OPTIMIZATION OF EXTRACTED CALCIUM FROM HELENCHA (*Enhydra fluctuans)* USING RESPONSE SURFACE METHODOLOGY

A THESIS

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A. B. M. SAID BIN SAIFULLAH

REGISTRATION NO.: 1705422

SESSION: 2017-2018

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FOOD SCIENCE AND NUTRITION

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DEPARTMENT OF FOOD SCIENCE AND NUTRITION
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The Author

ABSTRACT

Optimum extraction conditions of calcium from Helencha (*Enhydra fluctuans*) using hydrochloric acid as the solvent were evaluated. The extractions were carried out using three process parameters such as hydrochloric acid concentration, temperature and time ranging from 1 to 2 M, 50 to 100 °C and 60 to 180 minutes respectively. Box–Behnken statistical design was applied as the response surface method using Design Expert DX11 to determine the optimum extraction parameters. The extracted calcium was measured using a modified UV/Visible spectrophotometric method. The highest response experimented in the study was 190.95 mg calcium per 100 g powder. Though changing any parameter significantly ($p < 0.05$) affect the extraction of calcium, the temperature is the most significant factor ($F = 1054$) among others. A quadratic regression equation describing the effects of independent process variables on calcium extraction can be used for finding optimum conditions to achieve desired extraction yield in similar conditions. Applying the equation, the processed parameters (concentration, temperature and time) were optimized within the experimental range respectively as 1.97683 M, 98.2929 °C and 167.617 minutes and the predicted yield increased from 88.48 to 91.50 % with the extraction of 196.49 mg calcium per 100 g powder.

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CHAPTER I

INTRODUCTION

Dietary pattern of Bangladesh is changing with decreasing rice dependency and increasing intake of fruits, vegetables, meat, and poultry products. With a lower average of energy consumption per capita (2210.4 kcal), the micronutrient intake deficiency in Bangladesh especially vitamin A, calcium, iron, zinc, folic acid are still prominent (Nahar *et al.*, 2013; BBS, 2017).

Calcium, the most abundant mineral in the body plays a vital role throughout the life cycle. It helps to strengthen the teeth and bones and also helps nerves and muscles to function properly. US Food and Drug Administration suggests that adults should intake 1000 mg of Calcium per day (IOM, 2011). A study found that average national dietary calcium intake all over the world ranges from 175 to 1233 mg/day (Balk *et al.*, 2017). For the past decades, Bangladesh has average dietary calcium intake less than 500 mg/day (BBS, 2017).

Intake of calcium supplements is another way to achieve the daily requirements. Dairy and fish sources are the main element of calcium supplements. But the vegan often avoids such supplements and prone to take ones of lime sourced. Many researchers claiming that supplemental calcium obtained from lime is associated with kidney stones in the urinary tract (Tracy *et al.*, 2014). So it may be a better concept to isolate calcium from plant sources to avoid any health hazards.

Helencha (*Enhydra fluctuans*) commonly known as Marsh herb or watercress is a hydrophytic plant and mostly found on wet roadside canals and marshy waste places between the months of November to January. It is highly prevalent in Bangladesh, India, Malaysia, China and the rest of South East Asia and Tropical Africa (Chakraborty *et al.*, 2012; Sarma *et al.*, 2014).

It is rich in protein and is a good source of β-carotene and minerals including calcium, magnesium, sodium, phosphorus, etc (Ali *et al.*, 2013). It has immense potential and many beneficial effects such as anticancer, antioxidant, anti-diabetic, anti-inflammatory, antimicrobial, anti-diarrheal and even hepato-protective effects as a medicinal plant (Sarma *et al.*, 2014).

In the last few decades, Helencha has established its importance and gained popularity over so many researchers (Ali *et al.,* 2013). In spite of this, the consumption of Helencha has been reduced significantly because of its bitter taste. Nowadays it is being used as a popular cattle feed among rural areas (Satter *et al.*, 2016).

It seems that researchers have a huge debate on the available calcium level of Helencha which lies between a very high range of 1.32 – 932.69 mg/100 g of dried Helencha (Table 2.1). To verify the actual calcium range or to provide the vegan a plant sourced calcium or even to utilize a potential waste product, this study is hypothetically important.

Design of Experiment is a general and flexible method providing a comprehensive understanding of developed processes and also serves as a useful assessment of input factors and their interactions necessary for the determination of the outputs (Goos and Jones, 2011). Studies suggested the benefit of Response Surface Method as a Design of Experiment to optimize and determine the least number of experimental observations needed to generate a thorough illustration of the outputs of the entire experiment (Khan, 2013; Kusuma *et al.*, 2016).

In this study, the important parameters (factors) needed in the extraction process were considered to be solvent concentration, temperature and time. Helencha was chosen in this experiment because there has been a remarkable debate about its calcium content (Table 2.1).

The objectives of the present study are –

- ✓ To extract dietary calcium from *Enhydra fluctuans*
- ✓ To evaluate the potential effects of the three main parameters
- ✓ To optimize the extraction using response surface methodology

CHAPTER II

REVIEW OF LITERATURE

Review of related literature in any study is necessary because it provides a scope for reviewing the stock of knowledge, primary concept and relevant information to the proposed study, and provide a guideline in designing and conducting the study successfully. It is essential for reviewing that gives proper instruction in designing future research problems and validating the new findings.

2.1 Calcium

Calcium is the most abundant metal and the fifth-most-abundant element in the human body needed in large quantities. The majority (~ 99%) of calcium present in the body is found in bone, with a smaller amount found in teeth. The remainder (< 1%) is found in soft tissues and body fluids. The Ca^{2+} ion acts as an electrolyte and is vital to the health of the muscular, circulatory, and digestive systems; and supports synthesis and function of blood cells. Calcium ions outside cells are important for maintaining the potential difference across excitable cell membranes as well as proper bone formation.

2.1.1 Dietary calcium

Dietary calcium refers to both food sources and supplements combined (although some researchers reserve the term dietary calcium to mean only food sources) and is most often referred to as total calcium intake for clarity. Bioavailability is generally increased when calcium is well solubilized and inhibited in the presence of agents that bind calcium or form insoluble calcium salts. Humans absorb about 30 percent of the calcium from dairy and fortified foods (IOM, 2011).

During the past 10 years, there has been increasing interest in the possibility of enhanced roles for calcium in human health. There is a concern that some may not be obtaining sufficient amounts given the foods they eat. Calcium has been increasingly added to foods, and calcium supplement use, particularly among older persons, is widespread. There is controversy concerning levels of nutrient intake, and at times the concept that “more is better” emerges.

Dietary reference intakes is a set of recommendations for the proper intake of nutrients to ensure good health. These recommendations are often used by nutrition practitioners, governments and non-government organizations that help to meet the nutritional needs and create a plan for good health.

Table 2.1 Dietary reference intake of Calcium (IOM, 2011)

Life Stage	Age *	EAR †	RDA †	RNI †	UL †
Children	0 – 1	260	–	300	1,500
	1 – 3	500	700	500	2,500
	4 – 8	800	1,000	700	2,500
Males	9 – 13	1,100	1,300	1,300	3,000
	14 – 18	1,100	1,300	1,300	3,000
	19 – 30	800	1,000	1,000	2,500
	31 – 50	800	1,000	1,000	2,500
	51 – 70	800	1,000	1,000	2,000
	70 +	1,000	1,200	1,000	2,000
Females	9 – 13	1,100	1,300	1,300	3,000
	14 – 18	1,100	1,300	1,300	3,000
	19 – 30	800	1,000	1,000	2,500
	31 – 50	800	1,000	1,000	2,500
	51 – 70	1,000	1,200	1,300	2,000
	70 +	1,000	1,200	1,300	2,000

* Years; † mg/day; EAR = Estimated Average Requirement; RDA = Recommended Dietary Allowance, RNI = Recommended Nutrient Intake; UL = Tolerable Upper Intake Level.

Balk et al, 2017 reviewed a total sum of 74 countries with data and found that the average national dietary calcium intake ranges from 175 to 1233 mg/day. Many countries in Asia including Bangladesh have average dietary calcium intake less than 500 mg/day. Countries in Africa and South America mostly have low calcium intake between about 400 and 700 mg/day. Only Northern European countries have national calcium intake greater than 1000 mg/day. Average calcium intake is generally lower in women than men which led to a greater threat like osteoporosis.

2.2 Sources of Calcium

A wide range of foods contain calcium; with the amount of calcium provided on a per 100 g or serving basis and its bioavailability varying considerably.

2.2.1 Milk and dairy products

The calcium content of milk is fairly constant and is virtually unaffected by the cow's diet, lactation stage or the climate. About two-thirds of the calcium present in milk is bound to the protein casein and other milk proteins, phosphorus, and citrate, while the remainder is unbound. Skimmed milk, dried skimmed milk powder, and yogurts retain essentially all the original calcium present in the milk prior to processing. About 80% of milk calcium remains in cheddar and other hard cheeses, but butter contains only about 18%. The calcium in milk and milk products has a high bioavailability.

2.2.2 Cereal products

Cereals are not considered a rich source of calcium. However, many cereal products make a valuable contribution to calcium intake after fortification. In 1943 the addition of calcium carbonate to flour was made compulsory following recommendations from the Medical Research Council's Accessory Food Factor Committee. According to Bread & Flour Regulations (1995), the standard fortified flours derived from wheat must have a calcium content of between 94 and 156 mg/100 g flour, whilst self-rising flour must contain not less than 200 mg/100 g (Theobald, 2005).

2.2.3 Plant foods

Plant sources together can provide as much as 400 mg of calcium per person per day. The calcium content of vegetables is little affected by methods of cultivation, although calcium bioavailability varies considerably between plants. Calcium is not lost during cooking, but concentrations will be diluted if boiled during cooking. Fortified soya products are often marketed as alternatives to dairy products, and they form an important part of the diets of many vegetarians and vegans. It has been estimated that the bioavailability of calcium from soya beans is approximately 30 to 40% which is similar to milk and many dark green leafy vegetables, but higher than many commonly consumed vegetables (Heaney *et al.*, 2000).

2.2.4 Additional sources of calcium

Eggs, some fish and animal products are all additional sources of dietary calcium. A whole raw egg provides 57 mg calcium/100 g, while the yolk provides 130 mg/100 g. Shellfish and small fish; such as whitebait, canned salmon and sardines, which are eaten with bones, are the significant sources of calcium (Theobald, 2005).

Another source of calcium is water. Tap water contains 1 to 160 mg calcium per liter and contributes up to 4% of total daily calcium intake (Nerbrand et al. 2003). The calcium content of mineral waters varies considerably, typically from 7 to 486 mg per liter. In recent years, a number of mineral waters have been fortified with additional calcium. It is estimated that highly mineralized water (486 mg/L) may contribute up to 25% of calcium intake if consumed regularly in sufficient quantities (Galan *et al.* 2002).

A number of food additives also contain calcium. Calcium carbonate used in flour; calcium phosphates, calcium chloride, calcium citrate, and several other calcium salts is used in small amounts in a variety of foods. Antacid remedies contain calcium carbonate and may provide up to 400 mg of calcium per day (Theobald, 2005).

Calcium supplements may contain one of many forms of calcium, including calcium carbonate, calcium gluconate, calcium lactate, and calcium chelates. The proportion of elemental calcium present in the derivatives ranges from 9 (gluconate) to 40 % (carbonate). Calcium in the form of chelates is more bioavailable than that in the form of carbonate as it has a lower solubility. However, the absorption of calcium from calcium salts and from milk is similar (Guéguen & Pointillart 2000). Typically, supplements contain calcium concentrations of between 133 to 800 mg/tablet, if consumed three times a day can supply 2400 mg of calcium per day. The Food Standards Agency recommends individuals not to consume more than 1500 mg of calcium per day from supplements, as taking high doses of calcium can cause stomach pain and diarrhea (Expert Group on Vitamins & Minerals 2003). It has been suggested that calcium from dietary supplements absorbed best when consumed at a dose not greater than 500 mg at a time (Dawson-Hughes, 1998) and that the timing of meals influences absorption.

2.3 The Helencha Crop

Helencha is an old world species, possibly of Indochinese origin, which occurs in tropical Asia and Africa. It is common to all countries of Southeast Asia. It grows annually as a trailing herb along ditches, watercourses, margins of fish ponds and rice fields in the open, from sea-level up to 1,800 meters. It is able to reproduce by fragmentation and may be so abundant that it clogs watercourses.

Usually, Helencha has a fleshy, hairy and branched stem 30 to 60 cm long, rooting at the lower nodes. Leaves are sessile, 2.5 to 7.5 cm in length, stalk-less and margins are distinctly dented. Flowers are white to greenish white in color.

Fig 2.1 Pieces of edible parts of Helencha crop

2.3.1 Classification

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Asteraceae

Genus: *Enhydra*

Species: *fluctuans* Lour.

2.3.2 Common names

Bengali: Helencha, Hingcha, Hincha, Hinche

English: Marsh herb, Watercress, Buffalo spinach

Sanskrit: Helanchi, Hilamochika, Himamochika, Bramhi, Chakrangi, Achari

2.3.4 Utilization

Helencha (*Enhydra fluctuans*) has abundant applications in treating life-threatening diseases and even has strong antioxidant, analgesic, anti-inflammatory, anti-diarrheal and antimicrobial activity (Sarma *et al.*, 2014). Leave juice and pastes of this ethnomedicinal plant mixed with or without milk is being used as a to cure for a sum of seven diseases such as Inflammation, leucoderma, bronchitis, biliousness, smallpox, gonorrhea and headache among the tribal people (Rahman, 2015).

2.3.5 Calcium in Helencha

Although Helencha contains calcium, there is no strong evidence of exact calcium concentration of Helencha. The following Table 2.2 is a compilation of findings on Helencha commonly grows in Indian subcontinent especially Bangladesh.

Table 2.2: Findings of the last 20 years on Helencha (*Enhydra Fluctuans*)

Serial	Calcium (mg/ 100g)	Method of Measurement	Reference
1	1.32	AAS	Bhowmik *et al.*, 2012
2	4.00 ± 0.06	FES	Narzary *et al.*, 2017
3	31	AAS	Shaheen *et al.*, 2013
4	110	Titration	Maitra *et al.*, 2014
5	224.45	UV/VIS	Hazra *et al.*, 2018
6	247.2 ± 23.1	AAS	Saikia *et al.*, 2013
7	400 – 700	AAS	Dewanji *et al.*, 1992
8	900	FES	Choudhury *et al.*, 2017
9	902.46 ± 0.85	AAS	Satter *et al.*, 2016
10	932.69	AAS	Linkon *et al.*, 2015

2.4 Extraction of Calcium

Calcium is an alkaline earth metal. The earth metals include beryllium, magnesium, strontium, barium, and radium which make up Group 2 (IIA) of the periodic table, a chart that shows how the elements are related. These metals are more chemically active than most metals, so it doesn't occur in elemental form in nature. Limestone is a naturally occurring mineral high in calcium carbonate ($CaCO_3$). It is possible to extract pure calcium from calcium carbonate through a multi-stage process utilizing some special equipments.

2.4.1 Extraction from plant sources

Calcium is found outside the cytoplasm is partly in the vacuoles and partly in the cell walls. It is usually bound to the anionic constituents of the cell walls such as pectins as a stabilizer in the middle lamella. Considerable amounts of Calcium are retained in the cell walls in the form of $CaCO_3$ (Kinzel, 1989).

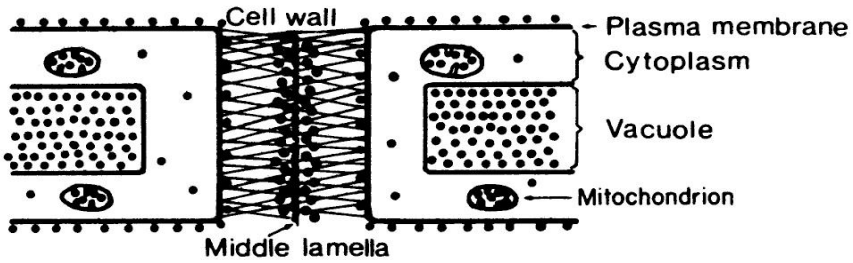

Fig 2.2 Schematic representation of two adjacent cells with a typical distribution of Calcium (•) (Marschner, 1986).

Calcium is distinguished from other major nutrient elements by its poor mobility and restricted recycling within the plant. One of the reasons often given for this limited movement is that the deposition of calcium in the form of oxalate or phytate, or binding of calcium in cell walls, effectively makes calcium unavailable for transport. This has led many workers to attempt identification of various fractions of calcium in plant tissues, these fractions usually being determined by the extraction of calcium in various solvents (Ferguson *et al.,* 1980).

Calcium fractionation procedures, probably originally based on soil extraction methods. The basic scheme has been to extract plant tissue in a sequence of solvents: water, acetic acid (to remove calcium bound in pectate), and hydrochloric acid (to

dissolve calcium oxalate), with the residue perhaps containing forms of calcium such as calcium silicate.

$$2CaCO_3 + H_2O \rightarrow CaCO_3 + Ca^{2+} + (CO_3)^{2-} + H_2O$$

$$2CH_3COOH + CaCO_3 \rightarrow (CH_3COO)_2Ca + CO_2 + H_2O$$

$$CaCO_3 + 2HCl \rightarrow CaCl_2 + CO_2 + H_2O$$

It seems that it is time to examine procedures for fractionating calcium by solvent extraction and to make some points on the chemical forms of calcium in the plant.

Miyazawa *et al.* (1984) found that a modified version of the hydrochloric acid extraction method was satisfactory for determining calcium in coffee, corn, grass leaf tissue, soybean, and sunflower. Their method involved digesting the finely ground plant samples (0.5 g) with 25 ml of 1 N hydrochloric acid at 80 °C in a water bath for 15 minutes on a horizontal shaker, followed by determination of the elements in the extract by an atomic absorption spectrophotometer.

Sahrawat (1987) described a non-digestion method for minerals to extract calcium and other elements in plant tissues of sorghum, pearl millet, chickpea, and pigeon pea. The method involves shaking 0.5 g of finely ground plant sample with 40 ml of 0.5 N HCl for 5 minutes at room temperature (25 °C). He mentioned that the HCl extraction method can be used for routine and rapid analysis of plant tissues for calcium content.

All these studies indicate that calcium recovery with dilute hydrochloric acid is preferable because they are likely associated with the dry matter of the plant.

2.5 Response Surface Methodology (RSM)

Response Surface Methodology is a collection of mathematical and statistical techniques useful for analyzing the effects of several independent variables (Myers and Montgomery, 2009). The method can help in investigating the interactive effect of process variables and in building an approximated models based on data collected during a physical examination, simulated by computer and experimented observations that accurately describes the overall process (Sarfarazi *et al.*, 2015).

In RSM, a set of mathematical-statistical designs are used for engineering and modeling procedures. The most common and efficient design used in response surface modeling is the Box–Behnken design. Compared to the central composite and Doehelrt designs, Box–Behnken presents some advantages such as requiring few experimental points for its application (three levels per factor) with higher efficiency (Ferreira *et al.,* 2007).

Several studies successfully used the Box–Behnken design such as the liquid-liquid extraction of calcium using ionic liquids in spiral microfluidics (Marsousi *et al.,* 2019), high pressure-assisted infusion of calcium into baby carrots part ii (Gosavi *et al.,* 2019), extraction process of plant-based gelatin replacer (Jaswir *et al.,* 2016), polysaccharides extraction from the fruiting bodies of Oyster Mushroom (Sun *et al.,* 2010), anthocyanins extraction from purple sweet potato (Fan *et al.,* 2008), and more.

2.6 Estimation of calcium

Volumetric methods are commonly used during the quantitative analysis of calcium in foodstuffs (Table 2.2). The analysis can be performed both directly and indirectly by using either titration or colorimeters, such as Atomic Absorption Spectrophotometer (AAS), Flame Emission Spectrophotometer (FES) and Ultra Violate Visible Spectrophotometer (UV/VIS).

Titration is the oldest method for estimating calcium (Fiske and Logan, 1931; Mason, 1952; Carr and Frank, 1956). High facility laboratories use AAS and FES for calcium estimation (David, 1959; Berry and Johnson, 1966; Ophel *et al.,* 1970).

Calcium content was measured using UV Visible spectrophotometer in several studies such as quantification of atorvastatin calcium in tablets (Mazurek *et al.,* 2009), determination of rosuvastatin calcium in tablets (Uyar *et al.,* 2007), flame synthesis of calcium ion (Grass and Stark, 2005), citrate and calcium determination in flavored vodkas (McCleskey *et al.,* 2003), design of a calcium-selective optode membrane based on neutral ionophores (Morf *et al.,* 1990), and more.

CHAPTER III

MATERIALS AND METHODS

This chapter deals with the materials and methods used that was carried out in several laboratories of Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh. The materials and methods adopted for the study are recorded in this section.

3.1 Sources of Materials

3.1.1 Helencha

Helencha (*Enhydra Fluctuans*) was sampled and harvested from four different sites of Chilmari [25° 26’ – 25° 40’ North longitude and 89° 36’ – 89° 50’ East longitude], Kurigram, Bangladesh (Fig 3.1). Since the experiment based on calcium content, the sites were selected by the Brahmaputra river basin where the plants grew naturally free from Ca salt sprays.

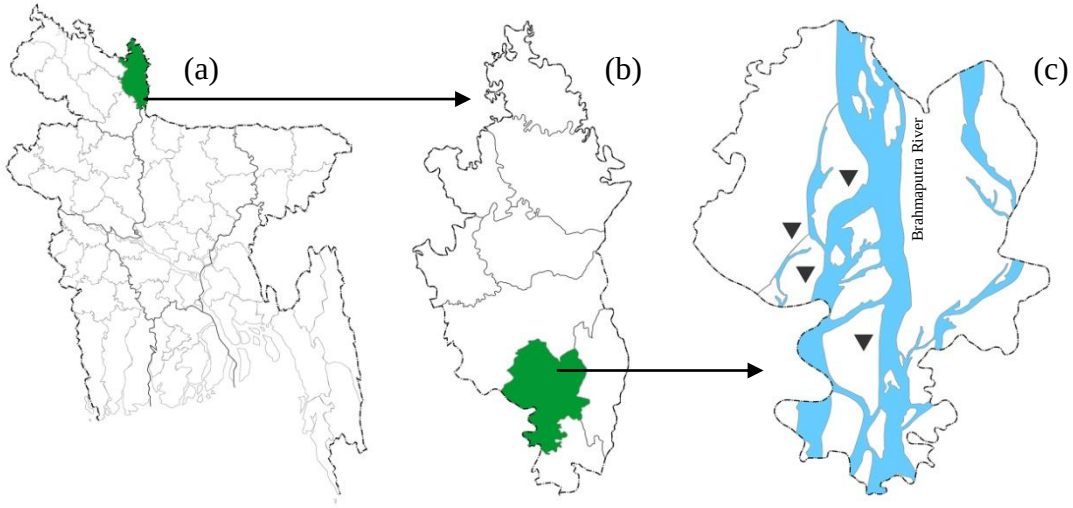

Fig 3.1: Location of (▼) sampling sites (a) Bangladesh, (b) Kurigram, (c) Chilmari.

3.1.2 Reagents

1. All the chemicals used in this study were Analytical-Grade reagent obtained from Merck, Darmstadt, Germany.
2. De-ionized distilled water was used throughout the study both as a reagent and cleansing agent.

3.1.3 Equipments

1. **Blender:** Jaipan Family Mate 850 W (Jaipan Industries Ltd, Mumbai, India)
2. **Muffle furnace:** Model-C (Navyug Udyog, Ambala, India)
3. **Oven Dryer:** CO-150 (Human Lab Inc, Suwon-Si, Korea)
4. **UV/Vis spectrophotometer:** UV-1800 (Shimadzu Corp, Kyoto, Japan)
5. **Vortex Mixer:** KMC-1300V (Vision Scientific Co. Ltd, Daejeon-Si, Korea)
6. **Water bath:** VS-1205SW1 (Vision Scientific Co. Ltd, Daejeon-Si, Korea)
7. **Water distiller:** Water Stills, Model 710 (Pobel S. A., Madrid, Spain)

3.2 Extraction of Calcium

3.2.1 Preparation of Helencha powder

Helencha (*Enhydra Fluctuans*) plant parts were first sorted and separated from dead and bruised leaves. Then thoroughly rinsed with de-ionized water until any sign of mud was present there.

The plant parts were chopped into small piece approximately 10 mm with a sharp knife and dried in a cabinet dryer at 55±5 °C for 24 hours. Subsequently, the dried Helencha plant was ground using a heavy-duty blender and sieved through a mesh (Mic–200) and obtained 0.2 mm of particle size.

Fig 3.2: Dried Helencha powder

An equal amount of ground Helencha powder (25 g) from each sample were mixed together to get the raw powder with balanced mineral contents (Fig 3.2). The powder was then packed in a high-density polyethylene bag and stored at the ambient condition to use as raw material for the entire study.

3.2.2 Design of Experiment

Response Surface Methodology was employed for the extraction of calcium from Helencha and the process parameters were optimized. The experimental levels for each variable were selected based on results from preliminary experiments. Three important process parameters including hydrochloric acid concentration (X_1), temperature (X_2) and time (X_3) were optimized to obtain the best yield of extracted calcium using the Box–Behnken statistical design respectively (Table 3.1).

Table 3.1 Independent variables and their levels used for Box–Behnken design.

Variables	**Factors**	**Level**		
	X	**-1**	**0**	**1**
Concentration of HCl (M)	X_1	1.0	1.5	2.0
Temperature (°C)	X_2	50	75	100
Time (min)	X_3	60	120	180

Coding of the variables was done according to the following equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X}$$

Where, x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and ΔX is the step change of the real value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable i. The number of experiments (N) needed for the development of Box–Behnken matrix is defined as $N = 2k(k - 1) + r$, where (k) is the factor number and (r) is the replicate number of the central point. A total of 17 experiments have been employed in this work to evaluate the effects of the three main independent parameters for calcium extraction efficiency.

3.2.3 Preparation of solvents

A series of solutions were prepared by diluting 37 %, fumed hydrochloric acid with de-ionized water to give three solutions with a concentration of 1 M, 1.5 M, and 2 M respectively.

3.2.4 The extraction process

In the present study, three important process parameters including hydrochloric acid concentration (1 to 2 M), temperature (50 to 100 °C) and time (60 to 180 minutes) were selected on the basis of trial and error based on the outline given by Bradfield (1977). The extraction solvents, process temperatures and reaction times were determined according to the experimental model designed by the Box–Behnken statistical method using Design Expert® DX11.

Helencha powder was mixed with a different hydrochloric acid solutions with a ratio of powder to the acid of 1:100 (w/v). Each mixture was incubated in a reciprocal shaking water bath with continuous shaking of 50 rpm. Then mixtures were cooled to room temperature and filtered through Whatman No. 42 filter paper (ash-free) using vacuum filter. Then UV/Vis spectrophotometer was used for the estimation of calcium. Three replications were done for each treatment.

3.3 Chemical analysis

The following parameters were studied for dried Helencha powder and extracted calcium of Helencha in the study. Proximate compositions and mineral contents (Calcium, Magnesium, and Potassium) for Helencha powder were determined by following standard procedures.

3.3.1 Moisture content

Moisture content was determined by oven drying method as described in AOAC (2006). The loss in weight due to evaporation from the sample at a temperature of 105 °C for 24 hours was taken into account to calculate the moisture content using the following equation.

$$\text{Moisture \%} = \frac{\text{Weight of raw sample} - \text{Weight of dried sample}}{\text{Weight of raw sample}} \times 100$$

3.3.2 Ash content

Ash content was determined according to the standard method as described in AOAC (2006) by combusting the samples in a muffle furnace at 550 °C for 6 hours. The sample was first treated with hot air oven for 24 hours to make free from moisture and then charred completely. The following equation was used to determine the ash content.

$$\text{Ash \%} = \frac{\text{Weight of ash with crucible} - \text{Weight of crucible}}{\text{Weight of sample}} \times 100$$

3.3.3 Fiber content

The crude fiber is the bulk of roughage in food. It was determined using the standard method as described in AOAC (2006). The raw sample was dried, defatted with serial digestion with 1.25% H_2SO_4 and 1.25% NaOH at 400 °C for 30 minutes each. Then the residue was first dried in a hot air oven at 100 °C for 1 hour and then charred in a muffle furnace at 550 °C for 4 hours. The following equation was used to determine the crude fiber content.

$$\text{Crude fiber \%} = \frac{\text{Loss in Weight}}{\text{Weight of sample}} \times 100$$

3.3.4 Calcium content

The calcium content of the sample was determined using the standard method as described in AOAC (2006). The sample was first digested at with diacid mixture at 195 °C for 1 hour. Then the digested mixture was mixed with 10% NaOH, masking reagents and calcon indicator and titrated with 0.01M Na_2EDTA. The following equation was used to determine the percent of calcium.

1ml of 0.01 M EDTA solution ≡ 0.2004 mg of Ca

$$\text{Calcium \%} = \frac{\text{mg of calcium obtained}}{\text{Weight of sample}} \times 100$$

3.3.5 Magnesium content

The magnesium content of the sample was determined using the standard method as described in AOAC (2006). The sample was first digested at with diacid mixture at 195 °C for 1 hour. Then the digested mixture was mixed with NH_3-NH_4 buffer solution, masking reagents and EBT indicator and titrated with 0.01 M Na_2EDTA. The following equation was used to determine the percent of magnesium.

1ml of 0.01 M EDTA solution ≡ 0.2432 mg of Mg

$$\text{Magnesium \%} = \frac{\text{mg of magnesium obtained}}{\text{Weight of sample}} \times 100$$

3.3.6 Potassium content

The potassium content of the sample was determined using the standard method as described in AOAC (2006). The sample was first acidified with 1 N HCl and cooled to 0-2 °C. Then Sodium tetraphenylboron solution (2.5%) was added dropwise to the swirled solution and allowed to stand for a further 10 minutes at 0 °C. The precipitate was collected using saturated solution of potassium salt and dried to constant weight at 110 °C . The following equation was used to determine the percent of potassium.

1 mg of precipitate ≡ 0.1091 mg of K

$$\text{Potassium \%} = \frac{\text{mg of potassium obtained}}{\text{Weight of sample}} \times 100$$

3.3.6 Estimation of Calcium by UV/VIS Spectrophotometer

3.3.6.1 Calcium stock solution

A stock solution of calcium standard (1000 mg/L) was prepared by dissolving 2.4973 g of dry $CaCO_3$ in 200 ml of distilled water containing 5ml of concentrated HCl. The solution was heated to drive out CO_2 and after cooling, it was made up to 1000 ml.

3.3.6.2 Lanthanum stock solution

Lanthanum chloride solution was used in this analysis as an ionization suppression agent. Stock solution (50 g/L) was prepared by dissolving 13.369 g of $LaCl_3.7H_2O$ in distilled water containing 1 ml of dilute HCl (0.1 N) and it was made up to 1000 ml.

3.3.6.3 Preparation of calibration curve

The shape of the standard calcium curve can be either linear, parabolic or even sigmoidal (Dybko *et al.,* 1998). Different amount of Calcium stock solution 0, 10, 20, 30, 40, 50, 60 70, 80 and 90 ml respectively was taken into a 100 ml beaker, followed by 10 ml of the lanthanum chloride stock solution and made up to 100 ml with de-ionized water. The solutions prepared in this way contained 100, 200, 300, 400, 500, 600, 700, 800 and 900 mg of calcium per liter respectively, and each contained 5 g of lanthanum per liter. The absorbance of the prepared solution was directly measured using a UV-Vis spectrophotometer with the wavelength set at 422.7 nm and plotted to achieve a standard parabolic calcium calibration curve (Fig 3.3).

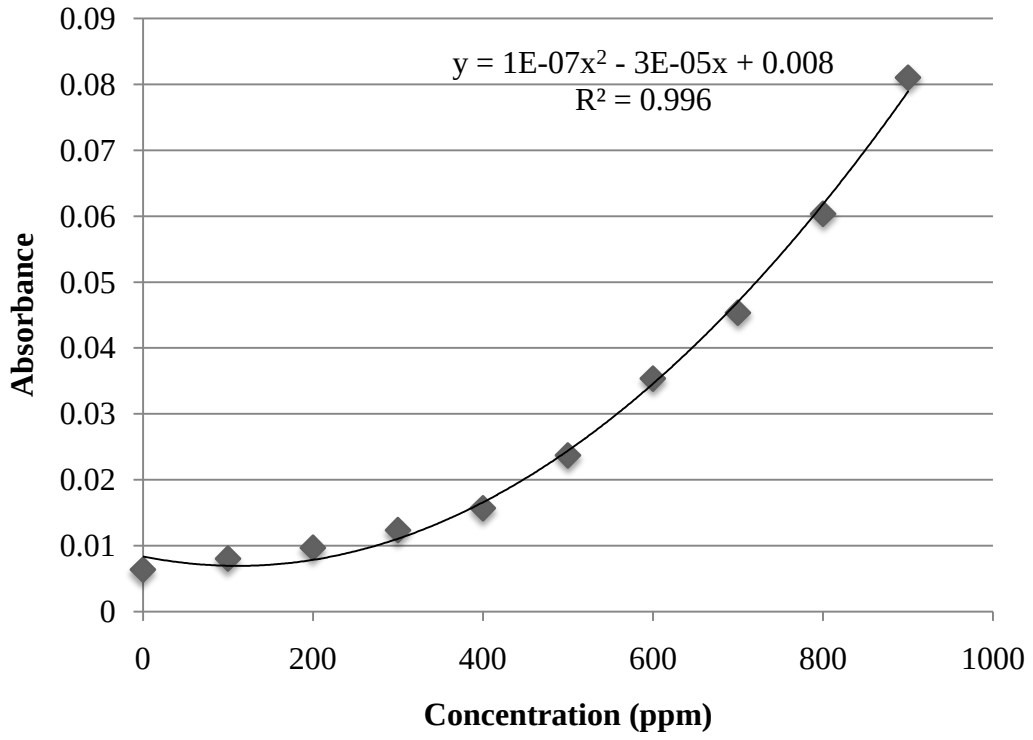

Fig 3.3: Calibration curve for calcium standards

3.3.6.4 Determination of calcium content

One ml of solvent extract and 1 ml of the lanthanum chloride solution was added in a 10 ml falcon tube. The mixture was made up to the mark with de-ionized water and mixed using a vortex mixer. The absorbance of the solution was directly measured using a UV-Vis spectrophotometer with the wavelength set at 422.7 nm. Data were stored for further analysis with triplications.

3.4 Statistical analysis

All experimental data of the study were compiled using Microsoft Excel 2007 (Microsoft Corporation, Washington, USA). Data related to the proximate analysis of Helencha powder were analyzed by IBM® SPSS® Statistics 25 (IBM Corporation, New York, USA). The design was based on Response Surface Methodology according to the Box-Benhken design using Design-Expert® DX11 (Stat-Ease, Inc., Minneapolis, USA). The first analysis in the Box-Benhken design was a statistical analysis with ANOVA using a quadratic model design to determine the significance of the model and the significant factors in the model. In the analysis R^2, adjusted R^2 and predicted R^2 were also determined. From these three values, a mathematical equation was generated from the numerical optimization process. This optimization process was used to plot the graph of the relationship between the three extraction parameters and the extracted calcium content of Helencha.

CHAPTER IV

RESULTS AND DISCUSSIONS

This study was carried out to find out the potential use of Helencha through the extraction of calcium. The effect of different treatments in relation to the extraction of calcium from Helencha powder and characterization of extracted calcium salt were studied in this research. The results obtained from the study are presented and discussed in this chapter.

4.1 Proximate composition of Helencha

Proximate compositions of Helencha powder before using it as the raw sample are shown in Table 4.1.

Table 4.1 Proximate composition of Helencha powder

Parameters	S_1 *	S_2 *	S_3 *	S_4 *	Mean ± SE
Moisture †	12.444 ^{b}	11.556 ^{c}	13.444 ^{a}	13.222 ^{a}	12.667 ± 0.236
Ash †	18.333 ^{b}	19.167 ^{a}	16.667 ^{c}	16.500 ^{c}	17.667 ± 0.350
Fiber †	15.013 ^{b}	14.270 ^{d}	15.367 ^{a}	14.510 ^{c}	14.790 ± 0.130
Calcium ‡	190.607 ^{d}	240.793 ^{a}	232.980 ^{b}	198.840 ^{c}	215.805 ± 6.471
Magnesium ‡	209.437 ^{b}	212.143 ^{a}	178.920 ^{c}	177.327 ^{d}	194.457 ± 4.937
Potassium ‡	813.600 ^{b}	781.267 ^{d}	817.633 ^{a}	787.500 ^{c}	800.000 ± 4.780

$S_{1, 2, 3, 4}$: Sample 1, 2, 3, 4; * Mean of triplicates; † g/100 g dried powder; ‡ mg/100 g dried powder.

4.1.1 Moisture content

Experimented moisture content was ranged between 11.556 to 13.444 % tabulated in Table 4.1. The mean moisture content (12.667 %) is slightly higher than the optimum moisture content (10 % or less) suggested by USDA Nutrient Database for edible powders in case of long term storage and shelf life (Ahuja *et al.*, 2015). Hazra *et al.* (2018) worked with a higher temperature (105 °C) and got almost optimum moisture content (10.06 %). This indicated that drying temperature could be slightly increased to produce low moisture content powder. Although it is very difficult and unnecessary to remove all moisture from dry foods.

4.1.2 Ash content

Ash content was ranged between 16.50 to 19.17 % tabulated in Table 4.1. The mean ash content (17.67 %) indicates that Helencha powder consisted of 18 % inorganic material. Dewanji *et al.* (1992) observed wide variation between seasons in ash content ranging from 13.7 to 18.3 % which supports the mean ash content of this study. A lower result (14 %) was found by Hazra *et al.* (2018) under *EX-SITU* conditions which indicates the river basin soil environment may have caused the increase in the ash content of the powder.

4.1.3 Fiber content

Fiber content was ranged between 14.27 to 15.37 % tabulated in Table 4.1. The mean fiber content (14.79 %) was found higher than the findings of Satter *et al.* (2016) and Hazra *et al.* (2018) ranged between 11.50 to 11.95 %. They used young leaves and shoots as raw materials. This study revealed that using whole edible portions of Helencha can increase the fiber content of the diet and fight against bowel troubles.

4.1.4 Calcium content

Calcium content was ranged between 190.61 to 240.79 mg/100g tabulated in Table 4.1. Previous studies illustrated a very wide range of calcium content found in Helencha ranged from 1.32 to 932.69 mg/100 g (Bhowmik *et al.,* 2012; Linkon *et al.,* 2015). This study found that the mean calcium content obtained by titration to be 215.81 mg/100 g which is almost similar to the findings of Hazra *et al.* (2018).

4.1.5 Magnesium content

Magnesium content was ranged between 177.33 to 212.14 mg/100g tabulated in Table 4.1. Previous studies illustrated that the magnesium content found in Helencha ranged from 6.50 to 134.3 mg/100 g (Saikia *et al.,* 2013; Narzary *et al.,* 2017). The mean magnesium content (194.46 mg/100 g) found in this study was much higher than the previous records for Helencha. The only possible cause for this is the concentration of metals and nutrients in soils increase the metal concentration within the roots, rhizomes and aerial biomass (Mendoza *et al.,* 2015).

4.1.6 Potassium content

Potassium content was ranged between 781.27 to 817.63 mg/100g tabulated in Table 4.1. Previous studies illustrated a wide range of potassium content found in Helencha ranged between 126.48 to 9166.26 mg/100 g (Narzary *et al.*, 2017; Linkon *et al.*, 2015). The concentrations were soil dependent because the waterborne Helencha contained lower potassium. The mean potassium content (800 mg/100 g) found in this study was within the range and closest to the value recorded by Satter *et al.* (2016).

4.2 Extraction of calcium

4.2.1 Experimental design and response analysis

Table 4.2 Box–Behnken experimental design matrix with experimental responses.

Run	X_1 (M)	X_2 (°C)	X_3 (min)	Calcium * (mg/100g)
1	2	50	2	136.48377
2	1	100	2	161.82306
3	1	75	1	131.72571
4	1.5	75	2	146.10951
5	2	75	3	166.07563
6	2	100	2	190.94964
7	2	75	1	149.25498
8	1	75	3	136.34619
9	1.5	75	2	149.25006
10	1.5	50	3	125.25851
11	1.5	75	2	147.75602
12	1.5	100	3	183.30019
13	1.5	75	2	145.73128
14	1.5	75	2	148.87777
15	1	50	2	115.45627
16	1.5	100	1	156.15032
17	1.5	50	1	120.15842

X_1: Concentration of HCl, X_2: Temperature, X_3: Time; * Mean of triplicates

4.2.2 Fitting Models for the extraction of calcium

The Box–Behnken responses were evaluated by analyzing the actual response and yield, and the results of ANOVA for the extraction of calcium are presented in Table 4.3.

Table 4.3 Regression summary and ANOVA for the extraction of calcium

Source	Sum of squares	df	Mean square	*F*-value	*p*-value
Model	6539.25	9	726.58	161.42	< 0.0001 [s]
X_1	1186.16	1	1186.16	263.52	< 0.0001 [s]
X_2	4746.61	1	4746.61	1054.52	< 0.0001 [s]
X_3	360.34	1	360.34	80.05	< 0.0001 [s]
X_1X_2	16.40	1	16.40	3.64	0.0979
X_1X_3	37.21	1	37.21	8.27	0.0238 [s]
X_2X_3	121.55	1	121.55	27.00	0.0013 [s]
X_1^2	11.24	1	11.24	2.50	0.1581
X_2^2	16.84	1	16.84	3.74	0.0944
X_3^2	46.63	1	46.63	10.36	0.0147 [s]
Lack of Fit	21.43	3	7.14	2.84	0.1700
R^2	0.9952	–	–	–	–
Adjusted R^2	0.9890	–	–	–	–
Predicted R^2	0.9454	–	–	–	–

X_1: Concentration of HCl, X_2: Temperature, X_3: Time; * Mean of triplicates; [S] Significant at 5% level of significance ($p < 0.05$).

The analysis of variance is essential to test the significance and adequacy of the model. It subdivides the total variation of the results in two sources of variation, the model and the experimental error, shows whether the variation from the model is significant when compared to the variation due to residual error (Segurola *et al.*, 1999). Fisher's F-test value, which is the ratio between the mean square of the model and the residual error, performs this comparison (Kasiri *et al.*, 2013).

As shown in Table 4.3, the model F-value (161.42) is greater than the F-value (2.84) obtained from the lack of fit test. The non-significant lack of fit confirmed the adequacy of the model for the present study. The significance of each term was determined by p-value ($p < 0.05$). As seen in this table the terms X_1, X_2, X_3, X_1X_3, X_2X_3, and X_3^2, were significant, with very small p-values ($p < 0.05$). The other term coefficients were not significant ($p > 0.05$).

Design of Experiment analysis of the actual experimental data in Table 4.2 showed that the data were normally distributed and that the quadratic model gave a highly significant response (Table 4.3), the R^2 was 0.9952. However, the difference between adjusted R^2 (0.9890) and predicted R^2 (0.9454) was 0.0436 and was within the recommended difference range of 0.2 or less for this type of experiment.

The quadratic model design also allows the generation of a quadratic equation which allows calculation of values in the region of the determined data.

$$\text{Calcium (mg/100 g)} = 147.54 + 12.18 \times X_1 + 24.36 \times X_2 + 6.71 \times X_3 + 2.02 \times X_1X_2 + 3.05 \times X_1X_3 + 5.51 \times X_2X_3 + 1.63 \times X_1^2 + 2.00 \times X_2^2 - 3.33 \times X_3^2$$

4.2.3 Factor-response relationship

Response surface analysis was carried out using 3D response surface plots, which explained the presence of interactions among the independent variables and their influences on the response variables. In all response surface plots, the third variable which is not mentioned is in their midpoints.

4.2.3.1 Concentration vs. Time

Fig. 4.1 present response surface plots of the effect of the relationship between the concentration of hydrochloric acid and time on the extraction of calcium. Concentration is more significant ($p < 0.05$) than time during the extraction. The plot shows that concentration and time together can obtain up to 166.08 mg calcium/100 g of dried powder during extraction. The reaction between hydrochloric acid amd calcium follows first order reaction. Following the “Collision Theory” extraction efficiency increased with increasing initial solvent concentration for a certain time (IUPAC, 1997). Depending on the half-life of HCl the reaction slows down because reaction time remains at a constant level and no other external catalyst was involved.

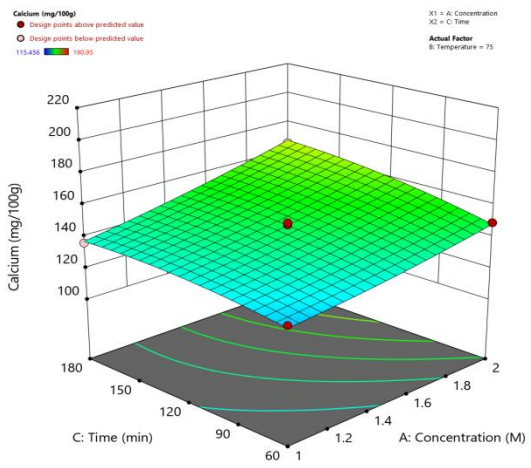

Fig 4.1 Response surface plots for the relationship between Temperature and Time during the extraction of calcium.

4.2.3.2 Temperature vs. Time

Fig. 4.2 present 3D-response surface plots of the relationship between temperature and time on the extraction of calcium. Temperature is more significant ($p < 0.05$) than the time during the extraction. The plot shows that time has a steady rate while increasing temperature has a sharp rising pattern during extraction. Temperature and time together obtained up to 183.30 mg calcium/100 g of dried powder during extraction. Hydrochloric acid breakdowns plant's calcium carbonate to calcium particles spontaneously over time. Heat works as a catalyst for this reaction, so the reaction rate speeds up while increasing the temperature.

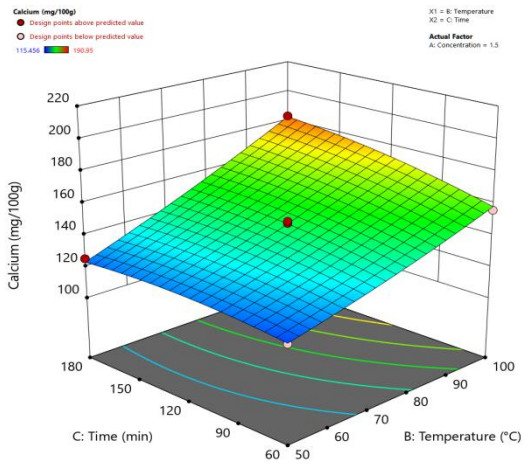

Fig 4.2 Response surface plots for the relationship between Temperature and Time during the extraction of calcium.

4.2.4 Optimization of process parameters

In order to achieve the highest extraction of calcium, the optimal conditions of the extraction process were evaluated. In order to assess the compliance of the optimal extraction conditions with laboratory conditions, solvent extraction took place in the optimal extraction conditions within the study range were provided by Design-Expert® DX11. The optimization changes the values of process parameters, such as hydrochloric acid concentration to 1.97683 M, temperature to 98.2929 °C and time to 167.617 minutes with a predicted extraction rate of 91.049 % and the expected yield of 196.49 mg calcium per 100 g powder.

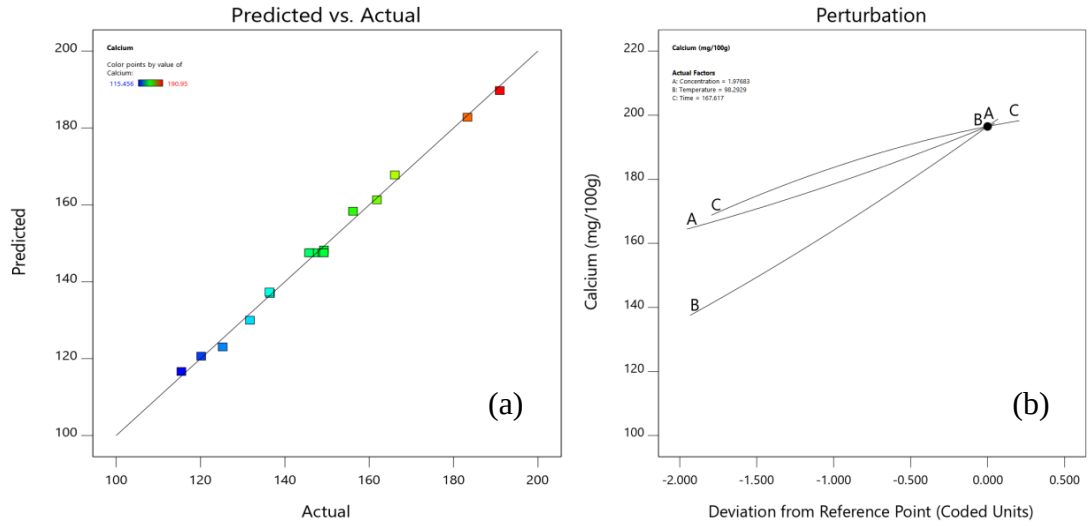

Fig 4.3 Optimization of process parameters during the extraction of calcium with Response Surface Methodology. (a) Predicted vs. Actual values of extracted calcium. (b) Perturbation of optimized parameters.

CHAPTER V

SUMMARY AND CONCLUSIONS

In this study, Box–Behnken statistical design is demonstrated to be effective and reliable in finding the optimal conditions for the extraction of calcium from Helencha. The quadratic polynomial model was fitted to the experimental data to predict the extraction rates. Using a range of measured conditions, the experimental and modeled results make it clear that attention to extraction conditions does influence the final extraction of calcium content. The response surface plots were used for estimating the interactive effect of three independent variables (hydrochloric acid concentration, temperature and time) on the response. Increasing temperature, regardless of the other variables, led to a constant increase in the total calcium content. The highest actual response experimented in the study was found 190.95 mg/100 g with 88.48 % yield to the source sample. Applying the method of the desirability function, optimization of hydrochloric acid concentration (1.97683 M), temperature (98.2929 °C) and time (167.617 minutes) gave a maximum of 91.049 % calcium extraction with the desirability of 196.488 mg/100 g. Compared with other extraction methods, hydrochloric acid proved to be the best choice for calcium extraction from Helencha as well as the other plant sources.

CHAPTER VI

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CHAPTER VII

APPENDICES

1. Proximate composition of Helencha powder

Parameter	Serial	N	Mean	SD	SE	Minimum	Maximum
Moisture	1	3	12.4443	.19283	.11133	12.33	12.67
	2	3	11.5557	.19283	.11133	11.33	11.67
	3	3	13.4443	.50921	.29399	13.00	14.00
	4	3	13.2220	.19226	.11100	13.00	13.33
	Total	**12**	12.6666	.81642	.23568	11.33	14.00
Ash	1	3	18.3333	.28868	.16667	18.00	18.50
	2	3	19.1667	.28868	.16667	19.00	19.50
	3	3	16.6667	.28868	.16667	16.50	17.00
	4	3	16.5000	.50000	.28868	16.00	17.00
	Total	**12**	17.6667	1.21231	.34996	16.00	19.50
Fiber	1	3	15.0133	.08622	.04978	14.92	15.09
	2	3	14.2700	.07550	.04359	14.19	14.34
	3	3	15.3667	.07506	.04333	15.29	15.44
	4	3	14.5100	.07550	.04359	14.43	14.58
	Total	**12**	14.7900	.45152	.13034	14.19	15.44
Calcium	1	3	190.6067	.49166	.28386	190.14	191.12
	2	3	240.7933	.53003	.30601	240.42	241.40
	3	3	232.9800	.24249	.14000	232.84	233.26
	4	3	198.8400	.42000	.24249	198.42	199.26
	Total	**12**	215.8050	22.41755	6.47139	190.14	241.40
Magnesium	1	3	209.4367	.40216	.23219	208.99	209.77
	2	3	212.1433	.28148	.16251	211.88	212.44
	3	3	178.9200	.39154	.22605	178.55	179.33
	4	3	177.3267	.29670	.17130	176.99	177.55
	Total	**12**	194.4567	17.10156	4.93679	176.99	212.44
Potassium	1	3	813.6000	.62450	.36056	812.90	814.10
	2	3	781.2667	1.45029	.83732	779.80	782.70
	3	3	817.6333	.83267	.48074	816.70	818.30
	4	3	787.5000	.65574	.37859	786.90	788.20
	Total	**12**	800.0000	16.55975	4.78039	779.80	818.30

2. Box-Behnken Analysis Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Cook's Distance	Influence on Fitted Value
1	136.48	136.97	-0.4880	0.750	0.063	-0.749
2	161.82	161.34	0.4880	0.750	0.063	0.749
3	131.73	130.01	1.71	0.750	0.782	3.269[(1)]
4	146.11	147.54	-1.44	0.200	0.014	-0.365
5	166.08	167.79	-1.71	0.750	0.782	-3.269[(1)]
6	190.95	189.74	1.21	0.750	0.391	2.031
7	149.25	148.27	0.9892	0.750	0.261	1.598
8	136.35	137.34	-0.9892	0.750	0.261	-1.598
9	149.25	147.54	1.71	0.200	0.020	0.442
10	125.26	123.06	2.20	0.750	1.291[(1)]	5.362[(1)]
11	147.76	147.54	0.2111	0.200	0.000	0.052
12	183.30	182.80	0.5012	0.750	0.067	0.770
13	145.73	147.54	-1.81	0.200	0.023	-0.474
14	148.88	147.54	1.33	0.200	0.012	0.337
15	115.46	116.67	-1.21	0.750	0.391	-2.031
16	156.15	158.35	-2.20	0.750	1.291[(1)]	-5.362[(1)]
17	120.16	120.66	-0.5012	0.750	0.067	-0.770

[(1)] Exceeds limits.

3. Sequential Model Sum of Squares

Source	Sum of Squares	df	Mean square	F-value	p-value	
Mean vs Total	3.708E+05	1	3.708E+05			
Linear vs Mean	6293.10	3	2097.70	98.22	< 0.0001	
2FI vs Linear	175.16	3	58.39	5.70	0.0154	
Quadratic vs 2FI	**70.99**	**3**	**23.66**	**5.26**	**0.0327**	**Suggested**
Cubic vs Quadratic	21.43	3	7.14	2.84	0.1700	Aliased
Residual	10.08	4	2.52			
Total	3.774E+05	17	22198.46			

4. Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	4.62	0.9577	0.9480	0.9130	571.38	
2FI	3.20	0.9844	0.9750	0.9261	485.79	
Quadratic	**2.12**	**0.9952**	**0.9890**	**0.9454**	**358.63**	**Suggested**
Cubic	1.59	0.9985	0.9939		*	Aliased

* Case(s) with leverage of 1.0000: PRESS statistic not defined.

5. Fit Summary

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	< 0.0001	0.0150	0.9480	0.9130	
2FI	0.0154	0.0507	0.9750	0.9261	
Quadratic	**0.0327**	**0.1700**	**0.9890**	**0.9454**	**Suggested**
Cubic	0.1700		0.9939		Aliased

6. Fit Statistics

Std. Dev.	2.12	**R^2**	0.9952
Mean	147.69	**Adjusted R^2**	0.9890
C.V. %	1.44	**Predicted R^2**	0.9454
		Adeq Precision	44.9054

7. Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Linear	267.58	9	29.73	11.80	0.0150	
2FI	92.42	6	15.40	6.11	0.0507	
Quadratic	**21.43**	**3**	**7.14**	**2.84**	**0.1700**	**Suggested**
Cubic	0.0000	0				Aliased
Pure Error	10.08	4	2.52			

8. ANOVA for Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	6539.25	9	726.58	161.42	< 0.0001	significant
X_1-Concentration	1186.16	1	1186.16	263.52	< 0.0001	
X_2-Temperature	4746.61	1	4746.61	1054.52	< 0.0001	
X_3-Time	360.34	1	360.34	80.05	< 0.0001	
$X_1 X_2$	16.40	1	16.40	3.64	0.0979	
$X_1 X_3$	37.21	1	37.21	8.27	0.0238	
$X_2 X_3$	121.55	1	121.55	27.00	0.0013	
X_1^2	11.24	1	11.24	2.50	0.1581	
X_2^2	16.84	1	16.84	3.74	0.0944	
X_3^2	46.63	1	46.63	10.36	0.0147	
Residual	31.51	7	4.50			
Lack of Fit	21.43	3	7.14	2.84	0.1700	not significant
Pure Error	10.08	4	2.52			
Cor Total	6570.76	16				

9. Final Equation

9.1 Coded Factors

$$\text{Calcium} = 147.54 + 12.18 \times X_1 + 24.36 \times X_2 + 6.71 \times X_3 + 2.02 \times X_1X_2 + 3.05 \times X_1X_3 + 5.51 \times X_2X_3 + 1.63 \times X_1^2 + 2.00 \times X_2^2 - 3.33 \times X_3^2$$

9.2 Actual Factors

$$\text{Calcium} = 113.50345 - 19.59772 \times \text{Concentration} - 0.189576 \times \text{Temperature} - 0.094414 \times \text{Time} + 0.161982 \times \text{Concentration} \times \text{Temperature} + 0.101668 \times \text{Concentration} \times \text{Time} + 0.003675 \times \text{Temperature} \times \text{Time} + 6.53404 \times \text{Concentration}^2 + 0.003200 \times \text{Temperature}^2 - 0.000924 \times \text{Time}$$