Addis Ababa Institute of Technology

School of Chemical and Bio Engineering

Parametric Optimization and Characterization of Ferulic Acid Produced from Brewery Spent Grains

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A thesis submitted to the School of Chemical and Bio Engineering, Addis Ababa Institute of Technology, in partial fulfillment of the requirements for the Degree of Master of Science in Chemical Engineering (Environmental Engineering)

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ABSTRACT

The utilization of agro-industrial wastes for the extraction of natural antioxidants such as Ferulic acid is currently getting a growing interest by the researchers around the world. This research study, therefore, aims at optimizing and characterizing the Ferulic acid extracted from brewery spent grains (BSG). Brewery spent grain was first treated with sulphuric acid before it was subjected to alkaline hydrolysis. The X-ray diffraction analysis of the raw and hydrolyzed BSG showed the evidence for a possible release of phenolic extractives after the hydrolysis. The effect of extraction time, extraction temperature, and alkali concentration on the Ferulic acid yield was studied at (60, 90, and 120 °C), (100, 120 and 140 min), and (1, 2 and 3% (w/v)) respectively. Optimization of these extraction parameters was carried out by the Box Behnken Design, using the Response Surface Methodology (Design experiment software version 11). It was observed that the Ferulic acid yield at different runs ranged from 6.62 mg/100 g BSG to 45.245±0.585 mg/100 g BSG. An optimum Ferulic acid yield of 46.85 mg/100 g BSG was observed at 119.83 °C, 97.04 min, and 2.21% (w/v) NaOH concentration. Both the Fourier Transform Infrared Spectroscopy (FT-IR) and High-Performance Liquid Chromatography (HPLC) analysis showed that the BSG was a good candidate for the extraction of the Ferulic acid. All the three main factors considered for this research study, namely the extraction time, extraction temperature and sodium hydroxide concentration had a statistically significant effect on the yield of Ferulic acid extracted. Spectrophotometric analysis of the produced Ferulic acid using DPPH radical scavenging method showed a strong capacity of its antioxidant activity. The result of the present research provides an alternative insight into the waste management of brewery spent grains (BSG) through the production of Ferulic acid.

Keywords: Ferulic acid, Brewery spent grains, Antioxidant, Extraction, Response surface methodology, Box Behnken Design, Optimization

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ACRONYMS AND ABBREVIATIONS

BSG	Brewery Spent Grain
Kg	Kilo Gram
h	Hour
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
TBHQ	Tertiary Butyl Hydroquinone
EDTA	Ethylenediaminetetraacetic Acid
mM	Milli Molar
GC/MS	Gas Chromatography/ Mass Spectroscopy
LC/MS	Layer Chromatography/Mass Spectroscopy
PC	Paper Chromatography
TLC	Thin Layer Chromatography
HPLC	High-Performance Liquid Chromatography
FT-IR	Fourier Transform Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
ANOVA	Analysis of Variance
BBD	Box -Behnken Design
CCD	Central Composite Design
RSM	Response Surface Methodology
DOE	Design of Experiment
RP	Reverse Phase
UV/VIS	UV/visible
DPPH	1,1-Diphenyl-1-picrylhydrazyl
TPC	Total Phenol Content

CHAPTER ONE

1. INTRODUCTION

1.1. Background

Next to tea, carbonates, milk, and coffee, beer is the fifth most consumed beverage in the world with an estimated annual world production exceeding 1.34 billion hectoliters in 2002 (Fillaudeau et al., 2006). In the manufacture of beer, various residues and by-products are generated. The most common ones are spent grains, spent hops and surplus yeast, which are generated from the main raw materials (Mussatto, 2009).

Brewer's spent grain (BSG) is a by-product of the mashing process corresponding to around 85% of total by-products generated in the brewery, consisting of the solid residue remaining after mashing and lautering (Mussatto, 2009; Xiros et al., 2012). BSG is lignocellulosic biomass that consists mainly of fibres such as cellulose and hemicellulose and other compounds such as protein, lipids, starch, and lignin (Aliyu et al., 2011).

The type of barley grains, its harvesting time, the addition of other adjuncts, the composition of the hops, and the brewing technology – all have direct effects on the chemical composition of BSG (Santos et al., 2003; Xiros et al., 2009). Since BSG high moisture content and high content in polysaccharides and proteins, it is prone to microbial contamination. This, in turn, makes its storage stability limited to up to 7–10 days (Aliyu et al., 2011). Consequently, this has an impact on the transportation of BSG, which becomes expensive due to high-water content, resulting in the discharge of BSG in landfills being a common practice which can lead to environmental problems (Xiros et al., 2012).

The characteristics of BSG discussed above, therefore, reduce its commercial value. For this reason, using it as livestock feed has been the most common practice aside landfilling for its treatment (Xiros et al., 2012). Currently, to solve this problem (the low commercial value of BSG and environmental issues of the uncontrolled emission in landfills), strong research interests are growing by different scholars around the globe with the purpose of both establishing techniques

that will improve its storage stability and evaluating new valorization schemes that will yield high-value-added products.

To this extent, various techniques have been applied for its storage and preservation (Mussatto, 2006), whereas applications such as human nutrition, energy production, charcoal production, adsorbents, and incorporation in biotechnological applications have been studied (Cooray et al., 2017). As for the biotechnological application, BSG has been studied as a substrate for enzyme production, biogas, ethanol fermentation, cultivation of mushrooms, and production of lactic acid (Mussatto, 2006). In addition, the presence of phenolic acids and mainly Ferulic acid in significant quantities, make BSG a promising material for many industrial applications (Charilaos et al., 2009). Ferulic acid (Figure 1-1), having a chemical formula $C_{10}H_{10}O_4$ and a chemical name 4-hydroxy-3-methoxycinnamic acid, is one of the phenolic acids group which are ubiquitous components of the primary cell walls of several plants (Hartley, 1973).

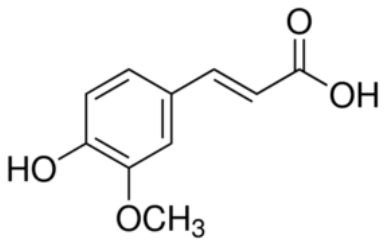


Figure 1-1: Structure of Ferulic acid (Young, 2003)

It naturally occurs in their seeds and leaves in both its free form and esterified to mono- and disaccharides forms as a plant cell wall polysaccharides, glycoproteins, polyamines, lignin and hydroxylated fatty acids (Smith & Hartley, 1983; Mueller-Harvey & Hartley, 1986; Saulnier & Thibault, 1999; Mathew & Abraham, 2004; Srinivasan et al., 2007; Shahidi & Chandrasekara, 2010). Ferulic acid, along with dihydroferulic acid, is a component of lignocellulose, helps to maintain cell wall rigidity by cross-linking lignin and polysaccharides.

Ferulic acid is considered one of the most important phenolic acids, having many physiological functions, including anti-oxidant, anti-microbial, anti-inflammatory, anti-thrombosis, and anti-cancer activities (Mussatto, 2007). It also protects against coronary disease, lowers cholesterol in serum and liver, and increases sperm viability (Ou and Kwok, 2004). Because of these properties, it is receiving increased attention regarding applications in the food, health, cosmetic, and pharmaceutical industries (Kroon and Williamson, 1999).

With the realization of this wide application of Ferulic acid, different methods have been tested for its extraction from cheap and abundantly available agro-industrial wastes like BSG. In this regard, numerous alkaline, acidic and enzymatic methods have been reported (Anvar et al., 2009; Kim et al., 2006, Mathew et al., 2005, Mathew et al., 2006, Soto et al., 2011, Xu et al., 2005) to produce Ferulic acid with an optimized process. Advanced methods such as microwave-assisted extraction, pressurized liquid extraction, ultrasonic extraction, and supercritical fluid extraction have also emerged since recent times.

Traditionally, the one-factor-at-a-time technique is used as an optimization method. However, these variables' optimization approach is not an effective one, mainly for two reasons: it is time-consuming and often easily confuses the alternative effects between the components. Besides the fact that it is resources intensive for it requires many experimental works to determine the optimum levels has made it, nowadays, the least preferable option. This drawback of the one-factor method can be eliminated by optimizing all the affecting parameters collectively by response surface methodology (Gopinadh et at al., 2015), which is the focus of this study.

1.2. Statement of the Problem

The brewery industry is a lucrative beverage market throughout the world and therefore generates enormous amounts of organic effluents - the major portion of which is BSG (Sachindra et al., 2018). In 2014 worldwide beer production was reported to be 193 billion liters, and for every 100 liters of beer produced roughly 20 kg of BSG was generated (Mussatto & Roberto, 2006). In the case of Ethiopia, as to the knowledge of the researcher, there is no published literature showing exact figures on the amount of this waste. However, having observed the rising levels in beer production in the country these days, it can be deduced that this sector is contributing its own share for the waste generation.

Generally, as reported by Gregory et al. (2013), waste utilization or disposal in beverage industries is a major problem in maintaining sanitation and avoiding pollution of land, air, and water. Most of the BSG-waste generated from the brewery industries is normally used as animal feed. But, when there is less demand by the cattle owners, or when the rate of generation is too much higher than the rate of consumption of BSG, it is just landfilled or simply disposed to the environment. Landfilling BSG usually contaminates groundwater due to the leachate formed when

microorganisms decompose the spent grain material. In addition, the degradation of BSG in these conditions produces methane, which is a harmful greenhouse gas. On the other hand, just giving away BSG -which can be used as a source of high-value bio-products such as Ferulic acid and others – as animal feed is an unwise use of resources, which is particularly important for Ethiopia.

Besides, since such residues are available in large amounts and usually at a low cost, they can be used to produce industrially applicable chemicals turning the production process economically profitable. Furthermore, since synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ) have restricted use in foods due to their toxicological effects on various species and suspected carcinogenic potential, the search of natural and safe antioxidants, especially of plant origin, has greatly increased in recent years. In addition to this, it solves the problem of waste disposal that would cause, otherwise, environmental pollution.

So far, lots of techniques have been put into practice to produce ferulic acid from agro-industrial wastes such as BSG. The most commonly used technique of extraction is alkaline hydrolysis. While this technique is reportedly very effective in extracting ferulic acid without affecting its properties, the extract is mostly viscous since it contains a mixture of other constitutes as well. Thus, there is always a need of undertaking some purification processes to get pure ferulic acid. What is normally being done in the scientific world so far is to use a chemically activated carbon for this purpose (Couteau, 1997), which is costly. Thus, a less costly but effective technique is needed, especially for poor countries like Ethiopia. Luckily enough, ferulic acid is soluble at concentrated ethanol (Anvar, 2009). Therefore, to contribute to the scientific community in this regard, in this thesis study, a purification mechanism was tested by dissolving the extract in 95% ethanol to fill this gap. Besides, the conversion of this waste into a highly applicable chemical in different industries, Ferulic acid, with a special focus on the optimization of the major parameters affecting the extraction process, was studied.

1.3. Objective

1.3.1. General Objective

The general objective of this work was to optimize the major parameters influencing the Ferulic acid production and to characterize the product (Ferulic acid).

1.3.2. Specific Objectives

The specific objectives of this study were to:

- characterize the raw material by analyzing its proximate composition and structure before and after hydrolysis using PXRD
- optimize the operating conditions namely NaOH concentration, reaction time and temperature to get a maximum Ferulic acid yield
- characterize the product by measuring the antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH), quantifying the total phenols spectrophotometrically, and carrying out FT-IR and HPLC analysis

1.4. Significance of the Study

The outcome of this research work will have a wide range of benefits to different segments of the community. The valorization of BSG can result into many other high-value-added bio-products including enzymes namely xylanase, β-xylosidase, and α-L-arabinofuranosidase and other bulk chemicals such as lactic acid, and pectin (Sachindra et al., 2018). Several reports also indicate that it can be applied in biogas (Ezeonu and Okaka, 1996), as a brick component (Russ et al., 2005), paper manufacture (Ishiwaki et al., 2000), as an adsorbent (Low et al., 2000), in charcoal production (Okamoto et al., 2002). Hence, this study may trigger other researchers to work on the wider aspect of this waste to investigate further the possible bio-products that are out of the scope of this study. When a complete 'lignocellulosic feedstock bio-refinery' process for the biochemical conversion of this waste into valuable bio-products is fully in the picture, it can be scaled up to large industry. This would have different benefits including creating job opportunity for the community around, saving foreign currency, minimizing the import of value-added chemicals that can be produced from this waste and others. Besides, the successful accomplishment of the study would have an important role in environmental protection by opening the way to alleviate the pollution problem the disposal of BSG would bring.

1.5. Scope of the Study

Regarding scale and coverage of available resources, the study was conducted at a laboratory scale with BSG industrial waste only. Besides the reason that this waste is rich with interesting nutritional elements for microorganisms, the researcher selected it for this study since it has got

little attention by other researchers so far. Furthermore, this thesis research was restricted to the investigation of only three major factors namely temperature, NaOH concentration, and reaction time.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Production of Brewer's Spent Grain

A typical beer manufacturing process comprises two main steps (1) brewing process and (2) fermentation process. Malted barley is the commonly used starting material in this process. Mussatto et al. (2006) describe the spent grain generation process as demonstrated in Figure 2-1, which refers to the important steps followed in beer manufacturing during the brewing process.

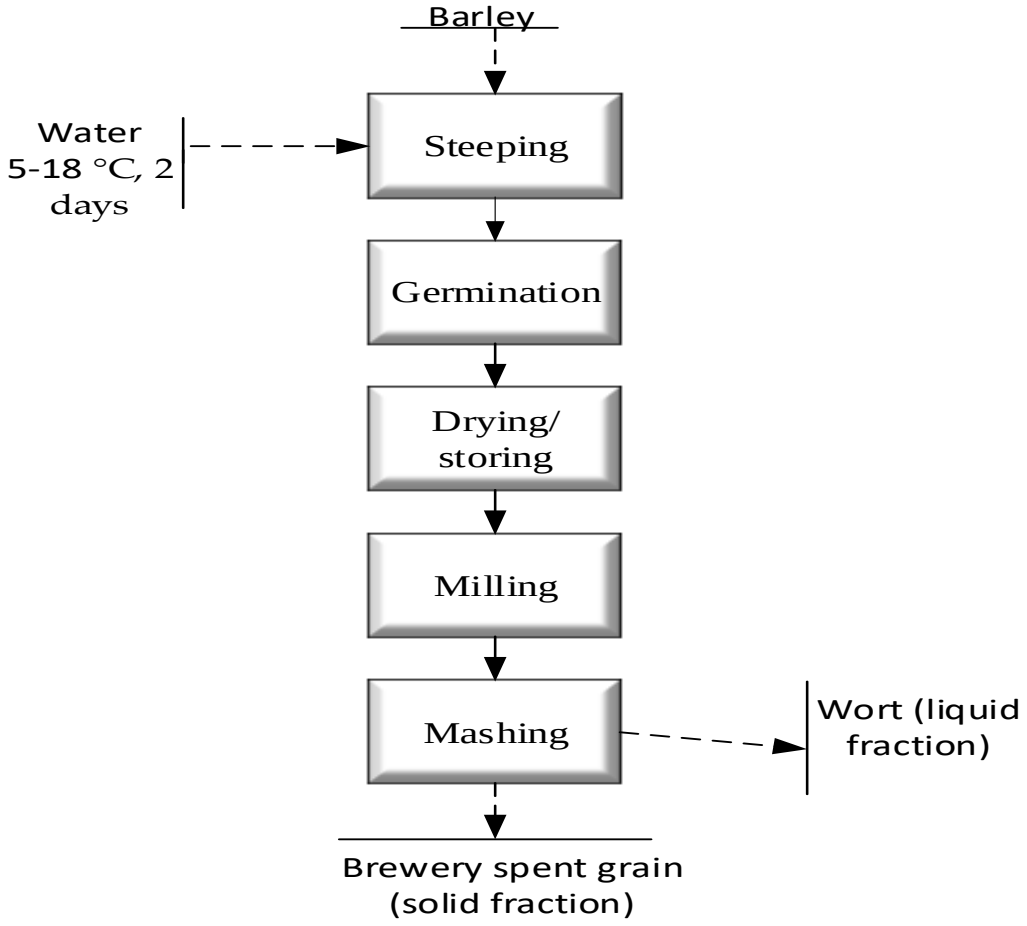


Figure 2-1: BSG generation as a by-product of beer production process (Mussatto et al., 2006)

Initially, the raw barley is kept for 4–6 weeks as a dormancy period prior to processing. Malted barley is produced by controlled germination, which enhances the grain's enzymatic content. Next, steeping is carried out by placing the cleaned barley in water tanks (5–18 ℃) for about 2 days. During steeping the moisture present in the grain reaches 42–48%, and this hydration triggers germination and activates aleurone metabolism. Afterward, the barley is transferred to a germination vessel (15–21 ℃) and held for about a week. During germination, the structure of the starchy endosperm is modified using enzymes such as amylases, proteases, and β-glucanases. Afterward, the malted barley is dried (40–60 ℃) till 4–5% moisture content is achieved. The drying helps to develop flavor components as well as to reduce microbial contamination. The homogeneity and equilibrium in the malted product are achieved by storing the barley for 3–4 weeks. The steps followed up to this point are referred to as the malting process. Next follows the brewing process in which the barley malt is converted to wort and spent grain. Sometimes the malted barley is mixed with other grains such as crushed corn or rice and milled. Afterward, this mixture is treated with hot water (70–74 ℃) to enzymatically hydrolyze the malt, which is referred to as mashing. During mashing, amylase from the malted barley converts the grain starch into fermentable and non-fermentable sugars, while proteins are degraded partially to amino acids and polypeptides. The mixture's filtrate is referred to as wort. This sugar-rich liquid is further processed to produce beer. The solid leftover solid fraction containing most of the proteins and fibers is referred to as brewer's spent grain (BSG) (Sachindra, 2018).

2.2. BSG Production Volume and its Environmental Impact

BSG accounts for about 85% of the by-products generated in the brewery industry (Mussatto & Roberto, 2006). Approximately, 31% of the original malt weight is converted to BSG. World beer production was reported at 193 billion liters in 2014 and China, the United States and Brazil are reported as global leaders in beer production (Statista, 2016). The same report showed that for every 100 liters of beer produced, 20 kg of BSG is generated. Therefore, a massive quantity of BSG is generated and thus industries are burdened with disposal methods for the generated BSG and one of the most common ways is to dump it in landfills. BSG accounts for 30-60% of BOD and suspended solids generated by a typical brewery (Aliyu and Bala, 2010). Therefore, direct disposal in landfills can lead to environmental problems because of organic waste. Moreover, BSG in its life cycle involves environment impacts like food crops such as deforestation, watering, pest

control, and erosion, resulting in a substantial carbon footprint. However, if it is not used till the fullest potential and discarded along with the leftover materials, the resources used in the production life cycle are lost.

2.3. BSG Structure and Content

Major constituents of BSG are the walls of husk, pericarp and the grain's seed coat that are abundant in lignin, cellulose and non-cellulosic polysaccharides. BSG may also consist of proteins and lipids. The starch content present is insignificant, as most of it will be used during wort production. Overall, BSG is a type of biomass abundant in protein and fiber (cellulose, arabinoxylan, and lignin) accounting for approximately 20% and 70% respectively (Mussatto, & Roberto, 2006). Hemicellulose, a branched heteropolymer, primarily consists of arabinoxylan (noncellulose polysaccharide). Lignin, which is useful in maintaining structural rigidity in plant cell walls, is a poly-phenolic macromolecule. Hemicellulose and lignin are responsible for the recalcitrant structure in biomass (Sachindra, 2018).

2.4. Antioxidants

An antioxidant is a substance, when present at a low concentration compared with that of an oxidizable substrate, inhibits oxidation of the substrate. Oxidation is a chemical reaction that transfers electrons from the substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that deteriorate foods. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions, so that extends the induction time of the foods. They do this by being oxidized themselves, so antioxidants are often called reducing agents such as thiols, ascorbic acid or Polyphenols. Addition of antioxidants after the end of this period tends to be ineffective in retarding rancidity development. The induction time (IT) is very sensitive to small concentrations of components that shorten it; the pro-oxidants, or lengthen; antioxidants. Metal ions are the most important prooxidants in foods, whereas antioxidants include compounds that act by radical scavenging, metal chelating or other mechanisms (Kammerer et al., 2005).

Antioxidants can inhibit or retard oxidation in two ways: either by scavenging free radicals, in which case the compound is described as a primary antioxidant, or by a mechanism that does not involve direct scavenging of free radicals, in which case the compound is a secondary antioxidant.

Primary antioxidants include phenolic compounds such as vitamin E (α-tocopherol). These components are consumed during the induction period. Secondary antioxidants operate by variety of mechanisms including binding of metal ions, scavenging oxygen, converting hydro peroxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen. Normally, secondary antioxidants only show antioxidant activity when a second minor component is present. This can be seen in the case of sequestering agents such as citric acid which are effective only in the presence of metal ions, and reducing agents such as ascorbic acid which are effective in the presence of tocopherols or other primary antioxidants (Makris et al., 2007).

2.4.1. Antioxidant Capacity Assays

The first international congress on antioxidant methods was held in Orlando, FL, in June 2004 for the express purpose of dealing with analytical issues relative to assessing antioxidant capacity (AOC) in foods, botanicals, nutraceuticals and other dietary supplements and proposing one or more analytical methods that could be standardized for routine assessment of AOC (Prior et al., 2005). Various antioxidants show substantially varying antioxative effectiveness in different food systems due to different molecular structure. The antioxidants should not impart any off-flavour and off colour. It should be able to get conviently incorporated to food or food systems and should be stable at pH of the food systems and during food processing. Various factors which affect the efficiency of antioxidants include activation energy of antioxidants, redox potential stability of pH and processing and stability, (Sharma & Singh, 2013)

A number of protocols have been proposed to determine the antioxidant capacity. Few use radicals and some use metal ions as the oxidizing agents. The wavelength at which measurement is done in the various protocols is tabulated in Table 2-1.

Table 2-1: Types of Assay with Operating Principles (Deepshikha, 2015)

Assay	Radical/Chromophore	Wavelength of Measurement	Mode of assay
DPPH	DPPH*	517 nm	Absorbance measurement
Total Phenolic Content	Mo^{6+}(yellow) ⟶ Mo^{5+}(blue)	765 nm	Absorbance measurement
ORAC	AAPH* (Fluoresein)	λ_{ex}=485 nm and λ_{em}=538 nm	Fluorescence decay measurement
Ferric ion Reducing	Chelated Fe^{3+} ions	595 nm	Absorbance

Antioxidant Power assay (FRAP)			measurement
Trolox equivalent Antioxidant capacity (TEAC)	ABTS*	734 nm	Absorbance measurement
Total Peroxyl Radical-Trapping Antioxidant Parameter (TRAP)	AAPH* (R-phycoerythrin/Luminol)	λ_{ex}=495 nm and λ_{em}=575 nm	Fluorescence decay measurement

2.5. Ferulic Acid

Ferulic acid was initially isolated from *Ferula foetida* for its structure determination and its name was based on the botanical name of plant (Hlasiwetz, 1866). In 1925, Ferulic acid was chemically synthesized and structurally confirmed by spectroscopic techniques, depicted the presence of an unsaturated side-chain in Ferulic acid, and the existence of both cis and trans isomeric forms (Dutt, 1925). The double bond present in the side-chain is subjected to cis–trans isomerization (Figure 2-2), and the resonance stabilized phenoxy radical accounts for its effective antioxidant activity. It catalyzes the stable phenoxy radical formation upon absorption of ultra-violet light, which gives the strength to Ferulic acid for terminating free radical chain reactions.

Ferulic acid is an enormously copious and almost ubiquitous phytochemical phenolic derivative of cinnamic acid, present in plant cell wall components as covalent side chains (Rosazza et al., 1995). Collectively with dihydroferulic acid, it is the component of lignocelluloses, where it confers rigidity to the cell wall by making the crosslink between polysaccharides and lignin. It has been found that Ferulic acid is linked with a variety of carbohydrates as glycosidic conjugates, different esters and amides with a broad range of natural products. It makes esters by binding with a variety of molecules such as polysaccharides, long chain alcohols, various sterols of plant, tetra-hydroisoquinoline-monoterpene glucoside, a cyanogenetic glycoside and an amino-hydroxy-cyclopentenone, flavonoids and different types of hydroxycarboxylic acids including gluconic, tartaric, malic, hydroxycitric, tartronic, quinic, and hydroxy fatty acids (Boven et al., 1994).

The biological usages of Ferulic acid came into notice when a group of Japanese researchers discovered antioxidant properties of steryl esters of Ferulic acid, which was extracted from rice oil (Yagi et al., 1979). Ferulic acid exhibits a wide range of biomedical effects including antioxidant, antiallergic, hepatoprotective, anticarcinogenic, anti-inflammatory, antimicrobial, antiviral,

vasodilatory effect, antithrombotic, and helps to increase the viability of sperms (Akihisa et al., 2000; Graf , 1992; Ou et al., 2004; Rukkumani et al., 2004). Also, it has applications in food preservation as a cross-linking agent (Okada, et al., 1982), a photoprotective constituent in sunscreens and skin lotions (Saija et al., 2000). An amide derivative of Ferulic acid, formed by the condensation of Ferulic acid with tyramine may be used as an indicator of environmental stress in plants. In the baking industry, amides of Ferulic acid with amino acids or dipeptides are commonly used for the purpose of preservation (Graf, 1992). In many countries, use of Ferulic acid as a food additive has been approved by their government as it effectively scavenges superoxide anion radical, and inhibits the lipid peroxidation (Srinivasan et al., 2007).

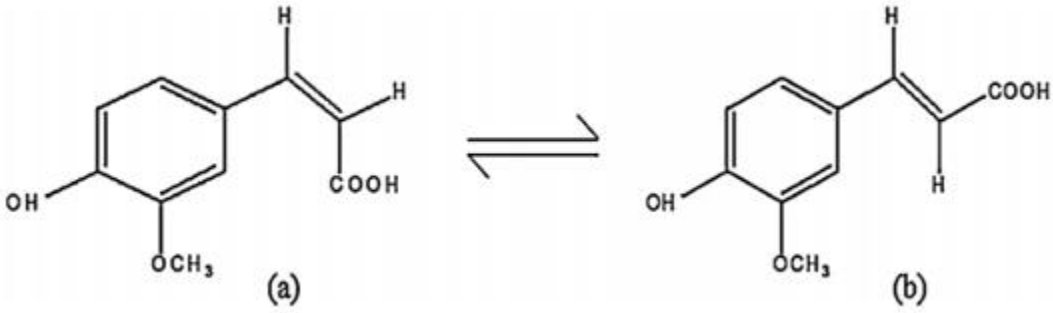


Figure 2-2: Schematic representation of two different isomeric forms of Ferulic acid found in nature (a) cis conformation and (b) trans conformation of Ferulic acid (Naresh, 2014)

2.5.1. Ferulic Acid Extraction

Ferulic acid is one of the major phenolic acids found in brewer's spent grain (Mussatto et al., 2007) and plant cell-walls such as cereal brans, corn hulls, corn kernel, grasses, maize bran, sugar beet pulp, wheat bran and woods (José et al., 2012; Saulnier et al., 1995; Ashwini et al., 2008) where it is usually found as ester cross-links with proteins and polysaccharides in the cell wall, such as arabinoxylans in grasses, pectin in spinach and sugar beet and xyloglucan in bamboo. For instance, José et al. (2012) tested seven lignocellulosic residues, namely, chestnut and pistachio shells, grass, leaf fruit, vine leaf, and red and white grape stems for the possible extraction of Ferulic acid by the method of alkaline hydrolysis under selected conditions 3 N NaOH (12%) in autoclave at 130 °C during 75 min. with a liquid/solid ratio of 10 (w/w). The result for each waste was then found to be as (mg/L): white grape stem (97.9), red grape stem (92.2), vine leaf (35.8), leaf fruit (64.0), grass (312.0), pistachio shell (43.0), and chestnut shell (19.5).

Ashwini et al. (2008) also investigated the potential of various agricultural wastes such as maize bran, rice bran, wheat bran, wheat straw, sugar cane bagasse, pineapple peels, orange peels, and pomegranate peels for the production of Ferulic acid. In this study, the extraction temperature and time were studied at (20, 25, 30, 35 and 40 °C), and (10, 16, 24, 32 and 37 h) respectively, and the results from each waste were reported as sugarcane bagasse (0.893% w/w), rice bran (0.972% w/w), rice husk (1.022% w/w), wheat bran (0.346% w/w), and wheat straw (0.867% w/w).

Ferulic acid with purity greater than 98% was obtained from extracts of *Radix Angelicae sinensis* after microwave-assisted extraction followed by highspeed counter-current chromatography by employing a solvent system consisting of n-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v) (Liu et al., 2006). The optimal condition, as reported by Liu, were 850 W of microwave power, 9 min of irradiation time, 90% of ethanol concentration, 6:1 ratio of liquid/solid, 140 min of herbal material soak time and particle sample size 250 ± 9.9 µm. Singh and Saldaña (2011) extracted eight phenolic compounds (gallic acid, chlorogenic acid, caffeic acid, protocatechuic acid, syringic acid, p-hydroxyl benzoic acid, Ferulic acid, and coumaric acid) from potato peel using subcritical water. Phenolic compounds were recovered highest (81.83 mg/100 g fresh wt.) at 180 °C and extraction time of 30 min. Mayanga-Torres et al. (2017) utilized two abundant coffee waste residues (powder and defatted cake) for extraction of total phenolic compounds using subcritical water under semi-continuous flow conditions. The highest total phenolic compounds (26.64 mg GAE/g coffee powder) was recovered at 200 °C and 22.5 MPa.

Ferulic acid was also extracted by Cantillo-Pérez et al. (2017) from corn bran using 0.25 mol/L NaOH in a 50 % aqueous ethanol solution at 75 °C for 2 h to obtain 8.47 g of Ferulic acid/kg of corn bran with 84.45 % purity. Likewise, Arabi et al. (2015) extracted Ferulic acid from the sugar beet pulp using three solvents: 0.5 and 1 M NaOH, 2 M methanol and a mixture thereof. The acid amount was quantified by the HPLC method, where the effects of solvent type, concentration and reaction time on the solubilization of Ferulic acid were evaluated. Results showed that a minimum amount of acid was obtained from the alcoholic extract, while a concentration of 957.4 mg/L was obtained with the highest concentration of NaOH and a reaction time of 12 h.

2.5.2. Antioxidant Properties of Ferulic Acid

The current definition for an antioxidant activity is given as the capability of a molecule or composition to inhibit oxidation of other molecules, avoiding their degradation (Roginsky and

Lissi, 2005). On the other hand, oxidation is defined as a reaction promoted by chemical and/or physical factors that result in a loss of electrons from a given substance (Battino et al., 2002). For this reason, oxidation may result in free radicals that start chain reactions and accelerate the oxidative degradation of biochemically important molecules such as lipids, proteins and nucleic acids, to mention few.

In the presence of antioxidants, however, they interrupt these chain reactions by removing free radical intermediates, and thus, inhibit further oxidation reactions. They do this by themselves becoming oxidized, so they are also reducing agents (Sies, 1997). Several literature works show that Ferulic acid is endowed with this antioxidant character. Its potent antioxidant activity is strongly associated with its phenolic hydroxyl group, which promptly terminates radical chain reactions by a radical scavenging mechanism (Toda et al., 1991; Graf, 1992; Kikuzaki et al., 2002). This is due to its potential to lose hydrogen radical to any reactive radical (R*) that it meets to regenerate a stable compound (R-H) and to afford a phenoxyl radical that is highly stabilized by resonance along both the entire aromatic ring and the unsaturated side chain.

2.6. Ferulic Acid Extraction Methods

2.6.1. Solvent Extraction

Extraction techniques need to consider the location of Ferulic acid in the sample. Most phenolic acid derivatives present in the plant matrix are stored in the vacuole and are commonly extracted in alcoholic or organic solvents. The exceptions are those bound to insoluble carbohydrates and proteins within the plant matrix. Often, saponification prior to extraction is employed to cleave the ester linkage to the cell walls. As an example, Kroon et al. (1997) incubated bran cell walls in 1 M NaOH for 24 h at 37 °C to remove the ester-linked Ferulic acid. The common solvents for extractions are hot water, methanol, ethanol, acetone, and ethyl acetate, the latter being the most common (Rebecca, 2003).

2.6.2. Hydrolysis

Hydrolysis of the ester to a carboxylic acid has been one strategy employed to simplify the analysis and gain a more specific picture of the phenolic acid profile in samples. There are two main procedures to cleave the ester bond reported in the literature, acidic hydrolysis, and saponification. A third, less prevalent technique is cleavage using enzymes (esterases) (Rebecca, 2003).

2.6.2.1. Dilute-Acid Hydrolysis

Acid hydrolysis is one of the most promising extraction methods with respect to industrial implementation. It is usually performed with mineral acids, but organic acids and sulfur dioxide are other options. Dilute sulfuric acid pretreatment has been studied for a wide range of lignocellulosic biomass (Yang and Wyman, 2008; Hu and Ragauskas, 2012). Acid ranges from 1 to 2 N aqueous HCl, and the reaction times range from 30 min (Escarpa et al., 2001) to 1 hr. Acid pretreatment has also some drawbacks, such as high cost of the materials used for the construction of the reactors, gypsum formation during neutralization after treatment with sulfuric acid, and formation of inhibitory by-products (Leif, 2016).

2.6.2.2. Mild Alkaline (Saponification) Hydrolysis

Saponification entails treating the sample with a solution of NaOH with reported concentrations ranging from 1 to 4 M. Alkaline treatment can be used for removing lignin and thereby increasing the digestibility of cellulose. Most of the reactions can stand at room temperature for a time ranging from 15 min (Leung et al., 1981) to overnight (Torres et al., 1987). Compared to acid processes, mild alkaline pretreatments lead to less solubilization of hemicelluloses and less formation of inhibitory compounds, and they can be operated at lower temperatures. Sodium hydroxide and potassium hydroxide are the most commonly used forms of alkali (Shahrzad and Bitsch, 1996).

2.6.2.3. Enzymatic Hydrolysis

Enzymatic reactions have been reported to release phenolic acids (mainly ferulic and p-coumaric acids). These enzymes (pectinases, cellulases, amylases) are employed for the degradation of the carbohydrate linkages. The mode of action by which these acids are released is the cleavage of an acetal or hemiacetal bond found between carbohydrate moieties and the hydroxyl groups of the aromatic ring, and not by ester cleavage reactions. Meyer et al. (Meyer et al., 1998, Landbo et al., 2001) discuss the release of phenolics using Grindamyl pectinase, a commercial enzyme having pectinolytic, cellulitic (cellulose) and hemicellulosic but no esterase activity. Andreasen et al. (1999) discuss and compare several different enzyme preparations for the release of phenolic acids from the cell wall of rye grains. Smith et al. (2001) used the enzyme Driselase (a commercial cellulase) to determine the location of the ester linkage of Ferulic acid. Driselase contains glycanases but no esterase activity. By isolating the feruloyl oligosaccharides released after

digestion, they established to which sugar Ferulic acid was esterified. Again, some acid derivatives are extracted from the cell walls, but it is not the ester bond that is cleaved.

2.6.3. Advanced Methods of Extraction

Due to some drawbacks related to the traditional methods of extraction discussed above such as requirements of a huge amount of solvents, long extraction time, and the possible degradation of target products, advanced methods of extraction have been developed since recent times. Among them, microwave-assisted extraction (Kyriakos et al., 2019) pressurized liquid extraction (Setyaningsih et al., 2015), ultrasonic extraction (Jasminka, 2018) and supercritical fluid extraction (Roberts, 1995) are the most reported methods.

Jasminka (2018) compared ultrasound-assisted and the conventional solid extraction methods of phenolics from olive leaves. As a result, it is reported that ultrasound-assisted extraction could improve the yield by 40%. Ultrasonic waves induce pressure and cavitation with disruption of cellular walls by facilitating the release of target components (Jasminka, 2018). Setyaningsih et al. (2015) also applied pressurized liquid extraction of phenolic compounds from rice grains where it was successfully applied for the analysis of a wide variety of rice grains. Microwave-assisted extraction of phenolics has also been reported. For instance, this method was applied by Kyriakos et al. (2019) to extract phenolics from pomegranate peels. The method was found to increase extraction yield, but mainly to shorten the treatment time by over 60 times compared to conventional extraction methods. These differences were attributed to the intense cell destruction of microwave-treated plant material observed by SEM analysis. Piantino et al. (2008) used pure CO_2 in a supercritical state to extract antioxidant fractions from *Baccharis dracunculifolia* with optimal conditions of 60 °C and 400 bar, resulted in 2-times higher yield (4 mg/g) compared to the yield of methanolic and ethanolic extracts.

However, there are some limitations of innovative technologies such as high investment costs, insufficient monitoring of the variables during processing, and a lack of regulative measures, as well as industrial implementation (Chemat, 2011). Thus, for this study, alkaline hydrolysis was selected because of its selectivity property in the release of phenolic compounds such as p-coumaric acid and Ferulic acid (Rodriguez and Diaz, 1994).

2.7. Factors Affecting Ferulic Acid Extraction

Many factors are reported to be affecting Ferulic acid extraction. The most important ones are reaction time, temperature, type of solvent extraction/hydrolysis method, and concentration of the alkaline/acid/solvent.

Effect of temperature: Temperature is always considered as one of the most important parameters that would affect the yield of Ferulic acid regardless of extraction techniques. Several authors have reported that significant amounts of phenolic acids are not released from the lignocellulosic structure with alkali at room temperature (Lozovaya et al., 1999; Sun et al., 2001). The report by Mussatto (2007) shows that a temperature of 120 ℃ is the optimum parameter to produce Ferulic acid from BSG by alkaline hydrolysis. This might be because the bond strength of ferulic acid which naturally occurs forming an ester bond to the polysaccharides of the plant cell walls is reached when the temperature of the medium reaches at around 120 °C. In other words, raising the temprature past that point will start breaking the bonds that naturally make ferulic acid so that it dissociates back into its elemental forms. On the other hand, a temprature lower than 120 °C will not be strong enough to break the bonds that naturally keep its esterified bonds intact with that of the plant cell wall polysaccharides.

Effect of reaction time: Time is the vital role in the extraction of Ferulic acid as agreed by Salleh et al. (2011), Tilay et al. (2008) and Li et al. (2006). Time was one of the significant factors in their optimization experiments on the extraction of Ferulic acid. Li et al. (2006) found that only 25 min was needed to complete the reaction when the operating temperature was 110 °C. This suggested that the extraction time would be shortened if the operating temperature is elevated. This proposition was supported by the experiments conducted by Salleh et al. (2011), Tilay et al. (2008) and Li et al. (2006). The extraction time for Salleh et al. (2011)'s experiment was 2.5 h when operated at 125 °C whereas the extraction time of Tilay et al. (2008)'s experiment was 24 h when operated at 21.6 °C.

Choice of solvents/hydrolyzing agents: The choice of solvents/hydrolyzing agents used for extracting important chemicals from lignocellulosic materials influences the effectiveness of the process regardless of the level of other operational parameters. Extraction yield of the compounds mainly depends on the solvent and the method of extraction (Goli et al., 2004). The solvents mainly used for the extraction of cereal polyphenolics are methanol, ethanol, propanol, acetone, ethyl

acetate, dimethylformamide, and their combinations (Naczk and Shahidi, 2006). In some cases, 1% HCl is used with the solvents for the extraction of polyphenols as it leads to acid hydrolysis of cell walls. This acid hydrolysis is mainly employed for the extraction of bound phenolics (Ramachandra et al., 1977; Sripriya et al., 1996). It is also reported that the extraction of polyphenols from plant material may be influenced by the ratio of solvent to sample volume (Naczk and Shahidi, 2006).

pH: The pH is an important factor for the extraction of phenolics. Usually, low pH value of the extraction solution can prevent the oxidation of phenolics. It has been reported that millet polyphenolics are stable at acidic conditions and feeble or labile at alkaline conditions. Millet polyphenolics may lose their identity by oxidation or could be removed by chelating with metal ions (Chethan and Malleshi, 2007). Alkaline hydrolysis has also been reported for the extraction of bound phenolic acids. Krygier et al (1982) reported that alkaline hydrolysis may lead to some degradation of hydroxycinnamic acid derivatives and a later report stated that this can be prevented by the addition of 1% ascorbic acid and 10 mM ethylenediaminetetraacetic acid, EDTA (Nardini et al., 2002).

The determination of optimum pH and solvent is an important factor for the extraction of polyphenols. The pH of the extraction medium depends on the nature of phenolic compounds to be extracted and the source of plant material. The solvent to be used for the extraction of compounds varies with the source and the type of phenolic compounds to be extracted. In fact, an integrated combination of pH and solvent can lead to enhanced extraction of polyphenols as pH determines the solubility of the polyphenols and the solvents address the efficient contact.

2.8. Determination of Ferulic acid

Quantification and identification of phenolic compounds mainly depend on different parameters, such as the chemical nature of the compounds, extraction method used, storage time and conditions, particle size, selection of standards, interfering substances and impurities, and assay methods. There are large numbers of spectrometric methods that have been used for the quantification of phenolic compounds. With the advent and advancement of analytical science, there has been an array of modern tools, such as HPLC, LC-MS, GC-MS, FT-IR, NMR, among others (Jenke, 1996).

2.8.1. Chromatographic Separation Methods

Paper Chromatography (PC)/Thin-Layer Chromatography (TLC): Early investigations used either paper chromatography (PC) or thin-layer chromatography (TLC). Paper chromatography consisted of Whatman filter paper as the stationary phase. For TLC, the stationary phase varied, including silica gel, cellulose, and polyamide layers (Ragazzi et al., 1973; Schulz et al., 1980). TLC was considered a convenient separation method. It is fast, inexpensive, and several samples can be examined at the same time, side by side. Although less used in the analysis of foods, TLC is still commonly used for the determination of phenolic acids in plant material in natural product analysis (Bylak et al., 2001). The main disadvantages of TLC are limited quantitation aspects. Estimations of recovery with preparatory TLC have, however, been reported including that of the total loss of Ferulic acid (45%) during sample preparation and analysis, 30% was lost in the preparative TLC step (Naim et al., 1988).

High-Performance Liquid Chromatography (HPLC): High-performance liquid chromatography (HPLC) is an essential analytical technique for qualitative and quantitative determinations in many fields, for research, diagnostic, and manufacturing purposes. HPLC has evolved dramatically in recent years, especially due to the development of new column technologies (Fekete, 2012). According to literature HPLC coupled with ultraviolet or diode array detection (PDA), is the most common analytical technique used for the separation and determination of phenolic compounds from different agro-industrial resources (Capriotti, 2014; Tasioula-Margari, 2015; Tasioula-Margari, 2001; Gómez, 2005).

Gas Chromatography-Mass Spectroscopy (GC-MS): GC-MS is another major chromatographic technique employed for the analysis of phenolic acids in plants. It is a hyphenated analytical technique that combines the separation properties of gas-liquid chromatography with the detection feature of mass spectrometry to identify different substances within a test sample. GC is used to separate the volatile and thermally stable substitutes in a sample whereas GC-MS fragments the analyte to be identified based on its mass (Sahil, 2001; Jenke, 1996).

2.8.2. Spectrophotometric Methods

The chromatographic techniques discussed above are highly precise and accurate with high informative value, but it is highly problematic to identify all the phenolic compounds in a specific sample at acceptable times and costs of analysis. Generally, it is necessary to analyze several

samples since the contents of phenolic substances can vary in different varieties and cultivars. In addition, contents of phenolic substances are influenced by many external factors such as agrotechnical processes, climatic conditions, and ripeness during harvest, postharvest manipulations, and time of consummation (Waterman et al., 1994). At the present time, enough information for the evaluation of the content of total phenolic compounds and total antioxidant activity as a criterion of the nutritional value of plant foodstuffs can be easily obtained from spectrophotometric measurements employing specific analytical reagents (Pavel, 2006).

2.8.2.1. UV/VIS Spectrophotometry: The Folin–Ciocalteu method is the most common method of determining phenolic compounds (Vinson et al., 1998). The Folin–Ciocalteu reagent which is a mixture of tungstates and molybdates works on the mechanism of oxidation-reduction reaction. The method strongly relies on the reduction of the mixture heteropolyphosphotungsates–molybdates by the phenolic compound which results in the formation of blue-coloured chromogen. The phenolic compounds react with Folin–Ciocalteu reagent only under basic conditions adjusted by sodium carbonate solution. Under Basic conditions it has been observed that the phenolic compound undergoes dissociation to form a phenolate anion which reduces the Folin–Ciocalteu reagent i.e. the mixture of tungstates and molybdates rendering a blue-coloured solution. The colour intensity of the formed blue chromogen can be measured by the absorbance readings using a spectrophotometer (Dejian et al., 2005).

2.9. Optimization

Optimizing refers to improving the performance of a system, a process, or a product to obtain the maximum benefit from it. The term optimization has been commonly used as a means of discovering conditions at which to apply a procedure that produces the best possible response (Araujo and Brereton, 1996). Traditionally, optimization has been carried out by monitoring the influence of one factor at a time on an experimental response. While only one parameter is changed, others are kept at a constant level. This optimization technique is called one-variable-at-a-time. Its major disadvantage is that it does not include the interactive effects among the variables studied. Consequently, this technique does not depict the complete effects of the parameter on the response (Lundstedt, 1998). Another disadvantage of the one-factor optimization is the increase in

the number of experiments necessary to conduct the research, which leads to an increase of time and expenses as well as an increase in the consumption of reagents and materials.

2.9.1. Response Surface Methodology Approach

To overcome the problem of one-variable-at-a-time optimization technique described above, the optimization of analytical procedures has been carried out by using multivariate statistical techniques. Among the most relevant multivariate techniques used in analytical optimization is response surface methodology (RSM).

RSM is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, which must describe the behaviour of a data set with the objective of making statistical previsions. It can be well applied when a response or a set of responses of interest are influenced by several variables. The objective is to simultaneously optimize the levels of these variables to attain the best system performance.

This is because statistical tools such as RSM recognize the interaction amongst the parameters and reduce their estimation error (Montgomery, 2014). These attributes favor the RSM as a better method to use when investigating the effect of operating variables during the production (N. Bari, 2009). In the RSM approach, statistical design of experiments (DOE) obtained via a standard design method are conducted systematically. Examples of the standard DOE are 2^3-full factorial, Box-Behnken Design (BBD), and central composite design (CCD). The BBD uses a spherical design with good certainty within the design space. It requires fewer experiments compared to other DOE and is rotatable or almost rotatable despite the number of variables under investigation (Nath et al., 2008). Besides, BBD is purposely built to fit a quadratic model. It does not have runs at the extreme combinations of all the factors, but compensates by having better prediction precision in the center of the factor space.

CHAPTER THREE

3. RESEARCH METHODOLOGY

3.1. Materials and Equipment

The raw material (BSG) was kindly provided by St. George Brewery, Addis Ababa, Ethiopia. Chemicals such as sodium carbonate (Na_2CO_3), Folin- Ciocalteu reagent (C_6H_6O), gallic acid ($C_7H_6O_5$), sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), potassium hydroxide (KOH), petroleum ether (C_6H_{14}), hydrochloric acid (HCl), and DPPH ($C_{18}H_{12}N_5O_6$) were available and used in the Center of Food Science and Nutrition, College of Natural Science, and School of Chemical and Bio Engineering, Addis Ababa Institute of Technology, Addis Ababa University, Ethiopia. Standard Ferulic acid ($C_{10}H_{10}O_4$) was bought from Charkos Market Center, and pure ethanol (C_2H_5OH) from the National Alcohol and Liquor Factory, Addis Ababa, Ethiopia. All extraction processes were carried out using distilled water.

Major equipment that were used in the whole experimental works were: X-ray diffraction (miniflex 300/600, Japan), laboratory autoclave, UV/Vis Spectrophotometer (model UV-752), condenser, sieves, volumetric flasks, vacuum rotary evaporator (Fisatom, model 801), drying oven (DAS 42000, 224.2007), coffee grinder (Mouliner,AR1044), tray, centrifuge (Labtech, model AVI-558) refrigerator (WestPoint, model WRES-358.X), soxhlet, Erlenmeyer flasks, small bottles (glass jars), sensitive balance (Electronic balance, FA2004B), pH meter, measuring beaker, quantitative filter paper which is equivalent to what man No. 42, spoon, pipette, micropipette, test tubes, desiccator, Kjeldahl flask (model KDN-102F), HPLC (Hitachi, USA), Perkin Elmer FTIR spectrometer (Perkin Elmer, 65, USA).

3.2. Methods

The overall scheme of this experimental investigation had the following structure.

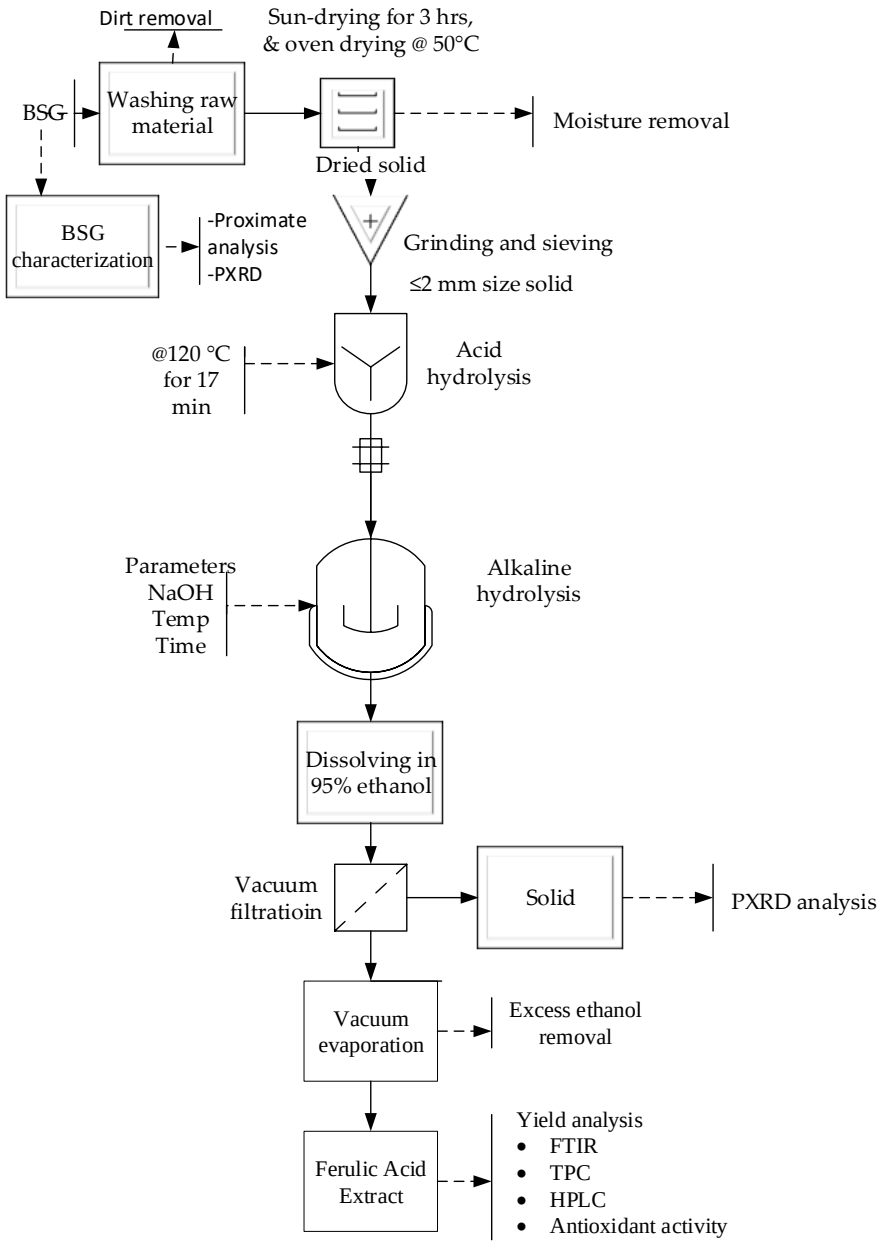


Figure 3-1: Conceptual framework of the experiment

3.2.1. Raw Material Characterization

3.2.1.1. Compositional Analysis

Moisture Content: The method was based on drying a 10 g sample in an oven at 50 °C for a day to a constant weight and determining moisture content by the weight difference between dry and wet material. The percentage of moisture content (wet basis) was then computed as follows:

$$\text{Mc}\left(\%\frac{\text{w}}{\text{w}}\right) = \frac{\text{W}_0 - \text{W}_\text{f}}{\text{W}_0} \times 100 \qquad (3.1)$$

Where Mc = Moisture content (%); W_0 = weight of the wet sample, g; W_f = weight of dry sample,g;

Ash Content: Ash content was determined according to the Technical Report NREL/TP 510-42622 (Sluiter et al., 2008). The dry sample (free of moisture) of 3 g BSG was incinerated at 575 °C for 3 h and then the ash content was calculated based on the following formula:

$$\text{Ash}\left(\%\frac{\text{w}}{\text{w}}\right) = \frac{M_1 - M_2}{M_3} \times 100 \qquad (3.2)$$

Where M_1 = mass of empty dish and ash, g; M_2 =mass of empty dish, g; M_3 = mass of the dry sample, g.

3.2.1.2. Crude Fiber Determination

Crude Fiber is the measure of indigestible cellulose, lignin and other components of this type in a sample. It is the residue remaining after acidic and alkali digestions.

A sample (3 g) was treated successively with boiling solutions of sulphuric acid (0.25 N) and potassium hydroxide (0.25 N). The residue was separated by filtration on a filter, washed, dried, weighed and ashed at 500 °C. The loss of weight resulting from ashing corresponds to the crude fibre present in the sample and was calculated as:

$$\%\ \text{Crude fibre} = \frac{\text{C}_1 - \text{C}_2}{\text{Weight of the original sample}} \times 100 \qquad (3.3)$$

Where C_1= weight of the crucible with dry residue and C_2 = weight of the crucible with ash.

3.2.1.3. Determination of Crude Lipid Contents

A clean, dried 500 cm^3 round bottom flask containing few anti-bumping granules was weighed (W_1) with 300 cm^3 petroleum ether (40 - 60 °C) for extraction poured into the flask filled with

Soxhlet extraction unit. The extractor thimble was fixed into the Soxhlet unit. The round bottom flask and a condenser were connected to the Soxhlet extractor and cold-water circulation was connected. The heating mantle was switched on and the heating rate adjusted until the solvent was refluxing at a steady rate. Extraction was carried out for 6 h. The solvent was recovered and the oil dried in an oven set at 70 °C for 1 h. The round bottom flask and oil were then weighed (W_2). The lipid content was calculated as a percentage.

$$\%\text{Crude Lipid (dry mass basis)} = \frac{W_2 - W_1}{\text{weight of the sample}} \times 100 \qquad (3.4)$$

Where W_1 = initial sample weight in grams and W_2 = weight of beaker and fat residue in grams

3.2.1.4. Protein Content Determination

For this task, Kjeldahl's method was used to evaluate the total nitrogen content of the sample. Three steps were carried out: digestion, distillation, and titration.

Digestion: The sample to be analyzed was weighed into a digestion flask and then digested by heating it in the presence of sulfuric acid (an oxidizing agent which digests the food), and anhydrous potassium sulfate (to speed up the reaction by raising the boiling point). This digestion converts any nitrogen in the food (other than that which is in the form of nitrates or nitrites) into ammonia and other organic matter to CO_2 and H_2O. Ammonia gas is not liberated in an acid solution because the ammonia is in the form of the ammonium ion (NH_4^+) which binds to the sulfate ion (SO_4^{2-}) and thus remains in solution. In this study, 1.6355 g of dry sample was weighed out into 300cm^3 Kjeldahl flask; then 1 g potassium sulfate, 0.7 g mercuric oxide, and 20 mL concentrated sulphuric acid were added. The sample was digested at a temperature slightly above the boiling point of sulfuring acid (340 ºC) until a clear green colour was obtained. The digest was cooled and diluted with 100 cm^3 with distilled water.

Distillation: The second stage followed was the distillation process. To change the ammonium ions to ammonia the pH of the sol was raised by adding 40 mL of 40% NaOH until the solution becomes highly alkaline. To proceed the boiling during digestion very smoothly and with minimum danger of bumping by providing an evolution of fine bubbles of hydrogen gas (Bradstreet, 1940) 0.5 g of zinc dust was added to each flask. The Kjeldahl flask was attached to a water condenser then heated to boil off the NH_3 gas from the digest. To trap the distilled NH_3 in

receiving solution the tip of the condenser was submerged in a flask of receiving boric acid solution.

Titration: Distillation collecting distillate was carried out in volumetric flask containing 50 cm^3 of 2% boric acid and about 150 cm^3 of distillate was collected. Boric was used a receiving solution to capture the ammonia gas forming an ammonium-borate complex. As the ammonia collects the color of the receiving solution was changed to slight whitish. Three drops of mixed indicator (methyl red-bromocresol green) indicator were added to this distillate. Finally, 20 mL of 0.1 N HCl until the solution has a slightly violet color. A 4.45 mL of HCl was also used to the reagent blank. Then protein content determination was then carried out by the following formula:

$$\%N_2 = \frac{(V_{standard\_acid} - V_{blank}) \times 14 \times N_{acid}}{weight\ of\ sample \times 1000\ mL} \times 100 \qquad (3.5)$$

Where N = normality, $V_{standard\_acid}$ = the titration volume of the acid, V_{blank} = volume of standard acid needed to titrate a reagent blank, and 14 is the molecular weight of nitrogen in g.

Then, to determine protein content, a nitrogen-to-protein conversion factor of 6.25 (most proteins contain 16% of nitrogen, and thus 1/0.16 = 6.25) was then used as follows (Hames, 2008):

% crude protein= $\%N_2$ * 6.25

3.2.1.5. Determination of Carbohydrate

The total carbohydrate was then determined by subtracting the sum percentage of moisture, ash, crude lipid, crude protein and crude fibre from 100 as follows.

$$\%TC = 100 - (\%\text{moisture content} + \%Ash + \%Lipid + \%Protein + \%Fiber) \qquad (3.6)$$

Where TC stands for total carbohydrate

3.2.1.6. Powder X-ray Diffraction (PXRD)

Evaluation of the phase and morphology of raw material was conducted using Miniflex 300/600, Japan, using Cu k_α radiation generated at 40 kV and a current of 15 mA. The samples were placed on a sample holder made up of silicon wafer and the measurements were taken continuously from 10° to 50° angles in 0.02 degree steps for 1 second per step.

3.2.2. Experimental Design and Optimization

In this study, a completely randomized design approach was followed. The randomization of the experimental runs was done through Design-Expert software version 11. A factorial design was chosen for planning the experiments and the evaluation of the experimental results was carried out by the Analysis of Variance (ANOVA).

3.2.3. Three-Level Factorial Design

The laboratory experiment was based on a factorial design where the different treatment factors (i.e. concentration of the hydrolyzing agent, reaction temperature and time) were analyzed for the different combinations of their test levels (hydrolyzing agent concentration (1-3%), reaction temperature (100-140 °C) and time (60-120 min)) as indicated in Table 3-1.

Table 3-1: Experimental Factors and Levels

Independent Variable	**Factor**	**Code levels**		
		-1	**0**	**+1**
Time of Hydrolysis (min)	X_1	60	90	120
Temprature of Hydrolysis (°C)	X_2	100	120	140
NaOH conc. (% w/v)	X_3	1	2	3

These ranges were selected based on the optimum results of Mussato (2007) report which states that the best alkaline hydrolysis conditions consisted in using a 2% NaOH concentration, at 120 °C for 90 min. When these levels of factors were randomized using BBD, 17 random experimental runs were generated (Table 3-2). Each of these three factors was evaluated for their effect at the specified levels and based on the result of the analysis, the optimum parameters were used to re-work the experiment for the validation of the optimum yield. The notation for a quadratic regression model having three predictor variables with interactions is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \in \quad (3.7)$$

where β_{0-3} are the cofficients of the model, X_1, X_2, and X_3 are the factors and $\in$ represents error.

Table 3-1: Randomized Experimental Runs

Std	Run	Factor 1: A-Time (min)	Factor 2: B-Temperature (°C)	Factor C: NaOH conc. % (w/v)
6	1	120	120	1
14	2	90	120	2
2	3	120	100	2
9	4	90	100	2
15	5	90	120	2
12	6	90	140	3
3	7	60	140	2
4	8	120	140	2
5	9	60	120	1
10	10	90	140	1
7	11	60	120	3
17	12	90	120	2
11	13	90	100	3
16	14	90	120	3
8	15	120	120	1
1	16	60	100	2
13	17	90	120	2

3.2.4. Experimental Setup

3.2.4.1. Factors Affecting Ferulic Acid Extraction

Reaction Temperature: As the temperature is one of the major factors that have a profound effect on chemical reactions, its effect on Ferulic acid extraction was studied by carrying out the

hydrolysis process at different temperatures ranging from 100 °C - 140 °C using steps of 20 for different reaction times and hydrolyzing agent concentrations.

Reaction Time: The other factor that was studied was the reaction time. To observe its influence on the hydrolysis reaction, the reaction time was studied at different times ranging from 60-120 min using steps of 30 for different temperature ranges and hydrolyzing agent concentrations.

Hydrolyzing Agent Concentration: The concentration of the specific hydrolyzing agent to be used for the hydrolysis has a significant effect on the accumulation of extraction of Ferulic acid. This was tested by varying the concentration within the range 1-3% (w/v) using steps of 1% at different reaction time and temperature.

3.2.5. Pre-treatment of BSG

Physical treatment: The BSG, that was collected from St. George Brewery, Addis Ababa, was immediately washed with distilled water until neutral pH, air-dried for 5 h and finally oven-dried at 50 °C to 4.5% moisture content. This procedure allows for the storage of BSG until processing and to determine the exact amount of the raw material used. The dried samples were then pulverized in a dry coffee grinder, passed through standard sieves with a pore size of 2 mm.

Chemical(acidic) treatment: The dried material was subjected to a pre-treatment with dilute sulfuric acid, under conditions optimized by Mussatto and Roberto (2005); 100 mg acid per gram of dry matter, a solid: liquid ratio of 1:8 (w/w), at 120 °C for 17 min. This procedure solubilizes the hemicellulosic fraction. The hemicellulose removal also increases the material porosity facilitating the diffusion and impregnation of the sodium hydroxide into the material (Zhao et al., 2002). At the end of the reaction, the solid residue was separated by centrifugation, washed with distilled water until neutral pH and dried at 50 °C to attain approximately 50% moisture content. This pretreatment of BSG helps to make the chemical constituents more accessible and the physical structure more susceptible to the hydrolysis step.

3.2.6. Alkaline Hydrolysis

All alkaline hydrolysis experiments were carried out with the acid pre-treated BSG. Extractions were performed in an autoclave using the pre-treated BSG (12.5 g) and the NaOH solution in a solid: liquid ratio of 1:20 (w/w) (Mussatto et al., 2007). Seventeen (17) runs of experiments as randomized by the Design-Experiment software were carried out, and at the end of each reaction

time, the samples were immediately cooled to room temperature and was neutralized by an addition of concentrated (35.4%) HCl. Due to the viscosity of alkaline extracts, 95% ethanol was added to precipitate the hemicellulose. The amount of added ethanol corresponded to two times of the original volume. The precipitates were separated from the liquid phase with filtration under vacuum with Buchner funnel. The supernatant was vacuum-evaporated to remove excess ethanol. This resulted in the development of a brown extract supposed to be containing Ferulic acid. The resulting supernatant was finally stored in a refrigerator until analysis.

3.2.7. Ferulic Acid Determination and Purification

The extraction yield is a measure of the solvent's efficiency to extract specific components from the original material and it was defined as the amount of extract recovered in mass compared with the initial amount of the sample. It is presented in percentage (%) and was determined for each experimental run. Since Ferulic acid is readily soluble in aqueous ethanol at concentrations ≥30% (Anvar U. et al., 3009), 95% ethanol was added to the brown extract. This resulted in the development of a murky solution. The murky solution was centrifuged at 6000 rpm for 20 min. The supernatant was further vacuum evaporated and the precipitated Ferulic acid became visible. To purify Ferulic acid further, it was dissolved by adding 6 mL anhydrous ethanol, resulting in a less intense murky solution which was again further centrifuged at 6000 rpm for 20 min. The supernatant (mostly ethanol) was vacuum evaporated to precipitate Ferulic acid. The obtained precipitate was finally weighed to determine the yield (mg/100 g BSG) of Ferulic acid. Equation (3.7) was applied to determine the yield percentage of Ferulic acid for each of the experimental runs.

$$\text{Extraction Yield (\%)} = \frac{\text{dried weight of extract}}{\text{total weight of BSG}} * 100 \qquad (3.8)$$

3.2.8. Ferulic Acid Characterization

3.2.8.1. Determination of Total Phenols

UV/Vis Spectrophotometry

The number of total phenols was determined using Folin – Ciocalteau reagent (Singleton et al., 1974). Briefly, 0.5 mL H_2O and 2 mL Folin–Ciocalteau reagent (1:5 H_2O) was added to the test sample (40 mL). After incubating it for 24 h, 10 mL of 10% (w/v) Na_2CO_3, 5 mL ethanol, and the

contents were mixed in a 250 mL flask and allowed to stand for 30 min. Absorbance at 765 nm was then measured in a UV/Vis spectrophotometer. A calibration curve was prepared using a standard solution of gallic acid (0, 1, 2, 4, 6, 8, 10, and 12 mg/mL). Finally, the amount was calculated as total phenolic acid content equivalent to gallic acid in mg per dry weight of the extract (mg GAE/g extract). The total phenol content was calculated using the following relationship:

$$TPC = \frac{CV}{W} \tag{3.9}$$

Where

C = gallic acid equivalent concentration obtained from the calibration curve (mg/mL)

V = volume of stock solution of extract (mL)

W = dry weight of extract found in the stock solution (g)

TPC = total phenol content (mg of GAE/g dry extract)

3.2.8.2. Determination of Antioxidant Activity of Ferulic Acid

Radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems. In this research study, the DPPH* test/method, which is one of the oldest and the most frequently used methods for total antioxidant potential/capacity of extracts, was used.

DPPH is stable free radical at room temperature and accepts an electron/hydrogen radical to become a stable diamagnetic molecule (David et al., 2004). The reduction capability of DPPH radical is determined by the decrease in its absorbance at 5l7 nm, induced by antioxidants. The decrease in absorbance of DPPH radical is caused by antioxidants, because of the reaction between antioxidant molecules and radicals, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in color from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidative activity. The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH. Determination of DPPH radicals scavenging activity was estimated with the method used by Barros et al. (2007). The radical scavenging (inhibition) activity was calculated as a percentage of DPPH discoloration using the following equation:

$$\text{DPPH radical scavenging (IA)}\% = \frac{\mathrm{A_{control}} - \mathrm{A_{sample}}}{\mathrm{A_{control}}} \times 100 \tag{3.10}$$

Where A_{sample} is the absorbance in the presence of the sample of the extracts and $A_{control}$ is the absorbance of the control samples without extract. The percent inhibition was plotted against concentration from which the IC_{50} was determined.

3.2.8.3. Fourier Transform Infrared Spectroscopy (FTIR)

The functional group analysis of the extracted Ferulic acid was carried out using FTIR spectroscopy. The FTIR spectra were recorded on spectrum 65 FT-IR (perkinElmer) equipped with KBr beam splitter. The sample was mixed with pre-dried KBr and the mixture was compressed to form a transparent pellet that was analyzed using transmission method of 400-4000 cm^{-1} at a spectral resolution of 4 cm^{-1}.

3.2.8.4. HPLC Analysis

The determination of Ferulic acid from the extract was conducted with the help of HPLC in Addis Pharmaceutical S.C, Adigrat, Ethiopia. This is an efficient method selected for the determination of Ferulic acid concentration. The estimation of the concentration was accomplished using An Elite LaChrom (Hitachi, USA) liquid chromatography consisting of a system controller, binary pump (L-2130), auto-injector (L-2200), column oven (L-2300) and photodiode array detector (DAD) (L2456).

The analyses were realized using a reverse-phase C-18 chromatography column with 5 μm particle size, 4.6 mm internal diameter and 250 mm length. The mobile phase was composed of acetonitrile and 0.5% acetic acid (37:63, v/v), eluted at a flow rate of 1.0 mL/min in isocratic mode (Isabela, 2017). The injection volume was 10 μL and the analyses were performed at 220 nm at 25.0±1.0 °C. A standard stock solution of Ferulic acid (1 mg/mL) was prepared in acetonitrile. The prepared solution was then injected into the HPLC and it was left for a certain period of time until its peak height was displayed. The same amount of the extracted Ferulic acid was also injected into the HPLC and its peak height was displayed. Detection and identification were performed using a photodiode array detector at 220 nm. The concentration of the extracted Ferulic acid was then calculated according to the formula:

$$\text{Ferulic acid } \left(\frac{\text{mg}}{\text{mL}}\right) = \frac{A_a}{A_s} \times \text{conc. of standard Ferulic acid} \qquad (3.11)$$

Where A_a is the peak area of the extracted Ferulic acid, and A_s is the peak area of the standard Ferulic acid.

3.3. Statistical Data Analysis

The Design-Expert software was used for the statistical analysis. Generation of the model equation, main and interaction effects of the independent variables, and surface plots from the effects of the factors were evaluated for the Ferulic acid yield. Based on their effect on the yield of the product, the factors were evaluated for their level of significance in the extraction process. A three-factor and three-level BBD design consisting of 17 runs for the total yield was employed, including four replicates at the center point (Table 4-2). The experimental design was analyzed and done by Design-Expert software. The quality of fit of the regression model expressed as the coefficients of determination (R^2), the statistical significance determined by ANOVA and the response surface plots were all studied to estimate the models as well as to determine the optimum levels. For each factor, an experimental range is adjusted based on the results of the literature data.

CHAPTER FOUR

4. RESULTS AND DISCUSSION

4.1. Compositional Analysis

As illustrated by Table 4-1, different compositions of the raw material (BSG) were determined. For the sake of comparison, literature results are tabulated alongside the results of this study. As expected, the moisture content of the raw material is high which makes it difficult for long transport and to store stably for a long time. The differences between the values obtained in this study and the literature are understandable when one takes into account that the proximate composition of the brewers' spent grain is a function of several factors, such as: barley variety, harvest time, grains used in the malting process, and the technological process used in the brewery (Sideney et al., 2017). The most important part here is the fiber content, from which Ferulic acid was extracted. It can, therefore, be concluded that BSG has considerable degree of the insoluble carbohydrates such as cellulose and hemicellulose content and thus is a good candidate for the extraction of phenolic compounds such as Ferulic acid.

Table 4-1: Proximate composition of BSG

Parameters	Present study (% wet basis)	Findings from literature (%)				
		Kanauchi et al. 2001	Santos et al. 2003	Carvalheiro et al. 2004	Mussatto and Roberto 2006	Meneses et al. 2013
Moisture (%)	57.5	n.d	n.d	n.d	n.d	n.d
Ash (%)	3.7	2.4	4	1.2	4.6	4.2
Protein (%)	12.4	24	31	24.6	15.2	24.7
Crude lipid (%)	5.6	10.6	3-6	n.d	n.d	n.d
Crude fibre (%)	15.3	n.d	n.d	n.d	n.d	n.d
Total Carbohydrate (%)	5.5	58	n.d	51.3	71	60

4.2. X-Ray Diffraction Analysis

X-ray diffraction (XRD) is a rapid analytical technique primarily used for phase identification of crystalline material. In this study, it was used to observe the changes in crystallinity. Figure 4-1 shows the diffraction pattern of the raw material before and after hydrolysis. The peaks correspond to the crystalline phase and the background corresponds to the amorphous phase. Sine BSG is composed of different polymers, mainly proteins, hemicellulose, lignin, etc., the structure is supposed to be of a semi-crystalline one. As such, the most obviously appearing bands of the raw BSG are broad with a highly unclear crystallinity index. The main diffraction pattern for the raw BSG, therefore, is displayed at diffraction angles 2θ around 29° (Figure 4-1). However, as the lignin and hemicelluloses are removed with the hydrolysis processes, the cellulosic crystallinity is expected to increase as can be noted from the XRD plot. This is explained by the sharper curves at diffraction angles of 32° and 46°. This marks the conversion of cellulose I to cellulose II (Cheng et al., 2011), which in turn marks the possible release of the extractives.

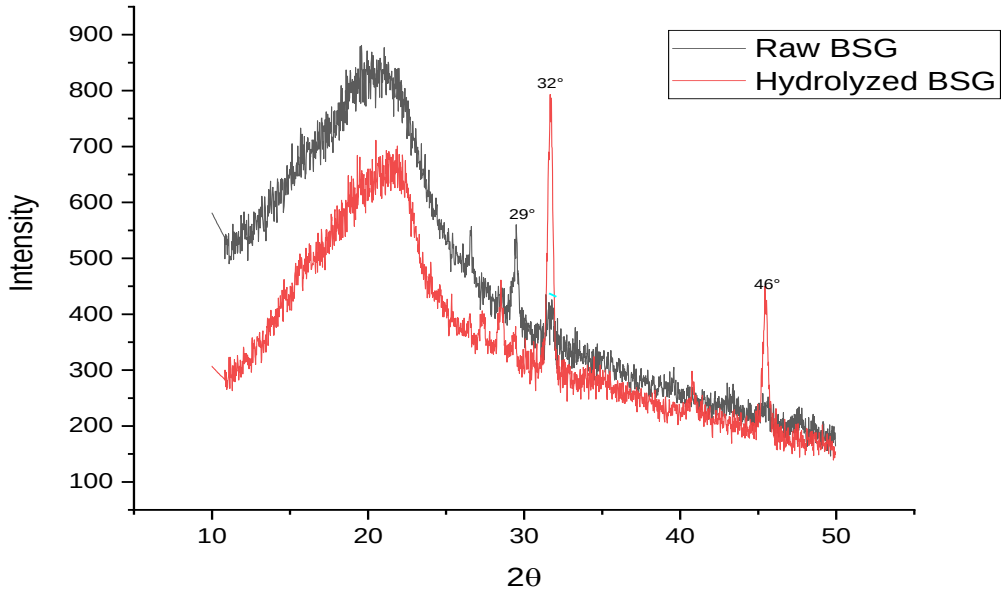


Figure 4-1: X-ray diffractogram of raw and hydrolyzed BSG

4.3. Statistical Analysis

Table 4-2 summarizes the yield of extracts obtained from BSG at each of the 17 experimental runs. Evidently, maximum extraction yields of Ferulic acid (45.245±0.585 mg/100 g BSG) were obtained at the run numbers of (13, 14, 15 & 17), which are the replicates at the experimental condictions (120 ºC, 90 min, and 2%(w/v) NaOH concentration). Thus, taking the averages of this values, it becomes a ferulic acid yield of 45.245±0.585 mg/100 g BSG. This value is lower than the report by Aarabi1 et al. (2016) who reported a maximum Ferulic acid yield of 1.29 g/100 g. Another study by Zhao (2008) had also reported a maximum Ferulic acid yield of 0.800 g/100 g, which still is higher than the maximum value obtained in this study. This difference might be due to reasons such as using a low performance equipment in the extraction process, and the difference in the factors and levels used in the extraction process. Some ferulic acid might also be extracted during the lautering stage of the mashing process that could resulted in lower yield. Data for the percentage yield were generated as per equation 3.8 given in section 3.2.6.

Table 4-2: Extracts yield at different processing conditions

Run №	Factors						Response: Yield of Ferulic acid	
	Coded value			Actual value				
	X_1	X_2	X_3	Time (min)	Temp (°C)	[NaOH] % (w/v)	Observed Ferulic acid (mg)	Yield (mg/100 g BSG)
1	1	-1	0	60	100	2	0.83	6.62
2	-1	0	1	120	100	2	4.85	38.81
3	0	+1	-1	60	140	2	4.41	35.28
4	+1	+1	0	120	140	2	3.63	29.07
5	0	0	0	60	120	1	1.25	9.96
6	0	-1	-1	120	120	1	4.66	37.25
7	+1	0	+1	60	120	3	4.06	32.50
8	0	0	0	120	120	1	4.76	38.05
9	0	0	0	90	100	2	3.59	28.69
10	+1	0	-1	90	140	1	5.60	44.78
11	-1	+1	0	90	100	3	4.31	34.51
12	0	0	0	90	140	3	3.94	31.49
13	-1	-1	0	90	120	2	5.63	45.04
14	0	-1	+1	90	120	2	5.57	44.56
15	0	0	0	90	120	2	5.65	45.21
16	0	+1	+1	90	120	3	5.17	41.35
17	-1	0	-1	90	120	2	5.77	46.17

Noticeably, the percentage yield varied within the range of 6.62 mg/100 g BSG to 45.245±0.585 mg/100g BSG. The minimum percentage yield was at 60 min, 100 °C, and 2% (w/v) NaOH

concentration at the run number (1) which was observed at the lowest time and temperature and average NaOH concentration. On the other hand, the maximum percentage yield was at 90 min, 120 °C, and 2% NaOH concentration, which are the average values of all the factors considered.

4.3.1. Experimental Design

The experimental result was analyzed using Box- Behnken (BBD) of Design-Expert software to develop a single model equation that can describe the significance of the extraction variables. The suggested choice of model was a quadratic model that fits the data.

Table 4-3: Model summary

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Linear vs Mean	585.23	3	195.08	1.64	0.2276	
2FI vs Linear	633.50	3	211.17	2.32	0.1367	
Quadratic vs 2FI	865.99	3	288.66	47.48	< 0.0001	Suggested
Cubic vs Quadratic	23.50	3	7.83	1.64	0.3141	Aliased
Residual	19.06	4	4.77			
Total	23794.32	17	1399.67			

The response of the analysis was Ferulic acid extraction yield. The response yield was arranged from 6.62 - 46.017 mg/100 g BSG. The aim of the model fit summary was to maximize the adjusted R-Squared and predicted R-Squared values. Model significance was checked for the model and model factors. Linear model factors such as extraction time (A), extraction temperature (B), and sodium hydroxide concentration (C) and, quadratic model factors, namely pure quadratic terms (A^2, B^2, C^2) and interaction quadratic terms (AB, AC, BC) based on the F and p-values were checked.

Table 4-4: Analysis of variance (ANOVA) for the Response of Ferulic acid from BSG

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3678.05	9	408.67	87.21	< 0.0001	significant
A-Time	95.76	1	95.76	20.44	0.0027	
B-Temperature	2626.41	1	2626.41	560.47	< 0.0001	
C-Sodium Hydroxide Concentration	180.09	1	180.09	38.43	0.0002	
AB	132.41	1	132.41	28.26	0.0011	
AC	0.1234	1	0.1234	0.0263	<0.0001	
BC	34.19	1	34.19	7.30	<0.0001	
A^2	29.06	1	29.06	6.20	<0.0001	
B^2	2.94	1	2.94	0.6274	<0.0001	
C^2	57.88	1	57.88	12.35	0.0098	
Residual	32.80	7	4.69			
Lack of Fit	20.28	3	6.76	2.16	0.2354	not significant
Pure Error	12.52	4	3.13			

Skimming along the last 2 columns of Table 4-4, it can easily be observed that the model is statistically significant for the p-value (<0.0001) is lower than 0.05. Values of " p-value" less than 0.05 indicate that model terms are significant. Hence, in this case, all model terms considered for this study (A, B, C, AB, AC, BC, A^2, B^2, and C^2) are significant. This shows that extraction time, extraction temperature, hydrolyzing agent concentration, the interaction between extraction time and temperature, temperature and time, and extraction temperature and hydrolyzing agent concentration, and the square of extraction time, extraction temperature, and hydrolyzing agent concentration affect the Ferulic acid extraction yield significantly.

The other important parameter that should be given attention here is the “lack of fit p-value” term, which shows the ANOVA statistics for unexplainable differences. This lack of fit p-value (0.2354) implies that lack of fit is not significant relative to the pure error. There is only a 23.54% chance

that lack of fit p-value this large could occur due to noise. The model fit summary statistics are listed in Table 4-5.

Table 4-5: Analysis of variance (ANOVA) for the Response of Ferulic Acid from BSG

Std. Dev.	2.16	R^2	0.9259
Mean	24.62	Adjusted R^2	0.9095
C.V. %	8.79	Predicted R^2	0.8771
		Adeq Precision	30.7316

The "Pred R-Squared" of 0.8771 is in reasonable agreement with the "Adj R-Squared" of 0.9095" because the difference is less than 0.2. The “Pred R-Squared is the measure of the extent this developed model can be used to predict ranges of data this study has not considered, which, therefore, means that this model has 87.771% precision in fitting to overall ranges of data. The R-squared, on the other hand, shows how much of the difference in the outcome is explained by the model. This model is, therefore, almost is fairly good in accomplishing this task as demonstrated through its high value (92.59%). “Adeq Precision" measures the signal-to-noise ratio. A ratio greater than 4 is desirable. In this case, the ratio of 30.7316 indicates an adequate signal. Hence, it can be concluded that this model can be used to navigate the design space.

Table 4-6: Coefficients in Terms of Coded Significant Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	27.90	1	1.04	25.45	30.35	
A-Time	4.12	1	0.9120	1.97	6.28	1.42
B-Temperature	19.76	1	0.8346	17.78	21.73	1.19
C-NaOH conc.	6.26	1	1.01	3.87	8.65	1.74
AB	5.75	1	1.08	3.19	8.31	1.0000
AC	0.2300	1	1.42	-3.12	3.58	1.61
BC	4.04	1	1.50	0.5034	7.58	1.40
A^2	-3.28	1	1.32	-6.40	-0.1657	1.57
B^2	0.9712	1	1.23	-1.93	3.87	1.36
C^2	-4.09	1	1.16	-6.85	-1.34	1.23

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the variance inflation factor (VIFs) is 1; VIFs greater than 1 indicate multi-collinearity. The higher the VIF, the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable. In this case, one can simply observe that a good multi-collinearity exists for all the significant factors.

4.3.2. Development of Regression Model Equation

A quadratic model equation in terms of coded factors (equation 4.1) and actual factors (equation 4.2) shown below was fitted to the data model for predicting the response (extraction yield). The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. In this case, therefore, the extraction temperature (B), extraction time (A), have increasing effects on the hydrolyzing agent concentration (C), and the interaction of extraction temperature and time (AB) have increasing effect on the extraction yield while the interaction of extraction time and hydrolyzing agent concentration (AC), extraction temperature and hydrolyzing agent concentration (BC) and all the quadratic terms have a decreasing impact. As implied by the

largest positive coefficient in the equation, the highest positive impact on the yield went to the interaction between the extraction time and temperature (+10.35), whereas quadratic terms of the extraction time had the highest negative impact (-10.37).

$$\text{Ferulic Acid Yield (\%)} = +46.23 + 4.76A + 7.21B + 2.52C + 10.35AB - 7.88AC - 8.18BC - 10.37A^2 - 8.42B^2 - 6.58C^2 \quad (4.1)$$

The equation in terms of actual factors (equation 4.2) can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space.

$$\text{Ferulic Acid Yield (\%)} = -398.29 + 0.69 \times \text{time} + 4.68 \times \text{temperature} + 101.55 \times \text{NaOH concentration} + 0.02 \times \text{time} \times \text{temperature} - 0.26 \times \text{time} \times \text{NaOH concentration} - 0.41 \times \text{temperature} \times \text{NaOH concentration} - 0.01 \times \text{time}^2 - 0.02 \times \text{temperature}^2 - 6.5 \times \text{NaOH concentration}^2 \quad (4.2)$$

4.3.3. Model Adequacy Check

It is always necessary to examine the fitted model to ensure that it provides an adequate approximation to the true system and verifies that none of the least square regression assumptions is violated. The quality of the model developed was, therefore, evaluated from their coefficients of correlation as summarized in Table 4-6 shown above. The coefficient of variance (CV), which is the ratio of the standard error of estimate to the mean value of the observed response is a measure of reproducibility of the model. As a rule, a model can be considered reasonably reproducible if its CV is not greater than 10% (Pinelo et al., 2005). In this case, the CV is 8.79% which means that the model is reasonably reproducible.

The regression model was found to be significant with the correlation coefficients of determination of R-Squared, adjusted R-Squared and predicted R-Squared which took the values 0.9259, 0.9095, and 0.8771 respectively. The highest percentage value of the R-squared term (92.95%) implies that only 8.05% of the total variation in the yield of extract was not explained by the model. The “Adeq Precision" measures the signal to disturbance ratio due to random error. A ratio greater than

4 is desirable. In this case, as indicated by Table 4-6, the value is found to be 30.7316, which is a measure of the highly adequate signal.

The normal probability plot of residuals is shown in Figure 4-2 (a). If data from experiments form a normal distribution, the residuals fall on a straight line, which implies that the errors are spread in a normal distribution. Here a residual means difference in the observed value (obtained from the experiment) and the predicted value or fitted value (a value that can be predicted by the developed model equation). Hence, observing this normal plot, it can be noticed that all the points line up well and the deviation of points for extract yield from normality is insignificant, which means that the Ferulic acid extract yield is precisely modelled. This can also be confirmed by the variations between the residuals and model predicted values analyzed through residual graphs as presented in Figure 4-2 (b). The plot is a random scatter (constant range of residuals across the graph). Thus, the model does not need any form of transformation as it is well fitted.

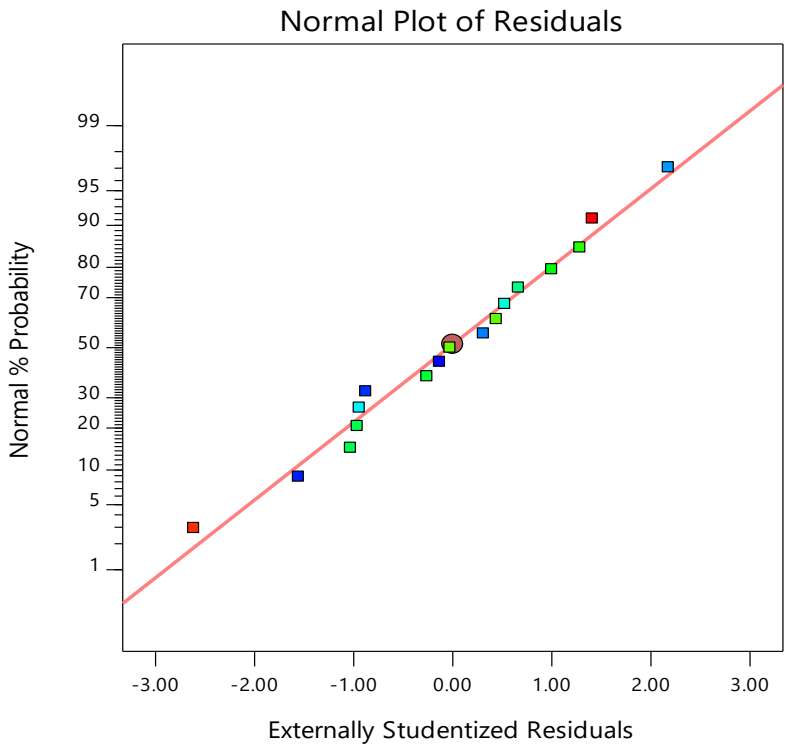
On the other hand, Figure 4-2 (c) shows the plot of residuals versus the experimental run order. It checks for lurking variables that may have influenced the response during the experiment. The plot shows a random scatter and thus no more blocking or any other solutions were needed. The plot of predicted versus actual (Figure 4-2 (d)), which is the graph of the predicted response values versus the actual response values, is also another important diagnostics plot that should be taken into consideration. The purpose is to detect a value, or group of values, that are not easily predicted by the model. Looking into the graph, all values fall along the straight line, which shows the high power of prediction of the model developed.

Design-Expert® Software

Ferulic Acid Yield

Color points by value of
Ferulic Acid Yield:
4.05 56.00

(a)



Design-Expert® Software

Ferulic Acid Yield

Color points by value of
Ferulic Acid Yield:
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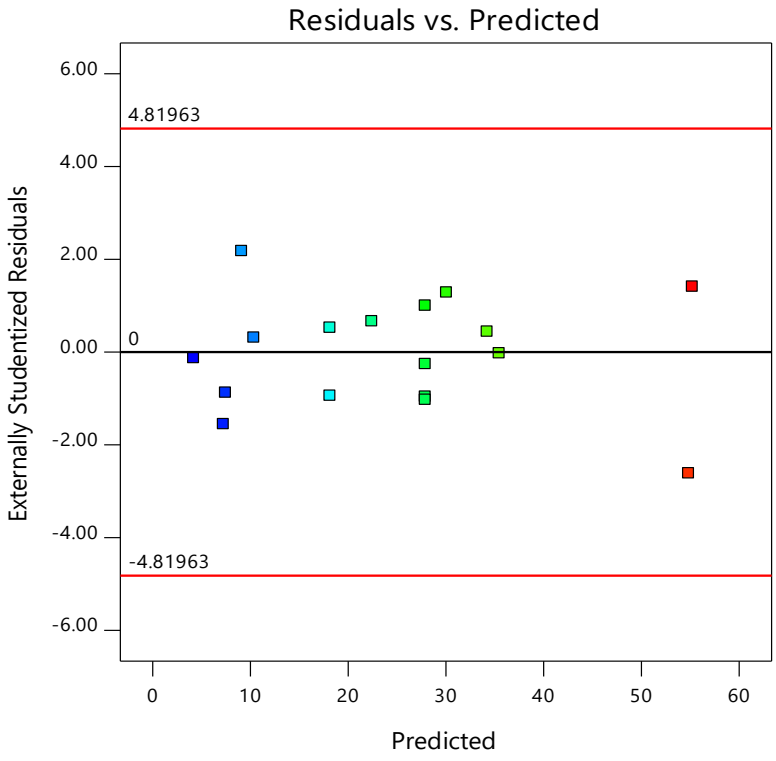
(b)



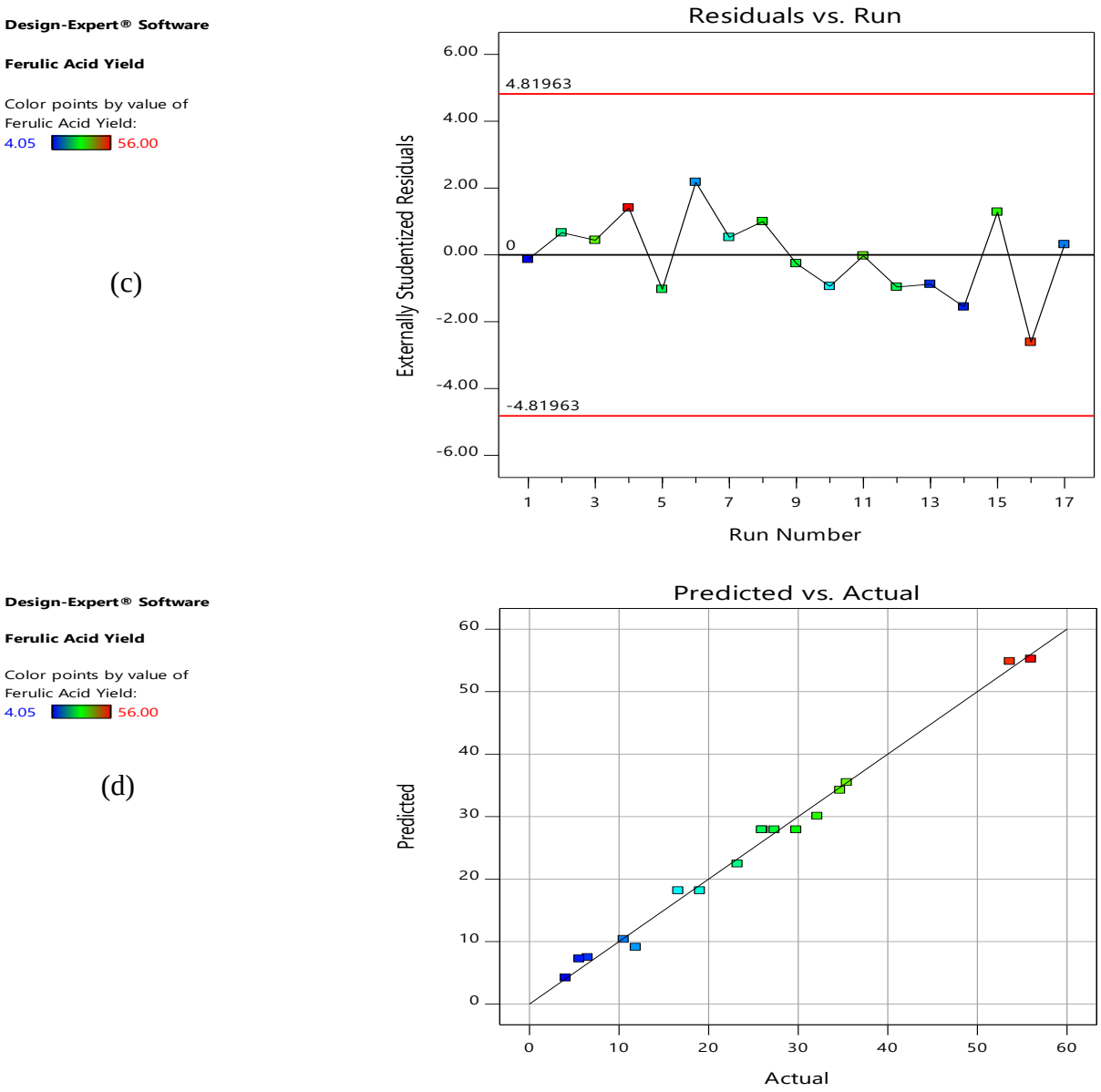


Figure 4-2: Normal plots of residuals yield (a); plot of residual versus predicted yield (b); plot of residuals against run order (c); plot of predicted versus actual (d)

4.3.4. Comparison of the Factor Effect

Figure 4-3 below is called a perturbation plot, which helps to compare the effects of all the factors at a point in the design space. The response is plotted by changing only one factor over its range while holding all the other factors constant. A steep slope or curvature in a factor shows that the response is sensitive to that factor. A relatively flat line shows insensitivity to change in that factor. If there are more than two factors, the perturbation plot could be used to find those factors that most affect the response. In this case, as it can be seen from the plot, the increase in three of the

factors has an increasing effect on the yield of the extract. This is especially true until the reference point is reached. Past the reference point, however, the effect of each factor on the extraction yield is reverted.

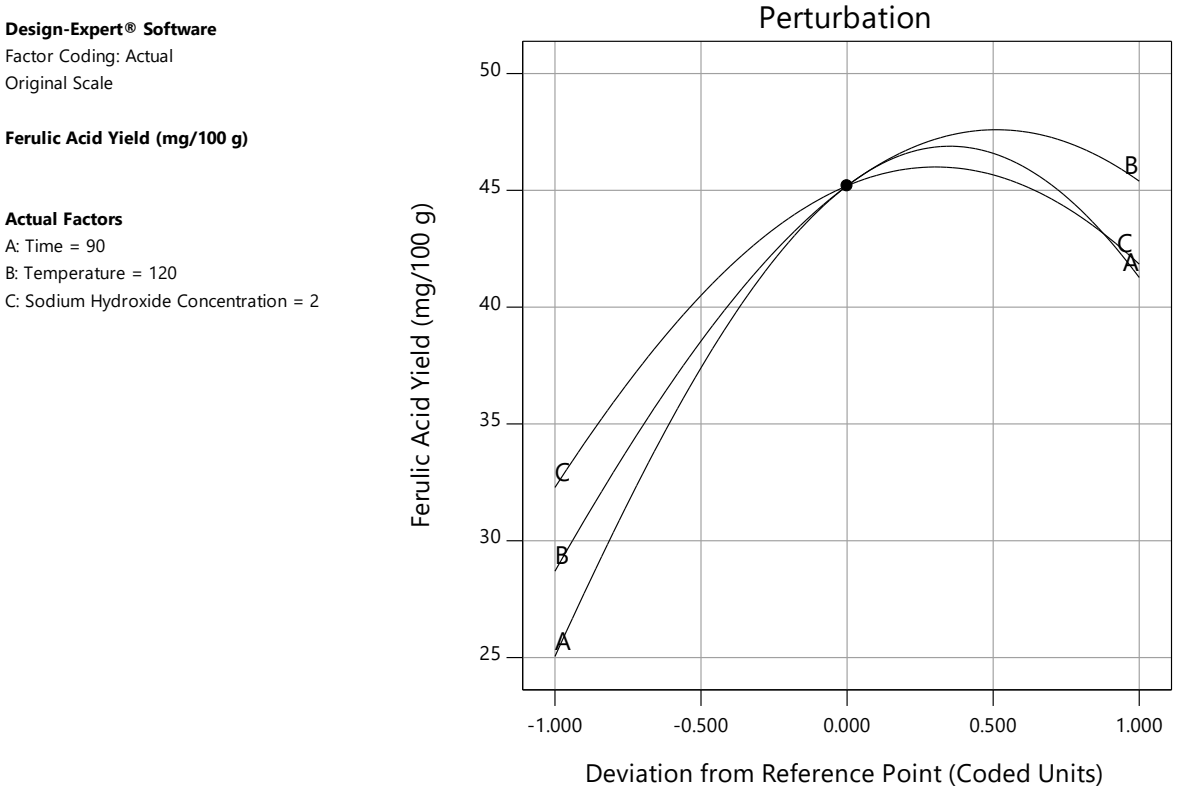


Figure 4-3: Perturbation plots comparing the effect of all factors at a point in the design space

4.3.5. Effect of Extraction Process Variables

Based on the ANOVA result, the yield extract is significantly affected by three of the factors involved in this study. Below is the discussion for each of these factors and how they affect the product.

4.3.5.1. Effect of Temperature on the Ferulic Acid Yield

Extraction temperature is one of the critical factors that have a profound effect on the extraction of Ferulic acid. In this study, a maximum Ferulic acid was found at around 130 °C as illustrated by Figure 4-4. Apparently, the lower temperature does not favour the extraction of Ferulic acid. This is because the lower temperature will not be strong enough to break the bonds crosslinking Ferulic acid in between. On the other hand, applying too high temperature oxidizes the antioxidant. In between, as temperature increases, so do the amount of Ferulic acid extracted since an increase

in the temperature of extraction contributes to the improvement of both the penetration of the hydrolyzing agent into the material and the diffusion coefficient. To be more specific, higher temperature increases the possibility of cleaving natural bonds that esterifies Ferulic acid to lignin and/or arabinoxylans and through alkali-labile cross-linkages (Scalbert, 1986). For this reason, in high temperatures, there is an increase in the content of extracted compounds. A report by Liyana-Pathirana et al. (2006) showed that heating favours the process of extraction of winery by-products, which agrees with the finding in this study. The report, when stated as a reason why this happens, claimed that an increase in temperature increases the solute diffusion ratio, accelerates the transference of mass, solubilizes compounds, and reduces solute–matrix interactions. Another report conducted by Xu (2005) also showed that an increase in alkaline treatment temperature had an important effect on the release of ester-linked p-coumaric and Ferulic acids from the cell walls of various cereal straws. This increase in yield is not infinite however. As it can observed from the same figure, there is a slight decrease in the yield pas the optimum point. This is because a too high temperature causes the oxidation and degradation of the desired compounds (Naczk, 2004).

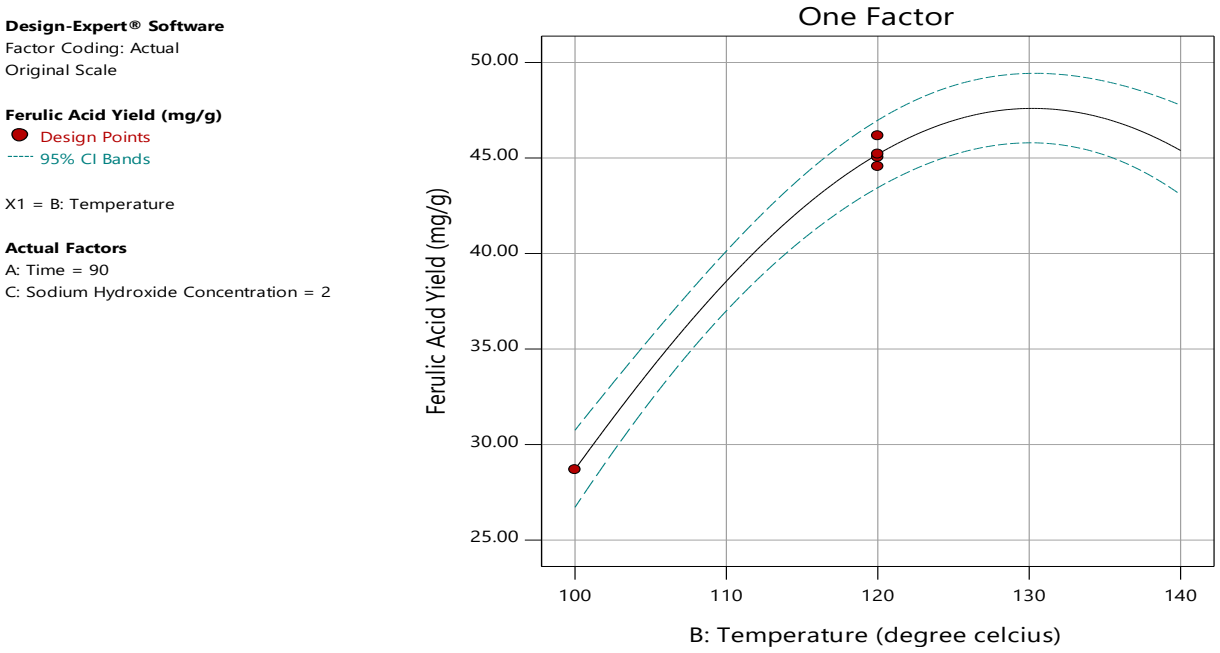


Figure 4-4: Effect of temperature on extraction yield

4.3.5.2. Effect of Time on Ferulic Acid Yield

Extraction time is crucial in solvent extraction of phenolic compounds as appropriate extraction time can result in time and cost-saving. From Figure 4-5, as expected, a longer extraction time led

to a higher percentage yield of extract. This is because the solute and solvent were in contact with each other for a longer amount of time which might have favoured the system to have a more mass transfer. As shown in the figure, the optimum extraction yield of Ferulic acid is obtained at an extraction time of 90 min with a value of 45 mg/100 g BSG. This direct relationship of time and the extraction yield is only true up to a certain point – the optimum level. Past this point, however, a further increase in time has led to a decrease in the Ferulic acid yield. This is because the application of the solvent for an extra time leads to oxidation of the Ferulic acid since it is sensitive to heat, temperature, and other oxidizing agents. This agreed with some literature results. Mussato et al. (2007) found out that time significantly affected the extraction yield of p-coumaric and Ferulic acid, where they were able to obtain optimum values of 138.18 mg/L and 145.3 mg/L in respective order at an optimum time of 90 min.

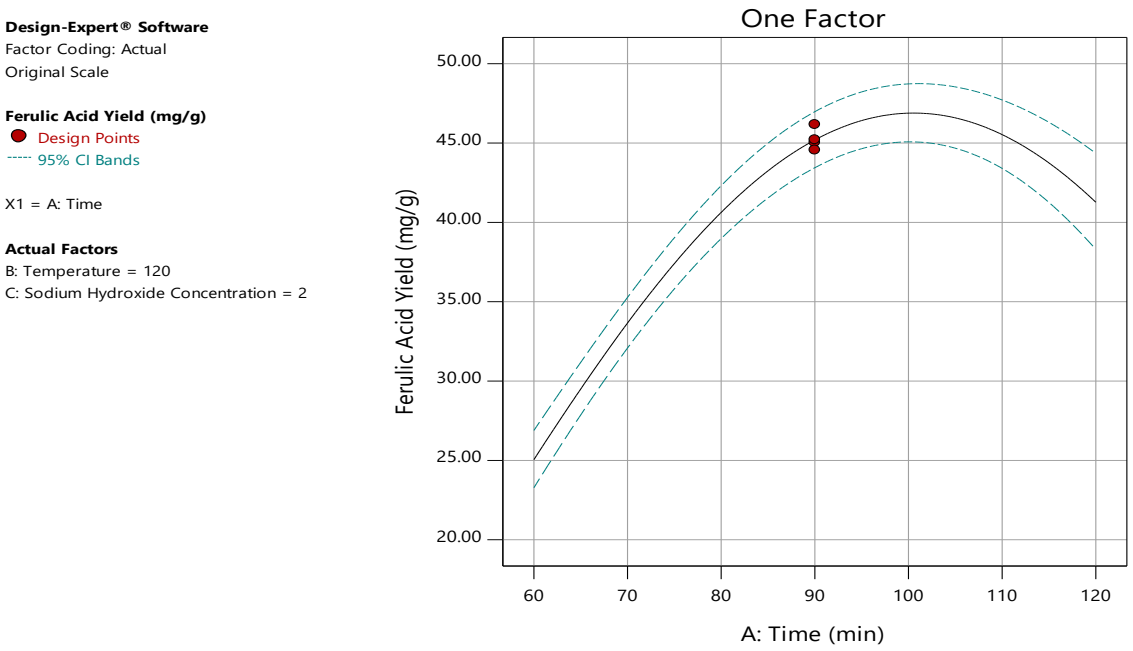


Figure 4-5: Effect of time on extraction yield

4.3.5.3. Effect of Sodium Hydroxide Concentration on Extraction Yield

As presented in Figure 4-6, the Ferulic acid yield significantly increased with sodium hydroxide concentration until a certain point and then it decreased. In other words, the plot shows that the yield of Ferulic acid is directly proportional to the concentration of sodium hydroxide until the concentration reaches 2%. This is because rising the alkaline treatments may dissolve lignin by cleavage of ester linkages in lignin–polysaccharide complexes, which leads to the release and

solubilization of phenolic acids (Aarabi1 et al., 2016). But past the optimum point, a further increase in the concentration might lead to unwanted oxidation reactions that counteract the extraction process (Aarabi1 et al., 2016). In other words, unlimited increase in the concentration of the hydrolyzing agent causes the degradation of polyphenolic compounds caused by hydrolysis, internal redox reactions and polymerization (Alonso-Salces et al., 2001). The same trend was also observed in Mussato et al. (2007) study where they found that the optimum yield was obtained at 2% hydrolyzing agent concentration.

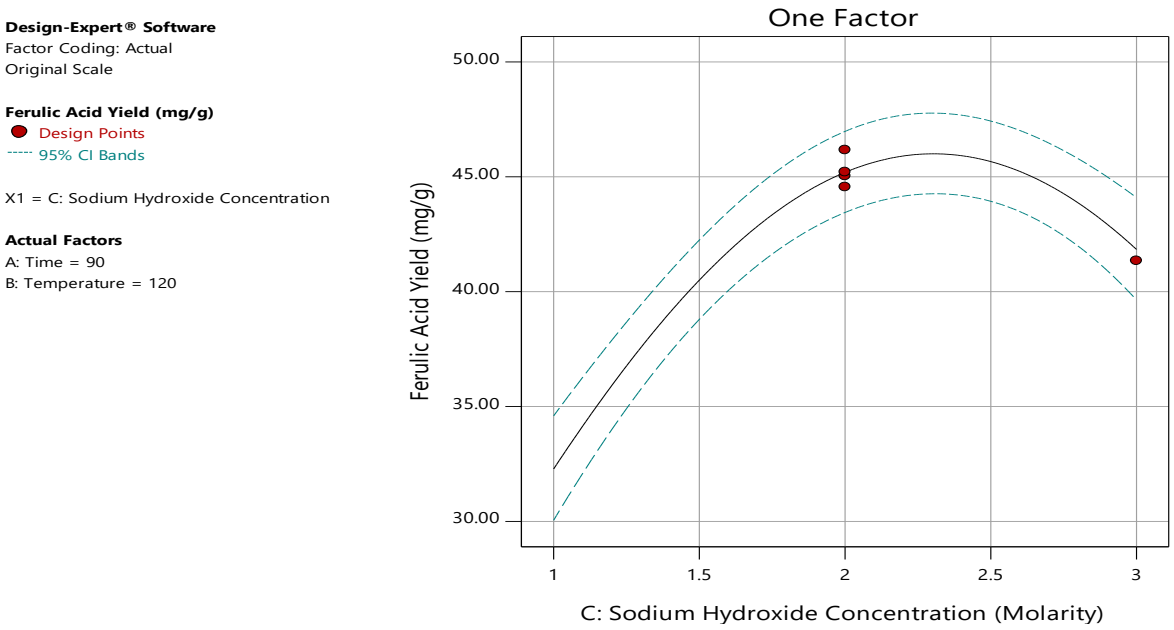


Figure 4-6: Effect of sodium hydroxide concentration on extraction yield

4.3.6. Effect of Interaction between Process Variables

The discussion of the single factors tells that each of the three factors has a significant effect on the response of extract yield. However, it was also observed that there is an interaction between each of the variables (Table 4-5). For graphical interpretation of the interactions between regressor variables, use of 3D plots of the regression equation is recommended. Interaction implies that the effect produced by changing one-factor level (for example, time) depends on the level of the other factor. In this research, to investigate the interactive effect of independent variables on the extraction yield from BSG, three dimensional (3D) plots were examined to understand the interactions between 3 of the variables and the optimum levels of each variable for maximum yield

of extract was determined. The interaction plots of extraction temperature and time, and extraction temperature and hydrolyzing agent concentration, and extraction time and sodium hydroxide concentration are shown in Figure 4-6 to 4-8 below.

4.3.6.1. Effect of Time and Temperature on Yield

Figure 4-7 shows how the interaction of extraction time and temperature affected the extraction yield of Ferulic acid when the concentration of NaOH was kept at a constant value of 2%. The plot, therefore, shows that the yield increased until it reaches the value 45.245±0.585 mg/100 g BSG as both the extraction time and temperature increased. This is because both time and temperature are key variables for the extraction of Ferulic acid from BSG. A temperature of 120 °C and 90 min time gave an optimum condition for the extraction solvent to act on the ester bonds that crosslink the extractives to the lignocellulosic material. As it was illustrated during the discussion on the effect of one-factor section, the increase of time (from 60 to 120 min) and temperature (from 100 to 140 °C) could only increase the yield to just over 40 mg/100 g BSG and 45 mg/100 g BSG respectively. But when these independent factors interact, their effect is maximized to have an additional increment value to rise the extract yield to around 45.245±0.585 mg/100 g BSG (Figure 4-7). This is because long time gives the high extraction temperature to have better chance to soften the plant tissues which, as a result, affects the cell membranes in such a way that phenolic compounds can be easily extracted into the solvent (Shi, 2003). However, the yield decreased to 45 mg/100 g BSG past the optimum point due to the reason that Ferulic acid is sensitive to extremely high temperature acting for too long time.

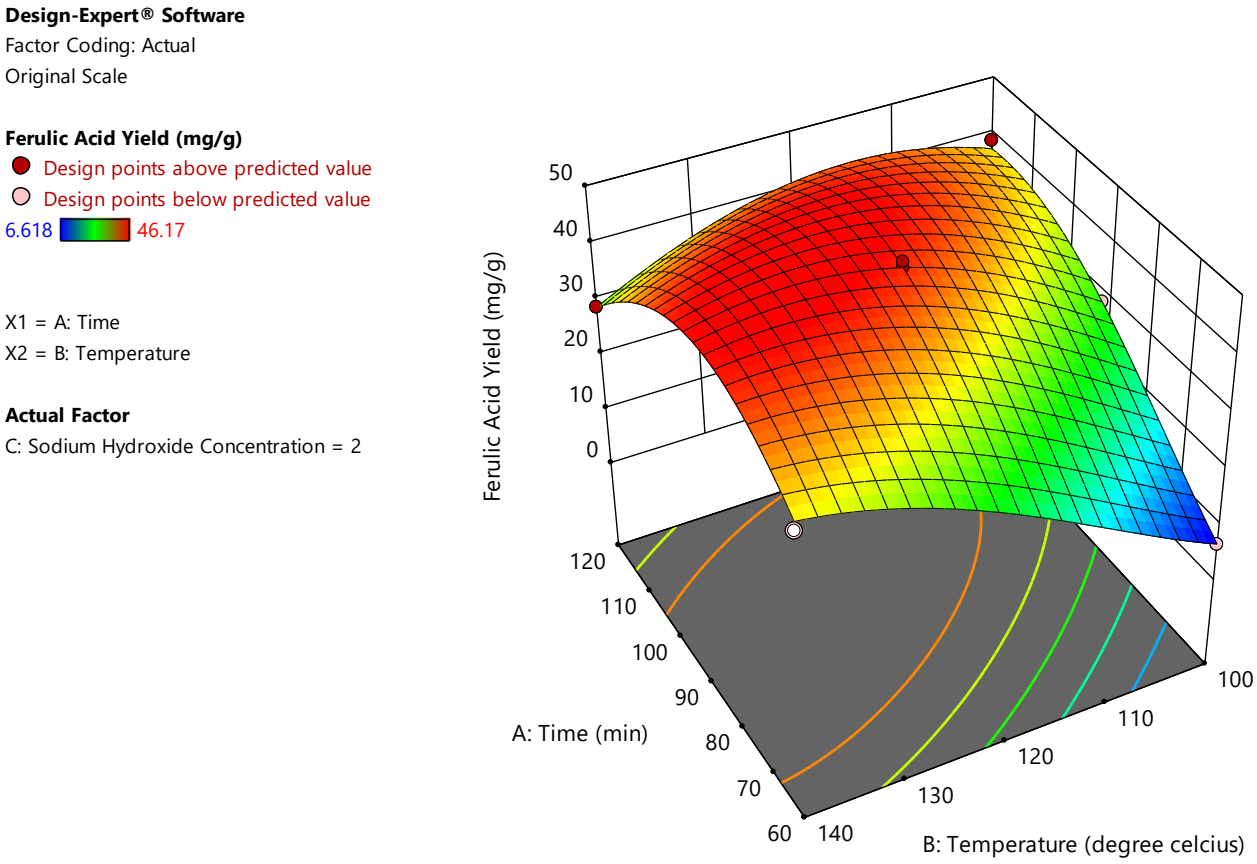


Figure 4-7: Surface plot of temperature and time interaction effect

4.3.6.2. Effect of Temperature and Hydrolyzing Agent Concentration on Ferulic Acid Yield

The same trend is also observed for the interaction effect of temperature and sodium hydroxide concentration on the yield at a constant value of time (90 min): the yield increased from 6.62 to around 46 mg/100 g BSG as temperature and alkali concentration increased from 60-120 mins and 1-3% (w/v) respectively. As discussed on the one-factor effect section, it is indicated that the sole effects of sodium hydroxide and extraction temperature could result in a maximum result of about 45 mg/100 g BSG. When these two most important factors interact with each other, as indicated in Figure 4-7, they could rise the extract yield to about 46 mg/100 g BSG, which means that the interaction effect of temperature and hydrolyzing agent concentration is significant. This can be explained by the fact that the high temperature makes the process of attacking the bonds that keep the structure of the BSG intact easier, which leads to a better chance of releasing Ferulic acid.

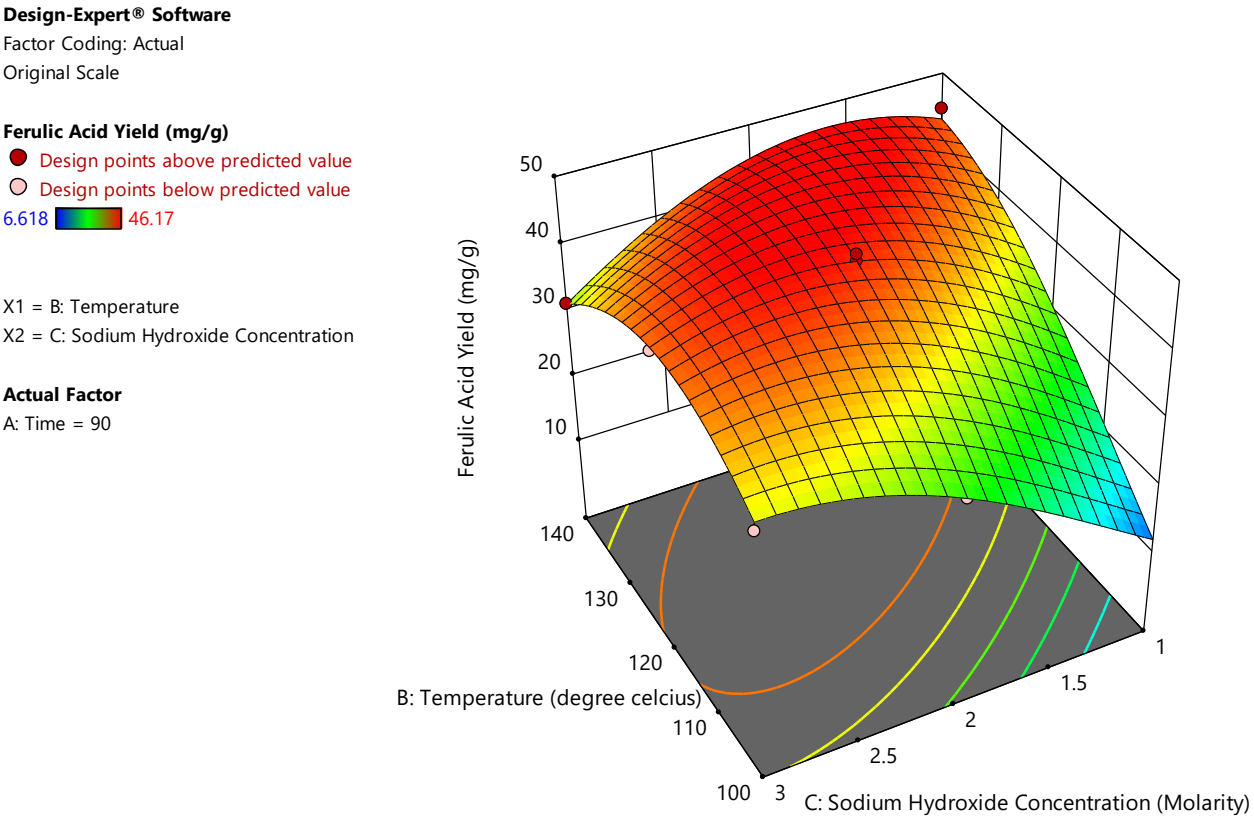


Figure 4-8: Surface plot of sodium hydroxide and temperature interaction effect

4.3.6.3. Effect of Time and Hydrolyzing Agent Concentration on Ferulic Acid Yield

Studying the plot given by Figure 4-9, it can be noted that Ferulic acid extracted at 90 min and 2% (w/v) alkali concentration values has the highest extraction yield. In other words, as both time and alkali concentration increased from 60-90 mins and 1-2% (w/v) alkali concentration respectively, the yield increased from 6.62-46 mg/100 g BSG. The yield then declines to around 40 mg/100 g BSG of Ferulic acid. The increase in yield of Ferulic acid is because prolonged exposure of the sample to the alkali allows sufficient time for the desired product to be extracted. But a too much alkali concentration acting on the process for a too long time degrades the Ferulic acid itself, which is the reason for the decline in the yield past the optimum point.

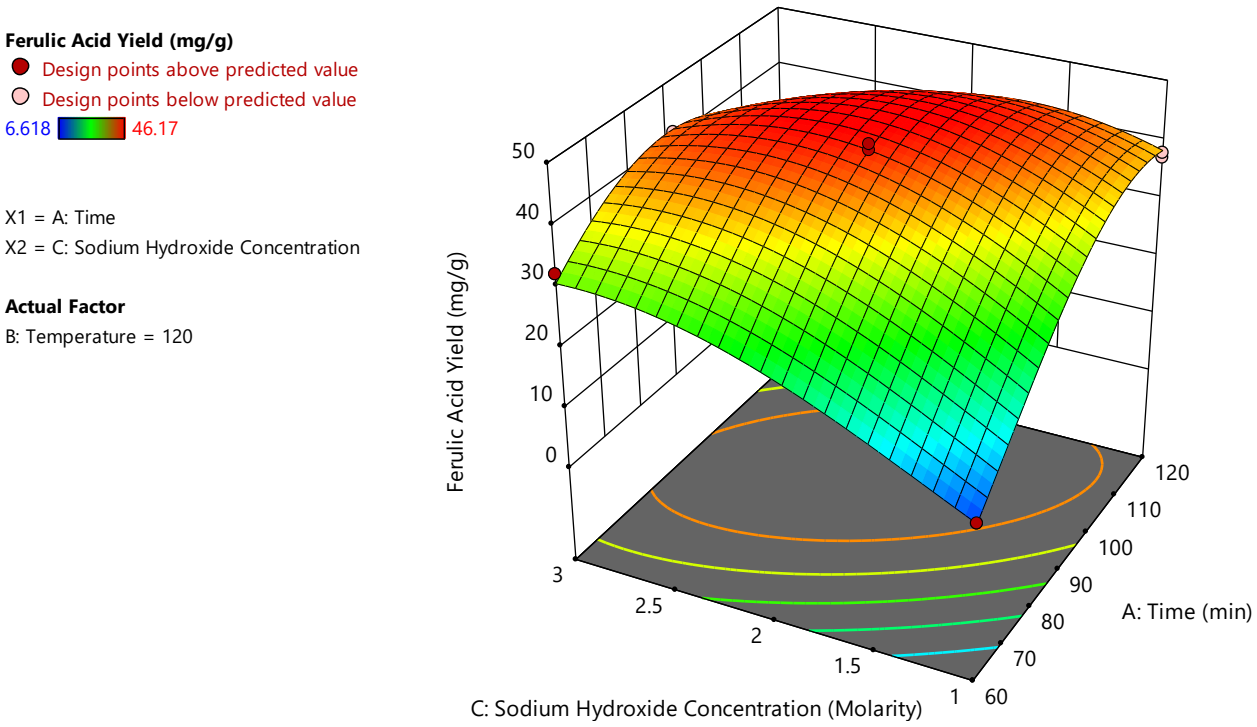


Figure 4-9: Surface plot of sodium hydroxide and time interaction effect

4.4. Optimization of Extraction Factors

One of the objectives of the present study was to find the optimal process variables for the best Ferulic acid yield. The process variables such as extraction time, extraction temperature and hydrolyzing agent concentration were optimized using the numerical optimization function of Design-Expert software. While doing so, all the factors were set to be in range while the Ferulic acid yield was the response that needs to be maximized. Table 4-5 shows the summary of factors, goals and the corresponding set of specific objectives that could optimize the process condition to have the highest response.

Table 4-7: Constraints Applied for Optimization

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A: Time	is in range	60	120	1	1	3
B: Temperature	is in range	100	140	1	1	3
C: Sodium Hydroxide Concentration	is in range	1	3	1	1	3
Ferulic Acid Yield	maximize	6.62	46.17	1	1	3

The design expert under numerical optimization gave 100 different optimization solutions. Depending on the parameters by compromising yield, economy and energy carrying, the best solution was selected among the alternatives. Undertaking the consideration of these constraints, 97.04 min, 119.83 °C and 2.205% (w/v) NaOH concentration were selected which gave a yield of 46.85 mg/100 g BSG as summarized in Figure 4-9.

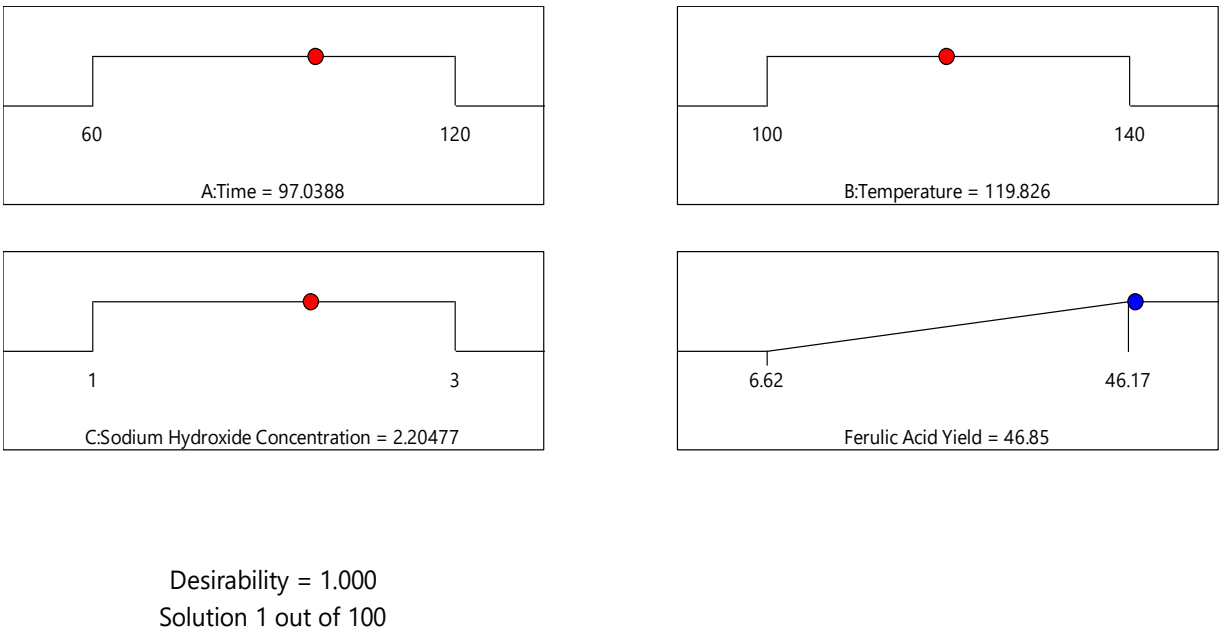


Figure 4-10: Ramp Plot of Optimization Solution for the Response

Hence, for verification of these conditions, an experimental investigation was conducted at these optimum parameters with four replicates and a close actual value to this result (Ferulic acid yield

of 45.25 mg/100 g BSG) was obtained. Further, the extract was subjected to the analysis of FTIR and HPLC.

4.5. Product Characterization

4.5.1. Determination of Total Phenols

To evaluate the extraction of phenolic compounds from BSG, the quantification of total phenolic content (TPC) was performed using the Folin-Ciocalteau method which gave the results summarized in Table 4-9. The concentration equivalent of gallic acid was calculated from the calibration curve given by Figure 4-11 ($y = 0.12x + 0.36$, $R^2 = 0.98$) after taking the absorbance for each sample concentrations.

Table 4-8: Concentrations of gallic acid standard solution and their corresponding absorbance

Gallic acid concentration (mg/mL)	Absorbance (λ_{max}=765nm) Mean ± SD
0	0
1	0.475 ± 0.123
2	0.536 ±0.152
4	0.8745 ±0.032
6	1.046 ±0.043
8	1.4155 ±1.202
10	1.588 ±1.54
12	1.683 ± 2.321

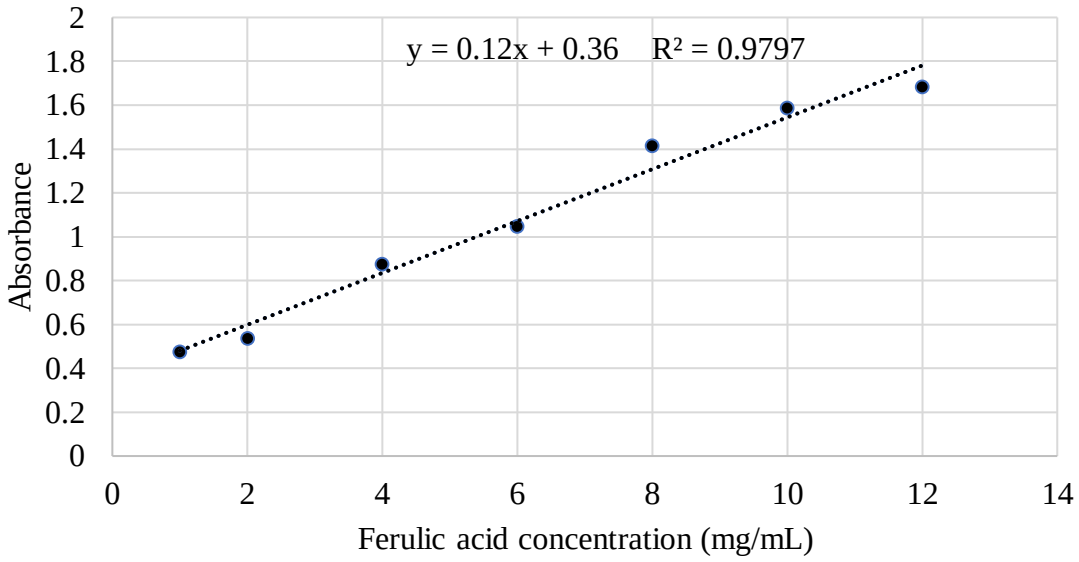


Figure 4-11: Gallic acid standard calibration curve

Finally, the total phenol content (TPC) was determined by using the equation (3.10) and was expressed as gallic acid equivalents/g (GAE/g) in the dry base. Referring to Table 4-9, the maximum TPC was found to be 118.50 mg GAE/100 g extract which was obtained at 12 mg/mL sample concentration.

Table 4-9: Amount of phenol content from a gram of dry BSG Extract

Sample concentration (mg/mL)	Absorbance (λ_{max}=765nm) Mean ± SD	Gallic acid equivalent Concentration (mg/mL)	Total phenol content (mg GAE/100 g extract)
1	0.075 ± 0.043	-*	-*
2	0.086 ±0.062	-*	-*
4	0.4725 ±0.052	0.935	18.7
6	0.556 ±0.173	1.641	32.82
8	0.6105 ±1.402	2.102	42.04
10	0.848 ±1.740	4.111	82.22
12	1.0623 ± 1.354	5.925	118.5

* only positive numbers that show meaningful values are included here because there is no negative concentration.

As observed in Table 4-9, only concentrations ≥ 4 mg/mL and their corresponding absorbances could satisfy the standard gallic acid calibration curve (linear regression curve) of BSG extract. The gallic acid equivalent concentrations at these absorbances were obtained using the gallic acid standard calibration curve ($y = 0.12x + 0.36$, $R^2 = 0.98$) as shown in Figure 4-12. Accordingly, the maximum gallic acid equivalent concentration obtained was 5.925 mg/mL (Table 4-9). Studying the trend of the results in Table 4-9, it can be observed that the total phenol obtained is directly proportional to the sample concentration. In other words, as the sample concentration was increased, so did the absorbance, which led to an increase in the total phenols finally obtained.

4.5.2. Determination of Antioxidant Activity

The antioxidant mechanism of Ferulic acid is complex, mainly based on the inhibition of the formation of reactive oxygen species or nitrogen, but also neutralization of free radicals. In addition, this acid is responsible for chelating protonated metal ions (Rice-Ecans et al., 1996). Ferulic acid is not only a free radical scavenger but also an inhibitor of enzymes that catalyze free radical generation and an enhancer of scavenger enzyme activity. Its antioxidant properties are primarily related to scavenging of free radicals, biding transition metals, and lipid peroxidation prevention.

Scavenging capacity of the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical was used to determine the antioxidant activity of samples as detailed by Brand-Williams et al. (1995). Results were expressed as the inhibition dose for 50% of reduction in the initial levels of DPPH free radical. IC_{50} value of an antioxidant is the concentration of the antioxidant required to give 50% inhibition of the DPPH assay. It was named as the ‘‘Inhibition Concentration” (IC_{50}) and determined by sequential sample dilutions versus the antioxidant activity scavenger percentage as described by Brand-Williams et al. (1995).

The free radical scavenging activity or inhibition activity of free radical DPPH percentage of standard ascorbic acid and BSG extract were calculated using the relationship written in the equation (3.9). The IC_{50} values of the standard ascorbic acid (Table 4-10) and that of the sample (Table 4-11) were determined from the plots as indicated in Figure 4-12 and Figure 4-13. Comparing this IC_{50} values we can see that the extract manifested the strongest capacity for neutralization of DPPH radicals.

Table 4-10: Average absorbance of ascorbic acid and corresponding concentration

Ascorbic acid concentration (mg/mL)	Absorbance (λ_{max}=517nm) Mean± SD	% IA	IC_{50} (mg/mL)
Control	1.001	-	-
1	0.723±0.032	27.7	3.257
2	0.496±0.034	50.4	
4	0.302±0.021	69.8	
6	0.081±0.035	91.9	
8	0.057±0.012	94.3	
10	0.040±0.037	96	
12	0.028±0.004	97	

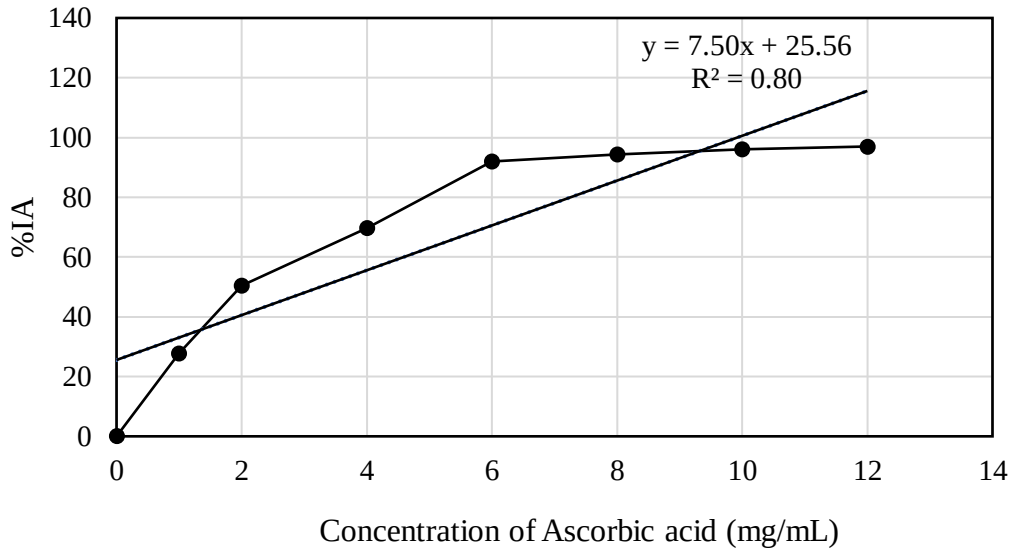


Figure 4-12: Inhibition activity (IA%) of the standard Ascorbic acid

Table 4-11: DPPH absorbance of sample concentration for BSG extract

Sample concentration (mg/mL)	Absorbance (λ_{max}=517nm) Mean± SD	%IA	IC_{50} (mg/mL)
0	0	-	-
1	0.495	50.5	1.743
2	0.34	66	
4	0.2	80	
6	0.021	97.9	
8	0.014	98.6	
10	0.012	98.8	
12	0.01	99	

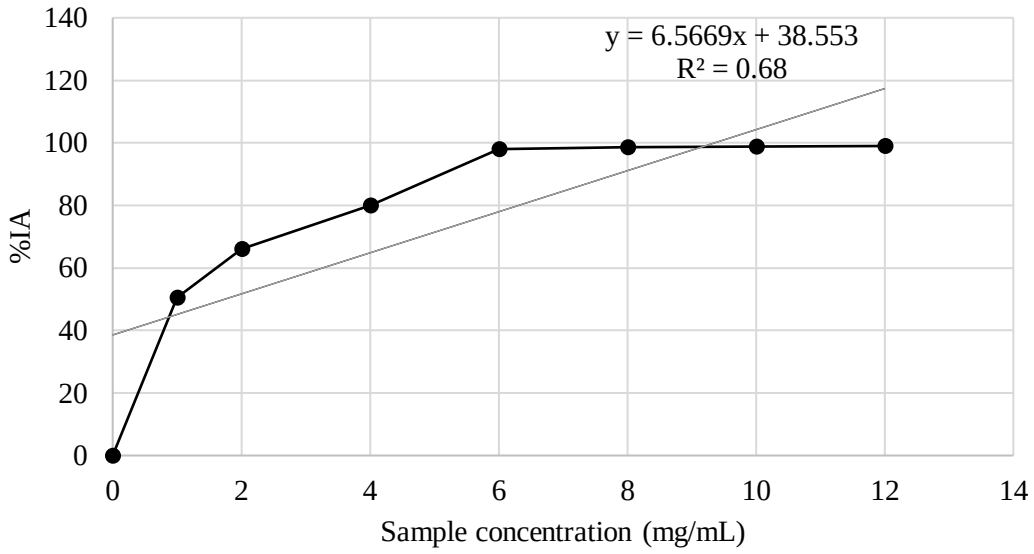


Figure 4-13: Inhibition activity (IA%) the sample

The strong antioxidant activity of Ferulic acid is supported by the literature. For instance, a study conducted by Kikuzaki et al. (2002) on 24 Ferulic acid-related compounds together with 6 gallic acid-related compounds using several different physical systems as well as their radical scavenging activity indicated that Ferulic acid was the most effective among the tested phenolic acids. This

property is attributed to its structural characteristics. Because of its phenolic nucleus and unsaturated side chain, it can readily form a resonance stabilized phenoxy radical, which accounts for its potent antioxidant activity (Clin, 2007). Pluemsamran et al. (2012) proved that human endothelial cells and keratinocytes are much less susceptible to UV-induced free radical damage when exposed to Ferulic acid prior to irradiation.

4.5.3. Fourier Transform Infrared Spectroscopy (FTIR)

The functional groups of the extracted Ferulic acid were characterized using FTIR spectroscopy (Figure 4-15). The FTIR spectrum of the extract was compared with literature for confirmation. The FTIR spectrum of the sample clearly showed the existence of main functional groups in the Ferulic acid structure, where the strong at around 3500 cm^{-1} region is a characteristic of the O-H functional group in the extracted compound.

The C-H stretching of the aromatic ring and C-H vibration on the methyl group were represented by the other strong and broad 3000-2800 cm^{-1} and 1330 cm^{-1} regions of the spectra depicted by the plot. Apart from this, the band in the range 1600-1700 cm^{-1} corresponds to that of the carbonyl group (C = O) while the vibration for C = C on the aromatic ring is at 690 cm^{-1} band. Results were compared with the finding of Robert et al. (1998) and a strong similarity was found out as compared with a pure Ferulic acid examined in the study.

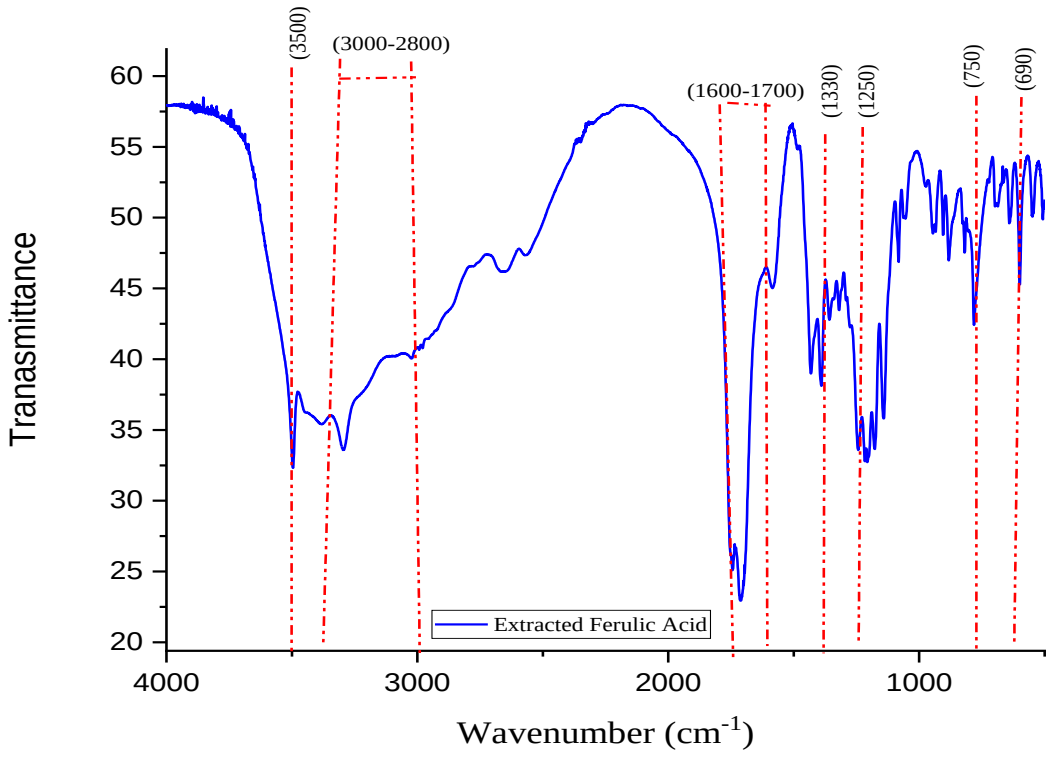


Figure 4-14: FTIR Spectra of Extracted Ferulic Acid

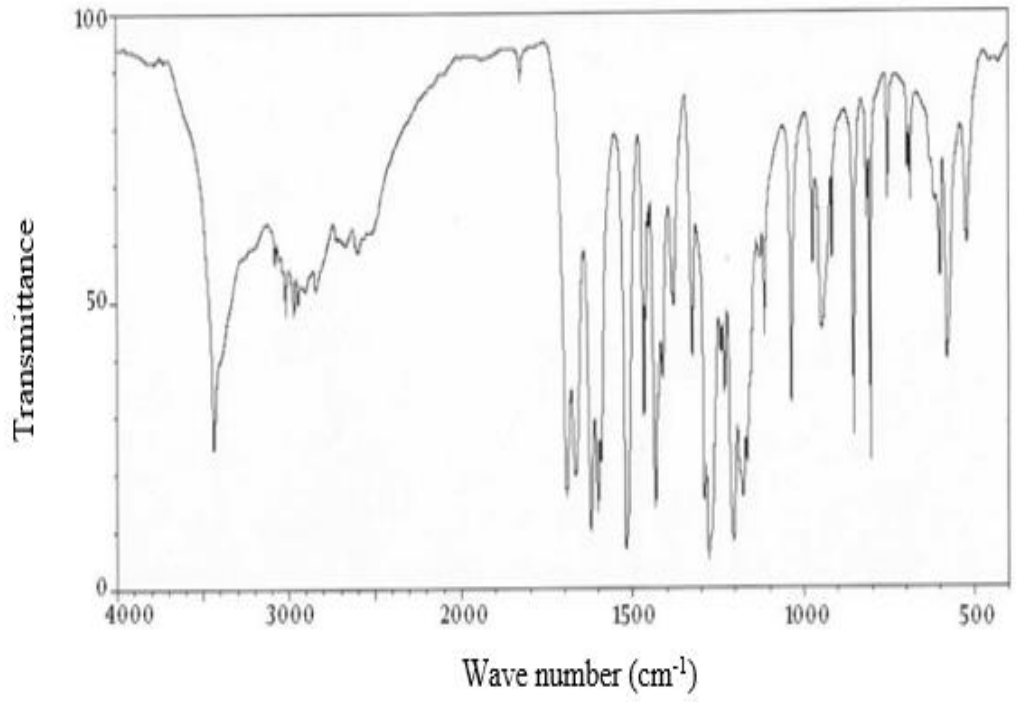


Figure 4-15: FTIR spectra of standard Ferulic acid (Robert et al., 1998)

4.5.4. High-Performance Liquid Chromatography (HPLC) Analysis

British Pharmacopoeia provides a method for quantifying Ferulic acid by HPLC, but its elution is by gradient, using phosphoric acid and acetonitrile (British Pharmacopoeia Commission, 2011). An isocratic elution methodology presents greater simplicity of execution, low cost and reduced time. Literature describes solvents, such as acetonitrile, methanol, acetic acid, orthophosphoric acid, acetate buffer solution and ultrapure water for Ferulic acid determination in plants, plasma and some lipid particles (Carbone et al., 2014; Li et al., 2008; Li, et al., 2004; Li & Bi, 2003; Lu et al., 2005; Seo et al., 2011; Trombino et al., 2013; Wang et al., 2015). In this specific work, Ferulic acid was detected at 8.0 min (Figure 4-17), which is confirmed by the retention time of its standard (Figure 4-16). Based on equation (3.11), the corresponding Ferulic acid concentration was found to be 30% (w/v). As it can be observed from this numerical value of the concentration and the figure below, the extracted Ferulic acid is not 100% pure. In fact, it occupies less than 50% of the region as explained by the percent of the peak area. This implies that the purification method followed in this work is not that efficient to obtain a pure Ferulic acid. However, it was a success to show that BSG is a good candidate for the extraction of Ferulic acid.

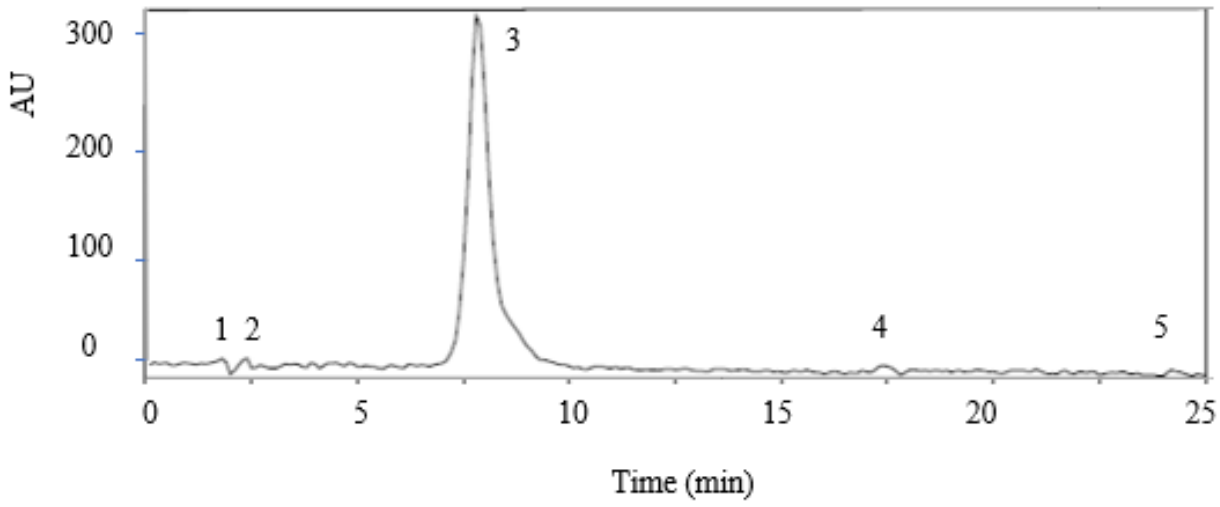


Figure 4-16: HPLC chromatograms of standard Ferulic acid

Table 4-12: Ferulic acid standard peak table

Peak Number	Ret. Time	Area	Area%	Height	Tailing Factor (USP)	Width	Number of Theoretical plates (USP)
1	2.042	37	0.476	232	-	0.320	226
2	2.454	32	0.405	263	-	0.240	579
3	8.000	7681	98.462	9453	1.045	1.225	236
4	17.5000	35	0.450	281	0.975	0.25	27146
5	24.000	16	0.207	202	-	0.16	124650

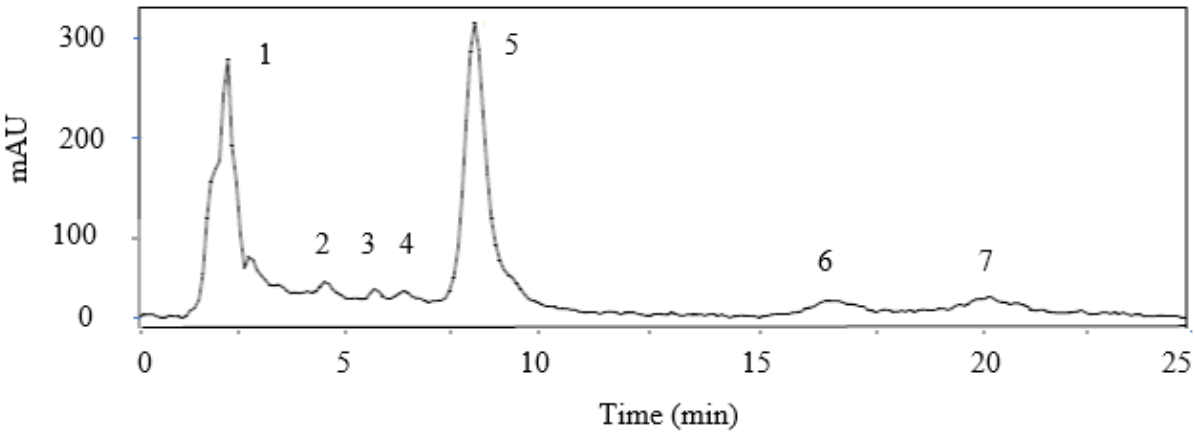


Figure 4-17: HPLC chromatograms of extracted Ferulic acid

Table 4-13: Extracted Ferulic acid peak table

Peak Number	Ret. Time	Area	Area%	Height	Tailing Factor (USP)	Width	Number of Theoretical plates (USP)
1	2.450	1951.875	39.026	3123	1.045	1.25	21
2	4.375	81.875	1.637	262	1.235	0.625	1086
3	5.854	102.610	2.052	331	0.985	0.62	12151
4	6.252	86.904	1.738	284	0.970	0.612	2217
5	8.000	2350.695	47.000	4678	1.050	1.005	227
6	16.752	205.875	4.116	183	-	2.25	1228
7	20.055	221.625	4.431	197	-	2.25	1761

CHAPTER FIVE

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

The utilization of agro-industrial wastes such as brewery spent grains for the production of natural antioxidants is both a wise waste management technique and a remedy for the use of synthetic antioxidants which are reported to have restricted use in foods due to their toxicological effects on various species. In this work, Ferulic acid, one of the most applicable antioxidants, was extracted using alkaline hydrolysis. The characteristics of the raw material, as revealed its high hemicellulose content which is reported to be containing extractives such as Ferulic acid crosslinked in its etheric bonds, indicated that the raw material is suitable for the extraction of Ferulic acid.

The results showed that a maximum percentage yield (45.245±0.585 mg/100 g BSG) was obtained at 90 min, 120 °C and 2% (w/v) NaOH concentration. The FTIR spectra of this extract indicated the presence of the main functional groups (O-H and C = O) that are responsible for the antioxidant characteristics of the Ferulic acid which was also confirmed by the HPLC result. The BBD of RSM was applied to optimize the extraction factors and it was found out that all the main factors and their interaction could affect the extraction process significantly where all three main factors and the interaction effect between temperature and time had an increasing effect on the extraction process whereas the rest had a decreasing effect. The optimum Ferulic acid extraction with an extraction yield of 46.85 mg/100 g BSG was extracted at 97.038 min, 119.826 °C and 2.205% (w/v) NaOH concentration.

The extract was tested for its antioxidant capacity using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical. Comparing the free radical scavenging activity or inhibition activity of the free radical DPPH standard ascorbic acid and BSG extract, it was observed that the extract manifested the strongest capacity for neutralization of DPPH radicals. With the vast availability different types of wastes from the continuously increasing brewery industries in the country, the researcher hopes that the results of this research study would give input to concerned bodies to consider the alternative waste management of agro-industrial wastes to produce value-added chemicals. This

will have a dual benefit – decreasing the environmental impact the free disposal of such wastes would bring and the possibility of reducing importing value-added chemicals. It can also help as a basis for other researchers to further study the extraction of Ferulic acid and other related antioxidants by considering other factors this research study did not cover.

5.2. Recommendations

Therefore, based on the outcomes of this research work and overall understanding of the extraction and characterization of Ferulic acid from brewery spent grains, the following recommendations are stated.

- To dry the raw material, a stove at 50 °C was used. This might have degraded some Ferulic acid molecules since this acid is sensitive to temperature. Hence, other less stressful drying methods such as freezing and spray drying can be tested for a better antioxidant activity of the extract.
- In this study, alkaline hydrolysis was used as a method of extraction. But other classical methods such as acidic hydrolysis, solvent extraction, and enzymatic hydrolysis methods are also recommended for investigation. It is also worth it considering other recently introduced extraction methods namely microwave-assisted extraction, pressurized liquid extraction, ultrasonic extraction, and supercritical fluid extraction.
- Other antioxidant assays such as FRAP, ORAC, (FRAP) etc. should also be applied for further investigation of the antioxidant activity of the extract.
- Ferulic acid is mostly applied in medical and food industries, which exhaustive testing mechanisms must be applied in the suitability of the extract before its application. Hence, other testing mechanisms besides to antioxidant potential are further recommended to be conducted prior to its application. The most important are: total flavonoid content, total anthocyanin, antimicrobial potential, preservative effect against oxidation, etc.
- Further investigations are also recommended regarding the suitability of the extract for further development as nutraceutical supplement or antioxidant remedies. Hence, future studies should focus on the assessments of economic benefits and in-vivo activities of these extracts before their commercial exploitation.
- A more sophisticated purification mechanism is also recommended to obtain a purer Ferulic acid.
- Future researches should pay attention to innovative food applications into meat, fish, cereal, fruit-based and dairy products, with a focus on the type of recovered ingredient, and dosage level achieved by the application of this brewery spent grains extract.

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APPENDICES

Appendix A: Proximate Analysis of BSG

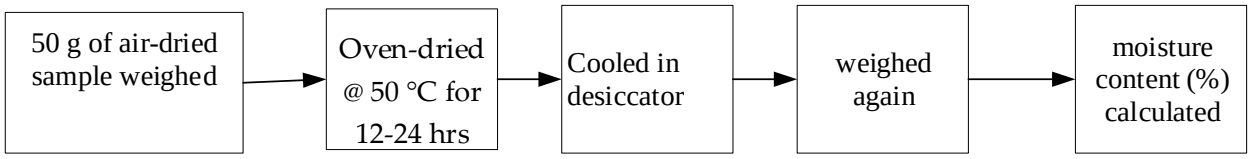

Figure A-1: Determination of Moisture Content in BSG

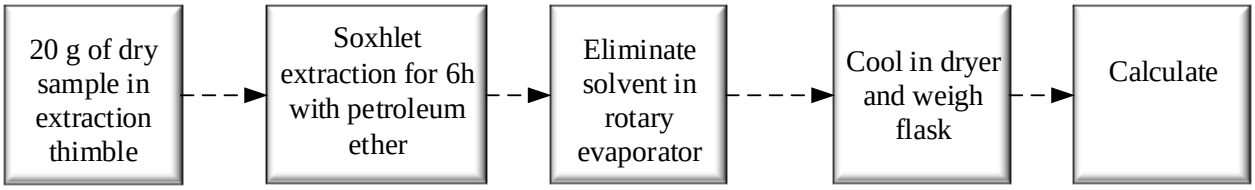

Figure A-2: Determination of lipids by Soxhlet method

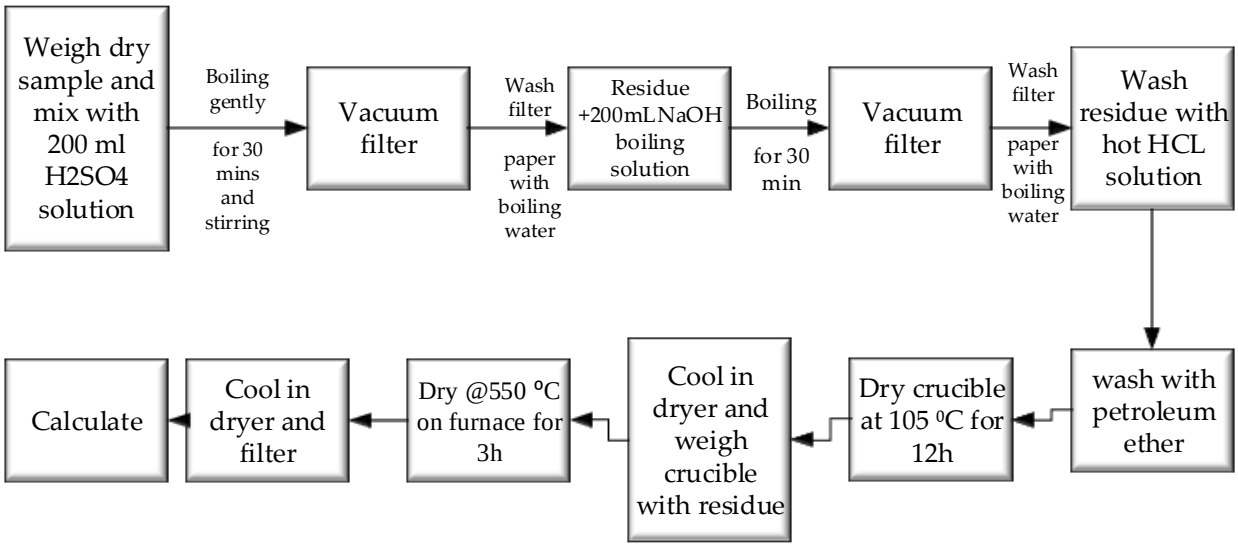

Figure A-3: Determination of Crude Fiber

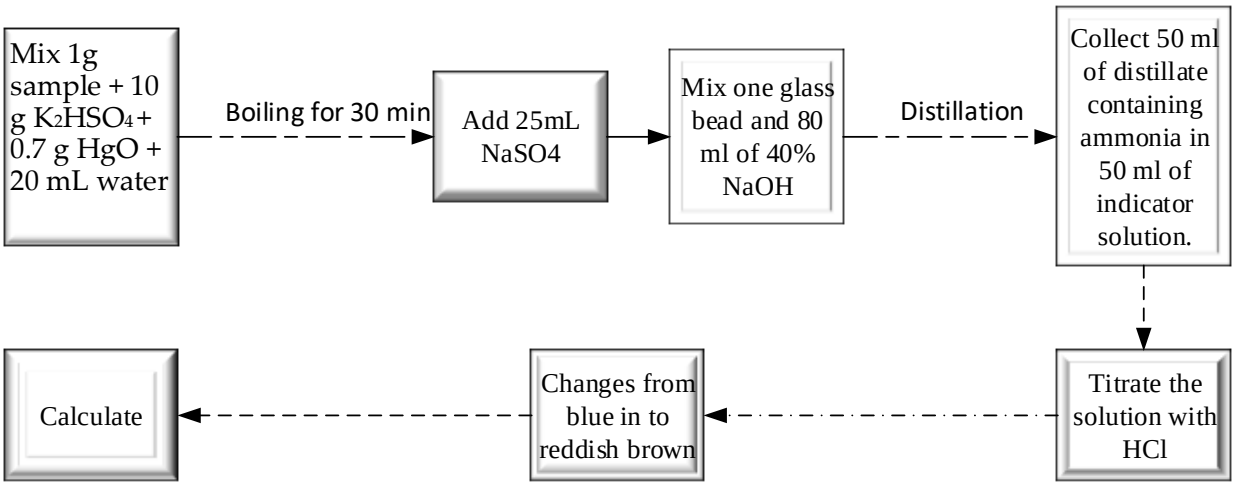

Figure A-4: Determination of crude protein by Kjeldahl method

Appendix B: Calculation Part

Alkaline Hydrolysis

A) Determination of added BSG weight: Based on the method developed by Mussatto et al. (2007), a solid to liquid ratio of 1:20 (w/w) was used during the alkaline hydrolysis. Thus, the weight of the sample taken was:

$\frac{1}{20} = \frac{X}{250\ mL}$, where x = the weight of BSG taken for each experimental run. Solving for x,

X = $\frac{250\ mL}{20}$ = 12.5 g.

Thus, 12.5 g of BSG was weighed and mixed together with each concentrations of sodium hydroxide to make up the 250 mL solution.

B) Determination of sodium hydroxide to be added: according to the design, the concentrations of sodium hydroxide were 1%, 2%, and 3%. This, therefore, will make up masses of 2.5 g, 4.5 g, and 6.5 g of sodium hydroxide, which all were weighed and added to make up the solution for each of the 17 experimental runs.

Amount of HCl used for the precipitation of lignin

Commercial HCl comes with 34.04% purity. The desired concentration for this study was 6 M. Thus, to obtain the corresponding volume:

$C_1V_1 = C_2V_2$

Where

C_1 = required HCl concentration, 6 M

C_2 = concentration of HCL in the solution

V_1 = corresponding volume to be taken

V_2 = total volume of the solution, 250 mL

Thus, substituting these:

(6 M) (V_1) = (C_2) (0.25 L)

To determine the concentration of HCl in the solution (i.e. C_2):

C_2 = n/V, where n is the number of moles of HCl dissolved in the solution and V is the volume of the solution.

C_2 = m/MwV, where m and MW are the mass and molecular weight of HCl dissolved in the solution. Since m/V is density;

C_2 = p /Mw

Substituting this into (6 M) (V_1) = $(C2)\ (0.25\ L)$ and solving for V_1:

$V_1 = \frac{(C2)(0.25\ L)}{6\ M} = \frac{\frac{p}{Mw} * 0.25\ L}{6\ M} = \frac{1.18\frac{g}{mL}}{36.47\frac{g}{mol}} * \frac{0.25\ L}{6\ M}$ = 1.35 L

But as indicated above, commercial HCl acid comes within 34.04% purity. Thus, the amount of pure HCl acid added is:

V_1 = 1.35 * 0.3402 = 0.5 L

Thus, 0.5 L of HCl was slowly added to the alkaline solution to lower the pH to an acidic value in the range 2-3.

Appendix C: Detail Procedures

Procedures for the determination of total phenols

A) Preparation of blank solution

A 1:1 of Folin-Ciocalteu's reagent solution and methanol were mixed together and incubated for 3 min after which 1 mL of saturated Na_2CO_3 solution was added. It was adjusted to 10 mL with distilled water and was left in the dark for 90 min at room temperature.

Preparation of standard solution

In this study, gallic acid was used as a standard for the determination of total phenolic content of the extract. A stock solution of gallic acid (20 mg/mL) was prepared by weighing 2 g of gallic acid by dissolving it in 5 mL methanol then diluting it to 50 mL volumetric measuring flask using distil water. Aliquots of 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, and 240 µL of standard gallic acid were withdrawn from the stock solution and mixed with 980, 960, 940, 920, 900, 880, 860, 840, 820, 800, 780 and 760 µL of methanol solvent to get gallic acid concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 mg/mL respectively in separate test tubes. Then 1 mL of Folin-Ciocalte's reagent was added, mixed well and incubated for 3 min. After 3 min, 1 mL of saturated Na_2CO_3 solution was added and adjusted the solution to 10 mL with distilled water, mixed well and incubated in the dark for 90 min at room temperature. After 90 min incubation, the absorbance was measured against the blank (the same mixture without the gallic acid) at 725 nm using a UV/Visible Spectrophotometer.

B) Preparation of sample solution

Fifty (50 mg/mL) sample stock solution of the extract was prepared by dissolving 2.0 g of extract into 40 mL of ethanol. From each stock solution, 20, 40, 80, 120, 160, 200 and 240 µL of sample solution were withdrawn and mixed with 980, 960, 920, 880, 840, 800 and 760 µL of methanol to get sample concentrations of 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 mg/mL respectively in separate test tubes. Then 1 mL of Folin-Ciocalteu's reagent was added, mixed well and incubated for 3 min. After 3 min, 1 mL of saturated Na_2CO_3 solution was added and adjusted the solution to 10 mL with distil water, mixed well and incubated in the dark for 90 min at room temperature. After 90 min incubation, the absorbance was measured against the blank (the same mixture without the extract) at 725 nm using a UV/Visible Spectrophotometer. The total phenol content was calculated

and expressed as milligrams of gallic acid equivalents (mg of GAL/g dry extract) using the gallic acid calibration curve and the curve used to determine the corresponding gallic acid concentration of the samples.

Procedures for the Determination of Antioxidant Activity of the Extract with the DPPH Radical Scavenging Method

A) Preparation of reagent and blank solution

DPPH (0.004%) was prepared by dissolving 0.01 g of DPPH in 250 mL of methanol. Then, 4 mL of this solution was added to the test tube containing 1 mL of methanol and mixed well.

B) Preparation of standard solution

For this study, the standard used was ascorbic acid. Two (2.0) g of this acid was dissolved into 40 mL of methanol to prepare a stock solution (50 mg/mL) of ascorbic acid. Aliquots were withdrawn from the stock solution to get ascorbic acid concentrations 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 mg/mL by mixing 20, 40, 80, 120, 160, 200 and 240 µL of standard ascorbic acid with 980, 960, 920, 880, 840, 800 and 760 µL of methanol solvent respectively in dissimilar test tubes. Then 4 mL of 0.004% DPPH was added to each sample concentrations in the test tubes and mixed well then incubated the samples for 30 min in the dark at room temperature. After 30 min incubation, the absorbance was measured against the blank (the same mixture without the ascorbic acid) at 517 nm using a UV/Visible Spectrophotometer.

C) Preparation of sample solution

Fifty (50 mg/mL) sample stock solution of the extract was prepared by 2.0 g of extract into 40 mL of ethanol. From each stock solution, 20, 40, 80, 120, 160, 200 and 240 µL of sample solution were withdrawn and mixed with 980, 960, 920, 880, 840, 800 and 760 µL of methanol to get sample concentrations of 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 mg/mL respectively in separate test tubes. Then 4 mL of 0.004% DPPH was added to each sample concentration in the test tubes and mixed well. Then it was incubated with the samples in the dark for 30 min at room temperature. After 30 min incubation, the absorbance was measured against the blank (the same mixture without the ascorbic acid) at 517 nm using a UV/Visible Spectrophotometer.

Appendix D: Images of Raw Material and Extraction Process

Figure C-1: Washed BSG sun-drying

Figure C-2: Weighing dried BSG

Figure C-3: Alkaline hydrolysis of BSG

Figure C-4: Alkaline-Hydrolyzed BSG

Figure C-5: Acidified hydrolyzed BSG

Figure C-6: Hydrolyzed BSG diluted with pure ethanol

Figure C-7: Vacuum Evaporation of ethanol from the hydrolyzed sample

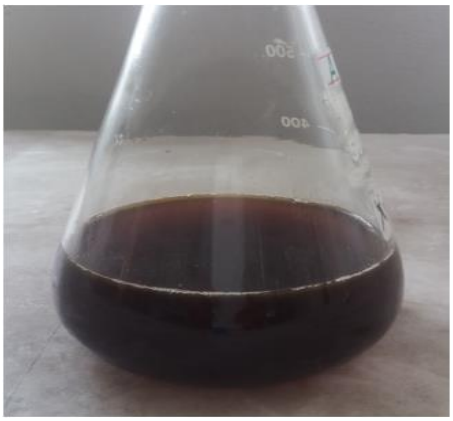

Figure C-8: Sample after vacuum evaporation

Figure C-9: Centrifugation of samples

Figure C-10: Extracted powder FA