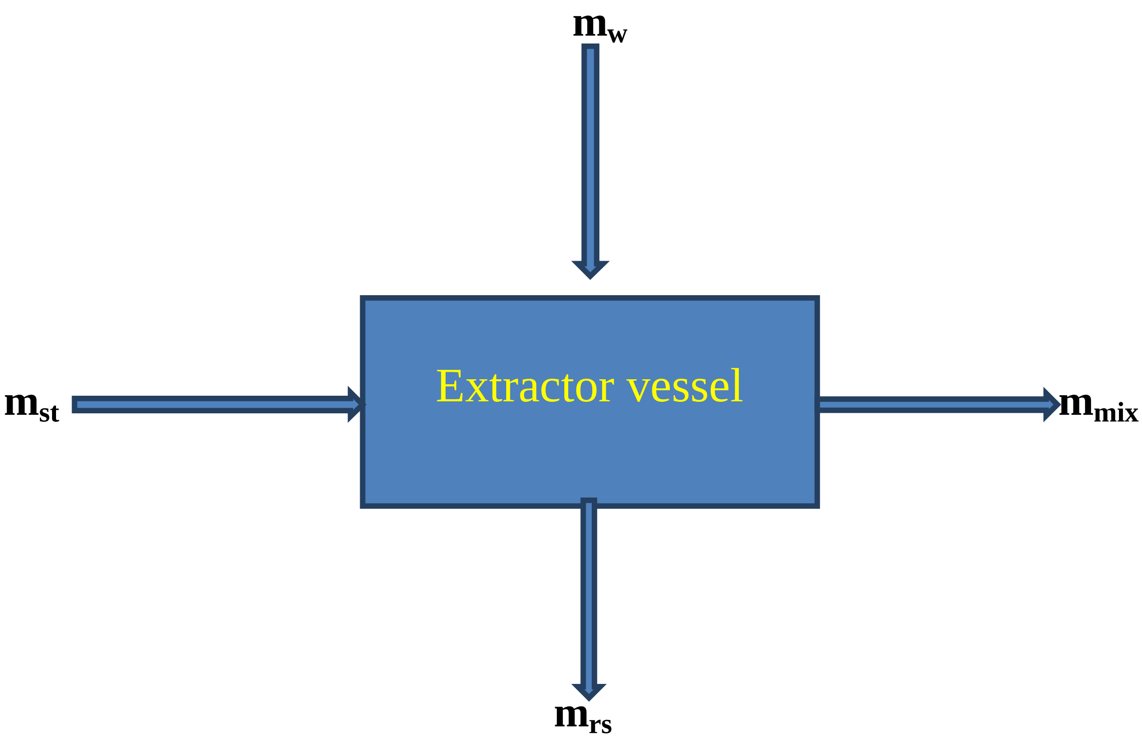
5. Material and Energy balance

In this section material and energy balance for the extraction process of the sweetener were investigated. Since there was no any chemical reaction occurred the material balance should be balance without chemical reaction.

5.1 Material balance on the extractor vessel

The extractor vessel was considered as the system for this experiment and the inside of the incubator shaker was the surrounding for the system.

5.1.1 Material balance on the first run when the ratio was 1:5, temperature 40°C and residence time at two hours.



The rule of mass conservation stated that

Input = output……………………………………………..…….5.1

Since there was no chemical reaction, i.e. no generation as well as consumption of matter,

$$m_{st} + m_w = m_{rs} + m_{mix} \quad \text{……….…………………………….5.2}$$

Where,

m_{st1} is the mass of the stevia powder used for a run 1 during the extraction process

m_{w1} is the mass of water (solvent) used for run1 during the extraction process

m_{rs1} is the mass of residue produced in run1 during the extraction process

m_{mix1} is the mass the mixture of the stevia sweetener and water produced in run 1 during the extraction process

- 5 grams of stevia leaf powder was also used for this run (m_{st1})
- 25 grams of distilled water was used (m_{w1})
- The mass of the residue that was produced in this run was 15.82 grams (m_{rs1})

The mass of the mixture of stevia sweetener that was produced in the above process condition calculated as follows.

From equation (5.2), i.e. mass conservation rule we have

$$m_{st1} + m_{w1} = m_{rs1} + m_{mix1}$$

by rearranging the above equation we get

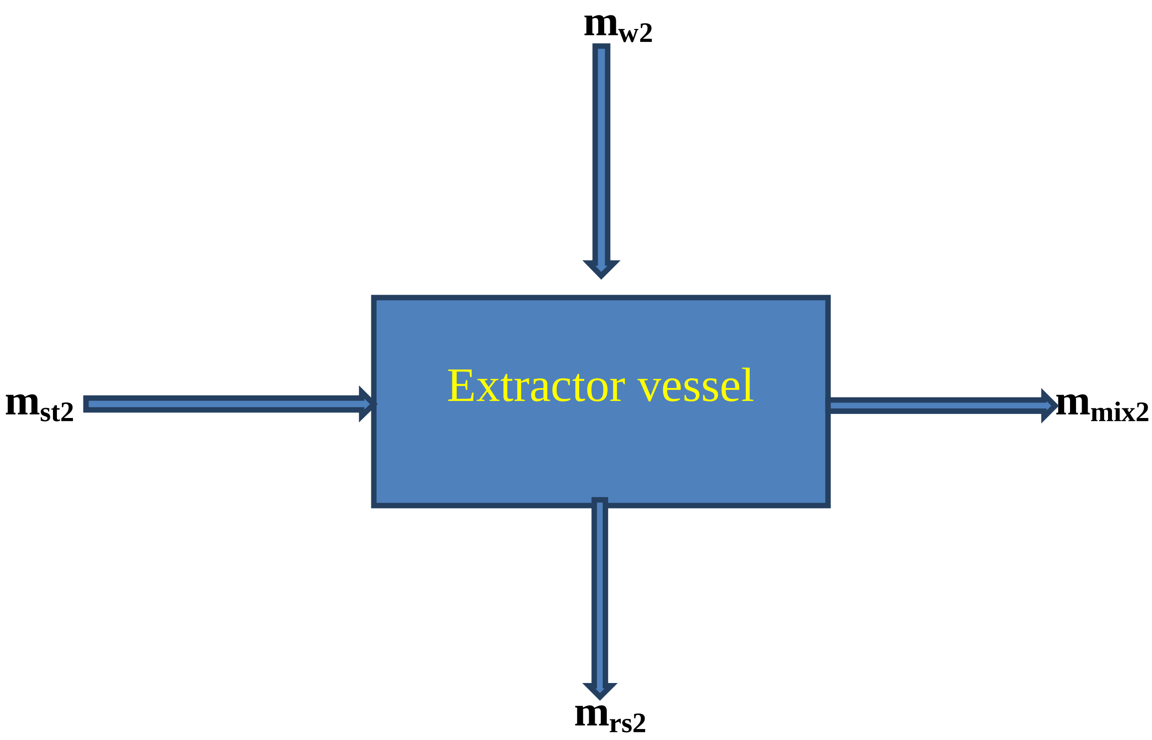
$$m_{mix1} = m_{st1} + m_{w1} - m_{rs1}$$

$$m_{mix1} = 5 \text{ grams} + 25 \text{ grams} - 15.82 \text{ grams}$$

$$m_{mix1} = 14.18 \text{ grams}$$

Therefore, the mass of the stevia sweetener mixture produced in the above process condition was 14.18 grams.

5.1.2 Material balance on a single extractor vessel at the optimal condition that was, when the ratio 1:10, temperature 50°C and residence time at three hours.



The rule of mass conservation stated that

Input = output

Since there was no chemical reaction i.e. no generation and consumption of matter

$$m_{st2} + m_{w2} = m_{rs2} + m_{mix2} \qquad \text{……….…………………………5.3}$$

Where,

m_{st2} is the mass of the stevia powder used for a run 2 during this extraction process

m_{w2} is the mass of water (solvent) used for a run 2 during this extraction process

m_{rs2} is the mass of residue produced in a run 2 during this extraction process

m_{mix2} is the mass of the mixture of the stevia sweetener and water produced in a run 2 during this extraction process

The amount of input that was feed and the amount of output that was measured

- 5 grams of stevia leaf powder was used for this run (m_{st2})
- 50 grams of distilled water was used (m_{w2})
- The mass of the residue that was produced in this ran was 27.5 grams (m_{rs2})

From equation 5.3, we have

$$m_{st2} + m_{w2} = m_{rs2} + m_{mix2}$$

By rearranging the above equation we can get

$$m_{mix2} = m_{st2} + m_{w2} - m_{rs2}$$

$$m_{mix2} = 5 \text{ grams} + 50 \text{ grams} – 27.5 \text{ grams}$$

$$m_{mix2} = 27.5 \text{ grams}$$

Therefore, the mass of the stevia sweetener mixture produced in the above process condition was 27.5 grams.

5.2 Energy balance for the extraction process on extractor vessel at the optimum condition run

Here also the extractor vessel considered as the system and the inside of the shaker as the surrounding. Since there was no mass transfer across the boundary of the system, the system was closed system.

The energy balance for the closed system states that

$\Delta U + \Delta E_K + \Delta E_P = Q\text{-}W$……………………………………5.4

Where,

ΔU is change internal energy for the system

ΔE_K is change kinetic energy for the system

ΔE_P is change potential energy for the system

Q is the heat energy of the system

W is work done on or by the system

Though there was no phase changes, no chemical reactions occur in the system and assume that pressure changes was insignificant, there was a temperature change on the system i.e. there was heat transfer from the incubator shaker to the extractor vessel. As a result,

$\Delta U \neq 0$

$\Delta E_K = 0$; the system did not accelerate

$\Delta E_P = 0$; the system did not rising or falling

$W = 0$; there was no movement of the system boundary against a resisting force and no the passage of an electrical current or radiation across the system boundary.

$Q \neq 0$; the system and the surrounding had no the same temperature or the system did not well insulated

The first law of thermodynamics for the closed system, equation 5.4 reduced to

$$\Delta U = Q$$

But, $Q = m * C_{p\ mix} * \Delta T$ ………………………………………………………….…….5.5

Where, m is the mass of the liquid mixture

$C_{p\ mix}$ is the specific heat capacity of the mixture of the liquid

ΔT is change in temperature of the liquid mixture

At the optimum condition run that was at the temperature level equal to 50°C, extraction time three hours and material ratio 1:10, mass of the mixture was 55 grams, the mixture temperature rise from 22°C to 50°C and ΔT became 28°C.

The specific heat capacity of the mixture could be calculated by the weighted arithmetic mean formula using the water specific heat capacity 4.186 J/g^{o}C and stevia leaf powder specific heat capacity 1.7 J/g.^{o}C and it became 3.958 J/g.^{o}C.

Then the heat energy (Q) of the mixture became

$Q = 55g * 3.958\ J/g.^{o}C * 22^{o}C = 4789.4$ joules

Therefore the amount of heat energy needed to rise the temperature of the mixture from 22^{o}C to 50 ^{o}C was 4789.4 joule.

6. Conclusion and Recommendations

6.1 Conclusion

This study showed that different factors had different effects on the yield of stevia sweetener product. The effects of treatment on the product also differ with in the treatment.

The effect of temperature was significant on the yield of stevia sweetener. It rapidly increase the yield when the temperature rise from 40^oC up to 50^oC but when the temperature beyond 50^oC the yield of the sweetener became decrease. When the extraction time rise from one hour up to three hours the yield also increase rapidly but further increase the time would gradually decrease the yield. The yield obtained at 1:10 material ratio was higher than the yield obtained at 1:5 material ratios.

Temperature and material ratio interaction showed significant effects on the yield of stevia sweetener. Residence time (extraction time) with material ratio interaction effect also high in the yield but the interaction effects of temperature with that of extraction time was not that much significant. As a result this interaction term should reduce from the model to increase the reliability of the model.

The analysis of variance data showed that the p-value of interaction of temperature with residence time was 0.1270 which was greater than the type one error 0.05. It implies that this term was insignificant in the yield as well as in the model. From the hypothesis testing the F-calculate was 1.79 which was less than the F- tabulate 1.90. This also indicated that the term had no effect on the yield. The predicted R-squared equals to 60.10% that was very far from adjusted R-squared 87.49%. This implies that model reduction would improve the reliability of the model. As a result the above term reduced from the model.

In the model adequacy checking, the normal probability plot of the residuals showed a straight line which implies that the residuals were normally distributed. The residuals verses predicted value plot did not show any regular pattern which implies the model was adequate.

As we seen from the analysis the highest yield, 49.90%, obtained at the treatment of the temperature 50^oC, residence time at the treatment of 3 hours and the ratio of stevia leaf with solvent (water) at 1:10 ratio. The second highest yield 49.88% also obtained at the combination of treatments 50^oC of temperature, 4 hours of extraction time and 1:10 material ratio. Both the report form as well as the ramp form outputs were enforces this conclusion.

6.2 Recommendations

Ethiopia should encourage the production of this valuable plant and value adds products of it for multi-directional purpose. The benefit of the products extends to health and socio-economic advantage. Among different sugar substitute sweetener, stevia sweetener is the most health sugar substitute. Different food complex factory in our country that are used sugar or other sugar substitute as a sweetener, have to use stevia sweetener by producing their own product in this fertile country.

Different studies show that, the imported sugar substitute to our country not only economic disadvantage but also different health problems. Nevertheless, any studies do not show the stevia sweetener negative side effect on our health. As a result others sugar substitute should be replaced by stevia sweetener as fast as possible.

The concerned body should introduce this very crucial plant to both small scale farm farmers as well as the investors to produce the plant in large scale. Since the plant is very expensive in the world market, the demand of the products highly increase day to day and no negative environmental impact, it will become the major export items for Ethiopia like flower in today. If this effort should be done, it would have significant positive effect on the Gross Domestic Product (GDP) of Ethiopia as well as the poverty and unemployment reduction.

Many researchers in our country should have dig a lot about this new idea for the country to produce the stevia sweetener in excellent quality as well as quantity. Different parameters (factors), even they will have low impact on the extraction process have to be investigate. This enables for the production of the products in the desire way.

Different governmental or private sectors that are interested in agro industry area recommend participating in this profitable area. Even they can cooperate with a large international company known as Pure Circle which is the main stevia sweetener supplier for the Pepsi and Coca Cola Company.

References

Alvarez, M.; Couto, A.C.C. Processo de fracionamento dos componentes das folhas da *Steviarebaudiana* (Bert.)Bertoni (FundaçãoUniversidadeEstadual de Maringá), Patent, Brazil, protocol No. 84 02 752 (1984).

Alvarez, M.; Kusumoto, I.T.; Arraes, L.S. Correlaçao entre o teor de carboidratostotais e o teor de glicosídeosditerpenicosnasfolhassecas de Stevia rebaudiana (Bert.)Bertoni, ArquivosBiologicosTecnologicos, 5, 19 (1986).

Correa, N.C.F. Estudo da cinetica de extraçao do óleo da semente de maracuja com CO_2supercitico. M.S. thesis, Universidade Federal doPara, Belem, PA, Brazil (1994).

Cramer, B.; Ikan, R. Progress in the chemistry and properties of rebaudiosides. In: Grenby, T. H. (Ed.), Developments in Sweeteners 3, Elsevier, New York, p.45 (1987).

Ferreira, S.R.S.; Nikolov, Z.; Doraiswamy, L.K.; Meireles, M.A.A.; Petenate, A.J. SCFE of Black Pepper (Piper nigrun) Essential Oil. J. Supercritical Fluids 14, No. 3, 235 (1999).

Jacobs, M.B. The chemical analysis of foods and food products, 3rd Ed., Robert Krieger Publishing Co, New York (1973).

Kienle, U. Method of making a natural sweetener based on *Stevia rebaudiana*, and use thereof. Int. Cl.5 A61K 35/78. U.S. Cl. 424/195.1, 586690 Sep. 24, 1990; May 12, 1992.

Pasquel, A. Extraçao dos Glicosideos da Estevia com FluidosPressurizados, Ph.D. diss., UniversidadeEstadual de Campinas, Campinas, SP, Brazil (1999.

Soejarto, D.D.; Compadre, C.M.; Medon, P.J.; Kamath, S.K.; Kinghorn, A.D. Potential sweetening agents of plant origin-II: Field search for sweet-tasting *Stevia* species, Econ. Bot. 37, 71 (1983).

McHugh, M.; Krukonis, V. Supercritical fluid extraction, 2d Ed., Butterworth-Heinemann, Boston (1994).

Phillips, K.C. Stevia: steps in developing a new sweetener. In: T. H. Grenby (Ed.), Developments in Sweeteners 3, Elsevier, New York, p. 1 (1987)

Sovova, H. Rate of the vegetable oil extraction with supercritical CO2: I.- Modeling of extraction curves, Chemical Engineering Science 49, 409 (1994).

Tan, S.; Shibuta, Y.; Tanaka, O. Isolation of sweetener from *Stevia rebaudiana*.*Jpn. Kokai* 63, 177, 764 (1988).

Tsanava, V.P.; Sardzhveladze, G.P.; Kharebava, L.G. Effect of technological procedures on the composition of volatile substances in *Stevia rebaudiana*, Subtrop.Kul't 3 64.*Apud*: Chemical Abstracts 116: 82387g (1991).

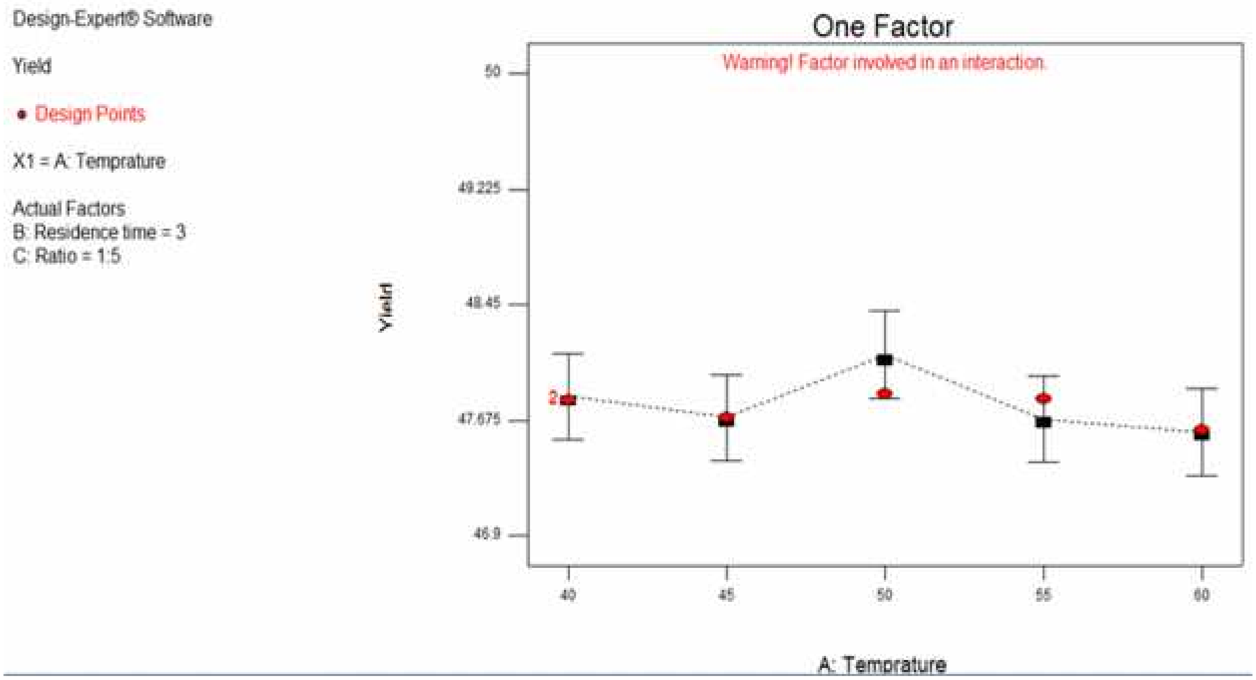
Appendixes

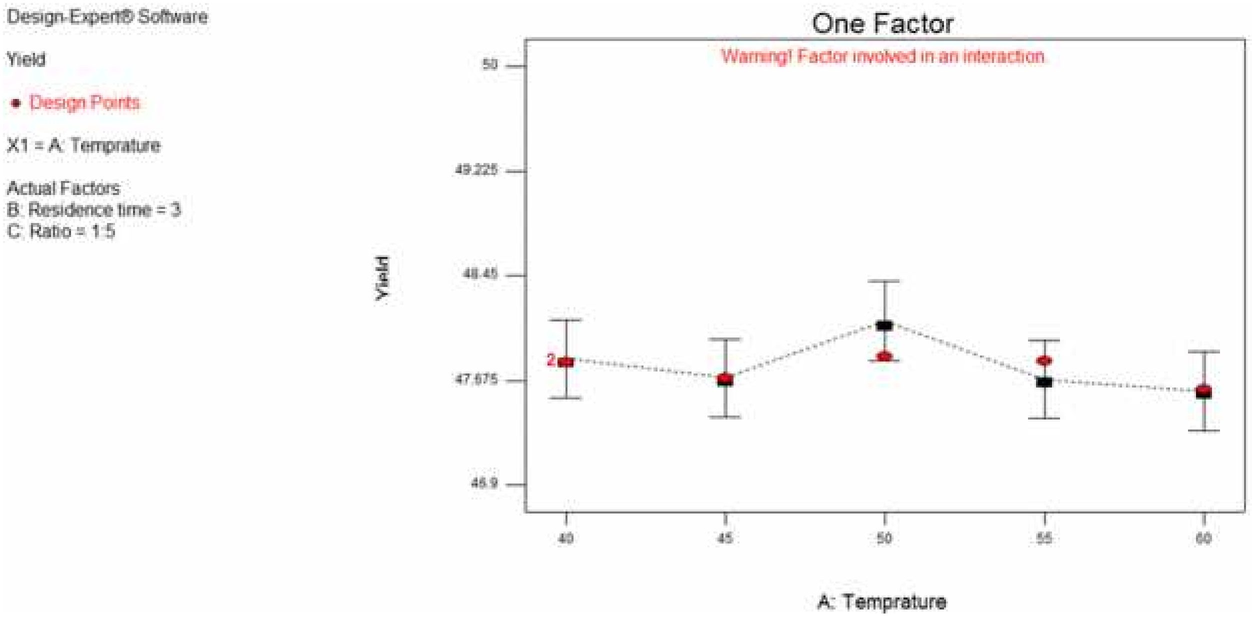
Appendix A: The design layout of the experiment designed by design expert software.

Std	Run	Block	Factor 1 A: Temperature ^oC	Factor 2 B: Time hours	Factor 3 C: Ratio	Response 1: yield %
6	1	Block 1	40	2	1:5	47.42
24	2	Block 1	55	5	1:5	47.72
19	3	Block 1	55	4	1:5	47.77
10	4	Block 1	60	2	1:5	47.57
1	5	Block 1	40	1	1:5	46.91
16	6	Block 1	40	4	1:5	47.82
29	7	Block 1	55	1	1:10	48.10
32	8	Block 1	45	2	1:10	48.09
2	9	Block 1	45	1	1:5	46.99
3	10	Block 1	50	1	1:5	47.59
21	11	Block 1	40	5	1:5	47.80
38	12	Block 1	50	3	1:10	49.90
4	13	Block 1	55	1	1:5	47.57
11	14	Block 1	40	3	1:5	47.81
28	15	Block 1	50	1	1:10	48.11
17	16	Block 1	45	4	1:5	47.69
23	17	Block 1	50	5	1:5	47.80
14	18	Block 1	55	3	1:5	47.82
33	19	Block 1	50	2	1:10	48.16
41	20	Block 1	40	4	1:10	48.85
50	21	Block 1	60	5	1:10	48.25
8	22	Block 1	50	2	1:5	47.74
28	23	Block 1	40	1	1:10	47.27
44	24	Block 1	55	4	1:10	48.29
34	25	Block 1	55	2	1:10	48.21
35	26	Block 1	60	2	1:10	47.88
12	27	Block 1	45	3	1:5	47.69
27	28	Block 1	45	1	1:10	48.01
13	29	Block 1	50	3	1:5	47.85
39	30	Block 1	55	3	1:10	48.29
40	31	Block 1	60	3	1:10	48.29
48	32	Block 1	50	5	1:10	49.50

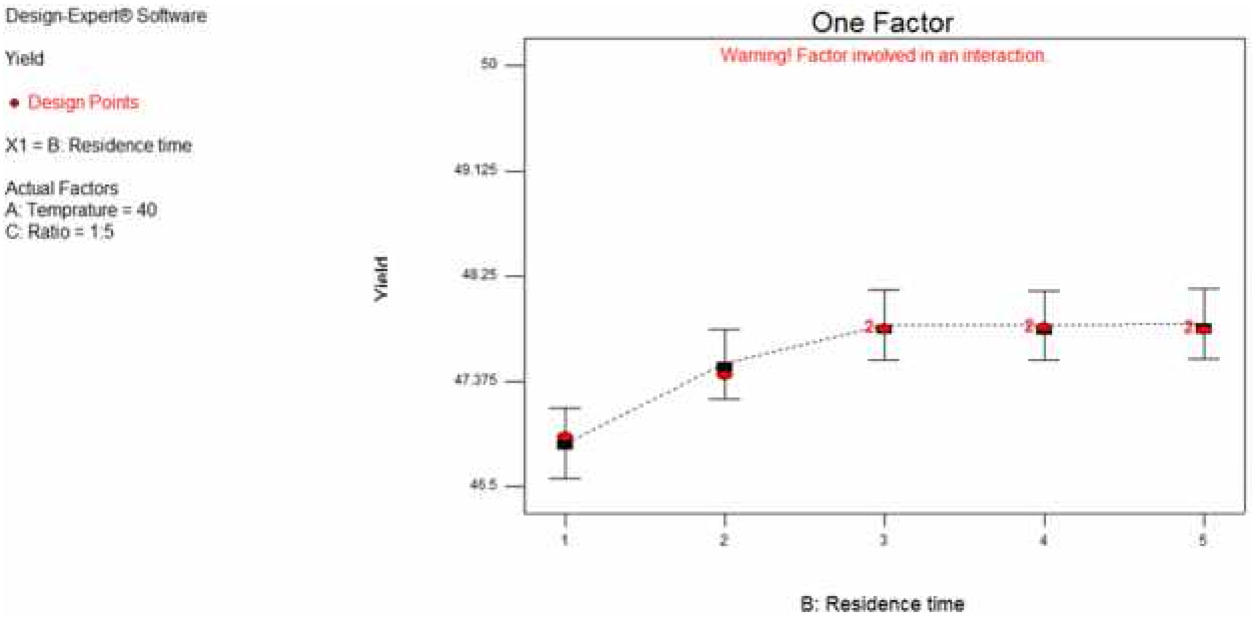
43	33	Block 1	50	4	1:10	49.81
7	34	Block 1	45	2	1:5	47.60
9	35	Block 1	55	2	1:5	47.62
15	36	Block 1	60	3	1:5	47.61
20	37	Block 1	60	4	1:5	47.60
31	38	Block 1	40	2	1:10	48.00
22	39	Block 1	45	5	1:5	47.64
48	40	Block 1	55	5	1:10	48.27
42	41	Block 1	45	4	1:10	49.01
18	42	Block 1	50	4	1: 5	47.80
47	43	Block 1	45	5	1:10	49.00
45	44	Block 1	60	4	1:10	48.25
36	45	Block 1	40	3	1:10	48.88
37	46	Block 1	45	3	1:10	49.01
30	47	Block 1	60	1	1:10	47.72
25	48	Block 1	60	5	1:5	47.60
5	49	Block 1	60	1	1:5	47.55
46	50	Block 1	40	5	1:10	48.85

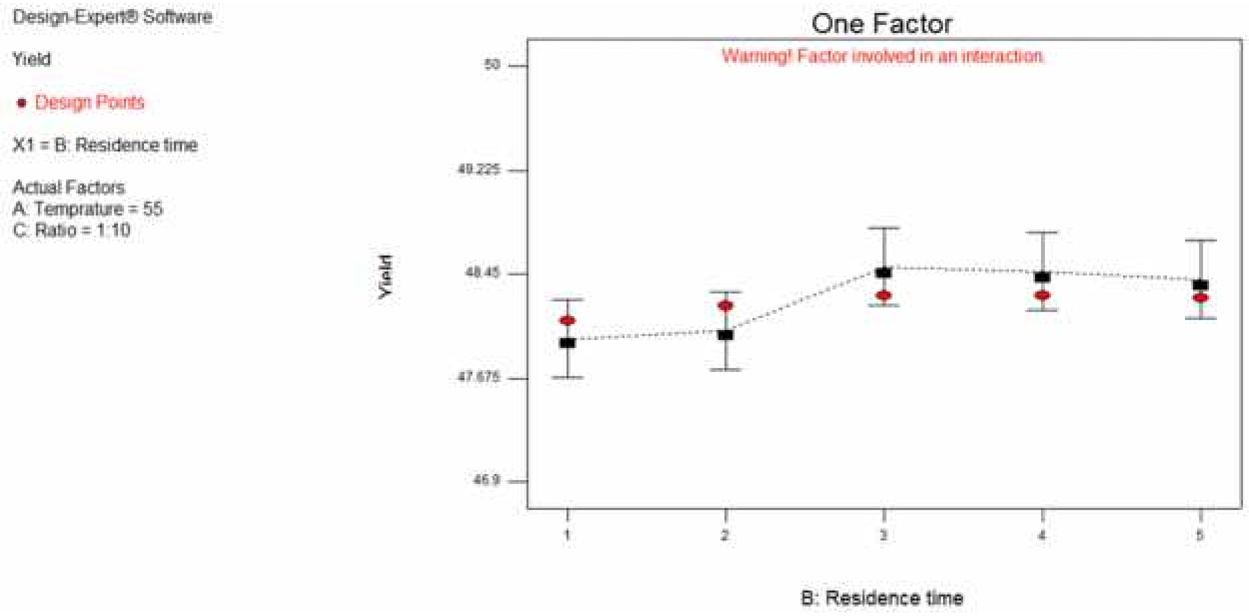
Appendix B: The effect of temperature on the yield at different level of residence time and material ratio.



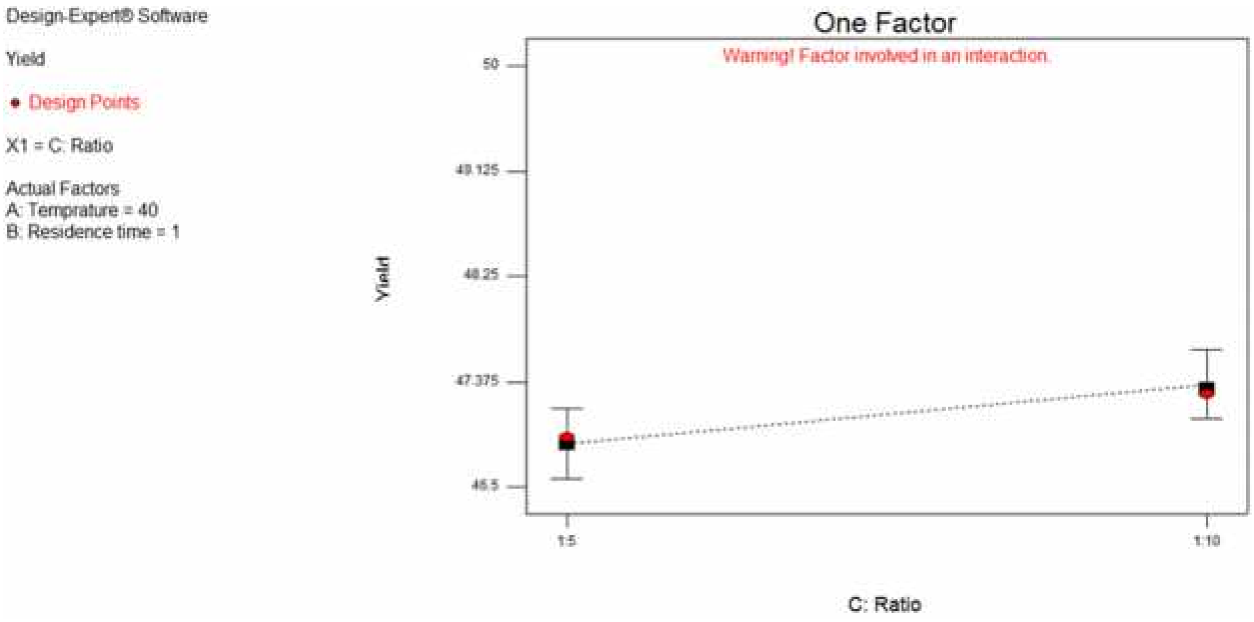


Appendix C: The effect of residence time on the yield at different level of temperature and material ratio.

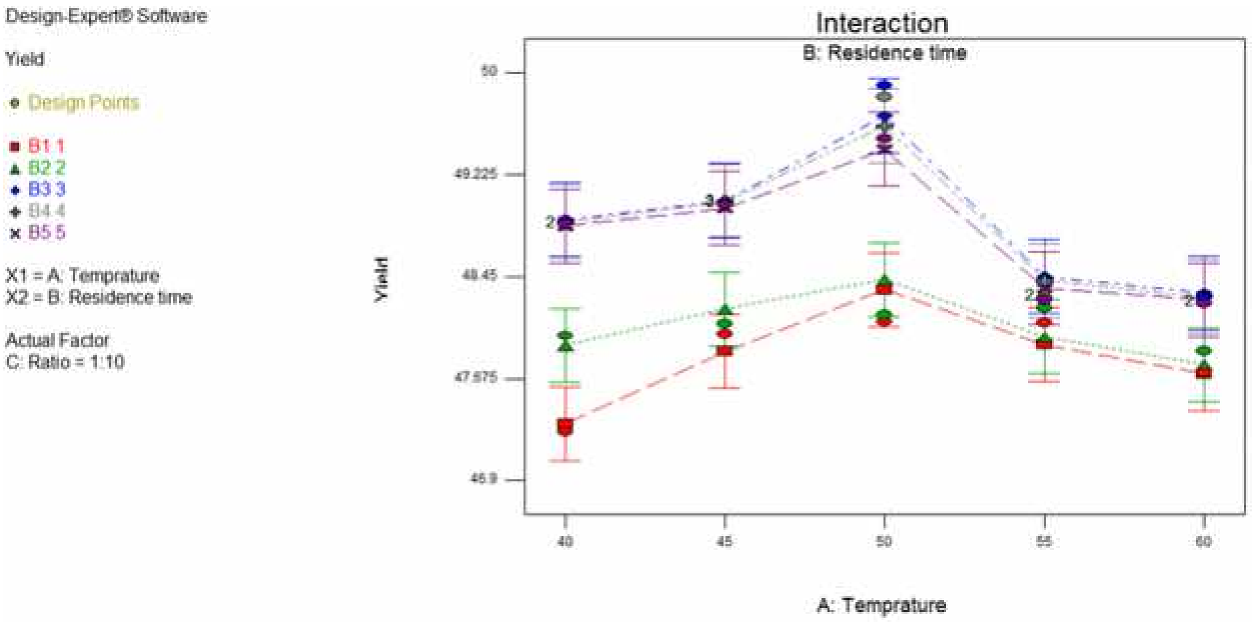




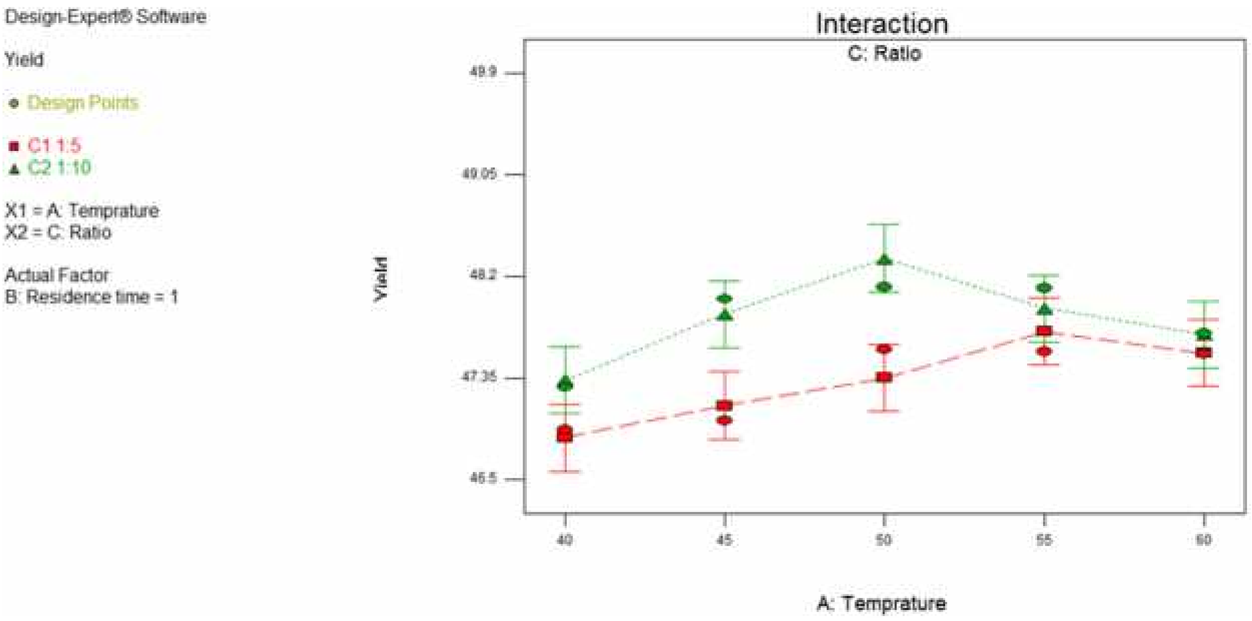
Appendix D: The effect of material ratio on the yield at different level of temperature and residence time.

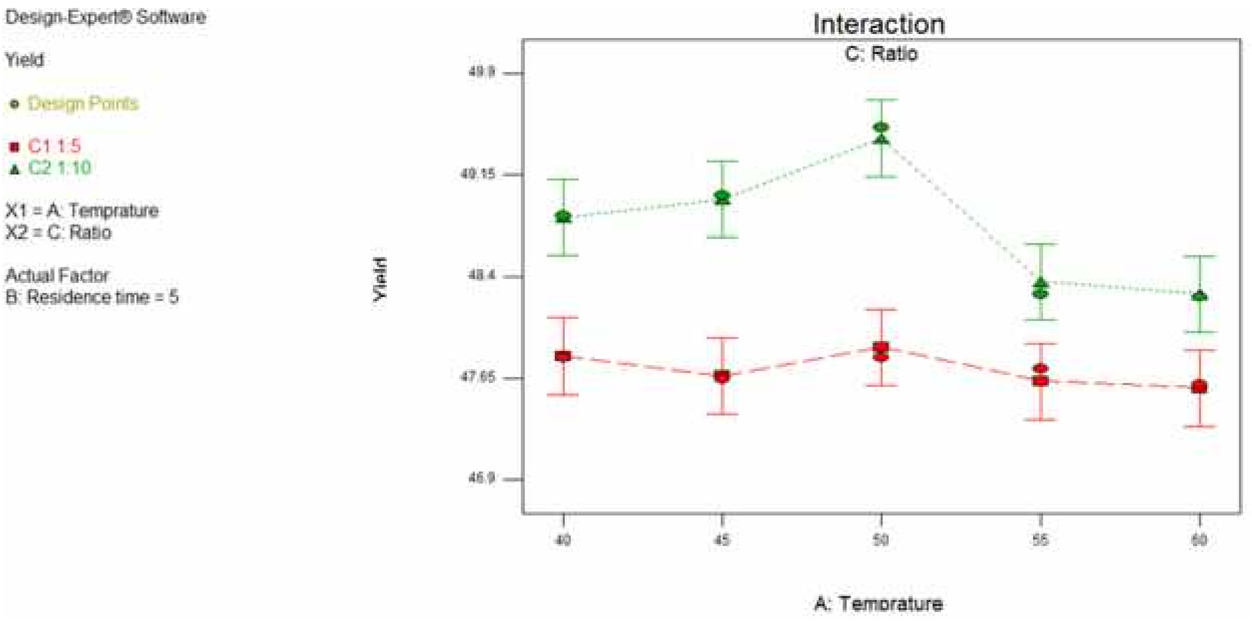


Appendix E: The interaction effect of temperature with residence time on the yield at 1:10 level of material ratio.



Appendix F: The interaction effect of temperature with material ratio on the yield at different level of residence time.





Appendix G: The interaction effect of material ratio with residence time on the yield at different level of temperature.