ULTRASOUND ASSISTED EXTRACTION OF THEOBROMINE FROM COCOA BEAN SHELL

By

FARHANA SHIRIN (2019-06-006)

MUBASHIR K.P. (2019-06-014)

SAJEER K (2019-06-017)

UMMUL ASNA K (2019-06-020)

VISHNU PRASAD J.R. (2019-06-021)

DEPARTMENT OF PROCESSING AND FOOD ENGINEERING

KELAPPAJI COLLEGE OF AGRICULTURAL ENGINEERING AND TECHNOLOGY

TAVANUR-679573, MALAPPURAM

KERALA, INDIA

2023

ULTRASOUND ASSISTED EXTRACTION OF THEOBROMINE FROM COCOA BEAN SHELL

By

FARHANA SHIRIN (2019-06-006)

MUBASHIR K.P. (2019-06-014)

SAJEER K (2019-06-017)

UMMUL ASNA K (2019-06-020)

VISHNU PRASAD J.R. (2019-06-021)

PROJECT REPORT

Submitted in partial fulfilment of the requirement for the degree

Bachelor of Technology

in

Food Engineering & Technology

Faculty of Agricultural Engineering and Technology

Kerala Agricultural University

DEPARTMENT OF PROCESSING AND FOOD ENGINEERING

KELAPPAJI COLLEGE OF AGRICULTURAL ENGINEERING AND TECHNOLOGY

TAVANUR-679573, MALAPPURAM, INDIA

2023

DECLARATION

We hereby declare that this project report entitled "**ULTRASOUND ASSISTED EXTRACTION OF THEOBROMINE FROM COCOA BEAN SHELL**" is a bonafide record of research work done by us during the course of research and that the report has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society

Place: Tavanur

Date:

Farhana Shirin (2019-06-006)

Mubashir K.P. (2019-06-014)

Sajeer K (2019-06-017)

Ummul Asna K (2019-06-020)

Vishnu Prasad J.R. (2019-06-021)

CERTIFICATE

Certified that this project entitled "**ULTRASOUND ASSISTED EXTRACTION OF THEOBROMINE FROM COCOA BEAN SHELL**" is a bonafide record of project work jointly done by Farhana Shirin (2019-06-006), Mubashir K.P. (2019-06-014), Sajeer K (2019-06-017), Ummul Asna K (2019-06-020) and Vishnu Prasad J.R. (2019-06-021) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to them.

Mrs. Sreeje R

Assistant Professor

Dept. of Processing and food Engineering

KCAET, Tavanur

Mr. Ramees P M

Assistant Professor (C)

Dept. of Processing and food Engineering

KCAET, Tavanur

ACKNOWLEDGEMENT

We hereby wish to acknowledge our gratitude to all the researchers and practitioners who have contributed towards our understanding and thoughts. We sincerely thank all of them, friends, near and dear ones.

We avail this opportunity to express our deep sense of gratitude and heartfelt indebtedness to **Dr. Prince M.V.**, Professor, Department of Processing and Food Engineering, K.C.A.E.T, Tavanur, for support, benevolent criticisms and encouragement during the course of project work.

We are immensely thankful to our project guide **Mrs. Sreeja R**., Assistant Professor, Department of Processing and Food Engineering K.C.A.E.T., Tavanur, for her constant support, encouragement and guidance that helped us to complete our Project successfully.

With extreme pleasure we express our whole-hearted gratitude to our co-guides **Dr Anila** and **Mr Ramees P M**, Assistant Professors (c), Department of Processing and Food Engineering K.C.A.E.T., Tavanur for their support and sincere help throughout the period of project work and also for their valuable suggestions and ideas.

Our special thanks to **Er Anjali M G** for her time-to-time help and kind co-operation throughout this work

We express our thanks to our **friends, members of Library, KCAET Tavanur** and **all the faculty members of KCAET** for their ever-willing help and co-operation. Our heartfelt thanks to **Kerala Agricultural University** in providing the favourable circumstances for the study.

We are greatly indebted to our parents for their blessings, prayers, and support without which we could not have completed this work. Ultimately, we bow down ourselves to the **ALMIGHTY** for leading us to the light at the end of the tunnel.

Farhana Shirin

Mubashir K.P.

Sajeer K

Ummul Asna K

Vishnu Prasad J.R.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	percentage
&	:	and
/	:	per
=	:	equal to
±	:	plus, or minus
≈	:	approximate
ºC	:	degree Celsius
µL	:	Micro litre
cm	:	centimetre
et al.	:	and others
etc.	:	et cetera
Fig.	:	figure
g	:	gram
Wt.	:	weight
h	:	hour
ha	:	hectare
hp	:	horse power
KAU	:	Kerala Agriculture university
kg	:	kilogram
kg/ha	:	kilogram per hectare
Ws/m^3	:	watt second per cubic metre

W	:	watt
W/m^2	:	watt per square metre
Kpa	:	kilo pascal
n.d.	:	not determined in the study
KHz	:	Kilo hertz
kN	:	kilo newton
L	:	litre
min	:	minute
ml	:	millilitre
mm	:	millimetre
m/s	:	metre per second
MB	:	methylene blue
MT	:	metric tonne
No.	:	number
CBS	:	cocoa bean shell
Tr	:	Traces
UAE	:	Ultrasound assisted extraction
MAE	:	Microwave assisted extraction
DES	:	Deep eutectic solvents
SFE	:	supercritical fluid extraction
PLE	:	Pressurised liquid extraction
HPLC	:	High performance liquid chromatography
FY	:	fiscal year
t/ha	:	tonne per hectare
kcal	:	kilo calorie
P	:	probability

DEDICATED TO

COCOA FARMERS

INTRODUCTION

CHAPTER I

INTRODUCTION

Cocoa (*Theobroma cacao L.*) is a perennial cash crop and its natural habitat is the humid tropics (Ndukwu, 2009). It is widely produced in West Africa and South America and is a great economic tree crop, with so many industrial as well as domestical use (known as "the food of the gods"). It is the main raw material in the production of chocolates, cosmetics, health drinks, pharmaceuticals etc. Cocoa beans (seeds of cocoa fruit) are processed to obtain chocolate liquor, cocoa butter and cocoa powder which are the main ingredients of chocolate and related products. These will impart a characteristic and distinctive flavour to the products. Cocoa is even used as medicine, for infectious intestinal diseases and diarrhea, asthma, bronchitis etc. Cocoa butter is also used as an ingredient in skin care products.

In India, there are numerous food processing industries to produce wide range of products along with the discharge of plenty of by-products. Both economically and ecologically, food industry waste management is a growing problem, that have to be considered throughout the food supply chain. Various standardized industrial waste treatments and technologies helps in reduction of environmental impact. Also, by-products in the food industry may contain valuable bioactive components and their utilization as raw material in some other production could result in less waste thereby less environmental side effects. The wastes can also be used as animal feeds. Considering a cocoa processing industry, about 80% of cocoa fruit is discarded as residual biomass, including cocoa pod husks, cocoa bean shells and cocoa sweatings. Usually these are generally used as fertilizers. Recently, these by-products are also used in the production of high-value-adding molecules with pharmaceutical, medical and cosmetic industries (Nguyen, 2017).

Cocoa bean shell (CBS) is a by-product of the chocolate industry, which is usually removed away from the beans after roasting and discarded as waste. Since the shells present as 12% - 20% of the bean, it can be considered as the largest waste generated by the processing of cocoa beans. The basic compositions are, polyphenols (ca. 1–2%), alkaloids such as theobromine (ca. 1–2%) and caffeine, vitamins such as Vitamin D, minerals such as calcium and phosphorus, amino acids, as well as soluble and insoluble dietary fibers (ca. 25–30%), etc. (Handoja et al., 2019). Significant amounts of Methylxanthines like theobromine and caffeine

confirm the use of CBS as a new source of those beneficial components. It is known that theobromine (3,7- dimethylxanthine) is a brain stimulant, diuretic with a potential in blood pressure reduction, cardiac stimulant etc. (Pavlovic et al., 2020). Caffeine is known to be a central nervous system stimulant even more than theobromine and also respiratory, diuretic and skeletal muscle stimulant. Still, there are various concerns about methylxanthine toxicity which depends on specific compound, type of living organism and their sensitivity to methylxanthines what may be genetically originated (Monteiro et al., 2016). However, our study is designed for the extraction of theobromine from cocoa bean shell along with ultrasound application.

Numerous methods have been evolved for the extraction of Methylxanthines like theobromine from Cocoa bean as well as CBS. Such a known method includes extraction using chlorinated hydrocarbon solvents such as chloroform, ethylene dichloride or tetrachloroethane. The conventional soxhlet extraction and stirring method of extraction can be also used to extract analytes from solid samples which requires more amount of solvents. In comparison with normal conventional methods there are various innovative technologies like super critical fluid extraction, ultrasound assisted, etc. with better yield and other advantages.

Ultrasound-assisted extraction (UAE) is a significant extraction method which involves the pre-treatment by sonication. Use of ultrasonic radiation shortens the steps of the process or time in comparison with other techniques. The method significantly reduces the time required to extract specific compounds, with higher yields and better quality of the extract while maintaining at a reduced temperature. It is also environment-friendly, clean, flexible, versatile and easy to use extraction method which requires low investment costs compared to some other novel green extraction technologies. The principle of UAE is acoustic cavitation (Tiwari, 2015). Under the influence of ultrasound, physical and chemical properties of the plant material change, what leads to releasing of extractable compounds.

By considering the above facts, the study named “Ultrasound assisted extraction of theobromine from Cocoa bean shell” was conducted under the following objectives:

1. To characterize the physico-chemical properties of cocoa bean shell
2. To optimize the process parameters for the ultrasound assisted extraction of theobromine.

3. To conduct comparative studies of traditional stirring method and ultrasound assisted extraction method of theobromine from cocoa bean shell in terms of extraction yield and physical characteristics.

REVIEW OF LITRATURE

CHAPTER II

REVIEW OF LITERATURE

This chapter contains reviews of researches related to ultrasound assisted extraction of theobromine from cocoa bean shell. This chapter provides general information about cocoa, theobromine and its benefits, extraction methods, and application of ultrasound, etc.

1.1 COCOA

Bean is the seed of cocoa tree (*Theobroma cacao*), a tropical plant indigenous to the equatorial regions of the America. The cocoa beans are processed to get cocoa mass/cocoa liquor, from which cocoa powder, cocoa butter and chocolates are made. The cocoa bean is greatly appreciated for its aroma and its nutrients (phosphorus, magnesium, iron, zinc, manganese, copper, potassium, selenium, vitamins B2 and B3). When fermented and dried, it contains 50 - 57% lipids, 10% proteins, 12% fibres, 8% carbohydrate (starch), approximately 5% minerals, etc. As a cash crop, a cocoa plantation can last between 15 and 40 years. Cocoa constitutes a significant source of income for the small-scale operators who are responsible for the majority of worldwide production. Cocoa production is also a source of foreign exchange for producing countries. Chocolate is the most sought after derived product of cocoa. Chocolate is sold directly to the consumer as solid bars of eating chocolate, as packaged cocoa, and as baking chocolate. It is also used by confectioners as coating for candy bars and boxed or bulk chocolates, by bakery product manufacturers and bakers as coating for many types of cookies and cakes, and by ice-cream companies as coating for frozen novelties. Cocoa powders, chocolate liquor, and blends of the two are used in bulk to flavour various food products and to provide the flavours in such “chocolate” products as syrups, toppings, chocolate milk, prepared cake mixes, and pharmaceuticals.

1.1.1 HISTORY

The cocoa tree is native to the Amazon Basin. It was domesticated by the Olmecs (Mexico). More than 4,000 years ago, it was consumed by pre-hispanic cultures along the Yucatán, including the Maya, and as far back as Olmeca civilization in spiritual ceremonies. It also grows in the foothills of the Andes in the Amazon and Orinoco basins of South America, in Colombia and Venezuela. Wild cocoa still grows there. Its range may have been larger in

the past; evidence of its wild range may be obscured by cultivation of the tree in these areas since long before the Spanish arrived. In 1828 C.J. Van Houten of the Netherlands patented a process for obtaining “chocolate powder” by pressing much of the cocoa butter from ground and roasted cocoa beans. In 1847 the English firm of Fry and Sons combined cocoa butter, a by-product of the pressing, with chocolate liquor and sugar to produce eating chocolate, and in 1876 Daniel Peter of Switzerland added dried milk to make milk chocolate. The proliferation of flavoured, solid, and coated chocolate foods rapidly followed. Cocoa trees grow in a limited geographical zone, of about 20°to the north and south of the Equator. Nearly 70% of the world crop today is grown in West Africa. The cocoa plant was first given its botanical name by Swedish natural scientist Carl Linnaeus in his original classification of the plant kingdom, where he called it Theobroma ("food of the gods") cocoa.

1.1.2 COCOA PRODUCTION

Globally, cocoa is grown in a narrow belt of around 20 degrees either side of the equator, as it offers the perfect conditions for growing cocoa. The cocoa tree needs high temperatures, humid conditions and plenty of rainfall to grow successfully. For these reasons, cocoa is produced predominantly in the hot and humid regions of Africa, but also in Asia, Central and South America and Australia.

Even temperatures between 21 and 23℃, with a fairly constant rainfall of 1,000 to 2,500 mm per year, are needed for cocoa cultivation without the presence of hot dry winds and drought (worldagroforestry.org).

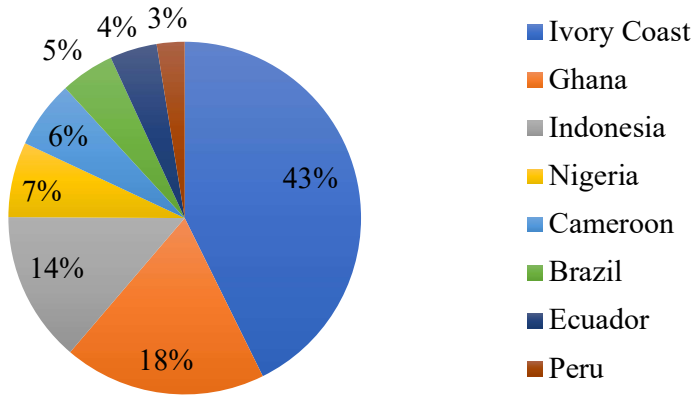

Figure 1.1. World cocoa production (%) in 2021-22.

Source*:* worldpopulationreview.com

Nearly two-thirds of the world‟s cocoa beans come from West Africa, with the Ivory Coast and Ghana being the two biggest producers. These two countries alone provide half of the world's cocoa. The following largest cocoa producing countries, in order, are Indonesia, Nigeria, Cameroon, Brazil, Ecuador, Peru and Dominican Republic (chocolatephyanak.com).

The Ivory Coast is the largest cocoa producing country in the world supplying over 40% of the world's cocoa beans at 2034,000MT in 2022**.**

1.1.2.1 Production scenario of cocoa in India

In India Cocoa is being cultivated in the States of Kerala, Karnataka, Andhra Pradesh and Tamil Nadu in an area of 1,03,376 ha with total production of 27,072 MT (2022). Andhra Pradesh ranks first having area 39,714 ha and production of 10,903 MT. The highest productivity is also in Andhra Pradesh which is 950 kg/ha. The average productivity of cocoa in Indian is 669 Kg/ha. (Directorate of Cashew nut and cocoa development, 2022).

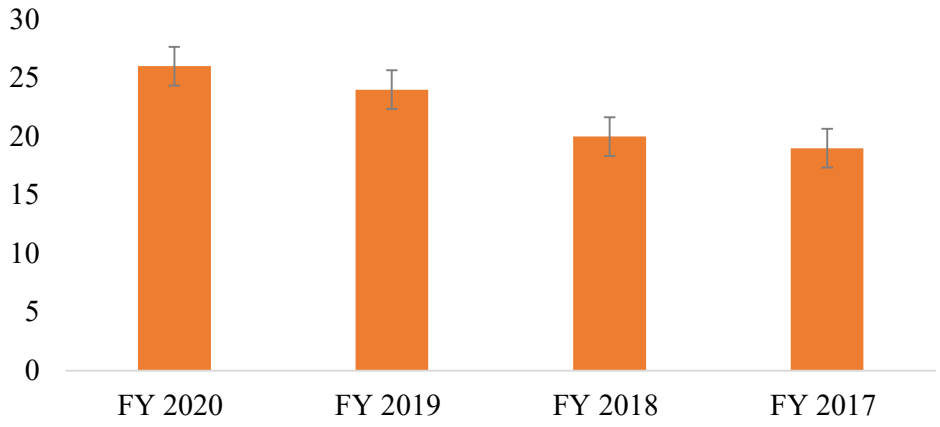

Figure 2.2. Cocoa production in India during FY 2017-2020 (in 1000 metric tons)

Source: Statista Research Department, 22 October 2020.

1.1.3 VARIETIES OF COCOA

The three large and distinct groups within the cocoa species are *Criollo, Forastero* and *Trinitario* (Adewumi, 1997).

Criollo native to Central America and considered the best flavoured cocoa. This variety has white to pale yellow cotyledon. The variety is also characterized by slender trees, green pods or pods coloured by anthocyanin pigments. Leaves are relatively smaller and more oval than the other types. The seed is cylindrical (in cross section) and plumb. It weighs around one

gram and is covered with sweet mucilage. Pods are soft, easy to break, and do not have the woody layer found in other varieties. Immature pod colour ranges from pale green to red. On fermentation and drying the cotyledon colour turns light brown. It is very susceptible to most pests and diseases of cocoa. It produces the best quality chocolate. With proper attention and care, the yield can be enhanced high as 1.0-1.5 t/ha.

Forastero is native to Venezuela and Northern Amazon Basin. It is commercially grown in Brazil, Central America, the Caribbean and West Africa. The group is characterized by green pods, absence of anthocyanin pigmentation, thick pericarp, strongly lignified mesocarp, plump but slightly flattened purple beans. The trees are vigorous, with leaves larger than those of *Criollo*.

Trinitario is a product of hybridization between *Criollo* and *Forastero* has its origin in Trinidad. It shows a range of characteristics possessed by both *Criollo* and *Forastero*. The trees are generally vigorous with a variable reaction to pests and diseases. Pods are green or pigmented. Beans colour varies from light to very dark purple. The most useful and valuable part of the crop is the bean. The highest percentages of cocoa beans produced in the developing countries are exported. The exported beans are processed abroad and the end products are imported back to the developing countries at a relatively high cost.

Figure 2.3. Types of cocoa fruits (Criollo, Forastero and Trinitario respectively)
Source: Gardner and Baker, (2020)

1.1.4 CONSTITUENTS OF COCOA

The cocoa fruit comprises of the pod or shell, beans or seeds, husk, cocoa bean shell and mucilaginous pulp which contains a sweet juice referred to as “sweating”. Generally, cocoa pods are oval-shaped and vary in size. The length is normally between 20 and 32 cm. Its colour ranges from yellow or green to red violet. The surface texture is warty and deep furrowed to nearly smooth in most cases. The husks appear appreciably in thickness. Each bean is surrounded by mucilaginous pulps. The number of beans per pod is usually between 30 and 40.

Each bean consists of two convoluted cotyledons and is enclosed in the testa. The cotyledon has its colour varying from white to purple.

When cocoa is processed, there are three types of co-products obtained: cocoa pod husk, cocoa bean shells and cocoa mucilage. These by-products are usually considered as "waste" and left to rot on the cocoa plantation, which can cause environmental problems, such as producing foul odours or propagate diseases (e.g., pod rot, because they are not composted (Martinez *et al.,* 2012; Barazarte *et al.,* 2008 and Sukha, 2003). Figure 2.4 shows the cocoa fruit, beans with the pulp and shell, as well as the pod or husk.

1.2 COCOA BEAN SHELL (CBS)

Cocoa production generates substantial quantities of waste. Indeed, only 10% of the total cocoa fruit weight is used for its commercialization, while the remaining 90% is discarded as waste or by-products (Battegazzore *et al.,* 2014). One of these by-products is the external tegument that cover the cocoa beans, also known as cocoa bean shells, which are generated during the cocoa bean roasting process. Generally, CBS constitute about 10%–17% of the total cocoa bean weight (Hashimoto *et al.,* 2018).

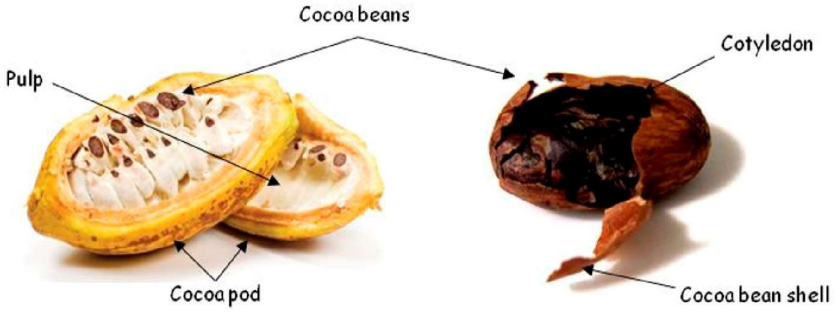

Figure 2.4. Cocoa beans and their processing by-products

Source: Rojo- Poveda *et al.* (2020).

Cocoa shell have high nutritional value owing to the presence of a variety of bio-compounds, such as phenolic compounds, dietary fibres, theobromine and a lipid profile similar to that of cocoa butter, besides its chocolate colour and flavour.

1.2.1 Nutritional and Chemical Composition of CBS

The proximate composition of CBS has been reported by several authors is summarized in Table 2.1 (Zeppa *et al.,* 2020). CBS proximate composition comprises proteins, fats, sugars, moisture, and ashes. Composition similar to cocoa beans, but CBS has less percentage of fats and higher number of fibers. CBS also have a higher content of proteins, fats, and carbohydrates

compared to other cocoa by-products, such as cocoa pods (Pérez *et al.,* 2015). Proximate composition of CBS depending several variable factors, such as the climatic conditions of the farming area, the cocoa variety, processing conditions (fermentation, drying, roasting temperature).

Table 2.1. Nutritional and chemical composition of cocoa bean shells.

Parameter	**Amount** [a]
Energy (kcal/100 g)	122.00
Moisture (%)	3.60–13.13
Ash (g/100 g)	5.96–11.42
Proteins (g/100 g)	10.30–27.40
Fats (g/100 g)	1.50–8.49
Carbohydrates (g/100 g)	7.85–70.25
-Starch (g/100 g)	0–2.80
-Soluble sugars (g/100 g)	0.16–1.66
Dietary fiber (g/100 g)	39.25–66.33
-Soluble fiber (g/100 g)	7.03–16.91
-Insoluble fiber (g/100 g)	28.34–50.42
Pectin (g/100 g)	7.62–15.59
Minerals	
-Calcium (g/100 g)	0.23–0.44
-Phosphorus (g/100 g)	0.58–1.00
-Magnesium (g/100 g)	0.48–1.29
-Potassium (g/100 g)	1.25–1.82
-Sodium (mg/100 g)	16.00–192.20
-Iron (mg/100 g)	27.60–80.50
-Manganese (mg/100 g)	4.53
-Copper (mg/100 g)	2.35–6.62
-Selenium (mg/100 g)	0.21

-Cobalt (mg/100 g)	0.10
-Zinc (mg/100 g)	2.75–19.00
-Chromium (mg/100 g)	0.67-4.86
Vitamins	
-B1 (μg/g)	0.70–3.10
-B2 (μg/g)	0.90–3.10
-B6 (μg/g)	Tr
-D (μg/g)	tr–0.53
-E (μg total tocopherols/g CBS fat)	1.02
Polyphenol content	
-Total phenolic content [b]	3.12–94.95
-Total flavonoid content [c]	1.65–40.72
-Total tannin content [c]	1.70–25.30
Flavanols	
-Epicatechin (mg/g)	0.21–34.97
-Catechin (mg/g)	0.18–4.50
-Procyanidin B1 (mg/g)	0.55–0.83
-Procyanidin B2 (mg/g)	0.23–1.38
Methylxanthines	
-Theobromine (g/100 g)	0.39–1.83
-Caffeine (g/100 g)	0.04–0.42
Volatile organic compounds (aromatics; μg/g)	4.92–16.10

([a] Data are referred to a CBS dry weight basis unless indicated differently. Intervals have been created, comprising all the values from the cited literature).

1.2.2 Cocoa Bean Shell Applications

According to Chronopoulos *et al.* (2011) It is possible to use milled cocoa shells, without any modifications, as well as to alkalize cocoa shells, and then use them as a food additive.

Relatively high values of dietary fiber together with phenolic compounds, imply that this by-product is interesting to the food industry (in the manufacturing of confectionery products and bakery products, or in the preparation of low calorie dietetic and fiber-rich products, etc.) (Zhong *et al.,* 2012 and Vıtola *et al.,* 2016) However, the most common use is still for feedstuff.

1.2.2.1 Use in Feedstuff

Silva *et al.* (2005) and Veloso *et al.* (2010) explored a number of studies to know the potential of cocoa shells to replace a part of a usual animal diet and investigated their influence on animals, because it contains theobromine, which may have a negative effect on some species.

The toxicity of a cocoa shell meal to broilers was examined by Day and Dilworth (1984). They added cocoa shell in amounts of 1, 2, 4, and 6% to the meal and concluded that 4 and 6% had a significant influence on the decrease of body weight of broilers. In a subsequent experiment, they added exactly the same amount of pure theobromine as there was in cocoa shells that were in the previous meals, but the broilers' weight was drastically decreased. Pure theobromine was more toxic than that furnished by the cocoa shell meal.

The economics of using cocoa shells as a food supplement for rabbits was examined by Ayinde *et al.* (2010). The authors concluded that untreated cocoa shells can be used at 100 g/kg inclusion in rabbit feed, while cocoa shells treated hot water can be included up to 200 g/kg in rabbit feed, for optimum growth performance and the highest cost–benefit ratio.

1.2.2.2 Use in Agriculture

It is possible to use cocoa husk mulch to suppress weed in perennial fruit crops, gardens, urban landscapes, and occasionally in vegetable crops in organic production systems (Bond and Grundy, 2001).

Arentoft *et al.* (2013) examined the difference between cocoa mulch and bark mulch in suppressing weed growth. Cocoa mulch was more effective, because when compared with

bark mulch, a thinner layer of cocoa mulch was needed to reduce the percentage of green pixels by 50% or 90% in relation to control plots.

Hale *et al.* (2013) investigated sorption and desorption of phosphate-P, ammonium-N, and nitrate-N in cocoa shell and corn cob biochar. The authors confirmed that biochar can add and slowly release essential nutrients to soil in order to improve agricultural properties, as the real-world biochar used here were able to release PO_4^{3-}–P and weakly exchange NH_4^{+}–N.

1.2.2.3 Use in Biofuels

Ethanol from lignocellulosic biomass, such as agricultural residue, is one of the important alternatives for fossil fuel. In their study, Awolu and Oyeyemi (2015) examined ethanol production from cocoa shells using acid hydrolysis and *Saccharomyces cerevisiae*. The result showed that pH had the highest effect on the cocoa shell ethanol yield, followed by fermentation time and yeast concentration; that cocoa shells are an excellent source for such production; and that response surface methodology is a promising tool in the optimization of ethanol production. Additionally, cocoa shells showed good potential for biogas production, with cumulative methane yields and this usage is performed by Mancini *et al.* (2016).

1.2.2.4 Use as an Adsorbent

Selvaraju *et al.* (2013) said that for the process of adsorption, agricultural waste products are used as natural or modified products through the activation process. Fioresi *et al.* (2017) investigated the grafting of aryl diazonium salt on cocoa shells. The authors concluded that modified cocoa shells can be used as a low-cost adsorbent to entrap pollutants such as heavy metal ions, gases, or industrial dye.

Ahmad *et al.* (2013) showed that cocoa shell-based activated carbons have the potential to be used as an adsorbent for 4-nitrophenol and methylene blue (MB) dye in water or wastewater treatments, and that acid treatment at a higher temperature and higher acid concentration resulted in the development of a new structure of cocoa shell-based activated carbon, which, through the elimination of carbonates and formation of amorphous silica, is highly mesoporous.

The application of microwave-assisted activated carbon from cocoa shells as an adsorbent for removal of sodium diclofenac and nimesulide from aqueous effluents was investigated by Saucier *et al.* (2015). It effectively removed approximately 95% of a mixture of different organic compounds in a medium with high salinity and sugar contents.

1.2.2.5 Use as a Dye

Tran *et al.* (2017), biofilaments based on cocoa shell waste and biodegradable polymer (ε-caprolactonethey are necessary) (PCL) have been prepared using a single-screw extruder. Using this simple and solvent-free fabrication technique, uniformly structured cocoa shell waste biofilaments can be produced in a very reproducible manner, and used in 3D printing of diverse objects with potential household and biomedical applications.

1.2.2.6 Use in Food Products

Jozinovic´ *et al.* (2017) produced corn snack products enriched with cocoa shells. They added milled shells to corn grits in 5%, 10%, and 15% d.m., and extruded in a laboratory single-screw extruder. The authors concluded that it can be successfully employed as nutritional fortification agent. Sanchez Mundo *et al.* (2017) used cocoa shell flour for production of muffins and biscuits.

1.2.2.7 Cocoa Bean Shell Extracts

Cocoa shells can also be used as a raw material for the production of extracts rich in fibers, polyphenols, antioxidants, and so on, which can then be used for further applications. The most abundant bioactive compounds in cocoa shell, according to some authors, are shown in Table 2.2., together with the applied extraction technique.

Table 2.2. The most abundant bioactive components in cocoa shells.

Researchers	Extraction method	Total phenols (mg/g)	Theobromine (mg/g)	Caffeine (mg/g)	Catechin (mg/g)	Epicatechin (mg/g)
Hernández-Hernández *et al.,* (2018)	Methanol-water extraction	14.64	10.20	n.d.	1.02	15.84
	Ethanol-acidified water extraction	49.46	11.00	n.d.	1.97	9.00
	Water extraction	5.77	8.47	n.d.	1.65	6.93
	Methanol-acidified water extraction	20.39	11.62	n.d.	4.00	17.70

	Acidified water extraction	9.40	6.6	n.d.	6.16	7.04
Barbosa-Pereira *et al.* (2018)	Pulsed Electric Field Extraction	24.93 – 32.30	4.64 – 10.92	1.59 – 4.21	n.d.	0.21 – 2.12
Arlorio *et al.* (2001)	Supercritical CO2 extraction	18.2	12.9	n.d.	n.d.	n.d.

Source: Balenti´c *et al.* (2018)

1.2.2.7.1 Polyphenol-rich cocoa shell extracts

As cocoa shells contain a certain proportion of phenolic components, which are stored in cocoa seed cotyledons, it is believed that they migrate from cotyledon cocoa beans in different chocolate production processes, such as fermentation, roasting, and alkalizing. This reduces the number of polyphenols in cocoa beans and gives polyphenol-enriched cocoa shells. The most common compounds are flavanols: epicatechin, catechin, and procyanidins. There are a few methods to obtain polyphenol-enriched extracts from cocoa shells. Generally, higher polyphenolic yields are expected from unfermented shells when compared with fermented ones, as well as from roasted shells when compared with unroasted shells.

1.2.2.7.2 Methylxanthine-rich cocoa shell extracts

During the processing of cocoa beans, in the fermentation stage, methylxanthines migrate from the bean into the shell. Theobromine is the most abundant methylxanthine in cocoa shells, followed by caffeine and theophylline. Theobromine is a white powder; a harsh and odourless component that can be stimulant in moderate amounts, while it may be poisonous in larger amounts. Theobromine gives bitterness to cocoa and chocolate products (Bentil *et al.,* 2015). Theobromine can be removed from the cocoa shell by extraction techniques, such as supercritical CO2 extraction. It is possible to completely remove theobromine from shells using supercritical CO2 and to obtain extracts rich in this component. Hot water treatment has also proved to be capable in reducing the theobromine content (Ayinde *et al.,* 2010). Bradbury & Kopp (2006) patented the production of two different extracts from cocoa shells; theobromine fraction and polyphenol enriched fraction.

1.2.2.7.3 Fiber-rich cocoa shell extracts

The proportion of fiber in cocoa shells depends on whether they are roasted or not. It has been reported that, in roasted seeds and shells, formation of Maillard compounds increases

the fiber content (Redgwell *et al.,* 2003). The optimal technique for extraction of pectins was given by Mollea *et al.* (2008) by hot acid extraction method. Redgwell *et al.* (2003) published that total dietary fiber content was approximately 40%, not as high as previous reports. A study by Castillejo *et al.* (2006) confirms the beneficial effect of a supplement of cocoa shells that are rich in dietary fiber (39.6 g of total fiber and 13.6 g of ß-fructosans per 100 g of product) on chronic idiopathic constipation in children. Cocoa shells have potential health effects on high cholesterol levels as well and it is reported by Ramos *et al.* (2008). He reported that the cocoa product obtained after enzymatic treatment of cocoa shells, rich in soluble dietary fiber and with appreciable amounts of antioxidant polyphenols, brought about remarkable hypocholesterolemic and hypotriglyceridemic responses in rats that were fed an atherogenic diet. It also decreased lipid peroxidation, thus diminishing several risk factors for cardiovascular diseases. It is also showed that the cocoa shell has nutritional effects, reducing food intake and body weight gain.

1.1 SOXHLET EXTRACTION

Soxhlet extraction is an exhaustive extraction technique widely applied to analytes that are sufficiently thermally stable. The extraction solvent is continuously cycled through the matrix, by boiling and condensation, with the sample being collected in the hot solvent.

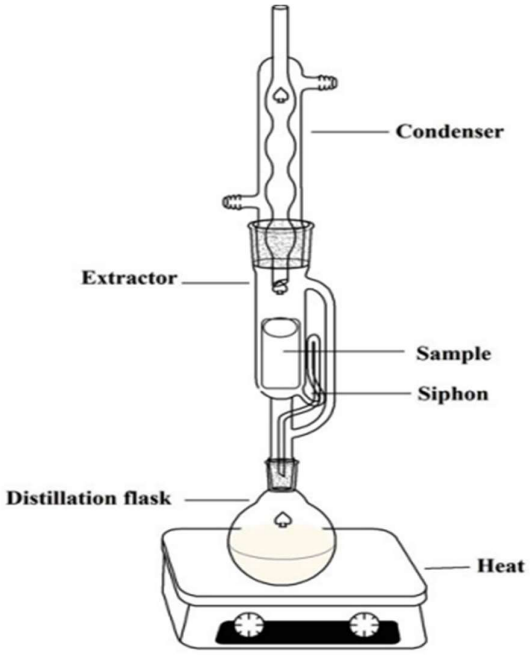

Figure 2.5. Soxhlet Apparatus

Source: Paulraj et.al., (2016)

A Soxhlet extractor is a piece of laboratory apparatus designed in 1879 by Franz von Soxhlet. The construction of the soxhlet extractor is shown in the Figure.2.5.

The soxhlet extractor setup consists of a round bottom flask, siphon tube, distillation path, expansion adapter, condenser, cooling water inlet, cooling water outlet, heat source and thimble. In this method, powdered sample is enclosed in a porous bag or "thimble" made from a strong filter paper or cellulose, which is placed, is in thimble chamber of the Soxhlet apparatus. Extraction solvent is taken in the round bottom flask and heated by using heating source like heating mantle. The heating temperature is built on the solvent employed to extraction. Due to heat the solvent in the bottom flask vaporizes into the condenser and then drip back to the sample thimble. When liquid content reaches the siphon arm, the liquid contents emptied into the bottom flask again and the process is the end of the process is indicated the clear solution in the siphon tube. The benefit of this system is possible that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method is not suitable for thermo labile compounds as extended heating may lead to degradation of compounds (Das *et al.,* 2009). This method maintains a relatively high extraction temperature with heat from the distillation flask. No filtration of the extract is required and the displacement of transfer equilibrium by frequently carrying fresh solvent into contact with the solid matrix (Kasiramar, 2019).

Solvent extraction is a diffusion process can be used for defatting the oil-bearing bio-materials wherein the solvent penetrates the oil-bearing cells of an oil-rich material; resulting in a solution of the oil-in-solvent (miscella). When dried grounded samples are immersed in a pool of solvent, the solvent percolates the oil-bearing cells and dislodges their contents (the oil) by molecular diffusion (Haque *et al.,* 2009 and Johnson *et al.,* 1983); reducing the residual oil content to less than 1% in the defatted material (Williams, 1996). In some cases, oilseeds are expanded or popped to improve percolation by the solvent and drainage of the miscella. A times, gentle agitation is required to increase area of contact between the oil-bearing material and the solvent. Where the oil-bearing material is fine and powdery (*e.g.,* rice bran), percolation may be obstructed, hence the usual practice in such instance is to process the material into crisp pellets.

The selection of the solvent for Soxhlet extraction is based on the phyto constituent isolation process. The solvent should be easy to remove and inert. Although Johnson and Lusas (1983) reported over 70 different solvents; hexane, petroleum ether, propanol and acetone are

the commonly used for Solvent oil extraction. Normally the solvent selection is based on the increasing polarity order like the order of acetone, petroleum ether, ethyl acetate, chloroform, methanol, ethanol and water. Safety and environmental concerns have stimulated interests in alternatives because hexane, though more preferred, is quite expensive. Heat assisted procedures is used at times for solvent extraction because the boiling point of most solvents is low and they dissolve oils and fats easily. Oil recovery from the miscella is usually achieved by evaporation and condensation of the solvent (Nagaraj, 2009). The petroleum ether is commonly used for the extraction of the steroids and fixed oils, and also used for the removal of the chlorophyll from the leaf powder; some of the researchers uses petroleum ether for defatting of the plant material. After defatting, main solvent like alcohol or aqueous extraction was performed. Some of the plant materials the defatting is essential because the waxy substances produce the emulsification process with the solvent and interferes the extraction process (Agarwal *et al.,* 2016). Methanol is the semi polar solvent which can extract many of the phytoconstituents and water is the polar solvent which is cheap solvent and nontoxic. Numbers of polar constituents are isolated by water and which is also suitable for the animal studies and human studies.

1.2 THEOBROMINE

Xanthine derivatives are the naturally occurring drugs which find use as central nervous system stimulants. Caffeine, theophylline and theobromine are methyl derivatives of xanthine. Chemically, caffeine is 1,3,7-trimethyl xanthine; theophylline 1,3-dimethyl xanthine and theobromine is 3,7-dimethyl xanthine. Theophylline, caffeine and theobromine have different bio-chemical effects and are present in different ratios in the different plant sources. Caffeine occurs naturally in coffee, tea, cola nuts, mate and guarana; theophylline is found in tea whereas theobromine is present in cocoa bean, cola nuts and tea. Theobromine is the main alkaloid in chocolate (2.8-3.5 % in cocoa), and caffeine is another major alkaloid (0.1-0.4 %). Cocoa bean shell possesses a high nutritive value, but due to the high theobromine and caffeine content, its utilization in animal feeds is limited. Still, it has been used as a goat feed, fish feed, pig feed, and as adsorbent for wastewater treatment. Some authors used it as a fortification agent in corn snack products whereas it has also been used as a flour in muffin and biscuit production. Theobromine was discovered in extracts from cocoa beans (*Theobroma cacao*) by Woskresensky in 1842 and its chemical structure (Figure 2.6) determined by Emil Fischer at the end of the 19th century. Theobromine is a colourless or white and odourless substance (melting point 357°C) with a slightly bitter taste that is naturally present in all parts of the seed

and in small quantities in the pod. Though theobromine considered as toxic compound, it is reported as possessing many pharmacological activities such as anti-cancer, diuretics, cardiac stimulants, hypocholesterolemic, smooth-muscle relaxants, asthma and coronary vasodilators (Bispo, 2002). Arlorio *et al*. reported that cocoa bean hull, principal by-product from cocoa industry, was commonly used as a secondary source of theobromine (1.29 g/100 g by dry

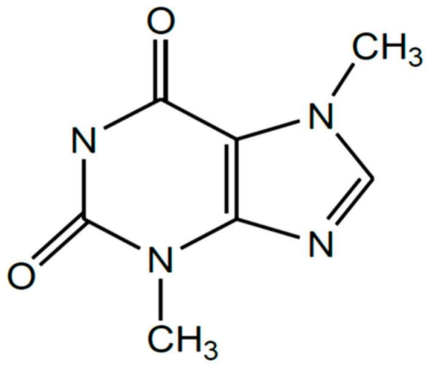

Figure 2.6. Chemical structure of theobromine.

weight), while Hammerstone and Chimel extracted theobromine from cocoa solids using various solvents, including isopropanol, methanol, and ethanol at different temperatures and found that the highest theobromine content was obtained with ethanol at 50°C.

Many works have reported the toxic effect of cocoa mainly due to its alkaloid contents. Manifestation of theobromine toxicity was first observed in poultry animals when un-decorticated cocoa meal cake fed to chicken resulted in loss of weight, poor egg production and death presumably from theobromine poisoning (Terperton and Dudley 1943). Musa *et al.* investigated the effect of theobromine on serum total protein, albumin, iron and transferrin in albino rats of wistar strain. There was a statistically significant increase in mean serum iron in the different test groups. However, serum transferrin levels showed a statistically significant decrease in the test group. Mean values of total serum protein and serum albumin showed no significant difference in the test. The consequence of elevated serum iron and lowered transferrin levels are discussed in relation to iron transport and erythropoiesis.

Theobromine, which is also a caffeine metabolite, has a much weaker action on the central nervous system since it has a 2- to 3-fold lower affinity for adenosine receptors than caffeine. Theobromine also possesses myorelaxant and cardiac stimulation properties and has been used as a coronary artery dilator or bronchodilator for asthma treatment. It is stimulant in moderate amount, while it may be poisonous in larger amounts. Theobromine is the most abundant methylxanthine present in cocoa bean shell. Bradbury & Kopp patented the

production of two different extracts from cocoa shells; theobromine fraction and polyphenol enriched fraction. The theobromine fraction can be rinsed with water, after which a polyphenolic fraction can be rinsed through a column with a low molecular weight solvent.

Premnath *et al.* conducted an in vitro study to evaluate and compare the re-mineralization potential of dentifrices containing theobromine, 0.21% sodium fluoride (NaF) with functionalized tricalcium phosphate (f-TCP) and amine fluoride on artificial enamel caries. The study resulted that theobromine containing dentifrice was effective in remineralizing lesions of enamel. However, theobromine demonstrated less remineralization potential in comparison to dentifrices containing NaF + f-(TCP) and amine fluoride. Based upon our comparative study between fluoride and theobromine by Alexander.U. *et al.* It has found that theobromine is a better alternative than fluoride. The ingestion of theobromine by lactating dams showed a decreased release of calcium and phosphorus ions from the enamel surface in the developing teeth of neonates in vivo. So, theobromine can be used as an ingredient of dentifrices and even if swallowed accidentally, there are no adverse effects. One of the adenosine receptor-independent effect of theobromine is demonstrated in cardiovascular protection by significant increases in HDL cholesterol plasma levels and decreases in LDL ones.

Various studies of theobromine toxicity also been studied in earlier days. Temperton & Dudley investigated the feeding of ground un-decorticated cocoa beans which contain 1.9 mg % theobromine to chickens at levels of 10, 20 and 30% of the cocoa mash followed by the death occurred in all groups fed cocoa beans. Then it was proved that the poisonous effect is due to the presence of theobromine. Theobromine toxicity also have been reported in dogs, cows, pigs etc. The reproductive toxicity of theobromine has received the attention of investigators in recent times. Acute structural toxicity on the testis and thymus tissue leading to fatality in rabbits, rats and mice. The developmental toxicity of theobromine results in reduction in thymus weight, slow heart rate, digestive complications and impaired growth. It is visible that the development of appropriate de-theobromination methodologies is necessary for utilization of cocoa by-products.

1.3 THEOBROMINE EXTRACTION

Pavlovic *et al.* (2020) reported on the extraction of CBS using deep eutectic solvents (DESs), microwave assisted extraction (MAE) and their combination. He used choline chloride-based solvents for the extraction of the bioactive compounds from CBS. The DES

extraction was compared to the DESs coupled with MAE, and the yields of the extracted compounds were higher for DES/MAE. For theobromine, the obtained yields for DES extraction were 2.145–4.682 mg/g, and for caffeine, were 0.681–1.524 mg/g, whereas for DES/MAE, the same compounds were obtained in 2.502–5.004 mg/g and 0.778–1.599 mg/g. The study demonstrated how extraction using DES and microwaves could be of a great importance in the future trends of green chemistry for the production of CBS extracts rich in bioactive compounds.

Jokic *et al.* (2018) separated active compounds from food by-product cocoa shell using subcritical water extraction. Subcritical water can extract polar and non-polar constituents with lesser time. It also informed that, the obtained extracts can be lyophilized and used in subsequent processes. It opens the possibility to achieve better product qualities and allow for the production of completely new products for use in the food, beverage, cosmetic and pharmaceutical industries as natural ingredients. Depending on applied extraction conditions, different concentration of theobromine, caffeine, theophylline, epicatechin, catechin, chlorogenic acid and gallic acid were extracted.

Kamal and Shamran (2006) performed extraction using liquid extraction solvents such as dimethyl chloride, chloroform, water, and water-chloroform system. De-theobromination of cocoa was also carried out by its extraction from aqueous solution by contacting the solution with a neutral activated carbon black. In this study effect of wt. ratio of chloroform to cocoa, extraction time and extraction temperature on theobromine yield was also investigated. The optimum condition is extraction time of 3 hours at 70°C and 20 wt. ratio of chloroform the results stated that it will be applicable in several fields for quality control procedures, including the pharmaceutical and food industries.

Hartati (2010) informed about hydrotropic extraction of theobromine from cocoa bean shell. She stated that high solubilization capacity and selectivity in solubilization by hydrotropy could be used for extraction of water in soluble bioactive compound such as piperin, limonin, curcuminoids and forskolin. Based on the promising result by hydrotropic extraction of natural product, thus it is a promising method in administering theobromine from cocoa bean shell.

Belwal *et al.* (2022) reviewed on bioactive compounds from cocoa husk. Extraction, analysis and applications in food production chain also reviewed. Various extraction methods were implemented for the preparation of extracts and the recovery of bioactive compounds. Besides conventional extraction methods, various studies have been conducted using advanced

extraction methods, including MAE, ultrasonic-assisted extraction (UAE), subcritical water extraction (SWE), supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE). To include cocoa husk waste products or extracts in different food products, various functional foods such as bakery products, jam, chocolate, beverage, and sausage were prepared. The review mainly focused on the composition and functional characteristics of cocoa husk waste products and their utilization in different food products. Moreover, recommendations were made for the complete utilization of these waste products and their involvement in the circular economy.

Nunez and Macias (2011) conducted stirring method by adding hot distilled water (80°C), Carrez I reagent and other chemicals for the extraction of theobromine and caffeine from cocoa. With the aim of improving the extraction efficiency, a focused microwave assisted method was developed. This method is optimised with 5min of irradiation time, 210W of power and 100 ml of the extractant and proved that focused microwave method was more efficient compared with a standard mechanic stirring method.

1.4 ULTRASOUND

Ultrasound is an emerging sustainable technology that enhances the rate of several processes in the food processing industry. Ultrasound is defined as sound waves exceeding the audible frequency range, greater than 20 kHz. Ultrasound propagate through a medium, they generate compressions and rarefaction (decompressions) in the medium particles.

Ultrasound waves are classified depending upon the sound power (W), sound intensity (W/m^2), or sound energy density (Ws/m^3) used in the food industry application is divided into two: low energy and high energy approaches. An ultrasound frequency higher than 100 kHz and intensity below 1 W/cm^2 is preferred for low-energy applications that normally do not change the physical or chemical properties of the material through which they propagate (Bhargava *et al.* 2021). These are normally used for analytical applications, such as the determination of the physico-chemical properties of the materials, composition, ripeness, firmness, sugar content, and acidity of fruits and vegetables (Qiu *et al.,* 2020). In high-energy (power ultrasound) applications use frequencies between 20 and 100 kHz, and intensities that are higher, in the range of 10–1000 W/cm^2 can alter the physicochemical properties or structure of a material (Charoux *et al.,* 2021). It can be used for enzyme inactivation, enhancement of drying, freezing and extraction processes, control of crystallization processes, and the degassing of liquid foods (Yao *et al.,* 2019). High-intensity ultrasound is characterized by the

induction of acoustic cavitation which is caused due to the production, subsequent growth, and sudden collapse of larger bubbles liberating a high amount of energy (Alzamora *et al.,* 2011)

1.4.1 Ultrasound Generation

Ultrasound production system comprises a generator, transducer and the application system. Generator generates electrical or mechanical energy and transducer transforms this energy into ultrasound of suitable frequencies. There are primarily three types of transducers: fluid-driven, magnetostrictive, and piezoelectric. The fluid driven transducers produce ultrasound energy by forcing liquid to a thin metallic blade. It is mainly used for mixing and homogenization of food products.

Magnetostrictive transducers are based on magnetostriction effect discovered by joule in 1874. These transducers were made of ferromagnetic material that changed dimensions when the magnetic field was applied, and these changes sought to generate mechanical vibrations after (Raichel, 2000). The efficiency of the system is quite poor, with a 60% conversion to acoustic energy. These setups are basically limited to 30 kHz.

Piezoelectric transducers work on the basis of the piezoelectric effect discovered in the 1800s by Pierre Curie. He reported that when mechanical pressure was applied to asymmetrical crystals like quartz and Rochelle salt electrical signals were produced. Conversely, mechanical vibrations can be produced by employing electrical oscillations to these salts. Paul Langevin performed the first practical examination in 1915 (Cruz *et al.,* 2014). The commonly used piezoelectric materials are lead zirconate titanate, barium titanate, and lead metaniobate. The piezoelectric transducers are most widely employed equipments and more energy efficient (80%- 90% transfer to acoustic energy).

1.4.2 Ultrasonic Processing Equipments

Ultrasound processing equipments are mainly divided into laboratory scale and large scale equipments. Large scale equipments are further divided into batch type and flow type devices.

Ultrasonic water baths have been widely accepted methods for cleaning, and sanitation in the food and beverage processing operations. Ultrasonic waves produced by the equipment into the cleaning fluid render the food safe for consumption, usually employed in degreasing processes. Ultrasonic cleaners transform low-frequency AC into high-frequency sound waves via the piezoelectric transducer, which is attached either to the bottom of the processing vessel

or submerged into the cleaning liquid. The transducer produces high-intensity waves into the solution, creating compression, which tears apart the liquid, resulting in millions of macroscopic cavities or bubbles, termed as 'cavitation' (Figure 2.7). These bubbles, violently collapse in the cleaning solution with enormous energy at high temperature and pressure.

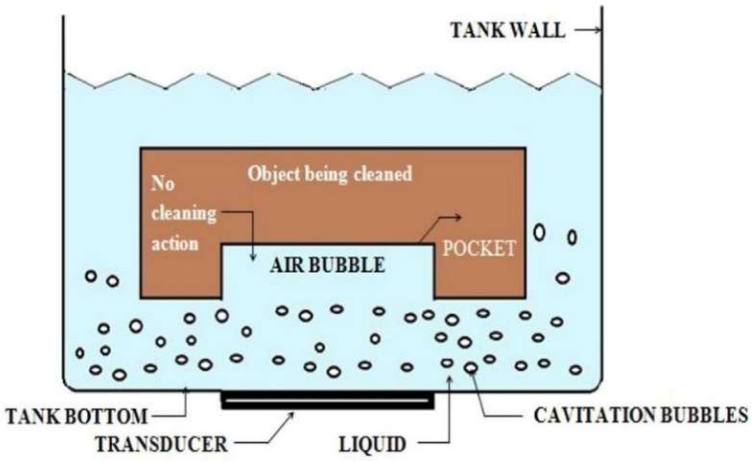

Figure 2.7. Ultrasonic water bath

Within a short span, this process eradicates the dirt from all products, submerged in the cleaning solution (Mason, 2016).

The commonly used another batch type equipment is ultrasonic probe (also known as sonotrode, ultrasonic horn). They are an immersion – type transducers. A sonotrode is a welder's tool, and thus in case of food processing is predominantly employed in cutting and slicing actions. Ultrasonic slicing of cheese, biscuits, fruits, and vegetables, etc. results in perfectly cut and sliced products. Also, contrary to conventional slicing techniques, ultrasonic technology minimizes loss of product and augments productivity. It is an essential part of the ultrasonic cutting system, which consists of a generator that produces alternating current with the required frequency of ultrasound. The transducer then converts electric oscillations to mechanical displacements. An amplifier then transfers the vibrations (mechanical) as a sound wave to sonotrode (Lucas *et al.,* 2014). The volume of sample to be treated, gap between probe apex and bottom of vessel, probe size, probe shape, and material of manufacturing are the major factors to be considered. Probes are generally manufactured using titanium, aluminum alloys or stainless steel. Probes are available in various shapes such as cylindrical, tapered, exponential, stepped, full wave and half wave (Cruz *et al.,* 2014).

Liquid whistle and resonating tube reactors are most common type of flow type reactors. Liquid whistle is the oldest type of device commonly used for emulsification. In liquid

whistle the medium is forced under pressure through an orifice using a powerful pump. The medium emerges and expands into a mixing chamber. There are no any other moving parts in a liquid whistle other than a pump so it is a highly durable device.

In resonating tube reactor, the liquid is conveyed through a pipe having ultrasonically vibrating walls. Ultrasound energy is readily transmitted to the moving liquid. Stainless steel is mainly used for commercial construction of resonating tube reactors. They have different cross section like rectangular, pentagonal, hexagonal, circular etc.

1.4.3 Mechanisms of Ultrasound Processing

Ultrasound moves through the material in the form of longitudinal waves. It induces alternate compression and rarefaction cycles in the medium particles (Povey and Manson, 1998). When power is sufficiently high the rarefaction cycles exceed the attractive forces of molecules of the medium and form cavitation bubbles. These bubbles grow over the cycles due to rectified diffusion. When the bubbles exceed critical size, they become unstable and leading to intense collapse. The process of bubble formation, growth and collapse is referred as cavitation. Cavitation produces mechanical, chemical and biochemical changes in the medium. It produces localized hot spots, which causes destruction of microorganisms and enzymes. The cavitation bubbles collide with each other and create shock waves which eventually cause inactivation of microorganisms by destruction of cell walls and cell membranes, and DNA denaturation through sonolysis of water. Cavitation also results microstreaming. Microstreaming is a phenomenon, in which the cavitation bubbles produce a vigorous circulatory motion and produce strong eddy currents in the medium. Microstreaming facilitates heat and mass transfer process (Zheng and Sun, 2006).

The effectiveness of cavitation depends on wave characteristics (frequency, intensity), product characteristics, treatment time and environment factors (temperature, pressure). The type of microorganism is also an important factor to be considered. The studies revealed that gram positive bacteria are comparatively resistant to ultrasound due to its cell wall composition and structure.

The microbial inactivation also depends on the environmental factors such as temperature and pressure. Generally, ultrasound is combined with other techniques in order to inactivate resistant microorganism.

1.4.4 Methods of Application of Ultrasound

1. Ultrasonication: Ultrasound is applied at low temperature.
2. Thermosonication: Ultrasound is combined with moderate heat in order to get more effective microbial inactivation than normal heat treatments (Manson *et al.,* 1996; Villamiel *et al.,* 1999).
3. Manosonication: Combination of ultrasound and pressure. Generally, use moderate pressure (100 to 300 kPa) at low temperature (Ercan and Soyal, 2013).
4. Manothermosonication: Heat, ultrasound and pressure applied simultaneously to the product. It can be used to inactivate microorganisms having high thermal tolerance (Chemat *et al.,* 2011).

1.4.5 Applications of Ultrasound in Food Processing

Ultrasound is widely employed in food processing sector for various unit operations such as degassing, crystallization, filtration, enhanced drying, microbial inactivation, enzyme inactivation, homogenization, emulsification, defoaming and meat tenderization.

1.4.5.1 Microbial inactivation

Microorganisms such as bacteria and fungus are responsible for food spoilage. Water and nutrients rich food products are suitable medium for microbial growth and multiplication. Ultrasound treatment is applied as a processing aid to inactivate microbes. Various textural and physiological changes such as thinning of cell membranes and the production of free radicals, are the main mechanisms by which microorganism inactivation takes place (Starek *et al.,* 2021) Transient cavitation will produce localized hot spots up to 4500–5000 K, and pressures > 199 MPa produce shock waves and free radicals, whereas stable cavitation will produce micro streaming accompanied by high shear (Silva *et al., 2020*). All these contribute to damage of the cell wall and membrane, resulting in cell death. It was reported that ruptured and disintegrated cells cannot be reviewed, which is advantageous over some other techniques in which damaged cells can recover if they encounter the right environmental conditions (temperature, pH, water activity, and nutrients). The resistance offered by five groups of microorganisms to the ultrasonic inactivation is in the order of spores > fungi > yeast > gram-positive cells > gram-negative cells. The resistance of viruses to ultrasound is high, but not enough data is yet available to compare it with the other microorganisms.

Kapturowska *et al.* (2011) carried out sonication and the count of live yeast cells decreases by 100 to 1000 times, compared to their initial count, expressed as Colony Forming

Units CFU/cm^3. The efficacy of microbial destruction is governed by amplitude and frequency of the ultrasonic waves, the exposure/contact time, the composition and volume of the food to be processed, and the conditions (Starek *et al.,* 2021). It was observed that the number of bubbles undergoing cavitation per unit of time increases at higher amplitudes, which resulted in a higher inactivation rate of the microorganisms.

According to studies performed by Valero *et al.* (2007), ultrasound treatment of orange juice (500 kHz, 240w for 15 min) reveals a small degree of microbial inactivation (about 1.08 log cfu/ml) without showing any adverse impact on the quality characteristics of juice such as limonene content, browning and colour change.

Microbial destruction can also be accomplished by combining ultrasound with other treatments, such as heat (thermosonication), low static pressure (monosonication), ultraviolet light, or antimicrobials. Sonication combined with high pressures and temperatures (manothermosonication at 400 kPa/59◦C) was applied by Lee *et al.* (2013) for the control of Escherichia coli (E. coli) K12 populations in apple cider, and they achieved a 5-log reduction in 1.4 min and in 3.7 min when sonication was combined with a lethal temperature.

Cabeza *et al.* (2005) conducted that thermosonication (54°C for 15 min) for pasteurization surface of intact eggs to eliminate *S. enteritidis.* The process did not affect its nutritional and functional properties such as shelf life, emulsifying and foaming capacities, stability, textural properties of egg white gel, breakage resistance of shell and sensory properties of cooked eggs.

1.4.5.2 Enzyme inactivation

Enzymatic reactions cause food spoilage. Generally, heat treatments are used for enzyme inactivation, but thermal treatments make undesirable effects such as unwanted flavours and nutrient losses which reduces consumer satisfaction.

Enzyme inactivation takes place due to cavitation in ultrasonic treatments. The first enzyme inactivated by sonication was pepsin around 60 years ago. Since then, sonication is used as a promising technique for enzyme inactivation. For the inactivation of enzymes such as glucose oxidase, peroxidase, pectin methyl esterase, protease and lipase, sonification alone or in conjunction with other techniques is used.

All enzymes are generally proteins in nature. Ultrasound creates cavitation bubbles in the medium or products. The violent collapse of these bubbles creates localized hotspots.

Cavitation also creates shock waves and microstreaming of liquid. All this effect induces structural changes in the protein's secondary and tertiary structures. Because of this, there will be enzyme denaturation. The free radicals formed due to sonolysis of water will react with amino acids of protein and destroy the biological activity of enzymes (Feng *et al., 2011)*.

Manas *et al.* (2006) reported that ultrasound treatment combined with temperature and pressure increased efficiency of inactivation of egg white lysozyme. Similarly, Villamiel and Dejong. (2000) observed that normal sonication treatment was not efficient for inactivation of endogenous milk enzymes such as alkaline phosphatase, G-glutamyl trans peptidase and lactoperoxidase at normal room temperature, but when combined with heat (60–70°C) sonication effectively denatured the proteins. Likewise, Lopez *et al.* (1998) observed that inactivation rate of endopolygalacturonases using Manosonication is higher than the heat treatment at 62.5°C.

The inactivation of enzymes by ultrasound depends on frequency, power, type and concentration of enzyme, pH and temperature (Potapovich *et al.,* 2003). In a buffer medium, catalase inactivation was reported to improve with increased power and decline with enzyme concentration. They also reported that inactivation rate increased with increase in frequency due to increased production of free radicals. Ultrasound and combined techniques can be used as a preservation tool to inactivate enzyme as a substitution to heat treatment.

1.4.5.3 Filtration

Filtration is one of the major unit operations in food industry. The conventional methods make problems of fouling or concentration polarization caused by the deposition of the filtrate or filter cake on the membrane surface. These problems cause a reduction in filtration efficiency. However, ultrasound energy is effective against this issue. Ultrasound energy increases the flux by reducing concentration polarization. This helps to preserve a frictionless filter without altering filter membrane permeability. It helps to maintain a frictionless surface without affecting permeability of filter membrane. Ultrasonically assisted filtration (usually referred to as acoustic filtration) enhances the life of filter. It is widely employed for waste water treatments and filtration of fruit juices and drinks. It is also broadly used in dairy industry for processing of milk and whey products and separation of milk components (Zisu and Chandrapala, 2015). Acoustic filtration reduces energy requirements for processing of whey solutions with high solid content (Koh *et al.,* 2012). Ultrasound, when combined with filters, enhances the life of the filter, by preventing the caking and clogging of

the membrane, enabled by continuous cavitation at the surface of the filter (Grossner *et al.* 2005).

1.4.5.4 Extraction

Extraction is a major unit operation used for the effective separation and production of various oils, bioactive compounds and molecules from their matrices. Soxhlet extraction, heat reflux, and maceration are the commonly used conventional techniques for extraction, which require large amounts of solvent. labour, and are energy and cost-intensive crystals (Ojha *et al.,* 2020). Ultrasonic implosion and cavitation rupture the cell walls, enhancing the mass transfer from solid to liquid phase. Also, within the tissues, microchannels are created on an ultrasound application which enhances the solvent penetration into the solid matrix which increases mass transfer (Yang *et al.,* 2017).

1.4.5.5 Drying

The application of ultrasound in the drying process can accelerate the rate of drying of fruit, vegetables, meat, and fish and reduces the drying time as well as enhances the rate of heat and mass transfer to preserve the product quality (Kowalski *et al.,* 2020). Ultrasonication has proven to be an effective alternative to the conventional drying process. The removal of water is facilitated by the phenomenon of "sponge effect", improving the water diffusion from the interior to the surface of the product (Riera *et al.,* 2006). New microchannels are formed due to intracellular and extracellular cavitation of water. Also, ultrasound generates air turbulence at the air product interface, to remove the moisture from the surface (Yao, 2016).

Research on the application of ultrasound in drying shows a reduction in drying time by about 20–30% at the low velocity of the air and reduced temperatures (Castillo *et al.,* 2019). In addition to convective drying, ultrasound is also applicable for freeze-drying (Cheng *et al.,* 2014) and vacuum drying (Tekin *et al.,* 2017) to enhance the rate of drying over conventional methods. Ultrasound treatment of eggplant before drying significantly decreases the duration of drying and preservation of microstructure (Simal *et al.,* 2014). In general, ultrasound treatment reduces water activity, enhances product colour, and decreases the loss of nutrients: flavonoid content, antioxidant activity, vitamin C, and total phenolic content (Huang *et al.,* 2019).

1.4.5.6 Emulsification

Emulsification is a unit operation in which two or more immiscible liquids are dispersed from a mixture together. The energy required to disperse a liquid phase in small droplets in a

continuous phase is provided by ultrasound. Collapsing cavitation bubbles induce shock waves with in liquid in the dispersing zone which result in high liquid velocity liquid jets forming. Emulsifiers (surface active substances, surfactants) and stabilizers are used in the emulsion to stabilize the newly formed droplets against coalescence.

The emulsification assisted by ultrasound requires less surfactant to form more stable emulsions. These methods are generally economically feasible, simple to use and integrate with existing manufacturing systems to increase the quality of the final product (Krasulya *et al.,* 2016). Riener *et al*. (2009) reported that the introduction of ultrasound reduced process temperature and time in homogenization. Similarly, chemat *et al*. (2011) found that ultrasonic homogenization at lower frequency ranges from 16 to 100 kHz produced smaller, evenly distributed and stable emulsions.

1.5 ULTRASOUND ASSISTED EXTRACTION (UAE)

UAE, also called sonication, uses ultrasonic wave energy during the extraction. Ultrasound produces cavitation, which accelerates the dissolution and diffusion of the cell ingredients: The ultrasound wave is propagated in the molecules of the medium and cavitation bubbles are formed at sufficiently high power. The disintegration of these bubbles generates energy, and the jets of solvent towards herbal particles extract the target compounds from them more efficiently (Vinatoru *et al.,* 2017). In addition, the heat transfer improves the extraction efficiency. The other advantages of UAE include low solvent and energy consumption and reduction of extraction temperature and time, which make UAE a simple and relatively low-cost technology (Rostagno *et al.,* 2003). Thus, Ultrasound aided extraction contributes to efficient recovery of compounds in lesser time, energy, and solvent requirements, add an added advantage of low-temperature extraction, for temperature-sensitive food products (Ojha *et al.,* 2020). Ultrasonic assisted extraction is usually performed under continuous wave mode and pulse mode technique is employed over long-term extraction. It is generally preferred for the extraction of bioactive compounds due to its versatility, easy operation, the scope of industrial application, with an ability to use less solvent and retain the biological activity (Duan *et al.,* 2020). However, the extraction efficiency is greatly affected by ultrasonic power, frequency, solvent, and the matrix to solvent employed (Rodsamran and Sothornvit, 2003).

Recently the ultrasonic assisted extraction has been extensively employed for the extraction of bioactive compounds from food and food wastes. Lu *et al.* (2015) optimized the operating conditions of UAE for total flavonoids from *Cryptotaenia japonica* Hassk using the

Box–Behnken design. Their results suggested UAE as a promising technique for this extraction. Ochoa *et al.* (2020) extracted anthocyanins from purple yam employing ultrasonic homogenizer at 750 W in pulse mode and observed that extraction performed at 30˚C for 10 min resulted in higher anthocyanin content than the conventional method. Bioactive compounds were extracted from grape pomace using ultrasound (250, 350, and 450 W) for 5, 10, and 15 min and it was observed that maximum extraction (45% of anthocyanins) took place at 10 min of exposure time (da Rocha and Noreña, 2020). Phenolics and anthocyanins were also extracted from jabuticaba peels in an ultrasonic water bath at 25 and 40 kHz and maximum extraction were observed when exposed for 10 min at 25KHz (Fernandes *et al.,* 2020). Bioactive compounds were extracted from a bitter gourd using ultrasound, employing different time, temperature, and solvent solid ratio combinations using water as a solvent and observed that the maximum extraction was observed at 68.4˚C for a 12 min exposure time (Chakraborty *et al.,* 2020).

1.6 UV SPECTROSCOPY

Li *et al.* (1990) individually determined the theobromine and caffeine in cocoa beans. Caffeine alone is completely extracted in to chloroform from an aqueous solution at a pH between 12.5 and 12.7, and can be determined by UV spectrophotometry at 275.9 nm. For the theobromine remaining the aqueous solution, a wavelength of 272.7 nm is used. The results were reproducible with a relative standard deviation of about 0.653.

Chaudhari and Gaikwad (2020) reported about simultaneous estimation of Caffeine and Quercetin by UV spectrophotometric method. The method involved estimation of Caffeine and Quercetin by simultaneous equation at 273nm and 372nm respectively in their solution in methanol. The Beer's law obeyed in the concentration range of 2-10 µg/ml for both Caffeine and Quercetin respectively. This method was validated with respect to linearity, accuracy, precision, Limit of detection and quantification as per ICH norms. The proposed method was found to be rapid, specific, precise and accurate for the routine analysis of Caffeine and Quercetin in niosomal formulation.

Xia *et al.* (2013) performed kinetic spectrophotometric method to determine the caffeine, theobromine and theophylline in food samples such as cola drinks, caffeine powder, cocoa powder and tea samples. This method was based on the different kinetic characteristics between the reactions of analytes with cerium sulfate in sulfuric acid and the associated change of absorbance at 320nm. Experimental conditions, the effects of sulfuric acid, cerium sulfate

and temperature, were optimized. Linear ranges (0.4-8.4 μg ml^{-1}) for all three analytes were established, and the limits of detection were: 0.30 μg ml^{-1} (caffeine), 0.33 μg ml^{-1} (theobromine) and 0.16 μg ml^{-1} (theophylline). The recorded data were processed by partial least squares and artificial neural network, and the developed mathematical models were then used for result. This research is strongly suggested that the differential kinetics method with the aid of chemometrics under established experimental conditions, could be successfully applied to the simultaneous determination of caffeine, theobromine and theophylline in food samples.

Hansen (1965) developed a method to determine the concentration of fat-free cocoa solids (true chocolate) in chocolate or chocolate-flavoured milk and ice cream by the absorbance of a trichloroacetic acid (TCA) filtrate of the product at wave length 275 μm, using either chocolate or theobromine as the standard reference. Results were within ± 4.5% of the expected values based on chemical analyses. The mean error for a series of duplicate determinations was ± 0.22%. The influence of milk or ice cream solids was not significant, provided the amounts were maintained constant in test and reference samples. The true chocolate content in 19 commercial chocolate ice creams averaged 2.1.3% and ranged from 1.3 to 3.1%.

Desai (2020) estimated the concentration of caffeine in soft drinks and energy drinks by using UV spectroscopy method using dichloromethane as an extracting solvent at 273nm wavelength. The method was found to be fast, simple, cost effective and environmentally friendly for the determination of caffeine in soft drinks with satisfactory results.

1.7 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography or High-pressure liquid chromatography (HPLC) is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds (Martin and Guiochon., 2005). HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes in synthetic procedures or scale up, evaluating new formulations and carrying out quality control / assurance of the final drug products (Dwivedia and Agarwal., 2015; Ahuja and Rasmussen.,2007). HPLC is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. HPLC is the most accurate analytical methods

widely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability (Rao *et al.,* 2015).

HPLC is basically a highly improved form of column chromatography. Instead of allowing the solvent to drip through a column under just the force of gravity, it is externally forced through the column under high pressures of up to 400 atm. This makes the chromatographic process a lot faster. It also allows the use of very small particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing through it. Thus, it allows a much better separation of the components of the mixture. Now a days, high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) is one of the most versatile analytical tools available due to its rapid and diverse growth in applications in the analysis of drugs and metabolites from biological fluids. In HPLC, the essential equipment consists of an eluent, reservoir, a high-pressure pump, and an injector for introducing the sample, a column containing the stationary phase, a detector and recorder (Taleuzzaman *et al.,* 2016). The development of highly efficient micro particulate bonded phases has increased the versatility of the technique and has greatly improved the analysis of multi component mixtures (Tamimi *et al.,* 2016).

HPLC principle is that solution of sample is injected into a column of porous material (stationary phase) and liquid phase (mobile phase) is pumped at higher pressure through the column. The principle of separation followed is the adsorption of solute on stationary phase based on its affinity towards stationary phase (Dwivedia and Agarwal., 2015). HPLC is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result, the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography and the use of much smaller particles of the adsorbent or support becomes possible increasing the column efficiency substantially (Sultana *et al.* 2016).

Ramli *et al.* (2000) determined methylxanthines and polyphenols levels in cocoa and chocolate products using HPLC method. Commercial cocoa and chocolate products such as cocoa powder, cocoa beans, cocoa liquor and chocolate were analysed for total polyphenols, (-)-epicatechin, (+)-catechin, theobromine and caffeine contents. The methylxanthines were identified and quantified with the use of -Bondapak column and mobile phase of methanol: water: acetic acid (20:79:1). Coefficient of variance values were relatively low (90%). Total

polyphenols ranged from 45.52 mg/g in cocoa liquor, 34.60 mg/g cocoa beans, 20.62 mg/g in cocoa powder. For (-)-epicatechin contents, the average is 3.81 mg/g in cocoa powder, 2.53 mg/g in cocoa liquor and 4.61 mg/g in cocoa beans. Whereas the average for (+)- catechin contents are 4.28 mg/g in cocoa powder, 3.49 mg/g in cocoa liquor and 3.02 mg/g in cocoa beans. Levels of caffein and theobromine in 32 samples of chocolate products averaged 0.62-1.14 mg/g and 0.026-0.153 mg/g respectively. The chocolate coating made from fat substitute had theobromine and caffeine levels ranged from 0.36-0.70 mg/g and 0.027-0.061 mg/g respectively. In local chocolate, the mean theobromine and caffeine levels respectively were 0.72 mg/g and 0.04 mg/g in milk chocolate, and 0.85 mg/g and 0.06 mg/g in dark chocolate. As in imported chocolate, the mean theobromine and caffeine levels respectively were 1.05 mg/g and 0.12 mg/g in dark chocolate; 0.76 mg/g and 0.04 mg/g in milk chocolate; and 0.74 mg/g and 0.03 mg/g in white chocolate. Compared with the local chocolate, they found that imported chocolate has higher level of theobromine and caffeine.

Coco *et al.* 2007 performed simultaneously by a rapid and selective reversed-phase high-performance liquid chromatography (HPLC) method with UV detection in by-products of cupuacu and cocoa seeds for determining theobromine, theophylline, and caffeine. The determination is carried out in the raw and roasted ground cupuacu seeds and in the corresponding powders obtained after pressure treatment. The by-products of both cupuacu seeds and cocoa seeds are obtained under the same technological conditions. The HPLC method uses isocratic elution with a mobile phase of methanol–water–acetic acid (80:19:1) (v/v) at a flow rate of 1 ml/min and UV absorbance detection at 275 nm. Total elution time for these analytes is less than 10 min, and the detection limit for all analytes is 0.1 mg/g. The amounts of theobromine and caffeine found in all the cupuacu samples are one or more orders of magnitude lower than those from cocoa. Theophylline is found in all cocoa samples except for the roasted ground paste, and it is only found in the roasted ground paste in the cupuacu samples.

Caudle *et al.* (2001) tried to improve the Association of Official Analytical Chemists (AOAC) official analytical method for analysing methylxanthines in cocoa-based food products. Theobromine and caffeine contents could be obtained by 12 reverse-phase HPLC. The AOAC method's degree of accuracy and precision was not reliable, especially for caffeine. In this study, the AOAC analytical method only showed recoveries of theobromine and caffeine to be 89.3 and 74.5%, respectively. The authors successfully changed from an organic extraction to an aqueous extraction and analysed the samples via reverse-phase HPLC to

improve the recoveries of theobromine and caffeine and obtained 99.6 and 103.4%, respectively.

Nishitani and Sagesaka (2004) developed an improved HPLC analytical method for simultaneously determining caffeine and the eight catechins as well as other phenolic compounds in tea. The proposed method provided additional ability to analyse phenolic compounds when compared with former HPLC methods. This procedure was based on an improved reverse-phase ODS column operated at 4°C, a binary gradient elution system of water-methanol-ethylacetate-phosphoric acid, and a photodiode array detector. The quantitative measurement of eight catechins and caffeine confirmed the validity of this proposed method. The detection limits of these analytes ranged from 1.4-3.5 ng per injection volume. The recovery rates of the analyses were in the range of 96-103%. The caffeine contents of Sencha, Matcha, Gunpowder, and Darjeeling determined in this study were 2.94±0.007, 3.62±0.005, 2.61±0.059, and 3.24±0.016% (dry weight), respectively.

Zuo *et al.* (2002) analysed various substances in several green, Oolong, black and pu-erh teas by HPLC. They used a methanol-acetate-water buffer gradient elution system and a C-18 column; detection utilized a photodiode array detector. After multiple extractions with aqueous methanol and acidic methanol solutions, four major catechins, gallic acid and caffeine could be simultaneously determined within 20 min. This improved the previous studies' problem of catechins and caffeine remaining in tea residues after a single extraction. The results demonstrated that green teas contain higher amounts of catechins than Oolong, pu-erh, and black teas due to their fermentation processes reducing the levels of catechins significantly. An interesting finding was a lower caffeine content in Oolong teas, especially in Fujian Oolong tea.

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

This chapter describes the ultrasound assisted extraction of theobromine from cocoa bean shell. The materials used for the extraction and analysis of product were explained. The optimization of process parameters for ultrasound assisted extraction of theobromine with maximum theobromine yield and maximum absorbance value.

2.1 RAW MATERIALS

Cocoa bean shell was procured from Cocoa Research Station, Vellanikkara, Thrissur. Cocoa bean shell was collected and transported to the laboratory with care. In the laboratory, cocoa bean shell was stored at ambient condition till the conduct of experiment.

2.1.1 Sample Preparation

Nibs were separated manually from cocoa bean shell, that are taken from Cocoa Research Station, Vellanikkara, Thrissur. Then samples were weighed and dried by using cabinet drier at 65°C. After it was kept in tightly closed plastic bag. Then it was followed to milling process to obtain uniform particle size by using mixer grinder.

Plate 3.1. Cocoa bean shell

Plate 3.2. Cocoa bean shell powder

2.2 DETERMINATION OF ENGINEERING PROPERTIES OF CBS

Prior to the extraction of theobromine from cocoa bean shell, the physiochemical properties of cocoa bean shell were studied. Engineering properties such as moisture content, pH, bulk density, fat content and ash content were determined by standard methods as explained in the following sections.

2.2.1 Bulk Density

The bulk density of a powder is the ratio of the mass of an untapped powder sample and its volume including the contribution of the inter-particulate void volume. The bulk density is expressed in kilogram per cubic meter, because the measurements are made using cylinders. The bulk density of cocoa bean shell powder is measured in 10 ml cylinder. The powder was filled in cylinder and the filled cocoa bean shell powder is weighted.

$$\text{Bulk density} = \frac{\text{Weight of sample,kg}}{\text{Volume of sample,m}^3} \quad \ldots\ldots. (3.1)$$

2.2.2 True Density

Known weight of cocoa bean shell was transferred into a measuring cylinder. Slowly add toluene into the measuring to fill the voids. Measure the amount of toluene added. True density of cocoa bean shell was determined using the following equation.

$$\text{True density} = \frac{\text{Weight of sample,kg}}{(\text{Bulk volume} - \text{Volume toluene}),\text{m}^3} \quad \ldots\ldots\ldots (3.2)$$

2.2.3 Porosity

Porosity of the cocoa bean shell was computed from the bulk and true density using a formula as explained by Mohsenin (1970). The reported values are means of 10 replications.

$$\text{Porosity} = \frac{\text{True Density} - \text{Bulk density}}{\text{True density}} \quad \ldots\ldots\ldots (3.3)$$

2.2.4 pH Measurement

pH is the negative logarithm of hydrogen-ion concentration in gram per litre. It is a measure of active acidity. The pH value of samples was determined by using a digital pH meter. A known quantity of sample was dissolved with known volume of distilled water and allowed stand for 30 min, then the solution was filtered to remove solid particles. The pH meter was calibrated using buffer solutions of pH 4, 7, and 9. The probe was then wiped dry with tissue and dipped in the prepared samples. The pH value of sample was recorded.

Plate 3.3. Digital pH meter

2.2.5 Moisture Content

The moisture content of the cocoa bean shell powder was determined by using infrared moisture analyser. Infrared moisture test instrument and can be used to measure the moisture of any material. The instrument is designed in accordance with the thermogravimetric principle. While the instrument measures the weight of samples, infrared heating units and water evaporation channels dries samples rapidly too. In the drying process, instrument measures continually and displays immediately the lost moisture (%) of the samples in the process. When the drying is completed, the finally measured moisture is locked. Press the display button, data such as the moisture value, the starting value of the weight, the initial value and measuring time can be observed.

Plate 3.4. Infra-red moisture meter

2.2.6 Fat Content

Fat content was determined using a Soxhlet apparatus (Model SCS 06 AS DLS TS SOCS PLUS). The Soxhlet apparatus works on the principle of Randall's Soxhlet chemistry.

Two sets of about 1g of cocoa mass was taken in a thimble made from thick filter paper. The thimble then loaded in the extraction tube of Soxhlet apparatus containing extraction

solvent (hexane). The fat present in the cocoa mass was extracted through siphoning of hexane through the apparatus and fat will settle at the bottom. This was transferred to a pre-weighed beaker and kept in a cabinet dryer for the hexane to evaporate. The cream-coloured substances left behind after evaporation of solvent was the fat which is weighed and expressed as percentage.

Fat content of the sample is obtained by following equation,

$$\text{Fat Content (\%)} = \frac{w1-w2}{w} \times 100 \qquad \ldots\ldots. (3.4)$$

where, w - weight of sample taken, g

w_1- initial weight of beakers, g

w_2 - final weight of beaker, g

Plate 3.5. SOCS PLUS Solvent/ Fat Extraction System

2.2.7 Ash content

Ignite the crucible in the muffle furnace at 600°C for 1 h. Place the crucible in the desiccator. Cool to room temperature and weigh to the nearest 0.1 mg. Weigh out to the nearest 0.1 mg, so that the estimated amount of ash will be 0.1 g. into the ignited crucible. Place the crucible in the furnace at 600°C. The process will take 2 hours. Place the crucible in the desiccator and allow to cool to room temperature When cool, admit air slowly to avoid loss of ash from the crucible. Weigh to the nearest 0.1 mg.

Ash content, $A_c = 100\frac{(F-G)}{(B-G)}$ ……. (3.5)

Where, G= mass of empty crucible in gram

B = mass of crucible plus dried sample in gram

F = mass of crucible plus ashed sample in grams

Plate 3.6. Muffle furnace

2.3 EXTRACTION OF THEOBROMINE

2.3.1 Conventional Method

20 g of defatted cocoa bean shell powder was weighed into a 250 ml conical flask using a weighing balance. 200 ml of chloroform, 20 ml of 10% NH_4OH were added into the conical flask and shaken for 10 minutes using a shaking incubator at 400 rpm. 50 g of anhydrous Na_2SO_4 was later added and shaken for 45 minutes at 400 rpm and the obtained solution left overnight, then it was filtered through a Whatman filter paper having 110 mm pore diameter into a 250 ml flask. The solution will be obtained and the residue is discarded. Chloroform will be removed from solution by applying heat using heating mantle at 60°C or keeping the solution open for few days to allow the evaporation of chloroform, thereby the precipitation of theobromine power. The Theobromine powder obtained was separated from conical flask and stored in suitable container.

2.3.2 Ultrasound-Assisted Extraction Method

In Ultrasound assisted extraction method, Ultrasonication was applied to sample after adding 10% NH_4OH solution and chloroform into the sample, as a pre-treatment. All other procedures are kept same as that of ordinary extraction method

2.3.2.1 Ultrasound water bath

Ultrasound is an oscillating sound wave which has a frequency greater than the human hearing threshold (> 20 kHz). It is considered to be a promising technology in food industry. High power US at lower frequencies (20 to 100 kHz) has an ability to produce cavitation, which can be utilized for disruption of microbial cell membranes, leading to microbial inactivation. US preserve nutritional and organoleptic qualities of food products. It also offers greater homogeneity and significant energy savings. To improve inactivation efficacy, US is combined with other treatments such as pressure, heat and antimicrobials.

Ultrasound production system comprises a generator, transducer and the application system. Generator generates electrical or mechanical energy and transducer transforms this energy into ultrasound of suitable frequencies.

Ultrasound treatment was carried out in an ultrasound bath with chiller (Athena technology, Mumbai, model ATSC–10) operating at a frequency of 33 kHz and output power 250W. The ultrasound bath (Plate 3.8) is made up of stainless steel (SS 304). The length, width and height of bath are 445 mm, 420 mm and 545 mm respectively with a capacity of 10 L.

The ultrasound water bath consists of advanced MOSFET based SMPS generator and five PZT transducer. High frequency electrical energy is converted into ultrasound waves of required frequency by using piezoelectric sandwich type transducers attached to the base of stainless-steel tank. These high-frequency sound waves will produce numerous microscopic vacuum bubbles that grow and collapse briskly. This process is called cavitation. Intense cavitation would result in microbial inactivation. Digital tuning of transducers with generator was applied to avoid frequency shifting during operation.

The compact in-build cooling system in the ultrasound water bath helps to maintain desired temperature from 10 to 30°C. The copper cooling coils around the outer surface of tank, used for refrigerant circulation are properly insulated and connected to a condenser, and compressor assembled in the system, which controls the bath temperature as per the requirement. A PT–100 simplicon sensor was employed for measurement of temperature. A

digital temperature controller with setting 10 to 30°C and timer with setting of 0 to 99 min is also provided to set the time and temperature of sonication.

Plate 3.7. Ultrasound bath with chiller

2.4 EXPERIMENTAL PROCEDURE

2.4.1 Defatting of Prepared Sample

Defatting of prepared sample was done by using soxhlet apparatus to remove the fat content from the sample. Otherwise, fat gets accumulate on extracted product and can affect the purity of the product.

A Soxhlet extractor has three main sections: a percolator which circulates the solvent, a thimble usually made of thick filter paper which retains the solid to be extracted, and a siphon mechanism, which periodically empties the thimble.

The prepared sample of cocoa bean shell powder containing the fat to be extracted is placed inside the thimble. The thimble is loaded into the main chamber of the Soxhlet extractor. The extraction solvent hexane to be used is placed in a distillation flask. The flask is placed on the heating mantle. Then the soxhlet extractor is placed at top of the flask. A condenser is placed at top the extractor. The hexane was heated to reflux. The hexane vapour travels up a distillation arm, and floods into the chamber housing the thimble of sample. The condenser ensures that any hexane vapour cools, and drips back down into the chamber housing the sample. The chamber containing the sample slowly fills with warm hexane. Some of the fat compound dissolves in the warm hexane. When the Soxhlet chamber is almost full, the chamber is emptied by the siphon. The hexane is returned to the conical flask. This cycle was

allowed to repeated up to six hours. During each cycle, a portion of the fat compound dissolves in the hexane. After many cycles the fat compound is concentrated in the conical flask.

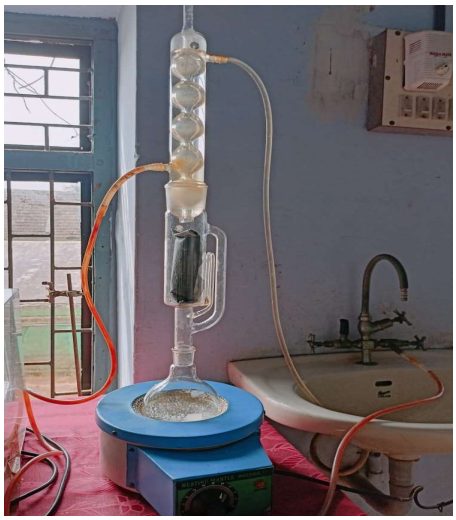

Plate 3.8. Defatting of sample with Soxhlet apparatus

2.4.2 Ultrasound treatment

After adding 200 ml of chloroform and 20 ml of 10% NH_4OH in to the defatted cocoa bean shell powder sample, it was placed in water bath ultrasonicator for 3 minutes at operating frequency of 33 kHz and output power of 250 W. Ultrasound treatment is done to improve the yield of extraction by cavitation principle.

2.4.3 Shaking

Shaking is done to ensure uniform mixing of solution and to increase the chemical reaction between reagents. Here shaking was done by using shaking incubator at room temperature, first it was done for 10 minutes at 400 rpm after ultrasonication. Then it was again shaken for 45 minutes at 400 rpm after adding 50 g of anhydrous Na_2SO_4.

Plate 3.9. Shaking incubator

2.4.4 Filtration

Filtration is a process in which components of a fluid mixture are separated based on their size during transfer through a porous material. The overnight kept sample were filtered by using Whatman filter paper of grade number 4 to obtain the filtered solution containing theobromine, and it was collected in conical flask. Then the theobromine was precipitated by evaporating chloroform from filtered solution.

Plate 3.10. Filtration of sample

2.5 SPECTROPHOTOMETER ANALYSIS

Spectrophotometer is used for the quantitative analysis of the sample, which measures the amount of light that a sample absorbs. It works by passing a light beam through a sample to measure the light intensity of a sample. Here analysis was done by using UV-VIS spectrophotometer. Spectrophotometer analysis were done for different samples. Transfer a few ml of chloroform extract into a l-cm quartz cell and observe the absorbance at 272.7 and 310 nm. Subtract the value at 310 nm from that at 272.2 nm to obtain a corrected absorbance value. The reading of samples in both nanometres are recorded.

Plate 3.12. UV Visible spectrophotometer

2.6 OPTIMISATION OF THE PROCESS PARAMETERS

Based on a detailed review of literature and the preliminary studies conducted,13 experiments, including 5 replicates or centre points, were performed according to Response Surface Methodology (RSM) and Central Composite Design (CCD). The process parameters which would influence the Theobromine yield and Absorbance in Spectrophotometer were chosen as independent variables. Yield of Theobromine and Absorbance in Spectrophotometer were taken as dependent variables.

Independent Variables:

1. Solute to Solvent ratio
    a) S_1 = 1:5
    b) S_2 = 1:10
    c) S_3 = 1:15
2. Time for Ultrasound Treatment (min)
    a) t_1 = 3
    b) t_2 = 5
    c) t_3 = 7

Dependent Variables:

1. Yield of Theobromine
2. Absorbance in Spectrophotometer

2.6.1 Determination of Theobromine Yield

$$\text{Theobromine yield (mg/g)} = \frac{\text{weight of Theobromine powder (mg)}}{\text{Weight of sample taken (g)}} \qquad \ldots\ldots(3.6)$$

Table 3.1. Different combinations of above chosen parameters

Sl. No.	Solute- solvent ratio	Time (minutes)
1	0.23	5
2	0.13	5
3	0.13	2
4	0.13	3
5	0.13	8
6	0.13	5
7	0.13	5
8	0.07	7
9	0.04	5
10	0.13	5
11	0.2	3
12	0.13	5
13	0.2	7

2.6.2 Absorbance in Spectrophotometer

Absorbance value of each sample is recording by using spectrophotometer. Absorbance value of each sample are used for further analysis (i.e., for obtaining optimised data).

2.7 HPLC (HIGH PERFORMANCE LIQUID CHROMATOGRAPHY)

The quantitative and qualitative analysis of product is done by high performance liquid chromatography. The theobromine obtained by optimised experimental procedure is utilised for high performance liquid chromatography. HPLC give quantitative and qualitative analysis of theobromine and also give confirmation about the product.

Theobromine content was extracted from cocoa bean shell powder was analysed using HPLC system (Plate 3.13) based on the previously developed method of Nguyen *et al.* (2015). Briefly, the extracts and standard theobromine solution (100 µg/ml in Dimethyl sulfoxide) were filtered through 0.45 µm nylon membranes, and 20 µL were then individually injected by an auto injector onto a column C18 (250 × 4.6 mm 5 µm), which was maintained at 35 ◦C by a column oven. The isocratic elution consisted of 0.05% phosphoric acid in distilled water (A) and 100% acetonitrile (B) (85:15). Flow rate was set at 1 ml/min. The theobromine compound was detected at 272 nm using a UV-VIS detector. Theobromine content in the extracts from

cocoa bean shell was quantified based on the calibration curve of standard theobromine by comparing retention time and peak area of standard theobromine.

Plate 3.13. HPLC System for quantification of theobromine

Theobromine content in the extracts from cocoa bean shell was quantified based on the calibration curve of standard theobromine having purity 98% by comparing concentration of standard theobromine in ppm and peak area of standard theobromine with those in the extracts from cocoa bean shell. The calibration curve of standard theobromine (concentration ranged from 0 to 1200 ppm) is indicated in Figure 3.1.

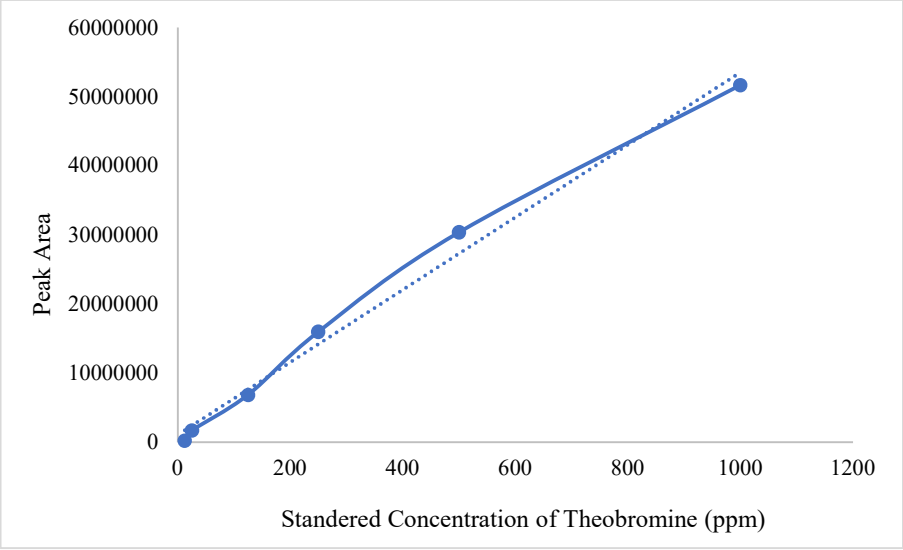

Figure 3.1. Calibration curve of standard theobromine.

2.8 DETERMINATION OF SOLUBILITY OF OBTAINED THEOBROMINE

To investigate the solubility of obtained theobromine in various solvents solubility test have been done. Chloroform, ethanol, distilled water and dimethyl sulfoxide (DMSO) were the solvents used for solubility determination which are frequently used solvents for determining solubility of alkaloids. Add 25 mg of a solid sample to 0.5 ml of above solvents in a conical flask followed by shaking at 250 rpm.

2.9 COMPARISON OF CONVENTIONAL EXTRACTION AND ULTRASOUND ASSISTED EXTRACTION METHOD

Compare the theobromine yield obtained from both conventional method of extraction and ultrasound-assisted method of extraction by using qualitative and quantitative analysis. The effect of ultrasound in extraction procedure were discussed.

2.10 STATISTICAL ANALYSIS

A CCD with two dependent factors was used for statistical analysis of the obtained parameters. The design consisted of 13 experiments with five replications at the center point. For the statistical analysis of obtained experimental data the commercial Design Expert® software, v.12 (Stat Ease Inc. Minneapolis, MN, USA) was used as well as the analysis of variance (ANOVA) to estimate the quality of the obtained models. The test of the statistically significant difference was based on the total error criteria with the level of confidence of 95.0%. The same software was used to generate the response plots in order to better understand the correlation of independent and response variables.

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

This chapter outlines the results obtained from various experiments conducted to determine some engineering properties of cocoa bean shell powder. Ultrasound assisted extraction of theobromine from cocoa bean shell have been evaluated along with the optimization of process parameters for maximum theobromine yield and maximum absorbance value. Also, the ultrasound extraction process is compared with conventional extraction process.

3.1 ENGINEERING PROPERTIES OF COCOA BEAN SHELL POWDER.

Prior to the extraction of theobromine from cocoa bean shell selected physical properties of cocoa bean shell powder viz., moisture content, bulk density, true density, porosity, ash content, pH, and fat content were investigated.

The average values of various physical properties of cocoa shell powder are presented in Table 4.1. The average moisture content of cocoa bean shell powder was 12.08 percent (wb). The true density and bulk density were 1333.33 and 480 kg/m^3, respectively. The porosity was 63.99 percent. Ash content of cocoa bean shell powder found as 10 percent. The pH found as 7.60. The Fat content of the sample was 2.75%.

Table 4.1. Engineering properties of cocoa bean shell powder

Sl. No.	Engineering properties	Value
1	Moisture content, %	12.08
2	True density, Kg/m^3	1333.33
3	Bulk density, Kg/m^3	480
4	Porosity, %	63.99
5	Ash content, %	10
6	pH	7.6
7	Fat content, %	2.75

3.2 ULTRASOUND ASSISTED EXTRACTION OF THEOBROMINE

In order to evaluate the extraction of theobromine from cocoa bean shell and for optimization of the process parameters, a series of experiments with solute-solvent ratios of 1:5, 1:10 and 1:15 and treatment times 3, 5 and 7 minutes as input variables were performed.

The experiments were performed as per the experimental procedure laid out in section 3.4. The results of the experiments conducted towards the ultrasound assisted extraction process with mean values of theobromine yield and absorbance in spectrophotometer are tabulated in Table 4.2.

Table 4.2 Effect of process variables towards extraction of theobromine from cocoa bean shell

Sl. No.	Sample	Yield, mg/g	Absorbance, au
1	S_1	1.217	0.074
2	S_2	3.153	0.954
3	S_3	5.714	1.05
4	S_4	5.23	0.698
5	S_5	2.923	0.085
6	S_6	3.153	0.953
7	S_7	1.077	0.352
8	S_8	3.429	1.712
9	S_9	7	2.562
10	S_{10}	3.384	0.586
11	S_{11}	1.45	0.098
12	S_{12}	2.769	0.495
13	S_{13}	1.65	0.071

Plate 4.1. Cocoa bean shell extract obtained through UAE method.

3.3 RESPONSE SURFACE ANALYSIS AND PROCESS OPTIMIZATION

To explore the influence of process parameters on the extraction yield, Absorbance of theobromine in CBS extracts, a response surface analysis was performed. The results showed that two responses (theobromine yield and Absorbance) (Table 4.2) were detected in all 13

experiments and they were included in further statistical analysis. The selected responses were evaluated by analysis of variance (ANOVA) and the results are summarized in Tables 4.3 and 4.4. The interaction between the extraction time and the solute to solvent ratio showed statistically significant effect for the yield (p= 0.0003 and 0.0064). The Model F-value of 10.79 implies the model is significant. There is only a 0.35% chance that an F-value this large could occur due to noise. The Lack of Fit F-value of 0.37 implies the Lack of Fit is not significant relative to the pure error. There is a 78.04% chance that a Lack of Fit F-value this large could occur due to noise. For Absorbance, a statistically significant effect was shown in quadratic term of solute to solvent ratio ($p < 0.0001$) not in time. The Model F-value of 18.64 implies the model is significant. There is only a 0.06% chance that an F-value this large could occur due to noise The Lack of Fit F-value of 0.66 implies the Lack of Fit is not significant relative to the pure error. There is a 61.69% chance that a Lack of Fit F-value this large could occur due to noise (Tables 4.3 and 4.4).

Table 4.3. Analysis of variance (ANOVA) for the response surface quadratic model for theobromine yield

Source	**Sum of Squares**	**Df**	**Mean Square**	**F value**	**p value**	
Model	34.62	5	6.92	10.79	0.0035	significant
A-Solute to Solvent ratio	28.50	1	28.50	44.43	0.0003	
B-Time	9.42	1	9.42	14.68	0.0064	
AB	4.21	1	4.21	6.57	0.0374	
A^2	2.77	1	2.77	4.32	0.0762	
B^2	3.68	1	3.68	5.73	0.0479	
Residual	4.49	7	0.6416			
Lack of Fit	0.9740	3	0.3247	0.3692	0.7804	not significant
Pure Error	3.52	4	0.8792			
Cor Total	39.12	12				

Table 4.4. Analysis of variance (ANOVA) for the response surface quadratic model for Absorbance

Source	Sum of Squares	df	Mean Square	F value	p value	
Model	5.97	5	1.19	18.64	0.0006	significant
A-Solute to Solvent ratio	5.23	1	5.23	81.65	< 0.0001	
B-Time	0.3222	1	0.3222	5.03	0.0598	
AB	0.0269	1	0.0269	0.4200	0.5376	
A^2	0.9916	1	0.9916	15.48	0.0056	
B^2	0.0060	1	0.0060	0.0933	0.7689	
Residual	0.4484	7	0.0641			
Lack of Fit	0.1489	3	0.0496	0.6629	0.6169	not significant
Pure Error	5.97	5	1.19	18.64	0.0006	significant
Cor Total	5.23	1	5.23	81.65	< 0.0001	

In general, the liquid/solid ratio showed the largest statistically significant effect for both tested responses (Absorbance and yield) ($p < 0.05$), while the extraction time show statistically significant effect on a response (yield). Regression models for both responses showed a statistically significant effect (p-values 0.00035 and 0.0006) with satisfactory coefficients of determination (R^2) 0.8852 and 0.9301. The Predicted R^2 of yield is of 0.7122 is in reasonable agreement with the Adjusted R^2 of 0.8032; i.e., the difference is less than 0.2. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. For this ratio is 11.1025, that indicates an adequate signal i.e., model can be used to navigate the design space. The Predicted R^2 of absorbance is 0.6816 and is in reasonable agreement with the Adjusted R^2 of 0.8802 and signal to noise ratio is 15.789. Given that p-value for all regression models were below 0.05 means that there is a statistically significant influence between the independent variables and the variables of the observed responses. The Lack of Fit in all cases was not statistically significant ($p > 0.05$), which means that the obtained second order polynomial equation is adequate for accurate estimation of experimental values and can be used to make predictions about the response for given levels of each factor. The high levels of the factors were coded as +1 and the low levels as -1, by default. The coded equations are

useful for identifying the relative impact of the factors by comparing the factor coefficients (Table 4.5).

Table 4.5. Polynomial equations calculated after implementation of CCD (in terms of coded factors)

Regresion Coefficient	2nd Order Polynominal Equation
Yield	2.6536 + -2.15159 * A + -1.09506 * B + 1.28789 * AB + 0.652682 * A + 0.676483 * B
Absorbance	0.60992 + -0.921735 * A + -0.202529 * B + 0.102912 * AB + 0.390351 * A + -0.0272667 * B

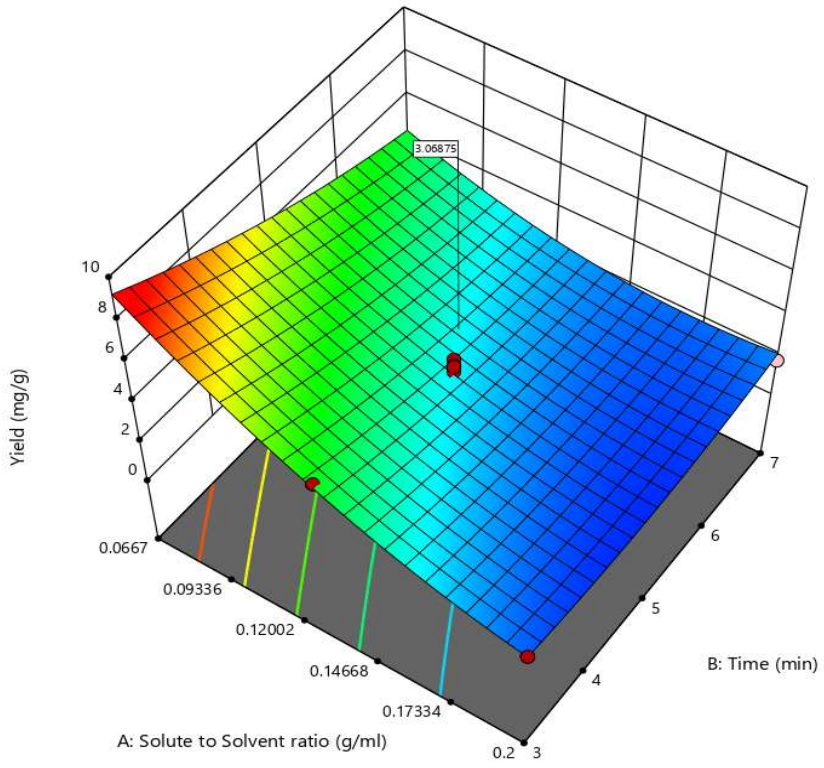

Figure 4.1. Three-dimensional plots for theobromine yield in CBS obtained by UAE

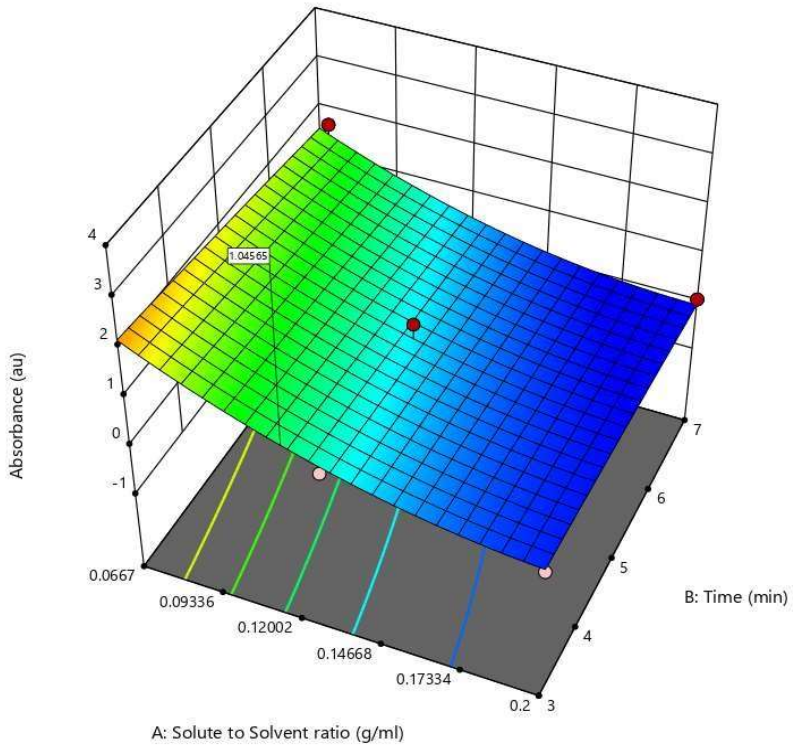

Figure 4.2 Three-dimensional plots for Absorbance in CBS obtained by UAE

Three dimensional plots for yield and absorbance shows slightly similiar shapes which was expected their slight correlation . Concentration of theobromine is increased by decreasing solute to solvent ratio from 1:10 to 1:15,and , also the theobromine yield will slightly decrease by increasing time for application of ultrasound. Highest yield and absorbance shows in 5 minute ultrasound application. So mean of total time range will give better yield (Figure 4.1). In case of absorbance, by increasing the solid to solvent ratio it is decreased. That is highest absorbance occur when the solute to solvent ratio lowest, while by further increasing of this parameter, Absorbance decreased. The graph also showed the slight increase of absorbance with increase in ultrasound time (Figure 4.2).

By looking 3D graph and ANOVA, solute to solvent ratio is highly significant in yield and less significant in absorbance, it may be due to spectrophotometr analysis at 272.7 nm and 310 nm. Time is marginally significant to both yield and absorbance, it may be due to extraction of other alkaloids such as caffeine, theophylline other than theobromine. While in case of absorbance, for theobromine have peak showed at 272.7 nm and 310 nm so absorbance of theobromine only showed in spectrophotometer (Berger and Hartland., 1990).

If we look at other similar studies, Esclapez et al. (2011) mentioned that Solute to solvent ratio, as one of the important UAE parameters, affects the yield of each compound such as theobromine, theophylline, Catechin, epicatechin, Caffeine acid, caffeine and etc. individually, due to improved mass transfer triggered by ultrasound. iménez and Cañizares-Macías (2013) proved that the UAE is more efficient in isolating caffeine (by 57.7%) and theobromine (by 43.6%) from cocoa beans in comparison to conventional extraction by mixing. As a reason for more efficient extraction, they pointed out the importance of ultrasonic waves that do not cause modification of the extract. Dent et al. (2015) also compared some conventional methods for extraction of phenolic compounds from sage using two different UAE techniques (ultrasonic device with direct mixing and direct sonication with a probe). Studies have shown that UAE with ultrasonic device with direct mixing achieved the highest yield of total and individual polyphenols while direct sonication was also more efficient than conventional extractions. Bamba et al. (2018) were the first who to investigate the influence of UAE conditions on the yield of phenolic compounds from blueberries (Vaccinium angustifolium) as well as the antioxidant activity of the obtained extracts. They proved that the efficiency of this extraction largely depends on the ethanol content in the aqueous extract, the solid/liquid ratio, temperature, time and pH. UAE with a 50% ethanol gave higher yields of flavonoids and anthocyanins.

The RSM in this study gave optimal conditions for the UAE extraction of theobromine from CBS from the desirability function approach, having a desirability of 0.969 taking into account in range 1:5 to 1:15 of solute to solvent ratio 0.067g/ml and an ultrasound application time of 3.000 minutes. Under these conditions, the predicted yield was calculated to be 8.517 mg/g and absorbance of 2.200 au which agrees with the experimentally obtained data (Table 4.6)

Table 4.6. Optimised values with a specific goal

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance	Optimized Value
A: Solute to Solvent ratio	is in range	1:15	1:5	1	1	3	1:15
B: Time	is in range	3	7	1	1	3	3.000
Yield	maximize	1.077	7	1	1	4	8.517
Absorbance	is in range	0	3	1	1	3	2.200

3.4 HPLC ANALYSIS FOR QUANTIFICATION

The presence of theobromine in CBS extracts of two samples having highest yield i.e., sample 9 (yield = 7 mg/g) and sample 3 (yield = 5.714 mg/g) was determined by high performance liquid chromatography method. The presence methylxanthine theobromine present in CBS was analysed by comparing with chromatogram of pure theobromine. The chromatogram of standard theobromine at 1000 ppm having 95% purity is shown in Figure 4.3. Chromatogram of sample 3 and 9 that are produced having solute to solvent ratio 0.13 and 0.04 g/ml and time 2 and 5 minutes respectively is shown in Figures 4.4 and 4.5.

From the Figures 4.4 and 4.5, it may be concluded that theobromine can be extracted in this method successfully and quantity of theobromine is highest in sample 9. It may be due to highest yield and absorbance of CBS extracts of sample 9. It shows that, might be able to get highest quantity of theobromine if we use optimized sample instead of 13 combinations of samples.

Table 4.7. Actual concentration of theobromine obtained through HPLC analysis

Samples conducted by HPLC	Peak Area	Dilution Factor	concentration of theobromine (ppm)	Actual concentration of theobromine (ppm)
Sample 9 10 mg/ml	89893954	1	1679.91665	1679.91665
Sample 9 4 times diluted	30560876	4	559.9993249	2239.9973
sample 3 4 times diluted	23803133	4	432.4463019	1729.785207

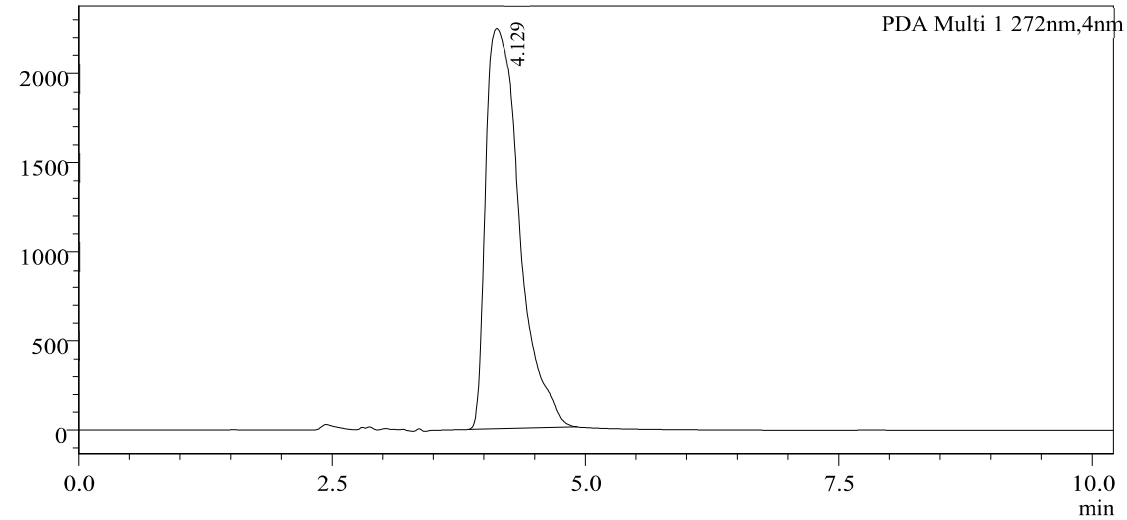

Figure 4.3. HPLC chromatograms of standard theobromine at 1000 ppm (95% purity)

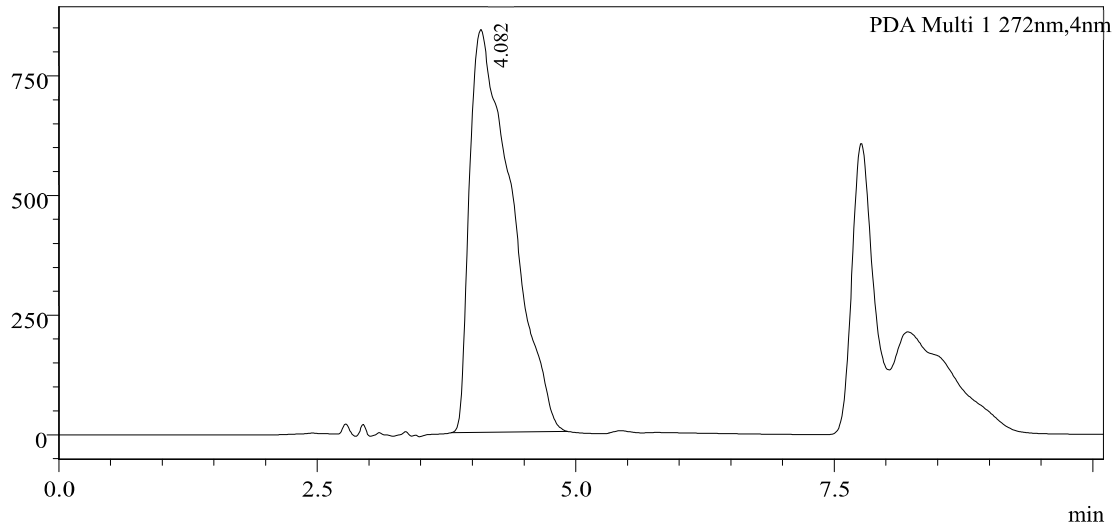

Figure 4.4. HPLC chromatograms of theobromine in CBS extracts of Sample 3.

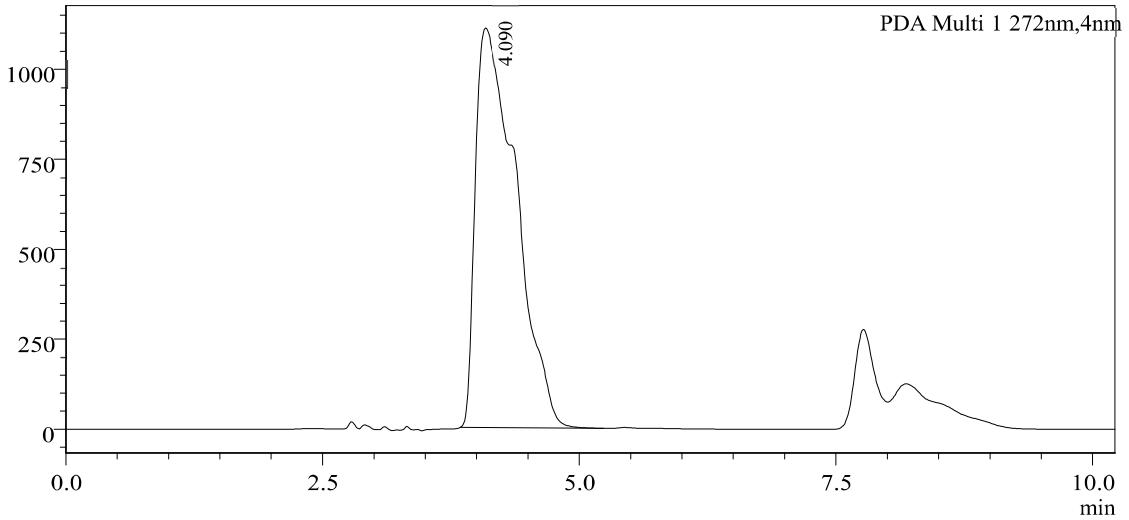

Figure 4.5. HPLC chromatograms of theobromine in CBS extracts of Sample 9.

3.5 SOLUBILITY TEST

From the selected solvents, extracted sample is found as soluble in Dimethyl Sulfoxide (DMSO). The standard theobromine also found as soluble in DMSO, where it's slightly soluble in chloroform and ethanol insoluble in water.

3.6 COMPARISON OF UAE WITH CONVENTIONAL EXTRACTION METHOD

The theobromine yield and absorbance of cocoa bean shell extracts at optimum parameter levels were found to be 8.517 mg/g and 2.200 au respectively whereas the same were found to be 4.75 mg/g and 0.372 respectively for conventional extraction process. By this we may be concluded that the application of ultrasound waves into the cocoa bean shell sample results in the higher yield of theobromine as well as other methylxanthines in the finished extracts, than the conventional extraction.

SUMMARY AND CONCLUSION

CHAPTER V
SUMMARY AND CONCLUSION

Theobroma cacao, also called the cocoa tree, which are usually found in tropical areas of South and Central America. Even there are over twenty species in the genus *Theobroma*, cocoa tree is the mostly cultivated one. The tree grows in wet lowland tropics, often in the shade of taller trees, and its thick trunk supports large leathery oblong leaves. Cocoa pods (outer husk) range in color from bright yellow to deep purple. Each pod holds 20 to 60 seeds or cocoa beans, arranged around the long axis of the pod. These cocoa beans are processed into chocolates, cocoa butter and cocoa powder, which will have the characteristic smell and flavor. In India, cocoa is being cultivated in Kerala, Tamil Nadu, Andhra Pradesh and Karnataka. The average productivity of cocoa in Indian is 669 Kg/ha. The global cocoa beans market is expected to grow at a compound annual growth rate (CAGR) of 7.3 percent from 2019 to 2025 and to reach USD 16.32 billion.

In the processing of cocoa beans to desired products, cocoa bean shell, cocoa pod and other undesired materials will be discarded. Cocoa bean shell (CBS) is the largest by-product obtained from a cocoa processing industry. CBS containing a number of bioactive compounds, can be convert into useful product by extraction of these compounds. The basic composition, includes dietary fibers, carbohydrates, methylxanthines, like theobromine (3,7-dimethylxantine), caffeine (1,3,7-trimethylxantine), and theophylline (1,3- dimethylxanthine), fats and some phenolic compounds. There are numerous methods that have been evolved for the extraction of Methylxanthines like theobromine from Cocoa bean as well as CBS. It involves extraction by using solvents like ethanol, chloroform etc. Recently, latest technologies like pressurized liquid extraction, super critical CO_2 extraction, ultrasound-assisted extraction, deep eutectic solvents etc.

In this study, we evaluate the ultrasound assisted extraction (UAE) of theobromine from cocoa bean shell. High frequency electrical energy is converted into ultrasound waves of required frequency and it will produce numerous microscopic vacuum bubbles that grow and collapse briskly. This process is called cavitation. For the evaluation, the process parameters which would influence the theobromine yield and absorbance were chosen as independent variables after preliminary studies. The solute – solvent ratio and treatment time are taken as

the independent variables. The levels of process parameters were fixed as solute-solvent ratios of 1:5, 1:10 and 1:15 and treatment time of 3, 5 and 7 minutes.

The optimized conditions of solute-solvent ratio and treatment time for extracting theobromine by ultrasound assisted process was found to be 0.067 g/ml and 3.542 minutes, respectively. Therefore, ultrasound assisted extraction of theobromine from cocoa bean shell could be considered as a best extraction technique that results in the production of theobromine in higher yield than conventional. Theobromine extracted by this study is quite a bit similar to the commercially available theobromine. CBS, a by-product obtained from cocoa industry can be utilized for the its higher nutritive compounds than simply discarding environment. Also, ultrasound having wider application in food industry is considered as a useful extraction technique for theobromine with higher yield.

REFERENCES

REFERENCES

Adamafio, N.A. 2013. Theobromine toxicity and remediation of cocoa by-products: an overview. *J. Biological Sci.* 13(7): 570-576.

Balentic, J.P., Ackar, D., Jokic, S., Jozinovic, A., Pavlovic, N. and Subaric, D. 2018. Cocoa shell: a byproduct with great potential for wide application. *J. Molecules.* 23(6): 1-14.

Bhargava, N., Mor, R.S., Kumar, K., Sharanagat, V.S. 2021. Advances in application of ultrasound in food processing: A review. *Ultrason. Sonochem*. 70: 1-14.

Bispo, M.S., Veloso, M.C., Pinheiro, H.L., De Oliveira, R.F.S., Reis, J.O.N. and De Andrade. J.B. 2002. Simultaneous determination of caffeine, theobromine and theophylline by high-performance liquid chromatography. *J. Chromatographic Sci.* 40(1): 45-48.

Chakraborty, S., Uppaluri, R. and Das, C. 2020. Optimization of Ultrasound-assisted Extraction (UAE) process for the recovery of bioactive compounds from bitter gourd using response surface methodology (RSM). *Food Bioprod. Process*. 120(5): 114-122.

Chaudhari, S.P. and Gaikwad, S.U. 2020. Development and validation of UV spectrophotometric method for simultaneous estimation of caffeine and quercetin in niosome formulation. *Int. J. Creat. Res. Thoughts* 8(4): 2320-2882.

Chavan, P., Sharma, P., Rattan, S., Mittal, T. C. and Jaiswal, A. K. 2022. Application of high intensity ultrasound to improve food processing efficiency: a review. *J. Foods.* 11(1): 1-18.

Chawla.G. and Chaudhary K.K. 2019. A review of HPLC technique covering its pharmaceutical, environmental, forensic, clinical and other applications. *J. Pharmaceutical Chem. Analysis.* 6(2): 27-39.

Chemat, F., Huma, Z. and Khan, M.K. 2011. Applications of ultrasound in food technology: processing, preservation and extraction. *Ultrason. Sonochem*. 18(4): 813-835.

Coco, F.L., Lanuzza, F., Micali, G. and Cappellano, G. 2007. Determination of theobromine, theophylline, and caffeine in by-products of cupuacu and cacao seeds by high-performance liquid chromatography. *J. Chromatographic Sci.* 45(5): 273-275.

Desai. S. 2020. Estimation of caffeine content from soft and energy drinks obtained from regional market by UV spectroscopy and TLC. *Int. J. Sci. Dev Res.* 5(4): 2455-2631.

Eteng, M.U., Eyong, E.U., Akpanyung, Agiang, M.A., Aremu, C.Y. 1997. Recent advances in caffeine and theobromine toxicities: a review. *Plant Foods for Human Nutri.* 51(3): 231-243.

Fioresi, F., Vieillard, J., Bargougui, R., Bouazizi, N., Fotsing, P. Woumfo, E., Brun, N., Mofaddel, N. and Derf, F. 2017. Chemical modification of the cocoa shell surface using diazonium salts. *J. Colloid Interface Sci.* 2017. 494: 92-97.

Gopalasatheeskumar, G. 2018. Significant role of soxhlet extraction process in phytochemical Research. *Mintage J. Pharmaceutical Medical Sci.,* 7(1): 43-47.

Hansen, P.M.T. 1965. Spectrophotometric Determination of Chocolate in Chocolate Products. *J. Dairy Sci.* 48(11): 1401-1405.

Hartati, I. 2010. Hydrotropic extraction of theobromine from cocoa bean shell. *Momentum.* 6(2): 17-20.

Jimenez, L.P. and Macias, M.P. 2012. Ultrasound-assisted method for extraction of theobromine and caffeine from cocoa seeds and chocolate products. *Food bioprocess Technol*. 6: 3522-3529.

Jokic, S., Gagic, T., Knez, Z. and Subaric, D. 2018. Separation of active compounds from food by-product (cocoa shell) using subcritical water extraction. *J. Molecules.* 23(6): 1-17.

Kapturowska, A., Stolarzewicz, I., Chmielewska, I. and Bialecka-Florjanczyk, E. 2011. Ultrasounds—A tool to inactivate yeast and to extract intracellular protein. *Food Sci. Technol. Qual.* 18: 160–171.

Kowalski, S.J., Mierzwa, D. and Stasiak, M. 2017. Ultrasound-assisted convective drying of apples at different process conditions, *Drying Technol*. 35(8): 939-947.

Krasulya, O., Bogush, V., Trishina, V., Potoroko, I., Khmelev, S., Sivashanmugam, P., and Anandan, S. 2016. Impact of acoustic cavitation on food emulsions. *Ultrason. Sonochem*. 30: 98-102.

Lee, H., Zhou, B., Liang, W., Feng, H.and Martin, S.E. 2009. Inactivation of Escherichia coli cells with sonication, manosonication, thermosonication, and manothermosonication: Microbial responses and kinetics modeling. *J. Food Eng*. 93: 354–364.

Li, S., Berger, J. and Hartlands, S. 1989. UV spectrophotometric determination of theobromine and caffeine in cocoa beans. *Anal. ChimActa.* 232: 409-412.

Manson, T.J., Paniwnyk, L., and Lorimer, J.P. 1996. The use of ultrasound in food technology. *Ultrason. Sonochem*. 3(5): 253 -260.

Martinez, E., Onatibia, A and Franco, R. 2015. The relevance of theobromine for the beneficial effects of cocoa consumption. *Frontiers in pharmacology.* 6(30): 1-5.

Martinez, R., Torres, P., Meneses, M.A., Figueroa, J.G., Pérez-Álvarez, J.A. and Viuda-Martos, M. 2012. Chemical, technological and in vitro antioxidant properties of cocoa (Theobroma cacao L.) co-products. *Food Res. Int*. 49: 39-45.

Nguyen, V. T., and Nguyen, N. H., 2017. Proximate composition, extraction, and purification of theobromine from cocoa pod husk. *J. Technol.* 5(2): 1 -10.

Nunez, L.P. and Macias, M.P. 2011. Focused microwaves-assisted extraction of theobromine and caffeine from cacao. *Food Chem*. 129: 1819-1824.

Patel, K., Panchal, N., and Ingle. P., 2019. Review of extraction techniques extraction methods: microwave, ultrasonic, pressurized fluid, soxhlet extraction, etc. *Int. J. Advanced Research in Chemical Sci.* 6(3): 6-21.

Pavlovic, N., Jakocljevi, M., Molnar, N. and Jokic, S. 2019. Green extraction technique of bioactive components from cocoa shell. *Croat. J. Food Sci. Technol.* 11(11): 11-20.

Pavlovic, N., Molnar, N. and Jokic, S. 2020. Green extraction method s for active compounds from food waste- cocoa bean shell. *J. Foods.* 9(2): 1-14.

Pavlovic, N., Molnar, N. and Jokic, S. 2021. Ultrasound-assisted extraction of active compounds from cocoa bean shell. *Food in Health and Disease.* 10(2): 77-88.

Premnath, P., John, J., Machanchery, N., Subbiah, K. S. and Prabhu, S. 2019. Effectiveness of theobromine on enamel re-mineralization: a comparative in-vitro study. *Cureus.* 11(9): 5686.

Ramli, N., Yatim, A.M., Said, M. and Hok, H.C. 2001. HPLC Determination of methylxanthines and polyphenols levels in cocoa and chocolate products. *Malaysian J. Analytical Sci.* 7(2): 377-386.

Redgwell, R., Trovato, V., Merinat, S., Curti, D., Hediger, S. and Manez, A. 2003. Dietary fibre in cocoa shell: Characterisation of component polysaccharides. *Food Chem.* 81(1): 103-112.

Rodsamran, P.and Sothornvit, R. 2019. Extraction of phenolic compounds from lime peel waste using ultrasonic-assisted and microwave-assisted extractions*, Food Biosci*. 28: 66-73.

Rojo- Poveda, O., Barbosa, L., Zeppa, G. and Stevigny, C. 2020. Cocoa bean shell-a by-product with nutritional properties and biofunctional potential. *J. Nutri.* 12(4): 1-29.

Saucier, C., Adebayo, M.A., Lima, E.C., Cataluña, R., Thue, P.S., Prola, L.D., Rosero, M.J., Machado, F.M., Pavan, F.A., Dotto, G.L. 2015. Microwave-assisted activated carbon from cocoa shell as adsorbent for removal of sodium diclofenac and nimesulide from aqueous effluents. *J. Hazard. Mater.* 289: 18-27.

Soares, T. F. and Beatriz, M. 2022. Cocoa by-products: characterization of bioactive compounds and beneficial health effects. *J. Molecules.* 27: 1-23.

Srdjenovic, B., Djordjevic-Milic, V., Grujic, N., Injac, R. and Lepojevic, Z. 2008.Simultaneous HPLC determination of caffeine, theobromine, and theophylline in food, drinks, and herbal products. *J. Chromatographic Sci.* 46:144-149.

Starek, A., Kobus, Z., Sagan, A., Chudzik, B., Pawłat, J., Kwiatkowski, M., Terebun, P. and Andrejko, D. 2021. Influence of ultrasound on selected microorganisms, chemical and structural changes in fresh tomato juice. *Sci. Rep*. 11: 1-12.

Tzanova, M., Atanasov, V., Yaneva, Z., Ivanova, D. and Dinev, T., 2020. Selectivity of current extraction techniques for flavonoids from plant materials. *Processes*. 8: 1-30.

Villamiel, M., Hamersveld, E.H., and DeJong, P. 1999. Effect of ultrasound processing on quality of dairy products. *Milchwissenschaft*. 54: 69-73.

Xia, Z., Ni, Y. and Kokot, K. 2013. Simultaneous determination of caffeine, theophylline and theobromine in food samples by a kinetic spectrophotometric method. *Food Chem.* 141(4): 4087-4093.

Yu, M., Wang, B., Qi, Z., Xin, G. and Li, W. 2019. Response surface method was used to optimize the ultrasonic assisted extraction of flavonoids from *Crinum asiaticum*., *Saudi J. Biological Sci.* 26(8): 2079–2084.

Zheng, L. and Sun, D.W. 2006. Innovative applications of power ultrasound during food freezing process – A review. *Trends Food Sci. Technol*. 17(1): 16-23.

ABSTRACT

ULTRASOUND ASSISTED EXTRACTION OF THEOBROMINE FROM COCOA BEAN SHELL

By

FARHANA SHIRIN (2019-06-006)

MUBASHIR K.P. (2019-06-014)

SAJEER K (2019-06-017)

UMMUL ASNA K (2019-06-020)

VISHNU PRASAD J.R. (2019-06-021)

ABSTRACT

Submitted in partial fulfilment of the requirement for the degree

Bachelor of Technology

in

Food Engineering & Technology

Faculty of Agricultural Engineering and Technology

Kerala Agricultural University

DEPARTMENT OF PROCESSING AND FOOD ENGINEERING

KELAPPAJI COLLEGE OF AGRICULTURAL ENGINEERING AND TECHNOLOGY

TAVANUR-679573, MALAPPURAM, INDIA

2023

ABSTRACT

Theobromine, also known as xantheose, is the principal alkaloid of *Theobroma cocoa.* During fermentation of cocoa bean, theobromine content increases and some are migrated to cocoa bean shell. Since cocoa bean shell is a by-product from cocoa processing industry, it is widely available and can't be used as animal feed, it is good for production of theobromine. Theobromine has many applications in pharmaceutical field such as it can be used as vasodilator, heart stimulant and as a treatment for asthma, uric acid nephrolithiasis, remineralisation of teeth enamel also can be used for production of caffeine.

In this study we are extracting theobromine from cocoa bean shell by normal conventional method and ultrasound assisted extraction method. For further evaluation, we made dependent and independent variables for extraction. The process parameters which would influence the theobromine yield and absorbance were chosen as independent variables after preliminary studies. The solute to solvent ratio and treatment time are taken as the independent variables. The levels of process parameters were fixed as solute-solvent ratios of 1:5, 1:10 and 1:15 and treatment time of 3, 5 and 7 minutes. Also, dependent variables are theobromine yield and absorbance. Using these variables 13 combinations were made by Design Expert® software, v.12 and central composite design. In this 13 experiments sample 9 and 3 shows first and second largest yield. Sample 9 has 7 mg/g yield and 2.562 au absorbance. Also sample 3 has 5.714 mg/g yield and 1.05 au absorbance. Based on this HPLC were conducted and found that concentration of theobromine in sample 9 has 2239.799 ppm and sample 3 has 1729.985 ppm. The optimized conditions of solute-solvent ratio and treatment time for extracting theobromine by ultrasound assisted process was found to be 1:15 and 3.000 minutes, respectively. The theobromine yield and absorbance of cocoa bean shell extracts at optimized levels were found to be 8.517 mg/g and 2.200 au respectively whereas the same were found to be 4.75 mg/g and 0.372 respectively for conventional extraction process. It was also observed that methylxanthine theobromine was found to be higher in ultrasound assisted extraction of theobromine from cocoa bean shell extracted under optimized condition compared to conventional extraction of theobromine. Therefore, ultrasound assisted extraction of theobromine could be considered as a best extraction technique that results in the production of theobromine.